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Studies on Glutaraldehyde Crosslinked Chitosan Hydrogel Properties for Drug Delivery Systems

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Chitosan was crosslinked with different amount of glutaraldehyde to prepare appropriate hydrogels to be used as drug delivery system. The swelling behavior of freeze-dried hydrogels in aqueous media at different temperature and pHs has been examined. The swelling, porosity and biocompatibility behavior of samples were investigated to check effects of polymer/polymer and polymer/drug interactions on these system characteristics. Obtained experimental results illustrates that with increasing crosslinking agent from 0.068 to 0.30, swelling of the prepared samples degrees from 1200% to 600% and pore diameters change from 100 to 500 μm . To investigate systems biocompatibility in gastric conditions, effects of crosslinker concentration on the pepsin enzyme activity have been studied using variation of relative viscosity of the system. Presented results also show that with increasing crosslinker agent concentration activity of enzyme reduces considerably and so crosslinker molar ratio to amine functional groups of chitosan must be less than 0.2.

Keywords: Amoxicillin, chitosan, drug delivery, glutaraldehyde, hydrogel

1. Introduction

Chitosan is the deacetylated derivative of chitin (Figure 1) which is a water insoluble polymer, (N-acetyl-D-glucosamine), found in nature, present in insect exoskeletons, outer shells of crabs, shrimps, lobsters and fungal cell walls etc. The difference between chitin and chitosan chemical structures lies in the degree of deacetylation [1,2].

Chitin and chitosan, after cellulose, are the second most plentiful natural biopolymers [3]. As shown in Figure 1, chitosan is a linear glucosamine biopolymer possessing an average molecular weight of 120000. Chitosan is a (1,4)-linked 2-amino-2-deoxy- β -D-glucan and can be prepared by N-deacetylation of chitin. Usually chitosan is not a homopolymer of D-glucosamine, but a copolymer containing less than 40% N-acetyl-D-glucosamine residues. Chitosan exhibits properties that make it desirable for biocompatible and blood-compatible materials with many superior properties [4]. It has been reported that chitosan plays a critical role in attachment and growth of selected cells: epithelial cells, vascular endothelial cells, and fibroblasts. However it was also shown that chitosan promotes surface-induced thrombosis and embolism. Moreover chitosan and chitin (as other natural polymers) are biodegradable in vivo by chitinase and lysozyme [5].

Chitosan hydrogels can be produced by crosslinking of chitosan macromolecular. These hydrogels can easily swell

in water or biological fluids therefore they have become a potential candidate for carriers of bioactive macromolecules, wound dressing, and controlled release of drugs in their swollen state. The most commonly used crosslinking agent is glutaraldehyde. Orton et al. [5], David et al. [6], and Keelara et al. [7] studied chitosan hydrogel behaviors such as crosslinking density and swelling in laboratory conditions (25°C and pH 7). However, they have not studied their hydrogel swelling behavior in gastric condition (37°C and pH 1.2), which is necessary if we want to use these hydrogel as matrix for antibiotic release control systems. In addition, their experiments have been done in absence of drug, which can deeply affect hydrogel crosslinking, porosity, and swelling behavior. Gupta et al. [8] have also done different research on glutaraldehyde and glyoxal crosslinked microspheres for sustained release of centchroman under physiological conditions.

To extend application of chitosan hydrogels for new application especially for oral usage control release systems, we have developed freeze-dried and crosslinked chitosan hydrogels containing amoxicillin antibiotic using different concentration of glutaraldehyde which acts as crosslinking agent. Effects of crosslinking density, porosity, pH, temperature, and crosslinker concentration on biocompatibility of hydrogel have been also studied.

