Bioconjugate Chemistry

Communication

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Axial PEGylation of Tin Octabutoxy Naphthalocyanine Extends Blood Circulation for Photoacoustic Vascular Imaging

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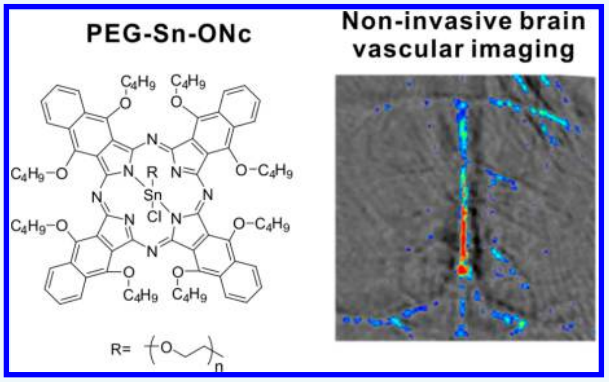
Supporting Information

ABSTRACT: Attachment of polyethylene glycol (PEG) can prolong blood circulation of biological molecules, a useful trait for a vascular imaging agent. Here, we present a route for modifying octabutoxy naphthalocyanine (ONc) with PEG, via axial conjugation following ONc chelation with Sn(IV) chloride (Sn-ONc). Tin chelation caused ONc absorbance to shift from 860 to 930 nm. Hydroxy terminated PEG was treated with sodium and then was axially attached to the tin, generating PEG-Sn-ONc. Unlike ONc or Sn-ONc, PEG-Sn-ONc was soluble in methanol. ONc and PEG-Sn-ONc were dissolved in polysorbate solutions and administered to mice intravenously. PEG-Sn-ONc demonstrated substantially longer blood circulation time than ONc, with a 4 times longer half-life and a nearly 10 times greater area under the curve. PEG-Sn-ONc gave rise to photoacoustic contrast and could be used for noninvasive brain vessel imaging even 24 h following injection. This work demonstrates that nonmetallic naphthalocyanines can be chelated with tin, and be axially modified with PEG for enhanced circulation times for long-term vascular imaging with photoacoustic tomography.



INTRODUCTION

Naphthalocyanine (Ncs) are hydrophobic and aromatic chromophores that exhibit intense absorbance in the near-infrared (NIR). Just like related porphyrins and phthalocyanines (Pcs), the two central hydrogen atoms of the tetrapyrrolic macrocycle are active and can be substituted by a wide range of elements (e.g., silicon, zinc, tin, gallium, aluminum, etc.) during the preparation process or via postchelation.[1−4] These central elements can modulate the optical properties of Nc.[5]

Good photostability and high NIR extinction coefficients make Ncs candidates for contrast agents for bioimaging techniques. For example, they have been used for NIR imaging,[6] photoacoustic tomography,[7,8] and positron emission tomography (PET),[8] making them candidates for multimodal imaging.[9] Ncs generally absorb at longer wavelengths than Pcs; they operate further in the NIR spectrum, where tissue scattering is decreased. However, their extreme hydrophobicity has probably limited their application in these fields. Researchers have attempted to address poor solubility issues by adopting strategies such as modification of aromatic rings with anionic functional groups.[10,11]

Due to differences in valency, elements chelated in the center of Pcs and Ncs have varying coordination properties. The most common are tetra- (Zn(II)),[12,13] penta- (Ga(III) and In (III)),[14−16] or hexacoordinate (Si(IV) and Sn(IV)).[17−19] To stabilize the penta- and hexacoordinate structure, chelation groups, such as chlorides or hydroxyls, are also attached to the axial positions. These axial ligands provide the possibility to modify Pcs and Ncs that do not have other reactive organic groups around the macrocyclic ring through chemical approaches.

