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Simple Biosensor with High Selectivity and Sensitivity: Thiol-Specific Biomolecular Probing and Intracellular Imaging by AIE Fluorogen on a TLC Plate through a Thiol–Ene Click Mechanism**

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Abstract: A handy, specific, sensitive bioprobe has been developed. Tetraphenylethene (TPE) was functionalized by a maleimide (MI) group, giving a TPE-MI adduct that was nonemissive in both solution and the solid state. It was readily transformed into a fluorogen showing an aggregation-induced emission (AIE) property by the click addition of thiol to its MI pendant. The click reaction and the AIE effect enabled TPE-MI to function as a thiol-specific bioprobe in the solid state. Thus, the spot of TPE-MI on a TLC plate became emissive when it had been exposed to L-cysteine, an amino acid containing a thiol group, but remained nonemissive when exposed to other amino acids that lack free thiol units. The thiol-activated emission was rapid and strong, readily detected by the naked eye at an analyte concentration as low as approximately 1 ppb, thanks to the "lighting up" nature of the bioprobing process. Similarly, the emission of TPE-MI was turned on only by the proteins containing free thiol units, such as glutathione. Clear fluorescence images were taken when living cells were stained by using TPE-MI as a visualization agent, affording a facile fluorescent maker for mapping the distribution of thiol species in cellular systems.

Keywords: aggregation-induced emissions · biological activity · biosensors · tetraphenylethene · thiols

Introduction

The development of fluorescent probes for biomolecular detection has emerged as an active area of research due to its importance to bioscience and applications in biotechnology as well as its impact on public health.[1] The fluorescent assay process offers a number of advantages over other analytical techniques, such as rapid response, high sensitivity, low background noise, and a wide dynamic working range.[2] Thanks to the enthusiastic efforts of scientists devoted to this area of research, a large variety of fluorescent bioprobes have been developed.[3] Many of the bioprobes work in a "turned off" mode; for example, the emission of a fluorophore is switched off when it interacts with a quenching species in a biological system through a mechanism of fluorescence resonance energy transfer.

Cellular thiols are essential biomolecules that play an important role in biological systems as key components of protein structures and metabolic intermediates. The thiol molecules are decisive in maintaining appropriate oxidation–reduction states of proteins, cells and organisms.[4] Glutathione (GSH), for example, is a thiol with the highest abundance in cellular systems. It is a small protein with a short sequence of Gly-Cys-Glu or GCE that resides in every cell of our body and is thus vital to life. GSH is so critical to cells that its deficiency results in cellular dysfunction.[5] Many health problems, such as Alzheimer's disease, leucocyte loss, liver damage, psoriasis, cancer, and AIDS are known to be associated with levels of cellular thiols.[6]

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[**] AIE = aggregation-induced emission; TLC = thin-layer chromatography.

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A sensitive and selective assay for thiols is thus of great biological importance. Much work has been done in the area of developing fluorescent sensors for thiol detection.[7] In almost all of the sensing systems, thiols have been assayed by measuring the changes in the fluorescence signals of the probes with the analyte concentrations in the solution state by using a spectrofluorometer.[7] For real-world applications, however, it is preferable to perform the bioassays on solid supports because it requires no complex and expensive equipment and is thus simple, quick and convenient.[8] To the best of our knowledge, no report, however, can be found in the literature about thiol assays in the solid state.

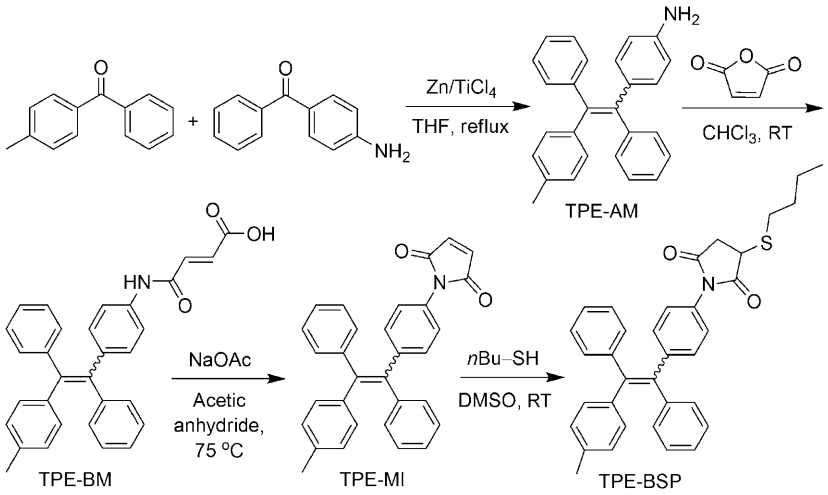
A thorny problem often encountered by traditional fluorophores is aggregation-caused quenching (ACQ) of their emissions in the solid state. When dispersed in aqueous media or bound to biomolecules, the fluorophore molecules are inclined to aggregate, which usually quenches their fluorescence processes and thus greatly limits their effective ranges as bioprobes. The ACQ effect also makes it difficult to assay low-abundance molecular species in biological systems because the fluorescence signals from the miniscule amounts of fluorophores matching the bioanalyte levels may be too weak to be determined accurately, while at the high fluorophore concentrations, the emissions are further weakened, rather than enhanced, due to the notorious ACQ effect.

