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REVIEW ARTICLE

Fluorescent Probes for Detection of Protein: From Bench to Bed

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Abstract: ***Background*:** Protein is a macromolecule, consisting of one or more long chains of amino residues. And it plays a vital role in the life system, especially for the evaluation of many diseases. Thus, the quantitative measurement of protein is benefit for the diagnosis and monitoring of diseases. Among the sensing methods currently developed, fluorescence detection is of particular concern as a technique with the potential to provide accurate and quantitative analyses. A wide variety of molecular probes for protein detection have been designed and synthesized for sensing, imaging, and biomedical applications.

***Conclusion*:** In this report, we provide an overview of the varied probes from small molecular to nanoparticle-based sensors which have been applied to detect a various proteins from bench to bed. Furthermore, some emerging trends, future directions as well as challenges in this rapidly growing field are discussed.

Keywords: Fluorescent probe, detection, molecular imaging, biosensor, optical imaging, molecular probes.

1. INTRODUCTION

Nowadays, understanding the physical and pathological processes of the human body at the molecular level is of major preclinical and clinical significance. Proteins, one kind of bio-macromolecules, consist of one or more long chains of amino acid residues. They are not only the final executants of life functions but also the key to understand the mechanisms of physiological, pathological and pharmacological processes [1]. Although the structures of many proteins has been successfully analyzed and confirmed, it remains difficult to understand their complex functions in living systems. Therefore, it is of great importance to develop effective bioimaging methods of proteins for the diagnosis and treatment of related diseases.

The common laboratory and clinical protocols to probe these disease-related proteins are the immunofluorescence technique (such as the Enzyme-Linked Immunosorbent Assay, ELISA) due to its modularity, reproducibility and high sensitivity [2]. However, this technique always requires complicated operation process, high detection cost and long detection time. Fluorescence spectroscopy has been a powerful tool in biochemical research by virtue of its high sensitivity, accuracy and non-invasiveness. With considerable attempts and efforts by researchers, a significant development of fluorescent probes for protein detection and imaging has been achieved during the past few decades. Fluorescence imaging could reveal the localization and distribution of protein in cells, even at the single-molecule level, which enables us to understand the roles of protein in biomedical systems.

Fluorescent probes are indispensable tools for protein detection, and a great diversity of fluorescent probes has been well developed so far. On the one hand, lots of efficient and effective small molecular probes were designed and synthesized according to a number of strategies, such as Excited-State Intramolecular Proton Transfer (ESIPT), Fluorescence Resonance Energy Transfer (FRET), Internal Charge Transfer (ICT), Photoinduced Electron Transfer (PET) and Aggregation-Induced Emission (AIE). On the other hand, some excellent fluorescent sensors were developed based on nanotechnology including Quantum Dots (QDs), Gold nanoparticles (Au NPs) and Magnetic Nanoparticles (MNPs) due to their unique physicochemical and optical properties. In fact, to date, fluorescent sensors were combined with High-Throughput Screening (HTS) technique such as planar microarrays (protein chips) and bead-based microarrays (suspension arrays) for rapid and precise detection of complex protein samples.

In this paper, we intend to provide a concise and timely minireview of the novel probes and their possible applications in protein detection and visualization. We will also discuss their implications for the development of future multifunctional agents by understanding the physical and pathological processes of the human body.

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2. SMALL MOLECULE-BASED FLUORESCENT PROBES FOR PROTEIN IMAGING

2.1. ESIPT-Based Fluorescent Probes

ESIPT is a photochemical process occurring with a fast proton transfer from a hydroxyl (or amino) unit to a carbonyl oxygen (or imine nitrogen) atom in the excited state of a fluorophore [3]. Considering the superior photophysical properties including high fluorescence quantum yield, dual-wavelength emission (emission of enol and ketone form), large Stokes shift and so on, the ESIPT-based fluorescent probe could be highly valuable for the biomolecular detection [4]. For example, as shown in Figure **1**, in the ground state, some benzoxazole derivatives exist preferentially in the enol form (E) and is stabilized in an intramolecular H-bonding motif. Upon excitation by UV-light, a rapidly transformation from the excited enol form (E*) into the excited keto tautomer (K*) occurs with a new longer emission band appearing because of the ESIPT effect. The proportion of two kinds of fluorescent intensities is closely related with the concentration of the analyte. In recent years, several other ESIPT fluorophores, including 3-hydroxychromones (3HCs), 2'-Hydroxychalcone (HC), 2-(2-benzoyloxy-3-methoxyphenyl) benzothiazole (HMBT), 2-(2'-hydroxyphenyl) imidazo [4,5-b]pyridine (HPIP), have been developed as fluorescent probes for protein detection. Yushchenko and co-workers [5] reported a 3HCs -based ESIPT probe (Probe 1) for the detection of α-synuclein. These compounds exhibit a rapid ESIPT process from 3-hydroxyl to 4-carbonyl, resulting in two emission bands from the initial "normal" excited state (N*) and the ESIPT phototautomer product (T*). The T*/ N*ratio is related to the H-bonding capacity and the polarity of the environment. Freire and co-workers [6] reported a HPIP-based ESIPT probe (Probe 2) for the detection of amyloid-β fibrils with high selectivity that is significant to the prevention and treatment of Alzheimer's disease. In other studies, a HMBT-based ESIPT probe (Probe 3) [7], where the hydroxyl group was protected by the benzoyl moiety, was found to be effective for the human carboxylesterase 1 (hCE1) detection (Figure **2**). Similarly, Song *et al*. [8] synthesized a ratiometric HC-based ESIPT probe (Probe 4) for ALP activity assay and visualization in living cells. As shown in Figure **3**, The probe only exhibits a fluorescence emission band in aqueous buffers because the hydroxyl group in this probe is protected by a phosphate group. In the presence of ALP, along with the removal of the phosphate group, the ESIPT effect could be recovered with the ratiometric fluorescent response, and the emission of probe change to deep red gradually.