Axial silicon Pcs substitutes have been widely studied and applied in both imaging and phototherapeutic applications in vitro and in vivo.[20−23] Polyethylene glycol (PEG) is a hydrophilic and biocompatible polymer chain, and axial conjugation has been used to improve the hydrophilicity of Pcs.[24] PEGylation has been shown to extend the blood circulation time by reducing reticuloendothelial cell uptake.[25−28] Axial modifications of Ncs for biological applications have not been frequently reported. In one example, Si-Nc was axially modified with hydrophobic oleate moieties to better confer loading into low density lipoproteins.[29] In another example, Si-Nc was modified with small hydrophilic PEG chains (molecular weight 1900 Da) for aqueous intra-

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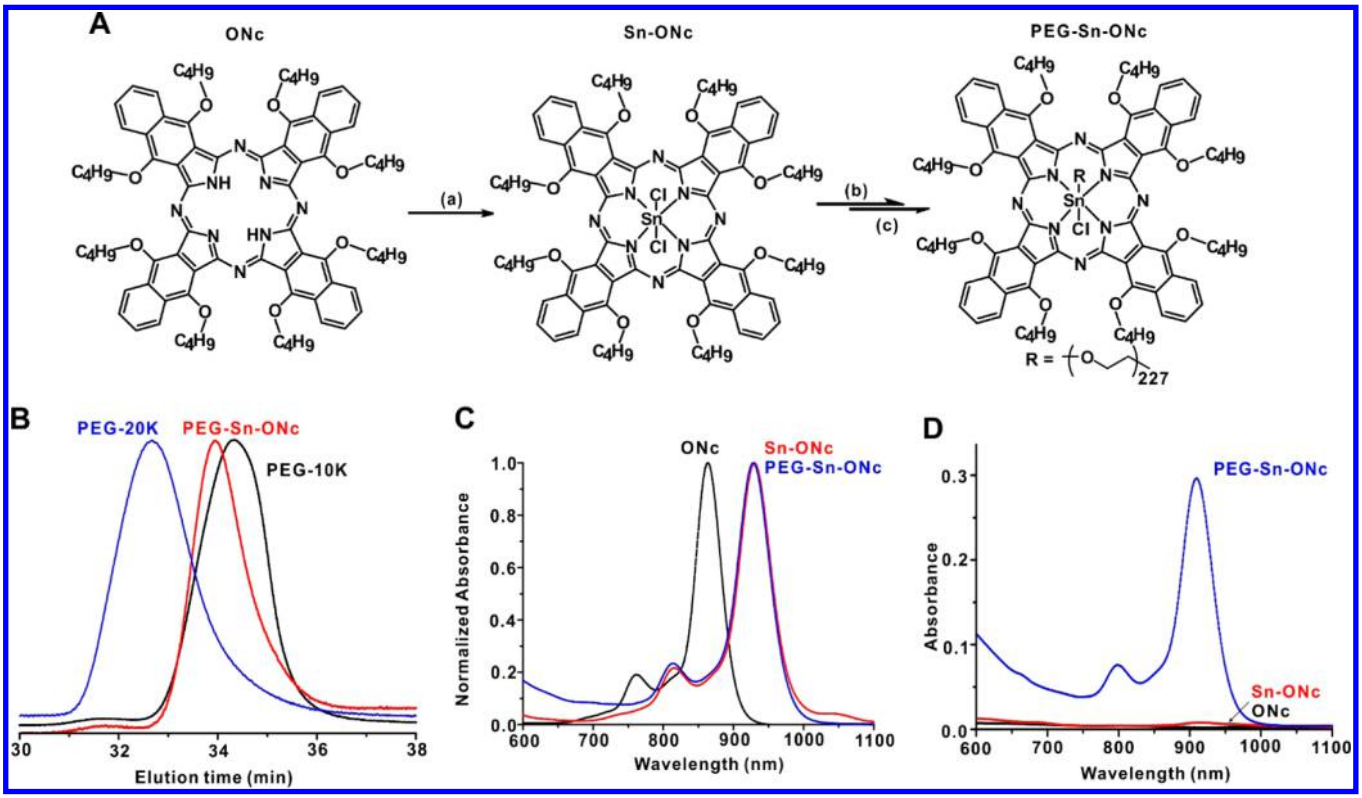

