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# A comparison of self-assembly and hydrogel encapsulation as a means to engineer functional cartilaginous grafts using culture expanded chondrocytes

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**Keywords** Self-assembly, agarose hydrogel, passaged chondrocytes, TGF-β3.

*Abstract*

Despite an increased interest in the use of hydrogel encapsulation and cellular self-assembly (often termed 'self-aggregating' or 'scaffold-free' approaches) for tissue engineering applications, to the best of our knowledge no study to date has been undertaken to directly compare both approaches for generating functional cartilaginous grafts. The objective of this study was to directly compare self-assembly (SA) and agarose hydrogel encapsulation (AE) as a means to engineer such grafts using passaged chondrocytes. Agarose hydrogels (5mm diameter x 1.5mm thick) were seeded with chondrocytes at two cell seeding densities (900,000 cells or 4 million cells in total per hydrogel), while self-assembled constructs were generated by adding the same number of cells to custom made moulds. Constructs were either supplemented with TGF-β3 for 6 weeks, or were only supplemented with TGF-β3 for the first 2 weeks of the 6 week culture period. The SA method was only capable of generating geometrically uniform cartilaginous tissues at high seeding densities (4 million cells). At these high seeding densities we observed that total sGAG and collagen synthesis was greater with AE than SA, with higher sGAG retention also observed in AE constructs. When normalized to wet weight however, SA constructs exhibited significantly higher levels of collagen accumulation compared to agarose hydrogels. Furthermore, it was possible to engineer such functionality into these tissues in a shorter timeframe using the SA approach compared to AE. Therefore, while large numbers of chondrocytes are required to engineer cartilaginous grafts using the SA approach, it would appear to lead to the faster generation of a more hyaline-like tissue, with a tissue architecture and a ratio of collagen to sGAG content more closely resembling native articular cartilage.

## *Introduction*

Cartilage damage can arise from degenerative diseases such as osteoarthritis or due to physical trauma to the articular surface. A large number of tissue engineering strategies have been proposed to repair such cartilaginous defects. Typical approaches involve the use of a scaffold or hydrogel for supporting and organising the cells in a three dimensional (3D) environment. Agarose hydrogels are commonly used for cartilage tissue engineering applications as they have been found to support the chondrogenic phenotype and the synthesis of cartilaginous extracellular matrix (ECM).[1-16] When seeded with primary chondrocytes, such hydrogels can be used to engineer tissues attaining native levels of compressive moduli and sGAG content.[17] However, as with many scaffolds or hydrogels, such an approach raises the issues of scaffold degradation products, inflammatory responses to the implanted materials, stress shielding of cells, and a reduction in cell to cell communication.[18,19] This has motivated research into scaffold-free techniques as a potential method for generating functional cartilage tissue.

One of the first reported uses of a scaffold-free or self-assembly (SA) (or self-aggregating) approach for engineering cartilage-like tissue involved directly seeding chondrocytes onto plastic dishes pre-coated with poly(2-hydroxyethyl methacrylate),[20-24] which leads to the development of a graft with a hyaline cartilage phenotype in terms of the expression of collagen type II and aggrecan.[22] Alternative SA approaches involve aliquoting chondrocytes into an agarose mould or similar, and allowing these cells to self-assemble over time.[18] After 12 weeks of culture, this SA approach has been shown to support the generation of a hyaline-like cartilaginous tissue with biochemical and mechanical properties approaching those of native articular cartilage. Numerous other studies have investigated the SA of chondrocytes,[25-33] with determination of the initial cell seeding number identified as a key parameter to successfully engineer a cartilaginous graft using the SA approach. Researchers

have also investigated the potential of generating cartilage grafts through self-assembly of mesenchymal stem cells (MSCs),[19,34] with some success reported in repairing chondral defects *in vivo* using this approach.[35,36] Furthermore, in terms of chondrogenic differentiation of human bone marrow derived MSCs, the SA method has demonstrated benefits over traditional pellet culture system.[37]

Despite the extensive research into scaffold-free cartilage tissue engineering, particularly in the area of chondrocyte SA, to the best of our knowledge no study to date has been undertaken to directly compare the SA approach to hydrogel encapsulation for engineering functional cartilaginous grafts. The objective of this study was to directly compare SA to agarose hydrogel encapsulation (AE) as a means to engineer such grafts. Passaged chondrocytes were encapsulated into agarose hydrogels at different cell seeding densities and maintained in a chemically defined media. The properties of these engineered tissues were then compared to those generated using a SA approach. As it is known that articular chondrocytes dedifferentiate after they attach to cell culture plastic,[3] the cells were allowed to self-assemble on an agarose bed that prevents cell attachment. Previous studies have shown that the SA of chondrocytes on a nonadhesive agarose coating leads to the development of a more smooth, flat, and hyaline-like construct, when compared to those assembled on culture treated plastic.[18] Constructs were seeded at two seeding densities; first a typical AE seeding density (approx. 900,000 cells per construct or 30 million cells/ml for a 5mm diameter x 1.5mm thick construct), and second a typical SA seeding density (4 million cells per construct). Finally, as transient TGF-β3 stimulation has been shown to enhance chondrogenesis in chondrocyte seeded agarose hydrogels,[17] we compared the effect of such media supplementation conditions on the development of cartilaginous grafts engineered using both SA and AE.

## *Materials and Methods*

### *Cell isolation and expansion*

Articular cartilage was aseptically harvested from porcine femoral condyles (4 months old), and the cartilage slices were rinsed thoroughly with Dulbecco's phosphate buffered saline (Sigma-Aldrich, Dublin, Ireland; PBS) containing penicillin (200 U/ml)-streptomycin (100µg/ml) (GIBCO, Invitrogen, Dublin, Ireland), and amphotericin B (2.5µg/ml) (Sigma-Aldrich, Dublin, Ireland). Chondrocytes were isolated from cartilage slices via digestion with high-glucose Dulbecco's modified Eagle's medium GlutaMAX (4.5 mg/ml D-Glucose, 200mM L-Glutamine; hgDMEM) (GIBCO, Invitrogen, Dublin, Ireland) containing collagenase type II (315 U/mg) (Worthington, Langanbach Services, Ireland) for 12-14 h under constant rotation at 37ºC. The resulting cell suspension was passed through a 40µm pore-size cell sieve (Fisher Scientific, Ireland) and the filtrate centrifuged and rinsed with PBS twice. Cell number and viability were determined using a haemocytometer and 0.4% trypan blue staining (Sigma-Aldrich, Dublin, Ireland), and the chondrocytes were then frozen in hgDMEM supplemented with 10% v/v foetal bovine serum (GIBCO, Invitrogen, Dublin, Ireland; FBS) and 10% dimethyl sulphoxide (Sigma-Aldrich, Dublin, Ireland; DMSO) and stored in liquid nitrogen. Before experiments were initiated cells were thawed and counted. Chondrocytes were plated at a seeding density of $5\times10^3$ cells/cm$^2$ in 500 cm$^2$ triple flasks (Thermo Fisher Scientific, Ireland) and expanded to passage two (P2) in a humidified atmosphere at 37°C and 5% $CO_2$. Chondrocytes were maintained in DMEM GlutaMAX supplemented with 10% v/v FBS, penicillin (100 U/ml)-streptomycin (100µg/ml) and 5ng/ml human fibroblast growth factor-2 (FGF-2; Prospec, Israel) during the expansion phase.

