

Biochemically functionalized silica nanoparticles

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In this report, we demonstrate the biochemical modification of silica based nanoparticles. Both pure and dye-doped silica nanoparticles were prepared, and their surfaces were modified with enzymes and biocompatible chemical reagents that allow them to function as biosensors and biomarkers. The nanoparticles produced in this work are uniform in size with a 1.6% relative standard deviation. They have a pure silica surface and can thus be modified easily with many biomolecules for added biochemical functionality. Specifically, we have modified the nanoparticle surfaces with enzyme molecules (glutamate dehydrogenase (GDH) and lactate dehydrogenase (LDH)) and a biocompatible reagent for cell membrane staining. Experimental results show that the silica nanoparticles are a good biocompatible solid support for enzyme immobilization. The immobilized enzyme molecules on the nanoparticle surface have shown excellent enzymatic activity in their respective enzymatic reactions. The nanoparticle surface biochemical functionalization demonstrates the feasibility of using nanoparticles for biosensing and biomarking applications.

Introduction

Nanotechnologies are now poised to revolutionize the electronic, chemical and biotechnology industries and biomedical fields. There are many interesting areas in nanotechnology.^{1,2} One of the most important aspects of this field is the preparation and development of nanomaterials, such as nanoparticles. There have been a variety of techniques for preparing different types of nanoparticles.^{3–7} The nanoparticles can be made with different materials and as small as 1 nm.^{1–15} There are a few important nanoparticle research areas of current interest, of which the following is especially important for biochemical analysis: preparation of nanoparticles with small and uniform sizes and the biochemical modification of nanoparticles for unique biochemical functionality. Biomolecular surface modification has added specific functions to a large variety of substrates.^{16–19} The surface modification of nanoparticles should also provide new opportunities for their effective applications in a wide variety of fields.^{1–4} Surface biochemical modification adds the desired functions to nanoparticles. There have been recent advances in nanoparticle surface modification.^{4,5,8–14} These nanoparticles are biochemically modified with interesting molecules for different surface properties.^{4–14} Recently, semiconductor nanocrystals (quantum dots) have been functionalized to couple biomolecules^{4,5} for biolabeling. Nanoparticles with biocompatible surfaces are useful for biochemical analysis, such as biosensors and biomarkers.

Out of the many techniques to prepare nanoparticles, the water-in-oil microemulsion is one most widely used method to prepare nanomaterials of small size.^{3,20–22} In this method, reversed micelles are formed, *i.e.* water nanodroplets are formed in an organic medium, and used as nanoreactors for the formation of nanoparticles. The water/oil ratio can be carefully adjusted to control the particle size. Particles prepared by the reverse microemulsion method show good promise in size control and further miniaturization.

Silica based solid support has been used effectively for the immobilization of various biomolecules such as enzymes, proteins, DNA *etc.*^{16–19, 23,24} It has been proven to be an excellent substrate suitable for many surface immobilization mechanisms. Biomolecules have been immobilized on silica

surface for applications ranging from biosensors to interfacial interaction studies.^{8–11,16–19,23,24} There is a rich database developed for silica surface modification. By combining the size advantage of the nanoparticles and the ease of surface modification of silica, one expects that nanometer scale biosensors could be prepared. Nevertheless, there has been limited research work on the development of nanoparticle-based biosensors or biomarkers. Even though there are various types of polymeric nanoparticles commercially available for biochemical applications,²⁵ there exist limitations that restrict their applications as biosensor arrays or individual biosensors. The polymeric nanoparticles have a severe self-aggregation in biological medium. Also they have a wide size distribution. As a result of these shortcomings, they have not been used effectively for sensitive analytical measurements.

