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ORIGINAL ARTICLE

Optimization of hydrogel containing toluidine blue O for photodynamic therapy in treating acne

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Abstract

Antibiotics and photodynamic therapy (PDT) are widely employed in curing acne. However, antibiotics as an effective treatment would lead to bacterial resistance and severe side effects. In this study, we aimed to develop a novel TBO hydrogel, which could prolong the retention time of photosensitizer (TBO) at the lesion site and improve therapeutic effect. In vitro antibacterial experiments (against *Staphylococcus aureus* and *Escherichia coli*), the response surface methodology was used to optimize the formulation of TBO hydrogel. The results indicated that the optimal formulation was 0.5% (*v*/*v*) carbomer, 0.01 mg/mL TBO, 0.5% (*v*/*v*) ethanol concentration, 0.5% (*v*/*v*) Tween 80, the mass ratio of NaOH to carbomer of 0.4 (*w*/*w*). The TBO hydrogel formulation showed the strong antibacterial activity for *Propionibacterium acnes*. The stability, pH, and antibacterial activity of TBO hydrogel did not significantly change under 4 °C, 25 °C, and 40 °C during 6-week storage. Furthermore, TBO combined with carbomer hydrogel showed the 51.28% (4 h) and 69.80% (24 h) release. In summary, the hydrogel TBO might be a vital therapeutic strategy to promote the PDT applied in the topical therapy of acne.

Keywords Acne · Photodynamic therapy · Topical therapy · Antibacterial activity · TBO hydrogel

Abbreviations

PDT	Photodynamic therapy
TBO	Toluidine blue O
CFU	Colony forming units
S. aureus	*Staphylococcus aureus*
E. coli	*Escherichia coli*
P. acnes	*Propionibacterium acnes*
ROS	Reactive oxygen species
PBS	Phosphate buffer solution
RSM	Response surface methodology
BBD	Box-Behnken design
LB	Lysogeny Broth
BHI	Brain heart infusion broth
RCA	Reinforced clostridial agar

Highlights • Optimization of hydrogel containing toluidine blue O for photodynamic therapy in treating acne.
• Photodynamic therapy with optimal TBO hydrogel showed antimicrobial activity including *Propionibacterium acnes*, *Staphylococcus aureus*, and *Escherichia coli*.
• The optimal TBO hydrogel was stable over time about appearance, pH, viscosity, and antimicrobial activity.

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Introduction

Acne is a common skin disease among youngsters, which demonstrates the incidence increase over years. Acne will not only influence the normal life of adults, but also damage their physical and psychological morbidity [1]. There are main causes of acne such as the development of inflammation, bacterial colonization, abnormal keratinization, and skin pore blockage [1, 2]. These interrelated factors will affect each other; for example, bacterial colonization can result inflammation. The major therapeutic strategies in clinic are antibiotic application [3, 4] including lincosamide and erythromycin, traditional herbal medicine, *etc* [5–7]. However, more and more patients treated by antibiotic strategies showed bacterial resistance and severe side effects [3]; the development of new no-antibiotic therapeutic strategies for acne is vital.

Photodynamic therapy (PDT) is a promising antibacterial strategy without side effects, which is applied for cancer, acne, and keratoacanthoma, *etc* [8–10]. Based on singlet oxygen and free radicals generating from photosensitizers, PDT showed a significant therapeutic advantage in various skin disease, especially in acne [11, 12]. The investigators reported that PDT might treat various human skin cancers [13]. In recent decades, 5-aminolevulinic acid and its derivatives as photosensitizer (PS) were widely employed in acne [14–18]. However, the 5-aminolevulinic acid and its derivatives had to transform into protoporphyrin IX (PpIX) in some time once applied on the skin, which is the vital active ingredient for the PDT reaction. Hence, therapeutic effect of above photosensitizers was limited [19]. Toluidine blue O (TBO) is a low-cost phenothiazinium salt, ideal tissue penetration, well-soluble in water, and different proportions of ethanol aqueous solution [20–22], which make it suitable for various diseases. For instance, as a potential drug candidate for Alzheimer's disease, antifungal therapy, and periodontal diseases [23–26], its main advantage was a high singlet oxygen yield that can inactivate gram-negative bacteria and gram-positive bacteria [27]. To be specific, they include *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *T. rubrum*, and *Escherichia coli*, *etc* [28–31].

However, it is difficult for TBO solution to maintain in the target sites for a long time, resulting in a low concentration of TBO, which limits its applications. A gel system with TBO was developed to overcome the above disadvantage. The gel is a semi-solid system, where the liquid phase is contained to the three-dimensional polymer matrix [32]. Due to their extremely high water content, hydrogels are considered to be the ideal platforms for photosensitizer delivery [33]. Chang et al. [34] have reported that a Carbopol 971P formulation with ALA resulted in a satisfactory PDT effect. In addition, Tween 80 was added as a penetration enhancer. Nawaz et al. [32] investigated the effect of almond oil and Tween 80 on the permeation of Clotrimazole in vitro. And the study reported that Tween 80 is considered as an outstanding non-ionic surfactant for its safe and increasing drug penetration [20].

Many reports supported that TBO solution applied in PDT showed satisfactory antibacterial activity in vitro [30, 35], but no studies reported about formulation of TBO applied on the skin disease. Our previous research showed that TBO hydrogel (A water mixture of TBO and gel) had better therapeutic effect than TBO solution and prolonged the retention time of photosensitizer (TBO) at the lesion site [36]; in this study, Tween 80 as penetration enhancer and ethanol aqueous solution as solvent were added to develop an improved TBO hydrogel formulation for curing acne. It would change the current single series of photosensitizers in the field of PDT applied to acne. The optimization of TBO hydrogel formulation was performed by response surface methodology (RSM). Antibacterial experiments were performed to verify the optimal TBO hydrogel by inactivating *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*) and *Propionibacterium acnes* (*P. acnes*). The in vitro release profile of TBO, stability study, and effectiveness test of the optimized hydrogel formulation was measured.

