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

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Abstract

Based on a nanocolloidal suspension of lipid-encapsulated, organically-soluble bivalent copper, a new site-targeted molecular imaging contrast agent has been developed. Concentrating high payload of bivalent copper ions per nanoparticle, this agent provides a high per-particle r1

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SUPPORTING INFORMATION. Description of experimental methods and analytical measurements. This material is available free of charge via the Internet at <http://pubs.acs.org>.

relaxivity allowing sensitive detection on T1-weighted MRI as is demonstrated herein targeted to fibrin clots *in vitro*. The particle also exhibits a defined clearance and safety profile *in vivo*.

Atherosclerotic plaque rupture induces thrombus formation, which can progress immediately to myocardial infarction and stroke.^{1–2} However, in many instances plaque ruptures are small and transiently elicit local myocardial symptoms or embolize to induce transient ischemic attacks. Despite recent improvements in high spatial resolution MR carotid imaging techniques, the detection of subtle intimal ruptures and microthrombosis in atherosclerotic vessels is complicated and not routinely performed in the clinic. However, MR molecular imaging of microthrombosis using targeted contrast agents could offer sensitive detection and rapid diagnosis.³

To date two types of MRI contrast agents have garnered the most clinical attention: those based on iron or gadolinium. Certainly gadolinium molecular imaging agents offer the best opportunity to achieve bright (T1-weighted), sensitive contrast enhancement shortly after intravenous administration, in contradistinction to most T2*-weighted agents, which require 24 or more hours for background interference from persistently circulating iron particles to abate. Unfortunately, the discovery of nephrogenic systemic fibrosis has created safety questions for Gd-based agents, including the contraindication of use for individuals with significant renal dysfunction or after liver transplant.⁴ These concerns have triggered reconsideration of alternative approaches based on non-lanthanide metals for T1-weighted imaging, such as manganese (Mn).⁵

Copper (Cu) is a non-lanthanide metal, which has remained mostly unexplored as an MRI contrast agent. Copper is paramagnetic and has intrinsic properties favorable for MRI contrast enhancement, specifically an unpaired electron in its outermost orbital. Copper is a critical nutrient and serves as a cofactor for many biological processes and, generally, is well tolerated in humans.^{6a} There are only a few reports on bivalent copper intoxication inducing pathological changes in cultured human skeletal muscle cells, but the mechanism is unclear.^{6b} To the best of our knowledge, only a single report describing the development of a “hard” copper-based nanoparticle is available.⁷ In that work, a Cu³⁺ ion in combination with gold (Au₃Cu₁) as nanoshells and nanocapsules were reported as a blood pool MR contrast agent. (Proton relaxivity was estimated in terms of molar relaxivity based on the number of nanoparticles per mM $\sim 3 \times 10^4$ mM⁻¹ s⁻¹ for nanocapsules.) Unfortunately, hard particles (>10 nm) are poorly eliminated in humans and often are very slowly or never biometabolized, which creates bio-safety questions. Our objective was to develop and characterize a new “soft” nanoparticle-based contrast agent platform comprising the paramagnetic bivalent copper with high paramagnetic metal capacity for sensitive MRI detectability which is rapidly bio-eliminated, preventing the bodily accumulation of copper, which can elicit unintended adverse effects.

In this work, we report for the first time the synthesis and MR characterization of copper oleate nanocolloids (NanoQ) based on divalent copper (Cu²⁺). We hypothesized that fibrin-targeted NanoQ nanoparticles could deliver millions of copper atoms specifically to thrombus and exhibit strong T1w MR contrast enhancement of the target. Importantly, NanoQ was designed for vascular retention, based on particle size (i.e., >200 nm), to avoid undesired extravasation and targeting of healed intraplaque hemorrhages or other nonvascular fibrin deposits. Moreover, the copper atoms are uniquely sequestered within the core matrix, a non-intuitive decision for paramagnetic agents intended to influence the spins of water protons in the surrounding medium, but important to avoid potential adverse blood contact with blood constituents, particularly complement, and to eliminate possible interactions with homing ligands on the functionalized surface.

