

The effect of bioactive hydrogels on the secretion of extracellular matrix molecules by valvular interstitial cells

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Abstract

Valvular interstitial cells (VICs) were encapsulated in enzymatically degradable, crosslinked hydrogels formed from hyaluronic acid (HA) and poly(ethylene glycol) (PEG) macromolecular monomers. Titration of PEG with HA allowed for the synthesis of gels with a broad compositional spectrum, leading to a range of degradation behavior upon exposure to bovine testes hyaluronidase. The rate of mass loss and release of HA fragments from the copolymer gels depended on the PEG content of the network. These hydrogels were shown to have the dual function of permitting the diffusion of ECM elaborated by 3D cultured VICs and promoting the development of a specific matrix composition. Initial cleavage of hydrogel crosslinks, prior to network mass loss, permit the diffusion of collagen, while later stages of degradation promote elastin elaboration and suppress collagen production due to HA fragment release. Exogenous HA delivery through the cell culture media further demonstrated the utility of delivered HA on manipulating the secretory properties of encapsulated VICs.

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1. Introduction

Tissue engineers are continually developing new types of scaffold materials that enable the 3D culture of cells in environments that facilitate tissue regeneration. One such class of scaffolds is based on hydrogels that allow direct encapsulation of cells; the gel properties are then tuned to enable cell proliferation and extracellular matrix production. Oftentimes, these design parameters involve tailoring the initial gel porosity, mechanics, and water content, as well as temporal changes in the gel properties through degradation to facilitate the elaboration and distribution of cell-secreted matrix molecules. Beyond engineering the physical and structural properties of the gels, the biofunctionality of the gel can also be altered to transform bioinert scaffolds, those that simply *permit* basic cell

function, to bioactive scaffolds that *promote* or *suppress* selected cellular activities.

Scaffolds formed from the chain polymerization of multi-vinyl macromolecular monomers are increasingly used to create covalently crosslinked hydrogels for cell encapsulation and delivery [1,2]. Since many macromolecules can be functionalized with vinyl groups and copolymerized to form gels, this approach provides a robust materials platform for systematic study of cellular interactions with specific matrix stimuli. For example, macromolecular monomers consisting of a poly(ethylene glycol) (PEG) core flanked by hydrolytically degradable blocks capped with vinyl end groups have been commonly utilized to create bioinert, degradable hydrogel environments for cell encapsulation and tissue engineering [3–7]. The degradation of hydrogel networks used for tissue engineering applications is not only necessary to eliminate synthetic components from regenerated tissue, but is also vital to permitting proper tissue formation.

Generally speaking, the initial mesh size of these covalently crosslinked PEG hydrogels is on the order of 10–100 Å,

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hindering the diffusion of large cell-produced extracellular matrix (ECM) molecules, and often resulting in the accumulation of ECM in the pericellular area and non-homogenous tissue formation [8]. To permit the diffusion of ECM components, investigators have engineered hydrolytically degradable hydrogel networks that erode at a pre-determined rate, leading to an increase in hydrogel diffusion properties with time and permitting matrix secretion and elaboration in 3D. For example, Bryant and Anseth copolymerized poly(ethylene glycol) dimethacrylate (PEGDM) macromonomers with PEG macromonomers containing hydrolytically degradable poly(lactic acid) blocks (PEG–LA–DM) for chondrocyte encapsulation, such that gels synthesized from these precursors degraded to varying extents [8]. After six weeks of culture, chondrocyte-produced collagen content was significantly higher in gels with an increasing amount of degradable crosslinks, but void spaces were visible in the cell–hydrogel constructs at high extents of degradation due to the loss of structural integrity of the gel, illustrating the difficulty in pre-engineering degradation to match tissue evolution [3].

As an alternative to hydrolytically degradable systems, investigators have developed enzymatically labile PEG hydrogels that degrade when exposed to cell-secreted or exogenously applied enzymes [9–15]. Because degradation is localized to the pericellular environment, a potential advantage of cellularly remodeled hydrogels is that the rate of hydrogel degradation may more closely match the rate of tissue formation. External control of degradation through exogenous administration of enzyme can be advantageous when combined with non-invasive monitoring methods for matrix deposition. Specifically, degradation can be accelerated or slowed by administering the enzyme in response to a measure of cellular activity and/or matrix production.

While degradation alters the gel's physical structure to facilitate matrix deposition, it is sometimes necessary to *promote* the rapid production of ECM by cells or to direct the production of a specific ECM component. In hydrogel cultures, this has been accomplished primarily through the use of growth factors. Delivery of growth factors has been achieved passively through supplementation of cell culture media [16] or co-encapsulation [17], sequestration by specific hydrogel chemistries [18–20], controlled release from encapsulated microparticles [21], and controlled release through degradable tethers incorporated into the hydrogel network [22,23]. While effective, growth factor delivery can be costly and requires specific design considerations to ensure protein stability.

As an alternative approach, one could envision a hydrogel network where the structural component is also a bioactive, ECM-promoting component. As the gel degrades, bioactive fragments are released, simultaneously promoting cellular production of matrix components, while allowing for the diffusion of these cell-secreted ECM components. In essence, the gel transitions from a passive environment that simply provides a 3D framework for tissue elaboration to an active environment that promotes cell secretory properties. One such molecule that can be readily modified to form hydrogels with

degradation products that are known to have biological activity is hyaluronic acid (HA) [24]. To exploit this concept, gels based upon hyaluronic acid (HA) were synthesized as carriers for valvular interstitial cells (VICs), the resident cells found in heart valve leaflets. VICs remodel a valve matrix primarily composed of collagen and elastin. Masters et al. demonstrated that when cultured on tissue culture polystyrene, VICs respond to the delivery of HA oligosaccharides by increasing elastin production and decreasing collagen production [25]. Similar effects of HA on other cell types have been observed. In a mouse wound healing model, poly(vinyl alcohol) (PVA) sponge implants releasing HA resulted in decreased granulation tissue deposition compared to control and hyaluronidase releasing sponges [26]. In addition to scarless wound healing, HA substrates have been shown to stimulate the production of an elastin-rich matrix by smooth muscle cells [27].

Here, hydrogels were synthesized through the photoinitiated chain copolymerization of HA-based and PEG-based macromers to produce gels of varying composition, and hyaluronidase (HAase) mediated network degradation was characterized as a function of the gel chemistry. HA:PEG gels formed from four different copolymer compositions (1.38 μM HA + 1.37–11.00 mM PEG) were degraded in the presence of 1 U/mL hyaluronidase. Mass loss, changes in equilibrium swelling, and the amount of HA released from the networks were characterized with time. The effect of network-released and exogenously administered HA on encapsulated VIC ECM production was investigated with specific attention to elastin and collagen production, two major protein components of heart valve tissue.

2. Materials and methods

2.1. Synthesis of methacrylated HA

The sodium salt of hyaluronic acid (HA, $\overline{M}_n \sim 4$ MDa by gel permeation chromatography) was methacrylated using fivefold excess methacrylic anhydride relative to HA primary alcohol groups following a procedure outlined in Smeds et al. [28]. The methacrylated hyaluronic acid (HA-MA) was precipitated twice in absolute ethanol and dialyzed for 3 days against deionized water. ^1H NMR analysis was performed on acid-degraded, HA-MA in deuterated water (Cambridge Isotopes, Andover, MA) to quantify the extent of methacrylation. Methacrylate protons at ~ 6.0 and ~ 5.6 ppm (methylene resonances) and ~ 1.8 ppm (methyl resonances) were integrated and normalized to carbohydrate protons. The product used in this work was functionalized with approximately 20 methacrylates per 100 disaccharide repeat units.

