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A heparin-mimicking reverse thermal gel for controlled delivery of positively charged proteins

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Abstract

Positively charged therapeutic proteins have been used extensively for biomedical applications. However, the safety and efficacy of proteins are mostly limited by their physical and chemical instability and short half-lives in physiological conditions. To this end, we created a heparin-mimicking sulfonated reverse thermal gel as a novel protein delivery system by sulfonation of a graft copolymer, poly(serinol hexamethylene urea)-*co*-poly(*N*-isopropylacrylamide), or PSHU-NIPAAm. The net charge of the sulfonated PSHU-NIPAAm was negative due to the presence of sulfonate groups. The sulfonated PSHU-NIPAAm showed a typical temperature-dependent sol-gel phase transition, where polymer solutions turned to a physical gel at around 32°C and maintained gel status at body temperature. Both *in vitro* cytotoxicity tests using C2C12 myoblast cells and *in vivo* cytotoxicity tests by subcutaneous injections demonstrated excellent biocompatibility. *In vitro* release tests using bovine serum albumin (BSA) revealed that the release from the sulfonated PSHU-NIPAAm was more sustained than that from the plain PSHU-NIPAAm. Furthermore, this sulfonated PSHU-NIPAAm system did not affect protein structure after 70-day observation periods.

Keywords

Injectable; reverse thermal gel; heparin; sulfonation; protein release

INTRODUCTION

The development of smart (or stimuli-responsive) biomaterials is a major research focus in the field of tissue engineering and biomolecule delivery^{1–9}. In particular, temperature-responsive reverse thermal gels (RTG) have gained much attention^{10–20}. At room temperature, RTG systems exist in a solution state (sol) with low viscosity that allows injection through a small gauge needle^{11,12}. Upon reaching body temperature, the RTG transitions from a low-viscosity sol to a semi-solid gel (sol-gel phase transition)^{13,21}. This unique characteristic may be used to deliver sensitive therapeutic agents, such as cells, drugs or proteins¹⁴, to a specific target site, avoiding side effects of invasive surgeries¹².

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Heparin, a naturally sulfated biopolymer with an intrinsic negative charge, stores, protects and stabilizes positively charged heparin-binding proteins in the extracellular matrix (ECM) and therefore plays an important role in the regulation of cellular proliferation and differentiation^{22–30}. Due to this, a vast number of heparin-conjugated protein delivery systems have been investigated^{31–36}. However, heparin has major limitations. It is difficult to modify, susceptible to desulfation, presents batch-to-batch variability in structure and biocompatibility³⁷ and has significant undesirable activity in other non-target biological pathways³⁸. Moreover, it may inhibit the normal growth of certain cells such as human umbilical vein endothelial cells and human dermal fibroblast^{39,40}.

A recent study reported that sulfonation (or sulfation) of polymeric materials may induce a biofunction similar to that of heparin^{41–44}. However, the nature of these bulk-sized scaffolds may still require invasive surgeries for implantation. A system that mimics the biofunction of heparin and delivers therapeutic proteins non-invasively to the zone of interest may be an ideal alternative to aforementioned system. In this study, we designed a unique heparin-mimicking sulfonated reverse thermal gel that not only undergoes typical sol-gel phase transition upon temperature changes and thus achieves a non-invasive treatment, but also acts like heparin for the delivery of therapeutic proteins while preserving their activity. In addition, we examined the biocompatibility of the sulfonated reverse thermal gel and further investigated its potential as a delivery matrix for positively charged proteins.

MATERIALS AND METHODS

Materials

N-Isopropylacrylamide (NIPAAm), anhydrous *N,N*-dimethylformamide (DMF), 4,4'-azobis(4-cyanovaleric acid) (ACVA), urea, *N*-BOC-serinol, hexamethylene diisocyanate (HDI), diethyl ether, trifluoroacetic acid (TFA), dichloromethane (DCM), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC-HCl), 1,3-propane sultone (PS), bovine serum albumin (BSA), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich, (St. Louis, MO, USA). *N*-Hydroxysuccinimide (NHS) and potassium tert-butoxide (t-BuOK) were purchased from Alfa Aesar (Ward Hill, MA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Invitrogen Corporation (Carlsbad, CA, USA). Fetal bovine serum (FBS), penicillin and DME/F-12 1:1 growth medium were purchased from Thermo Scientific (Logan, UT, USA). Trypsin EDTA 1× was purchased from Cellgro (Manassas, VA, USA). C2C12 (ATCC® CRL1772™) cell was purchased from the America Type Culture Collection (Manassas, VA, USA). Monoclonal antibody (host/isotype: IgG1 kappa) for ED-1 staining was purchased from Millipore (Temecula, CA, USA). Spectra/Por dialysis membranes (MWCO: 3500–5000 and 12,000–14,000 Da) were purchased from Spectrum Laboratories (Rancho Dominguez, CA, USA).

