

A Dual Ligand Targeted Nanoprobe with High MRI Sensitivity for Diagnosis of Breast Cancer^{*}

Fa-ming Gong^{a†}, Zuo-quan Zhang^{b, c†}, Xiao-dong Chen^b, Lu Zhang^a, Xing-su Yu^d, Qi-hua Yang^b,
Xin-tao Shuai^{a, d}, Bi-ling Liang^{b**} and Du Cheng^{a**}

^aPCFM Lab of Ministry of Education, School of Chemistry and Chemical Engineering, Sun Yat-sen University, Guangzhou 510275, China

^bDepartment of Radiology, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou 510630, China

^cDepartment of Radiology, The Fifth Affiliated Hospital, Sun Yat-sen University, Zhuhai 519000, China

^dCenter of Biomedical Engineering, Zhongshan School of Medicine, Sun Yat-Sen University, Guangzhou 510080, China

Abstract Antibody targeted delivery is an effective strategy to improve the diagnostic imaging outcome of nanoscale imaging agents in the focal areas. Dual targeting micelles encapsulating superparamagnetic iron oxide were prepared from the amphiphilic block copolymer poly(ethylene glycol)-poly(ϵ -caprolactone) (PEG-*b*-PCL) with different targeting ligands cRGD and scFv-ErbB single chain antibody conjugated to the distal ends of PEG block. The breast cancer animal model was established by subcutaneous injecting the BT474 cells into the BALB/c-nu female nude mice and then employed to assess the potential of the dual ligand targeted magnetic micelles as a novel MRI contrast agent on a 1.5 T clinical MRI scanner. The T_2 signal intensity of the tumor in animals receiving the dual ligand targeted magnetic micelles *via* tail vein decreased more significantly than the single ligand targeted and nontargeted magnetic micelles. These results indicate that the dual ligand targeted magnetic micelles, cRGD/scFv-ErbB-PEG-PCL-SPION, have great potential to act as a new type of effective nanoscale MRI contrast agent for early diagnosis of breast cancer.

Keywords: Block copolymer micelle; Superparamagnetic iron oxide (SPIO); Breast cancer; Magnetic resonance imaging (MRI); Herceptin; c(Arg-Gly-Asp-d-Phe-Lys) (cRGDFK).

INTRODUCTION

It was reported that about 500000 women died of breast cancer each year^[1, 2]. Over the past 20 years, both the incidence and mortality of breast cancer have increased very quickly in most developing countries^[3]. Admittedly, early diagnosis is the key to reduce mortality in breast cancer patients. Compared to conventional radiographic examinations such as X radiography which have low sensitivity and big limitation in assessing cancer invasion, magnetic resonance imaging (MRI) possesses great advantages including high soft tissue and spatial resolutions,

^{*} This work was supported by the 863 Programs of China (No. 2009AA03Z310), National Natural Science Foundation of China (Nos. 21174166, 30973419), the Ph.D. Programs Foundation of Ministry of Education of China (No. 20100171110011), the Postdoctoral Foundation (No. 201003370), Natural Science Foundation (Nos. 9351027501000003, S2011020003140) and S&T Programs of Guangdong Province (Nos. 2010B031500011, 2009B030803003, 2009B030801107, 2012B031800135), and SYSU Projects for Promotion of Key and Emerging Interdisciplinary Researches (10ykjc18) and Young Teachers (11lgpy44).

^{**} Corresponding authors: Du Cheng (程度), E-mail: chengdu@mail.sysu.edu.cn

Bi-ling Liang (梁碧玲), E-mail: liangbl@vip.tom.com

[†] These authors contributed equally to this work.

Received May 13, 2013; Revised July 26, 2013; Accepted August 7, 2013

doi: 10.1007/s10118-014-1399-8

high sensitivity, no radiation damage, which make it a much better tool for real time detection of breast cancer. Indeed, MRI is becoming one of the most important diagnostic techniques^[4] for breast tumor. Furthermore, application of the exogenous imaging probes, *i.e.* the contrast agents, is currently regarded as the most effective approach, which not only further increases the MRI sensitivity towards early tumor detection but also significantly improves the detection specificity. MRI probes bearing targeting ligands are able to recognize certain types of tumors *via* a process mediated by ligand directed binding to specific cancer cells.

Among different MRI contrast agents, superparamagnetic iron oxide (SPIO) is a nanoscale MRI T_2 contrast agent with excellent biocompatibility and high sensitivity. In order to improve the physiological stability and to prolong blood circulation time of SPIO, amphiphilic copolymers such as poly(ethylene glycol)-block-poly(ϵ -caprolactone) (PEG-*b*-PCL), poly(ethylene glycol)-block-poly(ethylenimine) (PEG-*b*-PEI), have been employed to modify the SPIO nanoparticles. In addition to its passive accumulation potential in tumor tissue *via* an enhanced permeability and retention (EPR) effect^[5], polymer-modified SPIO nanoparticles decorated with targeting ligands such as monoclonal antibodies (mAbs) have the specific binding affinity to certain cancer cells. Accumulation of SPIO in the cancer tissue and cells will further increase the MRI sensitivity and resolution in tumor diagnosis. Recently, many studies have shown that the ability of SPIO as an effective MRI nanoprobe for tumor detection can be significantly improved by surface modification with polymer and targeting ligands. Despite a great number of progresses^[6–10], currently the greatest challenge in tumor detection with MRI is still the development of effective contrast agents with high MRI sensitivity and excellent tumor cell affinity.

The present work aims at developing a highly sensitive MRI nanoprobe for breast tumor detection based on SPIO. To this end, SPIO nanoparticles were encapsulated in polymeric micelle of PEG-*b*-PCL, and the micelle surface was decorated with two different types of targeting molecules, *i.e.* the single chain HER-2 antibody fragment (scFv-ErbB) for human epidermal growth factor receptor-2 overexpressed in breast tumor cells and cyclic pentapeptide c(Arg-Gly-Asp-d-Phe-Lys) (cRGDFK) binding to the $\alpha_v\beta_3$ integrin of endothelial cells of tumor neovasculature, to enhance the targeting delivery ability towards breast tumor. Such dual-ligand targeting strategy is based on the fact that $\alpha_v\beta_3$ integrin was overexpressed only in the newly formed tumor blood vessels rather than in the static ones of normal tissues, which makes it an ideal target for detection and antiangiogenesis therapy of solid tumors. Meanwhile, scFv-ErbB modification may further enhance the accumulation of the micelle in tumor tissue *via* specific interaction with breast cancer cells^[11–13].

EXPERIMENTAL

Reagents

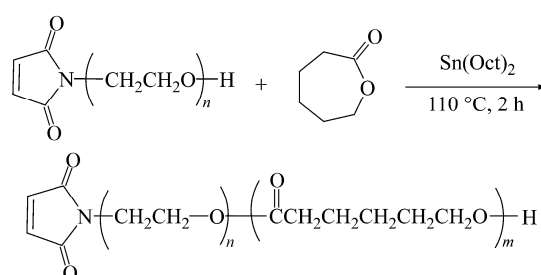
Maleimide-terminated polyethylene glycol (MAL-PEG-OH) (MWCO = 2000 Da), Monomethoxy polyethylene glycol (*m*PEG-OH) (MWCO = 2000 Da), ϵ -caprolactone, stannous(II) octoate were purchased from Fluka. Ethylenediaminetetraacetic acid (EDTA) was purchased from Guangzhou Chemical Reagent Factory. Chloroform (CHCl₃) and tetrahydrofuran (THF) were dried over CaH₂, and distilled before use. Phosphate Buffered Saline (PBS pH 7.4) was prepared. Superparamagnetic iron oxide (SPIO) measuring 6 nm in averaged diameter was synthesized *via* multistep synthesis as described in our publication. c(Arg-Gly-Asp-d-Phe-Lys) (cRGDFK), Herceptin were purchased from Genentech (South San Francisco, CA, USA).

