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Microstructured fluorescent biosensor based on energy migration for selective sensing of metalloprotein†

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Fluorescent microspheres consisting of a conjugated phenylenevinylene 3B2B doped with a fluorenone derivative DSFO were prepared by a reprecipitation method. The DSFO-doped 3B2B microspheres (DMPs) exhibited significantly reduced fluorescence from 3B2B (donor) and strongly enhanced emission from DSFO (acceptor), which is highly sensitive to the concentration of metalloprotein.

Recently, there has been significant interest in developing fluorescent probes that can simply and quantitatively respond to individual proteins because of their fundamental importance in proteomics, medical diagnostics, and pathogen detection.[1] The high sensitivity and selectivity of fluorescent sensors offers a distinct advantage in the early diagnosis of diseases. Several fluorescent reagents that target proteins have been developed for the detection of proteins; these include *o*-phthaldialdehyde, fluorescamine,[2] cyanine dyes,[3] acridizinium[4] and NanoOrange[2] which can detect proteins in solution. Recently, Thayumanavan and co-workers have utilized water-soluble polyelectrolyte to generate fluorescence-based patterns for both metalloprotein and non-metalloprotein sensing using solution-based detection schemes.[5–7] Although the specially designed amphiphilic sensors exhibited selectivity and high sensitivity to metalloprotein in solution, the hydrophobicity of most fluorescent organic dyes limited their applications such as in fluorescent probes for protein detection in aqueous media. Therefore, research into simple approaches to create water-soluble biosensors for solution-based protein detection is still needed.

Fluorescent organic nano/microparticles, as a result of their large diversity in molecular structure and optical properties that are of potential use in optoelectronics and biologics, have become the subject of ever-increasing attention in recent years.[8] Reprecipitation, one kind of solvent displacement method, has become a popular technique for the preparation of water-soluble nano/microparticles due to its easy and versatile operation. Compared to small organic molecules, the organic nano/microparticles, which generally disperse in aqueous media, simultaneously provide efficient fluorescence,[9] a great reduction in photobleaching,[10] low nonspecific adsorption,[11] colloidal stability and biocompatibility in a variety of bioenvironments. These nano/microparticles will be able to be directly used as fluorescent biosensors without modification. This method opens up a new way of making use of organic nano/microparticles in biological applications. Förster resonance energy transfer (FRET) is a physical phenomenon in which energy absorbed by a fluorophore is transferred to another molecule through a nonradiative pathway. Doping techniques based on FRET have been proven to be an effective way to improve the luminescence efficiency and tune the emission color of fluorescent materials and are widely used in the fabrication of advanced functional materials, such as molecular beacon biosensors[12] and optoelectronic devices.[13] However, little attention has been paid to doped organic nano/microparticles, which may potentially serve as energy donors and acceptors in a variety of FRET-based biological studies. Herein we report energy-transfer-mediated fluorescence from **DSFO**-doped **3B2B** microspheres (**DMPs**) and its application to metalloprotein sensing.

All the microparticles, including pure and doped ones, were prepared through the reprecipitation method. The conjugated phenylenevinylene **3B2B**, with a coplanar configuration, was employed as the doping host. The fluorenone derivative **DSFO** served as the dopant dye. Rapid addition of a solution of **3B2B** and **DSFO** in THF to water (PBS), followed by mixing, led to the formation of microparticles because of the sudden change of the environment. The formation process of the **DMPs** is shown in Fig. 1a. The major driving force during the formation of the **DMPs** maybe the intermolecular π–π interaction between the conjugated **3B2B** and **DSFO** molecules in cooperation with other noncovalent interactions, such as van der Waals, hydrogen bonding and hydrophilic/hydrophobic interactions. The size and shape of the as-prepared microparticles were observed using field emission scanning electron microscopy (FESEM) and transmission electron microscopy (TEM). Representative images of the **3B2B** microparticles with a **DSFO** doping level of 2% (molar) are presented in Fig. 1b and c. The microparticles are approximately spherical with a size in the range of (1.0 ± 0.3) μm (Fig. S1†). Fluorescence microscopy is a useful tool to investigate the morphology of the microstructures and the solid state luminescent properties. Fig. S2† shows the fluorescence microscopic images of microparticles of **3B2B**, 2% **DSFO**-doped

