AIEgen-enhanced protein imaging: Probe design and sensing mechanisms

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Abstract
Proteins are the building blocks of life, regarded as one of the most complex and crucial biomacromolecules in biological systems, and playing a significant role in executing genes and transferring genetic information. According to recent research, due to the structural intricacy of proteins and their sensitivity to physical and chemical degradation processes, they could be utilized as biomarkers or therapeutic agents in the diagnosis, treatment, or even prevention of different diseases. Therefore, modern pathways have been developed for understanding the function of proteins, resulting in intriguing approaches in the field of protein-related diseases. The diagnostic strategies to deal with such diseases, including protein analysis, protein quantification, and protein imaging, were argued in depth. Meanwhile, the aggregation-induced emission (AIE) concept and its potential applications for real-time imaging make AIE luminogens (AIEgen) attractive for protein imaging. In general, AIEgens refer to those luminogenic chemicals that are nonluminescent in solution, but luminescent in either the aggregated or solid states. This review is focused on the emergence of AIE materials in protein tracking, detecting, and imaging for medical applications.

KEYWORDS
AIE materials, confocal microscopy, fluorescence imaging, real-time protein imaging, theranostic imaging

INTRODUCTION

Biological processes are crucial for life and defined as a series of biochemical reactions and molecular functions in living organisms. Any irregularities in these processes could result in biological disorders as well as different diseases. Proteins, which have macromolecular structures and perform a wide variety of functions, are classified as an inevitable part of biosystems. The roles of proteins inside living organisms include but are not limited to building up and maintaining the body structure, promoting catalytic activities and carrying out most metabolic reactions, transporting nutrients and other substances between cells and different tissues, and boosting the immune as well as nervous systems. Any abnormalities in the proteins’ structure, shape, and functions have health consequences and could be considered as an indicator of different pathologies for the clinical diagnosis of disease onset and progression. Although monitoring inside living things has recently been developed and various types of techniques have been found for commercial applications, achieving accurate and correct data still faces several difficulties and a lack of completeness. The body’s exposure to X-ray or gamma radiation and injection of contrast agents are required for accurate results. On the other hand, low-risk diagnostic methods usually result in low-resolution images or unreliable responses. This issue is apparent in studying proteins as well.

The story of the protein analysis began with the gel electrophoresis technique to separate proteins. The movement toward protein imaging originated with blotting proteins with stains and development in proteomics. Thanks to modern detection and microscopy technologies, protein analysis has paired with imaging, and a novel diagnostic approach has been introduced under the title of protein imaging. This technique is defined as methods of analyzing the structure of proteins and may be conducted by scanning tunneling microscopy (STM), transmission electron microscopy (TEM), and atomic force microscopy (AFM). Moreover, owing to not relying on electron beams, fluorescence microscopy has caught a great deal of attention for diagnostic...
AGGREGATE

SCHEME 1 Schematic comparison between ACQ phenomenon in luminescent materials and AIE phenomenon in AIEgens. The aggregation stat in planer luminogens leads to accumulating disc-like components with strong π–π stacking bonds. This interaction makes the luminogen OFF. On the contrary, AIE materials, with special molecular structures, emit light in aggregation form. This phenomenon comes from the restricted intramolecular rotation at the molecular scale. Reproduced with permission: Copyright 2009, Royal Society of Chemistry

applications[13–15] and is known as an affordable and accessible method for real-time protein imaging.[16]

Fluorescence imaging is a relatively pioneer diagnostic and authentic technique, which visualizes biomolecules as well as living cells. Furthermore, biocompatibility, chemical stability in physiological serum, photobleaching resistance, and low cytotoxicity of the fluorescent agents are of indispensable emphasis in bioapplications.[17] Although organic fluorochromes, quantum-dot probes, and fluorescent proteins have successfully found commercial applications, there are still several issues that prevent developing fluorescence imaging. The weak fluorescent quantum yield (QY), inevitable decomposition by enzymes, as well as the cytotoxicity effects of quantum dots originated from heavy-metal ions, have made researchers look for an alternative.[18,19]

The aggregation-induced emission (AIE) hypothesis has been propounded to rationalize the emission behavior of some luminescent compounds in that their aggregate state leads to intensive emission. According to the given figure representing the AIE mechanism by Hong et al.,[20] AIE originates from the restriction in intramolecular rotation of the specific moieties, whereas aggregation-caused quenching (ACQ) is a consequence of disc pile up of a fluorescent dye due to strong π–π stacking interactions, see Scheme 1. Accordingly, AIE materials’ characteristics in comparison to those of ACQ materials promise a significant development for fluorescence imaging, either in amending image brightness or in boosting the photostability of fluorescent delegates.[21–26] However, taking advantage of AIE materials or the AIE concept for protein imaging via fluorescence microscopy still poses challenges to address high photostability, good biocompatibility, high QY, no ACQ, and low cytotoxicity.

The recent developments of AIE lumingens (AIEgens) coupled with fluorescence microscopy and their applications for real-time protein imaging are discussed here. For this aim, different human proteins, which have been studied in the literature are introduced first. Then, the molecular structures of some AIE materials and their requirements for changing to an emissive probe are surveyed. Finally, a summary and an ambitious proposal for real-time protein imaging are made.

DETECTION OF MISCELLANEOUS PROTEINS VIA AIE-PROMOTED PROTEIN IMAGING

Early diagnosis of diseases is one of the hottest issues in medicine. Cancer detection and treatment via targeting cell growth and apoptosis are developing. Moreover, different cell survival pathways are attracting major attention for screening and imaging the cell life and death. Such information has made pathologists aware of how cancer cells behave.[27] A fast growth rate, abnormal shape, anomalous membrane, and release of cytoskeletal proteins are some of the characteristics of cancer cells.[28] When some specific abnormal expressed proteins are released, to find the causes of disorder in biosystems, protein types are identified and changes in their structures are determined. For instance, the integrin protein is an integral cell membrane receptor, the heparin protein is made and secreted by activated mast cells and basophils, and caspases are a group of enzymes that serve as a cell death initiator if activated.[29–31] On the other hand, the concentration of a specific protein in tissue could be deemed as a sign of diseases. Amyloid, serum albumin, and alkaline phosphatase (ALP) are some examples of such proteins.[32–34] Furthermore, in some specific cancer types or tumors, overexpressed proteins such as integrins and cathepsins could be considered as their unique characteristic.[35,36] Accordingly, different strategies could be utilized for cell monitoring based on their protein moieties. The sensitivity of proteins to physical and chemical parameter variations has marked the development of their applications in this field. In other
FIGURE 1 The mechanism of the Ac-DEVD-TPS-cRGD probe. (A) In the case of the low level of integrin receptors, that is, in apoptotic cells, the probability of conjugation between cRGD and the cell wall is not high enough to be considered. (B) Healthy cells with overexpressed integrin receptors have a strong tendency to adsorb cRGD; so RGD-containing probes path through the cell membrane but remain in their original form. (C) In a cancer cell with overexpressed integrin receptors, RGD-containing probes path through the cell membrane, the caspase enzymes inside the cell cleave the Ac-DEVDS tail of the Ac-DEVDS-TPS-cRGD probe; so TPS moiety could be released and emit. (B) MTT assay to show that this compound is a biocompatible probe with no side effects on healthy cells. (C) Molecular structure of the synthesized compound, which is specifically matched with NMR as well as HPLC results. (D–F) Confocal laser scanning microscope and (G–I) fluorescence/transmission overlay images of healthy (D and G), cancer (E and G), and apoptotic (F and I) U87MG cells. These images approved the status of the probe in different positions, which is explained in part (A). Reproduced with permission: Copyright 2014, Royal Society of Chemistry.

words, proteins are increasingly being considered to diagnose diseases.

Integrin

As one of the most famous families of cell–cell or cell–matrix adhesion receptors, integrins are heterodimer transmembrane glycoproteins of α and β subunits. They play a significant role in miscellaneous disease diagnosis, particularly cancer, congenital muscular dystrophy (CMD), immunodeficiency, and inflammatory diseases. They are classified into cellular signal mediators and represent events at the cell membrane. The existence of different ligands inside integrins (i.e., fibronectin, vitronectin, collagen, and laminin) has led researchers to real-time monitoring of cells, single-photon emission tomography (SPECT), and positron emission tomography (PET). Integrin receptors, which have been studied for 20 years, have recently been utilized in theranostic applications.

