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Title: Incorporation of TGF-beta 3 within collagen-hyaluronic acid scaffolds improves their chondrogenic potential

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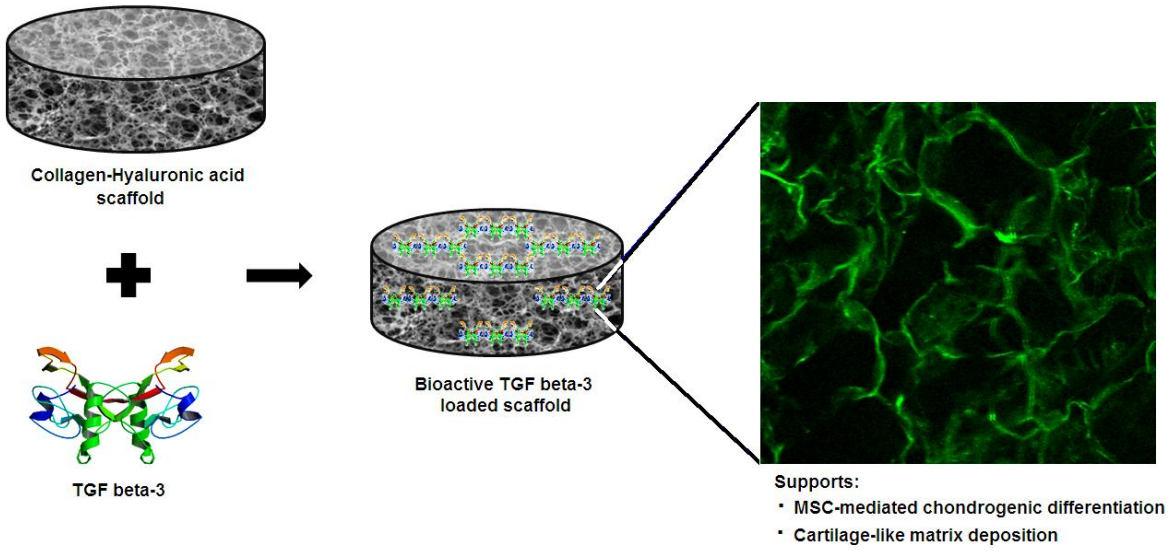
The potency of the combined biomaterial and drug-mediated treatment has been well recognized through the use of scaffolds as carrier systems for a range of therapeutic proteins, thereby offering the promise to expedite *in vivo* regeneration particularly for large defects. Accordingly, this study focused on the use of growth factors as therapeutics to enhance the biofunctionality of a scaffold with a proven capacity for tissue repair. Transforming growth factor-beta 3 (TGF-β3), known to mediate mesenchymal stem cell (MSC) chondrogenic differentiation, was successfully incorporated within a collagen-hyaluronic acid (CHyA) scaffold, developed within our laboratory for articular cartilage repair, using two distinct methods. The first method involved direct incorporation into a colloidal suspension of CHyA prior to fabrication and the second method involved soak-loading of the protein onto the scaffold post-fabrication. It was evident that TGF-β3 was released significantly quicker (approximately 6-fold quicker) when soak-loaded compared to direct incorporation which still contained approximately 37% of the protein after 14 days. However, it was clear that both groups supported enhanced *in vitro* MSC chondrogenic gene expression and subsequent cartilage-like matrix production compared to unloaded scaffolds. Such scaffold-delivery systems can easily be modified for the incorporation of multiple proteins, demonstrating their potential for use in a range of tissue repair applications requiring spatio-temporal release of therapeutics.

Articular cartilage is responsible for providing uncompromised joint movement throughout an individual's lifetime but due to its avascular nature, the regenerative capacity is significantly diminished following injury and thus, presents a grand challenge in clinical repair [1]. The use of therapeutic biomolecules has recently emerged as an approach to achieve enhanced cartilage repair. The focus of research over the last decade has been in developing methods of

