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Facile Functionalization of Ag@SiO₂ Core-Shell Metal Enhanced Fluorescence Nanoparticles for Cell Labeling

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Abstract

We describe a versatile approach for functionalizing core-shell Ag@SiO₂ nanoparticles for live-cell imaging. The approach uses physical adsorption and does not need covalent linkage to synthesize antibody-based labels. The surface orientation is not controlled in this approach, but the signal enhancement is strong and consistent. Antibodies were then attached using a non-covalent process that takes advantage of biotin-avidin affinity. Metal-enhanced nanoparticles doped with rhodamine B were used as the luminescent reporter. The enhancement of rhodamine B was between 2.7–6.8 times. We demonstrated labeling of CD19+ Ramos B lymphocytes and CD4+ HuT 78 T lymphocytes using anti-CD19 and anti-CD4 nanocomposite labels, respectively. This physical adsorption process can accommodate a variety of fluorophore types, and has broad potential in bioanalytical and biosensing applications.

Keywords

Metal-Enhanced Fluorescence; core-shell Ag@SiO₂ nanoparticle; rhodamine B; single molecule spectroscopy; cell labeling

Introduction

Metal-enhanced fluorescence (MEF) has emerged as a powerful method to improve fluorophore performance. Enhanced fluorescence systems hold promise to increase the sensitivity of many fluorescence techniques. In addition, metal-enhanced optical effects can potentially open new avenues of spectroscopic applications. In recent years, the pioneering work of Lakowicz and co-workers [1] has led to several metal-fluorophore systems for fluorescence enhancement. Both metal island films [2] and core-shell nanoparticles [3] have been developed for MEF. Nanoparticle-based approaches are particularly attractive; as mobile labels can be produced for flow measurements, cell labeling, and other applications [4–10].

MEF nanoparticles using silica shells have been developed in recent years as efficient luminescent reporters [11–14]. The silica shell maintains an optimum distance between the fluorophores and the metal core to produce efficient fluorescence enhancement [15–16]. Silica also provides a convenient surface for chemical coupling to produce different fluorescent probes and labels. Since Ag@SiO₂ nanoparticles—silver metal cores with silica sol-gel shells—are amenable to silane modification, Ag@SiO₂ nanoparticles can be covalently conjugated to antibodies and biomarkers. Covalent linkage is irreversible in most cases, but requires multiple steps and carefully tuned chemistry. However, it is also possible to take advantage of other approaches to attach fluorophores to the nanocomposites. Table 1

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shows a comparison of covalent and noncovalent bioconjugation methods for silica surface nanoparticle functionalization. Compared to covalent methods, noncovalent conjugation of nanoparticle is straightforward and non-toxic, although is not as stable over long time frames (weeks) as covalent conjugation method [17–19].

In previous work, we have developed simple conjugation steps to functionalize glass and silica surfaces and microchannels with antibodies and aptamers for cell capture [20]. This approach is noncovalent, conducted at room temperature in aqueous buffers, and results in stable surface coatings (Figure 1). Compared to the work done by Lakowicz and co-workers [1], which was focus on fabrication of Ag@SiO₂ fluorescent nanoparticles, we are studying the bio-application of core-shell nanoparticle surface functionalization. We want to illustrate that Ag@SiO₂ fluorescent nanoparticles can be used as cell labels. To achieve this goal, we used a straightforward method to functionalize the nanoparticle surface with antibodies. We have translated this surface coating approach to noncovalently functionalize MEF nanoparticles with antibodies to use as specific cell labels. In this work we use this sandwich conjugation approach to attach anti-human CD19 or anti-human CD4 antibodies to MEF nanoparticles for cell labeling. We demonstrate specific labeling with cell fluorescence that is markedly higher than control labels.

Experimental

Materials

Silver nitrite and ammonium hydroxide were purchased from Acros Organics (Pittsburg, PA, USA). Rhodamine B, bovine serum albumin, biotin labeled bovine serum albumin, sodium chloride and ethanol were purchased from Sigma-Aldrich (Milwaukee, WI, USA). Neutravidin was obtained from Pierce and biotinylated mouse anti-human CD71 and anti-CD4 were purchased from eBioscience. Trisodium citrate and tetraethyl orthosilicate (TEOS) were purchased from Alfa Aesar (Ward Hill, MA, USA). All solutions and samples were freshly prepared prior to each experiment.

