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Review

Advances in the study of luminescence probes for proteins

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Abstract

Spectral probes (or labels) have been widely used for the investigation and determination of proteins and have made considerable progress. Traditional luminescence probes include fluorescent derivatizing reagents, fluorescent probes and chemiluminescence probes which continue to develop. Of them, near infrared (NIR) fluorescent probes are especially suitable for the determination of biomolecules including proteins, so their development has been rapid. Novel luminescence probes (such as nanoparticle probes and molecular beacons) and resonance light scattering probes recently appeared in the literature. Preliminary results indicate that they possess great potential for ultrasensitive protein detection. This review summarizes recent developments of the above-mentioned probes for proteins and 195 references are cited.

Keywords: Reviews; Luminescence probe; Derivatization; Proteins

Contents

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1. Introduction

The development of novel methods and new techniques for protein determination is very important in a number of areas, such as chemical and biochemical analysis, immunodiagnostics and biotechnology. Spectral methods are widely used due to their high sensitivity and selectivity. In this paper, fluorescence, chemiluminescence and resonance light scattering methods are summarized. Absorption spectrometry is not included because of its low sensitivity. Among them, fluorescence has become one of the most sensitive methods for protein detection, especially when the method was incorporated into HPLC and CE [1,2]. It's well known that only proteins possessing Phe, or Trp, or a combination thereof, exhibit natural fluorescence. But it's too weak to be applied for the analysis of proteins at low concentration. In order to solve this problem, protein can be converted into suitable derivatives by chemical derivatization, typically using a derivatization reaction or spectral probes (or labels). Selection of an excellent probe is the key to this procedure. The probes can be achieved by different detection methods with different reactions. Choice of the right way to label the desired proteins depends on the stability of the probes in solution, the stability of the products, and on its sensitivity. There have been many publications reporting the use of derivatization methods for improving a protein's detectability [1,3–5], and some reviews have also summarized the luminescence method based on the derivatized proteins. Koller and Eckert [6] focused attention on some organic derivatizing reactions in chromatography. In another review [7], the selection of probes for different research was summarized. Our work covers spectral probes used in the protein analysis and focuses on fluorescence detection, such as derivatizing reagents, rare earth ions and complexes, the dyes and near-infrared dyes. In addition, chemiluminescence, resonance light scattering technique and some novel probe methods (nanoparticle and molecular beacon) are also summarized briefly in this paper.

2. Fluorescent derivatizing reagents reacting with protein at the N-terminus

Proteins have at least two functional groups where a derivatization may take place: the amino group and the carboxyl group. Whereas the carboxylic group at the C-terminus is less active and it must first be activated itself before derivatization, it is rarely used in protein labeling procedures. On the contrary, amino groups at the N-terminus are easily derivatized. But it is not neglected that proteins have a three-dimensional structure, which makes it difficult for some amino groups to be fully accessible to the reagents. As such, only the primary or secondary amino groups can be readily tagged. As the fluorimetric method has high sensitivity, only fluorescent derivatization of proteins at the N-terminus is described in this paper.

No spectroscopic technique is more widely used in peptide and protein chemistry than fluorescence for detecting proteins separated by HPLC and CE. Comparing fluorescence detection to other spectral detection, it is obvious that the sensitivity of fluorescence is some orders of magnitude higher than that of other spectral detection (UV, for example) [8]. In fluorescence detection, two wavelengths are involved: excitation wavelength and emission wavelength, which both characteristically depend on the chemical structure of the fluorescent residue. When the laser is applied, the systems can offer a higher sensitivity, a better signal-to-noise ratio, and a very high photon flow [9]. Because the fluorescence emitted from the native protein [10–12] can be intensive, not all proteins generate a useful native fluorescent signal. Therefore, the emphasis of fluorescence methods for the detection of proteins is focusing on the labeling of proteins. By selecting better reagents

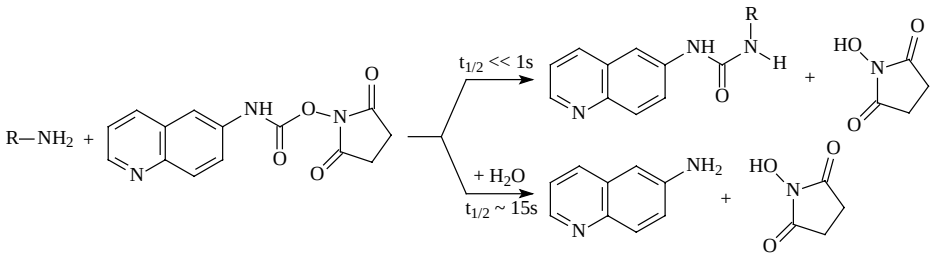

Fig. 1. The derivatizing reaction of 6-AQC.

(labels or probes), the derivatized proteins can emit more intense fluorescence than the aromatic groups of protein.

2.1. The reagents reacting at primary and secondary amino groups

During the past 60 years, ninhydrin has been an ideal derivatizing reagent for amino acids [13–18]. Later on, fluorescein-5-isothiocyanate (FITC) was widely accepted [19,20]. Since FITC itself can emit strong fluorescence, the excess of FITC must be removed, resulting in a troublesome operation. In recent years, some new derivatizing reagents have been used for the determination of proteins. These reagents themselves do not fluorescence or have only weak fluorescence, but upon binding to proteins through the derivatizing reaction, the fluorescence intensity of the derivative is considerably enhanced. Therefore, these reagents have great potential for protein detection. Of them, some reagents reacting at primary and secondary amino groups are below.

2.1.1. 6-Aminoquinolyl N-hydroxysuccinimidyl carbamate (6-AQC)

In 1997, a new derivatizing reagent was synthesized and first used for determining amino acids from proteins' total hydrolysis [21]. As an activated carbamate, it could easily react with primary and secondary amino groups to form highly fluorescent ureas, as described in Fig. 1. This product can be detected at an emission wavelength of 395 nm after excitation by 250 nm. The advantage of this method is low background noise because the difference between excitation and emission is larger than 100 nm. 6-AQC even reacts fast enough to form uniquely disubstituted products with lysine and cystine [22].

2.1.2. Fluorenylmethyloxycarbonyl chloride (FMOC-Cl)

Traditionally, FMOC-Cl is well known as an amino-protecting group in peptide synthesis. It easily reacts with primary and secondary amino groups, and even with lactams [23–28] to form highly fluorescent urethanes (see Fig. 2). Belonging to the same family, PEOC [29] and AEOC [30] are also successfully employed for the fluorescent detection of amino acids.

2.2. The reagents reacting at primary amino groups only

o-Phthaldialdehyde (OPA) is widely used for labeling peptides and proteins with a primary amino group [31–34]. However, the poor stability of its labeling derivatives limits its application. In recent years, some new but similar reagents have been synthesized. In comparison to OPA, these reagents are excellent derivatizing reagents for proteins with primary amino groups and are described below.

2.2.1. Naphthalene-2,3-dialdehyde/cyanide (NDA)

NDA is similar to OPA as a fluorescent label for protein. However, in this case the co-reagent is not a thiol but the cyanide ion (CN^-). The products formed by reacting NDA/CN^- with primary amino groups were identified as N-substituted 1-cyanobenz[*f*]-isoindoles by Koning et al. [35] (see Fig. 3). The derivatizing product CBIs are more stable than OPA/thiol derivatives, but the cyanide concentration has to be controlled in order to gain an optimal fluorescence yield. Excess reagents do not interfere with the subsequent HPLC-separation of the proteins. Recently, De-Silva et al. [36] successfully measured NDA/CN^- labeled dipeptides by LIF.

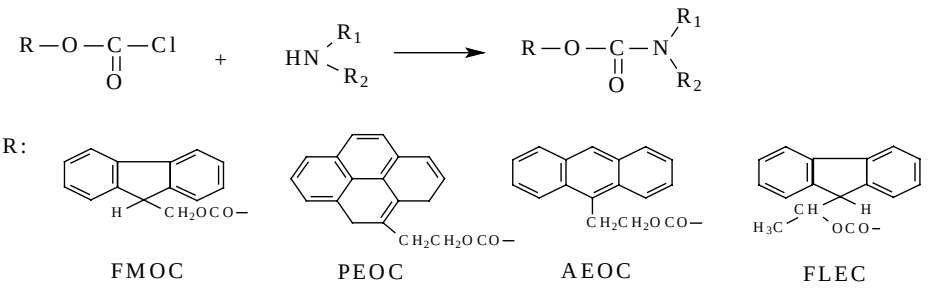

Fig. 2. The derivatizing reaction of FMOC-Cl and its family reagents.

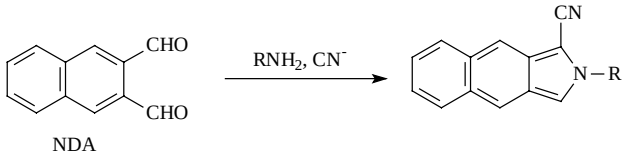

Fig. 3. The derivatizing reaction of NDA.

