Surface Modification of Silica Nanoparticles to Reduce Aggregation and Nonspecific Binding

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In this article, a systematic study of the design and development of surface-modification schemes for silica nanoparticles is presented. The nanoparticle surface design involves an optimum balance of the use of inert and active surface functional groups to achieve minimal nanoparticle aggregation and reduce nanoparticle nonspecific binding. Silica nanoparticles were prepared in a water-in-oil microemulsion and subsequently surface modified via cohydrolysis with tetraethyl orthosilicate (TEOS) and various organosilane reagents. Nanoparticles with different functional groups, including carboxylate, amine, amine/phosphonate, poly(ethylene glycol), octadecyl, and carboxylate/octadecyl groups, were produced. Aggregation studies using SEM, dynamic light scattering, and zeta potential analysis indicate that severe aggregation among amine-modified silica nanoparticles can be reduced by adding inert functional groups, such as methyl phosphonate, to the surface. To determine the effect of various surface-modification schemes on nanoparticle nonspecific binding, the interaction between functionalized silica nanoparticles and a DNA chip was also studied using confocal imaging/fluorescence microscopy. Dye-doped silica nanoparticles functionalized with octadecyl and carboxylate groups showed minimal nonspecific binding. Using these surface-modification schemes, fluorescent dye-doped silica nanoparticles can be more readily conjugated with biomolecules and used as highly fluorescent, sensitive, and reproducible labels in bioanalytical applications.

Introduction

In the past few years, nanoparticle-based techniques have shown great promise in bioanalysis and biomedical applications, specifically in ultrahigh throughput screening, chip-based technology, multitarget detection systems, diagnostic screening, and in vitro and in vivo diagnosis inside intact biologic systems (e.g., tissues, blood, and single cells).[1−11] In microarray and microspot techniques, the spatial resolution of individual reactive sites on a chip is extremely important. At the same time, improved labeling and detection technologies are required to analyze smaller sample volumes and to measure samples on a limited solid-phase area. The use of fluorescent labels that facilitate high specific activity and have minimal nonspecific binding is a prerequisite before optimal miniaturization of microarrays can be realized.[12]

Among fluorescent labels, dye-doped silica nanoparticles show distinct advantages over quantum dots, fluorescent dyes, up-converting phosphors, and plasmon resonant particles because of their high quantum yield, photostability, water dispersibility, and ease of surface modification with different functional groups for subsequent bioconjugation due to well-known silica chemistry. In addition, the size and fluorescence of these silica nanoparticles can be tailor made according to the specific needs of the biological application.[13] However, the high sensitivity provided by the fluorescence signal enhancement, selectivity, and reproducibility of nanoparticle-based bioassays can be inhibited by the tendency of the silica nanoparticles to agglomerate irreversibly and cause nonspecific binding. These phenomena can be attributed to the large hydrodynamic radii (>10 nm) and large surface area of the nanoparticles, as compared to those of dye molecules. Following surface modification, excess active functional groups, which are capable of binding to or interacting with various chemical and biological species, can lead to false positive/negative signals. Thus, a crucial factor in the design of surface-modified nanoparticles for the subsequent immobilization of biomolecules is the controlled covalent attachment of desired functional groups onto the particle surface. To obtain successful and reproducible detection of biological targets using these fluorescent labels, the silica nanoparticles must be well dispersed in aqueous solution with minimal to no aggregation and nonspecific binding to biomolecules or substrates.

To date, very few studies have been carried out that focus on the surface functionality of the particles and its effect on the efficiency of nanoparticle label−bioanalyte interactions in a systematic manner.[14] Hence, the objective of this study was threefold: (1) to develop a simple preparation procedure for the introduction of different functional groups onto the silica nanoparticle surface, (2) to minimize nanoparticles aggregation and nonspecific binding by introducing an optimum balance of