In this work, we demonstrate the surface modification of silica nanoparticles for biochemical functionality. We have adopted two approaches, aqueous solutions reaction²⁶ and water-in-oil microemulsions,^{3,20–22} for the preparation of uniform silica nanoparticles, including pure silica nanoparticles and dye-doped silica nanoparticles. The freshly prepared pure silica nanoparticles have been modified with two different enzymes, glutamate dehydrogenase (GDH) and lactate dehydrogenase (LDH). These enzymes are involved in similar enzymatic reaction with their co-substrates glutamate and lactate respectively.^{27,28} The dye, ruthenium(II) bipyridyl, (tris(2,2'-bipyridyl)dichlororuthenium(II) hexahydrate, Rubpy), doped silica nanoparticle surface has been modified with membrane binding reagents. The dye-doped nanoparticles have been used for cell membrane staining.

Experimental

Materials

Lactate dehydrogenase from hog muscle (550 units mg^{−1}) and glutamate dehydrogenase from beef liver (120 units mg^{−1}) were purchased from Roche Molecular Biochemicals (Germany). Lactate and glutamate were purchased from Sigma Chemical

Co. (St. Louis, MO). Nicotinamide adenine dinucleotide (NAD⁺) was purchased from Acros Organics (Fairlawn, NJ). Water soluble 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (WSC), tris(2,2'-bipyridyl)dichlororuthenium(II) hexahydrate (Rubpy), tetraethylorthosilicate (TEOS), lauroyl chloride and succinic anhydride were purchased from Aldrich Chemical Co. (Milwaukee, WI). Trimethoxysilylpropyldiethylenetriamine (DETA), a silanization reagent was purchased from United Chemical Technologies (Bristol, PA). All other chemicals were of analytical reagent grade. Distilled deionized water (Easy pure LF) was used for the preparation of all solutions. Commercial microspheres were purchased for comparison experiments with the nanoparticles produced in this report. Carboxylate modified microspheres were obtained from Bangs Laboratory.²⁵ Enzyme immobilization was carried out following the company's protocol. Microspheres were approximately 1–1.5 μm in size.

Nanoparticle synthesis

Synthesis of the pure silica nanoparticles was carried out following the Stober synthesis.²⁶ 5.0 ml of ethanol (95% v/v) was taken in a conical flask and mixed with 100 μl of TEOS. The flask was then cooled to 0 $^{\circ}\text{C}$ and placed floating in an ice cooled ultrasonic bath. 5.0 ml of ammonium hydroxide (NH₄OH, 28–30 wt.%) was added to the solution while ultrasonication. After 1 h sonication, nanoparticles were centrifuged out of solution, and were washed thoroughly (5–6 times) with both water and acetone, and finally air-dried. The dye-doped particles were synthesized using water-in-oil microemulsion [Triton X-100/cyclohexane/n-hexanol (4.2:1:1 v/v)/water] technique. Rubpy was chosen as a dye in this specific application. The water to surfactant molar ratio was 10 and the final dye concentration in the mixture was 0.1 M. TEOS and NH₄OH were used for the silica particle synthesis with volume ratio of TEOS to NH₄OH at 1.7. The dye-doped nanoparticles were isolated from microemulsion with acetone.

