Evaluation of Photografted Charged Sites Within Polymer Monoliths in Capillary Columns Using Contactless Conductivity Detection.

Damian Connolly[a], Vincent O'Shea[b], Paul Clark[b], Brendan O'Connor[b] and Brett Paull[a*]

Centre for Bioanalytical Sciences, School of Chemical Sciences[a], School of Biotechnology[b], Dublin City University, Glasnevin, Dublin 9, Ireland

***Corresponding author**

Email: Brett.Paull@dcu.ie

Fax: +353-(0)1-7005503

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Abstract.

Capacitively coupled contactless conductivity detection (C^4D) is presented as a novel and versatile means of visualising discrete zones of charged functional groups grafted onto polymer based monoliths. Monoliths were formed within 100 µm UV transparent fused silica capillaries and photografting methods were subsequently used to graft a charged functional monomer, 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS) onto discrete regions of the "generic" monolith using a photomask. Post-modification monolith evaluation involves scanning the C^4D detector along the length of the monolith to obtain a profile of the exact spatial location of grafted charged functionalities with millimetre accuracy. The methodology was extended to the visualisation of several zones of immobilised protein (bovine serum albumin) using photografted azlactone groups to enable covalent attachment of the protein to the monolith at precise locations along its length. In addition, the extent of non-specific binding of protein to the ungrafted regions of the monolith due to hydrophobic interactions could be monitored as an increase in background conductivity of the stationary phase. Finally, the technique was cross-validated using fluorescence microscopy by immobilising green fluorescent protein (GFP) in discrete zones and comparing the profiles obtained using both complementary techniques.

1. Introduction.

A number of recent **publications** [1 2 3 4] have demonstrated the use of photolithographic methodologies for the modification of pre-formed monolithic stationary phases. Essentially, this procedure involves filling the pores of a monolith with a reactive functional monomer in the presence of a free radical initiator and an inert solvent. By blocking off all but a specific region of the monolith with a photo-mask, irradiation of that particular unmasked region through the UV transparent capillary walls with UV light, will excite the initiator and trigger the covalent grafting of functional groups onto an otherwise inert monolith backbone. This technique thereby results in the simple preparation of monoliths with co-contiguous chemistries, with for example, a generic hydrophobic monolith backbone in one region of the column, and a separate region of the monolith possessing a different grafted functional group such as an anion exchange moiety. Grafted groups can be anion exchange, cation exchange, or protein immobilised via grafted reactive functional monomers such as vinyl azlactone. **[REF 4].** The benefits of photografting are numerous and have been discussed in a recent comprehensive review by Svec[5] **[and 3]**

Modification of polymer monoliths with protein for use as affinity chromatography phases is generating considerable interest. Examples include the immobilisation of protein A, for the separation of immunoglobulins[6 78], lectins such as concavalin A, for the extraction of glycoproteins[9], enzymes such as trypsin, for use as micro-bioreactors in LC-MS proteomic applications **[4]** and the immobilisation of immunoglobulins[10]. These varied applications of polymeric monolithic stationary phases for affinity chromatography has been the subject of a recent review by **Mallik**

et al[11]**,** in which the numerous immobilisation strategies which have previously been reported are discussed. The most common approaches result in covalent attachment of the protein to monolith and include the epoxy method[12 13], the Schiff base method [10,12],[7] the glutaraldehyde method[7][14], the carbonyldiimidazole method[7,10,12], the disuccinimidyl method, the hydrazide method[10], and the cyanogen bromide method.

A number of the above immobilisation strategies suffer from a range of disadvantages, including long reaction times, multiple reaction steps, low amounts of immobilised ligand, unwanted side reactions, the use of harsh reagents (which can often cause partial unfolding or complete denaturation of certain proteins) and the use of toxic reagents (cyanogen bromide). Recently, a number of research laboratories have reported the use of vinyl azlactone as an intermediate functional group for the safe and rapid immobilisation of proteins on monolithic surfaces. Vinyl azlactone has previously been covalently attached to monolithic surfaces using the photografting techniques previously discussed, and used as a scavenger for excess amines in reaction mixtures[15].[16] Leading on from this, other reports have demonstrated the ability of the azlactone functionality of these monoliths to react readily with nucleophiles such as the amino or thiol groups of proteins thereby enabling their rapid and efficient immobilization via a dipeptidic linker. **[3].** The advantage of using azlactone chemistry for immobilisation of protein, is that the procedure is rapid (grafting of azlactone takes up to 10 minutes, and the subsequent protein immobilisation takes less than three hours), and the procedure is separated into to steps such that the protein is never in contact with potential denaturants. Considerably higher activity of immobilised ligand has also been reported.

Obtaining qualitative and quantitative information regarding immobilised protein on surfaces is certainly a challenge. The ability to validate the exact spatial location of immobilised protein and the immobilised quantity thereof on a surface is important and techniques such as surface plasmon resonance[17 18] ellipsometry,[19] optical waveguide lightmode spectroscopy[20] and quartz crystal microbalance[21] have been previously reported. These techniques quantitatively measure the amount of protein immobilised on flat, planar surfaces such as glass, gold and plastic substrates, but these methods cannot easily be utilised for macroporous monolithic materials, because of the huge disparity between the surface area of a monolith compared with a flat surface.

