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Second Generation Gold Nanobeacons for Robust K-Edge Imaging with Multi-Energy CT

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Abstract

Spectral CT is the newest advancement in CT imaging technology, which enhances traditional CT images with the capability to image and quantify certain elements based on their distinctive K-edge energies. K-edge imaging feature recognizes high accumulations of targeted elements and presents them as colorized voxels against the normal grayscale X-ray background offering promise to overcome the relatively low inherent contrast within soft tissue and distinguish the high attenuation of calcium from contrast enhanced targets. Towards this aim, second generation gold nanobeacons (GNB₂), which incorporate at least five times more metal than the previous generation was developed. The particles were synthesized as lipid-encapsulated, vascularity constrained (>120 nm) nanoparticle incorporating tiny gold nanoparticles (2–4 nm) within a polysorbate core. The choice of core material dictated to achieve a higher metal loading. The particles were thoroughly characterized by physicochemical techniques. This study reports one of the earlier examples of spectral CT imaging with gold nanoparticles demonstrating the potential for targeted *in vitro* and *in vivo* imaging and eliminates calcium interference with CT. The use of statistical image reconstruction shows high SNR may allow dose reduction and/or faster scan times.

1. Introduction

Recent advances computed tomography towards energy-resolving imaging systems have garnered considerable interest. In particular the advent of photon-counting detectors with fast readout electronics has added a new dimension to computerized tomography. One exciting new imaging technique is referred to as K-edge imaging. While conventional CT provides information about the overall attenuation of the emitted x-ray beam, photon counting detectors allow retrieving spectral information across the bandwidth of the emitted photons. With this additional information it is possible to discriminate between different physical effects caused by the scanned object.^{1–4} Of particular interest is the capability to quantify certain elements utilizing their K-edge in the absorption spectrum. Elements with high atomic numbers such as bismuth (Z = 83), gold (Z = 79), and gadolinium (Z = 64), have their K-edge located well within the X-ray energy bandwidth of diagnostic CT, which

is typically between 20 and 150 keV, and are not profuse in humans naturally, thus lend themselves ideal as contrast enhancing substances.^{5–7} In short, K-edge imaging enables selective and quantitative imaging of administered contrast agents equipped with appropriate K-edge materials, making spectral CT with its high spatiotemporal resolution a quantitative molecular imaging technology.

We^{8–9} and others¹⁰ have recently shown that spectral CT in combination with suitably targeted contrast agent can be used successfully to visualize coronary artery plaques vulnerable to rupture. It is now well established that ruptured atherosclerotic plaques are the foundation of 70% of heart attacks affecting millions all over the world.^{11–12} We have demonstrated that nanometer sized particles carrying K-edge metal bismuth can be homed to thrombi and using a prototype spectral CT scanner illuminate the location of the vulnerable plaques *in vivo*.⁸ A number of other heavy metals for K-edge imaging have also been investigated, in phantom study, e.g. iodine ($Z = 53$) having a K-edge energy $E_K = 33.2$ keV and gadolinium.¹³ Although iodine is the basis of conventional blood pool CT contrast agents, for spectral CT applications in humans the low K-edge energy of iodine and the corresponding photon starvation in the surrounding energy range is foreseen to yield a K-edge image with poor noise properties. Our preliminary results with vascular-constrained bismuth nanoparticles (c.a. 160 nm) are promising and we have had early success in imaging *in vitro*, *ex vivo* and *in vivo* targets. However, with a high K-edge energy of 89.5 keV, the absorption profile of bismuth is less favorable for K-edge imaging than gold ($Z = 79$) which has been historically used as an x-ray contrast agent and with a K-edge energy at 80.7 keV. While gold nanoparticles have been considered previously as blood pool and nontargeted CT imaging agents,^{14–16} the particle size of the metal component were uniformly greater than the normal renal filtration threshold (i.e., 6 nm), posing potential bio-elimination issues and related regulatory approval challenges. Their whole body elimination is also questionable and has not been explored heavily. The goal of the present study is to introduce a new class of molecular imaging agents, based on second generation gold (Au) Nanobeacon (GNB₂), of “hybrid” organic-inorganic nature, to detect microthrombus in ruptured atherosclerotic plaque in combination with an advanced statistical image reconstruction method for improved signal-to-noise ratio (SNR).

