Dye-doped nanoparticles for bioanalysis

Understanding biological processes at the molecular level with accurate quantitation needs advanced bioanalysis. In this review, we describe dye-doped silica nanoparticles (NPs), their synthesis, bioconjugation, and applications in different bioanalysis formats. Silica-based nanomaterials have been developed with optical-encoding capabilities for the selective tagging of a wide range of biomedically important targets, such as bacteria, cancer cells, and individual biomolecules. We also briefly review other closely related nanomaterials, such as quantum dots, Au NPs, and magnetic NPs. We envisage that further development of these NPs will provide a variety of advanced tools for molecular biology, genomics, proteomics, drug discovery, and diagnosis and therapy of infectious disease and cancer.

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The identification and characterization of intracellular events and protein-receptor interactions are of great importance for basic medical studies and therapeutic applications. Optical analysis based on fluorescence labeling has been extensively used to study these interactions. Detection and *in vivo* imaging of a variety of biological samples, such as living cells and tissues, can be carried out in this way. Of the many fluorescent labeling reagents, organic fluorophores are used most often in these cases. The fluorophores usually have high quantum yield and can be easily used in many different applications. However, they usually suffer from limited sensitivity and photostability. Moreover, most fluorophores present a certain level of toxicity that hinders their application in *in vivo* cellular studies and imaging.

In the last ten years or so, significant advances have led to a large variety of labeling reagents based on nanomaterials with controlled size and shape. These nanomaterials represent an exciting and often more

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effective alternative to the use of organic fluorophores. The superior properties of such nanomaterials provide multiple possibilities for highly sensitive detection of various targets under different conditions, from the single-molecule level to human body applications, from *in vitro* diagnosis to *in vivo* real-time imaging[1,2].

Several types of NPs are currently being used for bioanalysis, including metallic (Au and Ag) NPs[3], quantum dots (QDs)[4], magnetic NPs, lanthanide NPs[5,6], silica NPs[7], and other types of nanomaterials. Different NPs have their own unique properties and have been adapted to different applications in the bioanalysis field.

Nanomaterials in bioanalysis

Au NPs

One of the most widely used nanomaterials in the bioanalysis field is Au NPs (commonly known as Au colloid or colloidal Au)[3,8]. The presence of a plasmon absorbance band, and their shape and size-dependent optical properties, make Au NPs suitable as colorimetric probes[9]. The plasmon-resonance spectra of free single particles differ significantly from those of aggregated ones. Au NPs have been used to develop highly sensitive detection schemes for many targets[10]. Ultrasensitive analysis of oligonucleotides, proteins, and other biomolecules has been achieved using Au NPs as biomarkers[11-13]. Au NPs are already in commercial products. One well-known example is the lateral flow strip developed for fast pathogen detection and point-of-care diagnosis[14,15].

Quantum dots

QDs are a category of semiconductor crystals (i.e. CdSe, CdTe, CdS, ZnSe, PbS, and PbTe)[4] with typical diameters that range between 2 nm and 10 nm (without outside coating). QDs provide a bright fluorescence (~10-20 fold higher than an organic fluorophore) and also present a higher photostability against photobleaching than conventional organic dyes. QDs have narrow emission bands that can be precisely tuned from blue (usually CdSe) to the near-infrared (PbS and PbSe) by varying the QD size and components. The near-infrared emission of QDs greatly expands their application, e.g. deep-tissue imaging in clinical and life science studies. QDs also have wide absorption spectra, which allow the simultaneous excitation of different QDs at the same time. These properties allow multicolor optical coding in gene-expression studies, high-throughput screening, and medical diagnosis[16-19]. One important application is high-throughput detection on microarray platforms[20,21].

Magnetic nanoparticles

NPs with superparamagnetic properties are ideal media for the manipulation of biological materials, targeting delivery of therapeutic compounds[22], and hyperthermia treatment[23]. Because of their superparamagnetism, magnetic NPs can also act as a contrast reagent in magnetic resonance imaging (MRI) for diagnosis[24-27].

