Fabrication of methylene blue loaded ovalbumin/polypyrrole nanoparticles for enhanced phototherapy-triggered antitumor immune activation

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Abstract

Background: Phototherapy-triggered immunogenic cell death (ICD) hardly elicit robust antitumor immune response partially due to low antigen exposure and inefficient antigen presentation. To address these issues, we developed a novel methylene blue loaded ovalbumin/ polypyrrole nanoparticles (MB@OVA/PPY NPs) via oxidative polymerization and π - π stacking reaction.

Results: The as-prepared MB@OVA/PPY NPs with outstanding photothermal conversion efficiency (38%) and photodynamic property could be readily internalized into cytoplasm and accumulated in lysosome and mitochondria. Upon 808 nm and 660 nm laser irradiation, the MB@OVA/PPY NPs not only ablated the tumor cells by inducing local hyperthermia, but also damaged residual tumor cells by generating a large amount of reactive oxygen species (ROS), finally triggered the release of large amount of damage associated molecular patterns (DAMPs). Moreover, the MB@OVA/PPY NPs synergized with DAMPs promoted the maturation and improved antigen presentation ability of DCs in vitro and vivo.

Conclusions: This work demonstrated that the MB@OVA/PPY NPs could be used as effective nanotherapeutic agents for eliminating the solid tumor and triggering powerful antitumor immune response.

Introduction

Phototherapy has received extensive attention in tumor treatment over the past few years, and provided greater safety and effectiveness than traditional therapies [1–2]. A great deal of research and effort has been devoted to developing non-invasive, targeted and highly effective treatments against the tumor [3–4]. For instant, photothermal therapy (PTT), as one of effective laser-based therapy, could convert near infrared light energy into heat to kill tumor cells based on the accumulation of photosensitizer in the tumor [5–6]. Compared to traditional chemotherapy, radiotherapy, surgery and others, PTT has strong, non-invasive and effective targeting characteristics, and does not cause severe side effects as traditional treatment [7–8]. Numerous studies have demonstrated that polypyrrole has excellent photothermal properties and good biocompatibility, which endowed them with great potential in the oncotherapy [9–11]. In addition, the photodynamic therapy (PDT) is another laser-based therapy in which the photosensitizer accumulated in tumors and produce reactive oxygen species (ROS) under the specific laser irradiation and then kill tumor cells [12–13]. PDT, as a rapidly developing treatment technology, has shown many advantages in the clinical treatment of various tumors, with the characteristics of less invasive, less side effects, and less drug resistance [14]. Methylene blue (MB) is a soluble heterocyclic aromatic dye in ethanol or water belonging to the thiophenazine family, and acts as high-efficient photosensitizer of PDT. Another advantage of MB in the biomedical fields is its good penetrating ability across the cell membrane due to the benzene ring, resulting in its accumulation in mitochondrial, lysosomes and double-stranded DNA [15–16]. Mounting evidences have shown that the combination therapy of PTT and PDT exhibited superior therapeutic effect in eradicating local tumors [17–19].

Recently, the effect of phototherapy on immune system has gradually become a hot research topic owing to its immune activation behavior. The possible mechanism is that phototherapy causes the immunogenic cell death (ICD) of tumor cell in situ to release tumor antigens and endogenous adjuvants (heat shock proteins, damage-associated molecular patterns (DAMPs) [20–21]. These molecules as ‘eat me’ signal are recognized and presented by antigen presenting cells (APCs) and induce antitumor immune response. During this process, the dendritic cells (DCs) as the strongest APCs exert the pivotal role in the major histocompatibility complex (MHC) mediated antigen cross presentation [22–23]. Li et al constructed a polydopamine (PDA) based core-shell nanoplatform loading CpG ODNs which has a remarkable synergistic treatment effect on inducing maturation of DCs and activation of T cells by the PTT combined with CpG ODNs [24]. Our previous studies reported a novel NIR responsive tumor vaccine in situ (HA-PDA@IQ/DOX HG) that promoted DC maturation, memory T cells generation in lymphatic node as well as cytotoxic T lymphocytes production in spleen after photothermal ablation [25]. However, the techniques of these studies suffer from many bottleneck such as complex preparation, insufficient time and inefficient immune activation.

Herein, we reported a simple two-step method for the preparation of the MB@OVA/PPY NPs to achieve ideal phototherapy and intense immune activation response. Firstly, ovalbumin and polyvinyl alcohol (PVA) were chosen as precursor to produce the OVA/PPY NPs by simple iron cation mediated oxidative polymerization. Secondly, MB was loaded onto the surface of OVA/PPY NPs via π - π stacking to obtain MB@OVA/PPY NPs. The chemical and physical properties of MB@OVA/PPY NPs was characterized and corresponding photothermal conversion efficiency was calculated. Then, MB@OVA/PPY NPs were used as dual photosensitizer to assess their influence on the growth of tumor cells. The ability of MB@OVA/PPY NPs to induce dendritic cells (DCs) maturation was studied using DC2.4 cell in vitro. In addition, specific peptide (SIINFEKL-H-2Kb) derived from OVA was chosen as indicator to detect the effect of MB@OVA/PPY NPs on antigen presentation ability of DCs. Finally, MB@OVA/PPY NPs was injected in situ into tumor site to investigate their influence on tumor growth and evaluate the DC maturation and antigen cross presentation, cytotoxic T-lymphocyte (CTL) and memory T cells in the major peripheral immune organs.

