JOURNAL OF PURE AND APPLIED MICROBIOLOGY, April 2013. Vol. 7(Spl. Edn.), p. 179-184

The Affection of the Vacuum Freeze-dry Temperature on Properties of Chitosan-gelatin Scaffolds

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(Received: 03 March 2013; accepted: 14 April 2013)

Generally, chitosan-gelatin scaffolds were fabricated with 0.25 wt% glutaraldehyde (GA) as a cross linker by twice vacuum freeze drying. Three kinds of different mixture volume of chitosan and glutaraldehyde were considered. Apparent density, porosity, water absorption and compress properties of scaffolds with different freeze-dry temperature (-55°C or -75°C) were studied to obtain scaffolds with more excellent performances. It was found that porosity, water absorption and compress properties increased by adding volume of GA, however, apparent density decreased. With the dropping of freeze-dry temperature (from -55°C to -75°C), the porosity and water absorption of scaffolds decreased, but increased the apparent density and compress properties. It can be summarized that apparent density, porosity, water absorption and compress properties of scaffolds were influenced by changing the volume of GA in scaffolds and freeze-dry temperature. Thus, scaffolds with excellent performances would be expected to fabricate by adjusting volume of GA in scaffolds and freeze-dry temperature.

Key words: Gelatin; Chitosan; Glutaraldehyde.

Tissue engineering and regenerative medicine have been used in clinical medicine successfully, biomaterials as porous 3D scaffolds is one of the most extensively used tissue engineering (Peng, *et al*., 2011; Drury *et al*., 2003).The morphology, density, swelling, biocompatibility, biodegradability and mechanical stability are important properties of biomaterials which could definite the uses of scaffolds (Martins, *et al*., 2009; Puleri, *et al*., 2008).

Natural polymers, such as gelatin and chitosan, have the unique advantages in avirulence, biocompatibility and biodegrability, which are usually used in preparations of porous three-dimensional scaffolds (Peng, *et al*., 2011; Peng, *et al*., 2010; Lin, *et al*., 2006). Chitosan is a polysaccharide derived from chitin, it has a structure of 1, 4-linked 2-amino-2-deoxy-â- D-glucan and has been investigated for several decades (Collins, *et al*., 2008; Jayakumar, *et al*., 2007). In consideration of its mechanical properties and other biological characters, chitosan has been put into used for skin regeneration and bone substitute (Kathuria, *et al*., 2009; Muzzarelli, *et al*., 2009).

Gelatin is a matrix of polypeptide hydrolyzed from collagen and it has been widely found in skin, bone and tendons (Wang, *et al*., 2011).Based on its excellent biocompatibility, gelatin has been used as medical dressing and adhesion in tissue engineering applications (Li, *et al*., 2005; Kim, *et al*.,2003). However, due to its quick biodegradation and low mechanical strength (Rathna, *et al*., 2008), gelatin always uses in combination with chitosan and with

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glutaraldehyde (GA) as cross-linking (Mubarak, *et al*., 2012; Yang, *et al*., 2005).

It has been reported that chitosan-gelatin could form membranes with smooth surface morphology (Mubarak, *et al*., 2012). The content of chitosan and GA could influence the properties of CG scaffolds such as density, porosity, swell and compress properties. Increasing content of chitosan could increase the swelling ratio and gelatin decreased the density of scaffolds. As the ratio of chitosan decreased, the compressive strength of chitosan-gelatin hydrogels decreased (Peter, *et al*., 2010) It can be summarized that chitosan and GA content adjusted the properties of scaffolds.

The fabrication of chitosan-gelatin (CG) scaffolds usually used vacuum freeze-drying (VFD) method undergoes twice (Peter, *et al*., 2010). The vacuum freeze drier common used are machine with -55°C or -75°C freeze-dried temperature. The affection of pre-freezd temperature on properties of chitosan-gelatin scaffolds has been reported, it can be summarized that the pore size decreased by dropping the pre-freezed temperature, however, the affection of dry temperature on properties of chitosan-gelatin scaffolds has not been studies. Thus, the research of properties comparisons of different freeze-dried temperature is necessary and useful to obtain scaffolds with more excellent performances.

