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Organically Modified Silica Nanoparticles with Intraparticle Heavy-Atom Effect on the Encapsulated Photosensitizer for Enhanced Efficacy of Photodynamic Therapy

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Abstract

We report a novel nanoassembly formulation for photodynamic therapy, which is composed of covalently iodine-concentrated organically modified silica (ORMOSIL) nanoparticles (diameter <30 nm) and a hydrophobic photosensitizer embedded therein. Comparative studies with iodinated and non-iodinated nanoparticles have demonstrated that the intraparticle external heavy-atom effect on the encapsulated photosensitizer molecules significantly enhances the efficiency of ¹O₂ generation, and thereby, the in vitro PDT efficacy.

Introduction

Photodynamic therapy (PDT) is a noninvasive and selective treatment for cancer and other diseases.¹ After parenteral administration and preferential accumulation of photosensitizers (PS) in malignant tissues, the therapeutic effect is triggered by light irradiation, through which the photon energy absorbed by PS is transferred to the environment to generate reactive oxygen species (ROS), resulting in death of the irradiated cells via apoptosis and/or necrosis.²

Among ROS, singlet oxygen (¹O₂), produced through the reaction between the long-lived triplet state of PS (³PS*) and surrounding molecular oxygen, is generally considered as the main cytotoxic agent.³ Since population of the ³PS* occurs as a result of intersystem crossing (ISC) from the photoexcited singlet PS (¹PS*), the quantity of generated ¹O₂, governing the efficacy of PDT, is depended on the efficiency of ISC (¹PS*→³PS*). It is known that the rate of ISC can be increased as a result of enhanced spin-orbit coupling by the presence of heavy atoms which are incorporated into the chromophore (internal heavy atom effect), or are external but close enough to the chromophore (external heavy atom effect).⁴

The heavy-atom effect has widely been utilized for PDT, mainly in an internal fashion by covalently incorporating heavy atoms into the PS chromophore.⁵ However, the intramolecular approach involves complicated synthetic steps, which are not general and not always possible. Furthermore, in some cases, it can also cause undesirable modifications in

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the PS characteristics, e.g. shortening in the triplet state lifetime, resulting in a drop in the $^1\text{O}_2$ productivity. In this respect, the utilization of the intermolecular external heavy-atom effect can be considered as a promising strategy for improving $^1\text{O}_2$ generation by PS.

Here we report a novel nanoassembly approach utilizing heavy atom-concentrated nanoparticles, as a simple and efficient pathway for the external heavy-atom effected PDT. Recently, we have developed an aqueous formulation of organically modified silica (ORMOSIL) nanoparticles encapsulating a hydrophobic PS, 2-devinyl-2-(1-hexyloxyethyl)pyropheophorbide (HPPH), using a silicate precursor, triethoxyvinylsilane (VTES).⁶ Along with providing porous matrix permeable to oxygen molecules, they have versatile properties, such as the possibility of surface modification for bioavailability and targeting, the flexibility to choose a variety of silicate precursors, and the capability to co-localize multiple functionalities in the same nanoparticle space. Therefore, one can expect that the proper choice and utilization of a heavy atom-substituted precursor will produce PDT-functional ORMOSIL nanoparticles co-localizing PS and concentrated heavy atoms. The proposed nanostructure will satisfy the requirements for efficient intraparticle external heavy-atom effect, i.e., enough quantity of heavy atoms, and close proximity between the heavy atoms and the PS chromophore. As a proof of concept, we have chosen (3-iodopropyl)trimethoxysilane (IPTMS) to produce heavy atom-concentrated ORMOSIL nanoparticles. Here, we report the preparation of iodinated ORMOSIL nanoparticles encapsulating HPPH as well as an experimental proof of the intraparticle external heavy-atom effect, drawn from comparative studies using iodinated and non-iodinated nanoparticles. Also presented is experiment showing the enhanced efficacy of in vitro PDT, to demonstrate the validity and potential of our proposed strategy utilizing the intraparticle external heavy-atom effect.

