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Review Article

Research progress of near-infrared fluorescence immunoassay

Abstract

Near-infrared fluorescence immunoassay has been widely studied in the bio analytical field. This review mainly introduces the basic principles of near-infrared spectroscopy and near-infrared detection technology, and summarizes the properties, characteristics and recent improvement of optical properties and signal intensity of three near-infrared fluorescence probes (i.e. organic fluorophores, quantum dots and rare earth compounds). We describe the applications of near-infrared fluorescence technology in immunoassay, and prospect the application potential of lateral flow assay (LFA) based on this probe in the rapid detection of pathogens. Our team intends to establish a new platform which has highly sensitive near-infrared fluorescence probes (NIFPs) combined with portable and simple immunochromatographic test strips (ICTSs) for rapid detection of food borne viruses. This will provide technical support for a rapid detection on the port.

Practical applications

The applications of near-infrared fluorescence probes (NIFPs) in immunological analysis and clinically important biomarkers were also elaborated. Moreover, NIFPs-based immunoassay adaptable for rapid detection of foodborne pathogens was also forecasted. In 2017, our team has developed an approach for detecting pathogens such as *Salmonella*, *Vibrio parahaemolyticus*, *Vibrio cholerae* and *Listeria monocytogenes* by near-infrared immunoassay.

Introduction

The infrared spectroscopy is an electromagnetic spectrum between the visible region and the microwave region. It is divided into three regions: near-infrared, mid-infrared, and far-infrared. The near-infrared band is between 780-2526 nm spectral sections of the electromagnetic spectrum [1]. Due to its high sensitivity and selectivity, fluorescence spectroscopy has a wide range of applications in analytical chemistry, especially in bioanalysis. Most biomolecules have no fluorescence or weak fluorescence, and their detection sensitivity is low. In order to detect them with high sensitivity, people use fluorescent labeling reagents or fluoregenic reagents to label or derivatize the analytes. The formation of a covalent or non-covalently bound material with high fluorescence intensity greatly decreases the detection limit.

Fluorescent reagents such as fluorescein, rhodamine or phthalaldehyde (OPA) currently used for labeling or derivatization. They have high fluorescence quantum yields, but their maximum absorption wavelength and fluorescence emission wavelength are mostly less than 600 nm. For biological samples, the sample matrix and some impurities also have light absorption or fluorescence in this area. In addition, the effect of light scattering will often cause more serious background interference, which limits the sensitivity of fluorescence analysis. Compared with the conventional fluorescence (λem<600nm) detection, the light absorption or fluorescence intensity of the biological sample matrix is small in the near-infrared fluorescence (λem>600nm) region. Therefore, the background interference is greatly reduced, and since the intensity of the scattered light is inversely proportional to the fourth power of the wavelength. As the wavelength increases, the Raman scattering rapidly decreases, and the scattering interference is also greatly reduced.

In recent years, near-infrared fluorescent labeling reagents and detection techniques based on diode lasers with compact structure, good stability and low price have higher sensitivity, and have been used for near-infrared fluorescence immunoassay, flow cytometry, fluorescence detection of biological active substances in high performance capillary electrophoresis separation. At the same time, with the continuous integration of various technologies, the development of many rapid detection devices such as laser fluorescence, sensors and immunodetection devices has promoted the development of near-infrared fluorescent marker detection and analysis in the biological field.

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NIR spectroscopy technology has many advantages which determine its wide application field. NIR spectroscopy could be used for food quality analysis, such as researching the freshness of agricultural products, testing fruit firmness and quality, detecting fruit sugar and acidity, controlling the quality of baked products and detecting alcohol during alcohol fermentation in food industry [2]. It also could detect changes in sugar content, prediction of different meat characteristics, and determination of nutrients in dairy products and identification of the authenticity of edible oil. In agriculture, it could be applied to crop quality analysis and evaluation, crop variety resource identification and quality breeding and crop resistance index analysis. Near-infrared spectroscopy has new developments in other fields such as microbiology. Currently, most of the research objects are bacteria and yeast and a few objects are fungi and algae.