incorporating these therapeutic biomolecules within biomaterials. These biomolecules include growth factors such as fibroblast growth factor-2 (FGF-2), insulin-like growth factor-1 (IGF-1), bone morphogenetic protein-6 (BMP-6) and transforming growth factor-beta (TGF-β) isoforms [2-5]. TGF-β is a chondro-inductive growth factor that has been shown to play a key modulatory role in mesenchymal stem cell (MSC) differentiation towards a chondrocytic lineage [6]. Indeed, encapsulation of TGF-β has been the primary focus in the development of bioactive biomaterials for cartilage defect repair applications [7]. However, the majority of such studies have encapsulated TGF-β within hydrogels with poor porosity or within microparticles which may impede the pore interconnectivity and porosity of the scaffolds [8-12]. In addition, most of the microparticles utilised hitherto have been based on synthetic materials such as poly (DL-lactide-co-glycolide) (PLGA) and poly (ethylene glycol) (PEG) which may have long term deleterious effects *in vivo* due to their toxic degradation products[8, 13]. Moreover, the encapsulation efficiency of the growth factors within such microparticles is generally very poor, leading to significant loss of the proteins during fabrication processes.

In our laboratory, we utilise collagen-glycosaminoglycan (CG) scaffolds for cartilage tissue engineering applications. Composites of collagen and glycosaminoglycans have been widely utilised for the development of biomimetic scaffolds [14, 15]. In particular, within our laboratory, we recently demonstrated that incorporation of hyaluronic acid (HyA) within collagen scaffolds had the effect of enhancing MSC chondrogenic differentiation and subsequent cartilage-like matrix deposition [16]. Such scaffolds have significant potential for the repair of chondral defects. However, for larger defects, an additional stimulus may be required to accelerate the repair

process *in vivo*. In that regard, the focus of this study was centred on the use of collagen-hyaluronic acid scaffolds as carriers for therapeutic proteins (**Scheme 1**).



Scheme 1: A schematic demonstrating the incorporation of TGF-β3 within a collagen-hyaluronic acid scaffold to produce a bioactive scaffold which supports mesenchymal stem cell chondrogenic differentiation and cartilage-like matrix deposition.

The ability to incorporate therapeutic proteins without the need for utilising microparticles may offer an advantage with regards to maintaining high scaffold porosity. The overall aim of this study was to develop a bioactive collagen-hyaluronic acid (CHyA) scaffold for the delivery of therapeutic biomolecules such as TGF-β3 in order to enhance the regenerative capacity of the scaffold. To achieve this, a model protein; bovine serum albumin (BSA) was initially used to establish optimal loading methods and to assess the distribution within the scaffolds. Having developed an optimised scaffold-based delivery system, TGF-β3 was loaded onto CHyA scaffolds and the release kinetics were analysed. Subsequently, the effect of the incorporated therapeutic protein on stem cell-mediated chondrogenic differentiation was elucidated.

In order to determine the distribution of the therapeutic protein when loaded by direct incorporation and soak-loading, fluorescently labelled BSA was used and it was evident that it was homogeneously distributed throughout the scaffolds when loaded with both methods (**Figure 1 a, b, d, e**). Based on the release kinetics, it was evident that soak-loading permitted greater release of the protein from the scaffolds in comparison to direct incorporation. Soak-loading permitted approximately 95% BSA release whilst direct incorporation permitted approximately 40% BSA release after 24 hours. By 48 hours, 100% of BSA was released from the scaffolds loaded with the soak-loading method whilst approximately 55% BSA was released from the scaffolds loaded with the direct incorporation method (**Figure 1 f**).