2.2. FRET-Based Fluorescent Probes

The molecules based on FRET properties are generally composed of two different fluorophores which act as the energy donors and acceptors, respectively, and linked by a non-conjugated spacer with energy transfer occurring through the non-radiative dipole-dipole interaction [9, 10]. The absorption of acceptor has a significant overlap with the emission of donor, and the distance between the two fluorophores is generally within the range of 1-10 nm [11,12]. To date, the probes with FRET effect are widely used for the detection of protein. For instance, a FRET-based probe (probe 5) was designed to trace the caspase-3 and monitor the apoptosis, as shown in Figure **4**, which consists of three parts: porphyrins pyropheophorbide (Pyro) as the caspase-3-responsive FRET accepter, 5-Carboxy-X-Rhodamine (Rox) as the organic fluorophore donors, and caspase-3 specific peptide GDEVDGSGK as the linker [13]. The overlap between ROX emission and Pyro absorption indicated that Pyro was a suitable accepter for ROX emission. Upon excitation of the ROX moiety, the excitation energy is transferred to the Pyro acceptor through a caspase-3 specific peptide GDEVDGSGK. However, in the presence of the caspase-3, the caspase-3 specific peptide GDEVDGSGK could be cleaved, thus separating the donor and acceptor; that led to the decrease of energy transfer efficiency with a ratiometric change in the fluorescent emission. In the following research, a probe with multiple-FRET effect could even detect a variety of substances through the interaction with different ligands. Li and co-workers [14] designed a dual-FRET-based probe (probe 6) for the specific detection of Matrix Metalloproteinases (MMPs) and caspase-3. 5(6)-carboxyfluorescein (FAM) and two 4-{[4- (dimethylamino)-phenyl]-azo}-benzoic acid (Dabcyl) moieties acted as the fluorescence donor and acceptor, respectively, and they were linked by MMP-2 specific peptide sequence Pro-Leu-Gly-Val-Arg (PLGVR) and a caspase-3 sensitive peptide unit Asp-Glu-Val-Asp (DEVD). As shown in Figures **5** and **6**, the fluorescence of FAM could be quenched by the two Dabcyl moieties, while the recovery of fluorescence could be observed in different degrees after the probe was incubated with the different enzymes (MMP-2 or caspase-3), and that made it easy to distinguish two types of protein. Moreover, the FRET between Fluorescent Protein (FP) variants, has been developed to monitor a series of protein and various biological phenomena [15, 16]. The specific substrate molecules fused with fluorescence protein lead to energy transfer upon cross-linking by linker; the increased analyte can be monitored as a FRET efficiency increase.

2.3. ICT-Based Fluorescent Probes

The probes with ICT effect are composed by an electron-donor and an electron-acceptor linked by the conjugated chain that give rise to a "push–pull" π-electron system in the excited state [17]. Upon the interaction of the electron-donor or the electron-acceptor with the analyte, the electronic effect of the system has changed greatly, resulting in the blue shift or red shift in the emission spectrum, respectively (Figure **7**). In addition, the changes in the fluorescence quantum yields also could be induced [18]. Recently, ICT-based probes have been extensively used for protein sensing. For instance, as shown in Figure **8**, Zeng and co-workers [19] designed and synthesized a colorimetric and ratiometric probe (probe 7) with ICT effect for the detection of Bovine Serum Albumin (BSA). The probe is composed of a 4-amino-naphthalimide fluorophore and a receptor of 4-hydroxy-2-butanone. As a protecting group, 4-hydroxy- 2-butanone could decrease the electron-donating character of the probe. When the BSA interacts with the 4-hydroxy-2-butanone unit, an apparent red shift could be observed in the emission spectrum due to the recovery of ICT effect. Similarly, Cui *et al.* [20] reported an ICT-based 4-aminonaph-

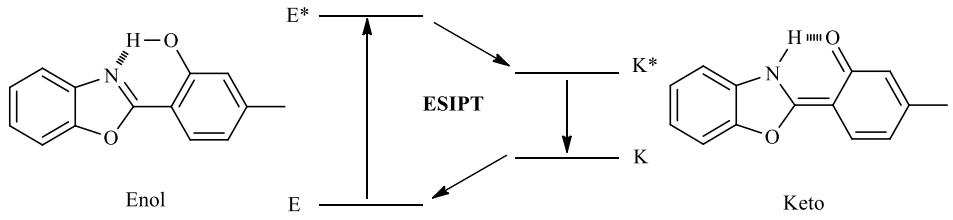

Figure 1. An illustration of ESIPT process that occur upon photoexcitation of benzoxazole derivatives.

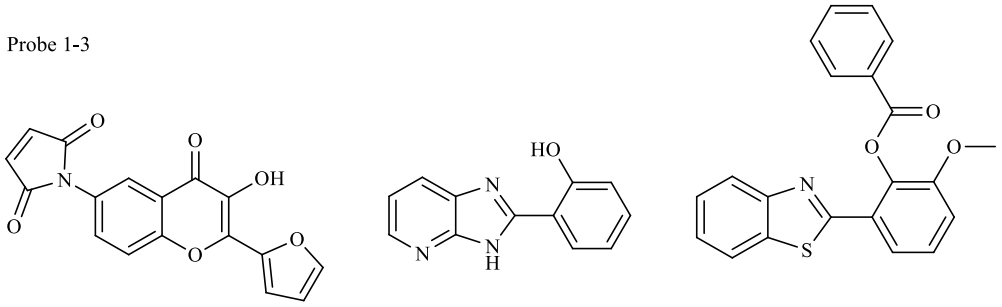

Figure 2. Chemical structures of ESIPT-based probes, probes 1-3.

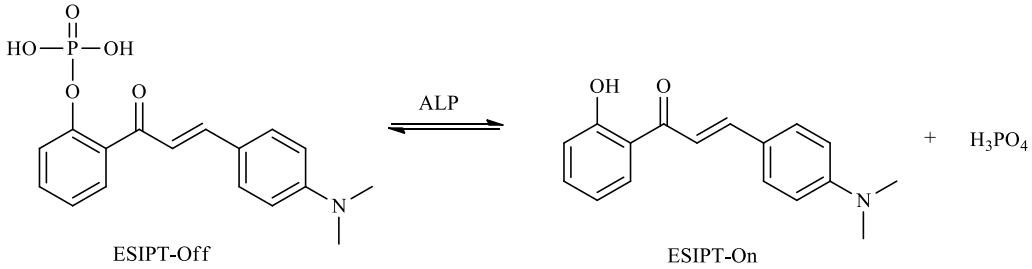

Figure 3. The schematic illustration of probe 4 for ALP activity assay.

