Formation of a Novel Heparin-Based Hydrogel in the Presence of Heparin-Binding Biomolecules

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An injectable, heparin-based hydrogel system with the potential to be gelled with cells was developed. First, heparin was modified to have thiol groups by the modification of carboxylic groups of heparin with cysteamine using carbodiimide chemistry. Thiol functionalization of heparin carboxylic groups was controlled from 10% to 60% of the available COOH groups, and the retained bioactivity of the modified heparin, characterized by its binding affinity to antithrombin III, decreased with increasing functionalization. Then, the thiol-functionalized heparin was reacted with poly(ethylene glycol) diacrylate to form a hydrogel. The gelation kinetics and mechanical properties of the final gel state could be tuned by controlling cross-link density. Fibroblast cell encapsulation using this hydrogel revealed the nontoxicity of the present system. Cell proliferation inside the hydrogel was observed, and it was significantly enhanced (more than 5-fold) by the addition of fibrinogen into the hydrogel during gelation.

Introduction

Heparin is a highly sulfated, anionic polysaccharide composed of repeating disaccharides of (1 → 4)-linked glucosamine and uronic acid residues. Heparin is best known for anticoagulant properties that are mediated through a pentasaccharide sequence that binds antithrombin III (AT-III).[1] Heparin also interacts with a variety of proteins that have heparin-binding domains, including various kinds of growth factors.[2−9] Using the highly specific binding properties of heparin for various growth factors, a number of systems with a small fraction of heparin attached inside the matrix have been developed for the controlled release of growth factors and/or their protection from in situ degradation.[5,10−13]

In addition, there have been attempts to use heparin as a main component of the hydrogel; Seal et al. covalently attached heparin-binding peptides to multiarm poly(ethylene glycol)s (PEGs), which were then physically cross-linked with heparin.[14] This gel had poor mechanical properties and exhibited a slow release of loaded other heparin-binding peptides. We also developed a cross-linked heparin-based hydrogel, formed by hydrazide-functionalized heparin (Hep-ADH) and the *N*-hydroxysuccinimidyl ester of poly(ethylene glycol)-bis-butanoic acid (SBA-PEG-SBA). The release of the loaded vascular endothelial growth factor (VEGF) from the hydrogel showed a slow, controlled release over a 3 week period without an initial burst, and the released VEGF maintained its biological activity.[15] For these reasons, it is projected that this cross-linked heparin gel can be used for the controlled release of growth factors that contain the heparin-binding domain. However, one problem is that the chemistry employed in this system is nonspecific, leaving potential for cross-linking reactions with the loaded biomolecules to occur. Thus, the heparin gel first needs to be prepared, and the target proteins subsequently need to be loaded; it cannot be used as an injectable gel or cell encapsulation matrix.

An injectable hydrogel that can gel in vivo, transforming from a sol state to a gel via a sufficiently gentle way in intimate contact with biological molecules, has several advantages in medical applications. For example, in vivo gelation of a hydrogel can match complex tissue shapes at the implantation site, and a large amount of materials can be delivered via minimally invasive surgery.[16] Also, an in situ forming hydrogel can safely encapsulate biological molecules such as proteins, DNA drugs, and living cells, meaning that this injectable gelling system can be used as an in vivo delivery carrier of these biological molecules as well as an in vitro cell or enzyme encapsulation matrix.[17]

We present here a heparin-based hydrogel system that can be cross-linked with cells. This injectable gelling system is prepared by modifying the carboxyl groups of heparin to have pendant thiol groups (Hep-SH) and reacting with PEG diacrylate (PEG-DA), which adds by Michael addition to form thioether linkages similar to those of the PEG-based hydrogel system reported by Hubbell and co-workers.[18] Since this hydrogel is heparin-based, it has the biological activities of heparin, and the biological activity can be further modified by adding heparin-binding biomolecules. As such, the proposed system can be applied as an in vitro cell encapsulating matrix, an in vivo cell delivery system or tissue regeneration scaffold, or an injectable delivery system for the controlled release of biological molecules that have heparin-binding affinity.

Experimental Procedures

Materials. Heparin (sodium salt, from porcine intestinal mucosa, M_w = 1.2 kDa) was purchased from Cellsus, Inc. (Cincinnati, OH). 1-Ethyl-3-[3-dimethylamino]propyl]carbodiimide (EDC), 1-hydroxy-

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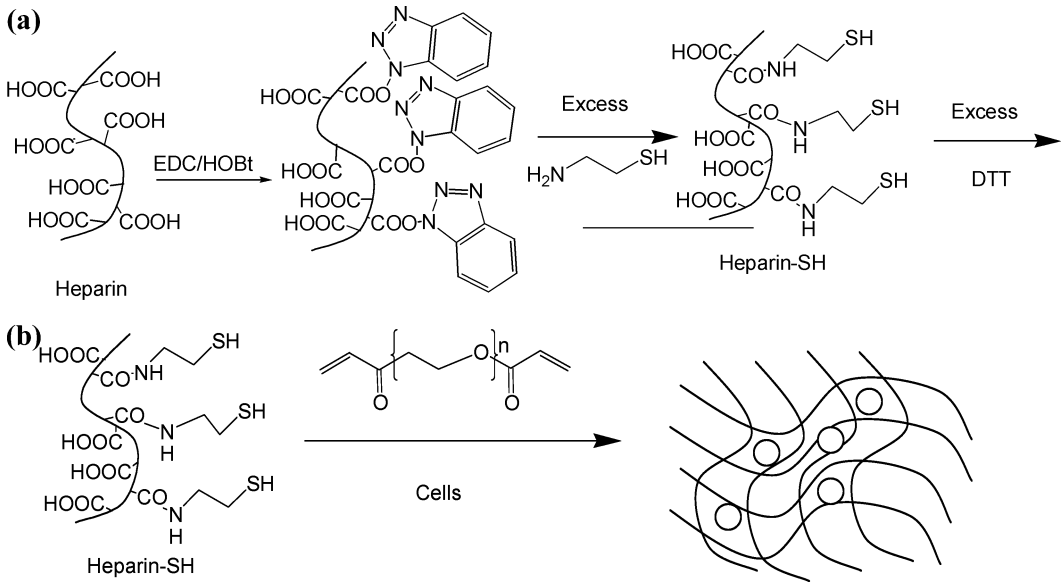

Figure 1. Reaction scheme. (a) Thiol-functionalized heparin (Hep-SH) from heparin and cysteamine. (b) In situ gelation of heparin-based gel from Hep-SH and poly(ethylene glycol) diacrylate (PEG-DA).