Lanthanide (Ln^{3+}) NPs

NPs prepared from Eu^{3+}, Sm^{3+}, Tb^{3+}, Gd^{3+}, etc. are attractive because they are photostable and usually present sharp emission spectra (<10 nm), large Stokes shifts (>150 nm), and long fluorescence lifetimes. The surface of the NPs can be easily modified without altering their properties significantly. These attributes make the NPs suitable for use as biolabels, since they enable the elimination of background and scattered fluorescence, allowing a delayed measurement on the scale of microseconds. These NPs also possesses excellent up-conversion fluorescence[6,28] and do not suffer from blinking. Lanthanide NPs have already been used in the development of immunoassays[29], time-resolved fluorescence imaging for quantitative histochemistry[30], specific targeting[31], and DNA detection[32].

Dye-doped silica NPs

Silica-based NPs are currently used in many areas of bioanalysis. Compared with polymer-based NPs, which have been used widely in bioanalysis and labeling, silica NPs show less aggregation and little dye leakage[33]. Using appropriate synthetic conditions, a large number of dye molecules (either organic or inorganic) can be incorporated inside a single silica particle (there can be tens of thousands of dye molecules) (Fig. 1). Even though there remains some fluorescence-quenching phenomena within an NP with a large amount of dye incorporated in a small volume, the goal of obtaining a particle with brighter luminescence is largely successful. Dye-doped NPs produce a highly amplified optical signal compared with a single dye molecule. If applied appropriately in bioanalysis, silica NPs can provide a great improvement in analytical sensitivity (Fig. 2). Moreover, as the dye is trapped inside the silica matrix, which provides an effective barrier keeping the dye from the surrounding environment, both photobleaching and photodegradation phenomena that often affect conventional dyes can be minimized[34]. The excellent photostability makes these NPs suitable for applications where high intensity or prolonged excitations are required. For example, intracellular optical imaging suffers severely from photobleaching; with silica NPs, this

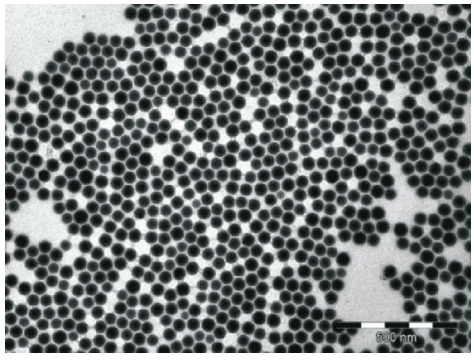

Fig. 1 Transmission electron microscopy (TEM) image of Rubpy dye-doped silica NPs. Scale bar = 500 nm. (Reprinted with permission from[36]. © 2004 American Chemical Society.)

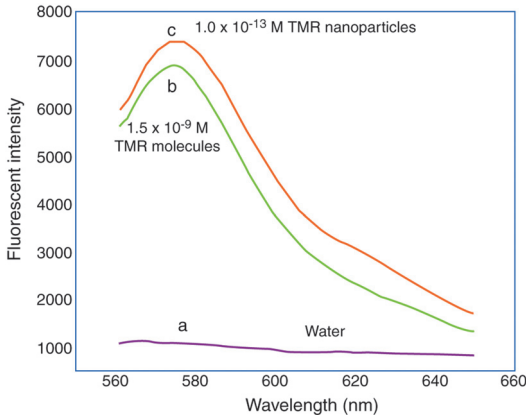

Fig. 2 Fluorescent signal amplification using dye-doped NPs: (a) pure water (control); (b) tetramethylrhodamine (TMR) dye, 1.5 x 10^{-9} M; (c) TMR-doped NPs, 1 x 10^{-13} M. (Reprinted with permission from[39]. © 2004 Wiley-VCH.)

problem could be minimized. The flexible silica chemistry also provides versatile routes for surface modification. Different types of functional groups can be easily introduced onto the NPs for conjugation with biomolecules. In addition, the silica surface makes these NPs chemically inert and physically stable[35]. All these properties make silica NPs excellent labeling reagents for bioanalysis and bioimaging[36-39].

