A universal strategy for the fabrication of single-photon and multiphoton NIR chromophore nanoparticles by loading organic dyes into water-soluble polymer nanosponges

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Abstract

The development of optical organic nanoparticles (NPs) is desirable and widely studied. However, most organic chromophores are water-insoluble, and therefore, the derivatization and modification of these chromophores are difficult. Herein, we demonstrated a simple platform for the fabrication of organic nanoemitters by loading ten different organic dyes (molar masses of 479.1-1081.7 g/mol) into water-soluble polymer nanosponges composed of poly(styrene-alt-maleic acid) (PSMA). This nanolization strategy enables the incorporation of small commercial dyes (3.7–50% loading) and newly synthesized large photosensitive molecules (> 85% loading) into PSMA NPs and prevents the spontaneous aggregation of these hydrophobic chromophores in aqueous solutions. These low-cytotoxicity organic nanoparticles exhibited tunable red to near-infrared fluorescence emission for cellular imaging and biological tracking *in vivo*. Moreover, PSMA NPs loaded with designed NIR-photosensitive molecules were fabricated, and photodynamic therapy with these dye-loaded PSMA NPs for the photolysis of cancer cells was achieved when coupled with 808 nm laser excitation. Indeed, our work demonstrates a facile approach for increasing the biocompatibility and stability of organic dyes by loading them into water-soluble polymer-based carriers, providing a new perspective of organic optoelectronic materials in biomedical theranostic applications.

Introduction

Over the past several decades, numerous organic molecules with near-infrared (NIR) absorption or emission properties have been synthesized for the development of efficient optoelectronic devices, such as organic light-emitting diodes (OLEDs) [1, 2], organic photovoltaics (OPVs) [3, 4], and dye-sensitized solar cells (DSSCs) [5, 6]. Compared to commonly used organic dyes, organic dyes applied in organic electronic devices are more suitable for biomedical applications due to their excellent photostabilities and high fluorescence quantum yields. Their self-assembling characteristics are beneficial for the formation of organic nanoparticles. Nevertheless, extending the π -conjugated system of a molecule results in an uncontrollable bulky structure, significantly hindering the fabrication of nanoparticles using these molecules [7–17]. In addition, the controlled nanolization of organic dyes is difficult, especially when -COOH or -NH₂ functional groups [18–21] are needed for subsequent biografting/derivatization without changing their optical properties after bioconjugation. Therefore, current applications of the nanolization of organic NIR dyes in biomedical applications are still limited.

To date, compared with the studies of NIR-absorbing organic materials, studies of organic materials as NIR emitters have been scarce. There are only a few examples of organic materials, such as rare earth (lanthanide) complexes [22], organic ion dyes [23], transition metal complexes [24], and low band gaps small molecules or polymers, that exhibit NIR electroluminescence (EL) properties [25–27]. For practical biomedical applications, developing NIR-active organic nanoemitters [10, 15, 17] allows deep-tissue bioimaging and laser therapy with low heating damage. Since the molecular structures of NIR organic emitters usually contain a well-extended π -conjugated system, the structural modification strategy for each organic emitter must be tailor-made, which increases the complexity of their organic synthesis [28].

Recently, many research teams have developed organic emitters for NIR nanophotonics [9, 13–16, 29–35]. They adopted methods such as coating [12], polymer grafting [13, 30, 31, 33], AIE self-assembly [16, 32], and coprecipitation [9, 14] to achieve individual NIR emitter formulations by nanofabrication. Scientifically formulated conjugation strategies [34, 35] by incorporating π -conjugated backbones or by chemically linking to nanocarriers have led to the synthesis of NIR semiconducting polymers. Unfortunately, these nanofabrication techniques cannot be universally applied to all types of organic emitters due to their different structural characteristics. The fabrication conditions may be affected by the charge and hydrophobicity of the organic molecular side chains such that they should be adjusted for different emitters. These obstacles impede the widespread use of OLED-, OPV-, and DSSC-functional molecules in nanomedicine. As a result, the development of a facile method for the nanolization of different organic emitters is desirable for the future exploration of π -conjugated chromophores through nanocondensable application characteristics.

