Monodispersed Magnetic Polystyrene Beads with Excellent Colloidal Stability and Strong Magnetic Response[a]

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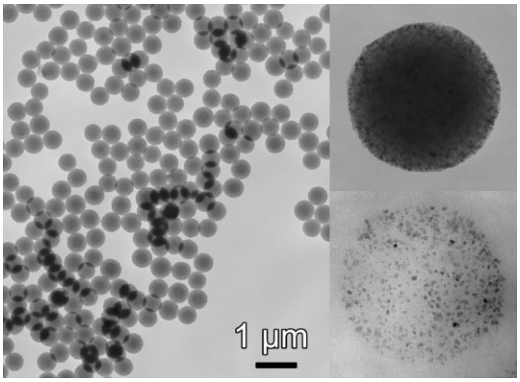
Monodispersed polystyrene beads incorporated with Fe_3O_4 nanoparticles are prepared via dispersion polymerization. The resultant magnetic beads present well-defined composite structures, excellent colloidal stability, and strong magnetic response. The formation mechanism for the monodispersed composite beads, incorporated with preformed Fe_3O_4 nanocrystals, was investigated. The potential applications of the monodispersed magnetic beads in bacteria capturing were demonstrated. After being coated with anti-Salmonella CSA-1 antibody, the magnetic beads show capturing efficiencies of $>$99.4% in isolating *Salmonella* sp.



Introduction

Magnetic beads have become a powerful tool in biological and biomedical fields nowadays. Upon proper surface engineering, magnetic beads are widely used in the purification and separation of proteins, nucleic acids, cells, and bacteria.[1] Furthermore, different biological sensors and analytical methods are being developed upon the use of magnetic beads as a facilely collectable platform.[2,3] In principle, for magnetic beads to be useful, narrow bead size distribution, satisfying colloidal stability, and strong magnetic response are essentially required. The monodispersed beads have the advantage of providing for a very uniform reproducibility of magnetic separation;[4] the colloidal stability is however, essential for fully utilizing the surface area of the magnetic beads to bind with specific targets in solution; while the strong magnetic response will guarantee an effective and fast collection of the targets captured. However, the colloidal stability and magnetic response of the beads are inimical to each other. Beads with poor colloidal stability are much easier to be magnetically collected in comparison with those with excellent colloidal stability, but they largely lose the usable surface area for binding with the targets. Therefore, in addition to the control over the bead size distribution, a proper balance between the colloidal stability and the magnetic response remains challenging but essential for developing high

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performance magnetic beads for bio-separation and purification purposes.

Although different methods have been developed for preparing magnetic beads,[5] the chemical synthetic method developed by Ugelstad in 1970s remains the most successful route so far and has successfully been used for creating commercialized magnetic beads,[6a] e.g., Dynabeads®. According to Ugelstad's synthetic route, macroporous polymeric beads are firstly synthesized according to a so-called "activated swelling method," and then $-NO_2$ or $-ONO_2$ groups are introduced onto the pore surface of the beads. By dispersing the macroporous beads in an aqueous solution of Fe^{2+} salts, Fe^{2+} ions loaded are hydrolyzed within the pores and subsequently converted to maghemite nanoparticles upon further reaction with $-NO_2$ or $-ONO_2$ groups at elevated temperature.[6b] However, governed by the principle of "activated swelling method," Ugelstad's synthetic route is more suitable for producing monodispersed polymer beads of 1–100 μm.[6c] However, beads in this size regime are prone to quick sedimentation due to gravity, which is unfavorable for the separation efficiency.

