Photosensitizer-Conjugated Albumin–Polypyrrole Nanoparticles for Imaging-Guided In Vivo Photodynamic/Photothermal Therapy

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Conjugated polymers with strong absorbance in the near-infrared (NIR) region have been widely explored as photothermal therapy agents due to their excellent photostability and high photothermal conversion efficiency. Herein, polypyrrole (PPy) nanoparticles are fabricated by using bovine serum albumin (BSA) as the stabilizing agent, which if preconjugated with photosensitizer chlorin e6 (Ce6) could offer additional functionalities in both imaging and therapy. The obtained PPy@BSA-Ce6 nanoparticles exhibit little dark toxicity to cells, and are able to trigger both photodynamic therapy (PDT) and photothermal therapy (PTT). As a fluorescent molecule that in the meantime could form chelate complex with Gd^{3+}, Ce6 in PPy@BSA-Ce6 nanoparticles after being labeled with Gd^{3+} enables dual-modal fluorescence and magnetic resonance (MR) imaging, which illustrate strong tumor uptake of those nanoparticles after intravenous injection into tumor-bearing mice. In vivo combined PDT and PTT treatment is then carried out after systemic administration of PPy@BSA-Ce6, achieving a remarkably improved synergistic therapeutic effect compared to PDT or PTT alone. Hence, a rather simple one-step approach to fabricate multifunctional nanoparticles based on conjugated polymers, which appear to be promising in cancer imaging and combination therapy, is presented.

1. Introduction

Conjugated polymers (CPs) are highly versatile materials that exhibit great potential to offer exciting opportunities in various areas including optoelectronics, photonics, and nanomedicine. Compared to many existing inorganic nanoparticles as well as small organic dyes currently explored in biomedicine, CPs show various advantages such as tunable properties, great optical and chemical stabilities, less toxicity, and better biocompatibility, making them highly attractive in biological applications. In recent years, many groups have developed various fluorescent CPs as a new generation of fluorescence probes for biological sensing and imaging. As far as therapy is concerned, CPs when complexed with photosensitizing molecules could produce reactive oxygen species (ROS) for rapid killing of neighboring bacteria and cancer cells upon photo-excitation. On the other hand, we and many other groups have demonstrated that several types of CPs with strong near infrared (NIR) absorbance could be utilized as promising photothermal therapy agents due to their excellent photo-stability and high photothermal conversion efficiency. The explorations of CPs for applications in various directions of biomedicine have become rather attractive nowadays.

One important step when fabricating CPs for biomedical applications is the surface coating of those CP-based nanoparticles. Since the majority of CPs are hydrophobic, surface modification is surely needed to make them soluble in aqueous solutions and stable in physiological environments to facilitate their biological applications. Unfortunately, many CPs currently being used in this area have few functional
groups available for further bioconjugation, making direct covalent modification of CPs a challenging task. Therefore, in order to develop CP-based nano-agents for biomedical applications, especially if we need to endow CPs with multiple functionalities, complicated materials design and chemistry is usually required. Hence, a simple method to synthesize and functionalize CPs to achieve great dispersity in physiological solutions, multiple imaging and therapy functionalities, and desired in vivo behaviors (e.g., tumor targeting ability) is still urgently needed to develop CP-based nanoscale theranostic agents.

Albumin as the most abundant serum protein (35–50 g L\(^{-1}\) human serum) has been widely used as a highly biocompatible drug carrier. In addition, albumin could also be utilized to coat various types of nanoparticles to enhance their biocompatibility. In this work, bovine serum albumin (BSA) preconjugated with chlorin e6 (Ce6) is utilized as the stabilizing agent in the polymerization of polypyrrole (PPy), which has been demonstrated to be a robust photothermal agent. Without any further modification, PPy@BSA-Ce6 nanoparticles obtained by such a one-step method show great stability in different physiological media, and can serve as an effective agent in both photodynamic and photothermal therapies. After being labeled with Gd\(^{3+}\) by forming a chelate complex with the Ce6, PPy@BSA-Ce6(Gd) offers strong contrasts in both fluorescence and magnetic resonance (MR) imaging. Motivated by the efficient tumor uptake of those PPy nanoparticles as revealed by such dual-modal imaging, in vivo combined photothermal and photodynamic therapy is then carried out with PPy@BSA-Ce6, achieving an obviously enhanced tumor growth inhibition effect compared with the respective mono-therapies. Our work develops a very simple strategy to fabricate CP-based multifunctional nanoparticles, which hold great promise for imaging-guided combination therapy of cancer.

