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Development of Receptor Targeted Magnetic Iron Oxide Nanoparticles for Efficient Drug Delivery and Tumor Imaging

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Abstract

The development of multifunctional nanoparticles that have dual capabilities of tumor imaging and delivering therapeutic agents into tumor cells holds great promises for novel approaches for tumor imaging and therapy. We have engineered urokinase plasminogen activator receptor (uPAR) targeted biodegradable nanoparticles using a size uniform and amphiphilic polymer-coated magnetic iron oxide (IO) nanoparticle conjugated with the amino-terminal fragment (ATF) of urokinase plasminogen activator (uPA), which is a high affinity natural ligand for uPAR. We further developed methods to encapsulate hydrophobic chemotherapeutic drugs into the polymer layer on the IO nanoparticles, making these targeted magnetic resonance imaging (MRI) sensitive nanoparticles drug delivery vehicles. Using a fluorescent drug doxorubicin (Dox) as a model system, we showed that this hydrophobic drug can be efficiently encapsulated into the uPAR-targeted IO nanoparticles. This class of Dox-loaded nanoparticles has a compact size and is stable in pH 7.4 buffer. However, encapsulated Dox can be released from the nanoparticles at pH 4.0 to 5.0 within 2 hrs. In comparison with the effect of equivalent dosage of free drug or non-targeted IO-Dox nanoparticles, uPAR-targeted IO-Dox nanoparticles deliver higher levels of Dox into breast cancer cells and produce a stronger inhibitory effect on tumor cell growth. Importantly, Dox-loaded IO nanoparticles maintain their *T2* MRI contrast effect after being internalized into the tumor cells due to their significant susceptibility effect in the cells, indicating that this drug delivery nanoparticle has the potential to be used as targeted therapeutic imaging probes for monitoring the drug delivery using MRI.

Keywords

Magnetic Iron Oxide Nanoparticles; uPAR; Targeted Nanoparticle; Breast Cancer; Drug Delivery Nanoparticle; Doxorubicin

1. INTRODUCTION

Breast cancer is the most common type of cancer and the second leading cause of death among women. In the United States alone, estimated new cancer cases in 2008 is 184,450 and about 40,930 cancer death are due to breast cancer. Over 2 millions of women have been treated for breast cancer and are at risk of cancer recurrence and/or metastasis.¹ Therefore, novel approaches for selective and efficient delivery of therapeutic agents into primary and metastatic breast cancers are in urgent need to improve the treatment efficacy and survival of breast cancer patients. Furthermore, new methods are highly desirable for timely monitoring therapeutic responses and optimization of therapeutic strategies.

One of the major challenges in cancer treatment is the inability to deliver sufficient amounts of therapeutic agents into the tumor cells in a tumor mass to maximize the therapeutic effect and minimize the side effects on normal tissues. Recent advances in nanotechnology have shown the feasibility of using nanoparticles for targeted drug delivery and simultaneous tumor imaging.²⁻⁸ With unique pharmacokinetics, nanoparticles with sizes between 10 and 100 nm have a prolonged circulation time compared with other small molecules since they are too large to be excreted by the kidney in a short time but are small enough to avoid significant uptake by the reticuloendothelial system within the liver or spleen.⁹ Additionally, it has been shown that tumor vasculatures are not well developed and usually leaky, which allow for the nanoparticles with sizes smaller than 100 nm passing through the endothelial cell layer and entering into the tumor mass.^{8, 9} Delivery of therapeutic agents using nanoparticles offers a chance to improve the water solubility and bioavailability of hydrophobic drugs. Because of large surface area and diverse surface chemistry, one of advantages of nanoparticles over other systems is that it potentially combines the delivery of several functionalities and applications simultaneously to a tumor mass.^{10, 11} Furthermore, targeting ligands that bind to cellular receptors highly expressed in tumor cells can be conjugated to the nanoparticles to facilitate selective and efficient delivery of drugs into tumor cells, which could potentially overcome drug resistance in tumor cells due to excretion of the drug out of tumor cells by multi-drug resistant mechanisms.¹²⁻¹⁵

Recently, magnetic iron oxide (IO) nanoparticles are becoming increasingly attractive in biomedical applications for developing magnetic resonance imaging (MRI) contrast agents as well as biocompatible and biodegradable drug carriers.^{2, 4, 6, 16-18} IO nanoparticles have unique paramagnetic properties, which generate significant susceptibility effect resulting in strong T_2 and T_2^* effects and MRI contrast. Non-targeted IO nanoparticle have been used in clinical settings and have proven to be safe for human use as MRI contrast agents for detecting liver tumor lesions or lymph node metastases in patients.^{19, 20} During the last several years, targeted superparamagnetic iron oxide nanoparticles for imaging of MUC1, folate receptor, or Her-2/Neu have been developed as MRI contrast agents and have shown the feasibility of MRI of tumors in animal tumor models.²¹⁻²³

In this study, we developed urokinase plasminogen receptor (uPAR) targeted IO nanoparticles by conjugating a recombinant protein containing the first 135 amino acids of the amino-terminal fragment (ATF) of the receptor binding domain of urokinase plasminogen activator (uPA) to the surface of the IO nanoparticles (ATF-IO). We further

developed a method to encapsulate hydrophobic drugs onto the ATF-IO nanoparticles. Our results show that ATF-IO drug delivery nanoparticles are able to specifically bind to and be internalized by uPAR-expressing tumor cells. Furthermore, uPAR-targeted IO nanoparticles can efficiently deliver chemotherapy drugs into breast cancer cells, producing cytotoxic effect and MRI contrast in the cells.

