Tissue Engineering by Molecular Disassembly and Reassembly: Biomimetic Retention of Mechanically Functional Aggrecan in Hydrogel

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In vitro assembly of key functional extracellular matrix constituents for tissue-engineered constructs may provide a tool to modulate the retention of proteoglycan (PG) aggregates, which are crucial to compressive biomechanical properties of connective tissues. This study tested the hypotheses that (1) biomimetic molecular reassembly of PG aggregates (native aggrecan [AGC] with hyaluronan [HA] $\pm$ link protein [LP]) affects AGC retention kinetics in hydrogel constructs, (2) the compressive properties of such hydrogel constructs are related to the content of retained AGC, and (3) the reassembly method is compatible with chondrocytes. Addition of HA to AGC in hydrogel constructs increased AGC retention in a dose-dependent manner, and the addition of LP to AGC + HA further enhanced AGC retention. The level of AGC retention, in turn, was associated with increased equilibrium compressive stress of the constructs. Chondrocytes could be included in the process, and maintained expression of the chondrogenic phenotype, secreting type II collagen but little type I collagen. Thus, by altering the assembly of PG aggregates with HA $\pm$ LP, which affects AGC retention, it may be possible to achieve the targeted levels of PG components to modulate the mechanical properties of the engineered construct for cartilage as well as other tissues containing PG and PG aggregates.

Introduction

THE CONTENT AND TURNOVER OF AGGREGAN (AGC) is central to the functional properties of a variety of connective tissues, including articular cartilage, intervertebral disc, meniscus, ligament, and tendon. The poly-anionic sulfated glycosaminoglycan (sGAG) moieties of AGC contribute to the compressive biomechanical properties of these tissues.^{1–4} This provides the tissue with a high osmotic pressure to retain water, a high resistance to fluid flow, and thereby resistance to compression, and prestress to the collagen network.⁵

Normally, AGC retention in cartilage is governed by the noncovalent aggregation of AGC monomers with hyaluronan (HA) as stabilized by link protein (LP). *In vivo*, up to ~ 100 AGC monomers, each with large numbers of sGAG chains of chondroitin sulfate (CS) and keratan sulfate (KS), noncovalently associate with a HA molecule to form a proteoglycan (PG) aggregate.^{6,7} LP binds to both AGC and HA to further stabilize their interaction and aids in the formation of more uniformly packed aggregates.⁵ This helps to retain the functionally important poly-anionic AGC within the tissue due to the large size of aggregates and their entrapment

within collagen network, especially under dynamic joint loading conditions.

In traditional approaches to cartilage tissue engineering, AGC accumulates in constructs over months as rates of biosynthesis by residing cells are gradually balanced by rates of loss.⁸ Various chemical and mechanical factors have been used to accelerate the accumulation and organization of PG within engineered cartilaginous constructs.^{9,10} To augment the AGC content and to enhance the AGC synthesis and retention by the residing cells, various hydrogels and scaffolds containing one or more PG components have been fabricated and studied, usually containing HA and/or CS and/or their derivatives.^{11–14} Less common are biomaterials containing LP or AGC, either in whole or peptide/truncated forms,^{14,15} and those containing whole AGC have not been targeted for cartilage applications.

In vitro, AGC can be extracted from cartilage and then biomimetically reassembled into aggregates with HA $\pm$ LP.¹⁶ The AGC-HA interaction has been shown to be highest at 0.8% HA (w/w AGC).¹⁷ Addition of LP to the AGC-HA mixture increased aggregate stability and AGC per aggregate, with 6%–8% (w/w AGC) LP (or 3–4:1 LP:AGC molar ratio) resulting in maximum aggregation of $\sim 80\%$ of initial

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AGC content.¹⁸ Various measures of viscoelastic properties in solutions have been shown to be significantly altered by the formation of PG aggregates with AGC and HA as compared to AGC alone, and further increased in the presence of LP, indicating one functional implication of the formation of PG aggregates.^{19,20}

