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Facile synthesis of high-magnetization γ-Fe_2O_3/alginate/silica microspheres for isolation of plasma DNA

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ABSTRACT

A novel process combining emulsification with sol–gel method is described to synthesize γ-Fe_2O_3/alginate/silica microspheres. The synthesis procedure consists of two steps: (1) synthesis of magnetic alginate microspheres via an emulsification technique; (2) in situ synthesis of magnetic silica microspheres by the hydrolysis of TEOS in the presence of ammonia solution. The as-synthesized composite microspheres with a typical average diameter of 4.4 μm were spherical and superparamagnetic. Moreover, they contained up to 31.7 wt% maghemite with a saturation magnetization of 15.1 emu g^{-1}. The application of these magnetic microspheres as adsorbents for isolation of plasma DNA has also been studied.

1. Introduction

Superparamagnetic microspheres consisting of magnetic nanoparticles (NPs) (e.g. γ-Fe_2O_3 or Fe_3O_4) encapsulated in polymer and/or inorganic oxide matrix have attracted intense attention in recent years due to their potential applications in biomedical fields such as cell separation [1], magnetic resonance imaging [2], drug delivery systems [3], and hyperthermia [4]. As specifically required in biological and biomedical applications, high magnetic sensitivity, biocompatibility, hydrophilic character, high stability against aggregation and versatility in surface modification are crucial [5]. So far, a lot of procedures have been developed to prepare such magnetic microspheres [6–9]. Among a variety of coating materials mentioned above, silica is most commonly used, providing magnetic microspheres with several benefits such as good hydrophilic character and high stability against aggregation. Moreover, its versatile surface functionality would allow for bioseparation, biolabeling and drug delivery. Recently, several approaches have been proposed for the synthesis of superparamagnetic silica microspheres [10–14]. Xu et al. described a new process to obtain monodispersed, nanoscale, superparamagnetic Fe_3O_4/polystyrene/silica spheres with 80 wt% of magnetite which exhibit a much higher saturation magnetization [15]. Nevertheless, these composite particles described above have some drawbacks, such as time-consuming preparation, complicated isolation and purification, low stability and so on, which restrict their further applications. Therefore, it is of fundamental importance to develop convenient, economic and efficient methods for the preparation of superparamagnetic microspheres with a high fraction of magnetic nanoparticles and hydrophilic surfaces.

Alginate, a naturally occurring polysaccharide, has attracted intense attention as an important class of biomaterial in recent years because of its unique properties including inexpensiveness, relatively inert hydrogel environment within the matrix, a mild room-temperature encapsulation process and biocompatibility. Furthermore, alginate can be ionically crosslinked in the presence of divalent cations such as Ca^{2+}, which has been extensively investigated for many biomedical applications including tissue engineering, drug delivery vehicles, and cell transplantation matrices [16–20]. It is well known that ionically crosslinked alginate gels dissolve in PBS buffer or biological environments due to the loss of divalent crosslinking cations into the surrounding medium, which is a critical aspect to control for further applications. Very recently, nanoorganized multilayer coatings involving chitosan/alginate, polylysine/alginate, polyethyleneimine/alginate and other polyelectrolyte coatings have been applied on alginate hydrogels using the layer-by-layer self-assembly technique in order to enhance the

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overall stability of the hydrogels in biological environments [19,21–23].

Herein, we describe a novel process combining emulsification with sol–gel method for the synthesis of magnetic silica microspheres, which may serve multiple purposes including stabilizing alginate hydrogels against dissolution in biological environments and providing versatility in surface modification, as well as making the structures useful for biological and biomedical applications. The synthesis procedure consists of two steps: (1) synthesis of magnetic alginate microspheres via an emulsification technique; (2) in situ synthesis of magnetic silica microspheres by the hydrolysis of TEOS in the presence of ammonia solution. The morphology, structure, and magnetic properties of the as-synthesized γ-Fe_2O_3/alginate/silica microspheres were characterized by transmission electron microscope, X-ray diffraction, thermogravimetric analysis and vibrating sample magnetometer. Furthermore, the utility of such magnetic microspheres is demonstrated by isolation of plasma DNA.

2. Materials and methods

2.1. Materials

Sodium alginate (1 wt%, viscosity $\geq$ 0.2 Pas, 20 °C), sorbitan trioleate (SPAN 85), polyoxyethylene sorbitan trioleate (TWEEN 85), tetraethoxysilane (TEOS), ferric chloride hexahydrate, ferrous sulfate heptahydrate, ammonia solution and calcium chloride were all purchased from Shanghai chemicals Co. Ltd. meso-2,3-dimercaptosuccinic acid (DMSA) was obtained from Sigma–Aldrich. Agarose (Biological grade) was purchased from Shanghai Haoyuan Co. Ltd. All chemicals were used as received without further treatment. PCR primers were synthesized by Takara. Other reagents used in DNA isolation and analysis were of analytical grade.