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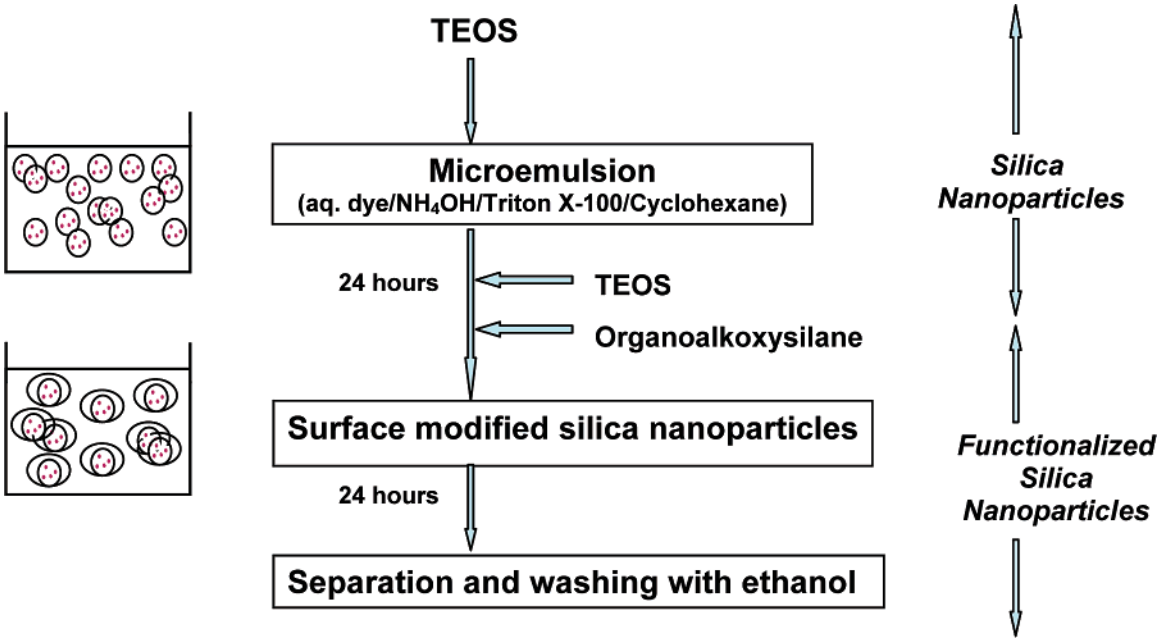


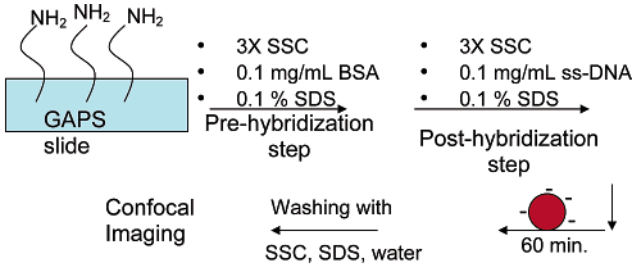
Figure 1. Procedure for the surface modification of dye-doped silica nanoparticles using a water-in-oil microemulsion. Following 24 h of synthesis, the surface of the silica nanoparticles is modified via cohydrolysis of tetraethyl orthosilicate and organosilane reagents in an additional 24 h postcoating step.

inert and active functional groups, and (3) to explore the mechanism of nanoparticles aggregation or flocculation prevention/reduction induced by the addition of inert functional groups along with active functional groups. To demonstrate the utility of the surface-modification strategy, fluorescent dye-doped silica nanoparticles, modified with inert alkyl groups and active carboxyl groups, were tested on amine-modified glass slides to simulate experimental conditions used in fluorescence-based DNA chip analysis.

Experimental Section

Materials. Unless otherwise noted, all reagent-grade chemicals were used as received, and Millipore water was used in the preparation of all aqueous solutions. Triton X-100 was purchased from Sigma-Aldrich (St. Louis, MO). Tetraethyl orthosilicate (TEOS), *n*-heptane, cyclohexane, hexanol, aqueous ammonia solution (NH_4OH, 71 wt % water, 29 wt % ammonia), and tris(2,2′-bipyridyl)dichlororuthenium(II) hexahydrate (RuBpy) were obtained from Aldrich Chemical (Milwaukee, WI). Organosilanes 3-(trihydroxysilyl)-propylmethylphosphonate (THPMP), 3-(aminopropyl)triethoxysilane (APTS), and octadecyltriethoxysilane were purchased from Aldrich (Milwaukee, WI), and carboxyethylsilanetriol, sodium salt (CTES, 25 wt % in water) was purchased from Gelest (Tullytown, PA). γ-Aminopropylsilane slides were purchased from Corning (Acton, MA). Saline sodium citrate (SSC) was purchased from Fisher chemicals (Fairlawn, NJ). Bovine serum albumin (BSA) and sodium dodecyl sulfate (SDS) were obtained from Sigma-Aldrich (St. Louis, MO). Sheared salmon sperm DNA was purchased from Eppendorf (Westbury, NY).