By modulating the assembly of aggregated PG, it may be possible to achieve the desired levels of PG components, which may modulate the mechanical properties of the engineered construct.^{21,22} Additionally, the ability to accelerate the formation of PG aggregates in the engineered tissue has the potential to save a significant amount of time compared to the biological replenishment and redistribution of AGC. Thus, AGC may be a useful component to include during the assembly of cartilaginous constructs—alone, +HA, or +HA+LP. Therefore, this study tested the hypotheses that (1) biomimetic molecular reassembly of AGC with HA $\pm$ LP affects AGC retention kinetics in hydrogel constructs, (2) the compressive properties of such hydrogel constructs are related to the content of retained AGC, and (3) the reassembly method is compatible with chondrocytes and formation of cartilaginous tissue with type II collagen deposition.

Materials and Methods

Preparation of PG components

AGC and LP were purified from bovine calf knee articular cartilage as described previously.^{23–25} Pharmaceutical-grade HA was obtained as Healon[®] (4 MDa) (Advanced Medical Optics). A1D6, containing LP, represents the least dense of the six fractions from the dissociative isopycnic centrifugation (D6) performed on the densest of the three fractions from the associative isopycnic centrifugation (A1). A1D6 fractions were subjected to size exclusion chromatography on a Sephacryl 300 column eluted with 4M guanidine-HCl. The protein-containing fractions were pooled and verified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) to contain LP by molecular size estimation (~44 and 48 kDa). A1D1, containing AGC monomers, represents the densest of six fractions from the dissociative isopycnic centrifugation (D1) performed on the densest of the three fractions from the associative isopycnic centrifugation (A1). A1D1 fractions were verified to contain high concentrations of sGAG that appeared as one sharp band on native agarose gel electrophoresis and had the ability to form large aggregates with HA as verified by native agarose gel electrophoresis and Sephacryl 1000 (S-1000) column chromatography.²⁶

Formation of PG-hydrogel constructs

Disk constructs (d = 4.8 mm, h = 1.5 mm; n = 10–20/group) were composed of 2% low-melting temperature agarose (SeaPlaque; Lonza) with variable amounts of AGC, HA, and LP (Table 1): group I, no AGC; group II, 2 mg/mL AGC; group III, AGC + 0.2% HA (by weight of AGC or 0.004 mg/mL); group IV, AGC + 1% HA (0.02 mg/mL); group V, AGC + 1% HA (0.02 mg/mL) + 5% LP (0.1 mg/mL); or group VI, AGC + 5% HA (0.1 mg/mL). The concentrated PG solutions for each experimental group were dialyzed (molecular weight cut-off [MWCO] 8–10 kDa) against phosphate-buffered saline (PBS), pH 7.0, at 4°C for 18 h to remove the guanidine-HCl to allow for aggregation,

TABLE 1. EXPERIMENTAL GROUPS

Group	In 2% agarose gel	Final concentration [mg/mL]		
		sGAG	HA	LP
I	Control	0	0	0
II	AGC	2	0	0
III	AGC + 0.2% HA	2	0.004	0
IV	AGC + 1% HA	2	0.02	0
V	AGC + 1% HA + 5% LP	2	0.02	0.1
VI	AGC + 5% HA	2	0.1	0

sGAG, sulfated glycosaminoglycan; HA, hyaluronan; LP, link protein; AGC, aggrecan.

which was verified by S-1000 column chromatography.²⁶ The percentage of sGAG contained in each fraction from the S-1000 column chromatography was plotted against K_{av} , the distribution coefficient; K_{av} is inversely related with the molecular size. The dialyzed PG solutions were warmed to 50°C and mixed well with a concentrated solubilized agarose solution, for a final agarose concentration of 2%. The PG-agarose solutions were gelled into 1.5-mm-thick sheets at 4°C for at least 1 h. Disk constructs were punched from the PG-agarose sheets and were placed into 300 μ L (~10 disk volumes) of PBS, pH 7.0, containing protease inhibitors (PIs) (0.5 mM ethylenediaminetetraacetic acid, 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 5 mM benzamidinium-HCl, and 5 mM N-ethylmaleimide) in a U-shaped bottom, 96-well plate with gentle nutation. PBS with PIs was changed and collected daily for subsequent analysis of released PG components. The hydrogel constructs at the end of day 3 were solubilized with 300 μ L of PBS at 80°C for 2 min and analyzed for retained PG components.

