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REVIEW ARTICLE

Fluorescent labels in biosensors for pathogen detection

Bianmiao Li[1], Qiaoling Yu[1,2], and Yixiang Duan[1]

[1]*Research Center of Analytical Instrumentation, Analytical & Testing Centre, College of Chemistry, Sichuan University, Chengdu, P. R. China and* [2]*Department of Environmental and Food Engineering, Liuzhou Vocational and Technical College, Liuzhou, P. R. China*

Abstract

Infectious diseases caused by pathogens have become a life-threatening problem for millions of people around the world in recent years. Therefore, the need of efficient, fast, low-cost and user-friendly biosensing systems to monitor pathogen has increased enormously in the last few years. This paper presents an overview of different fluorescent labels and the utilization of fluorescence-based biosensor techniques for rapid, direct, sensitive and real-time identification of bacteria. In these biosensors, organic dyes, nanomaterials and rare-earth elements are playing an increasing role in the design of biosensing systems with an interest for applications in bacterial analysis.

Keywords

Carbon nanotube, carbon quantum dot, fluorescence-based biosensor, fluorescent labels, organic dye, pathogen detection, rare-earth elements, semiconductor quantum dots

History

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Introduction

Pathogenic bacteria, fungi and viruses are ubiquitous in human surroundings. The presence of these pathogens poses a considerable risk to human health especially the sick people, pregnant women, babies, young children and others with compromised immune systems. The most prominent pathogens (García, 2001) are *Escherichia coli O157:H7* (*E. coli O157:H7*) which brings mycotoxins, exotoxins and enterotoxins, *Staphylococcus aureus* strains (*S. aureus*), *Bacillus anthracis* (produces anthrax toxin), *Shigella spp.*, *Clostridium perfringens*, *Campylobacter jejuni*, and *Clostridium botulinum*, resulting in a powerful paralytic toxin botulin, *Listeria monocytogenes* (*L. monocytogenes*), *Yersinia enterocolitica*, *Coxiella burnetii*, *Vibrio cholera* and *Salmonella spp*.

Food or food products as the real proviso for healthy life are the potent transmitting media of more than 250 known diseases and the yearly cost of food borne diseases is approximately $5–6 billion in the United States (Arora et al., 2011). Drinking water sources contaminated by microorganisms have increased rapidly in recent years, and the annual expense of industrial water treatment systems in the United States is estimated to be billions of dollars, which added an additional burden to economic development (Chuang et al., 2001). Public concern has dramatically increased on food and water safety over the past decades. Therefore, it is of paramount importance to monitor these microorganisms for the prevention of nosocomial infections, the maintenance of general public health and complying with legislative and quality standards.

Various pathogen detection techniques have been developed in recent years. Traditional methods containing latex agglutination, immuno-diffusion, impedance microbiology and immuno-precipitation (Liu & Duan, 2006) were cumbersome, multi-step and time consuming, requiring several days to confirm the presumptive results (Jofre et al., 2005). This made them unsuitable for fast and direct analysis of pathogens without pre-enrichment. Although some of them could be very sensitive, inexpensive and supply both quantitative and qualitative information. Enzyme-linked immunosorbent assays (ELISA) and polymerase chain reaction (PCR) techniques were frequently used to detect pathogens, but there were some shortcomings to limit their utilization. Specifically, ELISA needs pre-enrichment steps and 2–3 h for the assay process (Beckers et al., 1988; Hayes et al., 1991). On the other hand, active enzymes require special storage and carcinogenic reagents are often required for these colorimetric assays (Giletto & Fyffe, 1998). PCR not only suffers the problem of false positives by amplifying dead cells and making data interpretation complex (Deisingh & Thompson, 2004), but also requires sophisticated instruments and expensive commercial reagents for routine and large-scale assays or point-of-care detection (Papillard-Marechal et al., 2011). In addition, it also involves multi-step processing, well-trained personnel, considerable time and expensive detection. Hence, it needs to develop suitable detection methods that permit accurate, rapid and sensitive analysis to monitor pathogens.

Address for correspondence: Yixiang Duan, Research Center of Analytical Instrumentation, Analytical & Testing Centre, College of Chemistry, Sichuan University, Chengdu 610064, P. R. China. E-mail: yduan@scu.edu.cn

Biosensor technology for pathogen testing has been rapidly developed over the past decades since it is a fast, direct, online and provides real-time safety assessment. Optical sensors, due to their high sensitivity and selectivity, are the most popular methods because they can adopt plenty of techniques to detect targeted analytes and are based on well-appointed methods including fluorescence, phosphorescence, reflectance, chemiluminescence, light absorbance, light polarization, etc. Advantages of compactness, flexibility, resistance to electrical noise and small probe size could be offered by optical sensors (Ansari et al., 2010). Molecular emission such as fluorescence and phosphorescence is about 1000 times superior in sensitivity than most spectrophotometric methods and at the same time maintaining lower limits of detection for the desired analytes (Guilbault, 1990; Yu et al., 2012).

In a typical fluorescence measurement, the fluorophore is firstly excited by a specific wavelength of light and then it emits light at a different wavelength. Reporter molecules, labeled with fluorescent dyes, are required in sensitive detection of the targeted analytes presented at a trace level in the sample. Fluorescence detection has several well-established advantages including (Alan, 2006): high detection sensitivity (such as single molecule detection); fast response times; a localized fluorescence signal; multiplexed assays using multicolor dyes; and the straightforward labeling process, which provided appropriate functional groups on the target.

Fluorescent labeling reagents are vital components for sensitive fluorescence detection. Fluorescent labeling reagents include: organic dyes, nanoparticles, rare-earth elements, etc.

Fluorescent labels

Organic dyes

Organic dyes were frequently used as labeled molecules in fluorescence biosensors (Panchuk-Voloshina et al., 1999), including: fluorescein, sulforhodamine, rhodamine dyes, cyanine dyes (carbocyanines Cy3, Cy5 and Cy7), and Alexa dyes (Alexa350, Alexa430, Alexa488, Alexa532, Alexa546, Alexa568 and Alexa594).

The fluorescein was the first dye used as a fluorescent label followed by rhodamine (Alan, 2006). Fluorescein, with a higher possible quantum yield, has been widely used in fluorescent assays although it considerably quenches on conjugation to biological molecules, particularly proteins. In addition, it is also sensitive to pH and has photobleaching problems (Benchaib et al., 1996). Fluorescein isothiocyanate (FITC) is one of the most frequently used fluorescent markers (Li et al., 2004). The pH insensitive rhodamine dyes have superior resistance to photodegradation than that of fluorescein analogs, but they are more difficult to use because of low water solubility, nonspecific binding of the labeled species due to their hydrophobic planar structures and fluorescence quenching resulted from their dimerization of bound multiple rhodamine labels (Alan, 2006) when they conjugated to proteins. Researchers began to develop new multicolor fluorescent labels for antibodies and DNA probes in the 1980s and the early 1990s and phycoerythrin was introduced in 1982 (Oi et al., 1982). This powerful multi-chromophore protein-labeling reagent permitted sensitive two-color lymphocyte subset analysis with a single argon ion laser and now three or four-color analysis with a single laser are commonplace with phycobiliprotein-based energy transfer reagents (Alan, 2006).