We have recently discovered an extraordinary phenomenon of aggregation-induced emission (AIE) that is exactly opposite to the ACQ effect discussed above.[9–11] A series of fluorogen molecules that are nonemissive in solutions, such as tetraphenylethene (TPE), are induced to emit efficiently by aggregate formation. The unique AIE effect has been utilized to develop new "turned on" type bioprobes,[11] which enjoy much higher sensitivity than their "turned off" counterparts. The selectivity of the AIE probes, however, needs to be improved. Herein, we aimed to develop an AIE bioprobe with high selectivity, while keeping its superb sensitivity. In particular, we worked on the design of a TPE-based AIE-active fluorogen and the preparation of its solid state for thiol-specific detection in small and large biomolecules ranging from amino acids to proteins and living cells.

Results and Discussion

TPE is one of the best-known AIE fluorogens, partially due to the great simplicity in its synthesis and derivatization.[10] TPE and its derivatives have been widely used in the development of bioprobe systems with high sensitivity, but selectivity remains an issue of concern. It is envisioned that introducing a functional group with orthogonal reactivity to the TPE structure will generate a TPE derivative with selectivity or specificity when it is used as an AIE-active bioprobe. Maleimide (MI) is known for its orthogonal reactivity to thiol in the hydrothiolation or thiol–ene click reactions under mild conditions.[12] We thus designed the molecular structure of an MI-functionalized TPE derivative and elaborated a reaction scheme for the synthesis of the TPE-MI adduct.

According to the synthetic route shown in Scheme 1, an aminated TPE derivative (TPE-AM) was first prepared by the McMurry cross-coupling reaction of 4-methylbenzophenone with 4-aminobenzophenone catalyzed by a mixture of $Zn/TiCl_4$.[13] The amino group in TPE-AM was then transformed to the maleamic acid group in TPE-BM by the reaction with maleic anhydride. Finally, TPE-BM was amidated and cyclized in the presence of sodium acetate and acetic anhydride to give the desired product of TPE-MI in a high yield. The clickable nature of TPE-MI was verified by its model reaction with 1-butanethiol, which afforded the thiolated product of TPE-BSP in a yield as high as about 90% after the reaction mixture had been stirred at room temperature for a short while (2 h). All of the reaction intermediates and products were characterized by standard spectroscopic methods, from which satisfactory analysis data were obtained (see the NMR spectra shown in Figure S1–S3 and the numerical spectral data given in the Supporting Information).



Scheme 1. Syntheses of functionalized tetraphenylethene derivatives used in this work.

TPE-AM is nonemissive when it is molecularly dissolved in solvents, such as THF, ethanol, chloroform, dichloromethane, and DMSO. The photoluminescence (PL) spectrum of a dilute solution of TPE-AM in THF is basically a flat line parallel to the abscissa (Figure 1), manifesting the nonemissive nature in the solution state. However, when large amounts of water ($f_w > 80$ vol %) are added to THF, intense emission spectra are recorded under identical measurement conditions. Since water does not dissolve TPE-AM, the fluorogenic molecules must have aggregated in the aqueous mixtures with high water fractions. The PL data thus prove that TPE-AM behaves just like its parent form of TPE, that is, it is AIE active.