*Formation and culture of self-assembled and agarose hydrogel constructs*

At P2 cells were trypsinized, counted and suspended in basic chondrogenic medium (basic CDM) consisting of hgDMEM supplemented with penicillin (100 U/ml)-streptomycin (100µg/ml) (both from GIBCO, Invitrogen, Dublin, Ireland), 100 µg/ml sodium pyruvate, 40 µg/ml L-proline, and 1.5 mg/ml bovine serum albumin (all Sigma-Aldrich, Arklow, Ireland). A custom built polydimethylsiloxane (PDMS) mould was used to create sterile, 3% agarose wells (Type VII, Sigma-Aldrich, Arklow, Ireland) of 5mm diameter and 3mm thickness. Self-assembled constructs were formed by adding either 900,000 cells (low seeding density; 46,000 cells/mm$^2$) or 4 million cells (high seeding density; 204,000 cells/mm$^2$) in 40 µl aliquots of defined CDM to the 5mm diameter agarose wells, seated in either 12 well plates (low seeding density constructs), or 6 well plates (high seeding density constructs) (Fisher Scientific, Ireland). Defined CDM consisted of basic CDM supplemented with 0.25 µg/ml amphotericin B, 1x insulin-transferrin-selenium, 4.7 µg/ml linoleic acid, 50µg/ml L-ascorbic acid-2-phosphate and 100nM dexamethasone (all Sigma-Aldrich, Arklow, Ireland). Self-assembled constructs were initially not supplemented with TGF-β3 to minimize cell contraction. Cells self-assembled within 12 h, upon which defined CDM supplemented with 10 ng/ml of transforming growth factor-beta 3 (TGF-β3) (ProSpec-Tany TechnoGene Ltd., Israel) was added to each well; t = 0 was defined at this time point.

Chondrocyte encapsulated agarose hydrogel constructs were formed by mixing the chondrocyte cell suspension in basic CDM with 4% agarose in sterile PBS. This solution was mixed at a ratio of 1:1 at ~40ºC, to yield a final gel concentration of 2% and a cell density of either 30 million cells/ml or 136 million cells/ml. The agarose/cell suspensions were cast in a stainless steel mould, allowed to cool for 30 min, and solid construct cylinders (5mm diameter x 1.5mm thick) were removed using a biopsy punch. Constructs were then placed in 6 or 12 well plates corresponding to their cell number, and immersed in defined CDM

supplemented with 10 ng/ml of transforming growth factor-beta 3 (TGF-β3). The low and high agarose cell seeding densities correspond to the self-assembly (SA) seeding density of 900,000 and 4 million cells respectively. The high seeding density of 4 million cells was chosen as this has been previously shown to be the optimal initial seeding number for chondrocyte self-assembled constructs.[31] The low seeding density of 900,000 cells (30 million cells/ml) was chosen to enable comparisons to be made with other chondrocyte agarose hydrogel studies previously undertaken in our laboratory. Typical values for SA thickness range from 0.8mm[18] to 1.4 mm;[19] therefore we chose a thickness value of 1.5mm for our agarose constructs in order to generate similarly sized constructs to the SA approach at the end of the experiment.

Constructs at low seeding density were maintained in 2.5ml fully supplemented CDM, with high seeding density constructs maintained in 11 ml (hence maintaining the ratio of media to cells constant). Medium was fully exchanged every 3 or 4 days, with 500µl samples taken from wells for each group (n=3) at each medium exchange for biochemical analysis (as described below). All agarose and self-assembled constructs were maintained for 2 weeks in fully supplemented CDM, upon which TGF-β3 was withdrawn from half the samples of all experimental groups for the remaining 4 weeks. In addition, all self-assembled constructs were removed from their agarose moulds after 2 weeks of *in vitro* culture, as this has been shown to enhance aggregate moduli and collagen organization in self-assembled constructs. [28]

*Biochemical analysis*

The biochemical content of constructs (n = 3-4) was assessed at each time point (0, 21 and 42 days). To gain an appreciation of the spatial accumulation of sulphated glycosaminoglycan (sGAG) and collagen, the core of high seeding density constructs was

removed using a 3mm biopsy punch and analyzed separately from the annulus. On removal from culture, construct diameter was measured, the wet mass of both the core and annulus was recorded and all samples were subsequently frozen at -85ºC for later analyses. Samples were digested with papain (125µg/ml) in 0.1M sodium acetate, 5mM L-cysteine-HCL, 0.05 M EDTA, pH 6 (all Sigma-Aldrich, Ireland) under constant rotation at 60ºC for 18 h. DNA content was quantified using the Hoechst Bisbenzimide 33258 dye assay as previously described.[38] Proteoglycan content was estimated by quantifying the amount of sGAG in each hydrogel core/annulus using the dimethylmethylene blue dye binding assay (Blyscan, Biocolor Ltd., Carrickfergus, UK), with a shark chondroitin sulphate standard. sGAG secreted to culture media at each media exchange was also analysed for each group (n=3). Total collagen content was determined by measuring the hydroxyproline content,[39] using a hydroxyproline to collagen ratio of 1:7.69.[40]

*Histology and immunohistochemistry*

At each time point, two or more samples per group were fixed in 4% paraformaldehyde (Sigma-Aldrich, Arklow, Ireland), dehydrated with a graded series of alcohol and embedded in paraffin. 5µm sections were produced of the cross section perpendicular to the construct face. Sections were stained with 1% alcian blue 8GX (Sigma-Aldrich, Arklow, Ireland) in 0.1M HCL for sGAG accumulation. Collagen type II deposition was identified by immunohistochemical analysis. Briefly, sections were treated with peroxidase, and then rinsed with PBS before treatment with chondroitinase ABC (Sigma-Aldrich, Arklow, Ireland) in a humidified environment at 37°C to enhance permeability of the ECM. The sections were rinsed in PBS, and then incubated with goat serum to block non-specific sites, before the primary antibody was applied to the sections for 1 h. A mouse monoclonal anti-collagen type II antibody (1:100; 1mg/ml; Abcam, Cambridge, UK) was

used as the primary antibody for collagen type II. Next, an anti-mouse IgG biotin conjugate secondary antibody (1:133; 2mg/ml; Sigma-Aldrich, Arklow, Ireland) was applied for 1 h, followed by incubation with ABC reagent (Vectastain PK-4000, Vector Labs, Petersbough, UK) for 45 mins. Finally, the samples were developed with DAB peroxidase (Vector Labs, Petersbough, UK) for 5 min. Positive and negative controls (porcine cartilage and ligament respectively) were included.

*Statistical Analysis*

Statistical analyses were performed using the software package MINITAB 15.1 (Minitab Ltd., Coventry, UK). Groups were analysed for significant differences using a general linear model for analysis of variance with factors of time point, scaffold type, culturing conditions, construct region, and interactions between these factors examined. Tukey's test for multiple comparisons was used to compare conditions. Significance was accepted at a level of $p<0.05$. Numerical and graphical results are presented as mean ± standard deviation (n=3-4 for each group at each time point), with graphical results produced using GraphPad Prism (San Diego, USA; Version 4.03).

***Results***

*Self-assembly (SA) using large numbers of chondrocytes leads to the development of a tissue with a more articular cartilage-like composition compared to hydrogel encapsulation*

The morphology of self-assembled constructs seeded at low and high seeding densities varied dramatically (Fig. 1). By week 6, constructs formed with 4 million cells were firm, smooth and flat with a hyaline-like appearance. This was in stark contrast to SA constructs engineered at the lower seeding density (900,000 cells), which were uneven in their appearance, with a significantly reduced diameter (Table 1). Hence it would appear that 900,000 cells is too low a cell number to generate a satisfactory self-assembled construct. Agarose constructs seeded with 4 million cells were found to significantly increase in thickness over 42 days, with evidence of bulging at the top and bottom surfaces. These constructs were found to weigh substantially more than self-assembled constructs (Figs. 2A, B).