However, the molecular weight of the phycobiliproteins are 1–1.5 times the molecular weight of IgG, which limits diffusion of the labeled proteins, antibodies or DNA probes into regions of cells, chromosomes and tissues where the structural matrix is tight. Multicolor cyanine dye labels such as Cy3, Cy5 and Cy7 appeared widely in immunological reagents and DNA probes (Kallioniemi et al., 1992). Negatively charged sulfonate groups can greatly improve water solubility, decrease the inherent tendency of molecules to aggregate, presumably as a result of the increased polarity imparted by the sulfonic acid moiety and reduce fluorescence-quenching dye-dye interactions that happened on the surface of antibodies (Mujumdar et al., 1993). They are directly added to the ring systems of fluorescent cyanine dyes, increasing the brightness of the dyes in aqueous media, and make Cy3 and Cy5 widely used for labeling DNA and RNA for gene expression studies (Schena et al., 1995). Using Cy3 as a label immobilized on a capillary tube and mesoporous chip to detect CEA has also been explored in our research group (Duan & Yu, 2012, Yu et al., 2013). Alexa fluorescent labels were developed by Richard Haugland and his team (Panchuk-Voloshina et al., 1999); since then there has been a noticeable trend to use new labeling reagents with longer wavelength fluorescence. Alexa dyes were used to conjugate to the primary amines of biomolecules in succinimidyl ester form. These dyes have superior, large unparalleled fluorescence emission covering the entire visible spectra and better photostability by conjugating to a new series of sulfonated compounds, lower background fluorescence at long wavelengths and pH-insensitivity over a very broad range. Haugland and colleagues demonstrated that Alexa dye conjugated antibodies, streptavidin, and other proteins tended to be brighter and more resistant to photobleaching than alternative low molecular weight fluorescent dyes with similar spectral properties. However, Alexa dyes carry a net negative charge that might cause a nonspecific electrostatic interaction with positively charged cell structures (Mahmudi-Azer et al., 1998). These organic dyes discussed above can also be incorporated into nanoscale particles to form more sophisticated dyes that can be used together with metal nanoclusters, quantum dots (QDs), lanthanide chelates, etc. (Demchenko, 2008).

Optical biosensors based on the measurement of the fluorescence emission of organic dyes or protein-based fluorophores have been reported (Curtis et al., 2008; Ko & Grant, 2006; Simpson-Stroot et al., 2008). Problems were encountered with the highly sensitive fluorescent detection with lower fluorescence output, pH-sensitive, photobleaching and the formation of nonfluorescent products. These shortcomings confined their utilization in a sensing system. Therefore, the alternative materials need to be developed urgently.

Nanoparticles

Nanotechnology is a center of advances in biological research. Nanostructured materials, typically with dimensions

smaller than 100 nm, own extraordinary electrical, physical, thermal, mechanical and multifunctional properties because of the following effects: their macro-quantum tunnel, quantum size, surface and mini size. Nanoparticles such as QDs and carbon nanotubes (CNTs) have gained considerable interest from biological researchers as immobilization platforms or labels for the sensitive recognition events (Ansari et al., 2010) owing to their unique properties including good biocompatibility, high specific surface area, non-toxicity, good electro-catalytic activity, high chemical stability, thermal stability and fast electron communication. Therefore, nanoparticles offer novel alternatives for bioanalysis in sensing and biosensing applications (Arben, 2010; Rosi & Mirkin, 2005). Meanwhile, the combination of nanoparticles with fluorescence has been an emerging research area and paved a new road in biology and medicine because of the high sensitivity, simplicity and diversity of the fluorescence-based method. Nanoparticles are promising compounds that offer new opportunities for biomolecule detection.

Quantum dots

Suitable labels are at the core of fluorescence sensing. The emerging development of QDs is one of the most exciting and controversial advances in labeling technology as a result of their unique optical and chemical properties including broad absorption with narrow photoluminescence spectra, low photobleaching, high quantum yield and resistance to chemical degradation. QDs are somewhat spherical semiconductor nanocrystals with physical dimension ranging from 1 to 10 nm (Sutherland, 2002), and they composed of elements from groups II–VI and III–V in the periodic table (Shen, 2011) such as CdSe, CdTe, PbS, HgTe, InP, PbSe, PbTe, InAs and GaAs (Klostranec & Chan, 2006; Smith & Nie, 2004). One of the most intriguing features of QDs is that changing the particle size by altering their synthesis procedures and chemical composition could tune their wavelength of fluorescence emission. The fluorescence emitted by QDs will be blue shifted as the decrease of their size due to the confinement effect. The size-tunable property of QDs is useful for simultaneous labeling and detection of multiple analytes (Rosenthal, 2001). The low photodegradation rate of QDs can be used for continuous or long-term monitoring of slow biological processes, which are superior to traditional organic fluorophores (Chan & Nie, 1998; Wu et al., 2003). Besides, the long fluorescence lifetime of QDs signals of the order of 10–50 ns, can be distinguished from background fluorescence and may be applied during highly sensitive detection (Bruchez et al., 1998). Recent researches have indicated that QDs can be covalently linked with biorecognition molecules such as antibodies, nucleic acids, peptides or small-molecule ligands as biological labels (Bailey et al., 2004). Now QDs have been increasingly utilized as labeling probes and have conferred greater advantages than traditional fluorophores such as organic dyes (Vinayaka & Thakur, 2010). QDs are 20 times brighter, 100 times more stable against photobleaching, 10–15 times larger than the extinction coefficients of the organic dyes and one-third the breadth of spectral lines (Chan & Nie, 1998). In recent years, luminescent QDs utilized as donors in fluorescence resonance energy transfer (FRET) measurements have led to a new era in nanotechnology for the optical detection of pathogens.

However, QDs also have three main shortcomings (Shen, 2011), which limit their biological applications. First, QDs are made from toxic elements against the aqueous organism. Second, QDs have no intrinsic solubility because they are usually prepared in organic solvents at high temperature and easily covered by small hydrophobic molecules. Third, QDs are easy to aggregate when transferred into water and this will decrease their fluorescence quantum yields. That is to say, their physical stability could be easily damaged through simple processing steps in water. Bruchez et al. (1998) testified that QDs were superior to existing fluorophores by using QDs as fluorescent labels in a single-excitation, dual-emission experiment on mouse fibroblasts. There are other reports about the utilization of QDs for microorganism detection and for *in vivo* imaging of tissues (Dubertret et al., 2002; Larson et al., 2003; Zhu et al., 2004).