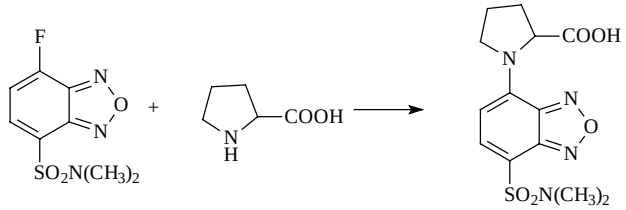

Fig. 5. The derivatizing reaction of DBD-F.

2.2.2. 5-Fluroylquinoline-3-carboxaldehyde (FQ)

Dovichi and co-workers [37] have recently developed a method for the pmol/l assay of native proteins using the fluorogenic amine-reactive probe FQ and sub-micelle concentrations of the anionic surfactant sodium dodecyl sulfate (SDS) added to the separation buffer. In this experiment, the SDS at sub-micelle concentration binds to proteins through ion pairing, thus the anionic head group will interact with positively charged lysine residues, which is similar to OPA and NDA. When derivatized with FQ, a lysine residue is converted into an uncharged isoindole. Recently, this method is developed for practical fluorescence detection of proteins separated by CE. The detection limits for a number of proteins are in the range of 0.01–1 mol/l.

2.2.3. 3-(4-Carboxybenzoyl)-2-quinolinecarboxaldehyde (CBQCA)

In recent years, CBQCA was reported as a reagent for fluorescent protein labeling [38]. According to Fig. 4, CBQCA and cyanide as a co-reagent react with primary amino functions. Using the laser-induced fluorescence detection technique, the limit of determination was found in the attomole range (10^{-18} mol). It was reported to be one of the most sensitive derivatization methods for proteins published so far. Unfortunately, different proteins require different pH for maximum yield, so preliminary tests have to be performed when unknown proteins are derivatized with CBQCA.

2.3. The reagents reacting at secondary amino groups

Almost all the derivatizing regents mentioned above react with primary or with both primary and secondary amines. Only 4-(*N*,*N*-dimethylamino-sulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F) can react with secondary amines [9]. In this experiment, DBD-F reacts with secondary amino groups faster than with primary amines. The procedure is described in Fig. 5. Based on this reaction mechanism, it can be seen that this reagent can be used in derivatization of biogenic peptide amides and is best applied to peptides with N-terminal proline. The product can be detected at an emission wavelength of 573 nm after excitation at 453 nm. The success of DBD-F offered a new thought for people to consider the activity of N-terminal and to select a suitable derivatizing reagent.

2.4. The reagents reacting with other functional groups

Generally, at a pH range of 7–10, arginine can be derivatized at the primary and secondary amino functions. Its guanidine function can be labeled only when the pH value of the reaction mixture exceeds 11. Ninhydrin and benzoin were reported during the last decade as selective labeling reagents for the Arg-guanidino group (see Fig. 6) [39]. These reagents can be successfully applied for these proteins, in which primary and secondary amines face some limitations, for example, an N-blocked synthetic protein bearing an arginine residue.

Introducing a fluorophore to thiol group is also an available method to form a highly fluorescent product. In 1986, Toyo'oka et al. [40] presented ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate to protect the SH-function of ovalbumin before its total hydrolysis with 6-*N*-hydrochloric acid. Rhodes et al. [41,42] picked up this idea in 1989 and performed the derivatization of synthetic vasopressin antagonists at sulfydryl groups. Recently, a fluoro compound was used again for the fluorometric HPLC analysis of Cys–Gly [43].

In addition, homogeneously derivatized proteins for highly sensitive analysis is promoted by Krull's group. Homogeneity means that all the free amino groups of proteins are labeled. In this approach, excess amounts of fluorescent probe are used in order to force a complete derivatization reaction. It appears that the protein must be denatured to

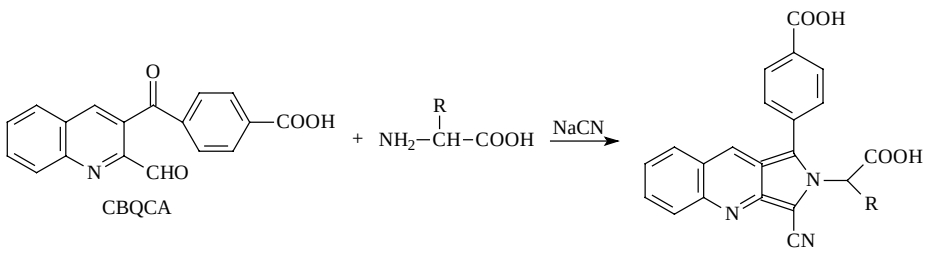

Fig. 4. The derivatizing reaction of CBQCA.

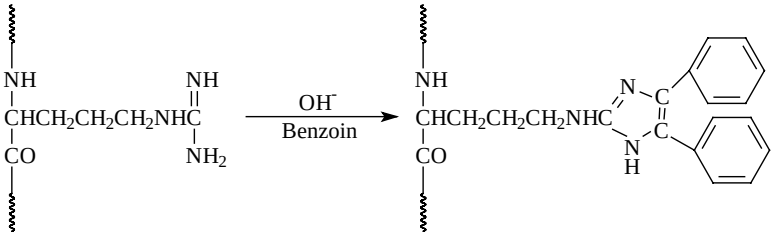

Fig. 6. The derivatizing reaction on guanidine function.

expose all groups to the probe. At first, the analysis of peptides and small proteins was reported by full derivatization with AQC [44–46]. This derivatization was simply done by mixing proteins with AQC with or without SDS present. In 2001, this method was successfully applied to larger proteins, such as α-chymotrypsinogen A (CTA) and bovine serum albumin (BSA) [47]. It was reported that CTA and BSA were derivatized with excess amounts of AQC. The samples were analyzed with matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) to determine the derivatization degree. HPLC and CE were used for characterizing these protein derivatives. Under the optimized fluorescence detection conditions, the detectability of the tagged proteins was 2400–6200 times better than that detected by UV at 280 nm, 170–300 times better than that detected by UV at 214 nm, and 150–420 times better than that with their native fluorescence.

The derivatizing reagents mentioned above associate with protein through covalent labeling. The labeling substantially improves the detectability and the sensitivity of protein determination. But there are some problems arising from inefficient chemistry and multiple derivatives for trace analysis of protein [2]. The resolution of these problems is emphasized in the future research.

3. Rare earth ions and their chelates as luminescent probes

3.1. Rare earth ions

Rare earth ions have luminescence characteristics such as narrow spectral width, long luminescence life-time, large Stokes shift and strong binding force with biological molecules. Up to now, studies based only on the luminescence of rare earth ions to detect protein were rare because of their low sensitivity. They are often used as fluorescence probes to study the property of large biological molecules. This is because rare earth ions are comparable in ionic radii to some other inorganic ions (Ca^{2+} and Mg^{2+}) which always exist in the body, and they can take the place of these inorganic ions without changing the structure of proteins. Of rare earth ions, Tb(III) has received the greatest attention as a fluorescent probe for Ca(II) binding in protein. These studies include the binding site, binding constant, the fold of protein and the energy transfer. It is well known that the Tb(III) ion could get energy from the protein and its fluorescence was enhanced. Martin [48] investigated the details of energy transfer from protein to Tb(III) and found that the Trp residue of protein was the main energy donor. This is the result of favorable overlapping between Tb(III) absorption bands and the region of tryptophan fluorescence. Therefore, some proteins and enzymes were investigated using Tb(III) as a fluorescent probe [49–51]. Our group [52] investigated the luminescence properties of the complex of Tb(III) with bovine pancreatic deoxyribonuclease (BPD), and bacillus subtilis α-amylase (BSα-A), and found that the complex ratios of Tb(III) with BPD and BSα-A were 2:1 and 4:1, respectively. The values of the critical distance for 50% energy transfer, Ro, of 0.336 nm and 0.390 nm were obtained using Forster theory. Their energy transfer distances estimated from the measured efficiency were 1.39 nm and 1.48 nm. It was inferred that the binding sites between Tb(III) and BPD, and BSα-A were Trp-178, Trp-188 and Trp-321, respectively. Yang [53] studied the action of Tb^{3+}, Eu^{3+} and Ca^{2+} on HSA using Tb(III) as a fluorescent probe and found that the distance between Trp-214 in HSA and a bound Tb(III) was about 1.38 nm.

Rare earth co-luminescence effect was first found by our research group in 1986 [54]. Recently, we [55,56] introduced this useful method to the analysis of nucleic acid and studied the luminescence mechanism. Recently, we found that this effect also existing in the system of proteins based on the denaturation of proteins by SDBS. In this experiment, SDBS exposes almost all of the Trp residues of protein to Tb^{3+} or Gd^{3+}, thereby promoting energy transfer. This system can be used for sensitive determination of proteins.