Shi et al. initiated designing AIEgen bioprobes fitted with a targeting ligand to integrin motifs. Cyclic arginine–glycine–aspartic acid tripeptide (cRGD) was used as a targeting agent conjugated with tetraphenyldisilole (TPS) as an AIEgen unit. Integrin receptors on cell membranes tend to bind to cRGD and induce binding of the probe. Ding et al. developed a probe using AIEgens to target integrin receptors and light up the cells for real-time imaging of apoptosis. To achieve this aim, two different peptide sequences were attached to TPS to compose an asymmetric bioprobe of Ac-DEVDS-TPS-cRGD. The cRGD was attached to TPS along one side whereas the acetyl protective N-terminal Asp-Glu-Val-Asp (Ac-DEVD) was conjugated to the opposite side. The cRGD peptide was utilized for targeting the integrin receptors while the DEVDS peptide could be significantly cleaved by caspase 3/7. This phenomenon led to preventing intramolecular rotations of the emission motifs, and consequently to light directed onto the aimed cells. The mechanism of this process and the confocal images are presented in Figure 1.

This strategy has been further developed by Yuan et al. who designed a probe, namely, TPETP-SS-DEVDS-TPS-cRGD, based on two AIEgens for cancer cell targeting in photodynamic therapy (PDT: a specific treatment, which is based on using a photosensitizer and irradiation of a light source; the photosensitizer becomes excited by light and releases reactive oxygen species [ROS]; ROS is a strong oxidizer and kills nearby microorganisms and cells). Tetraphenylethenethiophene (TPETP) and tetraphenylsaphyrin (TPS) had distinguishable emissions and both could be excited with a 405-nm laser. Due to the existence of cRGD, cellular uptake only occurred in cancerous cells and led to the cleavage of the S–S bonds, which subsequently converted TPETP-SS-DEVDS-TPS-cRGD into
FIGURE 2 A brief description of the DSPE-PEG2000-Mal-cRGD-TTD probe. (A) The procedure of AIEgen encapsulation inside a DSPE-mPEG shield with a tailed cRGD peptide. The cRGD part helps the compound to find integrin receptors of tumors, a path through the membrane. In the intercellular environment, ROS could be generated, and TTD could be released. The first one acts as the PDT agent and the second one provides the real-time imaging condition. (B) The adsorption edge and PL behavior of the synthesized compound approved its capability for both tasks. (C) Fluorescent images result from different cell lines incubated with the synthesized compound; blue for nuclei, green for cytoskeleton, and red for AIEgen agents. This figure approved the existence of TTD moiety in QBC939 but not in L-O2 and HK-2 cell lines. (D–E) The feasibility of targeting potential of the synthesized compound by tumor cells rather than healthy organs based on in vivo experiments. Reproduced with permission: Copyright 2017, American Chemical Society.

Li et al. took advantage of an AIEgen on one hand and a cRGD targeting ligand on the other hand to fabricate a compound for image-guided PDT. The significance of this compound was its stability in the vicinity of ROS. For this aim, a unique compound was prepared via conjugating DSPE-PEG2000-Mal (1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000]) to cRGD. Then, a mixture of TTD (2-(2,6-bis((E)-4-(phenyl(4′-(1,2,2-triphenylvinyl)-[1,1′-biphenyl]-4-yl)amino)styryl)-4H-pyran-4-ylidene) malononitrile) as an AIEgen and DSPE-PEG2000-Mal-cRGD drove the self-assembly process and formed an encapsulated structure with TTD core and cRGD shell, as shown in Figures 2(A) and 2(B). This shell not only led to active targeting but also increased the uptaking of cancer cells sixfold more than normal cells through integrin receptor–mediated endocytosis, see Figures 2(C)–2(E).

Jin et al. have reported a promising compound for RNA (ribonucleic acid) interference cancer therapy and...
FIGURE 3 (A) Schematic illustration of the TTD loaded and cRGD conjugated siVEGF-TTD nanoparticles (cRGD-siVEGF-TTD NPs). DSPE-PEG-
siVEGF is firstly prepared from DSPE-PEG2000-NH2 and HS-siVEGF. Then, TTD as the AIE agent is covered by the synthesized compound and conjugated to the RGD peptide. (B) UV-vis. and photoluminescence spectroscopy patterns in water (λex = 428 nm). The adsorption edge was centered at 430 nm, which means that ROS could be generated via visible light irradiation. Moreover, the photoluminescence band peaked at about 525 nm with the intensity of 20 Candela, which is a remarkable result for brightening the fluorescence imaging. (C–F) Confocal images of human breast cancer cell line after incubation with the synthesized compound, related to green BTPETD, Cy3-siVEGF, bright field image, and overlay image, respectively. The effects of AIEgens on brightening cells’ wall in fluorescent images is obvious. (G–I) Confocal images of three different human breast cancer cell lines after incubation with the synthesized compound, illustrating high-resolution images thanks to the existence of the AIEgens. Reproduced with permission: Copyright 2016, Royal Society of Chemistry[50]

Protopam and heparin

Heparin, having anti-inflammatory characteristics and a potent antiviral effect, is a natural polysaccharide with negative charges that could be clinically used as an anticoagulant.[52,53] In clinical medicine, protamine is known as a heparin counteractive with an arginine-rich polycationic protein and an average molecular weight of 4.3 kDa. It is a biologically active molecule that has a significant function in physiological processes such as tissue formation, gene expression, and transmission. Furthermore, it could be applied in the clinical therapy of cardiovascular and cerebrovascular diseases, diabetes, hemodialysis, and decreases the accumulation of antibiotics.[54–56]

Gao et al.[57] reported the heparin detection capability of an AIEgen containing four amino groups, that is, TPE-NH2. The mechanism was due to the sulfate and carboxylate groups with negative charge existing on the heparin peptide, promoting electrostatic interactions with the amino groups. This phenomenon led to a strong emission, but protamine might have caused quenching via disengaging amino functional groups and rigidifying their bonds with tetraphenylethene (TPE) benzene rings.

Wang et al.[58] have designed an amphiphilic pH-responsive tetraphenylenbenzene derivative (6-(1,3-dioxo-4,5,6,7-tetraphenylisoindolin-2-yl) hexanoic acid) named
TABLE 1  Integrin targeted bioprobes for imaging

| Ref. | Cell model      | AIE         | Probe modification | Mechanism of working                                      | Imaging target (tracking sensitivity)          |
|------|-----------------|-------------|-------------------|----------------------------------------------------------|-----------------------------------------------|
| 46   | HT-29           | TPS         | cRGD              | Targeting and cleavage by the integrin receptors         | Cancer cell wall (0.5 μg/mL)                   |
| 47   | MCF-7, U87MG    | TPS         | cRGD, Ac-DEVD     | Targeting by the integrin receptors. Cleavage by caspase enzyme | Cancer cell wall and inside cells (80 ng/mL for caspase) |
| 48   | MDA-MB-231      | TPETP, TPS  | cRGD              | Targeting by the integrin receptors. Release the TPETP    | Cancer cell wall (2.3×10^{-12} M for caspase)  |
| 49   | QBC939          | TTD         | cRGD, DSPE-PEG2000-Mal | Targeting and pass through the cell membrane by the integrin receptors | Inside cells                                    |
| 50   | MDA-MB-231, MCF-7 | TTD      | cRGD/PK           | Targeting by the integrin receptors                       | Cancer cell wall                               |

TPHA for detecting protamine and heparin, as shown in Figures 4(A)–4(C). The fluorescence power of TPHA in a mixture of acidic THF/water improved dramatically when the water content increased due to the formation of the aggregates, while it coped with feeble fluorescence in basic THF/water mixtures. The researchers stated that aggregates formation in acidic conditions might be related to the intermolecular π–π stacking interaction, hydrophobic effect, and hydrogen bonding of the carboxyl moieties. On the other hand, a molecularly dispersed system without any aggregation in basic pH could be due to the deprotonation of carboxyl groups. They achieved 45 times higher fluorescence intensity of TPHA in water than that in THF and 4.18% QY of TPHA in the molecular state versus 53.87% in the aggregate state. The addition of protamine to basic media resulted in a significant increase in the fluorescence intensity and aggregates formation through the electrostatic interplay of protein molecules and the deprotonated carboxyl groups, whereas heparin was a striking quenching agent of the TPHA–protamine system, as shown in Figure 4(D).