2. Experimental

2.1 Materials

Chitosan with 85% degree of deacetylation was purchased from Sigma. Glutaraldehyde 25% solution in water was

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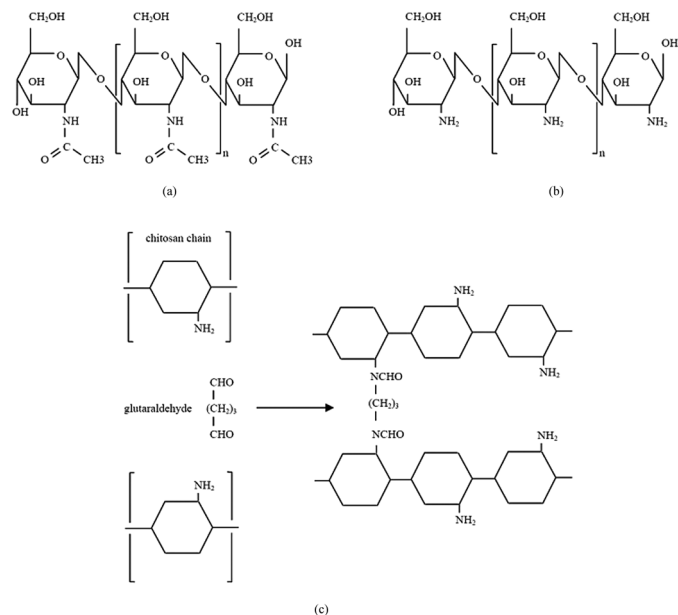


Fig. 1. Structure of (a) chitin and (b) chitosan, as well as (c) crosslinked with glutaraldehyde.

obtained from Merck (Schuchardt). Amoxicillin trihydrate was obtained from Zakariya Co. (Pharmacist). Acetic acid and hydrochloric acid were both purchased from Merck. Dried milk was obtained from Biomill [6,7].

2.2 Preparation of Chitosan Solutions

Chitosan was dissolved in 1% aqueous acetic acid at room temperature and left overnight in the shaker with rotation rate of 250 rpm to obtain a 1% (w/v) solution. The viscous, pale yellow chitosan solution was filtered through the filter paper to remove any undissolved and impure matters.

2.3 Hydrogel Preparation

1% aqueous solution of glutaraldehyde in different mole ratios was added to the clear, pale yellow chitosan solution. The solution was stirred for 30 min at room temperature until it became increasingly viscous. The viscous solution was poured into a Petri dish. Finally, gels were freeze-dried. Five samples with different concentration of crosslinker as presented in Table 1 are prepared. After freeze-dried process, all samples present foam structure but with different porosity diameter size.

2.4 Scanning Electron Microscopy Studies

Surface morphology of the films was studied using a scanning electron microscope (SEM). The individual dried films were gold coated (gas pressure of 0.5 mbar, current of 10 mA, and coating time of 200 s) and the SEM micrographs were observed at 20–25 kV (JEOL, model JXA-840).

2.5 Swelling Experiments

The swelling behavior of the crosslinked and noncrosslinked freeze-dried hydrogels was measured by swelling the films in

Table 1. Composition and insigne chitosan: glutaraldehyde hydrogels

Insigne	Volume of chitosan (cm ³)	Volume of crosslinker (cm ³)	Molar ratio (chitosan: crosslinker)
Uncrosslinked chitosan (G0)	25	0	1:0
Uncrosslinked chitosan (G1)	25	1	1:0.068
Uncrosslinked chitosan (G1.5)	25	1.5	1:0.102
Uncrosslinked chitosan (G3)	25	3	1:0.205
Uncrosslinked chitosan (G4.5)	25	4.5	1:0.307

media of different pHs (desirable swelling at simulated gastric fluid [SGF]; pH 1.2) at temperature of 37°C and in deionized water. Freeze-dried hydrogel films (approximately 100 mg) were floated in buffer solutions ranging from pH 2 to 7. The immersion time was 120 min. The films were withdrawn from the solutions at different time intervals and their wet weight was determined after preliminary blotting with a filter paper followed by blowing with a stream of air to remove the surface water and immediately weighing the films. The swelling ratio (% SW) was determined according to the following formula:

$$SW (\%) = ((W_s - W_d) / W_d) \times 100\% \quad (1)$$

where SW is the water absorption (%wt) of the films, W_d and W_s are the weights of the samples in the dry and swollen states, respectively. The equilibrium water content (EW) was calculated from the following equation:

$$EW (\%) = ((W_e - W_d) / W_e) \times 100\% \quad (2)$$

where W_e represents the weights of the swollen state at equilibrium.

