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Surface modified superparamagnetic iron oxide nanoparticles: as a new carrier for bio-magnetically targeted therapy

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Abstract Amino-functionalized superparamagnetic iron oxide nanoparticles (SPION) were synthesized by coprecipitation method. The particles were characterized by X-ray diffraction (XRD), vibrating sample magnetometer (VSM), scanning electron micrographs (SEM), transmission electron micrographs (TEM) and atomic force micrographs (AFM). The size of the modified particles varied in the range 10–15 nm and did not change significantly after modification. Hepama-1, an excellent humanized monoclonal antibody directed against liver cancer, was conjugated to the SPION to prepare immuno-magnetic nanoparticles (IMN). A direct labeling method was employed to radiolabel IMN with rhenium-188. The radiolabeling efficiency was about 90% with good in vitro stability. ^{188}Re labeled IMN could markedly kill SMMC-7721 liver cancer cells. Such SPION might be very useful for bio-magnetically targeted radiotherapy in liver cancer treatment.

Introduction

Cancer is considered the most dangerous threaten to human health. Currently cancer therapy involves chemotherapy followed by surgery or radiotherapy. Effectiveness of the treatments is directly related to ability of a treatment to target and kill the cancer cells while affecting as fewer healthy cells as possible. However, side effects occur with chemotherapy, as the drugs are usually non-selective to the cells.

Advanced treatments of cancers are in progress in terms of new agents against cancer and new ways of delivering drugs. Among applicable drug targeted delivery methods for cancer therapy, non-viral nanoparticlate systems have gained increasing interest within therapeutics. Due to their low toxicity in comparison with viral systems, they are good candidates for targeting tissue and cells with different agents. Chemotherapy with magnetically controlled nanoparticulates has received special attentions because it can not only prolong residence time of drugs in the blood circulation but also reduce the amount of systemic distribution of cytotoxic drugs and the dosage required by more efficient localized targeting of the drug.

In a typical magnetically targeted drug delivery system, superparamagnetic iron oxide nanoparticles (SPION) are always imbedded in a matrix material, which is then functionalized with biologically active species. These species may be extremely specific, such as antibodies, or may have a general affinity to proteins. The magnetic particles are intravenous infected, followed by applying a magnetic field gradient in an area where the delivery is desired.

Superparamagnetic iron oxide nanoparticles using as an effective magnetic drug carrier have received increasing attention due to their multifunctional properties, such as small size, superparamagnetism, and low toxicity [1–5]. At first, it was used to target cytotoxic drugs (doxorubicin) to sarcoma tumors implanted in rat tails [6–9]. Then several research groups succeeded in cytotoxic drug delivery and tumor remission using different animal models including swine, rabbits and rats. The technique has also been employed to target cytotoxic drugs to brain tumors [10, 11].

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On the other hand, improved understandings of the biology underlying the radiation response and the use of chemotherapy and molecularly targeted therapy to enhance radiation sensitivity have contributed significantly to the improved outcome of patients undergoing radiotherapy. Moreover, if the radionuclide is targeted to a region nearby the tumor site and stays there, it can overcome the release limit of the drug from the carrier caused by removal of the external magnetic field. Häfeli et al. [12–17] have demonstrated effectiveness of the technique in both animal and cell culture studies using yttrium-90 and rhenium-188.

Rhenium-188 is a good radionuclide for radiotherapy. Its half-life of 16.9 h is suitable for tumor treatment but of benefit to minimize toxicity to whole body; the ß-emissions of 2.12 MeV (71.6%) and 1.96 MeV (25.1%) are suitable for therapy with an average penetration range of 2.6 mm, and the γ-emission of 155 keV (15%) allows imaging with a γ-camera.

In this study, we synthesized SPION and modified them with amino-silane, and Hepama-1, a well proved monoclonal antibody in China, against liver cancer, was conjugated to functional SPION by the cross-linker of glutaraldehyde to prepare immuno-SPION. The modified particles were radiolabeled with rhenium-188 for biomagnetically targeted therapy.

