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Swelling behavior of gelatin-based hydrogel cross-linked with microbial transglutaminase

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Abstract: Hydrogels are chemically or physically crosslinked hydrophilic networks which do not dissolve in water but swell considerably in an aqueous medium. Since their response to changing environmental conditions such as temperature, pH and solvent composition, these materials have been attracting much attention in pharmaceutical, medical and mechanical engineering fields. At present, majority of hydrogels are prepared from a limited number of synthetic polymers and their derivatives such as copolymers of methacrylic acid, acrylamide and N-isopropylacrylamide, which are of more or less toxicity. Recent advances in hydrogel technology have been focused on finding more biocompatible, non-toxic materials intended for pharmaceutical and biomedical applications. Gelatin is a biocompatible protein substance derived from collagen, and when it is applied in living body, it shows low antigenicity and very high bioabsorptivity. Although the natural materials are more biocompatible than synthetic polymers, the use of toxic cross-linking agent (e.g. gluteraldehyde or formaldehyde) is still a threat. Transglutaminase is an enzyme that catalyzes an acyltransfer reaction which induces a crosslink between lysine and glutamine residues, thus the polymerization of proteins can be achieved as a result of the formation of intermolecular or intramolecular e-(g-glutamyl) lysine bonds. In this work, a novel gelatin-based hydrogel was prepared using gelatin in the presence of microbial transglutaminase (mTG). The swelling behavior of the hydrogels obtained with different Bloom value gelatins was measured. Subsequently, the swelling kinetics was investigated. The swelling ratios in various salt solutions (NaCl, $CaCl_2$, $FeCl_3$, $CrCl_3$) and at different temperatures were also determined. Additionally, the swelling of hydrogels was measured in solutions with pH ranged 1-12. Finally, the morphology of the samples was examined by scanning electron microscopy (SEM).

Keywords: Gelatin-based hydrogel; Microbial transglutaminase; Swelling behavior

1 Introduction

Hydrogels are chemically or physically crosslinked hydrophilic networks which do not dissolve in water but swell considerably in an aqueous medium. The use of hydrogels as biomaterials has recently gained great interest in view of ease of fabrication , good viscoelastic properties and high biocompatibility[1]. Hydrogel has many biomedical and bioengineering applications such as drug delivery, wound care material, and tissue engineering, etc . They can be made from chains of natural polymers such as collagen, alginate or from synthetic polymers such as poly (vinyl alcohol) (PVA) or poly(acrylic acid) (PAA) or from their hybrids. Because of more or less toxicity [2, 3] of synthetic polymers, recent advances in hydrogel technology have been focused on finding more biocompatible, non-toxic materials intended for pharmaceutical and biomedical applications. The most abundant naturally occurring polysaccharides such as cellulose, starch, alginates and protein [4-6] have attracted medical and pharmaceutical interests.

Gelatin is composed of polydisperse polypeptides obtained through either acid, alkaline and mixed processes from different collagen types present in natural sources, like bovine hides and pig and fish skins. It is a biocompatible protein substance, and when it is applied in living body, it shows low antigenicity [7] and very high bioabsorptivity. In the case of gelatin, physical cross-linking by heating and ultraviolet irradiation and chemical crosslinking by several agents such as formaldehyde, glutaraldehyde water-soluble carbodiimide, diepoxy compounds, diisocynate, and dextran dialdehyde have been performed. Although the natural materials are more biocompatible than synthetic polymers, the use of toxic cross-linking agent (e.g. gluteraldehyde or formaldehyde) is still a threat. Microbial Transglutaminase (mTG) is most often used as crosslinking agent in enzymatic modifications of protein-based hydrogels [8]. The enzyme could catalyse the formation of the covalent bonds between γ-carboxyamide groups of peptide-bound glutamine residues and ε-amino groups of lysine or primary amino groups of the peptide chain.

This research intended to prepare a novel gelatin-based hydrogel using microbial transglutaminase (mTG) as cross-linking agent, and evaluate the swelling behavior of the hydrogels obtained under different preparation conditions, in solutions with pH ranging from 1 to 12, in various salt solutions and different temperatures.

