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Directed molecular assembly into a biocompatible photosensitizing nanocomplex for locoregional photodynamic therapy

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ABSTRACT

Methylene blue (MB), a water-soluble cationic dye widely used in the clinic, is known to photosensitize the generation of cytotoxic singlet oxygen efficiently, and thus, has attracted interest as a potential drug for photodynamic therapy (PDT). However, its use for the *in vivo* PDT of cancer has been limited due to the inherently poor cell/tissue accumulation and low biological stability in the free molecular form. Here, we report a simple and biocompatible nanocomplex formulation of MB (NanoMB) that is useful for *in vivo* locoregional cancer treatment by PDT. NanoMB particles were constructed through the self-assembly of clinically usable molecules (MB, fatty acid and a clinically approved polymer surfactant) directed by the dual (electrostatic and hydrophobic) interactions between the ternary constituents. The nanocomplexed MB showed greatly enhanced cell internalization while keeping the photosensitization efficiency as high as free MB, leading to distinctive phototoxicity toward cancer cells. When administered to human breast cancer xenograft mice by peritumoral injection, NanoMB was capable of facile penetration into the tumor followed by cancer cell accumulation, as examined *in vivo* and histologically with the near-infrared fluorescence signal of MB. The quintuple PDT treatment by a combination of peritumorally injected NanoMB and selective laser irradiation suppressed the tumor volume efficaciously, demonstrating potential of NanoMB-based PDT as a biocompatible and safe method for adjuvant locoregional cancer treatment.

1. Introduction

Photodynamic therapy (PDT) is a non-invasive modality of light-activated cancer treatment that can alleviate the invasiveness of current standards based on surgery, radiation therapy and chemotherapy [1–5]. PDT involves a photosensitizer (PS), such as Photofrin®, which accumulates at the tumor systemically and induces singlet oxygen ($^1O_2$) generation upon selective light irradiation to cause irreversible oxidative destruction to peripheral lipids, proteins or nucleic acids of cancer cells [6]. The precise destruction of cancer by PDT can offer benefits of better therapeutic outcomes with minimal collateral damages on normal tissues [7].

Since Photofrin® was approved for clinical use in the 1990s, many porphyrinoid photosensitizers with longer-wavelength absorption, effective $^1O_2$ generation, and high tumor selectivity have been investigated [8]. However, most candidates have minute water solubility which often causes undesired self-aggregation and nonspecific tissue accumulation, hampering their uses in physiological milieu [7]. Water-soluble phenothiazinium compounds such as methylene blue (MB) are another potent family of photosensitizer. With high water solubility and biosafety, MB has widely been used in the clinic for a variety of purposes [9–11]. As a potential PDT drug, it has merits of high molar extinction coefficient and suitable $^1O_2$ generation quantum yield in the therapeutic near-infrared (NIR) optical window as well as minimal dark toxicity [12,13]. However, it also retains issues to be solved such as poor cell/tissue penetration and low stability against the reducible biological condition [14–16].

To complement these flaws, various nanoformulations of MB have been explored. For instance, MB was electrostatically loaded on the surfaces of gold, silica, or carbon-based nanostructures [17–21]; however, these inorganic nanomaterials themselves were revealed to be potentially toxic *in vitro* and *in vivo* [22–25]. More biocompatible polymer-based materials, such as a polyacrylamide, Aerosol OT (AOT)-alginate complex, and poly(lactic-co-glycolic acid) (PLGA), were also utilized for nanoformulation of MB [11,16,26–32]. Although these polymeric MB nanoparticles showed promising photodynamic efficacy compared to MB itself, most studies were limited to *in vitro* demonstration rarely with actual performance of *in vivo* tumor suppression [11,16,26–30].

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In the present study, we devised a simple and biocompatible colloidal nanoformulation of MB (NanoMB), *i.e.*, a nanoscale ternary complex that is physically constructed through the directed assembly of clinically usable molecules composed of 1) MB as a cationic photosensitizer, 2) sodium oleate as an anionic salt of fatty acid, and 3) Pluronic F-68 as a biocompatible polymeric surfactant (U.S. FDA approved as a local/i.v. injectable pharmaceutical ingredient) [9,33,34]. Physical integration of clinically usable ingredients without chemical modification offers an opportunity to take full advantage of the clinical potential of MB for *in vivo* PDT. The improved cell/tissue penetration of NanoMB and thereby enhanced tumor delivery and phototoxicity, compared to the molecular solution of MB (free MB), were evaluated *in vitro* and *in vivo* on an aggressive and resistant model of triple-negative breast cancer (TNBC, MDA-MB-231) that has poor prognosis with no standard of targeted therapy [35,36]. To prove the *in vivo* utility of NanoMB for the locoregional PDT application, peritumoral injection near the tumor tissue was employed, which is known to afford high tumor-to-normal organ drug uptake ratios [37,38]. We demonstrate that local PDT treatment by a combination of peritumorally injected NanoMB and selective laser irradiation is indeed operative for the efficacious tumor suppression on a MDA-MB-231 tumor-bearing mouse model.

2. Materials and methods

2.1. Materials

All chemical reagents were purchased from commercial suppliers and used without further purification. All cell lines, MDA-MB-231, MCF-7, PC-3, MRC-5, NIH-3T3 and clone 1-5c-4, were obtained from Korean Cell Line Bank (Seoul, Korea) and cultivated in Roswell Park Memorial Institute (RPMI) 1640 medium and Dulbecco's modified Eagle's medium (DMEM) medium supplemented with 10% FBS and 1% antibiotic (penicillin-streptomycin) in a humidified 5% CO_2 incubator at 37 °C.

