Over-1000 nm Near-infrared Fluorescence and SPECT/CT Dual-modal in vivo Imaging Based on Rare-earth Doped Ceramic Nanophosphors

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In this study, we aimed to develop a dual-modal in vivo imaging based on over-1000 nm near-infrared (OTN-NIR) fluorescence and single photon emission computed tomography (SPECT)/computed tomography (CT). As an OTN-NIR nanophosphor material, Ytterbium and erbium ion co-doped yttrium phosphate nanoparticles (YPO4 NPs) was synthesized by a hydrothermal synthesis method. Biocompatible poly(ethylene glycol) (PEG)/polycation block copolymer and radioactive 111In were introduced on the YPO4 NPs surface (PEG-111In-YPO4 NPs). The PEG-111In-YPO4 NPs showed high dispersion stability in physiological saline and excellent radioactivity in any of PBS and FBS solutions. Furthermore, in vivo OTN-NIR fluorescence and SPECT/CT imaging of live mice was performed. Strong OTN-NIR emission from the blood vessel and organs of live mice were observed. Moreover, SPECT/CT images clearly showed the three dimensional images of the live mice. Therefore, PEG-111In-YPO4 NPs is an attractive candidate for the OTN-NIR fluorescence and SPECT/CT dual-modal imaging probe, and the concept can be served as a novel platform for real-time imaging and three dimensional quantitative determinations.

Keywords: bioimaging, near-infrared, SPECT, nanoparticle, poly(ethylene glycol)

1. Introduction

Near-infrared (NIR) nanophosphors are particularly promising for in vivo imaging probe because of high penetration depth and low autofluorescence of NIR light [1,2]. Most of traditional fluorescence probes suffer from problems such as low penetration depth and significant autofluorescence, due to the ultraviolet (UV) or short-wavelength visible (VIS) excitation. Instead, the NIR light solves these problems and offers new opportunities to observe deep part of the body with low background. The NIR wavelength region (700-1800 nm) is called as the “biological window”[3]. Especially, NIR light in over-1000 nm (OTN-) NIR wavelength region (also called as “second biological window”(1000-1350 nm) and “third biological window”(1550-1870 nm)) can penetrate into the living tissues more deeply than commonly used NIR light in the “first biological window” (700-900 nm) wavelength region [4]. Therefore, OTN-NIR fluorescence imaging has recently attracted much attention. In 2010 and 2011, our group have first reported and developed the OTN-NIR in vivo
fluorescence imaging based on rare-earth ion doped ceramic nanophosphors (RED-CNPs) [5,6]. RED-CNPs are known to show strong OTN-NIR emission under NIR excitation [7,8]. Since then, other groups have reported the in vivo OTN-NIR fluorescence imaging by using RED-CNPs [9-14].

However, the OTN-NIR imaging based on RED-CNPs possesses several weak points for in vivo imaging. The observation of deep areas whose depths are longer than several millimeters is usually difficult even by OTN-NIR fluorescence. Furthermore, Quantitative evaluation of images is also difficult by optical imaging. To solve these problems, we have focused on combination of optical imaging and radionuclide one. Especially, three dimensional radionuclide images obtained by single photon emission computed tomography (SPECT) would be useful tools to provide physiological information of deep areas of the living body with high sensitivity and excellent quantitativity [15,16]. SPECT images reflect total counts during rather long acquisition time while OTN-NIR fluorescence imaging can observe real-time images. Therefore, it would be expected that the combination of OTN-NIR fluorescence and SPECT might be useful by considering complementary information provided by each imaging test. Recently, a SPECT/computed tomography (CT) combined scanner is getting popular because the superimposition of SPECT images on CT ones can provide physiological information with anatomical markers.

