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REVIEW

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FloDots: luminescent nanoparticles

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Abstract Luminescent dye-doped silica nanoparticles (FloDots) have been developed for ultrasensitive bioanalysis and diagnosis in the past several years. Those novel nanoparticles are highly luminescent and extremely photostable. In this paper, we review the preparation, characterization, bioconjugation and bioapplication of FloDots. All the results clearly demonstrated that FloDots have many advantages over currently used luminescent probes, such as traditional fluorophores and quantum dots.

Keywords Dye-doped silica nanoparticles · Nanotechnology · Bioanalysis

Introduction

Luminescent probes for biomolecular analysis and recognition are of great importance and have been widely used in the fields of chemistry, biology, medical science and biotechnology. Introduction of various fluorescent probes and the technological advancements have greatly improved the detection and identification ability of these molecules. All these efforts are focused to enhance the sensitivity and stability in detecting chemical and biological species. Most of the luminescent probes used now are traditional fluorescent dyes like FITC, TMR, Cy3 and Cy5, which have greatly helped the understanding of many chemical and biological problems. However, in addition to the relatively low fluorescence intensity, the photobleaching of these dye molecules has been a severe problem, so those probes are unsuitable for sensitive detection and real-time monitoring where the probes need to be exposed to continuous light excitation.

Recently, some new formats of luminescent probes [1–5] have been developed and have shown their unique properties, among which are quantum dots (Q-dots), fluorescent latex particles and dye-doped nanoparticles (FloDots). Q-dots are composed of atoms of groups II–VI or II–V of the periodic table of elements and are defined as particles with physical dimension smaller than the exciton Bohr radius [1]. Q-dots possess several fancy qualities and have been suggested to be better than traditional dyes: broad excitation spectra, size-tunable fluorescence properties, long fluorescence time and photostability. Nonetheless, owing to their poor solubility, agglutination, blinking and low quantum yield, Q-dots have not been used extensively. Fluorescent latex particles, such as fluorescent polystyrene particles and fluorescent polymethacrylic nanoparticles, have also been employed in some biological applications [4, 5]. However, because of agglomeration, swelling and dye leaking, these latex particles are also not very suitable for bioanalysis.

FloDots are dye-doped silica nanoparticles, which consist of luminescent organic or inorganic dye molecules dispersed inside the silica matrix. The preparation and application of these dye-doped nanoparticles have been extensively studied by our research group at the University of Florida, so we named them FloDots. Besides the facts that silica is not subject to microbial attack and has no

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swelling or porosity change with the change of pH, FloDots have many advantages over other luminescent probes:

1. *High emission intensity*. A large number of luminescent dyes are encapsulated inside a single FloDot, which produces a strong emission signal when the FloDot is properly excited. At the corresponding optimal excitation and emission wavelengths, the luminescence intensity of a single 70-nm Rubpy FloDot is equivalent to that of 39 particles of Q-dot QD 605 (obtained from Quantum Dot Corp., Hayward, CA, USA), 1,290 molecules of Taxas Red, and 72,413 molecules of Rubpy dye [6]. Experiment also found that the intensity ratio of one TMR–dextran FloDot to that of one TMR dye molecule was 10,000 [7]. Clearly, FloDots emit extremely bright light.
2. *Excellent photostability*. Photobleaching is one of the major problems in using traditional dyes for bioanalysis, especially for surface studies such as biosensors or bioimagings. Owing to the silica matrix shielding effect, the doped dye molecules are well protected from environmental oxygen, enabling the fluorescence to be constant and thus providing an accurate measurement for bioanalysis. The photostabilities of the pure organic dyes and the organic dye doped FloDots were compared by continuous illumination for 1,000 s and monitoring with solid-state spectrofluorometry [8]. As shown in Fig. 1, the intensity of pure rhodamine 6G (R6G) decreased rapidly, whereas the fluorescence intensity of the same R6G inside the FloDots did not change significantly under the same conditions [8]. Inorganic dye (Rubpy) doped FloDots were also studied by comparing them against Q-dot QD 605, Rubpy dye and Texas red [6]. Each luminescent probe was excited continuously at its optimal excitation wavelength for 50 min with a Spectrafluor microplate reader (Tecan), and the luminescence intensity was measured every 2 min. The results shown in Fig. 1 clearly demonstrate that the FloDot is as photostable as QD 605, and much more photostable than Texas red and Rubpy dye.
3. *Water solubility and efficient conjugation*. Silica is a desirable matrix for its role in the dispersion of particles in water. The silica surface can also be modified to contain functional groups and to serve as a universal biocompatible and versatile substrate for the immobilization of biomolecules. Biochemically modified FloDots have been developed for various applications using well-developed immobilization protocols based on the silica surface [6, 9–12].

