Published in final edited form as:

Nanomedicine. 2008 December ; 4(4): 318–329. doi:10.1016/j.nano.2008.05.004.

Nanoimmunoliposome Delivery of Superparamagnetic Iron Oxide Markedly Enhances Targeting and Uptake in Human Cancer Cells *In Vitro* and *In Vivo*

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Abstract

To circumvent the problem of reduction of the supermagnetic properties of superparamagnetic iron oxide (SPIO) nanoparticles after chemical modification to conjugate targeting molecules, we have adapted a tumor-targeting nanoimmunoliposome platform technology (scL) to encapsulate and deliver SPIO (scL-SPIO) *in vitro* and *in vivo* without chemical modification.

Scanning probe microscopy, confocal microscopy, and Prussian blue staining were employed to analyze the scL-SPIO nanoparticles and assess intracellular uptake and distribution of SPIO *in vitro*. *In vivo* targeting and tumor-specific uptake of scL-SPIO was examined using fluorescent-labeled SPIO.

We demonstrated that SPIO encapsulation in the scL complex results in approximately an 11 fold increase in SPIO uptake in human cancer cells *in vitro*, with distribution to cytoplasm and nucleus. Moreover, the scL nanocomplex specifically and efficiently delivered SPIO into tumor cells after systemic administration, demonstrating the potential of this approach to enhance local tumor concentration and the utility of SPIO for clinical applications.

Keywords

Superparamagnetic iron oxide nanoparticles; nanoimmunoliposome; Tumor targeting; intracellular uptake; systemic delivery

Introduction

Due to their unique properties (1, reviewed in 2) superparamagnetic iron oxide (SPIO) have found application in increasingly diverse areas of biotechnology and biomedical sciences (2–5). Currently, SPIO are most extensively used as magnetic resonance imaging (MRI) contrast agents, where they have advantages over conventional paramagnetic Gadolinium-based

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contrast agents, including low toxicity, subnanomolar range detection limits, exceeding that of Gadolinium by a factor of 100 and, due to their superparamagnetic properties, the potential to provide higher contrast enhancement in MRI (6–8). However, their lack of specificity reduces accumulation in target tissues thus generally limiting their current use as therapeutics and as a diagnostic agent to principally liver, lymph nodes and the gastrointestinal tract (9–11).

To enhance their diagnostic and therapeutic potential, SPIO nanoparticles require surface coating that would ensure biocompatibility while decreasing RES clearance. Current approaches employ various coatings or encapsulation in untargeted liposomes (12–16), which lead to increasing circulation times in the blood. Commercially available Dextran coated SPIO nanoparticles are currently in clinical use or in clinical trials as MRI contrast agents (17,18). However, these contrast agents still have low intracellular uptake.

An alternative strategy to improve tumor targeting and intracellular uptake is to surface modify the SPIO with ligands, a variety of which, including folic acid (19), monoclonal antibodies (20,21) and Luteinizing hormone releasing hormone (LHRH) (6), have been conjugated to the surface of SPIO nanoparticles,. However, in most instances the ligands can not be directly conjugated to the SPIO nanoparticles. The surface modification or coating required can result in loss of the superparamagnetic properties of the SPIO nanoparticles. Furthermore, the low efficiency of ligand conjugation leads to manufacturing difficulties.

Hence, low transfection efficiency, poor tissue penetration, and nonspecific delivery have significantly hindered the wide application of SPIO nanoparticles in diagnosis and therapy. The current challenge is to develop a novel technology for improving SPIO nanoparticle specificity and uptake into tumor cells.

Our laboratory has developed a tumor specific delivery system for use in gene medicine. This nanosized complex consists of a therapeutic or diagnostic payload (22–26) encapsulated within a cationic liposome the surface of which is decorated with an anti-transferrin receptor single chain antibody fragment (TfRscFv) which serves to target the complex to the transferrin receptor, the level of which is elevated on tumor cells (27). Here we adapt this platform technology to specifically and efficiently deliver SPIO nanoparticles into human tumor cells.

Methods

Chemicals

Sodium 3'-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonate (XTT) were purchased from Polysciences (Warrington, PA, USA). Ferric chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), ferrous chloride tetrahydrate ($\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$), ammonium hydroxide ($\text{NH}_4\text{-OH}$) (28% wt.% NH_3 in water) and ethanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and dioleoylphosphatidyl-ethanolamine (DOPE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). 3-Aminopropyl triethoxysilane (APTS), N-methyl dibenzopyrazine methyl sulphate (PMS) and 0.1% poly-L-lysine (PLL) solution were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fluorescein conjugated streptavidin was purchased from Invitrogen Molecular Probes, Inc. (Eugene, OR, USA). Sulfosuccinimidyl-6-(biotin-amido) hexanoate (Sulfo-NHS-LS-Biotin) was purchased from Pierce (Rochford, IL, USA). All other reagents and solvents were common analytical grade reagents.

