Preparation of collagen burn-healing membranes

Hua Yang, Ying Jiang, Shengli Ding, Hui Wang, Jing Liu, Linman Bi and Zibin Shu*

College of Chemistry and Materials Science, Sichuan Normal University, 5 Jing An Road, Jin Jiang District, Chengdu, Sichuan, China

_____

ABSTRACT

Collagen protein is obtained from fresh pigskin through hydrolysis with trypsin. By further adding extracts from scutellaria baicalensis (a Chinese herb), glutaraldehyde and etc., a novel type of collagen burn-healing membrane can be prepared. This novel type of membrane has a porous cross-linked netlike structure. The degree of crosslinking and mechanical properties of the membrane are examined; the optimal formulation and process parameters within preparation of the membrane are summarized. Based on the results from an in vitro test, the membrane enables its embedded medicines to possess a long-acting and slow-releasing property.

Keywords: Collagen protein; trypsin; burn wounds; membrane; baicalin

_____

INTRODUCTION

Collagen protein, which is widely used in biomedical materials, has excellent biocompatibility and low antigenicity. It is nontoxic, biodegradable, and induces no rejection reaction. Covering the burn wounds with collagen protein membranes can help absorb wound secretions. As the attachment and support of cell growth, collagen induces proliferation, differentiation and migration of cells, promotes the growth of epithelial cells, and reduces the degree of contraction of the wounds [1]. Without protection from the skin, the internal tissues of the burn wounds are more vulnerable to infection. There are a number of studies in the literature applying natural herbal extracts to promote wound healing [2][3]. After comparing and filtering, we adopt scutellaria baicalensis extracts to help prevent wounds from infection. Scutellaria baicalensis contains a large amount of baicalin, which belongs to flavonoids. It is anti-bacterial, anti-virus, anti-tumor, anti-inflammatory, and anti-allergy. It has a broad antibacterial spectrum and can inhibit a variety of bacteria, for example, skin fungus, leptospira, and staphylococcus aureus (which is commonly seen in burn wounds). Thus, we add scutellaria baicalensis extracts to collagen protein and prepare a novel type of collagen burn-healing membranes. The obtained membranes are examined to possess a long-acting and slow-releasing property.

EXPERIMENTAL SECTION

2.1 Reagents and instruments
Collagen protein powder (laboratory-prepared); soluble starch (AR); glutaraldehyde (AR); glycerol (AR); sodium carboxymethyl cellulose (CP); absolute ethyl alcohol (AR).

N-1001 Rotary evaporator (Shanghai Ai Lang Instruments Co., Ltd); TDZ5-WS low speed multi-pipe automatic balancing centrifuge (Xiang Yi Centrifuge Instrument Co., Ltd); DF-101S heat collection constant temperature magnetic stirrer (Zhengzhou Greatwall Scientific Industrial and Trade Co., Ltd); SHZ-C multi-purpose water circulation vacuum pump (Gongyi Ying Yu Yu Hua Instrument Co., Ltd); DHG-9070A electric heating constant temperature drying oven (Shanghai Jing Hong Laboratory Instrument Co., Ltd).

2802 UV/VIS spectrophotometer (Shanghai UNICO), 1100/1200 High performance liquid chromatography system

(Agilent Technologies Inc., U.S.) – combined with 18 angle laser light scattering instrument (Wyatt Technology Corporation, U.S.) and Optilab rEX differential refractive index detector; chromatographic column is 100,000 aqueous phase linear column (Shodex OHpak SB-803 HQ, Japan); guard column is Shodex OHpak SB-G.

2.2 Preparation and measurement of collagen protein
2.2.1 Preparation of collagen protein
Collagen protein was extracted from fresh pigskin. First, fat residues were removed from the pigskin by a knife. Second, the pigskin was cut into pieces and de-fatted by alkali solution. Third, the de-fatted pigskin was hydrolyzed at a low temperature using trypsin, separated using centrifuge, frozen, and dried. Finally we obtained the collagen protein powder [4][5]. When hydrolyzing the pigskin, a higher than needed temperature will denaturalize the resulting collagen protein, while a lower than needed temperature leads to a low extraction rate. The length of hydrolysis time not only affects the extraction rate of collagen protein, but also affects its molecular weight. Even if the extraction rate is high, a small molecular weight is less favorable for forming the netlike structure in the burn-healing membranes. A large molecular weight is beneficial for forming the netlike structure, but causes a low extraction rate. Taking all factors into consideration, we chose a hydrolysis temperature of 40°C and time of 2 hours.

