Electronic Supplementary Material (ESI) for Polymer Chemistry.

Supporting Information

A nanoparticle-preparation kit using ethylene glycol-based block copolymers with a common temperature responsive block

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1.1. Material

Two-(2-methoxyethoxy)ethyl methacrylate (MEO_2MA) and oligo(ethylene glycol) methacrylate (OEGMA, M_n = 475 g/mol) were purchased from Sigma-Aldrich and purified by passing through basic alumina column. All other chemicals and solvents were used as received. Distilled water used in this study was purified with a Millipore Milli-Q system.

1.2. Preparation of poly(MEO_2MA-*co*-OEGMA)

The reversible addition-fragmentation chain transfer (RAFT) polymerization was employed to synthesize the copolymers with a narrow distribution of molecular weight. MEO_2MA (3.16 g, 16.8 mmol), OEGMA (3.42 g, 7.2 mmol), 4-cyanopentanoic acid dithiobenzoate (CTP) (27.94 mg, 0.1 mmol), and 4,4'-azobis-4-cyanovaleric acid (ACVA) (11.00 mg, 0.044 mmol) ($[MEO_2MA]_0/[OEGMA]_0/[MAAmBO]_0/[CTP]_0/[ACVA]_0$ = 168/72/1/0.44) were dissolved in 10 mL of methanol. After degassing with nitrogen gas for 30 min, the mixture was allowed to polymerize for 6 h at 60 °C. The resulting poly(MEO_2MA-*co*-OEGMA) was purified by reprecipitation using diethyl ether and was dried under reduced pressure.

1.3. Preparation of poly(MEO_2MA-*co*-OEGMA)-*b*-poly(MEO_2MA)

MEO_2MA (1.00 g, 5.31 mmol), ACVA (2.48 mg, 9.92×10^{-3} mmol), and poly(MEO_2MA-*co*-OEGMA) as the macro-CTP (524.53 mg, 2.66×10^{-2} mmol) ($[MEO_2MA]_0/[\text{macro-CTP}]_0/[ACVA]_0$ = 200/1/0.37) were dissolved in 8 mL of methanol. After degassing with nitrogen gas for 30 min, the mixture was allowed to polymerize for 6 h at 60 °C. The resulting poly(MEO_2MA-*co*-OEGMA)-*b*-poly(MEO_2MA) was purified by dialysis against ethanol and acetone and was dried under reduced pressure.

1.4. Preparation of poly(MEO_2MA-*co*-OEGMA)-*b*-poly(MEO_2MA)-Cy5.5

The chain end of the poly(MEO_2MA-*co*-OEGMA)-*b*-poly(MEO_2MA) was modified by a fluorescent Cy5.5. Poly(MEO_2MA-*co*-OEGMA)-*b*-poly(MEO_2MA) (17 mg) and Cy5.5-maleimide (5 mg) were dissolved in 3 mL of tetrahydrofuran (THF). Two-

hydroxyethylamine (1.68×10^{-1} mg) was dissolved in 3 mL of THF, and was added to the polymer solution. After degassing with nitrogen gas for 30 min, the reaction was allowed for 20 h at 25 °C under dark. The resulting poly(MEO_2MA-*co*-OEGMA)-*b*-poly(MEO_2MA)-Cy5.5 was purified by dialysis against ethanol and water and was collected via freeze-drying.

1.5. Characterizations

^1H NMR spectra of copolymers were taken with a JNM-GSX300 spectrometer operating at 300 MHz (JEOL, Tokyo, Japan) to confirm successful synthesis and determine the chemical composition of the synthesized copolymers.

Molecular weight and polydispersity of the synthesized copolymers were determined by gel permeation chromatography (GPC) at 40 °C (DMF including 10mM LiBr, 1 mL/min) with a TOSOH TSK-GEL a-2500 and a-4000 and (Tosoh, Tokyo, Japan) connected to a RI-2031 refractive index detector (JASCO International Co., Ltd., Tokyo, Japan).

Transmittance of a copolymer solution at 500 nm was continuously recorded at a heating rate of 2.0 °C/min by a UV-Vis spectrometer V-650 (JASCO International Co., Ltd., Tokyo, Japan) to measure the lower critical solution temperature (LCST). Synthesized copolymers were dissolved in aqueous solution at the given concentration. LCSTs of copolymers were determined at 50% transmittance.

