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Light-up Endoplasmic Reticulum Probe based on Rational Design of Red-emissive Fluorogens with Aggregation-Induced Emission

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Fine-tuning the electron-acceptors through changing one cyano group to amide generates a more stable and emissive fluorophore with characeter of aggregation-indcued emission. Conjugation between the new fluorophore and CFFKDEL generated an excellent ER targeting light-up probe with high specificity and good photostability.

The tunable optical properties and structural diversity of organic fluorophores are of great interest in biomedical research.[1-6] Light-up probes based on organic fluorophores are especially attractive because of their reduced false-positive responses as compared to their light-off counterparts. Due to the intrinsic fluorescence of most fluorophores as molecular species, most of the light-up probes operate through photo-induced electron transfer[7] or charge transfer mechanisms[8]. Recently, fluorogens with aggregation-induced emission characteristics (AIEgens) have attracted significant research interest. These molecules are not emissive in molecularly dissolved state, but can be induced to be highly emissive in the aggregate state.[9-12] This character is opposite to that of conventional fluorophores. The manipulation of aggregation and de-aggregation processes of these fluorophores provides a unique approach to design light- up probes for cellular imaging and continuous monitoring of biological processes.[13-17]

Endoplasmic reticulum (ER) is a dynamic organelle with fundamental importance presented in all eukaryotic cells.[18] The majority of synthesized proteins undergo post-translational modification, oligomerization and folding in the ER lumen, allowing them to perform their physiological functions. Therefore, it is imperative to maintain ER homeostasis and function for proper cellular function. Pathological (e.g. stroke, diabetes, cancer, heart disease and neurodegenerative disease[19-22]) and physiological conditions (e.g. hypoxia, glucose deprivation, oxidants or reductants, viral infection, expression of aberrant proteins, and altered calcium regulation[23]) can destroy ER homeostasis and incur ER-stress induced cell death. As a consequence, both safety and efficiency should be considered when developing ER-targeting light-up probes.

Under light illumination, some organic fluorophores could generate reactive singlet oxygen species which consume the surrounding oxygen to cause hypoxia.[24] Both oxidants and hypoxia could cause ER stress and potentially induce cell death.[23] Therefore, to achieve safety, the development of fluorophores with minimized ability to generate singlet oxygen is of critical importance for ER probe design. In addition, to achieve high imaging efficiency, fluorophores with long wavelength absorption and emission are highly desirable to minimize the interference of autofluorescence. Specific targeting and aggregation of most conventional fluorophores in certain organelles may also induce aggregation-caused quenching (ACQ) effect to yield low fluorescence.[25] Taking all these considerations into account, in this study, we develop an ER targeting probe using AIEgens with long-wavelength absorption and emission for high contrast and specific imaging.

To realize both long wavelength absorption and emission, introducing donor-acceptor structures into a single molecule is an effective strategy. However, due to the charge transfer characteristics of these molecules, they often show very low fluorescence in aqueous media. Therefore, to balance the donor-acceptor strength to realize both high fluorescence and long wavelength emission is of key importance to the development of red emissive AIEgens. Due to their small size and strong electron withdrawing property, dicyanovinyl group (DCV) is a very popular electron acceptor for realizing red-emitting molecules. So far, different tetraphenylethylene (TPE) derivatives have been used as the electron donor to form donor-acceptor compounds with DCV.[26-28] Many of these compounds show great reactivity to biothiols (cysteine, homo-

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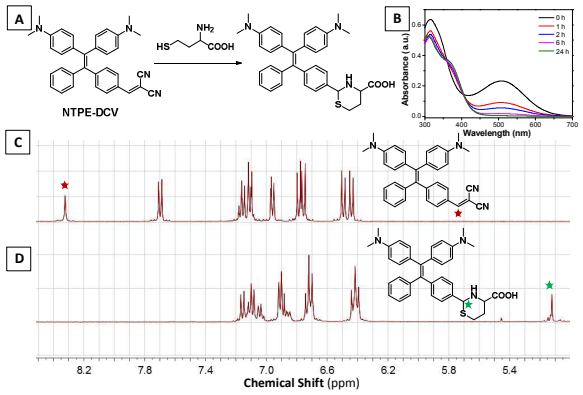