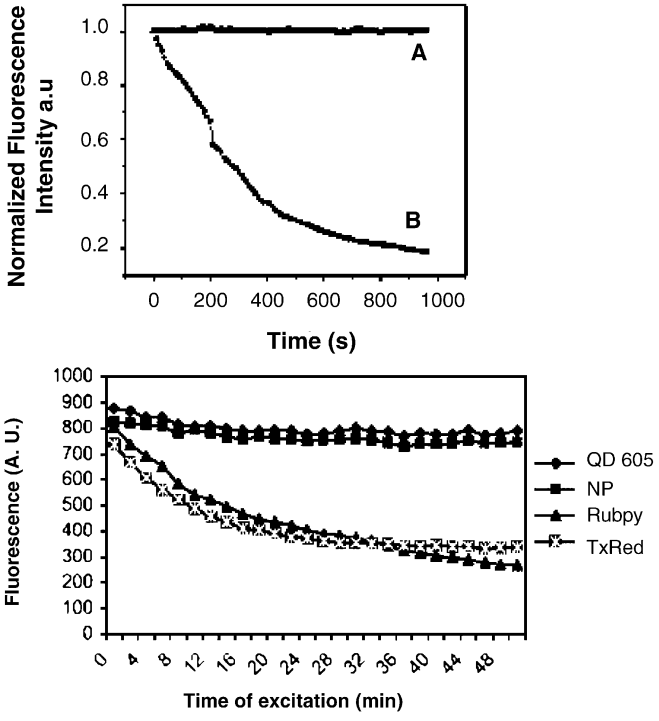

Fig. 1 Photostability comparison. *Left*: Comparison of rhodamine 6G (*R6G*) doped FloDots (*A*) with pure R6G (*B*). *Right*: Comparison of Rubpy-doped FloDots with QD605, Rubpy and Texas red

Preparation of FloDots

FloDots can be easily synthesized through two general routes. One is the reverse microemulsion method [7, 9–11, 13], primarily for the preparation of hydrophilic dye-doped FloDots, and the other is the Stöber method [8, 14], mainly for the preparation of hydrophobic dye-doped FloDots.

A reverse microemulsion is an isotropic and thermodynamically stable single-phase system consisting of water, oil and surfactant. As shown in Fig. 2, nanodroplets of water surrounded by surfactant are dispersed in the continuous bulk oil phase. The water nanodroplets will serve as confined nanomedia for the formation of discrete nanoparticles. The size of the FloDots synthesized is determined by the size of those nanodroplets, which is controlled by the water-to-surfactant molar ratio (W_0). By changing the W_0 value, inorganic dye Rubpy doped FloDots of different sizes have been successfully prepared through ammonia-catalyzed hydrolysis of tetraethylorthosilicate (TEOS) in those water-in-oil microemulsions [9, 10]. The fluorescence spectra, particle size and size distribution of Rubpy-doped FloDots were examined

