A Small Molecular Multifunctional Tool for pH Detection, Fluorescence Imaging, and Photodynamic Therapy

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multifunctional probe shows good pH response across a broad range of pH values. It also exhibits excellent fluorescence in vivo for mapping its biodistribution. Additionally, it produces enough ${}^{1}O_{2}$ radicals for in vivo PDT. The combination of these functionalities into a single small-molecule platform, rather than

a bulky nanoconstruct, offers unique possibilities for molecular imaging and therapy.

KEYWORDS: multifunction probe, ultrasensitive NMR, pH distribution MRI, fluorescence imaging, photodynamic therapy

INTRODUCTION

A long-standing problem in biomedicine is to combine therapeutic and diagnostic capabilities into a single, smallmolecule platform.¹ Small molecules are preferred over more bulky nanoconstructs to penetrate the blood-brain barrier or leak out of the vasculature and perfuse into the surrounding tissues. The ability to map the local accumulation of therapeutic molecules is important to ensure the correct dose reaches its target and real-time feedback methods at the lesion location have been shown to be useful in increasing the effectiveness of therapy.² The following three functions are desirable: (1) the therapeutic molecules should be detectable by common medical imaging techniques such as magnetic resonance, fluorescence, ultrasound, etc.; (2) the therapeutic agent should have the ability to target cells or tissues of interest, minimize side effects, and increase the accumulation of the biosensor in situ; (3) the therapeutic agent should be capable of treating disease locally. In recent years, a variety of nanoplatforms with multiple functionalities have been reported.^{1,3-5} Although nanoplatforms can be used to load multiple drugs or contrast agents, they are generally bulky, which leads to suboptimal transport properties. Most nanoparticles will be recognized and eliminated by the immune system as a foreign substance; less than 0.0014% injected dose nanoparticles were delivered to targeted nidus.⁶ Particles larger than a few nanometers are often regarded as ineffective therapeutically because of their limited transport capabilities,

including their inability to penetrate the blood-brain barrier.⁷ It would instead be preferable to develop strategies based on single small molecules because of their ability to perfuse through biological tissues. The selection of a proper vehicle remains an important challenge.

Imaging of targeted tumor

After

Photodynamic therapy

A small molecular multifunctional probe based on porphyrin -functionalized cryptophane-A has been developed to address these limitations. Porphyrins play significant roles in the metabolism of living organisms and have been widely used in biomedicine and clinic.^{8–12} Cryptophane-A is a cagelike molecule featuring high binding affinity (4000 M⁻¹, in organic solution) and suitable exchange kinetics (~30–300 ms, in aqueous solution) for the xenon atom.¹³ The xenon polarization can be increased via the spin-exchange optical pumping (SEOP) technique.^{14–16} This leads to a ¹²⁹Xe NMR signal enhancement that is 50,000-fold larger than thermal polarization,¹⁷ allowing for ultrasensitive NMR detection. The xenon-cryptophane host–guest system can be used to develop a hyperpolarized ¹²⁹Xe NMR probe for biomolecules and metal ion ultrasensitive detection.^{18–30}

Received: November 23, 2019 Accepted: January 10, 2020 Published: January 10, 2020



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Figure 1. Schematic illustration of different functions of the SMFT, reported in this manuscript. Our SMFT can map (via MRI or fluorescence) local accumulation and pH values and target tumor cells and shows promising capabilities for PDT.



Figure 2. Hyper-CEST test of SMFT in different pH buffers and cells. (A) Hyper-CEST spectra of SMFT in different pH buffers. (B) Hyper-CEST images of SMFT in different pH buffers. The Hyper-CEST images were reconstructed by MATLAB. (C) Hyper-CEST spectra of SMFT ($30 \mu M$) in A549 cells in different pH buffers. (pH 7.4, pH 5.1). (D) Hyper-CEST effect of SMFT in A549 cells in different pH buffer. Error bars are standard deviations between two replicates.