2.6 Characterization of Hydrogels by Infrared Spectroscopy

A Mattson FTIR Paragon 1000 (MB Series) infrared spectrometer was used to investigate progress of crosslinking of chitosan by glutaraldehyde. The scan range was 500–4000 cm⁻¹. Each spectrum was automatically averaged over 3 scans obtained at a spectral resolution of 4 cm⁻¹. Samples were thoroughly ground with exhaustively dried KBr and pellets were prepared by compression under vacuum. A sample cup filled with dried KBr powder was used as the background dataset.

2.7 Pepsin Activity Assay

The first effect of pepsin on protein solutions is a very rapid decrease in viscosity accompanied by a slower decrease in the quantity of nitrogen present in the form of protein and by

a still slower increase in the number of titrable carboxyl (or amino) groups. All of the previously mentioned parameters can be used for determination of pepsin activity or biocompatibility of system but viscosity measurements are advantageous due to rapidity and accuracy of measurements. So, effects of the pepsin on the viscosity of protein solutions were used to determine the cytotoxicity of hydrogels. In this assay, G0 was used as control [11].

2.7.1 Selection of Protein and Standard Protein Solution

Gelatin, edestin, casein, and a standardized solution of dried milk can be used for the activity tests because these solutions represent widely different classes of proteins and are the most easily obtainable in preparations of more or less constant properties. However, we have used dried milk as protein source in this study. Solutions prepared from dried milk powder "Biomil" are quite reproducible when prepared from the same original sample; even they do not vary considerably when prepared from different samples. 50 gm. of the milk powder is stirred into 500 mL of water at about 40°C. 50 mL of M/1, pH 5 acetate buffer added, and the solution is kept in the ice box until usage time. Great care must be taken to avoid contamination with the slightest amount of active enzyme as exceedingly minute quantities of pepsin could cause significant changes in the viscosity of the solution. The specific viscosity of the solution should be about 1.7. This solution may be kept for several weeks in the icebox or several hours at 35.5°C without any variation.

2.7.2 Viscosity Measurements

Ostwald type viscosimeters are used for all viscosity measurements. Since the capillaries are rather fine in these instruments, we have filtered all solutions through hard filter paper to avoid blocking of capillary by any small particles including shreds of filter papers.

5 mL of the protein solution pipetted into a 50 mL test-tube and allowed to remain in the water bath until it has reached the temperature of the bath. 0.2 mL of the solution to be tested is then added to the 5 mL protein solution and the time of addition is noted. The tube is shaken and contents poured into the wide arm of the viscosimeter. The liquid is drawn up into the bulb with gentle suction to avoid air bubbles and the time of the outflow measured with a stopwatch.

The specific viscosities corresponding to these readings are then calculated using the following equation.

$$\text{Specific Viscosity} = \frac{t''_s}{t''_{H_2O}} - 1 \quad (3)$$

Although different enzyme activity can be defined, we use one of them that used by Northrop et al. [11]. According to this definition one unit of activity is defined as a change in the specific viscosity of the standard protein solution at 35.5°C, at the rate of 1% per minute, and the quantity of material possessing one unit of activity is the quantity which will cause this rate of change in the viscosity when contained in 5.2 mL of the standard digestion mixture. This value is determined from the initial slope of the viscosity-time curve according to Equation (4). The activity is expressed as percent change

since this value is nearly independent of the protein concentration. The temperature is fixed at 35.5°C.

$$\text{Pepsin Unit} = [\text{P.U.}]^{\text{protein}}$$

$$= \text{Change in viscosity per min.}\% = \frac{100(t''_{\theta_0} - t''_{\theta_t})}{(t''_{\theta_0} - t''_{H_2O})t} \% \quad (4)$$

where t''_{θ_0} is outflow time for solution at beginning, which is determined by extrapolating; t''_{θ_t} is outflow time for solution at time t ; t''_{H_2O} is outflow time for water; t is time in minutes since enzyme solution is added; and t''_s is outflow time for test solution.