2.2. Synthesis of PEG dimethacrylate

Poly(ethylene glycol) (PEG, $\overline{M}_n \sim 4600$ Da) was dimethacrylated following a synthetic procedure outlined in Sawhney et al. [6]. Briefly, PEG was dissolved in methylene chloride and placed on ice. Under an inert atmosphere, a catalytic amount of triethylamine was added to the flask following the dropwise addition of methacryloyl chloride. After addition, the reaction was removed from ice and allowed to proceed at room temperature overnight. The product was precipitated two times in ethyl ether and dialyzed against deionized water for 3 days. ^1H NMR analysis in deuterated chloroform was used to calculate the degree of methacrylate substitution by comparing the area under the methacrylate peaks (~ 5.6 and ~ 6.1 ppm) to the area under the peak from PEG backbone hydrogens (~ 3.64 ppm). PEG macromer ends were $\sim 85\%$ substituted with methacrylate groups.

2.3. Hydrogel preparation

Hydrogels with an initial macromer solution concentration of 1.38 μM HA and a PEG concentration ranging from 1.37 to 22.0 mM (Table 1) were formed by photoinitiated chain copolymerization. The photoinitiator 1-[4-(2-hydroxyethoxy)-phenyl]-2-hydroxy-2-methyl-1-propanone (Irgacure 2959, Ciba Specialty Chemicals, Tarrytown, NY) was added at a solution concentration of 0.0375 wt%. Solutions were exposed to UV light ($\lambda = 365$ nm) at 5 mW/cm² for 7 min. Hydrogels (~5 mm in diameter, ~1 mm in thickness) were transferred to well-plates containing PBS and incubated at 37 °C until equilibrated.

2.4. Hydrogel degradation

Hydrogels were degraded for up to 30 days at 37 °C with 0, 1, and 10 U/mL bovine testes hyaluronidase (Sigma) in PBS. During this degradation period, samples were removed periodically, freeze-dried for 24 h and weighed to determine mass loss. For calculations of mass loss, the dry mass at time, t , was compared to the average initial dry mass of hydrogels of the same composition. The initial dry mass was determined by freeze-drying hydrogels after swelling in PBS for 2–3 days to remove any extractable sol fraction. The equilibrium mass swelling ratio, q , was then calculated with time by measuring the equilibrium swollen mass of the hydrogels prior to freeze-drying and dividing the swollen hydrogel mass by the dry mass. Enzyme solutions were changed at least every 4 days to ensure retention of enzyme activity.

2.5. Determination of HA release with time

To ascertain how much HA was released with time during copolymer network degradation, a colorimetric assay for uronic acid content was performed on the solution surrounding the hydrogels during degradation. Uronic acids encompass a variety of carboxylic acid sugars. In the case of HA, the uronic acid component is D-glucuronic acid. Using a protocol developed by van den Hoogen et al., 40 μL samples of HA released from the degrading networks were completely hydrolyzed in 200 μL of 80% (v/v) sulfuric acid containing 120 mM sodium tetraborate for 1 h at 80 °C [29]. The cooled, hydrolyzed product was then mixed with 40 μL of *m*-hydroxydiphenyl reagent (100 μL of 100 mg/mL *m*-hydroxydiphenyl dissolved in dimethyl sulfoxide, mixed with 4.9 mL 80% (v/v) sulfuric acid just before use). After incubation at room temperature for 15 min, the absorbance of the samples was read at 520 nm.

Table 1
Composition of HA:PEG macromonomer solutions and equilibrium swollen hydrogels

Monomer formulation				
[HA] (μM)	[PEG] (mM)	Concentration of double bonds		HA (% dry mass)
		[C=C] _{HA} (mM)	[C=C] _{PEG} (mM)	
1.38	1.37	2.75	2.68	46.6
1.38	2.76	2.75	5.35	30.2
1.38	5.50	2.75	10.7	17.9
1.38	11.00	2.75	21.4	9.8
1.38	22.00	2.75	42.8	5.2
Equilibrium swollen gel composition				
Water content, q (mass swelling)	HA in gel (wt%)	PEG in gel (wt%)	[HA] in gel ($\mu\text{g/mL}$)	
37.3 \pm 0.9	1.14	1.30	11,640	
30.6 \pm 1.1	0.99	2.28	10,210	
26.5 \pm 1.0	0.67	3.10	7000	
15.7 \pm 0.4	0.62	5.74	6670	
12.4 \pm 0.1	0.42	7.65	4520	

2.6. VIC isolation and culture

Fresh porcine hearts were acquired from Quality Pork Processors, Inc. (Austin, MN) and used within 24 h of slaughter. Aortic valve leaflets were excised from the hearts and subjected to a 30-min incubation in Earle's balanced salt solution containing 250 U/mL collagenase to remove endothelial cells, followed by a 60-min incubation in fresh collagenase solution (250 U/mL) to yield VICs. The VIC suspension was poured through a 100- μm cell strainer and centrifuged at 1000 rpm for 10 min. The cell pellet was re-suspended in VIC culture medium, consisting of 15% FBS, 50 U/mL penicillin–streptomycin, 20 $\mu\text{g/mL}$ gentamicin, 0.5 $\mu\text{g/mL}$ amphotericin B and 2 mM HEPES in Medium 199 (Invitrogen, Carlsbad, CA) and plated onto tissue culture dishes. VICs were cultured at 37 °C in a 5% CO₂ environment and used between passages 1–3 for all experiments.

2.7. VIC encapsulation within PEG:HA copolymers

VICs were added to a sterile macromer solution of 1.38 μM HA, 1.37 mM PEG, and 0.0375 wt% 1-[4-(2-hydroxyethoxy)-phenyl]-2-hydroxy-2-methyl-1-propanone (Irgacure 2959, Ciba Specialty Chemicals, Tarrytown, NY) in PBS at a final density of 22 million cells/mL. The cell–macromer suspension was transferred to sterile molds (40 $\mu\text{L}/\text{mold}$) and exposed to UV light ($\lambda = 365$ nm) at 5 mW/cm² for 7 min. Photopolymerized cell–gel constructs (~5 mm in diameter, ~1 mm in thickness) were transferred to well-plates containing cell culture medium and placed on an orbital shaker for the duration of the study.

2.8. Histological staining for collagen

Cell–gel constructs were fixed at 4 °C in 10% buffered formalin for 24 h after two and four weeks of culture. After a 30-min incubation in 30 wt% sucrose at room temperature, constructs were mounted in Cryo-Gel embedding medium (Instrumedics, Inc.) and cryosectioned into 8 μm sections. Slides were incubated in Scott's tap water for 10 min and stained using a standard Masson's Trichrome method [30].

2.9. Immunohistochemical staining for elastin

Cell–gel constructs were fixed and sectioned as described above. Sections were post-fixed in 10% buffered formalin to increase adhesion to the slides. Pepsin antigen retrieval was performed for 5 min prior to blocking and staining. Anti-elastin mouse monoclonal antibody, recognizing insoluble elastin, α -elastin, and tropoelastin, was used at a dilution of 1:50 (Abcam). Biotinylated anti-mouse IgG secondary antibody and streptavidin–HRP were applied from a ready-to-use VectaStain Elite ABC kit (Vector Laboratories). Vector NovaRed (Vector Laboratories) was used as the peroxidase chromogen, resulting in a reddish-brown color to indicate positive staining.