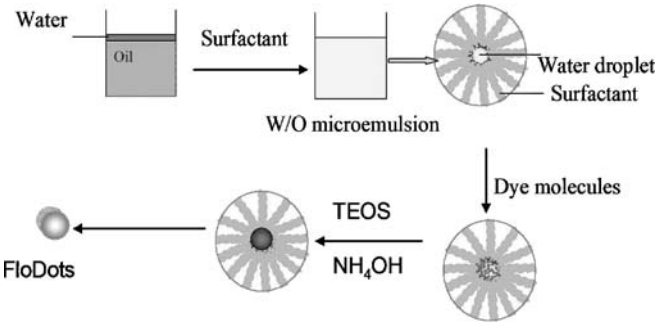

Fig. 2 The synthesis of FloDots through the reverse microemulsion method

systematically as a function of reactant concentrations (TEOS and ammonium hydroxide), the nature of surfactant molecules, W_0, and molar ratios of cosurfactant to surfactant [15]. The results show that the particle size and fluorescence spectra were dependent upon the type of microemulsion system chosen. The particle size was found to decrease with an increase in the concentration of ammonium hydroxide and with an increase in W_0 or the cosurfactant-to-surfactant molar ratio.

Compared with the quantum yield of organic dyes (more than 90 %), inorganic dyes usually have low quantum yields (60–70 %). However, most organic dye molecules are hydrophobic and hence are hard to dissolve in the water nanodroplets in the reverse microemulsion. Two approaches have been used to address the problem [7, 16]. The first one is to dissolve the organic dyes in acid solution. Addition of acetic acid or HCl into the aqueous core was found to improve the solubility of the organic dye inside the reverse microemulsion. The second approach is to use the water-soluble organic dye–dextran complex as the dye source.

The Stöber method is a relatively simple procedure to make silica nanoparticles [14], and starts from the hydrolysis of a silica precursor by HCl or ammonium hydroxide, and is followed by self-polymerization and nanoparticle formation. In the method, TEOS is added to a solution of ethanol, water and ammonia. The ammonia catalyzes the hydrolysis of the TEOS to silicic acid above its solubility in ethanol. The silicic acid homogeneously nucleates as nanometer-size particles which grow to a size determined by the amount of TEOS, water and ammonia. In general, the lower the concentration of water and ammonia, the smaller the particle size. Both hydrophilic and hydrophobic dyes have been incorporated in silica matrices via this method to produce dye-doped microspheres [17].

The Stöber method has been adapted to prepare nanosized FloDots. Tapec et al. [8] used a combination of hydrophobic phenyltriethoxysilane (PTES) and hydrophilic TEOS as the silica sources, which can produce both hydrophobic and hydrophilic sites inside the silica matrix. The organic dye (R6G) doped inside the silica matrix was found to be proportional to the amount of PTES. R6G-doped FloDots prepared by this approach exhibited high fluorescence intensity, excellent photostability, and minimal dye leakage after prolonged storage in aqueous solution [8]. However, a high quantity of PTES was found to make the particle hydrophobic and caused difficulties in surface modification for bioconjugation, and the particles formed using this approach has a broad size distribution.

Another modification on the Stöber method to prepare FloDots is to conjugate the organic dye to a silane molecule as the silica source (unpublished data). Succinimidyl esters or isothiocyanates are reactive with amine groups, and maleimides are reactive with thiol groups. Dyes with these reactive groups can be easily purchased commercially. Amine silanes such as aminopropyltriethoxysilane or thiol silanes such as (3-mercaptopropyl)triethoxysilane should be used with the corresponding dye reactive groups [18, 19].

Characterization of FloDots

The freshly prepared FloDots are often characterized for size and optical property, which are the significant factors to determine whether the FloDots can be used in bioanalysis. FloDot sizes are generally evaluated by transmission electron microscopy, scanning electron microscopy or light scattering. As shown in Fig. 3, FloDots from several nanometers to 100 nm could be successfully prepared. The results of the size measurements also revealed that FloDots prepared through the reverse microemulsion method have better uniformity than those prepared through the Stöber method.

