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Porphyrin Nanocage-Embedded Single Molecular Nanoparticle as Cancer Nanotheranostics

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Abstract

Single molecular nanoparticles (SMNPs) integrating imaging and therapeutic capabilities exhibit unparalleled advantages in cancer theranostics, ranging from excellent biocompatibility, high stability, prolonged blood lifetime to abundant tumor accumulation. Herein, we synthesize a sophisticated porphyrin nanocage that is further functionalized by twelve polyethylene glycol arms to prepare SMNPs (**porSMNPs**). The porphyrin nanocage embedded in **porSMNPs** can be utilized as a theranostic platform. Positron emission tomography imaging provides dynamic bio-distribution of **porSMNPs**, confirming their excellent circulation time and preferential accumulation at the tumor site attributing to the enhanced permeability and retention effect. Moreover, the cage structure significantly promotes the photosensitizing effect of **porSMNs** by inhibiting the π - π stacking interactions of the photosensitizers, ablating of the tumors without relapse by taking advantage of photodynamic therapy.

A sophisticated porphyrin nanocage is synthesized, which can be utilized as a functional platform to develop single molecular nanoparticles. Positron emission tomography (PET) imaging and photodynamic therapy demonstrate their superior capability in cancer nanotheranostics attributing to the unique topological structure of the porphyrin nanocage. This study paves a distinctive way to fabricate smart nanomaterials as cancer theranostics.

Graphical Abstract

Keywords

nanotheranostics; photodynamic therapy; imaging; cancer treatment; singlet oxygen

Nanotheranostics, nano-enabled amalgamation of therapy and diagnosis, offer promising opportunities in precise diagnosis and effective therapy of cancers.^[1] Benefiting from their excellent biocompatibility and biodegradability, organic theranostic platforms especially those fabricated from functional copolymers, such as micelles, nanoparticles, vesicles and liposomes, exhibit brilliant future in *the next-generation theranostics to meet the challenges in cancer management*.^[2] However, one practical challenge impeding the clinical trials of the conventional polymeric nanosystems is their low stability *in vivo* arising from the large dilution volume, shearing force and the interactions with biological components in the blood after intravenous injection, thus leading to undesirable premature release, off-target toxicity and diminished theranostic performances.^[3] Additionally, the imaging signals from the nanotheranostics hardly reflect the real delivery and excretion behaviors of the loaded therapeutic agents through physical encapsulation, because the imaging agents are always conjugated on the polymer backbones, thus making the theranostic results confusing. Therefore, it is urgently desirable to develop single molecular nanoparticles (SMNPs) integrating therapeutic and diagnostic/imaging abilities to solve these issues.

Another fatal obstacle impeding the clinical applications of the chemotherapeutic nanomedicines is the inevitable side effects towards normal tissues. Different from chemotherapy, photodynamic therapy (PDT) as a rapidly developing therapeutic modality can eradicate cancer cells with negligible drug resistance, low side effects, minimal invasion, and less damage to marginal tissues.^[4] Upon irradiation with light at an appropriate wavelength, the photosensitizer in its excited state interacts with molecular oxygen to generate highly reactive singlet oxygen (1O_2), a cytotoxic species that causes necrosis/apoptosis of the cancer cells.^[5] However, the 1O_2 generation quantum yield of the commonly used photosensitizers are unsatisfactory arising from their severe aggregation in aqueous solution, thus greatly lowering the PDT efficacy.^[6] Furthermore, low accumulation efficiency in tumors of the photosensitizers after systemic administration impairs the clinical applications of PDT and raises challenges for the development of suitable pharmaceutical formulations.

