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Targeted Magnetic Iron Oxide Nanoparticles for Tumor Imaging and Therapy

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1. Introduction

Nanoparticles and nanotechnology have been increasingly used in the field of cancer research, especially for the development of novel approaches for cancer detection and treatment (Majumdar, Peng et al. 2010; Davis, Chen et al. 2008). Magnetic iron oxide (IO, i.e. Fe_3O_4, γ-Fe_2O_3) nanoparticles (NPs) are particularly attractive for the development of biomarker-targeted magnetic resonance imaging (MRI) contrast agents, drug delivery and novel therapeutic approaches, such as magnetic nanoparticle-enhanced hyperthermia. Given the unique pharmacokinetics of nanoparticles and their large surface areas to conjugate targeting ligands and load therapeutic agents, biodegradable IO nanoparticles have many advantages in targeted delivery of therapeutic and imaging agents. IO nanoparticles possess unique magnetic properties with strong shortening effects on transverse relaxation times, i.e., T_2 and T^*_2, as well as longitudinal relaxation time, i.e., T_1, at very low concentrations, resulting in contrast enhancement in MRI. Together with their biocompatibility and low toxicity, IO nanoparticles have been widely investigated for developing novel and biomarker-specific agents that can be applied for oncologic imaging with MRI. In addition, the detectable changes in MRI signals produced by drug-loaded IO nanoparticles provide the imaging capabilities of tracking drug delivery, estimating tissue drug levels and monitoring therapeutic response in vivo. With recent progress in nanosynthesis, bioengineering and imaging technology, IO nanoparticles are expected to serve as a novel platform that enables new approaches to targeted tumor imaging and therapy. In this chapter, we will review several aspects of magnetic nanoparticles, specifically IO nanoparticles, which are important to the development and applications of tumor-targeted imaging and therapy. An overview of general approaches for the preparation of targeted IO nanoparticles, including common synthesis methods, coating methodologies, selection of biological targeting ligands, and subsequent bioconjugation techniques, will be provided. Recent progress in the development of IO nanoparticles for tumor imaging and anti-cancer drug delivery, as well as the outstanding challenges to these approaches, will be discussed.

2. Preparation of IO nanoparticles

Typical IO nanoparticles are prepared through bottom-up strategies, including coprecipitation, microemulsion approaches, hydrothermal processing and thermal decomposition (**Figure 1**) (Gupta and Gupta 2005; Laurent, Forge et al. 2008; Laurent, Boutry et al. 2009; Xie, Huang et al. 2009). The advantages and disadvantages of these conventional nanofabrication techniques are important and need to be taken into account in designing and developing a nanoparticle construct for specific cancer models and applications.
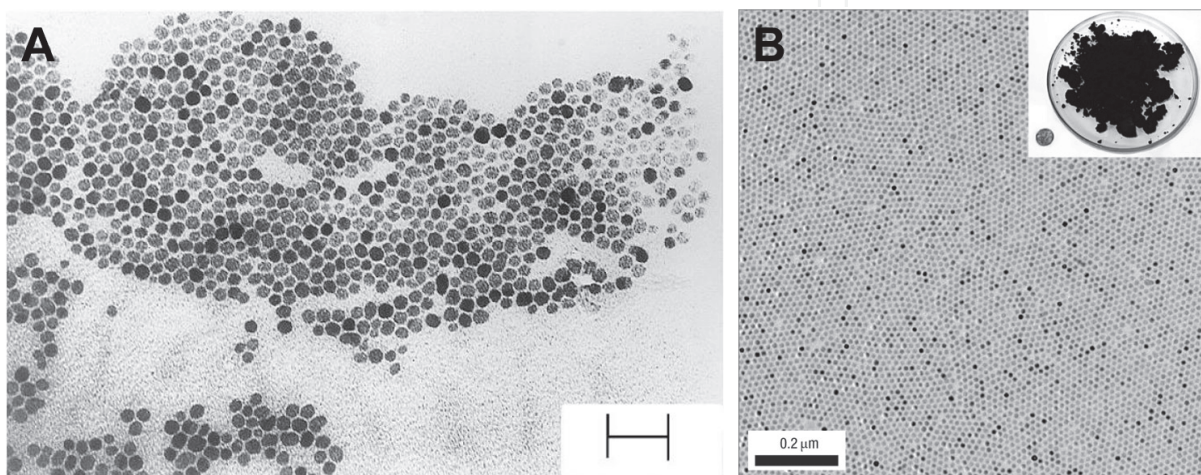

Fig. 1. (A) Fe_3O_4 NPs synthesized by coprecipitation method, the scale bar is 30 nm;
(B) Fe_3O_4 NPs prepared by thermal decomposition of iron oleate $Fe(OA)_3$.
(Reproduced with permission from Kang, Y. S., S. Risbud, et al. (1996).
"Synthesis and characterization of nanometer-size Fe_3O_4 and gamma-Fe_2O_3 particles."
Chemistry of Materials 8(9): 2209-2211and Park, J., K. J. An, et al. (2004).
"Ultra-large-scale syntheses of monodisperse nanocrystals." Nature Materials 3(12): 891-895.

Coprecipitation is the most commonly used approach due to its simplicity and scalability. It features coprecipitating Fe(II) and Fe(III) salts in the aqueous solution by adding bases, usually NH_4OH or NaOH (Massart 1981). The resulting IO nanoparticles are affected by many synthetic parameters, such as pH value, concentrations of reactants, reaction temperature etc. In addition, small molecules and amphiphilic polymeric molecules are introduced to enhance the ionic strength of the medium, protect the formed nanoparticles from further growth, and stabilize the colloid fluid (Kang, Risbud et al. 1996; Vayssieres, Chaneac et al. 1998). Though this method suffers from broad size distribution and poor crystallinity, it is widely used in fabricating IO-based MRI contrast agents (such as dextran-coated IO nanoparticles), because of its simplicity and high-throughput (Sonvico, Mornet et al. 2005; Muller, Skepper et al. 2007; Hong, Feng et al. 2008; Lee, Li et al. 2008; Agarwal, Gupta et al. 2009; Nath, Kaittanis et al. 2009). A modification of the coprecipitation method is the reverse micelle method, in which the Fe(II) and Fe(III) salts are precipitated with bases in microemulsion (water-in oil) droplets stabilized by surfactant. The final size and shape of

the nanoparticles can be precisely tuned through adjusting the surfactant concentration or the reactants concentration (Santra, Tapec et al. 2001; Zhou, Wang et al. 2001; Lee, Lee et al. 2005; Hong, Feng et al. 2009). The disadvantages of this method are its low yield and poor crystallinity of the product, which limit its practical use. A hydrothermal method is also considered a promising synthetic approach for IO nanoparticles towards biomedical applications, which is performed in a sealed autoclave with high temperature (above solvent boiling points) and autogenous high pressure, resulting in nanoparticles with narrow size distribution (Daou, Pourroy et al. 2006; Liang, Wang et al. 2006; Taniguchi, Nakagawa et al. 2009).

