Effect of Doxorubicin on the Near-Infrared Optical Properties of Indocyanine Green

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ABSTRACT: In recent years, chemo-photothermal therapy (chemo-PTT) has been extensively studied for the upgradation of cancer treatment. The combined therapeutic approach reduces the overall cytotoxicity and enhances the therapeutic effect against the cancerous cells. In chemo-PTT, Indocyanine green (ICG) dye, a near-infrared chromophore, is used for PTT in combination with doxorubicin (DOX), a chemotherapeutic drug. ICG and DOX work very efficiently in synergy against cancer. However, the effect of DOX on the optical properties of ICG has not been studied yet. Here, for the first time, we report the effect of DOX on the optical properties of ICG in detail. DOX interacts with ICG and induces the aggregation of ICG even at a low concentration. The coincubation of both the molecules causes H and J aggregations in ICG. However, the J aggregation becomes more prominent with an increasing DOX concentration. These findings suggest that the optical properties of ICG change upon incubation with the DOX, which might affect the efficacy of PTT.

1. INTRODUCTION

Cancer has multiple root causes, and its progression pattern varies atypically.1−3 This unpredictability in its inception and molecular alteration of the pathways throughout its progression makes the treatment very strenuous.1,4 Some therapies such as radiation, photothermal, immuno, and chemo show remarkable results in managing the disease. However, they fall back in the effectiveness due to multiple drug resistance, toxicity, and so forth.5−10 In this context, the combined photothermal and chemotherapy approach via a delivery system shows promising therapeutic potential against cancer.11,12 Photothermal therapy (PTT) uses a photosensitizer to generate heat upon laser irradiation, which locally kills the cancerous cells effectively. In PTT, the photosensitizer absorbs energy irradiated from the laser source and converts it into thermal energy, creating local hyperthermia at the targeted region. However, the PTT alone is ineffective against treating metastatic cancers and has not shown any inhibition of recurrence of cancer.13,14 On the other hand, chemotherapy kills the fast-growing cells of our body and is found to be effective against both primary and metastatic cancers.15 Currently, chemotherapy is one of the most effective ways to prevent the rapid proliferation of cancerous cells. However, the chemotherapeutic drugs show a fast clearance from the cellular system, which reduces the effective dosage.16

A combined photothermal and chemotherapy would destroy the tumor locally and remove the metastatic cancer cells more efficiently.7,10 Therefore, the current progress in combined PTT and chemotherapy has gradually overcome individual therapy’s limitations. A near-infrared (NIR) chromophore, Indocyanine green (ICG), and doxorubicin (DOX), a chemotherapeutic drug, have been used for combined PTT and chemotherapy to combat cancer more efficiently.7,16 ICG produces a photothermal effect upon irradiation by a NIR wavelength laser (~808 nm), which leads to the killing of the targeted cells leaving the non-targeted cells unaffected. In the absence of ICG, the NIR laser irradiation remained non-destructive, and no detectable photothermal damage occurs to the targeted tissue.7,14 On the other hand, DOX is an anthracycline antibiotic used for chemotherapy against various cancers, such as acute lymphoblastic leukemia, liver cancer, kidney, acute myeloblastic leukemia, and so forth.7,17 DOX is provided intravenously (IV); it acts as an intercalating agent, binds to the DNA duplex or tRNA, and prevents further macromolecular biosynthesis.7,18−21 In addition, DOX also inhibits topoisomerase II progression, an enzyme that relaxes supercoils in DNA for transcription.7,18−21

