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De Novo Design of Saccharide−Peptide Hydrogels as Synthetic Scaffolds for Tailored Cell Responses

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Abstract: A new class of functional saccharide−peptide copolymer-based hydrogels was synthesized and investigated as synthetic extracellular matrices for regenerative medicine applications. The polymer was composed entirely of natural building blocks, namely, galactaric acid and lysine on the backbone, with tyrosine grafted onto the side chain as a handle for enzyme-catalyzed hydrogelation. The resulting hydrogels are degradable under simulated physiological conditions and exhibit minimal cytotoxicity on dermal fibroblast and PC-12 cells. As a demonstration of the versatility of the system, the mechanical properties of the gels can be independently controlled without changing the polymer chemical composition. Using an identical copolymer solution, by simply allowing different lengths of cross-linking time, a series of hydrogels was obtained with different mechanical moduli at constant chemical structure. The moduli of the resulting hydrogels varied stepwise from 1.7, 4.1, 6.9, and 12.5 kPa to allow for systematic studies on the effects of modulus on cell behavior. It was exciting to observe that a simple change in hydrogel physical properties could induce a direct phenotypic change in cell adhesion and proliferation. Depending on the substrate mechanical modulus, the cell morphology changed and proliferation rate differed by an order of magnitude for different cell lines. These data suggest our saccharide−peptide hydrogels as promising synthetic extracellular matrices for cell culture and tissue regeneration.

Introduction

Hydrogels are important materials for many biomedical applications including drug delivery, biosensors, diagnostics, cell culture, and tissue regeneration.[1−8] One exciting direction in this area is to develop hydrogels as functional scaffolds that possess the appropriate chemical, physical, and biological cues so that they can interact effectively with living cells and encourage the development of new tissues.[9−11] Many criteria should be met for an effective scaffold for daily cell culture expansion as well as for advanced biomedical applications. The ideal scaffold should be biocompatible, nonimmunogenic, pathogen free, and biochemically defined for reproducibility and support the regeneration of the specific tissue type. In addition, for tissue regeneration applications, the mechanical property of the scaffolds should be tunable to match various tissues in the body having moduli ranging from 0.5 kPa in the brain, 10 kPa in muscle, to >30 kPa found in the bone.[12]

A variety of hydrogels derived from natural biopolymers, including agarose, alginate, collagen, silk, fibrin, and hyaluronic acid (HA),[13−23] have been explored as synthetic extracellular matrices (sECMs) for cell culture and tissue regeneration applications. Advantages of naturally derived sECMs over

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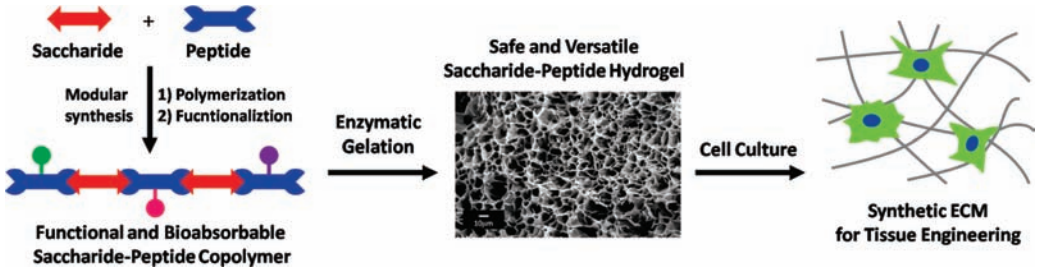

Figure 1. Design of fully naturally derived saccharide−peptide hydrogels as synthetic extracellular matrix (sECM) for cell culture.

synthetic analogs include their innate biocompatibility and biodegradability. However, extracted proteins and polysaccharides from animals are often expensive and have batch-to-batch variations.[10,20] Furthermore, possible chronic immunogenic responses, such as macrophage activation and chronic inflammation, may result when used inside the body.[24,25] In addition, the complex structures of natural biopolymers have often limited their design versatility for fine structural modification to tailor specific functions.

Synthetic polymer-based hydrogels, on the other hand, offer great versatility in controlling chemical structure, molecular weight, mechanical strength, and specific functions. This provides an exciting opportunity to design and tailor macromolecular chemistry and material properties to satisfy specific biomedical applications.[10] However, many synthetic hydrogels are composed of non-natural building blocks that are not biocompatible or biodegradable, limiting their ultimate in vivo biomedical applications. Among many synthetic systems, poly(ethylene glycol) (PEG)-based hydrogels have received particular attention recently due to their hydrophilicity, nontoxicity, and protein-resistant properties.[26,27] PEG hydrogels provide an inert surface onto which signaling motifs for adhesion, survival, proliferation, and differentiation can be incorporated to control cell adhesion, migration, and differentiation. Various chemistries have been developed for preparing PEG hydrogels as both 2D and 3D sECMs for cell culture and tissue engineering applications.[28−38] Despite the many excellent features, PEG hydrogels exhibit poor cell viability without the incorporation of adhesion ligands.[26,39,40] In addition, PEG hydrogels have limited versatility because the PEG backbone lacks sites for functionalization; therefore, the incorporation sites for functional groups, such as adhesion ligands, are limited to the termini of PEG. To address the limitations of PEG, hybrid PEG and peptide or protein gels have been reported for tissue engineering applications.[41]

Another important family of synthetic hydrogels is based on synthetic peptides and peptide-based amphilphiles that can self-assemble into nanofibers in the presence of cells.[42−46] One unique feature of these self-assembled hydrogels is their dynamic nature in the cellular environment, and some of them have been shown to be successful in neuronal differentiation. One potential limitation of these self-assembled systems, however, is that these self-assembled systems usually form relatively "weaker" gels, making them less amenable for a high-modulus environment such as in cartilage and bone repairs.