3.2. The chelates of rare earth ions

Using the simple rare earth ions as fluorescent labels for proteins is limited by low sensitivity. Some rare earth ions (especially Eu^{3+} and Tb^{3+}) when bound to the chelator, such as β-diketone, can emit strong fluorescence when excited by ultraviolet light. Further studies showed that the excitation wavelength varies with the chelating agents. The emission wavelength dose not change, and depends only on the native fluorescence of the rare earth ion. The wide range of excitation wavelengths promotes the absorption of high

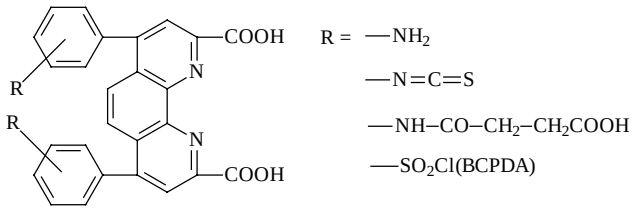

Fig. 7. The structure of phenanthroline derivatives.

energy, and narrow emission wavelength ranges are good to differentiate between the different rare earth ions. A large Stokes shift (250–350 nm) also alleviates the interference therein. So it is realized that the multi-analysis is simultaneously detected after labeled with different rare earth ions. In addition, the greater advantage is that some new and available experimental method can be introduced in the study. The time-resolved fluoroimmunoassay (TRFIA) is established based on the vast difference of the fluorescence life, the life time of the complex is always 100–1000 μs, while the background mainly caused by the natural fluorescence of the proteins only survives for 1–10 ns. According to this fact, a suitable delay for detection can eliminate the background. This technique has been widely used as an excellent tool for ultrasensitive analysis in the clinic during past years.

The choice of the chelator is the key to probe preparation. Some available chelating agents have been reported in recent years. These chelating agents always contain aromatic groups of high energy. This energy can be transferred to the rare earth ion, causing the enhancement of its fluorescence. At present, there are three major kind of chelators for rare earth ions: phenanthroline derivatives, salicylic acid derivatives and β-diketone. Their chelates can be used as fluorescent labels for proteins and are applied to immunofluorometric assays.

3.2.1. Phenanthroline derivatives

In 1988, Evangelista et al. [57] synthesized a series of phenanthroline derivatives (in Fig. 7), of which 4,7-bis(chlorosulfophenyl)-1,10-phenantroline-2,9-dicarboxylic acid (BCPDA) was an excellent chelator of Eu^{3+} and the chelate could emit strong intrinsic fluorescence of Eu^{3+}. Resichstein et al. [58] labeled BSA with the Eu^{3+}–BCPDA chelate and reported a new detection system for time-resolved fluoroimmunassay of cortisol in the sample. Later, Diamandis et al. [59] labeled a conjugate of streptavidin (SA) and thyroglobulin (TA) with BCPDA (shown in Fig. 8) and found that the fluorescent Eu–BCPDA chelate could be used for multiple fluorescence labeling without any fluorescence quenching. When 160 BCPDA molecules were incorporated into one tryroglobulin molecule, the fluorescence emitted by the labeled protein in the presence of excess Eu^{3+} was equivalent to that emitted by approximately 900 molecules of unconjugated BCPDA–Eu^{3+} complexes. On the basis of the complex mentioned above, a macromolecular complex was prepared, which consisted of SA(TG)$(BCPDA)_{160}$, TG$(BCPDA)_{160}$ and Eu^{3+} in a ratio of 1:3.3:480, and which was very stable and could be preserved for several years. This complex represents a novel universal detection reagent, which is suitable for time-resolved fluorometric application, using biotinylated reactants as complementary reagents. This reagent system yields a 33–90-fold improvement in detection limits for seven different immunofluorometric assays in comparison to directly labeled streptavidin.

3.2.2. Salicylic acid derivative

Bailey et al. [60] described the use of the terbium complex of a chelator derived from diethylenetriaminepentacetic acid and 4-aminosalicyclic acid (DTPA–pAS–Tb) in an immunoassay for human albumin. In order to improve the sensitivity, Canfi et al. [61] proposed multiple labeling of proteins with fluorescent DTPA–pAS–Tb chelates. An exceptionally large amount of label, on the order of a few hundred moles of chelates per mole of analyte, could be conjugated to the proteins tested through poly-lysine (PLL), which is shown as follows in Fig. 9.

For DTPA–pAS–Tb, its excitation and emission spectra do not overlap, and self-quenching is also small. The detection

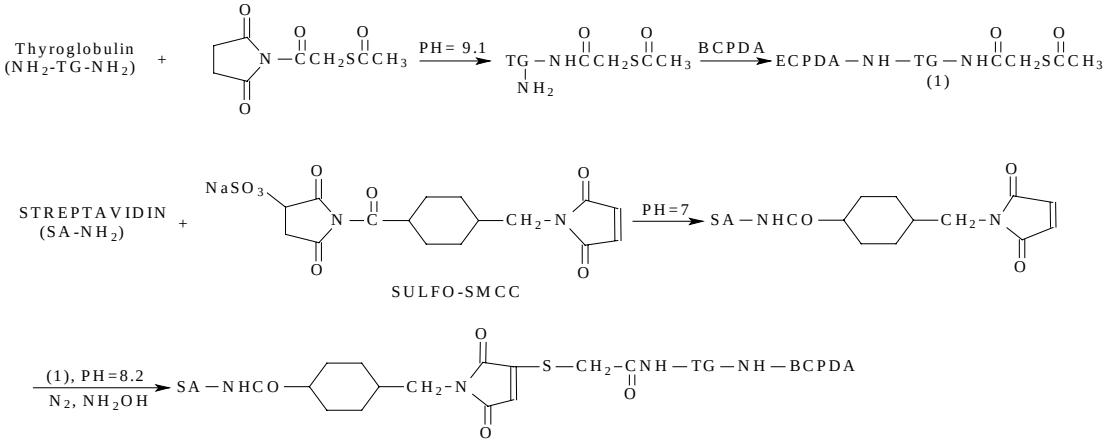

Fig. 8. The conjugate reaction between SA and BCPDA.

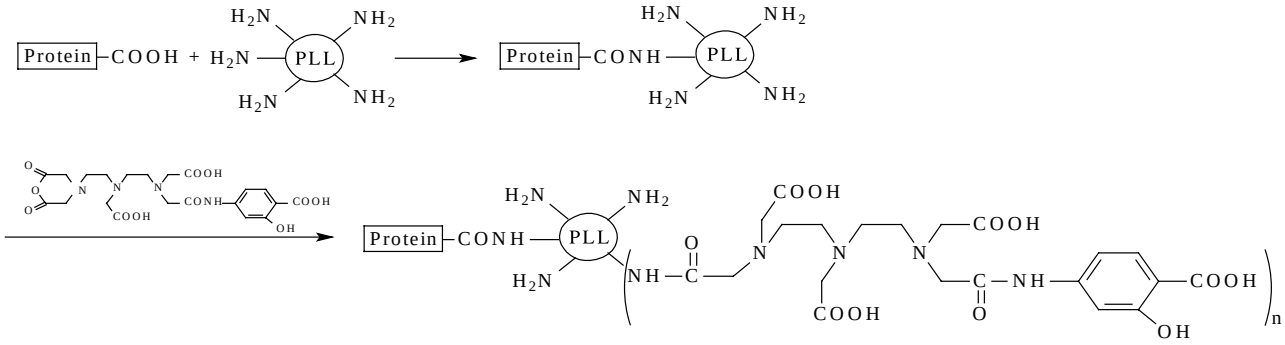

Fig. 9. The preparation of protein–PLL–DTPA.

limit of this method for human albumin is on the order of 10 mg/l (100 nmol/l), which is approximately 100-fold lower than that for single labeling [60].

3.2.3. β-Diketone

It is well known that β-diketone ligands are suitable for efficient energy transfer from the ligand to Eu^{3+} and for high fluorescence quantum yields of its complexes. In 1994, Ci et al. [62] synthesized a new europium chelator, 5-chlorosulforyl-2-thenoyl trifluoro acetone (CTTA), and proposed multiple labeling of protein with CTTA. The CTTA-labeled solid-phase immunocomplexes were transferred into solution with a dissolution solution, which consisted of sodium dodecyl sulfate, Eu^{3+}, and tri-*n*-octylphosphine oxide. The fluorescent complexes thus formed and were measured using a time-resolved fluorescence technique. This method was applied to the determination of cortisol, and its detection limit was 0.5 ng/ml. Its application to immunoassay did not yield a large improvement of the sensitivity [63].

Recently, Matsumoto's group [64] synthesized a new chlorosulforylated tetradentate β-diketone, 4,4-bis(1″,1″,1″,2″,2″,3″,3″-neptafluoro-4″,6″-hexanedion-6″-yl)chlorosulfo-*o*-terpheryl (BHHCT), which can be covalently bound to protein under mild conditions, and which forms a strongly fluorescent chelate with Eu^{3+}. The utility of the fluorescent label was exemplified by the detection of BSA in 0.1 mol/l Tris–hydrochloride and 10 μmol/l TOPO/0.05% SDS/0.1 M $NaHCO_3$, for which the corresponding detection limits were 0.67 pmol/l and 0.093 pmol/l [65]. Streptavidin was labeled with BHHCT–Eu^{3+} and was used for time-resolved fluoroimmunoassay of α-fetoprotein (AFP) in human sera. The detection limit was 4.1×10^{-3} pg/ml, which corresponds to an improvement of about 4–5 orders of magnitude, compared to conventional immunoassays [66].

