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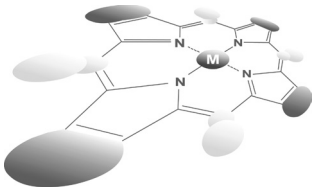

Synthesis, properties and near-infrared imaging evaluation of glucose conjugated zinc phthalocyanine *via* Click reaction

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ABSTRACT: In order to develop a novel near infrared fluorescence agent, glucose conjugated zinc phthalocyanine, [2,9(10),16(17),23(24)-tetrakis((1-(β-D-glucopyranose-2-yl)-1H-1,2,3-triazol-4-yl)methoxyl)phthalocyaninato]zinc(II), was synthesized *via* Click reaction. Their chemical structures were characterized by mass spectrometry, nuclear magnetic resonance spectrum. Their light stability and fluorescence quantum yield were evaluated by UV-visible and fluorescent spectroscopic method. Optical imaging *in vivo* was performed with this saccharide conjugated phthalocyanine as probe on liver tumor-bearing nude mice. Near-infrared imaging effect, organ aggregation as well as distribution of probe *in vivo* were evaluated by *in vivo* fluorescence imaging technique. Results show that glucose conjugated zinc phthalocyanine has favorable water solubility, good optical stability and high emission ability in near infrared region. Imaging results demonstrate that saccharide conjugated phthalocyanine has possess obvious imaging effect *in vivo*, which implies its potential in cancer diagnosis as near infrared optical probe.

KEYWORDS: near infrared fluorescence probe, zinc phthalocyanines, saccharide conjugated, Click reaction.

INTRODUCTION

Molecular imaging *in vivo* can display the biological processes at the cellular and molecular level, reveal the mechanism of physiological and pathological processes, and provide effective methods of detecting and tracking the diseases treatments. The applications of molecular imaging include the early clinical disease diagnosis, qualitative and quantitative evaluation of curative effect and so on [1, 2]. Comparing with CT, PET, MRI imaging modality, optical imaging has many advantages, such as low cost, non-ionic low-energy radiation, high sensitivity, continuous real-time monitoring, non-invasive or minimally invasive [3, 4]. Furthermore, the fact that organisms have low scattering effect and background interference in near-infrared region(NIR), increases near-infrared image sensitivity and penetrating depth in biological tissues [5, 6]. So near-infrared fluorescence imaging can be used in deep tissues and organs for detection and imaging owning to its specific advantages [7–9].

However, imaging events *in vivo* by fluorescence is limited by the probes available. As optical probe several properties are required including light transmission in depth, the luminescence quantum yield, good biocompatibility as well as targeting ability, so a great scientific effort has been focused on the development of near-infrared fluorescence probe. Phthalocyanines fluorescent dyes have large visible to NIR absorption molar extinction constant, acceptable fluorescent quantum yields, and good biocompatibility [10, 11]. Up to now, several kinds of phthalocyanines compounds with emission in NIR region have been developed [12, 13]. However, their low water solubility and no cell specificity were some obstacles for biomedical applications. The combination of carbohydrate moieties with macrocycles can not only improve the substrate solubility and

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biocompatibility [14], but also increase tumor targeting function of imaging probes or Photodynamic Therapy (PDT) sensitizer [15, 16]. In NIR optical imaging field, glucose conjugated phthalocyanine as NIR fluorescence probe has not been reported before. In this paper, near-infrared fluorescent probe was designed to meet molecular probes function and improve imaging effects by combining phthalocyanine with saccharide.