With the aim to combine the advantages from both natural and synthetic systems, namely, the biocompatibility and biodegradability from natural components and the versatility from synthetic chemistry, our group has been exploring new strategies to design synthetically simple, highly functional, and biocompatible novel biomaterials from natural building blocks. We previously reported our design and investigation of a novel sacchride-derived side-chain ether polymer as a protein-resistant material[47−49] and a family of cationic saccharide−peptide hybrid copolymers as vectors for plasmid DNA transfection.[50] Herein we describe our recent development of a novel family of hydrogels derived from anionic saccharide−peptide hybrid copolymer-based hydrogels as sECMS for cell culture and tissue engineering applications (Figure 1). Saccharides and amino acids are abundant natural building blocks used in nature to construct many important biological polymers including proteins, polysaccharides, and glycoproteins. Natural ECMs are intricate networks of proteins and polysaccharides that give mechanical, chemical, and biological cues to direct cell function.[51] We envision that hydrogels constructed from natural saccharrides and amino acids should not only more likely be safe (biocompatible, biodegrad-

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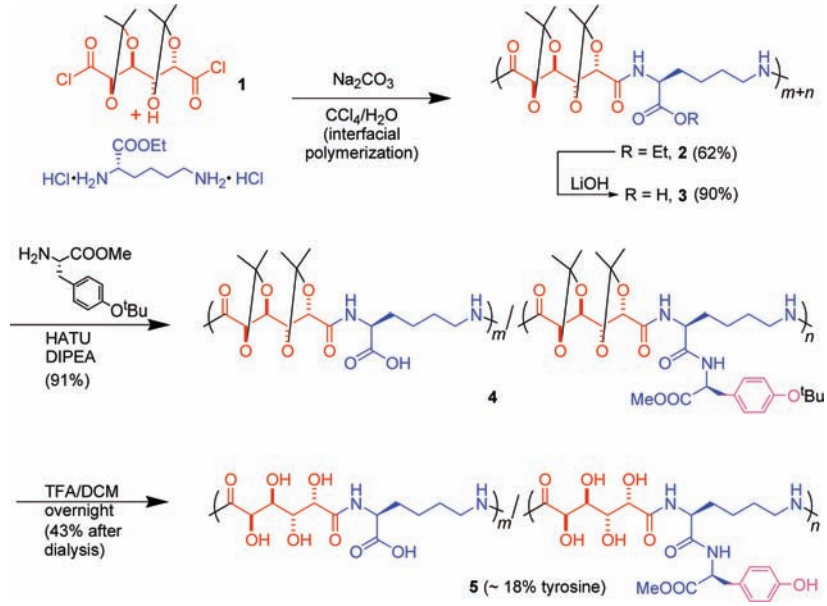
Scheme 1. Synthesis of Saccharide−Peptide Copolymer



able, and bioabsorbable) but also possess high functionality and versatility so that their structure and properties can be tailored. Compared to other synthetic hydrogels, the saccharide−peptide hydrogels described here may offer the following advantages: (1) they are fully composed of natural building blocks and will eventually degrade into natural, nontoxic, and bioabsorbable metabolites; (2) the hydrogels can be formed by cross-linking using a natural enzyme; (3) the hydrogels are highly functional, which offers a unique versatility to incorporate signaling motifs to control cell function; and (4) the modular synthetic strategy offers the advantage to combine structural precision with versatility, which offers the potential to independently control the physical, chemical, and biological properties of hydrogels for cellular studies. Specifically, in this paper we report our design and synthesis of a representative saccharide−peptide-derived hydrogel system as well as our investigation of their biodegradability, hydrogel morphology, cytocompatibility, and manipulation of their mechanical properties to control cell phenotypic behavior.

Results and Discussion

Synthesis and Characterizations of Anionic Saccharide−Peptide Copolymers. In this study, a highly water-soluble anionic galactaric acid−lysine copolymer was first synthesized that is composed of galataric acid and lysine on the main chain with tyrosine moieties grafted onto the side chains to effect hydrogellation by enzyme-mediated cross-linking using horseradish peroxidase (HRP). The copolymer carries anionic carboxylate groups for enhancing solubility in water while partially mimicking natural ECM polysaccharides such as alginate and HA that carry many anionic charges.[14,17,19,20] The carboxylate groups also served as convenient sites for further functionalization.

Interfacial condensation polymerization of an acetonide-protected galactaric acid dichloride (**1**) and an ethyl ester of L-lysine at the CCl_4/H_2O interface afforded the hybrid copolymer **2** with high molecular weight (M_n = 18.1k and PDI = 1.49) (Scheme 1). It should be noted that due to the nonregioselectivity of the polymerization reaction, a few possible isomeric repeat units coexist in the copolymer **2**, and for simplicity only one representative repeat unit is shown in Scheme 1. Nevertheless, this subtle structural heterogeneity is not critical for the current hydrogel studies. The ester side groups in polymer **2** were then hydrolyzed in LiOH solution to give polymer **3** with a carboxylic acid group.

