One-step and high-density protein immobilization on epoxysilane-modified silica nanoparticles

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Silica nanoparticles are most commonly modified with amino-silanes, followed by post-modification activation for protein immobilization. In this work, epoxy-functionalized silica nanoparticles were prepared by modification with glycidyloxypropyl trimethoxysilane (GPTMS) for direct protein immobilization. Silica nanoparticles possessed an average size of 46 nm, but increased to 63 nm after GPTMS modification. Reaction time, reaction temperature and GPTMS content had no significant effect on particle size. Zeta potential of SiO₂ changed from -26mV to +38mV after modification. Fourier-transformed infrared spectroscopy revealed alkyl C—H bending and stretching bands at 2944 cm⁻¹, 1343 cm⁻¹ and 1465 cm⁻¹, respectively, for the modified nanoparticles. Fluorescein cadaverine was found to bind to GPTMS-modified SiO₂, but not to bare SiO₂, indicating the chemical reactivity of epoxy groups on the modified nanoparticle with amines. Finally, fluorescently labeled bovine serum albumin (BSA) was used as a model protein to investigate the capacity of epoxy-SiO₂ nanoparticles for protein immobilization. The results showed that more proteins were immobilized on the particle with longer reaction time, higher NaCl concentration, lower pH, and less GPTMS content. More importantly, proteins bound to epoxy-SiO₂ nanoparticle were highly stable. Under optimized reaction conditions, as much as 25 mg BSA/g nanoparticle was covalently attached to the nanoparticle. The epoxy silane modification of silica nanoparticles offers a reactive surface for one-step and high-density protein immobilization.

epoxysilane, silica nanoparticle, protein immobilization

In the past few years, nanomaterials of various shapes (particles, wires, rods, tubes, etc.) have been synthesized and characterized. They have been found to possess unique size-dependent properties in terms of quantum effect, specific surface area, electric conductivity, mechanical strength, and catalytic reactivity^[1]. Consequently, nanomaterials have been found wide applications in the field of biological analysis and detection, biological imaging and drug delivery^[2,3]. Among them, silica nanoparticles have been studied extensively due to their easy synthesis, readily available surface modification, good dispersibility and stability in water, and biocompatibility^[4]. Mesoporous silica nanoparticles can be employed as drug delivery vehicles for controlled release^[5]. Surface-coated silica nanoparticles act as a

carrier for multiple optical^[6,7] and electrochemical^[8] labels to enhance detection signal. Dye-doped silica nanoparticles are more resistant to photo-bleaching than fluorescent dye molecules and much less toxic than quantum dots. They therefore offer distinct advantages when applied to *in vivo* fluorescence imaging^[9,10]. Ruthenium tris(bipyridyl)-doped silica nanoparticles^[11–13] have been used as electrochemiluminescent labels to provide large quantities of Ru(bpy)₃²⁺ molecules for signal detection. Furthermore, silica coating on other

Received December 10, 2008; accepted February, 18, 2009

doi: 10.1007/s11434-009-0210-7

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Supported by the National Basic Research Program of China (Grant No. 2006CB403303) and the National Natural Science Foundation of China (Grant Nos. 20621703, and 20825519)

nanoparticle materials (core-shell structure) allows these materials to retain the desired magnetic or optical properties. In the mean time, the silica shell makes them more biocompatible and easier to be functionalized^[14–16].

For biomolecule immobilization, silica nanoparticles need to be functionalized by surface modification to present reactive groups to biomolecules. To date, the most commonly employed method for surface modification of silica nanoparticles is silanization. Aminosilanes are coupled to the silica surface, and the terminal amino group is activated by reacting with glutaraldehyde. Proteins or amino-modified DNAs can then be immobilized irreversibly on the activated particles via the covalent bond between the amino group on the biomolecule and the aldehyde^[17–19]. Alternatively, the aminosilane surface can be transformed into carboxylic acid by reacting with succinic anhydride, followed by NHS/EDC activation^[20,21]. However, both procedures require a post-modification activation step before biomolecule immobilization, which adds considerable time and complexity. In addition, glutaraldehyde content and reaction sequence need to be controlled carefully in order to minimize nanoparticle crosslinking and aggregation.