2. Results and Discussion

In this work, we synthesized uniform PPy@BSA nanoparticles by an in situ chemical oxidative polymerization reaction (Figure 1a). In this process, FeCl\(_3\)-6H\(_2\)O was used as
an oxidant to initiate the polymerization, while BSA was introduced as the stabilizer to coat the formed PPy. As the control, polyvinyl alcohol (PVA) coated PPy nanoparticles were synthesized using sodium dodecylbenzenesulfonate (SDBS) and PVA (molecular weight = 9–10 kDa) as emulsifiers and stabilizers following our previously established protocol.[18] In order to optimize the ratio between BSA and PPy in our nanocomposites, the reactions were performed by adding different ratios of BSA and pyrrole monomer. The hydrodynamic sizes of those different PPy@BSA samples in water and phosphate buffered saline (PBS) were measured by dynamic light scattering (DLS) (Figure 1b,c). It was found that the nanoparticle sizes increased as the rise of pyrrole:BSA weight ratios. Notably, although all formulations of PPy nanoparticles regardless of BSA or PVA coating were soluble in water, PVA coated PPy as well as PPy@BSA with a low BSA:pyrrole ratio (1:2) aggregated and precipitated in phosphate buffered saline (PBS), while other PPy@BSA samples with sufficient BSA coating showed great stability in physiological buffers (Figure 1d). From the transmission electron microscope (TEM) images (Figure 1e), when the pyrrole:BSA ratio was 0.25:1 or 0.5:1, the obtained nanoparticles exhibited jumbled morphology even though they show great stability in PBS. The other two formulations of PPy@BSA nanoparticles with pyrrole:BSA ratios at 1:1 and 2:1, as well as PVA coated PPy nanoparticles, on the other hand showed quite uniform morphology (Figure 1e). PPy@BSA nanoparticles prepared under the pyrrole:BSA ratio at 1:1, with great physiological stability and well-defined morphology, were thus chosen in our following experiments.

To acquire additional imaging and therapy functions, chlorin e6 (Ce6), a fluorescent photosensitizing molecule, was conjugated to BSA, obtaining BSA-Ce6 (3 Ce6 per BSA in the product) that was then used as the stabilizer during the polymerization of polypyrrole following the same protocol at the feeding pyrrole:BSA ratio of 1:1 (Figure 2a). The yielded PPy@BSA-Ce6 nanoparticles showed similar diameters as PPy@BSA at ~35 nm (polydispersity index PDI = 0.13) (Figure 2b). The zeta potentials of PPy@BSA and PPy@BSA-Ce6 were measured to be −7.88 mV and −5.43 mV, respectively.
respectively. From the TEM images, PPy@BSA-Ce6 nanoparticles were well distributed in water and showed quite uniform morphology. Moreover, PPy@BSA-Ce6 nanoparticles without further modification exhibited great stability in different physiological media including phosphate buffered saline (PBS), fetal bovine serum (FBS), and cell medium (Figure S1a, Supporting Information), allowing them to be further used in biological systems.