Synthesis and Characterization of Saccharide−Peptide-Based Hydrogels. To allow for hydrogel formation, the linear, water-soluble polymers then need to be functionalized with a cross-linker. Ideally, gel formation should be viable in aqueous solution under mild conditions so that cross-linking can be carried out in the presence of cells. In addition, if a cross-linker derived from a natural building block is used, we could avoid the introduction of non-natural moieties that might have toxicity concerns. With these considerations in mind, we have been investigating a native cross-linking chemistry, i.e., oxidative coupling of tyrosine (Y) for cross-linking proteins in nature, for our hydrogel formation.[52] Tyrosine is an ideal cross-linker for our purpose because it is a naturally occurring amino acid, it can be functionalized to the polymer with simple coupling, and it can be efficiently cross-linked enzymatically. Other mechanisms of enzymatic hydrogelation have been reported in the literature.[53] As shown in Scheme 1, about 18% of the free carboxylic acid groups on polymer **3** were coupled with L-tyrosine-OMe to give tyrosine-functionalized polymer **4** via a standard peptide coupling protocol using HATU (2-(7-aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) as the coupling reagent. Global deprotection by TFA solution finally afforded the highly functional copolymer **5** with excellent water solubility.

For hydrogelation, polymer **5** was dissolved in 250 mM PBS buffer at a concentration of 50 mg/mL, which was subsequently

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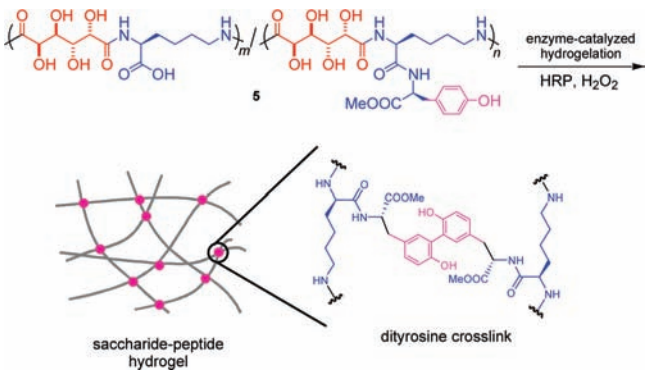
Scheme 2. Enzymatic Hydrogel Synthesis through Dityrosine Cross-Linking



cross-linked through HRP and H_2O_2 to form dityrosine as cross-linkers (Scheme 2). Hydrogels gelated by enzyme-mediated cross-linking using HRP have been reported previously.[54−57] Tyrosine- and tyramine-conjugated polymer was able to covalently cross-link via HRP and hydrogen peroxide. Moreover, dityrosine is a naturally occurring cross-linker utilized by nature in proteins such as camodulin, lysozyme, myoglobin, hemoglobin, insulin, RNase, and chymotrypsin,[58] making this new design of polymer totally natural. This avoids the introduction of unnatural moieties such as acrylate and diacrylate that might be cytotoxic if left unreacted.[59] The gelation kinetics can be controlled by experimental conditions such as the concentrations of HRP and H_2O_2. To demonstrate the versatility of our system, the mechanical properties of the gels were independently controlled without changing other molecular parameters. A series of identical solutions of polymer **5** were added the same amount of HRP and H_2O_2 to effect gelation. At different lengths of gelation time (8, 13, 17, and 60 min), excess Na_2SO_3 solution was added to quench the cross-linking reaction, resulting in a series of hydrogels having the same polymer structure and initial density but different mechanical properties. Following characterization of their chemical and physical properties, these hydrogels were further used for our subsequent cell culture studies.

To determine the cross-linking density in the resulting hydrogels, the hydrogels were completely hydrolyzed by heating in 6 N HCl solutions for 5 days. The digestion products of the hydrogel samples with different gelation times (8, 13, 17, and 60 min) were analyzed by ^{1}H NMR. The integrations of the proton peaks in the aromatic range (6−8 ppm) were used to calculate the percentage of tyrosines that form dityrosine cross-links (Figure 2). Discrete dityrosine standard was also synthesized according to the literature procedure to guide ^{1}H NMR spectral assignment.[60] From the integration of the proton on tyrosine (a_1) and dityrosine (b_1, b_2), the cross-linking percentage of tyrosine ($b_1/((a_1/2) + b_1)$) has been calculated and is shown in Figure 2. The percentages of tyrosine forming dityrosine cross-links are 49%, 54%, 58%, and 68% for cross-linking times