Herein, we demonstrated a universal strategy to fabricate optically active nanoparticles by loading commercially available and newly synthesized photosensitive molecules (i.e., designed OPV-, DSSC- and OLED-structured chromophore) into metal-free poly(styrene-alt-maleic acid) (PSMA) nanoparticles (Scheme 1a, b). The PSMA organic nanoparticles exhibit sponge properties for the direct adsorption of various optical molecules (i.e., commercial emitters) (3.7–50% loading) and designed chromophores (over 85% loading). This strategy provides design flexibility for the emission of fluorescence wavelengths from the visible to NIR band. These PSMA-based nanosponges showed physisorption ability, which prevented the aggregation of the organic molecules in water, biocompatibility [11] and conjugation/complex capability [8, 36] due to their amphiphilic nature [7, 8, 36–39] in aqueous solution. Our technique not only satisfies the need for the self-assembly of dyes to facilitate the formation of a micelle configuration, which has been shown to be susceptible to environmental dissociation [40], but is also not limited to adsorbing positively charged dyes. [36, 41] Owing to the single-photon and multiphoton NIR fluorescence properties of the organic dyes, NIR bioimaging *in vitro* and *in vivo* using these organic nanoparticles was achieved (Scheme 1c). In addition, the NIR-triggered photodynamic therapy (PDT) of bladder cancer cells was demonstrated by using PSMA NPs loaded with NIR dye.

Materials And Methods

Materials

Poly(styrene-alt-maleic acid) sodium salt solution (PSMA; Sigma–Aldrich), CuCl_2 (J.T. Baker), N_2H_4 (Alfa Aesar), hydrochloric acid (HCl, 37%; Fluka), $\text{H}_2\text{N-PEG-NH}_2$ (MW = 3500; JenKem Technology), 1-ethyl-3-(3-dimethylamioethyl)carbodiimide hydrochloride (EDC; Matrix scientific), N-hydroxysuccinimide (NHS, 98%+; Alfa Aesar), 4-carboxyphenylboronic acid (CPBA; Aldrich), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT assay reagent; Alfa Aesar), 2,7-dichlorofluorescein diacetate (DCFH-DA; Sigma–Aldrich), N,N-dimethyl-4-nitrosoaniline (RNO; Alfa Aesar), imidazole (Acros organics), dimethyl sulfoxide (DMSO; Fisher scientific), sodium iron chlorophyllin salt (Chl-Fe), rhodamine 6G (R6G; Sigma–

Aldrich), cyanine 5 (Cy5; Tocris Bioscience), synthesized OLED- (ADF1 ~ 3), OPV- (242), and DSSC- (YI-1, YI-3, YI-8) based photosensitive molecules (see organic chromophore syntheses in the SI experiment section), tetrahydrofuran (THF; Macron Fine Chemicals), ethanol (J.T. Baker), and 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine (JC-1; Sigma–Aldrich). 4,8-Dibromo-6-(2-ethylhexyl)-5*H*[1, 2, 5]thiadiazolo[3,4-*f*]isoindole-5,7(6*H*)-dione (**TID-Br**) was prepared according to the literature [42]. For the synthesis of DTDPTID, please see the SI experiment section.

Characterization

Transmission electron microscopy (TEM, Hitachi H7500 TEM instrument at 80 kV) was utilized to determine the structures and sizes of the PSMA-dye NPs. The absorption spectra of the PSMA-dye NP samples were measured by a V-730 UV–Vis spectrophotometer from Jasco (USA). The absorption rates of the PSMA dye samples were quantified by a fluorescence microplate reader (Biotek Synergy H1, USA). The zeta potentials (HORIBA, Ltd., Japan) of the PSMA-dye NP samples dispersed in an aqueous solution were measured. The FT-IR spectra obtained by Fourier transform infrared spectroscopy (JASCO FT/IR-4700) with a KBr plate were used for the vibrational signal analyses of the PSMA-dye NP samples. A fluorescence microscope (Olympus IX73) was employed for fluorescent cell imaging with UFUNA (420–460 nm), UFBWA (510–550 nm), and UFGW (> 575 nm) filters for blue, green, and red fluorescence observation, respectively. A Nikon Inverted Multiphoton Microscope Eclipse (A1MP + Eclipse Ti-2E, Nikon instrument Inc., Japan) with a 40 × NA = 1.15 water-immersion objective was used for *in vitro* and *in vivo* nonlinear photonics analysis.

PSMA NP preparation

The PSMA NPs were prepared through the etching of Cu@PSMA NPs by HCl solution. In brief, 1 mL of CuCl₂ solution (6 mM) was first mixed with 10 mL of PSMA solution (6 mg/mL) and then 22 μL of HCl (2 M) and 0.1 mL of N₂H₄ hydrate were subsequently added to the mixture before being sealed in a 23 mL Teflon-lined autoclave reactor. The sealed reactor was subjected to hydrothermal treatment at 158°C for 3 h in an oven to form Cu@PSMA NPs. The Cu@PSMA NPs were etched by 1.5 M HCl for 1.5 h to remove the copper, resulting in PSMA NPs. Finally, the resulting product was collected by centrifuging at 11,000 rpm for 10 minutes to collect the PSMA NPs, followed by three centrifugation/resuspension processes for product purification.