Cell culture

Human pancreatic (PANC-1) cell line was obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). Human breast cancer cell line MDA-MB-231 was provided

by the Georgetown University Medical Center Lombardi Comprehensive Cancer Center tissue culture core facility. Cell culture media was obtained from Invitrogen (Carlsbad, CA, USA). Both MDA-MB-231 and PANC-1 cells were maintained at 37 °C in a 5% CO₂ atmosphere in improved minimum essential medium (IMEM; Invitrogen [Carlsbad, CA, USA]) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA), 2 mM L-glutamine, and 50 µg/mL each of penicillin, streptomycin, and neomycin.

Preparation of SPIO nanoparticles

The iron oxide nanoparticles were prepared by a co-precipitation method as previously reported (28,29). Briefly, 8.6 g FeCl₂·4H₂O and 23.5 g FeCl₃·6H₂O were added to 600 ml autoclaved deionized water in a 2.0 L beaker under nitrogen gas and vigorously stirred at 85 °C until dissolved, after which 34 mL NH₄·OH was added to the solution. After 1 h of stirring at 85 °C, the SPIO nanoparticles precipitated and were isolated from the solution by a permanent magnet (12300 Gauss, Master Magnetics, Inc., Castle Rock, CO, USA). The SPIO nanoparticles were washed with approximately 1 L autoclaved deionized water 4 times, using the magnet to collect the particles between the washes. Finally, the SPIO nanoparticles were dispersed in autoclaved deionized water which was stored at 4 °C.

Surface modification of SPIO nanoparticles by APTS

SPIO nanoparticles were surface modified with Aminopropyl triethoxysilane (APTS) via a silanization reaction with the hydroxyl groups on the surface of the SPIO nanoparticle (30, 31). The scheme for the modification of SPIO nanoparticles is shown in Figure 1A.

Briefly, SPIO nanoparticles (206 µmol) were pelleted, the supernatant removed, washed 5 times with absolute ethanol to remove the remaining water, and diluted to 75 mL with ethanol. The suspension was sonicated for 10 min without heat in a sonicating water bath (FS9H, Fisher Scientific, Pittsburgh, PA, USA). Subsequently, 3.7 mmol of APTS was added and sonicated for an additional 10 min. 1 mL of autoclaved deionized water was added as a catalyst, and the solution was sonicated for an additional 30 min. The solution was then incubated with shaking (250 rpm) for 6 h at 60 °C. Finally, the solid magnetic nanoparticles were isolated using the magnet, washed with ethanol once and autoclaved deionized water 3 times. The APTS modified SPIO nanoparticles were stored at 4 °C.

The dried samples of SPIO or APTS modified SPIO nanoparticles were grounded with KBr and compressed into a pellet. The Fourier Transform Infrared (FTIR) spectrum was recorded in the transmission mode on a Vector 22 spectrometer (Bruker Optik GmbH, USA). The amount of amino groups on the APTS modified SPIO nanoparticles was also quantitatively measured by a surface chemical reaction method as previously described (32).

Fluorescein labeled SPIO nanoparticles

Sulfo-NHS-LS-Biotin was coupled to the APTS modified SPIO nanoparticle surface through a reaction between the Sulfo-NHS and amino groups on the SPIO-APTS particles as shown in Figure 1B. 5.5 mg Sulfo-NHS-LS-Biotin was dissolved in 1 mL of autoclaved deionized water according to the manufacturer's directions, to make a Sulfo-NHS-LS-Biotin solution of 10 µmol/mL which was kept on ice for use. 1.0 µmol of APTS modified SPIO nanoparticles were added to the Sulfo-NHS-LS-Biotin solution and incubated at room temperature for 2 h. The solid particles were isolated via the magnet and washed 3 times with autoclaved deionized water. Following this, 0.5 mL autoclaved deionized water was added to make the biotin conjugated SPIO suspension. The amount of conjugated biotin on the SPIO nanoparticles was quantitatively measured using the Biotin Quantitation Kit (Pierce, Rockford, IL, USA) at 500 nm.

Subsequently, based on the high affinity of biotin for streptavidin (33), fluorescein conjugated streptavidin was immobilized on the biotin labeled SPIO nanoparticles (Figure 1C). Fluorescein conjugated streptavidin solution was added to the above biotin modified SPIO suspension at a 1:1 molar ratio of streptavidin to biotin, and incubated at room temperature for 2 h. The resultant fluorescein labeled SPIO nanoparticles were isolated via magnet and washed 3 times with autoclaved deionized water, and stored at 4 °C.