Dynamic light scattering (DLS) was performed with a DLS-8000 seriese (Otsuka Electronics Co., Ltd., Osaka, Japan) using a light scattering apparatus equipped with He-Ne laser and temperature controller. All samples were kept at given temperatures to reach the equilibrium prior to the measurements. We obtained the diameter data using the Marquardt method (cumulative number = 80).

Fluorescence intensity was recorded using a IVIS imaging system (IVIS Lumina II Imaging System Lumina-FS-TH1, Xenogen co., US). As the wavelengths, an excitation wavelength of 640 nm and an emission wavelength of 700 nm were selected.

The zeta-potential was measured using an Electro Phonetic Light Scattering ELS-6000 (Otsuka Electronics Co., Ltd, Osaka, Japan). The sample (0.1wt% in PBS) was kept at 37^{o}C to reach the equilibrium prior to measurement.

The image of nanoparticles was observed using scanning electron microscopy (SEM) (JSM-IT 100, JEOL, Tokyo, Japan) at an acceleration voltage of 15.0 kV. Two drops of the sample solution (0.1 wt% in PBS) was added on a silicon wafer using the spin coating and allowed to dry under atmospheric pressure. The sample was then sputter coated with Au.

1.6. In vivo biodistribution

All animal experiments were performed in accordance with the guidelines of the National Institute for Materials Science (NIMS) and approved by the Animal Care and Use Committee at NIMS. A mixture of NCI-H23 cells (5×10^7 cells/120 µL saline) and Matrigel (60 µL) was inoculated to each CB17/SCID mouse in the right flank to establish lung tumor models. After about 45 days, the NCI-H23 tumor was established and the mice bearing the NCI-H23 tumor were treated via the poly(MEO_2MA-*co*-OEGMA)-*b*-poly(MEO_2MA)-Cy5.5 nanoparticles. The poly(MEO_2MA-*co*-OEGMA)-*b*-poly(MEO_2MA)-Cy5.5 was completely dissolved in PBS at 4 ^{o}C (0.1 wt%). After filtration via 0.22 µm filter, the polymer solution was incubated at 37 ^{o}C for 1h to form the stable nanoparticles. The nanoparticle suspensions (200 µL) were administered via heart injection. The mice were sacrificed after 36 h and their tumors and main organs such as hearts, livers, spleens, lungs, and kidneys were collected and weighted after treatment of heparin Na solution via the blood vessel. The Cy5.5 fluorescence intensity in the tumor tissue and organs were measured with the IVIS at an excitation wavelength of 640 nm and an emission wavelength of 700 nm.

1.7 Blood compatibility

1.7.1 Blood compatibility studies

Blood was collected from healthy consenting donors in 3.2% sodium citrated vacutainer tube at Centre for Blood Research, University of British Columbia, Canada. For serum preparation whole blood was collected in non-anticoagulated vacutainer tube. Platelet-rich plasma (PRP) was prepared by centrifuging citrated whole blood samples at 150xg for 15 min in an Allegra X-22R centrifuge (Beckman Coulter, Canada). Platelet-poor plasma (PPP) was prepared by centrifuging citrated whole blood samples at 2000xg for 20 min. Serum was prepared by centrifuging non-anticoagulated whole blood samples at 2000xg for 20 min after resting 30 min at room temperature to clot.

The polymer samples were dissolved in isotonic saline 0.9% solution. Both the polymer samples and all the reagents were pre-incubated at 37°C for 20 min before mixing. The Reagents Actin FSL and Dade Innovin, were purchased from Siemens Healthcare Diagnostics. Anti-CD62pPE was purchased from Beckman Coulter. GVB2+ (0.1% gelatin, 5 mM Veronal, 145 mM NaCl, 0.025% NaN_3 with 0.15 mM $CaCl_2$, and 0.5 mM $MgCl_2$, pH 7.3), GVB with EDTA, GVB^0 and antibody-sensitized sheep erythrocytes (EA) were purchased from CompTech (Tyler, TX).