The optical properties of our albumin-based agents were then carefully characterized. UV–vis-NIR spectra of PPy@BSA and PPy@BSA-Ce6 showed a broad absorption band extending from the visible to the NIR region. The absorption peaks of PPy@BSA-Ce6 at 404 nm and 660 nm corresponded to the characteristic peaks of Ce6 (Figure 2c). The partial quenching of Ce6 fluorescence could be owing to the close interaction between Ce6 and PPy (Figure S1b, Supporting Information). To prove the potential use of PPy@BSA-Ce6 as an effective photothermal agent, PPy@BSA-Ce6 solutions of different concentrations of PPy and PPy@BSA solution were exposed to an 808 nm NIR laser at the power density of 0.8 W cm⁻² for 5 min (Figure 2d and Figure S2, Supporting Information). The temperature of the PPy@BSA-Ce6 solution rapidly increased even at rather low concentrations, while pure water and free Ce6 solution showed little change. On the other hand, to demonstrate the use of PPy@BSA-Ce6 for PDT, we measured the ¹O₂ production under excitation of the 660 nm light by a singlet oxygen sensor green (SOSG) based on its fluorescence recovery in the presence of ¹O₂ (Figure 2e). It was found that PPy@BSA, H₂O, BSA, and PPy-PVA made little contribution to the light-induced ¹O₂ generation, while PPy@BSA-Ce6 could effectively produce ¹O₂ under light exposure, although its efficiency showed a slight decrease compared to free Ce6, likely due to the interaction between Ce6 and PPy that partially quenched the light-induced ¹O₂ generation. Therefore, PPy@BSA-Ce6 nanoparticles developed here could be utilized as a potential agent for both PTT and PDT.

Next, we studied the interactions of PPy@BSA-Ce6 nanoparticles with cancer cells. We first incubated murine breast cancer 4T1 cells with PPy@BSA-Ce6 nanoparticles for different periods of time to monitor the cellular uptake of the nanoparticles. From confocal fluorescence images, the cellular fluorescence intensity of Ce6 for PPy@BSA-Ce6 treated cells could be clearly observed after incubation for 6 h, confirming effective cellular uptake of PPy@BSA-Ce6 nanoparticles (Figure 3a). To study the phototoxicity of PPy@BSA-Ce6 nanoparticles, 4T1 cells were incubated with various concentrations of free Ce6 and PPy@BSA-Ce6 nanoparticles for 6 h, exposed to the 660 nm light for 30 min, and then re-incubated for additional 24 h. The relative viabilities of 4T1 cells were then measured by the standard thiazolyl tetrazolium (MTT) assay. Negligible cell toxicity under dark conditions (Figure 3b) was found for cells treated with either Ce6 or PPy@BSA-Ce6, demonstrating that PPy@BSA-Ce6 had great biocompatibility and low toxicity. In contrast, the relative viabilities of cells exposed to the 660 nm light decreased as the increase of PPy@BSA-Ce6 concentrations (Figure 3c). The in vitro photodynamic cancer cell killing efficacy of PPy@BSA-Ce6 appeared to be comparable to that of free Ce6, although the ¹O₂ generation ability of the former one was slightly lower.

The combined effect of photothermal and photodynamic therapies was then studied in vitro. 4T1 cells were incubated with various concentrations of PPy@BSA-Ce6 for 6 h, and then treated by PDT (660 nm, 5 mW cm⁻², 10 min), PTT (808 nm, 0.5 W cm⁻², 5 min), PDT first and then PTT (PDT + PTT), or PTT first and then PDT (PTT + PDT) (Figure 3d). Cell viabilities were determined after 24 h of further incubation in fresh cell medium. When treated by single PDT or PTT, only partial cell death was found under our tested conditions. However, after being treated by the combined PDT and PTT, regardless of the sequence of PDT and PTT, a highly synergistic effect in destructing cancer cells was achieved. According to a number of reports, mild photothermal heating (e.g., to ~43 °C) could accelerate the cellular uptake of either chemotherapeutic drugs or photodynamic agents, and thus enhance the overall therapeutic efficacy in the combined therapy. On the other hand, it has also been hypothesized that after being treated by PDT, the sensitivity of cancer cells to heating could be enhanced, leading to more effective cancer cell killing. Therefore, it seems that these two types of therapeutic approaches would be able to enhance the efficiency of each other, resulting in the obvious synergistic effect to destruct cancer cells.