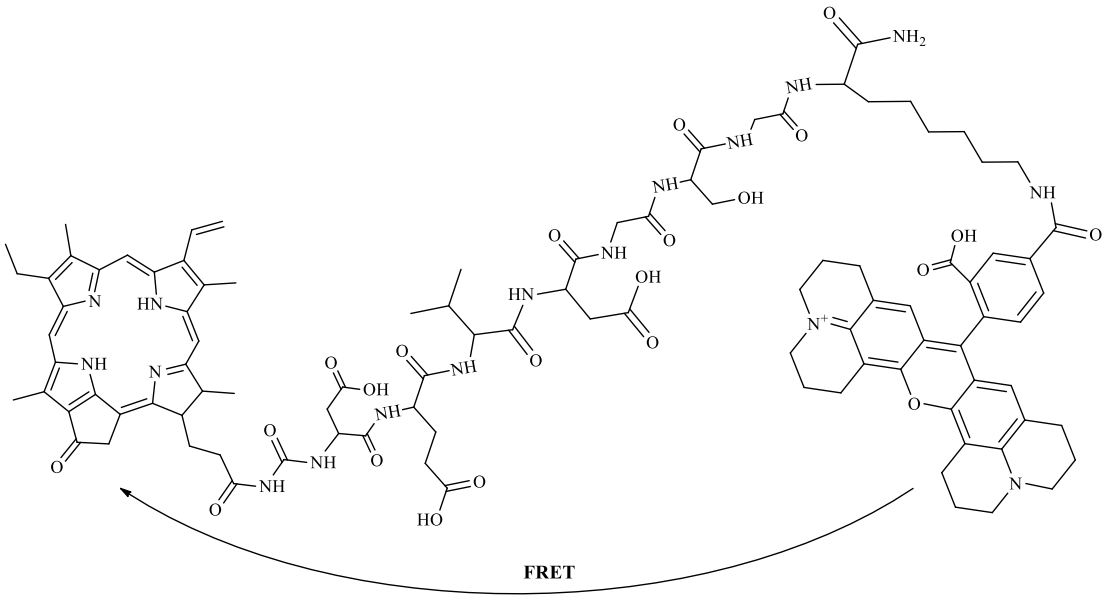

Figure 4. The chemical structure and FRET process of probe **5**.

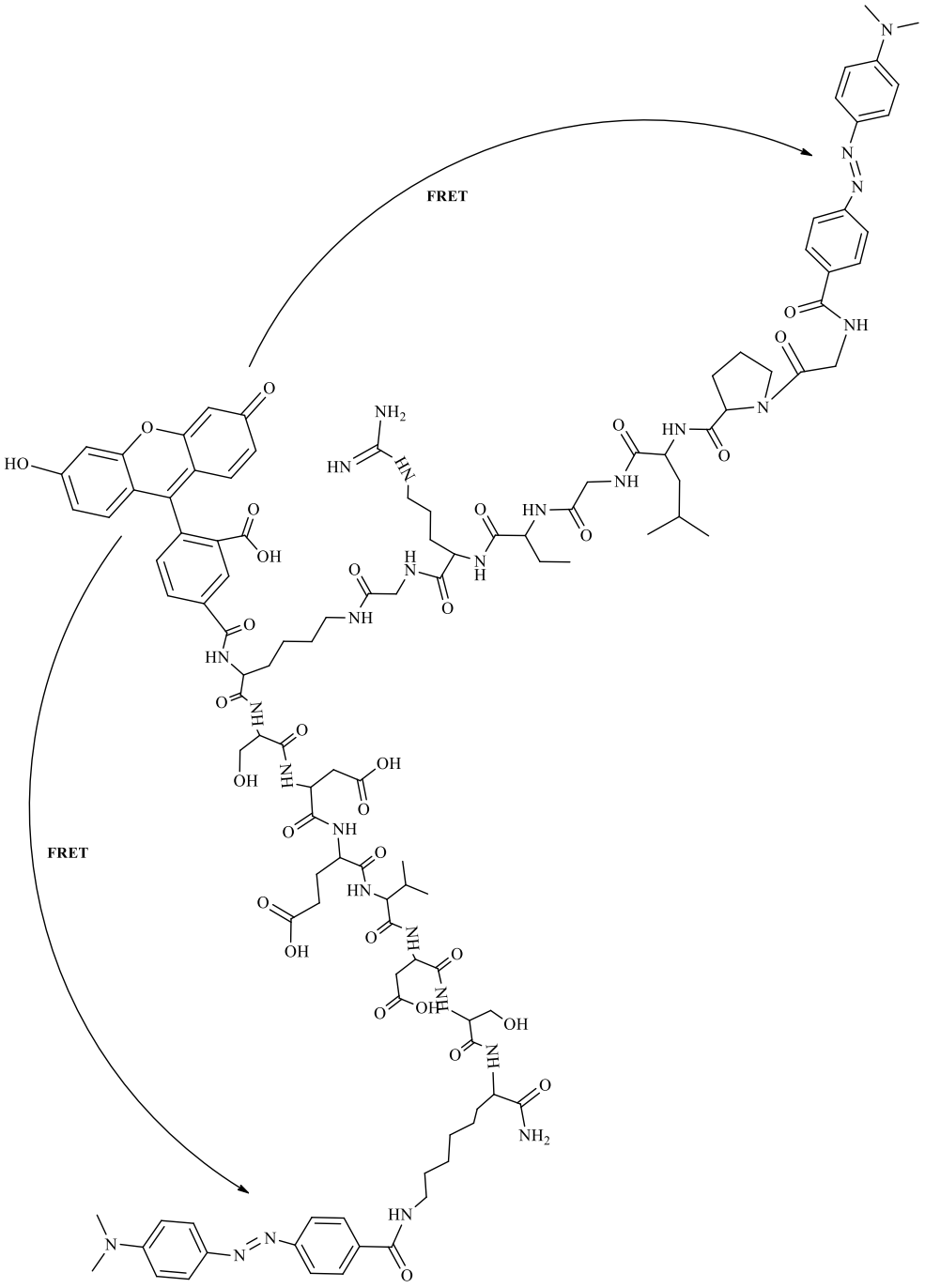

Figure 5. The chemical structure and FRET process of probe **6**.

