Facile synthesis and characterization of polyethylenimine-coated Fe₃O₄ superparamagnetic nanoparticles for cancer cell separation

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Abstract. The detection of cancer cells in clinical samples is of great interest for a range of diagnostic applications, and separation and enrichment of cancer cells in low concentrations from complex sample matrices is necessary for efficient cancer diagnostics. In the present study, new surface-modified iron oxide nanoparticles were synthesized for the separation of lung cancer cells by simple precipitation of Fe(II) and Fe(III) salts in an aqueous ammonia solution, followed by the addition of polyethylenimine (PEI). The modified nanoparticles were characterized by X-ray diffractometry (XRD), transmission electron microscopy (TEM), Fourier transform infrared spectroscopy (FTIR), X-ray photoelectron spectroscopy (XPS) and vibrating sample magnetometry (VSM). XRD and TEM revealed that the particles were ~10 nm in diameter, while FTIR and XPS showed that their surfaces were well coated with PEI. VSM results confirmed the superparamagnetic nature of PEI-coated Fe3O4 nanoparticles. The separation and enrichment of lung cancer cells from sputum samples was demonstrated using the synthesized developed PEI-coated Fe3O4 magnetic nanoparticles. Exfoliative cytopathology showed that the percentage of positive cells increased from 6.3% (38/600) in untreated sputum samples to 38.5% (231/600) in sputum samples treated with PEI-coated Fe3O4 magnetic nanocomposites. This finding indicated that PEI-coated Fe3O4 magnetic nanocomposites can be used to efficiently enrich lung cancer cells from sputum for subsequent cytopathological analysis.

Introduction

Magnetic nanoparticles (MNPs) are currently under investigation for their high potential for *in vitro* and *in vivo* diagnostic applications, such as in cellular therapy (cell labeling, targeting

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and as a tool for cell-biology research to separate and purify cell populations), tissue repair, drug delivery, magnetic resonance imaging (MRI), hyperthermia and magnetofection (1). Superparamagnetic particles are suitable for all these applications, since they do not retain magnetism after removal of the magnetic field.

In the last few decades, cancer has become one of the major human diseases that ultimately result in death. Accurate, sensitive rapid and facile diagnostic methods for collection/isolation of cancer cells are of critical importance for the investigation, prevention and treatment of cancer. A number of methods (2) are used for isolation of cells, including high-speed fluorescence-activated cell sorting, dielectrophoresis, parallel-plate flow chambers, immunoadsorption columns and immunomagnetic selection. Apart from methods of chemical separation of cells, mechanical methods, such as size-based separation and differential sedimentation also exist. Among these methods, MN-based separation of cells has several advantages in comparison to other techniques. Superparamagnetic nanoparticles are particularly suitable for cell enrichment, since they are of the same size scale as cell particles themselves and provide a larger overall surface area of interaction for the same bead volume. As an alternative to micrometer magnetic bead-based selection (3,4), the small size and increased relative surface area of nanoparticles provide enhanced extraction capacity. This allows target cells to be directly isolated from crude samples and is a relatively simple, inexpensive and fast method; to a certain degree, it may be considered as a sample enrichment step prior to subsequent analysis.

The magnetism of MNPs commonly comes from magnetic metallic elements or compounds, which are nevertheless easy to aggregate. In addition, since the metallic elements or oxides are reactive in a biological context, they are easily etched in practice. Therefore, inorganic or organic protective coatings, including silica, polyelectrolyte, lipid layers and micelles, have been developed for these particles (5). Among these, polymer wrapping, that is, encapsulation of already fabricated magnetic nanoparticles into polymer micro- or nanoparticles, is widely used. The magnetic cores ensure a strong magnetic response and the polymeric shell provides favorable functional groups and features that are suitable for various applications. These particles are stable in aqueous suspensions and can

