Synthesis of Silica-coated Iron Oxide Nanoparticles: Preventing Aggregation without Using Additives or Seed Pretreatment

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Abstract

The Stober process is frequently used to prepare silica-coated iron oxide nanoparticles. This is usually achieved by seeding a reaction mixture consisting of water, ethanol and a catalyst with iron oxide particles and adding a silica precursor. The hydrolysis and condensation of precursor monomers results in the deposition of a silica layer on iron oxide particles. However, this process is accompanied by an increase in the ionic strength of the medium which promotes the rapid aggregation of iron oxide particles. A number of methods have been developed to prevent seed aggregation during the coating process. The majority of these methods include a pretreatment step in which the surface of iron oxide particles is modified in a manner that increases their stability in aqueous solutions. Here we suggest that by decreasing the initial concentration of the catalyst for a short period to minimize nucleation by reducing precursor hydrolysis rate and then gradually increasing the concentration to the optimum level to allow silica formation to proceed normally it may be possible to prevent aggregation without surface modification. The properties of the resulting nanoparticles as analyzed by transmission electron microscopy and magnetometry as well as their efficiency at extracting genomic DNA from different bacterial strains compared to that of a commercial extraction kit are also reported.

Keywords: Nanoparticle; Magnetite; Silica; Sol-gel; Aggregation.

Introduction

Nanoparticles (NPs) comprising a magnetic iron oxide core encapsulated in an appropriate organic or inorganic coat have been successfully used in a wide range of biomedical applications from macromolecule separation to magnetic resonance imaging (1-3). Among the various coating materials, silica is probably the best studied and most commonly used substance (1). The silica coat not only improves the stability of magnetic NPs in aqueous solutions, but also provides a scaffold for the attachment of different functional groups (4-6). Such silica-coated iron oxide (Fe\textsubscript{3}O\textsubscript{4}@SiO\textsubscript{2}) NPs can be prepared by several methods (4). One of the most widely employed methods for the synthesis of Fe\textsubscript{3}O\textsubscript{4}@SiO\textsubscript{2} NPs is a procedure based on the sol-gel process described by Stober et al. (7). This method, known as the Stober process, was originally designed to prepare monodisperse suspensions of silica NPs through the alkaline hydrolysis and subsequent condensation of
tetraethyl orthosilicate (TEOS) in a mixture of ethanol and water. It was later modified to produce Fe$_3$O$_4$@SiO$_2$ NPs by seeding the reaction mixture with Fe$_3$O$_4$ nuclei (8). However, when added to the reaction mixture, Fe$_3$O$_4$ particles tend to aggregate and form large clusters entrapped in a silica matrix (6, 8). A number of approaches have been used to address this problem. These methods usually rely on the use of either an appropriate additive or some sort of pretreatment to improve the stability of Fe$_3$O$_4$ seeds in the water-ethanol reaction mixture. For example, Philipse et al. applied a thin layer of silica to Fe$_3$O$_4$ particles by exposing them to a dilute alkaline solution of sodium silicate for two hours before using them as seeds (8). Hui et al. used a combination of citrate and nitrate salts to produce hydrophilic citrate-capped Fe$_3$O$_4$ particles (9). Xu et al. also included a pretreatment step although in this case their primary goal was to increase the affinity of Fe$_3$O$_4$ particles to silica (5). Other methods such as sonochemical and template-driven coating have also been reported in the literature (6, 10). Alternatively, some researchers have suggested that Fe$_3$O$_4$@SiO$_2$ NPs can be obtained by coating the untreated Fe$_3$O$_4$ NPs under normal conditions and then separating aggregates with prolonged centrifugation at relatively high speeds (11). All these methods, despite their apparent advantages, introduce additional steps and thus increase the complexity of the process.

Although the mechanisms governing the formation of NPs in the Stober reaction are not completely understood (12, 13), it is known that the nature, concentration and molar ratios of reactants greatly influence the morphology and size of resulting particles (4, 7). For example, Bhakta et al. showed that decreasing the volumetric ratio of water to ethanol below certain levels results in the formation of a gel network rather than particulate structures (14). Similar results have also been reported for core-shell structures prepared by modifications of the Stober method. Using different chemical compounds as core-forming ligands, for example, Massé et al. showed that above certain ligand to TEOS ratios large particles with an increased tendency toward aggregation are formed while particles produced at lower ratios are noticeably more stable (12, 15). It may thus be hypothesized that by adjusting the reaction conditions during the coating process aggregate formation can be minimized or probably totally avoided. In this paper, we propose that by careful adjustments in the concentrations of reagents and the order and rate at which they are added to the reaction mixture it is possible to prevent aggregation without employing any additional procedures. Also, in order to evaluate the practicability of this approach, we compare the yield and quality of genomic DNA samples extracted from several bacterial strains using NPs produced by this method with those purified by a commercial DNA extraction kit.

