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BSA Immobilization on Amine-Functionalized Superparamagnetic Iron Oxide Nanoparticles

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Immobilization of bovine serum albumin (BSA) on surface-modified superparamagnetic iron oxide nanoparticles (SPION) has been performed by two different double-step immobilization approaches. The first approach consists of preparation of SPION by controlled chemical coprecipitation in the presence of BSA solution, whereas the second approach includes preliminary surface modification of SPION with an amine group using a coupling agent of 3-aminepropyltrimethoxysilane (APTMS). Both procedures are followed by 1-ethyl-3-(3-dimethylaminepropyl) carbodiimide hydrochloride (EDC) activation with sequential immobilization of the layer of BSA. Additionally, an attempt to modify the surface of SPION with amine and carboxylic groups is undertaken by using L-aspartic acid (LAA). TEM shows that the particle size varies in the range 10−15 nm and does not change significantly after the coating process. The presence of BSA and amine groups on the surface of SPION is confirmed by FT-IR. Magnetic properties are investigated by VSM and results indicate that the superparamagnetic properties are retained for BSA-coated SPION while reducing the value of saturation magnetization (M_s). The binding capacity is estimated from thermogravimetric and chemical analyses. APTMS-coated SPION show higher BSA binding capacity compared to that of coprecipitated SPION in the presence of BSA. In vitro tests have been performed after the functionalization of SPION with LAA and BSA. Human dermal fibroblasts are incubated with the surface-modified SPION for 6 and 24 h to observe cell behavior, morphology, cytoskeletal organization, and interactions between cell and SPION. BSA-coated SPION incubated with cells demonstrated a cell response similar to that of control cells, with no adverse cell damage and no endocytosis, whereas LAA-coated SPION show partial endocytosis without cytoskeletal disorganization.

1. Introduction

Superparamagnetic iron oxide nanoparticles (SPION) receive increasing attention in different fields of physics, medicine, biology, and materials science due to their multifunctional properties such as small size, superparamagnetism, and low toxicity, etc.[1−3] Functionalization and modification of the surface of SPION with various biocompatible and biodegradable materials has been widely examined in biomedicine fields for cell separation[2], diagnostic magnetic resonance imaging (MRI),[4] drug delivery systems (DDS),[5] and vaccine administration.[6] Proteins, which can provide biocompatibility, high solubility, and hydrophilic properties, are one of the most promising materials among several potential biocompatible and biodegradable substances serving as protective layers on SPION. Consequently, these can be used in DDS, neuroscience, DNA and cell separation, and also as model systems. Recently, several proteins such as dispase, chymotrypsine, and streptokinase have been immobilized on the surface of SPION. Streptokinase is considered as a material which can be used for the treatment of coronary thrombosis and in peripheral arterial occlusions.[7] Dextran-, albumin-, and transferrin-coated SPION have been tested in vitro using human dermal fibroblasts cells. Dextran-coated SPION show endocytosis with eventual cell death. A partial endocytosis has occurred for albumin-coated SPION with cells proliferation. Magnetic par-

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ticles coated with transferrin are not taken up by cells in this particular case, and demonstrate an increase of cell number.[8] SPION conjugated with antibodies, enzymes, and proteins have also been tested to specific targeted cells as DDS.[9] However, there are several significant demands which should be considered for in vivo applications. The particle size of uncoated SPION has to be less than 20 nm and may not exceed 50 nm after coating due to the limitation of extra-cellular space and biological capillary dimensions. The large surface area and specific magnetic properties of SPION may cause formation of clusters before they reach the target cell. Therefore, the colloidal stability of SPION is one of the important parameters to consider for biomedical applications. Several materials have been introduced as coating agents to improve colloidal stability of the ferrofluid, namely BSA, starch, poly(ethylene glycol) (PEG), and poly(vinyl alcohol) (PVA).[2,3,10] However, the stabilization mechanism after modifying the surface of SPION has not yet been intensively studied by considering the required factors for in vivo and in vitro applications. Currently, several types of SPION-like magnetic particles are commercially available, but their properties do not always satisfy the requirements for biomedical applications, such as monodispersed particle, intrinsic magnetization, and surface functionality.

BSA was selected from among several types of protein molecules for immobilization on the surface of SPION. The special functional groups are required on the surface of the SPION to covalently attach the BSA molecules. Because the SPION do not have such functional groups, they must be introduced. The phagocytic uptake of SPION has been investigated by using surface-modified SPION with butylamine and ethanolamine.[11] Recently, SPION functionalized with different numbers of amine groups have been tested for DNA extraction processes.[12]

Aspartic and glutamic acids, nonessential amine acids, are two of the most promising candidates to introduce the functional amine groups on the surface or SPION for in vivo applications. Aspartic acid is reported as a helpful chemical to control mental capacities and is essential in the urea cycle for proper elimination of waste products from dietary protein, whereas glutamic acid is significant in the metabolism of sugars and fats, and aids in transportation of potassium across the blood−brain barrier (BBB). Recently, the adsorption of aspartic and glutamic acids on maghemite nanoparticles has been demonstrated.[13] These particles also show a high colloidal stability in physiological media.

In this study, an attempt to simplify and optimize the process of SPION surface modification with amine groups is mainly focused for second functionalization with biocompatible substances. The synthesis of SPION, introduction of amine groups via silanization with APTMS, and chemisorption of LAA on the surface of SPION are briefly summarized. The binding capacity is estimated on the basis of magnetic measurements as well as thermal and chemical analyses. Furthermore, BSA−APTMS coated SPION were incubated with human dermal fibroblasts in vitro to investigate the cell response. Light microscopy was used to visualize cell morphology. In addition, fluorescent observations of the cell cytoskeleton and intracellular clathrin localization have also been evaluated.

2. Experimental Section

Ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$ > 99%), ferrous chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$ > 99%), sodium hydroxide (0.5 M in water), hydrochloric acid (diluted in water from HCl >37%), sulfuric acid (96%), nitric acid (65%), hydrogen peroxide (30%), 3-aminepropyltrimethoxysilane (APTMS), 1-ethyl-3-(3-dimethylaminepropyl) carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), bovine serum albumin (BSA), sodium azide and BCA kit for protein analysis, Natriumdihydrogenphospate-1-hydrate and Natriumdihydrogenphospate anhydrous, and L-aspartic acid (LAA) were purchased from Aldrich. All chemicals were used as received except methanol which was dried before use in the silanization process. Milli-Q purified water was used for all experiments.

Synthesis of Superparamagnetic Iron Oxide Nanoparticles (SPION). An aqueous suspension of SPION with an average particle size of 8−10 nm was prepared using controlled coprecipitation as reported before.[3] Briefly, 25 mL of 1 M $FeCl_3 \cdot 6H_2O$, 0.5 M $FeCl_2 \cdot 4H_2O$, and 0.4 M HCl was prepared as a source of iron by dissolving the respective chemicals in Milli-Q water under vigorous stirring. The coprecipitation of SPION was carried out in a reactor with high-speed mechanical stirring (2000 rpm) by adding the iron solution to 250 mL of 0.5 M NaOH, which was preheated to 80 °C before the coprecipitation reaction. N_2 was used during the reaction to prevent critical oxidation.[3] Black powder was collected by sedimentation with a help of an external magnetic field and washed several times with Milli-Q water until stable ferrofluid was obtained. Finally, the particles were redispersed in an aqueous solution by changing the pH to 11 with TMAOH (5 wt %).