Synthesis of fluorescent silica NPs

There are two main methods for the synthesis of dye-doped silica particles. The first is the Stöber method, which consists of the hydrolysis of a silica alkoxide precursor (such as tetraethyl orthosilicate, TEOS) in an ethanol and aqueous ammonium hydroxide mixture. Silicic acid is produced during hydrolysis and, when its concentration is above its solubility in ethanol, it nucleates homogeneously and forms silica particles of nanometer size. The method is comparatively simple and both organic and inorganic dyes can be incorporated using this method[40]. Nevertheless, because of the hydrolysis procedure, the NPs often have a relatively large size distribution. Some modifications of the procedure have been introduced and optimized for the incorporation of more hydrophobic dyes inside the silica NPs. One is based on the combination of both hydrophobic and hydrophilic precursors in making the NPs, such as phenyltriethoxysilane (PTES, hydrophobic) and TEOS (hydrophilic). Rhodamine 6G (R6G) has been successfully incorporated into silica NPs using this method[41]. The hydrophobic component keeps the organic dye in the silica matrix while the hydrophilic component allows the resulting NP to be dispersed in aqueous solutions. Another method for dye incorporation uses direct coupling of the organic dye to the silane reagent. Using this approach, van Blaaderen *et al.*[42] have covalently linked fluorescein isothiocyanate (FITC) to 3-aminopropyltriethoxysilane, and successfully incorporated the dye into the silica matrix.

The second main technique for dye-doped silica NP synthesis is the reverse microemulsion method. This is based on the formation of a water-in-oil reverse microemulsion system. Three main components make up the main reaction mixture: water, surfactant, and oil (an organic solvent, which is present in a high proportion relative to water). The stabilized water nanodroplets formed in the oil solution act as small microreactors, where silane hydrolysis and the formation of NPs with dye trapped inside take place[43]. The size of the NP is determined by the nature of surfactant, the hydrolysis reagent, and some other parameters, such as the reaction time, oil/water ratio, etc.[36]. The NPs produced by this method usually show a high degree of uniformity and are well dispersed in water. One of the disadvantages of this method is that, in most cases, it can only be used to incorporate inorganic dyes, some of which have lower quantum yields compared with organic fluorophores.

Organic-dye-doped silica NPs are difficult to prepare through this method because of the hydrophobic properties of the organic dye compared with the hydrophilic surface of the NPs. A modification of the protocol has been reported that increases the amount of organic dye incorporated in the particle[39]. Organic dyes are coupled to a dextran group, which is hydrophilic and can help keep the linked dye molecule within the silica. Fluorescent dyes such as tetramethylrhodamine (TMR), fluorescein, and Alexa Fluor 647 have been successfully doped into the silica NPs without leakage.

Besides single-dye doping, multiple-dye incorporation into the silica matrix has been reported. This can help provide more information upon detection. A method has been developed for the simultaneous doping of NPs with two inorganic dyes, tris(2'2-bipyridyl)dichlororuthenium(II) (Rubpy) and tris(2'2-bipyridyl)dichloroosmium(II) (Osbpy). These two dyes share a broad, overlapping excitation spectrum but have distinct maximum emission wavelengths. This makes them an ideal tool for a two-wavelength signaling approach where the intensity ratio can be used[44]. By precisely controlling and varying the concentrations of the dyes within the NP, excitation with a single wavelength leads to different emission signatures, permitting simultaneous and sensitive detection of multiple targets. In other work, Wang *et al.*[45] created dye-doped silica NPs that use fluorescence resonance energy transfer (FRET) as the emission scheme. Three different organic dyes, FITC, R6G, and 6-carboxyl-X-rhodamine (ROX) are incorporated into the same silica matrix. These dyes were carefully chosen to have overlapping emission and excitation spectra in order to allow efficient fluorescent energy transfer to occur. By doping with different combinations of the three types of dye, barcode tags can be produced for multiplexed, targeted FRET under single wavelength excitation (Fig. 3).

Surface functionalization of silica NPs

Once formed, silica NPs can be further modified with silane reagents. Because of the versatility of Si chemistry, various functional groups can be easily introduced onto the particle surface. Typically, the process requires an additional coating of the silica surface with the alkoxysilane reagent, such as carboxyethylsilanetriol for introduction of carboxylic