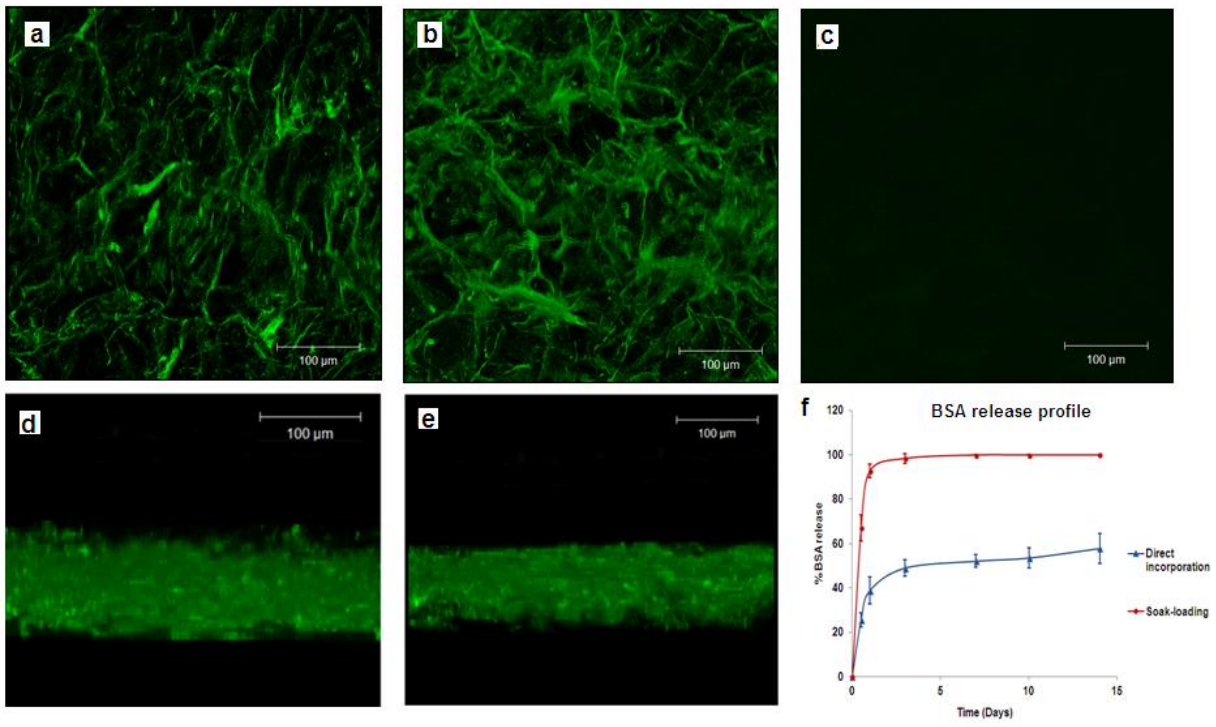


Figure 1: The figure shows presence of BSA incorporated within CHyA scaffolds. Bovine serum albumin-fluorescein isothiocyanate (green) was evident on the surface of scaffolds loaded with

direct incorporation **(a)** and soak-loading **(b)** methods. Blank (un-loaded) scaffolds did not show any evidence of BSA **(c)**. There was also evidence of BSA on the cross-sections of the scaffolds loaded with direct incorporation **(d)** and soak-loading **(e)** methods. The release profile of BSA from the scaffolds was quantified and demonstrates that 100% of the protein was released within 48 hours of the soak-loaded scaffolds whilst the direct incorporation led to approximately 55% release within the same time period **(f)**.

Similar to BSA, the method of TGF-β3 incorporation also affected the release kinetics as expected. Soak-loading permitted approximately 100% TGF-β3 release whereas direct incorporation permitted approximately 63% TGF-β3 release from CHyA scaffolds within the 14 day time frame **(Figure 2 a)**. On the contrary, direct incorporation permitted an initial release to approximately 15% after 24 hours followed by a lag phase which may be attributed to the breakdown of bonds which subsequently enabled further release of TGF-β3 from the scaffold as the ionic bonds and hydrogen bonds between the growth factor and scaffold were broken down [17, 18]. The direct incorporation and soak-loading methods investigated here thus provide two distinct release profiles of the therapeutic biomolecule thereby offering two distinct systems for different applications depending on the release profile desired.

