

A TRILAYER DERMAL EQUIVALENT CONTAINING SILVER NANOPARTICLES WITH ENHANCED ANTIBACTERIAL PROPERTY*

Ai-bin Huang^a, Shao-jun Xu^b, Guo-yang Wei^a, Lie Ma^{a**} and Chang-you Gao^{a**}

^aKey Laboratory of Macromolecular Synthesis and Functionalization, Ministry of Education, and Department of Polymer Science and Engineering, Zhejiang University, Hangzhou 310027, China

^bCollege of Clinical Medical, Hangzhou Normal University, Hangzhou 310036, China

Abstract A dermal equivalent having a trilayered structure was designed by combining a silver nanoparticles incorporated chitosan film with a bilayer collagen-chitosan/silicon membrane dermal equivalent (BDE). The silver nanoparticles prepared at different conditions were characterized by UV-Vis and transmission electron microscopy (TEM). The macroscopic shape and the microstructure of the trilayer dermal equivalent (TDE) were also studied. Then, the *in vitro* antibacterial property of TDE was evaluated by the antibacterial zone test. The effect of the incorporated silver nanoparticles on the resistance of wound infection was further studied by the *in vivo* animal test. The results prove that the silver nanoparticles incorporated TDE has a better antibacterial property, thus may be potentially applied to a broader field in skin repair such as full thickness defect and burn.

Keywords: Scaffold; Silver nanoparticles; Antibacterial; Dermal equivalent; Skin regeneration.

INTRODUCTION

Skin, the biggest organ of human body, plays many pivotal roles in maintaining the normal functions such as protection of bacteria infiltration, water balance and temperature control. Due to the exposure to the environment, skin loss occurs frequently as a result of burn, trauma and diseases. The wounds are normally classified into superficial wounds, partial thickness wounds and full thickness wounds according to the tissues involved^[1]. Traditional split-thickness skin grafts for the resurfacing of large burns are the gold standard and provide permanent wound closure. Indeed, most of the full thickness burn wounds are satisfactorily closed as quickly as possible with the split thickness auto-graft^[2]. However, the split thickness auto-graft, an imperfect replacement for the full thickness skin, may be limited in quantity and is associated with donor site morbidity. Therefore, skin substitutes have been developed during the past century for the requirement. Conceptually, the skin substitutes are temporary or permanent; epidermal, dermal or composite, either biologic or synthetic.

Up to present, many kinds of dermal equivalents have been developed to treat the full-thickness skin defects^[3]. In particular, the dermal equivalents, which possess the ability to induce the *in situ* cell infiltration and regeneration of the damaged dermal layer such as Integra, are more attractive due to their lower production and management fees. In our previous works, we have developed a bilayer dermal equivalent (BDE) composed of collagen-chitosan porous scaffold and silicone membrane^[4, 5]. *In vitro* cell culture demonstrated that the collagen-chitosan scaffold could effectively support the infiltration and proliferation of human fibroblasts, with trivial

*This work was financially supported by the Science and Technology Program of Zhejiang Province (No. 2007C23014), the Major State Basic Research Program of China (No. 2005CB623902) and the National Science Fund for Distinguished Young Scholars of China (No. 50425311).

**Corresponding authors: Lie Ma (马列), E-mail: liema@zju.edu.cn

Chang-you Gao (高长有), E-mail: cygao@zju.edu.cn

Received October 6, 2008; Revised November 30, 2008; Accepted December 12, 2008

shrinkage of the scaffold size even after a long-term culture. *In vivo* animal experiment found that this bilayer equivalent has a strong ability to *in situ* induce the regeneration of dermal layer. Fully vascularization of the implanted BDE was always observed after 4 weeks.

However, when we applied this equivalent onto burned defects, the healing time became longer. Among the various reasons accounting for this phenomenon, more extent of infection of the burned defects may take the major role. It is known that the use of antimicrobial drugs can effectively reduce the wound's microbial load^[6]. More recently, use of silver for antibacterial wound care is particularly interesting^[7]. Indeed, there are a growing number of silver-incorporated dressings available. Although each preparation claims different advantages, the common effect is attributed to the antibacterial action of silver^[8]. Besides its antimicrobial activity, silver was also proven to have other beneficial effects on the wound bed and to down-regulate the levels of matrix metalloproteinase (MMPs), which in turn facilitates wound healing^[9]. Despite of these merits, in view of the potential dangers of the silver ions^[10], more attention has been paid to silver nanoparticles. The wound dressings containing silver nanoparticles can significantly reduce the bacterial infiltration, which are used for prevention of sepsis of skin wounds like burns and ulcers. Moreover, incorporation of the silver nanoparticles is beneficial to prevention of cross-infection as well^[11, 12].