We combined ¹²⁹Xe MRI with fluorescence imaging on a small molecule, which can not only map the pH distribution but also target cancer cells. Because the pH of tumor cells is different from that of normal cells, the detection of pH and targeted detection of tumors can achieve precise detection of tumors together, which makes the small molecule multimodal probe for tumor detection. When tumors are accurately detected by MRI and fluorescence imaging, the tumor can be treated by PDT additionally without further in dose. These functions were integrated in a small molecule platform to construct multifunctional probe, which has the potential to overcome the weakness of conventional theranostics probes and provide specificity, high sensitivity, contrast, and spatial resolution.

We will refer to our multifunctional probe as a smart small molecular multifunctional tool (SMFT). The versatility of this small molecule, which combines ¹²⁹Xe MRI, fluorescence imaging, specificity, and PDT into a single molecule, is demonstrated in the series of experiments below (Figure 1).

RESULTS AND DISCUSSION

Detection of pH by Hyper-CEST. We first studied the effects of pH on the ¹²⁹Xe NMR signal of encapsulated xenon in cryptophane-A. However, although the concentration of SMFT is high (25 μ M), the signal of cryptophane-A caged xenon cannot be observed directly because of poor signal-to-noise (SNR). Indeed, as shown in Figure S1A, signal from dissolved ¹²⁹Xe appears at 236 ppm, there is no directly observable signal near 70 ppm. To circumvent this problem, we employed the hyper-CEST technique. Hyper-CEST is a method that combines hyperpolarization and the chemical exchange saturation transfer (CEST).³¹ After hyper-CEST was employed, a signal peak appears at 72 ppm (Figure S1B).

Hyper-CEST spectra of SMFT in different pH buffers were acquired. For pH 3.0 and pH 4.0, the CEST signal both appears at 72 ppm and the CEST effects are nearly identical (Figure S2A). Interestingly, when the pH of the buffer reaches 5.1, a new signal appears upfield (70 ppm) (Figure S2B), suggesting that the probe likely generates a new complex or alters its structure. Protonation of the iminonitrogens of the two pyrrolenine-like rings has been reported to occur at a pH

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Figure 3. Studies of optical spectra in solution and fluorescence imaging in cell of SMFT. (A) Adsorption spectrum of SMFT ($5 \mu M$) in PBS buffer (including 50% DMSO). (B) Fluorescence spectra of SMFT changes from pH 3.0 to pH 10.3. ($\lambda_{ex} = 430 \text{ nm}$; $\lambda_{em} = 600-800 \text{ nm}$, slit, 4 nm/4 nm) (C) Fluorescence intensity at 660 nm changed trend of SMFT with pH promoted from 3.0 to 10.3. (D) Fluorescence image for DAPI. (E) Fluorescence intensity for SMFT ($50 \mu M$) in cells. ($\lambda_{ex} = 402 \text{ nm}$; $\lambda_{em} = 665-735 \text{ nm}$). (F) Bright field. (G) Merge of D–F. (H, I) Quantization analysis of the fluorescence intensity in cells. Scale bars, 10 μm .



Figure 4. Fluorescence images of A549 and WI-38 cells incubated with SMFT (50 μ M). DAPI (blue) was used as nucleus dye.

of $5.0.^{32}$ When the pH is under 5.0, the two pyrrolenine-like rings are all protonated. Therefore, at lower pH (<5), SMFT only exhibits a single signal (resonance). Interestingly, the

CEST effect of the ¹²⁹Xe signal at 70 ppm increases almost linearly with pH from pH 5.1 to pH 9.3 (Figure 2A and Figure S2D). When increasing the pH to 10.3, the signal at 70 ppm



Figure 5. Studies of fluorescence imaging in vivo and ex vivo of SMFT. (A) Time-dependent fluorescence images of A549 tumor model nude mice after injection of SMFT (1 mM, 100 μ L) intravenously. (B) Time-course fluorescence signal intensity of the tumor post injection. (C) Average fluorescence intensity of major organs and tumor dissected from nude mice 24 h post injection of SMFT (n = 3), *p < 0.05.



