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A facile fabrication of spherical and beanpod-like magnetic-fluorescent particles with targeting functionalities

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Abstract
Magnetic-fluorescent particles with targeting functionalities were fabricated by a modified Stöber method and two shapes (spherical and beanpod-like) were obtained by simply tuning the reaction temperature. The two multifunctional probes combined the useful functions of magnetism, fluorescence and FA (folic acid)-targeting recognition into one entity. The products were characterized by scanning electron microscopy, transmission electron microscopy, photoluminescence spectroscopy, confocal laser scanning microscopy, by a superconducting quantum interference device and by Fourier transform infrared spectroscopy. The experimental results show that the products possessed rapid magnetic response, relatively strong fluorescent signal, higher photostability and FA-targeting recognition as well as good water-dispersibility, suggesting that they would have potential medical applications in biolabeling and bioimaging.

1. Introduction
In recent years, multifunctional particles with magnetism, fluorescence and target navigation, which have been explored extensively at the stage of conceptual demonstration in biomedical applications, have gained more and more attention [1–8]. They have been developed as an all-in-one biomedical platform for simultaneous fluorescence imaging [3, 9], manipulation of proteins [10, 11] and cells [11–13], and drug delivery tools [1, 14–16] etc. Most of the magnetic-fluorescent nanocomposites are core–shell structured particles composed of Fe3O4 cores and biocompatible shells incorporated with fluorescent markers (e.g. organic dyes, quantum dots (QDs) or rare-earth-based luminescent nanoparticles). Among the various multifunctional particles, dye-doped silica magnetic-fluorescent composites are very promising biomedical materials due to the following advantages: (1) the high photostability and relatively strong fluorescent signal owing to the high dye-incorporation capabilities and stabilization in a protective silica matrix with biocompatibility and less toxicity; (2) the superparamagnetic magnetic oxide iron nanoparticles with fast magnetic response make it easy to manipulate via an external magnetic field and this ‘remote control’ provides considerable advantages for many applications [17].

Up to now, several methods have been developed for preparing the silica-coated magnetic-fluorescent nanoprobe, including a sol–gel approach [18], a Stöber method [19], a surface modification method [5, 20, 21], a reverse micro-emulsion technique [22, 23] and hydrolysis of hydrophobic silane [24]. However, their fabrication still suffers from problems such as complicated multi-step syntheses, fluorescent quenching arising from the direct
contact of fluorophores with the magnetic iron oxide particles, the weak fluorescent signal due to the strong light absorption by the black magnetic cores and the low dye-doped concentration. Moreover, most previous studies focused on the preparation of silica-coated magnetic-fluorescent particles in the shape of a sphere instead of the elongated shape (e.g. nanorods, nanoworms) which may be far more effective in biological applications [25–27]. For example, Park et al [27] found that the elongated peptide-modified nanoworms have the following advantages over their spherical counterparts: (1) the prolonged blood half-life due to the low phagocyte uptake; (2) the larger surface area of the elongated shape resulting in multiple targeting ligands with cooperative interaction with cell surfaces; (3) the linearly aggregated iron oxide cores leading to an improved $T_2$ relaxivity for MR imaging. To the best of our knowledge, the shape influence of nanomaterials on biological interaction has not yet been thoroughly investigated, and the fabrication of comparable model samples with minimal interference is still a challenge. However, little work has been done on the synthesis of the spherical and the elongated ones with matchable size, charge, and surface chemistry. In addition, to increase targeting recognition, it is necessary to couple the specific targeting agent to the surface of the magnetic-fluorescent multifunctional particles.