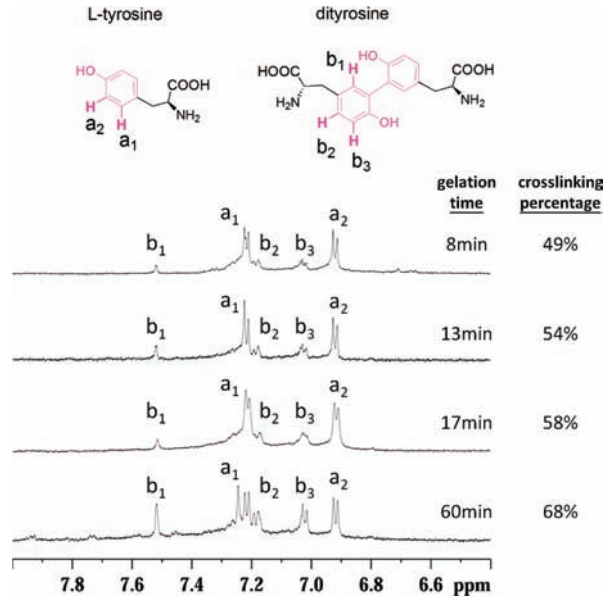


Figure 2. ^{1}H NMR spectra (the aromatic region) of the fully digested hydrogels.

of 8, 13, 17, and 60 min, respectively. Apparently, longer cross-linking time afforded gels with a higher cross-link percentage, which, consequentially, led to higher modulus.

Degradability of the Saccharide−Peptide Hydrogels. Following successful syntheses of the saccharide−peptide hydrogels, we first investigated their general properties as hydrogel scaffold materials, such as biodegradability, matrix morphology, and mechanical properties, before carrying out cell culture studies. Both the degradability and the degradation rate of sECM are important for tissue engineering applications. Compared to nondegradable material, matrix that can be degraded and be replaced by the host tissue is preferred. Biodegradable sECM would eliminate long-term immunoresponses as well as fibrous capsule formation. The degradation rate should match that of natural extracellular matrix production, so that similar mechanical properties can be retained over time. The saccharide−peptide copolymers are connected by peptide linkages, allowing for full biodegradability under physiological conditions. To study the rate of degradation, hydrogels were cross-linked in molds and swollen in PBS buffer for 24 h. The buffer was then decanted, and either 0.01 mg/mL trypsin solution in PBS buffer (pH 7.4) or pure PBS buffer was added. Trypsin was chosen for the degradation study because it is a common natural protease for digesting proteins. At regular time intervals, hydrogels in a 37 °C incubator were patted dry and weighted in triplicate. The swelling ratios were calculated by normalizing the swollen weight after addition of trypsin or buffer with the initial weight (W_t/W_o). The degradation profiles of the hydrogel with and without the presence of trypsin are shown in Figure 3. For trypsin-facilitated degradation, the degradation profile began with an initial increase in the swelling ratio, which resulted from initial partial degradation of the hydrogels that led to increased swelling by water. A similar phenomenon has been observed for other synthetic hydrogels.[61−63] As degradation continued with time, eventually the network started to lose short segments

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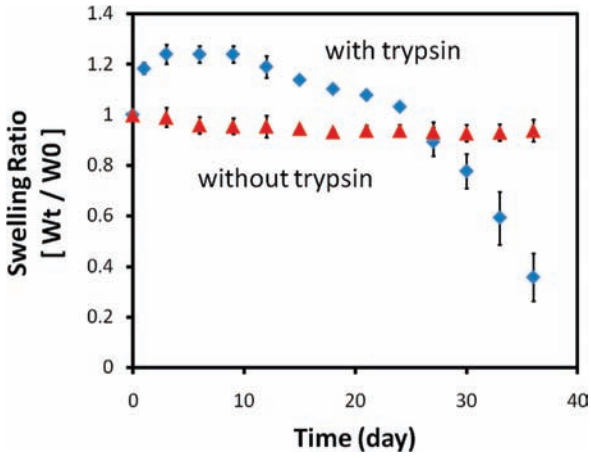

Figure 3. Swelling ratio (W_t/W_0) profiles of cross-linked hydrogel (60 min cross-linking time) prepared with 4% (w/v) polymer concentration in PBS buffer with or without the presence of 0.01 mg/mL trypsin ($n = 3$). Hydrogels (12.5 kPa) were kept in a 37 °C incubator and rotated at 50 rpm.

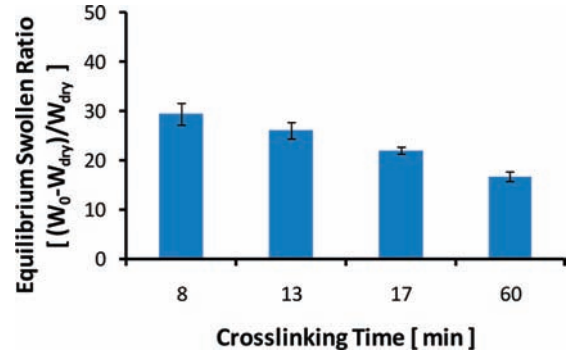

Figure 4. Equilibrium swelling ratios for hydrogels (calculated from the weight of swollen hydrogels (W_o) in PBS buffer (pH 7.4) and the weight (W_{dry}) of the corresponding lyophilized dry polymer).