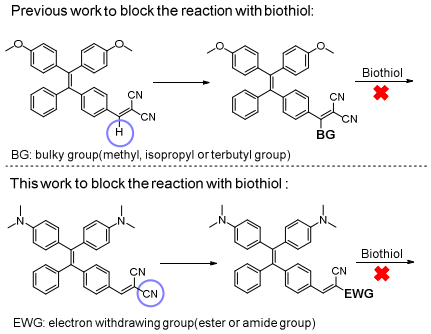
Figure 1 Reaction between NTPE-DCV and homo-Cysteine. A, Reaction scheme for NTPE-DCV and homo-Cysteine; B, Time-dependant absorbance change for the mixture of NTPE-DCV (20 μM) and homo-Cysteine (100 μM) in media of DMSO/water (10/1, v/v); C-D, ^{1}H-NMR spectra of NTPE-DCV before (C) and after (D) reaction with homo-Cysteine.

cysteine and glutathione (GSH)), making them difficult to be incorporated into molecular probes for imaging applications.[27,28]

In this communication, we report the design and synthesis of a series of red-emissive AIEgens through fine-tune of the electron withdrawing capabilities of the acceptors. We further study how the substituents affect their reactivity to biothiols. Based on an optimized fluorophore, its functional derivative was synthesized, which allowed us to develop a specific ER probe with low toxicity, high specificity and good photo-stability for real-time ER imaging.

Decoration of TPE core with dimethylamine and DCV generates NTPE-DCV which possesses high molar absorption coefficient in the range greater than 500 nm (ESI Fig S1†). However, the emission of NTPE-DCV is rather dim both as molecular species and as nano-aggregates. In addition, NTPE-DCV shows reactivity to biothiols. Interestingly, NTPE-DCV reacts with homo-cysteine or cysteine to generate a product with thiazolidine moiety (Fig 1, ESI Fig S2 and S3†) while it reacts with GSH to yield a Michael-addition product (ESI Fig S4†). These reactions could easily change the optical properties of NTPE-DCV. In addition, considering the homocysteine, cysteine and GSH are important protective constituents of the antioxidant systems in the body, the reaction with biothiol could induce their level deficiency and incur various diseases. As the nucleophilic center of thiol is also present in the catalytic sites of various enzymes, the reactivity between DCV and thiol could greatly affect the enzyme functions.[29] Therefore, both the non-emission and the instability of compound NTPE-DCV should be addressed before it can be used to develop probes for bioimaging.

Despite of its long wavelength absorption, the weak fluorescence of NTPE-DCV and its reactivity with biothiols could be ascribed to the interaction between the strong electron-withdrawing property of DCV group and strong electron-donating property of dimethylamine group. This property induces a strong process of intra-molecule charge



Scheme 1. Strategies to block the reaction between DCV and biothiols. A, previous approach; B, the approach used in this study.

transfer (ICT) that consumes the excited state energy of NTPE-DCV. Moreover, DCV makes the carbon next to it very electrophilic and reactive towards nucleophiles. In our previous study, we introduced steric hindrance by attaching an methyl group to the DCV moiety, which successfully blocked its reaction with biothiols (Scheme 1A).[30] Further modification on the methoxyl group generated a series of bioprobes for bioimaging and photodynamic therapy.[31,32] If the same method is employed for NTPE-DCV, it will be quite complicated to modify the resulting fluorophore to further introduce other functional groups. Therefore, in this study, we introduce a new strategy by changing one cyano group to other carbonyl groups with slightly lower electron-withdrawing ability, such as ester and amide groups (Scheme 1B). On one hand, the modification could decrease the ICT process and make the carbon next to the double bond less electrophilic, so that the fluorescence will increase and the reactivity towards nucleophiles (e.g. biothiols) will decrease. On the other hand, both ester and amide groups could be used for further functionalization to yield desired bioprobes.