Preparation of the ligand-liposome-SPIO nanocomplex

Cationic liposome (DOTAP:DOPE) (Lip) was prepared by the ethanol injection method as previously described (23). The targeting moiety used in these studies is an anti-transferrin receptor single-chain antibody fragment (TfRscFv) (23). The TfRscFv/Lip/SPIO complex (scL-SPIO) was prepared by simple mixing of the components essentially as previously described (34). The size (Intensity value) and the zeta-potential of the scL-SPIO nanocomplex was determined by dynamic laser light (DLS) scattering on a Malvern Zetasizer Nano-ZS (Malvern Instruments, Malvern, UK). For the preparation of scL nanocomplex carrying the fluorescein labeled SPIO the SPIO nanoparticles consisted of 20% fluorescein labeled (14 nmol) and 80% none-fluorescein labeled (56 nmol) particles. For the animal injections, the scL-SPIO nanocomplex is in 5% (w/v) dextrose.

Scanning probe microscopy

Preparation of substrates for attachment of scL-SPIO was done by carefully tuning the substrate charge. A freshly cleaved mica substrate was immersed in a 0.01% poly-L-lysine (PLL) solution diluted from 0.1% stock solution for 30 minutes and blown dry. Tuning the surface Zetapotential can be accomplished by exposing fresh PLL-mica to UV/ozone. The substrate is placed 1 cm from a UVP-51 lamp in a sealed metal box located in a fume hood containing ambient air for 0 to 30 minutes. This accelerates the PLL-mica aging process from weeks to minutes and the process is calibrated for a given system by employing the rotating disk method.

SPM fluid imaging was performed with a Veeco MultiMode AFM and Nanoscope IV controller (Veeco Metrology, Inc. Chadds Ford, PA, USA). Nanoscope Version 6 software (Veeco Metrology, Inc. Chadds Ford, PA, USA) was used for data acquisition. Dry imaging was performed in TappingMode using Veeco OTESP cantilevers (Veeco Metrology, Inc. Chadds Ford, PA, USA). For fluid imaging, a TappingMode fluid cell, without an O-ring, and Veeco OTR8 'B' cantilevers (24-kHz nominal resonance frequency in air) (Veeco Metrology, Inc. Chadds Ford, PA, USA) were used for fluid imaging by oscillating the cantilever in the low-frequency acoustic mode region, ca. 7–9 kHz. Imaging buffer was usually 5 mM MgCl₂. SPM calibration was performed using a series of negatively charged, citrate-stabilized gold nanoparticles with nominal sizes of 10 nm, 30 nm, 50 nm, and 80 nm. Particle size analysis using SPM image data was performed using resources available in Nanoscope Version 5 (Veeco Metrology, Inc. Chadds Ford, PA, USA), ImageJ (Wayne Rasband, National Institutes of Health, USA <http://rsb.info.nih.gov/ij/>), and SigmaPlot (Systat Software, Inc, San Jose Ca, USA) software.

Magnetic force microscopy (MFM) was performed with Co/Cr-coated Veeco MESP cantilevers (Veeco Metrology, Inc. Chadds Ford, PA, USA) in LiftMode. In order to enhance detection sensitivity of small super-paramagnetic aggregates, the sample substrate was mounted on a 2-kG permanent magnet with the magnetization direction normal to the probe tip and substrate. Since the magnetic probe tip, sample, and magnet are all aligned, the SPIO aggregates locally intensify magnetic field lines between the probe tip and the external magnet, resulting in a net attractive gradient on the probe tip. Using commercially available magnetic SPM probes, typical threshold detection of SPIO aggregates is in the range of 20 nm to 30 nm in diameter.

Confocal microscopy

MDA-MB-231 cells (2×10^4 cells/well) were seeded on cover glass in a 24-well tissue culture plate for 24 h after which the medium was removed, and 1.2 mL Earle's Balance Salt Solution (EBSS) was placed in each well. The free or scL-complexed SPIO nanoparticles [20% fluorescein labeled SPIO (14 nmol) and 80% non-fluorescein labeled SPIO (56 nmol)] in a volume of 300 μ L were added per well to a total volume of 1.5 mL/well. After incubation for 4.5 h at 37 °C, the EBSS was removed, and 1.0 mL of fresh complete medium was added and the cells incubated for an additional 1.0 h. The medium was then removed, the cells washed twice with PBS, fixed with 4% paraformaldehyde for 15 min, and washed twice with PBS. Subsequently, 100 μ L of 600 nM DAPI solution (Molecular Probes Invitrogen, Eugene, OR, USA) was added, and incubated at room temperature for 2 min. The cells were again washed twice with PBS. The cover glasses were mounted using the Prolong Antifade Kit (Molecular Probes Invitrogen, Eugene, OR, USA) and the samples were imaged with an Olympus 1X-70 laser confocal scanning microscope imaging system (Olympus Center Valley, PA, USA) equipped with an upright confocal microscope at a magnification of 60X.