A relatively new methodology which has not yet been explored as a means of measuring immobilised protein on monoliths is capacitively coupled contactless conductivity detection (referred to hereafter as C^4D). The principles of C^4D have been well studied[22 2324252627], and the electronic circuitry has been found to be simple and of low cost. The advantage of C^4D over conventional conductivity detection for chromatography is that the cell design is extremely robust and there is a lack of physical contact with the eluent. This eliminates possible fouling of the electrodes and reduces to zero the extra-column band broadening that would be observed with a conventional conductivity flow cell.

Building upon results recently reported by this research group, **[28 29],** this paper reports for the first time the use of C^4D as a novel means of (a) non-invasively inspecting the longitudinal homogeneity of monolithic structure and porousity within

capillary formats, (b) visualising the exact location of discrete zones of photografted charged functionalities or immobilised protein on polymer monoliths and (c) the semi-quantitative evaluation of non-specific binding of protein to hydrophobic monoliths before, during and after column washing steps.

2. Experimental.

2.1 Instrumentation.

The pump used for washing and equilibration of monoliths, for immobilisation of proteins (BSA and GFP-His) and during the taking of conductivity readings was a Dionex Ultimate 3000 LPG 3000 pump (Dionex, Sunnyvale, California). The operational flow rate was 1 µL/min. The pump used to prepare monolithic columns and to fill the pores of pre-formed monoliths with reactive monomers for photo-grafting was a Knauer pump Model K120 operated at 10 µL/min. The detector used was a TraceDec capacitively coupled contactless conductivity detector (Innovative Sensor Technologies GmbH, Innsbruck, Austria). The exact position of the detection cell along the length of the monolithic column was varied by hand, using a ruler as a position indicator. The monolithic column itself was passed through the radial capillary detector cell, which was programmed with the following settings: frequency, 3X HIGH; voltage, -12 dB; gain, 50% and offset, 0. A GFL water bath was used for monolith production, and the operational temperature was 60 ^{o}C. A Labnet Spectrafuge centrifuge was used to eliminate particulate matter during the preparation of monomer solutions and protein solutions. The UV lamp used was a Bondwand (Electro-Lite Corporation, Conneticut, USA) for production of monoliths with differing regions of porosity, and for photo-grafting of 2-acrylamido-2-methyl-1-

propanesulfonic acid onto pre-formed monoliths. The operational wavelength of this lamp was 350 nm with an irradiation intensity of 10 mW/cm^2. The UV lamp used for photografting of 4,4-dimethyl-2-vinylazlactone onto methacrylate monoliths was a battery operated handheld UVP lamp, model UVG4 with an operational wavelength of 254 nm with an irradiation intensity of 170 μW/cm^2 at 3". The photomask used for production of monoliths with differing zones of porosity was simply some black electrical tape stuck down over the capillary such that the photomask was only one layer thick (of tape). For all other photomasking events, the capillary monolith was passed through short pieces of 1/16" PEEK tubing (i.d. 380 μm). SEM images were taken with a Hitachi S-3000N scanning electron microscope. Fluorescence microscopy images were taken with the aid of a Vilber Lourmat UV Transluminator at wavelength of 254 nm and with a Panasonic NV-GS500 40 Megapixel digital video camera with 12X optical zoom.

2.2 Reagents.

Butyl methacrylate (99%, BuMA), ethylene dimethacrylate (98%, EDMA), benzophenone (99+%, BP), 3-(trimethoxysilyl)- propyl methacrylate (98%), AIBN, 1,4-butanediol, *tert*-butyl alcohol (99.5%), 2-acrylamido-2-methyl-1-propanesulfonic acid, sodium sulphate, sodium carbonate, bovine serum albumin (BSA) and sodium hydroxide pellets were purchased from Sigma-Aldrich (Tallaght, Dublin, Ireland). Hydrochloric acid was purchased from Fluka. Vinyl azlactone (4,4-dimethyl-2-vinylazlactone) was purchased from TCI Europe. Methanol, acetone and 1-propanol were purchased from Labscan (Stillorgan, Dublin, Ireland). All other reagents were of the highest available purity and used as received without additional purification or

distillation before use. UV transparent teflon coated fused silica capillary (100 µm i.d.) was obtained from Polymicro Technologies (Phoenix, AZ). Deionised water was produced with a Millipore Direct-Q™ 5 (Millipore, Bedford, MA, USA) water purification system, and eluents were vacuum filtered through a 0.2 µm filter (Supelco, Supelco Park, Bellefonte, PA, USA) and degassed by sonication. The eluent used for immobilisation of BSA and GFP-His was 0.5 *M* sodium sulphate, 0.1 *M* sodium carbonate at a flow rate of 1 µL/min. Histidine tagged green fluorescent protein (GFP-His) was expressed in *E. coli* and purified using nickel affinity chromatography.