thalimide probe (probe 8) to detect N-Acetyl Transferase 2 (NAT2) through enzyme-specific activation. In the other research, many ICT-based fluorescent probes have been successfully developed to detect protein via non-covalent bonding. As shown in Figure **9**, a series of microenvironment-sensitive fluorescent probes (probe 9-14) were designed to detect Human Serum Albumin (HSA). These probes were characterized by a charge transfer-excited singlet state, and it could be rapidly inactivated by intramolecular rotation about the donor–acceptor bond. After the binding onto the hydrophobic pocket of HSA, probes could exhibit strong fluorescence due to the restricted rotation of the fluorescent rotor and the change of Twisted ICT (TICT) effect [21-25]. In some recent studies, there are many TICT-based probes (probe 15-17) which consist of both a TICT-based fluorescent group and a highly affinitive binding site also have been reported for the detection of some specific protein [26]. For example, amyloid fibrils could be monitored by fluorescence molecular rotor *via* non-covalent bonding between fibrils and the fibrils-affinity unit N,N'-substituent in probe [27-29]. In the presence of amyloid fibrils, bond rotation of the probe is restricted, thereby triggering the emission of strong fluorescence.

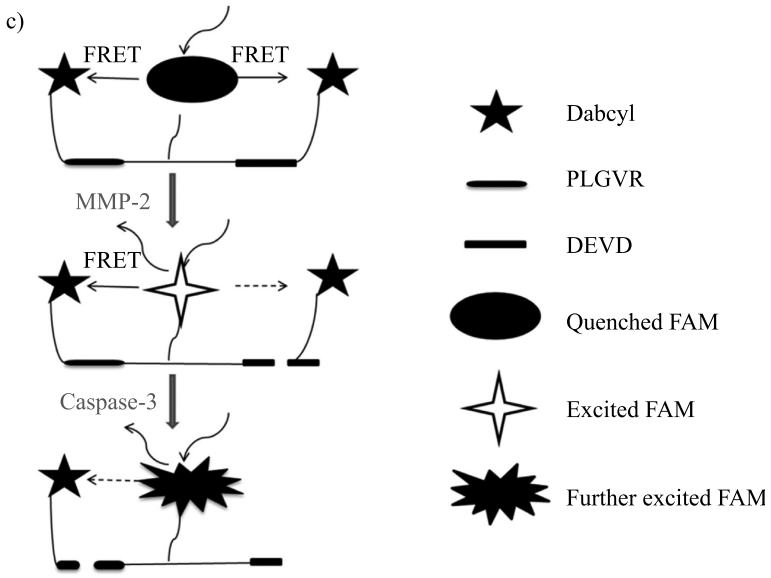

Figure 6. The mechanism of the probe **6** for the special MMP-2 and caspase-3 detection.

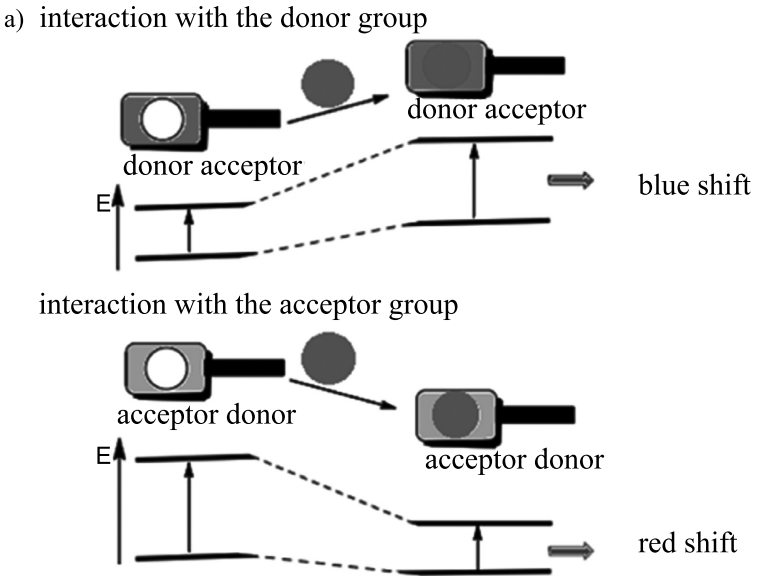

Figure 7. Schematic illustration of the mechanism based on ICT.

2.4. PET-Based Fluorescent Probes

PET-based fluorescent probe is composed of three parts: recognition group (electron-donor), linker, and fluorophore (electron-acceptor) [30]. As shown in Figure **10**, PET, in which electron transfer from the electron-donor to the electron-acceptor under the photo-excitation, resulting in fluorescence quenching process. After the interaction of the recognition group and the analyte, the electron-donor ability of recognition group will decrease, so electron transformation will be interrupted, and led to the fluorescent recovered again [31]. In addition, since PET efficiency is less sensitive to surrounding polarities, PET-based probes are candidates to measure strain in bimolecules and they have been extensively developed for protein sensing. For example, as shown in Figure **11**, 4,4-Difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY)-based probes (probe 18-19), which are generally designed by connecting the analyte to recognition groups, have attracted much attention in protein detection and exploit the PET effect to monitor the analyte by fluorescence changes [32, 33]. Besides, Peng's group [34,35] reported a series of PET-based probes (probes 20-21) to detect cyclooxygenase-2 (COX-2) *via* the specific interaction with the inhibitor of COX-2 (Indomethacin) (Figure **12**). In aqueous buffer solution, the probe is present in the folded state with quenching fluorescence because of the PET effect between the fluorophore and the inhibitor of COX-2. However, the fluorescence of probe is turned on due to the binding of probe to COX-2 could transform the structure of probe from folded to unfolded and restrain the PET effect. In addition, the result showed that the probe 18 could distinguish inflammation and tumor effectively; this is important for early diagnosis of cancers.

