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Genipin-cross-linked chitosan-based hydrogels: Reaction kinetics and structure-related characteristics

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ABSTRACT: The main aim of this work is the synthesis and characterization of cross-linked chitosan systems. Chitosan hydrogels can be prepared by physical or chemical cross-linking of polymer chains. Chemical cross-linking, leading to the creation of hydrogel networks possessing improved mechanical properties and chemical stability, can be achieved using either synthetic agents or natural-based agents. In this work, the cross-linker Genipin, a naturally derived compound, was selected because of the lower acute toxicity compared to many other commonly used synthetic cross-linking reagents. In particular, the chemical stabilization of chitosan through genipin cross-linking molecules was performed and characterized by calorimetric analyses (differential scanning calorimetry), swelling measurements in different pHs, and ionic strength. The reaction kinetics was carried out by means of rheological measurements, and both the activation energy (E_a) and the reaction order (m) were calculated. The hydrogel analyses were carried out at different concentrations of genipin (GN1 and GN2). The results were used to evaluate the possibility to use the chemical cross-linked chitosan–genipin hydrogel for biomedical applications. © 2015 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2015**, *132*, 42256.

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INTRODUCTION

Chitosan is considered as a valuable natural biocompatible polymer because it is nontoxic, biodegradable,[1,2] mucoadhesive,[3,4] antimicrobial,[5] easily bioabsorbable,[6,7] and it also possesses gel-forming ability at low pH.[8] All the aforementioned properties make chitosan an ideal element for several biomedical purposes, e.g., formulation of drug delivery devices,[9–14] gene therapy,[15] gene delivery,[16] for sutures and wound healing materials,[17] skin culture,[18,19] vascular grafting,[20] vaccine delivery,[21–23] tissue regeneration,[24–28] and making contact lenses.[29] Besides pharmaceutical application, chitosan has been used in pollution control to remove toxic metals[30] and dyes,[31] in photography to improve quality of film,[32] in cosmetics[33] due to fungicidal and fungistatic properties, and as a food and nutrition supplement.[34,35]

Chitosan (CS) is a copolymer of β-(1–4)-linked 2-acetamido-2-deoxy-D-glucopyranose and 2-amino-2-deoxy-D-glucopyranose, generally obtained by alkaline deacetylation from chitin, a mucopolysaccharide forming the exoskeleton of crustaceans, such as shrimps and crabs (Figure 1).[36] Chitin is composed of β-(1–4)-linked 2-acetamido-2-deoxy-D-glucopyranose units and its *N*-deacetylation is carried out by alkaline hydrolysis with NaOH at 120°C for 1–3 h; in general, the treatment produces 40–80% of deacetylated chitosan.[37] The deacetylation degree (DD) is an important parameter that determines the polymer solubility and chemical properties.[7] Chitosan-based hydrogels are stabilized by different methods in order to modulate their properties and to create a stable network for a period of time suitable for the selected application.[38] The cross-linking is achieved by forming chemical bridges through reactive amine side groups of polymer chain. The cross-linking mechanism may be ionic or covalent,[38] depending on the selected cross-linking agent. In fact, chitosan is present in solution as a polycationic form; hence, it may react with negatively charged molecules (anions and polyanions), forming ionic complexes.

Ionic cross-linkers (phosphates and sulfates) are safe from the toxicity point of view, but their cross-linked products have a limited stability due to the nature of the chemical bounds, that involve weak interactions such as electrostatic, dipole–dipole, as well as hydrogen and hydrophobic interactions.[38]

Conversely, covalent cross-linkers (formaldehyde and glutaraldehyde) produce hydrogels with stable chemical and physical characteristics; nevertheless, they may exhibit a certain degree of toxicity due to the presence of unreacted molecules both of cross-linker and by-products formed during the reaction.[56,57]

The main challenge of these systems is the chemical stabilization *via* nontoxic reagents and a modulation of the properties for a6prolonged period of time. Hence, it is desirable to use an

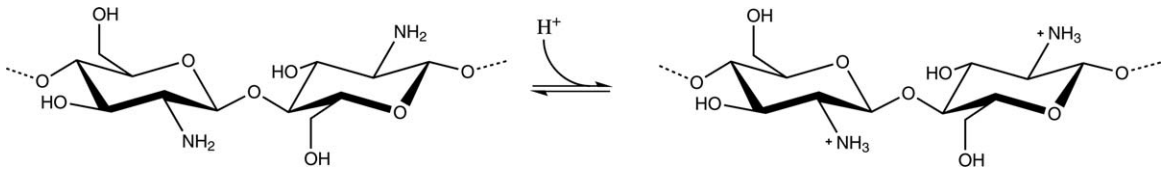

Figure 1. Chitosan structure in acidic environment.

