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Serum Albumin−Peptide Conjugates for Simultaneous Heparin Binding and Detection

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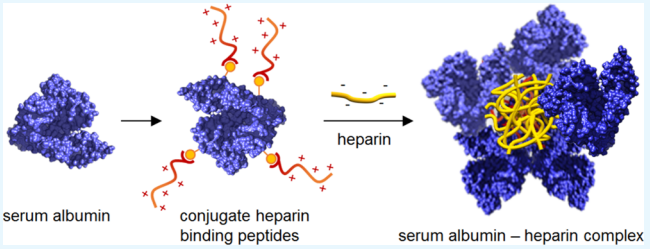
Supporting Information

ABSTRACT: Heparin is a polysaccharide-based anticoagulant agent, which is widely used in surgery and blood transfusion. However, overdosage of heparin may cause severe side effects such as bleeding and low blood platelet count. Currently, there is only one clinically licensed antidote for heparin: protamine sulfate, which is known to provoke adverse effects. In this work, we present a stable and biocompatible alternative for protamine sulfate that is based on serum albumin, which is conjugated with a variable number of heparin-binding peptides. The heparin-binding efficiency of the conjugates was evaluated with methylene blue displacement assay, dynamic light scattering, and anti-Xa assay. We found that multivalency of the peptides played a key role in the observed heparin-binding affinity and complex formation. The conjugates had low cytotoxicity and low hemolytic activity, indicating excellent biocompatibility. Furthermore, a sensitive DNA competition assay for heparin detection was developed. The detection limit of heparin was 0.1 IU/mL, which is well below its therapeutic range (0.2−0.4 IU/mL). Such biomolecule-based systems are urgently needed for next-generation biocompatible materials capable of simultaneous heparin binding and sensing.



INTRODUCTION

Heparin is a highly charged glycosaminoglycan (GAG) mainly composed of uronic acid and glucosamine subunits.[1] Ever since its first medical use in the 1930s, heparin has found widespread applications as the first naturally occurring polysaccharide-based drug.[2] The medical role of heparin is primarily based on its anticoagulant ability. It can complex with thrombin inhibitors such as antithrombin III with high affinity. This further inactivates thrombin, factor Xa, and other coagulation factors leading to an interrupted blood-clotting cascade.[3] Commonly, the dosage of heparin needs to be maintained within 2−8 IU/mL during cardiovascular surgery and 0.2−1.2 IU/mL in postoperative and long-term care. Maintaining heparin concentrations at sufficient levels will prevent thrombosis while avoiding the risk of serious side effects, including heparin-induced thrombocytopenia, caused by excessive heparin.[4−6] Hence, it is of vital importance to monitor heparin concentrations and also neutralize its anticoagulant effect when blood clotting needs to be recovered. Currently, heparin neutralization is predominantly achieved by an arginine-rich shellfish protein, protamine sulfate (PS), which is also the only clinically licensed antidote. Anti-Xa assay and activated partial thromboplastin time assay (aPTT) are the most desirable methods to monitor blood coagulation.[7−9]

Protamine sulfate binds to heparin efficiently through the electrostatic interaction between its cationic arginine groups and anionic sulfonate groups of heparin.[10] Despite the high binding efficiency using PS involves serious drawbacks that should not be overlooked. For example, it can cause severe adverse effects including anaphylactic reactions, and it is ineffective in the removal of low molecular weight heparin.[11−14] Therefore, great effort has been devoted to the development of new heparin antidote candidates.[2] Most of these rely on the development of cationic molecules since heparin has the highest negative charge among all known biomacromolecules.[15] Methylene blue is one of the earliest and simplest compounds that have been studied.[16] However, its binding efficiency is largely hindered in physiological conditions due to the low charge density.[17] Other small molecular systems have also been reported, such as surfen, delparantag, and foldamers.[18−21] Owing to their multivalency effects, cationic oligopeptides,[22,23] synthetic polymers,[24−27] and self-assembled systems[28−31] have drawn attention.

As mentioned above, another important area in maintaining sufficient heparin levels is the detection technique. Traditional clotting time-based assays have been proven accurate, but they are rather time-consuming. Therefore, real-time heparin detection methods would be highly desirable, and numerous luminescent,[32] colorimetric,[33,34] and fluorescent[35−38] sensors have been developed along these lines. Fluorescence-based