Herein, in order to improve the antibacterial properties and the transplantation effect of our dermal equivalent, a chitosan layer containing the silver nanoparticles is embedded between the collagen-chitosan porous scaffold and the silicone membrane, forming a trilayer dermal equivalent (TDE). Characterization of the silver nanoparticles, the equivalent as well as the biological performance shall be performed too.

MATERIALS AND METHODS

Materials

Chitosan (M_n : 1.0×10^5 – 1.7×10^5 , deacetylation degree: 75%–85%) was purchased from Sigma. Glutaraldehyde (GA, 25% aqueous solution) was purchased from Shanghai Pharm Co. (China). Silicone membrane (thickness: 150–200 μ m) is a medical grade product from Shanghai Xincheng Co. (China). Collagen type I was isolated from fresh bovine tendon by a method of trypsin digestion and acetic acid dissolution^[13]. Carboxymethyl chitosan (M_n : 0.8×10^5 – 1.5×10^5 , 80% substitution degree) was purchased from Qingdao Heppe Biology Co. (China). AgNO_3 and NaBH_4 were purchased from Sinopharm Chemical Reagent Co. (China). Silicone adhesive was purchased from Guangzhou Biomax fine Chemical Co. (China). Common burn-wound pathogens, namely methicillin-sensitive and -resistant staphylococcus aureus (MRSA) (ATCC 25923) was obtained from the American Type Culture Collection (Manassas, VA). Nutrient broth (NB) and nutrient agar were used as the culture medium of the bacteria. Bama miniature pigs were obtained from the animal laboratory (license number: SYXK (Hu) 2004-0004). All other reagents and solvents were of analytical grade and used as received.

Preparation of Chitosan Films Containing Silver Nanoparticles

3.6 g carboxymethyl chitosan was dissolved in 180 mL distilled water at 60°C under vigorous agitation to form a homogeneous solution. The silver-ammonia solution was obtained by dissolution of 3 mmol AgNO_3 in 1 L 1% $\text{NH}_3 \cdot \text{H}_2\text{O}$. Then the carboxymethyl chitosan solution and the silver-ammonia solution were mixed according to the ratios listed in Table 1. After stirring for 10 min and suitable amount of water was supplemented, NaBH_4 solution with a concentration of 18 mmol/L was added to obtain the silver nanoparticles, namely, NS-1, NS-2 and NS-3. The chitosan film containing the *in situ* formed silver nanoparticles was prepared by casting the mixed solution on a glass plate at room temperature.

Table 1. The experimental conditions for fabricating the silver nanoparticles

Sample code	Silver-ammonia solution (mL)	Carboxymethyl chitosan solution (mL)	NaBH_4 solution (mL)	Deionized water (mL)
NS-1	10.0		5.0	–
NS-2	5.0	15.0	2.5	7.5
NS-3	2.5		1.5	11.0

Characterization of Silver Nanoparticles

The silver nanoparticles prepared were characterized by UV-Visible spectroscopy (UV-1601, SHIMADZU). The sizes and morphology of the silver nanoparticles were observed by transmission electron microscopy (TEM, JEOL, Japan).

Preparation of TDE

Collagen and chitosan were dissolved in 0.5 mol/L acetic acid solution to form a 5 g/L solution with a mass ratio of 9:1. The collagen and chitosan composite was injected into a round mould and freeze-dried to obtain the collagen-chitosan porous scaffolds. The collagen-chitosan porous scaffolds were further crosslinked by 0.25% GA. TDE was prepared by sequentially binding the collagen-chitosan porous scaffold, chitosan film containing the silver nanoparticles and a silicone membrane with a silicone adhesive in a dosage of 10 mg/cm².