Epoxy-silane agents are classical compounds applied widely to a variety of polymer composite materials to enhance the stability and integrity of polymer/inorganic interfaces^[22,23]. Epoxy activated surfaces are also very attractive systems for covalent biomolecule immobilization. Epoxy is reactive under mild experimental conditions toward many nucleophiles on proteins including amine, hydroxyl and thiol groups. The chemical bond thus formed is very stable. Epoxy-activated surface is also stable under normal storage conditions. Therefore, epoxy-silane modification provides a chemically reactive surface for covalent protein immobilization without additional activation. Unfortunately, although epoxy-silanes have been used extensively for the modification and activation of glass slides, glass beads and other silica materials, to our knowledge, there has been no report on the use of epoxy-silane on silica nanoparticles.

The goal of the present work was to exploit epoxy-silane chemistry as a convenient and one-step method for the activation of silica nanoparticles and protein immobilization. Epoxy-silane-modified silica nanoparticles were fully characterized. Experimental conditions for surface modification and protein immobilization were investigated and optimized. It was found that as much as

25 mg bovine serum albumin was immobilized on 1g activated nanoparticle with high stability.

1 Experimental procedure

1.1 Chemicals

Fluorescein cadaverine was purchased from Invitrogen (Carlsbad, CA). Fluorescein isothiocyanate (FITC) was purchased from Amresco (Solon, OH), Biotinyl-N-hydroxy-succinimide (BT-NHS), Triton X-100 and glycidyloxypropyl trimethoxysilane (GPTMS) were from Sigma Aldrich (St. Louis, MO). Bovine serum albumin fraction V (BSA), mouse IgG and goat anti-mouse IgG were from Xijingke Corp (Beijing, China). Tetraethyl orthosilicate (TEOS) was from Beijing Chemical Reagent Corp. (Beijing, China). Other reagents are all of analytic purity. All buffers were prepared in high-purity water from a Millipore Milli-Q (Biocel) purification system.

FITC labeled BSA (FITC-BSA) was obtained by mixing BSA with FITC at a molar ratio of 10 FITC: 1 BSA in 0.1 mol/L sodium bicarbonate (pH 9.15) for 4 h at room temperature with stirring. After the reaction, the remaining free FITC was separated from the antibody by gel filtration on a D-SaltTM polyacrylamide desalting column from Pierce (Rockford, IL). The protein and FITC concentration of the labeled BSA were determined by absorbance at 280 nm and 495 nm respectively on a UNICO UV-2800 spectrophotometer (Shanghai, China). And the labeling ratio was determined to be 2 FITC per BSA. Biotinylated goat anti-mouse antibody (BT-Ab) was obtained according to the previously described method^[24]. The labeling ratio was 1.9 biotins per antibody. The labeled proteins were stored at 4°C in dark till use.

1.2 Preparation of various SiO₂ nanoparticles

Bare SiO₂ nanoparticles were prepared via modified reverse micro-emulsion method^[25]. In brief, 15 mL cyclohexane, 3.6 mL *n*-hexanol, 4 mL Triton X-100, and 1 mL deionized H₂O were mixed. Then 150 µL TEOS and 100 µL ammonia hydroxide were dropped into the micro-emulsion to react under stirring for 24 h at room temperature. The reaction mixture was added into acetone to break the micro-emulsion. Pellets were obtained by centrifugation at 12 000 rpm for 30 min at 4°C. Supernatant was discarded and pellets were washed with water and ethanol by centrifugation. The particles

were freeze dried in vacuum and stored at 4°C.

