Magnetite Nanocrystals Loaded Biocompatible Diblock Copolymer Micelles as Mouse Liver MRI Contrast Agents

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Abstract. Monodisperse magnetite nanocrystals have been synthesized in organic phase at high temperatures in the presence of oleic acid and oleylamine. These hydrophobic nanocrystals were transferred into water with the help of amphiphilic block copolymer poly (ε-caprolactone)-poly(ethylene glycol) (PCL-PEG). The composite structure is about 70 nm in diameter, containing clusters of magnetite nanocrystals per micelle. Under a 1.5T clinical scanner, the T2 relaxivity of composite micelles is 125 FmM⁻¹s⁻¹. The magnetite nanoparticles loaded PCL-PEG micelles have good cell biocompatibility as evaluated by the MTT cell proliferation assay. The T2-weighted signal intensity in mouse liver decreased drastically after intravenous administration, and slowly recovered to about 20% after 5 days. It may be used as a long-term MRI contrast agent in liver disease diagnosis.

Keywords: MRI, Magnetite, PCL-PEG, Micelle, Liver

1. Introduction

Poly(ethylene glycol) (PEG) is widely used in the field of biomaterials, tissue engineering and drug delivery due to its excellent biocompatibility [1-3]. PEG has simple chemical structures. It contains -OH functional group which makes it reactive to form diblock or triblock copolymer [2]. Amphiphilic diblock copolymer has both hydrophobic and hydrophilic segments. It undergoes phase separation during processing and can form core-shell micelle structure in aqueous solution to obtain a steady state [3]. The core can be used to load hydrophobic drugs while the hydrophilic PEG corona can improve the blood biocompatibility [4]. When used as drug carriers, micelles can contain poorly soluble drugs, increase their bioavailability, and permit them to accumulate in certain body regions [5]. Micelles have been used mostly to load chemotherapeutic drugs, such as paclitaxel [6-8] and doxorubicin [9]. Recently, micelles have extended their applications in nanocrystal encapsulation such as superparamagnetic iron oxide (SPIO) nanoparticles and quantum dots [5, 10, 11].

SPIO nanoparticles and their dispersions in various media have long been of scientific and technological interest [12]. SPIO agents are often composed of both active inorganic components (iron oxide) and organic coating materials [13]. SPIO nanoparticle based agents have been widely used for liver magnetic resonance imaging (MRI) in the last several years [14].

At present, MRI is one of the most important diagnostic imaging tools in medicine, with demonstrated feasibility as a non-invasive, non-radioactive technique [15]. However, the contrast between pathological and normal tissues is far from ideal in many situations. By using contrast agent, image contrast between normal and pathological tissues can be further increased. In this study, we first synthesized SPIO (Fe₃O₄) nanocrystals in organic phase at high temperatures. Amphiphilic copolymer poly(ε-caprolactone)-poly(ethylene glycol) (PCL-PEG) was prepared to form micelles and stabilize hydrophobic SPIO nanocrystals in water phase. Clusters of SPIO nanocrystals can be self-assembled inside the PCL cores and protected by the PEG corona. The biocompatibility was investigated using mouse macrophage cell line RAW

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264.7 and human hepatocarcinoma cell strain HepG2 in vitro. The contrast effect in liver tissue was assessed in mouse under a 3.0T Philips MRI scanner.