Experimental section

Materials

All chemicals were of reagent grade from commercial source. Ferric chloride, ferrous chloride, and tetramethylammonium hydroxide (TMAOH) were supplied by Aldrich. N-(2-aminoethyl)-3-aminopropyltrimethoxysilane (AEAPS), tetraethoxysilane (TEOS), avidin, sodium gluconate and stannous chloride ($SnCl_2$) were purchased from Sigma-Aldrich Chemical Co. Hydrochloric acid (diluted with water from HCl >37%). Carrier-free ^{188}Re-perrhenate was freshly eluted with saline from an alumina-based $^{188}W/^{188}Re$ -generator (Amersham-Kexing Radiopharm. Co, Ltd., P. R. China). Other materials were of analytical grade and used without further purification.

Synthesis of superparamagnetic iron oxide nanoparticles (SPION)

Chemical reaction of Fe_3O_4 precipitation is given by

$$Fe^{2+} + 2Fe^{3+} + 8OH^- \rightarrow Fe_3O_4 + 4H_2O \quad (1)$$

According to the results of thermodynamic modeling of this system [5], a complete precipitation of Fe_3O_4 is expected in pH value of 7.5–14 while maintaining a molar ration of Fe^{2+}/Fe^{3+} = 1:2 under a non-oxidizing environment. Under oxidizing conditions, Fe_3O_4 may be oxidized as given by the following equations.

$$Fe_3O_4 + 1/4O_2 + 4.5H_2O \rightarrow 3Fe(OH)_3 \quad (2)$$

$$Fe_3O_4 + 1/4O_2 \rightarrow 1.5Fe_2O_3 \quad (3)$$

Aqueous dispersion of SPION was prepared by adding of an aqueous mixture of ferric and ferrous salts to a strong alkaline solution (NaOH or NH_4OH) at room temperatures. In the present study, a solution of NaOH was used as alkali source instead of ammonia. Oxygen is eliminated from the solution by using N_2 flow through the reaction medium in a closed system during the synthesis.

Typically, an aqueous suspension of SPION with an average particle size of 10 nm was prepared using controlled coprecipitation according to the following procedures. Briefly, 25 mL of 1 M $FeCl_3 \cdot 6H_2O$, 0.5 M $FeCl_2 \cdot 4H_2O$, and 0.4 M HCl was prepared as a source of iron by dissolving the respective chemicals in Milli-Q water under vigorous stirring. The coprecipitation of SPION was carried out in a reaction with high-speed mechanical stirring (2,000 rpm) by adding the iron solution to 250 mL 0.5 M NaOH, which was preheated to 60 °C before the coprecipitation reaction. N_2 was used during the reaction to prevent critical oxidation. Black powder was collected by sedimentation with help of an external magnetic-field and washed several times with Milli-Q water until stable ferrofluid was obtained. Finally, the particles were redispersed in an aqueous solution by adjusting the pH to 11 with TMAOH (5 wt%).

Surface modification of SPION with AEAPS

After the above preparation, 100 mg of the SPION was washed several times with analytical grade methanol (99.5%). A strong magnet was used to remove the SPION from the residual water. The amount of AEAPS required to coat the 100 mg SPION of 10 nm in diameter was calculated, taking into account the surface area of a single particles. A solution of 3.5 nM AEAPS solution was prepared in 100 mL of a toluene/methanol(1:1v/v) mixture. AEAPS in the toluene/methanol solution (200 µL) was added to the SPION suspension. The ferrofluid suspension was transferred into a three-necked flask with a water cooled condenser, a temperature-controller, and a N_2 flow. The silanization was performed at 120 °C for 24 h under vigorous stirring, AEAPS acts as a coupling agent, where silanization took place on the particle surfaces bearing hydroxyl groups in the organic solvent. As a result, a three

dimensional polysiloxane network was formed. The silanization, during the reflux of AEAPS resulted in the formation of an AEAPS coating with a thickness of tens of molecular layers tightly cross-linked with a large surface density of amines. The powder was collected by applying an external magnetic field after the silanization process, washed with methanol several times, and finally redispersed in an aqueous medium. The silane-coated SPION were prepared in a similar process with coupling agent of TEOS.