Preparation of Silica Nanoparticles. Silica nanoparticles were synthesized using a water-in-oil (W/O) or reverse microemulsion method. The microemulsion consisted of a mixture of 1.77 g of Triton X-100, 1.6 mL of hexanol, 7.5 mL of cyclohexane, 80 μL of a 0.1 M aqueous dye solution, 400 μL of water, and 100 μL of aqueous ammonia that was stirred for 30 min at room temperature, and then 100 μL of TEOS was added. The aqueous ammonia served as both a reactant (H_2O) and a catalyst (NH_3) for the hydrolysis of TEOS. The mixture was allowed to stir for 24 h, followed by the addition of appropriate ratios of TEOS and organosilanes for particle post-coating and surface modification. The mixture was further reacted for 24 h, and the silica particles were released from the microemulsion by the addition of ethanol. The particles were separated from the reaction mixture by centrifugation at 4000 rpm for 15−30 min and washed two times with ethanol and one time with water. Figure 1 shows a schematic diagram of this procedure.



GAPS= Gamma-aminopropylsilane; SSC = Saline sodium citrate; SDS = Sodium dodecyl sulfate; BSA = Bovine serum albumin; ss-DNA = Salmon sperm DNA

Figure 2. Schematic diagram of a nonspecific binding experiment using dye-doped silica nanoparticles and γ-aminopropylsilane-modified glass slides (GAPS).

Nonspecific Binding Study. Rubpy dye-doped silica nanoparticles modified with different functional groups such as amine (NH_2), carboxylate (COOH), octadecyl (C-18), and poly(ethylene glycol) (PEG) were used to study the effect of surface modification on the degree of nanoparticle nonspecific binding to γ-aminopropylsilane (GAPS)-modified glass slides. The experimental conditions and procedures used were similar to those for a typical gene chip analysis using fluorescent dyes, as described in the literature.[17] For gene or DNA chip analysis, a GAPS slide was incubated in 3x saline sodium citrate (SSC), 0.1 mg/mL BSA, and 0.1% SDS for 30 min. The slide was then treated with a blocking buffer containing 3x SSC, 0.1% SDS, and 0.1 mg/mL sonicated salmon sperm DNA, with shaking for 30 min, and was then dried in compressed air. An aliquot of 2 mL of 1 mg/mL dispersed nanoparticles in 0.1 M PBS buffer, pH 7.4, was deposited onto the glass slide, allowed to react for 60 min, and then washed several times in SSC and SDS solutions. The slides were imaged using a confocal microscope (Fluoview 500 scanning

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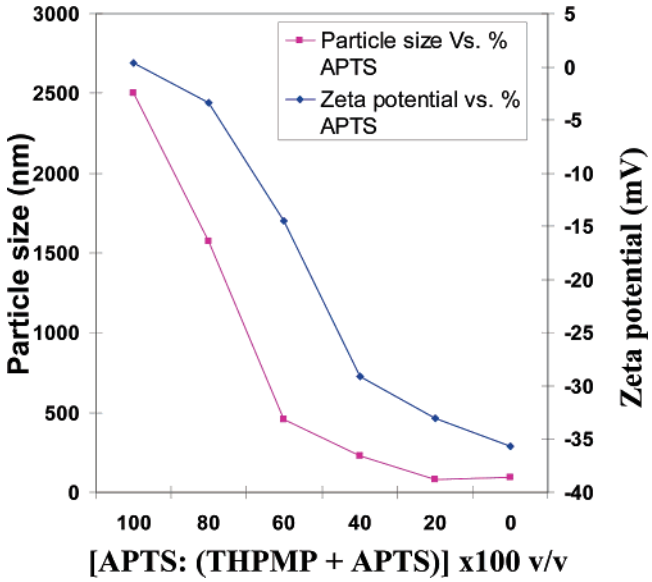

Figure 3. Effect of amine (3-(aminopropyl)trimethoxysilane (APTS)) and phosphonate (3-(trihydroxysilyl)propylmethylphosphonate (THPMP)) functionality on the silica nanoparticle surface charge and hydrodynamic size. The overall surface charge, as measured by its zeta potential (mV), and average hydrodynamic particle size (nm), as measured by DLS, become more negative and smaller, respectively, as the ratio of amine to phosphonate groups on the surface of silica nanoparticles decreases (i.e., the nanoparticles have more electrostatic repulsion and are more highly dispersed as the number of phosphonate groups is increased).

unit on an Olympus IX81). Figure 2 shows a schematic diagram of this procedure.