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be easily redispersed after agglomeration in the presence of a magnetic field. In addition, the structure, stability and physical properties of nanoscale-size materials can exhibit a strong dependence on the particle surface. Numerous polymers are biocompatible and may be used as MNP coating for biomedical applications. Polyethylenimine (PEI) is a branched polymer with a high-density amine group, (CH₂CH₂NH)_n. The ratio of primary to secondary to tertiary amines is 1:2:1. In each PEI molecule, one nitrogen atom is protonated per two carbon atoms. Due to the different pKa values of the primary, secondary and tertiary amino groups, PEI has the ability to capture protons at different pH conditions, which is known as the 'proton sponge' mechanism. PEI was developed to condense DNA via the electrostatic interaction between its positive and negative charge of the phosphate group of DNA (6). A PEI-modified MALDI plate was successfully used to concentrate DNA and protein digestion products (7). Due to its unique properties, PEI appears to be one of the most appropriate molecules for the surface modification of MNPs for biomedical applications.

Cytopathological analysis of sputum is one of the most promising and effective methods for early diagnosis of lung cancer. Currently however, there is one main disadvantage of this method: its high false-positive rate (up to 20-40%). This is typically caused by the loss of cells during sample preparation, the poor quality of the smear, complication of cells and the non-uniform distribution of lung cancer cells in sputum samples. Therefore, the present study aimed to develop a new method for enrichment of lung cancer cells from sputum for cytopathological analysis with increased positive rate, by adopting superparamagnetic PEI-coated Fe₃O₄ nanoparticles as convenient vehicles to isolate and enrich lung cancer cells. A detailed study of the preparation and characterization of the PEI-coated MNPs is presented below. Our study demonstrated a highly specific method for the enrichment of lung cancer cells using a simple approach based on the strong protonating capacity of PEI (~20% of nitrogen atoms are protonated under physiological conditions) (8).

Materials and methods

Materials. Chemical reagents were of analytical grade and were used without further purification. Iron(III) chloride hexahydrate (FeCl₃·6H₂O), iron(II) ferrous chloride (FeCl₂·4H₂O), aqueous ammonia and an ethyl alcohol solution of PEI (molecular weight, 20,000) were used as purchased from Shanghai Baijin Chemical Group Co., Ltd. (Shanghai, China). De-ionized water was used in all the experiments.

Nanoparticle synthesis. Magnetic particles were prepared by coprecipitation, by adding aqueous ammonia solution into a mixed solution of 0.25 mol/l ferrous chloride and 0.5 mol/l ferric chloride (molar ratio 1:2) at 70°C for 1 h until a pH 11.0 was achieved. The entire process was protected by inactive gas. The precipitate was alternately washed with distilled water and ethyl alcohol three times, and separated with centrifugation. It was then dried at 50°C for 16 h in a vacuum-drying chamber. Following this step, the magnetite and polyethylenimine were left to mix (mass ratio 1:2) at room temperature for 24 h at pH 9.0. This procedure was performed under ultrasonic

waves, so that the magnetic nanoparticles could homogenously disperse in the solution. The PEI-coated magnetic nanoparticles were thoroughly washed alternately with distilled water and methyl alcohol at least three times, and were then separated by centrifugation. The modified nanoparticles were then dried at 60°C for 24 h. Polyethylenimine molecules bound to particles by electrostatic interaction and the negative charges on the surface of the particles were converted to positive charges.

Characterization of the nanoparticles. The crystalline phases were studied by X-ray diffractometry (XRD) operating at a scanning rate of 10°/min from 10° to 85° (D/max2550VB3; Rigaku International Corporation, Tokyo, Japan). Identification of the synthesized iron-oxide was based on the position of characteristic peaks in the diffractograms using the Joint Committee on Powder Diffraction Standards (JCPDS) database. The XRD patterns were evaluated to determine the lattice spacing (d_{hkl} values) with the Bragg equation and the Miller (hkl) indices corresponding to the crystalline phases in the samples. The particle size and morphology of the naked and modified particles were examined with a transmission electron microscope (TEM; FTIR, HYPERION2000, Bruker, Ettlingen, Germany). The Fourier transform infrared (FTIR) spectrum (500-4,000 cm⁻¹) from the KBr pellet containing the PEI-coated magnetic nanoparticles was recorded on a FTIR spectrometer. The samples were also examined by X-ray photoelectron spectroscopy (XPS; Kratos, Tokyo, Japan). The magnetic properties were measured with a vibrating sample magnetometer (VSM; Lakeshore Cryotronics, Westerville, OH, USA) at room temperature in magnetic fields of up to 20 kOe.