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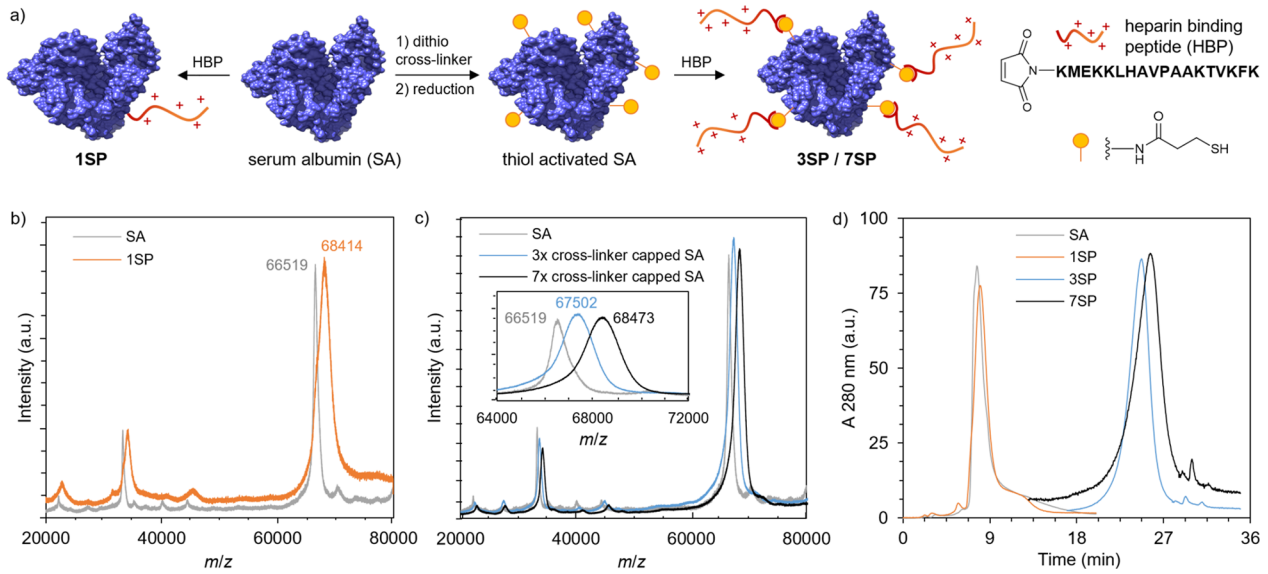

Figure 1. (a) Schematic presentation of the preparation of single (1SP) and multiple (3SP and 7SP) peptide-modified SAs. (b) MALDI-TOF spectra of SA and 1SP: M_{SA} = 66,519 g/mol and M_{1SP} = 68,414 g/mol (theoretical molecular weights: M_{SA} = 66,430 g/mol[57] and M_{1SP} = 68,636 g/mol); (c) MALDI-TOF spectra of cross-linker-capped SAs: $M_{5\times}$ = 67,502 g/mol and $M_{10\times}$ = 68,473 g/mol. Therefore, the estimated number of cross-linkers attached to SAs for 5 and 10 equiv syntheses was 3 and 7, respectively; (d) HPLC analysis of purified 1SP, 3SP, and 7SP using a heparin affinity column. SA was analyzed for comparison.

methods have been established using both turn-on[35,37] and turn-off[36] approaches.

Serum albumin (SA) is broadly recognized as a biocompatible vector for the delivery of drugs.[39−41] In addition, many SA-based conjugates have been reported to be nontoxic and nonimmunogenic.[40,42−45] In 2005, an albumin-based anticancer drug, Abraxane, was first approved by the U.S. Food and Drug Administration (FDA), and continuous effort has been put into the development of albumin-based drugs.[46,47] In this work, albumin-based heparin-binding compounds were developed. They are based on SA proteins that are conjugated with a variable number of heparin-binding peptides (HBPs). The heparin-binding peptide is known from the fibroblast growth factor (FGF) and has a dissociation constant of ∼134 pM with heparin.[48] Single (1SP) and multiple (3SP and 7SP) peptide-conjugated SAs were synthesized, and the binding ability of the products to heparin was evaluated with methylene blue (MB) displacement assay, dynamic light scattering (DLS), and anti-Xa assay in selected buffers and human blood plasma. It was found that the heparin-binding capacity increased with the amount of peptides attached. Under physiological conditions, 3SP and 7SP maintained moderate heparin-binding ability, which could be attributed to the multivalency effect,[49−51] while 1SP showed negligible heparin binding. The complexes were also visualized with atomic force microscopy (AFM). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye assay and hemolysis assay results revealed negligible toxicity to human dermal fibroblasts (HDF) cells and human red blood cells (RBCs) within the tested concentration range. Finally, a fluorescence-based heparin detection method was developed through complexing 7SP and chemically modified DNA (22 nucleotide (nt)-long DNA with a fluorescein (6-FAM) and black hole quencher (BHQ1) at the 5′ and 3′ ends, respectively). A calibration curve for quantitative heparin level detection was constructed, and it was found that the detection limit is <0.1 IU/mL, which is well below the concentration required during cardiovascular surgery (0.2−1.2 IU/mL).

RESULTS AND DISCUSSION

Preparation of SA−Peptide Conjugates. For conjugating SA and peptides, an efficient and selective thiol maleimide reaction was used.[52−54] Bovine serum albumin used in this work has a free and solvent-exposed cysteine (Cys34), which acts as a suitable and easily accessible conjugation handle.[55] Additional advantages including stability, low toxicity, and long circulation time make SA an ideal candidate as a scaffold and shielding group for bioconjugates.[45] For the preparation of single peptide-conjugated SA (1SP), an *N*-terminal maleimide-modified peptide was mixed with SA and left reacting overnight (Figure 1a). According to the analysis of the crude 1SP mixture with semipreparative high-performance liquid chromatography (HPLC) using a heparin affinity column (Figure S1a), the conversion was approximately 50%.[56] Peak fractions were collected, desalted, and finally lyophilized. The purity of 1SP was confirmed by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF, Figure 1b) and HPLC (Figure 1d).