Equipment

Morphological characterization was carried out by scanning electron microscopy (SEM) using a Field Emission SEM JEOL JSM 7401F. The low critical solution temperature (LCST) was determined using a UV-Visible spectrophotometer with a temperature-

controlled cell holder. Molecular weight was determined via gel permeation chromatography (GPC) on an Ecosec GPC system using a TOSOH TSK gel column, anhydrous DMF with 0.1% LiBr as mobile phase, and polystyrene as standard. Proton nuclear magnetic resonance (^1H NMR) spectra were recorded on a Bruker Avance 500 NMR. Zeta potential measurements were carried out in a Zetasizer Nano Z (Malvern, Westborough, MA) using a DTS1060 cell and analyzed with a zetasizer software. The secondary structure of BSA was determined using a Chirascan-plus circular dichroism (CD) spectrometer (Applied photophysics) at 25°C and a 1 mm quartz cuvette, by scanning from 200–260 nm with 2 nm increments.

Synthesis of SRTG

Synthesis of poly(serinol hexamethylene urea) or PSHU—N-BOC-serinol (1.147 g, 6 mmol) and urea (0.36 g, 6 mmol) were dissolved in 6 ml of anhydrous DMF in a 25 ml round bottom flask at 90°C under a nitrogen atmosphere. HDI (2.018g, 12 mmol) was added slowly, and the polymerization was performed for 7 days. After cooling down to ambient temperature, the mixture was precipitated into excess anhydrous diethyl ether. The precipitate was dissolved again in anhydrous DMF and precipitated into excess anhydrous diethyl ether. The purification process was carried out thrice. Unreacted urea was removed by washing with water, and the polymer was lyophilized at –45°C for 24 h.

De-protection of PSHU (PSHU-NH₂)—PSHU (1.5 g) was dissolved in 10 ml of a TFA/DCM (1:1, v/v) mixture, and the de-protection was performed for 30 min at room temperature. After removing TFA and DCM using a rotary evaporator, the resulting polymer was re-dissolved in DMF and purified by three precipitations into diethyl ether. Finally, the polymer was decanted in water and lyophilized at –45°C for 24 h.

Synthesis of PNIPAAm-COOH—NIPAAm (5 g, 800 mmol) and ACVA (0.06 g, 4 mmol) were dissolved in 10 ml of anhydrous DMF in a 25 ml round bottom flask. The solution was purged with pure nitrogen for 30 min. The polymerization was performed for 3 h at 68°C. After cooling down to ambient temperature, the mixture was precipitated into hot water (60°C). After washing twice in hot water, the polymer was dissolved in milli-Q water and dialyzed (MWCO: 3500–5000 Da) against 1 l water for 24 h at room temperature. The product was lyophilized at –45°C for 48 h.

Conjugation of PNIPAAm-COOH onto PSHU-NH₂ (PSHU-NIPAAm)—PNIPAAm-COOH (0.75 g, 1.21 mmol) was dissolved in 5 ml of anhydrous DMF with three molar excess of EDC-HCl and NHS in a 25 ml round bottom flask at room temperature under a nitrogen atmosphere and the mixture was stirred for 24 h. 1 ml of PSHU-NH₂ solution (0.125 g/ml) prepared in anhydrous DMF was added slowly and the reaction was performed for 24 h at room temperature under a nitrogen atmosphere. The mixture was precipitated into excess diethyl ether thrice. The polymer was dissolved in milli-Q water and dialyzed (MWCO: 12,000–14,000 Da) against 1 l water for 24 h at room temperature and the product was lyophilized at –45°C for 48 h.

Sulfonation of PSHU-NIPAAm—PS (0.034 g, 5 mmol) and t-BuOK (0.032 g, 5 mmol) were dissolved in 3 ml of anhydrous DMF in a 25 ml round bottom flask at 50°C under a nitrogen atmosphere. A solution of 3 ml PSHU-NIPAAm (0.1 g/ml) in anhydrous DMF was added slowly to the flask and the sulfonation was performed for 3 days at 60°C under a nitrogen atmosphere. After cooling down to ambient temperature, the mixture was precipitated into excess diethyl ether thrice. Finally, the polymer was dissolved in milli-Q water and dialyzed (MWCO: 12,000–14,000 Da) against 1 l water for 48 h at room temperature and lyophilized at –45°C for 48 h.