The shortcomings of QDs make it worthwhile to search for more benign nanomaterials with similar optical properties. Recently, newly emergent carbon-based nanomaterials, fluorescent carbon dots (CDs) with interesting photoluminescence properties, have attracted increasing concerns and considerable research interest. CDs, whose size is below 10 nm, were first obtained in 2004 by purifying single-walled carbon nanotubes through preparative electrophoresis (Xu et al., 2004). CDs possess several favorable characteristics similar to the traditional semiconductor QDs, like high photostability against photobleaching, good biocompatibility, tunable excitation and emission wavelength, relative small size, and low molecular weight. In addition, CDs have no intrinsic toxicity, elemental scarcity or complex, rigorous preparation process (Mao et al., 2010). One significant feature of CDs is that they do not contain any heavy metals, and therefore are more environmental friendly. At the same time, they are much safer in biological applications, making them superior to semiconductor QDs (Larson et al., 2003; Yang et al., 2009). According to these properties, CDs are amenable to serving as fluorescence probes in optical bioimaging (Yang et al., 2009).

To date, various progresses of CDs regarding their synthesis, property, and applications were reported as reviewed by Shen et al. (2012). In general, methods such as arc discharge (Xu et al., 2004), laser ablation (Hu et al., 2009), thermal oxidation of carbon precursors (Bourlinos et al., 2008), microwave synthesis (Wang et al., 2011), electrochemical oxidation (Long et al., 2012), etc. were developed to synthesize CDs. Chou et al. (2012) synthesized highly emissive CDs via pyrolysis process from a glycerol solvent as a single precursor for cell imaging and drug release. Lin et al. (2012) synthesized blue fluorescent CDs by microwave assisted one-step procedure without surface passivation. A newest tendency to prepare CDs was introduced with so-called ''green'' raw materials, e.g. coffee grounds (Hsu et al., 2012), pomelo peel (Lu et al., 2012), chitosan (Yang et al., 2012), eggshell membrane (Wang et al., 2012) and orange juice (Mohapatra, 2012) as carbon source, which have no need of complicated post-treatment processes and can avoid using large amounts of strong acid or expensive raw materials. Consequently, CDs have gradually become a rising

star in bioanalysis due to their benign, abundant and inexpensive nature (Baker & Baker, 2010).

Carbon nanotubes

CNTs have become a worldwide research hotspot due to their high surface/volume ratio, unique electronic and mechanical properties, strong adsorbability and microwave absorptivity (Davis et al., 1998), when they were first noticed and characterized in 1991 by Iijima (1991) of NEC Corporation in Japan. CNTs usually exist in two formats: single-wall CNTs (SWCNTs) and multi-wall CNTs (MWCNTs). CNTs have high bacterial adsorption capacity and are capable of concentrating different types of pathogens (Deng et al., 2008; Srivastava et al., 2004; Upadhyayula et al., 2009) because of its high aspect ratio and large specific surface area that imparts high antimicrobial nature to the material (Kang et al., 2007). CNTs have the ability to be used in biological applications due to their tunable emission, from 900 nm to 1400 nm, at near-infrared (NIR) wavelengths, where tissue and blood are most transparent and, at the same time, exhibit a low autofluorescent background signal (O'Connell et al., 2002; Wray et al., 1988). In addition, CNTs exhibit strong Raman signals as well as fluorescence emissions in the near infrared region without photobleach under prolonged excitation, which is an advantage in optical nanobiomarker applications (Xu et al., 2009).

Rare-earth elements

Rare-earth elements have gained extensive attention and been used as the fluorescent labeling reagents for microsecond time-resolved fluorescence bioassay (Yuan & Wang, 2005) in recent years owing to their unique spectroscopic characteristics including large Stokes shift, long fluorescence lifetime and sharp line-like emission bands occurring at long wavelengths. These specific properties overcome light scattering, enable spectral and temporal discrimination against background fluorescence compared with common fluorophores, leading to excellent detection sensitivity.

There are many elements in rare-earth elements, but the best studied is europium (Eu). It is easy to label oligonucleotides for time-resolved fluorescence bioanalysis (Qin et al., 2010) because of its larger Stokes shift, red emission and exclusion of second-order scattering interference in the maximum fluorescence intensity (Ai et al., 2009), and the fluorescence of Eu^{3+} can be dramatically enhanced when it is coordinated with appropriate organic ligands (Yuan & Matsumoto, 1996).

So far, a number of ultrasensitive bioanalytical assays, based on time-resolved fluorescent techniques using lanthanide complexes as fluorescent labels, have been developed (Härmä et al., 2001; Yuan & Wang, 2005), some of them were based on FRET technology (Härmä et al., 2007; Valanne et al., 2009).

Combination between fluorophores and biorecognition

One significant development in fluorescent immunoassay was the conjugation of biorecognition and fluorescent labels. Several commonly used methods were introduced during this study.

Commercially available fluorophores with a succinimidyl ester or maleimide reactive group were the most commonly used reagents for site-specific biolabeling that targets the primary amino or thiol groups, respectively, on biomolecules such as proteins or DNA (Sapsford et al., 2006). The labeling occurred between organic dyes such as AlexaFluor via the binding of a succinimidyl ester moiety of the AlexaFluor to the primary amines of the proteins, which resulted in the formation of antibody-AlexaFluor (Ko & Grant, 2006). All Alexa dyes were used with their succinimidyl ester form to conjugate the primary amines of biomolecules. Because the reactive portion of the Alexa dye hydrolyzes rapidly, the conjugate process took place by dissolving dyes at 10 mg/ml anhydrous dimethylformamide (DMF) and immediately added antibody to the solutions to obtain the desired dye-to-antibody complex while stirring (Panchuk-Voloshina et al., 1999). Having poly(ethylene glycol) (PEG) chains on the surface of chelated Eu(III) incorporated core-shell fluorescent nanosphere proved to be suitable for highly sensitive immunoassays. A hetero-crosslinker was adopted to converted primary amino groups from the distal end of the PEG into maleimide groups, then the Fab fragments of antibodies were covalently bound to the surface via their -SH functional group (Seydack, 2005).