Several studies have reported the codelivery of ICG and DOX via nanoparticle-mediated drug dye delivery systems for combined anticancer therapy. Lipid polymer [PLGA–
lecithin—poly(ethylene glycol) (PEG)-based nanoparticles,\textsuperscript{16} polydopamine ICG—PEG nanocarriers loaded DOX,\textsuperscript{25} liposome-coated thermo-sensitive nanogel [poly(N-isopropylacrylamide-co-acrylamide)],\textsuperscript{26} poly(γ-glutamic acid)-γ-poly (lactic-co-glycolic acid) (γ-PGA-g-PLGA)-based polymeric pH-sensitive nanocarrier,\textsuperscript{27} and phospholipid-calcium-carbonate-based hybrid nanoparticle (PL/ACC-DOX&ICG)\textsuperscript{7} were fabricated for the codelivery of ICG and DOX to the targeted cancer site. These nanocarriers showed remarkable anticancer effects and helped to bring down the effective DOX concentration for cancer treatment upon studying the photothermal properties of ICG has never been studied, which is crucial for combined therapy. However, the effective drug concentration to reduce the side effects to many folds\textsuperscript{7,16,25–27} These studies suggest that the combined PTT-chemotherapy became more effective and efficient compared to single therapies (PTT or chemotherapy) against cancer.

The recent inclination of researchers toward the DOX—ICG-based nanocarrier confirms the immense potency of this combined therapy. However, the effect of DOX on the optical properties of ICG has never been studied, which is crucial for studying the photothermal effect of ICG. In this paper, for the first time, the effect of DOX on the optical properties of ICG has been studied in detail. Our study would help the researchers and clinicians to optimize the DOX-ICG concentrations for achieving the best outcome of combined Chemo-PTT. It was observed that DOX interacts with ICG and induces the aggregation of ICG even at a low concentration. Furthermore, the incubation with DOX causes both H and J aggregations in ICG. However, J aggregation becomes more prominent with increasing DOX concentration and shows the emergence of a new absorption peak in the NIR region (at ∼835 nm). This emergence of the third peak at ∼835 nm for ICG in the presence of DOX might help us with deeper tissue theranostics due to a significant absorption at a higher wavelength than free ICG. Thus, our findings of change in the NIR optical properties of ICG upon incubation with the DOX can extensively be used to increase the efficacy of ICG—DOX-based chemo-PTT applications toward cancer cell eradication.

2. RESULTS AND DISCUSSION

Figure 1 represents the absorbance spectra of free ICG and DOX in an aqueous solution. DOX shows absorption in the visible range with absorption maxima at ∼480 nm. ICG shows two distinct characteristic absorption peaks at ∼720 and ∼780 nm. No overlapping of two spectra (DOX and ICG) was observed in the 600–900 nm range. Figure 2a shows the absorption spectra of free ICG (4 μM) and in combination with DOX at different molar concentrations (2, 5, 10, 15, and 20 μM). An increase in the absorption peak of DOX at ∼480 nm is observed with the increasing concentrations of DOX in the samples. It could be seen that in free ICG, the ∼720 nm peak had a lower value in comparison to the ∼780 nm peak, which with increasing the concentration of DOX at a fixed ICG concentration becomes more intense (an increase in 720 nm and a decrease in the corresponding ∼780 nm absorption peaks are observed upon dose-dependent addition of DOX). This suggests the aggregation of ICG with an increasing concentration of DOX in the samples. At higher DOX concentrations, the ICG absorption bands also show the peak widening and peak shifting. Specifically, a blue shift is observed in ∼720 nm peak with increasing DOX concentration (highlighted by the brown bar), which predominantly represents the H aggregation in ICG molecules. Similarly, a red shift was observed in the ∼780 nm peak (highlighted by a blue bar) due to the J aggregation of ICG molecules. In addition to these spectral changes, a new absorption peak at ∼835 nm (shown with the gray bar) is also observed at the higher DOX concentrations, which was not prominently visible at a lower concentration of DOX (2 μM). This new absorption peak at ∼835 nm also suggests the formation of the J aggregation of ICG molecules in the presence of DOX, which becomes prominent beyond 5 μM concentration of DOX in the aqueous solution of ICG (4 μM). Earlier, it was reported that the aggregation in ICG was a function of concentration,\textsuperscript{28} that is, ICG undergoes aggregation at a higher concentration.\textsuperscript{29} However, our results suggest that increasing the DOX concentration induces the aggregation of ICG molecules even at low concentrations. Figure 2b shows the change in the absorption ratio of 780 and 720 nm peaks of ICG with respect to increasing concentrations of DOX. A decline in the peak intensity of the 780 nm peak in comparison to the 720 nm peak of ICG (purple line) is seen with an increase in the DOX concentration, which gets stabilized at the higher concentrations of DOX. However, in Figure 2a, a continuous increase in ∼835 nm peak intensity of ICG is observed with increasing DOX concentration. This confirms the increase in aggregation of ICG with the increase in the DOX concentration.