Next, we carried out in vivo experiment to track PPy@BSA-Ce6 nanoparticles after being administrated into animals. Taking advantage of the Ce6 fluorescence, mice bearing 4T1 murine breast cancer tumors were intravenously (i.v.) injected with PPy@BSA-Ce6 (200 μL of 0.8 mg mL⁻¹ PPy) and then spectrally imaged by a Maestro EX in vivo fluorescence imaging system (CRI, Inc.) (Figure 4a). With widely distributed PPy@BSA-Ce6 fluorescence at early time points, it was found that PPy@BSA-Ce6 tended to be enriched in the tumor over time, with prominent uptake of nanoparticles observed in the tumor at 6 h p.i. By measuring Ce6 fluorescence in the collected blood samples, the blood circulation half-life of PPy@BSA-Ce6 was determined to be ~3.11 h, which appears to be reasonably long among various nanoparticle carriers, and is favorable for passive tumor homing of nanoparticles via the enhanced permeability and retention effect (EPR) (Figure 4b). To quantitatively analyze the biodistribution of PPy@BSA-Ce6 in major organs or tissues, mice bearing 4T1 murine breast cancer tumors were sacrificed 6 h after intravenously injected with PPy@BSA-Ce6. Major organs or tissues were wet weighed and solubilized by a lysis buffer. The obtained homogenized tissue lysates were diluted and measured by a fluorometer to quantitatively determine the PPy@BSA-Ce6 concentrations. 6 h after intravenously injected, tumor uptake of PPy@BSA-Ce6 was measured to be as high as 7.2% of injected dose per gram tissue (ID g⁻¹), which was comparable or slightly lower than that in the spleen and liver, the reticuloendothelial systems responsible for clearance of foreign nanoparticles (Figure 4c).

Fluorescence imaging is suitable for small animal imaging but has many shortcomings in clinical applications because of the limited tissue penetration of light. MR imaging, together with other 3D whole body imaging tech-
Techniques, would be preferred in the clinic to determine the exact tumor locations, sizes, and shapes, which are valuable information particularly important for the treatment planning in phototherapies.\(^{40-46}\) We and others have found that porphyrin structures could serve as the chelate ligand to capture metal ions such as Mn\(^{2+}\) and Gd\(^{3+}\) to offer contrast in MR imaging.\(^{11}\) We thus took advantage of Ce\(_6\) to capture Gd\(^{3+}\) for T1-weighted MR imaging. By simply mixing PPy@BSA-Ce6 with GdCl\(_3\) and removing excess Gd\(^{3+}\) (Figure 5a), we could obtain Gd-labeled PPy@BSA-Ce6(Gd) nanoparticles with a Ce\(_6\):Gd\(^{3+}\) ratio of 1:1.\(^{11}\) Fluorescent spectra of Ce6, Ce6(Gd), BSA-Ce6, BSA-Ce6(Gd), PPy@BSA-Ce6, and PPy@BSA-Ce6(Gd) aqueous solutions were taken at the same Ce6 concentration, showing a slightly quenched fluorescent intensity after chelating Gd\(^{3+}\) (Figure S3, Supporting Information). T1-weighted MR images of a series of concentrations of different solutions revealed the concentration-dependent whitening effect (Figure 5b). The transverse relaxivity \(r_1\) of PPy@BSA-Ce6(Gd) was calculated to be 14.2 \(\times 10^{-3}\) m\(^{-1}\) S\(^{-1}\), which appeared to be much higher than that of BSA-Ce6(Gd) \((8.55 \times 10^{-3}\) m\(^{-1}\) S\(^{-1}\)) and free Ce6(Gd) \((3.51 \times 10^{-3}\) m\(^{-1}\) S\(^{-1}\)) (Figure 5c), as well as that of the clinically used T1-contrast agent Magnevist (DTPA-Gd) \((4.3 \times 10^{-3}\) m\(^{-1}\) S\(^{-1}\))\(^{11,47}\). The high \(r_1\) value of PPy@BSA-Ce6(Gd), which is resulted by the increased local concentration of Gd\(^{3+}\) in the nanoparticle formulation,\(^{48-50}\) suggests its superior contrasting ability in T1-weighted MR imaging.