Recently, Ye et al [28] reported the fabrication of superparamagnetic Fe$_3$O$_4$/SiO$_2$/TiO$_2$ peapod-like nanostructures by using Fe$_3$O$_4$/SiO$_2$ core/shell particles as building blocks and TiO$_2$ as the adhesive without the need of any hard or soft templates. In this paper we report the fabrication of spherical and beanpod-like magnetic-fluorescent composites with FA (folic acid) targeting functionality through a simple synthetic route on the basis of our previous works [29, 30], as shown in scheme 1. We find that the beanpod-like products can be simply fabricated using the same Fe$_3$O$_4$/SiO$_2$ precursor and synthetic procedure as the spherical ones but changing the reaction temperature from 50 °C to room temperature (RT), which does not need another adhesive like TiO$_2$. The study has the following objectives: (1) to fabricate the spherical and beanpod-like magnetic-fluorescent particles with identical surface properties, which can provide two model samples for biological experimental comparison in later work; (2) to eliminate or alleviate fluorescence quenching and increase the photostability by embedding the dye into the silica shell; (3) to enhance the fluorescent signal by the high dye-incorporation capabilities into silica and by changing the black Fe$_3$O$_4$@SiO$_2$ into brick red magnetic Fe$_3$O$_4$@SiO$_2$ via calcination; (4) to add the receptor-mediated mechanism by the FA active targeting recognition as well as the magnetic guiding. As mentioned above, the spherical and the beanpod-like luminomagnetic particles with water-dispersibility, good magnetic response, fluorescence probe property and FA-targeting recognition would have potential applications in bioimaging detection.

2. Experimental section

2.1. Materials

Ferric chloride (FeCl$_3$·6H$_2$O), ethanol, ethylene glycol (EG), and ammonium hydroxide aqueous solution were obtained from Beijing Chemical Reagent Corporation, China. Tetraethyl orthosilicate (TEOS), sodium acetate (CH$_3$COONa), and polyethylene glycol (PEG molecular weight = 2000) were purchased from Beijing Yili Chemicals Limited Company, China. Fluorescein, N-hydroxysuccinimide (NHS) and folate were purchased from Sinopharm Chemical Reagent Co. Ltd, China. (3-aminopropyl)-triethoxysilane (APS) and dicyclohexylcarbodiimide (DCC) were provided by Sigma-Aldrich, St Louis, MO, USA. All of the chemical agents used in this experiment were of analytical grade and used directly without further purification.

2.2. Preparation of magnetic Fe$_3$O$_4$ particles

The magnetic particles were synthesized using a solvothermal reaction as reported previously [31]. Typically, FeCl$_3$·6H$_2$O (5.13 g, precursor) and sodium acetate (5.00 g, pH regulator) were dissolved in ethylene glycol (80 ml, reducing agent) under magnetic stirring, followed by the addition of PEG (1.25 g, stabilizer). The obtained homogeneous solution was transferred to a Teflon-lined stainless-steel autoclave (120 ml) and heated at 200 °C for 6 h. The autoclave was cooled to room temperature naturally. The obtained black magnetite particles were separated using an NdFeB magnet and then

Scheme 1. Schematic illustration of the synthesis strategy of the spherical and the beanpod-like luminomagnetic particles (denoted as SP and BP respectively).
washed with ethanol and deionized water several times and finally dried at 60 °C.

2.3. Preparation of magnetic Fe$_2$O$_3$@SiO$_2$ particles

The Fe$_2$O$_3$@SiO$_2$ core–shell particles were prepared via a modified Stöber sol–gel process [32]. In a typical procedure, as-prepared Fe$_2$O$_4$ (30 mg) particles were treated with 0.1 mol 1$^{-1}$ HCl aqueous solution (25 ml) by ultrasonication for 10 min, then separated and washed with deionized water. Subsequently, the treated particles ultrasonically dispersed in a solution containing ethanol (160 ml), deionized water (40 ml) and concentrated ammonia aqueous solution (10 ml, 28 wt%, catalyst). TEOS (0.4 ml, silica precursor) was then added dropwise to the solution under sonication. After stirring at room temperature for 3 h, the products were separated using an NdFeB magnet and washed with deionized water and ethanol several times before drying at 60 °C. The obtained powder was calcined at 400 °C overnight.

