Encapsulation of Photosensitizer into Multilayer Microcapsules by Combination of Spontaneous Deposition and Heat-Induced Shrinkage for Photodynamic Therapy

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Annealing of PDADMAC/PSS multilayer microcapsules assembled on PSS-doped CaCO₃ particles at 80 °C for 30 min reduces their size dramatically from 6.9 ± 0.3 to 3.1 ± 0.5 μm. Methylene blue molecules are encapsulated by spontaneous deposition and post-annealing with a concentration of 22 mg·mL⁻¹, which is 1000 times higher than the feeding value. The unreleased MB molecules are retained stably for a long time, which are then protected by the capsules against reductive enzymes and keep their photodynamic activity. The viability of HeLa cells incubated with the MB-loaded capsules decreases sharply from≈75 (dark cytotoxicity) to≈20% after irradiation with a laser at 671 nm and 60 J·cm⁻² for 75 s.

1. Introduction

Recently, photodynamic therapy (PDT) has emerged as an alternative therapeutic way for cancer and other diseases.¹ Light, photosensitizer and oxygen are three major factors in PDT. Near-IR lasers (600–900 nm) in the therapeutic window with deep tissue penetration and little damage to normal tissues are the ideal light source for PDT. Upon light irradiation, the photosensitizer is activated to its excited state, and the energy is then transferred to ground state molecular oxygen to generate singlet oxygen (¹O₂) and other reactive oxygen species (ROS), which can effectively kill cells.²,³ Therefore, PDT efficiency is largely determined by the production efficiency of ¹O₂, which is further mainly decided by the characteristics of the photosensitizer used. Methylene blue (MB) is one of the traditional photosensitizers that have been widely used in a variety of applications, including PDT.⁴ The high quantum yield of ¹O₂ generation (Φₐ ≈ 0.5) via excitation in the therapeutic window, coupled with its low toxicity, makes MB a promising candidate for PDT.⁴,⁵ However, in biological environment, MB is easily reduced by the enzyme diaphorase to colorless leukomethylene blue (LMB) which has negligible photodynamic activity.⁶ So carriers are needed to effectively encapsulate and further protect MB against reduction. Furthermore, the carriers are also helpful for the delivery of PDT agents. Encapsulation of MB into colloidal carriers such as polymeric nanoparticles,⁶ silica nanoparticles⁷ as well as protein nanocages⁸ has been demonstrated for successful protection of activity and delivery of MB for PDT.

As one of the recently developed new colloidal carriers, nanoengineered multifunctional capsules assembled through a layer-by-layer (LBL) method⁹ have attracted much attention for biomedical applications.⁹ Using the LBL technique capsules with well-controlled size and shape,
finely tuned wall thickness and variable wall compositions can be obtained. The ability of precise manipulation of capsule structures enables the tailoring of their loading and release properties, thus making them ideal candidates as drug carriers. Photosensitizers have been loaded into the capsules for PDT applications. For example, Wang et al. reported the encapsulation of water-insoluble photosensitizer, hypocrellin B, into the hollow cavity of multilayer capsules for killing cancer cells. While Tao and co-workers assembled the photosensitizers, such as phthalocyanine and Rose Bengal-grafted polyelectrolyte into the shells of the multilayer capsules through the LBL method. Both of the capsules show high killing efficiency against cancer cells. But the encapsulation of low-molecular-weight water-soluble photosensitizers such as MB into the multilayer capsules with high retention is still a big challenge due to the semi-permeability of the multilayer shells.

Recently, Köhler et al. demonstrated that poly(diallyldimethylammonium chloride)/poly(styrene sulfonate) (PDADMAC/PSS) capsules with PSS as the outermost layer can shrink dramatically at elevated temperature. Using this feature, macromolecules such as dextran (Mw ≈ 10 and 70 kDa) and poly(acrylic acid) (Mw ≈ 30 kDa) have been successfully encapsulated, with a slightly higher concentration than the feeding one. But the challenge still remains to develop a more efficient and facile method for encapsulation of low-molecular-weight drugs. In our previous study, high efficient loading and controlled release of low-molecular-weight drugs are achieved facilely by heat-shrinkage of PDADMAC/PSS multilayer capsules with a spontaneous deposition feature. The capsules with free PSS encapsulated have a strong ability to accumulate the positively charged drugs inside (the so-called “spontaneous deposition” property). These capsules shrink drastically at elevated temperature, accompanying with thickening of the capsule walls.