The fluorescence of FloDots was compared with that of the corresponding pure dye molecule. The FloDots have optical spectra very similar to those of the encapsulated dyes. As shown in Fig. 4, the excitation spectra remain the same for pure Rubpy and the FloDot in aqueous solution, but the emission maximum of the FloDot shifts by 7 nm towards longer wavelength when compared with the pure Rubpy dye owing to aggregation of dye molecules inside the nanoparticle [9]. Experiments also confirm that there is a general trend with the FloDot for the emission to be shifted compared with the free dye (unpublished data). For TMR-doped FloDots, the emission is at 577±1 nm, while for the TMR dye in pH 7.4 phosphate buffer it is at 572–573 nm. For rhodamine B, the emission of the FloDot is at 579–582 nm and that of the pure dye is at 576–577 nm. For FITC, the FloDots have maximum emission between 509 and 514 nm and the pure dye fluoresces between 516–518 nm, a blueshift instead of a redshift. It has also been noticed that the highly doped rhodamine B FloDots generally have a greater redshift than the lightly doped FloDots.

In addition, as shown already, the optical characterization confirms that the fluorescence intensity of FloDots is thousands of times that of pure the dye, and the FloDots do not suffer from photobleaching as the pure dyes do over a long period of continuous intense light exposure. These

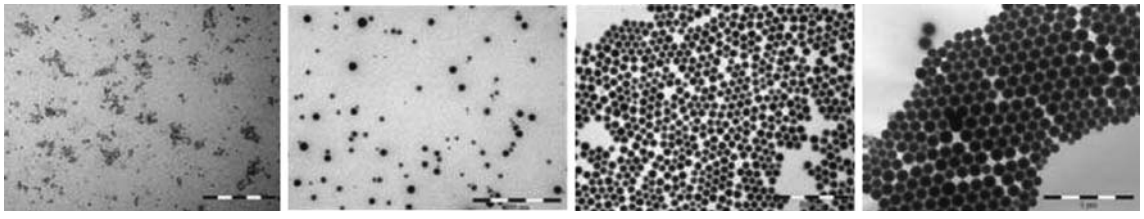

Fig. 3 Transmission electron microscopy images of FloDots. From *left* to *right*, the average size was 10, 30, 60 and 120 nm

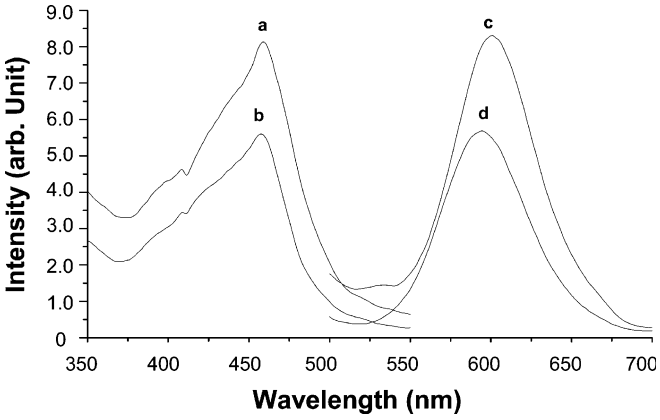

Fig. 4 Optical properties of FloDots. Excitation and emission spectra recorded in the aqueous phase for pure Rubpy (*b*, *d*) and nanoparticles (*a*, *c*)

Table 1 Chemical binding for bioconjugation of FloDots

FloDot surface-modification method	Functional group on FloDots	Functional group on biomolecules	Bioconjugation method
MPTS/pH 5.5, ethanol	–SH	–S–S–	Thiol–disulfide exchange
CNBr/CH_3CN	–OCN	$–NH_2$	Direct reaction
DETA/CH_3COOH	$–NH_2$	$–NH_2$	Glutaraldehyde cross-link
CEST or TMSP or NH_2-FloDot + succinic anhydride/DMF	–COOH	$–NH_2$	Carbodiimide chemistry

MPTS 3-mercaptopropyltrimethoxysilane, *DETA* trimethoxysilylpropyldiethylenetriamine, *CEST* carboxyethylsilanetriol, *TMSPN*-(trimethoxysilylpropyl)ethylenediamine, *DMF* dimethylformamide

superior properties suggest that FloDots are excellent probes for bioanalysis.