**Experimental**

**Reagents and bacterial strains**

All chemicals were used without further purification. FeCl$_3$·4H$_2$O, FeCl$_2$·6H$_2$O, TEOS, poly(ethylene glycol) (PEG, MW = 8000), NaCl, Tris-HCl, ethylene diaminetetraacetic acid (EDTA), sodium dodecyl sulphate (SDS), NaOH, HCl, calf thymus DNA (CT-DNA) were purchased from Sigma Aldrich (USA). EZ-10 Spin Column Bacterial DNA Mini-Preps Kit and proteinase K were purchased from Bio Basic (Canada). Primers for 16s rDNA amplification were synthesized by Boineer (Republic of Korea). RedSafe nucleic acid staining solution was purchased from iNtRON Biotechnology (Republic of Korea). Bacillus licheniformis was a kind gift from Dr. Faramarzi (School of Pharmacy, Tehran University of Medical Sciences). Escherichia coli, Staphylococcus epidermidis, S. aureus, Yersinia enterocolitica, and Pseudomonas aeruginosa were obtained from the culture collection of the Department of Medical Bacteriology of Tarbiat Modares University.

**Preparation of Fe$_3$O$_4$@SiO$_2$ NPs**

The Fe$_3$O$_4$ NPs were prepared according to a protocol described earlier (16). Briefly, a mixture of FeCl$_3$·6H$_2$O (1.3 g), FeCl$_2$·4H$_2$O (0.48 g) and polyvinyl alcohol 15000 (300 mg) in 30 mL deionized water was heated at 80 °C for 30 min under N$_2$ with vigorous stirring. After this period, 5 M NaOH (4.5 mL) was slowly added
to produce a black precipitate. The mixture was then stirred at 60 °C for 2 h. The precipitate was collected using a permanent magnet and washed several times with deionized water and then absolute ethanol. The resulting Fe₃O₄ NPs were kept in ethanol. In order to apply the silica coat, Fe₃O₄ NPs (100 mg) were suspended in a mixture of anhydrous ethanol (80 mL) and deionized water (20 mL) and sonicated in a water bath for 5 min. Subsequently, 0.10 mL of TEOS was added under N₂ and vigorous stirring and the dispersion was left under stirring at room temperature. After 10 min, 1.0 mL of NaOH solution (2 M) was added in 0.1 mL portions over a period of 2 h and the mixture was stirred for an additional 6 h at room temperature. Finally obtained Fe₃O₄@SiO₂ NPs were separated by an external magnet, washed several times with deionized water and then absolute ethanol until the supernatant was clear and stored in ethanol until required.

**Characterization of Fe₃O₄@SiO₂ NPs**

The size and morphology of magnetic NPs were investigated by transmission electron microscopy (TEM) using an EM10C-80 KV electron microscope (Zeiss, Germany). The magnetic properties of NPs were evaluated by a BHV-55 vibrating sample magnetometer (VSM) (Riken Denshi, Japan) at room temperature.

**Determination of DNA binding and elution conditions using CT-DNA**

In order to determine binding conditions, CT-DNA (12μg), Fe₃O₄@SiO₂ NPs (0.5 mg), NaCl (final concentration 0-2 M) and PEG 8000 (final concentration 0-20 % w/v) were added to 10 mM Tris-HCl buffer, pH 8 (final volume 100 μL) and incubated on a benchtop shaker at room temperature and 500 rpm for 5-45 min. Fe₃O₄@SiO₂ NPs were then collected with an external magnet, washed twice with 50 μL of ethanol (90 %) and allowed to dry at 40 °C. DNA was eluted with 100 μL TE buffer (10 mM Tris–HCl, 1 mM EDTA), pH 8.0 at 50 °C for 60 min. Genomic DNA was also extracted using a commercial DNA extraction kit following the manufacturer’s standard protocol.

