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Stability enhancement of fluorophores for lighting up practical application in bioimaging†

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The practical application of organic fluorophores in bioimaging is severely limited due to their generally poor stability. In this Highlight, we emphasize several representative strategies including nanoparticle-encapsulating dyes, dye-doped nanoparticles and molecular engineering for stabilizing fluorophores, especially with a breakthrough in photostability for visualizing disease therapy, tumor and biological processes.

Introduction

Fluorescence imaging is a powerful tool for visualizing biological processes such as genetic expression and biological information transmission at the molecular level, and tracing the real-time physiological processes *in vivo via* staining tissue, cells, or cellular compartments with fluorescent dyes.[1,2] Compared with traditional imaging methods, which are deeply obsessed with either low spatial resolution or expensive equipment, fluorescence imaging exhibits distinct advantages such as high sensitivity and non-invasive *in vivo* imaging without radiopharmaceuticals.[3,4] Moreover, fluorophores can be applied not only as biomarkers but also as probes for detecting intrinsically non-fluorescent species or parameters such as the pH value and activity of enzymes.[5–9] Therefore, the development of highly efficient fluorescent sensors has attracted highly focused research.[1]

Photostability is a particularly crucial criterion in the practical application of fluorophores as fluorescent markers in a complex biological environment, which asserts a claim to observe markers for extended periods of time against the background of intrinsic cellular emissions.[10] Unfortunately, the vast majority of commercially available contrast agents such as fluorescein isothiocyanate (FITC) and the United States Food and Drug Administration (FDA) approved indocyanine

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green (ICG) suffer from severe photobleaching. The poor stability of fluorophores is generally ascribed to the interaction between the fluorophore molecules and dissolved oxygen, which hinders their practical application to a large extent.[11,12] Hence, exploring methods to inhibit the photodegradation agents is of great significance for their practical applications in the fields of biological imaging, medical diagnosis and disease therapy.

Although the stability enhancement of fluorophores is still a challenging subject, chemists have a treasure chest of methods to prevent fluorophores from photobleaching and thus improve their efficiency. To date, the approaches to constructing fluorescent nanoparticles (NPs) or polymers that encapsulate or dope fluorophores have been identified as very promising strategies for photostability enhancement, especially for achieving some infusive accomplishment in both stability and efficiency. Moreover, a great deal of endeavour has concentrated on the molecular engineering of fluorophores that eliminate or weaken their activity towards dissolved oxygen. Various chromophores with higher stability and better photophysical properties have been explored as contrast agents instead of commercially available biomarkers. In this highlight, we illustrate some representative examples in recent studies on the stability enhancement of organic fluorophores, emphasizing the importance and significance of photostability as well as the practical applications of the judiciously modified fluorophores.

Nanoparticle-encapsulating fluorophores based on block copolymers

Previously, the nano-micelle-based drug-delivery systems have been well established, in which hydrophobic or photo-sensitive drugs (such as doxorubicin) are packaged to achieve dispersity in the aqueous system and better photostability.[13–15] Similarly, the strategy of encapsulating fluorophores in micelles is realized by the self-assembly of biocompatible amphiphilic block copolymers. In 2004, Saxena *et al.* encapsulated the FDA-approved ICG in the poly(lactic-*co*-glycolic acid) (PLGA) particles, successfully realizing a distinct improvement in the photo-, thermal- and aqueous-stability of ICG.[16] The protective effect of nanoparticles is attributed to the isolation of ICG from the surrounding environment by the polymeric-envelope. Micelles formed by various diblock copolymers such as poly(styrene-*alt*-maleic anhydride)-*b*-poly(styrene) (PSMA-*b*-PS) and poly(ethylene glycol)-*b*-poly(D,L-lactide) (PEG-*b*-PLA) provide a similar protective effect on encapsulated dyes.[17–19] Besides the improved stability, both water solubility and biocompatibility can be simultaneously increased due to the hydrophilicity and low toxicity of the outer hydrophilic block. The micelle-encapsulating dyes are capable of retaining or even enhancing response sensitivity and selectivity (Fig. S1 in ESI†).[18] However, the micelles might inevitably suffer from not only dye leakage, but also spontaneous dissociation at concentrations below their critical micelle concentration (CMC), which are still non-ignorable disadvantages against the practical application in medical diagnosis and biological processes.[16]