In the classical EDC method, for example, the conjugation of antibodies onto carboxyl group modified QDs, EDC(1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide) first activated the carboxyl group to produce a reactive O-acylisourea intermediate, which then reacted with the nucleophile of NHS (N-hydroxy succinimide). Eventually, the antibody can be immobilized through a labile Schiff's base formation between its amine group and the aldehyde group of NHS. In addition, QDs conjugated to biomolecules can also be achieved by covalent binding, electrostatic binding, noncovalent biotin–avidin binding and nickel-based histidine tagging as described in Figure 1 (Xing et al., 2007). These strategies were frequently utilized during the combination of QDs and antibodies (Goldman et al., 2002; Liu et al., 2007; Mukundan et al., 2009; Zhang et al., 2010). Ki Rahm & Ik-Joong (2009) conjugated antibody to QDs by covalent binding using glutaraldehyde. Briefly, the mixture of glutaraldehyde and QDs was achieved by adding 10 mM glutaraldehyde and 0.08 ml CdS-quantum-dot-encapsulated PAMAM dendrimer in PBS solution and stirred for 30 min, followed by adding various concentrations of AlexaFluor 488 goat anti-rabbit antibody to form antibody-QDs conjugates.

Semiconductor QDs were explored as fluorescence labels in immunoassays for simultaneous detection of *E. coli* O157:H7 and *Salmonella*. Different emission wavelengths of QDs (525 nm and 705 nm) were conjugated to anti-*E. coli* O157 and anti-Salmonella antibodies using the coupling strategy between streptavidin and biotin. In detail, 10 ml biotinylated anti-Salmonella antibody and 10 ml biotinylated anti-*E. coli* antibody were added into 100 ml of 0.2 mM of QDs 705 and QDs 525 with 30 min incubating at room temperature, therefore, the QD705-anti-Salmonella antibody and QD525-anti-*E. coli* antibody were formed and ready for further use (Yang & Li, 2006). In the experiment of human

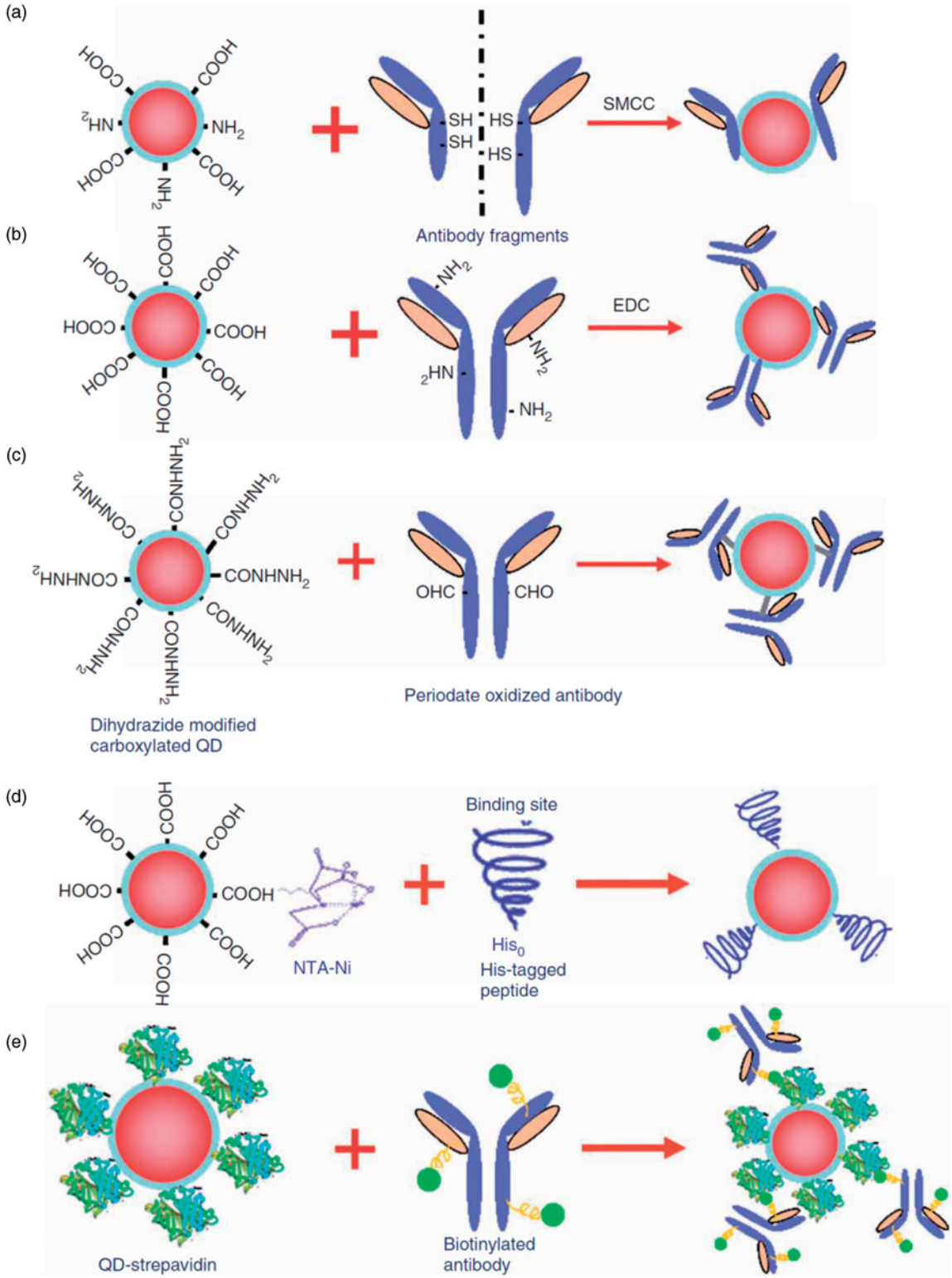

Figure 1. Schematic diagrams showing various methods for QD-antibody (QD-Ab) bioconjugation. (a) QD conjugation to antibody fragments via disulphide reduction and sulfhydryl-amine coupling; (b) covalent coupling between carboxylic acid (–COOH) coated QDs and primary amines ($-NH_2$)on intact antibodies using EDAC as a catalyst; (c) site-directed conjugation via oxidized carbohydrate groups on the antibody Fc portion and covalent reactions with hydrazide-modified QDs; (d) conjugation of histidine-tagged peptides or antibodies to Ni-NTA modified QDs; and (e) noncovalent conjugation of streptavidin-coated QDs to biotinylated antibodies. Reprinted by permission from Nature Protocols, Xing et al., Bioconjugated quantum dots for multiplexed and quantitative immunohistochemistry, 2, 1152–65. Copyright 2007.

serum albumin (HSA) detection, streptavidin-conjugated CdSe/ZnS QDs were mixed with biotinylated anti-HSA solution in PBS, due to the affinity of streptavidin–biotin chemistry, anti-HAS was successfully attached to QDs to form anti-HAS-QD complex in the mixed solution (Tu et al., 2012).

Kerman et al. (2005) prepared carboxyl modified SWCNTs and covalently immobilized oligonucleotides probe to

Table 1. Fluorescent labels in biosensors used for pathogen detection.