Preparation of Ag@SiO₂ Nanoparticles

To prepare the nanoparticles (Figure 1), 9 mg of silver nitrite was dissolved in 49 mL DI water and heated to boiling while stirring [1]. 10 mg of trisodium citrate dissolved in 1 mL DI water was then added and the mixture was kept boiling and stirring for 30 minutes until the solution became green-brown in color. After cooling to room temperature, the solution was centrifuged at 500 rpm for 1 hour to remove large particles and was then dispersed in 200 mL ethanol. Afterward, 4 mL of ammonium hydroxide (28–30%) was added to adjust the pH to ~9. At this point, a sol-gel coating was fabricated to cover the Ag nanoparticles with SiO₂. To generate the sol-gel layer, 22.5 μ L of TEOS in 10 mL ethanol was added to the silver colloid solution at a rate of 2.5 mL/h under stirring. The solution was then kept overnight to form the core-shell Ag@SiO₂ nanoparticle. Finally, the nanoparticle solution was centrifuged at 3500 rpm for 30 minutes three times and resuspended in ethanol for future use.

Preparation of Rhodamine B labeled Ag@SiO₂ Nanocomposites

A solution of Ag@SiO₂ nanoparticles was centrifuged for 30 minutes at 3500 rpm (2800 g-force), and the pellet was resuspended in 40 μ L of 200 μ M rhodamine B in ethanol and incubated for 2 hours. After incubation, the mixture was centrifuged and washed with 1 mL DI water twice. The final pellet was dispersed in 1 mL DI water and used for fluorescence analysis.

Preparation of Antibody-Conjugated Rhodamine B Ag@SiO₂ Nanocomposites

The rhodamine B Ag@SiO₂ nanoparticle solution was centrifuged as discussed above. The resulting pellet was then resuspended in 100 μ L biotin-conjugated albumin (1 mg mL⁻¹ albumin in 10 mM HEPES buffer, pH 7.2). The solution was incubated for 2 hours to allow the biotinylated albumin to adhere to the SiO₂ surface [20]. Afterwards BSA-coated nanoparticles were incubated with neutravidin, and finally with biotinylated mouse anti-human CD71 (Human Transferrin Receptor). Since nanoparticle concentration is difficult to measure directly, each batch of labels should be titrated against a standard number of cells.

Synthesis of Nanobubbles from Fluorescent Ag@SiO₂ Nanocomposites

Nanobubble controls were synthesized by adding sodium chloride to etch silver cores. To prepare nanoparticle controls, 48 mg of sodium chloride was added to 500 μ L of a fluorescent Ag@SiO₂ nanocomposite solution described previously. The final concentration of sodium chloride was 1.5 mM. The reaction mixture was kept stirring overnight in order to dissolve the silver within the nanoparticles while maintaining the fluorophore conjugation. When the reaction was completed, the resulting nanobubble sample was used for single molecule analysis directly. The surface chemistry was retained [1,3] and nanoparticles were also stirred in same amount of water for same length to keep all other conditions consistent.

Single Particle Fluorescence Measurements

The confocal single-molecule fluorescence microscope used in this work has been described previously [22]. Briefly, a 488 nm Argon-ion laser (Melles Griot) was used for excitation. The laser was directed into the back port of a custom-modified inverted Olympus microscope (IX 51) via a mirror periscope. Laser radiation was then reflected onto the back aperture of an oil-immersion 100 $\times$ objective (1.3 NA, Olympus) by a long-pass dichroic mirror (Semrock). The fluorescence emission was collected by the same objective, transmitted through the dichroic mirror, and spatially filtered using a 100 μ m pinhole (Newport). The fluorescence was then spectrally filtered using an interference filter (Omega Optical) matching the emission of rhodamine B, and detected by a single-photon avalanche photodiode. A handheld power meter (Laser Check, Edmund Optics) was used to measure laser power just before the sample.

For analysis, a 30 μ L droplet of nanocomposite suspension was placed on a 150 μ m coverslip. The coverslip was coupled to the microscope objective with immersion oil. This procedure was repeated for every measurement. Nanoparticle or nanobubble samples were then exposed to laser light ranging from 150 μ W to 1.5 mW. Evaporation of the sample droplet was not observed during the short sample analysis time. Data were acquired using a high speed counting board (PCI-6602, National Instruments) and LabView software (v.8, National Instruments). Data were then exported as text files and analyzed in Origin software (version 8.0, Origin Lab.). Fluorescence bursts of 1 ms duration were counted above the background using a signal to noise ratio of 3 [23–24]. The average baseline intensity was calculated from 70 consecutive signals in the absence of single particles for each sample.