Bioconjugation of FloDots

Coupling the FloDots to a biomolecule for target recognition or signal generation is critical for their use in bioanalysis. The silica matrix provides a versatile substrate for the surface immobilization. A variety of biomodification methods have been utilized to conjugate FloDots to various biomolecules, including nucleic acids, antibodies, enzymes and other proteins. Among these methods, physical absorption and chemical binding are two commonly used ones for surface modification of FloDots.

Physical absorption is mainly employed to modify the FloDots with an avidin molecule [8, 13]. Avidin is a glycoprotein containing four identical subunits, each of which can bind with one biotin molecule through the noncovalent biotin–avidin interaction. Biotin is a small molecule and it can be easily conjugated with other biomolecules without significantly changing their biological properties. The avidin molecule has a positive charge and therefore it can easily attach to the negatively charged silica surface of the FloDot through electrostatic coupling. The absorbed avidin layer is usually stabilized by cross-linking it with glutaraldehyde, followed by incubating it in tris(hydroxymethyl)aminomethane–HCl buffer to remove the unbound avidin.

The chemical binding method takes advantage of well-developed silica chemistry, and biomolecules are conjugated to the FloDots through the interaction of thiol (–SH) [20], cyanate ester (–OCN) [9], amine ($–NH_2$) [9, 12] or carboxyl (–COOH) [6, 16] groups. These binding methods are listed in Table 1. Thiol or disulfide coupling is a simple and efficient procedure to directly immobilize disulfide-containing biomolecules on the surface of FloDots through a thiol–disulfide exchange reaction, but it cannot be used under strong reducing conditions where the disulfide bond is very unstable. However, most biomolecules have amino groups, so cyanate ester, amine and carboxyl coupling are commonly used bioconjugation methods for FloDots.

Hydrophobic interaction is also used for the biomodification of FloDots. Experiments have been performed to treat the amine-modified FloDots with lauroyl chloride in dry tetrahydrofuran in an argon atmosphere [9, 12]. The lauroyl-functionalized FloDots are useful as membrane probes to bind the hydrophobic part of the membrane which consists mainly of phospholipids bilayers.

Bioanalysis application of FloDots

Bioimaging

Bioimaging is very important in biological and biomedical research. The most widely used technique with luminescent probes is probably fluorescence microscopy for immunocytochemistry and immunohistochemistry. Immunolabeling of the cell surface with fluorescent probes is commonly used in cell biology, immunology and clinical laboratories. Unlike the conventional immunoassay where each antibody is labeled with at most several fluorophores for signaling, every biomodified FloDot can cause significant signal amplification from thousands of fluorescent dye molecules doped inside each particle. The bioimaging application of FloDots has been demonstrated by the recognition of human leukemia cells with mouse antihuman CD10 antibody [9]. Leukemia cell suspensions were incubated with bare FloDos or antibody-modified FloDots, and were imaged by both optical microscopy and fluorescence microscopy. As shown in Fig. 5, the fluorescence images correlated well with the optical images and the leukemia cells could be identified easily, clearly and with high efficiency using these antibody-labeled FloDots.

Cell detection

Using a similar strategy in bioimaging, Zhao et al. [11] developed a new immunoassay for precise and rapid