Table 1. Control of Thiolation of the Heparin Carboxyl Group by Reaction Conditions[a]

	molar ratio					
sample	Hep(10)	Hep(20)	Hep(30)	Hep(40)	Hep(50)	Hep(60)
heparin (−COOH)	1	1	1	1	1	1
HOBT	0.75	1	1.5	1.75	2	2.5
EDC	0.75	1	1.5	1.75	2	3.5
cysteamine	2	2	2	2	3	3
conversion of COOH to SH (%)	9.6 ± 2.2	20.6 ± 3.2	31.5 ± 3.9	40.8 ± 2.9	50.0 ± 2.9	57.0 ± 11.6

[a] Numbers are the relative molar ratios among reactants. The last row represents the conversion of carboxylic groups to thiol groups ($n > 6$).

benzotriazole hydrate (HOBT), cysteamine, dithiotreitol (DTT), triethylamine, dichloromethane, potassium carbonate, magnesium sulfate, and acryloyl chloride were obtained from Sigma (St. Louis, MO). PEGs (M_w = 3.4 and 6 kDa) were obtained from Fluka (Switzerland). 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) was purchased from Acros (Houston, TX). Toluene and deuterated chloroform were obtained from Merk (Darmstadt, Germany). All chemicals were used without further purification. Slide-A-Lyzer dialysis cassettes (7 kDa M_w cutoff) were purchased from Pierce (Rockford, IL), and PD-10 desalting columns were obtained from Amersham Biosciences Corp. (Piscataway, NJ).

As a cell culture medium, Dulbecco's modified Eagle's medium (DMEM) with D-glucose and glutamine (4500 mg/L), fetal bovine serum (certified) (10%), penicillin G (50 U/mL), streptomycin sulfate (50 μg/mL), and amphotericin B (0.125 μg/mL) were used (all from Gibco, Grand island, NY); trypsin ethylenediaminetetraacetic acid (EDTA) (0.25%) was also purchased from Gibco. A fibroblast cell line (NIH/3T3, American Type Culture Collection) was used as the cell source. Potassium phosphate monobasic, sodium phosphate dibasic, acridine orange, propidium iodide, fibrinogen, and lysozyme were obtained from Sigma, and sodium chloride and potassium chloride were from purchased Merk. Phosphate-buffered saline (PBS), 0.01 mol/L PBS solution with 0.138 mol/L NaCl and 0.0027 mol/L KCl, pH = 7.4, was prepared with potassium phosphate monobasic and sodium phosphate dibasic. WST-1 was purchased from Roche Ltd. (Basel, Switzerland). To minimize the effect of cells attached to the bottom of the plate in the cell proliferation assay inside the hydrogel, cell culture plates with an ultralow attachment surface were used (Corning, VWR, Fontenay-sous-Bois, France). Ellman's reagent was obtained from Pierce (Rockford, IL), and a Coatest Heparin assay kit was purchased from DiaPharma (West Chester, OH).

Thiol-Functionalized Heparin (Hep-SH). Carboxylic acid groups of heparin were modified to include thiol groups (Figure 1a), similar to the method developed to modify hyaluronic acid.[19] Briefly, heparin was dissolved in double-distilled, deionized water at a concentration of 10 mg/mL. To this solution, EDC, HOBt, and an excess amount of cysteamine were added. Note that the molar ratios among reactants were varied to control the modification of carboxylic groups (Table 1). The pH of the reaction mixture was adjusted to 6.8 with 0.1 M NaOH and/or HCl solution, and the reaction was allowed to continue for 5 h with stirring at room temperature. Next, the reaction solution was exhaustively dialyzed using a dialysis cassette to remove all small molecules not attached to heparin and lyophilized. After that, a 10-fold molar (moles per COOH of heparin) excess of DTT was added to reduce the oxidized disulfide groups in order to free thiol groups. This solution was allowed to react for 3 h at pH 7.5 and then adjusted to pH 3.5 by the addition of 1.0 N HCl. The acidified solution was dialyzed against dilute HCl (pH 3.5) containing 100 mM NaCl, followed by dialysis against dilute HCl at pH 3.5. Hep-SH was further purified by running a PD-10 desalting column and lyophilized. The amount of the thiol group attached to heparin was measured using the molar absorptivity of Ellman's reagent at 412 nm. Finally, the anticoagulant bioactivity of the modified heparin was assayed using Coatest Heparin, which measures the binding affinity of heparin to AT-III.

Gelation, Swelling Ratio, and Protein Adsorption of Heparin-Based Hydrogel. PEG-DA was reacted with Hep-SH in PBS (degassed by N_2 purging) to form a cross-linked heparin gel (equal molar ratio of acrylate groups of PEG-DA and thiol groups of Hep-SH) (Figure 1b). PEG-DA was prepared by reacting PEG with acryloyl chloride.[20] The conversion of acrylation, calculated using 300 MHz ^{1}H NMR spectroscopy (JEOL JNM-LA300WB FT-NMR spectrometer, Japan) by comparing peaks at 3.5 ppm from the PEG backbone and at 5.9 ppm from the acrylate group, was above 95%.

The gelation process was monitored using a rheometer (Gemini, Malvern Instruments), equipped with a temperature controller at 37 °C and a solvent trap to suppress the drying of the polymer solution during gelation. Samples were analyzed with sandblast parallel plate

geometry and a 500 μm gap thickness. An angular frequency of $\omega = 1$ rad/s and strain of $\gamma = 0.1\%$ were selected to ensure a linear regime of oscillatory deformation. In this study, the total concentration of polymer, molar ratio between Hep-SH and PEG-DA, conversion of the thiolation of heparin, and molecular weight of PEG-DA were varied to characterize their effects on gelation.

