Photostabilization of Indocyanine Green Dye by Energy Transfer in Phospholipid-PEG Micelles

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Indocyanine Green (ICG) is one of the most common fluorescent dyes that emits in the near-infrared (NIR) region, with extensive use in the medical field. However, this dye is susceptible to photobleaching, thermal degradation and oxidation in acidic conditions. The major pathway by which ICG photobleaches involves sensitization to form singlet oxygen, which can react with the dye’s backbone, resulting in decomposition to non-fluorescent debris. In this paper we introduce the concept of using energy transfer (ET) for the protection of NIR dyes against photodecomposition. The dye IR-1061 was chosen as an ET pair due to its spectral overlap with ICG. First, it was shown that the presence of the former in solution reduced disintegration of the latter by absorbance and fluorescence spectroscopy. A singlet oxygen-reactive fluorescent indicator was employed to demonstrate that the production of this reactive species is also greatly reduced. This photoprotective effect was improved by encapsulation of the dyes in phospholipid-PEG micelles, which reduces the distance between them, thus enhancing the ET efficiency. The micelles were characterized for their optical properties and their size was determined to be about 10 nm with dynamic light scattering (DLS) techniques. The ET particles displayed greater fluorescence over 1000 nm compared to either dye encapsulated alone. The micelles proved to be superior than the free dye in terms of chemical, thermal and photo-stability. Moreover, the system demonstrated improved heating due to a greater photothermal effect compared to ICG dye in free or encapsulated form.

Keywords: FRET, polymer, fluorescence, NIR, ICG, nanoparticles

1. Introduction

The use of near-infrared (NIR) radiation in the field of non-invasive imaging for diagnostic applications is gaining increased scientific attention recently. This technique utilizes electromagnetic radiation in the NIR range to visualize living tissues with great temporal and spatial resolution. A great advantage of the use of NIR light is its low absorption by body tissues which allows for greater penetration in living subjects [1]. It is customary to divide the NIR range into three regions, or windows, whereas the first region resides roughly between 800 and 1000 nm [2]. The second and third windows are collectively known as “over-1000-nm NIR” (OTN-NIR) and offer very high penetration to living tissues, up to several centimeters in depth. One of the most common NIR dyes currently in use is indocyanine green (ICG), with absorption and emission in the first window, around 785 and 800 nm, respectively [3]. Other than its use for NIR
imaging, this dye is also used in photothermal therapy, generation of singlet oxygen for photodynamic therapy (PDT) and photoacoustic imaging [4,5]. ICG can be injected intravenously for angiography purposes due to the fact it has very low toxicity and its pharmacokinetics have been studied extensively [6]. ICG can also be easily integrated into nanoparticles (NPs), for example polymeric NPs have been prepared for photothermal- and chemo-therapy; and ICG-loaded ceramic/silica particles for up-conversion, photoacoustic and photothermal-therapy were reported [7,8].

However, this dye is highly unstable and is susceptible to photobleaching, thermal degradation and oxidation in acidic conditions [9]. The most probable route of ICG photodegradation involves sensitization to form reactive singlet oxygen \( \text{O}_2^\bullet \), which in turn reacts with the dye’s backbone, resulting in oxidative C-C cleavage [10]. Among the methods to reduce ICG deterioration are introduction of electron-withdrawing moieties; reduction of oxygen from the environment; addition of triplet quenchers; and isolation of the dye in nanoparticles [11–14].

Another tool commonly utilized in the field of bio-optical research is Förster resonance energy transfer (FRET) [15]. This physical process occurs when an excited fluorophore donor transfers its excess energy to an adjacent fluorophore acceptor and causes it to emit a photon. This energy transfer is non-radiative and occurs through dipole-dipole coupling, at distances of several nanometers. The efficiency of this process is proportional to the sixth power of the distance between the two molecules. This fact was previously exploited in our research to produce dye-integrated polymer that can change its emission profile upon application of external strain [16]. It has also been observed that FRET can decrease the donor’s excited-state lifetime and fluorescence quantum yield, thus reducing its photobleaching rate [17]. For example, Gadella et al. have used the distance-dependent reduction in donor photobleaching to assess the aggregation states of the epidermal growth factor receptor in epidermoid carcinoma cells [18]. In this research we take advantage of the photoprotective effect of the ET acceptor IR-1061 on ICG to prepare robust phospholipid-based micelles. The particles showed good chemical stability and reduced photobleaching, in addition to enhanced photothermal activity and excellent NIR emission.