Figure 6. Generation of singlet oxygen by SMFT and TPPS4 in buffer. (A) Time-course generation of singlet oxygen by SMFT at different concentrations in buffer. (B) Concentration-dependent generation of singlet oxygen by SMFT after irradiated for 20 min by 650 nm Laser. (C) Generation of singlet oxygen by SMFT (10 μ M) and TPPS4 (10 μ M) in buffer. (D) pH-dependent generation of singlet oxygen by SMFT (10 μ M) after irradiated for 20 min by 650 nm Laser. ΔA is the absorbance decrease of *p*-nitrosodimethylaniline at 440 nm. Error bars are based on the standard deviations (SD) of triplicated samples.

was larger than the one at 72 ppm (Figure S2C, D). This may be due to the three hydroxyl groups of SMFT, which are deprotonated one by one when the pH value of the solution was changed from acid to basic. We verified this by treating SMFT using aqueous NaOH followed by obtaining the ¹²⁹Xe NMR spectrum. Only a single resonance was observed, at 70 ppm (Figure S3A, B), which suggests that the three phenolic hydroxyl groups of SMFT may all be transformed into phenolate groups.

The SMFT can map pH in solution via hyper-CEST MRI. We found that the signal intensity of CEST images increased with pH (Figure 2B). Additionally, the average CEST effect as a function of pH (Figure S4) and associated trends were similar to the CEST spectra shown in Figure S2D, except for the absolute values. The latter differ because image intensities are related to several factors such as the MRI hardware settings, choice of imaging pulse sequence, etc.

More importantly, the SMFT exhibit good pH response in cell suspensions. We incubated the SMFT with cells for 4 h, treated the cells gradually, and resuspended them in buffers (pH 7.4 and pH 5.1).¹⁸ We found that for both pH 7.4 and pH 5.1, the CEST signal appears at 74 ppm, which we attribute to intracellular caged ¹²⁹Xe (Figure 2C). For pH 5.1, the CEST effect is 13%, whereas when the pH increased to 7.4, the CEST signal increased 33% compared to pH 5.1 (Figure 2D). These results demonstrate that SMFT has a good response to pH.

Fluorescence Imaging in Cell and in Vivo. To study the photophysical properties of SMFT, absorption spectra were obtained in solution. The Soret band of SMFT appeared at 426 nm, while the four Q bands appeared at 524 nm, 564 nm, 597 and 656 nm, respectively (Figure 3A). The molar extinction coefficient (4.04 \times 10⁵ M⁻¹ cm⁻¹) (Figure S5) and the fluorescence quantum yield ($\Phi = 0.137$) (Figure S6) of SMFT in DMSO were also obtained. The fluorescence spectra of SMFT were measured in buffers with different pH values. The fluorescence intensity decreases almost linearly with pH as the buffer was changed from 5.1 to 9.3 (Figure 3B, C); this change interval remained consistent with hyper-CEST, which suggests that hyper-CEST is a reliable and effective method for pH mapping. Importantly, SMFT has a strong fluorescence signal in a broad range of pH values (from pH 3.0 to pH 9.3) (Figure 3C), suggesting that SMFT could have potential applications in biological systems.

Because the fluorescence signal is strong when the pH value of the solution in a broad range (from pH 3 to pH 9.3), SMFT could be a promising fluorescence probe for imaging provided there is sufficient uptake by cells. We investigated SMFT uptake by lung cancer cells (A549) via confocal laser scanning microscope (CLSM). The fluorescence signal is uniformly distributed throughout the A549 cells. In addition, we found that the SMFT localized to the cytoplasm of cells (Figure 3D-G). Moreover, the fluorescence intensity of SMFT across the line in Figure 3H shows high red fluorescence intensity with high SNR (Figure 3I). These results show that SMFT could be transfected into A549 cells effectively.

