Supplementary Information

Sensitive and efficient detection of thrombus with fibrin-specific manganese nanocolloids

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Comparison of stability of the nanocolloids

The stability of the nanocolloids were tested for variation by measuring particle diameters (DLS), zeta potential and polydispersity changes (DLS) over a 10 months period. A less than 5% change of hydrodynamic diameter over time has been observed when stored at 4°C under argon in sealed serum vials, which is within the error of measurements.

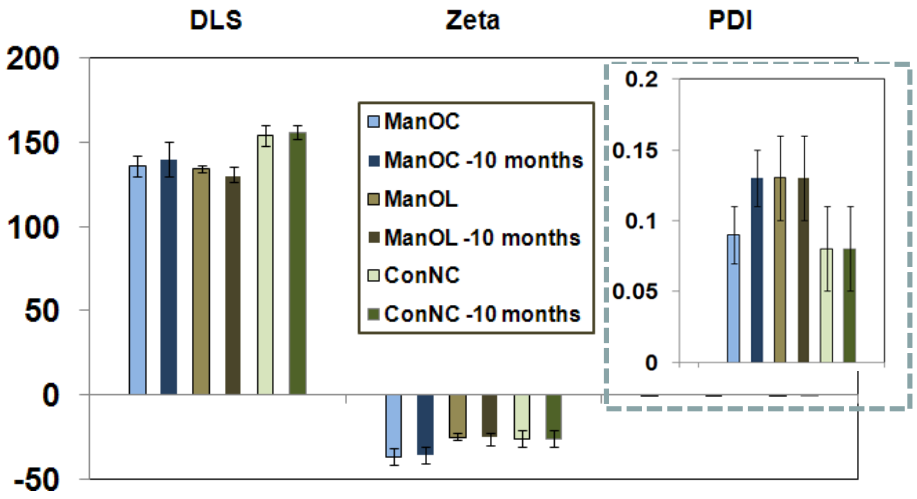

Figure 1S. Stability comparison of the manganese and control nanocolloids: (left) Number-averaged hydrodynamic diameter (from DLS) of colloids immediately after preparation and after 10 months storing at 4°C under argon in sealed serum vials; (middle) zeta potential of colloids immediately after preparation and after 10 months storing at 4°C under argon in sealed serum vials; (right) poly dispersity indexes (PDI, from DLS) of colloids immediately after preparation and after 10 months stored at 4°C under argon in sealed serum vials;

Characterisation

Manganese-Oleate was characterised by FT-IR, ESI-MS and thermo gravimetric (TGA) analyses. The molecular bonding of the metal complex was investigated using FT-IR spectroscopy. The infrared (IR) spectra revealed the presence of characteristic peaks of the metal-oleate[1]. The peaks at 1460 and 1550 cm^{-1} were assigned to the symmetric ν_s (COO^-) and asymmetric ν_{as}(COO^-) vibrational stretch modes, respectively.

Managnese-Oleate gave a strong positive ion electrospray-MS spectrum with $(M+H)^+$ pseudomolecular ion at m/z 618.5 and the associated $(M+Na)^+$ at m/z 640.4 corresponding to the expected $Mn(Oleate)_2$.

The thermal stability of the complex was observed by TGA analysis. Figure 2S shows the TGA curves that illustrated the decomposition of the Mn-oleate. A sharp drop in weight loss was observed in the 246-300°C region. The TGA patterns revealed that one oleate ligand dissociates from the precursor at 246.2 °C and the remaining oleate ligand dissociate at 291.4 °C. The first step began at approximately 246.2 °C and accounted for 43.76% of the mass loss, which correspond to the loss of one oleate group (nominal mass loss for one oleate functionality =45%). The second weight loss step began at about 291.4 °C.

The molecular bonding of the metal complex after the thermal decomposition was investigated using FT-IR spectroscopy. TEM images of the manganese oxide revealed the formation of relatively uniform nanoparticles with sizes ranging from 8 nm to 12 nm. These observations were similar to the previously reported procedures.[2] The final nanocolloids were characterized by DLS, AFM, TEM, electrophoretic measurements and ICP-MS.