High quality IO nanoparticles with perfect monodispersity and high crystallinity can be fabricated by the state of the art thermal decomposition method. Iron precursors, usually organometallic compounds or metal salts (e.g. $Fe(acac)_3$, $Fe(CO)_5$, and $Fe(OA)_3$), are decomposed in refluxing organic solvent in the presence of surfactant (e.g. oleic acid, and oleic amine) (Hyeon, Lee et al. 2001; Sun and Zeng 2002; Park, An et al. 2004; Sun, Zeng et al. 2004; Park, Lee et al. 2005; Lee, Huh et al. 2007). In this method, the size and morphology of the nanoparticles can be controlled by modulating the temperature, reaction time, surfactant concentration and type of solvent. Using smaller nanoparticles as growth seed, Hyeon and co-workers prepared 1-nm IO nanoparticles through additional thermal decomposition growth (Park, Lee et al. 2005). The obtained nanoparticles are usually hydrophobic, dispersible in organic solvent, which requires further phase transfer procedures to make them water-soluble. Recently, several studies have demonstrated that directly thermal decomposing iron precursors in strong polar solvents (e.g. DMF, 2-pyrrolidone) resulted in hydrophilic IO nanoparticles, which could be readily dispersed in water, as preferred in biomedical applications (Liu, Xu et al. 2005; Neuwelt, Varallyay et al. 2007; Wan, Cai et al. 2007).

Coating materials play an important role in stabilizing aqueous IO nanoparticle suspensions as well as further functionalization. Appropriate coating materials can effectively render the water solubility of the IO nanoparticles and improve their stability in physiological conditions. The coating of IO nanoparticles can be achieved through two general approaches: ligand addition and ligand exchange (Gupta, Gupta 2005; Xie, Huang et al. 2009). In ligand addition, the stabilizing agents can physically adsorb on the IO nanoparticle surface as a result of various physico-chemical interactions, including electrostatic interaction, hydrophobic interaction, and hydrogen bonding, etc. Besides physical adsorption, coating materials with abundant hydroxyl, carboxyl, and amino groups can readily and steadily absorb on the surface of the bare IO nanoparticle core, as the active functional groups are capable of coordinating with the iron atoms on the surface to form complexes (Gu, Schmitt et al. 1995). Even for nanoparticles with pre-existing hydrophobic coating, newly added amphiphilic agents could also stick on the surface physically or chemically to complete phase transfer. Various materials, including natural organic materials (e.g. dextran, starch, alginate, chitosan, phospholipids, proteins etc.) (Kim, Mikhaylova et al. 2003; Peng, Hidajat et al. 2004; Kumagai, Imai et al. 2007; Muller, Skepper et al. 2007; Nath, Kaittanis et al. 2009; Zhao, Wang et al. 2009) and synthetic polymers (e.g. polyethylene glycol (PEG), poly(acrylic acid) (PAA), polyvinylpyrrolidone (PVP), poly(vinyl alcohol) (PVA), poly(methylacrylic acid) (PMAA), poly(lactic acid) (PLA), polyethyleneimine (PEI), and block copolymers etc.) (Lutz, Stiller et al. 2006; Narain, Gonzales et al. 2007; Mahmoudi, Simchi et al. 2008; Hong, Feng et al. 2009; Yang, Mao et al.

2009; Yang, Peng et al. 2009; Hadjipanayis, Machaidze et al. 2010; Huang, Bu et al. 2010; Namgung, Singha et al. 2010; Vigor, Kyrtatos et al. 2010; Wang, Neoh et al. 2010) have been demonstrated to successfully coat the surface of IO nanoparticles through ligand addition. Alternatively, ligand exchange refers to the approach of replacing the pre-existing coating ligands with new, higher affinity ones. One such example is that of dopamine (DOP)-based molecules, which can can substitute the original oleic acid molecules on the surface of IO nanoparticles, as the bidentate enediol of DOP coordinates with iron atoms forming strong bonds (Huang, Xie et al. 2010; Xie, Wang et al. 2010). Dimercaptosuccinic acid (DMSA) and polyorganosiloxane could also replace the original organic coating by forming chelate bonding (De Palma, Peeters et al. 2007; Lee, Huh et al. 2007; Chen, Wang et al. 2010). After ligand addition and ligand exchange, surface-initiated crosslinking might be performed for further coating stabilization, yielding nanoparticles with great stability against agglomeration in the physiological environment (Lattuada and Hatton 2007; Chen, Wang et al. 2010).

3. Surface modification and functionalization of IO nanoparticles

Surface modification and functionalization play critical roles in the development of any nanoparticle platform for biomedical applications. However, the capacity of the functionalization may be highly dependent on the diversity and chemical reactivity of the surface coating materials as well as the functional moieties used for biological interactions and targeting. Commonly used functional groups, i.e., carboxyl -COOH, amino -NH_2 and thiol –SH groups, are ideal for covalent conjugation of payload molecules or moieties. However, there is an increased application of non-covalent interactions, such as hydrophobic and electrostatic forces, to incorporate the payload molecules.

Recently developed theranostics IO nanoparticles, i.e., multifunctional nanoparticles capable of both diagnostic imaging and delivery of therapeutics, often consist of small molecules (e.g, chemotherapy drugs, optical dyes) or biologics (e.g., antibodies, peptides, nucleic acids) to achieve effective targeted imaging and drug delivery. These functional moieties have high affinity and specificity for biomarkers, such as cell surface receptors or cellular proteins, which can enhance specific accumulation of IO nanoparticles at the target site. Major techniques for the functionalization of IO nanoparticles include the selection of biomarker-targeting ligands and the conjugation of targeting ligands on the nanoparticle surface (**Figure 2**). Targeting moieties can be obtained via screening of synthetic combinatorial libraries and subsequent amplification through an in vitro selection process (Yang, Peng et al. 2009; Hadjipanayis, Machaidze et al. 2010; Lee, Yigit et al. 2010). The selection process usually starts with a random moieties library generated through chemical synthesis, and polymerase chain reaction (PCR) amplification or cloning of the identified targeting moiety through transfected/infected cells. Purification is acheived by incubating the library with target molecules or target cells, so that the high affinity moieties can be captured, separated from those unbound moieties, and eluted from the target molecule or cells. In addition, counter selection might be performed to enhance the purity of the isolated targeting moiety. Amplification via PCR or cloning throguh transfected/infected cells will result in new libraries of targeting moieties enriched with higher affinity ones. The selection process may be repeated for several rounds, and the targeting moieties with the highest affinity to the target can be obtained for further functionalization of magnetic IO nanoparticles.
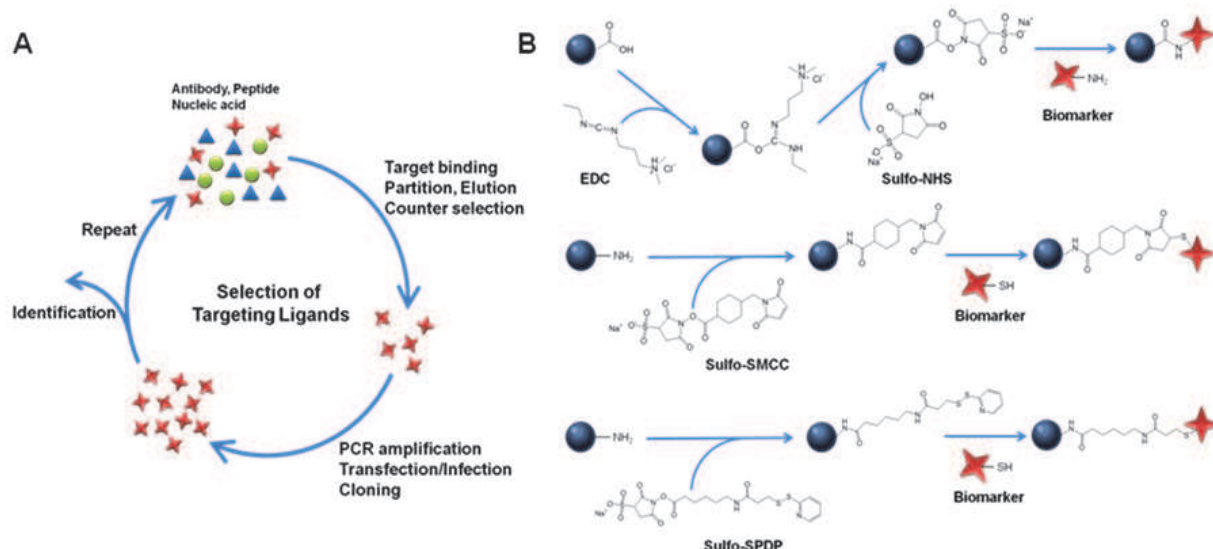
Fig. 2. (A) A schematic example of the selection process of targeting moieties. (B) Conjugation of IO nanoparticles with targeting ligands through maleimide reactions.