Instruments

A polarized Zeeman Atomic Absorption Spectrophotometer (Model: Z-2000 series) was used to determine the encapsulation efficiency of SPIO in nanoparticles, the chemical structure of products was identified by Varian INOVA 500NB superconducting NMR analysis. Nanoparticle size was determined by Brookhaven instrument (Brookhaven, NY, USA) at 25 °C and scattering light was detected at 90° angle. The magnetization property of SPIO-loaded micelles was determined using a MPMS XL-7 Quantum Design SQUID (Ningbo Permanent Magnetism Co., Ltd China) magnetometer at 10 K and 300 K. MRI measurements were performed on a clinical 1.5 T MRI scanner (Philips Intera, Netherland, B.V.) at room temperature. An animal coil (Chenguang Medical Technologies, Shanghai, China) was used for all the *in vitro* and *in vivo* MRI studies.

Synthesis of PEG-*b*-PCL

The mPEG_{2k}-*b*-PCL_{1k} copolymer was synthesized according to our reported method^[14]. The copolymer, MAL-PEG_{2k}-*b*-PCL_{1k} was synthesized as shown in Scheme 1. Briefly, 0.5 g MAL-PEG-OH (MWCO = 2000 Da) was dried at 70 °C in vacuum for 2 h, then 0.1 mL Sn(Oct)₂ was added into the dried MAL-PEG-OH, then 0.25 mL ϵ -caprolactone was added in, the reaction continued for 5 h at 110 °C, then the product was collected by precipitation into diethyl ether and filtration and was dried in vacuo. Fe₃O₄ nanoparticles (SPIO) were synthesized according to a reported method^[15]. In brief, iron(III) acetylacetonate (2 mmol), 1,2-hexadecanediol (10 mmol), oleic acid (6 mmol), oleylamine (6 mmol) and benzyl ether (20 mL) were mixed and magnetically stirred under a flow of nitrogen. The mixture was heated to 200 °C for 2 h and then, under a blanket of nitrogen, heated to reflux 300 °C for 1 h. The black-colored mixture was cooled to room temperature by removing the heat source. The product, 6 nm Fe₃O₄ nanoparticles, was then precipitated with ethanol, centrifuged (6000 r/min, 10 min) to remove the solvent, and redispersed into hexane. A black-brown hexane dispersion of 6 nm nanoparticles was then produced.



Scheme 1 Synthesis of MAL-PEG-*b*-PCL

SPIO Encapsulation in MAL-PEG-*b*-PCL

SPIO-encapsulated MAL-PEG-*b*-PCL nanomicelles were prepared by the hydrophobic interaction method^[16]. 30 mg of MAL-PEG-*b*-PCL and 1 mg of hydrophobic SPIO were co-dissolved in THF (1.0 mL). The above solution was then slowly added to 10 mL of Phosphate Buffered Saline (PBS pH 7.4) under sonication and quickly filtered in an Amicon cell (regenerated cellulose membrane, MWCO = 10000) to wash away the THF. The product magnetic nanoparticle MAL-PEG-*b*-PCL-SPION was obtained.

Synthesis of scFv-ErbB and cRGD Conjugated Magnetic Nanoparticles

The synthetic process of scFv-ErbB and cRGD conjugated magnetic nanoparticles MAL-PEG-*b*-PCL-SPION was outlined in Fig. 2. The antibody bearing free sulfhydryl groups was prepared for conjugation to nanoparticles according to our literature report^[17]. The 2-mercapto ethylamine (50 mg) dissolved in 500 μ L PBS was mixed with 50 μ g scFv-ErbB dissolved in 10 μ L EDTA aqueous solution (0.5 mol/L). After incubation for 1 h at 37 °C, the scFv-ErbB bearing sulfhydryl groups was collected and purified by ultrafiltration in an Amicon cell (regenerated cellulose membrane, MWCO = 5 kDa) along with three PBS washes (each 500 μ L containing 10 μ L of 0.5 mol/L EDTA solution, pH 7.4). The cRGD (1 mg) bearing free sulfhydryl groups was obtained through using hydroxylamine (200 μ L) to unlock disulfide bond of cRGD. Both of the cRGD and scFv-ErbB, which bearing free sulfhydryl groups, were added into the MAL-PEG-*b*-PCL-SPION magnetic nanoparticle, the final product scFv-ErbB and cRGD conjugated magnetic nanoparticle MAL-PEG-*b*-PCL-SPION was obtained. The ¹H-NMR spectrum of the freeze-dried micelles in D₂O provided the information of micelles. To determinate whether the scFv-ErbB has been successfully conjugated to the nanoparticle, 2 μ L goat-anti-mouse IgG antibody labeled by fluorescein isothiocyanate (FITC) (Wuhan Boster Biological Technology, LTD. China) was added to 100 μ L micelles bearing the scFv-ErbB ligand with 0.9% bovine serum, and the mixture was allowed to stand for 1 h at room temperature. The resultant solution containing scFv-ErbB-functionalized micelles was washed three times with PBS (pH 7.4) with the aid of ultrafiltration using an Amicon cell (MWCO = 5 kDa). Finally, the obtained micelles dispersed in 20 μ L PBS were observed using a Carl Zeiss Aviox-1 inverted fluorescence microscope (Carl Zeiss Co., Ltd., Gottingen, Germany).

Dynamic Light Scattering (DLS) and Transmission Electron Microscopy (TEM) Measurements

A Brookhaven laser light scattering system consisting of a BI-200SM goniometer and a BI-9000AT digital correlator was used to measure particle size in water. The measurements were carried out at 90° scattering angle at 25 °C. We obtain images of TEM on a JEOL JEM-2010HR (Japan) operated at 160 kV at room temperature. Samples were prepared by drying a dispersion of the particles on a copper grid coated with amorphous carbon.

Measurements of SPIO-loading Contents and Magnetization

The SPIO loading contents and magnetization data of particles were determined by a polarized Zeeman Atomic Absorption Spectrophotometer (Model: Z-2000 series) and an MPMS XL-7 Quantum Design SQUID magnetometer at 10 K and 300 K. We determine the iron concentration at the specific Fe absorption wavelength (248.3 nm) based on a calibration curve $y = 27.27x - 0.16$, $r^2 = 0.999$, y means Fe concentration (mg/L), x means absorbance value. In order to generate hysteresis loops, the applied magnetic field was varied from 1.6×10^6 A/m to -1.6×10^6 A/m.

