Photocurable Composite with Antimicrobial Activity for Wound-Healing

Ming-Hsiang Chang, Fan-Chun Ding, Wen-Yu Ju and Ping-Shan Lai*

Department of Chemistry, National Chung Hsing University
No. 250, Kuo-Kuang Road, Taichung 402, Taiwan, pslai@email.nchu.edu.tw

ABSTRACT

Wound infection is the process of colonization of replicating microorganisms within a wound that may extend the duration of wound-healing and even cause systemic infections such as sepsis or death. To develop an appropriate approach that can accelerate the wound healing rate or prevent other microorganisms contamination is necessary for wound healing. In this study, the combination of antimicrobial activity and photodynamic antimicrobial chemotherapy of photocurable wound dressing for wound healing were evaluated in *Staphylococcus aureus* infected wound. The wound dressing with photocurable gelatin /chitosan/methylene blue mixed solution could be formed fast after UV light irradiation. A better appearance of wounds on the dorsal of mice could be observed on photocruable wound dressing group after 13 days. These results suggested that the photocurable composite may not only exhibit good antimicrobial activity but show the potential to be an ideal wound dressing for wound healing.

Keywords: photodynamic antimicrobial chemotherapy, wound healing, chitosan, gelatin, methylene blue

1 INTRODUCTION

During the wound therapy, bacterial infection and wound sepsis are the major clinical problems which causes to mortality and morbidity in patients [1]. To alleviate these problems, antimicrobial agents can be used to destroy the normal function of bacterial proteins or damage the integrity of cell membranes and therefore it may lead to bacterial growth inhibition and cell death [2-5].

Natural heteropolysaccharide chitosan act as a good biomaterial with biodegradable and great biocompatible properties for medical and antibacterial applications [6-7]. Chitosan with its positive charge shows antibacterial mechanism that can disturb the permeability of cell walls and can form a stable complex with DNA after cell intrernalization then inhibiting RNA biosynthesis and reducing cell viability [8-10].

A strategy based on photodynamic therapy (PDT) named photodynamic antimicrobial chemotherapy (PACT) was investigated for topical antimicroorganism activity [11]. PDT is a photochemical process which is used to generate localized cell death involves the activation of a photosensitizing drug in the target area with light in the presence of molecular oxygen [12]. For wound therapy, PACT has been demonstrated successfully to kill various microbes and rapidly control topical wound infection in vivo [13-14] .

Thus, in this study PACT was used in combination with chitosan in topical wounds acting for antibacterial therapy. In addition to, a rapid wound dressing which can provide a moist environment leading to rapid granulation and re-epithelialization [15]. After UV irradiation, GE-BTHE (a modified liquid-phase gelatin (see materials)) can cross-link with poly(ethylene glycol) diacrylate rapidly [16]. The fast-film-forming property also can be served as a good wound dressing. In this study, a chitosan/photosensitizer GE-BTHE photocurable matrix as a rapid wound dressing material for wound-healing in *Staphylococcus aureus* infected wound was evaluated.

2 MATERIAL AND METHOD

2.1 Materals

Gelatin (food grade, type A, 225 Bloom, Mw = 50000), poly(ethylene glycol) diacrylate (PEGDA ave. Mn=700), chitosan (75-85% deacetylated, Mw=100000) and methylene blue (MB) were obtained from Sigma-Aldrich (St. Louis, MO,USA), 2-hydroxyethyl methacrylate (HEMA 96%, N,N'-dicyclohexyl-carbodiimide (DCC 99%), 4-dimethylamino- pyridine (DMAP 99%), 3,3',4,4'-benzophenone tetracarboxylic dianhydride (BTDA 98.5%, Mw = 322.23), methanol (99.5%), and acetone (98%) were purchased from ACROS Organics (Fair Lawn, NJ, USA). DPBS was purchased from Invitrogen© (Carlsbad, CA, USA). All chemicals are reagent grade and used without further purification unless otherwise noted. GE-BTHE was synthesized as described in detailed in our previous report [16].

