Improving drug accumulation and photothermal efficacy in tumor depending on size of ICG loaded lipid-polymer nanoparticles

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A B S T R A C T

A key challenge to strengthen anti-tumor efficacy is to improve drug accumulation in tumors through size control. To explore the biodistribution and tumor accumulation of nanoparticles, we developed indocyanine green (ICG) loaded poly (lactic-co-glycolic acid) (PLGA)-lecithin-polyethylene glycol (PEG) core-shell nanoparticles (INPs) with 39 nm, 68 nm and 116 nm via single-step nanoprecipitation. These INPs exhibited good monodispersity, excellent fluorescence and size stability, and enhanced temperature response after laser irradiation. Through cell uptake and photothermal efficiency in vitro, we demonstrated that 39 nm INPs were more easily absorbed by pancreatic carcinoma tumor cells (BxPC-3) and showed better photothermal damage than that of 68 nm and 116 nm size of INPs. Simultaneously, the fluorescence of INPs offered a real-time imaging monitor for subcellular locating and in vivo metabolic distribution. Near-infrared imaging in vivo and photothermal therapy illustrated that 68 nm INPs showed the strongest efficiency to suppress tumor growth due to abundant accumulation in BxPC-3 xenograft tumor model. The findings revealed that a nontoxic, size-dependent, theranostic INPs model was built for in vivo cancer imaging and photothermal therapy without adverse effect.

1. Introduction

Indocyanine green (ICG), a tricarbocyanine dye with substantial absorption and fluorescence in the near-infrared wavelength region (NIR) and effective photothermal response, has been widely utilized as a probe for diagnostic and therapeutic applications [1,2]. However, the applications of ICG in photothermal therapy and NIR imaging were restricted due to unstable optical properties, quick degradation and clearance in living body [3]. Various nanocarriers have been developed to encapsulate ICG and implement its enhanced penetration and retention (EPR) effect and fluorescence stability in vivo [4,5].

Cancer nano-therapeutics is expected to solve limitation of conventional drug delivery system, such as improving drug accumulation in tumor tissue [6]. Recently, great progress has been achieved in improving drug pharmacokinetics, biodistribution and drug penetration in tumor tissue through versatile nanocarriers, considering multiple factors such as size, shape, surface charge and water solubility [7]. Therein, the size of nanomedicines shows a critical effect on passive targeting, and accumulation in tumor, which would influence their therapeutic efficacy [8,9]. Additionally, the size control is also important in poorly permeable tumors like pancreatic carcinoma and colon carcinoma, which would induce limited drug distribution in tumors [6,10,11]. It has been proved that inorganic and polymer NPs with big size only accumulated near the vasculature while NPs with small size could rapidly diffuse throughout tumor matrix and provide a better penetration effect [12–16]. Therefore, it was necessary and urgent to validate the size control for improving drug biodistribution in vivo and EPR effect with enhanced therapeutic efficacy.

In this study, we developed ICG-loaded polymer-lipid NPs (INPs) with three different hydrodynamic dimensions of 39 nm, 68 nm, and 116 nm via single-step self-assembly, which integrated near-infrared imaging and photothermal therapy properties of ICG. Their physiochemical characters were systematically evaluated. In vitro endocytosis, subcellular localization, in vivo metabolic distribution and accumulation were directly observed utilizing the NIR fluorescence of ICG. The cytotoxic effects of different INPs based on
drug uptake were also investigated and compared in BxPC-3 cells. Moreover, the photothermal efficacy of three types of INPs to BxPC-3 xenograft tumors was evaluated in vivo.

2. Experimental section

2.1. Chemicals and materials

The following chemical reagents and materials were used in our experiment. PLGA (MW = 5000–15,000 Da, [lactide acid]: [glycolic acid] = 50:50), indocyanine green (ICG), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), heme, and eosin were obtained from Sigma–Aldrich (USA). Soybean lecithin and 1, 2-di-octanoyl-sn-glycero-3-phosphatidylcholine (DOPC) were obtained from Avanti (USA). Hoechst 33258, from Invitrogen (USA). Amicon ultra-4 centrifugal filter with a molecular weight cutoff of 10 kDa was purchased from Merck Millipore (USA). RPMI 1640 medium, fetal bovine serum, trypsin EDTA, penicillin and streptomycin were obtained from GIBCO (USA). Rat tail collagen was purchased from ICN Biomedicals (USA). 488 Annexin V and Propidium Iodide (PI) were obtained from Invitrogen (USA). Amicon ultra-4 centrifugal filter with a molecular weight cutoff of 10 kDa was purchased from Merck Millipore (USA). In vivo experiments were performed in accordance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH). Mice were purchased from animal facilities in Beijing. The ICG biodistribution analysis was obtained at indicated time after injection using the ex/in vivo imaging system (Maestro, USA) with a 704 nm excitation wavelength and 735 nm filter to collect the fluorescence. The mice were sacrificed immediately and major organs were fixed immediately and major organs and tissue were examined in a histological microscope.
2.13. In vivo tumor photothermal treatment

