Nanomedicine: Engineering of a Tri-Imageable Nanoparticle for Cancer Diagnostics

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ABSTRACT

We have created a potential targeted drug delivery platform with three imaging reporters by coupling the magnetic properties of USPIOs with near infrared fluorescence of Cy5.5 and γ-emissions of ^{111}In that is chelated to a conjugated antibody. The nanoparticle will allow for not only triple verification of localization, but also quantification. During each phase of development, the nanoparticles have been characterized for surface charge and structure by transmission electron microscopy and dynamic light scattering. Magnetic properties including hysteresis measurements and field cooling analyses were conducted using a superconducting quantum interference device. *In vitro* analyses of flow cytometry and cell viability as well as *in vivo* imaging studies have been conducted.

Keywords: Cancer Diagnostics, Imaging, Nanoparticle, Tri-Imaging

1 INTRODUCTION

Ultrasmall superparamagnetic iron oxide nanoparticles (USPIOs) are a class of probes that provide strong T2 relaxation for magnetic resonance imaging (MRI) and can be useful in a number of biomedical applications: immunochromatographic tests, targeted drug delivery, localized thermotherapy, conversion of a pro-drug to its active form, and, of course, diagnostic imaging. While MRI provides exquisite anatomic resolution, limitations of using USPIOs for imaging are the concentration needed to achieve significant contrast and the inability to quantify the number of particles at the target. Other technologies such as optical imaging have the advantage of high spatial and temporal resolution but have limited depth penetration due to light diffusion through tissue. Imaging of radioisotopes using single photon emission computed tomography (SPECT) allows quantification but it lacks spatial and temporal resolution. Combining the three imaging techniques could provide a most effective diagnostic tool. An USPIO that is labeled by both a radioisotope and optical contrast agent would permit high resolution imaging and quantification with the ability to verify that the particle has reached its target through three imaging modalities. For *in vitro* studies, utilization of a fluorescent agent would provide convenience with typical analysis tools such as confocal microscopy and flow cytometry, whereas the magnetic properties would allow for ease of separation by use of a strong magnet. Particularly if it is optional or removable, having the third imaging agent, the radioisotope, could provide great advantages for *in vivo* studies, such as for quantification of delivered construct through biodistribution studies. Furthermore, targeting these particles with biomolecules such as antibodies would create a noninvasive reporting tool used to monitor biological responses and provide valuable information regarding physiology and pathophysiology.

In vivo applications of nanoparticles are greatly affected by their size and outer coating. Larger iron oxide particles with diameters ranging from 300nm to 3.5μm and that are coated with an insoluble layer are used to image the gastrointestinal tract [1]. Smaller particles are better for prolonged circulation and are often taken up by the reticuloendothelial system and rapidly appear in the liver and spleen [2]. Outer functionality plays a highly significant role in particle interaction with the *in vivo* environment. For instance, a strong negative charge is desired at physiological pH to imitate other biomolecules and prevent aggregation. Silica is a biocompatible material with silanol groups that give the particle surface a strong and stable negative charge across changes in pH or electrolyte concentrations [3]. These functional groups also allow for straightforward covalent linkage to other groups and ligands. A silica layer can preserve the intrinsic properties of USPIO cores by preventing degradation and aggregation of the inner core. Silica is well known for its optical transparency [4], and an advantage it offers is its controllable thickness. The work presented describes a novel nanoparticle optimized for biomedical application. USPIOs of 9nm diameter with 2-5nm thin layers of silica embedded with optical dye have been synthesized, characterized, and attached to chelated antibody.

2 SYNTHESIS

Based on mechanisms described by LaMer [5] and Massert [6], USPIOs were synthesized by co-precipitation of ferrous and ferric salts in alkaline and acidic aqueous solutions. The iron oxide cores were then coated with thin layers of silica using the Stöber method [7] where

tetraorthoxysilane (TEOS) was hydrolyzed, followed by condensation of the alcohol and water [8]. By using catalysts such as ammonia and carefully controlling reagent ratios, reaction volume, pH, and reaction time, silica deposition can be controlled to form anything in the range from 200nm silica spheres embedded with a number of USPIOs to single USPIOs coated with 2nm-thick layers of silica. The linker 3-aminopropyltriethoxysilane (APTES) was used to covalently link and embed Cy5.5 within the silica shell of the particles.

To conjugate antibody (Ab) to the surface of the fluorescent silica-coated iron oxide nanoparticles (SCION), the particles were maleimide-functionalized with a conjugate of APTES and sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (s-SMCC). The antibodies were activated by conjugation to Traut's reagent [9], yielding approximately 1.8 thiols per antibody. The particle maleimides then formed strong covalent bonds with the antibody thiols. A number of antibodies have successfully been conjugated to the particles including L243, trastuzamab (Herceptin), and cetuximab (Erbitux). L243 is an anti–HLA-DR monoclonal antibody (mAb) that could be used to direct the particles to cells from the inflammatory foci in the brain of multiple sclerosis patients [10, 11]. Herceptin and Erbitux respectively target HER2 and HER1, membrane bound receptors that are over-expressed in a variety of epithelial cancers, including breast, ovarian, pancreatic, and colorectal carcinomas [12, 13]. The chemistry used for ligand conjugation can be applied to other proteins and biomolecules as well.

