Supplementary Information

Anticoagulant sodium alginate sulfates and their mussel-inspired heparin-mimetic coatings

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Supplementary Information

Materials

Sodium alginate (very low viscosity) (SA) was purchased from Alfa Aesar (China) Chemical Co. Ltd. 3, 4-Dihydroxyphenethylamine (DA·HCl, 99%), N, N'-Dicyclocarbodiimide (DCC), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), and N-hydroxysuccinimide (NHS) were purchased from Aladdin reagent Co. Ltd. (China). Polyethersulfone (PES, Ultrason E6020P, BASF) was used as received. Bovine serum albumin (BSA, fraction V, 95%) and Bovine serum fibrinogen (FBG) were obtained from Sigma Aldrich, USA. Micro BCA™ Protein Assay Reagent kits were the products of PIERCE. Phosphate-buffered saline (PBS, pH = 7.2−7.4) solution was a buffer solution commonly used to dissolve BSA and FBG. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma, US. Other reagents were obtained from Aladdin reagent Co. Ltd. (China) with analytical grade and used as received unless otherwise stated. All the aqueous solutions were prepared with deionized (DI) water. Dialysis membranes (MWCO = 3 500 Da) were obtained from Solarbio (Canada).

Preparation of PES membranes

Homemade PES membranes used in this study were prepared by a phase inversion technique. 16 wt. % PES was dissolved into N, N-Dimethylacetamide (DMAc) solvent and then with vigorous stirring until clear homogeneous solution was obtained. After vacuum degassing, the casting solution was prepared into membranes by spin coating coupled with a liquid–liquid phase separation technique at room temperature. The membranes were rinsed with de-ionized water thoroughly to remove the residual solvent, which were confirmed by the UV scanning. Then, the membranes were dried overnight in a refrigerant vacuum-desiccator, and with uniform thickness of about 70 μm.

GPC test

Two TSK gel columns in series (TSKgel G2000SWxl - TSKgel G3000SWxl

6.0×300mm, HLC-8320, Tosoh Corp. Japan) were used for GPC test. Columns, injector and detectors were maintained at 40 °C. An aqueous solution of 0.1 M $NaNO_3$ pre-filtered on 0.22 μm filter (Millipore) was used as the mobile phase at a flow rate of 1 mL/min. The system was calibrated with the PEO narrow and broad standards of known Mw, polydispersity, and intrinsic viscosity. The narrow standard was particularly useful for determining detector volume offsets and peak broadening parameters, whereas the broad standard was useful for checking the calculations for correct molecular weight distribution and Mark-Houwink parameters, etc. The processes were aided by Chromatography Manager Software, with its GPC option.

APTT and TT

Healthy human fresh blood was collected in vacuum tubes containing sodium citrate as the anticoagulant (anticoagulant to blood ratio, 1:9, V/V), and the platelet-poor plasma (PPP) was obtained after centrifuging at 4 000 r.p.m for 15 min. APTT and TT tests were measured by exposing PPP to the prepared aqueous solutions and membranes, and the results were analyzed by statistical methods Synchronously, the SA, SAS, DA-g-SA, and DA-g-SAS were dissolved in PBS buffer (pH 7.4) for a series of concentrations, such as 0.2, 0.5, 1, 2, and 5 mg/mL, respectively. The APTT test method was described as follows: 100 μL of PPP and 5 μL of the SA (or DA-g-SAS) PBS solution were incubated at 37 °C for 30 min; then, 50 μL of the incubated PPP was added to a test cup, followed by the addition of 50 μL of APTT agent (Dade Actin Activated Cephaloplastin Reagent, Siemens; incubated 10 min before use) and incubated at 37 °C for 3 min. Thereafter, 50 μL of 0.025 M $CaCl_2$ solution was added, and the APTT was measured. At least three measurements were averaged to get a reliable value, and the results were analyzed by a statistical method.

The TT test was carried out in a similar process as the APTT test. 100 μL of PPP and 5 μL of the solution were incubated at 37 °C for 30 min; then, 50 μL of the incubated PPP was added to a test cup, followed by the addition of 100 μL of Test Thrombin Reagent (Siemens; incubated 15 min before use) and incubated at 37 °C for 2 min. Then, the TT was measured.

