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Tuning Hydrogel Properties for Applications in Tissue Engineering

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Abstract—**Biomaterial design is an important component towards tissue engineering applications. There are many parameters that may be adjusted including physical properties (i.e., degradation and mechanics) and chemical properties (e.g., adhesion and cellular interactions). These design components may dictate the success or failure of a tissue engineering approach. Our group is particularly interested in the use of swollen hydrogels as cell carriers. One material that is used to fabricate hydrogels is hyaluronic acid (HA), which is found in many tissues in the body. Here, we show the control over hydrogel degradation, both in the bulk and locally to cells to control both the distribution of extracellular matrix by cells and whether or not a cell spreads in the hydrogels. These signals are important in the final structure and mechanical properties of engineered tissues, and potentially the differentiation of encapsulated stem cells.**

I. Introduction

Tissue engineering is a widely advancing field with a focus on improving the quality of life for patients with damaged or diseased tissues by developing replacement tissues using engineering principles. Typically, combinations of biomaterials, cells, and biological molecules are used synergistically for repair, but this is dependent on the tissue type and native environment [1]. One class of materials being developed includes highly-swollen hydrogels that can be used as cell carriers due to their tunable properties and high water content.

Our laboratory is specifically interested in combinations of mesenchymal stem cells (MSCs) and HA hydrogels for the repair of musculoskeletal tissues (e.g., cartilage). MSCs can be derived from adult tissues, such as bone marrow, and are pluripotent, so they are able to differentiate into a number of cell types, including chondrocytes. MSCs are widely used in tissue engineering and respond to many signals from their microenvironment, including the chemistry, topography, and mechanics of their surroundings [2].

HA is an attractive material for tissue engineering applications since it is a molecule found in many types of tissues, cells can bind to it with surface receptors (e.g., CD44), many cells are able to remodel HA due to the presence of enzymes (i.e., hyaluronidases), and it is easily modified to form crosslinked hydrogels for cell encapsulation [3]. One specific method for modification and cell encapsulation involves the introduction of reactive methacrylates and subsequent photopolymerization [3]. This technique provides some spatial control over the polymerization and supports viable cell encapsulation. Here, we present further modification of HA hydrogels to increase the functionality of the materials, including hydrogels that have controlled hydrolysis to facilitate ECM distribution and hydrogels that allow for MSC spreading.

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II. Materials and Methods

A. Polymer Synthesis

HA was modified to give various macromers containing either acrylate or methacrylate reactive groups (Figure 1). Methacrylated HA (MeHA) was synthesized as previously reported [3]. Briefly, methacrylic anhydride (Sigma) was added to a solution of 1 wt% HA (Lifecore, MW = 74 kDa) in deionized water, adjusted to a pH of 8 with 5 N NaOH, and reacted on ice for 24 hours. The macromer solution was purified via dialysis (MW cutoff 6-8k) against deionized water for a minimum of 48 hours with repeated changes of water.

Methacrylated lactic acid HA (MeLAHA) and methacrylated caprolactone HA (MeCLHA) were synthesized as previously reported [4], with modifications (Fig. 1A). Briefly, 2-hydroxyethyl methacrylate (HEMA) (Acros organics) was reacted with dl-lactide or ε-caprolactone (Sigma) via a ring opening polymerization in the presence of stannous octoate (Sigma) at 130°C for 1hr. The end group was then functionalized into a carboxylic acid (MeLA-COOH, MeCL-COOH) via reaction with succinic anhydride (Sigma) in the presence of N-methylimidazole at 65°C in dichloroethane for 13 hrs. The sodium salt form of HA was converted to a tetrabutylammonium (TBA) salt by acidic ion exchange with Dowex 50 W x 8-200 resin, followed by resin filtration and neutralization with aqueous TBA hydroxide for solubilization in dimethyl sulfoxide (DMSO). MeLA-COOH and MeCL-COOH were individually coupled to TBA-HA via esterification reactions with di-t-butyl dicarbonate (BOC_2O) as an activating agent with dimethylaminopyridine (DMAP) [5] for 20 hrs at 45ºC. The final products (MeLAHA and MeCLHA) were precipitated and washed in acetone, lyophilized, and stored at -20ºC in powder form prior to use.

Acrylated hyaluronic acid (AHA) was synthesized as previously reported [6]. Briefly, 2-hydroxyethyl acrylate (HEA) (Sigma) was functionalized with a carboxylic acid via reaction with succinic anhydride in the presence of N-

methylimidazole at 65°C in dichloroethane for 13 hrs to form HEA-Succinate (HEA-Suc). HEA-Suc was then coupled with HA-TBA by esterification with BOC_2O and DMAP for 18 hrs at 45ºC. The reaction mixture was dialyzed extensively against DI H_2O and lyophilized to yield the dry product. ^{1}H NMR was used to confirm intermediate and final polymer products.