Magnetic T_2 Relaxation of SPION-loaded Nanoparticles

MRI measurements were performed on a clinical 1.5 T MRI scanner (Philips Intera, Netherland, B.V.) at room temperature with an animal coil (Chenguang Medical Technologies, Shanghai, China). The micelles were suspended in 2% gelatin solution (Sigma-Aldrich Trading, Shanghai, China) at a series of pre-defined concentrations. T_1 relaxation data were acquired by using a single-section mixed inversion-recovery spin-echo sequence that was initiated with an inversion recovery pulse 10 (4000/160 and inversion time msec, 400) followed by a spin-echo pulse (3500/20) and a single-section multi-spin-echo (2000/160). A single-section multi-echo T_2 -mapping sequence was performed to acquire the T_2 relaxation time of the micelles with the following parameters: TR 2000 ms, first echo time, 20 msec; echo spacing, 20 msec; echo number, eight steps. Others parameters of both sequences were as followed: FOV, 60 mm $\times$ 90 mm; matrix, 256 $\times$ 384; section thickness, 2 mm. T_1 and T_2 relaxation time images were automatically generated. The longitudinal and transverse relaxivities (r_1 and r_2) of the particles were calculated by linear least squares regression analysis from the slope of the linear plots of T_1 and T_2 relaxation rates ($1/T_1$ and $1/T_2$) versus Fe concentration.

In vivo MR Imaging

Experiments have been reviewed and approved by the Animal Ethical and Welfare Committee (AEWC) of Sun Yat-sen University. BALB/c-nu female nude mice (weight of 18 ± 3 g) were anesthetized with 10% chloral hydrate and received subcutaneous injections of 200 μ L of serum-free culture medium containing 1×10^7 BT474 cells to produce the subcutaneous xenografts. The *in vivo* MR imaging was performed while the subcutaneous tumor xenografts reached a diameter of 0.3–0.5 cm. After anesthesia, The SPIO-loaded nanoparticle PBS solution was injected into the nude mouse bearing the BT474 tumor *via* the tail vein with a dose of 10 mg Fe/kg body weight.

The animals were scanned under a 1.5 T MRI scanner (Philips Intera, Netherland, B.V.) at room temperature before and after injection of the SPIO-loaded nanoparticles. The sequences included a fast field echo sequence (FFE, TR/TE = 5000/100 ms) and a T_2 -mapping sequence concerned above. Other acquisition parameters were FOV 80 mm $\times$ 60 mm, matrix 336 $\times$ 252, slice thickness 1.5 mm. The signal intensities of the tumor were measured using an ellipse 30 mm² region of interest. The signal intensity of the tumor after injection of SPIO-loaded micelles was compared with pre-contrast and the relative signal intensity was calculated.

Hemolysis Assay

Hemolytic test was performed using rabbit erythrocyte suspension *in vitro*. Briefly, 0.5 mL of dual ligand targeted micelles (5 mg/mL) was added into 2.5 mL of rabbit erythrocyte suspension (2%) in normal saline and incubated at body temperature under mild shaking for 3 h. The erythrocyte suspensions were centrifuged at 2000 r/min for 5 min, and then the supernatants of the erythrocyte suspension were collected and were detected on a UV-Vis spectrophotometer at 545 nm to determine the hemolytic rate.

Acute Toxicity in vivo

The dual ligand targeted micelles were intravenously injected to BALB/c-nu mice at a dose of 75 mg/kg, 125 mg/kg and 250 mg/kg. The mice's blood biochemical parameters were adopted as observation indexes to evaluate the acute toxicities.

Statistical Analysis

All data were analyzed with the Statistical Package for the Social Sciences version 16.0 (SPSS Inc, Chicago, IL, USA). The results were expressed as the means $\pm$ SE, and $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

As shown in Fig. 1, resonances of both the PEG (mainly at $\delta = 3.64$) and PCL protons ($\delta = 1.38, 1.65, 2.31$, and 4.06) were clearly observed. The vinylic protons of maleimide group was shown around $\delta = 6.74$. Figure 1 shows the ^1H -NMR spectrum of the freeze-dried cRGD-PEG-*b*-PCL micelle in D_2O . Due to the insufficient solubility/mobility of hydrophobic PCL core in D_2O , proton resonances of the PCL block were significantly reduced. In contrast, the resonance peaks of the phenyl protons around $\delta = 7.2$ attributing to cRGD was detected and meanwhile maleimide resonance signal around $\delta = 6.74$ disappeared, which verified the conjugation of cRGD to the PEG distal ends.

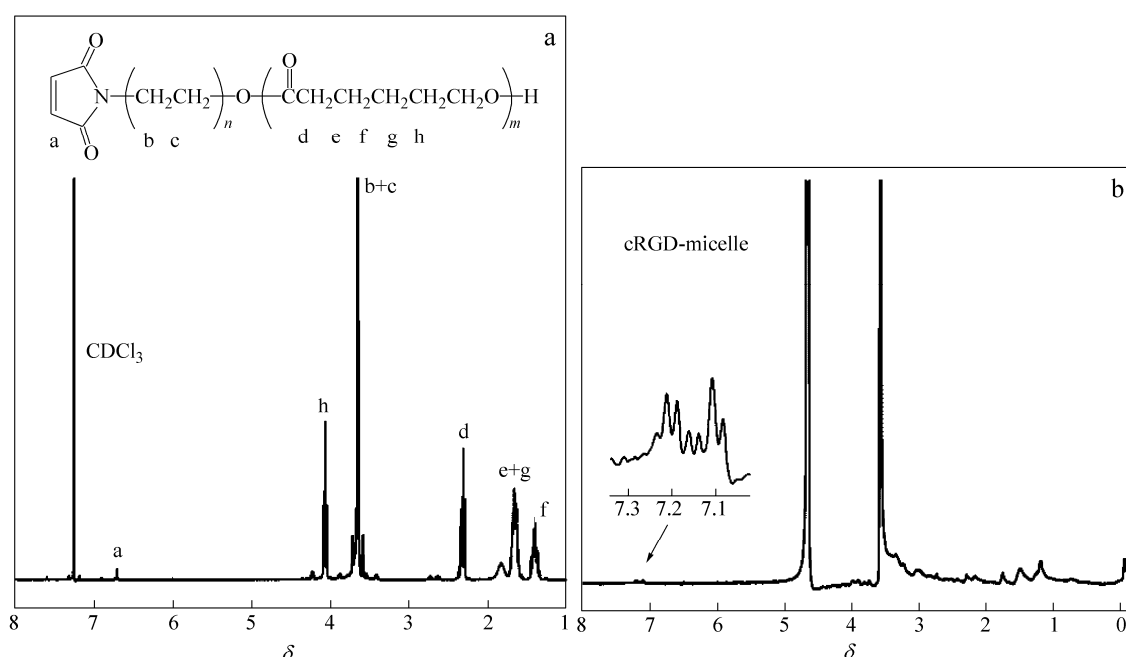


Fig. 1 ^1H -NMR spectra of MAL-PEG-*b*-PCL in CDCl_3 and cRGD-micelle in D_2O

The dual ligand targeted magnetic micelles were prepared as outlined in Fig. 2. To verify that scFv-ErbB was successfully conjugated to the PEG distal ends. The FITC labeled secondary antibody for scFv-ErbB was added to the micelle solution. After removing the excessive free secondary antibody by ultracentrifugation ($\text{MWCO} = 1 \times 10^5$), the micelle solution was observed under the inverted fluorescence microscope. As shown in Fig. 3, the existence of a great number of green fluorescent dots in the scFv-ErbB-PEG-*b*-PCL-SPION and cRGD/scFv-ErbB-PEG-*b*-PCL-SPION particle comparing with none fluorescent showed in cRGD-PEG-*b*-PCL-SPION demonstrated that scFv-ErbB was conjugated on the micelle shell, which enable the micelle to interact with the fluorescent secondary antibody to show dotted green fluorescence^[18].