As a matter of fact, the preparation of monodispersed magnetic polymer beads of several hundreds of nanometers remains challenging nowadays although different types of dispersed phase polymerization techniques have widely been tested. The major difficulties originate from the mutual incompatibility between inorganic particles and the polymer matrices,[7a] which often gives rise to poor encapsulation of the inorganic nanoparticles.[7b–7d] Moreover, the presence of inorganic nanoparticles strongly disturb the systems which generate monodispersed polymer beads, consequently the size distribution of the resultant composite beads is dramatically increased no matter what types of polymerization techniques are used.[7] Therefore, there is only a very limited number of reports on magnetic polymer beads of a few hundreds of nanometers. For example, by a two-step procedure, including the formation of ferrofluid droplets dispersed in water with the aid of surfactant, and the subsequent swelling of droplets with styrene and cross-linking agents for further polymerization, Montagne et al.[8] reported magnetic beads of 200 nm with extremely high inorganic content, up to 60%. By similar method, the inorganic content in the magnetic beads of 133 nm was further raised to 80%.[9] But the polymer content in such beads seems unreasonably low.

Different from dispersed-phase polymerization methods, the initiation and propagation of dispersion polymerization occur in a continuous phase. Only after a critical chain length is reached, the resultant polymers precipitate forming nuclei. Upon further adsorption of oligomeric radicals, monomer, as well as the coagulation of primary nuclei, stable polymeric particles are formed. As the dispersion polymerization follows a homogenous nucleation mechanism, it is widely used for producing polymeric beads with low polydispersity index (PDI).[10] Although the dispersion polymerization was previously used for creating magnetic polymer beads,[11] the resultant polymer beads either present a broad size distribution or poorly defined composite structure. In the current investigations, the dispersion polymerization was for the first time used for preparing highly monodispersed magnetic polymer beads with well-defined composite structure. The formation mechanism for the monodispersed composite beads, incorporated with preformed Fe_3O_4 nanocrystals, was investigated. The potential applications of the monodispersed magnetic beads in bacteria capturing were demonstrated. A capturing efficiency up to 99.4% was achieved in capturing *Salmonella* sp. with the number of *Salmonella* sp. ranging in three orders of magnitude, which is the highest capturing efficiency reported so far.[1b]

Experimental Part

Materials

2,2′-azobis(2-methylpropionamidine)dihydrochloride (V50, 2997-92-4, 97%) was purchased from Sigma–Aldrich. Styrene and polyvinylpyrrolidone (PVP, $\overline{M}_w = 30\,000$) were purchased from Beijing Fine Chemicals Co., Ltd. Styrene was used after reduced pressure distillation. Anti-Salmonella CSA-1 antibody (Cat. #: 01-91-99) was purchased from Kirkegaard & Perry Laboratories, Inc., *Salmonella* sp. (Strain No. 1.1552) was obtained from Beijing Center for Physical & Chemical Analysis. All other chemicals of analytical grade were used as received.

Synthesis of Magnetic PSt Beads

The magnetic polystyrene (PSt) beads were prepared by dispersion polymerization and the PVP-coated Fe_3O_4 nanocrystals used were prepared according to literature.[12] The average size of the Fe_3O_4 nanocrystals is of 5.8 nm determined by transmission electron microscopy (TEM). In detail, 1.8 mL styrene, 90 mg V50, 300 mg PVP, 25 mL ethanol, and 3 mL deionized water were added into a 50-mL flask. Nitrogen was introduced to purge the reaction mixture for 30 min and then the flask was immersed in a hot water bath. The reaction temperature was set to 70 °C. In the meantime, 2.4 mL ethanol solution containing 4.8 mg Fe_3O_4 nanocrystals was prepared and intermittently injected into the reaction system by six times with a time interval of 1 h. The first portion was introduced after the polymerization reaction had taken place for 2 h at 70 °C and the whole reaction process was lasted for 9 h. The final product as well as those extracted during the polymerization were washed three times with ethanol and then collected by centrifugation at 8 000 rpm for 10 min.

