Engineering cartilage or endochondral bone: A comparison of different naturally derived hydrogels

Eamon J. Sheehy[1,2], Tariq Mesallati[1,2], Tatiana Vinardell[3], Daniel J. Kelly[1,2,4]*

[1]Trinity Centre for Bioengineering, Trinity Biomedical Sciences Institute, Trinity College Dublin, Dublin, Ireland.

[2]Department of Mechanical and Manufacturing Engineering, School of Engineering, Trinity College Dublin, Dublin, Ireland.

[3]School of Agriculture and Food Science, University College Dublin, Belfield, Dublin 4, Ireland.

[4]Advanced Materials and Bioengineering Research Centre (AMBER), Royal College of Surgeons in Ireland and Trinity College Dublin, Dublin, Ireland

***Corresponding author**

E-mail address: kellyd9@tcd.ie

Address: Department of Mechanical and Manufacturing Engineering

School of Engineering

Trinity College Dublin

Dublin 2

Ireland

Telephone: +353-1-896-3947

Fax: +353-1-679-5554

Abstract

Cartilaginous tissues engineered using mesenchymal stem cells (MSCs) have been shown to generate bone *in vivo* by executing an endochondral program. This may hinder the use of MSCs for articular cartilage regeneration, but opens the possibility of using engineered cartilaginous tissues for large bone defect repair. Hydrogels may be an attractive tool in the scaling-up of such tissue engineered grafts for endochondral bone regeneration. In this study, we compared the capacity of different naturally derived hydrogels (alginate, chitosan, and fibrin) to support chondrogenesis and hypertrophy of MSCs *in vitro* and endochondral ossification *in vivo*. *In vitro*, alginate and chitosan constructs accumulated the highest levels of sGAG, with chitosan constructs synthesising the highest levels of collagen. Alginate and fibrin constructs supported the greatest degree of calcium accumulation, though only fibrin constructs calcified homogenously. *In vivo*, chitosan constructs facilitated neither vascularization nor endochondral ossification, and also retained the greatest amount of sGAG, suggesting it to be a more suitable material for the engineering of articular cartilage. Both alginate and fibrin constructs facilitated vascularization and endochondral bone formation as well as the development of a bone marrow environment. Alginate constructs accumulated significantly more mineral and supported greater bone formation in central regions of the engineered tissue. In conclusion, this study demonstrates the capacity of chitosan hydrogels to promote and better maintain a chondrogenic phenotype in MSCs and highlights the potential of utilizing alginate hydrogels for MSC-based endochondral bone tissue engineering applications.

Keywords: mesenchymal stem cell; hydrogel; alginate; chitosan; fibrin; endochondral ossification.

1. Introduction

Tissue engineering involves using a combination of cells, three-dimensional scaffolds and signaling molecules to repair or regenerate damaged or diseased tissues [1, 2]. The use of a supporting scaffold or hydrogel facilitates the scaling-up of tissue engineered grafts to clinically relevant sizes. Furthermore, understanding how cell-scaffold interactions regulate the terminal phenotype of the cell is critical in the development novel tissue regeneration strategies. To date, cell-based bone tissue engineering applications have generally focused on the direct osteogenic priming of mesenchymal stem cell (MSC) seeded scaffolds in a process resembling intramembranous ossification [3]. This approach however, has been hampered by insufficient vascularization of the graft following *in vivo* implantation, thus preventing the necessary delivery of oxygen and nutrients required to ensure cell survival [4]. For example, *in vitro* osteogenic priming of engineered constructs has been shown to occlude the pores of a scaffold with calcified matrix, resulting in the development of a necrotic core upon implantation into bony defects [5]. Core necrosis is a well-documented challenge in the field of tissue engineering, and will be exacerbated by the scaling-up of such constructs to treat critically sized bone defects.

In an attempt to address these challenges, there has been a recent shift away from classical tissue engineering paradigms, towards strategies aimed at recapitulating the natural mechanisms which drive tissue development during skeletogenesis [6]. The long bones of the body form by a process termed endochondral ossification, whereby chondrocytes in a developing cartilaginous rudiment undergo hypertrophy and direct vascularization and remodeling of the cartilaginous template into bone [7]. An endochondral approach to bone tissue engineering may circumvent many of the issues associated with the intramembranous method, as cells progressing down the endochondral route are equipped to survive hypoxic conditions and release pro-angiogenic factors for the conversion of avascular tissue to

vascularized tissue [8]. Indeed, it has been demonstrated that MSC-based cartilaginous grafts can be used to generate bone *in vivo* in both ectopic and orthotopic sites by executing such an endochondral program [8-15].

Hydrogels are water swollen cross linked polymers capable of forming large, abstract shaped constructs [16]. They can be derived from natural materials which are either components of, or have macro-molecular properties similar to, native extra-cellular matrix [17], and a number of naturally derived hydrogels have been shown to support chondrogenesis of MSCs *in vitro* [18-22]. Previous studies have compared the chondrogenic capabilities of MSC-seeded hydrogels *in vitro* [23-26], and also the potential of chondrogenically primed MSC-seeded hydrogels to maintain a stable chondrogenic phenotype *in vivo* [27-29]. However, little is known about the capacity of different MSC-seeded hydrogels to support the development of either phenotypically stable cartilage or endochondral bone *in vitro* and *in vivo*.

Therefore, the objective of this study was compare the capacity of different naturally derived hydrogels (alginate, chitosan and fibrin) to support chondrogenesis and hypertrophy of MSCs *in vitro,* and subsequent endochondral ossification *in vivo.* In the first phase of the study, MSCs were encapsulated in alginate, chitosan, and fibrin hydrogels and cultured in chondrogenic conditions (5 weeks) in order to promote chondrogenesis and cartilaginous matrix production. Thereafter, constructs were switched to hypertrophic conditions (3 weeks) in order to promote a hypertrophic phenotype and the development of calcified cartilaginous tissue. In the second phase of the study MSCs were encapsulated in alginate, chitosan, and fibrin hydrogels and subjected to a shorter *in vitro* culture period (6 weeks) prior to subcutaneous implantation into nude mice for an additional 6 weeks.

2. Materials and methods

2.1. Isolation and expansion of MSCs

Bone marrow derived MSCs were isolated from the femoral shaft of 4 month old pigs and expanded according to a modified method for human MSCs [30] in high glucose Dulbecco's modified eagle's medium GlutaMAX (hgDMEM) supplemented with 10% v/v foetal bovine serum (FBS), 100 U/mL penicillin – 100 µg/mL streptomycin (all Gibco, Biosciences, Dublin Ireland) and 2.5 µg/mL amphotericin B (Sigma-Aldrich, Dublin, Ireland) at 20% pO_2. Following colony formation, MSCs were trypsinised, counted, seeded at density of 5 x 10^3 cells/cm^2 in 500 cm^2 triple flasks (Thermo Fisher Scientific), supplemented with hgDMEM, 10% v/v FBS, 100 U/mL penicillin – 100 µg/mL streptomycin, 2.5 µg/mL amphotericin B, and 5 ng/mL human fibroblastic growth factor-2 (FGF-2; Prospec-Tany TechnoGene Ltd., Israel) and expanded to passage 2.