Surface Modification of SPION with APTMS. The SPION suspension was prepared as described above. Afterward, 100 mg of SPION was washed several times with analytical grade methanol (99.5%) with the help of a strong magnet to remove the SPION from the residual water. The amount of APTMS required to coat 100 mg of SPION with a diameter of 8−10 nm was calculated, taking into account the surface area of single particles. A 3 mM APTMS solution was prepared in 100 mL of a toluene/methanol (1:1 v/v) mixture. APTMS in the toluene/methanol solution (200 μL) was added to the SPION suspension. The ferrofluid suspension was transferred into a three-necked flask with water-cooled condenser, temperature controller, and N_2 gas flow. The silanization was performed at 110 °C for 24 h under vigorous stirring. APTMS acts as a coupling agent, where silanization takes place on the particle surfaces bearing hydroxyl groups in the organic solvent. As a result, a three-dimensional polysiloxane network was formed. The silanization taking place during the reflux of APTMS results in the formation of an APTMS coating with a thickness of two or three molecular layers tightly cross-linked with a large surface density of amines. The powder was collected by applying an external magnetic field after the silanization process, washed with methanol several times, and finally redispersed in an aqueous medium.

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Chemisorption of LAA on the Surface of SPION. A 500-mg portion of uncoated SPION was washed with deionized water and mixed with 200 mL of 0.05 M LAA solution prepared in nitric acid (pH ≈ 2). The mixture was sonicated for 10 min and vigorously stirred for 5 h at room temperature (RT). The particles were recovered by applying an external magnetic field and washed several times with Milli-Q water.

Immobilization of Bovine Serum Albumin (BSA) on SPION: Double-Step Immobilization Process via 1-Ethyl-3-(3-Dimethylaminepropyl) Carbodiimide Hydrochloride (EDC) Activation. The first step of the immobilization process represents the possibility of direct immobilization of BSA on the surface of SPION to produce either partially or fully covered SPION. As a result, SPION were coated with a thin layer of BSA. The second step includes the coupling of SPION with a second layer of BSA through the activation of a cross-linking agent. The stock solution of 10 mg/mL BSA was prepared in a phosphate buffer solution (PBS, pH 7.3) and stored at 4 °C. The mixture of iron salts solutions and BSA in the ratio of 1:1 was added dropwise into alkaline solution under continuous vigorous stirring. The product was washed several times with Milli-Q water and then placed in a dialysis membrane against 5 L of Milli-Q water. The dialysis was carried out for 48−72 h and finally BSA-coated SPION were redispersed in a weak alkaline solution in the presence of NaN_3. In the second step, 13 mg of SPION partially coated with BSA was redispersed by sonication in 75 mL of PBS. Appropriate amounts of EDC, NHS, and BSA were dissolved in 25 mL of PBS. The mixture of preliminary BSA-coated SPION, BSA, EDC, and NHS was finally shaken for 24 h. The suspension was then dialyzed against 5 L of Milli-Q water at RT. Subsequently, the particles were collected by applying an external magnetic field and conserved in buffer solution at 4 °C.

Immobilization of BSA on APTMS-Modified SPION. BSA (5 mg), EDC (5 mg), and NHS (6 mg) were dissolved in a 3 mM phosphate buffer solution. This mixture was added to 10 mg of SPION modified with APTMS (as described above). The mixture was then sonicated for 10 min at 4 °C and shaken for 24 h at RT. The particles were collected under a strong external magnetic field and washed several times with PBS. The SPION were recovered by applying a magnetic field and redispersed in aqueous media.

Cell Culture. Infinity Telomerase Immortalized primary human fibroblasts (hTERT-BJ1, Clonetech Laboratories, Inc., Palo Alto, CA) were seeded onto glass coverslips ($D = 13$ mm) at a density of 1×10^4 cells per disk in 1 mL of complete medium. The medium used was 71% Dulbeccos Modified Eagles Medium (DMEM) (Sigma, UK), 17.5% Medium 199 (Sigma, UK), 9% fetal calf serum (FCS) (Life Technologies, UK), 1.6% 200 mM L-glutamine (Life Technologies, UK), and 0.9% 100 mM sodium pyruvate (Life Technologies, UK). The cells were incubated at 37 °C with a 5% CO_2 atmosphere for 24 h. At this time the cells were incubated in complete medium supplemented with 0.05 mg mL^{-1} BSA-coated SPION for a further 6−24 h. All control cells were cultured in the absence of any particles.

X-ray Diffraction (XRD). The crystal structure of the precipitated and coated powders was obtained by analyzing the XRD pattern of each sample recorded with a Philips PW 1830 diffractometer, using a monochromatized X-ray beam with nickel-filtered Cu Kα radiation. The lattice constants were estimated using CELREF, a crystal cell parameters refinement program for powder X-rays, using at least squares refinement method.

Transmission Electron Microscopy (TEM). The size and morphology of the particles were measured by a JEOL-2000EX TEM. The specimen was prepared by dispersing the SPION in Milli-Q water by using sonication for 3 min. After the dispersion, 1 mL of SPION suspension was centrifuged for 5 min at 14 000 rpm. A drop of well-dispersed supernatant was placed on a carbon-coated 200-mesh copper grid, followed by drying the sample under ambient conditions.

ζ-Potential Measurements. Surface charge measurements were performed with a Matec Applied Sciences (ESA-9800) system. The dynamic mobility, also called the acoustic pressure amplitude (ESA), was generated by colloidal particles in alternating high-frequency electric fields. A 250-mL portion of 1.0 M NaOH solution was placed in a Teflon sample container. Iron stock solution (1mL) was pumped by a MICROLAB 500 Digital buret and stabilized for 10 s before measurements were taken. The electro-acoustic signal, electrical conductivity, and temperature were recorded as a function of pH while using the titration cell. The stirring rate was kept at a maximum value.

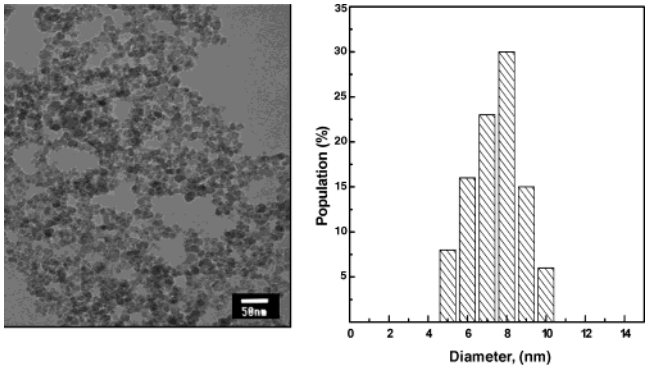


Figure 1. Transmission electron micrograph (left) and size distribution (right) of uncoated SPION.

Table 1. Particle Size Determined by TEM and Magnetization Analysis

sample	D_{TEM} (Å)	D_{MAG} (Å)
uncoated SPION	80	109
LAA-coated SPION	92	
APTMS-coated SPION	100	
BSA-coated SPION	110	
BSA−APTMS-coated SPION	120	108

Fourier Transform Infrared (FT-IR) Spectroscopy. FT-IR spectra were recordered by using a Nicolet Avatar 360 FT-IR spectrometer with the Smart DuraSamplIR Diamond ATR Accessory. Spectra were recordered with a resolution of 1 cm^{-1}.

Thermal Study. Thermogravimetric analysis (TGA) was performed for powder samples (∼10 mg) with a heating rate of 10 °C/min using a Perkin-Elmer TGA 7 thermogravimetric analyzer in synthetic air atmosphere up to 700 °C.