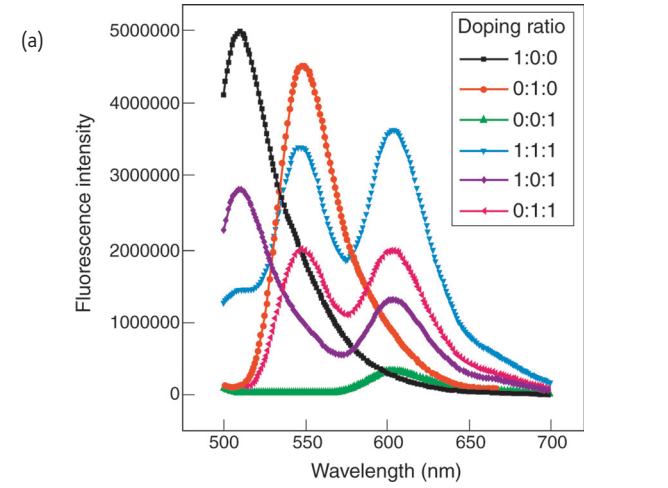

Fig. 3 Fluorescence emission of NPs with various FITC, R6G, and ROX dye-doping ratios: (a) emission spectra of NP samples; and (b) color of the NP samples under 300 nm ultraviolet excitation for FITC:R6G:ROX dye-doping ratios of 1:0:0, 0:1:0, 1:0:1, 4:1.5:3, 0.5:0.5:0.5, 2:2:2, 0:1:1, and 0.5:0.5:4 (from left to right). (Reprinted with permission from[45]. © 2006 American Chemical Society.)

acid groups, 3-aminopropyltriethoxysilane for amino groups, or 3-mercaptopropyl trimethoxysilane for thiol groups[46,47].

Rather than direct modification through covalent binding, there are alternative approaches based on the formation of noncovalent interactions. One of the most commonly adopted strategies is the attachment of avidin (which has an overall positive charge) to the negatively charged NP surface through electrostatic interactions[41].

Once the silica surface has been modified, biomolecules such as proteins, enzymes, antibodies, oligonucleotides, etc. can be linked to the NP following standard conjugation protocols[48]. Fig. 4 shows some conjugation procedures[49]. These surface modification schemes enable silica NPs to be conjugated with a large variety of biological molecules.

Silica NP applications

Silica NPs possess three important properties that have made them highly useful in bioanalysis: extremely high optical intensity, high photostability, and easy bioconjugation. We briefly discuss below a few interesting applications of silica NPs.

Silica NP-based immunoassays

The affinity and specificity associated with the antigen-antibody recognition process has been widely exploited in developing immunochemical techniques. Silica NPs can be used as a superior signaling element in an immunoassay by conjugating them to an antibody. Different types of targets, including proteins, cells, and bacteria, have been detected by these NPs[44,50-55].

Santra *et al.*[50] covalently attached mouse antihuman CD10 antibody to surface-modified, Rubpy-doped silica NPs. The antibody-modified NPs were then incubated with mononuclear lymphoid target cells. After washing away the unbound NPs, target leukemia cells could be clearly detected. Control experiments without antibody and specificity studies with nontarget cells show the effectiveness of this method to detect leukemia cells selectively.

Houser *et al.*[51] have developed a method for the recognition and detection of choline acetyltransferase (ChAT), a marker that only exists in cholinergic neurons in the brain. Goat anti-ChAT antibody was

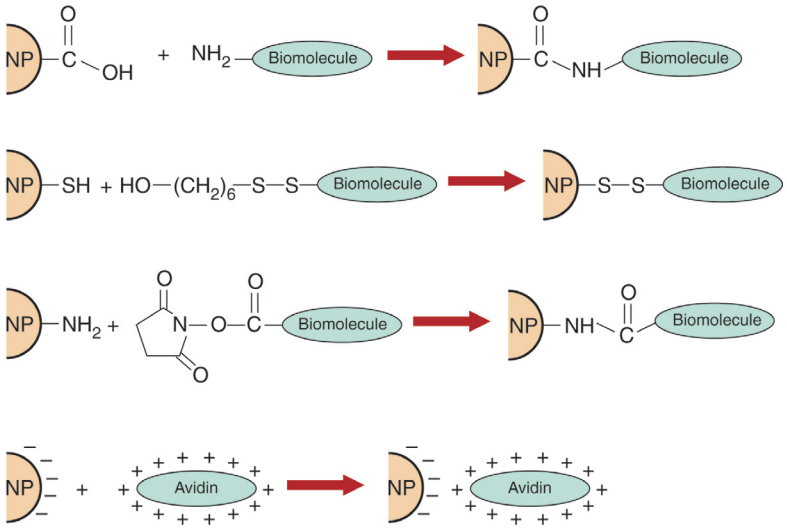

Fig. 4 Common bioconjugation protocols for the attachment of biomolecules onto the surface of silica NPs. (Reprinted with permission from[49]. © 2006 American Chemical Society.)