2.2. Synthesis of magnetic alginate microspheres (MAMs)

γ-Fe_2O_3 NPs, used as magnetic cores, were prepared by chemical coprecipitation. The stable ferrofluid was obtained via surface modification with DMSA according to our previous procedure [24,25]. The synthesis of MAMs was a modification of the conventional emulsification technique [21,26]. Briefly, 50 g aqueous solution of DMSA-coated γ-Fe_2O_3 NPs (200 mg) and sodium alginate (500 mg) was dispersed in 75 g isooctane containing 1.696 g SPAN 85, ultrasonicating and stirring for 10 min. Then, a solution of 5 g isooctane containing 0.904 g TWEEN 85 was added to the emulsion under stirring and ultrasonication at the same power for 20 min. After that, another 30 min stirring was proceeded to achieve stable water-in-oil emulsion droplet. Subsequently, 20 mL of aqueous solution containing 10 wt% of calcium chloride was added to form ionic crosslinks. Finally, the products were washed with water by magnetic decantation for four times and redispersed into water at room-temperature.

2.3. Synthesis of silica-functionalized magnetic alginate microspheres (MAMs@SiO_2)

The suspensions of the MAMs used for synthesis of MAMs@SiO_2 were prepared at a maghemite concentration of 0.84 mg/mL in 20 mL of aqueous solution. In a typical process, to the obtained suspension was added 0.3 mL of ammonia aqueous solution under stirring. After 5 min, 20 μL TEOS was added and the mixture was allowed to stand at room-temperature for 3 h. The resulting composite microspheres were collected by magnetic separation and washed several times with water.

2.4. Isolation of plasma DNA using MAMs@SiO_2

2 mL of vein blood was centrifuged at 1800 rpm for 10 min at room-temperature. The supernatant was added to a 1.5 mL microcentrifuge tube, and was centrifuged at 17000 rpm for another 10 min at 4 °C. Then a 200 μL aliquot of the supernatant (plasma) was aspirated and stored at −70 °C. In a typical experiment for the isolation of DNA from plasma, 100 μL of lysis buffer (0.1 M Tris–HCl, 0.05 M EDTA, and 0.5 M NaCl, pH 8.0) were added to the plasma and left at room-temperature for 10 min. Subsequently, 10 μL of magnetic microspheres (maghemite concentration: 1.674 mg/mL) and 150 μL of binding buffer (20% PEG_{8000}–2 M NaCl) were added and DNA binding to the microspheres completed by 5 min of incubation at room-temperature. After the magnetic separation of the microspheres, the supernatant was removed. The magnetic microspheres were washed with 80% 2-propanol, followed by 70% ethanol. The immobilized DNA was eluted from the magnetic microspheres by 30 μL of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.8) and was incubated at 50 °C for 5 min. A 5 μL aliquot of the eluted DNA was used as DNA matrix in PCR amplification. PCR was performed in a 25 μL reaction volume containing 10 μL of PCR buffer, 400 μM of dNTPs, 200 nM of primers 5′-GGACCTGACTGACCTCATGAA-3′ and 5′-CTTAATGTCACGCACGATTTCC-3′, and Taq-polymerase (0.025 U/L). The amplification was carried out in one cycles of 300 s at 95 °C; 30 s at 94 °C; 30 s at 58 °C; 45 cycles of 40 s at 72 °C. PCR products were detected using gel electrophoresis on 1% agarose.