Materials and methods

Materials

Four different kinds of carbomer including carbomer 934, carbomer 941, carbomer 940, and carbomer 934P were bought from J&L® (Shanghai, China). Isotretinoin Erythromycin Gel was bought from Sinopharm (China). Toluidine blue O was gained from J&K®. Tween 80 was purchased from Aladdin Chemistry Co., Ltd. Microbial culture media lysogeny broth (LB), reinforced clostridial agar (RCA), brain heart infusion broth (BHI), and agar were purchased from Sinopharm Chemical Reagent Co., Ltd. *Propionibacterium acnes* (ATCC 6919) was obtained from BNCC (Subei, China); *Staphylococcus aureus* (CMCC 26003) and *Escherichia coli* (ATCC 25922) were supplied by the Institute of Microbiology (Zhejiang University of Technology). Moreover, other chemicals were analytical reagent grade. The light source (CMC Dental, in Denmark) applied in the study is a kind of diode laser with an effective wavelength of 630 nm, which is equipped a perio tip 23 mm. Its output power and maximum output intensity were 5 W and 4 mW/cm^2, respectively.

The process of preparing TBO gel

The dry carbomer powder was dispersed in ethanol solution. Then, TBO and Tween 80 were incorporated into the gel. Finally, NaOH aqueous solution was added then mixed completely to pH 6.0–8.0. Before the further experiments, TBO gels were allowed to stand for 48 h in the dark condition. To optimize the formulation, TBO hydrogels were prepared on the basis of these parameters: types of gel (carbomer 934P, carbomer 934, carbomer 940, carbomer 941), the concentration of carbomer (0.1–0.9% *w*/*v*), the concentration of TBO (0.01–0.7 mg/mL), ethanol solution (0.5–9% *v*/*v*), Tween 80 (0.5–7% *v*/*v*), the mass ratio of NaOH to carbomer (0.2–0.4). The total volume of every sample was 15 mL.

Growth conditions of aerobic bacteria and anaerobic bacteria

S. aureus and *E. coli* were incubated in LB medium under aerobic condition at 37 ± 0.5 °C for 18 h. *P. acnes* was incubated in BHI medium under anaerobic condition at 37 ± 0.5 °C for 48 h. Subsequently, the bacterial culture was allowed to centrifuge at 4000 rpm for 5 min, removing supernatant and sterile phosphate buffer solution (PBS, pH 7.4) that

was added into deposit for resuspension; the final concentration of bacterial suspensions was 1.5×10^8 viable cell mL^{-1}. Prior to experiment, the number of bacteria in PBS (pH 7.4) was determined at 600 nm and the optimal density was 0.31.

Photodynamic therapy

In all experiments, 100 μL of bacterial suspension (1.5×10^8 viable cell mL^{-1}) was added to 96-well microtiter plate. Afterward, 100 μL of TBO hydrogels was added to the sample groups (PDT with TBO hydrogel). PBS of 100 μL was added into bacterial suspension in the negative control (NC) group. TBO of 0.1 mg/mL (dissolve in ethanol aqueous solution) was added into bacterial suspension in the positive control (PC) group. Then, the 96-well microtiter plates were allowed to shake for 15 min in the dark. In all PDT groups, laser irradiation (630 nm) was illuminated continuously for 30 s. In order to avoid light effecting the nearby wells, sterile black lid was covered on each well in the experiments. And 96-well microtiter plate was kept in the dark for 10 min. Then, PBS (pH 7.4) was added to bacterial suspensions to serially diluted. For evaluating antibacterial activity, 100 μL of different dilution was grown on LB medium under aerobic condition at 37 ± 0.5 °C. After 18 h, the number of colonies forming units per milliliter (CFU mL^{-1}) was counted. The results were expressed as log-transformed. All experiments were conducted in triplicate.

Response surface analysis

Response surface methodology (RSM) is a valid data analysis method in various fields [37], especially in pharmaceutical research. Cong et al. [38] reviewed a novel controlled drug delivery system that was optimized by RSM. The three terms (TBO concentration, ethanol concentration, and mass ratio of NaOH to carbomer) showed more significant effect on antibacterial activity according to single factor experiment results. Hence, the concentration of TBO (X_1), the concentration of ethanol solution (X_2), and the mass ratio of NaOH to carbomer (X_3) were selected as independent variables in the following optimization of the TBO hydrogel formulation by RSM. Meanwhile, response variable was the number of colonies forming units per milliliter (CFU mL^{-1}). The related experimental results of independent variables and response variables were analyzed to obtain the second-order polynomial model, which exhibited in the following equation.

$$Y = b_0 \sum_{i=1}^{3} b_i X_i + \sum_{i=1}^{3} b_{ii} X_i^2 + \sum_{i<j=1}^{3} b_{ij} X_i X_j$$

where Y represents the response variable, b_0, b_i, b_{ii}, and b_{ij} mean the intercept, linear, quadratic, and interaction coefficients, respectively. X_i and X_j are the independent variables.

Comparisons of the optimal TBO hydrogel, light, and antibiotics

Antibacterial tests by the TBO hydrogel or light were evaluated by inactivating *S. aureus* and *E. coli.* Meanwhile, the bactericidal effects of PDT with optimal TBO hydrogel were compared with Isotretinoin Erythromycin on *P. acnes.* Subsequently, 100 μL of bacterial suspension (1.5×10^8 viable cell mL^{-1}) was transferred to the sterile well of 96-well plate, and 100 μL of the optimal TBO hydrogel was added in the PDT groups and TBO hydrogel groups (without light). In the control groups and light group, equivalent volume of PBS was added. In the antibiotic group, 100 μL of the Isotretinoin Erythromycin Gel was added. For the photosensitizer and antibiotic to penetrate fully into the bacteria cells, the 96-well microtiter plates were allowed to shake for 15 min in the dark. Both in PDT and light groups, laser irradiation (630 nm) was illuminated continuously for 40 s. In order to avoid light effecting the nearby wells, sterile black lid was covered on each well in the experiments. And the 96-well microtiter plates were kept in the dark for 10 min. Then, the suspensions were added PBS (pH 7.4) to obtain a serially multiple of dilution. In order to evaluate antibacterial activity, 100 μL of each dilution was grown on LB medium under aerobic condition at 37 ± 0.5 °C for 18 h or reinforced clostridial agar medium under anaerobic condition at 37 ± 0.5 °C for 48 h. Then, the number of CFU mL^{-1} was measured. The results were expressed as log-transformed. All experiments were conducted in triplicate.