Figure 1. Synthesis of PEG-Sn-ONc: (A) Synthesis procedure. Conditions: (a) $SnCl_2 \cdot 2H_2O$, DMF, 163 °C reflux, 4 h; (b) $AgPF_6$, anhydrous toluene, 60 °C overnight; (c) PEG-10K and NaH, anhydrous toluene, refluxed 3 h, and transferred to the $AgPF_6$-reacted Sn-ONc solution and refluxed for 48 h. (B) Gel permeation chromatography of PEG-Sn-ONc (red), PEG-10K (10 kDa, black), and PEG-20K (20 kDa, blue). (C) Normalized absorbance spectra of ONc (black), Sn-ONc (red), and PEG-Sn-ONc (blue) in methylene chloride. (D) Absorbance of soluble fraction (supernatants) of dyes dissolved in methanol. ONc (black), Sn-ONc (red), and PEG-Sn-ONc (blue). Supernatants were diluted 100 times prior to measurement.

venous administration to rodents, although little dye remained in serum by 24 h.[30] To the best of our knowledge there have not yet been reports involving axial modifications of Sn(IV) Ncs for biomedical imaging applications.

While tetrapyrroles can be conjugated to polymers through a variety of convenient routes such as amidation,[31] the axial route provides an elegant and practically intrinsic handle for conjugation. Here, we adopt a postchelation approach involving insertion of Sn into the preformed Octabutoxy naphthalocyanine (ONc) macrocycle to generate Sn(IV) octabutoxy-naphthalocyanine (Sn-ONc). We then demonstrate an axial PEGylation strategy that extends circulation time for long-term vasculature imaging with photoacoustic computed tomography (PACT).

PACT is an emerging diagnostic technique providing the potential for monitoring contrast in biological tissues with high contrast and depth.[32−35] PACT can penetrate much deeper into living bodies than other optical techniques, and detect biological objects ranging from organelles to organs, such as kidney,[36] brain,[37] and tumors.[38] Phosphorus Pc has been used as a PACT contrast agent and was detected through an entire 5 cm human arm.[39] ONc has been encapsulated into Pluronic micelles and used for a variety of imaging applications.[8,40,41] It is known that after introducing Sn (IV), Sn-ONc possesses a longer absorbance close to 930 nm, compared to its original 863 nm.[4]

RESULTS AND DISCUSSION

The ONc tin chelation and axial PEGylation process is shown in Figure 1A. $SnCl_2$ chelation to ONc followed the method reported by Bae and co-workers.[4] ONc was reacted with $SnCl_2$ in anhydrous DMF for 4 h and then was purified with a size exclusive column to yield Sn-ONc. Without further modification, the axial chloride of Sn-ONc was replaced by reaction with silver hexafluorophosphate in toluene overnight. PEG-10K (with one terminal hydroxyl group) was reacted with NaH in anhydrous toluene to generate Na-PEG-10K. Na-PEG-10K was combined with the hexafluorophosphate Sn-ONc solution and further reacted for 48 h to obtain the final PEGylated compound, PEG-Sn-ONc. The NMR data of all of the compounds are shown as Figure S1 and Figure S2. IR data are shown in Figure S3.

With gel permeation chromatography, the product demonstrated a similar but slightly earlier elution time as PEG-10K alone, but a much later elution time than PEG-20K (Figure 1B). This indicates there was only one PEG-10K chain successfully conjugated to the axial position. Because of the long PEG chain used, steric hindrance from the PEG may have blocked reactive groups on one side of the dye. PEG-Sn-ONc had a similar absorbance spectrum as Sn-ONc, demonstrating that PEGylation did not alter electronic configurations of the Nc (Figure 1C). ONc, Sn-ONc, and PEG-Sn-ONc were adjusted to the same molar concentration and were dissolved in methanol. Following centrifugation to remove aggregates, the supernatants were diluted 100 times to test their absorbance. As shown in Figure 1D, PEG-Sn-ONc demonstrated high methanol solubility compared to the other two compounds, showing that PEG addition improves solubility in a polar solvent.