In vitro Prussian blue assay

MDA-MB-231 or PANC-1 cells (8×10^4 cells/well) were seeded in a 6-well tissue culture plate. 24 h later the culture medium was removed, and 1.2 mL EBSS was placed in each well. The scL-complexed or free SPIO nanoparticles, at 70 nM SPIO was added to each well. After incubation for 4.5 h at 37 °C, the EBSS was removed, fresh complete media was added, and cells were incubated for an additional 1.0 h. The medium was removed, and the cells were washed with PBS three times after which they detached from the culture plate using a cell scraper, and washed three times with PBS. For the Prussian blue assay, 100 μ L of 1.0 M HCl was added to the pellet, and the mixture incubated at 37 °C for 30 min. Equal volumes of the prepared acidic samples and potassium ferrocyanide were mixed and incubated at 37 °C for 30 min (35). The absorption of the samples was determined at a wavelength of 711 nm using a Beckman DU640 spectrophotometer (Beckman Coulter, Inc. Fullerton, CA, USA). Iron determinations were performed in duplicate.

In vivo tumor targeting studies

Human pancreatic PANC-1 cells (1×10^5 cells per mouse) suspended in Matrigel™ collagen basement membrane matrix (BD Biosciences, Bedford, MA, USA) were subcutaneously inoculated into female athymic nude (nu/nu) mouse. When the tumor were at least 0.5 cm³, the mouse was intravenously injected once with the scL-SPIO complex carrying a total of 349 μ g of SPIO, 44.7 μ g of which was fluorescein labeled SPIO. 5% dextrose was included as an excipient. 4.5 h after injection, the tumor, liver, spleen and lung were excised and examined under a fluorescence microscope with an exposure time of 5 seconds (Nikon SMZ-1500 EPI-Fluorescence stereoscope). The organs were fixed in 10% formaldehyde, embedded and sectioned. The mounted sections were stained by Prussian blue using the Sigma Prussian blue stain kit as per the manufacturer's directions. All animal experiments were conducted under humane conditions and in accordance with the approved Georgetown University Animal Care and Use Committee policies and guidelines.

Statistical analysis

Each *in vitro* experiment was performed three times, with four wells/sample/experiment. SigmaStat statistical analysis software (Systat Software, Inc, San Jose Ca, USA) was used to analyze the experimental data by the Student's t-test. The results are presented as mean $\pm$ SD.

Results

Encapsulation of SPIO within the scL complex

Our previous studies have shown that the payload (plasmid DNA, siRNA) is encapsulated within the liposome of the scL nanocomplex (26,36). To confirm that this is also the case with SPIO, scanning probe microscopy (SPM), including fluid SPM, phase contrast and magnetic force microscopy (MFM) was performed using the conditions described above in Methods.

The SPM images surface topography in tapping mode by oscillating the tip and cantilever to which it is attached close to the cantilever resonance frequency. A feedback circuit maintains the oscillation of the cantilever at constant amplitude. This constant amplitude is given by a set point that is somewhat smaller than that of the freely oscillating cantilever. Because the SPM tip interacts with the surface through various small forces, there is a phase shift between the cantilever excitation and its response at a given point on the surface. For an inhomogeneous surface, the tip-surface interactions will vary according to surface charge, steep topographical changes, and mechanical stiffness variations, for example.

When examined using fluid SPM, intact liposomes encapsulating the SPIO exhibited a comma-like deformation in the topographical image due to the interaction of the complex with the SPM probe tip (data not shown). However, when adsorbed onto a freshly cleaved mica substrate the cationic liposomes rupture on the strongly negatively charged mica. The phase image shown in Figure 2A reveals the presence of an aggregate surrounded by a lipid patch. The lipid appears dark since the interaction of the negatively charged Si_3N_4 SPM probe tip and cationic lipid is attractive. Encapsulation is further confirmed upon examination of the scL-SPIO complex by MFM after drying of the samples on the substrate. Comparison of the simultaneously obtained topographical (Figure 2B) and MFM images (Figure 2C) demonstrate that a magnetic field due to the SPIO corresponds to the particles seen on the topographical image.

scL-SPIO nanocomplex sizing

As previously reported, the magnetic iron oxide nanoparticles were about 10 nm in diameter and displayed superparamagnetic properties (28,29). These SPIO nanoparticles were encapsulated within the cationic liposome, the surface of which is decorated with TfRscFv that serves to target the nanocomplex to the tumor cells (scL-SPIO). DLS (mean $\pm$ S.E. of 4–6 measurements) was used to assess the difference in size between the TfRscFv/liposome without the SPIO (scL), and the full scL-SPIO complex. The size (Intensity average) of the scL was 132.0 ± 25.1 nm, while the size of the full scL-SPIO complex was 157.4 ± 12.5 nm. The polydispersity indexes, a measure of the size distribution within the sample, were 0.282 ± 0.02 and 0.210 ± 0.02 , respectively, confirming the uniform size of the complex. The Zeta potentials of the samples were positive, with values of 32.4 ± 2.2 mV and 29.4 ± 1.2 mV for the scL and scL-SPIO, respectively. Students t-test confirmed that there was no statistical difference in either size or zeta potential between the scL and scL-SPIO complexes. Thus, encapsulation of the SPIO did not appreciably alter the size or charge of the scL complex. Hence, the scL-SPIO complex is clearly in the nanosize range and maintains a positive charge which is important for binding to negatively charged cells.