TBO in vitro release studies

TBO release from the optimized TBO hydrogel formulation was measured using the Franz diffusion cell. Its effective diffusion area was 4.9 cm^2. Regenerated cellulose dialysis membrane was placed between the donor and receptor compartment. Then, PBS (pH 7.4) was applied on the receptor compartment. The experiment was carried out at 37 °C, the diffusion cell was put statically within a water bath to keep receptor medium and constantly stirred at 300 rpm. One milliliter optimal hydrogel was replaced to the donor compartment; 200 μL of samples in the receptor compartment were collected in certain time intervals. Afterward, the content of TBO in samples was measured at 630 nm through spectrophotometer. Meanwhile, 200 μL of fresh PBS (pH 7.4) was added into the receptor compartment. All experiments were evaluated in triplicate.

Stability and effectiveness of the optimal TBO hydrogel formulation in storage

MCR 302 was used to evaluate the rheological of the optimized hydrogel. Gap was 0.5 mm and the apparent viscosity was gained by the progressive increase (from 0.1 to 60.0 1/s) in the shear rate at a 25 ± 2 °C. Subsequently, the apparent

viscosity was plotted against the shear rate. The optimal TBO hydrogels were put into brown containers and stored at three different temperatures (4 ± 2 °C, 25 ± 2 °C, and 40 ± 2 °C) for 6 weeks, visually inspected every week for the color, homogeneity, consistency, and phase separation during the storage. Meanwhile, the pH of optimal hydrogels stored in different temperature were determined using a previously calibrated pH meter (PHS-3G, China). Afterward, their apparent viscosity was measured by MCR 302. The antibacterial activity of TBO hydrogel stored in three different temperatures was performed by calculating CFU mL^{-1}. All the experiments continued 6 weeks and were evaluated in triplicate.

Data analysis

All experiments were repeated three times. Results were shown as the mean ± SD (standard deviation). The data was subjected to analysis of variance (ANOVA), and Student's *t* test was performed to examine the statistical significance of the measurements. When the *p* value was less than 0.05, the measured data was considered to be statistically significant.

Results

Determination of carbomer types based on the antibacterial activity

Antibacterial activity by PDT of the formulation using the four types of carbomer (carbomer 934, carbomer 940, carbomer 941, and carbomer 934P) is shown in Fig. 1a. All TBO hydrogel formulations by PDT promoted an effective reduction in the bacterial number of *S. aureus* compared with the negative control (NC) ($p < 0.05$), but they showed slight reduction for the CFU mL^{-1} of *E. coli*. TBO hydrogel formulations also showed that carbomer 934 and carbomer 940 reduced more the CFU mL^{-1} than the other groups. Because higher viscosity of carbomer 934 is favorable to improve the viscosity of the formulation, carbomer 934 would be used for the base of the further formulation.

The influence of carbomer concentration

Figure 1b shows that the 0.1% (*w*/*v*) or 0.3% (*w*/*v*) carbomer 934 reduced effectively the CFU mL^{-1} of *E. coli* and *S. aureus* compared with the negative control (NC) ($p < 0.05$). However, the 0.1% (*w*/*v*) or 0.3% (*w*/*v*) carbomer 934 with the low viscosity were not suitable for clinical therapeutics. In addition, the CFU mL^{-1} of *S. aureus* and *E. coli* were also increased with the increase of carbomer 934 concentration, which might be the higher viscosity of hydrogel obstructed the diffusion of TBO and decrease the production of ROS. According to the antibacterial activity and enough apparent viscosity values, 0.5% (*w*/*v*) carbomer 934 was used to the further tests.

The influence of Tween 80 concentration

Tween 80 as a kind of effective penetration enhancers could provide a successful mean for achieving a desirable percutaneous absorption of hydrogel molecule. The influence of Tween 80 concentration on its antibacterial activity is shown in Fig. 1c; 0.5% (*v*/*v*) proportion of Tween 80 showed most effective antibacterial activity (compared with NC and PC) ($p < 0.05$). The CFU mL^{-1} of *S. aureus* and *E. coli* was also increasing slightly with increased concentration of Tween 80, because the higher concentration of Tween 80 could make the drug difficult to diffuse out from the hydrogel. Meanwhile, the previous studies [20] showed that Tween 80 could be used to improve the penetration of the TBO. Therefore, 0.5% (*v*/*v*) was used for the further experiments as the optimum concentration of Tween 80.