Cell separation and exfoliative cytopathology. One hundred milligram of PEI-coated Fe₃O₄ magnetic nanoparticles were suspended in 10 ml of 0.01 mol/l PBS (pH 7.2). The concentration of PEI-coated Fe₃O₄ magnetic nanoparticles in the solution was $\sim 3x10^{13}$ /mol. The solution was then mixed with 0.5 ml of staphylococcal protein A (SPA) protein solution. The nanocomposite solution was obtained following uniform stirring. Sputum (5 ml) was collected by expectorating from five lung cancer patients in the morning and lysed by 30 ml alkaline lysate (CytoLyt Solution). All patients provided written informed consent and the study was approved by the Ethics Committee of Tongji University, Shanghai, China. The lysed sputum was mechanically stirred for 15 min and then centrifuged for 5 min. It was suspended in preservation solution and then 1 ml of the nanocomposite solution containing the magnetic particles was added. After stirring, the mixed solution was kept still for 30 min. The ThinPrep test was used in cytological preparations, with hematoxylin and eosin staining. The prepared specimens were observed using a Nikon 90i optical microscope (Nikon Instruments Inc., Melville, NY, USA) and 600 cells were counted per sample.

Results

Fig. 1 shows the XRD patterns of the naked and the PEI-coated magnetic nanoparticles. The characteristic peaks of magnetite were detected at Miller indices 111, 220, 331, 400, 422, 511 and 440. The positions of characteristic peaks did not shift but showed limited broadening, indicating that the nanoparticles



Figure 1. X-ray diffraction patterns of naked magnetic nanoparticles (MNPs) and polyethylenimine (PEI)-coated MNPs.



Figure 2. Transmission electron microscope (TEM) photographs of (A) naked and (B) polyethylenimine (PEI)-coated iron oxide (magnetic) nanoparticles. Selected area electronic diffraction patterns are shown in the enclosed boxes.

have small crystalline sizes. Using the Debye-Scherrer equation for spherical particles, this was translated into an average grain size of 10.7 nm.

The size and morphology of the naked and PEI-coated nanoparticles were investigated by TEM due to their large surface area and high surface energy (nano effect). Fig. 2 shows the bright-field images and the corresponding selected area electronic diffraction patterns (SAED) of naked (Fig. 2A) and PEI-coated nanoparticles (Fig. 2B). Particles with an approximate spherical shape were observed. The estimated average diameter of naked nanoparticles was ~10 nm. This result was in accordance with that of X-ray diffraction analysis. However, the average size for PEI-coated Fe₃O₄ nanoparticles in solution was determined to be ~12.7 nm.

The surface chemical structure of PEI-coated Fe_3O_4 MNPs was characterized by FTIR spectroscopy. Fig. 3 shows the FTIR spectrum for the PEI-coated MNPs from observations in the infrared spectrometer using the KBr tabletting method. The absorption peaks at ~563/cm correspond to the characteristic absorption of Fe-O (9). The absorption peaks of PEI appear at 649/cm (-NH wagging vibration), 1384-1659/cm (NH₂-scissoring vibration and C-H stretching vibration), 2348 and 2372/cm (C-H₂ symmetry shrinkage). The characteristic



Figure 3. Fourier transform infrared (FTIR) spectra for polyethylenimine (PEI)-coated magnetic nanoparticles.

peaks corresponding to PEI were clearly observed at 649, 1560, 2850 and 2372/cm in the FTIR spectrum. We therefore confirmed that the PEI layer was coated on the surface of the Fe_3O_4 magnetic nanoparticles.