Near-infrared fluorescent probe types

Near infrared fluorescence immunoassay is a novel lateral flow assay (LFA) that combines near-infrared fluorescence probes (NIFPs) with immunoassay. In view of its outstanding advantages of small background interference and strong tissue penetration, NIFPs have attracted more and more attention in recent years (Table 1). Due to the limited sensitivity of labeled probes based on color signals such as colloidal gold, enzymes and electrochemical signal generation probes are expensive and cumbersome to operate, and it is difficult to achieve one-step detection. NIFPs have become one of the most popular signals molecules are widely used in various bioanalytical fields [3]. NIFPs with emission spectra in the near-infrared region (wavelength 650~1100 nm), which attracts attention in the field of analysis owing to their high signal-to-noise ratio and ideal detection sensitivity [4]. First, the biomatrix rarely fluoresces spontaneously in the near-infrared region, allowing NIFPs-based assays to be protected from background fluorescence interference. Second, because the intensity of the scattered light is inversely proportional to the fourth power of the wavelength, NIFPs with emitted light in the long-wave region are subject to its interference is small. The strong penetrability and small damage to biological tissues is another major advantage of NIFPs, which has been widely used in non-destructive testing [5] and biography [6].

Near-infrared fluorescent material: Although new forms of NIFPs are being synthesized, traditional organic fluorescent dyes are still a mainstream of current near-infrared fluorescent probes. Traditional organic fluorescent dyes such as cyanine, rhodamine, and other thiazide organic dyes, are widely developed as NIFPs in which cyanine dyes are most favored for good biocompatibility (Stoyanov, 2000). Cyanine dye-labeled biological samples mainly have non-covalent, covalent forms of electrostatic/hydrophobic interactions and bioconjugates with some biomolecules through reactive groups for labeling nucleic acids, proteins, immunoassays and contrast.

Lee et al. [7], further improved and derivatized it using the DOFLA (different-oriented fluorescence library approach), and obtained more than 40 NIFPs with excellent physicochemical properties. The light stability of AZA396 was 60 times higher than that of BodipyFl. Researchers not only synthesize new near-infrared organic probes, in order to better apply it to biological analysis, but also pay attention to the traditional near-infrared fluorescent dye water solubility, quantum yield, chemical and light stability, biocompatibility improvements in key traits [8]. Aggregation of dyes or dye combinations can lead to severe quenching of fluorescence [9], and many studies have focused on improving the water solubility of dyes. Since it was first discovered and reported by Waggoner et al. in 1993 [10], attachment of sulfonate groups to the aromatic ring is effective in increasing the water solubility of NIFPs. This conclusion was also confirmed by Cheng et al. [11]. The well-known commercialized near-infrared organic molecules Cy5.5 and Cy7 from Amersham Biosciences are all sulfonate phthalocyanine dye structures. In addition, studies have shown that the ideal water solubility can also be achieved by coating a hydrophobic dye onto a single layer of hydrophilic phospholipids on the surface of liposomes [12]. In order to effectively increase the fluorescence intensity of dyes to determine trace target analytes, researchers continue to explore efficient signal amplification strategies. For example, a large amount of fluorescent dye is wrapped in nanoparticles to form a near-infrared nanoparticle probe with higher fluorescence intensity, which has been widely confirmed to effectively improve the detection signal intensity while improving the chemical and photostability of the labeled molecule [13]. Furthermore, based

Table 1: Types and characteristics of near-infrared fluorescent probes.