After tumor model was performed and tumor sizes reached to 50 mm³, mice were divided into six groups (five per group). The mice were separately injected with 200 μL PBS, 200 μL PBS, 200 μL free ICG, 200 μL INP-1, 200 μL INP-2, and 200 μL INP-3. Total groups except the first one were irradiated by the 808 nm laser (0.8 W/cm²) for 10 min after drug squirt for 24 h, and the PBS-only group was set as control. The changes of tumor size and body weight of every mouse were recorded within 4 weeks. Mice with tumor volumes exceeding 600 cm³ would be euthanatized according to animal protocol, and the slow-growth and malignance of this tumor model remained above 60% (Fig. S2). These data indicated the stability of ICG was apparently improved when it was isolated from surrounding environment.

To further survey the photothermal efficiency in vivo, appropriate size sections of tumors at 12 h and major organs (heart, liver, spleen, lung and kidney) at 28 d after treatment were collected, and then were stained with hematoxylin-eosin (H&E) and examined by biological inverted microscope.

Table 1

Size distribution, surface charge, and drug encapsulating efficiency (EE) and loading efficiency (LE) of INP-1, INP-2 and INP-3. The data were shown as mean ± SD (n = 3).

<table>
<thead>
<tr>
<th>Particle type</th>
<th>Particle size</th>
<th>Surface potential</th>
<th>EE (%)</th>
<th>LE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>INP-1</td>
<td>39.4 ± 2.8</td>
<td>−53.6 ± 0.3</td>
<td>28.41 ± 0.05</td>
<td>6.71 ± 0.01</td>
</tr>
<tr>
<td>INP-2</td>
<td>67.9 ± 4.0</td>
<td>−52.8 ± 2.4</td>
<td>31.30 ± 0.29</td>
<td>7.39 ± 0.07</td>
</tr>
<tr>
<td>INP-3</td>
<td>115.8 ± 1.5</td>
<td>−50.9 ± 0.6</td>
<td>39.30 ± 0.19</td>
<td>6.73 ± 0.03</td>
</tr>
</tbody>
</table>

3. Results and discussion

3.1. Formulation and characterization of INPs

In order to investigate the size-dependent biodistribution in nude mice and drug accumulation behavior of INPs in pancreas carcinoma (BxPC-3) xenograft tumors, three types of INPs (INP-1, INP-2 and INP-3) were successfully developed according to previous works [17,18]. Afterward, diameter and surface potential of three INPs were measured by dynamic light scattering (DLS) and shown in Table 1. The average size of three INPs in water was 39.4 nm, 67.9 nm and 115.8 nm respectively, and average zeta potential were −53.6 mV, −52.8 mV and −50.9 mV. Transmission electron microscopy (TEM) was also utilized to investigate the morphology of three types of INPs. The images vividly demonstrated that INPs were divided into three types distribution and each type of INPs exhibited an excellent monodispersity (Fig. 1A).

The size of INPs was slightly small than corresponding DLS results because TEM images only gave the dehydration morphology of NPs, and DLS overestimate mean particle size due to high scattering intensities of large objects [19,20].

The size stability of three different INPs was investigated by DLS within 4 weeks. The diameter of INP-1, INP-2 and INP-3 after 4 weeks remained their size without evident variation. ICG fluorescence intensity (FL) of free ICG and three types of INPs was also detected by fluorescence spectroscopy for 30 d to evaluate the fluorescence stability of ICG. The results showed that the fluorescence of free ICG deceased to 13.4% compared to initial intensity, while the fluorescence of ICG in INP-1, INP-2 and INP-3 still remained above 60% (Fig. S2). These data indicated the stability of ICG was apparently improved when it was isolated from surrounding environment.