For the mussel-inspired coating membranes, the APTT and TT were also measured, and the test method was as follows: Synchronously, the modified membranes (0.5 cm × 0.5 cm, four pieces) were immersed in PBS (200 μL, pH = 7.4) for 30 min. Then the PBS was removed and 100 μL of fresh PPP was introduced. After incubating at 37 ºC for 30 min, 50 μL of the incubated PPP was added into the test cup, followed by the addition of 50 μL of APTT agent (incubated 10 minutes before use) and incubated at 37 ºC for 3 min. Thereafter, 50 μL of 0.025 M $CaCl_2$ solution was added, and then the APTT was measured. For the TT test, 100 μL of TT agent was added into the test cup (containing 50 μL of the incubated PPP) after 2 minutes incubation, and then the TT was measured.

Water contact angle

A piece of 1×1 cm^2 membrane was attached on a glass slide and mounted on the goniometer. For the static contact angle measurements, a total of 3 μL double distilled water was dropped on the airside surface of the membrane at room temperature and relative humidity of 80 %. At least five measurements were averaged to get a reliable value. The measurement error was ± 3°.

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, Hyclone, USA), 2 mM L-glutamine and 1% (V/V) antibiotics mixture (10000 U penicillin and 10 mg streptomycin). Cultures were maintained in humidified atmosphere of 5% CO_2 at 37 ^oC (Queue Incubator, Paris, France). Confluent cells were detached from the culture flask with sterilized 0.05% trypsin PBS solutions, and the culture medium was changed every day.

The PES and coated membranes were cut into 1×1 cm^2 for each piece to suit the size for 24-well cell-culture polystyrene plates, and pre-wetted by immersion in the culture medium for 3 h in a 37 ^oC incubator. And then the membranes were placed into the cell-culture plates, rinsed with PBS and sterilized by γ -ray.

Cell Morphology

The HUVECs were seeded onto the membranes at a density of approx. 1.25×10^4 cells/cm^2. For SEM observation, the seeded membranes after culturing for 5 days were rinsed with PBS and fixed with 2.5% (wt. %) glutaraldehyde in PBS at 4 ^{o}C for 12 h. For the morphology observation, the fixed samples were subjected to a drying process by passing them through a series of graded alcohol-PBS solutions (30, 50, 70, 80, 90, 95, and 100%, 15 minutes for each time) and then dehydrated through isoamyl acetate–alcohol solutions (30, 50, 70, 80, 90, 95, and 100%). The critical point drying of the specimens was done with liquid CO_2. The specimens were sputter-coated with a gold layer and examined by a FE-SEM (JSM-7500F, JEOL, Japan). For confocal laser scanning microscopy (CLSM) observation, the seeded membranes after culturing for 5 days were rinsed with 37 ^{o}C PBS and fixed in 4 wt. % paraformaldehyde for 10 min. Then the membranes were washed with PBS and further treated with 0.5 % Triton X-100 in PBS for 5 min. The actins were stained with Rhodamine-phalloidin (100 nM) in the dark for 30 min. The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (5 μg/mL) for 5 min. Subsequently, the stained cells were imaged with a confocal laser scanning microscopy (CLSM, Leica TCS SP8, Germany).

MTT Assay

After cell culture for 2, 4, and 6 days, the viability of the HUVECs was determined by MTT assay. The HUVECs were seeded onto the membranes at a density of approx. 1.25×10^4 cells/cm^2. In this study, the cells cultured in the wells without membranes were served as the control. After determined time intervals, 50 μL of MTT solution (1 mg/mL in the test medium) was added to each well and incubated for 4 h at 37 ^{o}C. Mitochodrial dehydrogenases of viable cells cleave selectively to the tetrazolium ring, yielding blue/purple formazan crystals. Then, 400 μL of ethanol was added to dissolve the formazan crystals. Therefore, the quantity of the formazan crystals dissolved in the ethanol could reflect the level of the cell metabolism. The dissolvable solution was jogged homogeneously for about 15 min by a shaker. The solution of each sample was aspirated on a microtiter plate and the optical density of the formazan solution was read on the Microplate reader (Model 550, Bio-Rad) at 492 nm. There are four samples for each kind of membrane to minimize the test error, and the

results were expressed as means ± SD.

Protein adsorption

Protein adsorption experiments were carried out with BSA and FBG solutions under static condition. Firstly, the membrane with an area of 1×1 cm^2 was immersed in PBS solution, containing BSA (or FBG) with the concentration of 0.5 mg/mL, and incubated at 37 °C for 1 h; then the membrane was rinsed slightly with PBS and ultrapure water. Then the membrane was placed in a washing solution (2 % sodium dodecyl sulfate (SDS), 0.05 M NaOH) at 37 °C, and shaken for 2 h to remove the adsorbed protein. The adsorption and desorption times were carefully determined in preliminary experiments. The protein concentration in the washing solution was determined by using the Micro BCA™ Protein Assay Reagent Kit (PIERCE). More than 95 % of the adsorbed protein could be eluted into the SDS solution, and then the adsorbed protein amount was calculated.

