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Protein Nanospheres: Synergistic Nanoplatfrom-Based Probes for Multimodality Imaging

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Abstract

No single clinical imaging modality has the ability to provide both high resolution and high sensitivity at the anatomical, functional and molecular level. Synergistically integrated detection techniques overcome these barriers by combining the advantages of different imaging modalities while reducing their disadvantages. We report the development of protein nanospheres optimized for enhancing MRI, CT and US contrast while also providing high sensitivity optical detection. Transferrin protein nanospheres (TfpNS), silicon coated, doped rare earth oxide and rhodamine B isothiocyanate nanoparticles, SiCGd₂O₃:Eu,RBITC, (NP) and transferrin protein nanospheres encapsulating SiCGd₂O₃:Eu,RBITC nanoparticles (TfpNS-NP) were prepared in tissue-mimicking phantoms and imaged utilizing multiple cross-sectional imaging modalities. Preliminary results indicate a 1:1 NP to TfpNS ratio in TfpNS-NP and improved sensitivity of detection for MRI, CT, US and fluorescence imaging relative to its component parts and/or many commercially available contrast agents.

Keywords

Protein nanospheres; multimodal imaging; gene delivery; nanoplatform; synergistic

1. INTRODUCTION

Single-modality imaging methods are often not sufficient for the elucidation of disease etiology ¹. Synergistic imaging allows more useful clinical information to be obtained via the combination of two or more modalities than may be obtained from the individual component images ². The unique attributes of each technique often has the potential to provide anatomical, function and/or molecular information that complements the other imaging modalities ³. The physico-chemical interactions involving each imaging method and their contrast agents may differ however. Positron Emission Tomography/Computed Tomography (PET/CT) has been the most useful clinical example of synergistic imaging. It provides excellent lesion localization via anatomical/functional registration, distinction between physiological and pathological uptake, and shorter PET scan time by using CT for attenuation correction ⁴. However, cost, radioisotope preparation issues, challenges associated with image co-registration, and increased ionizing radiation dose resulting from

both radionuclide and X-ray exposure highlight the need for the development of alternatives. The main advantage of using multiple modalities for imaging is the potential to combine optimal sensitivity and specificity of disease detection with high spatial resolution. High sensitivity imaging methods such as PET and Single Photon Emission Computed Tomography (SPECT), which have poor spatial resolution, must often be combined with high resolution techniques such as Magnetic Resonance Imaging (MRI), CT and Ultrasound (US), which have poor sensitivity and specificity of disease detection. Integration of optical techniques with these conventional medical imaging methods should increase sensitivity, specificity, spatial resolution and provide lower cost options for the detection, monitoring and treatment of disease⁵.

An important step in being able to compare imaging results is the development of multimodality imaging agents capable of providing contrast across several imaging modalities simultaneously. It is desirable that these agents act synergistically in providing optimal image enhancement in each imaging modality⁶. In so doing, these agents could potentially function as imaging reference standards, facilitating image comparison via probe co-localization, simplified image co-registration and decreased intra- and inter-modality variability⁷. Improved sensitivity of probe detection may also result in the use of less contrast material⁸. This could decrease toxicity and lead to reduced ionizing radiation dose/exposure during procedures such as X-ray CT due to improved certainty of probe location⁹.

Numerous dual-modality imaging agents have been reported in the literature, exploiting MRI-optical¹⁰, PET-optical¹¹, SPECT-optical¹² and PET-MRI¹³ technologies. These agents often combine the use of paramagnetic gadolinium chelates, organic dyes, metallic gold nanoparticles, semiconducting quantum dots and magnetic nanoparticles into single probes¹⁴. Molecular targeting is often accomplished by conjugation of the imaging agents to macromolecules such as DNA, peptides and antibodies as well as viruses and carbon nanotubes^{15, 16}. Towards this aim we have developed a prototype multimodality contrast agent, transferrin protein nanospheres (TfpNS) encapsulating silicon coated, doped rare earth oxide and rhodamine B isothiocyanate nanoparticles ($\text{SiC}\text{Gd}_2\text{O}_3\text{:Eu,RBITC}$), TfpNS-NP. In previous studies we demonstrated enhanced specificity for transferrin receptor-mediated uptake and gene delivery in cancer cells *in vitro* utilizing transferrin protein nanospheres¹⁷. In the present study phantoms were constructed to test the utility of a single probe, TfpNS-NP, in enhancing MRI, CT, US and fluorescent imaging contrast. These preliminary studies also investigate whether TfpNS-NP can improve sensitivity for probe detection utilizing conventional imaging modalities and fluorescence imaging.