2. MATERIALS AND METHODS

2.1. Production and Purification of Recombinant ATF Peptides

A cDNA fragment encoding amino acids 1–135 of mouse uPA, isolated by PCR amplification, was cloned into pET101/D-TOPO expression vector (Invitrogen, Carlsbad, CA). Recombinant ATF peptides were expressed in *E. coli* BL21 (Invitrogen) and purified from bacterial extracts under native conditions using a Ni²⁺ NTA-agarose column (Qiagen, Valencia, CA).

2.2. Preparation of ATF-IO Nanoparticles

IO nanoparticles were prepared using iron oxide powder as the iron precursor, oleic acid as the ligands, and octadecene as the solvent as described.²⁴ The core size and hydrodynamic size of the IO nanoparticles were measured using transmission electron microscopy (TEM), and light scattering scan, respectively. We used IO nanoparticles with 5 or 10 nm core size for this study. As shown in Scheme, the particles were coated with amphiphilic triblock polymers reported previously,⁸ which stabilizes IO nanoparticles in water and provides reactive carboxyl groups on the particle surface for bioconjugation. To reduce nonspecific binding and uptake by cells, short PEG chains with hydroxyl groups were conjugated to a portion of the carboxyl side groups on the polymers. ATF peptides were conjugated to the surface of the IO nanoparticles via cross-linking of carboxyl groups to amino side groups on the ATF peptides as shown in Scheme. Briefly, the polymer-coated IO nanoparticles were activated with ethyl-3-dimethyl amino propyl carbodiimide (EDAC, Pierce, Rockford, IL) and sulfo-NHS for 15 min in 20 mM pH 5.0 Borate buffer. After purification using Nanosep 100 k OMEGA (Pall Corp, Ann Arbor, MI), activated IO nanoparticles were reacted with ATF peptides at a molar ratio IO:ATF of 1:20 in 10 mM pH 8.5 Borate buffer and at 4 °C overnight, generating ATF-IO nanoparticles. The final ATF-IO conjugates were purified using Nanosep 100 k column filtration.

2.3. Conjugation of IO Nanoparticle with PEG-Diamine

PEG-Diamine (MW. 2,000 Scientific Polymer Products, Inc) was added to amphiphilic polymer coated IO nanoparticle and rocked at room temperature for 1 hour. After adding EDAC at room temperature for overnight, IO nanoparticles with PEG-Diamine (IO-NH₂) were collected by centrifugation at 45,000 rpm for 1 hour, and washed with Borate buffer (pH 5.0) twice.

2.4. Encapsulation of Chemotherapy Drugs onto IO Nanoparticles

Doxorubicin HCl (Polymed Therapeutics, Houston, TX) was dissolved in methanol and then added to non-targeted IO nanoparticles with 5 or 10 nm core size and either carboxyl or amine surface functional groups, or 10 nm ATF-IO nanoparticles at a ratio of 1 mg Dox to 3

mg of iron (Fe). After rotating at room temperature for 4 hrs, free Dox was separated from the encapsulated Dox using Nanosep 100 k column filtration. Hydrodynamic sizes of various IO-Dox nanoparticles were determined using Zetasizer Nano (Malvern Instruments Inc., Southborough, MA). To determine the amount of Dox in each IO nanoparticle, three standard curves of Dox were generated to correct the quenching effect of the IO particles on the fluorescence intensity of Dox detected using an excitation wavelength of 480 nm and emission wavelength of 580 nm (Spectra Max, Molecular Devices, Sunnyvale, CA). The first standard curve contains the fluorescence intensity of Dox alone at different concentrations. From this curve, we find that the concentrations of Dox in the samples should be diluted to 2 to 70 $\mu\text{g/ml}$ to obtain adequate Dox concentrations since the fluorescence intensity of Dox can be quenched at higher Dox concentrations ($>150 \mu\text{g/ml}$). The second standard curve was done by changing the concentrations of Dox while using a constant concentration of IO nanoparticles to quantify the quench effect of IO nanoparticles on Dox fluorescence. The third standard curve was generated using a constant Dox concentration while increasing the concentration of IO nanoparticles. Concentration of Dox encapsulated onto the IO nanoparticle was determined by measuring fluorescence intensity of Dox and calculating from the standard curve of Dox in the presence of IO nanoparticles. Total number of IO nanoparticles in each sample was derived from measuring absorption optical density (OD) at 500 nm for Fe concentration and then calculating the numbers of IO nanoparticles using a standard formula. For example, 1 mg of Fe equals to 0.906 nmol of 10 nm IO nanoparticles or 7.255 nmol of 5 nm IO nanoparticles. The numbers of Dox molecules in each IO nanoparticle were calculated by dividing total number of Dox with the total number of IO nanoparticles in each sample.