In this work, CG scaffolds were fabricated with changing content of chitosan and GA, the scaffolds were freeze-dried with different temperature by using two Vacuum Freeze Drier. We studied the inûuence of changing GA dosage on properties of scaffolds and also the affection of freeze-dried temperature on properties of CG scaffolds.

EXPERIMENTAL

Materials

Chitosan (pharmaceutical grade, 99% deacetylated) was supplied by Qingdao Ruicang Chemical Co., Ltd., China. Gelatin and phosphate buffer solution (PBS) were supplied by Sigma, USA. Glacial acetic acid, glutaraldehyde (GA), sodium borohydride, sodium hydroxide and sterile water for injection were procured from Tianjin Fengchuan Reagent Co., China.

Fabrication of chitosan-gelatin scaffolds

Chitosan was dissolved in 1wt% acetic acid and stirred for 2 h with a magnetic bar to prepare 2 wt% chitosan solutions. 2wt% gelatin solution was prepared by dissolving gelatin in sterile water for injection at 50°C until it complete dissolution and then filtered by 100 mesh filter paper. Then the two solutions were mixed with the same volume, stirred for 60min, and then placed into Water Bath at 40°C for 12 h to obtain homogeneous solutions. GA stock, 50 wt%, was diluted to achieve a 0.25 wt% GA solution. The 0.25 wt% GA solution was added under volume of 3%, 6%, 8% and then stirred for 30 min. Further in the sequence, the solution was poured into the self-made plastic moulds and cross linked for 12 h at room temperature statically. Following, the solution was pro-freezed at -20°C for 24 h, some of the hydrogels were vacuum freeze-dried at -55°C for 24 h by Vacuum Freeze Drier (LGJ-18, Beijing fourth-ring scientific instruments Factory, China) and the others were dried at -75°C for 24 h by Vacuum Freeze Drier (FD-1D-80 Shanghai Tianfeng industrial co., LTD). Then the scaffolds were soaked in 1% NaOH to neutralize residual acetic acid and treated with 5% NaBr, followed by further washing with sterile water to pH = 7.0. After that the scaffolds were again freeze dried by the Vacuum Freeze Drier.

Apparent density

In order to measure the apparent density of scaffolds, ten dry scaffolds were selected from each group. The diameter (D) and height (H) were measured by using a vernier caliper three times for averaging. Weight (W) was measured by using an electronic balance (BS210s, Sartorius Co., China). The apparent density ρ of scaffolds is equal to:

$$\rho = W / [\pi \times (D/2)^2 \times H] \quad ...(1)$$

Apparent density was recorded as mean ± S.D. (n =10).

Porosity

In order to determine the porosity, fluid-discharge therapy was used. Five dry scaffolds from each group were selected and weighed (W_0) by using the electronic balance. Diameter (D) and height (H) were measured by using the vernier caliper three times for averaging. Then each scaffold was injected into absolute ethyl alcohol at room temperature about 20°C for a predetermined time (1 h) until scaffolds saturated completely. Final

wet weight was recorded as W1. The density of absolute ethyl alcohol is ρa = 0.7893 g/cm3. The porosity of scaffolds could be calculated by the following formula:

$$P = (W_1 - W_0)/[\rho_a \times \pi \times (D/2)^2 \times H] \quad ...(2)$$

The porosity was recorded as mean ± S.D. (n =5).

Swelling studies and water content

To determine the percentage of water absorption and content, six dry scaffolds were selected from each group and placed into PBS (pH 7.4) at temperature of 20°C for a totle time (35 min) until until scaffolds saturated completely. The scaffolds were carefully removed from PBS after immersing for 1 min; they were weighed for determining the wet weight (Wt) after wiping off solution excess on the surface with ûlter paper, and then took 2 min as the interval measuring time. The dry weight of the scaffolds was recorded as (W1), and the final wet weight was noted (W2). Swelling ratio was calculated by using the following formula:

$$Swelling\ ratio = (W_w - W_d)/W_d \quad ...(3)$$

The water content was determined by applying the following formula:

$$Water\ content = (W_2 - W_1)/W_2 \times 100\% \quad ...(4)$$

Each experiment was repeated three times for averaging. Swelling ratio and water content were expressed as mean ± S.D. (n = 6) respectively.