Surface modification and fluorescent linkage

To confirm that the scL complexed SPIO nanoparticles were indeed internalized by the target cancer cells rather than simply bonding to the surface of the cells, and to visualize the localization of SPIO nanoparticles in the cells, the SPIO nanoparticles were surface modified and labeled with fluorescein as shown in Figure 1. The FTIR spectra of unmodified and APTS modified SPIO nanoparticles are shown in Figure 3A and 3B, respectively. The FTIR spectrum of the APTS modified SPIO nanoparticles shows an increase in the band at 1035 cm^{-1} which

indicates Si-O bonding on the SPIO nanoparticles surface (37). A new band at 1131 cm^{-1} may be attributed to C-N stretching modes (13). The peaks at 1350 and 1550 cm^{-1} indicate the presence of the primary amine on the SPIO nanoparticle surface (37). Further, the peak at 2930 cm^{-1} indicates the $-\text{CH}$ stretch present on the SPIO nanoparticle surface. To confirm that we had in fact surface modified the SPIO particles with APTS, we also determined the number of amino groups on the modified SPIO particles by the surface chemical reaction method (32). The results indicated that there were $399\text{ }\mu\text{mol}$ of NH_2/g SPIO particles after APTS modification. No amino groups would be present on unmodified SPIO nanoparticles. Therefore, both FTIR spectroscopy and the quantitative assay confirmed that APTS successfully surface modified the SPIO nanoparticles.

Next we conjugated biotin to the SPIO-APTS particles using Sulfo-succinimidyl-6-(biotin-amido) hexanoate (Sulfo-NHS-LS-Biotin), the most commonly used biotinylation reagent, which reacts efficiently with primary amine groups ($-\text{NH}_2$) to form stable amide bonds (38). Quantitative measurement of the conjugated biotin on the SPIO nanoparticles revealed that the amount of biotin on the SPIO nanoparticles was $318\text{ }\mu\text{mol}/\text{g}$ SPIO nanoparticles. Subsequently, fluorescein conjugated streptavidin was immobilized on the biotin labeled SPIO particles.

Figure 4A shows the fluorescence image of the fluorescein labeled SPIO nanoparticle obtained by confocal microscopy. The SPIO nanoparticles show a very strong fluorescence signal. To demonstrate that the fluorescence observed in Figure 4A was associated with the SPIO nanoparticles, a comparison between the level of fluorescence and SPIO amount was assessed. The fluorescence of increasing amounts of fluorescein labeled SPIO nanoparticles in a total volume of $50\text{ }\mu\text{L}$ of PBS was measured at an excitation wavelength of 485 nm and emission wavelength of 535 nm using a fluorescence spectrophotometer [VICTOR 2 1420 Multilabel Counter, (Wallac/PerkinElmer, Shelton, CT, USA)]. The results shown in Figure 3B suggest that the fluorescence is associated with the SPIO nanoparticles. Thus, we were able to successfully bind the fluorescence to the SPIO.

Assessment of cellular uptake by confocal microscopy

Confocal imaging was used to compare the cellular uptake of the SPIO when delivered as either scL-SPIO, liposome-SPIO without the targeting moiety (L-SPIO) or free SPIO. MDA-MB-231 human breast cancer cells were incubated with free SPIO, scL-SPIO, or L-SPIO at 70 nmol SPIO nanoparticles, 20% of which (14 nmol) were fluorescein tagged. Untreated (UT) cells were used as control. As shown in Figure 5, at 4.5 h post-incubation, there is a significantly higher level of uptake of fluorescently labeled SPIO by the cells incubated with the ligand targeted scL-SPIO complex as compared to either the unliganded (L-SPIO) or free SPIO, where little if any fluorescence is observed. Differential interference contrast (DIC) images revealed the morphology of the cells and demonstrated that the fluorescence seen after scL-SPIO incubation was associated with the cells. Moreover, with the scL-SPIO complex the fluorescence distribution was observed throughout the cells, in both the cytoplasm and the nucleus. These results show that complexing SPIO nanoparticles with our targeted delivery system can significantly increase the level of SPIO uptake by tumor cells.