2.5. pH Sensitive Drug Release in Solution

10 μl of various IO-Dox nanoparticles were placed in 100 μl of PBS buffer with pH 4, 5, 6, or 7. After incubating at 37 $^{\circ}\text{C}$ for 2 hrs, free Dox molecules in the buffer were separated from the IO nanoparticles using Nanosep 100 k column filtration. Dox fluorescence intensity in the solution was measured as described previously and the amount of free Dox was calculated from the Dox-only standard curve. The percentage of drug release was obtained using the total amount of Dox from input IO-Dox nanoparticles as 100%.

2.6. Breast Cancer Cell Lines

Mouse mammary carcinoma cell line 4T1 was kindly provided by Dr. Fred R. Miller in the Barbara Ann Karmanos Cancer Institute, Detroit, MI. Human breast cancer cell line MDA-MB-231 was obtained from American Type Culture Collection (ATCC, Rockville, MD). Both cell lines were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum.

2.7. Detection of the Binding Specificity of ATF Peptides Using Immunofluorescence Labeling

Acetone fixed 4T1 mouse mammary tumor cells were incubated with 10 $\mu\text{g/ml}$ of His-tagged recombinant mouse ATF peptides for 1 hr and then with anti-his tag mouse monoclonal antibody for 1 hr. After incubating with Alexa-fluor 555 labeled polyclonal rabbit anti-mouse antibody for 30 min, the cells were examined under a fluorescence

microscope (Zeiss Axioplan with Axiovision software, Carl Zeiss MicroImaging, Inc, Thornwood, NY).

2.8. ATF-Mediated Internalization of IO-Dox Nanoparticles in Cancer Cells

Cancer cells were cultured in 96 well tissue culture plate for 24 hrs and then incubated with 20 pmol/ml of non-targeted IO, ATF-IO, ATF-IO-Dox or non-targeted IO-Dox nanoparticles at 37 °C inside 5% CO₂ tissue culture incubator for 24 hrs. After washing with PBS buffer, the cells were examined under an inverted fluorescence microscope (Olympus IX-50). Some wells were washed and fixed with 2% formaldehyde. Prussian blue staining was performed to determine the presence of the IO nanoparticles inside the cells using a staining solution containing 1:1 mixture of 5% potassium ferrocyanide and 5% HCl acid at 37 °C for 60 min.²⁵ The cells were then examined under an inverted microscope.

2.9. Cell Proliferation Assay

3×10^3 of mouse mammary tumor 4T1 and human breast cancer MDA-MB-231 cells were plated in 96-well culture plates for overnight. The culture medium was then replaced with serum free medium containing IO-Dox or ATF-IO-Dox nanoparticles at a Dox concentration of 0.25 or 0.5 μ M. Control groups were treated with non-targeted or targeted IO nanoparticles without Dox but have equal concentration of the IO nanoparticle as ATF-IO-Dox, or with 0.25 or 0.5 μ M of free Dox. Cells were incubated with above mentioned agents for 2 hrs at 37 °C in 5% CO₂ tissue culture incubator. After replacing the culture medium and IO nanoparticles with fresh culture medium, the cells were cultured for 48 hrs and then examined using inverted fluorescence microscope to determine the presence of Dox fluorescence in the cells. The percentage of tumor cell growth inhibition was determined by Crystal Violet cell proliferation assay. Briefly, the cells were fixed in 96 well plate using 4% formaldehyde in PBS for 20 min and then washed with PBS. 0.5% crystal violet in H₂O was added to the wells for 20 min and unstained dye was washed away with H₂O. After air-dry, 100 μ l of Sorenson's solution containing 30 mmol/L sodium cit-rate, 0.02 mol/L HCl, and 50% ethanol at room temperature for 20 min was added to the well to elute the dye, and the optical density was read at 590 nm using a microplate reader (SpectroMax, Molecular Devices). Absorbance value was normalized to the value of the control cell group without treatment to obtain the percentage of viable cells. Each treatment group was performed in triplicate and similar results were obtained in three separate experiments.

2.10. In Vitro MRI Scan

Human breast cancer MDA-MB-231 cells were incubated with serum free medium containing 20 pmol of ATF-IO or ATF-IO-Dox nanoparticles at 37 °C for 2 hrs. After washing with PBS buffer, the cell pellets were placed in 200 μ l PCR tubes and then scanned on a 4.7T MRI scanner (Philips Medical Systems, Bothell, WA) using a multi-echo T_2 weighted fast spin echo sequence which simultaneously collects a series of data points at different echo times (TE_i , $i = 6$) for T_2 relaxometry measurement. T_2 relaxation time of each sample was calculated by fitting the decay curve of MRI signals (M_i) over different TE points on a pixel-by-pixel basis using the non-linear mono-exponential algorithm of $M_i = M_0 \cdot \exp(-TE_i/T_2)$.

3. RESULTS

3.1. Production and Characterization of uPAR-Targeted IO-Dox Nanoparticles

In this study, we synthesized the magnetic IO nanoparticles with uniform core sizes of 5 nm or 10 nm (Fig. 1(A)). The purity of the purified recombinant ATF peptides was confirmed by sodium dodecyl sulfate (SDS)-PAGE gel and the peptides appeared as monomers (17 KDa) or dimers (35 KDa) (Fig. 1(B)).