2. Experimental

2.1. Materials

All materials were purchased from Wako Chemicals (Japan) unless stated otherwise. 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-Poly(ethylene glycol), MW=5000 (DSPE-PEG), was purchased from NOF Corporation (Japan). IR-1061 and ICG were purchased from Sigma-Aldrich (MO, USA).

2.2. Physical and optical characterization

Absorption was measured with a V-770 spectrometer (Jasco, Japan). Fluorescent emission in the visible and NIR ranges was measured with suitable spectrometers (FLAME-S-VIS-NIR-ES, Ocean Optics, USA and NIR-256-1.7, Avantes, Netherlands, respectively). Irradiation was done with 808 and 980 nm fiber-coupled laser diodes (FL-FCSE08-7-808, Focus Light, China and K976AB2RN-9.000W, BWT, China, respectively). Hydrodynamic diameter of micelles was measured by dynamic light scattering (LB-250, Horiba, Japan). Encapsulation efficiency was measured by absorption of dye in nanoparticles.

2.3. Detection of singlet oxygen

Singlet oxygen sensor green reagent (SOSGR) was purchased from Molecular Probes (OR, USA) and used according to the manufacturer’s instructions. Solution of 1 µM ICG with 2.5 µM SOSGR was prepared in 2 mL water and irradiated with 808 nm laser (1.5 W). The fluorescence of the solution was measured at different time intervals (excitation/emission = 495/525 nm) with RF-5300PC spectrofluorometer (Shimadzu, Japan).

2.4. Preparation of dye-loaded micelles

Dyes at different concentrations were added to DSPE-PEG solution (3.75 mg/mL) in tetrahydrofuran (THF) and slowly added to 9 mL of distilled water. The mixture was stirred in a dark environment for 12 hours, during which time the THF has evaporated and micelles were formed. The particles were then washed and purified by centrifuge filtration (MWCO 10 kDa, Merck, NJ, USA; 2600 g, 15 min, 3 times).

2.5. Stability and photothermal effect

Micelles were submitted to various chemical environments including PBS (10 mM, Thermo Fisher, MA, USA), NaOH and HCl solutions (10 and 0.1 mM, respectively) while being gently shaken at room temperature under dark conditions. Heating of samples to 80 °C was done in a sand bath
inside an oven (DOV-450, As One, Japan). Photobleaching was performed under irradiation at 808 nm (2.8 W) while monitoring fluorescence with a spectrometer. Results are shown as percent of original intensity. Heating of samples due to photothermal effect was done under irradiation at 808 nm (2 W) while stirring and measuring the temperature of the cuvette with a thermocouple (TC-2000, As One, Japan).

3. Results and discussion

3.1. Photoprotection of ICG in solution

The dye IR-1061 was chosen as a ET-protective agent due to the fact it has some spectral overlap with ICG. The emission and absorption spectra of both dyes are given in Fig. 1. It should be noted that the two dyes have opposite electrical charges, which might cause attraction between them, thus reducing the donor-acceptor distance. To assess the photobleaching of ICG alone, the dye was first dissolved in water and irradiated with an 808 nm laser. Figure 2A shows the decomposition of the dye, which is manifested in a decline of the absorption peak at 780 nm and an increase in the peaks of 310 and 380 nm. This is probably due to the photodecomposition of ICG into smaller, non-fluorescent molecules with absorption in the ultraviolet and visible range [10].

Experiments were also carried out in ethanol (EtOH) to rule out the possibility of IR-1061 absorbing and preventing some radiation from decomposing ICG. ICG alone or with IR-1061 was irradiated while the decline in fluorescence was monitored and charted in Fig. 2C. While ICG’s emission intensity decreases to 20% of its original one after 6 minutes, as more IR-1061 is added the trend is greatly slowed. Adding double or quadruple amounts of IR-1061 compared to ICG preserves 60% of emission after 6 minutes. This trend is also preserved in water (Fig. S2).

It is hypothesized that since the two dyes have a spectral overlap, that is ICG emits light at a frequency where IR-1061 can absorb it, non-radiative energy transfer can occur. This process is mainly manifested by the fact that the emission from ICG is reduced upon addition of its ET pair. However, it is possible that due to this energy transfer and subsequent reduction of excited state lifetime, the donor is less available to react with triplet oxygen to create the reactive singlet form. In order to monitor the creation of $1O_2$ by ICG in the presence and absence of IR-1061, a commercial singlet oxygen detection kit was used. Upon reaction with $1O_2$, the reagent (SOSGR) in the kit is oxidized to form a fluorescent molecule that emits...
light at 525 nm upon excitation at 495 nm. The integrated SOSGR signal is charted in Fig. 3 and demonstrates that the creation of singlet oxygen is reduced as bigger quantities of IR-1061 are added. For example, while the initial fluorescent signal is intensified by 90% when 1 µM ICG is irradiated in solution, addition of a similar amount of IR-1061 increases the signal by only 20%.

