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Indocyanine Green-Coated Polycaprolactone Micelles for Fluorescence Imaging of Tumors

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Supporting Information

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TEM images of free ICG and free PCL (MW 14 K), ICG-PCL (MW: 5 K) micelle characterization, lyophilization effect on micelles size and fluorescence property, micelle cellular uptake, and H&E-stained organ sections (PDF)

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Abstract

Recently, near-infrared (NIR) fluorescent dyes such as indocyanine green (ICG) have received tremendous interest as contrast agents for use in fluorescence-guided, intraoperative cancer resection surgery. However, despite showing great promise, ICG has many shortcomings such as rapid clearance and poor tumor accumulation. To improve the selective accumulation of ICG within tumors, numerous groups have formulated ICG into nanoparticles, but these approaches can suffer from rapid leakage of ICG, use of materials that exhibit poor or incomplete excretion, or complex chemistries that are not easily amenable to scale up for clinical use. Here, we developed a simple one-step method to prepare ICG-based fluorescent micelles that are composed solely of unmodified ICG and polycaprolactone (PCL), two clinically used materials with well-characterized safety profiles. The ICG-PCL micelles are prepared via oil-in-water emulsions, and the resulting micelles exhibit a uniform size, good reproducibility, and high loading efficiency. In vivo fluorescence imaging demonstrated that the ICG-PCL micelles led to a significant improvement in the accumulation and retention of ICG, in four different tumor models, compared with free dye, making them an attractive option for image-guided surgery.

Keywords

indocyanine green; polycaprolactone; fluorescence imaging; accumulation; retention

INTRODUCTION

Surgical resection is one of the primary treatment approaches for patients with tumors. The main purpose of surgery is to remove all of the visible cancer tissues. Although the primary tumor mass is often readily detectable during surgery, the tumor margin can be difficult to identify.¹ Visual inspection and manual palpation are often used by surgeons in most common surgical procedures. Unfortunately, residue cancer cells are left in a significant portion of tumor resections, which could cause local recurrence.^{2,3} Intraoperative pathologic evaluation of the margins may aid in the determination of the microscopic status of tumor margins; however, this approach has not been proven to be of clinical utility in terms of sensitivity, specificity, timeliness, and cost.⁴ The current standard for margin assessment still remains postoperative histopathology.⁵ Therefore, real-time intraoperative oncologic imaging with tumor-targeted imaging agents could offer the possibility to more accurately identify the tumor margin, increase the likelihood of complete resection, and reduce the chance of tumor recurrence.⁶

X-ray, magnetic resonance imaging (MRI), computed tomography (CT), and ultrasound have been considered in assisting surgical procedures,^{7–11} but these methods have several shortcomings for intraoperative applications such as low sensitivity, poor specificity, long time for imaging, and risk of ionizing radiation exposure. In recent years, NIR imaging methods have attracted great interest for use in real-time optical imaging during surgery because of short imaging time, use of non-ionizing radiation, relatively low equipment costs,

portability, and intuitive operation.⁶ There have already been numerous clinical trials on the use of the FDA-approved ICG for image-guided tumor resection.^{12,13} By using an NIR imaging system, the fluorescence signal within the tumor after injection of ICG could be easily identified from the surrounding healthy tissue. However, free ICG was found to have low specificity for many tumors in its ability to differentiate tumor from surrounding inflammatory tissue, and redistribution (or clearance) of free ICG during tumor resection was found.^{14–16} In addition, the circulation half-life of ICG is only 150–180 s,¹⁷ which can limit the amount of ICG delivered to the tumor site and therefore provide poor contrast enhancement. Thus, it is highly desirable and clinically important to improve currently used approaches for intraoperative image-guided surgery to improve tumor contrast and margin delineation.