2.2. Preparation and characterization of NanoMB

Methylene blue trihydrate (20 mg, 0.05 mmol) and sodium oleate (30 mg, 0.10 mmol) were added in THF (100 mL). The mixture was repeatedly heated to 65 °C for 5 s and cooled down to room temperature (3 times), and was filtered to remove precipitate by 5 μm syringe filter (Millipore, Germany). After evaporating the filtrate with nitrogen blowing, the obtained solid was dried under reduced pressure overnight to obtain the methylene blue-oleic acid binary complex (MBO, 34 mg) with organic solubility. The complex composition and the yield were calculated based on the total feed amount of MB and sodium oleate, the weight gain of the MBO and the MB amount therein estimated from the UV absorbance at 645 nm in methanol (complexed molar ratio of MB/oleate = 1/1.4; complexation yield: 68%). For ternary complexation, MBO (0.1 mg) and Pluronic® F-68 (20 mg) were homogeneously mixed in THF (1.2 mL). After solvent evaporation by air blowing, the dried residue was mixed with 2 mL of deionized water and agitated by vortexing for a few seconds, to yield a clear aqueous dispersion of NanoMB particles. The shape and size of NanoMB were characterized using a CM30 transmission electron microscope (TEM, FEI/Philips, USA) operated at 200 kV. For the TEM sample preparation, a drop of NanoMB dispersion was dried on a 300-mesh copper grid coated with carbon and negatively stained with 2 wt.% uranyl acetate solution. The particle size was measured by counting at least 200 nanoparticles from the TEM image. The number-weighted mean hydrodynamic size distribution of NanoMB dispersed in deionized water was measured at 25 °C using a zeta-sizer (Nano-ZS, Malvern, UK). UV–vis absorption and fluorescence emission spectra were measured by a UV–visible spectrometer (Agilent 8453 UV–visible spectroscopy system, Agilent Technologies, USA) and a fluorescence spectrophotometer (Hitachi F-7000, Japan), respectively.

2.3. Singlet oxygen generation measurement

Singlet oxygen generation was evaluated by monitoring a decrease in the absorbance of the *p*-nitrosodimethylaniline (RNO) at 440 nm under excitation of 655 nm laser (200 mW/cm^2, Changchun New Industries Optoelectronics Tech. Co., Ltd., China). The mixture solution was prepared in deionized water by mixing 0.21 mL of NanoMB (10.05 mg/mL) or free MB solution of equivalent absorption at 655 nm with 0.11 mL of RNO stock solution (0.12 mM), 0.7 mL of histidine (0.03 M), and 0.18 mL water. Laser excitation at 655 nm was performed by irradiating the collimated laser beam through the sample. The relative quantum yields of $^1O_2$ generation were estimated by using an aqueous solution of free MB as a reference and comparing the derived slopes of RNO absorbance plots ($-\ln A_{440}$) as a function of laser irradiation time.

2.4. Cellular uptake

For the cellular uptake experiments, MDA-MB-231, MCF-7, PC-3, MRC-5 and clone 1-5c-4 cell lines were cultured in RPMI 1640 medium supplemented with 10% FBS and 1% antibiotic (penicillin-streptomycin) in a humidified 5% CO_2 incubator at 37 °C. NIH-3T3 cells were cultured in DMEM with the same supplement and condition. The tested cells (1×10^5 cells/dish) were seeded onto 35 mm cover glass bottom dishes and allowed to grow until a confluence of 70%. Prior to the experiment, cells were washed twice with PBS (pH 7.4) to remove the remnant growth medium, and then incubated for 1 h in a serum-free medium (2 mL) containing 200 μL of NanoMB (10.05 mg/mL) or aqueous free MB of equivalent absorption at 655 nm. The cells were washed with PBS and fixed with glutaraldehyde of 1% for 5 min. After fixation, the fluorescent images of each cell were obtained using a LEICA DMI3000B equipped with a Nuance FX multispectral imaging system (CRI, USA).

2.5. Flow cytometry analysis

Each cell line was seeded on 12-well culture plates at a density of 2×10^5 cells/mL and cultured for 48 h. After removal of medium, cells were further incubated for 1 h in a serum-free medium (2 mL) containing 200 μL of NanoMB (10.05 mg/mL) or aqueous free MB of equivalent absorption at 655 nm and washed twice with PBS (pH 7.4). Cell suspensions were transferred to conical tubes and centrifuged for 3 min at 2500 rpm. Supernatant was removed, and the cell pellets were resuspended in 1 mL of PBS. Fluorescence intensity of the cells was estimated by flow cytometry (Guava easyCyte™ Flow Cytometers, Merk Millipore, Germany). Samples of at least 1000 cells were analyzed in triplicate.

2.6. In vitro dark and phototoxicity assay

All cells were cultured in 96-well plates (1×10^4 cells/well) for 24 h, and then treated with 10 μL of NanoMB (10.05 mg/mL) or aqueous free MB of equivalent absorption at 655 nm for 1 h. After washing with fresh media, cells were treated with or without laser irradiation (655 nm, 200 mW/cm^2, each well for 5 min) and then further incubated for another 24 h. The resulting dark or phototoxic effects on cells were evaluated *via* MTT assay.

2.7. Tissue penetration assay

NanoMB (10.05 mg/mL) or aqueous free MB of equivalent absorption at 655 nm (500 μL) was dropped on one side of a chicken breast tissue (3 cm × 3 cm × 3 cm in width × length × height). After incubation for 1 h at 37 °C and washing with PBS, NIR fluorescence snapshots were imaged with a 12-bit CCD camera (Kodak Image Station 4000 MM, USA) equipped with a 150 W quartz halogen illuminator (Fiber-Lite, PL900, Dolan-Jenner, USA) and a filter set of excitation (625 nm) and emission (700 nm).