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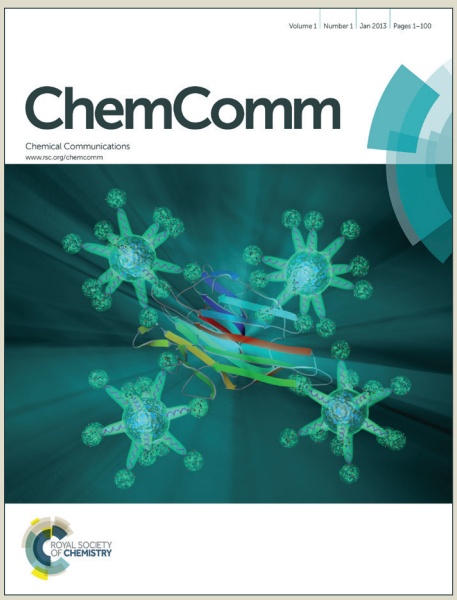

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Aggregation-induced emission fluorogen as biomarker to assess viability of microalgae in aquatic ecosystem

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Microalgae can be a valuable indicator in water pollution monitoring due to their sensitivity to the change of pollutants in the environment. In this study, aggregation-induced emission fluorogen was used as a novel tool to differentiate dead and live microalgae and quantify the link between live algal concentration and fluorogen intensity. Protein in cell protoplasm is the key component contributing to fluorescence emission in algae.

For the past few decades the oceans have been regarded as a giant dumping area for all types of disposal, from organic carbon, plastic, toxins and heavy metals to radioactive waste with the misconception that the gigantic size of the oceans is sufficient to dilute pollutants and render harmless for any materials dumped in [1]. In fact, the ocean ecosystem is currently under enormous stress from a variety of pollution sources. The oceans have a considerable self-purification function but that ability is not infinite. By dumping excessive pollutants into the oceans, humans may permanently alter the ocean ecosystem. Moreover, a number of manufactured chemicals can threaten human health, including cancer, immune deficiency, nerve disorders and low fertility.

Biological monitoring uses biological responses to evaluate changes in the environment with the intention of establishing a qualitative control program. Through systematic and regular monitoring, the responses of organisms are used to assess the impact of pollutants on the health and function of an aquatic ecosystem. Microalgae are vitally important to the food web in the aquatic ecosystem and can be a valuable indicator for monitoring water pollution due to their sensitivity to chemical changes in the environment. Moreover, microalgae are pivotal in the biogeochemical cycling of nutrients and pollutants in the ocean [2]. Microalgae have been referred to as a ''green liver'' of the ocean, acting as an important sink for chemical compounds. *Nannochloropsis* is a genus of small green microalgae and is well known for its nutritional value and ability to produce valuable lipophilic and lipophobic materials. In the aquaculture industry, *Nannochloropsis* is extensively used as live feed to grow small zooplankton such as rotifers and copepods in fish hatcheries. *Nannochloropsis oculata* is one of the six species in this genus. *Nannochloropsis* is a unicellular green alga having a spherical shape and 2-5 μm in diameter. In this study, this ubiquitous species was used to test whether a new and unique luminogen can be a biomarker to assess the viability of microalgae.

Various species of microalgae have been used for bioassessment of pollution in water [3]. Recently, some methods have been developed to assess phytoplankton viability in marine and coastal environments, such as SYTOX green [4, 5], fluorescein diacetate (FDA) [6-9], FDA + 5-chloromethylfluorescein diacetate (CMFDA) [10] and neutral red [11-12]. Among these assays, fluorescein formed by intracellular hydrolysis of FDA leaks rapidly from cells [13] and SYTOX green does not reveal the mechanism causing cell death. Auto-fluorescence in chlorophyll pigments overlaps with the fluorescence of propidium iodide (PI) [14] whereas neutral red is not effective for staining some phytoplankton species due to leaching or cell shrinkage [15, 16]. So far, no efficient staining method has been found to easily differentiate live from dead algae [17].