The swelling ratios of hydrogels prepared at various conditions with the same total concentration (10 wt %) were measured in both deionized water and various dilution ratios of PBS as well as in DMEM. Fully swollen hydrogels at 37 °C immersed for 2 days were weighed (W_s) immediately after the removal of excess water by rolling them in wet tissue papers; the hydrogels were lyophilized and weighed (W_d). The swelling ratio was calculated by W_s/W_d ($n = 4$).

The loading capacities of heparin-based hydrogels were characterized. Hep-SH with various degrees of thiolation (20%, 30%, 40%, and 50%) and 6 kDa PEG-DA (1:1 molar ratio of thiol groups to acrylate groups) were dissolved in PBS (degassed by N_2 purging) to make a 10 wt % solution and filtered through a 0.2 μm sterile filter to sterilize the precursor solution. Then, 40 μL of the precursor solution was poured into each well of a 96-well plate and incubated at 37 °C for 2 h for gelation. Freshly prepared 200 μL of fibrinogen or lysozyme solution (10, 20, 30, or 40 mg/mL) was added onto the hydrogel at 37 °C. After 1 day of incubation, the supernatant was removed, and the hydrogels were washed twice with PBS. The amount of unbound fibrinogen was measured using a Bicinchoninic Acid (BCA) Protein Assay Kit (Pierce, Bonn, Germany), and the adsorbed amount was calculated from it. All experiments were performed in triplicate.

Viability and Proliferation of Encapsulated Cells in Heparin-Based Hydrogels. Approximately 40% thiolated Hep-SH and PEG-DA (1:1 molar ratio of thiol groups to acrylate groups) were dissolved in a cell culture medium to make a 10 wt % solution and filtered through a 0.2 μm sterile filter to sterilize the precursor solution. Then, 50 μL of the solution was poured into each well of a 96-well plate, and 10 μL of fibroblast cell suspension was added to each well to make a final cell density of 1×10^6 cells/mL, which was then mixed by pipetting. All of these procedures were done in less than 3 min after mixing the two precursors. After 30 min of incubation at 37 °C, more cell culture medium (200 μL) was added to the gel-formed wells and cultured in a standard cell culture condition. In the case of the fibrinogen-added hydrogel, a cell culture medium containing various concentrations of fibrinogen was used to make the hydrogel.

Cell viability inside the hydrogel was determined by a double-staining procedure using acridine orange (AO) and propidium iodide (PI).[21] Briefly, 1 mL of PBS containing 0.67 μM AO and 75 μM PI was added to the sample containing the cells and incubated in the dark at room temperature for 30 min. The quantification of cell proliferation was performed with WST-1.[22] At each measurement point, WST-1 was added for 2 h, and the colorimetric absorbance of the produced formazan at 450 nm, directly proportional to the total number of viable cells, was measured using a scanning multiwell spectrophotometer (FL600, Bio-Tek, Winooski, VT) ($n = 4$).

Results

Thiol-Functionalized Heparin (Hep-SH). The method previously used to modify the COOH group of hyaluronic acid[19] was used to modify the COOH group of heparin (Figure 1). The disulfide bonds formed during the reaction and dialysis were reduced to free thiol groups using DTT. By varying the molar ratios among the reactants, the degree of conversion of the heparin COOH group to the thiol group, measured by the molar absorptivity of Ellman's reagent, could be systematically controlled (Table 1).

Bioactivity of the modified heparin, as measured by its binding affinity to AT-III, showed that the higher the degree of modification of the carboxyl groups, the lower the activity of the modified heparin (Figure 2a). However, still significant anticoagulant activity remained, even after quite extensive modification of the carboxyl groups to thiol (e.g., ca. 40% of the remained active after ca. 60% modification of the carboxylic groups of heparin), which supports previous reports stating that the sulfate group of heparin is more responsible for its anticoagulant activity.[23,24] Thus, it could be seen that the direct modification of the carboxylic groups of heparin did not result in the entire loss of anticoagulant biological activity in heparin.

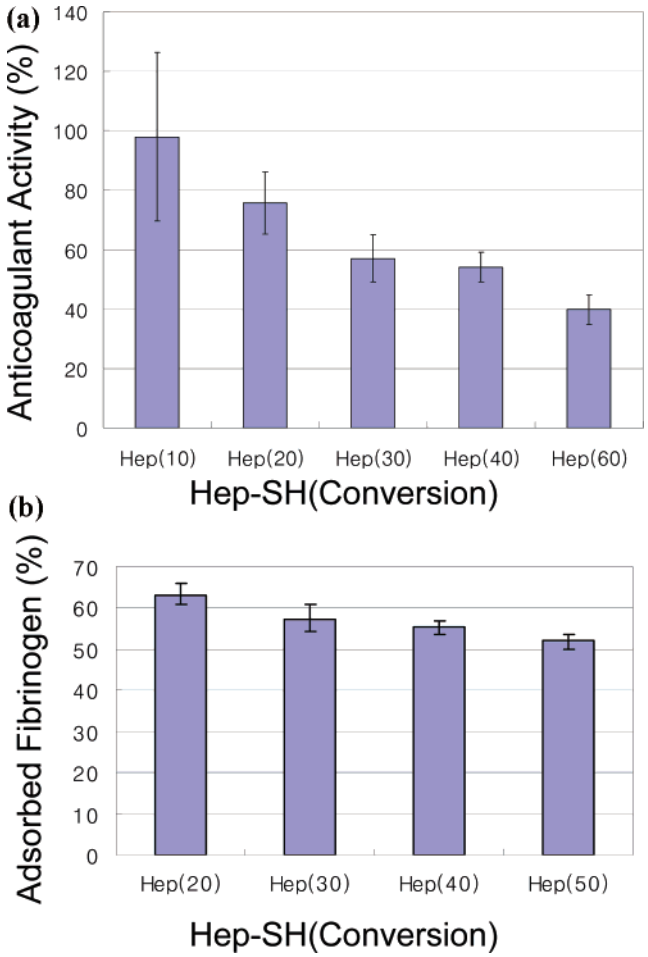

Figure 2. (a) Anticoagulant activity of thiol-functionalized heparin (Hep-SH) measured by its binding affinity to AT-III. Hep(*X*) denotes the Hep-SH that has *X*% conversion of COOH groups to SH. (b) Fibrinogen adsorption onto the heparin-based hydrogels. Approximately 200 μL of fibrinogen solution (20 mg/mL) was added to 40 μL of 10 wt % hydrogel and incubated at 37 °C for 1 day.