Cell Imaging and Analysis

For cell staining with the anti-CD71 rhodamine B nanocomposites, Ramos B lymphocytes (American Type Culture Collection) were stained for 40 minutes in phosphate buffered saline (pH 7.4) and washed before analysis. HuT 78 T lymphocytes (ATCC) were stained with anti-CD4 rhodamine B nanocomposites under the same conditions as Ramos cells. Cells were analyzed using an inverted, epifluorescence microscope (IX71, Olympus) and a cooled CCD camera. Excitation was performed using a metal halide lamp with appropriate

excitation and emission filters for rhodamine B. Images were analyzed using ImageJ (v1.43, National Institutes of Health); background was subtracted prior to cell intensity analysis.

Results and Discussion

Metal-Enhanced Fluorescent Nanocomposites

Synthesis of core-shell Ag@SiO₂ nanoparticles was a two-step process where nanocolloids were generated and then silica was grown over the colloids. The process of etching away the silver to achieve nanobubble controls ensures that dye aggregation effects are accounted for when evaluating MEF [1, 5]. Figure 2A shows a representative TEM image of core-shell Ag@SiO₂ nanoparticles dispersed in ethanol. In this figure no fluorophores have been added. The nanoparticles were uniform in size with a diameter of 120 ± 20 nm; the thickness of the silica shell was 15–20 nm. Figure 2B shows SiO₂ nanobubbles after etching with silver chloride. In Figure 2B incomplete etching can be seen in some nanobubbles. This artifact can be eliminated with longer etching times. The empty nanobubbles had diameters of 120 ± 20 nm.

The diffusion of individual nanocomposites through the microscope's laser beam is shown in Figure 3. Nanobubbles with etched cores showed minimal fluorescence signal, indicating that dye aggregation effects were minimal. This is a critical issue when dealing with enhancement effects, as the presence of multiple fluorophores on a single nanoparticle could be misconstrued as enhanced fluorescence. The fluorescence intensity of individual nanobubbles was low (mean intensity < 40 counts). However, the metal core nanoparticles showed higher fluorescence, with nanoparticle signals as high as 270 counts, with most nanoparticle intensities >75 counts. Each fluorescence burst corresponded to a single nanoparticle containing multiple fluorophores undergoing metal-enhanced fluorescence. The highest signal enhancement obtained was 6.8-fold and the average enhancement of the nanoparticles over the controls was on the order of 2.7 times, which is consistent with this type of nanocomposite [21].

Labeling of CD71 (Human Transferrin Receptor) and CD4 Using Antibody-Conjugated Nanoparticles

To demonstrate the facile functionalization of the nanocomposites, we used anti-Human CD71 antibodies to label the transferrin receptors of Ramos cells and anti-Human CD4 to label HuT 78 cells. CD71 is highly expressed on Ramos cells and can be used as a marker for cell proliferation. Figure 4A–B shows typical microscopy images of Ramos cells stained with rhodamine B Ag@SiO₂ nanoparticles conjugated to anti-CD71 using our sandwich attachment method. Control experiments were conducted using rhodamine B nanobubbles conjugated with anti-CD71 (Figure 4C–D).

The conjugation methods produce a stable coating mean cell fluorescence intensity of 5000 ± 380 counts (mean $\pm$ standard deviation), while the core-shell particles had a mean cell fluorescence of 8700 ± 1900 counts. To make sure protein exchange did not occur during the process, Ramos B cells were stained with rhodamine B nanoparticles conjugated to anti-CD4. Since Ramos B cells are CD4-, we expected a low fluorescence signal after staining. The mean cell fluorescence intensity was 1900 ± 1800 counts, indicating that protein exchange interference was negligible. Blank cells (no particle labeling) had intensities of 660 ± 14 counts. The cells were from the same culture and same point in time, so the anti-CD71 expression was uniform across experiments. The labeled cell intensities indicate that Ag@SiO₂ nanoparticles can be easily conjugated for different cell analyses.