In the optimized procedure for silica nanoparticle modification, the nanoparticle prepared above was re-dispersed in dry toluene by sonication for 1 h. Then 1% (V/V) GPTMS was added into the nanoparticle solution. After reaction with shaking for different time (from 1 h to 24h) and at different temperatures (20°C, 37°C and 65°C), nanoparticles modified with epoxy groups were obtained after 12,000 rpm centrifuging for 30 min at 4°C. The pellets were washed sequentially with dry toluene, acetone and ethanol, then freeze dried in vacuum, and stored at 4°C.

Fluorescein cadaverine (NH₂-Fluo), a bifunctional chemical containing an amino group and a fluorescein, was reacted with epoxy SiO₂ to produce dye-coated nanoparticles. In the reaction, 0.5 mg epoxy nanoparticles and 649 µmol/L NH₂-Fluo dissolved in phosphate buffer (138 mmol/L NaCl, 2.7 mmol/L KCl, 8.1 mmol/L Na₂HPO₄, 1.1 mmol/L KH₂PO₄, pH 7.0, PBS) were incubated at 37°C, 240 rpm with shaking for 16 h. After incubation, nanoparticles were washed with washing buffer (PBS buffer with 2% Tween 20, PBST) and centrifuged at 4°C, 12,000 rpm, for 25 min. After washing for three times, the nanoparticles were resuspended in 0.1 mol/L PB buffer (0.1 mol/L Na₂HPO₄, 0.1 mol/L KH₂PO₄, pH 8.0, PB) and fluorescence intensity was measured on an LS55 luminescence spectrometer (Perkin Elmer, USA) excitation 498 nm, slit 5 nm, emission 525 nm, slit 5 nm, scan rate 600 nm/s).

In the optimized procedure for protein immobilization on epoxy-modified silica nanoparticles, 0.2 mg epoxy-SiO₂ and 3.4 mg/mL FITC-BSA dissolved in phosphate buffer containing different concentrations of NaCl were incubated for 24 h at 37°C, 240 rpm with shaking. After incubation, the nanoparticles were washed with PBST buffer and centrifuged at 4°C, 12,000 rpm for 25 min. The nanoparticles were resuspended in 0.1 mol/L PB buffer and fluorescence intensity was measured (excitation 494 nm, slit 5 nm, emission 525 nm, slit 5 nm, scan rate 600 nm/s).

1.3 Characterization of silica nanoparticles

Unmodified or epoxysilane-modified SiO₂ nanoparticles were re-dispersed in ethanol by sonication for 1 h. The particles were detected by transmission electron microscopy (TEM) (H-7500, Hitachi, Japan) for particle size and morphology.

For zeta potential measurement, the nanoparticles

were re-suspended in pure water after 1h sonication. The supernatants were injected into a Zetasizer 2000 zeta potential detector (Malvern Co., United Kingdom).

Fourier transformed infrared (FT-IR) spectra were measured on a Vector 22 FT-IR spectrometer (Brucker, Germany) using a KBr disc for sample preparation. Sixteen scans were collected at a resolution of 4 cm⁻¹.

2 Results and discussion

2.1 Epoxysilane modification of silica nanoparticles

It is well known that, compared with Stöber method^[26], silica nanoparticles prepared by the reverse micro-emulsion method have smaller size and better homogeneity. Therefore, reverse micro-emulsion method was employed in our work to synthesize SiO₂ nanoparticles, using a water-to-surfactant molar ratio (W/O value) of 8.15. TEM graph in Figure 1(a) shows that the synthesized nanoparticles were spherical in shape and uniform in size, with an average diameter of 46.2 ± 5.7 nm.