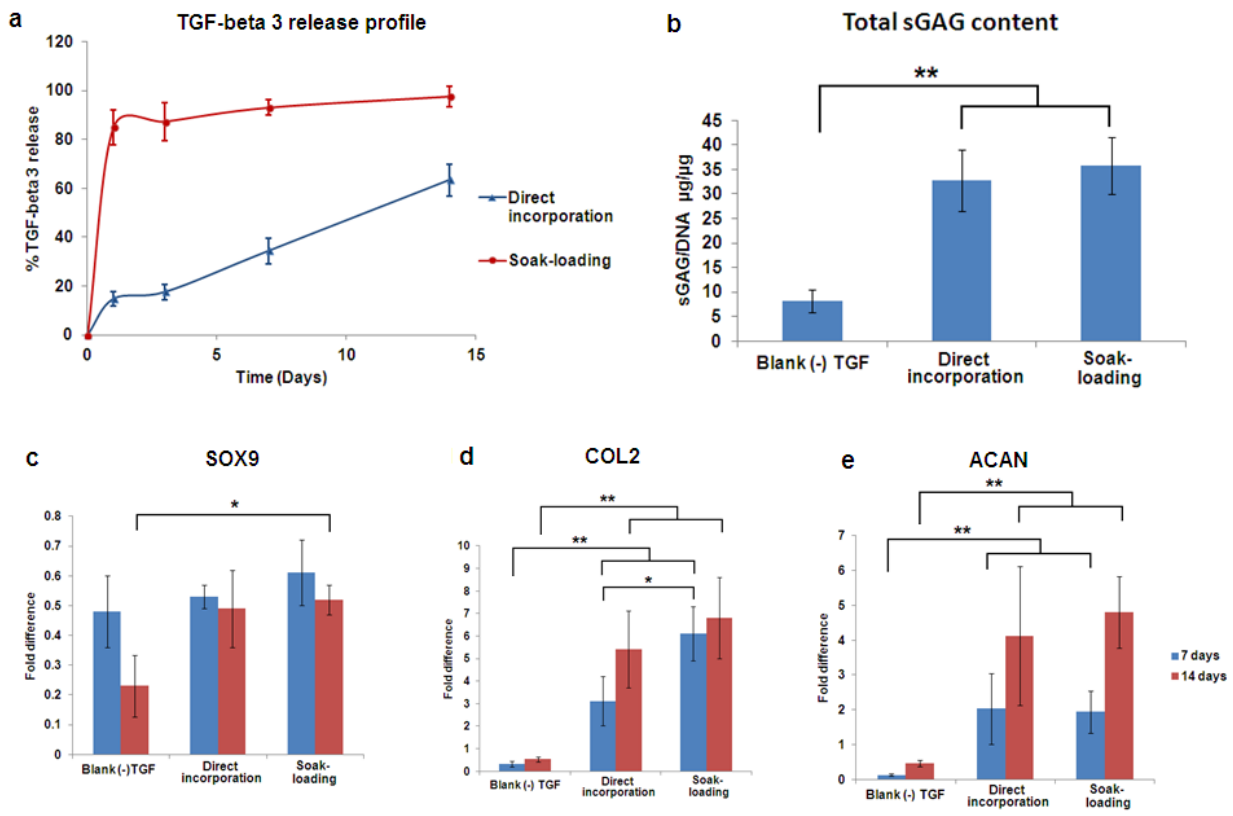


Figure 2: The cumulative release profile of TGF-β3 from CHyA scaffolds was quantified and it was evident that 100% of the protein was released when incorporated with the soak-loading method and approximately 63% with the direct incorporation by 14 days **(a)**. Synthesized sulphated GAG content was assessed after 14 days culture with TGF-β3-loaded scaffolds supporting greater synthesis than blank scaffolds **(b)**. Gene expression was assessed for SOX9 **(c)**, COL2 **(d)** and ACAN **(e)** with TGF-β3-loaded scaffolds supporting greater expression by 14 days. * denotes $p<0.05$ statistical significant difference, ** denotes $p<0.01$ statistical significance difference.

Having developed bioactive scaffolds containing TGF-β3 using two distinct methods, the effect of TGF-β3 released from the scaffolds on MSC-mediated chondrogenic differentiation was assessed and compared to unloaded (blank) scaffolds. At day 14, soak-loaded scaffolds

stimulated significantly higher SOX9 gene expression (approximately 100% higher) than the unloaded (blank) scaffolds **(Figure 2 c)**. COL2 gene expression was significantly higher within the TGF-β3 loaded groups in comparison to the blank group **(Figure 2 d)**. The direct incorporation and soak-loaded groups stimulated approximately 10-fold ($p<0.01$) and 20-fold ($p<0.01$) higher COL2 gene expression in comparison to the blank scaffolds respectively. Consistent with COL2 gene expression, TGF-β3 loaded scaffolds stimulated significantly higher ACAN gene expression (approximately 10-fold higher, $p<0.01$) in comparison to the blank scaffolds after 14 days (**Figure 2 e**). This shows that the TGF-β3 remained bioactive following release, which is crucial in demonstrating that the lyophilisation process had no deleterious effect on the protein.

