Supporting Information for

Detecting Vascular Bio-signatures with a Colloidal, Radio-opaque Polymeric Nanoparticle

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METHODS

Materials. Unless otherwise listed, all solvents and reagents were purchased from Aldrich Chemical Co. (St. Louis, MO) and used as received. Anhydrous chloroform and methanol were purchased from Aldrich Chemical Co. Poly(styrene-*b*-acrylic acid)[1] (PS-*b*-PAA) was purchased from Polymer Source Inc. (Montreal, Canada). Ethiodol™ was purchased from Savage Laboratories, Inc. (distributor: Beverly Hills Pharmacy, St Louis, MO).[2] Sorbitan monolaurate was purchased from Aldrich. Argon and nitrogen (Ultra High Purity: UHP, 99.99%) were used for storage of materials. The Spectra/Por membrane (Cellulose MWCO: 20 000 Da) used for dialysis was obtained from Spectrum Medical Industries, Inc. (Laguna Hills, CA).

Synthesis of biotinylated *c*ROMP nanoparticle. Typical procedure for the preparation of biotinylated *c*ROMP(I) nanoparticle (19 wt%): In a typical experimental procedure, Ethiodol™ (iodostearic acid ethyl ester and ethyldiiodostearate, Savage Laboratories, Inc. (distributor: Beverly Hills Pharmacy, St Louis, MO), 0.5mL, 50 v/v%) were suspended in sorbitan monolaurate (0.5mL, 50 v/v%) and vigorously vortexed to homogeneity. The suspension was filtered through a small bed of cotton. The amphiline PS-*b*-PAA[1] ($Mn \times 10^{-3}$: 0.8-b-29.3 polydispersity index: PDI=1.18, 0.0033 mmoles, 101.0 mg, 0.5mole%) was dissolved in a mixture of methanol and chloroform (4:1), filtered through a small bed of cotton, evaporated under reduced pressure at 50°C, dried in a 40°C vacuum oven for 6h, and dispersed into water by probe sonication until a clear suspension was obtained. This suspension (10ml) was combined with the Ethiodol-suspended polysorbate mixture (1mL, 5mole%), distilled de-ionized water (8.45ml, 0.2 μM), and glycerin (0.45ml). The mixture was then briefly probe sonicated at

ambient temperature followed by continuous processing at 20,000 PSI (137.9 MPa) for 4 minutes with an S110 Microfluidics emulsifier (Microfluidics) at 4°C.

The non-crosslinked particles were recovered from the microfluidizer and preserved in a sealed serum vial under nitrogen. To this, was added EDCI (1-(3'-dimethylaminopropyl)- 3-ethylcarbodiimide methiodide, (1.2 equiv. of -CO_2H groups) and was allowed to stir for 10 min at room temperature. 2,2'-(ethylenedioxy) bis(ethylamine) (0.5 equiv) was introduced next, followed by the addition of biotin hydrazide (5mg, 0.016 mmol, Aldrich, Inc.). The mixture was allowed to react for 16h at ambient temperature. The cross-linked and functionalized *c*ROMP particles were transferred to a 20,000 Da MWCO cellulose membrane dialysis tubing and dialyzed against water for 3 days with multiple exchange of water. The nanoparticles were recovered and passed through a 0.45 µm Acrodisc Syringe filter. To slow microbial growth the colloids were stored under an argon atmosphere typically at 4°C. DLS (Dav)/nm =162±03 nm; Zeta (ζ)/mV = -20±06 mV; TEM (D_{ah})/nm=97±30; AFM (H_{av})/nm =78±24 nm, PDI=0.26±0.01, ICP-MS= 12±03mg of iodine /ml.

Characterization of *c*ROMP.