alternative cross-linking reagent that could lead to the formation of stable and biocompatible cross-linked products. Starting from these considerations, in this work, the possibility of employing the natural extract of *Gardenia Iasminoides Ellis* (Genipin) as chemical cross-linker was evaluated. Genipin (GN) could overcome the aforementioned problems; in fact, it has been demonstrated that GN has a reduced cytotoxicity with respect to other cross-linkers such as glutaraldehyde, formaldehyde, and other agents.[39–42] It has also anti-inflammatory properties[43] and is folklorically used for the treatment of jaundice, headache, edema, fever, hepatic disorders, and hypertension.[44]

Genipin is obtained from a compound traditionally used in Chinese medicine, geniposide, which may be isolated from the fruits of *Gardenia jasminoides* Ellis.[45] It is commonly manufactured by using β-glucosidase[46] and is extracted by a direct chemical procedure or by a microbiological process involving *Peniccilium nigricans* that produces β-glucosidase, which in turn hydrolyzed geniposide into aglycone genipin.[47,48]

Polymerization reaction of chitosan/genipin system is induced in the presence of oxygen radicals, and it is strongly affected by temperature and the presence of H^+.[49]

The aim of this work is to study the kinetic reaction of chitosan and genipin at different thermal conditions and with different cross-linker concentrations in the absence of other solvents (i.e., ethanol or dimethyl sulfoxide) used for genipin dissolution. From this preliminary study, some kinetic parameters such as activation energy, rate constant, and reaction order will be extracted. In addition to this, chemical and physical characteristics of the cross-linked hydrogels will be evaluated such as thermal stability (differential scanning calorimetry (DSC) analysis) and swelling properties. In particular, the knowledge of these properties will be useful to understand the possible applications of the gel, for example, in the biomedical field, drug delivery, or packaging.

EXPERIMENTAL

Materials

Chitosan powder (medium Mw, 75% DD, CAS 9012-76-4) and acetic acid (purity 98%) was purchased from Sigma-Aldrich, and genipin [purity 98% (HPLC)] from Wako chemicals USA, Inc. The NaCl and HCl used for swelling measurements were purchased from Sigma-Aldrich and the buffer solution from Fluka. All the products were used without any further purification.

Preparation of Chitosan Hydrogel

Chitosan solutions at 1.5% w/v were prepared using water from reverse osmosis purification system (Waters, Italy) previously filtered through a 0.45 mm Milipore syringe filter. Briefly, the samples were prepared by suspending 1.5 g of chitosan in 100 mL of 0.1 M acetic acid solution in a glass flask and stirred for 6 h at 4°C till complete dissolution. Afterward, genipin was added to solution and stirred for another hour. Batches with different genipin concentrations were prepared in order to obtain samples with different degrees of cross-linking and properties. In particular, two different concentrations of genipin were used: 3,7% w/w dry chitosan (0.25 M of final solution) and 7,5% w/w dry chitosan (0.50 M of final solution). The resulting solutions were poured into round moulds with 22.5 mm diameter (2 g of solution/mold) and thermally treated in oven at the three different temperatures: 25, 37, and 50°C.

Rheological Characterization of Swollen Hydrogels and Kinetics Studies

The rheological characterization of chitosan/genipin hydrogels was performed in order to assess the effect of the genepin concentration on the mechanical properties of the chitosan cross-linked hydrogels. In this study, the sample was tested in the linear viscoelastic response range, identified through preliminary tests performed in stresses sweep condition ($f = 1$ Hz). The cross-linking process was monitored at fixed time points (every hour) by measuring the elastic moduli (G') of GN1 and GN2 hydrogels. For each hydrogel, the same starting solution was sampled during the thermal process at different time points. The rheological characterization was carried out in a dynamic controlled Rheometer (Ares Rheometric Scientific Inc.). The tests were performed with a plate and plate flow geometry (radius = 12.5 mm and gap of 3 mm) in a dynamic state mode at room temperature (25°C), by a frequency sweep rate ranging from 0.1 to 10 Hz and 1% of strain. The plate surfaces were properly modified through a fabric-based adhesive tape (Leukoplast (2.5 cm × 5 m), BSN Medicals, IT) to prevent hydrogel.[50] Previously, a "strain sweep test" was performed to select an appropriate strain amplitude at which the linear viscoelastic behavior is observed. All the experiments were carried out in triplicate, and the average and standard deviation values were evaluated. The results were recorded in terms of storage and loss moduli, respectively, G' and G''. The complex modulus $G*$ was calculated by means of the relationship $G* = \sqrt{\left(G'^2 + G''^2\right)}$ at 1 Hz. From the rheological measurements, the kinetic and mechanical parameters such as the activation energies were extracted.

