Near-infrared fluorescence tumor imaging using nanocarrier composed of poly(l-lactic acid)-block-poly(sarcosine) amphiphilic polydepsipeptide

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A nanocarrier, lactosome, which is composed of poly(l-lactic acid)-block-poly(sarcosine), as a contrast agent for the liver tumor imaging was examined using the near infrared fluorescence (NIRF) optical imaging technique. Lactosome labeled with indocyanine green (ICG) showed a high escape ability from the reticulo-endothelial system (RES). Lactosome was found to be stable in a blood circulation, and gradually accumulated specifically at a model liver tumor site, which was obtained by graft of HepG2/EF-Luc cells at a mouse liver. The high tumor/liver imaging ratio is due to the enhanced permeation and retention (EPR) effect of lactosome. The fluorescence intensity at the tumor site was correlated with the degree of malignancy. Tumor imaging using lactosome as a nanocarrier is therefore a potential candidate for a facile and general tumor imaging technique.

1. Introduction

Molecular imaging is a non-invasive visualization technique of normal and/or abnormal cellular processes. By monitoring the behavior of probes in the living organisms, molecular dynamism within the cell is traceable. Therefore, its utilization is emerging not only in the fundamental research areas for the elucidations of living system mechanisms, but also in applicative areas such as medicine and pharmaceutics [1–5]. In designing molecular probes for tumor imaging, low toxicity of the molecules is bare minimum. Additionally, methodology how to deliver the probes to the targeted tumor region is important. In tumor tissues, submicron-sized defects exist on the vascular wall because of the rapid angiogenesis, enabling permeation of macromolecules through the wall. Further, the lymph system around tumor grows too slowly to exclude foreign compounds from the tumor region, and nanocarriers of the size in the range of 30–100 nm are considered to be passively accumulated into tumor (the enhanced permeation and retention (EPR) effect) [6–8]. The EPR effect is one of the most widely utilized strategies for the tumor-targeting drug delivery system. However, foreign particles are likely to be captured by the cells at reticulo-endothelial system (RES) of liver and spleen. In order to obtain an escape ability, the stealth property in other words, from RES and a prolonged life time in blood circulation, chemical modification of molecular probes with poly(ethylene glycol) (PEG) is frequently adopted [9–11]. It is considered that PEG modification contributes to the inhibition from the macrophage recognition due to the good hydration property. However, PEGylated molecular probes are shown to be recognized to some extent as foreign substances by RES and accumulated in liver [12–14]. Complete suppression from the RES detection is yet to be solved out. This is one of the reasons for the tumor imaging with chemical probes to be limited to other sites from liver and spleen. Therefore, an alternative hydrophilic nonionic polymer with low toxicity and a good escape ability from RES is expected to be developed.

Polypeptides are considered to be a powerful candidate for a substitute of PEG because they have a possibility to be degraded by endogenous proteinases. Vesicular molecular assemblies composed of amphiphilic block polypeptide having a nonionic poly(sarcosine) (PS) as a hydrophilic block showed a long life time in blood circulation and named “peptosome” [15,16]. The high density of hydrophilic PS chains so called polymer brush around the molecular assemblies contributes to the inhibition from RES recognition similarly to PEG modification. PS is considered to have advantages against PEG on biodegradability and the equipped metabolic pathway for sarcosine. Another specificity of peptosome
is a helical structure of hydrophobic block. The block forms a more robust membrane via hydrophobic interaction than liposome composed of small lipids [17]. Poly(β-lactic acid) (PLLA), which is one of the most widely used biodegradable and bioinert materials [18,19], is known to form 3_10 helical structure [20]. From amphiphilic block polymers composed of PLLA and PS, molecular assemblies with various morphologies such as micelle, vesicle, and lamella were formed and named as “lactosome” [21]. In the present study, lactosome was examined for imaging the liver cancer using the near infrared fluorescence (NIRF) optical imaging technique [22]. As a result, accumulation of lactosome at normal liver is greatly suppressed to make liver cancer imaging possible.

2. Materials and methods

2.1. Materials

All reagents and solvents were purchased commercially and used as-received unless otherwise noted. Near infra-red fluorescence compound, ICG (indocyanine green)-OSu, was purchased from Dojin Laboratory Ltd. Dy-776 and Dy-750 carboxylic acid type were purchased from DynoMic GmbH. They were used as-received.

2.2. Measurements

NMR and UV spectra were recorded with a Bruker DFX 400 and Shimadzu UV-2450 spectrophotometer, respectively. The hydrodynamic diameters of the nanocarriers were measured by a dynamic light scattering spectrophotometer, Photal DLS-2450 spectrometer, respectively. The hydrodynamic diameters of the nanocarriers were measured by a dynamic light scattering spectrophotometer, Photal DLS-2450 spectrometer, respectively.