Stabilizing the micelles by locking the micellar structure with a cross-linking agent such as a silane source has been developed as an attractive strategy to improve the micellar stability (Fig. S2†).[20,21] Therefore, highly stable, not only for the content but also for the micelles, fluorescent nanoparticles have been constructed *via* cross-linking the micelles that encapsulate fluorophores. Recently, we have loaded naphthalimide dye into the polystyrene-*block*-polyacrylic acid (PS-*b*-PAA) micelles which are subsequently cross-linked by the silane source of MPTMS (Fig. 1a).[22] The hydrophilic nanoparticles, whose hydrophilicity is a result of the outermost hydrophilic block (PAA), can exactly ensure complete water solubility and further eliminate the solvatochromism. The nanoparticle-encapsulating naphthalimide in the aqueous system possesses a similar fluorescence quantum yield and lifetime as the free dye in CH_2Cl_2 solution, highlighting the complete elimination of the solvent effect. Impressively, after the cross-linking, the leakage of encapsulated dye molecules is reduced substantially because of the compact silica shell, thus achieving high long-term fluorescence stability (Fig. 1a and Fig. S3†). The excellent effectiveness in living cell imaging, resulting from the well-controlled particle size, defines this strategy as a fairly promising method for converting the hydrophobic species to hydrophilic ones, resulting in a highly stable contrast agent for bioimaging. Notably, here the complicated pre-treatments are unnecessary, and also the concentration of entrapped dyes and the size of particles can be easily tuned to a great extent.[23,24]

Cyanine dye is one of the most common near-infrared (NIR) fluorophores, which is deeply plagued by poor photo- and chemical stability.[25,26] Based on a similar strategy, cyanine was utilized for constructing NIR fluorescent nanoparticles (Fig. 1b). Generally, the NIR fluorescence of cyanine dyes is severely quenched by water or polar solvents. The encapsulation increases its fluorescence quantum yield by 450-fold in the aqueous system, along with changing the original hydrophobic species into hydrophilic nanoparticles.[27] Moreover, the encapsulated fluorophores possess a 95-fold longer half-life period than the FDA-approved ICG under continuous high density illumination due to the effective protection from the nanoparticle shell. Besides, the addition of reactive oxygen species (ROS) such as the hypochlorite anion which is also lethal to free cyanine dyes has almost no effect on nanoparticle-encapsulating cyanine dyes. More importantly, after injection into the tumor tissue, the nanoparticles exhibit bright NIR fluorescence in *in vivo* tumor imaging with long retention time (more than 24 h). The excellent performance in bioimaging highlights the significance of the enhanced stability, especially for the enhanced permeability and retention (EPR) effect derived from the proper size of nanoparticles (Fig. S4†).[28]

When reducing the cross-linking density, this strategy can also be utilized for fabricating the fluorescent hybrid nanosensor system. In our group, a pH-responsive naphthalenediimide derivative was immobilized into spherical PS-*b*-PAA micelles and discreetly cross-linked by low density of MPTMS, exhibiting several promising features including complete solubility in water