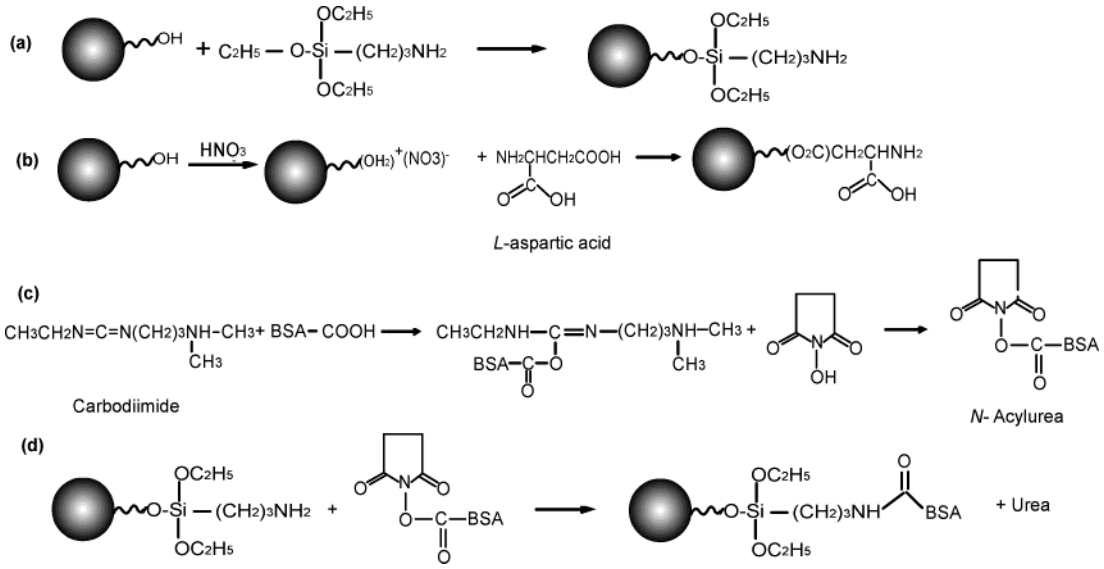
Magnetic Measurements. Magnetic properties were measured with a Princeton Applied Research vibrating sample magnetometer (VSM) at RT, with magnetic fields up to 15 Oe.

Chemical Analysis. The total concentration of iron was measured by using a SpectrAA-200. The amount of BSA immobilized on the surface of SPION was calculated by measuring the BSA concentration in solution before and after the reaction. The concentration of BSA was analyzed by using a BCA kit designed for protein analysis.

Light Microscopy Staining for Cell Morphology. The fibroblasts were cultured in the presence of SPION for 6 and 24 h. At each time point, the cells were washed in PBS and then fixed in 4% formaldehyde/PBS for 15 min at 37 °C. The cells were subsequently stained for 2 min in 0.5% Coomassie blue in a methanol/acetic acid aqueous solution, and washed with double distilled water to remove excess dye. Samples could then be observed by light microscopy, and digital images were captured using a Hamamatsu Argus 20 for image processing. Control cells were cultured in complete medium only.

Clathrin Immunofluorescence and Cytoskeletal Observation. After both 6 and 24 h culture with the SPION, cells were fixed in 4% formaldehyde/PBS with 1% sucrose at 37 °C for 15 min. The samples were then washed with PBS, and permeabilizing buffer was added at 4 °C for 5 min, followed by incubation at 37 °C for 5 min in 1% BSA/PBS. This was followed by the addition of anti-tubulin or anti-clathrin primary antibody (1:100 in 1% BSA/PBS, monoclonal anti-human raised in mouse (IgG1), Sigma, Poole, UK) for 1 h (37

Scheme 1. Schematic View of (a) Silanization Process, (b) Chemisorbtion Process, (c) Carbodiimide Activation, and (d) Immobilization of BSA on APTMS-modified SPION



°C). Simultaneously, rhodamine-conjugated phalloidin was added for the duration of this incubation (1:100 in 1% BSA/PBS, Molecular Probes, Eugene, OR). The samples were then washed in 0.5% Tween 20/PBS, and a secondary, biotin-conjugated antibody (1:50 in 1% BSA/PBS, monoclonal horse anti-mouse (IgG), Vector Laboratories, UK) was added for 1 h (37 °C), followed by more washing. A FITC-conjugated streptavidin third layer was added (1:50 in 1% BSA/PBS, Vector Laboratories, UK) at 4 °C for 30 min, and given a final wash. Samples were then observed by fluorescence microscopy (Vickers M17).

3. Results and Discussion

The phase identification of all samples was performed by XRD. The analysis of diffractograms (data not shown) indicated an inverse cubic spinel structure for uncoated SPION, and SPION coated with LAA, APTMS, BSA, and BSA−APTMS. The lattice constants are calculated to be a = 8.392, 8.389, 8.391, 8.388, and 8.390 Å for uncoated, and LAA-, APTMS-, BSA-, and BSA−APTMS-coated SPION, respectively. These values are close to the value of crystalline magnetite (a = 8.396 Å). The peak broadening for all samples is due to the small size of the crystallites. The broadening is slightly increased for APTMS- and BSA−APTMS-coated SPION.

The particle size and size distribution are calculated by an image analysis program from TEM images for all prepared samples on at least 500 particles. SPION prepared by controlled coprecipitation have almost spherical morphology with a particle size of 8 nm as shown in Figure 1. Generally, spherical shapes are formed because the nucleation rate per unit area is isotropic at the interface between the SPION. A roughly spherical or ellipsoidal-shaped particle with some irregularities has the characteristic crystalline order of magnetite. The size distribution is calculated using the following equation, based on a log-normal function:[14]

$$p(D) = \frac{1}{D\sigma_d\sqrt{2\pi}} \exp\left(-\frac{1}{2\sigma_d^2}\left(\ln\frac{D}{D_0}\right)^2\right) \qquad (1)$$

where σ_d is the diameter standard deviation and D_0 is the mean diameter. These results given for a volume-metric standard deviation of $\sigma_v = 3\sigma_d$ and a mean volume of $V_m = (\pi/6)\, D_m^3$. Table 1 shows the particle size for uncoated SPION calculated from TEM images with standard deviation of $\sigma \approx 0.2$.

The introduction of amine groups on the surface of SPION has been performed by two procedures depicted in Scheme 1: (a) silanization and (b) chemisorption processes. The silanization process is based on the covalent binding of APTMS to SPION. The silane coupling agent, usually called an organosilane, has the following structure: $R_xSiY_{(4-x)}$. Silicon is located at the center of the molecule and contains organic functional groups R (vinyl, amine, chloro, etc.) and other functional groups Y (methoxy, ethoxy, etc.). The inorganic groups, Y, of the molecule hydrolyze to silanol and form a metal hydroxide or siloxane bond with the inorganic material.[15,16] The organic group can be connected covalently with organic materials, such as protein, PEG, biomolecules, etc. The surface modification with LAA is based on the chemical adsorption process. LAA is an amine acid and used as a chelating agent of SPION. It has small intermediary molecules that can ensure the availability of complementary attachment of functional biomolecules. After LAA is chemisorbed onto the surface of SPION, the coupling between amine acid and the biomolecules can involve chemical bonds. With this procedure, a number of biomolecules can be grafted on the SPION. Scheme 1 (c) and (d) also demonstrate a schematic view of the BSA immobilization process. The BSA-coated SPION prepared by direct coprecipitation is based on the adsorption of protein followed by covalent binding via carbodiimide activation (Scheme 1 (c)). An attempt to immobilize BSA on APTMS-functionalized SPION has also been performed (Scheme 1 (d)).