Our drug loading approach takes advantage of hydrophobic interaction between the hydrophobic inner layer of the IO nanoparticles and the hydrophobic drug molecules. We found that Dox can be efficiently incorporated onto the amphiphilic block polymer coated IO nanoparticles by simply mixing the IO nanoparticles with appropriate concentrations of Dox. We found that a Dox to IO (Fe) ratio of 1 mg Dox: 3 mg of Fe results in IO-Dox nanoparticles with a high level of encapsulated Dox molecules while avoids aggregation of particles at a high Dox concentration. Using the method described in the Materials and Methods, over 85% (5 nm IO) and 95% (10 nm IO) of added Dox were incorporated onto the IO nanoparticles. Moreover, we found that the core size of IO nanoparticles affects the amount of encapsulated Dox molecules since a 10 nm IO nanoparticle incorporate nearly eight times more Dox molecules than that of a 5 nm IO nanoparticle (Table I). However, the percentages of Dox dry weight compared to either the weight of the IO nanoparticles or weight of Fe in 5 nm IO nanoparticles are very close to those of 10 nm IO-Dox nanoparticles since there are more 5 nm IO nanoparticles in a given IO or Fe weight (Table I). Additionally, it seems that surface modification affects the efficiency of Dox encapsulation. For example, modifying the surface carboxyl groups of the IO nanoparticles with PEG-diamine reduced the number of incorporated Dox from 652 molecules detected in IO nanoparticles with the carboxyl surface to 296 molecules, which is about 50% decrease. Conjugation of ATF peptides to the surface of the IO nanoparticles also decreases the drug encapsulation by 26% (Table I).

As drug delivery vehicles, it is important for the nanoparticles to have a compact size so that they can navigate through immature and leaky tumor vessels and efficiently enter into the tumor mass. We examined the hydrodynamic sizes of the Dox-loaded IO nanoparticles. Our result shows that IO nanoparticles encapsulated with Dox have relatively compact and uniform size distribution (Table II). ATF-IO-Dox nanoparticles display a hydrodynamic size of $33 \mu\text{m} \pm 7 \text{ nm}$, which is within the desired size range for targeted drug delivery nanoparticle (between 10 to 100 nm) (Table II). Result of Zeta potential (ξ) measurement indicates that Dox-loaded, carboxyl surface IO nanoparticles are slightly negatively charged with ξ values around -15 mV (10 nm) or -18 mV (5 nm) (Table II). However, PEG-diamine modified IO-Dox nanoparticles have a ξ value that is close to neutral (-3.9 mV).

3.2. Examination of the Binding and Internalization of ATF Peptide or ATF-IO-Dox Nanoparticles to uPAR-Expressing Breast Cancer Cells

Next, we determined the efficiency of uPAR-targeted nanoparticle for drug delivery using uPAR expressing mouse mammary tumor cells (4T1) and human breast cancer MDA-MB-231 cells. Although it has been shown that the binding of ATF to uPAR receptor has

species specificity and human ATF peptides have a low binding affinity to mouse uPAR, our result showed that human cancer cells can interact with mouse ATF peptides. Therefore, the use of mouse ATF peptides has an advantage for future preclinical studies for determining target specificity and biodistribution of the drug-loaded nanoparticles in mouse tumor models derived from either mouse tumor or human tumor cells. First, we demonstrated that mouse ATF peptides bind to uPAR-expressing mouse mammary tumor cells using an anti-histidine tag antibody and immunofluorescence labeling (Fig. 1(C)). We then examined the ability of ATF peptides to mediate the internalization of IO nanoparticles. Incubation of human breast cancer MDA-MB-231 cells with the nanoparticles for 2 hrs resulted in the binding and internalization of ATF-IO into the cells but not non-targeted IO nanoparticles (Fig. 1(D)). After incubating 4T1 mouse mammary tumor cells with 1 μ M free Dox, equivalent Dox concentration of IO-Dox or ATF-IO-Dox nanoparticles for 24 to 48 hrs, the amount of Dox in each treatment group was examined under an inverted fluorescence microscope, given strong orange-red fluorescence produced by Dox. We found that encapsulation of Dox onto the IO nanoparticles markedly increases the intracellular concentration of Dox in the cells treated with non-targeted or uPAR-targeted IO-Dox nanoparticles for 24 hrs, compared to free Dox-treated cells. However, a much higher level of Dox fluorescence was detected in the cells treated with uPAR targeted ATF-IO-Dox nanoparticles (Fig. 1(E)). At 48 hrs following treatment, the majority of ATF-IO-Dox nanoparticle-treated cells had Dox-fluorescence in the cell nucleus and underwent cell death, suggesting that Dox may enter and incorporate into cellular DNA (Fig. 1(E)). In contrast, we did not detect cell death in the group treated with IO-Dox, despite of the presence of an intermediate level of Dox in the cytoplasm of the cells at 48 hrs (Fig. 1(E)).

3.3. pH Sensitive Release of Dox from IO-Nanoparticles

One of the most important aspects for a drug delivery vehicle is its ability to release the payload drug efficiently into cells. The amine group of Dox may facilitate its conversion to a charged molecule at low pH and becomes soluble in water. Therefore, we examined whether Dox can be released from IO nanoparticles in acidic pH, which resembles pH of intracellular vesicles such as endosomes (pH 5.5–6.0) and lysosomes (pH 4.5–5.0). Our result shows that Dox molecules can be efficiently released from the non-targeted or uPAR-targeted ATF-IO-Dox nanoparticles under acidic conditions after 2 hr incubation. Incubation of above nanoparticles in pH 6.0 buffer enables the release of 20–30% of Dox molecules from the drug loaded nanoparticles (Fig.2). At pH 5.0, 30 to 40% of encapsulated Dox were released from the nanoparticles. Furthermore, under pH 4.0 conditions, about 70 to 80% of encapsulated Dox were released into the buffer (Fig.2). Importantly, conjugation of ATF peptides to IO nanoparticles did not affect the drug release efficiency (Fig.2).

