Effects of Processing pH on Emission Intensity of Over-1000 nm Near-Infrared Fluorescence of Dye-Loaded Polymer Micelle with Polystyrene Core

Masakazu UMEZAWA,† Mae HARUKI, Moe YOSHIDA, Masao KAMIMURA, and Kohei SOGA†

Department of Materials Science and Technology, Faculty of Industrial Science and Technology, Tokyo University of Science, 6-3-1 Nitaku, Katsushika, Tokyo 125-8585, Japan

Fluorescence imaging using the over-thousand-nanometer (OTN) near-infrared (NIR) light is an emerging method for an in vivo imaging analysis of deep tissues without physical sectioning. Polymer micelle nanoparticles (PNPs) composed of organic polymers encapsulating an OTN-NIR fluorescent dye, IR-1061, in their hydrophobic core are expected to be biocompatible probes. Because IR-1061 quickly quenches due to the vibration of polar hydroxyl bonding in its surroundings, the influence of hydroxyl ions should be minimized. Herein, we investigated the effect of the hydrogen ion concentration during the preparation process using IR-1061 and an organic polymer, poly(ethylene glycol)-block-polystyrene (PEG-b-PS), on the emission properties of the obtained OTN-PNPs. The OTN-PNP has a hydrodynamic diameter of 20 – 30 nm and emits 1110-nm fluorescence that is applicable to angiography. The loading efficiency of IR-1061 in the OTN-PNPs increased when prepared in an aqueous solution with a low hydroxyl ion concentration. In this solution (pH 3.0), highly emissive OTN-PNPs was obtained with IR-1061 at lower nominal concentrations. Decreasing the hydroxyl ion concentration during the preparation process yields highly emissive OTN-PNPs, which may improve the in vivo imaging analysis of biological phenomena in deep tissues.

Keywords Near infrared, fluorescence bioimaging, polymer micelle, organic dye, pH

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Introduction

The noninvasive analysis of biological phenomena in deep live tissues has been a fundamental challenge in the biomedical field. Computed tomography (CT), magnetic resonance imaging, positron emission tomography, single-photon emission CT,¹¹ ultrasound,²¹,²²,²³ and optical imaging²⁴⁻²⁶ are emerging techniques for deep tissue imaging without any physical sectioning of objects. The transparency and penetration of signals from inside tissues are crucial factors in deep imaging analyses. In addition to the currently used near-infrared (NIR) (700 – 900 nm) method in deep imaging analyses,¹¹⁻¹³,¹⁵⁻¹⁷ the use of over-thousand-nanometer OTN-NIR light is employed in order to overcome low scattering attributes in biological tissues, and it has garnered attention as the second “biological window” in optical imaging.⁹ The use of OTN-NIR expands the observation depth from several millimeters in the visible and short-wavelength NIR region to 1 – 2 cm.¹⁰

Both imaging systems and probes have been studied for more refined and more functional OTN-NIR fluorescence imaging.¹¹ Following several reports of prototypes of imaging systems,¹²⁻¹³ a portable in vivo OTN-NIR fluorescence imaging system was commercially released from Japan in 2014.¹⁴ Three-dimensional (3D) OTN-NIR fluorescence microscopic imaging was realized by setups of confocal¹⁵⁻¹⁷ and light-sheet excitation microscopy systems.¹⁸ Recently, a novel 3D imaging system based on OTN-NIR fluorescence CT was reported for small animals (mice).¹⁹ An OTN-NIR fluorescence time-gated imaging system was also developed for the lifetime-based multiplex imaging of deep tissues.²¹⁻²³ Because the fluorescence lifetime is sensitive to temperature, the analysis technique of the fluorescence lifetime of OTN-NIR phosphors is a novel contactless temperature sensing approach for deep tissues.²⁴

Rare-earth-doped nanoparticles,¹⁰,¹²⁻¹⁴,¹⁹⁻²³,²⁷⁻³⁷ rare-earth ion chelates,²⁸ single-walled carbon nanotubes,²⁹⁻⁴⁵ quantum dots,⁴⁶⁻⁵³ and organic dye-based phosphors⁵⁴⁻⁶³ have been reported as OTN-NIR fluorescent molecular dyes are expected to be highly biocompatible (due to the lack of solid surface) and excretable probes⁶⁶⁻⁶⁹ for future biomedical applications.