2.2 *In Vitro* Colony Assay

To evaluate the antibacterial activity of each material, *Staphylococcus aureus* 71 (*S. aureus*; ~10^9 cells/mL) was incubated with various concentration of chitosan, GE-BTHE or MB and all the procedures were carried out in subdued light. For MB mediated PACT efficacy evaluation, MB groups were divided into irradiated and non-irradiated group. In irradiated group, *S. aureus* were exposed to light irradiation (660 nm, 19.5 mW) for 15 minutes. After ligth exposure, irradiated samples diluted serially, transferred to nutrient agar plates and then cultured at 37 ^{o}C for 24 hours for CFU (colony-forming unit) observation. All experiments were carried out in triplicate.

GE-BTHE		Chitosan		MB(L+)		MB(L-)	
Conc. (μg/mL)	Viable count (CFU/mL)	Conc. (mg/mL)	Viable count (CFU/mL)	Conc. (μg/mL)	Viable count (CFU/mL)	Conc. (μg/mL)	Viable count (CFU/mL)
4000	5.16×10^8	3.3	3.30×10^6	8	6.75×10^8	8	8.42×10^8
1600	7.78×10^8	0.33	1.00×10^6	4	6.67×10^8	4	8.89×10^8
800	8.32×10^8	0.0033	8.20×10^8	0.08	8.69×10^8	0.08	9.18×10^8

Table 1. Antibacterial efficacy of differing concentrations of MB, chitosan and GE-BTHE for *S. aureus*.

2.3 Preparation of Antibacterial and Photocurable Polymer solution

The photo-curable polymer solution was prepared as a mixture of GE-BTHE/chitosan/MB/PEGDA. Samples of various GE-BTHE, chitosan, MB ratios were prepared for antibacterial studies. For bacteriostasis *in vitro* test, the best ratio based on the optimum antibacterial results of GE-BTHE, chitosan and MB were found 48.19 wt%; 51.63 wt% and 0.18 wt% individually. For *in vivo* study, the photo-curable polymer solution for wound healing was prepared with a ratio of 1.46 wt%; 1.56 wt%; 0.0056 wt%; 92 wt% and 4.48 wt% of GE-BTHE, chitosan, MB, PEGDA and PBS, respectively. After mixing, all of samples were stored at 4 ^{o}C.

2.4 Bacteriostasis *In Vitro*

Inhibition zone analysis was used to examine antimicrobial effect (*S. aureus*) of MB-mediated PACT. According to the disc agar diffusion (DAD) method, 40 μL of photocurable polymer solution (various of GE-BTHE, chitosan, MB ratios) was transferred to a paper disc (8 mm in diameter) then attached to the inoculated agar and cultured at 37 °C for 24 hours. The efficiency of bacteriostasis of the mixed polymer solution *in vitro* was assessed by measuring the diameter of the inhibition zone with caliper. The larger diameter of inhibition zone indicates the stronger bacteriostasis efficacy.

2.5 Wound Dressing (*In Vivo*)

The animal experimental protocols were reviewed and approved by the National Science Council of the Republic of China. Mice weighing 25 g (female, 8–12-weeks-old) were used in this study. After being anesthetized with Balanzine®, three round wounds (about 25 mm^2) were prepared on the dorsum of each mouse using a sharp pair of scissors and tweezers. The photocurable polymer solution (PEGDA, chitosan and MB) was then placed onto each wounds about 20 μL as wound-dressing glue. Wounds were monitored by photo records in 21 days duration.

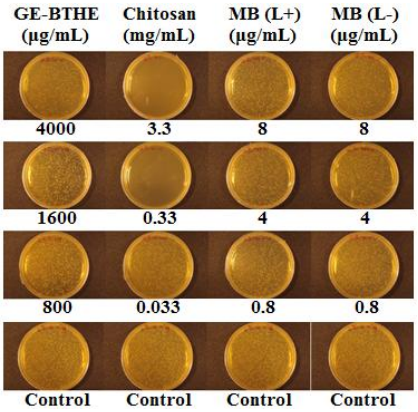

Fig. 1. The toxicity testing of differing concentrations of MB, chitosan and GE-BTHE on S. aureus (~10^9 cfu/mL).