of degraded chains and the hydrated mass decreased. After 33 days, the hydrogel disintegrated and finally completely dissolved. In the absence of trypsin, our amide-based hydrogel showed resistance to hydrolysis within the period of study, as seen by the unchanging swelling ratio (Figure 3). Trypsin significantly accelerated degradation of the polymer network, indicating the biodegradability of the polymer network in a natural environment exposed to many native protease enzymes. This novel polymer structure exhibited a significantly longer degradation time in the presence of trypsin than fibrinogen−PEG-based hydrogel under the same enzyme concentration.[61] Another hybrid hydrogel, PEG−PLA (PEG-poly(lactic) acid), exhibits complete degradation in 35 days in PBS buffer at a 10 wt % concentration.[64] Pure PEG hydrogel exhibits a longer degradation time than its PEG−PLA hydrogel analog due to the lack of cleavage sites on the PEG chains.[65] Overall, our saccharide−peptide hydrogels exhibit a longer degradation time than other PEG−peptide hydrogels but a shorter degradation time than pure PEG hydrogels. The observed degradation rate is in the suitable range for tissue engineering applications. It should be noted that the degradation profile can be further tuned by the choice of different peptide sequence, the concentration of polymer solutions for hydrogelation, and the cross-linking density.

Equilibrium Swelling Ratios. Swellability, as characterized by the equilibrium swelling ratio (ESR), is an important basic parameter for hydrogels. We measured the ESR values for all four hydrogel samples having cross-linking times of 8, 13, 17, and 60 min, respectively. The data showed that the swellability of the hydrogel has a moderate dependence on the cross-linking density (Figure 4). ESR decreases as the cross-linking density and modulus increase, and a total of 43% decrease in ESR was observed between the least and the most densely cross-linked hydrogels. This indicates a decrease in water uptake and a moderate increase in polymer density at higher cross-linking density. For comparison, other systems that utilize different initial polymer concentrations to achieve an increase in mechanical properties can have concentrations that differ by 100% or more.[38,66−68] Furthermore, the difference in final polymer density at equilibrium swelling in those systems is even larger after taking the ESR difference into account. Comparatively, our polymer system affords the opportunity to achieve hydrogels of dramatically different mechanical stiffness but with a relatively constant polymer density at the fully swollen state.

Morphology of the Saccharide−Peptide Hydrogels. Matrix morphology is another important physical parameter of hydrogels. For eventual 3D cell culture and in vivo tissue repairing, it is important for hydrogel matrices to have microporous structures so that mass transfer through the matrices is efficient. In this study, the morphology of our saccharide−peptide hydrogels was visualized by scanning electron microscopy (SEM) on freeze-dried hydrogels. Hydrogels were cross-linked in molds with predetermined dimensions and then equilibrated in PBS for 24 h. The freeze-drying procedure was shown previously as an effective method to preserve the native morphology of hydrogels.[62,69−73] Under normal drying conditions, capillary forces would cause the microporous structures to collapse so the native microstructures for hydrogels under hydration would be perturbed during the drying process. For freeze drying, the microstructures of the hydrogels were preserved at −50 °C and sublimation under high vacuum would leave the matrix morphology intact. As shown in Figure 5, our hydrogels exhibit microporous features with an average pore size above 10 μm.

Mechanical Properties for the Saccharide−Peptide Hydrogels. The mechanical properties of the matrices play an important role in controlling cell behavior. The versatility of our saccharide−peptide hydrogel system allows us to independently control the cross-linking density and accordingly the mechanical properties of the hydrogels without significant change of other properties. This offers a unique opportunity to

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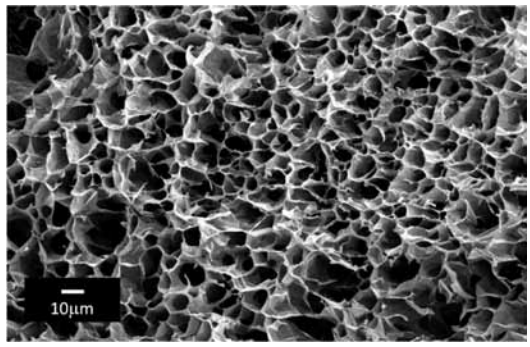

Figure 5. Representative SEM images of freeze-dried hydrogel prepared in PBS buffer (pH 7.4) at 4% (w/v) polymer concentration (60 min cross-linking time).

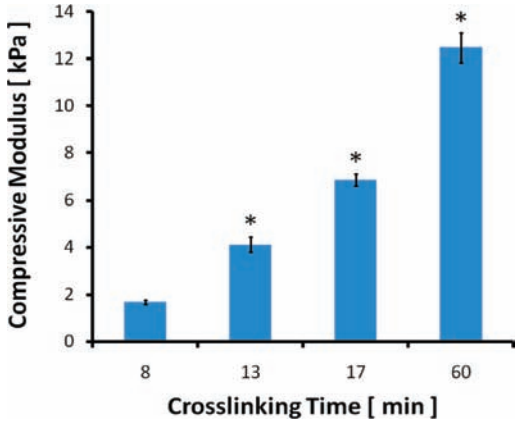

Figure 6. Compressive modulus of hydrogels as determined by a MTS machine ($n = 3$). Cross-linking time was controlled at 8, 13, 17, and 60 min (asterisks denote $p > 0.05$).

dissect the impact of the chemical and mechanical properties on cell function in future studies. To demonstrate this versatility, we prepared a series of saccharide−peptide hydrogels that are identical in chemical structure, charge density, and initial polymer density but differ only in the cross-link density and the resulting modulus. As discussed previously, this was achieved by controlling the cross-linking time (8, 13, 17, and 60 min) for identical polymer **5** solutions. The rheometry experiment was first conducted to determine the gelation process as a function of time (Figure S1, Supporting Information). It was observed that gelation occurred shortly after the addition of HRP, while the storage modulus (G') continues to increase with time. After 30 min, the modulus reaches the maximal value. The aforementioned cross-link times were then selected to obtain incremental increases in modulus. To correlate the hydrogel mechanical property with the cross-link time, the compressive modulus of hydrogels at different cross-link times were determined using MTS (Figure 6).