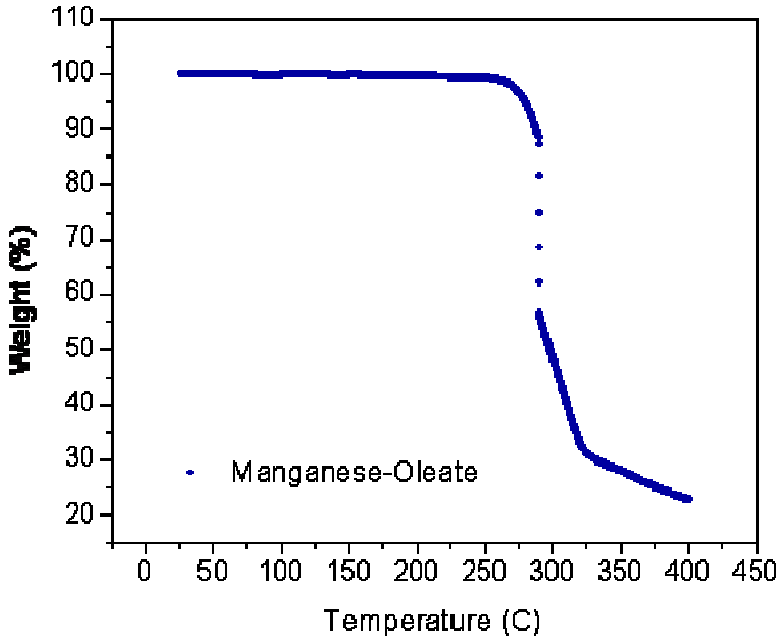

Figure 2S. TGA results for manganese oleate complex. The decomposition rate is maximum at the 246-300°C region.

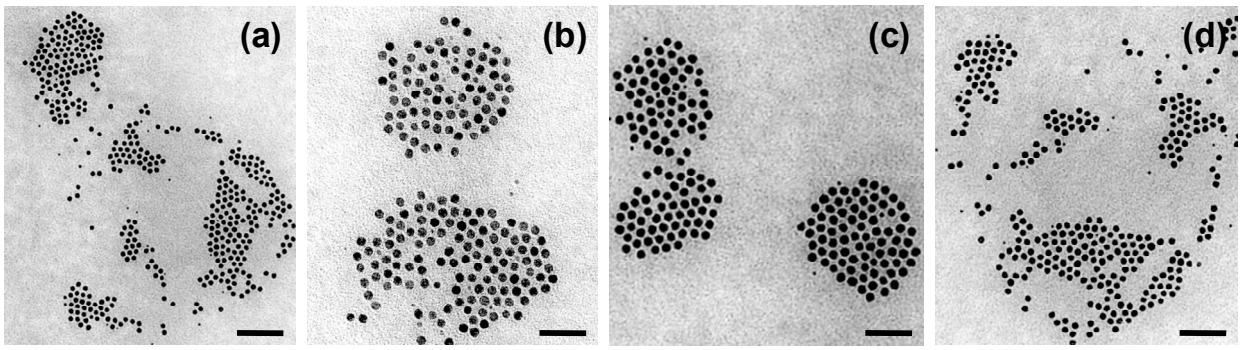

Figure 3S. Representative TEM images of the manganese oxide show highly monodispersed nature of the particles in terms of size and shape [scale bar (a), (c) =110nm; (b), (d) =70nm.]

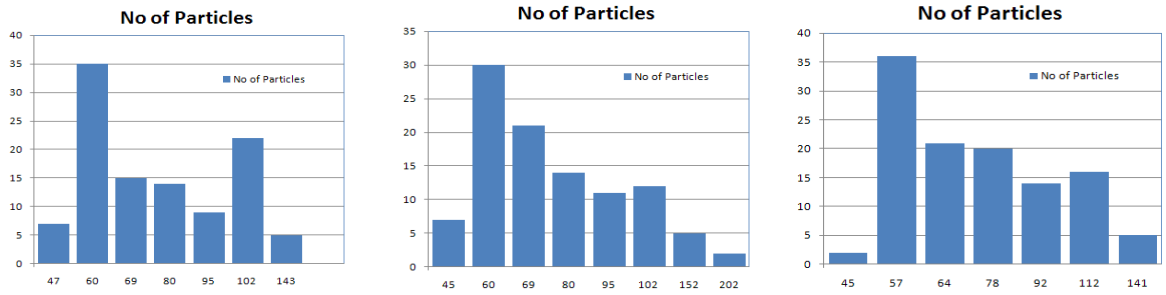

Figure 4S. Particle height distributions of ManOC (left), ManOL (middle) and ConNC (right) from dried state AFM.

