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Enhanced photodynamic therapy efficacy of methylene blue-loaded calcium phosphate nanoparticles

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ABSTRACT

Although methylene blue (MB) is the most inexpensive photosensitizer with promising applications in the photodynamic therapy (PDT) for its high quantum yield of singlet oxygen generation, the clinical use of MB has been limited by its rapid enzymatic reduction in the biological environment. To enhance PDT efficacy of MB by preventing the enzymatic reduction, we have developed a new mineralization method to produce highly biocompatible MB-loaded calcium phosphate (CaP-MB) nanoparticles in the presence of polymer templates. The resulting CaP-MB nanoparticles exhibited spherical shape with a size of under 50 nm. Fourier transform infrared (FT-IR) and zeta-potential analyses confirmed the insertion of MB into the CaP-MB nanoparticles. The encapsulation of MB in CaP nanoparticles could effectively protect MB from the enzymatic reduction. In addition, the CaP-MB nanoparticles exhibited a good biocompatibility in the dark condition and significantly enhanced PDT efficacy due to apoptotic cell death against human breast cancer cells as compared with free MB, implying that CaP-MB nanoparticle system might be potentially applicable in PDT.

1. Introduction

Photodynamic therapy (PDT) has emerged as an important treatment modality for a variety of cancers, cardiovascular and ophthalmic diseases [1,2]. This modality involves the delivery of light-sensitive molecules called photosensitizers to target sites followed by irradiation with appropriate wavelength of light. Upon irradiation, the activated photosensitizers transfer their excess energy to the surrounding oxygen to form reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$) or free radicals, which will cause irreversible damage of diseased cells and tissues. PDT is also expected to be a potential method of overcoming multidrug resistance (MDR) because cytotoxicity mechanism of photosensitizers onto cancer cells is different from that of other chemotherapy agents [3].

However, the clinical use of many photosensitizers has been hampered by their significant side effects including nonspecific damage to normal tissues due to low accumulation selectivity to specific cells or tissues, environmental degradation and hydrophobicity [4]. To solve these problems, various biocompatible nanocarrier systems for delivering photosensitizers such as liposomes, polymeric micelles and nanoparticles have been investigated [5–7]. These nanocarriers have promoted uptake of photosensitizers into target sites and reduction of nonspecific damage to normal tissues caused by free photosensitizers [8]. In addition, nanocarrier systems have offered stable aqueous dispersion of photosensitizers by surface modification and protected photosensitizers from environmental degradation [9]. Despite many advantages of the nanocarrier systems, the use of liposomes and micellar systems was limited because of their lower drug loading capacity and severe side effects like anaphylactic shocks [1,10].

Methylene blue (MB) is a phenothiazinium photosensitizer that has been employed in a variety of applications including PDT [9,11]. The high quantum yield of $^1O_2$ generation by MB in the excitation of the therapeutic window (600–900 nm) makes MB a reasonable candidate for PDT. However, the clinical use of MB has been hindered by its propensity for rapid chemical alteration when systemically applied. MB is usually converted by accepting electrons from nicotinamide adenine dinucleotide (NADH)/nicotinamide adenine dinucleotide phosphate (NADPH) in the biological environment and the formed colorless leucomethylene blue has negligible photodynamic activity [12]. The presence of a transmembrane thiazine dye reductase at the cell surface is commonly recognized as the initial factor for MB reduction [13]. After cellular uptake of MB, the reduction also can be catalyzed by NADH/NADPH dehydrogenases. In this respect, the major obstacle for the use of MB in PDT applications is the difficulty in preparing pharmaceutical formulations that enable their facile administration. Therefore, biocompatible nanoparticles have received attention as an adequate means of encapsulating and delivering MB for PDT [9,11].

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Calcium phosphate (CaP) materials have been widely used in biomedical applications as useful drug carriers due to their excellent biocompatibility and bioactivity [14,15]. Among them, photosensitizers-incorporated CaP nanoparticles, which were synthesized in the presence of ionic polymers such as poly(ethylene imine) and poly(styrene sulfonic acid), exhibited a good phototoxicity against murine macrophages and bacteria [15]. In addition, CaP is absorbable in specific cellular environments (endosome/lysosome) as non-toxic ionic species [16]. Thus, the employment of controlled mineralization technology using self-assembled polymer templates would lead to the successful development of biocompatible and biodegradable nanocarriers of photosensitizers. The major purpose of this study was to enhance PDT efficacy of MB by increasing biostability such as the prevention of environmental degradation and enzymatic reduction under the biological conditions. For this reason, a new mineralization method was explored to produce highly biocompatible MB-loaded CaP (CaP-MB) nanoparticles in the presence of alginic acid sodium salt (alginate) and poly(ethylene glycol)–*block*–poly(propylene glycol)–*block*–poly(ethylene glycol) (PEG–PPG–PEG) triblock copolymer (Pluronic F-68) as polymer templates (Fig. 1). The prepared MB-loaded CaP nanoparticles were systematically examined by considering their morphologies, chemical structures and particle size. The enzymatic reduction and yield of singlet oxygen generation were thoroughly investigated. Furthermore, the cellular internalization behavior and phototoxicity onto human breast cancer cell line (MCF-7) were evaluated via fluorescence microscopy and MTT assay.