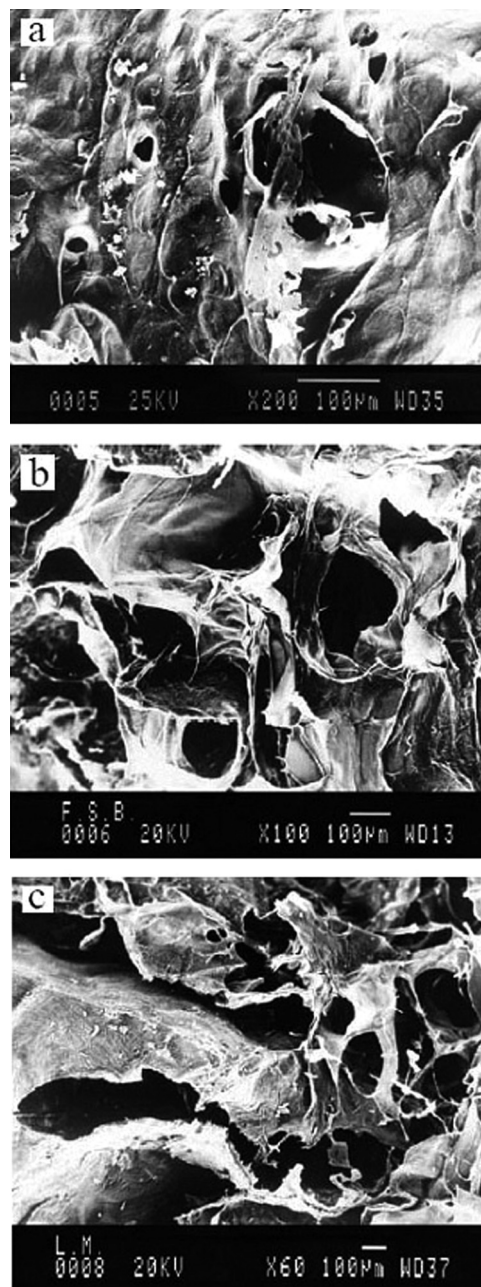


Fig. 2. Scanning electron microscopy of freeze-dried chitosan-based hydrogels.

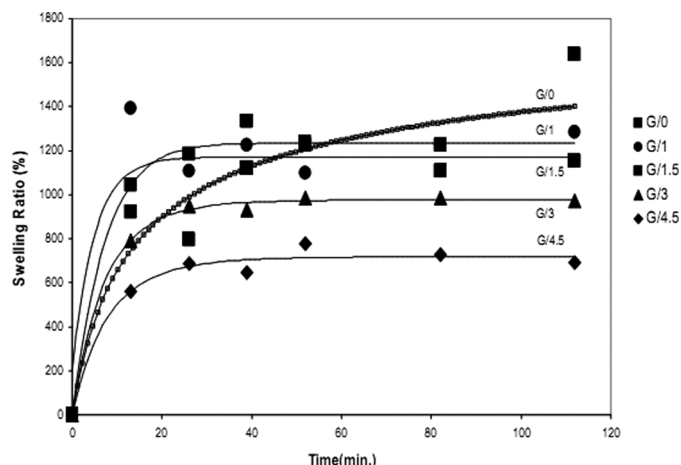


Fig. 3. Swelling behaviors of chitosan hydrogels at 37°C and pH 7.

3. Results and Discussion

3.1 Scanning Electron Microscopy

The degree of crosslinking affects on bulk and surface morphology of freeze-dried hydrogels. Scanning electron microscopy has been used for investigate morphology and porosity of produced crosslinked chitosan hydrogels samples. SEM photos show an increase in the pore average size from 25 μm for non-crosslinked sample to 500 μm for G/3 samples. Also, hydrogels prepared with pure chitosan (G/0) are smooth in morphology (see Figure 2) comparing to those of crosslinked with glutaraldehyde [9]. This difference in morphology might elucidate the distinction in swelling behavior and drug release of hydrogels in SGF. As shown in Figure 2, distribution of pore size, consists of 25 $\mu\text{m} < D < 100 \mu\text{m}$ for G/0, 100 $\mu\text{m} < D < 300 \mu\text{m}$ for G/1.5, and 200 $\mu\text{m} < D < 500 \mu\text{m}$ for G/3. According to Figure 2, increase in extent of crosslinker causes the growth of pore size and decrease in distribution of them. Also the contact surface of hydrogel and SGF decreased (that is important element in diffusion of SGF into hydrogels), that is elucidate the swelling decrease, with increasing the extent of crosslinker.