Free lysines on the SA surface were further used for the synthesis of two different types of antidote: SA with approximately three or seven peptides (3SP or 7SP). A cross-linker, di(*N*-succinimidyl) 3,3′-dithiodipropionate containing a disulfide bond, was first added to SA in 5 or 10 equiv of excess. Successful conjugation was confirmed by HPLC and MALDI-TOF, which further revealed that the estimated number of cross-linkers attached to SA for 5 and 10 equiv syntheses was 3 and 7, respectively (Figure 1c and Figure S1b,c). Surprisingly, no cross-linked SA was observed in either reactions (Figure S1b,d), which is most likely due to the relatively low protein and high cross-linker concentrations. Next, the disulfide bonds were cleaved using a reducing reagent, tris(2-carboxyethyl)phosphine hydrochloride (TCEP), yielding reactive sulfhydryl groups. Excess TCEP

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and cleaved cross-linkers were removed with HPLC using a desalting column (Figure S2a,b). The reactive elution fractions were subsequently combined with an *N*-maleimide-modified HBP to yield 3SP and 7SP. From HPLC data, it was found that the conversion in both cases was quantitative and the products were pure (Figure 1d and Figure S2c,d).

Heparin-Binding Study. Heparin-binding ability was first evaluated using MB displacement assay in a 2 mM Tris-HCl buffer. MB is a known heparin-binding dye with an absorbance peak at 664 nm, which shifts to 568 nm when bound to heparin. In the presence of another but stronger heparin-binder, MB can be displaced from heparin and the absorbance peak at 664 nm is restored (Figure S3a). Therefore, the ratio between *A* (664 nm)/*A* (568 nm) can be used to evaluate the heparin-binding efficiency of conjugates (Figure 2) as a

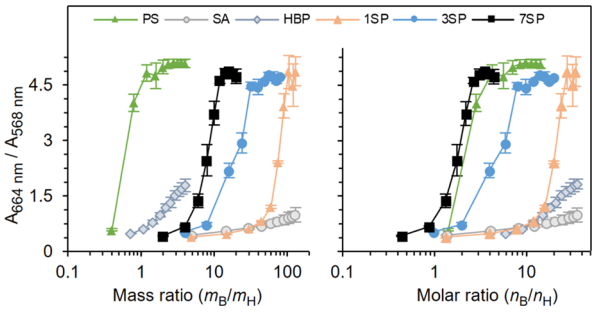

Figure 2. MB displacement assay results in (left) mass and (right) molar ratios of the binder to heparin, measured by the *A* (664 nm)/*A* (568 nm) ratio. Heparin concentration: 0.017 mg/mL (0.9 μM). Protamine sulfate was plotted for comparison. Measurements were performed using triplicate samples, and the averaged results with standard deviation are presented.

function of the mass (m_B/m_H) or molar (n_B/n_H) ratios between the binder and heparin.[34] SA showed minor heparin-binding efficiency within the tested concentration range, while that of a pure peptide was higher but not saturated. The slight binding of SA could be attributed to electrostatic attraction to heparin due to the large pool of lysines on the surface.[58,59] When one peptide was attached to SA (1SP), the heparin-binding ability was significantly enhanced and saturated at a mass ratio of ∼100 equiv (∼28 equiv in the molar ratio, Figure 2). This binding efficiency is even higher than that of plain SA or the plain peptide alone, which indicates a synergistic effect between these two components.[60] We can also conclude that the number of conjugates needed to fully release MB was significantly reduced with the increasing number of attached peptides. The saturation point shifts from ∼100 equiv of 1SP to ∼32 equiv of 3SP and ∼12 equiv of 7SP (Figure 2). In Table 1, c_{50}, the concentration required for 50% displacement of MB from its complex, and the positive/negative charge ratio of binder/heparin for 50% MB displacement are compared. The data indicates that all binders achieve MB displacement at comparable ratios and 7SP showed similar heparin-binding capacity with PS when comparing the molar ratios.

Next, dynamic light scattering (DLS) was employed to confirm the heparin-binding efficiency in the absence of added electrolytes (Figure 3a,b) as well as at a physiological salt concentration (150 mM NaCl, Figure 3c,d). Heparin solutions (0.02 mg/mL) were titrated with the conjugates, and the increased scattering intensity (derived count rate) and hydrodynamic diameter (D_h) were used as indicators for

Table 1. Data of 1SP, 3SP, 7SP, and PS Derived from MB Displacement Assay

binder	solvent	nominal charge	c_{50} [μM][a]	+/− ratio[b]
1SP	2 mM Tris-HCl	6+	111.1 ± 3.1[c]	1.01 ± 0.03
3SP	2 mM Tris-HCl	18+[d]	26.0 ± 2.1	0.71 ± 0.06
7SP	2 mM Tris-HCl	42+[d]	9.7 ± 0.6	0.61 ± 0.04
PS	2 mM Tris-HCl	24+[e]	11.1 ± 0.1	0.41 ± 0.01