2.3 Vinylisation of fused silica capillaries for monolith production.

A suitable length of fused teflon-coated silica capillary (100 µm i.d.), was rinsed with acetone using a pump at 10 µL/min, and dried in a stream of nitrogen for 10 minutes at room temperature. The inner walls of the capillary were then activated with 0.2 mol/*L* sodium hydroxide for 30 minutes by pumping at 10 µL/min. The capillary was then washed in the following order at 10 µL/min: water for 5 minutes, 0.2 mol/*L* HCl for 30 minutes, water for 5 minutes, and finally with acetone for 5 minutes. After drying the capillary again with nitrogen gas, a 50 wt % solution of 3-(trimethoxysilyl)propyl methacrylate in acetone was prepared. A 435 µL length of PEEK tubing was then filled with the solution using a disposable syringe, one end of the filled loop was attached to the empty capillary, and the other end to the outlet of a piston driven pump delivering methanol at 10 µL/min. The reagent was pumped through the capillary at 10 µL/min until all air was expelled from the capillary and the entire capillary length was full of reagent. The capillary was then end-capped with pieces of rubber septa, and immersed in a water bath at 60 ^{o}C for 20 hours. Finally,

the column end-plugs were removed, the column was flushed with acetone to remove excess 3-(trimethoxysilyl)propyl methacrylate, and the column was dried with a stream of nitrogen and stored indefinitely.

2.4 Preparation of methacrylate monolithic capillary columns.

A polymerization mixture was prepared comprising 24 % BuMA, 16 % EDMA, 34 % 1-propanol, 26 % 1,4-butanediol, and 0.4 % AIBN as free radical initiator (all w/w). The solution was sonicated for 20 minutes to dissolve the AIBN, and then centrifuged for 10 minutes at 13,000 r.p.m to eliminate particulate matter. The supernatant was degassed by allowing nitrogen gas to bubble through the solution for 10 minutes. The de-aerated monomer solution was introduced into the capillary using a PEEK loop as described above, and each end of the capillary again sealed with a piece of rubber. Monoliths were prepared using either thermal or photochemical initiation. The former was carried out by immersing the capillaries in a water bath kept at 60 ^{o}C for 20 hours, while photoinitiated polymerizations in the UV transparent Teflon-coated capillaries required irradiation using a Bondwand UV lamp at 350 nm and a distance of 5 centimetres for one hour. After removing the seals, the columns were flushed with methanol at a flow rate of 1 µL/min for 60 min to remove porogen and unreacted monomer using the Dionex LC pumping system.

2.5 Photografting of porous methacrylate monoliths with 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS).

A mixture comprising 15 wt% AMPS and 0.22 wt% benzophenone in a porogen comprising 75/25 t-butanol/water was prepared and vortexed vigorously to dissolve the solid material. After centrifuging at 13,000 r.p.m for 10 minutes, the supernatant was degassed with nitrogen for 10 minutes. The de-aerated solution was pumped through the methacrylate monolith to completely fill the pores using the PEEK loop and pump as previously described. The column was then end-capped with rubber and photo-grafting was achieved by irradiating the column through a number of precisely positioned PEEK photo-masks (of approximately 10 mm in length) at a wavelength of 350 nm at a distance of 5 cm for one hour. The capillary was then washed with methanol at a flow velocity of 1 μL/min for 12 hours, followed by water for 3 hours.

2.6 Photografting of porous polymer monoliths with 4,4-dimethyl-2-vinylazlactone.

The same procedure was used as for photografting of 2-acrylamido-2-methyl-1-propanesulfonic acid, except that the monomer solution comprised 15 wt% 4,4-dimethyl-2-vinylazlactone and 0.22 wt% benzophenone in a porogen comprising 75/25 t-butanol/water. The UV lamp used was a UVP battery operated lamp at 254 nm, with the face of the lamp in constant contact with the region of capillary not covered by the photomask. The time of irradiation was 30 minutes. The capillary was then washed with methanol at a flow velocity of 1 μL/min for 2 hours, followed by water for 3 hours.

2.7 Immobilisation of proteins on azlactone functionalised monoliths.

For immobilisation of each particular protein (bovine serum albumin or GFP-His), each protein was prepared as a 1 mg/ml solution in 0.5 *M* sodium sulphate and 0.1 *M* sodium carbonate. A washed 435 µL loop was filled with the protein solution (BSA or GFP-His) using a manual syringe, and one end of the loop connected to the azlactone functionalised monolith, with the opposite end connected to the Dionex LC pumping system which was primed with 0.5 *M* sodium sulphate / 0.1 *M* sodium carbonate. The protein solution was pumped through the monolith at 1 µL/min for three hours at room temperature to allow covalent attachment of the protein to the azlactone functionalities via native lysine or cysteine groups on the protein surface. After immobilisation, the column was washed with water for up to 3 hours at 1 µl/min.

2.8 Verification of the presence of charged groups on functionalised monoliths using C^4D.

While pumping water through the functionalised monolith at 1 µL/min, the detection cell was physically scanned across the length of the column at millimetre increments to allow the detection of localised regions of charge within the monolithic stationary phase. The conductivity value (reported as mV) at each millimetre location along the column was recorded. C^4D was also used in the same manner to locate the presence of voids/regions of diffuse porosity within a length of otherwise homogeneous monolithic stationary phase.