Consistent with gene expression data, it was evident that the TGF-β3 loaded scaffolds stimulated significantly higher sGAG production than the blank scaffolds. There was approximately 3-fold higher ($p<0.01$) sGAG/DNA in the TGF-β3 loaded scaffolds than the blank scaffolds. Since it is still not clear in the literature as to the most optimal duration that TGF-β3 is required *in vitro* and subsequently *in vivo* to stimulate MSC chondrogenesis and cartilage repair [5, 13, 19-21], both soak-loading and direct-incorporation were chosen for the *in vitro* analysis due to the differing release kinetics. From the results, it was evident that by day 7 there was a significantly higher COL2 gene expression in the soak-loaded scaffold group in comparison to the direct incorporation scaffold group which may be attributed to the higher initial release of TGF-β3. However, there was no difference between the two methods with regards to the synthesis of sulphated GAGs after 14 days. Taken together these results demonstrate the effectiveness of the techniques used

to produce functionalised scaffolds and showed that TGF-β3 released from the scaffolds remained bioactive and elicited a therapeutic response.

Depending on the application of the delivery system, either of the loading methods (direct incorporation and soak-loading) presented in this study could be utilised. Although there is currently significant interest in the development of scaffold delivery systems that can achieve sustained release of therapeutics over time [22, 23], this study demonstrates that an initial burst release can provide the required cues to support chondrogenic differentiation of MSCs with resultant equivalent production of cartilage-like matrix to the sustained TGF-β3-releasing scaffold. A recent study demonstrated that a brief exposure to a high dose of TGF-β3 was sufficient to maintain MSC chondrogenesis over a 9 week period [24]. Collectively, the methods utilised in the present study demonstrate two distinct release kinetics (burst release and sustained release) could be achieved from scaffolds composed of natural ECM components.

The ECM has been widely known to modulate the spatial presentation of growth factors thereby regulating consequential cellular response [25]. The presence of hyaluronic acid in the current study may play a defining role in the interaction of the growth factor. A study demonstrated that the hyaluronic acid plays a role in sequestering and presenting TGF-β1 and thereby affecting cellular proliferation. They also suggest that hyaluronic acid may offer protection from tryptic degradation and may subsequently modulate the activity of the protein [26]. Other GAGs such as heparin sulphate have been widely used for the delivery of growth factors including bFGF, VEGF and BMPs [27-29]. One major advantage of the scaffold system utilised in this study is that

their composition can easily be adapted to include other GAGs or growth factors depending on the application.

In conclusion, the study presented in this article demonstrates that incorporating a growth factor within a collagen-glycosaminoglycan scaffold can support a desired cellular response with no detriment to the protein. In particular, we have shown that two distinct methods can be utilised to incorporate TGF-β3 within CHyA scaffolds thereby enabling two distinct release profiles. It was evident that such TGF-β3-loaded scaffold could support MSC chondrogenic differentiation with resultant cartilage-like matrix deposition. By incorporating the therapeutic protein within the highly porous scaffold, there is greater control over desired cellular response. In addition, the potential to sustain the release of the therapeutic protein over a particular period of time offers a significant advantage clinically especially for the repair of large defects. Ultimately, the ability to elicit such desired responses without the use of microparticles presents noteworthy cost saving in biomaterial development. Whilst this study focuses on articular cartilage, the ability of the methods presented in this article to be easily adapted for different therapeutic proteins targeted for a range of other prospective clinical applications represents a considerable promise.

Experimental section

Scaffold fabrication: Collagen-hyaluronic acid (CHyA) scaffolds were fabricated by lyophilisation as described previously [16]. Briefly, a suspension was made of 0.5% (w/v) collagen type I from bovine Achilles tendon (Collagen Matrix, USA), 0.05% (w/v) hyaluronic acid derived from streptococcus equi (Sigma-Aldrich, Ireland) dissolved in 0.5M acetic acid and

blended at 15,000 rpm (IKA Works Inc, USA) at 4°C. The suspension was subsequently freeze-dried (Virtis Genesis 25EL, Biopharma, Winchester, UK) at a constant cooling rate of 1°C/min to a final temperature of -20°C with an additional annealing step (-10°C held for 24 hours) before sublimating the ice phase at 0°C under a vacuum (200 mTorr).