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† Electronic Supplementary Information (ESI) available: materials, algae strain and culture conditions, BSPOTPE monitoring live and dead *N. oculata*, monitoring PL intensity of live and dead *N. oculata* according to different time profiles, measurement of *N. oculata* staining by BSPOTPE under the control of salinity, evaluating PL intensity of BSPOTPE with different density of *N. oculata* in the PBS buffer, live and dead *N. oculata* staining with BSPOTPE or propodium iodine and elucidation of staining mechanism of *N. oculata* by BSPOTPE. See DOI: 10.1039/x0xx00000x

We previously discovered a group of unique luminogens which are nonluminescent when molecularly dissolved but highly fluorescent when aggregated [18]. Aggregation-induced emission (AIE) describes this novel phenomenon and the restriction of intramolecular motions is the main cause of the AIE phenomenon [19]. Over the past decade, AIE luminogens have been successfully employed in a variety of biological applications [19-21], such as long-term cell tracking, non-self-quenching DNA labelling, inhibition of amyloid fibrillation, differentiation of protein monomer, oligomer and fibril [22], monitoring of cell apoptosis and long-term bacterial viability assays [23]. AIE luminogens exhibit high quantum efficiency, good biocompatibility and appreciable photostability, and these features have motivated us to further explore their application in the thriving field of aquatic ecology.

Recently we discovered an AIE-active molecule, 1,2-Bis [4-(3-sulfonatopropoxyl) phenyl]-1,2-diphenylethene salt (BSPOTPE), for staining live particles [24]. BSPOTPE is a water-soluble and biocompatible fluorogen without fluorescence in physiological buffers. In the current study, we discovered an important function of BSPOTPE as a fluorescent probe to quantitatively differentiate live and dead *N. oculata* in algal culture.

Dead *N. oculata* (10×10^6/ml) was obtained after preservation in 75% ethanol for 30 min, followed by BSPOTPE/PI staining for 20 min. Our major finding was that BSPOTPE induced an increase of fluorescence (>300-fold) at BSPOTPE (200 µM) by binding with live *N. oculata*, whereas dead cells released minimum emission stained with BSPOTPE (Fig. 1a). To obtain stable emission, optimising the binding condition with an increment of dye concentration was conducted. The photoluminescence (PL) intensity of BSPOTPE-bound live *N. oculata* increased with the increase of BSPOTPE concentration (Fig. 1A) and reached a plateau at 200 - 500 µM. No obvious change in PL intensity was observed in BSPOTPE-bound dead *N. oculata*. The 200 µM BSPOTPE was chosen to further differentiate dead and live cells. Live *N. oculata* could be instantly stained by BSPOTPE after gentle mixing, which is much quicker than that by the neutral red staining method (1-2 h) [12, 15]. Staining time with FDA usually takes <10 min, but not all organisms can be stained, and the fluorescent signal is not stable [7, 25]. Peperzak assessed the vitality of phytoplankton with six dyes by flow cytometry. Only Calcein-AM stained *Nannochloro*psis, but the signals were not strong [26]. Flow cytometry has been used for particle quantification in aquatic ecosystems since the 1980s. Despite its high sensitivity and rapid quantification in cell densities [27], it is unlikely to differentiate dead algal cells from live algae.

The time profiles of PL intensity of BSPOTPE binding with live and dead *N. oculata* were compared. The PL intensity of BSPOTPE with live *N. oculata* was very high at the beginning and showed little variation over time (Fig. 1B). The combination of dead *N. oculata* with BSPOTPE binding led to very low but constant PL intensity. As BSPOTPE had high stability for staining live *N. oculata* for at least 7 days, BSPOTPE could provide a long-term viability assay for staining live *N. oculata*. Furthermore, algal cell shape remained intact after verification with trypan blue staining. Salinity may affect the binding efficiency of BSPOTPE on live *N. oculata*. We controlled salinity by fixing a total of 500 µl of *N. oculata*/seawater and 2 ml of phosphate-buffered saline (PBS) at 13.8‰ salinity. The PL intensity was stable in a high concentration of live *N. oculata* (Fig. 1C). BSPOTPE could measure live algae concentration under a light condition regardless of salinity.