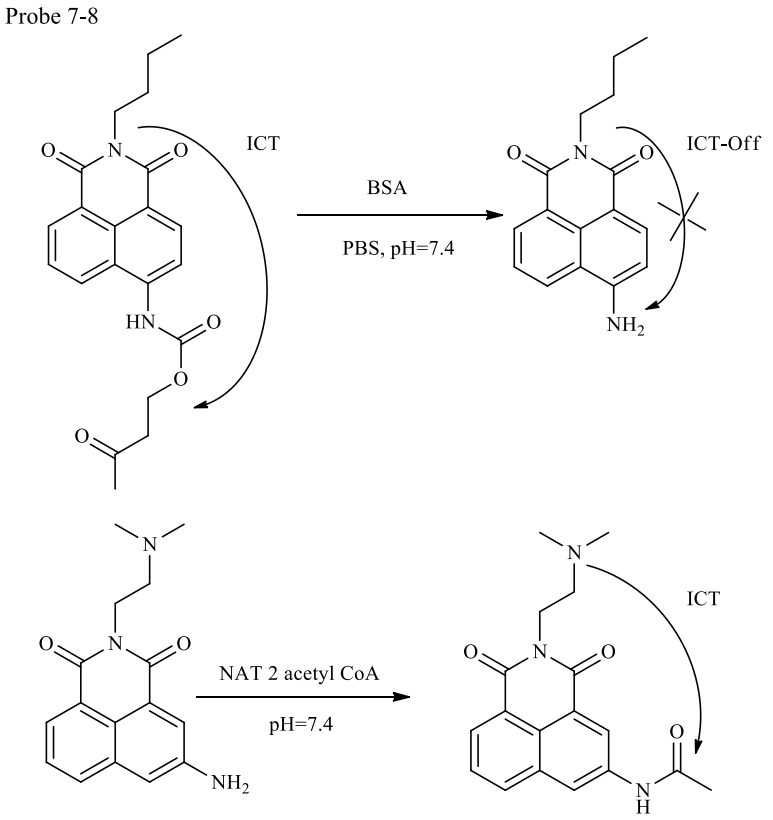

Figure 8. Chemical structures and ICT process of probe **7** and **8**.

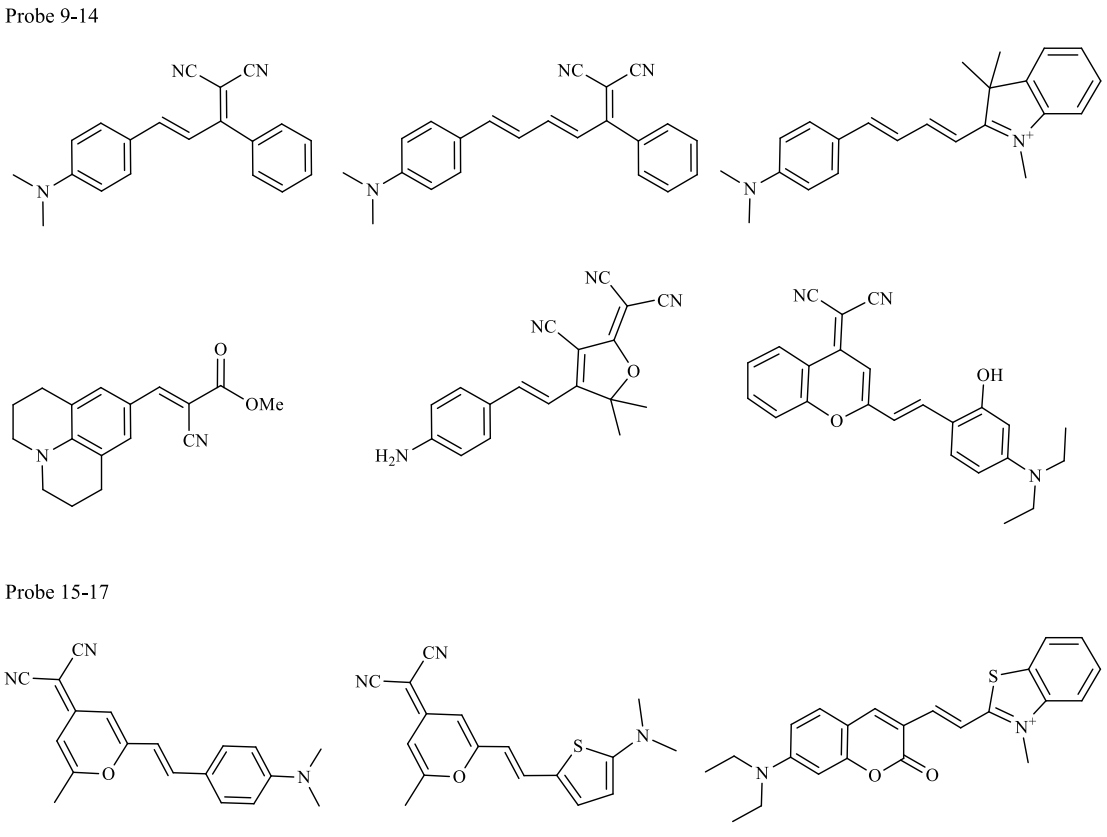

Figure 9. Chemical structures of probe **9-17**.

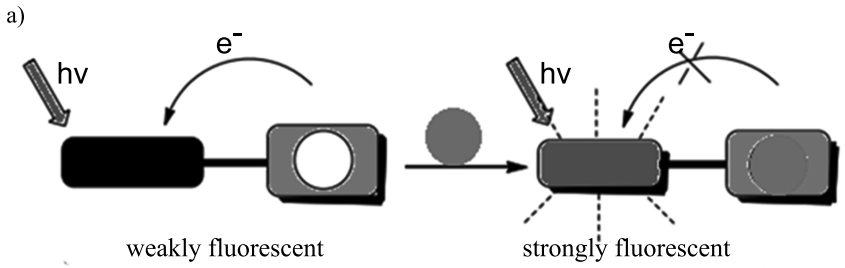

Figure 10. Schematic illustration of the mechanism based on PET.