Fig. 4A shows the XPS spectrum of Fe(2p) for the PEI-coated Fe₃O₄ magnetic nanoparticle powder. The binding energy values for the main peak maxima 2p3/2 and 2p1/2 were estimated at 711.1 and 724.5 eV, respectively. Fig. 4B shows the typical XPS spectrum of the N1s region. Notably, one peak was detected for N1s, at 399.8 eV.

The magnetization hysteresis loops of PEI-coated and naked Fe_3O_4 nanoparticles were measured by VSM at room temperature (Fig. 5). From Fig. 5, it is evident that the saturation magnetization values for naked and PEI-coated magnetic nanoparticles were ~58.3 and 53.4 emu/g, respectively. The variation of magnetization M, on an applied field H, showed no hysteresis in the two samples; that is, both the remanence and coercivity of the samples were zero.

Fig. 6 shows images from optical microscopy of magnetic nanocomposites adsorbed on the surface of lung cancer cells. As seen in Fig. 6A, after staining, the oval/round-shaped cells were dispersed and showed strong light refraction. The core located at the center of cells and the nucleolus can be clearly observed. Fig. 6B shows high numbers of magnetic nanoparticles attached on the surface of the cells, which rendered the enrichment and the separation of the cells, either by magnetic or by gravity separation, easy and convenient. Exfoliative cytopathology analysis showed that the percentage of positive cells increased from 6.3% (38/600) in the untreated sputum samples to 38.5% (231/600) in the sputum samples treated with PEI-coated Fe₃O₄ magnetic nanocomposites.

Discussion

The diffraction peaks of PEI-coated magnetic nanoparticles agree well with standard Fe_3O_4 powder diffraction data (JCPDS card 72-2303), and indicate that there is no crystallographic change after coating, while no additional peaks are detectable except for those corresponding to magnetites.



Figure 4. XPS spectra of A) Fe(2p) and (B) N1s for the polyethylenimine (PEI)-coated Fe₃O₄ magnetic nanoparticles.



Figure 5. Hysteresis loops for naked magnetite (Fe₃O₄) and polyethylenimine (PEI)-coated Fe₃O₄ nanoparticles.

The size of the modified nanoparticles as determined from TEM images is expected to reflect their actual size in solution. In addition to dispersal, the naked nanoparticles also exhibited aggregated morphology to a certain degree (Fig. 2A) due to their large specific surface area and the high energy of this surface. By contrast, after PEI coating, the dispersibility of the cells showed an obvious improvement (Fig. 2B). Since polyethylenimine is a linear molecule, the ζ potential of the MNPs is reduced after functional processing (coating) with PEI, leading to a steric effect, resulting in disaggregation of the MNPs (9). Therefore, PEI coating can reduce the aggregation and improve particle dispersion.

The surface specificity of XPS renders it a useful analytical technique for the direct characterization of iron oxide nanoparticles (10,11). The observed position of the magnetites is consistent with reported iron assignments (12,13). The appearance of a satellite peak of Fe 2p3/2 in the XPS spectra is an important feature for discrimination of magnetite from maghemite (14,15). As seen in Fig. 4, the absence of the satellite peak further substantiates the formation of magnetite in our experiments. In addition, the difference in the binding energies of the main peaks dE, was 13.4 eV, that is, very similar to that reported for Fe_3O_4 (13.5 eV) (16). It is notable that the XPS analysis of N1s detected one peak at 399.8 eV. This has been commonly interpreted as the formation of nitrogen-coordinated metal complexes (17,18). Therefore, the peak position of N1s at 399.8 eV indicates that the nitrogen from the amino groups of PEI coordinates with the magnetic nanocrystals.

