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Morphology and characterization of 3D micro-porous structured chitosan scaffolds for tissue engineering

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Abstract

This research studies the morphology and characterization of three-dimensional (3D) micro-porous structures produced from biodegradable chitosan for use as scaffolds for cells culture. The chitosan 3D micro-porous structures were produced by a simple liquid hardening method, which includes the processes of foaming by mechanical stirring without any chemical foaming agent added, and hardening by NaOH cross linking. The pore size and porosity were controlled with mechanical stirring strength. This study includes the morphology of chitosan scaffolds, the characterization of mechanical properties, water absorption properties and in vitro enzymatic degradation of the 3D micro-porous structures. The results show that chitosan 3D micro-porous structures were successfully produced. Better formation samples were obtained when chitosan concentration is at 1–3%, and concentration of NaOH is at 5%. Faster stirring rate would produce samples of smaller pore diameter, but when rotation speed reaches 4000 rpm and higher the changes in pore size is minimal. Water absorption would reduce along with the decrease of chitosan scaffolds' pore diameter. From stress–strain analysis, chitosan scaffolds' mechanical properties are improved when it has smaller pore diameter. From in vitro enzymatic degradation results, it shows that the disintegration rate of chitosan scaffolds would increase along with the processing time increase, but approaching equilibrium when the disintegration rate reaches about 20%.

Keywords: Chitosan scaffold; Biodegradable polymer; Lysozyme degradation; Tissue engineering

1. Introduction

Biomedical polymers were widely used as medical service materials such as surgical sutures, wound dressing, braces/fixation materials, etc., because of its biocompatibility, bioabsorbability, that it will not induce antibody from the immune system, hypoallergenic and that it does not cause inflammation to human tissue [1–2]. These biomedical polymers used include natural polymers such as collagen, hyaluronic acid, chitosan and alginate; microbial synthesized polymers such as poly(3-hydroxybutyrate) [P(3HB)], poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)], poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)]; and artificial synthesized polymers such as poly(lactic acid), poly(lactic-*co*-glycolic acid) [3–8]. Since about 20 years ago in the 1980s, many researchers have utilized these biomedical polymers to manufacture human body tissue in organ shape for implant, growing new tissues by molding in the shapes of organs for use in repairing body tissue imperfection or damages [9]. Therefore, biomedical polymers have become the extremely popular material in this new research domain of tissue engineering. In order to effectively and massively cultivate the tissue cells, these biomedical polymers usually designed as three-dimensional structure (scaffolds) of interconnected pores with high porosities for easily implant into the human body. Hence, this kind of scaffolds must have good biocompatibility, biodegradability, high porosity, and possess appropriate mechanical properties [10–15].

Chitosan is extracted and purified from the shells of shrimp, crab and other crustaceans, and from some of the fungi cell walls.

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It is an abundant deposit of natural cationic polysaccharides second only to cellulose on earth's reserves. The amino and hydroxyl groups in its molecular structure can be easily modified chemically to produce other derivatives. Chitosan can be made into gelatin, orb, fiber and membrane shapes for various uses. Moreover, because chitosan can be easily obtained and confirms to tissue engineering application requirements; can be implanted into human body and causes no harm; it is a notably suitable material for use in tissue engineering [16]. At present time, there are not many related research for chitosan direct application in 3D scaffolds. Furthermore, most of these researches are mainly on 3D composite scaffolds that mixed with other materials for permanent implant in human body [17,18].

This research uses chitosan as the only crude materials, utilize mechanical agitation to produce foaming bubbles, and through a simple liquid hardening method to produce the chitosan scaffolds. The purpose is to avoid any excessive chemical additives in the manufacturing processes that could possibly cause harmful effects to the human body. We observed the produced sample's formation status through SEM, and investigated the influence of various concentrations of chitosan and hardening agent, stirring rate of homogenizer and other variables to the pore sizes production and pore distribution of the chitosan scaffolds samples. In addition, we measure water content/uptake ratio and use compression tensile test to investigate the influence of structural variation to chitosan scaffolds' physical properties. Lastly, we conduct in vitro enzymatic degradation to observe the condition while chitosan scaffolds being disintegrated by enzyme so we could assess the feasibility of chitosan scaffolds' use for cell culture in tissue engineering application.

