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Enhanced Photodynamic Selectivity of Nano-Silica-Attached Porphyrins Against Breast Cancer Cells

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Abstract

The synthesis and characterization of bare silica (4 nm in diameter) nanoparticle-attached *meso*-tetra(N-methyl-4-pyridyl)porphine (SiO₂-TMPyP, 6 nm in diameter) are described for pH-controllable photosensitization. Distinguished from organosilanes, SiO₂ nanoparticles were functionalized as a potential quencher of triplet TMPyP and/or singlet oxygen (¹O₂) at alkaline pH, thereby turning off sensitizer photoactivity. In weak acidic solutions, TMPyP was released from SiO₂ surface for efficient production of ¹O₂. By monitoring ¹O₂ luminescence at 1270 nm, quantum yields of ¹O₂ production were found to be pH-dependent, dropping from ~ 0.45 in a pH range of 3–6 to 0.08 at pH 8–9, which is consistent with pH-dependent adsorption behavior of TMPyP on SiO₂ surface. These features make bare SiO₂-attached cationic porphyrin a promising candidate for use in PDT for cancer treatment in which efficient ¹O₂ production at acidic pH and sensitizer deactivation at physiological pH are desirable. The enhanced therapeutic selectivity was confirmed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) tests and trypan blue exclusion tests of cell viability in breast cancer cell lines. Bimolecular quenching rate constants of ¹O₂ by free TMPyP, SiO₂ and SiO₂-TMPyP nanoparticles were also determined.

Introduction

Photodynamic therapy (PDT) has developed into an emerging cancer treatment that has now become an FDA-approved therapy for different malignancies, in which singlet oxygen (¹O₂) plays a key role in light-induced cell death.^{1–4} Unfortunately, this therapeutic method is not highly selective. One of the side effects of PDT is prolonged skin photosensitivity due to sensitizer accumulation in normal cells. The molecular fabrication of an on/off switch in a sensitizer could overcome some of the limitations associated with sensitizer spatial localization and achieve, in turn, a higher level of PDT selectivity. The basic design of an on/off switch system is to incorporate certain triggers (such as pH, bioaffinity, temperature, etc.) into a conventional sensitizer to control the competition between the production and

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quenching of $^1\text{O}_2$ and/or triplet state of a sensitizer, which has been the subject of much recent work.^{5–11} The quenching process could occur through a variety of mechanisms,¹² including vibrational energy transfer.^{13, 14} The vibrational deactivation of $^1\text{O}_2$ has been used in stereoselective control of enecarbamate photooxidation.¹⁴ We herein propose a new system in which bare SiO_2 nanoparticles were functionalized as a potential quencher of triplet states and/or $^1\text{O}_2$ at alkaline pH. The enhanced photosensitization at acidic tumor pH is achieved directly on the interaction of a cationic porphyrin *meso*-tetra(*N*-methyl-4-pyridyl)porphine (TMPyP) with SiO_2 nanoparticles.