Due to the unique pharmacokinetics and large quantity of dyes inside the nanometer-sized particles compared with free dyes, it is expected that NIR fluorophore-loaded nanoparticles can achieve sufficient sensitivity and selectivity for intraoperative molecular imaging. It has been found that fluorescently labeled nanoparticles (e.g., Cy5-labeled iron oxide nanoparticles) can not only provide accurate demarcation of the tumor margin but can also limit diffusion through the interstitial space during surgery.¹⁸ Recently, there has been a growing interest in the development of ICG-loaded nanoparticles for biomedical applications, including lipid-, polymer-, magnetic-, and silica-based nanoparticles.¹⁹ Our recent work showed that the image-guided surgery with ICG-incorporated superparamagnetic iron oxide nanoparticle (SPION) nanoclusters (NCs) led to an improvement in gross tumor resection and significantly increased survival rates of tumor-bearing mice.²⁰

Although most ICG-loaded nanoparticles utilized in pre-clinical studies can solve many problems of using free ICG, most of these nanoparticles still face many challenges in their clinical translation, including biocompatibility and safety, large-scale manufacturing, and government regulations, etc. To address this issue, we used a new approach to prepare ICG-based nanoparticles that consist solely of two clinically used materials with well-characterized safety profiles, ICG, and polycaprolactone (PCL). PCL is one of the most used synthetic biomaterials due to its biocompatibility and biodegradation, which is approved by the Food and Drug Administration (FDA) for use in humans. Moreover, these micelles are prepared via a simple one step oil-in-water emulsion method that is scalable. These micelles were fully characterized in terms of their imaging contrast enhancing capabilities in multiple solid tumor models.

MATERIALS AND METHODS

Materials

Indocyanine green (ICG) and polycaprolactone (PCL) with different molecular weights (5, 14, 45 K) were purchased from Sigma-Aldrich. Dulbecco's Modified Eagle Medium, streptomycin, penicillin, and heat-inactivated fetal bovine serum (FBS) were purchased from Gibco Life Technologies, Inc. All the buffer solutions were prepared with deionized water.

Synthesis of ICG-PCL Micelles

PCL (4 mg, different MW: 5, 14, or 45 K) was dissolved in 150 μL toluene, with the addition of ICG (4, 2, or 1 mg) in 50 μL DMSO, and then vortexed for 3 min. Next, the mixture of ICG and PCL was added into 4 mL of water followed by sonication until a homogeneous sample was formed. Prior to dialysis, the glass vial was placed in the dark overnight and left open to allow toluene to evaporate, since the toluene solvent is not compatible with the dialysis membrane. Afterward, the sample was transferred into a dialysis tubing (3500 MWCO, Fisher brand) and left in 4 L of water overnight to remove DMSO and most unloaded ICG from the sample. To further remove free ICG from the samples, the prepared ICG-PCL micelles were purified via Superdex 200 chromatography columns (GE Healthcare, Piscataway, NJ).

Characterization of ICG-PCL Micelles

The hydrodynamic diameter and size distributions of the ICG-PCL micelles were measured with dynamic light scattering (DLS, Malvern, Zetasizer, Nano-ZS). The morphology of the micelles was characterized using transmission electron microscopy (TEM) (JOEL 1010) using a negative-staining technique (i.e., phosphotungstic acid). To determine the loading efficiency of ICG, UV absorbance of ICG-PCL micelles were measured in DMSO at 795 nm. The ICG encapsulation efficiency and loading efficiency were determined using the following equations:

$$\begin{aligned} \text{encapsulation efficiency (\%)} \\ = \frac{\text{weight of ICG in the micelles}}{\text{weight of ICG in feeding}} \times 100 \end{aligned} \quad (1)$$

$$\text{loading efficiency (\%)} = \frac{\text{weight of ICG in the micelles}}{\text{weight of ICG-PCL micelles}} \times 100 \quad (2)$$

ICG-PCL Micelles Stability Studies

Synthesized ICG-PCL micelles were stored in water at 4 °C. The size of micelles was monitored by DLS over a 1 month period. UV absorbance and fluorescence spectroscopies of micelles were also determined over the same period of time.

Cell Culture

The lung cancer cell line A549, skin/epidermis cell line A431, mouse breast cancer cell line 4T1, and brain cancer cell line U251 (ATCC) were cultured in DMEM containing 10% FBS, supplemented with penicillin/streptomycin at 37 °C with 5% CO₂.