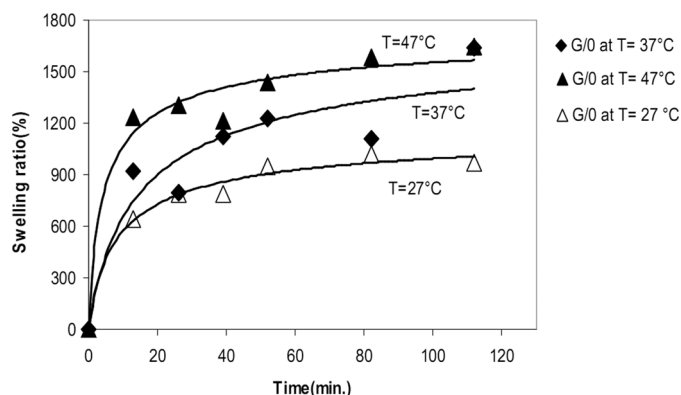


Fig. 4. Swelling behaviors of G/0 at pH 7 and various temperatures.

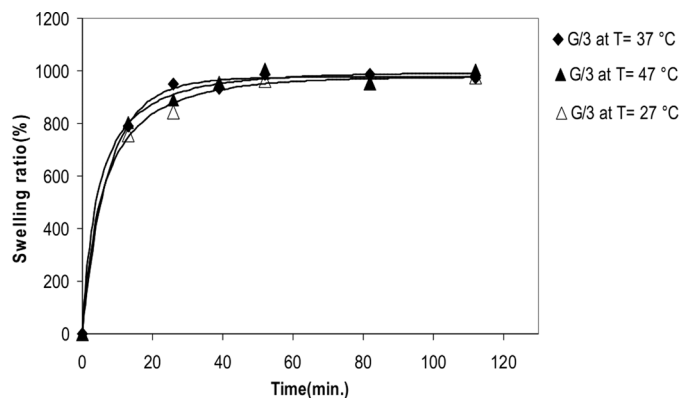


Fig. 5. Swelling behaviors of G/3 at pH 7 and various temperatures.

3.2 Swelling Study

All swelling behavior is plotted on the average of three trials. The time-dependent swelling behavior of chitosan hydrogels in deionized water (pH 7) at 37°C are given in Figure 3. As shown in Figure 3, the G/0 sample has the highest swelling ratio, while G/4.5 has the lowest. Since the G/0 was not crosslinked, the polymer chains could freely move where increase in the extent of crosslinker caused restricting the movement of the polymer chains, and can prospect that swelling ratio decreases with increasing in extent of crosslinker. The porous nature of the freeze-dried hydrogel may be responsible for offering a larger surface area and, therefore, a better matrix solvent interaction, allowing faster solvent diffusion and efficient and rapid swelling [10].

The temperature dependent swelling behavior of two hydrogels (G/0, G/3) in deionized water (pH 7) at 27°C, 37°C, and 47°C is shown in Figures 4 and 5. The swelling ratio for G/0 at 27°C was at its minimum and increased with temperature. Since the G/0 was not crosslinked, the polymer chains were flexible and increase in temperature caused breaking of secondary interactions, creating more space for water diffusion in the gel matrix and producing gel with more than 1400% water. This indicates that G/0 structure is becoming mechanically very fragile and easily fractured.

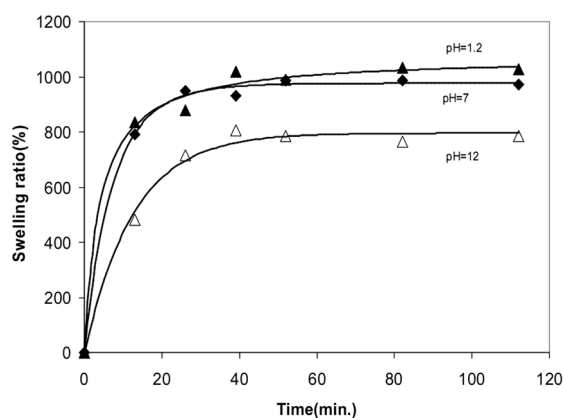


Fig. 6. Swelling behaviors of G/3 at 37°C and various pH.

