Development of an oral drug delivery platform formulation for the targeted delivery of celecoxib for the chemoprevention and treatment of colorectal cancer

A dissertation submitted for the degree of Ph.D.

By

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Declaration

‘I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy is entirely my own work, and that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work’

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Abstract

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Bernard McDonald

The anti-inflammatory drug celecoxib (CLX) has been shown to exert protective effects in colorectal cancer (CRC) therapy. The primary objective of this study was to develop and characterize a novel CLX multiparticulate drug delivery technology suitable for use in the treatment and prevention of CRC which has the potential to minimize the side effects associated with CLX. Liquid CLX formulations were developed as precursors to CLX microbeads and the effect of formulated CLX samples on the viability and motility of a CRC cell line was examined. CLX liquid formulations were shown for the first time to have an enhanced effect in comparison to the marketed CLX product Celebrex®. Liquid CLX formulations were translated into an optimized CLX microbead formulation which met a number of pre-defined critical quality attributes. A sustained release coat was applied to the beads. An *in-vivo* study was performed to compare the effect of the coated CLX microbeads versus Celebrex® in the attenuation of CRC tumours and inflammation in a CRC mouse model. Whilst the level of CRC tumour attenuation and inflammation was comparable between both formulations, the CLX microbead statistically outperformed Celebrex®. Microbead production was scaled-up and subsequent coating optimisation studies were performed resulting in products that met pre-defined target product profiles for both murine and human colon delivery. Finally a screening study to assess the applicability of the platform formulation to a range of APIs other than CLX was performed with 50% of the actives screened being successfully incorporated into microbeads. In summary, the *in-vitro* and *in-vivo* results described in this thesis present a significant step forward in CRC therapy using CLX, as the microbead formulation developed poses the possibility of presenting CLX in a format that has the potential to minimize GI and CV side effects whilst enhancing the effectiveness of the treatment.

Publications

Publications

- **McDonald, B.F.,** Coulter I.S., and Marison, I.W. 'Microbeads: A novel multiparticulate drug delivery technology for increasing the solubility and dissolution of celecoxib'. *Pharm Dev Technol* 2013 Nov 27. [epub ahead of print].

- **McDonald, B.F.,** Quinn, A.M., Devers, T., Cullen, A., Coulter I.S., Marison, I.W., and Loughran, S.T. '*In-vitro* characterization of a novel celecoxib microbead formulation for the treatment and prevention of colorectal cancer'. *Journal of Pharmacy and Pharmacology*: Accepted for publication on the 2nd of November 2014.

Patent Filings

- **McDonald, B.F.,** Aversa, V., Coulter, I.S. **'**Celecoxib Formulations Useful for Treating Colorectal Cancer'. PCT/EP2014/060750 2014 May 23.

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CHAPTER 1

Introduction: Colorectal cancer, celecoxib and oral drug delivery

1.1 Background

Colorectal cancer (CRC) is the third most common cause of cancer mortality worldwide with more than 1 million new cases of CRC diagnosed each year (Siegel *et al*., 2014). CRC is a heterogeneous disease, including at least three major forms; hereditary, sporadic, and colitis-associated CRC. Together with familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC), chronic inflammation is among the top three high risk conditions for CRC (Wang and Dubois 2010). Significant research has been dedicated to identify novel drug targets for CRC prevention and treatment. Non-steroidal anti-inflammatory drugs (NSAIDs) are one group of compounds that have been found to decrease the risk of CRC (Ruder *et al*., 2011). NSAIDs target and inhibit the cyclooxygenase (COX) enzymes, COX-1 and COX-2. Since elevated COX-2 expression has been found in approximately 50% of colorectal adenomas and 85% of colorectal adenocarcinomas (Wang and Dubois 2010), it is hypothesised that NSAIDs may exert some of their anti-inflammatory and anti-tumour effects through inhibition of COX-2. Given this hypothesis, and the fact that many of the unwanted GI side effects associated with NSAIDs are related to COX-1 inhibition, there has been a focus on the use of COX-2 selective NSAIDs for the treatment and prevention of CRC. Celecoxib (CLX) is a COX-2 selective inhibitor. CLX has also demonstrated significant chemopreventative activity in colon carcinogenesis (Maier *et al*., 2004, Reddy *et al*, 2000 and Kawamori *et al*., 1998), however the administration of CLX is associated with the potential risk for serious CV side effects and also some serious GI adverse events despite being COX-2 selective (Sostres *et al*., 2010 and Pfizer Important Safety Information for Celebrex®, 2013).

The overall objective of this project was to develop a novel CLX formulation for use in the treatment and prevention of CRC that would offer a more effective and safer

alternative to the currently marketed CLX product Celebrex®. The five subsequent results chapters broadly describe the following work packages involved in meeting the stated objective; a) pre-formulation development of lipid based CLX formulation, b) an assessment of the CLX lipid formulations in an *in-vitro* CRC cell culture model, c) translation of the lipid-based CLX formulations into a multiparticulate microbead, d) colonic targeting of the microbeads and a subsequent *in-vivo* assessment in an animal model and e) optimisation of the microbead including an assessment of the platform formulation with respect to other active ingredients. Each chapter outlined above includes a detailed introduction on the subject matter of that chapter, therefore this initial introduction will serve as an overview of CRC, CLX and the use of CLX in the treatment and prevention of CRC and finally the challenges with respect to the oral drug delivery of CLX.

1.2 Colorectal cancer (CRC)

CRC develops in the colon or the rectum which is also known as the large intestine (See Figure 1.1 below). The colon and rectum are parts of the digestive system, also called the gastrointestinal (GI) tract. The digestive system processes food for energy and also eliminates solid waste from the body. After food is chewed and swallowed, it travels through the oesophagus to the stomach. In the stomach, food is partially broken down before entering the small intestine via the pylorus. In the small intestine (consisting of the duodenum, jejunum and ileum) digestion continues and most of the nutrients are absorbed. The small intestine joins the large intestine in the lower right abdomen at the ileocecal junction. The first and longest part of the large intestine is the colon, a muscular tube which is approximately 1.5 meters in length. Water and mineral nutrients are absorbed from the food matter in the colon, whereas the leftover waste (the faeces)

passes into the rectum (the final 15 cm of the large intestine) and is then expelled from the anus. Cancer develops much less often in the small intestine than in the colon or rectum (American Cancer Society, 2014). The colon is divided into four sections; a) the ascending colon (extends upward on the right side of the abdomen), b) the transverse colon (crosses the body from the right to the left side), c) the descending colon (descends on the left side) and d) the sigmoid colon were the colon joins the rectum (sigmoid colon is in the shape of an "S"). The ascending and transverse sections are collectively referred to as the proximal colon, while the descending and sigmoid colon are referred to as the distal colon. Colorectal cancers have different characteristics based on their location within the colon or rectum (Iacopetta, 2002). In the case of women and older patients, proximal tumours are more prevalent, whereas distal tumours are more common among men and younger patients (Matanoski *et al.*, 2006 and Nawa *et al.*, 2008).

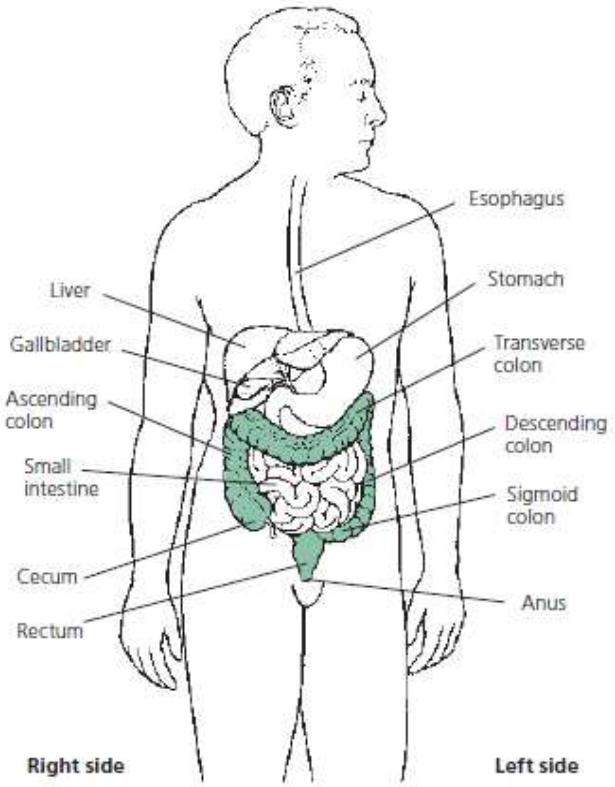

Figure 1.1 Diagram depicting the gastrointestinal tract, including the colon and rectum. (Adapted from American Cancer Society, 2014).

Most cancers of the colon begin as a noncancerous growth called a polyp that develops on the inner lining of the colorectum (colon/rectum). The most common kind of polyp is called an adenomatous polyp or adenoma. Adenomas arise from glandular cells in the mucosa (which produce mucus to lubricate the colorectum) as a result of a multistep process in which normal crypts are initiated to form foci of aberrant crypts (ACF) that proliferate by crypt fission to form microadenoma (De Robertis *et al*., 2011). The microadenomas enlarge to give macroscopic adenomas and eventually adenomatous polyps (Figure 1.2). It is estimated one-third to one-half of all individuals will eventually develop one or more adenomas (Bond, 2000 and Schatzkin *et al*., 1994). Some people are more likely to develop polyps than others such as those with a family history of polyps and/or colorectal cancer. Despite the fact that adenomas have the capacity to become cancerous, less than 10% are estimated to progress to invasive cancer (Levine and Ahnen, 2006 and Riso, 2010), however there is an increased tendency for cancers to evolve as adenomas become larger (Pickhardt *et al*., 2013). These cancers are refered to adenocarcinomas (Figure 1.2) and account for 96% of all colorectal cancers (Stewart *et al*., 2006). Adenocarcinomas can ultimately metastasize and spread to other organs via the blood or lymph system. The colorectal cancer description outlined above relates to the most common colorectal cancer sequence (i.e., adenoma-carcinoma sequence type or sporadic cancer), however as eluded to in Section 1.1, there exists a number of other colorectal sequence types (i.e., hereditary and colitis-associated cancer types) with their own unique histopathological features (Tanaka, 2012). For example, in contrast to the involvement of adenomas in the case of sporadic cancer, colitis associated cancer involves another precancerous condition of the

colorectal tissue known as dysplasia which is characterised by abnormal cell growth and inflammation. Tissue exhibiting dysplasia can often be benign but similar to adenomas it can also turn malignant (Johns Hopkins Medicine Colorectal Cancer, 2014).

In addition to understanding the histopathology of CRC (sporadic, hereditary and colitis associated), scientists have also been able to trace colon cancer progression at a molecular level. Despite differences in the stepwise mutations in oncogenes and tumour suppressor genes and the expression of key proteins and enzymes, there is considerable overlap in the genetic and signalling pathways involved in the pathogenesis of the different types of colorectal cancers. For example the expression of the following genes and associated proteins, *K-ras, APC, p-53 and β-catenin*, and also the enzymes such as COX-2 (cyclooxygenase 2) have been shown or suggested to play a role play in both sporadic and colitis-associated CRC (Tanaka, 2012, Terzic *et al*., 2010 and De Robertis *et al*., 2011).

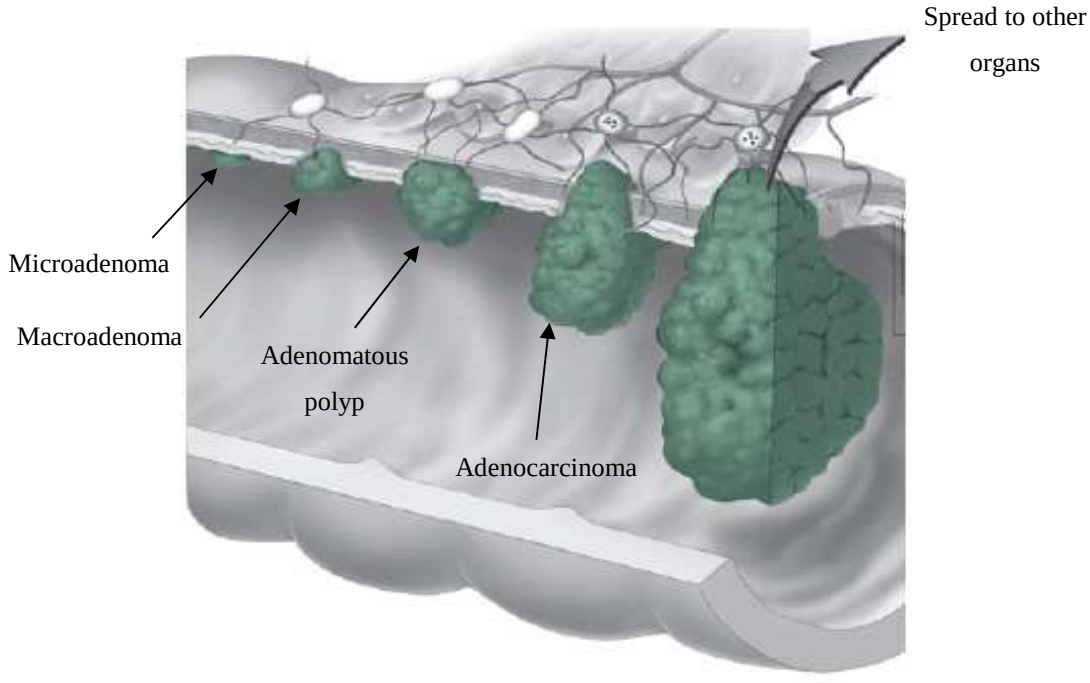

Figure 1.2 Diagram depicting development of CRC from small adenoma (polyp) to metastatic adenocarcinoma (Adapted from American Cancer Society, 2014).

Whilst a small percentage of human colorectal cancers are associated with defined familial syndromes (e.g., FAP and HNPCC), the vast majority of malignant colorectal cancers arise out of benign adenomatous polyps over a course of several decades (Johnson and Fleet, 2013). In the case of non-familial colorectal cancers, malignancy can develop spontaneously or as a late complication of a chronic inflammatory state (De Robertis *et al*., 2011). Many of the risk factors (including environmental causes) associated with cancer (including CRC) are associated with some form of chronic inflammation. Up to 20% of cancers are linked to chronic infections, 30% can be attributed to tobacco smoking and inhaled pollutants and 35% can be attributed to dietary factors (De Robertis *et al*., 2011). In the case of CRC, chronic inflammation as a result of inflammatory bowel disease (IBD) greatly increases the risk of the disease (van Hogezand *et al*., 2002). IBD is a complex class of immune disorders that have been grouped into two major forms, ulcerative colitis (UC) and Crohn's disease (CD). Colitis-associated CRC (CACRC) is the subtype of CRC that is associated with IBD, it is difficult to treat and has a high mortality (Feagins *et al*., 2009). More than 20% of IBD patients develop CACRC within 30 years of disease onset and >50% will die from CACRC (Lakatos and Lakatos, 2008).

It is important to note that although the clearest link between inflammation and colon cancer is seen in patients with IBD (Fukata *et al.*, 2007), colorectal tumours not associated with IBD have also been shown to display robust inflammation and increased expression of proinflammatory cytokines, which highlights the key role that inflammation plays regardless of the colorectal cancer type (Terzic *et al*., 2010).

1.3 Celecoxib and CRC

Given the role that inflammation plays in CRC, there has been a lot of focus on the use of anti-inflammatory drugs for the treatment and prevention of CRC and in particular there has been a focus on the role of COX and specifically COX-2 with respect to inflammation and CRC progression. Two isoforms of COX have been identified: COX-1 and COX-2. Both of these enzymes are encoded by separate genes located on different chromosomes and catalyze the conversion of arachidonic acid and other fatty acids to prostaglandins (lipid inflammatory mediators) (Gonzalez-Angulo *et al*., 2002). Evidence has revealed that although both COX-1 and COX-2 catalyze the same reaction, COX 1 produces metabolites that play a central role in maintaining homeostatic functions, including platelet aggregation, renal blood flow and gastric cytoprotection, whereas, COX-2 is an inducible enzyme expressed in response to a variety of physiological stimuli such as inflammation, fever, wound healing, and neoplasia (Gonzalez-Angulo *et al*., 2002). COX-2 is also understood be be induced physiologically in the heart and therefore plays a vital role in opposing platlet ahhesion and aggregation (Funk and Fitzgerald, 2007). The mechanism of action of COX-1 and COX-2 is illustrated in Figure 1.3 below.

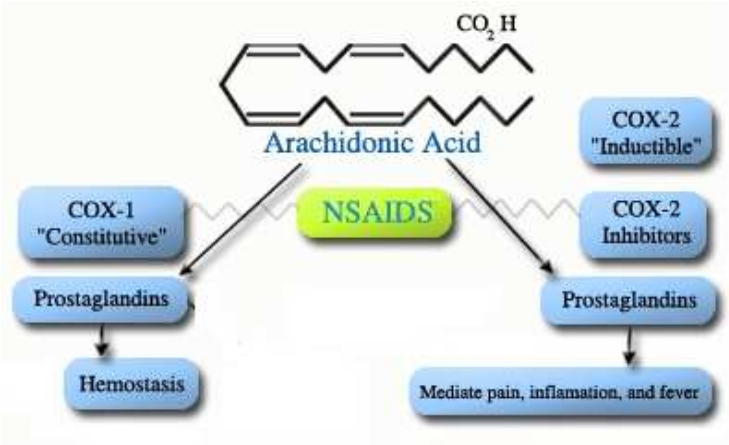

Figure 1.3 Mechanism of action of COX-1 and COX-2 enzymes. COX-1 and COX-2 catalyse conversion of arachidonic acid into different prostaglandins. Prostaglandins resulting from the COX 1 pathway are responsible for homeostatic functions whereas COX-2 prostaglandins primarily play a role in inflammation, fever and pain. COX-1 is referred to as constitutive enzyme as it is produced by cells under all types of physiological conditions, whereas COX-2 is an inducible enzyme produced only under specific conditions such as inflammation.

Expression of COX-2 has been found to be increased at sites of inflammation and also in approximately 85% of CRCs and 50% of colorectal adenomas (Eberhart *et al*., 1994, Marnett and Dubois, 2002, Wang and Dubois 2010). The COX-2 protein is found in the cytoplasm of neoplastic colonic epithelial cells and to a lesser extent in stromal cells, whereas normal epithelium is negative for COX-2 (De Robertis *et al*., 2011). It is postulated that COX-2 may contribute to tumour development by modulating apoptosis, angiogenesis, and tumour invasiveness and also that COX-2 has a role in the progression of cancer via activation of metalloproteases, thereby increasing the invasiveness of colon cancer cells (Tsujii and Dubois, 1995). The tumorigenic effect of COX-2 on the development of colorectal tumours has been well documented not only in the sporadic (Oshima *et al.,* 1996) but also in the colitis-associated model of CRC (Tanaka *et al.,* 2003 and Kim *et al.,* 2008). It is however noted that there exists some discord with respect to this as a number of other studies cast doubt on the role that COX-2 plays in the progression of CRC (Grosch *et al.,* 2001, Ishikawa and Herschman,

2010, Arber, 2008, Sade *et al*., 2012 and Sacchetti, 2013). Although the mechanism for cancer protection by COX-2 inhibitors is unknown, it is speculated that it may relate to an altered synthesis of arachidonic acid metabolites which, as previously discussed, may play a role in apoptosis, angiogenesis, and tumour invasiveness, however it is also possible that COX-2 inhibitors act by COX-2 independent mechanisms with respect to colorectal cancer (Arber, 2008). Regardless of the role that COX-2 plays in CRC progression there is a large body of evidence that anti-inflammatory drugs and specifically COX inhibitors have a role to play with respect to the prevention and treatment of CRC. The past three decades have witnessed more than 200 well-conducted, randomized, single-blind, placebo-controlled animal studies that showed the consistent preventive effect of NSAIDs on carcinogen-induced colorectal neoplasia in rodents (Arber, 2008). In a review by Gonzalez-Angulo and colleagues (Gonzalez-Angulo *et al.*, 2002), a list of twenty three COX inhibitors successfully used (to varying degrees) in animal studies for the prevention of colon carcinogenesis is provided. The review includes two studies by Kawamori and colleagues (Kawamori et al., 1998) and Reddy and colleagues (Reddy et al., 2000) on the chemopreventative effects and the chemotherapeutic effects of CLX (selective COX-2 inhibitor) with respect to CRC. These studies demonstrated the inhibitory activity of CLX during the initiation and post-initiation stages of carcinogenesis (Kawamori *et al*., 1998) but also that CLX can inhibit tumour growth during the promotion/progression stage of carcinogenesis when premalignant lesions have developed (Reddy *et al*., 2000). The study by Reddy and colleagues is of particular interest as it suggests that colon tumour development can also be achieved even when the treatment is delayed and it subsequently prompted the clinical use of CLX in secondary prevention of colon cancer in patients with FAP and also patients with sporadic polyps (details of these clinical studies are outlined later). As eluded to above, in addition to a review of animal studies, Gonzalez-Angulo and

colleagues also cite several epidemiological studies and clinical trials involving aspirin, sulindac (non-selective COX-1 and COX-2 inhibitors) and CLX (selective COX-2 inhibitor) which illustrate the anti-cancer effect of these NSAIDs. The studies cited reported a) a decreased risk of CRC (epidemiological study involving aspirin) and b) a suppression of adenomatous polyp formation/regression of existing polyps (FAP clinical studies involving CLX and sulindac). Despite evidence regarding the chemopreventative effects of aspirin and other conventional nonselective NSAIDs (e.g., ibuprofen, sulindac, naproxen and diclofenac), their long-term use may result in serious side effects, with the most significant side effect being their serious gastrointestinal toxicity which includes dyspepsia, peptic ulcer disease, and significant bleeding with associated increased mortality These side effects associated with nonselective NSAIDs are thought to result from gastric cytoprotective COX-1 inhibition (Gonzalez-Angulo *et al.,* 2002). Therefore a lot of attention has focused on the use of selective COX-2 inhibitors (which display reduced GI side effects) with respect to their ability to prevent the development and progression of CRC. As previously described CLX, is an example of one such selective COX-2 inhibitor. CLX is weakly acidic (pKa 11.1), hydrophobic in nature (Log P 3.5) and has a low aqueous solubility of 3—7 μg/ml at 20 °C (Avrahami *et al.,* 2007). It is categorised as a BCS (biopharmaceutical classification system) class II drug because of its poor aqueous solubility and high membrane permeability (Morgen *et al*., 2012). The structure of CLX is shown in Figure 1.4 below. CLX has been shown to be 30 times more selective than COX-2 than COX-1, in contrast to other COX-2 inhibtors such as rofecoxib and valdecoxib (both of which have been withdrawn from the market) which are 300 times more selective (Marino, 2011).

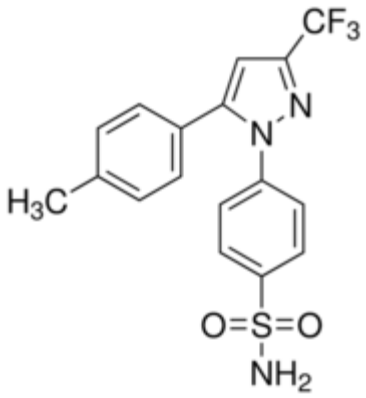

Figure 1.4 Structure of Celecoxib (adapted from Sigma Aldrich celecoxib product page, 2014)

CLX exhibits anti-inflammatory, analgesic and anti-pyretic activities in animals and is routinely administered for human therapy as the marketed product Celebrex® in the treatment of osteoarthritis, adult rheumatoid arthritis and ankylosing spondylitis. Whilst CLX is extensively hepatically metabolised by the liver and these metabolites are in-active, CLX does not undergo extensive first pass metabolism with approximately 50% of the drug remaining in its active form three hours after administration (Paulson *et al*., 2000). Although there is no absolute bioavailaity data for Celebrex® in humans due to the absence of an intravenous dose, bioavailability studies in dogs have demonstrated an absolute bioavailability in the region of 30 %. (Paulson *et al.,* 2000). As previously mentioned, clinical trials have been performed to assess the effect of CLX (Celebrex®) on the prevention of both FAP and sporadic (non FAP related) intestinal polyps. In the FAP trial, six months of twice-daily treatment of FAP patients with 400 mg of CLX led to a significant reduction (~ 30%) in the number of colorectal polyps and was found to be more effective than a 100 mg twice daily dose (Steinbach *et al*., 2000). This subsequently resulted in the European approval of Pfizer's CLX Onsenal®) product for the treatment of FAP. At the same time, Celebrex® was approved by the FDA (Food and Drug Administration) for the treatment of FAP (Cancer Network, 2002). In the EMA

(European Medicines Agency) approval report for Onsenal® the following mode of action for the product was described; *'the active substance in Onsenal®, celecoxib, is an NSAID that belongs to the group of COX-2 inhibitors. It blocks the COX-2 enzyme, resulting in a reduction in the production of prostaglandins, substances that are involved in processes such as inflammation and the activity of smooth muscle (muscle that performs automatic tasks such as the opening and closing of blood vessels). COX-2 is found at high levels in adenomatous colorectal polyps. By blocking the activity of COX-2, celecoxib helps to slow down the formation of polyps by stopping them developing their own blood supply and by increasing the rate of cell death'* (EMA Onsenal® Approval Report, 2003 *and* Pfizer Withdrawal Notice for Celebrex for FAP indication, 2012*).* The approval for Onsenal® and Celebrex® (for the treatment of FAP only) was subsequently withdrawn in 2011 as a result of Pfizer being unable to recruit sufficient patients to support clinical trials to prove the clinical benefits of the product (EMA Onsenal® Withdrawal Notification, 2011). Two further clinical trials, referred to as the APC (Adenoma Prevention with Celecoxib) and the PreSAP (Prevention of Spontaneous Adenomatous Polyps) trials were launched in 2000 to assess the effect of Celebrex® in reducing the proportion of subjects with new colorectal adenomas post baseline polypectomy after 1 and 3 years of study drug administration. The trials targeted patients at a high risk of recurrent adenomas. The APC trial (CLX 200 mg or 400 mg twice daily) and the PreSAP trial (CLX 400 mg once daily) tested the efficacy and safety of CLX against placebo. The two trials were discontinued in 2004 based on an analysis that revealed that patients taking CLX were at increased risk for cardiovascular (CV) events (Soloman *et al*., 2006). For APC and PreSAP trials combined, 83 patients (out of a total of 3853 patients) experienced cardiovascular death, non-fatal myocardial infarction, non-fatal stroke, or heart failure (Solomon *et al*., 2006), however it is important to note that no excessive cardiovascular toxicity was observed

for the PreSAP trial where the relative risk on 400 mg of CLX one a day compared to the placebo was not significant (Arber, 2008). It is also worth noting that the 200 mg twice-daily dosing in the APC trial also demonstrated much less risk than 400 mg twice-daily compared with the placebo (Solomon *et al.*, 2006). Despite the discontinuation of the trials, the data analysis for the trials demonstrated that in the PreSaP trial (in which 80% of patients had completed their 3 year treatment and follow up period) that the use of 400 mg of CLX once daily was shown to significantly reduce the occurrence of colorectal adenomas within three years after polypectomy (Arber *et al*., 2006). In the APC trial (in which 77% of patients had completed their 3 year treatment and follow up period) it was demonstrated that the use of CLX by patients at high risk for colorectal neoplasia significantly reduced the proportion of patients with adenomas detected during a three-year study. Additionally the trial documented a prevention of premalignant adenomas with CLX, although the trial was not designed to assess effectiveness of the drug for the prevention of colorectal cancer (Bertagnolli *et al*., 2006). Although the trials were discontinued due to CV toxicity, it has been reported that the trials exhibited a dose-related increase in CV events and blood pressure which therefore raises the possibility that lower doses or other dose intervals may be associated with less CV risk (Solomon *et al*., 2006).

1.4 Oral drug delivery and Celecoxib

Based on the information outlined above, it was planned to develop a colonic specific CLX formulation for the treatment and prevention of CRC, in the belief that targeting a lower dose of the drug to the colon would allow for a safe and more effective therapy. There are many ways to deliver drugs into the body including; oral delivery (through swallowing), sub-mucosal delivery (through buccal and sublingual mucosa), parenteral

delivery (through injection), transdermal delivery (through skin) and pulmonary delivery (through inhalation). Among these routes of delivery, oral delivery is the most widely accepted by patients with respect to compliance (Gupta *et al.,* 2009). As previously stated CLX is a poorly soluble drug. Poorly water-soluble drugs are becoming more prevalent as candidates for oral drug delivery and it has been estimated that approximately 60–70 % of newdrug molecules are insufficiently soluble in aqueous media and/or have very low permeability to allow for their adequate and reproducible absorption from the gastrointestinal tract (GIT) following oral administration (Gupta *et al.,* 2013). Drug development scientists have adopted various strategies to enhance the solubility of these molecules to ultimately enhance their absorption. These strategies can be broken down into two broad methods. The first method involves chemically altering the structure of the molecule in order to render it more soluble. The second method is described as a 'formulation approach' in which the active material is physically altered or combined with other materials that result in the solubilisation of the active, but critically the chemical structure of the molecule is not altered. In the case where the chemical structure of the molecule is altered, the outcome is a new chemical entity with its own toxicological profile. When addressing the solubility of a poorly soluble drug, the 'formulation approach' is usually the preferred option, primarily due to the significant regulatory implications involved in bringing a new chemical entity to the market. An example of a 'formulation approach' involves particle size reduction of crystalline compounds via micronisation (e.g., Elan's Nanocrystal® technology) which can increase the surface area and hence the dissolution rate of the drug, however this approach may not be desirable in situations where poor wettability and handling difficulties are experienced for very fine powders (Gupta *et al.,* 2013). Another 'formulation approach' is that of 'amorphous formulations' including 'solid solutions' which can be formed using a variety of technologies including spray drying and melt

extrusion in which the drug is rendered amorphous and therefore more soluble. A drawback of 'amorphous formulations' is that they can display stability issues over time as they revert back to the crystalline form (Singh *et al.,* 2011). Lipid based drug delivery systems (LBDDSs) are one of the most promising 'formulation approaches' for addressing the challenges associated with poorly soluble drugs and confers many advantages over other drug delivery systems (Gupta *et al*., 2013). Although the intention of this project was not to enhance the absorption of CLX, one of the key formulation objectives was to enhance the solubility of CLX to allow the drug to be available in a freely molecular form in order for the drug to be optimally available to interact with colonic tissue. The efficacy of an anti-cancer agent in a patient depends both on its potency but also on the application of an effective drug delivery system to ensure the drug is targeted to the site of action (Venkatesan *et al*., 2011). On this basis, a LBDDS in which the drug is delivered in a pre-solubilized form was selected as the most appropriate formulation approach for this project (refer to Chapter 2 for further details on LBDDSs). The second key formulation objective was to present the drug in a multiparticulate pellet format based on the following advantages of multiparticulate dosage forms (Sharma and Chaurasia, 2013, Asghar and Chandran, 2006 and Porter, 2013);

- Multiparticulates/pellets are less susceptible to dose dumping than single unit bolus formulations (e.g., tablets, soft gelatine capsules or powder filled hard gelatine capsules)

- Multiparticulates reduce intra and inter-subject variability by reducing variations in gastric residence times and GI transit times

    - Particles smaller than 2 mm can pass through the constricted pyloric sphincter even during the gastric phase of the digestion process and distribute themselves more readily throughout the distal part of the gastrointestinal tract
    - Multiparticulates are also retained longer in the colon (thereby allowing for a more prolonged period of action)

- Multiparticulates disperse freely in the GI tract thereby allowing for more widespread interaction with GI tissue

- Multiparticulates can minimise irritant effects
    - Single unit dosage forms can potentially lodge in restrictions within the gastrointestinal tract, causing the release of drug to be localized and thus cause mucosal damage should the drug possess irritant effects. This potentially harmful effect can be minimized with multiparticulates since their small size reduces the likelihood of such entrapment, while the drug concentration is spread out over a larger number of discrete particles.

- The spherical nature of pellets allows for the improved application of controlled release coating and also enhances flow properties

The combination of these key formulation objectives required the development of a multiparticulate formulation that was amenable to the encapsulation of lipids and the application of controlled release polymers. Conventional LBDDSs such as soft gelatine capsules were therefore not considered given that they represent a single unit dosage form and are not broadly amenable to coating. The selection and development of a

suitable formulation technology to meet the requirements outlined is described in detail in Chapter 4.

As referred to earlier, CLX is currently administered for the treatment of osteoarthritis, adult rheumatoid arthritis and ankylosing spondylitis as the marketed formulation Celebrex® and was previously administered for the treatment of FAP as the marketed product Onsenal® (both Pfizer products). Based on a review of the available literature it would appear that Celebrex® and Onsenal® are equivalent products marketed under different brand names (FDA Oncologic Drugs Advisory Committee Briefing Document, 2005 and MHRA Safety Advisory Notice, 2011). In the EMA scientific discussion for the approval of Onsenal®, the manufacturing method is outlined. It describes a product which is wet granulated (to improve its flow properties) followed by milling (to reduce the particle size and increase the surface area to aid dissolution) (EMA Scientific Discussion for the Approval of Onsenal®, 2004). The manufacturing method and product description for Onsenal® corresponds to the visual appearance of Celebrex® (a fine powder filled into hard gelatine capsule). Although Celebrex® is not approved for use in the prevention or treatment of CRC, its efficacy has been shown in the various clinical trials previously described. The primary drawback of Celebrex® is its CV side effects and despite being COX-2 selective, Celebrex® is also associated with GI toxicity (Silverstein FE *et al*., 2000, Pfizer Celebrex® Monograph 2014). The CV side effects associated with COX-2 inhibitors is understood to be a result of the inhibition of the physiological induction of COX-2 in the heart (Funk and Fitzgerald, 2007). In the case of GI side effects, COX-2 is produced in the intestinal epithileum in response to tissue injury which accounts for aspects of the toxicity associated with drugs such as CLX when administered in combination with non-slective COX inhibitors such as aspirin which supress the mucosal defence (Wallace, 2000). A literature review in relation to Celebrex® identified the unwanted side effects of Celebrex® to be a) dose related (CV

and GI side effects) and b) dosage form related (GI side effects) (Sacchetti, 2013, Soloman *et al*., 2005 and FDA labelling Revision for Celebrex® Capsules, 2008). Aside from COX inhibition, direct damage to the mucus layer and cytotoxicity to the gastrointestinal epithelia has been observed with different NSAIDs, including CLX (Tomisato *et al.,* 2004). In the case of COX-2 inhibitors such as CLX, it is postulated that this direct toxicity might occur in spots of higher vulnerability where the protective mucus is weakened by COX-1 inhibition, pre-existing lesions and incipient damage (Tomisato *et al.*, 2004, Lichtenberger *et al*., 2006, Scarpignato and Hunt, 2010 Pfizer Celebrex® Monograph 2014).

A central hypothesis of this study was that by presenting pre-dissolved CLX in multiparticulate form, the GI irritation effects of the current dosage form could potentially be alleviated and in tandem by addressing the solubility issues associated with the drug it would potentially allow for the opportunity of administering a lower dose thereby reducing the potential for both GI and CV side effects. Celebrex® was used throughout the project as a formulation comparator with respect to physicochemical performance (e.g., *in-vitro* release testing) and efficacy (*in-vitro* cell line and *in-vivo* animal studies) in the belief that the development of an enhanced formulation with respect to these parameters would then warrant an enhanced safety assessment in human volunteers in future studies.

1.5 Nomenclature

Table 1.1 List of abbreviations which are listed according to their appearance in the text.

Abbreviation	Definition
CRC	Colorectal cancer
FAP	Familial adenomatous polyposis
HNPCC	Hereditary nonpolyposis colorectal cancer
NSAIDs	Non-steroidal anti-inflammatory drugs
COX	Cyclooxygenase
CLX	Celecoxib
GI	Gastrointestinal
ACF	Aberrant crypt foci
IBD	Inflammatory bowel disease
UC	Ulcerative colitis
CD	Crohn's Disease
CACRC	Colitis associated colorectal cancer
CV	Cardiovascular
BCS	Biopharmaceutics classification system
EMA	European medicines agency
LBDDS	Lipid based drug delivery system
MHRA	Medicines and healthcare products regulatory agency
FDA	Food and drug administration

1.6 References

American Cancer Society. Cancer Facts & Figures 2014. Atlanta: American Cancer Society; 2014

Arber, N. 2008, "Cyclooxygenase-2 inhibitors in colorectal cancer prevention: point", *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology,* vol. 17, no. 8, pp. 1852-1857.

Arber, N., Eagle, C.J., Spicak, J., Racz, I., Dite, P., Hajer, J., Zavoral, M., Lechuga, M.J., Gerletti, P., Tang, J., Rosenstein, R.B., Macdonald, K., Bhadra, P., Fowler, R., Wittes, J., Zauber, A.G., Solomon, S.D., Levin, B. & PreSAP Trial Investigators 2006, "Celecoxib for the prevention of colorectal adenomatous polyps", *The New England Journal of Medicine,* vol. 355, no. 9, pp. 885-895.

Asghar, L.F. & Chandran, S. 2006, "Multiparticulate formulation approach to colon specific drug delivery: current perspectives", *Journal of Pharmacy & Pharmaceutical Sciences : a publication of the Canadian Society for Pharmaceutical Sciences, Societe canadienne des sciences pharmaceutiques,* vol. 9, no. 3, pp. 327-338.

Avrahami, M., Aserin, A. & Garti, N. 2007, "Crystallization of Celecoxib in Microemulsion Media", *J. Dispersion Sci Technol,* vol. 28, no. 8, pp. 1228-1235.

Bertagnolli, M.M., Eagle, C.J., Zauber, A.G., Redston, M., Solomon, S.D., Kim, K., Tang, J., Rosenstein, R.B., Wittes, J., Corle, D., Hess, T.M., Woloj, G.M., Boisserie, F., Anderson, W.F., Viner, J.L., Bagheri, D., Burn, J., Chung, D.C., Dewar, T., Foley, T.R., Hoffman, N., Macrae, F., Pruitt, R.E., Saltzman, J.R., Salzberg, B., Sylwestrowicz, T., Gordon, G.B., Hawk, E.T. & APC Study Investigators 2006, "Celecoxib for the prevention of sporadic colorectal adenomas", *The New England Journal of Medicine,* vol. 355, no. 9, pp. 873-884.

Bond, J.H. 2000, "Polyp guideline: diagnosis, treatment, and surveillance for patients with colorectal polyps. Practice Parameters Committee of the American College of

Gastroenterology", *The American Journal of Gastroenterology*, vol. 95, no. 11, pp. 3053-3063.

De Robertis, M., Massi, E., Poeta, M.L., Carotti, S., Morini, S., Cecchetelli, L., Signori, E. & Fazio, V.M. 2011, "The AOM/DSS murine model for the study of colon carcinogenesis: From pathways to diagnosis and therapy studies", *Journal of Carcinogenesis*, vol. 10, pp. 9-3163.78279.

Eberhart, C.E., Coffey, R.J., Radhika, A., Giardiello, F.M., Ferrenbach, S. & DuBois, R.N. 1994, "Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas", *Gastroenterology*, vol. 107, no. 4, pp. 1183-1188.

Feagins, L.A., Souza, R.F. & Spechler, S.J. 2009, "Carcinogenesis in IBD: potential targets for the prevention of colorectal cancer", *Nature reviews.Gastroenterology & Hepatology*, vol. 6, no. 5, pp. 297-305.

Funk, C.D. & Fitzgerald, G.A., 2007, “COX-2 inhibiotrs and cardiovascular risk”, *J Cardiovasc Pharmacol.*, vol 50, no 5, pp.470-9.

Fukata, M., Chen, A., Vamadevan, A.S., Cohen, J., Breglio, K., Krishnareddy, S., Hsu, D., Xu, R., Harpaz, N., Dannenberg, A.J., Subbaramaiah, K., Cooper, H.S., Itzkowitz, S.H. & Abreu, M.T. 2007, "Toll-like receptor-4 promotes the development of colitis-associated colorectal tumors", *Gastroenterology*, vol. 133, no. 6, pp. 1869-1881.

Gonzalez-Angulo, A.M., Fuloria, J. & Prakash, O. 2002, "Cyclooxygenase 2 inhibitors and colon cancer", *The Ochsner Journal*, vol. 4, no. 3, pp. 176-179.

Grosch, S., Tegeder, I., Niederberger, E., Brautigam, L. & Geisslinger, G. 2001, "COX-2 independent induction of cell cycle arrest and apoptosis in colon cancer cells by the selective COX-2 inhibitor celecoxib", *FASEB journal : Official Publication of the Federation of American Societies for Experimental Biology*, vol. 15, no. 14, pp. 2742-2744.

Gupta, H., Bhandari, D. & Sharma, A. 2009, "Recent trends in oral drug delivery: a review",*Recent Patents on Drug Delivery & Formulation,* vol. 3, no. 2, pp. 162-173.

Gupta, S., Kesarla, R. & Omri, A. 2013, " Formulation Strategies to Improve the Bioavailability of Poorly Absorbed Drugs with Special Emphasis on Self-Emulsifying Systems ", *ISRN Pharm.,* vol. 848043, pp. Epub ahead of print.

Iacopetta, B. 2002, "Are there two sides to colorectal cancer?", *International Journal of Cancer,* vol. 101, no. 5, pp. 403-408.

Ishikawa, T.O. & Herschman, H.R. 2010, "Tumor formation in a mouse model of colitis-associated colon cancer does not require COX-1 or COX-2 expression", *Carcinogenesis,* vol. 31, no. 4, pp. 729-736.

Johnson, R.L. & Fleet, J.C. 2013, "Animal models of colorectal cancer", *Cancer Metastasis Reviews,* vol. 32, no. 1-2, pp. 39-61.

Kawamori, T., Rao, C.V., Seibert, K. & Reddy, B.S. 1998, "Chemopreventive activity of celecoxib, a specific cyclooxygenase-2 inhibitor, against colon carcinogenesis", *Cancer Research,* vol. 58, no. 3, pp. 409-412.

Kim, H.S., Kundu, J.K., Lee, J.S., Oh, T.Y., Na, H.K. & Surh, Y.J. 2008, "Chemopreventive effects of the standardized extract (DA-9601) of Artemisia asiatica on azoxymethane-initiated and dextran sulfate sodium-promoted mouse colon carcinogenesis", *Nutrition and Cancer,* vol. 60 Suppl 1, pp. 90-97.

Lakatos, P.L. & Lakatos, L. 2008, "Risk for colorectal cancer in ulcerative colitis: changes, causes and management strategies", *World Journal of Gastroenterology: WJG,* vol. 14, no. 25, pp. 3937-3947.

Levine, J.S. & Ahnen, D.J. 2006, "Clinical practice. Adenomatous polyps of the colon", *The New England Journal of Medicine,* vol. 355, no. 24, pp. 2551-2557.

Lichtenberger, L.M., Zhou, Y., Dial, E.J. & Raphael, R.M. 2006, "NSAID injury to the gastrointestinal tract: evidence that NSAIDs interact with phospholipids to weaken the hydrophobic surface barrier and induce the formation of unstable pores in

membranes", *The Journal of Pharmacy and Pharmacology*, vol. 58, no. 11, pp. 1421-1428.

Maier TJ, Schilling K,Schmidt R, Geisslinger G, Grösch S. 2004, "Cyclooxygenase-2 (COX-2)-dependent and -independent anticarcinogenic effects of celecoxib in human colon carcinoma cells", *Biochemical Pharmacology*, vol. 67, no. 8, pp. 1469-1478.

Marino, J., 2011. “Celecoxib” in *The Essence of Anelgesia and Analgesics*, ed. Sinatra, R.S., Jahr, J.S., and Watkins-Pitchford, J.M., Cambridge University Press, Cambridge, pp 238-243.

Marnett, L.J. & DuBois, R.N. 2002, "COX-2: a target for colon cancer prevention", *Annual Review of Pharmacology and Toxicology*, vol. 42, pp. 55-80.

Matanoski, G., Tao, X., Almon, L., Adade, A.A. & Davies-Cole, J.O. 2006, "Demographics and tumor characteristics of colorectal cancers in the United States, 1998-2001", *Cancer*, vol. 107, no. 5 Suppl, pp. 1112-1120.

Morgen, M., Bloom, C., Beyerinck, R., Bello, A., Song, W., Wilkinson, K., Steenwyk, R. & Shamblin, S. 2012, "Polymeric nanoparticles for increased oral bioavailability and rapid absorption using celecoxib as a model of a low-solubility, high-permeability drug", *Pharmaceutical Research*, vol. 29, no. 2, pp. 427-440.

Nawa, T., Kato, J., Kawamoto, H., Okada, H., Yamamoto, H., Kohno, H., Endo, H. & Shiratori, Y. 2008, "Differences between right- and left-sided colon cancer in patient characteristics, cancer morphology and histology", *Journal of Gastroenterology and Hepatology*, vol. 23, no. 3, pp. 418-423.

Oshima, M., Dinchuk, J.E., Kargman, S.L., Oshima, H., Hancock, B., Kwong, E., Trzaskos, J.M., Evans, J.F. & Taketo, M.M. 1996, "Suppression of intestinal polyposis in Apc delta716 knockout mice by inhibition of cyclooxygenase 2 (COX-2)", *Cell*, vol. 87, no. 5, pp. 803-809.

Paulson, S.K., Vaughn, M.B., Jessen, S.M., Lawal, Y., Gresk, C.J., Yan, B., Maziasz, T.J., Cook, C.S. & Karim, A. 2001, " Pharmacokinetics of Celecoxib after Oral Administration in Dogs and Humans: Effects of Food and Site of Absorption", *Journal of Pharmacology and Experimental Therapeutics*, vol. 297, no. 2, pp. 638-645.

Paulson, S.K., Hribar, J.D., Liu, N.W.K., Hadju, E., Bible, R.H., Piergies, A. and Karim, A., 2000, "Metabolism and Excretion of [14C] Celecoxib in Healthy Male Volunteers", *Drug Metabolism and Deposition*, vol. 28, no. 3, pp. 308-314.

Pickhardt, P.J., Kim, D.H., Pooler, B.D., Hinshaw, J.L., Barlow, D., Jensen, D., Reichelderfer, M. & Cash, B.D. 2013, "Assessment of volumetric growth rates of small colorectal polyps with CT colonography: a longitudinal study of natural history", *The Lancet.Oncology*, vol. 14, no. 8, pp. 711-720.

Porter, S.C. 2013, "Coating of Tablets and Multiparticulates" in *Aulton's Pharmaceutics, 4th Edition The Design and Manufacture of Medicines*, ed. K. Taylor, Amsterdam, Elsevier, pp. 567-582.

Reddy, B.S., Hirose, Y., Lubet, R., Steele, V., Kelloff, G., Paulson, S., Seibert, K. & Rao, C.V. 2000, "Chemoprevention of colon cancer by specific cyclooxygenase-2 inhibitor, celecoxib, administered during different stages of carcinogenesis", *Cancer Research*, vol. 60, no. 2, pp. 293-297.

Risio, M. 2010, "The natural history of adenomas", *Best Practice & Research.Clinical Gastroenterology*, vol. 24, no. 3, pp. 271-280.

Ruder, E.H., Laiyemo, A.O., Graubard, B.I., Hollenbeck, A.R., Schatzkin, A. & Cross, A.J. 2011, "Non-steroidal anti-inflammatory drugs and colorectal cancer risk in a large, prospective cohort", *The American Journal of Gastroenterology*, vol. 106, no. 7, pp. 1340-1350.

Sacchetti, A. 2013, "Cancer cell killing by Celecoxib: reality or just in vitro precipitation-related artifact?", *Journal of Cellular Biochemistry*, vol. 114, no. 6, pp. 1434-1444.

Sade, A., Tuncay, S., Cimen, I., Severcan, F. & Banerjee, S. 2012, "Celecoxib reduces fluidity and decreases metastatic potential of colon cancer cell lines irrespective of COX-2 expression" *Bioscience Reports*, vol. 32, no. 1, pp. 35-44.

Scarpignato, C. & Hunt, R.H. 2010, "Nonsteroidal antiinflammatory drug-related injury to the gastrointestinal tract: clinical picture, pathogenesis, and prevention", *Gastroenterology Clinics of North America*, vol. 39, no. 3, pp. 433-464.

Schatzkin, A., Freedman, L.S., Dawsey, S.M. & Lanza, E. 1994, "Interpreting precursor studies: what polyp trials tell us about large-bowel cancer", *Journal of the National Cancer Institute*,vol. 86, no. 14, pp. 1053-1057.

Sharma, A. & Chaurasia, S. 2013, "Multiparticulate drug delivery system: pelletization through extrusion and spheronization", *IRJP*, vol. 4, no. 2, pp. 6-9.

Siegel, R., DeSantis, C. & Jemal, A. 2014, "Colorectal cancer statistics, 2014", *CA: A Cancer Journal for Clinicians*, vol. 64, no. 2, pp. 104-117.

Singh, S., Baghel, R.S. & Yadav, L. 2011, "A review on solid dispersion", *IJPLS*, vol. 2, no. 9, pp. 1078-1095.

Silverstein, F.E., Faich, G., Goldstein, J.L., Simon, L.S., Pincus, T., Whelton, A., Makuch, R., Eisen, G., Agrawal, N.M., Stenson, W.F., Burr, A.M., Zhao, W.W., Kent, J.D., Lefkowith, J.B., Verburg, K.M. & Geis, G.S. 2000, "Gastrointestinal toxicity with celecoxib vs nonsteroidal anti-inflammatory drugs for osteoarthritis and rheumatoid arthritis: the CLASS study: A randomized controlled trial. Celecoxib Long-term Arthritis Safety Study", *JAMA*, vol. 284, no. 10, pp. 1247-1255.

Solomon, S.D., McMurray, J.J., Pfeffer, M.A., Wittes, J., Fowler, R., Finn, P., Anderson, W.F., Zauber, A., Hawk, E., Bertagnolli, M. & Adenoma Prevention with Celecoxib (APC) Study Investigators 2005, "Cardiovascular risk associated with celecoxib in a clinical trial for colorectal adenoma prevention", *The New England Journal of Medicine*, vol. 352, no. 11, pp. 1071-1080.

Solomon, S.D., Pfeffer, M.A., McMurray, J.J., Fowler, R., Finn, P., Levin, B., Eagle, C., Hawk, E., Lechuga, M., Zauber, A.G., Bertagnolli, M.M., Arber, N., Wittes, J. & APC and PreSAP Trial Investigators 2006, "Effect of celecoxib on cardiovascular events and blood pressure in two trials for the prevention of colorectal adenomas", *Circulation,* vol. 114, no. 10, pp. 1028-1035.

Sostres, C., Gargallo, C.J., Arroyo, M.T. & Lanas, A. 2010, "Adverse effects of non-steroidal anti-inflammatory drugs (NSAIDs, aspirin and coxibs) on upper gastrointestinal tract", *Best Practice & Research.Clinical Gastroenterology,* vol. 24, no. 2, pp. 121-132.

Steinbach, G., Lynch, P.M., Phillips, R.K., Wallace, M.H., Hawk, E., Gordon, G.B., Wakabayashi, N., Saunders, B., Shen, Y., Fujimura, T., Su, L.K., Levin, B., Godio, L., Patterson, S., Rodriguez-Bigas, M.A., Jester, S.L., King, K.L., Schumacher, M., Abbruzzese, J., DuBois, R.N., Hittelman, W.N., Zimmerman, S., Sherman, J.W. & Kelloff, G. 2000, "The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis",*The New England Journal of Medicine,* vol. 342, no. 26, pp. 1946-1952.

Stewart, S.L., Wike, J.M., Kato, I., Lewis, D.R. & Michaud, F. 2006, "A population-based study of colorectal cancer histology in the United States, 1998-2001", *Cancer,* vol. 107, no. 5 Suppl, pp. 1128-1141.

Tanaka, T. 2012, "Development of an inflammation-associated colorectal cancer model and its application for research on carcinogenesis and chemoprevention", *International Journal of Inflammation,* vol. 2012, pp. 658786.

Tanaka, T., Kohno, H., Suzuki, R., Yamada, Y., Sugie, S. & Mori, H. 2003, "A novel inflammation-related mouse colon carcinogenesis model induced by azoxymethane and dextran sodium sulfate", *Cancer Science,* vol. 94, no. 11, pp. 965-973.

Terzic, J., Grivennikov, S., Karin, E. & Karin, M. 2010, "Inflammation and colon cancer", *Gastroenterology,* vol. 138, no. 6, pp. 2101-2114.e5.

Tomisato, W., Tsutsumi, S., Hoshino, T., Hwang, H.J., Mio, M., Tsuchiya, T. & Mizushima, T. 2004, "Role of direct cytotoxic effects of NSAIDs in the induction of gastric lesions", *Biochemical Pharmacology*, vol. 67, no. 3, pp. 575-585.

Tsujii, M. & DuBois, R.N. 1995, "Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2", *Cell*, vol. 83, no. 3, pp. 493-501.

van Hogezand, R.A., Eichhorn, R.F., Choudry, A., Veenendaal, R.A. & Lamers, C.B. 2002, "Malignancies in inflammatory bowel disease: fact or fiction?", *Scandinavian Journal of Gastroenterology.Supplement*, vol. (236), no. 236, pp. 48-53.

Venkatesan, P., Puvvada, N., Dash, R., Prashanth Kumar, B.N., Sarkar, D., Azab, B., Pathak, A., Kundu, S.C., Fisher, P.B. & Mandal, M. 2011, "The potential of celecoxib-loaded hydroxyapatite-chitosan nanocomposite for the treatment of colon cancer", *Biomaterials*, vol. 32, no. 15, pp. 3794-3806.

Wallace, J. L., 2000, “How do NSAIDs cause ulcer cancer”, *Baillieres’s Clinical Gastroenterology*, vol. 14, no. 1, pp. 147-159.

Wang, D. & DuBois, R.N. 2010, "The role of COX-2 in intestinal inflammation and colorectal cancer", *Oncogene*, vol. 29, no. 6, pp. 781-788.

Celebrex is approved for polyp reduction in FAP patients, 2000.
Available: http://www.cancernetwork.com/articles/celebrex-approved-polyp-reduction-fap-patients [Oct/03, 2014].

EMA Onsenal® Withdrawal Notification, 2011.
Available:http://www.ema.europa.eu/docs/en_GB/document_library/Public_statement/2011/04/WC500104950.pdf [2014, Oct/03].

EMA Onsenal® Approval Report, 2003.
Available: http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Summary_for_the_public/human/000466/WC500044631.pdfOct/03].

EMA scientific discussion for the approval of Onsenal®, 2004.
Available: http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Scientific_Discussion/human/000466/WC500044630.pdf [2014, Oct/03].

FDA Oncologic Drugs Advisory Committee Briefing Document, 2005.
Available: http://www.fda.gov/ohrms/dockets/ac/05/briefing/2005-4191B1_06_Pfizer-Celebrex.pdf [2013, Oct/03].

FDA Labelling Revision for Celebrex® Capsules, 2008. Available:
http://www.accessdata.fda.gov/drugsatfda_docs/label/2008/020998s026lbl.pdf [2013, Oct/03].

Johns Hopkins Medicine Colorectal Cancer. Available:
http://www.hopkinscoloncancercenter.org/ [2014, Oct/24].

MHRA Safety Advisory Notice, 2011.
Available:
http://www.mhra.gov.uk/Safetyinformation/DrugSafetyUpdate/CON125965 [2013, Oct/03].

Non-Steroidal Anti-inflammatory Drugs (NSAIDS) & COX-2 Inhibitors, 2014.
Available:http://www.marceldumont.com/reels/interactive/Pain_Mngmt_1/Nsaids_Cox-2_Inhibitors.html [2014, Oct/03].

NSAIDs – Types, Uses and Contraindications, 2014. Available:
http://medicapharm.com [2014, Oct/03]

Pfizer Important Safety Information for Celebrex® 2014.
Available: http://www.celebrex.com/isi.aspx [2013, Dec/12].

Pfzier Celebrex Monograpgh, 2014.
Available: http://www.pfizer.ca/en/our_products/products/monograph/125 [2014, Oct/03].

Pfizer, Inc.; Withdrawal of Approval of Familial Adenomatous Polyposis Indication for Celebrex, 2012.
Available: https://www.federalregister.gov/articles/2012/06/08/2012-13900/pfizer-inc-withdrawal-of-approval-of-familial-adenomatous-polyposis-indication-for-celebrex [2014, Oct/03].

Sigma Aldrich Celecoxib Product Page.
Available:http://www.sigmaaldrich.com/catalog/product/sigma/pz0008?lang=en&region=IE [2014, Oct/02].

CHAPTER 2
Pre-formulation

Publication Status: Elements of this work, in addition to data from chapter 4, have been published in the following article: McDonald, B.F., Coulter I.S., and Marison, I.W. Microbeads: A novel multiparticulate drug delivery technology for increasing the solubility and dissolution of celecoxib. *Pharm Dev Technol* 2013 Nov 27. [epub ahead of print].

2.1 Abstract

The purpose of this phase of the project was to develop a lipid-based celecoxib (CLX) liquid formulation which would act as a precursor for the development of a suitable oral drug delivery formulation designed to deliver pre-solubilised CLX to the gastrointestinal (GI) tract and more specifically to the colon for the treatment of colorectal cancer (CRC). The solubility of CLX in a range of lipids, surfactants and cosolvents was evaluated. CLX was solubilised in mixtures of these vehicles to produce liquid formulations. The *in-vitro* release of these liquid formulations was assessed and compared to the marketed CLX product Celebrex® with optimised liquid CLX formulations demonstrating a greater release performance to Celebrex®. A successful feasibility study was performed in which the potential for converting liquid CLX formulations into CLX microbeads was assessed.

2.2 Introduction

2.2.1 Background

The challenges of orally delivering poorly soluble drugs such as CLX to the colon for the treatment and prevention of CRC were outlined in Chapter 1, which concluded that a multiparticulate lipid based drug delivery system (LBDDS) in which the drug is delivered in a pre-solubilized form was the optimal formulation type for CLX for this indication. Over the past several decades, LBDDSs (emulsions, microemulsions, mixed micelles etc.) have been explored for resolving a variety of drug delivery challenges (Cannon and Long, 2008, Kalepu *et al*., 2013). Most frequently LBDDSs for oral use are designed to present a poorly soluble drug in a solubilized format to eliminate dissolution of crystalline material as the rate limiting step of absorption (Pouton, 2000).

For this project the intention was to develop a lipid based formulation in which the dissolution of CLX in the GI tract would be eliminated as a rate limiting step, thereby allowing for the drug to be delivered to colonic tissue in a free molecular form.

2.2.2 Lipid based formulations

Lipids are one of the most versatile excipient classes available to formulation scientists for improving the solubility of poorly water soluble drugs. The formulation options include lipid suspensions, solutions, emulsions, microemulsions, mixed micelles, SEDDs (self-emulsifying drug delivery systems), SMEDDS (self-microemulsifying drug delivery systems), thermo-softening matrices and liposomes. When designing one of these lipid based drug delivery systems there are hundreds of potential excipients from which to choose, however despite the number of possibilities there are only a relatively small subset of lipids which have found application in clinical development due to a lack of regulatory approval (Gibson, 2007). Typically lipid classes used for pharmaceutical applications include the following; a) fatty acids, b) natural oils/fats, c) semi-synthetic mono-, di- and triglycerides, d) mixtures of glycerides/glyceride polyethylene glycol (PEG) derivatives and fatty acid esters of PEG and e) polyglyceryl fatty acid esters. A brief background to each of these lipid classes is included here. Surfactants and co-solvents play a key role in lipid formulations and are also described. Finally, a brief overview of the most common lipid based formulations is provided.

2.2.2.1 Lipids

A. Fatty acids

Fatty acids are monocarboxylic acid derivatives of saturated or unsaturated (carbon-carbon double bond) aliphatic hydrocarbons. Saturated fatty acids with eight or fewer carbons are flowable liquids at room temperature whilst those fatty acids of 10 or more carbons in chain length are semi-solid at room temperature and possess melting points that increase in proportion to the hydrocarbon chain length but which decrease with increasing degree of unsaturation. Fatty acids find pharmaceutical application primarily as solubilizing vehicles for poorly water soluble drugs (Gibson, 2007 and Saxena *et al*., 2013). The structure of oleic acid is provided in Figure 2.1 below as an example of a fatty acid. The abbreviated name for oleic acid is C18:1, which describes a fatty acid of 18 carbons containing one carbon-carbon double bond.

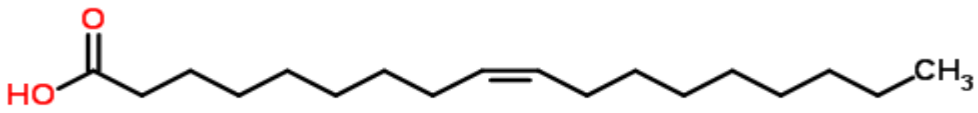

Figure 2.1 Structure of oleic acid (C18:1) (Adapted from Chemspider oleic acid technical information page, 2014)

B. Natural oils and fats

Naturally occurring oils and fats are comprised of mixtures of various triglycerides (TG) and are more commonly (but rarely) referred to as triacylglycerols since chemically they are fatty acid tri-esters of glycerol. Naturally occurring triglycerides contain fatty acids of varying chain lengths and degrees of unsaturation. Based on the length of their

component fatty acids, triglycerides can be classified as short (< 5 carbons), medium (6-12 carbons) or long chain (> 12 carbons) (Gibson, 2007 and Boyd *et al*., 2011). The structure of a glyceryl trioleate (a major component of olive oil) is provided in Figure 2.2 below as an example of a long chain triglyceride.

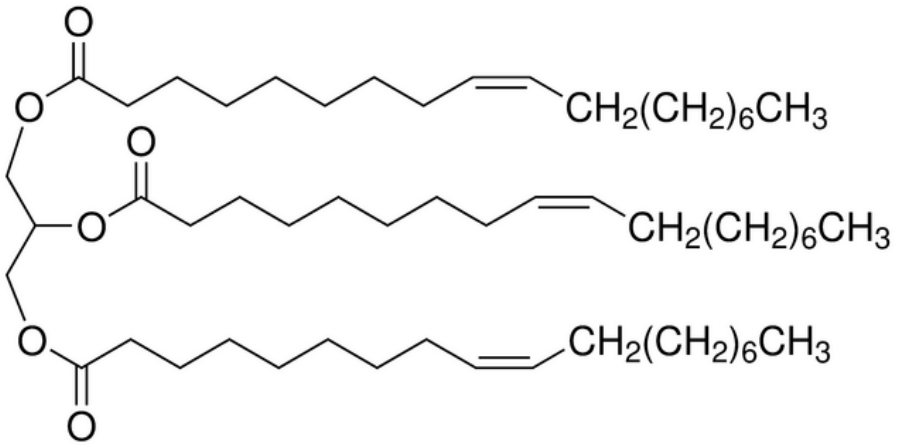

Figure 2.2 Structure of a glyceryl trioleate (Adapted from Sigma Aldrich glyceryl trioleate product page 2014).

C. Semi-synthetic mono-, di-, and triglcerides

In addition to naturally occurring triglycerides, there also exists a wide range of semi-synthetic mono-, di- and triglycerides which contain one, two and three fatty ester groups respectively. The primary advantage of semi-synthetic glycerides compared to naturally occurring glycerides is that they offer more uniform compositions with respect to fatty acid content, however it should be acknowledged that semi-synthetic glycerides still exhibit a certain amount of compositional variability (e.g., with respect to fatty acid content and the position of fatty acids on the glycerol backbone). The compositional variability of semi-synthetic glycerides can vary depending on the excipient brand and from batch to batch, therefore careful consideration should be employed as part of the selection process. In Table 2.1, the composition of Miglyol® 810N is shown as an example of a semi-synthetic medium chain triglyceride (Gibson, 2007).

Table 2.1 Composition of Miglyol® 810N (Gibson, 2007).

Fatty Acid	Composition (%)
Caproic (C6)	≤ 2
Caprylic (C8)	65-80
Capric (10)	20-35
Lauric (C12)	≤ 2
Myristic (C14)	≤ 1

D. Mixtures of glycerides/glyceride PEG derivatives and fatty acid esters of PEG

Another common class of lipid excipients are mixtures of mono-, di- and triglycerides/ glyceride PEG derivatives and fatty acid esters of PEG. These excipients are commonly used as solubilising vehicles, surfactants, wetting agents and as emulsifiers in SEDDS/SMEDDS. Some typical examples of this excipient class are detailed in Table 2.2 below. These excipients range from being highly lipophilic (e.g., Labrafil® M 1944 CS – HLB of 3–4) to being water soluble (e.g., Cremophor® RH-40 – HLB of 14–16). See Section 2.2.2.2 for further details on hydrophile-lipophile balance (HLB).

Table 2.2 Examples of mixtures of glycerides/glyceride PEG derivatives and fatty acid esters of PEG

Chemical name	Composition	Trade name
PEG-32 glyceryl laurate	Mono-, di-, and trilauric (C12:0) acid esters of glycerol plus mono- and difatty acid esters of PEG 1500	Gelucire® 44/14
PEG-32 glyceryl palmitosterate	Mono-, di-, and tripalmitic acid (C16:0) and steraic acid (C18:0) esters of glycerol plus mono- and difatty acid esters of PEG 1500	Gelucire® 50/13
PEG-8 glyceryl caprylate/caprate	Mono-, di-, and tricaprylic acid (C8:0) and capric acid (C10:0) esters of glycerol plus mono- and difatty acid esters of PEG 1500	Labrasol®
PEG-6 glyceryl oleate	Mono-, di- and trioleic acid (C18:1) esters of glycerol and mono and diesters of PEG 300	Labrafil® M 1944 CS
PEG-6 glyceryl linoleate	Mono-, di- and trilinoleic acid (C18:2) esters of glycerol and mono and diesters of PEG 300	Labrafil® M 2125 CS
PEG-35 castor oil	Mixtures of glyceryl PEG ricinolate with fatty acid esters of PEG, free PEGs and ethoxylated glycerol	Cremophor® EL
PEG-40 hydrogenated castor oil	Hydrogenated glyceyl PEG ricinoleate with 40 moles of ethylene oxide per mole of castor oil	Cremophor® RH-40

E. Polyglyceryl fatty acid esters

The final lipid classification that was considered as part of this project was polyglyceryl fatty acid esters. Polyglyceryl fatty acid esters are composed of a chain of glycerol molecules linked together by ether linkages, which are esterified with one or more fatty acid molecules. Similar to the previous category described, these excipients find application as solubilising vehicles and surfactants and also as crystallisation inhibitors. An example of a polyglyceryl fatty acid ester is Plurol® Oleique which is an octasteraic acid (18:0) ester of a 6 glycerol unit chain (Gibson, 2007).

