Heparin Reversal by an Oligoethylene Glycol Functionalized Guanidinocalixarene

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1. Materials and methods

Chemicals. All the reagents and solvents were commercially available and used as received unless otherwise specified purification. Eosin Y (EY) was purchased from Sigma-Aldrich. Heparin sodium (UFH) was purchased from Meilun Biotechnology. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) was supplied by Amresco. DMEM Cell culture medium, penicillin-streptomycin (PS), trypsin, phosphate buffered saline (PBS) and fetal bovine serum (FBS) were purchased from Gibco (Carlsbad, CA, USA). The HEPES buffer solution of pH 7.4 was prepared by dissolving 2.38 g of 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) in approximate 900 mL double-distilled water. Titrate to pH 7.4 at 25 °C with NaOH and make up volume to 1000 mL with...
double-distilled water. The pH value of the buffer solution was then verified on a pH-meter calibrated with three standard buffer solutions. Milli-Q water was purified using a Milli-Q Integral system from Merck Millipore.

**Instrumentation.** $^1$H and $^{13}$C NMR data were recorded on a Bruker AV400 spectrometer. Mass spectra were performed on a Varian 7.0T FTICR-MS (MALDI-TOF). Steady-state fluorescence measurements were recorded in a conventional quartz cuvette (light path 10 mm) on a Cary Eclipse equipped with a Cary single-cuvette peltier accessory. MTT assay and hemolysis test were with assistance of a FlexStation 3 microplate reader.

**Cell culture.** 4T1 (Breast cancer cell line of mouse) and HEK293T (Human embryonic kidney cells) were obtained from America Type Culture Collection (ATCC, Shanghai, China). The above cell lines were maintained in culture medium DMEM supplemented with 10% FBS and 1% PS, incubated in a humidified environment at 37 ℃ with 5% CO$_2$. The medium was changed every 2 days. The cells were sub-cultured when the density reached 80%.

**MTT Assay.** The cytotoxicity of GC4AOEG against cancer cell lines and non-cancerous cells were investigated by MTT assay. Briefly, cells were seeded into 96-well plates at a density of 4000-5000 cells/well with 100 μL culture medium. After 24 h incubation, the culture medium was replaced with 100 μL of a fresh medium containing various concentrations of GC4AOEG (6.25, 12.5, 25, 50, 100 and 200 μM) and the cells were incubated for another 24 h. After 24 h treatment, MTT solution (final concentration 1 mg/mL) was added to each well, followed by incubation at 37 ℃ for 4 h. The culture medium was removed by aspiration, and 100 μL of DMSO was added to dissolve blue formazan in living cells and the absorbance was measured at 570 nm with a microplate reader. The cells incubated with only the culture medium were considered to be 100% viable. Cell viability (%) = the OD value of each treated group/OD value of the control group × 100%.

**Hemolysis assay.** The hemolysis assay was conducted according to a method reported previously.$^1$ Fresh blood containing sodium citrate (3.8 wt.%) in the ratio of 9:1 from healthy Balb/c mouse was diluted with normal saline and centrifuged at 1500 rpm for 10 min. The erythrocyte pellets were collected at the bottom of centrifuge tube and washed for another four times with normal saline. Subsequently, erythrocyte pellets diluted with normal saline to 2% (v/v) concentration. 1.5 mL of GC4AOEG or GC4A-6C solutions with different concentrations were respectively placed in a 2 mL tube which containing 50 μL of 2% erythrocyte pellets solution. Normal saline solution was used as the negative control (NC) and distilled water was used as the positive control (PC). All the tubes were gently mixed and the mixtures were incubated for 1 h at 37 ℃. After that, all the tubes were centrifuged at 3000 rpm for 5 min and the supernatant was transferred to the 96-well plates for the estimation of free hemoglobin. -Spectroscopic analysis at 545 nm. The hemolysis rate was calculated using the mean OD value for each group as follows:
Hemolysis rate (%) = \( \frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{negative control}}}{\text{OD}_{\text{positive control}} - \text{OD}_{\text{negative control}}} \times 100\% \) \\
where \( \text{OD}_{\text{sample}} \) corresponds to the OD value obtained in the presence of GC4AOEG solution, \( \text{OD}_{\text{negative control}} \) and \( \text{OD}_{\text{positive control}} \) were obtained from the positive control and negative control tests as mentioned above, respectively.

**Hemagglutination assay.** Red blood cells (RBCs) agglutination assay was performed on freshly obtained erythrocytes in the presence of an anticoagulant. Firstly, triplicates of different concentrations (6.25, 12.5, 50, 100, 200 µM) of GC4AOEG were diluted in normal saline onto a 96 well V-shaped plates (100 uL/well). Normal saline only group as the negative control group. And leave for 15-20 minutes. Then RBCs were dispensed onto a 96 well V-shaped plates with a final v/v concentration of 2.0%. The results were visually accessed for agglutination after 1 h at 37 °C after the RBCs suspension adsorbed had fully sedimented.