The surfactant mixture for the targeted nanoparticles was comprised of phosphatidylcholine (lecithin-egg PC, 99 mole% of lipid constituents), and biotin-caproyl-PE (1 mole%). To achieve a loading more than 2 w/v%, commercially available Cu(II)-oleate was suspended with sorbitan sesquioleate to produce a homogenous suspension of the inner matrix. Self-assembled NanoQ particles were formed by high-pressure homogenization (137 MPa, i.e., ~20,000 PSI $\times$ 4 minutes) at 4°C in presence of the aqueous dispersed surfactant mixture (Figure 1). Presence of the caproylbiotin moiety onto the NanoQ presented nominally 18,000 biotin molecules per nanoparticle, which were used for avidin-biotin coupling of the homing ligand to the nanoparticles in a demonstration of concept. The concentration of copper in the nanoparticle was determined by ICP-OES as 67.2mg/L of colloidal suspension of NanoQ corresponding to ~14,000 copper atoms per particle. A non-targeted control nanoparticle was synthesized in a similar way which excluded the incorporation of biotin in the surfactant mixture. For details on the synthesis and characterization please see supporting information.

Multiple analytical techniques were employed to characterize these particles. Hydrodynamic particle sizes for the biotinylated-NanoQ were 210 ± 6 nm with narrow distribution (polydispersity indexes, PDI: 0.17). The anhydrous state properties were determined by atomic force microscopy (AFM) analyses by drop depositing the aqueous suspension over a glass slide. The particle height was calculated to be 78 ± 12 nm. UV-Vis spectroscopy confirmed the absorbencies at 650–750 nm, corresponding to the presence of multiple Cu atoms in oleate complexes. The particle stability (with time and within a physiological pH range 5.9–9.5; Figure 1S, 2S) and successful phospholipids-encapsulation were confirmed based on light scattering measurements and the presence of negative electrophoretic potential (ζ) values, i.e., -15 mV. These uniquely constructed nanocolloids possess long shelf-life stability and retain the particle integrity for viable clinical translation. In order to test the stability of the copper binding in the particle, a dissolution experiment was undertaken. In this study, only a less than 2% total release was observed over 3 days against an infinite sink. These results indicate a loading efficiency of 98–99% with excellent retention in dissolution and without significant *in vitro* immediate release of copper in presence rabbit plasma, saline or human plasma albumin at 37 °C. The release of the copper oleate was monitored by UV spectroscopy at 600–700nm wavelength range. (see Figure 1d and supporting information stability data.) The study further confirms that copper is well retained within the particle membrane.

The MR properties of serially diluted NanoQ were characterized in triplicate. By adding ultrapure water, nine dilutions of the particles were prepared ranging from the concentration as prepared, i.e., 6.82×10^{-5} mM NanoQ to 4.24×10^{-7} mM NanoQ. The nanoparticle concentration was estimated from nominal particle size determined by laser light scattering and the total volume of polysorbate-Cu(II) incorporated into the NanoQ formulation. The dilutions were scanned at room temperature on clinical scanners with a transmit-and-receive birdcage head coil (Achieva, Philips Healthcare) to measure relaxation rates, R1 & R2. A single slice inversion recovery sequence (i.e., the Look-Locker technique)⁸ was employed to calculate the ionic (per mM copper) and particulate (per mM NanoQ) r_1 relaxivities at 1.5T and 3.0 T (resolution = $1 \times 1 \times 10$ mm³, 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). Similarly, r_2 relaxivity was measured using a multi echo-spin echo technique,⁹ resolution = $1 \times 1 \times 10$ mm³, 15 echoes at 8 ms intervals, TR=750 ms, 2 averages). The relaxivities (mean $\pm$ std.err.) for the agent based on Cu concentrations are $r_1 = 4.26 \pm 0.14$ mM⁻¹s⁻¹ and $r_2 = 8.7 \pm 0.18$ mM⁻¹s⁻¹ for 1.5T, and $r_1 = 4.02 \pm 0.19$ mM⁻¹s⁻¹ and $r_2 = 10.43 \pm 0.34$ mM⁻¹s⁻¹ for 3T. The ionic relaxivity values of NanoQ were comparable to commercially available gadolinium based contrast agents (e.g. magnevist, gadovist, multihance).¹⁰ The more relevant particulate relaxivities were $r_1 = 66000 \pm 2200$ mM⁻¹s⁻¹