PSMA-dye NP preparation

Small molecular dyes and OLED-, OPV-, and DSSC-based photosensitive molecules were prepared in THF with 5 mM stock solutions. The prepared PSMA NPs (OD = 2 at 230 nm) were mixed with 0.2 mM of each dye in a 1:1 volume ratio and kept in the dark at 4°C overnight for dye adsorption. The synthesized PSMA-dye NPs were then centrifuged at 11,000 rpm for 10 minutes to remove the nonadsorbed dyes and washed with water three times. The absorption rates of the immobilized dyes were determined by using a fluorescent microplate reader.

CPBA-conjugated PSMA-dye NP preparation

To enhance the *in vitro* PDT of bladder cancer cells, we prepared PSMA-dye NPs conjugated with the target ligand CPBA. Briefly, 4 mg of $\text{NH}_2\text{-PEG-NH}_2$ was mixed with 200 μL of PSMA-dye NPs (0.1 mM of dyes and ultrasonicated in the dark for 5 minutes. Then, 0.7 mL of NHS/EDC mixture (1.5 mg/1.5 mg) was added to induce PEGylation, followed by mixing with CPBA (0.05 mM, 50 μL) in the dark at 4°C for 1 h. The sample was then centrifuged at 9500 rpm for 10 minutes and washed with DI water 3 times to obtain CPBA-modified PSMA-dye NPs for the *in vitro* PDT test.

Singlet oxygen detection

Different dyes and dye-immobilized PSMA NPs (0.1 mM) were thoroughly mixed with RNO (0.05 mM) and imidazole (0.05 mM) and then exposed under a 660 nm LED board with a 75 mW/cm^2 power density or an 808 nm continuous-wave (CW) laser with a power density of 0.9 W/cm^2 for 10 minutes. For singlet oxygen detection, the decay of the RNO absorption peak at 440 nm was measured every 2 minutes during the exposure time.

Temperature examination under 808 nm laser irradiation

The temperature elevation of the dye-immobilized PSMA NPs was measured by placing the material solutions (0.1 mM) in 96-well plates and irradiating them with an 808 nm CW laser with a power density of 0.9 W/cm^2 . A thermocouple was immersed in the material solutions to monitor the temperature and record the thermal curve.

Cell culture and cell viability assays

MB49 mouse bladder cancer cells were cultured in Roswell Park Memorial Institute (RPMI) medium 1640 supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics in a humidified incubator at 37°C with air containing 5% CO_2 . MTT assays were performed to estimate cell viability after the cells were treated with different PSMA-dye NPs without light irradiation. All experiments were performed with 8×10^3 cells/well in a 96-well culture plate overnight. Then, the cells were treated with different types and concentrations of PSMA-dye NPs and incubated for 24 h. The medium was then replaced with fresh medium containing MTT reagent (0.5 mg/mL), and the cells were incubated at 37°C for another 1 h. The medium was then removed, and the converted formazan was dissolved in DMSO and quantified by optical absorbance at 565 nm to determine the cell viability.

Mitochondria membrane potential loss analysis

The membrane potential status of mitochondria was detected using JC-1 reagent. The aggregate form of JC-1 in normal mitochondria has absorption and emission maxima at 585 and 590 nm, respectively, while the maxima for its monomer form in damaged mitochondria occur at 510 and 527 nm. A stock solution of JC-1 was prepared by dissolving 5 mg of JC-1 in 1 mL of DMSO followed by storage at -20°C . Cells were seeded at a density of 8×10^4 cells in 3.5 cm dishes without PSMA-dye NPs and coincubated in JC-1-containing medium (5 $\mu\text{g/mL}$) for 30 minutes at 37°C . After washing three times with culture medium, the cells were observed and analyzed by fluorescence microscopy (Olympus IX73).

Characterization of multiphoton fluorescence for PSMA-242, PSMA-YI-1, PSMA-ADF1, and PSMA-DTDPTID

To evaluate the multiphoton fluorescence emission features of NPs prepared with different NP formulations (PSMA-242, 72 ppm; PSMA-YI-1, 118 ppm; PSMA-ADF1, 82 ppm; PSMA-DTDPTID, 217 ppm), these nanoparticles were characterized by a CCD-cooled spectrometer (iDus 401 plus shamrock 193i, ANDOR, Oxford Instruments) under laser radiation at different wavelengths.