2.9 Cross-validation of the C^4D methodology using fluorescence microscopy.

Monolithic columns containing a number of zones of immobilised GFP-His, were flushed with methanol to remove non-specifically bound protein, and placed on the plate of a Vilber Lourmat UV transluminator, which irradiated the monolithic columns from below with light of 254 nm. Photographic images of monoliths illuminated in this manner were taken with a Panasonic digital video camera as described.

3. Results and discussion.

3.1 Evaluation of monolith quality using C^4D.

The monolithic stationary phase was produced using the procedure of Geiser et al[30]. Butyl methacrylate was specifically chosen as a monomer since it exhibits appreciable hydrophobic character, and therefore should result in non-specific binding of protein in ungrafted regions of the monolith. Butyl methacrylate would also not significantly contribute to background conductivity measurements since it does not contain ionisable groups. Prior to monolith formation, capillaries were wall-modified as described in Section 2.3. Monolith formation was carried out as detailed in Section 2.4. The hydrodynamic properties of the monolith formed were adequate for the purposes of this work, with for example a 12 cm x 100 µm monolith producing a backpressure of 4 bar while pumping water at 1 µL/min. The above recipes for generic monolith production were found to be both robust and reproducible. The hydrodynamic properties were not extensively studied since the primary aim of this work was to use C^4D to detect zones of different charged functionalities or zones of differing porosity *within the same single monolith.*

Determination of monolith pore size and the location of monolith breakages or voids is an important quality control test for laboratories producing monolithic columns. The pore size dictates the hydrodynamic properties of a monolith, with large pore sizes resulting in lower backpressures at a given flow rate for a given solvent, but with concomitantly lower surface area. Typically, the pore structure of a monolith is determined using two complementary methodologies. The first involves the use of scanning electron microscopy which is a destructive technique in that the column needs to be sliced into cross-sections before microscopic analysis, with the images typically used to confirm that the monolith is well bound to the wall. In addition, SEM images are generally used to directly compare two or more monoliths produced under different conditions such as porogen type, porogen concentration, reaction time, reaction temperature etc. Secondly, for the determination of actual average pore size within a monolith or the pore size distribution, such techniques as mercury-intrusion porosimetry are often applied. **[4]**

Neither of the above common techniques allows a non-invasive evaluation of the longitudinal homogeneity and structural uniformity of the monolith within the capillary column. However, C^4D can be recorded along the entire length of a column non-invasively, with the recorded response directly proportional to sum of conducting elements within the 'virtual electrode area' [30-35] **[45].** The presence of non-conducting monolithic phase filled with a background solution, housed within a fused silica capillary of uniform character, will provide a conductive response proportional to the fluid volume. Hence, where irregularities within the monolith structure exist, these will be apparent in the C^4D capillary profile. In this study a monolithic column

was produced containing four separate zones in which the monolith was drastically diffuse relative the remainder of the monolith. This was achieved using a photo-initiated polymerisation technique whereby the fused silica capillary containing the monomer mix, was masked in four discrete regions with pieces of black electrical tape, with each masked region measuring approximately 10 to 13 millimetres. After irradiating the UV transparent capillary for one hour at a distance of 5 centimetres, a monolith was obtained containing four highly porous zones (it was expected that no monolith at all would form under the masked zones, however partial formation of monolith under each masked region was observed).

Figure 1 illustrates the C^4D response profile obtained by scanning the detector along the length of the capillary column at one millimetre intervals while pumping deionised water through at 1 μL/min. The zones where there was "less" monolith are clearly evident and result in higher C^4D responses. There are also present small zones of increased monolithic density at the interface between the photo-masked zones and unmasked regions. Although here these zones represent a minor irregularity in the monolith structure, the C^4D evaluation clearly identifies their presence. Two cross-sectional slices of the monolith were made at approximately 25 mm (sample A) along the monolith and 36 mm (sample B) along the monolith, corresponding to regions of low and high C^4D response respectively, and subjected to SEM evaluation to validate the above C^4D profile. Figure 2 clearly illustrates that sample B contains large voids, consistent with an incomplete polymerisation, whereas sample A exhibits the normal uniformly structured monolith.

3.2 Determination of the spatial location of precisely grafted charged functionalities (AMPS) using C^4D.

Until now, the visualisation of grafted zones of different functionalities on a single monolith has been challenging and time consuming. If the grafted functional groups are fluorescent, or can be reacted with a fluorescent tag in a second step after photo-grafting is complete, then fluorescence microscopy can be utilised to visualise the grafted zones. **Rohr et al [1]** used this approach recently to visualise grafted zones of vinyl azlactone by tagging the azlactone functionalities with Rhodamine 6G via its secondary amino groups. This methodology also allowed **Rohr et al** to evaluate the sharpness of the boundaries between the grafted and ungrafted zones, which ideally should be perpendicular to the capillary axis. In this manner the irradiation time and the physical geometry of the photomasks employed could be accurately optimised.