Biochemical analysis of released and retained PG components

To determine the sGAG retained and released from the constructs, the dimethylmethylene blue assay was performed on portions of solubilized constructs and PBS from day 1 to 3.²⁷

To quantify the released and retained HA content, portions of solubilized constructs and pooled PBS from day 1 to 3 were digested overnight at 60°C with 0.5 mg/mL proteinase K to remove possible interference from HA binding regions of AGC in the HA assay. The samples were incubated with 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride for 2 h at 37°C to inhibit proteinase K and assayed using HA test kit (Corgenix).²¹ HA was analyzed in PBS pooled from day 1 to 3, because the amount released was small in comparison to sGAG release as determined from a pilot study (Supplemental Material, available online at www.liebertonline.com/ten).

To determine the release and retention of LP in group V, SDS-PAGE was performed on portions of solubilized constructs and PBS pooled from day 1 to 3. The samples for SDS-PAGE were reduced with 10 mM dithiothreitol for 30 min at 37°C, alkylated with 55 mM iodoacetamide for 30 min at 37°C, and separated in 4%–20% gradient Tris-glycine gels. A constant voltage of 125 V (15.6 V/cm) was applied for 105 min in Tris-glycine SDS running buffer (25 mM Tris base, 192 mM glycine, and 0.1% SDS, pH 8.3). The gels were

subsequently stained overnight for proteins with SYPRO Ruby[®] (Invitrogen) and imaged using Storm imaging system (GE Healthcare). The densities of the stained bands of ~40–50 kDa were quantified using ImageQuant software (GE Healthcare) to determine the percentage of LP retained in and released from the constructs.

Histology

To assess the spatial distribution of the sGAG and HA within the agarose constructs, the constructs from groups I, II, and IV–VI were fixed in 4% paraformaldehyde for 3 h, snap-frozen with optimal cutting temperature compound (Sakura Finetek) in isopentane cooled with liquid nitrogen, and cryosectioned across the height of the constructs at 30 μ m thickness. The sections were assessed for retained sGAG with Alcian blue,²⁸ and for retained HA with hydrogen-peroxide-conjugated HA binding protein (Corgenix) and reaction with Vectastain VIP substrate. The sections were imaged using brightfield microscopy (Eclipse TE300; Nikon).

Biomechanical analysis of PG-hydrogel constructs

For compression testing, larger disk constructs ($d = 9.6$ mm; $n = 6$ –8) for groups I, II, and IV–VI were punched from the PG-agarose sheets described above. The constructs were placed in 1.2 mL (~10 disk volumes) of PBS, pH 7.0, containing PIs with gentle nutation for 3 days with daily change of PBS with PIs. At the end of day 3, a stack of two hydrogel constructs was placed in a radially confining chamber between two porous platens filled with PBS + PIs and tested at 30%, 60%, 75%, and 90% compressive strains of initial thickness to equilibrium relaxation times using a mechanical spectrometer as previously described.¹ The relaxation times were sufficient for samples to reach equilibrium, based on stresses that were within $1.4\% \pm 0.6\%$ of the equilibrium values predicted by fits of the load data to a logistic model $y = (A - C)/(1 + e^{-b(x-M)})$. The testing protocol was validated to achieve similar results for an individual sample and a stack of two samples of the same total height, and also to maintain integrity of the constructs during and after the test (Supplemental Material). The high strains were applied to reach higher GAG concentrations, after volume adjustment at each strain, to allow for electrostatic repulsion. At each strain, the peak stress and equilibrium stress, calculated from the measured load at end of ramp compression and at end of relaxation divided by the circular confining chamber area, were analyzed. To assess the contribution to the compressive stress from the retained PGs apart from the contribution from the agarose, assuming the equilibrium stress contributions were additive, the equilibrium compressive stress ascribed to PG at each strain was calculated by subtracting the equilibrium stress of the agarose-only constructs. The secant modulus at equilibrium at each strain was also calculated by taking the slope of the stress–strain data with respect to the origin. The constructs and the PBS bath solution from the compression tests were analyzed for sGAG content after mechanical testing.