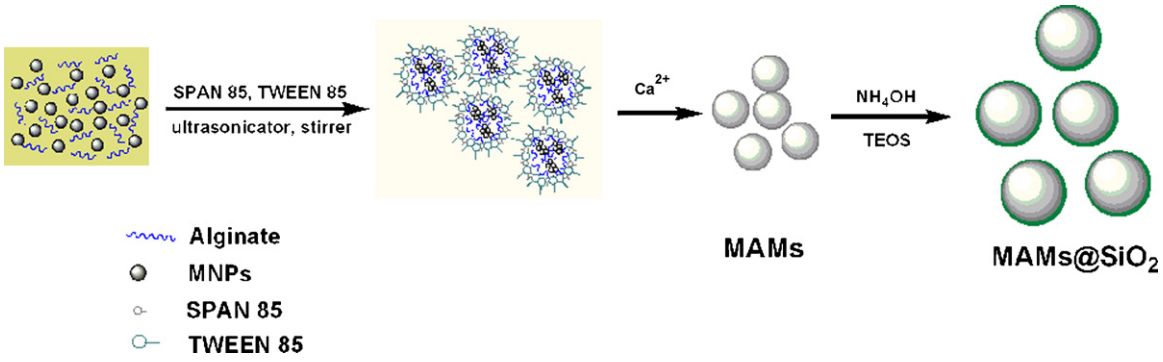
2.5. Characterization

The size and morphology of the mcirospheres were characterized by transmission electron microscope (TEM, JEOL, JEM-2000EX). The samples were prepared by dropping 6 μL of solution on the carbon-coated copper grids and allowing the solution to dry in the air. Energy-dispersive spectroscopy (EDS) spectrum was collected from the core-shell regions. The samples were prepared by depositing a drop of solution on the aluminium substrate. The shape of the samples was determined by an inverted optical microscope (Zeiss, Axioscop200). Particle size distribution was measured by quasi-elastic light scattering (Malvern Mastesizer 2000).The surface charge of the products were investigated through a ζ-potential analyzer (Beckman Coulter, Delsa 440SX). Powder X-ray diffraction (XRD, Rigaku, D/MaxRA, λ=1.5405 × 10^{-10} m, CuKα) and selected-area electron diffraction (SAED) were used to determined the crystal structure of the products. Thermogravimetric analysis (TGA) was performed on a PerkinElmer Pyris-1 series thermalanalysis system under a flowing nitrogen atmosphere at a scan rate of 10 °C/min from 100 °C to 700 °C. Magnetic properties were determined with vibrating sample magnetometer (VSM, Lakeshore 7407) at room-temperature in a field up to 5 kOe. The PCR reaction mixture was amplified on a thermal cycler (ABI 7500, Applied Biosystems). Agarose gel electrophoresis was carried out using a 3000 Xi power supply (Bio-Rad).

3. Results and discussion

3.1. Synthesis of MAMs and MAMs@SiO_2

Scheme 1 illustrates the preparation procedure. Firstly, aqueous solution containing alginate and DMSA-coated γ-Fe_2O_3 NPs was added to isooctane with surfactants SPAN 85 and TWEEN 85. The mixture was treated ultrasonically to obtain water-in-oil emulsion. Secondly, calcium ions were added to the emulsion to form MAMs. Finally, the MAMs were coated with a silica layer via the hydrolysis and condensation of TEOS onto the surface of the microspheres. It is worthy mentioning that the presence of DMSA-coated γ-



Scheme 1. Schematic illustration of the synthesis of MAMs@SiO_2 particles.

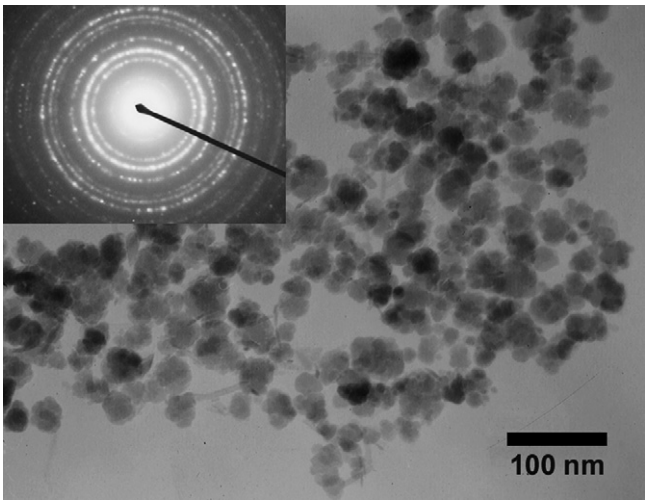


Fig. 1. TEM image of DMSA-coated γ-Fe_2O_3 NPs. The insert shows a typical SAED pattern.

Fe_2O_3 NPs is an important factor in the formation of water-in-oil emulsion. Under otherwise identical conditions, the solution with uncoated γ-Fe_2O_3 NPs can hardly produce stable water-in-oil emulsion. In this case, the DMSA-coated γ-Fe_2O_3 NPs has an average diameter of 18 nm (Fig. 1). ζ-Potential measurements as a function of pH value indicate that the DMSA-coated γ-Fe_2O_3 NPs have high negative potential at a wide range of pH value (Fig. 2).