An active targeting approach in nanomedicine involves the direct conjugation of targeting ligands to the surface of nanoparticles rather than adsorption encapsulation. A variety of bioconjugation reactions have been developed by the incorporation of functional groups (e.g. carboxyl group, and amino group, thiol group) at the IO nanoparticle surface and in the targeting ligands. Besides affinity interactions, click chemistry, and streptavidin biotin reactions (Yang, Mao et al. 2009; Cutler, Zheng et al. 2010; Vigor, Kyrtatos et al. 2010), bioconjugation can be achieved by using linker molecules with carboxyl-, amine- or thiol-reactive groups, such as glutaraldehyde, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), N-succinimidyl-3-(2-pyridyldithio)-propionate (SPDP), etc. (Lee, Huh et al. 2007; Lee, Li et al. 2008; Bi, Zhang et al. 2009; Yang, Mao et al. 2009; Yang, Peng et al. 2009; Hadjipanayis, Machaidze et al. 2010; Kumar, Yigit et al. 2010; Vigor, Kyrtatos et al. 2010; Yang, Park et al. 2010). For example, Yang et al. conjugated amphiphilic polymer-coated IO nanoparticles with amino-terminal fragment peptides via cross-linking of carboxyl groups to amino side groups through an EDC/NHS approach (Yang, Peng et al. 2009). The well developed bioconjugation methodologies advance the surface engineering of IO nanoparticles and expand the functionalities of IO nanoparticles.

4. Recent progress using IO nanoparticles for tumor imaging and therapy

With the emphasis on personalized medicine in future clinical oncology practices, the potential applications of biomarker-targeted imaging and drug delivery approaches are well recognized. Tumor-targeted IO nanoparticles that are highly sensitive imaging probes and effective carriers of therapeutic agents are the logical choice of a platform for future clinical development. Increasing evidence indicates that the selective delivery of nanoparticle therapeutic agents into a tumor mass can minimize toxicity to normal tissues and maximize bioavailability and cell killing effects of cytotoxic agents. This effect is mainly attributed to changes in tissue distribution and pharmacokinetics of drugs. Furthermore, IO nanoparticle-

drugs can accumulate to reach high concentrations in certain solid tumors than free drugs via the enhanced permeability and retention effect (EPR). However, the EPR facilitates only a certain level of tumor targeting, while actively tumor-targeted IO nanoparticles may further increase the local concentration of drug or change the intracellular biodistribution within the tumor via receptor-mediated internalization.

4.1 Targeted IO nanoparticles for tumor imaging

Passive targeting of tumors with IO nanoparticles via the EPR effect plays an important role in the delivery of IO nanoparticles in vivo and can be used for tumor imaging. However, the biodistribution of such IO nanoparticles is non-specific, resulting in insufficient concentrations at the tumor site, and thus low sensitivity and specificity. The development of tumor-targeted IO nanoparticles that are highly sensitive and specific imaging probes may overcome such problems.

Various genetic alterations and cellular abnormalities have been found to be particularly distributed in tumors rather than in normal tissues. Such differences between normal and tumor cells provide a great opportunity for engineering tumor-targeted IO imaging probes. Antibodies, peptides and small molecules targeting related receptors on the surface of tumor cells can be conjugated to the surface of IO nanoparticles to enhance their imaging sensitivity and specificity. Many studies have reported using targeted IO nanoparticles for tumor imaging in vitro and in vivo, and such nanoparticles may have the potential to be used in the clinic in the near future.

Antibodies are widely used for engineering tumor targeted IO nanoparticles for *in vivo* tumor imaging due to their high specificity. The conjugation of antibodies to IO nanoparticles can maintain both the properties of the antibody and the magnetic particles. Monoclonal antibody-targeted IO nanoparticles have been well studied in vivo (Artemov, Mori et al. 2003; Serda, Adolphi et al. 2007; Kou, Wang et al. 2008; Chen, Cheng et al. 2009).

One well-known tumor target, the human epidermal growth factor receptor 2 (Her-2/*neu* receptor), has been found overexpressed in many different kinds of cancer such as breast, ovarian, and stomach cancer. Yang et al (Yang, Park et al. 2010) conjugated the HER2/neu antibody (Ab) to poly(amino acid)-coated IO nanoparticles (PAION), which have abundant amine groups on the surface. After conjungation, the diameter of PAION-Ab was 31.1 ± 7.8 nm, and the zeta-potential was negative (-12.93 ± 0.86 mV) due to the shield of amine groups by conjugated Her-2 antibodies. Bradford protein assay indicates that there are about 8 HER2/neu antibodies on each PAION. The T_2 relaxation times showed a significant difference between the PAION-Ab-treated (37.7 ms) and untreated cells (79.9 ms) in positive groups (SKBR-3 cells, overexpressing HER-2), while no significant difference was founded in T_2-weighted MR images of negative groups (H520 cells, HER-2 negative). The results demonstrated that HER2/*neu* antibody-conjugated PAION have specific targeting ability for HER2/*neu* receptors. Such HER2/*neu* antibody-conjugated PAION with high stability and sensitivity have potential to be used as an MR contrast agent for the detection of HER2/*neu* positive breast cancer cells. Herceptin, a well-known antibody against the HER2/*neu* receptor, which has been used in the clinic for many years, can also be conjugated to the IO nanoparticles for breast cancer imaging. Using such herceptin-IO nanoparticles, small tumors of only 50 mg in weight can be detected by MRI (Lee, Huh et al. 2007).

However, the relatively large size of intact antibodies limits their efficient conjugation because of steric effects. The specificity of antibody-conjugated IO nanoparticles may also decrease due

to the interaction of antibody with Fc receptors on normal tissues. In addition, the expensive cost of intact antibodies further limits the application of antibody-targeted IO nanoparticles. Recently, more and more studies have reported engineering targeted IO nanoparticles using single chain antibodies (scFv) or peptides with small molecular weight and size. Compared with intact antibodies, there are many advantages of using scFv as tumor targeting ligands, 1) relatively small molecular weight and size; 2) no loss of antigen binding capacity; 3) no immune responses due to lack of Fc constant domain; 4) low cost and easily obtained.

The epidermal growth factor receptor (EGFR) signaling pathway is involved in the regulation of cell proliferation, survival, and differentiation, and it has been found overexpressed in many different kinds of cancer such as breast, ovarian, lung, head and neck cancer. By using a high-affinity single-chain anti-EGFR antibody (scFvB10, $K_D = 3.36 \times 10^{-9}$ M), Yang et al. has developed a EGFR-targeted amphiphilic triblock polymer coated IO nanoparticle for in vivo tumor imaging (Yang, Mao et al. 2009) (**Figure 3**). ScFvEGFR was conjugated to IO nanoparticles by crosslinking carboxyl groups to amino groups of the ScFvEGFR proteins mediated by ethyl-3-dimethyl amino propyl carbodiimide (EDAC). The in vitro results showed that the ScFvEGFR IO nanoparticles specifically bind to EGFR, which was demonstrated by Prussian blue staining and MRI (**Figure 4**). The EGFR-targeted or non-targeted IO nanoparticles were administrated via the tail vein to nude mice bearing orthotopical human pancreatic cancer xenograft. The results showed that the ScFvEGFR-IO nanoparticles could selectively accumulate within the pancreatic tumors, which was evidenced by a decrease in MRI signal in the tumor site and confirmed by histological examination of the pancreatic tissue, while non-targeted IO nanoparticles did not cause MRI signal changes in tumor.