Characterization. The fluorescent dye-doped silica nanoparticles were characterized with respect to particle size, degree of aggregation, overall surface charge, and fluorescence properties. The samples were imaged on a Hitachi S-4000 FE-SEM to assess the particle size and shape. To prepare the samples for SEM studies, silica particles were dispersed in water, and the resulting suspension was vortexed and sonicated for 2 min. A drop (~1−10 μL) of the silica nanoparticles suspension was then placed on a piece of a microglass slide and dried overnight in a desiccator.

The mean diameter and standard deviation of the nanoparticles were determined by dynamic light scattering using a BI 90 particle sizer (Brookhaven Instruments Corp., Holtsville, NY). The particle size was analyzed using a dilute suspension of particles in Millipore water.

The zeta potential or overall surface charge of each nanoparticle sample in solution (~1 mg/mLin Millipore water) was determined using a Zeta Plus zeta potential analyzer (Brookhaven Instruments Corp. Holtsville, NY).

Fluorescence measurements were conducted on a Fluorolog Tau-3 spectrofluorometer (Jobin Yvon Spex Instruments, S. A. Inc., Edison, NJ) to verify the attachment of amine groups on the surface of the silica nanparticles. Silica nanoparticles were added to 2 mL of Millipore water and sonicated for 5 min. The 2 mL aliquot was diluted in 1 mL of water, and 300 μL of a 5 mM fluorescamine solution in methanol was added. The mixture was then reacted on a vortex mixer for 3 min at room temperature. The fluorescence of the fluorescamine solution, indicative of surface amine groups, was measured using an excitation wavelength of 420 nm.

Results and Discussion

Surface Modification of Fluorescent Dye-Doped Silica Nanoparticles. The synthesis and surface modification of silica nanoparticles is very simple, and the particle size and polydis-