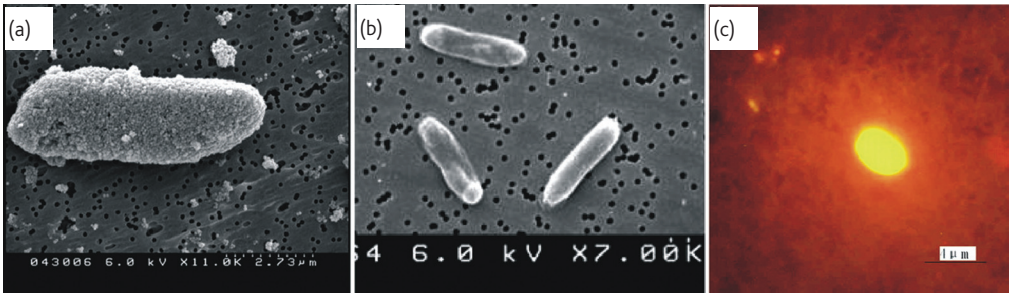

Fig. 5 Images of bacterial cells. (a) Scanning electron microscopy (SEM) image of an E. coli O157:H7 cell incubated with NPs conjugated with an antibody for E. coli O157:H7. (b) SEM image of a E. coli DH5 cell incubated with NPs conjugated with an antibody for E. coli O157:H7 (negative control). (c) Fluorescence image of E. coli O157:H7 after incubation with the antibody-conjugated NPs. (Reprinted with permission from[54]. © 2004 National Academy of Sciences.)

biotinylated and attached to streptavidin-modified NPs through biotin-streptavidin conjugation. The antibody-labeled NPs provide a powerful tool for imaging of cholinergic neurons, which could be beneficial in Alzheimer's disease research.

Deng *et al.*[52] have doped the silica matrix with a near-infrared fluorescent dye, methylene blue. The hydrophobic core enables the dye to exhibit enhanced fluorescence and shows improved stability to dye leaching and exogenous quenchers. The NPs were functionalized with monoclonal anti-alpha fetoprotein (AFP, a cancer marker) antibody. Detection of AFP was demonstrated with fluorescence-anisotropy measurements. The low interference in the near-infrared region makes it possible to perform fluorescence-anisotropy measurements directly on whole blood samples.

Eu-doped silica NPs have been developed by Tan *et al.*[53]. The NPs were first modified with streptavidin and then biotinylated antibodies were conjugated to the surface. The antibody-NP complexes have been used in a sandwich-type time-resolved fluoroimmunoassay (TR-FIA) for the detection of carcinoembryonic antigens (CEA) and hepatitis B surface antigens (HBsAg). Results with human sera samples correlate well with a comparative study using an established method.

Zhao *et al.*[54] have developed a silica NP-based method for the detection of single bacterium. The silica NPs were doped with Rubpy and surface modified with a specific antibody against *E. coli* O157:H7. Under optimized conditions, a single pathogenic *E. coli* O157:H7 bacterium could be detected within 20 mins in a complex matrix, such as spiked beef samples (Fig. 5). High-throughput and multiple-sample bacteria detection was also demonstrated using a 384-plate format.

Silica NPs for cellular imaging

He *et al.*[55] have constructed FITC-doped silica NPs labeled with antihuman liver cancer monoclonal antibody HAb18. Liver cancer cells can be easily distinguished from other cells with this NP-antibody labeling scheme and be selectively detected in a mixed sample. Silica NPs can be adopted as a useful tool for further cell-based studies.