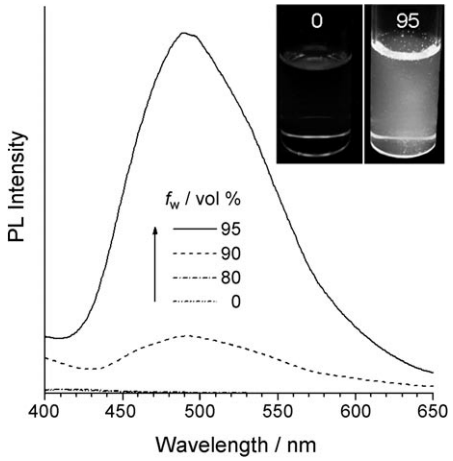


Figure 1. PL spectra of TPE-AM in THF/water mixtures with different fractions of water (f_w). Inset: TPE-AM in pure THF ($f_w = 0$ %) and a THF/water mixture with $f_w = 95$ %; photographs taken under illumination of a 365 nm UV light.

The aggregates of the TPE-AM molecules are nanodimensional in size, as proven by the example of TEM images shown in Figure S4 in the Supporting Information and by the leveled-off tails in the long-wavelength region in the absorption spectra of TPE-AM in the THF/water mixtures with high f_w values (Figure S5 in the Supporting Information). The photographs given in the inset of Figure 1 clearly show the nonemissive and emissive nature of the molecular species and aggregative particles of TPE-AM in the THF solution and aqueous suspension, respectively. When the molecules of TPE-AM are aggregated in the THF/water mixture with 95 % water, its emission intensity is increased by around 200 fold, in comparison to that of the solution in THF.

Whereas the nanoaggregates of TPE-AM are highly emissive, the TPE-MI congener is nonfluorescent in either solution or solid state (Figure 2). The fluorescence of TPE-MI is probably quenched by the exciton annihilation process associated with the n–π electronic conjugation of the carbonyl (C=O) and olefinic (C=C) groups in its MI unit.[14] If this is the case, breaking the n–π conjugation should help to recuperate the AIE activity. The MI unit is known to readily undergo selective electrophilic reaction with a thiol group.[12,15] When a thiol group is added across the C=C double bond in the MI unit, the n–π electronic conjugation is destroyed. Thus, investigation of fluorescence behavior of the thiolated TPE derivative, namely, TPE-BSP, would help clarify whether the MI unit is responsible for the PL quenching in the TPE-MI system.

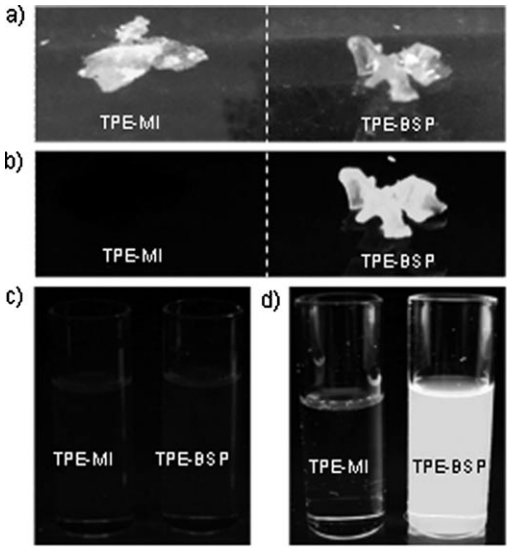


Figure 2. TPE-MI (a) and TPE-BSP (b) in the solid state, c) a solution in THF, and d) in a THF/water mixture with 95 % water fraction. Photographs taken under normal laboratory lighting (a) and 365 nm UV illumination (b–d).

As can be seen from Figure 2, TPE-BSP is clearly AIE active: the solution in THF is nonemissive but a solid powder and aggregate suspension emit very bright sky-blue light. The UV and PL spectra as well as the photographs shown in Figures S6 and S7 in the Supporting Information further substantiate the AIE nature of its light emission. These results are exciting because they show that the emission of TPE-MI can be rejuvenated by an orthogonal click reaction with thiol in the aggregate state, which constitutes the foundation for the development of solid strips of fluorescent probes for thiol-specific detection.

TLC has been widely used as a handy tool in chemical research, especially for monitoring propagation of chemical transformation. The combination of TLC with spectrometric methods has led to the development of a variety of convenient analytical techniques.[16] We have been interested in expanding the usefulness of TLC[17] and in this work, we tried to use TLC plates as matrix strips to develop solid-state fluorescent probes for the detection of thiol-containing biomolecules. The probe strips were prepared by dropping tiny aliquots of a solution of TPE-MI in dichloromethane (≈ 5 μM) onto the TLC plates. The plates were then dipped into solutions of the bioanalytes in DMSO for 1 s. After partial evaporation of solvent, the TLC plates were examined under UV illumination.

