Published in final edited form as:

Langmuir. 2006 April 25; 22(9): 4357–4362.

Surface modification of silica nanoparticles to reduce aggregation and non-specific binding

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Abstract

In this paper, 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 non-specific binding. Silica nanoparticles were prepared in a water-in-oil microemulsion and subsequently surface modified via co-hydrolysis with tetraethylorthosilicate (TEOS) and various organosilane reagents. Nanoparticles with different functional groups, including carboxylate, amine, amine/phosphonate, polyethylene 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 non-specific 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 non-specific 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 ultra-high throughput screening, chip-based technology, multi-target 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, 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

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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 nanoparticles based bioassays can be inhibited by the tendency of the silica nanoparticles to agglomerate irreversibly and cause non-specific binding. These phenomena can be attributed to the large hydrodynamic radii (> 10 nm) and large surface area of the nanoparticles, as compared to dye molecules. Following surface modification, an excess of active functional groups, which are capable of binding to or interacting with various other chemical and biological species, can lead to false positive/negative signals. Thus, a crucial factor in the design of surface modified nanoparticles for 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 non-specific 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 non-specific binding by introducing an optimum balance of 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

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. Lois, MO). Tetraethylorthosilicate (TEOS), n-heptane, cyclohexane, hexanol, aqueous ammonia solution (NH₄OH, 71 wt % water, 29 wt % ammonia), and tris(2,2'-bipyridyl)dichlororuthenium (II) hexahydrate (RuBpy), were obtained from Aldrich Chemical (Milwaukee, WI). The organosilanes 3-(trihydroxysilyl)propylmethylphosphonate (THPMP), 3-aminopropyltriethoxysilane (APTS), octadecyltriethoxysilane, were purchased from Aldrich (Milwaukee, WI), and carboxyethylsilanetriol, sodium salt (CTES, 25 wt. % in water) was purchased from Gelest (Tullytown, PA). Gamma-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. Lois, 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 grams of Triton X-100, 1.6 mL of hexanol, 7.5 mL of cyclohexane, 80 μ L of 0.1 M aqueous dye solution, 400 μ L of water, and 100 μ L of aqueous ammonia that was stirred for 30 minutes at room temperature, and then 100 μ L of TEOS was added. The aqueous ammonia served as both as a reactant (H₂O) and a catalyst (NH₃) for the hydrolysis of TEOS. The mixture was allowed to stir for 24 hours, 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 hours 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 minutes and washed two times with ethanol and one time with water. Figure 1 shows a schematic diagram of this procedure.

Non-specific binding study

Rubpy dye-doped silica nanoparticles modified with different functional groups such as amine (NH_2), carboxylate (COOH), octadecyl (C-18), and polyethylene glycol (PEG) were used to study the effect of surface modification on the degree of nanoparticle non-specific binding to gamma-aminopropylsilane (GAPS)-modified glass slides. The experimental conditions and procedures used were similar to that 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 $3\times$ saline sodium citrate (SSC), 0.1 mg/mL BSA, and 0.1 % SDS for 30 minutes. The slide was then treated with a blocking buffer containing $3\times$ SSC, 0.1% SDS, and 0.1 mg/mL sonicated salmon sperm DNA, with shaking for 30 minutes, and dried in compressed air. An aliquot of 2 mL of 1 mg/mL dispersed nanoparticles in 0.1 M PBS buffer, pH 7.4 were deposited onto the glass slide, allowed to react for 60 minutes, and then washed several times in SSC and SDS solution. The slides were imaged using a confocal microscope (Fluoview 500 scanning 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 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 minutes. A drop ($\sim 1\text{--}10\ \mu\text{L}$) of the silica nanoparticles suspension was then placed on a piece of micro glass 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 ($\sim 1\text{ mg/mL}$ in 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 nanoparticles. Silica nanoparticles were added to 2 mL millipore water and sonicated for 5 minutes. The 2 mL aliquot was diluted in 1 mL water, and 300 μL , 5 mM fluorescamine solution in methanol was added. The mixture was then reacted on a vortex mixer for 3 minutes 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 polydispersity 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) on the surface of the primary dye-doped silica nanoparticles by condensation of tetraethylorthosilicate (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; absence of tetraethylorthosilicate leads to no surface modification. Further, 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 minutes. This time interval is important because otherwise most of the active functional groups would be buried inside the particles, as the condensation reaction of the organosilane is faster than that of tetraethylorthosilicate. 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-aminopropyltriethoxysilane) to 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 test¹⁶ for particles prepared under same condition but without dye molecules. The results showed a strong fluorescence peak at 455 nm in all cases.