Herein, we synthesize a *porphyrin nanocage via a template-directed strategy and further fabricate it into SMNPs (porSMNPs)* through post modification by polyethylene glycol chains. The size of the resultant SMNPs locates the optimal range for the enhanced permeability and retention (EPR) effect, and the dense PEG corona significantly prolong their circulation time, both of which are favorable to increase the tumor accumulation of

porSMNPs. The *porphyrin nanocage acting as the theranostic platform can be labeled by radioactive ^{64}Cu for positron emission tomography (PET) imaging*, allowing to monitor the delivery, pharmacokinetic behaviors, biodistribution and excretion of **porSMNPs** in real time. More interestingly, the rigid structure of the *porphyrin nanocage* significantly inhibits the π - π stacking interactions between the photosensitizers, thus boosting the PDT efficacy of **porSMNPs**. Benefiting from the outstanding pharmacokinetic behaviors and photosensitizing effect, **porSMNPs** exhibit superior anti-tumor performance that ablate the tumors without recurrence and systemic toxicity, showing unparalleled advantages over the clinically used chemotherapeutic nanomedicine (doxorubicin hydrochloride liposome, Doxil).

The tetraalkene-derived zinc porphyrin derivative **2** was synthesized from the reaction of **1** and pyrrole in propionic acid at reflux for 2 h followed by the coordination with zinc acetate (Scheme 1). After further reaction with methyl bromoacetate, zinc porphyrin monomers **3** were preorganized together to form a triangular-prism trimer **3₃·4**, in which 2,4,6-tris(pyridin-4-yl)-1,3,5-triazine (**4**) acted as a template. The porphyrin nanocage **5** was prepared through an olefin metathesis reaction using Grubbs' II catalyst with a yield of 48%. The template and zinc metals were removed by treating **5** with trifluoroacetic acid. The twelve ester groups were further modified by PEG chains via an amidation reaction by refluxing a mixture of **6** and excessive methoxypolyoxyethylene amine (mPEG-NH₂, 5 kDa) in toluene. The product **7** was finally obtained by removing the free mPEG-NH₂ using a dialysis bag with a molecular weight cut-off of 50 kDa. Various characterizations including ¹H NMR spectroscopy, ¹³C NMR spectroscopy, electrospray ionisation mass spectrometry and gel permeation chromatography (GPC) were utilized to provide convincing evidences for the successful syntheses of these compounds (Figure S1–S19). The characteristic peaks related to the protons of the porphyrin nanocage and PEG chains were detected in the spectrum of **7** (Figure S15), indicating the coexistence of these two components. The molecular weight and polydispersity index of **7** were determined to be 69.7 kDa and 1.12 according to the GPC curve (Figure S17), which demonstrated that all of the ester groups were functionalized by the PEG chains.

The solubility of the porphyrin nanocage was significantly improved due to the existence of PEG chains, more than 500 mg of **7** could be solubilized in water (1 mL) to afford a transparent aqueous solution. An intense absorption at 423 nm was observed related to the typical Soret band, and the Q-bands appeared at 523, 561, 595 and 655 nm in the UV-vis spectra of **7** (Figure 1a). Excitation of the aqueous solution of **7** at 450 nm gave a strong emission peak at 678 nm (Figure 1a). The fluorescence quantum (QY) yield of **7** was determined to be 6.9% in aqueous solution by using tetra-*n*-propylporphycene as a reference (QY = 38%). Transmission electron microscopy (TEM) was utilized to reveal the morphology and size of **7**. As shown in Figure 1b, sphere nanoparticles were observed around 20 nm in diameter. Dynamic light scattering (DLS) indicated that the average diameter of PEGylated nanocage was 34.6 ± 1.8 nm (Figure 1b), bigger than the size observed in TEM image, which was attributed to the existence of flexible PEG layer with low contrast on the nanoparticle surface as well as the swelling effect in aqueous solution. During the experiments, we noticed that the size of the nanoparticles maintained well by

gradually diluting the solution, which indicated that no dissociation occurred. Considering the sterically hindered PEG chains on the porphyrin platform, the nanoparticles formed by **7** are SMNPs. Importantly, the **porSMNPs** exhibited high stability in physiology environment, negligible changes in diameter was detected by DLS in PBS containing fetal bovine serum (10%) over 72 h (Figure S20).