2.10. Biochemical assay for hydroxyproline content

Constructs were freeze-dried for 24 h and digested in 100 U/mL hyaluronidase (Sigma) solution for 1 h at room temperature prior to overnight digestion in a papain solution, containing 125 $\mu\text{g/mL}$ papain (Worthington Biochemical), 10 mM L-cysteine (Aldrich), 100 mM phosphate, and 10 mM EDTA at pH 6.3 and 60 °C. Total collagen content was determined indirectly using a hydroxyproline assay [31]. Hydroxyproline accounts for ~10% of the chemical content of collagen [32]. Due to differences in cell proliferation within hydrogel constructs, results were normalized to DNA content. DNA content was measured using Hoechst 33258 dye (Polysciences, Inc.) [33].

2.11. Western blotting for elastin content

After digestion of cell–gel constructs as described above, digests were analyzed for total protein content using the Micro BCA Protein Assay (Pierce). Equal protein quantities were separated by SDS-PAGE (10% Ready Gel Tris–HCl gels, BioRad) and electroblotted onto PVDF membranes (BioRad).

Anti-elastin mouse monoclonal antibody (1:200, Abcam), goat anti-mouse-HRP (1:1000, Invitrogen), and the Opti-4CN Detection Kit (BioRad) were used to detect elastin bands. After scanning the blot on a flatbed scanner, ImageJ software (NIH) was used to analyze band intensity. The intensity values for elastin bands were normalized to construct DNA content to obtain a value for elastin production on a per cell basis.

3. Results and discussion

Copolymer hydrogels based on multimethacrylated HA and dimethacrylated PEG provide many unique advantages for VIC encapsulation. Previous studies have shown that a range of HA concentrations exists to support VIC survival when encapsulated in HA:PEG copolymer gels [34]. Specifically, HA was found to promote VIC survival up to two weeks at concentrations greater than 1.38–5.50 μM . The synthetic PEG component enables well-characterized, systematic control over the final network properties and composition. By manipulating either the macromer structure or relative comonomer concentration, gels are readily synthesized with a range of properties, including water content, stiffness, diffusivity, and degradation profiles. Understanding and tailoring the degradation-dependent properties of these gels are critical for tissue engineering applications to permit the diffusion and distribution of extracellular matrix proteins; further, hyaluronidase-mediated degradation of HA:PEG hydrogels results in the release of bioactive HA fragments that have the ability to influence the composition of the ECM elaborated by 3D cultured VICs.

3.1. Hydrogel characterization

The specific gel compositions explored in this work are outlined in Table 1. HA, $\overline{M}_n \sim 4$ MDa, was functionalized with approximately 1 methacrylate per 5 disaccharide repeat units. PEG, $\overline{M}_n \sim 4600$ Da, was end-capped with methacrylate groups (Fig. 1A and B). From the perspective of network connectivity and crosslinking density, the initial HA:PEG co-macromer formulations ranged from a composition with an equivalent carbon–carbon double bond contribution from each macromer to a composition where the carbon–carbon double bond contribution by the PEG macromer was roughly 16 times greater than that of the HA macromer. As illustrated in Fig. 1, the molecular weight of the kinetic chains (i.e., polymethacrylate chains) that result from the subsequent polymerization of the HA and PEG macromers increases with increasing carbon–carbon double bond concentration. In addition, as the number of carbon–carbon double bonds contributed by PEG increases, more PEG is incorporated into the growing kinetic chains, and the network connectivity is dramatically different. Both the network composition and structure directly influence the initial gel properties and the rate and extent of degradation with time.

By manipulating the initial macromer content, the relative amount of HA within the final hydrogel networks was manipulated. Here, with increasing PEG macromer content (1.37–22.00 mM), the total macromer mass increased, but the dry

mass contribution from HA (expressed as a percentage of the total dry mass) decreased from 46.6% to 5.2%. Increasing the PEG concentration and the total concentration of carbon–carbon double bonds also resulted in more densely crosslinked gels with lower equilibrium water contents, q (mass swelling ratio). As summarized in Table 1, q varied from 12.4 to 37.3, which corresponds to gels containing 91.9–97.6 wt% water. The equilibrium water content depends on several factors: the gel composition, the charge density, and the number of elastically active crosslinks. Here, the predominant factor is the crosslinking density, as PEG and HA have similar interaction parameters with water, and the ionically charged groups on HA are shielded by the buffer salts in the swelling medium [34].

Finally, the equilibrium composition of the gels is reported in terms of the wt% of the PEG and HA, as well as the equilibrium concentration of HA in the gel (prior to degradation), which ranges from ~ 4500 to $\sim 11,600$ $\mu\text{g}/\text{mL}$. These latter values are important as previous studies in 2D VIC culture have shown that soluble HA, even at concentrations as low as 3 $\mu\text{g}/\text{mL}$, influences VIC secretory properties [25].

3.2. Characterization of hydrogel degradation

An examination of hydrogel mass swelling ratios with time, in addition to mass loss profiles, provides insight into the degradation mechanism. If bulk degradation is occurring, the mass swelling will increase due to the cleavage of hydrogel crosslinks and a decrease in the overall crosslinking density. When sufficient crosslinks are cleaved from the network, resulting in the insoluble gel transitioning to a collection of highly branched soluble chains, a point of reverse gelation is reached and all mass is lost from the system. If surface degradation dominates, mass swelling will remain relatively constant and complete degradation results when the surface eroding fronts converge.

3.2.1. General degradation and mass loss behavior of PEG:HA gels

Fig. 2 shows the mass swelling and mass loss profiles as a function of time for the copolymer composition containing 1.140 wt% HA exposed to 1 U/mL hyaluronidase. Fig. 2A shows that q increases with time, indicative of a bulk degradation mechanism where the rate of enzyme diffusion is much faster than the rate of reaction. Significant mass loss in this system is not observed until after ~ 6 days of enzyme treatment (Fig. 2B). This delay is due to insufficient substrate cleavage to produce releasable fragments because of the high connectivity within the gel (Fig. 2C). Significant mass loss takes place between 6 and 14 days of hyaluronidase treatment, which is primarily released HA fragments, and reverse gelation occurs at ~ 14 days. The HA release profile (Fig. 2B, secondary axis) tracks well with the mass loss profile, as HA accounts for $\sim 47\%$ of the dry polymer mass and is the only cleavable substrate.