3.4. uPAR-Targeted IO-Dox Nanoparticles Produce a Strong Cytotoxic Effect on Breast Cancer Cell Lines

To evaluate the feasibility of the targeted IO-Dox nanoparticles for the treatment of breast cancer, we examined the cytotoxic effect of the targeted or non-targeted IO-Dox nanoparticles on MDA-MB-231 and 4T1 cell lines. Current data suggest that a low level of non-specific uptake of IO-Dox nanoparticles was present following continuous incubation with the cells for over 24 hrs (Fig.1(E)). For this experiment, we incubated the cells with

free Dox, IO-Dox or ATF-IO-Dox nanoparticles for 2 hrs, which allows for uPAR-targeted IO nanoparticles to be internalized by cells. The culture medium was then replaced with fresh medium and the cells were cultured without additional treatment for 48 hrs. We found that ATF-IO nanoparticles deliver a much higher level of Dox into tumor cells, compared to the equal concentration of free Dox or IO-Dox treated cell groups. For example, treatment of 4T1 cells with 0.5 μM of free Dox or non-targeted IO-dox did not show the accumulation of Dox fluorescence nor any cytotoxic effects on the cells (Figs.3(A and B)). However, strong Dox fluorescence was detected in the cells treated with 0.25 to 0.5 μM of ATF-IO-Dox nanoparticles (Fig.3(A)). High magnification fluorescence image shows that Dox fluorescence is located in the cell nucleus. A marked inhibition of tumor cell growth was also found in ATF-IO-Dox nanoparticle treated cells (Fig.3(B)). Similarly, 0.5 μM of free Dox did not induce death of human breast cancer cells. A low level of non-targeted IO-Dox was seen in MDA-MB-231 cells after 2 hr incubation, causing 50% of growth inhibition in the cells (Figs. 3(C and D)). However, over 90% of growth inhibition of MDAMB-231 cells was detected after treating the cells with 0.25 to 0.5 μM of Dox in ATF-IO-Dox nanoparticles. Our results demonstrate that the receptor targeted IO nanoparticles have a high efficiency in delivery of payload drugs into tumor cells to produce a strong anti-tumor effect.

3.5. Encapsulation of Dox Molecules onto ATF-IO Nanoparticles Does Not Affect T_2 Contrast of the IO Nanoparticle

A major goal of our research is to develop multifunctional nanoparticles for simultaneous drug delivery and tumor imaging. Our preparation of IO nanoparticles has strong T_2 shortening effect that leads MRI signal drop or “darken” contrast using T_2 weighted imaging. However, effect of MRI contrast after ATF-IO-Dox nanoparticles being internalized into cancer cells was not clear. To determine the feasibility of using IO-Dox nanoparticles as a MRI contrast agent, we examined MRI contrast effect of ATF-IO-Dox nanoparticles. MDA-MB-231 cells were incubated with ATF-IO or ATF-IO-Dox nanoparticles for 2 hrs and unbound nanoparticles were washed away by centrifugation. Results from MR imaging of ATF-IO-Dox or ATF-IO nanoparticle treated cell pellets showed that the ATF-IO-Dox labeled cells have a similar T_2 value as the cells labeled with ATF-IO particles without Dox (Fig. 4(A)). T_2 values in ATF-IO or ATF-IO-Dox labeled cells were four times lower than that of unlabeled cells, suggesting that ATF-IO-Dox nanoparticles can be used as a targeted drug delivery vehicle as well as a MRI contrast agent.

4. DISCUSSION

One of the most pressing needs in clinical oncology is the development of novel approaches for targeted delivery of therapeutic agents and timely assessment of therapeutic response of a given therapy in primary and metastatic tumor sites. The goal of this study is to develop multifunctional biodegradable nanoparticles for targeted delivery of therapeutic agents and for monitoring of drug delivery and treatment response using non-invasive tumor imaging. Up to date, exploring and developing the unique capability of such multifunctional nanoparticles have been challenging. Only limited numbers of studies have demonstrated the

possibility of using targeted nanoparticles for therapy and tumor imaging.^{2, 4, 6, 26} Specific delivery of chemotherapy drugs using targeted IO nanoparticles has the potential to increase the anti-tumor effect while decreases toxicity effect on normal tissues and organs. Doxorubicin (Dox) is one of the most frequently used chemotherapy drugs in treating breast cancer. However, it is well known that doxorubicin has a serious side effect: damaging the heart muscle that can lead to congestive heart failure and sometimes death.^{27,28} A higher risk for heart damage has been shown when patients received the combination therapy of doxorubicin with Herceptin, a monoclonal antibody to Her-2/neu that is given to Her-2/neu positive breast cancer patients.²⁹ However, a result of a clinical trial shows that encapsulation of Dox into pegylated liposomes produces equal efficacy but significantly less cardiotoxicity than conventional doxorubicin in breast cancer patients treated with the combination therapy of Dox and Herceptin.²⁹ Therefore, the development of targeted nanoparticles for delivering Dox may further enhance the selectivity and increase the amount of the drug locally in primary and metastatic breast cancer lesions, resulting in an enhanced therapeutic effect on tumor cells while reduce systemic side effects. The use of biodegradable magnetic iron nanoparticles as a drug carrier makes it possible for the development of cancer treatment in human since it requires multiple administrations in a long period of the time, or for monitoring the therapeutic response of primary and metastatic breast tumor lesions using MRI, which is a clinically relevant imaging modality.