Following the new strategy, we designed two novel fluorophores 3 and 5. The synthetic routes are shown in Scheme 2. Fluorophores 3 and 5 were synthesized by aldol condensation between aldehyde and methyl 2-cyanoacetate or 2-cyano-*N,N*-diethylacetamide. Notably, during the synthesis of 3, we serendipitously found that a small portion of 3 was hydrolyzed by the solvent of isopropyl alcohol (IPA) to generate 4. In addition, reaction between the aldehyde 2 and 2-cyano-*N,N*-diethylacetamide yielded a mixture of *cis* and *trans* isomers (5 and 6) which were separated by silica gel column. All the fluorophores were purified carefully and the correct structures were verified by NMR and high resolution mass (ESI Fig S5-S15). The specific structures of the isomers 5 and 6 were identified by heteronuclear multiple bond correlation (HMBC). As shown in Fig S16, for the major product 5, the coupling constant between the proton (coloured in black) and the carbon (coloured in purple) from the cyano group is 14.6 Hz, while it is 5.6 Hz between the same proton and the carbon (coloured in blue) from the amide group. The coupling data indicates that the proton and the cyano groups

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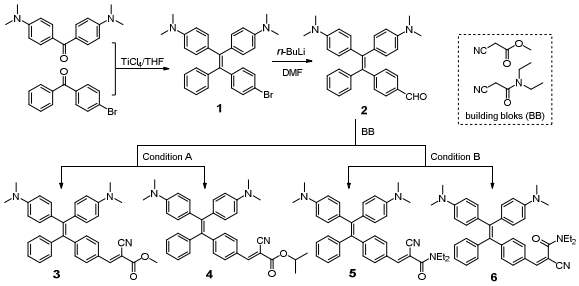
are located at the opposite sides of the double bond and 5 is in the



Scheme 2 Synthetic scheme for fluorophores 3-6. Condition A: DCM/IPA/piperidine, 20°C; Condition B: DCM/IPA/piperidine, 60°C. Compounds 4 and 6 were obtained unexpectedly as side products. DCM: dichloromethane; IPA: isopropyl alcohol.

trans form. The minor isomer 6 is thereby with *cis* form.

With the four new fluorophores in hand, we firstly measured their photo-physical properties at room temperature. The absorption maxima are 536, 496, 496, 454 and 436 nm for fluorophores of NTPE-DCV, 3, 4, 5, 6, respectively in aqueous media (Fig 2A). This observation is consistent with the calculated energy gap between the HOMO and LUMO energy levels for each compound (ESI Fig S17†). As shown in Figure S17, energy gap increased gradually for fluorophores of NTPE-DCV, 3-6. As large energy gap usually indicates small ICT effect, their emission may differ greatly. We then measured the emission of all the fluorophores in the aqueous media. As shown in Figure 2B and the inset, NTPE-DCV is almost non-emissive in aqueous solution. Fluorophores 3 and 4 have very dim emission while bright red fluorescence is observed from 5 and 6. The emission maxima are 663 and 640 nm for 5 and 6, respectively. At the same concentration of 12.5 μM, the emission intensity from 5 is 57-fold higher than that from 3. In addition, both 5 and 6 show typical property of aggregation-induced emission (Fig 2C and ESI Fig S18†). The difference in the absorption and emission spectra of these compounds indicates that reducing the electron withdrawing ability of the electron acceptor decreases the ICT effect and makes the fluorophore more fluorescent in aggregated state.