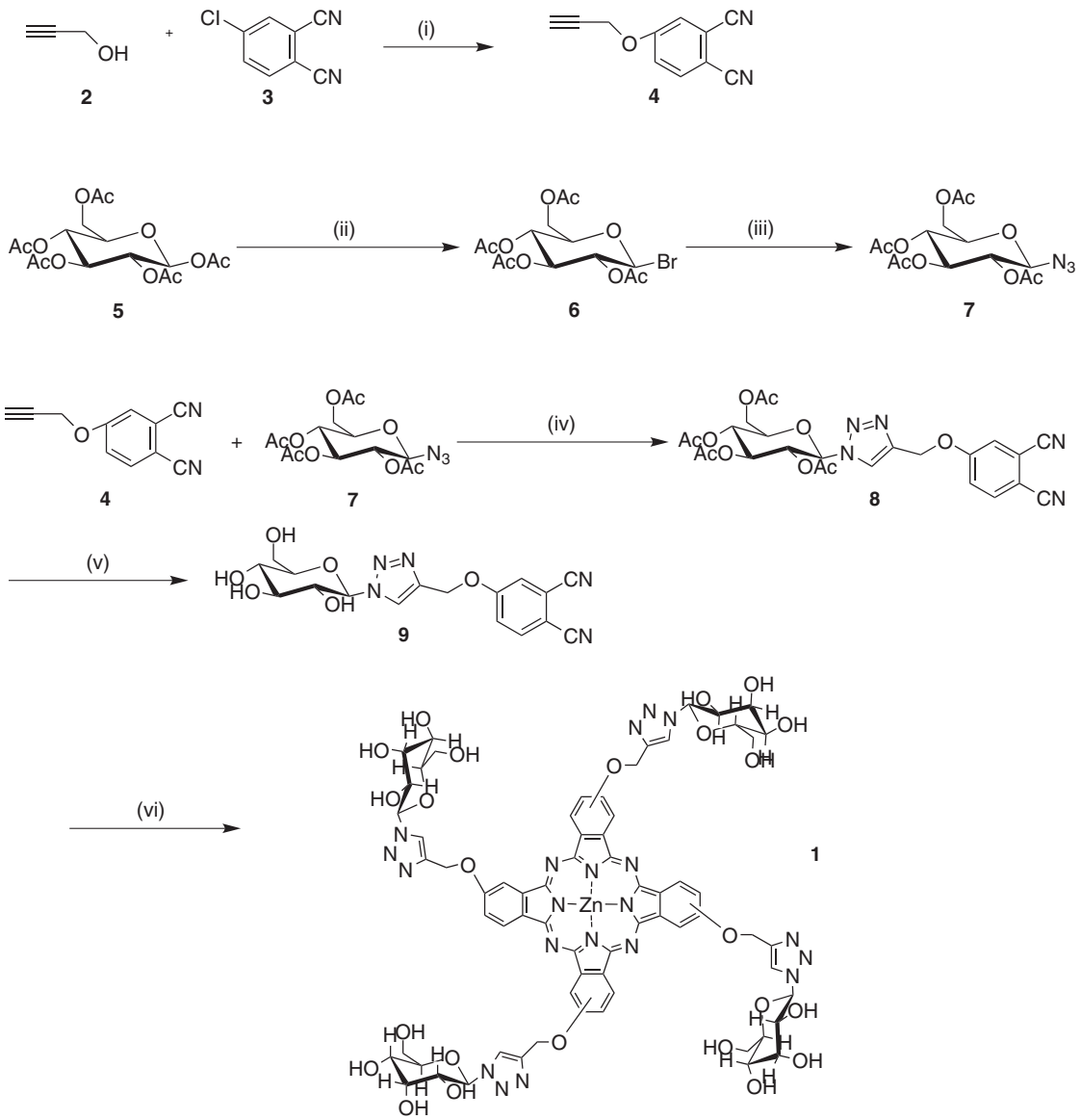
Saccharide conjugated macrocyclic compounds usually were achieved using coupling methods such as esterification, amidation or etherification [17, 18]. Recently, saccharide decorated macrocyclic compounds including porphyrin or phthalocyanine were synthesized by Click reaction as a novel method instead of traditional synthesis method [19, 20]. Click chemistry has been exploited for the generation of neoglycoconjugates and been applied in a wide variety of research areas, including material science, polymer chemistry, and pharmaceutical sciences because of the simplicity of this reaction and the easy workup procedure for the resulting products [21]. The characteristics of tolerance of typical biological conditions, tolerance of most functional groups makes click reaction particularly ideal for bioconjugations [22, 23]. In carbohydrate chemistry, propargyl glycosides have thus been widely utilized with alkyl azides. Based on this approach, we synthesized glucose conjugated phthalocyanine [2,9(10),16(17),23(24)-tetrakis((1-(β-D-glucopyranose-2-yl)-1H-1,2,3-triazol-4-yl) methoxyl) phthalocyaninato]zinc(II). The fluorescent imaging *in vivo* with glucose conjugated phthalocyanine as probe was reported with liver tumor-bearing athymic nude mice as animal model.

