Supporting Information for

Synthesis of NanoQ, a Copper-Based Contrast Agent, for High-Resolution Magnetic Resonance Imaging Characterization of Human Thrombus

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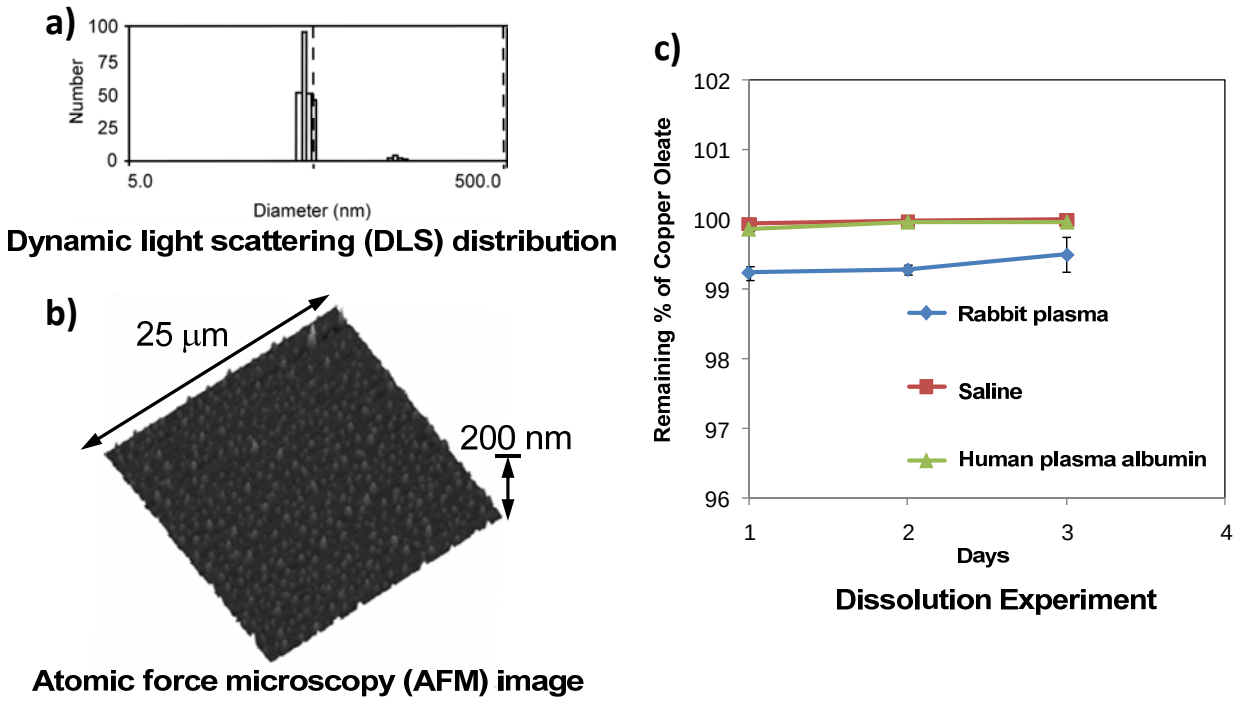

Figure S1. (a) Number-averaged hydrodynamic distribution of NanoQ from dynamic light scattering measurements in aqueous state. (b) Atomic force microscopy (AFM) image of biotinylated NanoQ in anhydrous state drop-deposited over glass. (c) Dissolution of copper from NanoQ against infinite sink over 3 days at 37°C.

Shelf-life stability of NanoQ

The stability of the NanoQ particles were tested for variation by measuring particle diameters (DLS), zeta potential and polydispersity changes over a 4 ½ 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.