2.4. Synthesis of the spherical and the beanpod-like luminomagnetic Fe$_2$O$_3$@SiO$_2$@fluorescein/SiO$_2$–NH$_2$ particles

The spherical Fe$_2$O$_3$@SiO$_2$@fluorescein/SiO$_2$–NH$_2$ luminomagnetic particles were prepared in accordance with the following procedure. Fluorescein was conjugated with APS by an esterification reaction of the carboxyl group with the amine group. APS (100 µl), NHS (8.0 mg) and DCC (70.0 mg) were added into 20 ml of the fluorescein (10$^{-4}$ mol l$^{-1}$) ethanol solution with an ultrasonic treatment for 10 min and kept in the dark. After 24 h, the above solution was injected into a mixing solution containing 160 ml ethanol, 30 ml deionized water and 2 ml concentrated ammonia aqueous solution (28 wt%) and then sonicated for 20 min. Next, TEOS (0.2 ml) was added dropwise to the solution under sonication, followed by stirring for another 4 h at 50 °C. The products were separated magnetically, washed with ethanol and water several times, and dried at 60 °C. The beanpod-like Fe$_2$O$_3$@SiO$_2$@fluorescein/SiO$_2$–NH$_2$ luminomagnetic particles were prepared by the same procedure but the reaction temperature was changed from 50° to RT.

2.5. Synthesis of the spherical and the beanpod-like luminomagnetic Fe$_2$O$_3$@SiO$_2$@fluorescein/SiO$_2$–FA particles

Folate was conjugated onto the Fe$_2$O$_3$@SiO$_2$@fluorescein/SiO$_2$–NH$_2$ particles by a covalently coupling technique. In brief, an NHS ester of folic acid (NHS-FA) was first prepared by esterification of folate (5 g) with NHS (2.6 g) in 100 ml dry dimethyl sulfoxide (DMSO) in the presence of DCC (4.7 g) and triethylamine (2.5 ml) as the catalyst overnight at room temperature. The by-product, dichlorohexylurea, was removed by filtration. The dried Fe$_2$O$_3$@SiO$_2$@fluorescein/SiO$_2$–NH$_2$ particles (10 mg) were dispersed in the above solution (10 ml), and then the mixture was sonicated and vortexed at room temperature for 12 h. Final products were collected by a magnet, washed with deionized water several times, and dried overnight at 60 °C.

2.6. Characterization

Scanning electron microscopy (SEM) images were performed on a field emission scanning electron microscope (FE-SEM, Hitachi, S4800). Transmission electron microscopy (TEM) images were obtained with a FEI Tecnai G2 S-Twin transmission electron microscope with a field emission gun operating at 200 kV. The Fourier transform infrared spectroscopy (FTIR) of the sample was detected on a Bio-Rad FTS135 Fourier transform infrared spectrometer using the KBr pellet technique with a wave number range of 4000–400 cm$^{-1}$. The excitation and emission spectra were recorded on a Hitachi F-7000 spectrophotometer equipped with a 150 W xenon lamp as the excitation source. Confocal microscopy images were taken using an Olympus FV1000 confocal microscope with laser excitation at 488 nm. The magnetic properties were measured using a Quantum Design magnetic properties measurement system (MPMS) XL-7 superconducting quantum interference device (SQUID). All the measurements were performed at RT.