RESULTS AND DISCUSSION

Glucose conjugated phthalocyanine [2,9(10),16(17), 23(24)-tetrakis((1-(β-D-glucopyranose-2-yl)-1H-1,2,3-triazol-4-yl)methoxyl)phthalocyaninato]zinc(II) (**1**) was achieved according to Ref. 24 by condensation of its precursor 4-((1-(3,4,5-trihydroxy-6-(hydroxymethyl)-tetra-hydro-2H-pyran-2-yl)-1H-1,2,3-triazol-4-yl-methoxy)phthalonitrile (**9**), which was achieved in a routine three step route from 4-propyne oxide phthalonitrile(4) and 1,2,3,5-tetra-O-acetyl-β-D-glycopyranosyl azide (**7**) through Click reaction (Scheme 1). 4-propyne oxide phthalonitrile (**4**) was achieved from reaction of 4-chlorophthalonitrile (**2**) and propiolic alcohol (**3**) in DMF for 24 h at 60 °C in the presence of potassium carbonate. After recrystallization from methanol, the compound (**4**) was obtained as a yellow-white solid in 65% yield. Hydrogen bromide in acetic acid reacted with 1,2,3,4,6-penta-O-acetyl-β-D-glucopyranose (**5**) in CH_2Cl_2 at 0 °C to give 2,3,4,6-tetra-O-acetyl-β-D-glycopyranosyl bromide (**6**) in 80% yield. Then compound (**6**) reacted with NaN_3 in water at 70 °C overnight with benzyltriethylammonium chloride as phase transfer catalyst to give corresponding 1,2,3, 5-tetra-O-acetyl-β-D-glycopyranosyl azide (7). After recrystallization from 95% ethanol, the compound (**7**) was obtained as a white solid in 89% yield. Reaction of 4-propyne oxide phthalonitrile (**4**) and 1,2,3,5-tetra-O-acetyl-β-D-glycopyranosyl azide (7) in the presence of anhydrous $CuSO_4$ and sodium ascorbate in dichloro-methane/methanol at room temperature for 48 h gave compound (**8**) 4-((1-(1,2,3-trihydroxy-4-(hydroxymethyl)-tetrahydro-2H-pyran-2-yl)-1H-1,2,3-triazol-4-yl)methoxy) phthalonitrile in 60%yield. Compound (**8**) was deprotected in dry MeOH and NaOMe to give compound (**9**) 4-((1-(3,4,5-trihydroxy-6-(hydroxymethyl)-tetrahydro-2H-pyran-2-yl)-1H-1,2,3-triazol-4-yl)methoxy)phthalonitrile. Without further purification, the deprotected 9 reacted in a mixture of DMAE and *n*-butanol, zinc chloride under N_2 for 24 h at 100 °C. After recrystallization from water and acetone, glucose conjugated phthalocyanine [2,9(10),16(17),23(24)-tetrakis((1-(β-D-glucopyranose-2-yl)-1H-1,2,3-triazol-4-yl)methoxyl)phthalocyaninato] zinc(II) (**1**) was obtained as a green solid in 62% yield.

The structures of products were characterized by NMR spectroscopy and MS. ^{1}H NMR (DMSO-d_6, 300 MHz) shows typical signals of phthalcyanine and glucose. Peak at 8.437 ppm (s, 12H) is assigned as aromatic proton, broad peak at 7.07 ppm as hydroxyl protons (s, 16OH), peak from 5.562 to 5.191 (s, 8H) assigned as triazole and related sugar protons. High resolution MS recorded by Agilent 6510 confirmed the target compound. The optical characters of target compound were evaluated by UV-vis and fluorescence spetra. The absorption and fluorescence spectra of glucose conjugated zinc phthalocyanines in DMSO and in water were shown in Fig. 1. The spectrum in DMSO shows no intermolecular aggregation. Characteristic sharp bands of the zinc phthalocyanines are seen at 618 nm and at 682 nm. However, the absorption spectrum in water differs remarkably from that in DMSO. In water, the intensity of absorption peak is much lower and broader than in DMSO, which can be attributed to cofacial aggregation of phthalocyanines in water [25, 26]. Consistent with the aggregation difference in DMSO and water, the emission ability in these two solvents also show large difference [27, 28]. Glucose conjugated zinc phthalocyanine in water has weak fluorescence signal due to its aggregation, while strong fluorescence at 690 nm with excitation wavelength at 618 nm ($\Phi_f = 0.482$) was detected in DMSO. However the following experiment *in vivo* demonstrated that the low fluorescent ability of glucose conjugated phthalocyanines in water does not limit its application as fluorescence probe *in vivo*.

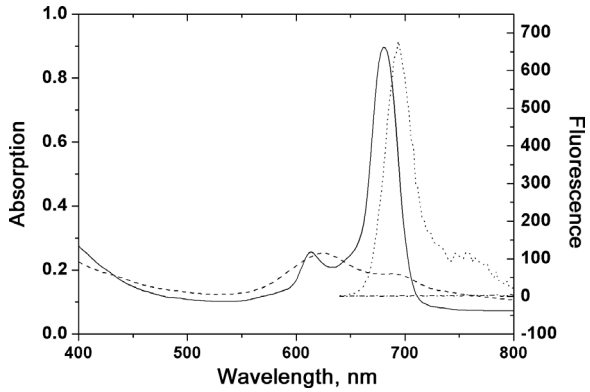
Photo-bleaching experiment demonstrated that glucose conjugated phthalocyanine had relative high photostability. After continuous irradiation by 0.1 w/cm^2/s laser with light emerge density of 60 J/cm^2 at 690 nm, its absorbance spectrum was recorded every 10 min irradiation. In first 10 min, its optical density decreased less than 10%, 20 min *vs.* 20%, 40 min to 50%, after continuous irradiation for 70 min, it decreased 70% or so.