Multiphoton cellular imaging

MDA-MB-231 cells (2×10^5 /mL, 1 mL) were seeded in confocal dishes and cultured with Dulbecco's modified Eagle's medium with 1% (v/v) penicillin/streptomycin and 10% (v/v) FBS for 24 h. Afterward, nanoparticles (PSMA-242, 2.4 ppm, 1 mL; PSMA-YI-1, 11.8 ppm, 1 mL) were added to the dishes, and the cells were cultured for another 16 h. Then, the cells were washed with medium three times and observed using a Nikon Inverted Multiphoton Microscope Eclipse (A1MP + Eclipse Ti-2E, Nikon instrument Inc., Japan) with a $40 \times$ NA = 1.15 water-immersion objective.

In vivo studies with a multiphoton microscope

For deep-tissue *in vivo* bioimaging, nanoparticles (PSMA-242, 72 ppm, 100 μ L; PSMA-YI-1, 118 ppm, 100 μ L) were injected intravenously into C57BL/6-C2J mice via the tail vein. During the injection, the ear was mounted on cover glasses and observed with a Nikon Inverted Multiphoton Microscope Eclipse (A1MP + Eclipse Ti-2E, Nikon instrument Inc., Japan) with a $40 \times$ NA = 1.15 water-immersion objective. During the experiment, anesthesia was achieved by injecting Avertin intraperitoneally.

In vivo PDT efficacy evaluation

The orthotopic bladder tumors were inoculated in the bladder of C57BL/6 mice with 1×10^6 MB49 cells 10 days before starting treatment for a total of 2 doses of irradiation every 5 days. The mice were randomly divided into 2 groups (3 mice in each group) that received PBS or 120 ppm CPBA-conjugated PSMA-YI-1 NPs combined with 30 minutes of 660 nm laser irradiation (75 mW/cm^2). The tumor volumes were estimated by a VisualSonics Vevo 770 High-Resolution Ultrasound Imaging System (Visual Sonics Inc, Toronto, Ontario, Canada) with a 25 MHz probe in B mode to identify the bladder tumor region. The tumor size changes were normalized to the initial tumor volume before treatment started and statistically analyzed by Prism (version 7; GraphPad, San Diego, CA, USA) with t test analysis (unpaired, one-tailed). The mice were anesthetized with 2% (v/v) isoflurane/O₂ during all of the procedures. All animal studies were performed according to protocols approved by the Institutional Animal Care and Use Committee of National Cheng-Kung University.

Histological examination

Histological analysis of mice tissues was performed after 20 days of treatment. Major organs (heart, liver, spleen, lungs, and kidneys) were collected and performed with hematoxylin and eosin stain. The H&E

stained samples were then visualized by microscope (at 20x objective).

Results And Discussion

Synthesis and characterization of the commercial dye-loaded PSMA NPs

We prepared PSMA nanoparticles with a diameter of 73.4 ± 11.9 nm (Fig. 1a). The single void ($\sim 17.1 \pm 6.6$ nm) in the central region of the PSMA nanoparticle was created by removing the metallic Cu core from the interior of the Cu@PSMA nanostructure (Fig. 1b) via the HCl etching process (Figure S1). Then, we loaded organic dyes with low molar mass (479.1-753.9 g/mol) into these nanosponges. The dye loading efficiency was $\sim 3.7\%$ for sodium iron chlorophyllin (FeChl), 50% for Cy5, and 26.5% for rhodamine 6 G (R6G). This phenomenon can be explained by the positively charged Cy5 and R6G dyes, which prefer to load into/onto the negatively charged PSMA NPs (-22.3 mV). These dye-loaded PSMA NPs exhibited fluorescence peak positions similar to those of their free-form molecules in the solution (Fig. 1c). Although the FeChl molecules were less attached to the PSMA NPs, the FeChl-loaded PSMA NPs could produce a prominent deep red to NIR emission. Without the loading of emitters, the PSMA NPs showed no significant emission signals upon excitation at 365–600 nm (Fig. 1d).

Photophysical properties of the PSMA NPs loaded with OLED-, OPV-, and DSSC-functional molecules