Polymers stock solutions at 1, 5, and 10 mg/mL, were prepared in 0.9% isotonic saline solution. The polymer samples were mixed in a 1/10 ratio (v/v) with plasma, whole blood, washed red blood cells or serum in order to achieve final polymer concentrations 0.1, 0.5 and 1 mg/mL of the polymer, respectively.

1.7.2 Determination of platelet activation by flow cytometry

Fresh blood from 3 different donors (N=3) was collected and platelet rich plasma (PRP) was prepared. Stock polymer solution (10 µL) (in duplicate) was incubated with 90 uL PRP in 1.5 mL of polystyrene tube for 1 h at 37°C. A small aliquot of this platelet suspension (5 µL) was mixed with 5 µL mouse antibody monoclonal CD62 PE in PBS buffer pH 7.4. The platelets activation was analyzed by flow cytometry (BD, FACS Canto II) and 10000 events were recorded for each run. The mouse monoclonal CD42a-FITC anti human antibody (Beckman Coulter) was incubated also in the same proportion with the

PRP to confirm the presence of the platelet in the flow gate. Adenine diphosphate (ADP) 10 µM was used as a positive control for platelet activation.

1.7.3 Complement activation assay using antibody sensitized sheep erythrocytes

A modified hemolytic assay was performed in human serum to measure the complement activation (consumption) induced by the presence of the polymer.[1] Two incubation steps were utilized. In the first step 10 µL of stock polymer solution was incubated with 90 µL of GVB2+ diluted human serum (20% dilution) at 37 °C. After one hour incubation the mixture was diluted with 200 µL more of GVB2+ buffer. In the second step, 100 µL of diluted sample was incubated for another hour with 100 µL of antibody-sensitized sheep erythrocyte (EA cells).

Heat-aggregated IgG (final concentration 2 mg/mL) was also incubated with GVB2+ human serum for 1 h at 37 °C and was used as the positive control. The normal control was isotonic saline and it was incubated with serum at similar conditions as that of polymer samples. The reaction was stopped by the addition of 400 µL cold GVB with EDTA. After one hour, the samples were centrifuge at 1000xg for 3 min and the optical density (OD) was measured at 540 nm. To determine the percent of cell lysis, a blank sample only with GVB^0 was compared with a sample of sheep erythrocyte that has been lysed 100% in H_2O. The absorbance of duplicate samples was measured for serum 3 different donors (N=3). The sheep RBC lysis was calculated as follows:

% complement consumed= 100 %- percent of sheep rbc lysis

% sheep rbc lysis = 100 x (OD sample - OD blank) / (OD 100% lysis sample - OD blank)

1.7.4 Activated Partial Thromboplastin Time (APTT) and Prothrombin Time (PT) analysis

The APPT and PT assay was used to monitor the effect of the polymers on the blood coagulation. The polymer stock solution (25 µL) was mixed with the platelet poor plasma (225 µL) at 37 °C. Saline solution was used as the normal control. Coagulation initiation

reagents Actin FSL and Innovin were used for APTT and PT analyses, respectively. Triplicate samples were tested on the STart4 coagulometer (Diagnostica Stago, France) using plasma from three separate donors (N=3) and the average ± SD was reported.

1.7.5 Thromboelastography Analysis

Thromboelastograph hemostasis system 5000 (TEG) (Haemoscope Corporation) instrument was used. Sodium citrate anticoagulated whole blood fresh collected (within 5 min) was mixed with the saline control or stock polymers solutions (9:1 v/v). The blood–polymer mixture (340 µL) was transferred into the TEG cup. The coagulation was initiated by the addition 20 µL of 0.2 M calcium chloride solution. The TEG clot's physical and kinetic properties were analyzed for 3 donors (N=3).

1.7.6 Red blood cells hemolysis and aggregation

Citrate anticoagulated whole blood or washed in phosphate buffer red cells (10% hematocrit) was used for these studies. polymers samples or saline control (20 uL) were incubated with 180 uL of blood samples, for one hour at 37°C.

For the red blood cell aggregation study, the cells were fixed with 2% glutaraldehyde (2 hours incubation at room temperature). The blood cells morphology was assessed using a bright field light microscope (Zeiss Axioskop 2 Plus, 40 x magnifications) with a digital microscope camera (AxioCam ICc 1, Carl Zeiss Microimaging Inc.) attached on it.