Scheme 1. Synthetic route of glucose conjugated zinc phthalocyanines. (i) DMF, K_2CO_3, 60 °C; (ii) CH_2Cl_2, 33% HBr/AcOH, 0 °C; (iii) $CHCl_3$, NaN_3, PTC, 70 °C; (iv) $CuSO_4$, Na ascorbate, CH_2Cl_2/MeOH (4:1), rt; (v) MeOH, NaOMe, rt; (vi) DMAE, *n*-butanol, $ZnCl_2$, N_2, 100 °C

As a NIR probe, stability of probe in physiological conditions such as salt or serum was important. The emission of probe was measured in different physiological conditions. It can be seen that emission fluorescence was greatly reduced in serum or salt solution owning to molecule aggregation. After incubation in DMSO, FCS or physiological salt solution at 37 °C for 1 h, the total fluorescence emission of probe showed a slightly increase in first 20 min and then maintained at a stable level for 40 min, which indicated that the probe was pretty stable in serum or salt solution. Considering that optical image recording costs just several minutes, the photo stability of this compound was good enough as probe.

The NIR fluorescent imaging was conducted with liver tumor-bearing athymic nude mice as animal model. Two groups of model mice, one group was injected with 200 μL of glucose conjugated phthalocyanines at 2×10^{-4} M through the tail vein, another was injected with equal amount of saline as control. The *in vivo* imaging at 12 h post injections were acquired respectively using a Kodak *In Vivo* FX Professional Imaging System equipped with fluorescent filter sets (excitation/emission, 625/700 nm) near optimal wavelength. As shown in Fig. 2, a significant luminescence signal was observed in the body after intravenous injection of probe for 12 h, whereas no significant luminescence signal was observed in the control group. Considerable fluorescence was detected in liver and kidney at experiment group after injecting near infrared fluorescence agent. These results indicate that *in vivo*, glucose conjugated zinc phthalocyanines are emissive and do not undergo substantial phase transitions that drive phthalocyanines aggregation or facilitate



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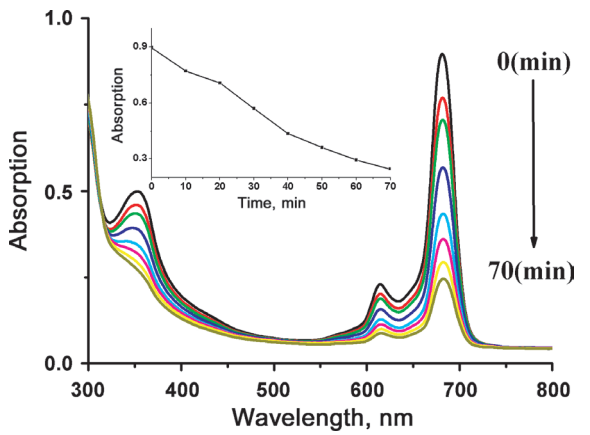

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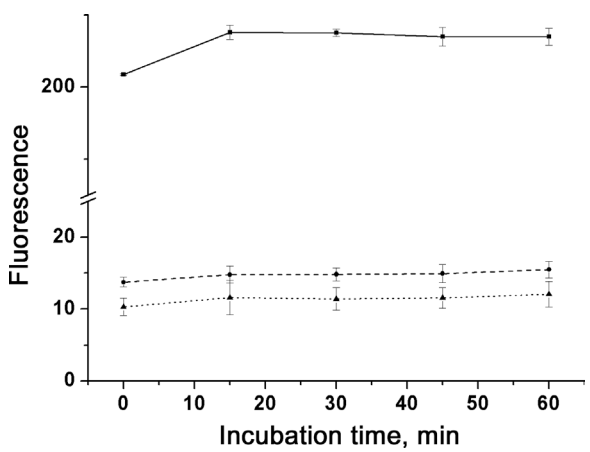


(c)

Fig. 1. (a) UV and FL spectrum of glucose conjugated phthalocyanines in different solvents (2×10^{-4} M; solid line UV: spectrum in DMSO; dot line: FL spectrum in DMSO; dash dot line: UV spectrum in H_2O; short dot line: FL spectrum in H_2O). (b) Photobleaching experiment recorded by UV-vis (light intensity was 0.1 w/cm^2/s and light emerge density was 60 J/cm^2. The photobleaching measurements was carried from 0 to 70 min). Inset: absorbance spectra data after different irradiation time. (c) Optical stability of glucose conjugated phthalocyanines incubation in DMSO, FCS or physiological salt solution at 37 °C (5×10^{-5} M, solid line: DMSO; dash line: DMSO:FCS = 1:1; dot line: DMSO:physiological saline = 1:1)