**Amplification of extracted DNA by PCR**

DNA samples extracted from bacterial strains by both methods were used as template for PCR amplification. A region of approximately 1500 pb of the 16S rDNA gene was amplified using primers 27F(5´-AGAGTTTGATCCTGGCTCAG-3´) and 1(5´-TACGGCTACCTTGTTACGACTT-3´)(17). The PCR reactions were carried out in a peqS TARthermocycler(PEQLABBiotechnologie, Germany) using the following program: initial denaturation at 95 °C for 300 s, 30 cycles of denaturation at 95 °C for 60 s, annealing at 60 °C for 60 s and extension at 72 °C for 90 s and final extension at 72 °C for 600 s.
Synthesis of Silica-coated Iron Oxide Nanoparticles

Fe₃O₄@SiO₂ NPs are usually prepared by dispersing Fe₃O₄ NPs in a mixture of water, a low molecular weight alcohol (used as cosolvent) and a catalyst (NH₄OH or NaOH), adding a small amount of TEOS (sometimes in batches) and stirring the mixture for 6 to 48 h at room temperature (4, 6, 8, 9, 14). When untreated Fe₃O₄ NPs are used, large aggregates form upon addition of TEOS (6, 8, 9). It has been suggested that the lower pH of the reaction mixture compared to that of the medium in which Fe₃O₄ NPs were originally dispersed and the increase in the ionic strength of the mixture resulting from the hydrolysis and condensation of TEOS units are the major contributors to this phenomenon (8). This means that, ironically, the same process that leads to the deposition of a silica coat on Fe₃O₄ NPs (and thus increases their stability in aqueous solutions) is also (indirectly) responsible for their flocculation. Whether coating or flocculation occurs is determined by the kinetics of the two pathways. Apparently, flocculation cannot be prevented simply by decreasing the rate of polymerization as this would also affect coating kinetics. One should also note that coating Fe₃O₄ NPs using the Stober method is a slow process that requires several hours to complete (6) and thus further decreasing the reaction rate would not be favorable from an economic point of view either. A better approach would be to minimize nucleation while sustaining the rate of growth (6, 9).

The formation of silica during the Stober method occurs via two distinct but related processes: nucleation and growth (19, 20). Nucleation is the process during which the first insoluble species, probably doubly hydrolyzed TEOS monomers, form and precipitate (21). Growth, on the other hand, results from both the addition of newly hydrolyzed monomers to these nuclei and the aggregation of small particles to form larger ones (not to be confused with Fe₃O₄ NP aggregation) (20, 22). Although these two processes are closely related and cannot be completely separated, their relative contribution to the whole reaction can be modified to some extent by manipulating steps. This was achieved by introducing a small change in the order and rate at which reagents are added to the mixture so that NaOH is added in the last step over a period of 2 h.

Fe₃O₄@SiO₂ NPs were prepared by FeCl₂ and FeCl₃ coprecipitation under alkaline conditions. (B) Fe₃O₄@SiO₂ NPs were prepared using the method described in the Experimental section (initial TEOS concentration 0.1 % v/v).