Gelation of Hep-SH with PEG-DA and Swelling Ratio of the Hydrogel. PEG-DA, having an acrylate group at both ends of PEG, was used to cross-link Hep-SH chains to form a gel structure. This Michael-type addition between vinyl groups and thiol groups has been previously reported to be fast and selective, allowing it to be used for protein or cell encapsulation without damaging the encapsulated biomolecules or surrounding environment.[18]

Gelation of Hep-SH and PEG-DA was characterized at various conditions using a rheometer (Figure 3). First, the degree of thiolation of Hep-SH was varied (Figure 3a). Results indicated that by increasing the degree of thiolation of Hep-SH stronger gels were obtained; the storage modulus after the cross-linking reaction was larger for Hep-SH with higher conversion. In fact, Hep-SH with much lower thiolation (~10%) did not produce a hydrogel with PEG-DA at all (data not shown), and Hep-SH with 20% conversion resulted in a very slow increase in storage

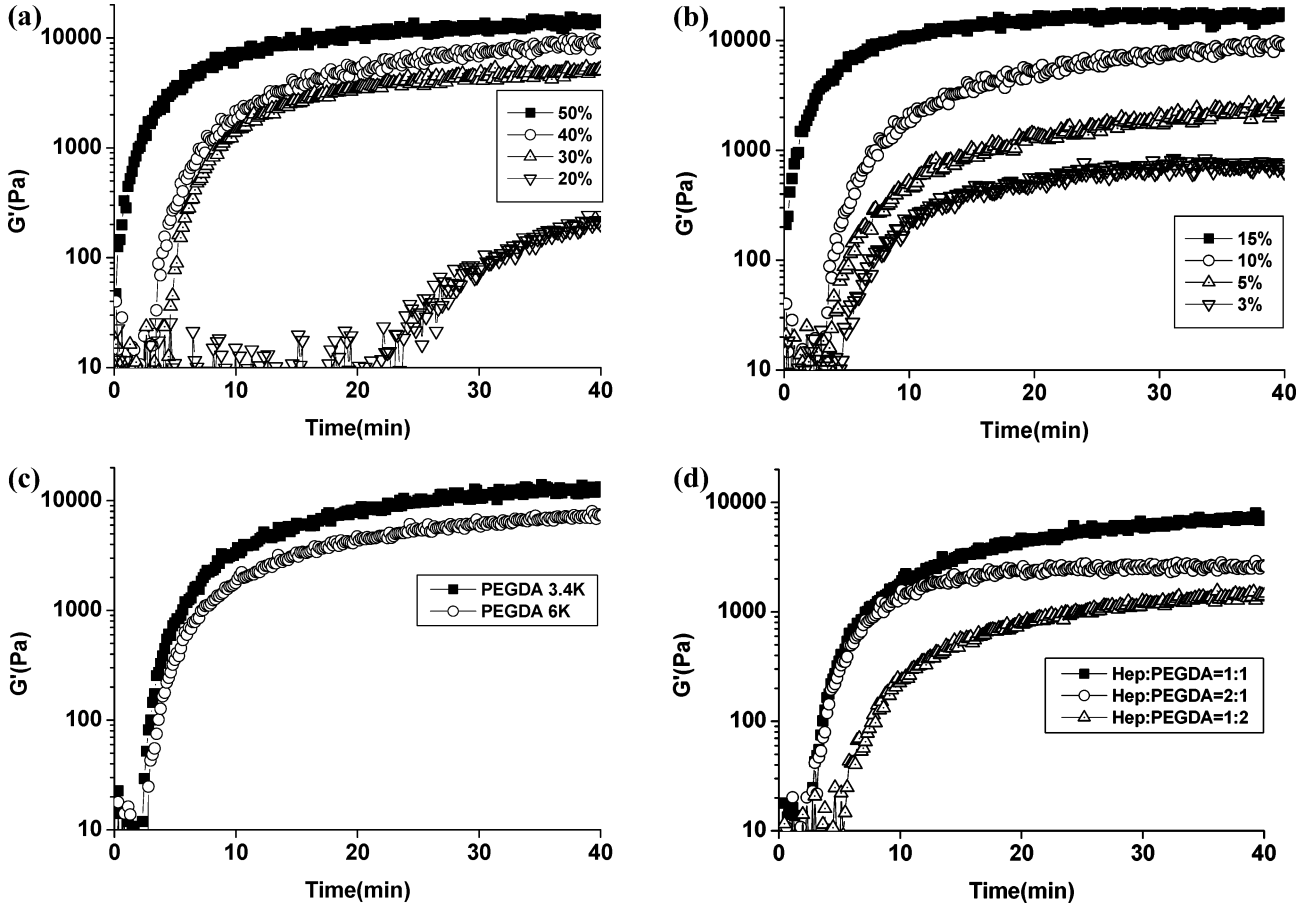

Figure 3. Gelation of Hep-SH and PEG-DA at various conditions in PBS. The default condition was the equimolar addition of Hep-SH and PEG-DA in terms of functional groups with 6 kDa PEG-DA, 40% thiloated Hep-SH, and 10 wt % total precursor concentration. (a) Effect of thiolation of Hep-SH. (b) Effect of total precursor concentration. (c) Effect of M_w of PEG-DA. (d) Effect of the molar ratio between Hep-SH and PEG-DA in terms of functional units.