2. Materials and method

2.1 Materials

The source of transglutaminase was a commercial product obtained from Yiming Biological Products Co., Ltd (JiangSu, China). As determined by a colorimetric hydroxamate method [9], the enzyme activity of mTG was 102 U/g of powder. Three kinds of gelatins with different Bloom strength were provided by Sigma (300 Bloom), a gelatin factory in Qufu, Shandong, China (172 Bloom), and Tianjin Chemical Reagent CO., LTD (90 Bloom), respectively. All other regents used were of analytical grade.

2.2 Preparation of gelatin-based hydrogel

Gelatin powder was mixed with distilled water to obtain a final concentration of 8% (w/v). The mixture was left at room temperature for 2 h to allow the gelatin to absorb water and swell, and the mixtures were then incubated at 45°C for 30 min in a temperature-controlled water bath with occasional stirring. The pH was adjusted to 6.5±0.1 with 0.2 mol/L NaOH. The mTG (4 U/g gelatin) was added after total dissolution of the dry gelatin in water. The gelatin-mTG solutions were incubated at 45°C for 4 h, followed by a heating step of 90°C for 5 min to inactive the mTG, and the gelatin-based hydrogel was formed. The hydrogel was cut into thin slices approximately 50 mm in length, 10 mm in width, with a thickness of 1.5~2 mm.The hydrogel slices were extensively washed with distilled water to remove slats and uncrosslinked gelatin chains. Dry hydrogels were obtained by keeping the wet hydrogel slices in a well ventilated place at room temperature for 3 days. Hydrogel slices were then weighed and placed into a vacuum oven at 60ºC for 24 h and then re-weighed. Virtually no change in dry hydrogel weight was found following vacuum drying. In this paper, all the hydrogel samples were preparation with the gelatin of 172 Bloom except the sample which was used to investigate the effect of Bloom strength on swelling capacity.

2.3 Swelling measurement

The swelling measurement of hydrogel was carried out as follows. Pieces of xerogel were immersed into 250 ml distilled water. The samples of swollen hydrogel were weighed after removal of surface water using filter paper at designed time intervals. Data presented in this experiment were the mean values of triplicate measurements. Results were calculated according to the following

equation:

$$Q = \frac{W_s}{W_d} \qquad (1)$$

Where W_s is the mass of the hydrogel in the swollen state, W_d is the mass of the hydrogel in the dried state and Q is equilibrium swelling ratio.

2.4 Influence of Bloom strength on swelling property

To investigate the effect of Bloom strength on hydrogel swelling, three kinds of gelatins with different Bloom (90, 172 and 300) were used. The methods of hydrogel preparation and swelling measurement were employed as described above. Pieces of xerogel were immersed in 250 ml distilled water at 25 °C. The samples of swollen were weighed after removal of surface water using filter paper. All samples were monitored in this way until the swelling equilibrium was reached.

2.5 Swelling at various temperature

The effect of temperature on swelling ratios was determined by placing samples in six temperature controlled water baths and equilibrated for 24 h. The temperature of the water baths were controlled at 25°C, 30°C, 35°C, 40°C, 45°C and 50°C, respectively.

2.6 Swelling at various values of pH

Individual solutions with acidic and basic pH values were prepared by dilution of NaOH (pH 12.0) and HCl (pH 1.0) solutions to achieve pH P 6.0 and pH < 6.0, respectively. The pH values were precisely checked by a pH-meter (Hanna Bench/pH 211, accuracy ±0.01). Then, the swelling ratios were measured at 25 °C and equilibrium time 12 h according to 2.3.

2.7 Swelling in various salt solutions

Hydrogel swelling was evaluated in solutions of 0.5 M NaCl, 0.25M $CaCl_2$, 0.167 M $FeCl_3$ and 0.167 M $CrCl_3$ according to the above method described for swelling measurement in distilled water. In addition, swelling ratio of the hydrogel was measured in different concentration of NaCl range from 0.05 to 0.5M. The measurement temperature was 25°C and the equilibrium time was 12 h.