2.2. Encapsulation of MSCs within alginate, chitosan and fibrin hydrogels

A 4% agarose-based mould was used to cast cylindrical (Ø5 x 3 mm) alginate, chitosan and fibrin hydrogels at a cell density of 20 x 10^6 MSCs/mL. Alginate constructs were fabricated by injecting passage 2 MSC-laden 2% w/v alginate (Pronova, FMC Biopolymer, Norway) into a 4% agarose/50 mM $CaCl_2$ mould, and allowing gelation to occur for 30 min. Chitosan constructs were fabricated by combining 1.5 mL of 3% w/v chitosan (Pronova) with 350 µL of β-glycerophosphate (β-GP; 600 mg/mL; Sigma-Aldrich, Dublin, Ireland), 360 µL of hydroxyethylcellulose (HEC; 25 mg/mL; Sigma-Aldrich) and 790 µL of MSC suspension and allowing gelation to occur at 37 °C for 30 min to yield a final concentration of 1.5% w/v chitosan, 7% w/v β-GP and 0.3% w/v HEC. Fibrin constructs were fabricated by dissolving 100 mg/mL bovine fibrinogen (Sigma-Aldrich) in 10,000 KIU/mL aprotinin (Norma Pharma, UK) containing 19 mg/mL sodium chloride (NaCl). This solution was laden with MSCs and

combined 1:1 with a 5 U/mL thrombin in 40 mM $CaCl_2$ solution and allowed to gel at 37° C for 30 min yielding a final concentration of 50 mg/mL fibrinogen, 2.5 U/mL thrombin, 5,000 KIU/mL aprotinin, 17 mg/mL NaCl and 20 mM $CaCl_2$.

2.3. In vitro culture conditions

The chondrogenic culture conditions applied in this study are defined as culture in a chondrogenic medium (CM) consisting of hgDMEM GlutaMAX supplemented with 100 U/mL penicillin/streptomycin (both Gibco), 100 µg/mL sodium pyruvate, 40 µg/mL L-proline, 50 µg/mL L-ascorbic acid-2-phosphate, 4.7 µg/mL linoleic acid, 1.5 mg/mL bovine serum albumine, 1×insulin–transferrin–selenium, 100 nM dexamethasone (all from Sigma-Aldrich), 2.5 µg/mL amphotericin B, and 10 ng/mL of human transforming growth factor-β3 (TGF-β3) (Prospec-Tany TechnoGene Ltd., Israel) at 5% pO_2. The hypertrophic culture conditions applied are defined as culture in a hypertrophic medium consisting of high glucose DMEM GlutaMAX supplemented with 100 U/mL penicillin/streptomycin, 100 µg/mL sodium pyruvate, 40 µg/mL L-proline, 50 µg/mL L-ascorbic acid-2-phosphate, 4.7 µg/mL linoleic acid, 1.5 mg/mL bovine serum albumine, 1×insulin–transferrin–selenium, 1 nM dexamethasone, 2.5 µg/mL amphotericin B, 1 nM L-thyroxine (Sigma-Aldrich) and 20 µg/mL β-GP at 20% pO_2.

2.4. Experimental design

The first phase of this study examined the *in vitro* development of MSC-seeded alginate, chitosan and fibrin cylindrical hydrogels. Constructs were maintained in chondrogenic culture conditions for a period of 5 weeks, and thereafter were switched to hypertrophic culture conditions for an additional 3 weeks. The second phase of the study investigated the capacity of MSC-seeded alginate, chitosan and fibrin hydrogels to undergo endochondral ossification *in vivo.* Constructs were maintained in chondrogenic culture conditions for a

period of 5 weeks, followed by an additional week in hypertrophic culture conditions. At the end of the 6 week *in vitro* culture period a single channel (Ø2 mm) was cored into the constructs prior to subcutaneous implantation in nude mice for 6 weeks.

2.5. In vivo subcutaneous implantation

MSC- seeded alginate, chitosan, and fibrin hydrogels were implanted subcutaneously into the back of nude mice (Balb/c; Harlan, UK) as previously described [31]. Briefly, 2 subcutaneous pockets were made along the central line of the spine, one at the shoulders and the other at the hips, and into each pocket 3 constructs were inserted. 9 constructs were implanted per group and constructs were harvested 6 weeks post-implantation. Mice were sacrificed by CO_2 inhalation and the animal protocol was reviewed and approved by the ethics committee of Trinity College Dublin.

2.6. Biochemical analysis

The biochemical content of alginate, chitosan and fibrin constructs were analyzed at weeks 5 and 8 of *in vitro* culture. Prior to biochemical analysis, constructs were sliced in half, washed in PBS, weighed, and frozen for subsequent assessment. One half of each construct was digested with papain (125 µg/mL) in 0.1 M sodium acetate, 5mM L-cysteine-HCL, 0.05 M ethylenediaminetetraacetic acid (EDTA), pH 6.0 (all from Sigma-Aldrich) at 60 °C and 10 rpm for 18 h. The amount of sulphated glycosaminoglycan (sGAG) was quantified using the dimethylmethylene blue dye-binding assay (Blyscan, Biocolor Ltd., Northern Ireland), with a chondroitin sulphate standard. Total collagen content was determined by measuring the hydroxyproline content, using a hydroxyproline-to-collagen ratio of 1:7.69. The other half was digested in 1 M hydrochloric acid at 60 °C and 10 rpm for 18 h. The calcium content was determined using a Sentinel Calcium kit (Alpha Laboratories Ltd, Uk). 3-4 constructs per group were analyzed by each biochemical assay.

2.7. Histological and immunohistochemical analysis

Constructs were fixed in 4% paraformaldehyde, dehydrated in a graded series of ethanols, embedded in paraffin wax, sectioned at 8 μm and affixed to microscope slides. Post-implantation constructs were decalcified in EDTA for up to 1 week. The sections were stained with haematoxylin and eosin (H&E) to assess bone formation, 1% alizarin red to assess calcium accumulation, and aldehyde fuschin/ alcian blue to assess sGAG content. Collagen types I, II and X were evaluated using a standard immunohistochemical technique as described previously [32].

2.8. Micro-computed tomography

Micro-computed tomography scans were performed on constructs using a Scanco Medical 40 μCT system (Scanco Medical, Bassersdorf, Switzerland). Constructs were scanned in PBS, at a voltage of 70 kVp, and a current of 114 μA. A Gaussian filter (sigma=0.8, support=1) was used to suppress noise and a global threshold corresponding to a density of 399.5 mg hydroxyapatite/cm^3 was applied. 3D evaluation was carried out on the segmented images to determine bone volume and density and to reconstruct a 3D image. The variance of mineralization with depth through cylindrical constructs was analyzed qualitatively by examining sections corresponding to a thickness of 96 μm at a depth of 25% from the top of the construct (quarter section), and a depth of 50% from the top of the construct (center section). 4 constructs were analyzed per group.

2.9. Statistical analysis

All statistical analyzes were carried out using Minitab 15.1. Results are reported as mean ± standard deviation. Groups were analyzed by a general linear model for analysis of variance with groups of factors. Tukey's test was used to compare conditions. Anderson-Darling

normality tests were conducted on residuals to confirm a normal distribution. Non-normal data was transformed using the Box-Cox procedure. Significance was accepted at a level of $p < 0.05$.