Table 2. Swelling Ratios of Heparin-Based Hydrogels at Different Precursor States in (a) Deionized Water, (b) Normal PBS, and (c) PBS at Various Ionic Strengths for Hydrogel Preprared from 40% Hep-SH and 6 kDa PEG-DA[a]

	thiolation of the heparin		
M_w of PEG-DA	30%	40%	50%
(a) Deionized Water			
6 kDa	150.0 ± 6.7	129.2 ± 1.7	94.4 ± 3.1
3.4 kDa	127.3 ± 7.2	81.1 ± 3.3	68.8 ± 1.2
(b) Normal PBS			
6 kDa	30.8 ± 3.3	23.7 ± 2.8	20.2 ± 2.9
3.4 kDa	28.4 ± 1.6	20.7 ± 1.5	14.6 ± 2.5

(c) PBS at Various Ionic Strengths

buffer	2.8 × PBS	2.0 × PBS	1.0 × PBS	0.5 × PBS	0.1 × PBS	0.02 × PBS	0.01 × PBS	D.I. water	DMEM
swelling ratio	19.1 ± 1.0	20.2 ± 0.7	23.7 ± 2.8	25.6 ± 1.5	38.0 ± 2.1	62.9 ± 1.2	76.6 ± 4.8	129.2 ± 1.7	27.1 ± 1.4

[a] Hydrogels were prepared by the equimolar addition of functional groups of precursors at 10 wt %. The swelling ratio was calculated by W_s (wet weight)/W_d (dry weight) ($n = 4$).

modulus over time. The molecular weight of heparin is ca. ~12 kDa, and the dimer unit of heparin has one carboxylic group. Thus, 10% thiolation represents about 2 thiol units per heparin molecule, and 30% thiolation represents about 6 thiol units per heparin molecule. Since a linear linker (PEG-DA) was added as a cross-linker, the minimum number of cross-linking sites per heparin molecule to make a hydrogel is 3. Therefore, it is obvious that 10% thiolated Hep-SH cannot lead to gel formation. Considering the requirement of a fast conversion from a sol to a gel state to be applied as an useful in situ forming gelation system, 30% thiolation is regarded as the minimum level of conversion of COOH of heparin to SH.

Second, the total precursor concentration including Hep-SH and PEG-DA was varied (Figure 3b). Modulation of the total precursor concentration resulted in the systemic control of the strength of the gel. By increasing the total concentration from 3% to 15% (w/v), the storage modulus after cross-linking increased more than 20-fold (from less than 10^3 to over 10^4 kPa).

Next, the molecular weight of PEG-DA was varied from 3.4 to 6 kDa (Figure 3c). Since the same total precursor concentration and equimolar ratio between Hep-SH and PEG-DA were employed, the use of a smaller molecular weight of PEG-DA implies a higher molar concentration of both Hep-SH and PEG-

DA. In this arrangement, a marginally higher storage modulus was obtained for PEG-DA with 3.4 kDa. Additionally, the molar ratio between Hep-SH and PEG-DA was also varied (Figure 3d), and the equimolar addition (in terms of reactive functional units) of Hep-SH and PEG-DA resulted in a stronger gel state than the excess addition of either one of the components individually.

The swelling ratios of the heparin hydrogels, which were prepared from different precursor states (different molecular weights of PEG-DA or different degrees of thiolation of Hep-SH) but with the equimolar addition of functional groups of precursors and at the same total concentration (10 wt %), are summarized in Table 2. As can be seen in the table, a remarkable change in the swelling ratio was observed by the change of medium with respect to hydrogel swelling; a much higher swelling ratio was obtained in deionized water than that in PBS from the same hydrogel. Due to the highly negatively charged state of heparin, the swelling ratio of the same hydrogel was greatly influenced and subsequently reduced by the presence of ionic salts.[25] As well explained in ref 25, at equilibrium, the swelling ratio of the hydrogel with ionizable groups is decided by the balance between $\Delta G_{mix} = -\Delta G_{elasticity} - \Delta G_{ionic}$, where ΔG_{mix} is the mixing free energy, $\Delta G_{elasticity}$ is the entropy elasticity of the network, and ΔG_{ionic} is the ionic free energy of the network. Thus, when the ion concentration of the solvent is low (e.g., deionized water), the net negative repulsion among the heparin part of the network induces an additional swelling. By increasing the ionic strength of solvent, the negative repulsion among the heparin part is shielded; thus, the additional contribution due to the ionic nature of the network diminishes as shown in Table 2c. The swelling ratio of the hydrogel in PBS was relatively similar to that in DMEM, which also has a high ion concentration. However, except for the difference in the absolute value of the swelling ratio, the hydrogels showed the same tendency for swelling ratios in both PBS and water. First, a modest increase in the swelling ratio of the hydrogels was observed with an increase in the molecular weight of the PEG cross-linker, as a smaller cross-linker length introduces a higher density of cross-linking, coinciding with a higher modulus of the hydrogel (Figure 3c). There was also a significant change in the swelling ratio based on the difference in thiolation of heparin; hydrogels with a larger thiolation of heparin showed a smaller swelling ratio due to the higher cross-linking site, also coinciding with a larger storage modulus (Figure 3a). Thus, it was possible to systemically control the swelling ratio of the hydrogel by modulating the degree of thiolation of Hep-SH or the molecular weight of PEG-DA, even at the same total concentration. Note that all hydrogels exhibited very high water content, which should facilitate the exchange of nutrients and small hydrophilic molecules between the hydrogels and encapsulated cells.

Adsorption of Heparin-Binding Proteins on the Heparin-Based Hydrogel. The loading amounts of heparin-binding biomolecules on the heparin-based hydrogels prepared at various conditions were analyzed by adding a highly concentrated protein solution to the hydrogels, followed by incubation for 1 day at 37 °C. The unbound proteins were washed out, and the amounts were analyzed to obtain the adsorbed protein amounts on the hydrogels. Since growth factors are very expensive, it is not practical to measure the loading capacity of growth factors. Instead, fibrinogen and lysozyme were used. As shown in Figure 2b, even though there is some effect, the loading capacity of the hydrogel does not strongly depend on the thiolation of Hep-SH used for gelation, compared to the anticoagulant activity