2. Experimental Section

2.1. 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 was distilled over calcium hydride prior to use. Biotinylated dipalmitoyl-phosphatidylethanolamine and high purity egg yolk phosphatidylcholine were purchased from Avanti Polar Lipids, Inc. Gold nanoparticle was obtained from Ocean NanoTech, Inc. Argon and nitrogen (UHP, 99.99%) were used for storage of materials. The Spectra/Por membrane (Cellulose MWCO: 20 000 and 50 000 Da) used for dialysis was obtained from Spectrum Medical Industries, Inc. (Laguna Hills, CA).

2.2 Typical procedure for preparation of control nanobeacon

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 is 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 is 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 is continuously processed

thereafter at 20,000 PSI for 4 minutes with an S110 Microfluidics emulsifier (Microfluidics) at 4°C. The nanobeacons are 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 nanobeacons are stored under argon atmosphere typically at 4°C. DLS (D_{av})/ nm = 165 ± 15 nm; Zeta (ζ)/ mV = -26 ± 05 mV.

2.3 Preparation of GNB₂

In a typical experimental procedure, octanethiol coated gold nanoparticles (2–4 nm, 2% w/v) in toluene (100 mg) are suspended in sorbitan sesquioleate (4 mL, 2 mole%) and vigorously vortexed to homogeneity. The suspension was filtered through a small bed of cotton. The solvent was evaporated under reduced pressure at 45°C. The surfactant co-mixture included high purity egg yolk phosphatidylcholine (91 mole%, 380 mg), cholesterol (8 mole%, 17.39 mg), and biotinylated-dipalmitoyl phosphatidylethanolamine (1 mole%, 6.2 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 gold nanoparticle-suspended sorbitan sesquioleate mixture (20% v/v), distilled de-ionized water (15.23 mL, 77.3% w/v), and glycerin (0.37 mL, 1.7%, w/v). The mixture was continuously processed thereafter at 20,000 PSI for 4 minutes with an S110 Microfluidics emulsifier (Microfluidics) at 4°C. The nanobeacons 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 nanobeacons were stored under an argon atmosphere typically at 4°C. DLS (D_{av})/ nm = 92 ± 13 nm; Zeta (ζ)/ mV = -55 ± 14 mV; AFM (H_{av})/ nm = 45 ± 10 nm, ICP-MS = 138 ± 11 of gold /g of 20% colloidal suspension.

2.4 Post preparative modification of GNB₂ for spectral CT imaging

GNB₂ synthesized as above were aliquoted in centrifuged tubes centrifuged at 40,000 rpm and 4°C for 20–30 min. Samples were removed from the rotor and the concentrated GNB₂ was collected carefully from the bottom. Supernatant was removed manually using a pipette. Sample was visually inspected and the heavier, denser particles with higher gold loading were removed from the supernatant comprising of lighter particles or particles with no metal.

2.5 Preparation of GNB₁

In a typical experimental procedure, gold nanoparticles (2–4 nm) in toluene (100 mg) are suspended in almond oil (4 mL) and vigorously vortexed to homogeneity. The suspension was filtered through a small bed of cotton. The solvent was evaporated under reduced pressure at 45°C. The surfactant co-mixture included high purity egg yolk phosphatidylcholine (91 mole%, 377.4 mg), cholesterol (8 mole%, 16.9 mg), and biotinylated-dipalmitoyl phosphatidylethanolamine (1 mole%, 5.8 mg). The surfactant co-mixture is dissolved in chloroform, evaporated under reduced pressure, dried in a 40°C vacuum oven overnight, and dispersed into water by probe sonication. This suspension is combined with the gold nanoparticle-suspended almond oil mixture (20% v/v), distilled de-ionized water (77.3% w/v), and glycerin (1.7%, w/v). The mixture is continuously processed thereafter at 20,000 PSI for 4 minutes with an S110 Microfluidics emulsifier (Microfluidics) at 4°C. The nanobeacons are 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 nanobeacons are stored under an argon atmosphere typically at 4°C. DLS (D_{av})/ nm = 154 ± 06 nm; Zeta ζ = -47 ± 08 mV; AFM (H_{av})/ nm = 101 ± 51 nm.