2. Experimental

2.1. Preparation of chitosan scaffolds

Chitosan [poly(β-(1-4-2-amino-2-deoxy-D-glucopyranase)] was purchased from Wako Pure Chemical Industries Co. (80° of deacetylation). Place 1–3% (C-1%, C-2%, C-3%) chitosan and evenly dissolved in 1N acetic acid solution to form thick chitosan solution. Use the Homogenizer (HG-300D + K12S, Shuang-Tai, Taiwan) set at 2000–6000 rpm speed to mechanically agitate and stir to foam bubbles in the thick chitosan solution. Then pour into a regulate container having 5% aqueous sodium hydroxide solution (NaOH, First Chemical, Taiwan) to conduct liquid hardening processes for chitosan porous scaffolds to slowly harden into formation. Wash with distilled water at least three times to remove remaining traces of alkali, and cure with Freeze Dryer (FD-3a2d, Fuyuan Co., Taiwan), then stored in desiccator as the samples for this research.

C-1%, C-2% and C-3% represent the chitosan scaffolds samples produced under 1%, 2% and 3% of chitosan concentration, 4000 rpm, 5% NaOH. The liquid hardening method we used is the above processes of producing the 3D micro-porous chitosan scaffolds, including the foaming by mechanical stirring without adding any chemical foaming agent, and the hardening process of cross-linked by NaOH.

2.2. Analytical methodology

2.2.1. Pore size and porosity

By using a scanning electron microscope (SEM) apparatus, the pore sizes of the cross section and the average pore diameter of samples were observed, we could derive the mean value and standard deviation ($N = 20$) of the pore sizes of the samples. However, porosity (P) is obtained from calculation formula below. It is calculated from the density of chitosan and the density of actual porous scaffolds sample produced. The density of chitosan (ρ) is 1.342 g/cm^3. So the calculation formula of porosity (P) is defined as follows:

$$\text{porosity}\,(\%) = \frac{V_\text{m} - V_\text{p}}{V_\text{m}} \times 100 = \frac{V_\text{m} - (W_\text{m}/\rho)}{V_\text{m}} \times 100 \quad (1)$$

V_m is the total volume of chitosan scaffolds (cm^3), V_p the actual volume of chitosan (cm^3) and W_m is the mass of scaffold (g).

2.2.2. Water absorption

The water absorption of the chitosan samples were obtained by following these steps: the chitosan scaffolds samples were prepared into round shape specimens with diameter about 2 cm. The absolute-dry weight (w_0) of the samples were measured and then were placed into 37 °C PBS buffer solution to saturate with liquid. The samples were then taken out quickly after 24 h and placed into the Automatic Water Content Measuring System (AWCMS) [19]. The measurements of the samples' saturated weight (w_t) were not taken until the weight displayed on AWCMS no longer changes. The water absorption (W) of these samples was calculated using the following equation:

$$\text{water absorption}\,(\%) = \frac{w_t - w_0}{w_0} \times 100 \quad (2)$$

2.2.3. Compressive strength and strain

The compression strength and strain data of the chitosan scaffolds samples were obtained from the following processes. First place 5 cm (L) × 1 cm (H) × 2 cm (W) (L, H, W denoted the length, height and width, respectively) size chitosan scaffolds samples into a 25 ± 2 °C, 65 ± 2% RH Programmable Temperature & Humidity Chamber (THS-A, KSON) for 24 h, then use Micro Hardness Tester (HMV-2T, SHIMADZU) to measure samples' compressive strength and strain. The compression rate was set at 10 mm/min.

2.2.4. In vitro enzymatic degradation

First the absolute-dry weight (w_0) of the chitosan scaffolds samples were measured, and the samples were placed into 37 °C PBS buffer solution, simultaneously add in 5 μg/ml of Lysozyme (PSTi Co., Taiwan). Then the samples were placed in an Orbital Shakers (SO-701, GMB Co.) with temperature set at constant 37 °C to do the time course weight-change experiment of enzymatic degradation. After the samples were washed with distilled water and freeze-dried, they were placed into a 25 ± 2 °C, 65 ± 2% RH Programmable Temperature & Humidity Chamber for 24 h and the weight loss ratio was then calculated. The weight loss ratio of these samples was calculated using the

following equation:

$$\text{weight loss } (\%) = \frac{w_0 - w_t}{w_0} \times 100 \quad (3)$$

3. Results and discussion

3.1. Morphology of the scaffolds structure

Fig. 1 shows different pore sizes of 3D porous scaffolds produced under various concentration of chitosan and stirring rate of homogenizer. Testing samples were produced by using 1, 2, and 3% concentration of chitosan solution, foaming using homogenizer with 2000, 4000, and 6000 rpm stirring rate, then add 5% NaOH to harden into shape. From the figure we could see that the samples' pore diameter decrease while the stirring rate increase. Take C-1% chitosan scaffolds for example, when stirring rate increases from 2000 rpm to 4000 rpm, its pore diameter rapidly decreases from 500 μm to about 400 μm. The reason is that for homogenizer the higher the stirring rate the greater the shear force of the cutting head. Nevertheless, when the stirring rate reaches 4000 rpm and higher, the change in porous diameter of the chitosan scaffolds is minimal. Both C-2% and C-3% chitosan scaffolds also have similar results.