2.8 Scanning electron microscopy

A hydrogel samples which had been swollen in distilled water in swelling measurement was selected. And it was dehydrated using a Freeze Dryer (Christ Alpha 1-2, German). The dehydrated sample was imbed in liquid nitrogen and randomly broken in order to investigate the cross-section surface of the sample. The sample was examined using an environmental scanning electron microscope (FEI Quanta 200, Holland) at 20 kV after sputter-coated with gold.

3. Results and discussion

3.1 Effect of Bloom strength

The gel strength is one of important criteria which determine the quality of gelatin as required by manufacturers and users. It indicates the hardness, stiffness, firmness and compressibility of the gel at a particular temperature and concentration. It is associated with the contents of proline and hydroxyproline in gelatin and molecular weight [10]. In general, a high molecular weight gelatin gives a high Bloom value[11].

In our experiment, hydrogel could not be obtained with the gelatin of 90 Bloom at gelatin concentration of 8% (w/w). It was supposed that molecular weight plays an essential role in the

formation of hydrogel cross-linked with mTG. We supposed that it is difficult to develop a firm network formation for low molecular weight protein chain, so as to hydrogel can not be formed. Swelling behavior of gelatin hydrogels obtained with 300 Bloom and 172 Bloom was shown in Fig. 1(a). The results clearly show that the Bloom strength has a significant influence on the swelling behavior of hydrogel. The swelling capability of 172 Bloom gel (about 14 in swelling equilibrium) was much higher than that of 300 bloom gel (about 5 in swelling equilibrium). Lower swelling ratio was observed at higher Bloom strength. This is most likely due to a higher molecular weight gelatin chain, which are able to interact with one another and are prone to form a more compact network, and hence a lower water holding capacity. In addition, the swelling equilibrium time between the two gels was also significantly different. As for 300 bloom gel, the swelling equilibrium time was about 4 h, compared to about 10h of 172 Bloom gel.

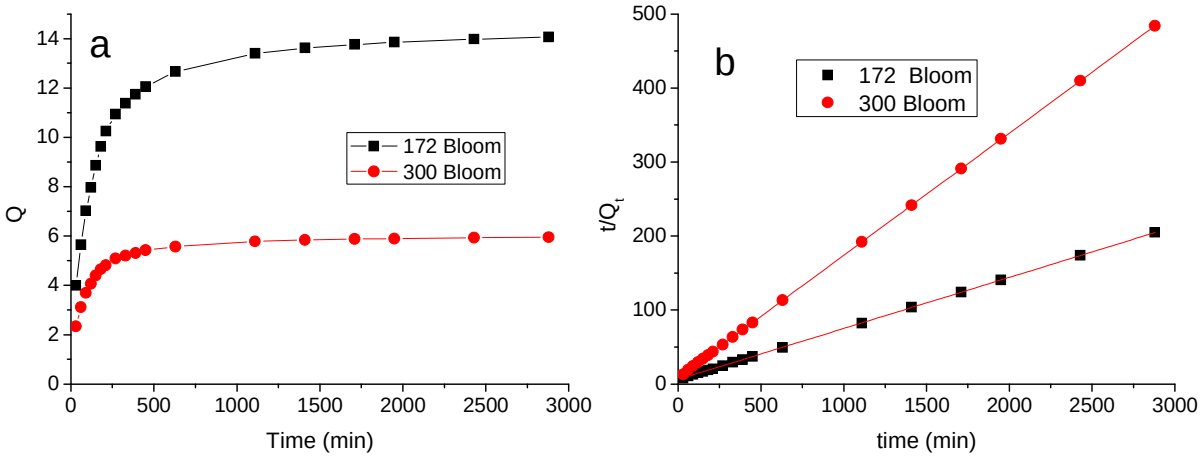

Fig.1 The swelling ratio Q (a) and t/Q_t (b) of gelatin-based hydrogels obtained with different Bloom strength gelatins versus the swelling time.