Incorporation of BSA and TGF-β3 within CHyA scaffolds: To assess the optimal method for incorporation of a therapeutic protein within the CHyA scaffolds, bovine serum albumin (BSA) was initially used as a model protein. The first method, direct incorporation of the protein prior to freeze-drying, involved addition of 0.1 g/ml BSA directly into the CHyA slurry to give a final concentration of 2 mg/ml. BSA was subsequently blended into the slurry at 15,000 rpm at 4°C before being freeze-dried. The second method of incorporation was based on soak-loading of freeze-dried CHyA scaffolds with a solution containing BSA. In this method, BSA was added evenly over the surface of a freeze-dried CHyA scaffold sheet to give an equivalent concentration of 2 mg per cm^3 of scaffold. To visualise the protein, fluorescently labelled BSA (2 mg/ml), bovine serum albumin-fluorescein isothiocyanate - BSA-FITC (Sigma-Aldrich, Ireland), was incorporated within CHyA as above. Similar loading methods (direct incorporation and soak-loading) were subsequently used to incorporate TGF-β3 within CHyA scaffolds. In both methods, 160 ng per 9.5 mm diameter scaffold was incorporated. The TGF-β3-loaded scaffolds were subsequently sterilised using ultraviolet-C radiation (UVC) at 254 nm for 4 hours.

BSA release study and detection using high performance liquid chromatography (HPLC): BSA-loaded scaffolds were incubated in 2 ml PBS in a 24-well plate and placed on a micro-plate shaker (PMS-1000, Grant-Bio, Cambridge, UK) within an incubator set at 37°C. After 12 hours,

1, 3, 7 and 14 days, the PBS was collected from each well for HPLC analysis and replaced with fresh PBS. The collected PBS from each sample was filtered through 0.45 μm filters (Millipore, UK) into HPLC glass vials (Millipore, UK). The HPLC system (Agilent 1120LC, Agilent Technologies, USA) was equipped with a Gemini-NX C18 column (Phenomenex, UK). The column was conditioned with an aqueous acetonitrile (ACN, Fisher Scientific, UK) mixture (50:50) at a flow rate of 1 ml/min followed by the mobile phase which consisted of 0.1% tri-fluoroacetic acid (TFA, Sigma-Aldrich, Ireland) in water: 0.08 % TFA in ACN (at a gradient of 80:20). 50 mL of the sample or standard was injected into the column which was set to 40°C and the detection was carried out at a wavelength of 220 nm. Based on a calibration curve, the concentration was determined.

TGF-β3 release study and detection using enzyme linked immunosorbent assay (ELISA): TGF-β3-loaded CHyA scaffolds were incubated in 2 ml of Dulbecco's modified eagles medium (DMEM, Sigma-Aldrich, Ireland) in a 24-well plate and placed on a micro-plate shaker (PMS-1000, Grant-Bio, Cambridge, UK) within an incubator set at 37°C. After 12 hours, 1, 3, 7 and 14 days, DMEM was collected for ELISA assessment. The ELISA assay was carried out according to the manufacturer's instructions (Human TGF-β3 ELISA, Komabiotech, S. Korea). Using a microtiter plate reader (Varioskan Flash Multimode reader, ThermoScientific, Ireland) the well plate was then read at an absorbance of 450 nm with correction at 570 nm. The readings were converted to a concentration using a standard curve generated.

Cell isolation and culture: Primary mesenchymal stem cells (MSCs) were isolated from rat femorae and tibiae following RCSI Ethics Committee approval (REC237). For the culture of

cells onto the TGF-β3-loaded and unloaded scaffolds, cells were seeded at a density of $0.5x10^6$ cells per scaffold and 2 mL of growth media added to each well. After 24 hours, the growth medium was replaced with chondrogenic medium (50 µg/mL ascorbic acid, 40 µg/mL L-proline, 100 nM dexamethasone, 1X ITS (Insulin, Transferrin, Selinium), and 0.11 mg/mL sodium pyruvate) in the absence of TGF-β3.

Gene expression analysis: To determine gene expression of the cultured cells within the scaffolds, real time polymerase chain reaction (RT-PCR) was carried out as described previously [16]. The relative expression of mRNA was calculated by the delta-delta Ct (ΔΔCt) method for the target mRNA (SOX9, COL2 and ACAN). These genes are specific to cells which have differentiated towards a chondrocytic lineage.

Sulphated GAG quantification: Cell-seeded scaffolds were assessed for sulphated GAG production using a solution of di-methyl-methylene blue dye binding assay (Blyscan, Biocolor, UK) according to the manufacturer's instructions.

Statistical analysis: Statistical differences were assessed by two-way ANOVA with Tukey's post hoc analysis. All results were reported as mean + standard deviation. A probability value of 95% ($p<0.05$) was used to determine significance.

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