F. Propylene glycol fatty acid esters

Propylene glycol fatty acid esters are another group of substances closely related to glycerides but which do not fit into the classical lipid classifications described in Section 2.2.2. They are chemically derived from propylene glycol and fatty acids. Although they have surfactant properties, they are most commonly employed as solubilisers. Some typical examples of this excipient class are detailed in Table 2.3 below.

Table 2.3 Examples of propylene glycol fatty acid esters.

Chemical name	Trade name(s)
Propylene glycol monolaurate	Lauroglycol ® FCC
	Capmul® PG-12
	Lauroglycol 90®
Propylene glycol dicaprylate/dicaprate	Labrafac® PG
Propylene glycol monocaprylate	Capryol® PGMC
	Capryol® 90

2.2.2.2 Surfactants

Lipid based formulations often contain surfactants to facilitate dispersion of the drug and formulation components after ingestion. Conventional or traditional surfactants are made up of distinct regions; a hydrophobic portion referred to as the 'tail' and a hydrophilic 'head' group. The hydrophobic portion of these surfactants is commonly made up of straight- or branched- hydrocarbon chains, which may include aromatic moieties. The hydrocarbon tails are normally flexible, and when aggregated present a fluid hydrocarbon environment for the solubilisation of hydrophobic materials Surfactant structures other than the conventional head/tail arrangement also exist, for example, bile salts have a less common planar structure where the steroidal backbone presents a hydrophobic face and the reverse face is hydrophilic (Liu *et al.*, 2008).

Surfactants are often classified according to their hydrophile-lipophile balance (HLB) number. The HLB balance of a surfactant is a measure of the degree to which it is hydrophilic or lipophilic. Surfactants with low HLB values (1–9) are more lipophilic and tend to be lipid soluble, whereas those with high HLB values (>10) are more hydrophilic and often form transparent micellar solutions when added to water. A blend of low and high HLB surfactants are often used in the preparation of oil-in-water emulsions to ensure a maximum resistance to phase separation and a high degree of dispersion (Gibson, 2007 and Cannon and Long, 2008).

In the case of traditional surfactants, the chemical nature (i.e., the charge) of the hydrophilic head group is also used to classify surfactants into four distinct groups; nonionic (no charge), anionic (negatively charged), cationic (positively charged) and zwitterionic (negatively and positively charged – pH dependent).

Nonionic surfactants are the most widely used surfactants in pharmaceutical systems due to their low toxicity and excipient compatibility and were therefore the focus with respect to surfactants in this project. The head groups of nonionic surfactant molecules contain no charged moieties, with their hydrophilc properties attributed to the presence of hydroxyl groups. All of the lipids listed in Section 2.2.2.1 D with the exception of Labrafil® M 1944 CS and Labrafil® M 2125 CS are classified as nonionic surfactants. Other nonionic surfactants include the following categories; sorbitan fatty acid esters, PEG-ylated sorbitan fatty acid esters, PEG fatty acid esters and vitamin E PEG esters. The most common nonionic surfactants used in the pharmaceutical industry (including their HLB values) are detailed in Table 2.4 below. The non-ionic surfactants from section 2.2.2.1 D are also included. Figure 2.3 below shows the structure of Solutol® HS-15, a PEG fatty acid ester. Solutol® HS-15 (also referred to as Kolliphor® HS-15) is a non-ionic solubiliser and surfactant obtained by reacting 15 moles of ethylene oxide with 1 mole of 12-hydroxy stearic acid. The resulting product consists of PEG mono- and di-esters of 12 hydroxystearic (primary lipophilic component) and of about 30% of free PEG. Solutol® HS-15 is a yellowish white paste at room temperature that becomes liquid at approximately 30ºC. It is soluble in water and its critical micelle concentration lies between 0.005 and 0.02% (BASF Solutol®HS-15 technical information sheet, 2012).

Table 2.4 Examples of nonioinc surfactants.

Classification	Chemical Name	HLB	Trade name
Sorbitan fatty acid esters	Sorbitan monooleate	4	Span® 80
	Sorbitan trioleate	2	Span® 85
	Sorbitan monolaurate	8	Span® 20
PEG-ylated sorbitan fatty acid esters	Polyoxyethylene sorbitan monolaurate	17	Tween® 20
	Polyoxyethylene sorbitan monopalmitate	16	Tween® 40
	Polyoxyethylene sorbitan monosterate	15	Tween® 60
	Polyoxyethylene sorbitan monooleate	15	Tween® 80
Polyethylene glycol fatty acid esters	Polyethylene glycol-15-hydroxystearate	15	Solutol® HS-15
Vitamin E PEG esters	Tocopherol PEG succinate	13	Vitamin E TPGS
Mixtures of glycerides/ glyceride PEG derivatives and fatty acid esters of PEG	PEG-32 glyceryl laurate	14	Gelucire® 44/14
	PEG-32 glyceryl palmitosterate	13	Gelucire® 50/13
	PEG-8 glyceryl caprylate/caprate	14	Labrasol®
	PEG-35 castor oil	13	Cremophor® EL
	PEG-40 hydrogenated castor oil	15	Cremophor® RH-40

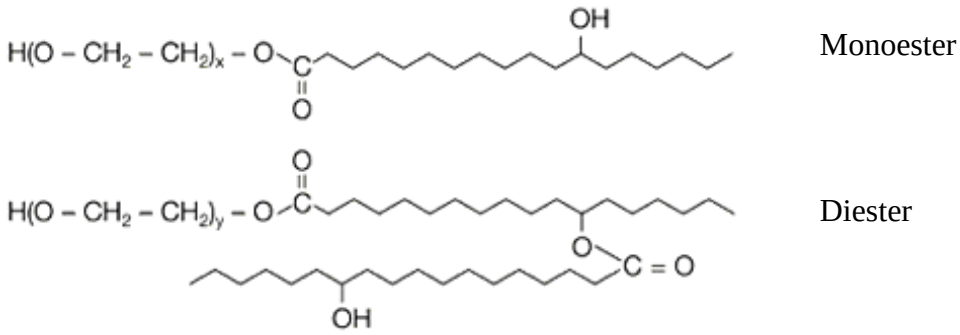

Figure 2.3 Structure of primary lipophilic component of Solutol® HS-15 (Adapted from BASF Solutol®HS-15 technical information sheet, 2012).

2.2.2.3 Cosolvents

Hydrophilic cosolvents may be used in lipid-based formulations to improve drug solubilisation. A second advantage of cosolvents is their ability to aid dispersion of lipid formulations by facilitating water ingress into the formulation. When cosolvents are employed on their own (i.e., single component systems), drug precipitation is likely due to rapid dissipation of the cosolvent, however when formulated with lipids, the lipids remain post cosolvent dissipation thereby preventing precipitation (Cannon and Long, 2008). The amount of cosolvent employed in a formulation is generally limited by its compatibility with other formulation excipients (e.g., gelatine in the case of soft gelatine capsules). Table 2.5 below lists some common cosolvents used in the pharmaceutical industry.

Table 2.5 Examples of cosolvents.

Chemical Name	Abbreviated or Trade Name
Ethanol	EtOH
Dimethyl Acetamide	DMA
Polyethylene Glycol 300	PEG 300
Polyethylene Glycol 400	PEG 400
Polyethylene Glycol 200	PEG 200
Polyethylene Glycol 1000	PEG 1000
Polyethylene Glycol 4000	PEG 4000
Diethylene glycol monoethyl ether	Transcutol® P

2.2.2.4 Lipid formulations

Of the various lipid formulations listed in Section 2.2.2, emulsions/SEDDS, microemulsions/SMEDDS and micelle formulations are among the most common formulation approaches (Hauss, 2007 and Lui, 2008). A brief description of these formulation types is provided below.

A. SEDDS and SMEDDS

SEDDS are drug delivery systems consisting of drug, oils and surfactants and may also include cosolvents. On addition to water (e.g., in the GI tract) and with gentle agitation, the system will form an emulsion (i.e., a dispersion of droplets of one liquid in another immiscible liquid (e.g., oil in water)) (Grove and Mullertz, 2007 and Cannon and Long, 2008). SEDDS are more practical for oral applications than ready-to-use emulsions (i.e., those that contain water) due to volume considerations, easier formulation into dosage

forms such as soft gelatine capsules and also enhanced chemical stability (due to the absence of water in the formulation) (Date *et al*., 2010). SEDDS are essentially emulsion preconcentrates. SMEDDS are those self-emulsifying systems that form microemulsions on addition to water and gentle agitation. The term microemulsion implies an emulsion with a very fine droplet size, however microemulsions are not actually emulsions (Cannon and Long, 2008). Microemulsions form spontaneously on mixing with little or no mechanical energy whereas for emulsions homogenisation is of critical importance. In comparison to emulsions, microemulsions have a much smaller droplet size (6-80 nm) and are visually transparent or translucent, whereas the drops of the dispersed phase in an emulsion are generally large (> 0.1 µm) so that they often take on a milky, rather than a translucent appearance (Cannon and Long, 2008). In contrast to emulsions, microemulsions are thermodynamically metastable. In emulsions the average drop size grows continuously with time so that phase separation ultimately occurs under gravitational force (i.e., they are thermodynamically unstable and their formation requires input of energy as described above). The marketed cyclosporine formulations Sandimmune® (SEDDS) and Neoral® (SMEEDS) are examples of formulations which yield emulsions and microemsulions respectively on dilution with the aqueous environment of the GI tract (Narang *et al*., 2007).

B. Micelles

The ability of surfactants to enhance the solubility of poorly water soluble compounds in an aqueous environment is widely known and is used in many aspects of drug formulation development (Liu *et al*., 2008). This enhancement of the aqueous solubility by surfactants occurs as a result of the dual nature of surfactant molecules (i.e., their discrete hydrophobic and hydrophilic regions). The term surfactant describes a surface-

active agent. The hydrophobic (nonpolar) and hydrophilic regions (polar) of these surface-active agents allow them to orientate at polar-nonpolar interfaces (e.g., water/air or water/oil). Once the interface is saturated, the surfactants then self-associate to form micelles and other aggregates, whereby their hydrophobic regions are minimized and shielded from aqueous contact by their hydrophilic regions. This creates a discrete hydrophobic environment suitable for solubilisation of many hydrophobic compounds (Lui *et al.,* 2008). Alternatively, the poorly aqueous soluble drug may be solubilized within the head group layer at the surface or in the palisade portion of the micelle (the portion between the surface and the core) (Rangel-Yagui *et al*., 2005). In the case of dilute surfactant solutions, the polar head groups of the surfactant molecules are generally arranged in an outer spherical shell, while the hydrocarbon chains are orientated towards the center forming a spherical micelle (Figure 2.4 A) which are usually between 3–50 nm in size (Paul and Prud'homme, 2001). Depending on the type of surfactant employed and the solution conditions, the aggregates may form structures other than micelles such as lamellar bilayers (Figure 2.4 B) or spherical bilayers (vesicles) containing an encapsulated aqueous phase (Figure 2.4 C). As micelles become larger they have been shown to become more asymmetric with their shape changing from spherical to cylindrical and lamellar. As stated above, surfactants self-associate in aqueous media to minimize the area of contact between their hydrophobic tails and the aqueous solution. The concentration of surfactant at which this phenomenon occurs is referred to as the critical micelle concentration (CMC) (Liu *et al,* 2008). At surfactant concentrations below the CMC, the surfactant molecules exist predominantly as monomeric units (no micelles) whereas at surfactant concentrations above the CMC, micelles exist.

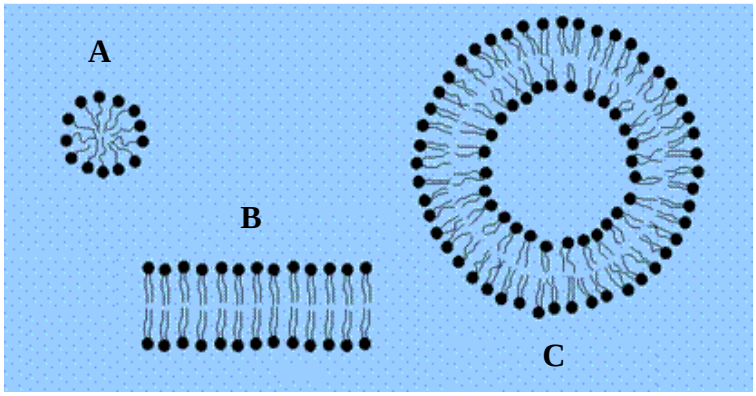

Figure 2.4 Structure of surfactant aggregates: A) spherical micelle, B) lamellar bilayer and C) spherical bilayer (vesicle) (Adapted from Mc Clements, 2007).

Surfactant mixtures (forming mixed micelles) are commonly used in pharmaceutical applications as they often perform better than a single surfactant system. In addition to enhancing drug solubilisation, the use of mixed micelles can result in a synergy whereby the total concentration of required surfactant required is reduced (Liu *et al*. 2008).

As with SEDDS and SMEDDS, micelle formulations are usually formulated in the absence of water (i.e., they are micelle pre-concentrates). Due to incompatibility with encapsulation materials (e.g., gelatine) in current technologies, micelle pre-concentrates are less common than SEDDS and SMEDDS however given the number of components in SEDDS and SMEDDS, it is expected that several dispersion mechanisms are operating in parallel in these type of formulations (including the presence of micelles and mixed micelles) (Narang *et al*., 2007 and Grove and Mullertz, 2007). Examples of LBDDS which are predominantly surfactant based and thereby could be referred to as micelle pre-concentrates are a) the amprenavir formulation Agenerase®, b) the cyclosporine formulation Gengraf®, c) the fenofibrate formulation Fenogal® and d) the ibuprofen formulation Solufen®-Gé (Liu *et al.*, 2008 and Strickley, 2007).

2.2.3 Characterization of release from LBBDS (release testing)

The characterisation of the release of an API (active pharmaceutical ingredient) is usually performed for a number of reasons; a) to compare formulation candidates during the development of a drug, b) to predict the performance of a formulation *in-vivo* and c) to act as a quality control (QC) tool (to distinguish between different batches, to assess formulation changes, to assess product quality during a products shelf life etc.). Characterisation of release from LBDDS is performed via *in-vitro* release testing. The primary focus of *in-vitro* release testing in the context of this stage of the project was to compare formulation candidates and to a lesser extent their potential *in-vivo* performance. LBDDS present special challenges in the design of release tests (Dressman, *et al*., 2007). Unlike the majority of other oral dosage forms in which the API is present in a solid format (e.g., tablets and granules) and which therefore undergo dissolution testing the drug in LBDDS is usually pre-dissolved, therefore despite the fact that the same medthodies are employed (i.e, use of dissolution test apparatus, dissolution media etc.), the use of standard dissolution terminology is not appropriate to describe the results of these experiments. In the cases of LBDDS, these tests are performed as a measure of how well the drug disperses or releases into the chosen media rather than a measure of how the drug dissolves[1]. When designing a release experiment for the testing of lipophilic dosage forms, the contents of the dissolution medium are important when trying to mimic *in-vivo* conditions. For example, in the GI tract, dispersion of the formulation will occur via emulsification in the stomach and small intestine (Dressman *et al*., 2007) and therefore it is important to mimic the

[1] Given that the methodologies involved include the use of standard dissolution apparatuses, dissolution media etc., the term ‘dissolution’ will be used to describe the methodologies where appropriate, however the term ‘release’ will be used to describe the release/dispersal of drug from the dosage various forms into the media.

components of the intestinal fluids to simulate this emulsification process (i.e., the use of biorelevant dissolution media). An alternative approach to the release testing of lipid based dosage forms and the approach that was adopted here is to test the lipophilic dosage forms in simple aqueous media such as Purified Water (PW), Simulated Gastric Fluid (SGF – pH 1.2) and Simulated Intestinal Fluid (SIF – pH 6.8) on the basis that the formulation should be as robust as possible with respect to the physiology of the GI tract (i.e., not reliant on GI conditions).

2.2.4 Objectives

Bioavailability studies have shown CLX to be a poorly soluble, highly permeable drug, (i.e., class II of the Biopharmaceutical Classification System), in which the bioavailability of the drug is limited by its poor solubility (Paulson *et al.*, 2001). This poor aqueous solubility and consequent poor dissolution in gastric fluids is considered to be a major drawback of celecoxib therapy (Rawat and Jain, 2004). The poor solubility of the drug reduces its effectiveness in treating the diseases for which it is indicated. In order to counteract this poor bioavailability the drug is administered in high doses, however these high doses result in a greater incidence of severe adverse side effects (FDA labelling revision for Celebrex®, 2008). It is also postulated that the effectiveness of CLX in the prevention and treatment of CRC is prohibited by the presentation of the current dosage form (powder filled capsule) which is not amenable to widespread interaction with colonic tissue in a free molecular form.

The primary goal of this phase of the project was to develop a formulation for improving the solubility of CLX using a combination of excipients that were suitable for oral administration and ultimately the incorporation of the resultant formulations

into a suitable drug delivery technology for oral delivery. The primary aims/objectives of this stage of the project to meet the stated goal were as follows:

A. The first objective of the study was to screen a range of vehicles (lipids, surfactants and co-solvents) and identify those in which CLX was readily soluble. It was proposed that by employing lipids and surfactants in the formulation, that the solubility of CLX would be enhanced, thereby increasing its oral effectiveness in the local treatment of CRC.

B. The second objective was to use data collected from the initial screening studies to form the basis for the design of liquid formulations. The drug release performance (*in-vitro* release testing) of these liquid formulations would then be compared to the currently marketed CLX product Celebrex®. The drug release performance of the liquid formulations would also be compared to each other to assess the impact of changing excipient types and ratios.

C. The third objective was to perform an initial feasibility trial on selected liquid formulations to assess if the liquid formulations selected were amenable to incorporation into microbeads (Refer to Chapter 4 for development and optimisation of microbeads).

D. The fourth and final objective was to select optimal liquid formulations for progression to a CRC cell culture study (Refer to Chapter 3).

2.3 Material and methods

2.3.1 Materials

Solubilisation studies were performed using a wide range of vehicles consisting of lipids, surfactants and cosolvents. The vehicles used here were as follows; corn oil, soybean oil, olive oil, oleic acid, linoleic acid, stearaic acid, Span® 80, Span® 85, Span® 20, (all Sigma Aldrich, USA), Incromega® TG3322 (Croda, UK) Mineral Oil, Tween®

20, Tween® 40, Tween® 60, Tween® 80, EtOH, DMA, PEG 300, PEG 400, PEG 200 (Merck, Germany), Miglyol® 810, Miglyol® 812, Miglyol® 829 (all Sasol, South Africa), Labrafrac®, Lipophile WL1349, Capryol® PGMC, Capryol® 90, Lauroglycol® FCC, Lauroglycol® 90, Labrafac® PG, Plurol Oleique® CC497, Gelucire® 44/14 Gelucire® 50/13, Transcutol® P, Labrafil® M2125, Labrafil® M1944 CS, Gelucire® 33/01, Labrasol®, Maisine® 35-1, Peceol (all Gattefosse, France), Captex® 300, Capmul® MCM, Capmul® PG-12 (all Abitec, USA), Imwitor® 308, Imwitor® 742 (both Cremer, Germany), Cremophor® RH40, Cremophor® EL, Solutol® HS-15 (all BASF, Germany) and Vitamin E TPGS (Eastman, USA). Microbeads were prepared using these vehicles in combination with porcine gelatin (Nitta Gelatin, Japan) and sorbitol (Neosorb®) (Roquette, Franace). A sample of CLX API was kindly provided by Erregierre (Italy). The purity of the API was 99.6% based on the COA provided by the supplier. All chemicals used for the release experiments, HPLC and UV testing were of laboratory grade.

2.3.2 Methods

2.3.2.1 Solubility measurements

CLX was added to measured quantities of a range of lipids, surfactants and cosolvents in glass vials (minimum of n =2 measurements). These mixtures were stirred at room temperature (20°C) (except in the case of excipients which were solid at room temperature, in which case the solubilisation measurements were performed at elevated temperatures) using a magnetic stirrer. Additional amounts of CLX were added to samples and were allowed to stir for periods of five min prior to observations being made.The solubility of CLX in the liquid vehicles was recorded as the range between which the samples transgressed from transparent to cloudy.

2.3.2.2 CLX liquid formulations

CLX liquid formulations were prepared by dissolving measured quantities of CLX into measured quantities of liquids. Formulations were prepared as one, two or three component systems (i.e., containing a single liquid vehicle or mixtures of liquid vehicles).

2.3.2.3 *In-vitro* release testing

Release testing of CLX API was performed at 37°C in SIF (pH 6.8), SGF (pH 1.2) and PW (pH 7) to determine whether pH had an impact on the dissolution of CLX. The protocols for SIF and SGF were taken from the United States Pharmacopoeia (United States Pharmacopeia, 2010). A high throughput drug release screening study (*n=1*) was then performed on CLX liquid formulations at 37°C in PW. For optimal liquid formulations, these release experiments were repeated (*n=3*). All release experiments were performed out using either a Varian/Vankel VK7010 dissolution apparatus (VanKel, USA) or a Distek Evolution 6300 (Distek, USA) equipped with standard glass vessels and USP type II paddles. Paddle rotating speed in all experiments was 75 rpm. Formulations containing 50 mg of dissolved CLX were weighed and added to 1000 mL of the relevant dissolution medium (SIF, SGF or PW). At specified times 1.8 mL samples were withdrawn, filtered through a 70 µm pore filter (QLA, USA) and analysed using either a high performance liquid chromatography (HPLC) method or an ultraviolet (UV) spectrophotometric method analysis. The % of drug released at particular time points was determined from peak areas which were calculated against a

single point external reference standard in the case of the HPLC method, whereas a standard curve was used for the UV method.

2.3.2.4 HPLC and UV Analysis

The HPLC method for the analysis of the release and assay samples was adapted from Saha and colleagues (Saha *et al.,* 2002). The HPLC column used was a reverse phase 4.6 x 250 mm Inertsil® C8 column (Inertsil, The Netherlands) with 5 µm particles. The mobile phase was acetonitrile:water (65:35). The isocratic method used a flow rate of 1.25 ml/min and ultraviolet (UV) detection at 230 nm. The injection volume was 20 µl and the retention time was 8 min. The HPLC apparatus that was used for the analysis were Thermo Finnigan (Thermo Electron Corporation, USA) and Waters (Waters, USA) HPLC systems (and associated Chromquest and Empower software). The UV method for the analysis of the release samples was also adapted from Saha and colleagues (Saha *et al*. 2002). The spectrophotometer used was a A Genesys 10 series UV-visible spectrophotometer (Thermo Electron Corporation, USA). Absorbance was read at a wavelength of 251 nm.

2.3.2.5 Microbead manufacture feasibility study

A microbead manufacture feasibility study was performed on the basis of the CLX liquid formulation produced. Microbeads containing CLX were prepared via a manual 'dripping' method. The microbeads were manufactured by combining the '*surfactant phase premix'* (drug dissolved in various combinations of surfactants, lipids and co-solvents) with a '*gelatine phase premix'* (mixture of gelatin, water and sorbitol) and mixing at approximately 60°C. Droplets of the mixture were then allowed to fall into a

bath of cooling/hardening oil (Miglyol® 810N) at approximately 10°C. The resultant beads were then air dried for 24 h (over this time the water in the formulation evaporated). Further details on microbead manufacture are provided in Chapter 4. Shape and surface morphology of freshly prepared and dried microbeads were observed under a Nikon (Nikon, Japan) Eclipse Ti optical microscope mounted with a digital camera.

2.4 Results and discussion

2.4.1 Solubility screening studies

Solubility screening studies were performed to identify excipients which had the capacity to dissolve CLX. The excipients included a range of lipids, surfactants and cosolvents. The range of lipids, surfactants and cosolvents were chosen based on a literature review of the various grades of vehicles available (Hauss, 2007 and Lui, 2008).

2.4.1.1 Excipient screening - lipids

The solubility of CLX in a range of lipids is provided in Table 2.6. Long chain and medium chain triglycerides demonstrated a poor capacity for dissolving CLX. Mixtures of monoglycerides and diglycerides proved to be efficient solubiliers for CLX. Capmul® MCM (glyceryl caprylate/caprate) was the best solubiliser within this class. The best solubilisers of CLX within the lipids tested were the propylene glycol esters. Capryol® 90 from Gattefosse had the best solubilising power. The solubility of CLX in Capryol® 90 was determined to be 58–64 mg/g.

Table 2.6 Solubility of CLX in a range of lipids.

Classification	Common name/Trade name	Measured Solubility (mg/g)
Fatty Acids	Oleic acid	1–1.6
	Linoleic acid	1–2
	Stearic acid	6–7
Natural Oils/Fats	Corn Oil	2–3
	Soybean Oil	2–3
	Olive Oil	1–2
	Incromega® TG3322 (Omega 3 oil)	< 7
	Mineral Oil	< 4
Semi-synthetic mono-, di- and triglycerides	Miglyol® 810	6–9
	Miglyol® 812	8–11
	Miglyol® 829	15–19
	Captex® 300	9–11
	Labrafrac® Lipophile WL1349	6–9
	Capmul® MCM	67–72
	Peceol®	21–28
	Maisine® 35–1	14–20
	Imwitor® 308	42
	Imwitor® 742	40
	Gelucire® 33/01	7–13
Propylene glycol fatty esters	Capryol® PGMC	49–56
	Capryol® 90	58–64
	Lauroglycol® FCC	22–28
	Lauroglycol® 90	27–32
	Labrafac® PG	14–21
	Capmul® PG-12	23–39
Polyglyceryl fatty acid esters	Plurol Oleique® CC497	21–28
Mixtures of glycerides/glyceride PEG derivatives and fatty acid esters of PEG	Labrafil® M 1944 CS	31–39
	Labrafil® M 2125 CS	30–60

2.4.1.2 Excipient Screening – surfactants

The solubility of CLX in a range of surfactants is provided in Table 2.7. The sorbitans (Spans®) and the vitamin E PEG ester demonstrated a poor capacity for dissolving CLX. The best results for the solubilisation of CLX were found within the glyceride/PEG ester mixtures, the PEG-ylated sorbitan fatty acid esters and the PEG esters. Among the excipients with the best solubilisation capacity were Cremophor® EL, Tween® 20 and Solutol® HS-15 (Solutol required melting at approximately 30°C) which had solubilisation capacities for CLX of 264–330, 233–269 and 320–356 mg/g respectively.

Table 2.7 Solubility of CLX in a range of nonionic surfactants.

Classification	HLB	Trade name	Measured solubility (mg/g)
Sorbitan fatty acid esters	4	Span® 80	<17
	2	Span® 85	<24
	8	Span® 20	<17
PEG-ylated sorbitan fatty acid esters	17	Tween® 20	270–300
	16	Tween® 40	280–310
	15	Tween® 60	248–275
	15	Tween® 80	233–269
PEG fatty acid esters	15	Solutol® HS–15	320–356
Vitamin E PEG esters	13	Vitamin E TPGS	41–59
Mixtures of glycerides/ glyceride PEG derivatives and fatty acid esters of PEG	14	Gelucire® 44/14	300-330
	13	Gelucire® 50/13	316-342
	14	Labrasol®	311-354
	13	Cremophor® EL	264-330
	15	Cremophor® RH-40	240-250

2.4.1.3 Excipient Screening – cosolvents

The solubility of CLX in a range of cosolvents is provided in Table 2.8. The solubility of CLX was found to be high in all of the solvents tested with the exception of ethanol where the maximum solubility was determined to be between 100–125 mg/g. CLX demonstrated high solubility in diethylene glycol monoethyl ether (Transcutol® P). The solubility of CLX in Transcutol® P was 345-390 mg/g. Although the solubility of CLX in the high molecular weight PEGs appear to be very high, these PEGs required the input of heat (~45°C for PEG 1000 and ~65°C for PEG 4000) therefore it is difficult to determine whether the solubility of CLX is full attributable to the excipient or to the heat input or a combination of both (Note: it was difficult to control the heat on the hotplate magnetic stirrer).

Table 2.8 Solubility of CLX in a range of cosolvents.

Chemical name	Trade name /Common name	Measured Solubility (mg/g)
Ethanol	EtOH	100–125
Dimethyl Acetamide	DMA	152–288
Polyethylene Glycol 300	PEG 300	310–362
Polyethylene Glycol 400	PEG 400	282–324
Polyethylene Glycol 200	PEG 200	313–362
Polyethylene Glycol 4000	PEG 4000	615–660
Polyethylene Glycol 1000	PEG 1000	601–625
Diethylene glycol monoethyl ether	Transcutol® P	345–390

2.4.2 CLX liquid formulations

Liquid formulations containing CLX dissolved in one, two and three component systems (i.e., in combinations oils/surfactants/cosolvents) were prepared on the basis of the results of the screening studies above. The composition of these liquid formulations is provided in the following sections. *In-vitro* release testing was performed on these formulations to assess and compare their performance. *In-vitro* testing was also performed on the CLX API and the marketed product Celebrex®.

2.4.2.1 *In-vitro* release testing of CLX API

A release test was performed on 50 mg of CLX API in PW, SIF and SGF (Figure 2.5). The release of the API was very poor in all media with a maximum release of 4 % after 6 hours in PW. The drug release performance of the drug was shown to be independent of the pH of the dissolution media.

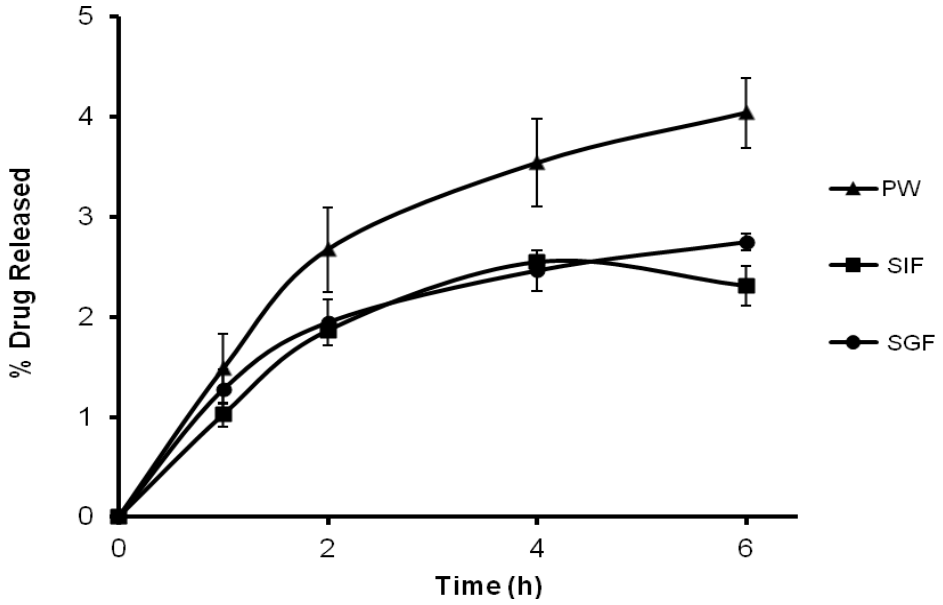

Figure 2.5 Percentage of drug released from release testing of CLX API in PW, SGF and SIF. The data presented are mean values ± STDEV (n=3).

2.4.2.2 *In-vitro* release of Celebrex®

A release test was performed on 50 mg Celebrex® capsules in PW, SGF and SIF (Figure 2.6). Celebrex® wassupplied as 100 mg capsules. The contents of each capsule was removed and weighed. Fifty percent of the contents were then placed into empty capsules so that the content of CLX was equal to 50 mg. The release of the Celebrex® was very poor in all three media (SGF, SIF and PW) with a maximum release of 6 % achieved (in PW). The drug release performance of the drug product was shown to be independent of the pH of the dissolution media in the range from pH 1.2 to 6.8

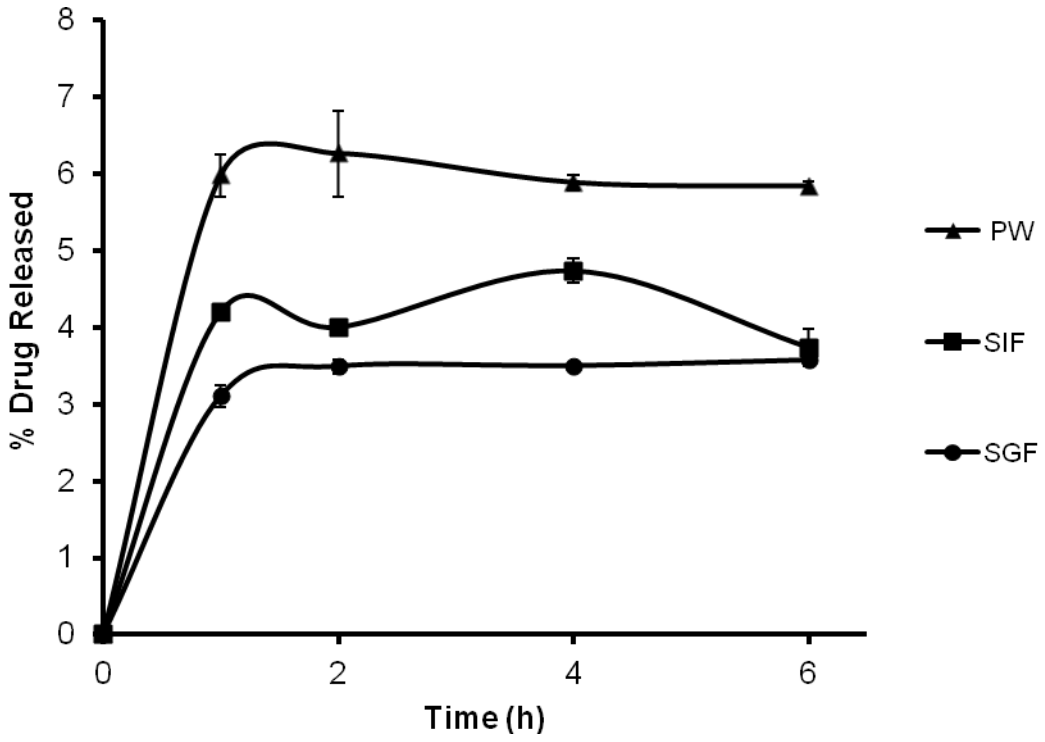

Figure 2.6 Percentage of drug released from release testing of Celebrex® in PW, SIF and SGF. The data presented are mean values ± STDEV (n=3).

2.4.2.3 *In-vitro* release performance of preliminary CLX liquid formulations in comparison to CLX API and Celebrex®

Two liquid formulations, CLX 001/L and CLX 002/L (Table 2.9) were prepared by dissolving CLX in Capryol® 90 and Tween® 20 respectively. A set quantity of both formulations equating to 50 mg of CLX were filled into empty gelatine capsules and were added to the dissolution medium. PW was chosen as the dissolution media as previous results demonstrated that the release of CLX was independent of the pH of the media (Figure 2.7).

Table 2.9 Comparison of the composition of formulations CLX 001/L and CLX 002/L.

Formulations		CLX 001/L	CLX 002/L
Component Concentration (mg/g)	**Celecoxib**	50.0	24.39
	Capryol® 90	950.0	-
	Tween® 20	-	975.61

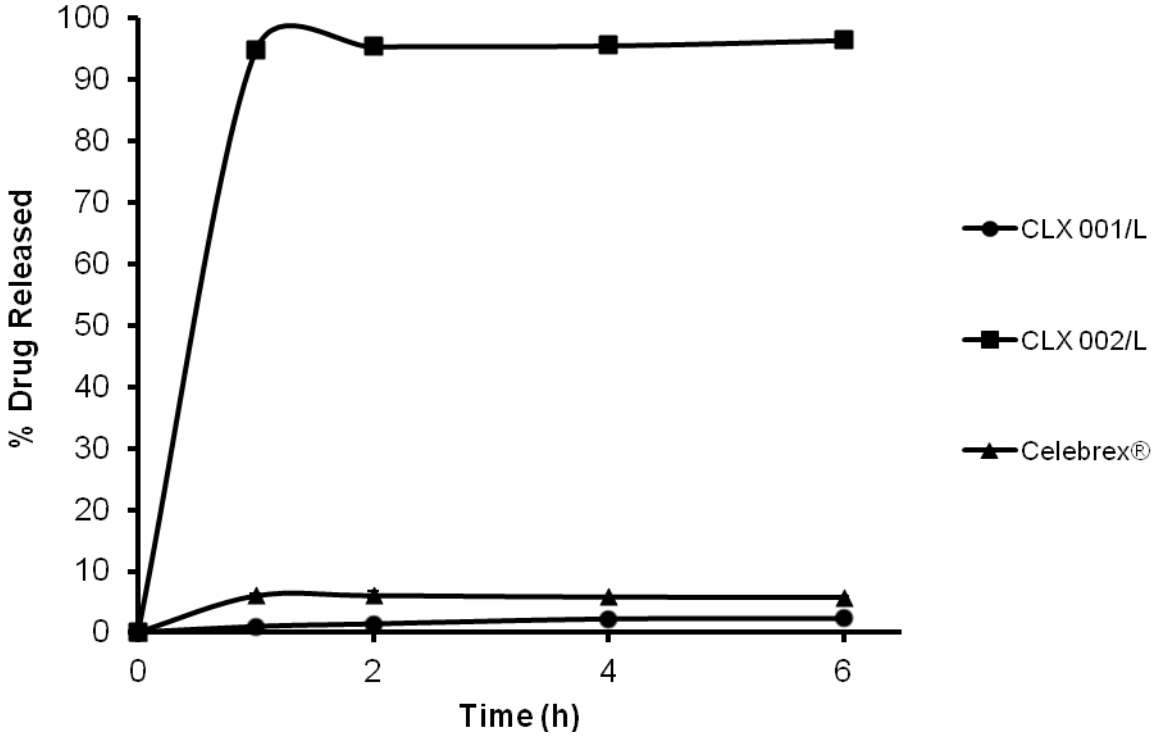

Figure 2.7 Percentage of drug released from release testing of CLX 001/L, CLX 002/L, Celebrex® and CLX API in PW. The data presented are mean values ± STDEV (n=3).

The release of the CLX 002/L was greaterthanCLX API and the Celebrex® capsules. The release of CLX 002/L was 92 % after 1 hour. This test demonstrated that the release of CLX could be dramatically increased by formulating the drug in a lipophilc format.

The results from formulation CLX 001/L demonstrated the importance of release testing with respect to assessing the performance of the liquid formulations. Although the CLX was completely dissolved in the Capryol® 90 in formulation CLX 001/L, this was not sufficient to enable the drug to disperse within the dissolution media. This is a common problem for drugs which are formulated as liquids in solid oral dosage forms (e.g., soft gelatine capsules). Although the drug is presented in a solubilised form, there is often no or insufficient components in the formulation to allow for the dispersal of the drug (e.g., a surfactant). This is due to the fact that the current technologies are not compatible with surfactants when used at significant levels.

2.4.2.4 *In-vitro* release comparison of CLX liquid formulations

A further six formulations (CLX 004/L to CLX 009/L) were prepared and their release performance was compared to that of CLX 002/L. These formulations consisted of CLX dissolved in various combinations of Transcutol® P, Capryol® 90, Tween® 20, Miglyol® 810N and Solutol® HS-15 (Table 2.10). Although the maximum release of these formulations (ranging from 5.5 to 14%) were greater than that of Celebrex® and CLX API, their performance were poor in comparison to CLX 002/L (Figure 2.8). The primary difference between these formulations and CLX 002/L was the level of surfactant employed (Table 2.10).

Table 2.10 Comparison of the composition of formulations CLX 002/L, CLX 004/L, CLX 005/L, CLX 006/L, CLX 007/L, CLX 008/L and CLX 009/L.

Formulations		CLX 002/L	CLX 004/L	CLX 005/L	CLX 006/L	CLX 007/L	CLX 008/L	CLX 009/L
Component Concentration (mg/g)	Celecoxib	24.39	320.51	141.84	141.84	319.49	194.17	233.64
	Transcutol® P	-	679.49	-	428.37	335.46	219.42	378.50
	Capryol® 90	-	-	429.79	429.79	-	388.35	-
	Tween® 20	975.61	-	428.37	-	345.05	198.06	-
	Miglyol® 810 N	-	-	-	-	-	-	154.21
	Solutol® HS-15	-	-	-	-	-	-	233.64

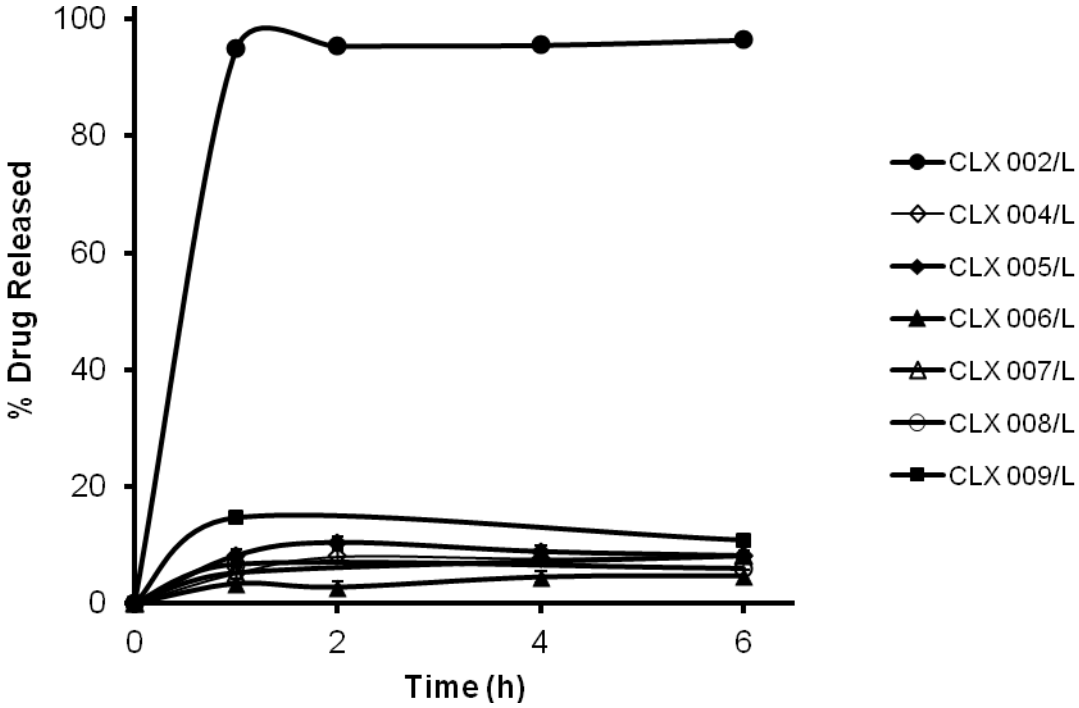

Figure 2.8 Percentage of drug released from release testing of CLX 004/L, CLX 005/L, CLX 006/L, CLX 007/L, CLX 008/L and CLX 009/L tested in PW. This was a high throughput screening study (n=1).

CLX 002/L comprised a surfactant concentration of 975.61 mg/g (Tween® 20), whereas the maximum surfactant concentration for the formulations described in Table 2.10 was for formulation CLX 005/L (428.37 mg/g of Tween® 20). The results also suggested that the type of surfactant employed was also of major importance as CLX 009/L achieved the highest % of drug released (aside from CLX 002/L) despite its surfactant (Solutol® HS-15) concentration being less than that of the other formulations tested. It was also observed and noted that precipitation of CLX was more prevalent in formulations containing Transcutol® P.

2.4.2.5 Comparison of CLX liquid formulations with increasing concentrations of Tween 20®

Liquid formulations CLX 010/L, CLX 011/L, CLX 012/L, CLX 013/L and CLX 014/L (Table 2.11) were all prepared by dissolving CLX in increasing concentrations of Tween® 20 to allow for comparisons with formulation CLX 002/L. It was observed (Figure 2.9) that the percentage of drug released was greater for formulations containing increasing concentrations of Tween® 20. These results demonstrated that there was a critical surfactant concentration required to fully disperse the drug in the dissolution media. In the case Tween® 20, this was > 961.54 mg/g (conc. of Tween® 20 in CLX 012/L).

Table 2.11 Comparison of the composition of formulations CLX 002/L, CLX 010/L, CLX 011/L, CLX 012/L, CLX 013/L and CLX 014/L

Formulations		CLX 002/L	CLX 010/L	CLX 011/L	CLX 012/L	CLX 013/L	CLX 014/L
Component Concentration (mg/g)	Celecoxib	24.39	150.00	90.91	38.46	142.86	30.30
	Tween® 20	975.61	369.00	909.09	961.54	857.14	969.70

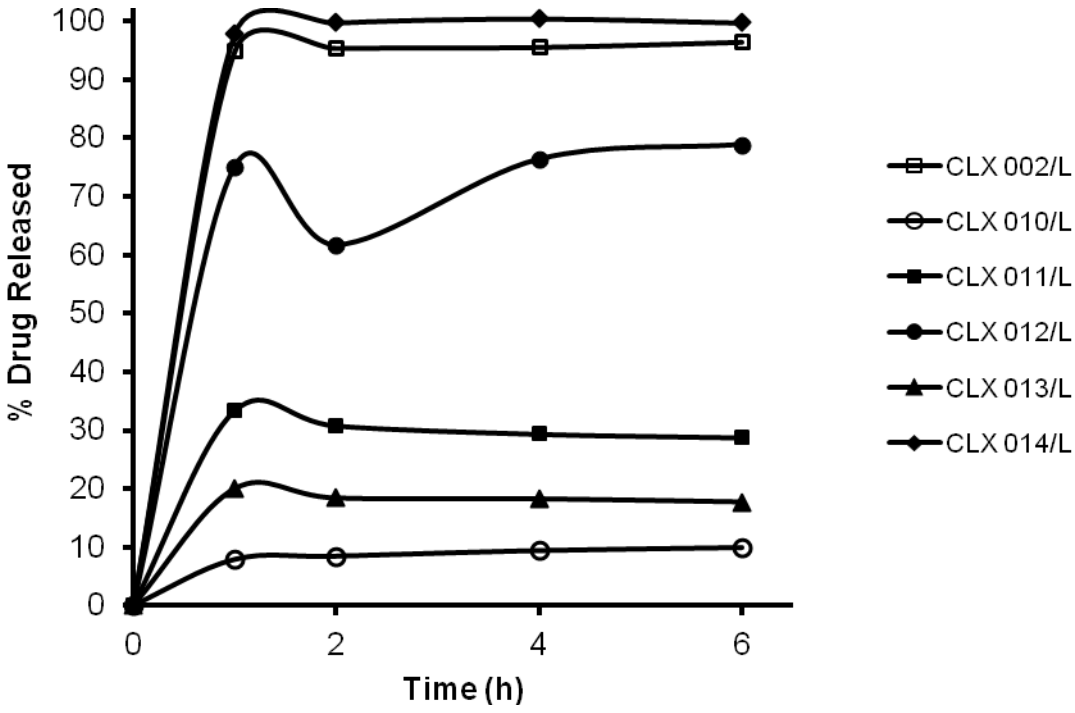

Figure 2.9 Percentage of drug released from release testing of CLX 002/L, CLX 010/L, CLX 011/L, CLX 012/L, CLX 013/L and CLX 014/L tested in PW. This was a high throughput screening study (n=1).

2.4.2.6 Comparison of CLX liquid formulations comprising different surfactants

Liquid formulations CLX 015/L, CLX 016/L and CLX 017/L were prepared by dissolving CLX in different concentrations of Solutol® HS-15 (Table 2.12). Formulations CLX 018/L, CLX 019/L and CLX 020/L were prepared by dissolving CLX in various concentrations of Labrasol® (Table 2.12). Release testing in PW was performed on all six formulations (Figure 2.10). Overall the percentage of CLX released was greater for formulations containing Solutol® HS-15 in comparison to those containing Labrasol®. These results demonstrated that the degree to which the CLX dispersed in the dissolution media was dependent on the type of surfactant employed and also the concentration of surfactant employed. For example, CLX 017/L and CLX 020/L comprised of an equal concentration of surfactant (i.e., 937.50 mg/g), however the maximum percentage of CLX released was 69 % in the case of CEL 017/L (formulation containing Solutol® HS-15), whereas it was 38 % in the case of CLX 020/L (formulation containing Labrasol®). In the case of the formulations containing Solutol® HS-15), when the concentration of Solutol® HS-15 was reduced to 800 mg/g (CLX 015/L), the maximum percentage of celecoxib released dropped to 24 %.

Table 2.12 Comparison of the composition of formulations CLX 015/L, CLX 016/L, CLX 017/L, CLX 018/L, CLX 019/L and CLX 020/L.

Formulations		CLX 015/L	CLX 016/L	CLX 017/L	CLX 018/L	CLX 019/L	CLX 020/L
Component Concentration (mg/g)	Celecoxib	200.00	100.00	62.50	200.00	100.00	62.50
	Solutol® HS-15	800.00	900.00	937.50	-	-	-
	Labrasol®	-	-	-	800.00	900.00	937.50

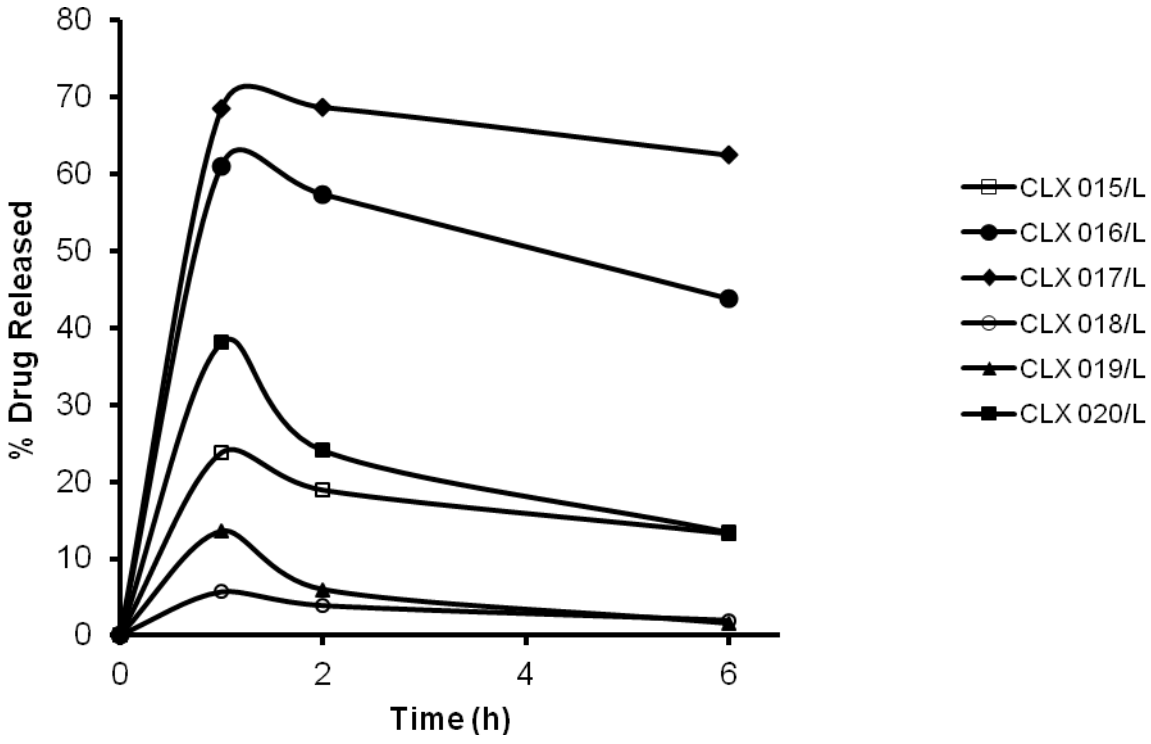

Figure 2.10 Percentage of drug released from release testing in PW of CLX 015/L, CLX 016/L, CLX 017/L, CLX 018/L, CLX 019/L and CLX 020/L. This was a high throughput screening study (n=1).

Following a review of the results obtained to date, it was observed that the most effective surfactants employed in the CLX liquid formulations to date were Solutol® HS-15 and Tween® 20. A further six formulations were prepared (CLX 021/L to CLX 026/L - Table 2.13) containing Solutol® HS-15 or Tween® 20 in combination with Miglyol® 810N and Transctol® P. Two additional CLX liquid formulations containing Cremophor® EL, which had previously been shown to be an effective solubiliser of CLX were also prepared (CLX 028/L and CLX 029/L - Table 2.13). Release testing in PW was performed on all eight formulations (Figure 2.11).

Table 2.13 Comparison of the composition of formulations CLX 021/L, CLX 022/L, CLX 023/L, CLX 024/L, CLX 025/L, CLX 026/L, CLX 028/L and CLX 029/L.

Formulations		**CLX 021/L**	**CLX 022/L**	**CLX 023/L**	**CLX 024/L**	**CLX 025/L**	**CLX 026/L**	**CLX 028/L**	**CLX 029/L**
Component Concentration (mg/g)	Celecoxib	66.67	90.91	90.91	66.67	90.91	90.91	260.00	260.00
	Solutol® HS-15	600.00	454.55	454.55	-	-	-	-	-
	Miglyol ® 810N	333.33	454.55	227.27	333.33	454.55	227.27	110.00	110.00
	Transcutol® P	-	-	227.27	-	-	227.27	400.00	-
	Tween® 20	-	-	-	600.00	454.55	454.55	-	-
	Cremophor® EL	-	-	-	-	-	-	230.00	630.00

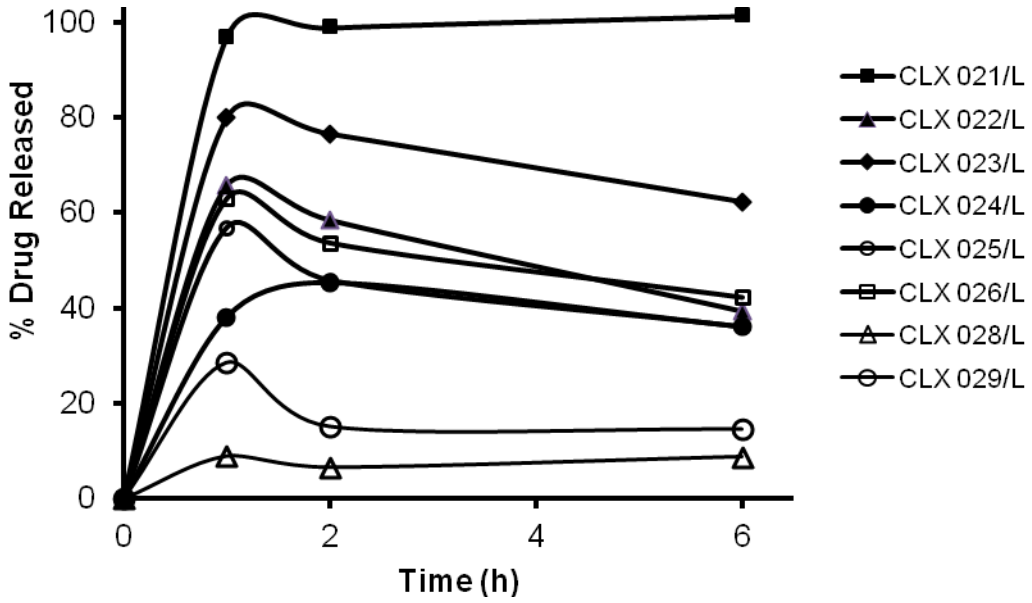

Figure 2.11 Percentage of drug released from release testing in PW of CLX 021/L, CLX 022/L, CLX 023/L, CLX 024/L, CLX 025/L, CLX 026/L, CLX 028/L and CLX 029/L. This was a high throughput screening study (n=1).

Overall the percentage of CLX released was greater for formulations containing Solutol® HS-15 in comparison to those containing Tween® 20 or Cremophor® EL. The results for CLX 021/L, CLX 022/L and CLX 023/L were particularly interesting when compared to that of CLX 015/L. In the case of CLX 015/L, the concentration of Solutol® HS-15 was 800 mg/g and the maximum percentage release was 24 %, whereas in the case of CLX 021/L, CLX 022/L and CLX 023/L the maximum release percentages were 100%, 66 % and 80 % respectively despite lower concentrations of Solutol® HS-15 (600 and 454.55 mg/g). These results demonstrated that the addition of a lipid component (CLX 021/L contained Miglyol® 810N in addition to Solutol® HS-15) improved the drug release performance of the lipophilic CLX formulations containing Solutol® HS-15 (compared to formulations in Figure 2.10).

2.4.3 CLX microbead production feasibility study

On the basis of the liquid formulations that were developed, a microbead production feasibility study was performed which involved the preparation of six formulation mixtures (mixture of surfactant phase premix and gelatine phase premix) with the intention of converting them to microbeads using the method described in section 2.3.2.5 above. These six formulations are described in Table 2.14 below. Formulations CLX 027/B, CLX 028/B, CLX 029/B, CLX 031/B and CLX 032/B were prepared as mixtures but were not progressed to microbeads as the drug precipitated in the mixture upon contact of the surfactant phase premix with the gelatine phase premix. The surfactant phase premix in these formulations contained 26% w/w CLX dissolved in various combinations of Solutol® HS-15, Miglyol® 810N, Cremophor® EL and Transcutol® P.

Table 2.14 Composition of CLX microbead formulations CLX 027/B, CLX 028/B, CLX 029/B, CLX 030B, CLX 031/B and CLX 032/B.

Formulations		**CLX 027/B**	**CLX 028/B**	**CLX 029/B**	**CLX 030/B**	**CLX 031/B**	**CLX 032/B**
Component Concentration (mg/g)	Celecoxib	100.00	104.00	98.79	23.03	100.41	98.02
	Gelatine	553.85	540.00	558.02	554.40	552.35	560.62
	D-Sorbitol	61.54	60.00	62.00	61.70	61.47	62.39
	Transcutol® P	153.85	160.00	-	-	-	-
	Miglyol® 810N	42.31	44.00	41.80	130.53	42.48	-
	Solutol® HS-15	88.46	-	-	230.34	243.29	278.97
	Cremophor® EL	-	92.00	239.38	-	-	-

One of the formulations (CLX 030/B) produced during this phase of experiments did not result in precipitation on contact and was progressed to the manufacture of microbeads. This formulation contained 6% w/w CLX dissolved in 60% w/w of Solutol® HS-15 and 34% w/w of Miglyol® 810N. The emulsion produced on the combination of the surfactant phase premix and gelatine phase premix was transparent, suggesting that it was a microemulsion (i.e., a clear, stable, isotropic liquid mixtures of oil, water and surfactant). During the manufacture of the beads it was observed that a portion of the microemulsion was dissipating into the cooling solution. The resultant beads were roughly spherical and robust and became opaque upon drying. Photographs of beads (multiple beads and a single bead) from this batch of microbeads is shown in Figure 2.12 below. This preliminary experiment illustrated that it was possible to make microbeads from a formulation consisting of CLX dissolved in Solutol® HS-15 and Miglyol® 810N. The liquid formulations produced up to this point were reviewed and the formulations described in Table 2.15 below were identified as potential candidates for an *in-vitro* CRC cell line study (Chapter 3) principally based on their *in-vitro* drug release performance. It should be noted that formulations containing Tween® 20, Cremophor® EL or Labrasol® were excluded from consideration as Solutol® HS-15 had been shown to be a better surfactant to these and also that formulations containing Transcutol® P were also excluded as precipitation appeared to be more extensive in the case of CLX 027/B than for CLX 031/B or CLX 032/B. A comparison of the quality attributes of the remaining formulations (Table 2.15) was made to select two formulations for progression.

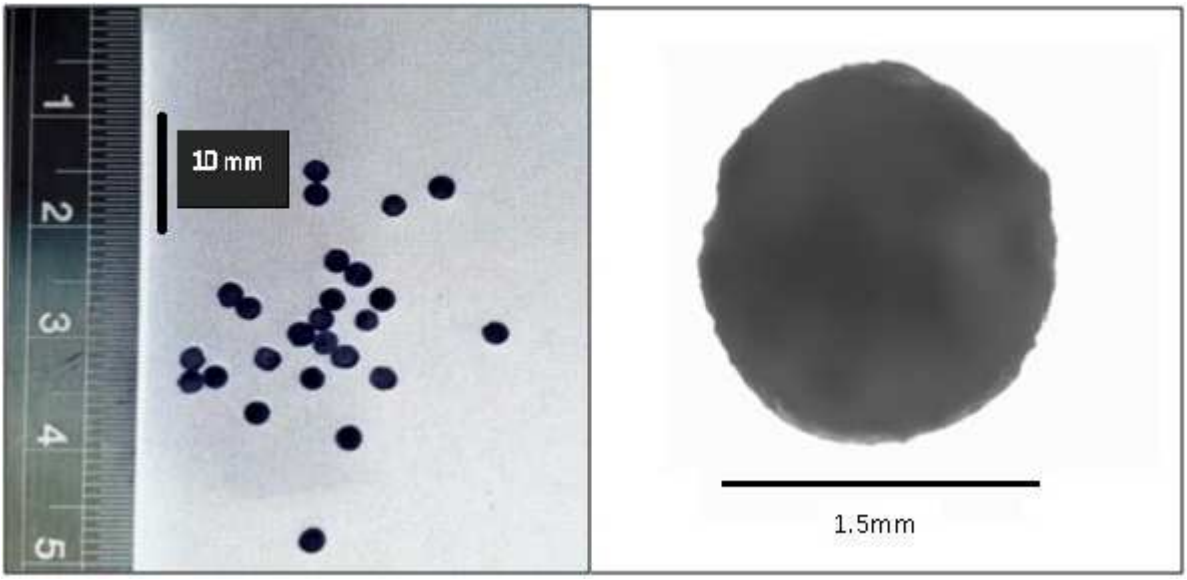

Figure 2.12 Photographs of CLX 030/B beads (multiple beads and a single bead).

Table 2.15 Comparison of the quality attributes of formulation candidates for progression to CRC cell culture study.

Formulations	% Release in PW at 1 h ≥ 50%	% Release in PW at 6 h ≥ 50%	High Drug Load [1]	Solutol® HS-15	Miglyol® 810N
CLX 016/L	✓	×	✓	✓	×
CLX 017/L	✓	✓	×	✓	×
CLX 021/L	✓	✓	×	✓	✓
CLX 022/L	✓	×	✓	✓	✓

[1] High drug load defined as ≥ 8% (w/w).

Two candidates were required for progression to the cell culture study. The primary criteria for selection for the first candidate was a formulation which demonstrated a high % drug release and which remained stable over a period of 6 h. Based on the comparison in Table 2.15, formulations CLX 017/L and CLX 021/L met this criteria. CLX 021/L was selected as the first candidate on the basis of its greater drug release performance compared to CLX 017/L. CLX 021/L represented a two component formulation. The selection criteria were revised for the selection of the second

formulation to exclude a) two component formulations and b) formulations with low drug loadings (i.e., < 8% w/w). These criteria were included to ensure a diverse sample set given the preliminary nature of the development work. CLX 016/L was the only remaining formulations that met these two criteria and therefore was selected for progression.

2.5 Conclusions

The solubility of CLX in a range of liquid vehicles was assessed. On the basis of this assessment, liquid formulations were produced. Optimal liquid formulations produced (e.g., CLX 016/L and CLX 021/L) were demonstrated to have a greater drug release performance than that of the marketed CLX product Celebrex®. These formulations were based on the nonionic surfactant Solutol® HS-15 and the semi synthetic medium chain triglyceride Miglyol® 810N. A feasibility study was performed to assess the potential for these optimal liquid formulations to be translated into gelatine based microbeads. Spherical and robust microbeads were successfully produced which represented the first step in the development of a suitable oral drug delivery formulation designed to deliver of pre-solubilised CLX to the colon for the treatment of CRC. Despite evidence that CLX is a potentially useful drug for the prevention or treatment of CRC, a number of questions regarding itssafety remain (Gonzalez-Angulo *et al.*, 2002). A major safety concern relating to CLX is the serious GI side effects associated with Celebrex®. It is proposed that the GI side effects are not only dose related but also a result of local irritation (Halen *et al.*, 2009) and therefore may be related to the presentation of the current dosage form (a powder filled capsule). The work presented here in this phase of the project constituted a preliminary but significant step towards the possibility of administering CLX for the prevention and/or treatment of CRC in a

free molecular form (i.e., fully dissolved) which had a) the potential to reduce the known GI side effects and b) the potential to be more efficacious than the current marketed dosage form on the basis that the pre-dissolving the drug would enable direct interaction with the colonic tissue. The next step of the project (Chapter 3) involved testing of this second hypothesis by administering the optimal liquid formulations produced here to a CRC cell line and assessing their impact on cell proliferation in comparison to cells treated with Celebrex®.

2.6 Nomenclature

Table 2.16 List of abbreviations which are listed according to their appearance in the text.

Abbreviation	Definition
CLX	Celecoxib
GI	Gastrointestinal
CRC	Colorectal cancer
LBDDS	Lipid based drug delivery system
SEDDS	Self emulsifying drug delivery system
SMEDDS	Self microemulsifying drug delivery system
PEG	Polyethyleneglycol
HLB	Hydrophilic-Lipophilic balance
QC	Quality control
API	Active Pharmaceutical ingredient
PW	Purified water
SIF	Simulated intestinal fluid
SGF	Simulated gastric fluid
HPLC	High performance liquid chromatography
UV	Ultraviolet
CLX xxx/L	Celecoxib liquid formulation numbering system where xxx is a sequential number and L is liquid
CLX xxx/B	Celecoxib bead formulation numbering system where xxx is a sequential number and L is liquid

2.7 Acknowledgements

I would like to express my gratitude to the analytical department at Sigmoid Pharma Ltd. for analytical support as part of this work.