4. Noncovalent fluorescent probes

There are noncovalent probes as well as covalent probes for protein detection. The dyes serving as noncovalent probes are almost all anionic dyes. These dyes can bind to the residues of proteins which carry positive charges, and so pH is an important parameter. Upon binding to proteins, the fluorescence intensity of the dyes may be enhanced or quenched.

The enhancement of dyes' fluorescence mainly comes from a change in the microenvironment in which those dyes exist. Generally, these probe reagents are nonfluorescent in water, but highly fluorescent in apolar media. Given the three-dimensional structure of proteins, these dyes can bind to the hydrophobic region of a protein through noncovalent binding and their fluorescence yields are enhanced greatly. Typical probes of this type are naphthalene derivatives [67], Sypro dyes [68] and Nile red [69]. In addition, the fluorescence peaks of complexes formed by Nile red bound with different proteins are not at the same wavelength [70]. In order to study the same character of these dyes, Takaji et al. [71] compared the absorption and fluorescence spectra of the complexes formed by human serum albumin (HSA) with 10 dyes similar to CAS in structure. They found that ionized carboxyl is essential to the formation of these fluorescent complexes. Recently, Shen and co-workers reported a new detection of protein using acridine orange (AO) as probe. In a solution with cationic surfactant (sodium dodecyl benzene sulfonate (SDBS)), AO could form a nonfluorescent dimer. When protein was added to this system, the intensity of fluorescence was enhanced. It was considered that the addition of protein broke the assembled structure of AO. Based on this, the sensitive detection of protein was established. For BSA, its detection limit was as low as 0.08μg/ml [72].

On the other hand, fluorescence quenching of dyes can also be used for the analysis of proteins. At pH 3.1, Eosine Y gives off strong green fluorescence. After bound to proteins, its fluorescence is quenched and this method can be used to detect proteins at a level of 1–100 μg [73,74]. Based on the above facts, proteins can be successfully detected using the dyes as probes. Especially, when AlS_4PC was used for detecting HSA, the site of the emission peak was far away from that of the excitation peak, and so the decrease in the background caused the detection limit to be as low as 0.04 mg/l. However, it is difficult to improve the sensitivity of this method through quenching.

Table 1
Some fluorescent dye methods for the determination of protein

Reagent	Determination object	λ_{ex} (nm)	λ_{em} (nm)	Linear range (mg/l)	Reference
Enhancement					
Vasoflavine	HAS	390	420	0–5	[75]
ANSA	Histone, HSA	375	500	1–300	[76]
CAS	Albumin	485	616	5–80	[71]
CAS	Albumin	538	619	5–60	[77]
ECR	HAS	308	423	0.5–12	[78]
bis-Ans	Protein	380	480	0.0–0.6	[67]
Hypochlorite	Protein	370	440	20 ng–2 μg	[79,80]
MTPT	Microstudule protein	350	425	–	[82]
EB	Protein	317	550	1.36–20.40	[83]
SYPRO O/R	BSA	300	–	<10	[68]
	Ovalbumin	300	–	10–40	
TCPP	Protein	425	655	0.09–12 μg	[84]
Oxalate	Heme protein	410	600	0.05–0.5	[87]
Phehalaldehyde	Protein	340	450	0.05–2 μg	[88]
Quenching					
Eosine Y	Protein N	518	540	0.06–6	[73]
Eosine Y	Protein	518	540	1–100 μg	[74]
EPQS	HAS	370	470	1–5	[81]
AlS_4Pc	Serum albumin	357	686	0.10–1.0	[85,86]

Note: Abbreviations: ANSA, 8-hydroxamino-naphthene sulfonic acid; CAS, chrome azurol S; ECR, eriochrome cyanine R; MTPT, 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)-tropone; EB, erythrosin B; TCPP, α,β,γ,δ-tetra(4′-carboxyphenyl)porphin; AlS_4Pc, aluminium tetrasulphonated phthalocyanine.

The fluorescent dye reagents used for protein detection are summarized and listed in Table 1.

5. Near infrared fluorescence probes

Spectrofluorimetry in the near infrared region (about 600–1000 nm) is a rapidly developing area. In comparison to more conventional measurements made in the ultraviolet and visible regions, NIR fluorescence has many advantages [89,90]: (1) The low levels of background interference, since few naturally occurring molecules can undergo electronic transitions in this low-energy region of the electromagnetic spectrum. (2) Scatter (Raman and Rayleigh) can be reduced at higher wavelengths due to its dependence on the wavelength of detection by $1/\lambda^4$. (3) Sample photodecomposition is reduced when longer excitation wavelengths are used. (4) Excitation in the NIR region can be accomplished by using cheap, stable and compact diode lasers, so the sensitivity can increase considerably. (5) Since the NIR wavelengths can effectively penetrate through skin and overlaying tissue, there is potential for the development of a noninvasive clinical diagnostic. From the above mentioned features, NIR fluorescence is especially suitable for the studies of biological samples.

NIR dyes as fluorescence probes should have favorable photophysical properties including high molar absorptivity, high quantum yield, large Stokes shift, thermal and photochemical stability, and low susceptibility to fluorescence quenching, but their availability remains limited. Recently, several new NIR fluorescence probes have been reported [91,92]. Emphasis is on three types of NIR dyes, which have received much attention in the recent literature, i.e., cyanines, squaraines, and thiazine and oxazine dyes.

5.1. Cyanine dyes

Cyanine dyes are widely used as fluorescent probes for biomacromolecules, and their representative structures [92–97] are shown in Fig. 10. The range of their maximum absorption is between 600–800 nm, some even exceed 800 nm. Their molar absorptivities are at the 10^5 l/mol cm level. When in aqueous solution, their quantum yields are very low. After binding to the analytes, the system has changes in the absorption wavelength and emission wavelength. Of importance is its increased lifetime. Up to now,

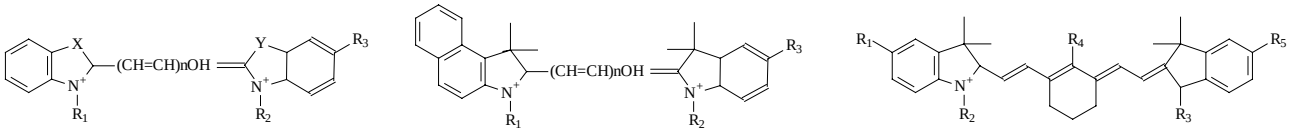

Fig. 10. The structures of dicarbocyanines dyes.

these reagents have been successfully used in the field of immunoassay and have received some attention in the area of separation science [98,99].

Cyanine dyes can label proteins through covalent or noncovalent binding. Noncovalent binding is especially useful for the detection of large compounds such as proteins and nucleic acids. The positive charge of the most common cyanine dyes can be used for noncovalent labeling of proteins. Indocyanine green (ICG) has labeled the protein in human serum and the complexes were separated by a gel filtration column [99]. Its detection limit was about two orders of magnitude better than the values obtained by conventional methods. ICG had also been used for diagnosing liver activity [100] and fluorescence NIR angiography [101]. In 1990, Wilberforce and Patonay [102] investigated the properties of four NIR laser dyes, viz. 3,3′-diethyloxaicarbocyanine iodide (DODCI), 3,3′-diethylthiadicarbocyanine iodide (DTDCI), 3,3′-diethyloxatricarbocyanine iodide (DOTCI) and 3,3′-diethylthiatricarbocyanine iodide (DTTCI) and its complex with albumin. The DTTCI complex with albumins showed a significant decrease in the NIR absorption while the other three dyes did not. These results suggest that the presence of the S heteroatoms as well as their distance from each other are determining factors in the observed specific binding. In application, DTTCI was used as a detection reagent to determine albumin protein, and detection limits were about 3 amol with LIF. An new water-soluble cyanine dye had been used as a NIR probe for the determination of proteins [103]. The reaction of the dye with serum proteins through noncovalent binding was carried out in 0.02 mol/l Hac–NaAc buffer, and the linearity of this method was over the range of 1.0×10^2 to 1.9×10^3 ng/ml for HSA and γ-globulin, and the detection limit was 50 ng/ml. A commercially available cyanine dye albumin blue 670 [104] has been applied as a NIR fluorescent probe for the determination of trace levels of human serum albumin. After the addition of albumin, the fluorescence intensity of albumin blue 670 is enhanced by several orders of magnitude. Therefore, the enhanced fluorescence intensity directly reveals the albumin concentration.

The cyanine derivatives with alkyl SO_3^- groups and active groups such as isothiocyanate, iodoacetamide and *N*-succinimidyl are rather promising for covalent binding [91,92]. Boyer et al. [105] used cyanine dyes as biomolecule labels for immunoassay. The NIR dye was derivatized with an isothiocyanate functional group and conjugated to goat anti-human immunoglobulins (GAHG). After purification by G-25 size exclusion chromatography, the conjugate was used to detect and quantitate human immunoglobulins (Ig), the antigen for GAHG.