Before dipping the TLC plates into the bioanalyte solutions, the TPE-MI spots are nonemissive under both 254

and 365 nm UV illuminations (Figure S8, panels a and b; Supporting Information). After being treated by a solution of L-cysteine, the dark spot of TPE-MI on the TLC strip turns to bluish under the illumination of a 254 nm UV light (Figure 3a). When excited by a 365 nm UV beam, the spot

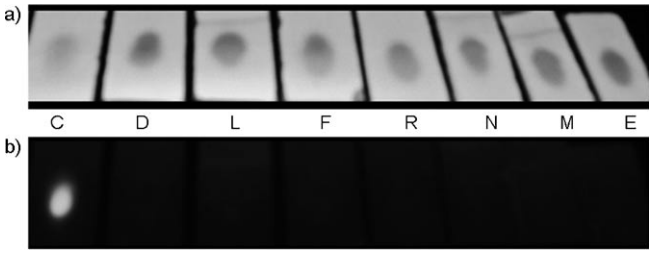


Figure 3. Selective detection of L-cysteine (C), a standard amino acid carrying a free thiol unit, by a TPE-MI spot on a TLC plate. Data for other standard amino acids containing no thiol unit, including L-aspartic acid (D), L-leucine (L), L-phenylalanine (F), L-arginine (R), L-asparagine (N), L-methionine (M), and L-glutamic acid (E), are shown for comparison. Photographs were taken under illuminations of a) 254 and b) 365 nm UV light after the TLC plates had been dipped into solutions of the amino acids (0.3 mg mL^{-1}) in DMSO with and without thiols.

emits a bright blue light (Figure 3b). In sharp contrast, the TPE-MI spots on the TLC plates treated with other amino acids not containing free thiol groups all remain nonfluorescent under the 365 nm UV illumination. Evidently, TPE-MI has readily and selectively reacted with L-cysteine to yield a fluorescent thiolated product in the solid state at room temperature, thanks to the click nature of the involved thiol–ene hydrothiolation reaction. The detection process is fast and the assay procedure is simple. Furthermore, the contrast between the spots of TPE-MI and its thiolated product on the TLC plates is very high under UV illumination, which is vividly discernable by the naked eye.

Selectivity and sensitivity are the two most important parameters that decide the goodness and usefulness of a bioprobe. It has become clear that the TPE-MI probe is highly selective: it works only for free thiol. To determine how sensitive the bioprobe was, we performed the TLC detection experiments using solutions of L-cysteine in different concentrations. The results are shown in Figure 4. The TLC plate treated with a solution of L-cysteine with $c=$ 1000 ng mL^{-1} ($\approx$1 ppm) shows a spot with very bright fluorescence. Decreasing the solution concentration from 100 and 10 ng mL^{-1} lowers the emission intensity, but the spots

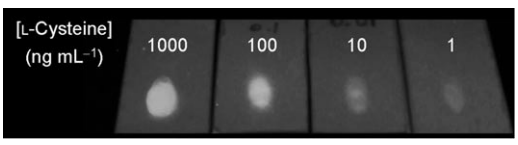


Figure 4. Sensitivity in fluorescent probing of L-cysteine by TPE-MI spots on TLC plates. Photographs were taken under 365 nm UV illumination after the TLC plates had been dipped into solutions of L-cysteine in DMSO with concentrations of 1–1000 ng mL^{-1}.

are still clearly visible under UV illumination. Although the spot on the TLC strip treated with the 1 ng mL^{-1} ($\approx$1 ppb) solution of L-cysteine is somewhat weak, it is still discernable by the naked eye, demonstrating the high sensitivity or low detection limit of the bioprobe in the solid state.[7]

An alternative way for assaying a trace amount of a bioanalyte is to first treat a blank TLC plate with a solution of TPE-MI and then drop an aliquot ($\approx$1 μL) of a solution of bioanalyte onto the pretreated TLC plate. Figure S9 in the Supporting Information shows the experimental results obtained from this alternative procedure. The data are very similar to those shown in Figure 4, once again proving the sensitive nature of the bioprobing system.