Cytotoxicity test

In vitro cytotoxicity test was performed by MTT assay using C2C12 myoblast cells by ISO 10993-5 guidelines. Briefly, C2C12 cells were seeded into 96-well plate (10,000 cells/well) and incubated with 100 µL of sequentially diluted polymer extract at 37°C for 24 h. The cell viability was examined by MTT assay.

In vivo cytotoxicity test was performed by subcutaneous injection into male Sprague-Dawley rats (~230 g). All animal experiments were performed under a protocol approved by the Institutional Animal Care and Use Committees (IACUC) at the University of Colorado Denver. The rats were anesthetized by isoflurane inhalation. Rats were injected subcutaneously with 0.5 ml of SRTG solution on the right and left side of their back. The animals were sacrificed 1, 7, and 10 days post-injection; tissues were fixed in 10% formalin and cryo-sectioned longitudinally to 8µm thickness. The sectioned tissues were fixed in acetone for 10 min, air dried, and incubated in mouse anti-rat ED1 primary antibody for 30 min at 37°C. After washing with PBS three times, the tissues were incubated in goat anti-mouse Cy3 secondary antibody for 30 min at room temperature. ED1-positive cells in square millimeters were imaged and quantified.

BSA release test

Two concentrations (10% and 15%, w/v) of the sulfonated PSHU-NIPAAm -BSA solution were prepared in pH 4.5 acetate buffer solution with a final BSA concentration of 0.5 mg/ml. The mixtures were stirred for 30 min at 4°C. A 300 µl of the sulfonated PSHU-NIPAAm-BSA solution was poured into a cylindrical mold, and allowed to form a disc-shaped gel at 37°C. Once the disc-shaped gel was formed, it was transferred into a vial containing 10 ml of pH 4.5 acetate buffer solution and incubated at 37°C. At pre-determined time points, 3 ml of sample was withdrawn, and the released BSA concentration was quantified by UV-Vis spectrometer at 280 nm and expressed as cumulative percent release. The same experiment was performed with the plain PSHU-NIPAAm for the comparison.

RESULTS AND DISCUSSION

Synthesis of sulfonated PSHU-NIPAAm and characterization

With an aim to create a heparin-mimicking polymer that possesses the biofunction of natural heparin and presents the advantages of an RTG, the sulfonated PSHU-NIPAAm was synthesized using PSHU-NIPAAm copolymer (Figure 1). While the primary amine in PSHU is initially protected by a BOC group, it is easily de-protected to a primary amine in

the mixture of TFA/DCM. These de-protected primary amines can then be used for further modifications. The presence of both sulfonate groups and PNIPAAm in sulfonated PSHU-NIPAAm was confirmed by FTIR (Figure 2) and ^1H NMR (Figure 3). FTIR showed sulfonate ($\text{O}=\text{S}=\text{O}$) vibrations at 995 cm^{-1} , while no signals of sulfonate groups were observed in plain PSHU-NIPAAm (Figure 2). In both plain PSHU-NIPAAm and sulfonated PSHU-NIPAAm amide amine (N-H) and amide carbonyl ($\text{C}=\text{O}$) peaks were observed at 1645 cm^{-1} and 1500 cm^{-1} , respectively. The presence of sulfonate group was further confirmed by ^1H NMR (Figure 3). The methylene proton adjacent to sulfur ($^-\text{O}_3\text{S}-\text{CH}_2$) was confirmed at 3.01 ppm ⁴⁵ that does not exist in plain PSHU-NIPAAm. In addition, the presence of methylene and methyl protons at 1.55 and 1.09 ppm , respectively, also confirmed PNIPAAm in the polymer. GPC was used to investigate the molecular weight (M_w) of sulfonated PSHU-NIPAAm and plain PSHU-NIPAAm, which were found to be $100,129\text{ Da}$ and $77,302\text{ Da}$, respectively, corroborating the further functionalization of amine groups.

The introduction of sulfonate groups onto PSHU-NIPAAm may lead to a shift in the net charge to more negative state³¹. The charge shifts before and after the sulfonation were verified by zeta potential analysis (Figure 4). The zeta potential of 10% and 15% (wt/v) plain PSHU-NIPAAm were 1.47 and 2.08 , respectively, while sulfonated PSHU-NIPAAm showed negative values due to the presence of negatively charged sulfonate groups. In addition, a higher concentration of sulfonated PSHU-NIPAAm resulted in higher negative charges (-0.56 mV and -7.05 mV for 10% and 15% , respectively), indicating that the negative charge intensity can be easily modulated by simple variations of polymer concentrations.