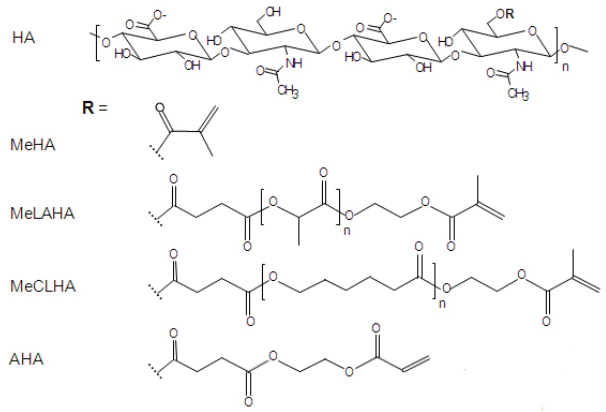

Fig. 1. Chemical structures of MeHA, MeLAHA, MeCLHA, and AHA. Each macromer forms distinct hydrogels with degradation based on the macromer chemistry. Copolymerization of the macromers leads to a wide variety of properties.

B. Hydrogel formation

For radical crosslinking, lyophilized HA macromers (e.g., MeHA, MeLAHA, MeCLHA and AHA) were dissolved in phosphate buffered saline (PBS) containing 0.05 wt% 2-methyl-1-[4-(hydroxyethoxy)phenyl]-2-methyl-1-propanone (I2959, Ciba) and exposed to UV light for 5 minutes. Addition (via monothiol cell adhesive and dithiol matrix metalloprotease-degradable peptides) crosslinking of AHA was performed in a 0.3M triethanolamine (TEOA) buffered saline with reaction for 15 minutes at 37ºC.

C. Hydrogel Characterization and Cell Encapsulation

For degradation studies, hydrogels were incubated in 1ml of PBS at 37ºC for up to 21 days. Degradation products were monitored using a uronic acid assay [7,8], and degradation is plotted as % uronic acid release. For matrix elaboration, MSCs were photoencapsulated in HA hydrogels (2 wt% MeHA, 1 wt% MeLAHA: 1wt% MeHA, and 1 wt% MeCLHA: 1wt% MeHA) and cultured *in vitro* under standard conditions for 14 days in chondrogenic media. Samples were fixed in 10% formalin for 24 hours, embedded in paraffin, and processed using standard histological procedures. The histological sections (7 μm thick) were stained for chondroitin sulfate distribution using the Vectastain ABC kit (Vector Labs) and the DAB Substrate kit for peroxidase (Vector Labs). Sections were predigested in 0.5 mg/ml hyaluronidase for 30 min at 37ºC and incubated in 0.5 N acetic acid for 4 hours at 4ºC to swell the samples prior to overnight incubation with primary antibodies (mouse monoclonal anti-chondroitin sulfate, Sigma) at a 1:100 dilution.

The degree of spreading of MSCs encapsulated in AHA networks crosslinked either through radical or addition mechanisms was assessed qualitatively, via visualization with a live/dead staining kit (Molecular Probes), and quantitatively, via measurement of cellular aspect ratios. For the latter, the maximum orthogonal width and length of $n \geq 75$ cells per condition were measured using NIH ImageJ, and the aspect ratio calculated as the longer length divided by the shorter length. Images and measurements were taken after 5 days in standard culture.

III. Results and Discussion

^{1}H NMR confirmed the successful synthesis of hydrolytically degradable HA macromers (MeLAHA and MeCLHA) with ~10.5% and ~13% methacrylate modification containing ~3 and ~3.8 repeat units of lactic acid and ε-caprolactone between the methacrylate and the HA backbone, respectively. Hydrolytic degradation of these polymers was confirmed with the complete degradation of 2 wt% MeLAHA and 3 wt% MeCLHA hydrogels in 5 and 18 days of incubation in PBS at 37°C without the addition of exogenous enzymes, while 2 wt% MeHA hydrogels remained relatively stable up to 21 days with only ~26% HA released (Fig. 1B).

When photoencapsulated MSCs were cultured in chondrogenic media for 14 days *in vitro*, the copolymerization of these hydrolytically degradable HA macromers with MeHA resulted in increased ECM deposition and distribution as illustrated in Figure 1C. Cell viability was high in all formulations as assessed with Live/Dead staining. Generally, the photopolymerization does not induce any loss of membrane integrity or mitochondrial activity to the cells during encapsulation. Purely enzymatically degradable MeHA hydrogels can hinder the diffusion of nutrients and ECM proteins within the hydrogel as visualized by the pericellular staining of