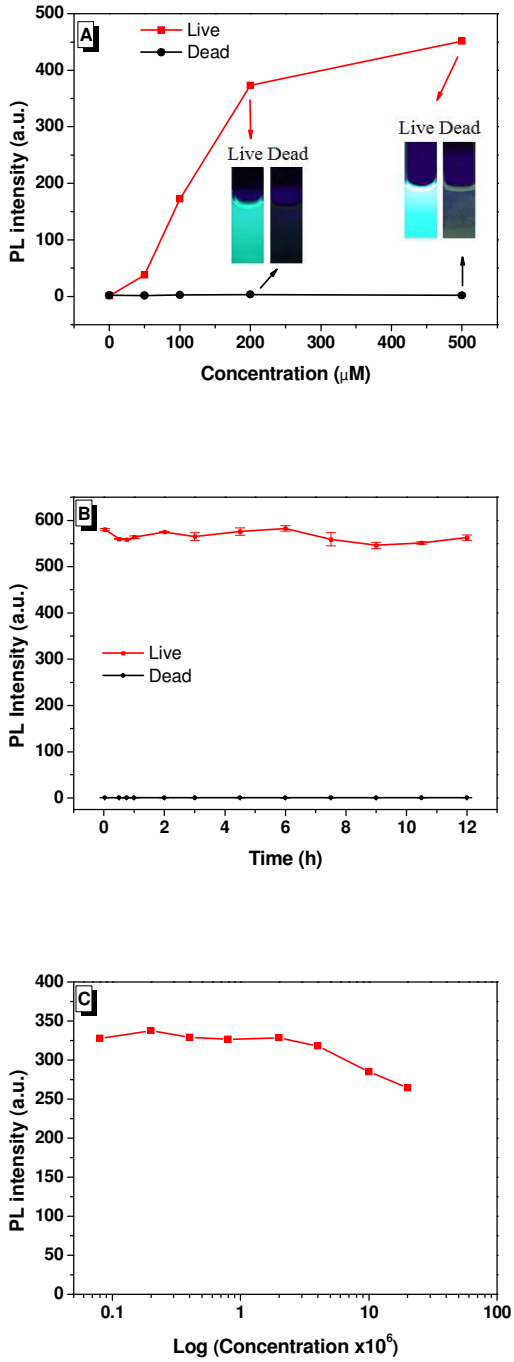

Fig. 1. Photoluminescence (PL) intensity of live and dead *N. oculata* at different BSPOTPE concentrations (A) and time elapse (B). The PL intensity of BSPOTPE at different *N. oculata* concentrations (C).

The binding of BSPOTPE to live cells can be visualised by fluorescence microscopy with strong blue fluorescence (Fig. 2B).

The binding shows no overlap with the auto-fluorescence of chlorophyll pigments. On the other hand, the dead cells are almost non-emissive after binding to BSPOTPE (Fig. 2F). It is possible that 75% ethanol may denature the proteins on the cell membrane that could have been bound by BSPOTPE. In the present study, strong red fluorescence was achieved by the binding of PI and dead cells (Fig. 2H), though PI was non-emissive binding to live cells (Fig. 2D). It is well known that PI can stain dead cells as it is an impermeant nucleic acid dye that only stains cells with a damaged membrane, and the enhanced fluorescence can thus indicate dead status of a cell. However, the method is not suitable for microalgae because of the overlap between auto-fluorescence of chlorophyll pigments and PI [14]. PI is not capable of passing through the membranes of intact cells, and therefore should not be able to stain live cells [28, 29], which explains why PI could not stain live *N. oculata.*