To test the stability of the nanolabels, we used the same batch of nanolabels conjugated to anti-CD19 to label Ramos B cells in 0, 24, and 48 hours (Figure 5). Cells were stained with

rhodamine B anti-CD19 nanoparticles and washed before analysis for each experiment. The mean fluorescence intensity for labeled cells was 9900 ± 2500 counts (0 hour), 9400 ± 3600 counts (24 hours), 8500 ± 2100 counts (48 hours). The consistent fluorescence signal demonstrated Ag@SiO₂ nanoparticles are stable and can last up for several days.

We also conjugated nanoparticles with anti-Human CD4 and tested them with a CD4⁺ cell line. As in the case with anti-CD71, the anti-CD4 nanoparticles show higher staining levels than the nanobubble controls conjugated to the same antibody. Control cells (labeled with anti-CD4 rhodamine B nanobubbles) had a mean intensity of 300 ± 50 counts. Cells labeled with anti-CD4 rhodamine B nanocomposites had an intensity of 1600 ± 100 counts. This enhancement is similar to what we observed by single particle microscopy. The antibody coating approach is flexible, in that the first two steps, coating with biotinylated-BSA and neutravidin, respectively, are universal for all of our nanoparticles. The final step, the addition of a biotinylated antibody, determines which receptor the nanoparticle has affinity for. Since other fluorophores can be incorporated into the nanoparticles, it is possible to make many different labels for cell imaging or flow cytometry. It is also possible to develop pairs of dyes for FRET transfer within the nanoparticle, further extending their use as cell labels. We will explore these and other applications of our conjugation method in the future.

Conclusion

In this work, we demonstrate a straightforward method for conjugating silica-shell nanoparticles for cell labeling. The process results in efficient coupling of antibodies or other affinity ligands to the nanoparticle surface. While we used metal-enhanced fluorescence nanoparticles, our conjugation approach can be used for any nanoparticle clad in a silica shell. The ease of conjugation makes this method an attractive tool for bioconjugation, and can be applied to a wide range of materials for many bioanalytical and biosensing applications.

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Figure 1.

Preparation of Ag@SiO₂ nanoparticles, dye-conjugated nanocomposites, and etched-core nanobubbles (controls). Silver ion was reduced to nanocolloids, and was coated with silica sol-gel to produce a surface for conjugation. The core-shell nanoparticles can be rapidly conjugated to organic dyes such as rhodamine B via surface adsorption or sol-gel intercalation. Antibodies were then added using a sandwich approach. To discount increases in fluorescence due to dye/protein aggregation effects, control nanobubbles were produced by etching the silver cores, leaving hollow silica shells.

Figure 2.

TEM images of Ag@SiO₂ nanoparticles (A) and hollow nanobubbles (B). The mean diameter of the particles was 120 ± 20 nm with silica layers that were 15–20 nm thick. The scale bar = 100 nm.

Figure 3. Single-particle fluorescence of core-shell Ag@SiO₂-rhodamine B nanocomposites (blue) and nanobubble-rhodamine B composites (red) at 270 μ W of excitation power. The nanocomposites were allowed to freely diffuse through the laser beam probe volume, generating fluorescence bursts. The enhancement ranged from 2.7- to 6.8-fold.

Figure 4.

White light and fluorescence images of Ramos B cells stained with rhodamine B nanoparticles (A–B) conjugated to anti-CD71 or rhodamine B nanobubble controls conjugated to anti-CD71 (C–D). The mean fluorescence intensity of nanoparticle labeled cells was 8700 ± 1900 counts and the mean fluorescence intensity of nanobubble control labeled cells was 5000 ± 380 counts, and the mean fluorescence intensity of rhodamine B anti-CD4 nanoparticles control was 1900 ± 1800 counts.

Figure 5. White light and fluorescence images of Ramos B cells stained with rhodamine B nanoparticles conjugated to anti-CD19 in 0 hour (A–B), 24 hour (C–D) and 48 hour (E–F). The mean fluorescence intensity of nanoparticle labeled cells was 9900 ± 2500 counts, 9400 ± 3600 counts, 8500 ± 2100 counts.

Table 1

Comparison of covalent and noncovalent bioconjugation methods for silica surface nanoparticle functionalization. Characters of synthesis process, materials and solvent, conjugation robustness and signal intensity for both methods are compared.

	Covalent conjugation	Noncovalent conjugation
Synthesis process	Usually multi-step and long reaction time required	Straightforward
Materials and solvent	Organic solvent increases toxicity	Only need aqueous solution
Conjugation robustness	Stable (days-weeks)	Stable (days)
Signal intensity	Strong	Strong