The cytotoxicity of PEG-Sn-ONc was examined in vitro in U-87 cancer cells (Figure S4). It generally demonstrated similar cytotoxicity as the commonly used NIR dye methylene blue. For in vivo studies, PEG-Sn-ONc or ONc was dissolved in polysorbate-20 (TWEEN 20) and intravenously administered

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to mice. After injection of 6 optical densities (O.D.) of sample (200 μL, with a NIR absorbance of 30 for a 1 cm path length cuvette after considering the dilution factor), PEG-Sn-ONc exhibited a much longer blood circulation time than ONc, as shown in Table 1. The area under the curve parameter was nearly

Table 1. Blood Circulation Parameters

	ONc	PEG-Sn-ONc
Injected Dose (O.D.)	6	6
$t_{1/2}$ (h)	1.7 ± 1.2	5.6 ± 1.2
Mean Residence Time (h)	2.5 ± 1.7	8.1 ± 1.8
Steady state volume of distribution (O.D./O.D)	4.2 ± 1.0	1.4 ± 0.25

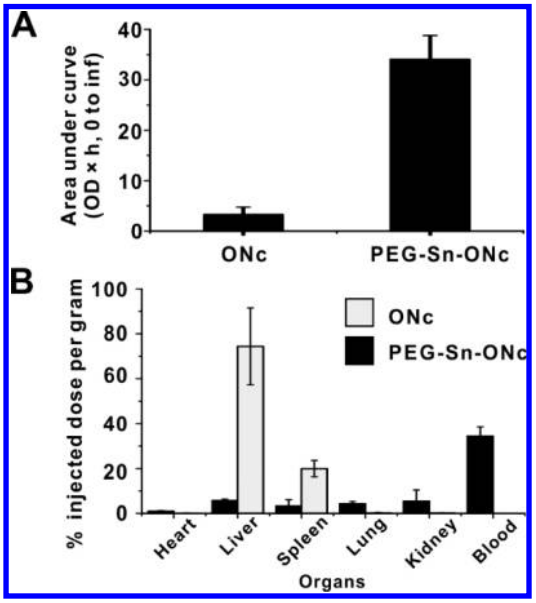

Figure 2. (A) Area under the blood concentration−time curve of ONc and PEG-Sn-ONc. (B) Biodistribution of PEG-Sn-ONc (black) and ONc (gray) 24 h after intravenous administration. Data show mean ± std deviation for n = 3 mice per group.

10 times greater for PEG-Sn-ONc than ONc (Figure 2A). Preliminary biodistribution was assessed using NIR optical absorption measurements of homogenized tissues. At the 24 h time point, a large proportion of PEG-Sn-ONc, but very little ONc, remained in the blood (Figure 2B). ONc was taken up mostly in the liver and spleen. This is consistent with the paradigm that PEGylation can induce evasion of macrophages in the reticular endothelial system, which is largely represented by the liver and spleen.[42] Similar results have been observed in other materials which were coated with longer chain PEGs. Lipka et al. found that gold nanoparticles coated with PEG-10K demonstrated longer blood circulation time with lower amounts of liver and spleen uptake relative to those coated with PEG-750.[43] There are also several other parameters that could affect macrophage uptake including the conformation of the biomaterials.[44]

The longer blood circulation time provides the possibility for long-term blood vessel monitoring. Because of its NIR absorbance, PEG-Sn-ONc was used as contrast agent for long-term blood vessel imaging by PACT. Six optical densities (O.D.) of ONc or PEG-Sn-ONc were injected into mice and the signals of brain vessels were monitored immediately and 24 h post injection. Photoacoustic imaging wavelengths were selected on the basis of the peak absorption of each dye in TWEEN 20 (Figure S5). Mice had fully intact skulls during the procedure. As shown in Figure 3, PEG-Sn-ONc illustrated a clearer photo-