Differential Scanning Calorimeter (DSC) Analyses

The thermal stability of chitosan samples was measured through DSC (Star and System, METTLER Toledo). The samples were obtained by casting chitosan/genipin hydrogels onto polystyrene petri dishes and then dried in an oven at 30°C for 48 h for film

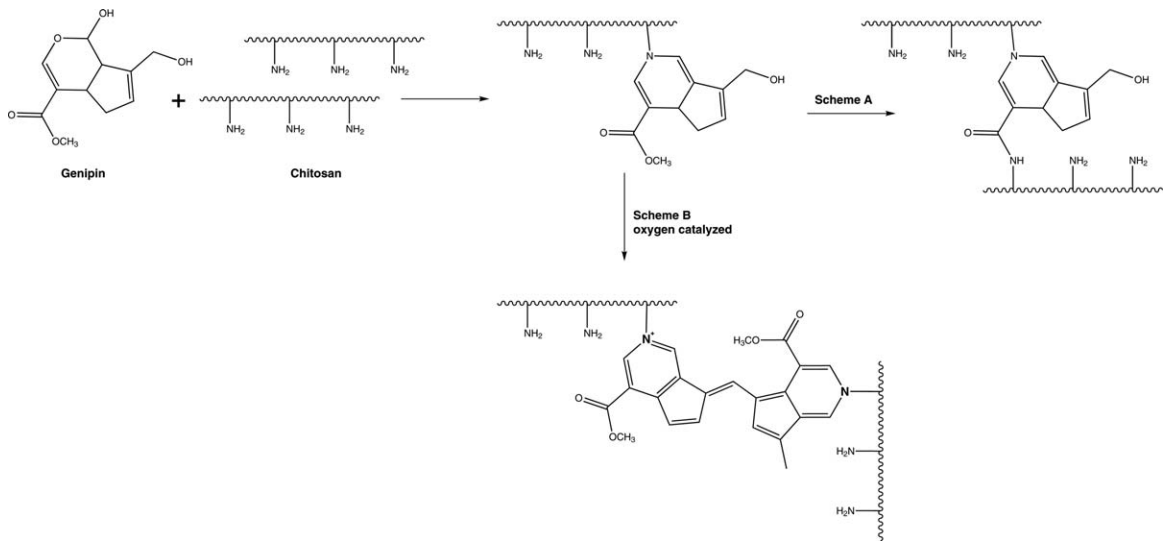

Figure 2. Scheme of cross-linking reactions between chitosan and genipin and a picture of a chitosan–genipin sample.

formation. The obtained films were peeled off gently, dried in a desiccator (with silica gel desiccant, under vacuum), and tested without any further modification. An empty pan was used as a reference. For this purpose, the samples were heated from 25°C up to 400°C at 10°C/min in nitrogen atmosphere.

Swelling Measurements

Equilibrium swelling measurements were performed in purified water at different ionic strength (range) and pH (range). The influence of the ion concentration on the hydrogel sorption capacity was evaluated by placing hydrated samples in a solution at different NaCl concentrations (0, 0.01, 0.1, and 1 M). Samples with 6 mm diameter and 3.7 mm thickness were obtained from hydrated hydrogel dishes by means of a surgical punch (biopsy punch—Kai Medical BB-807). All tests were performed in triplicate. The same procedure was followed for the analysis of the swelling capacity at different pH (0–12). The buffer solutions was used to create systems at different pHs [1 M H_3PO_4/NaOH at different ratio (pH 1–12), and 1 M HCl (pH 0)]. The swelling ratio (SR) was measured by weighing samples before and after their immersion in water for about 24 h. The SR is defined as follows:

$$SR = \frac{W_f - W_i}{W_i} \times 100 \quad (1)$$

where W_f is the weight of the swollen sample and W_i is the weight of the starting sample.[51,52] All the measurements were performed at room conditions and in triplicate. Samples were weighed with a Mettler balance (10^{-5} sensitivity).