NIFP Types	NIFPs	NIFP advantages	Reference
Near-infrared fluorescent material	AZA396	higher stability than BodipyF1	Lee et al, 2013
	Cy5.5、Cy7	Water solubility increased	Cheng et al., 2015
	near-infrared nanoparticle probe	Higher signal intensity ,chemical and photostability	Christian et al., 2007
	Silver island films resonance	signal intensity increased	Malicka et al., 2007
	Gold nano-shells resonance	signal intensity increased	Tam et al., 2007
	nano-microspheres with a multi-polymer materia	improve the biocompatibility	Kim et al., 2006
Near-infrared fluorescent quantum dot	semiconductor nano-microcrystals	high quantum yield, strong anti-photobleaching ability and concentrated emission spectrum	Kairdolf et al., 2013
Near-infrared fluorescent rare earth complex	Lanthanides containing Nd^{3+}, Er^{3+}, Yb^{3+} and Tm^{3+}	large stock's displacement; long fluorescence lifetime; no photobleaching	Aita et al., 2010 Yu et al., 2007 Korovin et al., 2010 Zhang et al., 2010
	MOFs	improve detection sensitivity	Foucault-Collet et al., 2013
	laser materials	improve optical properties	Duan et al., 2006
	nanocrystals	improve optical properties	Wei et al., 2007

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on the surface plasmon resonance of the metal nanostructure, the fluorescence intensity of the near-infrared fluorescent dye can also be significantly improved. As studies have shown, by using rough metal surfaces such as silver island films or gold nano-shells, the signal intensity of phthalocyanine green can be increased by 20 and 50 times respectively [14,15]. In addition, coating the nano-microspheres with a multi-polymer material coated with a near-infrared fluorescent dye has been indicated to improve the biocompatibility of the dye. For example, Kim et al., has improved the compatibility of dyes with cells by encapsulating Cy5.5 in a hydrophilic polymer. NIFP could be used to monitor the imaging changes of cell structure in early stage of apoptosis [16].

Near-infrared fluorescent quantum dot: Quantum dots (QDs) known as semiconductor nano-microcrystals, have been widely used in bio analysis and medical diagnostics recently as a new class of fluorescent probes due to their excellent optical properties [17]. The fluorescence emission spectrum of this probe with adjustable particle size and composition ensures its feasibility as a near-infrared labeled probe. Near-infrared quantum dots refer to quantum dots with emission wavelengths between 650 and 900 nm, and have the dual characteristics of near-infrared and quantum dots. Compared with traditional organic fluorescent dyes, QDs have unparalleled advantages such as high quantum yield, strong anti-photo bleaching ability and concentrated emission spectrum. As an emerging biological probe, applications of quantum dots are still expanding in scope. However, it cannot replace the traditional organic small molecule fluorescent probe, and can only be used as a powerful supplement to the existing organic small molecule fluorescent probe. Biocompatibility remains to be further explored because of its potential toxicity to living tissue. Currently, quantum dot-based chromatographic test strips have been widely used in food safety, environmental monitoring, medical diagnosis and other fields [18-20].

Near-infrared fluorescent rare earth complex: Complexes of rare earth elements (lanthanides) containing Nd^{3+}, Er^{3+}, Yb^{3+} and Tm^{3+} in the near-infrared region have been widely developed in recent years [21-24]. Near-infrared fluorescent rare earth complexes have unique advantages such as large stock's displacement, long fluorescence lifetime, and no photobleaching relative to NIFPs such as organic dyes and semiconductor nanocrystals [25]. The use of free lanthanides is often hindered by the need for a photonic converter to handle vibrational overtone spectroscopy induced by -OH, -NH and -CH due to the low extinction coefficient [26]. In order to overcome the difficulties, many researchers are committed to the further optimization of NIF lanthanides. For example, Foucault-Collet et al. [27], developed a unique NIF rare earth metal-organic frameworks (MOFs) that encapsulate a large number of NIF-emitting Yb^{3+} ions with the sensitizer phenylenevinylene dicarboxylate (PVDC) in a small volume. This structure not only provides a new way for sensitization and protection of lanthanides, but also greatly improves the detection sensitivity due to the increase in the number of probes carried per unit volume. In addition, incorporation of rare earth elements into laser materials [28] or nanocrystals has also been shown to effectively improve their optical properties [29].