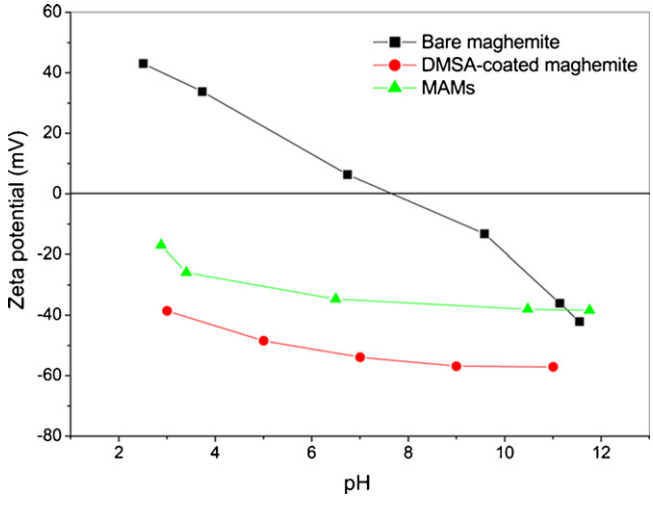


Fig. 2. ζ-potential measurements for the bare γ-Fe_2O_3 NPs, DMSA-coated γ-Fe_2O_3 NPs and MAMs at different pH value.

This results in the strong electrostatic repulsion to protect the particles from agglomeration, which is also confirmed by TEM. Moreover, due to a large number of carboxyl groups attributed to the DMSA molecules, the DMSA-coated γ-Fe_2O_3 NPs and alginate could be simultaneously crosslinked by Ca^{2+}, which to some extent improve the encapsulation efficiency of γ-Fe_2O_3 NPs. Fig. 3 shows the typical optical microscopy image of the MAMs and the corresponding diameter distribution. It can be found that spherical particles, with mean diameter of 2.54 ± 0.36 μm, were obtained. However, TEM images of the MAMs reveal that their average diameter was estimated to be ~600 nm (Fig. 4a). Within each particle, the γ-Fe_2O_3 NPs are randomly embedded in the polymer matrix (Fig. 4b). Note that the shrinkage of the particles took place when prepared for TEM measurement, and this explains why the size of the particles in the TEM image is significantly smaller than that in the optical microscopy image. As shown in Fig. 2, ζ-potential measurements of the MAMs as a function of pH value confirm the negatively charged surface of the particles. The as-synthesized MAMs exhibit high negative potential at basic medium, which is probably attributed to the ionization of carboxyl (pK_{a1} = 4.21, pK_{a2} = 5.64) and hydroxyl (pK_a = 8.76) groups on alginate molecules with increasing pH. Such a rich water region on the surface of the MAMs will therefore facilitated the silica nucleation and coating onto the surface of the particles with the catalysis of ammonia. Fig. 4c reveals that the MAMs@SiO_2 particles with an average diameter of 4.4 μm are largely spherical and essentially free from clustering.

It is well known that the alginate-based microspheres crosslinked with Ca^{2+} exhibited controlled-release-function due to the pH-sensitivity of Ca^{2+}/COO^- linkage, which can be only stable in acidic condition [27,28]. Thus, the alginate-based microspheres can swell with increasing pH. As shown in Fig. 5, with the addition of ammonia solution, the size of the MAMs increased from 2.5 μm to 4.1 μm. And this is the reason why the size of the MAMs is much smaller than that of MAMs@SiO_2. Expectedly, the size of the latter increased with increasing ammonia solution volume. When the volume of ammonia solution was increased from 0.1 mL to 0.6 mL, the size of the MAMs@SiO_2 was 3.23 ± 0.46 μm, 4.14 ± 0.32 μm, 5.65 ± 1.26 μm respectively (see Fig. 6). In addition, a series of MAMs@SiO_2 particles were synthesized by varying the initial amount of TEOS from 5 μL to 200 μL. Especially, when the TEOS content was increased below 150 μL, some core-free silica spheres derived from the condensation of TEOS molecules themselves were hardly observed. However, when the TEOS content was increased to 200 μL, many agglomerates were observed, most likely because of the formation of some core-free silica spheres. Unfortunately, we are unable to show whether the silica thickness increased with increasing TEOS content, because the alginate and silica have similar lower electron density compared with γ-Fe_2O_3 NPs. On the basis of the hydrolysis of TEOS, the thickness of the silica shell can