It was observed that the compressive modulus of the hydrogels increased with increasing cross-linking time: ranging from 1.7, 4.1, 6.9, and 12.5 kPa for cross-linking times of 8, 13, 17, and 60 min, respectively. This trend correlates well with the tyrosine cross-link percentages quantified by ^{1}H NMR.

The hydrogel moduli encompassed the range corresponding to very soft matrices (1.7 kPa) for neuronal tissue to intermediate stiffness (12.5 kPa) suitable for connective tissues. Instead of varying modulus by changing polymer concentration, which leads to changes in the density of the underlying substrate, we demonstrate the ability of obtaining different modulus by

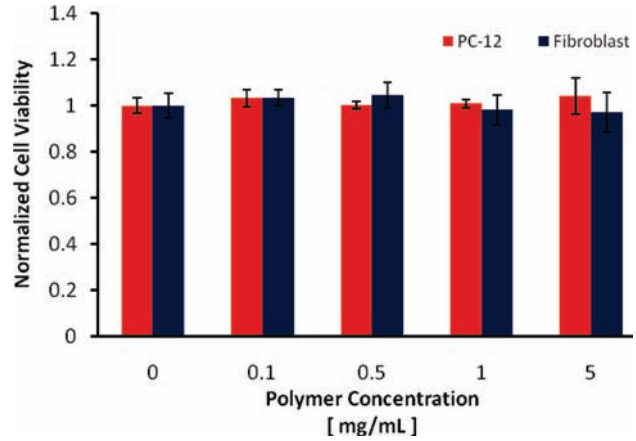

Figure 7. Polymer cytotoxicities for fibroblast (dark blue) and PC-12 neuronal cell line (red) are shown with 5000 cells per well in 48-well plates. Data are normalized to cell number without any added polymer. Comparable morphology and number were indicated for up to 5 mg/mL of 18% tyrosine-functionalized polymer **5** solution ($n = 3$) after 24 h of incubation.

allowing different times for cross-link formation. The ease of tunability for our system allows for independent control of the chemical structure and physical and functional properties of the hydrogels. As a simple demonstration, in this study we independently manipulate the mechanical properties of the hydrogels to investigate its effects on cell adhesion and proliferation (vide infra).

Cytotoxicity of Saccharide−Peptide Copolymers. Following investigation of their basic chem-physical properties, we started cell culture studies using the new hydrogels. In our initial studies, we chose two representative cell lines, PC-12 neuronal cell lines and NHDF dermal fibroblasts; one prefers a softer matrix, while the other prefers a stiffer matrix. First, the cytotoxicity of the anionic saccharide−peptide copolymers was assayed by determining cell viability. The cell cytotoxicity assay demonstrated excellent cytocompatibility for the saccharide−peptide copolymer with fibroblasts and PC-12 cells in culture for up to 5 mg/mL of polymer in solution, showing no significant differences between cell number in the presence or absence of the copolymer in solution (Figure 7). Given the polydisperse nature of the soluble polymer, this result implies a high tolerance to our polymers that even if the whole construct disintegrates completely, its fragments poses minimal cytotoxicity. In addition, as shown in our further investigation (vide infra), fibroblasts adhered on hydrogel surfaces to the same degree as on a standard tissue culture surface that has been optimized for adherent-dependent cell types. This demonstrates the cytocompatibility and that our polymer system is suitable for cell culture studies.

Cell Adhesion and Proliferation on Saccharide−Peptide Hydrogels. After confirming cytocompatibility, our saccharide−peptide hydrogels were subjected to cell adhesion and proliferation studies. For initial studies, we focused on investigating the impact of the mechanical properties of our hydrogels on cell adhesion and proliferation. To dissect the impact of mechanical stiffness on cell behavior from other parameters, we prepared a series of hydrogels having identical chemical structures and surface charges but with different modulus. The hydrogel modulus ranges from very low (1.7 kPa) to intermediately stiff (12.5 kPa). When seeded on hydrogel with 12.5 kPa modulus, fibroblasts attached well and spread out, giving the same morphology as those observed on tissue culture polystyrene (Figure 8). When seeded on hydrogels with lower moduli, they