The cancer cell microenvironment is more acidic than normal cells, which causes the porphyrin to be uptaken selectively by cancer cells.³³ Since the SMFT were endowed with a porphyrin moiety, we can investigate whether SMFT could target lung cancer cells selectively or not. A549 cells and WI-38 cells (human normal lung cells) were incubated with SMFT for 4 h, followed by fluorescence imaging on CLSM. As seen in Figure 4, fluorescence is uniformly distributed throughout A549 cells, and the red fluorescence intensity was weak in WI-38 cells, suggesting that transfection of SMFT into WI-38 cells was low. These data show that SMFT uptake by A549 cells is more effective than by WI-38 cells, suggesting that SMFT could be targeted to lung cancer cells.

The SMFT features strong fluorescence signals and Stokes shifts in solution, making it a potential fluorescent probe for



Figure 7. Studies of PDT efficiency of SMFT. (A) MTT assay of the A549 cells in the presence of different concentrations of SMFT with or without irradiation by 650 nm Laser (100 mW/cm², 10 min). Error bars are based on the standard deviations (SD) of 5 samples. (B) Photographs of A549 tumor bearing nude mice with different treatments, before and after. (C) Time-dependent tumor growth curves after treatments. Error bars are based on the standard deviations (SD) of 3 or 4 mice. (E) Mice body weight curves with relevant treatments. Error bars are based on the standard deviations (SD) of 3 or 4 mice. (E) Mice body weight curves after different treatments. Scale bar, 50 μ m.

animal imaging. The fluorescence imaging ability of SMFT were tested in a tumor model of nude mice. After injection of SMFT intravenously, the fluorescence images were obtained at different time points. The fluorescence intensity of the tumor from the SMFT increased within the time promoted (Figure SA, Figure S7). Twenty-four hours after injection, the ex vivo fluorescence image was obtained. The SMFT were mainly localized to the tumor, with a tiny amount of SMFT localized to the liver and kidney (Figure SB, C). This demonstrates that the SMFT can target tumor tissue and be imaged by fluorescence in vivo.

Generation of Singlet Oxygen in Solution. To evaluate the photosensitizing property of porphyrin we studied the singlet oxygen (¹O₂) production capability of SMFT via UVvis spectrometry. The generation of ¹O₂ was detected via pnitrosodimethylaniline in PBS buffer. The p-nitrosodimethylaniline absorbance at 440 nm is known to bleach in the presence of ${}^{1}O_{2}$. As shown in Figure 6A, the quantity of ${}^{1}O_{2}$ generated by SMFT increases with irradiation time. The quantity of ¹O₂ generated by SMFT increased linearly with probe concentration (Figure 6B). More importantly, the ${}^{1}O_{2}$ production ability of SMFT with 5,10,15,20-tetra (4sulfanatophenyl)-porphyrin (TPPS4) under the same condition is shown in Figure 6C. TPPS4 is a second-generation PDT sensitizer, which implies that SMFT may also be a good photosensitizer. We also studied the ¹O₂ production ability of SMFT in buffers at different pH values. We found that SMFT

has good ${}^{1}O_{2}$ production ability for pH values between 4 and 8 (Figure 6D). Thus, we conclude that SMFT could be a useful PDT agent for cancer therapy.

Photodynamic Therapy (PDT) In Cell and In Vivo. Encouraged by the good ${}^{1}O_{2}$ generation efficiency, first, we tested the PDT efficacy of SMFT on A549 cells by MTT assays. The cell viability with irradiation was found to decrease with SMFT concentration increased (Figure 7A). The IC50 value for SMFT in A549 cells with irradiation was calculated to be 15 μ M. Although cell viability without irradiation is close to 95% (Figure 7A), our results suggest that the key features of SMFT include good biocompatibility and low dark toxicity.