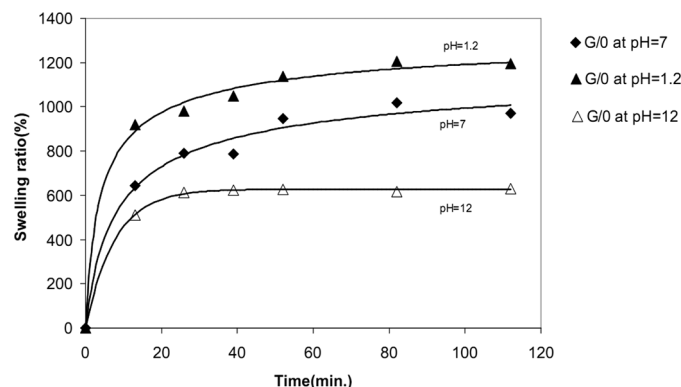


Fig. 7. Swelling behaviors of G/0 at 37°C and various pH.

This sample reached equilibrium swelling up to 120 min. However, the swelling ratio for G/3 remained relatively unchanged at the previously mentioned temperatures (see Figure 5). Certainly, induced crosslinking in this gel have reduced chain flexibility, and reduced water absorption and water absorption dependent to temperature. The swelling kinetics show that G/3 sample attained equilibrium in approximately 30 min at all temperatures. That is too rapid comparing to G/0 gel.

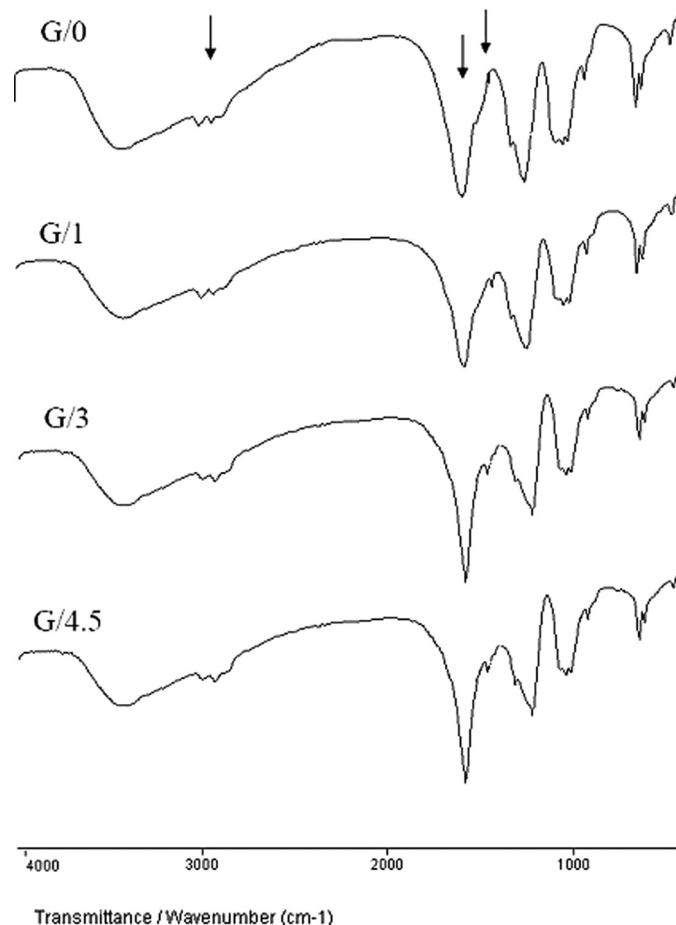


Fig. 8. FTIR spectra of freeze-dried hydrogels (G/0, G/1, G/3, G/4.5).

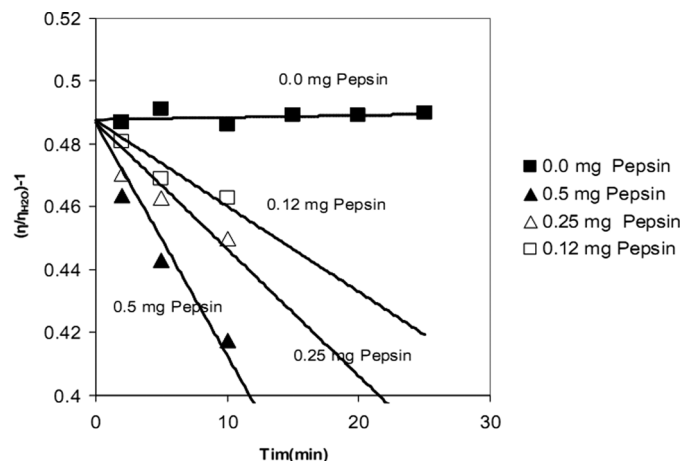


Fig. 9. Change in specific viscosity of milk, with different quantities of crystalline pepsin.