A sensitizer that produces $^1\text{O}_2$ at an acidic pH but is inactive at physiological pH would be of great benefit to therapeutic selectivity in cancer treatment because the pH of growing malignant tumors tends to be somewhat lower than that of surrounding normal tissue.^{15, 16} Various pH-responsive polymers designed for drug release have been developed for this purpose.^{17, 18} However, there are very few reports regarding the development of pH-controlled sensitizers. O'Shea's group prepared a supramolecular agent containing an amine functional group in which the pH-based reversibility of $^1\text{O}_2$ generation was achieved.¹⁹ At pH values greater than the pK_b of amines, intramolecular electron transfer from the adjacent amine quenched the excited sensitizer, hence preventing the energy transfer that leads to $^1\text{O}_2$ production. Upon the protonation of amines, however, electron-transfer quenching of the chromophore was precluded, and the photosensitized production of $^1\text{O}_2$ could ensue. Lee and co-workers prepared a polysaccharide/drug conjugate in which glycol chitosan was grafted with 3-diethylaminopropyl isothiocyanate, chlorin e6 and poly(ethylene glycol).¹¹ At higher pH chlorin e6 is deactivated via autoquenching. However, in tumor acidic conditions, polysaccharide/drug conjugate undergoes conformational change into an uncoiled structure for $^1\text{O}_2$ production. Our recent work showed that imidazole-modified porphyrin, 5,10,15,20-tetrakis(*N*-(2-(1H-imidazol-4-yl)ethyl)benzamide)porphyrin, produced twice as many $^1\text{O}_2$ molecules at pH 5.0 than at pH 7.4, whereas $^1\text{O}_2$ quenching rate was reduced by a factor of 2.5 for a pH change from 7.4 to 5.0.²⁰ Imidazole moieties were employed as a pH-sensitive trigger for controllable $^1\text{O}_2$ release. Wang, Zhang and co-workers prepared a pH-responsive nanoparticle-5 based platform for controllable $^1\text{O}_2$ production where both hydrophobic porphyrin and pH-indicator bromocresol purple (BCP) were encapsulated in organically modified silica nanoparticles.¹⁰ The activation of porphyrin molecules and subsequent $^1\text{O}_2$ production were only observed at acidic pH. In alkaline solutions, BCP absorbed light competitively, thereby restricting porphyrin excitation. In other pH-controlled photosensitization studies, Ogilby, Gothelf and co-workers reported pH-based reversible control of photosensitized $^1\text{O}_2$ production with a DNA i-motif.⁹ This i-motif, a four-stranded DNA structure, could deactivate the sensitizer by holding it close to a quencher at $\text{pH} < 5$. Under alkaline conditions, however, the cytosine residues in DNA were fully deprotonated and the i-motif was no longer stable. In turn, the sensitizer was released from the quencher and rescued its ability to generate $^1\text{O}_2$. Our work showed that the strong adsorption of 5,10,15,20-tetrakis(4-carboxyphenyl) porphyrin molecules onto a TiO_2 surface at a low pH favored the electron transfer reaction and hence resulted in inefficient $^1\text{O}_2$ production,²¹ which indicated the possibility of modulating $^1\text{O}_2$ production directly on the type of support media.

Silica-based nanoparticles for PDT applications have been previously studied.^{14, 22} In those work, the hydrophobic sensitizers could be encapsulated in organosilane nanoparticles in monomeric form without loss of activities. Rossi et al. developed an organosilane nanocarrier to load protoporphyrin IX that possessed a higher Φ_Δ in a silica matrix than in solution due to the formation of porphyrin monomers.²³ Prasad's group utilized organically modified silica-based nanoparticles for the aqueous dispersion of hydrophobic porphyrins for $^1\text{O}_2$ production.^{24–26} These nanoparticle systems showed enhanced two-photon absorption and could be excited at longer wavelengths within the therapeutic window.²⁵

Apparently, the use of organically modified silica nanoparticles retains both photosensitization efficacy and selective accumulation in tumor cells due to the nanopermmeability. We herein propose a new system, in which, instead of organosilanes, the bare SiO₂ nanoparticles were employed as a pH-based trigger for pH-controlled photosensitization. Based on photosensitization mechanism, a sensitizer is promoted by the absorption of light to an electronically excited singlet state that subsequently undergoes intersystem crossing to generate a triplet state. ¹O₂ is then produced by triplet energy transfer to ground state molecular oxygen (³O₂). SiO₂ colloids show a zero zeta potential at pH 2–4 but negative zeta potential at higher pH due to the dissociation of silanol protons.^{27–29} The pH at the point of zero charge also varies in different SiO₂ media.³⁰ The strong adsorption of positively charged dyes onto SiO₂ surfaces at higher pH could lead to charge transfer at nanointerface³¹ and a low yield of triplet formation,³² subsequently inhibiting ¹O₂ production. However, ¹O₂ photosensitization can be rescued when dyes are desorbed from SiO₂ surface at lower pH, which is desirable in PDT. The present work focuses on the preparation and characterization of pH-responsive bare SiO₂ nanoparticle-attached TMPyP (SiO₂-TMPyP). SiO₂ nanoparticles were functionalized as a potential quencher of triplet TMPyP and/or ¹O₂ in weak alkaline solutions, thereby turning off sensitizer photoactivities. However, TMPyP was released from SiO₂ surface at weak acidic pH for efficient production of ¹O₂. This pH-controlled therapeutic selectivity was confirmed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) tests and trypan blue exclusion test of cell viability in breast cancer cell lines. The quenching of ¹O₂ by free TMPyP, SiO₂ and SiO₂-TMPyP nanoparticles was also studied.