Nanoparticle characterization

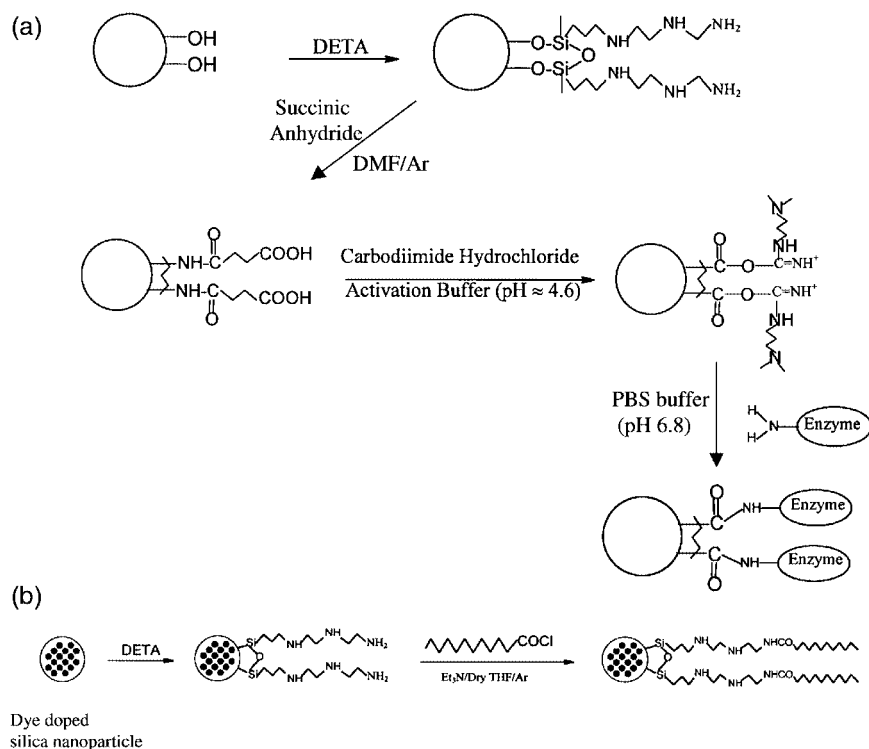
Silica nanoparticles have been characterized by a Hitachi transmission electron microscope, model H7000. Optical and fluorescence images of the dye-doped silica nanoparticles were taken by an inverted Olympus fluorescence microscope (model IX708F).

Silica nanoparticle surface modification

Silanization of nanoparticles were performed by immersion in freshly prepared 1% (v/v) solution of distilled DETA and 1 mM acetic acid for 30 min at room temperature (23 $^{\circ}\text{C}$). The DETA modified silica nanoparticles were thoroughly rinsed with deionized water to remove excess DETA (pure silica nanoparticles needed to be centrifuged). The silanized particles were then treated with 10% succinic anhydride in dimethylformamide solution under an argon atmosphere and stirred for 6 h. The resulting carboxylate modified particles were washed with deionized water and centrifuged two to three times. Using the Bangs Laboratory²⁵ protocol the carboxylate modified particles were functionalized with enzyme. Scheme 1a shows the surface modification procedures for the pure silica nanoparticles.

Enzyme immobilization

LDH and the GDH solution with concentration of 1×10^{-5} M were prepared in 0.1 M PBS at pH 6.8. The nanoparticles were immersed in the LDH/PBS or GDH/PBS solution for 23 h at room temperature to allow the enzymes to be immobilized on the surface (Scheme 1a). The particles were subsequently washed and ultrasonicated to remove any physically adsorbed enzyme molecules on the silica surface. The particles were either used immediately for testing or stored in a PBS buffer at 4 $^{\circ}\text{C}$ for later usage.



Scheme 1 Surface modification of the pure silica nanoparticles for enzyme immobilization (a), and surface modification of the dye doped silica nanoparticles for membrane probing (b).

Biochemical modification of the dye-doped silica nanoparticles

The silica surface had been silanized by DETA as described above. The amine-functionalized particles were then treated with lauroyl chloride in dry tetrahydrofuran in presence of argon atmosphere for 6 h.²⁸ These functionalized nanoparticles, used as a membrane probe, were then tested with PTK2 cells and leukemia cells.²⁹ The cells were obtained in a dish with approximately 2.0 ml culture medium. 20 μ l of functionalized dye-doped particles in DMSO [1% (v/v)] was added to the cell dish and incubated for 6 h. After thoroughly rinsing the cells with a PBS buffer (pH 6.8), images were taken by a fluorescence microscope.

Spectroscopic measurement

Preliminary spectroscopic measurements were done on the pure silica nanoparticles to test if the nanoparticles were functionalized with enzyme. Emission spectra were recorded on an LS50B Perkin Elmer Spectrofluorometer. Measurements of the GDH activity were carried out in a 3 ml cuvette. The substrate solutions of glutamate with 2 mM NAD⁺ in a PBS buffer (pH 6.8) were used for the enzymatic activity study. A known amount of the enzyme-immobilized nanoparticles was added to the cuvette. The reaction was allowed to take place and, upon 350 nm excitation, NADH fluorescence was monitored at 465 nm with time. All measurements were carried out at room temperature (23 °C).