A high affinity scFv reactive to carcinoembryonic antigen (CEA), sm3E, was covalently conjugated to superparamagnetic iron oxide nanoparticles (SPIONs), and the functionalized SPIONs could bind specifically to CEA while unmodified SPIONs did not show any binding ability. The ability of the targeted-SPIONs to specifically target and image CEA was further demonstrated by using the colorectal cancer cell line LS174T (CEA-expressing) and adherent melanoma cell line A375M (CEA negative). MR images showed 57% reduction in T_2 values compared with the 11% reduction induced by non-targeted SPIONs (Vigor, Kyrtatos et al. 2010).

Peptides that target specific receptors on the tumor cell surface can be used for engineering targeted IO nanoparticles for tumor imaging due to their small size and molecular weight. The urokinase plasminogen activator receptor (uPAR) is expressed in many different human cancers, and may play important roles in the tumor metastasis. The amino-terminal fragment (ATF) of urokinase plasminogen activator (uPA) can bind to uPAR on the cell surface, thus the ATF peptide is ideal for constructing uPAR-targeted IO nanoparticles for *in vivo* tumor imaging. Yang et al. purified the ATF peptide and conjugated it to amphiphilic polymer-coated IO nanoparticles (Yang, Mao et al. 2009). These uPAR-targeted IO nanoparticles showed selective accumulation at the tumor mass in orthotopical xenografted human pancreatic cancer model. More importantly, such uPAR-targeted IO nanoparticles could be internalized by both uPAR-expressing tumor cells and tumor-associated stromal cells, to further increase the amount and retention of the IO nanoparticles in a tumor mass, which increased the sensitivity of tumor detection by MRI. Pancreatic tumors as small as 1 mm³ could be detected by a 3T clinical capable MRI scanner using the targeted IO nanoparticles. After labeling the ATF peptide with the near infrared (NIR) dye Cy5.5, the targeted IO nanoparticles enabled the detection of a 0.5 mm³ intraperitoneal pancreatic cancer lesion by NIR optical imaging. Further study showed that NIR optical imaging

detected tumor cell implants with only 1×10^4 tumor cells while MRI detected tumor cell grafts containing 1×10^5 labeled cells (**Figure 5**).
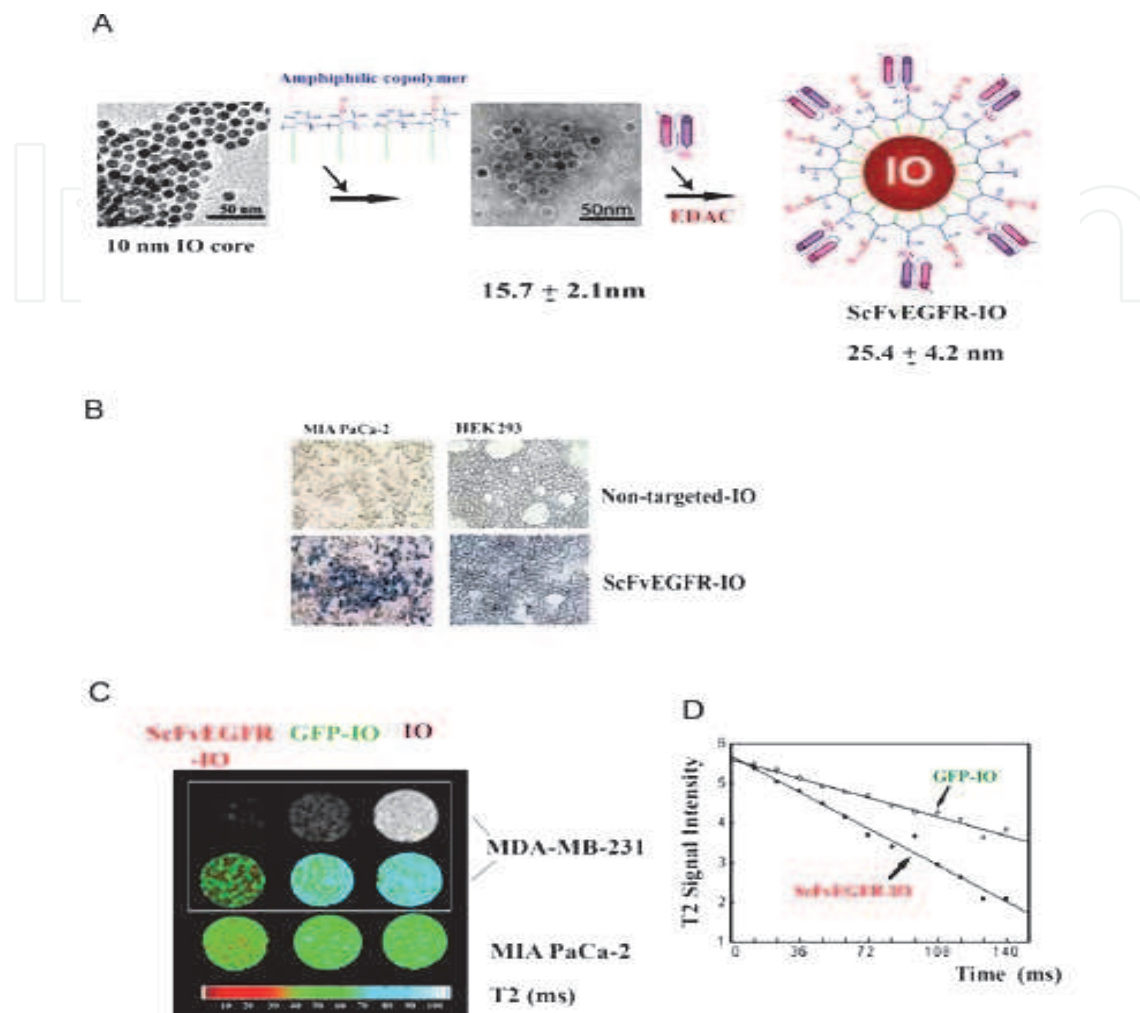