3. Results and discussion

3.1. Morphologies of the products

Two types of magnetic-fluorescent materials with a FA-targeting agent, the spherical particles (SP) and the beanpod-like particles (BP), were prepared. The exterior morphologies and interior structures of the samples at different fabrication stage were investigated by SEM and TEM, respectively. Figure 1(a) shows a representative SEM image of Fe$_3$O$_4$ particles. These Fe$_3$O$_4$ particles are sub-microspheres with an average size of 570 nm. A high-magnification SEM image (inset in figure 1(a)) reveals that the sub-microspheres with rough appearance are self-assembled from small Fe$_3$O$_4$ nanoparticles. Such structure endows the Fe$_3$O$_4$ particles with higher magnetization saturation, negligible coercivity and remanence because the nanocrystals size decreased to below a critical value, generally around 20 nm [33]. The SEM image of the resulting Fe$_2$O$_3$@SiO$_2$ composite particles is shown in figure 1(b). Compared with the Fe$_3$O$_4$ particles they have smoother surface and larger sizes, indicating that the Fe$_2$O$_3$ particles have been coated by silica successfully. The thickness of the silica shell can be precisely controlled by simply changing the TEOS concentration [34]. After calcination at 400 °C, the black powder changed into brick red, but the morphologies and magnetic response almost remained unchanged (note: the SEM image of the red powder is not shown). SEM images (figures 1(c) and (e)) show that the SP and the BP all have granular surface morphologies, which implies that the fluorescein/SiO$_2$ has deposited on the surface of the Fe$_3$O$_4$@SiO$_2$ particles. The structural features of SP and BP were further characterized by TEM (figures 1(d) and (f)). The core–shell structures can be easily recognized due to the good contrast between the cores and the shell regions. The black cores are magnetic particles and the gray part is the silica shell. The thickness of the shell of the SP and the BP was about 100 nm and 200 nm, respectively. Observing
carefully, we can find the silica layer with irregular margins and small gray nanoparticles, which are consistent with those of SEM images. Although the fluorescein molecules incorporated in the silica layer are too small to be tested by TEM, the fluorescent measurements can prove the existence of fluorescein in the complex.

As shown in scheme 1, the BP and the SP can be synthesized using the same Fe$_3$O$_4$@SiO$_2$ precursor and synthetic procedure but changing the reaction temperature. However, the beanpod-like luminomagnetic particles (BP) can be only obtained at lower reaction temperature (e.g. RT), while the spherical luminomagnetic particles can be prepared at higher reaction temperature (50°C). Apparently, the temperature difference is responsible for the phenomenon under the present conditions. However, we guess that the key factor may be the adhesive force between several neighboring precursor particles resulting from the condensation reaction of surface silanol groups on the growing silica surface. A possible formation mechanism is suggested as follows. The Fe$_2$O$_3$@SiO$_2$ particles can produce magnetic dipoles induced by the magnetic stirrer. They may be linearly linked together by self-assembly of the magnetic Fe$_2$O$_3$@SiO$_2$ particles under magnetic attraction at room temperature [28]. Meanwhile, they were slowly fixed together by the hydrolyzed silica species from the silanol group condensation reaction and the coating process also restrained the formation of a matrix. Contrarily, under the higher temperature the process could be difficult because the random thermal motion of Fe$_2$O$_3$@SiO$_2$ particles counteracts the bondage, and the individual spherical luminomagnetic particles (SP) were produced. It should be pointed out that the formation mechanism of the particles (both SP and BP) is very complicated due to the existence of many factors such as reaction conditions (e.g. temperature, concentration, solvent etc) and characteristics of the particles (e.g. diameter, shell thickness, magnetism etc) [35], thus it is difficult to give an accurate explanation of the formation mechanism. More detailed and in-depth investigations of the formation mechanism of these products are still in progress.

### 3.2. Fluorescent properties of the products

Photoluminescence (PL) spectra of the free fluorescein and the fluorescein-doped core–shell SP/BP are shown in figure 2. The spectrum testing results prove that fluorescein has been incorporated into the silica shell successfully ($\lambda_{em} = 520$ nm) [19]. Although fluorescein was doped inside the silica matrix, the emission peak feature is still
similar to that of the free fluorescein, which indicates that the modification process has no apparent influence on the spectra characteristics of fluorescein. To investigate the fluorescence imaging of SP/BP particles, confocal laser scanning microscopy (CLSM) was used. The samples in CLSM images (figures 3(b) and (e)) all exhibited strong green fluorescence. Their corresponding location and distribution of the particles well matched up to those in the bright-field images (figures 3(a) and (d)). The overlay images (figures 3(c) and (f)) demonstrate that the fluorescence originated from the magnetic-fluorescent microspheres themselves rather than the background. Additionally, there are obviously some dark spots in the merged photos, because the uneven surface of the samples makes some emission light out-of-focus and undetectable. Thus, the samples with strong enough fluorescence have potential applications in biomedical imaging, fluorescent labeling etc.