Pathogen	Fluorescent label	Detection method	The detection limit or detection time	Reference
S. aureus	FITC	Sandwich immunoassay	24 h	Chang et al., 1996
SEB	Cy5	Sandwich immunoassay	20 ng/mL, less than 20 min	Rowe-Taitt et al., 2000
SEB	CdSe-ZnS QDs	Sandwich fluoroimmunoassay	15 ng/mL	Goldman et al., 2002
Staphylococcal enterotoxin C1	silica-Ru(bpy)$_3$Cl$_2$ (Rubpy)	Sandwich immunoassay	0.3 ng/mL	Hun & Zhang, 2007
Salmonella	Cy5	Sandwich immunoassay	10^4 CFU/mL	Zhou et al., 1997
S. typhimurium	QDs	Immuno-magnetic beads	10^3 CFU/mL, 10^4 CFU/mL	Yang & Li, 2005
S. typhimurium	Alexa Fluor 546 and Alexa Fluor 594	Fret	10^5 CFU/g, 5 min	Ko & Grant, 2006
E. coli O157	Cy5	Sandwich immunoassay	10 cells/capillary, 3 h	Zhu et al., 2005
E. coli O157:H7	CdSe/ZnS QDs	Sandwich immunoassay	2.3 CFU/mL, 30 min	Liu et al., 2007
L. monocytogenes	Cy5	Sandwich immunoassay	4.3×10^3 CFU/mL, 2.5 h 4.1×10^8 CFU/mL	Geng et al., 2004; Tims et al., 2001
L. monocytogenes	QDs 605	Immuno-magnetic beads	2–3 CFU/mL, 1.5 h	Wang et al., 2007
BT	Zn/CdSe QDs	Not mentioned	10^3 CFU/mL	Ikanovic et al., 2007
B. anthracis spores	Eu (III)	Not mentioned	0.2 nM CaDPA	Ai et al., 2009

functionalize SWCNTs through their amino group linked at the 5′ end of oligonucleotides, CDs used as biocompatible optical nanoprobes successfully targeted cancer cells for *in vitro* cancer diagnostics by conjugating with transferrin. In this protocol, an appropriate amount of transferrin was added to ultrapure water followed by the mixing of EDC solution and stirred for 30 min. Next, the NH_2 terminated CDs were added to this mixture and incubated at room temperature for 2 h to allow the covalent bonding of protein and CDs (Li et al., 2010).

Applications

Most prominent pathogens including *E. coli* O157:H7, *Staphylococcus aureus*, *Salmonella* spp., *L. monocytogenes* can lead to severe health consequences in animals and humans. Hence, there is an urgent need to develop techniques for diverse pathogenic bacteria detection. Biosensors offer advantages over current analytical methods. Besides their good selectivity and low cost, they are portable to use in working sites and have the ability of measuring samples with minimal sample preparation. Fluorescence-based biosensors have been classified in the category of most popular biosensors with the detection purposes for their high selectivity and sensitivity. Fluorescent labels in biosensors used for pathogen detection are summarized in Table 1.

Staphylococcus

A fiber-optic biosensor used for the detection of *S. aureus* was developed in 1996 (Chang et al., 1996). It involved a sandwich immunoassay in the biosensor with FITC conjugated with anti-protein A (a product secreted only by *S. aureus*) immunoglobulin G to produce fluorescent signal of the antigen–antibody reaction and the detection time was shortened to 24 h.

The above method was adopted to detect *Staphylococcal enterotoxin B* (*SEB*) by using cyanine dye Cy5 as label (Rowe-Taitt et al., 2000). A planar waveguide captured *SEB* present in samples, a small diode laser excited the fluorophore and a CCD camera detected the pattern of fluorescent antibody–*SEB* complexes on the waveguide surface. The detection limit was 20 ng/mL and the total time for the biochemical assay and extraction procedure was less than 20 min.

Goldman et al. (2002) used the same fluoroimmunoassay method in which the avidin-bridged CdSe–ZnS core shell nanocrystal QDs as fluorescence label to detect *SEB*. A SpectraFluor Plus microtiter plate reader was used to detect the signal. The lowest concentration of *SEB* that gave a useful signal over the background was approximately 15 ng/mL. This method has been successfully applied in the detection of other protein toxins (e.g. *cholera toxin*). In the sandwich immunoassay developed by Hun & Zhang (2007), silica-Ru(bpy)$_3$Cl$_2$ (Rubpy) dye core-shell nanoparticles were added to generate fluorescence and the fluorometric measurement was carried out by the instrumentation system containing a spectrofluorophotometer with xenon discharge excitation source and a 4.0 mm diameter bifurcated glass optical bundle at the common end. The detection limit of *Staphylococcal enterotoxin C1* was 0.3 ng/mL. The sandwich fluoroimmunoassay is described in Figure 2.

Salmonella

A compact immunosensor used Cy5-labeled antibody for the detection of *Salmonella* (Zhou et al., 1997). A sandwich format was formed over the surface of a fiber tip on which the antibody was immobilized and the fluorescent signal was detected by a CCD fiber spectrometer with a detection limit of 10^4 CFU/mL. Yang & Li (2005) also applied immunomagnetic beads for separation and QDs as fluorescence labels for detection of *S. typhimurium* or simultaneous detection of *E. coli* O157:H7 and *Salmonella* (Yang & Li, 2006). Targeted bacteria were separated from samples by using specific antibodies coated with magnetic beads. QDs with different emission wavelengths conjugated to antibodies and reacted with bead-cell complexes to form bead-cell-QD complexes. The detection limit was 10^4 CFU/mL and 10^3 CFU/mL, respectively, within 2 h. It was expected that this method would be suitable for detecting 3–4 species simultaneously with well-separated emission spectra.