Materials And Methods

2.1. Materials

Polyvinyl alcohol (PVA), $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, pyrrole (Py), methylene blue (MB) were purchased from Shanghai Aladdin Reagent Co., Ltd. (China). OVA were purchased from Shanghai Sangon Biotechnology Development Co., Ltd. (China). All reagents were received without further purification and the water used in the experiment was deionized water.

2.2. Synthesis of MB@OVA/PPY NPs

MB@OVA/PPY NPs were prepared based on the publications with appropriate modifications [7, 15]. Briefly, 0.5 g of PVA was dissolved in 10 mL deionized water, and then heated to 70°C for 30 min to gain a uniformly viscous solution. After cooling down to room temperature, 0.5 g of OVA and 0.5 g of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ was dissolved in the PVA solution and stirred for 30 min. Then, 140 μL of pyrrole was added dropwise and stirred for 4 h. The supernatant was centrifuged at 2500 RPM for 10 min and packaged in a dialysis bag (MW = 3500 Da) to remove residual unreacted raw agents. The purified solution underwent a typical lyophilization process for 48 h to obtain the OVA/PPY NPs powder. Next, 0.5 mg of methylene blue was added into 20 mL of OVA/PPY NPs solution (0.25 mg/mL) and kept stirring for about 24 h. Finally, MB@OVA/PPY NPs were collected after dialysis and lyophilization.

2.3. Characterization

The particle size and morphology of MB@OVA/PPY NPs was analyzed using a Zetasizer particle size analyzer and Transmission electron microscope (TEM). The elemental composition of MB@OVA/PPY NPs was characterized by EDX energy spectrum. The absorption spectrum was characterized by UV-Vis spectrophotometry. The chemical structure and surface groups of MB@OVA/PPY NPs was detected by Fourier transform infrared spectroscopy (FTIR). The components and content of MB@OVA/PPY NPs were detected by X-ray photoelectron spectroscopy (XPS). The loading of OVA in MB@OVA/PPY NPs was determined using MERITON SMA1000 ultra-micro UV spectrophotometer.

2.4. OVA loading and release

In order to remove the interference of MB, 1 mL of MB@OVA/PPY NPs solution in a dialysis bag (MWCO 3500 Da) was immersed in 10 mL DI water under magnetic stirring. After 24 hours of dialysis, OVA content in MB@OVA/PPY NPs solution was measured with the ultra-micro UV spectrophotometer and then the OVA loading rate was calculated. The OVA release in vitro profiles from MB@OVA/PPY NPs were investigated as above-mentioned method. Briefly, 1 mL of MB@OVA/PPY NPs solution in a dialysis bag (MWCO 500 kDa) was immersed in 10 mL of different buffer (pH 5.4, 6.4, 7.2) under magnetic stirring. At the predetermined time points, 1 mL of PBS was collected and equal volume of fresh PBS was supplied. The content of OVA was measured by the ultra-micro UV spectrophotometer.

2.5. Photothermal performance

MB@OVA/PPY NPs aqueous solutions with different concentrations (200, 400, 600, 800 mg/mL) was irradiated with 808 nm near-infrared light at different power densities (0.5, 1.0, 1.5, 2.0, 2.5 W/cm^2). The solution temperature was measured by a hand-held near-infrared thermal camera. The temperature of the solution was recorded every 30 s. Meantime, the photothermal repeatability and stability of MB@OVA/PPY NPs were studied by using five cycles of laser on and off. The photothermal conversion efficiency was calculated by linear regression data of a heating and cooling cycle.

2.6. Fluorescent staining

The 4T1 cells suspension cells (1.0×10^4 cells) were seeded onto a 24-well plate. The cells were washed with PBS for 3 times at 37°C in 5% CO₂ incubator overnight. The FITC-labeled MB@OVA/PPY NPs (100 µg/mL) was transferred into the 24-well plate, and washed with PBS for 3 times after incubation. Mitochondria, lysosomes and endoplasmic reticulum were stained with Tracker red fluorescent probe, respectively. After washed with PBS for 3 times, The 4T1 cells were fixed using the paraformaldehyde, and DAPI dye solution was added. Laser scanning confocal microscope (Leica SP8SSTED3X) was used to observe after washed with PBS for 3 times.

2.7. Photodynamic performance

Under 660 nm laser irradiation, the released MB could be utilized as a competent PDT photosensitizer to produce singlet oxygen ($^1\text{O}_2$) and induce cell death. Singlet oxygen ($^1\text{O}_2$) generated from the MB@OVA/PPY NPs were evaluated by detecting the absorption intensity of a ROS indicator 1, 3-diphenylisobenzofuran (DPBF) at 410 nm [26]. Different concentrations of MB@OVA/PPY NPs aqueous solution (100, 200, 300, 400, 500 µg/mL) in DPBF mixed with H₂O₂ (as O₂ source) was irradiated under 660 nm laser (50 mW/cm²) for 5 min. Afterwards, the absorbance of the solution at 410 nm was detected by an UV-vis spectrophotometry.

2.8. Biocompatibility

The hemocompatibility of MB@OVA/PPY NPs was assessed by hemolytic assay. Briefly, the MB@OVA/PPY NPs with different concentrations (50, 100, 200, 400 µg/mL) and mouse blood were co-incubated for 2 h. Water was used as the positive control and PBS was used as the negative control. The UV-Vis absorbance of the supernatant at 541 nm was detected to calculate the hemolysis rate.