Experimental

Materials

Triton X-100 [polyoxyethylene (10) isooctylphenylether, 4-(C₈H₁₇)C₆H₄(OCH₂CH₃)_nOH, n~10], tetraethyl orthosilicate (TEOS) (99%), NH₃.H₂O (30 wt%) and Trypan blue solution (0.4% in Phosphate Buffered Saline) were purchased from Thermo Fisher Scientific. Cyclohexane and *n*-pentanol were purchased from Acros Chemicals. *Meso*-tetra(*N*-methyl-4-pyridyl)porphine tetratosylate (TMPyP), *N*-benzoyl-DL-methionine, 9,10-anthracenedipropionic acid, chloroform-*d*₃, acetonitrile-*d*₃, D₂O (99% atom), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. *meso*-Tetra(4-sulfonatophenyl)porphine dihydrochloride (TSPP) was purchased from Frontier Scientific. The human adenocarcinoma breast cell line SK-BR-3, McCoy's 5A Medium, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay kit (30-1010k), trypsin EDTA, fetal bovine serum (FBS) and F-12K medium 30-2004 were purchased from American Type Culture Collection (ATCC). Penicillin/streptomycin and phosphate-buffered saline (PBS) were purchased from Fisher Scientific, Inc. All reagents and solvents were used as received without further purification. Deionized water was obtained from a Barnsted deionization system.

Instrumentation

A pulsed Nd:YAG laser (Polaris II-20 Hz from New Wave Research, Inc) and a liquid-N₂-cooled germanium photodiode detector (Applied Detector Corporation) equipped with a 1270 nm interference filter and a 800 nm cut-on filter were used to monitor time-resolved ¹O₂ luminescence. The UV-visible absorption was recorded on a BioMate 3 UV-Vis spectrophotometer (Thermo Fisher Scientific, Inc.) or a SolidSpec-3700 UV-VIS-NIR Spectrophotometer (Shimadzu Corp.). Fluorescence spectra were measured on a FluoroMax-3 spectrofluorometer. A Multiskan Ascent 354 Plate Reader from Labsystems was used for absorbance measurements at 540 nm for the microplate colorimetric assay. The

morphologies of the SiO₂ and SiO₂-TMPyP were measured by transmission electron microscopy (TEM, JEOL 1200 and 2010). SiO₂-TMPyP treated breast cancer cells were irradiated under simulated solar light at an average intensity of 7.3 mW/cm² (68911 300-W Arc lamp from Oriel Instruments and 67005 Arc Lamp Housing from Newport Corporation equipped with a 500 nm cut on filter). The light intensity was measured with an Optronics OL754 Spectroradiometer (Optronics Laboratories, Orlando, FL). A 200 mW continuous wavelength Diode-Pumped Solid-State (DPSS) laser system (LUD532, Laserglow Technology, Toronto, Ontario, Canada) operating at 532 nm was used as a irradiation source in trypan blue exclusion tests of melanoma skin cancer cell viability. Bright field inverted microscope (Olympus CKX41, Olympus, Center Valley, PA) images of SiO₂-TMPyP were taken after being stained with Trypan Blue.

Preparation of SiO₂ nanoparticles and colloidal SiO₂ solutions

SiO₂ nanoparticles were synthesized according to a modified literature method³³ with quaternary microemulsion compositions of Triton X-100, cyclohexane, pentanol and deionized H₂O. Briefly, H₂O (7.5 mL) was added to a mixture of cyclohexane (100 mL), Triton X-100 (6.0 mL) and pentanol (4.0 mL), followed by 30 minutes sonication. Then, TEOS solution (7.5 mL) was added dropwise to the above microemulsion solution. The mixture was agitated by sonication for 2–3 hours then stirred for 2–4 hours at pH 8 adjusted by NH₃·H₂O solution (10 wt%). SiO₂ nanoparticles were washed with ethanol to the point where the sedimentation of white material ceased with the addition of AgNO₃. SiO₂ powders were dried in an oven at 80°C for 6 hours and then placed in a vacuum desiccator for three days. The sizes of SiO₂ nanoparticles were measured by TEM. A drop of colloidal nanoparticle solution was deposited on a Formvar-covered carbon-coated copper grid (Electron Microscopy Sciences, PA). Particle sizes were analyzed using a Nanotracer particle size analyzer (Microtrac, Inc.). SiO₂ nanoparticles were uniform, with ~70% of the particle diameters between 3 nm to 5 nm (see Supporting Information). Upon adsorption of TMPyP, the sizes of ~70% SiO₂-TMPyP particle increased to 5–7 nm. An average diameter of 4.0 nm for SiO₂ nanoparticles and 6.0 nm for SiO₂-TMPyP were therefore used for all calculations in this work. To prepare colloidal SiO₂ solutions, SiO₂ powder (0.10 g) was dissolved in H₂O or D₂O (10.0 mL) under sonication.