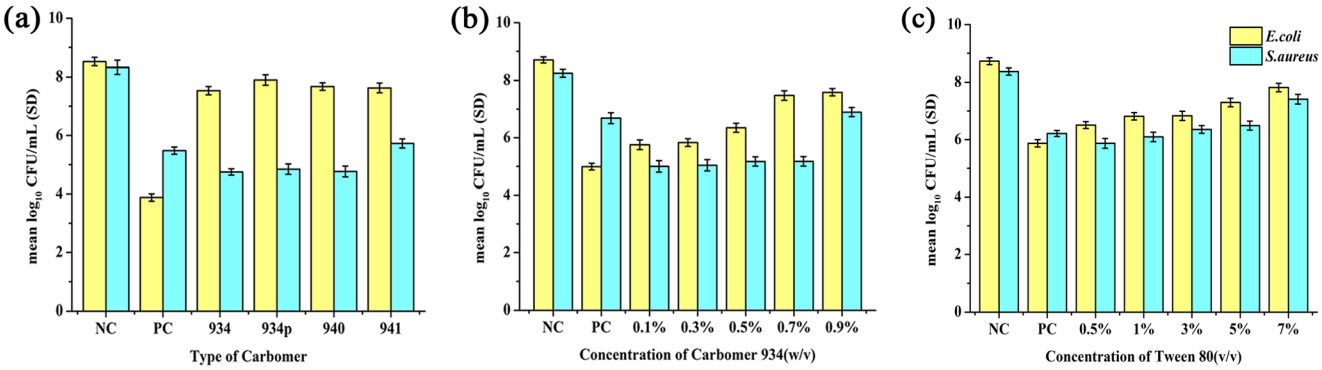

Fig. 1 In vitro antibacterial effects of single-factor. **a** Four types of carbomer concentration of 0.5% (*w*/*v*), Tween 80 concentration of 5% (*v*/*v*), TBO concentration of 1 mg/mL, ethanol concentration of 5% (*v*/*v*), the mass ratio of NaOH to carbomer of 0.30 and irradiation time of 30 s for the PDT groups and the PC group. **b** The concentration of carbomer 934 from 0.1% (*v*/*v*) to 0.9% (*v*/*v*), Tween 80 concentration of 5% (*v*/*v*), TBO concentration of 1 mg/mL, ethanol concentration of 5% (*v*/*v*), the mass ratio of NaOH to carbomer of 0.30 and irradiation time of 30 s for the PDT groups and the PC group. **c** The concentration of Tween 80 from 0.5% (*v*/*v*) to 7% (*v*/*v*), carbomer concentration of 0.5% (*w*/*v*), TBO concentration of 1 mg/mL, ethanol concentration of 5% (*v*/*v*), the mass ratio of NaOH to carbomer of 0.30 and irradiation time of 30 s for the PDT groups and the PC group

The influence of TBO concentration

The concentration of photosensitizer affected significantly the treatment photodynamic therapy. Antibacterial activity of the formulation with different TBO concentration (compared with NC and PC) is shown in Fig. 2a; the CFU mL^{-1} of *S. aureus* and *E. coli* apparently increased when the TBO concentration raised, which might be that the absorbed energy per unit mass of TBO was reduced and led to the reduction of ROS or fluorescence quenching effects that led to the reduction of ROS. According to economic benefits and antibacterial activity, 0.01 mg/mL was used for the further experiments as the optimum TBO concentration. The optimization formulations by the RSM and the TBO concentration of 0.010, 0.055, and 0.100 mg/mL were applied in the further tests.

The influence of the ethanol concentration

The antibacterial activity of PDT with TBO hydrogel was observed in different concentration of ethanol in Fig. 2b, and a significant microbial reduction was observed compared to NC and PC ($p < 0.05$) when the ethanol concentration solution was 0.50% or 1.0% (*v*/*v*). Moreover, *E. coli* seemed to be more sensitive to the 0.5% and 1.0% ethanol solution. Therefore, the ethanol concentration of 0.50%, 4.75%, and 9.0% (*v*/*v*) were used for further tests of RSM.

The influence of the mass ratio of NaOH to carbomer

To evaluate the effect of pH on antimicrobial activity of the formulation, the different mass ratio of NaOH to carbomer (0.2, 0.25, 0.3, 0.35, 0.4) was investigated (Fig. 2c) that demonstrated that both *S. aureus* and *E. coli* were sensitive to the