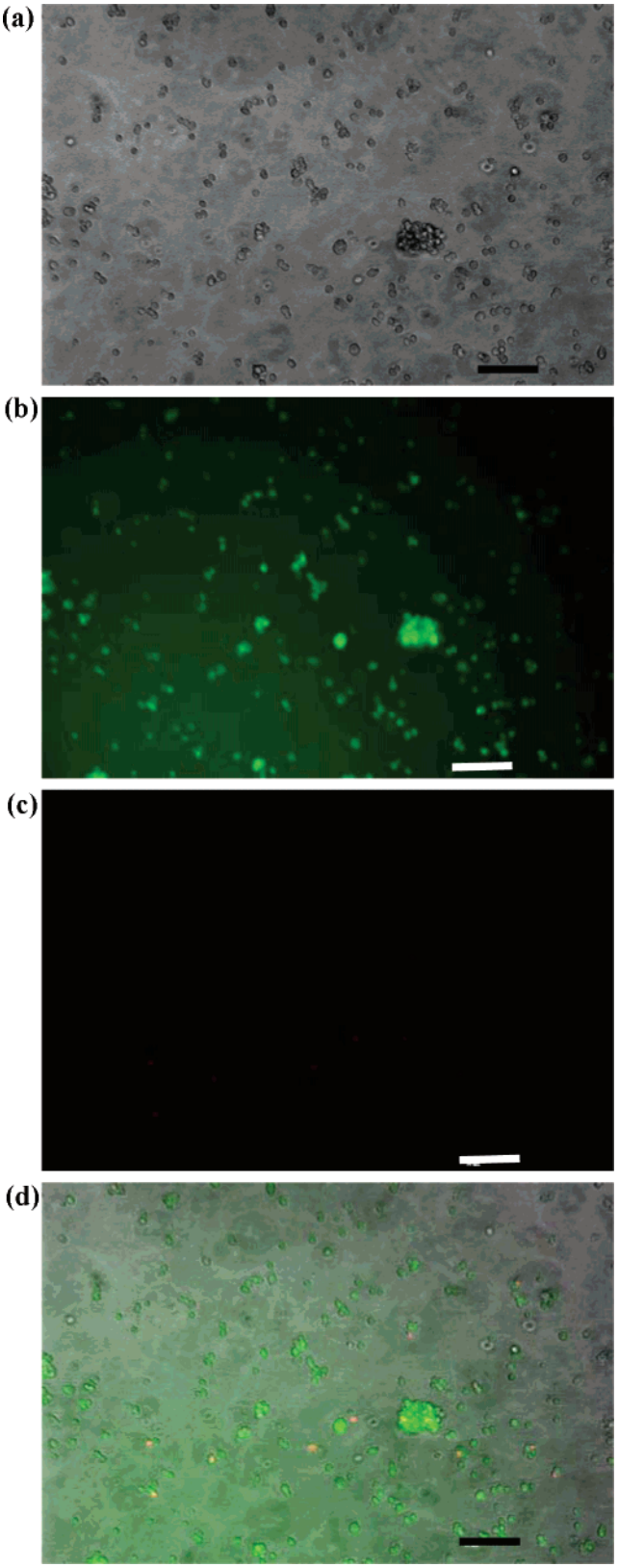

Figure 4. Cell (NIH/3T3) viability after cell encapsulation (in 2 h) measured by double staining with acridine orange (AO) and propidium iodide (PI). (a) Phase contrast image (b) Live cell image (c) Dead cell image (d) Merged image. Cell density: 1×10^6 cells/mL. Scale bar: 100 μm.

(Figure 2a). Also, the amounts of adsorbed fibrinogen were very high, ranging from ~0.5 mg/mg of polymer for the hydrogel with 50% thiolation of Hep-SH to ~0.6 mg/mg of polymer for the hydrogel with 20% thiolation of Hep-SH. Therefore, it can