3. Results

3.1. The in vitro development of hypertrophic cartilaginous grafts engineered using MSC-seeded alginate, chitosan and fibrin hydrogels.

3.1.1. Biochemical analysis

~~MSCs were encapsulated in alginate, chitosan, and fibrin hydrogels, and maintained in chondrogenic culture conditions for a period of 5 weeks, followed by an additional 3 weeks in hypertrophic culture conditions.~~ After 5 weeks of chondrogenic culture, sGAG accumulation was significantly higher within MSC-seeded alginate hydrogels (1.29 ± 0.07 mg) compared to chitosan ($p=0.0451$) and fibrin ($p<0.0001$) hydrogels, see Figure 1a. During this period, collagen synthesis was significantly higher in chitosan constructs (620 ± 32 μg, $p<0.05$), and collagen synthesis continued to increase in both chitosan and fibrin constructs during the hypertrophic culture period (Figure 1b). Calcium accumulation also increased during the hypertrophic culture period within all engineered tissues, with alginate and fibrin constructs accumulating significantly more calcium compared to chitosan constructs ($p<0.01$), see Figure 1c. The switch from chondrogenic to hypertrophic culture conditions at week 5 did not result in significant changes in sGAG content within the engineered constructs by week 8 (Figure 1a). However, sGAG release to the media from alginate constructs increased dramatically during this period, with more modest releases observed in the chitosan and fibrin constructs (Figure 2).

Alginate constructs had a significantly higher wet weight at all time points (Figure 3a), and so when normalized to %wet weight, sGAG and collagen accumulation was highest in chitosan constructs (Figures 3b and 3c), whereas calcium accumulation was highest in fibrin constructs (Figure 3d).

3.1.2 Histology and immunohistochemistry

Histology and immunohistochemistry performed at week 8 indicated that all engineered constructs supported robust chondrogenesis, with aldehyde fuschin/ alcian blue staining demonstrating the production of sGAG and immunohistochemical staining demonstrating the synthesis of collagen type II (Figure 4). Alizarin red staining demonstrated the deposition of a ring of calcium around the periphery of alginate and chitosan constructs, whereas fibrin constructs supported more homogenous calcium production throughout the engineered tissue.

3.2 Endochondral bone formation following subcutaneous implantation of MSC-seeded alginate, chitosan and fibrin hydrogels

3.2.1 Histology and immunohistochemistry

MSC-seeded alginate, chitosan, and fibrin hydrogels were cultured in chondrogenic conditions for a period of 5 weeks, followed by an additional week in hypertrophic culture conditions, prior to subcutaneous implantation in nude mice for 6 weeks. ~~A single axially aligned channel (Ø2mm) was cored into these construct cylinders immediately before implantation to better mimic the geometry of the mid-section of a long bone and to provide an additional conduit for vascularization.~~ Pre-implantation, all constructs stained positive for sGAG and collagen type II, but weakly for collagen type I (Figure 5). Alginate constructs also stained weakly for collagen type X pre-implantation, whereas chitosan and fibrin constructs stained more strongly for collagen type X. Post-implantation, a loss of chondrogenic phenotype was observed in alginate and fibrin constructs, as evidenced by a dramatic increase in the intensity of collagen type I and type X immunostaining and a decrease in sGAG staining. Chitosan appeared to better support the maintenance of a chondrogenic phenotype *in vivo,* as demonstrated by more intense staining for sGAG, and weaker staining for collagen type X post-implantation. It should be noted however, that a reduction in type II collagen deposition was also observed in chitosan hydrogels *in vivo*.

H&E staining was used to assess spatial bone tissue formation post-implantation (Figure 6). Alginate and fibrin constructs both supported bone formation at the top of the construct (Figure 6a,c). Central sections of fibrin constructs demonstrated the presence of a marrow component consisting of a mixture of hematopoietic foci and marrow adipose tissue, though less bone formation was observed at this depth (Figure 6f). Alginate constructs appeared to support greater bone formation in the central region of the engineered construct, with evidence of trabecular strut formation and bone marrow foci accompanied by blood vessel infiltration (Figure 6d). The formation of fibrovascular and bone marrow tissue within alginate and fibrin constructs appeared to correlate with the regions within these hydrogels undergoing dissolution/degradation. Chitosan hydrogels supported the development of a bony collar on the periphery of the construct, although there was no evidence of bone formation or vascularization within the body of the hydrogel at any depth (Figure 6e,f).

3.2.2. Micro-computed tomography

Prior to implantation, all constructs had begun to mineralize around their periphery (Figure 7a). Micro-computed tomography (μCT) analysis of engineered tissues post-implantation revealed that, to different degrees, continued calcification occurred within all constructs *in vivo* (Figure 7b). In general, calcification appeared more confined to the periphery of chitosan constructs, but more diffuse with alginate. For all constructs, mineralization decreased with depth through the tissue. Macroscopic observation suggested that chitosan and fibrin constructs better retained their pre-implantation shape, with the cylindrical core still evident throughout the depth of the tissue, whereas in alginate constructs this core region was only partially retained with the overall shape and geometry of the construct becoming distorted (Figure 7c). This macroscopic assessment also suggested that alginate and fibrin constructs facilitated higher levels of vascularization. Alginate constructs appeared to support the greatest degree of mineralization, which was confirmed by μCT quantification, with bone

volume being significantly higher in alginate constructs (43.08 ± 8.6 %BV/TV) as compared to chitosan (p=0.0073) and fibrin (p=0.04) constructs (Figure 7d). The local density of the newly formed bone in alginate constructs (748.42 ± 42.26 mg HA/cm^3) was significantly (p=0.005) higher when compared to chitosan constructs (Figure 7e). The total bone density was also significantly higher in alginate constructs (Figure 7f).

4. Discussion

MSC based articular cartilage tissue engineering strategies require the identification of hydrogels that support the maintenance of a stable chondrogenic phenotype. In contrast, the scaling-up of engineered hypertrophic cartilaginous grafts to treat bone defects of a clinically relevant size requires the use of a suitable scaffolding material tailored to facilitate chondrogenesis and the transition from engineered hypertrophic cartilage into bone. The objective of this study was to compare the capacity of different naturally derived hydrogels (alginate, chitosan, and fibrin) to support the *in vitro* development of either articular or hypertrophic cartilaginous tissues using MSCs, as well as the capacity of such hydrogels to facilitate endochondral bone formation *in vivo*. *In vitro*, alginate and chitosan constructs accumulated the highest levels of sGAG, with chitosan constructs synthesising the highest levels of collagen. Alginate and fibrin constructs supported the greatest degree of calcium accumulation *in vitro*, though only fibrin constructs calcified homogenously. *In vivo*, both alginate and fibrin constructs facilitated vascularization and endochondral bone formation as well as the development of a bone marrow environment, with alginate constructs accumulating significantly more mineral and supporting greater bone formation in central regions of the engineered tissue. Chitosan hydrogels appeared to best maintain a stable

chondrogenic phenotype, although some mineralization of the engineered tissue was still observed *in vivo*.