Salmonella sp. Capturing Experiments

Firstly, magnetic beads covered by anti-Salmonella CSA-1 antibody were prepared by incubating 2 mg beads and 500 μg antibody in

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PBS buffer for 2 h at room temperature. The resultant beads were then dispersed in 10 mL 1% BSA (bovine serum albumin) to block the non-specific binding site. Then the resultant immuno-magnetic beads were collected by centrifugation and redispersed in 10 mL PBS buffer. For *Salmonella* sp. capturing experiments, four *Salmonella* sp. solutions with different numbers of bacteria, i.e., 33, 57, 631, and 19 500, were prepared and incubated with 200 μg immuno-magnetic beads in 1 mL PBS buffer for 30 min. Subsequently the magnetic beads were collected by a 0.5 T permanent magnet. The supernatant was transferred by a pipet to a 2-mL Eppendorf tube. The resultant beads were redispersed in 1 mL PBS buffer. Colony-counting method was adopted to determine the number of bacteria presented in both supernatants and redispersed beads solutions after a cultivation of 48 h at 37 °C.

Characterizations

The PSt beads were characterized by TEM (JEM-100CX II, 100 kV), dynamic light scattering (Malvern Zetasizer Nano ZS), thermal gravimetric analysis (TGA, Perkin-Elmer), Brunauer–Emmett–Teller (BET) method (Autosorb-1 MP, Quantachrome, USA). The magnetic properties of the resultant beads were also characterized by vibrating sample magnetometer (JDM-13, China). UV-Vis absorption spectrometer (Cary 50, Varian) was used to monitor the colloidal stability of magnetic beads suspended in water.

Results and Discussion

Uniform PSt beads incorporated with Fe_3O_4 nanocrystals were prepared by dispersion polymerization of styrene in the presence of pre-formed PVP-coated Fe_3O_4 nanocrystals. V50 was used as initiator. A typical sample of the magnetic PSt beads are shown in Figure 1a. The average bead size was determined to be 468 ± 24 nm by TEM by counting more than 300 beads. The dynamic light scattering (DLS) measurements revealed that the average bead size was of 469 nm with a PDI of 0.042 in pure water. The perfect match between the TEM size and DLS size suggests that no agglomeration occurs in the aqueous suspension of the resultant beads. Such monodispersed magnetic beads can also be obtained in a size range of 400–900 nm, by manipulating the synthetic parameters. Further TEM results (Figure 1b) demonstrate that a large quantity of Fe_3O_4 nanocrystals is loaded in the each bead and the nanoparticles are evenly distributed throughout the bead matrix as shown in a representative cross-sectional TEM image presented in Figure 1c. The magnetic properties of the composite beads shown in Figure 1a were investigated by vibrating sample magnetometer (VSM). The room-temperature magnetization measurements reveal that the saturation magnetization of the magnetic beads is around 7.38 $emu \cdot g^{-1}$. The zero coercivity and the reversible hysteresis behavior, shown in Figure S1 in Supporting Information (SI), indicate the magnetic beads are superparamagnetic, similar to the original magnetic nanocrystals whose saturation magnetization is of 42.4 $emu \cdot g^{-1}$. In combination with TGA results shown in the SI the weight percentage of the Fe_3O_4 nanocrystals was estimated to be around 15.0 wt-%.