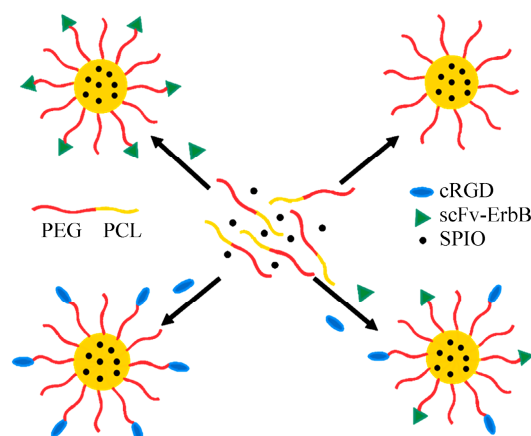


Fig. 2 Synthesis of dual ligand targeted magnetic micelle

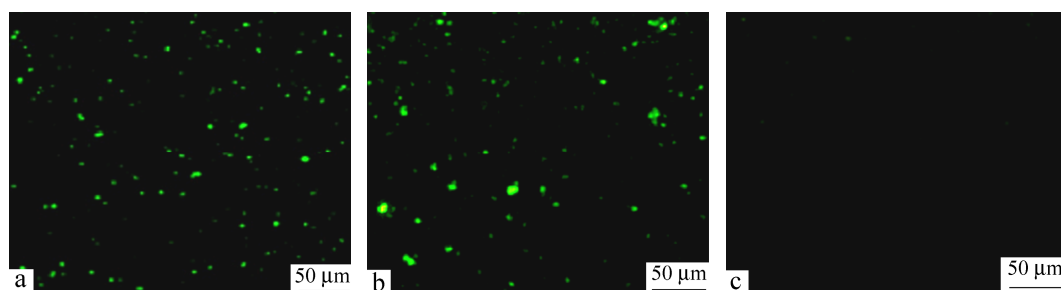
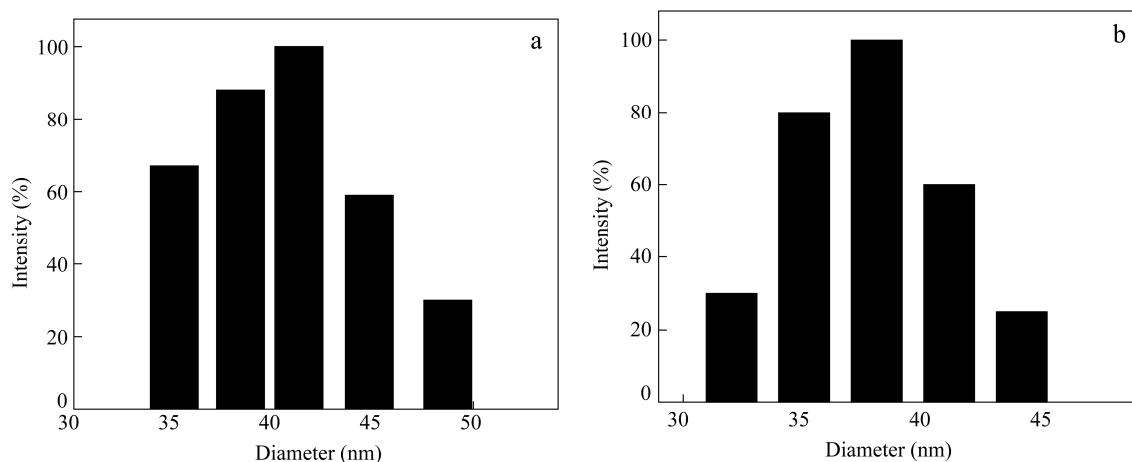


Fig. 3 Magnetic micelle binding to FITC-labeled secondary antibody for scFv-ErbB showing fluorescent dots under inverted fluorescent microscopic in scFv-ErbB-PEG-*b*-PCL-SPIO (a), cRGD/scFv-ErbB-PEG-*b*-PCL-SPIO (b) and none-fluorescent in cRGD-PEG-*b*-PCL-SPIO (c)

It is well-known that the size of nanocarriers affects its cellular uptake^[19–21]. We measured the morphology and size of the magnetic micelles. As detected by dynamic light scattering (DLS) (Fig. 4), the mean diameter of the dual ligand targeted micelle (39.9 ± 1.9 nm) was very close to that of the cRGD-alone targeted (38.4 ± 3.1 nm), scFv-ErbB-alone targeted (40.2 ± 2.3 nm) and non-targeted (41.2 ± 1.6 nm) micelles. SPIO clustering was observed in the measurement of transmission electron microscopy (Fig. 5), which was regarded as an underlining reason for high T_2 relaxivity of SPIO loaded micelles^[22].



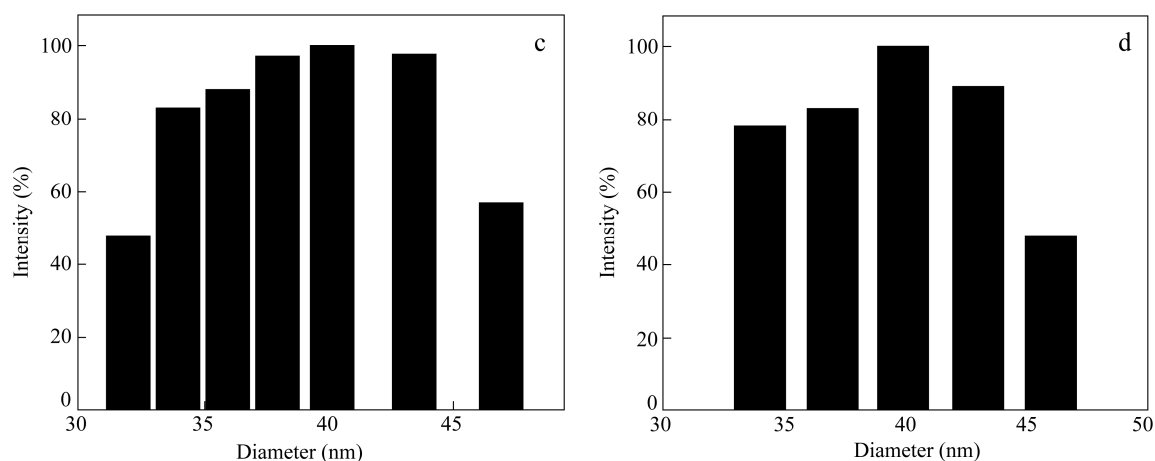


Fig. 4 Size distribution of mPEG-*b*-PCL-SPION (a), cRGD-PEG-*b*-PCL-SPION (b), scFv-ErbB-PEG-*b*-PCL-SPION (c) and cRGD/scFv-ErbB-PEG-*b*-PCL-SPION (d) micelles