Dynamic light scattering measurements (DLS)

Instrument and method: Hydrodynamic diameter distribution averages for cROMP and controls in aqueous solutions were determined by dynamic light scattering. Hydrodynamic diameters were determined using a Brookhaven Instrument Co. (Holtsville, NY) Model Zeta Plus particle size analyzer. Measurements were made following dialysis (MWCO 50 kDa dialysis tubing, Spectrum Laboratories, Rancho Dominguez, CA) of *c*ROMP suspensions into deionized water

(0.2 μM). Nanoparticles were dialyzed into water prior to analysis. Scattered light was collected at a fixed angle of 90°. A photomultiplier aperture of 400nm was used, and the incident laser power was adjusted to obtain a photon counting rate between 200 and 300 kcps. Only measurements for which the measured and calculated baselines of the intensity autocorrelation function agreed to within +0.1% were used to calculate nanoparticle hydrodynamic diameter values. All determinations were made in multiples of five consecutive measurements.

Electrophoretic potential measurements (Zeta Potential)

Instrument and method: Zeta potential (ζ) values for the *c*ROMP were determined with a Brookhaven Instrument Co. (Holtsville, NY) model Zeta Plus zeta potential analyzer. Measurements were made following dialysis (MWCO 50 kDa dialysis tubing, Spectrum Laboratories, Rancho Dominguez, CA) of *c*ROMP suspensions into water. Data were acquired in the phase analysis light scattering (PALS) mode following solution equilibration at 25°C. Calculation of ζ from the measured nanoparticle electrophoretic mobility (μ) employed the Smoluchowski equation: $\mu = \varepsilon\zeta/\eta$, where ε and η are the dielectric constant and the absolute viscosity of the medium, respectively. Measurements of ζ were reproducible to within ±4 mV of the mean value given by 16 determinations of 10 data accumulations.

Fluorescence Spectroscopy

Fluorescence spectroscopy, performed as a function of pH, exemplify the pH dependence of the emission intensity from fluorescein thiosemicarbazide (FTSC) labeled *c*ROMP. *c*ROMP nanoparticle suspension were excited at λ_{ex} =496 nm (λ_{em} =510 nm).

Instrument and method: Fluorescence spectroscopic measurements were made with a Cary Eclipse fluorescence spectrophotometer equipped with Cary Eclipse modular software.

Transmission Electron Microscopy Measurements (TEM)

Instrument and method: Glow discharged carbon/formvar coated nickel grids were floated on a drop of sample for 2 mins. Grids were blotted, rinsed quickly in water, and stained in 1% aqueous uranyl acetate (UA) for 1 min. Samples were blotted, air dried, and viewed on a Zeiss 902 Electron Microscope, and recorded with Kodak E.M. film. Micrographs were collected at 100,000x magnification. The number-average particle diameter (D_{ah}) values and standard deviations were generated from the analyses of a minimum of 100 particles from three micrographs.

Atomic Force Microscopy Measurements (AFM)

Instrument and method: A Digital Instruments Dimension 3000 series AFM (calibration date 08/2008) and standard Veeco tapping mode silicon probes w/PtIr coating were used for scanning the samples.

In a typical methodology, aqueous suspensions of *c*ROMP samples were dried in a class 10000-clean room on a clean glass slide for 3h. Once dried, samples were placed on the AFM and scanned. Pertinent scanning parameters were as follows: Resonant frequency (probe): 60-80 kHz; Example of tip velocity: (4 um/s for 2um), (15 um/s for 5um), (30 um/s for 10 um). Aspect ratio: 1:1; Resolution: 512 samples/line, 256 lines. The average particle height (H_{av}) values and standard deviations were generated from the analyses of a minimum of 100 particles from three micrographs.

Inductively coupled plasma-optical emission spectroscopy (ICP-OES)

Instrument and method: The iodine and bismuth contents of *c*ROMP were analyzed by inductively coupled plasma-optical emission spectroscopy (ICP-MS, SOP7040, Rev 9) conducted at the Bodycote, West Coast Analytical Service (WCAS), Santa Fe Springs, CA. Briefly, the samples were analyzed by a Leeman Labs Direct Reading Echelle ICP-MS, or a DRE (Direct Reading Echelle) instrument which was designed to handle sub-ppm to percent level metal concentrations. DRE consists of a 2 dimensional, high resolution Echelle grating which precisely and reliably locate any peak in the ICP spectrum.