A fiber optic biosensor (Ko & Grant, 2006) based on FRET to detect *S. typhimurium* in ground pork was fabricated using Alexa Fluor 546 as donor fluorophore, and Alexa Fluor

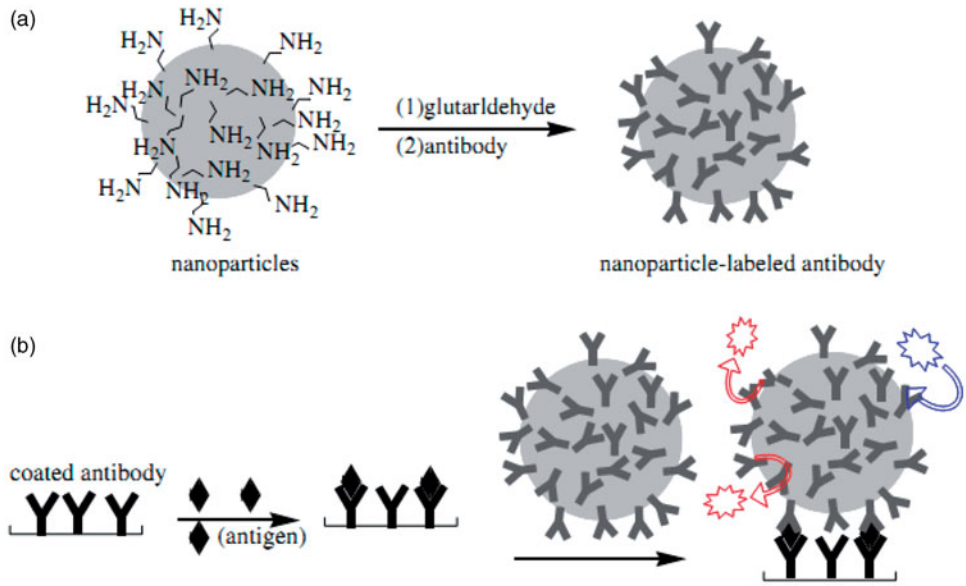

Figure 2. Schematic representation of antibody immobilization process onto functionalized fluorescent core-shell nanoparticles (a). Principle of the sandwich fluoroimmunoassay (b). Reprinted from Food Chemistry, Vol. 105, Hun Xu and Zhang Zhujun, A novel sensitive Staphylococcal enterotoxin C1 fluoroimmunoassay based on functionalized fluorescent core-shell nanoparticle labels, pages No.1623–1629. Copyright 2007 with permission from Elsevier.

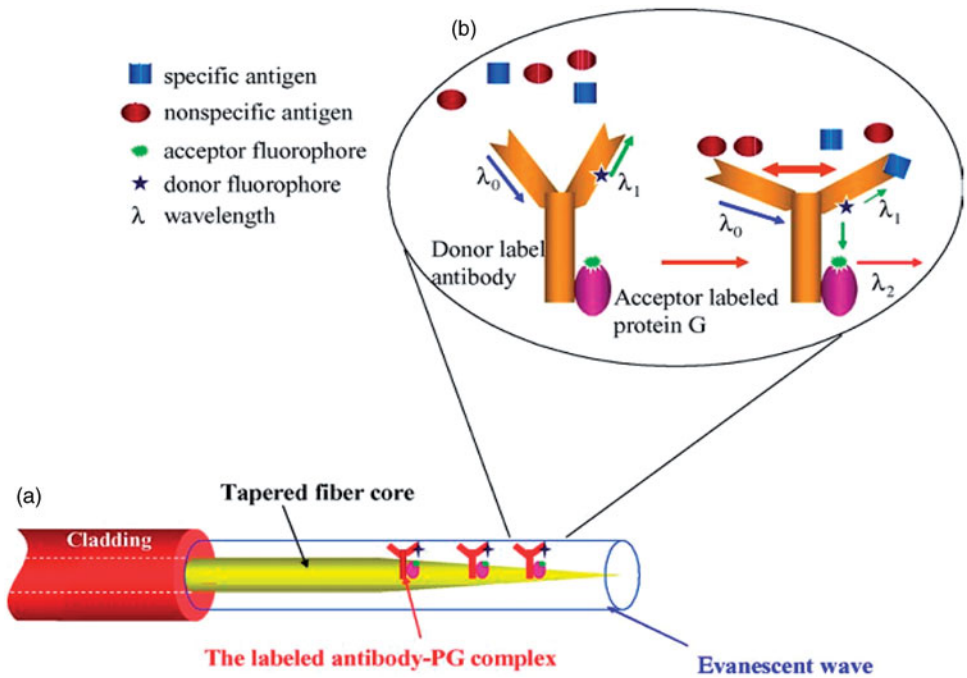

Figure 3. (a) The evanescent wave penetrates pass the fiber core surface and excites the labeled antibody–protein G complexes. Only the interaction between the analyte and the immobilized molecular complexes within the evanescent wave region can be sensed. (b) Conceptual schematic of a FRET immunosensor. When the antibody binds with the target antigen, a conformational change in the 3D structure of an antibody will occur, resulting in non-radiative energy transfer from the donor to acceptor fluorophores. Reprinted from Biosensors and Bioelectronics, Vol. 21, Sungho Ko and Sheila A. Grant, A novel FRET-based optical fiber biosensor for rapid detection of Salmonella typhimurium, pages No.1283–1290. Copyright 2006 with permission from Elsevier.

594 as acceptor fluorophore since they have high spectral overlap and energy transfer (Panchuk-Voloshina et al., 1999). This biosensor integrating FRET with antibody whose conformation changed as it bound to antigens on the surface of the fiber, which made it possible to detect analytes while drastically reducing false positives because conformational changes were measured. Figure 3 shows the interaction between analyte and immobilized molecular complexes, and Figure 4 details the schematic of the detection device. The packing density of 0.033 mg/mL gave the best energy transfer data in PBS solution and the detection limit was 10^5 CFU/g of *S. typhimurium* in homogenized pork sample solutions within a 5 min response time. The observed experimental results showed that this biosensor system appeared to represent an efficient technology to direct, fast, sensitive and on-site analysis of food samples without complex sample preparation.

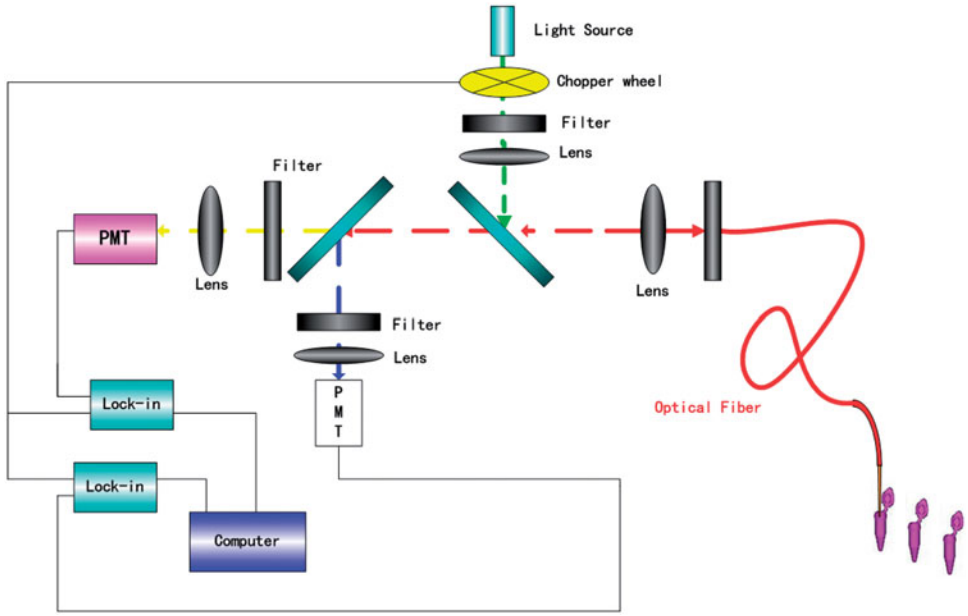

Figure 4. Schematic of detection device. Reprinted from Biosensors and Bioelectronics, Vol. 21, Sungho Ko and Sheila A. Grant, A novel FRET-based optical fiber biosensor for rapid detection of Salmonella typhimurium, pages No. 1283–1290. Copyright 2006 with permission from Elsevier.