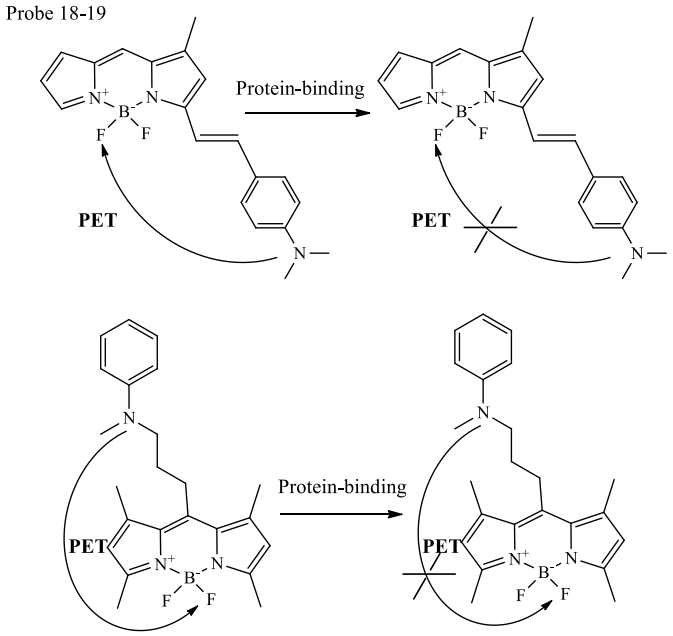

Figure 11. Chemical structures and the PET process of probe **18** and **19**.

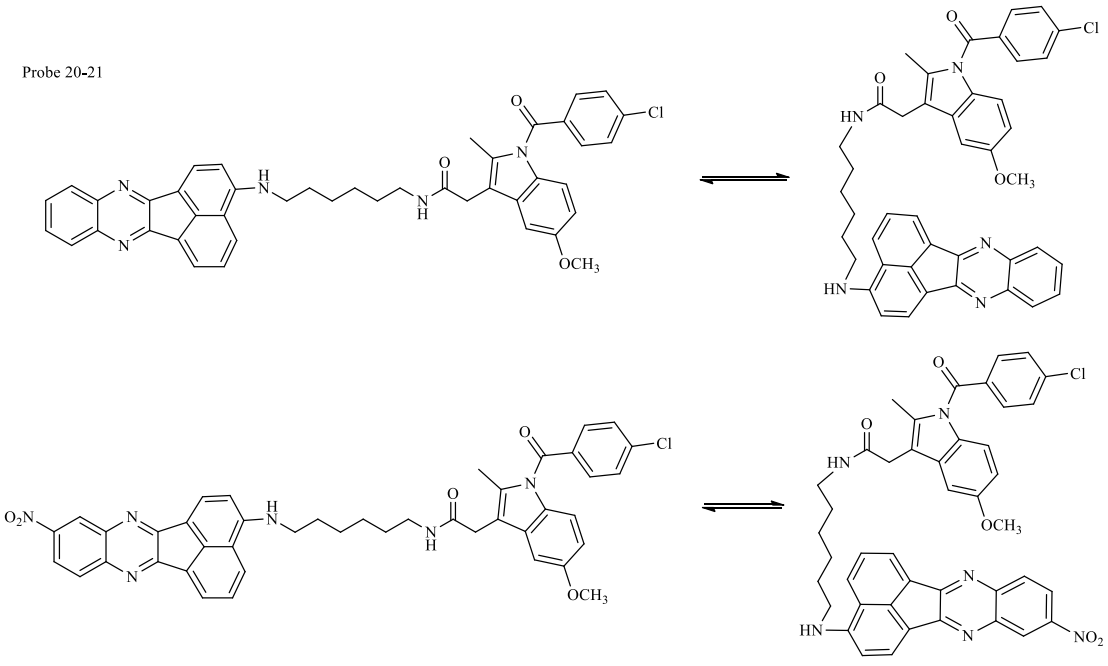

Figure 12. Chemical structures of probe **20-21**.

2.5. AIE- Based Fluorescent Probes

Fluorescence probes have been deeply developed because their superior properties; however, a many traditional fluorescence probes have a obvious shortcoming which is that they are easy to aggregate through the π-π interaction resulting in the fluorescence quenching. The Aggregation-Caused Quenching (ACQ) has significantly limited the degree of labeling of fluorophores to analytes in the solid-state [36, 37]. In contrast, the AIE property which is opposite to the ACQ effect has been founded in many fluorescent probes and has attracted much attention of researchers due to its superior emission intensity in the solid-state [38, 39]. In 2006, two AIE-active cationic Tetraphenylethylene (TPE) derivatives were reported to have been used for the protein sensing. The emissions were switched on by their bindings with proteins [40]. The AIE-based probes have weaker fluorescence in aqueous solutions due to the free intramolecular rotation, and excellent "turn-on" fluorescent behavior after specific binding to the high affinity site because the intramolecular rotation is restricted. In recent years, the AIE-based probes have attracted more and more attention for protein detection because of their unique properties. For example, a number of tetraphenylethylene (TPE) [41]; 2,2',4,4'-tetrahydroxy-benzophenone azine [42]; 1,2,5-triphenylpyrrole(TPP) [43]; and squaraine dye derivatives [44, 45] (probe 22-26) have been rapidly developed for the detection and quantitation of HSA because they can form highly fluorescent aggregates with the hydrophobic pocket of HSA (Figure **13**). In addition to binding to the hydrophobic pockets, some AIE-based probes with site-selective group also could combine with the special binding sites of protein, for instance, as shown in Figure **14**, the TPE probe with triphenylphosphonium (probe 27) exhibits the best performance in distinguishing the a-Syn fibrils because of the restriction of intramolecular motions (RIM) through interactions with a-Syn fibrils [46]; 4-((1Z,3Z)-1,4-bis (4-(methoxycarbonyl) -phenyl)-4-(pyridin-4-yl) buta-1,3-dien-1-yl)- 1- methylpyridin-1-ium hexafluorophosphate (TABD-Py-PF6) as a fluorescence bioprobe to monitorγ-globulins *via* specific interactions in a certain high affinity binding site (probe 28) [47]. Besides, the probe (probe 29) with cyclic arginine–glycine-aspartic acid (cRGD) could selective bind to the integrin αvβ3 [48]. To be noted, these applications lay the foundation for the development of the specific protein tracing in clinic.