2.6 Preparation of GNB (rod)

GNB (rod) particles were synthesized similarly by incorporating gold nanorods (axial diameter: 25 ± 5 nm, length: 80 ± 8 nm). The surfactant co-mixture included high purity egg yolk phosphatidylcholine (90 mole%, 559 mg), cholesterol (8 mole%, 21 mg), and biotinylated-dipalmitoyl phosphatidylethanolamine (2 mole%, 15 mg). The mixture was microfluidized at 20,000 psi for 4 min with an S 110 Microfluidics emulsifier (Microfluidics) at 4 °C. The nanobeacons were purified further by dialysis against water using a 50,000 Da MWCO cellulose membrane for a prolonged period of time. DLS (D_{av})/nm : 139 ± 10 nm; Zeta (ζ) : -41 ± 12 mV; AFM (H_{av})/nm: 60 ± 20 nm; ICP-MS = 8.1 μ g of gold/g.

2.7 Dynamic light scattering measurements

Hydrodynamic diameter distribution and distribution averages for the GNB 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 GNB suspensions into deionized water (0.2 μ M). Nanobeacons were dialyzed into water prior to analysis. Scattered light was collected at a fixed angle of 90°. A photomultiplier aperture of 400 nm 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.

2.8 Electrophoretic potential measurements

Zeta potential (ζ) values for the GNB₂ 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 GNB 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: $\mu = \epsilon \zeta / \eta$, 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.

2.9 UV-visible Spectroscopy

Absorption measurements were made with a Shimadzu UV-1601 P/N 206–67001 spectrophotometer using Shimadzu-UV probe 2.21 software.

2.10 Transmission Electron Microscopy Measurements

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.

To image GNB, nanoparticles were first fixed with 2.5% glutaraldehyde (Electron Microscopy Sciences) in PBS for 30 min on ice and were spun at high speed in a table top microfuge to form a pellet. After a thorough rinsing, the pellet was sequentially stained with

osmium tetroxide, tannic acid (Sigma-Aldrich), and uranyl acetate, dehydrated and embedded in Polybed 812 (Polysciences). Tissue was thin sectioned on a Reichert-Jung Ultracut, post-stained in uranyl acetate and lead citrate, viewed on a Zeiss 902 electron microscope, and recorded with Kodak E.M. film.

2.11 Atomic Force Microscopy Measurements

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 GNB 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 $\mu\text{m/s}$ for 2 μm), (15 $\mu\text{m/s}$ for 5 μm), (30 $\mu\text{m/s}$ for 10 μm). 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 100 particles from three micrographs.

2.12 Inductively coupled plasma-optical emission spectroscopy

After imaging, the gold content of GNB₂ 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.

2.13 *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)²³ 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 $\mu\text{g/mL}$ PBS) for 30 minutes, followed by biotinylated GNB (30 $\mu\text{L/mL}$ PBS) for 30 minutes. The control clots were treated similarly with control nanoparticle (30 $\mu\text{L/mL}$ PBS).

2.14 *In vivo* imaging

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 body weights ranging from 200–300 g were used for the *in vivo* studies. Initial anesthetization of the rat was done using a mixture of ketamine (85 mg/kg) and xylazine (15 mg/kg). The animal was maintained with 1.5–3% isoflurane in oxygen on a ventilator for approximately 2 h. Spectral CT images were acquired after the administration of GNB₂. 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. Given the long scan durations preventing *in-vivo* imaging under sedation, the animal was scanned immediately after euthanization.