Gillespie et al [44] have previously reported the use of C^4D to detect the presence of hydrophobically adsorbed ionic surfactants on capillary C_{18} silica monoliths. The same approach was used in this work, but in this case ionisable functional monomers were photo-grafted onto a butyl methacrylate monolith using techniques previously discussed. AMPS (2-acrylamido-2-methyl-1-propanesulfonic acid) was selected as a suitable candidate for grafting since it contains a strong acid sulfonate group which is ionised across the pH range, and because the grafting of this monomer onto polymer monoliths has been well studied**. [1,2]** Figure 3 shows the C^4D profile of a butyl methacrylate monolith containing two zones of photografted AMPS. The generic "backbone" monolith yields a low conductivity response as expected. Since it is typically desirable to have a sharp graft boundary, it is advantage

of this proposed method that the detector can be scanned across the column length with millimetre precision. This results in a very sharp and rapid increase in the conductivity response as the pair of C^4D electrodes pass across the region of monolith where the grafted groups begin, and an equally rapid return to baseline once the moving detector cell has left the tail edge of the grafted zone behind. The uniform baseline conductance shown in Figure 3 surrounding the zones of AMP, illustrates the quality of the pre-formed monolith, whereas the contribution to the conductance from the presence of the charged AMP groups is equally clear. The two separate zones were grafted also to show how C^4D can be used to readily monitor the reproducibility of the photografting process, which in this case is highly reproducible. Although not shown here, it is readily possible to correlate the conductance signal recorded and the density of AMP groups upon the monolith and so also utilise this simple technique to determine 'zone capacity'.

3.3 Determination of the precise spatial location of immobilised protein and non-specifically bound protein using C^4D.

One of the few non-invasive methodologies for both detection and quantification of adsorbed protein on monolithic surfaces has been recently reported by Stachowiak et al[31]. They described a fluorescence assay that was performed "on-monolith" for semi-quantitative determination of fluorescein-labeled bovine serum albumin (BSA), which was allowed to hydrophobically adsorb onto a range of monoliths, the surfaces of which were each grafted with progressively more hydrophilic functional monomers. This allowed them to evaluate the most suitable hydrophilic graft monomers to use for the reduction of unwanted non-specific binding

of proteins to monolithic surfaces. Stachowiak et al. pointed out that a limitation of the methodology was that the fluorescence intensity was not directly proportional to the absolute amount of adsorbed protein and so the method was not strictly quantitative, but could nevertheless be used for making simple comparisons between monolithic surfaces using the same model protein.

Here, having successfully visualised the exact spatial location of grafted charged functional groups on an otherwise electrically neutral monolith using C^4D, it follows that the same methodology should permit the detection of immobilised protein on a surface. All immobilised proteins will carry a net charge at given pH due to the presence on their surface of amino acids with ionisable R-groups such as lysine, arginine, glutamic acid and aspartic acid. It follows therefore that the passage of the C^4D detector cell across a monolithic surface on which protein is immobilised at specific locations should result in an increase in signal at each protein immobilisation zone due to the native net charge of that protein. Therefore, a monolith containing two 9 mm long photografted zones of vinyl azlactone was flushed with a solution of 1 mg/ml bovine serum albumin (BSA) in 0.5 *M* sodium sulphate, 0.1 *M* sodium carbonate at a flow rate of 1 µL/min for 3 hours. A high salt carrier solution was used to facilitate covalent attachment of BSA to the azlactone modified support, based on the findings of **Drtina et al.**[32] After washing the column extensively with water, the C^4D detector was scanned across the column as described, and a profile obtained as shown in Figure 4 (Profile a). Again, the presence of two narrow well-defined zones of immobilised BSA can be clearly seen, with the width of each zone matching perfectly with the original width grafted azlactone zones (9 millimetres). A significant advantage of this technique over that developed by Stachowiak et al. is that it is not