Quantitation of in vitro cellular uptake

Using the fluorescently labeled SPIO nanoparticles we can compare and correlate the amount of fluorescence and SPIO in the same cells. Thus, the level of fluorescence (via fluorescence spectroscopy) and amount of iron (via Prussian blue) were measured in MDA-MB-231 cells incubated with either scL-SPIO or L-SPIO. The results (mean $\pm$ SD) are shown in Figure 6A and 6B. Similar results were obtained with both the fluorescence and Prussian blue assays. There was a significant difference between the complex with and without the targeting moiety. Inclusion of the TfRscFv in the complex increases the uptake by 2–3 fold whether assessed by

fluorescence or iron content with p values as determined by the Students t-test of $p \leq 0.001$ for both.

To demonstrate that these results were not tumor cell line specific, we also compared the *in vitro* uptake of scL-SPIO or L-SPIO in both human breast and human pancreatic cancer cells using the Prussian blue reaction. Cellular uptake with free SPIO was also assessed in these experiments. As shown in Figures 7A and 7B, the level of iron detected in both the breast and pancreatic cell lines after incubation with the complete complex was at least 2.5 fold greater than obtained with the complex minus the TfRscFv. Moreover, delivery of SPIO by the scL nanocomplex resulted in ~11 fold increase in SPIO uptake as compared to free SPIO in both the breast and pancreatic cancer cell lines. In all of the above assays the differences between scL-SPIO and either L-SPIO or Free SPIO were statistically significant ($p \leq 0.001$). Taken together these studies demonstrate that inclusion of the SPIO nanoparticles in the nanoimmunoliposome complex can lead to enhanced uptake of the particles by tumor cells. Moreover, this efficient uptake is mediated by the addition of the TfRscFv on the surface of the liposome.

***In vivo* tumor targeting**

As initial proof-of-principle studies to assess the applicability of this approach for potential clinical use we examined the ability of the scL-SPIO complex to specifically target and deliver the scL-SPIO to tumors in an *in vivo* mouse model of human pancreatic cancer after systemic administration. Mice bearing PANC-1 subcutaneous tumors were intravenously (via the tail vein) injected with the scL-SPIO complex carrying 349 μg of SPIO, 12.7% of which was fluorescently labeled. 4.5 h post-injection, the animal was humanely euthanized and the tumor, liver, spleen and lung excised and examined with a fluorescence microscope. Figure 8 shows the same field photographed in bright-field and with fluorescence imaging. It can be clearly seen that the tumor cells display a very strong fluorescence signal. However, only very weak, or no, fluorescence was evident in the other tissues examined. After fixing, embedding and mounting, the same tissues were also stained by Prussian blue to assess the presence of SPIO nanoparticles in the cells. The red color indicates nuclei, and the pink is cytoplasm, while the SPIO nanoparticles stain blue. Very strong blue color can be seen throughout the tumor in the tumor cells, both in the cytoplasm and in the nucleus. However, while blue stained macrophage-like (Kupffer) cells are evident throughout the liver, no blue staining was evident in the lung aveolar, liver hepatocytes and spleen cells themselves in the normal tissues. These results confirm the efficient tumor specific uptake of SPIO after systemic administration when incorporated into the liganded liposome complex.

Discussion

The development of SPIO nanoparticles that specifically interact with tumor cells has been a field of increasing interest over the last few years. We have succeeded in developing a tumor-targeting nanoimmunoliposome technology to deliver superparamagnetic iron oxide (SPIO) nanoparticles *in vitro* and *in vivo*. The results described above establish that we have successfully encapsulated SPIO particles within our immunoliposome nanocomplex. This is demonstrated by the SPM studies, particularly the phase contrast images, where the SPIO particles are shown surrounded by the ruptured liposomal shell. Encapsulation is further established by comparison of the topographical and MFM images where the magnetic signal precisely coincided with the nanocomplexes identified by topography. Furthermore, the SPM imaging confirms the nanosize of the scL-SPIO complex obtained by DLS. The particle shown by phase imaging (Figure 2A) is less than 200nm.

Due to the presence of the anti-transferrin receptor single chain antibody fragment as the targeting moiety, the SPIO nanoimmunoliposome complex resulted in a significant increase

in SPIO uptake as compared to free SPIO and the unliganded complex *in vitro*. This increase in intracellular uptake of SPIO when encapsulated within the scL complex was shown in two different human cancer cell lines, indicating the general applicability of this approach for use in efficient delivery of SPIO to various cancers.