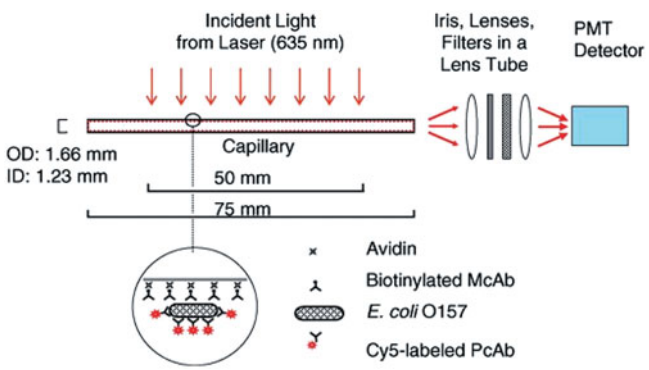

Figure 5. Side view of waveguide capillary and experimental configuration of the integrating waveguide biosensor. A sandwich immunoassay is performed on the inner surface of capillary using Cy5-labelled anti-E. coli O157 antibody for generation of the specific fluorescent signal. Lines with letters 1.66, 75 and 50 mm indicate the diameter, length and laser excitation area of the capillary, respectively. Reprinted from Biosensors and Bioelectronics, Vol. 21, Peixuan Zhu, et al., Detection of water-borne E. coli O157 using the integrating waveguide biosensor, pages No. 678–683. Copyright 2005 with permission from Elsevier.

However, there were problems such as washing, filtration, enrichment step, sandwich assay and immunomagnetic separation that required comparably long reaction time and multi-step processing. Therefore, the methods for more accurate, inexpensive, simple and rapid monitoring, as well as being capable of detecting microorganisms with a minimum of manipulations are needed.

Escherichia coli O157:H7

Zhu et al. (2005) described a Cy5 labeled sandwich fluorescence biosensor to detect water-borne *E. coli* O157 inside a glass capillary with a 635 nm, 12 mW diode laser (Figure 5). The limit of detection was 10 cells per capillary (0.075 mL volume) for quantitative detection with an assay time of 3 h. Another interesting sandwich immunoassay to detect *E. coli* O157:H7 was described whereby CdSe/ZnS core/shell dendron nanocrystals and a flowing chamber with a microporous immunofilter (Liu et al., 2007). The detectable level was as low as 2.3 CFU/mL and the assay time decreased to 30 min without any enrichment and incubation. The result demonstrated that this biosensor was better than those other published biosensor systems, which had a detection limit of 10^3 CFU/mL in 2 h utilizing immunomagnetic separation and a CCD-array spectrometer (Su & Li, 2004), and 10^4 CFU/mL for simultaneous detection of *E. coli* O157:H7 and *S. typhimurium* by using QDs as fluorescent labels (Yang & Li, 2006). El-Boubbou, et al. (2007) carried out a method to monitor *E. coli* O157 in which surface functionalized magnetic nanoparticles incubated with solutions of PicoGreen stained *E. coli*.

Laser-induced fluorescence coupled with flow cytometry was also used to detect *E. coli O157:H7* in ground beef (Johnson et al., 2001) with many advantages like intrinsically automatic, sensitive only to selected bacteria and capacity to examine a large volume of food or water in real time.

Listeria monocytogenes

A sandwich immunoassay was carried out to detect *L. monocytogenes* cells by an enrichment step (Tims et al., 2001), which employed Cy5-labeled anti-Listeria IgG on a polystyrene fiber to generate a specific fluorescent signal collected by a photodetector. The detection limit was approximately 4.1×10^8 CFU/mL for a pure culture of *L. monocytogenes*. Geng et al. (2004) improved this assay with a detection limit of 4.3×10^3 CFU/mL within 2.5 h using better quality antibodies.

QDs and magnetic nanobeads were also used in biosensors for a higher capture efficiency for rapid detecting *L. monocytogenes* (Wang et al., 2007). Magnetic nanobeads were used to separate the bead-cell conjugates from the solution and the QDs 605 was utilized to produce fluorescence. A laptop-controlled portable system was employed to monitor the fluorescence signals. The detection limit was 2–3 CFU/mL *L. monocytogenes* in a pure culture with total detection time 1.5 h. Multiple bacterial species in one sample may be detected simultaneously by using different sizes of QDs as fluorescent labels for each different bacterial genus/species.

Other pathogens

A novel fluorescence detection strategy for *Bacillus thuringiensis* (BT) spores was reported utilizing ZnS capped CdSe QDs as fluorescence labels (Ikanovic et al., 2007). The assay is based on the fluorescence observed after binding an aptamer-QD to BT spores. In this system, ZnS/CdSe QDs attached to aptamer sequences that are specific for BT spores and a Cary Eclipse Fluorescence Spectrophotometer was used to obtain the fluorescence spectra. The aptamer-QD complexes were excited at 400 nm and the fluorescence read between 430 and 700 nm. The assay is semi-quantitative, specific and can detect BT at concentrations of about 1000 CFU/mL.

Human enterovirus 71 (*EV71*) in homogeneous solution was determined through a new dual-color fluorescent immune ensemble probe consisting of a conjugated lower toxic water-soluble CdTe:Zn^{2+} QDs and $Ru(bpy)_2$(mcbpy-O-Su-ester)$(PF_6)_2$ antibody complex (Chen et al., 2011). Figure 6 illustrates the schematic of *EV71* detection. *EV71* was detected at subnanogram per milliliter concentrations in the presence of 160 μg/mL bovine serum albumin; moreover, this strategy could be used as a universal method for other proteins or viruses by changing conjugated antibodies.

Goldman and his colleagues (Goldman et al., 2004) had developed a system in which antibodies were labeled with different sizes of CdSe/ZnS QDs and a sandwich immunoassay format was formed to detect *Cholera toxin*, *Ricin*, *Shiga-like toxin 1* and *Staphylococcal enterotoxin B* simultaneously in single wells of a microtiter plate.