NN382 is a novel NIR fluorescent sulfocyanine dye with an isothiocyanate group, which has absorption and emission maxima in water around 780 and 800 nm, respectively. The dye was evaluated as an ultra sensitive peptide labeling reagent for use with CE [89]. Derivatization of six angiotensin I (Ang-I) variants was achieved in aqueous solution. The fluorescence response was linear over a 200-fold range for Ang-I samples with concentration between 1×10^{-5} and 5×10^{-8} mol/l. The detection limits ranged from 100 to 300 zmol for the six Ang-I variants, so NN382 is a viable alternative fluorophore for CE methods requiring high sensitivity.

Large red-shifts of about 100 nm in absorption maximum are possible by adding a CH=CH entity to the polymethine chain of cyanine (as shown in Fig. 10) [91]. Therefore, the dicarbocyanine dyes ($n = 2$) as NIR labels have been the subjects of many studies [92,106–108]. The investigations indicated that the dicarbocyanine dyes with a succinimidyl ester functionality were suitable for covalent labeling of molecules with primary and secondary amino groups under slightly alkaline conditions and had been used for sensitive determination of amino acids and peptides with LIF detection. A detection limit of 0.1 amol was achieved [106].

5.2. Squaraine dyes

The squaraines are 1,3-bis-substitution products obtained by the reaction between squaric acid or its derivatives and some donors of electrons. These dyes have structures which are similar to those of cyanine dyes, but also contain a central squarate bridge. The squarate residue shifts the absorption and emission maxima to longer wavelengths relative to the comparable cyanine dye, and is expected to increase the photostability of the dyes. The squaraines have two types—symmetrical and unsymmetrical squaraines. The representative structure of symmetrical squaraines [92,109,110] is shown in Fig. 11.

Terpetschnig et al. [109] studied the absorption and emission spectra of 10 squaraines and indicated that the most suitable probes for use in a biological application were found to be the symmetrical indolenine derivatives of the squaraines, which display the highest photostability. Importantly, their lifetime and quantum yield increase significantly upon noncovalent binding to bovine serum albumin, suggesting that a conjugatable derivative of these indolenine squaraines will be suitable for use in labeling proteins. When bound to BSA, the nanosecond lifetimes displayed by the indolenine derivatives are long enough to allow the use of simple phase modulation instrumentation, which can be practical in a clinical environment. Ozinskas and co-workers [110] studied the fluorescence characteristics of a series of squaraine derivatives and their complexes with BSA and indicated that the lifetime and fluorescence intensity of the squaraine derivative

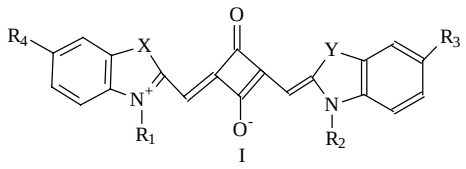

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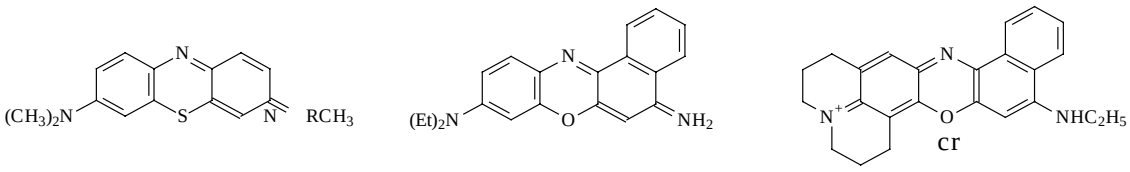
Fig. 11. The structure of symmetrical squaraines.



Azure B: R=H
Methylene blue: R=CH3

Nile Blue

Oxazine 750

Fig. 12. The structures of phenoxazine and oxazine dyes.

bound to BSA increased 31- and 28-fold, respectively. The covalent association of the squarylium dye with BSA was investigated by using fluorescence-detected circular dichroism (FDCD) [111]. It was found that the FDCD signal of the dye–BSA complex was diminished upon addition of urea, which denatured the protein, but no change occurred to the spectral properties of the dyes in urea. This indicated that the FDCD spectral feature arose from covalent interaction of the dye and the secondary structure of the protein.

5.3. Thiazine and oxazine dyes

Both thiazines and oxazine dyes have amine groups that can be used for labeling protein. Methylene blue, Azure B, Nile blue andoxazine 750, which were shown in Fig. 12, are the most used dyes for NIR fluorescent probes of proteins.

Methylene blue can emit fluorescence when excited at 670 nm. It has been used as a noncovalent probe for the determination proteins [112] and amino acids [113]. The detection limit of the latter is 1 pmol. Nile red is an uncharged phenoxazine dye. It is photochemically stable and is known to be a good hydrophobic probe because its fluorescence maximum varies depending on the relative hydrophobicity of its surrounding environment. This property facilitates its use as a solvatochromic probe in the measurement of changes in solvent polarity. As a NIR dye, Nile blue was added to four proteins, viz., bovine albumin, α_1-acid glycoprotein, β-lactoglobulin and ovomucoid, and showed an enhancement in fluorescence and a shift in emission wavelength, suggesting that it was bonding hydrophobically to these proteins [114]. Their detection limits could reach 0.1 ng/ml.

In oxazines and thiazines, Azure B, Nile blue and Oxazine 750 have been used as covalent labels for proteins and amino acids [92,113]. By means of a bifunctional reagent such as a water soluble carbodiimide, oxazine 750 can be bound to albumin through covalent binding and the detection limit is 0.13 pmol for HSA [115]. In order to make azure B suitable for covalent labeling, a new labeling reagent is synthesized, which consists of an azure B chromophore for fluorescence detection and a succinimidyl ester for combination with proteins and amino acids. The labeled amino acids are clearly resolved by capillary zone electrophoresis, the detection limit being at the 10 pmol level [113]. However, the studies indicated that thiazines and oxazines provide limited prospects for the development of appropriate labels, because the reaction yield is rather low [92,113]. It is considered that among the above mentioned three types of NIR dyes, cyanines and squaraines are the best prospects for the development of covalent labels.

In addition, the photophysical features of other NIR fluorescent probes, such as naphthalocyanines, and new luminescent complex of lanthanide and ruthenium ions, have been investigated [93,116–118]. Unfortunately, they have not yet been used for the determination of proteins.

Further, emphasis for the studies of NIR fluorescence probes for proteins should be focused on the exploration of novel probes, and the improvement and utilization of the present fluorescent dyes. The new NIR fluorescent probes should have high quantum yields in aqueous solution, long lifetimes for time-resolved fluorescence detection, long wavelength absorption suited for excitation by laser light sources (such as diode lasers), and should be easy to derivatize.

6. Chemiluminescence probes

Chemiluminescence is a type of luminescence, but its energy comes from a chemical reaction [119]. No excitation is required for sample radiation, so problems encountered frequently in photoluminescence (such as light scattering or source instability) are absent in chemiluminescence. Also, high background due to unselective photoexcitation do not appear; thus, there is no need for time-resolved detection. There have been many excellent papers that reviewed the chemiluminescence method [120–123]. They summarized the main advantages of chemiluminescence labeling and its application to biomolecular analysis and immunoassay. It is pointed out that the linear response reaches up to six orders of magnitude; that there is fast emission of light especially when it is generated in a single flash; that there is high stability of several reagents and most of the conjugates (increased stability is often observed after conjugation); and that there is a low consumption of expensive reagents. Based on these findings, chemiluminescence detection has become one of the most sensitive methods in the fields of chemical and biochemical analysis. In recent years, several papers dealing with new chemiluminogenic compounds and more than 1500 per year dealing with applications in immunoassays and biomedical research have been published.

The most common chemiluminescence methods require derivatization of proteins by the addition of a chemilumino-

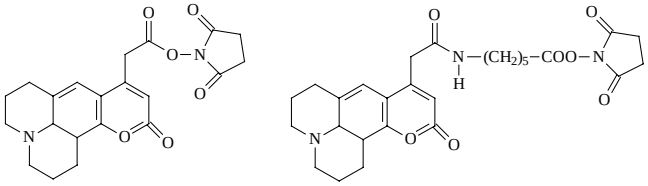


Fig. 13. The structure of luminarins 1 and 2.

genic label. In 1991, a chemiluminescence method for protein and amino acid detection was reported [124]. In this case, the AZT-amino acids were labeled by a ring-opening procedure with thiazoles for chemiluminescence detection. It is known that fluorescein isothiocyanate (FITC), when oxidized by some oxidizers such as NaClO, can emit weak chemiluminescence in alkaline solution. Recently, it was found that upon binding to protein through covalent labeling, the chemiluminescence intensity of the FITC–NaClO system is considerably enhanced by a cationic surfactant such as cetyltrimethylammonium bromide (CTMAB) [125]. Based on the relationship between chemiluminescence intensity and protein concentration, a flow injection assay has been established for the determination of BSA. The linear range is 0.08–16.0 mg/l and the detection limit is 0.032 mg/l. The enhancement effect of chemiluminescence that is characteristic of protein labeled with FITC is also discussed.