The sol-gel phase transition of sulfonated PSHU-NIPAAm was determined by estimating its low critical solution temperature (LCST) (Figure 5). The transmittance of an aqueous solution of sulfonated PSHU-NIPAAm decreased slowly upon heating from 20°C to 31°C , reached almost zero at 32°C , and turned to an opaque solid upon further heating over 33°C , indicating that the aqueous solution turns to a physical gel as the temperature increases. This phase transition is driven purely by the thermodynamic competition between hydration of the polymer chains and hydrophobic interactions between polymer molecules¹¹. At low temperatures (below the LCST, 32°C in this case), hydration is thermodynamically favored and the polymer molecules are maintained in a solution state (Figure 5B). Above the LCST, hydrophobic interactions between the polymer chains are favored and the polymer molecules interact to form a self-standing physical gel (Figure 5C).

The morphological characterization of 10% and 15% (wt/v) sulfonated PSHU-NIPAAm was achieved by SEM (Figure 6). Cross-sectional images of the gels revealed that both concentrations resulted in a highly porous configuration. It is also noticeable that a higher concentration of the polymer solution formed smaller pores than the lower concentration ($0.48 \pm 0.1\text{ }\mu\text{m}$ and $1.11 \pm 0.3\text{ }\mu\text{m}$ for 15% and 10% , respectively), indicating that the pore size of sulfonated PSHU-NIPAAm could be easily tuned by polymer concentrations, which may be beneficial for modulating protein release from the matrix.

Cytotoxicity test

In vitro cytotoxicity was investigated using an MTT assay with C2C12 myoblast cells, a well-documented method for measuring cell viability and providing a general indication of cell health^{46–48}. Figure 7 shows no statistical difference between the absorbance (directly related to cellular metabolic activity) of pure medium (positive control) and the polymer extract, while the presence of 10% DMSO significantly reduced the level of absorbance. The similar absorbance levels of the extract and the medium is good evidence that the sulfonated PSHU-NIPAAm is non-cytotoxic. In this study, we did not include plain PSHU-NIPAAm as a control group, since in our previous study plain PSHU-NIPAAm proved to have good biocompatibility⁴⁹.

In vivo cytotoxicity was further investigated by subcutaneous injection of sulfonated PSHU-NIPAAm solution into male Sprague-Dawley rats (~230 g). We injected 27% (wt/v, the highest possible concentration in water) sulfonated PSHU-NIPAAm solution using a 25-gauge needle. The inflammatory reaction was examined using ED1 staining to estimate the macrophage activity triggered by injection of the gel (Figure 8). At day-1 (Figure 8A), a large number of ED1-positive macrophages were found, indicating an acute inflammatory reaction caused by a non-specific inflammatory reaction⁵⁰. The density of ED1-positive cells decreased rapidly after 7 days (Figure 8B), and most of the ED1-positive cells disappeared after 10 days (Figure 8C) indicating a mild inflammatory reaction. ED1-positive macrophages were quantified using 10 randomly chosen images around the tissue-gel interface. The sequential decrease in the number of macrophages was observed (Figure 8D), indicating excellent biocompatibility even at the highest dose.

BSA release test

The potential of sulfonated PSHU-NIPAAm as a protein delivery system was studied using BSA with different concentrations of sulfonated PSHU-NIPAAm and plain PSHU-NIPAAm (Figure 9). The sulfonated PSHU-NIPAAm showed remarkably more sustained release behavior than plain PSHU-NIPAAm, indicating that the negatively charged sulfonate groups in the sulfonated PSHU-NIPAAm may effectively hold BSA in the matrix (Note that BSA shows positive charge at pH 4.5). Moreover, the fact that BSA release was more sustained at higher concentrations well supports that the release rate can be easily modulated by changing the sulfonated PSHU-NIPAAm concentration. A possible explanation is that higher concentrations of the sulfonated PSHU-NIPAAm leads to a higher sulfonate group density in a given volume of the gel matrix, resulting in more negative charge (as confirmed by zeta potential measurement in Figure 4), which causes enhanced ionic interaction between sulfonated PSHU-NIPAAm and BSA. Although not presented here, currently we are applying this system for *in vivo* fibroblast growth factor (FGF) delivery for the treatment of a cardiac disease. For this on-going study, we preliminarily examined *in vitro* FGF release behavior to decide an appropriate formulation for *in vivo* injection, with similar FGF release behavior in physiological condition as we observed with BSA release in pH4.5. Thus, the sulfonated PSHU-NIPAAm system is expected to be a promising tool for controlled and sustained protein delivery.