2.8 References

Boyd, B.J., Nguyen, T. & Mullertz, A. 2011, "Lipids in Oral Controlled Release Drug Delivery" in *Controlled Release in Oral Drug Delivery*, eds. C.G. Wilsom & P.J. Crowley, Springer, New York, pp. 299-328.

Cannon, J.B. & Long, M.A. 2008, "Emulsions, Microemulsions and Lipid-Based Drug Delivery Systems for Drug Solubilisation and Delivery" in *Water-Insoluble Drug Formulation*, ed. R. Lui, 2nd edn, CRC Press, Florida, pp. 227-254.

Date, A.A., Desai, N., Dixit, R. & Nagarsenker, M. 2010, "Self-nanoemulsifying drug delivery systems: formulation insights, applications and advances", *Nanomedicine (London, England)*, vol. 5, no. 10, pp. 1595-1616.

Dressman, J., Schamp, K., Beltz, K. & Alenz, J. 2007, "Characerizing Release from Lipid-Based Formulations" in *Oral Lipid-Based Formulations – Enhancing the Bioavailability of Poorly Water-Soluble Drugs*, ed. D.J. Hauss, Informa Healthcare, New York, pp. 241-256.

Gibson, L. 2007, "Lipid-Based Excipients for Oral Drug Delivery" in *Oral Lipid-Based Formulations – Enhancing the Bioavailability of Poorly Water-Soluble Drugs*, ed. D.J. Hauss, Informa Healthcare, New York, pp. 33-62.

Gonzalez-Angulo, A.M., Fuloria, J. & Prakash, O. 2002, "Cyclooxygenase 2 inhibitors and colon cancer", *The Ochsner Journal*, vol. 4, no. 3, pp. 176-179.

Grove, M. & and Mullertz, A. 2007, "Liquid Self-Microemulsifying Drug Delivery Systems - Enhancing the Bioavailabilty of Poory Water-Soluble Drugs" in *Oral Lipid-Based Formulations*, ed. D.J. Hauss, Informa Healthcare, New York, pp. 107-128.

Halen, P.K., Murumkar, P.R., Giridhar, R. & Yadav, M.R. 2009, "Prodrug designing of NSAIDs", *Mini Reviews in Medicinal Chemistry,* vol. 9, no. 1, pp. 124-139.

Hauss, D.J. 2007, *Oral Lipid-Based Formulations – Enhancing the Bioavailability of Poorly Water-Soluble Drugs,* Informa Healthcare, New York.

Kalepu, S., Manthina, M. & Padavala, V. 2013, "Oral lipid-based drug delivery systems – an overview", *Acta Pharmaceutica Sinica B,* vol. 3, no. 6, pp. 361-372.

Liu, R., Dannenfelser, R. & Li, S. 2008, "Micellization and Drug Solubility Enhancement" in *Water-Insoluble Drug Formulation*, ed. R. Lui, 2nd edn, CRC Press, Florida, pp. 255-306.

Lui, R. 2008, *Water-Insoluble Drug Formulation,* 2nd edn, CRC Press, Florida.

Mc Clements, D.J. 2007, *Food Biopolymers and Colloids Research Laboratory - Food Emulsions Short Course*. Available: http://people.umass.edu/mcclemen/Group.html [2014, Oct/06].

Narang, A.S., Delmarre, D. & Gao, D. 2007, "Stable drug encapsulation in micelles and microemulsions", *International Journal of Pharmaceutics,* vol. 345, no. 1-2, pp. 9-25.

Paul, E.J. & Prud'homme, R.K. 2001, "Material Synthesis by Polymerization in Surfactant Meophases" in *Reactions and Synthesis in Surfactant Systems*, ed. J. Texter, Marcel Dekker Inc., New York, pp. 525-528.

Paulson, S.K., Vaughn, M.B., Jessen, S.M., Lawal, Y., Gresk, C.J., Yan, B., Maziasz, T.J., Cook, C.S. & Karim, A. 2001, " Pharmacokinetics of Celecoxib after Oral Administration in Dogs and Humans: Effects of Food and Site of Absorption", *Journal of Pharmacology and Experimental Therapeutics,* vol. 297, no. 2, pp. 638-645.

Pouton, C.W. 2000, "Lipid formulations for oral administration of drugs: non-emulsifying, self-emulsifying and 'self-microemulsifying' drug delivery systems", *European Journal of Pharmaceutical Sciences*vol. 11 Suppl 2, pp. S93-8.

United States Pharmacopeia, 2010, 33rd edn, USP/NF, Rockville.

Rangel-Yagui, C.O., Pessoa, A.,Jr & Tavares, L.C. 2005, "Micellar solubilization of drugs", *Journal of Pharmacy & Pharmaceutical Sciences*, vol. 8, no. 2, pp. 147-165.

Rawat, S. & Jain, S.K. 2004, "Solubility enhancement of celecoxib using beta-cyclodextrin inclusion complexes", *European Journal of Pharmaceutics and Biopharmaceutics*vol. 57, no. 2, pp. 263-267.

Saha, R.N., Sajeev, C., Jadhav, P.R., Patil, S.P. & Srinivasan, N. 2002, "Determination of celecoxib in pharmaceutical formulations using UV spectrophotometry and liquid chromatography", *Journal of Pharmaceutical and Biomedical Analysis*, vol. 28, no. 3-4, pp. 741-751.

Saxena, S., Singh, H.N., Agrawal, V.K. & Chaturvedi, S. 2013, "Lipid Excipients in Self Emulsifying Drug Delivery Systems", *AJBPS*, vol. 3, no. 22, pp. 16-22.

Strickley, R.G. 2007, "Currently Marketed Oral Lipid-Based Dosage Forms: Drug Products and Excipients" in *Oral Lipid-Based Formulations – Enhancing the Bioavailability of Poorly Water-Soluble Drugs*, ed. D.J. Hauss, Informa Healthcare, New York, pp. 1-32.

BASF - Solutol HS-15 technical information sheet, 2012. Available: http://www.pharma-ingredients.basf.com/Statements/Technical%20Informations/EN/Pharma%20Solutions/03_111149e_Kolliphor%20HS%2015.pdf [2014, Oct/06].

FDA labelling revision for Celebrex capsules, 2008. Available: http://www.accessdata.fda.gov/drugsatfda_docs/label/2008/020998s026lbl.pdf [2014, Oct/08].

Chemspider - Oleic Acid technical information page, 2014. Available: http://www.chemspider.com/Chemical-Structure.393217.html?rid=d7ff7393-3c2a-4c48-8a7e-2078a07eb41c [2014, Oct/06].

Sigma Aldrich - Glyceryl trioleate product page 2014. Available: http://www.sigmaaldrich.com/catalog/product/sigma/t7140?lang=en&region=IE [2014, Oct/06].

CHAPTER 3

In-vitro cell study

Publication Status: The work presented in this chapter in addition to data from chapters 5 and 6 has been accepted for publication (02-11-14) in the Journal of Pharmacy and Pharmacology under the following title '*In-vitro* characterization of a novel celecoxib microbead formulation for the treatment and prevention of colorectal cancer'.

3.1 Abstract

The purpose of this phase of the project was to assess the effect of lipid based celecoxib (CLX) liquid formulations on the viability of a cyclooxygenase 2 (COX-2) expressing colorectal cancer (CRC) cell line (HT29) and to compare the inhibitory effect to that of the marketed CLX product Celebrex®. *In-vitro* cell viability (measured via MTT and flow cytometry assays) and motility (measured via a scratch wound healing assay) were shown to be significantly reduced after treatment with CLX liquid formulations relative to the control, whereas the results for treatment with Celebrex® were comparable to the control. Release experiments and correlation analysis demonstrated that the formulations with enhanced and stable drug release (i.e., greater drug release profiles) resulted in reduced cell viability and motility.

3.2 Introduction

3.2.1 Background

A study by Eberhart and colleagues was among the first to demonstrate a significant elevation of cyclooxygenase 2 (COX-2) expression in most human colorectal carcinomas and also in fifty percent of adenomas (Eberhart *et al.*, 1994). Following this observation by Eberhart and colleagues, COX-2 expression was also demonstrated in induced tumours from CRC mouse and rat models (Shao *et al.,* 1999 and Boolbol *et al.,* 1996). These findings, in addition to others as described in Chapter 1, regarding the relationship between the COX-2 inhibitor CLX and CRC, have stimulated research focusing on studying the effects of COX-2 inhibitors (including CLX) on CRC cell lines (Buecher *et al.,* 2005 and Sade *et al.,* 2012).

At the conclusion of Chapter 2, a hypothesis was presented that the liquid CLX formulations developed had the potential to be more efficacious than the current marketed CLX dosage form (Celebrex®) on the basis that pre-dissolving the drug would enable direct interaction with the colonic tissue as the drug would be presented in a free molecular form (i.e., fully dissolved). The primary aim at the outset of this phase of the project (Chapter 3) was to test this hypothesis by administering the optimal liquid formulations developed in Chapter 2 (CLX 016/L and CLX 021/L) to a COX-2 expressing CRC cell line and assessing their impact on cell viability in comparison to cells treated with Celebrex®.

3.2.2 HT29 CRC carcinoma cell line

CRC cell lines are useful models for understanding the underlying biological and molecular basis for colon cancer and are also useful for assessing the impact of chemotherapeutics on colon cancer cells (Rutzky, L.P., and Moyer, M. P., 1990). In a review by Ettarh and colleagues (Ettarh *et al.*, 2010), a variety of CRC cell lines that have been used in *in-vitro* investigations on the effects of NSAIDs (non-steroidal anti-inflammatory drugs) on several aspects of tumour initiation and progression were summarised. A review of this summary identified the COX-2 expressing cell line HT29 as one of the most widely used cell lines for this type of studies. The HT29 cell line was also included in the studies cited in Section 3.2.1 (Buecher *et al*., 2005 and Sade *et al*., 2012). HT29 is a human CRC adenocarcinoma cell line with epithelial morphology. The cell line was cultured by Jorgen Fogh, and was established in 1964 from the primary tumour of a 44 year old female with CRC adenocarcinoma (HT29 Cell Line Summary, Memorial Sloan Kettering Cancer Centre, 2014). In addition to the use of the HT29 cell line for *in-vitro* chemotherapeutic studies (such as that described above), the

HT29 cell line is also used as a xenograft tumor model for CRC and also as an *in-vitro* model to study absorption, transport, and secretion by intestinal cells (HT29 Cell Line Summary, Memorial Sloan Kettering Cancer Centre, 2014)

3.2.3 Cell viability (MTT Assay)

As described in Section 3.2.1, the primary aim at the outset of this phase of the project was to assess the impact of the optimised CLX formulations on the viability of HT29 cells following drug treatment and to compare their effect to that of Celebrex®. There are a number of assays available which are designed to measure the viability and cytotoxicity of cells in culture after treatment with various stimuli. Assays for cell viability may monitor the number of cells over time, the number of cellular divisions, metabolic activity, or DNA synthesis (Frei, 2011). An example of an assay which measures cell viability via metabolic activity and the assay which was used for this study is the MTT (3-[4,5-di**m**ethyl**t**hiazol-2-yl]-2,5 diphenyl **t**etrazolium bromide) assay. The MTT assay is based on the conversion of the tetrazolium salt (MTT) into formazan crystals by living cells, which determines mitochondrial activity. This conversion is thought to be facilitated by NADPH (nicotinamide adenine dinucleotide phosphate-oxidase) or NADH (nicotinamide adenine dinucleotide phosphate) produced by dehydrogenase enzymes in metabolically active cells (Berridge and Tan, 1993). Since for most cell populations the total mitochondrial activity is related to the number of viable cells, this assay is broadly used to measure the *in-vitro* cytotoxic effects of drugs on cell lines or primary patient cells (van Meerloo *et al*., 2011).

MTT is a water soluble tetrazolium salt. Dissolved MTT is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by dehydrogenase enzymes. This water insoluble formazan can be solubilized using solvents (e.g., dimethyl sulfoxide

(DMSO)) and the dissolved material is measured spectrophotometrically yielding absorbance as a function of concentration of converted dye. The cleavage and conversion of the soluble yellow dye to the insoluble purple formazan has been used to develop a cell viability assay system as active mitochondrial dehydrogenases in living cells will cause this conversion whereas dead cells do not cause this change (Frei, 2011). The reduction of MTT to coloured formazan compounds only occurs in metabolically active cells. The primary disadvantage of the MTT assay is that the living cells must be metabolically active in order to cause the cleavage, therefore the assay cannot distinguish between dead cells and living cells that are not metabolically active and therefore viable cells which are not metabolically active can be excluded from the cell number result. In light of this deficiency of the MTT assay, a second cell viability assay (apoptosis assay) was performed to support findings from the MTT assay.

3.2.4 Cell viability and apoptosis (Flow cytometry assay)

Flow cytometry can be defined as a technology to measure properties of cells as they flow in a liquid suspension. The majority of flow cytometers can measure two kinds of light from cells; a) light scatter and b) fluorescence. Light scatter is the interaction of light and matter. All materials, including cells, will scatter light. In the flow cytometer, light scatter detectors are located opposite the laser (relative to the cell), and to one side of the laser, in-line with the fluid-flow/laser beam intersection. The measurements made by these detectors are called forward light scatter and side light scatter, respectively. Forward light scatter provides information on the relative size of individual cells, whilst side light scatter provides information on the relative granularity of individual cells (Loughran, 2007). Fluorescence is the property of a molecule to absorb light of a

particular wavelength and re-emit light of a longer wavelength. The wavelength change relates to an energy loss that takes place in the process. By gathering fluorescence information, flow cytometry can be used to assess cell viability and more specifically apoptotic and necrotic processes in conjunction with commercially available molecular tools. Forward scatter and side scatter are used to focus on (referred to as gating) on a population of cells which are simultaneously analysed for fluorescence (Loughran, 2007).

Apoptosis, or programmed cell death is a normal physiologic process for the removal of unwanted cells. Necrosis is defined as unscheduled cell death, usually as a result of injury or disease. Apoptosis is a genetically programmed process that occurs during embryonic development, as well as in maintenance of tissue homeostasis, under pathological conditions, and in aging (Hingorani *et al.*, 2011). The term apoptosis, from the Greek word for "falling off" of leaves from a tree, is used to describe a phenomenon in which a cell actively participates in its own destructive processes. The process is characterized by specific morphologic features, including loss of plasma membrane asymmetry and attachment, plasma membrane blebbing, condensation of the cytoplasm and nucleus, and internucleosomal cleavage of DNA. Loss of plasma membrane asymmetry is one of the earliest features of apoptosis (Hingorani *et al.*, 2011). In normal healthy live cells (viable cells), phosphatidyl serine (PS) is located on the cytoplasmic surface of the cell membrane (Figure 3.1 A). However, in apoptotic cells, PS is translocated from the inner to the outer leaflet of the plasma membrane, thus exposing PS to the external cellular environment. The human anticoagulant, Annexin V, is a 35–36 kDa Ca^{2+} -dependent phospholipid-binding protein that has a high affinity for PS and can be used to identify apoptotic cells by binding to PS. Annexin V can be conjugated to fluorochromes (e.g., fluorescein isothiocyanate (FITC)) whilst maintaining its high affinity for PS and therefore can serve as a sensitive probe for flow cytometric analysis

of cells undergoing apoptosis (Figure 3.1 B). PS translocation precedes the loss of membrane integrity, which accompanies the later stages of cell death resulting from either apoptotic or necrotic processes, hence, staining with Annexin V is usually used in conjunction with a fluorescent dye (referred to as a vital dye) such as propidium iodide (PI) that binds to nucleotides and which only penetrates damaged cellular membranes. Intercalation complexes are formed by PI with double-stranded DNA (deoxyribonucleic acid), which results in an amplification of the fluorescence. This vital dye is used in order to distinguish between early apoptosis (Figure 3.1 B), late apoptosis/necrosis (Figure 3.1 C) and necrosis (Figure 3.1 D). Since PS translocation and loss of membrane integrity are features of both apoptotic and necrotic processes, albeit at different stages, (Hingorani *et al.*, 2011 and Kawamura and Ye., 2007) a combination of these dyes are required to distinguish between the four events (viable cells, early apoptosis, late apoptosis/necrosis and necrosis).

In summary, when molecular tools such as Annexin V-FITC and PI are used in combination the following interactions occur; viable cells with intact membranes exclude Annexin V-FITC and PI (i.e., negative for both), whilst cells in the early stage of apoptosis stain positive for Annexin V-FITC and negative for PI. In the case of cells that stain positive for both Annexin V-FITC and PI, these cells are classified as late apoptotic or necrotic as it is possible that they may have died via necrosis or apoptosis. In the case of late apoptotic/necrotic events, it is possible to gain more information about the mechanism of cell death by measuring apoptosis over time, as it allows cells to be tracked from Annexin V-FITC and PI negative (viable, or no measurable apoptosis), to Annexin V-FITC positive and PI negative (early apoptosis with intact membranes), and finally to Annexin V-FITC and PI positive (end stage apoptosis and death). The presence of cells with these three phenotypes within a mixed cell

population, or the “movement” of a synchronized cell population through these three stages, suggests apoptosis (Hingorani *et al*., 2011).

Finally, it is proposed by Kawamura and Ye that cells that are undergoing or have recently underwent necrosis stain negative for Annexin V-FITC but positive for PI. It is possible to distinguish these cells from apoptotic cells as a result of the fact that the cell membrane of cells undergoing necrosis being much more permeable to PI than apoptotic cells, resulting in PI staining prior to PS externalizing on the outer surface of the cell membrane (Kawamura and Ye., 2007). In flow cytometry using Annexin V-FITC and P), this necrotic event is not cited to same extent as the other three events (viable, early apoptotic and late apoptotic/necrotic), however the event warrants reporting under the following circumstances; a) where there is a specific Annexin V-FITC negative/PI positive result for a particular treatment group in comparisons to other groups (i.e., the event is not attributable to a sample preparation artefact) and b) where the results are gated for size and granularity consistent with cells (i.e., unwanted particles such as debris from necrotic events is eliminated).

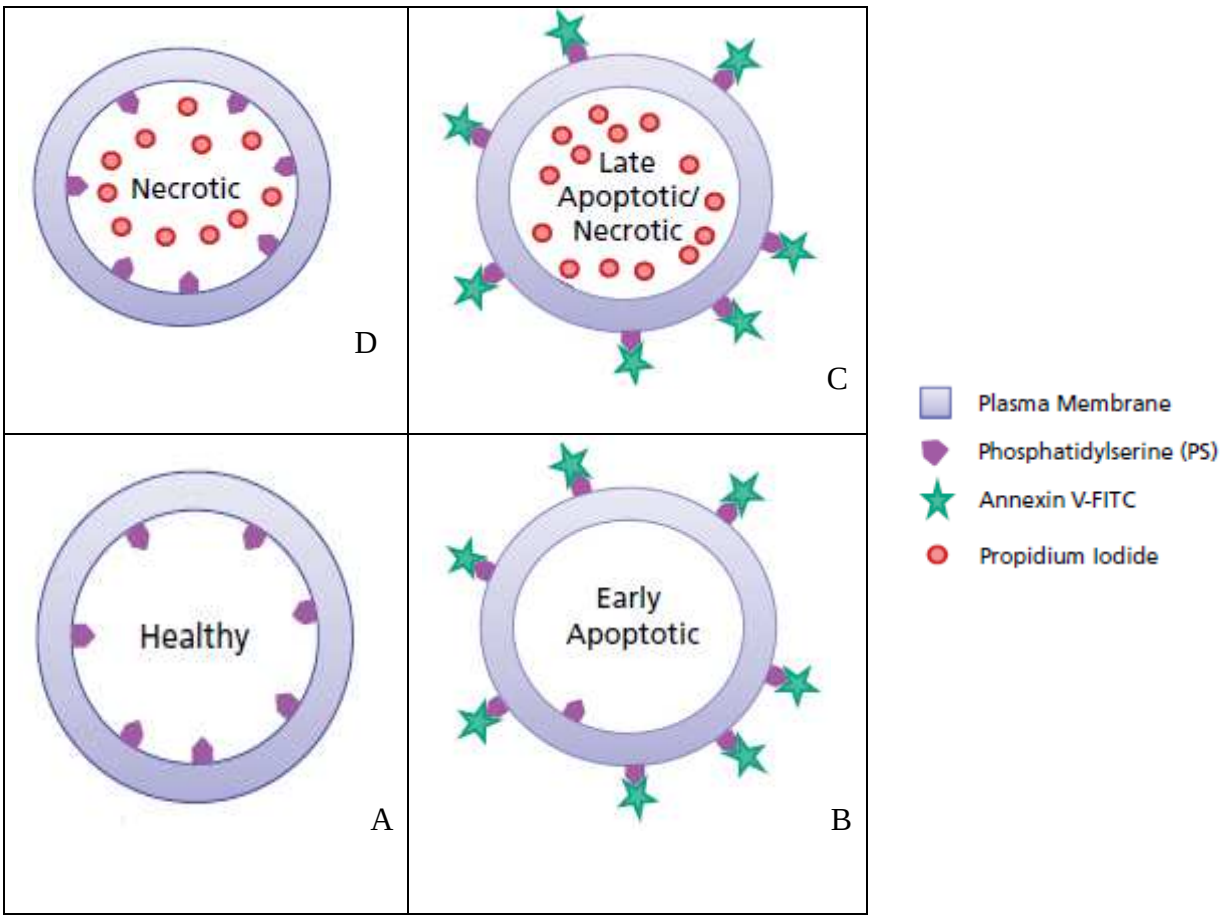

Figure 3.1 Diagram showing viable healthy cells (Annexin V-FITC$^-$ and PI$^-$), early apoptotic cells (Annexin V-FITC$^+$ and PI$^-$) late apoptotic/necrotic cells, (Annexin V-FITC$^+$ and PI$^+$) (Hingorani *et al*., 2011) and necrotic cells (Annexin V-FITC$^-$ and PI$^+$) (Kawamura *et al*., 2007). The diagram includes the markers for the detection of apoptosis and necrosis. The diagram is presented in a quadrant which correlates to the position of these cells in flow ctyometry dot plots (see Figure 3.6). This figure has been adapted from Hingorani and colleagues (Hingorani *et al* 2011).

3.2.5 Cancer metastasis (scratch wound healing assay)

Metastatic disease is the major cause of death in patients with colorectal cancer, and depending on the tumour stage, liver metastases occur in 20% to 70% of patients, while lung metastases occur in 10% to 20% of cases (Shibayama *et al.*, 2011), therefore the metastatic potential of tumour cells (i.e., their ability to migrate and invade) is an extremely important factor for formation of solid tumours and necessary for their spread to distant organs. COX-2 expression is a hallmark of increased metastatic potential in colon cancer cells (Greenhough *et al.,* 2009) and NSAIDs have been shown to abrogate this invasiveness (Tsujii *et al.,* 1997). Cell migration is defined as the movement of individual cells, cell sheets and clusters from one location to another. It is central to a variety of different pathologic and physiologic processes across many disciplines of biology including wound healing, cancer, cell growth and differentiation. (Hulkower and Herber, 2011). There are many complex mechanisms underlying the processes of cell migration and invasion including angiogenesis (physiological process through which new blood vessels are formed from pre-existing vessels). Interestingly, COX-2 inhibition has been suggested to block angiogenesis and CLX has been successfully used in combination with other compounds to block tumour cell migration and invasion *in-vitro* (Zengel *et al.,* 2010).

Scratch wound healing assays have been widely used to assess the effect of drugs, including CLX, on cell migration and proliferation (Erdog *et al*., 2013, Sade *et al*., 2012). The basic principle of the assay is that a ‘scratch’ is inflicted on a monolayer of cells which creates a ‘wound’, followed by monitoring of the ‘healing’ of the wound as a result of cells migrating/growing towards the centre of the wound, thereby closing the wound (Figure 3.2). In the event that the addition of drug (treated cells) prevents cell

migration and proliferation, the % wound closure would be expected to be less than for untreated cells or control-treated cells.

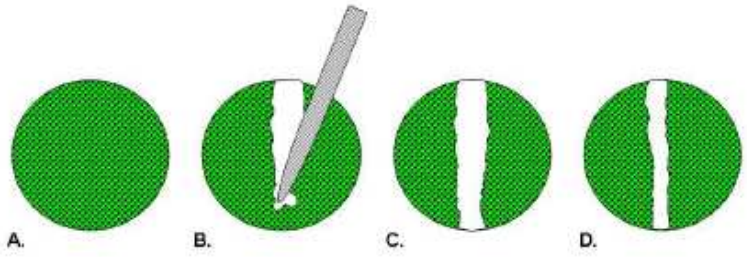

Figure 3.2 Scratch wound healing assay. A) a confluent monolayer of cells are grown in a well, B) a scratch wound is inflicted with a pipette tip, C) the width of the wound is measured prior to incubation with drug and D) the width of the wound is measured after incubation for a specified number of hours. Image adapted from Hulkower and Herber (Hulkower and Herber, 2011).

3.2.6 Objectives

As described in Chapter 1, CRC is the third most common cause of cancer mortality and significant research has been dedicated to identify novel drug targets for CRC prevention and treatment, including the use of COX-2 inhibitors such as CLX (Siegel, *et al.*, 2014). In the context of the overall aim of this project to develop a CLX formulation (in which CLX was pre-solubilised) for the prevention and treatment of CRC, the work presented in this chapter focused on the *in-vitro* evaluation of the effect of liquid CLX formulations (i.e., pre-solubilised CLX) on the viability of the COX-2 expressing colon cancer cell line HT29, compared to the marketed product Celebrex®. The primary aims/objectives of this stage of the project were as follows:

A. Firstly to assess and compare the effects of CLX liquid formulations and Celebrex® on the viability of HT29 using an MTT assay.

B. Secondly to use flow cytometry as a secondary assay to confirm the cell viability results of the MTT assay and also to assess the cell death pathway (i.e., apoptosis and necrosis).

C. The third objective was to assess and compare the effect CLX of liquid formulations and Celebrex® on the metastatic potential of HT29 via a scratch wound healing assay.

D. The fourth objective was to analyse the data obtained from A, B and C above in order to assess if there existed any correlation between the solubility of the formulations and the anti-carcer effect observed and to perform additional drug release studies where applicable to further investigate any possible correlation identified.

E. The fifth and final objective of this stage of the project was to confirm that the liquid formulations tested were suitable precursors for the development of optimal microbead formulations (Chapter 4).

3.3 Materials and methods

3.3.1 Materials

3.3.1.1 Formulation materials

CLX liquid formulations were made using the following materials; Solutol HS-15® (BASF, Germany) and Miglyol® 810N (Sasol, South Africa). A sample of CLX active pharmaceutical ingredient (API) was kindly provided by Erregierre (Italy). The purity of the API was 99.6% based on the COA (certificate of analysis) provided by the supplier.

All chemicals used for the release experiments and HPLC testing were of laboratory grade. Celebrex® was manufactured by Pfizer (USA).

3.3.1.2 *In-vitro* model materials

McCoy's 5A modified medium, L-glutamine, 10% FBS (fetal bovine serum), 1% penicillin/streptomycin, molecular grade DMSO, phosphate buffered saline (PBS) (all Sigma Aldrich, USA), VybrantMTT assay kit (Invitrogen, USA) and Annexin V-FITC/PI Apoptosis Detection Kit (BD Biosciences, USA).

3.3.2 Methods

3.3.2.1 Preparation of liquid formulations

CLX liquid formulations were prepared by dissolving measured quantities of CLX into measured quantities of liquids. Formulations were prepared as one or two component systems (i.e., containing a single liquid vehicle or mixtures of two liquid vehicles).

3.3.2.2 *In-vitro* release testing

Release testing of CLX formulations were performed ($n=3$) at 37°C in purified water (PW). All release experiments were carried out using either a Varian/Vankel VK7010 dissolution apparatus (VanKel, USA) or a Distek Evolution 6300 (Distek, USA) equipped with standard glass vessels and USP type II paddles. Paddle rotating speed in all experiments was 75 rpm. Formulations containing 50 mg of CLX were weighed and added to 1000 ml of the relevant dissolution medium. At specified times 1.8 ml samples

were withdrawn, filtered through a 70 µm pore filter (QLA, USA) and analysed using a high performance liquid chromatography (HPLC) method. The % of drug released at particular time points was determined from peak areas which were calculated against a single point external reference standard.

3.3.2.3 HPLC method

The HPLC method for the analysis of the release and assay samples was adapted from Saha and colleagues (Saha *et al*., 2002). The HPLC column used was a reverse phase 4.6 x 250 mm Inertsil® C8 column (Inertsil, The Netherlands) with 5 µm particles. The mobile phase was acetonitrile:water (65:35). The isocratic method used a flow rate of 1.25 ml/min and ultraviolet (UV) detection at 230 nm. The injection volume was 20 µl and the retention time was 8 min. The HPLC apparatus that was used for the analysis were Thermo Finnigan (Thermo Electron Corporation, USA) and Waters (Waters, USA) HPLC systems (and associated Chromquest and Empower software).

3.3.2.4 Cell culture

The HT29 cell line was grown in McCoy's 5A modified medium supplemented with 1.5 mM L-glutamine, 10% FBS (fetal bovine serum) and 1% penicillin/streptomycin. The cells were grown in a humidified atmosphere containing 5% CO_2.

3.3.2.5 Cell viability – MTT assay

Cell viability was measured using the VybrantMTT assay kit according to the manufacturer's guidelines. HT29 cells (10,000) were plated in 100 μl of complete McCoy's 5A media in 96-well tissue culture dishes. After 24 h cells were treated with CLX (CLX liquid formulations, Celebrex® and CLX dissolved in DMSO) at 20, 30, 50 and 100 μM (*n=6* for each concentration). In the case of CLX dissolved in DMSO, the CLX was dissolved overnight in DMSO before treatment. The working concentration of DMSO in all treatments involving DMSO was <0.1%. Two control groups were employed, one which involved no treatment (referred to as media) and the other which involved treatment with DMSO and media (referred to as DMSO). After 72 h, the MTT labelling reagent (10 µl) was added and incubated for 3 h, followed by solubilisation with DMSO. The absorbance (*A*) was determined in a BioRad 680 microplate reader (BioRad, USA) at 550 nm. The effect of a placebo formulation (i.e., no CLX) on the viability of HT29 cells was also assessed (using the procedure described above) by treating cells with excipient mixtures equating to quantities of excipients that were present in the equivalent 20 and 50 μM CLX liquid formulations. The same passage number of cells was used for both the active and placebo experiments. In the case of both experiments treatments were compared to controls which were set to 100% to reveal relative viability.

3.3.2.6 Cell viability and apoptosis assay (flow cytometry)

HT29 cells were seeded at 5 x 10^5 cells/mL in 10% FBS-supplemented medium prior to treatment with CLX at 50 µM (CLX liquid formulations, Celebrex® and CLX dissolved in DMSO) for a period of 72 h. Two control groups were employed, one which involved

no treatment (referred to as media) and the other which involved treatment with DMSO dissolved in media (referred to as DMSO). Cell viability and the onset of apoptosis was monitored using an AnnexinV-FITC/PI Apoptosis Detection Kit which contains recombinant Annexin V-fluorochrome FITC conjugate and the vital dye (PI) followed by flow cytometry on a FACSCalibur system using CellQuest software (BD Biosciences, USA) . Data for at least 10,000 events were collected for each treatment made and two-dimensional plots of Annexin V-FITC versus PI were generated from gated populations consistent with size and granularity of HT29 cells (as determined using forward and side light scatter).

3.3.2.7 *In-vitro* scratch wound healing assay

Cellular motility was measured by an *in-vitro* scratch wound healing assay. HT29 cells were seeded in six-well plates and incubated until they were 90% confluent. The monolayer of cells was scratched vertically down the plate with a sterile pipette tip and debris was removed from the culture by washing twice with PBS. Images were captured immediately after wounding, with a Nikon Eclipse Ti optical inverted microscope with 4X objective (Nikon, Japan). The cells were then incubated in complete medium with or without CLX at 50 μM (CLX liquid formulations, Celebrex® and CLX dissolved in DMSO). Two control groups were employed, one which involved no treatment (referred to as media) and the other which involved treatment with DMSO dissolved in media (referred to as DMSO). Wound closure was monitored microscopically after the wound persisted for 72 h. Scratch width before and after healing was measured (n=9) and compared to the controls. The percentage wound closure between the wound edges were analysed using Nikon NIS microscope imaging software. The experiments were performed with a minimum of three replicates.

3.3.2.8 Statistical analysis

Statistical analysis was performed using GraphPad Prism (La Jolla, USA). Results are presented as mean ± SEM (n=3-9). Statistical significance was determined by ANOVA using the Dunnett's test for multiple comparisons to the relevant control (*** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$).

3.4 Results and discussion

3.4.1 CLX liquid formulations

Based on the findings detailed in Chapter 2, two liquid formulations (CLX 016/L and CLX 021/L) were selected for progression to the *in-vitro* cell culture study described here. The composition of formulations CLX 016/L and CLX 021/L are detailed in Table 3.1 below.

Table 3.1 Comparison of the composition of formulations CLX 016/L and CLX 021/L

Formulations		**CLX 016/L**	**CLX 021/L**
Component Concentration (mg/g)	**Celecoxib**	100.00	66.67
	Solutol HS-15	900.00	600.00
	Miglyol 810N	-	333.33

3.4.2 Effects of CLX formulations on the viability of HT29 cells

The effect of CLX liquid formulations, CLX 016/L and CLX 021/L, on the viability of HT29 colorectal cancer cells was assessed and compared to that of the marketed CLX product Celebrex®. In addition to the two control groups (media and DMSO), cells were treated with CLX formulations CLX 016/L and CLX 021/L, Celebrex® and CLX API (dissolved in DMSO) at 20, 30, 50 and 100 μM for a period of 72 h. The results for the two control groups were comparable. It was observed that formulations CLX 016/L and CLX 021/L resulted in a reduction in cell viability by approximately 60–70% (compared to media) in the concentration range of 20–100 μM whereas in contrast CLX API (compared to DMSO) and Celebrex® (compared to media) reduced cell viability by only 30–40 % across the same concentration range (Figure 3.3).

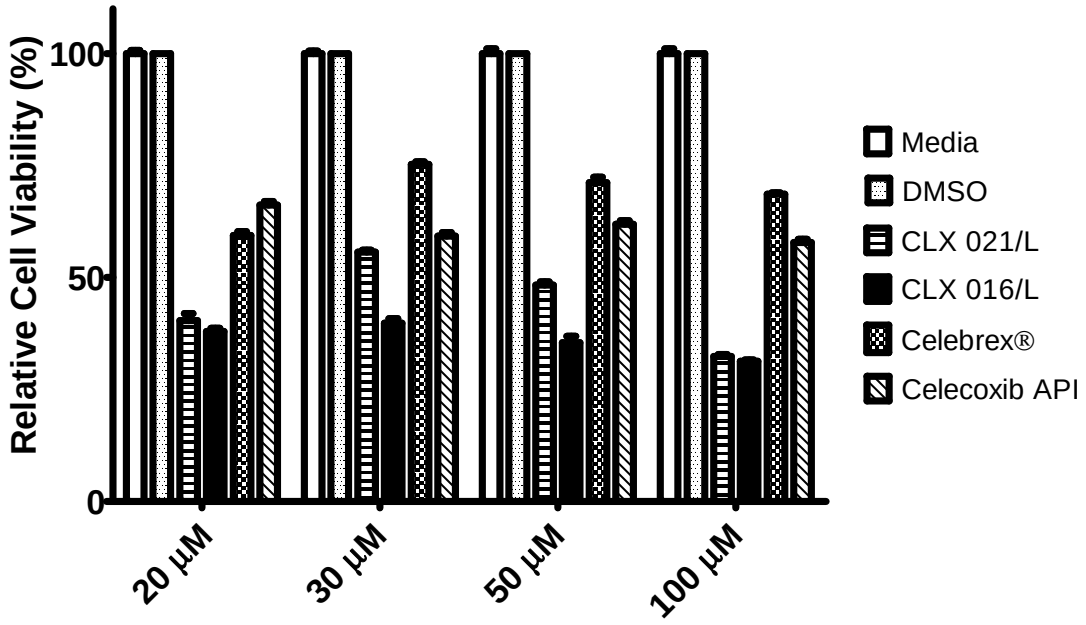

Figure 3.3 The effect of CLX on the viability of HT29 cells. (A) HT29 cells were plated in 96-well plates and after 24 h the cells were treated with CLX (CLX liquid formulations, Celebrex® and CLX dissolved in molecular grade DMSO) at 20, 30, 50 and 100 μM (*n=6* for each concentration) for 72h. Cellular viability was determined by MTT assay.

As outlined in the objectives, the first aim of this phase of the project was to compare the effects of treatment with liquid CLX formulations to that of Celebrex® on the viability of a COX-2 expressing CRC cell line, HT29. CLX liquid formulations were used as they represented a precursor to microbead formulations and were also amenable for direct application to the cells (in contrast to testing with microbeads which was unviable due to the quantity of media required to hydrate and dissolve the microbeads). The primary hypothesis of this phase of the project was that presenting CLX to the cells in a stable soluble form would allow for maximum distribution of CLX to the cells (and hence interaction) and consequently would allow for a greater inhibition of cellular viability. The results observed from the MTT assay (Figure 3.3) agree with this hypothesis, wherein both CLX liquid formulations (CLX 021/L and CLX 016/L) inhibited HT29 viability (approximately 60–70% across the range from 20 µM to 100 µM) to a much greater extent than the current marketed product Celebrex® (reduction of cell viability in the region of 30–40% across the same concentration range). It has been reported that maximum anti-cancer effects of Celebrex® are enhanced at higher doses such as 800 mg/day (Steinbach *et al*., 2000) whereas the recommended dosage of Celebrex® for its anti-inflammatory indication is 200–400 mg/day (Celebrex® Rxlist, 2014) and that the risk of GI and CV side effects are greater at higher doses (discussed at length in Chapter 1). The MTT assay results presented here reveal the possibility for using a reduced dose of CLX to exert an anti-cancer effect with a consequent reduction or elimination of these unwanted side effects. The study also revealed that CLX dissolved in DMSOhad an enhanced inhibitory effect compared to Celebrex®. IC_{50} concentrations were not established for the formulations across the concentration range tested, however the primary objective was to observe differences in the inhibitory effect of the various formulations rather than establishing the IC_{50} (which based on the results would require a broader range of drug concentrations).

In order to confirm that the inhibitory effect of the CLX liquid formulations were related to the activity of the drug (CLX) as opposed to the excipients, a placebo formulation (excipient mixture of Solutol® HS-15 and Miglyol 810N®) was prepared and cells were treated with an excipient mixture equating to quantities of excipients that were present in the equivalent 20 and 50 μM formulation of CLX 021/L (see Table 3.1 for concentration of excipients employed). Formulation CLX 021/L was selected for comparison on the basis that it contained both Solutol® HS-15 and Miglyol 810N®. Although there was some inhibitory effect observed for the placebo formulation, it is shown in Figure 3.4 that the placebo formulation did not have the same inhibitory effect as the formulations containing CLX, with the placebo formulation resulting in approximately a 1.25 fold decrease in the number of viable cells whereas formulation CLX 021/L resulted in approximately a 2.5 fold decrease.

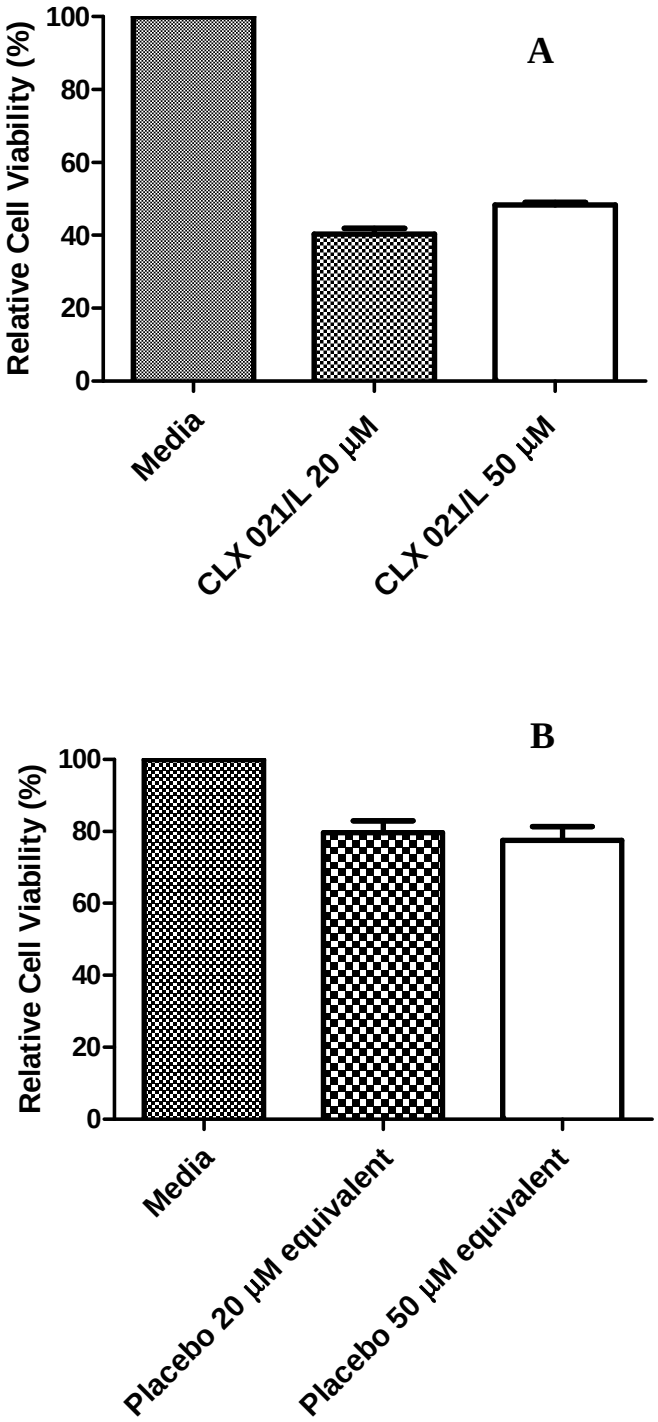

Figure 3.4 A comparison of the effect of placebo and CLX formulations on the viability of HT29 cells (the same cell passage number was used for both placebo and active experiments). HT29 cells were plated in 96-well plates and after 24 h the cells were treated with (A) CLX liquid formulation CLX 021/L at 20 and 50 μM (*n=6* for each concentration) for 72h and (B) a placebo excipient mixture at equivalent 20 and

50 μM concentrations (*n=6* for each concentration) for 72h. Cellular viability was determined by MTT assay. The placebo formulation resulted in a 1.25 fold decrease in the number of viable cells, whereas formuation CLX 021/L resulted in a 2.5 fold decrease.

3.4.3 Effects of CLX formulations on cell viability and apoptosis

Cell viability and the onset of apoptosis were assessed via flow cytometry. In addition to the two control groups (media and DMSO), cells were treated with CLX formulations, CLX 016/L and CLX 021/L, Celebrex® and CLX API (dissolved in DMSO) at 50 μM for a period of 72 h (*n=6*). Relative to the controls (media in the case of CLX 016/L, CLX 021/L and Celebrex® and DMSO in the case of CLX API), it was observed that the % of viable cells for formulations CLX 021/L and CLX 016/L were significantly reduced whereas in the case of Celebrex® there was no significant reduction in the % of viable cells (Figure 3.5). The % viable cells in the case of formulation CLX 021/L (59.6 ± 2.59%) was lower than that for formulation CLX 016/L (70.51% ± 3.99%). The % viable cells for CLX API (69.02 ± 2.71%) was higher than that for formulation CLX 021/L but was comparable to formulation CLX 016/L, however the % of necrotic death for CLX API (13.96 ± 2.48%) was significantly higher than for any of the other formulations tested. The % of cells undergoing early apoptosis for formulation CLX 021/L and formulation CLX 016/L were 22.08 ± 0.92% and 10.77 ± 1.84% respectively, which was higher than for CLX API (6.30 ± 1.42%) and notably higher than that achieved for Celebrex® (3.91 ± 0.83%). An example of one of the dot plots from which this data was obtained is shown in Figure 3.6. It was noted that in the case of formulations CLX 021/L and CLX 016/L, that the cell culture media remained transparent following treatment with the drug but that the media was or became cloudy in the case of treatments with Celebrex® and CLX API (dissolved in DMSO). In the case of CLX API, the media was not initially cloudy on application of the drug but

appeared cloudy when observed at 72 h as result of drug precipitating out of solution (the onset time for precipitation is unknown) whereas in the case of Celebrex®, the media became cloudy immediately on application of the treatment as Celebrex® did not dissolve in the media.

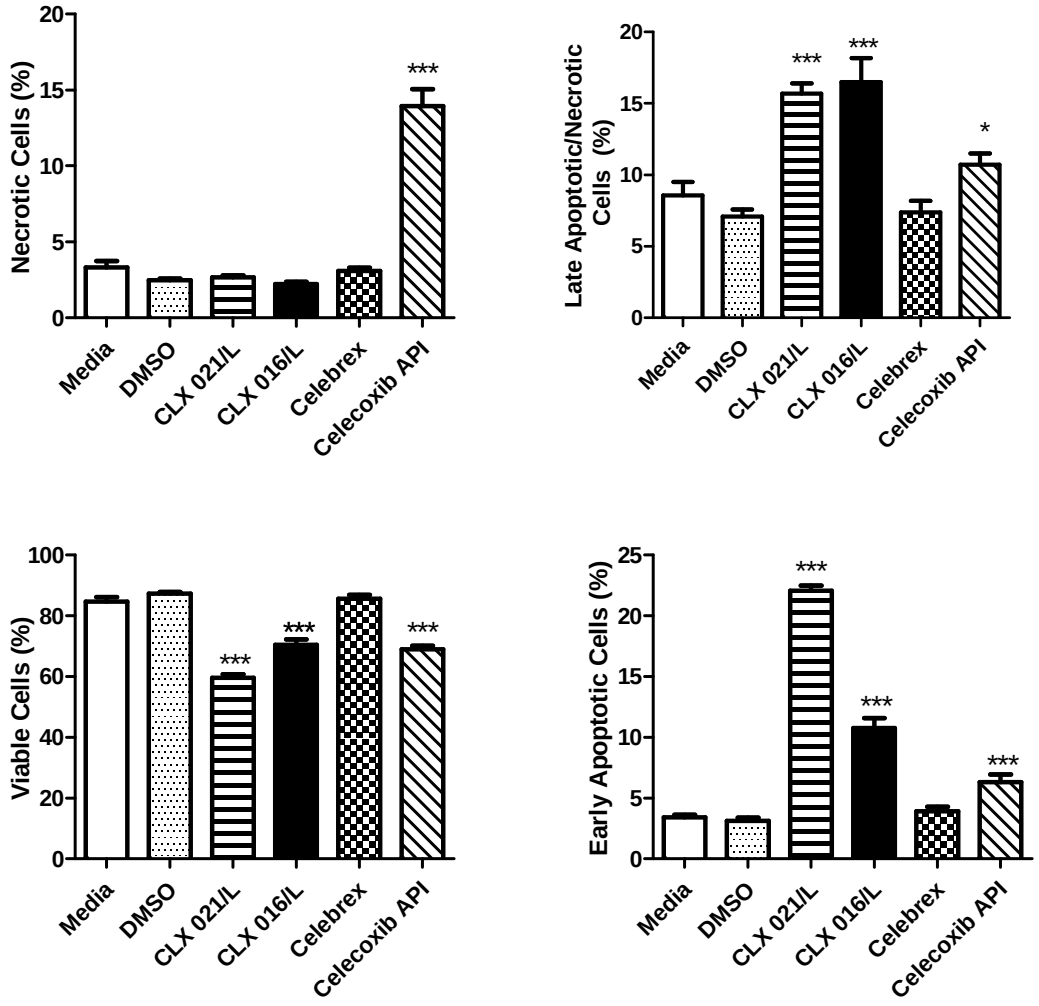

Figure 3.5 HT29 cells were plated in 24-well plates 24 h prior to treatment with CLX at 50µM (CLX liquid formulations, Celebrex® and CLX dissolved in DMSO). Seventy two hours later, cells were double stained with recombinant Annexin V-FITC conjugate and PI and survival profiles monitored by flow cytometry. Viable cells (Annexin V-FITC$^-$ and PI$^-$), early apoptotic cells (Annexin V-FITC$^+$ and PI$^-$), late apoptotic/necrotic cells (Annexin V-FITC$^+$ and PI$^+$) (Hingorani *et al*., 2011) and necrotic cells (Annexin V-FITC$^-$ and PI$^+$) (Kawamura *et al*., 2007), were plotted as a percentage of the total population for each

treatment (n = 6). Data for 10,000 events was collected for each replicate. Statistics were performed relative to media for formulations CLX 021/L, CLX 016/L and Celebrex® and relative to DMSO in the case of CLX API.

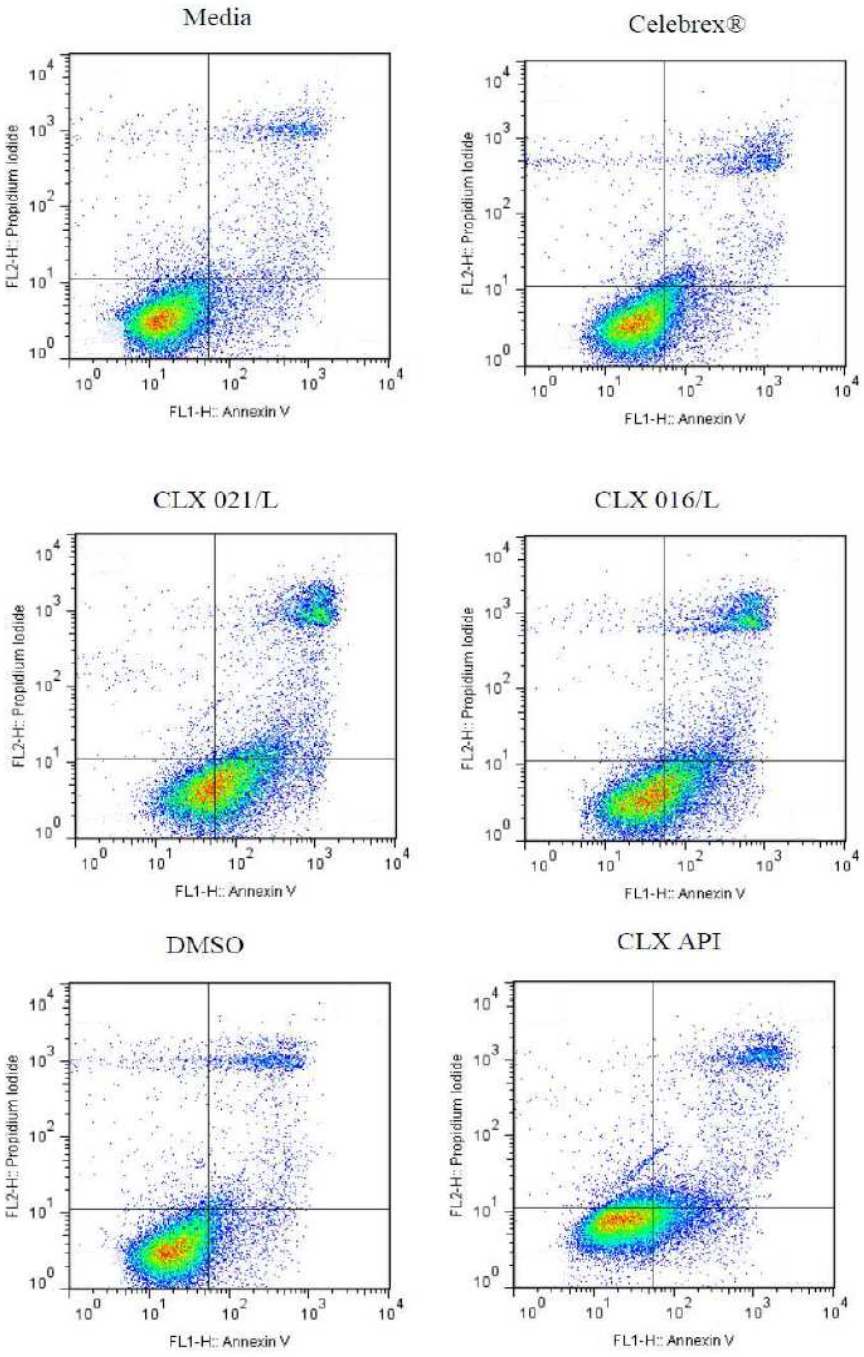

Figure 3.6 2-D dot plots of Annexin V FITC-PI flow cytometry of HT29 cells following treatment with CLX at 50µM (Celebrex, CLX 021/L, CLX 016/L and CLX API (dissolved in DMSO)). There were two controls (media alone and DMSO). Seventy two hours after treatment, cells were double stained with recombinant Annexin V-FITC conjugate and PI. The lower left quadrant represents viable cells cytometry

(Annexin V-FITC$^-$ and PI$^-$), the lower right hand quadrant represents early apoptotic cells (Annexin V-FITC$^+$ and PI$^-$), the upper right quadrant represents late apoptotic/necrotic cells (Annexin V -FITC$^+$ and PI$^+$) and the upper left hand quadrant represents necrotic cells (Annexin V-FITC$^-$ and PI$^+$). One representative experiment is shown.

One of the aims of the cell viability and apoptosis assay was to verify the MTT assay results described in Section 3.4.2. CLX formulations CLX 016/L and CLX 021/L were again demonstrated to exert a greater effect than Celebrex® with respect to their inhibitory effect on HT29 cells relative to a control (Figures 3.5 and 3.6). The data also revealed interesting findings with respect to the mechanisms of inhibition. In the case of Celebrex®, in addition to the observation that there was no significant impact on cell viability, there was no significant necrotic (passive cell death) or apoptotic (controlled cell death) effect observed. There exists a theory that the cancer killing effect of CLX on CRC cell lines may be related to direct cytotoxicty (resulting from irreversible binding and damage to the plasma membrane by CLX precipitates) as opposed to molecular toxicity (Sacchetti, 2013). Based on this theory, it could be argued that the concentration of Celebrex® used (50 µM) was not sufficient to result in direct cytotoxicty. In contrast, in the case of CLX API dissolved in DMSO, a significant necrotic effect was observed which is consistent with the direct cytotoxicity theory. It is postulated that by pre-dissolving CLX in DMSO that the drug was allowed to seed onto the cells prior to exerting a cytotoxic effect for example via direct damage to the plasma membrane. The capacity of precipitates of CLX to damage cellular membranes has previously been shown by Sacchetti [14]. Given that the CLX API formulation (pre dissolved in DMSO) was observed to precipitate in the cell culture media, it is proposed that the precipitation of the drug allowed it to exert a strong necrotic effect in contrast to formulations A and B (neither of which were observed to precipitate in the media) and

also Celebrex® which was never in solution and therefore did not get an opportunity to seed onto the monolayer of cells In strong contrast to the results obtained for Celebrex® and CLX API there was a significant inhibitory effect observed for both formulations CLX 021/L and CLX 016/L without exerting a significant necrotic effect. The strong apoptotic effect for formulations CLX 021/L and CLX 016/L is very important as it suggests that these formulations caused HT29 cell death via molecular mechanisms and thereby is a new development on the data presented by Sacchetti in which it was found that *in-vitro* cell death for CRC cell lines only occurred at insoluble concentrations of CLX. The data presented here suggests that a molecular toxicity effect is possible for CLX if the drug is optimally presented to the cells (i.e., in a stable solubilised state). In the case of the CLX API dissolved in DMSO, an early apoptotic effect was also observed (albeit less pronounced than for formulations CLX 021/L and CLX 016/L – see Figure 3.5) which suggests some of the API which remained in solution may have been able to exert a toxic effect by molecular mechanisms. The finding that formulations CLX 016/L and CLX 021/L did not exhibit a significant necrotic effect is an important finding given that previous research by Tomisato and colleagues (Tomisato *et al.,* 2004) has shown that NSAIDs including CLX kill cells by both necrosis and apoptosis and that necrosis is linked to unwanted GI side effects.

As the assay performed cannot distinguish between late apoptotic and necrotic cells in the case of cells staining positive for both Annexin V-FITC and PI (see Section 3.2.4), these results are not discussed as it would be necessary to perform a time course experiment to track the movement of cells through the stages of viable cells, early apoptotic cells and late apoptotic cells in order to fully distinguish late apoptotic cells from necrotic cells. Also while the necrotic effect of the various formulations are discussed here, it is acknowledged that further studies involving a time course annexin V/PI apoptosis assay, microscopic analysis or an alternative apoptotic assay (e.g.,

TUNEL - terminal deoxynucleotidyl transferase dUTP nick end labelling) could be performed to support the proposed necrotic effect observations for CLX API.

3.4.4 Effects of CLX formulations on the motility of HT29 cells

In order to examine the effects of CLX liquid formulations CLX 016/L and CLX 021/L on the motility of HT29 cells in comparison to Celebrex®, an *in-vitro* scratch wound healing assay was performed. In addition to the two control groups (media and DMSO), cells were treated with CLX formulations, CLX 016/L and CLX 021/L, Celebrex® and CLX API (dissolved in DMSO) at 50 μM for a period of 72 h after the scratch wound was inflicted. Figure 3.7 displays a sample of images of the wound on the day of application and after 72 h of incubation for illustrative purposes. As seen in the histogram in Figure 3.8, treatment with formulations CLX 021/L and CLX 016/L significantly reduced the % wound closure relative to the control (media in the case of CLX 016/L, CLX 021/L and Celebrex®); which indicated a loss in the motility of the HT29 cells after 72 h. In contrast the % wound closure for Celebrex® and CLX API was not significant. The data also revealed a difference in the effect observed for formulations CLX 021/L and CLX 016/L. As previously stated in Section 3.2.5, the high mortality associated with CRC is related to its ability to spread beyond the large intestine and invade distant sites. Therefore the metastatic potential of tumour cells (i.e., their ability to spread) is an extremely important factor for formation of solid tumours and necessary for their spread to distant organs. As described in the present study, the motility of HT29 cells were examined as a measure of their metastatic potential via the scratch wound healing assay. As with the other assays performed, in contrast to Celebrex®, relative to the control, the CLX liquid formulations CLX 021/L and CLX 016/L had a significant effect, whereby the % wound closure was markedly reduced for

formulations CLX 021/L and CLX 016/L (Figure 3.8), illustrating that these formulations had the potential to reduce the likelihood of CRC cells to metastazize.

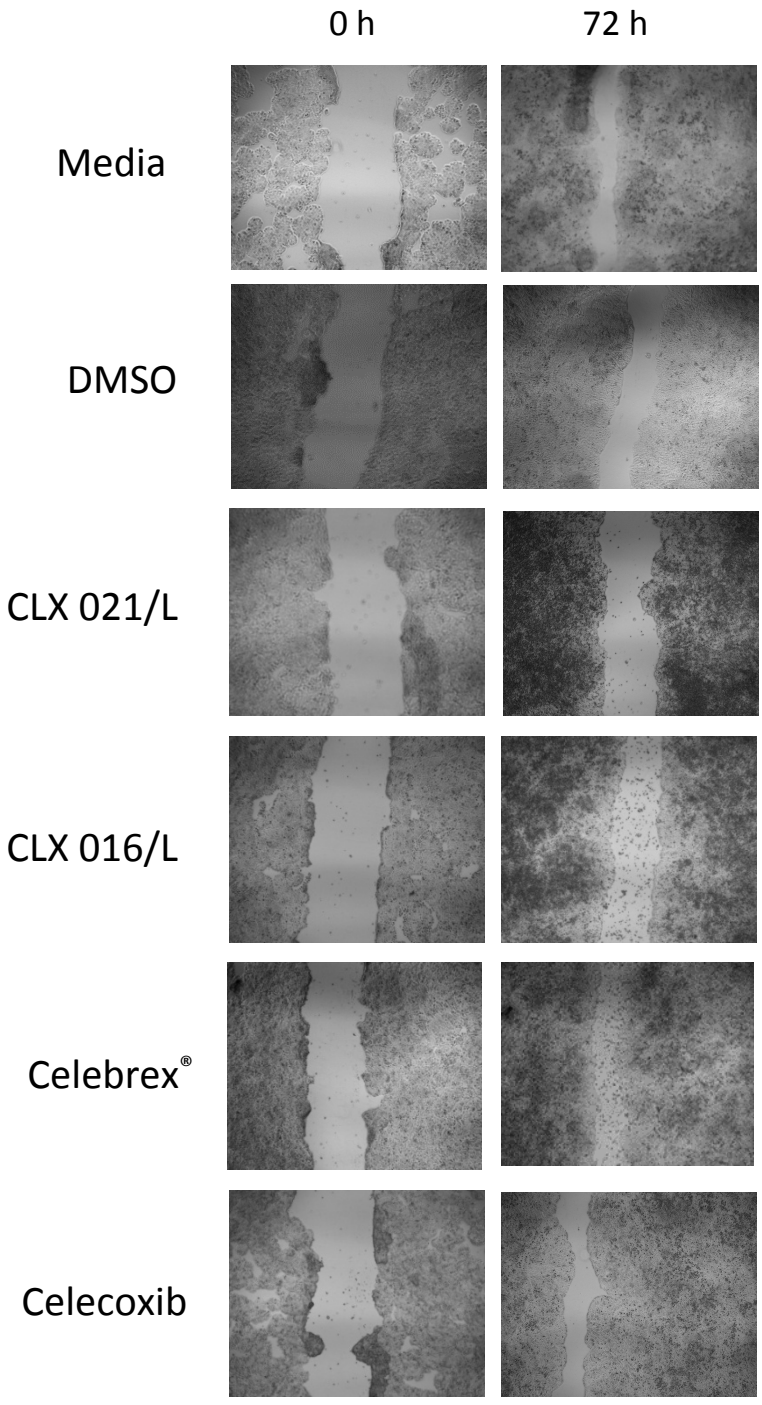

Figure 3.7 The effect of CLX on the motility of HT29 cells. Scratch wound healing assay was conducted and inverted microscope images were captured and analysed (including measurements) immediately after the wound (0 h) and after treatment with CLX at 50 µM (CLX liquid formulations CLX 016/L and CLX 021/L, Celebrex® and CLX dissolved in molecular grade DMSO) (*n=9* for each group) for 72 h. Images for the control group are also shown.

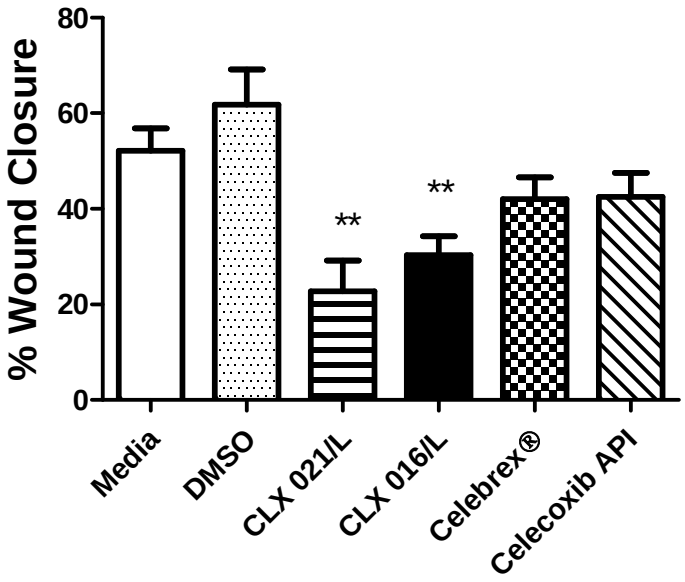

Figure 3.8 The effect of CLX on the motility of HT29 cells. The histogram shows the percentage wound closure for the controls and for the CLX treatments 72 h after the wound persisted. Statistics were performed relative to media for formulations CLX 021/L, CLX 016/L and Celebrex® and relative to DMSO in the case of CLX API.

3.4.5 *In-vitro* drug release studies on CLX liquid formulations

Given the differences in the effects observed for the CLX formulations compared to Celebrex® with respect to the *in-vitro* cell model parameters examined and also the enhanced effect for formulation CLX 021/L compared to formulation CLX 016/L, an *in-vitro* drug release study was performed over a period of 12 h to study the release of CLX and to assess whether a correlation existed between drug release performance and the performance of the various formulations in the *in-vitro* cell model (previous to this, release analysis had only been performed over a period of 6 h). Figure 3.9 shows the *in-vitro* CLX release profiles of formulation CLX 016/L, formulation CLX 021/L,

Celebrex® and CLX API (pre dissolved in DMSO). Consistent with the *in-vitro* cell model data, formulations CLX 016/L and CLX 021/L markedly outperformed both Celebrex® and CLX API. Interestingly the % release for formulation CLX 021/L remained steady over the 12 h period at 98.83 ± 0.75%, whereas in the case of formulation CLX 016/L, CLX was observed to have started to precipitate by 6 h and at 12 h the % of drug which remained dispersed in the mediahad reduced to 59.23 ± 9.90%. CLX API (pre dissolved in DMSO) was also observed to precipitate almost immediately upon contact with the dissolution media.