The third imaging modality was attached using the chelate 2-(p-isothiocyanatobenzyl)-cyclohexyl-diethyl enetriaminepentaacetic acid (CHX-DTPA). Chelate isomer CHXA"-DTPA provides efficient labeling with ^{111}In and demonstrates maintenance of integrity and immunoreactivity of its radioimmunoconjugates [14, 15]. In the case of particles that were being radiolabeled, the chelate was attached to the antibody before thiol-activating it with Traut's reagent. The chelation chemistry is described by [16], where on average 1.9 chelates were attached per antibody. High yields of 77% have been achieved during radiolabeling of chelated Ab-SCION with Indium-111.

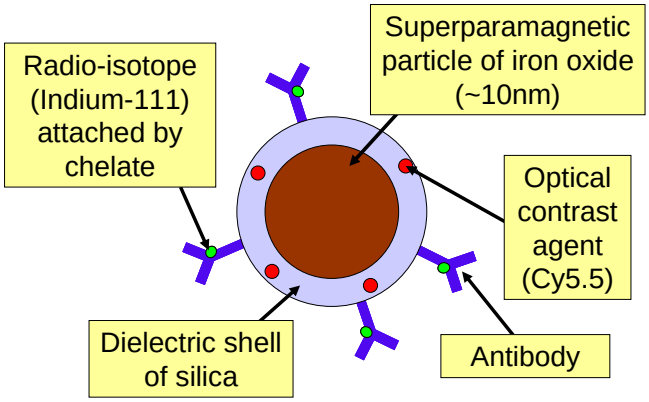

Figure 1: Tri-imageable nanoparticle

The final structure of the developed particle is shown in Figure 1.

3 PARTICLE CHARACTERIZATION

Based on transmission electron microscopy (TEM), the USPIO core has an average diameter of 9.2nm (s=1.4nm). The protocol for silica layer deposition was optimized to generate shells with thicknesses as thin as 2nm.

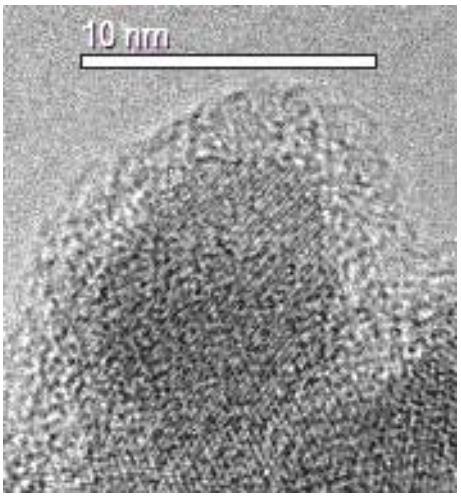

Figure 2: Transmission electron microscopy of SCION

TEM diffraction pattern analyses confirmed the crystalline structures of iron oxide and silica. Samples were further characterized for size by dynamic light scattering where the SCION hydrodynamic diameter was approximately 18nm. ζ-potential measurements were taken to study surface charge across the pH range. The point of zero charge (PZC) of uncoated USPIO was at 7.0, whereas for SCION it was below pH 2.0. Coating with silica made the sol anionic across the working pH range. The stable negative charge in the pH range of 6-7 is desirable because it imitates the negative charge of most biomolecules in physiological conditions [17] and prevents the particles from flocculating because of stable electrostatic repulsion.

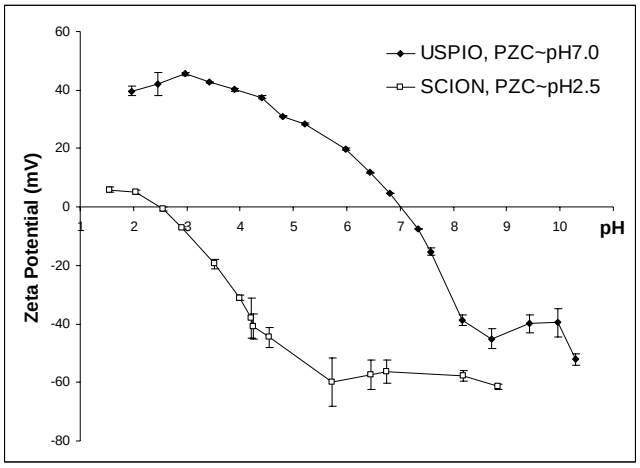

Figure 3: Surface charge characterization of USPIO and SCION particles.

Using a superconducting quantum interference device (SQUID), the magnetic properties of USPIOs and silica-coated USPIOs were analyzed. The SCION particles demonstrated superparamagnetic behavior with tight hysteresis curves and no losses. The blocking temperature where material begins to demonstrate superparamagnetic properties dropped from 162.4K for the USPIO sample to 82.2K for silica-coated USPIOs. The results indicated that silica was effectively suppressing magnetic dipolar interaction between particles. The blocking temperature of single particles is always lower than that of agglomerated nanocrystals. Uncoated particles may agglomerate because their large surface-area-to-volume ratio enables strong magnetic dipolar interaction.