2.15 Spectral CT Imaging

The spectral CT prototype scanner (Philips Healthcare, Eindhoven, The Netherlands) employed in our studies has been previously reported^{5–6} for imaging phantoms. The scan parameters were set to: tube voltage 130 kV, tube current 50 μ A, threshold energies 25–33–50–81–91–110 keV, views per turn 1250, rotation time 100s, slice increment 0.33 mm. The acquired energy-resolved data was decomposed into the basis components photoelectric effect, Compton effect and gold. For reconstruction of the separated gold signal, a recently reported 2D iterative reconstruction technique (ML-GV) was used for improved image quality.²¹ Reconstruction parameters were: FOV 60×60 mm², pixel size 0.1×0.1 mm².

3. Results and Discussion

Very recently we have synthesized and reported gold nanobeacons (GNB) by incorporating tiny 2–4 nm sized octanethiol-coated gold nanoparticles within a bigger vascularly-constrained nanoparticle, stabilized by phospholipids. By the virtue of their unique optical properties in the near infrared region, these nanoparticles were used for sensitive imaging with photoacoustic tomography (PAT).¹⁷ In a series of works we demonstrated the potential of this platform for imaging atherosclerosis,¹⁷ sentinel lymphnodes¹⁸ and for the first time finding nascent sprouting angiogenic blood vessels.¹⁹ The design of the nanoparticle allowed us to incorporate 2 w/v% of gold nanoparticles suspended within a vegetable oil core matrix. Although CT imaging with gold particles have been demonstrated recently by other investigators^{20–22}, this platform represents a unique blend of “hybrid” nanomaterial of organic-inorganic nature. We presume that the encapsulation of multiple copies of tiny gold nanoparticles will help to further amplify the radioopacity offered by an individual gold particle. The encapsulation with phospholipids surface coating will ensure the biocompatibility of the platform and the small particle diameter of the gold nanoparticles will promise a rapid clearance. The enhanced optoacoustic sensitivity can be attributed to the presence and close interaction of multiple copies of tiny gold particles encased within the larger nanoparticle. These particles also exhibited remarkable shelf-life stability and a defined biological clearance.^{17–18}

The promising results with the first generation gold nanobeacons prompted us to use them for the spectral CT application. In the first phase of our experiment, the potential of targeted spectral CT imaging with first generation GNB₁ [D_{av} (DLS): av. Diameter 150 ± 20 nm, ICP-OES: ~6000 Au atoms / nanobeacons] was tested *in vitro*. (Figure 1a) However, even with maximum GNB₁ (600 nM), *in vitro* spectral CT imaging was not successful (see supporting information Figure 1S for more details). The study produced overall unsatisfactory spectral CT detection sensitivity. The result was not unexpected and it was realized that for effective molecular imaging, nanoparticles designed for CT must incorporate and deliver very high concentration of elements, at least 5×10^5 metal atoms per nanoparticle (e.g., gold atoms) for a 200 nm -sized agent.⁸

Our preliminary methodology to resolve this issue was to encapsulate higher (>2 w/v%) amount of metal within the vegetable oil (almond oil) core of the colloidal nanobeacons. In short, the synthesis process entails the suspension of AuNPs (2–4 nm, 10 w/v%) in peanut oil and then microfluidized as a 20% (v/v) colloidal suspension with 2% (v/v) phospholipids surfactant in nanopure (0.2 μ m) water (Figure 1b). This synthesis resulted in micron sized particles [D_{av} (DLS): 1020 ± 320 nm] with very high polydispersity index (PDI = 0.72) and low zeta potential ($\zeta = -2$ mV), indicating an overall poor colloidal stability of the system.