The surface of the particles was functionalized with epoxy groups by reacting with GPTMS for protein immobilization. As depicted in Figure 1(b), after only 1 h reaction, particle size increased significantly to 63.1±4.5 nm. But the shape remained spherical. Since the thickness of a GPTMS monolayer is reported to be 0.8 nm^[27], it can be inferred from the 17 nm increase in particle size that an equivalent of about 20 layers of GPTMS was formed on the surface of silica nanoparticles. Higher GPTMS concentration (5% and 10%) and longer reaction time up to 24 h did not increase the particle size any further (Figure 1(c)), suggesting that the surface silanization reaction was complete within 1h even with only 1% GPTMS. Three different reaction temperatures, 20°C, 37°C and 65°C, were investigated. Although average particle size did not change with the temperature, size distribution was slightly more uniform for particles produced at 65°C. Therefore, in the optimized procedure for silica nanoparticle modification, 1% (v/v) GPTMS was reacted with the particle at 65°C for 1 h.

Zeta potential is a function of surface charge, and is very sensitive to the chemical groups on the surface of a solid material. The zeta potential of both bare and modified silica nanoparticles was measured. Bare SiO₂ was found

Figure 1 TEM graphs of (a) bare SiO₂ nanoparticles; (b) epoxy-modified SiO₂ after 1 h reaction at 20°C; (c) epoxy-modified SiO₂ after 24 h reaction at 20°C; (d) epoxy-modified SiO₂ after 1 h reaction at 65°C. GPTMS concentration was 1% (v/v). Scale bar was 100 nm.

to have a potential of -26.27 ± 0.71 mV in pure water (pH 6.8), due to the negative charge of hydroxyl groups on the particle surface. The potential is similar to the values reported in other publications^[28,29]. After epoxysilane reaction, the nanoparticles turned into positive charge ($+38.07 \pm 0.55$ mV), indicating conversion of hydroxyl groups into other chemical functionalities.

The silica nanoparticles were characterized further by FT-IR. Figure 2 shows the FT-IR spectra of bare nanoparticles and the nanoparticles reacted with

GPTMS of different concentrations. Although the characteristic band of epoxy group at around 1150 cm^{-1} overlapped with strong absorption of the bare silica, alkyl C—H stretching vibration band at 2944 cm^{-1} and its bending vibration bands at 1343 cm^{-1} and 1465 cm^{-1} ^[30–34] were clearly visible in the spectra of the modified particle. The results indicate that epoxysilane was attached successfully to the surface of the silica nanoparticles. To investigate the chemical reactivity of the epoxy groups on the epoxysilane-modified silica nanoparticles, fluorescein cadaverine reacted with the nanoparticles. The fluorescent dye contains an amino group, and can be immobilized on the nanoparticle by forming a covalent bond with the epoxy group. As shown in Figure 3, after reacting with NH₂-Fluo, fluorescence intensity of bare silica nanoparticles was only slightly higher than the background. The slight increase is most likely due to some non-specifically bound dyes on the particle. By contrast, GPTMS-modified nanoparticles emitted approximately 20-fold more fluorescence after dye attachment. The results demonstrate that the epoxy functionalities on the modified SiO₂ nanoparticle were reactive toward amine groups, and could be used directly (without activation) for covalent protein immobilization, as described below.

2.2 Protein immobilization on epoxysilane-modified silica nanoparticles

Most proteins contain lysine residues which react readily with epoxy groups through the primary amine of the

Figure 2 FT-IR spectra of (a) bare silica nanoparticles; (b) silica nanoparticles reacted with 1 % GPTMS; (c) silica nanoparticles reacted with 10% GPTMS. The scale of wavenumber of graph is from 1700 cm^{-1} to 400 cm^{-1} (left) and from 2500 cm^{-1} to 4000 cm^{-1} (right), respectively.

Figure 3 Fluorescence intensity of various SiO₂ nanoparticles dispersed in 0.1 mol/L PB buffer. Excitation at 498 nm, emission at 525 nm. Error bar represents standard deviation ($n=3$).

amino acid. In our work, BSA was employed as a model protein to investigate protein immobilization on epoxy-functionalized silica nanoparticles. The protein was labeled with a fluorescent dye, FITC, for protein quantification on the particle. To immobilize FITC-BSA, the protein was incubated with GPTMS-modified nanoparticle for a specified time. The particle was then washed, and re-suspended in a phosphate buffer for fluorescence measurement. Figure 4 shows that the fluorescence intensity of FITC-BSA-immobilized silica nanoparticle increased steadily with incubation time. The signal did not reach a plateau even after 24h incubation, suggesting a slow reaction rate for protein immobilization.

