Nanomedicine: Engineering of a Tri-Imageable Nanoparticle

A. Bumb[*], M.W. Brechbiel[**], P. Choyke[**], L. Fugger[***] and P.J. Dobson[***]

[*]University of Oxford, Oriel College, Oxfordshire OX14EW, UK, ambika.bumb@eng.ox.ac.uk
[**]National Cancer Institute, Bethesda, MD, USA, martinwb@mail.nih.gov, pchoyke@mail.nih.gov
[***]University of Oxford, Oxfordshire, UK, lars.fugger@imm.ox.ac.uk, peter.dobson@eng.ox.ac.uk

ABSTRACT

A potential targeted drug delivery platform with three imaging reporters has been developed by coupling the magnetic properties of ultra-small superparamagnetic iron oxide nanoparticles (USPIOs) with near infrared fluorescence of Cy5.5 and gamma emissions of Indium-111 that is chelated to a targeting antibody. The nanoparticles have been characterized for surface charge by zeta potential measurements, structure by transmission electron microscopy, magnetic properties with a superconducting quantum interference device, and optical response by fluorescence spectrophotometry and flow cytometry. To test the generalizability of this technology, the particle was conjugated to different antibodies for targeting different diseases. High efficiency was found during *in vitro* studies for multiple sclerosis-targeting and cancer-targeting particles.

Keywords: tri-imaging, USPIO, molecular imaging, nanoparticle

1 INTRODUCTION

At the junction of nanomaterials and medical diagnostics, USPIOs are emerging as a class of novel probes to be used for *in vitro* and *in vivo* cellular and molecular imaging. Maghemite/magnetite, γ-Fe_2O_3/Fe_3O_4, particles have face-centered cubic packing of oxygen that allows electrons to jump between iron ions occupying interstitial tetrahedral and octahedral sites, giving the molecules half-metallic properties that can be used for magnetic resonance imaging. Along with their ability to provide MR contrast, USPIOs present unique properties than can be useful in a great number of biomedical applications: targeted delivery of therapeutic agents, development of immunochromatographic tests, localized thermotherapy (i.e. hyperthermia), and conversion of a pro-drug to its active form [1].

As all technologies have their limitations, the concern with using MRI to image USPIOs is the concentration needed to achieve sufficient contrast [2]. This issue is coupled with the problem that the number of particles within the target cannot be readily quantified. 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) is useful for quantification purposes 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 allow for high resolution imaging and quantification with the ability to verify that the particle has reached its target through three images. 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. Nitin et al have reported the development of micelle-encapsulated SPIOs attached to a fluorescent dye and have used the particle for *in vitro* studies [3]. An optimal and MR imageable particle is useful for *in vitro* purposes, but attaching a radioisotope as a third mode of imaging would provide great advantages for quantification of delivered construct and biodistribution studies *in vivo*. Furthermore, targeting these particles with biomolecules such as antibodies would create a noninvasive reporting tool used to monitor a variety of specific biological responses while providing valuable information regarding physiology and pathophysiology.

For *in vivo* applications of these particles, two key factors play an important role: size and outer coating. The diameter of superparamagnetic iron oxide nanoparticles (SPIOs) greatly affects their localization *in vivo*, even without targeting ligands bound on the surface. Larger particles with diameters ranging from 300nm to 3.5μm that are coated with an insoluble layer are used to image the gastrointestinal tract [4]. Particles ranging in size from 60 to 150nm are taken up by the reticuloendothelial system and rapidly appear in the liver and spleen [5]. Particles in the range of 10 to 40 nm, including ultrasmall SPIOs (USPIOs), are optimal for prolonged blood circulation, can cross capillary walls, and are often taken up extensively by lymph nodes and bone marrow [5].

The particle outer functionality greatly affects its interaction with the *in vivo* environment. Silica is a non-toxic and biocompatible material that can preserve the intrinsic properties of USPIO cores by preventing degradation and aggregation of the inner core. The immobilization of an oxide such as silica allows for tailoring of the particles by straight-forward linkage of functional groups. The surface of silica is coated with silanol groups that easily react with alcohols and silane coupling agents [6] to produce dispersions that are stable in

NSTI-Nanotech 2007, www.nsti.org, ISBN 1420061836 Vol. 2, 2007

non-aqueous solvents and are ideal for strong covalent bonding with ligands such as biomolecules or drugs. The silica shell also plays a role in maintaining stability for particle suspensions during changes in pH or electrolyte concentration, for instance during synthesis, due to silanol groups that make the surface lyophilic [7]. Several layers of water form, preventing coagulation of the particles even at the point of zero charge. Amorphous silica is a heat-resisting material, with a low specific gravity, and good mechanical strength. It is well known for its optical transparency [8], and the advantage silica offers for this application is its controllable thickness. Optimized for biomedical application, 9nm USPIOs coated with 2-5nm thin layers of silica have been synthesized and characterized.