The in vivo anticancer efficacy of SMFT was tested on the A549 tumor mouse model. A549 tumor mouse models were divided in four groups after the tumors volume reached a size of ~50-70 mm³. Four mice were injected with SMFT (100 μ L, 1.15 mM) intratumorally. At 2 h postinjection, each tumor was irradiated with 650 nm laser (100 mW/cm²) for 40 min (240 J/cm²). Three other groups including (1) PBS (n = 3), (2) PBS+Laser (n = 3), (3) SMFT (n = 3) used as controls. After the group of mice injected with SMFT and irradiated by a 650 nm laser, the tumor growth could be completely inhibited. Conversely, the control groups with laser irradiation alone or with SMFT injection alone did not exhibit any tumor growth inhibition (Figure 7B, C). The tumor volume of the SMFT-injected with irradiation was smaller than the other groups (Figure 7D). Meanwhile, the body weight of all mice

did not exhibit any changes following treatment (Figure 7E). On the other hand, hematoxylin and eosin (H&E) staining of tumor slices confirmed that only experiment group tumors exhibited apoptosis/necrosis (Figure 7F). Furthermore, the SMFT did not cause any damage to the viscera, such as liver, heart, spleen, and kidney (Figure S8). Therefore, we conclude from these results that SMFT is an efficient photosensitizer for in vivo PDT of cancer.

CONCLUSIONS

How to combine multifunction in one single platform is challenging work. Most of the reported multifunctional probes are based on nanoparticles. In comparison, functional smallmolecule systems have obvious advantages in tumor uptake and metabolism in vivo. Our work, for the first time, introduces the hyperpolarized ¹²⁹Xe NMR into a small molecular multifunctional tool (SMFT), this probe can map pH values, be used for imaging of cells and tumor in vivo, and show good ability for PDT. The detection of pH and targeted imaging of tumors can improve the precision of tumors detection and guide the tumors treatment by PDT. This SMFT shows great promise as a highly versatile platform. We have thus expanded the multifunctional probe toolbox to include the multifunction in one single small molecule. Moreover, combining different detection method could obtain more abundant information on nidus than one single technique alone.³⁴⁻³⁸ Hyperpolarized ¹²⁹Xe magnetic resonance is a new promising technology. There are still many problems to be solved before hyperpolarized ¹²⁹Xe molecular probes can be used in the clinic, but this method has bright prospects and needs further study. Combining this method with other imaging techniques, such as fluorescence, is expected to be used for high sensitivity detection of clinical diseases in the future.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsabm.9b01080.

Experimental details, additional data including synthetic scheme of SMFT, ¹²⁹Xe NMR spectra, hyper-CEST spectra, in vivo fluorescence images, ¹H NMR spectra, ¹³C NMR spectra, and HRMS (PDF)

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Author Contributions

[†]Q.Z. and Q.G. contributed equally. X.Z., and Q.G. designed the study. Q.Z., Q.G., Y.Y., X.Z., W.J., S.X., and B.Z. completed the experiments. Q.Z. and Q.G. wrote the original draft. X.Z., X.L., C.Y., M.L., and L.-S.B. edited the manuscript. X.Z. supervised the project.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work is supported by National Key R&D Program of China (2018YFA0704000), National Natural Science Foundation of China (81625011, 91859206, 21921004), Key Research Program of Frontier Sciences, CAS (QYZDY-SSW-SLH018), and Hubei Provincial Natural Science Foundation of China (2017CFA013, 2018ACA143).

ABBREVIATIONS

NMR, nuclear magnetic resonance

MRI, nagnetic resonance imaging

PDT, photodynamic therapy

PTT, photothermal therapy

PET, positron emission computed tomography

SEOP, spin-exchange optical pumping

SMFT, smart small molecular multifunctional tool

SNR, signal-to-noise

CEST, chemical exchange saturation transfer

H&E, hematoxylin and eosin

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