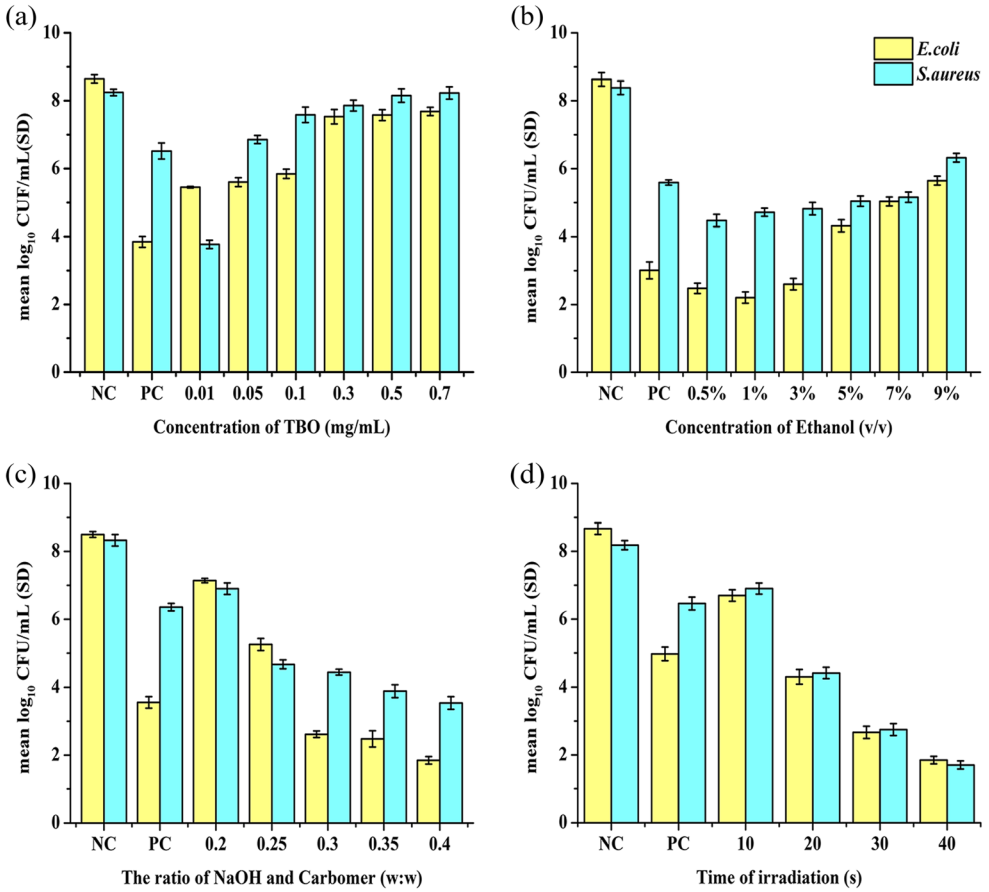

Fig. 2 **a** The concentration of TBO from 0.01 to 0.7 mg/mL, carbomer concentration of 0.5% (*v*/*v*), Tween 80 concentration of 0.5% (*v*/*v*), ethanol concentration of 5% (*v*/*v*), the mass ratio of NaOH to carbomer of 0.30 and irradiation time of 30 s for the PDT groups and the PC group. **b** The concentration of ethanol from 0.5% (*v*/*v*) to 9% (*v*/*v*), Tween 80 concentration of 0.5% (*v*/*v*), TBO concentration of 0.01 mg/mL, carbomer concentration of 0.5% (*v*/*v*), the mass ratio of NaOH to carbomer of 0.30 and irradiation time of 30 s for the PDT group and the PC group. **c** The mass ratio of NaOH to carbomer from 0.20 (*w*/*v*) to 0.40 (*w*/*v*), carbomer concentration of 0.5% (*w*/*v*), TBO concentration of 0.01 mg/mL, Tween 80 concentration of 0.5% (*v*/*v*), ethanol concentration of 1% (*v*/*v*) and irradiation time of 30 s for the PDT groups and the PC. **d** The irradiation time from 10 s to 40 s, carbomer concentration of 0.5% (*w*/*v*), TBO concentration of 0.01 mg/mL, Tween 80 concentration of 0.5% (*v*/*v*), ethanol concentration of 1% (*v*/*v*), the mass ratio of NaOH to carbomer of 0.4 and irradiation time of 30 s for the PC group

change of the mass ratio of NaOH to carbomer in the formulation (compared with NC and PC), and the CFU mL^{-1} of *S. aureus* and *E. coli* had a pronounced effect when the ratio of 0.4 (specifically for *E. coli*) ($p < 0.001$). Hence, a ratio of 0.4 was used to further study, and a ratio of 0.2, 0.3, and 0.4 was used for further tests of the RSM.

The influence of irradiation time

To evaluate the influence of the irradiation time on antimicrobial activity of the formulation, the different irradiation time of PDT was investigated (Fig. 2d) that showed that the antimicrobial activity of the irradiation 40 s by PDT was the best effect ($p < 0.001$). These results showed the long-time irradiation favored improving antimicrobial activity of the formulation, but pain in clinic under too long-time irradiation limited the use of PDT. Therefore, 40 s was used for the further experiments.

Response results

The TBO hydrogel formulation in this study was the topical semi-solid dosage form according to the International Pharmacopoeia (Seventh Edition, 2017). The TBO hydrogel formulation was different from the other drugs semi-solid dosage forms; many factors might influence the production and half-life of the singlet oxygen from TBO and further affect the antibacterial activity of the TBO hydrogel formulation by PDT, so we chose the antibacterial activity as main indicator in our optimization process. Experimental design was performed by Box-Behnken design of RSM, and 17 experiments were designed and executed about the concentration of ethanol, the concentration of TBO, and the mass ratio of NaOH to carbomer, which has established models. In order to evaluate the accuracy of the quadratic (2nd degree) model, the analysis of variance (ANOVA) was used. For the optimal hydrogel formulations, the results of CFU mL^{-1} in the design of experiments are shown in Table 1; the responses were determined using variable limit values for the experimental design. The experiment results analyzed by the multiple quadratic regression; the data were correlated using the second-order polynomial equation function.

$$CFU_{S.aureus} = 1.56388 + 12.33206X_1 + 99.05934X_2 - 4.49073X_3 - 87.01411X_1X_2 + 17.50859X_1X_3 + 145.01290X_2X_3 - 7.70354{X_1}^2 - 617.20252{X_2}^2 - 35.36893{X_3}^2$$

$$CFU_{E.coli} = 5.88723 + 14.33068X_1 + 64.45374X_2 - 22.15333X_3 - 113.93580X_1X_2 + 13.90065X_1X_3 + 231.47389X_2X_3 - 8.34886{X_1}^2 - 457.09192{X_2}^2 - 11.02368{X_3}^2$$

According to the result of antibacterial activity, the effects of the ethanol concentration, the concentration of TBO, and the mass ratio of NaOH to carbomer are shown in Fig. 3, which were useful to determine both the main and interaction effects of the factors on the responses at one time. Three statistical evaluation functions were measured in order to assess the reliability of the model; the analysis of variance, F-test, and p value are presented in Table 2. The p value and F-value of *E. coli* (*S. aureus*) were 0.0003 (< 0.0013) and 20.68 (13.18), so the model was adequate statistical significance and applicable to optimize the hydrogel formulations. The optimized formulation was composed of 0.01 mg/mL TBO concentration, 0.5% ethanol, and the mass ratio of NaOH to carbomer of 0.4. Specifically, the rank order of standardized coefficient was $X_3 > X_1 > X_2$, the results indicated that sequence of influence of three factors on yield was the mass ratio of NaOH to carbomer, the TBO concentration and the concentration of ethanol. From Table 2, the value of determination coefficients (R^2) of this model for *S. aureus* (*E. coli*) was 0.94 (0.96), respectively, so the results implied that the model was precise ($P < 0.05$).

Verification tests

In order to verify the predictive value from RSM analysis, the verification test was designed with the optimal hydrogel formulations as follow: TBO concentration of 0.01 mg/mL, ethanol concentration of 0.5% (*v*/*v*), carbomer concentration of 0.5% (*w*/*v*), the proportion of Tween 80 of 0.5% (*v*/*v*), and the mass ratio of NaOH to carbomer of 0.4 (*w*/*w*). The log-transformed of CFU mL^{-1} of *S. aureus* (*E. coli*) was 0.69 (0.70), which was close to predicted value of 1.90 (0.30) (Fig. 4b). Hence, the model was reliable and reasonable. All experiments were evaluated in triplicate.

Comparisons of the optimal TBO hydrogel, light, and antibiotics

To verify the effectiveness of the optimal TBO hydrogel formulation, antibacterial test of the optimal TBO hydrogel, antibiotics, or light was tested (Fig. 4b, c). The number of *S. aureus* and *E. coli* was performed with the optimal TBO hydrogel alone or light (630 nm) alone did slightly affect the microbial viability, and a significant antibacterial activity was observed when TBO hydrogel was irradiated by 630 nm light (PDT). In addition, PDT groups showed 2.13 log10 reduction in the CFU/mL of *P. acne* (Fig. 4c). Similar researches were also obtained by the application of PDT regimes causing a 2–3 log decrease in the bacterial population [39]. Meanwhile, PDT treatment is considered as a kind of effective therapy for acne vulgaris.