Understanding how cell-material interactions regulate stem cell fate is a key challenge in developing successful tissue engineering therapies. Cells encapsulated in alginate cannot directly adhere to the hydrogel and hence adopt a spherical morphology known to promote chondrogenesis, which coupled with the significant construct swelling and capacity of this hydrogel to retain synthesized matrix components (Fig. 2), may explain the large accumulation in sGAG during culture in chondrogenic conditions. Unlike alginate, fibrin permits cell mediated integrin binding [33] and a more spread MSC morphology [34], which in turn may support robust osteogenic [35] and/or endochondral differentiation. This may explain why in contrast to alginate and chitosan constructs, which calcified preferentially around their periphery upon culture in hypertrophic conditions, fibrin constructs facilitated a homogenous deposition of calcium *in vitro* while accumulating the highest levels of calcium when normalized by % wet weight. Previous studies have suggested that the development of a low oxygen micro-environment within core regions of MSC-seeded hydrogels will suppress hypertrophy and calcification in this region of the engineered tissue [31]. In the context of MSC seeded fibrin gels, it may be that the combination of a spread cell shape and such low oxygen conditions are supporting a sub-population of MSCs undergoing direct osteoblastic differentiation in response to the β-GP (an osteogenic inducer) within the hypertrophic medium, as previous studies have demonstrated enhanced calcium deposition by MSCs at an oxygen tension of 5% pO2 compared to 20% pO2 [30, 36]. Previous studies have also demonstrated that MSCs cultured in a chondrogenic medium containing β-GP accumulated more calcium in hypoxic conditions than in normoxic conditions when seeded onto an electrospun fibrous polymer scaffold [37], a scaffold which also promotes an elongated

morphology in MSCs [38]. Further work is required to determine the role of cell shape and oxygen tension in regulating the endochondral phenoype of MSCs.

Chitosan constructs appeared to generate the most stable cartilage-like tissue over the 8 week *in vitro* culture period, as demonstrated by the highest levels of collagen and sGAG accumulation (as a % of tissue wet weight) and lowest calcium accumulation. A number of previous studies have investigated the use of chitosan hydrogels for cartilage tissue engineering applications [39-43]. A linear polysaccharide, chitosan consists of β (1→4) linked D-glucosamine residues with a variable number of randomly located *N*-acetyl-glucosamine groups and is structurally similar to a number of sGAGs and hyaluronic acids present in articular cartilage [44]. Furthermore, the cationic nature of chitosan facilitates the formation of ionic complexes with anionic polysaccharides such as sGAGs [45]. The chitosan hydrogel may therefore be supporting a favourable environment for the chondrogenesis of MSCs; firstly, by providing a structural framework containing cartilage specific biochemical cues, and secondly, by facilitating the development of a dense cartilaginous matrix through the entrapment of generated sGAGs, a mechanism which may be playing a further role in maintaining the chondrogenic phenotype. Interestingly, chitosan constructs experienced only a modest 2 fold increase in sGAG release to the culture media upon transition to hypertrophic conditions, as opposed to the dramatic 125 fold increase from alginate constructs during the same period. This polycation-polyanion structural relationship between the positively charged chitosan hydrogel and negatively charged sGAG may be the regulatory mechanism behind sGAG retention within the constructs.

When comparing the performance of various biopolymers, the purity of the different materials must be considered. The ultrapure alginate and chitosan polymers utilized in this study contained very low levels of endotoxins (≤ 100 EU/g), which may have had a beneficial effect on matrix accumulation within these hydrogels. The fibrinogen and thrombin utilized

to form fibrin hydrogels had not been tested for endotoxins and therefore we are unable to confirm if they are of the same high purity as the alginate and chitosan hydrogels.

To assess the capacity of the alginate, chitosan and fibrin hydrogels to generate endochondral bone *in vivo*, engineered hypertrophic constructs were implanted subcutaneously into nude mice. Both alginate and fibrin constructs supported endochondral bone formation, vascularization, and the development of a hematopoietic marrow component. Interestingly both these hydrogels demonstrated levels of dissolution/degradation following implantation with vascularization and bone marrow formation apparently localised to the regions of scaffold degradation. Of the two hydrogels, alginate underwent the greater remodelling and facilitated the development of the highest levels endochondral bone formation. Interestingly, this occurred despite alginate constructs having demonstrated the lowest levels of hypertrophy pre-implantation (specifically these constructs stained weakest for type X collagen), with robust hypertrophic differentiation only evident following *in vivo* implantation. This was would suggest that relative levels of type X collagen production pre-implantation are not necessarily predictive of the extent of endochondral bone formation *in vivo*. The more rapid degradation rate of the alginate *in vivo* is likely favoring vascularization and oxygen availability within this hydrogel, and subsequently driving hypertrophy [30, 46]. Tailoring the degradation characteristics of a hydrogel may therefore be of critical importance in promoting endochondral ossification of engineered grafts [47-50]. Alternatively, or perhaps in conjunction, inflammatory cytokines may be leveraged to direct more efficient resorption of a large cartilaginous template [51]. Hydrogels for endochondral applications may also benefit from the incorporation of channeled arrays, in order to provide additional conduits for vascularization [52].

Chitosan constructs did not undergo any degradation following *in vivo* implantation and only supported a collar of bone formation on the periphery of the hydrogel, nor was there

any evidence of vascularization or marrow formation. The preservation of the chitosan hydrogel *in vivo* would seem to be playing a role in inhibiting bone formation and vascularization. While chitosan appeared to best support the chondrogenic phenotype of MSCs *in vivo*, with retention of the sGAG matrix again perhaps occurring through the polycation-polyanion mechanism described above, a sufficiently stable chondrogenic phenotype was not achieved as evident by a reduction in collagen type II and an increase in collagen types I and X production and matrix calcification. A previous study comparing the capacity of various hydrogels to support stable chondrogenesis of adipose derived stem cells *in vivo* reported suppression of calcification by Matrigel [26]. However, when the source of stem cells was changed from adipose tissue to bone marrow, as used in this study, Matrigel calcification was also reported. Furthermore, cartilaginous tissues engineered using stem cells isolated from different sources have been shown to be phenotypically different [31]. Therefore, MSC-based articular cartilage repair therapies would appear to depend greatly on the source from which the MSCs are isolated and the hydrogel in which they are encapsulated. Chitosan hydrogels, provided they're loaded with the appropriate stem cell source, may be an attractive material for the engineering of phenotypically stable and functional articular cartilage for use in orthotopic defects. Further studies are required to test this concept in appropriate models.

4. Conclusions

This work examined the capacity of different naturally derived hydrogels to support chondrogenesis and hypertrophy of MSCs *in vitro* and endochondral ossification *in vivo*. All hydrogels supported, to differing degrees, the development of mineralized cartilage *in vitro*, though only alginate and fibrin hydrogels facilitated endochondral bone formation *in vivo*, with alginate constructs accumulating significantly more mineral and supporting bone

formation in central regions of the engineered tissue. Chitosan hydrogels better maintained the chondrogenic phenotype of MSCs, suggesting it to be a promising material for the engineering of articular cartilage. Future work will explore the capacity of alginate hydrogels to support endochondral bone formation in large bone defects, and furthermore, to form an anatomically shaped tissue engineered long bone as a paradigm for whole bone regeneration through endochondral ossification.