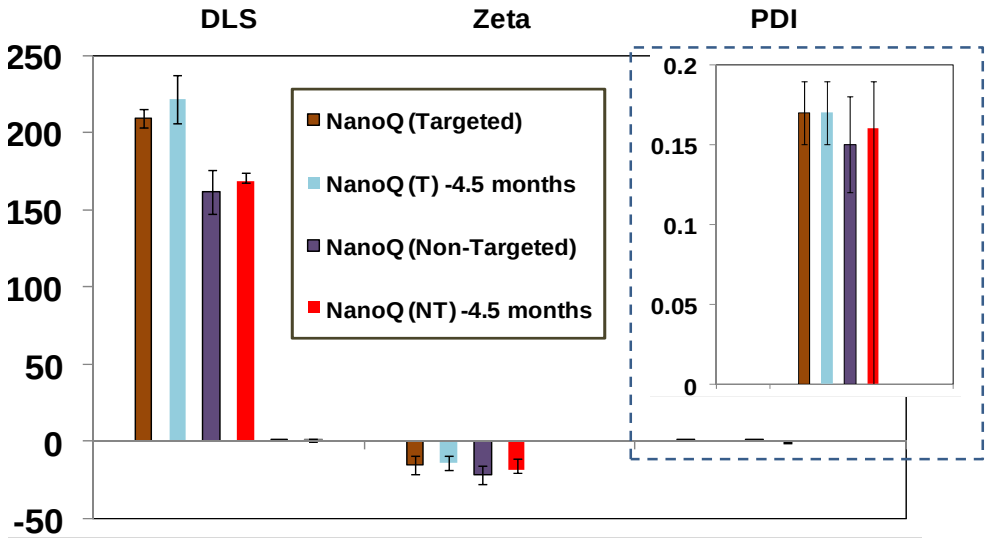

Figure S2. Stability comparison of the copper NanoQ and control nanocolloid: (left) Number-averaged hydrodynamic diameter (from DLS/ nm) of colloids immediately after preparation and after 4 ½ months storing at 4°C under argon in sealed serum vials; (middle) zeta potential (mV) of colloids immediately 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 4 ½ months stored at 4°C under argon in sealed serum vials. T: targeted; NT: non-targeted.