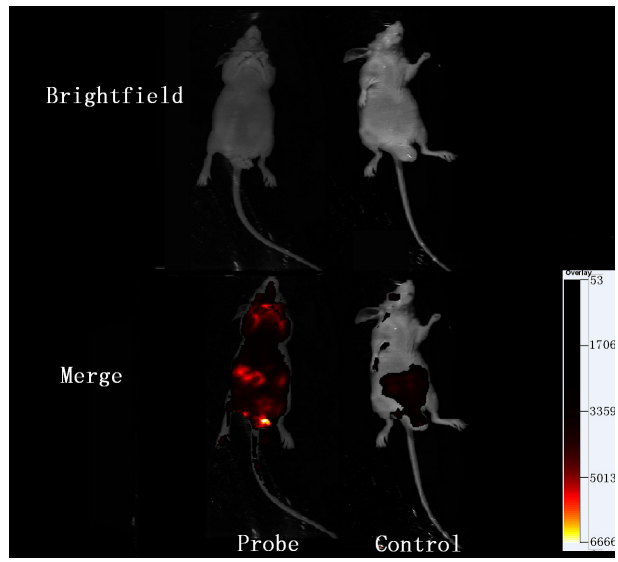


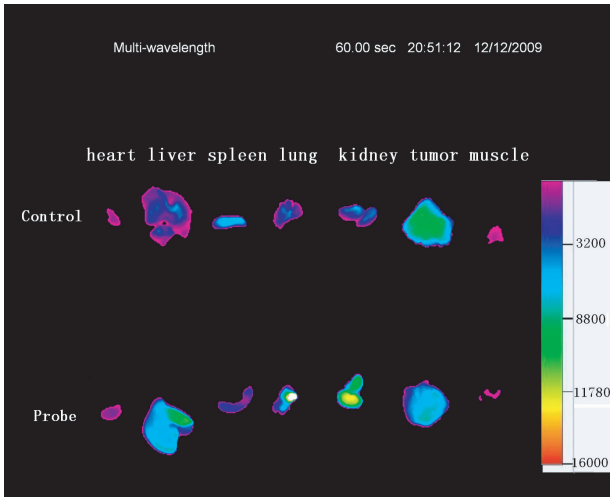
Fig. 2. Optical imaging *in vivo* with glucose conjugated zinc phthalocyanines as probe, mice bearing cancer as model (injected with 200 μL of 2×10^{-4} M with an exposure time of 1min (filters: excitation 625 nm, emission 700 nm)

intermembranous fluorophore transfer to surrounding biological structures.

To further investigate the probe distribution in various organs, the animals were sacrificed immediately after intravenous injection of probe at 12 h time point, and the kidney, heart, liver, lung, spleen, muscle and tumor were harvested and subjected to the imager. Fluorescence could be detected clearly in the lung, kidney and liver, a little in the heart and muscle (Fig. 3). Comparing with the control group, the intensity of sample group was about two-fold higher. This further confirmed that the probe was accumulated in these organ after enough circulation time. Because the lung, kidney and liver are the main metabolism organs, the probe as external matter was accumulated at relative large amount in these organs.

In order to investigate the influence of glucose substituted zinc phthalocyanines to organ, histological analysis were performed. Large amount of fluorescence agent aggregated in the liver and kidney (Fig. 4), while little fluorescence was found in the other organs, which was in accordance with the result of NIR fluorescent imaging of organs. These results further revealed the distribution of fluorescence agent. Toxic effects are an important issue for probe. Histological analysis showed that the glucose conjugated phthalocyanines had no damage to the organ according to the HE staining, which proved the safety of fluorescence agent as the near infrared fluorescence probe *in vivo*.

In summary, a novel water-soluble glucose conjugated zinc phthalocyanines was synthesized. With mice bearing liver cancer as animal model *in vivo* fluorescent imaging was conducted. Near-infrared imaging effect, distribution in organs as well as its histological analysis were also assessed. The results proved that glucose conjugated phthalocyanines has obvious imaging effect



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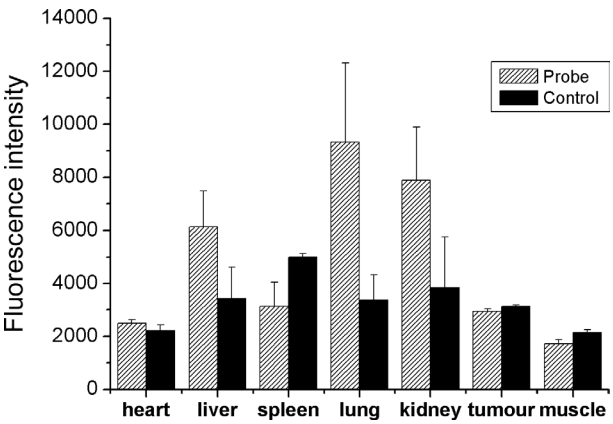


(b)

Fig. 3. (a) Distribution and fluorescent intensity of dissected organs. (a) *Ex vivo* imaging with an exposure time of 1 min (filters: excitation 625 nm, emission 700 nm). (b) Distribution and fluorescent intensity of dissected organs. B: Quantitative analysis

and exhibits its potential as near infrared optical probe in the diagnosis of cancer in future.