Fig. 3. ScFvEGFR-conjugated IO nanoparticles show high specificity to EGFR-overexpressing tumor cells and induce MRI signal changes in IO nanoparticle-bound tumor cells in vitro. A) ScFvEGFR-IO nanoparticle construct consists of uniform IO nanoparticles (10 nm core size) coated with amphiphilic copolymers modified with short PEG chains. ScFvEGFR proteins were conjugated to the IO nanoparticles mediated by EDAC. B) Prussian blue staining confirmed the specific binding of the ScFvEGFR-IO nanoparticles to tumor cells with EGFR overexpression . C) T_2 weighted MRI and T_2 relaxometry mapping showed significant decreases in MRI signals and T_2 relaxation times in the cells bound with ScFvEGFR-IO nanoparticles but not with GFP-IO nanoparticles or bare IO nanoparticles. MDA-MB-231 breast cancer cells and MIA PaCa-2 pancreatic cancer cells have different levels of EGFR expression and different levels of T_2 weighted contrast. A low T_2 value correlates with a higher iron concentration (red color), indicating higher level of specific binding of ScFvEGFR-IO nanoparticles to tumor cells. D) Multi-echo T_2 weighted fast spin echo imaging further confirmed the fastest T_2 value drop in MDA-MB-231 cells after incubation with ScFvEGFR-IO but not with control GFP-IO nanoparticles. Reproduced with permission from Yang, L., H. Mao, et al. (2009). "Single chain epidermal growth factor receptor antibody conjugated nanoparticles for in vivo tumor targeting and imaging." Small 5(2): 235-43.
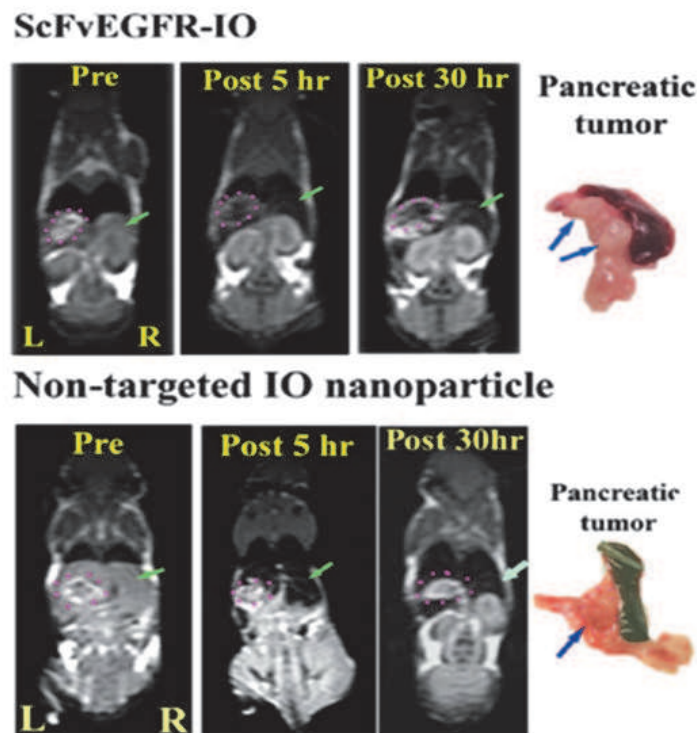
Fig. 4. Examination of target specificity of ScFvEGFR-IO nanoparticles by MRI using an orthotopic human pancreatic xenograft model, the areas of the pancreatic tumor were marked as pink dash-lined circle. Right is the picture of tumor and spleen tissues, showing sizes and locations of two intra-pancreatic tumor lesions (arrows) that correspond with the tumor images of MRI. Reproduced with permission from Yang, L., H. Mao, et al. (2009). "Single chain epidermal growth factor receptor antibody conjugated nanoparticles for in vivo tumor targeting and imaging." Small 5(2): 235-43.

The approach of using optically sensitive small dye molecules along with MRI-capable IO nanoparticles not only provides a potential multi-modal imaging capability for future application but also a way to validate and track the magnetic IO nanoparticles to investigate tumor targeting and biodistribution of nanoparticle constructs in animal models. Underglycosylated mucin-1 antigen (uMUC-1) is overexpressed in more than 50% of all human cancers and is located on the surface of tumor cells. The EPPT1 peptide, which is able to specifically bind to uMUC-1, has been synthesized and used by Moore et al. to fabricate uMUC-1-targeted superparamagnetic IO nanoparticles with dextran coating, their results showed that such targeted CLIO nanoparticles could induce a significant T2 signal reduction in uMUC-1-positive LS174T tumors compared with that of uMUC-1-negative U87 tumors in vivo (Moore, Medarova et al. 2004).

The luteinizing hormone releasing hormone (LHRH) (Chatzistamou, Schally et al. 2000) is a decapeptide, and more than half of human breast cancers express binding sites for receptors for LHRH. Leuschner et al synthesized LHRH-SPIO nanoparticles, and both in vitro and in vivo data showed that the IO nanoparticles selectively accumulated in both primary tumor cells and metastatic cells. The LHRH-conjugated SPIO nanoparticles may have potential to be used for detecting metastatic breast cancer cells *in vivo* in the future (Leuschner, Kumar et al. 2006).
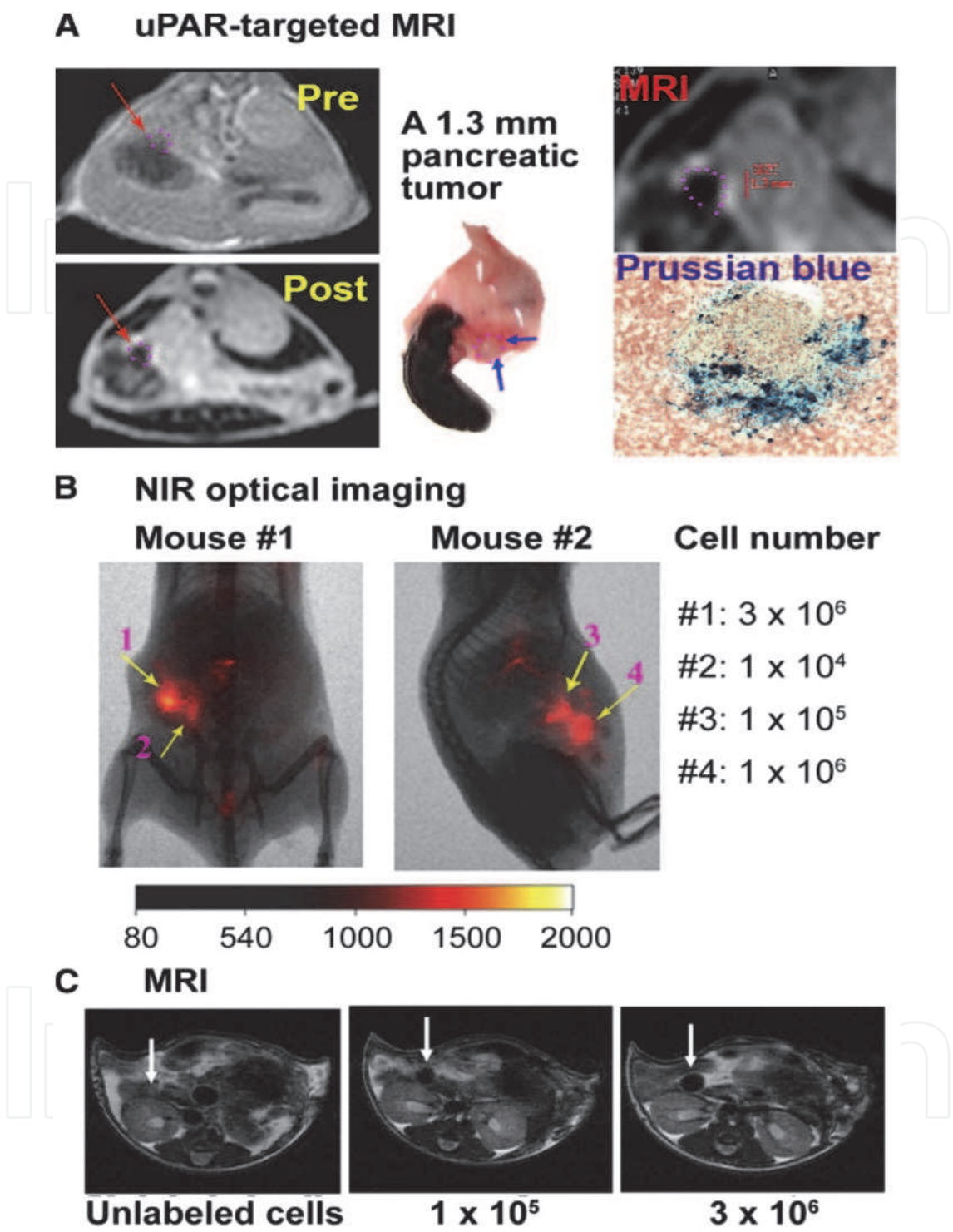
Fig. 5. Examination of sensitivity of in vivo tumor imaging. (A) uPAR-targeted MRI of an orthotopic pancreatic cancer. Tumor is marked as pink dotted circle. Prussian blue staining revealed the presence of IO nanoparticles in the tumor lesion with strong staining in tumor stromal areas. (B) NIR optical imaging and (C) MRI of injected labeled cells and nonlabeled cells in mouse pancreas. Reproduced with permission from Yang, L., H. Mao, et al. (2009). "Molecular imaging of pancreatic cancer in an animal model using targeted multifunctional nanoparticles." Gastroenterology 136(5): 1514-25.