RESULTS

Chitosan/genipin cross-linking produces a dark-blue hydrogel, whose coloration is associated with the genipin reaction with chitosan amino groups as shown in Figure 2. The cross-linking mechanism between chitosan and genipin may occur with different pathways (i.e., Scheme A and B).[53,54] According to Scheme A represented in Figure 2, under mild acidic or neutral conditions, cross-linking reaction involves two different steps. The first is the nucleophilic attack of amino groups of chitosan on the olefinic carbon atom at C3 of genipin, followed by opening the dihydropyran ring and carbonyl group attacked by the secondary amino group newly formed. The second step is a nucleophilic attack of amino group of chitosan to carboxyl group of genipin with amide formation. According to scheme B, the oxygen radical-induced polymerization of genipin might be produced between genipin molecules already linked to amino groups of chitosan, which could lead to the cross-linking of chitosan chains by genipin molecules or even by genipin copolymers that have a high conjugation of C–C double bond, and therefore, it is responsible of dark-blue color.[53–55]

Rheological Characterization of Swollen Hydrogels and Kinetics Study

Kinetics Characterization. In Figure 3(A,B), the complex elastic moduli $G*$ acquired at different reaction temperatures (i.e., 25, 37, and 50°C) were plotted as a function of reaction times for both systems GN1 and GN2.

The gelling time point t_{gel} was determined from the intersection of two linear region observed during reaction times at different temperatures: the resulting values are summarized in Table I for each tested sample and temperature.

The gelation time t_{gel} is related to the activation energy and the reaction order by the following equation:[55]

$$1/t_{gel} \propto C^{(m-1)} e^{(E_a/RT)} \quad (2)$$

in which t_{gel} is the gelation time, m is the order of the cross-linking reaction, and E_a is the overall activation energy, which includes the activation energy in the hydrolysis, condensation, and diffusion processes, derived from Arrhenius equation.

$$k = Ae^{-E_a/RT} \quad (3)$$

In Figure 4, ln t_{gel} was plotted as a function of $1/T$ for both systems GN1 and GN2, where T is the absolute temperature (K)

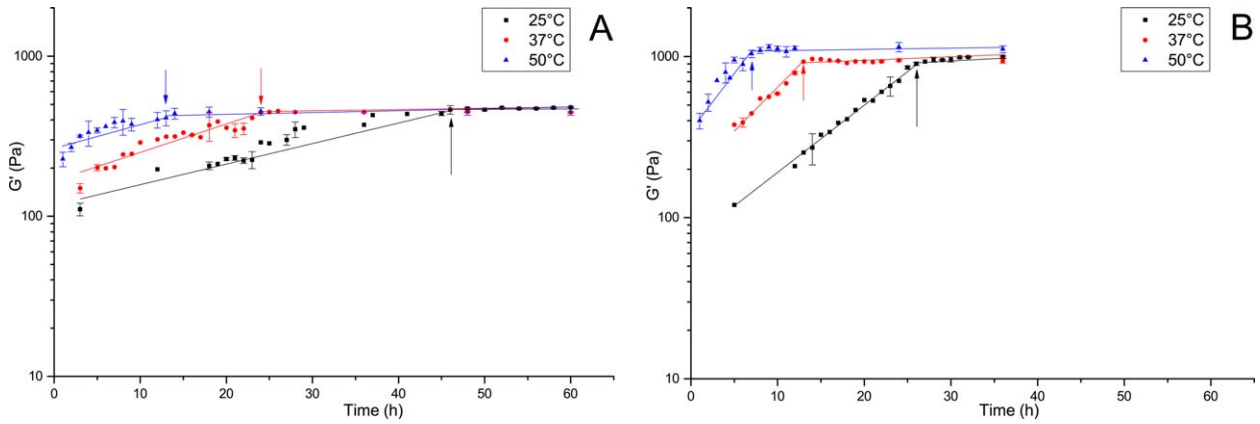

Figure 3. Plot of complex elastic moduli $G*$ vs reaction time at different temperature conditions for (A) GN1 and (b) GN2. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

and E_a is the activation energy of the rate-limiting step in the cross-linking process. From these results for chitosan/genipin system, at fixed genipin concentration, an Arrhenius relationship was observed. The value of E_a that was measured from this plot was 41 kJ/mol, and it is reported in Table II. This value must be related to the rate-limiting step in the formation of the gel, which is the nucleophilic substitution of the ester group on the genipin molecule by the secondary amide linkage between chitosan and genipin (Figure 2, Scheme A) or the oxygen radical-induced polymerization of genipin already linked to chitosan (Figure 2, Scheme B). Moreover, activation energy of cross-linking process is very low when compared with value of activation energy for thermal degradation of chitosan chloride (109–114 kJ/mol),[56] and other glycosidic compounds such as methyl-2-acetamido-2-deoxy-13-D-glucopyranose (119 kJ/mol) and methyl-2-amino-2-deoxy-13-D-glucopyranose (151 kJ/mol),[57] and then cross-linking reaction is predominant process over thermal degradation even at high temperature. A value of reaction order m from 1.83 for 25°C to 1.90 for 50°C was measured as slope of plot of $1/t_{gel}$ vs genipin concentration as shown in Figure 5 and reported in Table II. These values were closer to 2, which is the value expected from an irreversible gelation process, according to scheme A in Figure 2.[55] The occurrence of the various side reactions, in particular, oxygen radical-induced polymerization of genipin already linked to chitosan, that gave the dark-blue color to the gels due to high delocalized double bond,[58] in according to scheme B in Figure 2, or polymerization of genipin not linked to chitosan, may be the reason for the deviation from second-order reaction kinetics.[55] Temperature plays an important role in this process, and it is evident that by increasing the temperature, the reaction order increases, and irreversible cross-linking processes over side reactions are favored.