The ineffective outcome impelled us to adopt a unique approach, which is based on the co-self-assembly of phospholipids and polysorbate in aqueous media to entrap high payloads of gold. (Figure 1c) The alternative approach followed a two step procedure. In the initial step,

oleate coated gold nanoparticles were synthesized following a modified procedure. Aqueous solutions of gold nanoparticles (2–4 nm) were obtained from Ocean NanoTech, Inc. Sodium oleate (2.2 μL of a 9.3 mM solution) was added to gold nanoparticles (1 mL, 0.8 OD at $\lambda_{\text{max}} = 520 \text{ nm}$ in H_2O) and stirred for 30 min at room temperature. In the final step, oleate coated AuNPs (10% w/v of inner matrix) were suspended within a polysorbate (sorbitan sesquioleate (20 vol%) core matrix and homogenized with the surfactant mixture at 20,000 psi (141 MPa) for 4 min, at 4°C to produce second generation gold nanobeacons (GNB₂). The surfactant mixture was comprised of phosphatidylcholine (lecithin-egg PC), (99 mol%), dipalmitoyl phosphatidylethanolamine caproyl biotin (approx. 1.0 mol% w/v, PE-biotin) for *in vitro* avidin–biotin coupling of homing ligands. GNB₂ was purified by exhaustive dialysis through 20 kDa MWCO cellulosic membrane against water. The Microfluidics emulsifier (Microfluidics) is capable of scaling up the formulation of as-synthesized GNB₂ from hundreds of milliliter up to a liter. As shown in Figure 1c, GNB₂ underwent a post preparative modification. Nanoparticles were aliquoted in centrifuged tubes and subjected to centrifugation at 40,000 rpm and 4°C for 20 min. Samples were removed from the rotor and the concentrated GNB₂ was collected carefully from the bottom. The sample was visually inspected and the heavier, denser particles with higher gold loading were removed from the supernatant comprising of lighter particles or particles with no metal. This unique post preparative modification helped us to further concentrate the nanoparticles for spectral CT application.

The hydrodynamic diameter D_{av} of GNB₂ was determined in hydrated state by dynamic light-scattering (DLS) measurements. The number averaged hydrodynamic diameters of control nanobeacons (no gold) and GNB₂ were $165 \pm 15 \text{ nm}$ and $92 \pm 13 \text{ nm}$, respectively. (Figure 1d) Electrophoretic potential values (ζ) of the nanocolloids were $-24 \pm 9 \text{ mV}$ and $-55 \pm 14 \text{ mV}$, respectively (Brookhaven Instrument Co.) indicating a stable surfactant coating around the core matrix. Gold content, determined by ICP-OES, was $138 \pm 11 \mu\text{g.g}^{-1}$ of the 20% colloid suspension, which corresponds to $\sim 580,000$ gold atoms/nanobeacons. Particles in suspension were drop-deposited over nickel grids and negatively stained with uranyl acetate and observed to be spherical in their anhydrous state by transmission electron microscopy (Figure 2e–f). Transmission electron microscopy confirmed that the oleate coated gold nanoparticles were exclusively retained in the hydrophobic phase rather than adhered to the outer surface or free within the hydrophilic phase. (Figure 2d–g) It is important to note that to simultaneously stain the phospholipids outer layer and the encapsulated gold nanoparticles within the particles, nanoparticles were first fixed with glutaraldehyde and was sequentially stained with osmium tetroxide, tannic acid and uranyl acetate. The particle height obtained from dried-state atomic force microscopy (AFM) images was $45 \pm 10 \text{ nm}$. (Figure 2i) The presence of surface plasmon band was detected by UV-vis spectroscopy at 520 nm, which is the characteristics of gold nanoparticles and further confirms their presence. (Figure 2h) Interestingly, our attempt to incorporate bigger sized gold nanoparticle resulted in an overall similar sized nanocolloids ($\sim 130 \text{ nm}$) with less number of gold particles inside. Hydrophobically coated gold nanorods (GNR; axial diameter from TEM: $25 \pm 5 \text{ nm}$, length: $80 + 8 \text{ nm}$) were incorporated following a similar protocol as mentioned above. This procedure resulted in an entrapment of ~ 100 gold atoms (ICP-MS: $8.1 \mu\text{g}$ of gold/g). Figure 2k clearly indicated the presence of multiple GNRs within the phospholipids encapsulated particles. AFM image (Figure 2j) confirmed the spherical nature of the particles. The overall stability of the GNB₂ particles was monitored over 3 months by observing the changes in particle hydrodynamic diameter and zeta potential. Only $<3\%$ change in the particle diameter, polydispersity and zeta potential were observed when these particles are preserved under argon atmosphere at 4°C . (Figure 2S)