Although several previous studies have shown that it is feasible to conjugate chemotherapy drugs onto different types of magnetic IO nanoparticles,^{3, 4, 6, 11, 30–33} several obstacles remain to be overcome to translate this approach into clinical applications. First, drug delivery nanoparticles should be stable and have a compact size to improve delivery efficiency. Traditional coating polymers for magnetic IO nanoparticles, such as dextran and PEG, often have weak core-shell interactions and can be easily detached from the surface of the nanocrystals, leading to the aggregation and eventually precipitation of the nanoparticles even under physiological conditions or simply during storage.^{34,35} In this study, we used a thin amphiphilic polymer layer to coat an IO nanoparticle resulting in a compact size and stable IO nanoparticle with active functional groups for bioconjugation. Next, to ensure the effectiveness of the therapy, it is important to incorporate sufficient amounts of the chemotherapy drugs onto the nanoparticles. Based on the properties of the drug, it can be covalently conjugated to the polymer coating of the nanoparticles or can be encapsulated into nanoparticles. In general, covalent linking may limit the amount of the drug molecules conjugated to each nanoparticle and have a low efficiency in releasing the drug upon entering into the cells. On the other hand, direct encapsulation of the drug molecules into the nanoparticles may carry more drug molecules into cells and release the drug efficiently. Since Dox is a hydrophobic molecule, we expect that it can penetrate the hydrophilic portion of the block polymer and reach to the hydrophobic surface of the IO nanoparticles and/or interact directly with the hydrophobic areas on the surface of the IO nanoparticles that are not covered entirely by the polymer. In this study, we showed that Dox molecules can be encapsulated into the amphiphilic polymer coated IO nanoparticles using a simple method. An advantage of using fluorescent Dox to optimize our nanoparticle drug delivery system is that it is easy to determine conjugation efficiency, to visualize specific targeting and therapeutic response in tumor cells, and to examine distribution of the targeted IO-dox

nanoparticles in normal and tumor tissues after systemic delivery. Our result suggests that the core size and surface areas of the IO nanoparticles affect amounts of encapsulated Dox molecules since a 5 nm IO nanoparticles have eight times lesser Dox molecules compared to that in a 10 nm IO nanoparticle. Importantly, Dox-loaded 5 or 10 nm IO nanoparticle preserves its compact size with an overall hydrodynamic size of 8 or 18 nm, respectively, which should facilitates intra-tumoral distribution as well as internalization of the nanoparticle by cells.

A critical aspect for engineering a drug delivery vehicle is the ability of the drug to be released from the drug carrier. The unique chemical structure of the Dox molecule makes it easier to convert to a charged molecule and become water soluble under lower pH condition, which facilitates the drug release in acidic intracellular vesicles such as endosomes and lysosomes. We demonstrated that high percentages of Dox molecules are released from the IO nanoparticle within 2 hrs of incubation at pH 4 to 5. Detection of high levels of Dox fluorescence in cytoplasm and nucleus of the ATF-IO-Dox nanoparticle treated tumor cells further supports the notion that Dox can be released from the targeted IO-Dox nanoparticles.

A major challenge for developing nanoparticle drug carrier is targeted delivery of the drug into tumor cells. Ideally, a targeting ligand should bind to a cell surface receptor that is upregulated in cancer cells with a high affinity and initiates efficient endocytosis that allows for the delivery of payload drug molecules into cells. uPA is a serine protease that converts plasminogen into plasmin to activate matrix metalloproteinases resulting in matrix degradation and promoting cell motility, metastasis and angiogenesis.³⁶³⁷ uPAR signaling pathway plays important roles in regulating tumor cell survival, migration, metastasis and tumor angiogenesis.³⁸ uPAR is highly expressed in many types of human tumor cells and active tumor stromal cells including intra-tumoral fibroblasts, tumor endothelial cells and macrophages.^{39–42} It has been shown that 60–90% of invasive breast cancer tissues express uPAR.³⁹ Elevated level of uPAR is associated with tumor aggressiveness, the presence of distant metastasis and poor prognosis of breast cancer patients.³⁶³⁹⁴³⁴⁴ Most importantly, internalization of uPAR upon binding to ATF of uPA allows targeted delivery of therapeutic agents into tumor cells by endocytosis, which may bypass the drug resistant machinery on the cell membrane.⁴⁵⁴⁶ Therefore, uPAR is an excellent cellular surface target for designing and engineering tumor targeting drug delivery and imaging nanoparticles. Previous study showed that uPAR is undetectable in the majority of normal tissues except for low levels in macrophages, granulocytes, thymus, kidney and spleen.⁴⁷ It has been shown that estimated levels of uPAR in breast cancer cells are 1 to 5×10^5 receptors/cell, while primary normal human mammary epithelial cells only have 2,500 receptors/cell.⁴⁸ Therefore, a significant difference in the level of uPAR between normal and tumor cells make it possible to selectively deliver IO-drug nanoparticles into tumor cells. In this study, we used a high affinity receptor binding domain of uPA (ATF, $K_d < 1$ nM)⁴⁹ to target IO-Dox nanoparticles and demonstrated that ATF-IO-Dox nanoparticles can be internalized into the uPAR-expressing tumor cells. Furthermore, Dox molecules can be released from the nanoparticles inside the cells and produce cytotoxic effects on breast cancer cells. Consistent with previous observations, non-specific uptake of IO-Dox nanoparticles at a high IO nanoparticle concentration is detected in cancer cells after incubation with the nanoparticles for a long time. However, the amount of Dox delivered by non-targeted IO-Dox