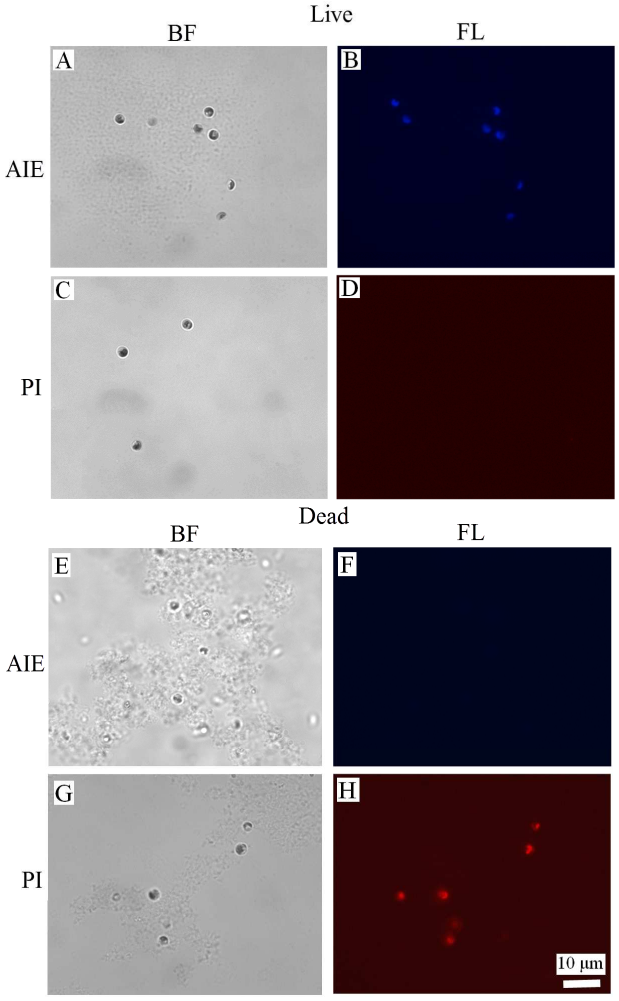

Fig. 2. (A, C, E, G) Bright-field (BF) and (B, D, F, H) fluorescent (FL) images of dead and live *N. oculata* stained with BSPOTPE (A, B, E, F) and PI (C, D, G, H) for 0.5 h in 200 μM BSPOTPE.

It is of interest to further explore the emission mechanisms in live *N. oculata* bound with BSPOTPE. The PL intensity was stable (within 300-350 a.u.) in a broad range of concentration of live *N. oculata* (Fig. 1C). Trypsin is a common enzyme that digests proteins into peptides and it only hydrolyses the peptide bonds in which the carbonyl group is contributed either by an arginine or lysine residue. BSPOTPE reduces fluorescence emission once it is combined with trypsin. The fluorescence was 157 a.u. prior to trypsin digestion and reduced to 128 a.u. after digestion by 1% trypsin for 2 h (Fig. 3A). Trypsin digestion reduced fluorescence by 18%. In contrast to trypsin, proteinase K is a broad-spectrum serine protease [28]. The predominant site of cleavage is the peptide bond adjacent to the carboxyl group of aliphatic and aromatic amino acids with blocked alpha amino groups. The fluorescence was 163 a.u. prior to proteinase K digestion and 28 a.u. after 0.5% proteinase digestion for 2 h. Proteinase digestion reduced fluorescence by 83%, suggesting that proteinase is stronger than trypsin in proteinlysis (Fig. 3B). Our data suggest that the fluorescence of live *N. oculata* may exist in cell protoplasm. We can thus assume that protoplasm proteins inside the cell are the main binding site of BSPOTPE, rather than the protein on the cell surface. The staining mechanism may be similar to neutral red which can be accumulated in the cytoplasm and/or vacuoles in plant cells [15].