Cell Viability Assay

The lung cancer cell line A549 were seeded in 96-well plates and incubated overnight. The free ICG and ICG-PCL micelles were incubated with A549 cells at five different concentrations ranging from 200 to 6.25 $\mu\text{g mL}^{-1}$ (200, 100, 50, 25, 12.5, and 6.25 $\mu\text{g mL}^{-1}$), and the cell viabilities were determined. After 24 h incubation, the cells grew in 100 μL of DMEM with 10 μL of MTT assay stock solution added to each well. After 4 h incubation,

100 μ L of detergent was added to each well. Finally, the absorbance of the dissolved product was measured on a Tecan microplate reader (Tecan) at 570 nm. Cell viability was determined using the following equation:

$$\text{cell viability (\%)} = \frac{\text{absorbance}_{\text{sample}}}{\text{absorbance}_{\text{control}}} \times 100 \quad (3)$$

Cellular Uptake Measured by Fluorescence Microscopy

A549 cells were incubated with ICG-PCL micelles at an ICG concentration of 5 μ g mL⁻¹ in DMEM for 1, 4, 8, and 20 h. The cells were then washed with PBS three times to remove excess micelles. Microscopy images were taken with an Olympus IX81 motorized inverted fluorescence microscope.

Animal Studies

Animal investigations were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. Studies were performed with ~6 weeks old female nude mice (Charles River Laboratory, Charles River, MS, USA). Mice were anesthetized by isoflurane, and then, A549 cells were injected subcutaneously into the mice (2×10^6 cells in 0.1 mL of PBS). When the tumor volume reached about 160 mm³, ICG-PCL micelles or free ICG were administrated at a dose of 5 mg ICG per kg body weight by retro-orbital injection. Pre-contrast and post-contrast optical images at 0.5, 1, 2, 6, 8, 24, and 96 h were acquired with a Perkin Elmer IVIS Spectrum In Vivo System using the following imaging parameters: excitation, 745 nm; emission, 800 nm; exposure time, 3 s; binning, medium; and $f=2$.

For the biodistribution study, mice were sacrificed at 24 h post-injection. Heart, liver, spleen, lung, kidney, and tumor were collected for ex vivo imaging. The relative fluorescence intensity was processed using ImageJ software.

Hematoxylin and Eosin (H&E) Study

H&E study was performed by the pathologist from the PCMD Histology Core, Department of Orthopaedic Surgery, University of Pennsylvania.

RESULTS AND DISCUSSION

As shown in Figure 1, ICG-coated PCL micelles were synthesized by simply mixing ICG and the biodegradable polymer PCL (MW 14 K) in DMSO/toluene, adding them directly to deionized water, and sonicating to form microemulsion. The mixture was then purified by dialysis against water and next subjected to size exclusion column purification. There was no any additional polymers or agents included. The ICG was used as an amphiphilic solubilizing agent that coated the surface of the hydrophobic PCL core.

When the weight ratio (w/w) of ICG to PCL (MW 14 K) was 1:1, the generated ICG-coated PCL micelles were highly water-soluble, with an average size of ~42.41 nm and a polydispersity index (PDI) of 0.184 in water based on DLS measurements (Figure 2a). In

addition, ICG-PCL micelles observed by TEM were approximately spherical in shape (Figure 2b) and 30.55 ± 0.35 nm in diameter (Figure 2c). As a comparison, use of ICG or PCL alone did not form micelles (see Figure S1, Supporting Information), suggesting that ICG-PCL micelles were formed only in the presence of both ICG and PCL.

Next, the effect of the ICG to PCL (14 K) weight ratio (w/w) on the physical–chemical properties of the ICG-PCL micelles was further investigated. As the ICG to PCL weight ratio increased from 0.25 to 1, the ICG encapsulation efficiency increased from 8.5 to 52.8%, ICG payload increased from 1.7 to 26.4% (weight of ICG/total weight), and the hydrodynamic diameter decreased from 72.56 to 42.41 nm (see Table S1, Supporting Information). This size decrease may be due to the smaller PCL core sizes in the presence of higher amounts of the ICG. In all cases, the polydispersity index (PDI) was <0.2 . We also tested the effect of the molecular weight of PCL (5, 14, or 45 K) and the ICG:PCL ratio on the encapsulation efficiency, loading efficiency, and size of the ICG-coated PCL micelles. It was found that ICG-PCL (45 K) micelles had a larger size than that of ICG-PCL(5 K) or ICG-PCL (14 K) micelles (see Table S1 and Figure S2, Supporting Information), likely due to its higher molecular weight and increased hydrophobicity. As the ICG:PCL ratio decreased from 1 to 0.25, both ICG encapsulation efficiency and loading efficiency were reduced for all three species of PCL. ICG-PCL micelles prepared with ICG to PCL (14 K) at weight ratio of 1 were utilized for all subsequent studies.