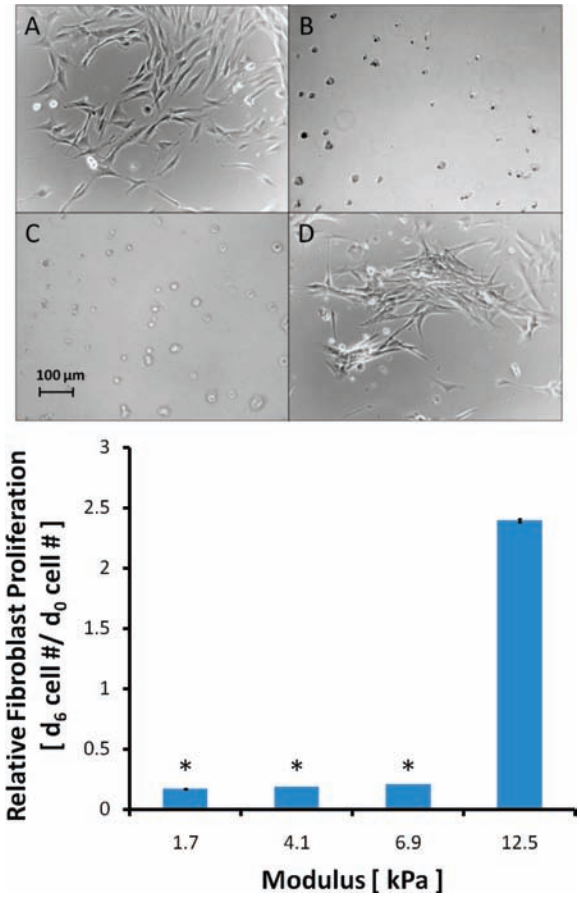

Figure 8. Images of fibroblasts seeded on (A) tissue culture polystrene, (B) 1.7 kPa gel, (C) 6.9 kPa gel, and (D) 12.5 kPa gel after 1 day of culture. The corresponding proliferation data for fibroblasts on hydrogels of different modulus is shown on day 6 (E). Fibroblast proliferation was observed on the stiffest hydrogel with a modulus of 12.5 kPa.

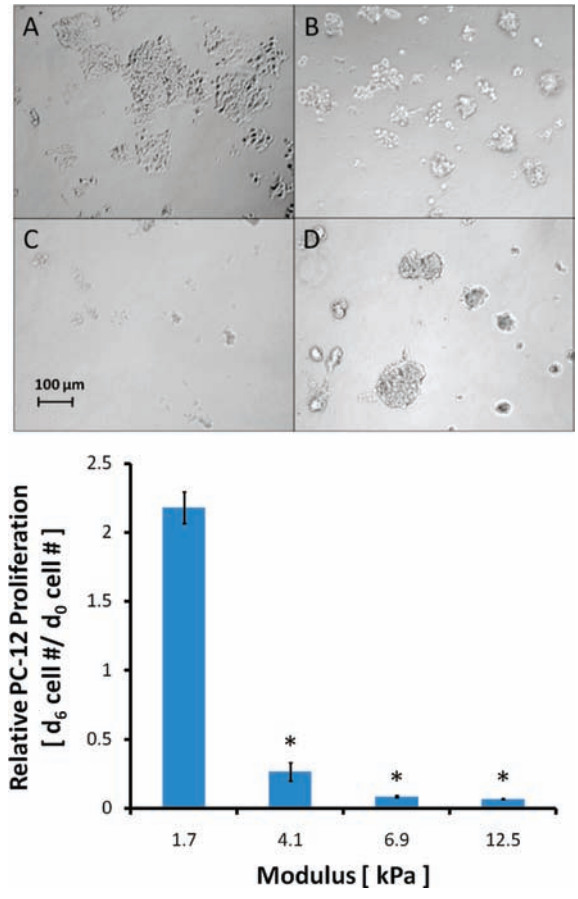

Figure 9. Images of PC-12 cell on (A) tissue culture polystyrene, (B) 1.7 kPa gel, (C) 12.5 kPa gel, and (D) Matrigel after 1 day of culture. The corresponding proliferation data for PC-12 cells on hydrogels of different modulus is shown on day 6 (E). PC-12 proliferated on the softest hydrogel with a modulus of 1.7 kPa.

remained rounded and would not spread, consistent with other literature findings.[74] The converse dependence on modulus was observed with PC-12 cells that are neuronal in origin and prefer softer matrices. When seeded on the 1.7 kPa hydrogel, PC-12 cells adhered well (Figure 9B), while minimal cell adhesion was observed on the 12.5 kPa hydrogel (Figure 9C). PC-12 morphology was observed to be different on tissue culture polystyrene and soft hydrogel, reflecting the 10^6 kPa difference in the modulus of polystyrene and neuronal tissues.[12,75] Matrigel, a commonly used soft tissue culture matrix with a modulus of ~0.65 kPa, was used as an additional control for PC-12 culture studies. PC-12 cells exhibit a similar morphology on Matrigel and on our 1.7 kPa hydrogel, indicative of a similar degree of cell support.[76−78] Despite the fact that Matrigel is a commonly used natural ECM for cell culture, especially for neuronal cells, its complex composition (a mixture of natural polymers such as fibronectin, collagen IV, heparin sulfate, and laminin and an undefined mixture of growth factors from mouse sarcoma source) makes it unsuitable for in vivo applications.[79−81]

Cell proliferation follows the same trend as cell adhesion. Cell proliferation was only observed on hydrogel that best matched the modulus of the natural ECM for each cell line (Figures 8E and 9E). These results agree with previous findings that demonstrated fibroblasts have a maximal spread area on a modulus of greater than 10 kPa[82] while neurons prefer low-modulus substrates.[83,84] The fact that the cells were able to proliferate on the desirable hydrogel of choice implied the substrate was not only able to support adhesion but supported the necessary activation of genes for proliferation. Depending on the modulus, cell number can vary 10-fold over 6 days for