Ghosh et al.\[59\] have recently designed a hybrid compound for fluorescent imaging of heparin using an AIE, which was conjugated with protamine peptide. For this aim, naphthalene diimide–based bola-type amphiphilic (NDI-1) was utilized to achieve the AIE characteristic. The synthesized compound, named NDI-1 FONP, consists of an AIEgen and protamine moiety with no emission, due to the low aggregation probability. NDI-1 FONP had a negative surface charge and tendency to adhere to protamine, which was naturally positively charged. This conjugation caused quenching of emission. Figure 5(A) shows how the existence of heparin could turn this probe on and lead to inducing the emission via providing the aggregation condition for AIEgens. This phenomenon originates from the high tendency of protamine and heparin peptides to combine and could be the principle of heparin sensing. Results are shown in Figures 5(B)–5(E).

Jiang et al.\[54\] introduced a probe with a similar performance to that of Ghosh et al.\[59\] but with higher accuracy in heparin or protamine detection to as low as 0.08 and 0.02 μg/mL, respectively. For this purpose, 4,4′,4′′,4″-(anthracene-9,10-diylbis(ethene-2,1,1-triyl))tetra-carboxybenzene (DSA-4COOH) was synthesized from distyryl-anthracene derivative (DSA). The functional carboxyl group in the probe played a remarkable role in this system, hydrolyzing heparin into small moieties and preventing its combination with protamine. This led to the recovery of the probe emission via rebinding with protamine and thus improved its sensitivity. Classified data regarding heparin–protamine probes are summarized in Table 2.

Caspases

Caspases are a family of enzymes that modulate cell death, inflammation, immune responses, biological functions, and homeostasis. Recent research has demonstrated that some have potential in developing drugs aimed at preventing osteoporosis, fatty liver diseases, or neurodegenerative disease.\[60-62\] Ding et al.\[47\] have synthesized a fluorescent bioprobe containing two peptides, caspase-specific Asp-Glu-Val-Asp (Ac-DEVD) and cRGD, onto TPS units. While the fluorescence intensity of this probe remarkably increased in the presence of caspase-3, however, the probe was not emissive in aqueous solutions. These researchers elucidated that caspase-3 was the main cleavage agent of the DEVD moiety and led to the release of the TPS–cRGD residues. The aggregation of the released residues could restrict the intramolecular rotations of TPS, resulting in the fluorescence turn on. As this probe has the capacity to perform well when interacting with the integrin αβ3 receptor, this is not only beneficial to cancer cell targeting but also could be an impressive fluorescent lighting probe for real-time apoptosis tracking and imaging.

Fürster resonance energy transfer (FRET) is known as a mechanism for energy transfer between two chromophores in which the donor chromophore could transfer energy to an acceptor chromophore via the nonradiative dipole–dipole coupling.\[63\] Yuan et al.\[64\] have introduced a triple fluorescent probe, which included coumarin (Cou) as the energy donor, TPETP as the energy quencher as well as AIE agent, and a caspase-3–specific peptide substrate, that is, DEVD. This probe was nonfluorescent due to the energy transfer and
decadence of the acceptor energy through the free motion of TPETP. The existence of caspase-3 led to the cleavage of the probe, consequently, severe green and red fluorescent signals were emitted simultaneously. This was related to the separation of the donor–acceptor in Cou-DEVD and the aggregation in the TPETP residue, respectively. These fluorescent signals could be utilized for the detection of caspase-3 with high signal-to-noise ratios and fluorescence amplifications.

In another work, Yuan et al. evaluated an AIEgen for the caspase-targeting performance. A probe with simultaneous green and red emission was designed containing TPS and tetraphenylethylene-thiophene (TPETH) as well as a caspase indicator; Asp-Glu-Val-Asp-Ile-Glu-Thr-Asp (DEVD IETD). Caspase 8 or caspase 3 cleaved the hydrophilic moiety of the probe and induced green or red fluorescence, respectively. This sequential color change has the potential for real-time monitoring of cellular enzyme behavior in a biosystem, as shown in Figure 6.

Han et al. have designed a gemcitabine (GEM)-based prodrug made up of a cRGD peptide for targeting overexpressed αvβ3 integrin in pancreatic cancer cells, GFLG (glycine-phenylalanine-leucine-glycine) peptide for the GEM prodrug hydrolysis and release in the presence of overexpressed cathepsin-B, and caspase-3 cleavable DEVD peptide. Subsequently, due to the hydrophobic TPE residues with AIE characteristics, an intense blue fluorescence was observed, which is applicable for the real-time monitoring of some
FIGURE 5 The mechanism of the NDI-1 FONP probe. (A) The heparin-sensing mechanism is based on the strong binding affinity between heparin and protamine. This probe consists of an AIEgen and protamine moiety with no emission, due to the low aggregation probability. The existence of heparin changes the status of the probe to ON via combining with protamine and providing the aggregation condition for AIEgens. (B and C) The emission intensity versus protamine concentration in the synthesized probe shows a linear inverse relationship. (D and E) Increasing the heparin concentration from 0.1 to 0.8 μg/mL leads to a 40% increase in the emission intensity. Reproduced with permission: Copyright 2019, American Chemical Society.
cancer cells, such as pancreatic. Different bioprobes for caspase sensing are briefed in Table 3.

**Amyloid β**

The proteolytic processing of amyloid precursor protein (APP) as a transmembrane protein, by β- and γ-secretases could result in the production of the amyloid β-peptide (Aβ). The transformation of soluble monomeric Aβ to filamentous aggregates is related to various diseases, from neurogenerative disorders such as Alzheimer’s disease to diabetes.[32,67–69] Zhang et al.[70] have constructed a bio-probe (DES) and fluorescent glycoprobes (DK1 or DK2) for Aβ detection. Due to the FRET between the two molecules, (DES) and fluorescent glycoprobes (DK1 or DK2) for Aβ detection. They used a design strategy for mapping Aβ comprising: (i) introducing a lipophilic π-conjugated thiophene-bridge for developing the emission wavelength to the NIR region with blood–brain barrier (BBB) penetrability, (ii) substituting ACQ for AIE building block QM, and consequently (iii) modifying the hydrophilic substituted position of the sulfonate group to guarantee the OFF state of the probe. This probe with a lipophilic tail shows a long-term AIE in water as well as protein fibrillogenesis and has a capability for the detection of Aβ plaques in in vivo experiments. The mechanism of Aβ detection relies on the probe hydrophilicity. N, N′-dimethylamino causes the probe to enter into a hydrophobic situation and facilitates its conjugation with Aβ, giving rise to the restriction of QM rotation and boosting the emission. Wang et al.[72] developed a series of probes to detect Aβ fibrillation for real-time monitoring of the progress of Alzheimer disease with a high level of accuracy. They focused on optical diffraction, which restricts the resolution of fluorescent images. Based on the AIE concept and boosting the emission by restricting intramolecular vibration, a series of probes were designed with the capability of Aβ sensing, as shown in Table 4.