pH stability data of NanoQ

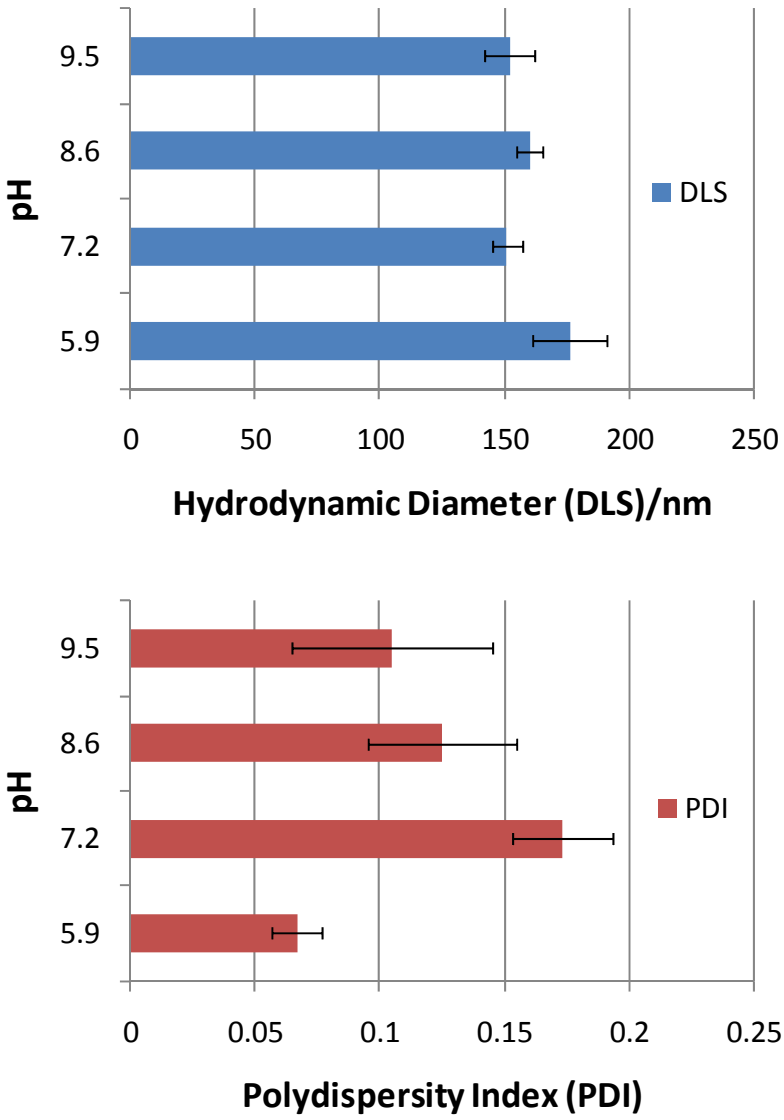

Figure S3. pH stability data (Top): variation of number averaged hydrodynamic diameter against changes of pH; (bottom): variation of polydispersity indexes against changes of pH. Nontargeted NanoQ particles were chosen (~180 days from formulation) and the pH was adjusted with sodium phosphate mono basic buffer 0.5M or sodium phthalate buffer 0.5M. The particles

retained their integrity in a physiological pH range of 5.9-9.5. Particles sediment at pH 4.9 and below (i.e. pH 4.0).

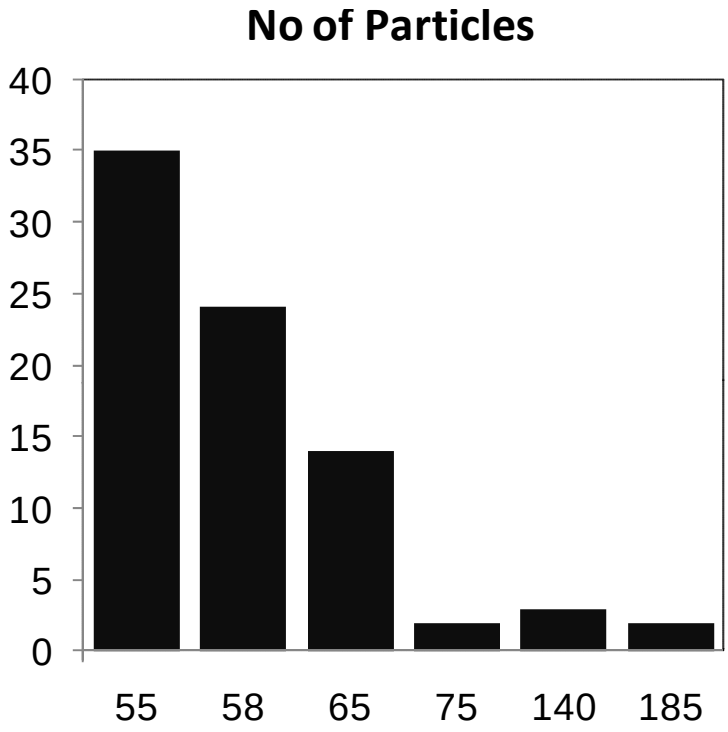

Figure S4. Particle height distributions of NanoQ from dried state AFM (horizontal axis: no of particles; vertical axis: particle height (nm)).

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 and sorbitan sesquioleate were purchased and used as received from Aldrich Chemical Co. (St. Louis, MO). Copper oleate was purchased and used as received from MP Biomedicals. (Solon, OH). 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 biotinylated NanoQ (Cu): In a typical experimental procedure, cupric oleate (0.253 g) was dissolved in anhydrous chloroform and mixed with sorbitan sesquioleate (4 mL), and vigorously vortexed to homogeneity. The anhydrous chloroform was evaporated gently under reduced pressure at 45°C, The surfactant co-mixture included high purity egg yolk phosphatidylcholine (99 mole%, 395.5 mg), biotinylated-dipalmitoyl phosphatidylethanolamine (1 mole%, 6.0 mg). The surfactant co-mixture was dissolved in anhydrous chloroform, evaporated under reduced pressure to form a thin film, dried in a 50°C vacuum oven overnight, and dispersed into water (5 mL) by probe sonication. This suspension was combined with the copper polysorbate mixture (20% v/v, 4 ml), distilled, de-ionized water (77.3% w/v, total 15.23 ml) and glycerin (1.7%, w/v, 0.37 ml). The mixture was continuously processed thereafter at 20,000 PSI for 4 minutes with an S110 Microfluidics emulsifier (Microfluidics) 0°C. The NanoQ particles were dialyzed against nanopure (0.2μm) 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 nanoparticles were stored under argon atmosphere typically at 4°C in order to prevent any bacterial growth.