In contrast, cost effective but high affinity small molecule targeting moities are not widely available or well tested. One exception is folic acid (FA), which targets the folate receptor, which is overexpressed on the surface of many human tumor cells and can thus be used as a target for tumor imaging. The vitamin FA has low molecular weight and has been widely studied as a targeting ligand. There are many advantages of using FA as a targeting ligand for synthesizing IO nanoparticles, 1) high binding affinity for its receptor ($K_d = 10^{-10}$ M), 2) low cost and easily obtained, 3) easy to be conjugated with the imaging agents, 4) lack of immunogenicity (Low, Henne et al. 2008). Sun et al constructed the FA-IO-nanoparticles, the *in vitro* experiments showed that FR-positive HeLa cells could uptake1.410 pg iron per cell after incubated with FR-targeted IO nanoparticles for 4 hrs, which was 12-fold higher than those cultured with non-targeted IO nanoparticles, and the increased internalization could be inhibited by increasing free FA concentration, and such targeting specificity of the FR-targeted IO nanoparticles could be further demonstrated by using FR-negative Human osteosarcoma MG-63 cells. The T_2-weighted MR phantom images of HeLa cells cultured with FR-targeted IO nanoparticles showed significantly lower T_2 values (23.5–14.2 ms) than those incubated with non-targeted IO nanoparticles (80.2–49.3 ms)(Sun, Sze et al. 2006). Another study also showed FA-targeted IO nanoparticles could selectively accumulate in human nasopharyngeal epidermoid carcinoma (KB) cells both in vitro and in vivo, which resulted in significant MRI signal changes (Chen, Gu et al. 2007).

Given the concerns regarding the delivery of fairly large nanoparticle constructs directly into the tumor, targeted imaging and drug delivery into the tumor vasculture, which is often associated with tumor angiogenesis, appears to be a feasible approach. Angiogenesis is essential for the development of tumors. As a marker of angiogenesis, the $\alpha_v\beta_3$ integrin locates on the surface of the tumor vessels and can be directly targeted via blood. The Arg-Gly-Asp (RGD) peptide, which can bind to the $\alpha_v\beta_3$ integrin receptor, has been well studied as a tumor vessel-targeted ligand. One study using RGD-USPIO nanoparticles for tumor vessel imaging showed that RGD-USPIO nanoparticles could target to the tumor vessels and resulted in a change in T_2 relaxation detected at the field strength of 1.5 T with a clinical MRI scanner, and the signal changes were correlated to the $\alpha_v\beta_3$ integrin expression level (Zhang, Jugold et al. 2007).

On the other hand, targeted delivery of biomarker-specific nanoparticle constructs to brain tumors needs to overcome the challenge of penatrating the intrinsic blood-brain barrier. Efforts have been made to identify the appropriate design of nanoparticle constructs for targeting brain tumors. It has been reported that matrix metalloproteinase-2 (MMP-2) is overexpressed in gliomas and other related cancers, and facilitates cancer invasion (Soroceanu, Gillespie et al. 1998; Deshane, Garner et al. 2003; Veiseh, Gabikian et al. 2007). The chlorotoxin (Cltx) is a small peptide (36-amino acid) which can recognize and bind to the MMP-2 endopeptidase, one study showed that Cltx-conjugated IO nanoparticles could be taken up in 9L glioma cells at significantly higher concentrations than that of their non-targeted counterpart, which further resulted in a significant difference in $R_2(1/T_2)$ relaxivity between Cltx-targeted IO nanoparticle- (5.20 mm^{-1}s^{-1}) and non-targeted IO nanoparticle- (0.22 mm^{-1}s^{-1}) treated tumor cells, and such R_2 change was also observed by MRI in vivo (Sun, Veiseh et al. 2008). One alternative and potential solution for overcoming the blood-brain barrier to deliver therapeutic IO nanoparticles is the use of conventional enhanced delivery, in which a magnetic IO nanoparticle suspension can be slowly infused into the

tumor site via a minimally invasive procedure (Hadjipanayis, C. G., R. Machaidze, et al. (2010)).

There are still many issues that need to be addressed in the study of IO nanoparticles for tumor imaging, and which must be throughly investigated in future studies. These include: 1) the optimal coating of the IO nanoparticles, which may avoid non-specific binding to normal cells, prolong the blood circulation time, and make the IO nanoparticles more stable in physiological conditions; 2) quantification of the density of targeted ligand on the surface of IO nanoparticles, which may affect the binding and internalization of IO nanoparticles, as well as their in vivo biodistribution; 3) the long-term fate and toxicity of targeted IO nanoparticles in vivo. Until now, most tumor-targeted IO nanoparticles have only been applied in vitro or in small animal models for tumor imaging, and are not yet ready for clinical use. The development of tumor-targeted IO nanoparticles with high specificity and sensitivity in vivo for early stage detection of tumors, monitoring of tumor metastasis and response to therapy is greatly needed.

4.2 Tumor-targeted IO nanoparticles as selective drug delivery vehicles

The selective delivery of therapeutic agents into a tumor mass may enhance the antitumor efficacy while minimizing toxicity to normal tissues (Brigger, Dubernet et al. 2002; Maillard, Ameller et al. 2005; Shenoy, Little et al. 2005; Bae, Diezi et al. 2007; Gang, Park et al. 2007; Lee, Chang et al. 2007). While the delivery of small molecule drugs to the tumor is often limited by fast secretion, drug solubility and low intra-tumor accumulation, nanoparticle delivery vehicles can alter the pharmacokinetics and tissue distribution profile in favor of tumor specific accumulation. It is widely considered that nanoparticle-drugs can accumulate to higher concentrations in certain solid tumors than free drugs via the enhanced permeability and retention effect (EPR). In addition, actively tumor-targeted nanoparticles may further increase the local concentration of drug or change the intracellular biodistribution within the tumor via receptor-mediated internalization. With magnetic IO nanoparticles, the imaging capability allows for monitoring and potential quantification of the IO nanoparticle-drug complex in vivo with MRI.

Therapeutic entities, such as small molecular drugs, peptides, proteins and nucleic acids, can be incorporated in the IO nanoparticles through either loading on the surface layer or trapping within the nanoparticles themselves. When delivered to the target site, the loaded drugs are usually released by 1) diffusing; 2) vehicle rupture or dissolution; 3) endocystosis of the conjugations; 4) pH-sensitive dissociation, etc. Such delivery carriers have many advantages, including 1) water-soluble; 2) low toxic or nontoxic; 3) biocompatible and biodegradable; 4) long blood retention time; 5) capacity for further modification. Futhermore, these therapeutic IO conjugations enable the simultaneous estimation of tissue drug levels and monitoring of therapeutic response (Lanza, Winter et al. 2004; Atri 2006).