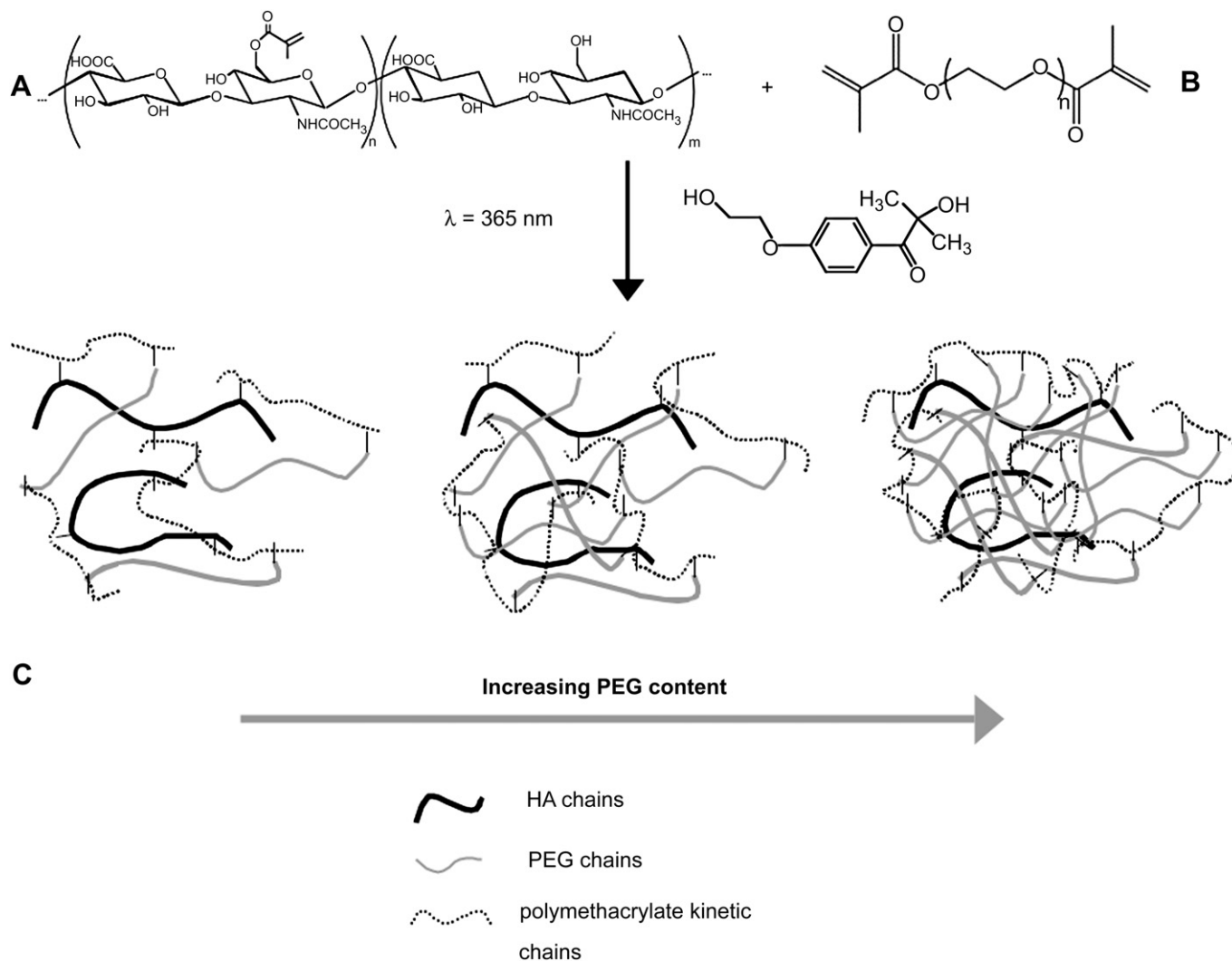


Fig. 1. A) Multimethacrylated HA with $m+n$ ($m+n \approx 10,000$) repeat units ($M_r \approx 400$ Da). In this study, ~ 1 in 5 disaccharide units was functionalized with a methacrylate group. (B) Dimethacrylated PEG chains with n repeat units ($M_r \approx 44$, $n \approx 105$) were end-capped with methacrylate groups. (C) Hydrogel networks formed by radical initiated chain copolymerization of HA with varying amounts of PEG. Primary radicals were produced by the dissociation of the photoinitiator, 1-[4-(2-hydroxyethoxy)-phenyl]-2-hydroxy-2-methyl-1-propanone, in the presence of UV light ($\lambda = 365$ nm).

3.2.2. Effect of altering copolymer composition on gel degradation

Increasing PEG content was introduced into the hydrogel networks as one means of altering the equilibrium concentration of HA within the networks. Fig. 3A shows changes in the copolymer gel mass swelling ratio with time and composition. Hydrogels containing equilibrium concentrations of 0.99 wt% HA + 2.28 wt% PEG and 0.67 wt% HA + 3.10 wt% PEG demonstrated an increase in q with time when treated with 1 U/mL hyaluronidase, implying a bulk degradation mechanism with 1.14 wt% HA + 1.30 wt% PEG gels. Hydrogels containing 0.624 wt% HA + 5.74 wt% PEG exhibited little overall change in the mass swelling ratio with degradation time. A statistical increase in q was seen between 0 and 6 days, but q did not change significantly between 6 and 30 days. This indicates that the enzyme can diffuse into these more highly crosslinked networks, but substrate limitations may be hindering or slowing degradation, which correlates to little changes in q with time.

With increasing PEG content and increasing total carbon-carbon double bond content (i.e., increasing crosslinking density), the rate of mass loss decreased, and the onset of reverse gelation, or complete dissolution of the gel, was delayed (Fig. 3B). Hydrogels containing 0.67 wt% HA + 3.10 wt% PEG demonstrated very little mass loss at early time points, although significant increases in q were observed during this time. Enzymatic cleavage of HA glycosidic bonds resulted in a decrease in the gel crosslinking density and corresponding increase in its water content, but sufficient cleavage to allow substantial release of network fragments and measure corresponding mass loss has not yet occurred. This observation is most likely due to the decrease in the concentration of potential degradation sites within these networks. Additionally, with increasing PEG content, the loss of PEG chains from the network will not be observed until later and later stages of gel degradation, as PEG is released as branches attached to the polymethacrylate chains (Fig. 2C).