In order to improve the sensitivity, a two-step derivatization process has been proposed [126–128]. First, the analytes are labeled by a fluorescent reagent and second, the derivatized analytes are reacted with certain oxalates and hydroperoxide as chemiluminogenic labeling reagents. It's known that there are dansyl chloride (Dns-Cl), 4-fluoro-7-nitro-2,1,3-benzontinezole (NBD-F), 4-chloro-7-nitro-2,1,3-benzontinezole (NBD-Cl), naphthalene-2,3-dialdehyde (NDA) and *o*-phthaldialdehyde as the derivatizing reagents. Meanwhile, *N*-(4-aminobutyl)-*N*-ethylisoluminol (ABEI) [129] and luminarins 1 and 2 [130] (see Fig. 13) are used as chemiluminogenic labeling reagents for the sensitive determination of amines.

Compounds belonging to five chemical classes—acylhydrazides, acridinum derivatives, dioxetanes, coelenterazines and peroxyoxalic derivatives—are currently used as chemiluminescent labels [131]. Each of them has advantages and drawbacks, so none of them is definitively preferred over the others. Acylhydrazides like (*iso*)luminol are the most frequently used chemiluminescence compounds in immunoassays and in oxygen metabolism studies. However, they need a catalyst for light emission and an enhancer to increase the sensitivity of the system, and this can result in higher background signals. Acridinium derivatives have high quantum yields even if coupled to proteins. The dioxetanes used for diagnostic applications are enzyme triggered dioxetanes; however, a rather long period of time is need to reach a constant signal, thereby limiting their application in immunoassays. In the presence of a fluorophore, oxalate derivatives have the main advantages that they are efficient and flexible for biomedical applications.

Alba and Daban [132] investigated the bis-(2,4,6-trichlorophenyl)oxalate (TCPO)–H_2O_2 chemiluminescence system for the detection of proteins labeling with various fluorescent dyes. Covalent labeling with 2-methoxy-2,4-diphenyl-3(2*H*) furanone (MDPF) gave the best results, and was suitable for the detection of proteins on western and slot blots. Down to 5 ng, protein could be detected for a 5 min exposure.

There are a large number of Ru–ligand complexes that can be used as potential chemiluminescence reagents. The physiochemical properties of these complexes have been studied in detail [133,134]. There have been some reports dealing with the detection of amino acids and amines using tris(2,2′-bipyridyl) ruthenium(III) $Ru(bpy)_3^{3+}$-based chemiluminescence [135–137]. Brune and Bobbitt [135] proposed a new technique for the detection of underivatized amino acids and amines using $Ru(bpy)_3^{3+}$ as a chemiluminescent reagent. In this technique, $Ru(bpy)_3^{3+}$ was shown to undergo a chemiluminescent reaction with free amino acids and the chemiluminescence was maximized at pH values near the pK_a of the N-terminal amine sites. The studies indicated that among a series of amines, tertiary amines luminesced with the most efficiency. Their detection limits are reported in the picomole to femole range (1–10 nmol/l) [136]. Recently, Waguespack et al. [137] evaluated the properties of four ruthenium complexes, viz. bis(bipyridyl) bipyrimidine ruthenium ($Ru(bpy)_2(bpym)^{3+}$), bis(bipyridyl)biimidazole ruthenium ($Ru(bpy)_2(biim)^{2+}$), tris(4,7-diphenyl-1,10-phenanthroline) ruthenium ($Ru(phen)_3^{2+}$) and tris(bipyridyl)ruthenium ($Ru(bpy)_3^{3+}$) and their efficiency as probes for chemiluminescence detection. It is indicated that among the four ruthenium complexes studied, $Ru(bpy)_3^{3+}$ exhibits the characteristics necessary for optimum chemiluminescence detection. A protocol for chemiluminescence detection of a protein labeled with a tertiary amine was also proposed. Two biotin derivatives were synthesized containing a tertiary amine functionality and then incubated with the protein, avidin. The addition of the tertiary amine moiety to biotin made the derivative more sensitive to $Ru(bpy)_3^{3+}$-based CL. Limits of detection for either derivative were determined to be about 50 nmol/l. The results showed the potential of this method for the determination of proteins using this tertiary amine labeling technique.

Chemiluminescence has become an essential tool in medical research as well as in routine analysis. But its disadvantages, such as chemical noise, quenching and background chemiluminescence, are still not avoided. In the future, studies should be focused on improving noise, sensitivity and selectively, developing new chemiluminescent probes, and using these probes in multianalyte and homogeneous immunoassays.

7. Resonance light scattering probes

Resonance light scattering (RLS) is a phenomenon of elastic light-scattering. In this case, when the scattering wavelength is near or within an absorption band of the scattering particles (such as molecules), the light scattering intensity at some wavelengths becomes much higher than that of usual light scattering and can be measured with a common spectrofluorometer. The RLS intensity of some organic compounds of small molecules can be substantially enhanced by biopolymers or inorganic ions, due to supramolecular aggregations and complex reactions. In recent years, RLS spectroscopy has gradually become a useful technique to study the interaction of biopolymers and their probes [138–140]. Yguerabide and Ygrerabide [141,142] also remarked that light-scattering submicroscopic particles could serve as highly fluorescent analogs and as trace labels in clinical and biological applications. The application of RLS technique has begun to appear in the determination and detection of proteins.

7.1. Organic dyes

When the pH is lower than their isoelectric point (pI), proteins are positively charged. As such, protein is easy to aggregate on acidic dyes to form a bigger complex. Therefore, the intensity of the resonance light scattering of dyes can be greatly enhanced after the addition of proteins. Based on the enhancement of RLS of organic dyes by proteins, a new sensitive RLS assay for proteins has been established. At present, organic dyes used for the determination of protein have two categories: porphyrin and acidic dyes. Their characteristics are briefly introduced.

7.1.1. Porphyrins

Huang et al. [143] reported a method of protein determination using a water soluble porphyrin, α,β,γ,δ-tetrakis(5-sulfothienyl) porphine (T(5-ST)P) with the resonance light scattering peak being located at the blue side of the absorption. It was found that J-aggregation occurs in the presence of proteins and gives the J-absorption band at 490.2 nm, whereas the maximum resonance light scattering of J-aggregation was found at 472.0 nm. Because of the protonation of the two pyrrolic nitrogen atoms in the porphyrin macrocycle, T(5-ST)P, and depending on the acidity of the solution, there can be three chemical species: the free base species, H_2P^{4-}, and the two protonated species, H_2P^{3-} and H_2P^{2-}. At pH 1.81, T(5-ST)P exists in the form of H_2P^{4-}, and has D_{4h} spectroscopic features, with the Soret and Q bands located at 451.2 and 690.5 nm, respectively. When proteins were added to T(5-ST)P solution, both the Soret and Q bands decreased, giving a new weak absorption band at 490.2 nm. Three isosbestic points can be observed at 438.0, 472.1 and 645.0 nm. These isosbestic absorption points indicate that species with a maximum wavelength at 490.2 nm originated from the species at 452.1 and 690.5 nm absorption bands. Since Beer's law holds for the absorption of T(5-ST)P at 452.1 nm, the species at 490.2 nm in the presence of proteins must be the aggregate of T(5-ST)P.

7.1.2. Acidic dyes

Acidic dyes are negatively charged and bovine serum albumin or human serum albumin is positively charged under acidic conditions. Therefore a large charge aggregate may be formed due to electrostatic forces. Based on the Rayleigh formula, the larger the volume of the particle, the stronger the signal of the scattered light. This possibly leads to great enhancement of the weak RLS signal of acidic dyes. The experiments performed verified this conjecture. Based on this, a series of different acidic dyes were used for the determination of proteins by RLS. They are shown in Table 2.

7.2. Anion surfactants

Anion surfactants have a negative charge, while both BSA and HSA are positively charged when the pH of the medium is lower than the protein's pI. Because of the electrostatic attraction forces, they can easily interact to form associates. Our group [162] found that in the medium of phosphoric acid (pH 2.6), the weak RLS of sodium dodecyl benzene sulfonate or sodium lauryl sulfate (SLS) can be greatly enhanced by proteins, owing to the interaction between protein and anion surfactant, and the formation of the aggregate. But the RLS intensity of the SDBS–protein system is much stronger than that of the SLS–protein system under the same experimental conditions. According to the traditional light scattering theory, the enhanced intensity of light scattering originates from the absorption of the probe. In the SLS–BSA system, SLS has no absorption in the studied wavelength range, and the enhanced RLS originated from the absorption of protein. However, in the SDBS–BSA system, both SDBS and protein have absorption at about 280.0 nm, so it is the synergetic resonance caused by their absorption that makes the I_{RLS} in the SDBS–BSA system far stronger than that of the SLS–BSA system.