Furthermore, to acquire more information about the interaction of ICG with DOX at different molar concentrations, Gaussian peak fitting was done to the measured absorption spectra. Peak fitting was done in the 580–900 nm range. Figure 3a shows the peak fitting to the ICG absorption curve. The absorption curve of the free ICG could be fitted with two Gaussian peaks with the intensity maxima at 726 and 782 nm, respectively. Figure 3b, c shows ICG absorption curves’ peak fitting with DOX concentrations of 10 and 20 μM, respectively. The Gaussian peak fitting affirmed the contribution of the three individual peaks in the absorption spectra of ICG when DOX was added to it at different molar concentrations. The deconvolution of the peak confirms the emergence of a new NIR absorption peak at ∼835 nm, which was absent in free ICG. The blue shift of ∼15 nm was observed in the ∼726 nm deconvoluted peak and a red shift of ∼10 nm
was observed in the \(\sim 782\) nm deconvoluted absorption peak of ICG when 20 \(\mu\)M DOX was mixed.

Similarly, the Gaussian peak fittings were done for all the samples, and the shift in the peak positions was calculated using the peak position of the fitted curves. Figure 4 shows the variation in the peak position of ICG with varying concentrations of DOX. Figure 4a shows the change in peak positions of \(\sim 720\) nm (blue curve) and \(\sim 780\) nm (red line) peaks of ICG after the addition of different molar concentrations of DOX. It shows that the \(\sim 780\) nm peak is red-shifted linearly with the increasing concentration of DOX in the sample and the \(\sim 720\) nm peak of ICG shows a blue shift. The blue shift in the \(\sim 720\) nm peak changes gradually till 10 \(\mu\)M concentration of DOX and gets saturated beyond this concentration of DOX (\(>10\) \(\mu\)M). This result suggests that the DOX quickly triggered H aggregation in ICG, which slowed down beyond 10 \(\mu\)M concentration of DOX.

Further to delineate the effect of solvent on the newly discovered DOX-induced aggregation in ICG, the experiment was reconducted in phosphate-buffered saline (PBS) at three different pH values (5.8, 7.0, and 8.0) values. As reported by Björnsson et al., ICG undergoes decomposition below pH 5 and above pH 11, but no changes are observed in the pH 6.0–8.0 range. Therefore, S, 7.0, and 8.0 pH values were chosen to study the effect of pH on the DOX-induced aggregation of ICG.
IGC. Figure 6 represents the absorption spectra of free ICG (4 μM) and ICG in combination with DOX at different molar concentrations (2, 5, 10, 15, and 20 μM) in PBS at different pH 5.8 (Figure 6a), 7.0 (Figure 6b), and 8.0 (Figure 6c). DOX-induced H and J aggregations of ICG were observed under all three pH conditions, similar to the experiment conducted in Milli-Q water. An increase in the absorption peak of DOX at ∼480 nm was observed with the increasing concentrations of DOX in the samples. At the same time, a decrease in the ∼780 nm absorption peak and a small increase in the ∼720 nm absorption peak intensities were observed with increasing concentrations of DOX in ICG. The blue and red shift of the ∼720 and ∼780 nm peaks, respectively, was observed in the ICG absorption spectra upon addition of DOX at ∼480 nm was observed with the increasing concentrations of DOX in the samples. At the same time, a decrease in the ∼780 nm absorption peak and a small increase in the ∼720 nm absorption peak intensities were observed with increasing concentrations of DOX in ICG. The blue and red shift of the ∼720 and ∼780 nm peaks, respectively, was observed in the ICG absorption spectra upon addition of DOX, in PBS at all the selected pH 5.8, 7.0, and 8.0. However, no peak shift or the emergence of a new peak was observed in the case of free ICG at all the pH values. A similar behavior was observed for free ICG, as shown in Figure 2, where ICG was dissolved in the DI water. Also, the emergence of a new absorption peak at ∼835 nm was observed in ICG at the higher DOX concentrations (in all the different pHs), suggesting the aggravation of the J aggregation of ICG molecules. This result indicates that the observed aggregation is independent of the solvent and pH of the solvent.