[a] c_{50} represents the concentration required for 50% displacement of MB from its complex in 2 mM Tris-HCl. [b]The +/− ratio indicates the positive/negative charge ratio of binder−heparin at corresponding c_{50} values. [c]The highest concentration of 1SP tested. [d]We assume that all the attached cross-linkers reacted with HBP and only the charges on HBP were included. [e]The positive charge of PS was used as reported.[29]

binding and complex formation. In the plain buffer, the hydrodynamic diameters of heparin titrated with SA or 1SP yielded complexes with sizes of ∼20 nm within the whole concentration range (Figure 3a). Much higher values were recorded when heparin was titrated with 3SP or 7SP. This is expected as the one binding site on 1SP results in complex coacervate core micelles, while the multiple binding sites on 3SP and 7SP can cross-link with heparin and therefore form larger complexes.[61] As for the derived count rate, solutions titrated with PS, 3SP, and 7SP increased sharply at the low mass ratios and decreased after a certain mass ratio was reached (Figure 3b), which is a typical phenomenon when oppositely charged polyelectrolytes are complexed.[62] These peaks indicate a neutral charge balance, and they agree well with the saturation points obtained from the MB displacement assay.

At the physiologically relevant salt concentration, the binding affinities of all compounds were slightly weakened because of the shielding effect of counter ions.[51] Nevertheless, both hydrodynamic diameter values and derived count rates remained high for heparin complexes formed with 3SP or 7SP (Figure 3c,d).[63] SA and 1SP showed a small increase in the count rate and no change in size, indicating minor complex formation with heparin at these conditions. The free peptide presented heparin-binding ability in the Tris-HCl buffer, as indicated by the increased diameter and count rate, but the binding was not observed in the presence of 150 mM NaCl (Figure S4).

The morphologies of heparin complexed with 3SP and 7SP were directly visualized with AFM (Figure 3e and Figure S5). Both complexes were well dispersed on the silica surface and adopted roughly spherical morphologies with sizes up to ∼1 μm. No obvious size change was observed in buffers with or without NaCl. The size of the complexes observed with AFM is slightly smaller than with DLS, which could be attributed to drying and consequent shrinking of the complexes.[64]

Finally, in vitro heparin neutralization efficiency was evaluated in plasma using a chromogenic anti-Xa assay (Figure 4). Again, it can be observed that the neutralization efficiency increased with the number of conjugated peptides. SA and 1SP showed negligible neutralization efficiency, which is consistent with the DLS results in 150 mM NaCl. Compared to SA and 1SP, 3SP and 7SP showed much better performance. 3SP reached as high as 60% neutralization at ∼50 equiv, while 7SP showed full heparin neutralization at ∼25 equiv.

Combining all the results from the binding assays, we found that the SA conjugated with multiple peptides (3SP and 7SP)

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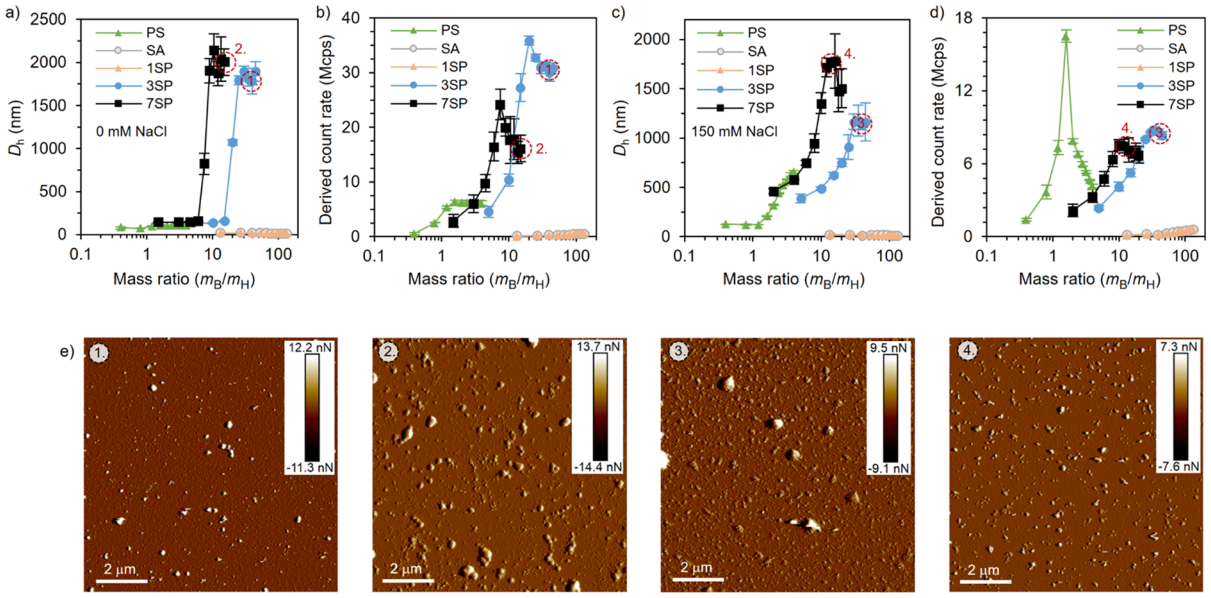

Figure 3. Titration of heparin (0.02 mg/mL) with binders (a, b) in the 2 mM Tris-HCl buffer and (c, d) at the physiological salt concentration (2 mM Tris-HCl, 150 mM NaCl) was monitored with DLS. The hydrodynamic diameter (a, c) and derived count rate (b, d) were used as indicators for the successful complexation. Titrations were performed using triplicate samples, and the averaged results with standard deviation are presented. (e) Peak force tapping mode AFM images (insets show the peak force error scale) of heparin neutralized with (1, 3) 3SP (m_B/m_H = 40) and (2, 4) 7SP (m_B/m_H = 15) in the presence of 0 mM (1, 2) and 150 mM (3, 4) NaCl. The corresponding DLS samples have been marked on the data.