DLS (D_{av})/nm =210 ± 06 nm; AFM (H_{av})/nm =78 ± 12nm; Zeta (ζ)/mV = -15 ± 06 mV; PDI: 0.17±0.05; ICP-OES [Cu]/mg.L^{-1}: 67.2.

Typical procedure for preparation of control NanoQ (non targeted): The control NanoQ (Cu) particles were prepared following a similar procedure as above with the exclusion of biotinylated lipid. In a typical experimental procedure, cupric oleate (0.257 g) was dissolved in anhydrous chloroform and mixed with sorbitan sesquioleate (4 mL), and vigorously vortexed to homogeneity. The anhydrous chloroform was evaporated gently under reduced pressure at 45°C, The surfactant co-mixture included high purity egg yolk phosphatidylcholine (100 mole%, 401.5 mg). The surfactant co-mixture was dissolved in anhydrous chloroform, evaporated under reduced pressure to form a thin film, dried in a 50°C vacuum oven overnight, and dispersed into water (5 mL) by probe sonication. This suspension was combined with the copper polysorbate mixture (20% v/v, 4 ml), distilled, de-ionized water (77.3% w/v, total 15.23 ml) and glycerin (1.7%, w/v, 0.37 ml). The mixture was continuously processed thereafter at 20,000 PSI for 4 minutes with an S110 Microfluidics emulsifier (Microfluidics) 0°C. The NanoQ particles were dialyzed against nanopure (0.2μm) 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 nanoparticles were stored under argon atmosphere typically at 4°C in order to prevent any bacterial growth.

DLS (D_{av})/nm =162 ± 14 nm; Zeta (ζ)/mV = -22 ± 06 mV; PDI: 0.15±0.06.

Measurements

Dynamic light scattering measurements: Hydrodynamic diameter distribution and distribution averages for the copper 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 copper 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: Zeta potential (ζ) values for the copper 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 copper 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 (AFM): 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 NanoQ 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.

Dissolution experiment

Summary

	Rabbit Plasma		saline		Human Plasma Albumin	
day	ave	std	ave	std	ave	std
0	97.70866	1.500037	97.70866	1.500037	97.70866	1.500037
1	99.24049	0.099444	99.93813	0.06447	99.86709	0.032888
2	99.28389	0.065101	99.98101	0.032888	99.96202	0.065776
3	99.50089	0.246469	100	0	99.96202	0.032888

Determine releasing percentage of copper from NanoQ

Material: Spectra for dialysis tubing, Biotech membranesgrade (regenerated cellulose) MWCO=50K, 8 mm X 5mm 0.25ml/cm 15m; lot #27007 from Spectrum Laboratories, Inc. Spin-x ® Microcentrifuge tube filters, 0.22 μm Cellulose Acetate in 2.0 ml Polypropylene Tube; 25 /Pack; 100/case; non-sterile from Corning Incorporated; NaCl, from Sigma, HSA, human serum albumin from Sigma; fatty acid free, globulin free; NaN_3 sodium azide; from Sigma; 4 ml amber autosampler vials with PTFE septa from Natioanal Scientific Co. Cat# c4015-21W;

Presterilized 500 ml Vacuum Driven disposable filtration system, 0.22 μm, GP Express PLUS Membrane; from Millipore Corporation Acrodisc® syringe filter 0.2 μm super®Membrane non-Pyrogenic REF:4612 from Pall Gelman laboratory.

Instrument and methods: Copper was analyzed by UV absorbance spectrometer at wavelength 600-700 nm for detection.