3.2 Swelling kinetics

As can be seen from Fig.1 (a), the swelling process could be divided into two stages: the fast swelling occurring before 10 h and the equilibrium swelling occurring after 10h. The second order swelling kinetic theory proposes that the swelling rate of a hydrogel is controlled by both the diffusion of solvent molecules and the relaxation of macromolecule chains [12], i.e., the swelling rate is directly proportional to the square of the remaining swelling capacity $(Qe–Q)^2$. Thus:

$$\frac{dQ_t}{dt} = K(Q_e - Q_t)^2 \qquad (2)$$

Equation (2) is integrated between the limits Q=0, when t=0 and Q for t and rearranged. The Schott's equation is obtained:

$$\frac{t}{Q_t} = \frac{t}{Q_e} + \frac{1}{K Q_e^2} \qquad (3)$$

Where Q_t is the swelling ratio of the gel at time t, and Q_e is the equilibrium swelling ratio of the hydrogel. K is the rate constant.

Fig.1 (b) shows the plots of t/SR as a function of time t in three kinds of salt solutions. The

swelling kinetics of gelatin-based hydrogels is in good agreement with the Schott's equation (3).

3.3 Effect of temperature

The effect of temperature on the weight equilibrium swelling ratio in distilled water for 172 Bloom hydrogel is reported in Fig. 2(a) and Fig. 2(b). The equilibrium swelling ratio is significantly different over the temperature range investigated. The results indicate that the swelling ratio increases with increasing the temperature. Moreover, it also implies from Fig.2 (a) and Fig. 2(b) that the swelling rate is not significantly affected when the temperature exceeds 30°C. On the other hand, the swelling curve at lower temperature (25°C to 30°C) shows a much lower swelling rate. The observed results can be explained by the fact that when the temperature increases from lower to 30°C, the water sorption capacity increases significantly due to an increased segmental mobility of hydrogel chains. Several previous studies [13, 14] have investigated that gelatin gel involves formation of triple helical sequences connected in an essentially random fashion by peptide sequences in disordered conformation. The gelatin chains in hydrogel revert partially to the tropocollagenic triple helix, from disordered to rather ordered status, depending on temperature, concentration and rate of cooling. This phenomenon occurs usually at around 28°C, where the coil to helix transition is observed. And when mTG was introduced the covalent cross-link between γ-carboxyamide groups of glutamine residues and ε-amino groups of lysine residues was formed. Thus the resulting gelatin hydrogel is composed of both chemical and physical cross-links. The result is that parts of either three chains or two chains in a physical ''hair-pin'' bond type [15] are stabilized mainly through hydrogen bonds, forming thus a basic physical network coupled to the covalent one (Fig.3, left). As temperature increased to 30°C, the hydrogen bonds within tropocollagenic triple helix break (Fig.3, right), and a majority of triple helix disappear. Therefore, the hydrogel chains become more mobile and the swelling of hydrogel is increased.

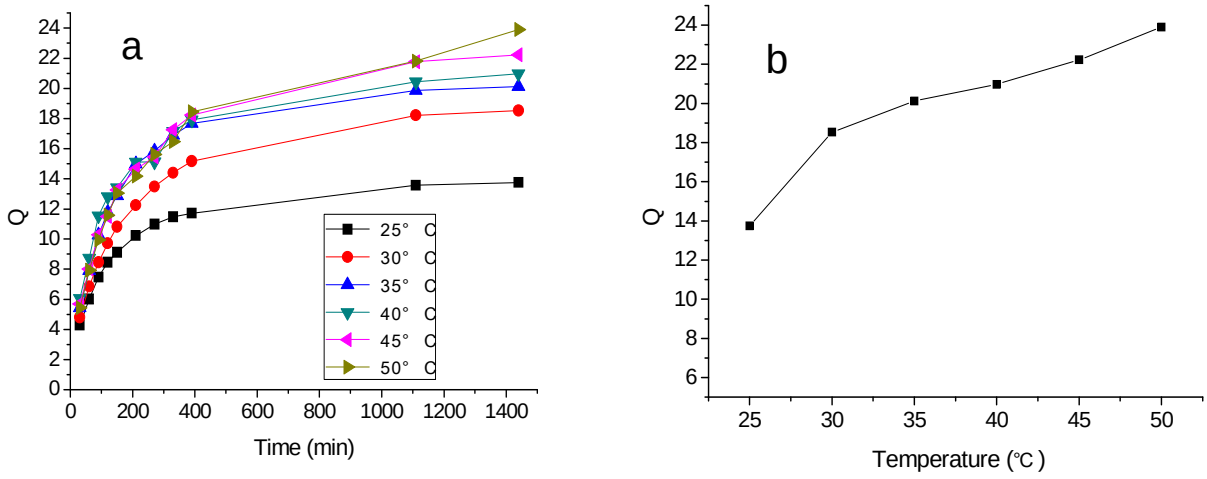

Fig.2 The effect of temperature on the weight equilibrium swelling ratio in distilled water. (a): Swelling behavior of hydrogels at different temperature. (b): Viability of swelling ratio immerging in distilled water for 24h at different temperature.