Silica nanoparticles for multiplexed bioanalysis

Two-dye encapsulated NPs have been adopted for multiplexed bacteria detection within a flow system[44,49]. Three different antibodies were

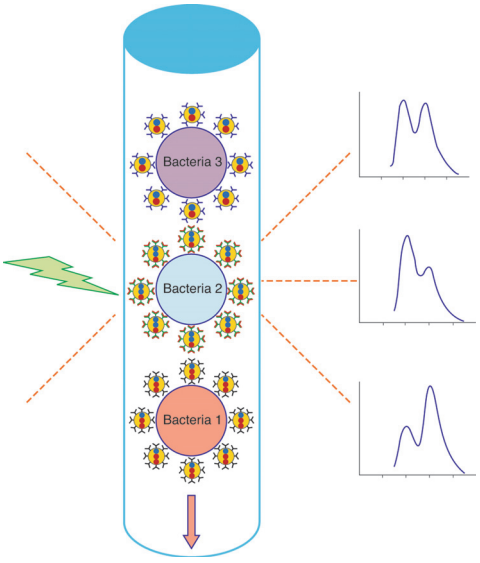

Fig. 6 Schematic of the dual-dye encoding system and its potential for multiplexed bacteria/cell detection within a flow system. Antibodies against different bacteria are labeled with NPs doped with different ratios of two dyes. When the bacteria pass through the fluidic channel, the fluorescence signal corresponds to the specific NP attached to the target cells. (Reprinted with permission from[49]. © 2006 American Chemical Society.)

conjugated to different NPs doped with varying intensity ratios of the two dyes. Each labeled NP specifically recognized and bound to the corresponding antigen-presenting bacteria. When the bacteria-NP mixture passed through a flow cytometer, each kind of bacterium-NP complex exhibited the unique fluorescence signature of the attached NPs. With this scheme, rapid, sensitive, and selective multiple bacteria or cell detection can be achieved (Fig. 6).

Silica NPs for nucleic acid analysis

Molecular recognition based on the hybridization of nucleic acid strands is now widely used in disease diagnosis, drug development, and many other biotechnology applications[56,57]. Dye-doped NPs have also been used as labels for increased sensitivity and throughput in this approach.

Zhao *et al.*[58] have devised a sandwich-assay setup for silica NP-based DNA detection. Three different DNA species were present in

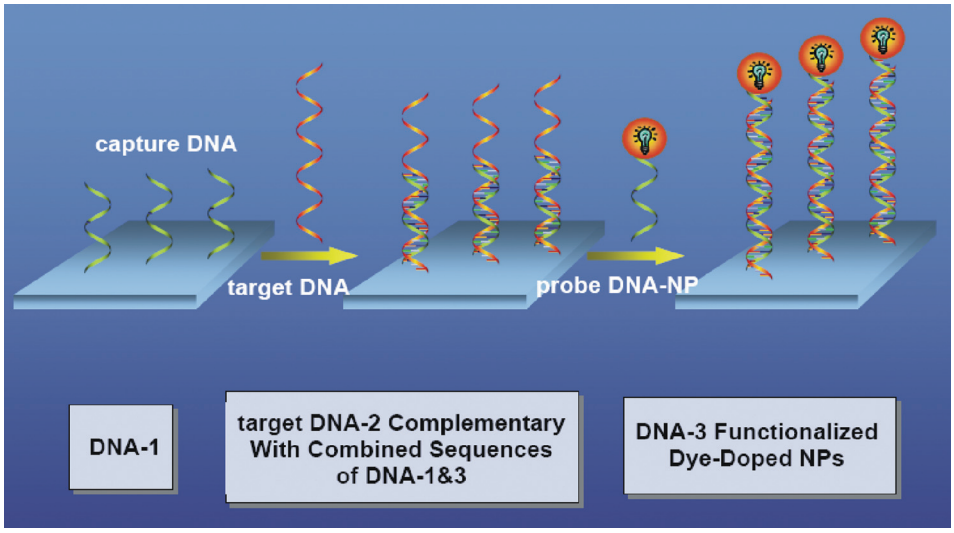

Fig. 7 Schematic of a sandwich DNA assay based on NPs. (Reprinted with permission from[58]. © 2003 American Chemical Society.)

the assay: capture DNA, which was immobilized on a glass surface; a probe sequence, which was attached to TMR-doped silica NPs; and the unlabeled target sequence, which was complementary to both the capture sequence and the probe sequence through different parts of the sequence (Fig. 7). The sandwich assay eliminates the need for labeling of the target, and the hybridization of target DNA also brings one dye-doped silica NP to the surface. This provides a large number of dye molecules on the surface for signaling. By monitoring the luminescent intensity from the surface-bound NPs, DNA target molecules can be detected with increased sensitivity (as low as 0.8 pM). This scheme has also been introduced into a microarray platform to obtain an improved signal[49]. NPs containing over 10 000 TMR dye molecules have been used with an Affymetrix GeneChip. The results show good correlation with the conventional single phycoerythrin labeling method, while both the photostability and detection sensitivity are greatly enhanced and a 20 times lower detection limit is obtained (50 fM).