Next, we examined the loading abilities of molecules with larger sizes and molar masses to demonstrate the versatility of the developed nanoformulation platform. We loaded PSMA NPs with an OPV-functional molecule, i.e., 242 (~ 2.5 nm in length; 772.4 g/mol), with a 4-dicyanomethylidenepyran-based core and 2-methylindoline-based donor moieties, and a DSSC-functional molecule, i.e., YI-1 (~ 3 nm in length; 1081.7 g/mol), with a donor- π bridge-acceptor structure using bis-(4-alkoxyphenyl)amine as the electron donor and indolium as the electron acceptor. In our previous report, we found that 242 was highly emissive in the deep red and NIR regions and that 242-based OLEDs exhibited high external quantum efficiency [43]. On the other hand, YI-1 exhibited both strong π - π transitions and intramolecular charge transfer (ICT) absorption bands ranging from the visible to NIR regions. Further details of the synthesis of 242 and YI-1 were described in the Supporting Information, including NMR and mass spectra results (Scheme S1 and Figure S2 ~ S15). The loading efficiencies of PSMA NPs for 242 and YI-1 were both larger than 85% (Figure S16). As shown in Fig. 2a, 242 exhibited a characteristic absorption band at 498 nm, which is redshifted by 12 nm (*J*-aggregation) [44] after loading into the PSMA NPs. On the other hand, a redshifted emission band for the PSMA-242 NPs was also observed when compared with that of 242 alone (Fig. 2b). A similar redshifting trend was found in the absorption and emission spectra for YI-1 and PSMA-YI-1 NPs (Fig. 2a and 2b).

In addition, we loaded three OLED-functional ADF-based molecules (Scheme 1b, Fig. 2c, and Figure S17) into PSMA NPs. Loading efficiencies of 85–88% were achieved for ADF1 (~ 2.8 nm in length; 954.5 g/mol), ADF2 (~ 2.1 nm in length; 754.5 g/mol), and ADF3 (~ 1.8 nm in length; 506.2 g/mol) (Figure S16). In terms of their fluorescence properties, ADF1-, ADF2-, and ADF3-loaded PSMA NPs exhibited a red emission band at 660 nm, green emission band at 515 nm, and blue emission band at 435 nm, respectively.

Analysis of multiphoton fluorescence spectra of dye-loaded nanoparticles

For deep-tissue imaging applications, we further investigated the multiphoton spectra of PSMA-242, PSMA-YI-1, and PSMA-ADF1 NPs (Fig. 2d-2f). The PSMA-242 NPs exhibited two-photon fluorescence peaks at approximately 700 nm under varying excitation wavelengths from 1000 nm to 1200 nm (Fig. 2d). Interestingly, the PSMA-YI-1 NPs have two fluorescence peaks at 600 and 800 nm (Fig. 2e). Both PSMA-242 and PSMA-YI-1 exhibited NIR-excited multiphoton NIR-fluorescence emission features. In contrast, PSMA-ADF1 NPs exhibit weak luminescence at ~ 605 nm when excited at 920 nm (Fig. 2f).

Surface characterization of dye-loaded PSMA NPs

Based on the FT-IR spectrum analysis (Figure S18), the typical absorption bands of 242, YI-1, and ADF1 overlapped with those of the PSMA NPs at 1720–1760 cm^{-1} resulting from C = O groups, 1601 – 1584 cm^{-1} resulting from C = C stretching within the ring of styrene, 1450 cm^{-1} resulting from $\nu_{\text{asym}}\text{COO}^-$ mode and 1340 cm^{-1} resulting from $\nu_{\text{sym}}\text{COO}^-$ mode. The IR spectra of the dye-loaded PSMA NPs and the bare PSMA NPs are almost the same, indicating that most organic molecules were loaded in the cores of PSMA NPs. The zeta potential of each of the dye-loaded PSMA NPs was approximately – 20 mV (Figure S19), indicating a negatively charged surface with carboxylate terminals exposed to water. This surface charge property is beneficial for the good dispersion of PSMA NPs in aqueous solutions.

The transmission electron microscopy (TEM) images revealed that PSMA NPs showed increased contrast after loading with photosensitive molecules with larger size and higher hydrophobicity (Figure S20). The increased contrast can be attributed to the high loading efficiencies of PSMA NPs for the photosensitive molecules (85% for ADF1, 99% for 242, and 87% for YI-1). The resulting particle size was slightly increased by 1.5–7.8 nm, and some particles had enlarged void sizes in the interior (Table S1), suggesting that the sponge-like swelling nature of the PSMA NPs allows the penetration of these photosensitive molecules into the cores of NPs (Scheme 1a) and not just the adsorption of the photosensitive molecules on the surfaces of NPs. Moreover, the potential leakage of the dyes was below 6.25% after two days of aging (Figure S21a).

Without help from carriers, hydrophobic 242, YI-1, and ADF1 photosensitive molecules will aggregate in the blood [45] and undergo undesired rapid clearance by the endothelial reticular system. One common solution for this is to carry these chromophores in a polymer through a phospholipid packaging method to form encapsulated nanoparticles (NPs) [46, 47]. The disadvantage of this method is a low loading rate.