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Figure legends

Figure 1. Phase 1. Biochemical analysis of alginate, chitosan and fibrin constructs at week 5 and week 8 of *in vitro* culture. (a) sGAG, (b) collagen and (c) calcium accumulation within constructs. Significance: $p < 0.05$, a vs. chitosan at same time point, b vs. fibrin at same time point, c vs. corresponding group at week 5. 3-4 constructs were analyzed biochemically at each time point.

Figure 2. Phase 1. sGAG content in the medium of alginate, chitosan, and fibrin constructs. Measurements were taken at week 4 and week 6 to capture the change in sGAG release kinetics upon transfer from chondrogenic to hypertrophic culture conditions. Significance: $p < 0.05$, a vs. chitosan constructs, b vs. fibrin constructs, c vs. corresponding group at week 4. 4 samples analyzed at each time point.

Figure 3. Phase 1. Biochemical content of alginate, chitosan and fibrin constructs at week 5 and week 8 of *in vitro* culture normalized to % wet weight (%ww). (a) Mass of the constructs (mg). (b) sGAG, (c) collagen and (d) calcium accumulation of the constructs normalized to % wet weight (%ww). Significance: $p < 0.05$, a vs. chitosan at same time point, b vs. fibrin at same time point, c vs. corresponding group at week 5, d vs corresponding group at day 0. 3-4 constructs were analyzed biochemically at each time point.

Figure 4. Phase 1. Histological and immunohistochemical analysis of alginate, chitosan and fibrin constructs at week 8 of *in vitro* culture. Constructs were stained for sGAG (aldehyde fuschin/ alcian blue), collagen type II, and calcium (alizarin red). Scale bar is 250 μm.

Figure 5. Phase 2. Histological and immunohistochemical analysis of alginate, chitosan and fibrin constructs at the end of the 6 week *in vitro* culture period (pre-implantation) and after the 6 week *in vivo* period (post-implantation). Constructs were stained for collagen type I, collagen type II, collagen type X and sGAG (aldehyde fuschin/ alcian blue). Scale bars are 500 μm.

Figure 6. Phase 2. H&E staining of alginate, chitosan and fibrin constructs post-implantation. Staining was performed on slices taken from two depths, the top and the center, of each construct. (a-c) Top slices. (d-f) Center slices. (a,d) alginate, (b,e) chitosan, and (c,f) fibrin constructs. Arrows indicate blood vessel structures. ‘at’ indicates marrow adipose tissue. Main image scale bars are 250 μm. Inset scale bars are 50 μm. Note: the intense pink staining

in chitosan constructs (images (b) and (e)) is as a result of the high eosinophilicity of the chitosan material.

Figure 7. Phase 2. Calcification of alginate, chitosan and fibrin constructs pre-implantation and post-implantation. (a) Alizarin red staining of alginate, chitosan and fibrin constructs pre-implantation. Scale bar is 500 µm. (b) µCT imaging of alginate (left column), chitosan (center column) and fibrin (right column) constructs post-implantation. Sections, corresponding to a thickness of 96 µm, were analyzed at a depth of 25% from the top of the construct (quarter section) and at a depth of 50% from the top of the construct (center section). Scale bar is 1 mm. (c) Macroscopic image of alginate (left), chitosan (center) and fibrin (right) constructs post-implantation. Scale bar is 3 mm. (d) % Bone volume per total volume (BV/TV), (e) local bone density and (f) total bone density of alginate, chitosan and fibrin constructs post-implantation. Significance: $p<0.05$, a vs. chitosan, b vs. fibrin. 4 constructs were analyzed per group by µCT. HA-Hydroxyapatite.

Figure 1

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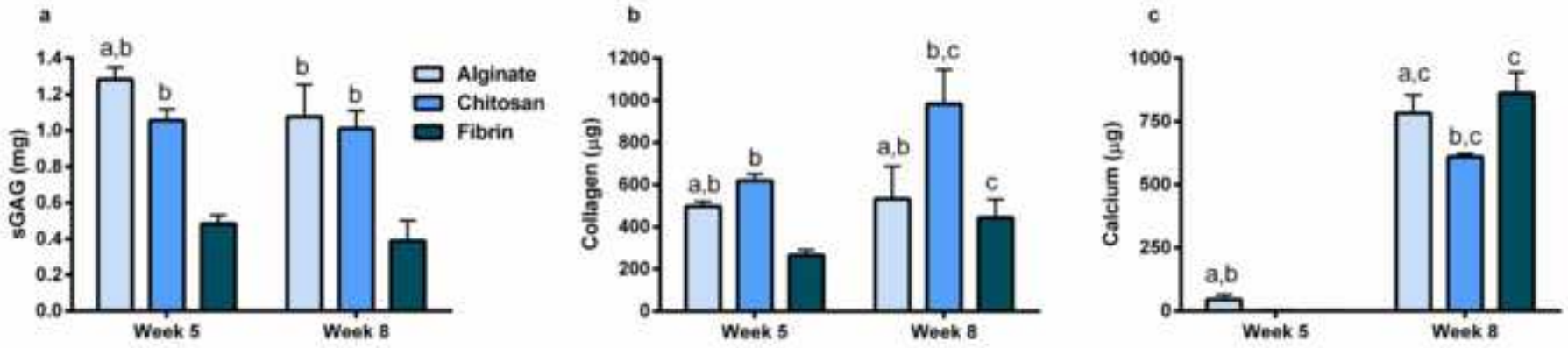