2. Materials and Methods

2.1. Synthesis and characterization of SPIO nanocrystals

All the following reagents were purchased from Sigma-Aldrich. SPIO nanocrystals were synthesized according to a method by Sun et al. [12]. Briefly, Fe(acac)_3 (2mmol), 1,2-hexadecanediol (10mmol) were first mixed with oleic acid (6mmol), oleylamine (6mmol), and benzyl ether (20ml). The mixture was stirred under a flow of Argon, then heated to 200°C for about 2 hours, and finally heated to 300°C for 1 hour under reflux. The black-brown mixture was cooled to room temperature. By adding 40ml ethanol to the mixture, black product precipitated to the bottom. The black precipitates collected by centrifugation were dissolved in hexane containing oleic acid (about 0.05ml) and oleylamine (about 0.05ml). The solution was centrifuged to remove the undispersed residue. Finally, the product, Fe_3O_4 nanocrystals were washed using ethanol and redispersed into hexane. Dynamic light scattering (DLS) was applied to measure size distribution of the Fe_3O_4 nanocrystals. DLS was performed simply by adding sample solution to a sample cell and measured using a Zetasizer Nano ZS (Model ZEN3600, Malvern Instruments, UK) directly.

2.2. Synthesis and characterization of PCL5k-mPEG5k

PCL5k-mPEG5k was synthesized by ring-opening polymerization of ε-caprolactone using monomethoxy-terminated PEG (mPEG) as a macroinitiator and stannous octoate (Sn(Oct)_2) as a catalyst [4, 16]. mPEG (M_w = 5kDa) was purchased from Fluka Chemical Co. ε-caprolactone and Sn(Oct)_2 were purchased from Aldrich Chemical Co. and used as received. ε-caprolactone was dried over calcium hydride (CaH_2) powder and purified by distillation under reduced pressure, while PEG was dried by azeotropic distillation method with toluene to constant weight under vacuum. The whole reaction system was protected by dried argon atmosphere.

The reaction was performed by adding an equal amount (1:1 w/w) of ε-caprolactone monomer under an argon atmosphere into a dried flask containing a pre-weighted amount of mPEG5k. One drop of Sn(Oct)_2 was added as catalyst. The whole flask was placed in oil bath at 105°C for 48hrs with magnetic stirring. After cooling at room temperature, the resulting block copolymers were dissolved in tetrahydrofuran (THF) and precipitated in excess amount of cold ether. Then the precipitates were dried at 40°C under vacuum.

^1H-NMR spectra was obtained with deuterium chloroform (CDCl_3) as solvent and TMS as internal standard, using a Bruker AM 400 apparatus at 25°C. The actual PCL content of copolymer was calculated from the integral height of hydrogen shown in ^1H-NMR.

Average molecular weight and its distribution were determined by gel permeation chromatography (GPC) (Waters ALC/GPC 244, USA) operating with THF as eluent and calibrated with polystyrene standards.

2.3. Formation and Characterization of SPIO Micelles

![Fig. 1 Scheme of SPIO/PCL-PEG micelle formation](image)

The process of SPIO micelle formation is illustrated in Fig.1. SPIO dispersed in hexane was dried by Argon flow and then dissolved in THF. PCL5k-mPEG5k powder was added to the mixture. Mass ratio of
SPIO and PCL5k-mPEG5k was 1:2. After complete dissolution, the mixture was added to water in the presence of sonication. Dynamic light scattering (DLS) was used to characterize size distribution of SPIO micelles. T₂ relaxivity was measured at 1.5T under a clinical MRI scanner (Siemens) at room temperature.

2.4. Biocompatibility test

Biocompatibility of SPIO micelles was tested by MTT cell proliferation assay [17, 18]. MTT test was performed on two different cell lines. Mouse macrophage cell line RAW 264.7 was cultured in RPMI-1640 medium containing 10% FBS (GIBCO). Human hepatocarcinoma cell strain HepG2 was cultured in DMEM medium with 10% FBS. After digestion by Trypsin-EDTA (GIBCO), cells were inoculated in 96 well culture plates and cultured at 37°C in a humidified atmosphere containing 5% CO₂ for 24 hours. SPIO micelles at iron concentration of 0.01, 0.1, 1, and 10 μg/ml were added to the medium and incubated for 4 hours. MTT reagent (Sigma) was added to the medium and incubated for 4 hours. Then medium was discarded and 200μl DMSO (dimethyl sulfoxide) was added per well. Absorbance was measured, including blanks, at 570 nm in a Microplate Reader (BIO-RAD, model 550). Linear relationship between cell viability and absorbance can be established. Cell viability was determined by the following equation: Cell Viability (%) = (Ni/Nc) × 100, Where Ni and Nc are the absorbance of surviving cells treated with and without SPIO-loaded micelles, respectively [8].