In addition, there are concerns about the undesired release of the dyes. Although synthetic methods, such as polymer grafting, AIE self-assembly, and coprecipitation methods, can be used to assemble light-emitting dye nanoparticles [9, 12–14, 16], the synthesis parameters of these approaches still require optimization. In our current work, the assembly of PSMA NPs is achieved by a straightforward strategy (Scheme 1a) to load small commercially available dyes and newly synthesized large dyes. It should be a more appropriate method to prepare organic nanoemitters without tedious synthetic procedures.

Cell viability of dye-loaded PSMA NPs

The cytotoxicity of these 242-, YI-1-, and ADF1-loaded PSMA NPs was further evaluated by MTT assay. The cell viability was over 80.2% throughout the concentration range of 0 ppm_[dye]–50 ppm_[dye] for all samples incubated with MB49 cancer cells (1 day), demonstrating the reduced cytotoxicity of these molecules (Fig. 3a) compared to that of free dye molecules (Figure S21b). Their low cytotoxicity implies the low leakage of dyes (Figure S21a). Direct treatment of these dyes at the same released concentration did not cause cell toxicity (Figure S21b). Using JC-1 staining, we further confirmed the unchanged bioactivity of the mitochondria in MB49 cells after treatment with dye-loaded PSMA NPs (Figure S22).

Single-photon cellular imaging of dye-loaded PSMA NPs

After observing the red-NIR emission characteristics of the dye-loaded PSMA NPs (Fig. 2b and 2c), we treated MB49 cells with PSMA NPs loaded with 242, YI-1, and ADF1 and explored their potential applications as biological probes. Fluorescence images (Fig. 3b) showed that all three nanoemitters appeared red within MB49 cells after 24 h of coculture. The 530–550 nm excited red fluorescence was detected by employing a longpass filter (Olympus, UFGW) with an edge wavelength of 575 nm. Based on the confocal microscopy image, these red fluorescent dots of PSMA-ADF1 (as a modeling nanoemitter) in cells were overlaid with the green fluorescence of the lysosome tracker, which is represented as yellow (S23a).

The wavelength tunability of the labeling can be easily achieved by choosing the emitter color of fluorescence. As a demonstration, we prepared ADF1-, ADF2-, and ADF3-loaded PSMA NPs that exhibited individual red, green, and blue fluorescence properties to stain MB49 cancer cells after 24 h of incubation (Figure S23b). Then, we codelivered both ADF1- and ADF2-loaded PSMA NPs to MB49 cancer cells. A fluorescence microscope could separately visualize the emission signals from the green and red emitters in the MB49 cells (Fig. 3c). In the merged image, most of the emission color manifested as yellow due to the colocalization of the ADF1- and ADF2-loaded PSMA NPs, and combined with the confocal microscopy images of PSMA-ADF1 and lysosomes, it showed a colocalized position (Figure S23a), suggesting the internalization of ADF1- and ADF2-loaded PSMA NPs in the endolysosomes. We believe that these nanoemitters, combining different colors of dye molecules with strong fluorescent properties, can replace small molecular dyes, thereby allowing the development of biological immunofluorescence staining technology with low concentrations of biomolecules.

Multiphoton bioimaging in vitro and in vivo

To demonstrate the multiphoton contrasts of dye-loaded PSMA NPs in biomedical imaging, we treated MDA-MB-231 cells with PSMA-242 and PSMA-YI-1 NPs for 16 h. The cells were observed using an inverted multiphoton microscope (Nikon, A1 MP + with Ti2 E). The multiphoton fluorescence signals from PSMA-242 (Fig. 3d) and PSMA-YI-1 NPs (Fig. 3e) were mainly distributed in the cytoplasm, demonstrating that the nanoparticles were biocompatible and able to enter cells through endocytosis. To demonstrate *in vivo* deep-tissue imaging, we tail-vein injected the PSMA-242 and PSMA-YI-1 NPs to circulate them in the blood of mice and successfully observed their presence in the blood vessels of mouse ears (Fig. 3f and 3g). These nanoparticles were able to circulate to the ear (white arrows) after tail-vein injection (see supplementary movies 1 and 2). These features validated that dye-loaded PSMA NPs can serve as NIR-active multiphoton contrast agents in deep-tissue biomedical imaging. We also loaded DTDPTID (Scheme S2) into PSMA NPs. At a threefold concentration of PSMA-242, a sixfold two-photon fluorescence intensity was obtained (Figure S26). PSMA-DTDPTID exhibited a better two-photon quantum yield due to its D-A-D-configured molecular structure, which can generate efficient charge transfer interactions and has a more promising detection ability for highly sensitive biolabeling *in vivo*.

