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Optimization of hydrogel containing toluidine blue O for photodynamic therapy by response surface methodology

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ABSTRACT

Photodynamic therapy with toluidine blue O (TBO) hydrogel exhibits antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* in this paper. The response surface methodology is employed to optimize formulations for antibacterial activity. The optimal formulations are carbomer concentration of 3% (w/v), TBO concentration of 0.1 mg/mL and the quality ratio of NaOH and carbomer of 0.4 (w/w). Under the optimized formulations, the log-transformed of CFU mL^{-1} on the *Staphylococcus aureus* and *Escherichia coli* are 0.84 and 1.26 (the log-transformed of CFU mL^{-1} of negative control groups on the *Staphylococcus aureus* and *Escherichia coli* are 8.21 and 8.47), respectively. In comparison with photodynamic therapy with TBO aqueous solution, the proposed formulations provide a much stronger antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*. TBO hydrogels are stable during 6 weeks at three different temperatures (4, 25 and 40 °C) with respect to no change of color, transparency, pH and viscosity. 50% and 68.26% of TBO are released from carbomer hydrogel after 4 h and 24 h, respectively. TBO hydrogel alone or light alone (630 nm) treatment is incapable of showing antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*. Therefore, photodynamic therapy with the novel optimized TBO hydrogel formulations is a promising treatment strategy for periodontitis.

1. Introduction

Periodontitis, known as a multifactorial disease, involves progressive damage to tooth supporting structure and tooth loss initiated and perpetuated by microorganism colonizing the subgingival area [1,2]. Various therapeutic strategies were used for the treatment of periodontitis, including scaling and root planing (SRP), antibiotics, disinfectant and surgical debridement [3–6]. Although SRP and other antibiotics make an important contribution to the clinical therapy in periodontitis, the disadvantages of both treatments are still remained. For instance, the complex anatomy of teeth (furcation involvements and root invaginations) impose significant limitations on the SRP efficiency of eliminating subgingival microorganism [7,8]. On the other hand, antibiotic resistance is occurring worldwide threaten the efficiency of antibiotics [9]. To overcome the above problems, antimicrobial photodynamic therapy has been proposed as a desirable treatment strategy for periodontitis.

Photodynamic therapy (PDT) is a physical treatment for various diseases, such as cancers, dental caries and skin diseases [10–12]. PDT involves in a combination of a photosensitizer, light and molecular oxygen. The certain wavelength of light is able to activate photosensitizer in the presence of molecular oxygen to generate reactive oxygen species (ROS) resulted in destroying microorganisms [8,12]. The advantage of PDT over other treatment strategies lies in reducing steric hindrance from complicated anatomy of teeth and preventing adverse side effect by the selectable activation of photosensitizer only when exposed to light [11]. Also, highly targeted into lesion by PDT reduces the overdose of antibiotics and the occurrence of antibiotics

Abbreviations: PDT, photodynamic therapy; TBO, toluidine blue O; CFU, colony forming units; *S. aureus*, *Staphylococcus aureus*; *E. coli*, *Escherichia coli*; SRP, scaling and root planing; ROS, reactive oxygen species; PBS, phosphate buffer solution; RSM, response surface methodology; BBD, Box-Behnken design; NC, negative control; PC, positive control; LB, lysogeny broth

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resistance [13]. A phenothiazinium salt, named as Toluidine blue O (TBO), has been widely used for the inactivation of microorganisms by the reason of non-toxic to human cell and high quantum yields of singlet oxygen [14–18]. TBO is capable of effectively inactivating a broad range of microorganisms including both Gram-positive and Gram-negative bacteria such as fungi, yeast, *Streptococcus mutans*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* [18–21].

In most case, photosensitizer in solution was applied for antimicrobial photodynamic therapy for the treatment of periodontitis [22–24]. However, the solution of photosensitizer is easy to remove by saliva and mechanical activities. The limited number of novel TBO formulations has been prepared to increase the retention time in treatment. Unfortunately, they were not shown desirable antibacterial activity. Chen et al. [25] have reported a chitosan/hydroxypropyl methylcellulose hydrogel containing TBO but without strong antibacterial activity. Hence, a novel dosage form of TBO should be developed to effectively apply on clinical practice.

Hydrogels are widely used as a dosage form in drug delivery to retain large amount of water, saline and physiological solutions [26–28]. Hydrogels also show a high adsorption, strong adhesiveness, and superior biocompatibility [29–31]. Hydrogels are three-dimensional hydrophilic polymers such as carbomer, chitosan and alginate [31]. Carbomers are polymers of high molecular weight polyacrylic acid cross-linked with poly alkenyl ethers of sugars or polyalcohols [32]. Due to its safety and high viscosity, carbomers have widely used in controlled release solid dosage formulations, bioadhesive and topical formulations [33]. Hence, carbomer was selected as the carrier loaded with TBO in our paper. A carbomer hydrogel incorporating TBO was conducted to extend the residence time of TBO in gums and improve the therapeutic effect at the target site.