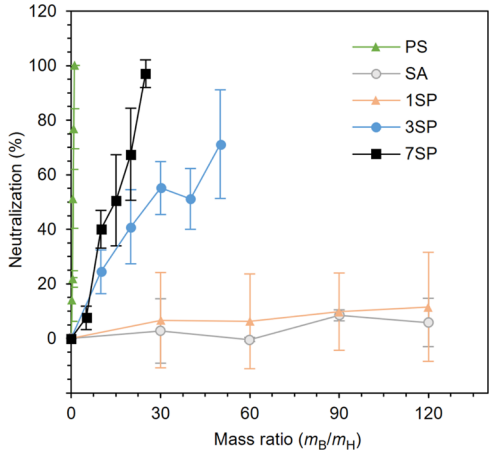

Figure 4. Heparin neutralization measured with the anti-Xa assay in plasma. Experiments were performed using triplicate samples, and the averaged results with standard deviation are presented.

showed significant multivalency effects, leading to excellent heparin-binding ability. 7SP showed better performance than that of PS when comparing the molar ratios, which underlines the potential of 7SP to substitute PS. On the other hand, the heparin-binding capacity of unmodified SA and the peptide was only moderate in the Tris-HCl buffer and negligible at the physiological salt concentration. When the two components were conjugated in a 1:1 ratio (1SP), significant enhancement was achieved but only in the absence of added electrolytes and plasma. Considering that there are 30−35 lysines available for modification on the SA surface, it is foreseeable that even more efficient heparin binders could be achieved through further optimization.

Biocompatibility Study. The biocompatibility of conjugates, pure PS, and heparin was first studied with an MTT cell viability assay. Human dermal fibroblasts (HDF) cells were incubated for 4, 14, or 24 h with the free compounds (PS, heparin, HBP, SA, 1SP, 3SP, and 7SP) and the same components complexed with heparin. As can be observed from Figure 5 and Figure S6, SA and heparin did not induce

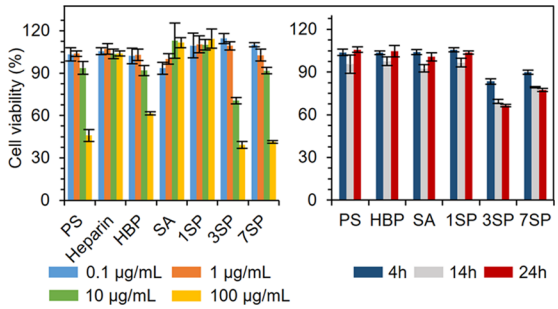

Figure 5. Effect of the heparin binders on cell viability evaluated with MTT assay using HDF cells. (left) Cells were incubated with free binders at four concentrations for 24 h. (right) Heparin of 1 μg/mL was first neutralized by binders and then incubated with the cells for 4, 14, or 24 h. Measurements were performed using triplicate samples, and the averaged results with standard deviation are presented.

any decrease in cell viability at 100 μg/mL after 24 h of incubation. Increased cytotoxicity was observed with PS as the incubation time and concentration increased: the cell viability dropped to 50% after 24 h of incubation (100 μg/mL PS). The free peptide followed a similar trend, but the drop at 100 μg/mL was not as drastic as in the case of PS. For 1SP, no toxicity was observed. However, when the number of peptides conjugated to SA was increased, cell viability decreased with the increasing concentration and incubation time. In general, 3SP and 7SP showed similar influence on fibroblasts as PS. When 1 μg/mL (~0.2 IU/mL) heparin was first neutralized by the compounds and subsequently incubated with fibroblasts, only minor toxicity was observed (Figure 5).

Aside from MTT assay, hemolysis assay was utilized to evaluate biocompatibility of the studied compounds under

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application-relevant conditions. Since heparin has been widely used in both surgery and blood transfusion, it is of vital importance to assess the influence of the heparin-binding conjugates on the stability of RBCs.[65,66] Hemolysis data in Figure 6 shows that the conjugates induced negligible

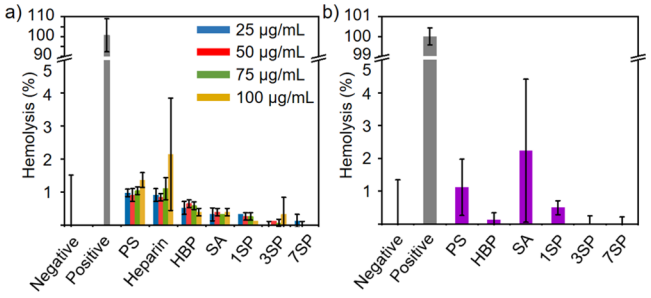

Figure 6. Hemolysis assay with RBCs; results indicate that both (a) binders alone and (b) binder–heparin (5 μg/mL) complexes cause negligible red blood cell hemolysis. Solutions of 1× phosphate-buffered saline (PBS) and 1% Triton X-100 were used as the negative and positive control, respectively. Measurements were performed using triplicate samples, and the averaged results with standard deviation are presented.

hemolysis on RBCs up to 100 μg/mL. When 5 μg/mL (1.1 IU/mL) heparin was first fully neutralized by 3SP or 7SP and thereafter incubated with RCBs, no noticeable hemolysis was observed. Combining the hemolysis and MTT assay results, we can conclude that the biocompatibility of the SA–peptide conjugates is comparable to that of the commercial PS.