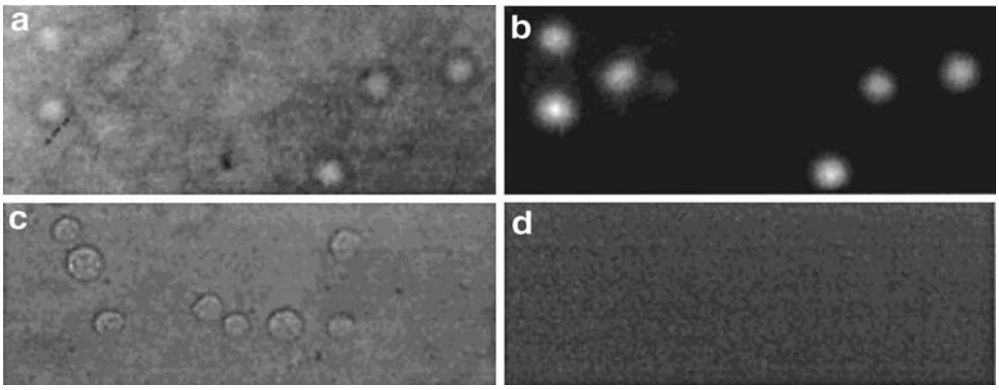

Fig. 5 Optical and fluorescence images of leukemia cells (5–7 μm in size): **a** optical and **b** luminescence images of leukemia cells incubated with antibody-immobilized FloDots, and **c** optical and **d** luminescence images of leukemia cells incubated with bare FloDots as a control

determination of a single bacteria cell with bioconjugated FloDots. The rapid and sensitive determination of pathogenic bacteria is extremely important in biotechnology, medical diagnosis and the current fight against bioterrorism. *Escherichia coli* O157:H7 was chosen as the target because it is one of the most dangerous agents of bloodborne disease. Owing to the low infectious dose of *E. coli* O157:H7 (approximately 10–100 cells), the presence of even a single bacterium in food may pose a serious health risk. Traditional methods for the detection of trace amounts of bacteria require amplification or enrichment of target bacteria in the sample, which is laborious and time-consuming because of complicated assay procedures. In the new method based on bioconjugated FloDots, each FloDot provides greatly amplified fluorescence intensity because of thousands of dye molecules doped inside. For a bacterium, many surface antigens are available for specific recognition using antibody-conjugated FloDots. Therefore, as shown in Fig. 6, it is possible to have thousands of FloDots bind to each bacterium (a few microns in size) for an extremely high signal. The single bacterium detection capability within 20 min was confirmed by the plate-counting method and was realized by using two independent optical techniques. Furthermore, this assay was employed to detect multiple samples with high throughput using a 384-well microplate format. In addition, accurate enumeration of 1–400 bacterial cells in spiked ground-beef samples was demonstrated. All these results clearly show the excellent utility of biologically conjugated FloDots can be adapted for the detection of a wide variety of bacterial pathogens, including bioterrorism agents, in food, clinical and environmental samples.

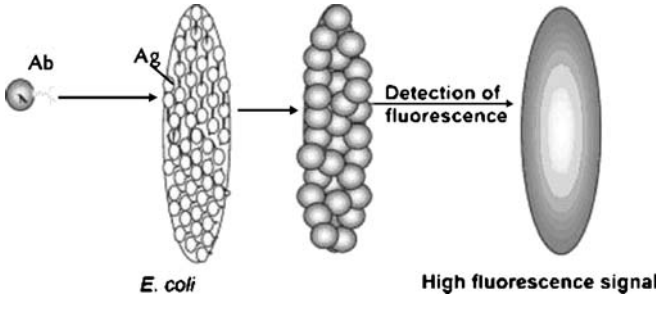

Fig. 6 Single bacterium detection scheme. A single bacterium will bind with many FloDots for signaling

Gene detection

Biological science is being revolutionized by the availability of more information regarding complete genome sequences for many different pathogenic and nonpathogenic microorganisms, plants, etc. DNA/RNA sequences provide the framework allowing investigation of biological processes by the use of comprehensive approaches. Gene detection is useful for disease identification and for providing accurate results whether or not the target disease gene sequences exist in the sample [21]. Recently, efforts have been made to develop new biotechnologies to improve the sensitivity and selectivity of gene analysis [22–24]. Nucleic acid hybridization, offering excellent selectivity by DNA base pairs coupled with optical detection, is most widely used [25–28]. In ultratrace gene analysis, signal amplification is most critical and is typically achieved by coupling fluorophores, such as organic dyes [25–28], to the DNA probes.