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both fibroblasts and PC-12 with opposite trends. Uniquely, no cell adhesion ligands were necessary for the adhesion and selective proliferation of these cells. As a simple example, this result has manifested the versatility of our saccharide−peptide hydrogel system: although having the same polymer structure and charge density and at similar polymer concentration, the hydrogel can be controlled to exhibit different cellular responses based on the mechanical modulus. This also illustrated the complexity of cellular behavior that is dependent on small changes in the underlying substrate and, in this case, presumably the modulus sensing ability through onmuscle myosin II. Through membrane-bound mechanosensors, e.g., integrins, cells can "sense" the modulus of the underlying substrate. According to the cellular tensegrity model,[85] cells exist in a state of prestress through myosin II contraction on the actin cytoskeleton. ECM adhesions and micrtotubules act as a counterbalance. Cell recognizes outside forces through integrin binding, and depending on the microenvironment, cells reorganize their cytoskeleton, reorganize focal adhesion on the surface of their lipid bilayer membrane, and affect substream signal cascade.[86] This in turn modulates cell morphology,[87] differentiation,[87] apoptosis,[88] migration,[89,90] and proliferation.[84,91]

Despite many elegant studies probing the mechanical effects of sECM on cell responses,[28,82–84,89] due to the constraints of chemical structures and the mechanisms for manipulating their mechanical properties, it is challenging to independently change the hydrogel mechanics without concurrently modifying other properties. While many biomaterials exist with tunable modulus, it is usually achieved through changes in polymer structures, polymer density, or structure of cross-links,[28,29,38,64,68,89,92,93] which often complicates the desired studies. Herein, we demonstrated that without changing the structure of the polymer backbone and cross-links, surface charge, or initial polymer concentration, the mechanical properties can be systematically controlled by cross-linking level. Whereas there is a moderate increase in polymer density at increasing cross-linking density, the overall change in polymer density is relatively small compared to other synthetic polymer hydrogels. This provides a unique opportunity to investigate the pure mechanical effects on cellular responses with minimal perturbation of other gel properties. Furthermore, the current hydrogels were prepared from a single saccharide−peptide copolymer with ∼18 mol % tyrosine functionalization at 4% (w/v) polymer concentration. Through modifying the tyrosine functionalization level, changing the cross-linking mechanism, and varying the copolymer concentration, a much larger range of mechanical properties can be explored. In addition, given their high versatility and functionality, biologically relevant ligands can be conveniently conjugated to the hydrogels to further optimize the properties for tissue engineering applications.

Conclusion

In summary, we developed a versatile hydrogel system using naturally occurring amino acids and saccharides as building blocks. Specifically, the tyrosine-functionalized galactaric acid−lysine copolymers were efficiently synthesized via interfacial condensation polymerization, and the copolymers gel efficiently under physiologic conditions by enzyme-catalyzed cross-linking. Using the same copolymer and at constant initial polymer concentration, a series of hydrogels was prepared with controlled cross-linking density and resulting mechanical properties by controlling the enzymatic cross-linking time. Quantified by ^{1}H NMR spectroscopy, the dityrosine cross-linking density increases with the cross-linking time. MTS mechanical testing confirms that the modulus of the hydrogels correlates well with the cross-linking density. SEM images of the freeze-dried hydrogels show that they exhibit microporous microstructures with pore sizes over 10 μm, suitable for cell culture and tissue engineering applications. Cytotoxicity assay proves that the saccharide−peptide copolymers are noncytotoxic at relatively high polymer concentration. The sacchride−peptide hydrogels are fully degradable under enzymatic conditions, and the final degradation products are simple natural metabolites (amino acids and saccharic acids).

Following the investigation of the basic hydrogel properties, the saccharide−peptide hydrogels were further used in cell culture studies where they induced a tailored cellular response to different cell lines for hydrogels of constant structure but different mechanical stiffness. As a simple demonstration of the versatility of our hydrogel system, the hydrogel modulus was manipulated by controlling the cross-linking density for one single polymer structure at constant initial polymer concentration. This provides a unique opportunity to dissect mechanical change from other factors and probe the impact of pure mechanical change on cell responses. We were excited to observe very different cell behavior for fibroblasts and PC-12 cells on hydrogels with different modulus for both cell adhesion and proliferation, with 10-fold increases in the cell proliferation rate on substrate that matched its native microenvironment. These data suggest that cell response in culture depends intimately on substrate modulus as well as the specific cell types. This demonstrates the ability of our system to select for adherent cell types based on mechanical modulus alone.

This study introduces a novel family of saccharide−peptide hydrogel as safe, versatile, and functional sECMs for cell culture and tissue regeneration purposes. In addition, this scaffold represents a clean slate free of growth factors and animal containments that can easily be purified and produced on a large scale. Since it is composed of all natural components, there is a high potential for further applications for tailored differentiation of stem cell and angiogenesis. In further studies, we plan to investigate the mechanisms by which our hydrogels influence cell behavior and also conduct studies on a 3-D culture of stem cells to investigate the ability of these novel materials to control cell differentiation.

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Supporting Information Available: Synthesis and characterization of monomers and polymers, experimental details for degradation and equilibrium swelling ratio, mechanical and rheological measurements, and cell culture experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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