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Photosensitizer encapsulated organically modified silica nanoparticles for direct two-photon photodynamic therapy and *In Vivo* functional imaging

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ABSTRACT

Nanoparticle-assisted two-photon imaging and near infrared (NIR) imaging are two important technologies in biophotonics research. In the present paper, organically modified silica (ORMOSIL) nanoparticles encapsulated with either PpIX (protoporphyrin IX) photosensitizers or IR-820 NIR fluorophores were synthesized and optically characterized. Using the former ORMOSIL nanoparticles, we showed: (i) direct excitation of the fluorescence of PpIX through its efficient two-photon absorption in the intracellular environment of tumor cells, and (ii) cytotoxicity towards tumor cells by PpIX under two-photon irradiation. The latter ORMOSIL nanoparticles can be used as efficient NIR fluorescent contrast agents for various types *in vivo* animal imaging. We applied IR-820 doped ORMOSIL nanoparticles in *in vivo* brain imaging of mice. We also demonstrated the applications of them to sentinel lymph node (SLN) mapping of mice. Finally, we showed that the nanoprobes could target the subcutaneously xenografted tumor of a mouse for long time observations. ORMOSIL nanoparticles have great potentials for disease diagnosis and clinical therapies.

1. Introduction

During past years, organically modified silica (ORMOSIL) nanoparticles [1] have shown their great potentials as an ideal nanoplatform for various multimodal bio-imaging and "theranostic" (diagnosis-therapy) applications [2–5]. ORMOSIL nanoparticles are mesoporous with big pores in their matrix, which can facilitate some controlled release of encapsulated biomolecules like drugs, proteins and reactive oxygen species, etc. ORMOSIL nanoparticles can be loaded with either hydrophilic or hydrophobic drugs/dyes, protecting them against denaturation by the extreme bio-environment: by changing the dye type, the fluorescent ORMOSIL nanoparticles can achieve good fluorescence quantum yield and tunable photoluminescence that spans the entire visible and IR spectrum; by changing the drug type (such as genetic materials, chemotherapeutic and photodynamic drugs), the fluorescent ORMOSIL nanoparticles can be used to treat different kinds of diseases. ORMOSIL nanoparticles can be surface-functionalized with various chemical groups (e.g., carboxyl/thiol/amino/hydroxyl), and can be further conjugated with different targeting biomolecules (such as proteins and antibodies) and additional functionalities (such as probes for MR/radio imaging). Chemically inert ORMOSIL nanoparticles are transparent to visible/NIR light, and possess good biocompatibility. Due to the aforementioned features, ORMOSIL nanoparticles have been widely applied in photodynamic therapy (PDT). Previously, Roy et al. [6] and Ohulchanskyy et al. [7] have used HPPH doped ORMOSIL nanoparticles for *in vitro* PDT of tumor cells. Our group have utilized ORMOSIL nanoparticles to encapsulate protoporphyrin IX (PpIX), and applied them in PDT of HeLa cells [8,9]. Furthermore, ORMOSIL nanoparticles have also been used in many *in vitro* bio-imaging examples [10–12].

Two-photon excitation induced bio-imaging has many unique advantages [13–16]. Due to a quadratic dependence of two-photon absorption on laser intensity [17,18], the sample region outside the beam focus cannot be excited, and it could reduce the possibility of photobleaching. The nonlinear excitation mode is also helpful to improve the spatial resolution of imaging, since only the site where the laser beam is focused can be efficiently excited. These two advantages of two-photon excitation are very important to long-term and selective imaging/PDT of biological specimen.

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Kim et al. used to co-encapsulate HPPH and a special type of two-photon absorbing fluorophores into ORMOSIL nanoparticles, and used them for indirect two-photon PDT of *in vitro* cells, which was based on the Foster resonance energy transfer (FRET) effect inside nanoparticles [19]. Furthermore, two-photon excitation has great potentials for deep-range tissue imaging by utilizing laser scanning microscopy. For one-photon bio-imaging, photosensitizers usually absorb light in the visible spectral region below 700 nm, where light penetration into the skin is only a few millimeters. On the other hand, the laser wavelength for two-photon excitation is usually in the range of 700–900 nm, which is typically considered as the transparent window of light for tissues [20], and thus the penetration of excitation light and the generation of deep-tissue signals can be improved.