Macroscopic Shape and Microstructure Observation

Macroscopic images of the chitosan films containing the silver nanoparticles and TDEs were taken by a digital camera (BENQ: DC-E520). The corresponding microstructures were observed by scanning electron microscopy (SEM, Cambridge stereoscan).

In Vitro Antibacterial Test

The antibacterial performance of TDE was assessed by an *in vitro* bacteria culture. 100 µL MRSA-NB suspension with a concentration of 10⁵–10⁷ CFU/mL was placed on a nutrient agar plate. The plate was then supplemented with different kinds of TDEs, and incubated further at 37°C for 12 h^[14]. The diameter of the antibacterial zone was then measured.

In Vivo Animal Test

2 Bama miniature pigs weighing 10–12 kg were selected for the study. Before implantation, the dorsal surface was shaved to remove hairs, followed by sterilization with 5% PVP-I. The pigs were anesthetized by celiac injection of 0.5 mL/kg pentobarbital sodium. Then 12 full-thickness skin defects with a diameter of 3.3 cm were made symmetrically on the back of each animal. The NS-1 incorporated TDE were sterilized by immersion in 75% (V/V) ethanol solution for 30 min, followed by sufficient washing with PBS (pH 7.4) (5 times × 5 min). In this experiment, polypropylene wound isolation rings were used to resist the automatic skin shrinkage. Then the sterilized TDEs were applied onto the skin defects. The samples were harvested after 16 d. The transplantation sites were cut in a full thickness manner. Paraffin sections were stained with hematoxylin-eosin (HE) reagent for histological observations.

RESULTS AND DISCUSSION

Morphology and Size of Silver Nanoparticles

UV-Visible spectroscopy is widely used to characterize the structure of silver nanoparticles, in which the absorption peak is related with the size and shape of the nanoparticles. The absorption spectra of the silver nanoparticles prepared at different conditions are shown in Fig. 1. The as-prepared samples, *i.e.* NS-1, NS-2 and NS-3, have maximum absorptions at 402, 403, and 412 nm, respectively. The maximum absorption at about 400 nm indicates the presence of nano-particles^[15].

According to the equations below^[16],

$$En_{r,l} = \frac{-h^2}{2\mu a^2} Xn_{r,l}^2 \quad n_{r,l} = (1, 2, 3, \dots)$$

$$\Delta E = \frac{-h^2}{2\mu a^2} (Xn_{r+1,l}^2 - Xn_{r,l}^2)$$

Where $En_{r,l}$ represents energy level, a represents quantum well radius, h and μ is constant. Therefore, along with the size of silver nanoparticles increases, the corresponding wavelength of the maximum absorption will shift to higher position, *i.e.* red-shift. In comparison of the three samples, NS-1 has the lowest wavelength of the maximum absorption, indicating that it should have the smallest size.

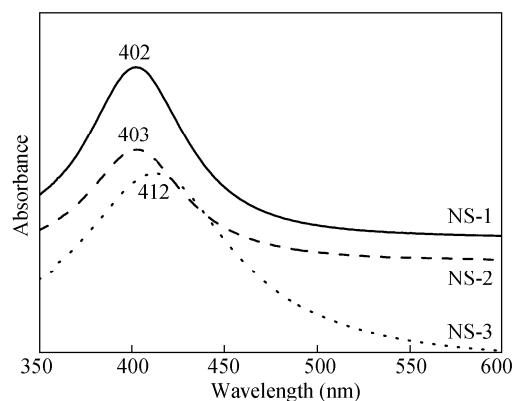


Fig. 1 UV-Vis spectra of the silver nanoparticles prepared at different conditions (see Table 1)

The morphology of the silver nanoparticles was observed by TEM (Fig. 2). Many spheres with a diameter of 8–10 nm distributed evenly for the NS-1 (Fig. 2a). For NS-2 and NS-3, the shapes of the particles became irregular. The particles with a larger size of about 50 nm (Fig. 2b) and about 150 nm (Fig. 2c) were observed, respectively.