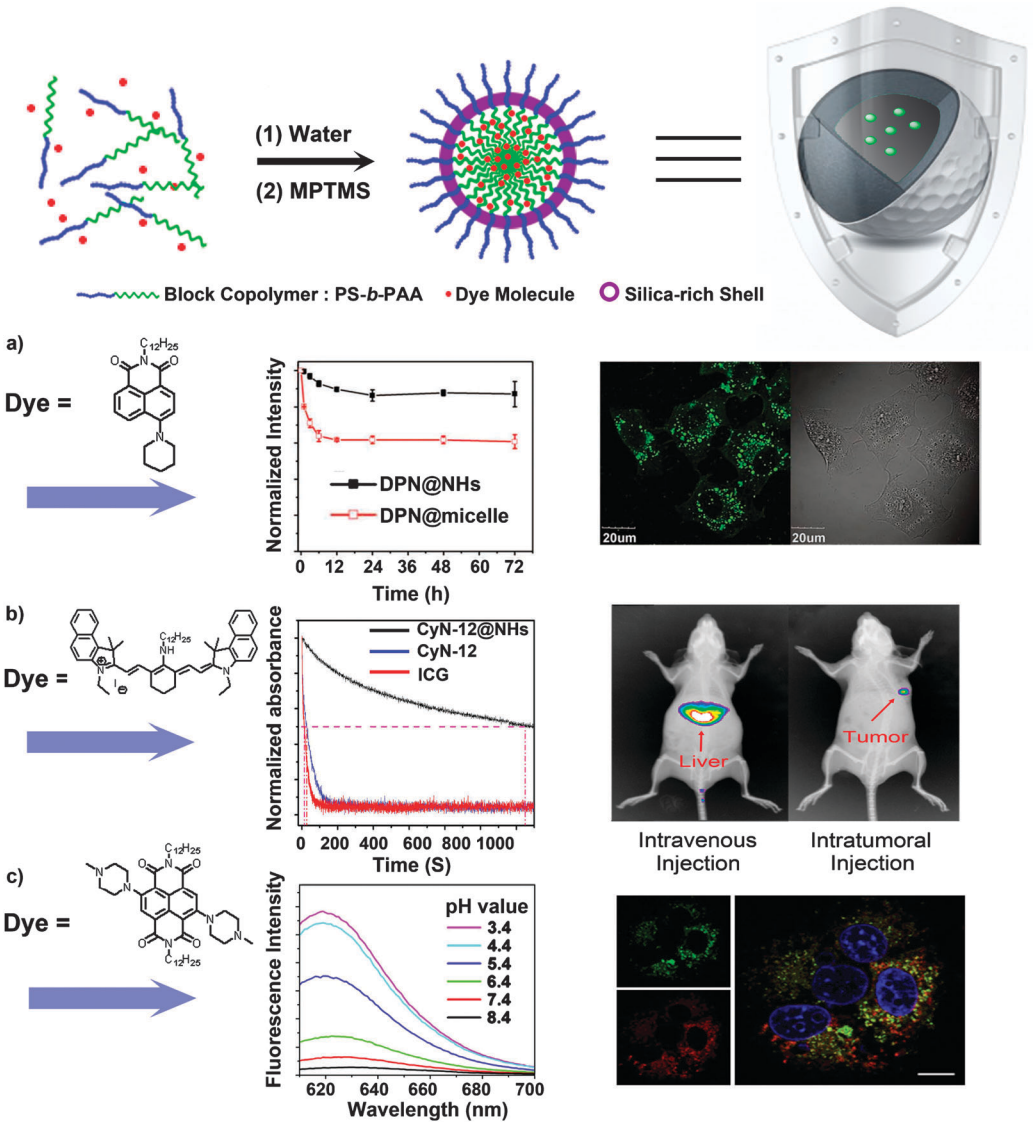

Fig. 1 Schematic diagram of nanoparticle-encapsulating dyes based on diblock copolymer and a silica-rich shell: (a) nanoparticle-encapsulating naphthalimide and its stability, including living cell imaging; (b) nanoparticle-encapsulating cyanine and its photostability, including *in vivo* bioimaging; and (c) nanoparticle-encapsulating naphthalenediimide including its pH response and living cell imaging.

in spite of the hydrophobicity of the free dye, a low level of dissociation or leakage of encapsulated fluorophores, fast and precise pH response along with extremely low cytotoxicity and outstanding intracellular tumor cell labeling (Fig. 1c and Fig. S5†).[29] Consequently, the core–shell nanostructured particles provide an effective approach to construct highly stable contrast agents for bioimaging.[30]