Furthermore, Gaussian peak fitting was carried out to the measured absorption spectra to confirm the aggregation of ICG molecules. Peak fitting was done in the 580–900 nm range. Figure 7a,d,g shows the peak fitting of the free ICG absorption curve at pH 5.8, 7.0, and 8.0, respectively, in PBS. The absorption curve of the free ICG could be fitted with two Gaussian peaks with the intensity maxima at 723 and 782 nm (Figure 7a), 723 and 782 nm (Figure 7d), and 726 and 782 nm (Figure 7g) at pH 5.8, 7.0, and 8.0, respectively. Figures 7b,e,h shows the peak fitting of ICG absorption curves with 10 μM DOX at pH 5.8, 7.0, and 8.0, respectively. Figure 7c,f,i shows the peak fitting of ICG absorption curves with 20 μM DOX at pH 5.8, 7.0, and 8.0, respectively, in PBS. The Gaussian peak fitting and deconvolution of the peak affirmed the contribution of the three individual peaks in the absorption spectra of ICG when DOX was added to it at different molar concentrations. At pH 5.8, a blue shift of ∼10 nm and a red shift of ∼3 nm were observed in 723 and 782 nm deconvoluted absorption peaks of ICG (4 μM) when 20 μM DOX was mixed with it. At pH 7.0, a blue shift of ∼12 nm and a red shift of ∼6 nm were observed in 726 and 782 nm deconvoluted absorption peaks of ICG when 20 μM DOX was mixed with it. At pH 8.0, a blue shift of ∼10 nm and a red shift of ∼6 nm were observed in 723 and 782 nm deconvoluted absorption peaks of ICG when 20 μM DOX was mixed with it. As discussed previously, this blue and red shift of ∼15 nm and ∼10 nm, respectively, was observed in ICG with 20 μM DOX, when the samples were prepared in water (Figure 3c). In all the abovementioned cases, the emergence of a new NIR peak at ∼835 nm was evidenced (absent in free ICG), which delineated the effect of pH and affirmed the role of DOX in the aggregation of ICG.

Figure 8 shows the fluorescence emission spectra of ICG upon 680 nm excitation at three different pHs. The emission spectra were recorded in the wavelength range from 735 to 900 nm. Similar to the observation made in Figure 5a, quenching in the characteristic fluorescence peak intensity of ICG was
observed with the increasing concentration of DOX at different pHs, 5.8 (Figure 8a), 7.0 (Figure 8b), and 8.0 (Figure 8c). No shifting in the emission peak was observed. This observation defined the role of pH and solvent in the quenching of the ICG emission upon the addition of DOX. ICG, like other cyanine dyes, has a tendency to self-aggregate at higher concentrations. This paper reports the aggregation in ICG at a lower concentration in the presence of DOX. This might be due to an active interaction between ICG and DOX. Furthermore, we have shown that the incubation of DOX with ICG causes both H and J aggregations in ICG, where J aggregation is more prominent. Thus, the NIR optical properties of ICG change upon interaction with DOX, which might help in improving the deeper tissue theranostics.

3. CONCLUSIONS

In the present work, the influence of DOX on the NIR optical properties of ICG was investigated. It was found that DOX alters the absorption–emission behavior of ICG molecules in an aqueous solution significantly, even at a lower concentration. The absorption spectra showed that DOX induces both the H and J aggregation of ICG, respectively, even at a low concentration of ICG molecules (4 μM). Furthermore, a new absorption peak in the NIR region at ∼835 nm was observed upon the addition of DOX, which is not reported yet. This emergence of a new peak at ∼835 nm and aggregation is independent of pH and the solvent properties. The fluorescence emission study also confirmed the active interaction between the ICG and DOX molecules, which reduces the fluorescence intensity due to molecular aggrega-
The overall findings suggest that the NIR optical properties of ICG change upon the incubation with DOX, which might enhance the efficacy of PTT in ICG−DOX-mediated combined chemo-PTT.