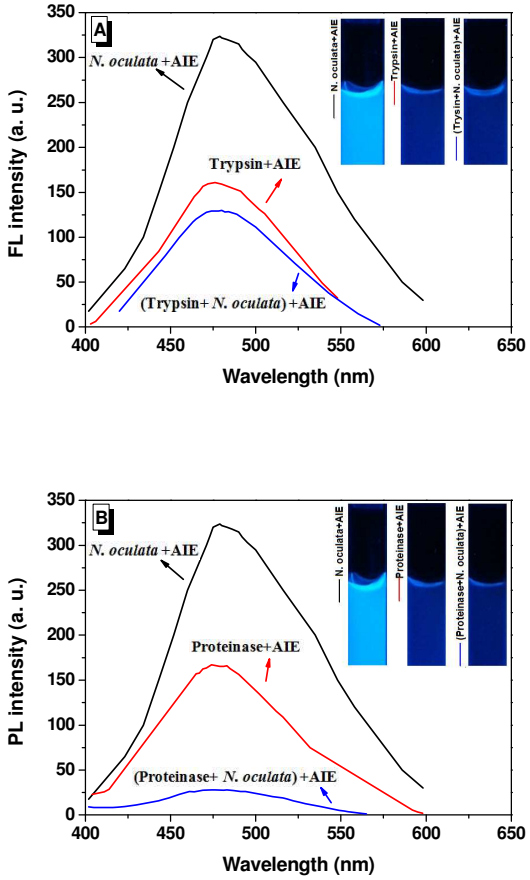

Fig. 3. Change of photoluminescence (PL) intensity in live *N. oculata* before and after (A) trypsin or (B) proteinase K digestion.

The BSPOTPE solution in PBS emitted dim luminescence at 390 nm in the absence of *N. oculata*. When the density of *N. oculata* was low in PBS, the BSPOTPE solution became luminescent. The FL intensity at 485 nm kept rising with an increase in the *N. oculata* concentration diluted with PBS. The rate of FL enhancement was fast at lower *N. oculata* concentrations and became almost constant at 20×10^6 *N. oculata*/ml (Fig. 4). At a *N. oculata*

concentration of 20×10^6/ml, the FL intensity was increased five-fold. The detection threshold was reduced to 0.2×10^6/ml of *N. oculata*. In the *N. oculata* range of $0.2\text{-}20\times10^6$/ml, the plot of FL enhancement as a function of *N. oculata* concentration is linear with a high correlation coefficient (r^2 = 0.989), indicating that the AIE luminogen can be used for a quantitative assay for *N. oculata* concentration in a PBS buffer.

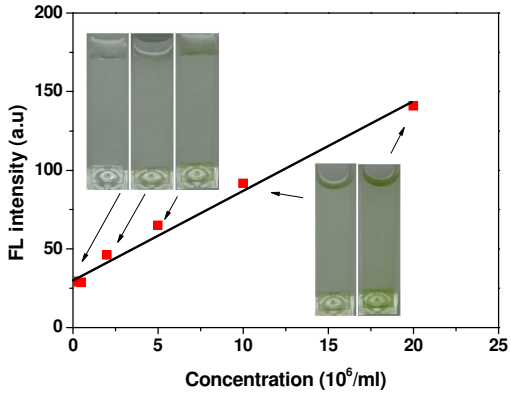

Fig. 4. Change in FL intensity at 475 nm at different *N. oculata* concentrations.

Cell density and chlorophyll *a* concentration are conventional measures for algal abundance but are insufficient to accurately reflect the physiological responses to ambient stress [30, 31]. For instance, algae may be deformed, decrease in cell size, or cease cell division in response to exposure to lethal metals or phytotoxic chemicals [30-32]. Furthermore, chlorophyll *a* concentrations may overestimate the viability of algae because of interference by other pigments (e.g. pheophytins) [33, 34]. This study contributes to understanding the accuracy, precision and utility of the BSPOTPE as a viability measure. Compared with commercial viability probes, this dye is highly emissive, photostable and ease to use, making it suitable for long-term microalgae viability assays. To our best knowledge, this is the first study reported using AIE fluorogen in microalgae. The great difference in fluorescence signals prompted us to conclude that BSPOTPE can be used for the *N. oculata* viability assay and may be employed as a biomarker for assessing the functional performance of other primary producers in aquatic ecosystems.

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