Compress test

The dry scaffolds with diameter (D) approximately 5 mm and height (H) close to 4.5 mm were placed into PBS for 40 min. Their compressive properties were measured by using the Instron 5544 (Instron, Co., England) equipped with a 5N load-cell at room temperature. Scaffolds were compressed at a speed of 1 mm/min for the experiment of ultimate compressive strength. Initial thickness (tc0), width (wc0) and length (lc0) were averaged after measuring three times with a vernier caliper. The compressive modulus in the elastic stage (CM) and compaction stage (CSCM) were the averages of six tests by using the following formulates:

$$CM = \Delta N_1/(t_{c0} \times w_{c0})/(\Delta l_1/l_{c0}) = k_{c1}/(t_{c0} \times w_{c0}) \times l_{c0} \quad ...(5)$$

$$CSCM = \Delta N_2/(t_{c0} \times w_{c0})/(\Delta l_2/l_{c0}) = k_{c2}/(t_{c0} \times w_{c0}) \times l_{c0} \quad ...(6)$$

Kc_1 was the slope of elastic range; Kc_2 was the slope of compaction stage. CM and CSCM were expressed as mean ± S.D. (n =6).

RESULTS

Morphology and apparent density of scaffolds

Scaffolds are uniform 3D pore light yellow cylindrical objects. Figure 1 plotted the differences of apparent density of scaffolds after -55°C and -75°C freeze-dried when the volume of 0.25 wt% GA solution increased from 3%, 6% to 8% in the combination scaffolds. It can be seen that the apparent density decreased with increasing the volume of GA which is in accordance with the previously reported literature (Peter, *et al.*, 2010); with decreasing of freeze-dry temperature, the apparent density increased. It can be calculated that the minimum differences of scaffolds among -75°Cfreeze-dried and that of -55°C was 4.47%, which can be seen as obvious difference. The increase of apparent density with decreasing of freeze-dry temperature may be due to the pore size of -75°C dried scaffolds were smaller than that of -55°C, which also lead to the decrease of permeability.

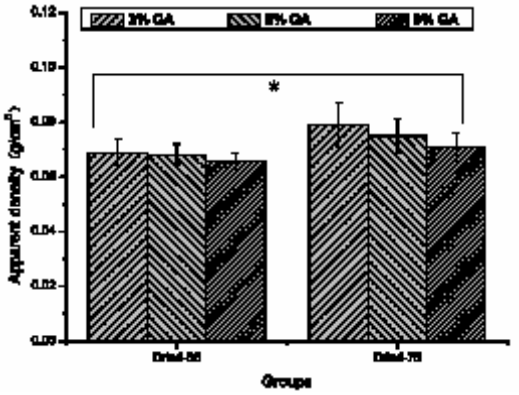

Fig. 1. Apparent density of scaffolds(*P<0.05, n = 5), the GA in the heading represents 0.25 wt% GA solution, 3%, 6%, and 8% are the volume ratios of 0.25 wt % GA solution and the composite scaffolds, dried-55 in the heading represents the scaffolds were dried at -55°C, dried -75 represents the scaffolds were dried at -75°C

Porosity of scaffolds

Porosity is a decisive factor to determine water absorption capacities of scaffolds and cells growth pattern in cells. The largest porosity reached to 64.75 ± 2.06 % which could solve the delay of wound healing and infection caused by

liquid ooze in clinical applications (Lin, *et al.*, 2006). The porosities of each group are summarized in Figure 2. The porosity increased with the volume of 0.25wt% GA solution changing from 3%, 6% to 8%. Porosity decreased obviously with dropping freeze-dry temperature from -55°C to -75°C.The pore structure was formed, after the crystallizable water was sublimed by vacuum freeze drying, so the adding of GA volume could lead to the increase of porosity by increasing volume of crystallizable water. The pore sizes of scaffolds decreased with dropping dried temperature which lead to the decrease of pore volume in unit volume