The cytocompatibility of MB@OVA/PPY NPs was detected by cell counting-8 kit (CCK-8) assay. 1.0×10^4 Mouse Embryonic Fibroblast (MEF) cells and 4T1 cells (Mouse Breast Cancer cells) were inoculated into 96-well plates for 24h. Then, different concentrations of MB@OVA/PPY NPs (0, 50, 100, 200, 400, 800 µg/mL) were added for 48 h. After CCK-8 reagent was added, the absorbance at 450 nm in each well was measured by enzyme plate analyzer.

2.9. Photothermal and photodynamic therapy in vitro

The MEF and 4T1 suspension cells (1.0×10^4 cells) were seeded onto a 96-well plate. After incubation for 24 h at 37 °C, the medium containing different concentrations of MB@OVA/PPY NPs (0, 50, 100, 200, 400, 800 µg/mL) was added. In order to achieve the desired effect, the near-infrared light aperture of the 808 nm laser was adjusted in order to match with the edge of each well of plate. The each experimental group was irradiated by 808 nm near-infrared laser with a power density of 2.0 W/cm² for 5 min. Then, these cells were washed by PBS and placed in the incubator overnight. Next, 10µl CCK-8 reagent was added to each well and kept incubating for 2h. The absorbance values of each well at 450 nm were determined by enzyme-labeled analyzer to calculate the cell viability. For PDT assessment, the procedure

was similar to the previous PTT. The 4T1 cells were cultured with different concentrations of MB@OVA/PPY NPs (0, 50, 100, 200, 400 $\mu\text{g/mL}$). The photodynamic therapy was conducted by using a 660 nm laser (50 mW/cm^2) for 5 min. CCK-8 assay was carried out to calculate cell viability.

2.10. Maturation and antigen presentation capability of DCs in vitro

DC2.4 cells were chosen to characterize the immune induction function of MB@OVA/PPY NPs in vitro. Firstly, DC2.4 cells were incubated with different concentration of MB@OVA/PPY NPs (10–400 $\mu\text{g/mL}$) for 24 hours, then stained with apoptosis Kit (Annexin V-FITC/PI) for 30 min. Then, these cells were washed and their activity was detected by flow cytometry. Then, DC2.4 cells were co-incubated with MB@OVA/PPY NPs (10–400 $\mu\text{g/mL}$) for 24 h. These cells were washed and stained with anti-CD86-PE and anti-CD80-FITC for 30 minutes. The cells were washed with FACS buffer, and sorted by flow cytometry [27].

The antigen cross presentation ability of DC cells was detected by detecting the expression profile of SIINFEKL-H2Kb on the surface. DC2.4 cells were incubated with different concentrations of MB@OVA/PPY NPs (10, 50, 100 $\mu\text{g/mL}$) for 24 h. Three replicates were set in each group, and flow cytometry was used for detection.

2.11. Mouse tumor model

In our experiment, female Balb/c mice were all purchased from Jiangsu ALF Biotechnology Co., LTD. Animal experiments were carried out in accordance with the protocol approved by the Experimental Animal Center of Jiangsu University. To establish a tumor model, 4T1 cells (1×10^6) in 50 μL PBS were subcutaneously injected into the lower left breast of each mouse. For in vivo combination therapy, 4T1 tumor-bearing mice were divided into four groups, including PBS, PPY NPs, MB@PPY NPs, MB@OVA/PPY NPs. After around one week, the average size of the tumor reached about 100 mm^3 , and then about 1 mg/mouse of each group of nanoparticles was injected in situ. The tumor was irradiated with 808 nm laser at a power density of 2 W/cm^2 for 5 min, and then irradiated with 660 nm laser at a power density of 100 mW/cm^2 for 5 min. During the laser irradiation, the temperature change of the tumor was recorded by an infrared thermal imaging camera (HT-19). After treatment, tumor size was monitored by vernier caliper to record the lengths and widths every two days for two weeks. The tumor volumes were calculated by “length $\times$ width²/2”.

2.12 Measurement of Memory T Cell, DC cell and CD8 + T cell populations.

After challenging mice with 4T1 tumor for seven days, the inguinal lymph nodes and spleen were isolated and dissociated into single cells by mashing through cell strainers (70 μm). The cell suspension of inguinal lymph nodes and spleen was stained with APC anti-mouse CD3 (Bio Legend), PE anti-mouse CD8a (Bio Legend), PE/Cy7 anti-mouse CD62L (Bio Legend), FITC anti-mouse/human CD44 (Bio Legend) and APC anti-mouse CD11c, FITC anti-mouse CD80, PE anti-mouse CD86 and APC anti-mouse CD3, PE anti-mouse CD8a, respectively. The cell suspension of inguinal lymph nodes and spleen was

stained with APC anti-mouse CD3 (Bio Legend), PE anti-mouse CD8a (Bio Legend). The percentage of CD3 + CD8 + CD44 + CD62⁻ cells, CD11c + CD80 + CD86 + cells, and CD3 + CD8 + corresponding to effector memory T cells, DC cells, CD8 + T cells was analyzed by flow cytometry.