3 RESULTS AND DISSCUSSION

3.1 Antibacterial Effects of Each Reagent

The antibacterial effects of each material in the polymeric matrix including GE-BTHE, chitosan, PEGDA (crosslinker) or MB (photosensitizer) with or without light irradiation were evaluated against *S. aureus* with various concentrations. As shown in Table 1 and Fig. 1 (Bacteria counting assay), a chitosan concentration at 3.3 mg/mL showed a significant inhibitory effect and GE-BTHE exhibited low bacterial inhibition at 0.16% (1600 μg/mL) concentration. But it was slightly toxic towards *S. aureus* when the concentration was increased to 0.4% (4000 μg/mL). It has been reported that chitosan has a higher antibacterial activity which markedly inhibits growth of most bacteria tested. MB-mediated PDT exhibits both the efficient antitumor efficacy and also photo-inactivated activities towards various microorganisms and viruses via ROS generation [14, 17-18]. However, our result showed that MB exhibited less antibacterial activity to *S. aureus* for all concentrations in the test. It may be due to the lower MB concnetration which we used in this study compare to *in vivo* test [19]. Based on the best bacteriostasis results, the concentrations of each component of the photocurable glue for wound dressing were determined as following description: chitosan, 3.3 mg/mL; MB, 8 μg/mL; and GE-BTHE, 0.16% (1600 μg/mL).

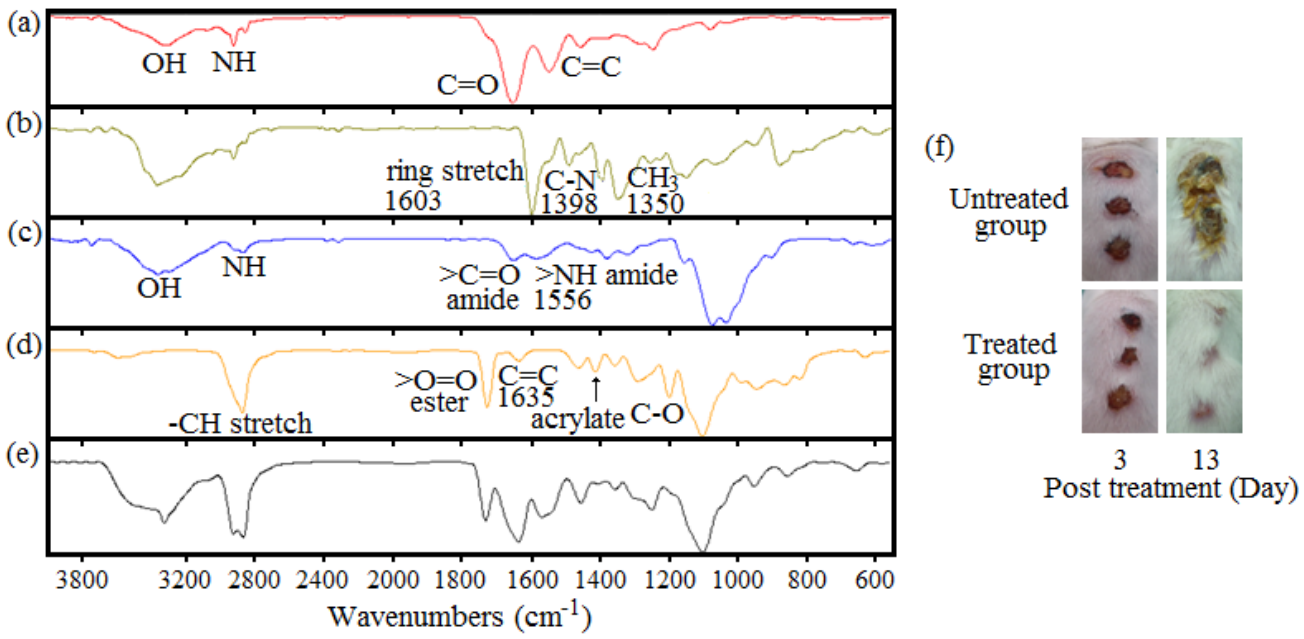

Fig. 2. FT-IR spectra of (a) GE-BTHE, (b) MB, (c) chitosan, (d) PEGDA and (e) the GE-BTHE mixture (including the above components) and macroscopic aspect of (f) wounds without or with photocurable composite-mediated wound dressing.