A lateral flow DNA biosensor was reported (Chua et al., 2011) for detecting foodborne *Vibrio cholerae* by using capture reagents coupled to carrier beads and fluorescein-labeled detector reagent bioconjugated to Au NPs. Denton et al. (2009) fabricated a portable, automated fiber optic evanescent wave biosensor using Cy5 as fluorescent label to detect *M. tuberculosis* from lung tissue. Eu (III)-based fluorescence biosensor was first proposed using Eu (III) as the fluorescent label to monitor *B. anthracis spores* in aqueous solution, and the detection limit was 0.2 nM calcium dipicolinate (CaDPA) which is a unique biomarker for *Bacillus spores* (Ai et al., 2009). A sensitive and rapid *Respiratory syncytial virus* real-time detection method using dual-color QDs or fluorescence energy transfer nanobeads was carried out by Agrawal et al., (2005). Biosensors for detecting *Mycobacterium tuberculosis*, *Cryptosporidium parvum* and *Giardia lamblia*, as well as *Campylobacter jejuni* were also reported (Ikanovic et al., 2007; Qin et al., 2008; Sapsford et al., 2006; Zhu et al., 2004).

Conclusion

Biosensor fields are growing rapidly and, as described throughout this review, fluorescent biosensor systems have been incorporated with different fluorescent labels and pathogen recognition agents. As a significant portion of the fluorescence-based biosensor, fluorescent labels play an increasing role in the design of biosensing systems. Several important fluorescent labels such as organic dyes, fluorescent NPs and rare-earth elements and their utilization in pathogen detection are described in this text. Among these biosensors, nanobiosensor technologies fit very well to user-friendly and in-field application devices. It is expected that future methods should be able to detect multifarious species of microorganisms simultaneously, and antibodies or aptamers functionalized multiple QDs or fluorescent probes can be excited at the same time, making multiplex detection of these signals possible (Tully et al., 2006). Some commonly used fluorescence biosensor systems were enumerated for the rapid detection of pathogens such as *S. typhimurium*, *E. coli* O157:H7 and so on.

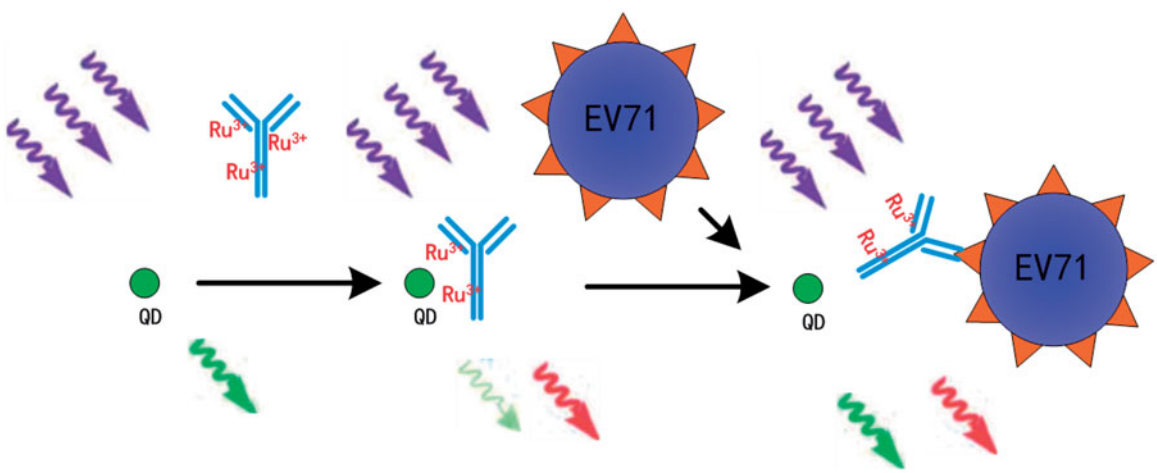

Figure 6. Schematic illustration of the QDs and Ru-Ab complex based EV71 biosensor. Reprinted with permission from Analytical Chemistry, 83, Lu Chen, et al., Dual-color fluorescence and homogeneous immunoassay for the determination of Human Enterovirus 71, 7316–7322. Copyright 2011 with permission from American Chemical Society.

Novel immunoassay protocols were carried out based on surface enhanced Raman scattering-fluorescence dual mode nanoprobes combined with magnetic nanobeads (Zong et al., 2013), or on-chip dual detection based on self-assembled magnetic bead patterns and quantum dots (Yu et al., 2013), which were suitable methods for pathogen detection.

The application of fluorescence-based biosensors could lead to significant improvement in quality control, food safety and traceability in industry. They can achieve very low detection limits even single cells. Besides, they offer possibilities of multiple analyses, ensure a high stability, reduce reagent volumes and detection times and maintain a high sensitivity. Nevertheless, only a few biosensors for bacterial detection are approaching the commercialization stage related to both technology and the market. Besides, it is a very complicated task to enhance the specificity and sensitivity of fluorescent biosensor systems within one bacterial biosensor device. This is the main reason why the penetration of biosensors onto the market is so slow!

The review is comprehensive, but incomplete, because of the extensive research effort in this area. Recent advances in nanotechnology have enabled a paradigm shift in fluorescence-based sensor technology, while significant challenges still exist including, but not limited to, reliable control of length, diameter, density, orientation, crystallization and hierarchical assembly. The interaction mechanism between nanomaterial and biomolecules has not yet been well clarified. In a word, processing, interface problems, characterization, tailoring of nanomaterial, availability of high quality nanomaterial, and the mechanisms governing the behavior of these nanoscale composites are also potential challenges for the current techniques.

Besides the efforts to explore new fluorescent materials, a latest ''blockbuster'', graphene, with salient and unique properties, has become an increasingly important nanomaterial and shown great prospect in the fields of material science and nanotechnology. Compared with these fluorescent nanoparticles, graphene has been shown to be an extraordinary quencher. It is frequently adopted together with fluorescent materials to quench their fluorescence, which is a significant improvement in fluorescent biosensors (Chang et al., 2010; Dong et al., 2010; Wang et al., 2012). Graphene is now at the forefront of almost all rapidly developing fields of science and engineering as reviewed by Yao et al. (2012) and Yang et al. (2012).

Clearly, the pioneering work carried out by researchers in the physical sciences has laid a solid foundation for understanding the properties of nanomaterials, and their continued contributions will give the next generation of engineers and technologists an excellent platform on which to design and build devices to solve a variety of problems in biology and medicine. Future trends will continue to implement fluorescence-based technologies into microdevices, ultimately permitting on-site analysis to be performed in a rapid, reliable and sensitive manner and the prospects of developing biosensors for diseases may help to fight from the epidemics at a glance. In addition, there is the possibility to open a commercial market of fluorescent biosensors within the food and drink industry, medical and clinical diagnostics and environmental control.

Declaration of interest

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