Short-term culture of PG-hydrogel constructs with chondrocytes

To determine the effect of the assembled PG on chondrogenic activity, disk constructs ($d = 3$ mm, $h = 1.5$ mm)

containing sterilized PG solutions (groups I, II, and V) in 2% SeaPlaque agarose were prepared at 40°C with the addition of isolated bovine calf chondrocytes at 4×10^6 cells/mL.^{29,30} The AGC and LP were sterilized by 0.45 μ m filtration before PG assembly. After 2 days of culture in Dulbecco's modified Eagle's medium with additives (100 U/mL penicillin, 100 g/mL streptomycin, 0.25 g/mL fungizone, 0.1 mM MEM nonessential amino acids, 0.4 mM L-proline, and 2 mM L-glutamine), 10% fetal bovine serum, and 25 g/mL ascorbate, the constructs were analyzed. Portions of constructs were assessed qualitatively by live/dead staining (Invitrogen) for chondrocyte viability, and after cryosectioning by immunohistochemistry for expression of collagen type I and II,³¹ and by Alcian blue staining for sGAG distribution.

Statistical analysis

Data are presented as mean $\pm$ standard error of the mean. The data expressed in percentages (percent sGAG released and retained and percent HA retained in constructs) were arcsine transformed to improve normality before subsequent analysis. The effects of construct type, along with time for released sGAG, were analyzed by analysis of variance with *post-hoc* Tukey test when significant variation ($p < 0.05$) was detected.

Results

The addition of HA $\pm$ LP to AGC in the dialyzed PG solutions for each experimental group resulted in increased amounts of PG aggregates, as indicated by the increased size of the peaks of sGAG-containing molecules at the lower K_{av} values (Fig. 1). Column chromatography on S-1000 demonstrated that the percentage of sGAG in PG aggregates increased in a dose-dependent manner with HA (2% for group II vs. 31% for group III vs. 34% for group IV vs. 64% for group VI) and increased further with the addition of 5% LP to AGC + 1% HA (81% for group V).

The retention of sGAG in the construct was markedly affected by the presence of HA or HA with LP (Fig. 2A, C). In constructs with AGC only (group II), sGAG was released extensively and rapidly, with 61% or 31 μ g of the sGAG

FIG. 1. Representative S-1000 chromatograms of percent sGAG content from concentrated PG solution of groups II–VI at day 0 to determine the levels of AGC in PG aggregates and in monomeric forms. sGAG, sulfated glycosaminoglycan; PG, proteoglycan; AGC, aggrecan; HA, hyaluronan; LP, link protein.

FIG. 2. Biochemical analysis on the effect of PG aggregation with HA and LP on the release (PBS days 1–3) and retention (construct day 3) of PG components. Released and retained sGAG (A, C) and HA (B, D) from groups I–VI constructs expressed as content (A, B) and percentage (C, D). Symbols + and – indicate the relative amounts of PG components present as compared to other experimental groups (* $p < 0.001$ vs. II, # $p < 0.001$ vs. III, ^ $p < 0.001$ vs. IV). PBS, phosphate-buffered saline.

released on day 1 ($p < 0.001$ vs. day 1 PBS for groups III–VI; $p < 0.001$ vs. days 2, 3 PBS for group II), and 82% total over 3 days, with retention of only 18% or 9 μg by end of day 3. The addition of aggregating components (HA) stabilized AGC in the constructs, increasing 3-day retention in a dose-dependent manner to 31% for 0.2% HA (group III), 61% for 1% HA (group IV), 84% for 5% HA (group VI) (all $p < 0.001$ vs. groups II, III, and IV). Retention was increased further with addition of 5% LP to 87% (group V) ($p < 0.001$ vs. group IV). The other PG components were mostly retained within the constructs at the end of day 3 when present initially. Most of the added HA was retained in constructs (>90% for groups III–VI, $p = 0.11$; Fig. 2B, D). Most of the added LP was also retained in the constructs when present initially ($90 \pm 2\%$ for group V; Fig. 3).