By day 42 in culture, agarose constructs seeded with 4 million chondrocytes accumulated higher levels of sGAG (2555.21±46.05 μg) compared to any other group (Figs. 2C, D). For both transient and continuous TGF-β3 supplementation, AE led to greater amounts of absolute sGAG accumulation (measured in μg) compared to SA. However when normalized to wet weight, SA constructs were found to accumulate comparable levels of sGAG to agarose hydrogels (Figs. 2E, F). Finally, when normalized to DNA content (Figs. 2G, H), sGAG accumulation was found to be significantly greater in agarose gels compared to self-assembled constructs for both seeding densities. In addition, a lower seeding density was more conducive to matrix synthesis (on a per cell basis) in agarose constructs.

Similar trends were observed in collagen accumulated per construct (Fig. 3), with continuously supplemented agarose hydrogels accumulating significantly more collagen than

other constructs by day 42 (Fig. 3B). When normalized to wet weight however, the SA constructs accumulated more collagen than corresponding AE constructs, for both low and high seeding densities (Figs. 3C, D). When normalized to DNA content (Figs. 3E, F), collagen accumulation was observed to follow similar trends to sGAG/DNA. Collagen synthesis (Collagen/DNA) did not appear to be dramatically affected by transient TGF-β3 supplementation for either SA or AE.

The temporal development of grafts engineered using hydrogel encapsulation and SA was also different. At high seeding densities, sGAG and collagen accumulation in AE constructs continued to increase from days 21 to 42 in continuously supplemented conditions (Figs. 2F, 3D). In contrast, ECM accumulation in SA constructs appeared to peak by day 21 (Figs. 2F, 3D), with smaller changes over the subsequent 21 days of culture. There were comparable levels of sGAG and greater levels of collagen accumulation (measured as %w/w) in day 21 SA grafts compared to day 42 agarose constucts (Figs. 2E, F; 3C, D).

In normal articular cartilage, the tissue contains approximately 3 times more collagen than sGAG as a percentage of wet weight.[41] To enable the comparison between the relative compositions of our engineered tissue with normal articular cartilage, we normalized collagen accumulation within all constructs to corresponding sGAG accumulation (Fig. 4). At higher seeding densities (Fig. 4B), SA constructs at day 42 displayed a ratio of approximately 1, significantly greater than that of agarose constructs (less than 0.5). This would suggest SA using a sufficient number of chondrocytes leads to the development of a tissue with a more cartilage-like composition compared to hydrogel encapsulation.

*The spatial accumulation of matrix components in tissues engineered using self-assembly and hydrogel encapsulation*

All constructs stained positively for sGAG and collagen type II (Fig. 5), with evidence of increased staining of collagen type II in constructs continuously supplemented with TGF-β3. SA at a higher seeding density resulted in the development of a more uniform tissue, with contraction and distortion of SA constructs witnessed at low seeding densities. High seeding density SA constructs exhibited a peripheral region with weak sGAG staining and strong collagen type II staining. At higher magnification it was observed that the structure and organization of self-assembled constructs mimics certain aspects of native articular cartilage (Fig. 6). Clustering of chondrocytes was observed in the deeper zones of the tissue. The superficial regions of the tissue stained intensely for type II collagen. Transiently supplemented SA constructs appeared more homogeneous than continuously supplemented constructs.

We decided to only investigate high seeding density constructs from this point forward in the experiment, as it was clear from our analysis that the low seeding density generated an inadequate SA construct.

Prevention of core degradation is an important challenge when scaling up engineered grafts. To gain an appreciation of spatial variations in matrix synthesis within constructs engineered using AE and SA, we next compared sGAG and collagen accumulation in the core and annular regions of these constructs (Fig. 7). In each region of the constructs, similar trends were seen between groups in terms of respective sGAG and collagen accumulation. Continuously supplemented AE constructs accumulated significantly more sGAG than other constructs in both the core (4.87±0.14 %w/w) and annulus (4.24±0.04 %w/w). AE constructs accumulated significantly more sGAG in their core compared to their annuli, as did

transiently supplemented SA constructs. In contrast to this, a more homogeneous sGAG distribution was observed in continuously supplemented SA constructs, with no significant difference found between core and annulus. As noted before, SA constructs accumulated significantly more collagen (measured as %w/w) than their corresponding AE constructs. Greater collagen accumulation was observed in the annular regions of all groups compared to their respective cores (although this was not significant in transiently supplemented SA constructs).

*Total matrix synthesis is greater in hydrogels than self-assembly*

We next wished to determine if the greater levels of sGAG accumulation within the agarose hydrogels (measured in µg) were due to greater total sGAG synthesis or enhanced retention of sGAG within the construct (Figs. 8, 9). Agarose constructs were found to accumulate significantly more sGAG than corresponding SA constructs, but they also released more sGAG to the culture media, with the highest levels of sGAG release observed in transiently supplemented agarose constructs (1447.56±61.88 µg) (Fig. 8A). Continuously supplemented agarose constructs synthesized the greatest overall levels of sGAG (3809.72±48.58 µg). The total amount of sGAG synthesis in these AE constructs was approximately double that of SA constructs. To ascertain whether this increased level of sGAG production was due to changes in cell number, we normalized our results to DNA content (Fig. 8B). We found almost identical trends, indicating that the greater sGAG production within AE constructs was mainly due to an enhanced matrix synthesizing capacity of the encapsulated cells. Agarose hydrogels were also more efficient at retaining sGAG within the construct (Fig. 9), with continuously supplemented constructs retaining

approximately 67% of synthesized sGAG (day 42 samples). All constructs were found to retain a lower % of sGAG at day 42 compared to day 21.

***Discussion***

The objective of this study was to directly compare the self-assembly (SA) method to agarose encapsulation (AE) for engineering cartilaginous grafts using passaged chondrocytes. Two seeding densities were chosen as it is known that a minimum number of cells are required to form stable self-assembled constructs.[31] We found that at a low seeding density of 900,000 cells (46,000 cells/mm$^2$), generating a uniform 5mm diameter SA construct proved difficult. Previous studies have shown that the minimum number of cells needed to generate a uniform 5mm diameter chondrocyte SA construct is 2 million cells (102,000 cells/mm$^2$).[31] At the high seeding density (4 million cells total or 204,000 cells/mm$^2$) we observed that sGAG and collagen synthesis was greater using AE compared to SA. When normalized to wet weight however, SA constructs accumulated significantly greater levels of collagen compared to agarose gels. Consequently, SA led to the formation of an engineered cartilaginous tissue with a ratio of collagen to sGAG more comparable to native articular cartilage. A further benefit of SA is that such grafts can be generated within a relatively short time frame (approximately 3 weeks), with comparable sGAG levels and higher collagen levels to AE constructs. Shortened culture times are important for clinical translation of tissue engineered products.