In this study, by combination of spontaneous deposition and heat-induced shrinkage of capsules the MB molecules are encapsulated in (PDADMAC/PSS)5 microparticles (Scheme 1), whose apparent concentration is as large as 1000 times higher than the feeding value. The highly efficient loading of the photosensitive dyes is one of the mandatory progresses toward the applications of multilayer microcapsules for PDT. Here PSS-doped CaCO3 microparticles are used as template, since CaCO3 microparticles have been widely used as template for the multilayer capsule fabrication and functionalization. The encapsulation and protection of MB by the capsule wall against enzyme reduction are studied. In vitro cell culture is performed to test the efficiency of encapsulated MB for killing cancer cells under laser irradiation.

2. Experimental Section

2.1. Materials

Sodium PSS (Mw ≈ 70 kDa), PDADMAC (medium molecular weight, typically Mw ≈ 200–350 kDa, 20 wt% in water), 2,7'-dichlorodihydroxydibenzosuberone (DCHS) from Sigma-Aldrich. Disodium ethylenediaminetetraacetate dihydrate (EDTA) was purchased from Guangdong Guanghua Chemical Factory Co. Ltd. Sodium chloride (NaCl) was obtained from Sinopharm Chemical Reagent Co. Ltd. β-Nicotinamide adenine dinucleotide, reduced dipotassium salt (β-NADH) was obtained from Roche. Dihydroxypropylamine dehydrogenase (diaphorase, 42.7 U·mg⁻¹) was purchased from Worthington. All chemicals were used as received. The water used in all experiments was prepared in a Millipore Milli-Q Reference purification system with a resistivity of 18.2 MΩ.

2.2. Preparation of Microcapsules

PSS-doped CaCO3 microparticles were prepared and used as the sacrificial template for capsule fabrication according to a previously reported method. Briefly, PSS was completely dissolved in 100 mL 0.05 M calcium nitrate solution in a 500 mL beaker under magnetic agitation (~600 rpm), into which an equal volume of 0.05 M sodium carbonate solution in another beaker was rapidly poured at room temperature. The final PSS concentration was 1 mg·mL⁻¹. Finally, the precipitated CaCO3 particles were collected and washed by centrifugation with water. The LBL assembly of PDADMAC and PSS onto PSS-doped calcium carbonate particles was carried out from 2 mg·mL⁻¹ polyelectrolyte solutions containing 0.5 M NaCl, starting with PDADMAC. After each layer assembly, the excess polyelectrolytes were removed through 3 cycles of centrifugation/washing/redispersion with water. After 5 bilayers were assembled, the multilayer-coated particles were incubated in 0.2 M EDTA (pH = 7.0, adjusted by NaOH solution) for 30 min under shaking to remove the CaCO3. The resultant PSS-(PDADMAC/PSS)5 capsules were washed with fresh EDTA solution 3 times, and then with water 3 times and finally dispersed in water. The obtained capsules with free PSS inside possess spontaneous deposition property.

Scheme 1. Schematic illustration to show the high efficient loading of MB into PSS-encapsulated (PDADMAC/PSS)5 multilayer capsules through the combination of spontaneous deposition and heat-induced shrinkage.
2.3. MB Encapsulation and Release

For MB encapsulation, PSS-(PDADMAC/PSS)$_3$ microcapsules (1.1 × 10$^9$ capsules) were dispersed in 20 mL of 0.02 mg · mL$^{-1}$ MB solution for 2 h with shaking. Then the tube with the suspension was immersed into a water bath at 80°C for 30 min. The excess MB dye was removed through 3 cycles of centrifugation/washing/redispersion with water. The shrunk capsules were assembled to another PDADMAC/BSA bilayer to improve their dispersibility in cell culture medium. The capsules with encapsulated MB were designated as CapMB. At each step, the supernatant was carefully collected, in which the MB concentrations was determined with UV-Vis spectrophotometer (UV-2550, Shimadzu, Japan) at 664 nm by referring to a calibration curve. All data were averaged from 3 parallel experiments.