2 SYNTHESIS

Though there are several methods to synthesize USPIOs, the most commonly used approaches are precipitation-based. These can be done either by coprecipitation of metal salts [9-10] or reverse micelle synthesis. Reverse micelle synthesis [11-12] can produce very uniform particles, however they are soluble only in organic solvents. Thus, for medical applications, co-precipitation is the more preferred route of synthesis. Based on mechanisms described by LaMer [13] and Massert [14], USPIOs were synthesized by co-precipitation of ferrous and ferric salts in alkaline and acidic aqueous.

The most commonly used method to prepare silica particles and shells is the Stöber method, developed by Stöber, Fink, and Bohn in 1968 [15]. The Stöber process involves hydrolysis of an alkoxy silane and condensation of alcohol and water [16]. By using catalysts such as ammonia and carefully controlling reagent ratios, reaction volume, pH, and reaction time, particle synthesis 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. Surface conjugation to antibodies was achieved by reacting thiol-functionalized particles with antibodies activated by sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (s-SMCC) [17]. Antibodies used thus far include mAbs L243 and Herceptin, though the conjugation chemistry can be applied to other proteins and biomolecules as well. L243, an anti–HLA-DR monoclonal antibody, can direct the particles to cells from the inflammatory foci in the brain for multiple sclerosis [18-19]. Herceptin targets HER2, a membrane bound receptor associated with tyrosine kinase activity that is over-expressed in a variety of epithelial cancers, including breast, ovarian, pancreatic, and colorectal carcinomas [20].

The third imaging modality was attached using the chelate, 2-(p-isothiocyanatobenzyl)-cyclohexyl-diethyl enetriaminepentaacetic acid (CHX-DTPA). CHX-DTPA provides efficient labeling with ^{111}In and demonstrates maintenance of integrity and immunoreactivity of its radioimmunoconjugates [21-22].

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

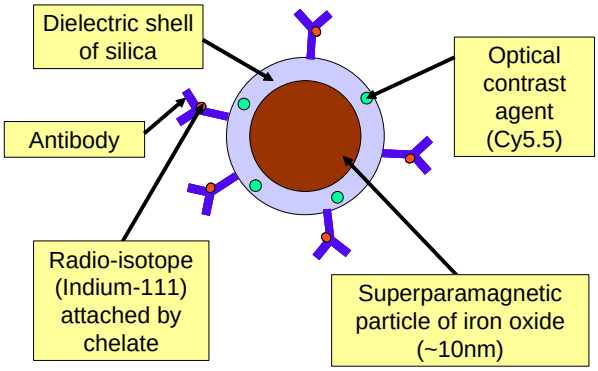

Figure 1: Tri-imageable nanoparticle

3 CHARACTERIZATION

Using transmission electron microscopy, particle structures along each step of synthesis were analyzed. The USPIO core of the particles had an average diameter of 9.2nm (*s*=1.4nm). Measurements of silica layers were used to determine the optimal conditions for the protocol to generate shells with thicknesses as thin as 2nm. Diffraction pattern analyses confirmed the crystalline structures of iron oxide and silica.

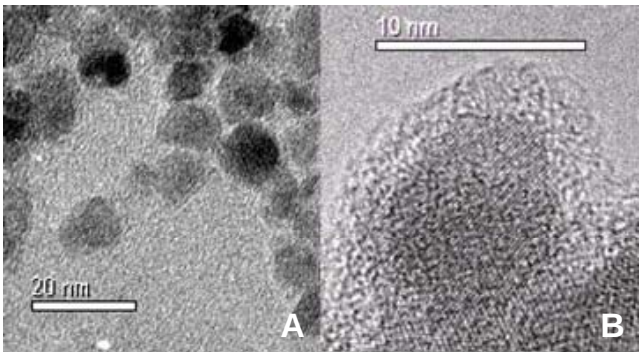

Figure 2: Transmission electron microscopy of A) USPIOs and B) thinly silica-coated USPIO

Samples were analyzed for ζ-potential and the results are presented in Figure 3. Depending on the USPIO preparation procedure, partial conversion of magnetite to maghemite was evident by the dip in ζ-potential near pH5. By re-characterizing surface charge after complete

conversion to maghemite, it was confirmed that the point of zero charge (PZC) of maghemite is lower than magnetite. 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 [23].