Results And Discussion

3.1 Preparation and characterization of MB@OVA/PPY NPs

MB@OVA/PPY NPs were successfully prepared using two-step method in Scheme 1. Firstly, the biological macromolecule OVA was integrated into the polypyrrole nanoparticles by a facile iron cation-mediated oxidative polymerization using water-soluble poly vinyl alcohol (PVA) as a stabilizer and FeCl₃ as an oxidizing agent, then, the photosensitizer MB was loaded via π - π stacking to form MB@OVA/PPY NPs. In the Fig. 1A, TEM image showed that MB@OVA/PPY NPs was spherical and uniformly dispersed without obvious aggregation. The integration of OVA and MB loading on the particle size had little effect. As shown in Fig. 1B, the MB@OVA/PPY NPs had an average particle size of 98 nm, which was similar to PPY NPs and PPY/OVA NPs. The loading of methylene blue on the surface of polypyrrole nanoparticles was verified using the UV-vis absorption spectrum. Figure 1C showed that the specific absorption peak of methylene blue was 660 nm, and there was no peak in the unloaded PPY NPs and PPY/OVA NPs. As we expected, there were obvious specific absorption peaks in MB@OVA/PPY NPs group at 660 nm. The data indicated that methylene blue was successfully loaded on the surface of PPY/OVA NPs.

The chemical composition of MB@OVA/PPY NPs was investigated using FTIR and EDX spectrum. In the Fig. 1D, the FTIR spectra of MB@OVA/PPY NPs showed that a wide absorption peak appeared near 3400 cm⁻¹, which was ascribed to -OH stretching vibration. The asymmetric stretching vibration peak of -CH₂ appeared near 2926 cm⁻¹, and the peak at 1647 cm⁻¹ belonged to the scissors bending vibration of C = C. The peak near 1283 cm⁻¹ corresponded to the bending vibration signal frequency of -CH, and the peak at 1094 cm⁻¹ corresponded to the vibration contraction of -C-O. The FTIR results showed that the surface of MB@OVA/PPY NPs was rich in a large number of hydrophilic groups, which endowed the MB@OVA/PPY NPs with good hydrophilicity and water dispersion. In addition, the peak at 1647 cm⁻¹ due to C = C stretching vibrations shifted slightly and intensity of the peak decreased, indicating the π - π interactions between MB and PPY/OVA NPs. The EDX spectrum showed that MB@OVA/PPY NPs was mainly composed of C, O, N and Fe, and the doping amount of Fe was about 5.44% (Fig. 1E), which indicated that iron element had been doped into nanoparticles. XRD results (Fig. 1F) showed that MB@OVA/PPY NPs had a maximum absorption peak around 19.6 °, which was consistent with polypyrrole.

Next, we investigated the OVA loading rate of MB@OVA/PPY NPs. It can be seen from Fig. 1G that both of OVA loading rate of PPY/OVA NPs and MB@OVA/PPY NPs were about 70%, demonstrating that OVA was successfully loaded into nanoparticles and the loading efficiency was high. Moreover, stable loading rate showed MB loading through a π - π stacking did not affect the combined OVA. Then, the OVA release profiles were studied in vitro at different pH values to assess the pH-responsive of MB@OVA/PPY NPs. As

shown in the Fig. 1H, MB@OVA/PPY NPs exhibited pH-related OVA release behavior. At pH 7.2, MB@OVA/PPY NPs shown low OVA release and the cumulative release was about 12% within 48 h. However, OVA release was significantly accelerated at lower pH, and the cumulative release was more than 30% at pH 5.4. This phenomenon might be attributed to the massive cleavage of coordination bond between Fe and OVA.

XPS results showed (Fig. 2A) that four peaks appeared near 710 eV, 530 eV, 399 eV and 284 eV, corresponding to four elements Fe, C, O and N, respectively. The Fe_{2p} XPS spectra suggested that MB@OVA/PPY NPs combined a mixed valence state (Fe^{3+} and Fe^{2+}). As shown in Fig. 2B, the peak value at 710 eV indicated the presence of Fe^{3+} , and the peak value at 714.5 eV indicated the presence of Fe^{2+} . Meanwhile, the peak value at 710 eV was stronger than that at 714.5 eV, which proved that the bound Fe in MB@OVA/PPY NPs mainly existed in the form of trivalence. The C_{1s} spectra (Fig. 2C) showed two main peaks of 286 eV and 284.6 eV, which corresponded to C-C and C-O, and C = C, respectively. The O_{1s} spectrum (Fig. 2D) had three distinct peaks at 532.3 eV, 530.3 eV and 539.9 eV, which were caused by O-C, Fe_3O_4 and H_2O . The N_{1s} spectrum (Fig. 2E) showed two peaks at 399.9 eV and 399.8 eV, indicating the presence of nitrogen atoms [28–29]. The above data were consistent with our expectation, which proved the prepared MB@OVA/PPY NPs containing Fe, C, O and N elements.