Results and discussion

Uniform silica based nanoparticles

Two different types of silica nanoparticles of uniform sizes have been prepared. The first type of nanoparticles prepared in this work is the dye-doped silica particles. The silica nanoparticle with membrane dye were prepared by the W/O microemulsion technique. The highly luminescent water-soluble dye, Rubpy, becomes water insoluble when doped inside the silica network of the nanoparticles. TEM image of the nanoparticles shows that the particles are 62 nm in size, and their size distribution is extremely uniform with a standard deviation of less than 2% (based on a calculation done on 30 nanoparticles shown in Fig. 1). Fluorescence emission spectra show that the dye-doped

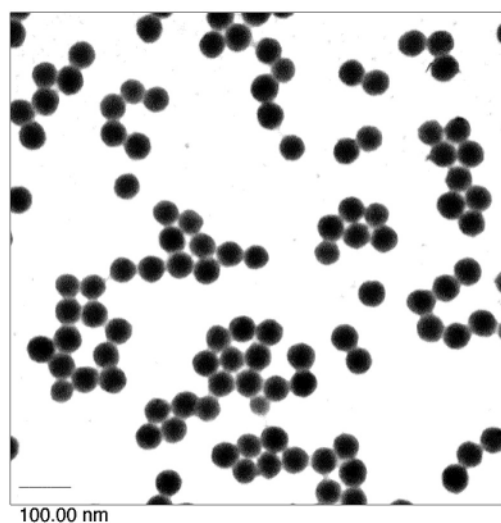


Fig. 1 TEM image of the dye doped silica nanoparticles. Particles size are determined to be about 60 nm. The relative standard deviation in size distribution is 1.6%.

particles have characteristic bright red emission at 595 nm when excited at 450 nm. The second type of nanoparticles is the pure silica nanoparticles. They have also been prepared with similar sizes and uniformity in size distribution as those for dye doped nanoparticles. We have also prepared 100–120 nm nanoparticles grown directly on a glass slide. These nanoparticles will increase the silica surface area of the slide.

Nanoparticle surface modification

The freshly prepared silica nanoparticles are functionalized with enzymes or membrane binding reagents. The nanoparticles prepared in this work all have a functionalizable silica surface. These surfaces can be modified with different reagents for a variety of applications. Two major modification mechanisms were used in this work as shown in Scheme 1. Both of these schemes rely on the creation of primary amine functional groups onto the particle silica surface. Fluorescamine, which becomes highly fluorescent upon reacting with primary amines, is used to test the efficiency of the silanization. The fluorescamine assay was used for the monitoring of the first two steps in both immobilization processes. We observed positive fluorescamine test in the first step confirming that DETA was successfully used for the silanization of the nanoparticles, leaving free primary aliphatic amine groups on the surface. After the succinic anhydride treatment in Scheme 1a, nanoparticles were carboxylated and negative fluorescamine test confirmed no free amine groups left on the nanoparticle surfaces. Once the nanoparticles were carboxyl modified, the particles were used for the immobilization of enzyme molecules (LDH and GDH). Similarly, in Scheme 1b, we show that the amine-functionalized particles can also be linked with a membrane functional group. We treated the nanoparticles with lauroyl chloride in dry tetrahydrofuran in presence of an argon atmosphere for 6 h.²⁷ Lauroyl-derivatized nanoparticles became poorly soluble in water because of the presence of hydrophobic lauroyl groups on their surface (Scheme 1b). They tend to aggregate in aqueous solution. These particles showed negative fluorescamine assay²⁷ indicating that amine groups on the particle surfaces had been consumed. In addition, the presence of the amide group ($-\text{NHCO}-$) was confirmed by the IR spectra as shown in Fig. 2. It is expected that the lauroyl group can bind to the lipid bilayer of the cell membrane. It is clear that the nanoparticles can be functionalized with desired molecules by providing the required functional groups on their surfaces.