**4. MATERIALS AND METHODS**

**4.1. Materials.** Indocycanine Green (ICG) was purchased from Sigma-Aldrich chemicals Pvt Ltd (21 980, >99% purity, St. Louis, USA). High-performance liquid chromatography
grade DOX hydrochloride (>95.0% purity) was obtained from Tokyo Chemical Industry Co. Ltd. (TCI, D4193, >95% purity, Japan). NaCl, KCl, Na2HPO4, KH2PO4, HCl, and NaOH were purchased from Merk (Germany). Ultrapure Milli-Q water (18.2 MΩ) was used to prepare the samples.

4.2. Sample Preparation. The ICG and DOX stock solutions were prepared using Milli-Q water with concentrations of 0.5 mg/mL (645 μM) and 1 mg/mL (1.75 mM), respectively. Stock solutions were stored in amber-colored microcentrifuge tubes (MCT) at −80 °C temperature for further use. Different sets of samples were prepared by mixing DOX at different molar concentrations while keeping the ICG concentration constant (4 μM) in all samples. The concentrations of DOX, added to ICG in different samples, were 2, 5, 10, 15, and 20 μM, respectively. ICG and DOX were mixed in MCT for 10–15 s and then the final volume was made up to 1 mL by Milli-Q water for each sample to maintain the concentrations mentioned above. Four micromolar (4 μM) ICG was taken as the control sample. All samples were freshly prepared in triplicate and were kept at 4 °C temperature.

In a similar fashion, the replica sample sets were carried out in the phosphate buffer saline (PBS) solution at three different pHs (5.8, 7.0, and 8.0). 1 X PBS buffer containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 1.8 mM KH2PO4 was prepared, and the required pH (5.8, 7.0, and 8.0) was achieved with the help of HCl and NaOH. PBS was selected as a buffering agent for its wide buffering range and because it contained NaCl. For sample preparation, DOX at varying molar concentrations, 2, 5, 10, 15, and 20 μM, was added to the fixed final ICG concentration (4 μM) to prepare different sample sets. ICG and DOX were mixed in MCT for 10–15 s and then the final volume was made up to 1 mL by 1X PBS for each sample to maintain the concentrations as mentioned above. Four micromolar (4 μM) ICG in PBS was taken as the control sample. All samples were freshly prepared in triplicate at a different pH range of PBS (5.8, 7.0, and 8.0) and were kept at 4 °C temperature.

4.3. Optical Spectroscopy Measurements. The absorption spectra of the samples were measured using a PerkinElmer Lambda-35 UV–Vis–NIR spectrometer. The spectra of all the samples were recorded in the 410–920 nm wavelength region with a fixed slit width of 1 nm. The scanning speed was kept at 480 nm/min for all the measurements. Ultrapure Milli-Q water was taken as a reference as the samples were prepared in it. Fluorescence emission study was done using a Fluorolog-3 (FL3-21), Jobin Yvon, spectrophuorimeter. In this spectrofluorimeter, a 450 W xenon lamp was used as the excitation source. For the fluorescence experiment, all the samples were freshly prepared and all the measurements were done immediately within 5 min of sample preparation. Fluorescence spectra were collected in the wavelength region of 735–900 nm with an excitation wavelength of 680 nm in a right-angle geometry and both the absorption and emission slit widths were 5 nm. To study the effect of DOX on the NIR excitation and emission of ICG, the EEM was measured. For EEM measurements, the samples were excited from 650 to 780 nm with an increment of 10 nm, and the emissions were collected in the wavelength range of 680–950 nm with an increment of 1 nm. Rayleigh masking of first order was enabled with a 10 nm Rayleigh masking slit width fixed. Absorption and emission spectra were analyzed using Origin software.
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