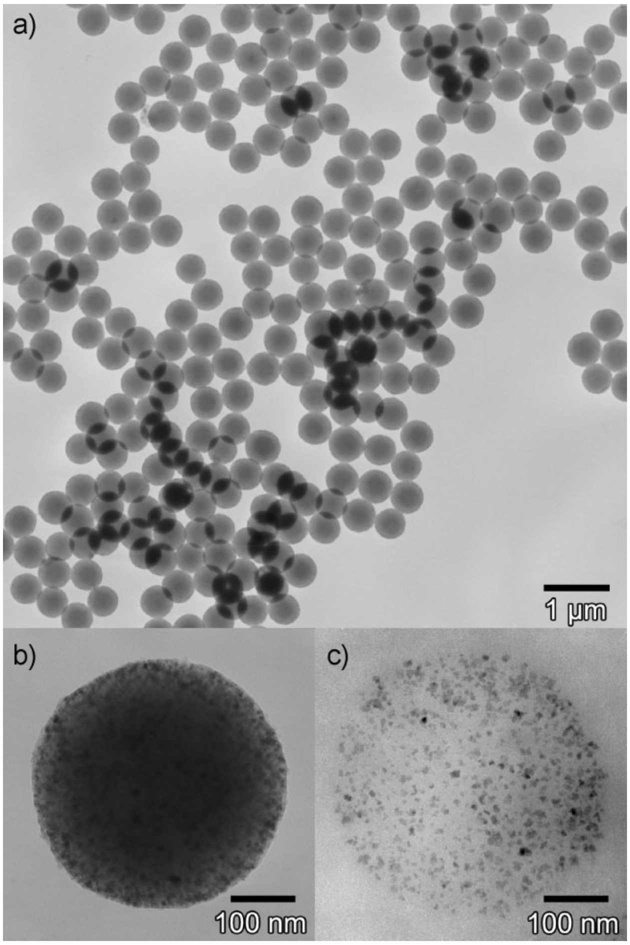

Figure 1. TEM images of the magnetic PSt beads by different magnifications (a, b), together with a cross-sectional image (c).

Although there have been a large number of investigations on magnetic polymer beads so far, the magnetic polymer beads with so narrow size distribution and such a well-defined composite structure have never been reported before. It was previously demonstrated that one of the most difficult points, faced by the preparations of magnetic polymer beads no matter what types polymerization techniques were employed, is the effective encapsulation of the inorganic nanoparticles, because in polymer matrices the inorganic nanoparticles tend to agglomerate and eventually are driven out of the polymer beads during polymerization.[7a] To further reveal the encapsulation process of the PVP-coated Fe_3O_4 nanocrystals in the PSt beads, samples of the composite beads were extracted at different time points of the polymerization. The TEM results

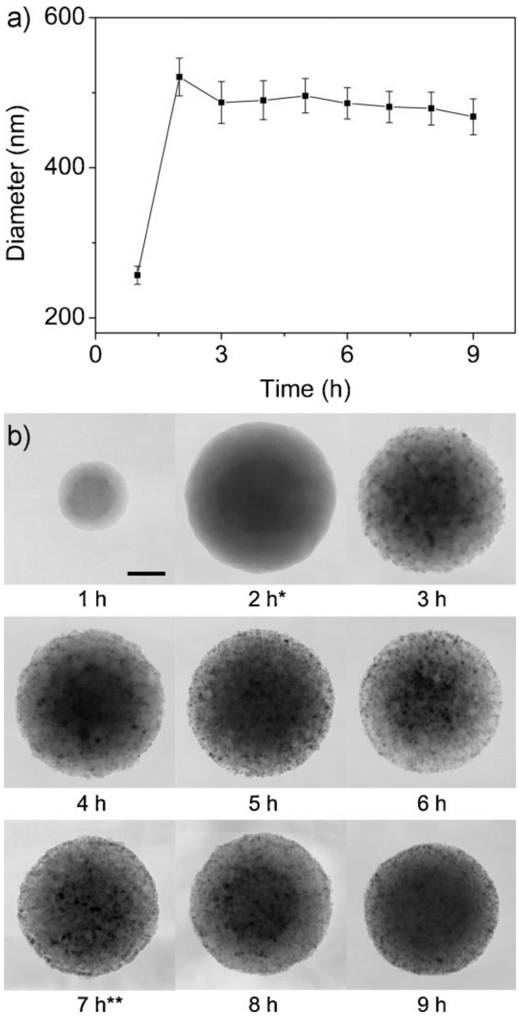

Figure 2. Panel a: the temporal size evolution of magnetic beads as a function of reaction time (extracted from the TEM measurements); Panel b: TEM images of representative single magnetic beads obtained by different reaction times shown below each image. The scale bar corresponds to 100 nm.