Most significantly, systemic administration of the scL-SPIO complex resulted in tumor specific accumulation of fluorescent-labeled SPIO. The tumor-targeting ability of the scL-SPIO nanocomplex is demonstrated in Figure 8, where neither fluorescence microscopy imaging, nor Prussian blue staining detected the presence of SPIO particles in the normal cells examined, including the liver. Previous studies using the scL complex to deliver other fluorescent payloads have shown that there is very little if any auto-fluorescence by PANC-1 tumors using the same conditions and settings used in these studies (39, unpublished data). These results correlate with our previously published results with our ligand-liposome complex carrying plasmid DNAs or siRNA as the payload (22,26,39,40). In these studies we also found that normal hepatocytes, lung alveolar, bone marrow or gut cells were not transfected, while the adjacent tumor cells were. These findings are attributed in part to the fact that although TfR expression has been observed on liver cells, albeit at a significantly lower level than on tumor cells (41), the “leakiness” of tumor vasculature serves to increase the accumulation of the complex in the tumor milieu as compared to the normal tissues. In addition, our previous *in vitro* optimization of the complex to preferentially bind to and transfect tumor over normal cells (22,23) also plays a role in the tumor specific uptake. However, since this is not a sterically stabilized liposome complex (i.e. does not include PEG), it is taken up by macrophage-like cells, including Kupffer cells in the liver. A number of such macrophage-like cells are seen in the liver in these studies. Although they exhibit a blue color indicative of the presence of iron, hepatocytes themselves do not, confirming that the scL-SPIO complex is not taken up by the non-tumor tissue cells. The small number of these macrophage-like cells among a preponderance of cells in the normal tissues would not be detectable by fluorescence.

The goal of the studies described here is to develop a tumor-targeting nanoimmunoliposome complex that can be used to enhance the utility of SPIO particles as magnetic resonance imaging agents or for use in anti-cancer hyperthermic therapy.

Free SPIO particles are currently in use as an MRI contrast agent (9–11). However, free SPIO is only taken up by the reticuloendothelial system (RES), not by tumor cells. Thus, the current imaging uses for this agent are for visualization of lymph nodes to show that they do not contain tumor, for MRI imaging of the GI tract [ferumoxsil, (oral solution) Mallinckrodt, Hazelwood, MO, USA], and for clinical visualization of the liver, used both for an evaluation of Kupffer cell function, as in the evaluation of cirrhosis, and for the detection of liver metastases (9–11). At least 2 formulations for liver imaging are FDA approved or in Phase II trials (18). When used as an MRI contrast agent, SPIO, which usually has T2 and T2* effects, results in a decrease in signal (pixel) intensity. For lymph node evaluation, it is injected intravenously and then accumulates in normal lymph nodes changing them from moderate intensity to decreased intensity, indicating uptake by the RES (17,42–44). When used as a selective liver contrast agent, the free SPIO is in colloidal suspension with a mean particle size of 60 nm and is covered by carboxydextran. In this instance also, the SPIO accumulates in the RES (Kupffer cells) of the liver, decreasing hepatic MRI signal. It thus shows Kupffer cell function in cirrhosis (45) and provides a decreased intensity background for the detection of metastases and hepatic carcinoma, both of which lack Kupffer cells and hence do not show any change with SPIO (46,47). However, as shown here scL encapsulated SPIO is taken up into the tumor cells themselves. This should result in persistent decreased signal intensity of the tumor cells on the MRI images, and would provide potential advantages for imaging tumors of multiple types and locations (e.g. metastases) beyond the current limited utility of the free SPIO.

Encapsulation in the tumor-targeting complex also enhances the potential therapeutic application of SPIO as hyperthermic agents. SPIO particles have an overall magnetic moment which undergoes fluctuations due to orientation of the molecules when exposed to an alternating external magnetic field. This external AC magnet induces magnetic moment fluctuations and the magnetic energy is subsequently converted to thermal energy (1). Jordan et al (48) and Ito et al (49) have reported that such magnetically induced hyperthermia can kill cancer cells after intratumoral injection of the SPIO particles and exposure of the tumor to a localized magnetic field. Maier-Hauff et al (50) have reported that this approach is in clinical trials for brain and prostate cancer in Germany. The ability to specifically and efficiently systemically deliver the iron oxide particles to the tumors, both primary and metastatic, would significantly enhance the efficacy of this therapeutic approach.

Our approach also has an advantage over the other methods currently being used to achieve tumor targeting. Currently, biodegradable polymers have been widely used to coat or encapsulate SPIO nanoparticles (51), followed by chemical coupling of ligands such as folic acid (19) LHRH (6,35) or anti-EGFR antibody (21,52) to their surface. These chemical modification processes are complicated and often result in loss of the supermagnetic properties of the SPIO nanoparticles. However, in our approach, the SPIO is encapsulated by simple mixing of the targeting moiety, liposome and SPIO particles. Thus, the requirement for chemical modification or conjugation is eliminated, preserving the superparamagnetic characteristics of the SPIO.

In conclusion, a specific and efficient tumor targeting superparamagnetic iron oxide (SPIO) nanoimmunoliposome delivery system was successfully produced without complicated chemical manipulations of the SPIO. Our results show that SPIO can be delivered to tumor specifically and much more efficiently when incorporated into our complex than as free SPIO, a simple and promising approach to increase the local concentration of the SPIO in the target tissue and thus enhance the utility of SPIO, particularly as a diagnostic or therapeutic agent.