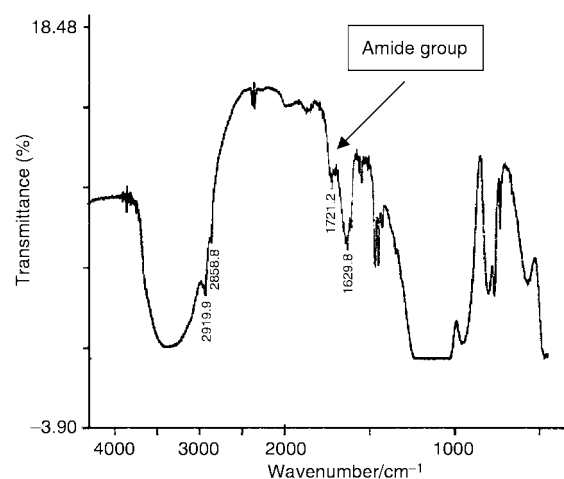


Fig. 2 IR spectra of the lauroyl derivatized membrane dye which is immobilized on nanoparticles.

Enzyme immobilized nanoparticles for glutamate and lactate analysis

LDH and GDH were both covalently immobilized onto the nanoparticles prepared in this work. The immobilized enzyme molecules display excellent enzymatic activity. The enzymatic activity of the immobilized LDH/GDH was monitored by following the reaction between lactate or glutamate with NAD^+ when the LDH/GDH immobilized nanoparticles were added to the substrate solution as shown in Fig. 3. The fluorescent signal from the product NADH was measured as a function of reaction time.

Detection limit for glutamate by the GDH immobilized nanoparticles was determined when 10 μl of a GDH immobilized nanoparticle suspension was added for a 3 ml of substrate solution. A series of glutamate solutions were tested as shown in Fig. 4. The detection limit is determined to be 0.5×10^{-6} M. Similar results were obtained with LDH for the detection of lactate. The detection limit for the enzyme immobilized nanoparticles can not be directly compared with results obtained by other means^{17,24} as the exact amount of enzyme molecules immobilized on the nanoparticle surfaces is not known. However, it can be used as a way to compare

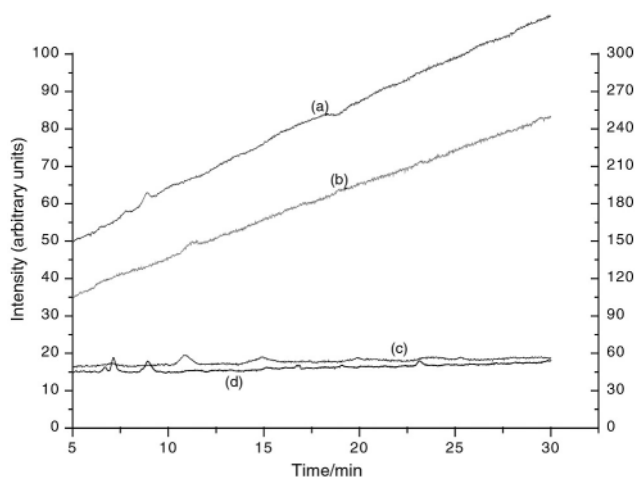


Fig. 3 Comparison of LDH- and GDH-immobilized nanoparticles vs. microspheres. NADH was monitored over time as a product of the enzymatic reaction catalyzed by the enzyme molecules immobilized on the surfaces of the particles. (a) GDH nanoparticles, (b) LDH nanoparticles, (c) LDH microspheres, (d) GDH microspheres.