Platelet adhesion

In order to eliminate the interference of other components in blood, such as erythrocyte and leucocyte, platelet-rich-plasma (PRP) was used for studying platelet adhesion on the membranes. Healthy fresh human blood (male, 25 years old) was collected using vacuum tubes containing sodium citrate as an anti-coagulant; the concentration of sodium citrate was 3.8 wt.%, and the ratio of the anticoagulant to blood was 10:90 vol. %. The blood was centrifuged at 1500 r.p.m. for 15 min to obtain platelet-rich plasma (PRP) or at 4000 r.p.m. for 15 min to obtain platelet-poor plasma (PPP).

The pristine and modified PES membranes were immersed in PBS and equilibrated at 37 °C for 1 h. Then, the PBS was removed and 1 mL of fresh PRP was introduced. The membranes were incubated with PRP at 37 °C for 2 h. Then, the PRP was decanted off and the membranes were rinsed three times with PBS. Finally, the membranes were treated with 2.5 wt. % glutaraldehyde in PBS at 4 °C for 1 day. The samples were washed with PBS, subjected to a drying process by passing them through a series of graded alcohol–PBS solutions (30, 50, 70, 80, 90, 95, and 100%)

and isoamyl acetate–alcohol solutions (30, 50, 70, 80, 90, 95, and 100%). Platelet adhesion was observed using a FE-SEM (JSM-7500F, JEOL, Japan). The number of the adherent platelets on the membranes was calculated from five SEM pictures at 500× magnification from different places on the same membrane.

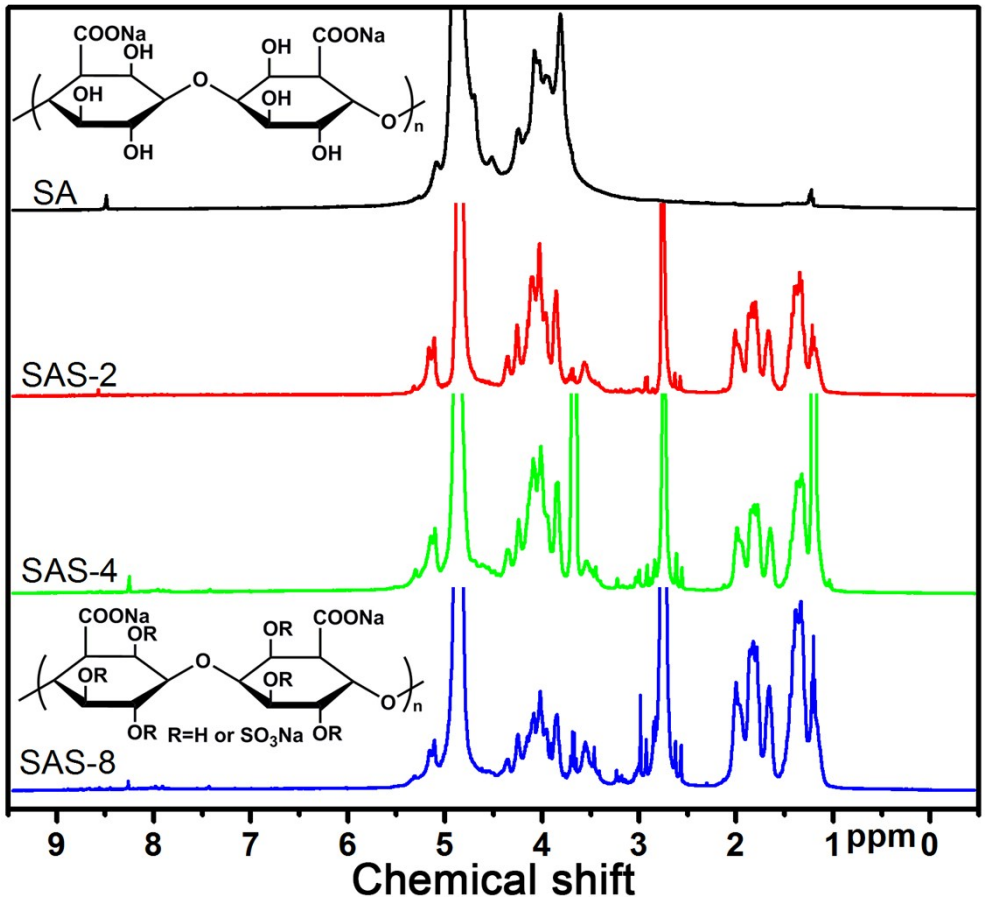

Figure S1. ^{1}H-NMR spectra for the SA, SAS-2, SAS-4, and SAS-8 molecules.