**Table 2** Heparin–protamine bioprobes for tracking and imaging

| Ref. | Cell model | AIE | Probe Modification | Mechanism | Imaging target (tracking sensitivity) |
|------|------------|-----|--------------------|----------|--------------------------------------|
| 57   | MCF-7      | TPE-NH₂ | -                  | Electrostatic interactions between amino groups of the probe and the sulfates and carboxylates groups of heparins. | Tracking (35 ng/mL for heparin) |
| 58   | TPHA       | -    | -                  | pH-responsive mechanism. Electrostatic interactions between the deprotonated carboxyl groups and protamine molecules. Heparin with a negative charge binds to protamine molecules and turns the probe OFF. | Tracking (4.8 ng/mL for protamine) |
| 59   | -          | NDI-1 | Protamine          | Nonmissive NDI-1-Pro probe binds heparin and changes to ON via the aggregation of the separated NDI-1 molecules. | Tracking (12 nM for heparin) |
| 54   | -          | DSA  | COOH               | Hydrolyzing heparin into small moieties by the carboxyl groups. Preventing combination of protamine and protamine. | Tracking (30 ng for protamine 37 ng/mL for heparin) |

TPE-NH₂: amino-tailored tetraphenylethene, TPHA: tetraphenylbenzene derivative (6-(1,3-dioxo-4,5,6,7-tetraphenylisoindolin-2-yl) hexanoic acid), NDI-1: naphthalene diimide-based bola-type amphiphilic, DSA-4COOH: 4,4″,4‴-(anthracene-9,10-diylbis(ethene-2,1,1-triyl))tetra-carboxybenzene.

**Table 3** Caspase sensitive bioprobe for imaging and tracking

| Ref. | Cell model | AIE | Probe Modification | Mechanism | Imaging target (tracking sensitivity) |
|------|------------|-----|--------------------|----------|--------------------------------------|
| 64   | MDA-MB-231, Hela | TPETP | Cou-DEVD | Cou-DEVD would be deconjugated from the probe via the existence of caspase molecules and makes the probe ON. | Cell wall |
| 65   | Hela       | TPS, TPETH | DEVD, IETD | The caspase-8 and caspase-3 activation during cell apoptosis lead to cleave the DEVD or IETD tails. The probe releases green then red emission. | Cell wall |
| 66   | BxPC-3     | TPE | GEM, cRGD, GFLG, DEVD | RGD conducts the probe toward aimed cells with α5β3 receptors and internalization occurs. Cathepsin-B cleaves the GFLG peptide. Gem could be released. Apoptosis occurs; so caspase-3 would be activated. Cleavage of DEVD leads to the probe changes to ON. | Tracking |

TPS: tetraphenyilsolole, TPETP: tetraphenylenethiophene, TPETH: tetraphenylethylene-thiophene, cRGD: cyclic Arg-Gly-Asp, ASVD: Asp-Glu-Val-Asp, Cou-DEVD: coumarin-Asp-Glu-Val-Asp, IETD: Ile-Glu-Thr-Asp, GFLG: glycine-phenylalanine-leucine-glycine, GEM: gemcitabine.

Fu et al.[71] have designed a near-infrared (NIR) AIE bio-probe (QM-FN-SO₃) based on quinoline-malononitrile (QM) for identifying Aβ. They used a design strategy for mapping Aβ comprising: (i) introducing a lipophilic π-conjugated thiophene-bridge for developing the emission wavelength to the NIR region with blood–brain barrier (BBB) penetrability, (ii) substituting ACQ for AIE building block QM, and consequently (iii) modifying the hydrophilic substituted position of the sulfonate group to guarantee the OFF state of the probe. This probe with a lipophilic tail shows a long-term AIE in water as well as protein fibrillogenesis and has a capability for the detection of Aβ plaques in in vivo experiments. The mechanism of Aβ detection relies on the probe hydrophilicity. N, N′-dimethylamino causes the probe to enter into a hydrophobic situation and facilitates its conjugation with Aβ, giving rise to the restriction of QM rotation and boosting the emission. Wang et al.[72] developed a series of probes to detect Aβ fibrillation for real-time monitoring of the progress of Alzheimer disease with a high level of accuracy. They focused on optical diffraction, which restricts the resolution of fluorescent images. Based on the AIE concept and boosting the emission by restricting intramolecular vibration, a series of probes were designed with the capability of Aβ sensing, as shown in Table 4.
Two functional groups, piperidine (PD) and dimethylamino (DM), were put inside the probe for donating electron and Aβ-targeting, respectively. Four different AIEgens, namely, PD-NA, PD-NA-TEG, DM-BZ, and PD-BZ, are represented in Figure 7. These compounds contain benzene ring (BZ) or naphthalene ring (NA), but NA-containing probes provide more rigid conjugation and show higher QY. Therefore, PD-NA and PD-NA-TEG have a higher potential for high-resolution imaging. Such a clear image of the mouse brain shows the Aβ plaques consist of radiant nanofibers, which
TABLE 4  Aβ bioprobes for tracking and high-resolution imaging

| Ref. | Cell model | AIE | Probe modification | Mechanism | Imaging target (tracking sensitivity) |
|------|------------|-----|--------------------|-----------|--------------------------------------|
| 70   | -          | DES | DK1, DK2           | Detaching the glycoprobes in the existence of Aβ and emission. Lectins lead to emission of different color. | Tracking |
| 71   | In vivo    | QM  | SO3                | The lipophilic π-conjugated thiophene-bridge changes the probe emission wavelength to NIR. QM changes the probe to an AIE probe. The sulfonate group provides the OFF state. | In-vivo tracking |
| 72   | HEWL, Tg6799 | BZ, NA | PD, DM | The probe binds with the Aβ. Restricts its intramolecular motion and Boosts AIE. Fluorescence switching due to reversible binding of AIE-active fluorogens and Aβ fibrils enables the probes detecting single molecular localization. | High-resolution imaging of cell wall and inside (63 nM) |

DES: silole-diyne, QM: quinoline-malononitrile, BZ: benzene ring, NA: naphthalene ring, Aβ: Amyloid beta peptide, DK1: galacto-dicyanomethylene-4H-pyran, DK2: manno-dicyanomethylene-4H-pyran, PD: piperidine, DM: dimethylamino.

FIGURE 7  (A) Molecular structure of different compound fabricated for Aβ imaging. DM-BZ and PD-BZ contain benzene rings, whereas PD-NA and P-NA-TEG contain naphthalene ring. (B and C) Fluorescent images of Aβ without and with using PD-NA. (D and E) Fluorescent images of Aβ without and with using PD-NA-TEG. More information could be achieved from the high-resolution images using the synthesized probes. (F–I) Confocal images of Aβ-containing cells stained with PD-NA and PD-NA-TEG probe. The resolution level is remarkably improved in comparison to conventional images. Reproduced with permission: Copyright 2018, Royal Society of Chemistry
are originated from the protein \( \beta \)-sheet transition. This could be important to monitor the growth pathway of \( \beta \)-amyloid in the early stages of Alzheimer’s disease.

In another prestigious work, Ding et al.[73] have reported a series of bioprobes consisting of electron-donating aniline groups and electron-withdrawing barbituric acid groups for amyloid detection. During conjugation with amyloid fibrils, the fluorescence intensity of some probes increases significantly (about 30 and 55 times in comparison to amorphous and monomer amyloid-containing solutions). These probes exhibited long-wavelength emission in the orange-red region that could be interesting in biological processes owing to avoid the auto-fluorescence and to reach an appropriate penetration depth.

**Esterase**

Esterases are a versatile family of hydrolase proteins that enable splitting ester bonds to generate acid and alcohol molecules.[74] They can serve as biosensors for early detection of a broad range of diseases from urinary tract infection (UTI)[75] to Alzheimer’s disease.[76] Choi et al.[77] have introduced different functionalized water-soluble AIEgens, based on benzyl bromoacetate, with various surface charges to discriminate proteins. Among them, probes that are functionalized with amine groups with positive or neutral charge indicated intensive fluorescence intensity by the addition of esterase. This phenomenon is related to the electrostatic interaction between the amine groups of the probe and the esterase, resulting in the limitation of intermolecular rotation of AIE materials. Furthermore, they also found out that in the concentration of \( 2 \mu \text{M} \) esterase, the fluorescence intensity of the probe is higher than that in \( 200 \text{ nM} \) esterase by a factor of 5. An AIE material with an excited-state intramolecular proton transfer (ESIPT) characteristic has been designed by Peng et al.[78] For this aim, salicyladazine has been attached to diethylamine and maleonitrile groups as a donor and acceptor of electrons, respectively. They figured out that the AIE behavior of this compound originates from interplanar spacing, whereas similar structures without interplanar spacing exhibit ACQ. However, the composed probe needs to be modified to apply in light-up sensing of esterase. This procedure was performed through the substitution of the hydroxyl group by an esterase reactive acetoxy. Due to the accumulation of the reaction product of the probe and esterase in mitochondria, the probe has the capability of detecting and imaging mitochondrial esterase in the range of 0.01–0.15 U/mL in vitro.