Table I. Values of the Gelling Times t_{gel} (h) Calculated at Different Temperatures as Intersection of Two Linear Regions in $G*$ vs Reaction Time Plot

Temperature (°C)	t_{gel} GN1 (h)	t_{gel} GN2 (h)
25	46	26
37	24	13
50	13	7

Mechanical Characterization. Figure 6 shows that both chitosan/genipin gels exhibited a mechanical behavior with G' higher than G'' in all studied ranges. Cross-linking degree (CD) plays a key role in defining the G' and G'' moduli of the hydrogel.[59] An hydrogel with a lower cross-linking degree (and, therefore, a low number of covalent bonds) has a greater length of the chitosan chains between links; thus, less force is required to deform the hydrogel. As the network is tightened by increasing the number of cross-links, the hydrogel becomes stiffer. Pendant-type modification of chains has little effect on the moduli because they do not form a cross-linked network. This behavior demonstrates an increased elastic modulus for the cross-linked chitosan hydrogels relative to network stabilization through the strong covalent cross-links, which reduce the intrinsic mobility of the chains and increase the relaxation time characteristic of motion.[60] Consequently, the polymer chains cannot release stress during the period of oscillation and show elastic behavior.

The mechanical behavior of hydrogels can be related to the gel strength.[61] In weak gels, the moduli have low frequency dependence, and the magnitude of G' is often 10 times smaller than the magnitude of G''. Conversely, strong gels exhibit a G' that is higher than G'' (Figure 6).

The experimental data obtained in the frequency sweep setup at the end of cross-linking reaction were reported as G^* and they correlated by means of a power law equation (eq. 4):

$$G^*(f) = \sqrt{(G'2 + G''2)} = A \cdot x^B \quad (4)$$

In the power law relationship, G^* is the complex modulus, x is the oscillation frequency in rad s^{-1}, A is the constant, and the exponent B is the slope in a log–log plot of G^* versus x. The A value can be correlated with cross-linking degree and the B values can be correlated with the bond strength of the gels. The value of $B = 0$ is typical for covalently cross-linked gels, whereas $B > 0$ corresponds to physical gels.[62]

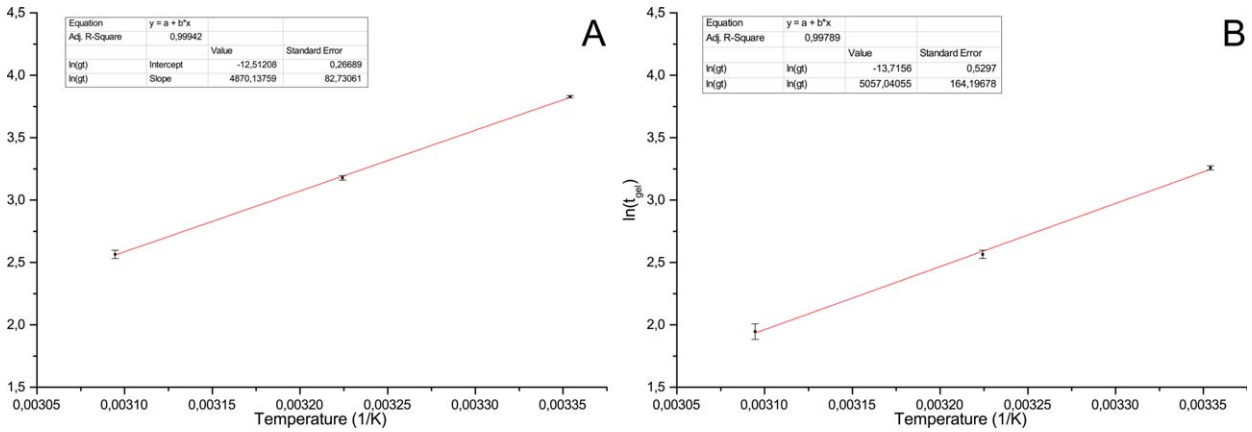

Figure 4. $ln(t_{gel})$ plot of gelling time reaction of chitosan/genipin solution at different temperatures (25, 37, and 50°C) vs $1/T$ (K^{-1}), the activation energy E_a is calculated from the slope. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table II. Kinetics Parameters Extracted from Rheological Measurements

E_a (GN1) kJ/mole	E_a (GN1) kJ/mole	E_a (average) kJ/mole	m 25°C	m 37°C	m 50°C
40.49	42.04	41.26 ± 1.10	1.83	1.86	1.90

The values of A and the slope B of the double log plot of G^* vs oscillation frequency are shown in Figure 7, and reported in Table III. These data show that the cross-linking degree of the hydrogels, represented by A values increases with increasing genipin concentration. The B values, the bond strength, do not change with genipin concentration, and suggest the typical behavior of covalently cross-linked hydrogels.