**Animals.** Balb/c mice with body weights of 24-28 g at 10-12 weeks of age (Animal Facility, University of Macau, Macau) were used for our mouse model studies. Mice were maintained at an ambient temperature of 20 ± 2 °C on a 12:12 light-dark cycle environment with free access to water and food. All animal experiments were conducted in accordance with the ethical guidelines of the Institute of Chinese Medical Sciences, University of Macau and the protocols were approved by the Animal Ethics Committee at the University of Macau.

**In vivo mouse tail transection bleeding model.** Thirty Balb/c mice (male and female, 24-28 g) were randomly divided into five groups, anesthetized by intraperitoneal injection of chloral hydrate aqueous solution (10% w/v, 0.005 mL/g) and then, mice were injected intravenously with saline, GC4AOEG (2.245 mg/Kg), or heparin sodium (UFH, 200 U/Kg) without and with GC4AOEG (2.245 mg/Kg), respectively. After 2 min, the scalpel was cleaned and disinfected, then each tail was transected at a site 5 mm from the end of tail with scalpel and immediately immersed in a tube containing normal saline at 37 °C. The blood draining out from the wound was collected in each tube. The tail bleeding time was counted until there was no blood flow for 1 min continuously. Total blood loss from each tail of the mice in tail transection bleeding model was measured according to a previously reported method by Genmin Lu et al.² The collected blood samples were frozen at −80 °C to lyse erythrocytes. The total blood volume in each tube was quantified by spectrophotometry (absorbance at 490 nm) from a standard curve that was constructed with known volumes of blood hemoglobin concentration and corresponding absorbance value.

**In vivo mouse liver injury model.** Balb/c mice (male and female, 24-28 g) were divided into five groups of six, and anesthetized. Subsequently, mice were injected intravenously with saline, GC4AOEG (2.245 mg/Kg), or heparin sodium (UFH, 200 U/Kg) without and with GC4AOEG (2.245 mg/Kg), respectively. After 2 minutes, mice were placed on their backs and the abdomen was opened exposing the liver. A wound with a length of 0.5 cm, 2 mm deep in the left lobe of the liver, was cut by razor
blade. The time for complete arrest of bleeding (no blood flow within 10 s) was recorded, blood was collected with cotton every 10 seconds until bleeding stopped.

**In vivo mouse femoral artery bleeding model.** Balb/c mice (male and female, 24-28 g) were utilized in this study, and divided into five groups of six. After anesthesia, then was separately applied saline, GC4AOEG (2.245 mg/Kg), or heparin sodium (UFH, 200 U/Kg) without and with GC4AOEG (2.245 mg/Kg) via i.v. injection. Subsequently, the skin of leg and the overlying muscles were removed to expose the femoral artery and sciatic nerve. Injury at the middle segment of the femoral artery was induced by a surgical scissors. The time for complete arrest of bleeding (no blood flow within 10 s) was determined, blood was collected with cotton every 10 seconds until bleeding stopped.

**Preliminary acute toxicity evaluations on GC4AOEG.** As a preliminary study on the safety profile of GC4AOEG, 12 male Balb/c mice (24-28 g) were randomly separated into two groups (n = 6 in each group). Specifically, GC4AOEG in saline was i.v. administered at a total dose of 2.245 mg/Kg. In the control group, mice were i.v. injected with normal saline. Body weight of mice was monitored every day at defined time point and their general behaviors were observed. After 3 weeks, animals were euthanized. Blood samples were collected for hematological analysis (Chemray 240, Rayto life and analytical sciences Co., China), like whole blood cell (WBC), red blood cell (RBC), platelet (PLT) and hemoglobin (HGB). The serum concentrations of biomarkers including alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), crea and urea were tested by kits. Major organs including heart, liver, spleen, lung and kidney were isolated and weighed. The organ index was calculated as the ratio of organ weight to the body weight of each mouse. In addition, histopathological sections of collected organs were prepared and stained with hematoxylin and eosin (H&E), followed by imaging with optical microscopy.