Adsorption of TMPyP onto SiO₂ nanoparticles

A mixture of SiO₂ colloids (10.00 mL 10 g/L) and TMPyP (0.0032 g) at pH 8 was stirred for 48 hours under dark conditions. Such prepared SiO₂-TMPyP nanoparticles were then separated from the solution by centrifugation, followed by washing with pH 8 NaOH solution and drying in a vacuum desiccator. The amount of adsorbed TMPyP was determined either as the difference between the quantity used in the starting solution and that remaining in the solution after filtration, or by calculation with Lambert-Beer's law using an extinction coefficient of $2.3 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ at 426 nm (See ESI). An average of 6.0 nm for SiO₂-TMPyP was used for calculations in this paper. Under our experimental conditions, an average of one TMPyP molecule was loaded per SiO₂ nanoparticle. A higher sensitizer loading ratio may be possible within solution phase preparation. However, in the present work, 1:1 SiO₂-TMPyP powders were dispersed into aqueous solutions at pH 8 to ensure the absence of any free porphyrin molecules.

Φ_{Δ} Measurement

Quantum yields were determined by time-resolved laser as previously described in which the ¹O₂ phosphorescence at 1270 nm was monitored, and the initial ¹O₂ intensity was extrapolated to time zero.³⁴ The data points of the initial ~ 5 μs were not used due to electronic interference signals from the detector. The intensity of the pulses at 532 nm ranged from 20 and 30 mJ. Φ_{Δ} were determined in aerated D₂O solutions by comparing the

intensity of $^1\text{O}_2$ phosphorescence at 1270 nm sensitized by SiO_2 -TMPyP with that sensitized by free TMPyP using the known value of Φ_{Δ} , TMPyP = 0.58 at pH 7.35. The absorbance of SiO_2 -TMPyP and the TMPyP reference was matched at an excitation wavelength of 532 nm, and was controlled to be between 0.8–0.9. Φ_{Δ} were calculated according to Eq. 1.

$$\frac{\Phi_{\Delta, \text{SiO}_2\text{-TMPyP}}}{\Phi_{\Delta, \text{TMPyP}}} = \frac{I_{\Delta, \text{SiO}_2\text{-TMPyP}}}{I_{\Delta, \text{TMPyP}}} \quad (1)$$

Here, $\Phi_{\Delta, \text{SiO}_2\text{-TMPyP}}$ and $\Phi_{\Delta, \text{TMPyP}}$ are the Φ_{Δ} from SiO_2 -TMPyP and TMPyP reference, respectively, and $I_{\Delta, \text{SiO}_2\text{-TMPyP}}$ and $I_{\Delta, \text{TMPyP}}$ are the $^1\text{O}_2$ intensities from SiO_2 -TMPyP and TMPyP reference, respectively.

Cell viability by MTT assay

Cell viability is measured by MTT assay to evaluate the effects of SiO_2 -TMPyP-initiated photodynamic therapy. The breast cancer cells were grown in McCoy's 5a medium supplemented with FBS (10%) and antibiotics (10 IU/mL penicillin) in 75-cm² tissue culture flasks at 37°C in a 5% CO_2 /95% air humidified incubator. The cells were collected and resuspended in PBS buffer (1×10^6 cells/ml), then seeded in 96-well plates (well diameter 6.4 mm) at a density of 10^6 cells/well and allowed to attach for 24 hours at 37°C in a 5% CO_2 incubator before treatment with SiO_2 -TMPyP. The medium at pH 6 was prepared from commercially available K-12 medium of pH 7.4 by addition of an appropriate amount of HCl (0.2 M). SiO_2 -TMPyP was added to pH 7.4 or pH 6 K-12 medium (containing 0.5% DMF) to produce a final concentration of 1.0 mg/ml. The above SiO_2 -TMPyP solution was then subjected to vortex or sonication or a combination of both. A solution (10.00 μL) of SiO_2 -TMPyP (1.0 mg/mL) was added to breast cancer cells. The mixture was then exposed to visible simulated solar light for 20 minutes at an average intensity of 7.3 mW/cm² for MTT tests. To perform MTT assay, MTT solution (50 μL , 5 mg/mL) was added to each well and incubated for 30 minutes. The cells were centrifuged, and the suspension was discarded. DMSO (200 μL) was added to each well and incubated for 10 min. The absorbance was read at 540 nm using a Multiskan Ascent Plate Reader.

