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Encapsulation of Methylene Blue in Polyacrylamide Nanoparticle Platforms Protects its Photodynamic Effectiveness

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Abstract

The ability to prevent methylene blue (MB), a photosensitizer, from being reduced by plasma reductases will greatly improve its efficacy in photodynamic therapy (PDT) applications. We have developed a delivery approach for PDT by encapsulating MB using nanoparticle platforms (NPs). The 30-nm polyacrylamide-based NPs provide protection for the embedded MB against reduction by diaphorase enzymes. Furthermore, our data shows the matrix-protected MB efficiently induces photodynamic damage to tumor cells. The unprecedented results demonstrate the significant *in vitro* photodynamic effectiveness of MB when encapsulated within NPs, which promises to open new opportunities for MB in its *in vivo* and clinical studies.

Keywords

methylene blue; leukomethylene blue; singlet oxygen; photodynamic therapy; polyacrylamide; nanoparticle platform; enzymatic reduction

INTRODUCTION

Photodynamic therapy (PDT) is a promising mode of treatment for a variety of cancers and other diseases. Briefly, the activation of a photosensitizer, with a specific wavelength of light in the presence of oxygen, promotes cellular damage [1–3]. Following the absorption of light, a photosensitizer is activated to its excited states and may undergo two well defined mechanisms for generating cytotoxic species [4,5]. Type I reactions produce free radicals or superoxide ions resulting from hydrogen or electron transfer; Type II reactions generate ¹O₂, singlet oxygen, via an energy transfer process that occurs during a collision of the excited sensitizer with oxygen. It is generally agreed that ¹O₂, produced via a Type II process, is the primary reactive oxygen species (ROS) that causes damage to nearby biomolecules. Due to its short lifetime and diffusion distance, photodynamic damage by singlet oxygen is often restricted to the surrounding cell/tissue, with minimal systemic toxicity. Currently, several research groups are actively developing PDT technology. Numerous investigations on a variety of photosensitizers, e.g., Photofrin, have been reported with promising results [1–3]. However, improvements in the quantum efficiency, reductions in toxicity and the ability to enable targeting of a highly effective payload remain a desirable therapeutic goal for PDT.

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Methylene blue (MB) is a widely used photosensitizer that has been employed in a variety of applications, including PDT [3,6]. The high quantum yield of $^1\text{O}_2$ generation ($\Phi_{\Delta} \sim 0.5$) by MB, via excitation in the therapeutic window (600–900 nm), coupled with its low toxicity, makes MB a promising candidate for PDT [7–10]. However, the clinical use of MB has been hampered by its propensity for rapid chemical alteration when systemically applied: MB in the biological environment is usually converted by accepting electrons from NADH/NADPH, and unfortunately, the formed colorless leukomethylene blue (LMB) has negligible photodynamic activity [11–13]. Recent studies suggested the presence of a transmembrane thiazine dye reductase at the cell surface as the initial factor for MB reduction [14–16]. Once within the cells, MB also can be reduced via NADH/NADPH dehydrogenases.

In view of the above, the major obstacle for the use of MB, as well as many other photosensitizers, in PDT applications is the difficulty in preparing pharmaceutical formulations that enable their facile administration. In recent years, with the advancement of nanotechnology, engineered nanoparticles have received attention as a possible means of encapsulating and delivering PDT agents. Research in our laboratories [17–22] and elsewhere [23,24] has demonstrated the utility of nano-structured materials, which employ biocompatible nanoparticle matrices encapsulated with efficient photo-active molecules, for PDT. Our concept of a nanoparticle platform (NP) was initiated by the design of Photonic Explorers for Biomedical use by Biologically Localized Embedding, termed PEBBLEs. Based on the previously demonstrated advantages of PEBBLEs [25], the development of NPs enables the use of PDT methods that differ significantly from conventional approaches. Rather than releasing photosensitizers, the NP is able to deliver only photo-generated cytotoxic species. Moreover, such a NP may be a modular system that permits a high degree of loading of drug molecules. Thus, concentrated (critical mass) packaging can be achieved by encapsulation with other incorporated sensitizers, probes or antibodies, through adsorption or covalent linkage, which together function as a synergistic unit for enhanced therapeutic efficacy [17].