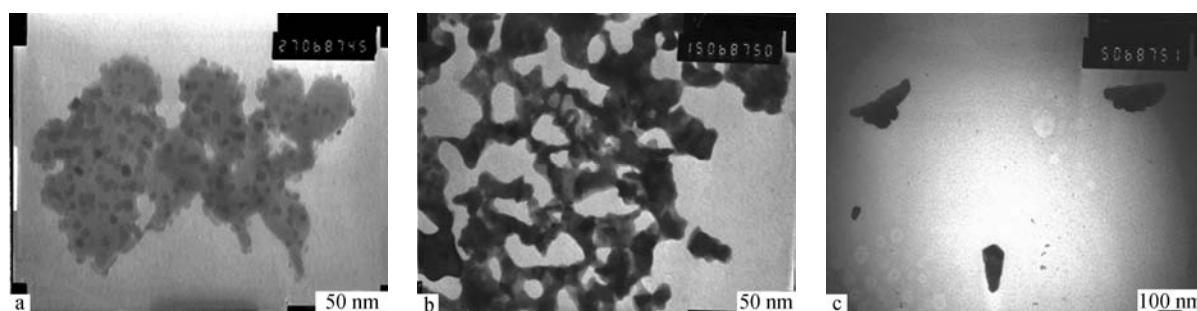


Fig. 2 TEM images of the silver nanoparticles: (a) NS-1, (b) NS-2 and (c) NS-3

Macroscopic Shape and Microstructure of the Films and Equivalent

The macroscopic shapes of the silver nanoparticles incorporated chitosan films and TDEs are displayed in Fig. 3. The films prepared at different conditions show different appearance (Fig. 3a). Figure 3(b) shows the gross view of the trilayer equivalents in which the silver nanoparticles containing chitosan films have been stacked.

The microstructure of a typical TDE is shown in Fig. 4. Figure 4(a) displays the surface morphology of TDE. The pores are clearly observed in the collagen-chitosan scaffold with a size ranging from 80–200 μm , which is suitable for fibroblast to infiltrate^[17]. The cross-section morphology of TDE is shown in Fig. 4(b). It shows that the thickness of the trilayer equivalent is about 1 mm, which contains collagen-chitosan scaffold, silver nanoparticles incorporated chitosan films (with the thickness of about 50 μm) and silicone membrane.

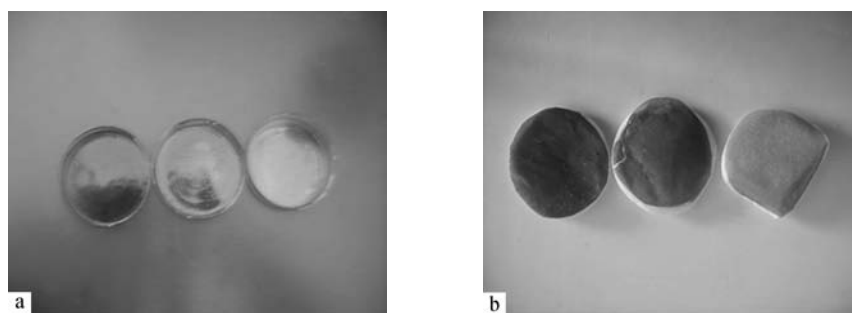


Fig. 3 Macroscopic images of the silver nanoparticles incorporated chitosan films (a) and the trilayer dermal equivalents (TDE) (b)
From left to right, the films and the equivalents were obtained by incorporating NS-1, NS-2, and NS-3.

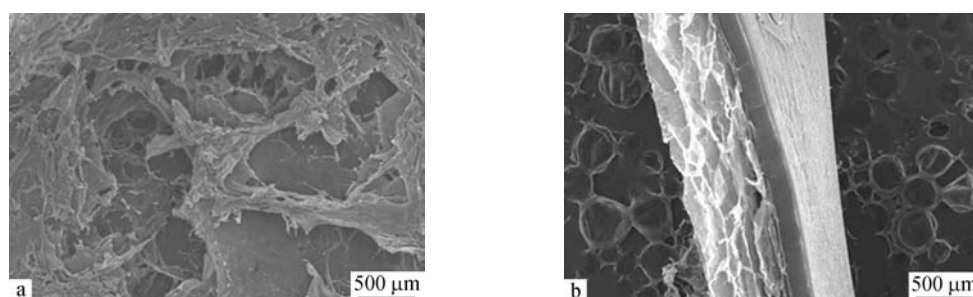


Fig. 4 SEM images of TDE obtained by incorporating NS-1
a) Top view; b) Cross-section view