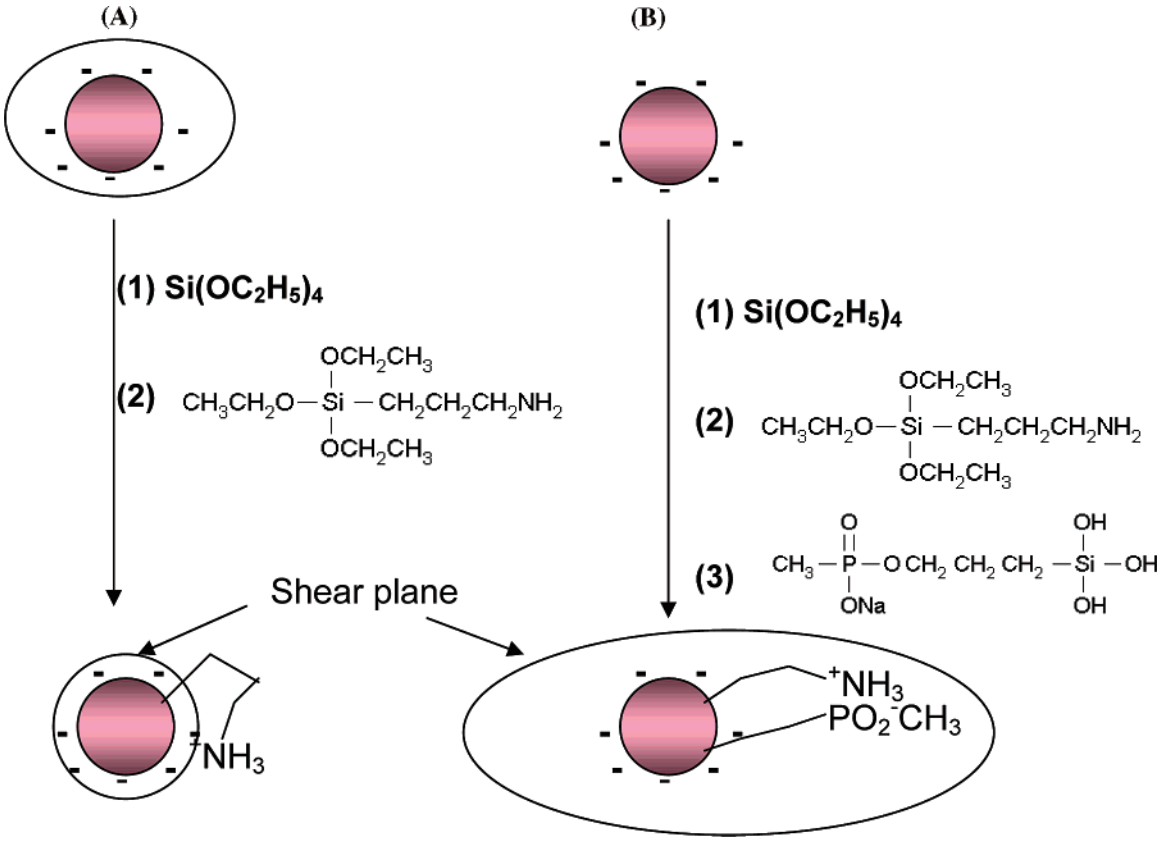

Figure 4. Schematic diagram showing the mechanism by which the back bonding of amine-modified silica nanoparticles is reduced by the addition of methyl phosphonate groups to the silica nanoparticle surface. (A) Back bonding of the NH_2 functional group to silanols on the particle surface. (B) Elimination of back bonding due to interactions between the amine and phosphonate groups on the particle surface, thus increasing the shear plane and electrostatic repulsion of the particles.

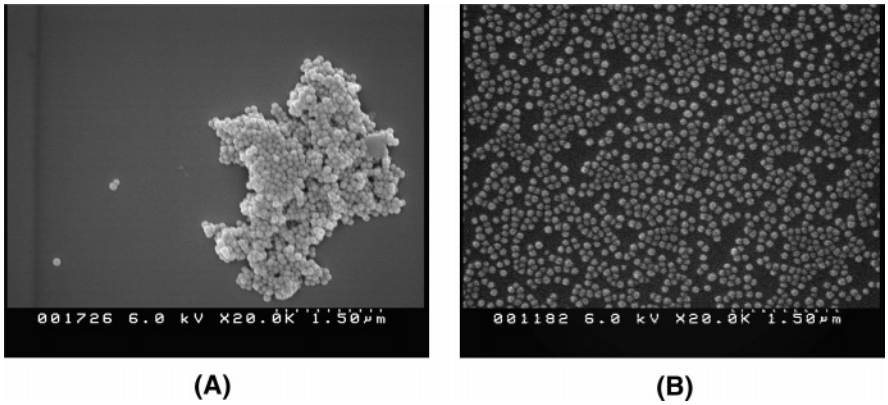

Figure 5. SEM images of amine-modified dye-doped silica nanoparticles. (A) Amine-modified particles. (B) Amine/phosphonate-modified particles. The overall charge and concentration of functional groups on the surface of the nanoparticles greatly affect the tendency of the nanoparticles to aggregate.