***In vitro* human plasma clot phantoms**

Method: In a typical procedure, whole porcine blood was obtained fresh and anticoagulated (9:1, vol/vol) with sterile sodium citrate. Plasma clots were produced by combining plasma and 100 mmol/L calcium chloride (3:1 vol/vol) with 5 U thrombin (Sigma-Aldrich, Inc.) in a (low density polyethylene) tube (~1 cc volume, I. D. ~6 mm). The plasma was allowed to coagulate slowly at room temperature. The clots were incubated individually with 150 µg biotinylated antifibrin monoclonal antibody (NIB5F3)[3] in 10 mL PBS with 1% crystalline BSA (Sigma Chemical Co) for 2 hours. The antibody-treated clots were then incubated with excess avidin (50 µg/mL PBS) for 30 minutes, followed by biotinylated cROMP (30 µL/mL de-ionized water) for 30 minutes. The control clots were treated similarly with control non-targeted nanoparticle (30 µL/mL de-ionized water).

Blood-smear experiment

A single smear was made per slide by putting a drop of fresh rabbit blood on the slide (near the end). The drop was spread by using another slide ("spreader"), placing the spreader at a 45° angle and backing into the drop of blood. The spreader catches the drop and it spreads by capillary action along its edge. Smear is allowed air dry for 1min and cover-slip before placed directly on the microscope and observed under 40 x magnifications. A ratio of 1:9 *c*ROMP (300 µl of 0.5% colloidal suspension of a 0.5-0-5% formulation) and rabbit blood was used for preparation of the smear.

Computed Tomography Imaging System

Instrument and method: High-resolution CT data were acquired on a Philips Precedence™ (Brilliance) 16 channel CT imaging system with X-ray tube settings of 90 kVp and 800 mA. With a 100 mm field of view and 0.80 mm slice thickness, the length of the scan was 234mm. The high resolution scans were acquired dynamically at baseline, 30min, 1h, 1.5h, 2h, 2.5h, 3h and 3.5h with a collimation of 16×0.75, pitch 0.313 and a rotation time 1.5 sec.

Animal and Drug Information: Guidelines on the care and the use of laboratory animals at Washington University in St. Louis were followed for all animal experiments. Adult Sprague Dawley rats with various body weights (250-350 g) were used for the experiments. Initial anesthetization of the rat was done using a mixture of ketamine (85 mg/kg) and xylazine (15 mg/kg) and maintained on 0.75-1.0% isoflurane delivered through a calibrated vaporizer. *c*ROMP (I) was administered (6ml/kg; total volume: 1.96ml for a 327g rat) intravenously through tail vein catheter. CT images were acquired before and after the administration of

*c*ROMP. During the image acquisition, anesthesia was maintained using vaporized isoflurane (1 L/min oxygen and 0.75% isoflurane, Euthanex Corp.), and a pulse oximeter (NONIN Medical INC., 8600V) was used to monitor the vitals. If needed, 8 ml of 0.9% saline was administered to the rat for hydration. After image acquisition, the animal was euthanized by pentobarbital overdose.

References

1. (a) Laruelle, G.; François, J.; Billon L. *Macromol Rapid Commun* **2004**, *25*(21), 1839-1844; (b) Huang, H.; Kowalewski, T.; Remsen, E. E.; Gertzmann, R.; Wooley, K. L. *J. Am. Chem. Soc.*, **1997**, *119* (48), 11653-11659; (c) Wu, J.; Eisenberg, A. *J. Am. Chem. Soc.*, **2006**, *128* (9), 2880-2884. (d) Xu J, Sun G, Rossin R, Hagooly A, Li Z, Fukukawa K. I., Messmore B. W., Moore D. A., Welch M. J., Hawker C. J., Wooley K. L. Macromolecules. **2007**, *40*(9), 2971-2973. (e) Avital A, Shapiro E, Doviner V, Sherman Y, Margel S, Tsuberi M, Springer C. *Am J Respir Cell Mol Biol.* **2002**, 27(4), 511-4.

2. Stanbury, J. B.; Barnaby, J.; Daza, G.; **2002**. *Recommendations for the use of iodized oil*, http://icb.usp.br/lats/STAMBURY.HTM, accessed on September 17.

3. Raut S.; Gaffney, P. J. *Thromb. Haemostasis*, **1996**, *76*, 56.