The CapMB suspension (1 mL, 3.1 × 10$^8$ capsules · mL$^{-1}$) was put into a dialysis bag with a molecular weight cut-off of 3.5 kDa, which was then placed in 49 mL phosphate-buffered saline (PBS) or water at 37°C. At a given time interval, 1 mL solution was withdrawn for UV-Vis analysis and 1 mL fresh PBS or water was added to keep the constant solution volume. The released amount of MB was determined with UV-Vis spectrophotometry by referring to a calibration curve. All data were averaged from 3 parallel experiments. The release of MB from PSS-(PDADMAC/PSS)$_3$ capsules before and after heat-treatment at 80°C for 30 min was investigated in the same way.

2.4. The Protection Test of Capsules for MB

To verify the protection role of capsules for the encapsulated MB, 0.45 μmol NADH and 0.05 mg diaphorase were sequentially added in 1 μg · mL$^{-1}$ free MB solution or CapMB suspension with same MB content. The decrease of MB absorbance at 664 nm reflects the reduction of MB, which was monitored at room temperature using a UV-Vis spectrophotometer. As a control test, the absorption of the capsule suspension with the same concentration (but without NADH and diaphorase) was monitored to adjust light scattering of colloids.

2.5. Cell Culture and Photodynamic Therapy

HeLa cells (supplied by the cell bank of the Typical Culture Collection of the Chinese Academy of Sciences, Shanghai, China) were cultured in Dulbecco’s modified Eagle medium (DMEM, Gibco, USA) containing 10% fetal bovine serum (FBS, Sijijing Inc., Hangzhou, China), 100 U · mL$^{-1}$ penicillin and 100 μg · mL$^{-1}$ streptomycin, and cultured at 37°C in a 5% CO$_2$ humidified environment. 10$^4$ cells were seeded in a well of 96-cell culture plates and incubated in a cell-culture incubator for 24 h. The culture medium was changed with fresh one containing 0.15 μg · mL$^{-1}$ free MB or CapMB (ratio of capsules to cells 50:1 with total MB amount of 0.15 μg) and the system was maintained for another 4 h. Then the cells were incubated with a 671 nm laser at 60 J · cm$^{-2}$ for 75 s. After the cells were further incubated for another 3–4 h, their viability was estimated by an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Sigma). Following the replacement of the culture medium with 120 μL MTT in fresh medium (0.5 mg · mL$^{-1}$) for 4 h, the resulting blue formazan was dissolved by 200 μL of dimethyl sulfoxide and the absorbance at 570 nm was measured with a microplate reader (Model 550, Bio Rad, USA). Each experiment was performed in triplicate.

2.6. Measurement of Intracellular ROS

The oxidation-sensitive probe DCFH-DA was employed for determination of the generation of ROS.[24] DCFH-DA is an amphiphilic non-fluorescent molecule that readily crosses cell membranes, is deacetylated by esterases and then oxidized to highly fluorescent 2,7’-dichlorofluorescein (DCF) in the presence of intracellular ROS. In this study, the HeLa cells (5 × 10$^4$ cells per well in a 24-well culture plate) were incubated with free MB or CapMB (ratio of CapMB to cell 50:1) for 24 h, washed and incubated with 10$^{-7}$ M of DCFH-DA for 30 min at 37°C in the dark. The cells were then irradiated with 671 nm laser at 60 J · cm$^{-2}$ for 5 min, followed by PBS washing. Pictures were taken immediately via a fluorescence microscope (Zeiss Axiocvert 200).

2.7. Scanning Electron Microscopy (SEM)

The samples were prepared by applying a drop of capsule suspension onto glass slides. After drying overnight, the samples were sputtered with gold and measured by a field-emission SEM (JEM-1230, FEI) at an operation voltage of 3 keV.

2.8. Transmission Electron Microscopy (TEM)

The capsules were washed with water thrice and then by graded ethanol/water solutions. The sample was embedded into epoxy resin and ultramicrotomed into thin sections, which were transferred onto a carbon film-coated copper grid and investigated by a JEM-1230 TEM.

2.9. Optical Microscopy

The CapMB suspension was dropped on glass slides and the images were taken with a Zeiss Axiocvert 200 microscope with a 100 × oil immersion objective.