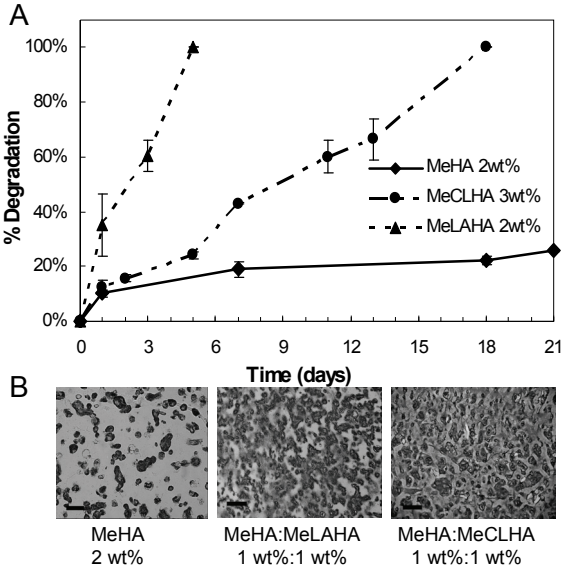

Fig. 2. (A) Degradation of MeHA, MeLAHA, and MeCLHA homopolymer hydrogels in PBS at 37°C over 21 days. (B) Immunohistochemical staining of chondroitin sulfate for 2 wt% MeHA, 1 wt% MeHA: 1 wt% MeLAHA, and 1 wt% MeHA: 1wt% MeCLHA.

chondroitin sulfate, while the addition of faster hydrolytically degrading components allow for enhanced distribution of ECM proteins, providing a more homogenous distribution of ECM proteins throughout the construct. It is also important to note that the rate of degradation can also influence matrix production and distribution, where a fine balance of hydrogel degradation with ECM deposition must be met to optimize tissue formation.

To further assess the capacity of HA-based scaffolds to control stem cell behavior, AHA was synthesized with ~38% acrylate modification, as confirmed by ^{1}H NMR. In contrast to methacrylation, acrylation enables chemical (addition) crosslinking with sufficiently short gelation times for uniform distribution of encapsulated cells, while still retaining the ability to undergo crosslinking via the radical mechanism. Figs. 3a and 3b illustrate the good agreement between predicted and observed morphology of MSCs encapsulated in AHA hydrogels crosslinked via each mechanism. Cells in addition-only crosslinked gels secrete MMPs that cleave degradable sites in the crosslinks, creating sufficient local volumes for spreading via focal adhesions with the tethered RGD domains. In contrast, the biologically inert radical crosslinks create mesh sizes in which cells remain completely rounded. These differences are quantified in Fig. 3c; while hMSCs encapsulated in radical-only gels remain completely rounded, with 100% of cells exhibiting an aspect ratio between 1 and 2, cells in addition-only gels exhibit a distribution toward significantly higher aspect ratios. An advantage to the system is the decoupling of mechanics (control of crosslink density by varying macromer concentration and MMP peptide concentration) and adhesion (control of cell spreading by varying RGD peptide concentration). Both are potential inputs to cellular differentiation [9-11], but are commonly mutually dependent in biomaterial scaffolds. This approach has also been extended to incorporate both radical and addition crosslink types in single gels, with the degree of outgrowth dependent on the ratio of each type used. This allows for spatial control of cellular spreading in a single construct, based on the spatial control afforded with light as a crosslinking mechanism. Taken together, these results illustrate the versatility of AHA and its potential usefulness in controlling stem cell behavior. Towards an implantable material, the spreading of cells could influence not only cellular differentiation, but the integration and interaction with surrounding cells and tissues.

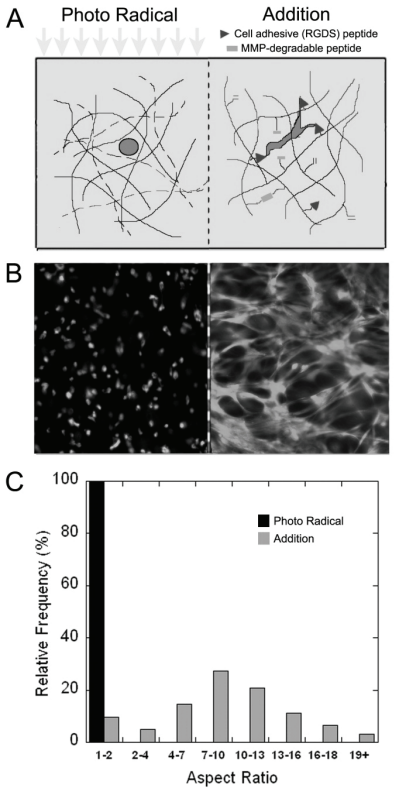

Fig. 3. (A) Schematic of predicted morphology of cells encapsulated in radical and addition crosslinked AHA gels, respectively. (B) Images of encapsulated cells (stained with calcein) in AHA hydrogels formed using radical and addition crosslinking, respectively. (C) Histogram of the cellular aspect ratio (longest to shortest dimension of encapsulated cells) for these same groups. All cultures were for five days.

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