Experimental Methods

Materials

Surfactant, Aerosol OT (AOT, sodium bis(2-ethylhexyl)-sulfosuccinate, 98%), triethoxyvinylsilane (VTES, 97%), and dimethyl sulfoxide (DMSO) were purchased from Aldrich. (3-Iodopropyl)-trimethoxysilane (IPTMS, 95.0%) was obtained from Fluka. Cosurfactant, 1-butanol, and NH_4OH (28.0-30.0%), are products of J.T. Baker. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was purchased from Sigma. The drug, HPPH, was provided by the Roswell Park Cancer Institute, Buffalo, NY. All the above chemicals were used as received without further purification.

Preparation of HPPH-Encapsulating ORMOSIL Nanoparticles

Both nanoparticles, INP and VNP, were prepared in the nonpolar core of AOT/1-butanol/water micelles, by using IPTMS and VTES, respectively. In a typical experiment, the micelles were prepared by dissolving the following amounts of AOT and 1-butanol in 10 mL of deionized water by vigorous magnetic stirring: 0.44 g of AOT/0.8 mL of 1-butanol for INP and 0.22 g of AOT/0.4 mL of 1-butanol for VNP. A 60 μL of HPPH solution in DMSO (5 mM) was added to both micellar solutions under magnetic stirring. 150 μL of neat silicate precursors, IPTMS and VTES, were added to each micellar system for INP and VNP, respectively, and the resulting mixtures were sonicated for about 5 min, or until they became homogeneous. After that, 40 μL of NH_4OH was added and both mixtures were magnetically stirred for about 20 hrs at room temperature, to ensure completion of sol-gel condensation. The residual DMSO, catalyst and surfactants were removed by dialyzing the nanoparticle dispersions against deionized water in a 12-14 kDa cutoff cellulose membrane (Spectrum Laboratories, Inc.) for 48 h.

Characterization

Transmission electron microscopy (TEM) was performed to determine the size and shape of the prepared nanoparticles, using a JEOL JEM-100cx microscope at an accelerating voltage of 80 kV. Absorption and fluorescence spectra were acquired using a Shimadzu UV-3600 spectrophotometer and a Jobin-Yvon Fluorog FL-3.11 spectrofluorometer, respectively.

Fluorescence lifetime measurements

EasyLife fluorescence lifetime system (Photon Technology International, Birmingham, NJ) was used to obtain decays of fluorescence from VNP and INP water suspensions. Origin 6.1 (OriginLab, Northampton, MA) was used to monoexponentially fit experimental decays.

Singlet oxygen phosphorescence detection

Time-resolved detection of the $^1\text{O}_2$ emission was used to distinguish singlet oxygen emission, which is known to have extremely low yield in water. A SPEX 270M spectrometer (Jobin Yvon) equipped with a Hamamatsu IR-PMT coupled to an Infinium oscilloscope (Hewlett-Packard) was used for recording singlet oxygen phosphorescence decay. The monochromator was tuned to 1270 nm. The second harmonic (532 nm) from a nanosecond pulsed Nd:YAG laser (Lotis TII, Belarus) operating at 20 Hz was used as the excitation source. The sample solution in a quartz cuvette was placed directly in front of the entrance slit of the spectrometer, and the emission signal was collected at 90° relative to the exciting laser beam. Additional long-pass filters (a 950LP filter and a 538AELP filter, both from Omega Optical) were used to attenuate the scattered light and fluorescence. Monoexponential fitting was performed using Origin software.