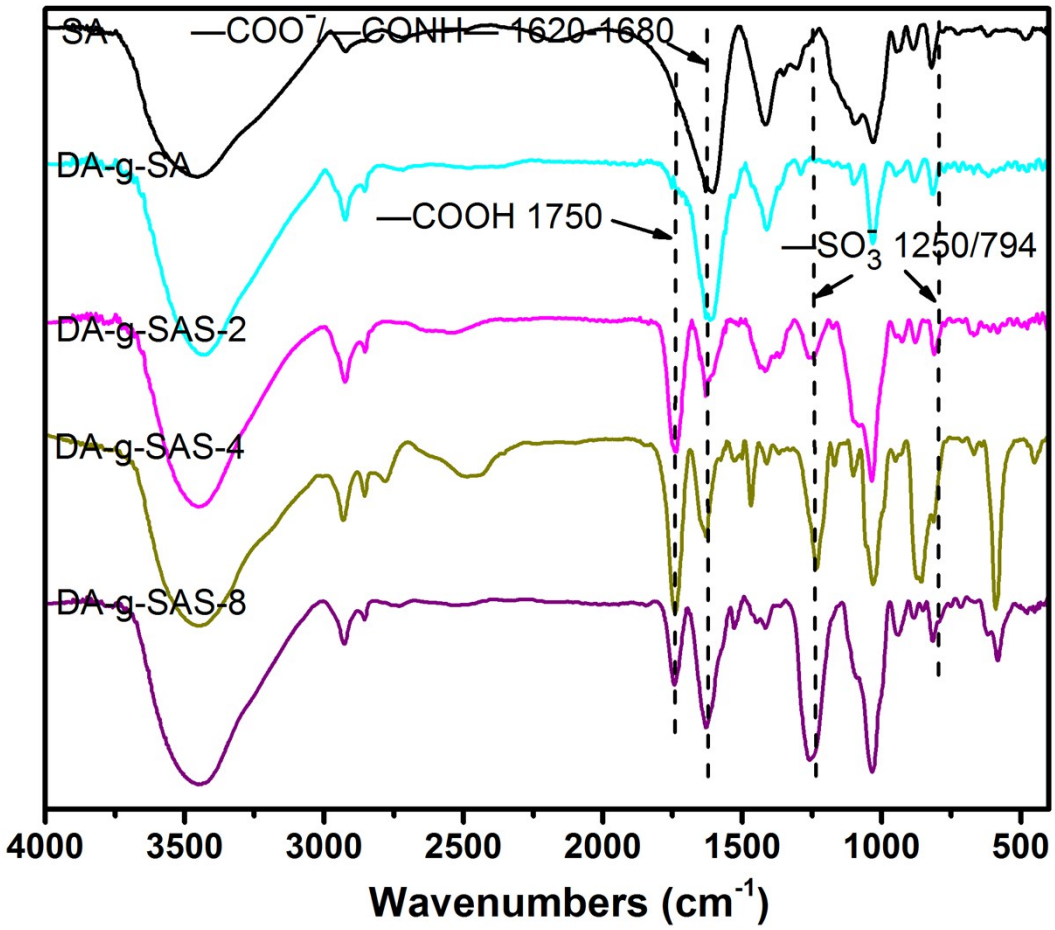

Figure S2. FTIR spectra for the SA, DA-g-SA, DA-g-SAS-2, DA-g-SAS-4, and DA-g-SAS-8 molecules.

Table S1. Relation between DS and the usage of sulfonation agent

Samples	Usage of sulfonation agent		N%	C%	H%	S%	DS[2]
	SA (g)	Mol ratio [1]					
SA	0	0	0.604	29.805	5.323	0.465	—
SAS-2	5	2:1	2.919	32.17	6.138	5.572	0.390
SAS-4	5	4:1	1.032	19.586	3.625	7.087	0.814
SAS-6	5	6:1	0.952	17.772	3.881	7.594	0.961
SAS-8	5	8:1	2.031	24.006	5.098	14.359	1.35
SAS-10	5	10:1	1.896	22.898	4.920	14.913	1.47
SAS-12	5	12:1	0.190	17.301	3.920	11.280	1.48

[1.] Mole ration of H_2SO_4/DCC added to the uronic acid unit.

[2.] The degree of substitution of sulfonic acid groups per uronic acid calculated according S%/C%.