Immobilization of Hepama-1 on amino-coated SPION

The experiments of Hepama-1 protein immobilization were conducted in batch by continuous shaking at 4 °C for 24 h, which proved to be a sufficient period for any run. In a typical experiment, glutaraldehyde was used as a cross-linker for the immobilization of Hepama-1 on SPION. For this purpose, the AEAPS-treated SPION were dispersed in phosphate buffered saline (PBS, 0.1 M, pH 8.0) with concentration of 5 g L^{-1}, Hepama-1 of different concentrations were added to 10 mL solutions of amino-coated SPION, respectively. The mixtures were incubated at 4 °C for 24 h. Then the magnetic supports were separated by permanent magnet (2000Oe). The amount of Hepama-1 immobilized onto magnetic supports was determined by measuring the initial and final concentrations of Hepama-1 from the absorbance at 280 nm of the supernatant using a calibration curve prepared previously. To remove the physically adsorbed Hepama-1, the sediments of the Hepama-1-immobilized magnetic supports were resuspended in a glycine buffer at pH 11.0 for 2 h. The desorbed amounts of Hepama-1 were determined from the absorbance at 280 nm of supernatants.

Radiolabeling of SPION-Hepama-1 with ^{188}Re

A direct labeling method, which was modified from the method described by Griffiths et al. [18], was used in this study. First, mAb was reduced by a 5,000-fold molar excess of 2-ME for 30 min in order to reduce disulfide bonds of the antibody to mercapto groups. The reduced antibodies were purified by Sephadex G-50. Purified and reduced antibodies were added to 0.2–1.0 mL of solution of $^{188}ReO_4^-$ (185–370 MBq) and stannous chloride ($SnCl_2$) solution dissolved in sodium gluconate solution. The mixtures were incubated at room temperatures for 2 h. The radioactivity was measured by a γ-counter. The labeling efficiency refers to the ratio of activity bound to the particles over the total activity added.

Cell culture

Human liver cancer lines SMMC-7721was kept in our laboratory. All cells were cultured in RPMI medium 1640 (Gibco, USA) supplemented with 10% fetal bovine serum (Sigma, USA) at 37 °C in a humidified 5% CO_2 incubator. MTT method [19] was used to observe in vitro tumor inhibition of ^{188}Re-IMN, ^{188}Re-Hepama-1, ^{188}Re-SPION, $^{188}ReO_4^-$, respectively, to SMMC-7721 liver cancer cell lines.

Characterizations

Elemental analysis (C, H, and N) was performed by Redox Spa (Milan, Italy) UV absorbance was measured on a Carry 100 Scan UV-vis spectrophotometer (Varian ltd., Oxford, UK). The absorbance of Hepama-1 was measured at 280 nm.

XRD characterization. Crystal structure of the precipitated and coated powders was obtained by analyzing X-ray diffraction (XRD) patterns of each sample recorded with Philips PW 1830 diffractometer, using a monochromatized X-ray beam with nickel-filtered Cu Kα ray. The lattice constants were estimated using CELREF, a crystal cell parameters refinement program for powder X-rays, using at least squares refinement method.

TEM/SEM characterization. Size and morphology of the particles were measured by scanning electron micrographs (SEM) and transmission electron micrographs (TEM). The specimen was sonicated for 3 min. After the dispersion, 1 mL of SPION suspension was centrifuged for 5 min at 14,000 rpm. A drop of well-dispersed supernatant was coated on a carbon-coated 300-mesh copper grid, followed by drying the sample under ambient conditions.

AFM characterization. Atomic force micrograph (AFM) was performed to study the shape, size, and surface appearance of the nanoparticles. The aqueous dispersion of the nanoparticles was put on a glass coverslip and the coverslip, which was air dried at room temperatures for 24 h. The dried samples were analyzed using an atomic force microscope (AFM, Nanoscope III, Digital Instruments, Santa Barbara, CA).

VSM characterization. Magnetic properties was checked using a vibrating sample magnetometer (VSM) (Model 155, EG&G Princeton Research, USA) on liquid samples.