nanoparticles is not sufficient enough to kill the tumor cells. In contrast, uPAR targeted ATF-IO-Dox nanoparticle-treated cells have a markedly higher level of Dox within cancer cells and kill the mammary tumor cells within 48 hrs *in vitro*. Our results also suggest that ATF-mediated uptake of IO-Dox is a fast and efficient process. Dox fluorescence is not detected in the cells after a short time incubation of free Dox and non-targeted IO-Dox. However, incubation of the cells with equal concentration of ATF-IO-Dox nanoparticles results in a high level of Dox in tumor cells and induction of cell death. Our result from cell proliferation assay further shows that treatment of breast cancer cells with ATF-IO-Dox nanoparticles markedly reduced viable cells, while treating the cells with an equivalent concentration of free Dox did not affect the growth of tumor cells. Therefore, uPAR-mediated delivery of the drug molecules using nanoparticle as vehicles is more efficient compare to free drug and non-targeted IO-Dox nanoparticles.

An important feature of using an IO nanoparticle as a drug carrier is its potential as a targeted MRI contrast agent. To determine whether incorporation of Dox molecules onto the IO nanoparticles changes T_2 relaxation time and produces T_2 weighted MRI contrast, we examine the T_2 signal in breast cancer cells labeled with ATF-IO or ATF-IO-Dox nanoparticles *in vitro* using MRI scan. Our result demonstrates that the MRI T_2 contrast of IO nanoparticles remains in the presence of Dox. Therefore, it is feasible to apply this targeted drug delivery nanoparticle as a MRI contrast agent for monitoring intratumoral drug delivery and therapeutic response using MRI.

In summary, we have developed a targeted nanoparticle that can be used for drug delivery as well as tumor imaging by MRI. Our research is the first of such a study that using uPAR as a cancer cell target for multifunctional nanoparticles capable of drug delivery and MRI contrast. Methods for efficient encapsulation of chemotherapeutics and demonstration of pH-sensitive release of the drug from biodegradable IO nanoparticles provide us with a novel platform for further developing this targeted therapeutic and imaging nanoparticle into clinical applications.

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Abbreviations

ATF	Amino-terminal fragment of urokinase plasminogen activator;
Dox	Doxorubicin;
MRI	Magnetic resonance imaging;
IO	Magnetic iron oxide nanoparticle;
uPAR	Urokinase plasminogen activator receptor.

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Fig. 1.

Examination of the binding specificity of the recombinant ATF peptide and its ability to target IO drug-nanoparticles into breast cancer cells. (A) IO nanoparticles synthesized have a uniform size of 10 nm as shown in transmission electron microscopy (TEM). (B) Examination of purified mouse ATF peptides by SDS-PAGE gel. ATF peptides appeared as monomers (17 KDa) or dimers (35 KDa). (C) Detection of the binding of mouse ATF peptides to mouse mammary tumor 4T1 cells. Red fluorescence is found on the surface of tumor cells after incubating ATF peptides followed by mouse anti-His tag antibody and Alexa-fluor 555 labeled anti-mouse antibody. However, red fluorescence is not detected in the cells incubated with anti-His tag and secondary antibodies without ATF-peptides. Blue: Hoechst 33324 background staining. (D) Mouse ATF conjugated-IO nanoparticles are able to bind to and be internalized by human breast cancer MDA-MB-231 cells. Prussian blue staining shows strong blue iron staining inside the cells incubated with ATF-IO nanoparticles for 2 hrs. Cells incubated with non-targeted IO only display a low level of non-specific iron staining. (E) Mouse mammary tumor 4T1 cells were incubated with 1 μ M of free Dox or equivalent Dox dosages of IO-Dox and ATF-IO-Dox for 24 to 48 hrs. Cells were examined under a fluorescence microscope. After incubating with free Dox, most cells showed weak fluorescence signal with a small percentage of the cells having a higher level of Dox. Non-targeted IO-Dox treated cells have an increased amount of Dox in all tumor cells, suggesting that non-specific uptake of IO nanoparticles at a higher concentration of IO nanoparticles and after longer incubation time can also deliver the drug into the cells. However, tumor cells treated with uPAR targeted, ATF-IO-Dox nanoparticles have a significant higher level of Dox that is located in the cytoplasm of the cells at 24 hrs and then moved to cell nucleus at 48 hrs. Interestingly, Dox fluorescence at 48 hrs showed fragmented nuclear staining, which is an indication of apoptotic cell death induced by incorporating Dox into cellular DNA (far right picture).

Fig. 2.