Swelling Measurements of Genipin/Chitosan Hydrogels

A monotonic decrease of the swelling ratio was observed increasing the ionic strength of the external solution, until negative values at 1 M NaCl for both the samples tested (sample shrinkage with water expulsion). This behavior was attributed to the polyelectrolyte nature of the polymer chains. In fact, fixed charges on the polymer backbone play an essential role in swelling mechanism. This is related both to the electrostatic repulsion between charges of the same charge, which contributes to expand the polymer structure, and to the "Donnan type" absorption contribution to water sorption, which promotes an osmotic pressure, π_I, favoring the water to enter the hydrogel.[63] Both these contributions to the hydrogel swelling depend on the difference in ion concentration between the internal of the gel (c) and the external solution (c^*), as reported in eq. (5).[64,65]

$$\pi_I = RT\left|\frac{ic_2}{z_k - n(c^* - c)}\right| \tag{5}$$

where $c_2$is the concentration of fixed ionic charges on the network, i is their dissociation degree, z_k is their charge, and n is the number of ions generated by the dissociation of one salt molecule.

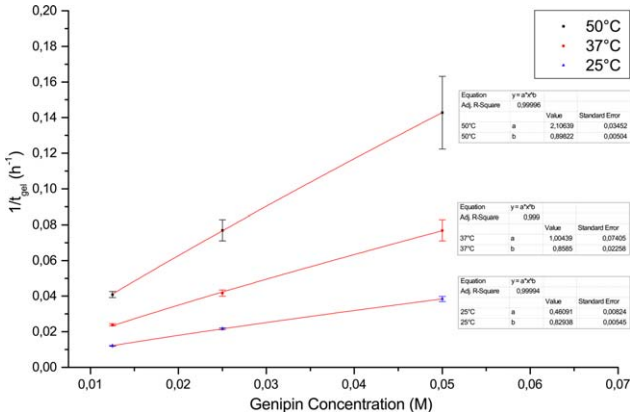

Figure 5. $1/t_{gel}$ vs genipin concentration of chitosan–genipin hydrogels, the reaction order m is calculated from the slope. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

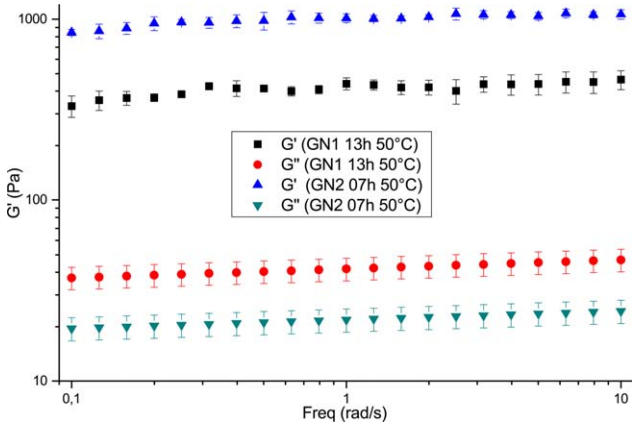

Figure 6. Double log plot of storage moduli G' and loss moduli G'' vs oscillation frequency of chitosan–genippin hydrogels. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

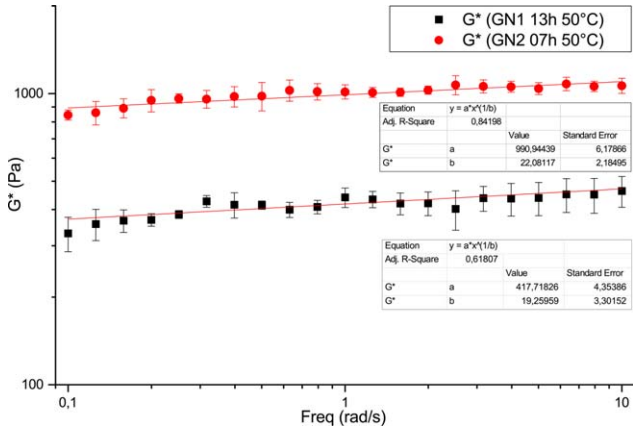

Figure 7. Double log plot of complex viscoelastic moduli G^* vs oscillation frequency of chitosan–genipin hydrogels. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

In particular, increasing the number of fixed charges on the polymer network increases the hydrogel swelling capacity, which, instead, decreases by increasing the ionic strength of the external solution. This is due to neutralization of the fixed charges by the free charges present in the outer solution, thus reducing both the repulsion effect between polymer chains and the osmotic contribution to the swelling due to the Donnan contribution.