The drug encapsulation efficiency (EE) and drug loading efficiency (LE) were important parameters for clinical application [17]. The EE of ICG encapsulated in INP-1, INP-2 and INP-3 was 28.41%, 31.30% and 39.30%; the LE was 6.71%, 7.39% and 6.73% (Table 1). These results exhibited increased EE due to enhanced polymer weight fraction [21] and similar LE of three types of INPs.

To estimate whether the photothermal efficiency of ICG were affected by NPs, the temperature changing profiles in vitro was investigated by continuous laser irradiation. After 1.6 W/cm² laser irradiation for 8 min, the maximum temperature of free ICG, INP-1, INP-2 and INP-3 aqueous solution rose to 56.0 °C, 57.1 °C, 57.3 °C and 57.4 °C respectively, while PBS-only rose to 32 °C with comparative unapparent increase (Fig. 1B). Moreover, the maximum temperature in each group turned stable after 5 min irradiation. The infrared thermo-graphic images proved that the maximum temperature of PBS, free ICG, INP-1, INP-2 and INP-3 at
8 min was 31.9 °C, 55.9 °C, 57.4 °C, 56.8 °C, and 56.9 °C respectively (Fig. 1C). The temperature of INPs was rapidly increased at the first 100 s. Moreover, the photothermal efficiency of INPs was even slightly enhanced compared with free ICG at 5–8 min.

Drug release of three types of INPs was also explored due to its importance for drug efficiency and safety. ICG was released from INP-1, INP-2 and INP-3 with a biphasic profile, where an initial burst release was seen in the first 12 h and more than 50% of ICG were released, followed by a sustained and uniformed release (Fig. 1D). These results demonstrated that the INPs showed similar drug release rates, which was also supported by Cabral’s work [9].

### 3.2. In vitro cell uptake

Tuning the size of NPs would greatly influence cell uptake [14,22]. The subcellular localization of ICG in BxPC-3 cells was detected by confocal microscopy. After 2 h incubation, highest NIR fluorescence signals could be observed in INP-1 group cells,
illustrating that ICG could efficiently distribute into cells via INP-1 transportation. The fluorescence signals in INP-2 group cells was not that sharp compared to INP-1 group, displaying a less ICG distribution into cells. While only faint fluorescence signals could be observed in INP-3 group cells and illustrated only a few ICG was observed in BxPC-3 cells (Fig. 2A). To confirm the differences of subcellular localization in three groups, Flow cytometry was further utilized. The results exhibited most amount of ICG were detected in BxPC-3 cells incubated with INP-1, while less ICG signals were obtained in the cells incubated by INP-2 and INP-3 (72.79% and 44.00% FL intensity of INP-1 group) (Fig. 2). It had been proven that most NPs were taken up by an endocytic process, and NPs of 20–50 nm were taken up more rapidly than larger particles [22–24]. Therefore, INP-1 could be most internalized by cells and the highest amount of ICG could accumulate in cells in INP-1 group. And it was obvious to conclude that the size of INPs had a significant effect on delivering drugs into BxPC-3 cells.

3.3. In vitro photothermal therapy

To explore its biomedical applications of INPs, the potential toxicity of INPs was first tested. The cytotoxicity of free ICG, empty NPs and three INPs to BxPC-3 cells was quantitatively evaluated through MTT assay. The cells treated with INPs containing even 80 μg/mL ICG still performed favorable cell viability after 24 h incubation, which indicated that INPs below the concentration were nontoxic (Fig. S2).

Next, INPs was employed as photothermal agent for in vitro tumor cell therapy. BxPC-3 cells exhibited different survive rate when incubated with various ICG concentrations of INP-1, INP-2 or...
INP-3 for 24 h and then irradiated by 808 nm laser (1.6 W/cm²). BxPC-3 cells in INP-1 group were killed most efficiently at each ICG gradient concentration, and only 5.76% cells could survive after photothermal treatment when the ICG concentration reached to 80 µg/mL (Fig. 3A). ICG concentration was a crucial factor for final photothermal efficiency [25], it was not surprising to indicate that the amount of ICG existed in cells determined the efficiency of photothermal treatment. The smaller size and better cell uptake of INP-1 could evidently increase intracellular ICG concentration in BxPC-3 cells, thus significantly improved the photothermal efficiency when treated with same dosage of ICG and irradiation.