Fig. 3. Generation of 1O2 by ICG alone or in the presence of IR-1061 under irradiation at 808 nm (1.5 W). 2.5 µM SOSGR was left to react in aqueous solutions of the dyes and its fluorescence was measured at certain intervals. Results are shown as increase in percentage of integrated signal at around 525 nm. SOSGR alone served as negative control.

3.2. Synthesis of ICG/IR-1061 micelles

While ICG is fairly stable in pure water, IR-1061 quickly oxidizes and loses its fluorescence. In order to protect IR-1061 from the aqueous environment and keep it in a closer proximity to ICG, we set out to load the dyes in polymeric micelles. Dye-containing DSPE-PEG micelles were synthesized using the solvent evaporation method described before [19]. Shortly, dyes and DSPE-PEG 5000 were dissolved in tetrahydrofuran (THF) and added dropwise to water for overnight evaporation of THF. The micelles were then washed in water by centrifuge filter purification. The size distribution of the particles is given in Fig. 4A, revealing the diameter of the micelles is around 10 nm. Adjusting the quantity of each dye in the range of 2-30 µg did not change the size of the particles considerably (Table S1). The absorption spectra of the different dyes are given in Fig. 4B. It is evident that the absorption of IR-1061 encapsulated in micelles is much lower than ICG loaded in similar conditions. This is probably due to the intrinsic higher extinction coefficient of ICG, but also due to lower loading of IR-1061 in micelles [20]. The absorption spectrum of micelles prepared with both dyes resembled a superposition of the two former spectra, indicating no chemical changes upon incorporation of the two molecules together. Both dyes displayed a red shift of the major absorption peak by about 20 nm, an effect already observed in the literature [14]. In contrast, in an article published by Tao et. al IR-1061 was loaded into DSPE-PEG micelle with the addition of poly(acrylic acid) and the resulting absorption of the dye was markedly changed [21].

Fig. 4. A) Size distribution of dye micelles. B) Absorption of different micelles. All micelles prepared with 5 µg/mL dye and dispersed in water.

The encapsulation efficiency of each dye is given in Fig. 5 and it is evident ICG loading is much more efficient, with about 80% loaded when 15 µg dye is used. In contrast, the loading of IR-1061 is much lower, reaching 10% for the same amount of dye. This is due to the fact that while ICG has a known affinity to lipids, IR-1061 is more lipophobic and probably cannot inhabit the lipid core, but rather resides in the more oxygen-rich portion of the surfactant molecule [22]. It should be noted that the hydrophobic portion of the surfactant, where dyes can be incorporated, contributes to only 15% of the total mass, which might explain the low w/w % loading.

Fig. 5. Encapsulation efficiency of each dye given in overall percentage and as percent of surfactant weight (bars, left scale; and lines, right scale, respectively).
The emission spectra of the different particles in the first NIR and the OTN-NIR windows are given in Fig. 6. As was the case with absorption, both dyes display a red shift in their emission, where ICG’s peak shifts to about 820 and IR-1061’s to 1100 nm. It is also noted that both dyes display much higher emission than when dissolved in water. On the other hand, ICG displays stronger emission in less polar media compared to water, which is provided by the phospholipid core of the micelles [3].

The integrated intensity of micelles is charted in Fig. 7. The first notable feature is that ICG alone emits more strongly than with IR-1061. This is evidence of energy transfer where ICG loses some of its excitation energy to the acceptor dye. The formula for calculation of energy transfer efficiency ($E$) is given in Eq. 1, where $F_{DA}$ is the intensity of the donor’s fluorescence in the presence of the acceptor and $F_D$ without it [23]. In the case of 5 µg/mL of each dye the efficiency is 55%, but efficiency of more than 75%, which is characteristic to FRET phenomena, could be achieved when bigger amounts of dyes are used and the distance between donor and acceptor is reduced (Fig. S3). Conversely, the emission form IR-1061 alone by direct excitation with 980 nm light is much lower than the secondary excitation through ICG with 808 nm, indicating absorption and emission of the energy provided by the donor dye. It should be noted that it has been recently discovered that ICG has an emission component in the OTN window, which might contribute to the overall emission of ICG/IR-1061 micelles [24].

\[ E = 1 - \frac{F_{DA}}{F_D} \] (1)