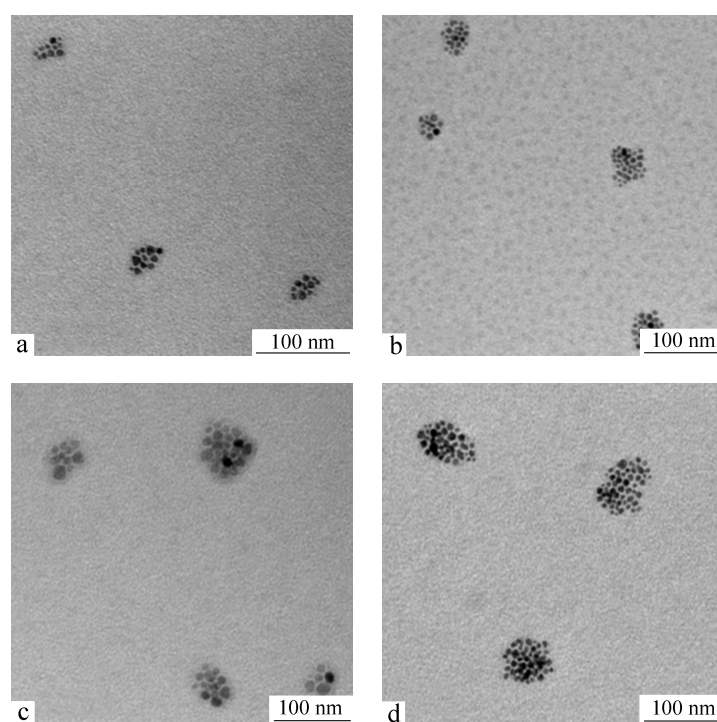


Fig. 5 TEM images of mPEG-*b*-PCL-SPION (a), cRGD-PEG-*b*-PCL-SPION (b), scFv-ErbB-PEG-*b*-PCL-SPION (c) and cRGD/scFv-ErbB-PEG-*b*-PCL-SPION (d) micelles

The SPIO-loading content of mPEG-*b*-PCL-SPION was 37.5%, as measured by AAS. The magnetization loops of SPIO and SPIO-loaded micelles measured at 10 K and 300 K are shown in Fig. 6. As reported previously^[23], the magnetization per Fe unit had no obvious loss when SPIO encapsulated in micelles. Both the mPEG-*b*-PCL-SPION and SPIO nanoparticles were ferromagnetic at 10 K. The micelles and SPIO showed a coercivity of 1.76×10^4 A/m and 1.92×10^4 A/m, respectively. However, at 300 K, both sample turned into superparamagnetic. That is, both samples showed typical behavior with zero coercivity and remanence. mPEG-*b*-PCL-SPION showed a saturation magnetization of $66.0 \text{ Fe emu} \cdot \text{g}^{-1}$, which is very close to that of SPIO ($64.7 \text{ Fe emu} \cdot \text{g}^{-1}$). These data demonstrated that the ideal magnetic properties of SPIO were not affected upon encapsulation in the mPEG-*b*-PCL micelles.

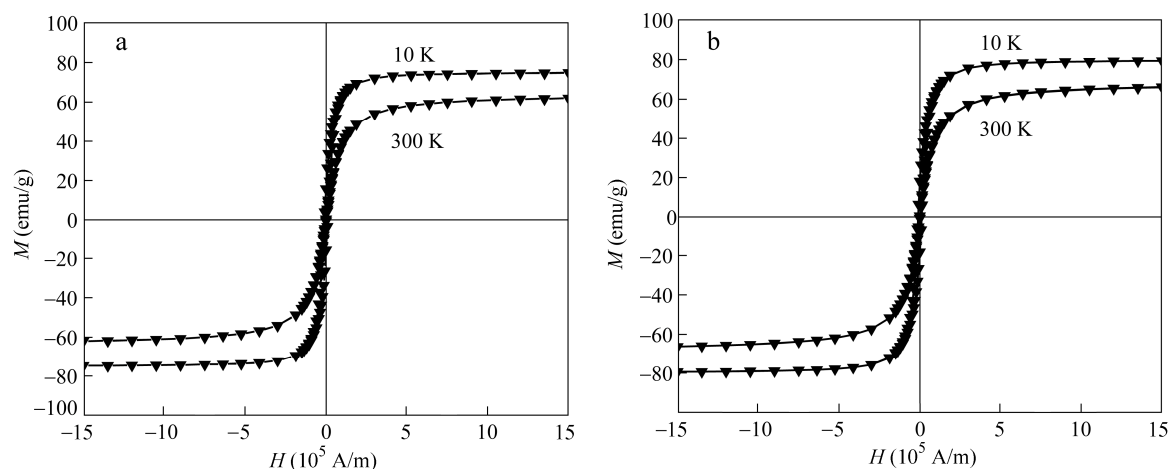


Fig. 6 Magnetization loops of SPIO nanoparticles (a) and mPEG-*b*-PCL-SPION (b) measured at 10 K and 300 K

In order to evaluate the MRI sensitivity of the magnetic micelles, we then measured the transversal relaxation times (T_1 and T_2) of the water protons of the aqueous micelle solutions on a 1.5 T MRI scanner at room temperature. For comparison, water soluble SPIO nanoparticles (WSPIO) were prepared by mixing the hydrophobic SPIO with a bipolar agent (etramethylammonium 11-aminoundecanoate). As shown in Fig. 7, encapsulation of SPIO in mPEG-*b*-PCL micelles increased the r_2 value ($102.7 \text{ Fe mmol}^{-1}\text{s}^{-1}$) whereas decreased the r_1 value ($1.7 \text{ Fe mmol}^{-1}\text{s}^{-1}$) as compared to WSPIO ($r_2 = 53.1 \text{ Fe mmol}^{-1}\text{s}^{-1}$, $r_1 = 3.4 \text{ Fe mmol}^{-1}\text{s}^{-1}$). The significantly increased r_2/r_1 value (60.4 versus 15.6) compared to WSPIO indicates that SPIO encapsulation in the mPEG-*b*-PCL micelles results in much higher MRI T_2 sensitivity *via* a mechanism of SPIO clustering effect as previously established^[24, 25].

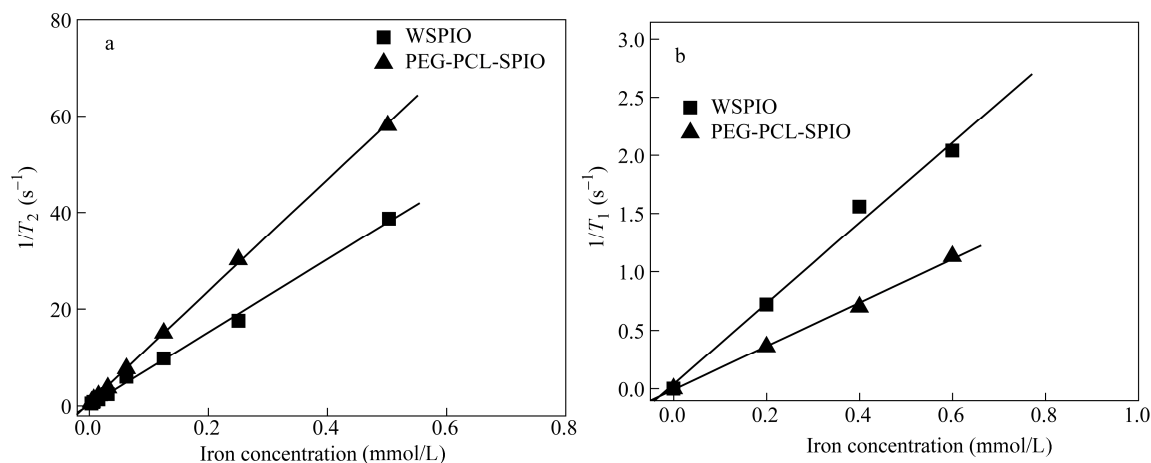


Fig. 7 Relaxation rates (s^{-1}) of T_2 (a) and T_1 (b) as a function of iron concentration (mmol/L) for magnetic micelle in comparison with WSPIO

As the T_2^* -weighted GRE (gradient-recalled echo) sequences is sensitive for detection of the signal changes induced by magnetic iron oxides^[26] and the T_2 -mapping sequence is used for qualitative analysis of SPIO nanoparticles *in vivo*^[27, 28], they were chosen for animal studies. The micelle solutions were injected into the nude mice bearing BT474 tumor at a dose of 10 mg Fe/kg body weight. As shown in Fig. 8, the FFE (T_2^* W) images acquired before and after the tail vein injection of magnetic micelles (Fig. 8). The signal of the tumor attenuated gradually along with the time delay (0.5 h, 3 h and 5 h). The signal intensity of tumor after injection of SPIO-loaded nanoparticles compared to pre-contrast indicated the signal changes caused by accumulation of

magnetic micelles. At the time of 5 h, it produced the maximum signal attenuation of the tumor and most obvious in the dual targeted group (Fig. 8b) with a maximum relative intensity decrease rate of 65.2% (Fig. 8c).