Recently, a smart probe has been designed based on benzothiazole (EP) to detect esterase specie with a high sensing accuracy, that is, \( 4.73 \times 10^{-5} \text{ U/mL} \), and high selectivity to the aimed proteins compared to other macromolecules. For this aim, 2-Cyano-6-Hydroxybenzothiazole and 2-aminobenzethiol were used for synthesizing the EP probe, which makes it capable of showing an intensive emission via reacting with esterase. The existence of the acetoxy group in the probe and the characteristic of esterase to cleave to the acetoxy lead to the fact that the emission behavior could be changed. In other words, acetoxy functional groups owing to the electron-withdrawing characteristic weaken the emission intensity of the EP. Any external agent, which could trig the probe by cleaving the acetoxy group, leads to rid it of the acetoxy effects and changes its emission behavior. The application of this feature for imaging is summarized in Figure 8.[79]

Wang et al.[80] have prepared a fluorescence nanosensor through electrostatic interaction between AIE amphiphile materials and gold nanoparticles (Au NPs) for sensing acetylcholine esterase (AChE), which plays a catalyst role in the conversion of acetylcholine into choline. This reaction is vital in the activation-resting cycle of neurons. The detecting mechanism is based on the interaction between Au NPs and acetyltihiocione (ATch) moiety of AChE, which leads to hydrolyze ATch into thiociono, anchors on AuNPs surface, resulting in the separation of AIE materials, and their subsequent aggregation and fluorescent emission. Utilizing this probe, a detection limit of 0.015 mU/mL for AChE has been reported. Furthermore, the AIE-Au fluorescence nanosensor could provide a sensitivity of 0.01–100 ng/mL for AChE inhibitor (carbaryl) with a correlation coefficient of 0.9967, indicating the application in inhibitor screening. Different probes and their performances for tracking esterase are foreshortened in Table 5.

**\( \beta \)-galactosidase**

GM1 gangliosidosis, a neurologic disorder, and morquio-B disease, a skeletal-connector tissue disorder result from a genetic deficiency of \( \beta \)-galactosidase, an enzyme with potential application for gene expression, transcription, and transfection efficiencies. It also could be utilized as a biomarker for ovarian cancer, colorectal cancer, and cancer senescence.[81–83] Yang et al.[84] have incorporated the \( \beta \)-galactopyranoside group (\( \beta \)Gal) to hydroxyphenylquinazolinone (HPQ) chromophore to design HPQ-\( \beta \)Gal as a luminescent probe for in vitro sensing of \( \beta \)-galactosidase. The general form of this probe is OFF, whereas the interaction between the probe and \( \beta \)-galactosidase makes the probe ON. This response originated from the restriction of HPQ intramolecular motions due to the generation of \( \beta \)Gal groups. The mentioned restriction gives rise to fluorescence emission. Upon 12.8 U/mL addition of \( \beta \)-galactosidase, the fluorescence intensity of the HPQ-\( \beta \)Gal probe was promoted about 13 times with a detection limit of 0.013 U/mL. An NIR fluorescent probe with AIE characteristics has been synthesized by Fu et al.[85] that could be activated by \( \beta \)-galactosidase. The probe is made up of QM as an AIE building block, a hydrophobic 2-(2-hydroxyphenyl) benzothiazole (HBT) moiety to improve the capability of the probe in the NIR wavelength, and hydrophilic galactose moiety to be provoked by \( \beta \)-galactosidase. During the presence of \( \beta \)-galactosidase, the hydrophobic AIEgen QM-HBT-OH is released. Subsequently, owing to the insolubility and aggregation of released QM-HBT-OH in water, the NIR fluorescence emission of the probe is observed. Two other \( \beta \)-galactosidase-activable fluorescent probes consist of a hydrophobic fluorophore (QM-OH) or dichloromethane hydroxide (DCMOH) as AIE agents and a hydrophilic galactose moiety as a \( \beta \)-galactosidase-triggered unit have been reported by Gu et al.[86,87] While the probes are initially nonemissive in aqueous media, it becomes highly fluorescent upon entering
FIGURE 8  (A) Molecular structure of the EP probe. (B) The cleavage of the probe by esterase enzyme and change the emission status to ON. Esterase is a hydrolase enzyme; so, it could react with the acetyl group, causes the limitation of its free rotation, and leads to boost the emission. (C) High selectivity performance of the probe and its sensitivity to the esterase. (D) Fluorescent images of living cells (d1), pretreated cells with 1 mM (d2) and 5 mM (d3) 4(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), an inhibitor of esterase activity. Healthy cells are apparent, and an intensive bright emission appears in imaging, due to the expression of esterase enzyme. Treatment of the cells caused to disrupt their life and reduce esterase secretion, so the emission of the probe is attenuated, and the quality of imaging is significantly reduced. Reproduced with permission: Copyright 2020, Royal Society of Chemistry[79]

TABLE 5  Esterase sensing bioprobe for tracking and cell imaging

| Ref. | Cell model | AIE | Probe modification | Mechanism | Imaging target (tracking sensitivity) |
|------|------------|-----|--------------------|-----------|--------------------------------------|
| 77   | -          | Benzylic bromoacetate | Amine  | Esterase molecules with negative charge bind with a probe, which is positively or naturally charged, due to the electrostatic interaction between the amine groups and the esterase. The limitation of intermolecular rotation of probe and boosting AIE. | Tracking (500 nM) |
| 78   | MCF-7, Hela | Salicyladazine | Diethylamin, maleonitrile, and acetoxyl | Salicyladazine is nonemissive. Modification by the addition of diethylamin and maleonitrile leads to ESIPT and the interplanar spacing leads to AIE. Modification by the addition of esterase reactive acetoxyl makes the probe sensitive to esterase. | Tracking and cell wall (0.01−0.15 U/mL) |
| 79   | MDA-MB-231 | Benzothiazole | Acetoxyl group | Esterase cleaves the acetoxyl group attached to the probe, so leads to the emission. | Tracking and cell wall (4.73 × 10−5 U/mL) |
| 80   | -          | Amphiphile | Au NPs | Au NPs causes to hydrolyze ATCh into thiocholine, thiocholine anchors on the AuNPs, then leads to AIE. | Tracking (0.015 mU/mL) |

Hong et al.[89] have presented a fluorescence bioprobe for HSA detection. This TPE derivative (sodium 1,2-bis[4-(3-sulfonatopropoxy)phenyl]-1,2-diphenylethene or BSPOTPE) is initially nonemissive and becomes luminescent in the presence of HSA due to docking in interdomain hydrophobic regions of the protein. The BSPOTPE probe is an Na-containing TPE salt, which is completely water soluble and consequently nonemissive. As a polypeptide chain, HSA owns three types of R-helical domains and some ligand-binding sites in the hydrophobic cavities. During the unfolding process of HSA near BSPOTPE, the probe gets involved in the hydrophobic cavities of HSA and becomes water insoluble. By inducing guanidine hydrochloride (GndHCl) as a denaturant, AIE materials leak from these interdomain regions, resulting in a nonluminescent state of the bioprobe.

In a prestigious work, Gao et al.[90] reported a lipophilic NIR-II fluorophore, which shows either twisted intramolecular charge transfer (TICT) or AIE behaviors. For this aim, 4,8-Bis(5-(4-(N,N-diphenylamino)phenyl)-2-thiophene) benzo[1,2-c:4,5-c′]bis[1,2,5] thiadiazole (BPBBT) has...

to the cells and conjugating with β-galactosidase because of specific enzymatic turnover and further liberation and aggregation hydrophobic QM-OH or DCM-OH. The performance of the QM-probe in β-galactosidase-overexpressed ovarian cancer cells is observable even after 12 h incubation, approving the intracellular retention of nanoaggregates and long-term tracking of β-galactosidase. The procedure of the preparation of the probe and their specific behaviors are summarized in Figure 9 and Table 6.