Effects of pH on the swelling behavior of two hydrogels (G/0, G/3) at pH 1.2, 7, and 12 are summarized in Figures 6 and 7. These figures show that hydrogels swell more in acidic pH than basic one (temperature unlike). These figures also show that swelling ratio of all hydrogels decreased as the pH increased and maximum swelling for all samples happen at minimum pH. Figure 6 shows that swelling ratio of G/3 decrease gradually over the entire pH range but swelling ratio of G/0 does not show significant changes until pH 7 and decreases drastically at pH above 7. At low pH, protonation of the amino groups of chitosan takes place. This leads to repulsion in the polymer chains, dissociation of secondary interactions such as intramolecular hydrogen bonding, allowing diffusion of more water into the gel network. As the pH increased, amino groups became deprotonated and repulsion in the polymer chains receded allowing shrinking. G/0 gel showed intense swelling and shrinking compared to G/3.

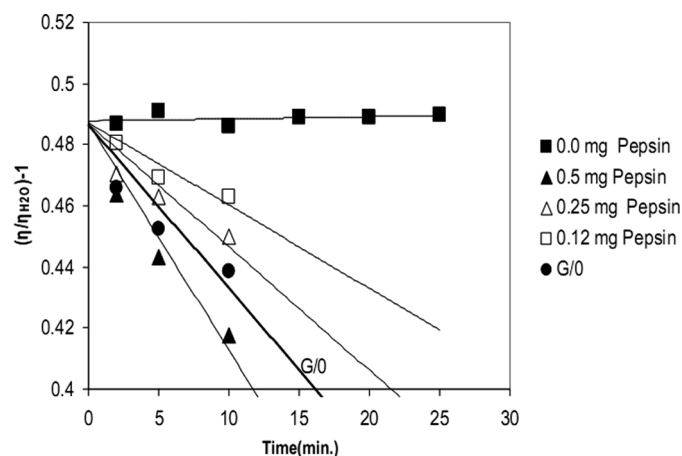


Fig. 10. Change in specific viscosity of milk that takes in G/0 vicinity.

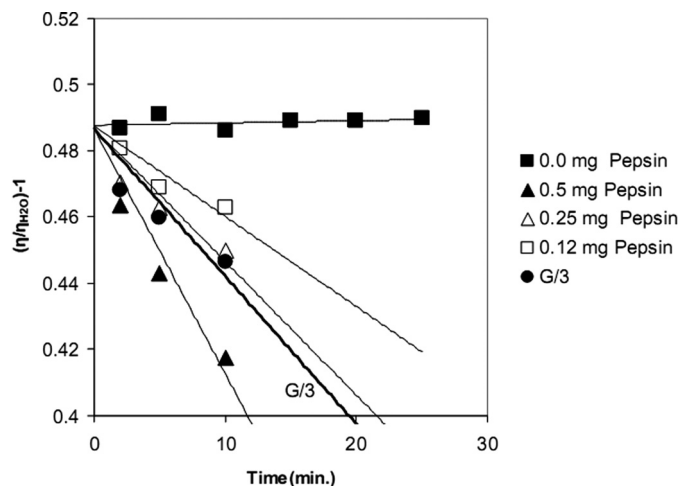


Fig. 11. Change in specific viscosity of milk that takes in G/3 vicinity.