For these reasons, GN1 hydrogel displays a swelling capacity greater than GN2 for each value of ionic strength, as observed in Figure 8(A).

Swelling measurements in water solutions at different pH show that both GN1 and GN2 samples display the highest swelling ratio at value of pH between 1 and 2. This was attributed to the protonation of amino groups on the chitosan chains, depending upon the pH of the surrounding environment. As the pH of the external solution decreases, the number of the amino groups protonated increases on the polymer backbone. Thus, increases the "Donnan type" contribution to water sorption due to the presence of fixed charges, increasing the swelling equilibrium capacity, as reported in Figure 8(B). In addition, the increasing

Table III. Values of A and the Slope B of the Double Log Plot of G^* vs Oscillation Frequency

	A (Pa)	B
GN1	417.72 ± 4.35	0.0519 ± 0.0089
GN2	990.94 ± 6.18	0.0453 ± 0.0045

of charges of the same sign increases the electrostatic repulsion between the polymer backbone, which repels and contributes to swelling. However, the supply of H^+ ions from the protonated groups on the backbone is limited; at a certain value of the pH (about pH 1), most of the amino groups will be protonated, giving rise to a fully charged network. At this point, the hydrogel equilibrium swelling capacity displays a maximum, as observed in the plot in Figure 8(B). Beyond this point, the further supply of H^+ ions from external solution determines an osmotic pressure from the inside outward of hydrogel (pH 0).

On the other hand, increasing the pH of the external solution, the number of amino groups protonated decreases; thus, the ionic contribution to the hydrogel equilibrium swelling capacity also decreases. At pH 6, most of the amino groups are protonated and both hydrogels have a similar behavior. From pH 6 to 12, the hydrogel presents negative values of swelling ratio and expels water solutions. Swelling capacity of GN1 and GN2 is different at the same values of pH and the highest difference between the hydrogels is about at pH 2, where swelling ratio of GN1 is one order of magnitude of GN2 as observed in the plot in Figure 8(B).

Differential Ccanning Calorimetry Results

DSC presented the characteristic peaks of genipin and genipin–chitosan hydrogel.

The analysis performed on genipin powder [Figure 9(A)] shows that two endothermic and one exothermic peaks are evident at 123°C (ΔH 570 J/g), 199°C (ΔH 270 J/g), and 245°C (ΔH −650 J/g). These peaks could correspond to physical changes of genipin; in particular, the first correspond to a melting transition,[66] and the second and third ones are associated to thermal rearrangement of cyclic rings of genipin.

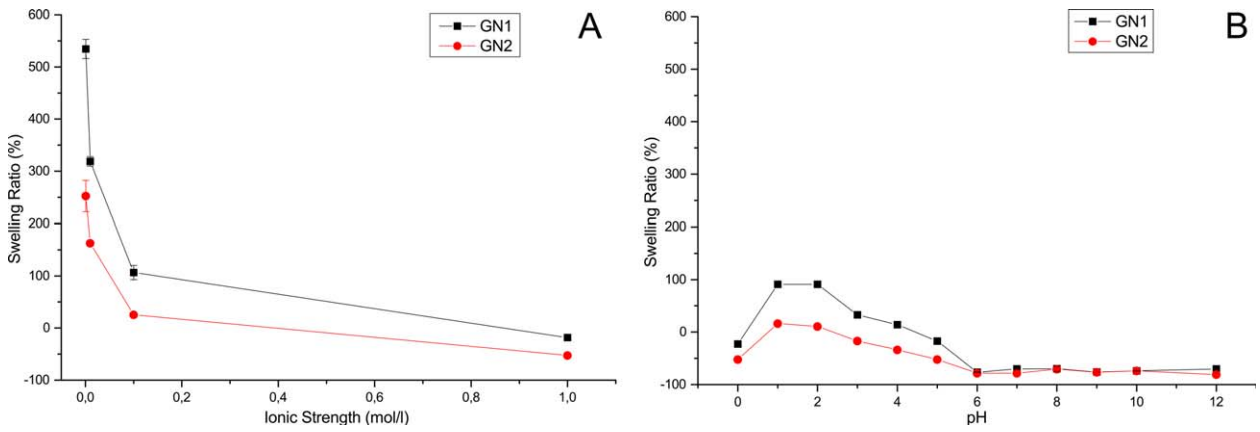

Figure 8. Swelling ratio of chitosan–genipin hydrogels (A) as function of the ionic strength of water solutions and (B) as function of pH of water solutions. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