2. MATERIALS AND METHODS

2.1 Contrast Agent Synthesis

Briefly, the $\text{SiC}\text{Gd}_2\text{O}_3\text{:Eu,RBITC}$ NP was synthesized via a modification of methods by Louis et. al.^{18,*}. 0.1 mol/L Gd (95%) and Eu (5%) in the presence of 13 mg RBITC was prepared in DEG salt and precipitated versus 3 mol/L NaOH at high temperature (140° C for 1 hr, 180° C for 4 hr). Activation via 0.1 M TEA aqueous solution followed by 88.5 uL APTES and 66.8 uL TEOS at low temperature facilitated formation of a polysiloxane shell around the $\text{Gd}_2\text{O}_3\text{:Eu,RBITC}$ core. Subsequent dialysis against ethanol and water provided the purified final product. Transferrin was purchased from Athens Research & Technology (Athens, GA). Unlabeled transferrin protein nanospheres (TfpNS) and TfpNS-NP were

*Disclaimer: The full description of the procedures used in this paper requires the identification of certain commercial products and their suppliers. The inclusion of such information should in no way be construed as indicating that such products or suppliers are endorsed by NIST or are recommended by NIST or that they are necessarily the best materials, instruments, software or suppliers for the purposes described.

synthesized via ultrasonic irradiation at 70 W/cm² and modification of methods previously described in detail ¹⁹.

2.2 Contrast Agent Characterization

Samples for AFM were imaged with a Bioscope II atomic force microscope (Veeco, Santa Barbara, CA) on a silicon substrate. AFM images were collected in tapping mode using silicon tips (Veeco) at resonance frequencies of 200 kHz - 300 kHz. Images were collected at a scan rate of 1 Hz and captured an 8 X 8 micron image, 2048 X 2048 pixels. The theoretical x,y resolution is 3.9 nm and Z resolution is 0.5 nm. AFM images were flattened and plane-fit before image analysis. All images were collected under ambient conditions. TfpNS and TfpNS-NP size and zeta potential were determined by dynamic laser light scattering (DLS) using a ZetaPALS particle size analyzer (Brookhaven Instruments, Holtsville, NY). Measurements were performed at 25°C in aqueous solution. Absorbance and fluorescence spectra were obtained using Carey Eclipse spectrometers (Varian, Palo Alto, CA). Gadolinium concentration was determined with the semi-quantitative mode of an Agilent model 7500cs inductively coupled plasma mass spectroscopy (Agilent Technologies, Santa Clara, CA).

2.3 Phantom Preparation

Phantoms were utilized in order to decrease variability secondary to NP agglomeration and facilitate image comparison. A dilution series of each contrast agent (TfpNS, NP and TfpNS-NP as well as control samples) was prepared in 8% gelatin in either eppendorf tubes for MRI and CT experiments or in a 24-well plate for optical imaging. Typically, the gelatin solution was heated to 95° C with stirring until it clarified. It was next cooled to 55°C with stirring and aliquoted with the appropriate concentration of contrast agent into eppendorf tubes. The tubes were then vortexed and placed in a refrigerator for faster cooling. Similarly, the still-liquid gelatin solution and contrast agents were then poured into 24-well plates and rapidly cooled in a refrigerator. Phantoms for US imaging were prepared by heating 4% agarose to 95°C in a waterbath with stirring, pouring it into a 250 mL container and leaving a void tube (a lithium greased metal rod) in place to make a 6 mL void volume ²⁰. The agarose was hardened in a refrigerator. Subsequently contrast agent in 8% gelatin, prepared as above, was poured into the agarose void volume. The gelatin/contrast agent inclusions were then hardened in a refrigerator. All imaging experiments utilizing phantoms were conducted with sample and measurement numbers of at least n = 3 and error less than 4% unless otherwise noted in the text.