Zhou *et al.*[59] have developed dye-doped core-shell NPs for microarray-based analysis. The NPs were prepared by attaching dye-alkanethiol $(dT)_{20}$ oligomers to the surface of colloidal Au particles. The Au NPs were then coated with a 10-15 nm silica layer through the thiol functional groups. Two-color DNA microarray-based detection was demonstrated using Cy3- and Cy5-doped NPs in sandwich hybridization. Detection limits of 1 pM for the target DNA are obtained following this scheme.

Bioanalysis based on other molecular recognition schemes

There are other affinity recognition schemes besides antibody-antigen interaction and nucleic acid hybridization. These schemes can also be adopted in silica NP-based assays. Santra *et al.*[60] have doped silica NPs with Rubpy and modified the surface with membrane-anchoring groups, which could be attached to cell membranes with a high affinity. Human leukemia cells have been successfully stained for imaging with this kind of NP. Santra *et al.*[61] have also modified FITC-doped silica NPs with TAT (a cell-penetrating peptide) for labeling of human lung adenocarcinoma and rat brain tissue. In another report, folic acid (folate) was covalently attached to amine-modified NPs. The NPs were then used for targeting of human squamous cancer cells (SCC-9), which was confirmed by laser confocal scanning microscopy[62]. By using avidin-modified NPs, Lian *et al.*[63] have developed a methodology for the detection of biotin-labeled immunoglobulin G (IgG) under different configurations. Biotinylated IgG could be distinguished from regular IgG and easily detected with this generic nanoprobe.

Recently, aptamers have appeared as a new and promising alternative to antibodies as affinity probes. In most cases, the aptamer is a single DNA or RNA strand evolved from systematic evolution of ligands by exponential enrichment procedure (SELEX)[64,65], and it can bind to the target with a specific steric conformation. Herr *et al.*[66] have modified two types of silica NP with an aptamer selected toward a specific type of leukemia cells. One NP was doped with Rubpy dye for fluorescence sensing and the other contained a magnetic core[67] for selective capture and magnetic collection. The two-particle assay was able to capture leukemia cells from a spiked blood sample. The method has also been adapted for multiple cell capture[68], where three types of cancer cells were selectively collected and detected. The approach has a great potential for clinical diagnostic applications.

Summary and future perspectives

Dye-doped silica NPs have been demonstrated to be sensitive labeling reagents for biosensing and imaging. Their flexible conjugation, excellent photostability, and ultrasensitivity make them a powerful tool in bioanalysis. Although there have been many reports on the basic research and application of NPs, they are far

from being fully optimized. There are a few remaining technical problems, of which the most difficult is nonspecific binding when NPs are intended for ultrasensitive detection. In spite of the efforts in NP surface modification, rendering them water soluble, chemically stable, and biocompatible in physiological media, more studies are needed to develop strategies to improve the properties of the NP support matrices and surfaces. This could help reduce nonspecific binding and facilitate the subsequent attachment of biological moieties that will improve the binding kinetics and affinities of the NPs for their target molecules. Nonspecific binding and NP aggregation are still major issues blocking or slowing our progress in realizing the power and ultrasensitivity of nanomaterials in bioanalysis. The lower the detection desired limit in bioanalysis, the bigger the problem in using NPs as there is always a small amount of nonspecific binding.

Total elimination of nonspecific binding is almost impossible, especially when NPs are used in a biological milieu. This problem demands a huge effort in designing new strategies to reduce NP background signal caused by nonspecific binding sufficiently to be able to detect ultratrace amounts of analytes. In addition, better doping techniques are also needed to avoid dye leakage and fluorescence quenching in order to obtain excellent signal reproducibility and sensitivity. Long-term biocompatibility and biotoxicity studies are also needed before this kind of nanomaterial can be used for *in vivo* sensing. With further optimization, dye-doped silica NPs will be widely accepted as labeling reagents in bioanalysis and bioimaging for their excellent intrinsic optical and chemical properties. nt

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