The stability of ICG-PCL micelles was evaluated by monitoring UV absorbance, fluorescence, and micelle size for 30 days. Free ICG was used for comparison. UV-absorption spectrum showed that both free ICG and ICG-PCL micelles at an ICG concentration of 1 mg mL^{-1} were relatively stable (Figure 3a). However, the fluorescence signal of ICG and ICG-PCL micelles gradually decayed over time (Figure 3b). After 1 month, ICG-PCL micelles maintained ~50% of their initial fluorescence intensity when they were stored in water, while free ICG maintained $<10\%$ of their initial fluorescence intensity. DLS measurements showed that there were no significant changes in the hydrodynamic diameter and PDI when ICG-PCL micelles were incubated in water for 30 days (Figure 3c). To improve their stability in long-term storage, ICG-PCL micelles were subjected to lyophilization using sucrose as a lyoprotectant. Our results indicated that the rehydrated micelles from the freeze-dried samples had a similar size compared with the original formulation of ICG-PCL micelles (see Figure S3, Supporting Information) when aqueous ICG-PCL micelles were lyophilized in the presence of 30% sucrose. However, in the absence of sucrose, the ICG-PCL micelles lost most of their structural integrity after lyophilization. Moreover, there was no significant change in the fluorescence property of ICG-PCL micelles after lyophilization (see Figure S3b, Supporting Information).

Although both ICG and PCL are currently FDA-approved for clinical applications, their combination could result in toxicity. Therefore, the cytotoxicity of the ICG-PCL micelles was evaluated in an MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide) cell proliferation assay. Various concentrations of ICG-PCL micelles were incubated with A549 cells for 24 h. It was found that the ICG-PCL micelles (Figure 4) had no obvious cytotoxicity on cells, even at relatively high concentrations of ICG (up to $200 \text{ } \mu\text{g mL}^{-1}$). In contrast, free ICG exhibited distinct cytotoxicity (~15%) even at a concentration of $100 \text{ } \mu\text{g}$

mL⁻¹, likely due to aggregation because of the hydrophobic domains of ICG. We further investigated the cellular uptake of ICG-PCL micelles by A549 cells using a fluorescence microscope. It was found that the fluorescence intensity gradually increased over the course of 20 h, suggesting that ICG-PCL micelles are taken up by cells over time. (see Figure S4, Supporting Information).

To further confirm the contrast-enhancing capabilities of ICG-PCL micelles as NIR fluorescence imaging probes in vivo, A549 lung cancer cells were injected into the flank of athymic nude mice. When the size of A549 tumor reached approximately 160 mm³, mice were retro-orbital injected with ICG-PCL micelles or free ICG at a dose of 5 mg ICG/kg. Fluorescence images of mice implanted with A549 tumors were acquired pre-contrast and at various times after sample injection (Figure 5). In the pre-contrast images, there was little intrinsic fluorescence signal in the implanted A549 tumor. At 0.5 h following the administration of the ICG-PCL micelles, a contrast enhancement was observed within the A549 tumor (Figure 5a). However, there was also high background fluorescence from surrounding normal tissues and organs due to micelles still in circulation. In this case, the tumor margin was difficult to identify by NIR imaging. The signal enhancement within the tumor increased significantly by 24 h, while the background fluorescence decreased, allowing the boundary of the tumor to be clearly demarcated. The tumor was also visible at 24 h following the injection of free ICG; however, the signal-to-noise ratio (SNR) was significantly higher ($p < 0.05$) for ICG-PCL micelles compared to free ICG at all time points (Figure 5b). At 24 h post-injection, the SNR values were 9.2 ± 2.5 with ICG-PCL micelles compared to 3.4 ± 2.1 with free ICG. After 96 h, the tumor-bearing mice injected with free ICG expressed almost no fluorescence signal while the signal from the mice injected with ICG-PCL micelles was still visible, with an SNR value of 5.3 (Figure 5b).

To assess the accumulation of ICG in the various organs, mice were sacrificed 24 h post-injection of ICG-PCL micelles or free ICG. Different organs were collected and then imaged (Figure 6). The fluorescence intensity of ICG-PCL micelles in tumors was significantly higher than that of free ICG, further indicating that the accumulation of ICG-PCL micelles in tumors is much more efficient (2.21 times higher) than that of free ICG. All organs were further examined by histology. Hematoxylin and eosin (H&E) staining confirmed that ICG and ICG-PCL micelles were safe, with no obvious toxic effects in the heart, liver, lung, spleen, and kidney (Figure S5, Supporting Information).