Results and discussion

Synthesis of SPION and amino-coated SPION

Superparamagnetic iron oxide nanoparticles were prepared by the co-precipitation method from ferrous and ferric ion solutions with a molecular ratio of 1:2. To obtain the stoichiometric ratio, both ferrous chloride and ferric chloride chemicals were used. Nitrogen gas was used to prevent oxidation of ferrous ions in aqueous solution. The Fe_3O_4

nanoparticles prepared had high crystallization and good magnetic properties. SEM images showed that the mean particle size was 10 nm (Fig. 1a) and the saturation magnetization was 71 emu/g with a small coercivity value of σ = 17Oe (Fig. 1b).

Transmission electron micrographs measurements revealed that amino-coated SPION were in a spherical, shape and uniform sizes around 10–15 nm in diameter (Fig. 2). And AFM image (Fig. 3) of the amino-modified nanoparticles after water evaporation shows size homogeneity of the nanoparticles, with an average diameter of about 15 nm, agreeing well with TEM results.

XRD measurement. XRD patterns of (B) uncoated, (E) silane-, and (G) amino-coated SPION are shown in Fig. 4. The patterns of all the samples show peaks corresponding to the spinel structure. The uncoated SPION prepared by the controlled chemical coprecipitation process can be expected as magnetite with a pure phase, based on the results from thermodynamic modeling [4]. From Fig. 4, it can be clearly seen that the particles do not show sharp diffraction peaks corresponding to extended crystalline structure. Instead, a broad band appears in each spectrum. This observation is typical for amorphous materials and also for ultrafine crystalline materials where diffraction peals cannot be well resolved. Although our bulk sample also does not show any sharp peak, still, diffraction peaks can be resolved. The diffraction pattern of our bulk magnetic sample is close to the standard pattern for crystalline magnetite (Fe_3O_4).

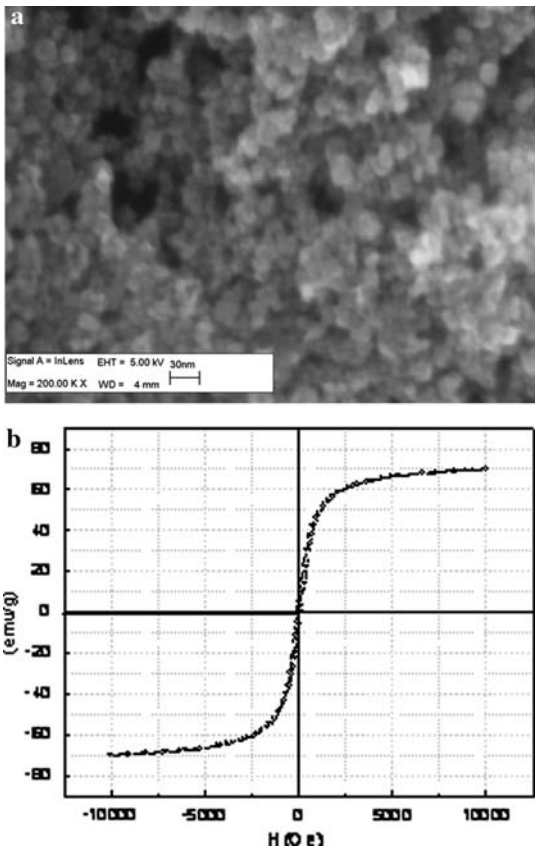

Fig. 1 (**a**) SEM image of SPION; (**b**) Magnetic hysteresis curve for SPION measured at room temperature