Another promising approach towards deep-range imaging is to use near infrared (NIR) excitation and emission [21,22]. Compared with visible light, NIR light (700–900 nm) is less absorbed and scattered by biological tissue and thus NIR excitation can greatly increase the penetration depth and emission. Furthermore, NIR light has lower energy than ultraviolet- and visible light, and less fluorophores in tissue can be stimulated if NIR excitation is adopted. Thus it can efficiently restrain the generation of auto-fluorescence and improve the contrast of the image. Different from two-photon excitation, NIR deep-tissue imaging is usually performed on a macro *in vivo* imaging system. Although ORMOSIL nanoparticles have been used in various types of *in vitro* bio-imaging, the reports about their *in vivo* applications are relatively rare. Recently, Kumar et al. [23] have utilized DY776-doped ORMOSIL nanoparticles for NIR *in vivo* animal imaging. In their work, NIR signals from ORMOSIL nanoparticles were helpful to investigate the biodistribution and clearance process of ORMOSIL nanopaticles in animal bodies. Histological analysis of the dissected organs illustrated that ORMOSIL nanopaticles produced no cytotoxic effects in animal tissues. However, no further applications of NIR fluorescent ORMOSIL nanoparticles have been demonstrated in their research.

In this paper, we report the synthesis of ORMOSIL nanoparticles, which are encapsulated with photosensitizer PpIX and NIR fluorophores IR-820 [24]. PpIX photosensitizers have been officially approved for use in clinical treatments and are commercially available [25,26]. Here we measure the one- and two-photon absorption/fluorescence properties of PpIX doped ORMOSIL nanoparticles, and investigate their feasibility in fluorescence imaging and PDT towards tumor cells under two-photon excitations. We then verify the deep-range imaging capacity of IR-820 encapsulated ORMOSIL nanoparticles in simulated tissue, and investigate their applications in *in vivo* functional imaging of mice [e.g., brain imaging, sentinel lymph node (SLN) mapping and tumor targeting]

2. Experimental section

2.1. Materials

Aerosol OT (98%), VTES (97%), APTES (98%), PpIX, IR-820 and O,O′-Bis[2-(N-Succinimidyl-succinylamino)ethyl] polyethylene glycol 3000 (NHS-PEG-NHS, 3000) were purchased from Sigma Aldrich. DMSO, 1-butanol (99.8%), acetone and sodium hydroxide (NaOH) were purchased from Sinopharm Chemical Reagent Co., Ltd, China. Cell-culture products, unless otherwise mentioned, were purchased from GIBCO. All the above chemicals were used without any additional purification, and DI water was used in all the experimental steps.

2.2. Synthesis of PpIX/IR-820 doped ORMOSIL nanoparticles

ORMOSIL nanoparticles with PpIX or IR-820 were synthesized in the nonpolar core of Aerosol-OT/DMSO/water micelles (shown in Fig. 1) [12]. Typically, the micelles were prepared by dissolving a certain amount of Aerosol-OT and 1-butanol in 10 ml of DI water by vigorous magnetic stirring. 400 μl of PpIX/IR-820 in DMSO (1 mM) was then added to the solution under magnetic stirring. Half an hour later, 100 μl of neat VTES was added to the micellar system, and the resulting solution was stirred for about 1 h. Next, ORMOSIL nanoparticles were precipitated by adding 15 μl of APTES and stirred for another 20 h at room temperature. After the formation of the nanoparticles, surfactant Aerosol-OT, cosurfactant 1-butanol, residual VTES and APTES were removed by dialyzing the solution against DI water in a 12–14 kDa cutoff cellulose membrane for 50 h. The dialyzed solution was then filtered through a 0.45 μm cutoff membrane filter to be used in later experiments.