NIR-based PDT

Finally, we examined the photodriven toxicity of the PSMA-YI-1 NPs by using a RNO/imidazole indicator [48]. The stability test revealed that the loading of YI-1 into PSMA NPs increased their optical stability in a physiological buffer (PBS, pH = 4 and 7) environment (Figure S27) compared to that of the dye molecules alone under the same conditions. Considering the broadened absorption range of the PSMA-YI-1 NPs (Fig. 2a), we utilized a 660 nm laser and 808 nm laser (Fig. 4a) to access the energy transfer from the triplet state of the PSMA-YI-1 NPs to $^3\text{O}_2$, leading to the production of $^1\text{O}_2$ species by the intersystem crossing pathway. We observed a decrease in the absorbance at 440 nm by the RNO molecule as a function of irradiation at 660 nm (75 mW/cm^2) and 808 nm (0.9 W/cm^2), indicating the successful production of $^1\text{O}_2$ molecules after the light excitation of the PSMA-YI-1 NPs. Approximately 40.5% at 660 nm and 54.2% at 808 nm of PDT activity decay were measured for the PSMA-YI-1 NPs compared to the performance of $^1\text{O}_2$ species generation from the free YI-1 photosensitive molecules (Figure S28). This decreased efficiency could be attributed to the tremendous adsorption of the YI-1 molecules and thus caused energy compulsion via intermolecular interactions [45]. The PSMA NPs did not contribute to the production of $^1\text{O}_2$ molecules when irradiated with 808 nm laser light.

On the other hand, we found that the NIR-response of $^1\text{O}_2$ produced by the PSMA-YI-1 NPs was larger than that of the other DSSC-based analog molecules (Figure S28). This result can be attributed to the strong absorbance of YI-1 in the NIR region ($\epsilon = 23,000 \text{ M}^{-1}\cdot\text{cm}^{-1}$ at 808 nm), which is advantageous for the generation of singlet oxygen through electron transfer in the excited states. Such an extended π -conjugated system also results in a small energy gap of YI-1 (1.29 eV) that could facilitate electron transfer from the sensitizer to oxygen. In addition, the efficient generation of $^1\text{O}_2$ by YI-1 can also be explained by its low fluorescence quantum yield, suggesting a decreased rate of fluorescence decay from

the excited state and therefore increasing the chance of the generation of triplet excitons through intersystem crossing.

Because of the exposure of the carboxylate groups in the dye-loaded PSMA NPs, 4-carboxyphenylboronic acid (CPBA) was conjugated to the nanoemitters to enhance targeted delivery to the glycoprotein receptors of the MB49 cells. Figure 4b shows an ~ 50% decrease in cell viability in PSMA-YI-1 NP-treated MB49 cells by 808 nm laser irradiation (0.9 W/cm^2 , 10 minutes). On the other hand, we found that the culture medium was maintained below 38°C during the same light incident (Figure S29), indicating successful PDT-elicited phototoxicity and low side effects by heating. By screening with the other dye-loaded PSMA NPs, they presented NIR-photoactive ROS (e.g., PDT) that were negligible with regard to cells (Figure S30).

To suggest the concept that NIR-PDT treatment is promising for the depletion of solid tumors, we further investigated the *in vivo* PDT efficacy of PSMA-YI-1 NPs in an orthotopic bladder cancer mouse model^[39]. Mice were first intrabladder inoculated with tumors of 1×10^6 MB49 cells at 10 days before starting treatment and received irradiation every 5 days (Fig. 4c). The mice receiving 120 ppm CPBA-conjugated PSMA-YI-1 NPs combined with 30 minutes of 660 nm laser irradiation (75 mW/cm^2) showed a significant tumor volume decrease after 2 rounds of PDT treatment according to ultrasound imaging observations, while the tumor size in the PBS-treated group continuously increased and eventually occupied almost the entire bladder cavity by Day 20 (Fig. 4d and 4e). The subsequent pathology assessment of mouse organs through H&E stain revealed that no obvious damage or inflammation in heart, liver, spleen, lung and kidney was observed in PSMA-YI-1 NPs-treated mice (Figure S31), demonstrated no systemic toxicity of PSMA-YI-1 NPs on mice model. Our preliminary results suggested that the PSMA-YI-1 NPs not only possess *in vitro* PDT properties but also exhibit successful *in vivo* PDT efficacy.