2.5. In vivo MRI

MR images were acquired under a 3T imaging system (ACHIEVA 3.0T, Philips) by using a mouse coil for transmission and reception of the signal. Multisection single-echo T₂-Weighted TSE sequence (TE = 80ms; TR = 1000ms; slice thickness = 1.6mm; flip angle = 90°) was used in all the study following ref [19]. Mouse was anaesthetized by pentobarbital sodium at a dose of 40 mg/kg body weight. MRI scan was performed before and 0.1, 0.5, 1, 2, 3, 4, 24, 48, 72, 96, and 120 hrs after SPIO micelle administration at a dose 5mg iron per kilogram body weight. Signal intensity was measured at each time point using tools of eFilm software.

3. Results and Discussion

3.1. Characterization of PCL5k-mPEG5k

In the 1H NMR spectra of block copolymers dissolved in CDCl₃, the peaks at 3.39 and 3.64 ppm are the characteristic chemical shifts corresponding to mPEG, while the peaks at 1.38, 1.65, 2.30, and 4.06 ppm are the characteristic chemical shifts corresponding to PCL. The composition of copolymer was calculated from the integral values of characteristic peaks of PEG (~ 3.39 ppm) and PCL (~ 2.31 ppm), using the known molecular weight of MPEGS (5000). And the result of the molecular weight of PCL block is about 4519. The molecular weight of PCL-PEG copolymer is 1.127×10⁴ according to the GPC result.

3.2. Characterization of Fe₃O₄ nanocrystals and SPIO micelles

![Graph showing number distribution of nanocrystals and micelles]
Amphiphilic block copolymers can usually form core-shell structures in aqueous solution due to their amphiphilic characteristic [6, 20]. SPIO agents are often composed of both an active SPIO component (iron oxide crystals) and a coating material. Sizes of iron oxide crystals are generally between 4-10 nm [13]. Standard SPIO MRI contrast agent is at approximately 60-150nm [21]. In this work, we used a sonication method to form PCL5k-mPEG5k loaded SPIO micelles (Fig.1). Size distribution of both SPIO crystals and SPIO micelles were determined by DLS. The diameter of SPIO nanocrystals was 9.0 ± 2.5 nm (Fig. 2, line a), slightly larger than TEM observed diameter (~ 6 nm) because organic coatings (oleic acid and oleylamine) were not visible under electron microscope. The size of SPIO micelles was 68.8 ± 23.5nm (Fig. 2, line b). It was found that the diameter of the micelles increased with an increase in the length of the PCL blocks [6]. In the study we found that mass ratio of SPIO and PCL 5k-mPEG5k also influenced diameter of micelles. A higher polymer/SPIO ratio led to a smaller micelle size.

Fig. 2 Size distribution of SPIO nanocrystals and SPIO micelles by DLS measurement. a: Size distribution of SPIO nanocrystals in hexane. b: size distribution of SPIO micelles in water.

T₂ relaxivity was measured using SPIO micelle samples of different iron concentrations in water under a 1.5T clinical scanner. The linear relationship between relaxation rate and iron concentration is displayed in Fig. 3. T₂ relaxivity of SPIO micelles is 125 Fe mM⁻¹s⁻¹. In comparison, AMI-25 (Feridex by Berlex Laboratories) has a T₂ relaxivity of 98.3 Fe mM⁻¹s⁻¹ with a larger particle size (80-150nm) [13, 22]. USPIO AMI-227 (Sinerem by Guerbet) has size of 20-40 nm, and its T₂ relaxivity is 53 Fe mM⁻¹s⁻¹ [23]. Higher T₂ relaxivity indicates stronger negative contrast effect [24]. Therefore micelles containing clusters of SPIO nanocrystals may be an effective MRI contrast agent.