3.2 Photothermal conversion performance and photodynamic behavior

The photothermal conversion performance of MB@OVA/PPY NPs was characterized under 808 nm NIR irradiation. As shown in the Fig. 3A, the temperature of MB@OVA/PPY NPs solution increased gradually with their increasing concentration (200–800 $\mu\text{g/mL}$) under irradiation of 808 nm NIR laser at the power density of 2.0 W/cm^2 . Meanwhile, the maximum temperature in each group went up from 51.2 $^{\circ}\text{C}$ (200 $\mu\text{g/mL}$) to 59.7 $^{\circ}\text{C}$ (800 $\mu\text{g/mL}$) after 10 min of irradiation. Subsequently, the MB@OVA/PPY NPs solution (800 $\mu\text{g/mL}$) was irradiated by different NIR at distinct power densities (0.5, 1.0, 1.5, 2.0, 2.5 W/cm^2). The Fig. 3C showed that there was similar gradual trend in temperature change. When the power density reached to 2.5 W/cm^2 , the peak temperature also exceeded 65.9 $^{\circ}\text{C}$. The photothermal repeatability and stability of MB@OVA/PPY NPs were tested by switching the laser on and off. The results showed that the temperature of MB@OVA/PPY NPs aqueous solution rised rapidly more than 60 $^{\circ}\text{C}$ after the laser switch on, and dropped rapidly after the laser switch off. The obtained temperature curves matched each other well in five cycles (Fig. 3D). The above results indicated that MB@OVA/PPY NPs has reliable photothermal conversion stability and repeatability. Finally, through a heating-cooling cycle, linear regression was used to calculate the integrated photothermal conversion efficiency of MB@OVA/PPY NPs was 38% at 808 nm, which was higher than those of approximate reported PTT agents [7].

Methylene Blue (MB) is a heterocyclic aromatic dye belonging to the thiophenazine family, and could be utilized as an efficient PDT photosensitizer for generating singlet oxygen ($^1\text{O}_2$) that was responsible for PDT-motivated cell death. To evidence the $^1\text{O}_2$ generation, DPBF as an indicator of ROS was used to

evaluate the capability of MB@OVA/PPY NPs to generate $^1\text{O}_2$ under 660 nm laser irradiation. The light absorption value of DPBF decreased in a step-by-step way following the increasing concentration of MB@OVA/PPY NPs. The maximum light absorption value of DPBF decreased about 73% after 5 min of 660 nm (50 mW/cm²) laser irradiation (Fig. 3F) under the concentration of 500 µg/mL, indicating that MB@OVA/PPY NPs had good ability to produce ROS, which was also proportional to the concentration and time of exposure. Based on these results, we speculated that MB@OVA/PPY NPs might be used as ideal photosensitizer for photodynamic therapy of tumor.

3.3 Intracellular distribution of MB@OVA/PPY NPs

The uptake and localization of MB@OVA/PPY NPs in cells was characterized using confocal fluorescence microscopy. As shown in Fig. 5, bright green fluorescence was observed in the cytoplasm of each group after 6 h incubation, indicating FITC-labeled MB@OVA/PPY NPs could be easily internalized by cells. In order to further explore their distribution in the cytoplasm, three kinds of fluorescent (ER, Lyso, Mito) probe were chosen to label specific organelles. Through gray scale analysis of the images, we found that MB@OVA/PPY NPs overlapped with the gray levels of lysosomes and mitochondria, but had poor overlapped with the endoplasmic reticulum, indicating that MB@OVA/PPY NPs was more easily located on lysosomes and mitochondria. The possible reason may be that the internalized MB@OVA/PPY NPs readily entered into the lysosome, then spread into the mitochondria through the lysosomal escape pathway. These findings revealed that MB@OVA/PPY NPs mainly distributed in the lysosome and mitochondria after the cytophagy.

3.4 Biocompatibility of MB@OVA/PPY NPs

The biocompatibility of biomedical materials is essential for clinical applications. Therefore, hemolytic test was conducted to access the blood compatibility of MB@OVA/PPY NPs. Briefly, the different concentrations of MB@OVA/PPY NPs solution was co-incubated in mouse blood for 2 h. Compared to the PBS treatment, no significant hemolysis of red blood cells was observed following increasing concentration (0-400 µg/mL) of the MB@OVA/PPY NPs (Fig. 5A). When the concentration was 400 µg/mL, the hemolysis rate was only 1.46%. It indicated that MB@OVA/PPY NPs had good blood compatibility.

Next, the cytotoxicity of MB@OVA/PPY NPs was evaluated using standard CCK-8 assays on normal cells (MEF cells) and cancer cells (4T1 cells). The Fig. 5B showed that the cell viability of both cells was not significantly affected after incubation with the MB@OVA/PPY NPs for 48 h at a wide concentration range (0, 50, 100, 200, 400, 800 µg/mL). Even at high concentration of 800 µg/mL, the cell viability of both cells remained about 80%. These findings indicated that the MB@OVA/PPY NPs possessed favorable cytocompatibility which could be as alternative biomaterials for biomedical applications in vitro and vivo.

3.5 PTT/PDT performance of MB@OVA/PPY NPs

The PTT therapeutic effect of MB@OVA/PPY NPs was explored by measuring the viability of cells under 808 nm NIR irradiation. With the concentration of MB@OVA/PPY NPs increasing, both of these cell viability decreased gradually under the 1.0 W/cm^2 of irradiation. Although there was similar concentration-dependent manner, 4T1 cell viability showed a more obvious trend of decline than that of MEF cell viability (Fig. 5C, D). When the concentration reached $800 \mu\text{g/mL}$, MEF cells viability still had a 74% survival rate, while 4T1 cells viability only had a 15% survival rate. These findings indicated that MB@OVA/PPY NPs had a good photothermal performance to inhibit the tumor cell growth. In addition, the sensitivity of tumor cells to temperature might be attributed to high metabolic activity of tumor cells. This phenomenon was beneficial to reduce the damage of PTT to normal tissues and enhance the selective killing of tumor cells.