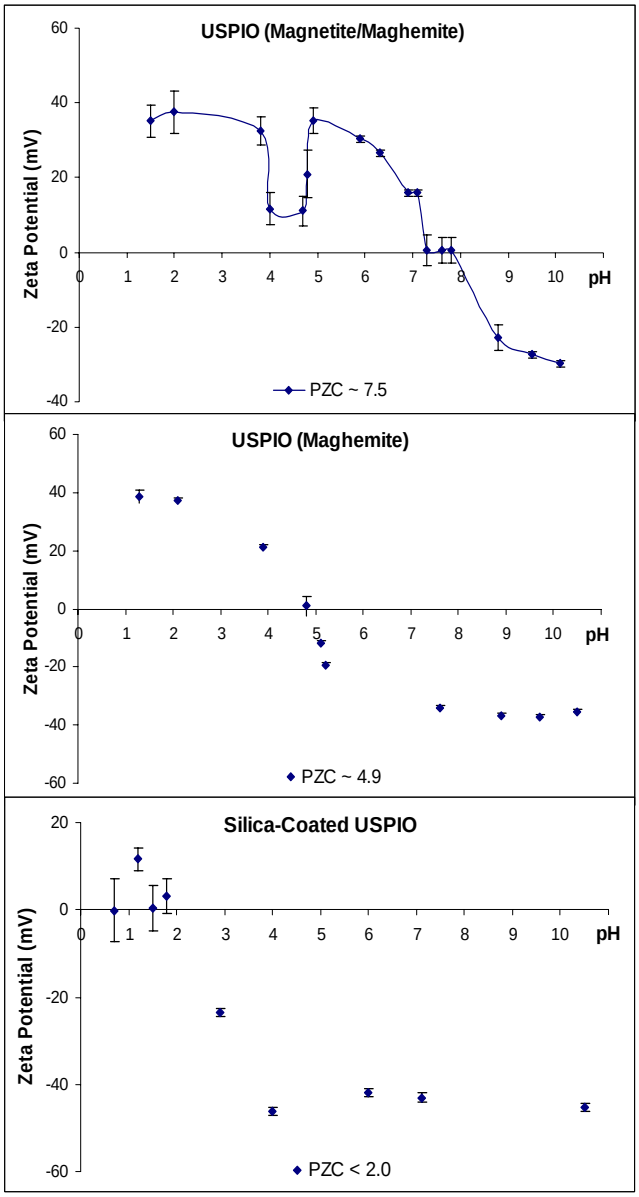

Figure 3: Surface charge characterization of USPIOs

Using a superconducting quantum interference device (SQUID), the magnetic properties of USPIOs and silica-coated USPIOs were analyzed. In order to confirm that the particles were in fact superparamagnetic, hysteresis curves were traced. The particles demonstrated superparamagnetic behavior with tight hysteresis curves and no losses (Figure 4). Based on saturation magnetizations, the mass composition of silica for the particle samples was calculated. To further characterize particle behavior, field cool and zero field cool analyses were used to determine that the blocking temperature for the USPIO sample was 162.4K and 82.2K for silica-coated USPIOs. The results indicate that the silica layer is effectively suppressing magnetic dipolar interaction between particles. Uncoated particles may agglomerate because their large surface-area-to-volume ratio enables strong magnetic dipolar interaction. The blocking temperature of single particles is always smaller than that of agglomerated nanocrystals.

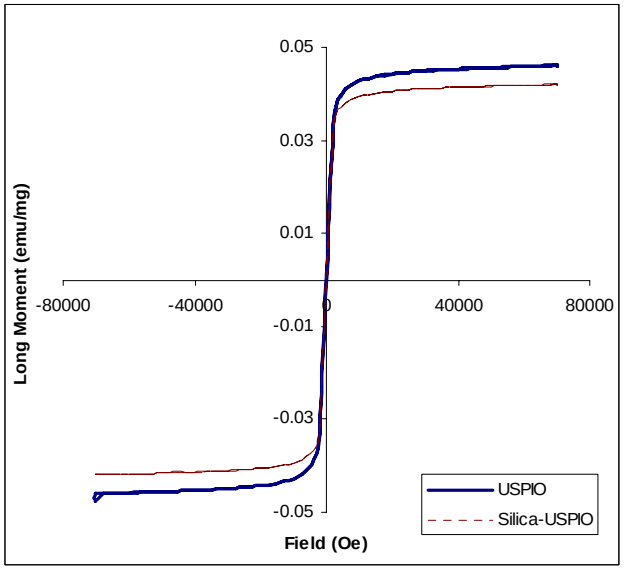

Figure 4: Magnetic hysteresis of uncoated and silica-coated USPIOs

Nanoparticles were conjugated to mAbs L243 and Herceptin. Negative control antibodies SPV-L3 (anti-HLA-DQ) and humanized HuM195, were also conjugated to nanoparticles. 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 that the antibody conjugation did not damage the antibody's interaction with its receptor.