Without showing noticeable cytotoxicity in vitro (Figure S4, Supporting Information), in vivo MR imaging was then carried out. After being intravenously injected with PPy@BSA-Ce6(Gd) for 6 h, the dramatic whitening effect in the tumor area could be found from the T1-weighted MR images of 4T1 tumor-bearing mice (Figure 5d). The quantified T1-weighted MR signals in the tumor also showed a significant increase by as much as 55% over 6 h postinjection of PPy@BSA-Ce6 (Gd), demonstrating the high tumor uptake of PPy@BSA-Ce6(Gd) (Figure 5e). The MR imaging results together with in vivo fluorescence imaging and ex vivo

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**Figure 3.** In vitro PDT and combination therapy. a) Confocal fluorescence images of 4T1 cells after incubation with free Ce6 or PPy@BSA-Ce6 ([Ce6] = 2 \(\times 10^{-6}\) M) for different periods of time. b,c) Relative viabilities of 4T1 cells treated by Ce6 and PPy@BSA-Ce6 in dark b) or with 660 nm light exposure c) (optical dose = 18 J cm\(^{-2}\), 30 min). d) Relative viabilities of 4T1 cells after incubation with PPy@BSA, free Ce6 or PPy@BSA-Ce6 at different concentrations for various treatments. Controls were cells treated only with materials and without light exposure. PTT and PDT were conducted by 808 nm (0.5 W cm\(^{-2}\), 5 min) and 660 nm light exposure (optical dose = 18 J cm\(^{-2}\), 10 min), respectively. Relative cell viabilities in comparison to untreated cells were presented in those data. Error bars were based on four parallel samples. \(P\) values were calculated by ANOVA with Tukey’s post-test: NS (nonsignificant difference) \(P > 0.05\), * \(P < 0.05\), ** \(P < 0.01\).
Figure 4. In vivo behaviors of PPy@BSA-Ce6 after intravenous injection. a) In vivo fluorescence images of 4T1 tumor-bearing mice taken at different time points postinjection of PPy@BSA-Ce6. The autofluorescence of the mouse was removed by spectral unmixing. b) Blood circulation curve of PPy@BSA-Ce6 after i.v. injection as determined by measuring Ce6 fluorescence in the blood at different time points postinjection. The unit is percentage of injected dose per gram tissue (% ID g⁻¹). c) Biodistribution of PPy@BSA-Ce6 in mice determined by the Ce6 fluorescence from diluted tissue lysates.

Figure 5. In vivo T1-weighted MR imaging. a) A scheme showing the fabrication PPy@BSA-Ce6 (Gd). b,c) T1-weighted MR images and T1 relaxation rates of Ce6 (Gd), BSA-Ce6 (Gd), and PPy@BSA-Ce6 (Gd) measured at different gadolinium concentrations. d) T1-weighted MR images of 4T1 tumor-bearing mice before injection and 6 h after i.v. injection with PPy@BSA-Ce6(Gd). White circles highlight the tumor site. e) The quantification of T1-weighted MR signals from the tumor before and 6 h after injection of PPy@BSA-Ce6(Gd).
biodistribution data all confirmed the efficient tumor homing of our nanoparticles upon systemic administration.

Motivated by the high tumor accumulation of PPy@BSA-Ce6, we then would like to use PPy@BSA-Ce6 for in vivo cancer treatment. For in vivo monitoring of the photothermal effect generated from PPy@BSA-Ce6, an infrared (IR) thermal mapping apparatus was used to record the temperature change in the tumor area under irradiation by the NIR laser. In the PTT treatment, the mice after i.v. injection with PPy@BSA-Ce6 (dose of PPy = 8 mg kg$^{-1}$) were irradiated with 808 nm laser at the power density of 0.5 W cm$^{-2}$. The tumor temperatures on nanoparticle-injected mice showed a rapid increase under laser irradiation and maintained at $\approx$46 °C for 5 min, while that on saline-injected mice showed no significant heating effect.