An inherent advantage to using agarose hydrogels is the ability to easily control the height and width of the engineered tissue. However depending on the cell seeding density, the construct can experience bulging at the top and bottom surfaces, as was observed in the high seeding density AE constructs. Self-assembled constructs reached a maximum thickness of

approximately 1.4mm. This is similar to previous studies[18] where a SA thickness of approximately 1mm was reached. Articular cartilage thickness is on average 2.4 ± 0.5 mm in human medial femoral condyles,[42,43] demonstrating that further optimization is required if SA constructs are to be used to treat full thickness cartilage defects.

sGAG accumulation was greater in agarose constructs compared to SA. To assess if this was simply due to greater retention of sGAG within hydrogels, or due to overall higher levels of sGAG synthesis, we evaluated sGAG release to the media. Both the amount of sGAG released and total sGAG retained was higher in the agarose hydrogels, clearly demonstrating that total sGAG synthesis was higher in this system. To determine if this greater sGAG synthesis was due to greater cell proliferation in the hydrogel environment, we normalized our sGAG data to DNA content. Even by this measure, sGAG synthesis was higher in agarose hydrogels, indicating that altered ECM synthesis and not simply greater proliferation in the hydrogel environment was responsible for this different level of sGAG accumulation. This may be considered an advantage to using agarose, with more sGAG synthesis on a per cell basis, and also a greater percentage of sGAG retained within the constructs. This occurs despite higher levels of collagen accumulation (%w/w) in the SA constructs, which presumably play an important role in proteoglycan retention in engineered tissues, highlighting the benefit of agarose for maintaining synthesized matrix components. The higher levels of cartilage specific ECM synthesis in the AE constructs may be due to the agarose promoting a more spherical chondrocyte morphology, which previously has been shown to support the re-establishment of a chondrogenic phenotype in passaged chondrocytes.[3] It could also be due to the physical separation of cells within the agarose hydrogel, whereas in SA constructs significant cell to cell contact occurs.

While chondrocytes appear more synthetically active in hydrogels, the composition of the engineered tissue (as a % of wet weight), as well as the relative amounts of collagen to

proteoglycans, are more like native articular cartilage in the SA constructs. It has been well documented that achieving native levels of collagen accumulation is more challenging than reaching native levels of proteoglycan accumulation in tissue engineered cartilage.[44-47] Indeed, rapid GAG synthesis has been hypothesized to be an impediment to collagen synthesis in chondrocyte seeded agarose hydrogels, with recent studies demonstrating that inducing enzymatic GAG loss during the early phase of culture can increase the ultimate collagen concentration and tensile properties of the engineered tissue.[48] The local environment within SA constructs would appear to suppress sGAG synthesis while maintaining collagen synthesis at levels approaching that found in the agarose hydrogels. Therefore, in spite of the fact that both sGAG synthesis and retention were lower in SA constructs compared to AE constructs, it would appear that the SA process generates a tissue with a composition more akin to that of native articular cartilage.

By spatially analyzing the biochemical composition of the engineered tissues, we observed greater collagen accumulation in the annulus of constructs compared to their corresponding cores (Fig. 7). This could be due to gradients in nutrients and other regulatory molecules developing within the constructs. It should be noted that sGAG levels were comparable between the core and annuli of continuously supplemented SA constructs. This would suggest that collagen synthesis may be more sensitive to nutrient availability than sGAG synthesis.

It was noted that collagen type II staining was more intense in superficial regions of self-assembled constructs, which is similar to native articular cartilage where staining is generally highest in the superficial tangential zone. Clustering of chondrocytes was also observed in the deeper zones of the SA tissues. Previous studies have demonstrated that organization of cartilaginous tissues generated by self-assembly of MSCs mimic certain aspects of the native articular cartilage architecture. Specifically these tissues stained

intensely for collagen type II, and weakly for proteoglycans, in the superficial region of the engineered tissue.[19] It may be that surface tension developing at the surface of self-assembled constructs is contributing to the higher level of collagen type II production in the superficial region of the developing tissue. Greater nutrient/growth factor availability in this region of the engineered tissue could also play a role.

As transient TGF-β3 stimulation has been shown to enhance chondrogenesis in bovine chondrocyte seeded agarose hydrogels,[17] we compared the effect of such media supplementation conditions on the development of cartilaginous grafts engineered using both the SA and AE approaches. We found no clear benefit to transient TGF-β3 supplementation for either SA or AE, apart from the fact that the fiscal cost of this approach is lower than continuous growth factor supplementation. The discrepancy between our findings and that of previous studies[17] may possibly be due to our use of expanded chondrocytes or species differences.

The lack of mechanical property data is a limitation of this study. The non-uniform shape of the self-assembled tissue (and indeed agarose constructs at high seeding densities, see Fig. 5) led to varying and possibly unreliable mechanical testing results in pilot studies, and such tests were not undertaken as part of the main study. The fact that chondrocytes were obtained from the femoral condyles of a 4 month old pig might also be considered a limitation of the study. At this age such animals have not reached skeletal maturity. Chondrocytes from such tissue would probably be more adept at producing cartilage-specific ECM than chondrocytes obtained from an older donor, as seen in bovine[49,50] and human donors.[51] As with many tissue engineering studies, cells were expanded and differentiated in high glucose (25mM) culture medium. One possible implication of this could be hyperglycaemic conditions leading to the copious production of hyaluronic acid (HA).[52] As rapid GAG synthesis has been hypothesized to be an impediment to collagen production,[48]

this additionally produced HA could inhibit collagen production of our tissue engineered constructs. Future studies will explore the influence of altered glucose conditions on tissue engineered cartilage.

In conclusion, a higher seeding density was required to develop robust cartilaginous grafts using a SA approach. If achieving such high numbers of chondrocytes is clinically feasible, the SA approach has many attractive attributes, including the generation of grafts with a high collagen content, and the development of a tissue with an architecture and a ratio of collagen to sGAG content more closely resembling native articular cartilage. The SA process also generated tissues with such high levels of ECM within a relatively short time-frame. Coupled with the inherent advantages of a scaffold-free approach, the results of this study provide strong support for the future use of the SA approach for engineering functional cartilaginous grafts for clinical applications.

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***Author Disclosure Statement***

No competing financial interests exist.

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**FIG. 1.** Self-assembled constructs at day 42. Scale bar = 5mm.

**FIG. 2.** Wet weight and sGAG content of agarose (AE) and self-assembled (SA) constructs for both transient (AE-, SA-) and continuous (AE+, SA+) TGF-β3 supplementation. **(A)** wet weight for low seeding density constructs (mg); **(B)** wet weight for high seeding density constructs (mg); **(C)** sGAG content for low seeding density constructs (μg); **(D)** sGAG content for high seeding density constructs (μg); **(E)** sGAG content normalized to wet weight for low seeding density constructs (%w/w); **(F)** sGAG content normalized to wet weight for high seeding density constructs (%w/w); **(G)** sGAG content normalized to DNA content for low seeding density constructs (μg/μg); **(H)** sGAG content normalized to DNA content for high seeding density constructs (μg/μg). a: $p<0.05$ vs. self-assembly with same culturing conditions at same time point; b: $p<0.05$ vs. continuous TGF-β3 with same scaffold at same time point. * denotes significant difference with $p<0.05$.

**FIG. 3.** Collagen content of agarose (AE) and self-assembled (SA) constructs for both transient (AE-, SA-) and continuous (AE+, SA+) TGF-β3 supplementation. **(A)** collagen content for low seeding density constructs (μg); **(B)** collagen content for high seeding density constructs (μg); **(C)** collagen content normalized to wet weight for low seeding density constructs (%w/w); **(D)** collagen content normalized to wet weight for high seeding density constructs (%w/w); **(E)** collagen content normalized to DNA content for low seeding density constructs (μg/μg); **(F)** collagen content normalized to DNA content for high seeding density constructs (μg/μg). a: $p<0.05$ vs. self-assembly with same culturing conditions at same time point; b: $p<0.05$ vs. continuous TGF-β3 with same scaffold at same time point. * denotes significant difference with $p<0.05$.