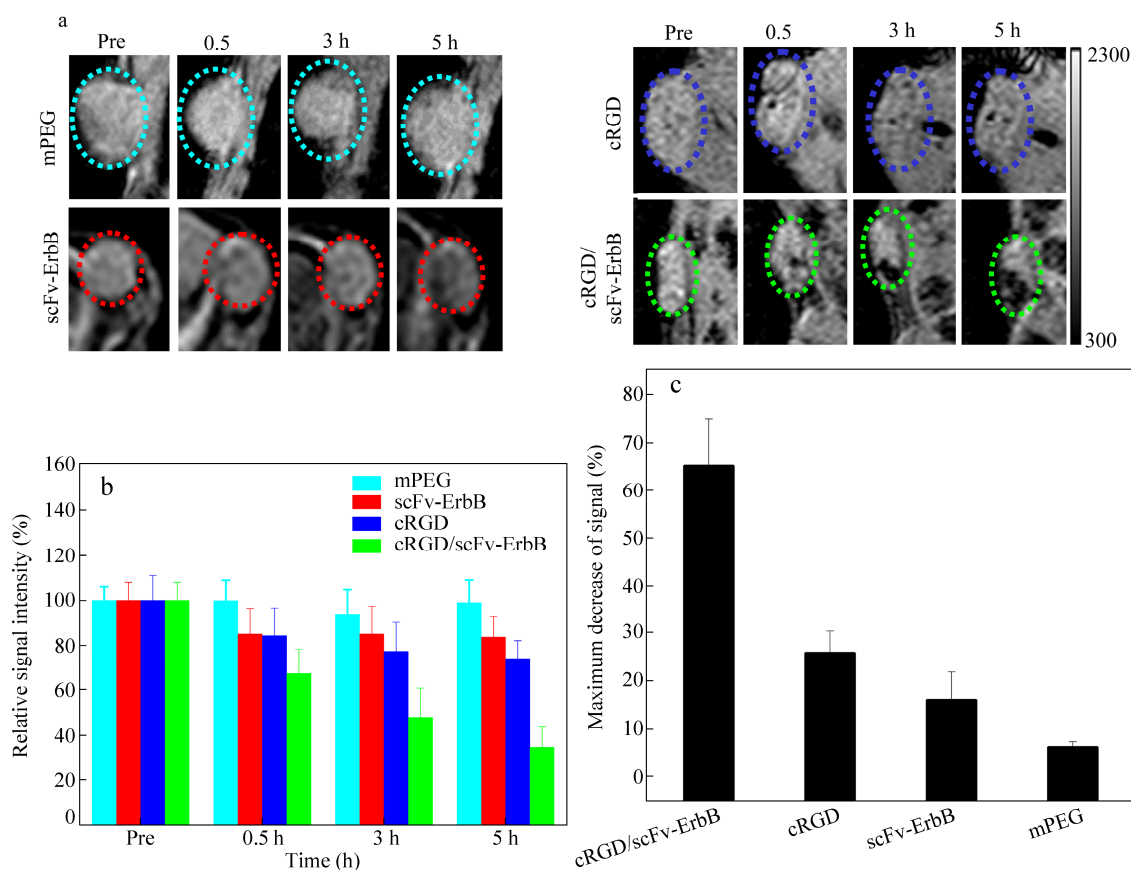


Fig. 8 (a) Animal MRI T_2^*W acquired before (Pre) and after tail vein administration of SPION loaded micelles, mPEG-*b*-PCL-SPION (mPEG), scFv-ErbB-PEG-*b*-PCL-SPION (scFv-ErbB), cRGD-PEG-*b*-PCL-SPION (cRGD) and cRGD/scFv-ErbB-PEG-*b*-PCL-SPION (cRGD/scFv-ErbB), into mice at 0.5 h, 3 h, 5 h; (b) the relative signal intensity of the tumor site and (c) the maximum decrease of the signal. The cyan, red, blue and green circle area marked the tumor area of mice receiving mPEG-*b*-PCL-SPION, scFv-ErbB-PEG-*b*-PCL-SPION, cRGD-PEG-*b*-PCL-SPION and cRGD/scFv-ErbB-PEG-*b*-PCL-SPION.

The T_2 relaxometry method was used to visualize the ability of micelles to shorten T_2 in the tumor sites. As shown in Fig. 9, a postinjection time-dependent T_2 shortening progress was detected in tumor sites. Injection of targeting micelles resulted in T_2 shortening in tumor. We compared the rates of signal intensity decrease from precontrast to 6 h upon injecting magnetic micelles which corresponded to T_2 attenuation. At 6 h after injection, the signal fell to the lowest among the three postinjection time points. The T_2 attenuation upon injecting nontargeted micelle (12.5%) is obviously less than that upon injecting any targeted one. Furthermore, the dual ligand targeted micelle (cRGD/scFv-ErbB-PEG-*b*-PCL-SPION) caused even higher T_2 attenuation (42.5%) than the single ligand targeted ones in tumor sites (33.8% for cRGD-PEG-*b*-PCL-SPION and 32.6% for scFv-ErbB-PEG-*b*-PCL-SPION). A partial MRI T_2 recovery was observed at 24 h after the injection of magnetic micelles, which was likely due to the metabolism of micelles *in vivo*. These data clearly show that the dual ligand targeting strategy is a highly effective approach to enhance the tumor accumulation of the magnetic micelles, which lays down the foundation for our magnetic micelle with high T_2 relaxivity (r_2) to be used as an effective MRI nanoprobe specific for breast cancer.