To underscore the versatility of the ICG-PCL micelles and to further confirm their ability to generate improved fluorescence contrast in tumors following systemic administration, compared with free ICG, fluorescence imaging was also carried out in mice implanted with three other cancer types, A431 (epidermoid carcinoma), U251 (glioblastoma), and 4T1 (mammary carcinoma) tumors (Figure 7). In all cases, the ICG-PCL micelles exhibited a statistically significant enhancement in fluorescent intensity at the site of the tumor, both in tumor-bearing mice and in the excised tumors, compared with free ICG. Since ICG has been extensively investigated to assist the surgical resection including head and neck cancer, gastric cancer, lung cancer, and breast cancer,²¹ it is expected that the newly developed ICG-PCL micelles could have a significant impact on fluorescence tumor imaging applications in the clinic in the near future.

CONCLUSIONS

In conclusion, we have successfully developed a new type of contrast agent ICG-coated PCL micelles. These micelles are unique in that they consist solely of clinically used materials. In addition, the method for producing these micelles is simple, scalable, and reproducible. In vitro studies demonstrate that the micelles are biocompatible and non-toxic. In vivo studies show that the fluorescence intensity and retention time of ICG-PCL micelles are significantly better than free ICG in four different tumor models. Thus, this agent offers a unique opportunity and tremendous potential for image-guided surgery applications with a direct clinical impact.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Schematic diagram of indocyanine green (ICG)-coated polycaprolactone (PCL) micelles. Micelles were formed through the co-assembly of the PCL and ICG. Due to the amphiphilic nature of ICG, the ICG were able to solubilize the hydrophobic PCL core in aqueous solvents.

Figure 2.

Characterization of ICG-coated PCL (MW: 14 K) micelles. (a) Dynamic light scattering (DLS) of the ICG-PCL micelles. (b) Transmission electron microscopy (TEM) image of the ICG-PCL micelles. (c) Quantitative analysis of ICG-PCL micelle size, based on TEM images.

Figure 3. Stability of free ICG and ICG-PCL (MW, 14 K) micelles (1 mg mL⁻¹). (a) UV absorption and (b) fluorescence of free ICG and ICG-PCL micelles over time in water at 4 °C. (c) ICG-PCL micelles were incubated in water at 4 °C, and size and PDI were monitored.

Figure 4. Viability of A549 cells after incubation with increasing concentrations of ICG-PCL micelles and free ICG. Statistical analysis was performed using one-way ANOVA. *, $p < 0.05$ for free ICG versus untreated cells.

Figure 5.

(a) Representative fluorescent images of mice with sub-cutaneous A549 lung tumors.

Images were acquired pre-contrast and 0.5, 1, 2, 6, 8, 24, and 96 h post-administration of

free ICG or the ICG-PCL micelles at an ICG concentration of 5 mg kg⁻¹ body weight. (b)

Semiquantitative analysis of tumor fluorescence as a function of time in mice injected with ICG or ICG-PCL micelles.

Figure 6.

(a) Fluorescence images of various organs of nude mice 24 h after injection of ICG or ICG-PCL micelles. (b) Semiquantitative analysis of ICG fluorescence in different organs and tumors. Statistical analysis was performed using one-way ANOVA. *, $p < 0.05$ for ICG-PCL micelles versus free ICG. $n = 4$ per group.

Figure 7.

(a) Fluorescent images of mice with A431, U251, and 4T1 tumors 24 h after injection of ICG or ICG-PCL micelles at an ICG concentration of 5 mg kg^{-1} body weight. The tumor location is indicated by a dotted black circle (top row). Some fluorescence can also be seen in the liver and at the injection site (eye). Bottom row: fluorescence images of excised tumors 24 h post-administration of free ICG or the ICG-PCL micelles (bottom row). (b) Semiquantitative analysis of tumor fluorescence from in vivo studies. (c) Semiquantitative analysis of tumor fluorescence from excised tumors. Statistical analysis was performed using one-way ANOVA. *, $p < 0.05$ for ICG-PCL micelles versus free ICG. $n = 4$ per group.