**FIG. 4.** Ratio of collagen to sGAG content of agarose (AE) and self-assembled (SA) constructs for both transient (AE-, SA-) and continuous (AE+, SA+) TGF-β3 supplementation at days 21 and 42. **(A)** low seeding density; **(B)** high seeding density. a: $p<0.05$ vs. self-assembly with same culturing conditions at same time point; b: $p<0.05$ vs. continuous TGF-β3 with same scaffold at same time point. * denotes significant difference with $p<0.05$.

**FIG. 5.** Alcian Blue staining for sGAG production, and type II collagen immunohistochemistry staining of agarose and self-assembled constructs for both transient and continuous TGF-β3 supplementation at day 42. Scale bar = 1mm

**FIG. 6.** High magnification images of type II collagen immunohistochemistry staining of self-assembled constructs for both transient and continuous TGF-β3 supplementation at day 42 (high seeding density constructs). Scale bar = 250μm

**FIG. 7.** Biochemical composition of core and annular regions of agarose (AE) and self-assembled (SA) constructs for both transient (AE-, SA-) and continuous (AE+, SA+) TGF-β3 supplementation at day 42. **(A)** sGAG content normalized to wet weight for high seeding density constructs (%w/w); **(B)** collagen content normalized to wet weight for high seeding density constructs (%w/w). a: $p<0.05$ vs. self-assembly with same culturing conditions at same time point; b: $p<0.05$ vs. continuous TGF-β3 with same scaffold at same time point. * denotes significant difference with $p<0.05$.

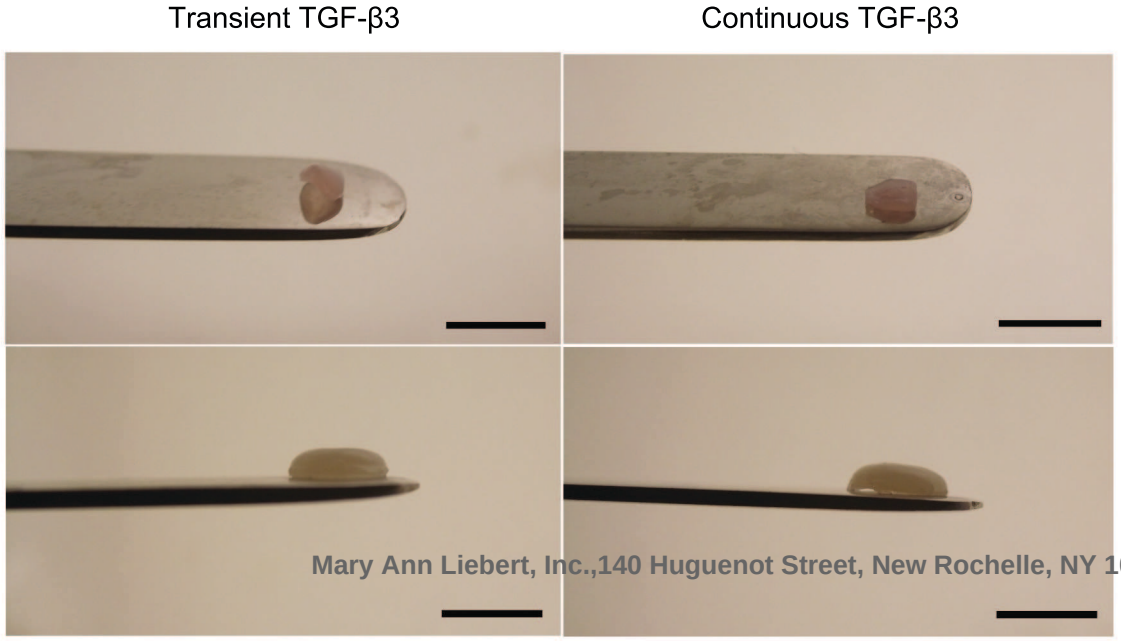
**FIG. 8.** Total sGAG accumulated, released, and produced for agarose (AE) and self-assembled (SA) constructs for both transient (AE-, SA-) and continuous (AE+, SA+) TGF-β3 supplementation over 42 days. **(A)** sGAG for high seeding density constructs (μg); **(B)** sGAG normalized to DNA content for high seeding density constructs (μg/μg). a: $p<0.05$ vs. self-

assembly with same culturing conditions at same time point; b: $p<0.05$ vs. continuous TGF-β3 with same scaffold at same time point. * denotes significant difference with $p<0.05$.

**FIG. 9.** Percentage of sGAG retained in agarose (AE) and self-assembled (SA) constructs for both transient (AE-, SA-) and continuous (AE+, SA+) TGF-β3 supplementation. a: $p<0.05$ vs. self-assembly with same culturing conditions at same time point; b: $p<0.05$ vs. continuous TGF-β3 with same scaffold at same time point. * denotes significant difference with $p<0.05$.

TABLE 1. CONSTRUCT PHYSICAL PARAMETERS OF DIAMETER (MM) AND THICKNESS (MM) FOR CHONDROCYTE ENCAPSULATED AGAROSE AND SELF-ASSEMBLED CONSTRUCTS FOR BOTH LOW AND HIGH SEEDING DENSITIES. A: $P<0.05$ VS. DAY 0; B: $P<0.05$ VS. DAY 21 WITH SAME CULTURING CONDITIONS; C: $P<0.05$ VS. CONTINUOUS TGF-β3 (SAME TIME POINT); D: $P<0.05$ VS. CORRESPONDING GROUP IN LOW SEEDING DENSITY; E: $P<0.05$ VS. CORRESPONDING AGAROSE GROUP.