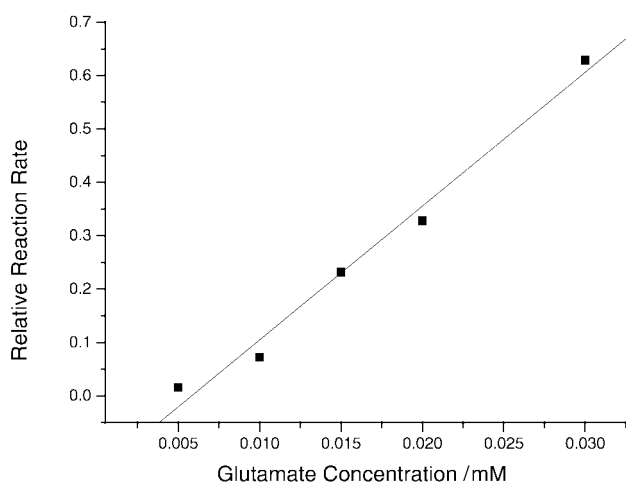


Fig. 4 Effect of glutamate concentration on the relative enzymatic reaction rate of GDH immobilized nanoparticles. Based on this curve, the detection limit of glutamate has been determined. 10 μl GDH nanoparticle (1 mg particle/ml particle concentration) is used and the NAD^+ concentration is 2 mM. The reaction was carried out at pH 6.8 PBS buffer.

relative capacity of different nanoparticle probes prepared with a similar procedure.

The lifetime of biosensors, especially those employing biologically active materials such as enzymes or antibodies, has been a major concern for the sensor's applicability. The nanoparticle-based lactate and glutamate nanoparticles were stored in a PBS buffer (pH 6.8) when not in use. The stability of the enzyme-immobilized particles was evaluated based on a daily response measurement. Both types of nanoparticles showed good stability over a two-week period. 93% of their enzymatic activities remained after a two-week period, with daily fluctuations of 4%. The enzyme-immobilized nanoparticles began to degrade after two weeks, which is most likely due to the regulatory denaturation process of the enzymes. The fast response time for the functionalized nanoparticles are in the millisecond range, which is probably due to the fact that the active sensing elements are on the nanoparticle surfaces for easy access.

Comparison between enzyme immobilized microspheres and nanoparticles

Nanoparticles prepared in this work were directly compared with commercially available microspheres with 0.5 μm in diameter. We used the same amount of enzymes for the immobilization on the same amount of particles (nano and micro). We then took an equal amount of particles for the same enzyme assay. We compared their enzymatic activities. In order to compare them fairly, we used optimized procedures for the immobilization of LDH (or GDH) on both particles. We took 1 mg of each type of enzyme-immobilized particles to run the enzyme assay. Microspheres purchased from Bangs Laboratories were already modified with carboxylate groups. LDH was covalently immobilized to these polystyrene-based microspheres following the company's protocol. Enzyme immobilized microspheres were always dispersed by ultrasonication before use. The enzymatic activity was monitored, as shown in Fig. 3c–d. It was clear that the microsphere-based probes have lower sensitivity, about one order of magnitude lower than that of the nanoparticle-based probes. This applied to both LDH and GDH nanoparticles. This is most likely due to the fact that the nanoparticles have a larger surface area than the microspheres, therefore more enzyme molecules are attached to the nanoparticle surface which gives higher activity. Moreover, the commercially available microspheres are difficult to handle. Due to the polymeric composition of the nanoparticles, they have a tendency to agglomerate, resulting in a higher fluctuation in their optical signal as shown in Fig. 3c and d. The microspheres also tend to stick to the centrifuge tubes, both glass and plastic, during centrifugation and ultrasonication. These problems did not occur to the silica nanoparticles prepared in this work.

Dye-doped nanoparticles for modification and application as a membrane probe

The biological membrane consists mainly of phospholipid bilayers. The interior of the membrane is hydrophobic in nature. Therefore, membrane probes should be designed in such a way that it can bind to the hydrophobic part of the membrane. We have modified the silica surface of the dye-doped nanoparticles. The nanoparticles are prepared with a suspension (5 mg ml^{-1}) in dimethylsulfoxide (DMSO) solution by ultrasonication. The dye concentration in the nanoparticles is approximately 32.6 wt.%. The surface modification of the membrane probe is shown in Scheme 1b. The functionality of the lauroyl-derivatized dye particles as membrane-probes have been tested with PTK2 cells.³⁰ As shown in Fig. 5, these nanoparticles can be used as effective membrane staining probes for PTK2 cells.