Procedure: Preparation of calibration copper solution: 1 mg / ml ethanol Cupric Oleate solution was diluted into 100 μg/ml; 10 μg/ml; solution with $CHCl_3$/ethanol (1:1) and the UV absorbance were measured measure at 600nm to generate a calibration standard. Dialysis tubing was cut in ~8 cm lengths and soaked in 2 L deionized (DI) water for at least 30 min. It was then rinsed well with DI water and tied knot at one end. Three releasing medium were used as human serum albumin medium (0.9% NaCl, 0.2mg/ml human serum albumin; 0.05% NaN_3 in 500ml), rabbit plasma releasing medium (2ml rabbit plasma in human serum albumin releasing medium) and Saline (0.9 % NaCl). 0.9% NaCl, 0.2mg/ml human serum albumin; 0.05% NaN_3 in 500ml. Dissolved 4.5 g ; 250 mg NaN_3, 100 mg human serum albumin in 500 ml DI water; filter through 0.22 μm, GP Express PLUS Membrane; A pipette was used to added 0.25 ml NanoQ to each of 3 dialysis tubings. The end was tied rinsed with DI water before placing in autosampler vial filled with 3.5 ml releasing medium. The cap was parafilmed to incubate samples on a rocker at 37 $^{\circ}$C. The samples were transferred to 3.5 ml fresh releasing medium each day and UV absorbance were measured at 600nm. At 0 day-1/2: 125 μl NanoQ was mixed with 375 μl cleanascite^{TM} (HC prod# LGC1050; Lot#103061, from CPG Inc 800-362-2740) and incubated for ~30min at RT, centrifuged 10 min at 5000rpm. 250 μl of supernatant was taken and dissolved into 0.75 ml saline, then UV absorbance were recorded.

Inductively coupled plasma-optical emission spectroscopy: The copper content of NanoQ particles were analyzed by inductively coupled plasma-optical emission spectroscopy (ICP-OES) using Perkin Elmer Optima 7000DV. Briefly, the samples were digested with conc. Nitric acid, conc. Hydrochloric acid and hydrogen peroxide mixture using Multiwave 3000 microwave under high pressure. The clear solution obtained was analyzed by the Optima 7000DV instrument which was designed to handle sub-ppm to percent level copper concentrations.

In vitro human plasma clot phantoms: 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)[1] 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 NanoQ or control NanoQ (non-biotin) (30 μL/mL PBS) for 30 minutes. The untreated clots were treated similarly with PBS (30 μL/mL PBS).

Magnetic Resonance Imaging (MRI) measurements: All images and scans were acquired on a 1.5T and 3.0T clinical scanner (Achieva; Philips Medical Systems, Best, Netherlands) with a quadrature (transmit/receive) volume head coil. Relaxivity measurements were obtained for the NanoQ (Cu) and controls at 1.5T and 3.0 T using turbo spin-echo (SE) and inversion recovery (IR) techniques. NanoQ 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 1 mm x 1 mm x 10 mm was

used using 64 samples of the inversion recovery signal starting at 21ms and spaced at 30ms, with 6° sampling flip angle, (TE=1.62ms, TR=4s, 6 averages). Test tubes with 1 mL of NanoQ suspension (concentration ranging from 1:2 to 1:40) were placed vertically in a clinical MRI scanner and imaged in cross section (to reduce through plane partial volume effects). T2 was calculated from multi echo SE images with resolution = 1 x 1 x 10 mm^3, 15 echoes at 8 ms intervals, TR=750 ms, 2 averages. T1 measurements were calculated using the complex numbers reconstructed of the images collected with an IR technique described by Look and Locker[2](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: NanoQ test phantoms were prepared in snap cap tubes. Selected volumes (1:2, 1:4, 1:8, 1:10, 1:20, 1:40) of the NanoQ samples were added to the phantom tubes and diluted by adding deionized water.

Targeted clot phantoms: All images and scans were acquired on a 1.5T and 3.0T clinical scanner (Achieva; Philips Medical Systems, Best, Netherlands) with a quadrature

(transmit/receive) volume head coil. For target specific imaging, the clots, in saline, were imaged using T1-weighted techniques (TR=25ms, TE = 6.2ms (at 1.54T) or 3.9ms (at 3T), flip angle=30, resolution=0.5mm x 0.5mm x 1.0mm) at 1.5T and 3.0T.

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. NanoQ (Cu) was administered (1ml/kg; total volume) intravenously through tail vein catheter. Blood samples were collected at different time points for pK studies. The major organs were independently frozen, ground to tissue homogeneity, weighed, and the entire specimen was analyzed for copper content using ICP-OES. No adverse effects were observed in these animals during the in-life phase of the study, which reflected the lack of free copper bioavailability from cupric oleate.

References:

1. S. Raut, P. J. Gaffney, *Throm. Haemostasos* 1996, **76**, 56–64.

2. D. C. Look, D. R. Locker, *Rev. Sci. Instrum.*1970, **41**(2):621-627.