and $r_2 = 135000 \pm 2900 \text{ mM}^{-1}\text{s}^{-1}$ for 1.5T, and $r_1 = 62000 \pm 3000 \text{ mM}^{-1}\text{s}^{-1}$ and $r_2 = 162000 \pm 5300 \text{ mM}^{-1}\text{s}^{-1}$ for 3T (Figure 2). The physical basis for the high particulate relaxivity of NanoQ is unproven, but hypothesized to reflect the intercalation of cupric oleate within the hydrophobic aspect of the phospholipid surfactant, which potentially affords an adequate paramagnetic influence on the immediately surrounding water medium.

To demonstrate the concept and effectiveness of fibrin-bound NanoQ, acellular fibrin clots were formed on a suture in saline (see supporting information) as a phantom target to which NanoQ was bound using a well-characterized and fibrin-specific (i.e., dog, human, pig) monoclonal antibody¹¹ and classic avidin-biotin chemistry. As controls, two clots were (i) not treated or (ii) treated with non-targeted contrast agent. The clots, in saline, were imaged using T1-weighted techniques (TR=25ms, TE = 6.2ms (at 1.5T) or 3.9ms (at 3T), flip angle=30, resolution=0.5mm × 0.5mm × 1.0mm) at 1.5T and 3.0T. Strong T1w signal enhancement was appreciated along the targeted phantom clot periphery. The particles were excluded by size from penetrating into the dense fibrin weave of the acellular thrombus, consistent with previous studies using this experimental model. In contradistinction, the phantom clot was essentially unseen following exposure to the nontargeted agent or saline alone, confirming that the enhancement was the result of the bound NanoQ alone (approx. corresponds to 400% signal enhancement over controls) (Figure 3). The quantitative MR measurement (as signal intensity (SNR) normalized to the SNR of the surrounding fluid) of the fibrin clots targeted with NanoQ presented homogeneous T1w contrast enhancement with signal intensities (at 1.5T): $1.41 \pm 0.28 \text{ a.u.}$; $1.08 \pm 0.06 \text{ a.u.}$ and $1.01 \pm 0.03 \text{ a.u.}$, respectively for targeted NanoQ, non-targeted NanoQ and un-treated clot. The results from 3T were similar and resulted in T1w contrast enhancement with signal intensities: $1.38 \pm 0.17 \text{ a.u.}$; $1.09 \pm 0.07 \text{ a.u.}$ and $0.99 \pm 0.09 \text{ a.u.}$, respectively for targeted NanoQ, non-targeted NanoQ and un-treated clot. The normalized signal intensity of the surrounding medium (nanopure water) was $1.00 \pm 0.06 \text{ a.u.}$ at both 1.5T and 3T.

Pharmacokinetic, biodistribution, and bioelimination of NanoQ was evaluated in rodents. Following intravenous administration, the concentration of copper in serial blood samples was determined by ICP-OES over time (Figure 4A). In all animals (n=3), a standard two compartment biexponential model fit the data well, with $R^2 > 0.99$. The closed-form solution to this model is the well-described: $C(t) = Ae^{-\alpha t} + Be^{-\beta t}$ with constants A and α describing the distribution phase, and B and β describing the blood clearance phase.¹² The half-life for the distribution phase was $5.04 \pm 1.1 \text{ min.}$ (mean $\pm$ standard error), while the elimination half-life was $99.2 \pm 10.7 \text{ min.}$