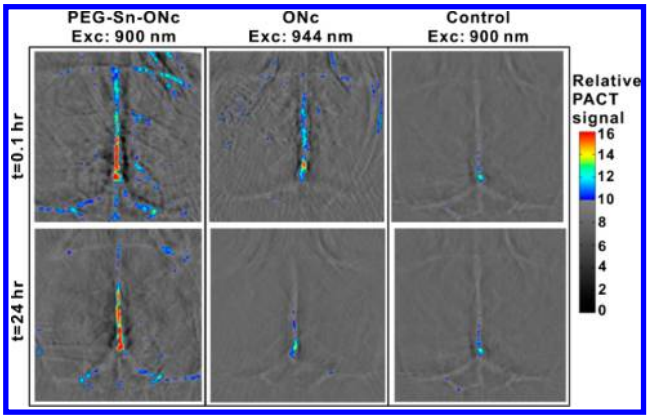

Figure 3. Noninvasive photoacoustic images of brain blood vessels of mice administered with 6 O.D. of PEG-Sn-ONc or ONc at the indicated time points following intravenous injection.

acoustic map of the brain vessels than ONc immediately after the injection. Twenty-four hours later, the PEGylated ONc still demonstrated strong photoacoustic signals while the signals from ONc group were low, and were almost the same as the untreated control group. The overall photoacoustic intensities of these three groups were quantified and are shown in Figure S6. By 24 h, the non-PEGylated ONc group had returned to the same photoacoustic intensity of the control mice, whereas the PEG-Sn-ONc group photoacoustic signal remained elevated.

In summary, the chelation of Sn (IV) to the center of ONc shifts its absorbance from 860 to 930 nm. Axial PEGylation was then performed with the Sn-ONc macrocycle intact and with unchanged NIR absorbance. The PEG chain provided improved solubility in polar solvents. After injection into mice, PEG-Sn-ONc exhibited longer blood circulation than ONc. Mice showed strong photoacoustic signals 24 h post injection to enable vascular brain imaging. In the future, other functional groups could be used to modify the axial position of these Ncs to achieve modulated biological properties.

EXPERIMENTAL SECTION

Materials. 5,9,14,18,23,27,32,36-Octabutoxy-2,3-naphthalocyanine (ONc) was from HPPE Co., tin dichloride was from Alfa Aesar, PEG-10K, PEG 20-K and silver hexafluorophosphate were from Sigma-Aldrich, TWEEN 20 was from Amresco, Biobeads SX-1 were from Bio-Rad Laboratories, XTT was from Biotium. ICR mice were ordered from Harlan Laboratories.

Postchelation of Tin Dichloride into ONc. 58 mg (0.045 mmol) ONc was mixed with 300 mg (1.33 mmol) $SnCl_2$ in anhydrous DMF and refluxed for 4 h. DMF was removed by rotary evaporator. The product was obtained by purification with Biobeads SX-1 size exclusive column in toluene. 40.2 mg of product was obtained (0.027 mmol) (60.5% yield). ^{1}H NMR (500 MHz, benzene-d_6): δ = 9.14 (m, 8H, 1,4-Nc H); 7.67 (m, 8H, 2,3-Nc H); 5.25 (t, 16H, Bu-α-CH_2); 2.24 (m, 16H, Bu-β-CH_2); 1.62 (m, 16H, Bu-γ-CH_2); 1.01 (t, 24H, Bu-CH_3). MS (Maldi-TOF): m/z = 1478.56 [M+] (calculated 1479).

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Synthesis of PEG-Sn-ONc. The axial chloride of $SnCl_2ONc$ (100 mg, 0.068 mmol) was removed using silver hexafluorophosphate (170.94 mg, 0.68 mmol) in anhydrous toluene at 60 °C overnight. In the meantime, polyethlyene glycol-10 000 (PEG-10K) (2040 mg, 0.204 mmol) was refluxed with NaH (60% dispersion, 8.16 mg, 0.204 mmol) in anhydrous toluene for 3 h. After the Na-PEG-10K toluene solution cooled to 60 °C, it was transferred to the $SnCl_2ONc$-hexafluorophosphate toluene solution, and refluxed at 115 °C for 48 h. Toluene was removed by rotary evaporation, and then the product was washed with ethyl ether and methanol to precipitate out unincorporated PEG-10K and $SnCl_2ONc$. The product was further purified by Biobeads SX-1 size exclusion column in toluene. 77.4 mg of product was obtained (10% yield). ^1H NMR (500 MHz, benzene-d_6): δ = 8.39 (m, 8H, 1,4-Nc H); 7.29 (m, 8H, 2,3-Nc H); 4.47 (t, 16H, Bu-α-CH_2); 3.48 (m, 938H, PEG-CH_2); 1.78 (m, 16H, Bu-β-CH_2); 1.47 (m, 16H, Bu-γ-CH_2); 0.88 (t, 24H, Bu-CH_3).