Histological stains for sGAG and HA on cryosections of the constructs at day 3 showed similar trends to the biochemical analysis, with more intense sGAG staining in the presence of HA and LP. Alcian blue staining for sGAG (Fig. 4A) on the cryosections of the constructs was lighter at the edges of AGC-containing constructs without LP (groups II, IV, and VI) and more uniform and intense when PG aggregate components were present (groups IV–VI). HA staining (Fig. 4B) was uniform and intense throughout the constructs containing HA (groups IV–VI).

Compressive properties of the constructs generally increased with the content of sGAG retained in the constructs, which was affected by presence of HA and LP. The peak and equilibrium stresses increased with the application of increasing strains for all constructs. The constructs with enhanced retention of AGC (groups IV, V, and VI) exhibited higher peak stresses and higher equilibrium stresses than did constructs with less retained AGC (groups I and II), which was most evident at higher strains of 75% and 90% (Fig. 5A, B). The sGAG dependence on the equilibrium compressive stress was evident after adjustment for stress contribution from agarose and adjustment in sGAG concentration for the volume changes at each strain (Fig. 5C). The secant modulus was generally higher for constructs with higher AGC retention (group IV, V, VI) and also increased with increasing strain-adjusted sGAG concentration (Fig. 5D).

Chondrocytes were compatible with varying degrees of assembled PG within agarose constructs as indicated by various cell metabolic markers. Chondrocyte viability was qualitatively high in the absence or presence of AGC alone or AGC+1% HA+5% LP (Fig. 6A). In addition, the cells expressed the chondrogenic phenotype as indicated by positive type II collagen immunostaining (Fig. 6B), which was enhanced in the presence of PG aggregates (group V), lack of

FIG. 3. Observation of sodium dodecyl sulfate–polyacrylamide gel electrophoresis for LP (~44 and 48 kDa) of construct at day 3, PBS from day 1 to 3, construct at day 0 for typical group V sample (AGC + 1% HA + 5% LP).

type I collagen staining (Fig. 6C), and pericellular Alcian blue staining indicative of sGAG (Fig. 6D).

Discussion

The tissue engineering approach addressed in the present study was the creation of cartilaginous constructs by biomimetically harnessing the biochemical processes to assemble one of the key functional constituents of the native cartilage, PG aggregate. Biomimetic molecular reassembly of AGC with HA $\pm$ LP enhanced the AGC retention in hydrogel constructs (Figs. 2 and 4), which in turn resulted in differential compressive properties of the constructs (Fig. 5). The addition of HA increased AGC retention in a dose-dependent manner, and the addition of LP to AGC+HA further enhanced AGC retention (Figs. 2 and 4). Additionally, chondrocytes in the PG-containing constructs were able to maintain chondrogenic functions after a short-term culture (Fig. 6). These results support the general concept that a matrix disassembly and reassembly approach is feasible for the engineering of cartilaginous tissue as well as a number of other tissues containing PG and PG aggregates.

PG components from various sources may be utilized to assemble the matrix containing PG aggregates. This study used AGC from immature bovine calf cartilage, which has different GAG profiles in chain length and type (higher CS:KS ratio) than AGC from more mature cartilage.³² While these differences may affect the fixed charge density of the tissue, the GAG content in cartilage is positively correlated with compressive modulus regardless of the level of maturation.¹ Since AGC monomers extracted from mature tissue maintain the ability to form aggregates *in vitro*³³ and PG aggregation is a highly conserved molecular mechanism across species, the approach described here is likely to be applicable to AGC from other sources, including human sources, and to other HA-binding PGs to create a biomimetic matrix that can be used as a homograft. Recombinant forms of AGC and LP, which have been produced and demonstrated to maintain the ability to form aggregates,^{34,35} along with commercially available HA, also may provide other sources of purified PG components.