In the development of hydrogel dosage form, it was crucial to design an optimized pharmaceutical formulation with appropriate antibacterial activity within minimum trails. Response surface methodology (RSM) is a collaboration of statistical and mathematical techniques for exact model building [34], which has widely applied in pharmaceutical research [35]. The main advantage of RSM is that it significantly reduces the number of experiment trails for optimization. Moreover, RSM can generate a mathematical model to explain the reciprocal interaction of experimental factors and obtain the optimal values [36]. In the work, the antibacterial activity of the TBO hydrogel formulation was optimized and enhanced by RSM.

In this paper, a novel TBO hydrogel for the treatment of periodontitis was prepared with carbomer and NaOH as base and neutralize, respectively. The semisolid formulations of TBO hydrogel were used as drug delivery platforms based on the demand of clinical treatments. The antibacterial activity of PDT treated with TBO hydrogel was performed on the *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*). The TBO hydrogel formulations were optimized by RSM. Furthermore, the release and stability of the TBO hydrogel were evaluated.

2. Materials and Methods

2.1. Materials

Toluidine blue O was purchased from J & K®. Carbomer 934P, carbomer 934, carbomer 940 and carbomer 941 were bought from J & L® (Shanghai, China). *Staphylococcus aureus* (CMCC 26003) and *Escherichia coli* (ATCC 25922) were provided by the Institute of Microbiology (Zhejiang University of Technology). Lysogeny broth (LB) and agar were purchased from Sinopharm Chemical Reagent Co., Ltd. All other chemicals were of analytical reagent grade and deionized water was used in all experiments. Light source (CMC Dental, in Denmark) is a diode laser equipped with perio tip 23 mm, an output power of 5 W, a maximum output intensity of 4 mW/cm^2 and a predominant wavelength of 630 nm.

2.2. 2.2 Preparation of TBO Hydrogels

TBO hydrogels were prepared by dispersing the carbomer powder in the sterile water and mixing completely. TBO aqueous solution and NaOH aqueous solution were added into carbomer solution and mixed until pH 6.0–8.0. All the TBO hydrogel samples were equilibrated in the dark for at least 48 h at room temperature before further experiments. Different TBO hydrogel samples were prepared by the following parameters: gel agent (carbomer 934P, carbomer 934, carbomer 940 carbomer 941), carbomer concentration (0.5%–10% w/v), TBO concentration (0.1–4 mg/mL), the quality ratio of NaOH and carbomer (0.2–0.4 w/w) and sterile water (the total volume of solution up to 15 mL).

2.3. Bacterial Strain and Growth Conditions

S. aureus and *E. coli* were aerobically incubated in lysogeny broth (LB) at 37 ± 0.5 °C for 18 h. The bacterial culture was centrifuged at 4000 rpm for 5 min. The supernatant was removed before cell pellets were resuspended with sterile phosphate buffer solution (PBS, pH 7.4) until the turbidity of McFarland (1.5×10^8 viable cell mL^{-1}) standard suspension reach 0.5. The confluence of cells in PBS (pH 7.4) was quantified at 600 nm (OD_{600} = 0.31) by a UV-spectrophotometer (759S, in China).

2.4. Antimicrobial Photodynamic Inactivation of S. aureus and E. coli

In the experiment, 100 μL of standard bacterial suspensions was added to each well of sterilize 96-well plate. In the PDT with TBO hydrogel groups, 100 μL of TBO hydrogel was added to well with suspension. In the negative control (NC) groups, 100 μL of PBS was added instead of TBO hydrogel. 100 μL of 0.1 mg/mL TBO aqueous solution was performed as the positive control (PC). It has been reported that 0.1 mg/mL aqueous TBO was effective in the inactivation of *S. aureus* biofilms [37]. Moreover, 0.1 mg/mL TBO aqueous solution showed the best antibacterial activity at 30 s irradiation time in our preliminary experiment. After all treatments were added into 96-well plate, the plate was shaken for 15 min on the micro oscillator in the dark to make the photosensitizer penetrate into the bacteria cells. In the PDT with TBO hydrogel groups and the PC groups, laser irradiation (630 nm) was performed under aseptic condition in a laminar flow hood for 30 s and kept in the dark for 10 min. On the contrary, the NC groups were not exposed to light. Each well was covered with a sterile black lid to avoid light scattering to the adjacent wells. After exposed to light, the suspensions were serially diluted in PBS (pH 7.4). 100 μL of each dilution was seeded onto LB agar and incubated aerobically at 37 ± 0.5 °C for 18 h. After incubation, the number of colony forming units per milliliter (CFU mL^{-1}) was calculated as log-transformed to evaluate bacterial viability. Each sample was carried out in triplicate.

2.5. Optimization TBO Hydrogel Formulations by Response Surface Methodology

An optimization process was carried out by RSM. Box-Behnken design (BBD) requires fewer numbers of experiments to determine the optimal formulations [38]. For this reason, it was employed to optimize formulation factors and access the interactions between factors on the basis of the single-factor experiment results. Carbomer concentration (X_1), TBO concentration (X_2) and the quality ratio of NaOH and carbomer (X_3) were independent variables. Response variable (Y) was the log-transformed of CFU mL^{-1}. The related experimental data of independent variables and response variables were analyzed to get the second-order polynomial model as shown in the following equation,