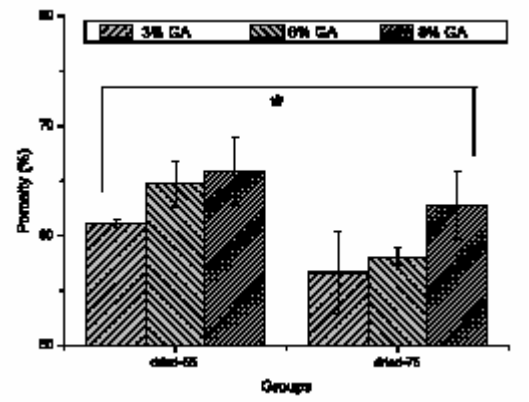

Fig. 2. Porosity of composite scaffolds (*P<0.05, n = 5), the GA in the heading represents 0.25 wt% GA solution, 3%, 6%, 8% and 10% are the volume ratios of 0.25 wt % GA solution and the composite scaffolds

Swelling studies and water content

The water absorption capacities of scaffolds is a significant property to characterize if the scaffolds are suitable for use as tissue engineered skin for absorbing inflammation liquid timely from malignant skin. The cylindrical composite scaffolds were light yellow at dry time and changed to semitransparent form after being soaked into PBS for 4min and swelled, but keep the cylindrical unchanged. The network structure of the scaffolds was compacted and had an opaque form (Rose *et al.*, 2011; Sokker, *et al.*, 2009), as the polymer chain of hydrophilic chitosan-gelatin was not spread in dry scaffolds. The gel network chain extended and appeared semitransparent form after numerous water molecules flocking into network when scaffolds were soaked into PBS.

Typical water absorption changes of the scaffolds are shown in Figure 3 for CG with same volume of chitosan-gelatin in combination with 0.25 wt% GA solution at volumes of 3% to 8%.Briefly, Fig. 3a and Fig. 3b indicated an initiative rapid absorbed dose of PBS in approximately 1min, followed by mass stabilization over 10min. The

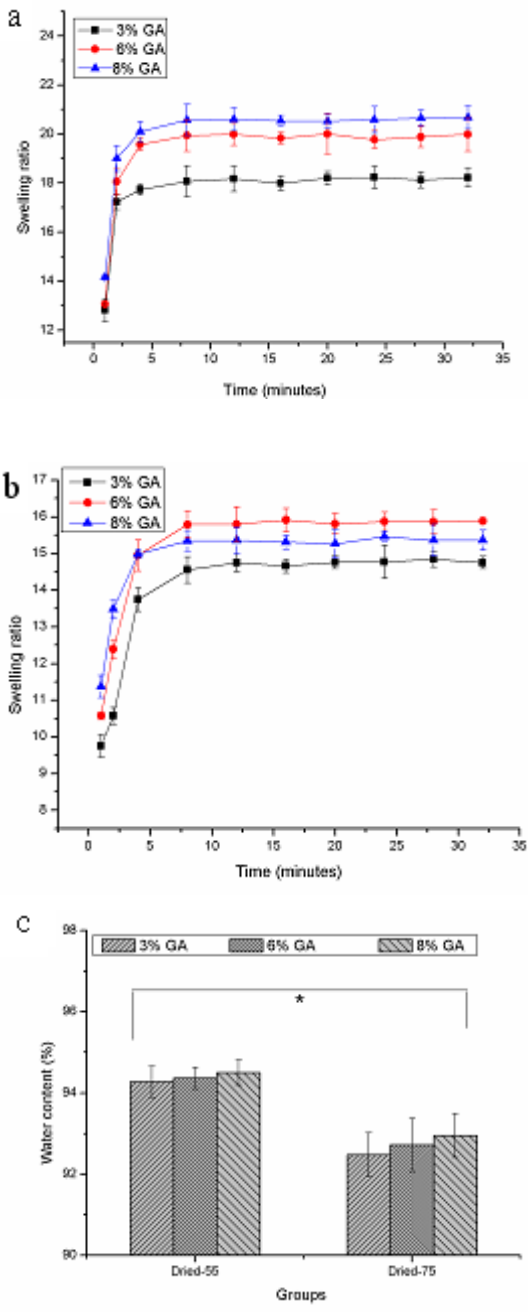

Fig. 3. (a) The swelling ratio of scaffolds freeze-dried at -55°C (b)The swelling ratio of scaffolds freeze-dried at -75°C(c)the water content of scaffolds at 35 min. (*P<0.05, n = 6)

initial and final swelling ratios were enlarged obviously of the same scaffolds. As the content of GA changed from 3% to 8%, the water content of scaffolds with the same dry temperature increased. With decreasing of dry temperature, the swelling ratios decreased. Due to the changes of pore size and porosity, the scaffolds had different swelling ratios which caused by water absorption through the porous structure allowed water into the center rapidly by capillary force.