2.3. Syntheses of amphiphilic block and NIRF labeled polymers

Amphiphilic polymers of PLLA90-block-PS90 and (Leu-Aib)_8-block-PS90 were synthesized as previously reported [21,22]. The molecular structures of the block polymers are shown in Fig. 1. The syntheses of all compounds were confirmed by H NMR and UV spectra were recorded with a Bruker DPX 400 and Shimadzu UV-2450 spectrophotometer, respectively.

2.3.1. ICG–PLLA, ICG–PS and ICG–PS-block–PLLA

Chemical modification of the polymer terminal end with indocyanine green (ICG) was carried out as follows (Fig. S1, Supporting information). ICG–OSu (1.0 mg) was added to the DMF solution of free amino group bearing PLLA (2.46 mg), whose amino group is designed as an initiator for the sarcosine NCA polymerization at the synthesis of amphiphilic PLLA-block–PS block polymers. The reaction mixture was stirred at room temperature overnight under light shielding condition (Fig. S1a). In a similar way with ICG–PLLA, ICG–OSu was reacted with the N-terminal end of PS, which was synthesized via NCA polymerization initiated from hexylamine or amino group in PLLA block (Fig. S1b and c). These reaction mixtures were purified with size exclusion chromatography, a Sephadex LH-20 column using DMF as eluant.

2.3.2. Dy-750–PLLA and Dy-776-(Leu-Aib)₈

Dy-750–COH (1.0 mg), 1-[Bis(dimethylamino)methyl]vinyl]-1-[4,5-bipyridine–3-oxa-hexafluorophosphate (HATU, 2.66 mg) and N,N-disopropyl-ethylene diamine (DIEA, 1.81 mg) were added to the DMF solution (2.0 mL) of free amino group bearing PLLA (3.22 mg) at 0 °C. The reaction mixture was stirred at room temperature for 12 h under the light shielding condition (Fig. S1d). Chemical modification of (Leu-Aib)_8 amino terminal end with Dy-776 was carried out in a similar way to Dy-750–PLLA (Fig. S1e). These reaction mixtures were also purified with size exclusion chromatography, a Sephadex LH-20 column using DMF as eluant.

2.4. Preparation of NIRF labeled molecular assemblies

A chloroform solution (1.0 mL) of polymer 1 (0.20 mmol) and NIRF labeled polymer (3.0 μmol) at a defined ratio (Table 1, entries 1–4) was dripped into a glass test tube. The solvent was removed under the reduced pressure and thin film was formed on wall surface of the tube. To the test tube, 10 μm Tris–HCl buffer (pH 7.4) (3.0 mL) was added and the solution was treated by bath-ultrasonic processor for 5 min. The resulting aqueous solution was purified by Sephacryl S-100 size exclusion chromatography eluting with 10 μm Tris–HCl buffer (pH 7.4) to obtain NIRF labeled micelle, lactosome. The hydrodynamic diameters of the molecular assemblies were determined by dynamic light scattering of the aqueous dispersions of the polymers without NIRF-labeled polymers to avoid the fluorescence influence on the measurements. The autocorrelation function was analyzed by the time interval method of photon correlation. The light scattering data were analyzed by the program of Contin [24,25].

2.5. Cell culture

SUIT-2 human pancreatic cancer cell line, HepG2 hepatocellular carcinoma cell line and NCI-H441 papillary adenocarcinoma cell line were purchased from the American Type Culture Collection. These cell lines that carry the luciferase reporter gene under control of pEF constitutive promoter were isolated by the same method as described elsewhere. SUIT-2/EF-Luc and HepG2/EF-Luc cell lines were maintained at 37 °C with 5% FBS-Dulbecco’s modified Eagle’s medium (Nacalai Tesque, Kyoto, Japan). NCI-H441/EF-Luc cell line was maintained in RPMI 1640 ( Gibco, Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 10% FBS (Biosource, USA), penicillin (100 U/mL), and streptomycin (100 μg/mL).

2.6. Tumor bearing mouse

SUIT-2/EF-Luc cells were subcutaneously inoculated at 1 × 10⁶ cells in 40 μL of phosphate-buffered saline (PBS) into front legs of 7-week-old male nude mice (BALB/c nu/nu; Japan SLC Inc., Hamamatsu, Japan). The tumor bearing mice after 14 days from the transplantation were used for the imaging. HepG2/EF-Luc cells were grafted at 1 × 10⁶ cells in 40 μL in the liver of 8-week-old male nude mice (BALB/c nu/nu; Japan SLC Inc., Hamamatsu, Japan). For the imaging, the tumor bearing mice of 7 days after the transplantation were used. NCI-H441/EF-Luc cells were injected at 1 × 10⁶ cells in 40 μL into the left lung through the left chest wall of 7-week-old male nude mice (BALB/c nu/nu; Japan SLC Inc., Hamamatsu, Japan) and the mice after 11 days after the transplantation were used.