In Vitro PDT Studies with Tumor Cells

The RIF-1 tumor cells, grown in alpha-minimum essential medium (α -MEM) with 10% fetal calf serum, L-glutamine, and penicillin/streptomycin/neomycin, were maintained in 5% CO₂, 95% air, and 100% humidity. These cells were plated in 96-well plates at a density of 5×10^3 cells/well in complete medium, as a means to determine PDT efficacy. The next day, the IPS-conjugated nanoparticles were added at a concentration corresponding to 0.06 μM of HPPH. After the 24 h incubation in the dark at 37 °C, the cells were replaced with fresh media and exposed to light at a dose rate of 3.2 mW/cm² at various light doses (0, 0.25, 0.5, 1, 2, 4, 8 J). The dye laser (375; Spectra Physics, Mt. View, CA) excited by an argon-ion laser (171 laser; Spectra-Physics, Mt. View, CA) was tuned to emit the drug-activating wavelength of 665 nm. Uniform illumination was accomplished using a 600 μm diameter quartz optical fiber fitted with a graded index refraction lens. Following illumination, the plates were incubated at 37 °C in the dark for 48 hours. Appropriate control experiments using identical light doses without any photosensitizer were also performed. After this, the plates were evaluated for cell viability using cell viability assay.

Cell Viability Assay

Cell viability was measured using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide (MTT) assay. Immediately following light treatment, the cells were incubated for 44 h in the dark at 37 °C. Then, 10 μL of 4.0 mg/mL solution of MTT dissolved in PBS was added to each well. After 4 h incubation with the MTT, the media were removed and 100 μL of dimethyl sulfoxide was added to solubilize the formazan crystals. The PDT efficacy was measured by reading the 96-well plate on a microtiter plate reader (Miles Inc., Titertek Multiscan Plus MK II) at an absorbance of 560 nm. The results were plotted as percent survival compared with the corresponding control experiments results (cells were not incubated with drug, but exposed to light). Each data point represents the mean from a typical experiment with six replicate wells.

Results and Discussion

Aqueous formulations of iodinated and non-iodinated ORMOSIL nanoparticles encapsulating HPPH (hereafter, denoted as INP and VNP, respectively) were synthesized with commercial precursors, IPTMS and VTES, respectively, in the nonpolar core of Aerosol OT (AOT)/1-butanol/water micelles, following the reported method.^{6a,b} For quantitative comparison, the corresponding feed amounts of HPPH and precursors were kept the same for the INP and the VNP samples. The nanoparticle sizes were controlled to be similar by choosing an appropriate amount of AOT for each sample.^{6b} Figure 1 shows the precursor structures used and the transmission electron microscopy (TEM) images of the nanoparticles after dialysis. The obtained nanoparticles are spherical, with diameters of 25.7 ± 3.5 nm (INP) and 24.5 ± 4.1 nm (VNP), when 4.4 and 2.2 wt% of AOT in water were used for each preparation.

The absorption spectra of the encapsulated HPPH are almost identical, except that INP gives higher scattering than VNP, even with a comparable size range (Figure 2). This difference is apparently associated with the change in the refractive index with change in composition of the nanoparticles. At the same time, all the characteristic bands of HPPH appear at the same peak positions for both nanoparticles, indicating that the hydrophobic HPPH has been embedded in the INP matrix in the same way as in VNP, which is known to encapsulate HPPH in the monomeric form.⁶ Aggregation of the HPPH molecules results in broadening and bathochromic shift of the longest-wavelength Q band in the absorption spectrum.⁷ In our case of encapsulation in both INP and VNP, we see no such manifestation which suggests that the HPPH is molecularly dispersed within both ORMOSIL matrices, without noticeable self-aggregation.