We then measured the reactivity of all the new fluorophores to biothiol. As shown in Fig 2D-E and ESI Fig S19†, different from NTPE-DCV, which reacts with all the tested biothiols quickly, the four new fluorophores do not react with GSH. For homo-cysteine and cysteine, 3 has moderate reactivity, while 4 and 6 show very low reactivity. The most stable fluorophore is 5, which does not show any reactivity to biothiols under the tested condition. Notably, the *trans*-form of 5 is slightly more stable than the *cis*-form of 6, as the double bond in *cis* form is exposed more to the attack by biothiols. It is clear that reducing the electron withdrawing ability of the acceptor in these fluorophores also makes them much more stable. It should be noted that some fluorophores with electron-withdrawing CN and amide group have recently been reported to show covalent and reversible reactivity with biothiols,[33,34] which is not observed in our study.

The phototoxicity of 5 was subsequently evaluated by

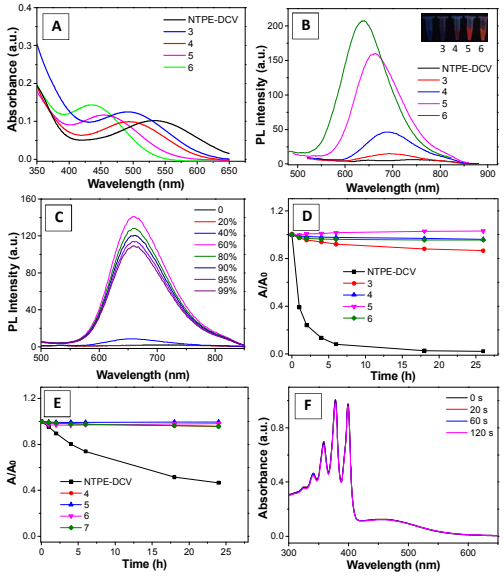


Figure 2 Optical properties of the fluorophores (20 μM each of NTPE-DCV, 3-6). (A) UV-vis absorption spectra in DMSO/Water (v/v = 1/99); (B) PL spectra in DMSO/Water (v/v = 1/99). The inset is the fluorescent photos; (C) Photoluminescence (PL) spectra of 5 (12.5 μM) in DMSO–water mixtures with different volume fractions of water (f_w). The absorbance change of fluorophores (NTPE-DCV, 3-6, 10 μM) upon incubation with six-fold homo-cysteine (D) or GSH (E). The absorption wavelengths used in the calculation of A/A_0 were 510 nm, 484 nm, 485 nm, 442 nm, 426 nm, for NTPE-DCV, 3, 4, 5, 6, respectively. A_0 is the initial absorbance value while A is the absorbance value at different reaction time. (F) The absorption spectra of ABDA (100 μM) in the presence of 6 (20 μM) after different durations of white light irradiation (100 mW/cm^{-2}).

indicator. As shown in Fig 2F, even after illumination of the solution for 2 minutes, the absorption of ABDA remains unchanged, which indicates that 5 does not produce singlet oxygen and therefore it has little photo-toxicity. In comparison, fluorophore 3 consumed 5% of ABDA under the same condition (ESI Fig S20†). The low phototoxicity together with the red-NIR emission and eliminated reactivity to biothiol make 5 a highly desirable AIEgen to be used for the development of fluorescent light-up probes for imaging applications.

KDEL is an ER-targeting peptide. We next conjugated KDEL to compound 5 in order to produce a probe which can be specifically light-up in ER. To facilitate the conjugation, we synthesized a new derivative (11) with a maleimide (MAL) group according to Fig 3A. The intermediates and 11 were characterized by NMR and high resolution mass (ESI Fig S21-28). Although two dimethylamine groups are connected to the TPE core, its absorbance remains stable in aqueous media with pH ranging from 4 to 11 (ESI Fig S29†). The probe of TPECA-ER obtained upon coupling between 11 and CFFKDEL through thiol-en reaction has been well characterized to show the correct structure (ESI Fig S30-31†). With the probe in hand, we firstly measured its photo-physical properties. TPECA-ER and 5 have roughly the same absorption profile (ESI Fig S32†) while

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TPECA-ER is much less emissive than 5 in aqueous media. This