**Activated partial thromboplastin time (aPTT) measurements.** The following procedure was carried out for aPTT measurements: Plasma was taken from 2.5 mL of citrated blood (2.25 mL blood + 0.25 mL sodium citrate 3.8%), via centrifuging citrated blood for 15 minutes at 4000 rpm at 4°C within 30 minutes of collection. Normal saline (1 µL, control group), UFH (1 µL of a 20 µg/mL solution), GC4AOEG (1 µL of a 51.97 µg/mL solution), UFH-GC4AOEG 1:1 (1 µL of a UFH 20 µg/mL + GC4AOEG 51.97 µg/mL solution), protamine (1 µL of a 37 µg/mL solution) and UFH-protamine 1:1 (1 µL of a UFH 20 µg/mL + protamine 37 µg/mL solution) were added to 99 µL of plasma and incubated for 1 min, respectively. The samples (100 µL) was added to 100 µL of aPTT reagent solution and incubated another 4 min. Subsequently, 100 µL of 0.02 M CaCl₂ solution (37°C) was added and the clotting time was recorded. For all aPTT samples, the incubation temperature was 37°C. All tests were performed in triplicate.

**Enzyme-linked immunosorbent assay.** Plasma level of coagulation factor X was quantified using commercially available ELISA kit (Molecular Innovations, Peary Ct, Novi; USCN Life Science, Wuhan,
Hubei, China). This kit is a sandwich enzyme immunoassay kit for *in vitro* quantitative measurement of factor X in plasma. Plasma was collected using 3.8% sodium citrate (sodium citrate :blood = 1: 9) as an anticoagulant. The plasmas was centrifuged for 15 minutes at 4000 rpm at 4°C within 30 minutes of collection and stored at -20°C until use. Samples were prepared by adding normal saline (control group), UFH (20 µg/mL, 3.7 U/mL), GC4AOEG (51.97 µg/mL), UFH-GC4AOEG 1:1 (UFH 20 µg/mL+ GC4AOEG 51.97 µg/mL), protamine (37 µg/mL) and UFH-protamine 1:1 (UFH 20 µg/mL+protamine 37 µg/mL), respectively, to plasma. These samples were diluted and ELISA kit’s reagents were reconstituted according to the manufacturer’s instructions. Linearity was assessed and confirmed by samples containing rat coagulation factor X standard plasma serially diluted with a calibrator diluent.

**Statistical Analysis.** Statistical analysis was performed by GraphPad Prism 6.01 using the one-way ANOVA test. The p < 0.05 is considered to be statistically significant.

### 2. Syntheses of GC4AOEG

Compound 1, 2, 3 and 4 was synthesized and purified according to the procedure reported previously.

**Compound 5:** Compound 4 (1.70 g, 1.43 mmol) was dissolved in a mixture of C2H5OH (120 mL) and AcOEt (120 mL), then the mixture was refluxed for 48 h after adding SnCl2·2H2O (4.50 g, 20 mmol). The reaction solution was cooled to room temperature and poured into ice water. The pH of the solution was adjusted to 8.0 by NaOH. Dichloromethane (100 mL) was added into the mixture, and the organic phase was separated, dried over anhydrous Na2SO4. The solvent was evaporated to obtain pale yellow oil 5 (1.20 g, 78%).

**1H NMR (400 MHz, CDCl3, δ):** 5.98 (s, 8H, ArH), 4.23 (d, J = 13.20 Hz, 4H, ArCH2Ar), 3.91 (t, J = 5.80 Hz, 8H, CH2OAr), 3.75 (t, J = 5.80 Hz, 8H, CH2CH2OAr), 3.54-3.59 (m, 24H, OCH2CH2OCH2CH2OCH3), 3.45-3.47 (m, 8H, CH2OCH3), 3.30 (s, 12H, OCH3), 2.83 (d, J = 13.24 Hz, 4H, ArCH2Ar).
Figure S1. $^1$H NMR spectrum of 5 in CDCl$_3$, 400 MHz, 25 °C.

**Compound 6:** To a solution of 5 (1.00 g, 0.94 mmol) in dry CH$_2$Cl$_2$ (100 mL), 1,3-bis(tert-butoxycarbonyl)-2-methyl-2-thiopseudourea (3.00 g, 10.30 mmol), AgNO$_3$ (1.75 g, 10.30 mmol) and Et$_3$N (1.50 mL, 10.82 mmol) were added and the mixture was stirred for 36 h at room temperature. The solvent was evaporated under reduced pressure and the residue was purified by column chromatography on silica gel to obtain pale yellow powder 6 (0.96 g, 51%).

$^1$H NMR (400 MHz, CDCl$_3$, $\delta$): 11.53 (s, 4H, NH), 9.78 (s, 4H, NH), 6.88 (s, 8H, ArH), 4.37 (d, $J = 13.10$ Hz, 4H, ArCH$_2$Ar), 4.03 (t, $J = 5.70$ Hz, 8H, CH$_2$OAr), 3.80 (t, $J = 5.70$ Hz, 8H, CH$_2$CH$_2$OAr), 3.56-3.58 (m, 24H, OCH$_2$CH$_2$OCH$_2$CH$_2$OCH$_3$), 3.46-3.48(m, 8H, CH$_2$OCH$_3$), 3.31(s, 12H, OCH$_3$), 3.08 (d, $J = 13.10$ Hz, 4H, ArCH$_2$Ar), 1.42 (s, 36H, Bu$^i$), 1.39 (s, 36H, Bu$^i$).
Figure S2. $^1$H NMR spectrum of 6 in CDCl$_3$, 400 MHz, 25 °C.