HSA (human serum albumin) and BSA (bovine serum albumin)

Serum albumin is the most abundant protein in blood plasma, its low amount is associated with emerging different diseases including coronary artery disease, heart failure, atrial fibrillation, stroke, and venous thromboembolism. Furthermore, ultralow or ultrahigh content of serum albumin could increase the risk of chronic kidney disease in general hypertensive patients.[33,88]
FIGURE 9 (A) DCM, 4-hydroxybenzaldehyde, and piperidine were used for DCM-OH preparation. Then, tetra-O-acetyl-α-D-galactopyranosyl-1-bromide was used to achieve DCM-βgal Ac. At the last step, sodium methylate in a methanol-based solution was applied for neutralization, purification, and preparation of DCM-βgal. (B) A similar procedure could lead to QM-βgal synthesizing. (C) The relatively high selectivity of β-galactosidase after exposure to various compounds and potentially interfering species. (D–G) Confocal microscopic images of SKOV-3 cells incubated with DCM-βgal (D and E) or QM-βgal probe (F and G) after 0.5 h (D and F) or 6 h (E and G). After 6 h, the emission of DCM-βgal decreases gradually due to its diffusion, whereas QM-βgal remains emissive even after 6 h. (H–J) High-resolution confocal microscopic images of SKOV-3 cells incubated with QM-βgal probe in bright, fluorescence, and merge modes, respectively. Activation of the probe by the intracellular β-galactosidase species and brightening of the cell are seen in the fluorescence mode. This might provide accurate information from inside or around the cells. Reproduced with permission: Copyright 2019, Royal Society of Chemistry.

TABLE 6 β-galactosidase bioprobes for high-resolution imaging

| Ref. | Cell model | AIE | Probe modification | Mechanism | Imaging target (tracking sensitivity) |
|------|------------|-----|--------------------|-----------|--------------------------------------|
| 84   | OVCAR-3    | HPQ | βGal               | β-galactosidase leads to disjoint the βGal group from the probe and makes it ON. | Tracking and cell wall (12.8 U/mL) |
| 85   | Hela, SKOV-3 | QM | HBT, βGal         | HBT for changing the probe to NIR fluorescent and hydrophilic βgal to be sensitive to β-galactosidase. It leads to disjoint βGal from the probe and changes it to QM-HBT-OH, which is emissive. | Tracking and cell wall |
| 86,87| SKOV-3     | QM, DCM | βGal | β-galactosidase interact with βgal and changes the probe to QM-OH or DCM-OH, which are strongly emissive and could be remained for several hours. | High-resolution imaging of cell wall and inside |

QM: quinoline-malononitrile, HPQ: hydroxyphenylquinazolinone, DCM: dichloromethane, HBT: hydrophobic 2-(2-hydroxyphenyl)benzothiazole.

been synthesized, as shown in Figure 10(A). Figure 10(B) shows that conjugation between the probe and HSA leads to an equivalence between AIE and TICT. These specific traits enable this compound to reveal some cancer cells and tumors based on the AIE concept (Figures 10(C)–10(F)). HSA causes a change in dihedral angles of BPBBT molecules and consequently restricts its intramolecular rotation. On the other hand, TICT might be induced by HSA but in the opposite direction. However, this dual-task probe has been utilized for high-resolution imaging of invisible tumor lesions during the photothermal treatment (PTT).

Gao et al. have fabricated a fluorescent luminogen TPE-IL by conjugating a functionalized TPE-OH with ionic liquid (IL) HOOCMIMBr. Due to the hydrophobic and hydrogen-bond interactions between the amino acid residues of HSA and TPE-IL, the latter is docked in the hydrophobic subdomain of the former, resulting in remarkable fluorescence emission of this probe. TPE-IL biosensors can detect
FIGURE 10  (A) The molecular structure of the BPBBT probe, which is designed based on the donor–acceptor fluorescent compounds (D-π-A-π-D).

The benzobis(thiadiazole) moiety plays an acceptor role, and two similar parts with donor characteristics attach to the core via π bond. The benzobis(thiadiazole) moiety plays an acceptor role, and two similar parts with donor characteristics attach to the core via π bond. (B) The BPBBT probe could be emissive via restricting its intramolecular rotation due to the HSA existence. Moreover, the equilibrium between AIE and TICT concepts and the role of HSA are shown. HSA leads to simultaneous emission and photothermal behavior. (C) The capability of the synthesized probe in the imaging of mouse colon tumors is obvious. The existence of the BPBBT compound provides more details from the cancerous cells inside the healthy tissue. Reproduced with permission; Copyright 2019, Springer Nature

HSA in the linear range of 0.02–10 μg/mL, sensitively and selectively. This probe could be applied successfully for the detection of HSA contents in human serum and urine samples. Shao et al. have introduced an AIE material based on QM but modified it via substitution of O atoms with N-ethyl groups. This probe is nonemissive in pure water due to its enough cavities or free volume to consume the radiative energy. During incorporation with BSA, sulfonate units of the probe as a conformation function group convert its initial loose unit to tight compact aggregation, resulting in abnormal AIE characteristics in pure water.

Li et al. have designed a TPE-sodium dodecyl sulfonate (TPE-SDS) dye with a long hydrophobic chain. Aggregation could be accomplished in these fluorescent molecules during hydrophobic interaction with proteins in polyacrylamide gel electrophoresis (PAGE). The amount of proteins directly affects the fluorescence intensity of the TPE-SDS-protein composite. This biosensor could be utilized as a novel protein-prestaining reagent in the PAGE analysis owing to its benefits such as rapid staining procedure, stability, and simplicity. Wang et al. have synthesized a new diketopyrrolopyrole-based compound (DPP) containing an ammonium salt with AIE property either in solid state or in the high viscosity system. The presence of ammonium groups promotes the solubility of the probe in water, whereas the interaction between the probe and BSA leads to boosting its emission. Tong et al. have reported a functionalized derivative of TPE, namely, 1,2-bis[4-(3-sulfonatopropoxy)-phenyl]-1,2-diphenylethene sodium salt, with nonluminescent property in the solution state and effective emission in the aggregation form. This probe does not disintegrate under ambient conditions after storage for a long time and has good stability; therefore, it could be an appropriate candidate for protein detection. Due to the hydrophobic interaction between this probe and BSA, their conjugation intensifies the fluorescence intensity of AIE material. This interaction results from the native folding structure of BSA that contains hydrophobic binding sites, so the rotation of AIE molecules is ceased, leading to its significant emission.

Sun et al. have presented a new 1,8-naphthalimide-based fluorescent, which is composed of a 4-triphenylamine-N-(3-hydroxyphenyl)-1,8-naphthalimide moiety (NapTpa) modified with 1,2-oxathiane 2,2-dioxide. The probe named NapTpa-bs and utilized for BSA detection. Assembly of NapTpa-bs and BSA due to the entrance of flexible long chains of NapTpa-bs into the cavities of BSA results in emission enhancement of this probe to sixfold at 629 nm for NapTpa-bs with 11 μM of BSA concentrations and inducing the light-up AIE characteristic. Wang et al. have synthesized a biosensor based on the cationic poly(diketopyrrolopyrole-co-ethynylfluorene) for fluorescence turn-on detection of BSA. This probe contains an ammonium group in the side chain to enhance its water solubility and biocompatibility, also provides positive charged binding sites for the electrostatic interaction between negative BSA and the probe. Incorporation of BSA into the probe leads to the improvement of fluorescence emission of the latter by about two- to threefold. Consequently, this fluorescence turn-on bioprobe could represent an essential role in BSA detection and imaging. A comparison between various
### Table 7: HSA or BSA biosensors for tracking applications