Conclusions

Based on the above TEM and optical analysis results, we verified that the PSMA structure was applicable not only for the loading of commercial dyes with small molar masses but also for the facile enhancement of the loading of photosensitive molecules with molar masses up to 1081.7 g/mol and molecular lengths of approximately 3 nm. Our results from synthetic design, fluorescence spectroscopy, biomedical imaging, and photodynamic therapy validated that the delicate PSMA nanosponge can serve as a biocompatible platform for assembling organic nanoemitters. Due to the flexibility of the synthetic strategy, we produced nanoparticles with multiple emission wavelengths ranging from the visible to NIR regions. These organic nanoemitters exhibited single-photon and multiphoton NIR luminescence for bioimaging applications and phototherapeutic activation under NIR stimulation. Based on the successful demonstration of the potential targeted-PDT of PSMA-YI-1 NPs in bladder cancer cells, the additional loading of chemo-drugs into the PSMA nanocarriers may result in better anticancer efficacy with lower dose administration for future chemotherapeutic strategies.

Declarations

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Author contributions

Li-Xing Yang, Yi-Tseng Tsai and Wei-Shiang Huang: performed the PSMA-dye synthesis, characterization and in vitro PDT experiments. Yu-Cheng Chin and Wei-Rou Jiang: performed the in vivo PDT experiments. Yu-Cheng Liu, Yin-Lin Lu, Zhiming Zhang and Tzu-Ming Liu: performed in vitro and in vivo multiphoton examination. Chang-Hui Cho, Yu-Lin Chiu and Yuan Jay Chang: performed synthesis and characterization of ADF-1, 2, 3 and 242 compounds. Yi-Rou Chen, Hsiu-Min Pan and Chih-Hsin Chen: performed synthesis and characterization of YI-1, 3, 8 compounds. Yu-Ting Huang, Li-Ming Chen and Ken-Tsung Wong: performed synthesis and characterization of DTDPTID compound. Jiashing Yu and Chun-Kai Sun: performed PSMA NPs production. Li-Xing Yang and Chih-Chia Huang: original draft preparation. Jiashing Yu, Ken-Tsung Wong and Chih-Chia Huang: review and editing this paper. Chih-Hsin Chen, Yuan Jay Chang, Chih-Chia Huang, and Tzu-Ming Liu: supervised and designed this research. All authors discussed the results and commented on the manuscript.

Competing interests

The authors declare no competing financial interest.

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Scheme

Scheme 1 is available in the Supplemental Files section.

Supplementary

Supplementary Movies 1 & 2 are not available with this version.

Figures

Figure 1

Transmission electron microscope (TEM) images of (a) PSMA NPs and (b) Cu@PSMA NPs. Fluorescent spectra of c) FeChl-, R6G-, and Cy5-loaded PSMA NPs and d) pure PSMA NPs.

Figure 2

Absorption and multiphoton fluorescence spectra of PSMA loaded with dyes with high molar masses. (a) UV–Vis spectra and (b) single-photon fluorescent spectra (excited at 512 nm) of 242, YI-1 (solid line), PSMA-242, and PSMA-YI-1 NPs (dotted line). (c) Fluorescence spectra of ADF1 and PSMA-ADF1 NPs excited at 512 nm. Multiphoton fluorescence spectra of (d) PSMA-242, (e) PSMA-YI-1, and (f) PSMA-ADF1 NPs.

Figure 3

(a) Biocompatibility of PSMA NPs loaded with different dyes. Fluorescent images of cells labeled with (b) PSMA-242, YI-1, ADF1 single staining or (c) PSMA-(ADF1 + ADF2) double staining and tracked by fluorescence microscopy. Scale bars = 20 μm . Multiphoton imaging of (d) PSMA-242 and (e) PSMA-YI-1 excited at 1080 nm. *In vivo* two-photon fluorescence (red color) and second harmonic generation (green color) imaging of dye-loaded PSMA NPs circulated in the blood vessels of a mouse ear. (f) PSMA-242; (g) PSMA-YI-1; excitation wavelength: 1080 nm. Fields of view: 317 $\times$ 317 μm .

Figure 4

(a) Singlet oxygen generation of PSMA NPs loaded with YI series dyes by RNO/imidazole assay under 10 minutes of 660 (solid lines) and 808 (dotted lines) nm laser irradiation (75 mW/cm^2 and 0.9 W/cm^2 , respectively). (b) PDT efficacy of PSMA-242, PSMA-YI-1, and PSMA-ADF1 NPs in MB49 cancer cells under 808 nm laser irradiation (0.9 W/cm^2) for 10 minutes. (c) Schematic of the *in vivo* treatment process. (d) Ultrasound images of a mouse bladder with tumors before and after treatment with PBS and CPBA-conjugated PSMA-YI-1 NPs combined with 30 minutes of 660 nm laser irradiation (75 mW/cm^2). Scale bar = 2 mm. (e) Tumor area changes quantified from (d). (*: $p < 0.05$, compared with PBS group)

Supplementary Files

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