**Compound GC4AOEG:** To a solution of 6 (0.80 g, 0.39 mmol) in 50 mL of AcOEt, SnCl$_4$ (0.50 mL, 4.28 mmol) was added quickly. The mixture was stirred for 12 h at room temperature and the solvent was evaporated under reduce pressure. The residue was dissolved in CH$_3$OH, then appropriate amounts of diethyl ether was added to obtain white powder GC4AOEG (0.43 g, 79%).

$^1$H NMR (400 MHz, D$_2$O, $\delta$): 6.66 (s, 8H, ArH), 4.43 (d, $J = 13.50$ Hz, 4H, ArCH$_2$Ar), 4.17 (t, $J = 4.40$ Hz, 8H, CH$_2$OAr), 3.90 (t, $J = 4.60$ Hz, 8H, CH$_2$CH$_2$OAr), 3.52-3.64 (m, 24H, OCH$_2$CH$_2$OCH$_2$CH$_2$OCH$_3$), 3.46-3.48 (m, 8H, CH$_2$OCH$_3$), 3.28 (d, $J = 13.50$ Hz, 4H, ArCH$_2$Ar), 3.24 (s, 12H, OCH$_3$).

$^{13}$C NMR (100 MHz, D$_2$O, $\delta$): 156.08, 155.78, 136.50, 128.37, 125.46, 73.40, 71.05, 70.91, 69.70, 69.67, 69.41, 58.00, 30.43.

MALDI-TOF MS $m/z$: [M + H - 4HCl]$^+$ calcd. for C$_{60}$H$_{93}$N$_{12}$O$_{16}$ 1237.6833, found 1237.6831; [M + Na - 4HCl]$^+$ calcd. for C$_{60}$H$_{92}$N$_{12}$O$_{16}$Na$^+$ 1259.6652, found 1259.6650.
Figure S3. (a) $^1$H NMR spectrum of GC4AOEG in D$_2$O, 400 MHz, 25 ºC; (b) $^{13}$C NMR spectrum of GC4AOEG in D$_2$O, 100 MHz, 25 ºC; (c) MALDI-TOF MS of GC4AOEG.

3. Chemical structures of EY and GC4A-6C

Figure S4. Chemical structures of EY and GC4A-6C.
4. Syntheses of GC4A-6C

GC4A-6C was synthesized and purified according to the procedure reported previously.\(^8\)

\( ^1\text{H NMR (400 MHz, CD}_3\text{OD, } \delta): \) 6.69 (s, 8H, ArH), 4.43 (d, \( J = 13.20 \text{ Hz, 4H, ArCH}_2\text{Ar} \)), 3.98 (t, \( J = 7.40 \text{ Hz, 8H, CH}_2\text{OAr} \)), 3.32 (d, \( J = 13.20 \text{ Hz, 4H, ArCH}_2\text{Ar} \)), 2.00 (m, 8H, OCH}_2\text{CH}_(2)(CH}_3\text{CH}_3), 1.42 (m, 24H, OCH}_2\text{CH}_(2)(CH}_3\text{CH}_3), 0.96 (t, \( J = 6.3 \text{ Hz, 12H, O(CH}_2\text{)}_3CH}_3 \)).

![NMR spectrum](image)

**Figure S5.** \(^1\text{H NMR spectrum of GC4A-6C in CD}_3\text{OD, 400 MHz, 25 °C}**

5. Hemolysis assay

![Hemolysis test](image)

**Figure S6.** Hemolysis test of GC4A-6C at different concentrations. Each data point represents the mean ± S.E.M. from a set of experiments (n = 3). NC = negative control, PC = positive control
6. Standard curve of blood loss

![Standard curve of blood loss](image)

**Figure S7.** Standard curve for quantification of blood loss volume.

7. aPTT activity measurement

![aPTT activity measurement](image)

**Figure S8.** *In vitro* aPTT time assays. Blood clotting time induced by UFH with or without GC4AOEG and protamine, GC4AOEG and protamine, respectively. Data presented are the mean ± S.E.M (n = 3). ***p < 0.005, ****p < 0.001, ns represents “no significant difference” between the experimental group and the control group.
8. Factor X assay

![Factor X assay](image)

**Figure S9.** Factor X levels in plasma were determined using ELISA. Data presented are the mean ± S.E.M (n = 3). ****p < 0.001, ns represents “no significant difference” between the experimental group and the control group.

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