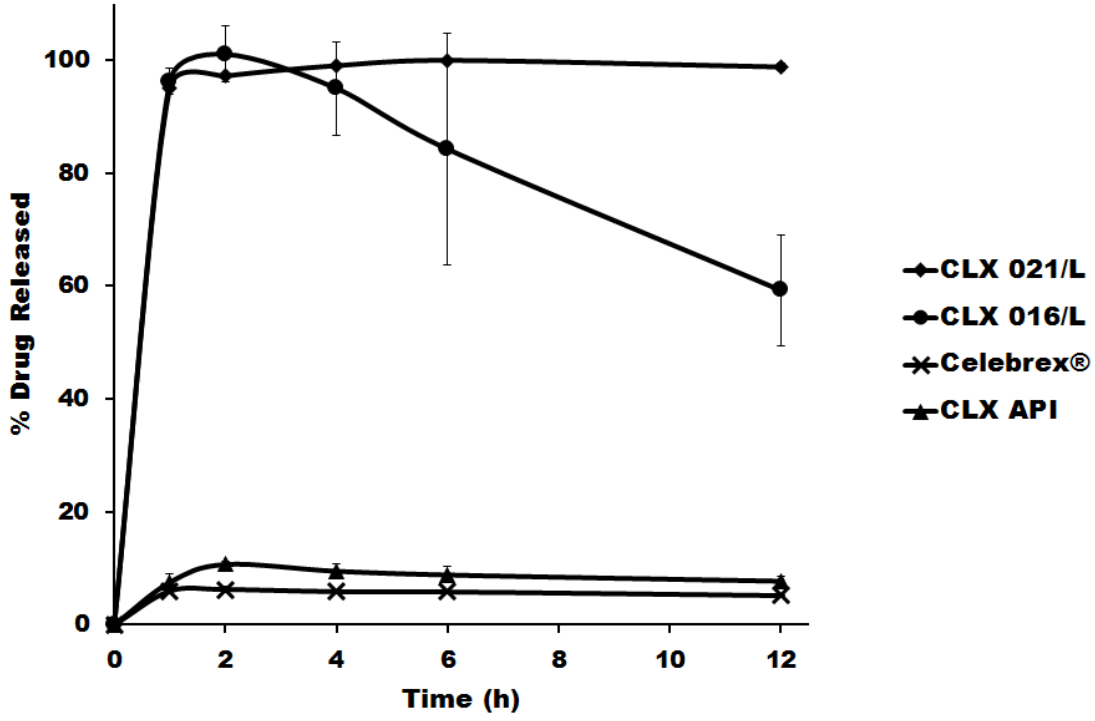

Figure 3.9 Percentage of drug released from release testing of formulation CLX 016/L, formulation CLX 021/L, Celebrex and CLX API (all tested in PW over a period of 12 h). The data presented are mean values ± STDEV (n=3).

In the case of both the cell viability/apoptosis assay and the scratch wound healing assay, formulation CLX 021/L was observed to have had a greater effect compared to formulation CLX 016/L. The *in-vitro* drug release test performed in PW therefore revealed a correlation between % of drug released (and which remained in solution) (Figure 3.9) and the performance of the formulation with respect to the *in-vitro* cell culture model. This is an important finding as it identifies the use of release testing in PW as a simple and effective tool for screening and selecting stable CLX formulations. It was also notable that CLX API, dissolved in DMSO, performed marginally better than Celebrex® with respect to its drug release performance which was also the case for the *in-vitro* cell culture experiments.

3.5 Conclusions

In this phase of the project it was shown that CLX liquid formulations performed significantly better than the marketed CLX product Celebrex® with respect to their ability to inhibit the viability and motility of a HT29 CRC cell line *in-vitro*. Whilst focusing on only one cell line (HT29), it was also demonstrated that liquid CLX formulations had an apoptotic effect on HT29 cells, whereas CLX API alone had both a necrotic and apoptotic effect, which was an important finding as it presented the opportunity for targeted CLX therapy with reduced GI side effects for which there is an obvious unmet clinical need. Although the exact mechanisms for the anticancer activity of CLX are unclear, a wide array of tumour-associated moleculat events have been shown to be modulated by CLX in *in-vitro* assays. It has been proposed that the mechanisms of action include the induction of apoptosis, cell arrest cycle, regulation of

angiogenesis and the induction of endoplasmic reticulum stress. This study demonstrated that the liquid CLX formulations developed had a greater cytotoxic effect on HT29 CRC cells in comparison to Celebrex® and that the mechanism of cell death was predominatly via apoptosis. It is proposed here that the administration of CLX to these cancer cells targets proteins (e.g., peroxisome proliferator-activated receptor (PPAR) involved in the apoptosis pathway and has the ability to to switch off the survival signals that these cells depend on (Yang and Frucht, 20001 and Gong *et al.*, 2012).

A correlation between the drug release performance of CLX formulations in PW and their ability to affect HT29 cells was also observed, thereby presenting an effective tool for formulation screening. CLX liquid formulations were used for this *in-vitro* cell study as they represented a precursor to microbead formulations and were also amenable for direct application to the cells. In Chapter 2, a feasibility study was performed to assess the potential of the liquid formulations produced to be converted into microbeads. The feasibility study described in Chapter 2 revealed that it was possible to make microbeads from a formulation consisting of CLX dissolved in Solutol® HS-15 and Miglyol® 810N. Given the performance of formulations CLX 016/L (formulation containing Solutol® HS-15) and CLX 021/L (formulation containing a mixture of Solutol® HS-15 and Miglyol® 810N) in the *in-vitro* cell culture study, their suitability with respect to excipient selection was confirmed. The next phase of the project (Chapter 4) focused on the translation of these formulations into optimal microbead formulations.

3.6 Nomenclature

Table 3.2 List of abbreviations which are listed according to their appearance in the text.

Abbreviation	Definition
CLX	Celecoxib
COX-2	Cycloxygenase 2
CRC	Colorectal cancer
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NADP	Nicotinamide adenine dinucleotide phosphate
DMSO	Dimethyl sulfoxide
PS	Phosphatidyl serine
FITC	Fluorescein isothiocyanate
PI	Propidium iodide
DNA	Deoxyribonucleic acid
API	Active pharmaceutical ingredient
COA	Certificate of analysis
PBS	Phosphate buffered saline
HPLC	High performance liquid chromatography
FBS	Fetal bovine serum
PW	Purified water
UV	Ultraviolet
SEM	Standard error of the mean
STDEV	Standard deviation
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
CLX xxx/L	Celecoxib liquid formulation numbering system where xxx is a sequential number and L is liquid

3.7 Acknowledgments

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3.8 References

Berridge, M.V. & Tan, A.S. 1993, "Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction", *Archives of Biochemistry and Biophysics*, vol. 303, no. 2, pp. 474-482.

Boolbol, S.K., Dannenberg, A.J., Chadburn, A., Martucci, C., Guo, X.J., Ramonetti, J.T., Abreu-Goris, M., Newmark, H.L., Lipkin, M.L., DeCosse, J.J. & Bertagnolli, M.M. 1996, "Cyclooxygenase-2 overexpression and tumor formation are blocked by sulindac in a murine model of familial adenomatous polyposis", *Cancer Research,* vol. 56, no. 11, pp. 2556-2560.

Buecher, B., Bouancheau, D., Broquet, A., Bezieau, S., Denis, M.G., Bonnet, C., Heymann, M.F., Jarry, A., Galmiche, J.P. & Blottiere, H.M. 2005, "Growth inhibitory effect of celecoxib and rofecoxib on human colorectal carcinoma cell lines", *Anticancer Research,* vol. 25, no. 1A, pp. 225-233.

Eberhart, C.E., Coffey, R.J., Radhika, A., Giardiello, F.M., Ferrenbach, S. & DuBois, R.N. 1994, "Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas", *Gastroenterology,* vol. 107, no. 4, pp. 1183-1188.

Erdog, A., Putra Limasale, Y.D., Keskin, D., Tezcaner, A. & Banerjee, S. 2013, "In vitro characterization of a liposomal formulation of celecoxib containing 1,2-distearoyl-sn-glycero-3-phosphocholine, cholesterol, and polyethylene glycol and its functional effects against colorectal cancer cell lines", *Journal of Pharmaceutical Sciences*, vol. 102, no. 10, pp. 3666-3677.

Ettarh, R., Cullen, A. & Calamai, A. 2010, "NSAIDs and Cell Proliferation in Colorectal Cancer", *Pharmaceuticals,* vol. 3, no. 7, pp. 2007-2021.

Frei, M. 2011, "Cell Viability and Proliferation", *Biofiles,* vol. 6, no. 5, pp. 17-21.

Greenhough, A., Smartt, H.J., Moore, A.E., Roberts, H.R., Williams, A.C., Paraskeva, C. & Kaidi, A. 2009, "The COX-2/PGE2 pathway: key roles in the hallmarks of cancer and adaptation to the tumour microenvironment", *Carcinogenesis,* vol. 30, no. 3, pp. 377-386.

Gong, L., Thorn, C.F., Bertagnolli, M.M., Grosser, T., Altman, R.B., and Klein, T.E., 2012, "Celecoxib pathways: pharmacokinetics and pharmacodynamics", *Pharmacogenet Genomics,* vol. 22, no. 4, pp. 310-318.

Hingorani, R., Deng, J., Elia, J., McIntyre, C. & Mittar, D. August 2011, "Detection of Apoptosis Using the BD Annexin V FTIC Assay on the BD FACSVerse™ System", *BD Biosciences Application Note,* .

Hulkower, K.I. & Herber, R.L. 2011, "Cell migration and invasion assays as tools for drug discovery", *Pharmaceutics,* vol. 3, no. 1, pp. 107-124.

Kawamura, K. & Ye, Y. 2007, "Recent progress in the studies of molecular mechanisms of apoptosis in mammalian preimplantation embryo" in *Cell Apoptosis Research Advances,* ed. C.R. Kettleworth, Nova Science Publishers Inc., New York, pp. 145-177.

Loughran, S.T. 2007, Expression and role of the human anti-apoptotic bfl-1 gene in Hodgkin's Lymphoma. PhD Thesis, Dublin City University, Dublin Ireland.

Rutsky, L.P. & Moyer, M.P. 1990, "7 - Human Cell Lines in Colon Cancer Research" in *Colon Cancer Cells,* ed. M.P.M.H. Poste, Academic Press, pp. 155-202.

Sacchetti, A. 2013, "Cancer cell killing by Celecoxib: reality or just in vitro precipitation-related artifact?", *Journal of Cellular Biochemistry,* vol. 114, no. 6, pp. 1434-1444.

Sade, A., Tuncay, S., Cimen, I., Severcan, F. & Banerjee, S. 2012, "Celecoxib reduces fluidity and decreases metastatic potential of colon cancer cell lines irrespective of COX-2 expression", *Bioscience Reports,* vol. 32, no. 1, pp. 35-44.

Saha, R.N., Sajeev, C., Jadhav, P.R., Patil, S.P. & Srinivasan, N. 2002, "Determination of celecoxib in pharmaceutical formulations using UV spectrophotometry and liquid chromatography", *Journal of Pharmaceutical and Biomedical Analysis,* vol. 28, no. 3-4, pp. 741-751.

Shao, J., Sheng, H., Aramandla, R., Pereira, M.A., Lubet, R.A., Hawk, E., Grogan, L., Kirsch, I.R., Washington, M.K., Beauchamp, R.D. & DuBois, R.N. 1999, "Coordinate regulation of cyclooxygenase-2 and TGF-beta1 in replication error-positive colon cancer and azoxymethane-induced rat colonic tumors", *Carcinogenesis,* vol. 20, no. 2, pp. 185-191.

Shibayama, M., Maak, M., Nitsche, U., Gotoh, K., Rosenberg, R. & Janssen, K.P. 2011, "Prediction of metastasis and recurrence in colorectal cancer based on gene expression analysis: ready for the clinic?", *Cancers,* vol. 3, no. 3, pp. 2858-2869.

Siegel, R., DeSantis, C. & Jemal, A. 2014, "Colorectal cancer statistics, 2014", *CA: A Cancer Journal for Clinicians,* vol. 64, no. 2, pp. 104-117.

Steinbach, G., Lynch, P.M., Phillips, R.K., Wallace, M.H., Hawk, E., Gordon, G.B., Wakabayashi, N., Saunders, B., Shen, Y., Fujimura, T., Su, L.K., Levin, B., Godio, L., Patterson, S., Rodriguez-Bigas, M.A., Jester, S.L., King, K.L., Schumacher, M., Abbruzzese, J., DuBois, R.N., Hittelman, W.N., Zimmerman, S., Sherman, J.W. & Kelloff, G. 2000, "The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis", *The New England Journal of Medicine,* vol. 342, no. 26, pp. 1946-1952.

Tomisato, W., Tsutsumi, S., Hoshino, T., Hwang, H.J., Mio, M., Tsuchiya, T. & Mizushima, T. 2004, "Role of direct cytotoxic effects of NSAIDs in the induction of gastric lesions", *Biochemical Pharmacology,* vol. 67, no. 3, pp. 575-585.

Tsujii, M., Kawano, S. & DuBois, R.N. 1997, "Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential", *Proceedings of the National*

Academy of Sciences of the United States of America, vol. 94, no. 7, pp. 3336-3340.
van Meerloo, J., Kaspers, G.J. & Cloos, J. 2011, "Cell sensitivity assays: the MTT assay", *Methods in Molecular Biology (Clifton, N.J.)*, vol. 731, pp. 237-245.

Yang, W-L and Frucht, H., 2001 “Activation of the PPAR pathway induces apoptosis and COX-2 inhibition in HT-29 human colon cancer cells” *Carcinogenesis*, vol. 22, no. 9, pp. 1379-1383.

Zengel, P., Ramp, D., Mack, B., Zahler, S., Berghaus, A., Muehlenweg, B., Gires, O. & Schmitz, S. 2010, "Multimodal therapy for synergic inhibition of tumour cell invasion and tumour-induced angiogenesis", *BMC cancer*, vol. 10, pp. 92-2407-10-92.

Celebrex Indications and Dosage – Rxlist 2014. Available: http://www.rxlist.com/celebrex-drug/indications-dosage.htm [2014, Oct/10].

HT29 Cell Line Summary - Memorial Sloan Kettering Cancer Centre 2014. Available: www.mskcc.org/research/technology/human-colorectal-adenocarcinoma-cell-line-ht-29 [2014, Oct/10].

CHAPTER 4

Microbead Development

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4.1 Abstract

The purpose of this phase of the project was to develop, optimise and characterise a lipid-based spherical multiparticulate celecoxib (CLX) formulation in which CLX was retained in a fully solubilised form. One of the primary objectives of the project to meet the stated purpose involved developing a robust spherical microbead with sufficient drug loading in which precipitation was absent both during processing and also in the final product. This objective was achieved through a combination of formulation approaches including; excipient substitution, inclusion of precipitation inhibitors and ratio alterations. *In-vitro* drug release and content assay (entrapment efficiency) analysis were the primary tools used to assess and compare formulations. An optimised microbead with a CLX loading of 6% w/w was produced, with an entrapment efficiency of 97% and an *in-vitro* drug release result of 80% over 6 h. The structure of these microbead formulations were characterised and compared using light microscopy which revealed a correlation between droplet size and drug release performance.

4.2 Introduction

4.2.1 Background

At the conclusion of Chapter 3, two CLX lipid-based liquid formulations (formulations consisting of CLX dissolved in Solutol® HS-15 and Miglyol® 810N) were identified as being better than the marketed CLX formulation Celebrex® with respect to both their *in-vitro* physicochemical performance (i.e., release) and *in-vitro* cell culture performance (i.e., inhibitory effect on colorectal cancer (CRC) HT29 cell line). The next phase of the project focused on the translation of these liquid formulations into optimal microbead formulations that would be amenable to colon targeting.

In Chapters 1 and 2, the advantages of oral drug delivery and lipid-based drug delivery systems were outlined respectively. The limitations of the use of conventional dosage forms such as soft gelatine capsules with respect to the delivery of lipid-based formulations were also described in Chapter 2. At the conclusion of Chapter 2, a feasibility study was described in which liquid lipid-based formulations were converted into microbeads via a manual '*dripping'* microencapsulation process. Microencapsulation is defined as a process, which involves the complete envelopment of pre-selected core material(s) within a defined porous or impermeable membrane (shell) using various techniques, to give miniature sized particles (Whelehan, 2010). Depending on the size of the resultant particles, various terminologies can be applied (microcapsules, macrocapsules etc.) which can lead to confusion, however, the term "microcapsule" is often defined as a spherical particle containing a core substance with the size of the microcapsule varying between 2-2000 µm (Singh *et al.,* 2010). This definition distinguishes microcapsules from smaller nanoparticles or nanocapsules (Singh *et al.,* 2010). There are many different examples of microencapsulation processes which include but are not limited to the following; spray drying, spray congealing, hot melt extrusion (HME), coacervation, supercritical CO_2 – assisted microencapsulation, concentric nozzle extrusion and prilling (Particle Sciences Technical Brief, 2010, Umer *et al*., 2011, Ghosh, 2006). A review of microencapsulation techniques specifically relating to lipid-based drug delivery systems is included below. The critical quality attributes (CQAs) of the desired CLX formulation/technology which formed the basis for the selection of the manual '*dripping'* microencapsulation process employed in this study for the production of CLX microbeads is also described. Finally the objectives for this phase of the project are detailed.

4.2.2 Microencapsulation

As described in section 4.2.1, microencapsulation processes yield miniature sized particles ranging in size from 2-2000 μm. These miniature-sized particles are usually referred to as microcapsules or microbeads/microspheres depending on their morphology. Microcapsules or microbeads may have regular or irregular shapes and on the basis of their morphology, they can be classified as mononuclear, polynuclear or matrix types (Figure 4.1).

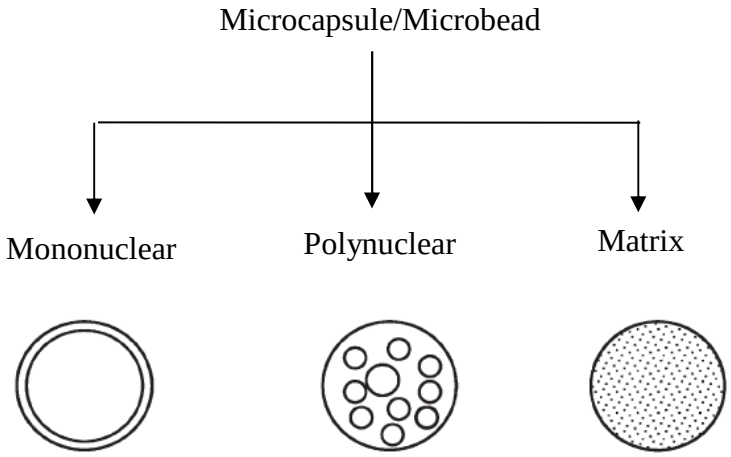

Figure 4.1 Morphology of microcapsules/microbeads. (Diagram adapted from Ghosh, 2006).

The term microcapsule is most commonly used for particles with mononuclear and polynuclear morphologies shown in Figure 4.1. Mononuclear microcapsules are also referred to as core-shell microcapsules as they contain a shell surrounding a single core. In contrast, in the case of matrix encapsulation, the core material is distributed homogenously in the shell material. Particles possessing the matrix morphology shown in Figure 4.1 are commonly referred to as microbeads or microspheres. Microencapsulation has the potential to pose a number of advantages as a drug delivery technology including the following; enhanced shelf life stability (protection of API from

the storage environment (oxygen, humidity etc.)), presentation of a multiparticulate amenable to further processing, potential for inherent controlled and/or targeted drug delivery, safe handling of potent materials (API entrapped from outer surface), taste masking and crucially the ability to handle liquids as solids (Ghosh, 2006, Singh *et al*., 2010, Umer *et al.,* 2011, Particle Sciences Technical Brief, 2010). The advantages of microencapsulation described above met the desired CQAs for a colon targeted CLX formulation as outlined in Chapter 1 (i.e., lipid-based multiparticulate), therefore the next step of the process focused on the selection of the most appropriate microencapsulation technique.

4.2.3 Microencapsulation techniques

There are many techniques available in which core materials can be encapsulated within a shell or matrix. These techniques are typically divided into chemical, physicochemical and mechanical processes although there is considerable overlap between the techniques. Chemical processes include interfacial and *in situ* polymerization methods. Physicochemical processes include coacervation, layer-by layer assembly, sol-gel encapsulation, supercritical fluid-assisted and solvent evaporation. Mechanical processes include spray drying, spray congealing, HME, concentric nozzle extrusion, and finally mechanically aided dripping techniques such as prilling (Ghosh, 2006, Singh *et al*., 2010, Umer *et al.*, 2011, Particle Sciences Technical Brief, 2010). Given that the aim of this project was to encapsulate a liquid lipid core containing CLX and ultimately to develop an acceptable pharmaceutical product and process (i.e., a low cost, high efficiency, reproducible and environmentally acceptable process) the focus of the review below was restricted to the mechanical technologies described as they were considered to have the greatest potential to meet these requirements. Most of the

technologies described are based on the principle of generating droplets from a polymer (liquid form) extruded through a nozzle (orifice) and they work on the basis that a mechanical force (cutting/vibration/atomisation) is applied at the nozzle to break-up the extruded polymer. In all cases with the exception of concentric nozzle extrusion (Section 4.2.3.4), the active drug is dispersed homogenously in the extruded polymer. It should be noted that spray drying, spray congealing and HME can also be used to produce particles consisting of only matrix material and active drug (i.e., no encapsulate), therefore the resultant products are collectively often referred to in terms of solid dispersions rather than using microcapsule terminology.

4.2.3.1 Spray congealing

Spray congealing or spray cooling involves a method in which molten material (e.g., lipid) is sprayed (via atomisation at the nozzle) into a cooling chamber and on contact with cool air, congeals into spherical solid particles (Kalepu *et al.,* 2013). In terms of microencapsulation, the molten material is usually the core, whereas the matrix consists of inert fillers such as lactose. Among the advantages of spray congealing are; a) it does not require the input of aqueous or organic solvents , b) it yields solid particles (often referred to as solid dispersions) that are directly amenable to coating and c) the solid dispersions produced presents an opportunity for presenting drug in an amorphous state thereby potentially increasing its solubility. Despite these advantages, the technology is limited with respect to the choice of acceptable excipients on two levels; a) a requirement for the molten excipients to instanteously solidify in the cooling chamber at the set temperature and b) a requirement for the core excipients to be solid at room temperature (i.e., the preclusion of liquid excipients). A review of the literature revealed that spray congealing is usually limited to waxy materials such as polyethylene glycol (PEG) 4000, PEG 1500, Poloxamer® 188, and Gelucire® 50/13 (Passerini *et al.,* 2006,

Mackaplow *et al.,* 2006, Cavallari *et al.,* 2005, Passerini *et al.,* 2002 and Martinsa *et al*., 2012).

4.2.3.2 Spray drying

Spray drying is a unit operation in which a drug solution (containing the drug, the core material (lipids) and the shell/matrix (solid carrier) dissolved in an organic solvent/water) is sprayed (atomised at the nozzle) into a hot chamber in which the organic solvent or water evaporates thereby giving rise to solid microparticles of the remaining materials. Although spray drying is a low cost, readily scalable and efficient process, there are a number of disadvantages of the technology; a) the solid particles produced are powder particles which have an irregular shape and have large size distribution which are directly not amenable to coating (i.e., an intermediate agglomeration stage is required) and b) organic solvents are routinely employed as solvents due to the limited selection of suitable water soluble shell/matrix materials (Gharsallaoui *et al.,* 2007).

4.2.3.3 Hot melt extrusion (HME)

HME involves pumping a mixture of raw materials at an elevated controlled temperature (temperature at which the mixture becomes molten) and pressure through a heated barrel into a product of uniform shape and density which is subsequently extruded through a nozzle and cut into rods of defined length (Maniruzzaman *et al.,* 2012, Andrews *et al*., 2009). Whilst HME is predominantly used to disperse APIs (active pharmaceutical ingredients) in a matrix at the molecular level, thus forming solid dispersions, it can also be used as a microencapsulation technology where the drug is dissolved/suspended in a lipid which is homogenously dispersed in the matrix. HME

offers many advantages compared with traditional solid oral dosage processing techniques namely; solvents are not required and it is amenable to continuous processing resulting in an environmentally friendly and cost effective process (Andrews *et al*., 2009). There are however also a number of disadvantages of HME; a) its use is limited to a narrow range of polymers/excipients due the high flow properties required and b) the technology is not suitable for all actives and excipients due to the prolonged thermal residence time involved which can cause degradation (Maniruzzaman *et al.*, 2012). Another disadvantage of the technology is that the extrudate from HME processes is in the form of a rod and therefore requires an extra spheronisation step prior to being amenable the application of a polymer coat.

4.2.3.4 Concentric nozzle extrusion

Concentric nozzle extrusion or co-extrusion is a process that was developed by the Southwest Research Institute in the USA (Ghosh, 2006). It involves the dual pumping of two liquids (core and shell) through a concentric vibrating nozzle which results in the shell material (generally hydrophilic) enveloping the core material (generally hydrophobic) in the form of a droplet. The droplet maintains its structure due to viscosity and crucially surface tension differences between the core and shell materials (Brandau, 2014). The shell of the droplet is then hardened in a hardening bath by one of a number of processes depending on the nature of the shell material (chemical crosslinking, cooling or solvent evaporation). A diagram depicting concentric nozzle extrusion is shown in Figure 4.2 below. It should be noted that in some instances the nozzle may be submerged in the hardening solution, in which case the surface tension difference between the shell material and the hardening liquid also plays a key role with respect to microcapsule formation.

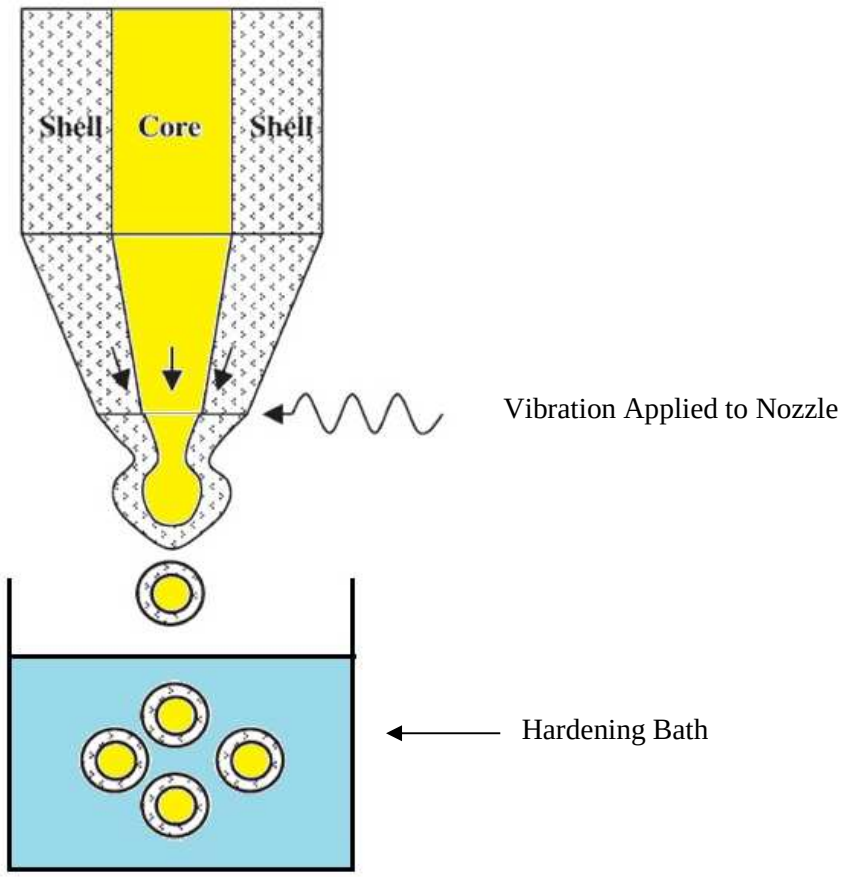

Figure 4.2 Diagram depicting concentric nozzle extrusion (Diagram adapted from Ghosh, 2006).

Although in theory co-extrusion technology has the potential to achieve all the benefits associated with encapsulation technology as outlined in Section 4.2.2, in practice co-extrusion poses a number of limitations in terms of a technology that is suitable for the colon delivery of pre-solubilised poorly soluble API. These limitations revolve around the limited choice and concentration of surfactant that can be encapsulated in the core. Because of the key role that surface tension plays in maintaining the concentric droplet following extrusion from the nozzle, the incorporation of a surface active agent (i.e., a surfactant) with a high HLB (hydrophilic-lipophilic balance – refer to Chapter 2 for further details) disrupts this surface tension thereby causing the droplet to collapse or the formation of 'leaky' microcapsules (Refer to Section 4.4.5 for further details on 'leaky' microcapsules) which are not amenable to further processing because of a lack

of robustness. The scalability of concentric nozzle extrusion also poses challenges due to mechanical/tooling difficulties of producing multiple concentric nozzles (both raised and submerged nozzle processes) and of applying an even flow of the hardening solution around each nozzle in the case of submerged nozzle process (Brandau, 2014).

4.2.3.5 Mechanically aided dripping extrusion techniques

Dripping involves a technique similar to the co-extrusion technique described in Section 4.2.3.4, whereby instead of extruding two liquids (core and shell) through a concentric nozzle, a single liquid (mixture of core and shell/matrix materials) is extruded through a single nozzle resulting in a matrix droplet in which the core material is homogenously dispersed within the matrix. Similar to co-extrusion, the matrix of the droplet is then hardened in a hardening bath to form a microsphere by one of a number of processes depending on the nature of the matrix material (chemical crosslinking, cooling or solvent evaporation). The principle of this process is based on natural gravity whereby when the liquid passes through the tip of the nozzle, a droplet grows and separates from the stream before falling into the hardening solution. A diagram depicting gravity dripping is depicted in Figure 4.3 below (Chavarri *et al.*, 2012 and Nisco gravity dripping, 2014).

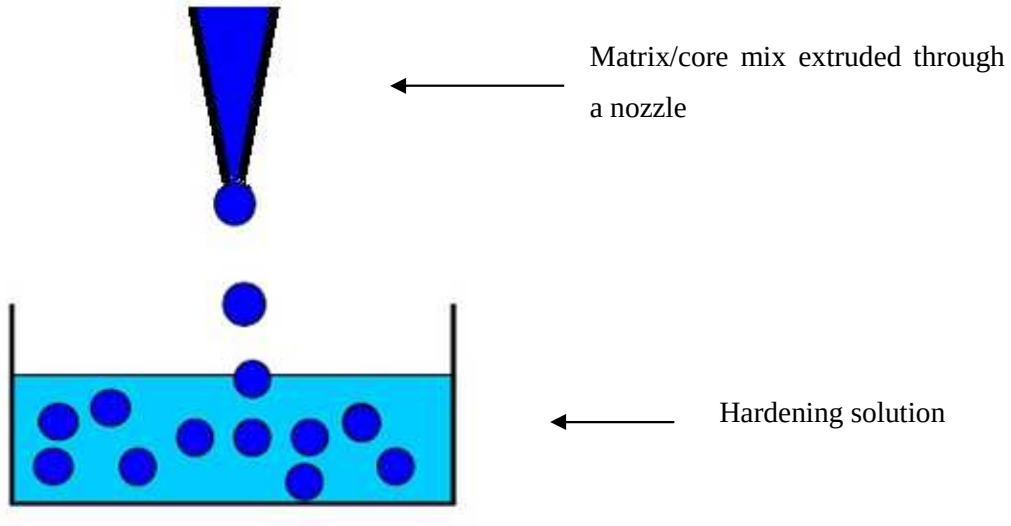

Figure 4.3 Diagram depicting gravity dripping extrusion (Diagram adapted from Nisco gravity dripping 2014).

Dripping techniques offer the advantage over co-extrusion in that surfactants can be easily incorporated into these formulations as long as the ratio of the matrix to core phase is appropriate. Gravity dripping represents the simplest method of making individual droplets and hence microspheres, however the size of the droplet is not easily controlled as it is determined by the weight and surface tension of the droplet (i.e., the formulation) and by the diameter of the nozzle. The flow rates for gravity dripping processes are also very slow thereby rendering them unsuitable for industrial applications (Chavarri *et al.*, 2012). The disadvantages associated with gravity dripping can be overcome by increasing the flow rate to the nozzle and via the application of a mechanical force to break up the stream of liquid as it flows from the nozzle (i.e., droplet formation is no longer reliant on the force of gravity). These mechanical forces give rise to the following techniques based on the principle of dripping; jet cutting technology, rotating (spinning) disc atomisation, electrostatic extrusion, coaxial air flow and prilling. A review of these mechanically aided dripping techniques is described in Chapter 6. A primary advantage of all of these dripping technologies is that they

represent a single step process (with the exception of a simple drying step) in which the end product is amenable to direct further processing (i.e., coating).

4.2.4 Desired CQAs of CLX formulation/technology

As outlined in Chapter 1, the overall objective of this project was to develop a spherical multiparticulate lipid-based oral drug delivery formulation/technology for the delivery of pre-solubilised CLX to the colon for the treatment and prevention of CRC. The desired CQAs of the final CLX formulation have been discussed in the previous chapters, however a summary of these CQAs and the rationale for them is included in Table 4.1 below. Three critical process requirements (CPRs) are also included.

Table 4.1 Summary of desired CQAs and CPRs for colonic delivery of CLX CRC formulation/technology

CQA	Rationale
Formulation that can incorporate a wide range and concentration of surfactants	As outlined in Chapter 2, the use of surfactants is required for an optimal formulation in which the drug is dissolved and which remains in solution on contact with dissolution media and gastrointestinal (GI) contents. Surfactants also have the potential to stabilise the formulation (e.g., prevention of recrystallization).
Formulation in which liquids can be encapsulated	One of the key objectives of the final formulation is the delivery of pre-solubilised CLX to the colon so that it is available to tissue in a free molecular form. Many of the excipients described in Chapter 2 required to dissolve CLX are liquid at room temperature hence it is a requirement of the final formulation to be amenable to liquid encapsulation.
Multiparticulate formulation	The benefits of presenting CLX in a multiparticulate format are described in Chapters 1 and 5. The key benefits include; ✓ Enhanced safety regarding GI irritation ✓ Reduced stomach residence time ✓ Even distribution of drug to the colon (including polyps and CRC tumours) ✓ Suitable for the application of controlled release polymers ✓ Application of consistent/uniform film coatings (particularly for spherical multiparticulates)
Formulation that is amenable to coating	Delivery to the colon requires the application of controlled release polymers hence in addition to being a multiparticulate the formulation/technology must be amenable to robust coating processes (high temperatures and high attrition).
CPRs	**Rationale**
Single step process	In order to have an efficient cost effective process, a single step process that yields a multiparticulate is desirable.
Readily scalable process	The formulation/technology must be readily scalable in order to be commercially viable.
Process that can be manually simulated at the bench	Based on the resources available at this stage of the project, it was necessary that the type of technology applied could be manually simulated at the bench without the requirement for complex process equipment.

4.2.4.1 Assessment of microencapsulation technologies against CQAs and CPRs

On the basis of the CQAs and CPRs described in Section 4.2.4, a review of all the technologies described in Section 4.2.3 was performed to assess and identify the technology which was most amenable to the needs of the desired CLX formulation. A summary of this assessment is outlined in Table 4.2 below.

Table 4.2 Summary assessment of microencapsulation technologies against CQAs and CPRs for desired CLX formulation/technology

CQAs/CPRs	**Technology**				
	Spray Congealing	Spray Drying	HME	Co-extrusion	Dripping Techniques
Amenable to surfactants	✓	✓	✓	×	✓
Amenable to liquids	×	✓ [1]	✓[1]	✓	✓
Multiparticulate	✓	✓ [2]	✓ [2]	✓	✓
Directly amenable to coating	×	×	×	✓	✓
Single step process [1]	✓	×	×	✓[3]	✓[3]
Scalable Process	✓	✓	✓	×	✓
Manually simulated process	×	×	×	×	✓

[1] These technologies have been determined to meet this requirement on the basis that a literature review identified some examples of liquids being encapsulated using these technologies, however this application appears limited.

[2] These technologies have been determined to meet this requirement on the basis that their end products in theory meet the definition of a multiparticulate (Porter, 2013) or can be translated into multiparticulates. It is noted that a technology that directly yields a multiparticulate is preferable.

[3] Although these technologies usually require a drying step after the formation of the microcapsule/microbead, they are considered here to be a single step process given the simple nature of the drying step in comparison to secondary steps required for some of the other technologies to yield a suitable multiparticulate (e.g., spheronisation in the case of HME).

Following a review of Table 4.2, dripping techniques were identified as the technology of choice. Although the eventual aim of the project was to employ one of the mechanically aided dripping techniques described in Section 4.2.3.5, for this phase of the project a manual dripping technique (gravity dripping) was employed. Details of this technique are described in Section 4.3.2.1.

4.2.5 Objectives

As outlined in Section 4.2.1, this phase of the project focused on the translation of liquid formulations developed in Chapters 2 and 3 into optimal CLX microbead formulations that would be amenable to colon targeting using the aforementioned manual dripping technique. The primary objectives in producing an optimal CLX microbead formulation were as follows;

A. The first objective was to produce ‘fit for purpose’ microbeads to meet the following pharmaceutical requirements;
    - ✓ High entrapment efficiency
    - ✓ Consistent drug content
    - ✓ Spherical beads amenable to coating
    - ✓ A robust process in which CLX was maintained in a soluble state (i.e., precipitation was avoided)
    - ✓ Sufficient drug loading to allow for intended dose to be filled into a reasonable sized capsule (maximum of size 00 capsule)
    - ✓ High % drug release in simple media including a greater performance to the marketed CLX product Celebrex®

B. The second objective for this phase of the project was to perform some initial physical characterisation of the optimised microbeads to develop an understanding of their internal structure and to predict how this internal structure might impact on their *in-vitro* (and consequently *in-vivo*) performance.

In the subsequent sections the terms ‘suitable’ and ‘unsuitable’ are used with respect to the manufacture of microbeads, where ‘suitable’ refers to robust spherical microbeads in which precipitation was absent during processing.

4.3 Materials and Methods

4.3.1 Materials

The vehicles used here were as follows; Solutol HS-15® and Cremophor EL® (both BASF, Germany), Transcutol® P (Gattefosse, France), Miglyol® 810N (Sasol, South Africa). Microbeads were prepared using these vehicles in combination with porcine gelatin (Nitta Gelatin, Japan) and sorbitol (Neosorb® - Roquette, France). An array of precipitation inhibitors were also investigated including Sodium Dodecyl Sulphate (SDS), Vitamin E acetate (both Merck, Germany), Vitamin E TPGS (Eastman, USA), Hydroxypropyl Methylcellulose (HPMC), Hydroxypropyl cellulose (HPC), polyvinyl alcohol (PVA), Pluronic® F127, hypromellose (all Sigma Aldrich, USA), Opadry® II and Opadry® White (both Colorcon, USA). A range of fluorescent dyes were sourced from Invitrogen. A sample of CLX API was kindly provided by Erregierre (Italy). The purity of the API was 99.6% based on the COA (certificate of analysis) provided by the supplier. All chemicals used for the release experiments, HPLC (high performance liquid chromatography) and UV (ultraviolet) testing were of laboratory grade.

4.3.2 Methods

4.3.2.1 Preparation of microbeads

Microbeads containing CLX were prepared via a manual gravity dripping method. The microbeads were manufactured by combining a '*surfactant phase premix'* (drug dissolved in various combinations of oils, surfactants and co-solvents) with a '*gelatin phase premix'* (mixture of gelatin, water and sorbitol) and mixing at approximately 60°C on a magnetic stirrer. Aliquots of the mixture were removed using a pipette for ejection of droplets of the mixture into a bath of cooling/hardening oil (Miglyol® 810N at 10°C). As the mixture droplets fall in air, coacervation occurs to form a coacervate suspension in which droplets of the surfactant/oil phase are surrounded by a layer of gelatine (multiple coacervates exist homogenously within the bead matrix). The dropping of the bead into a cooling/hardening bath allows the beads to harden before being recovered for drying. The bead formation occurs in air prior to impaction with the cooling oil, thereby preventing interaction of the cooling oil with the internal surfactant/oil phase droplets due to the presence of the layer of gelatine at the surface of the coacervates and hence the bead. A range oils were investigated (olive oil, mineral oil and other medium chain triglycerdies) for use as the cooling/hardening oil in the production of microbeads. Miglyol® 810N was selected as it was observed to perform optimally in the production of spherical beads, it has been shown to be stable against oxidation and is listed on the FDA's inactive ingredient (IIG) database (FDA IIG database, 2014). The beads were then air dried for 24 h (over this time the water in the formulation evaporated). In some of the formulations other components were added to the gelatine phase to prevent precipitation of drug during manufacturing and also to help

maintain the bead structure. Gelatine was selected as shell encapsulation material as it fulfilled a number of criteria namely; it is predominantly hydrophilic and therefore suitable for encapsulation of hydrophobic materials (e.g., oils), it forms a thermal hydrogel which melts when heated but solidifies when cooled again (i.e., suitable for extrusion process described), it is water soluble and generally melts in the region of 35°C (i.e., melts at body temperature in GI fluids) and it is also a well-known and acceptable pharmaceutical polymer (Rowe *et al.*, 2006)

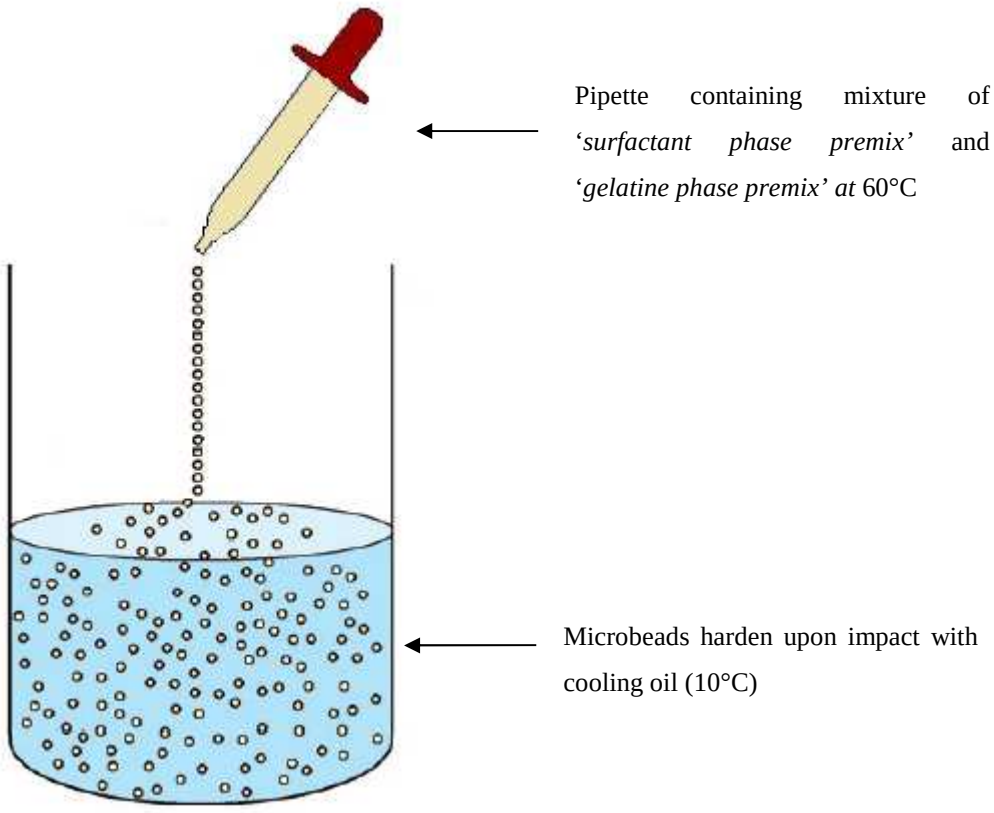

Figure 4.4 A schematic of the manual microbead gravity dripping process. The emulsion/micelle formulation contains the drug (CLX) dissolved in a mixture of the gelatine phase (gelatine, sorbitol water and other components) and the surfactant phase (drug dissolved in mixtures of surfactants, oils and co-solvents). The mixture was maintained at 60°C on a magnetic stirrer and aliquots were removed using a pipette for ejection of droplets of the mixture into the cooling oil bath (Miglyol® 810N at 10°C). The pipette was moved in a circular motion above the cooling oil bath to prevent the droplets from coalescing. The resultant beads were maintained in the cooling oil for 20 min before being separated and dried (at ambient conditions) to remove water from the beads.

4.3.2.2 *In-vitro* Release Testing

Release of CLX formulations (CLX microbeads and Celebrex®) were performed (*n=3*) at 37°C in purified water (PW). Release experiments were carried out using either a Varian/Vankel VK7010 dissolution apparatus (VanKel, USA) or a Distek Evolution 6300 (Distek, USA) equipped with standard glass vessels and USP type II paddles. Paddle rotating speed in all experiments was 75 rpm. Formulations containing 50 mg of CLX were weighed and added to 1000 mL of PW. At specified times 1.8 mL samples were withdrawn, filtered through a 70 µm pore filter (QLA, USA) and analysed using either a HPLC method or an UV spectrophotometric method. The % of drug released at particular time points was determined from peak areas which were calculated against a single point external reference standard in the case of the HPLC method, whereas a standard curve was used for the UV method.

4.3.2.3 HPLC and UV Analysis

The HPLC method for the analysis of the release and assay samples was adapted from Saha and colleagues (Saha *et al*. 2002). The HPLC column used was a reverse phase 4.6 x 250 mm Inertsil® C8 column (Inertsil, The Netherlands) with 5 µm particles. The mobile phase was acetonitrile:water (65:35). The isocratic method used a flow rate of 1.25 ml/min and ultraviolet (UV) detection at 230 nm. The injection volume was 20 µl and the retention time was 8 min. The HPLC apparatus that was used for the analysis were Thermo Finnigan (Thermo Electron Corporation, USA) and Waters (Waters, USA) HPLC systems (and associated Chromquest and Empower software). The UV method for the analysis of the release samples was also adapted from Saha and colleagues (Saha *et al*. 2002). The spectrophotometer used was a Genesys 10 series UV-

visible spectrophotometer (Thermo Electron Corporation, USA). Absorbance was read at a wavelength of 251 nm.

4.3.2.4 Evaluation of Entrapment Efficiency

The amount of incorporated CLX was determined in the optimised microbead formulations produced. A quantity of beads (*n=2*) with a theoretical potency between 5 mg and 50 mg (depending on the quantity of sample available) were sonicated for 2 h in a mixture of acetonitrile:water 65:35 (HPLC method) or acetonitrile:phosphate buffer 50:50 (UV method) to extract the drug from the microbeads. The resultant solution was passed through a 0.45 µm filter prior to absorbance analysis. Where required the samples were diluted prior to analysis. The concentration of CLX was determined by absorbance measurements at 230 or 251 nm via the HPLC or UV analysis methods described above. CLX content (%) was calculated as the amount of determined CLX with respect to the total mass of dried microbeads. The entrapment efficiency (%) of CLX was expressed as a percentage of the determined CLX with respect to the total amount of CLX used in the preparation of the microbeads.

4.3.2.5 Microbead Characterization using Light Microscopy

Shape and surface morphology of freshly prepared and dried microbeads were observed under a Nikon (Nikon, Japan) Eclipse Ti optical microscope mounted with a digital camera. Pictures were taken of sliced sections of dried microbeads. Thin films of selected formulations were also prepared and viewed under the microscope in an effort to understand the internal structure of the beads. A number of fluorescent dyes (Nile red, Bodipy 505/ 515, Sudan orange, Dextran Alexa Fluor 546, Dextran Rhodamine Green and Dextran Cascade Blue – all Invitrogen, USA) were incorporated into some of

these formulations in an attempt to distinguish between the surfactant/lipid and gelatine phases of the formulations.

4.4 Results and discussion

4.4.1 Development and optimisation of CLX microbeads

In Chapter 2, the details of a microbead production feasibility study were outlined in which one formulation (CLX 030/B) was found to be amenable to manufacture. The composition of CLX 030/B is detailed in Table 4.3 below.

Table 4.3 Composition of formulation CLX 030/B

Components	(mg/g)
Celecoxib	23.03
Gelatine	554.40
D-Sorbitol	61.70
Miglyol® 810N	130.53
Solutol® HS-15	230.34

A review of formulations CLX 027/B to CLX 032/B from the feasibility study, revealed that five of the formulations were unsuitable due to precipitation. It also revealed that the only suitable formulation (CLX 030/B) had a much lower drug loading (2.3% w/w) than the five unsuitable formulations (drug loadings of 9.8–10.4% w/w). Based on these observations,the first aim of the microbead development and optimisation study presented here was to overcome precipitation whilst increasing the drug loading to commercially viable levels.

Whilst formulation development focused primarily on formulations containing Miglyol® 810N and/or Solutol HS-15® in the ‘surfactant phase’ due to the promising *in-*

vitro cell culture results described in Chapter 3, based on the observation above regarding the drug loading, the scope of the formulation development was also widened to look at other 'surfactant phase' components including Transcutol® and Cremophor® EL.

4.4.1.1 Overcoming precipitation during manufacture and optimising loading of CLX

A number of formulation aspects were investigated with the aim of overcoming precipitation and optimising the loading, including an assessment of the impact of the following; a) increasing the CLX concentration and changing excipient composition, b) modifying the 'gelatine phase' and c) incorporation of precipitation inhibitors.

4.4.1.1.1 Increasing CLX concentration and changing excipient composition

Two formulations (CLX 033/B and CLX 034/B – Table 4.4) were produced on the basis of formulation CLX 030/B. CLX 033/B and CLX 034/B contained increasing concentrations of 10% and 15% w/w CLX in their respective 'surfactant phases', equating to corresponding final loadings of 3.8% and 5.8%. In the case of CLX 033/B, there was no precipitation evident and bead manufacture was successful (i.e., suitable beads were manufactured). In the case of CLX 034/B there was slight evidence of precipitation and whilst bead manufacture was still possible there was tailing evident (see Figure 4.5 below).

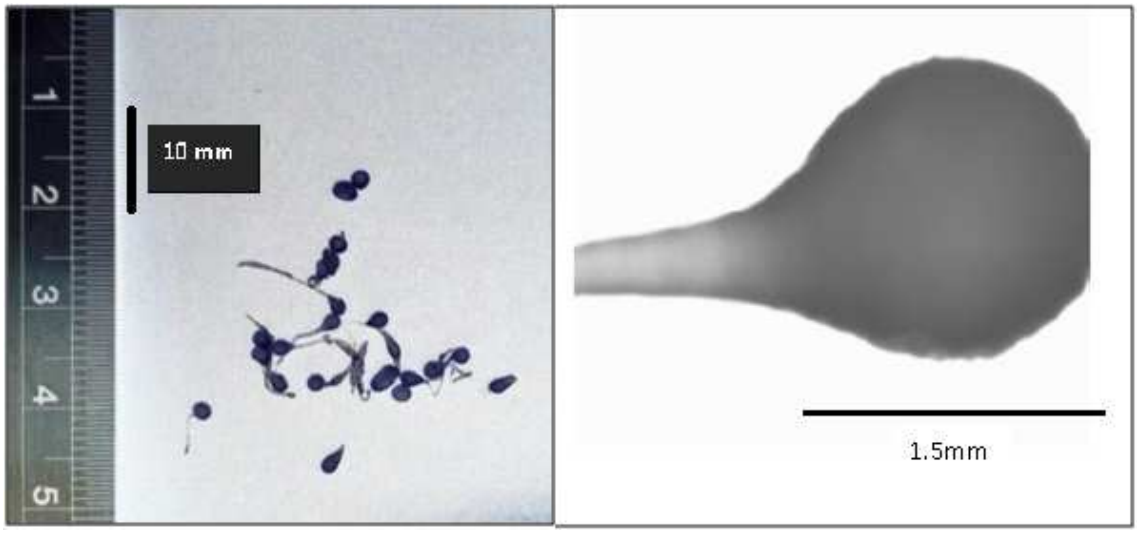

Figure 4.5 Photographs (using Inverted Microscope (Nikon Eclipe Ti) of representative microbeads from CLX 034/B demonstrating the 'tailing effect'

It was concluded from these formulations that the loading of CLX played a crucial role with respect to precipitation. Dissipation of the emulsion into the cooling oil was evident for both of these formulations. It was proposed that the inclusion of Miglyol® 810N in the formulations could be contributing to the dissipation. Given that Miglyol® 810N was also employed as the cooling oil, it was proposed that the Miglyol® 810N in the formulation could have a high affinity for the cooling oil resulting in dissipation of the formulation. Three further formulations were produced to assess the impact of removing Miglyol® 810N from the formulation. The three formulations contained increasing concentrations of CLX in their respective 'surfactant phases'. The concentration of CLX was 15%, 20% and 25% w/w in the 'surfactant phases' of formulations CLX 036/B, CLX 037/B and CLX 038/B respectively (Table 4.4). Dissipation of the emulsion into the oil component was not observed for any of these formulations which confirmed that Miglyol® 810N was the cause of the dissipation. An increasing level of precipitation was observed for the three formulations relative to their increased loading of CLX which also reaffirmed the conclusion regarding precipitation (i.e., precipitation was related to the concentration of the drug).

Table 4.4 Composition of CLX microbead formulations CLX 033/B, CLX 034/B, CLX 036/B, CLX 037B and CLX 038/B

Formulations		CLX 033/B	CLX 034/B	CLX 036/B	CLX 037/B	CLX 038/B
Component Concentration (mg/g)	Celecoxib	38.46	57.69	57.25	73.86	96.01
	Gelatine	553.85	553.85	552.55	567.63	555.32
	D-Sorbitol	61.54	61.54	61.39	63.07	60.63
	Miglyol® 810N	115.38	107.69	-	-	-
	Solutol® HS-15	230.77	219.23	328.81	295.44	288.04

The entrapment efficiency for formulations CLX 036/B and CLX 037/B was determined by analysing quantities of microbeads equating to 5 mg doses of CLX. The % entrapment was 94.95 % ± 0.04% and 95.73% ± 0.21% for formulations CLX 036/B and CLX 037/B respectively.

Two further microbead formulations (CLX 039/B and CLX 040/B – Table 4.5) were prepared to investigate whether the type of excipients employed impacted on the occurrence or levels of precipitation when drug loadings were high. The Solutol® HS-15 employed in formulations CLX 036/B and CLX 037/B was replaced with a combination of Transcutol® P, Cremophor® EL and Miglyol® 810N. In the case of both CLX 039/B and CLX 040/B precipitation was evident in the respective emulsions overtime. The level of precipitation for both formulations was greater than that observed for the corresponding formulations containing Solutol® HS-15 indicating that excipient choice employed played a role with respect to the precipitation of the drug. As Transcutol® P was present in the highest concentration; it is likely that it had the largest impact.

Table 4.5 Composition of CLX microbead formulations CLX 039/B and CLX 040/B

Formulations		CLX 039/B	CLX 040/B
Component Concentration (mg/g)	Celecoxib	78.24	56.98
	Gelatine	548.80	552.89
	D-Sorbitol	59.99	61.67
	Transcutol P	163.60	171.05
	Miglyol 810N	49.79	103.05
	Cremophor EL	99.58	54.36

4.4.1.1.2 Modifying the 'gelatine phase' – Stage 1

In the formulations described in Table 4.4, the % of gelatine was in the region of 55% w/w. Formulations CLX 041/B and CLX 042/B (Table 4.6) were produced to assess the impact of reducing the concentration of gelatine from 55% to 52% w/w. It was evident from these formulations that the level of precipitation was slightly greater for the formulation containing 55% gelatine (CLX 041/B) than for that containing 52% gelatine (CLX 042/B). This was the first indication that the level of water and/or gelatine in the formulation impacted on the level of precipitation.

Table 4.6 Composition of formulations CLX 041/B and CLX 042/B (including wet bead compositions)

Formulation Components	CLX 041/B		CLX 042/B	
	Dry Bead Composition mg/g	Wet Bead Composition mg/g	Dry Bead Composition mg/g	Wet Bead Composition mg/g
Celecoxib	77.85	22.46	84.67	25.42
Solutol® HS 15	307.04	88.57	330.55	99.25
Gelatine	554.05	159.83	521.20	156.50
D-Sorbitol	61.05	17.61	63.58	19.09
Purified Water	-	711.53	-	699.73

4.4.1.1.3 Inclusion of Precipitation Inhibitors

It was shown in the previous section that it was possible to affect the level of precipitation of CLX in the formulations by using different excipients and that it was possible to eliminate precipitation by employing very low concentrations of the drug. These formulations were however not viable for further progression as the concentration of drug was insufficient. It was required to produce a formulation in which precipitation of the drug was eliminated and also in which the drug loading was at a viable level. A number of known precipitation inhibitors for CLX (Guzman *et al.,* 2007) and some other recognised pharma excipients that were known to act as precipitation inhibitors were selected and investigated with respect to their ability to inhibit precipitation in the CLX formulations described here.

A. Hydroxypropyl methylcellulose (HPMC)

HPMC is widely used in the pharmaceutical industry as an excipient in oral formulations. It is primarily used as a binder, in film-coatings and also as a matrix for extended release formulations. HPMC has also been widely shown to prevent the precipitation of poorly soluble drugs *in-vitro* and *in-vivo* (Brouwers *et al.,* 2009, Rowe *et al.,* 2006). In the formulations presented in Table 4.7 below, the HPMC that was used was Methocel® E15 LV (referred to as HPMC E-15) from Colorcon®. In all formulations the HPMC was added to the 'gelatine phase' during the processing of the microbeads. Concentrations of 3% (CLX 050/B), 6% (CLX 051/B) and 8% (CLX 052/B) w/w of HPMC were attempted and although the increasing concentration of HPMC did reduce the level of precipitation it was not sufficient to eliminate it. It was also observed that the processing of the microbeads became increasingly difficult as the

concentration of HPMC increased due to an increase in the viscosity of the emulsions. Formulations containing Trancutol® P, Miglyol® 810N and Cremophor® EL were assessed as they represented a worst case in terms of levels of precipitation observed up to this point.

Table 4.7 Composition of formulations CLX 050/B, CLX 051/B and CLX 052/B

Formulations		**CLX 050/B**	**CLX 051/B**	**CLX 052/B**
Component Concentration (mg/g)	Celecoxib	74.44	72.32	71.42
	Gelatine	537.20	521.90	503.29
	D-Sorbitol	60.12	58.41	55.92
	HPMC E-15	30.65	58.26	83.88
	Transcutol® P	156.18	151.74	149.84
	Miglyol® 810N	48.44	47.06	46.48
	Cremophor® EL	92.96	90.31	89.18

B. Vitamin E (alpha tocopherol)

Alpha tocopherol is primarily recognised as a source of Vitamin E. Alpha tocopherol has three chiral centres, giving rise to eight isomeric forms. The naturally occurring form is known as *d*-alpha tocopherol. The synthetic form is known as *dl*-alpha tocopherol or simply alpha tocopherol and it occurs a racemic mixture containing eqimolar quantities of all the isomers. It is a highly lipophilc compound and is an excellent solvent for many poorly soluble drugs (Rowe *et al.*, 2006). There are many related substances to alpha tocopherol which include *d*-alpha tocopherol polyethylene glycol succinate (vitamin E TPGS) and *dl*-alpha tocopherol acetate (vitamin E acetate). The appearance of vitamin E TPGS is a white waxy solid, while vitamin E acetate is a yellow viscous oil. Vitamin E is widely used in the pharmaceutical industry as an

excipient in oral formulations. It has been used as a solvent for many poorly soluble drugs and also as a surfactant. It has also been shown that vitamin E TPGS can prevent the precipitation of celecoxib *in-vivo* (Guzman *et al.*, 2007). For the purposes of this work, the two forms of vitamin E described above (Vitamin E TPGS and Vitamin E acetate) were investigated (the formulations are presented in Table 4.8 below). Although the literature pointed towards vitamin E TPGS being effective as a precipitation inhibitor, it was decided to also investigate vitamin E acetate to assess any differences in the liquid (acetate) and solid (TPGS) form of vitamin E. Initial concentrations of 6% w/w of vitamin E acetate and vitamin E TPGS were included in formulations CLX 053/B and CLX 054/B respectively. The formulation containing the vitamin E TPGS (CLX 054/B) was very viscous and was difficult to process into microbeads and any microbeads that were produced showed evidence of 'tailing'. The viscosity of CLX 053/B (vitamin E acetate) did not pose any processing difficulties, however 'tailing' was again observed during the microbead manufacturing process. There was no precipitation evident with this formulation over a period of 3 h stirring at 60 °C, however the drug did precipitate overnight. In an effort to combat the 'tailing' issues observed in the case of CLX 053/B, another formulation (CLX 055/B) was produced in which the concentration of vitamin E acetate was reduced to 1.6% w/w. It was observed that the reduction in the concentration of the vitamin E acetate resulted in more precipitation but also a reduction in the 'tailing' phenomenon. A further formulation (CLX 056/B) was produced consisting of a vitamin E acetate concentration of 3.3% w/w. This formulation exhibited properties that were a median of those observed for CLX 053/B and CLX 055/B. An attempt was also made to make Solutol® HS-15 based microbeads incorporating vitamin E acetate (CLX 059/B and CLX 061/B) in the formulation (the previous formulations described in this section all contained a combination of Miglyol® 810N, Cremophor® EL and Transcutol® P in the surfactant

phase). Precipitation was observed to be reduced for formulations CLX 059/B and CLX 061/B compared to formulations CLX 053/B – CLX 057/B with no precipitation evident in the case of CLX 061/B. Manufacturing however again posed some difficulties with 'tailing' evident. These results confirmed that vitamin E acetate had the potential to be a precipitation inhibitor in the formulation but that its high viscosity impacted on the ability to produce spherical microbeads.

Table 4.8 Composition of formulations CLX 053/B, CLX 054, CLX 055/B, CLX 056/B, CLX 057/B and CLX 059/B

Formulations		**CLX 053/B**	**CLX 054/B**	**CLX 055/B**	**CLX 056/B**	**CLX 057/B**	**CLX 059/B**	**CLX 061/B**
Component Concentration (mg/g)	Celecoxib	71.84	72.40	76.01	74.17	75.34	75.90	73.64
	Gelatine	518.80	515.55	542.13	534.84	542.77	543.43	526.28
	D-Sorbitol	58.59	59.22	61.58	61.30	62.20	63.01	58.85
	Vitamin E Acetate	63.61	-	16.45	33.21	-	15.28	47.53
	Vitamin E TPGS	-	63.42		-	18.52	-	-
	Transcutol® P	150.72	151.89	159.47	155.61	158.07	-	-
	Miglyol® 810N	46.75	47.11	49.46	48.26	49.03	-	-
	Solutol® HS-15	-	-	-	-	-	302.38	293.40
	Cremophor® EL	89.70	90.40	94.91	92.61	94.08	-	-

When lower concentrations of vitamin E acetate were employed, it was observed that the bead manufacture process posed fewer difficulties. On this basis, an attempt at producing a microbead formulation using vitamin E TPGS using a lower concentration to that previously employed for CLX 054/B (6% w/w) was performed. Formulation CLX 057/B which contained 1.8% w/w of vitamin E TPGS was produced. In this instance, the microbead manufacturing process was feasible (although the beads had a tendency to float on the top of the oil bath) and the level of precipitation was reduced.

The formulations described in this section highlighted the potential of vitamin E (both forms) to act as a precipitation inhibitor of CLX, however its incorporation into microbeads was shown to pose difficulties form a manufacturing perspective. The CLX content of formulations CLX 055/B and CLX 056/B were determined via the entrapment efficiency assay. Quantities of microbeads equating to 5 mg doses of CLX were weighed and analysed. The % entrapment was 78.85 % ± 0.30% and 99.48 % ± 0.18% for formulations CLX 055/B and CLX 056/B respectively. These assay results reaffirmed the observation that the level of precipitation was related to the concentration of vitamin E acetate employed. The level of vitamin E acetate in formulation CLX 055/B was approximately half of that employed in formulation CLX 056/B and there was approximately a 20% difference in the amount of CLX entrapped in the resultant microbeads. It is proposed that the % of entrapment for formulation CLX 055/B was low because some of the drug had precipitated prior to bead manufacture and therefore was not available during microbead manufacture (precipitated drug tended to accumulate on the sides of the mixing vessel).

C. Opadry® II

Opadry® II is an excipient manufactured by Colorcon® and is widely used in the pharmaceutical industry in oral formulations. It is primarily used for film coating (Rowe *et al.*, 2006). Opadry® II contains polyvinyl alcohol (PVA), PEG 3000, titanium dioxide and talc (Colorcon Opadry® II product specification, 2010). Opadry® II was selected on the basis that PVA had been shown to prevent the precipitation of CLX *in-vivo* (Brouwers *et al.*, 2009). The first formulation produced that contained Opadry® II was CLX 062/B (Table 4.9). It was observed that Opadry® II greatly facilitated the microbead manufacturing process. It was difficult to assess its impact on precipitation

as Opadry® II did not dissolve in the formulation, but instead formed a suspension. The next formulation produced containing Opadry II® was CLX 063/B (Table 4.9). The primary difference between formulations CLX 062/B and CLX 063/B was the inclusion of Solutol® HS-15 alone and a combination of Miglyol®/ 810N Transcutol® P/ Cremophor® EL in their respective 'surfactant phases'. Precipitation was evident in the case of CLX 063/B which again pointed towards the role different excipients play with respect to the onset of precipitation. A further formulation was then produced (CLX 067/B) which involved increasing the CLX concentration employed for formulation CLX 062/B from 7% w/w to 9% w/w. Again microbead manufacture was without difficulty; however some precipitation was evident after stirring overnight. This experiment reaffirmed the criticality of the drug concentration employed with respect to precipitation (regardless of whether or not precipitation inhibitors were included). In the case of Opadry® II, it was not possible to increase its concentration above the levels employed here as the formulations became too difficult to process (i.e., too viscous).