To study the efficacy of in vivo combination therapy, mice bearing 4T1 tumors were randomly divided into four groups, with one group injected with saline as a control group and the other three groups i.v. injected with PPy@BSA-Ce6 (dose of PPy = 8 mg kg$^{-1}$, dose of Ce6 = 1.2 mg kg$^{-1}$). The corresponding treatments were conducted 6 h after i.v. injection. The PTT treatment was carried out by the 808 nm laser at 0.5 W cm$^{-2}$ for 5 min, while the mice for the PDT-treated group were irradiated with 660 nm light at the power density of 5 mW cm$^{-2}$ for 1 h (optical dose = 18 J cm$^{-2}$). Note that while PTT is able to exert its effect within a short period of time by hyperthermia, PDT relying on the generation of singlet oxygen to kill cancer usually requires a long period of irradiation with low light powers (otherwise the diffusion of oxygen could be the rate-determining step in PDT). After various treatments, tumor volumes and body weights of each group were monitored every other day (Figure 6c and Figure S5, Supporting Information). Compared to the control group, tumors on mice after single PTT or PDT treatment although showed slightly delayed growth within the first few days post treatment, regained the rapid growth speed after Figure 6. In vivo combination therapy. a) IR thermal images of 4T1 tumor-bearing mice under 808 nm laser irradiation (0.5 W cm$^{-2}$), which was conducted 6 h after i.v. injection of saline (upper row) or PPy@BSA-Ce6 (lower row). b) Tumor temperature changes of mice monitored by the IR thermal camera during laser irradiation as indicated in (a). c) Tumor growth curves of different groups of mice (five mice per group) after various treatments indicated. Saline injected mice were used as the control. PTT (808 nm, 0.5 W cm$^{-2}$, 5 min), PDT (660 nm, 5 mW cm$^{-2}$, 1 h), and PDT+PTT were conducted 6 h after i.v. injection of PPy@BSA-Ce6. d) Photos of tumors after various treatments taken at day 14. e) H&E stained slices of tumors from different groups of mice taken 1 d after various treatments.
BSA-Ce6. Considering the biodegradability of albumin, acute side effect of our combination therapy based on PPy@Ce6 (Figure S5, Supporting Information), indicating no apparent prolongation of blood circulation half-life, and efficient in vivo tumor homing ability. Ce6 modification on BSA further renders the nano-platform highly enriched functionalities for both imaging and therapy. On the other hand, by anchoring Ce6 on nanoparticles, the in vivo pharmacokinetics of Ce6, both imaging and therapy, are improved. The combined PDT and PTT treatment delivered by the single agent, PPy@BSA-Ce6, results in a remarkable synergistic anti-tumor effect, as compared to the PDT agent, is also improved. The combined PDT and PTT treatment delivered by the single agent, PPy@BSA-Ce6, results in a remarkable synergistic anti-tumor effect, as compared to the PDT and PTT treatments, hematoxylin and eosin (H&E) staining assays were introduced to study the morphology of tumor cells. The tumor slices of each group 1 d after treatments were compared (Figure 6e). As expected, severe damages were noted in the tumor from the combination therapy group, while no notable or much less damage could be found in other control groups, further demonstrating the great therapeutic efficacy of our combined PDT and PTT in cancer treatment.

In this system, the BSA coating offers PPy@BSA-Ce6 nanoparticles great stability in physiological environments, prolonged blood circulation half-life, and efficient in vivo tumor homing ability. Ce6 modification on BSA further renders the nano-platform highly enriched functionalities for both imaging and therapy. On the other hand, by anchoring Ce6 on nanoparticles, the in vivo pharmacokinetics of Ce6, the PDT agent, is also improved. The combined PDT and PTT treatment delivered by the single agent, PPy@BSA-Ce6, results in a remarkable synergistic anti-tumor effect, as compared to the PDT and PTT treatments, hematoxylin and eosin (H&E) staining assays were introduced to study the morphology of tumor cells. The tumor slices of each group 1 d after treatments were compared (Figure 6e). As expected, severe damages were noted in the tumor from the combination therapy group, while no notable or much less damage could be found in other control groups, further demonstrating the great therapeutic efficacy of our combined PDT and PTT in cancer treatment.