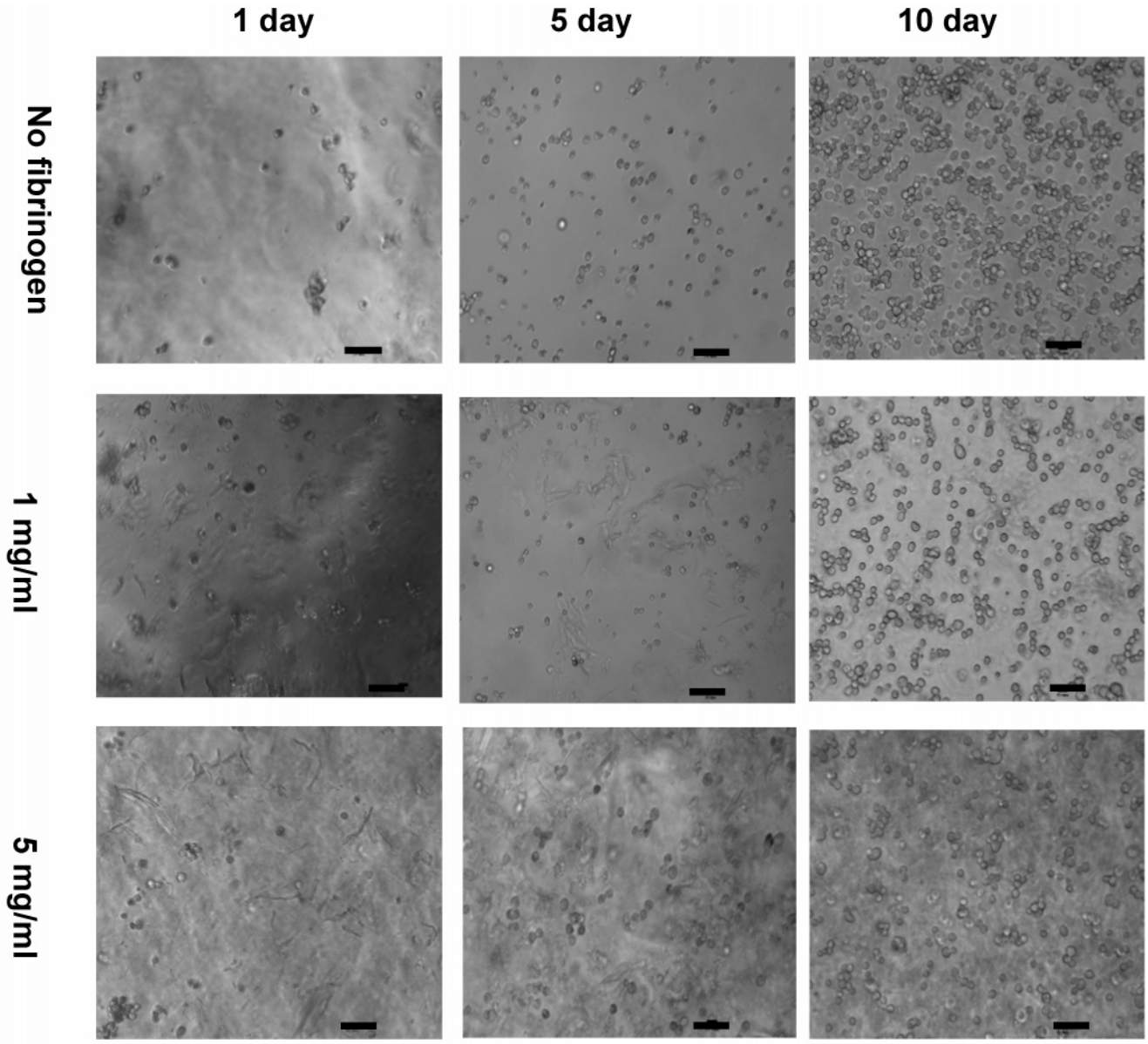

Figure 5. Phase contrast images of fibroblast cells (NIH/3T3) inside the heparin gels with no fibrinogen, 1 and 5 mg/mL of fibrinogen added during gelation after 5 and 10 days of incubation. Cell density: 1×10^6 cells/mL. Scale bar: 100 μm.

be concluded that the binding of fibrinogen to the heparin moiety is less specific than that of AT-III. Also, considering a very large adsorbed amount and weak dependency on thiolation of Hep-SH, the modified and cross-linked heparin entities seem to interact with proteins having heparin-binding domains. There was an increase in the adsorbed amount by increasing the protein concentration of the added solution to the hydrogel during adsorption from 10 to 20 mg/mL. However, above 20 mg/mL, the adsorbed amount did not increase by further increasing the protein concentration of the added solution during adsorption up to 50 mg/mL, which supports that the obtained values were close to the maximum loading capacities of the hydrogels. Similar adsorbed amounts and adsorption tendencies were observed in the case of lysozyme, too.

Cell Viability and Proliferation inside the Heparin-Based Hydrogel. The initial viability of fibroblast cells after encapsulation using the present gelation system was assayed, and it was determined that most of the cells were still alive (Figure 4). Cell viability calculated by counting live cells and dead cells was found to be over 95%. Thus, it reconfirms that the gelation scheme used here is so specific that the cross-linking reaction predominantly occurs between the gel-forming components; consequently, this gelation method can be used in vivo, based on the assumption that biomolecules (e.g., growth factors) do not have any exposed SH groups.[18]

Cell proliferation inside the hydrogel was characterized and compared with the case of the fibrinogen-added hydrogel. After incubation, the increase in cell number became apparent from the internal images of the heparin gel (Figure 5); at a later time point, the budding of cells from the existing cells was also observed. The cells inside the hydrogels, conversely, were round-shaped at all time points, and no distinct sign of fibroblast cell attachment to the heparin gel was observed, even after 10 days. Fibrinogen, a cell-adhesion protein that also has heparin-binding domains, was added into the hydrogel before gelation, with the expectation that added fibrinogen could promote the attachment of fibroblast cells into the heparin gel. However, no apparent change of cell morphology due to the presence of fibrinogen in the gel-forming materials was observed (Figure 5).

To quantitatively analyze the cell proliferation inside the hydrogels, a WST-1 assay[22] of the cell-encapsulated heparin

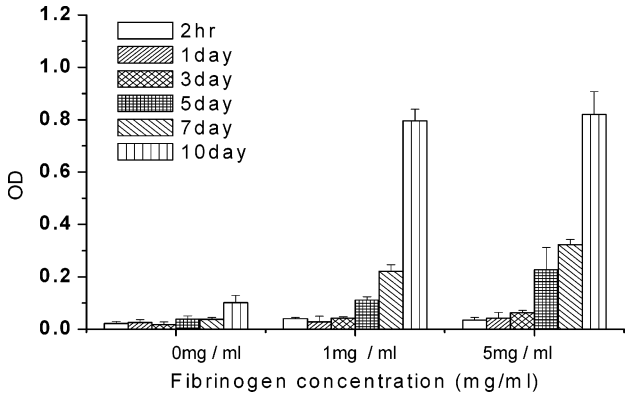

Figure 6. Fibroblast cell proliferation measured by WST activities inside the fibrinogen-added hydrogel, 1 and 5 mg/mL of fibrinogen added during gelation. Cell density: 1×10^6 cells/mL ($n = 4$).

gel was employed (Figure 6). It can be seen that in the case of the heparin-based hydrogel itself modest cell proliferation inside the hydrogel was observed, about a 70% increase at day 7 and about a 360% increase at day 10. In contrast, cell proliferation was greatly enhanced by the addition of fibrinogen inside the hydrogel; about a 170% increase in cell numbers at day 5, about a 4-fold increase at day 7, and over an 18-fold increase at day 10 were observed from the heparin hydrogel initially containing 1 mg/mL fibrinogen. In addition, a higher amount of fibrinogen in the hydrogel resulted in more effective proliferation; about a 5-fold increase in cell numbers at day 5, about an 8-fold increase at day 7, and a much greater than 20-fold increase were observed from the heparin hydrogel that initially contained 5 mg/mL fibrinogen.

The OD value of the WST-1 assay in this experiment was found to be linearly proportional with respect to cell numbers only up to 0.6, and then it gradually became saturated. Thus, rather similar values between the 1 mg/mL fibrinogen sample and the 5 mg/mL fibrinogen sample at day 10 did not result from the actual cell numbers but from the insensitivity of the assay at such high cell densities. Thus, the added fibrinogen was effective enough to significantly (more than 5-fold) enhance the cell proliferation, probably due to more efficient cell attachment to the hydrogel via the adsorbed fibrinogen on the heparin backbone of the hydrogel. Therefore, this suggests that the bioactivity of the present hydrogel system can be simply modulated by the addition of biomolecules with heparin-binding affinity.