| Ref. | Cell model | AIE | Probe modification | Mechanism | Imaging target (tracking sensitivity) |
|------|------------|-----|--------------------|-----------|--------------------------------------|
| 89   | -          | BSPOTPE | -                  | The probe docks in interdomain hydrophobic regions of HSA and emit due to its restriction. | Tracking (1 nM) |
| 90   | In vivo    | BPBBT | -                  | HSA causes a change in dihedral angles of the probe, restricts its intramolecular rotation, and leads to the emission. Conversely, TICT might be induced by HSA. | In-vivo tracking and photothermal treatment |
| 92   | -          | TPE   | IL, HOOCMIMBr      | The interactions between HSA and TPE-IL lead to a formation of aggregation and restrict the probe, resulting in remarkable fluorescence emission. | Tracking (0.02–10 μg/mL) |
| 94   | Hela       | QM    | N-ethyl group      | Nitration between BSA and sulfonate unites of the probe leads to convert its initial loose unit to tight compact aggregation and consequently emission. | Tracking and cell wall |
| 93   | PAGE analysis | TPE | SDS                | The formation of the TPE-SDS aggregates inside the hydrophobic pocket of BSA leads to a boost as well as a blue shift of the emission peak. The distorted conformation of TPE-SDS in the hydrophobic cavities of BSA is the origin of this behavior. | Tracking (0.2 μg) |
| 95   | Hela       | DPP   | Ammonium group     | BSA adheres to the probe due to electrostatic interactions and leads to aggregate its particles and emit. | Tracking and cell wall |
| 96   | -          | TPE   | Sodium ethoxide    | BSA molecules are attracted by the functional tails of the probe and restrict its rotation. This leads to a strong emission. | Tracking (500 ng/mL) |
| 97   | -          | NAPTPA | 1,2-oxathiane 2,2-dioxide | Long chains of the probe enter the cavities of BSA. This forms a huge molecule with high AIE capability. Results in emission | Tracking (11 μM) |
| 98   | Hela       | DPP   | ammonium group, positively charged sites | The ammonium group helps the probe to be soluble in water and positive charged binding sites generate electrostatic adsorption onto the BSA. This phenomenon leads to emission. | Tracking (20 μM) |

**BSPOTPE:** sodium 1,2-bis[4-(3-sulfonatopropoxy)phenyl]-1,2-diphenylethene, **BPBBT:** 4,8-Bis[5-(4,4'-di(4-diphenylamino)phenyl)-2-thiophene]benzol[c][1,2,5]thiadiazole, **TPE:** tetraphenylethylene, **DPP:** diketopyrrolopyrrole, **NAPTPA:** 4-triphenylamine-N-(3-hydroxyphenyl)-1,8-naphthalimide, **IL HOOCMIMBr:** ionic liquid 1-carboxymethyl-3-methylimidazolium bromide, **SDS:** sodium dodecyl sulfonate.

### Table 8: ALP bioprobes for tracking as well as imaging

| Ref. | Cell model | AIE | Probe modification | Mechanism | Imaging target (tracking sensitivity) |
|------|------------|-----|--------------------|-----------|--------------------------------------|
| 118  | HeLa, HepG2 | TPE | CN, pho            | pho as hydrophilic groups caused the probe to be solved in water and CN as electron-withdrawing groups adsorb ALP in aqueous solutions, which results in a green emission. | Cell walls |
| 99   | Hela       | TPEQH | Pho               | ALP causes the decomposition of phosphate bonds and changes the probe to TPEQH, so a green emission could be achieved. | Tracking and cell walls |
| 100  | Hela       | DQM-ALP |                 | The rapidliberation of DQM-OH aggregates in the presence of ALP resulted in aggregation-induced fluorescence. | Monitor spatially heterogeneous ALP activity |

**TPE:** tetraphenylethylene, **TPEQH:** methyl(E)-2-(7-hydroxy-2-oxo-3-(4,1,2,2-triphenylvinyl)quinoxalin-1(2H)-yl)acetate, **CN:** cyano group, **Pho:** phosphate group.

mechanisms in imaging based on HSA or BSA is provided in Table 7.

### Alkaline phosphatase (ALP)

ALP is an enzyme in tissues and could be a biomarker throughout the body, because its abnormal decrease or increase is related to many diseases, from liver dysfunction to bone disease and aplastic anemia.\[^{34}\] A “turn-on” probe with AIE characteristics has been synthesized by Lin et al.\[^{34}\] for ALP detection consisting of TPE, a cyano group in the middle as an electron-withdrawing group, and a phosphate group at the end as the hydrophilic recognition moiety. The probe TPE-CN-pho has a high affinity for conjugation with ALP in an aqueous solution. During this conjugation, the water solubility of the probe decreases due to the elimination of the hydrophilic phosphate group and formation of TPE-CN-OH, which results in the activation of AIE characteristic and intense green emission. Furthermore, its high biocompatibility makes it a successful candidate for cell imaging so that in ALP-overexpressed HeLa cells and HepG2 cells, strong green fluorescence can be observed, as shown in Figure 11 and Table 8.

Guan et al.\[^{99}\] have introduced a fluorescent probe, based on methyl(E)-2-(7-hydroxy-2-oxo-3-(4,1,2,2-triphenylvinyl)quinoxalin-1(2H)-yl)acetate, CN: cyano group, Pho: phosphate group.
fluorophore (i.e., 2-(7-hydroxy-2-oxo-3-(4-(1,2,2-triphenylvinyl)styryl)quinoxalin-1(2H)-yl)acetate or TPEQH), then attached a phosphate group onto the phenolic hydroxyl group for detecting ALP. In the presence of ALP, due to the decomposition of phosphate bonds, the probe transforms into TPEQH. Further precipitation and aggregation of TPEQH lead to strong green fluorescence. Moreover, Li et al.\textsuperscript{[100]} have designed an AIEgen probe (DQM-ALP) consisting of QM, and hydrophilic phosphate groups to recognize ALP. The probe contains a hydrophobic QM core as an AIE building block and hydrophilic phosphate groups for ALP detection. During activation of the probe by ALP, its ALP-sensitive moieties are cleaved. Consequently, releasing and then the aggregation of DQM-OH result in restriction of molecular rotation and eventually turn-on AIE fluorescence. The results of long-term fluorescence cell imaging indicate that DQM-ALP probe could be visualized up to 13 h in cancer cells. Owing to the ALP overexpression in cancer cells, the mentioned probe could distinguish normal tissues from cancerous tissues with high contrast.

### Transferrin receptor (TfR) and soluble transferrin receptor (sTfR)

As a transmembrane glycoprotein, TfR is involved in iron transport and overexpressed in cancer cells. The cleaved form of TfR that could be released into the blood is sTfR, and both of them are utilized to detect iron deficiency anemia (IDA).\textsuperscript{[35,101]} Zhang et al.\textsuperscript{[102]} have reported a light-up probe named TPETH-2T7 composed of an AIE material conjugated by a TfR-targeting peptide, that is, alkyne-HAIYPRH (alkyne-T7). This probe is nonluminescence in aqueous media owing to the hydrophilic T7 moiety and could be a red-emissive sensor by an emission intensity increase of nearly 90-fold during TfR detection with a detection limit of 0.45 μg/mL due to the restricting of the intramolecular motion of TPETH. Zhang et al.\textsuperscript{[103]} have also synthesized a water-soluble probe TPE-2T7 for the light-up detection of sTfR. It consists of a functionalized TPE with azide groups and a functionalized peptide moiety T7 with alkyne groups, both for further covalent conjugation. During interacting with
sTfR, the probe indicates green fluorescence with a detection limit of 0.27 \( \mu \)g/mL, as shown in Table 9. The results of confocal microscopy showed that this probe could also turn on the cancer cell membrane due to the conjugation with overexpressed sTfR.

**Cathepsin-B**

Investigations of Alzheimer’s disease, traumatic brain injury (TBI), and related brain disorders have demonstrated that cathepsin-B, a cysteine lysosomal protease, is extensively involved. Additionally, it could be overexpressed under various pathological conditions such as inflammation, infection, and cancer.$^{[36,104,105]}$ Yuan et al.$^{[106]}$ have developed a bioprobe (TPECM-2GFLGD-cRGD) that consists of an optimized TPE, that is, 2-(1-(4-(1,2,2-triphenylvinyl)phenyl)ethylidene)malononitrile (TPECM), two cleavable GFLG peptides by cathepsin-B, three Asp (D) units to provide the hydrophilicity, and a cRGD peptide for integrin \( \alpha_v \beta_3 \) targeting. Owing to the presence of hydrophilic peptides, AIE is nonemissive in the solution state. After conjugation to intracellular cathepsin-B and subsequent cleavage of the GFLG peptide substrate, probe aggregation, and intense fluorescence occur, so the probe could play its role as a turn-on fluorescence biosensor.