Agarose was used in the present study as a hydrogel to retain PG components and aggregates, and as model for cartilage tissue engineering. Agarose is somewhat different than articular cartilage in that it lacks the biomechanical characteristics of the collagen network in the latter, providing a high resistance to PG swelling and allowing tissue pressurization. Since agarose has a relatively low tensile modulus, the constructs were fabricated with sGAG concentrations at free swelling state that were relatively low compared to those found in articular cartilage and were not significantly altered in dimension or wet weight by the inclusion of sGAG. However, with application of high compressive strains (e.g., to 90%), the agarose concentration reached ~200 mg/mL, and the sGAG concentration (~15 mg/mL tissue for group VI) is in the range of that in articular cartilage where electrostatic repulsion by sGAGs is marked³⁶ relative to the contribution of agarose to the compressive properties. The biomimetic assembly approach could be extended to PG concentrations approaching physiological levels with biomaterials with higher tensile stiffness closer to that of the target tissue.

FIG. 4. Histological staining on constructs at day 3 to assess distribution of PG components. Alcian blue staining for sGAG distribution (A) and purple staining for HA distribution (B) on sections of constructs from groups I (control), II (AGC), IV (AGC + 1% HA), V (AGC + 1% HA + 5% LP), and VI (AGC + 5% HA). Scale bar = 250 μ m. Color images available online at www.liebertonline.com/ten.

Agarose can provide a reasonable approximation to the pore size of the collagen network in articular cartilage, and thus was useful to assess transport properties. The pore size of 2% agarose is estimated to be ~ 200 nm,³⁷ similar to the ~ 100 nm interfibrillar spacing of collagen fibrils in middle and deep zones of articular cartilage.³⁸ Similar to cartilage, 2% agarose appears to allow for release of unbound AGC monomers and entrapment of AGC in PG aggregates.

The transport of the PG components out of the 2% agarose constructs in this study was consistent with a diffusion process. On the basis of the hydrodynamic radius (R_H) estimations in solution, AGC monomers ($R_H = 57$ nm for 2.6 MDa AGC monomer) should be able to diffuse out slowly over time while much larger PG aggregates ($R_H = \sim 370$ nm for PG aggregate with ~ 30 AGC monomers) should be almost immobile.³⁹ The high HA retention in the constructs may be due to large size of HA (~ 4 MDa), which is larger than those in native cartilage⁴⁰ but similar to those in synovial fluid.⁴¹ The large size of HA also may enhance the AGC retention as compared to HA with smaller molecular weight due to the formation of larger PG aggregates. Since the very small LP molecules (~ 4 – 5 nm) can readily diffuse out and were present in higher molar ratio than AGC, the formation of LP-HA complexes without AGC likely aided in the high LP retention in the constructs.^{42,43}

Such *in vitro* molecular engineering to preset AGC, HA, and LP levels of a matrix to a desired level may markedly quicken the tissue engineering process by avoiding the time normally needed for *de novo* matrix deposition by indwelling cells. AGC turnover in tissues generally takes a long time, with the half-life of PGs estimated to be ~ 150 days in adult dog articular cartilage⁴⁴ and 60–70 days in guinea pig costal cartilage⁴⁵ in *in vivo* studies, and 300–800 days in adult human articular cartilage in explant cultures.⁴⁴ Typically, traditional cartilage tissue engineering constructs take weeks, if not months, to culture and fabricate to reach near physiological levels of AGC accumulation by indwelling cells.⁴⁶ Since the relative amounts of HA or LP to AGC can affect the level of AGC in aggregates,^{18,47} the relative concentrations of AGC, HA, and LP may be varied to modulate the retention of AGC in tissue-engineered constructs. Therefore, the ability to accelerate the formation of PG aggregates in the biomimetically engineered tissue has the potential to save significant amount of time compared to the biological replenishment and redistribution of AGC.