Cell Viability. Human glioblastoma (U87) cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin and were maintained at 37 °C with 5% CO_2. Cells were seeded in a 96-well plate at a concentration of 2×10^4 cells/well 24 h before the addition of dyes. PEG-Sn-ONc or methylene blue was added to wells containing DMEM with serum to achieve a final concentration as indicated in the figure. Sample containing media was removed 24 h later and the wells were washed with PBS before adding fresh media with FBS. To perform the XTT assay, media was aspirated out and cells were washed with 100 μL of PBS gently. 100 μL of PBS containing XTT (50 μg/mL) and of PMS (*N*-methyl dibenzopyrazine methyl sulfate) (60 μg/mL) was added to each well. Plate absorbance was read 2 h after XTT treatment at 450 and 630 nm (background). Cell viability was calculated after subtracting blank values sets. All measurements were made in triplicate.

In Vivo Studies. Animal studies were carried out in accordance with the University at Buffalo IACUC. Ten mg of PEG-Sn-ONc was dissolved in 100 μL TWEEN 20 and the mixture was sonicated and centrifuged at 14 000 rpm for 15 min to remove aggregates. The solution was diluted with water to obtain a NIR absorbance of 30, the solution was centrifuged again to remove any aggregates, and the absorbance of the solution was confirmed. Female ICR mice were injected with 200 μL solution (for NIR O.D. = 6). Blood samples were collected at 1, 2, 4, 6, 8, 12, and 24 h post injection. Serum was obtained following centrifugation for 15 min at 2000 rpm and was diluted in water to measure the NIR absorbance. PKSolver software was used to determine pharmacokinetic parameters. Organs including heart, liver, spleen, lung, kidney, and blood were collected for biodistribution. Organ pieces were mixed with 500 μL nuclear lysis buffer [0.25 mol/L sucrose, 5 mmol/L TriseHCl, 1 mmol/L $MgSO_4$,1 mmol/L $CaCl_2$ (pH 7.6)] and were homogenized with a steel bead-based Bullet Blender homogenizer according to manufacturer recommendation. Supernatants were centrifuged and absorbance was measured. Absorbance was tested and compared to the original absorbance of administrated solution to calculate the biodistribution. For the mice administrated ONc, the same organs were collected and ONc distribution was determined using previously published methods involving dichloromethane extraction.[8]

PA Imaging. An Nd:YAG pumped OPO laser (SureliteTM OPO Plus, Continuum) was chosen as the excitation light source. The laser beam was routed to the mouse brain through a 1.2-cm-diameter fiber bundle. The maximum light intensity at the scalp was around 14 mJ/cm^2, which was far below the American National Standards Institute (ANSI) safety limit of 50 mJ/cm^2 for 900 and 60 mJ/cm^2 for 940 nm. The photoacoustic signal was detected with a customized 3/4 ring transducer array with 128 elements and 5 MHz central frequency. The radius of the ring array was 40 mm and each element formed an elevation focus at 35 mm. Thus, elevation resolution and receiving sensitivity are relatively uniform at the central 10 mm radius region. The received PA signals were amplified (by 54 dB) and digitized by a 128-channel ultrasound data acquisition system (Vantage, Verasonics). The raw channel data was reconstructed and displayed in real time using the universal back-projection algorithm.[45] Two ICR mice were imaged immediately and 24 h after injecting 6. O.D. ONc or PEG-Sn-ONc, respectively. A control mouse without injection was also imaged for comparison.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.6b00280.

NMR, FTIR spectra, cell viability, absorption spectra, and photoacoustic signal analysis (PDF)

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Notes

The authors declare no competing financial interest.

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