EXPERIMENTAL

General methods and materials

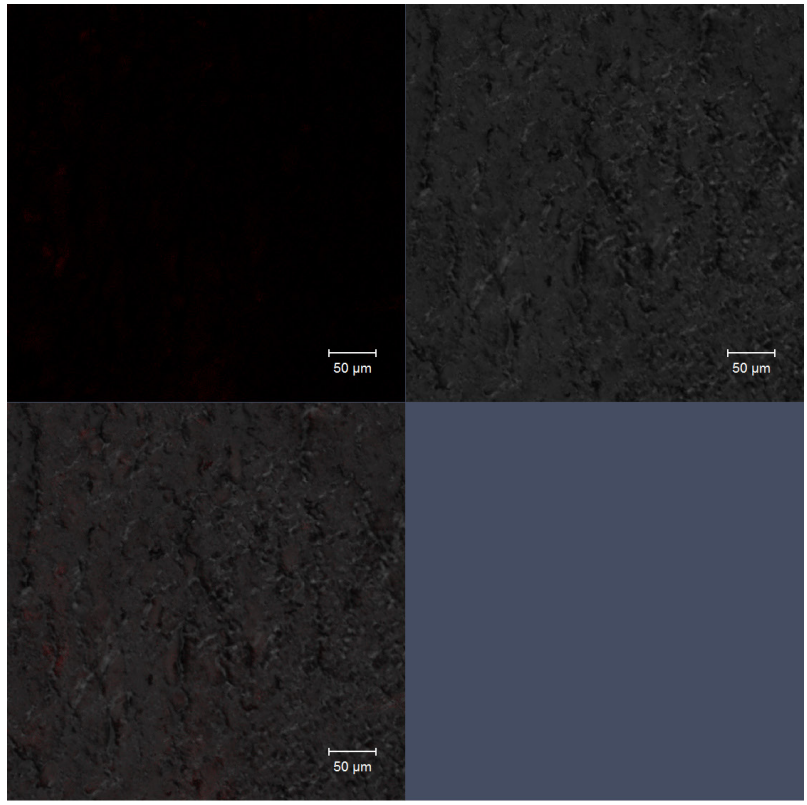
The ^{1}H NMR spectra were recorded on Varian Mercury at 300 MHz using $CDCl_3$ or DMSO-d_6 as solvent and TMS as internal reference. MS were recorded on Varin FT-ICR-MS and Agient 6510 Q-Tof instrument. The UV-vis and fluorescence spectra were recorded on a ThermoFisher scientific Varioskan TM Flash multimode microplate spectra photometer. All purchased materials were used without further purification.

Synthesis

4-propyne oxide phthalonitrile (4). 4-chlorophthalonitrile (1.638 g, 10 mmol) and propiolic alcohol (1.2 g, 20 mmol) were stirred for 24 h in DMF (40 mL) at 60 °C in the presence of potassium carbonate (8.3 g, 60 mmol). The reaction was then followed to complete by TLC. The reaction mixture was poured into ice-water to give a green-brown precipitate, which was extracted with dichloromethane. The organic extracts were dried over anhydrous $MgSO_4$ and concentrated under vacuum to give a yellow-brown solid. After recrystallization from methanol, the title compound was obtained as a yellow-white solid. Yield 1.2 g (65%), mp 112.3–114.1 °C. ESI-MS: *m/z* ($C_{11}H_6N_2O$) 183.0826143 (calcd. for $[M + H]^+$ 183.0553). IR (solid): ν, cm^{-1} 3288.3 (C≡H); 2232.9 (CN); 1593.1, 1494.3 (C=C phenyl). ^{1}H NMR ($CDCl_3$, 300 MHz): ™, ppm 7.762 (d, 1H, J = 8.7 Hz), 7.373 (d, 1H, J = 2.4 Hz), 7.314 (dd, 1H, J = 2.4, 8.7 Hz), 4.816 (d, 2H, J = 2.4 Hz), 2.636 (t, 1H, J = 2.4,2.7 Hz).