Acknowledgements

Grant support: NCI grant 5R01CA132012-02 (Chang, E.H.) Research grant from SynerGene Therapeutics, Inc. (SGT) (Pirolo K.F.). These studies were conducted in part using the Microscopy and Imaging, Histopathology and Tissue, and Animal Core Facilities supported by NCI Cancer Center Support grant and U.S. Public Health Service grant 2P30-CA-51008 and 1 S10 RR 15768-01. This investigation was performed in part in a facility constructed with support from Research Facilities Improvement grant C06RR14567 from the National Center for Research Resources, NIH.

The following lists any competing financial interests for the authors of this manuscript:

Chengli Yang	None
Kathleen Pirolo	Research funded by SynerGene Therapeutics, Inc (SGT)
John A. Dagata	None
Natalia Farkas	None
Antonina Rait	Consultant for SGT
Esther H. Chang	Consultant for SGT in which she has significant personal financial interest.

SGT has no involvement in study design; in the collection, analysis, interpretation of data; in the writing of the report; or in the decision to submit the report for publication

Abbreviations List

TfRscFv, anti-transferrin receptor single chain antibody fragment; SPIO, superparamagnetic iron oxide; scL, TfRscFv/Liposome; L, Liposome.

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Figure 1. Surface modification scheme to produce Fluorescent-labeled SPIO nanoparticles
(A) = Aminopropyl triethoxysilane (APTS) was used to modify the SPIO nanoparticles via a silanization reaction with the hydroxyl groups on the SPIO nanoparticle surface;
(B) = Sulfo-NHS-LS-Biotin was used to bind biotin to the resulting amino groups on the SPIO nanoparticle via the reaction between Sulfo-NHS and the amino groups;
(C) = Fluorescein conjugated streptavidin was immobilized on the biotin labeled SPIO nanoparticles.

Figure 2. Scanning probe microscopy (SPM) of scL—SPIO nanocomplex

(A) = Phase contrast image obtained by fluid SPM imaging of the scL-SPIO nanocomplex adsorbed onto freshly cleaved mica substrate; (B) = Topographic image of the scL-SPIO nanocomplex after drying on an aged poly-L-lysine mica substrate; (C) = simultaneously obtained MFM image of the same sample as in B.

Figure 3. FTIR spectra of unmodified and APTS modified SPIO nanoparticles
The spectrum was taken from 4000 to 500 cm^{-1} . (A) = Unmodified SPIO nanoparticles. (B) = APTS modified SPIO nanoparticles

A **B**

Figure 4. Relationship between fluorescence and amount of SPIO

(A) Fluorescein labeled SPIO nanoparticles in solution were mounted on glass slides and the fluorescence imaged using confocal microscopy.

(B) The fluorescence of increasing amounts of fluorescein labeled SPIO nanoparticles in 50 μ L of PBS was measured (excitation wavelength of 485 nm and emission wavelength of 535 nm).

Figure 5. *In vitro* localization of scL-complexed Fluorescein labeled SPIO nanoparticles in MDA-MB-231 cells by confocal microscopy

Confocal microscopy shows the localization of Fluorescein labeled SPIO nanoparticles in MDA-MB-231 cells after incubation with scL complexed SPIO (**scL-SPIO**); the complex without the targeting moiety (**Lip-SPIO**); uncomplexed SPIO (**Free SPIO**). Untreated (**UT**) cells were used as a control. **FITC** = fluorescence signal in the cells; **DAPI** = intercalation of DAPI in the chromosomal DNA imparts a blue color to identify the nucleus; **DIC** = differential interference contrast images

Figure 6. Quantitative comparison of scL-SPIO and L-SPIO uptake in MDA-MB-231 human breast cancer cells via Fluorescence and Prussian blue staining

(A) = The level of fluorescence obtained from half of the treated cells was measured by fluorescence spectrophotometry.

(B) = The iron content of half of the treated cells was measured using the Prussian blue assay with absorption at 711 nm. **L-SPIO** = complex minus the TfRscFv targeting moiety

Figure 7. *In vitro* uptake of SPIO in two human cancer cell lines via Prussian blue
(A) = Comparison of iron content between, scL-SPIO, L-SPIO (complex minus TfRscFv targeting moiety), and Free SPIO in MDA-MB-231 breast cancer cells.
(B) = Comparison of iron content between scL-SPIO, L-SPIO (complex minus TfRscFv targeting moiety) and free SPIO in PANC-1 pancreatic cancer cells.

Figure 8. *In vivo* tumor targeting by scL delivered fluorescently labeled SPIO nanoparticles
4.5 h after i.v. injection the tumor, lung, liver and spleen were excised and examined using a Nikon SMZ-1500 EPI-Fluorescence stereomicroscope. The samples were subsequently fixed, and stained with Prussian blue. The Brightfield and Fluorescence images show the identical field of the sample.