2.2.2 Measurement of collagen protein
GPC-LS-RI was used to measure the molecular weight of the collagen protein.

Testing conditions were as follows. The mobile phase was Na_2HPO_4 + NaH_2PO_4 + NaN_3 + H_2O; flow rate was 1.0mL/min; Temperature is 35°C.

2.3 Extraction of baicalin
We put 12.0g scutellaria baicalensis into a round-bottom flask and added 120mL 75% ethanol solution. The mixture was heated under reflux for 1 hour with the temperature of 85°C. Then the solution was filtered. Again, 60mL 75% ethanol solution was poured into the filter residue [6], heated under reflux for 1 hour, and filtered. The filtrate was combined with the former one. The solvent was removed using the rotary evaporator. A slurry liquid was obtained. The liquid was put into a watch glass and dried at a constant temperature of 60°C in the oven, and baicalin crystals were obtained.

2.4 Preparation of collagen burn-healing membranes
2.4.1 Determination of the amount of collagen
Experiments have proved that adding collagen protein with a mass fraction of 1% can achieve favorable results. However, a small amount of collagen protein prevents the forming of a good netlike structure. This intended netlike structure is beneficial for improving the mechanical properties of the membranes. Furthermore, it helps embed the medicines in the membranes, and thus enables the medicines to release slowly on burn wounds.

We used single factor experiment method to determine the proper amount of collagen protein. Based on the formulation and dosages listed in Table 1, we prepared base membranes by the following steps. Then the membranes were soaked in distilled water, which had a temperature of 37°C. Degrees of dissolution were recorded.

2.4.2 Determination of the amount of glutaraldehyde
Commonly used crosslinking agents are glutaraldehyde, glyoxal, carbodiimide, epoxy compounds, and etc. Glutaraldehyde has good water solubility, low toxicity, and a low price. Thus, in this experiment, glutaraldehyde was chosen to be the crosslinking agent. If the amount of crosslinking agent is too small, the degree of crosslinking will be small. However, if the amount of the agent is too big, it is not good for embedding the medicines because the formed net will be too dense due to an increase degree of crosslinking. Based on the proportions described in Table 1, No. 5, we only changed amounts of glutaraldehyde and prepared base membranes. Dissolution of these membranes in 37°C distilled water was studied. It was found that when the amount of glutaraldehyde was 2mL, membranes swelled after soaking. When the amount was smaller than 2mL, membranes dissolved. Thus, the optimal amount of glutaraldehyde was set to be 2mL.

2.4.3 Determination of the amount of humectant
Adding humectant, glycerine, to the collagen protein membranes can improve and adjust at some level the moisturizing effect of the membranes. It enables the membranes to have some certain humidity and toughness, so that the membranes will not be easily torn or broken. Through experiments, when the amount of glycerine was 2.0mL, the burn-healing membranes had a relatively high moisturizing effect.

2.4.4 Determination of the amount of medicines
Scutellaria baicalensis extracts can dissolve in the solution for preparing base membranes. However, when the

amount of the medicine is too big, it will be separated out from the membranes. Thus, the optimal amount of baicalin was set to be 0.1g.

2.5 The in vitro test of medicine release in collagen burn-healing membranes
2.5.1 Measurement of the UV standard curve of scutellaria baicalensis extracts
The scutellaria baicalensis extracts were dissolved in 50% ethanol solution and made into 0.025mg/mL solution. The solution was scanned with ultraviolet at 200-400nm using UV-Vis spectrophotometer. UV absorption spectrum was measured. It was found that the maximum absorption wavelength was 276nm. The standard curve of the scutellaria baicalensis extracts was drawn. First, 50% ethonal-water solution was added to scutellaria baicalensis extract samples and prepared 1mg/mL solution. Second, pipettes were used to transfer 0.50, 0.75, 1.00, 1.25, 1.50, and 2.00mL solutions to 50mL volumetric flasks, respectively. Third, these solutions were diluted with 50% ethanol solution to the corresponding marks, shaked well, and made into standard solutions with concentrations 0.010, 0.015, 0.020, 0.025, 0.030, and 0.040mg/mL. Finally, with the 50% ethanol solution serving as a reference solution, the absorbance of each solution was measured at 276nm using the UV-Vis spectrophotometer. A standard curve was drawn based on the results.