2. Materials and methods

2.1. Materials

Methylene blue (MB), poly(ethylene glycol)–*block*–poly(propylene glycol)–*block*–poly(ethylene glycol) (PEG–PPG–PEG triblock copolymer, Pluronic F-68, M_n = 8400), alginic acid sodium salt (alginate), calcium nitrate tetrahydrate ($Ca(NO_3)_2{\cdot}4H_2O$), ammonium phosphate dibasic ($(NH_4)_2HPO_4$), ammonium hydroxide solution (NH_4OH), β-nicotinamide adenine dinucleotide, reduced dipotassium salt (NADH), diaphorase from *C. kluyveri*, paraformaldehyde and 9,10-dimethylanthracene (DMA) were purchased from Sigma–Aldrich (USA) and used without further purification. Human breast cancer cell line (MCF-7) was received from the Korean Cell Line Bank (KCLB, Korea). RPMI-1640, fetal bovine serum (FBS) and penicillin–streptomycin were obtained from Gibco BRL (USA). Slowfade gold antifade reagent and Live/Dead Viability/Cytotoxicity assay kit were purchased from Molecular probes (USA). Annexin V-fluorescent isothiocyanate (FITC) fluorescence microscopy kit was from BD Biosciences (USA) and DePsipher kit was from Trevigen (USA). Human cytochrome c Quantikine ELISA kit was purchased from R&D Systems (USA). Other reagents and solvents were commercially available and were used as received.

2.2. Synthesis of MB-loaded CaP nanoparticles

A synthesis of MB-loaded CaP (CaP-MB) nanoparticles is as follows. 1 w/v% MB solution was first added dropwise to 70 mL of

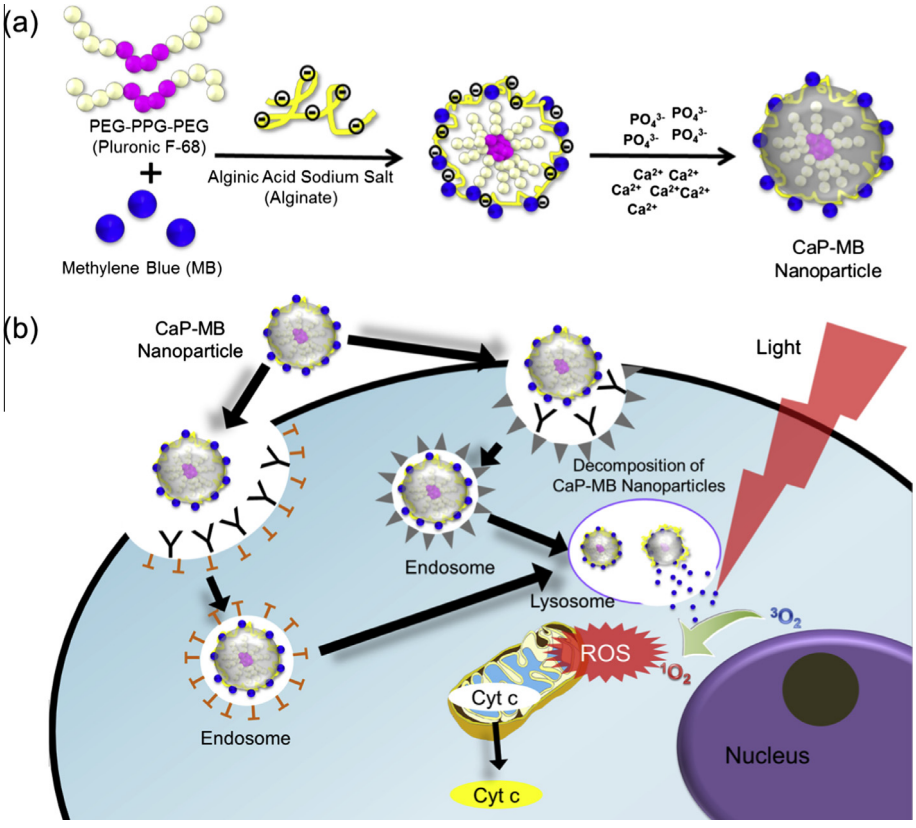

Fig. 1. Schematic images of methylene blue-loaded calcium phosphate (CaP-MB) nanoparticles for photodynamic therapy. (a) Synthetic scheme for CaP-MB nanoparticles and (b) schematic illustration of photoactivity mechanism for CaP-MB nanoparticles. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)