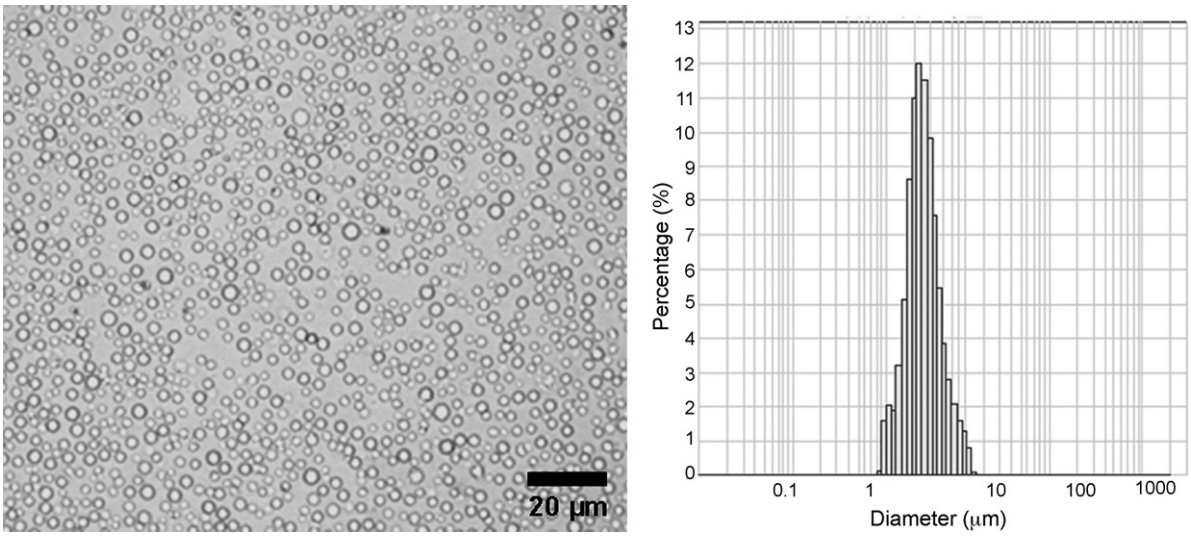


Fig. 3. Optical microscopy image of the MAMs and its corresponding diameter distribution.

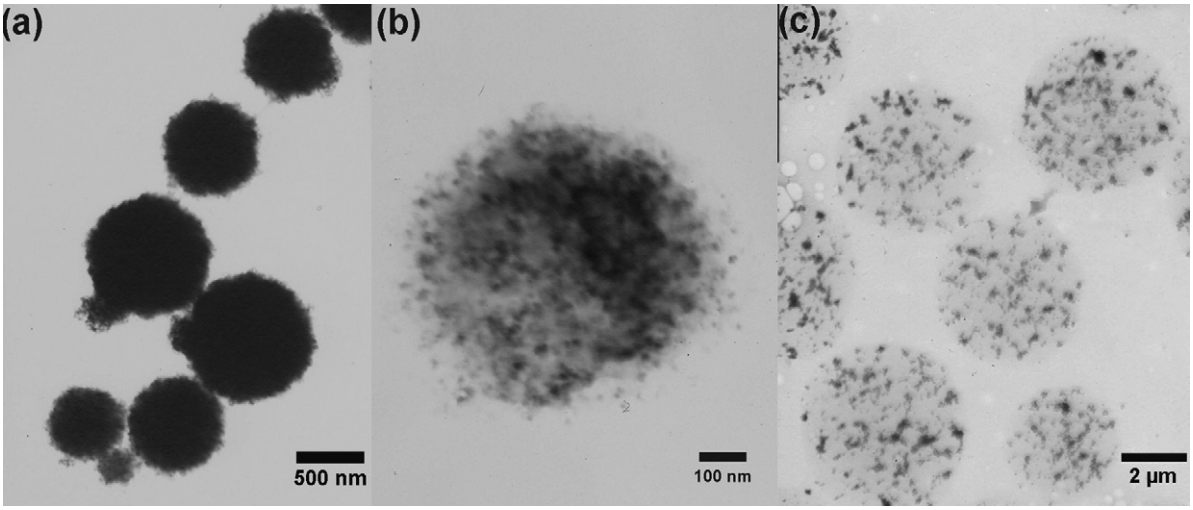


Fig. 4. TEM image of MAMs (a), a representative microsphere in high magnification (b), and MAMs@SiO_2 particles (c).