Fig1




Mary Ann Liebert, Inc.,140 Huguenot Street, New Rochelle, NY 10801

Fig2

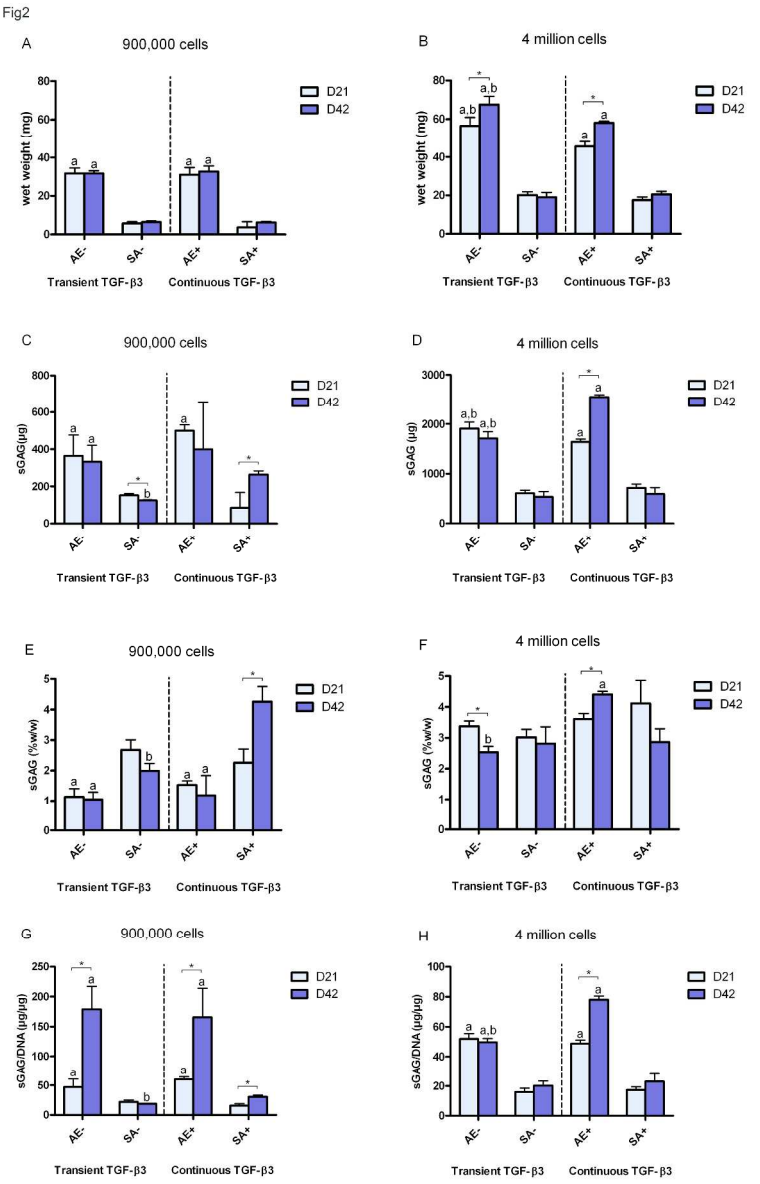



FIG. 2. Wet weight and sGAG content of agarose (AE) and self-assembled (SA) constructs for both transient (AE-, SA-) and continuous (AE+, SA+) TGF-β3 supplementation. (A) wet weight for low seeding density constructs (mg); (B) wet weight for high seeding density constructs (mg); (C) sGAG content for low seeding density constructs (µg); (D) sGAG content for high seeding density constructs (µg); (E) sGAG content normalized to wet weight for low seeding density constructs (%w/w); (F) sGAG content normalized to wet weight for high seeding density constructs (%w/w); (G) sGAG content normalized to DNA content for low seeding density constructs (µg/µg); (H) sGAG content normalized to DNA content for high seeding density constructs (µg/µg). a: $p<0.05$ vs. self-assembly with same culturing conditions at same time point; b: $p<0.05$ vs. continuous TGF-β3 with same scaffold at same time point. * denotes significant difference with $p<0.05$.
270x414mm (300 x 300 DPI)

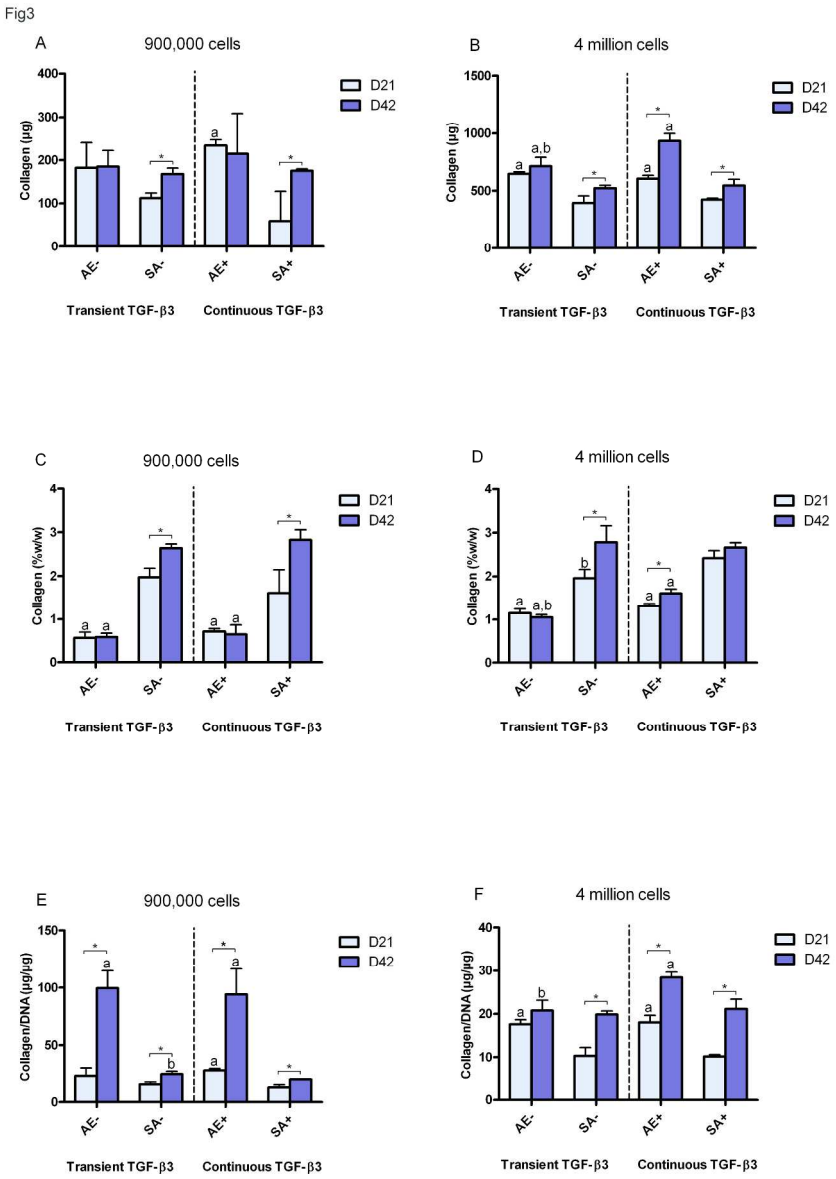



FIG. 3. Collagen content of agarose (AE) and self-assembled (SA) constructs for both transient (AE-, SA-) and continuous (AE+, SA+) TGF-β3 supplementation. (A) collagen content for low seeding density constructs (µg); (B) collagen content for high seeding density constructs (µg); (C) collagen content normalized to wet weight for low seeding density constructs (%w/w); (D) collagen content normalized to wet weight for high seeding density constructs (%w/w); (E) collagen content normalized to DNA content for low seeding density constructs (µg/µg); (F) collagen content normalized to DNA content for high seeding density constructs (µg/µg). a: $p<0.05$ vs. self-assembly with same culturing conditions at same time point; b: $p<0.05$ vs. continuous TGF-β3 with same scaffold at same time point. * denotes significant difference with $p<0.05$.
245x337mm (300 x 300 DPI)