2.6. Others

Besides the mechanism reviewed in the above paragraphs, the protein also could be detected through a variety of other methods, such as the electrostatic interaction [49], the metal ion reactions [50], and the interaction with high affinity and selectivity between the aptamer and the ligand of probe [51, 52] and so on. However, it is difficult to protein sensing *in vivo* due to the small molecule-based fluorescent probes could be influenced by many factors, such as pH, temperature, and so forth. Thus, it is necessary to develop more useful strategy to generate small molecule fluorescent probe for protein sensing in clinical.

3. NANOPARTICLES-BASED FLUORESCENT PRO-BES FOR PROTEIN IMAGING

3.1. QDs Based Probes

Quantum Dots (QDs) are the semiconductor nanocrystals which have physical dimensions close to or smaller than the exciton Bohr radius [53]. Considering the adaptable photophysical properties including a very broad absorption spectrum and large effective Stokes shifts of more than 100 nm [54-58] and the superior stability of QDs, they could be used for super-resolution microscopy and single-particle monitoring. In 1998, two papers appeared almost simultaneously in Science [59, 60] describing the first applications of QDs to biological imaging. After that, Wu and co-workers [61] described the QDs which linked to immunoglobulin G (IgG) and streptavidin for nuclear antigens inside the nucleus detection through imaging the breast cancer marker Her2 on the surface of fixed and liver cancer cell, and staining actin and microtubule fibers in the cytoplasm. Interestingly, using QDs conjugated to IgG and streptavidin, two cellular targets with one excitation wavelength could be detected due to their different emission spectrums exhibited. To develop more rapid and precise detection of proteins, scientists attempted to integrate QDs and High Throughput Screening (HTS) technology such as microfluidic chip and microarrays. For example, Hu and co-workers developed a microfluidic protein chip for a multiplexed and ultrasensitive detection of cancer biomarkers [62]. Microfluidic biochips are hopeful tools to detect protein with simple rapid and high sensitivity for the reason that the ambulatory fluid could catalyze the reaction between QD probes and targets, and prevent QDs from nonspecific deposition at surfaces. More recently, Chang and co-workers [63] not only developed an optical cross-reactive sensor arrays based on a number of intrinsic dual-emitting Mn-doped quantum dots capped with different organic functional groups for detection of protein, but also designed a ratiometric strategy, which used signal ratios of emission intensities at two different wavelengths to detect proteins.

3.2. Au NPs Based Probes

Gold Nanoparticles (Au NPs) are sol or colloidal suspension of submicrometre-size nano- particles of gold in a fluid. In the recent decades, Au NPs have been universally used for applications both in photonics and bio-imaging due to their unique optical properties which are conferred by the interaction of light with electrons on the Au NP surface. In 2009, Hong and his coworkers [64] reported a AuNP-based probe. It could monitor the evolution of Superoxide Dismutase (SOD1) aggregates with highly sensitive and colorimetric, which was very important to discuss the pathology of Amyotrophic Lateral Sclerosis (ALS). And a novel gold nanoparticle (AuNP)-based colorimetric detection system also was proposed by the same group for the visualization and characterization of protein aggregates sensitively using their own monomer-conjugated AuNPs. For high throughput discrimination of proteins, Zhang and his coworkers [65] developed the array-based sensing strategy which provided differential fluorescence changing patterns that can be used for protein

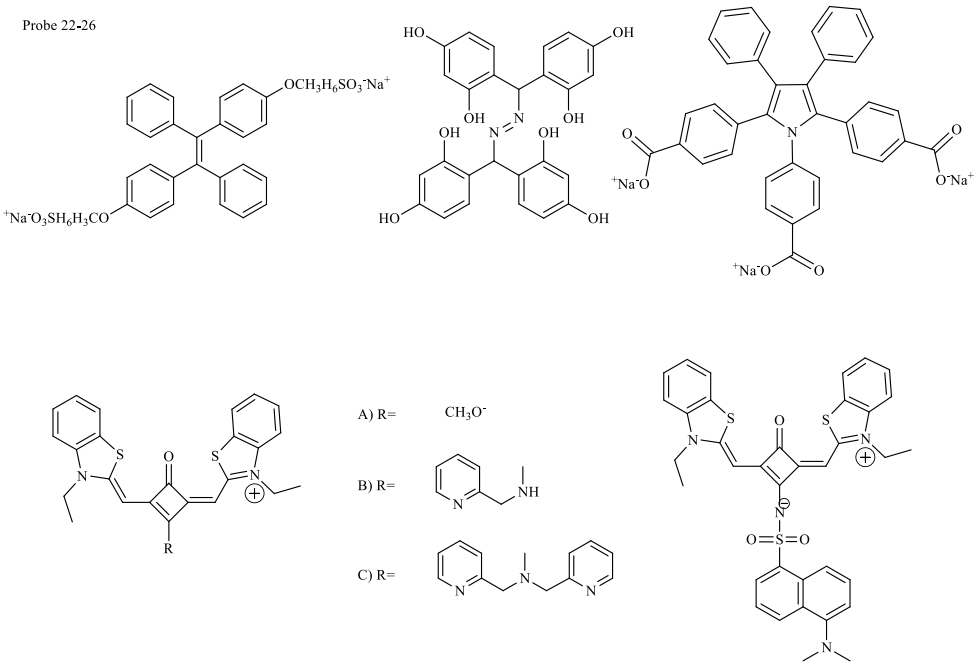

Figure 13. Chemical structures of AIE-based probes **22-26**.