Fig. 1 also shows that the porous diameter decreases along with the concentration of chitosan solution decreases. Higher chitosan solution concentration also easier to obtain smaller diameter foam bubbles. In our laboratory preparation experiments we discovered that when chitosan solution concentration is lower than 1%, the sample's viscosity is too low for foaming, on the other hand, when the chitosan solution concentration is higher than 3%, the sample's viscosity is too high thus the sample would stick to the homogenizer's cutting heads and were unable to mix homogeneously.

The pore diameters of C-3% chitosan scaffolds are smaller than those of C-2% and C-3%. The reason is that C-3% has higher viscosity therefore it is easier to maintain the smaller pore diameter after foaming. To sum it up, we are certain that chitosan solution concentration and homogenizer stirring rate both variables have great influence to the pores' formation in scaffolds.

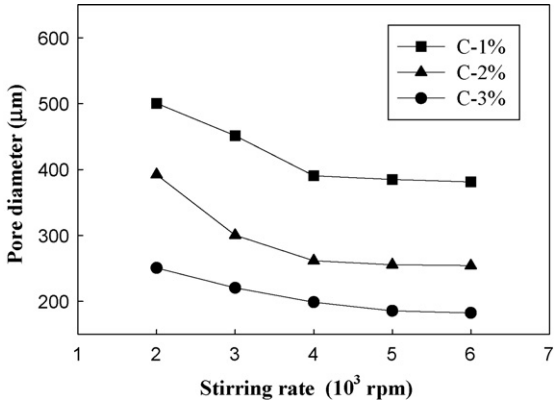

Fig. 1. The pore sizes of chitosan scaffolds at various concentration of chitosan and various stirring rate (5% NaOH).

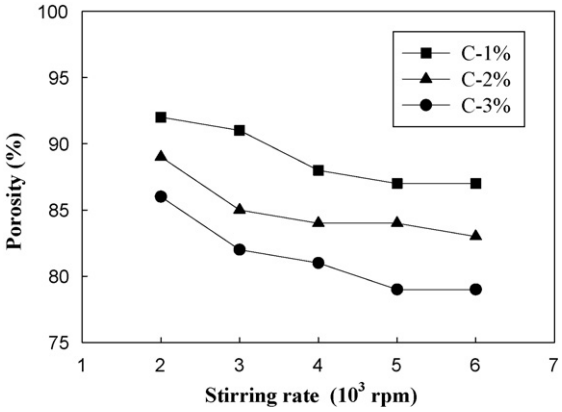

Fig. 2. The porosity of chitosan scaffolds at various concentration of chitosan and various stirring rate (5% NaOH).

Fig. 2 is the porosities for chitosan scaffolds of various chitosan solution concentrations. Porosity is calculated from Eq. (1) in Sections 2 and 3. As shown in the figure, porosity would decrease along with the increase of both homogenizer stirring rate and chitosan solution concentration. In addition, that under these set conditions, no matter how high the concentration or stirring rate is, the porosity of chitosan scaffolds constantly maintains in high level above 80%. The formation of chitosan scaffolds samples produced were always inspected and observed with SEM.

Fig. 3 is the SEM photo taken from a 2% chitosan scaffolds after the 5% NaOH liquid hardening processes. From Fig. 3 it can be seen that although no chemistry foaming agent was added in the manufacture process, yet the 3D scaffolds samples with pore sizes of 200–500 μm^{-1}, and 80% porosity could still be obtained by controlling the concentration of chitosan and the stirring rate of homogenizer.