Conventional anti-cancer agents, such as doxorubicin, cisplatin, and methotrexate, have been conjugated with tumor-targeted IO nanoparticles to achieve effective delivery. Recently Yang et al (Yang, Grailer et al. 2010) developed folate receptor-targeted IO nanoparticles to deliver doxorubicin (DOX) to tumor cells. As shown in **Figure 6**, the hydrophillic IO nanoparticles were encapsulated in the multifunctional polymer vesicles in aqueous solution, the long hydrophilic PEG segments bearing the FA targeting ligand located in outer layers, while the short hydrophilic PEG segments bearing the acrylate

groups located in inner layers. The anticancer drug (DOX) was conjugated onto the hydrophobic polyglutamate polymer segments via an acid-cleavable hydrazone bond, and could release at low pH value. The loading efficacy of DOX was about 14 wt %. The FA-conjugated SPIO/DOX-loaded vesicles demonstrated higher cellular uptake and cytotoxicity compared with FA-free vesicles due to folate receptor-mediated endocytosis.
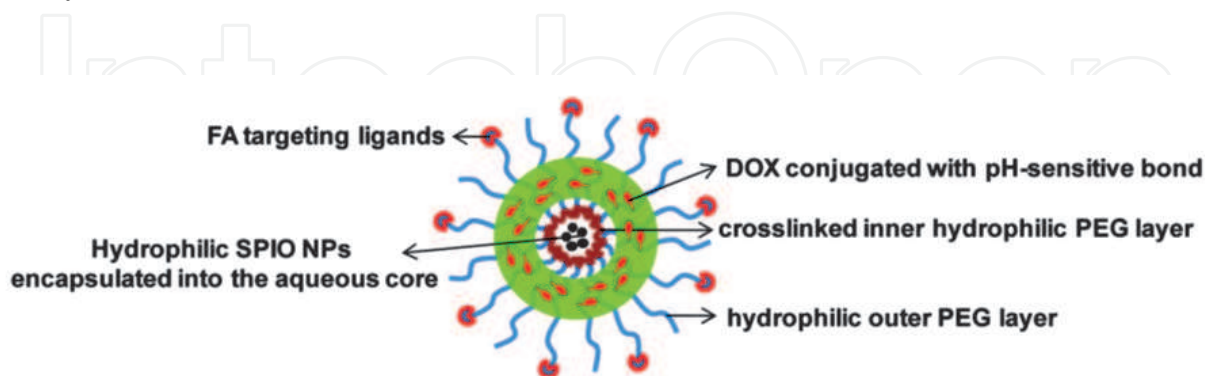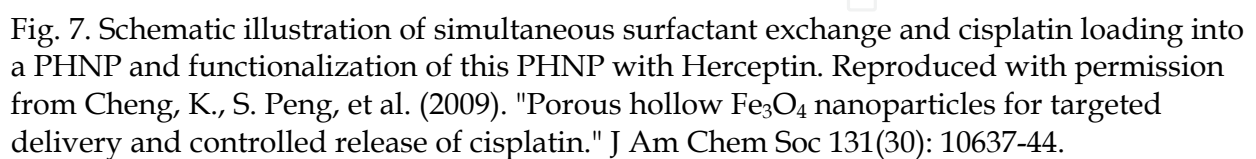

Fig. 6. Synthetic scheme of the amphiphilic triblock copolymers and the preparation process of the SPIO/DOX-loaded vesicles with cross-linked inner hydrophilic PEG layers. Reproduced with permission from Yang, X., J. J. Grailer, et al. (2010). "Multifunctional stable and pH-responsive polymer vesicles formed by heterofunctional triblock copolymer for targeted anticancer drug delivery and ultrasensitive MR imaging." ACS Nano 4(11): 6805-17.

Cisplatin (DDP) is one of the most widely used chemotherapy drugs in the treatment of cancers, including head and neck, testicular, bladder, ovarian, and non-small lung cancer. However, the major dose limiting toxicity of DDP is cumulative nephrotoxicity; severe and irreversible damage to the kidney will occur in about 1/3 of patients who receive DDP treatment. The selective delivery of DDP to tumor cells would significantly reduce drug toxicity, improving its therapeutic index. Recently, IO nanoparticles have been used as DDP carriers for targeted therapeutic applications. Sun's group (Cheng, Peng et al. 2009) reported DDP porous could be loaded into PEGylated hollow NPs (PHNPs) of Fe_3O_4 by using the nanoprecipitation method (**Figure 7**), which resulted in 25% loading efficacy. Herceptin was covalently attached to the amine-reactive groups on the Pt-PHNP surface, and such conjugation did not change the Herceptin activity. Results showed that DDP could release from the Her-Pt-PHNPs in the acidic endosomes or lysosomes after internalization by cells, and could significantly increase the cytotoxicity of DDP.

Methotrexate (MTX) can be used as both a targeting molecule for folate receptor (FR) and a therapeutic agent for cancer cells overexpressing FR on their surface. Its carboxyl end groups provide the opportunity to be conjugated on the IO nanoparticles with amine groups. Kohler et al. have demonstrated that the uptake of MTX-IO nanoparticles by FR-overexpressing cancer cells was significant higher than that of FR-negative control cells. This system showed high drug loading efficiency, about 418 MTX molecules could be loaded onto each IO nanoparticle with a core size of 10 nm diameter. Loaded MTX was only released inside the lysosomes at low pH condition after internalization by the targeted cells, and the drug delivery system could be monitored in vivo by MRI in real-time (Kohler, Sun et al. 2005).

Fig. 7. Schematic illustration of simultaneous surfactant exchange and cisplatin loading into a PHNP and functionalization of this PHNP with Herceptin. Reproduced with permission from Cheng, K., S. Peng, et al. (2009). "Porous hollow Fe_3O_4 nanoparticles for targeted delivery and controlled release of cisplatin." J Am Chem Soc 131(30): 10637-44.

RNA interference (RNAi) has become a promising molecular therapeutic tool due to its high specificity. One of the big challenges for its in vivo application is that small interfering RNA (siRNA) cannot reach the target tissue at sufficient concentrations due to RNase degradation and inefficient translocation across the cell membrane. IO nanoparticles are expected to be applicable for delivering siRNA and monitoring the efficacy of therapy because of their unique characteristics as described above. It has been reported that BIRC5 could encode the antiapoptotic survivin proto-oncogene, and can be used as a good target for tumor therapy. The knockdown of BIRC5 by RNAi may mediate a therapeutic effect by inducing necrotic/apoptotic tumor cell death. Kumar et al (Kumar, Yigit et al. 2010) synthesized a novel tumor-targeted nanodrug (MN-EPPT-siBIRC5), which consists of 1) peptides (EPPT) that specifically target the antigen uMUC-1; 2) IO nanoparticles; 3) the NIR dye, Cy 5.5 and 4) siRNA that targets the tumor-specific antiapoptotic gene BIRC5 (**Figure 8**). Systemic delivery of MN-EPPT-siBIRC5 to nude mice bearing human breast adenocarcinoma tumors showed significant decrease of T_2 relaxation time of the tumor, which remained significantly lower than the preinjection values over time, suggesting that the concentration of nanodrug within the tumor tissue could be maintained. While this demonstrated that it is feasible to follow the accumulation and retention of drug-IO nanoparticles in vivo with MRI, the in vivo data also showed that MN-EPPT-siBIRC5 therapy can led to a 2-fold decrease in the tumor growth rate compared with the MN-EPPT-siSCR-treated group. The efficacy of MN-EPPT-siBIRC5 in the breast tumors was evaluated by H&E staining and TUNEL assay, which showed a 5-fold increase in the fraction of apoptotic nuclei in tumors in MN-EPPT-siBIRC5 treated mice via the MN-EPPT-siSCR group.