Afterward, the combined photothermal and photodynamic performance of MB@OVA/PPY NPs was investigated to evaluate their tumor killing capability. The 4T1 cells incubated with MB@OVA/PPY NPs was irradiated successively by 808 nm NIR (1.0 W/cm^2) and 660 nm laser (50 mW/cm^2). When the concentration of MB@OVA/PPY NPs reached $100 \mu\text{g/mL}$, all cell viability was significantly reduced after 660 nm and 808 nm dual laser irradiation in the Fig. 6C. It was worth noting that cell viability after 808 nm laser irradiation was lower than that of 660 nm laser irradiation, indicating that PTT had better therapeutic effect than PDT. Furthermore, the dual laser irradiation exhibited a stronger inhibition rate of tumor cell viability than single 660 or 808 nm laser irradiation. When the concentration of MB@OVA/PPY NPs reached $400 \mu\text{g/mL}$, the inhibition rate of the dual laser irradiation was about 82%, and that of single 660 and 808 nm laser irradiation which was 32% and 55%. Therefore, the combination of photothermal and photodynamic therapy has a stronger killing effect on tumor cells especially under low concentration.

3.6 The effect of MB@OVA/PPY NPs on DC maturation and antigen presentation in vitro

DCs are the most important APC in the body, which can not only activate the immune response, but also induce immune tolerance, and play an important role in maintaining the immune balance of the body. Immature DCs can take up antigen through endocytosis and mature DCs have strong antigen presentation function. During this process, the antigens are recognized and decomposed into polypeptides, and the major histocompatibility complex (MHC) signals juvenile T cells in the draining lymph nodes, ultimately inducing an immune response [30]. Mature DCs express high levels of MHC class II and costimulatory molecules, such as CD40, CD80, and CD86, which provide the signals required for T cell activation. The maturity of DC is an important indicator reflecting the immune response in the immunotherapy, which is determined by the expression level of CD80 and CD86 on the cell membrane [27].

In order to exclude the autofluorescence interference from dead cells, the DC cell viability after incubating with MB@OVA/PPY NPs was detected. As can be seen from Fig. 6A, DC cell viability was no less than 95% after incubating with MB@OVA/PPY NPs of different concentrations for 24 hours, indicating its negligible effect on cell viability. Next, we detected the effect of MB@OVA/PPY NPs on the maturation of DCs using flow cytometry. After 24 hours of co-incubation, CD80 and CD86 expression levels in

MB@OVA/PPY NPs group were significantly increased even at a low concentration of 10 µg/mL (20.4%), compared with control group (8.2%) in the Fig. 6A. Meanwhile, the CD80 and CD86 expression also increased with the increase of MB@OVA/PPY NPs concentration, indicating there was a concentration dependent manner. These data indicated that MB@OVA/PPY NPs has the ability to promote the maturation of DCs, which was benefit to further elicit a robust anti-tumor immune response in vivo.

Previous publications have reported that mature DC cells have superior ability of antigen cross presentation [31–32]. Therefore, we chose specific peptide (SIINFEKL-H-2Kb) derived from OVA as indicator to detect the effect of MB@OVA/PPY NPs on antigen cross presentation ability of DCs. It was proved that DC cells captured the OVA and so increased the expression level of SIINFEKL-H-2Kb on their surface accordingly. As shown in Fig. 6C, the expression of SIINFEKL-H-2Kb was positively correlated with the concentration of MB@OVA/PPY NPs. At the same concentration, the results in the MB@OVA/PPY NPs group (46.6%–73.8%) were more than twice high as those in the free OVA group (22.8%–30.0%). This indicated that the MB@OVA/PPY NPs could notably enhance the antigen presentation ability of DCs, which might be ascribed to their larger specific surface area, increasing solubility, and nanoscale size [34–35].

3.7 Immune responses assessment in vivo

In view of the excellent performance in vitro, we further studied the immunomodulatory function of MB@OVA/PPY NPs in vivo. The maturation of DC cells in the inguinal lymph nodes and spleen of mice with breast cancer was detected by flow cytometry. As shown in Fig. 7B, the ratio of CD11c⁺ and CD80⁺/CD86⁺ cells in the spleen after PBS, PPY NPs and MB@PPY NPs treatment was 0.6%, 0.9%, 1.2%, respectively. Notably, the ratio of CD11c⁺ and CD80⁺/CD86⁺ cells in MB@OVA/PPY NPs group were 3.8%, which was higher than other groups. In lymph node, the ratio of CD11c⁺ and CD80⁺/CD86⁺ cells in MB@OVA/PPY NPs group (30.6%) was higher than that of PBS (8.4%), PPY NPs (8.8%) and MB@PPY NPs (10.8%) treatment. These data in vivo were consistent with the results in vitro, which further confirmed that MB@PPY NPs possessed the potential to promote DCs maturation in vivo.