Here, we have explored the preparation and evaluation of RED-CNPs based novel probe for the OTN-NIR fluorescence and the SPECT dual-modal in vivo imaging (Fig. 1). Because of the limited half-life of the 111In (2.8 days), the probe preparation must be conducted with several hours after obtaining the hot 111In. For fulfilling this criterion, a relatively simple and quick method was developed for building up the surface structure in Fig. 1 on the YPO₄ NPs. Since the NIR nanophosphor, rare-earth ion-doped yttrium phosphate nanoparticles (YPO₄ NPs) have negatively charged surface at around neutral pH, radio-active 111In³⁺ was introduced on the YPO₄ NPs surface via electrostatic interactions for the SPECT imaging. Furthermore, for the improvement of stability of NPs, the surface of YPO₄ NPs was modified with poly(ethylene glycol) (PEG)/polycation block copolymer, PEG-b-poly((2-N,N-dimethylamino) ethyl methacrylate) (PAMA) via electrostatic interactions. PEG is well known to improve the dispersion stability of NPs [17-20]. Moreover, the polyvalent effect of the cationic PAMA segment in PEG-b-PAMA on the negative charged YPO₄ NPs surface was anticipated. Additionally, the OTN-NIR fluorescence and the SPECT/CT in vivo imaging of live mice was also carried out.

![Fig. 1. Schematic illustration of PEG-111In-YPO₄ NPs.](image)

2. Materials and Methods

2.1. Materials

Yttrium nitrate hexahydrate (Y(NO₃)₃·6H₂O), erbium nitrate pentahydrate (Er(NO₃)₃·5H₂O), ytterbium nitrate hydrate (Yb(NO₃)₃·H₂O), cetyltrimethylammonium bromide (CTAB), and ammonium dihydrogenphosphate (NH₄H₂PO₄) were purchased from Wako Pure Chemicals (Osaka, Japan). Radioactive 111InCl₃ was purchased from Nihon Medi-Physics (Tokyo, Japan). Non-radioactive InCl₃ was purchased from Sigma-Aldrich (St. Louis, US). All reagents were used as received.

2.2. Synthesis and characterization of Yb- and Er-doped YPO₄ NPs

Ytterbium and Erbium ion-doped YPO₄ NPs were synthesized by a hydrothermal synthesis method [21,22]. Y(NO₃)₃·6H₂O (12.285 mmol), Yb(NO₃)₃·H₂O (0.675 mmol), Er(NO₃)₃·6H₂O (0.54 mmol), and CTAB (0.3375 mmol) were dissolved in 15 mL of
distilled water. \( \text{NH}_4\text{H}_2\text{PO}_4 \) (13.5 mmol) was dissolved in 15 mL of distilled water. These two aqueous solutions were poured into a polytetrafluoroethylene vessel. The vessel was sealed and placed in a stainless steel DAB-2 autoclave (Berghof GmbH, Germany), and kept at 200°C for 6 h under autogenously pressure. The obtained \( \text{YPO}_4 \) NPs were separated by centrifugation (1.5 \times 10^4 g, 15 min, 3 times) with distilled water and dried at 80°C. Subsequently, the obtained \( \text{YPO}_4 \) NPs were calcinated at 900°C for 30 min in air in order to get rid of crystal water and improve the luminescence intensity. The obtained \( \text{YPO}_4 \) NPs was characterized by using powder X-ray diffraction (XRD) (XRD-6100, Shimadzu, Japan).