3.3. Magnetic properties of the products

The magnetic properties of Fe$_3$O$_4$, Fe$_3$O$_4$@SiO$_2$, SP and BP were recorded using a SQUID magnetometer with fields up to 1 T. The hysteresis loops and values of saturation magnetization ($M_s$), remanent magnetization ($M_r$) and coercivity ($H_c$) are shown in figure 4 and table 1, respectively. As revealed in the hysteresis loops, all the samples show strong magnetism with weak coercivity and remanence at room temperature, which would be strong enough to meet biomedical applications. All of their $M_s$ values are lower than that of bulk magnetite (92 emu g$^{-1}$) [36], because of the existence of a large amount of crystal defects inside the bare magnetite particles and some non-magnetic stabilizer (e.g. PEG) adsorbed on the surface. In addition, there are two main reasons resulting in the decrease of the $M_s$ of SP and BP. On the one hand, the Fe$_3$O$_4$@SiO$_2$ sample...
was oxidized to form $\gamma$-Fe$_2$O$_3$@SiO$_2$, while the $M_s$ value of 74 emu g$^{-1}$ of commercial $\gamma$-Fe$_2$O$_3$ powder [31] is lower than that of bulk magnetite (92 emu g$^{-1}$). On the other hand, there are many non-magnetic substances (e.g. silica, FITC, FA), which decreased the mass fraction of the Fe$_2$O$_3$ magnetic substance in the SP/BP composite [29, 37]. Moreover, the non-magnetic shell of BP (about 200 nm) is thicker than that of SP (about 100 nm). Thus the magnetic core has a smaller mass fraction in the BP composite, which could be responsible for the smaller saturation magnetization of the BP sample.

3.4. Dispersity and magnetic response of the products

The SP/BP sample has good dispersibility in aqueous medium and responds quickly to an external magnetic field, as shown in figures 5(a) and (c). The SP/BP microspheres were dispersed uniformly in the absence of magnetic field. When a magnet was placed beside the glass vial, the particles accumulated at the vial wall near the magnet within 30 s (figures 5(b) and (d)). After removal of the external magnetic field, the aggregates could be rapidly redispersed by gentle shaking.

3.5. Modification with folic acid

These SP/BP composites not only effectively keep the photochemical stability of dyes, but also provide a platform for grafting other functional or targeted moieties via the remaining amino functional groups on the silica surface [38]. Folic acid (FA), a high affinity ligand to folate receptor (FR), is known as a promising targeting agent for many biomedical applications. This is because FRs are overexpressed in many human cancer cells, which provides a distinguishable marker from normal cells. Compared with the single clone antibody, FA is not only cheap, but also has many other advantages such as non-immunogenicity, high stability, and its faster internalization kinetics through the cellular membrane [39]. Thus, based on the above reasons, FA was chosen to modify covalently the surface of the SP/BP particles. Since the SP and the BP have similar surface properties and the same $\text{--NH}_2$ active groups, herein we take, for example, the SP particles to demonstrate the modification of FA. FA was first activated by NHS and then reacted with the amino groups of the SP/BP, the NHS-FA reacted with primary amine in high yield [40]. Figure 6 gives the FTIR spectra of Fe$_3$O$_4$@SiO$_2$@fluorescein/SiO$_2$--NH$_2$ (SP-NH$_2$), FA and SP-FA. By comparison with their FTIR spectra, the
appearance of the absorption peak at 1607 cm$^{-1}$ in the SP-FA FTIR spectrum confirms the existence of folate moieties. The result is similar to that of a previous report [41].

4. Conclusions

In summary, a facile method was developed to prepare two different shapes of the magnetic-optical dual-modal probes (SP and BP). The strong fluorescence properties and magnetic responsiveness of the SP and BP can contribute to remotely manipulating proteins and cells, fluorescence imaging, biomedical detection applications and so on. They can be also used as comparable model samples for biomedical experiments. The preparation procedure is an environmentally friendly and low cost synthetic route. We predict that it can be applied to synthesize other organic dye doped magnetic-fluorescent materials.

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