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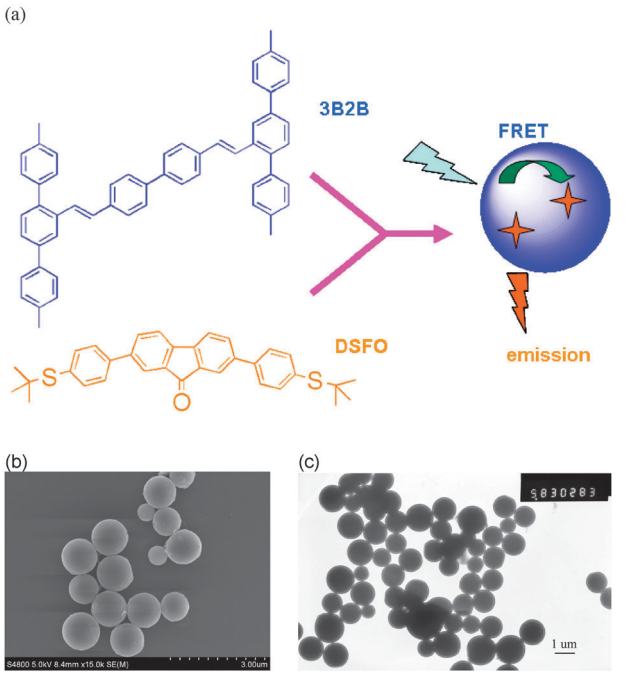

Fig. 1 (a) Schematic illustration of the formation of the **DMPs** for metalloprotein sensing. (b) FESEM and (c) TEM images of the **DMPs** with a **DSFO** doping content of 2% (molar).

3B2B and **DSFO** on excitation with UV light (330–380 nm). Fig. S2a presents clear spherical particles with the blue luminescence of **3B2B** and Fig. S2c exhibits microparticles with the yellow luminescence of **DSFO**. The green luminescence of the 2% **DSFO**-doped **3B2B** is observed in Fig. S2b,† which suggests that **DSFO** molecules have been doped into **3B2B** particles during the reprecipitation process.

The absorption spectrum of **DSFO** microparticles shows good overlap with the fluorescence emission spectrum of **3B2B** in the 400–550 nm wavelength range (Fig. 2a), which is essential for the occurrence of energy transfer in the doping systems. The emission spectrum of **3B2B** shown in Fig. 2a was obtained at an excitation wavelength of 369 nm where the absorption of **3B2B** is at a maximum while that of **DSFO** is weak. The suspension of pure **DSFO** particles, which has the same concentration as **DSFO** in the suspension of the **DMPs**, is non-emissive. When compared to the undoped **3B2B** particles, the doped particles exhibited reduced **3B2B** fluorescence and strongly enhanced emission from **DSFO**

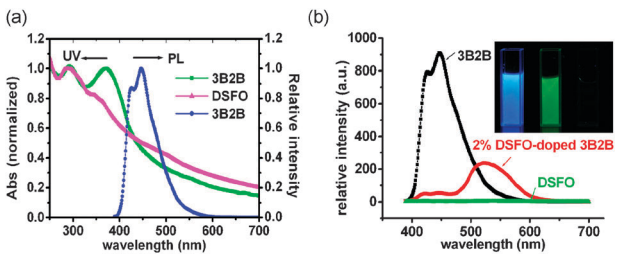

Fig. 2 (a) Normalized absorption and emission spectra of aqueous suspensions of **3B2B** and **DSFO** microparticles. (b) Emission spectra of the **3B2B**, 2% **DSFO**-doped **3B2B** and **DSFO** aqueous dispersions with an excitation wavelength of 369 nm. Inset (from left to right): photoimages of **3B2B**, 2% **DSFO**-doped **3B2B** and **DSFO** suspensions under a UV lamp (365 nm).