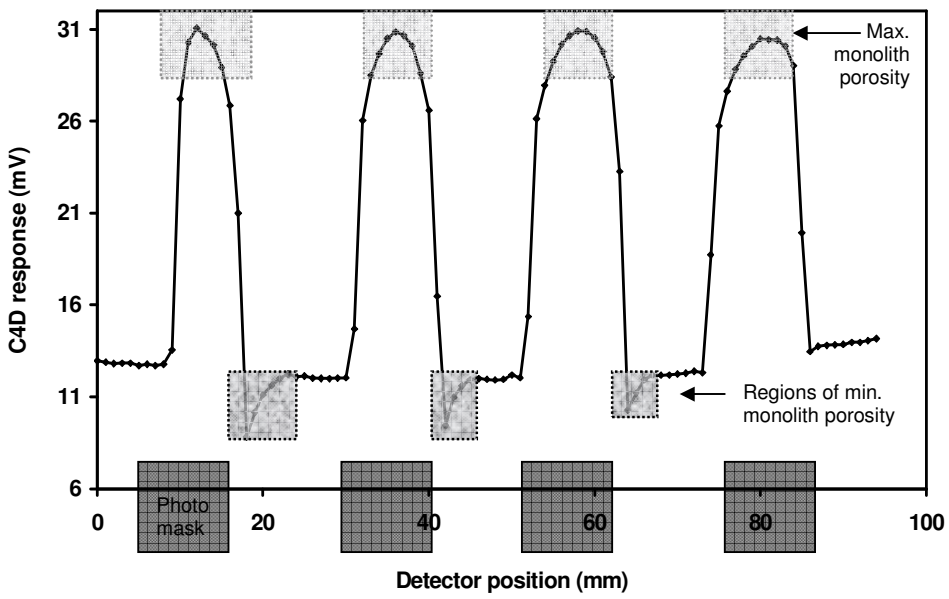
420 necessary to fluorescently tag the protein prior to immobilisation, which may
421 negatively impact secondary interactions in affinity applications.
422
423 In the evaluation of bound protein on affinity monoliths, produced in the
424 above manor, it is important to be able to readily distinguish non-specifically
425 bound/adsorbed protein. C^4D offers a simple and semi-quantitative method to evaluate
426 the spatial location of non-specifically bound protein on such a monolith. As
427 previously discussed, butyl methacrylate was used as a monomer to produce the
428 backbone monolith, since it has appreciable hydrophobic character that would
429 promote non-specific hydrophobic binding of protein across the entire length of the
430 column. Bovine serum albumin is well known as an adhesive protein that binds to a
431 wide variety of surfaces. Figure 4 illustrates that non-specific binding of protein has
432 taken place. The profile with the lowest conductivity (Profile d) was obtained after the
433 azlactone groups had been photo-grafted to the butyl methacrylate support followed
434 by copious washing with water, but *before* BSA had been immobilised. After
435 immobilisation of BSA, Profile (a) was obtained. Profile (a) demonstrates that as well
436 as the presence of two distinct zones of covalently immobilised BSA via azlactone
437 groups, the overall conductivity of the stationary phase across the entire length of the
438 column has shifted higher (300 to 400 mV). This is clearly due to non-specific
439 binding of BSA to the non-grafted regions of the butyl methacrylate monolith through
440 hydrophobic interactions. Profile (b) shows that the amount of non-specifically bound
441 BSA had been reduced after further copious washing with water. Profile (c) was
442 obtained after washing the column with methanol and shows that the conductivity of
443 the stationary phase had been reduced down to the original value prior to
444 immobilisation of BSA (Profile d). This clearly indicates that the high conductivity

445 value across the length of the column was due to hydrophobic interactions, which
446 were disrupted by flushing the column with an organic solvent. It is also indicative of
447 the fact that the immobilised zones of BSA were covalently bound to the grafted
448 azlactone functionalties, with this covalent bond undisturbed by the presence of
449 organic modifiers. It is proposed that the absolute conductivity of both immobilised
450 zones of BSA was reduced due to excessive washing with water and finally methanol,
451 because some of the BSA present in those zones was non-specifically bound, causing
452 a net increase in conductivity in that particular zone when combined with the
453 covalently immobilised BSA via the azlactone moiety.
454
455 **3.3 Cross-validation of the C^4D methodology using fluorescence microscopy.**
456
457 The use of C^4D detection for the profiling of monolithic columns containing
458 discrete zones of immobilised protein was cross-validated with an orthogonal
459 methodology. Green fluorescent protein (GFP) was immobilised on a monolith which
460 had three separate zones of grafted azlactone groups, and then extensively washed
461 with methanol to remove any non-specifically bound protein due to hydrophobic
462 interactions. While pumping water at 1 μL/min, the C^4D response profile was
463 collected and is shown as Figure 5(b). After the C^4D profile was acquired, the
464 monolithic column (with attached scale bar) was placed on the bed of a UV
465 translumninator set to a wavelength of 254 nm and a digital image was captured,
466 which is shown aligned with the C^4D response as Figure 5(a). The position of three
467 sharp zones of immobilised GFP are clearly visible, represented as bright fields within
468 the monolith. The sharp boundary between the regions of the monolith containing
469 grafted zones of azlactone and therefore immobilised GFP, and the ungrafted

monolith regions is clear from both Figure 5(a) and 5(b). However, the C^4D profile provides additional information about the protein zones, including the longitudinal homogeneity of the protein band itself, which here clearly shows the highest protein concentration towards the centre of the zone, and secondly the means for quantitative evaluation of each bound protein zone, in terms of peak area and height measurements. Here, the C^4D profiles shown allow a simple and rapid determination of an approximate 5-10% lower amount of bound protein in zone three in comparison with zones 1 and 2.

4 Concluding remarks.

A novel use of a simple, inexpensive commercially available technology (C^4D) has been presented as a means of rapidly profiling the presence of charged groups covalently bound to monolithic stationary phases housed within capillary columns. The use of this technique has been demonstrated to be particularly useful as a means of detecting the precise location and homogeneity of photografted immobilised protein, together with the precise location and extent of non-specific binding of unwanted protein. Application of C^4D in this way should therefore facilitate future evaluation of affinity monolith fabrication techniques by allowing a potentially quantitative scanning profile of a monolith to be established in a matter of minutes. The universal nature of this technique is particularly interesting, since the visualisation of grafted zones of protein is not restricted merely to proteins that fluoresce.