A TEM image and the particle size distribution of BSA-coated SPION synthesized by direct precipitation

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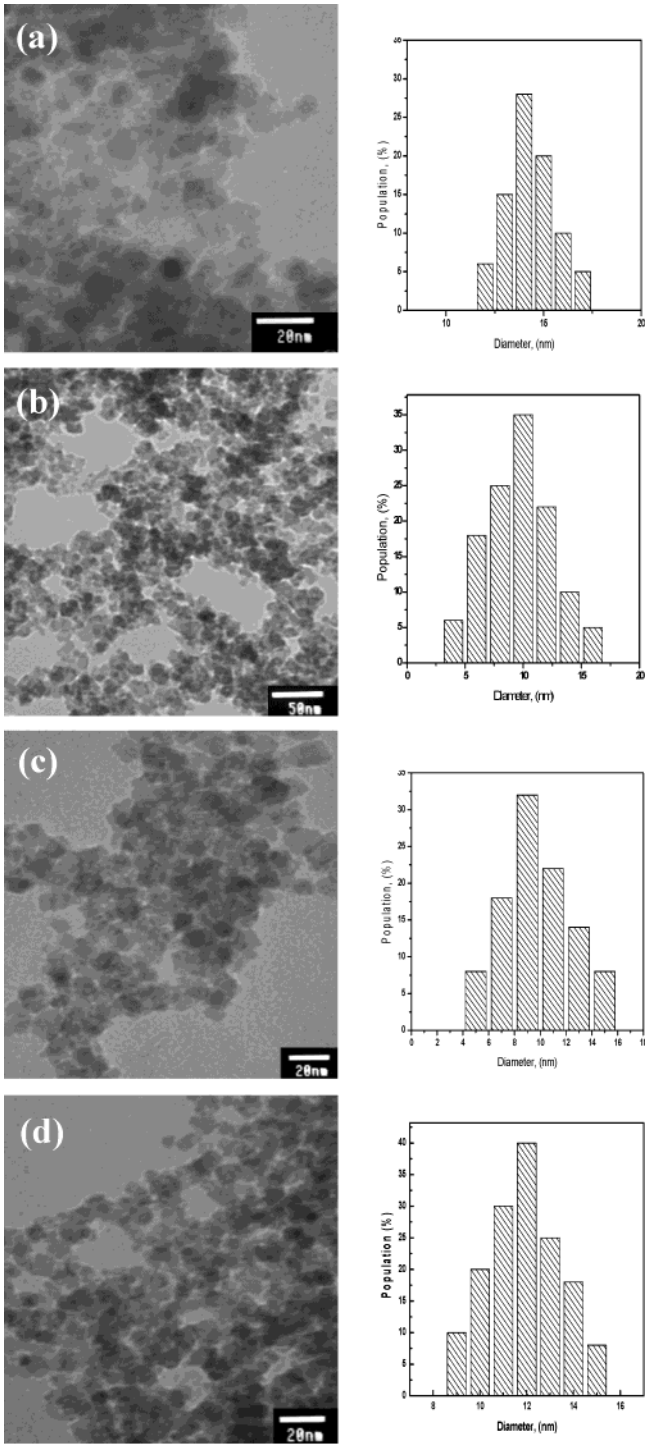

Figure 2. Transmission electron micrographs (left) and size distribution (right) of (a) BSA-, (b) LAA-, (c) APTMS-, (d) BSA−APTMS-coated SPION.

are shown in Figure 2 (a). The particle size increases slightly after the coating with BSA (Table 1). A scarcely visible thin layer of BSA can be observed on the surface of SPION due to the low electron density of BSA. The BSA-coated SPION are spherical with a mean diameter of 11 nm. Figure 2 (b), (c), and (d) display the TEM micrographs and particle size distribution for the SPION modified with APTMS, LAA, and BSA−APTMS layers, respectively. The majority of these particles also show a spherical shape. The volume-weighted size distribution can be calculated by a log-normal distribution with standard deviation of $\sigma \approx 0.2$, assuming the particles are a spherical shape (Table 1).

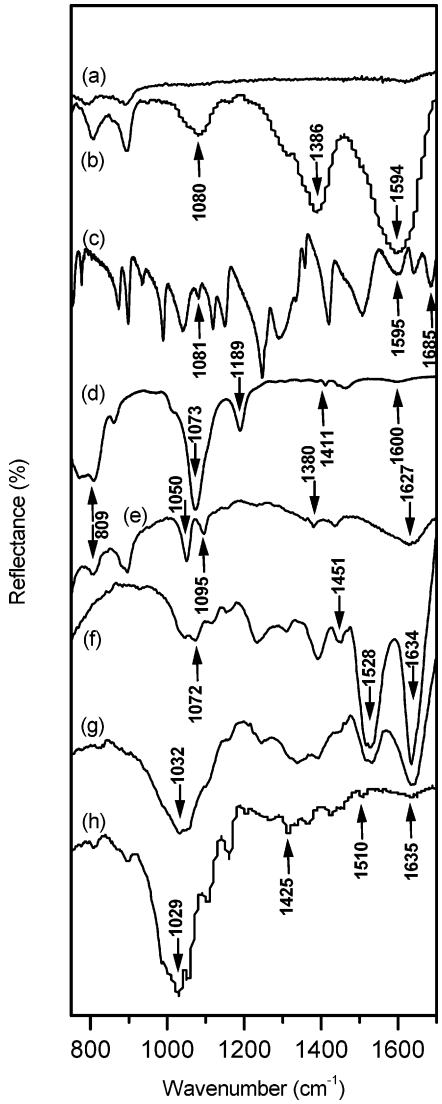

Figure 3. FT-IR spectra of (a) uncoated SPION, (b) LAA-coated SPION, (c) LAA, (d) APTMS, (e) APTMS-coated SPION, (f) BSA, (g) BSA-coated SPION, and (h) BSA−APTMS-coated SPION.

Coating and functionalization of SPION with different organic substances were monitored by FT-IR spectroscopy. Figure 3 shows the FT-IR spectra for uncoated SPION, and APTMS-, LAA-, BSA-, and BSA−APTMS-coated SPION, and for pure BSA, LAA, and APTMS. The method is especially convenient because SPION (magnetite) has no strong IR bands in the frequency region above 650 cm^{-1} (Figure 3, spectrum 1). The spectrum of LAA-coated SPION exhibits only a few absorption bands indicating the nature of interactions (Figure 3, spectrum 2). The bands that can be assigned to vibrations of zwitterions are observed at 1594 cm^{-1} (ν_a of dissociated COO^- group) and 1080 cm^{-1} (charged NH_3^+ in amine acids). They do not change their positions in comparison with those of the spectrum of pure amine acid (1595 and 1081 cm^{-1} correspondingly). Hence, neither α-carboxyl nor the amine group take part in the interaction with SPION. The new band appears at 1386 cm^{-1}. It can be assigned to symmetrical stretching of the dissociated carboxylic group attached to the radical of the amine acid. The spectrum of the initial