In the hemolysis study, RBCs were incubated with distilled H_2O and was used as positive control (100% lysis). The percent of RBC lysis was measured on a 96 well plate Spectra Max 190 spectrophotometer by the ferricyanide-cyanide (Drabkin's) method.[2,3]

RBC/polymer mixture (5 µL) was added to 295 µL of Drabkin's solution and the optical density (OD) at 540 nm of the solution was measured. The remaining RBC/polymer mixture was centrifuged and 50 µL of the supernatant was added to 250 µL of Drabkin's solution.

The OD of the solution was measured using Drabkin's reagent as a blank. The percent of

lysed cells was calculated from the ratio of hemoglobin in the present in the supernatant and the total hemoglobin. The values reported were from duplicates measurements of 3 separate donor RBCs, and the average ± SD values were reported.

References

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1.8 Statistical analysis

All hemocompatibility data is reported as mean values from three donors ± standard error of mean unless otherwise noted. Statistical analysis students t-test was performed using Excel for two group comparisons. Comparisons were considered significant if $p < 0.05$.

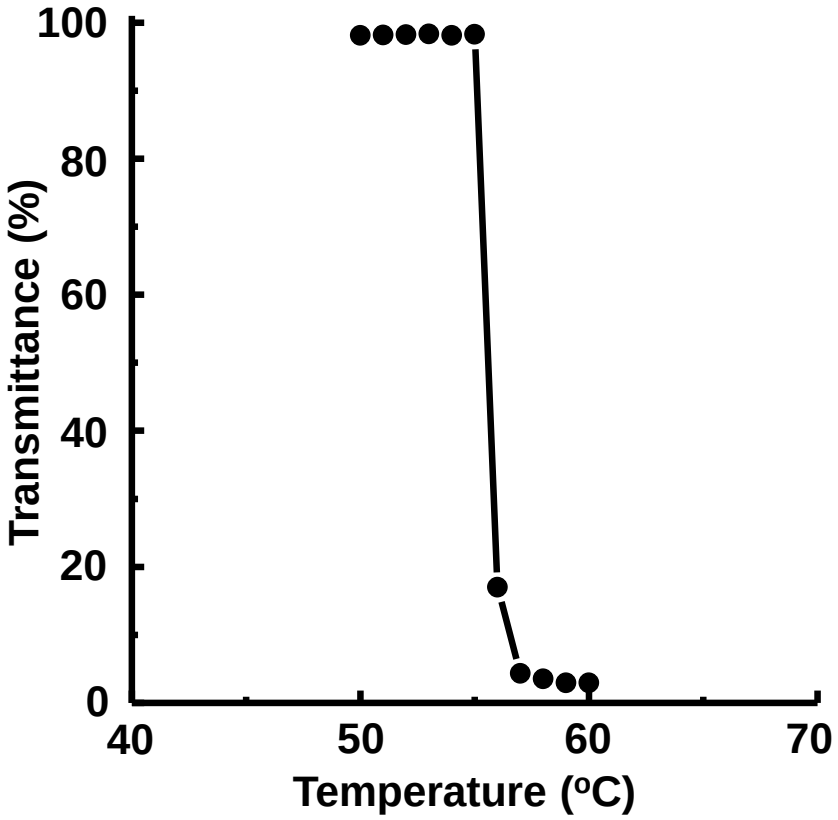

Figure S1. Transmittance change of 0.5 wt% solution of poly(MEO_2MA_{50}-*co*-$OEGMA_{21}$) as a function of temperature in pH 7.4 PBS.