3.2. Water absorption

The water absorption property of the raw materials influences not only the maintaining of the chitosan scaffolds' shape

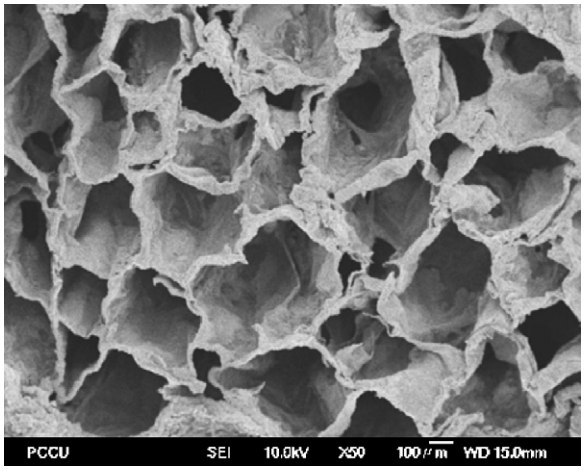

Fig. 3. Morphology of chitosan scaffold produced by liquid hardening method (2% chitosan, 4000 rpm, 5% NaOH).

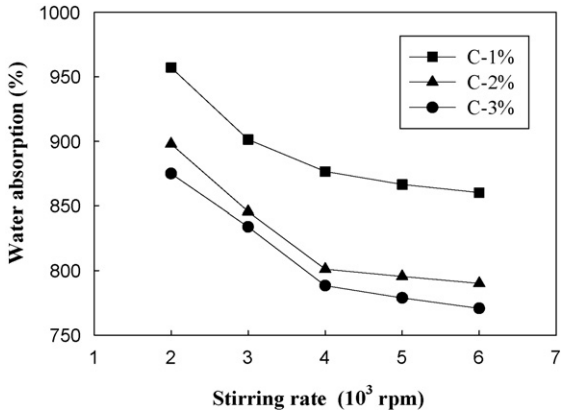

Fig. 4. The water absorption diagram of chitosan 3D porous scaffolds at various concentrations and various stirring rates.

and form, but also affects the cells' growth. In the cell cultivation processes of tissue engineering, if long culture time were required and high water absorption materials were used, the scaffolds might saturated with water and expand, causing deform and affecting the proliferation and division of cells. Fig. 4 shows the diagram of water absorption for 1–3% chitosan scaffolds under 2000–6000 rpm stirring rates. The change ratio of water absorption is the water absorption in relation to the homogenizer stirring rates, which would show the influence of stirring rates to the amount of water absorbed. As the diagram illustrated, along with the increase of both chitosan solution concentration and homogenizer stirring rate, the change ratio of water absorption reveals a reducing trend. Regardless of the chitosan scaffolds sample is C-1%, C-2% or C-3%, if the homogenizer stirring rate is between 2000 and 4000 rpm, the change ratio of water absorption decrease rapidly, as shown in Table 1 as the variation are between 8.5 and 10.8%. If the stirring rate reaches above 4000 rpm the decrease of the change ratio of water absorption tend to slow down and not as rapidly, also shown in Table 1 as the variation are between 1.4 and 2.2%. When compared these experiment results with Figs. 1 and 2, we could find that under the manufacturing condition of 2000–4000 rpm stirring rate, pore sizes would quickly become smaller and porosity lower, result in the change ratio of water absorption rapidly reduced. On the other hand, when samples are manufactured under the condition of 4000–6000 rpm stirring rate, the above-mentioned shrink in pore sizes and porosity tend to slow down, thus reducing the change ratio of water absorption. Therefore, we believe that the samples' ratio of water absorption can be changed not only by the samples' material properties, but also by varying the pore sizes and porosity of the samples as well as change the structure of chitosan scaffolds and control the samples' water absorption property.

Table 1
The water absorption (changing ratio) for chitosan scaffolds at the manufacturing conditions of 2000–6000 rpm stirring rate

Samples	Stirring rate	
	2000–4000 rpm	4000–6000 rpm
C-1%	957–876 (−8.46%)	876–860 (−1.83%)
C-2%	898–801 (−10.80%)	801–790 (−1.39%)
C-3%	875–788 (−9.94%)	788–771 (−2.16%)

As far as the influence of chitosan concentration changes to the chitosan scaffolds samples' ratio of water absorption, shown in Table 1, apparently we could see that along with the concentration of chitosan solution increase the water absorption tends to decrease. Relatively to 6.2–8.1% between C-1% and C-2%, the difference between C-2% and C-3% is minimal (about 1.6–2.6%). From Figs. 1, 2 and 4 we could find that the ratio of water absorption of chitosan scaffolds has very high relevance to pore sizes, porosity and water absorption. When chitosan solution concentration is within the range of 1–3%, larger pore sizes having higher porosity thus would have higher ratio of water absorption. Obviously the scaffolds has higher ratio of water absorption as it has more water storage space (than surface area). While mechanical stirring rate of homogenizer is under 4000 rpm, along with the increase of stirring rate the pore size and porosity decrease. The decrease of pore size may causes capillary phenomenon and increases some water absorption [20,21]. However, we believe the main reason for the water absorption to decrease should be the decrease of the whole sample's porosity that reduces the sample's specific surface area for water absorption. We could also see similar results from other correlated literatures [22].