In Vitro Antibacterial Property

The results from the antibacterial zone tests are given in Fig. 5. The diameter of the zone of inhibition and the amount of diffusion from the edge of each hole in the agar plate can be obtained. The ability of the control collagen-chitosan/silicone equivalent against bacteria growth is not obvious (Fig. 5a). Also, the NS-3 showed very limited ability to resist bacteria growth (Fig. 5b). In contrast, MRSA have been obviously antiblastic for NS-1 and NS-2 (Fig. 5b). These results indicate that the silver nanoparticles-containing equivalent indeed show antibacterial property, whose ability is related with the silver particle size and concentration too^[18]. Nonetheless, the equivalent having the NS-1 nanoparticles has the best antibacterial performance, and thus is subject to further *in vivo* animal experiment.

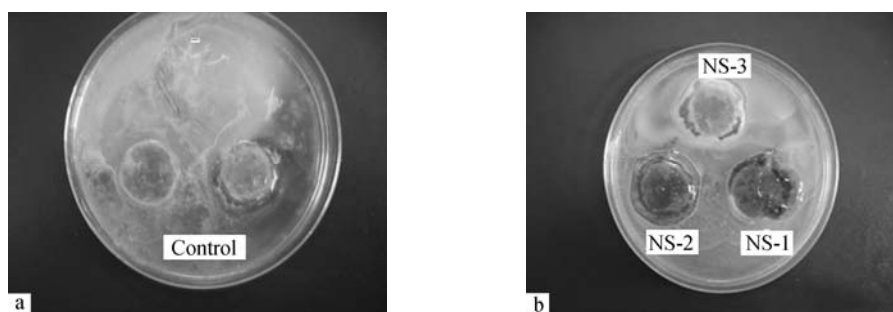


Fig. 5 Antibacterial zone test of (a) control collagen-chitosan/silicone bilayer equivalent (BDE) and (b) the trilayer dermal equivalents (TDE)

In Vivo Animal Test

The macroscopic appearances of the wounds after transplantation of TDE and the BDE control are displayed in Fig. 6. Here only normal gauzes were used to cover on the equivalents. For the group without silver nanoparticles (Fig. 6a), the wound has been suffered from serious infection. However, in the group incorporated with NS-1 (Fig. 6b), the wound presented a reddish and relative smooth surface, indicating a slight infection.

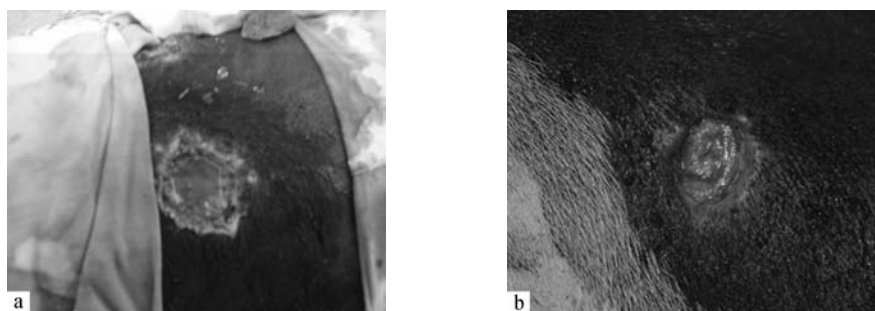


Fig. 6 Macroscopic appearance of the wounds after transplantation of (a) control collagen-chitosan/silicone bilayer equivalent (BDE) and (b) the NS-1 incorporated TDE after 16 d

The histological analysis of the above two wounds are shown in Fig. 7. On the wound without the silver nanoparticles, more inflammatory cells can be detected, confirming the occurrence of infection (Fig. 7a). In contrast, new-formed blood vessels can be found in the silver nanoparticles incorporated group. The neovascularization can effectively improve the oxygen concentration on the wound. Due to the anaerobic characters of most bacteria, few bacteria can survive on this wound.