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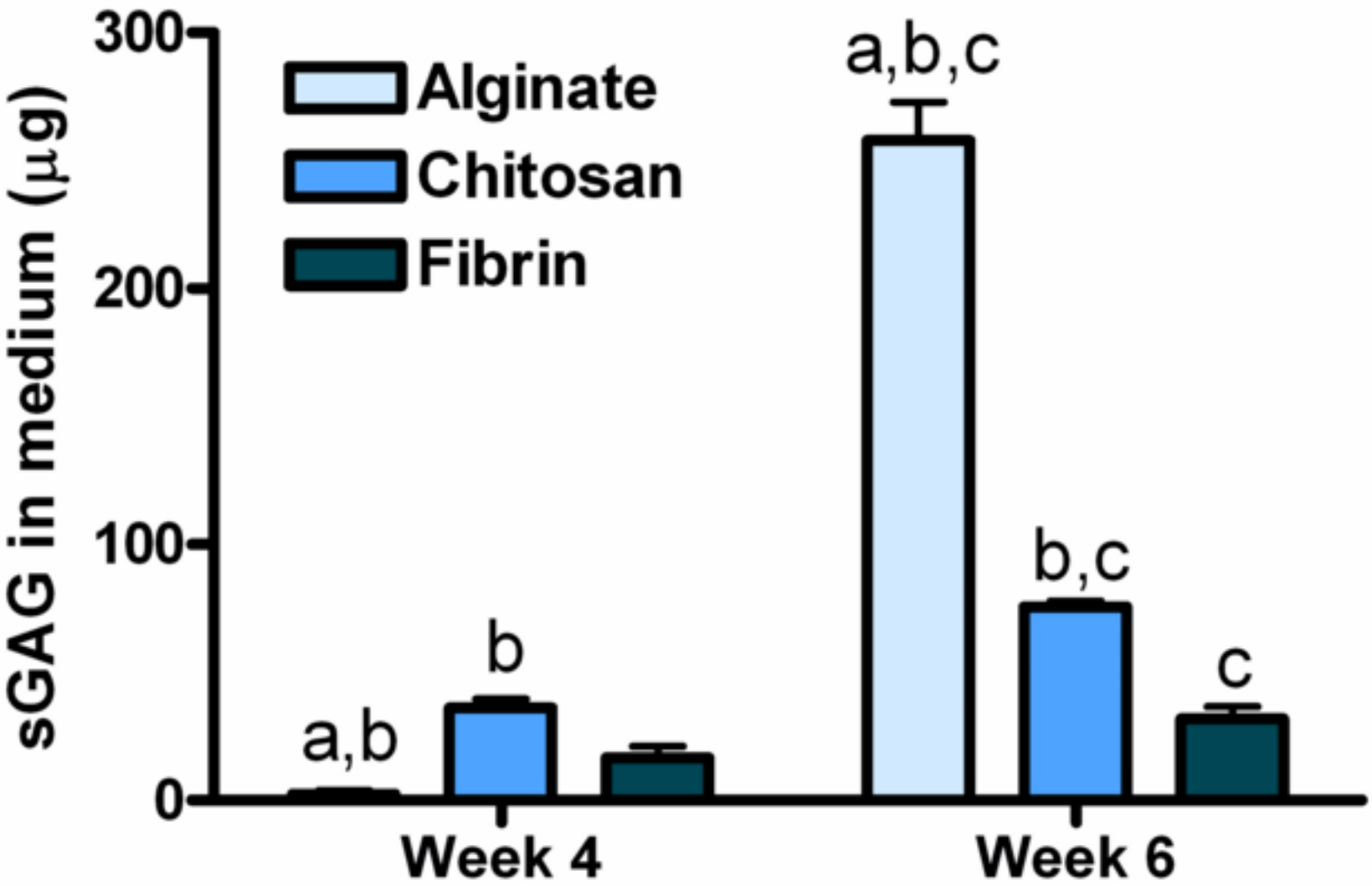

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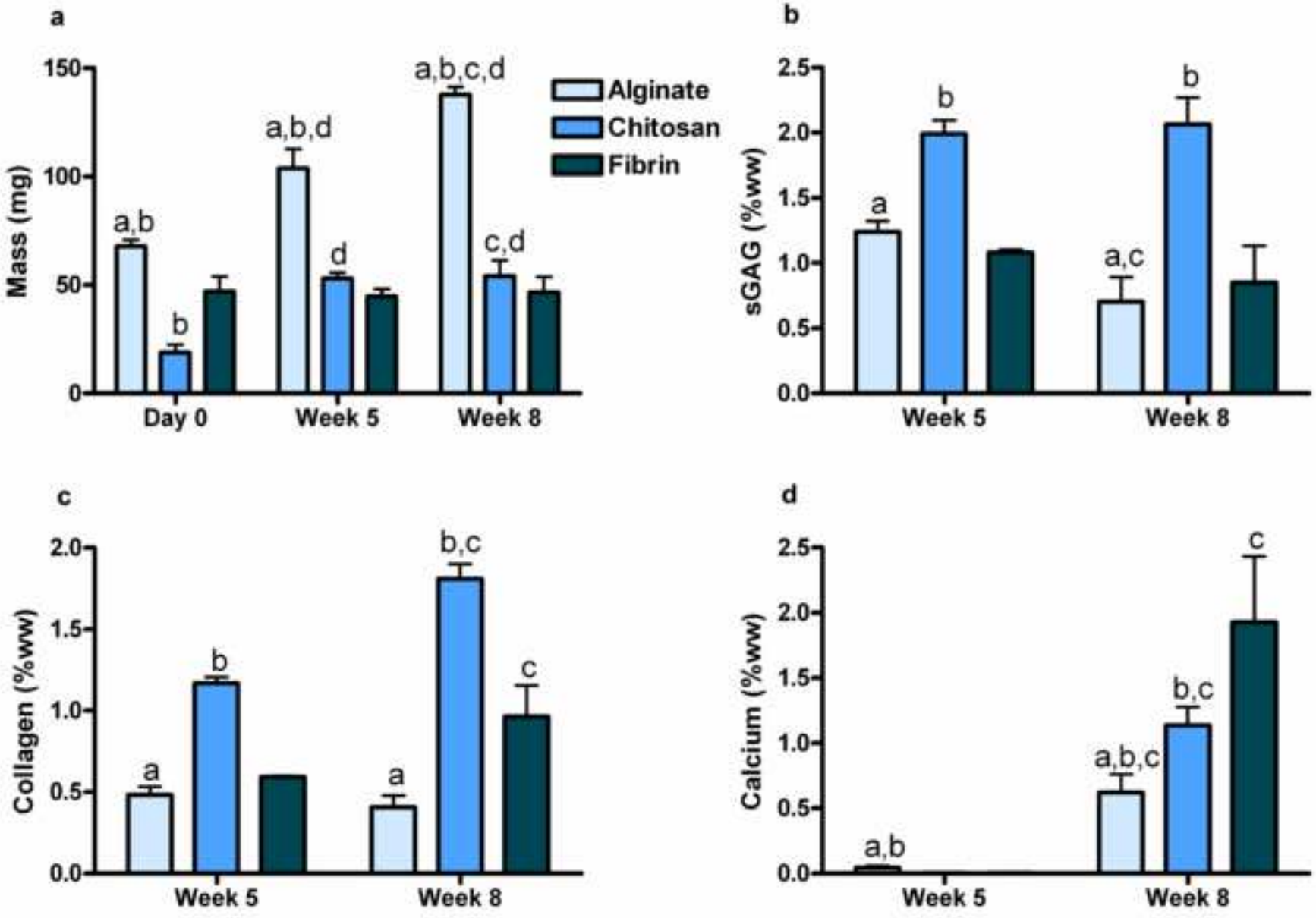