The PEI-coated nanoparticles were uniformly dispersed in aqueous solution in the absence of a magnetic field. A suspension of PEI-coated Fe_3O_4 magnetic nanoparticles is deep brown. When a magnet was placed under the glass vial, the particles accumulated on the bottom of the vial near the magnet within a few minutes. After removal of the external magnetic field, the aggregates were rapidly redispersed by gentle stirring. The magnetization hysteresis loops of PEI-coated and naked Fe_3O_4 nanoparticles were measured by VSM at room temperature (Fig. 5). The magnetic data are described by the Langevin equation (19), which indicates that the magnetic nanoparticles are single-domain, while the samples exhibit superparamagnetic behavior at room temperature, as expected by the nanoscale dimension of the particles



Figure 6. Optical microscopy observation of lung cancer cells labeled with polyethylenimine (PEI)-coated Fe₃O₄ nanoparticles. Magnification (A) x100; (B) x400.

[the critical size for superparamagnetic behavior of magnetite is ~20 nm (20)]. The saturation magnetization values for naked and PEI-coated magnetic nanoparticles are lower than the reported saturation magnetization of their bulk counterparts (magnetite, 92-100 emu/g at 300 K). This result is consistent with that observed for magnetic iron oxide nanoparticles coated with poly(methacrylicacid) (20). This phenomenon has been attributed to the presence of nonmagnetic or 'dead' surface layers resulting from the chemical reaction between the stabilizing surfactant and the ferrite particles (21). Such dead surface layers make the magnetic diameter of the particles smaller than its physical diameter. Nanoparticles coated with PEI showed an even lower value of saturation magnetization (Ms, 53.4 emu/g). Davies et al (22) suggested that particles containing sufficient concentrations of functional groups allow spin pinning of the iron oxide surfaces, which gives rise to a non-collinear spin structure and is known to produce reduced magnetic moments for the particles (23,24).

The fabricated PEI-coated Fe_3O_4 magnetic nanocomposites were used for separation and enrichment of lung cancer cells in the present study. The magnetic nanocomposites can be easily aggregated using an external magnetic field and can be uniformly dispersed when the magnetic field is removed, owing to their superparamagnetic properties. Following PEI coating, the Fe_3O_4 magnetic nanoparticles were functionalized with amino groups on their surfaces. This feature renders them attractive to cells with pathological alterations and thus, a nanoparticle-PEI-pathologically modified cell composite is formed. This composite can then be easily disaggregated by applying an external magnetic field.

In our experiments, the PEI-coated Fe_3O_4 magnetic nanoparticles effectively attracted pathologically modified cells and the magnetic nanocomposites had a measurable effect on the separation and enrichment of cancer cells. The exfoliative cytopathology results indicated that using PEI-coated Fe_3O_4 magnetic nanocomposites can increase the percentage of positive cells, indicating that these nanocomposites are very effective in cell separation and enrichment of lung cancer cells.

In summary, we synthesized the superparamagnetic Fe_3O_4 magnetic nanoparticles coated with PEI by a simple precipitation method. The average grain size of PEI-coated Fe₃O₄ magnetic nanoparticles is ~12.7 nm while that of naked nanoparticles is ~10 nm. The PEI layer was successfully coated on the surface of Fe₃O₄ magnetic nanoparticles according to XPS and FTIR analyses. Magnetic measurement showed that the nanoparticles are superparamagnetic. The synthesized PEI-coated Fe₃O₄ magnetic nanocomposites were used for separation and enrichment of lung cancer cells. Exfoliative cytopathology analysis showed that the percentage of positive cells increased from 6.3% (38/600) in untreated sputum samples to 38.5% (231/600) in sputum samples treated with PEI-coated Fe₃O₄ magnetic nanocomposites. This finding indicates that PEI-coated Fe₃O₄ magnetic nanocomposites can be used to efficiently enrich for lung cancer cells from sputum for cytopathology analysis.

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