2.5.2 The in vitro test of medicine release in collagen burn-healing membranes
The prepared collagen burn-healing membranes were soaked in a conical flask which contained 37°C distilled water. Every two hours 1mL soaking solution was sampled out to get its absorbance measured. Using the standard curve, a corresponding concentration was determined. The cumulative percentage of the released medicine was computed [7].

RESULTS AND DISCUSSION

3.1 Measurements of collagen protein

Collagen protein M_w = 7933; M_n = 7399; Polydispersity M_w / M_n = 1.072.

3.2 The formulation and preparation of collagen burn-healing membranes
3.2.1 Determination of the amount of collagen protein
Table 1 lists the experiment results.

According to the data shown in Table 1, when the amount of collagen protein was small, there was a smaller degree of crosslinking because membranes dissolved quickly in the water. When the amount of collagen protein became bigger, the degree of crosslinking became bigger and it became more difficult for the membranes to dissolve. When the amount reached 0.5g, the membranes did not dissolve but swelled in the water. Thus, in order to form a good netlike structure, a proper amount of collagen protein is 0.5g.

Table 1 Evaluation of netlike structure formed with different amount of collagen

No.	1	2	3	4	5
Sodium carboxymethyl cellulose* (g)	9.0	9.0	9.0	9.0	9.0
Starch (g)	0.1	0.1	0.1	0.1	0.1
Glutaraldehyde (mL)	2.0	2.0	2.0	2.0	2.0
Glycerin (mL)	2.5	2.5	2.5	2.5	2.5
Collagen protein (g)	0.1	0.2	0.3	0.4	0.5
Dissolution in the water	Fastest	Fast	Medium	Almost insoluble	Swelled

The ratio of sodium carboxymethyl cellulose to water is 1:18.

3.2.2 Formulation of collagen burn-healing membranes
Experiments showed that the optimal formulation of collagen burn-healing membranes was: 0.5g collagen, 0.1g starch, 2.0mL glycerin, 2.0mL glutaraldehyde, 9g sodium carboxymethyl cellulose (mass ratio to water: 1:18).

3.2.3 Preparation of collagen burn-healing membranes
First, collagen powder, starch, glycerol, sodium carboxymethyl cellulose solution and glutaraldehyde were mixed together in a flask. Stirring at a temperature of 40°C, a viscous evenly distributed liquid is obtained. Second, 0.1g scutellaria baicalensis was added, and the liquid became yellow. Third, the liquid was spread flat on a template. After freeze-drying and peeling off, collagen burn-healing membrane was obtained. Freeze-drying helps decrease the degree of denaturation of protein and helps form a porous structure. A porous structure lets the embedded medicine to release slowly, and it also absorbs wound secretions.

3.3 Results of the in vitro medicine release test of the membranes

Figure 1 shows the standard curve of scutellaria baicalensis extracts. The x-axis represents the concentration of scutellaria baicalensis extracts. The y-axis represents the absorbance. A linear regression was fitted and the estimated model was: y = a + bx, where a = -0.0306 and b = 21.354. R^2 = 0.9944, meaning that the estimated model fitted the data points well.
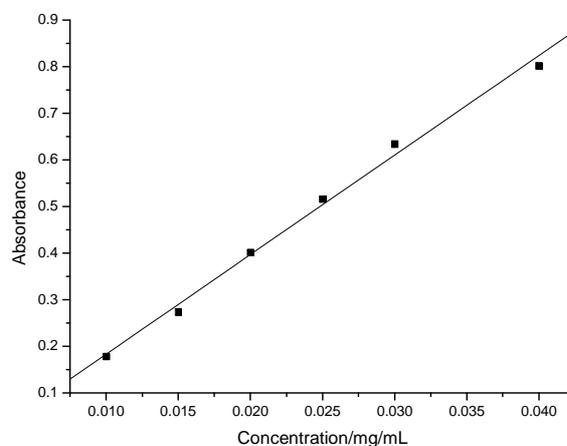