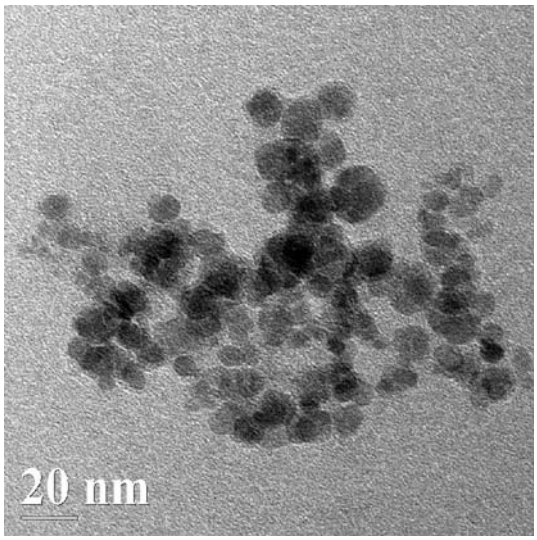

Fig. 2 TEM image of amino-coated SPION

The amino group density can be calculated from the nitrogen amount measured from C, H, and N analysis of the treated magnetic nanoparticles. Elementary analysis results of amino-coated magnetite nanoparticles treated with AE-APS yield 3.6 wt% C, 1.3 wt% H, and 1.6 wt% N. The amino group density is therefore about 0.5 umol/mg solid, which is sufficient to immobilize biomolecules.

Labeling of particles with ^{188}Re

Radiolabeling results show that the ^{188}Re was deoxidized by stannous chloride ($SnCl_2$) well on the surface of the magnetic nanoparticles. Figure 5 shows the relationship between radiolabeling efficiency and reaction time. The reaction time affects the radiolabeling efficiency similarly between 30 min and 50 min. However, the release of ^{188}Re at 37 °C in bovine serum albumin from the particles labeled in 30 min was about 25% at 24 h and 10.7% labeled in 50 min respectively. The labeling efficiency was above 90% at 50 min with less than 25% release of radioactivity in 24 h. For this reason, we chose 50 min as the time for further reaction optimizations.

In vitro studies

MTT colorimetric assay was used for determining in vitro tumor inhibition of ^{188}Re-IMN, ^{188}Re-Hepama-1, ^{188}Re-SPION, $^{188}ReO_4^-$ to SMMC-7721 liver cancer cell lines.