EXPERIMENTAL SECTION

Materials

Unless otherwise listed, all solvents and reagents were purchased from Aldrich Chemical Co. (St. Louis, MO) and used as received. Anhydrous chloroform was purchased from Aldrich Chemical Co. and distilled over calcium hydride prior to use. Biotinylated dipalmitoyl-phosphatidylethanolamine and high purity egg yolk phosphatidylcholine were purchased from Avanti Polar Lipids, Inc. Cholesterol, manganese chloride and sorbitan sesquioleate were purchased and used as received from Aldrich Chemical Co. (St. Louis, MO). Sodium oleate was purchased and used as received from TCI America. (Portland, OR). Argon and nitrogen (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).

Methods

Typical procedure for preparation of Mn-Oleate: Manganese-oleate was prepared and characterized following a modified procedure reported by Na Bin et al.[2] Briefly, manganese chloride tetrahydrate (9.9g, 51 mmol) was weighed in a 250mL round bottomed flask. To this, 30 mL of deionized water (0.2μM) was added. Sodium oleate (32g, 105 mmol) was weighed separately and added to the manganese chloride mixture. Additional 20 mL of de-ionized water and 40mL of anhydrous ethanol were added. The mixture was vortexed well, followed by the addition of n-hexane (~90mL). The mixture was allowed to reflux at 75-80°C for 14h. With the progression of reaction, the reaction mixture turned pink and transferred to the top organic layer. The solution was then allowed to come to the room temperature and further allowed to stir for additional 4h at ambient temperature. The organic layer was separated using a separatory funnel, washed (3x100 mL) with water, dried (Na_2SO_4) and evaporated to produce Mn-Oleate as a waxy sticky solid. The complex was re-dissolved in chloroform-ether (2:1 v/v) mixture and evaporated to produce purified solid.

ESI-MS $(M+H)^+$ m/z 618.5, $(M+Na)^+$ m/z 640.4; FT-IR ν_{max} 1096, 1120, 1231, 1312, 1338, 1420, 1458 ($\nu_s COO^-$), 1527, 1532, 1550 ($\nu_{as} COO^-$), 2854 ($-CH_3$), 2926 ($-CH_2$) cm^{-1}.

Typical procedure for preparation of MnO: 8-12nm sized manganese oxide nanoparticles were synthesised by thermally decomposing manganese-oleate complex.[2] Briefly, Mn-oleate complex (2g, 3 mmol) was weighed in a 100mL round bottomed 2-necked flask and dissolved in 20 mL of anhydrous 1-octadecene. The mixture was charged with a stirring bar and degassed under vacuum for 2 hr. The round bottom flask was then fitted with a refluxing condenser and slowly heated to 325 °C with vigorous stirring. With progression of the reaction, the colored

mixture turned from colorless to dark green, indicating the thermal decomposition of the complex. The reaction was allowed to go for 70 minutes at this temperature, followed by the gradual decrease to ambient temperature. At ambient temperature the mixture was allowed to stir for ~1h followed by diluting the mixture with 30 mL of hexane. Acetone (excess amount) was added to this to precipitate the nanoparticles from the solution. The precipitate was recovered by centrifugation and repeated treatment with acetone. The waxy precipitate thus obtained, was purified by re-dissolving in a mixture of acetone and diethyl ether (5:1). The purified manganese oxide nanoparticles were vacuum dried at 40°C for 1h and at ambient temperature for overnight. The powder was recovered and dissolved in anhydrous chloroform before using in the next step. TEM (D_{ah}): 10±2nm; FT IR ν_{max} 568 (Mn-O), 627, 721, 876, 1038, 1371, 1460 ($\nu_s COO^-$), 1538($\nu_{as} COO^-$), 2854, 2926, 2959 cm^{-1}.