3.3. Mechanical properties

During cell cultivation, 3D porous scaffolds need to have appropriate strength for cells attachment. Fig. 5 shows the chitosan scaffolds' compressive strength–strain curves of C-1%, C-2% and C-3%. In the initial compression phase (strain < 0.4), chitosan scaffolds exhibits poor resistance to tension stress, but demonstrates better resistance to tension stress when strain is greater than 0.4. Moreover, C-3% has stronger compressive strength than C-2% and C-3%. Chitosan is essentially a non-water-soluble material, even soaked in water for a long period (such as in PBS for cell culture) should have minimal influence to the compressive strength of chitosan scaffolds. Therefore, the deciding factor of chitosan scaffolds' mechanical properties

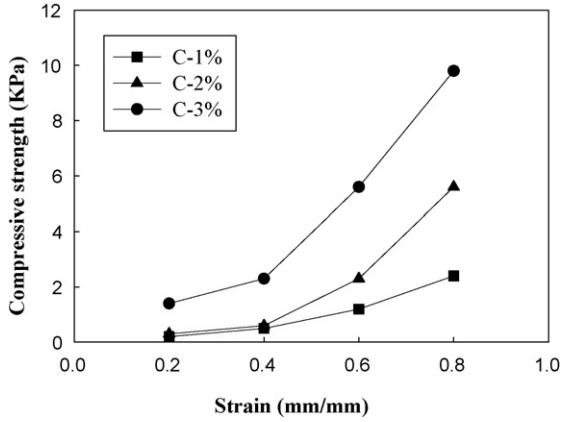

Fig. 5. The compressive strength and strain diagram of chitosan 3D porous scaffolds at concentration of chitosan 1–3% (4000 rpm, 5% NaOH).

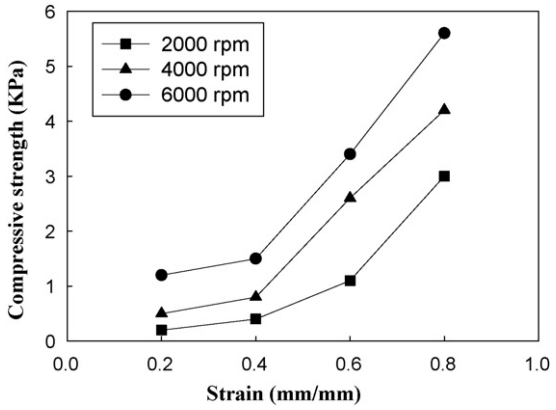

Fig. 6. The compressive strength and strain diagram of chitosan scaffolds at manufacturing conditions of stirring rate 2000–6000 rpm (2% chitosan, 5% NaOH).

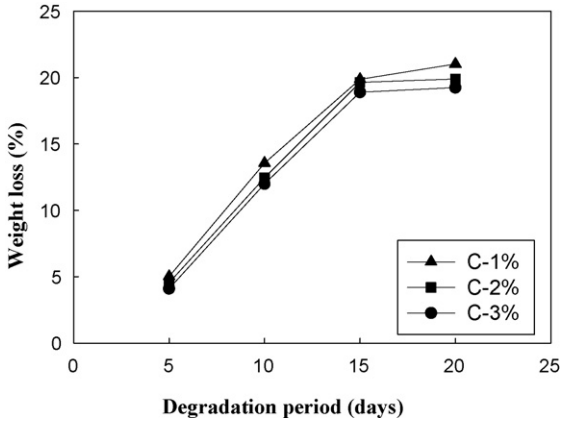

Fig. 7. The weight loss curves of chitosan scaffolds samples under the time course of degradation (4000 rpm, 5% NaOH).

is the concentration of chitosan added in the solution. Fig. 6 is the compressive strength–strain diagram for chitosan scaffolds manufactured under various homogenizer stirring rates. From the diagram, we could see that chitosan scaffolds would have stronger mechanical strength along with the increase of homogenizer stirring rate. The reason is that higher homogenizer stirring rate produces smaller pore diameter in the scaffolds, and smaller pore sizes could gain larger surface area, which could withstand stronger compression force. In summary, we found that chitosan scaffolds manufactured under the conditions of higher chitosan concentration and higher homogenizer stirring rate would possess better mechanical properties.