Except for the block copolymer-based nanoparticles, the enhancement of photostability can also be achieved by the encapsulation of fluorophores into the biological macromolecules such as proteins and liposomes.[31,32] The unique microenvironment inside the macromolecules is capable of shielding the inner fluorophores against the attack by ROS or other active species. However, the structurally specific effect of protein encapsulation cannot guarantee it as widely applicable as the aforementioned block polymer systems.[30] In addition, the enhancement of fluorophore stability can also be realized by tedious covalent conjugation with polymer or polymeric nanoparticles.[33,34]

Fluorophore-doped nanoparticles based on inorganic matrices

Fluorophore-doped materials, in which organic dyes are doped during the growth of inorganic nanoparticles, represent another widely used strategy for the stability enhancement of traditional dyes.[35] Based on the well-developed Stöber method, the resultant dye-doped silica nanoparticles generally have diameters ranging from tens to hundreds of nanometers, along with efficiently encapsulating several dye molecules per nanoparticle. The constituent fluorophores are endowed with enhanced photostability and

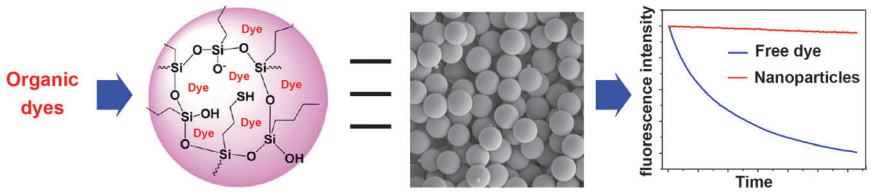

Fig. 2 Schematic diagram of dye-doped nanoparticles, its representative TEM image, and the enhanced stability of doped fluorophores.

longer fluorescence life-time or stronger brightness (Fig. 2).[36–39] Notably, the easy surface modification and biocompatibility of the silica-rich structure substantially reduce the nanoparticle cytotoxicity, making it suitable for living cell imaging and flow cytometry.[40]

The dye nano-encapsulation system based on calcium phosphate (CP), which is "generally regarded as safe" by the FDA, has also been established as an effective platform for enhancing fluorophore photostability.[41] In one approach, ICG was doped by CP surface modified with carboxylate or PEG termination for early stage *in vivo* tumor imaging. The fluorescence of dye-doped CP nanoparticles is little sensitive to solvent polarity, resulting in an increase in fluorescence quantum efficiency by 2-fold with respect to the free fluorophore. Importantly, their photostability is 5-fold higher at clinical imaging excitation power than that of the corresponding free dye. Here the enhanced stability and prolonged circulation times *in vivo* with passive tumor accumulation are highly desirable for diagnostic imaging (Fig. S6†).[42] As well demonstrated, CP nanoparticles can efficiently provide improved stability to a variety of organic dyes.[43]

Generally, nanoparticles based on both the organic block copolymer and inorganic matrix (such as silica and calcium phosphate) have a similar capacity to provide the encapsulated or doped dye molecules with better photostability. Compared with the micelle-based ones, the structure of the inorganic nanoparticles is more stable, but their surface functionalization is limited to some extent because of the relatively simple functional group on the surface. On the other hand, the easy modification of the copolymer-based nanoparticles not only eliminates the micelle dissociation by cross-linking, but also endows the nanoparticles with a variety of functionalities.[44]

Molecular engineering of fluorescent chromophores

The key element of the enhancement of photostability is to hinder the energy or electron transfer between the excited fluorophores and ROS, which can also be achieved by the direct structural modification of fluorophores. As a matter of fact, the delicate decoration of molecular structures can induce great change in the photostability of fluorophores (Fig. 3).[45,46] For example, the simple replacement of oxygen by the nitrogen atom in the traditional dicyanomethylene-4*H*-pyran (DCM) moiety creates a new family of fluorophores for realizing particular red light aggregation-induced emission (AIE) with significant stability.[47–50] An early and efficient approach to enhancing the