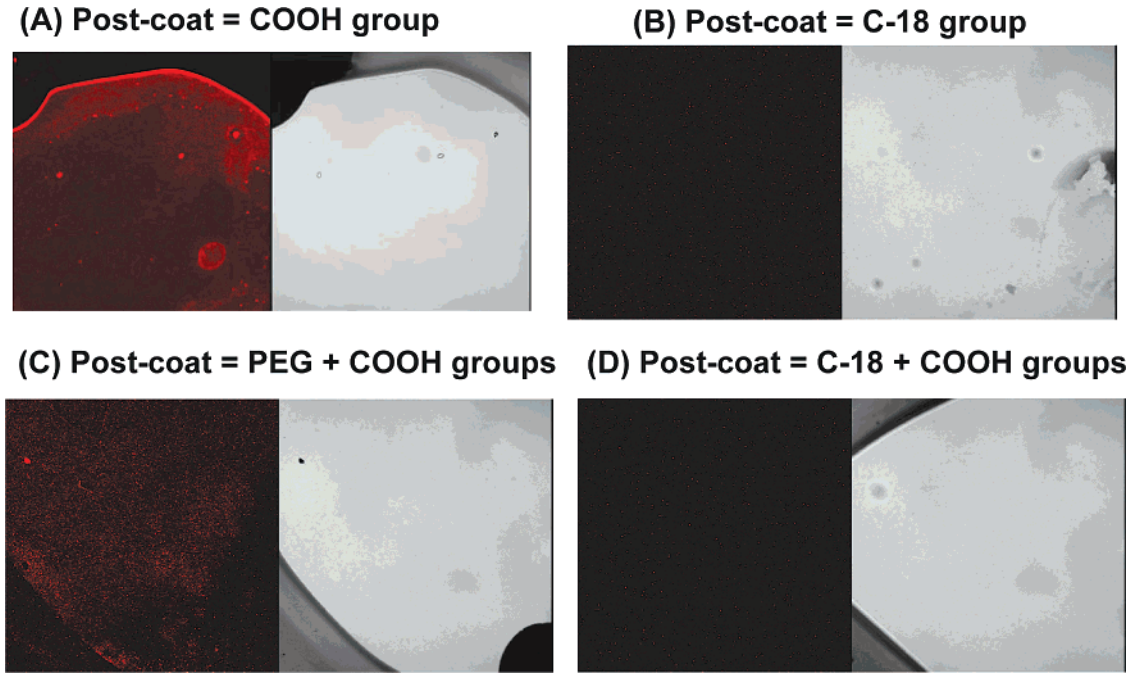

Figure 6. Nonspecific binding study of surface-modified dye-doped silica nanoparticles and γ-aminopropylsilane (GAPS) slides using confocal microscopy. (A) Silica nanoparticles functionalized with the carboxylate group. (B) Silica nanoparticles functionalized with the octadecyl group. (C) Silica nanoparticles functionalized with poly(ethylene glycol) and carboxylate groups. (D) Silica nanoparticles functionalized with octadecyl and carboxylate groups. Minimal nonspecific binding is observed with the octadecyl/carboxylate-modified RuBpy-doped silica nanoparticles.

persity can be easily controlled by tuning the microemulsion properties as has been demonstrated in previous studies.[13] In the first step, primary fluorescent dye-doped silica nanoparticles are formed inside the water-in-oil microemulsion. The second step involves the introduction of the desired functional group(s) onto the surface of the primary dye-doped silica nanoparticles by condensation of tetraethyl orthosilicate (TEOS) and organosilane reagents containing the active functional groups. In preliminary experiments, it was determined that the addition of TEOS along with the functionalized organosilane is necessary during the second step; the absence of tetraethyl orthosilicate leads to no surface modification. Furthermore, the time interval between the addition of TEOS and the organosilane reagent(s) in the second post-coating step needs to be at least 20 to 30 min. This time interval is important because otherwise most of the active functional groups would be buried inside the particles because the condensation reaction of the organosilane is faster than that of tetraethyl orthosilicate. This observation was found to be in agreement with the work done by Deng et al.,[15] who performed a systematic study of the effect of the addition of organosilanes at different stages on silica particle growth in microemulsions.

Aggregation Studies of Fluorescent Silica Nanoparticles. The effect of varying the volume ratio of amine (3-(aminopropyl)-triethoxysilane) to the phosphonate group (3-(trihydroxysilyl)-propylmethylphosphonate) on the particle size and zeta potential value is shown in Figure 3. When high concentrations of amine groups were added to the surface, nanoparticle agglomeration (as shown by the measured hydrodynamic particle size) was very high, and the zeta potential value was very low The presence of amine groups on the surface was confirmed using the fluorescamine test[16] for particles prepared under the same condition but without dye molecules. The results showed a strong fluorescence peak at 455 nm in all cases.

As the inert functional group, methyl phosphonate, was added to the surface, the zeta potential value became more highly negative, and the particle size decreased because of strong