Cytotoxic T lymphocytes (called CTL cells or CD8⁺ T cells) play an essential role in anti-tumor immune response where they are activated by tumor-derived antigens and directly destroyed target cells. Therefore, we analyzed the CD8⁺ T cells in lymph node and spleen after MB@OVA/PPY NPs treatment. In this experiment, each group of inguinal lymph node cells and spleen cells were co-stained with anti-CD3 and antiCD8a antibodies and was measured by flow cytometry. As shown in Fig. 7B, the percentage of CD3⁺CD8⁺ T cells from spleen after MB@OVA/PPY NPs treatment (42.4%) was higher than that of PBS, PPY NPs, MB@PPY NPs (16.1%, 16.3%, 17.0%). Meanwhile, the percentage of CD3⁺/CD8⁺ T cells from inguinal lymph node in PBS, PPY NPs, MB@PPY NPs and MB@OVA/PPY NPs and group were 24.6%, 24.9%, 25.1%, and 49.7%, which showed similar trend to that of spleen. It was worth noting that the percentage of CD3⁺CD8⁺ T cells from inguinal lymph node and spleen after MB@OVA/PPY NPs treatment were more than two times as that of other treatments. This enhancement of CTL might be

ascribed to the introduction of OVA, which remarkably stimulated the DC cells maturation and enhanced the tumor-derived antigens presentation function.

Finally, we explored the effect of MB@OVA/PPY NPs on effector memory T cells ($CD3^+CD8^+CD44^+CD62^-$) in inguinal lymph nodes to study the potential utility of this treatment strategy for the long-term prevention of tumor recurrence. As shown in Fig. 7E, the ratio of memory T cells of PPY NPs group and MB@PPY NPs group improved not much. However, the ratio of memory T cells reached 25.8% under MB@OVA/PPY NPs treatment, which was more than 8 times compared with control group (PBS). These results demonstrated that MB@OVA/PPY NPs had the ability to promote memory T cells production.

3.8 Anti-tumor effect of MB@OVA/PY NPs in vivo

In order to evaluate the photothermal performance in vivo, the temperature changes of tumor site were measured after in-situ injection of MB@OVA/PPY NPs under 808 nm laser irradiation, meanwhile, the photodynamic performance was measured under 660nm laser irradiation. As shown in the Fig. 8A, the temperature of tumor sites was significantly increased over time in all groups except PBS group, which was ascribed to the introduction of polypyrrole nanoparticles. Notably, the tumor temperature in the MB@OVA/PPY NPs group reached the highest (even reached 57.5°C) after laser irradiation of 5min in the Fig. 8B, but the temperature in PBS did not changed obviously (only reached 19.7 °C). Afterward, irradiation was performed every other day for 3 times over 22 days, and corresponding tumor volumes were recorded at certain time intervals. As shown in the Fig. 8C, compared with PBS group, tumor volume decreased significantly under photothermal action after 808nm laser irradiation, meanwhile, due to the photothermal and photodynamic effect, the MB/PPY NPs group showed stronger anti-tumor effect than the PPY NPs group under dual laser irradiation at 808nm and 660nm, the growth of tumor volume in the MB@OVA/PPY NPs group was significantly suppressed compared with other groups and the tumor disappeared on day 8. These results demonstrated that MB@OVA/PPY NPs had outstanding antitumor efficacy under dual laser irradiation and immunity activation, the immunological effect was greatly strengthened. Then the combined Laser therapy and antitumor immune response resulted in improved tumor suppression[32–33]. In addition, there was no significant change in body weight among all groups in Fig. 8D. Finally, we further explored the potential risk of MB@OVA/PPY NPs to major organs in vivo. After treatment, the heart, liver, spleen, lung and kidney of mice were harvested for H&E staining analysis. As shown in Fig. 8E, the images showed that there was no inflammatory lesions or obvious tissue damage.

Conclusions

In summary, we successfully synthesized multifunctional MB@OVA/PPY NPs using iron ion mediated oxidation polymerization and π - π stacking interaction. The prepared MB@OVA/PPY NPs with uniform size distribution exhibited favorable water dispersibility and biocompatibility. Under 808 nm NIR irradiation, the photothermal conversion efficiency was up to 38%. Meanwhile, MB@OVA/PPY NPs could

generate large amounts of ROS under 660 nm laser irradiation. The combination of PDT and PTT mediated by MB@OVA/PPY NPs significantly suppressed the 4T1 cells growth in vitro via inducing the immunogenic death of tumor cells. More importantly, MB@OVA/PPY NPs significantly promoted maturation and antigen presentation capability of DCs, elicited strong and persistent anti-tumor immune response in vivo. These MB@OVA/PPY NPs had the potential to serve as a novel nano-platform to synergize the combined PDT/PTT with immunotherapy, which provided a new idea for tumor therapy.

Declarations

Ethics approval and consent to participate

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Medical College of Jiangsu University (NO. UJS-IACUC-AP-20190314002).

Consent for publication

Each coauthor has read the manuscript and approves its submission, and this work is being submitted exclusively to your journal.

Availability of data and material

The data sets supporting the results of this article are included within the article.

Competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors' contributions

Xiao Xu and Huafen Mao are responsible for experimental operation. Yunchao Wu, Suwan Liu, Jingjin Liu, Qianzhe Li are responsible for data collection and analysis. Mengyu Yang, Jinqian Zhu, Shengqiang Zou are responsible for data processing and drawing. Fengyi Du are responsible for experimental design and writing.

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Scheme

Scheme 1 is available in the Supplementary Files section.