However, on increasing the temperature from 30°C to 50°C, the hydrogel chains must have acquired complete relaxation, so that with a further increase in temperature, they do not loosen and, as a consequence, no appreciable change in swelling behavior could be observed at a higher

temperature.

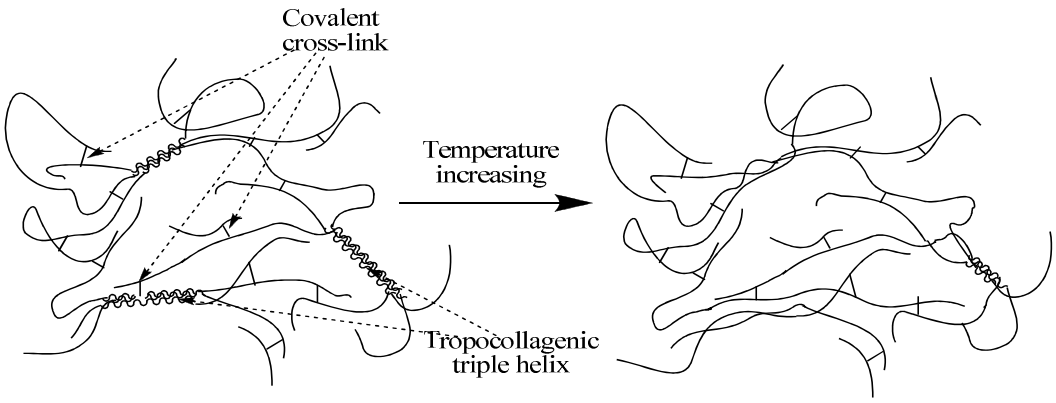

Fig.3 Scheme illustrating a gelatin hydrogel network.

3.4 Effect of pH

Ionic hydrogels exhibit swelling changes at a wide range of pH values. Since the swelling capacity of all "ionic" hydrogels is appreciably decreased by addition of counter ions to the swelling medium [16], without any buffer solutions.Therefore, a series solutions with acidic and basic pH values were prepared by dilution of NaOH (pH 12.0) and HCl (pH 1.0) solutions to achieve pH>6.0 and pH < 6.0, respectively.

Fig.4 shows the effect of pH on the swelling ratio of hydrogel. The results clearly indicate that the hydrogel exhibits extensive swelling in the swelling medium of pH<2 and pH>8, whereas the hydrogel demonstrates low degree of swelling in the medium between pH 3 to pH 7.

A polyampholytic gelatin hydrogel cross-linked with mTG presents a network that contains positive and negative ionizing groups. In acidic medium, gelatin acts as base and takes up H^+ ions from the medium forming-NH_3^+ and-COOH and proteins become positively charged. In alkaline medium, protein acts as an acid gives H^+, forming -COO^- and –NH^2groups and proteins become negatively charged.

In an acidic environment, the swelling is controlled mainly by the -NH_3^+, in basic medium by COO-, and between pH 3.0 and 7.0 by -NH_3^+ and COO-. In Fig. 4, we can observe that in basic medium the swelling is higher; this behavior is due to the presence of the hydrophilic functional groups (mainly COO-) in the gelatin structure. Moreover, the gelatin hydrogels begin hydrolyze in a pH close to 11.0, giving up carboxy groups, which also result in increasing of swelling ratio. The swelling datum of pH12 was not present in Fig.4 because the hydrolysis degree of hydrogel sample was too severe to measure the swelling ratio accurately.