493

494

495 **Figure 1.** Porosity profile of a monolith containing four zones of incomplete polymerisation.
496 Profile acquired while pumping water at 1 µl/min.
497

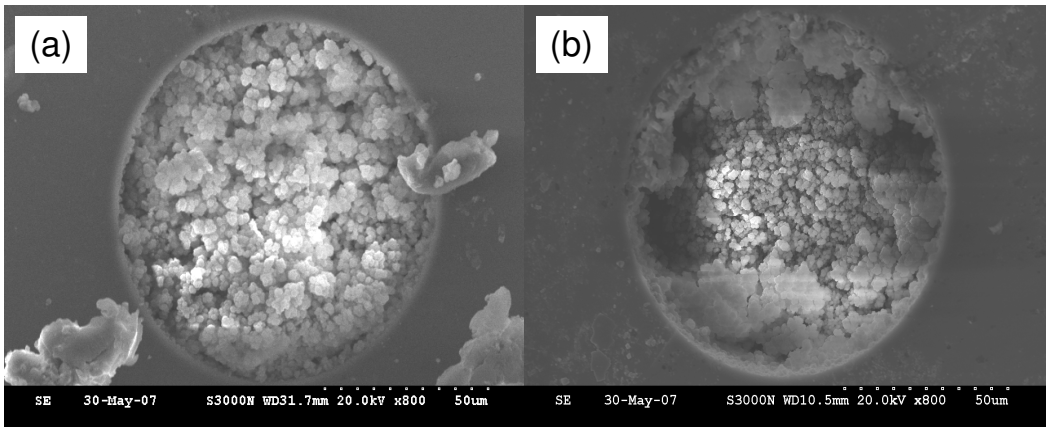
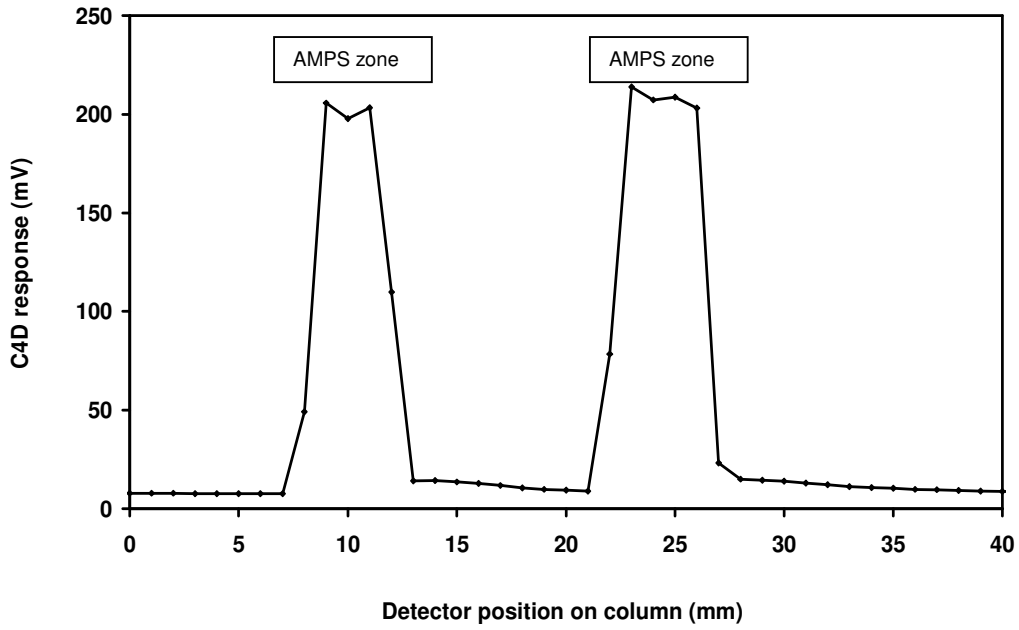