Spectral CT imaging experiments were performed with GNB₂ particles in suspension (20% v/v). Centrifuge tubes filled with serially diluted GNB₂ particles (in water) were scanned.

Figure 3a shows a representative conventional CT image of a cross-section generated from the spectral data. Varying x-ray absorption/CT numbers as a function of concentration generated from centrifuge tubes (internal diameter, i.d., 2.8 mm; outer diameter, o.d., 4.9 mm; vol. 0.34 cc) filled with GNB₂ (gold) with varying concentration of metal and CaCl₂ is clearly demonstrated. In Figure 2b, the K-edge contrast from gold is readily separated and, thus, provides a quantitative image of the GNB₂ particles. GNB₂ provided much higher contrast in comparison to the other gold nanoparticles previously used for spectral computed tomographic application.¹⁰ Spectral CT is quantitative and correspondingly the K-edge image values of GNB₂ formulations varied linearly as a function of metal concentration ($r^2=0.9944$) as estimated from the four regions in Fig. 3b.

For *in vitro* targeting, biotinylated GNB₂ (~150 nm) were designed and synthesized by concentrating a high payload of Au nanoparticles (2–3 nm) within a vascularly constrained bigger particle. As written above, a flat circular container (Figure 4a) was created by suspending acellular fibrin-rich clots in agarose contained in sealed polystyrene test tubes (75 mm). Fibrin-rich clots were targeted with biotinylated GNB₂ (n=3) or the control nanocolloids (n=3) using a well-characterized fibrin-specific monoclonal antibody (NIB5F3).^{23,25} In a separate tube CaCl₂ (500 mM) was introduced to simulate arterial wall calcifications, a common feature of plaque complicating traditional CT coronary imaging applications. CaCl₂ probes also should provide spectral attenuation effects similar to rib cage and spine. Spectral CT readily detected the targeted GNB₂ bound at physiologic fibrin presentation density. K-edge images were formed from material-specific sinograms using iterative image reconstruction (ML-GV).²⁴ The figure shows a conventional CT image of the phantom (a) and gold-specific image using ML-GV after 25 iterations (b). By virtue of its vascular-constrained nature, GNB₂ precluded deep penetration through the tight weave of fibrin fibrils, which is uniform with prior examples in this phantom model.²⁵

The comparative sensitivity of spectral CT using conventional filtered Back projection versus iterative reconstruction technique ML-GV was further evaluated in a non targeted *in vitro* imaging of acellular clot in a dedicated rat phantom, see Fig. 5. The phantom consists of a circular body (50 mm diameter) made of polymethyl methacrylate (PMMA) polymer mimicking attenuation of soft tissue and tubes in the periphery filled with CaCl₂ (3 mM) resembling the attenuation of the rib cage. A tube containing the clot pre-admixed with a small portion of the non targeted GNB₂ (50 μ l), is inserted in the center of the phantom. The conventional CT image (a) shows the complete phantom with an increase in signal due to GNB particles (in set box) while only the gold signal can be appreciated in the decomposed gold image using spectral CT in Figure 5b.

We further investigated whether GNB₂ could be used for sentinel lymph node delivery to improve cancer staging. Rats (n=3) were injected intradermally (foot paw) with 150 μ l GNB₂ (2 ml/kg) and imaged at 2 h after injection. The position of the lymph node and injection site is indicated by the arrows in Figure 6a. Regional sentinel lymph nodes were clearly contrasted (Figure 6b) in conventional CT images and selectively imaged with the K-edge method. As shown in Figure 6c, the strong signal of accumulated gold in the lymph node was selectively imaged with spectral CT using iterative reconstruction and proofed a complete separation from the bone or soft tissue.