Switch-On Heparin Detection. With the help of a dye-modified DNA oligonucleotide and the 7SP, we were able to design a detection system for ultralow amounts of heparin. A non-self-complementary 22 nt-long DNA sequence (0.2 μM), modified with a fluorescent dye (6-FAM) at the 5′ end and a quencher dye (BHQ1) at the 3′ end, was first complexed with 7SP through electrostatic interactions. This led to a significant drop in 6-FAM fluorescence intensity due to formation of the complex where both dyes are at close proximity (blue squares in Figure 7). It was found that the maximum quenching effect was reached at 9 μg/mL 7SP already, resulting in roughly one-

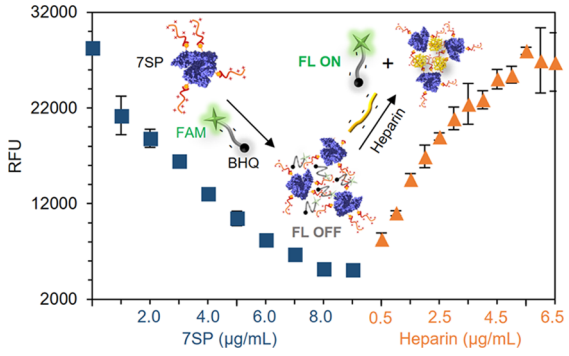

Figure 7. Fluorescence switch-on detection of heparin in the 2 mM Tris-HCl buffer with the 7SP–DNA complex. Blue squares (on the left-hand side) show the titration of 0.2 μM DNA with 7SP. Orange triangles (on the right-hand side) show the fluorescence recovered upon adding heparin to the solution of the 7SP–DNA complex. The insets show schematic steps of the process. Titrations were performed using triplicate samples, and the averaged results with standard deviation are presented.

sixth of the initial fluorescence intensity (FL, off). When heparin was introduced, DNA in the 7SP–DNA complex was replaced with heparin owing to the high binding efficiency between heparin and 7SP, yielding free unbound DNA strands and 7SP–heparin complexes. As a result, the fluorescence intensity increased as a function of heparin concentration (orange triangles in Figure 7). It can be observed that, within the range of 0–5.5 μg/mL heparin, the fluorescence intensity rises almost linearly with respect to the increasing heparin concentration. The initial fluorescence level is regained at 5.5 μg/mL (FL, on), showing that quantitative detection of heparin is feasible with this system. Moreover, the detection limit reaches 0.5 μg/mL (~0.1 IU/mL), which is well below the lowest therapeutic level (0.2 IU/mL), thus making the 7SP–DNA complex a highly sensitive heparin detector.[4−6] In addition, the selectivity of the detector complex toward heparin was compared to two other GAG analogues, hyaluronic acid and chondroitin sulfate (Figure S7). As expected, in the same concentration range, no clear fluorescence change was observed with chondroitin sulfate, while hyaluronic acid showed only a moderate increase (~2-fold enhancement). Plausibly, this difference in the obtained fluorescence changes can be attributed to the charge densities as the charge density of the hyaluronic acid is higher than that of chondroitin sulfate. Nevertheless, according to these results, the detector complex is very selective toward heparin (~6-fold fluorescence enhancement with heparin and only ~2-fold enhancement with hyaluronic acid).

CONCLUSIONS

In this study, we expanded the application space of SA to heparin binding. Assays showed that the heparin-binding ability of the three heparin neutralizers kept mounting as the number of conjugated peptides increased (7SP > 3SP > 1SP). 3SP and 7SP exhibited excellent binding affinity in both the buffer and blood plasma. All SA–peptide conjugates as well as conjugate–heparin complexes showed low or negligible cytotoxicity to HDF cells in application-relevant concentrations. Additionally, hemolysis assay results indicated that no hemolysis was induced on RBCs even with high conjugate concentrations (0.1 mg/mL). Finally, with the help of a dye complex containing 7SP and FAM- and BHQ-modified DNA, we were able to quantitatively detect heparin down to 0.1 IU/mL, which is well below the lower limit of clinically relevant dosage. In summary, all obtained results indicate that these multifunctional (neutralization/detection) biomolecule-based heparin binders are promising alternatives to the commercially available PS, which is arguably efficient but rather contentious due to its adverse effects.