2.3. Conjugating polyethylene glycol (PEG) with IR-820 doped ORMOSIL nanoparticles

As illustrated in Fig. 1B, 18 mg NHS-PEG-NHS (MW: 3000) was added to 2.5 ml aqueous dispersion of IR-820 doped ORMOSIL nanoparticles, and NaOH solution was added drop by drop to keep the pH value of the solution around 8. Since amino groups were grafted on the surfaces of ORMOSIL nanoparticles, PEG molecules could conjugate with nanoparticles through specific NHS-NH_2 bonds in the weak alkali solution. 3 h later, the reaction solution was dialyzed against DI water for 24 h to remove the unreacted excess NHS-PEG-NHS molecules.

2.4. Characterization

The structures of two kinds of ORMOSIL nanoparticles were taken by a JEOL JEM-1200EX transmission electron microscope (TEM) operated at 160 kV in bright-field mode. The absorption/transmission spectra of nanoparticles within the wavelength region of 300 nm–900 nm were recorded by a Shimadzu 2550 UV–vis scanning spectrophotometer. One-photon excited fluorescence spectrum of PpIX doped ORMOSIL nanoparticles was obtained by a Fluorescence Spectrophotometer (F-2500, HITACHI, Japan). Two-photon fluorescence of PpIX doped ORMOSIL

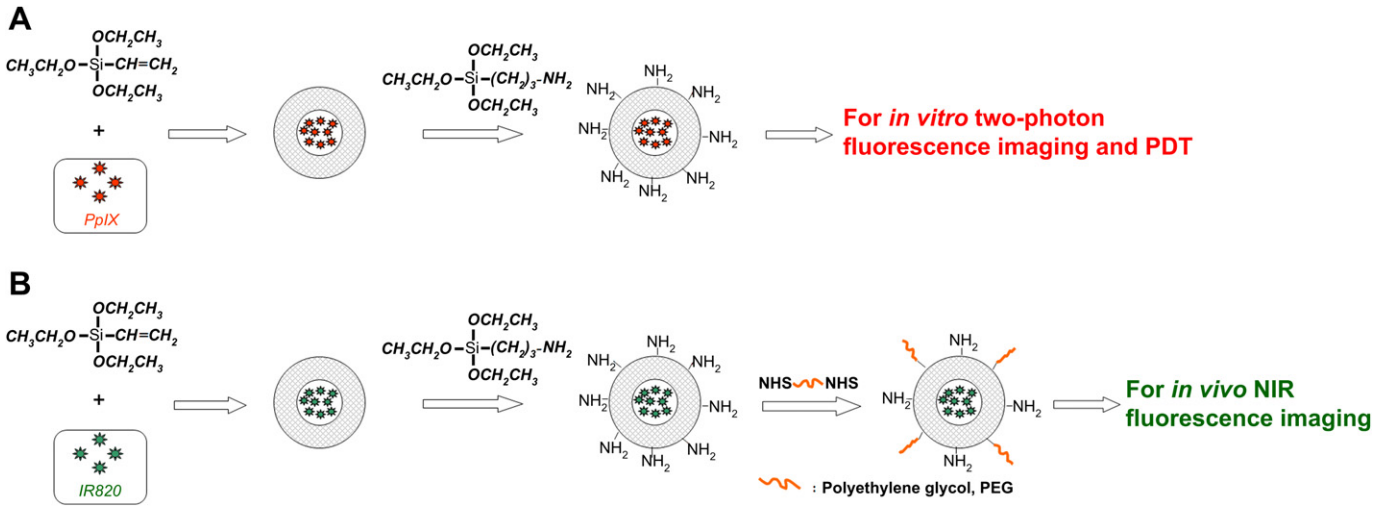

Fig. 1. Synthesis illustration of PpIX doped ORMOSIL nanoparticles (A) and PEG modified IR-820 doped ORMOSIL nanoparticles (B).