The biomimetic basis of the approach described here, using native molecules and macromolecular structures, is likely to foster biocompatibility. Native PG molecules in cartilage have been suggested to have bioactive functions where they may play a role in binding of other matrix proteins and other biochemical factors that regulate cell function.⁴⁸ In tissue engineering applications, scaffolds containing PG components have been shown to enhance chondrogenesis and

FIG. 5. Compression properties of day 3 constructs from groups I (control), II (AGC), IV (AGC + 1% HA), V (AGC + 1% HA + 5% LP), and VI (AGC + 5% HA). Peak stress (A) and equilibrium stress (B) at each strain (30%, 60%, 75%, 90%). Equilibrium stress less than that of constructs with no AGC (group I) (C) and secant modulus (D) at all strains relative to strain-adjusted sGAG concentration. * $p < 0.05$ and ** $p < 0.01$ versus group I. # $p < 0.05$ and ## $p < 0.01$ versus group II.

FIG. 6. Histo- and immuno-chemical staining on constructs containing chondrocytes after 2 days of culture. Live/dead staining for cell viability (**A**). Collagen type I and II (**B**, **C**) immunochemical staining for chondrogenic function, and Alcian blue staining for sGAG distribution (**D**) on sections of constructs from groups I (control), II (AGC), and V (AGC + 1% HA + 5% LP). Scale bar = 300 μ m. Color images available online at www.liebertonline.com/ten.

matrix production by the indwelling cells, whether they are chondrocytes or stem cells.^{10,49} The use of native PG molecules in tissue-engineered constructs may foster a more native extracellular matrix (ECM) turnover processes to take place within the constructs. The short-term culture of constructs containing immature chondrocytes along with assembled PG aggregates of the native AGC and LP along with HA indicated cell compatibility and chondrogenic activity. Longer-term culture and use of different cell types (in terms of maturity and differentiation state) would help to further elucidate the effect of the addition of assembled native PG components in tissue-engineered constructs. Incorporating substantial quantities of native PG components into a collagenous matrix populated by chondrocytes has the potential to provide grafts with improved mechanical function.

Higher sGAG concentration, as achieved from enhanced AGC retention by aggregation with HA $\pm$ LP and from decreased volume from increasing compressive strain, positively related with compressive resistance apart from the contribution from the agarose. Previous studies with cultured chondrocytes encapsulated in agarose have shown higher compressive stress with increasing sGAG content with time in culture.^{30,50} Depletion of PG in immature car-

tilage has been shown to increase tensile strength²¹ and may alter biomechanical properties in tissue-engineered constructs as well.²² Studies of *in vitro* collagen fibril assembly have shown that the presence of PG, such as CS or AGC, alters size and rate of fibril formation and the mechanical properties of the resulting collagen-PG material as compared to collagen alone.^{51,52} Additionally, the presence of PG in a tissue-engineered construct may aid in the retention of newly synthesized collagen monomers from the indwelling cells (Fig. 6). Thus, alterations in PG content in tissue or engineered constructs may significantly affect the biomechanical properties of the tissue or construct. By modulating the assembly of aggregated PG, which affects AGC retention, it may be possible to create the desired balance between PG and collagen components, to modulate the mechanical properties of the engineered construct.

This article describes the utilization of biomimetic molecular reassembly of native molecules into macromolecular structures, using AGC with HA $\pm$ LP, to modulate AGC retention in hydrogel constructs and, as a result, the compressive properties of the constructs. *In vitro* assembling of key functional ECM constituents of native cartilage has the potential to improve retention of the matrix constituents,

modulate the biomechanical properties of the engineered construct, and significantly reduce the time to produce a mature ECM in tissue-engineered constructs.

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Disclosure Statement

No competing financial interests exist.

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