3.3. Biocompatibility test
The cytotoxicity of SPIO loaded PCL5k-mPEG5k micelles was evaluated by MTT assay on both mouse macrophage cell line RAW 264.7 and human hepatocarcinoma cell line HepG2. Cell viability for each treatment group is not significantly different from the control group (P > 0.05). The results indicate that SPIO PCL5k-mPEG5k micelles have good biocompatibility on both cell lines within the measured concentrations (Fig. 4).
Fig. 4 Biocompatibility study of SPIO micelles at different iron concentrations by MTT test (n = 3). (A): Cell viability investigation on the human hepatocarcinoma cell line HepG2. (B): Cell viability investigation on the mouse macrophage cell line RAW 264.7.

3.4. In vivo MRI

![Signal Intensity vs Time after SPIO Administration](image)

Fig. 5 Mouse liver signal intensity at different time points after administration of SPIO micelles at a dose of 5 mg Fe/kg animal body weight (n = 3). Control indicates signal intensity before SPIO micelle administration.

MRI contrast agent can enhance the image contrast of tissues at the location where the agent accumulates, providing functional or blood flow information [15]. MRI was performed following a T2-weighted TSE sequence with TE = 80 ms and TR = 1000 ms. Fig. 5 shows signal intensity change at different time points before and post intravenous administration of SPIO micelles. The MRI signal intensity of normal liver tissue is 403 ± 36 and it dropped significantly to 40 ± 11 immediately after SPIO micelle administration. The obvious drop of MRI signal intensity may due to phagocytosis of MnFe2O4 micelles by liver Kupffer cells of reticuloendothelial system (RES) [25]. It can be seen from Fig. 5 that signal intensity recovers gradually with the elapse of time. Even 120 hours after SPIO micelles administration, the signal intensity only increases to 93 ± 49, still much lower than the controlled one. Corresponding MRI images are displayed in Fig. 6, and we can see that liver images are much darker for post contrast agent administration (B - L) than the controlled sample (A).

Briley-Saebo et al. suggested that coating materials significantly influence the rate of iron oxide...
clearance in rat liver [26]. After administration, the half-life of iron oxide particles in rat liver was 8 days for dextran-coating, 10 days for carboxydextran coating, 14 days for unformulated oxidized-starch and 29 days for formulated oxidized-starch [26]. Besides, administration of different dose per body weight also has effects on MRI contrast and higher dose can induce longer MRI effect in liver [26, 27]. While, for long-term MRI effect, elevated liver R2* values may be attributed to the compartmentalization of the breakdown products within the cells [26-28].

Fig.6 T2-weighted TSE images of mouse liver before and after administration of SPIO micelle at the dose of 5 mg Fe/kg animal body weight. A: before SPIO micelles administration; B: 0.1 hour after administration; C: 0.5 hour after administration; D: 1 hour after administration; E: 2 hours after administration; F: 3 hours after administration; G: 4 hours after administration; H: 24 hours after administration; I: 48 hours after administration; J: 72 hours after administration, K: 96 hours after administration, L: 120 hours after administration

4. Conclusion

Monodisperse magnetite nanocrystals have been synthesized in organic phase and transferred into water with the help of amphiphilic polymer PCL-mPEG. The composite structure is about 70 nm in diameter, containing clusters of magnetite nanocrystals per micelle. Under a 1.5T clinical scanner, the T2 relaxivity of composite micelles is 125Fe mM⁻¹s⁻¹. The cell biocompatibility of magnetite nanoparticles loaded PCL-PEG micelles have been evaluated by the MTT cell proliferation assay and no cytotoxicity was discovered. The T2-weighted signal intensity in mouse liver decreased to 10% immediately after intravenous administration, and slowly recovered to about 23% after 5 days. This composite micelle may be used as a long-term MRI contrast agent in liver disease diagnosis.

5. Acknowledgement

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6. References