Examination of pH sensitive release of Dox from non-targeted or ATF-targeted IO nanoparticles *in vitro*. Non-targeted and targeted-IO-Dox nanoparticles containing 400 to 600 pmol of Dox were placed in the buffer with pH 4, 5, 6 and 7 for 2 hrs at 37 °C. The amount of released Dox molecules was determined by measuring fluorescence intensity in solution and then calculated from the Dox standard curve. The total amount of Dox added to IO-Dox or ATF-IO-Dox nanoparticles is used as 100%. Result shown is the mean of three repeat experiments.

Fig. 3.

Detection of effect of drug delivery using ATF-IO-Dox on breast cancer cells *in vitro*. Cells cultured in 96 well plates were incubated with the medium containing 0.25 or 0.5 μM of free Dox, IO-Dox or ATF-IO-Dox nanoparticles as well as control IO and ATF-IO nanoparticles for 2 hrs. IO nanoparticles with 10 nm core size were used from this study. After removing the drug and nanoparticles, the cells were cultured for 48 hrs and then fixed with 4% formaldehyde in PBS buffer. (A and B) Examination of the viable cells and Dox fluorescence in 4T1 cells using phase contrast and fluorescence microscopy and cell proliferation assay. Treatment of the cells with 0.5 μM of free Dox and IO-Dox nanoparticles did not induce cell death in 4T1 cells. Dox fluorescence was absent or present in a very low level in those cells (A). However, a high level of Dox was detected in the tumor cells after 0.25 to 0.5 μM of ATF-IO-Dox treatment. Higher magnification fluorescence image (40 $\times$ lens) shows the presence of Dox in the cell nucleus. Cell proliferation assay shows that treatment of 4T1 cells with ATF-IO-Dox nanoparticles at 0.5 μM of Dox concentration induces cell death in 75% of the cells, while the same drug concentration of free Dox or IO-Dox, or same IO concentration of IO or ATF-IO treated cells did not show any cytotoxic effects on the cells (B). In human breast cancer MDA-MB-231 cells, 0.5 μM of free Dox treatment did not induce cell death and Dox fluorescence was not detected in the cells (C and D). Our result showed non-specific uptake of IO-Dox by MDA-MB-231 cells also induced cell death. However, a higher level of Dox fluorescence and 40% more cell death were found in ATF-IO-Dox treated cells compared to IO-Dox treated cells (C and D). A slightly high percentage of MDA-MB-231 cells detected in ATF-IO treated group is due to the internalization of IO nanoparticles into cells resulting in an enhanced Crystal Violet staining. Results shown are the mean of four repeat samples. Similar results were obtained from three separate studies.

Fig. 4.

Examination of effect of drug encapsulation on the T_2 weighted MRI contrast produced by IO nanoparticles. Human breast cancer MDA-MB-231 cells were incubated with 20 pmol of ATF-IO or ATF-Dox-IO nanoparticles for 4 hrs. After washing with PBS, the cell pellets were examined using 4.7T MRI scanner. (A) MRI T_2 relaxometry demonstrated that ATF-IO and ATF-IO-Dox labeled tumor cells have similar T_2 values of 112 ± 10 and 118 ± 10 ms, respectively. However, T_2 value in ATF-IO or ATF-IO-Dox labeled cells is three times lower compared to unlabeled cells (470 ± 10 ms), suggesting that ATF-IO-Dox nanoparticle may be used as a targeted MRI contrast agent. T_2 values of each sample/well were calculated from multi-echo images by fitting the decay curve on a pixel-by-pixel basis using the non-linear mono-exponential algorithm of $M_i = M_0 \cdot \exp(-TE_i/T_2)$. Echo time (TE) has a unit in million seconds as shown in the figure. Orange-red color in the scale bar indicates a high T_2 value and green color represents a low T_2 value. (B) ATF-IO-Dox nanoparticle-labeled MDA-MB-231 cells, but not un-labeled cells, showed strong Dox fluorescence inside the cells.

Scheme.

Schematic illustration of the procedure for production of receptor-targeted drug delivery nanoparticles. Magnetic iron oxide (IO) nanoparticles with core sizes of 5 or 10 nm were coated with amphiphilic copolymers modified with short PEG chains. Recombinant ATF peptides were conjugated to the carboxyl side groups mediated by EDAC. Dox molecules were then encapsulated onto the surface of the IO nanoparticles.

Table I

Encapsulation efficiency of Dox in IO nanoparticles with different sizes and surface modifications.

Drug encapsulation	10 nm IO-Dox (-COOH surface)	5 nm IO-Dox (-COOH surface)	10 nm ATF-IO-Dox
Numbers of Dox per IO particle	652±253	84±33	480±71
Percentage of IO (weight %)	2.3±0.73	2.6±1.4	1.7±0.25
Percentage of Fe ⁺⁺ (weight %)	21±6.7	23±12	15.6±2.3

Table II

Characterization of doxorubicin encapsulated magnetic iron oxide nanoparticles.

Nanoparticle types	Hydrodynamic size (nm)	Zeta potential (mV)
IO-Dox (5 nm)	7.9±2.5	−18.4
IO-Dox (10 nm)	17.9±6.3	−15
IO-Dox (NH ₂) (10 nm)	28.8±6.6	−3.9
ATF-IO-Dox (10 nm)	33.4±7.7	−13