Table 4.9 Composition of formulations CLX 062/B, CLX 063/B and CLX 067/B

Formulations		**CLX 062/B**	**CLX 063/B**	**CLX 067/B**
Component Concentration (mg/g)	Celecoxib	72.87	74.55	92.35
	Gelatine	518.98	521.29	524.25
	D-Sorbitol	60.35	60.35	61.18
	Opadry® II	57.47	45.80	45.16
	Transcutol® P	-	156.41	-
	Miglyol® 810N	-	48.51	-
	Solutol® HS-15	290.33	-	277.06
	Cremophor® EL	-	93.09	-

D. Opadry® White 20A28380

Opadry® White 20A28380 is an excipient manufactured by Colorcon and widely used in the pharmaceutical industry in oral formulations. It is primarily used for film coating (Rowe *et al.,* 2006). It contains talc, hydroxypropyl cellulose (HPC), hypromellose and titanium dioxide (Colorcon Opadry® White 20A28380 product specification, 2010) and was selected for screening as it contained HPC which has been shown to prevent the precipitation of CLX *in-vivo* (Guzman *et al.,* 2007). CLX 066/B (Table 4.10) was produced with a concentration of 4.5% w/w of Opadry® White and a high loading of CLX (9.4% w/w). As was the case for the formulations containing Opadry® II, the inclusion of Opadry® White greatly assisted the microbead manufacturing process. Opadry® White also produced a fine suspension which made the detection of precipitation difficult. For formulation CLX 066/B, there was only slight evidence of precipitation which was encouraging given the high loading of CLX. CLX 068/B (Table 4.10) was produced to assess the impact of Opadry® White on a formulation containing Miglyol® 810N/ Transcutol® P/ Cremophor® EL. In this case, precipitation was evident but to a lesser extent than with formulation CLX 063/B (similar formulation containing Opadry II) which suggested that Opadry® White was a greater precipitation inhibitor for CLX formulations in comparison to Opadry® II.

Table 4.10 Composition of formulations CLX 066/B and CLX 068/B

Formulations		CLX 066/B	CLX 068/B
Component Concentration (mg/g)	Celecoxib	94.25	74.22
	Gelatine	520.03	523.82
	D-Sorbitol	57.43	59.25
	Opadry® White	45.56	45.99
	Transcutol® P	-	155.73
	Miglyol® 810N	-	48.30
	Solutol® HS-15	282.74	-
	Cremophor® EL	-	92.68

E. Hydroxypropyl cellulose (HPC)

Although the exact composition of Opadry® White 20A28380 is unknown, it is known to include HPC and it is believed that HPC is a predominant component as HPC is widely used as a film coating agent in tablet formulations. HPC has been shown to prevent the precipitation of CLX *in-vivo* (Guzman *et al.*, 2007). It was found that it was very difficult to make microbeads which incorporated HPC, as it was hard to disperse the HPC in the 'gelatine phase' which consequently made the formulations very viscous. Two formulations containing HPC were attempted (CLX 070/B and CLX 072/B – Table 4.11), however neither of these were viable options for further consideration due to processing difficulties. If HPC is indeed the primary component of Opadry® White, then it is proposed that Colorcon® (the manufacturer) includes a processing step (e.g., milling) in their material manufacture that makes it more amenable for inclusion in this type of formulation.

Table 4.11 Composition of Formulations CLX 070/B and CLX 072/B

Formulations		CLX 070/B	CLX 072/B
Component Concentration (mg/g)	Celecoxib	74.63	94.09
	Gelatine	523.21	519.68
	D-Sorbitol	58.49	57.57
	HPC	46.34	46.41
	Solutol® HS-15	297.33	282.26

F. Pluroinc® F127 and other precipitation inhibitors

Pluronic® F127 was also assessed with respect to its effectiveness at preventing precipitation. Pluronic® F127 is a poloxamer polyol (a block copolymer of ethylene oxide and propylene oxide) and is typically used in pharmaceutical industry as an emulsifying and solubilizing agent. Pluronic® F127 has also been shown to prevent the precipitation of CLX *in-vivo* (Guzman *et al.*, 2007). In the case of formulation CLX 075/B (Table 4.12), it was found to have no effect on the onset of precipitation and therefore was not pursued further. Other potential precipitation inhibitors that were investigated (CLX 111/B and CLX 112/B – Table 4.12) but which were ultimately unsuitable were hypromellose and PVA.

Table 4.12 Composition of formulation CLX 075/B, CLX 111/B and CLX 112/B

Formulations		CLX 075/B	CLX 111/B	CLX 112/B
Component Concentration (mg/g)	Celecoxib	92.08	54.35	54.35
	Gelatine	276.23	521.74	521.74
	D-Sorbitol	58.54	57.97	57.97
	Pluronic® F127	43.98	-	-
	Hypromellose	-	57.97	-
	PVA	-	-	57.97
	Solutol® HS-15	276.23	307.97	307.97

4.4.1.1.4 Modifying the 'gelatine phase' – Stage 2

A number of approaches were made to modify the gelatine phase of the CLX microbead formulations with the dual intention of increasing the loading and eliminating precipitation. These approaches involved a) addition of talc to the 'gelatine phase' and b) reduction of the 'gelatine phase' component (i.e., changing the ratio between the gelatine phase and surfactant phase components).

A. Formulations containing Talc

During the course of some of the experiments described above (Sections 4.4.1.1.3 C and D), it was observed that two of the potential precipitation inhibitors investigated (Opadry® II and Opadry® White) offered the potential benefit of modifying the formulation to the extent that microbead manufacture posed few difficulties. It was proposed that the incorporation of these components helped to maintain the structure of the emulsion droplet on impact with the cooling oil. The components of Opadry® II and

Opadry® White were examined to identify similarities. The one excipient common to both materials was talc. The impact of including talc in formulations similar to those in Section 4.4.1.1.3 B (i.e., formulations containing vitamin E) was investigated as these formulations containing vitamin E had previously exhibited difficulties with respect to microbead manufacture. Formulation CLX 081/B (Table 4.13) was produced, which was a similar formulation to CLX 055/B and CLX 056/B with the addition of talc (7.3% w/w). It was observed that the bead manufacture process was without difficulty which added to the hypothesis that the incorporation of talc helped to maintain the structure of the emulsion droplet on impact with the cooling oil.

Table 4.13 Composition of Formulation CLX 081/B

CLX 081/B	
Formulation Components	**mg/g**
Celecoxib	68.53
Transcutol® P	143.78
Cremophor® EL	85.57
Miglyol® 810	44.60
Gelatine	499.07
Vit E Acetate	28.14
Talc	73.67
D-Sorbitol	56.66

B. Reduction of the 'gelatine phase' component

In all of the formulations presented to this point, the concentration of gelatine contributed in the region of 55% w/w of the entire dried formulation. Given the observation that increasing the concentration of CLX in the 'surfactant phase' generally led to increased precipitation, the possibility of reducing the 'gelatine phase' component in the formulation was investigated as an alternative method of

increasing the loading of CLX. An initial attempt (CLX 090/B – Table 4.14) was made in which the final gelatine concentration was reduced to approximately 40% w/w, which resulted in an increase in the CLX concentration up to 11% w/w. The formulation preparation was identical to that of formulations CLX 037/B, CLX 041/B and CLX 042/B with the exception that the 'surfactant phase': 'gelatine phase' ratio was 1:4 for CLX 090/B whereas it was 1:9, 1:8 and 1:7 for CLX 037/B, CLX 041/B and CLX 042/B respectively. The concentration of CLX in the 'surfactant phase' (prior to the microbead processing stage) was 20% w/w. It was found that microbead manufacture was possible for this formulation; however some precipitation of the drug was observed which suggested that a loading of 20% w/w CLX in the surfactant phase was excessive. Formulation CLX 098/B (Table 4.14) was produced containing a loading of 15% w/w of CLX in the 'surfactant phase' and a final concentration of 40% w/w gelatine in the final microbeads (i.e., the 'surfactant phase': 'gelatine phase' ratio of 1:4 was maintained). There was only slight precipitation evident overtime. This was a major advance on the previous formulation produced. In Chapter 2, formulation CLX 032/B was presented in which the gelatine concentration was 65% w/w and the CLX loading was 9.8% w/w. In the case of CLX 032/B, this formulation could not be progressed to microbead manufacture as the CLX precipitated on impact of the 'surfactant phase' with the 'gelatine phase'. Formulation CLX 098/B had a drug concentration of 8.2% w/w (comparable to that of CLX 032/B), however in contrast to CLX 032/B, it was shown to be easily converted into microbeads. It was postulated that the improvement with respect to the precipitation of the drug was as a result of the reduced number of water molecules (in the gelatine phase) available for interaction with the drug.

Table 4.14 Composition of Formulations CLX 090/B and CLX 098/B

Formulations		CLX 090/B	CLX 098/B
Component Concentration (mg/g)	Celecoxib	111.38	82.97
	Gelatine	399.69	400.76
	D-Sorbitol	45.19	46.54
	Solutol® HS-15	443.75	469.73

4.4.2 Optimised CLX microbead formulations

Based on the data and observations from all the formulations described above and Section 4.4.1.1.4 B in particular, optimised CLX microbead formulations were developed. This optimisation process is described below.

4.4.2.1 Inclusion of SDS as a precipitation inhibitor

Following the important findings presented in Section 4.4.1.1.4 B, all subsequent experiments focused on formulations containing gelatine in the range of 30-40% w/w. In an effort to eradicate the slight precipitation observed for formulation CLX 098/B (Table 4.14), another potential precipitation inhibitor, sodium dodecyl sulfate (SDS), was investigated. The inclusion of 3.2% w/w SDS resulted in a formulation (formulation CLX 099/B – Table 4.15) in which precipitation was initially absent. Part of the mixture was converted into microbeads, while the remaining fraction was kept stirring at 60°C for a period of 1 week. After 1 week the emulsion was examined and some precipitate was observed. A further formulation CLX 101/B (Table 4.15) was manufactured in which the concentration of SDS was increased to 5.3% w/w. No precipitation occurred initially but again some precipitate became evident over a prolonged period of time.

Table 4.15 Composition of Formulations CLX 099/B, CLX 101/B, CLX 102/B, CLX 104/B, CLX 105/B and CLX 115/B

Formulations		CLX 099/B	CLX 101/B	CLX 102/B	CLX 104B	CLX 105/B	CLX 115/B
Component Concentration (mg/g)	Celecoxib	80.65	79.33	105.53	76.91	88.50	51.72
	Solutol® HS-15	456.99	449.15	420.46	435.46	501.07	293.10
	Gelatine	387.10	374.29	377.54	369.12	309.48	496.55
	SDS	32.26	52.81	53.69	76.22	65.54	103.45
	D-Sorbitol	43.01	44.42	42.78	42.28	35.41	55.17

The CLX content of formulation CLX 101/B was determined by weighing quantities of microbeads equating to 5 mg doses of CLX and analysing for CLX following extraction. The average % entrapment was 91.77 % ± 0.04%. Formulations CLX 099/B and CLX 101/B both represented formulations in which the CLX loading in the 'surfactant phase' was 15% w/w. In an effort to increase the overall loading of the drug, a formulation (CLX 102/B – Table 4.15) was attempted in which the loading of the drug in the surfactant phase was increased to 20% w/w. This formulation was however unsuitable as the drug was observed to precipitate. It was concluded that despite the reduction of the gelatine concentration and the inclusion of SDS as a precipitation inhibitor that there was still a maximum concentration of CLX that could be included in the formulation in order to avoid precipitation. Formulation CLX 104/B (Table 4.15) was prepared in which the SDS concentration was increased to 7.6% w/w. The concentration of CLX in the 'surfactant phase' was also reduced back to 15% w/w. There was some precipitation evident over time but was reduced compared to formulations that contained lower concentrations of SDS, confirming that the SDS level had an impact with respect to precipitation. The CLX content of formulation CLX

104/B was determined to be 93.21% ± 0.73%. In an effort to further increase the drug loading in the overall formulation, whilst keeping a constant drug concentration in the 'surfactant phase' (i.e., 15% w/w), a further reduction of the gelatine concentration was attempted (Formulation CLX 105/B – Table 4.15). The gelatine concentration was reduced from an average of 37% w/w to approximately 30% w/w. Although it was possible to produce microbeads it was found that the mixture was viscous and difficult to process, indicating that a gelatine concentration of 37% w/w was the limit for the gelatine phase at this loading of CLX (15% w/w in the surfactant phase). Microscopic analysis was performed on microbead formulation CLX 104/B. The analysis was performed on beads produced prior to the onset of any visible precipitation. Thin slices of the beads were obtained by cutting the beads with a blade and these slices were viewed under the inverted microscope (Figure 4.6). This analysis revealed what appeared to be needle like crystals in the microbeads. These needles were characteristic of CLX needle crystals (Chawla *et al*., 2003) which suggests that some precipitation may have occurred that was not visible to the naked eye. This was an important finding in the context of the requirement for the formulation to present CLX in a solubilised state (i.e., no precipitation).

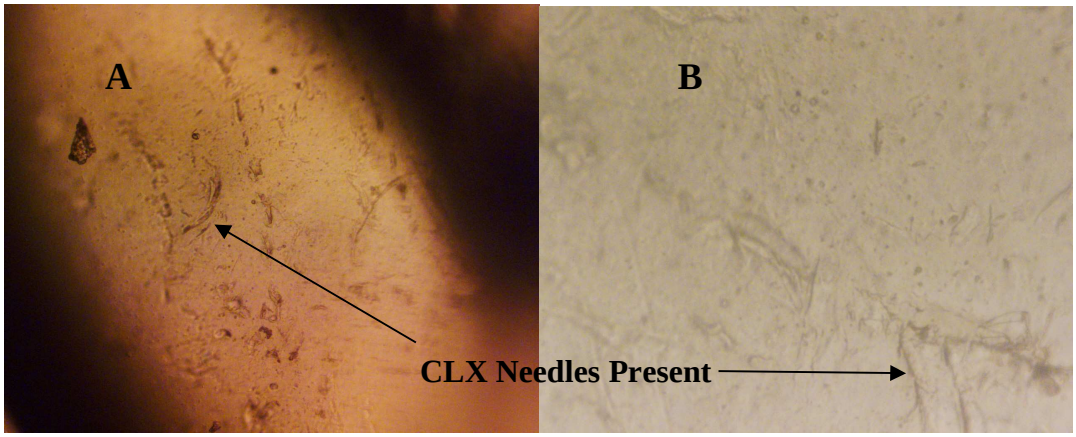

Figure 4.6 Photographs (using Inverted Microscope (Nikon Eclipe Ti) of a 'slice' of formulation CLX 104/B at (A) 10X magnification and (B) 40X magnification.

In order to assess the impact of including SDS in the formulation at the original gelatine concentration of 55%, formulation CLX 115/B (Table 4.15) was prepared. Significant precipitation was evident in this formulation which confirmed that a combination of reducing the gelatine concentration and the inclusion of SDS was required to eliminate precipitation.

4.4.2.2 Impact of 'surfactant phase excipients' on reducing the gelatine phase concentration

Following on from the important finding in section 4.4.1.1.4 B, regarding the reduction of the gelatine phase concentration, the role of the 'surfactant phase' excipients in these formulations was further investigated. A formulation similar to CLX 101/B (Table 4.15) was prepared in which the Solutol® HS-15 'surfactant phase' was substituted with a 'surfactant phase' containing 20% w/w CLX dissolved in a mixture of Transcutol® P, Cremophor® EL and Miglyol® 810 (Formulation CLX 119/B – Table 4.16). There was no precipitation for this formulation originally; however there was significant precipitation evident after a number of hours. It was noted that the level of precipitation was significantly less compared to a corresponding formulation (CLX 028/B – Chapter 2) in which the gelatine concentration was 54% w/w. It should also be noted that there was no SDS in formulation CLX 028/B which may also have contributed to the improvement with respect to CLX 119/B. The most significant observation with respect to CLX 119/B was however regarding its processing conditions and the morphology of the resultant microbeads. It was found that it was very difficult to process the beads when Miglyol® 810N/Cremophor® EL/Transcutol® P were used instead of Solutol® HS-15. In the case of the beads that were produced, they had a very irregular bead shape (not spherical) and appeared to be leaking oil. It was proposed that the waxy nature of

Solutol® HS-15 (solid at room temperature) helped to retain the integrity of the bead. This was a very important observation regarding the choice of excipients when attempting to formulate microbeads with low gelatine concentrations (i.e., in the region of 30–40% w/w).

Table 4.16 Composition of Formulation CLX 119/B

CLX 119/B	
Formulation Components	**mg/g**
Celecoxib	105.08
Transcutol	220.47
Cremophor EL	131.21
Miglyol 810	68.38
Gelatin	377.01
SDS	54.91
D-Sorbitol	42.95

4.4.2.3 First generation CLX microbead formulations in which precipitation was eliminated

The following formulations (CLX 123/B, CLX 125/B and CLX 127/B – Table 4.17) represent the first generation of CLX formulations which were produced on the basis of all the findings previously described and crucially in which there was no precipitation evident (immediately or overtime). The formulations containing SDS were transparent (both the original mixture and the resultant beads) which suggests that they were potentially micellar solutions. The formulation containing Opadry® White had a white appearance which was likely attributable to the Opadry® White powder forming a fine suspension.

Table 4.17 Composition of formulations CLX 123/B, CLX 124/B CLX 125/B and CLX 127/B

Formulations		CLX 123/B	CLX 124/B	CLX 125/B	CLX 127/B
Component Concentration (mg/g)	Celecoxib	54.68	88.61	56.79	58.49
	Solutol® HS-15	492.13	501.97	511.14	526.38
	Gelatine	396.71	282.52	298.56	305.75
	SDS	-	95.36	99.80	75.35
	Opadry White	11.98	-	-	-
	D-Sorbitol	44.51	31.54	33.70	34.04

Release analysis was performed on 50 mg doses of these three formulations (CLX 123/B, CLX 125/B and CLX 127/B). The dissolution medium employed was PW. The reader is reminded that in the case of the Celebrex™ (marketed CLX product), the maximum % of CLX released in PW was 6 % (Refer to Chapter 2). In contrast the maximum % drug release for the three optimised formulations ranged between 69 and 84 % release (Figure 4.7). It is important to note that the concentration of drug in the 'surfactant phase' was reduced to 10% in all of these formulations. CLX 124/B (formulation containing 15% CLX in the 'surfactant phase') was produced in direct comparison to CLX 125/B and it was observed that precipitation occurred 2–3 h after the mixture was prepared which again emphasised the criticality of drug loading with respect to precipitation.

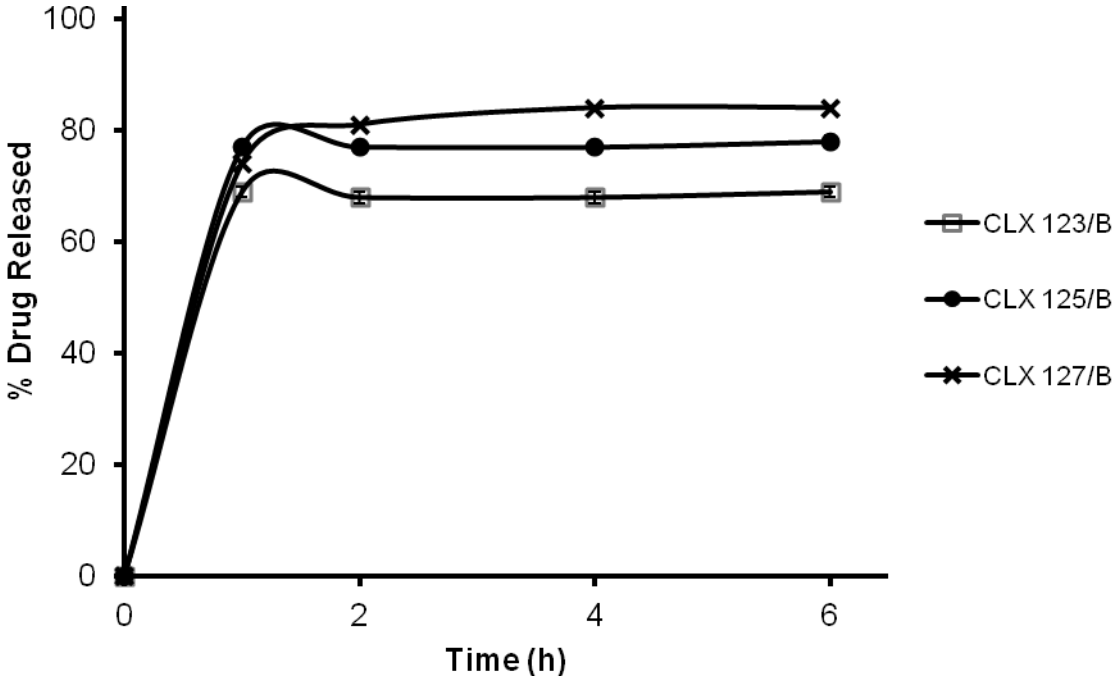

Figure 4.7 Percentage of CLX released from formulations CLX 123/B, CLX 125/B and CLX 127/B tested in PW. The data presented are mean values ± STDEV (n=3).

4.4.3 Physical characterisation of optimised microbead formulations

In order to compare and understand the performance of the formulations described above, it was required to characterise the internal structure of these formulations. Although image analysis had previously been performed on slices of microbeads (e.g., for formulation CLX 104/B - Figure 4.6), it was found that this method had the potential to be destructive (the integrity of the bead could be compromised as a result of the slicing process). An alternative approach involving the preparation of 'thin films' of the microbead formulations was attempted. A number of fluorescent dyes were incorporated into some of these formulations in an effort to distinguish between the 'surfactant phases' and 'gelatine phases' of the formulations. These dyes are described

in Table 4.18 below. It was postulated that the amphiphilic dyes might have a higher affinity for the 'surfactant phase' whereas the water soluble dyes would have a higher affinity for the gelatine/water phase.

Table 4.18 List of amphiphilic and water soluble dyes incorporated into microbead formulations

Amphiphilic Dyes	Water Soluble Dyes
Nile Red	Dextran Alexa Fluor 546
Bodipy 505/515	Dextran Rhodamine Green
Sudan Orange	Dextran Cascade Blue

Based on a number of trials it was found that a combination of Dextran Alexa Fluor 546 and Nile Red were found to be the best dyes in distinguishing between the two phases and therefore these dyes were incorporated into all formulations produced for the purpose of microscopic analysis.

In order to understand the structure of optimised formulations such as CLX 125/B, a number of formulations were prepared to act as controls and/or to assess the impact of the addition and removal of certain formulation components (e.g., SDS). The formulations prepared are listed in Table 4.19 below. Two of these formulations (CLX 130/B and olive oil formulation) were prepared as placebos (i.e., no CLX API was included). The purpose of the olive oil formulation was to assess the impact of employing no surfactant within the formulation whilst CLX 130/B was prepared to assess the impact of removing API from CLX 125/B. CLX 131/B was prepared to assess the impact of substituting SDS with an alternative surfactant/precipitation inhibitor (Vitamin E TPGS). Formulation CLX 134/B was prepared to assess the impact of removing SDS from the formulation and also the impact of employing a smaller concentration of the 'surfactant phase' in comparison to CLX 125/B (i.e., less Solutol® HS-15).

Table 4.19 Composition of Formulations; Gelatine Control Formulation, CLX 130/B, CLX 131/B, CLX 134/B and olive oil formulation.

Formulations		Gelatine control formulation	CLX 130/B	CLX 131/B	CLX 134B	Olive oil formulation
Component Concentration (mg/g)	Celecoxib	-	-	58.45	28.35	-
	Solutol® HS-15	-	412.32	526.08	255.15	-
	Gelatine	903.61	445.50	303.94	644.33	314.89
	SDS	-	94.79	-		
	D-Sorbitol	96.39	47.39	34.27	72.16	34.24
	Vit E TPGS	-	-	77.25	-	-
	Olive Oil	-	-	-	-	520.29

Thin films of each of these formulations were prepared and photographs of each film were taken using an inverted microscope (Nikon Eclipse Ti). The microscope was predominantly used in its light microscope setting as opposed to fluorescent light (greater clarity was observed using the light microscope). At this time, the measurement function on the microscope was not operational therefore it was not possible to take measurements, however images were taken at similar levels of magnification (where possible) to allow for direct comparisons to be made. In Figure 4.8 A, the structure of a film of the gelatine control formulation represented in Table 4.19 is shown. It appeared to have consistent matrix appearance in which there were distinct regions (light blue and dark blue regions). This was not unexpected as gelatine is a polydisperse system comprising different lengths of protein chains that in turn consist of long hydrophobic chain segments and short hydrophilic segments (i.e., it has amphiphilic properties). In this instance Figure 4.8 A was acting as a background control for comparison with the other films.

In Figure 4.8 B, the structure of formulation CLX 134/B is shown. This formulation is similar to that previously described for CLX 125/B with the exception that SDS is not included in the formulation and also the concentration of Solutol® HS-15 is reduced. Figure 4.8 B illustrated a system in which there was evidence of the presence of large polydisperse vesicles. Figure 4.8 C illustrated the internal structure of a film of CLX 131/B, a formulation again similar to CLX 125/B but where SDS was substituted with Vitamin E TPGS. In this instance, it was evident that there were large vesicles or oil droplets present in the formulation. Similarly, Figure 4.8 D (olive oil formulation) displayed evidence of large oil droplets. Figures 4.8 C and D were both taken at a lower magnification (10X), which illustrated the large size of their respective oil droplets/vesicles in comparison to formulation CLX 134/B (Photograph 4.8 B). In Figures 4.8 E (CLX 130/B) and F (CLX 125/B), there was no evidence of vesicles present which suggested that their sizes were too small to be visible (i.e., mixed micelles could be present). This was a very important finding as it suggests that formulation CLX 125/B was a micellar solution (which corresponds with the fact that the formulation is transparent) in comparison to other formulations such as CLX 134/B, CLX 131/B or the olive oil formulation which appeared to be emulsions (they had a milky appearance – refer to Chapter 2 for emulsion description).

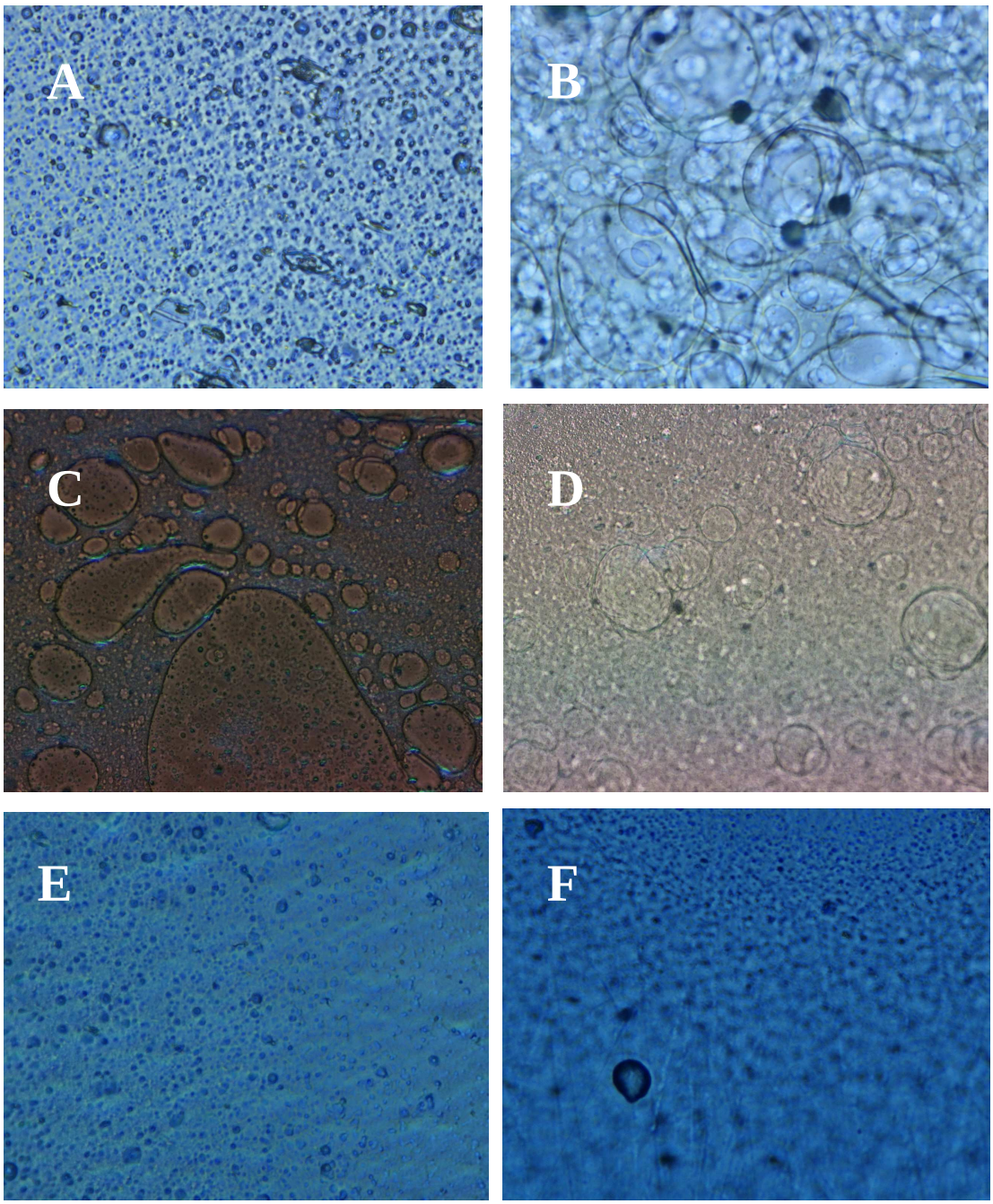

Figure 4.8 Photographs of thin films of various formulations taken using an Nikon Eclipse Ti Inverted Microscope**;** A) 40X image of gelatine formulation, B) 40X image of CLX 134/B, C) 10X image of CLX 131/B, D) 10X image of olive oil formulation, E) 40X image of CLX 130 and F) 40X image of CLX 125/B. All formulations included a combination of Dextran Alexa Fluor 546 and Nile Red dyes.

This is important as the *in-vivo* performance of formulations with a fine droplet size (e.g., microemulsions, micellar solutions etc.) have in cases been shown to be superior (better and more consistent absorption, less impact of food effects etc.) in comparison to equivalent large droplet formulations (e.g., emulsions). An example of this is the

improved performance of the Novartis® microemulsion Cyclosporine preparation Neoral® in contrast to the equivalent emulsion formulation Sandimmune® (Mueller *et al.*, 1994).

4.4.4 Selection of optimal CLX microbead formulation

Following a review of formulations CLX 123/B, CLX 125/B and CLX 127/B, the development of an optimal formulation focused on formulations containing SDS (i.e., formulations similar to CLX 125/B and CLX 127/B). This decision was made on the basis that formulations containing SDS were transparent therefore it was definite that there was no precipitation evident. In addition the entrapment efficiency for CLX 125/B (97.55 % ± 0.85 %)) was higher than that for CLX 123/B (91.06 % ± 0.58 %)

Formulation CLX 136/B (Table 4.20) was developed on the basis of CLX 125/B and CLX 127/B, with an enhanced drug loading (6%) and also a lower concentration of SDS. It was desirable to lower the quantity of SDS in the formulation given that the equivalent quantity of SDS in a 50 mg dose of formulation CLX 127/B (64.41 mg) was higher than the maximum quantity listed for SDS in a solid oral dosage form (51.69 mg) on the FDA's IIG database (FDA IIG Database, 2014). In order to build on the characterisation studies outlined in Section 4.4.3, the impact of removing SDS from formulation CLX 136/B was assessed in formulation CLX 135/B (Table 4.20). Release analysis was performed on 50 mg doses of formulations CLX 135/B and CLX 136/B (Figure 4.9) to evaluate the impact of removing SDS with respect to *in-vitro* drug release (in addition to its impact with respect to precipitation). The removal of SDS resulted in a dramatic decrease in the release, with a maximum of 46% release for formulation CLX 135/B in comparison to 80% release for formulation CLX 136/B. In order to gain a better understanding of the impact of removing SDS from the

formulation, thin films of these formulations (including Dextran Alexa Fluor 546 and Nile Red dyes) were prepared and photographs were taken using a Nikon Eclipse Ti inverted microscope (Figure 4.10). Similar to Figure 4.8A, in photograph A of figure 4.10, the structure of a film of a gelatine formulation is again represented for comparison purposes as a background control. In photograph 4.10B, the structure of formulation CLX 135/B is represented. Photograph 4.10B illustrated a system in which there was evidence of the presence of large polydisperse surfactant phase droplets/vesicles. In photograph 4.10C, the structure of formulation CLX 136/B is represented. The image in photograph 4.10C was similar to that for the gelatine control in photograph 4.10A. It is again proposed that the surfactant phase droplets/vesicles present in formulation CLX 136/B were too small to be visible (i.e., mixed micelles could be present). Similar to the finding in Section 4.3.3, this is an important point as it suggests that formulation CLX 136/B was a micellar solution (which also corresponds with the fact that the liquid formulation was transparent) in comparison to formulation CLX 135/B which was opaque. It is suggested that the greater *in-vitro* release performance of formulation CLX 136/B in comparison to formulation CLX 135/B which contained less surfactant (i.e., no SDS) was related to the droplet size of the corresponding formulations.

Table 4.20 Composition of formulations; CLX 135/B and CLX 136/B

Formulations		**CLX 135/B**	**CLX 136/B**
Component Concentration (mg/g)	Celecoxib	63.06	60.63
	Solutol® HS-15	567.50	545.66
	Gelatine	396.71	316.13
	SDS	-	42.51
	D-Sorbitol	38.05	35.07

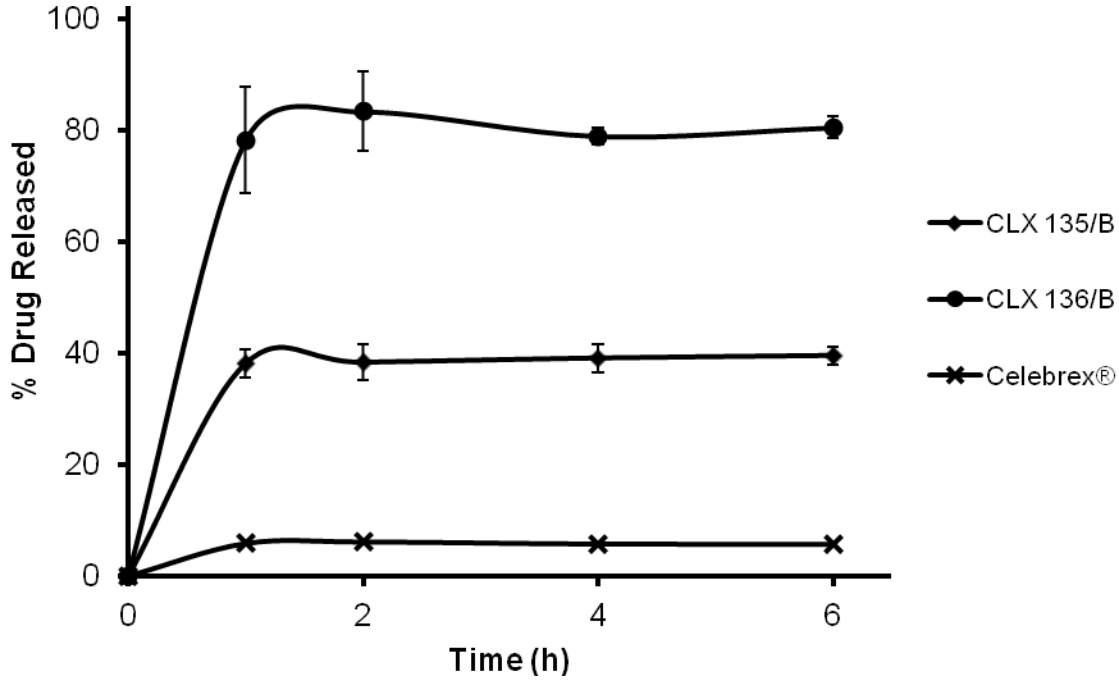

Figure 4.9 Percentage of CLX released from formulations CLX 135/B, CLX 136/B and Celebrex® tested in PW. The data presented are mean values ± STDEV (n=3).

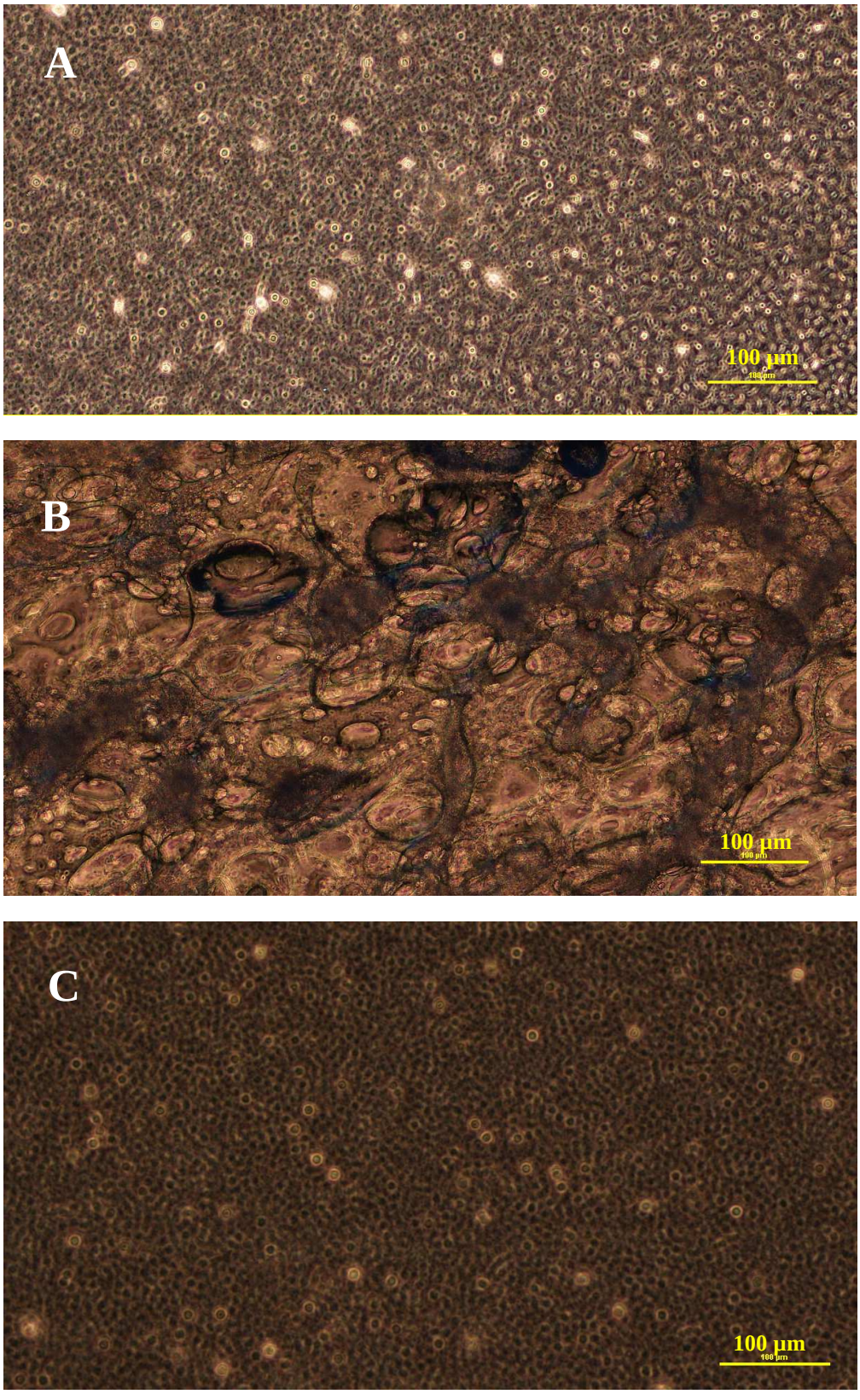

Figure 4.10 Photographs of thin films of three formulations taken using an Nikon Eclipse Ti Inverted Microscope; A) 10X image of gelatine formulation, B) 10X image of formulation 135/B and C) 10X image of formulation 136/B. All formulations included a combination of Dextran Alexa Fluor 546 and Nile Red dyes.

Some further formulations were also produced to assess the impact of changing the Solutol® HS-15 concentration in the formulation. Formulation CLX 138/B (Table 4.21) was produced in which the Solutol® HS-15 concentration was decreased by approximately 50% in comparison to CLX 136/B (Table 4.20). It is noted that the decrease in the Solutol® HS-15 also results in a corresponding increase in gelatine concentration. Although the release profiles for both formulations were similar (Figure 4.11), microscope analysis of a 'thin film' of CLX 138/B revealed the presence of large droplets/vesicles (Figure 4.12). Given that the drug concentration in CLX 138/B was relatively low at 2.8% w/w, it is not surprising that the release profile was similar to that of CLX 136/B, however the larger droplet/vesicle is indicative of a formulation in which release could pose problems at a higher drug loading. Formulation CLX 141/B (Table 4.21) was produced to test this theory. Release testing of CLX 141/B was performed in PW over a period of 6 h, with a maximum of 40 % release of CLX achieved (Figure 4.11). Formulation CLX 141/B had a milky white appearance therefore it was difficult to see vesicles/ droplets when viewed under the microscopic however needles of CLX were clearly visible (Figure 4.12 B). As a result of these findings, formulations with lower levels of Solutol® HS-15 (<45% w/w) were excluded from further assessment.

Table 4.21 Composition of formulations; CLX 138/B and CLX 141/B

Formulations		CLX 138/B	CLX 141/B
Component Concentration (mg/g)	Celecoxib	28.00	54.55
	Solutol® HS-15	252.01	218.18
	Gelatine	615.26	619.83
	SDS	36.30	38.02
	D-Sorbitol	68.43	69.42

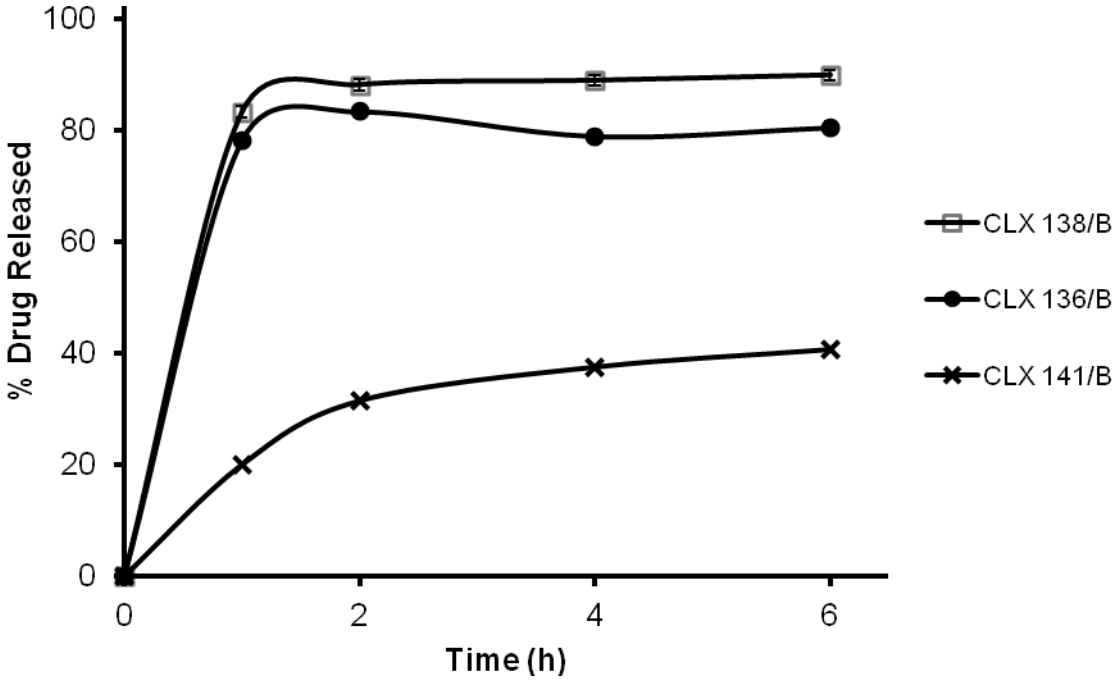

Figure 4.11 Percentage of CLX released from formulations CLX 136/B, CLX 138/B and CLX 138/B tested in PW. The data presented are mean values ± STDEV (n=3).

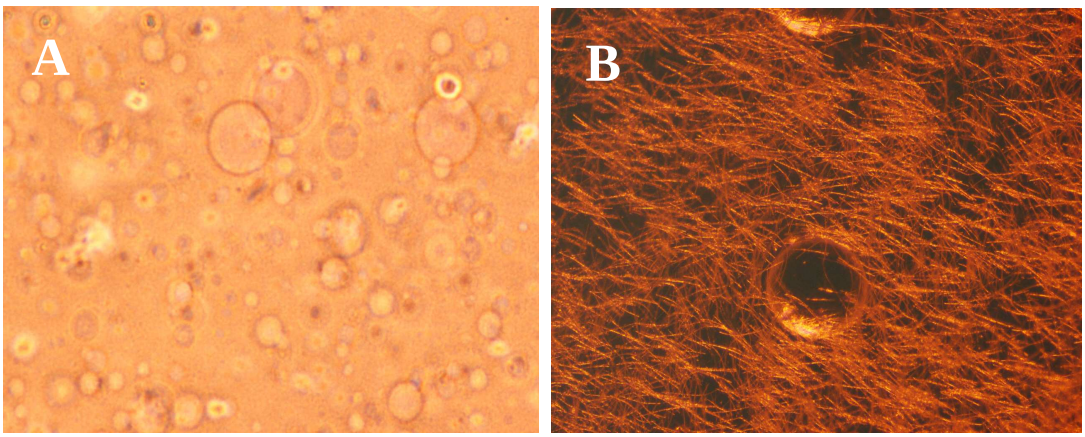

Figure 4.12 Photographs of thin films of two formulations taken using an Nikon Eclipse Ti Inverted Microscope; A) 40X image of formulation CLX 138/B and B) 10X image of formulation CLX 141/B. All formulations included a combination of Dextran Alexa Fluor 546 and Nile red dyes.

Following a review of all the formulations produced, CLX 136/B was considered to be the optimal formulation for progression to coating development and ultimately an *in-vivo* animal study. The 'surfactant phase' of formulation CLX 136/B constituted the liquid formulation CLX 016/L described in Chapter 3 (i.e., 10% CLX dissolved in Solutol® HS-15). Given that the other formulation described in Chapter 3, CLX 021/L (6.7% CLX dissolved in Solutol® HS-15: Miglyol® 810 (2:1)), had out performed CLX 016/L in the *in-vitro* cell study and subsequent release experiment (refer to Chapter 3), a final round of drug release experiments was conducted to understand the basis for this greater performance and to provide extra assurance that CLX 136/B (i.e., a formulation without Miglyol® 810N) was the optimal formulation for progression. Two liquid formulations, CLX 151/L and CLX 158/L (Table 4.22) were produced. The intention of formulation CLX 151/L (6.7% CLX dissolved in Solutol® HS-15) was to allow a direct comparison with formulation CLX 021/L in the absence of Miglyol® 810N, whereas CLX 158/L (10% CLX dissolved in Solutol® HS-15 and Miglyol® 810N (2:1) was

produced to allow for a direct comparison with CLX 016/L with the addition of Miglyol® 810N.

Table 4.22 Composition of formulations; CLX 016/L, CLX 021/L, CLX 151/L and CLX 158/L

Formulations		CLX 021/L	CLX 151/L	CLX 016/L	CLX 158/L
Component Concentration (mg/g)	Celecoxib	66.67	66.67	100.00	100.00
	Solutol® HS-15	600.00	933.33	900.00	600.00
	Miglyol® 810N	333.33	-	-	300.00

Release analysis in PW over a period of 12 h was performed on formulations CLX 151/L and CLX 158/L and a comparison made to previous release experiments on formulations CLX 016/L and CLX 021/L (Figure 4.13). The results illustrated that although the inclusion of Miglyol® 810N in the formulation had some initial stabilisation effect, that the % of drug still in solution at 12 h was comparable for formulations CLX 016/L and CLX 158/L. The results also demonstrated that the primary difference in formulations CLX 016/L and CLX 021/L was the loading of CLX employed, as when the loading of CLX in Solutol® HS-15 was decreased to 6.7% w/w in formulation CLX 151/L, the % of drug release was comparable to CLX 021/L over a period of 12 h. These results provided an added assurance that formulation CLX 136/B (i.e., formulation excluding Miglyol® 810) was the correct formulation choice for the next phase of the project.

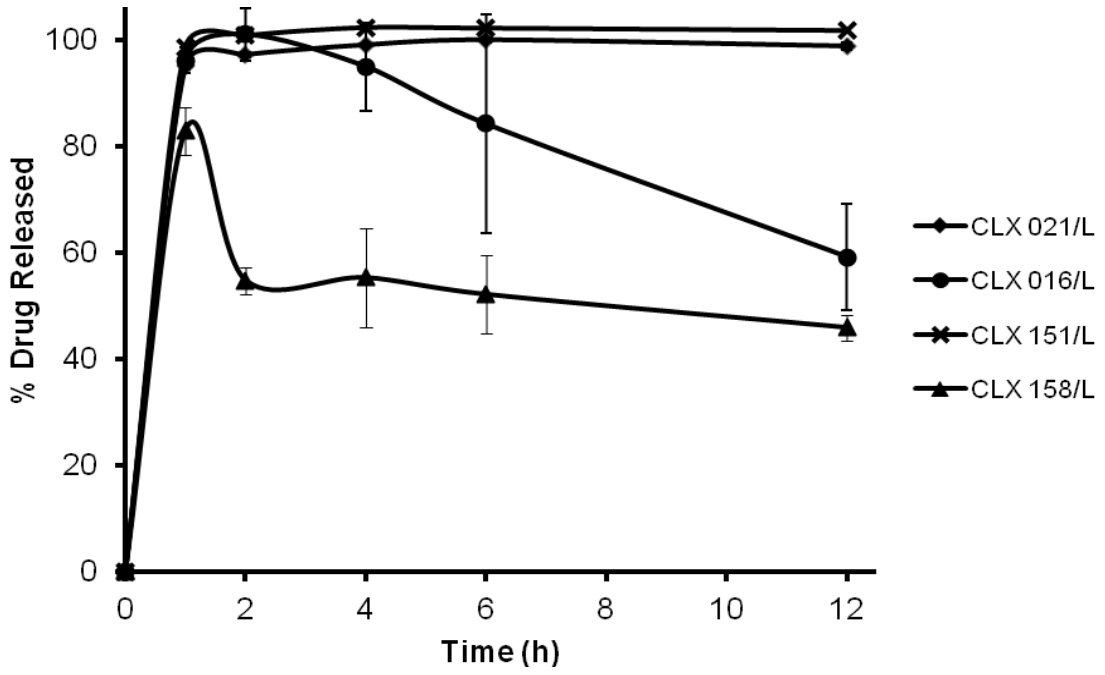

Figure 4.13 Percentage of CLX released from formulations CLX 021/L, CLX 016/L, CLX 151/L and CLX 158/L tested in PW. The data presented are mean values ± STDEV (n=3).

4.4.5 CLX 136/B – optimal CLX microbead formulation

Microbead formulation CLX 136/B represented the development of an optimal oral lipophilic drug delivery system for CLX. The inclusion of such a high level of surfactant (>50% w/w of the entire formulation) precludes the formulation from incorporation into conventional oral dosage forms such as soft gelatin capsules and also microencapsulation technologies such as shell/core microcaspules, due to interactions between the inner capsule contents and the capsule shell. Lipophilic CLX formulations have previously been developed, such as the nanoemulsion formulation presented by Shakeel and Faisal (Shakeel and Faisal , 2010), however as nanoemulsions have a high water content they have been shown to be unsuitable for incorporation into soft gelatin,

hard gelatine or HPMC capsules for oral delivery. Also the high water content of these type of formulations promotes hydrolysis and/or precipitation of certain drugs on long-term storage, which ultimately affects their utility in oral delivery (Date *et al.,* 2010). Based on current marketed technologies, the only suitable mode of administration of such an emulsion would be as an oral solution, however oral solutions have an inherent disadvantage in that they are not amenable to further processing to allow for targeted or sustained delivery (e.g., for colon delivery). It is acknowledged that Self Emulsifying Drug Delivery Systems (SEDDS) and Self Micro-Emulsifying Drug Delivery Systems (SMEDDS) are suitable for incorporation into soft gelatine capsules and a number of CLX SEDDS/SMEDDS have been reported (Subramanian *et al*., 2004, and Song *et al*., 2013). Although the delivery of CLX SEDDS/SMEDDS has many advantages including improved solubility and bioavailability, one of their primary disadvantages is that they are currently delivered as single dosage units (e.g., in soft gelatine capsules) and as eluded to in Section 4.2.4 there are a number of advantages of delivering drugs in a multiparticulate format and in the context of this project (delivery of CLX to the colon), a multiparticulate format is essential.

Multiparticulate formulations containing CLX SMEDDS/SEDDS have been attempted, however entrapment efficiencies have ranged between 60-82% (Homar *et al.,* 2009) in contrast to an entrapment efficiency of 97.02 % ± 0.99% in the case of CLX 136/B. It is also worth noting that the shape of CLX 136/B beads (robust spherical beads) compared favourably with dried CLX microcapsules reported by Homar and colleagues, in which the microcapsules produced were irregular in shape and in many cases were leaky resulting in a low % of entrapment efficiency.

As shown in Figure 4.9, release analysis on 50 mg doses in PW resulted in a maximum release of 80% for formulation CLX 136/B over a period of 6 h. In addition to outperforming Celebrex®, this data also represents an increase in the release of CLX

from microcapsules compared to that reported in studies by Homar and colleagues (Homar *et al*., 2007 and 2009). In the latter study, CLX microcapsule formulations with a maximum release ranging from 9–16% were reported (the release experiments were performed on 3 mg doses). In the 2007 study, CLX microcapsule formulations with a maximum release in the range of 60–80 % were reported however the dissolution media employed contained a surfactant (2 % Tween 80) to facilitate the release of the drug and the release experiments were again performed on 3 mg doses. This data illustrates that the microbead approach presented here is advantageous to the microcapsule approach presented by Homar and colleagues.

4.5 Conclusions

Chapters 2 and 3 described the development of liquid CLX formulations which were tested in an *in-vitro* cell culture study and yielded positive results. In this phase of the project (Chapter 4), these liquid formulations were successfully translated into gelatine based microbeads via the use of a gravity 'dripping' technique. An optimal microbead formulation (CLX 136/B) with a commercially viable loading of 6% w/w CLX was produced. Formulation CLX 136/B produced spherical and robust microbeads with a high drug content of 97% and 80% drug release in PW, thereby meeting the formulation objectives set out at the start of the study. The viable drug loading of 6% w/w was ultimately achieved by eliminating the onset of precipitation in the formulations by including SDS as a precipitation inhibitor and also by reducing the gelatine concentration within the formulation. The inclusion of SDS was also shown to improve the *in-vitro* drug release performance of the formulation. CLX 136/B was demonstrated to have a greater drug release performance than the marketed CLX product Celebrex®

and is believed to represent a micellar solution based on the transparent appearance of the original liquid and image analysis of the resultant beads.

The optimised beads produced in this phase of the study ultimately provided a platform for the treatment of CRC as it allowed the possibility of presenting CLX to the colon in a pre-solubilised multipariculate format, however the next challenge was to target these multiparticulates to the colon and to then to assess their potential for the treatment and prevention of CRC using an appropriate animal model. This challenge is described in Chapter 5.

4.6 Nomenclature

Table 4.23 List of abbreviations which are listed according to their appearance in the text.

Abbreviation	Definition
CLX	Celecoxib
CRC	Colorectal cancer
HME	Hot melt extrusion
CQA	Critical quality attribute
PEG	Polyethylene glycol
API	Active pharmaceutical ingredient
HLB	Hydrophilic lipophilic balance
STDEV	Standard deviation
SDS	Sodium dodecyl sulphate
HPMC	Hydroxypropyl cellulose
HPC	Hydroxypropyl cellulose
COA	Certificate of analysis
HPLC	High performance liquid chromatography
UV	Ultraviolet
GI	Gastrointestinal
IIG	Inactive ingredient
PW	Purified water
TPGS	Tocopherol polyethylene glycol succinate
PVA	Polyvinyl alcohol
SEDDS	Self-emulsifying drug delivery system
SMEDDS	Self-microemulsiying drug delivery system
CLX xxx/L	Celecoxib liquid formulation numbering system where xxx is a sequential number and L is liquid
CLX xxx/B	Celecoxib bead formulation numbering system where xxx is a sequential number and B is bead

4.7 Acknowledgements

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4.8 References

Andrews, G.P., Jones, D.S., Diak, O.A., Margetson, D.N. & McAllister, M.S. 2009, "Hot-melt extrusion: an emerging drug delivery technology", *Pharmaceutical Technology Europe*, vol. 21, no. 1, pp. 18-23.

Brandau, T. 2014, "Annular Jet-Based Processes" in *Microencapsulation in the Food Industry: A Practical Implementation Guide*, eds. A. Gaonkar, N. Vasisht, A. Khare & R. Sobel, Elsevier, San Diego, pp. 99-110.

Brouwers, J., Brewster, M.E. & Augustijns, P. 2009, "Supersaturating drug delivery systems: the answer to solubility-limited oral bioavailability?", *Journal of Pharmaceutical Sciences*, vol. 98, no. 8, pp. 2549-2572.

Cavallari, C., Rodriguez, L., Albertini, B., Passerini, N., Rosetti, F. & Fini, A. 2005, "Thermal and fractal analysis of diclofenac/Gelucire 50/13 microparticles obtained by ultrasound-assisted atomization", *Journal of Pharmaceutical Sciences*, vol. 94, no. 5, pp. 1124-1134.

Chávarri, M., Marañón, I. & Villarán , M.C. 2012, "Encapsulation Technology to Protect Probiotic Bacteria" in *Probiotics*, ed. E.C. Rigobelo, Intech, Winchester, pp. Ebook.

Chawla, G., Gupta, P., Thilagavathi, R., Chakraborti, A.K. & Bansal, A.K. 2003, "Characterization of solid-state forms of celecoxib", *European Journal of Pharmaceutical Sciences*vol. 20, no. 3, pp. 305-317.

Colorcon Opadry® White 20A28380 product specification, 2010.

Colorcon Opadry® II product specification, 2010.

Date, A.A., Desai, N., Dixit, R. & Nagarsenker, M. 2010, "Self-nanoemulsifying drug delivery systems: formulation insights, applications and advances", *Nanomedicine (London, England)*, vol. 5, no. 10, pp. 1595-1616.

Gharsallaoui, A., Roudaut, G., Chambin, O., Voilley, A. & Saurel, R. 2007, "Applications of spray-drying in microencapsulation of food ingredients: An overview", *Food Research International,* vol. 40, no. 9, pp. 1107-1121.

Ghosh, S.K. 2006, "Functional Coatings and Microencapsulation: A General Perspective" in *Functional Coatings,* ed. S.K. Ghosh, Wiley, New Jersey, pp. 1-28.

Guzman, H.R., Tawa, M., Zhang, Z., Ratanabanangkoon, P., Shaw, P., Gardner, C.R., Chen, H., Moreau, J.P., Almarsson, O. & Remenar, J.F. 2007, "Combined use of crystalline salt forms and precipitation inhibitors to improve oral absorption of celecoxib from solid oral formulations", *Journal of Pharmaceutical Sciences,* vol. 96, no. 10, pp. 2686-2702.

Homar, M., Dreu, R., Kerc, J. & Gasperlin, M. 2009, "Preparation and evaluation of celecoxib-loaded microcapsules with self-microemulsifying core", *Journal of Microencapsulation,* vol. 26, no. 6, pp. 479-484.

Homar, M., Ubrich, N., El Ghazouani, F., Kristl, J., Kerc, J. & Maincent, P. 2007, "Influence of polymers on the bioavailability of microencapsulated celecoxib", *Journal of Microencapsulation,* vol. 24, no. 7, pp. 621-633.

Kalepu, S., Manthina, M. & Padavala, V. 2013, "Oral lipid-based drug delivery systems – an overview", *Acta Pharmaceutica Sinica B,* vol. 3, no. 6, pp. 361-372.

Mackaplow, M.B., Zarraga, I.E. & Morris, J.F. 2006, "Rotary spray congealing of a suspension: effect of disk speed and dispersed particle properties", *Journal of Microencapsulation,* vol. 23, no. 7, pp. 793-809.

Maniruzzaman, M., Boateng, J.S., Snowden, M.J. & Douroumis, D. 2012, "A review of hot-melt extrusion: process technology to pharmaceutical products", *ISRN Pharmaceutics,* vol. 2012, pp. 436763.

Martinsa, R.M., Siqueiraa, S. & Freitasa, L.A.P. 2012, "Spray Congealing of Pharmaceuticals: Study on Production of Solid Dispersions Using Box-Behnken Design", *Drying Technology: An International Journal,* vol. 30, no. 9, pp. 935-945.

Mueller, E.A., Kovarik, J.M., van Bree, J.B., Tetzloff, W., Grevel, J. & Kutz, K. 1994, "Improved dose linearity of cyclosporine pharmacokinetics from a microemulsion formulation", *Pharmaceutical Research,* vol. 11, no. 2, pp. 301-304.

Passerini, N., Albertini, B., Perissutti, B. & Rodriguez, L. 2006, "Evaluation of melt granulation and ultrasonic spray congealing as techniques to enhance the dissolution of praziquantel", *International Journal of Pharmaceutics,* vol. 318, no. 1-2, pp. 92-102.

Passerini, N., Perissutti, B., Moneghini, M., Voinovich, D., Albertini, B., Cavallari, C. & Rodriguez, L. 2002, "Characterization of carbamazepine-Gelucire 50/13 microparticles prepared by a spray-congealing process using ultrasounds", *Journal of Pharmaceutical Sciences,* vol. 91, no. 3, pp. 699-707.

Porter, S.C. 2013, "Coating of Tablets and Multiparticulates" in *Aulton's Pharmaceutics, 4th Edition The Design and Manufacture of Medicines*, ed. K. Taylor, Amsterdam, Elsevier, pp. 567-582.

Rowe, R.C., Sheskey, P.J. & Owen, S.C. 2006, *Handbook of Pharmaceutical Excipients,* 5th Edition edn, Pharmaceutical Press, London.

Saha, R.N., Sajeev, C., Jadhav, P.R., Patil, S.P. & Srinivasan, N. 2002, "Determination of celecoxib in pharmaceutical formulations using UV spectrophotometry and liquid chromatography", *Journal ofPharmaceutical and Biomedical Analysis,* vol. 28, no. 3-4, pp. 741-751.

Shakeel, F. & Faisal, M.S. 2010, "Nanoemulsion: A promising tool for solubility and dissolution enhancement of celecoxib", *Pharmaceutical Development and Technology,* vol. 15, no. 1, pp. 53-56.

Singh, M.N., Hemant, K.S., Ram, M. & Shivakumar, H.G. 2010, "Microencapsulation: A promising technique for controlled drug delivery", *Research in Pharmaceutical Sciences,* vol. 5, no. 2, pp. 65-77.

Song, W.H., Park, J.H., Yeom, D.W., Ahn, B.K., Lee, K.M., Lee, S.G., Woo, H.S. & Choi, Y.W. 2013, "Enhanced dissolution of celecoxib by supersaturating self-

emulsifying drug delivery system (S-SEDDS) formulation", *Archives of Pharmacal Research,* vol. 36, no. 1, pp. 69-78.

Subramanian, N., Ray, S., Ghosal, S.K., Bhadra, R. & Moulik, S.P. 2004, "Formulation design of self-microemulsifying drug delivery systems for improved oral bioavailability of celecoxib", *Biological & Pharmaceutical Bulletin,* vol. 27, no. 12, pp. 1993-1999.

Umer, H., Nigam, H., Tamboli, A.M. & Nainar, M.S.M. 2011, "Microencapsulation: Process, Techniques and Applications", *IJRPBS*, vol. 2, no. 2, pp. 474-481.

Whelehan, M. 2010, Liquid-core microcapsules: A mechanism for the recovery and purification of selected molecules in different environments. PhD Thesis, Dublin City University, Dublin, Ireland.

FDA - Inactive Ingredients Database, 2014. Available: http://www.accessdata.fda.gov/scripts/cder/iig/index.cfm [2014, Oct/13].

Nisco- Gravity Dripping, 2014. Available: http://www.nisco.ch/working_principle_dripping_by_gravity.htm [2014, Oct/13].

Particle Sciences Technical Brief – Encapsulation, 2010. Available: http://www.particlesciences.com/docs/technical_briefs/TB_2010_7.pdf [2014, Oct/13].

CHAPTER 5

Colon targeting of microbeads for an *in-vivo* animal study

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5.1 Abstract

The purpose of this phase of the project was to develop a colon targeted celecoxib (CLX) microbead formulation, on the basis of formulation CLX 136/B (described in Chapter 4) for use in an AOM (azoxymethane)/DSS (dextran sodium sulphate) colorectal cancer (CRC) mouse model. One of the primary objectives of the project was to apply a sustained release coating polymer to the beads to allow for colon specific delivery to a mouse according to a defined target product profile (TPP). A diffusion based polymer coat (Surelease®) was applied to the microbeads at a weight gain (w/g) of 8% and despite not meeting the TPP, this partially colon targeted microbead formulation was selected for progression to the AOM/DSS CRC mouse model. In the murine model, the effect of the microbead formulation on the attenuation of CRC tumours was compared to that of Celebrex® and was found to be marginally better. The anti-inflammatory effects for both formulations were also assessed, with the microbead formulation being found to have a significant effect compared to the control in the case of colon length and histology scoring whereas, the effect of Celebrex® did not meet significance.

5.2 Introduction

5.2.1 Background

At the conclusion of Chapter 4, CLX 136/B, a lipid based CLX microbead formulation was identified as having met all the desired critical quality attributes (CQAs) for an intermediate CLX product designed for the treatment and prevention of CRC. Details of all the CQAs for the intermediate product are detailed in Chapter 4, however one of the

key requirements was that the formulation would provide a suitable substrate for the application of controlled release polymers in order to allow for colon targeting. The next phase of the project focused on developing the intermediate product described in Chapter 4 to yield a colon specific finished product via the application of controlled release polymers to CLX 136/B microbeads. This phase of the project also involved the subsequent testing of the coated microbeads in an *in-vivo* CRC animal study to assess and compare their anti-cancer effects to that of the marketed CLX product Celebrex®.