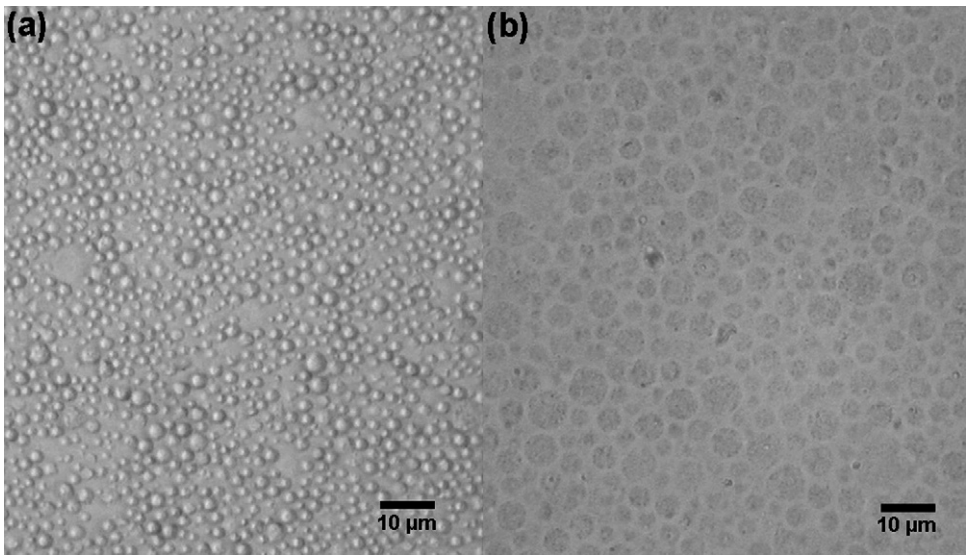


Fig. 5. Optical microscopy images of as-synthesized MAMs before (a) and after (b) addition of ammonia solution.

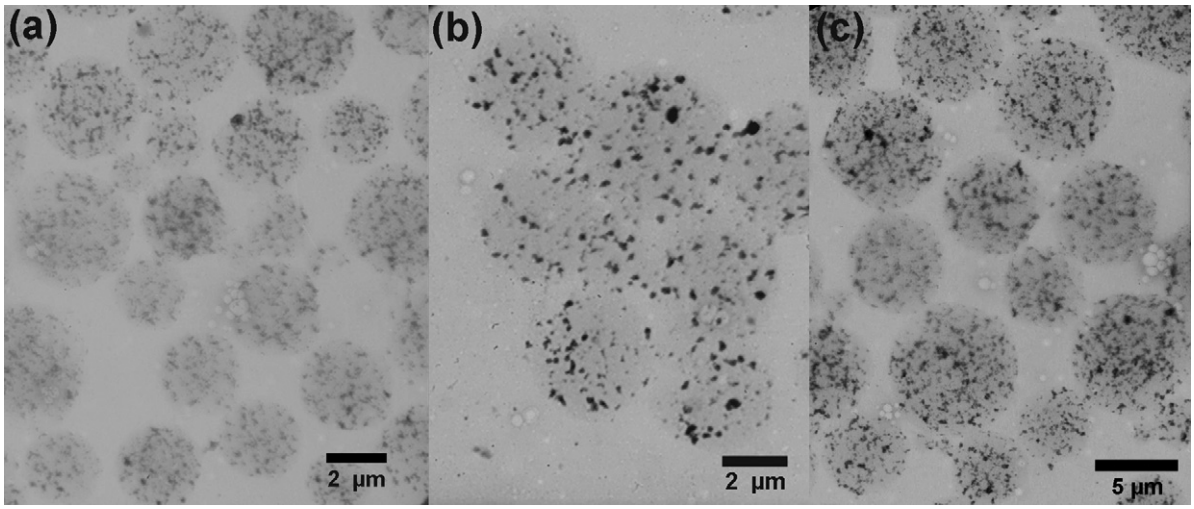


Fig. 6. TEM image of MAMs@SiO_2 particles obtained with the addition of ammonia solution of 0.1 mL (a), 0.3 mL (b), and 0.6 mL (c). Fixed TEOS content (20 μL) was used.

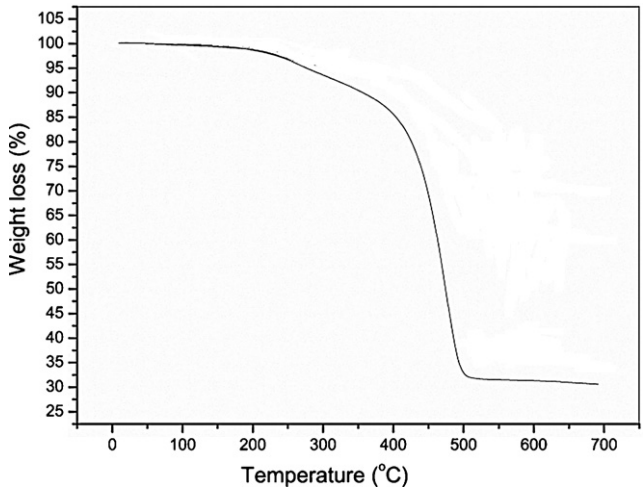


Fig. 7. A typical TGA curve of as-synthesized MAMs.