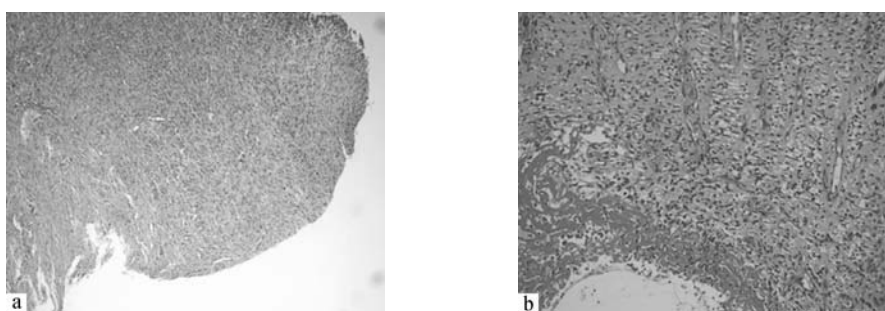


Fig. 7 Histological images of the wounds after transplantation of (a) control collagen-chitosan/silicone bilayer equivalent (BDE) and (b) the NS-1 incorporated TDE after 16 d

CONCLUSIONS

A trilayer dermal equivalent (TDE) with enhanced antibacterial property was fabricated by incorporating silver nanoparticles. Formation of the silver nanoparticles is demonstrated by UV-Vis spectroscopy and transmission electron microscopy (TEM). The results of *in vitro* antibacterial test and *in vivo* animal test indicate that the incorporation of silver nanoparticles can restrict the growth of bacteria, and thereby reduce the potential infection risk. In conclusion, the TDE can be served as a more promising dermal equivalent for uses in broader types of skin defects.

REFERENCES

- 1 Rowland, K., Publication of National Healing Corporation, 2005, 2(5): 1
(<http://www.nationalhealing.com/downloads/nhcwhpFall05.pdf>)
- 2 Sheridan, R.L. and Ronald, G.T., Burns, 1999, 25(2): 97
- 3 Balasubramani, M., Kumar, T.R. and Babu, M., Burns, 2001, 27(5): 534
- 4 Shi, Y.C., Ma, L., Zhou, J., Mao, Z.W. and Gao, C.Y., Polym. Adv. Technol., 2005, 16: 789
- 5 Ma, L., Shi, Y.C., Chen, Y.X., Zhao, H.G., Gao, C.Y. and Han, C.M., J. Mater. Sci. Mater. Med., 2007, 18: 2185
- 6 Yoko, N. and Mohamud, D., Emerg. Med. Clin. North. Am., 2007, 2(1): 159
- 7 Leaper, D.J., Int. Wound J., 2006, 3(4): 282
- 8 Vermeulen, H., van Hattem, J.M., Storm-Versloot, M.N. and Ubbink, D.T., Cochrane Database Syst. Rev., 2008, 3:1
- 9 Atiyeh, B., Costagliola, M., Hayek, S. and Dibo, S., Burns, 2007, 33(2): 139
- 10 Pal, S., Tak, Y.K. and Song, J.M., Appl. Environ. Microbiol., 2007, 4: 1712
- 11 Dunn, K. and Edwards, J.V., Burns, 2004, 30(1): S1
- 12 Chen, X. and Schluesener, H.J., Toxicol. Lett., 2008, 176(1): 1
- 13 Ma, L., Gao, C.Y., Mao, Z.W., Shen, J.C., Hu, X.Q. and Han, C.M., J. Biomater. Sci. Polym. Edn., 2003, 14: 861
- 14 Yin, H.Q., Langford, R. and Burrell, R.E., J. Burn Care Rehabil., 1999, 20(3): 195
- 15 Lee, S.Y., Kim, H.J., Patel, R., Im, S.J., Kim, J.H. and Min, B.R. Polym. Adv. Technol., 2007, 18: 562
- 16 Ji, S.L., Si, M.C. and Miao, R.C., Acta Photonica Sinica, 1999, 128(1): 93
- 17 Yannas, I.V., J. R. Soc. Interface, 2005, 2: 403
- 18 Lok, C.N., Ho, C.M., Chen, R., He, Q.Y., Yu, W.Y., Sun, H.Z., Tam, P.K.H., Chiu, J.F. and Che, C.M., J. Biol. Inorg. Chem., 2007, 12: 527