amine acid contains a band at 1685 cm^{-1} corresponding to stretching of C═O in the protonated carboxylic group. This frequency does not appear in the spectrum of LAA-coated SPION. Consequently, LAA interacts with surface of SPION through the second carboxylic group. This conclusion agrees with observations previously reported by others authors.[13] Covalent binding of APTMS to the surface of SPION is also confirmed by FT-IR. The spectrum of initial ATPMS exhibits a number of bands corresponding to the different vibrations of Si (Figure 3, spectrum 3). These bands can be observed in the spectrum of APTMS-coated SPION (Figure 3, spectrum 4). However, their frequencies are shifted to lower values, indicating strong bonding of Si. Particularly, the band at 1411 cm^{-1} ($Si-CH_2$ scissoring vibrations)[17] is shifted to 1380 cm^{-1}, the band at 1189 cm^{-1} (ν_a Si−O)[18] is shifted to 1095 cm^{-1}, and the band at 1073 cm^{-1} (assigned to vibrations of $-SiOCH_2-$ structure)[19] is shifted to 1050 cm^{-1}. The band at 809 cm^{-1} (ν_s Si−O)[18] is not shifted. The line 1600 cm^{-1} can be assigned to deformations of the $-NH_2$ group.[17,19,20] This line is shifted to 1627 cm^{-1}. This shift can be explained by the dissociation of the $SiO^-\cdots H\cdots NH_3^+$ intermolecular hydrogen bond structure that can be found in the initial APTMS.[21] Initial BSA (Figure 3, spectrum 5) exhibits a number of characteristic spectral bands. The most characteristic of them are at 1634 (amide I),[22] 1528 (amide II),[23,24] and 1451 (amide III) cm^{-1}. All these bands (that are characteristic for pure BSA) are present in the spectrum of BSA-coated SPION (Figure 3, spectrum 6), while their intensity ratios are altered due to the interactions with the surface. The spectrum of BSA−APTMS-coated SPION (Figure 3, spectrum 7) does not exhibit all the bands that are observed in the spectra of pure BSA and BSA-coated SPION. The characteristic bands 1635 (amide I),[23] 1510 (amide II),[23,24] and 1425 (amide III) cm^{-1} can be recognized. But they are very weak. In contrast, the band at 1029 cm^{-1} is much stronger than that of the pure BSA (1072 cm^{-1}). In the case of BSA−APTMS-coated SPION this band has intermediate intensity and position (1032 cm^{-1}) between those of pure BSA and BSA-coated SPION. This band can correspond to the structure responsible for binding the BSA to the surface of SPION.

ζ-potential measurements (Figure 4) of uncoated SPION (a), and BSA−APTMS- (b), APTMS- (c), and LAA-(d) coated SPION as a function of pH have been recordered to confirm the presence of amine groups and BSA on the surface of SPION. All samples are prepared at high pH (pH 13) and remained under stirring overnight before the pH titration started. According to Figure 4, an isoelectric point (IEP) for the uncoated SPION with 10 mM NaCl electrolyte concentration is

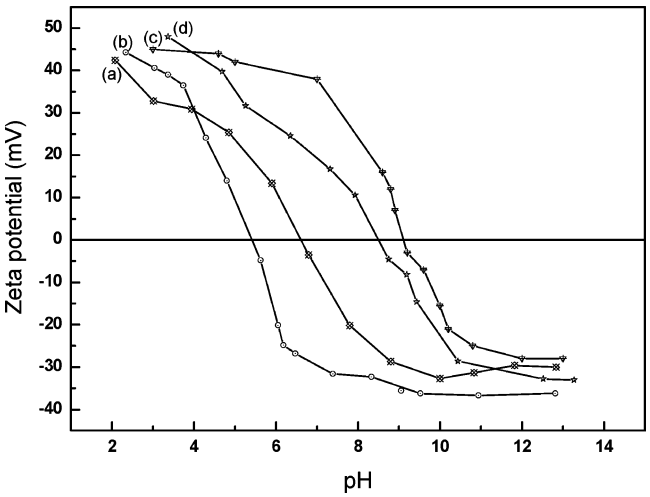

Figure 4. ζ-potential measurements of (a) uncoated SPION, (b) BSA−APTMS-, (c) APTMS-, and (d) LAA-coated SPION.

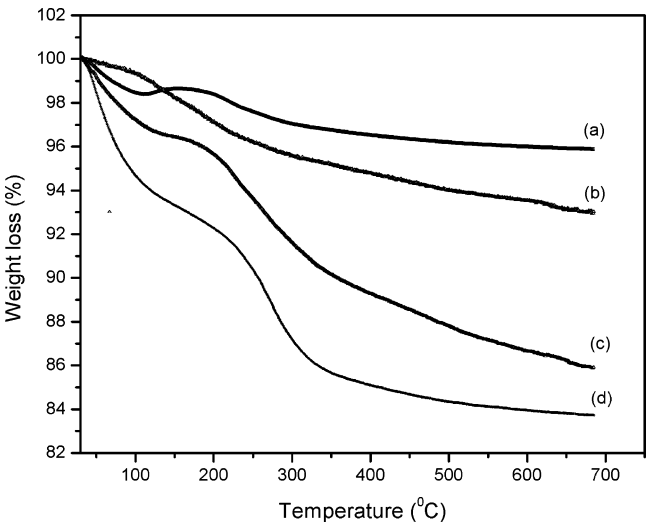

Figure 5. TGA curves of (a) uncoated SPION, (b) LAA-, (c) APTMS-, and (d) BSA−APTMS-coated SPION.

at $pH_{iep} = 6.6$. Even though applying relatively strong external force, it is difficult to break up the agglomerations formed when the surface charge density is low (pH range between 4 and 8). At the high and low pH range, ζ-potential shows the highest values because hydrodynamic force introduced by sonication should be strong enough to break the weak bonding. LAA- and APTMS-coated SPION show rather higher IEP of pH_{iep} 8.5 and 9.1, respectively, than that of uncoated SPION. The adsorption of amine groups changes the interfacial properties of the colloidal SPION in solution. The IEP is about pH_{iep} 5.4 for BSA−APTMS-coated SPION. This value is slightly higher than that for BSA (pH_{iep} 4.7).[25] Thus, by properly adjusting the pH we are able to specify the conditions necessary to obtain a stable ferrofluid in the matrix medium.

Thermal analysis has been performed to confirm the coating formation and estimate the binding efficiency on the surface of SPION. Figure 5 shows the weight loss for uncoated SPION, and APTMS-, LAA-, and BSA−APTMS-coated SPION. The major weight loss of un-