3.4. In vitro degradation

The ultimate goal for the application of chitosan 3D porous scaffolds in tissue engineering were the hope that it could disintegrated naturally along the cells growth. As a result, the time to degradation would affect the condition of the cells growth. Lysozyme could hydrolyze the bindings between *N*-acetylmuramic acid and *N*-acetylglucosamine in some bacteria's cell wall. Therefore, this research uses lysozyme as degradation enzyme to investigate through the time course of degradation conditions of chitosan 3D porous scaffolds. Fig. 7 shows the enzymatic degradation experiments of scaffolds with various chitosan concentrations. From the diagram, the degradation rates of chitosan C-1%, C-2% and C-3% all increase as time increases; moreover, degradation would decelerate slightly as chitosan concentration increases. Take chitosan scaffolds C-2% as example, first 15 days presents fast degradation rate nearly in 45° angle, but the degradation decelerate after 15 days, and almost horizontal when degradation rate reaches 20%. Possible explanation for enzyme degradation rate of chitosan scaffolds were only about 20% are related to the function active sites on lysozyme. Its function active sites are primarily β-(1–4)-glycosidic bonds between polysaccharide. The chitosan used in this research has about 80% degree of deacetylation. Because it only has about 20% β-(1–4)-glycosidic bond, the chitosan scaffolds reach equilibrium when it has 20% degradation rate. Fig. 8 shows SEM micrograph of chitosan scaffold (2% Chitosan, 4000 rpm, 5% NaOH) after

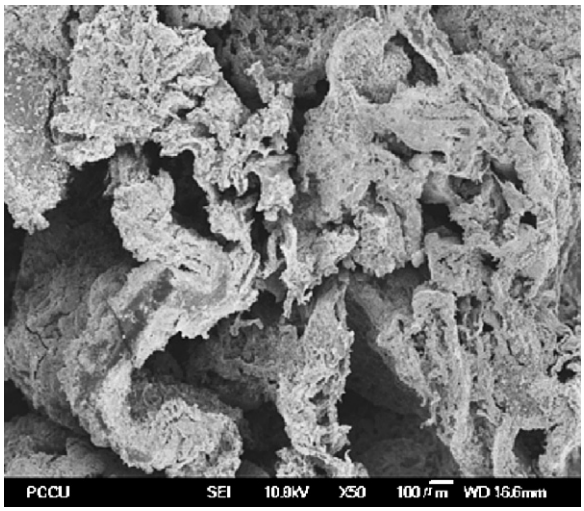

Fig. 8. SEM micrograph of chitosan scaffold after 20 days enzymatic degradation at 37 °C (2% chitosan, 4000 rpm, 5% NaOH).

20 days enzymatic degradation at 37 °C. As you can see from the picture, after enzyme degradation for 20 days the chitosan scaffolds obviously has partially disintegrated on the surface.

4. Conclusion

In this research we create a simple liquid hardening method to manufacture 3D chitosan porous materials. The main purpose is to investigate the feasibility of chitosan scaffolds application in cell culture of tissue engineering. The following conclusions were reached.

(1) With chitosan concentration in 1–3% range, chitosan porous scaffolds could obtain good formability. When chitosan concentration is smaller than 1%, there's insufficient viscosity thus scaffolds forming is not good, on the other hand, chitosan concentration higher than 3% the viscosity too high to cause difficulty for formation.
(2) The pore size and porosity will reduce along with the increase of chitosan concentration. Higher chitosan con-

centration is easier to obtain smaller pore diameter foaming bubbles. The pore size and porosity also will reduce along with the increase of homogenizer's stirring rate. But when the stirring rate is greater than 4000 rpm, the pore size change of chitosan scaffolds is minimal.

(3) The higher the chitosan concentration, the lower the water absorption of chitosan scaffolds; similarly, increase the homogenizer's stirring rate also reduce the water absorption of the chitosan scaffolds. By changing the concentration of chitosan and homogenizer's stirring rate, the water absorption of chitosan scaffolds samples can be controlled.

(4) From the mechanical property test of the chitosan scaffolds, we could see that the compressive strength of chitosan scaffolds increases along with chitosan concentration increases; in addition to that, the compressive strength of chitosan scaffolds also increases along with homogenizer's stirring rate.

(5) In the enzymatic degradation tests, weight loss of chitosan scaffolds increases along with time of degradation, but stop further weight loss when the loss reaches 20% of chitosan scaffolds weight.

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