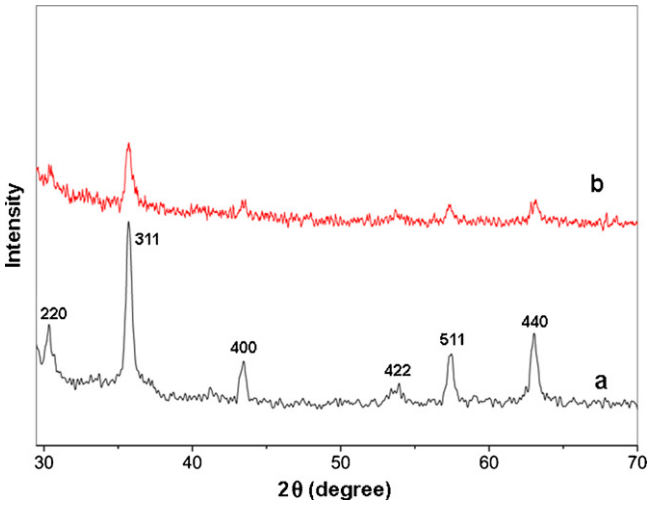


Fig. 8. XRD patterns of as-synthesized DMSA-coated γ-Fe_2O_3 NPs (a) and MAMs@SiO_2 particles (b).

be tuned from a few to several hundreds of nanometers by simply varying the initial amount of TEOS [8].

A typical TGA curve of the as-synthesized MAMs is shown in Fig. 7. The weight loss of 68 wt% at 500 °C is mainly due to the decomposition of polymer matrix. The result implies at least 32 wt% of γ-Fe_2O_3 content in the MAMs, much higher than reported previously [11]. Fig. 8 is the XRD patterns of the products. In Fig. 8a, the position and relative intensity of main diffraction peaks match well with standard maghemite (JCPDS no. 391346) and in Fig. 8b, the crystal structure of γ-Fe_2O_3 is well retained in the MAMs@SiO_2 particles. Furthermore, a broad band was detected at the angle below 35°, which is the characteristic for amorphous materials. The EDS image in Fig. 9 shows that the components of the MAMs@SiO_2 particles are C, O, Fe, Ca, and Si. The Al signal is attributed to the aluminium substrate for EDS. The presence of Si confirms that there exists silica in the composite particles.

Fig. 10 shows the room-temperature hysteresis loops of the as-synthesized γ-Fe_2O_3 NPs and MAMs@SiO_2 particles. The typical saturation magnetization (Ms) value of the MAMs@SiO_2 (15.1 emu g^{-1}) is less than those of MAMs (16.3 emu g^{-1}), DMSA-coated γ-Fe_2O_3 NPs (47.7 emu g^{-1}), and uncoated γ-Fe_2O_3 (50.9 emu g^{-1}), which could be explained by the nonmagnetic

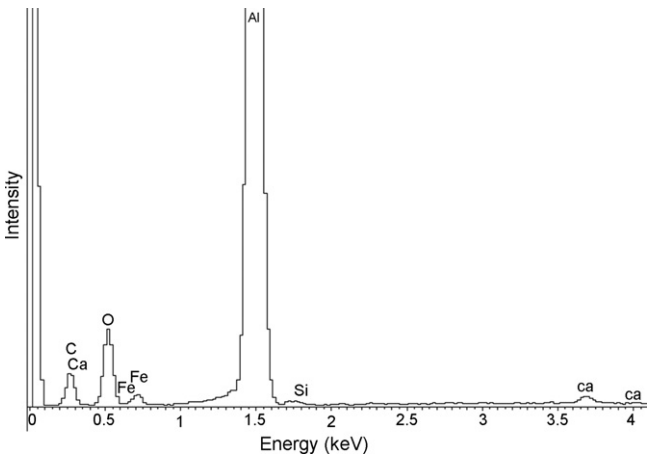


Fig. 9. EDS data from as-synthesized MAMs@SiO_2 particles. The Al signal is attributed to the aluminium substrate.

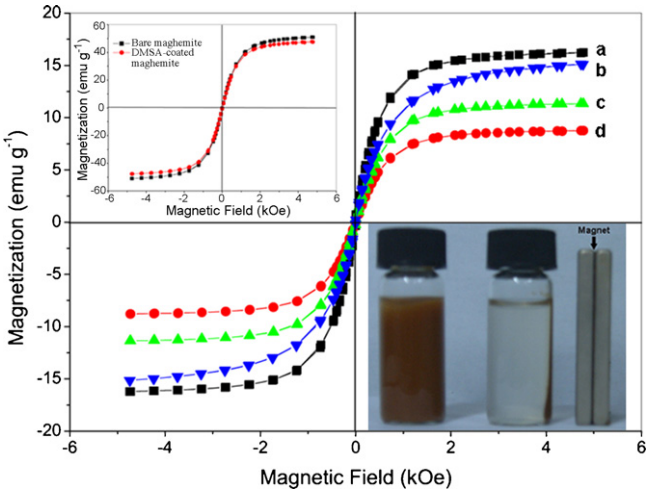


Fig. 10. Room-temperature hysteresis loops of the MAMs (a) and MAMs@SiO_2 particles synthesized with TEOS content of 20 μL (b), 50 μL (c) and 100 μL (d), respectively. Fixed ammonia solution volume (0.3 mL) was used. The insets show room-temperature hysteresis loops of bare γ-Fe_2O_3 NPs and DMSA-coated γ-Fe_2O_3 NPs and the photographic image of the as-synthesized MAMs@SiO_2 particles in the presence of an external magnet.