TBO in vitro release studies

The release experiment was carried out to evaluate the release of TBO from TBO hydrogel, and the results are shown in the

Table 1 Experimental parameters of Box-Benhnken design and the antibacterial activity in photodynamic therapy

Run	Factor			Antibacterial activity (log_{10} CFU mL^{-1})	
	[a]X_1 (% mg/mL)	[a]X_2 (% *v*/*v*)	[a]X_3 (*w*/*w*)	*S. aureus*	*E. coli*
1	0.100	9.00	0.30	5.47 ± 0.10	7.48 ± 0.19
2	0.055	9.00	0.40	5.12 ± 0.10	5.01 ± 0.06
3	0.055	4.75	0.30	6.47 ± 0.12	6.71 ± 0.11
4	0.055	4.75	0.30	6.47 ± 0.20	6.53 ± 0.19
5	0.055	4.75	0.30	6.46 ± 0.15	6.28 ± 0.16
6	0.055	4.75	0.30	6.49 ± 0.18	7.33 ± 0.14
7	0.010	4.75	0.40	1.47 ± 0.13	3.21 ± 0.13
8	0.010	9.00	0.20	7.75 ± 0.20	7.20 ± 0.26
9	0.010	4.75	0.30	2.77 ± 0.12	6.93 ± 0.24
10	0.100	0.50	0.20	6.69 ± 0.10	8.01 ± 0.15
11	0.010	0.50	0.30	2.47 ± 0.12	3.32 ± 0.17
12	0.055	9.00	0.20	6.50 ± 0.12	8.02 ± 0.17
13	0.100	0.50	0.30	6.25 ± 0.12	6.64 ± 0.18
14	0.055	4.75	0.30	6.46 ± 0.14	6.71 ± 0.15
15	0.055	0.50	0.20	7.69 ± 0.17	7.04 ± 0.11
16	0.055	0.50	0.40	5.56 ± 0.21	1.70 ± 0.22
17	0.100	4.75	0.40	6.00 ± 0.16	2.70 ± 0.19

[a] X_1, [a] X_2, and [a] X_3 represent the concentration of TBO, the concentration of ethanol, and the quality ratio of NaOH and carbomer, respectively

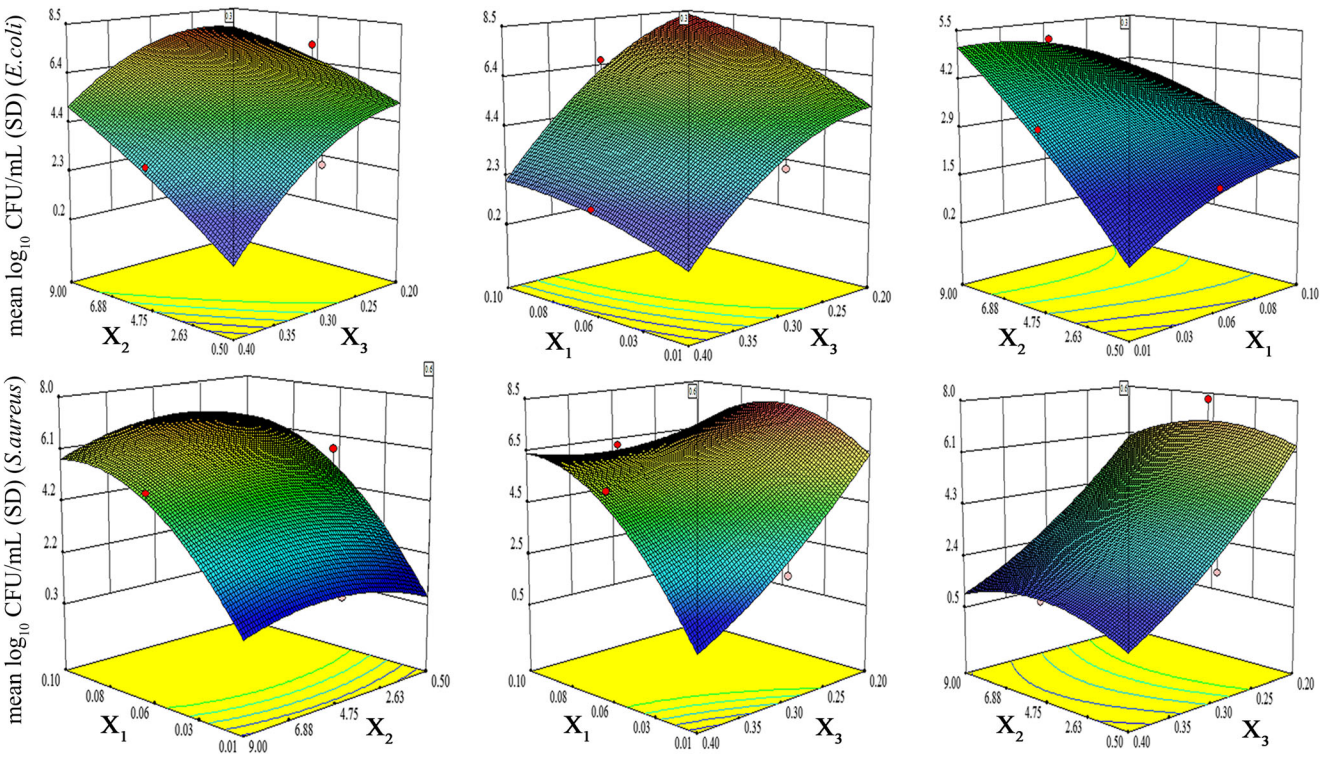

Fig. 3 Response surface for the interactions of independent variables on antibacterial activity (X_1 for TBO concentration; X_2 for ethanol concentration; X_3 for the quality ratio of NaOH and carbomer)

Table 2 ANOVA for responses of the fitted second-order polynomial model for *S. aureus* and *E. coli*