4. Conclusion

This study reports one of the earlier examples of spectral CT imaging with gold nanoparticles demonstrating the potential for *in vivo* imaging and eliminating calcium interference with CT. The use of statistical image reconstruction shows high SNR which may allow dose reduction and/or faster scan times. With this preliminary data, additional

studies, including in-depth pharmacokinetics, bio-distribution and clearance behaviour and time-course histological examination of susceptible organs will be warranted to fully assess the safety profile of second generation gold nanoparticles.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Synthesis and post-preparative modification of GNB: (a) previous synthetic approach to first generation GNB particles from octanethiol coated AuNP with a final gold loading c.a. 2 w/v %: (i) gold nanoparticles suspended in vegetable oil, (ii) preparation of phospholipids thin film, (iii) microfluidization of gold nanoparticle-vegetable oil with surfactants in water; (b) new strategy to increase final gold loading up to 10 w/v%: oleate coated gold nanoparticles were suspended in sorbitan sesquioleate and microfluidized with phospholipids thin film mixture as a 2-0-20 formulation. reaction condition: (i) gold nanoparticles suspended in polysorbate, (ii) preparation of phospholipids thin film, (iii) microfluidization of gold nanoparticle-polysorbate with surfactants in water; 20,000 psi (141 MPa), 4°C, 4 min; (d) schematic representation of the procedure to concentrate GNB₂ prior to spectral CT imaging.

Fig. 2. Characterization of GNB: (a) number-averaged hydrodynamic diameter of GNB₂ from dynamic light scattering measurements; TEM images of (b) oleate coated gold nanoparticles, scale bar= 20 nm, (c) control nanobeacons with no gold incorporated, scale bar= 100 nm, (d) first generation GNB (scale bar= 100 nm) and (e–g) second generation GNB revealing the gold clusters entrapped inside the phospholipids membrane (scale bar= 100 nm); (h) UV-vis spectrum of GNB₂ in water confirming the presence of gold nanoparticle cluster and their surface plasmon resonance. AFM images of GNB₂ (i) and GNB (j, rod); (k) TEM image of GNB (rod) showing the presence of discrete gold rod nanoparticles of larger dimension within a phospholipids encapsulation (scale bar= 100 nm).

Fig. 3.

Spectral CT imaging of a rodent phantom with serially diluted GNB₂ particles: (a) CT opacity as a function of concentration generated from centrifuge tubes (internal diameter, i.d. 2.8 mm; outer diameter, o.d. 4.9 mm; vol, 0.34 cc) filled with GNB₂ (gold) with varying concentration of metal and CaCl₂; (b) cross sectional gold K-edge images of the same ROI; (c) variation of the image values (Hounsfield unit) in (b) (gold, 10 w/v %).

Fig. 4.

In vitro targeting: (a) a cartoon showing the phantom set up and the position of the targeted clots and calcium; (b) representative spectral CT images of human plasma clots treated with GNB₂ (note, both calcium and gold signals are prominent); (c) K-edge contrast of the gold is completely separated from calcium signal. The clots treated with control GNB₂ particles are impossible to distinguish from the surrounding water (c, scale bar 10 mm).

Fig. 5. Sensitive *in vitro* targeted imaging with iterative reconstruction technique: (a) representative spectral CT images of a tiny human plasma clot admixed with GNB₂ (note, the bright gold signals at the periphery of the tube is prominent in the photoelectric image); (b) K-edge image shows high contrast of gold to background noise.

Fig. 6.

In vivo noninvasive spectral CT imaging of sentinel lymph nodes. (a) A cartoon illustrating the site of injection and the area of interest. 150 μL of nanobeacons were injected intradermally in all the cases; (b) regional sentinel lymph nodes were clearly contrasted in conventional CT; (c) K-edge contrast of accumulated gold in the lymph node was selectively imaged with spectral CT.