Circular dichroism (CD) spectroscopy

In order for the sulfonated PSHU-NIPAAm to serve as a protein delivery matrix, it should not affect protein structure or possibly lead to the denaturation of proteins. To address it, the secondary structure of released BSA at the last day of observation was determined by CD spectroscopy and compared with native BSA solution (Figure 10). No significant difference was observed in all samples with a typical α -helix conformation^{51–53}, confirming well-preserved protein structure.

CONCLUSION

A novel heparin-mimicking injectable sulfonated PSHU-NIPAAm was successfully synthesized, demonstrating typical sol-gel phase transition upon temperature changes and remarkably sustained BSA release profile compared to a plain PSHU-NIPAAm with capacity to preserve protein structure. We expect that this sulfonated PSHU-NIPAAm system can serve as a platform for biomimetic injectable biomaterials for controlled and sustained protein delivery in biomedical applications.

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Figure 1.

Synthesis of sulfonated PSHU-NIPAAm by Step 1: synthesis of PSHU, Step 2: deprotection of PSHU, Step 3: conjugation of PNIPAAm-COOH to PSHU, and Step 4: sulfonation of PSHU-NIPAAm using 1,3-propane sultone.

Figure 2.

FTIR spectra of sulfonated PSHU-NIPAAm and plain PSHU-NIPAAm. The signals represent (from right to left) sulfonate groups (995 cm^{-1}), amide carbonyl (1500 cm^{-1}) and amide amine (1645 cm^{-1}).

Figure 3.

^1H NMR spectra of sulfonated PSHU-NIPAAm and plain PSHU-NIPAAm. Successful sulfonation of PSHU-NIPAAm was confirmed by the presence of a methylene proton adjacent to sulfur ($\text{O}_3\text{S}-\text{CH}_2$) at 3.01 ppm. In addition, the conjugation of PNIPAAm was confirmed by the presence of methylene and methyl protons at 1.55 and 1.09 ppm, respectively.

Figure 4.

Zeta potential of sulfonated PSHU-NIPAAm and plain PSHU-NIPAAm. The plain PSHU-NIPAAm showed positive zeta potential: 1.47 mV for 10% (■) and 2.08 mV for 15% (▲). In contrast, the zeta potential of sulfonated PSHU-NIPAAm showed negative values due to the negatively charged sulfonate groups: -0.56 mV for 10% (◆) and -7.05 mV for 15% (●).

Figure 5. Temperature-dependent phase transition of sulfonated PSHU-NIPAAm. A) The sulfonated PSHU-NIPAAm undergoes a sharp, reversible phase transition around 32°C as determined by UV-Visible spectroscopy. B) An aqueous solution of sulfonated PSHU-NIPAAm at room temperature C) turns to physical gel at 37°C.

Figure 6.
SEM images of sulfonated PSHU-NIPAAm cross-section. A) 10% (w/v) and B) 15% (w/v) of SRTG, with larger pore size in the lower concentration of sulfonated PSHU-NIPAAm.

Figure 7.

In vitro cytotoxicity of sulfonated PSHU-NIPAAm by MTT assay. Results demonstrated no cytotoxic effects of sulfonated PSHU-NIPAAm on C2C12 cells after exposure to the polymer extract in medium, while 10% DMSO shows significant cytotoxicity. There is no statistical difference between medium and extracts. Data represent mean $\pm$ SD. * indicates p value <0.05 (Student's test).

Figure 8.

Immunohistochemically stained ED1-positive macrophages (200 $\times$, scale bar = 70 μ m).

Tissues were harvested after (A) 1 day, (B) 7 days, and (C) 10 days. (D) The decrease in the number of ED1-positive macrophages with time indicates a reduction in the inflammatory response. Data represent mean $\pm$ SD.

Figure 9.

BSA release profiles from sulfonated PSHU-NIPAAm and plain PSHU-NIPAAm: ◆ sulfonated PSHU-NIPAAm 10%, ● sulfonated PSHU-NIPAAm 15%, ■ plain PSHU-NIPAAm 10%, ▲ plain PSHU-NIPAAm 15%. BSA release was more sustained from sulfonated PSHU-NIPAAm than that from plain PSHU-NIPAAm, with a more sustained profile at higher concentrations. Data represent mean $\pm$ SD.

Figure 10.

Circular dichroism spectra (CD) of native and released BSA. The similarity of the protein conformation between samples indicates that the secondary structure of the BSA protein was well preserved by the system.