Most importantly, in the present case, we hypothesized the nanoparticle matrix would protect the embedded active form of photosensitizers from enzymatic or environmental degradation. Hence this would widen the choice of photosensitizers to include “reject” molecules, such as MB, which would otherwise be unlikely candidates for systemic administration in PDT treatment. Our recent research has reported the synthesis and characterization of a NP made of polyacrylamide, which encapsulates MB for its use in PDT applications [21]. Here we show for the first time that this polymeric matrix can prevent the embedded MB from being reduced by diaphorase enzymes, thereby retaining the photoactive form of MB for efficient PDT treatment. We furthermore demonstrate that following light exposure, the MB-containing NP can induce photodynamic effects on rat C6 glioma cells. Thus, this work emphasizes the potential for using MB, when encapsulated within nanoparticles, as a simple but effective photosensitizer for PDT.

MATERIALS AND METHODS

Chemicals

MB (certified 97% dye content) was acquired from Aldrich without further purification. Acrylamide, N, N'-methylenebis(acrylamide) (BIS), N,N,N',N'-tetraethylmethylenediamine (TEMED), polyoxyethylene (4) lauryl ether (Brij 30), and dioctyl sulfosuccinate (AOT) were also purchased from Aldrich. Ammonium persulfate (APS), β -Nicotinamide adenine dinucleotide, reduced dipotassium salt (NADH) and Diaphorase from *Clostridium kluyveri* were purchased from Sigma. Ethanol (200 proof) was obtained from Pharmco Products, Inc., and hexane from Fisher. Calcein acetoxymethyl ester (Calcein-AM) and Propidium Iodide (PI) were purchased from Molecular Probes. All solutions were prepared from 18 M Ω water purified by a Barnstead 1 Thermolyne Nanopure II system.

Preparation of MB-containing polyacrylamide NPs

The polymerization procedure of acrylamide was adapted from previously described work [21,26]. The polymerization solution consisted of 0.711 g acrylamide, 0.231 g BIS, and 2 mL of distilled water. A 2-mL portion of this aqueous solution was added to an oil phase that contained 43 mL deoxygenated hexane, 1.6 g AOT, and 3.1 g Brij 30, and was stirred vigorously to produce a microemulsion. 4 mg MB was added before the polymerization was initiated by adding 30 μ L of 10% (w/v) APS and 15 μ L of TEMED. The solution was then stirred under argon at room temperature for 2 hours. At the completion of polymerization, hexane was removed by rotary evaporation and the particles were precipitated by addition of ethanol. The surfactant, unreacted monomers and excess dye molecules were washed away from the particles with ethanol in an Amicon ultra-filtration cell (Millipore Corp.), using a 100 kDa filter membrane under 15 psi of pressure. The particle suspension was then collected by a suction filtration system (Fisher) with a 0.02 μ m Whatman Anodisc filter membrane. The MB-containing NPs were protected from light during the production process.

Enzymatic reduction test of MB

Solutions of 3 mg/mL MB-containing NPs and 1 μ g/mL (0.3 μ M) free MB were prepared respectively in Dulbecco's Phosphate buffered saline (PBS) (pH 7.2). 0.45 μ mol NADH and 0.05mg diaphorase were sequentially added into the free MB solution. The decrease in MB emission at 680 nm due to the reduction of MB was measured at room temperature with incubation time up to an hour, using a Jobin Yvon FluoroMax-3 Spectrofluorometer. The solution of the MB-containing NPs was treated in the same way, and its fluorescence spectra were also recorded for comparison. Additionally, as a control test, the emission of the same particle solution (without NADH and diaphorase) was monitored to adjust for the photobleaching effect.

Cell Culture

Rat C6 glioma cells were cultured in Dulbecco's modified Eagle's medium containing 400 mg L⁻¹ D-glucose, 2 mM L-glutamine and 10% fetal bovine serum, and incubated at 37 °C in a 5% CO₂ environment. Cells were plated on uncoated 22-mm glass cover slips 2 days prior to experimentation.

Laser-induced in vitro PDT effect

The glass cover slips with glioma cells were removed from the culture medium, rinsed carefully with Hanks 1x balanced salt solution and placed in a microscope chamber at 37 °C. The cells were covered with 2 mL Hanks 1x balanced salt solution containing 10 mM pH buffer HEPES, 0.1 μ M Calcein-AM, 10 μ M PI, and 1 mg/mL MB-containing NPs. Calcein-AM, a cell-permeant dye, was used to determine cell viability both before and after the photodynamic treatment. In live cells the non-fluorescent Calcein-AM is converted to a green-fluorescent Calcein, after acetoxymethyl ester hydrolysis by intracellular esterases. Meanwhile, PI marks the nuclei once the integrity of the cell membrane is compromised, i.e. detecting dead or dying cells. Cell images were acquired with a Perkin Elmer UltraView confocal microscope system equipped with an Argon-Krypton laser. Pre-exposure images were taken with an oil immersion 60x objective lens in two channels (488 nm excitation of the Calcein converted from Calcein-AM, 568 nm excitation of the PI). The cells were exposed for five minutes at 647 nm resulting in an estimated optical energy dose of 52 J/cm². Images were taken every two minutes, for up to two hours, to monitor cell death. Finally, a 20x objective was used to broaden the field of view and allow concurrent comparison of the cells exposed to light along with those that had not been exposed.