Trypan blue exclusion tests with SiO_2 -TMPyP

The breast cancer cells were grown in a 5% CO_2 incubator at 37 °C in DMEM medium supplemented with 10% premium FBS and antibiotics (10 IU/mL penicillin G and streptomycin). Before use, the cells were resuspended at a concentration of 1×10^6 cells/mL in cell medium. A 100.0 μL of cell solutions (1×10^6 cells/mL) was seeded into each well and was treated with 38.0 μL of 1×10^{-6} M SiO_2 -TMPyP in a 5% CO_2 incubator at room temperature for 2 hours followed by 200 mW light exposure at 532 nm for 30 minutes. To determine the amount of cell death, 25.0 μL 5-times diluted trypan blue solution was added into each well. The SK-BR-3 cells were kept under darkness in the presence of trypan blue dyes for 20 minutes. The bright field inverted microscope images were then taken. In trypan blue exclusion tests, the dead cells take up the dyes and appear blue under microscope, whereas living cells exclude the dyes and appear translucent.

Results and Discussion

Adsorption of TMPyP onto SiO_2 nanoparticles

The adsorption of cationic porphyrins, such as TMPyP³⁶ onto glass³⁷ and metallo-TMPyP³⁸ onto mesoporous silica, has been reported. The zeta potential gives an indication of the change in surface charge.³⁹ Since electrostatic attraction between positively charged organic

molecules and SiO₂ nanoparticles is more pronounced at higher pH due to the prevalence of SiO⁻ anion sites, the preparation of SiO₂-TMPyP was performed at pH 8. A 1:1 loading ratio was obtained for TMPyP molecules adsorbed onto SiO₂ nanoparticles. Considering an average diameter of 4.0 nm for the SiO₂ nanoparticles and 2 nm for the porphyrin molecules, the percent coverage of TMPyP on SiO₂ surface is approximated as 6%, assuming round SiO₂ nanoparticles and cyclic porphyrin molecules.

Figure 1 shows the absorption spectra of SiO₂-TMPyP at different pH. The absorption features at pH 6.0 are more related to free porphyrins with two electronic transitions in visible region: a Soret band at 423 nm and four Q-bands (520, 557, 583 and 641 nm), which is characteristic of monomeric TMPyP.^{36, 40} The noticeable adsorption of TMPyP onto SiO₂ nanoparticles occurs at weak basic solutions. The spectrum of SiO₂-TMPyP at pH 9 is red-shifted to a Soret band of 426 nm and Q-bands of 523, 560, 587 and 643 nm, with the absorbance decreased by ~ 30% at Soret band when compared to that at pH 6. Examples of fluorescence spectra of SiO₂-TMPyP at different pH are shown in Figure 2. A broad fluorescence band from 660 nm to 720 nm was observed for free TMPyP at a wide range of pH, e.g., pH 3 (solid red line) and 7 (dot red line) in Figure 2. The fluorescence spectra of SiO₂-TMPyP split into two distinct bands centered at 658 nm and 710. The interaction between TMPyP and SiO₂ nanoparticles at a higher pH of 7 (black dot line) caused a decrease in fluorescence intensity when compared to these at pH 3 (solid black line) and 5 (dash black line) where the adsorption between cationic TMPyP and SiO₂ nanoparticles was somewhat inhibited and the sensitizers were desorbed from SiO₂ surface. The maximum emission wavelength of 658 nm is consistent with the literature reports of 650-665 nm.³⁶ Similar observations in the fluorescence spectra were also demonstrated for free TMPyP bound to negatively charged DNA⁴⁰ or adsorbed onto trace solid materials⁴¹ or silica.⁴² A variation in absorption and fluorescence bands as a function of pH reflected the features of charge transfer at nanointerface and clearly indicated the adsorption of TMPyP onto SiO₂ surfaces at higher pH.

Desorption of TMPyP from SiO₂ surface

Free TMPyP is soluble in a wide pH range of 2–10 in water. To test pH-triggered desorption of TMPyP from SiO₂ surface, SiO₂-TMPyP was precipitated by centrifugation at different pH. The supernatant was analyzed by absorption spectroscopy to quantify the remaining TMPyP concentrations at 423 nm using an extinction coefficient of $2.3 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$. As shown in Figure 3, the extent of desorption increases with a decrease of pH. The desorption occurred when pH was just below neutral and was complete at pH 2 (100%) where the adsorption of cationic TMPyP onto positively charged SiO₂ surface was inhibited. In alkaline solutions, the electrostatic attraction favored the association of positively charged pyridinium groups in TMPyP and negatively charged SiO₂ nanoparticles.