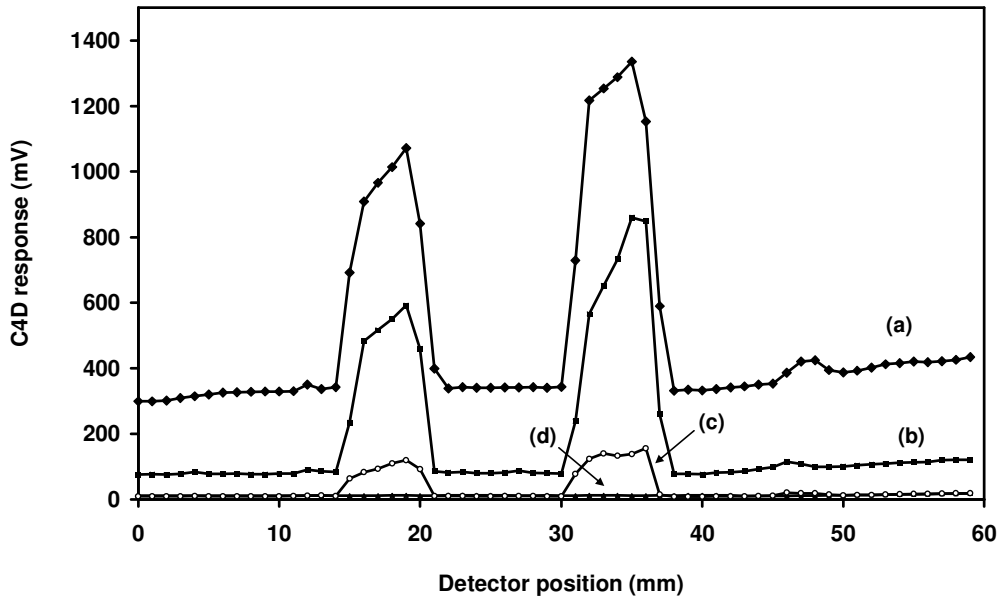
Figure 2. Scanning electron microscopy images of monolith cross-sections taken (a) 25 mm and (b) 36 mm along the length of the capillary column.



503

504

505 **Figure 3.** Conductivity profile of a monolith containing two sharp zones of grafted AMPS.
506 Profile acquired while pumping water at 1 µl/min.
507



508

509

510 **Figure 4.** Conductivity profile of a monolith containing two zones of immobilised bovine serum
511 albumin (BSA) via a dipeptide linkage via photgrafted vinyl azlactone. Profile (a)
512 was obtained after washing the BSA immobilised monolith with water for one hour.
513 Profile (b) was obtained after washing the monolith for a further 24 hours with water.
514 Profile (c) was obtained after then washing the monolith for one hour with 100 %
515 methanol. Profile (d) was obtained before immobilisation of BSA.
516

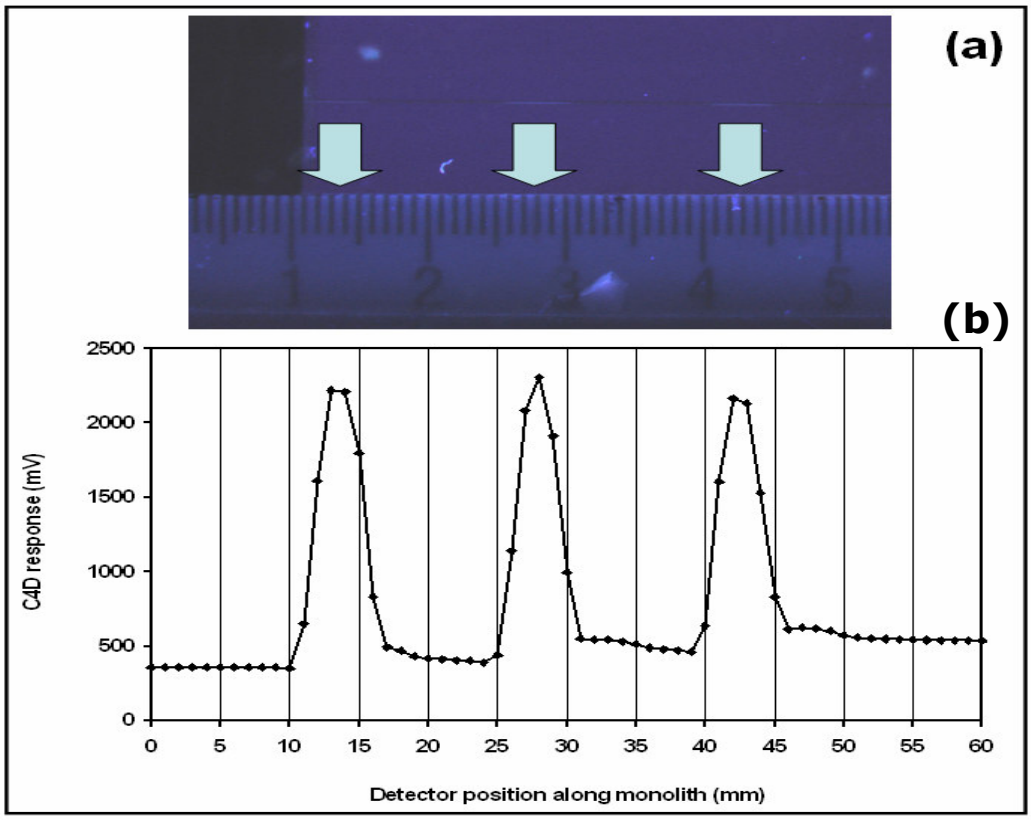


Figure 5. Comparison of fluorescence microscopy image with C^4D conductivity profile for a monolith with three zones of immobilised GFP. Figure 5 illustrates (a) the fluorescent zones of GFP, overlaid and aligned with (b) the zones of increased C^4D response. The dark region in 5(a) left is a section of the C^4D cell, the centre of which is positioned at the zero millimetre location on the capillary.

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To be updated

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