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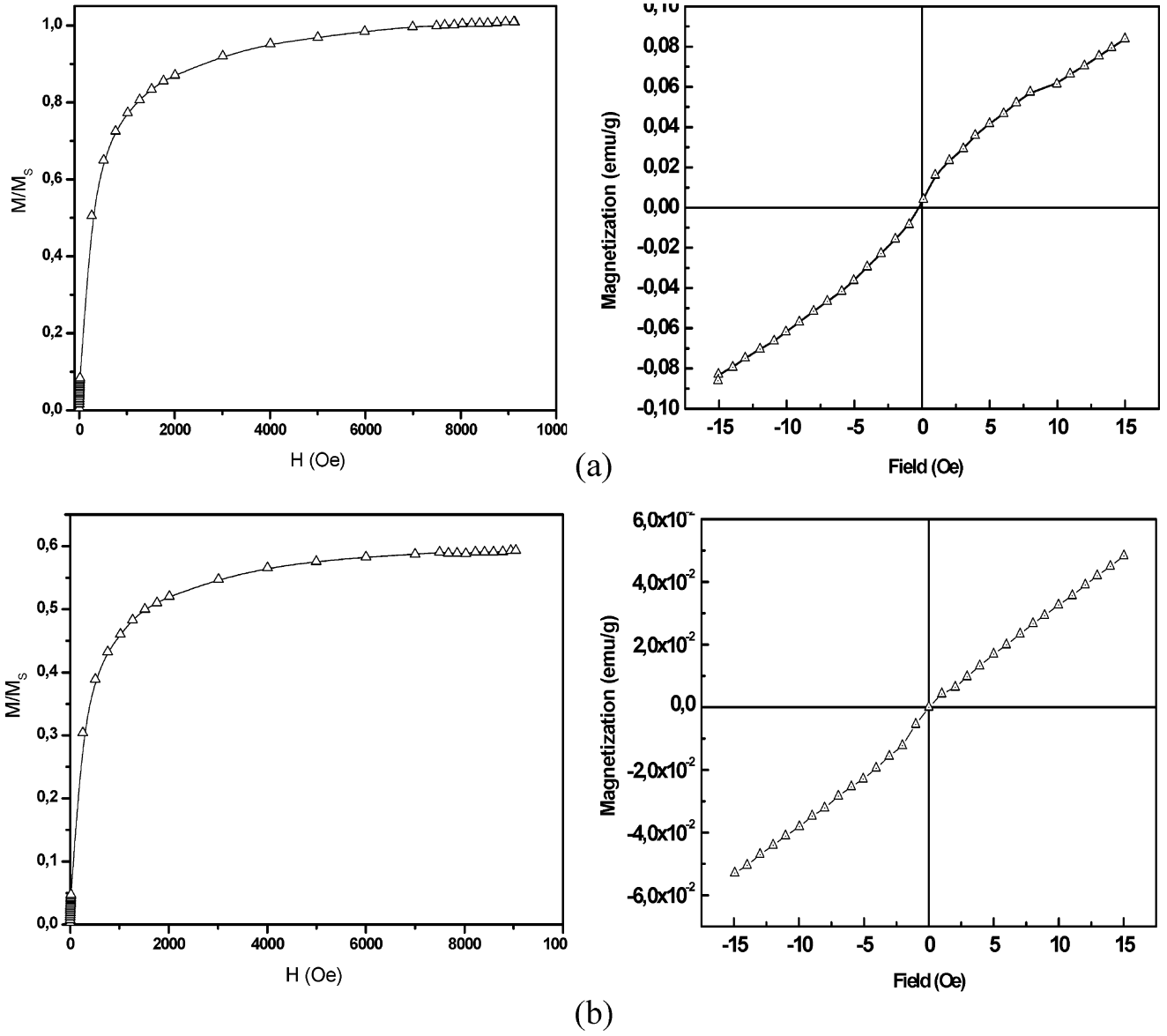

Figure 6. Relative magnetization M/M_S (left) and magnetization versus applied magnetic field (right) for (a) uncoated and (b) BSA−APTMS-coated SPION.

coated SPION, which corresponds to approximately 2%, is in two ranges. First ,weight loss is observed between the temperatures of 50−110 °C due to the evaporation of physically adsorbed water on the surface of SPION. The second range can be assigned to the dehydroxylation of lepidocrocite from the surface of SPION in the temperature range 200 to 300 °C. The dehydroxylation of lepidocrocite to maghemite is observed at 248 °C.[26] A small augmentation of weight can be seen between the temperatures of 120 and 210 °C for uncoated SPION, which can be assigned to the surface oxidation of the main phase of SPION, e.g., magnetite to maghemite in the synthetic air atmosphere. The weight loss of APTMS-modified SPION between 50 and 600 °C shows two different regimes: the first regime from 50 to 150 °C can be attributed to the evaporation of physically absorbed water on the surface, and second regime corresponds to the decomposition of APTMS. The major weight loss of LAA-coated SPION is about 7% in the temperature range between 120 and 350 °C due to the decomposition of adsorbate. This value is significantly lower than that of APTMS-coated SPION. The weight loss up to 150 °C for BSA−APTMS-coated SPION can be attributed to the evaporation of physically adsorbed water. The decomposition of BSA starts at approximately 150 °C, is moderate between 200 and 250 °C, and becomes significant at about 300 °C.

Figure 6 shows the relative magnetization curve as a function of an external magnetic field (H_{ext}) for the uncoated (a) and BSA−APTMS-coated SPION (b) recordered at RT. Relative magnetization M/M_S was calculated according to the Langevin function. The characteristic superparamagnetic behavior is indicated by zero coercivity and zero remanence on the magnetization curve. The saturation magnetization (M_S) of the uncoated sample is 76.9 emu/g at RT, which is smaller than that of bulk magnetite (92 emu/g).[27] The value of M_S may decrease due to the presence of the nonmagnetic

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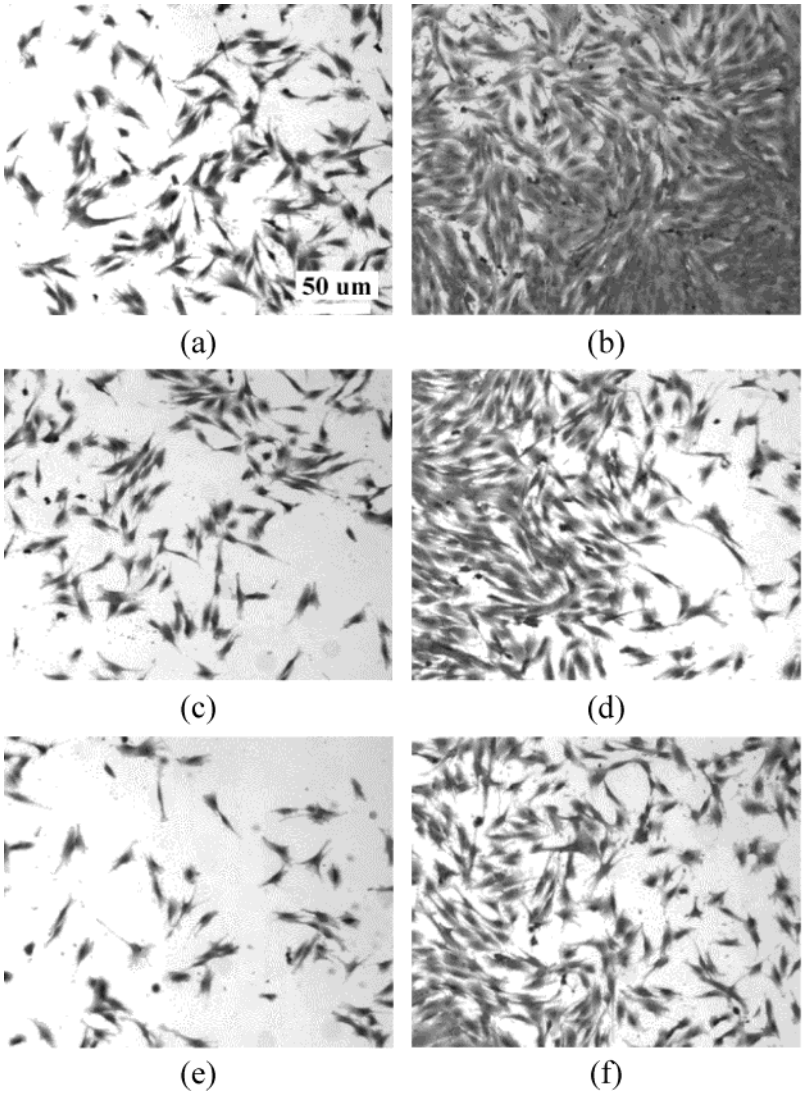

Figure 7. Coomassie images of (a and b) control cells after 6 and 24 h culture, (c and d) cells incubated with LAA-coated SPION, and (e and f) cells incubated with BSA−APTMS-coated SPION, respectively.