As the amount of inert functional group, methyl phosphonate, was added to the surface, the zeta potential value became more highly negative and the particle size decreased due to strong electrostatic repulsion forces between nanoparticles. It should be noted here that during the surface modification step, a fixed concentration of tetraethylorthosilicate was added in addition to the varying concentrations of 3-aminopropyltriethoxysilane and 3-(trihydroxysilyl)propylmethylphosphonate. However, for simplicity, the graph in Figure 3 is labeled as a function of the volume ratio of 3-aminopropyltriethoxysilane and 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 form back-bonding to surface silanol groups, as shown in Figure 4(A). 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 on the surface, most of the amino groups on the silica surface interact with methylphosphonate groups as shown in Figure 4(B), 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) more representative of the actual size of the nanoparticles. (The silica nanoparticles were 80–100 nm in diameter, as determined by TEM; the data is 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.

Non-specific binding studies of fluorescent silica nanoparticles with GAPS glass slides

In gene chip analysis, non-specific adsorption of nanoparticles onto the substrate is the major source of false positive/negative signal. Due to 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 percent effective. Thus, these active functional groups can facilitate nanoparticle aggregation and/or substrate/sample non-specific binding. The goal of this experiment was to show that the non-specific 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 that 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 polyethylene 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 signal, respectively, indicating non-specific binding. Octadecyl and octadecyl/carboxylate-functionalized nanoparticles showed minimal non-specific binding. However, the hydrodynamic particle size, as determined by dynamic light scattering, for octadecyl-modified nanoparticles had an average hydrodynamic particle size of 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. Based on 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 desired size and surface functionality at room temperature. Colloid stability studies, based on particle sizing and zeta potential, indicate that the addition of appropriate ratios of inert functional groups (e.g. methyl phosphonate) 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. Non-specific 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 non-specific binding to the glass slide as compared to particles having only either carboxylate, octadecyl or PEG groups on the nanoparticles 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.

Acknowledgements

This work was partially supported by a NSF NIRT grant, by a NIH grant and by a Packard Foundation Science and Technology Award.

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Figure 1. Procedure for surface modification of dye-doped silica nanoparticles using a water-in-oil microemulsion

Following 24 hour synthesis, the surface of the silica nanoparticles are modified via co-hydrolysis of tetraethylorthosilicate and organosilane reagent(s) in an additional 24 hour post-coating step.

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 non-specific binding experiment using dye-doped silica nanoparticles and gamma-aminopropylsilane-modified glass slides (GAPS).

Figure 3. Effect of amine (3-aminopropyltrimethoxysilane (APTS)) and phosphonate (3-(trihydroxysilyl)propylmethylphosphonate (THPMP)) functionality on 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, becomes more negative and smaller, respectively, as the ratio of amine to phosphonate group on the surface of silica nanoparticles decreases (i.e., the nanoparticles have more electrostatic repulsion and are more highly dispersed as the amount of phosphonate group is increased).

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 on the silica nanoparticle surface

(A) Back-bonding of 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.

(A) Post-coat = COOH group **(B) Post-coat = C-18 group** **(C) Post-coat = PEG + COOH groups** **(D) Post-coat = C-18 + COOH groups**

Figure 6. Nonspecific binding study of surface modified dye-doped silica nanoparticles and gamma-aminopropylsilane (GAPS) slides using confocal microscopy

(A) Silica nanoparticles functionalized with carboxylate group. (B) Silica nanoparticles functionalized with octadecyl group. (C) Silica nanoparticles functionalized with polyethylene glycol and carboxylate groups. (D) Silica nanoparticles functionalized with octadecyl and carboxylate groups. Minimal non-specific binding is observed with the octadecyl/carboxylate modified RuBpy-doped silica nanoparticles.

Figure 7. Schematic diagram showing 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.