Figure 3a shows the fluorescence spectra of INP and VNP, encapsulating the same amount of HPPH. Both nanoparticles exhibit typical HPPH fluorescence peaking at 667 nm, further confirming no occurrence of aggregation.⁶ However, it is clearly seen that the fluorescence intensity is significantly reduced in INP with respect to that in VNP. Fluorescence decays can be fitted monoexponentially, indicating shortening of HPPH fluorescence lifetime from 7.8 ns in VNP to 5.4 ns in INP (Figure 3b). A decrease in both fluorescence intensity and lifetime of HPPH entrapped in INP in a comparison to those of HPPH in VNP, in the absence of other noticeable quenching effects (e.g., aggregation), can be caused by the presence of the excess iodine atoms in close proximity to the HPPH chromophore. The ratio between iodine and HPPH is $\sim 2.6 \times 10^3$, according to the feeding amounts. This implies that molecularly dispersed HPPH molecules in the iodopropyl-rich regions within INP nanoparticles are surrounded with large excess of iodines within the nanoscopic distance, which can lead to enhanced spin-orbit coupling and, correspondingly, an increase in the probability of the intersystem crossing.⁴ In this case, with an increase in population of the triplet level of the photosensitizer, an increase in singlet oxygen sensitization may also occur, which can further support that the intraparticle external heavy-atom effect does take place in the iodinated nanoparticles.

We have monitored $^1\text{O}_2$ generation by its characteristic phosphorescence peaked at 1270 nm.⁶⁻⁸ Figure 4 shows the detector decays of the spectral signal at 1270 nm for INP and VNP, under nanosecond pulsed excitation at 532 nm. Along with a fast component coming from the scattered excitation light, one can see a slow decay component with characteristic lifetime in the microsecond range for both nanoparticle formulations, which is definitely the $^1\text{O}_2$ phosphorescence, suggesting that HPPH retains the functionality as PS for PDT, in both ORMOSIL matrices. Importantly, the quantity of $^1\text{O}_2$, generated by INP and estimated from the area under decay curve, is found to be increased by ~ 1.7 times over that in the case of VNP. In contrast to this generation efficiency change, the lifetimes of $^1\text{O}_2$, sensitized in

both nanoparticles, are close enough to each other ($\sim 4.3 \mu\text{s}$ for VNP and $\sim 4.6 \mu\text{s}$ for INP) and to that in water ($4 \mu\text{s}$)⁹. The close values of the $^1\text{O}_2$ lifetimes indicates that the sensitized oxygen molecules are mostly deactivated outside the nanoparticles, which, along with the enhanced generation efficiency, would result in enhanced phototoxic efficacy of the nanoparticles.⁸ To check this, we have performed a comparative study of the in vitro photosensitizing activity of IVP and INP.

Cell viability (MTT) assay^{6a,7,8} was carried out for RIF-1 tumor cells treated with INP and VNP; the latter is known to be avidly internalized into tumor cells and exert phototoxic effect.^{6a} As shown by the light dose response of cell viability in Figure 5, INP exhibits significantly enhanced phototoxic effect over VNP, well elucidating the potential of our PS/heavy atom nanoassembly for improving the actual efficacy of PDT.

Conclusion

We present here the first example of an iodine-concentrated ORMOSIL nanoparticle formulation for PDT, in which the intraparticle external heavy-atom effect on the encapsulated PS molecules significantly enhances the efficiency of $^1\text{O}_2$ generation, and thereby, the in vitro PDT efficacy.

Acknowledgments

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Figure 1. TEM images of HPPH-encapsulating ORMOSIL nanoparticles, prepared with (a) (3-iodopropyl)trimethoxysilane (IPTMS) and (b) triethoxyvinylsilane (VTES), respectively. Scale bars: 100 nm. Insets show the structures of each precursor used.

Figure 2.
Absorption spectra of VNP (blue) and INP (red), entrapping the same amount of HPPH.

Figure 3.

Fluorescence spectra (a) and decays (b) of the same amount of HPPH, entrapped in water-dispersed VNP (blue) and INP (red). Colored solid lines in (b) indicate monoexponential fitting. Instrument response function (IRF) is shown as black line.

Figure 4.
 $^1\text{O}_2$ phosphorescence decays for VNP (blue) and INP (red). Green lines show monoexponential fitting of the decays. IRF is shown as black line.

Figure 5.

Comparative in vitro photosensitizing efficacy of VNP (blue) and INP (red). RIF-1 cells were treated with nanoparticles to have the same concentration of HPPH in every well (0.06 μ M).