1,2,3,5-tetra-O-acetyl-β-D-glycopyranosyl azide (7). To a stirring solution of 1,2,3,4,6-penta-O-acetyl-β-D-glucopyranose (2.4 g, 6.1 mmol) in CH_2Cl_2 (20 mL) was added HBr/HOAc (10 mL, 33%) solution at 0 °C. The resulting solution was stirred for 7 h until 1,2,3,4,6-penta-O-acetyl-β-D-glucopyranose completely disappeared. The solution was neutralized with saturated $NaHCO_3$ aqueous solution before being dried ($MgSO_4$), filtered, and evaporated under vacuum to give the crude glycosyl bromide as a yellow oil. After recrystallization from ethanol, the compound was obtained as a white solid (2.0 g, 80%). 2,3,4,6-tetra-O-acetyl-β-D-glycopyranosyl bromide (0.5 g, 1.2 mmol) was dissolved in $CHCl_3$ (20 mL) and stirred with NaN_3 (0.3 g, 4.6 mmol) in water (20 mL) at 70 °C overnight in the presence of benzyltriethylammonium chloride (0.1 g, 0.4 mmol). The reaction mixture was diluted with water and extracted with $CHCl_3$. The combined organic layers were dried ($MgSO_4$), filtered, and concentrated under vacuum to give an orange oil. After recrystallization from 95% ethanol, the title compound 1,2,3,5-tetra-O-acetyl-β-D-glycopyranosyl azide was obtained as a white solid. Yield 0.4 g (89%), mp 129.7–130.5 °C. ESI-MS: *m/z* ($C_{14}H_{19}N_3O_9$) 396.1013 (calcd. for $[M + Na]^+$ 396.1014). IR (solid): ν, cm^{-1} 2233.3 (N=N=N), 1737.8 (C=O). ^{1}H NMR ($CDCl_3$, 300 MHz):™, ppm 5.247 (t, 1H, J = 9.3, 9.6 Hz), 5.133 (t, 1H, J = 9.6, 9.9 Hz), 4.982 (t, 1H, J = 8.7, 9.6 Hz), 4.661 (d, 1H, J = 8.7Hz), 4.301 (dd, 1H, J = 4.8, 12.6 Hz), 4.184 (dd, 1H, J = 2.1, 12.3 Hz), 3.821–3.764 (m, 1H), 2.165 (s, 3H, CH_3), 2.099 (s, 3H, CH_3), 2.074 (s, 3H, CH_3), 2.027 (s, 3H, CH_3).

4-((1-(1,2,3-trihydroxy-4-(hydroxymethyl)-tetrahydro-2H-pyran-2-yl)-1H-1,2,3-triazol-4-yl)methoxy)-phthalonitrile (8). A mixture of 4-propyne oxide phthalonitrile (**4**) (500 mg, 2.7 mmol), 1,2,3,5- tetra-O-acetyl-β-D-glycopyranosyl azide (**7**) (1100 mg, 2.9 mmol), anhydrous $CuSO_4$ (45 mg, 0.28 mmol), and sodium ascorbate (110 mg, 0.56 mmol) in dichloromethane/methanol = 4:1 (15 mL) was stirred at room temperature for 48 h. After being filtered under vacuum, washed with water, extracted with dichloromethane, the organic extracts were dried over anhydrous $MgSO_4$ and concentrated under vacuum. The residue was purified



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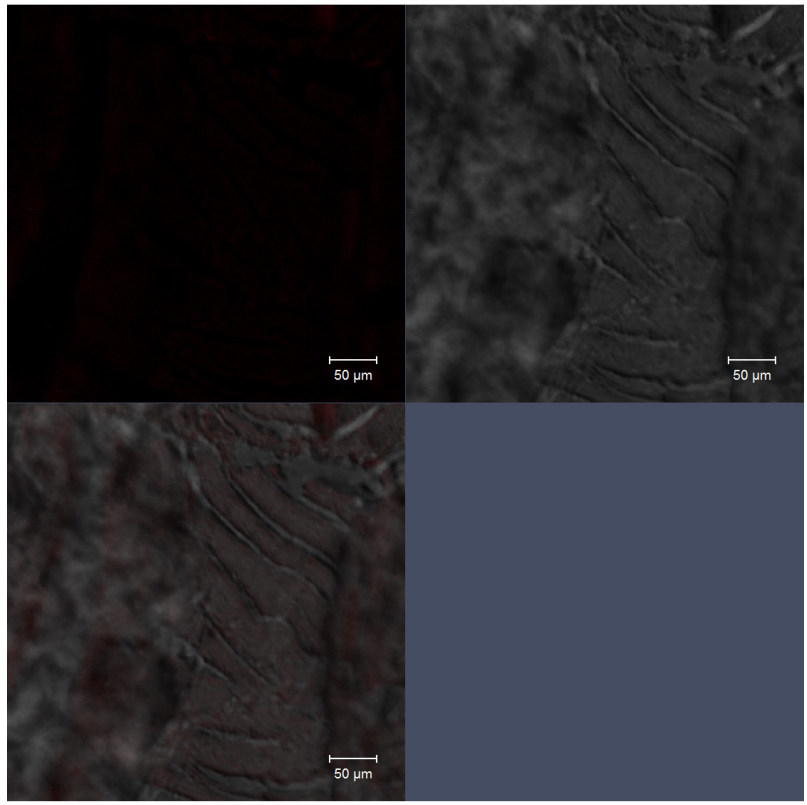

(b)

Fig. 4. Fluorescent image of liver (a) and kidney (b) tissue slice recorded by confocal laser microscopy

by column chromatography (silica gel: ethyl acetate-petroleum ether, 1:1), to yield a white solid. Yield 899 mg (60%), mp 161.5–162.8 °C. ESI-MS: *m/z* ($C_{25}H_{25}N_5O_{10}$) 578.2507 (calcd. for [M + Na]$^+$ 578.1494). IR (solid): ν, cm^{-1} 2233.3 (CN), 1737.8 (C=O). ^{1}H NMR ($CDCl_3$, 300 MHz):™, ppm 7.907 (s, 1H), 7.751 (d, 1H, *J* = 8.7 Hz), 7.383 (d, 1H, *J* = 2.4 Hz), 7.359 (dd, 1H, *J* = 2.7, 8.7 Hz), 5.908 (d, 1H, *J* = 9.3 Hz), 5.476–5.206 (m, 5H), 4.359 (dd, 1H, *J* = 5.1, 12.6 Hz), 4.184 (dd, 1H, *J* = 2.1, 12.9 Hz), 4.058–4.007 (m, 1H), 2.178 (s, 3H, CH_3), 2.090 (s, 3H, CH_3), 2.079 (s, 3H, CH_3), 2.038 (s, 3H, CH_3).