Results and Discussion

Typical TEM images of Fe₃O₄ and Fe₃O₄@SiO₂ NPs prepared using our simplified method are shown in Figure 1. The mean diameter of Fe₃O₄ NPs is about 8 nm which is in accordance with previous reports (6, 8, 18). Fe₃O₄@SiO₂ NPs, as expected, are larger with an average diameter of ca. 18 nm. The whole synthesis process can be completed in less than 12 h and it does not include any pretreatment or additional treatment. TEM micrographs of Fe₃O₄ (A) and Fe₃O₄@SiO₂ (B) NPs. (A) Fe₃O₄ NPs were prepared by FeCl₂ and FeCl₃ coprecipitation under alkaline conditions. (B) Fe₃O₄@SiO₂ NPs were prepared using the method described in the Experimental section (initial TEOS concentration 0.1 % v/v).
reaction conditions (23). The choice between nucleation and growth is determined by the rates at which hydrolyzed monomers are produced and consumed (20). If these hydrolyzed monomers are produced at a higher rate than they can be incorporated into existing particles, new nuclei are formed. Otherwise, simple growth occurs. Therefore, nucleation can be minimized by either increasing the concentration of seed particles or reducing TEOS hydrolysis rate (20). Increasing the concentration of Fe₃O₄ NPs, however, would also promote flocculation. So there is a limit to the concentration of Fe₃O₄ NPs beyond which flocculation would occur. Philipse et al. showed that in order for silica growth to outpace flocculation the concentration of seed particles should be kept below 12 mg/L (8). However, other teams were able to achieve comparable results at concentrations of as high as 0.4 to 1 g/L (9, 10, 24). Our preliminary experiments at a range of 0.5-5 g/L revealed that a seed concentration of 1 g/L is acceptable. We were thus left with only one option: reducing the rate of TEOS hydrolysis. However, TEOS hydrolysis is the rate-limiting step in silica formation during the Stober process (25). Any factor that negatively affects this step would also hamper the coating process. We hypothesized that by first reducing the rate of hydrolysis to allow the formation of a thin silica layer around Fe₃O₄ NPs under suboptimal conditions for a short period and then, after NPs have been stabilized enough to withstand variations in medium ionic strength, gradually increasing the rate to promote further silica deposition it may be possible to address this problem. The rate of TEOS hydrolysis is determined by the concentrations of TEOS and the catalyst (25). Although theoretically either one can be used to control hydrolysis rate, in practice it is much easier to use the catalyst as it is not consumed during the process. The reaction thus would start at low pH and (relatively) high TEOS concentrations. In principle, this stage is analogous to the pretreatment step described by Philipse et al. (8). However, since in that case sodium silicate was used instead of TEOS, nucleation was avoided by increasing medium pH. It has been shown that using NaOH instead of NH₄OH as the catalyst allows for more precise control over the process (14). It is also used at lower concentrations (10-20 mM) (14). So we decided to use NaOH. Several experiments were performed to determine the optimal final concentration of NaOH (10-200 mM) and the time span over which it should be added (5-120 min) to the mixture. We obtained the best results with a 2 M solution added in ten 0.1 mL batches over 2 h. The first three batches (0.3 mL) can be added at once without any detrimental effect. At higher NaOH concentrations or when NaOH was added over a shorter period visible aggregation occurred. We also notice that even when NaOH concentration is gradually increased, the initial concentration of TEOS should be carefully adjusted as even small deviations results in homogeneous nucleation. For example, Figure 2 shows the electron micrograph of Fe₃O₄@SiO₂ NPs prepared using a TEOS concentration of 0.2% (v/v). As described in the text, large homogenous silica particles without a magnetic core are formed at this increased TEOS concentration.

![TEM micrograph of Fe₃O₄@SiO₂ NPs](image)

Figure 2. TEM micrograph of Fe₃O₄@SiO₂ NPs prepared using a TEOS concentration of 0.2% (v/v). As described in the text, large homogenous silica particles without a magnetic core are formed at this increased TEOS concentration.
Synthesis of Silica-coated Iron Oxide Nanoparticles

In order to investigate whether Fe$_3$O$_4$@SiO$_2$ NPs prepared using the method described in this paper possess the characteristics required for biomedical applications, we evaluated their performance for isolating genomic DNA from bacterial samples. We began by determining the optimum binding and elution conditions for DNA using a standard CT-DNA solution. Results are shown in Figure 4. Maximum adsorption was achieved in 10 mM Tris-HCl buffer (pH 8.0) containing NaCl (1 M) and PEG 8000 (5% w/v). It was also noticed that incubation periods longer than 20-35 min did not result in higher yields. Under optimal conditions, ca. 90% of CT-DNA was adsorbed onto the surface of Fe$_3$O$_4$@SiO$_2$ NPs. For elution experiments, the only factor that required optimization was the incubation time as the composition of the elution solution is quite simple and usually consists of TE buffer or water (27, 29-31). As shown in Figure 5, elution in 10 mM TE buffer (pH 8.0) was complete after 50-60 min. It should be noted that although in theory adsorption and elution can be achieved simply by adjusting the pH of the medium (32), which is also consistent with our results presented in Figure 6, we noticed that especially at lower bacterial loads when larger amounts of starting...
NPs. Samples containing 0.5 mg Fe$_3$O$_4$@SiO$_2$ NPs with bound DNA from binding experiments in 100 μL 10 mM Tris-HCl buffer (pH 8) containing NaCl (1 M) and PEG 8000 (5% w/v) were incubated at room temperature and 500 rpm for different time periods.

Different concentrations of NaCl were added to 10 mM Tris-HCl buffer (pH 8) and samples were incubated for 30 min at room temperature and 500 rpm. (B) Different concentrations of PEG 8000 were added to 10 mM Tris-HCl buffer (pH 8) containing NaCl (1 M) and samples were incubated for 30 min at room temperature and 500 rpm. (C) Samples prepared in 10 mM Tris-HCl buffer (pH 8) containing NaCl (1 M) and PEG 8000 (5% w/v) were incubated at room temperature and 500 rpm for different time periods.