3.3 IR Studies

Infrared spectra of all prepared hydrogels are shown in Figure 8. Comparison of these spectra shows that two new bonds appeared in samples with crosslinking agent. The first one at 1655 cm^{-1} can be attributed to an imine bond ($\text{N}=\text{C}$) and the second at 1562 cm^{-1} is associated with an ethylenic bond ($\text{C}=\text{C}$). On the other hand, there is no evidence of the characteristic bond related to free aldehydic group near 1720 cm^{-1} in the infrared. The increase of glutaraldehyde in the sequence of these crosslinked chitosans caused a successive increase in intensity of ethylenic bond frequency at 1562 cm^{-1} . The same behavior was observed for the C-H stretching vibration frequency at 2936 cm^{-1} . This fact can be attributed to the increase of the contribution of the glutaraldehyde molecule in the chitosan–glutaraldehyde reaction to cause an increase in the crosslinking chain. The spectra included in the G/3–G/4.5 interval of comparison are very similar, indicating that the chitosan glutaraldehyde reaction ended at the G/3 ratio.

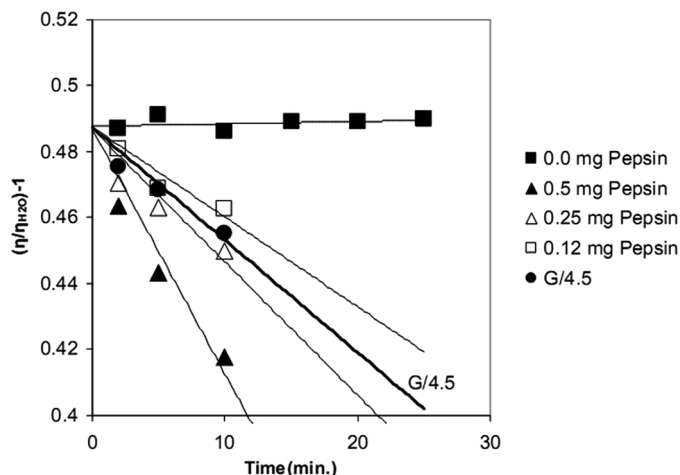


Fig. 12. Change in specific viscosity of milk that takes in G/4.5 vicinity.

Table 2. Pepsin activity result according to Eq. (3) (P.U. = a/t)

Insigne	a	r ²
0.0 mg Pepsin	0.8155	0.5829
0.12 mg Pepsin	2.7521	0.6770
0.25 mg Pepsin	4.7189	0.9571
0.50 mg Pepsin	6.6794	0.4001
G/A/0	5.8809	0.8643
G/A/1	6.0576	0.8908
G/A/1.5	7.8350	0.9936
G/A/3	5.2656	0.9572
G/A/4.5	3.7403	0.9419

3.4 Pepsin Assay Studies

Standard curves for various amount of enzyme, has drawed in Figure 9. In this figure, change in specific viscosity of milk is shown with different quantities of pepsin. These lines are used for biocompatibility reference of hydrogels.

Then, repeat this stage with tacking definite extent of enzyme on propinquity of hydrogels and elapse of definite time. The enzyme activity result (see Figures 10, 11, and 12) by Equation (3) that extract in Table 2. These data show that for the G/1, G/1.5, and G/3 samples, enzyme activity did not change. But for G/4.5 sample, pursuant to extent of a, decreasing in enzyme activity was illustrated. As a result of IR studies for G/4.5, we await further glutaraldehyde free molecules in its structure. That is effective on enzyme activity.

Therefore suitable and biocompatible sample molar ratio is lower than G/3 sample that all of glutaraldehyde reacted with chitosan and biocompatibility quantity for this confine is suitable.

4. Conclusions

In this research, after preparation of hydrogels, we tried to illustrate and survey parameters involved in actualizing of hydrogel use for drug delivery systems. Pore size of hydrogel which can significantly change gel swelling and so its drug delivery behavior, according to SEM results, increases as concentration of crosslinking agent increases. Also the contact surface of hydrogel and SGF decreased as concentration of glutaraldehyde increase. Many parameters have influence on hydrogel swelling, including crosslinker concentration, temperature, and pH. Scrutiny illustrates that increase in the extent of crosslinker caused restricting the relaxation of the polymer chains, and can prospect that swelling ratio decrease with increasing in extent of crosslinker. Crosslinker agent reduces chain flexibility and temperature effects. All prepared hydrogels show their maximum swelling at the lowest pH. Hydrogel samples with about 20 mol% crosslinker present the best swelling behavior for drug release and more concentration of crosslinking agent have negative effects on pepsin enzyme activity and so biocompatibility of hydrogel.

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