**Mouse double minute-2 (Mdm2)**

Mdm2 protein is the primary negative regulatory factor of the p53 protein; hence, its overexpression is associated with a wide variety of diseases from chronic viral infections (HBV, HCV, and HIV) and metabolic syndrome to liver, lung, breast, esophageal, gastric, and colorectal cancer.$^{[107,108]}$ Geng et al.$^{[109]}$ have developed a fluorescent light-up probe to detect Mdm2 at a cellular level. This specific probe is formed of TPECM, which is conjugated with a specific p53-derived peptide (MPRFMDYWEGLS, 12.1Pep) for targeting Mdm2. While this probe is nonemissive in its isolated state, its fluorescence intensity enhances significantly upon binding with Mdm2 proteins due to the docking of 12.1Pep into the hydrophobic binding pocket of Mdm2 and the limitation of rotating TPECM molecules. The fluorescence intensity of the probe/protein in the presence of 2500 nM Mdm2 is 13 times higher than that without Mdm2, as shown in Table 10.

**Lysosomal-associated transmembrane protein 4B (LAPTM4B)**

LAPTM4B is an integral membrane protein, which is upregulated during the outbreak of different cancers,
extrahepatic cholangiocarcinoma, or ovarian carcinoma.[110,111] Huang et al.[112] have designed a bio-probe composed of TPE and a small peptide (IHHGHIISVG or AP2H) for the detection of LAPTM4B. Due to the restriction of molecular rotations in TPE structure, the interaction between the TPE-AP2H probe and target proteins, either in solution or at the cellular level, results in turn-on fluorescence of the probe. Moreover, a decrease in pH values of tumor cells causes higher fluorescence intensity of TPE-AP2H. Accordingly, it could apply as a pH sensor of tumor cells as well as recognition of LAPTM4B proteins.

PROSPECTIVE OF THE AIE-PROMOTED PROTEIN IMAGING

Tracking cancer cells is a major focus of research in biology and biochemistry. One approach is to exploit organic dyes for marking targeted cells or tissues.[113] The second strategy comes from fluorescent proteins. They have been inspired by the camouflage in animals and were widely utilized in the current decade for biolabeling, drug screening, ROS production, and detecting.[13] Inorganic fluorescent semiconducting compounds or quantum dots is the third option for theranostic applications.[114] Fluorescence microscopy has progressed and been more accurate throughout the labeling target species via the mentioned mechanisms, but some restrictions, such as cytotoxicity, photobleaching, and lower QY, convinced researchers to look for alternatives, which could satisfy the following criteria: (i) biocompatibility, (ii) photostability, (iii) brightness, (iv) targeting efficiency and specificity, and (v) manageable emission. Unlike conventional fluorophores, which follow the ACQ mechanism, AIE materials exhibit some marvelous responses when subjected to different protein levels and fortunately have the potential for addressing most weaknesses of using organic dyes, quantum dots, and fluorescent proteins in protein imaging.

The first and foremost advantage of AIE materials is the probability of performing molecular engineering to manage required characteristics, such as emission wavelength, protein targeting, and ON/OFF switching.[115] Cancer cell hallmarks and structural information of proteins and peptides conduct a molecular design of AIE materials with previously unfavorable responses. In other words, molecular engineering is a novel but genuine technique to tackle barriers in protein imaging. For example, (i) autofluorescence as a prevalent obstacle in protein imaging could be easily dealt with via changes in the excitation wavelength as well as emission color.[16,17] (ii) Active tumor targeting is a deterministic issue in imaging due to the damaging effects of fluorophores on healthy cells. Tumor tracers or cancer cells targeting ligands can also be easily conjugated to AIE materials. Pre-requisites of this attitude are basic knowledge of the existed functional groups on the AIE structure as well as the tracer. This feature could lead to the assumption that each protein needs a specific AIEgen for obtaining images with enhanced details and information. Based on molecular engineering, the required functional group could be joining the AIE core without affecting its initial emission behavior. In the case of cRGD as the integrin tracer, an azide functional group is required so the best option for this probe is an AIE, which has been functionalized with azide.[46–48] (iii) Manageable emission is a stunning feature in AIE materials and develops their applications in various fields. The existence of some moieties in the probe makes another precondition for emission, in other words, the conjugated moiety makes the probe OFF and emission does not occur unless in the vicinity of a specific chemical. Regarding caspase, DEVD, IETD, and GFLG could be conjugated to the probe as cleavage sites. These sites quench AIEs, but different caspase levels cleave them and make the probe ON,[64,65] and (iv) tuning the emission wavelength is another feature of the manageable emission. In vivo protein imaging in the NIR region becomes more favorable, due to higher transparency of tissues as well as less light absorbance by skin.[71] In a case study, with the aim of extension to the NIR wavelength range, a lipophilic π-conjugated thiophene-bridge was put on a ThT-based probe and shifted its emission to a wavelength of around 700 nm.[71] However, different aspects of protein imaging have been studied, and comparing them could be led to interesting results. A systematic assay for profiling data is making a comparison between critical viewpoints. Based on this model, some points would be raised: (i) the most popular proteins for cancer targeting are integrin receptors and caspase. The first one has an intriguing performance, which enables it to pass cRGD through the cell membrane and obtain data from inside the cell. (ii) Heparin, protamine, and esterase with a high density of surface charge could be tracked via a simple electrostatic mechanism with a high degree of accuracy. This theme can be easily accessed via attaching a carboxyl or acetoxyl group to an AIEgen. (iii) Aβ or β-galactosidase-based imaging has progressed and its in vivo results are very realistic and promising, even for detecting single molecular localization, and (iv) BSA and HSA are considered to be monitored and measured via AIEgens. This feature finds some initial applications for anticipating heart failure as well as stroke.

Briefly, early-stage diagnosis of disease and disorders via simple and cheap methodologies is an unmet need in medical science. Thanks to the specific features of AIEgens and molecular engineering, designing, and manufacturing such modern diagnostic devices is not far from the mind.

CONCLUSION AND OUTLOOK

In this review, we have endeavored to summarize recent progress on AIE materials focusing on their applications in protein imaging. Miscellaneous proteins as case studies were introduced with respect to the molecular structure of the probe, real-time protein detection mechanism, and probe sensitivity. As mentioned above, outstanding achievements have been represented that require to be improved in terms of sensitivity, photostability, QY, biocompatibility, and fluorescence intensity. Certainly, amelioration of these characteristics will inject new blood into the diagnosis, treatment, and prevention of a wide variety of diseases.

AIE-based probes combine the novel and unique features of AIE materials and fluorescent microscopy to track, monitor, and diagnose existential symptoms of diseases. These probes light up targeted position and improve imaging outcomes and analyzing biological processes without the need for increasing dye concentration. The molecular structure of AIE materials are highly desirable and could be managed by various parameters, such as pH, temperature, pressure,
and chemical stimulants. Therefore, the autonomous navigation of probe in vivo, which is a limitation of protein, live-cell, RNA, and DNA imaging, could be overcome by getting molecular engineering involved in probe designing. Furthermore, specific responses of organs and tissues to diseases, abnormal glandular activities, extreme cell growth, and secretion of the immune system enzymes could be clarified and utilized as irritant agents. Although tracking and diagnosis of Alzheimer, diabetes, and tumors are the most popular topics in biochemistry, biomedicine, and pharmaceutical chemistry, early detection of symptoms of diseases and disorders has a high potential in developing AIE-based probes.

Precancerous lesions and conditions become the center of attention for decreasing the risk of developing into cancer. Therefore, propelling the concept of AIE-based probes toward stem cells might open a promising path for real-time or even anticipating the potential disorders. Heart attack is the main consequence of coronary artery disease and has the potential to be detected using an AIE-based probe. Changes in hydrostatic blood pressure, as well as secretion of troponin, are the signs, which could be monitored in the early stages. The ambitious idea of using the AIE-based probe concept for understanding neurological disease mechanisms might lead to figuring out reliable treatment methods for multiple sclerosis disease (MS), autism, and down syndrome. However, the AIE concept provides visualization capability for different phenomena in the visible and NIR spectral region. It is required to identify the target behavior and design the probe according to that specific criterion.

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