EXPERIMENTAL SECTION

SA–Peptide Conjugate Synthesis. For the syntheses of 1SP, 1.6 mg (0.72 μmol) of an *N*-terminal maleimide-modified peptide (sequence: KME KKL HAV PAA KTV KFK, GenScript) was mixed with 40 mg (0.6 μmol) of SA (1.2:1 in a molar ratio) in 1 mL of a 1× phosphate-buffered saline (PBS) buffer and let to react overnight. The crude product was purified with HPLC using a HiTrap Heparin HP 5 mL column (GE Healthcare Life Sciences, elution buffers: buffer A: 10 mM PB buffer, pH 6.0 and buffer B: 10 mM PB buffer, 300 mM NaCl, pH 6.0). The collected fraction was concentrated and dialyzed against Milli-Q water for three days (dialysis tubing

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cellulose membrane, molecular weight cut-off (MWCO) of 14 kDa, Sigma-Aldrich). The final product was lyophilized, weighed, and dissolved in Milli-Q water for further use.

For the synthesis of 3SP and 7SP, 5 μmol (5×) and 10 μmol (10×) of di(*N*-succinimidyl) 3,3′-dithiodipropionate in 100 μL of dimethyl sulfoxide (DMSO) were added to 900 μL of PBS buffer solutions containing 1 μmol (66 mg) of SA. These solutions were kept at room temperature overnight. The successful attachment of cross-linkers was determined by HPLC and MALDI-TOF. Before peptide conjugation, 0.3 μmol of each cross-linker-capped SA was first washed three times with PBS using centrifugal filters with an MWCO of 10 kDa (Amicon Ultra). Thirty and 60 μmol of tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (20×× cross-linker, respectively) in 500 μL PBS were added to reduce dithiols. Afterward, the solutions were desalted with a HiTrap desalting column (GE Healthcare Life Sciences, elution buffer: Milli-Q water). Collected fractions were immediately mixed with 1.5 μmol (5×) and 3 μmol (10×) of the *N*-maleimide-modified peptide and let to react overnight. After the reaction, crude products were purified with HPLC using a HiTrap Heparin HP 5 mL column (GE Healthcare Life Sciences, elution buffers: buffer A: 10 mM PB buffer, pH 6.0; buffer B: 10 mM PB buffer, 1 M NaCl, pH 6.0.). Collected fractions were concentrated and dialyzed against Milli-Q water for three days (dialysis tubing cellulose membrane, MWCO of 14 kDa, Sigma-Aldrich). Final products were lyophilized, weighed, and dissolved in Milli-Q water for further use.

Methylene Blue (MB) Displacement Assay. Eighty microliters of 0.013 mg/mL MB was first mixed with 20 μL of 0.1 mg/mL heparin in a 2 mM Tris-HCl buffer, pH 7.3. Different concentrations of compounds in 20 μL in volume were added to the MB/heparin solution and allowed to equilibrate for 5 min on a shaker. Next, absorption spectra of the samples (400−750 nm) were measured with a BioTek Cytation 3-microplate reader in a 96-well plate at room temperature. The absorbance intensity ratio *A* (664 nm)/*A* (568 nm) was used to determine the heparin-binding ability. Measurements were performed using triplicate samples.

Dynamic Light Scattering (DLS) Measurement. DLS measurements were carried out with a Zetasizer Nano ZS device (Malvern Instruments) with a 4 mW He−Ne ion laser at the wavelength of 633 nm and an Avalanche photodiode detector at an angle of 173°. Zetasizer software (Malvern Instruments) was used to attain the data. Cumulant analysis was used to obtain the intensity mean value of the complex size, that is, the hydrodynamic diameter. Experiments were carried out at 25 °C. Heparin solutions were prepared by diluting 10 mg/mL heparin stock solution to 0.02 mg/mL in 0.3 mL of the buffer. The heparin solutions were titrated with 2 μL of sample solutions (different compound concentrations) resulting in total sample volumes of 20 μL. Measurements were carried out in 2 mM Tris-HCl buffer at pH 7.3 with 0 and 150 mM NaCl. After every addition, the samples were allowed to equilibrate for 1 min. Each titration series was carried out three times, and all titration points were measured three times.

Atomic Force Microscopy (AFM) Imaging/Measurement. AFM imaging was carried out with Dimension Icon (Bruker) in the ScanAsyst Fluid mode. To prepare the samples for imaging, 15−20 μL of the sample solution (heparin at 0.1 mg/mL) was incubated on a freshly cleaved mica surface for 5 min and allowed to dry. Then, ∼250 μL of the corresponding buffer (2 mM Tris−HCl with or without 150 mM NaCl) was carefully pipetted on the mica to form a meniscus between the AFM scanner and sample surface. During the imaging, the tip scanning velocity was limited to 2−5 μm/s to minimize the drifting of the particles on surface caused by the probe. The images were taken in 256 × 256 resolution, processed, and exported with NanoScope Analysis software ver. 1.9 (Bruker).

Anti-Xa Assay. Heparin neutralization with compounds in plasma was evaluated using a commercial two-stage kit, BiophenTM Anti-Xa (221005). Conjugates of different concentrations were first lyophilized and redissolved in human plasma. Dissolved compounds were then added to the heparin solution in 150 mM NaCl (0.1 mg/mL), giving a final heparin concentration of 0.075 IU/mL and compound/heparin ratios similar to those of the MB replacement assay and DLS measurement. Kit reagents were utilized according to the manufacturer's instructions. To run the calorimetric assay, 40 μL of the sample solution was added to a 96-well microplate followed by the addition of 40 μL of antithrombin and incubation for 2 min. Then, 40 μL of factor Xa was added and incubated for another 2 min. Afterward, 40 μL of the factor Xa-specific chromogenic substrate was added to the solution and let to react for 2 min. Finally, the reaction was quenched by introducing 80 μL of 2% citric acid. The absorbance at 405 nm was recorded immediately using a BioTek Cytation 3-microplate reader. The anticoagulant activity is inversely proportional to the measured absorption intensity, and the percentage of neutralization was determined using a calibration curve constructed according to the manufacturer's instructions (Figure S8). Measurements were performed using triplicate samples.