The excellent biocompatibility of **porSMNPs** was confirmed by 3-(4',5'-dimethylthiazol-2'-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay using U87MG cell line. The relative cell viability maintained higher than 90% in dark when the concentration of **porSMNPs** reached 0.64 mg/mL (Figure S22). Additionally, the *in vivo* safety of **porSMNPs** was evaluated using a hemolysis assay. Figure S23 demonstrated that **porSMNPs** was *not hemolytic*, as indicated by the negligible changes in hemolysis percentage in the test concentration range. The cellular internalization of **porSMNPs** was verified by confocal laser scanning microscopy (CLSM). A red fluorescence was observed in cytoplasm for cells incubated with **porSMNPs** (Figure 2a). The endocytic pathways of **porSMNPs** were evaluated using flow cytometry by applying different endocytosis inhibitors. As shown in Figure 2b, the internalization of **porSMNPs** by the cells was significantly inhibited at 4 °C, suggesting the cellular uptake was energy-dependent. Treatment of the cells with amiloride-HCl (AMD), *chlorpromazine* (CPZ) or genistein (Gen) led to 18.5%, 56.8% or 30.7% decrease in the cellular uptake, respectively, indicating the **porSMNPs** were mainly internalized *via* clathrin-mediated endocytic pathway with the assistance of macropinocytosis- and caveolae-mediated endocytosis.

Apart from the enhancement of solubility and biocompatibility, the PEG chains endow the **porSMNPs** with a stealth character to protect them from being absorbed by proteins and degraded by enzymes, thus imparting the PEGylated nanocage a prolonged circulation time.^[7] In order to investigate the pharmacokinetics and biodistribution of **porSMNPs**, PET imaging was employed to monitor the delivery and excretion of **porSMNPs** by labeling the porphyrin nanocage with ⁶⁴Cu through the coordination interactions (Figure 3a). Compared with other diagnostic modalities, PET imaging provides a quantitative readout of the tissue targeting efficiency and pharmacokinetics of the nanotheranostics with deep tissue penetration, highly sensitivity and non-invasivity.^[8] Based on thin layer chromatography, the stability of the chelation was 99.2 ± 0.9% even after culturing the ⁶⁴Cu@**porSMNPs** in mouse serum for 24 h (Figure S24), confirming the PET imaging accurately disclosed the *in vivo* behaviors of the ⁶⁴Cu@**porSMNPs** rather than the dissociated ⁶⁴Cu.

Whole-body PET images were collected at various time post intravenous (*i.v.*) injection of ⁶⁴Cu@**porSMNPs** (150 µCi/mouse) into U87MG tumor-bearing mice (Figure 3b). The circulation time of ⁶⁴Cu@**porSMNPs** was roughly calculated to be 11.3 h by detecting the signal changes in heart. Quantitative region-of-interest analysis indicated that the ⁶⁴Cu@**porSMNPs** quickly accumulated in tumor site, 4.1% ID/g (percentage injected dose per gram) uptake was detected at 4 h post injection, which was further increased to 4.8% ID/g at 24 h post injection. The signal was maintained 2.4% ID/g even at 48 h post injection (Figure 3c). Such an efficient tumor localization was attributed to the EPR effect.^[9] Due to the capture by the reticuloendothelial system, the radioactive signal in liver increased from 16.3% ID/g at 1 h post injection to 20.4% ID/g at 4 h post injection. The excretion of

^{64}Cu @**porSMNPs** was also traced using PET imaging. As shown in Figure 3c, the signal in liver decreased from 23.6% ID/g at 24 h to 18.1% ID/g and 48 h post injection. Additionally, high signal in intestine was observed at 4 h post injection, while little radioactivity was detected in bladder and kidneys, indicating the ^{64}Cu @**porSMNPs** was excreted through the hepatobiliary route.