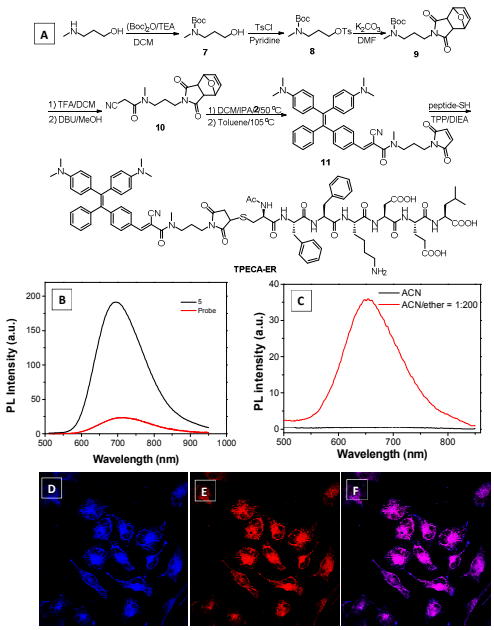

Figure 3 Synthesis of ER-targeting probe. (A) Synthetic route to the probe TPECA-ER; (B) PL spectra of 5 and TPECA-ER (10 μM) in DMSO–PBS buffer(1 ×) mixtures (v/v = 1/100), λ_{ex} = 460 nm; (C) PL spectra of TPECA-ER (12.5 μM) in acetonitrile or acetonitrile and ethyl ether mixture (v/v = 1/100), λ_{ex} = 460 nm; (D-E) Confocal images of HeLa cells treated with ER tracker (ER-Tracker™ Blue-White DPX, λ_{ex} = 405 nm, λ_{em}= 420 - 460 nm) and TPECA-ER (λ_{ex} = 470 nm, λ_{em}= 560 - 750 nm) for half an hour. (F) Overlay of images D and E.

endows the probe with good solubility in aqueous media. TPECA-ER also dissolves well in acetonitrile with low fluorescence, but it becomes highly emissive when the poor solvent of ether was added (Fig 3C). This result indicates that TPECA-ER is also AIE active.

As mentioned previously, safety is of great concern when developing imaging probes for ER. We next measured the cytotoxicity of TPECA-ER. As shown in ESI Fig S33†, the probe shows no obvious inhibition on cell growth at the concentration of 6 μM in dark or under light irradiation (0.25 W cm^{-2}, 3 min). However, more than 20% of cells were killed when commercial ER tracker was used under the same condition. Moreover, TPECA-ER also showed higher photostability than commercial ER tracker (ESI Fig S34†). Finally, HeLa cells were selected to assess the intracellular distribution profile of TPECA-ER. As shown in Fig. 3D-F, TPECA-ER displays a characteristic ER localization pattern, which is consistent with that of the ER-tracker. The Pearson's correlation co-efficient between the probe and a commercial ER tracker is 0.95, which confirms that KDEL can effectively drive the accumulation of TPECA-ER into ER. The high stability, low toxicity and specific ER localization makes TPECA-ER a successful imaging probe for ER.

In summary, we found that DCV group in NTPE-DCV reacted differently with different biothiols. It formed thiazolidine with cysteine and homo-cysteine, but it underwent Michael addition with GSH. To develop a chemically and photo-physically stable AIEgen with red emission, we changed one cyano group of DCV to amide group. This change has led to a 57-fold fluorescence intensity increase, which also completely expunged its reaction with biothiols. Conjugation of the new AIEgen with ER targeting peptide CFFKDEL generated an excellent ER targeting light-up probe. This study highlights the importance of fine-tuning the interaction between electron donors and acceptors to adjust the ICT process, which has significantly affected the photophysical and chemical properties of fluorescent dyes and their corresponding probes.

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TOC

Fine-tuning the interaction between electron donors and acceptors generates a red-emissive AIEgen which was further developed into an ER targeting imaging probe for specific ER imaging with high selectivity.