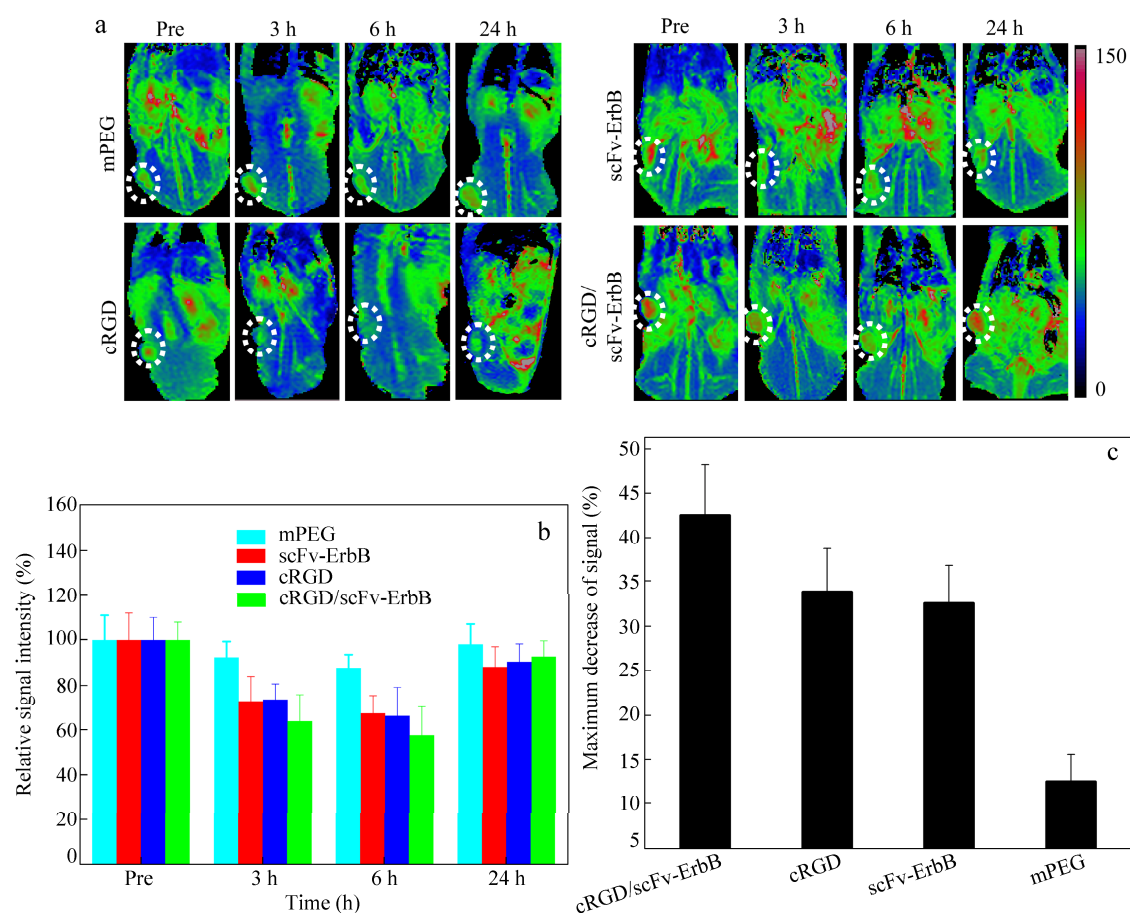


Fig. 9 (a) Animal MRI T_2^*W acquired before (Pre) and after tail vein administration of SPION loaded micelles, *m*PEG-*b*-PCL-SPION (*m*PEG), scFv-ErbB-PEG-*b*-PCL-SPION (scFv-ErbB), cRGD-PEG-*b*-PCL-SPION (cRGD) and cRGD/scFv-ErbB-PEG-*b*-PCL-SPION (cRGD/scFv-ErbB), into mice at 3 h, 6 h, 24 h. (The white circle indicated the tumor area.); (b) the relative signal intensity of the tumor site and (c) the maximum decrease of the signal corresponding to the decrease of T_2 value

As shown in Fig. 10, the hemolytic rate of the dual ligand targeted micelle solution at 5 mg/mL was lower than 10%, which implied that the micelles could be safely applied by intravenous injection. No mice died during the whole acute toxicity observation period. The body weight of mice has no significantly difference before and after injection in various doses in 14 days. As shown in Table 1 and Table 2, the results demonstrated that prepared dual ligand targeted MRI probe was safe *in vivo* for micellar concentrations of less than 250 mg/kg.

Table 1. The organ coefficient of BALB/c-nu mice of acute toxicity (%), Mean $\pm$ SD

	NS	30 mg/kg	50 mg/kg	100 mg/kg
Heart	0.58 $\pm$ 0.06	0.56 $\pm$ 0.05	0.58 $\pm$ 0.04	0.58 $\pm$ 0.08
Liver	6.27 $\pm$ 0.95	6.10 $\pm$ 0.23	5.57 $\pm$ 0.16	5.71 $\pm$ 0.30
Spleen	0.63 $\pm$ 0.20	0.63 $\pm$ 0.08	0.38 $\pm$ 0.02	0.53 $\pm$ 0.05
Lung	0.77 $\pm$ 0.10	0.69 $\pm$ 0.09	0.72 $\pm$ 0.08	0.72 $\pm$ 0.07
Kidney	1.63 $\pm$ 0.11	1.61 $\pm$ 0.10	1.33 $\pm$ 0.11	1.40 $\pm$ 0.09
Brain	1.90 $\pm$ 0.28	1.88 $\pm$ 0.10	2.20 $\pm$ 0.10	2.24 $\pm$ 0.16

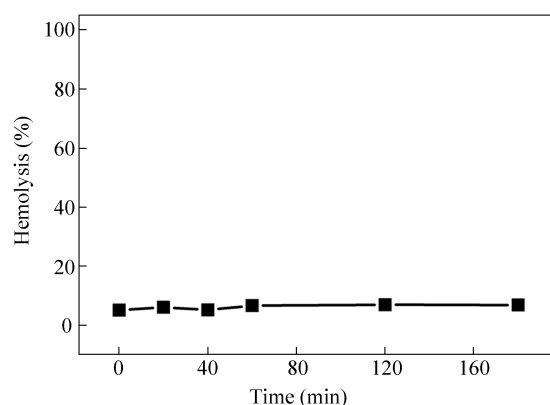


Fig. 10 Hemolytic test of the dual ligand targeted micelles using 2% rabbit erythrocyte suspension *in vitro* (micelles concentration: 5 mg/mL)

Table 2. Blood routine examination of BALB/c-nu mice of acute toxicity (Mean $\pm$ SD)

	NS	75 mg/kg	125 mg/kg	250 mg/kg
WBC (10^9 /L)	6.06 $\pm$ 1.10	6.42 $\pm$ 0.78	6.07 $\pm$ 0.62	6.27 $\pm$ 0.93
RBC (10^{12} /L)	9.55 $\pm$ 0.52	10.69 $\pm$ 0.37	10.73 $\pm$ 0.61	10.40 $\pm$ 0.17
HGB (g/L)	138.50 $\pm$ 9.19	156.20 $\pm$ 4.92	165.60 $\pm$ 4.56	159.60 $\pm$ 6.50
PCV (%)	42.85 $\pm$ 1.63	48.06 $\pm$ 1.77	49.70 $\pm$ 2.66	48.54 $\pm$ 1.11
MCV (fL)	44.90 $\pm$ 0.71	44.94 $\pm$ 0.59	46.32 $\pm$ 0.54	46.66 $\pm$ 0.44
MCH (pg)	14.50 $\pm$ 0.14	14.60 $\pm$ 0.40	15.46 $\pm$ 1.15	15.34 $\pm$ 0.44
MCHC (g/L)	323.50 $\pm$ 9.19	325.20 $\pm$ 6.06	334.00 $\pm$ 22.15	328.60 $\pm$ 8.20
BPC (10^9 /L)	656.00 $\pm$ 120.21	1074.00 $\pm$ 161.61	1130.40 $\pm$ 133.57	753.00 $\pm$ 154.37
RDW-SD (fL)	21.75 $\pm$ 0.35	21.90 $\pm$ 0.31	21.46 $\pm$ 0.59	21.00 $\pm$ 0.51
RDW-CV (%)	14.10 $\pm$ 0.12	14.10 $\pm$ 0.29	13.52 $\pm$ 0.45	13.02 $\pm$ 0.29
PDW (fL)	5.80 $\pm$ 0.03	5.74 $\pm$ 0.05	5.46 $\pm$ 0.05	5.76 $\pm$ 0.15
PCT (fL)	6.45 $\pm$ 0.07	6.36 $\pm$ 0.09	6.14 $\pm$ 0.11	6.48 $\pm$ 0.15
P-LCR (%)	3.40 $\pm$ 0.14	2.76 $\pm$ 0.63	2.36 $\pm$ 1.01	3.56 $\pm$ 1.06
PCT (%)	0.43 $\pm$ 0.08	0.68 $\pm$ 0.10	0.69 $\pm$ 0.08	0.49 $\pm$ 0.10
GRAN (%)	24.95 $\pm$ 7.14	18.78 $\pm$ 7.52	13.12 $\pm$ 4.29	14.38 $\pm$ 4.36
LYM (%)	44.05 $\pm$ 9.26	55.08 $\pm$ 3.96	48.36 $\pm$ 7.49	57.10 $\pm$ 6.55
MONO (%)	29.60 $\pm$ 1.98	25.02 $\pm$ 5.48	36.40 $\pm$ 4.23	27.30 $\pm$ 6.01
EOS (%)	1.40 $\pm$ 0.14	1.12 $\pm$ 0.38	1.90 $\pm$ 1.95	1.22 $\pm$ 0.29
BAS (%)	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.22 $\pm$ 0.49	0.00 $\pm$ 0.00
RET (%)	4.37 $\pm$ 1.21	3.69 $\pm$ 0.40	3.09 $\pm$ 1.44	3.02 $\pm$ 0.34