The application of FloDots for gene detection is based on the fact that each FloDot has the fluorescence intensity of thousands of dye molecules; therefore, each gene hybridization will be reported by thousands of fluorophores. An ultrasensitive assay was thus achieved using FloDots as shown in Fig. 7 [13]. Typically, a biotinylated capture DNA is immobilized through avidin–biotin linkage to the glass slide. This capture DNA is the complement of a portion of the analyte sequence. Hybridization between the capture DNA and the analyte sequence is carried out while the remaining target sequence hybridizes with a probe DNA conjugated with the FloDot. One probe DNA hybridizes one target DNA, and thus brings one FloDot to the surface, leaving a large number of dye molecules on the surface for signaling. By monitoring the fluorescence intensity from these surface-bound FloDots, DNA target molecules can be detected with high sensitivity. Using this assay method, oligonucleotides ranging from 12 to 100 base pairs will be used as capture and detection probes to determine the detection limit. The shortest probes with the highest detection sensitivity will be adopted for future use.

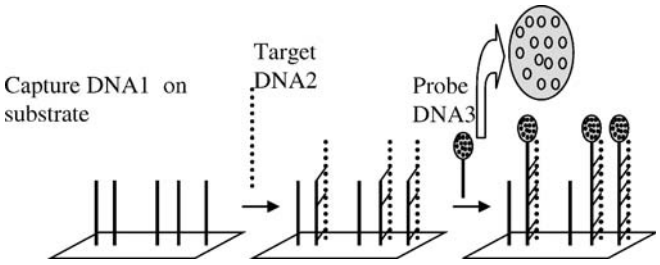

Fig. 7 A sandwich DNA analysis assay based on FloDots. The substrate is a glass slide coated with an avidin layer

Protein array

A protein array has become an increasingly important tool in functional genomics and proteomics. Those arrays have great potential in global studies of protein expression, protein profiling, and protein–protein interaction. Compared with conventional fluorescent labeling, which is based on single dye molecule, the bioconjugated nanoparticles that gather thousands of dye molecules in each particle are brighter and thus more useful.

Serial dilutions of human immunoglobulin G (IgG)–biotin along with regular human IgG were manually spotted on microarray slides, followed by incubation with avidin-labeled FloDots. As shown in Fig. 8, there were strong signals in the upper four rows where human IgG–biotin was spotted, while no signal was detected in the lower four rows where human IgG was spotted (negative controls), indicating a high labeling specificity. The fluorescence intensity from each spot was proportional to the concentration of human IgG–biotin applied, demonstrating the possibility to quantify the amount of the target by measuring the fluorescence. Experiments were also performed to compare the sensitivity of FloDots with that of conventional fluorescent dyes. These results clearly showed enhanced fluorescent intensity and hence increased detection sensitivity.

Multiplexed bioanalysis

Dual-luminophore-doped FloDots with different surface modifications have been successfully developed for simultaneous multiplex signaling and bioanalysis [29]. These functional FloDots can be easily labeled with biomolecules and possess optical encoding capability. Two luminophores, Osbpy and Rubpy, were entrapped together inside silica nanoparticles at precisely controlled ratios, with desirable sizes and required surface functionality. Single-wavelength excitation with dual emission endows the FloDots with optical encoding capability for rapid and high-throughput multiplexed detection.

A proof-of-concept flow cytometry experiment was conducted for the detection of mixtures of analytes with two kinds of those dual-luminophore-doped FloDots, which had intensity ratios (610 nm to 710 nm) of 9:1 and 2:1, and labeled with human IgG and mouse IgG, respectively. The same number of antimouse IgG and antihuman IgG coated microspheres (1.62×10^{-17} mol, respectively) were mixed with these two kinds of IgG-coated nanoparticles (8.1×10^{-15} mol, respectively) to form a cocktail. Figure 9 shows a two-dimensional dot plot illustrating classification of the two microsphere sets based on simultaneous analysis of logarithmic orange luminescence (FL2) and red luminescence (FL3) in a flow cytometer. The dots in the R1 region represent antimouse IgG microspheres coated with mouse IgG nanoparticles (2:1 ratio), and those in the R2 region represent antihuman IgG microspheres coated with human IgG nanoparticles (9:1 ratio). The number of dots in the two regions was counted. Experimental results show that the population distribution was 46.56 and 53.42 %, respectively, in regions R1 and R2, which correlates well with the expected value. This result demonstrated a model for the multiplexed detection of microspheres by bioconjugated FloDots, and provided the possibility to apply these smart FloDots to multiplexed target recognition.