CRC development is a long term process beginning in normal epithelial cells via aberrant crypts and progressive adenoma stages to carcinomas *in situ* and then metastasis (De Robertis *et al.,* 2011) (see Chapter 1 for further details). The goal of modelling human CRC in animals is to recapitulate the molecular etiology, pathology, and clinical progression of the disease and to provide a tool for advancing our understanding of the tumour response to novel chemopreventative and therapeutic strategies (Johnson and Fleet, 2013 and De Robertis *et al.,* 2011). A variety of models of CRC have been developed that mimic human CRC, a summary of which is provided in Section 5.2.2. Colon delivery has for a long period of time been exploited for the topical treatment of intestinal pathological conditions such as irritable bowel disease (IBD) (Maroni *et al*., 2012). For example in the case of Crohn´s disease (CD) and ulcerative colitis (UC), dosage forms of mesalazine (a 5-aminosalicylic acid (5-ASA) anti-inflammatory drug used to treat these conditions) have been designed to achieve high concentrations of the drug at the local inflamed areas (lower small intestine (SI) and colon) while minimizing release in the stomach and upper SI so as to avoid premature absorption and consequent drug wastage and systemic side effects (Klein *et al.,* 2002). Given the requirement to target CLX to the colon in the treatment of CRC, a review of formulation options for colon targeting is included in Section 5.2.3. Finally the objectives for this phase of the project are detailed in Section 5.2.4.

5.2.2 Animal models for CRC

The available animal models for CRC constitute three broad categories; a) spontaneous cancers in various animal species (e.g., dogs, sheep and rodents), b) chemically induced cancers in rodents and c) cancers resulting from genetically modified mice (Johnson and Fleet, 2013 and De Robertis *et al.*, 2011). It is noted that there also exist other animal models which involve a cross over between these broad categories (e.g., chemical induction on genetically modified mice).

Animal models involving spontaneous cancers are not widely used for a variety of reasons including, low prevalence of CRC in animals, long latency of carcinogenesis, cost and non-correlation with human CRC (e.g., tumours often occur in the SI of the animals described rather than the colon). This review of animal models for CRC is therefore restricted to chemically and genetically induced tumour models.

5.2.2.1 Chemically induced tumour rodent models

Given that spontaneous incidence of CRC in rodents is low (1–4%) (Karim and Huso, 2013), many chemicals have been used to induce CRC. These carcinogens include (a) heterocyclic amines (HCAs) (b) aromatic amines (AAs), (c) alkylnitrosamide compounds and (d) dimethylhydrazine (DMH) and AOM (De Robertis *et al.,* 2011). These four categories of carcinogens are described below in addition to a fifth category describing enhanced AOM models.

A. Heterocyclic amines (HCAs)

HCAs include 2-amino-1-methyl-6-phenylimidazol (4,5-b) pyridine (PhIP) and 2-amino-33-methylimidazo[4,5-f] quinolone (IQ) which were identified in broiled and grilled meat and fish and have since been introduced into experimental CRC as they

have been demonstrated to be highly mutagenic and tumorigenic in rodent models, with respect to colon cancer induction. It has been also shown that the incidence of tumours in rodents arising from treatment with HCAs increases when co-administered with a high fat diet which is important given the links between CRC and dietary factors (De Robertis *et al.,* 2011 and Rosenberg *et al*., 2009). Although much attention has been focused on the use of HCAs such as PhIP and IQ for chemically inducing CRC tumours in rodents, primarily due to the fact that epidemiologic evidence links PhIP from cooked meat to increased CRC risk (Johnson and Fleet, 2013), there are a number of disadvantages associated with the model. These disadvantages include; a) the carcinogens demonstrate multi-target-organ specificity resulting in prostate and mammary tumours in addition to colon tumours, b) tumour incidence is generally low (5–28% for 52 week studies), c) very lengthy studies (>100 weeks) are required for higher rates (43–55%) of tumour incidence, d) lengthy studies (>100 weeks) are associated with severe toxicity and e) the studies are not cost effective due to the length of the studies required (De Robertis *et al.,* 2011 and Rosenberg *et al*., 2009).

B. Aromatic amines (AAs)

AAs such as 3,2'-dimethyl-4-aminobiphenyl (DMBA) have been shown to chemically induce both benign (adenomas) and malignant (adenocarcinomas) tumours in rodents. Similar to HCAs, the co-administration of AAs with a high fat diet results in a greater incidence of CRC in rodents (Rosenberg *et al*., 2009). There are a number of disadvantages of DMBA induced CRC rodent models inducing the following; a) a large number of injections (up to 20) are required to induce colonic tumours, b) similar to HCAs, DMBA demonstrates multi-target-organ specificity resulting in neoplasms in other tissues including mammary glands, the stomach and the bladder and c) the studies

are not cost effective due to the number of injections required (Karim and Huso, 2013, De Robertis *et al.*, 2011 and Rosenberg *et al*., 2009).

C. Alkylnitrosamide compounds

One of the features of both HCAs (e.g., PhIP) and AAs (DMBA) which was not described above is a requirement for these compounds to undergo metabolic activation prior to exerting their carcinogenic effect *in-vivo.* Methylnitrosourea (MNU) and N-methyl-N-nitro-N-nitrosoguanidine (MNNG) are examples of directly acting alkylating agents that do not require metabolic activation and therefore are considered to be potent topical carcinogens (Rosenberg *et al*., 2009). The direct acting nature of these agents is considered to be the primary advantage with respect to use in CRC models as they enable the modifying effects of xenobiotics (e.g., chemotherapy drugs) to be studied without consideration of the metabolism of the initiating carcinogen (i.e., it removes an unwanted variable). The direct acting feature of these agents is however also a disadvantage as it is necessary to administer the carcinogen via an intrarectal injection which whilst ensuring local action, is a technically difficult procedure (Karim and Huso, 2013 and Rosenberg *et al*., 2009).

D. DMH and AOM

DHM (1,2-dimethylhydrazine) or its metabolite azoxymethane (AOM) have been widely used to induce CRC in rodents. DMH is a precursor of methylazoxymethanol (MAM), a carcinogen found in cycad flour. Both DMH and AOM require several metabolic activation steps, some of which are postulated to be favourable with respect to specifically targeting the colon (Rosenberg *et al*., 2009). The majority of recent studies have focused on the use of AOM rather than DMH due to a number of practical

advantages associated with AOM including the following; high potency, simple mode of application, excellent stability in solution and lower cost. The primary advantage of DMH or AOM CRC rodent models is that the chemically induced tumours share many of the histopathological features of human CRC and therefore are considered to be good models. Some disadvantages of the model include infrequent *APC* (adenomatous polyposis coli) or *p53* gene mutations which are common in human CRC. The primary disadvantage of both DMH and AOM models is however the latency periods required prior to tumour formation which can range from 21 to 40 weeks and also as many as 10 injections are required (De Robertis *et al.,* 2011).

E. Enhanced AOM models

Based on the information described in Sections 5.2.2.1 A-D above, rodent models involving AOM are deemed among the most favourable models for a variety of reasons including the route of administration, relevance of tumours with respect to human CRC tumours and the colon specific nature of the carcinogen. In an effort to circumvent the disadvantages of AOM-only models described in Section 5.2.2.1 D (namely long latency periods and multiple injections), a lot of attention has been focused on enhancing these AOM models by combining AOM with the inflammatory agent DSS, which has resulted in a dramatic shortening of the latency time and reduction in the number of intraperitoneal (i.p.) injections. In two studies, involving a single injection of AOM (7.4–10 mg/kg body weight) in combination with a) 3 cycles (7 day cycle) of administering 3% DSS in the drinking water and b) one cycle (7 day cycle) of administering 2% DSS in the drinking water, latency periods were demonstrated to be reduced to 12 weeks (De Robertis *et al.,* 2011). AOM/DSS has also been shown to be a useful tool in the evaluation of CRC chemopreventative strategies (Karim and Huso,

2013), including its use for assessing the chemopreventative effects of CLX (Lee *et al*., 2005), which was of particular relevance in the context of this study.

5.2.2.2 Genetic tumour models

As discussed in Chapter 1, familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC) are two genetic conditions which are risk factors for the development of CRC. Strains of mice have been genetically modified in order to mimic cancers caused by FAP and HNPCC. These genetic models are discussed below.

A. Genetic mouse model for FAP

Patients with FAP carry a germline mutation in the *APC* gene. They develop hundreds to thousands polyps within the large intestine and they are at high risk for developing CRC. A mouse model, referred to as APC^{Min} mouse, which possess a similar mutation to that observed in FAP patients, has been extensively used to study the development, treatment and prevention of CRCs that contain somatic *APC* mutations (Johnson and Fleet, 2013). The primary disadvantage of the APC^{Min} mouse model is with respect to tumour location, as *APC* mutant mice usually develop more adenomas in their small intestine than their large intestine and also only on rare occasions do the adenomas progress to invasive adenocarcinomas which is in stark contrast to FAP patients (i.e., large number of adenomas in the large intestine which progress to invasive adenocarcinomas) (Karim and Huso, 2013).

B. Genetic mouse model for HNPCC

In the case of HNPCC, patients possess mutations in one of the DNA mismatch repair (MMR) genes; *MSH2, MLHJ, PMSJ, and PMS2*. The development of mouse models for HNPCC has proved challenging as the heterozygous deletion of the MMR genes involved is not sufficient to predispose to cancer and as a result mice with homozygous deletions have been used as disease models for HNPCC-like cancers. Although these models do result in intestinal tumour formation, they also have a tendency to result in lymphoma development in which is not reflective of HNPCC (De Robertis *et al.,* 2011). A novel mouse model has been developed in which *MSH2* is knocked down in villin-expressing tissues (i.e., the small and large intestine) but normal MMR activity is preserved in the rest of the mouse (Karim and Huso, 2013). Although this model is advantageous over the homozygous deletion models described in that the mice do not develop lymphomas, the disadvantage of the model is that tumour formation occurs predominantly in the small intestine (Johnson and Fleet, 2013).

5.2.2.3 CRC model selection

On the basis of the information described in Sections 5.2.2.1 and 5.2.2.2, the AOM/DSS mouse model was selected as the appropriate model for this study primarily on the basis that it was the model that most accurately recapitulated the histopathology and pathogenesis observed in sporadic human CRC but also because it represented an inflammation-driven CRC model which is important in the context of the links between inflammation (including colitis) and CRC as outlined in Chapter 1. Experimental details for the model are detailed in Section 5.3.2.5, however a detailed overview of the model focusing on its applicability with respect to CRC and intervention studies for COX-2 inhibitors is provided below.

5.2.2.3.1 AOM/DSS mouse model

As referred to earlier, a key advantage of the AOM/DSS mouse model is that the development of cancer in the model is highly reflective of that seen in humans. Aside from exhibiting histopathological features similar to human CRC, the tumours produced in the model also accurately reflect the pathogenesis observed in human CRC (e.g., tumours are very frequent in the distal part of the colon, which is also the predominant location of spontaneous CRC in humans). In addition, the AOM/DSS mouse model has been shown to be particularly applicable to tumour progression driven by colitis (Oshima and Oshima, 2012).

By using a combination of AOM (tumour-inducing agent) and DSS (tumour-promoting agent), the AOM/DSS model reproduces colorectal carcinogenesis promoted by an initial acute inflammation phase (the DSS dissolved in drinking water is toxic to the epithelial lining of the colon and produces severe colitis), and has a much shorter latency period than models based on only AOM or DSS administration. AOM only models have already been described, however there also exists DSS-only models (widely used as IBD models). These DSS models typically require many cycles of DSS administration and also result in a low incidence of tumours (De Robertis *et al.*, 2011 and Becker *et al.*, 2005). CRC development following DSS-induced inflammation supports the hypothesis that chronic inflammation in IBD plays a crucial role in malignant epithelial neoplasia in the colon (Rosenberg *et al.*, 2009).

In Chapter 1, the possible tumourigenic effect of cyclooxygenase-2 (COX-2) on the development of CRC tumours (in both sporadic CRC and CACRC (colitis associated CRC)) was discussed. In addition to displaying similar histopathological features and pathogenesis as human CRC, the tumours induced in the AOM/DSS model also display very similar characetristics to human CRC at a molecular level. Included among these

molecular similarities is an observation of increased levels of COX-2 enzymes in AOM/DSS induced tumours (De Robertis *et al.*, 2011), which further vindicates its selection in the context of this project. It is noted however (as per Chapter 1) that other studies involving AOM/DSS treatment on COX-$2^{2/2-}$ knockout mice (i.e., mice in which COX-2 is not expressed) have also resulted in tumour formation (Ishikawa and Herschman, 2010).

Regardless of the role that COX-2 plays in CRC, as described in Chapter 1 there is a large body of evidence illustrating the anti-cancer effects of CLX including a number of chemopreventative studies for CLX using the AOM/DSS CRC model (Lee *et al*., 2005, Coudry *et al.*, 2004 and Li *et al*., 2009).

In summary, the AOM/DSS mouse model was selected as the model of choice for the CLX intervention studies on the basis that it is reflective of human CRC and also because it is considered to be a highly reproducible, potent and affordable model for studying colon carcinogenesis.

5.2.3 Colon targeting

With respect to colon delivery, several different formulations have been attempted, which largely rely on selected physiological parameters exhibiting typical variation patterns along the gastrointestinal (GI) tract to enable colon targeting. These physiological parameters include; composition of GI microflora, pH of intestinal fluids and transit/residence times within particular segments of the gut. Although the primary purpose of this phase of the project was to develop a product to target the colon in the selected animal model (i.e., colonic targeting in a mouse), given that ultimate aim is to develop a product for human therapy, a comparison of the physiology of mouse vs human GI physiology is detailed below. A review of the various technologies available

for the colon targeting of multiparticulates and the decision making process regarding the selection of the most suitable technology is also included. Finally the TPP for the product to enable colonic targeting in the mouse model is described in the context of the current stage of this project.

5.2.3.1 Comparison of human and mouse GI physiology

Although intense research efforts have been focused on understanding human GI physiology, there is a lack of agreement with respect to some of the key parameters such as transit times, pH and microflora content as highlighted in a review by Mc Connell and colleagues (McConnell *et al.*, 2008 A). This discord is largely as a result of variations due to food effects, diseased states and inter/intra subject variability (including gender). In the absence of a consensus in relation to these parameters, the information presented here in this review of human GI physiology represents median figures quoted by Mc Connell and others. In contrast to human physiology, based on a review of the literature, there is very limited research and data in relation to murine GI physiology. The information presented here summarises a review of the literature that is available in relation to mouse GI physiology.

A. Transit times in human and murine GI tracts

Gastric emptying in humans (i.e., emptying of stomach contents into the small intestine) of dosage forms is a highly variable process which primarily depends on whether the subject is fed or fasted and on the properties of the dosage unit (e.g., size and density). Whilst there are a large number of studies which reference different times for gastric emptying, a median figure of 2 h (<1 h (fasted) and >3 h (fed)) is most often quoted for

multiparticulate formulations (Patel *et al*., 2011, Garbacz and Klein 2012 and Asghar and Chandran, 2006). In contrast to gastric residence time, transit time in the small intestine has been shown to be more consistent and less dependent on food intake. The transit time of dosage forms is predominantly quoted at 3–4 h regardless of the dosage form (pellets or tablets) (Garbacz and Klein 2012, McConnell *et al.*, 2008 A, Patel *et al*., 2011, Varum *et al*., 2010 and Asghar and Chandran, 2006). Prior to entering the colon, both single-unit and multiple unit dosage forms accumulate and stagnate at the ileocaecal junction for a variable period of time which is affected by food intake. In contrast to small intestine transit times, transit times in the colon do vary with respect to the dosage form with longer transit times being quoted for multiparticulates (Varum *et al.*, 2010). Although there is evidence of colon transit times ranging from 6 h to greater than 70 h (Varum *et al*., 2010), the transit time is often quoted to be in the region 20-30 h (Patel *et al*., 2011, Oh and Lee, 2014). Disease can also impact on GI transit times, for example, patients with UC have been shown to have significantly faster colon transit times compared to controls (McConnell *et al*., 2008 A).

In the case of mice, the reporting of transit times is very limited and in addition published GI transit times vary considerably. In the work presented by both Padmanabhan (Padmanabhan *et al*., 2013) and Ashok (Ashok *et al*., 2012), the total GI transit time is estimated at 6 h, whereas in the case of work presented by Bellier (Bellier *et al*., 2005) the total transit time is estimated at 10–12 h. Padmanabhan and colleagues also reported residence transit times of 1 h for the stomach and 3 h for the SI.

B. pH of human and murine GI tracts

Similar to transit times, the pH in the different regions of the human GI tract is subject to variation and is heavily reliant on inter/intra subject variability but also on food

effects. In addition it has been reported that pH changes in disease (McConnell *et al*., 2008 A). In the case of healthy subjects, the predominant reported pH range for the various regions of the human GI tract (fed and fasted) is as follows; stomach (pH: 1–5) duodenum/jejunum (5.5–7) and the ileum/colon (pH 7–8) (Patel *et al*., 2011 and McConnell *et al*., 2008 A). In the case of mice, pH values across the murine GI was measured as part of a comprehensive study by McConnell and colleagues (McConnell *et al*., 2008 B). The results of study reported pH ranges for the various regions of the murine GI tract (fed and fasted) as follows; stomach (pH: 3.0–4.0) duodenum/jejunum (pH 4.7–5.0) and the ileum/colon (pH 4.4–5.2). A comparison of the pH profiles of humans and mice illustrate that the mouse has a much lower intestinal pH which has implications with respect to *in-vivo* testing (discussed later).

C. Microflora of human and murine GI tracts

Bacteria are ubiquitous along the human GI tract, although some regions (e.g., the colon) are more heavily colonised than others. The high bacterial content of the colon (1 x 10^{12} CFU/g contents) distinguishes it from other regions of the GI tract (e.g., stomach and SI bacterial content ranges from $1x10^{3}$ to 1 $x10^{4}$ CFU/g contents) (McConnell *et al*., 2008 A). The difference between bacterial concentrations in the upper and lower gut can be exploited with respect to colon specific drug delivery (discussed in Section 5.2.3.2 C). As in the case of both pH and transit times, intra and inter individual variability does occur with respect to microflora populations and levels. These fluctuations can also be caused as a result of disease, either by the disease itself (e.g., in UC and CD) or due to drug therapy (e.g., antibiotics in the case of infections) (McConnell *et al*., 2008 A).

Although the GI physiology of mice and humans are quite different, as are their dietary habits, a common feature of both species is that anaerobic bacteria (e.g., bacteroides, and eubacterium) are the predominant populations in the colonic flora. It has also been shown that lactic acid bacteria (e.g., bifidobacteria and lactobacilli), enterobacteria (e.g., *E. coli*) and enterococci are common to the colon of both species (Haeberlin and Friend, 1992). It is notable that the levels of bacteria in mice (including most of the species listed above) are however much higher in the SI and stomach compared to humans. This is due to the fact that mice are coprophagic (i.e., eat their own faeces) and therefore they ingest large numbers of bacteria (Haeberlin and Friend, 1992).

5.2.3.2 Technologies for colonic targeting of multiparticulates

As described earlier, the common approaches available for colon-specific delivery of multiparticulates include; a) design of time dependent delivery systems, b) coating with pH-dependent polymers and c) the use of polymers that are degraded exclusively by colonic bacteria (Krishnaiah *et al.*, 2002). A review of these three approaches is detailed below.

A. Time dependent delivery systems

The time dependent approach to colon delivery relies on the relatively consistent transit time through the SI (3–4 h in humans - Section 5.2.3.1 A). By combining this consistent SI transit time with the use of multiparticulates, which allow for a shorter gastric residence time (<1 h – Section 5.2.3.1 A), it is possible to design a time based colon delivery system that preserves the release of the drug from the formulation through the stomach and SI until reaching the ileum/colon (i.e., a lag time of 4–6 h). There are a number of mechanisms through which time delayed release can be achieved including

erodible systems, rupturable systems and diffusive systems. All of these systems can be used to achieved a delayed release (i.e., lag period), however diffusive systems may be considered advantageous over erodible and rupturable systems in that they also allow for sustained release of the drug over a prolonged period of time following the initial delay which results in a sigmoidal release pattern (Maroni *et al*., 2012). Diffusion controlled systems are based on the application of a diffusive coating to the dosage form. The lag time achieved is reliant on the thickness of the coat as it corresponds to the time required for media (e.g., GI fluid) to penetrate the coat to reach the core via channels created in the polymer coat. The sigmoidal profile is achieved on the basis of the coat becoming more permeable over time as more channels are created. The most common diffusion based polymer coats are Eudragit® RS and RL (pH independent polymethacrylates manufactured by Evonik) and Surelease® (ethylcellulose based coating manufactured by Colorcon). Surelease® is used as the time dependent control mechanism for Pentasa® (marketed Mesalamine product for the treatment of UC and CD) (Klein *et al*., 2002).

B. pH dependent delivery systems

As described in Section 5.2.3.1 B, the pH in the ileum and colon is higher than in any other region of the GI tract. Based on this physiology, dosage forms that disintegrate at high pH levels (e.g., pH 7) have the potential for colon specific delivery. The most commonly used pH dependent coating polymers for oral delivery are methacrylic acid copolymers, Eudragit L100 and Eudragit S100, which dissolve at pH 6 and 7 respectively. It has been reported that the use of Eudragit S alone may not be suitable for colon delivery as studies in healthy subjects have shown a pH drop from 7 at the terminal ileum to pH 6 in the ascending colon and as a result such systems sometimes

fail to release the drug (e.g., if the dosage form is not exposed to pH 7 for a sufficient length of time). In order to overcome this problem, a combination of these two polymers at various ratios can be used to release drug within a pH range of 6–7 (Asghar and Chnadran, 2006). There are a number of products on the market for the treatment of IBD that rely on pH as their mechanism for release. These products include the following; Asacol® and Salofac® (coated Mesalamine tablets) which dissolve at pH 7 (Eudragit-S) and pH 6 (Eudragit-L) respectively (Klein *et al*., 2002).

C. Microflora dependent delivery systems

Microflora dependent delivery systems exploit the ability of colonic bacteria to catalyse enzymatic reactions on substrates that undergo no major dissolution, degradation or absorption phenomena in the GI tract. Included in this approach are the use of prodrugs, such as that described by Ruiz and colleagues (Ruiz *et al*., 2011) in which prednisone (steroid used for the treatment of UC) is released from a prodrug as a result of colon specific azoreductase-activated mechanism. Given that the use of prodrugs was not considered for this project (due to the regulatory implications of developing new chemical entities), the assessment of the use of microflora dependent delivery systems solely focused on coating systems. These coating systems are based on the use of naturally occurring polymer materials (e.g., amylose, pectin, guar gum and chitosan) which are susceptible to selective degradation in the lower GI tract based on microflora catalysed enzymatic reactions (Maroni *et al*., 2012). The use of polysaccharides for coating purposes has been tried with limited success due to the poor film properties of the majority of non-starch polysaccharides but also due to their tendency to swell in the GI tract resulting in porosity and consequent early drug release (Asghar and Chnadran, 2006). Some of these problems can however be overcome by the combination of the

polysaccharides with other polymers, such as a combination of amylose and ethylcellulose (Siew *et al*., 2000 and Freire *et al*., 2010). One of the disadvantages of microflora dependent coating polymers is the lack of commercially approved products which rely on this mechanism of release. Alizyme's COLAL-PRED® technology which involves a prednisone multipartculate coated with Amylose/Surelease® for the treatment of UC represents the only such product that has progressed to Phase III clinical trials, however it did not meet one of its primary clinical endpoints (Alizyme Product Information, 2014).

D. Selection of most suitable technology

The decision making process for selecting the most suitable technology for the colonic delivery of the CLX multiparticulates was based on three key criteria;

1. That the technology selected would be directly applicable for colon delivery in the mouse model but also for colon delivery in humans (on the basis that the ultimate aim of the research was to act as a stepping stone towards the development of a commercial product).

2. That the technology had a proven track record of use in humans (i.e., that there are approved products using the technology on the market).

3. That the technology could circumvent the potential for the diseased state of patients to affect GI physiology to such an extent as to compromise the release of the drug.

Table 5.1 Comparison of coating technologies with respect to technology requirements

Coating Technology	Technology Requirement		
	Directly applicable to both mouse and humans	Track record of use in humans	Drug release not affected by disease state
pH dependent delivery systems	✗[1]	✓	✗[4]
Time dependent delivery systems	✓[2]	✓	✗ [4]
Microflora dependent delivery systems	✗[3]	✗	✗ [4]

[1] The technology was not considered to meet this requirement as the pH dependent polymers required for colonic delivery in humans (i.e., dissolve at pH >7) were unlikely to dissolve across the pH range in the mouse GI tract (i.e., a different pH dependent polymer that dissolved in the pH range of 4.5–5 would be required).

[2] Although the transit times in mice and humans are different, the technology was considered to be directly applicable to both given that the same polymer could be used albeit probably at different weight gains (i.e., higher weight gains likely required to achieve required lag for humans).

[3] Given that differences in stomach and upper SI microflora in mice compared to humans, this technology was not considered to be appropriate given the likelihood of early drug release in mice due to the action of microflora.

[4] Disease in patients has the potential to affect pH, microflora content and transit time in the GI tract therefore none of the technologies were considered to meet this requirement.

Following a review of Table 5.1, it was concluded that a time dependent delivery system was the most appropriate system for investigation. Although there is evidence to suggest that disease (e.g., UC) could result in faster transit times in patients, the impact was considered to be less than with pH or microflora dependent delivery systems due to the use of multiparticulates which by default allow for both longer colon residence times and a greater distribution across the colon, thereby negating some of the impacts of rapid transit times caused by disease.

5.2.3.3 TPP for mouse model

Given that the primary aim for this stage of the project was to develop a product suitable for colon delivery to a mouse, a TPP was designed prior to the initiation of coating studies. The TPP for the product to allow for maximum colonic targeting in the mouse was as follows; <10% release at 2 h and >50% release at 6 h. The TPP was based on published GI transit times for mice described in Section 5.3.2.1 A. On this basis, a two point TPP was stipulated to a) ensure minimal drug release in the stomach and SI prior to reaching the colon (i.e., <10% release at 2 h) and b) to ensure sufficient drug was released prior to expulsion of the multiparticulate (i.e., >50% release at 6 h).

5.2.4 Objectives

As described in Section 5.2.1, the primary goal of this phase of the project was to develop a colon specific targeted CLX microbead formulation and to assess and compare the anti-cancer effect of the targeted microbead to that of the marketed CLX product Celebrex® in an *in-vivo* CRC animal study. The aims/objectives set out to meet this goal were as follows:

A. The first objective was to apply a controlled release polymer to the microbeads with the intention of meeting the TPP described in Section 5.2.3.3 and to select the preferred coated microbead formulation for progression to an animal study using the AOM/DSS CRC mouse model (refer to Section 5.2.2.3.1 for details).

B. The second objective of the study was to assess and compare the effects of the selected CLX microbead formulation to that of Celebrex® on the attenuation of CRC tumours in the AOM/DSS CRC mouse model.

C. Given the association of CRC and in particular CACRC with inflammation, the third objective involved an assessment of the anti-inflammatory effects of both the CLX microbead formulation and Celebrex® in the mouse model as a means of further monitoring their effects with respect to CRC pathology.

5.3 Materials and Methods

5.3.1 Materials

5.3.1.1 Formulation materials

CLX microbeads (formulation CLX 136/B) were made using the following materials; Solutol HS-15® (BASF, Germany), Miglyol® 810N (Sasol, South Africa), porcine gelatin (Nitta Gelatin, Japan), sorbitol (Neosorb® - Roquette, France) and Sodium Dodecyl Sulphate (SDS) (Merck, Germany). Coating trials were performed with the following sustained release coating polymers; Surelease® (Colorcon®, USA), Eudragit® RS30D/RL30D (Evonilk®, Germany). A sample of CLX active pharmaceutical ingredient (API) was kindly provided by Erregierre (Italy). The purity of the API was 99.6% based on the COA (cert of analysis) provided by the supplier. All chemicals used for the release experiments and HPLC (high performance liquid chromatography) testing were of laboratory grade. Celebrex® was manufactured by Pfizer (USA).

5.3.1.2 In-vivo model materials

Azoxymethane (AOM), methylene blue (MB), formalin, ethanol, eosin, Phosphate-buffered saline (PBS) and hematoxylin were obtained from Sigma-Aldrich (USA). Dextran sodium sulphate (DSS) was purchased from MP Biomedicals (UK).

5.3.2 Methods

5.3.2.1 *In-vitro* release testing

Release testing of coated CLX microbeads was performed (*n=3*) at 37°C using a two-step release method (2 h in 0.1 M HCl (750 ml) followed by 10 h in phosphate buffer, pH 6.8 (1000ml)). Release experiments were carried out using either a Varian/Vankel VK7010 dissolution apparatus (VanKel, USA) or a Distek Evolution 6300 (Distek, USA) equipped with standard glass vessels and USP type II paddles. Paddle rotating speed in all experiments was 75 rpm. Microbeads equating to 25 mg of CLX were weighed and added to the media. At specified times 1.8 mL samples were withdrawn, filtered through a 70 µm pore filter (QLA, USA) and analysed using the HPLC method. The % of drug released at particular time points was determined from peak areas which were calculated against a single point external reference standard. The % of drug release was adjusted to take into account the content assay of the formulation. Release analysis of Celebrex® (25 mg doses) was also performed using the method described above.

5.3.2.2 HPLC analysis

The HPLC method for the analysis of the release and assay samples was adapted from Saha and colleagues (Saha *et al*., 2002). The HPLC column used was a reverse phase 4.6 x 250 mm Inertsil® C8 column (Inertsil, The Netherlands) with a particle size of 5

µm.. The mobile phase was acetonitrile:water (65:35). The isocratic method used a flow rate of 1.25 ml/min and ultraviolet (UV) detection at 230 nm. The injection volume was 20 µl and the retention time was 8 min. The HPLC apparatus that was used for the analysis were Thermo Finnigan (Thermo Electron Corporation, USA) and Waters (Waters, USA) HPLC systems (and associated Chromquest and Empower software).

5.3.2.3 Content assay

The content of CLX in coated microbead formulations was determined. A quantity of beads (*n=2*) with a theoretical potency of 5 mg wassonicated for 2 h in a mixture of acetonitrile:water 65:35 to extract the drug from the microbeads. The resultant solution was passed through a 0.45 µm filter prior to absorbance analysis. Where required the samples were diluted prior to analysis. The concentration of CLX was determined by absorbance measurements at 230 nm via the HPLC method described above. CLX content (%) was determined from peak areas which were calculated against a single point external reference standard.

5.3.2.4 Fluid bed coating of microbeads

Coating solutions/suspensions of Surelease® and Eudragit® RS30D/RL30D were prepared as per the manufacturer's instructions. The starting weight of microbeads (CLX 136/B) for coating was approximately 6 g. Given that the substrate microbeads were manually produced, there was limited availability of material for coating trials; therefore approximately 5 g of coloured placebo beads were added to 1 g of active microbeads to enable a batch size that was sufficient for coating. An MFL01 fluid bed system (Vector Corporation, USA) equipped with a Wurster insert was used for coating.

Microbeads were coated at an inlet air temperature setting of 65°C and a product temperature of 40°C. The volume of fluidizing air was maintained at 179 LPM (litres per minute) to ensure optimum fluidizing of microcapsules. A nozzle air pressure of 1.7 bar and a solution flow rate of 3-4 g/min was applied. At the end of the coating process, the coated microbeads were dried for 5 min in the fluid bed system at a product temperature of 40°C. The weight gain of the beads was calculated based on the starting (pre-coating) and end (post- coating) weights of the beads.

5.3.2.5 AOM/DSS-induced CRC model

Six week-old female C57BL/6 mice, purchased from Jackson Laboratories (Bar Harbor, USA), were housed in a specific pathogen-free (SPF) facility in individually ventilated and filtered cages (Tecniplast, Italy) under positive pressure. All animal experiments were performed in compliance with the Irish Department of Health and Children regulations and approved by the Trinity College Dublin's BioResources ethical review board (Ref:B100/325 Feb 2012). CRC was induced by the co-administration of AOM and DSS using a hybrid of the methods described in Section 5.2.2 E. Briefly, colon tumours were induced by i.p. injection of 10 mg/kg AOM at day 0 and day 14. After both injections, mice underwent two cycles of 2 % DSS in drinking water for 5 days (week 1 and week 3). AOM and DSS were administered as per the protocol described by Neufert and colleagues (Neurfert *et al.,* 2007). Mice were treated with vehicle (PBS) or drug three times *per* week for the following 12 weeks, and monitored daily for morbidity/mortality (Figure 5.1). Mice were randomly divided into experimental groups receiving the following treatments: Group 1) PBS (0.1 ml/mouse) (*n=10*), Group 2) Celebrex® (0.25 mg/mouse) (*n=10*), Group 3) Coated CLX microbeads (1 bead/mouse) (*n=10*). Group 4 (n=6), the negative control, did not receive AOM/DSS administrations,

but only PBS (0.1 ml/mouse). Celebrex® powder (0.25 mg/mouse) was suspended in 0.1 ml PBS and administered orally using a stainless steel gavage needle. Coated CLX microbeads were administered orally by means of a modified gavage needle (Figure 5.2). One microbead containing the same dose of CLX present in 0.25 mg of Celebrex® powder (i.e., 0.1 mg) was loaded in the PVC tube fixed on the needle and a 1 ml-syringe pre-loaded with 0.1 ml PBS and 0.9 ml air. PBS was used as a delivery medium for easy passage through the oesophagus.

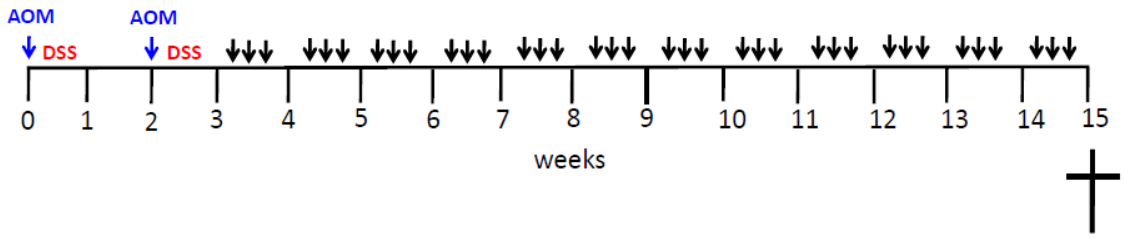

Figure 5.1 Experimental protocol for AOM/DSS CRC model. Colon tumours were induced by i.p. injection of 10 mg/kg AOM at day 0 and day 14. After both injections, mice underwent two cycles of 2 % DSS in drinking water for 5 days (week 1 and week 3). Mice were treated with vehicle (PBS) or drug three times *per* week for the following 12 weeks. At the termination of the study (week 15), mice were euthanized by cervical dislocation.

Figure 5.2 Modified gavage needle for administering coated CLX beads to the mice in AOM/DSS CACRC model

5.3.2.6 Assessment of inflammation (body weight, colon length and histology scoring)

Body weight was recorded weekly as index of pathology by using a digital scale (VWR, UK). Body weight change was expressed in percentage (%) and calculated for each mouse according to the formula: BWn/BWi*100 where BW*i* is the *initial* body weight (week 0) and BW*n* is the *n-th* body weight recorded every week for 15 weeks.

At the termination of the study (week 15), mice were euthanized by cervical dislocation and colon length were measured as a macroscopic index of tissue inflammation (Diaz-Granados *et al.*, 2000). The entire colon (from ileocecal junction to the anus) was excised and the length was measured and expressed in mm.

Histology scoring was also performed as an assessment of inflammation. Approximately 1 cm segments of excised distal colonic tissue were fixed in 10% buffered formalin (pH, 7.4; PBS buffered) and embedded in paraffin. Sections (5 μm) were cut and stained with heamatoxylin and eosin (H&E) and graded blind using a cumulative score ranging from 0 to 3–4 (Aveillo *et al.*, 2014). An arbitrary maximum combined score of 10 was determined on the basis of the following parameters: severity of inflammatory cell infiltration, extent of injury, and crypt damage. Photomicrographs were taken using a Leica® microscope (Leica® DM 3000 LED) equipped with Leica® DFC495 camera (Leica® Microsystem, Germany). Images were visualized by LAS v4.0 and ultra-scanned with ScanScope® (Aperio® ePathology Solutions, Oxford, UK).

5.3.2.7 Tumour assessment (tumour number, tumour size and tumour location)

The formation of tumours in the colon was evaluated as described by Aviello and colleagues (Aviello *et al.*, 2012). Colons were excised, fixed flat in 10% buffered

formalin for 24 h and then stained with 0.2% MB. The total number of tumours *per* mouse was scored stereo-microscopically (5X magnification) using a Leica® microscope equipped with Leica® DFC495 camera. Focal lesions of the colonic mucosa were easily detectable prior to MB staining of fixed whole-mount preparations of colon as they presented enlarged dysmorphic crypts, compression of surrounding epithelium, darkly coloured staining and raised circle-like masses. The size of each tumour mass identified was measured by a micrometer system included in the Leica Application Suite (LAS) version 4.0 (Laboratory Instruments & Supplies Ltd., Ashbourne, Ireland) software used for the visualization of tumours. Tumour size was used as an indicator of disease progression, in that smaller tumours would be indicative of a positive effect of the treatment on tumour progression. In order to study the anatomic tumour distribution, after being fixed flat, colons where cut in three segments (approximately 3 cm long), namely proximal, central and distal (referred to the distance from the ileo-cecal valve junction). The location of each tumour identified was recorded.

5.3.2.8 Statistical analysis

Statistical analysis was performed using GraphPad Prism (La Jolla, USA). Results are presented as mean ± SEM (standard error of the mean) (6–10). Differences, indicated as two-tailed *P* values, were considered significant when $P < 0.05$ as assessed by unpaired Student's t test.

5.4 Results and Discussion

5.4.1 Coated CLX microbead formulations

As described in the introduction, microbead formulation CLX 136/B was chosen for progression for coating trials and ultimately for the AOM/DSS CRC murine study. A fluid bed wurster coating process was used to apply sustained release polymers with the intention of targeting the beads to the colon of the mouse. A number of attempts were made to apply Eudragit RS30D/RL30D sustained release coating polymers to the beads however these attempts proved unsuccessful due to blocking of the spray gun by the coating suspensions. An ethyl cellulose dispersion (Surelease®) was attempted and was successfully applied to the beads without any gun blocking issues. The TPP for the product to allow for maximum colonic targeting in the mouse was as follows; <10% release at 2 h and >50% release at 6 h. Formulation CLX 136/B was initially coated to yield two formulations (Table 5.2); formulation C-01 (CLX 136/B C-01 - 5% w/g of Surelease®) and C-02 (CLX 136/B C-02 - 8% w/g of Surelease®). Despite the use of a pH independent polymer (Surelease®), a two-step pH change release method was employed to mimic the transition from the stomach (acid step) to the SI/colon (phosphate buffer step). It can be seen from Table 5.2 and Figure 5.3 (*in-vitro* release data and profiles for formulation C-01 and formulation C-02) that neither formulation met the desired TPP at the 2 h time point. Given that the availability of sufficient material for coating trials was limited (formulation CLX 136/B was manually produced), formulation C-02 was chosen as a partially colonic targeted CLX microbead formulation for progression to the *in-vivo* AOM/DSS model of murine CRC. The processing conditions for formulations C-01 and C-02 are detailed in Table 5.3 below. The drug release of Celebrex® 25 mg capsules was also performed using the two-step release method for comparison purposes (see Figure 5.3).

Table 5.2 Percentage CLX released (± standard deviation (STEDV) from release testing of formulations C-01 and C-02 (two-step release method: 2 h in 0.1 M HCl (750 ml) followed by 10 h in phosphate buffer, pH 6.8 (1000ml)).

Time point (h)	% CLX Released	
	C-01 (5% weight gain of Surelease®)	**C-02 (8% weight gain of Surelease®)**
0	0	0
1	36.35 (± 0.77)	20.09 (± 0.28)
2	70.63 (± 1.48)	39.78 (± 0.21)
4	86.46 (± 1.13)	62.65 (± 0.56)
6	86.72 (± 1.34)	71.34 (± 1.76)
12	85.08 (± 0.70)	78.61 (± 0.98)

Table 5.3 Fluid bed processing conditions for formulations C-01 and C-02

Parameter	C-01	C-02
Product temperature (°C)	59–63	59–60
Exhaust temperature (°C)	37–41	37–41
Atomisation pressure (Bar)	1.7	1.7
Air flow (LPM)	196–197	196–198
Average spray rate (g/min)	4	4
Coating time (min)	37	52
Oven curing temperature (°C)	40	40
% Weight gain after curing	5.4	8.3

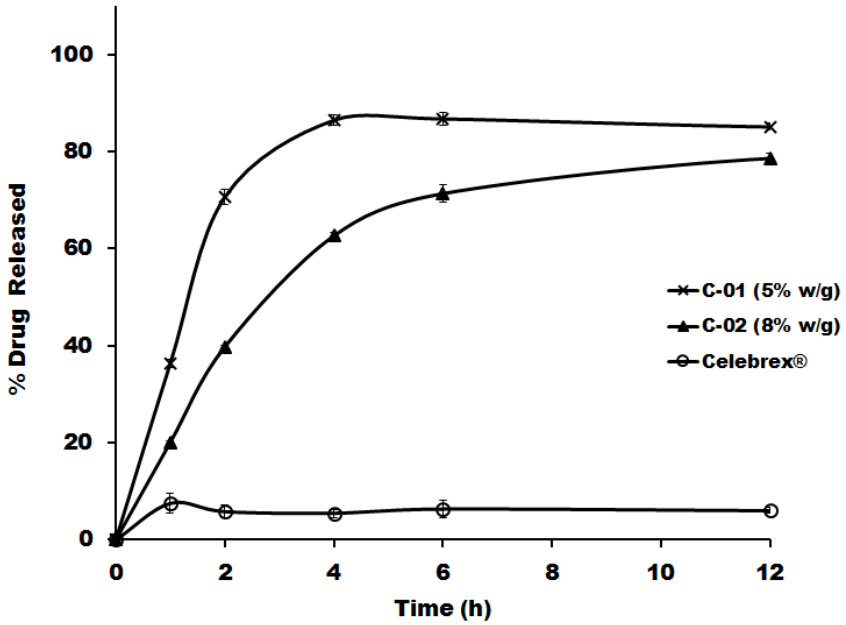

Figure 5.3 Percentage of drug released from release testing of formulations C-01, C-02 and Celebrex® (two-step release method: 2 h in 0.1 M HCl (750 ml) followed by 10 h in phosphate buffer, pH 6.8 (1000ml)). The data presented are mean values ± STDEV (n=3).

5.4.1.1 Effects of CLX formulations on extent of inflammation

Mean body weight (index of pathology) and colon length (index of tissue inflammation) of mice administered Celebrex® (group 2) and CLX microbeads (group 3 - formulation C-02) were compared to that of group 1 (PBS) and/or group 4 (untreated). The body weight change of groups 1, 2 and 3 was not significantly different (Figure 5.4).

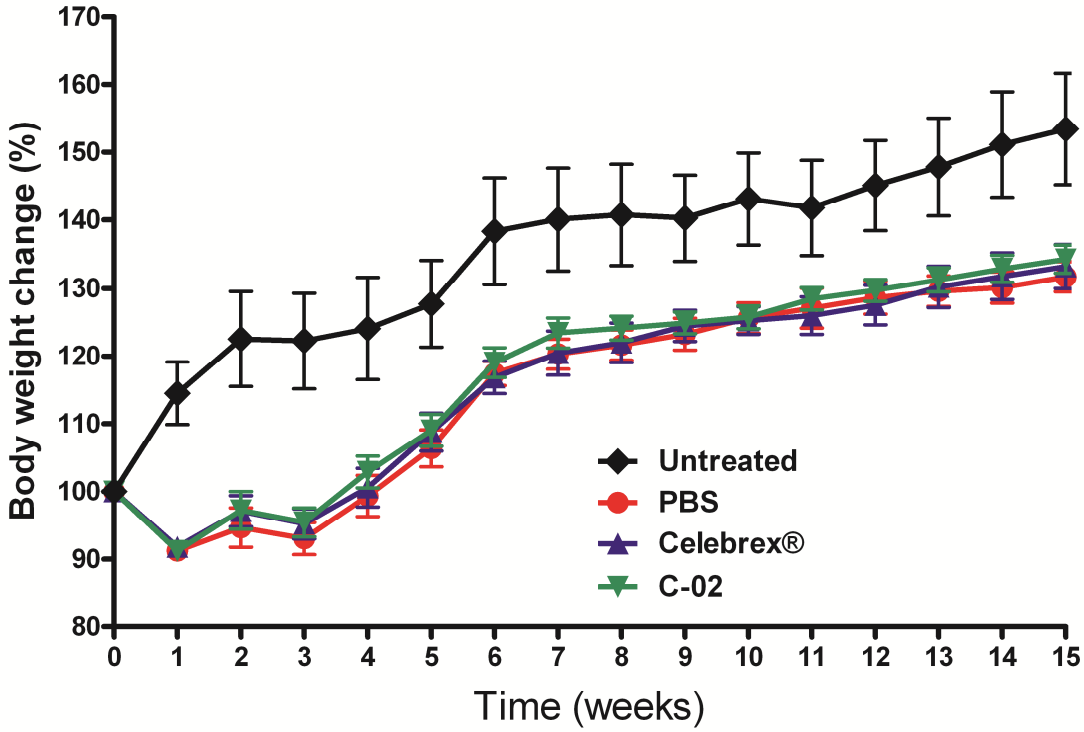

Figure 5.4 Average percentage body weight change of mice (±SEM) from each group (untreated negative control (n=6), PBS (n=10), Celebrex® (n=10) and coated microbead formulation C-02 (n=10)) from weeks 0–15 of the study.

On the contrary, at autopsy mice treated with formulation C-02 for 12 weeks showed a significant (*p=0.0330*) reduction in colon length compared to the PBS treated mice (Figure 5.5). In Celebrex® treated mice, although the colons were shortened, this difference was not statistically significant (*p=0.2470*).

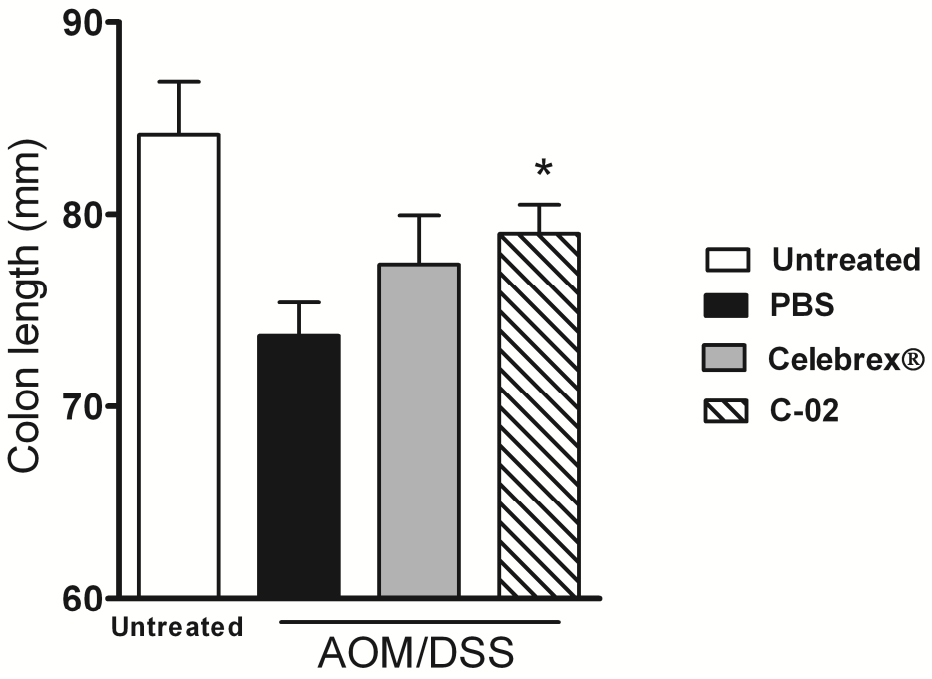

Figure 5.5 The effect of CLX on inflammation in the AOM/DSS CRC murine model included an assessment of colon length. The entire colon was excised and the length was measured and expressed in mm. The average colon length (±SEM) for each group (untreated negative control (n=6), PBS (n=10), Celebrex® (n=10) and CLX microbead formulation C-02 (n=10)) are shown. Statistics were performed relative to PBS for formulations C-02 and Celebrex®.

Histology scoring was also performed as an assessment of inflammation. It can be seen from Figure 5.6 that whilst histology scores for formulation C-02 and Celebrex® were comparable, the histology score for formulation C-02 was statistically significant ($p = 0.0446$), compared to the PBS control, in contrast Celebrex® was not significant ($p = 0.0628$).

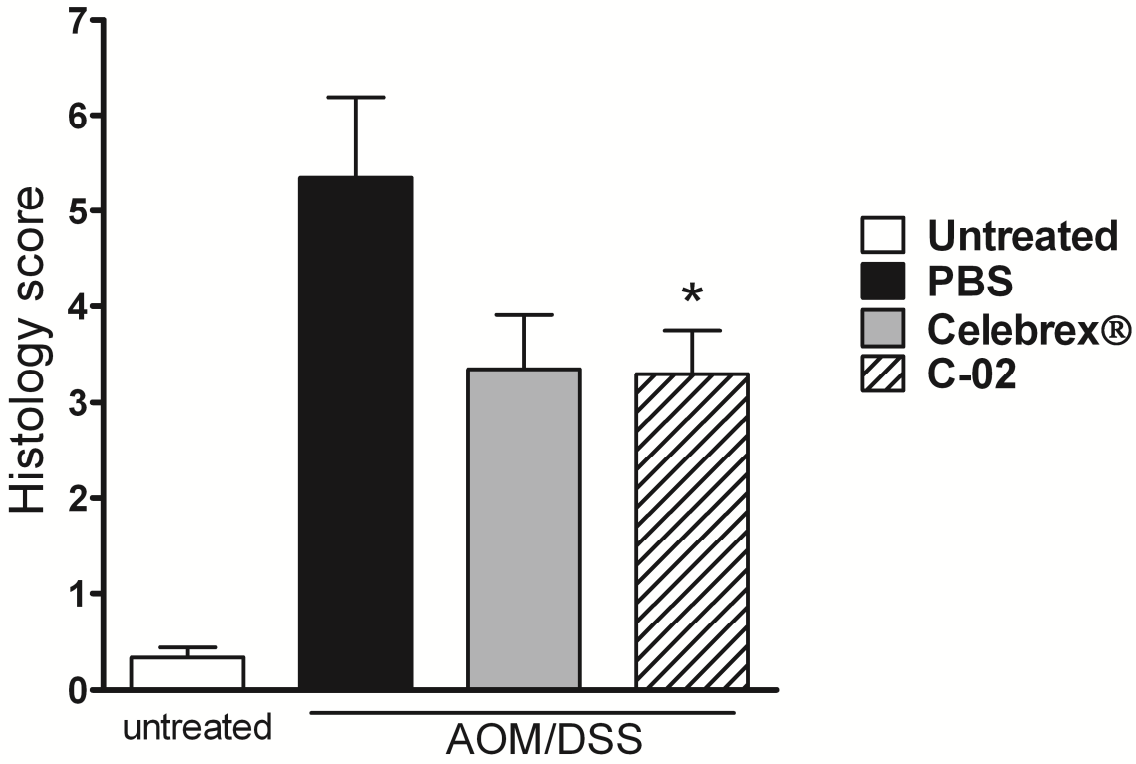

Figure 5.6 The effect of CLX on inflammation in the AOM/DSS CRC murine model included an assessment of colon histology. Excised distal colonic tissue (1 cm-segments) of were fixed in 10% buffered formalin (pH, 7.4; PBS buffered) and embedded in paraffin. Sections (5 μm) were cut and stained with H&E and graded blind using a cumulative score ranging from 0 to 3–4 (Aveillo *et al*., 2014). The average histology score (±SEM) for each group (untreated negative control (n=6), PBS (n=10), Celebrex® (n=10) and CLX microbead formulation C-02(n=10)) are shown. Statistics were performed relative to PBS for formulations C-02 and Celebrex®.

5.4.1.2 Effects of CLX formulations on CRC tumour attenuation

The effect of CLX microbeads (group 3 – formulation C-02) and Celebrex® (group 2) on tumour size, tumour location and number were compared to that of group 1 (PBS) and/or group 4 (untreated). Figure 5.7 illustrates a representative pictomicrograph of normal cells (A) and a tumour mass (B). There was no difference in tumour size among groups 1–3 (Figure 5.8) with tumour masses observed to have been homogenously distributed along the colon tissues.

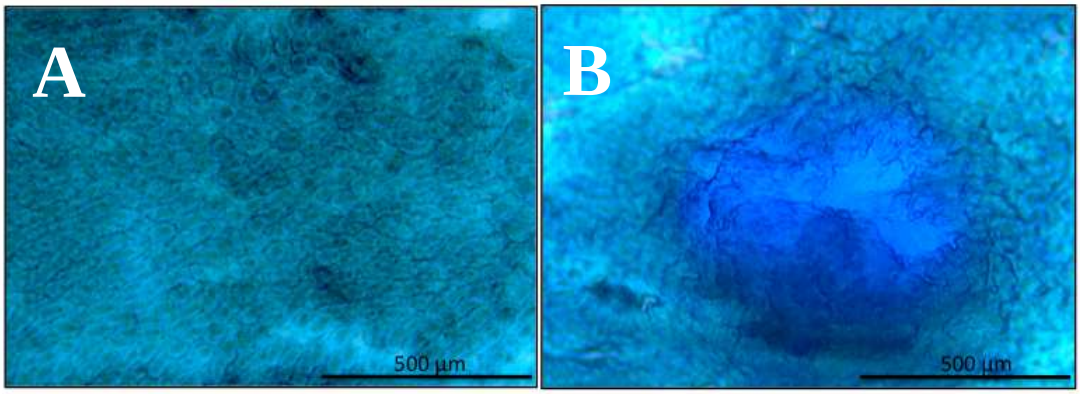

Figure 5.7 Photomicrographs of (A) normal cells and (B) tissue mass, taken using a Leica® microscope equipped with Leica® DFC495 camera (Leica® Microsystem, Germany). Images were visualized by LAS v4.0 software.

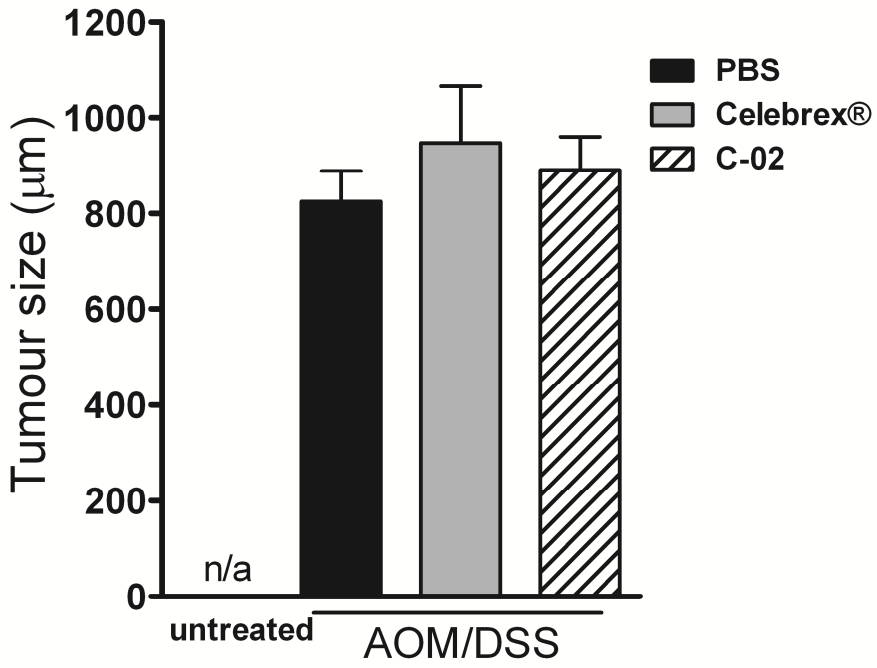

Figure 5.8 The effect of CLX on tumour size in the AOM/DSS CRC murine model was assessed. Following identification of tumours (see Figure 5.9 for details), the size of each tumour mass identified was measured by a micrometer system included in the LAS version 4.0 software used for the visualization of tumours. The average size (±SEM) of tumours for each group (untreated negative control (n=6), PBS (n=10), Celebrex® (n=10) and CLX microbead formulation C-02(n=10)) are shown.

Mice treated with formulation C-02 did however show a reduction in the number of AOM/DSS-induced tumours compared to the PBS control group approaching a statistical significance (*p=0.0558*) (Figure 5.9). In contrast, the *p* value for Celebrex® compared to the control was 0.1500 (i.e., it did not approach statistical significance).

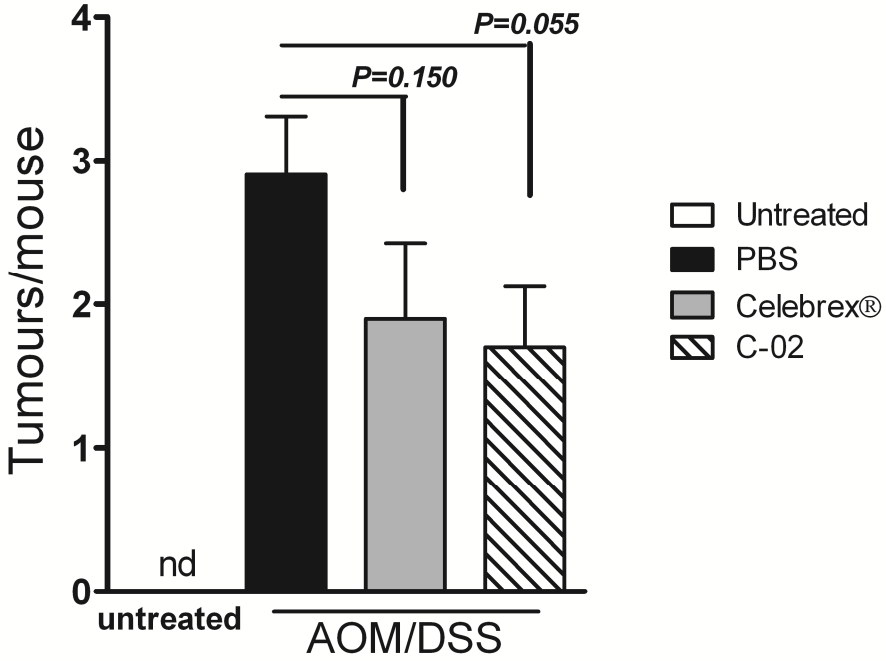

Figure 5.9 The effect of CLX on tumour number in the AOM/DSS CRC murine model was assessed. Colons were excised, fixed flat in 10% buffered formalin for 24 h and then stained with 0.2% MB. The total number of tumours *per* mouse was scored stereo-microscopically (5X magnification) using a Leica® microscope equipped with Leica® DFC495 camera. The average number of tumours for each group (untreated negative control (n=6), PBS (n=10), Celebrex® (n=10) and CLX microbead formulation C-02 (n=10)) are shown. Statistics were performed relative to PBS for formulations C-02 and Celebrex®.

5.4.1.3 Formulation analysis of *in-vivo* animal results

As described in Section 5.2.3.3, the TPP for the coated formulation included a requirement for <10% release at 2 h in order to ensure minimal drug release in the

stomach and SI prior to reaching the colon. Due to the limited quantity of manually produced microbeads available for coating trials, a partially colonic targeted formulation comprising a sustained release polymer coat, which allowed in the region of 50% of the drug to be available in the colon, was chosen for the subsequent *in-vivo* animal study (Figure 5.3 – formulation C-02).

In this phase of the project the therapeutic efficacy of the partially colonic targeted microbead formulation (formulation C-02) was evaluated compared to Celebrex® with respect to the attenuation of colonic inflammation and the development of colorectal tumours in the AOM/DSS murine model of CRC. Relative to the control, the CLX microbead formulation was found to score significantly better compared to Celebrex® with respect to the level of indicators for inflammation (Figures 5.5 and 5.6) and whilst the level of tumour attenuation was comparable between the CLX microbead formulation and Celebrex®, the CLX microbead formulation approached significance (*p=0.0558)* whereas Celebrex® did not (*p=0.150)* (Figure 5.9).

As described in Chapter 1, whilst it is generally accepted that the GI side effects of NSAIDs (Non-steroidal anti-inflammatory drugs) are mainly caused by cyclooxygenase-1 (COX-1) inhibition, which blocks the production of the protective mucus at the epithelial layer in the GI tract, COX-2 inhibitors such as CLX are still associated with GI toxicity (Silverstein *et al*., 2000). This GI toxicity is partly attributable to local effects on the GI tract, including local irritation (Halen *et al.,* 2009). Multiparticulate drug delivery systems have been shown to be less likely than single unit dosage forms to cause local irritation (Tang *et al.*, 2005) as they allow for a greater distribution of the drug. Similarly, micelle formulations allow for an even distribution of drug in the GI tract and can reduce the toxicity caused by the administration of a neat drug (Lui *et al*. 2008). The results obtained in this phase of the project represented a

significant step forward in that it allowed the possibility of presenting CLX in a micellar format within a multiparticulate, a combination that has the potential to minimize GI side effects whilst maintaining the effectiveness of the treatment.

A further analysis of the data obtained from the *in-vivo* study highlighted the potential for the microbead formulation to have an even more enhanced effect than Celebrex® over that presented in Figures 5.5, 5.6 and 5.9. The release profile for formulation C-02 (Figure 5.3) shows that in the region of 50% of CLX is likely to have been released prior to the microbead formulation reaching the colon based on a 3 h SI transit time (Padmanabhan *et al.,* 2013). A number of *in-vivo* studies conducted in both animals and humans have shown that when CLX is delivered in a soluble form that the extent of drug absorption is significantly greater when compared to Celebrex® (Paulson *et al*., 2001 and Subramanian *et al*., 2004). The study by Paulson and colleagues also demonstrated that when a CLX solution was delivered directly to the stomach and the duodenum, that the T_{max} (time at which serum levels of drug are at their highest) for drug absorption was approximately 0.69 and 1.13 h respectively. Based on these studies, it could be hypothesied in this study that in the region of 50% of the CLX from formulation C-02 could have been rapidly absorbed within 3 h of administration and therefore may not have been available in luminal side of the colon. In contrast, the low solubility of CLX in Celebrex® has been shown to prolong the absorption process, with a terminal half life of 11 h being reported (FDA Clinical Pharmacology and Biopharmaceutics Review for Celebrex®, 1998). Based on this information and the release profile for Celebrex® shown in Figure 5.3, it is also reasonable to assume that the majority of the Celebrex® dose administered in this study would have been available to the colon 3 h after administration. A hypothesis is therefore presented that the chemopreventative effects of the CLX microbead formulation achieved in this study may have been achieved with a lower concentration of drug locally available in the

lumen of the colon than that which may have been locally available (in the colon lumen) in the case of Celebrex®. Should this hypothesis be proven in future studies (i.e., by repeating the study using a CLX microbead formulation that meets the desired TPP), it would represent a significant breakthrough for the use of CLX in the treatment and prevention of CRC as it would afford the opportunity to administer CLX at lower doses and thereby reduce the incidence of cardiovascular (CV) and GI side effects associated with high doses of Celebrex® (Soloman *et al*., 2005 and FDA labelling Revision for Celebrex Capsules, 2008).

5.5 Conclusions

Chapter 4 described the development of an optimised CLX microbead which culminated in formulation CLX 136/B, a formulation which met all the CQAs required for the intermediate product (i.e., a robust microbead which contained a sufficient loading of dissolved CLX). In this phase of the project (Chapter 5), a sustained release polymer (Surelease®) was applied to CLX 136/B microbeads with the intention of meeting a defined TPP to allow for colon specific delivery in a mouse model. Despite the successful application of Surelease® to the microbeads at 5% (C-01) and 8% (C-02) w/g, the TPP at 2 h time point was not achieved for either formulation. Due to the limited quantity of product available for coating optimisation studies, coated microbead formulation C-02, which represented a partially colonic targeted formulation, was selected for progression to an AOM/DSS CRC murine model. In the murine model, the effect of formulation C-02 on the attenuation of CRC tumours was compared to that of Celebrex® and was found to have a marginally greater effect. The anti-inflammatory effects of both formulations were also assessed with formulation C-02 exhibiting a significant effect compared to the control in the case of colon length and histology

scoring whereas Celebrex® did not achieve significance. A hypothesis was also presented to suggest that the anti-cancer and anti-inflammation effects of formulation C-02 may have been achieved with a lower concentration of drug locally available in the lumen of the colon than that which may have been available in the case of Celebrex®.. The results for the partially colonic formulation presented here (C-02) enables the possibility of presenting CLX in a micellar format within a multiparticulate, a combination that has the potential to minimize GI side effects whilst maintaining the effectiveness of the treatment. In order to build upon these results, a next generation fully colonic targeted formulation was required in order to allow for hypothesis described to be tested in a future animal study. Chapter 6 describes the development of this next generation product.

5.6 Nomenclature

Table 5.4 List of abbreviations which are listed according to their appearance in the text.