3. Conclusions

In summary, we develop a one-step simple approach to fabricate PPy@BSA-Ce6 nanoparticles, in which BSA-Ce6 is utilized as a stabilizer to control the nanoparticle morphology during synthesis, a biocompatible coating, as well as a multifunctional imaging and therapy agent. The as-prepared PPy@BSA-Ce6 nanoparticles without further surface modifications show great stability in different physiological environments. It is found that such PPy@BSA-Ce6 nanoparticles, while being effective as both photodynamic and photothermal agents, could serve as a fluorescent probe as well as an MR contrast agent (with Gd3+ labeling). With a long blood circulation half-life, PPy@BSA-Ce6 nanoparticles show high in vivo tumor uptake by the EPR effect after intravenous injection as revealed by in vivo fluorescence imaging and MR imaging. Moreover, a remarkable synergistic therapeutic effect has been achieved in our in vivo combined PDT and PTT cancer treatment utilizing systemically administrated PPy@BSA-Ce6. Therefore, PPy@BSA-Ce6 nanoparticles developed in this work may be a safe and rather effective agent for cancer theranostics. Moreover, utilizing modified proteins with pre-existing functionalities to facilitate the synthesis of CPs by one step may be a promising chemistry approach to develop multifunctional CP-based nano-agents for various applications in biomedicine.

4. Experimental Section

Materials: Pyrrole, N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), and chlorin e6 (Ce6) were purchased from Sigma-Aldrich. Bovine serum albumin (BSA) was purchased from J&C Chemical Co. Ferric chloride hexahydrate (FeCl3·6H2O) was purchased from Sinopharm Chemical Reagent Co., Ltd. All cell-culture-related reagents were purchased from Hyclone.

Preparation of PPy@BSA: To obtain PPy@BSA, 20 mg BSA was dissolved in 6 mL water and then added with various volumes of pyrrole monomer (5, 10, 20, 40 µL). After being stirred at room temperature for 1 h, ferric chloride hexahydrate (5, 10, 20, and 40 mg, respectively) was added to the reaction mixtures. The reactions were preceded for 24 h. The final products were purified by filtration through 100 kDa molecular weight cut-off (MWCO) filters and redissolved in water for future use. The concentration of PPy was quantified by its mass extinction coefficient, which was measured to be ~62 L g⁻¹ cm⁻¹ at 808 nm. The concentration of BSA in the PPy@BSA complex was determined by bicinchoninic acid (BCA). For the selected PPy@BSA formulation synthesized at the pyrrole:BSA ratio of 1:1, the PPy:BSA ratio in the final product was determined to be 1:3.5.

Preparation of PPy@BSA-Ce6 and PPy@BSA-Ce6(Gd): To prepare PPy@BSA-Ce6, Ce6 (0.596 mg) was dissolved in 40 µL anhydrous dimethyl sulfoxide (DMSO) and added with NHS and EDC (molar ratio of Ce6:EDC:NHS = 1:1:1). The mixture was vibrated for 30 min and then added with the 8 mL phosphate buffered saline (PBS) (pH = 7.4) containing 20 mg BSA. After being gently stirred overnight, the reaction mixture was purified by filtration through 10 kDa MWCO filters (Millipore) for two times to remove excess Ce6 and catalyst molecules. Based on the UV–vis spectra, it was estimated that each BSA was conjugated to 2.7 Ce6 molecules.

The obtained BSA-Ce6 (20 mg) dissolved in 6 mL water was then added with a 20 µL pyrrole monomer. The mixture was stirred for 1 h and then added with 20 mg ferric chloride hexahydrate. The reaction was continued for another 24 h. After being purified by filtration through 100 kDa MWCO filters, the final product, Py@BSA-Ce6, was dissolved in water for further use.

To prepare PPy@BSA-Ce6(Gd), gadolinium chloride (GdCl3) was added to the solution containing PPy@BSA-Ce6 at a molar ratio of 1:5:1 (GdCl3:Ce6). Excess GdCl3 was removed through 100 kDa MWCO filters three times.

