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Folate-bovine serum albumin functionalized polymeric micelles loaded with superparamagnetic iron oxide nanoparticles for tumor targeting and magnetic resonance imaging

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ABSTRACT

Polymeric micelles functionalized with folate conjugated bovine serum albumin (FA-BSA) and loaded with superparamagnetic iron oxide nanoparticles (SPIONs) are investigated as a specific contrast agent for tumor targeting and magnetic resonance imaging (MRI) *in vitro* and *in vivo*. The SPIONs-loaded polymeric micelles are produced by self-assembly of amphiphilic poly(HFMA-co-MOTAC)-g-PEGMA copolymers and oleic acid modified Fe_3O_4 nanoparticles and functionalized with FA-BSA by electrostatic interaction. The FA-BSA modified magnetic micelles have a hydrodynamic diameter of 196.1 nm, saturation magnetization of 5.5 emu/g, and transverse relaxivity of 167.0 $mM^{-1} S^{-1}$. *In vitro* MR imaging, Prussian blue staining, and intracellular iron determination studies demonstrate that the folate-functionalized magnetic micelles have larger cellular uptake against the folate-receptor positive hepatoma cells Bel-7402 than the unmodified magnetic micelles. *In vivo* MR imaging conducted on nude mice bearing the Bel-7402 xenografts after bolus intravenous administration reveals excellent tumor targeting and MR imaging capabilities, especially at 24 h post-injection. These findings suggest the potential of FA-BSA modified magnetic micelles as targeting MRI probe in tumor detection.

1. Introduction

Morbidity and mortality caused by cancer is increasing and early detection is the key to effective treatment [1,2]. As one of the powerful techniques in cancer diagnosis, magnetic resonance imaging (MRI) offers the advantages of non-invasive, multiparametric imaging as well as deep soft tissue penetration [3]. Tumor-specific targeted MR imaging [4–6] thus has large potential and nanoparticle encapsulated contrast agents can enhance the contrast between tumors and normal tissues [7]. In this respect, superparamagnetic iron oxide nanoparticles (SPIONs) are sensitive and negative MRI probes possessing the ability to noninvasively monitor events occurring on the cellular and even molecular levels *in vivo* [8,9]. However, biological applications of SPIONs are limited because of the high surface hydrophobicity making them prone to being engulfed by macrophages and rapidly removed from circulation [10]. In order to prolong the circulation time, it is essential to modify the surface of these magnetic iron oxide nanoparticles. Several methods have been explored to convert hydrophobic SPIONs into hydrophilic ones, for instance, ligand exchange [11] and amphiphilic copolymer encapsulation [12]. Amphiphilic copolymers have drawn much interest because of their self-assembling properties [13]. Amphiphilic copolymers consisting of both hydrophobic and hydrophilic segments can self-assemble into hydrophobic core–hydrophilic shell structures in an aqueous medium [14]. The hydrophobic SPIONs can form small clusters on the hydrophobic core of the polymeric micelle to produce high MRI T_2 contrast [15], whereas the hydrophilic segments of the polymer derivatized with a ligand endows them with targeting ability [16]. For example, self-assembled fluorine-containing amphiphilic poly(HFMA-g-PEGMA) copolymeric micelles loaded with SPIONs have an organized core–shell structure and show excellent stability and loading efficiency [17] and the cationic monomer methacryloxyethyl

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trimethyl ammonium chloride (MOTAC) serves as a binding site due to the positive charge [18].

Bovine serum albumin (BSA), a negatively charged plasma protein, offers advantages such as non-toxicity, good biocompatibility, and excellent biodegradability [19]. It has been used as a carrier for targeting agents such as folate [20,21] to improve the water solubility and prolong circulation in the blood. Moreover, the negatively charged BSA can serve as a stabilizing agent to bind cationic particles [22] since a polyelectrolyte complex can be formed by the electrostatic attraction between the cationic polymeric micelles and negatively charged BSA in a solution. However, *in vivo* application of water-soluble SPIONs-loaded polymeric micelles has been hampered by lack of specificity toward a pathological site [23]. The folate receptor (FR) is a specific tumor marker and overexpressed in many forms of cancer [24,25]. The folate receptor also has a high binding affinity to folic acid (K_d ~190 pM) [26] and is thus an attractive target for site-specific delivery of folate modified contrast agents into proliferating cells. In fact, folate has been conjugated with nanoprobes to improve the sensitivity and specificity of tumor diagnosis [27]. When cationic magnetic polymeric micelles are functionalized with folic acid using BSA as the carrier and stabilizing agent, the encapsulated SPIONs can be taken up by the cancer cells *via* a folate receptor mediated endocytic pathway. In this study, FA-BSA modified and SPIONs-loaded polymeric micelles are prepared and the use of the materials in folate-receptor overexpressed cancer targeting and MR imaging are investigated *in vitro* and *in vivo*.