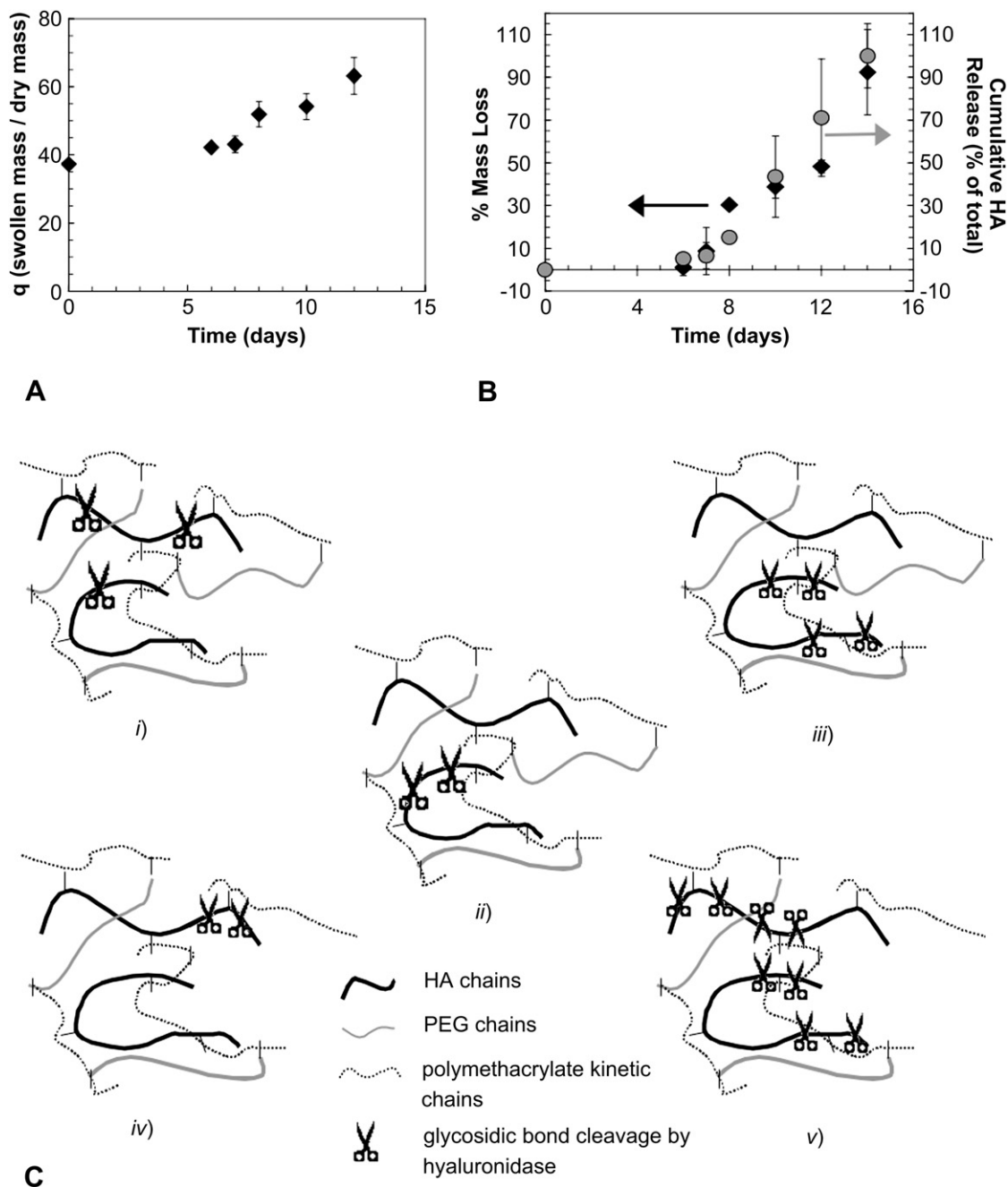


Fig. 2. Hydrogels containing 1.14 wt% HA and 1.30 wt% PEG were treated with 1 U/mL hyaluronidase. (A) Hydrogel mass swelling ratio, q , was measured with degradation time. (B) Mass loss (diamonds) and cumulative HA release (circles) from the hydrogels was also assessed. Cumulative HA release was expressed as a percentage of total HA contained within the network (secondary axis, B). (C) The cleavage of one bond between reacted HA methacrylate groups (*i*) is insufficient to release mass, but will increase the network mesh size and swelling. Mass loss is possible when (*ii*) two glycosidic bonds are cleaved between consecutive HA methacrylate groups, when (*iii*) degradation occurs on either side of two consecutive HA methacrylates reacted into a kinetic chain, and (*iv*) when degradation occurs on either side of a HA methacrylate and all other linkages to the same kinetic chain have been broken. PEG will be released from the network (*v*) when HA chains connected to the same kinetic chains have been freed from other network connections.

Gels containing 0.62 wt% HA + 5.74 wt% PEG exhibited very little mass loss overall ($\sim 11\%$) with no statistically significant mass loss observed past day 10 of the study (Fig. 3B). Initially, mass loss did occur in the bulk as indicated by swelling data (Fig. 3A). The cessation of mass loss is most likely due to the exhaustion of degradation sites (i.e., HA content). This hypothesis was further supported by degradation

of 0.62 wt% HA + 5.74 wt% PEG systems with 10 U/mL hyaluronidase. After 30 days of degradation, gels treated with 10 U/mL hyaluronidase exhibited slightly higher degrees of swelling than gels treated with 1 U/mL hyaluronidase ($q = 24.9 \pm 1.5$ compared to 20.9 ± 0.3), but did not lose any additional mass (%mass loss = 13.1 ± 1.4 compared to 11.6 ± 1.7). The higher enzyme concentration cleaved more

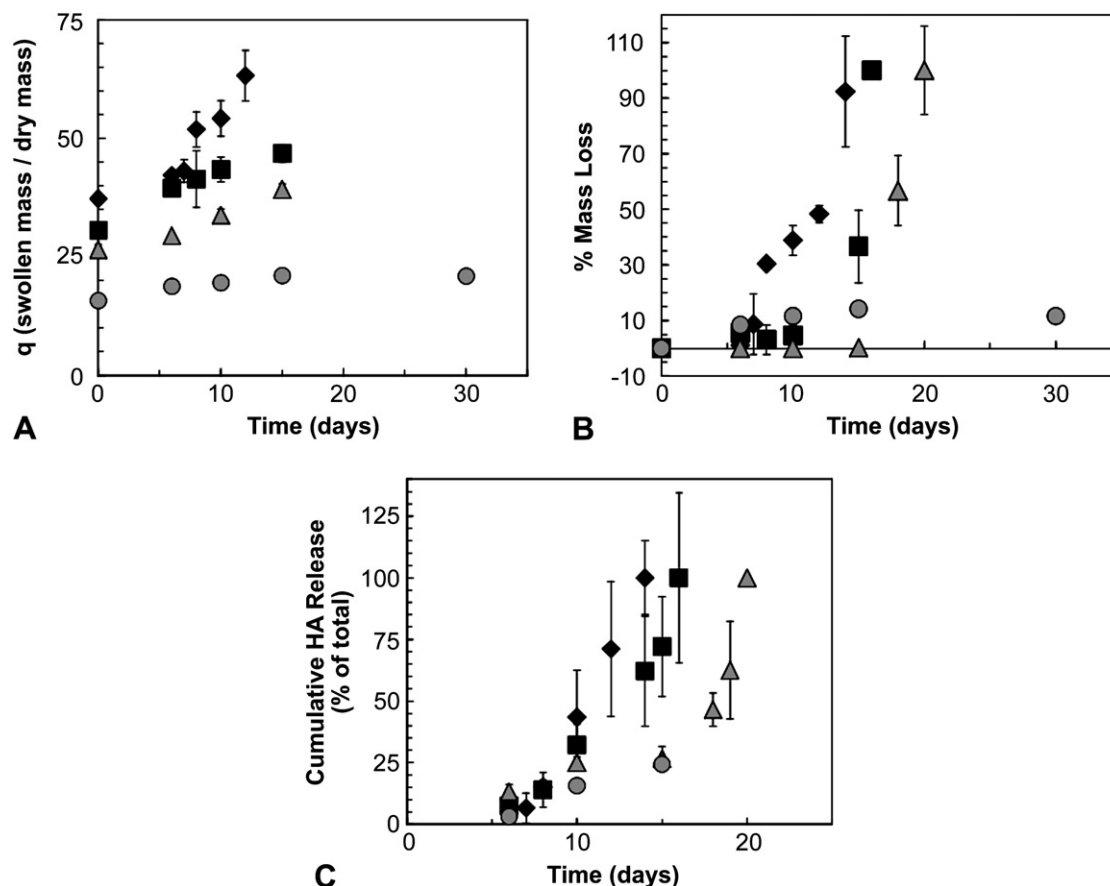


Fig. 3. Effects of 1 U/mL hyaluronidase on the degradation characteristics of HA:PEG hydrogels. Specifically, (A) mass swelling ratio, (B) percent mass loss, and (C) cumulative HA release were characterized. Data in A–C correspond to gels containing 1.14 wt% HA + 1.30 wt% PEG (diamonds), 0.99 wt% HA + 2.28 wt% PEG (squares), 0.67 wt% HA + 3.10 wt% PEG (triangles), 0.62 wt% HA + 5.74 wt% PEG (circles).

substrate within the same amount of time, but these sites were not in a configuration that enabled their release from the network. This result brings to light that not all copolymer compositions will reach a reverse gel point, or critical point of complete solubility.

With the goal of using degrading HA:PEG gels as a dual encapsulation and ECM-promoting delivery vehicle, the rate of HA released from the networks was characterized. Because these networks are copolymer systems of HA and PEG, mass loss cannot be attributed to one specific component. An assay was used to determine the uronic acid content, specifically the glucuronic acid content, in the degradation solution. These results are shown in Fig. 3C. In general, the HA release profiles closely match that of mass loss, and is dependent on the copolymer compositions. Specifically, increasing PEG content slows the release of HA fragments and alters the overall HA release profiles. Once detectable mass loss was achieved, HA release was sustained in 1.14 wt% HA + 1.30 wt% PEG and 0.99 wt% HA + 2.28 wt% PEG copolymer gels over an 8–10-day period; 0.67 wt% HA + 3.10 wt% PEG copolymers showed a period of low-level, sustained HA release, followed by a period of negligible release, and finally a period of increased release up to reverse gelation. Copolymers containing the highest amount of PEG released the lowest amount of HA

during the course of the study, which is consistent with the low release of mass from these systems.