Molecular dyes, such as IR-1061 and IR-26, are available as OTN-NIR fluorophores. However, it is difficult to dissolve these organic dyes in aqueous solutions; hence, they need to be conjugated or incorporated into biocompatible polymers. Previously, our group reported an easier method, the “one-pot” procedure, for preparing OTN-NIR fluorescence PNP-s (OTN-PNPs) by encapsulating a molecular dye, IR-1061, with an amphiphilic block copolymer containing the chain of poly(ethylene glycol) (PEG).⁵⁹ PNPs formed by the self-assembly of block copolymers composed of PEG and hydrophobic segments have been used as carriers of drug delivery systems because it can encapsulate small molecules in...
the hydrophobic core. Previous studies have investigated the \textit{in vivo} behavior of PNP encapsulating hydrophobic molecules as therapeutic drugs. Encapsulating water-insoluble molecular fluorescent dyes also provide OTN-PNPs that can be applied to the imaging analysis of deep live tissues. Because OTN-NIR dyes easily quench by the vibration of polar hydroxyl bonding in aqueous and physiological environments, minimizing the influence of surrounding hydroxyl ions on the dyes during the preparation process may improve the optical properties of the obtained OTN-PNPs. Herein, we aim to investigate the effect of the hydrogen ion concentration (pH) during the preparation process using IR-1061 and a block copolymer, PEG-block-polystyrene (PEG-b-PSt), on the emission properties of the obtained OTN-PNPs.

**Materials and Methods**

**Materials**

PEG-b-PSt ($M_w = 5000/1600$; P13141-SEO) was purchased from Polymer Source (Montreal, QC, Canada). IR-1061 and sodium hydroxide were purchased from Sigma-Aldrich (St. Louis, MO) and Kanto Chemical (Tokyo, Japan), respectively. Acetonitrile (ACN) and hydrochloric acid were purchased from Wako Pure Chemical Industries (Osaka, Japan). All of the reagents were used without further purification.

**Preparation of OTN-PNPs**

PEG-b-PSt (4 mg) and IR-1061 (0.63 – 30 $\mu$g; 0.84 – 80 nmol) were mixed in ACN (1.25 mL), and the resultant mixture was dropped into 2 mL of distilled water (pH 5.6), an aqueous solution of hydrochloric acid (pH 3.0), or sodium hydroxide (pH 11.0). Thus, the nominal concentration of IR-1061 corresponds to 0.32 – 30 $\mu$g mL$^{-1}$ (0.42 – 40 $\mu$M) in a solution of pH 3.0, 5.6, or 11.0. ACN was removed by evaporation from the aqueous OTN-PNP suspension while stirring at room temperature for 9 h (Fig. 1). The obtained OTN-PNPs were purified by centrifugation on a spin-column (MWCO 10 kDa, 27000g, 15 min, 3 times) and then dispersed in distilled water.

**Characterization of OTN-PNPs**

The hydrodynamic diameter of OTN-PNPs was analyzed by dynamic light scattering using an LB-550 (Horiba, Kyoto, Japan). Furthermore, the absorption spectra of the OTN-PNPs were analyzed using a spectrometer, V-770 (Shimadzu, Kyoto, Japan). The emission spectra of OTN-PNPs were analyzed using a spectrometer (NIR-256-1.7; Avantes, Apeldoorn, Netherlands) equipped with a fiber-coupled laser diode (SP-976-5-1015-7; Laser Components, Olching, Germany) as the 980 nm excitation source. The total concentration of IR-1061 loaded into OTN-PNPs was determined by the absorption.