2.3. Preparation of PEG and \( ^{111}\text{In} \) co-immobilized \( \text{YPO}_4 \) nanoparticles

\( \text{YPO}_4 \) NPs (20 mg) were dispersed in 0.72 mL of distilled water and sonicated for 10 min at room temperature. Next, 0.8 mL of \( ^{111}\text{InCl}_3 \) aqueous solution (0.04 M, \( ^{111}\text{In} : 50 \text{ MBq} \)) was added to 0.084 mL of acetate buffer (2 M, pH5.5). These two aqueous solutions were mixed and stirred for 1 h at 75°C. The free excess \( ^{111}\text{In} \) in the solution was removed by centrifugation (1.5 \times 10^4 g, 15 min, 3 times), and 20 mg of \( ^{111}\text{In} \)-immobilized \( \text{YPO}_4 \) (\( ^{111}\text{In-YPO}_4 \)) NPs were dispersed in 10 mL of distilled water. Then, PEG-b-PAMA (20 mg) in 30 mL of distilled water was added to the \( ^{111}\text{In-YPO}_4 \) NPs dispersion. The pH value of the mixture was controlled by adding HCl or NaOH, and the mixture solution with a predicted pH values (pH5.3 - 10.5) were stirred for 1 h at 50°C. The free excess polymer in the solution was removed by centrifugation (1.5 \times 10^4 g, 15 min, 3 times), and the solvent was changed to pure water. The obtained PEG and \( ^{111}\text{In} \) co-immobilized \( \text{YPO}_4 \) (PEG-\( ^{111}\text{In-YPO}_4 \)) NPs were dispersed in pure water. PEG and non-radioactive In co-immobilized \( \text{YPO}_4 \) (PEG-In-YPO_4) NPs were also prepared by same procedure.

2.4. Evaluation of PEG and \( ^{111}\text{In} \) co-immobilized \( \text{YPO}_4 \) NPs

Chemical and optical properties of PEG and/or In co-immobilized \( \text{YPO}_4 \) NPs were characterized by using non-radioactive In-immobilized NP samples (In-YPO_4 NPs and PEG-In-YPO_4 NPs). The surface charge of the \( \text{YPO}_4 \) NP samples were measured by using \( \xi \)-potential analyzer (ZetasizerNano ZSP, Malvern Instruments, Ltd., U.K.) in 10 mM NaCl solution. The PEG density on the \( \text{YPO}_4 \) NPs surface was evaluated by thermal gravimetric analysis (TGA) (SHIMADZU DTG-60). The particle size distribution of \( \text{YPO}_4 \) NP samples in physiological saline was evaluated by dynamic light scattering (DLS) (LB-550, HORIBA). Turbidity of the \( \text{YPO}_4 \) NP samples in physiological saline were measured by ultraviolet-visible spectrophotometer (V630, JASCO, Japan). Time dependent changes of relative absorbance of sample solutions at 550 nm were measured. The OTN-NIR emission spectra of PEG-In-YPO_4 NPs was measured by NIR fluorescence spectrometer (AvaSpec-NIR256-1.7, Avantes, Nederland) under an excitation of 980 nm laser diode (L9418-04, Hamamatsu Photonics, Japan) equipped with a temperature controller (TED200, Thorlabs, US). The OTN-NIR fluorescence image of PEG-In-YPO_4 NPs was observed by portable OTN-NIR imaging system (SAI-1000, Shimadzu). Radio activity of radioactive \( ^{111}\text{In-YPO}_4 \) NPs and PEG-\( ^{111}\text{In} \)-YPO_4 NPs were measured by using gamma counter (2480 WIZARD2 auto gamma counter, Perkin Elmer, US).

2.5. OTN-NIR fluorescence and SPECT imaging of live mice

Male Slc:ddY mice (8-10 weeks old) were purchased from SLC Japan. The PEG-\( ^{111}\text{In-YPO}_4 \); Yb, Er NPs were dispersed in physiological saline and it was injected into mice via tail vein at a dose of 4 mg. The \( \text{in vivo} \) OTN-NIR fluorescence imaging was carried out by using the home-made NIR imaging system at 0 - 300 sec after intravenous injection of samples. The OTN-NIR fluorescence imaging system consists of 980 nm laser excitation and InGaAs CCD camera [6,7]. SPECT/CT fusion images were obtained using a SPECT/CT combined scanner (NanoSPECT/CT, Bioscan Inc., Washington, D.C., USA) [15,16]. Tube voltage and tube current of CT scans were 45 kV and 177 \( \mu \text{A} \), respectively. The obtained CT data were reconstructed by a cone-beam.
filtered back-projection method. Then, SPECT data were acquired in six steps over 90° (300 s per step) with four detectors covering 360°. Energy peaks were set to 171 and 245 keV to detect two kinds of gamma rays emitted from \(^{111}\)In. The acquired SPECT data were reconstructed by an ordered subsets-expectation maximization algorithm. Both CT and SPECT reconstructed images were automatically superimposed by using the built-in software and their fusion images were expressed using a maximum intensity projection method.