3.5 Effect of salts

The swelling ratio is mainly related to the characteristics of the external solution, such as charge number, ionic strength and polymer nature, i.e. network elasticity, presence of hydrophilic functional groups, and extent of crosslinking density [17].

In the study, the electrolyte effect has been observed by adding univalents salts to the external solution in the concentration range 0.05M to 0.5 M and the results are depicted in Fig.5. It could be noted that the swelling ratio of the hydrogel increased in the concentration range investigated. This situation between the swollen hydrogel and the surrounding aqueous phase is usually described as a

Donnan equilibrium where the biopolymer network acts as its own membrane [18, 19] thus preventing the diffusion of the attached ionizing groups toward aqueous phase. Therefore, the osmotic pressure resulting from the mobile ion concentration difference between the gel and aqueous phase increased in a certain range of NaCl concentration, and consequently, the swelling ratio increased. A similar type of increase has also been reported by Baker [20] and Bajpai [21] in ampholytic and non-ionic acrylamide based hydrogels.

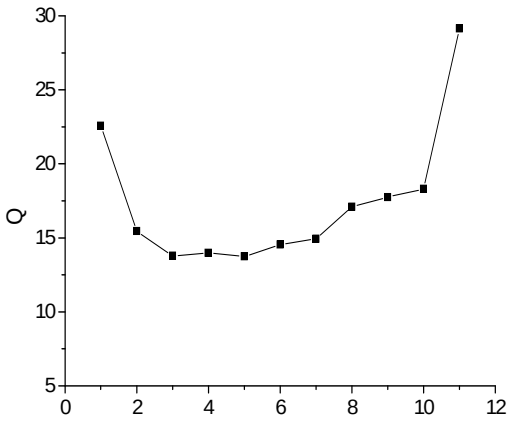

Fig.4 Effect of pH of solutions on swelling capacity of the hydrogel at 25°C for 12h.

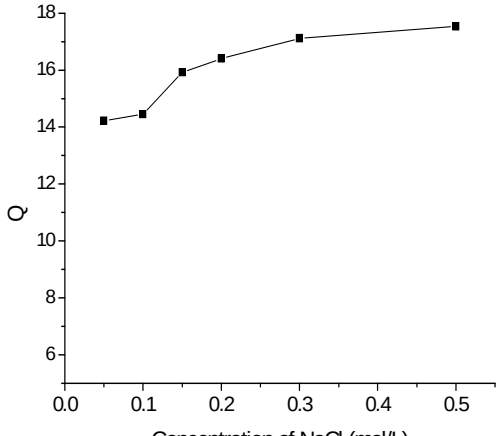

Fig.5 The swelling ratio of the hydrogel in NaCl solution with the concentration range 0.05M to 0.5M. 25°C for 12h.

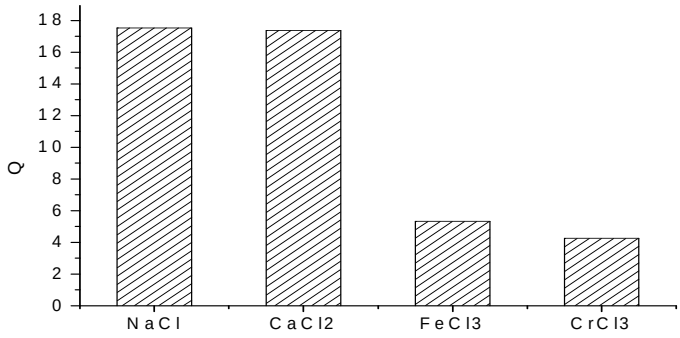

Fig.6 Swelling capacity of hydrogel in differentchloride salt solutions at 25°C for 12h.