2.4 Magnetic Resonance Experiments

MRI was acquired using a Philips Achieva 1.5T SE (Philips Medical Systems, Andover MA). A spin-echo (SE) imaging sequence was used to obtain T1-weighted images. The imaging parameters were as follows: echo time (TE) 15 milliseconds, repetition time (TR) 434 milliseconds, Flip angle 69°, slice thickness 3.0 mm, field of view 200 mm, zoom 328%, 256 X 256, 20 frames. A turbo spin echo (TSE) imaging sequence was utilized for T2 experiments. The T2 measurement parameters were as follows: TE 100 milliseconds, TR 3.2 seconds, Flip angle 90°, slice thickness 3.0 mm, field of view 220 mm, zoom 205%, 256 X 256, 20 frames. A Philips 8 Channel SENSE Head Coil was used for both T1 and T2 imaging experiments.

2.5 Computed Tomography Experiments

Experiments were performed to determine the relative CT attenuation of the NP. The Gd containing contrast agent MultiHance was used for comparison of X-ray attenuation. Data were acquired at ambient temperature. Samples were positioned in the center of the gantry

of a Siemens Somatom Definition Flash CT scanner (Siemens, Baltimore MD) and imaged under the following constant scanning parameters: slice thickness 0.750 mm, 120 keV, 220 mA, scan time 1 second, 300 mm field of view, W: 100 L: 45. Attenuation measurements were recorded as mean Hounsfield units obtained from region-of-interest squares approximately 300 mm² using syngo CT software and plotted against contrast agent concentration using linear regression analysis.

2.6 Ultrasound Experiments

Ultrasound imaging experiments were conducted with a ATL HDI® 4000 Ultrasound System in 2D imaging mode, 256 (8 bits) greyscale, Two different ultrasound transducers were used for imaging studies: 1) a 5-2 MHz curvilinear array transducer and 2) a 12-5 MHz 256 element high resolution linear array transducer. Both were operated at a MI of 0.7 and 13 Hz frame acquisition rate.

2.7 Fluorescence Imaging Experiments

The contrast agents were quantified with respect to fluorescent intensity (radiance, photons/s/cm²/sr) using a Caliper IVIS Spectrum imaging system (Caliper Life Sciences, Hopkinton, MA) with excitation/emission filters at 570/620 nm and the following parameters: 5 second exposure, F/stop = 2, binning = 16 pixels per superpixel, manual focus @ 0.5cm above stage, FOV = 12.9cm.

3. RESULTS AND DISCUSSION

3.1 Contrast Agent Characterization

As indicated in Table 1, the NP has a positive zeta potential at pH 7.4 making it more likely to agglomerate in solution²¹. NP agglomeration was further substantiated by DLS and AFM studies (Fig. 2). Both TfpNS and TfpNS-NP have negative zeta potentials larger than -30 mV at physiological pH, favoring dispersion in solution. Experiments conducted in which NP was added to TfpNS did not result in a change in zeta potential until more than 3/4 of the volume contained NP, at which point a precipitous increase in charge was observed. Similarly, adding TfpNS to NP did not initially result in a change in zeta potential. These results support the hypothesis that the NP is encapsulated within TfpNS in the case of TfpNS-NP. Size and charge analysis (as determined by DLS and AFM measurements) indicate that it is likely that the NP to protein ratio in TfpNS-NP is 1:1. We are currently exploring the use of flow cytometry to differentiate and/or separate NP (positively charged and strongly fluorescent) from TfpNS-NP (negatively charged and strongly fluorescent) and TfpNS (negatively charged and weakly fluorescent) and as means of improved characterization.