shown in Figure 2a demonstrate that the beads almost reach the maximum size after 2 h of polymerization, then slightly decrease in size upon the introductions of the PVP-coated Fe_3O_4 nanocrystals until the preparation was stopped. Along with this change, the PSt beads undergo a quasi-porous stage before they eventually become condensed, indicating that the magnetic composite beads were formed by sucking the PVP-coated Fe_3O_4 nanocrystals into the bead matrix while the blank beads were nearly formed. Nevertheless, such a mechanism needs two prerequisites to be valid, i.e., the PSt beads are porous and there are strong interactions between the bead matrix and the PVP-coated Fe_3O_4 nanocrystals. As a matter of fact, in Ugelstad's synthetic route, macroporous polymeric beads were synthesized via complicated procedures called "activated swelling method." However, no porous polymer beads prepared by dispersion polymerization were ever reported before. Therefore, BET experiments were carried out to further characterize the resultant composite beads together with the blank PSt beads prepared by using V50 as initiator instead of the conventional AIBN. Banett–Joyner–Halenda (BJH) curves extracted from the BET measurements shown in Figure S2 demonstrate that both PSt beads and the magnetic beads are mesoporous with an average pore size of 11 nm, which is larger than the average particle size of Fe_3O_4 nanocrystals. The formation of unusual mesoporous structure in the resultant PSt beads can then be understood as follows. Different from AIBN which is a commonly used initiator for styrene, V50 is a hydrophilic initiator. The thermal decomposition of V50 initiates the polymerization of styrene by bringing in a -2-methylpropanimidamide group to the end of PSt chain, consequently leading to the formation of hydrophilic domains in the hydrophobic PSt matrix. Due to the interaction between the -2-methylpropanimidamide-ending groups and water presented in the reaction system, the mesoporous structures are created. To provide more experimental proofs on this hypothesis, a control sample of PSt beads were prepared by replacing V50 with identical amount of AIBN whilst keeping all other experimental conditions unchanged. TEM results demonstrated that no Fe_3O_4 nanocrystals were encapsulated, as shown in Figure S3. In addition, no porous structure is presented in the resultant beads, as shown in Figure S4.

To meet the second prerequisite mentioned above, the surface of the pores has to have strong enough interactions with the PVP-coated Fe_3O_4 so as to hold the particles inside. To experimentally prove this, the following experiments were designed and carried out. First, a control solution was prepared by the same recipe as that for preparing the magnetic PSt beads except that no styrene was presented in the system. Then the solution was divided into two portions. One was stored at room temperature under stirring (solution I), while the other one was kept at 70 °C according to the conditions used for preparing the magnetic beads (solution II). For comparison with solution II, solution III was prepared with no initiator being presented, and solution II and solution III were treated the same way in the hot bath. DLS was used to monitor the agglomeration of the PVP-coated Fe_3O_4 nanoparticles in all these three systems. The results shown in Figure S5 demonstrate that the initiator, especially the decomposed initiator can greatly induce the agglomeration of the Fe_3O_4 nanoparticles, which strongly supports that the PVP-coated nanoparticles are fixed in the pore channel via interactions between the -2-methylpropanimidamide-ending groups of PSt chains and PVP-coated Fe_3O_4 nanocyrstals, which also interprets the slight size decrease of the PSt beads upon loading of the Fe_3O_4 nanoparticles, as shown in Figure 2.

The magnetic beads shown in Figure 1 present a very strong magnetic response. Typically 20 min were long