To determine the biodistribution of ^{64}Cu @**porSMNPs**, the mice were sacrificed at 48 h post injection and the radioactivity in different organs was measured via γ counting, which indicated that the uptake of ^{64}Cu @**porSMNPs** by heart, liver, spleen, stomach, kidneys, lung, pancreas, intestine, bladder, bone, muscle and tumor was 1.41 ± 0.32 , 20.6 ± 3.70 , 11.8 ± 1.91 , 3.25 ± 0.47 , 0.73 ± 0.11 , 2.27 ± 0.36 , 0.72 ± 0.16 , 1.17 ± 0.14 , 2.13 ± 0.26 , 1.68 ± 0.25 , 0.56 ± 0.18 and $2.84 \pm 0.36\%$ ID/g, respectively (Figure 2d). Moreover, inductively coupled plasma mass spectrometry was used to verify the accuracy of PET quantification analysis by labeling the porphyrin nanocage with Au. According to Figure 3e, the circulation half-life of Au@**porSMNPs** was calculated to be 5.69 ± 0.36 h, which was much higher than most of the nanomaterials,^[10] firmly confirming the prolonged circulation time in blood stream. Time-dependent biodistribution of Au@**porSMNPs** in the main organs also supported the results from PET imaging (Figure 3f).

Excitingly, the cyclic structure of **7** greatly enhanced the photosensitizing effect of **porSMNPs** by inhibiting the π - π stacking between the porphyrins. Tetrakis(4-carboxyphenyl)porphyrin modified by four PEG chains (**TCPPEG**) was utilized as a control. Different from **porSMNPs**, **TCPPEG** self-assembled into nanoparticles around 200 nm attributing to the stacking of the porphyrin core (Figure S25). Singlet oxygen sensor green (SOSG) was used to trace the generation of $^1\text{O}_2$, the characteristic peak at 532 nm increased rapidly upon irradiation in the presence of **porSMNPs** or **TCPPEG** at the same molar concentration of porphyrin photosensitizer (Figure S26). Notably, **porSMNPs** caused more significant enhancement in fluorescence intensity than that of **TCPPEG** under the same conditions, which demonstrated the photosensitizing effect was boosted by the formation of a confined nanocage (Figure S27 and Figure S28). In sharp contrast with the commercially used photosensitizer (indocyanine green, ICG), **porSMNPs** were highly photostable, negligible changes in fluorescence intensity were detected after several irradiation circles (Figure S29). The intracellular generation of $^1\text{O}_2$ was disclosed by dichlorofluorescein diacetate (DCF-DA), a non-fluorescent dye that became brightly emissive after being oxidated into DCF. Strong green fluorescence was detected in the cells treated with **porSMNPs** followed by laser irradiation (Figure 4a), an evidence for the generation of $^1\text{O}_2$ inside the cells. Pretreatment the cells with vitamin C, a reactive oxygen series scavenger, resulted in the attenuation of the DCF signal, further verifying the successful production of $^1\text{O}_2$. MTT assay indicated that the cytotoxicity against different types of cancer cell lines was activated by laser, and the anticancer efficacy depended on the laser density and irradiation time (Figure S22 and Figure S30–S32). The high phototoxicity index of **porSMNPs** facilitated to promote the therapeutic performances and minimize the side effects.

Inspired by the prolonged circulation time, high tumor accumulation and excellent anticancer efficacy, *in vivo* anti-tumor studies was conducted. A commercially used

chemotherapeutic nanomedicine Doxil was employed as a control. When the tumor volume reached $\sim 100 \text{ mm}^3$, the mice were divided randomly and treated with PBS, laser (L), Doxil, **porSMNPs** and **porSMNPs** + L, respectively. For photodynamic therapy, the mice only received one laser irradiation (671 nm, 0.1 W/cm^2 , 30 min) at 24 h post the first injection considering the relatively high tumor accumulation of **porSMNPs** at this time point. Compared with the PBS-treated group, limited anti-tumor effect was observed for the mice treated with laser or **porSMNPs** (Figure 4b). Doxil moderately suppressed the tumor growth with a inhibition rate of 53.4% after three injections (Figure S34). However, tumor relapsed after 12 days, because single chemotherapy hardly eliminate all cancer cells in tumor sites. Excitingly, **porSMNPs** + L showed superior anti-tumor efficacy, the tumors were completely ablated without recurrence during the treatment period. Transferase-mediated dUTP nick end-labeling (TUNEL) staining indicated that **porSMNPs** + L caused 90.6% cell apoptosis in tumor sites, which was much higher than those of the other formulations (Figure 4d and Figure S35). Hematoxylin and eosin (H&E) staining confirmed that the PDT resulted in the highest level of apoptosis and necrosis in tumor tissues (Figure 4d).