Time-resolved ¹O₂ luminescence

The luminescence of ¹O₂ was monitored at 1270 nm after laser pulse-excitation at 532 nm. Examples of ¹O₂ kinetic decay from SiO₂-TMPyP are shown in Figure 4. The challenge associated with the direct detection of ¹O₂ results from its weak luminescence signal at 1270 nm.^{43, 44} This detection difficulty can be further exacerbated by interference signals and the presence of quenchers that reduce the lifetime of ¹O₂. In Figure 4 ¹O₂ decay was displaced relative to the signals deriving from other rapid events coincident with the laser pulse (e.g., electronic interference from the detector). Such a background signal was usually observed from colloidal or heterogeneous systems (red line, Figure 4). Signals of ¹O₂ were therefore corrected for the interference by using the same but N₂-saturated sample as a control. The intensity of ¹O₂ was sensitive to oxygen concentration, and the 1270 nm luminescence decay was exponential. The observed first-order solvent deactivation rate constant of ¹O₂

(k_d) in D₂O was calculated to be $1.6 \times 10^4 \text{ s}^{-1}$, which is consistent with the literature value of $1.5 \times 10^4 \text{ s}^{-1}$.⁴⁵

Quantum yield of ¹O₂ production (Φ_Δ)

The Φ_Δ values measured from a colloid are less precise than that from a homogeneous solution because light scattering by suspended particles makes absorption measurements difficult. Nonetheless, Φ_Δ can be approximated using Eq. 1 by comparing the initial ¹O₂ intensity with that from a well-established reference sensitizer, such as free TMPyP that has a Φ_Δ value of 0.58 at pH 7.³⁵ The intensity of ¹O₂ signals was corrected for background interference with a N₂-saturated sample as a control. The Φ_Δ values from SiO₂-TMPyP revealed a sigmoidal curve, indicating pH-dependent photosensitization (Figure 4). The maximum ¹O₂ production by released TMPyP was observed in acidic solutions, which is consistent with the desorption behavior of TMPyP from SiO₂ surface (Figure 3). For comparison, the Φ_Δ values of free TMPyP were also determined as a function of pH, showing relative insensitivity to pH change.

The presence of charge transfer sites on SiO₂ surface has been reported based on the decrease in fluorescence lifetime and quantum yields of pyrene, perylene, coronene as well as other polyaromatic adsorbates on SiO₂ gel.³¹ The fluorescence and triplet quantum yields of several arenes on SiO₂ and other oxides were also found to be markedly lower than those in solution due to the formation of charge transfer complexes.³² Similar to these systems, the strong adsorption of TMPyP onto SiO₂ nanoparticles in alkaline solutions potentially facilitates the charge separation at nanointerface as indicated by the changes in absorption and fluorescence spectra, which could reduce the formation yield of triplet state. Moreover, it is reasonable to assume that the quenching of triplet TMPyP and/or ¹O₂ by SiO₂ nanoparticles would be more efficient when they are kept in close proximity to one another at alkaline pH. These factors could lead to a low Φ_Δ . The efficient ¹O₂ production under acidic conditions resulted from the desorbed TMPyP from SiO₂ surface. The lower Φ_Δ values obtained from SiO₂-TMPyP relative to those from free TMPyP in acidic solutions might be due to the possible aggregation and quenching of triplet TMPyP and/or ¹O₂ by SiO₂ nanoparticles (see discussion below). The aggregation is known to reduce the quantum yield and lifetime of the excited triplet states of porphyrins, thereby adversely affecting Φ_Δ .^{46, 47} Although molecular aggregation is assumed to be absent or minimized with cationic porphyrins, such as TMPyP because of delocalized positive charge over the porphyrin ring,⁴⁸ the formation of H-dimer⁴⁹ and H-tetramer⁵⁰ from TMPyP solutions was observed. For SiO₂-TMPyP sensitizers, porphyrin aggregation might depend on several factors, such as loading concentration, relative sizes of porphyrin molecules to SiO₂ nanoparticles, pH, etc., and could, to certain extent, reduce Φ_Δ .