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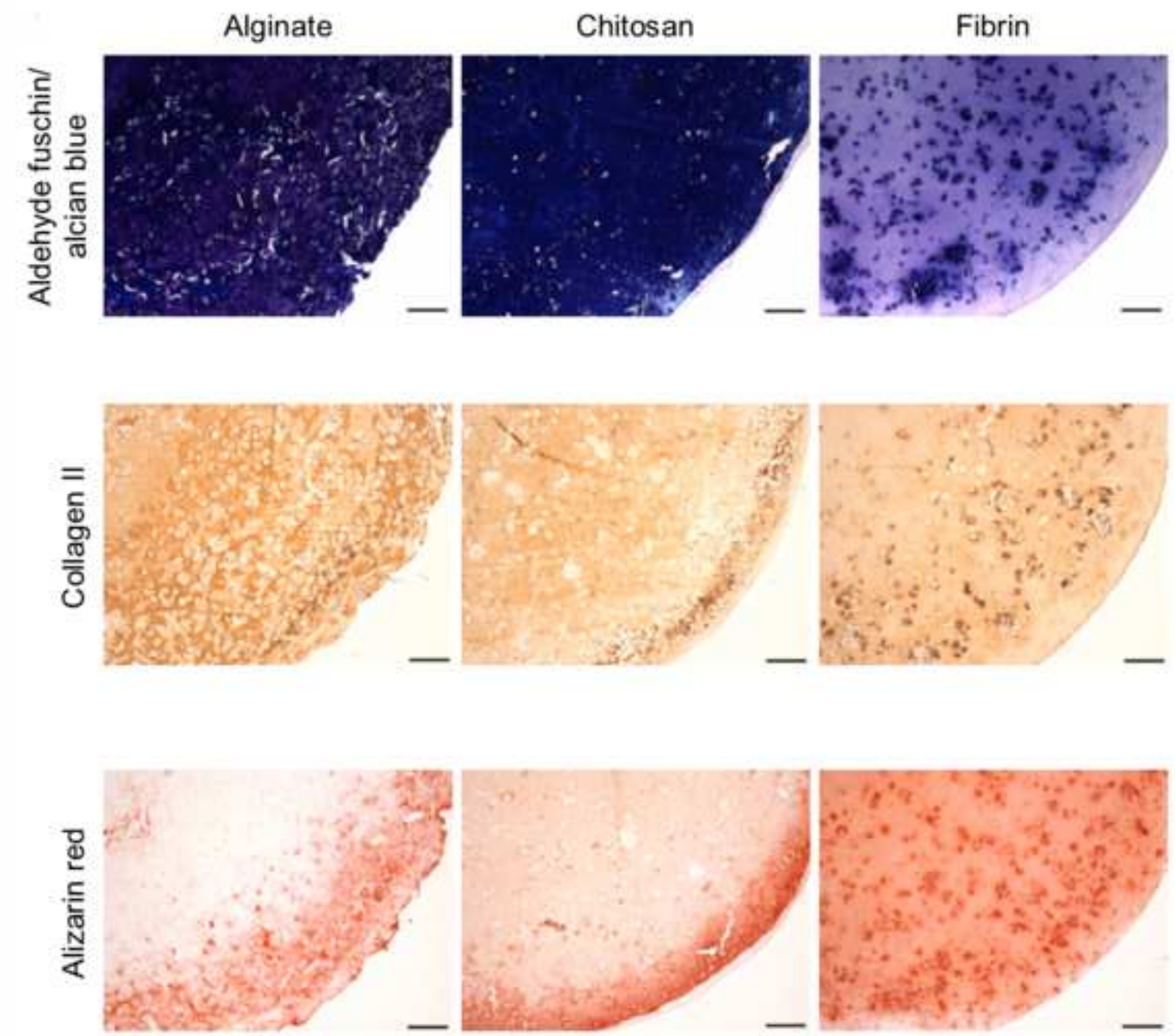

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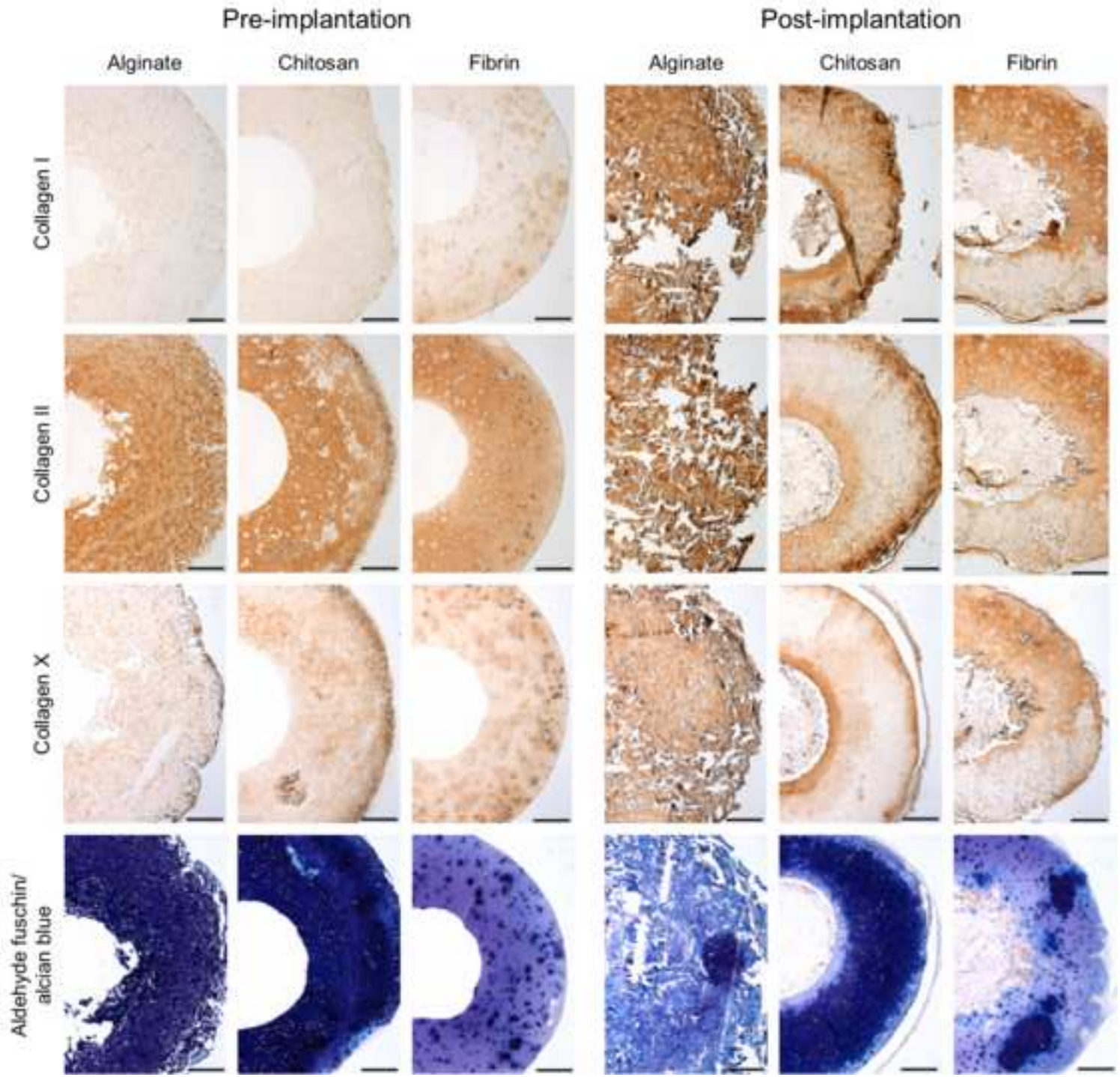