4 DISCUSSION

Targeted delivery of therapeutic drugs is a major goal of pharmaceutical development. Accurate imaging of drugs permits confirmation that the drug is "hitting" the target. In some cases, the imaging agent can be used to convert a pro-drug from its inactive to active form by local application of energy. Though many techniques exist, few allow live imaging and control of drug release at the cellular level. We have created a triple-reporting nanoparticle that will

NSTI-Nanotech 2007, www.nsti.org, ISBN 1420061836 Vol. 2, 2007

allow for exactly these facilities of simultaneous diagnostics and drug delivery. The nanoparticle is imageable by three separate and distinct properties through magnetic resonance (MR), optical, and radioisotope imaging.

We have coupled the magnetic properties of ultra-small superparamagnetic iron oxide nanoparticles (USPIOs) with the optical dye Cy5.5, and to an antibody that is chelated to the radioisotope Indium-111. The synthesized USPIOs have a diameter of approximately 9nm and are small enough to circulate without being rapidly removed by the body. 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.

The new nanoparticles allow for not only triple 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. Though the small size of the particle is one of its greatest advantages for delivery, in order to direct it to a specific location, the attached antibodies recognize receptors that are specifically expressed on the target cell surfaces. To assure flexibility of this technology, the particle has been conjugated to two different antibodies to study two different diseases. One is being used to direct the particles to cells to detect multiple sclerosis, while the other targets cancer cells especially those related to breast, ovarian, pancreatic, and colorectal carcinomas.

As currently developed, the nanoparticle has many applications for detection and diagnostics; nevertheless, it can also be developed into a method of targeted drug delivery. Once the delivery particle is traced to its target location, the superparamagnetic properties of the USPIOs can then be exploited to rupture and release a therapeutic drug payload. Superparamagnetic particles can also be used to heat locally and kill cancer cells. This technology has great potential to be a sensitive, minimally invasive, and highly specific method to trace biomolecules and deliver therapy.

REFERENCES

[1] Shinkai M., J. Biosci. Bioeng. **94,** 606, 2002.

[2] Shapiro, E.M., Skrtic, S. & Koretsky, A.P., Magn Reson.Med, **53**, 329-338, 2005.

[3] Nitin, N., LaConte, L.E.W., Zurkiya, O., Hu, X. & Bao, G, J. Biol. Inorg. Chem., **9**, 706-712, 2004.

[4] Wang, Y.X., Hussain, S.M. & Krestin, G.P., Euro. Rad., **11**, 2319-2331, 2001.

[5] LaConte, L., Nitin, N. & Bao, G., Mat. Today, **8**, 32-38, 2005.

[6] Ulman, A., Chem.Rev. **96**, 1533-1554, 1996.

[7] Mulvaney, P., Liz-Marzan, L.M., Giersig, M. & Ung, T., J.Mater.Chem., **10**, 1259-1270, 2000.

[8] Liu, D.M. & Chen, I.W., Acta Materialia, **47**, 4535-4544, 1999.

[9] Shen, T., Weissleder, R., Papisov, M., Bogdanov Jr, A. & Brady, T.J., Magn. Res. Med., **29**, 599-604, 1993.

[10] Kim, D.K. et al., Chem. Mat., **15**, 4343-4351, 2003.

[11] Pileni, M.P., Nat. Mat., **2**, 145-150, 2003.

[12] Seip, C.T. & O'Connor, C.J., Nanostr. Mat., **12**, 183-186, 1999.

[13] LaMer, V. & Dinegar, R., J. Amer. Chem. Soc., **72**, 4847-4854, 1950.

[14] Massart, R., IEEE Trans. Magn., **17**, 1247-1248, 1981.

[15] Stober, W. & Fink, A., J. Col. Interf. Sci., **26**, 62-69, 1968.

[16] Bardosova, M. & Tredgold, R.H., J. Mat. Chem., **12**, 2835-2842, 2002.

[17] Wolcott, A. et al., J. Phys. Chem. B, **110**, 5779-5789, 2006.

[18] Krogsgaard, M. et al., J. Exper. Med., **191**, 1395-1412, 2000.

[19] Madsen, L.S. et al., Nat Genet ,**23**, 343-347, 1999.

[20] Milenic, D.E.D.E. & Garmestani, Clin. Canc. Res., **10**, 7834-7841, 2004.

[21] Huneke, R.B. et al, Canc. Res., **52**, 5818-5820, 1992.

[22] McDevitt, M.R. et al., Canc. Res., **60**, 6095-6100, 2000.

[23] Vroman, L., Science, **184**, 585-586, 1974.