**OTN-NIR fluorescence in vivo imaging**

All animal experiments were conducted according to the national and institutional guidelines on the care and use of laboratory animals with the approval of the Tokyo University of Science’s Animal Care and Use Committee. Female 4-week-old ICR mice were purchased from Japan SLC (Hamamatsu, Japan) and fed the AIN-76A diet (Research Diets, New Brunswick, NJ) for 2 weeks to weaken the autofluorescence of the body. The mice (6 weeks of age) were anesthetized and their hair was removed to avoid light scattering. Then, 0.2 mL of the OTN-PNP dispersion in physiological saline (containing 50 $\mu$g mL$^{-1}$ of IR-1061) was intravenously injected \textit{via} the tail vein. OTN-NIR fluorescence images were collected using a portable in vivo OTN-NIR fluorescence imaging system (SAI-1000, Shimadzu).

**Results and Discussion**

The OTN-PNPs were prepared by dropping a mixture of PEG-b-PSt (4 mg) and IR-1061 (15 – 60 $\mu$g) in ACN into 2 mL of water (pH 5.6), followed by stirring to remove ACN for micellization. The hydrodynamic diameter of the obtained OTN-PNPs was approximately 25 nm. The fluorescence intensity of the OTN-PNPs composed of 2 mg mL$^{-1}$ of PEG-b-PSt in water was highest when they were prepared with IR-1061 at a nominal concentration of 12.5 $\mu$g mL$^{-1}$ (Fig. 2a). The OTN-PNPs composed of PEG-b-PSt and IR-1061 emitted the OTN-NIR wavelength peaked at 1110 – 1120 nm under 980 nm excitation, and successfully visualized the whole-body blood vessels after intravenous injection in mice (Fig. 2b). While the blood vessels were visualized for 3 h post-injection, the representative OTN-NIR fluorescence image of mice acquired 1 min post-injection is shown in Fig. 2b.

To investigate the effect of the hydrogen ion concentration during the micellization process on the properties of the obtained OTN-PNPs, the OTN-PNPs were prepared by dropping a mixture of PEG-b-PSt (4 mg) and IR-1061 (25 $\mu$g) in ACN into...
2 mL of an aqueous solution of hydrochloric acid or sodium hydroxide (the nominal IR-1061 concentration was 12.5 μg/mL) with different hydrogen-ion concentrations (pH 3.0, 5.6, or 11.0), followed by stirring to remove ACN. The obtained OTN-PNPs showed hydrodynamic diameters of 21 ± 5, 27 ± 7, and 25 ± 7 nm when prepared at pH 3.0, 5.6, and 11.0, respectively (Fig. 3a). The optical absorbance of IR-1061 encapsulated in the PNP increased when it was prepared under acidic conditions.
(pH 3.0), whereas the absorbance decreased when prepared under alkaline conditions (pH 11.0) (Fig. 3b). By calculating the loading amount of IR-1061 in the PNP from the concentration-dependent change in the optical absorbance (Fig. 3c), the final concentration of IR-1061 was found to be 7.4, 3.84, and 1.01 μg/mL when they were prepared in aqueous solutions of pH 3.0, 5.6, and 11.0, respectively; thus, the loading efficiencies of IR-1061 into the PNPs were 59.3, 30.7, and 8.1%, respectively. The results indicate that the loading efficiency of IR-1061 changes with the hydrogen ion concentration during the micellization process. The PNPs showed OTN-NIR fluorescence peaks at 1110 – 1120 nm, which are available for \textit{in vivo} deep imaging, under 980 nm NIR excitation (Fig. 3d). The fluorescence intensity changed with the condition (pH) during the micellization process. The OTN-PNP prepared at pH 11.0 showed only low fluorescence intensity due to its low loading efficiency of IR-1061. Physicochemically, we first hypothesized that the ionization of the cationic dye, IR-1061, is less in an environment with fewer protons (high pH), and thus would be more encapsulated into the hydrophobic polystyrene core. However, the results were opposite, and suggested that the influence of vibration quenching by hydroxyl ions on the electron state of IR-1061 was more dominant. The high concentration of hydroxyl ions (high pH) affected the electron status and the optical properties of IR-1061, which decreased the loading efficiency of the emissive form of the dye. Although the OTN-PNP prepared at pH 3.0 showed a higher loading efficiency of IR-1061 than the other two products, its fluorescence intensity was lower than that of the OTN-PNP prepared at pH 5.6 and 11.0 (Fig. 3d). The decrease in the fluorescence intensity was likely due to the concentration quenching of IR-1061 in the hydrophobic core of the OTN-PNP. Because of the spontaneous mobility of molecules forming OTN-PNPs by self-organization, IR-1061 in the core may leak out while being suspended in water under neutral conditions (pH 5.6) for dozens of hours. This leakage caused a decrease in the fluorescence intensity when the OTN-PNPs were prepared at pH 5.6 and 11.0, whereas it showed a transient increase in the fluorescence intensity at 24 – 96 h after preparation when prepared at pH 3.0 (Fig. 3e). The decrease in the fluorescence may also be caused by quenching of the dye by the influence of invaded water molecules into the micelle cores. The results suggest that the optimal nominal concentration of IR-1061 may be dependent on the pH during preparation.