Typical procedure for preparation of ManOC: In a typical experimental procedure, manganese oxide nanoparticles (8-12 nm, 100mg, ~2w/v%) was dissolved in anhydrous chloroform (5 mL), filtered through a small bed of cotton to remove any dust particles and suspended in almond oil (4.5 mL) and vigorously vortexed to homogeneity. The suspension was filtered through a small bed of cotton. The solvent was evaporated under reduced pressure at 50°C. For phospholipid encapsulation, the surfactant co-mixture included high purity egg yolk phosphatidylcholine (90 mole%, 376.2 mg), cholesterol (8 mole%, 20.3 mg), and biotinylated-dipalmitoyl phosphatidylethanolamine (1 mole%, 5.9 mg). The surfactant co-mixture was dissolved in chloroform, evaporated under reduced pressure, dried in a 40°C vacuum oven overnight, and dispersed into water by probe sonication. This suspension was combined with the manganese oxide nanoparticle-suspended almond oil mixture (4 mL, 20% v/v), distilled de-ionized water (77.3% w/v), and glycerin (1.7%, w/v). The mixture was continuously processed

thereafter at 20,000 PSI for 4 minutes with an S110 Microfluidics emulsifier (Microfluidics) at 0°C.

The nanocolloids were dialyzed against water using a 20,000 Da MWCO cellulose membrane for a prolonged period (>48h) of time and then passed through a 0.45 μm Acrodisc Syringe filter. To prevent bacterial growth the nanocolloids were stored under an argon atmosphere typically at 4°C.

DLS (D_{av})/nm =136±06 nm; AFM (H_{av})/nm =74±42nm; Zeta (ζ)/mV = -37±05 mV; PDI: 0.09±0.02; ICP [Mn]/mg.ml^{-1}:0.37±0.02.

Typical procedure for preparation of ManOL: In a typical experimental procedure, manganese oleate (0.5g) was suspended in sorbitan sesquioleate (5mL), and vigorously vortexed to homogeneity. The surfactant co-mixture included high purity egg yolk phosphatidylcholine (90 mole%, 374.5 mg), cholesterol (9 mole%, 17.4 mg), biotinylated-dipalmitoyl phosphatidylethanolamine (1 mole%, 5.5 mg).

The surfactant co-mixture was dissolved in chloroform, evaporated under reduced pressure, dried in a 50°C vacuum oven overnight, and dispersed into water by probe sonication. This suspension was combined with the bismuth polysorbate mixture (20% v/v), distilled, de-ionized water (77.3% w/v) and glycerin (1.7%, w/v). The mixture was continuously processed thereafter at 20,000 PSI for 4 minutes with an S110 Microfluidics emulsifier (Microfluidics) 0°C.

The nanocolloids were dialyzed against water using a 20,000 Da MWCO cellulose membrane for prolonged period of time and then passed through a 0.45 μm Acrodisc Syringe

filter. The nanocolloids were stored under argon atmosphere typically at 4°C in order to prevent any bacterial growth.

DLS (D_{av})/nm =134±02 nm; AFM (H_{av})/nm =86±32nm; Zeta (ζ)/mV = -25±02 mV; PDI: 0.13±0.03; ICP [Mn]/mg.ml^{-1}: 0.49±0.02.

Typical procedure for preparation of control nanocolloid (ConNC): The control nanocolloid was prepared following a similar procedure as above with the exclusion of metal. In a typical experimental procedure, the surfactant co-mixture included high purity egg yolk phosphatidylcholine (90 mole%, 558.6 mg), cholesterol (8 mole%, 26.3 mg), and biotinylated-dipalmitoyl phosphatidylethanolamine (2 mole%, 16.2 mg). The surfactant co-mixture was dissolved in chloroform, filtered and evaporated under reduced pressure, dried in a 40°C vacuum oven overnight, and dispersed into water by probe sonication. This suspension was combined with the almond oil mixture (20% v/v), distilled de-ionized water (77.3% w/v), and glycerin (1.7%, w/v). The mixture was continuously processed thereafter at 20,000 PSI for 4 minutes with an S110 Microfluidics emulsifier (Microfluidics) at 0^{o}C. The nanocolloids were dialyzed against water using a 20,000 Da MWCO cellulose membrane for a prolonged period of time and then passed through a 0.45 μm Acrodisc Syringe filter. To prevent bacterial growth the nanocolloids were stored under argon atmosphere typically at 4°C.

DLS (D_{av})/nm =154±06 nm; AFM (H_{av})/nm =71±40nm;Zeta (ζ)/mV = -26±05 mV; PDI: 0.08±0.03.

Measurements

Electrospray Ionization Spectrometry

Instrument and method: Electrospray ionization spectrometry was performed using a Sciex Q-Star/Pulsar. After dissolving in a solution of chloroform:methanol (95:5) v/v, sample aliquots of 1 μL were introduced into the instrument source via a nanospray needle.