Source	Y_1 (*S. aureus*)			Y_2 (*E. coli*)		
	F value	*p* value Prob > F		F value	*p* value Prob > F	
Model	13.18	0.0012	Significant	20.68	0.0003	Significant
$^aX_1$	30.77	0.0009		6.85	0.0345	
$^aX_2$	1.39	0.2772		29.36	0.0010	
$^aX_3$	34.22	0.0006		120.71	< 0.0001	
X_1X_2	0.73	0.4223		5.96	0.0447	
X_1X_3	19.48	0.0031		1.47	0.2642	
X_2X_3	0.34	0.5786		4.19	0.0798	
X_1^2	23.12	0.0019		1.89	0.2120	
X_2^2	5.81	0.0468		0.73	0.4210	
X_3^2	2.53	0.1560		13.84	0.0075	
R^2			0.94	0.96		

$^aX_1$, $^aX_2$, and $^aX_3$ represent the concentration of TBO, the concentration of ethanol, and the quality ratio of NaOH and carbomer, respectively. Y_1 and Y_2 are *S. aureus* and *E. coli* antibacterial activity ($\log_{10}$ CFU mL^{-1})

Fig. 5a. The TBO release kept stable basically after 7 h (after 8 h was 61.8%).

Stability and effectiveness examination of optimal TBO hydrogel formulation

The results in Fig. 4a showed that the optimal hydrogel had adequate rheological property (pseudoplastic behavior), which presented the decreased viscosity with increasing shear rate and facilitating the spreading of hydrogel over tissue. The pH values and the rheology of the optimal TBO hydrogel were investigated at 4 ± 5 °C, 25 ± 5 °C, and 40 ± 5 °C for 6 weeks. Figure 5b shows that the pH of TBO hydrogels were also stable at a range of 7.4–7.9. The viscosity of TBO hydrogels was no obvious changes at three different temperatures (data not shown). Furthermore, the appearance properties of all the optimized TBO hydrogels kept stable under different temperatures. As we could find in the Fig. 5c, d, it also showed that the antibacterial activity of PDT with the optimal TBO hydrogels was sustainably significant effect within different temperatures during 6 weeks.

Discussion

In recent years, PDT has been emerged as a novel therapeutic method in treating many diseases. Under the dermatology, it played a vital role in curing acne. Its effectiveness was attributed to the direct destruction mediated by reactive oxygen species (ROS), even reducing the hyperkeratosis and the formation of acne precipitating factor [16]. As the development of photosensitizers and applied in acnes or other skin treatments increases, TBO as a kind of phenothiazinium photosensitizers which give a reasonable yield of singlet oxygen has already been demonstrating its potential as an antimicrobial therapy [28–31]. Moreover, the effect of PDT using TBO inhibited both gram-positive and gram-negative bacteria [27].

Our previous research reported that TBO hydrogel (a water mixture of TBO and gel) had better therapeutic effect than TBO solution and prolonged the retention time of photosensitizer (TBO) at the lesion site [36]. Tween 80 as penetration enhancer and ethanol aqueous as solvent were added to our previous TBO hydrogel to optimize the new formulation; the new TBO hydrogel formulation in this study showed improved properties.

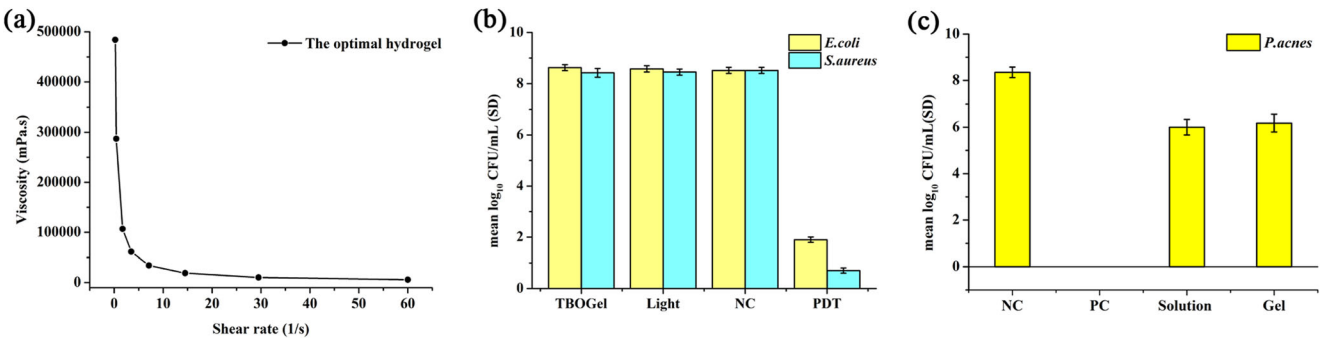

Fig. 4 **a** Apparent viscosity values as a function of shear rate. **b** Antibacterial activity of the optimized TBO hydrogel with light, the optimized TBO hydrogel alone and light alone. NC represents negative control. **c** Antibacterial activity of the optimized TBO hydrogel with light. PC represents antibiotic group (positive control)