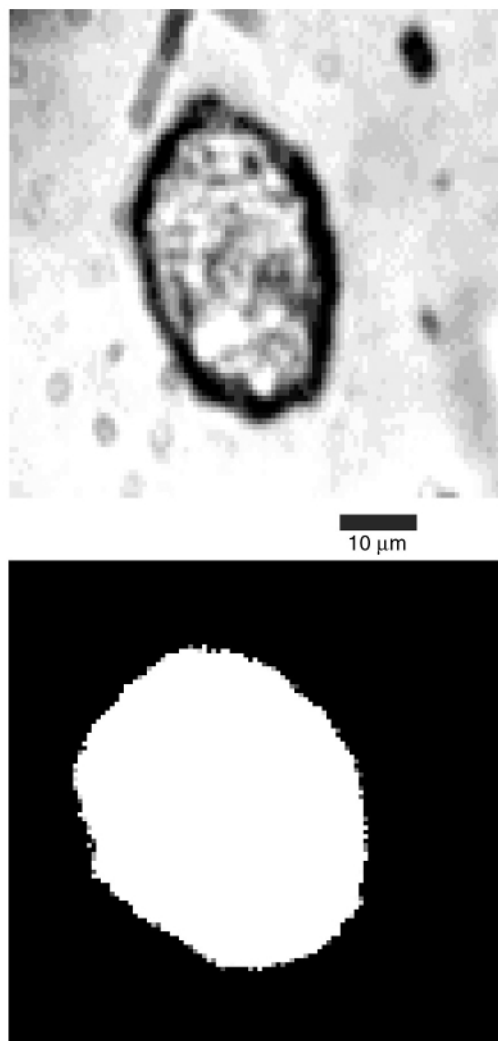


Fig. 5 Cell staining images by optical and fluorescence microscopy: top panel, optical images of PTK2 cell; bottom panel, fluorescence image of PTK2 cell. After staining with the dye-doped nanoparticles with membrane functional group, the cells have strong fluorescence. The cell size is around 20 μm .

In Fig. 5, the optical image (top panel) corresponds well to the fluorescence image (bottom panel). Multiple staining experiments showed that the results were reproducible.

The dye-doped nanoparticles can be used for cell staining with some advantages. The dye molecules are photostable. Since they are doped inside the silica network, there will not be any direct contact between the dye molecules and the surrounding environment that could affect their emission properties. The nanoparticle dimension is approximately three orders of magnitude smaller than that of the cell. It is thus essentially similar to the dye molecules for non-invasive staining. The dye-doped nanoparticles can also be versatile for specific and non-specific labeling and tracing of biocomponents.

Conclusion

Two different types of silica nanoparticles of uniform sizes have been synthesized and biochemically modified for specific function. They can be functionalized with enzyme molecules and biomembrane probes. The surface modification of the nanoparticles has added unique biofunctionality for these nanoparticles. We have used lactate dehydrogenase and glutamate dehydrogenase for enzyme immobilization onto the nanoparticles. The immobilized nanoparticles have shown excellent enzymatic activities and excellent detection capability. Direct comparisons with commercially available micro-

spheres have shown that the nanoparticles have at least one order of magnitude better enzymatic activities due to larger surface areas for enzyme immobilization. To further demonstrate that nanoparticle's bioapplicability, we have also used dye-doped silica nanoparticles as biomarkers for cell staining. The dye molecules inside the nanoparticles are stable, and there is no direct contact between the dye molecules and their surrounding environments that could affect their optical properties. Our work demonstrates an easy approach of combining nanotechnology with biomolecules for biochemical analysis and biotechnology applications.

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