3.2 Characterization of the Photocurable Polymer Solution

According to the effective concentration of each component as description above, the photocurable polymer solution was prepared as antibacterial wound-dressing material. Fig. 2 shows the ATR-FTIR spectra for analysis of the structural formula of the five materials (GE-BTHE, MB, chitosan, PEGDA and the photocurable glue). Fig. 2(a) indicates the un-irradiated GE-BTHE, showing –OH, –NH, and C=O group absorptions at 3300, 2900, and 1680 cm^{-1}, respectively. The C=C bond stretching shows at 1480 and 1550 cm^{-1}. Fig. 2(b) shows the IR spectra of MB, which exhibits a ring stretch at 1603 cm^{-1}, symmetric stretch of C–N at 1398 cm^{-1} and symmetric deformation of –CH3 at 1354 cm^{-1}. Fig. 2(c) shows the IR absorptions of the –OH and –NH group of chitosan at 3360 cm^{-1} and 2890 cm^{-1}, respectively. The absorption of the amide C=O bond shows at 1650 cm^{-1} and –NH bending at 1556 cm^{-1}. The characteristic peaks for the acrylate groups of PEGDA (i.e., those at about 812 cm^{-1}, 1190 cm^{-1} and 1410 cm^{-1}) shows in Fig. 2(d). The bottom panel Fig. 2(e) shows the ATR-IR spectra of all components, GE-BTHE, MB, chitosan, and PEGDA. The C=C stretching of GE-BTHE at 1480 and 1550 cm^{-1} disappeared owing to cross-linking with PEGDA. The acrylate group (1410 cm^{-1}) of PEGDA also disappeared in the ATR-IR spectra of the GE-BTHE mixture. The specific absorptions of MB (1603 cm^{-1} and 1354 cm^{-1}) and chitosan (1000-1100 cm^{-1}) were not clear observed because the cross-linked structure covering MB and chitosan and both specific absorptions were shielded.

GE-BTHE/chitosan ratio	Bacteriostasis Diameter (mm)
3 / 3	15
1 / 3	14
3 / 1	10

Table 2. Bacteriostasis of *S. aureus* in different chitosan /GE-BTHE ratio.

3.3 Bacteriostasis *In Vitro*: Disc Agar Diffusion Method (DAD)

Tables 2 showed the relationship between the antibacterial effect and the GE-BTHE/chitosan ratio. Caminos et al. mentioned that PDT only can work on the suspension bacteria, because the photosensitizer can't completely conjugate the membrane of bacteria if the bacteria are cultured on agar plates.[20] Also our result showed there was no cellular toxicity toward *S. aureus* up to 8 μg/mL of MB. Owing to these reasons, the bacteriostasis diameter experiment was conducted in the absence of MB in the componet. The bacteriostasis diameter was increased with the increasing amount of chitosan, and GE-BTHE also showed a mild effect, so the increasing GE-BTHE/chitosan ratio caused a slight decrease in the bacteriostasis diameter. This result also exhibited showed good agreement with the result in our colony assay.

3.4 Wound Dressing *In Vivo*

Chitosan and gelatin were most investigated substances for both the artificial skin and the wound dressing. During integrating dressing functionalities, the material needs to be

modified so that it has a better antibacterial property or a faster healing effect. The purpose of adding MB in this study is to kill bacteria and reduce inflammation. In addition, the rapid bactericidal effect will assist the wound to achieve rapid wound healing. If the wound is not healing, it is easy to contract invasive infections caused by bacteria, which leads to complications, potentially life-threatening.

Fig. 2(f) showed a comparison of without and with photocurable composite-mediated wound dressing treatment. The irradiated GE-BTHE mixture can be converted quickly into artificial scabs to protect wounds from infection, whereas an untreated wound forms a scab naturally after about 3–7 days. Trauma can stop plenty of bleeding then form an important scab. Although we did not have any cases of heavy bleeding in this study, but the scab formed sooner and wound can heal itself as well. The most important thing is to stop bleeding by quickly transforming a wound dressing glue into a scab.