Figures

Figure 1

Characterization of MB@OVA/PPY NPs. (A) TEM image. (B) Particle-size distribution. (C) UV-Vis absorbance spectra. (D) FTIR spectrum. (E) EDX energy spectrum. (F) XRD spectrum. (G) Content of OVA. (H) Cumulative release of OVA at different pH.

Figure 2

(A) The full-scan XPS spectrum of MB@OVA/PPY NPs. (B) Fe_{2p} spectrum. (C) C_{1s} spectrum. (D) O_{1s} spectrum. (E) N_{1s} spectrum.

Figure 3

(A) Photothermal-heating curves of various concentrations of MB@OVA/PPY NPs solution under 808 nm NIR laser irradiation at the power density of (2 W/cm²). (C) Photothermal-heating curves and (B) the corresponding infrared thermal image of MB@OVA/PPY NPs solution (800 µg/mL) under 808 nm NIR laser irradiation with varied power density. (D) Heating curves of the MB@OVA/PPY NPs solution for five laser on/off cycles under the irradiation of 808 nm NIR laser (2 W/cm²). (E) Photothermal effect of MB@OVA/PPY NPs solution under 808 nm NIR laser irradiation (black dots), and then the laser was turned off (red dots). The time constant (τ_s) for the heat transfer from the system was determined by applying the linear time data from the cooling period (blue line). (F) ¹O₂ production efficiency of the different samples with different treatments. (1. DPBF, 2. DPBF+laser, 5-9. DPBF+laser+MB@OVA/PPY NPs 100, 200, 300, 400, 500 µg/mL).

Figure 4

LSCM images of 4T1 cells treated with MB@OVA/PPY NPs-FITC for 6h. (A-C) The nucleus and organelles (endoplasmic reticulum, lysosomes, mitochondria) were stained with DAPI and specific organelle probe (ER, Lyso, Mito), respectively. (D-E) Intensity profile within the regions of interest (yellow line in each Merge) of MB@OVA/PPY NPs (green line) and specific organelle probe Track-red (red line). Scale bar: 10 µm.

Figure 5

(A) Hemolytic profile of red blood cells after incubated with various concentrations of MB@OVA/PPY NPs for 2h. (1, PBS. 2, water. 3-5, 50-400 $\mu\text{g/mL}$). (B) The cytocompatibility of MB@OVA/PPY NPs. The effect of PTT mediated by MB@OVA/PPY NPs on MEF (C) and 4T1 cells (D) viability under 808 nm NIR at 1.0 W/cm^2 power intensity for 5 min. (E) The effect of photothermal and photodynamic treatment mediated by MB@OVA/PPY NPs on 4T1 cell viability in vitro (1. Control, 2. 808nm laser, 3. 660 nm laser, 4. 808 nm+660 nm laser).

Figure 6

(A) Flow cytometry for viability of DCs incubated with different concentrations of MB@OVA/PPY NPs (10, 50, 100, 200, 400 $\mu\text{g/mL}$) for 24 h and (B) corresponding statistical data. (C) Flow cytometry of expression levels of CD80⁺ and CD86⁺ on the surface of DCs incubated with different concentrations of MB@OVA/PPY NPs (10, 50, 100, 200, 400 $\mu\text{g/mL}$) for 24 h and (D) corresponding statistical data. (E) Flow cytometry of SIINFEKL- H-2Kb expression on the surface of DC2.4 cells after co-culture with different treatment, (1. control, 2. OVA, 3. MB@PPY 10 $\mu\text{g/mL}$ +OVA, 4. MB@PPY 50 $\mu\text{g/mL}$ +OVA, 5. MB@PPY 100 $\mu\text{g/mL}$ +OVA, 6. MB@OVA/PPY 10 $\mu\text{g/mL}$, 7. MB@OVA/PPY 50 $\mu\text{g/mL}$, 8. MB@OVA/PPY 100 $\mu\text{g/mL}$) and (F) corresponding statistical chart.

Figure 7

Immune responses assessment in vivo. (A) Scheme and timeline of the experimental design to evaluate the in vivo immune responses triggered by MB@OVA/PY NPs treatment. (B) Representative flow cytometry plots and statistic data of CD80+ and CD86+ cells among CD11c+ DCs extracted from the inguinal lymph nodes (LNs) and Spleens. (C, D) Representative flow cytometry plots and statistic data of CD3+CD8+T cells in inguinal LNs and spleen. p values were calculated by the t test. (E) Representative

flow cytometry plots and statistic data illustrated memory T cell in inguinal LNs. p values were calculated by the Student's t test. Mean $\pm$ SD (n=3) of three independent experiments, *p<0.05, **p<0.01, ***p<0.001, ****p<0.001.

Figure 8

In vivo antitumor effect of MB@OVA/PPY NPs using the 4T1 subcutaneous tumor-bearing mice model. (A) Infrared thermal images of mice at varied time points of treatment under different conditions by 808 nm laser irradiation. (B) Corresponding temperature change curves at the breast tumor sites of mice in different treatment groups upon laser irradiation. (C) Tumor volume change curves and (D) the body-weight change of mice in the different treatment groups. Data are expressed as mean $\pm$ S.D. (n=4) (***P<0.001, ****P<0.0001 by t-test). (E) H&E-stained images of the major organs in the different treatment groups. Scale bar: 100 μ m.

Supplementary Files

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- [Scheme1.png](#)