Figure 4 Fluorescence intensity of FITC-BSA-immobilized epoxy-SiO₂ nanoparticle as a function of protein incubation time. Incubation was carried out in PBS buffer. Fluorescence was measured by dispersing the particles in 0.1 mol/L PB buffer. Excitation is at 494 nm, and emission at 525 nm. Error bar represents standard deviation ($n=3$).

It was found that salt had some dramatic impact on BSA immobilization on the epoxy nanoparticle. Figure 5 shows that as NaCl was added into the reaction buffer, the amount of BSA immobilized on the nanoparticle increased significantly. At the highest concentration investigated (1 mol/L NaCl), fluorescence signal increased almost 5-fold. It is well known epoxy groups are stable and not very reactive under mild experimental conditions. However, it has also been found that high concentrations of salt facilitate protein immobilization on epoxy-functionalized chromatographic supports^[35,36]. In the hypothesized mechanism, hydrophobic interactions are enhanced in the presence of high-concentration salt. Proteins are first adsorbed hydrophobically on the surface, followed by the formation of covalent bonds between nucleophiles of the protein and the epoxy groups on the surface. The salt effect observed in our experiment with epoxy-modified nanoparticles can also be interpreted by the same mechanism.

Figure 5 Fluorescence intensity of FITC-BSA-immobilized epoxy-SiO₂ nanoparticle as a function of NaCl concentration in protein immobilization buffer. FITC-BSA was dissolved in 10 mmol/L phosphate buffer (pH 7.0) containing different concentrations of NaCl. Fluorescence was measured by dispersing the particles in 0.1 mol/L PB buffer. Excitation is at 494 nm, and emission at 525 nm. Error bar represents standard deviation ($n=3$).

In the optimized procedure for protein immobilization, epoxy-modified silica nanoparticles were incubated with BSA in 10 mmol/L phosphate buffer with 1 mol/L NaCl (pH 7.0) at 37°C for 24 h. Under these experimental conditions, the amount of immobilized BSA correlated very well with its solution concentration until the concentration reached 3.4 mg/mL, as depicted in Figure 6. On bare silica nanoparticles, some FITC-BSA proteins were adsorbed non-specifically on the surface. The weakly

Figure 6 Amount of FITC-BSA immobilized on various silica nanoparticles as a function of protein concentration. The nanoparticles are bare SiO₂ (circle), bare SiO₂ washed with BSA (inverse triangle), epoxy SiO₂ (square), and epoxy SiO₂ washed with BSA (triangle).

bound proteins could be removed by washing in a BSA-containing buffer. By contrast, the proteins immobilized on the epoxy-functionalized nanoparticles were very stable, and could not be washed off with the same buffer. The strong binding reflects the covalent nature of the interaction between BSA and surface. At high protein

concentrations, as much as 25 mg BSA was immobilized on 1 g nanoparticles.

3 Conclusion

In this study, silica nanoparticles were functionalized with epoxysilane for direct protein immobilization. Silica nanoparticles prepared by reverse micro-emulsion were examined by TEM for particle size and size distribution. Zeta potential and FT-IR were employed to verify the modification of the nanoparticles. Chemical reactivity of the epoxy group on the modified particle was confirmed by its reaction with an amine-containing fluorescent dye. Fluorescently labeled BSA was used as a model protein to investigate experimental conditions for protein immobilization on the modified nanoparticles. Under the optimized reaction conditions, as much as 25 mg BSA/g nanoparticle was immobilized on the nanoparticle with high stability. The epoxy-functionalized silica nanoparticles are very stable under normal storage conditions, and are reactive toward many nucleophilic groups present in most proteins. They therefore offer an attractive material for one-step and high-density protein immobilization.

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