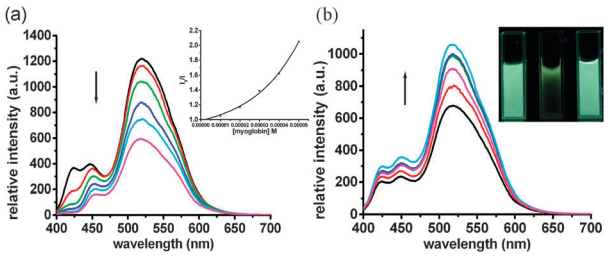

Fig. 3 (For details see Fig. S3†). (a) Fluorescence spectra of the **DMPs** with various concentrations of metalloprotein (myoglobin) in 10 mM PBS (pH 7.4). Inset: corresponding Stern–Volmer plot. (b) Fluorescence spectra of the **DMPs** with various concentrations of non-metalloprotein (BSA) in 10 mM PBS (pH 7.4). Inset: (from left to right): photoimages of a suspension of the **DMPs**, the **DMPs** with myoglobin, and the **DMPs** with BSA under a UV lamp (365 nm).

(Fig. 2b), which is consistent with energy-transfer-mediated fluorescence in the doped particles. The energy transfer efficiency for 2% **DSFO**-doped **3B2B** microparticles obtained from fluorescence spectra (Fig. 2b) was approximately 93%. Irradiation of aqueous microparticle dispersions with a UV lamp (365 nm) illustrates a clear difference in the fluorescence color of undoped **3B2B**, 2% **DSFO**-doped **3B2B** and **DSFO** microparticles (Fig. 2b inset).

To verify and quantify the selective response to metalloprotein, we monitored the fluorescence behavior of the doped microspheres (**DMPs**) in the presence of myoglobin (a metalloprotein) and BSA (a non-metalloprotein) as a function of concentration. The results are shown in Fig. 3. It is clear that the fluorescence from the **DMPs** reduced with the increasing concentration of myoglobin, while addition of similar concentrations of BSA to the solution led to a slight enhancement of the fluorescence of the **DMPs**. Myoglobin (p*I* 6.8)[14] and BSA (p*I* 4.8–4.9) are negatively charged at neutral pH. Therefore, it is supposed that the interactions of the **DMPs** with these two proteins would be similar. The efficient quenching effect of the **DMPs** in the presence of metalloprotein myoglobin is primarily due to energy or electron transfer of the **DMPs** with heme portions within the protein, which have the appropriate energy or electron-donating/accepting functionalities (Fig. 3a). The slight increase of the fluorescence of the **DMPs** upon addition of BSA suggests the π–π stacking of the **DMPs** was reduced by the negatively charged BSA through repulsion forces (Fig. 3b).[15]

The fluorescence dynamic quenching sensitivity can be quantified through measurements with the Stern–Volmer equation:[16]

$$\frac{I_0}{I} = 1 + K_{sv}[Q] \qquad (1)$$

where I_0 and I are the fluorescence intensities in the absence and presence of protein, respectively; K_{sv} is the Stern–Volmer quenching constant, and [Q] is the concentration of protein. The quenching data are usually presented as plots of I_0/I *versus* [Q] with a slope equal to K_{sv}. The constant K_{sv} thus provides a direct measure of the quenching sensitivity. For myoglobin, the plot is shown in Fig. 3a inset and K_{sv} was 4.2×10^4. Superlinear behavior was observed due to a sphere of action mechanism (ref. S3†).[17]