3.2 Magnetic Resonance Experiments

Representative T1- and T2-weighted MR images of TfpNS and NP are shown in Fig. 3 and 4. At the relatively low Gd concentrations studied, a nonlinear increase in T1 and T2 signal intensity with increasing NP and TfpNS-NP concentration was observed (Tables 2 and 3). However, despite this non-linearity, the possibility of detecting less than 0.04 mM gadolinium in TfpNS-NP is demonstrated. This represents a significant improvement in sensitivity of probe detection in comparison to many commercially available MRI contrast agents²². TfpNS-NP exhibit a larger increase in T1 and T2 relaxation than either the NP or MultiHance, a clinically used MRI contrast agent, when matched for NP concentration and/or gadolinium concentration. This may be due to the increased rotational correlation time associated with the size of the NP and encapsulation of the NP within TfpNS, which is anticipated to have an effect on local viscosity²³. Prolonged water residence time proximal to the gadolinium oxide nucleus²⁴, due to water compartmentalization/sequestration within

the protein nanosphere, may also influence the relaxation rate. Relaxivity as a function of time, r_1 and r_2 , were estimated via a non-linear model²⁵. r_1 and r_2 were 28.6 and 38.0 s⁻¹ mM⁻¹, respectively, for TfpNS-NP and 11.3 and 18.8 s⁻¹ mM⁻¹, respectively for the NP. MultiHance when prepared in the same manner had r_1 and r_2 values of 6.4 and 7.3 s⁻¹ mM⁻¹, respectively. This difference from the manufacturer reported values²² may be due to our measurements having been conducted in 8% gelatin and/or variability associated with preparation of the gelatin phantoms i.e., evaporation of water during phantom preparation. The presence of varying amounts of water was shown to exert a significant influence on $1/T_1$ and $1/T_2$ even in the absence of contrast media.

3.3 Computed Tomography Experiments

A plot of X-ray CT attenuation (HU) versus gadolinium concentration (Fig. 5) demonstrates the potential for detection of the NP at a concentration of less than 0.2 mM gadolinium. Iodine-based contrast agents are typically given to patients at molar concentration²⁶, i.e., the NP is detectable at 4 orders of magnitude lower contrast agent concentration than is used clinically. On an equimolar basis, gadolinium exhibits approximately double the X-ray attenuation of iodine at 120 keV²⁷. In addition, gadolinium's higher density (7.41 g/cm³ for gadolinium oxide vs. 2.2 g/cm³ for iodine) and the packaging of hundreds of thousands of gadolinium atoms per nanoparticle may account for the significantly greater X-ray CT attenuation observed with the NP vs. MultiHance, a monomeric gadolinium chelate. However, X-ray CT attenuation of the NP is non-linear, indicating it is poorly dispersed. Phantom variability and NP clustering (Table 1) may also contribute to the observed increase in sensitivity for detection.

3.4 Ultrasound Experiments

Fig. 6 demonstrates the 2-fold increase in US backscatter at 7 MHz observed for TfpNS-NP relative to TfpNS, NP and a control. The experiments controlled for NP and protein nanosphere concentration making it more likely that an interaction between the NP and the wall of the protein nanosphere accounts for the increased signal intensity seen in TfpNS-NP. In previous studies we investigated the use of protein (sub)microspheres as US, optical and photoacoustic imaging agents¹⁷. The present experiment demonstrates the utility of NP encapsulation within TfpNS. In addition to providing high specificity for disease targeting and a means of delivering therapeutic genes and drugs into cells, encapsulation of the NP is essential for extending the functionality of protein nanospheres to US imaging.

3.5 Fluorescence Imaging Experiments

Fig. 9 demonstrates linear increase in fluorescence intensity observed with increasing NP and TfpNS-NP concentration. Among the potential advantages of using NPs vs. dye molecules, such as RBITC, for optical bioimaging are broader spectrum excitation, longer fluorescence lifetime, decreased quenching, and lower cytotoxicity; all of which are influenced by the protection of RBITC (and Gd₂O₃:Eu) within the porous silicon structure²⁸. TfpNS-NPs exhibit 3-fold greater fluorescence intensity relative to the NP although matched for NP and gadolinium concentration. The overall sensitivity for detection utilizing fluorescence imaging is greater than 2×10^{-8} M gadolinium. The cause for TfpNS-NPs increased fluorescence signal relative to the NP is still under investigation. A broadened increase in TfpNS fluorescence intensity is observed between 300 nm and 500 nm. This may correspond to the intrinsic fluorescence of native transferrin reported by other investigators²⁹. It is possible that intrinsic fluorescence contributes to both the TfpNS fluorescence signal observed in Fig. 8 and 9 and to the increased signal intensity seen for TfpNS-NP as compared to NP despite equivalent NP concentration. We are actively investigating a means of quantifying the contribution of RBITC versus europium doping/trivalent lanthanide ion luminescence (Gd₂O₃:Eu) to NP fluorescence.