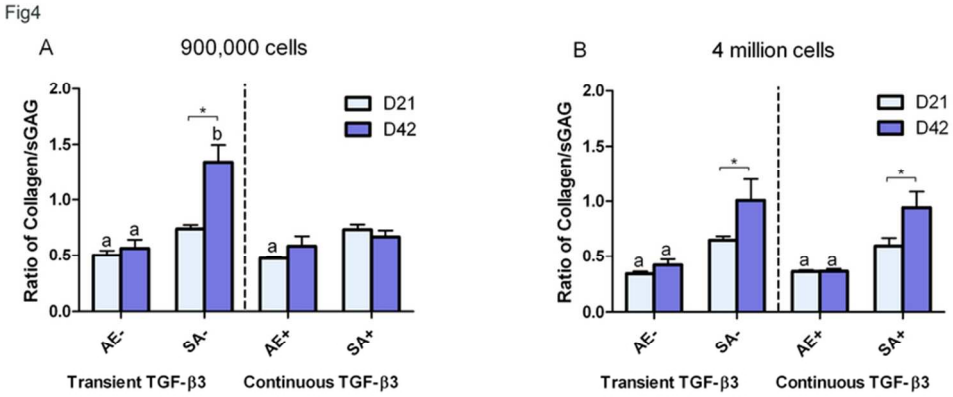
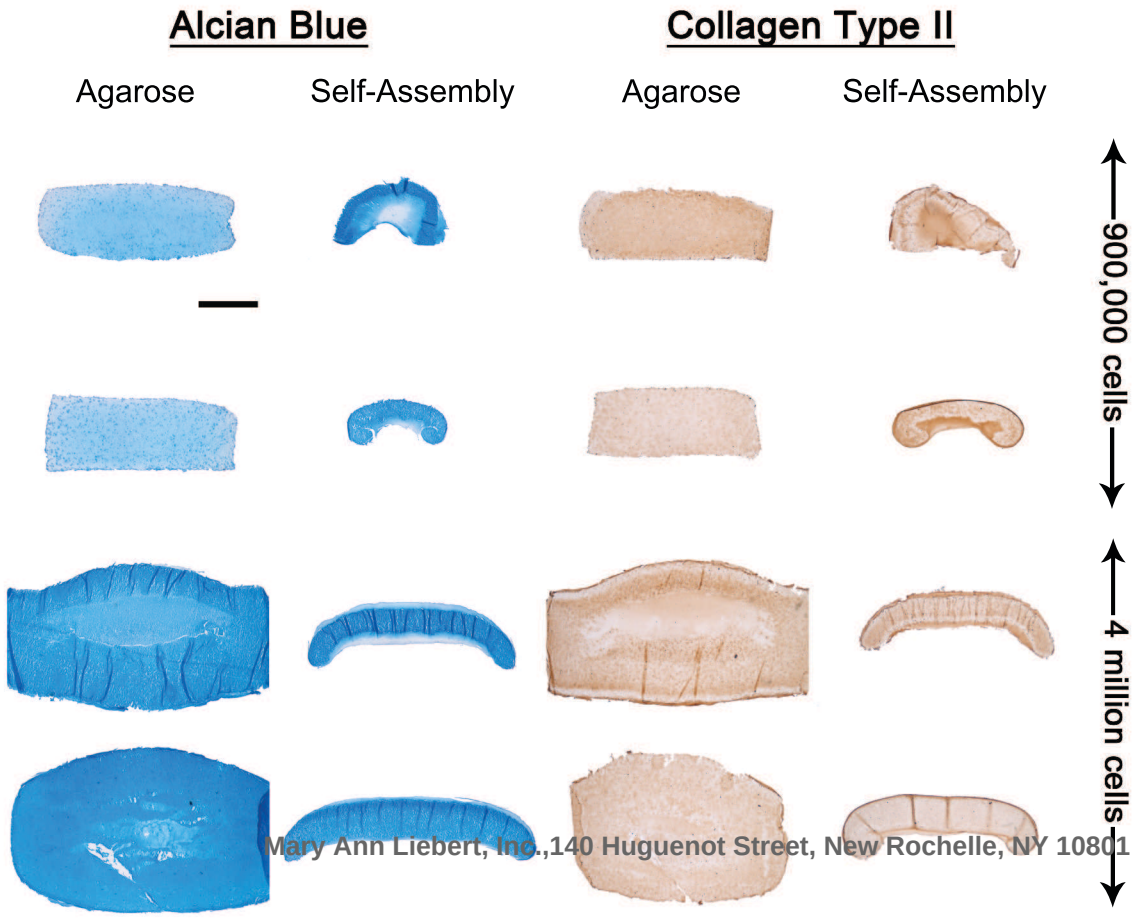



FIG. 4. Ratio of collagen to sGAG content of agarose (AE) and self-assembled (SA) constructs for both transient (AE-, SA-) and continuous (AE+, SA+) TGF-β3 supplementation at days 21 and 42. (A) low seeding density; (B) high seeding density. a: $p<0.05$ vs. self-assembly with same culturing conditions at same time point; b: $p<0.05$ vs. continuous TGF-β3 with same scaffold at same time point. * denotes significant difference with $p<0.05$.
72x31mm (300 x 300 DPI)

Fig5



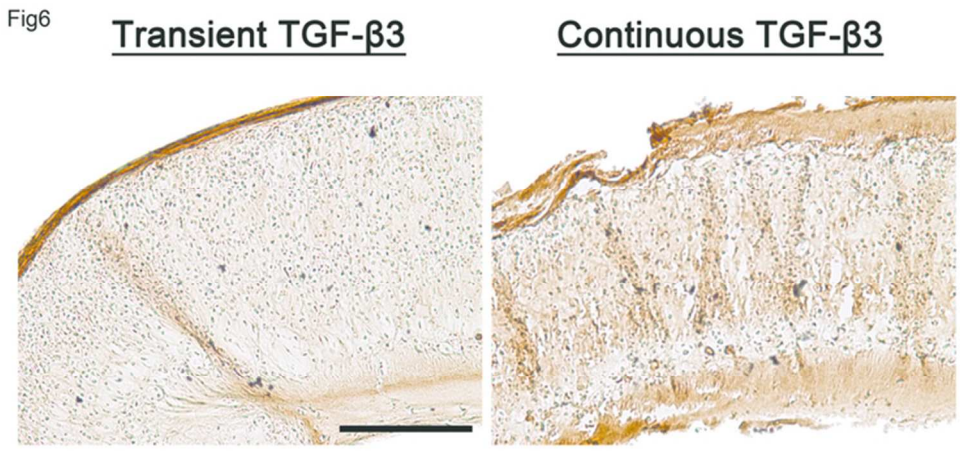



FIG. 6. High magnification images of type II collagen immunohistochemistry staining of self-assembled constructs for both transient and continuous TGF-β3 supplementation at day 42 (high seeding density constructs). Scale bar = 250μm
64x30mm (300 x 300 DPI)

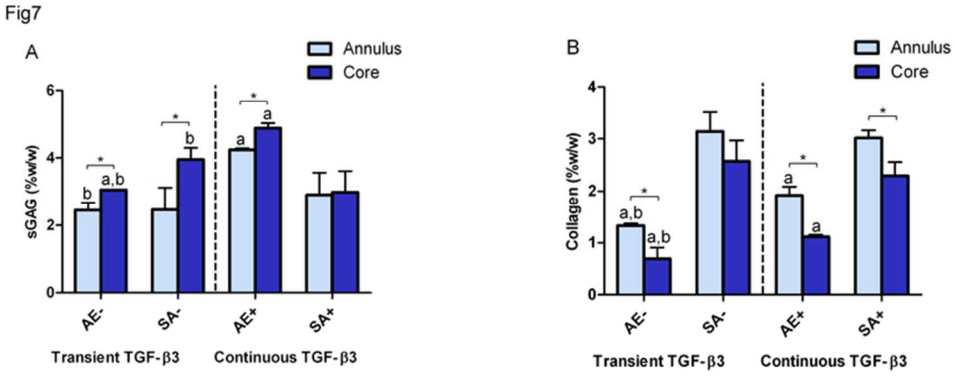



FIG. 7. Biochemical composition of core and annular regions of agarose (AE) and self-assembled (SA) constructs for both transient (AE-, SA-) and continuous (AE+, SA+) TGF-β3 supplementation at day 42. (A) sGAG content normalized to wet weight for high seeding density constructs (%w/w); (B) collagen content normalized to wet weight for high seeding density constructs (%w/w). a: $p<0.05$ vs. self-assembly with same culturing conditions at same time point; b: $p<0.05$ vs. continuous TGF-β3 with same scaffold at same time point. * denotes significant difference with $p<0.05$.
68x27mm (300 x 300 DPI)