Figure 4. Effect of NaCl concentration (A), PEG concentration (B) and incubation time (C) on CT-DNA adsorption on Fe$_3$O$_4$@SiO$_2$ NPs. In all experiments, 0.5 mg Fe$_3$O$_4$@SiO$_2$ NPs and 12 μg CT-DNA in a final volume of 100 μL were used. (A) Different concentrations of NaCl were added to 10 mM Tris-HCl buffer (pH 8.0) and samples were incubated for different time periods at 50 °C.

Figure 5. Effect of incubation time on the elution of CT-DNA from Fe$_3$O$_4$@SiO$_2$ NPs. Samples containing 0.5 mg Fe$_3$O$_4$@SiO$_2$ NPs with bound DNA from binding experiments in 100 μL 10 mM TE buffer (pH 8) were incubated for different time periods at 50 °C.

Figure 6. Effect of pH on CT-DNA adsorption on Fe$_3$O$_4$@SiO$_2$ NPs. Samples containing 0.5 mg Fe$_3$O$_4$@SiO$_2$ NPs and 12 μg CT-DNA in 100 μL buffer (10 mM citrate, pH 4-6; 10 mM Tris-HCl, pH 7-9) were incubated at room temperature and 500 rpm for 30 min.
using Fe$_3$O$_4$@SiO$_2$ NPs can be directly used for PCR amplification without further processing, we used extracted DNA samples to amplify a region of the 16S rDNA gene. As shown in Figure 7, all samples were successfully amplified.

The results presented in this paper suggest that the requirement for Fe$_3$O$_4$ pretreatment in the preparation of Fe$_3$O$_4$@SiO$_2$ NPs using the Stober process can be bypassed by introducing minor modifications to the reaction conditions so that a short period of slow silica growth is allowed to proceed in the absence of noticeable nucleation. This goal can be achieved by reducing the rate of TEOS hydrolysis by limiting the initial concentration of NaOH in the reaction mixture and then gradually increasing its concentration as the reaction proceeds. Fe$_3$O$_4$@SiO$_2$ NPs produced by this method show good physicochemical properties can be used for biological applications such as genomic DNA extraction with a separation quality comparable to that of commercial kits.

**Acknowledgment**

This work was supported by a grant from the Shahid Beheshti University of Medical Sciences (SBMU) and forms part of a thesis to be submitted to the SBMU School of Pharmacy by Zeinab Sharafi in partial fulfillment of the requirements for the degree of Ph.D.

### Table 1. Yield and quality of genomic DNA samples extracted from different bacterial strains using Fe$_3$O$_4$@SiO$_2$ NPs and a commercial genomic DNA extraction kit. Results are shown as mean ± standard error of the mean.

| Bacterial strains | Fe$_3$O$_4$@SiO$_2$ NPs | EZ-10 Bacterial DNA Kit |
|-------------------|-------------------------|-------------------------|
|                   | Yield (μg) | A$_{260}$/A$_{280}$ | Yield (μg) | A$_{260}$/A$_{280}$ |
| E. coli           | 53.70 ± 3.48 | 1.93 ± 0.02 | 60.00 ± 2.64 | 2.00 ± 0.01 |
| S. epidermidis    | 28.00 ± 4.04 | 1.73 ± 0.05 | 34.33 ± 2.96 | 1.85 ± 0.03 |
| S. aureus         | 12.00 ± 1.15 | 1.74 ± 0.02 | 14.67 ± 1.55 | 1.70 ± 0.03 |
| B. licheniformis  | 17.00 ± 1.15 | 1.80 ± 0.01 | 15.33 ± 1.20 | 1.77 ± 0.01 |
| Y. enterocolitica | 19.67 ± 1.45 | 1.83 ± 0.02 | 23.00 ± 2.08 | 1.85 ± 0.03 |
| P. aeruginosa     | 26.67 ± 0.88 | 1.86 ± 0.02 | 21.33 ± 1.86 | 1.92 ± 0.02 |

**Figure 7.** Agarose gel electrophoresis of PCR products. Extracted genomic DNA samples were amplified using 16S rDNA gene-specific primers. From left to right: E. coli, S. epidermidis, S. aureus, DNA ladder, B. licheniformis, Y. enterocolitica, P. aeruginosa.
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