coating materials surrounding the magnetic cores. The Ms of the MAM@SiO_2 decreases with increasing TEOS content due to the increase of silica fraction in each microsphere, conforming that the thickness of the silica shell can be tuned by simply varying the initial amount of TEOS. The zero coercivity and the reversible hysteresis behavior at room-temperature indicate the superparamagnetic nature of the MAMs@SiO_2. The magnetic content of the MAMs@SiO_2 is estimated to be ~31.7 wt%, which is consistent with the above-mentioned TGA result. Furthermore, the magnetic separability of the microspheres was tested by placing an external magnetic field (Nd–Fe–B magnet, 0.1 T) near the glass bottle. The microspheres were attracted toward the magnet within 30 s (see the inset in Fig. 10), demonstrating that the microspheres have strong magnetic responses to an external magnetic field, and this presents an easy and efficient way to separate the microspheres from a suspension system under an external magnetic field.

3.2. Isolation of plasma DNA

The isolation protocol used consists of lysis to release DNA and binding of the DNA to magnetic microspheres, followed by elution of the immobilized DNA. The mechanisms of selective binding and elution of DNA using silica particles have been extensively studied [29–31]. PCR products were detected using gel electrophoresis on 1% agarose and were compared with those obtained from commercially available kits based on the use of magnetic beads. Fig. 11 shows the separation of the isolated DNA using gel electrophoresis, from which it can be seen that the molecular weights of the purified DNA are about 90 bp. The integrity and purity of the isolated DNA using the new magnetic silica particles are comparable to those obtained from commercial kits. However, the yield of the isolated DNA is about 30%, less than that obtained with commercial kits (62.9%). To obtain high throughput of the isolated DNA, additional studies are needed to optimize the synthesis of magnetic silica microspheres and the extraction procedures.

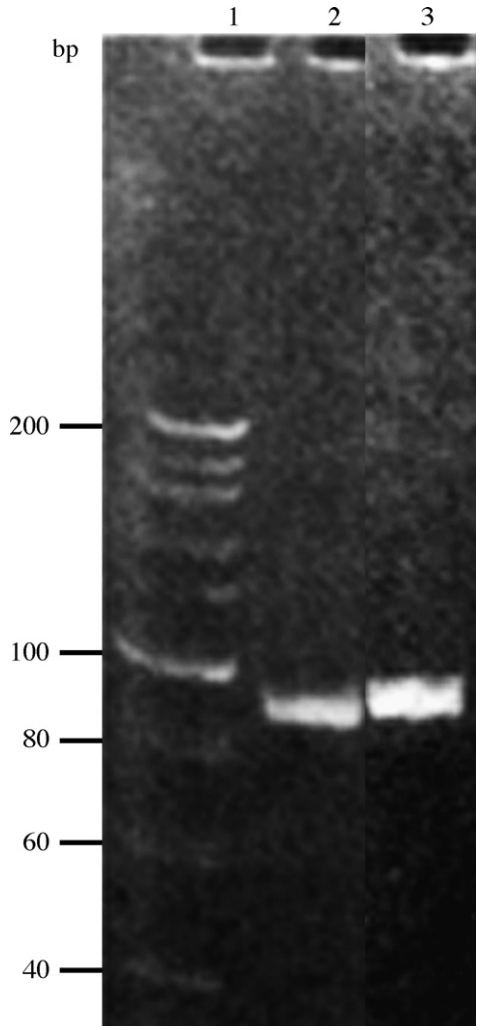


Fig. 11. 1% Agarose gel electrophoresis of isolated plasma DNA. Lane 1: DNA 20 bp ladder; lane 2: DNA isolated using the magnetic silica microspheres; lane 3: DNA obtained from commercially available kits.

4. Conclusions

In conclusion, we have described a novel process combining emulsification with sol–gel method to synthesize superparamagnetic γ-Fe_2O_3/alginate/silica microspheres which exhibit a high saturation magnetization. The utility of such magnetic microspheres in biomedical applications is demonstrated by isolation of plasma DNA. The microspheres for DNA isolation as employed in this study offers several advantages such as convenient to use, reliable, inexpensive, amenable to automation, and more importantly, the reduction of processing time, which will allow them to serve as ideal candidates for isolation of DNA, cancer diagnosis and treatment and drug delivery system.

Acknowledgments

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