It can be observed from Figure 3c that as concentration of GA increased the water content of the scaffolds also increased. There are significant differences on water content between scaffolds of different freeze-dry temperature when the content of GA was fixed. The reasons of the above phenomenon may be because the cross-link between chitosan and gelatin formed by GA could increase the hydrophilicity of scaffolds.

Compress properties of scaffolds

When the tissue engineered scaffolds skin is applied on skin diseases, it should have the ability to resist stresses through the process of wound healing. In compress experimental process, the scaffold first undergoes elastic stage, with a linear relation between stress and strain. The scaffold then exceeds yield deformation and reaches to compression stage because of the enlargement of cross sectional area.

The variations of CM and CSCM were displayed in Figure 4. As the volume of 0.25wt% GA solution increased from 3%, 6% to 8%, the CM and CSCM of scaffolds increased significantly, when the freeze-dry temperature was fixed. With the dropping of freeze-dry temperature, the CM and CSCM increased, when the content of GA was fixed. The scaffolds with minimum CM was 6.80±0.98 can be used as artificial skin which usually under pressure of 1.33-6.65Kpa after grafting onto skin (Mubarak, *et al*., 2012). The CSCM changed from 0.93±0.18Mpa to 1.93±0.51Mpa which within the limits of experimental data of human skin 1.05-2.98Mpa. The CM was a elastic modulus when the scaffolds contained water and meaningful in physiological application, but after full compaction the CMCS reflected the performance of the material itself, thus the CMCS was much larger than the CM of the same scaffolds.

In the compression test, scaffolds recovered to initial height with the water gone after unloading which demonstrated that the scaffolds had outstanding elasticity due to the cross linking of chitosan and gelatin. However, the dropping of freeze-dry temperature could decrease porosity to enhance the compress properties.

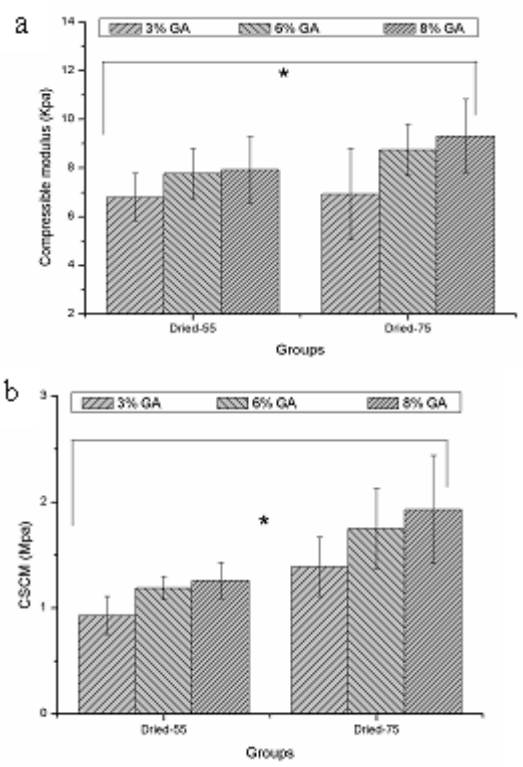

Fig. 4. Compress properties of scaffolds.
(a) The TM of scaffolds. (*P<0.05, n =8).
(b) The MTS of scaffolds. (*P<0.05, n =8).

CONCLUSIONS

Chitosan-gelatin scaffolds of 1:1 volume ratio were fabricated with using 3%, 6% and 8% volume of 0.25 wt% GA solution as cross linker by vacuum freeze drying at -55°C or -75°C. Porosity, swelling ability, water content, CM and CSCM all increased with adding content of GA, when freeze-dry temperature was fixed, however apparent density was changed inversely. With dropping freeze-dry temperature, porosity, swelling ability, water content, CM and CSCM all decreased when the content of GA was fixed. Due to the increase of apparent density and the decrease of porosity the scaffolds of dried at -75°C had superior compress properties than that of -55°C. The changes of

properties among each group were obvious, which demonstrate that scaffolds with excellent performances could be fabricated by adjust the content of GA and freeze-dry temperature.

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