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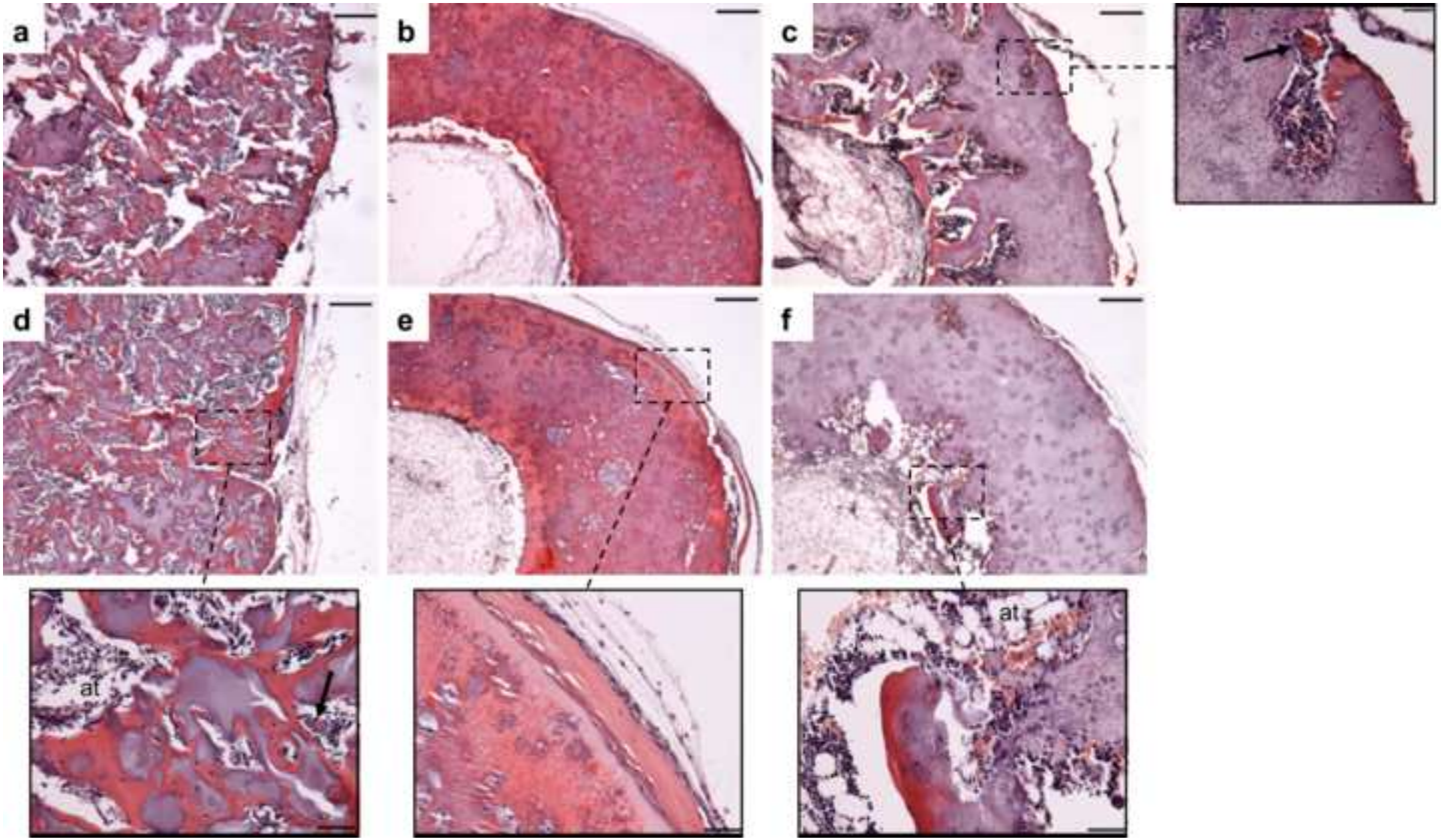

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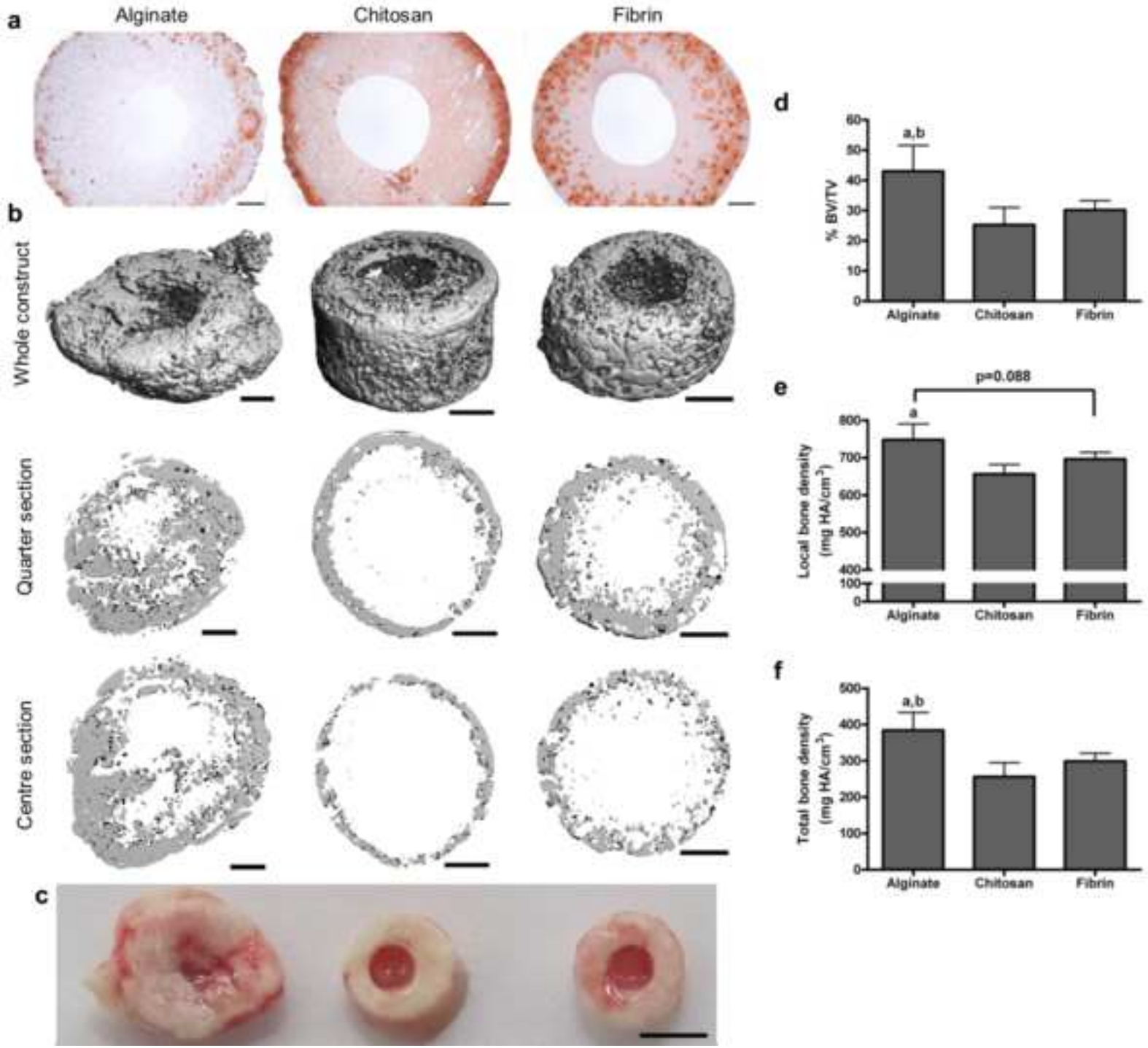

Graphical Abstract