Table 1

The type of NIRF mixed in the molecular assemblies and tumor/liver NIRF intensity ratio after 24 h from administration to tumor bearing mice.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Nanocarrier</th>
<th>NIRFa</th>
<th>Tumor-liver NIRF intensity ratio (24 h)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Lactosomé</td>
<td>ICG–PS–PLLA</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>2</td>
<td>Lactosome</td>
<td>ICG–PLLA</td>
<td>2.3 ± 0.4</td>
</tr>
<tr>
<td>3</td>
<td>Lactosomé</td>
<td>Dy750–PLLA</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>4</td>
<td>Lactosomé</td>
<td>ICG–PS</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>Lactosome</td>
<td>ICG–PS</td>
<td>0.68 ± 0.07</td>
</tr>
</tbody>
</table>

a The amount of mixed NIRF-labeled polymer into the amphiphilic block polymers is 1.5 mmol.
b SUIT-2 human pancreatic cancer cell at front legs, n = 3.

c Lactosome was consisted of PLLA_n–PS_m. The hydrodynamic diameter of lactosome determined by DLS measurement was 37 nm.

d Peptosome was consisted of (Leu-Aib)_n–PS_m and the hydrodynamic diameter was 32 nm.

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2.7. In vivo near-infrared fluorescence (NIRF) cancer imaging

β-Luciferin solution (200 μL, 10 mg/mL in PBS; Promega Corp., Madison, WI) was intraperitoneally injected to tumor-bearing mouse in order to take bioluminescence images from the SUIT-2/pEF-Luc. After 15 min, the mice were set in an optical imaging device. From UV spectra, concentration of ICG, Dy-750, and Dy-776 in lactosome or peptide micelle solution was calculated using corresponding molar absorbance coefficients at 795 nm, 757 nm, and 771 nm, respectively. The administered dose of lactosome was normalized by the NIRF intensity. Each solution of NIRF labeled lactosome (100 μL) was injected to every three tumor-bearing mice (n = 3), and their NIRF images were taken by Clairvivo OPT (Shimadzu corp.) or IVIS™-200 (Xenogen Corp.). For the measurement, a filter set (ex. at 785 nm and em. at 845 nm for Clairvivo OPT) was used for the measurement of ICG, Dy-750 labeled lactosome and Dy-776 labeled peptide micelle. Another filter set (ex. at 735 nm, and em. at 842.5 nm for IVIS™-200) was used for the measurement of ICG and ICG–PS. During the imaging process, the mice were kept on the imaging stage under anesthetized condition with 2.5% of isoflurane gas in oxygen flow (1.5 L/min). The intensity ratio of fluorescence at tumor against liver was calculated from the photon counts as average of three mice.

3. Results and discussions

The PLLA-block-PS amphiphilic polymer (1) (Fig. 1), was synthesized as previously reported [21]. The number-average molecular lengths of hydrophobic PLLA and hydrophilic PS blocks were determined to be 30 mer and 90 mer, respectively. As near infra-red fluorescence (NIRF) agents, indocyanine green (ICG, ex: 768 nm, em: 807 nm) and Dy-750 (ex: 747 nm, em: 771 nm) were used. NIRF groups were connected to the terminal of PS or PLLA using a peptide coupling reactions (Fig. S1, supporting information). Micellar molecular assemblies were prepared from a mixture of polymer 1 and NIRF labeled polymers as following. A chloroform solution of the mixture of polymer 1 and NIRF labeled polymer (Table 1, entries 1–5) was dripped into a glass test tube. By evaporating the solvent under the reduced pressure, thin film was formed on wall surface of the tube. To the test tube, 10 mM Tris–HCl buffer (pH 7.4) was added and the dispersion was sonicated. The resulting aqueous dispersion was purified by Sephacryl S-100 size exclusion chromatography to obtain NIRF labeled micelle, lactosome. The elution profiles clearly indicated that all the NIRF-labeled compounds except ICG alone and ICG–PS were integrated in the molecular assemblies. The hydrodynamic diameters of the molecular assemblies were determined by DLS on the dispersions of the amphiphilic polymers without the NIRF-labeled compounds, because the fluorescence from the sample interfered the data. The amount of the NIRF-labeled compound in the molecular assemblies was chosen to be 1.5 mol%, which is a small number not to change the morphology and the diameter of the molecular assemblies. For example, the diameter of lactosome was unchanged upon inclusion of poly(-lactic acid) (30 mer) at a concentration as high as 5 mol%.