Since the free thiol group in L-cysteine can readily react with TPE-MI and selectively turn on the emission of its spot on the TLC plate in the solid state at room temperature, we examined whether the TLC plate could be used to detect small proteins or oligopeptides containing free thiol groups under ambient conditions, in an effort to widen the applicability of the bioprobe system. We used GSH (with a sequence of GCE) as the bioanalyte and performed the bioassay by following the experimental procedure described above. As depicted in Figure 5, the TPE-MI spot becomes

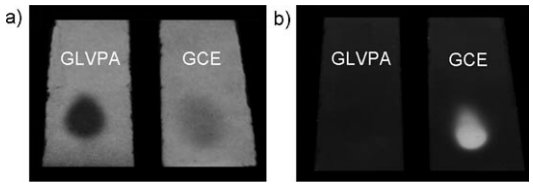


Figure 5. Detection of protein containing free thiol groups by TPE-MI coated on TLC plates. Photographs were taken under a) 254 and b) 365 nm UV illumination after the TLC plates had been dipped into solutions of proteins (0.3 mg mL^{-1}) in DMSO without (GLVPA) and with thiol unit (GCE or GSH).

emissive after the TLC plate has been dipped into a solution of GSH with a concentration as low as about 0.3 ppm. When treated with a protein (GLVPA) that lacks of free thiol group, the spot of TPE-MI remains nonemissive under UV illumination.

While the fluorescence detections of thiol-containing amino acids and proteins are of interest, monitoring thiol levels in living cells is more exciting. To explore the possibility of utilizing TPE-MI as a bioprobe for cell imaging, we tried to map the thiol levels in HeLa cells using a standard cell-staining protocol. After incubating the living HeLa cells in the culture buffer in the presence of the TPE-MI nanoaggregates for 5 min, bright fluorescence images of the stained HeLa cells were recorded (Figure 6). Closer scrutinization of the cell images reveals that the TPE-MI aggregates predominantly visualize the cytoplasmic regions. Weaker fluorescence signals are seen in the nucleus regions of the HeLa cells, probably due to the hydrophobic nature of the fluorogen.

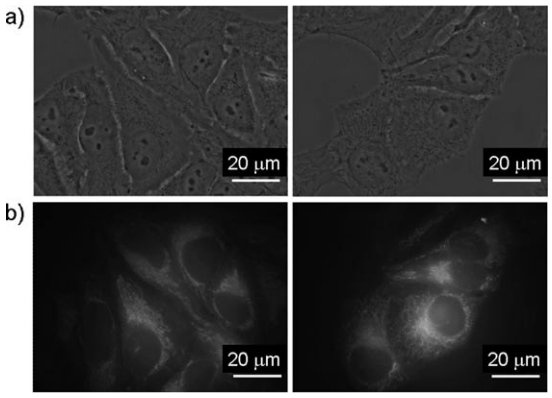


Figure 6. a) Bright field and b) fluorescent images of HeLa cells stained by TPE-MI nanoaggregates.

The cell-imagining results show that the TPE-MI nanoaggregates can easily penetrate the cell membranes, possibly through an endocytosis process, and readily realize fluorescence labeling under the standard cell incubation conditions. All of the living cells stained by the TPE-MI nanoaggregates look healthy, indicative of good biocompatibility of the AIE fluorogen. Taking into consideration the relatively high levels of GSH in the cytosolic regions and the relationship between the cytosolic GSH levels and many health problems,[6] this thiol-specific bioprobe may find an array of biomedicinal and biotech applications as a simple visualization tool for mapping cytosolic GSH levels in the living systems.

Conclusion

AIE fluorogens commonly show very high sensitivity because the emissions are from their collective assemblies, often in the form of nanoaggregates. There is, however, much room for improvement in terms of selectivity or specificity. Herein, an AIE bioprobe with not only high sensitivity but also high selectivity has been described that utilizes a thiol–ene click reaction mechanism. Functionalization of TPE by an MI group completely quenches the fluorescence process of TPE in both solution and in the solid state. The AIE activity, however, is recovered by the orthogonal hydrothiolation reaction of the MI unit with the free thiol group in the aggregate state under ambient conditions.

This unique process has enabled the development of a solid-state, thiol-specific bioprobe system based on TPE-MI spots on TLC plates. Thus, thanks to the remarkable click nature of the thiol–ene addition, the bioprobe plates selectively respond to the amino acid (L-cysteine) and protein (GSH) containing thiol groups at concentrations down to approximately 1 ppb, but not to other small and large biomolecules that lack free thiol units. The TPE-MI nanoaggregates show excellent performance as a visualization agent for mapping thiol distributions in living cells under normal cell incubation conditions. The biosensing process is simple and convenient, in addition to being highly sensitive and selective. We are currently working on the expansion of the scope of its applications in biomolecular sensing and cellular imaging systems.

Acknowledgements

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