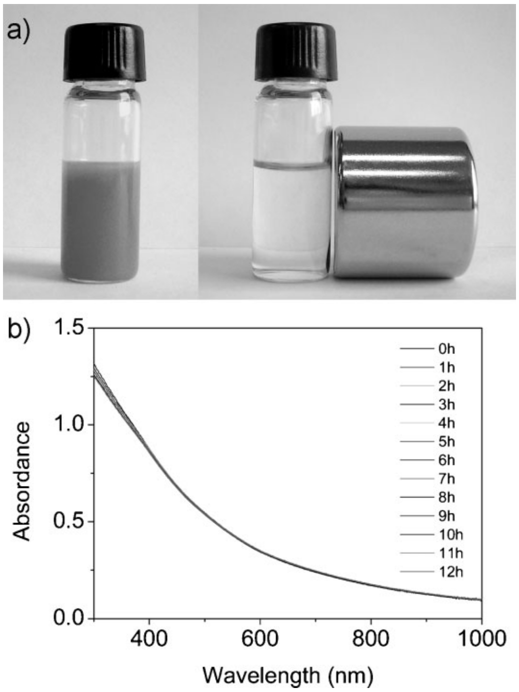

Figure 3. Panel a: photographs of an aqueous dispersion of the magnetic PSt beads taken in the absence (left) or in the presence of a 0.5 T magnet (right), respectively; Panel b: the temporal evolution of the absorption spectrum of the aqueous dispersion.

enough to completely collect the beads, suspended in pure water in a bottle of 12 mm in diameter, by a permanent magnet of 0.5 Tesla (Figure 3a). Much quicker collection can be realized by using a magnetic separation column and the recycling efficiency of the magnetic beads is higher than 95%. The colloidal stability of the magnetic beads suspended in pure water was monitored by UV-Vis absorption spectroscopy. From the results shown in Figure 3b, it can be concluded that within the inspection time window, almost no agglomeration takes place as the absorbance in long wavelength range remains nearly unchanged, while the slight decrease in short wavelength range suggests that a very little amount of the magnetic beads may fall out of the light beam due to gravity. In general, the results shown in Figure 3 suggest that the magnetic beads are highly colloidally stable due to their high Zeta-potential, around +30 mV. All these superior properties make these magnetic beads potentially useful for bioseperation applications.

A preliminary experiment was carried out by using the magnetic beads to capture *Salmonella* sp. To provide specific binding affinity of the magnetic beads to *Salmonella* sp., anti-Salmonella CSA-1 antibody was pre-absorbed on the bead surface simply via electrostatic attraction. Four *Salmonella* sp. solutions were prepared with the number of *Salmonella* sp. ranging from 33 to 19 500. In a typical capture experiment, the antibody covered magnetic beads were incubated with *Salmonella* sp. for 30 min at room temperature, then the beads were magnetically collected by a 0.5 T magnet. After the magnetic beads were re-dispersed in PBS buffer, a solution of *Salmonella* sp. captured by the magnetic beads was obtained. By conventional colony-counting method, the number of *Salmonella* sp. captured by the magnetic beads and the number of those remained in the supernatant were determined. The results tabulated in Table 1 shows that the capturing efficiency of *Salmonella* sp. with respect to those remained in supernatants are higher than 99.4%, with the number of *Salmonella* sp. ranging in three orders of magnitude.

Conclusion

In summary, monodispersed PSt beads incorporated with Fe_3O_4 nanoparticles have successfully been prepared via dispersion polymerization by using preformed PVP-coated Fe_3O_4 nanoparticles. Detailed experimental results demonstrate that the magnetic composite beads are formed by loading the PVP-coated Fe_3O_4 nanocrystals into the mesoporous PSt beads formed upon the use of V50 as initiator. The resultant magnetic beads present excellent colloidal stability because of their high surface potential. Nevertheless, due to the effective loading of the superparamagnetic Fe_3O_4 nanoparticles, they also present strong enough magnetic response in the magnetic fields. All these remarkable features together with the perfect monodispersity of the resultant beads will make them greatly desirable for bioapplications, which is demonstrated by *Salmonella* sp. capturing experiments.

Table 1. The number of *Salmonella* sp. in the initial solutions, together with those in the supernatants and dispersions of the magnetic beads carrying *Salmonella* sp. obtained after magnetic separation. The numbers were obtained by colony-counting method.

Initial number of *Salmonella* sp.		**33 ± 2**	**57 ± 3**	**631 ± 19**	**19 500 ± 254**
Capturing efficiency (%)	Captured by beads	85 ± 12	89.5 ± 1.7	80.2 ± 1.0	80.7 ± 0.4
	Remained in supernatant	0	0	0.6 ± 1.1	0.4 ± 0.04

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