Thermo gravimetric (TGA) analysis

Instrument and method: The TGA data were collected using a TA Instruments 951 TGA controlled by Instrument Specialists Inc (ISI) data collection software. Data were then analyzed using ISI's analysis program. The sample was heated from 25 °C to 400 °C at a rate of 10 °C/min. Nitrogen was flowing through the sample chamber of the TGA at a rate of 25 mL/min. A 15-minute purge time was used for the platinum pan tare. Decomposition of a material is indicated by a reduction in mass (reduced weight %) as a function of increasing temperature.

Dynamic light scattering measurements

Instrument and method: Hydrodynamic diameter distribution and distribution averages for the Manganese colloids 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 10 kDa dialysis tubing, Spectrum Laboratories, Rancho Dominguez, CA) of manganese colloid suspensions into deionized water (0.2 μM). Nanocolloids were dialyzed into water prior to analysis. Scattered light was collected at a fixed angle of 90°. A photomultiplier aperture of 400 mm 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

Instrument and method: Zeta potential (ζ) values for the manganese colloids were determined with a Brookhaven Instrument Co. (Holtsville, NY) model Zeta Plus zeta potential analyzer. Measurements were made following dialysis (MWCO 10 kDa dialysis tubing, Spectrum Laboratories, Rancho Dominguez, CA) of manganese colloids 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.

Atomic Force Microscopy Measurements

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 manganese colloid 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; Lift height: 20 nm; 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 80 particles from three micrographs.

Transmission Electron Microscopy Measurements

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 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.

Inductively coupled plasma-optical emission spectroscopy

Instrument and method: After imaging, the manganese content of manganese colloids was 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 instrument which was designed to handle sub-ppm to percent level metal concentrations.

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 ManOC, ManOL or ConNC (30 µL/mL PBS) for 30 minutes. The control clots were treated similarly with control nanoparticle (30 µL/mL PBS).

Magnetic Resonance Imaging (MR) measurements

All images and scans were acquired on a 3.0T clinical scanner (Achieva; Philips Medical Systems, Best, Netherlands) with a quadrature (transmit/receive) volume head coil. Relaxivity measurements were obtained for the manganese colloids and control at 3.0 T using turbo spin-echo (SE) and inversion recovery (IR) techniques. Manganese colloids and control nano-colloids were diluted with distilled de-ionized water in ratios of 1:2, 1:4, 1:8, 1:10, 1:20, 1:40. An image resolution of 0.95 mm x 0.95 mm x 6 mm was used. Test tubes with 1 mL of manganese colloids suspension (concentration ranging from 1:2 to 1:40) were placed vertically in a clinical MR scanner and imaged in cross section (to reduce through plane partial volume effects). T2 was calculated from multi echo SE images with a range of echo times (TSE es/shot (ms) 3.9/78; number echoes =20). T1 measurements were calculated using the complex numbers reconstructed of the images collected with an IR technique described by Look and Locker[4](Look DC, Locker DR. Time saving in measurement of NMR and EPR relaxation times. Rev. Sci. Instrum.1970; 41(2):621-627.); following a 180 degree inversion pulse, the magnetization recovery was sampled 55 times every 21ms with a gradient echo imaging technique using a flip

angle of 10 degrees. The time between successive inversion pulses was 1.5 seconds. The resulting images were analyzed using custom software which calculates relaxation rates on a pixel-by-pixel basis. The relaxivities (i.e., r_1 and r_2) were calculated from the slope of the linear least squares regression of relaxation rate vs. metal, i.e. ion relaxivity, or nanoparticle, i.e. particle relaxivity, concentrations and are reported in units of $(s \bullet mM)^{-1}$. Parameters were estimated as mean % std dev within regions of interest drawn within the test tubes of various concentrations.

Nanocolloid suspension phantoms: Nanocolloid test phantoms were prepared in snap cap tubes. Selected volumes (1:2, 1:4, 1:8, 1:10, 1:20, 1:40) of the nanocolloid samples were added to the phantom tubes and diluted by adding deionized water.

Targeted clot phantoms: All images and scans were acquired on a 3.0T clinical scanner (Achieva; Philips Medical Systems, Best, Netherlands) with a quadrature (transmit/receive) volume head coil. For target specific imaging, clots were imaged at 3.0 T (TSE es/shot (ms) 4.8/155; TR=1200ms; number echoes: 32) using 2D TSE and a reconstructed pixel dimension was 0.73mm × 0.73mm × 5mm slice thickness.

References:

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