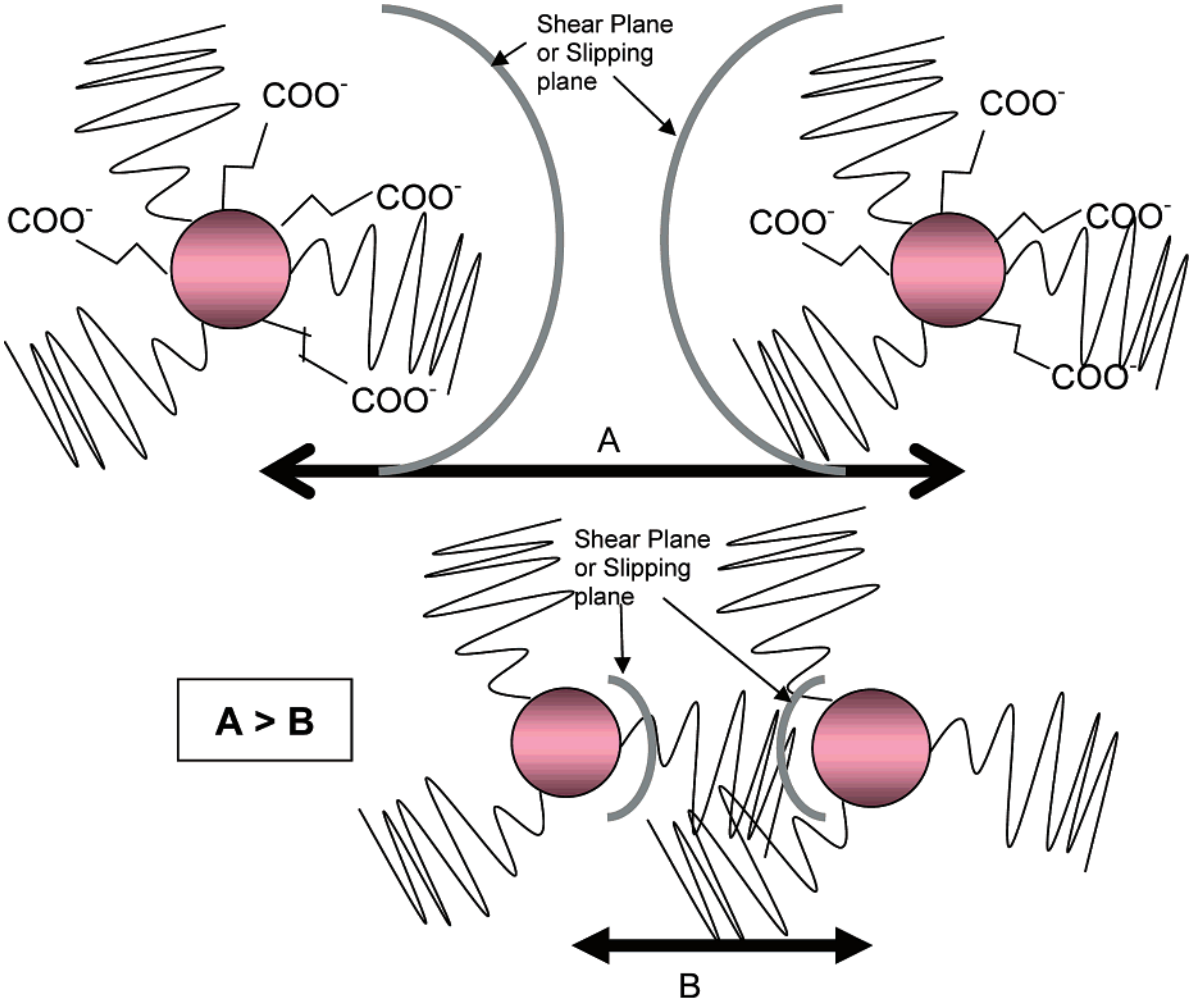

Figure 7. Schematic diagram showing the mechanism of electrostatic repulsion or steric hindrance-based stabilization for the prevention of hydrophobic octadecyl-modified silica nanoparticle agglomeration. When combined with carboxylate functionality, the octadecyl groups on the nanoparticle surface have an increased shear or slipping plane due to a more electrostatic and steric environment.

electrostatic repulsion forces between nanoparticles. It should be noted here that during the surface-modification step a fixed concentration of tetraethyl orthosilicate was added in addition to the varying concentrations of 3-(aminopropyl)triethoxysilane and 3-(trihydroxysilyl)propylmethylphosphonate. However, for simplicity, the graph in Figure 3 is labeled as a function of the volume ratio of 3-(aminopropyl)triethoxysilane to 3-(trihydroxysilyl)propylmethylphosphonate.