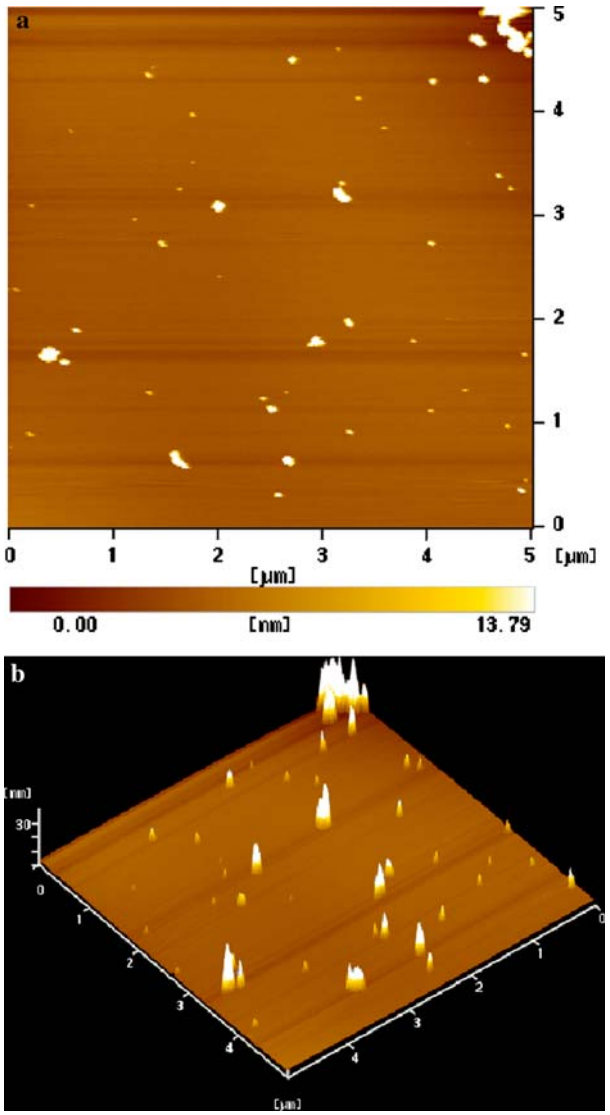

Fig. 3 (**a**, **b**) AFM image of amino-coated SPION

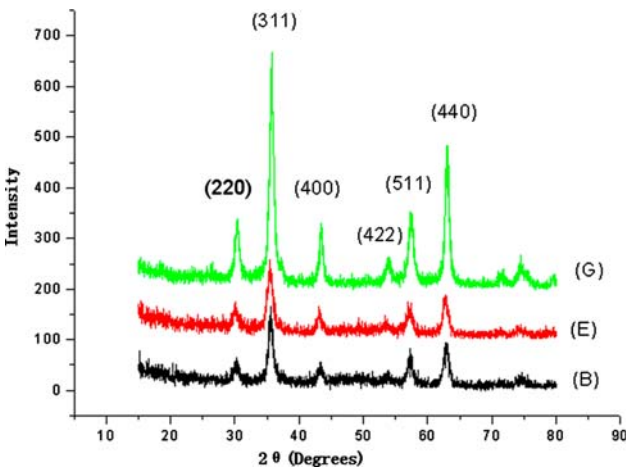

Fig. 4 X-ray powder diffraction patterns of (B) uncoated-, (E) silane-, and (G)amino-coated SPION, respectively

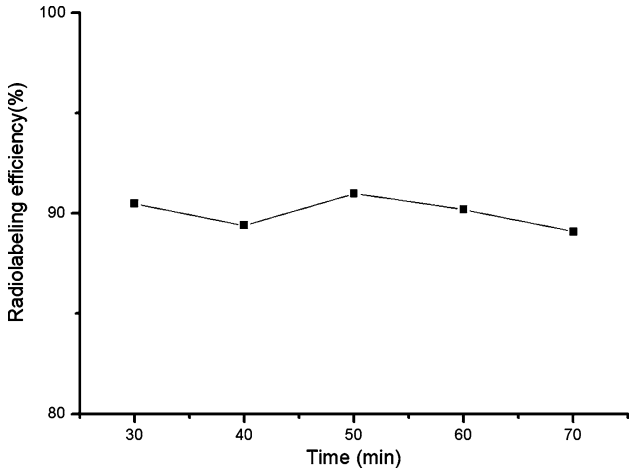

Fig. 5 Relationship between radiolabeling efficiency and reaction time (with 37 °C reaction temperature and 100 uL reaction volume)

The corresponding IC_{50} values were $5.3 \times 10^5 Bq{\cdot}L^{-1}$, $7.9 \times 10^4 Bq{\cdot}L^{-1}$, $1.6 \times 10^6 Bq{\cdot}L^{-1}$, $1.9 \times 10^6 Bq{\cdot}L^{-1}$. Therefore, the results showed that ^{188}Re labeled IMN could markedly kill SMMC-7721 cells with dose dependence.

Conclusion

Bio-magnetically targeted radioimmunotherapy is a new therapeutic approach that uses mAbs directed against tumor-associated antigens to carry cytotoxic radionuclides to antigen-expressing tumor tissues, and is promising for cancer therapy. In this work, a convenient and effective method of preparation of modified magnetite nanoparticles with AEAPS in order to produce a superparamagnetic material with the required properties for technological applications is described. The results showed that these SPION were roughly spherical in shape and around 10 nm in diameter, which is close to the size of magnetite crystallite, suggesting the formation of a continuous and very fine layer of amino on the surface of the magnetite core. For liver cancer treatment, Hepama-1 was conjugated on the surface of amino-modified SPION. In order to radiolabel the particles with ^{188}Re, $^{188}ReO_4^-$ was used and labeled directly to the surface of modified magnetite nanoparticles. For 0.1 mCi $^{188}ReO_4^-$ and 5 mg magnetic nanoparticles, the best labeling condition was incubated the nanoparticles in a volume of 100 uL for 50 min at 37 °C. The labeling efficiency was about 90% and the stability in serum albumin at was 80% after 10 h. And the results showed that ^{188}Re labeled IMN could markedly kill SMMC-7721 cells.

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