4. CONCLUSIONS

In conclusion we have developed a multimodal imaging contrast agent, TfpNS-NP, capable of permitting high sensitivity of probe detection utilizing MRI, CT, US and optical imaging with a single contrast injection. These preliminary studies represent an attempt to characterize imaging sensitivity in advance of initiating toxicity and probe metabolism/excretion studies. As little as 10^{-5} M Gd can be detected by MRI, 10^{-4} M Gd by X-ray CT and 10^{-8} M Gd by fluorescence imaging. In addition, there is a 2-fold increase in TfpNS-NP US backscatter relative to the agents' component parts (NP and TfpNS). Because the probe itself is the targeting moiety protein nanospheres represent a unique and flexible imaging and therapeutic nanoplatform. They may be easily rerouted to diverse disease types simply by creating protein nanospheres from protein disease markers capable of binding to their respective ligand/receptor. In addition to providing a means of delivering nanoparticles, drugs and genes to specific cells it is anticipated that nanomaterials that closely mimic endogenous proteins, such as protein nanospheres, may eventually play a role in monitoring protein metabolism *in vivo*, serving as an entry point for the integration of proteomics and personalized medicine into multimodal molecular imaging³⁰.

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Fig. 1. Schematic depicting experimental contrast agents: SiC₂Gd₂O₃:Eu,RBITC nanoparticles (NP), transferrin protein nanospheres (TfpNS) and transferrin protein nanospheres encapsulating SiC₂Gd₂O₃:Eu,RBITC nanoparticles (TfpNS-NP).

Fig. 2.

A representative Atomic Force Microscope scan of multiple $\text{SiC Gd}_2\text{O}_3\text{:Eu,RBTC}$ nanoparticles (NPs) (a) with detailed AFM size distribution data of five NPs (b).

Fig. 3.

T1- and T2-weighted magnetic resonance images of phantoms containing SiCGd₂O₃:Eu,RBITC nanoparticles (NP) at a Gd concentration of 0 mM (a), 0.002 mM (b), 0.050 mM (c), 0.200 mM (d), and 0.500 mM (e). The NPs were suspended in 8% gelatin.

Fig. 4.

T1- and T2-weighted magnetic resonance images of phantoms containing transferrin protein nanospheres encapsulating $\text{SiCGd}_2\text{O}_3\text{:Eu,RBITC}$ nanoparticles (TfpNS-NP) at a Gd concentration of 0 mM (a), 0.002 mM (b), 0.040 mM (c), 0.200 mM (d), and 0.400 mM (e). The TfpNS-NPs were suspended in 8% gelatin.

Fig. 5. $\text{SiCGd}_2\text{O}_3\text{:Eu,RBITC}$ nanoparticles (NP) and MultiHance X-ray CT attenuation (HU) plotted as a function of gadolinium concentration. Both agents were suspended in 8% gelatin.

Fig. 6.

Magnetic resonance (a) and ultrasound images (b-g) of gelatin/agarose phantoms in coronal view (a), axial view at 5 MHz utilizing a curvilinear ultrasound transducer (b) and at 7 MHz utilizing a linear ultrasound transducer (c). An arrow originating from the 8% gelatin inclusion (c) indicates the ROI used for backscatter determination in a control phantom (d) and phantoms containing SiCGd₂O₃:Eu,RBITC nanoparticles (NP) (e), transferrin protein nanospheres (TfpNS) (f) and transferrin protein nanospheres encapsulating SiCGd₂O₃:Eu,RBITC nanoparticles (TfpNS-NP) (g). The gadolinium concentration for the NP (e) and TfpNS-NP (g) is 0.200 mM. TfpNS and TfpNS-NP were prepared at equivalent protein nanosphere concentration (as indicated by A₂₈₀ protein concentration measurements).

Fig. 7.