Fig8

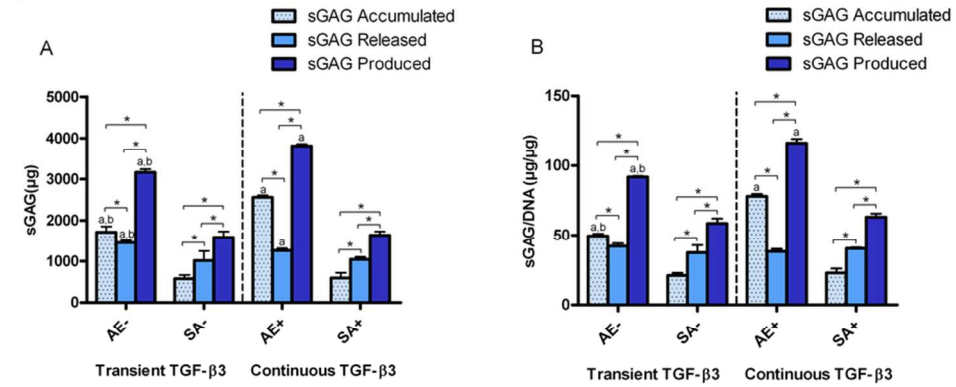



FIG. 8. Total sGAG accumulated, released, and produced for agarose (AE) and self-assembled (SA) constructs for both transient (AE-, SA-) and continuous (AE+, SA+) TGF-β3 supplementation over 42 days. (A) sGAG for high seeding density constructs (μg); (B) sGAG normalized to DNA content for high seeding density constructs (μg/μg). a: $p<0.05$ vs. self-assembly with same culturing conditions at same time point; b: $p<0.05$ vs. continuous TGF-β3 with same scaffold at same time point. * denotes significant difference with $p<0.05$.
81x36mm (300 x 300 DPI)

Fig9

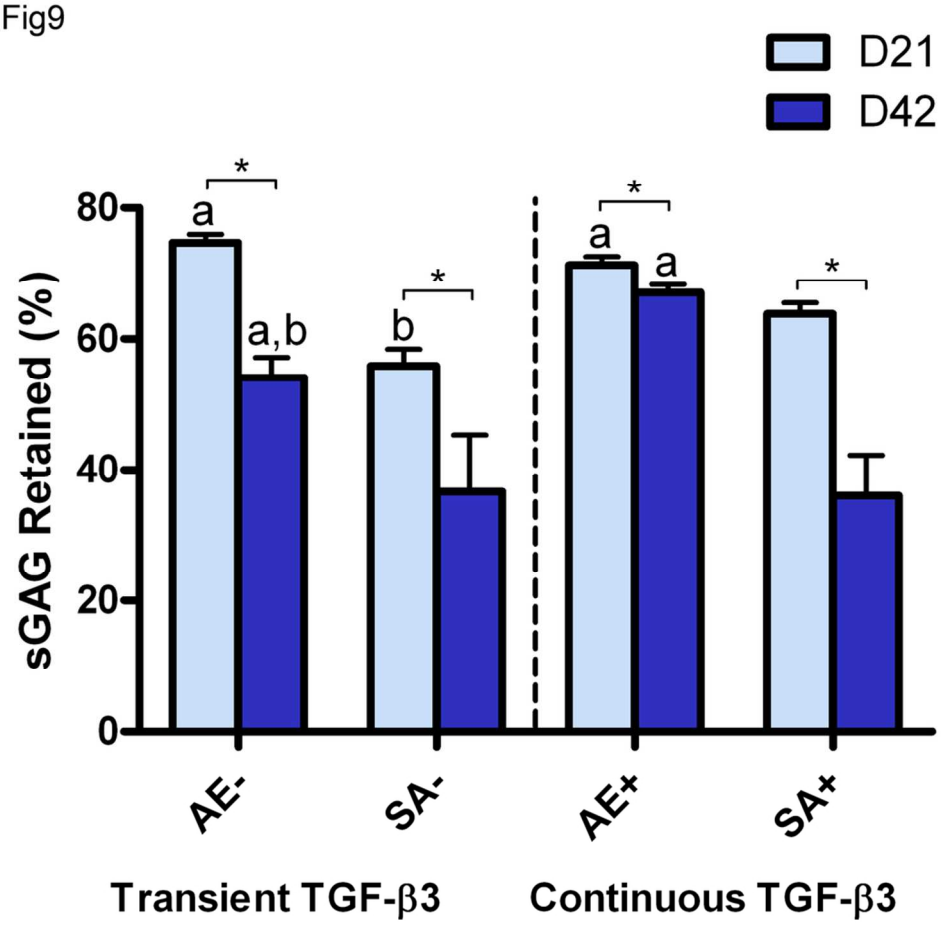



FIG. 9. Percentage of sGAG retained in agarose (AE) and self-assembled (SA) constructs for both transient (AE-, SA-) and continuous (AE+, SA+) TGF-β3 supplementation. a: $p<0.05$ vs. self-assembly with same culturing conditions at same time point; b: $p<0.05$ vs. continuous TGF-β3 with same scaffold at same time point. * denotes significant difference with $p<0.05$.
93x90mm (300 x 300 DPI)

| Construct Type | Parameter | Low Seeding Density | | | | | High Seeding Density | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | D21 | | D42 | | | D21 | | D42 | |
| | | D0 | Transient TGF-β3 | Continuous TGF-β3 | Transient TGF-β3 | Continuous TGF-β3 | D0 | Transient TGF-β3 | Continuous TGF-β3 | Transient TGF-β3 | Continuous TGF-β3 |
| Agarose | Diameter (mm) | 4.97±0.08 | 5.04±0.1 | 5.08±0.04 | 5.08±0.03 | 5.05±0.08 | 5.06±0.18 | 5.53±0.05[a,d] | 5.39±0.19[a] | 5.52±0.06[a,d] | 5.54±0.1[a,d] |
| | Thickness (mm) | 1.56±0.02 | 1.57±0.06 | 1.64±0.01 | 1.62±0.05 | 1.69±0.06[a] | 1.61±0.01[d] | 2.86±0.23[a,c,d] | 2.32±0.16[a,d] | 3.86±0.47[a,b,c,d] | 3.08±0.03[a,b,d] |
| Self-Assembly | Diameter (mm) | 3.62±0.1[e] | 3.03±0.39[e] | 2.31±0.75[a,e] | 3.24±0.3[e] | 3.1±0.18[e] | 4.67±0.29[d] | 5.13±0.04[a,d,e] | 5.04±0.05[a,d,e] | 5.1±0.36[d,e] | 5.18±0.14[a,d,e] |
| | Thickness (mm) | 0.13±0.01[e] | 0.99±0.27[a,e] | 0.69±0.59[a,e] | 1.1±0.13[a,e] | 1.11±0.2[a,e] | 0.23±0.02[d,e] | 1.47±0.38[a,e] | 1.31±0.06[a,e] | 1.35±0.14[a,e] | 1.38±0.1[a,e] |