Tumor-targeted IO nanoparticles can also be used to "rescue" some anticancer drugs which show severe toxicity, low solubility or low antitumor efficacy in vivo. One example is the targeted delivery of noscapine, an orally available plant-derived anti-tussive alkaloid which shows antitumor activity by targeting tubulin, however, related preclinical studies did not exhibit significant inhibition of tumor growth even using high dosage (450 mg/kg), which may result from the shorter circulation time and lower drug uptake by tumor cells. Abdalla et al (Abdalla, Karna et al. 2010) have developed uPAR-targeted IO nanoparticles for selective delivery of noscapine to prostate cancer by conjugating the human ATF to the IO

nanoparticles, and encapsulated about 80% of noscapine onto the uPAR-targeted nanoparticles via the interaction between the hydrophobic noscapine molecules and the hydrophobic segment of the amphiphilic polymer coating of nanoparticles. Their data showed the nanoparticles were uniformly sized and stable at physiological pH, while about 80% of drug molecules were efficiently released at pH 4 due to the onset of polymer degradation at lower pH, the breakage of hydrophobic interactions between polymer and drug molecules or hydrogen bonding. The hATF-Cy5.5-IO-Nos nanoparticles could significantly inhibit the proliferation of uPAR-positive human prostate carcinoma PC-3 cells compared with the nontargeted IO-Nos and free drug at the same concentration (10 μM). The uPAR-targeted NPs also delivered a significantly higher concentration of noscapine to the receptor positive cells, which led to a 6-fold enhancement in cell death compared to the free drug.
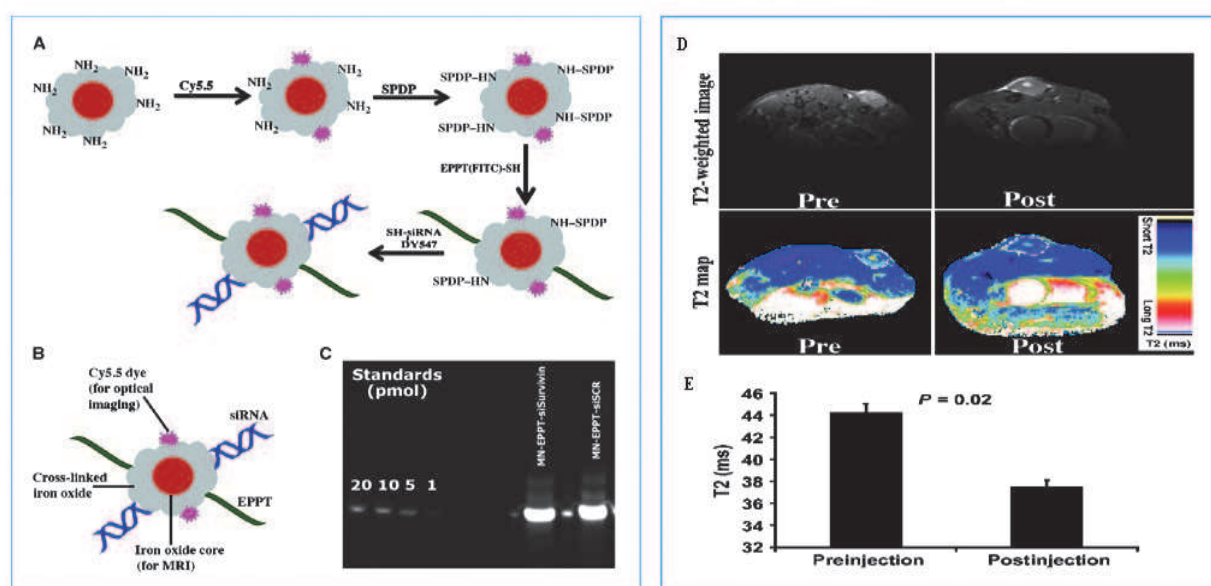
Fig. 8. A, flowchart of the synthesis. B, nanodrug characterization. The nanodrug consisted of dextran-coated MNs triple labeled with Cy5.5 dye, EPPT peptides, and synthetic siRNA duplexes. C, gel electrophoresis showing dissociation of siRNAs from the nanoparticles under reducing conditions. D, representative precontrast and postcontrast T_2-weighted images (top) and color-coded T_2 maps (bottom) of tumor-bearing mice injected i.v. with MN-EPPT-siBIRC5 (10 mg/kg iron). The tumors (outlined) were characteristically bright (T_2 long) before contrast. At 24 h after injection, there was a loss of signal intensity (T_2 shortening) associated with the tumors, indicative of nanodrug accumulation. E, quantitative analysis of tumor T_2 relaxation times. T_2 map analysis revealed a marked shortening of tumor T_2 relaxation times 24 h after nanodrug injection, indicating accumulation of MN-EPPT-siBIRC5.Reproduced with permission from Kumar, M., M. Yigit, et al. 2010 "Image-guided breast tumor therapy using a small interfering RNA nanodrug." Cancer Res 70(19): 7553-61.

Although much progress has been made in the development of tumor-targeted IO nanoparticles for the delivery of anticancer agents, there are still many obstacles to be overcome. First, the conjugation process during the synthesis of nano-drugs may induce a

change in the chemical properties of the drugs or a loss in magnetization of the core magnetic material. Second, the drug loading efficiency is not high as expected for most nano-drugs. Third, controlling the drug release at the proper compartment within the tumor is still quite challenging, since most of the loaded drugs in nanoparticles release either prematurely or at a low rate from the nanoparticles. In this regard, novel strategies such as the development of magnetic IO nanoparticles for hyperthermia treatment and heating-induced drug release are under investigation and are expected to provide solutions for future clinical applications.

5. Conclusions and perspectives

Intensive investigations and the development of magnetic IO nanoparticles in the past decade have led to the much better understanding of the biological significances and potential biomedical applications of IO nanoparticles and a wide range of novel IO nanoparticle constructs designed for tumor targeted imaging and drug delivery. However, when constructing magnetic nanoparticles for tumor imaging and drug delivery, there are several goals that remain challenging to achieve, such as 1) specific accumulation in the tumor but minimal uptake in normal tissue and organs by selecting ideal tumor-targeted ligands; 2) modification of the surface and control of the size and charge of nanoparticles for adequate delivery; 3) regulation of blood circulation time; 4) stability of IO nanotherapeutics; 5) construction of smart tumor-targeted IO nanoparticles such that loaded drugs release only within tumor cells.

Recently, increasing concerns are focused on the safety of IO nanotherapeutic delivery systems. Although many animal studies have not shown visible toxicities, most of the available data come from mice with only a few studies conducted in rats, dogs and monkeys, and the sub-chronic and chronic toxicity studies for most IO nanoparticles have yet to be performed. Little is known about the long-term fate of IO nanoparticles and the pharmacokinetic/pharmacodynamic (PK/PD) changes in IO nanotherapeutics in vivo. The EPR effect constitutes only part of the drug targeting mechanism, and accumulating evidence has shown that tumor-targeted nanotherapeutics can internalize into tumor cells to a significantly higher concentration than their non-targeted counterparts. The majority of nanotherapeutic delivery systems are non-targeted, thus intensive studies using tumor-targeted nanoparticles as drug delivery carriers are needed.

6. Acknowledgment

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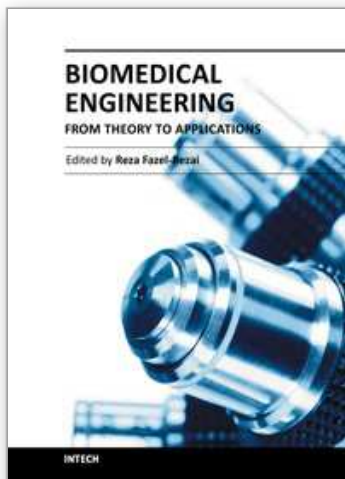
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Edited by Prof. Reza Fazel

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