3.3. Stability of dye micelles

While the encapsulation of dyes often renders them more resistant to various environmental conditions, the effect of IR-1061 addition was also evaluated [14]. First, micelles were tested for their durability in different chemical environments, shown in Fig. 8A. While ICG dissolved in water lost most of its fluorescence after 5 days in all 3 treatments, exposure to mildly alkaline conditions had the least effect on it. The encapsulation of ICG with IR-1061 greatly improved its stability in PBS and alkaline solution. In contrast, the acidic environment proved to be as devastating as it was to the dye in its free form. To circumnavigate this, ICG/IR-1061 micelles were prepared with addition of polyethylenimine (PEI), which is a polycationic polymer. This addition improved the dye’s stability in all 3 chemical environments. It is believed the polymer serves as a physical buffer with its amine moieties free to react with protons and protect ICG’s conjugated backbone from oxidation.

Next, the encapsulated dye was exposed to 80 °C for an hour and the change in its fluorescent emission upon irradiation was monitored. Figure 8B shows that while the free dye loses 35% of its initial intensity, the encapsulated dye alone or with the second dye loses only 20%. It is not surprising that the addition of IR-1061 had little influence on ICG’s stability, since this decomposition pathway doesn’t include excitation or change in the electronic state of the latter. As was the case with chemical stability, addition of PEI greatly improved the micelles’ robustness.

Most importantly, the encapsulation of the two dyes greatly improved ICG’s stability upon irradiation, as can be seen in Fig. 9. ICG in
micelles proved slightly more resilient to photodecomposition compared to the dye in free form, retaining 15% of its initial emission, compared to 5%. In contrast, the influence of IR-1061 addition was quite dramatic, causing it to retain almost 90% of initial fluorescence. The different protective roles of IR-1061 in photo- and thermal decomposition is consistent with the findings of Engel et. al that the two degradation mechanisms follow a different route [10]. This stark photoprotective effect is yet another proof of the efficient ET between the two dyes, which is intensified when the two dyes are in proximity inside a particle measuring at about 14 nm. It should be noted that the actual size the dyes occupy inside the hydrophobic core is much smaller than the overall hydrodynamic diameter measured by DLS. Since the calculated length of the extended PEG chain composes about 85% of the overall length, it is safe to assume the dye molecules reside within an area of less than 5 nm in diameter, making the distance between donor and acceptor molecules suitable for FRET.

One final aspect of the stabilizing consequence of IR-1061 addition was its contribution to heat generation by ICG. ICG has already been used as a photothermal agent in the experimental treatment of cancer, but its use can be limited by its short lifetime due to photo- and thermal decomposition [7,14]. To determine whether the addition of an acceptor dye improves ICG’s heat generating performance, free ICG, ICG- and ICG/IR-1061 micelles were irradiated while the temperature was measured with a thermocouple. Figure 10 shows the rise in temperature of the different treatments during 10 minutes of irradiation. In the case of the dye dissolved in water, the bigger the concentration, the longer time heat is generated. For all dye quantities the heating behavior can be divided into two periods: an initial one when heat rises at a rate between 2.5-4 °C/min and a second where the rate slows down to about 0.2 °C/min. However, while 20 µg/mL free dye and 10 µg/mL ICG in micelles behave similarly and decrease their heating activity after about 5 minutes, ICG with IR-1061 in micelles keeps heating for over 7 minutes. Additionally, the latter slows down to 1 °C/min, which is a significantly faster rate than the others’ second heating stage. Overall ICG/IR-1061 micelles increased the surrounding temperature by 30° C in 10 mins, compared to only 20 °C for double the amount of free ICG. This is another evidence of the increased stability of ICG in this ET micellar system which allows it to keep generating heat without decomposing.

4. Conclusion

In this research we set out to improve the stability of ICG by introducing the compatible ET dye IR-1061. Addition of the latter in solution was shown to increase ICG’s resistance to photodecomposition.
This is believed to be due to energy transfer from the exited ICG to IR-1061 which shortens its excitation life-time, thus preventing it from reacting with oxygen and creating destructive singlet oxygen. This was confirmed by directly demonstrating that the introduction of IR-1061 greatly reduces the rate of production of this reactive oxygen species. In order to improve this protective effect, dyes were encapsulated in DSPE-PEG micelles. These particles proved to have superior emission in the OTN region compared to each dye in its free form. Of special interest was to increase the overall robustness of ICG and it was shown that not only micelles proved extremely resistant to photobleaching, but also their chemical and thermal stability were greatly improved. Finally, the heat generation of the system was shown to be better than ICG alone, either in micellar or free form.

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