Abbreviation	Definition
AOM	Azoxymethane
DSS	Dextran sodium sulphate
CLX	Celecoxib
CACRC	Colitis associated colorectal cancer
TPP	Target product profile
w/g	Weight gain
CRC	Colorectal cancer
CQA	Critical quality attribute
IBD	Irritable bowel disease
CD	Crohn´s disease
UC	Ulcerative colitis
5-ASA	5-aminosalicylic acid
SI	Small intestine
HCAs	Heterocyclic amines
AAs	Aromatic amines
DMH	Dimethylhydrazine
PhIP	2-amino-1-methyl-6-phenylimidazol (4,5-b) pyridine
IQ	2-amino-33-methylimidazo[4,5-f] quinolone
DMBA	3,2'-dimethyl-4-aminobiphenyl
MNU	Methylnitrosourea
MNNG	N-methyl-N-nitro-N- nitrosoguanidine
DMH	1,2-dimethylhydrazine
MAM	methylazoxymethanol
i.p.	Intraperitoneal
APC	Adenomatous polyposis coli
FAP	Familial adenomatous polyposis
HNPCC	Hereditary non-polyposis colorectal cancer
MMR	mismatch repair
COX-2	cyclooxygenase-2
GI	Gastrointestinal
SDS	Sodium dodecyl sulphate
API	Active pharmaceutical ingredient
COA	Certificate of analysis
HPLC	High performance liquid chromatography
MB	methylene blue
PBS	Phosphate-Buffered Saline
UV	ultraviolet

Abbreviation	Definition
LPM	Litre per minute
H&E	Heamatoxylin and eosin
SEM	Standard error of the mean
STDEV	Standard deviation
NSAIDs	Non-steroidal anti-inflammatory drugs
COX-1	cyclooxygenase-1
T_{max}	Time at which serum levels of drug are at their highest
CV	cardiovascular
CLX xxx/B	Celecoxib bead formulation numbering system where xxx is a sequential number and B is bead

5.7 Acknowledgements

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5.8 References

Asghar, L.F. & Chandran, S. 2006, "Multiparticulate formulation approach to colon specific drug delivery: current perspectives", *Journal of Pharmacy & Pharmaceutical sciences*, vol. 9, no. 3, pp. 327-338.

Ashok, B.K., Bhat, S.D. & Ravishankar, B. 2012, "Screening of intestinal transit time of *Euphorbia fusiformis* Buch.- Ham. ex D. Don in Swiss albino mice", *Indian Journal of Natural Products and Resources*, vol. 3, no. 4, pp. 547-550.

Aviello, G., Corr, S.C., Johnston, D.G., O'Neill, L.A. & Fallon, P.G. 2014, "MyD88 adaptor-like (Mal) regulates intestinal homeostasis and colitis-associated colorectal cancer in mice", *American Journal of physiology.Gastrointestinal and Liver Physiology*, vol. 306, no. 9, pp. G769-78.

Aviello, G., Romano, B., Borrelli, F., Capasso, R., Gallo, L., Piscitelli, F., Di Marzo, V. & Izzo, A.A. 2012, "Chemopreventive effect of the non-psychotropic phytocannabinoid cannabidiol on experimental colon cancer", *Journal of Molecular Medicine (Berlin, Germany)*, vol. 90, no. 8, pp. 925-934.

Becker, C., Fantini, M.C., Wirtz, S., Nikolaev, A., Kiesslich, R., Lehr, H.A., Galle, P.R. & Neurath, M.F. 2005, "In vivo imaging of colitis and colon cancer development in mice using high resolution chromoendoscopy", *Gut*, vol. 54, no. 7, pp. 950-954.

Bellier, S., Da Silva, N.R., Aubin-Houzelstein, G., Elbaz, C., Vanderwinden, J.M. & Panthier, J.J. 2005, "Accelerated intestinal transit in inbred mice with an increased number of interstitial cells of Cajal", *American Journal of Physiology.Gastrointestinal and Liver Physiology*, vol. 288, no. 1, pp. G151-8.

Coudry, R.A., Cooper, H.S., Monique, G., Lubet, R.A., Chang, W., L. & Clapper, M.L. 2004, *Correlation of inhibition of colitis-associated dysplasia by celecoxib with degree of inflammation in the mouse model of DSS-induced colitis*, Abstract 548, AACR Meeting Abstracts.

De Robertis, M., Massi, E., Poeta, M.L., Carotti, S., Morini, S., Cecchetelli, L., Signori, E. & Fazio, V.M. 2011, "The AOM/DSS murine model for the study of colon carcinogenesis: From pathways to diagnosis and therapy studies", *Journal of Carcinogenesis*, vol. 10, pp. 9-3163.78279.

Diaz-Granados, N., Howe, K., Lu, J. & McKay, D.M. 2000, "Dextran sulfate sodium-induced colonic histopathology, but not altered epithelial ion transport, is reduced by inhibition of phosphodiesterase activity", *The American Journal of Pathology*, vol. 156, no. 6, pp. 2169-2177.

Freire, C., Podczeck, F., Ferreira, D., Veiga, F., Sousa, J. & Pena, A. 2010, "Assessment of the in-vivo drug release from pellets film-coated with a dispersion of high amylose starch and ethylcellulose for potential colon delivery", *The Journal of Pharmacy and Pharmacology*, vol. 62, no. 1, pp. 55-61.

Garbacz, G. & Klein, S. 2012, "Dissolution testing of oral modified-release dosage forms", *The Journal of Pharmacy and Pharmacology*, vol. 64, no. 7, pp. 944-968.

Haeberlin, H. & Friend, D.R. 1992, "Anatomy and Physiology of the Gastroinestinal Tract: Implications for Colonic Drug Delivery " in *Oral Colon-Specific Drug Delivery*, ed. D.R. Friend, : , 1992: 1-44. edn, CRC Press, New York, pp. 1-44.

Halen, P.K., Murumkar, P.R., Giridhar, R. & Yadav, M.R. 2009, "Prodrug designing of NSAIDs", *Mini Reviews in Medicinal Chemistry*, vol. 9, no. 1, pp. 124-139.

Ishikawa, T.O. & Herschman, H.R. 2010, "Tumor formation in a mouse model of colitis-associated colon cancer does not require COX-1 or COX-2 expression", *Carcinogenesis*, vol. 31, no. 4, pp. 729-736.

Johnson, R.L. & Fleet, J.C. 2013, "Animal models of colorectal cancer", *Cancer Metastasis Reviews*, vol. 32, no. 1-2, pp. 39-61.

Karim, B.O. & Huso, D.L. 2013, " Mouse models for colorectal cancer ", *Am J Cancer Res.*, vol. 3, no. 3, pp. 240-250.

Klein, S., Rudolph, M.W. & Dressman, J.B. 2002, "Drug release characteristics of different mesalazine products using USP apparatus 3 to simulate passage through the GI tract", *Dissolution Technologies*, vol. 01, pp. 6-12.

Krishnaiah, Y.S., Satyanarayana, V., Kumar, B.D. & Karthikeyan, R.S. 2002, "Studies on the development of colon-targeted delivery systems for celecoxib in the prevention of colorectal cancer", *Journal of Drug Targeting*, vol. 10, no. 3, pp. 247-254.

Lee, J., Kim, H.S., Oh, T., Yeo, M., Hahm, K., Bang, Y. & Surh, Y. 2005, *Chemopreventive effects of a specific COX-2 inhibitor (Celecoxib) and a novel HDAC inhibitor (SK-7041) on azoxymethane-initiated and dextran sulfate sodium-promoted mouse colon carcinogenesis*, Abstract 580a, ACCR Meeting Abstracts.

Li, Y., Niu, Y., Wu, H., Zhang, B., Sun, Y., Huang, H., Li, Q., Fan, L., Liu, L. & Mei, Q. 2009, "PC-407, a celecoxib derivative, inhibited the growth of colorectal tumor in vitro and in vivo", *Cancer Science*, vol. 100, no. 12, pp. 2451-2458.

Lichtenberger, L.M., Zhou, Y., Dial, E.J. & Raphael, R.M. 2006, "NSAID injury to the gastrointestinal tract: evidence that NSAIDs interact with phospholipids to weaken the hydrophobic surface barrier and induce the formation of unstable pores in membranes", *The Journal of Pharmacy and Pharmacology*, vol. 58, no. 11, pp. 1421-1428.

Liu, R., Dannenfelser, R. & Li, S. 2008, "Micellization and Drug Solubility Enhancement" in *Water-Insoluble Drug Formulation*, ed. R. Lui, 2nd edn, CRC Press, Florida, pp. 255-306.

Maroni, A., Zema, L., Del Curto, M.D., Foppoli, A. & Gazzaniga, A. 2012, "Oral colon delivery of insulin with the aid of functional adjuvants", *Advanced Drug Delivery Reviews*, vol. 64, no. 6, pp. 540-556.

McConnell, E.L., Basit, A.W. & Murdan, S. 2008 A, "Measurements of rat and mouse gastrointestinal pH, fluid and lymphoid tissue, and implications for in-vivo experiments", *The Journal of Pharmacy and Pharmacology*, vol. 60, no. 1, pp. 63-70.

McConnell, E.L., Fadda, H.M. & Basit, A.W. 2008 B, "Gut instincts: explorations in intestinal physiology and drug delivery", *International Journal of Pharmaceutics*, vol. 364, no. 2, pp. 213-226.

Neufert, C., Becker, C. & Neurath, M.F. 2007, "An inducible mouse model of colon carcinogenesis for the analysis of sporadic and inflammation-driven tumor progression", *Nature Protocols*, vol. 2, no. 8, pp. 1998-2004.

Oh, D.A. & Lee, C.H. 2014, "Physiological Barriers in Advanced Drug Delivery: Gastroinsteinal Barrie" in *Advanced Drug Delivery*, eds. A.K. Mitra, C.H. Lee & K. Cheng, Wiley, New Jersey, pp. 3-20.

Oshima, H. & Oshima, M. 2012, "The inflammatory network in the gastrointestinal tumor microenvironment: lessons from mouse models", *Journal of Gastroenterology*, vol. 47, no. 2, pp. 97-106.

Padmanabhan, P., Grosse, J., Asad, A.B., Radda, G.K. & Golay, X. 2013, "Gastrointestinal transit measurements in mice with 99mTc-DTPA-labeled activated charcoal using NanoSPECT-CT", *EJNMMI research*, vol. 3, no. 1, pp. 60-219X-3-60.

Patel, A., Bhatt, N., Patel, K.R., Patel, N.M. & Patel, M.R. 2011, "Colon Targeted Drug Delivery System: A Review System", *JPSBR*, vol. 1, no. 1, pp. 37-49.

Paulson, S.K., Vaughn, M.B., Jessen, S.M., Lawal, Y., Gresk, C.J., Yan, B., Maziasz, T.J., Cook, C.S. & Karim, A. 2001, " Pharmacokinetics of Celecoxib after Oral Administration in Dogs and Humans: Effects of Food and Site of Absorption", *Journal of Pharmacology and Experimental Therapeutics*, vol. 297, no. 2, pp. 638-645.

Rosenberg, D.W., Giardina, C. & Tanaka, T. 2009, "Mouse models for the study of colon carcinogenesis", *Carcinogenesis*, vol. 30, no. 2, pp. 183-196.

Ruiz, J.F., Kedziora, K., Windle, H., Kelleher, D.P. & Gilmer, J.F. 2011, "Investigation into drug release from colon-specific azoreductase-activated steroid prodrugs using in-vitro models", *The Journal of Pharmacy and Pharmacology*, vol. 63, no. 6, pp. 806-816.

Saha, R.N., Sajeev, C., Jadhav, P.R., Patil, S.P. & Srinivasan, N. 2002, "Determination of celecoxib in pharmaceutical formulations using UV spectrophotometry and liquid chromatography", *Journal of Pharmaceutical and Biomedical Analysis*, vol. 28, no. 3-4, pp. 741-751.

Siew, L.F., Basit, A.W. & Newton, J.M. 2000, "The potential of organic-based amylose-ethylcellulose film coatings as oral colon-specific drug delivery systems", *AAPS PharmSciTech*, vol. 1, no. 3, pp. E22.

Silverstein, F.E., Faich, G., Goldstein, J.L., Simon, L.S., Pincus, T., Whelton, A., Makuch, R., Eisen, G., Agrawal, N.M., Stenson, W.F., Burr, A.M., Zhao, W.W., Kent, J.D., Lefkowith, J.B., Verburg, K.M. & Geis, G.S. 2000, "Gastrointestinal toxicity with celecoxib vs nonsteroidal anti-inflammatory drugs for osteoarthritis and rheumatoid arthritis: the CLASS study: A randomized controlled trial. Celecoxib Long-term Arthritis Safety Study", *JAMA*, vol. 284, no. 10, pp. 1247-1255.

Solomon, S.D., McMurray, J.J., Pfeffer, M.A., Wittes, J., Fowler, R., Finn, P., Anderson, W.F., Zauber, A., Hawk, E., Bertagnolli, M. & Adenoma Prevention with Celecoxib (APC) Study Investigators 2005, "Cardiovascular risk associated with celecoxib in a clinical trial for colorectal adenoma prevention", *The New England Journal of Medicine*, vol. 352, no. 11, pp. 1071-1080.

Subramanian, N., Ray, S., Ghosal, S.K., Bhadra, R. & Moulik, S.P. 2004, "Formulation design of self-microemulsifying drug delivery systems for improved oral bioavailability of celecoxib", *Biological & Pharmaceutical Bulletin*, vol. 27, no. 12, pp. 1993-1999.

Tang, E.S.K., Chan, L.W. & Heng, P.W.S. 2005, "Coating of multiparticulates for sustained release", *Am J Drug Deliv*, vol. 3, no. 1, pp. 17-28.

Tomisato, W., Tsutsumi, S., Hoshino, T., Hwang, H.J., Mio, M., Tsuchiya, T. & Mizushima, T. 2004, "Role of direct cytotoxic effects of NSAIDs in the induction of gastric lesions", *Biochemical Pharmacology*, vol. 67, no. 3, pp. 575-585.

Varum, F.J., Merchant, H.A. & Basit, A.W. 2010, "Oral modified-release formulations in motion: the relationship between gastrointestinal transit and drug absorption", *International Journal of Pharmaceutics*, vol. 395, no. 1-2, pp. 26-36.

Alizyme Product Information for COLAL-PRED®2014. Available: http://ww7.investorrelations.co.uk/alizyme/products/colalpred/ [2014, Sept/02].

FDA Labelling Revision for Celebrex® *Capsules* 2008. Available:http://www.fda.gov/ohrms/dockets/ac/08/briefing/2008-4344b1_07_06_Celebrex%20Label.pdf [2011, Mar/10].

FDA Clinical Pharmacology and Biopharmaceutics Review for Celebrex® 1998. Available: http://www.accessdata.fda.gov/drugsatfda_docs/nda/99/21156-S007_Celebrex_biopharmr.pdf [2013, Dec/12].

CHAPTER 6

Encapsulation scale-up, coating optimisation and application of formulation platform to other actives

Publication Status: Elements of the work presented in this chapter in addition to data from chapters 3 and 5 has been accepted for publication (02-11-14) in the Journal of Pharmacy and Pharmacology under the following title '*In-vitro* characterization of a novel celecoxib microbead formulation for the treatment and prevention of colorectal cancer'.

6.1 Abstract

The primary purpose of this phase of the project (Chapter 6) was to overcome the limitations of the partially colonic targeted microbead celecoxib (CLX) formulation described in Chapter 5 by scaling-up microbead production and subsequently optimising the coating applied to the microbeads. Microbead production scaled-up was achieved via automated prilling (using an Inotech IE-50 R encapsulator) which resulted in robust microbeads with a very narrow size distribution, a high % entrapment efficiency and, critically, a high and consistent level of drug release in release experiments using purified water (PW) as the dissolution media. Subsequent coating optimisation studies resulted in a microbead formulation coated with 20% weight gain (w/g) of Surelease® (SR) that was deemed suitable for use in a future colorectal cancer (CRC) mouse study. Further coating optimisation was performed which included the deployment of a HPMC (hydroxypropyl methylcellulose) based sub-coat (Opadry® White) resulting in a product suitable for human colon delivery. Finally a screening study was performed which illustrated that the optimised CLX microbead formulation (CLX 136/B) was suitable as a platform formulation for a range of alternative actives with similar physicochemical characteristics.

6.2 Introduction

6.2.1 Background

Chapter 5 described the application of a sustained release polymer (SR) to CLX 136/B (the optimised CLX microbead formulation developed in Chapter 4) and an assessment of the anti-cancer and anti-inflammatory effects of the coated formulation versus that of Celebrex® in an AOM (azoxymethane)/DSS (dextran sodium sulphate) CRC murine model. It was intended that the coating applied to the microbead would result in a

product capable of specifically targeting the colon of a mouse, however the target product profile (TPP) for the product was not met, with the consequence that the coated microbead formulation (C-02) used for the animal study was partially rather than fully colonic targeted due to an early release of the drug, with approximately 50% of the drug being released after 3 h (i.e., before reaching the colon). Whilst formulation C-02 was observed to have out-performed Celebrex® with respect to both its anti-tumorigenic and anti-inflammatory effects, it was postulated in the conclusion to Chapter 5 that the use of the partially colonic targeted formulation (C-02) may have resulted in a sub-optimal result for formulation CLX 136/B. The focus of this phase of the project (Chapter 6) was therefore to optimise the SR coating applied to formulation CLX 136/B with the intention of developing a next generation coated CLX microbead formulation to meet the TPP for colon specific delivery to a mouse for use in a repeat of the AOM/DSS CRC mouse model in the future (Note: the repeat of this animal study is outside the scope of the current project).

It was necessary to use formulation C-02 for the animal study described in Chapter 5 due to the limited availability of substrate beads for coating optimisation as the beads were manually produced via a manual gravity dripping method (see Chapter 4 for details). In order to produce sufficient quantities of substrate microbeads for the required coating optimisation studies, the first phase of this stage of the project involved the automated scale-up of microbead production using a suitable mechanically-aided dripping technology. A review of available mechanically aided dripping technologies are included in Section 6.2.2 below.

As previously described, the primary aim of this phase of the project was to develop an optimised coating formulation in order to meet the TPP for colon targeting in a mouse (Section 6.2.3.1). Given that the ultimate objective for this formulation is the development of a commercial product for use in human therapy, the TPP for a human

product is also described (Section 6.2.3.2). In order to meet the TPP for human administration and in particular to ensure sufficient release at the latter end of the release profile, a number of possible formulation approaches to help achieve this target are described (Section 6.2.3.3).

Finally, although the primary focus of the project was on the development of a CLX formulation suitable for the treatment and prevention of CRC, it is desirable for any formulation/technology to be broadly applicable to a range of actives, therefore as a final step in the project, the suitability of the core formulation for the incorporation of a range of APIs (active pharmaceutical ingredients), in a solubilised form, across a broad physicochemical spectrum was assessed (See section 6.4.4 for details).

A summary of the objectives of the final phase of the project (Chapter 6) are detailed in section 6.2.4.

6.2.2 Mechanical-aided dripping techniques

As described in Chapter 4, dripping involves a technique where a single liquid (mixture of core and shell/matrix materials) is extruded through a nozzle resulting in a matrix droplet in which the core material is homogenously dispersed within the matrix. The principle of this process is based on gravity whereby when the liquid passes through the tip of the nozzle, a droplet grows and separates from the stream before falling into the hardening solution. Gravity dripping represents the simplest method of making microbeads, however it has a number of limitations including very slow flow rates (refer to Chapter 4 for further details). The disadvantages associated with gravity dripping can be overcome by increasing the flow rate to the nozzle and via the application of a mechanical force to break-up the stream of liquid as it flows from the

nozzle (i.e., droplet formation is no longer reliant on the force of gravity). These mechanical forces give rise to the following techniques based on the principle of dripping; jet cutting technology, rotating (spinning) disc atomisation, electrostatic extrusion, coaxial air flow and prilling. A review of these mechanically aided dripping techniques detailed below and a diagram depicting the techniques is shown in Figure 6.1.

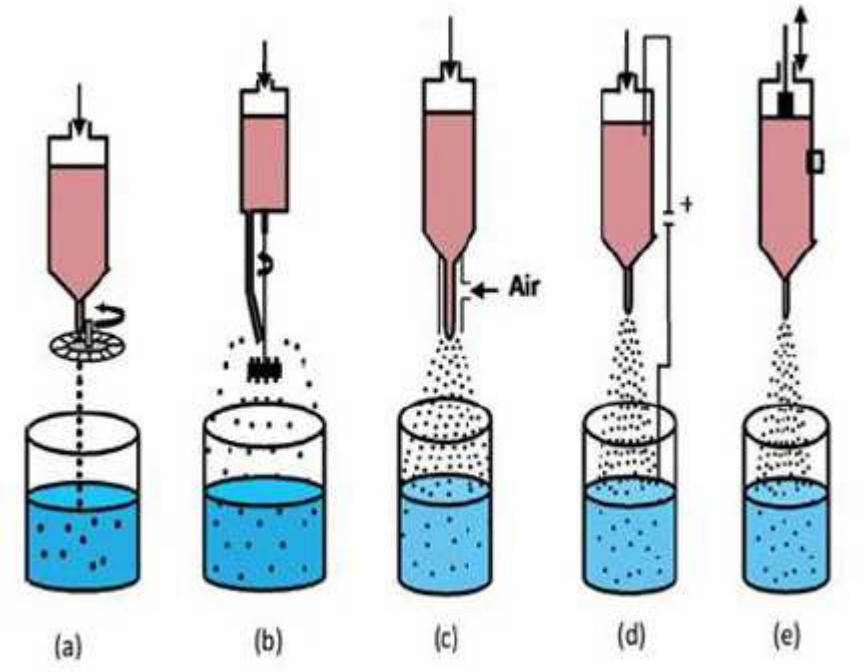


Figure 6.1 Schematic diagram of different mechanically aided dripping techniques; (a) Jet cutting technology, (b) spinning disc atomisation, (c) coaxial air flow, (d) electrostatic extrusion and (e) prilling. Diagram adapted from Krasaekoopt (Krasaekoopt, 2013).

6.2.2.1 Jet cutting technology

High throughput production of microbeads can be achieved by cutting the jet of liquid extrudate (from the nozzle) into cylindrical segments via a rotating micrometric cutting tool (i.e., cutting wire) (Figure 6.1 (a)). Due to surface tension, these cylindrical segments then form spherical droplets/beads when passing through the air before dropping into the hardening solution. The JetCutter® from (GeniaLab®, Germany) is

based on this technology (GeniaLab® Technology overview, 2014). The primary advantage of the JetCutter® are a) an ability to process very viscous liquids, b) high flow rates (up to 5L/h), c) ability to produce microbeads across a wide range of bead sizes and d) a very narrow bead size distribution. Despite these advantages, the technology also poses a number of challenges, namely; a) a high loss percentage associated with the cutting technique and b) scaling up to a multi nozzle process is difficult (Chavarri *et al*., 2012). In addition to these technical challenges, the author could not find any references for the use of the JetCutter® with respect to either gelatine or for pharmaceutical production.

6.2.2.2 Spinning disk atomisation

Spinning disk atomization constitutes a technique in which the liquid extrudate is fed onto a high velocity spinning disc, which in turn produces droplets due to the centrifugal force at the edge of the disc. Similar to the jet cutting technology, these droplets are then dropped into a hardening solution thereby forming solid beads (Figure 6.1 (b)). The method is capable of producing beads in a size range of a few hundred microns up to several millimetres (Krasaekoopt, 2013), however bead deformation is high due to the impact speed of the droplets into the hardening solution and satellite beads (i.e., a second population of very small beads) are also known to form resulting in a wider bead size distribution (Teunou and Poncelet, 2005).

6.2.2.3 Coaxial air flow

Coaxial air flow technology involves applying a stream of compressed air around the extrusion nozzle with the result of pulling the liquid droplets away from the nozzle at a

faster rate than that achieved for gravity dripping (Figure 6.1 (c)). Coaxial air flow is also referred to as the concentric air-jet technique as the equipment consists of a concentric nozzle; an inner orifice (through which the liquid flows) and an outer orifice (through which the air flows). An ability to produce beads with a diameter from a few microns to 1mm is considered to be the main advantage of the technology (Chavarri *et al*., 2012). Interestingly however, this generally perceived advantage is actually considered to be a disadvantage in the context of this project as the target bead size is in the region of 1–2 mm (suitable bead size for fluid bed coating). Another disadvantage of the technology is a very slow flow rate (< 30 ml/h) which poses challenges on scale-up (Chavarri *et al*., 2012).

6.2.2.4 Elecrostatic extrusion

Similar to coaxial air flow, the principle of electrostatic extrusion is based on a force pulling liquid droplets off the orifice at a faster rate than that achieved for gravitational dripping, however an electrostatic force is applied rather than air flow. The electrical potential is applied to the extruded polymer solution by passing it through a charged nozzle (usually a hypodermic needle) with the produced droplets subsequently falling into a hardening solution which has been earthed or holds an opposite charge, thereby creating electrostatic potential between the nozzle and the hardening bath and hence drawing droplets from the nozzle (Figure 6.1 (d)) (Dormer, Berkland and Singh, 2014). The technology is capable of producing beads across a wide size range (1 µm to several millimetres), however its limitations include the following; a) low production rates, b) a wide size distribution and c) safety concerns due to the use of electrostatic charge (personal injury and fire hazard) (Dormer, Berkland and Singh, 2014).

6.2.2.5 Prilling

Prilling, which is also referred to as the vibrating-jet, vibrating nozzle, vibrational drip casting technique or annular jet, is one of the most widely used methods for the production of microspheres/microbeads and microcapsules (Whelehan and Marison, 2011). The technique involves extruding a liquid through a nozzle at a given flow rate to create a laminar jet. The laminar jet is then broken up into short lengths by the application of a vibrational frequency with defined amplitude (i.e., a sinusoidal force) to the jet. The short segments, in turn, form spherical droplets in the air due to the surface tension of the extruded liquid. The characteristics of the drops formed are dependent on the nozzle diameter, the flow rate of the laminar jet, the size of the frequency at defined amplitude and the viscosity of the extruded liquid. The most common application of the sinusoidal force involves either vibrating the nozzle (vibrating nozzle technique) or by pulsating the polymer before passing through the nozzle (vibrating liquid technique) (Whelehan and Marison, 2011, Brandau 2014). Based on a review of the literature there is no collective agreement with respect to the terminology used to describe the laminar jet break-up techniques, however the term prilling is being increasingly used with respect to pharmaceutical applications (Vervaeck *et al*., 2014 and Pivette *et al.*, 2012) and therefore is the term being employed here. A diagram depicting prilling is shown in Figure 6.2.

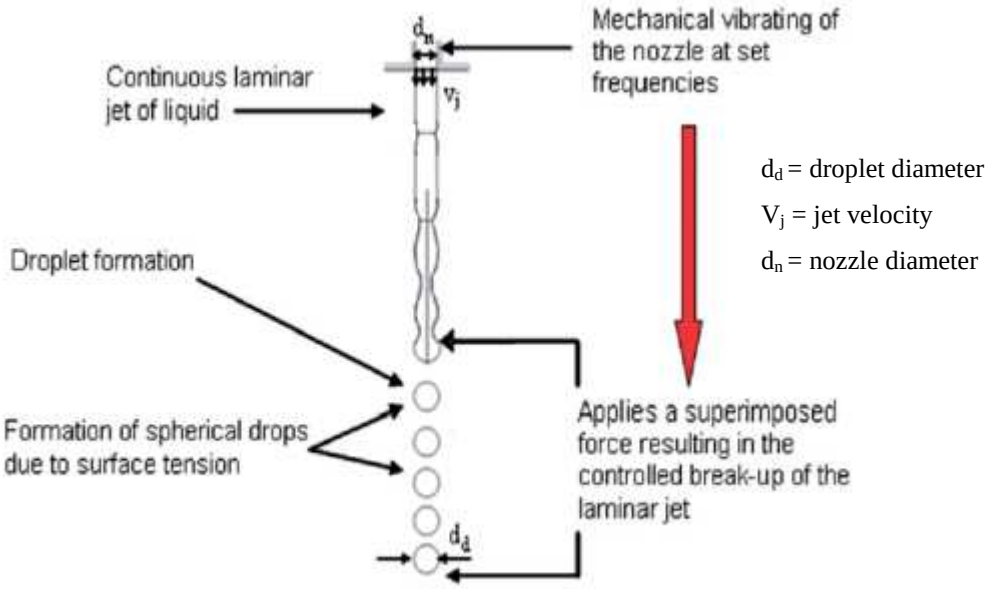


Figure 6.2 Schematic diagram depicting microbead formation via a prilling technique. Microbead formation is achieved by applying a sinusoidal force to the extruded jet, in this case by mechanically vibrating the nozzle at a set frequency with a defined amplitude. Diagram adapted from Whelehan and Marison (Whelehan and Marison, 2011).

There are a number of advantages associated with prilling processes. Compared to other microencapsulation techniques and indeed other dripping techniques, prilling results in perfect regular shaped microbeads with a smooth surface and excellent flow properties and which are capable of 100% drug encapsulation (Vervaeck *et al*., 2014 and Pivette *et al*., 2012). The process also allows for continuous manufacture and can be easily scaled-up using multiple nozzle configurations (Brandau, 2014). The primary disadvantage associated with the technology is that processing is usually restricted to low viscosity liquids, however in terms of gelatine formulations this can usually be circumvented via the addition of water. Given that most of the water is eventually removed during drying of the microbeads, the addition of extra water to facilitate processing does not impact on the final formulation composition.

Compared to the other mechanically aided dripping technologies described, a distinct advantage of prilling processes is the availability of both lab scale and commercial scale equipment. In terms of lab scale equipment, the options include the Var-D series of encapsulators from Nisco Engneering (Switzerland), Inotech encapsulators from Encap Biosystems (now Buchi, Switzerland) and the Spherisator series from Brace GmBH (Germany). Brace GmbH also supply commercial scale equipment as does the Freund Corporation (Japan).

Based on the advantages associated with prilling processes and also access to one of the pieces of lab scale equipment described (i.e., an Inotech IE-50 R encapsulator), prilling was selected as the process of choice for the scale-up manufacture of formulation CLX 136/B.

6.2.3 Coating Optimisation

As described in the introduction, the primary intention of the coating optimisation study was to develop a next generation fully colon targeted CLX microbead formulation that would meet the TPP for colon specific delivery in a mouse model. As a reminder for the reader, the TPP for delivery in the mouse is repeated in Section 6.2.3.1 below and a brief outline of the intended formulation approach to meet the desired TPP is described. Given that the ultimate aim of this research is the development of a product for human therapy, a secondary aim of the coating optimisation study was to develop the release profile of the product to meet a TPP for human administration. Section 6.2.3.2 outlines the TPP for human dosing and also highlights the challenges of meeting the TPP based on the data generated up to this point (i.e., Chapter 5). Finally some formulation approaches to facilitate the regulatory requirements associated with the human TPP are described in Section 6.2.3.3.

6.2.3.1 Mouse TPP

The TPP for the product to allow for maximum colon targeting in the mouse described in Chapter 5 was as follows; <10% release at 2 h and >50% release at 6 h. The TPP was based on published GI transit times for mice (described in Chapter 5). On this basis, the two point TPP described intended to a) ensure minimal drug release in the stomach and small intestine prior to reaching the colon (i.e., <10% release at 2 h) and b) to ensure sufficient drug was released prior to expulsion of the multiparticulate (i.e., >50% release at 6 h). A review of the release profiles for the two coated formulations produced up to this point (Figure 6.3) illustrated that neither formulation met the desired TPP at the 2 h timepoint. Following a review of the data presented in Figure 6.3, it was clear that by increasing the w/g of SR that a longer lag time could be achieved therefore the primary focus of the initial coating optimisation work involved the application of increasing levels of SR.

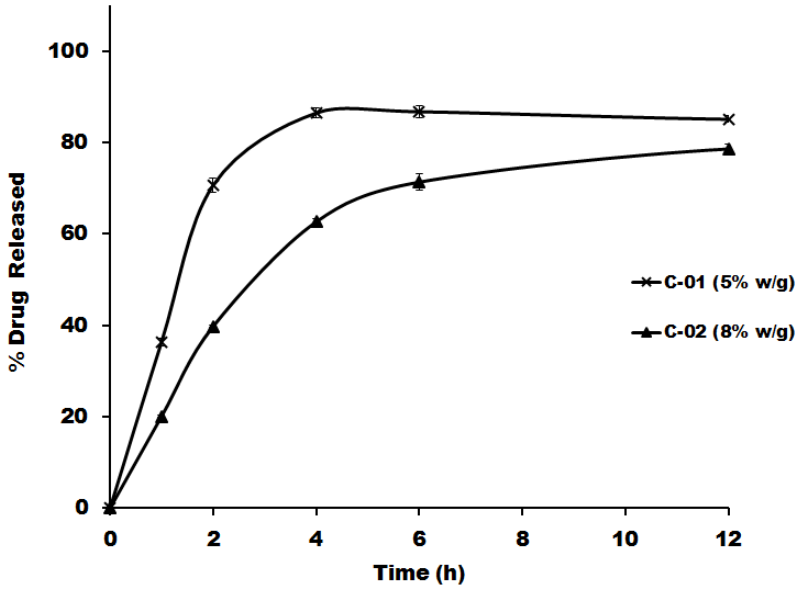


Figure 6.3 Percentage of drug released from release testing of formulations C-01 and C-02 (two-step release method: 2 h in 0.1 M HCl (750 ml) followed by 10 h in phosphate buffer, pH 6.8 (1000ml)). Note: This is a repeat of Figure 5.5 from Chapter 5. The figure is presented again here for the benefit of the reader.

6.2.3.2 Human TPP

When defining a TPP for human administration there are two key points for consideration; a) the TPP to enable a maximum therapeutic/clinical effect and b) a TPP to meet regulatory requirements for the quality control testing of the final product. It is possible to define two separate TPPs to meet these dual requirements, however this usually requires the development of two separate analytical methods with associated cost implications therefore where possible it is desirable to have a TPP that meets both sets of requirements. With respect to this product, the clinical TPP was defined on the basis of the description of human gastrointestinal (GI) physiology described in Chapter 5. The primary focus was therefore to develop a formulation that would preserve the formulation during its passage through the stomach and small intestine, to start release of the drug at the ileocecal junction and thereafter to release drug in a sustained release manner to enable maximum exposure throughout the colon. On the basis of the transit times described in Chapter 5, it was considered that this clinical TPP would equate to the following; < 10% release at 2 h (to minimise release in the stomach), < 30% release at 4 h (to minimise release in the small intestine), approximately 50% release at 6 h (to ensure that sufficient drug is released as the beads start to move through the ascending colon and > 80% release after 18 h (to ensure full release of the drug prior to exiting the colon/rectum). In terms of a regulatory TPP, the FIP (International Pharmaceutical Federation) guideline and the European Pharmacopeia require at least 3 specifications points when setting a drug release specification for a sustained/extended release product. These specifications points are as follows; 1) NMT (not more than) 20–30% after 1–2 h (to provide an assurance against premature drug release), 2) a second specification at approximately 50% release to define the release pattern and 3) > 80% release at the end of the release profile (to ensure almost quantitative release) (Sievert

and Siewert, 1998). Based on a review of the clinical and the regulatory TPPs described above, an overall TPP was derived to meet all requirements. The number of specification points was limited to three to meet commercial pressures to keep the number of time points to a minimum (i.e., less time points means less samples to be tested and also less opportunity to generate out of specification results). The overall human TPP derived was as follows; < 20% release at 2 h, 40-60% release at 6 h and > 80% release after 18 h.

6.2.3.3 Functional excipients to enhance drug release

In section 6.2.3.1, a strategy of applying increasing levels of SR was presented as a means of achieving the 2 h lag time required. It is a well-known phenomenon that applying greater levels of SR can in some cases result in difficulties in achieving full release (i.e., > 80%) at the latter end of the release profile (Rege *et al*., 2005 and Levina *et al.,* 2007). Some of the strategies that can be employed to modulate the release profile to order to achieve full release when using increasing levels of SR are described below.

A. Pore formers

Functional coatings (e.g., SR) are applied to multiparticulate drug delivery systems to achieve desired release profiles. These release profiles can be controlled by varying the composition and levels of the sustained release polymer. One method of varying the release profile from polymers is via the inclusion of a pore former which on contact with liquid results in the formation of channels in the polymer and hence enhanced release (Teng and Qui, 2010). Pore formers can act on the basis of a number of different principles including the following; a) an ability to dissolve at a specified pH, b)

enzymatic activation and c) water solubility. A review of enzymatic and solubility activated pore formers is included below. Pore formers activated via pH changes were omitted from consideration due to GI pH fluctuations (particularly in disease) as previously described in Chapter 5.

A1. Solubility activated pore formers

The primary component of SR is ethyl cellulose (EC). EC is a water-insoluble polymer, having a relatively small degree of swelling due to its hydrophobicity. Incomplete drug release, as well as a long lag time, has been reported in some instances from EC coated multiparticulates, even at low coating weight gains. As a result there have been a number of studies performed to assess the use of HPMC as a water soluble pore-former to enhance the release of drugs form dosage forms coated with SR (Dias *et al*., 2010 and Levina *et al.*, 2007). It has been shown in the studies cited that by including various concentrations of HPMC as pore formers resulted in an enhancement of both the rate and extent of release from dosage forms over a period of 24 h and ultimately ensured complete terminal release. It was postulated that the HPMC in the coat hydrated thereby producing water-logged regions (pores) within the film. Some of the HPMC was then believed to have migrated into the dissolution medium, thereby creating regions with higher film permeability to the drug.

A2. Enzymatic activated pore formers

As described in Chapter 5, colon microflora is recognised as a triggering component in the design of colon specific drug delivery systems. For a number of reasons the use of a predominantly enzymatic activated coating system was not pursued for this project (refer to Chapter 5 for details), however it was felt that the use of enzymatic pore

formers did warrant investigation on the basis of a lower risk than that associated with systems that primarily relied on enzymatic activation. The risk is considered to be less due to the purpose of the pore former in such formulations, in that the primary function of the pore former is ensure maximum release at the end of the release cycle. For example in the case of a diseased patient in which the necessary colonic microflora is not present to activate the pore former then it is reasonable to expect that the patient might still receive a minimum of 70–80% of the intended dose assuming the purpose of the pore former was to ensure release of the remaining 20–30%. As referred to in Chapter 5, there are a number of polysaccharides that have been studied in the context of colon specific delivery. Of these polysaccharides, the use of pectin as a pore former has been the focus of a number of studies involving SR (Wei *et al.,* 2007, Wei *et al*., 2008, Ahmed, 2005 and Wakerly *et al*., 1997). Since pectin is water soluble, it is not considered to be an ideal carrier for colon delivery (when used alone), however it is deemed suitable for use as a pore former when used in combination with an insoluble polymer (e.g., ethylcellulose). In this context an added benefit of pectin is that in the event of the failure of pectin to breakdown enzymatically (e.g., in the absence of sufficient levels of *Bacteroides thetaiotaomicron* (bacteria primarily involved in breaking down of dietry pectin (Dongowski *et al.,* 2000)), the pectin may form pores in the coat as a result of its water solubility in a similar fashion to that postulated for HPMC (Section 6.2.3.3 A1).

B. Sub-coats

A second strategy to modulate the release profile to achieve full release of active when using increasing levels of SR involves the application of an Opadry® (HPMC) sub-coat underneath the functional SR layer (i.e., the outer EC coat). This strategy has previously

been shown to be successful in achieving full release of active drug from microbeads coated with SR in an unpublished patent from Sigmoid Pharma (Coulter, 2013). HPMC sub-coats have previously been employed in combination with EC outer coats in rupturable pulsatile drug delivery systems where it is postulated that the release of the drug at the end of the release cycle corresponds with a rupturing of the EC coat perhaps due to an interaction between the inner coat and/or the core on the outer coat. There are many examples in the literature of rupturable systems (Maroni *et al.,* 2012 and Yadav *et al*., 2011), however the system described in the Sigmoid Pharma patent is unique in that it 1) allows for the maintenance of a significant lag period (<10% release over 2 h), 2) it allows for drug release to be modulated across the entire release profile (i.e., the burst effect seen in pulsatile delivery systems is not observed) and 3) it has been shown to improve batch to batch repeatability (Coulter, 2013).

6.2.4 Objectives

As described in the introduction (Section 6.2.1), the primary goal of this phase of the project was to optimise the SR coating applied to formulation CLX 136/B with the intention of developing a next generation coated CLX microbead formulation that meet the TPP for colon specific delivery to a mouse for use in a repeat of the AOM/DSS CRC mouse model in the future. A secondary goal was to develop the coating formulation with respect to its ultimate use as a finished product for human therapy. The final goal was to assess the suitability of the core formulation for incorporation of a range of APIs (in a solubilised form) across a broad physicochemical spectrum. The objectives set out to fulfil these three goals were as follows:

A. Firstly to scale-up the manufacture of formulation CLX 136/B using an Inotech IE-50 R encapsulator to allow for a sufficient quantity of beads for coating optimisation studies.
B. Secondly to apply increasing levels of SR to CLX 136/B beads with the intention of securing a 2 h lag time and therefore meeting the mouse TPP.
C. In the expectation that by applying increasing levels of SR that > 80% release would not be achieved at the latter end of the release profile, the third objective was to incorporate release enhancers (e.g., pore formers) in the coating composition in order to meet the human TPP.
D. The fourth and final objective was to screen the solubility of a range of actives in the surfactant phase of formulation CLX 136/B and attempt to manually manufacture beads of actives deemed to be soluble in an effort to assess the broader application of the platform formulation.

6.3 Materials and methods

6.3.1 Materials

CLX microbeads (formulation CLX 136/B) were made using the following materials; Solutol HS-15® (BASF, Germany), Miglyol® 810N (Sasol, South Africa), porcine gelatin (Nitta Gelatin, Japan), sorbitol (Neosorb® - Roquette, France) and Sodium Dodecyl Sulphate (SDS) (Merck, Germany). Coating trials were performed using Surelease®, Methocel® E5, Opadry® White 20A28380 (all Colorcon®, USA) and Apple Pectin USP (Sigma Aldrich, USA). A sample of CLX API was kindly provided by Erregierre (Italy). A solubility screening study was performed using the following APIs; Nimodipine (Allchem, India), Budesonide (Crystal Pharma, Spain), Zolpidem Tartrate (Farmak, Czech Republic), Naproxen Sodium (Teva, Israel), Cyclosporine (Euticals,

Italy), Tacrolimus (Biocon, India) Aspirin, Ibuprofen, Sulindac, Naproxen, Tramadol HCl, Diclofenac Sodium salt and Theophyline (all Sigma Aldrich, USA). All chemicals used for the release experiments and HPLC (high performance liquid chromatography) testing were of laboratory grade. *Aspergillus niger* pectinase (Sigma Aldrich, USA) was used for release testing of some formulations comprising pectin.

6.3.2 Methods

6.3.2.1 Automated microbead manufacture

A vibrating nozzle, jet break-up encapsulator (Inotech model IE-50 R, Switzerland) equipped with a 1 mm diameter single nozzle, an air pressure solution delivery system and a temperature controlled nozzle jacket was used to prepare microbeads. The details of the formulation preparation, the cooling oil used and the cooling oil temperature were as previously described for manual microbead production in Chapter 4. The nozzle jacket was set to 65°C. Heat wire set at 65°C was wrapped around the solution tube to maintain the solution at 65°C. Microbeads were produced at a solution flow rates of 8-10 g/min. Microbeads were removed from the cooling oil and were allowed to dry at ambient temperature for 18-24 hours before being sieved. Batch sizes in the region of 50 g were produced.

6.3.2.2 Fluid bed coating of microbeads

The following coating solutions were prepared with PW as per the manufacturer's instructions; SR (15% solids), SR:Pectin (98:2 w/w – 15% solids), SR: Methocel® (90:10 w/w – 15% solids) and Opadry® White 20A28380 (10% solids). The starting weight of microbeads for all coating trials was approximately 6 g. Microbeads with an

average size range of approximately 1.4 mm were used for coating trials. An MFL01 fluid bed system (Vector Corporation, USA) equipped with a Wurster insert was used for coating. Microbeads were coated at an inlet air temperature setting of 65–70°C and a product temperature of 39–45°C. The volume of fluidizing air was maintained at 179 LPM (litres per minute) to ensure optimum fluidizing of microbeads. A nozzle air pressure of 1.7 bar and solution flow rates of 3–4 g/min were applied. At the end of the coating process, the coated microbeads were dried for 5 min in the fluid bed system at a product temperature of 40°C. The weight gain of the beads was calculated based on the starting (pre-coating) and end (post- coating) weights of the beads.

6.3.2.3 *In-vitro* release testing

Release testing of uncoated CLX microbeads (50 mg doses) was performed (*n=3*) at 37°C in PW. Release of coated CLX microbeads (25 mg doses) were performed (*n=3*) at 37°C using a two-step release method (2 h in 0.1 M HCl (750 ml) followed by 16 h in phosphate buffer, pH 6.8 (1000 ml)). For some formulations containing pectin, pectinase (0.02 %) was added to the dissolution media after approximately 16 h. Release experiments were carried out using either a Varian/Vankel VK7010 dissolution apparatus (VanKel, USA) or a Distek Evolution 6300 (Distek, USA) equipped with standard glass vessels and USP type II paddles. Paddle rotating speed in all experiments was 75 rpm. Microbeads equating to 25 or 50 mg of CLX were weighed and added to the release media. At specified times 1.8 ml samples were withdrawn, filtered through a 70 µm pore filter (QLA, USA) and analysed using a HPLC method. The % of drug released at particular time points was determined from peak areas which were calculated against a single point external reference standard. The % of drug release was adjusted to

take into account the content assay (coated beads)/entrapment efficiency (uncoated beads) result for the formulation.

6.3.2.4 HPLC analysis

The HPLC method for the analysis of the release and assay samples was adapted from Saha *et al*. (2002). The HPLC column used was a reverse phase 4.6 x 250 mm Inertsil® C8 column (Inertsil, The Netherlands) with 5 µm particles. The mobile phase was acetonitrile:water (65:35). The isocratic method used a flow rate of 1.25 ml/min and ultraviolet (UV) detection at 230 nm. The injection volume was 20 µl and the retention time was 8 min. The HPLC apparatus that was used for the analysis was a Waters (Waters, USA) HPLC systems (and associated Empower software).

6.3.2.5 Evaluation of entrapment efficiency

The amount of incorporated CLX was determined in selected CLX microbead formulations produced using the Inotech encapsulator. A quantity of beads (*n=2*) with a theoretical potency equating to 5 mg were sonicated for 2 h in a mixture of acetonitrile:water 65:35 to extract the drug from the microbeads. The resultant solution was passed through a 0.45 µm filter prior to absorbance analysis. Where required the samples were diluted prior to analysis. The concentration of CLX was determined by absorbance measurements at 230 nm via the HPLC method described. CLX content (%) was calculated as the amount of determined CLX with respect to the total mass of dried microbeads. The entrapment efficiency (%) of CLX was expressed as a percentage of the determined CLX with respect to the total amount of CLX used in the preparation of the microbeads.

6.3.2.6 Particle size analysis

Particle size analysis (PSA) was performed for selected CLX microbead formulations produced using the Inotech encapsulator. The entire batch size of dried microbeads produced was sieved using 100 mm stainless steel sieves (Retsch, Germany). The sieve sizes used were as follows; 1 mm, 1.25 mm, 1.4 mm, 1.6 mm, 2 mm and 2.5 mm. The resultant fractions were separated and weighed. Approximate determinations of the size of selected wet beads was also performed using a calibrated vernier calipers (Sigma Aldrich, USA).

6.3.2.7 Content assay

The content of CLX in coated microbead formulations was determined via the same method described in Section 6.3.6. The concentration of CLX in the beads tested was determined by absorbance measurements at 230 nm via the HPLC method previously described. CLX content (%) was determined from peak areas which were calculated against a single point external reference standard.

6.3.2.8 API solubility screening

The solubility of a range of APIs in Solutol® HS-15 was investigated. API was added to a measured quantity of Solutol® HS-15 at concentration of 10% w/w (minimum of n=2 measurements). These mixtures were stirred at approximately 35°C on a hotplate/magnetic stirrer. Mixtures that appeared cloudy after overnight stirring were categorised as ‘insoluble’ whereas those which resulted in transparent solutions were categorised as ‘soluble’.

6.3.2.9 Manual manufacture of microbeads

Following on from the API solubility screening described above, the feasibility of manufacturing microbeads using selected APIs was performed via the manual manufacturing method described for CLX microbeads in Chapter 4. The microbead formulation was identical to that employed for CLX 136/B with the exception of the API.

6.4 Results and discussion

6.4.1 Encapsulation scale-up

A total of 9 batches of CLX 136/B were made on an Inotech IE-50 R vibrating jet encapsulator. The batch numbers for these batches were CLX 136/B IN (Inotech) 01–09. The composition for all batches was as outlined in Table 6.1 below.

Table 6.1 Composition of scaled-up formulation CLX 136/B

CLX 136/B Scale-up		
Components	**g**	**mg/g**
Celecoxib	2.86	60.43
Solutol® HS-15	25.70	543.88
Gelatin	15.00	317.39
SDS	2.00	42.32
D-Sorbitol	1.70	35.97
Total	**47.26**	-

The processing conditions for these batches are outlined in Tables 6.2 and 6.3 below. The first batches attempted were CLX 136/B IN-01 and CLX 136/B IN-02 and although it was possible to make beads, the process was very inefficient (process yields of 32.4%

and 34.3% respectively) with the nozzle blocking on numerous occasions as a result of the solution not being sufficiently hot by the time it reached the nozzle. For all subsequent experiments, heated wire was wrapped around the solution tube to ensure that the solution remained at approximately 65°C prior to reaching the nozzle. Where processing was possible, the mechanism of jet 'break-up' was observed to be a 'dripping' process in the case of these two batches (IN-01 and IN-02) which resulted in large beads (all of the beads were >2 mm after drying). The next batch produced, CLX 136/B IN-03, proved more efficient with a process yield of 69.3 % but again the process was characterised as 'dripping'. CLX 136/B IN-04 represented the first batch in which 'prilling' was observed and it was noted that a very fine adjustment in the flow rate resulted in a change from a 'dripping' process to a 'prilling' process. The adjustments to the flow rate resulted in a very low process yield (17.7 %) as it proved difficult to find the correct combination of process conditions that were amenable to prilling-induced 'break-up' of the liquid stream. Importantly, IN-04 represented the first batch in which dried beads with a diameter of < 2 mm were produced.

Table 6.2 Processing conditions for Inotech batches IN-01, IN-02, IN-03 and IN-04

Parameter		IN-01	IN-02	IN-03	IN-04
Heat wire set point (°C)		N/A	N/A	65 °C	65 °C
Cooling oil temperature (°C)		8–10 °C	8–10 °C	8–10 °C	8–10 °C
Air Pressure (bar)		NR	NR	0.5	0.5
Nozzle jacket temperature (°C)		65 °C	65 °C	65 °C	65 °C
Liquid flow observations/ recordings	**Liquid break up process**	Dripping	Dripping	Dripping	Dripping/Prilling
	Approximate Flow rate	NR	NR	NR	NR
Nozzle vibration	**Frequency (Hz)**	NR	NR	NR	45
	Amplitude	NR	NR	NR	**7**
Distance from nozzle to cooling bath (cm)		NT	NR	NR	NR
Size of wet beads produced (mm)		NR	NR	NR	NR
Size of dried beads produced (mm)		2-2.5	2-2.5	2-2.5	1-2.5
% Yield		32.4	34.3	69.3	17.7

NR = Not recorded or not measured

Following the observations for batch CLX 136/B IN-04, two further batches (CLX 136/B IN-05 and IN-06) were produced to examine the impact of adjusting the flow rate and ultimately, the size of the beads produced. IN-05 and IN-06 were the first batches for which the flow rate of the delivery solution was measured. IN-05 was manufactured via a 'dripping' process with a recorded flow rate of approximately 8 g/min. As with previous dripping processes, all the beads (dried) produced were >2 mm. A sample of wet beads were also measured for IN-05 and were found to be within the size range of 3-3.5 mm. The flow rate for batch IN-06 was increased and measured to be approximately 8.5 g/min. It was observed that the process alternated between 'dripping'

and ‘prilling’ with the flow rate adjusted throughout the process in an attempt to maintain a ‘prilling’ process. The resultant dried beads were found to be within a range of 1–2.5 mm which was reflective of the dual process. A sample of wet beads were also measured for IN-06 and were found to be within the size range of 1.5–3 mm. For the next two batches produced, CLX 136/B IN-07 and CLX 136/B IN-08, the flow rate increased toapproximately 10 g/min and although both processes were observed to be predominantly ‘prilling’ processes, the process flow rate required continual adjustment throughout the run to ensure that ‘prilling’ was maintained. These fine adjustments resulted in a variation in the size of beads (dried) produced as illustrated in Figure 6.4. In the case of IN-08, a phenomenon of ‘doubling’ was observed in which wet beads were colliding in the cooling oil and therefore resulted in a larger faction of beads > 2mm. Based on a review of the processing parameters the ‘doubling’ phenomenon was attributed to a reduced nozzle height been employed (height from the nozzle tip to the top of the cooling bath). The process yields for batches IN-07 and IN-08 (71.1% and 73.1% respectively) were higher than that previously achieved which was indicative of the increased level of control compared to the six previous batches.

Table 6.3 Processing conditions for Inotech batches IN-05, IN-06, IN-07, IN-08 and IN-09

Parameter		IN-05	IN-06	IN-07	IN-08	IN-09
Heat wire set point (°C)		65 °C	65 °C	65 °C	65 °C	65 °C
Cooling oil temperature (°C)		8–10 °C	8–10 °C	8–10 °C	8–10 °C	8–10 °C
Air Pressure (bar)		0.5	0.5	0.5	0.5	0.5
Nozzle jacket temperature (°C)		65 °C	65 °C	65 °C	65 °C	65 °C
Process/ flow rate	**Process type**	Dripping	Dripping/ Prilling	Prilling	Prilling	Prilling
	Approximate Flow rate	8 g/min	8.5 g/min	10 g/min	10 g/min	10 g/min[1]
Nozzle vibration	**Frequency (Hz)**	45	45	45	45	45
	Amplitude	**7**	**7**	**7**	**7**	**7**
Distance from nozzle to cooling bath (cm)		29	29	29	20	29
Size range of wet beads produced (mm)		3–3.5	1.5–3	NR	NR	NR
Size of dried beads produced (mm)		2–2.5	1–2.5	See Figure 6.4	See Figure 6.4	See Figure 6.4
% Yield		NR	NR	71.1	73.1	NR

NR = Not recorded or not measured
[1] = Flow rate regulator with enhanced sensitivity used for this trial

The final batch produced was CLX 136/B IN-09. In the case of IN-09, the optimal process conditions from the previous batches were employed. Crucially a flow rate regulator with enhanced sensitivity was fitted for the manufacture of this batch which resulted in a much tighter control of the delivery flow rate of the solution to the nozzle and which consequently did not require adjustment during the process. The consistency introduced to the process as a result of this change resulted in a much tighter distribution of dried micobeads with 98.6% of microbeads being within the desired 1.25–2 mm size range (Figure 6.4).

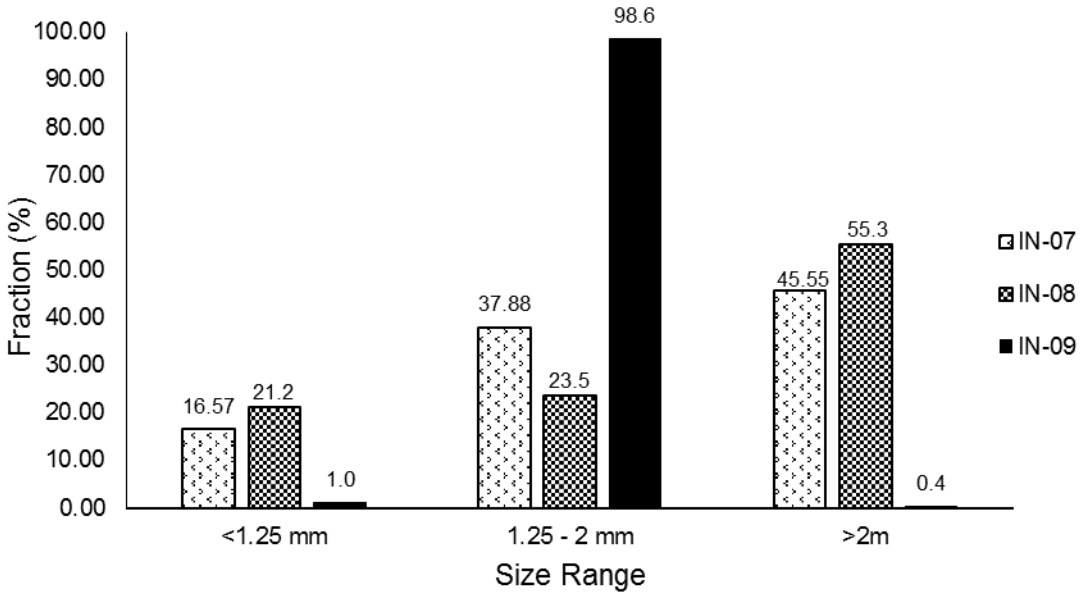


Figure 6.4 Fractions (%) of beads <1.25 mm, 1.25–2 mm and > 2mm following sieving of batches IN-07, IN-08 and IN-09.

Further PSA was performed on IN-09 (Figure 6.5) which revealed a very narrow size distribution with 98.6% of the beads falling with a size range of 1.25–1.6 mm and 73.4% being with a 1.25–1.4 mm.

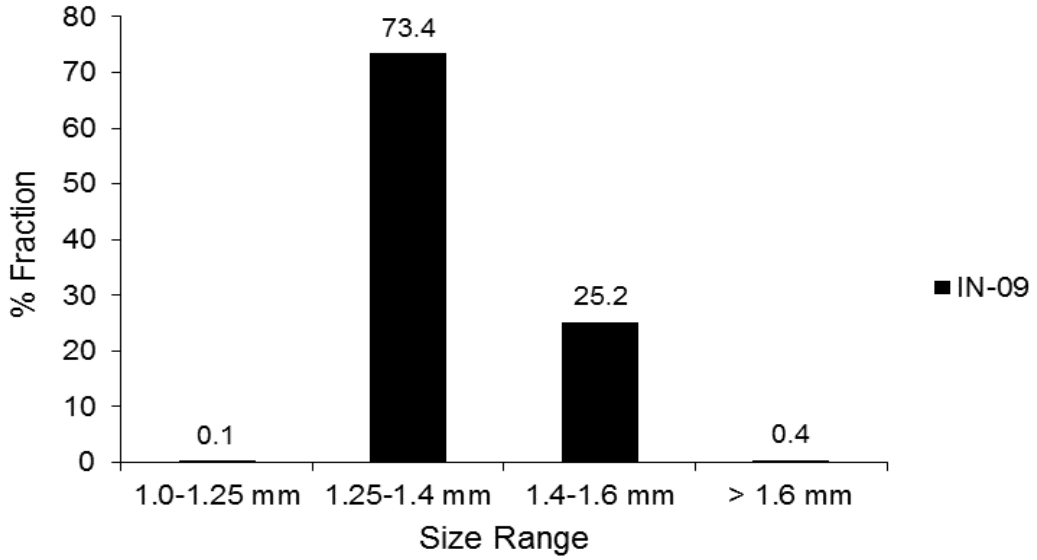


Figure 6.5 Fractions (%) of beads 1–1.25 mm, 1.25–1.4 mm, 1.4–1.6 mm and > 1.6 mm following sieving of batch IN-09.

All of batches produced (with the exception of batch IN-05) were tested with respect to their entrapment efficiency. In the case of batches IN-06, IN-07, IN-08 and IN-09, the fraction of beads with the smallest diameter were used for entrapment efficiency testing in order to preserve quantities of beads with diameters greater than 1.25 mm for coating trials. A comparison of the bead size used for analysis against the % entrapment efficiency revealed that larger beads (> 2mm) had a higher % entrapment which is likely attributable to a smaller surface area compared to small beads. This highlights a requirement to tightly control the bead size on scale-up to ensure that the entrapment efficiency is optimal. It is noted that for batch IN-09 that the % entrapment of the fraction tested (i.e., 80.4%) was at the higher end of that reported by Homar and colleagues for the encapsulation of CLX (Homar *et al*., 2009 – See Chapter 4 for further details).

Table 6.4 % Entrapment efficiency and bead size used for analysis for batches IN-01 to IN-09 (excluding IN-05).

Batch Number	% Entrapment Efficiency (± % STDEV (n=2))	Fraction of bead size analysed
CLX 136/B IN-01	92.08 ± 1.0%	> 2 mm
CLX 136/B IN-02	91.9 ± 0.1%	> 2 mm
CLX 136/B IN-03	93.70 ± 0.6%	> 2 mm
CLX 136/B IN-04	89.70 ± 0.8%	> 2 mm
CLX 136/B IN-06	75.6 ± 0.6%	< 1.25 mm
CLX 136/B IN-07	69.3 % ± 0.5%	< 1.25 mm
CLX 136/B IN-08	75.0% ± 1.3%	< 1.25 mm
CLX 136/B IN-09	80.4% ± 0.2%	1.25–1.4 mm

Release analysis in PW over a period of 24 h was performed on IN-09. The release profile shown in Figure 6.6 illustrates that the % release of formulation CLX 136/B described in Chapter 4 was maintained after scale-up on the Inotech IE-50 R.

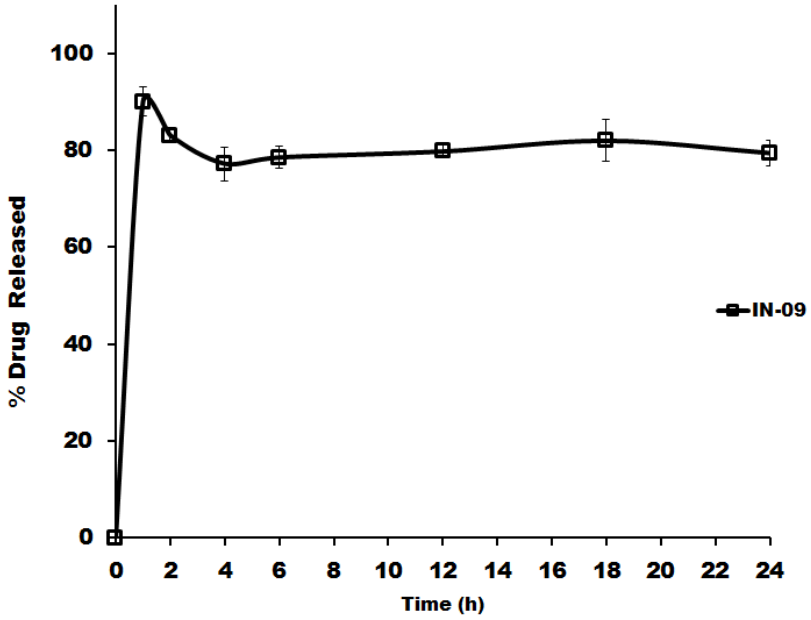


Figure 6.6 Percentage of drug released from release testing of formulation IN-09 in PW. The data presented are mean values ± STDEV (n=3).

6.4.2 Coating optimisation to meet mouse TPP

As described in the introduction, the formulation used for the mouse model study (CLX 136/B C-02) did not meet the desired TPP requirement of <10% release at 2 h. As a result of the successful scale-up of formulation CLX 136/B (Section 6.4.1), there was now a sufficient quantity of beads available to allow for a coating optimisation study with the aim of meeting the mouse TPP. This coating optimisation study involved the application of increased levels (weight gains) of SR in order to extend the lag time.

6.3.3.1 Increased levels of Surelease®

Chapter 5 described the application of SR to formulation CLX 136/B at weight gains of 5% (C-01) and 8% (C-02). Neither formulation met the TPP with in excess of 40% of the drug being released before 2 h. The primary challenge of this coating optimisation study was therefore to achieve a 2 h lag time in which of <10% of drug would be released. In an attempt to meet this challenge, four coating trials were performed in which SR weight gains of 17% (CLX 136/B C-03), 20% (CLX 136/B C-04), 27% (CLX 136/B C-05) and 32% (CLX 136/B C-06) were achieved. It can be seen from Table 6.5 and Figure 6.7 (*in-vitro* release data and profiles for formulations C-03 – C-06) that in excess of 30% of CLX was released from formulation C-03 (17% w/g SR) after a period of 2 h and therefore did not meet the TPP at the 2 h time point. In contrast, formulations C-04 (20% w/g SR), C-05 (27% w/g SR) and C-06 (32% w/g of SR) did not release any CLX (0%) after 2 h and therefore met the TPP at this time point (in addition to meeting the 6 h specification). The processing conditions for formulations C-03 to C-06 are detailed in Table 6.6 below.

Table 6.5 Percentage CLX released (± standard deviation (STDEV) from release testing of formulations C-03, C-04, C-05 and C-06 (two-step release method: 2 h in 0.1 M HCl (750 ml) followed by 16 h in phosphate buffer, pH 6.8 (1000 ml)).