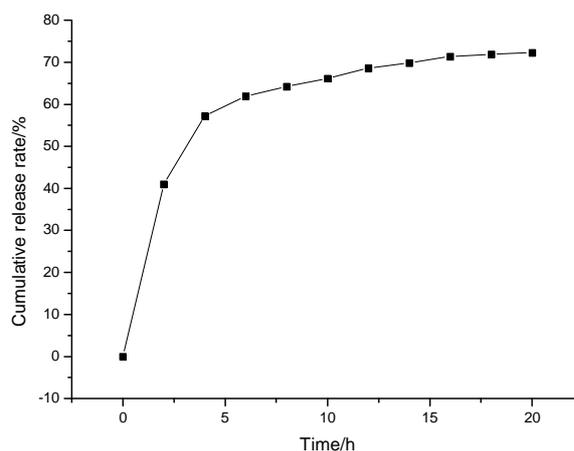**Figure 1 The standard curve of scutellaria baicalensis extracts**

Figure 2 Medicine release curve

Medicine release curve of collagen burn-healing membrane is shown in Figure 2.

According to Figure 2, the medicines embedded in the membranes released faster in the beginning. This matches our intuition because at first, the medicines were more concentrated in the membranes, while in the solution there was a smaller concentration of the medicines. The difference was huge, so in the frist 2 hours, the rate of releasing was large. As the medicines continued to release, the concentration of the medicines in the solution became bigger, and the difference of concentrations between the solution and membranes decreased. Thus, we observed a smaller medicine releasing rate. On the other hand, during the release of medicines, there were not only medicines diffusing from inside of the membranes to the outside, but there were also medicines in the solution re-absorbed by the membranes. This re-absorption process led to a decreased medicine releasing rate as well. After a certain amount of time, a banlance was reached between the release rate and re-absorption rate. According to the experiment, the final stablized proportion of medicines that were released was 70%. Of course, when the membranes are applied to huamn wounds, the embedded medicines will be absorbed by human bodies nonstop, so the body fluid at the wound will be constantly updating. Thus, the rate of medicine releasing on human will be bigger than what was seen in the static experiment, which prevents reaching a balance between the realease rate and re-absorption rate as in our experiment. On human wounds, the medicines in the membranes will release completely in the end. This fast-in-the-begininng and slow-in-the-end property matches well with burn wounds' time-varying need for the concentration of anti-bacterial medicines. Through the above experiment, it is found that the collagen burn-healing membranes have a long-acting and slow-releasing property.

___

CONCLUSION

In this paper, we conducted a series of experiments to study the optimal techniques to prepare a novel type of collagen burn-healing membrane which involves adding scutellaria baicalensis to collagen protein. To summarize the findings, the optimal formulation and preparation procedure is: first, mix 0.5g collagen protein, 0.1g starch, 2.0mL glycerol, 2.0mL glutaraldehyde and 9.0g sodium carboxymethyl cellulose solution together; second, stir the mixture at a temperature of 40°C until obtain a viscous evenly-distributed liquid; third, add 0.1g extract of scutellaria baicalensis to the liquid, and spread the stirred liquid flat on a template; finally, freeze-dry and peel the membrane off. Then, the collagen burn-healing membrane is obtained.

This new type of membranes has excellent biocompatibility and cell adaptability. Furthermore, it is anti-bacterial, anti-inflammatory, and anti-allergy; it can also absorb the wound secretions. Medicines embedded in the membranes can release slowly for a long time. These membranes can also boost the growth of the epithelial cells, and reduce the degree of contraction for the wounds. This novel type of membranes makes an ideal healing material for burn wounds.

REFERENCES

[1] ZR Gao; Y Li, *Burns*, **1992**, 18(6), 492.
[2] MLA Bastos; RLS Houly; LM Conserva; VS Andrade; EMM Rocha; RPL Lemos, *J. Chem. Pharm. Res*., **2011**, 3(4), 213-222.
[3] AA Attama; PF Uzor; CO Nnadi; CG Okafor, *J. Chem. Pharm. Res*., **2011**, 3(3), 718-724.
[4] H Yang; XX Chen; ZB Shu; XF Guo; RH Gao; SY Zhou; YCH Yan, *J. Chem. Pharm. Res.,* **2013**, 5(11), 655-658.
[5] H Yang; ZB Shu, *J. Chem. Pharm. Res*., **2014**, 6(2), 683-687.
[6] YN Lei; XB Zhang, *J. Shangluo University*, **2012**, 26(6), 51-54.
[7] L La; XT Jing; QL Huo, *Academic Journal of Guangdong College of Pharmacy*, **2002**, 18(1), 16-18.