Collectively, these results demonstrate how the addition of PEG to HA hydrogel networks can be used to achieve a variety of mass loss and HA delivery profiles in the presence of a constant hyaluronidase concentration of 1 U/mL and that bulk degradation can be achieved for a variety of copolymer gel compositions. Previous work to characterize the hyaluronidase-mediated degradation of homopolymer HA networks has shown that surface erosion profiles are prevalent [35]. If exogenous delivery of hyaluronidase is the primary mechanism of controlling gel degradation, the surface degrading hydrogel formulations are undesirable for 3D VIC culture, as encapsulated cells would be released along with gel mass. Bulk degrading systems that release HA fragments to encapsulated cells is much more desirable. Alternatively, if cell-secreted hyaluronidase is used as the primary degradation modality, then a much broader range of gel compositions can be employed.

3.3. Delivering HA to encapsulated VICs – effects on matrix synthesis

Hydrogels HA (1.14 wt%) + PEG (1.30 wt%) exposed to 1 U/mL hyaluronidase provide a strategic composition in

which to observe the effect of degradation-induced changes in hydrogel diffusive properties on VIC ECM production, almost entirely separate from the effect of released HA fragments. Acellular gels exposed to 1 U/mL hyaluronidase demonstrated significant increases in equilibrium mass swelling ratio after one week of degradation (q ranging from 37.3 ± 0.9 to 43.1 ± 2.4), but little mass loss and HA release were observed (Fig. 2). After two weeks of enzyme treatment, acellular gels completely eroded, releasing significant amounts of HA during the one- to two-week period. To investigate the effects of the erosion of network crosslinks and soluble HA delivery on encapsulated VICs, cell-laden constructs were exposed to three categories of treatments: (1) exposure to 1 U/mL hyaluronidase for zero or one week to alter the network crosslinking density and diffusive properties of the gel without substantial release of HA, (2) exposure to hyaluronidase treatment for two weeks resulting in a combination of increasing gel diffusive properties with the simultaneous release of HA, and (3) a combination of enzyme treatment for zero, one or two weeks to alter the gel diffusive properties, followed by exogenous HA supplementation through the cell culture media at a concentration of 1.2 mg/mL during weeks three and four. The temporal delivery profiles are outlined schematically in Table 2.

3.3.1. VIC ECM production in response to changes in network structure

The ECM production of VICs encapsulated within HA-PEG networks and treated with 1 U/mL hyaluronidase for zero or one week was evaluated histologically. Networks degraded with hyaluronidase for one week experienced a decrease in crosslinking density, but very little HA fragment release. Histological data provides the opportunity to observe how changes in network structure facilitate the distribution of encapsulated VIC ECM. Masson's Trichrome staining was used to visualize the collagen distribution within cell-gel constructs as a function of hyaluronidase exposure. As can

be seen in Fig. 4A, constructs that did not receive hyaluronidase treatment show intense staining for collagen immediately around the cell body, but no staining throughout the construct. Gels subjected to hyaluronidase-mediated degradation for the first week of culture show a better distribution of collagen away from the cell body, indicating that degradation permitted the diffusion of collagen throughout the scaffold. Additionally, an increase in cell number is apparent in the degrading constructs.

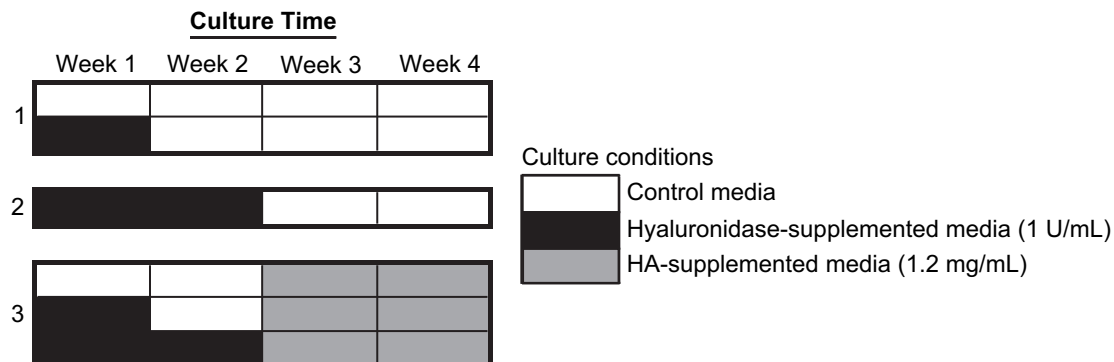
Elastin production within cell-gel constructs after two and four weeks of culture was assessed by immunohistochemistry for elastin distribution. Elastin staining was not seen in constructs after two weeks of culture, but constructs cultured for four weeks provided more insight into the effects of gel degradation on elastin elaboration. Immunohistochemical staining of elastin production within non-degraded cell-gel constructs documented no apparent elastin staining, while positive staining for elastin was observed in constructs treated with 1 U/mL hyaluronidase during the first week of culture (Fig. 4D). Here, hydrogel degradation promoted the elaboration of elastin and permitted its distribution within the scaffold.

3.3.2. VIC ECM production in response to changes in network structure and HA release

Cell-gel constructs treated for two weeks with 1 U/mL hyaluronidase experienced high levels of erosion and release of HA fragments, potentially affecting both the distribution and quantity of cell-produced collagen. Histological analysis of constructs after two weeks of enzyme treatment and two weeks of culture revealed an increase in cell number relative to non-degraded constructs (Fig. 4A and C). Degradation encourages encapsulated cell proliferation [36], but the delivery of HA fragments to VICs has also been shown to promote proliferation [25]. Staining of collagen within the highly eroded constructs was visible, but differed from the type of staining seen in constructs that had received one week of enzyme treatment (Fig. 4B and C). Collagen staining was less prevalent and

Table 2

HA treatment profiles for VIC encapsulation experiments. To investigate the effects of changes in network structure and the release of soluble HA on VIC secretory properties, specifically elastin and collagen production, cell-gel constructs were divided into to one of three treatment groups. Treatment group #1 was subjected to zero or one week of enzyme treatment to investigate how changes in network structure affect cell ECM production, treatment group #2 was subjected to two weeks of enzyme treatment resulting in changes in network structure and exposure to soluble HA fragments released from the hydrogel itself, and treatment group #3 received HA-supplemented media during weeks three and four to further investigate the effect of soluble HA on 3D cultured VICs