Cell Culture and MTT Assay. Human dermal fibroblasts (HDF) cells (Gibco) were purchased from Fisher Scientific. The cells were then expanded in Dulbecco's Modified Eagle Medium (DMEM) substituted with 10% fetal bovine serum (FBS) and 100 U/mL penicillin and 100 μg/mL streptomycin. The cells were kept in humidified conditions with 5% CO_2 at 37 °C. Once reached the 90% confluency the cells were split using 0.25% ethylenediaminetetraacetic acid (EDTA)-trypsin. Cell passages between 3 and 5 were used for the cell culture studies. Before MTT assay, cells were split into 96-well culture plates (approximately 10,000 cells/well) and incubated for 24 h. After the incubation, the culture media was replaced with 100 μL of compound solutions (0.1−100 μg/mL) or compound/heparin complex solutions in DMEM supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin. Based on the DLS data, the amount of PS, 3SP, and 7SP was chosen to fully neutralize heparin, while the highest mass ratios were used for HBP, SA, and 1SP (PS = 2 μg/mL, HBP = 5 μg/mL, SA = 140 μg/mL, 1SP = 140 μg/mL, 3SP = 40 μg/mL, 7SP = 15 μg/mL, and heparin = 1 μg/mL). The cells were then kept in humidified conditions with 5% CO_2 at 37 °C for 4, 14, or 24 h. After that, the sample solutions in each well were replaced with 100 uL of complete media and 10 uL of MTT solution (5 mg/mL in PBS). After 4 h of incubation at 37 °C with 5% CO_2, the MTT solution was replaced with 100 μL DMSO in each well to dissolve formazan crystals before reading. The absorbance was measured with a microplate reader (Cytation 3, BioTek) at the wavelength of 570 nm. Measurements were carried out using triplicate samples.

Hemolysis Assay. The detailed procedure for the hemolysis assay has been previously reported.[67] Generally, freshly donated human red blood cells were purchased

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(Cambridge Bioscience Ltd., the United Kingdom) and stored at 4 °C. Before samples were added, 1 mL of blood was centrifuged at 500 × *g* for 5 min and the plasma was removed gently. The remaining red blood cells were washed with 1× PBS three times and redispersed to the initial volume in 1× PBS. The red blood cells were diluted 50× and split into 96-well culture plates (190 μL/well). The concentrated sample (10 μL) or compound/heparin solutions in 1× PBS were added to each well, resulting in the desired final compound concentrations (25−100 μg/mL) or compound/heparin complex concentrations (PS = 10 μg/mL, HBP = 25 μg/mL, SA = 700 μg/mL, 1SP = 700 μg/mL, 3SP = 200 μg/mL, 7SP = 75 μg/mL, and heparin = 5 μg/mL; based on the DLS data, the amount of PS, 3SP, and 7SP was chosen to fully neutralize heparin, while the highest mass ratios were used for HBP, SA, and 1SP). Ten microliters of 20% Triton X-100 in 1× PBS and 10 μL of 1× PBS were added as positive and negative controls, respectively. After incubation at 37 °C for 1 h, the plates were centrifuged for 5 min at 500 × *g* to pellet intact erythrocytes, and 100 μL of the supernatant from each well was delicately transferred into a clear 96-well plate. The resulting hemoglobin in the supernatant was measured at 540 nm with a microplate reader (Cytation 3, Biotek). The percentage of hemolysis was calculated as follows:

$$\%\text{Hemolysis} = [(A_{\text{sample}} - A_{\text{negative control}})/(A_{\text{positive control}} - A_{\text{negative control}})] \times 100$$

The measurements were performed using triplicate samples.

Switch-On Heparin Detection. The quenching effect of 7SP on FAM- and BHQ- modified 22 nt DNA was evaluated by titrating 0.2 μM DNA with 0−9 μg/mL 7SP in 2 mM Tris-HCl buffer. The fluorescence intensity stabilized after 9 μg/mL 7SP. For the heparin replacement assay, 0.2 μM DNA was first quenched with 9 μg/mL 7SP, which was followed by the titration of heparin.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.9b02883.

Supplementary details on reagents and materials, procedures for HPLC and MALDI-TOF, characterization of cross-linker capped SA, deprotection of cross-linker-capped SA, purification of 1SP, 3SP, and 7SP, absorbance spectra of SA, HBP, 1SP, 3SP, and 7SP titrated with heparin in MB displacement assay, DLS results of heparin titrated with HBP, AFM characterization in Tris-HCl buffer, MTT assay of compounds (incubation time of 4 and 14 h), control experiments of titration with hyaluronic acid and chondroitin 4-sulfate in Tris-HCl buffer, and a calibration curve for anti-Xa assay (PDF)

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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