4 CONCLUSION

In this study, GE-BTHE mixed with poly(ethylene glycol) diacrylate (PEGDA, a cross-linker) were applied to increase the speed of wound dressing. The GE-BTHE mixture was found to have fast film-forming, antibacterial, bacteria-killing and wound-healing properties. Therefore, GE-BTHE can be transferred as a good dressing support by the UV-light irradiation, which can be combined with the antibacterial chitosan and MB as the photodynamic therapy agent. In the future, the GE-BTHE mixture able to use clinically in the wide range of wound infection and healing situations.

REFERENCES

[1] S. Yendamuri, G.J. Fulda, G.H. Tinkoff, The Journal of Trauma, 55, 33-38, 2003.

[2] H.-L. Su, C.-C. Chou, D.-J. Hung, S.-H. Lin, I.C. Pao, J.-H. Lin, F.-L. Huang, R.-X. Dong, J.-J. Lin, Biomaterials, 30, 5979-5987, 2009.

[3] H. Tang, P. Zhang, T.L. Kieft, S.J. Ryan, S.M. Baker, W.P. Wiesmann, S. Rogelj, Acta Biomaterialia, 6, 2562-2571, 2010.

[4] Y. Dong, X. Li, L. Tian, T. Bell, R.L. Sammons, H. Dong, Acta Biomaterialia, 7, 447-457, 2011.

[5] E.I. Rabea, M.E.T. Badawy, C.V. Stevens, G. Smagghe, W. Steurbaut, Biomacromolecules, 4, 1457-1465, 2003.

[6] Felt O., Carrel A., Baehni P., Buri P., G. R., Journal of Ocular Pharmacology and Therapeutics, 16, 261-270, 2000.

[7] H.K. No, N. Young Park, S. Ho Lee, S.P. Meyers, International Journal of Food Microbiology, 74, 65-72, 2002.

[8] M. Kong, X.G. Chen, K. Xing, H.J. Park, International Journal of Food Microbiology, 144, 51-63, 2010.

[9] M. Jumaa, F.H. Furkert, B.W. Müller, European Journal of Pharmaceutics and Biopharmaceutics, 53, 115-123, 2002.

[10] J. Vinsova and E. Vavrikova, Current Pharmaceutical Design, 14, 1311-1326, 2008.

[11] S. Ferro, G. Jori, S. Sortino, R. Stancanelli, P. Nikolov, G. Tognon, F. Ricchelli, A. Mazzaglia, Biomacromolecules, 10, 2592-2600, 2009.

[12] T.J. Dougherty, C.J. Gomer, B.W. Henderson, G. Jori, D. Kessel, M. Korbelik, J. Moan, Q. Peng, Journal of the National Cancer Institute, 90, 889-905, 1998.

[13] M.R. Hamblin, D.A. O'Donnell, N. Murthy, C.H. Contag, T. Hasan, Photochemistry and Photobiology, 75, 51-57, 2002.

[14] J.P. Tardivo, A. Del Giglio, C.S. de Oliveira, D.S. Gabrielli, H.C. Junqueira, D.B. Tada, D. Severino, R. de Fátima Turchiello, M.S. Baptista, Photodiagnosis and Photodynamic Therapy, 2, 175-191, 2005.

[15] W. Paul, C.P. Sharma, Trends Biomater. Artif. Organs, 18, 6, 2004.

[16] F.-C. Ding, S.-h. Hsu, W.-Y. Chiang, Journal of Applied Polymer Science, 109, 589-596, 2008.

[17] B.B. Noodt, G.H. Rodal, M. Wainwright, Q. Peng, R. Horobin, J.M. Nesland, K. Berg, International Journal of Cancer, 75, 941-948, 1998.

[18] M.N. Usacheva, M.C. Teichert, M.A. Biel, Lasers in Surgery and Medicine, 29, 165-173, 2001.

[19] P. Zolfaghari, S. Packer, M. Singer, S. Nair, J. Bennett, C. Street, M. Wilson, BMC Microbiology, 9, 27, 2009.

[20] D.A. Caminos, M.B. Spesia, E.N. Durantini, Photochemical & Photobiological Sciences, 5, 56-65, 2006.

*Corresponding author: Ping-Shan Lai, Department of Chemistry, National Chung Hsing University, No. 250, Kuo-Kuang Road, Taichung 402, Taiwan,
Ph: (886) 4-22840411 ext. 428, Fax: (886) 4-22862547,
Email: pslai@email.nchu.edu.tw