Characterization: Transmission electron microscopy images were taken using a Philips CM300 transmission electron microscope operating at an acceleration voltage of 200 kV. Fluorescence spectra were obtained on a FluoroMax 4 spectrometer (Horiba Jobin Yvon). UV–vis–NIR spectra were acquired by using a PerkinElmer Lambda 750 UV–vis spectrophotometer. The sizes of nanoparticles were measured using ZEN3690 zetasizer (Malvern, USA). Laser irradiation was performed using an optical-fiber-coupled power-tunable diode laser (continuous wave) (maximal power = 10 W, Hi-TechOptoelectronics Co., Beijing, China). The generation of singlet oxygen was determined by the singlet oxygen sensor green (SOSG) dye following the standard procedure.[51]

Cell Toxicity Assay: For confocal fluorescence imaging, 4T1 cells (1 × 10⁵ cells) were cultured in 35 mm culture dishes containing free Ce6 and PPy@BSA-Ce6 with the same concentration of Ce6 (2 × 10⁻⁸ M). After washing with PBS (pH = 7.4) for three times, confocal fluorescence images were taken by a Leica SP5 confocal laser scanning microscope.
4T1 cells preseeded into 96-well plates (1 × 10^4 per well) were incubated with series concentrations of free Ce6 and PPy@BSA-Ce6. After incubation for 24 h, the standard thiazolyl tetrazolium (MTT, Sigma-Aldrich) test was conducted to measure the relative cell viabilities compared with the untreated cells.

For photodynamic therapy, 4T1 cells preseeded into the 96-well plates were added with series concentrations of PPy@BSA-Ce6 and free Ce6. After 6 h of incubation, the cells were exposed to 660 nm light with a power density of 5 mW cm^-2 for 30 min. After being further cultured for another 24 h, the relative cell viabilities were then measured by the MTT assay. Cells without light exposure were used as the control.

For combination therapy, PPy@BSA-Ce6 at different concentrations of Ce6 were added to 4T1 cells. After 6 h of incubation, cells of the PDT group were irradiated by 660 nm light for 10 min at the power density of 5 mW cm^-2, while cells of the PTT group were irradiated by an 808 nm laser for 5 min at the power density of 0.5 W cm^-2. After additional incubation for 24 h, the standard MTT test was conducted to determine the relative viabilities of cells.

In Vivo Imaging: In vivo fluorescence imaging, 200 µL PPy@BSA-Ce6 with a 0.12 mg mL^-1 Ce6 equivalent concentration was i.v. injected into each mouse. In vivo fluorescence imaging was conducted using the Maestro in vivo fluorescence imaging system (CRI Inc.). The autofluorescence was removed by the spectrum unmixing software. For in vivo MR imaging, mice bearing 4T1 murine breast cancer tumors after i.v. injection with PPy@BSA-Ce6(Gd)([Ce6] = 0.12 mg mL^-1, 200 µL) were imaged under a 3-T clinical MRI scanner equipped with a small animal imaging coil.

In Vivo Combination Therapy: Mice bearing 4T1 tumors were randomly divided into four groups (n = 5 per group). The control group of mice was i.v. injected with 200 µL of saline, while the rest three groups of mice were i.v. injected with 200 µL of PPy@BSA-Ce6 (dose of Ce6 = 1.2 mg kg^-1, dose of PPy = 8 mg kg^-1). 6 h after injection, PTT, PDT, and the combination therapy were conducted. For the PTT group, the tumors were irradiated with the 808 nm laser at a power density of 0.5 W cm^-2 for 5 min. The temperatures of the tumor site were monitored through an IR thermal camera (Infrared Cameras Inc.). For the PDT group, the tumors were irradiated with the 660 nm light at a power density of 5 mW cm^-2 for 30 min. For the PDT plus PTT group, the mice were first exposed to the 660 nm light for 1 h, and then irradiated with the 808 nm laser for 5 min. After various treatments, the tumor sizes and body weights were monitored every 2 d for 14 d. The tumor volume was calculated according to the following equation: width^2 × length/2.

Histological Examination: Hematoxylin and eosin (H&E) staining assay were used to stain the tumor slices. Tumor tissues from each group which were harvested 1 d after different treatments and fixed in 10% neutral buffered formalin embedded into paraffin and sliced into 8 µm thick sections. The obtained tumor slices were then stained with H&E and examined under a digital microscope (Leica QWin).

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