[2,9(10),16(17),23(24)-tetrakis((1-(β-D-glucopyranose-2-yl)-1H-1,2,3-triazol-4-yl)methoxyl) phthalocyaninato]zinc(II)(I). 4-((1-(1,2,3-trihydroxy-4-(hydroxymethyl)-tetrahydro-2H-pyran-2-yl)-1H-1,2,3-triazol-4-yl)methoxy)phthalonitrile (**8**) (600 mg, 1.1 mmol) was suspended in dry MeOH (10 mL). NaOMe (300 μL) was added and the solution was stirred for 4–5 h at room temperature. The ion exchanger was added to neutralize the solution, then filtered off and the solvent evaporated. Without further purification, to the compound (**9**) 4-((1-(3,4,5-trihydroxy-6-(hydroxymethyl)-tetrahydro-2H-pyran-2-yl)-1H-1,2,3-triazol-4-yl) methoxy)phthalonitrile dissolved in a mixture of DMAE (1 mL) and *n*-butanol (0.5 mL), zinc chloride (70 mg, 0.5 mmol) were added. The reaction mixture was stirred under N_2 for 24 h at 100 °C. After cooling, the solid was reprecipitated by adding acetone and collected after filtration. Yield 1.08 g (62%), mp > 300 °C. ESI-HRMS: *m/z* ($C_{68}H_{68}N_{20}O_{24}Zn$) 1635.3891 (calcd. for [M + Na]$^+$ 1635.3899). ^{1}H NMR (DMSO, 300 MHz): ™, ppm 8.437 (s, 12H), 7.076 (s, 16OH), 5.562 (d, 4H, *J* = 9 Hz), 5.439 (s, 4H), 5.301 (s, 4H), 5.191 (s, 8H), 3.797–3.016 (m, 28H).

Optical measurement

The optical characters of target compound were evaluated by UV-vis and fluorescence spectrum. The photobleaching measurements were carried out in Multimode Microplate Spectraphotometer (VarioskanTM Flash, *ThermoFisher Scientific*). Dye and buffer solutions were prepared immediately prior to measuring. 200 μL 2×10^{-4} M in 96 wells was used as sample and sealed by cover slips to avoid evaporation. The light intensity was 0.1 w/cm^2/s and light emerge density was 60 J/cm^2. The photobleaching measurements was carried from 0 to 70 min, every 10 min record one datum, with irradiation wavelength at 690 nm. Optical stability of glucose conjugated phthalocyanines incubation in DMSO, FCS or physiological salt solution at 37 °C was measured using 200 μL 5×10^{-5} M in 96 wells as sample.

In vivo imaging and distribution of glucose conjugated zinc phthalocyanines

Athymic nude mice (seven weeks old, 20–25 g) were used. All the animal experiments were performed in compliance with the Guiding Principles for the Care and Use of Laboratory Animals, Peking Union Medical College, China. Animals can access free to food and water. Tumor-bearing mice were prepared by injecting a suspension of 1×10^6 liver tumor cells in physiological saline (100 μL) into the subcutaneous left flank. Tumors develop within a periods of 1 week. Athymic nude mice were randomly assigned to perform as follows: glucose conjugated zinc phthalocyanines, control group (n = 6 for each group). In experimental group, near infrared fluorescence agent was injected into the tail vein of the liver tumor-bearing mice at 200 μL of 2×10^{-4} M. Images were taken using a Kodak Image Station *in vivo* FX (filters: excitation 625 nm, emission 700 nm) with an exposure time of 1 min. At the end of the imaging, anesthetized mice were sacrificed and images of organs were made to evaluate the distribution of near infrared fluorescence agent. Fluorescence images of organ were analyzed using the Kodah Image Analysis Software. After imaging, organ tissues were immediately immersed into 4% formaldehyde in phosphate-buffered saline of pH 7.4 at 4 °C for 24 h. After fixation, the samples were embedded in paraffin and sectioned to 5-μm-thick slices. Routine staining was performed with hematoxylin-eosin.

Acknowledgements

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Supporting information

Experimental details for the synthesis, measurement method, MS data and H NMR data are given in the supplementary material. This material is available free of charge *via* the Internet at http://www.worldscinet.com/jpp/jpp.shtml.

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