Ultrasound backscatter intensity at 7 MHz of control phantoms and phantoms containing $\text{SiCGd}_2\text{O}_3\text{:Eu,RBITC}$ nanoparticles (NP), transferrin protein nanospheres (TfpNS) and transferrin protein nanospheres encapsulating $\text{SiCGd}_2\text{O}_3\text{:Eu,RBITC}$ nanoparticles (TfpNS-NP). The gadolinium concentration for the NP and TfpNS-NP is 0.200 mM. TfpNS and TfpNS-NP were prepared at equivalent protein nanosphere concentration (as indicated by A_{280} protein concentration measurements).

Fig. 8.

Sample image from a 24-well plate containing 8% gelatin (control), 8% gelatin and transferrin protein nanospheres encapsulating $\text{SiCGd}_2\text{O}_3\text{:Eu,RBITC}$ nanoparticles (TfpNS-NP), $\text{SiCGd}_2\text{O}_3\text{:Eu,RBITC}$ nanoparticles (NP), and transferrin protein nanospheres (TfpNS). The gadolinium concentrations in the TfpNS-NP and NP rows are 0 mM (a), 1.59×10^{-5} mM (b), 1.59×10^{-4} mM (c), 1.59×10^{-3} mM (d), 1.59×10^{-2} mM (e) and 1.59×10^{-1} mM (f). TfpNS and TfpNS-NP were prepared at equivalent protein nanosphere concentration (as indicated by A_{280} protein concentration measurements).

Fig. 9. Plot of fluorescence intensity (expressed as the average radiance) versus Gd concentration from a 24-well plate containing 8% gelatin and transferrin protein nanospheres encapsulating $\text{SiCGd}_2\text{O}_3\text{:Eu,RBITC}$ nanoparticles (TfpNS-NP), $\text{SiCGd}_2\text{O}_3\text{:Eu,RBITC}$ nanoparticles (NP), transferrin protein nanospheres (TfpNS) and a control. TfpNS and TfpNS-NP were prepared at equivalent protein nanosphere concentration (as indicated by A_{280} protein concentration measurements)

Table 1

Summary of Characterization Results for SiCGd₂O₃:Eu,RBITC nanoparticles (NP), transferrin protein nanospheres (TfpNS) and transferrin protein nanospheres encapsulating SiCGd₂O₃:Eu,RBITC nanoparticles (TfpNS-NP)

	NP (SiCGd ₂ O ₃ :Eu,RhB)	TfpNS-NP	TfpNS
Gd, Eu Concentration (ICP-MS)	Gd=0.31 mg/mL Eu=0.025 mg/mL	Gd=0.25 mg/mL Eu=0.020 mg/mL	
UV-Vis	550–590 nm	550–590 nm	280nm
Fluorescence	580–700 nm	580–700 nm	
DLS	(unstable, micron sized)	63–80 nm	79–91 nm
ZetaPotential @ pH 7.4	2.06 ± 0.97 mV	−45.05 ± 7.39 mV	−47.37 ± 0.87 mV
AFM	25–68nm		

Table 2

Mean T1 and T2 $\pm$ SD values calculated from phantoms (n = 3) containing SiC:Gd₂O₃:Eu,RBITC nanoparticles (NP) in 8% gelatins at ambient temperature.

	0 mM Gd	0.002 mM Gd	0.05 mM Gd	0.20 mM Gd	0.5 mM Gd
T1 (msec $\pm$ SD)	156 $\pm$ 14.5	255 $\pm$ 21	605 $\pm$ 33	454 $\pm$ 24	758 $\pm$ 66
T2 (msec $\pm$ SD)	667 $\pm$ 31	472 $\pm$ 63	310 $\pm$ 32	260 $\pm$ 15	78 $\pm$ 11

Table 3

Mean T1 and T2 $\pm$ SD values calculated from phantoms (n = 3) containing transferrin protein nanospheres encapsulating SiC Gd₂O₃:Eu, RBITC nanoparticles (TfPNS-NP) in 8% gelatins at ambient temperature.

	0 mM Gd	0.002 mM Gd	0.04 mM Gd	0.16 mM Gd	0.4 mM Gd
T1 (msec $\pm$ SD)	349 $\pm$ 44	671 $\pm$ 57	949 $\pm$ 142	1130 $\pm$ 194	1263 $\pm$ 203
T2 (msec $\pm$ SD)	2270 $\pm$ 113	1753 $\pm$ 82	1240 $\pm$ 54	853 $\pm$ 46	433 $\pm$ 38