Furthermore, dose-dependent PDT studies were performed using the MB-containing NPs at final concentrations of 0.1 mg/mL, 1 mg/mL and 5 mg/mL, respectively. Pre-exposure images were taken with a 20x objective lens. The cells were then exposed continuously for 45 minutes, using 647 nm laser light with a power density of 0.32 W/cm². Afterwards, images were taken to compare the induced cell damage.

RESULTS AND DISCUSSION

Prevention of enzymatic reduction of MB by the matrix

Characterization of the MB-containing polyacrylamide NPs was reported in previous publication [21]. The particle size of these NPs was determined by SEM to be approximately 30 nm in diameter. The chemical measurements using Anthracene-9, 10-dipropionic acid, disodium salt, also showed significant photo-generation of ¹O₂ for PDT. Here, to verify the hypothesis that encapsulation of MB in NPs could prevent the reduction of MB, and thus protect its photodynamic effectiveness, the MB-containing NPs were tested in solution with a diaphorase from *Clostridium kluyveri* (molecular weight 24 kDa), and the cofactor NADH. Figure 1 shows the fluorescence emission of MB and MB-containing NPs, respectively, when mixed with NADH and the diaphorase at room temperature. Under these conditions, we confirmed the reduction of free MB by monitoring the disappearance of fluorescence signal at 680 nm, the characteristic emission peak of MB. In contrast to the approximately 60% decrease of fluorescence intensity in free MB solution, the MB-containing NPs exhibited minimal fluorescence change within up to an hour (after the subtraction of a small photobleaching effect for both curves). These results suggest that the embedded MB stayed mostly intact during the treatment, while free MB in solution was rapidly reduced.

The fact that addition of NADH and diaphorase severely depleted levels of free MB indicates the necessity for separation of the photosensitizer from biological reduction systems in maintaining photodynamic activity. In the case of the MB-containing NPs, penetration of the diaphorase into the particle matrix is hypothesized to fail because of its large molecular size. Previous work on both polyacrylamide-based and sol-gel based PEBBLE sensors also demonstrated that the matrix, as expected, prevented macromolecules such as proteins (e.g. bovine serum albumin) from diffusing through [27–29]. The matrix protected entrapped dyes from the intracellular environment, thus diminishing interference with the fluorescent properties of the dyes [25]. Our findings here are in accordance with the previous results on polyacrylamide and sol-gel silica matrix, demonstrating the shielding effect of the matrix in such a designed NP. The shielding effect indeed is of critical importance for preserving MB in its valid chemical formula and thus potentially extending its medical use in PDT. Furthermore, in general, the matrix should also significantly reduce any potential toxicity of the encapsulated drug, excluding any perturbation of the nearby biological system. Therefore, a large variety of drugs could be incorporated by taking advantage of the dual-protection by the matrix.

In vitro PDT studies on tumor cells treated with MB-containing polyacrylamide NPs

The photodynamic mechanism of free photosensitizers in general is fundamentally different from the work presented in this paper and the previous reports on our NPs [17–21]. As illustrated by the minimal enzymatic reduction of the encapsulated MB discussed above, the polymeric matrix of the NPs should be capable of protecting the embedded photosensitizers from direct interaction with biological macromolecules *in vivo*. Also, due to the “shielding” of NPs, Type I pathways in which the excited photosensitizer reacts directly with a substrate, such as the cell membrane or a molecule, should be hampered or totally inhibited, leaving the ¹O₂ generated via Type II pathways as the key ROS responsible for cytotoxicity. In addition, incubation time in PDT may be shortened when using such NPs, as they are not designed to

always internalize into target cells but to deliver $^1\text{O}_2$ to them, thus requiring only external contact with the cell membrane. In practical PDT treatment, the intracellular aggregation of free photosensitizers and their being pumped back out of cells by multidrug resistance proteins may also cause problems. In contrast, our NPs are anticipated to minimize these complications often encountered with free photosensitizers, especially MB, in clinical trials, thus resulting in higher cell killing efficiency.