4 BIOMEDICAL APPLICATION

Nanoparticles were conjugated to mAbs L243, Herceptin, and Cetuximab, as well as their negative control antibodies SPV-L3 (anti-HLA-DQ) and humanized HuM195. Furthermore, the same antibodies attached directly to Cy5.5 were used as controls to compare if the conjugation to nanoparticles was altering antibody activity. For proof of principle, cells expressing the appropriate receptors were stained with the antibody-conjugated nanoparticles and analyzed using flow cytometry. High signal-to-noise efficiencies, greater than even the Ab-Cy5.5 controls, were found indicating strong cellular uptake. Viability of human B lymphocytes for up to 65 hours was not affected.

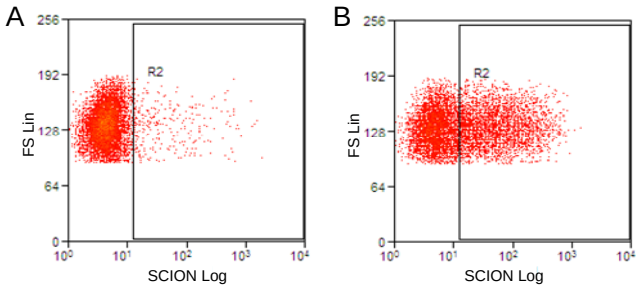

Figure 4: Example of specific uptake of targeted SCION. Flow cytometry analysis of L243 receptor expressing L cells stained with (A) untargeted SCION and (B) mAb L243-conjugated SCION.

Studies with tumor bearing and non-tumor bearing mice are currently being conducted with Erbitux-conjugated SCION. Initial *in vivo* studies of untargeted SCION reveal quick blood clearance and high uptake in the liver and spleen.

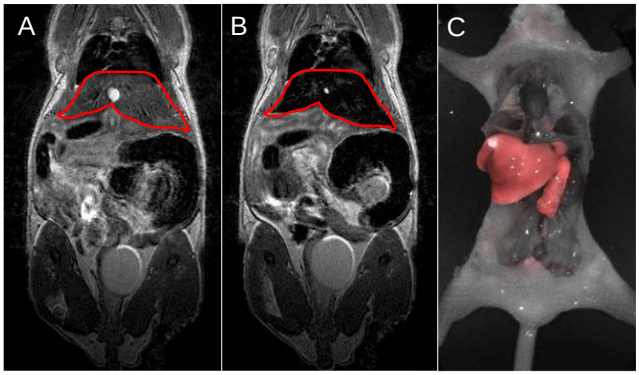

Figure 5: Athymic mouse given i.v. injection of untargeted and non-radiolabeled SCION. (A) T2-weighted MR image of mouse pre-injection and (B) at 20hr post-injection. (C) Composite optical image of mouse at 21 hour post-injection. High uptake by the liver and spleen confirmed by both imaging modalities.

5 DISCUSSION

Targeted delivery of therapeutics is a major goal of pharmaceutical development. Accurate imaging of drugs permits confirmation of the drug "hitting" the target. Though many techniques exist, few allow *in vivo* imaging and control of drug release at the cellular level. The nanoparticle we have synthesized is a triple-reporting contrast agent that allows for not only verification of location but also quantification of the delivered structures. When the radiolabeled antibody that targets delivery is at the same location as the optical and magnetic resonance signal, the stability of the entire compound can be confirmed. The nanoparticle core is an USPIO with a

diameter of ~9nm and the silica coating allows for the conservation of superparamagnetic properties of the iron oxide cores and inhibits agglomeration that could lead to ferromagnetic behavior if the separating layer is not present. It also improves the fluorescence stability of the Cy5.5 dye, ensures biocompatibility, and provides a surface for antibody attachment.

Although the particle size is advantageous for passive delivery, to actively direct it to a specific location antibodies are conjugated to the particle surface to recognize targeted cell surface receptors. We have demonstrated successful attachment of a number of antibodies to SCION and conducted a series of *in vitro* studies that confirm uptake. The chelator CHXA"-DTPA is conjugated to the antibody for radioisotope labeling. The entire nanoparticle-antibody construct has been successfully labeled with ^{111}In.

These particles can be used for a number of biomedical applications. Though our current work has been focused on imaging of diseases such as multiple sclerosis and cancer by antibody-directed uptake after i.v. injection, the tracking of preloaded cells is another highly relevant use. For example, loading T cells before adoptive transfer for the experimental allergic encephalomyelitis model would allow for the observation of how demyelination and lesion formation occurs. As currently developed, the nanoparticle can be used for diagnosis; however, it can also be developed into a method of targeted drug delivery. Once the delivery construct is traced to its target location, the USPIO's superparamagnetic properties can be exploited by irradiation with alternating magnetic fields to heat tissue and rupture the vehicle, leading to controlled release of a payload at the target site Thus, with conjugates of this nanoparticle, it should be possible to target specific tissues, verify localization and then non-invasively activate multimodal therapies using extrinsic fields.

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