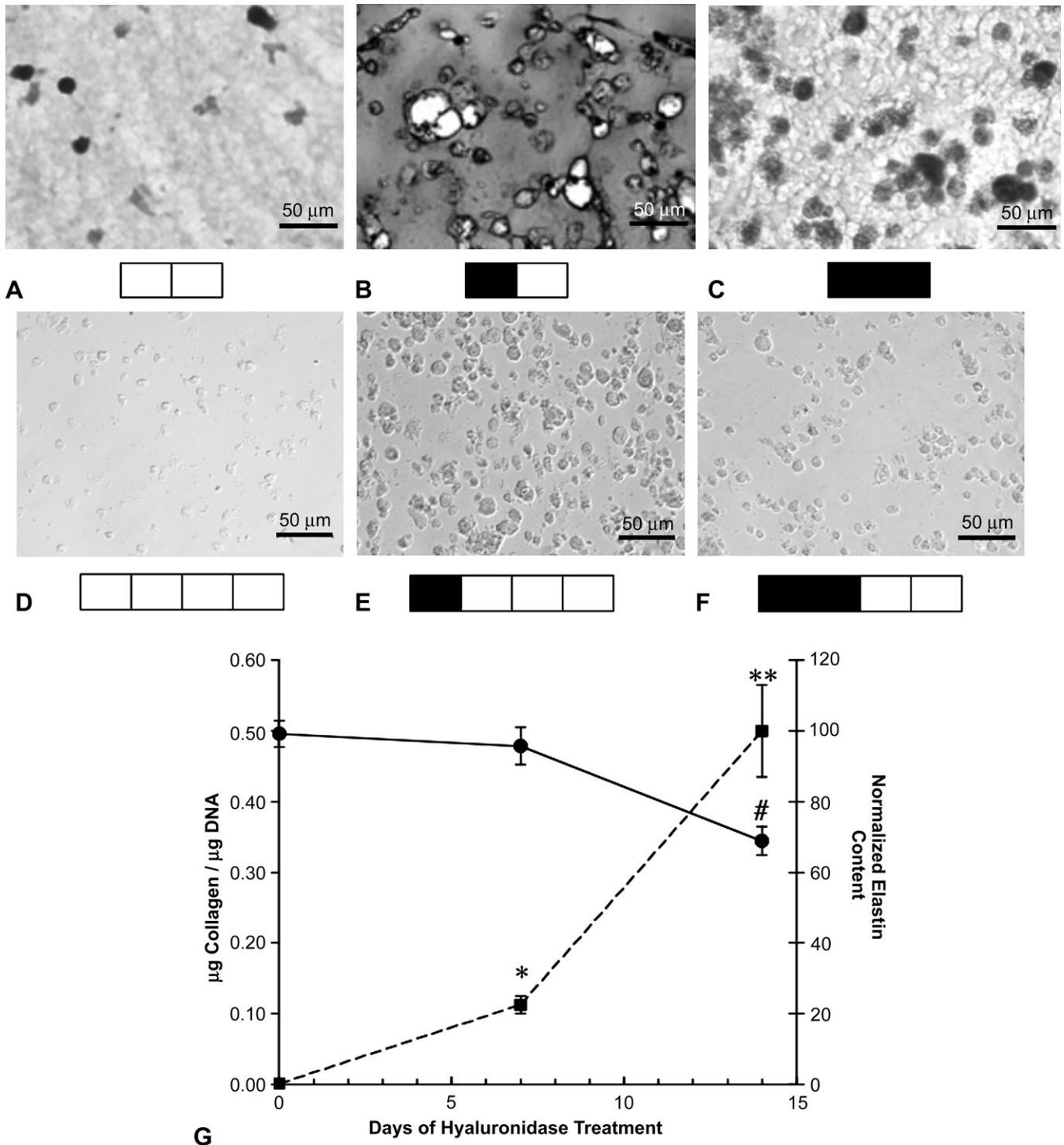


Fig. 4. (A–C) Collagen distribution and production in cell–gel constructs as shown by Masson’s Trichrome method after two weeks of culture. (A) Collagen distribution in gels that had not been degraded by hyaluronidase was limited, while (B) collagen diffusion is seen throughout constructs that had received enzyme treatment for one week. (C) Gels treated with hyaluronidase for two weeks did not stain as intensely for collagen as hydrogels treated with enzyme for just one week. (D–F) Elastin distribution and production in cell–gel constructs as shown by immunohistochemistry after four weeks of culture. (D) Elastin staining was not seen in non-degraded constructs, while (E) positive staining for elastin was seen in constructs that had been degraded with hyaluronidase for one or (F) two weeks. Scale bar equals 50 μm . (G) VIC collagen and elastin production as a function of days of hyaluronidase treatment. Hydroxyproline assay results show that VICs in gels that only experienced changes in network structure (7 days of hyaluronidase treatment) produced a similar amount of collagen per cell as VICs in non-degraded gels. VICs that experienced changes in network structure and release of HA fragments produced significantly less collagen compared to all other conditions (#). Meanwhile, VIC elastin production was significantly increased in constructs degraded for 7 days compared to non-degraded constructs (*) and further increased in constructs degraded for 14 days compared to all other conditions (**).

much lighter in constructs that had been degraded with hyaluronidase for two weeks. Because the extent of degradation was sufficient to permit collagen distribution, the decrease in overall staining intensity in constructs degraded for two weeks indicated a decrease in total collagen produced by encapsulated VICs. A hydroxyproline assay was used to quantify the total collagen content of the cell–gel constructs and confirmed that VICs in gels subjected to two weeks of hyaluronidase degradation produced significantly less collagen on a per cell basis compared to constructs that had only received one week of enzyme treatment or no enzyme treatment at all (Fig. 4G). This result demonstrates that the release of bioactive HA fragments could be used to suppress VIC collagen production or prevent further elaboration of collagen after a desired content is achieved in tissue engineering strategies. This observation agrees with previous findings of a decrease in VIC collagen production upon treatment with soluble HA fragments in 2D culture [25].

Cell–gel constructs were also analyzed for elastin distribution and content after two weeks of enzymatic degradation. Immunohistochemical staining for elastin was faint after only two weeks of culture (results not shown), but significant staining was observed after four weeks of culture in hydrogels where alterations in network structure and HA release had occurred. Specifically, two weeks of enzyme treatment resulted in significantly increased levels of cellular elastin production compared to constructs that received no enzyme treatment or one week of enzyme treatment (Fig. 4D–F). Because significant elastin production was not seen immediately in these constructs after the second week of culture when HA fragments were released, changes in hydrogel structure are likely the major driving force behind the increased elastin production seen here.

3.3.3. Further altering encapsulated VIC ECM production using HA-supplemented media

To better assess the effects of soluble HA on encapsulated VICs, HA at a concentration of 1.2 mg/mL was delivered to the cell–gel constructs through the cell culture media for weeks three and four of *in vitro* culture. The delivery of exogenous HA to constructs hindered the production of collagen in hydrogels treated with hyaluronidase during the first week of culture and those exposed to hyaluronidase for the first and second weeks of culture. This was observed histologically as a decrease in staining intensity compared to similarly degraded constructs that had not received HA-supplemented media during weeks three and four. Hydroxyproline results confirmed that exogenous HA delivery to constructs that had been enzyme treated during week one showed a statistically significant decrease in collagen production relative to similar constructs that had not received exogenous HA delivery. Exogenous HA delivery to constructs that had received enzyme treatment for weeks one and two of culture also resulted in a significant reduction of collagen production by encapsulated VICs compared to similarly degraded constructs that had not received HA-supplemented media. Non-degrading constructs showed no change in collagen production with the delivery

of exogenous HA (Fig. 5). This lack of response to HA delivery may be due to the inability of the delivered, soluble HA to interact with cells in non-degrading constructs as efficiently as in degrading constructs.

While HA-supplemented media decreased encapsulated VIC collagen production, long-term soluble HA supplementation stimulated elastin production in both non-degraded and degraded hydrogel constructs (Fig. 6). The combination of hydrogel degradation and media supplementation with soluble HA resulted in the highest levels of elastin secretion by encapsulated VICs demonstrating that both network alterations allowing for elastin diffusion and high concentrations of soluble HA positively influence VIC ECM secretion. These results demonstrate that while VICs do not significantly increase elastin production when exposed to low concentrations of HA fragments released during 1.14 wt% HA + 1.30 wt% PEG hydrogel degradation, VICs do respond to high soluble concentrations of HA by increasing their elastin production.