CONCLUSIONS

An amphiphilic block copolymer PEG-*b*-PCL was synthesized and assembled into a core-shell structural micelle with clustered magnetic SPIO nanoparticles encapsulated inside the core. The saturation magnetization of SPIO was not changed upon micellar encapsulation, and the magnetic micelle showed high MRI T_2 sensitivity which is essential for diagnostic application. Two targeting ligands (cRGD and scFv-ErbB) were introduced jointly to the PEG shell of micelle. Owing to this dual ligand targeting strategy, delivery efficacy of the magnetic micelle to the BT474 breast cells was significantly improved *in vivo*. These results indicate that the dual ligand targeted SPIO micelle has the potential to be used as a novel nanoprobe for breast tumor detection.

REFERENCES

- 1 Ferlay, J., Shin, H.R., Bray, F., Forman, D., Mathers, C. and Parkin, D.M., *Int. J. Cancer*, 2010, 127(12): 2893
- 2 Jemal, A., Bray, F., Center, M.M., Ferlay, J., Ward, E. and Forman, D., *CA Cancer J. Clin.*, 2011, 61(2): 69
- 3 Jemal, A., Center, M.M., DeSantis, C. and Ward, E.M., *Cancer Epidemiol. Biomarkers Prev.*, 2010, 19(8): 1893
- 4 Shen, J., Zhou, C.P., Zhong, X.M., Guo, R.M., Griffith, J.F., Cheng, L.N. and Liang, B.L., *Radiology*, 2010, 254(3): 729
- 5 Greish, K., *Cancer Nanotechnology Methods in Molecular Biology*, 2010, 624: 25
- 6 Guo, Y., Chen, W., Wang, W., Shen, J., Guo, R., Gong, F. and Shuai, X., *ACS nano*, 2012, 6(12): 10646
- 7 Shen, M., Gong, F., Pang, P., Zhu, K., Meng, X., Wu, C. and Shuai, X., *Int. J. Nanomed.*, 2012, 7: 3319
- 8 Yang, X., Chen, Y., Yuan, R., Chen, G., Blanco, E., Gao, J. and Shuai, X., *Polymer*, 2008, 49(16): 3477
- 9 Hong, G., Yuan, R., Liang, B., Shen, J., Yang, X. and Shuai, X., *Biomed. Microdevices*, 2008, 10(5): 693
- 10 Chen, G., Chen, W., Wu, Z., Yuan, R., Li, H., Gao, J. and Shuai, X., *Biomaterials*, 2009, 30(10): 1962
- 11 Zutter, M.M., Mazoujian, G. and Santoro, S.A., *Am. J. Pathol.*, 1990, 137(4): 863
- 12 Nahta, R., Yuan, L.X., Zhang, B., Kobayashi, R. and Esteva, F.J., *Cancer Res.*, 2005, 65(23): 11118
- 13 Nasongkla, N., Shuai, X., Ai, H., Weinberg, B.D., Pink, J., Boothman, D.A., and Gao, J., *Angew. Chem.*, 2004, 116(46): 6483
- 14 Yang, X., Deng, W., Fu, L., Blanco, E., Gao, J., Quan, D. and Shuai, X., *J. Biomed. Mater. Res. A*, 2008, 86(1): 48
- 15 Sun, S., Zeng, H., Robinson, D.B., Raoux, S., Rice, P.M., Wang, S.X. and Li, G., *J. Am. Chem. Soc.*, 2004, 126(1): 273
- 16 Wang, W., Cheng, D., Gong, F., Miao, X. and Shuai, X., *Adv. Mater.*, 2012, 24(1): 115
- 17 Zhou, J., Huang, L., Wang, W., Pang, J., Zou, Y., Shuai, X. and Gao, X., *Chinese Sci. Bull.*, 2009, 54(18): 3137
- 18 Liao, C., Sun, Q., Liang, B., Shen, J. and Shuai, X., *Eur. J. Radiol.*, 2011, 3(80): 699
- 19 Lu, J., Ma, S., Sun, J., Xia, C., Liu, C., Wang, Z. and Gu, Z., *Biomaterials*, 2009, 30(15): 2919
- 20 Shuai, X., Ai, H., Nasongkla, N., Kim, S. and Gao, J., *J. Control. Release*, 2004, 98(3): 415
- 21 Shuai, X., Merdan, T., Schaper, A.K., Xi, F. and Kissel, T., *Bioconjugate Chem.*, 2004, 15(3): 441
- 22 Ai, H., Flask, C., Weinberg, B., Shuai, X.T., Pagel, M.D., Farrell, D. and Gao, J., *Adv. Mater.*, 2005, 17(16): 1949
- 23 Sun, Q., Cheng, D., Yu, X., Zhang, Z., Dai, J., Li, H. and Shuai, X., *J. Mater. Chem.*, 2011, 21(39): 15316
- 24 Nasongkla, N., Bey, E., Ren, J., Ai, H., Khemtong, C., Guthi, J. S. and Gao, J., *Nano Lett.*, 2006, 6(11): 2427
- 25 Tromsdorf, U.I., Bigall, N.C., Kaul, M.G., Bruns, O.T., Nikolic, M.S., Mollwitz, B. and Weller, H., *Nano Lett.*, 2007, 7(8): 2422
- 26 Cheng, D., Hong, G., Wang, W., Yuan, R., Ai, H., Shen, J. and Shuai, X., *J. Mater. Chem.*, 2011, 21(13): 4796
- 27 Imai, Y., Kaneko, E., Asano, T., Kumagai, M., Ai, M., Kawakami, A. and Shimokado, K., *J. Atheroscler. Thromb.*, 2007, 14(2): 65
- 28 Langley, J., Liu, W., Jordan, E.K., Frank, J.A. and Zhao, Q., *Magn. Reson. Med.*, 2011, 65(5): 1461