Quenching of ¹O₂ by free TMPyP, SiO₂ and SiO₂-TMPyP nanoparticles

Bimolecular total quenching rate constants of ¹O₂ removal (k_T) by free TMPyP, SiO₂ and SiO₂-TMPyP nanoparticles were determined at different pH by Stern-Volmer analysis using TSP as a sensitizer (see ESI). The k_T values for SiO₂ nanoparticles were calculated in terms of particle concentrations. As shown in Table 1, the highest k_T was obtained from SiO₂ nanoparticles ($1.8 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ at pH 8.0 and $1.3 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ at pH 6.0) and was reduced when their surfaces were coated with TMPyP molecules ($6.2 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ at pH 8.0). For a 1:1 molar loading ratio of TMPyP to SiO₂ nanoparticles, the surface coverage of SiO₂ nanoparticles by TMPyP was calculated to be ~ 6%. Thus, the SiO₂ in SiO₂-TMPyP complex contributed to the majority of ¹O₂ quenching. Our results also explain the efficient photosensitization from sensitizers encapsulated in organosilane nanoparticles,²² where the quenching of ¹O₂ was considerably reduced due to the coverage of organic functional groups on silica surface. The k_T for SiO₂-TMPyP ($6.2 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$) was three times higher

than that for free TMPyP ($2.0 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$). The high quenching rate by SiO_2 nanoparticles could account for the inefficient production of $^1\text{O}_2$ by SiO_2 -TMPyP in alkaline solution where TMPyP and SiO_2 were kept in close proximity. It is reasonable to assume that the quenching of $^1\text{O}_2$ by SiO_2 nanoparticles is mainly via physical reactions due to the extreme stability of SiO_2 . Less than 30% decrease in quenching rate constants was observed for free TMPyP and SiO_2 nanoparticles at pH 6.0 than at pH 8.0, which might be attributed to the protonation of porphyrin and silica.

MTT assay and Trypan blue exclusion tests of cell viability

MTT is a yellow dye that is taken up by viable cells and reduced to an insoluble purple-colored formazan salt by enzymes in the endo-plasmic reticulum, cytosol, and mitochondria. The amount of chromophores produced is proportional to the number of viable cells. The human adenocarcinoma breast cell line SK-BR-3 was used to test photodynamic selectivity at both physiological pH 7.4 and acidic tumor extracellular pH 6.0. The results in Figure 6 reveal that SiO_2 -TMPyP exhibited pH-sensitive responses. For the same amount of sensitizer, the cytotoxicity was significantly enhanced at pH 6.0 compared to that at pH 7.4. When the pH was reduced from 7.4 to 6.0, the cell viability decreased from 60% (grey bar in samples 4) to 35% (red bar in samples 4) after 20 minute visible irradiation of SiO_2 -TMPyP in the breast cancer cell lines. Control experiments were performed under darkness in the absence (samples 1) and presence (samples 2) of SiO_2 -TMPyP at pH 7.4 (grey bars) and pH 6.0 (red bars), and under 20 minute visible irradiation in the absence of TMPyP at both pH (samples 3). Samples 1 were used as references for measurements at pH 7.4 (grey bars) and pH 6.0 (red bars), and were normalized to 100% cell viability. SiO_2 nanoparticles used in this work showed potential as selective drug carriers for PDT in cancer treatment.

A decrease in cell viability under dark control as well as light irradiation at pH 7.4 (grey bars in samples 2 and 4) suggested certain levels of toxicity or PDT activity from SiO_2 -TMPyP. Similar phenomenon was observed with polymer-conjugated pH-responsive sensitizers.¹¹ These issues may adversely affect therapeutic selectivity and should be taken into consideration for the practical application of pH-responsive sensitizers. Comparing to the pH-dependent Φ_{Δ} (Figure 5), a significant drop in cell viability was not obtained when pH was decreased from 7.4 to 6.0. A possible explanation might be the difference in experimental conditions. The lifetime of $^1\text{O}_2$ depends largely on the surrounding environment.⁵¹ Φ_{Δ} values were determined in D_2O solutions where $^1\text{O}_2$ has a much longer lifetime, e.g., $\sim 67 \mu\text{s}$ ⁵² in comparison to $\sim 3.5 \mu\text{s}$ in H_2O ⁵³ and $\sim 3 \mu\text{s}$ in cells.^{54, 55} The efficient decay of $^1\text{O}_2$ in cells would greatly limit its utility.