3. Results and Discussion

3.1. Fabrication of PSS-(PDADMAC/PSS)$_3$ Capsules and Encapsulation of MB

The PSS-(PDADMAC/PSS)$_3$ capsules were prepared on negatively charged PSS-doped CaCO$_3$ microparticles by starting assembly with the positively charged polyelectrolyte PDADMAC. Their diameter (6.9 ± 0.3 μm) was slightly larger than that of the template (6.7 ± 0.4 μm). The capsule swelling is attributed to the release of PSS
molecules from the template, which create an osmotic pressure difference across the capsule wall pointing outward.\cite{25} Our previous studies have demonstrated that this kind of capsules encapsulated with free negatively charged polyelectrolytes such as PSS can induce the spontaneous deposition of water soluble and positively charged low-molecular-weight substances into the capsules.\cite{23,26} This unique property has good reproducibility and high efficiency, which has been used for the facile encapsulation of diverse substances into multilayer capsules.\cite{27} More recently, it is found that besides the spontaneous deposition property, these capsules show temperature-dependent shrinkage. The capsules shrink drastically upon heating, accompanied with thickening of the capsule walls. Thus the encapsulated low-molecular-weight drugs can be well retained.\cite{19} As shown in Figure 1, before heat treatment, there are a lot of wrinkles and folds on the microcapsule surface in a dry state (Figure 1a). The TEM image of ultramicrotomed capsule section (Figure 1b) shows that the capsule wall is very thin, and thereby its mechanical strength is not strong enough to preserve the spherical shape at the dry state. After annealing at 80 °C for 30 min, the capsules shrunk greatly and could keep the spherical shape with smooth surface (Figure 1c). This is due to the dramatic thickness increase after capsule shrinkage. The size decreased from $6.9 \pm 0.3$ to $3.1 \pm 0.5 \mu m$, and the shell became very thick after heat treatment (Figure 1d), leading to disappearance of cavities in some capsules.

By combination of spontaneous deposition and heat-induced shrinkage of this kind of multilayer capsules, positively charged MB molecules were successfully encapsulated (Figure 2). The CapMB were well dispersed in water with a blue color, which can be only attributed to the loading of MB (Figure 2a). MB molecules are distributed inside the capsule, in the shrink thick shell and also possibly on the outermost negative surface due to the electrostatic interaction. The UV-Vis spectrum of CapMB suspension shows a typical absorption peak of MB at 664 nm (Figure 2b), which is identical to that of free MB (Figure 2c). The amount of loaded MB was calculated as $3 \times 10^{-7}$ μg per capsule. Taking the size of the shrunk capsule into consideration, the apparent MB concentration in the capsule was about 22 mg · mL$^{-1}$, which is more than 1000 times higher than the feeding value (0.02 mg · mL$^{-1}$). This efficient loading is attributed to the virtues of spontaneous deposition.\cite{19} It is worth mentioning that the calculated apparent MB concentration is the sum of the MB inside the capsule, in the thick shell as well as on the outermost surface.

### 3.2. Retention and Prevention of Enzymatic Reduction of MB by the Capsules

In order to effectively protect the photodynamic activity of MB molecules, it is required to preserve them inside the capsules, thus the high-molecular-weight enzyme cannot react with MB due to the semi-permeability of capsule wall. The retention of encapsulated MB was first tested by incubation of CapMB in PBS or water at 37 °C. Compared with that in water, in PBS the MB molecules were released...
faster from the CapMB at the initial stage. 4 h later, the release was stopped and no longer occurred during the investigation period of time (Figure 3). The retained amount of MB in capsules is about 60% in PBS, which is lower than that in water (≈90%). The ionic strength of PBS can weaken the electrostatic attraction not only between the PDADMAC and PSS in the capsule wall but also between the bound MB molecules and PSS on the capsule surface, leading to the larger release of MB in PBS. The MB molecules adsorbed on or near the outer surface of the capsules should mainly contribute to the initial burst release, whereas the interior MB molecules could be well preserved due to the thick capsule wall. It is worth mentioning that both the capsule shrinkage and assembly of additional PDADMAC/BSA bilayer on the shrunk capsules contribute to the retention of the low-molecular-weight MB inside the capsules, because the released amount of MB from CapMB was much lower than that from PSS-(PDADMAC/PSS)₅ after heat treatment, while the original PSS-(PDADMAC/PSS)₅ capsules released almost all the MB in PBS after about 50 h (Figure S1).