In this method, the enhanced intensity of RLS is proportional to the concentration of protein. If SDBS is used as the probe, the linear range is 7.5×10^{-9} to 1.5×10^{-5} g/ml for BSA, and 1.0×10^{-8} to 1.0×10^{-5} g/ml for HSA. The detection limits are 1.8 and 2.8 ng/ml, respectively. When SLS is used as the probe, the linear range is 2.0×10^{-8} to 1.0×10^{-5} g/ml, and 2.5×10^{-8} to 1.0×10^{-5} g/ml for BSA and HSA, respectively. Their detection limits are 12.8 and 21.6 ng/ml, respectively.

7.3. Dye–nonionic surfactants

Our group [163] found that in the citric acid–NaOH (pH 2.35) buffer, the RLS of the Resorcinol yellow (RY)–protein system can be greatly enhanced by addition of nonionic surfactant OP, owing to the interaction between OP and RY–protein. The enhanced RLS is in proportion to the

Table 2
Different probes of protein by RLS

Probe	Wavelength (nm)	Linear range (mg/l)	Detection limit (mg/l)	Reference
BPB	334	0.34–18.7	–	[144]
TIPSP	334	0.34–12.24	–	[145]
CAS	350	0–1	0.02	[146]
AG25	347	0.136–10.2	–	[147]
DBHPF	586	0.05–0.75	0.013	[148]
AlS_4Pc	413	0.05–2.0	0.016	[149]
PR	332	0.136–6.8	–	[150]
BPR	360	0–5	–	[151]
ACAP	337	0.2–4.0	0.068	[152]
ACBK	345	0.136–10.88	0.136	[153]
TB	470	0–5	–	[154]
Sulfonazo III	602	0.3–30.5	0.3	[155]
SPAPNS	340	0.198–14.9	0.005	[156]
Amaranth	364	0–0.5	0.022	[157]
Arsenazo III	400	0.085–21.52	0.085	[158]
DBM-CPA	411.6	0.065–40.05	0.030	[159]
CPA-mK	410	0.5–35	0.104	[160]
QT	400	0–3.0	0.032	[161]

Note: Abbreviations: BPB, bromophenol blue; TIPSP, tetraiodophenolsulfo-naphthalein; CAS, chrome azurol S; AG25, acid green 25; DBHPE, dibromohydroxyphenylfluorone; AlS_4Pc, tetra-substituted sulphonated aluminum phthalocyanine; PR, pyrogallol red; BPR, bromopyrogallol red; ACAP, 4-azochromotropic acid phenylfluorone; ACBK, acid chrome blue K; TB, trypan blue; DBM-CPA, dibromomethylchlorophosphonazo; CPA-mK: *m*-carboxychlorophosphonazo.

concentration of protein in the range 0.02–4.0 μg/ml for both BSA and bovine hemoglobin (HEM), and the detection limits were 10.4 ng/ml for BSA and 11.4 ng/ml for HEM.

Because the pI of BSA is 4.7, BSA is positively charged when the pH is lower than 4.7, leading to interaction with negatively charged RY and the formation of an association complex by electrostatic attraction. Therefore, RLS enhancement is observed in the RY–protein system. In addition, the experiment indicated that OP could also enhance the RLS intensity of positively charged protein, which suggested that OP could bind BSA. It is considered that the ether oxygen atom in OP possesses electronegativity, which resulted in the interaction between OP and BSA through both electrostatic force and hydrophobic interaction between the hydrocarbon tail of OP and BSA's hydrophobic region. Therefore, it can be postulated that both RY and OP bind to the protein, which acts as a template. RY molecules bind to BSA through electrostatic attraction, whereas OP molecules bind to BSA through a combination of electrostatic and hydrophobic interactions and form micelle-like clusters. The formation of the large RY–BSA–OP complex can result in very strong RLS.

7.4. Resonance double scattering (RDS) method

Double scattering (DS) is a kind of light scattering existing at the double incident wavelength. DS may be the influencing factor in fluorescence determination. It has always been eliminated as a bad phenomenon. Liu et al. [164,165] found that the intensity of DS can be enhanced greatly when the wavelength of the incident beam is close to that of the absorption band of the molecular particles that exist as aggregates. This phenomenon is called resonance double scattering. Based on this phenomenon, Liu and Liu [166] have developed a method for determination of cationic surfactants. This phenomenon has not been applied to biomacromolecule study.

Our group [167] first used the RDS technique for the determination of proteins in morin–CTMAB–proteins systems and studied the reaction mechanisms. Morin (3,5,7,2′,4′-pentahydroxyflavone) is one kind of polyhydroxyflavone and is most frequently used as an analytical reagent. It is a negatively charged dye at pH 7.30. Both BSA and HSA are biomacromolecules. They are negatively charged when the pH of the medium is higher than their pI. Because of the electro-repulsion, morin cannot interact with protein, but can enhance the intensity of RDS at pH 7.30. Experimental results proved that protein acted as the template in the system. The positively charged CTMAB will continue to assemble on protein to form positively charged pre-micelles. The latter promote the assembly of negatively charged morin on the pre-micelle to form the three component protein–CTMAB–morin aggregate. During the formation of aggregate, the surfactant acts as the mediator, which shortens the distance between protein and morin, thus enhancing their interaction and causing enhanced RLS. In addition, the merit of this method is that the system can be excited with near ultraviolet or visible light and detected in the near infrared region. This characteristic greatly alleviates interference of background.

Since the enhanced intensity of DS is in proportion to the concentration of protein, this method can be used for the determination of protein. Under the optimum conditions, the linear range is 7.5×10^{-8} to 1.0×10^{-5} g/ml for BSA, 2.5

$\times 10^{-8}$ to 5.0×10^{-6} g/ml for HSA. The detection limits are 66.0 ng/ml for BSA and 23.0 ng/ml for HSA, respectively. So, this method provided a new method for the determination of proteins.

Therefore, we can conclude that the RLS method is a sensitive technique for protein studies, but its selectivity is poor. We consider that the RLS method will have good prospects if it can be coupled with chromatographic techniques in the future.

8. Novel luminescent probes

8.1. Molecular beacons (MBs)

The molecular beacon is a novel fluorescent probe for nucleic acid, and it was first developed by Tyagi and Kramer [168] in 1996. MB is a kind of single-stranded oligonucleotide composed of a probe sequence embedded within complementary sequences that form a hairpin stem with a fluorophore and quencher attached to opposite termini. When the fluorophore and quencher are in close proximity, the fluorescence of the former is quenched by the latter through energy transfer. If the probe encounters a target DNA molecule, the MB undergoes a spontaneous conformational reorganization that forces the stem apart, resulting in the separation of both the fluorophore and the quencher, and the restoration of fluorescence [169]. Although MB probe was originally designed for nucleic acid studies, the hairpin structure of MB can be disturbed upon binding to some proteins and the fluorophore is distant from the quencher, resulting in fluorescence enhancement. Therefore, MB probes can also be used for protein studies [169].

Recently, Fang and co-workers [170] applied MBs to study DNA–protein interactions and to quantify protein molecules. It was found that an interaction between an *E. coli* single-stranded DNA binding protein (SSB) and an MB molecule results in significant fluorescence enhancement. Using MB-SSB binding, it is possible to detect SSB at a concentration as low as 2×10^{-10} mol/l using a common spectrofluorimeter. In addition, it was found that MB binding affinity with the different proteins decreased in the order: SSB > lactate dehydrogenate (LDH)-5 > histone (calf) > *E. coli* RecA protein > LDH-1 > bovine serum albumin, which would lead to selective binding investigations of the proteins. LDH is a common intercellular enzyme, which has five isoenzymes. All the isoenzymes' activities in serum are critically important in supporting a host of diagnoses [171]. MB probe has also been used for a detailed binding investigation with LDH [172]. It was found that different LDH isoenzymes had different single-stranded DNA binding abilities, and the binding ratio of LDH-5 to MB and the binding constant were 1:1 and 1.9×10^{-7} l/mol, respectively. This method is able to detect 1.8×10^{-8} mol/l LDH-5.

In comparison to other fluorescent probes, MBs have significant advantages of high sensitivity, excellent specificity and use in situations where it is not possible or desirable to isolate the probe-target hybrids from an excess of the unhybridized probes. But using MB probes for protein studies are still initial attempts and only nonspecific DNA binding proteins have been investigated so far. New MB probes for real-time specific protein detection will be further explored. We believe these MB probes will become a potential tool for genomics and proteomics studies, diagnoses of diseases, and new drug development.

8.2. Nanoparticle probes

As particle size approaches molecular dimensions, all properties of a material change, making nanomaterials useful for particular applications. Nanomaterials constitute an emerging subdiscipline in the chemical and materials sciences [173,174]. With the development of this nanoscience, the nanomaterials have numerous commercial and technological applications, including analytical chemistry [175–178]. Many papers have reported nanometer-sized luminescent particles linked to DNA or proteins as the detection probe. Its high sensitivity makes single-molecule detection (SMD) possible. Three types of nanoparticles are potentially useful as single-molecule probes, in particular, latex nanospheres, luminescent quantum dots and optically active metal nanoparticles. Using nanoparticles as probes in bioanalysis offers several potential advantages. First, suspensions of nanoparticles do not appreciably scatter light. Second, the low background results in low detection limits. In addition, nanoparticles form more stable suspensions and are therefore less susceptible to self-agglomeration.