3. Results and Discussion

The YPO\(_4\) NPs (\(d = 100\) nm) was synthesized by a hydrothermal synthesis method. The crystal phase of the YPO\(_4\) NPs was identified by XRD measurement (data not shown). Next, for the evaluation of surface character of YPO\(_4\) NPs, \(\xi\)-potential value of YPO\(_4\) NP samples was evaluated. Fig. 2 shows the \(\xi\)-potential measurement results of YPO\(_4\) NP samples with pH ranging from 2 to 12. The \(\xi\)-potential of bare-YPO\(_4\) NPs was about -30 mV at pH 7.0 and it increased with decreasing pH value. From this result, isolectric point of YPO\(_4\) NPs was determined as about 4.0 and YPO\(_4\) NPs has negatively charged surface at around neutral pH value. Thus, PEG/polycation block copolymer, PEG-b-PAMA was used as the surface modification agent. For the surface characterization of PEGylated NPs, PEG-b-PAMA and non-radioactive In-co-immobilized YPO\(_4\) (PEG-In-YPO\(_4\)) NPs was used as measurement samples. In order to confirm the surface modification of the YPO\(_4\) NPs, TGA measurements of the YPO\(_4\) NP samples were carried out. Table 1 shows the TGA results for the PEG-In-YPO\(_4\) NP samples. From these data, PEG chain densities on the YPO\(_4\) NPs surface was increased with increasing of PEGylation pH values. The maximum PEG chain density on the YPO\(_4\) NPs surface was estimated to be 0.45 chains nm\(^{-2}\), which was obtained at pH10.5. This tendency is probably due to that the amine groups in PAMA segment possess anion charge and thus it adsorbed on Y\(^{3+}\) of YPO\(_4\) NPs via electrostatic interactions under high pH conditions. Therefore, we have mainly focused on PEG-In-YPO\(_4\) NPs prepared under high pH condition (pH10.5) for further characterization.

To confirm the surface modification of the YPO\(_4\) NPs, the surface charge on the In-YPO\(_4\) NPs and PEG-In-YPO\(_4\) NPs (PEGylated at pH10.5) was also estimated (Fig. 2). After surface modification of the YPO\(_4\) NPs with In and PEG-b-PAMA, the free In and polymer in the solution was removed by centrifuge purification, and the NPs were dispersed in 10 mM NaCl solution with various pH values. The \(\xi\)-potential of the In-YPO\(_4\) NPs was almost same as bare-YPO\(_4\) NPs. On the other hand, the \(\xi\)-potential value of PEG-In-YPO\(_4\) NPs were close to zero regardless of the environmental pH values. These results suggest that the PAMA segment in the block copolymer was adsorbed on the YPO\(_4\) NPs surface and non-ionic PEG segment shielded the surface charge. Thus, the surface of In-YPO\(_4\) NPs was effectively modified by PEGylation.

![Fig. 2. Change in the \(\xi\)-potential of YPO\(_4\) NP samples with pH ranging from 2 to 12. \(\xi\)-potential measurement condition: 10 mM NaCl solution; sample concentration, 0.1 mg/mL.](image)