The effect of cation type (cations with different radius and charge) on swelling behavior is shown in Fig. 6. From these data, a distinct effect on swelling ratio could be found for trivalent cations ($FeCl_3$, $CrCl_3$) comparing to monovalent (NaCl) and divalent ($CaCl_2$). Electrostatic screening effect and ionic crosslinking are the main explanations for the intense loss of swelling [17]. The electrostatic screening effect of salt can weaken the electrostatic interaction and de-swell hydrogel through causing a non-perfect anion-anion electrostatic repulsion[22]. As for ionic crosslinking, it is well known that gelatin hydrogels consisting of carboxylic groups can chelate with multivalent cations, such as Cr^{3+}, and Fe^{3+} ions, which leads to network contraction and decreases porosity of the gel network, therefore, results in the deswelling of hydrogels. It could be

noted from Fig.6 that $CaCl_2$ affect the swelling behavior of hydrogel nonsignificantly, and the swelling ratio was slightly higher than that in distilled water. Moreover, the swelling ratio increases when NaCl concentration range is from 0.05M to 0.5 M. Therefore, the effect of the ionic crosslinking acts as more effective factor against swelling rather than the electrostatic screening effect of the cation.

3.6 Morphology of hydrogel

A characteristic morphology of the freeze-dried samples of the water-swollen hydrogel was observed by scanning electron microscopy. The sample showed a relatively regular network structure with 20μm sized pores connected to each other, as shown in Fig.7. It is supposed that the pores are the regions of water permeation and interaction sites with the hydrophilic groups of the hydrogel chains. These pictures verify that gelatin-based hydrogel cross-linked with mTG have a porous structure, which assure the swelling capacity of the gelatin hydrogel.

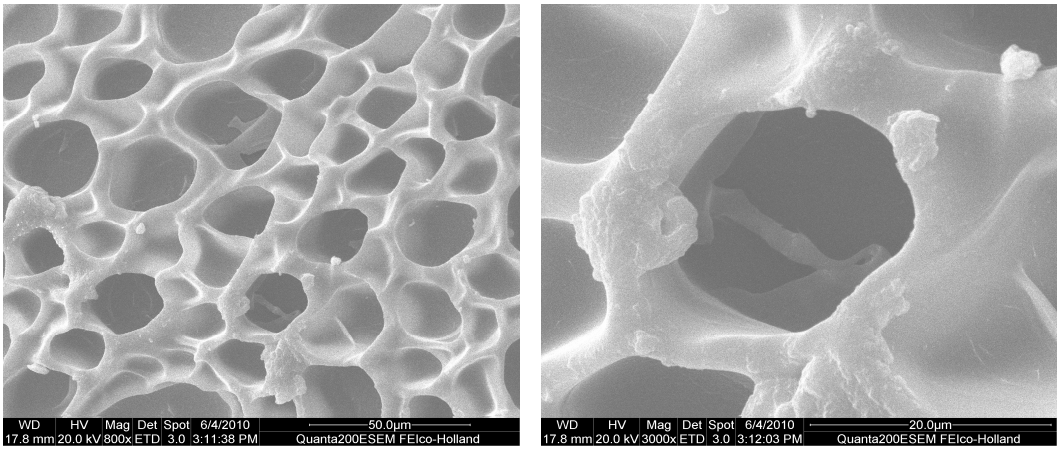

Fig.7 Scanning electron microscope images of transversal sections of swollen hydrogel dehydrated with freeze drying method. left: 800×; right: 3000×

4. Conclusions

In this paper, a gelatin-based hydrogel cross-linked was synthesized using microbial transglutaminase as cross-linking agent. The Bloom strength has a significant influence on the swelling behavior of hydrogel and the higher the Bloom strength the smaller the swelling capacity. The swelling kinetics of gelatin-based hydrogels is in good agreement with the Schott's equation. Due to the tropocollagenic triple helix structure including in gelatin-based hydrogel, the swelling of hydrogel is increased dramatically when the temperature increased from 25°C to 30°C. The curve of swelling ratios on the function of pH at 25°C is saddle-like, and in basic medium the swelling is higher contributed to the presence of the hydrophilic functional groups and the hydrolysis of gelatin hydrogels. An increase in the ionic strength (NaCl) of the swelling medium from 0.05M to 0.5M resulted in an increase in the degree of swelling. In addition, the influence of trivalent cations ($FeCl_3$, $CrCl_3$) on swelling ratio is more significant than monovalent (NaCl) and divalent ($CaCl_2$) because of the ionic crosslinking. The gelatin-based hydrogel had a certain swelling capacity and swelling ratio because of the high porosity and interconnection among some of the pores within the hydrogel that approved by the SEM images.

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