layer on the surface of SPION. According to the XRD and TGA data, the small amount of lepidocrocite phase, which is nonmagnetic, can coexist with uncoated SPION. The total magnetic moment can be decreased due to thermal fluctuations when the energy of small magnetic particles becomes comparable to thermal energy.[28] Therefore, the decrease of particle size and increase of the surface area causes decrease of M_s. The mean magnetic diameter can be calculated with Langevin function based on the magnetization data.[14]

$$D_{vm} = \left[\frac{18kT}{\pi M_s'}\sqrt{\frac{\chi}{3\rho M_s' H_0}}\right]^{1/3} \qquad (2)$$

where D_{vm} is the volume fraction median diameter, M_s is the saturation magnetization of the bulk material (in emu/cm^3), χ is the initial susceptibility, $1/H_0$ is the extrapolation of the abscissa of a plot of M versus $1/H$ at high field, k is the Boltzmann constant, T is the temperature, and ρ is the density of magnetite (5.18 g/cm^3). By assuming that SPION have a log-normal size distribution, the particle size calculated from eq 2 was found to be 10.9 nm. The M_s value is 47.6 emu/g for BSA−APTMS-coated SPION, which is nearly 40% lower than that for uncoated SPION (Figure 6 (b)). There are several approaches that can explain the reduction of the M_s for coated magnetic nanoparticles.[29−31] Currently, the presence of a nonmagnetic BSA layer on the surface of SPION or the excess of BSA can lead to a decrease of the M_s. Meanwhile, the SPION maintain their superparamagnetic properties after the immobilization of BSA. The magnetic particle size of BSA−APTMS-coated SPION is calculated according to the Langevin function (eq 2) and the particle size is found to be 10.8 nm. The binding efficiency can be estimated from the results of magnetic measurements. The value of magnetization can be reduced by the presence of shielding by the coating layer. If we assume that all particles are covered by BSA, the value of magnetization has to be about 30% less than that for uncoated SPION. In our case, we have a 40% difference in the value of M_s between uncoated

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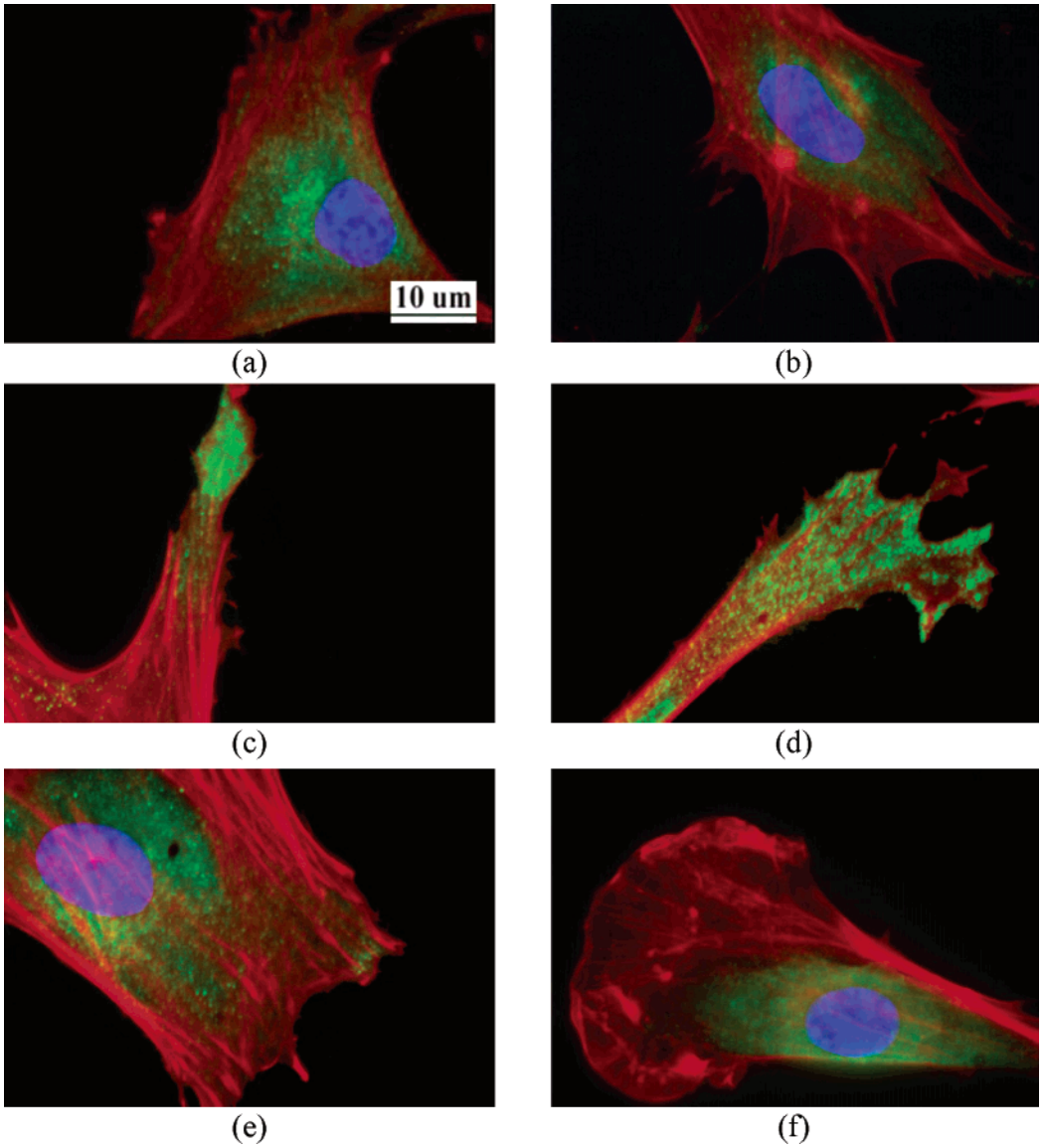

Figure 8. Fluorescent F-actin (red) and tubulin (green) images of (a and b) control cells after 6 and 24 h culture, (c and d) cells incubated with LAA-coated SPION, and (e and f) cells incubated with BSA−APTMS-coated SPION, respectively.

and BSA−APTMS-coated SPION. Because the presence of the excess BSA is observed, it can be expected that only 60% of BSA is bound to SPION according to the magnetic measurements. Two independent analytical methods have also been applied to check the binding of BSA. The concentration of BSA and total concentration of iron were determined before and after the immobilization process as well as in the unreacted fraction. For BSA−APTMS-coated SPION, the percentage of binding is found to be 90%, whereas this value is reduced to 76% for coprecipitated SPION in the presence of BSA.

Recently, uncoated SPION have been tested in vitro with human dermal fibroblast cells.[32] The results point to the cells' response to uncoated SPION with massive internalization of the particles. This process occurred through endocytosis and resulted in eventual cell death. The introduction of a biocompatible layer on the surface of SPION decreases the probability of cytotoxicity and demonstrates a further advantage for biomedical applications. Figure 7 shows the Coomassie images of control cells after (a) 6 and (b) 24 h culture, cells incubated with LAA-coated SPION for (c) 6 and (d) 24 h, and cells incubated with BSA−APTMS-coated SPION for (e) 6 and (f) 24 h. According to the results, there is an increase in cell numbers observed with an increase in culture time, suggesting that the cells are proliferating normally (with the control appearing to exhibit the highest number of cells). In addition, cells incubated with BSA-modified SPION have gross cell morphologies similar to those of control cells. This indicates that there is no adverse cell reaction in the presence of the BSA−APTMS-coated SPION. The information about cytoskeletal future and cell response is provided by fluorescent observation. Fluorescent F-actin (red) and tubulin (green) images of control cells after (a) 6 and (b) 24 h culture, cells incubated with LAA-coated SPION for (c) 6 and (d) 24 h, and cells incubated with BSA−APTMS-coated SPION for (e) 6 and (f) 24 h are shown in Figure 8. All cells appear well spread with defined cytoskeletal features at both 6 and 24 h. Cells incubated with the