To define the *in vivo* biodistribution and acute safety profile in rats, copper-loaded NanoQ particles were injected intravenously at 1 ml (20 volume% emulsion)/kg body weight. Tests for free hemoglobin in the urine with urine strips (Vetstrip; ARJ Medical) revealed no evidence of marked hemolysis. At two hours post NanoQ injection, kidney and feces accumulated 16.9 ± 2.4 and $11.6 \pm 0.5 \text{ \%ID/g}$ of copper, whereas lung and liver took up 10.9 ± 2.0 and $11.2 \pm 1.8 \text{ \%ID/g}$ of copper of NanoQ. Spleen, heart, muscle and lymph accumulated smaller amounts of NanoQ as well (3.7 ± 0.5 (spleen), 5.1 ± 0.5 (heart), 2.8 ± 0.9 (muscle) and $2.5 \pm 0.5 \text{ \%ID/g}$ of NanoQ). Interestingly, some accumulation of NanoQ has been noticed at 2h in lung, which is typically not observed in similar systems. Further detailed investigation is warranted to study the *in vivo* biodistributive properties of these particles. At twenty four hours after NanoQ injection, feces and kidney were the sites of major accumulation (Figure 4B). Almost all other organs showed negligible copper content at that time. These data suggest NanoQ presumably follows a typical biodistribution of particles into the reticuloendothelial system, but the copper organocomplexes were rapidly bio-eliminated primarily through both renal and biliary routes, suggested by the metal concentrations of the kidney and feces. Collectively, these data indicate that NanoQ has an

optimal circulatory half-life for targeting intravascular thrombus. The particle is cleared from blood as expected through normal RES organs and the copper metal complexes are rapidly eliminated from the body.

In conclusion, we report an efficient, commercially amenable synthesis of paramagnetic bivalent copper nanocolloid targeted to fibrin. The incorporation of copper as a metal complex allowed very high loading without resorting to the use of large, hard solid metal particles, which greatly contributed to the desired pharmacokinetic, biodistribution, and bioelimination profiles in rats. The agent provided strong T1w contrast in vitro that exhibited nearly three times higher longitudinal relaxivity as compared to previously reported gold-copper alloy nanoparticles. The high relaxivity and detection sensitivity of the copper nanocolloids are in the low nanomolar range, which point towards the potential utility of these agents for detection of microthrombi. NanoQ offers an effective, non-gadolinium based T1w molecular imaging agent that could be applied to the diagnosis of ruptured unstable plaque in accessible vascular structures such as carotid arteries, which might be useful for delineating unstable lesions in acute vascular syndromes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

a) Synthesis and characterization of NanoQ. i) Cu(II)-oleate (anhydrous chloroform solution) suspended with sorbitan sesquioleate (>2 w/v%), vortex, mixing, evaporation of chloroform under reduced pressure, 45 °C; ii) thin film formation from phospholipids mixture at 40 °C; dried under vacuum overnight at 45 °C; iii) self-assembly by homogenization at 20,000 psi (137.9 MPa), 4 min at 0 °C; b) number averaged hydrodynamic distribution of NanoQ from dynamic light scattering measurements in aqueous state; c) atomic force microscopy (AFM) image of biotinylated NanoQ in anhydrous state drop deposited over glass; d) dissolution of copper from NanoQ against infinite sink over three days at 37°C.

Figure 2.

Relaxivity measurements of NanoQ. Relaxivities, r_1 and r_2 , are calculated from the measured relaxation rates as a function of contrast agent concentration. The graph (A) is the data for longitudinal relaxation rate (R_1) and transverse relaxation rate (R_2) at 1.5T in replicate. Below, the image (B) shows the T1-weighted MRI of the contrast agent diluted in water from lowest concentration tested (left) to highest (right).

Figure 3.

MRI detection of fibrin clots *in vitro*. On T1-weighted cross-sectional images, the clot with Targeted NanoQ (A) shows marked signal enhancement, whereas the controls of Non-Targeted Contrast Agent (B) or No Treatment (C) show little or no enhancement above the background water signal. (D) Normalized contrast-to-noise measurements of targeted and control NanoQ bound to clots with respect to the surrounding fluid.

Figure 4.

In vivo pharmacokinetics and bio-distribution of NanoQ. (A) pharmacokinetic profile of targeted NanoQ with a bi-exponential fitting ($y=0.5903*\exp(-0.1374*t) + 0.5205*\exp(-0.0070*t)$). (B) Organ distribution of NanoQ based on copper estimation of major organs by ICP-OES at 2h and 24h following NanoQ injection (1mg/ml) i.v.