MB is easily reduced by diaphorase to LMB which has negligible photodynamic activity. The thick capsule wall cannot only preserve the MB molecules inside the capsules but also may prohibit the access of diaphorase. To verify the protection role of the capsule wall, CapMB were dispersed in a medium containing diaphorase and the cofactor β-NADH. UV-Vis spectroscopy shows that the absorption at 664 nm decreased instantaneously upon incubation with either free MB or CapMB, and then leveled off (Figure 4). However, the capsules show significant protection effect: the reduced amount of free MB (70%) was doubled compared with that in capsules (35%) within 30 min. The reduced amount of MB in capsules (≈35%) is very close to the released amount of MB in the retention test (≈38%) (Figure 3), confirming that the retained MB (more than 60%) in the capsules can be well protected against enzyme reduction.

3.3. In vitro PDT Studies on Tumor Cells Treated with CapMB

The MTT assay was utilized to estimate the in vitro PDT effect of CapMB on HeLa cells. The ratio of CapMB to cell was fixed at 50:1 and they were co-incubated for 24 h. Most of the capsules adhered on the cell surface (Figure S2). Although the CapMB may kill cells more effectively if they can enter into the cells, the ROS still can be generated from the encapsulated MB upon light irradiation and diffuse out of the capsules to interact with cells. It is known that the excess ROS kill cells by peroxidizing lipids and disrupting structural proteins, enzymes and nucleic acids. In this study, the cells were irradiated with 671 nm laser at 60 J cm⁻² for 75 s. Under dark condition, the viability of cells incubated with CapMB was ≈75% of the drug-free control, revealing some extent of dark cytotoxicity (Figure 5). However, this value was higher than that of the cells (≈60%) incubated with same amount of free MB (0.15 μg mL⁻¹). After laser irradiation, the viability of control cells dropped slightly to ≈80%. In sharp contrast, the viability of cells incubated with CapMB and free MB decreased to ≈20%. In the current in vitro cell experiments the CapMB show a similar PDT effect to that of free MB. The main reason is that in the cell culture experiments it is difficult to mimic the real biological environment and the content of NADH and diaphorase is too low to affect MB. However, in the experiments mimicking the higher reductive environment in vivo the CapMB show better
protection of MB against reductive enzyme compared with the free MB (Figure 4).

During the PDT process, ROS are generated to kill the cells. To visualize the intracellular ROS, a cell permeable oxidation-sensitive probe DCFH-DA was used. This probe can readily cross cell membranes to be deacetylated by esterases and then oxidized to highly fluorescent DCF in the presence of intracellular ROS. The bright green fluorescence emitted from cells incubated with free MB or CapMB and irradiated with a laser verified the ROS generation (Figure 6b,d). These results are consistent with the MTT assay. The excess ROS can kill cells by peroxidizing lipids and disrupting structural proteins, enzymes and nucleic acids. By contrast, without laser irradiation the cells incubated with free MB or CapMB showed negligible fluorescence (Figure 6a,c). All these results demonstrate the success of CapMB as a good photodynamic therapeutic anti-cancer agent.

4. Conclusion

LBL-assembled PSS-(PDADMAC/PSS)_5 capsules can effectively induce the spontaneous deposition of water-soluble and positively charged low-molecular-weight photosensitizer MB. Combined with heat-induced capsule shrinkage, most of the encapsulated MB (more than 60%) are retained for long time and protected by capsule wall against reductive enzyme to keep the photodynamic activity. The preliminary in vitro results suggest that the viability of HeLa cells incubated with MB-loaded capsules is reduced significantly under laser irradiation at 671 nm. Generation of ROS is confirmed, which is believed the main reason for cell death. It is expected that further conjugation of targeting ligands onto the capsules may result in more effective adhesion of the CapMB to cancer cells, leading to a more efficient PDT effect.

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Figure 5. Viability of HeLa cells incubated with CapMB and free MB with or without laser irradiation at 671 nm and 60 J cm⁻² for 75 s. The ratio of CapMB to cell was 50:1, and the amount of MB in CapMB was equal to 0.15 μg free MB.

Figure 6. Fluorescent microscopy images of the cells after incubated in mediums containing (a,b) free MB and (c,d) CapMB without (a,c) and with (b,d) laser irradiation at 671 nm and 60 J cm⁻² for 5 min, respectively. The fluorescence intensity determined by DCFH-DA is proportional to the amount of ROS. The scale bar is 200 μm.

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