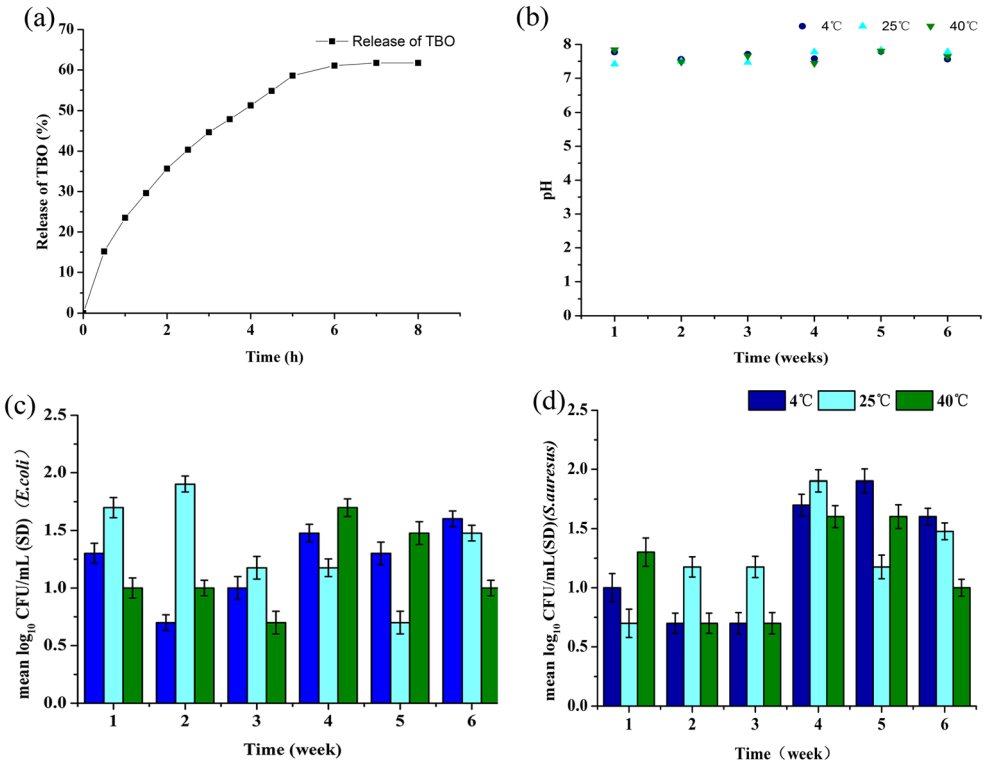

Fig. 5 **a** Amount of TBO released from the optimized TBO hydrogel. **b** Influence of temperature on pH of the optimized TBO hydrogel during 6 weeks. **c** Influence of temperature on antibacterial activity of the optimized TBO hydrogel during 6 weeks (*E. coli*). **d** Influence of temperature on antibacterial activity of the optimized TBO hydrogel during 6 weeks (*S. aureus*)

Our results suggested that TBO as an appropriate photosensitizer could be used for photodynamic therapy of dermatology, and TBO dissolved in different concentration of ethanol showed the different antibacterial activity, which is consistent with the previous researches [40, 41]. The TBO dissolved in ethanol can influence the production and half-life of the singlet oxygen. Prochnow et al. [40] observed PSs dissolved in ethanol aqueous solution improved the antibacterial activity in PDT. Due to increase in the half-life, it could provide more time for ROS to interact with bacteria. Pillusky et al. [41] demonstrated that PDT with methylene blue dissolved in ethanol aqueous solution showed better therapeutic effect than that dissolved in aqueous solution.

Carbomer was used as a hydrogel matrix in our study. Carbomer as drug carrier system has been extensively studied for topical delivery, since it has the no toxicity and no drug resistance into the skin. It has been reported that the carbomer used as a gelling agent was able to deliver photosensitizer (PS) into the stratum corneum or deeper skin layers. One example of carbomer hydrogel-based was provided by Dragicevic-Curic et al. [42], who developed a topical mTHPC-loaded liposomal hydrogel able to deliver mTHPC.

To determine the efficacy of single factors, antibacterial activity was performed to evaluate the effect in the experiments. The optimal formulation results obtained were successfully designed with RSM, which had greatly verified the single-factor experiment. As shown above, the optimal TBO hydrogel formulation was 0.5% (*w*/*v*) CP 934, TBO concentration of 0.01 mg/mL, ethanol concentration of 0.5% (*v*/*v*), the proportion of Tween 80 of 0.5% (*v*/*v*), and the mass ratio of NaOH to carbomer of 0.4 (*w*/*w*). The results (Fig. 4a) demonstrated that the optimal TBO hydrogel showed suitable rheological properties. Moreover, the efficacy of this hydrogel with PDT was evaluated and the results are shown in Fig. 4b. *S. aureus* and *E. coli* were inhibited significantly, which suggested a strong antibacterial activity effect (bacterial number of *S. aureus* and *E. coli* was reduced by 7.82 log10 and 6.70 log10, respectively). The CFU mL^{-1} of *P. acnes* on the optimal formulation and antibiotic were 6.17 and 0, respectively (Fig. 4c). Over the years, antibiotics remain the major therapeutic strategies in the clinic of acne vulgaris now [3]. In this study, although antibiotic showed the strong antibacterial activity compared with the TBO hydrogel, the antibiotic resistance and severe side effect limited the use of antibiotics.

The accelerated stability tests of gels play the key role in pharmaceutical product market. Furthermore, hydrogel should be easily applied onto the skin and be stable along the time. The stability tests from the TBO hydrogel showed that pH, antibacterial activity, and viscosity kept stable for 6 weeks. Obtained results were similar with other similar studies using carbomer hydrogels [43].

In topical skin treatment, photosensitizers are diffused through the skin barrier. Hence, a good release of TBO in the hydrogel is essential for a successful PDT treatment. In the vitro TBO release studies, the optimal hydrogels showed the release of the PS after 4 h about 51.28% (Fig. 5a) and after 24 h about 69.80% (data not shown). In this study, in vitro release rate of the hydrogel containing Tween 80 was faster

than that in the previous research [36]. PDT as a fast clinic therapy, improving release rate of photosensitizer is beneficial to increase the therapeutic effect. Therefore, it could promote the penetration of TBO in skin.

Conclusions

The new developed TBO hydrogel for PDT in this paper could be a promising treatment against acnes. The optimal TBO hydrogel was 0.5% (*w*/*v*) carbomer 934, TBO concentration of 0.01 mg/mL, ethanol concentration of 0.5% (*v*/*v*), the proportion of Tween 80 of 0.5% (*v*/*v*), and the mass ratio of NaOH to carbomer of 0.4 (*w*/*w*). The properties of TBO hydrogel, such as appearance, clarity, viscosity, antibacterial activity, and pH, were generally stable at 4 °C, 25 °C, and 40 °C within 6 months. It also showed effective antibacterial activity for *P. acnes*, *S. aureus*, and *E. coli.* All the above results supported the novel TBO hydrogels that were feasible for acne treatment, and further studies on cell toxicity and animal studies would be done.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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