Body weight loss and survival time of the mice received different formulations were carefully evaluated. No weight loss was found for the mice treated with **porSMNPs** + L (Figure S37), indicating negligible systemic toxicity of **porSMNPs**. Moreover, no apparent signs of toxic effects were observed during the treatment, including drinking, eating, urination, activity and neurological status. The median survival time for the mice treated with PBS, laser, **porSMNPs** and Doxil was 30, 34.5, 36 and 37.5 days, respectively, while the median survival of the mice treated with **porSMNPs** + L was remarkably prolonged over 60 days without a single death (Figure 4c). Urine and blood were collected at different days during the treatment period for biochemistry assay. The level of clinical chemistry parameters were all in the normal range compared with the health mice (Figure S38), which demonstrated that no nephrotoxicity and hepatotoxicity were caused by **porSMNPs**. Other vital hematology markers also located in the health ranges for the mice treated with **porSMNPs** + L (Figure S39). Unlike chemotherapy with systemic delivery of anticancer drug, PDT activate the anticancer efficacy localizedly, thus significantly reducing side effects towards normal tissues.

In conclusion, we synthesized a novel porphyrin nanocage and used it as a platform to prepare SMNPs, which could be applied in cancer theranostics. Different from traditional photosensitizers, the cyclic structure of the nanocage greatly boosted the photosensitizing effect by inhibiting the π - π stacking between the porphyrins in aqueous solution, making the **porSMNPs** excellent candidates for PDT. Benefiting from the unique structure and rational modification, prolonged circulation time and high tumor accumulation were achieved, both of which were extremely important for precise diagnosis and effective cancer therapy. *In vitro* studies demonstrated that **porSMNPs** were highly biocompatible, while their anticancer efficacy was activated specifically by laser irradiation, which was favorable to reduce side effects towards normal tissues. Compared with clinically used Doxil, **porSMNPs** exhibited superior anti-tumor performances, and the tumors were completely

ablated by PDT without tumor recurrence. This pioneering work provides a theranostic agent with promising potential for clinical translation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Figure 1.

a) UV-vis absorption and fluorescence spectra of **porSMNPs** in aqueous solution. Inset: photo of the **porSMNPs** solution under UV light (365 nm). b) TEM image of the **porSMNPs**. Inset: DLS intensity-weighted size distributions of **porSMNPs** in aqueous solution.

Figure 2.

a) CLSM images of U87MG cells incubated with **porSMNPs** for 8 h. Scale bar = 50 μm . b) Probing the mechanisms of cellular internalization by using various inhibitors.

Figure 3.

a) Radiolabeling of **porSMNPs** by ^{64}Cu . b) PET images of the mice bearing U87MG tumor at 1, 4, 24 and 48 h post i.v. injection of ^{64}Cu @porSMNPs. White circle indicates the location of tumor. c) Time-activity curves of ^{64}Cu @**porSMNPs** in the main organs ($n = 3$). d) Biodistribution of ^{64}Cu @**porSMNPs** in the main organs at 48 h post injection. e) Plasma concentration versus time after injection of Au@**porSMNPs** ($n = 4$). f) Biodistribution of Au@**porSMNPs** in the main organs at different time post injection ($n = 4$). H: heart, Li: liver, St: stomach, Lu: lung, K: kidney, Sp: spleen, In: intestine, Bl: bladder, Pa: pancreas, Bo: bone, Mu: muscle, T: tumor, Sk: skin.

Figure 4.

a) CLSM images of the cells treated with **porSMNPs** and DCF-DA with/without laser irradiation (671 nm, 0.2 W/cm², 5 min) in the absence or presence of vitamin C. Scale bar = 50 μm . b) Tumor growth inhibition curves and c) survival rate of the mice bearing U87MG tumors after different treatments ($n = 8$), *** $P < 0.001$. d) H&E and TUNEL staining of the tumor tissues from the mice treated with different formulations. Scale bar = 200 μm .

Scheme 1.

Synthetic route to the porphyrin nanocage-embedded polymer 7.