The efficiency of three types of INPs was further evaluated through Calcein-AM and PI staining after 24 h incubation of three different INPs and followed by laser irradiation (1.6 W/cm²). Viable cells were stained green with Calcein-AM, while dead or later apoptosis cells were stained red with PI. In vitro treatment efficiency was directly visualized through the staining, and the images illustrated that laser irradiation alone was quite safe for BxPC-3 cells. When ICG concentration increased to 80 µg/mL, cells in INP-1 group were almost entirely dead after 24 h incubation; nearly 20% cells were still alive in INP-2 group and about 70% survival cells in INP-3 group, which was consistent with the cytotoxicity results of photothermal therapy (Fig. 3B).

### 3.4. In vivo biodistribution

The biodistribution results of three different INPs were observed in BxPC-3 tumor bearing mice by ex/in vivo imaging system. ICG FL intensity was obtained after injected with PBS, free ICG, INP-1, INP-2 or INP-3 after 24 h Fig. 4A demonstrated that free ICG was metabolized and quickly excreted from living body and weak FL intensity could only be detected in liver after 24 h. This was because after binding to plasma proteins, ICG was cleared away by initial hepatic localization and eventual total clearance through biliary tree with minimal acute renal involvement [1,2,26], simultaneously showed the inefficient passive targeting of free ICG.

However, our results demonstrated that three types of INPs could be reserved in living body for a longer time. In INP-1, INP-2 and INP-3 groups, FL intensity of ICG could hardly be detected in heart, indicating the high biological safety of three INPs for heart. While ICG fluorescence could be detected in liver, spleen, lung and kidneys, which indicated that pulmonary endothelial cells uptake...
was also a pathway to remove INPs, except for hepatic clearance effect [2]. No statistical fluorescence difference was detected except in spleen, where the highest fluorescence intensity was found in INP-3 group. Since liver, spleen and lung were the organs of reticuloendothelial system [2,27], and the total fluorescence in these organs of each INPs group were analogous, a similar biodistribution could be deduced in three INPs groups. Therefore, these three different INPs apparently extended ICG circulation time in living body, exhibited similar distribution, and provided a noninvasive and visible method to analyze the biodistribution of NPs in vivo.

Contemporarily, ICG FL intensity was detected in tumor of mice after injected with INP-1, INP-2 and INP-3. Compared with the hierarchy of arterioles, capillaries and venules in normal blood vessels, tumor blood vessels were disorganized, irregularly shaped and leaky to obtain sufficient nutrition from blood [27,28]. This vascularity allowed preferential extravasation of circulating NPs. And polymersome have been used to encapsulate hydrophilic and hydrophobic drugs for passive targeting of tumors, and demonstrated tumor passive-targeting of ICG was enhanced through EPR effect through the encapsulation of lipid/polymer NPs [27,29]. The highest ICG FL intensity in tumor tissue was clearly observed in INP-2 group, which indicated INP-2 possessed best passive-targeting efficiency and highest ICG accumulation in tumor tissue.

In order to achieve efficient extravascular accumulation in tumor tissue, NPs must leave tumor blood vessel efficiently and maintain retention in the tissues [6,30]. It was reported that pancreatic carcinoma was a low-permeable tumor, whose median diameter of vessel pores in the sinusoids was around 110 nm [9,31]. Since the size of INP-1, INP-2 and INP-3 was 39.4 nm, 67.9 nm and 115.8 nm, the pores would obviously restrict the convection of INP-3 and it would only accumulate at or near the surface of tumor blood vessel. While INP-1 and INP-2 could easily pass through the vessel pores because their size was smaller than 100 nm.

Secondly, the particles diffused to tumor tissue must obtain efficient retention, and the retention was influenced by both cell metabolism and lymphatic drainage. According to previous reports, smaller particles could diffuse to the deep tissue of tumor much faster [9,32–34]. Decreased pO2 and hypoxic cells were also discovered in the deep region of tumor [6], which meant that smaller particles would be metabolized by tumor cells more quickly. Moreover, smaller particles could more easily be cleared away from tumor through lymphatic drainage and further enter sentinel lymph node [35–38]. Therefore, INP-1, the particle with the smallest size, was very easily to be metabolized by tumor cells, and then eliminated from tumor through lymphatic drainage after the diffusion from vessel (Fig. 4C). And due to relatively shallower diffusion and slower cell metabolism, INP-2 could be better reserved in tumor tissue.