The nanoparticles prepared under these conditions were found to be stable for more than 8 months in aqueous solution. This observation can be explained by considering the pK_a values of amine, phosphonate, and silica groups on the surface of nanoparticles dispersed in water, which are 9.0, 2.0, and 7.0, respectively. At physiological pH 7.4, the amine groups have a positive charge, and the methylphosphonate and silica groups have negative charges. The amine-modified silica nanoparticles can back bond to surface silanol groups, as shown in Figure 4A. Hence, the overall charge on the surface is very low, as shown by the low zeta potential value, and the particles tend to aggregate because there is no driving force on the surface of the nanoparticles to keep them apart. As methylphosphonate groups are introduced onto the surface, most of the amino groups on the silica surface interact with methylphosphonate groups as shown in Figure 4B, preventing back bonding. Consequently, the nanoparticles were highly dispersed as indicated by the high zeta potential value (−35 mV) and hydrodynamic particle size (115 nm) that is more representative of the actual size of the nanoparticles. (The silica nanoparticles were 80−100 nm in diameter, as determined by TEM; the data are not shown.) This result was further confirmed using SEM as shown in Figure 5. The amine-modified silica nanoparticles were highly agglomerated, whereas the amine- and phosphonate-modified silica nanoparticles remained highly dispersed.

Nonspecific Binding Studies of Fluorescent Silica Nanoparticles with GAPS Glass Slides. In gene chip analysis, the nonspecific adsorption of nanoparticles onto the substrate is the major source of false positive/negative signals. Because of their very small size and high surface area, modified and bioconjugated nanoparticles contain large numbers of functional groups, some of which are bound to the DNA/oligonucleotides of interest and others that are not. Blocking buffers are used to inactivate unreacted functional groups but are not 100% effective. Thus, these active functional groups can facilitate nanoparticle aggregation and/or substrate/sample nonspecific binding. The goal of this experiment was to show that the nonspecific binding between nanoparticles and amine-modified glass slides can be minimized when inert and active functional groups are attached to the nanoparticles. The experimental conditions used were similar to those for gene chip analysis using fluorescent dye molecules.

Four batches of RuBpy-doped silica nanoparticles, differing in surface properties, were prepared. The nanoparticles were

post-coated and modified with the following functional groups: (1) carboxylate, (2) octadecyl, (3) a combination of poly(ethylene glycol) (PEG, MW 5000)/carboxylate, and (4) a combination of octadecyl/carboxylate. Figure 6 shows the confocal microscope images of the amine-modified glass slides reacted with the four types of particles. The carboxylate-functionalized and PEG/carboxylate-functionalized nanoparticles showed strong and weak fluorescence signals, respectively, indicating nonspecific binding. Octadecyl and octadecyl/carboxylate-functionalized nanoparticles showed minimal nonspecific binding. However, the average hydrodynamic particle size, as determined by dynamic light scattering, for octadecyl-modified nanoparticles was 888 nm, indicating a strong degree of agglomeration, whereas the octadecyl/carboxylate-functionalized nanoparticles had a hydrodynamic diameter size of 140 nm in 0.1 M PBS buffer, pH 7.4. This observation can be explained by considering the mechanism shown in Figure 7. The silica nanoparticles functionalized with only octadecyl groups have a much smaller shear or slippage plane, and the octadecyl tails extending from the particle surface are beyond the shear plane. Hence, there is a strong hydrophobic interaction between two nanoparticles, leading to agglomeration. The introduction of a carboxylate-containing silane with three methyl groups increases the shear plane boundary, and the octadecyl chains lie within the shear plane, resulting in more stable suspensions. On the basis of these results, the carboxylate/octadecyl-modified fluorescent dye-doped silica nanoparticles can be conjugated with probe DNA for ultrasensitive DNA detection on the GAPS slide.

Summary and Conclusions

A water-in-oil microemulsion-based surface modification method has been used to successfully prepare uniform fluorescent dye-doped silica nanoparticles of the desired size and surface functionality at room temperature. Colloid stability studies, based on the particle size and zeta potential, indicate that the addition of appropriate ratios of inert functional groups (e.g., methyl phosphonate (Typo)) to active functional groups (e.g., amino groups) to the surface of silica nanoparticles results in a highly negative zeta potential, which is necessary to keep the particles well dispersed and at the same time enable amine-based bioconjugation. Nonspecific binding studies, using confocal imaging of amine-modified glass slides with fluorescent silica nanoparticles, indicate that silica nanoparticles modified with a combination of carboxylate and octadecyl groups have less agglomeration and nonspecific binding to the glass slide as compared to particles having only carboxylate, octadecyl, or PEG groups on the nanoparticle surface. Using these surface-modification schemes and similar strategies, fluorescent dye-doped silica nanoparticles can be more readily conjugated with biomolecules and used as highly fluorescent, sensitive, and reproducible labels in DNA chip analysis and various other types of bioanalytical applications.

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