8.2.1. Latex nanospheres

Early in the mid-1950s, Singer and Poltz [179] invention of latex agglutination tests, which used suspended latex microparticles that were chemically derivatized with a desired antibody. The analyte is an antigen, which binds to more than one antibody molecule. This resulted in agglutination (or clumping together) of the particles into what looks like curdled milk. Latex agglutination tests have been developed for more than 100 analytes, including infectious disease antigens and drugs [180]. Up to now, these micrometer-sized particles have been replaced by the nanoparticles [176,178,180]. Medcalf et al. [180] have developed an immunoturbidimetric assay for urine albumin, and indicator of kidney problem. In this study, poly(vinylnaphthalene) particles (40 nm) were coated with an outer layer of a chloromethylstyrene polymer, which was used to immobilize an antibody. Agglutination in the presence of urine albumin was detected by measuring the change in absorbance caused by light scattering at 340 nm [180]. In 2000, Nie and co-workers [181] used 20 nm fluorescent latex particles that are conjugated to proteins through amide bond formation. Unlike single dye molecules, each nanoparticle contains about 100–200 molecules of an em-

bedded dye that is protected from the outside environment. As such, these fluorescent nanoparticles are highly resistant to photobleaching and emit bright and steady (no blinking) fluorescence.

8.2.2. Luminescent quantum dots (QDs)

Luminescent quantum dots are also named semiconductor nanocrystals. These particles as probes are used in biological staining, diagnostics and fluorescent analysis. Compared with conventional fluorophores, the luminescent quantum dots have a narrow, tunable, symmetric emission spectrum and are photochemically stable. In these nanocrystals, the absorbance onset and emission maximum shift to shorter wavelength with decreasing size [182]. The excitation tracks the absorbance, resulting in a tunable fluorophore that can be excited efficiently at any wavelength shorter than the emission peak and that can emit with the same characteristic, narrow and symmetric spectrum regardless of the excitation wavelength. However, the use of semiconductor nanocrystals in a biological context is potentially more problematic because the high surface area of the nanocrystal might lead to reduced luminescence efficiency and photochemical degradation. By enclosing a core nanocrystal of one material with a shell of another having a larger bandgap, one can efficiently confine the excitation to the core, eliminating nonradiative relaxation pathways and preventing photochemical degradation. In addition, the water-solubility of these nanocrystals also should be considered. Weiss and co-workers [183] used a series of silica-coated core (CdSe–ZnS) nanocrystal probes in aqueous buffer to fluorescently label biological molecules and concluded that these nanocrystal probes are thus complementary and in some cases may be superior to existing fluorophores.

Facing the problem that these luminescent QDs are prepared in organic solvents and are not suitable for biological application, Chan and Nie [184] proposed their method to solve this problem. They used mercaptoacetic acid for solubilization and covalent protein attachment. When reacted with ZnS-capped CdSe QDs in chloroform, the mercapto group binds to a Zn atom and the polar carboxylic acid group renders the QDs water-soluble. The free carboxyl group is also available for covalent coupling to various biomolecules (such as proteins, peptides and nucleic acids) by cross-linking to reactive amine groups [185]. In addition, this mercaptoacetic acid layer is expected to reduce passive protein adsorption on QDs. This “blinking” behavior may seem fascinating from a fundamental point of view but can cause signal intensity fluctuations in ultrasensitive detection. The fluorescence QD labels have been used for sensitive immunoassay by QD-immunoglobulin G conjugated with bovine serum albumin. The result showed that well-dispersed and primarily single QDs were detected in the presence of BSA and the attached immunomolecules can recognize specific antibodies or antigens. This is similar to latex immunoagglutination.

8.2.3. Optically active metal nanoparticles

Extremely small clusters of metal are generally not long-lived in aqueous solution and ambient temperature [186]. Only stable intermediate colloidal metals have practical significance. It’s well known that the colloidal particles are large enough (>1 nm) to have the property of a metal. In the cases of silver and gold, the metal property is readily recognized by the presence of a band (Ag, 380 nm; Au, 520 nm) in the absorption spectrum, which is caused by surface plasmon absorption of the electron gas. With these two characteristic visible absorption bands, Ag and Au are mostly used as the probes of biological molecules. In fact, the particles with truly nanoscopic dimensions are gold nanoparticles, which are applied in tests of latex agglutination [178]. Garter-Wallace used conventional micrometer-sized latex particles conjuncted with gold nanoparticles (less than 50 nm diameter), where gold nanoparticle acted as an indicator.

Study also found that direct adsorption of proteins, such as enzymes, onto bulk metal surfaces frequently results in denaturation of the protein and loss of bioactivity. In contrast, when such proteins are adsorbed onto metal nanoparticles, bioactivity is often retained. Crumbliss [187] found that they could adsorb redox enzymes to colloidal gold with no loss of enzymatic activity. In addition, Natan and co-workers [188] found that cytochrome c retained reversible cyclic voltammetry when deposited onto 12 nm diameter gold particles attached to a conductive substrate. These results demonstrate another unique feature of metal nanoparticles-biocompatibility.

The optically active metal nanoparticles are widely used in the technique of surface enhanced Raman spectroscopy (SERS). This concept was first introduced by Van Duyne et al. [189], showing that attomole mass sensitivity could be achieved using micrometer-sized sampling areas. SERS uses roughened metal surfaces to enhance the Raman scattering of surface-adsorbed molecules, which can be as much as 10^6 over the molecule’s native Raman scattering in solution. Taking advantage of the giant local field enhancement observed in SERS, it has become a part of the family of single molecular spectroscopies (SMS). Recently, it has been reported that SMS was obtained on silver aggregates [190,191] and silver particles [192,193]. The key to using SERS as an analytical technique is to obtain a reproducible and uniform surface roughness. Natan’s group has studied SERS surfaces prepared by self-assembling gold and silver nanoparticle on glass and other substrates. Such surfaces have shown excellent reproducibility, for both different locations on a single surface and different but identically prepared surfaces [194]. If binding with biological molecules, use of Au nanoparticle tags leads to a more than 1000-fold improvement in sensitivity [195]. Thus, the ultrasensitive detection technique is established.

Nanoparticles have unique chemical and physical properties that offer important possibilities for analytical

chemistry. This field is in its infancy, and many new opportunities for nanomaterials will arise in the future.

9. Conclusions

Spectral probes (or labels) have been used for the investigation and determination of proteins and have made considerable progress in recent years. Lots of useful methodologies have been summarized in this review. Each has its advantages and disadvantages.

Fluorescent derivatizing reagents suitable for labeling the N-terminus or another functional group of proteins have been successfully used because of their high sensitivity, especially in HPLC and CE separations. However, major problems arising from inefficient chemistry, multiple derivatives, and the reaction conditions, such as time, temperature and concentration, must be overcome through further studies.

Rare earth ions and their chelates have good luminescence characteristics and strong binding with biological molecules. In the theoretical study of proteins, rare earth ions are used for detecting the binding site and studying the conformation of protein. The co-luminescence effect solves the problem of low sensitivity caused by rare earth ions and enriches the energy transfer theory. The multiple labeling with suitable rare earth chelates associated with time-resolved fluorescence technique can be used for the immunoassay with very high sensitivity.

The high sensitivity obtained from the noncovalent binding between organic dyes and proteins makes this method attractive to biologists, and it is very simple, fast and cheap. The binding effect between organic dyes and proteins is mainly based on electrostatic forces, so the pH must be strictly controlled in the detection.

Compared with the fluorescence methods, the problems encountered in photoluminescence, such as light scattering or source instability, are absent in chemiluminescence. The high sensitivity, fast reaction and low consumption of expensive reagents are also advantages of this method. However, the shortage of multianalytes, homogeneous analysis and selectivity of coupling and triggers are need to be considered in the future.

Near infrared fluorescence detection and resonance light scattering techniques are hot spots in recent years. They enable scientists to design better probes suitable for protein detection. The resonance light scattering technique possesses the advantages of a short reaction time, easy operation and high sensitivity, whereas the advantages of new infrared fluorescence methods are low background and high sensitivity. Therefore, they will be more important and will become an essential tool in medical and biochemical research as well as in routine analysis.

The molecular beacon is a novel fluorescent probe first used for nucleic acid. Recently, MB had been used for protein detection and studies. As a fluorescent probe, it has significant advantages of high sensitivity and excellent specificity over common fluorescent probes. Therefore, it will become a potential tool for genomics and proteomics studies. The luminescent nanoparticle is a new luminescent probe for protein, which is brighter and more stable against photobleaching in comparison with single organic dyes. We believe that nanoparticle probes will carry bio-analytical chemistry in to a new and wonderful world.

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