Our previous report has verified the photo-generation of $^1\text{O}_2$ by the MB-containing polyacrylamide NPs [21]. Here, to confirm the photodynamic cytotoxicity of the NPs for PDT, their *in vitro* experiments were performed on rat C6 glioma tumor cells. Confocal microscopy was used to obtain a series of images of cells treated with the NPs without incubation, under red light irradiation. The sample solutions also contained two fluorescent stains, Calcein-AM and PI, to monitor live and dead cells, respectively. Figure 2 shows the overlay images of cells treated with 1 mg/mL MB-containing NPs. Before light irradiation, all the cells in Figure 2A were stained by Calcein showing green fluorescence, which verified the cell viability. A series of images of the cells (Figure 2B–E) were sequentially taken within two hours, after a 5-min irradiation period with 647 nm laser light. The cytoplasm of the cultured cells lost its green fluorescence in a time dependent manner, while the nuclei of the compromised cells were observed to increase in their uptake of PI, indicated by the red fluorescence. Internal controls were obtained by capturing images afterwards, using a 20x (rather than 60x) objective lens with the irradiated area in the center (Figure 2F). It can be seen that the local illumination induced damage only to the cells located in the beam of light; while other cells, in contact with MB-containing NPs but not illuminated by the laser, remained intact, as indicated by their green fluorescence. These observations suggest that exposure of cells to the combination of MB-containing NPs and light resulted in producing $^1\text{O}_2$ in sufficient quantities to cause damage to tumor cells in culture.

Control experiments using only red light, or blank NPs (not MB-containing) with the same light exposure, were described in previous studies [21]. Neither revealed significant cell damage in the illumination area. Furthermore, the same polyacrylamide-based matrix has been reported to be non-toxic *in vitro*, based on results using MTT assay [22]. Therefore, under current conditions, neither the red light nor the polymeric matrix by itself is cytotoxic to the glioma cells; light and photosensitizers, in combination, are required for the photodynamic cytotoxicity.

Additionally, the dose-dependence of PDT with the MB-containing NPs was evaluated *in vitro*. A series of experiments on C6 glioma cells were performed during treatment by red light at a laser power density of $\sim 0.32 \text{ W/cm}^2$, where three different concentrations of the NPs were applied. Cells were stained with both Calcein-AM and PI, and their confocal images before light exposure (Figures 3A–3C) were acquired using a 20x objective lens. After 45 min of continuous illumination by 647 nm laser light, images were taken again, as displayed in Figures 3D–3F. We observed that higher NP concentration induced more cell damage, as indicated by an increase in both the loss of Calcein (green) and the incorporation of PI (red). These results thus demonstrate the dose-dependent PDT efficiency of the MB-containing NPs.

In conclusion, a novel design, using a NP for the encapsulation of MB for PDT, has been developed; it provides a stable polyacrylamide-based formulation and can deliver $^1\text{O}_2$ to cells in the vicinity. We demonstrated that MB can be protected by the polymeric matrix from enzymatic reduction to its non-photoactive form, LMB. This protection by encapsulation should, therefore, greatly enhance the efficiency and potency of MB as a photodynamic agent after intravenous injection. As illustrated in the *in vitro* studies above, encapsulated MB permits not only sufficient light-stimulated generation of $^1\text{O}_2$, but also adequate diffusion out of the matrix to cause damage to the tumor cells. Furthermore, based on recent successful *in vivo*

work with the similar polyacrylamide-based NPs, using the less efficient photofrin [17–20], rather than MB, as the PDT agent, we expect the MB-containing polyacrylamide NPs to be equally or more efficient in *in vivo* targeted treatment of glioma.

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Figure 1.

Fluorescence emission at 680 nm vs time (after subtraction of photobleaching for both curves): (A) 3 mg/mL MB-containing NPs and (B) 1 μ g/mL MB, respectively, when mixed in PBS (pH 7.2) with 0.45 μ mol NADH and 0.05 mg diaphorase. The inset is the reduction of MB to its non-photoactive form.

Figure 2.

Composite confocal images of the rat C6 glioma cells stained with Calcein-AM (green) and PI (red), after treatment with 1 mg/mL MB-containing NPs: (A) before light exposure, using a 60x objective; (B–E) within two hours after 5-min light exposure with a dose of 52 J/cm^2 , using a 60x objective; (F) two hours after 5-min light exposure, using a 20x objective.

Figure 3.

Composite confocal images showing rat C6 glioma cells stained with Calcein-AM (green) and PI (red), using a 20x objective, after treatment with 0.1, 1 and 5 mg/mL MB-containing polyacrylamide NPs, respectively: (A–C) before light exposure; (D–F) after 45 min light exposure.