Applications of near-infrared fluorescence immunoassay

Antibiotic test: In 2016, Chen et al., developed a multiple lateral flow immunoassay based on near-infrared fluorescence by combining near-infrared labeling with broad-spectrum-specific monoclonal antibody/receptor as a detection complex. The method can simultaneously detect four antibiotic families in milk such as β-lactams, tetracyclines, quinolones and sulfonamides within 20 minutes [30]

Medical diagnostic marker molecular detection: Compared with a few reports of near-infrared fluorescence immunoassay for antibiotic test, this method is more widely used in immunoassay of important marker molecules in diagnostics. So far, near-infrared fluorescent probes have been successfully developed for immunotiter plates, fiber-optic immunosensors [31], capillary blotting [32], capillary electrophoresis immunoassays [33] and immunochromatographic assays. A variety of different immunoassay models such as strips [34], are used to detect key proteins for medical diagnosis.

In the early 1990s, NIR fluorescent probes were first applied in immunoassays. Researchers achieved quantitative determination of human immunoglobulin by adding an excess of NIR-labeled antibody and subsequent fluorescence detection in an antigen-coated polyethylene microtiter plate [35]. Daneshvar et al. [36], designed and developed a fluorescent fiber-optic immunosenseor (FFOI) for near-infrared labeling of human IgG. An antibody is immobilized on the sensing end of FFOI for the identification and capture of trace specific antigens. The immune mode is sandwich type, which can be completed within 10~15 min with a detection limit of 10 ng/mL. In a follow-up study, the Dye1 used in the above studies was replaced by a water-soluble NIR dye. The FFOI system was further confirmed to efficiently quantify human IgG and effectively sensitize one order of magnitude, while FFOI can also be used in the detection of *Legionella pneumophila* [31]. The detection sensitivity of the FFOI system is comparable to that of ELISA, and it has many advantages such as short operative time, low detection cost and suitable for on-site detection. In addition, Silva et al. [37], developed an optical immunosensor based on the near-infrared dye Cy5 for the determination of disease infection in sheep Brucella sp., which can achieve Brucella sp. antibody in serum of sick sheep (0.005~0.11 mg/mL) quantitative analysis. According to the difference in electrophoretic behavior between antigen-antibody complexes and free antigens and antibodies, Cy5 was also used for capillary electrophoresis immunoassay of IgA secreted in human saliva.

In recent years, novel NIFPs other than organic fluorescent dyes have also been introduced into near-infrared fluorescent immunoassay systems. For example, Deng et al. [38], prepared a novel core/shell NIF nanoparticle by encapsulating the inexpensive near-infrared fluorescent dye methylene blue in a hydrophobic silica gel shell. The immunoglobulin was used to determine the alpha fetus in the whole blood sample protein. This special structure exhibits higher fluorescence intensity and better stability than conventional dye-coated silicon nanoparticles, thereby preventing interference from dye leakage and exogenous quenching factors. In addition,

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dual stabilizer-modified CdTe [39], CdTe/CdS core (thin) / shell (thick) [40] and CdTe [41] and CdSeTe/CdS/ZnS with mercaptopropionic acid as stabilizers [42] quantum dot near-infrared electrochemiluminescence immunosensors have also been developed for the detection of fetal protein antigens, human IgG and carcinoembryonic antigens respectively. The above system utilizes a near-infrared fluorescence resonance energy transfer system to measure the distance effect caused by the immune reaction between a near-infrared quantum dot-labeled protein and another probe (such as gold particles), and the fluorescence is caused by the induced energy transfer. The change in intensity enables high-sensitivity quantitative detection of target analytes. In addition to NIR-QDs, the emerging NIR fluorescent material SWCNTs have also been used in IgG immunoassays by Iizumi et al. [43]. By detecting co-immunoprecipitation between IgG-bound SWCNTs and immunomagnetic beads linked to protein G, the system can measure target analytes at concentrations as low as 600 pmol/L.