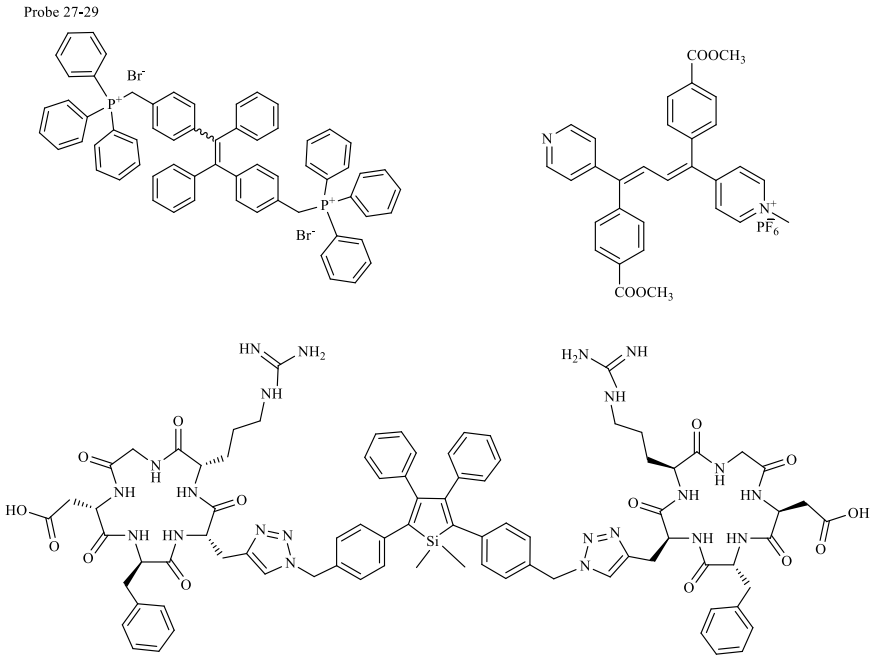

Figure 14. Chemical structures of AIE-based probes **27-29**.

detection. Recently, Chen and his team [66] designed a novel fluorescence probe to trace PSA in patient serum at pg/mL level. This probe has superior sensitivity because of the high loading efficiency of RBITC onto AuNP surfaces and high fluorescence "turn-on" characteristic of RBITC-AuNPs.

3.3. Magnetic Nanoparticles Based Probes

Magnetic Nanoparticles (MNPs) always refer to the nanomaterials containing Cobalt (Co) or Iron (Fe) as well as their oxides and alloys which become superparamagnetic at room temperature on condition that their size is below a critical value. Due to their large constant magnetic moment, MNPs have shown many interesting potential applications in protein detection and purification, such as biomarker enrichment and detection, indicated protein separation and analyses, labeling and dynamic tracking, fluorescence or Magnetic Resonance (MR) imaging. For instance, The Au-doped Magnetic Silica Nanotubes (Au-MSNTs) [67], acted as a receptor, were prepared by Jung and co-workers for the selective detection of proteins containing cysteine and other

biomolecules. In 2010, Chen and his team [68] proposed a new method to detect phosphorylated species. They designed a probe by fixing Riboflavin-5'-Monophosphate (RFMP), a fluorescence molecules, onto the surface of alumina-coated magnetic nanoparticles (RFMP-Fe_3O_4@Al_2O_3 MNPs) through Al-phosphate chelating. The magnetic features of the MNPs lead to fast isolation of the MNPs-target species from the solution. Recently, Liu and his team [69] designed and synthesized a simple, rapid and sensitive aptamer-based probe to detect protein using bare MNPs as the fluorescence quenchers. The principle of the sensor is the fact that the bare MNPs can adsorb the fluorescent dye modified thrombin aptamer (CY3-aptamer) in binding buffer, resulting in fluorescence quenching.

4. ASSEMBLED FLUORESCENT PROBES FOR PROTEIN IMAGING

Another nanotechnology for protein imaging is self-assembly, in which a disordered system of pre-existing components forms an organized structure or pattern as a consequence of specific, local interactions among the components themselves, with no external direction. These small-molecule organic dyes usually assembled into nanoparticles directly without nanocarriers. They often exhibit good photostability, favorable biocompatibility and specific response to many biological analytes. In addition, they have great flexibility, variety and functionality in molecular design and good stability in optical properties. Palapuravan Anees and his team [70] demonstrated a fluorescent dye which can be empowered to form a selective nonspecific protein probe with a thiol sensitive near-IR Squaraine (SQ) dye. In order to improve the stability of the aggregates of squaraine dyes, Fan and co-workers [71] designed and synthesized a new pyrene-conjugated squaraine fluorescent sensor SQ-P for serum albumin detection in the Near Infrared (NIR) region with a turn-on response. In their design, the dyes could self-assemble into nanoparticles in aqueous solution via multiple interactions. And an additional intermolecular π-π and π interactions were constructed through pyrene segment which was conjugated to the SQ fluorophore. Recently, Yu and his team [72] developed a C-H small molecule-based nanosensor to monitor the conformational changes and the site-specific interaction of human serum albumin. When acting with albumin, the sensor showed fast "turn-on" fluorescence response with the dismantling of the nanostructure. This phenomenon proved that the nanosensor have highly specific and sensitive response to HSA, without interference by other endogenous biomolecules.

CONCLUSION AND PERSPECTIVES

Over the past decades, the area of protein fluorescence imaging is an impressive field of research pushed by the increased interest into their important role in the life systems, especially for the evaluation of many diseases. To date, a variety of molecular probes for protein detection have been explored, including small molecule-based fluorescent probes, nanoparticle-based fluorescent probes, assembled fluorescent probes and so on. In this review, the currently available methods for protein imaging and detection, as well as trends and challenges have been discussed.

However, before these probes can be applied in clinical protein detection and disease diagnosis, several challenges need to be well addressed. Up to now, there are only a few probes could detect protein in clinical trial. The challenge is their poor biocompatibility and short emission wave-length which makes them difficult to enter the specific target site and deeper tissues. In addition, the long incubation time greatly limits the *in vivo* application of protein detection. Furthermore, it's a great challenge to combine with some specific protein due to the complexity of the probe structure. The problem may be settled by several approaches as follow. Firstly, increasing the conjugate length and introducing the chromophore will help to the emission wavelengths of probes shift to long wavelength. Additionally, surface modification techniques may improve their biocompatibility and decrease the incubation time, thereby enabling real-time protein detection *in vivo*. Besides, since the reactions of antigen-antibody are highly specific, more and more specific ligands could be explored and combined into the probes so that the protein could be detected and quantified precisely. In a word, these approaches will be meaningful in the development of novel fluorescence probes for protein imaging both *in vitro* and *in vivo*.

In conclusion, the detection of protein has always been an attractive field filled with both challenges and opportunities. We believe that protein imaging techniques will be used not only for basic research in cellular biology, but also for the analysis and treatment of disease in the not too distant future.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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