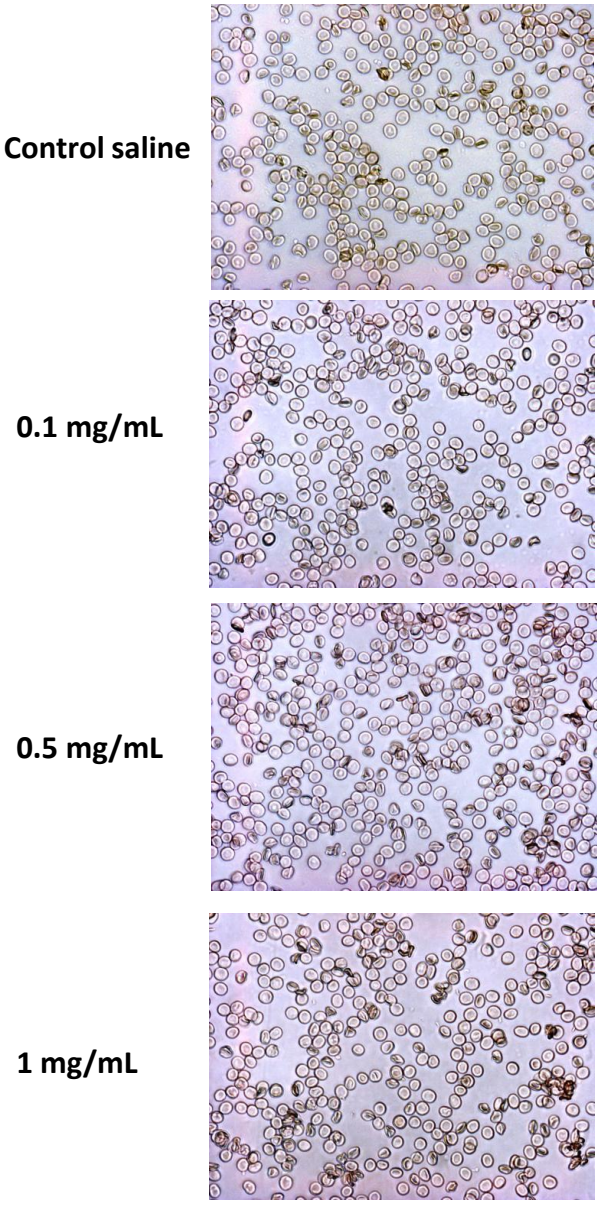

Figure S2. Red blood cell aggregation in presence of poly(MEO_2MA_{50}-*co*-$OEGMA_{21}$)-*b*-poly(MEO_2MA_{42}) nanoparticles at different concentrations measured at 37°C in human whole blood. Nanoparticles were incubated with whole blood (1:9 v/v) ratio at different concentration and optical images were taken. Saline is used as a control. Results show that the polymer does not induce non-specific aggregation of the red blood cells.

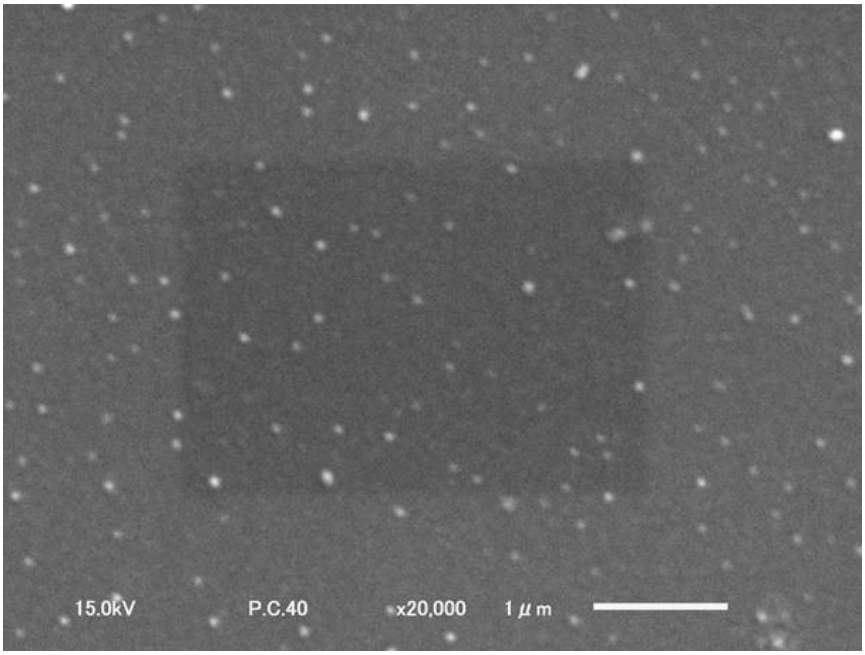

Figure S3. The image of poly(MEO_2MA_{50}-*co*-$OEGMA_{21}$)-*b*-poly(MEO_2MA_{42}) nanoparticles via SEM measurement. The nanoparticles were prepared at 37°C in PBS.