Time point (h)	% CLX Released			
	C-03	C-04	C-05	C-06
0	0	0	0	0
1	2.67 (±0.60)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)
2	33.87 (±0.15)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)
4	61.00 (±0.60)	44.23 (±1.06)	29.95 (±0.83)	12.06 (±0.94)
6	69.07 (±0.22)	72.65 (±0.07)	56.02 (±0.65)	53.53 (±1.32)
12	67.74 (±0.15)	70.71(±0.38)	69.44 (±0.18)	71.33 (±0.54)
18	64.58 (±0.07)	65.59 (±0.15)	66.71 (±0.18)	66.0 (±0.36)

Table 6.6 Fluid bed processing conditions for formulations C-03, C-04, C-05 and C-06

Parameter	C-03	C-04	C-05	C-06
Product temperature (°C)	41–42	40–41	40–41	39–40
Inlet temperature (°C)	65	65–70	65–72	65–70
Atomisation pressure (Bar)	1.7	1.7	1.7	1.7
Average spray rate (g/min)	3–4	3–4	3–4	3–4
Coating time (min)	100	105	117	134
Curing temperature (°C)	40	40	40	40
% Weight gain after curing	17.3	20.8	27.2	32.4

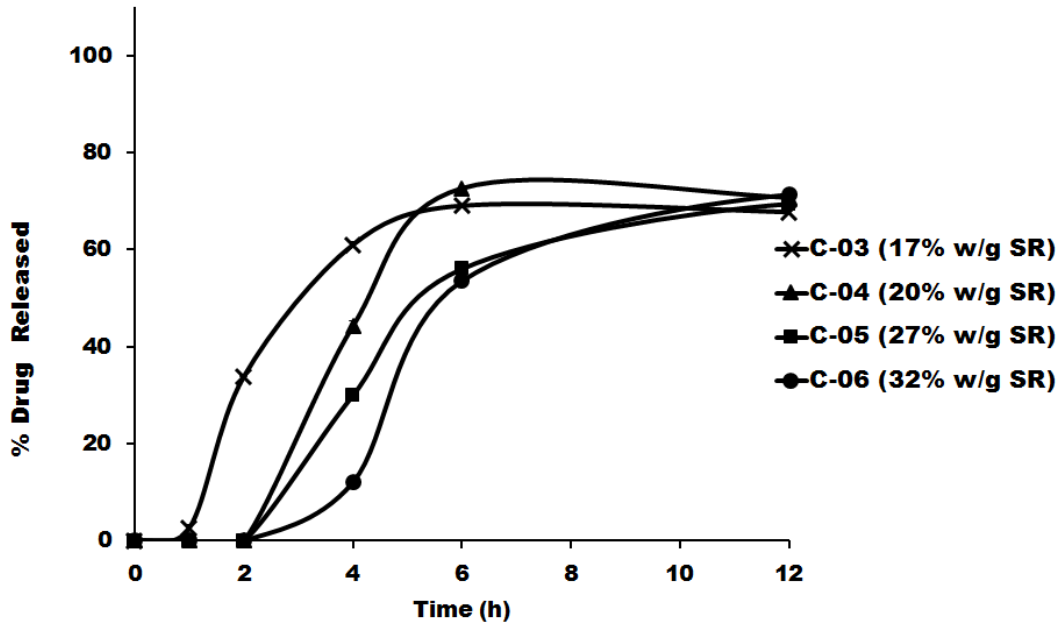


Figure 6.7 Percentage of drug released from release testing of formulations C-03, C-04, C-05 and C-06 (two-step release method: 2 h in 0.1 M HCl (750 ml) followed by 16 h in phosphate buffer, pH 6.8 (1000 ml)). The data presented are mean values ± STDEV (n=3).

Note: Only 12 h of the release profile is represented on the graph as the transit time in mice extends to a maximum of 12 h.

It was concluded that whilst formulations C-04, C-05 and C-06 all met the mouse TPP, that formulation C-04 (20% w/g SR) was the most suitable for use in a follow on animal study as it represented the highest percentage release at the 6 h time point (72.65%) which would ensure a maximum possible release in the event of a shorter transit time.

6.4.3 Coating optimisation to meet human TPP

Having produced a product which met the desired mouse TPP, the focus of the coating optimisation study turned towards developing a product to meet the human TPP outlined in Section 6.2.3.2, that is, < 20% release at 2 h, 40-60% release at 6 h and >

80% release after 18 h. Based on a review of Table 6.5, it was observed that none of the formulations met the human TPP at the 18 h time point, that C-05 (27% w/g SR) and C-06 (32% w/g SR) met the TPP at the 6 h and 2h, that C-04 (20% w/g SR) only met the TPP at 2 h and that C-03 (17% w/g SR) did not meet the TPP at any of the specification points. Based on the observation that none of the formulations met the 18 h specification, the primary focus was to include excipients into the formulation that would enhance release at the end of the cycle.

6.5.3.1 Inclusion of pore formers

The initial attempts to enhance release at the latter end of the release profile involved the incorporation of pore formers into the SR coating. Two pore formers, Apple Pectin USP and Methocel® E5 were investigated. The details of these attempts are described below.

A. Pectin

In an attempt to assess the suitability of pectin as a pore former to meet the TPP at the 18 h time point, a coating solution comprising 98:2 SR:pectin (SR:P) solids was prepared and applied to CLX 136/B. This ratio was chosen based on previous experiences at Sigmoid Pharma. The target weight gain for the formulation was 20% on the basis that the effect of the pore former might be more evident in an initial trial for a product with a lower overall percentage weight gain of SR than that for those weight gains that more closely reflected the desired human TPP (i.e., 27% and 32% w/g SR). Due to a lower coating efficiency for this coating solution, an even lower actual weight gain of 15% was achieved (CLX 136/B C-07). Formulation C-07 (15% w/g SR:P) was

compared to formulation C-03 (17% w/g SR) given that C-03 was the closet formulation to C-07 with respect to the coating weight gain. Formulation C-07 was also tested in the presence of pectinase. It can be seen from Figure 6.8 below, that the inclusion of pectin as a pore former at this concentration did not have any effect with respect to enhancing the % drug released at 18 h.

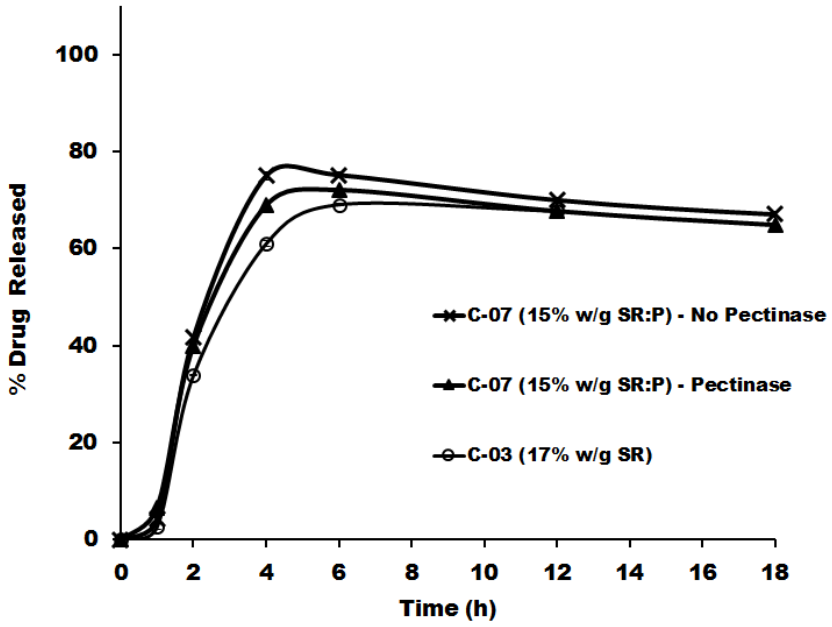


Figure 6.8 Percentage of drug released from release testing of formulations C-03 and C-07 (two-step release method: 2 h in 0.1 M HCl (750 ml) followed by 16 h in phosphate buffer, pH 6.8 (1000 ml) ± addition of pectinase at 16 h). The data presented are mean values ± STDEV(n=3).

B. Methocel® E5

The use of Methocel® E5 as a pore former to meet the TPP at the 18 h time point was also investigated. Methocel® is a grade of HPMC widely used in the pharmaceutical industry and is recommended by Colorcon® for use as a pore former with SR coatings. A coating solution comprising 90:10 SR: Methocel® E5 (SR:M) solids was prepared

and applied to CLX 136/B. This ratio was chosen based on a recommendation from the manufacturer (Colorcon®). In this case, a target weight gain of 27% was set for this formulation, given that formulation C-05 (27% w/g SR) met the TPP at the 2 h and 6 h time points and that less coating polymer was required in comparison to formulation C-06 (which also met the TPP at 2 h and 6 h). A slightly higher coating efficiency for this coating dispersion, resulted in an actual weight gain of 29% (CLX 136/B C-08). C-08 (29% w/g SR:M) compared to formulation C-05 (27% w/g SR). It can be seen from Figure 6.9 below, that whilst formulation C-08 did result in enhanced % drug released at 18 h, the use of the pore former at this ratio resulted in a much higher rate of drug release and consequently the ability of the formulation to meet the 2 h and 6 h specifications was lost.

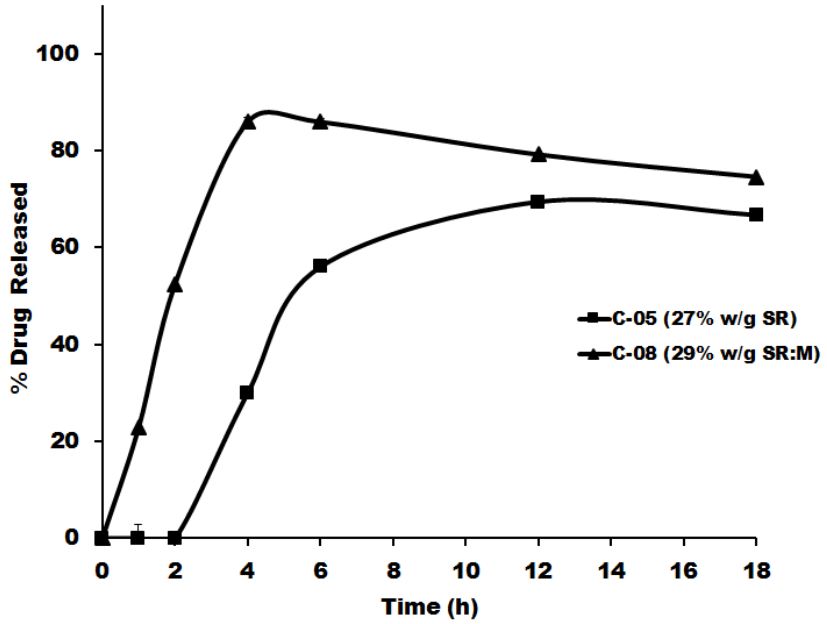


Figure 6.9 Percentage of drug released from release testing of formulations C-05 and C-08 (two-step release method: 2 h in 0.1 M HCl (750 ml) followed by 16 h in phosphate buffer, pH 6.8 (1000ml)). The data presented are mean values ± STDEV(n=3).

6.5.3.2 Inclusion of Opadry® sub-coat

Although the use of pore formers to meet the desired TPP had not been conclusively deemed unviable, for example, it is possible that the TPP could have been achieved by altering the concentrations of pectin or Methocel® employed, however the investment of time to assess this possibility was not warranted at this stage. Instead an alternative approach involving the application an Opadry® sub-coat to achieve the TPP at 18 h was investigated. A subcoat of 8% w/g Opadry® (OP) was applied to CLX 136/B beads prior to the application top coat of 27% w/g SR which yielded formulation C-09 (10% w/g OP + 27% w/g SR). A w/g of 27% SR was selected for the same reasons as outlined in Section 6.4.3.1 B. The % weight gain of Opadry® was based on previous experiences from Sigmoid Pharma. Formulation C-09 (8% w/g OP + 27% w/g SR) was compared to formulation C-05 (27% w/g SR) given that C-05 was the closest formulation to C-09 with respect to SR coating weight gain. It can be seen from Figure 6.10 and Table 6.7 below, that the inclusion of the Opadry® sub-coat modulated the release profile at both ends of the release profile and ultimately resulted in a product that met the desired human TPP at all three specification points. The processing conditions for both the Opadry® and SR coatings for C-09 are outlined in Table 6.8 below.

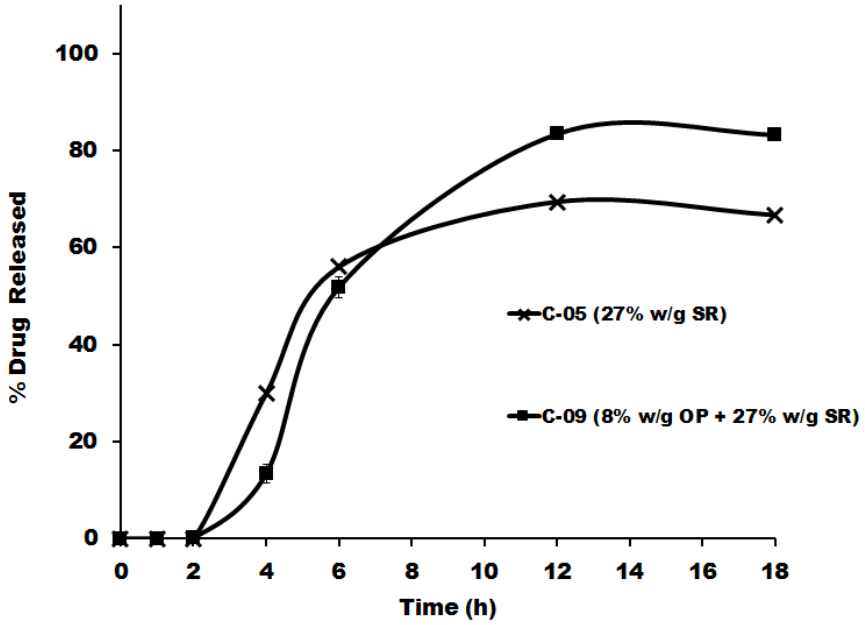


Figure 6.10 Percentage of drug released from release testing of formulations C-05 and C-09 (two-step release method: 2 h in 0.1 M HCl (750 ml) followed by 160 h in phosphate buffer, pH 6.8 (1000 ml)). The data presented are mean values ± STDEV(n=3).

Table 6.7 Percentage CLX released (± standard deviation (STDEV) from release testing of formulations C-05 and C-09 (two-step release method: 2 h in 0.1 M HCl (750 ml) followed by 16 h in phosphate buffer, pH 6.8 (1000 ml)).

Time point (h)	% CLX Released	
	C-05	C-09
0	0	0
1	0.00 (±0.00)	0.0 (±0.00)
2	0.00 (±0.00)	0.2 (±0.00)
4	29.95 (±0.83)	12.1 (±1.84)
6	56.02 (±0.65)	50.2 (±2.26)
12	69.44 (±0.18)	84.2 (±1.13)
18	66.71 (±0.18)	84.0 (±1.13)

Table 6.8 Fluid bed processing conditions for Opadry® and SR coating of formulation C-09

Parameter	Opadry® Coating	SR Coating
Product temperature (°C)	39–41	45
Inlet temperature (°C)	65—70	70
Atomisation pressure (Bar)	1.7	1.7
Average spray rate (g/min)	3	4
Coating time (min)	110	90
Curing temperature (°C)	40	40
% Weight gain after curing	8.0	26.9

6.4.4 Application of the platform formulation to other APIs

As described in the introduction, the final objective of the project was to assess the broader application of microbead formulation CLX 136/B with respect to other APIs. As the name suggests, formulation CLX 136/B was the 136th formulation produced in this study which ultimately resulted in a robust spherical microbead in which precipitation of the drug was not evident during processing and which yielded both a good release performance in PW and a high % entrapment efficiency. Given that the latter two quality attributes would require the development of API-specific analytical methods, a high throughput API screening experiment was designed to focus on critical quality attributes at the point of manufacture. The first critical quality attribute (CQA 1) involved an assessment as to whether Solutol® HS-15 (major component of CLX-

136/B) had the ability to dissolve the given APIs at a concentration of 10% w/w (i.e., 10% drug/90% Solutol® HS-15 – surfactant phase concentration of CLX 136/B). The second CQA (CQA 2) involved an assessment of the ability of those actives which were deemed to be 'soluble' in Solutol® HS-15 to be translated into robust (non-leaking) spherical microbeads in which precipitation was not evident during processing. The composition of the beads attempted was identical to CLX 136/B with the exception of the API. Finally a review of the formulations attempted was performed in an effort to predict the type of actives that might be suitable for incorporation into the platform formulation.

6.5.4.1 Solubility assessment in Solutol® HS-15

The following APIs were screened with respect to their solubility in Solutol® HS-15 at 10% w/w; Nimodipine, Aspirin, Ibuprofen, Busedonide, Sulindac, Diclofenac Sodium salt Naproxen, Zolpidem Tartrate, Naproxen-Sodium, Theophylline, Tacrolimus, Cyclosporine and Tramadol-HCl. The actives were selected from those available in the laboratory in addition to some anti-inflammatory agents which were specifically purchased for the screening study. The categorisation of the actives with respect to being 'soluble' or 'insoluble' is detailed in Table 6.9 below. An active which was 'soluble' was considered to have met CQA 1 as described in Section 6.4.4.

Table 6.9 List of actives that were deemed 'soluble' or 'insoluble' at 10% w/w in Solutol® HS-15

Soluble Actives	Insoluble Actives
Nimodipine Aspirin Ibuprofen Busedonide Sulindac Diclofenac Sodium salt Naproxen	Zolpidem Tartrate Naproxen-Sodium Theophylline Cyclosporine Tramadol-HCl Tacrolimus

The first observation based on a review of Table 6.9 is that out of 14 actives (including CLX) that 8 (i.e., >50%) were 'soluble' at 10% w/w which illustrates the powerful solubilising potential of Solutol® HS-15 and its suitability as a first line solubility screening agent for future projects. It is also noted that one of the other actives in the 'insoluble' category (Tacrolimus) was almost fully solubilised in Solutol® HS-15 at 10% w/w.

6.5.4.2 Microbead feasibility manufacture

All of the actives categorised as 'soluble' in Table 6.9, were progressed to a microbead feasibility study with the aim of assessing their ability to meet CQA 2 described in Section 6.4.4. A summary of these microbead formulation attempts is provided in Table 6.10.

Table 6.10 List of actives which were successful and unsuccessful in achieving CQA 2

Active	Comment	CQA 2 Achieved?
Naproxen	Robust spherical white beads formed	✓
Ibuprofen	Beads produced had poor bead shape	✓
Nimodipine	Robust spherical yellow beads formed	✓
Busedonide	Robust spherical yellow beads formed	✓
Diclofenac Sodium Salt	Robust spherical transparent beads formed	✓
Aspirin	Leaky beads formed, poor bead shape	×
Sulindac	Leaky beads formed, poor bead shape	×

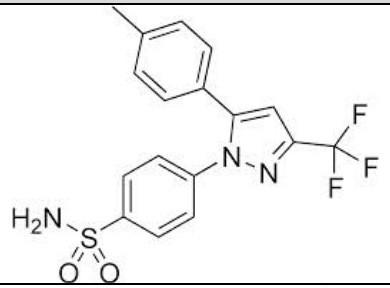
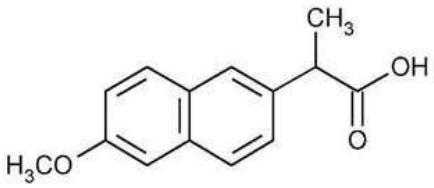
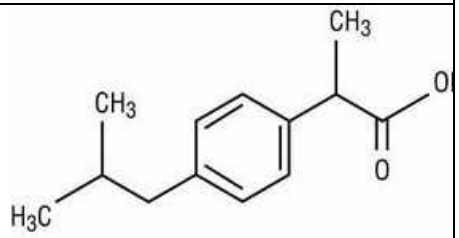
The initial observation based on a review of Table 6.10 is that out of 8 actives (including CLX) that 6 (75%) were successfully translated into microbeads which

illustrates the broad application of the formulation developed. It is also noted that it was possible to translate Tacrolimus (almost soluble in Solutol® HS-15 at 10% w/w – Refer to Section 6.4.4.1) into microbeads.

6.5.4.3 Formulation review/predictability assessment

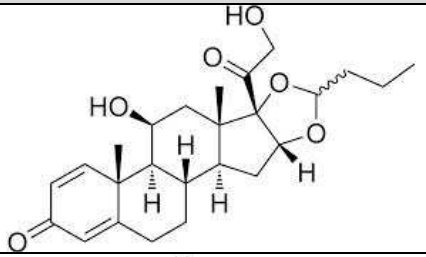
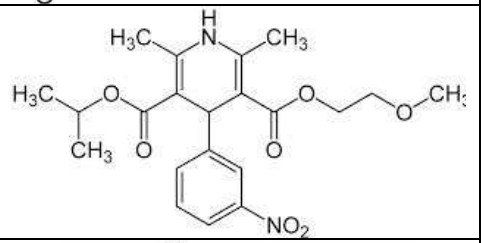
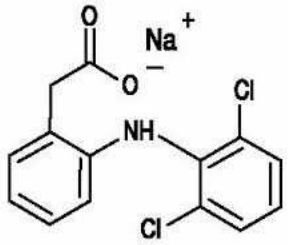
The results outlined in Sections 6.4.4.1 and 6.4.4.2 describe a high throughout two-step screening tool for the assessment of the platform formulation (CLX 136/B) with respect to other APIs. Given the high success rate of the platform illustrated, in that 75% of actives which were shown to be 'soluble' in Solutol® HS-15 were subsequently successfully translated into microbeads, there does not exist a strong case for a theoretical prediction tool for assessing the use of the platform formulation. The physicochemical characteristics of all the actives were however analysed in order to elucidate whether any correlations exist. This assessment was based on two key questions; a) are there any physicochemical similarities between the actives that met CQA 1 and b) are there any physicochemical similarities between the actives that met CQA 2? A review of the physicochemical properties (including their chemical structures) of all the actives tested (including CLX) is provided in Tables 6.11 and 6.12 below. Table 6.11 includes all the actives that were 'soluble' in Solutol® HS-15 whereas Table 6.12 includes all the actives that were 'insoluble' in Solutol® HS-15. Unless otherwise indicated the references for this table were from either the European Pharmacopeia (European Pharmacopeia, 2014), the United States Pharmacopeia (United States Pharmacopeia, 2010) or the Drug Bank database (Drugbank, 2014).

Table 6.11 Comparison of the physicochemical properties of all actives that were 'soluble' in Solutol® HS-15

Active (including CQA status)	Molecular weight	Log P	pKa	Water Solubility	Molecular Formula	Chemical Structure
Celecoxib CQA 1 ✓ CQA 2 ✓	381.37	3.9	11.1	Practically insoluble in water (3.3 mg/L)	$C_{17}H_{14}F_3O_7N_3\ O_2S$	
Naproxen CQA 1 ✓ CQA 2 ✓	230.25	3.18	4.15	Practically insoluble in water (15.9 mg/L)	$C_{14}H_{14}O_3$	
Ibuprofen CQA 1 ✓ CQA 2 ✓	206.28	3.97	4.91	Practically insoluble in water (21 mg/L)	$C_{13}H_{18}O_2$	

Note: USP and EP definition of 'practically insoluble in water': ≥ 10,000 parts solvent required to dissolve 1 part solute

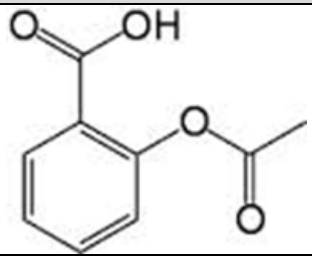
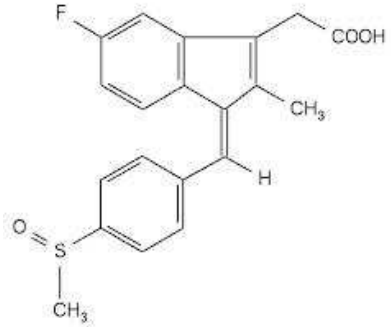
Table 6.11 contd. Comparison of the physicochemical properties of all actives that were 'soluble' in Solutol® HS-15

Active (including CQA status)	Molecular weight	Log P	pKa	Water Solubility	Molecular Formula	Chemical Structure
Busedonide CQA 1 ✓ CQA 2 ✓	430.53	1.9	N/A	Practically insoluble in water	$C_{25}H_{34}O_6$	
Nimodipine CQA 1 ✓ CQA 2 ✓	418.44	3.05	N/A	Practically insoluble in water (24 mg/L) [1]	$C_{21}H_{26}N_2O_7$	
Diclofenac Sodium Salt CQA 1 ✓ CQA 2 ✓	296.14	4.57	4.15	Sparingly soluble in water	$C_{14}H_{10}Cl_2NO_2$. Na	

[1] Santa Cruz Biotech Product Datasheet, 2014 N/A = Not available

Note: USP and EP definition of 'sparingly soluble in water': 30–100 parts solvent required to dissolve 1 part solute

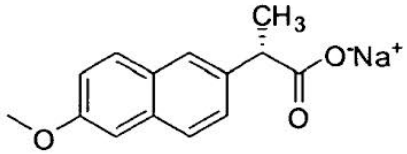
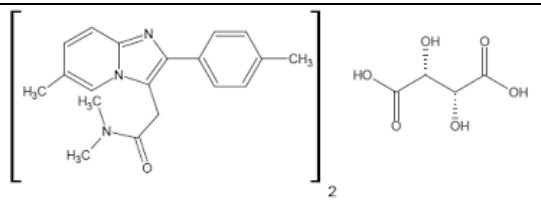
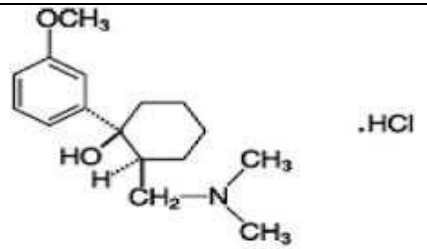
Table 6.11 contd. Comparison of the physicochemical properties of all actives that were 'soluble' in Solutol® HS-15

Active (including CQA status)	Molecular weight	Log P	pKa	Water Solubility	Molecular Formula	Chemical Structure
Aspirin CQA 1 ✓ CQA 2 ×	180.15	1.19	3.49	Slightly soluble in water (4600mg/L)	$C_9H_8O_4$	
Sulindac CQA 1 ✓ CQA 2 ×	356.41	3.42	4.7	Very slightly soluble in water	$C_{20}H_{17}FO_3S$	

Note: USP and EP definition of 'slightly soluble in water': 100–1000 parts solvent required to dissolve 1 part solute

USP and EP definition of 'very slightly soluble in water': 1000–10,000 parts solvent required to dissolve 1 part solute

Table 6.12 Comparison of the physicochemical properties of all actives that were 'insoluble' in Solutol® HS-15

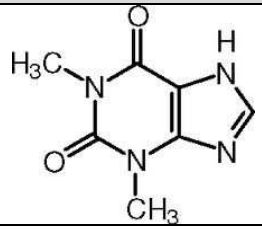
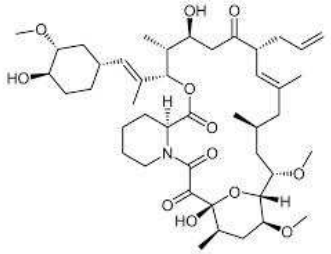
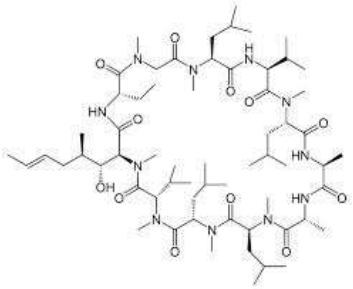
Active (including CQA status)	Molecular weight	Log P	pKa	Water Solubility	Molecular Formula	Chemical Structure
Naproxen Sodium CQA 1 ×	252.24	3.18[1]	N/A	Soluble in water (250 g/L[1])	$C_{14}H_{13}NaO_3$	
Zolpidem Tartrate CQA 1 ×	764.82	3.85 [2]	6.2 [2]	Slightly soluble in water	$[C_{19}H_{21}N_3O]_2 . C_4H_6O_6$	
Tramadol-HCl CQA 1 ×	299.83	2.4	9.41	Freely soluble in water	$C_{16}H_{25}ClNO_2 . HCl$	

[1]Roche Product Datasheet, 2014 [2] Soine, 2008 N/A = Not available

Note: USP and EP definition of 'soluble in water': 10–30 parts solvent required to dissolve 1 part solute

USP and EP definition of 'freely soluble in water': 1–10 parts solvent required to dissolve 1 part solute

Table 6.12 contd. Comparison of the physicochemical properties of all actives that were 'insoluble' in Solutol® HS-15

Active (including CQA status)	Molecular weight	Log P	pKa	Water Solubility	Molecular Formula	Chemical Structure
Theophylline CQA 1 ×	180.12	-0.02	8.81	Slightly soluble in water (7360 mg/L)	$C_7H_8N_4O_2$	
Tacrolimus CQA 1 ×	804.01	3.3	N/A	Practically insoluble in water (4–12mg/L)[1]	$C_{44}H_{69}NO_{12}$	
Cyclosporine CQA 1 ×	1202.61	4.12	Non-ionizable	Practically insoluble in water	$C_{62}H_{111}N_{11}O_{12}$	

[1] Patel *et al*., 2012 N/A = Not available

In terms of CQA 1 (i.e., the ability of Solutol® HS-15 to dissolve the active) it is important to refer back to the structure of Solutol® HS-15 as described in Chapter 2. Solutol® HS-15, is a PEG (polyethylene glycol) fatty acid ester consisting of PEG mono- and di-esters of 12 hydroxystearic (primary lipophilic component) and of about 30% free PEG. In Chapter 2, the solubility of CLX in stearic acid and a range of PEGs (i.e., the primary components of Solutol® HS-15) was examined and it was found that CLX was 40–95 times more soluble in the range of PEGs tested in comparison to stearic acid therefore it is reasonable to assume that the solubilising power of Solutol® HS-15 in relation to CLX is predominantly PEG related. In spite of its name, PEG has hydroxyl groups only at each end of the polymeric chain, therefore, except for these ends, the polymer is essentially non-polar and hence is more amenable for dissolving non-polar compounds.

Based on a review of the 'insoluble' compounds in Table 6.12, it was noted that three out of six of the insoluble compounds are salts. These are highly polar compounds with pure ionic bonds and therefore would not readily dissolve in PEG or PEG 15 hydroxystearate (i.e., Solutol® HS-15). Of the remaining 'insoluble' compounds, Tacrolimus and Cyclosporine are much larger molecules than any of the other actives screened (aside from Zolpidem tartrate (another 'insoluble' compound) and therefore it is proposed that they are too bulky to interact with the hydrophobic structure of Solutol® HS-15 (i.e., the molecules of the active and the solvent are too big to have an interaction). It is notable that Tacrolimus which is smaller and less bulky than Cyclosporine had a better solubility in Solutol® HS-15. Finally in the case of Theophylline, it has a pKa of 8.8 and therefore is also quite polar and will become ionized at pH<8 and hence is unlikely to solubilise in Solutol® HS-15. With respect to the actives that were 'soluble' in Solutol® HS-15 (Table 6.11), it would appear that the majority of these compounds are pushing towards the non-polar side of the spectrum

and therefore are suitable for dissolving in PEG or PEG 15 hydroxystearate. This includes CLX, which is unionized between pH 1-11 and would only lose a proton from the sulfonamide group at a very high pH. The main exception in Table 6.11 to the theory proposed would appear to be the one salt in the list (i.e., Diclofenac Sodium salt), however when compared to the other salts that were 'soluble' in Solutol® HS-15 (Table 6.12), Diclofenac Sodium salt (sparingly soluble in water) has a lower water solubility than Tramadol HCl (freely soluble in water) or Naproxen Sodium (soluble in water) therefore one could reasonably argue that it is still a very non-polar compound and hence soluble in Solutol® HS-15. It is also noted that it is a much smaller compound compared to Zolpidem Tartrate.

In terms of CQA 2, as previously stated 75% of the actives that were 'soluble' were found to be suitable for conversion into microbeads. A review of the suitable versus unsuitable actives with respect to CQA 2 (Table 6.11) highlighted that the two unsuitable actives had a higher water solubility compared to the other six actives.

In summary, it is concluded that the platform formulation is very versatile but that non-polar small molecules with very poor water solubility are most suited to the application.

6.5 Conclusions

Whilst Chapter 5 described the assessment of the anti-cancer and anti-inflammatory effects of a coated microbead formulation in a CRC murine model, the limitation of the formulation, that is that it was not fully colon targeted was also identified. The primary focus of this phase of the project (Chapter 6) was therefore to overcome these limitations by scaling-up microbead production and subsequently optimising the coating applied to the microbeads. Microbead production was successfully scaled-up via automated prilling (using an Inotech IE-50 R encapsulator) which resulted in robust

microbeads with a very narrow size distribution, a high % entrapment efficiency and critically a high and consistent level of drug release in release experiments using PW as the dissolution media. Importantly the scaled-up encapsulation process allowed for batch sizes in the region of 50 g which made significant coating optimisation studies a possibility. These coating optimisation studies initially focused on optimising the coating in order to meet the desired TPP for colon delivery in a mouse. This target was achieved by applying increased levels of the SR polymer (20%, 27% and 32% weight gains). The focus of the project then switched to meeting the desired TPP for human colon delivery and specifically to enhance release at the end of the release cycle to meet clinical and regulatory needs. This aim was achieved by applying a HPMC based sub-coat (Opadry® White) under the SR coat. The final aspect of the project focused on assessing the broader application of the platform formulation using alternative actives to CLX. This assessment illustrated the versatility of the formulation in that out of a total of 14 actives tested (including CLX), 50% were successfully converted into robust spherical microbeads in which there was no evidence of precipitation during processing. A review of the physicochemical characteristics of all the actives also identified that non-polar, poorly water soluble small molecules were most suited to the platform formulation.

6.6 Nomenclature

Table 6.13 List of abbreviations which are listed according to their appearance in the text.

Abbreviation	Definition
CLX	Celecoxib
CRC	Colorectal cancer
w/g	Weight gain
PW	Purified water
HPMC	Hydroxypropyl methylcellulose
AOM	Azoxymethane
DSS	Dextran sodium sulphate
API	Active pharmaceutical ingredient
TPP	Target product profile
GI	Gastrointestinal
FIP	International Pharmaceutical Federation
NMT	Not more than
EC	Ethylcellulose
HPLC	High performance liquid chromatography
SDS	Sodium dodecyl sulphate
LPM	Litres per minute
PSA	Particle size analysis
SR	Surlease®
STDEV	Standard deviation
SR:P	Surelease®:Pectin
SR:M	Surelease®:Methocel E5
OP	Opadry®
PEG	Polyethylene glycol
CQA	Critical quality attribute
CLX xxx/B	Celecoxib bead formulation numbering system where xxx is a sequential number and B is bead

6.7 Acknowledgements

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6.8 References

Ahmed, I.S. 2005, "Effect of simulated gastrointestinal conditions on drug release from pectin/ethylcellulose as film coating for drug delivery to the colon", *Drug Development and Industrial Pharmacy*, vol. 31, no. 4-5, pp. 465-470.

Brandau, T. 2014, "Annular Jet-Based Processes" in *Microencapsulation in the Food Industry: A Practical Implementation Guide*, eds. A. Gaonkar, N. Vasisht, A. Khare & R. Sobel, Elsevier, San Diego, pp. 99-110.

Chávarri, M., Marañón, I. & Villarán, M.C. 2012, "Encapsulation Technology to Protect Probiotic Bacteria" in *Probiotics*, ed. E.C. Rigobelo, Intech, Winchester, pp. Ebook.

Coulter, I.S. Unpublished Formulation Patent; GB1319791.8.

Dias, V.D., Ambudkar, V., Steffenino, R., Farrell, T. & Rajabi-Siahboomi, A.R. 2010, *The Influence of Pore-Former on Drug Release from Ethylcellulose Coated Multiparticulates*.

Dongowski, G., Lorenz, A. & Anger, H. 2000, "Degradation of pectins with different degrees of esterification by Bacteroides thetaiotaomicron isolated from human gut flora", *Applied and Environmental Microbiology*, vol. 66, no. 4, pp. 1321-1327.

Dormer, N.H., Berkland, C.J. & Singh, L. 2014, "Monodispersed Microencapsulation Technolgy" in *Microencapsulation in the Food Industry: A Practical Implementation Guide*, eds. A. Gaonkar, N. Vasisht, A. Khare & R. Sobel, Elsevier, San Diego, pp. 111-124.

European Pharmacopeia, 2014, 8.0th edn, EDQM, Strasbourg.

Homar, M., Dreu, R., Kerc, J. & Gasperlin, M. 2009, "Preparation and evaluation of celecoxib-loaded microcapsules with self-microemulsifying core", *Journal of Microencapsulation*, vol. 26, no. 6, pp. 479-484.

Krasaekoopt, W. 2013, "Microencapsulation of probiotics in hydrocolloid gel matrices: a review", *Agro Food Industry Hi Tech*, vol. 24, no. 2, pp. 76-86.

Levina, M., Vuong, H. & Rajabi-Siahboomi, A.R. 2007, *The Effect of Hypromellose as a Pore-Former on Drug Release from Aqueous Ethylcellulose Film-Coated Dipyridamole-Loaded Non-Pareil Beads* , Poster edn, Controlled Release Society Annual Meeting.

Maroni, A., Zema, L., Del Curto, M.D., Foppoli, A. & Gazzaniga, A. 2012, "Oral colon delivery of insulin with the aid of functional adjuvants", *Advanced Drug Delivery Reviews*, vol. 64, no. 6, pp. 540-556.

Patel, P., Patel, H., Panchal, S. & Mehta, T. 2012, "Formulation strategies for drug delivery of tacrolimus: An overview", *International Journal of Pharmaceutical Investigation*, vol. 2, no. 4, pp. 169-175.

Pivette, P., Faivre, V., Mancini, L., Gueutin, C., Daste, G., Ollivon, M. & Lesieur, S. 2012, "Controlled release of a highly hydrophilic API from lipid microspheres obtained by prilling: analysis of drug and water diffusion processes with X-ray-based methods", *Journal of Controlled Release* vol. 158, no. 3, pp. 393-402.

United States Pharmacopeia, 2010, 33rd edn, USP/NF, Rockville.

Rege, P.R., Fegely, K.A., Scattergood, L.K. & Rajabi-Siahboomi, A.R. 2005, *Predictability of Drug Release from Multiparticulate Systems Coated with an Aqueous Ethylcellulose Dispersion*, Poster edn, Controlled Released Society Annual Meeting.

Saha, R.N., Sajeev, C., Jadhav, P.R., Patil, S.P. & Srinivasan, N. 2002, "Determination of celecoxib in pharmaceutical formulations using UV spectrophotometry and liquid chromatography", *Journal of Pharmaceutical and Biomedical Analysis*, vol. 28, no. 3-4, pp. 741-751.

Sievert, B. & Siewert, M. 1998, "Dissolution tests for ER products", *Dissolution Technology*, vol. 5, no. 4, pp. 1-7.

Soine, W. 2008, "Sedative-Hypnotics" in *Foye's Principles of Medicinal Chemistry*, eds. T.L. Lemke & D.A. Villiams, 6th edn. edn, Lippincott, Williams and Wilkins, Baltimore, pp. 504-520.

Teng, Y. & Qui, Z. 2010, "Fluid Bed Coating and Granulation for CR Delivery" in *Oral Controlled Release Formulation Design and Drug Delivery: Theory to Practice*, eds. H. Wen & K. Par, Wiley, New Jersey, pp. 115-128.

Teunou, E. & Poncelet, D. 2005, "Rotary disc atomisation for microencapsulation applications—prediction of the particle trajectories", *Journal of Food Engineering*, vol. 71, no. 4, pp. 345-353.

Vervaeck, A., Monteyne, T., Saerens, L., De Beer, T., Remon, J.P. & Vervaet, C. 2014, "Prilling as manufacturing technique for multiparticulate lipid/PEG fixed-dose combinations", *European journal of Pharmaceutics and Biopharmaceutics*vol. 88, no. 2, pp. 472-482.

Wakerly, Z., Fell, J.T., Attwood, D. & Parkins, D. 1997, "Studies on drug release from pectin/ethylcellulose film-coated tablets: a potential colonic delivery system", *International Journal of Pharmaceutics*, vol. 153, no. 2, pp. 219-224.

Wei, H., Qing, D., De-Ying, C., Bai, X. & Fanli-Fang 2007, "Pectin/Ethylcellulose as film coatings for colon-specific drug delivery: preparation and in vitro evaluation using 5-fluorouracil pellets", *PDA Journal of Pharmaceutical Science and Technology / PDA*, vol. 61, no. 2, pp. 121-130.

Wei, H., Qing, D., De-Ying, C., Bai, X. & Li-Fang, F. 2008, "In-vitro and in-vivo studies of pectin/ethylcellulosefilm-coated pellets of 5-fluorouracil for colonic targeting", *The Journal of Pharmacy and Pharmacology*, vol. 60, no. 1, pp. 35-44.

Whelehan, M. & Marison, I.W. 2011, "Microencapsulation using vibrating technology", *Journal of Microencapsulation*, vol. 28, no. 8, pp. 669-688.

Yadav, D., Survase, S. & Kumar, N. 2011, "Dual coating of swellable and rupturable polymers on glipizide loaded MCC pellets for pulsatile delivery: formulation design and in vitro evaluation", *International Journal of Pharmaceutics*, vol. 419, no. 1-2, pp. 121-130.

Drugbank database, 2014. Available: www.drugbank.ca [2014, Oct/20].

GeniaLab Technology overview, 2014.
Available: http://www.genialab.com/TechJetCutter.php [2014, Oct/20].

Roche Product Datasheet - Naproxen Sodium, 2014.

Available: http://www.roche.com/pages/csds/english/out/0490628.20110225.8049.pdf [2014, Oct/20].

Santa Cruz Biotech Product Datasheet, 2014.

Available: http://www.scbt.com/datasheet-201464-nimodipine.html [2014, Oct/20].

CHAPTER 7

Conclusions and perspectives

7.1 Conclusion and perspectives

Colorectal cancer (CRC) is the third most common cause of cancer mortality worldwide with an estimated 1,360,000 new cases of CRC diagnosed worldwide in 2012, (Cancer Research UK, 2013). In Ireland, CRC is the second most common type of cancer (as it is in Europe) and it is predicted that the number of cases of colorectal cancer will double between 2015 and 2040 (National Cancer Registry Ireland 2013 and 2014).

As with other cancers, currently, the most effective treatments for colorectal cancer (combinations of surgical resection, radiation, and/or chemotherapy) depend on the detection of the cancer at a very early-stage. Despite the more widespread use of colorectal cancer screening (e.g., via colonoscopy procedures for patinets presenting with positive faecal occult blood tests), unfortunately, it has not been possible to identify all individuals at the earliest stages of disease. In fact, most patients present to their physician with advanced cancer when standard treatments for solid malignancies result in a much lower 5-year survival (Wang and Dubois, 2010). Thus, an effective approach for this disease must include prevention and targeted therapy. It is generally agreed that an effective way to control cancer is to find better ways of preventing it and/or detecting the disease at its earliest stage (Wang and Dubois, 2010). This is particularly important in the case of colitis associated cancer as the entire colon is considered to be at a heightened risk of dysplasia which ultimately requires surgical removal of the entire colon, therefore chemopreventive approaches present obvious benefits. Since elevated cyclooxygenase-2 (COX-2) expression was found in most colorectal cancer tissue and is associated with worse survival among CRC patients, investigators have sought to evaluate the effects of nonsteroidal anti-inflammatory drugs (NSAIDs) for CRC prevention and treatment. The epidemiologic studies, clinical trials and animal experiments indicate that NSAIDs are among the most promising

chemopreventive agents for this disease. NSAIDs exert their anti-inflammatory and anti-tumour effects primarily by reducing prostaglandin production via inhibition of COX-2 activity, therefore there has been a particular focus on the use of COX-2 inhibitors for CRC prevention and treatment (Wang and Dubois, 2010). As described in Chapter 1, there is a significant body of research illustrating the anti-CRC effects of COX-2 inhibitors such as Celecoxib (CLX). The significant drawbacks of current therapy with the marketed CLX product Celebrex®, that is the serious cardiovascular (CV) and gastrointestinal (GI) side effects associated with the drugs, were also outlined. Chapter 1 revealed that the unwanted side effects of Celebrex® are a) dose related (CV and GI side effects) and b) dosage form related (GI side effects) (Sacchetti, 2013, Soloman *et al*., 2005 and FDA labelling Revision for Celebrex Capsules, 2008). The core objective of this project was to develop a more effective CLX formulation in which the solubility issues associated with the drug would be addressed, thereby ultimately allowing for the unwanted side effects associated with Celebrex® to be eliminated or reduced. A five step plan was developed to meet this core objective. These five steps were as follows;

1. Development of a lipid-based formulations to enhance the solubility of CLX

2. An assessment of the anti-cancer effects of selected lipid-based formulations in an *in-vitro* cell culture model and a comparison to the anti-cancer effects of Celebrex®

3. The translation of the optimal lipid-based formulation into a multiparticulate lipid-based drug delivery system (LBDDS), a microbead formulation, and the optimisation of the same microbead formulation

4. The application of controlled release polymers to the optimal microbead formulation to enable colonic delivery in a mouse, an assessment of the anti-cancer effects of the colonic targeted formulation a comparison to the anti-cancer effects of Celebrex®

5. The optimisation of the coated microbead formulation to allow for the development of a more colon specific formulation for use in future *in-vivo* mouse and human studies. The phase of the project also involved a scale-up of microbead manufacture and the application of the platform formulation to other active ingredients.

The primary conclusions from each of these phases of the project are outlined below. References are also made to links between these conclusions and the various stages of a 'parallel screening model' which was unexpectedly developed through the course of this work and which is described later in the chapter.

In the first phase of the project (Chapter 2), lipid-based liquid formulations were produced, formulations CLX 016/L and CLX 021/L, which were demonstrated to have a greater drug release performance to that of Celebrex®. These liquid formulations were based on the non-ionic surfactant Solutol® HS-15 and the semi-synthetic medium chain triglyceride Miglyol® 810N (parallel screening - stage 2). An initial feasibility study also illustrated that these formulations were amenable to translation into spherical and robust gelatine based microbeads. This result was an important first step in the project as it demonstrated the potential to meet the two key critical quality attributes (CQAs) of

the desired formulation outlined in Chapter 1; 1) A LBDDS in which CLX was fully dissolved and 2) the translation of the LBDDS into a multiparticulate form..

The second phase of the project (Chapter 3) involved testing a hypothesis that the lipid-based CLX formulations had the potential for a more efficacious inhibitory effect than Celebrex® in a CRC cell line on the basis that pre-dissolving the drug would enable direct interaction with colon cancer cells (parallel screening – stage 3). The testing of this hypothesis demonstrated that CLX liquid formulations (CLX 016/L and CLX 021/L) performed significantly better than the marketed CLX product Celebrex® with respect to their ability to prevent the proliferation but also motility of a HT29 CRC cell line *in-vitro*. The CLX liquid formulations were also shown to significantly reduce the motility (a marker for the metastatic potential of cancer cells) of the HT29 CRC cell line, whereas Celebrex® did not have a significant effect. One of the key findings of this phase of the project involved a demonstration that the liquid CLX formulations employed had an early apoptotic effect on HT29 cells, whereas CLX alone had both a necrotic and an early apoptotic effect, which was a very significant finding as it presented the opportunity for targeted CLX therapy with reduced GI side effects on the basis that the GI side effects related to Celebrex® are as a result of direct epithelial toxicity and are associated with the current presentation of the drug as a powder filled capsule (refer to Chapters 1 and 3 for further details). A correlation between the drug release performance of CLX formulations in PW and their ability to affect HT29 cells was also observed, thereby presenting an effective tool for formulation screening (parallel screening - stage 2). CLX liquid formulations were used for this *in-vitro* cell study as they represented a precursor to microbead formulations and were also amenable for direct application to the cells.

In the third stage of the project (Chapter 4), the CLX liquid formulations developed in the first stage of the project and tested in the second stage of the project were used a precursor for the development of gelatine based microbead formulations using a gravity 'dripping' technique. A spherical robust microbead formulation (CLX 136/B) with an entrapment efficiency of 97% and a maximum drug release in PW of 80% was produced, which represented a formulation that had the potential to consistently deliver CLX in a pre-solubilised state (parallel screening - stage 4). CLX 136/B was demonstrated to have a greater drug release performance to that of Celebrex®. A correlation between the droplet size and drug release performance was also established for optimal and sub-optimal formulations (parallel screening - stage 5).

The proposition for the fourth stage of the project (Chapter 5) was to target the optimal microbead formulation developed in the previous phase of the project (CLX 136/B) to the colon of a mouse and to assess their potential, and that of Celebrex® for the treatment and prevention of CRC using an AOM (azoxymethane)/DSS (dextran sodium sulphate) mouse model for CRC. Surelease® (ethylcellulose polymer) was applied to the microbeads (batch size of 6 g) at a weight gain of 8% and despite not meeting a pre-defined target product profile (TPP) for colonic specific delivery in a mouse (parallel screening stage 5), the partially colonic targeted formulation (C-02) was progressed to the CRC model (parallel screening stage 6). The effect of formulation C-02 on the attenuation of CRC tumours was compared to that of Celebrex® and was found to be marginally better. The anti-inflammatory effects of both formulations were also assessed, with formulation C-02 being found to have a significant effect compared to the control in the case of colon length and histology scoring (markers for inflammation) whereas Celebrex® did not meet significance. A hypothesis was also presented to suggest that the anti-cancer and anti-inflammatory effects of formulation C-02 may

have been achieved with a lower dose that that required to exert the effect seen for Celebrex® given that in the region of 50% of the drug from C-02 was likely released before the microbead reached the colon and therefore would not have been available in the colon to interact with colonic tissue.

Given the limitations of the formulation tested in the mouse model, with respect to it being only partially colonic targeted, the focus of the fifth and final phase of the project was to optimise the coating applied to formulation CLX 136/B with the aim of producing a fully colonic targeted final product. This coating optimisation study necessitated the automated scale-up of microbead manufacture to allow for a sufficient quantity of microbeads for the coating. Microbead production was successfully scaled up via an automated prilling process (using an Inotech IE-50 R encapsulator) which resulted in robust microbeads with a very narrow size distribution, a high % entrapment efficiency and critically, a high and consistent level of drug release in release experiments using PW as the dissolution media. The application of increased levels of the Surelease® polymer (20%, 27% and 32% weight gains) resulted in a product that met the predefined mouse TPP. Having achieved the primary objective of the fifth phase of the project, the focus of the project then switched to meeting the desired TPP for human colonic delivery and specifically to enhance release at the latter end of the release profile to meet clinical and regulatory needs. This aim was achieved by applying a HPMC (hydroxypropyl methylcellulose) based sub-coat (Opadry® White) under the Surelease® coat. The final part of the project focused on assessing the broader application of the platform formulation using alternative actives to CLX. A total of 14 actives tested with 50% of these successfully converted into microbeads thereby illustrating the versatility of the platform formulation.

Parallel screening is defined as a cost-effective approaching for selecting a suitable drug delivery system to enhance the chances of achieving the required effects (e.g., bioavailability) for a given drug in the clinic (Chow and Kane, 2011). The requirement for parallel screening approaches is as a result of a major change in the types of new chemical entities (NCEs) being presented to formulation scientists. Literature shows that there is an increasing number of poorly soluble compounds in the drug discovery pipeline with BCS (biopharmaceutical classification system) Class II drugs (poorly soluble/highly permeable drugs such as CLX) and Class IV drugs (poorly soluble/poorly permeable representing 90% of NCEs, whereas BCS Class II and Class IV drugs only account for approximately 36% of the existing top 200 marketed drugs (Figure 7.1) (Chow and Kane, 2011 and Hauss, 2007). Current discovery programmes have the potential to create a significant loss of economic and therapeutic opportunity due to product attrition resulting from poor bioavailability (primarily due to poor solubility), however it is believed that applying suitable technologies and approaches to increase the bioavailability of poorly soluble molecules will reduce the attrition rate, will increase the number new drugs getting to the market and consequently will bring better healthcare to patients (Chow and Kane, 2011).

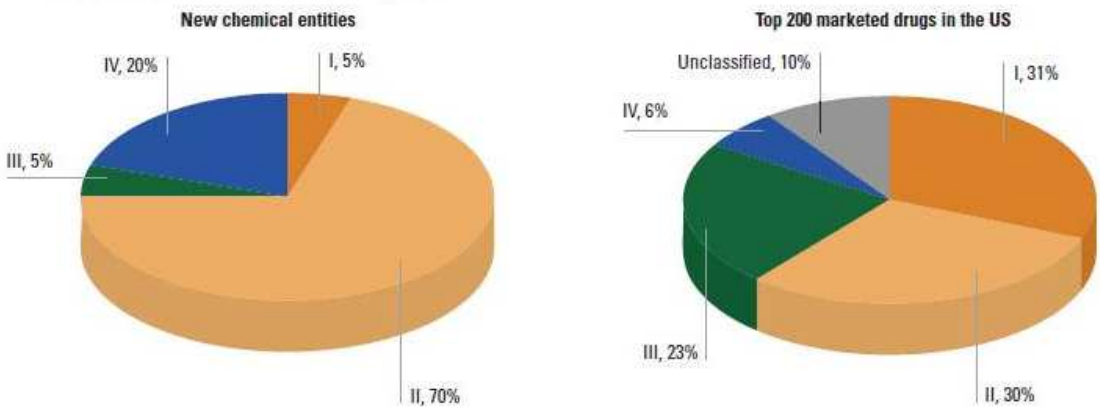

Figure 7.1 Breakdown of NCEs and existing drugs with respect to their BCS classification.

Class I: highly soluble/highly permeable, Class II: poorly soluble/highly permeable, Class III: highly soluble/poorly permeable and Class IV: poorly soluble/poorly permeable. (Adapted from Chow and Kane, 2011)

As outlined in Chapter 1, there are a number of formulation approaches which can be utilised to address the poor solubility of drug candidates and historically pharmaceutical companies have assessed the available technologies in an uncertain fashion that has resulted in a significant investment of time and money (See Figure 7.2).

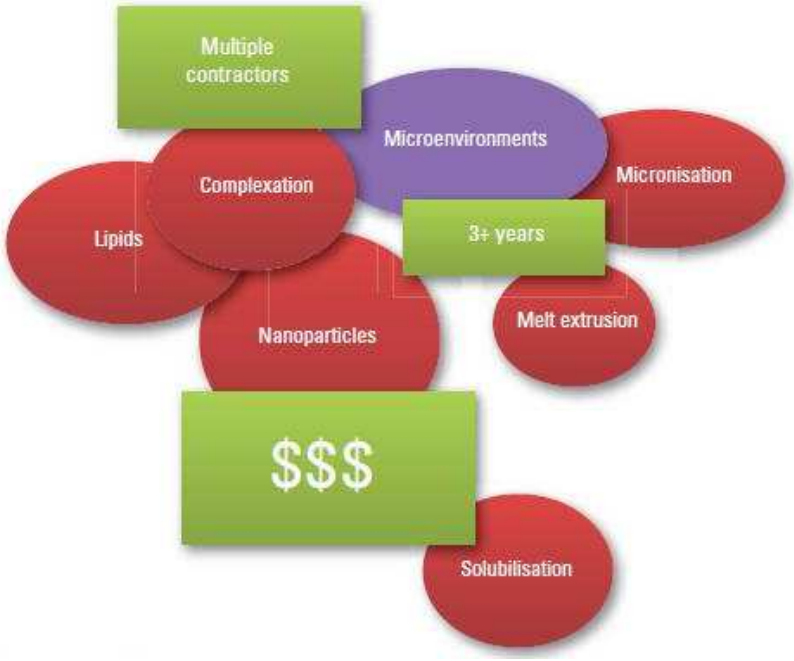

Figure 7.2 Uncertain pathway historically adopted by pharmaceutical companies to assess available technologies which has resulted in a drain on resources (Adapted from Chow and Kane 2011).

A parallel screening strategy is advocated by Chow and Kane in order to increase the speed of screening drug candidates and to identify a suitable technology to progress the molecule to a proof-of-concept stage. The parallel screening strategy proposed by Chow and Kane involves the six steps outlined in Table 7.1 below. The equivalent six steps executed in the development of the CLX formulation are also described for comparison purposes.

Table 7.1 Six step parallel screening strategy advocated by Chow and Kane (Chow and Kane, 2011) and comparison to equivalent CLX formulation development steps

Parallel Screening Step	Chow and Kane	Equivalent CLX Formulation Development Steps
1	Upfront scientific assessment including an assessment of the physicochemical characteristics of the drug	Documentation of CLX API physicochemical characteristics (Chapter 1) and latterly the physicochemical characteristics of the various APIs screened (Chapter 6)
2	Screening experiments including solubility screening, thermal analysis etc.	Excipient solubility screening (Chapter 2) and drug release screening experiments in PW
3	*In-vitro* assessments such as permeation studies involving Caco-2 cell lines	Cell proliferation studies involving HT29 cell lines and correlation with drug release performance in PW (Chapter 3)
4	Preparation of pilot formulations using inexpensive miniaturised (API sparing) equipment	Manual preparation of microbeads (Chapter 4) and latterly encapsulation using lab scale Inotech encapsulator (Chapter 6). Coating of small batches of microbeads on MFL01 fluid bed coater (Chapters 5 and 6).
5	Predictive analytical test methods to select pilot formulation for bioavailability studies	Release studies on coated microbeads using two step release media (acid followed by phosphate buffer to mimic GI conditions) and development of model specific TPPs (Chapters 5 and 6).
6	Suitable animal model for bioavailability assessment	Attenuation of CRC tumours in studies involving AOM/DSS induced mice (Chapter 5)

As eluded to earlier, it was not an intended aim of this project to develop a parallel screening model, however based on the results presented and in particular the API screening study described in Chapter 6, it is clear that a very robust model has been developed for the screening of molecules linked to the prevention and treatment of CRC. In addition to a CRC parallel screening model, despite increased bioavailability not being a focus of this project, stages 1, 2, 4 and 5 of the model are deemed to be directly transferable to a model screening for enhanced bioavailability whereas stages 3 (*in-vitro* cell model) and 5 (*in-vivo* animal model) could easily be adapted to suitable models where bioavailability enhancement is the aim of an alternative project (i.e., this model has the potential for a much broader application than CRC).

In terms of the next stages of development for the CLX product, a number of key milestones have been identified. These include;

- Modulation of the release profiles for the formulations designed for colonic targeting in a mouse (formulations C-04, C-05 and C-06) in order to ensure >80% release at 12 h to guarantee the maximum possible dose of drug is made available.

- A repeat of the AOM/DSS CRC mouse model described in Chapter 5 using a fully colonic targeted microbead formulation. It is also recommended that this study would involve an assessment of CLX levels in the blood (i.e., a pharmacokinetic (pK study)) for both CLX microbeads and Celebrex®. This is important so as to verify that the CLX released from the microbead formulation is exerting its effect via luminal interaction rather than via systemic delivery but also to understand if CLX in the microbeads has an alternative mode of action compared to Celebrex®. It is known that CLX is rapidly eliminated from the blood which thereby might limit its therapeutic concentration at the tumour site (Paulson et al., 2000). It is also recommended that tissue samples be taken as part of any animal study to assess levels of markers such as PGE2

- It is also recommended to perform a pK study on the uncoated CLX formulation to assess its potential to improve the bioavailability of CLX compared to Celebrex® for the treatment of osteoarthritis, adult rheumatoid arthritis and ankylosing spondylitis.

- Performance of a drug loading study for formulation CLX 136/B to assess the boundaries of the formulation with respect to its physicochemical performance (e.g., will a higher drug loading affect its release performance).

- Performance of excipient compatibility studies in order to establish an impurity profile for the product.

- Performance of a packaging study to elucidate the optimal packaging (e.g., bottles, blister packaging) for the product.

- Performance of stability studies at ICH (International Conference on Harmonization) conditions in order to establish the stability of the formulation and ultimately to set a shelf-life for the product.

- Performance of clinical studies to assess the efficacy and safety of the formulation.

Although the focus of clinical cancer research and indeed of this project with CLX has predominantly been on chemoprevention, the potential chemotherapeutic use of CLX in cancer is also attracting considerable attention. Chemotherapeutic agents and radiation therapy have been shown to enhance COX-2 protein expression in human cancer cells, which in turn results in resistance to therapy, therefore there is a desire to determine whether CLX enhances the chemo-sensitivity and radio-sensitivity of tumour cells (Wang and Dubois, 2010). Preclinical studies have shown that CLX potentiates the effects of radiotherapy (Davis *et al*., 2004) and that a combination treatment of CLX with oxaliplatin had synergistic effects on inhibition of tumour growth in a mouse

xenograft model of human colon cancer (Zhao *et al*., 2009). Combination products in general are in fact an area of increased attention both from a chemotherapeutic but also a chemopreventative perspective with recent evidence illustrating that a combination of CLX and erlotinib (an EGFR tyrosine kinase inhibitor) had more effective prevention of polyp formation in $APC^{Min/+}$ mice and more significant inhibition of tumour growth in a xenograft model than either drug individually (Buchanan *et al*., 2007). The combination therapies cited leverage on the principle that by combining CLX with the synergistic effects of other drugs that a lower dose of CLX may be used thereby reducing the undesired side effects associated with CLX (Wang and Dubois, 2010).

In conclusion, despite a large body of evidence illustrating the chemopreventative effects of CLX, the identification of adverse CV side effects associated with the drug has understandably made it difficult for the development of new formulations in this field, however to ignore the potential benefits of chemoprevention with CLX is to continue to accept a higher than necessary death rate from colorectal cancer is patients who do not have access to or are not fully compliant with colorectal cancer screening and it also ignores the needs of a subset of patients for whom routine colorectal cancer screening is not as an effective detection mechanism (i.e., patients with colitis associated dysplasia) (Wang and Dubois, 2010). It is unlikely that chemoprevention will completely replace screening, but its success may lead to fewer screening exams and to fewer cancer-related deaths, especially in high risk groups (Arber, 2008). This project has resulted in the development of a colonic targeted microbead formulation in which CLX is presented in a pre-dissolved micellar format within a multiparticulate, a combination that has been shown to greater than the marketed CLX formulation Celebrex® from a physicochemical perspective (*in-vitro* release testing), from an anti-inflammatory perspective (*in-vivo* CRC animal study) and an anti-cancer perspective

(*in-vitro* CRC cell line study and *in-vivo* CRC animal study). Importantly this combination (i.e., pre-dissolved micellar format within a multiparticulate) has the potential to minimize GI and CV side effects associated with the current marketed formulation and thereby presents the prospect of a formulation that can capitalise on the anti-adenoma and anti-cancer effects described in previous clinical studies whilst addressing the serious side effects that ultimately led to the discontinuation of these studies. In summary the formulation developed here has the potential to put colorectal cancer prevention, using a safer more effective CLX formulation, back on the table for consideration.

7.2 Nomenclature

Table 7.2 List of abbreviations which are listed according to their appearance in the text.

Abbreviation	Definition
CRC	Colorectal cancer
NSAIDs	Non-steroidal anti-inflammatory drugs
COX-2	Cyclooxygenase-2
CLX	Celecoxib
GI	Gastrointestinal
CV	Cardiovascular
LBDDS	Lipid-based drug delivery system
PW	Purified water
SDS	Sodium dodecyl sulphate
AOM	Azoxymethane
DSS	Dextran sodium sulphate
TPP	Target product profile
HPMC	Hydroxypropyl methylcellulose
ICH	International conference on harmonisation

7.3 References

Arber, N. 2008, "Cyclooxygenase-2 inhibitors in colorectal cancer prevention: point", *Cancer Epidemiology, Biomarkers & Prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology,* vol. 17, no. 8, pp. 1852-1857.

Buchanan, F.G., Holla, V., Katkuri, S., Matta, P. & DuBois, R.N. 2007, "Targeting cyclooxygenase-2 and the epidermal growth factor receptor for the prevention and treatment of intestinal cancer", *Cancer Research,* vol. 67, no. 19, pp. 9380-9388.

Chow, K. & Kane, A. 2011, "Parallel Screening", *European Pharmaceutical Contractor,* vol. 158, pp. 100-104.

Davis, T.W., O'Neal, J.M., Pagel, M.D., Zweifel, B.S., Mehta, P.P., Heuvelman, D.M. & Masferrer, J.L. 2004, "Synergy between celecoxib and radiotherapy results from inhibition of cyclooxygenase-2-derived prostaglandin E2, a survival factor for tumor and associated vasculature", *Cancer Research,* vol. 64, no. 1, pp. 279-285.

Hauss, D.J. 2007, *Oral Lipid-Based Formulations – Enhancing the Bioavailability of Poorly Water-Soluble Drugs,* Informa Healthcare, New York.

Paulson, S.K., Zhang, J.Y., Breau, A.P., Hribar, J.D., Liu, N.W., Jessen, S.M., Lawal, Y.M., Cogburn, J.N., Gresk, C.J., Markos, C.S., Maziasz, T.J., Schoenhard, G.L. & Burton, E.G. 2000, "Pharmacokinetics, tissue distribution, metabolism, and excretion of celecoxib in rats", *Drug Metabolism and Disposition: the Biological Fate of Chemicals,* vol. 28, no. 5, pp. 514-521.

Sacchetti, A. 2013, "Cancer cell killing by Celecoxib: reality or just in vitro precipitation-related artifact?", *Journal of Cellular Biochemistry,* vol. 114, no. 6, pp. 1434-1444.

Solomon, S.D., McMurray, J.J., Pfeffer, M.A., Wittes, J., Fowler, R., Finn, P., Anderson, W.F., Zauber, A., Hawk, E., Bertagnolli, M. & Adenoma Prevention with Celecoxib (APC) Study Investigators 2005, "Cardiovascular risk associated with celecoxib in a clinical trial for colorectal adenoma prevention", *The New England Journal of Medicine,* vol. 352, no. 11, pp. 1071-1080.

Wang, D. & DuBois, R.N. 2010, "The role of COX-2 in intestinal inflammation and colorectal cancer", *Oncogene*, vol. 29, no. 6, pp. 781-788.

Zhao, S., Cai, J., Bian, H., Gui, L. & Zhao, F. 2009, "Synergistic inhibition effect of tumor growth by using celecoxib in combination with oxaliplatin", *Cancer Investigation*, vol. 27, no. 6, pp. 636-640.

Cancer Research UK - Bowel Cancer Incidence Statistics, 2013. Available: http://www.cancerresearchuk.org/cancer-info/cancerstats/types/bowel/incidence/uk-bowel-cancer-incidence-statistics [2014, Oct/01].

FDA Labelling Revision for Celebrex® Capsules, 2008. Available: http://www.fda.gov/ohrms/dockets/ac/08/briefing/2008-4344b1_07_06_Celebrex%20Label.pdf [2011, Mar/10].

National Cancer Registry Ireland - Cancer Projections for Ireland 2015-2040, 2014. Available: http://www.ncri.ie/publications/cancer-trends-and-projections/cancer-projections-ireland-2015-%E2%80%93-2040 [2014, Oct/07].

National Cancer Registry Ireland - Cancer in Ireland 2013: Annual Report of the National Cancer Registry, 2013. Available: http://www.ncri.ie/sites/ncri/files/pubs/CancerinIreland2013AnnualReportoftheNationalCancerRegistry.pdf [2014, Oct/07].