3.5. In vivo photothermal efficiency and treatments

After intravenous injection of PBS, free ICG, INP-1, INP-2 or INP-3 through tail vein for 24 h, the tumor region was irradiated by 0.8 W/cm² near-infrared laser for 10 min. Then the temperature variation of tumor in 5 different groups was observed utilizing infrared thermal imaging camera. The maximum temperature of tumors in each group was 41.1 °C, 41.6 °C, 46.9 °C, 50.1 °C and 44.1 °C respectively (Fig. 5). The results indicated that when treated with INPs, tumors would probably acquire adequate temperature to cause destruction, while treated with PBS or free ICG would be insufficient to achieve tumor destruction, owing to previous work that efficient tumor cell destruction would be induced when temperature increased over 43 °C [39–41]. It demonstrated that the highest accumulation in tumor could bring about the greatest temperature increase in INP-2 group.

To identify the anti-tumor effect of passive targeted INPs to mice, BxPC-3 xenograft tumors were collected and stained with hematoxylin and eosin. In PBS plus laser and ICG plus laser groups, poorly differentiated histology and thick fibrotic tissue in the interstitium was discovered, and no vasculature could be observed inside tumor cell nests (Fig. 6A), which was corresponded with the characteristics of BxPC-3 xenograft tumor tissue described in
earlier references [9–11]. According to such structure, tumor cell nests were probably the barrier against the penetration of drugs and nanocarriers [11], and destroying nests structure was essential to inhibit tumor growth and relapse. In Fig. 6A, the BxPC-3 tumor tissue in PBS plus laser group showed the same features compared with control group, which indicated that a moderate and safe laser irradiation (0.8 W/cm²) was offered without local toxicity or evident systemic toxicity. Meanwhile, the tumor cells in three INPs groups exhibited coagulative necrosis and pyknosis. Particularly, the histological features in INP-2 group proclaimed an irreversible destruction to tumor nests and blood vessels.

The efficiency of photothermal treatment was further investigated in nude mice bearing BxPC-3 tumors. The results showed that tumors treated with PBS, PBS plus laser or free ICG plus laser grew rapidly, straightly confirming the laser irradiation and free ICG almost had no effect on tumor growth. While the growths of tumors treated with INP-1 and INP-3 were strongly inhibited during the first week after treatment, but then relapsed and grew rapidly. However, the tumors treated with INP-2 plus laser in this group were successfully suppressed. Notably, the tumor growth with INP-2 plus laser treatment was much more efficient than that of INP-1 or INP-3 plus laser treatment, even though the survive rate of mice within INP-1 or INP-3 groups were improved in a short-term (Fig. 6B and C). Our results demonstrated that adequate ICG accumulation in tumor tissue with INP-2 plus laser treatment could trigger sufficient temperature to obtain complete remission of tumors.

Side-effect of anti-tumor therapy is greatly concerned throughout clinical applications, and it proved that the loss of weight was an indicator for treatments-induced toxicity [17]. On 28 d, the weight of mice bearing BxPC-3 tumors increased 1.9 ~ 8.9% in the control groups treated with PBS or PBS plus laser, and the
weight of nude mice bearing BxPC-3 tumors in other groups treated with ICG plus laser or INPs plus laser increased 4.8~15.6% (Fig. 5S). These results indicated all treatments were nontoxic in our experiment. In the meantime, the major organs of mice treated with INP-2 plus laser were collected for histological staining to compare with normal organs. No noticeable abnormality was observed in heart, liver, spleen, lung and kidneys. The results indicated that photothermal therapy of INPs was tolerable and safe in vivo.

4. Conclusions
We successfully fabricated ICG-loaded lipid-polymer nanoparticles with three size distributions (39 nm, 68 nm and 116 nm) using single-step nanoprecipitation method. NIR fluorescence imaging and photothermal therapeutic efficiency of ICG was perfectly integrated in these three types of INPs for cancer theranostics. These three types of INPs exhibited excellent size stability and fluorescence stability, and enhanced temperature response compared to free ICG. Size could not only obviously influence endocytosis and further photothermal efficiency of INPs in vitro, but also affect their tumor accumulation and photothermal therapeutic efficiency in vivo. INP-2 showed the strongest efficiency to suppress tumor growth in BxPC-3 xenograft tumors. Hence, we offered a nontoxic and size-dependent theranostic model for passive targeting and NIR imaging guided photothermal therapy with minimized adverse effect.

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Appendix A. Supplementary data
Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2014.04.019.

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