Therefore, the dependence of the fluorescence intensity of OTN-PNPs on the nominal concentration of IR-1061 was investigated in the case of micellization in aqueous solutions of pH 3.0 and 11.0. The intensity showed peaks at the nominal concentrations of 5.0 and 14 μg/mL when the OTN-PNPs were prepared at pH 3.0 and 11.0, respectively (Fig. 4a), while it was highest at the nominal concentration of 12.5 μg/mL with 2 mg/mL PEG-b-PSt when they were prepared at pH 5.6 (Fig. 2a). The hydrodynamic diameters of OTN-PNPs prepared under both conditions were approximately 20 – 30 nm (Fig. 4b).
final concentration of IR-1061, determined by optical absorbance (Fig. 4c) along with the calibration curve (Fig. 3c), was 3.05 and 1.00 μg/mL when prepared with the optimal nominal concentrations for aqueous solutions at pH 3.0 and 11.0, respectively; therefore, the loading efficiencies of IR-1061 into the PNPs were 60.9 and 7.1%. The fluorescence intensity of OTN-PNP prepared at pH 3.0 with optimal nominal concentration (Fig. 4d) was 1.34-times higher than that of the original one prepared at pH 5.6 (Fig. 3d). Since the concentration quenching of IR-1061 in the OTN-PNPs prepared at pH 3.0 was suppressed, a transient increase in the fluorescence intensity at 24 – 96 h after preparation, due to leakage of the dye molecule from the hydrophobic core, was not observed (Fig. 4e). The results showed that increasing the hydrogen ion concentration quenching of IR-1061 in the OTN-PNPs prepared at pH 3.0 was suppressed, a transient increase in the fluorescence intensity remained at about 80% for 24 h after preparation, it decreased to 28% after 160 h. Although the fluorescence intensity remained at about 70% for 24 h at pH 5.6, the fluorescence intensity increased under low pH (mildly acidic) conditions because of the low concentration of hydroxyl ions that quench the OTN-NIR dye in the reaction system. The optimized protocol presented herein for the preparation of OTN-PNPs will provide highly emissive probes, and is expected to open up further OTN-NIR fluorescence imaging applications to analyze biological structures and phenomena in deep live tissues.

Conclusions

Highly emissive OTN-PNPs composed of PEG-b-PSt were obtained by lowering the pH during micellization by encapsulating IR-1061 dye. The loading amount of fluorescent dye into PNPs increased under low pH (mildly acidic) conditions because of the low concentration of hydroxyl ions that quench the OTN-NIR dye in the reaction system. The optimized protocol presented herein for the preparation of OTN-PNPs will provide highly emissive probes, and is expected to open up further OTN-NIR fluorescence imaging applications to analyze biological structures and phenomena in deep live tissues.

Conflicts of Interests

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