One of the early pathological markers of Alzheimer's disease (AD) is the deposition of amyloid-beta (Aβ) plaques in the brain. Optical imaging and particularly near-infrared fluorescence (NIRF) imaging has become a safe, low-cost, real-time and widely available technique that provides an attractive method for in vivo detection of Aβ plaques in many different imaging techniques. In 2015, Tong et al. briefly outlined the latest developments in NIRFAβ probes and their applications in vitro and *in vivo* [44].

Despite its high sensitivity, applications of NIFPs in immunoassays are still lacking in simplicity, so its user friendliness needs further improvement. To overcome this shortcoming, Swanson and D Andrea developed quantitative immunochromatographic test strips (ICTSs) based on NIFPs for single- and multiple-synchronous detection of interleukin-6 and C-reactive protein. In 2013, Swanson developed a quantitative ICTSs based on a near-infrared fluorescent probe. The NIR dye was coupled to the selected antibody and integrated into the LFA. The test strip can detect single and multiple simultaneous detection of interleukin-6 and C-reactive protein [34]. The high signal-to-noise ratio of NIFPs makes the detection limit of the test strip as low as pg/mL .That is equivalent to ELISA. In summary, NIR-labeled ICTSs provide a powerful tool for the evaluation of biomarker proteins in a real-time assay environment.

Applications in microorganism: Although current in vitro and in vivo bio-imaging is still the main application field of NIFPs, its application in immunoassay has never stopped for many years since Boyer was the first researcher in 1992 [35]. With continuous exploration of new NIFPs and continuous development of immunoassay technology, the combination of the two has become increasingly popular in many analytical fields. LFA is the most powerful immunoassay for immediate testing due to its simple operation and portability. The application of NIFPs in LFA will undoubtedly provide a valuable platform on-site, high-sensitivity bio analysis in the near future.

In 2013, Cheng et al., extracted the anti-pulmonary Legionella LP antigen to prepare immunofluorescent LP antibody kit, and explored application value of near-infrared fluorescence detection of *Legionella pneumophila* (LP) antigen [45]. It was found that this method is not related to other common bacteria. Cross-reactivity occurs with a minimum of 10 ng/ml, with good stability and repeatability. In recent years, some scholars have combined near-infrared technology with immunomagnetic bead coupling method for quantitative detection. Zhou et al. [46], labeled the monoclonal antibody targeting larabinomannan (LAM) with a near-infrared fluorescent dye, and the LAM method for detecting *Mycobacterium tuberculosis* by targeting the multi-antibody of LAM coated on the surface of the nanomagnetic beads. They used a double antibody sandwich method to magnetically separate the conjugate and the free substance, and then used a portable near-infrared fluorescence detector to detect the fluorescence intensity of the magnetic conjugate, thereby detecting the LAM content in the sample to be tested, and found that the minimum detection limit of the method was 0.5 ng/mL. In 2018, Lin Chen et al. developed a new lateral flow assay (LFA) based on near-infrared (NIR) fluorescent dyes to detect anti-dengue virus (DENV1) IgG antibodies. The results of NIR-LFA were compared to those of Panbio Dengue IgG ELISA and the Dengue Duo IgM / IgG Kit. They identified 19 confirmed DENV1 positive samples by NIR-LFA with 95% sensitivity [47].