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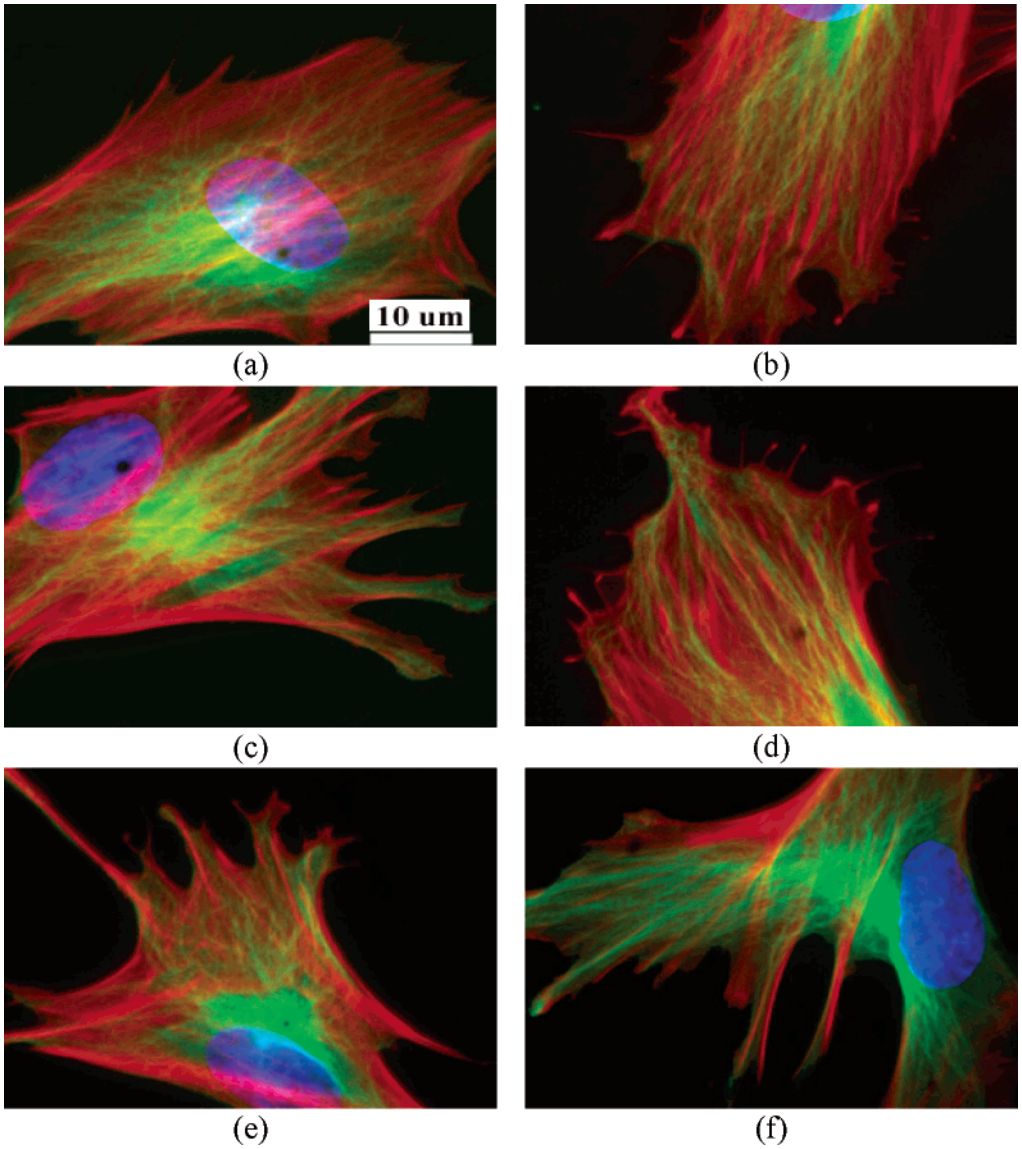

Figure 9. Fluorescent F-actin (red) and clathrin (green) images of (a and b) control cells after 6 and 24 h culture, (c and d) cells incubated with LAA-coated SPION, and (e and f) cells incubated with BSA−APTMS-coated SPION, respectively.

BSA−APTMS-coated SPION exhibit prominent stress fiber formation and tubulin clearly radiating out from the tubulin organizing center by the nucleus, exactly as the control cells. Figure 9 displays fluorescent F-actin (red) and clathrin (green) images of control after (a) 6 and (b) 24 h culture, cells incubated with LAA-coated SPION for (c) 6 and (d) 24 h, and cells incubated with BSA−APTMS-coated SPION for (e) 6 and (f) 24 h. The cells incubated with BSA−APTMS-coated SPION appeared to show background levels of clathrin similar to that of the control cells at both time points. A cell response similar to that of control cells is demonstrated with no adverse cell damage and no endocytosis. However, the cells incubated with the LAA-coated SPION demonstrated an increase in clathrin production, particularly at the cell filopodia. This suggests that the particles are partially being up-taken into the cell body via endocytosis.

4. Conclusions

The synthesis of SPION and its surface modification with amine and carboxylic groups, and immobilization of BSA have been studied. Uncoated SPION with a particle size of 8 nm and roughly spherical shape are prepared by controlled chemical coprecipitation. The surface modification of SPION with an amine group is achieved by using $-NH_2$-bearing APTMS producing a stable suspension at pH 7. Chemical adsorption process achieved for the surface modification of SPION with LAA resulted in the presence of a high population of amine and carboxylic groups on the surface of SPION. An attempt at direct immobilization of BSA is also performed during coprecipitation. Preliminary APTMS-modified SPION and SPION coprecipitated in the presence of BSA are successfully functionalized with a second layer of BSA. Mean particle diameters measured by TEM are 10 nm for APTMS, 9.2 nm for LAA, 11 nm for BSA, and 12 nm for BSA−APTMS-coated SPION. FT-IR results confirm the presence of amine group and BSA on the surface of SPION. According to the ζ-potential measurements, the IEP of APTMS- and LAA-coated SPION is found to be at pH_{iep} 9.1 and 8.5, respectively, which is in an agreement with the value reported for $-NH_2$ group. In the case of BSA−APTMS-

coated SPION, the IEP is determined to be at pH_{iep} 5.4. The coating yield was estimated from chemical and thermal analysis and magnetic measurements. BSA−APTMS-coated SPION show 90% binding efficiency. Magnetic properties of uncoated and BSA−APTMS-coated SPION have been studied at RT and showed superparamagnetic behavior with magnetic core sizes of 10.9 and 10.8 nm, respectively. The SPION coated with BSA retained superparamagnetic behavior with reduction of the M_s, which can be explained by the presence of the excess of BSA and nonmagnetic coating layer on the surface of SPION. The LAA- and BSA−APTMS-coated SPION were incubated with human fibroblast for 6 and 24 h for an in vitro cytotoxicity test. Although the LAA-coated SPION do appear to be endocytosed into the cell body, there is no cell damage caused after 24 h culture. In addition, as there is no cytoskeletal disorganization, with no evidence of vacuoles in the cell body,endocytosis is suggested to be only partial. The BSA−APTMS-coated SPION, however, demonstrate a cell response similar to that of control cells, with no adverse cell damage and no endocytosis. Therefore, these two materials can be evaluated for further medical applications.

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