The trypan blue exclusion tests were performed at least three times to ensure the reliability. Examples are shown in Figure 7. SK-BR-3 cells were incubated with SiO_2 -TMPyP for 2 hours and irradiated at 532 nm for 30 minutes at different pH. Trypan blue only binds with dead cells, showing blue color in cell monolayer, whereas living cells are colorless. The cell viability was determined by microscopic examination. Figure 7 indicates that cell death was observed only in the presence of SiO_2 -TMPyP upon visible irradiation (right two images) but not under darkness (left two images) or in the absence of SiO_2 -TMPyP with visible irradiation (middle two images). Efficient cell death was confirmed at pH 6.1 (top right image) but not at pH 7.7 (bottom right image). The mechanism of light-induced cell death requires further investigations.

Conclusions

We propose a new system to improve the selectivity of PDT in which pH-controllable photosensitization was achieved directly upon the interaction of cationic TMPyP with bare SiO_2 nanoparticles. SiO_2 -TMPyP has the advantage of being highly dispersed in aqueous

solutions. The desorption of TMPyP from SiO₂ surface at acidic pH resulted in a higher Φ_{Δ} . The utility of SiO₂-TMPyP was, however, restricted in alkaline solutions due to the strong adsorption of TMPyP onto SiO₂ surfaces, which favored charge separation at nanointerface and efficient quenching of triplet TMPyP and/or ¹O₂ by SiO₂ nanoparticles. These features make bare SiO₂-attached cationic porphyrin a promising candidate for use in PDT for cancer treatment in which efficient ¹O₂ production at acidic pH and sensitizer deactivation at physiological pH are desirable. This pH-triggered therapeutic selectivity was further confirmed by MTT cytotoxicity tests and trypan blue exclusion tests of cell viability in breast cancer cell lines. Our work opens a simple path toward the modulation of photosensitization directly on support media.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Absorption spectra of 6.0×10^{-6} M TMPyP adsorbed onto SiO_2 nanoparticles at pH 6.0 (black line) and pH 9.0 (red line). The insertion is the enlarged view of the Q-bands absorption.

Figure 2. Fluorescence spectra of 1.0×10^{-6} M free TMPyP at pH 3 (red solid line) and 7 (dot red line), and SiO₂-TMPyP at pH 3 (solid black line), 5 (dash black line) and 7 (dot black line) upon excitation at 420 nm

Figure 3.
Effects of pH on 5.0×10^{-5} M TMPyP desorption from SiO₂ nanoparticle surface

Figure 4. Time-resolved $^1\text{O}_2$ luminescence at 1270 nm recorded upon irradiation of SiO_2 -TMPyP (OD = 0.8 at an excitation wavelength of 532 nm) in pH 5 O_2^- (blue line), air- (black line) and N_2 -saturated (red line) D_2O solutions. The insertion shows decays corrected with N_2 -saturated sample.

Figure 5. Variation of Φ_{Δ} with pH for free TMPyP (black line) and SiO₂-TMPyP (red line) in D₂O solutions, the error bars represent the standard deviations from three replicated experiments.

Figure 6. SK-BR-3 cell viability upon 20 minutes incubation with 1×10^6 breast cancer cells per mL for dark control in the absence (samples 1) and presence (samples 2) of 1.0 mg/mL SiO_2 -TMPyP, and for samples under 20 minute visible irradiation (at an average intensity of 7.3 mW/cm^2) in the absence (samples 3) and presence (samples 4) of 1.0 mg/mL SiO_2 -TMPyP. Samples 1 were used as references for measurements at pH 7.4 (grey bars) and pH 6.0 (red bars), and were normalized to 100% cell viability. The error bars represent the standard deviations from three replicated experiments (See ESI).

Figure 7.

Trypan blue exclusion tests of cell viability at pH 6.1 (top three images) and pH 7.7 (bottom three images): bright-field inverted microscope images of SK-BR-3 cells under darkness in the presence of SiO₂-TMPyP (left two images), under 200 mW laser irradiation at 532 nm for 30 minutes in the absence (middle two images) and presence (right two images) of SiO₂-TMPyP.

Table 1

Quenching rate constants of total $^1\text{O}_2$ removal by free TPyP, SiO_2 and SiO_2 -TPyP nanoparticles at different pH

$^1\text{O}_2$ Quenching by	TPyP	SiO_2	SiO_2 -TPyP	pH
$k_T \times 10^{-8}, \text{M}^{-1} \text{s}^{-1}$	2.0 ± 0.1	18 ± 2.1	6.2 ± 0.1	8.0
	1.5 ± 0.2	13 ± 4.1		6.0