Our team's research on near-infrared fluorescence immunoassay: Foodborne pathogens are one of the most important threat factors for food poisoning incidents worldwide. However, traditional microbial culture-based assays are time consuming and labor intensive, failing to provide timely data to effectively reduce the incidence of foodborne illness. Therefore, whether it is from the control of product quality by food companies, or the government's effective supervision of food safety, so as to protect public health, we urgently need a faster and independent method for detecting food-borne pathogenic microorganisms. Although current colloidal gold-based LFA are still the gold standard for rapid detection of pathogens, the labeling technique is still limited by its low sensitivity and inability to accurately quantify defects. Fluorescent probes are methods that use optical properties of fluorescent molecules to study some of the physical, chemical, and physical properties of a particular environmental material at the molecular level. It has high sensitivity and wide dynamic response range, so it is widely used in biological macromolecules. In the near-infrared region, biomolecules have weak self-fluorescence and small background interference, and high sensitivity is obtained in this region. Therefore, the research of NIFPs has become a research hotspot in recent years, showing great potential in biological analysis.

At present, our team has developed an approach for detecting pathogens such as *Salmonella, Vibrio parahaemolyticus, Vibrio cholerae* and *Listeria monocytogenes* by near-infrared immunoassay. In 2017, we used near infrared fluorescent marker of *Vibrio parahaemolyticus* monoclonal antibody, *Vibrio parahaemolyticus* polyclonal antibody and Goat anti-mouse IgG polyclonal antibody was coated on nitrocellulose membrane

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as the detection line and the control line. We has developed the detection of *Vibrio parahaemolyticus* near infrared later flow assay strips and supported the standard substance. The results showed that the near infrared spectroscopy technique has good specificity and high sensitivity for *Vibrio parahaemolyticus*, the lowest detection limit is $1.2*10^2$ CFU/mL. No cross-reaction with *Salmonella*, *Staphylococcus aureus*, *Escherichia coli* and *Listeria monocytogenes*. Compared with the traditional detection method, the detection time of near infrared fluorescence method is the shortest, and the detection limit is close to that of RT-PCR method. This near infrared immunochromatographic method is ompleted in 45 minutes . It could be used for efficient detection of *Vibrio parahaemolyticus* in food and provide reliable technical support for food safety supervision.

Conclusions and Prospects

At present, near-infrared spectroscopy technology has developed with development of different fields such as computer science, chemometrics, photomaterial science and measuring instruments, and has become a widely used analytical tool to solve some difficulties for quick detection challenges. This technology requires qualitative and quantitative analysis of unknown samples by establishing a calibration model. Because the absorption spectrum is a superposition of the absorption spectra of the contained compounds, and the map has a certain similarity, the map is complicated and difficult to resolve. The further popularization of near-infrared spectroscopy in the field of detection will be a great challenge. Therefore, near-infrared spectroscopy technology needs to be combined with characteristics of actual detection to develop near-infrared spectroscopy special information of each system and the processing technology of other disciplines, so that it can be better applied. With the development of NIR analysis technology and other fields of technology and the continuous expansion of its application, it will play an important role in analytical tools modernization.

Since labeled molecules play a decisive role in the development of immunochromatography technology platforms, it is particularly important to develop ideal probes with high quantum yield, good stability, small background interference and easy labeling. The existing probe development mostly relies on simplicity to obtain high sensitivity and accuracy, which to some extent undermines the superiority of ICTSs in the field and in real-time detection. Therefore, more innovative research should be devoted to the development of highly accurate probes. At present near-infrared fluorescent probe has excellent potential, but it is mainly used for biological imaging research, and the application on the ICTSs is almost blank. Previous studies have shown that near-infrared fluorescent probe labels are 100 times more effective than traditional colloidal gold test strips in detecting HIV.

In recent years, our team has developed a method for detecting pathogens included *Salmonella*, *Vibrio parahaemolyticus*, *Vibrio cholerae* and *Listeria monocytogenes* by near-infrared immunoassay. Our team is working on food-borne viruses such as rotavirus, norovirus and hepatitis A virus, and we could develop near-infrared ICTSs using NIFPs-labeled antibodies to detect these foodborne viruses. Our team intends to establish a new platform which has a highly sensitive near infrared probe combined with portable and simple ICTSs for rapid detection of food borne viruses. This will provide technical support for a rapid detection on the port.

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