Polypeptide micelle was prepared as a reference sample from the mixture of (Leu-Aib)8-block-PS amphiphilic polymer (2) (Fig. 1) and Dy-776 (ex: 771 nm, em: 793 nm) labeled (Leu-Aib)8 (Table 1, entry 6) similarly to lactosome [23]. The chain length of the PS block was set at 60 mer to obtain a micellar molecular assembly with a diameter of 30–40 nm as large as the lactosome. The block polypeptide having a longer PS chain did not form stable micelles due to the hydrophilic property of the block polypeptide in total.

SUIT-2/pEF-Luc cells that carry the luciferase reporter gene under control of pEF constitutive promoter were used as a tumor model and were grafted in front legs of mouse. Location and size of tumor are confirmed by bioluminescence image of β-luciferin upon oxidation by luciferase from the SUIT-2/EF-Luc. The buffered solutions of NIRF-labeled lactosomes were injected to tumor bearing mice from the tail vein and NIRF images were taken by Shimadzu Clairvivo OPT or Xenogen IVIS™-200 (Fig. 2). The NIRF probes spread over the whole body through the blood circulation to show the maximum intensity in the beginning 1–3 h after the injection, and the intensities at normal regions quickly decreased. In entries 1–3, the remaining fluorescence after 24 h was mostly observed from the tumor sites (both front legs). This observation was independent on the kind of the NIRF probes which were used for labeling the lactosome. The NIRF probes are considered to be concealed in the hydrophilic PS layer of the molecular assemblies. In entry 3, the fluorescence intensity ratio (tumor/liver) still increased after 48 h, suggesting that some NIRF-labeled nanocarriers remained in the blood circulation to accumulate at the tumor site. These high tumor/liver accumulation ratios of lactosome should be due to the EPR effect. Indeed, in vivo retention in
The fluorescence intensity ratio at tumor against liver after 24 h sites gradually increased with time probably due to the EPR effect. In the case of lactosome, however, the fluorescence from the tumor (entry 6), fluorescence from liver is detected much stronger than that of the peptide micelle. Blood test of lactosome confirmed a highly escape ability from RES of NCI-H441 papillary adenocarcinoma cell bearing mouse at lung (data not shown). The hydrophilic property of PS (Fig. 2a) is therefore prevailing despite of the grafted tumor type and site.

Fig. 3. Bright field, luciferin bioluminescence, and ICG fluorescence images of HepG2 hepatocellular carcinoma cell bearing mouse at liver (a–e) and its isolated liver (f–h). Strong signal was observed from the NIRF image of tumor bearing mouse at liver (b, e, h), where is the same place with luciferin bioluminescence (a, d, g). Isolated lung images of NCI-H441 papillary adenocarcinoma cell bearing mouse at lung (i–k).

The high tumor/liver accumulation ratio of lactosome is further examined by in vivo optical imaging of liver cancer. HepG2 (Human hepatocellular liver carcinoma cell line) was used as a liver tumor model. Lactosome was labeled with ICG–PLLA, which is considered to be better than ICG–PS–PLLA as labeling of lactosome, because the ICG moiety should be concealed deeply in lactosome. The optical images were taken after 48 h from the administration of lactosome to the liver tumor bearing mouse, showing that the ICG fluorescence site overlapped at the luciferin bioluminescence site, which was a marker of HepG2 (Fig. 3a–e). When the liver was taken out from the tumor bearing mouse, notably, ICG fluorescence image is clearly observed at the same place as the luciferin bioluminescence (Fig. 3f–h). It is confirmed that liver tumor can be precisely detected for example by esterases due to the facile ester linkages.

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4. Conclusions

The high tumor/normal tissue accumulation ratio of lactosome is therefore prevailing despite of the grafted tumor type and site.
Lactosome is a powerful molecular probe candidate for tumor imaging at liver and lung without gene manipulations. Further, nanocarriers composed of amphiphilic block polymers offer multiple advantages. Various kinds of functional groups can be easily introduced to the nanocarrier. Morphology of lactosome is shown to be controlled by the hydrophilic–hydrophobic balance of the amphiphilic block polymer to provide micelles and vesicles. Various types of pharmaceutical agents can be encapsulated into a proper nanocarrier. Lactosome is a potential nanocarrier not only for molecular imaging but also for drug delivery system.

Acknowledgements

This study is a part of joint research, which is focusing on the development of the basis of technology for establishing COE for nanomedicine, carried out through Kyoto City Collaboration of Regional Entities for Advancing Technology Excellence (CREATE) assigned by Japan Science and Technology Agency (JST).

Appendix

Figures with essential colour discrimination. Figs. 2–4 in this article are difficult to interpret in black and white. The full colour version can be found in the on-line version at doi:10.1016/j.biomaterials.2009.05.046.

Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.biomaterials.2009.05.046.

References