<table>
<thead>
<tr>
<th>PEGylated pH value</th>
<th>PEG brush density (chains/nm(^2))</th>
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<tbody>
<tr>
<td>5.3</td>
<td>0.13</td>
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<tr>
<td>8.3</td>
<td>0.26</td>
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<tr>
<td>9.2</td>
<td>0.38</td>
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<tr>
<td>10.5</td>
<td>0.45</td>
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The dispersion stability of PEG-In-YPO\(_4\) NPs in physiological saline was then investigated by DLS measurement. Fig. 3(i) shows the particle size distribution of
PEG-In-YPO₄ NPs in physiological saline. The average particle size of bare-YPO₄ NPs was about 1100 nm, indicating the agglomeration of NPs. In the case of PEGylated NPs, with increasing the PEGylation pH value, the average particle size of the PEG-In-YPO₄ NPs decreased and reached saturation at about 100 nm (PEGylation pH ≥ 8.3). Furthermore, the long term dispersion stability of YPO₄ NP samples in physiological saline was also evaluated by measurement of turbidity of the NP solutions. Fig. 3(ii) shows the turbidity of the physiological saline solution of bare-YPO₄ NPs and PEG-In-YPO₄ NPs with various PEGylation pH conditions. The YPO₄ NP samples were dispersed in physiological saline. However, the bare-YPO₄ NPs and PEG-In-YPO₄ NPs (PEGylated at pH5.3) were immediately agglomerated within 1 h. Although the dispersion stability of the YPO₄ NPs was increased by increasing of PEGylation pH value (pH8.3 and pH9.2), NPs were also agglomerated. In contrast, PEG-In-YPO₄ NPs (PEGylated at pH 10.5) were not agglomerated, and it showed high dispersion stability over 12 h. From these results, surface modification of YPO₄ NPs by PEG-b-PAMA at high pH conditions affords much higher dispersion stability than that prepared at low pH conditions. From these findings, it was concluded that PEG-b-PAMA was adsorbed and formed PEG layer on the YPO₄ NPs surface (PEGylated at pH10.5). The OTN-NIR emission of PEG-In-YPO₄ NPs was then evaluated in physiological saline. As shown in Fig. 4 (i), PEG-In-YPO₄ NPs showed strong 1550 nm emission under 980 nm excitation. Furthermore, strong OTN-NIR emission of PEG-In-YPO₄ NPs was also observed by using portable OTN-NIR imaging system (Fig. 4 (ii)).

Next, for the SPECT imaging application, radio-active ¹¹¹In- and PEG-b-PAMA were immobilized on the YPO₄ NPs surface (PEG-¹¹¹In-YPO₄ NPs). Effect of PEGylation against the labeling yield of radio-active ¹¹¹In on the ¹¹¹In-YPO₄ NP surface was evaluated by using gamma counter. From this result, the labeling yield of ¹¹¹In on the ¹¹¹In-YPO₄ NPs and PEG-¹¹¹In-YPO₄ NPs surface were determined as 91.4% and 86.7%, respectively. Although this labeling yield of ¹¹¹In of PEG-¹¹¹In-YPO₄ NPs was lower than that of ¹¹¹In-YPO₄ NPs, labeling yield of ¹¹¹In was enough to use for the SPECT imaging. Moreover, retention rate of ¹¹¹In of the PEG-¹¹¹In-YPO₄ NPs was determined in PBS and FBS solutions (Fig. 5). From this result, PEG-¹¹¹In-YPO₄ NPs displayed high retention rate in both PBS and FBS solutions. Although the retention rate of ¹¹¹In of the PEG-¹¹¹In-YPO₄ NPs was decreased in FBS solutions, retention rate of PEG-¹¹¹In-YPO₄ NPs kept 90 % over a period of initial 4 h incubation. Therefore, PEG-¹¹¹In-YPO₄ NPs possess sufficient potential as SPECT imaging probes.

Fig. 3. Stability evaluation of PEG-In-YPO₄ NPs under physiological saline. (i) Particle size distribution of PEG-In-YPO₄ NPs in physiological saline. DLS measurement condition: sample concentration, 10 mg/mL. (ii) Relative turbidity of PEG-In-YPO₄ NPs in physiological saline. Absorbance measurement condition: laser wavelength, 550 nm; sample concentration: 10 mg/mL.
Fig. 4. OTN-NIR emission measurement of PEG-In-YPO₄ NPs (PEGylated at pH10.5). (i) Emission spectrum of PEG-In-YPO₄ NPs in pure water. Excitation wavelength, 980 nm; laser strength, 150 mW; sample concentration, 10 mg/mL. (ii) Observation of OTN-NIR emission of PEG-In-YPO₄ NPs in pure water. Excitation wavelength, 980 nm; laser strength, 150 mW.