Discussion

Comparison with Hyaluronic-Acid-Based Hydrogels. Other glycosaminoglycan-based hydrogel systems, especially hyaluronic-acid-based systems, have been developed.[26−31] However, heparin has unique biological and physicochemical properties compared to those of other glycosaminoglycans. In terms of biological aspects, hyaluronic acid can bind with positively charged growth factors by simple electrostatic interaction, whereas heparin has specific binding affinities to manygrowth factors and extracellular matrix (ECM) proteins related to cell binding. In addition, synergy between heparin and growth factors[32] or ECM proteins[33] in biological activities has been reported. In this report, we characterized that a significantly modified heparin to make heparin-based hydrogels still maintains its biological properties. Therefore, the present heparin-based hydrogel itself has heparin activity, whereas heparin was chemically attached additionally to a hyaluronic-acid-based system to further regulate the release of growth factors.[26,31] Also, we demonstrated that a simple addition of fibrinogen during gel preparation was enough to significantly enhance the cell proliferation of the heparin-based hydrogel via physical adsorption of fibrinogen to the hydrogel, whereas the fibronectin domain was chemically attached to the hyaluronic-acid-based hydrogel to control the biological properties of the matrix.[30]

In terms of physicochemical aspects, heparin has much smaller molecular weight than hyaluronic acid. Unfractionated hyaluronic acid has about 100 times larger molecular weight than heparin, and even low molecular weight hyaluronic acid (LMHA) has more than 10 times larger molecular weight than heparin. Thus, physical properties of two systems are also very different. Due to its smaller molecular weight, heparin-based hydrogel does not have physical cross-linking by entanglement and can be made even at above 30 wt % of precursor solution. Also, the additional cross-linker PEG-DA was necessary to make a hydrogel state; Hep-SH alone did not induce a hydrogel state, as no apparent change in viscosity was observed; instead, some white precipitates formed. In contrast, all of the hyaluronic-acid-based hydrogels were prepared at very low concentrations, probably due to the difficulty to dissolve high molecular weight hyaluronic acid, and thiol-functionalized hyaluronic acid itself can make a hydrogel state under certain physiological conditions via disulfide bond formation, without any additional cross-linker,[27,29] probably due to a significant level of physical entanglement present in hyaluronic acid solution even at low concentrations (∼1 wt %). Thus, the formation of a disulfide linkage among hyaluronic acid in addition to its physical entanglement is enough to produce a stable hydrogel state.

Degradation and Application of the Heparin-Based Hydrogels. The heparin-based hydrogels prepared by the proposed method are degraded slowly in physiological buffer by the hydrolytic degradation of ester bonds present in PEG-DA. Thus, the degradation of the hydrogels is controlled by the density of the ester bonds, which is a function of total polymer concentration as well as the ratio of PEG-DA added, which is determined by the degree of thiolation of Hep-SH. For example, the hydrogel prepared with 10 wt % total concentration and 30% thiolated Hep-SH is mostly degraded in PBS at 37 °C in 6 weeks. In addition to the hydrolytic degradation of the present hydrogel by a single ester bond in cross-linked chains, it is also possible to make the hydrogel degaradable in more explicit ways, thus controlling the degradation rate of the hydrogel; more effective hydrolytic degradation of the hydrogel can be obtained by attaching α-hydroxy acid units at the ends of PEG-DA,[34] or proteolytically degraded hydrogel can be achieved by adding an appropriate short peptide sequence into the cross-linked network.[35,36]

The loading capacity of heparin-binding proteins to the present hydrogel is about 10 times larger (∼0.5 mg of protein/mg of polymer) than the fibrinogen added during gel formation to enhance cell proliferation (5 mg/mL in 10 wt % polymer, corresponding to 0.05 mg of protein/mg of polymer). Also, dangling heparin residues present inside the hydrogel must be potentially more active than those cross-linked to the hydrogel networks. The existence of dangling heparin residues in the hydrogel can be deduced. First, the fraction of elastically effective chains can be estimated using G/nRT, where G is the plateau modulus of hydrogel, n is the theoretical chain molar concentration, which can be calculated from the polymer concentration and the effective number-average molecular weight between cross-links, R is the gas constant, and T is the temperature. To be a dangling bond state, only one of the thiol

groups in Hep-SH must be connected to the network. On the basis of a simple and rough assumption that thiol groups have the same probability of reaction and it is comparable to G/nRT, the amounts of heparin moieties present as dangling bonds are ~15% for 10 wt % hydrogel prepared from Hep-SH with 40% thiolation and ~1.5% for that with 50% thiolation. Thus, there is enough room in the hydrogel to add other heparin-binding biomolecules such as growth factors or other proteins for providing additional biofunctionalities by simple adsorption. Previously, we reported that the chemically modified heparin-based cross-linked hydrogel still maintains a specific binding affinity for heparin-binding growth factors such as VEGF.[15] We also observed that the heparin-based hydrogel efficiently retained the immonoreactive osteoprotegerin (OPG), promoting vascularization of the OPG-loaded hydrogel in animal experiments.[37] Therefore, the present gelation system has potential for use as a tissue regeneration scaffold with cellular interactions via the heparin and heparin-binding factors as well as an injectable delivery system for the heparin-binding growth factors.

Conclusion

A new, injectable, heparin-based hydrogel system that can be cross-linked with cells was developed. This injectable gelation system is achieved via a Michael-type addition between thiol-derivatized heparin and PEG diacrylates in physiological conditions. It was determined that mechanical properties, gelation kinetics, and swelling ratios of the hydrogels can be controlled by modulating the precursor concentration and degree of substitution of functional groups. Fibroblast cells were encapsulated in situ using the proposed gelation method, and cells remained viable after gelation. Proliferation of fibroblast cells was also observed inside the heparin gel, and the simple addition of fibrinogen during gelation was found to enhance the cell proliferation inside the hydrogel more than 5-fold. Thus, the present gelation system has a potential for use as a tissue regeneration scaffold with heparin bioactivity as well as an affinity-based, injectable delivery system.

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