2. Materials and methods

2.1. Materials

2,2,3,4,4,4-hexafluorobutyl methacrylate (HFMA) purchased from Xeogia Fluorine-Silicon Chemical Company (Harbin, China) was distilled at reduced pressure before use and methoxy poly (ethylene glycol) monomethacrylate (PEGMA) (average molecular weight of 950 g/mol), 75 wt.% methacryloxyethyl trimethyl ammonium chloride (MOTAC) solutions, and folic acid were obtained from Aldrich. 2,2′-azobisisobutyronitrile (AIBN) was purified by recrystallization in ethanol and oleic acid, iron (III) chloride hexahydrate ($FeCl_3{\cdot}6H_2O$), iron (II) chloride tetrahydrate ($FeCl_2{\cdot}4H_2O$), ammonium hydroxide ($NH_3{\cdot}H_2O$, 25–28%), dimethyl sulfoxide (DMSO), ethanol, hexane, hydrochloric acid (HCl), 1-ethyl-3-(3′-dimethylaminopropyl) carbodiimide (EDC), and tetrahydrofuran (THF) were purchased from Sinopharm Chemical Reagent Co. Ltd., China. Bovine serum albumin (BSA) and 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma–Aldrich.

2.2. Sample preparation

2.2.1. Preparation of cationic SPIONs-loaded polymeric micelles

Mono-dispersed SPIONs were synthesized by chemical co-precipitation and modified with oleic acid according to the procedures described previously [28]. The cationic amphiphilic poly (HFMA-co-MOTAC)-g-PEGMA copolymers were synthesized by free radical polymerization [17,29]. Briefly, 1.02 g of PEGMA, 0.91 g of HFMA, and 0.12 g of MOTAC were dissolved in 15 mL of THF in a 50 mL round bottomed flask with a magnetic stirrer. After adding 0.068 g of AIBN as a radical initiator, the mixture was deoxygenated under vacuum and backfilled with nitrogen several times in an ice bath. Polymerization proceeded at 75 °C for 24 h and the cationic amphiphilic poly(HFMA-co-MOTAC)-g-PEGMA copolymers were collected by precipitation in hexane. Cationic SPION-loaded polymeric micelles denoted as unmodified magnetic micelles were prepared by self-assembly. 0.20 g of SPIONs were dissolved in 5 mL of hexane and manually mixed with 25 mL of distilled water containing 0.21 g of the cationic amphiphilic poly (HFMA-co-MOTAC)-g-PEGMA copolymers prior to sonication for 15 min. Hexane was evaporated at 70 °C in a water bath during sonication and the solution containing the cationic magnetic micelles was purified and separated from the large particles and free copolymers by centrifugation and filtration, respectively.

2.2.2. Preparation of FA-BSA modified magnetic micelles

FA-BSA modified magnetic micelles were synthesized by functionalizing the cationic magnetic micelles with FA-BSA by electrostatic complexation [30]. Conjugation of folate with the bovine serum albumin was carried out according to the previously reported method [20,31]. 10 mg of folic acid and 10 mg of EDC were mixed in 10 mL of DMSO under stirring at room temperature for 2 h to modify the terminal carboxylate group. 50.0 mg of the BSA dissolved in 10 mL of distilled water was added to the above solution and stirred at room temperature in the dark for 4 h. The folate and other reactants in excess were removed from the conjugated protein using Sephadex G-25. 1 mL of FA-BSA and 2 mL of purified cationic magnetic micelle solutions were mixed and reacted for 8 h at room temperature under continuous agitation. The solution was dialyzed against ultrapure water for 3 days. The FA-BSA modified magnetic micelles were re-dispersed and stored at 4 °C for further studies.

2.3. Characterization

^{1}H NMR was conducted to investigate the chemical structure of the amphiphilic poly(HFMA-co-MOTAC)-g-PEGMA copolymers using the UNITY INVOA 600 MHz spectrometer (Varian, USA) with $CDCl_3$ containing 0.03% v/v tetramethylsilane (TMS) as the solvent. The structures of the SPIONs, amphiphilic poly(HFMA-co-MOTAC)-g-PEGMA copolymers, and folate-modified magnetic micelles were assessed by Fourier transform infrared spectroscopy (FTIR, Perkin-Elmer Spectrum One, USA). The morphology of the samples was examined by transmission electron microscopy (TEM, Tecnai G20, FEI Corp., USA) at 200 kV. The hydrodynamic size and size distribution were measured using a dynamic light scattering instrument (DLS, Zetasizer NanoZS90, Malvern Instruments Ltd., Worcestershire, UK) at 25 °C at a scattering angle of 90°. The zeta potential of the particles was determined by DLS. The total iron concentration was determined by fast sequential atomic absorption spectroscopy (SpectrAA240FS, Varian, Palo Alto, USA) and the thermogravimetric analysis was performed on the Perkin Elmer TGA-7. The magnetic properties were studied on a vibrating sample magnetometer (VSM, HH-15, China) at 298 K under an applied magnetic field.

The transverse relaxivity (r_2) of the magnetic micelles was determined using a 3.0-T whole body MR scanner (MAGNETOM Trio, A Tim System 3 T, Siemens, Munich, Germany) in combination with an 8-channel wrist joint coil. The particles were diluted by 300 μL 0.5% agarose gel on a 96-well plate with iron concentrations in the range of 0–0.10 mmol/L and were tested by T_2-weighted multi-echo spin echo sequence. The parameters were as follows: field of view (FOV) = 120 mm, base resolution = 384 × 384, slice thickness = 1.5 mm, multiple echo time (TE) = 20, 40, 60, 80, 100, 120, and 140 ms, repetition time (TR) = 2000 ms, and scanning time = 13–14 min. The transverse relaxation time (T_2) of each suspension was quantified using the in-house software. The transverse relaxation rates ($1/T_2$) were plotted versus iron concentrations and the transverse relaxivity (r_2) was computed based on linear regression (Origin 7.5).

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