Fig. 5. Retention rate of ¹¹¹In of the PEG-¹¹¹In-YPO₄ NPs (PEGylated at pH10.5). Measurement condition: sample concentration, 20 mg/mL.

Fig. 6. OTN-NIR and SPECT/CT in vivo dual modal imaging of a living mouse with intravenous injection of PEG-¹¹¹In-YPO₄ NPs (PEGylated at pH10.5). (i) Interval changes of OTN-NIR images of a living mouse. Emission measurement: room temperature; excitation wavelength, 980 nm; laser strength, 2.88 W. (ii) SPECT/CT images of a living mouse.

Finally, the OTN-NIR fluorescence and SPECT/CT imaging of live mice was demonstrated. PEG-¹¹¹In-YPO₄ NPs were injected into mice via tail veins and the live mice were observed by the OTN-NIR imaging system and SPECT/CT imaging system. Fig. 6 (i) shows interval changes of OTN-NIR image of live mouse just after injection (0 - 300 sec). From these results, OTN-NIR emission of PEG-¹¹¹In-YPO₄ NPs was observed from blood vessel just after injection (30 sec). Furthermore, strong OTN-NIR emission of PEG-¹¹¹In-YPO₄ NPs from the liver and spleen was also confirmed at 180 and 300 sec after injection. These results indicated the OTN-NIR fluorescence imaging can observe in early stage of imaging experiment just after injection of probe samples without dissection. The SPECT/CT imaging was also carried out to observe the same mice. Fig. 6 (ii) shows SPECT/CT images of a live mouse with various angles. These SPECT/CT images were acquired between 15 and 45 minutes after the injection of samples. These images clearly showed the three dimensional information of the organs of the live mouse. From these results, three dimensional images were successfully obtained from SPECT/CT imaging.
imaging. In this study, we used relatively large size YPO₄ NPs (d = 100 nm) and thus these NPs non-specifically accumulated mainly in the liver and spleen. To visualize specific tissues or organs by in vivo OTN-NIR fluorescence and SPECT imaging, small sized RED-CNPs would be required. The development of such kinds of probes is now under investigation.

4. Conclusion

We present here the first report of the dual-modal in vivo imaging of living mice based on OTN-NIR fluorescence and SPECT/CT. The OTN-NIR nanophosphor, YPO₄ NPs was synthesized by the hydrothermal synthesis method. YPO₄ NPs co-immobilized with biocompatible PEG and radioactive ^{111}In was successfully prepared. The pH value of PEGylation condition strongly affected on the PEG brush density on the NPs surface. PEG-b-PAMA interacted with the YPO₄ NPs through the electrostatic interaction between tertiary amino group of PAMA segment and Y³⁺ on the NPs surface. The PEG-^{111}In-YPO₄ NPs showed excellent dispersion stability in high ionic strength. The obtained PEG-^{111}In-YPO₄ NPs also showed high radioactivity in any of PBS and FBS solutions. Additionally, in vivo OTN-NIR fluorescence and SPECT/CT imaging of live mice was carried out. The strong OTN-NIR emission from the blood stream and organs of live mice were observed in vivo. Furthermore, SPECT/CT images of live mice were also detected by a SPECT/CT combined scanner. The obtained SPECT/CT images clearly showed the three dimensional view of the organs of live mice. This dual-modal imaging technique based on the obtained PEG-^{111}In-YPO₄ NPs possess strong advantages for successful in vivo imaging of both real-time imaging with the OTN-NIR imaging and three dimensional quantitative determination by the SPECT.

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References