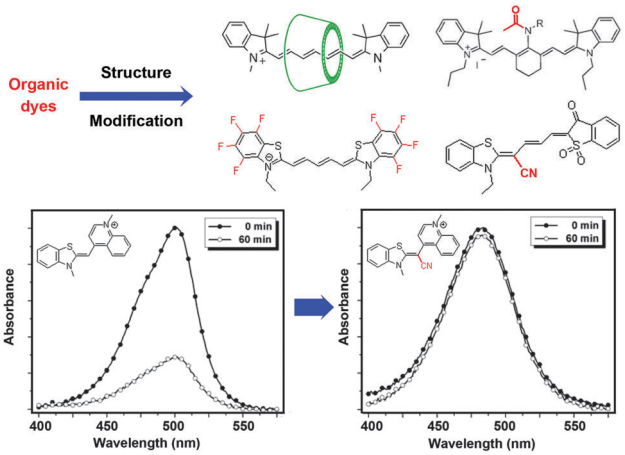

Fig. 3 Schematic diagram of structural modification of cyanine dyes including cyclodextrin complex, acetyl modification, fluoro- and cyano-substitution, and the enhanced photostability of the cyano modified cyanine.

stability of fluorescent dyes was demonstrated by incorporating a cyclodextrin (or cucurbituril) ring onto cyanine fluorophores (Fig. 3).[51–53] The crystal structure of the cyanine dye rotaxane demonstrated by Anderson *et al.* exhibits tight encapsulation as well as protection for the dye molecules, explaining the enhanced photostability.[53] Recently, triplet-state quenchers such as cyclooctatetraene and 4-nitrobenzyl alcohol have been covalently conjugated with cyanine fluorophores to distinctly decrease the triplet state occupancy and thus the rate of singlet-oxygen generation and photo-oxidation.[54] In this way, the newly developed supramolecular-like fluorophores realize an efficient decrease of the blinking and photobleaching in both deoxygenated and oxygenated environments, extremely valuable for *in vivo* applications.[55] Additionally, introducing a strong electron-withdrawing unit such as the acetyl or cyano group,[56–58] and fluorine,[59,60] into the tricarbocyanine backbone can also improve the stability sharply (Fig. 3), which can be successfully utilized in protein labelling and cancer bioimaging.[61–63] As constructed with fluorophore cores, dendrimers are also an efficient approach to enhancing the photostability and water solubility of the core dyes due to the protection of the hydrophilic dendritic shell.[64,65] For instance, Zimmerman *et al.* conjugated the hydrophobic dye (KFL-3) with water-soluble and biocompatible polyglycerol dendrimers (PGD) to fabricate the far-red fluorescent probe PGD-KFL. With the help of the dendritic shell, PGD-KFL exhibits comparable photostability to Cy5 and especially long-lasting fluorescence emission with a fairly low level of blinking in single-molecule imaging (Fig. S7†).[66]

To date, novel NIR fluorophores equipped with better stability and efficacy are highly preferable as contrast agents. For example, a derivative of the well-known laser dye DCM, with 19-fold better photostability than ICG, has been successfully utilized to construct NIR theranostic prodrug. It exhibits highly specific tumor-activatable drug release, resulting in the turn-on NIR fluorescence for *in vivo* imaging as well as excellent antitumor activity with lower side effects than free drugs (Fig. S8†).[67] Now more and more successful examples open a new window to design highly photostable fluorophores for visualizing disease therapy, tumor and biological processes.

Summary and outlook

The stability enhancement of fluorophores benefits their practical applications in the fields of biological and biomedical imaging. In this highlight, we focused on the recent noteworthy efficient strategies to improve chromophore stability including the encapsulation and doping of fluorophores by nanoparticles, and structural modification of fluorophores. With the help of these strategies, numerous highly stable fluorescent nanoparticles, polymers and fluorophores have been particularly developed. However, there are still severe barriers against the practical application of the newly developed contrast agents, especially for the nanoparticle-based ones in *in vivo* imaging. For example, the mononuclear phagocyte system (MPS) clearance presents challenges of effectively removing a large fraction of available nanoparticles from circulation and leading to long retention times of potentially toxic nanoparticle components or metabolites.[68] Although most of the newly developed fluorescent contrast agents could still only be applied in the laboratory due to the slight lack of comprehensive research, it is expected that these strategies will pave a promising way for fabricating highly stable contrast agents for real bioimaging.

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