Conclusion

We have discussed here the properties, preparation, characterization, surface modification and application to bioanalysis of the newly developed FloDots, or luminescent dye doped silica nanoparticles. Each FloDot consists of thousands of dye molecules throughout the silica network, and can be made uniformly in a variety of

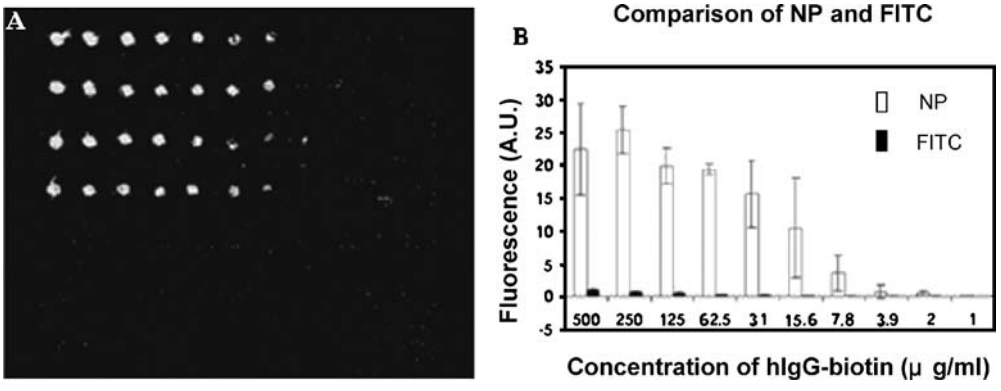

Fig. 8 Labeling of protein chips with FloDots or FITC. **a** The top four rows were spotted with serial dilutions (from *left* to *right*) of biotinylated human immunoglobulin G (*hIgG*), while the lower four rows were spotted with serial dilutions of hIgG. Avidin–FloDot was used to detect the fluorescence. **b** Comparison of fluorescence signal intensities of protein chips labeled with either FloDots or FITC

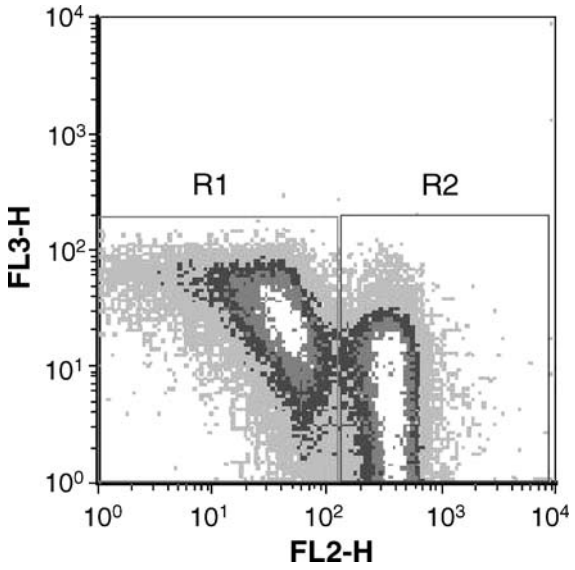

Fig. 9 Two-dimensional dot plot showing the classification of the two microsphere sets based on simultaneous analysis of logarithmic orange luminescence (*FL2-H*) and logarithmic red luminescence (*FL3-H*)

sizes. Those FloDots are highly luminescent and are highly photostable in comparison with most commonly used fluorescent probes, so they can be employed in many areas of bioanalysis after surface modification and bioconjugation.

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