To further verify this sensing mode, we also studied the fluorescence response of the **DMPs** in PBS to 6 other different proteins. Cytochrome *c* (cyt *c*) and ferritin are iron metalloproteins, while the

other four proteins (lysozyme, thrombin, pepsin, α-chymotrypsin) are non-metalloproteins. The fluorescence spectra of the **DMPs** with various concentrations of ferritin and cyt *c* and corresponding Stern–Volmer plots are illustrated in Fig. S4.† The fluorescence intensity decreases proportionally with the increase in ferritin concentration (Fig. S4a†). Similar behavior was observed with cyt *c* (Fig. S4c†). Superlinear behavior appears with increasing concentration due to the sphere of action mechanism for ferritin (Fig. S4b†). For cyt *c*, a linear plot emerges (Fig. S4d†). The different behaviors of the plots imply a different quenching mechanism for the proteins, *i.e.*, static quenching *via* complex formation and/or dynamic quenching due to random collisions between the PL emitter and acceptor.[17] We believe the quenching mechanism of the **DMPs** in the presence of ferritin is due to electron transfer since there is no chromophore that could accept the excited-state energy from the **DMPs**.[7] For cyt *c*, a heme-containing protein, the electron transfer and energy transfer would be possible.

The sensitivity, K_{sv}, of the **DMPs** was found to be 5.7×10^5 and 2.9×10^4 for ferritin and cyt *c*, respectively. Ferritin exhibits the highest K_{sv}, which is likely to be due to the fact that a single protein binding event brings hundreds of bound Fe^{2+}.[7] Comparing with cyt *c*, myoglobin presented a relatively large quenching sensitivity for the **DMPs**, which is demonstrated with the Stern–Volmer constants. Myoglobin (p*I* 6.8) is neutral/slightly negatively charged at pH 7.4, whereas cyt *c* (p*I* 10.6)[14] is positively charged at this pH. Though the **DMPs** and cyt *c* are oppositely charged at neutral pH and thus readily form complexes, which will facilitate rapid energy/electron transfer and high quenching efficiency, the higher quenching efficiency of myoglobin compared to that of cyt *c* was not surprising. The net negative charge of the **DMPs** plays a significant role but is not the only factor in the interaction of proteins with these spheres.[7,18] On the other hand, not only binding affinity but also inherent ability of the protein to quench the fluorophore contributes to the quenching efficiency.[18] A similar result was also obtained in Bunz's research,[11] in which a higher quenching efficiency of myoglobin compared to that of cyt *c* was obtained. In the presence of four other non-metalloproteins, *viz.*, lysozyme, thrombin, pepsin and α-chymotrypsin, a slightly increased fluorescence of the **DMPs** was obtained (Fig. S5†).

In summary, we have developed a new water-soluble biosensor (**DMPs**) by a reprecipitation method and reported energy-transfer-mediated fluorescence from the **DMPs** and its application to metalloprotein sensing. Fluorescent spectroscopy indicates highly efficient energy transfer from the host **3B2B** to the dopant dye **DSFO**, which results in significantly enhanced emission from the dopant dye **DSFO** that is highly sensitive to the concentration of metalloprotein. In the presence of metalloprotein, highly quenched emissions of the **DMPs** were observed with increasing concentrations of metalloprotein, whereas slightly increased emissions were observed with non-metalloprotein. The mechanism of quenching for metalloprotein ferritin is likely to be based on electron transfer, since there is no chromophore that could accept the excited-state energy from the **DMPs**. The quenching effects for cyt *c* and myoglobin are a result of energy and/or electron transfer processes between porphyrin cofactor and the fluorescent **DMPs**. The increase in fluorescence with non-metalloprotein is due to the induced decrease in π–π stacking of the microspheres. We noticed that the aqueous suspension of pure **3B2B** also has selective fluorescent signals in the presence of metalloprotein (Fig. S6†). The motivation to set up a FRET scaffold between **3B2B** and **DSFO** is to increase the fluorescence intensity of **DSFO** and expand the applications of **DSFO**. In our opinion, **3B2B** efficiently "transfers" the function to **DSFO** due to the FRET scaffold between them.

The light-harvesting properties *via* energy transfer and the water-solubility, together with the quantitative selectivity for sensing metalloprotein of the **DMPs** indicate that the new biosensors have the potential to be used in bioanalysis and sensor fabrication. The reprecipitation method is proved to be a simple and versatile method to obtain water soluble biosensors of fluorescent organic molecules with hydrophobicity, which will expand their biological applications.

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