By incorporating an enzymatically degradable, bioactive component into hydrogel networks for VIC encapsulation, the relative amount and composition of the matrix produced can be controlled through systematic hydrogel degradation. Hydrogel degradation alone, without the release of HA fragments, was shown to permit the diffusion of collagen through the hydrogel networks and promote the secretion of elastin. Hydrogel degradation accompanied by HA fragment release suppressed collagen production and further encouraged elastin production. The increased elastin production seen in hydrogels treated with hyaluronidase for two weeks could not be directly attributed to the release of soluble HA, but when treated with a higher concentration of soluble HA, VIC elastin production was increased while collagen production was restrained. Collectively, these results suggest that this PEG:HA material platform may prove useful in tuning cell-secreted matrix composition for heart valve regeneration by simple changes in the gel chemistry and/or degradation profile.

4. Conclusions

HA:PEG gels with varying compositions and degradation profiles were readily synthesized by the photoinitiated chain copolymerization of methacrylate functionalized macromolecular monomers. Specifically, increasing the PEG content of the hydrogels resulted in a delay or prevention of reverse gelation, while increasing the gel's HA content resulted in more rapid mass loss and HA fragment release. For example, acellular 1.14 wt% HA + 1.30 wt% PEG hydrogels reached reverse gelation after 14 days of treatment with 1 U/mL hyaluronidase, while 0.67 wt% HA + 3.10 wt% PEG hydrogels achieved no mass loss after 14 days of enzyme treatment. Towards their application as a bioactive cell matrix, VICs were encapsulated in a gel fabricated from a 1.14 wt% HA + 1.30 wt% PEG comonomer solution, and their ECM production was shown to be influenced by the degradation of the gel and release of HA. When degraded in the presence of 1 U/mL hyaluronidase, physical changes in the gel structure permitted collagen diffusion after one week, while later stages of degradation promoted

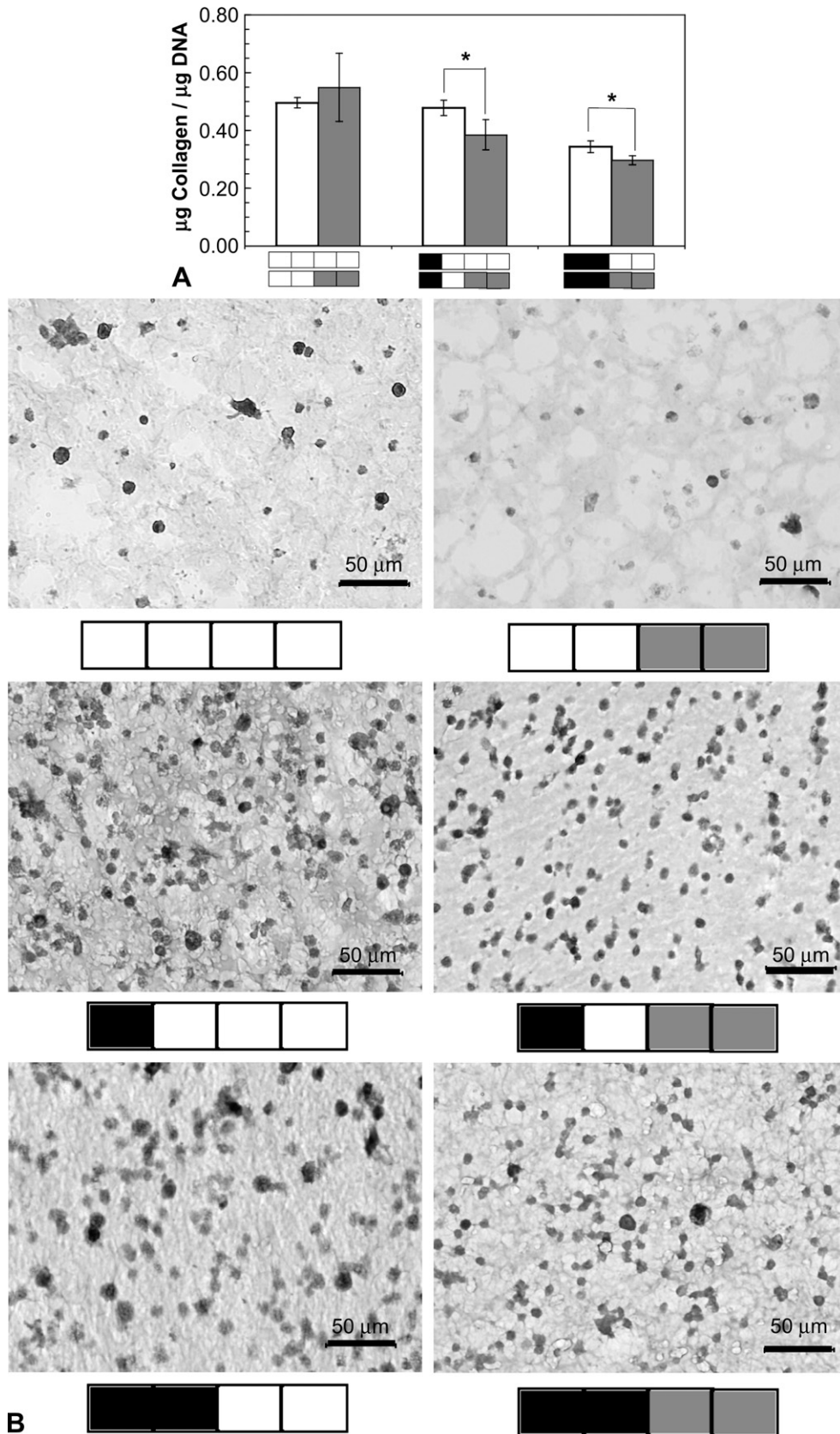


Fig. 5. (A) Collagen production, as a function of HA treatment profile (Table 2), was assessed using an assay for hydroxyproline and normalized to DNA content. Collagen production was significantly decreased (*) in constructs that had been enzymatically degraded for one or two weeks and treated with exogenous HA compared to similar constructs that had only been degraded. (B) Cryosections (8 μm) of similar hydrogel constructs were stained using Masson's Trichrome. Scale bar equals 50 μm .

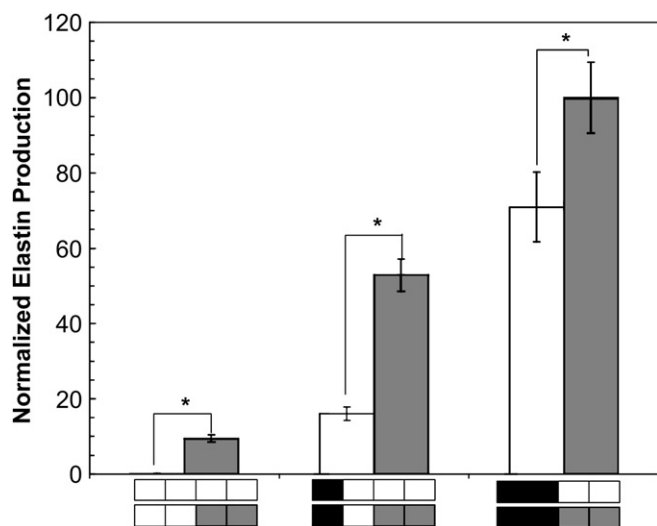


Fig. 6. Elastin production, as a function of HA treatment profile (Table 2), was assessed by western blot and normalized to DNA content. The administration of exogenous HA through the cell culture media significantly increased (*) elastin production in all constructs compared to similar constructs that had not received exogenous HA.

elastin synthesis and suppressed collagen production as bioactive HA fragments were released from the hydrogel network concurrent with further increases in the gel mesh size. Finally, exogenous delivery of 1.2 mg/mL of HA to the VIC–gel constructs further demonstrated that HA hinders collagen production and promotes elastin production in 3D VIC cultures. The dual utility of using HA as both structural and bioactive component to direct VIC secretory functions is promising as a material for heart valve tissue engineering strategies where the ECM composition must be finely tuned.

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