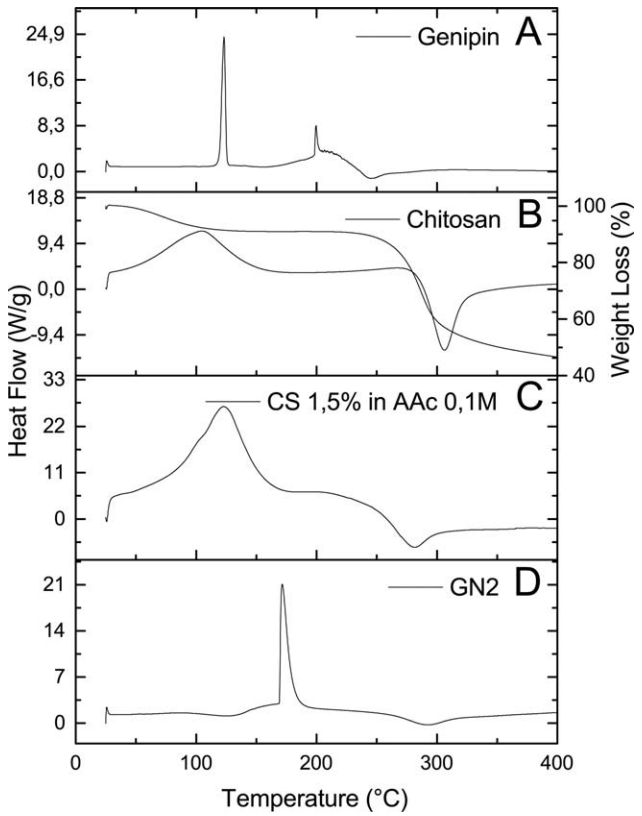

Figure 9. Differential scanning calorimetry analysis on (A) genipin powder, (B) chitosan powder, (C) dry chitosan at 1.5% in acetic acid 0.1 M, and (D) dry genipin cross-linked chitosan GN2.

The analysis shows that chitosan powder [Figure 9(B)] is characterized by an endothermic peak at 105°C (ΔH 3114 J/g), due to evaporation of residual water adsorbed on the surface of the flakes and an exothermic peak at 306°C (ΔH −3058 J/g), associated with degradation of glucosamine rings.

Dry uncross-linked chitosan sample [Figure 9(C)] displays an endothermic peak at 122°C (ΔH 6100 J/g), due to the evaporation of acetic acid (boiling point at 118°C) and an exothermic one at 281°C (ΔH −3100 J/g). The fact that the latter peak is shifted to lower temperatures, but the value of ΔH remains the same than chitosan powder, which indicates that acetic acid probably produces a breaking of chitosan chains, therefore its partial degradation.[56,57]

The dry chitosan/genipin (GN2) hydrogel [Figure 9(D)] displays a different behavior; as observing in the plot, there is an exothermic peak at 126°C (ΔH −235 J/g) and an endothermic peak at 171.5°C (ΔH 765 J/g); this is the peak associated with melting transition of genipin, shifted to higher temperature, due to covalent interaction with chitosan's chains, and an exothermic one at 293°C (ΔH −835 J/g). As expected, the degradation peak for dry genipin–chitosan sample is at 293°C; this could indicate that genipin cross-linking stabilizes the hydrogel structure, because it could create chemical bridges between side backbone of chitosan.

CONCLUSION

Chitosan hydrogels were cross-linked with a genipin compound at different concentrations and analyzed from kinetic and physical–chemical points of view by means of rheological measurements, DSC, and swelling measurements. The kinetic analysis showed that the chitosan–genipin reaction is characterized by an activation energy (E_a) of 41 KJ/mol and a reaction order close to 2. These properties depend both on the concentration of cross-linker and on the temperature. As a result, by increasing either of these two parameters, it is possible to modulate the t_{gel} parameter. This represents a crucial aspect for potential applications of these hydrogels in many technological fields.

The DSC analyses showed that the cross-linking of chitosan with genipin produces a stabilization of the chains of chitosan with a significant change of the spectrum of genipin.

Swelling measurements were carried out in solutions at different ionic strength and pH, and high sensitivity to the external solution was displayed by GN1 and GN2 in proportional manner to their degree of cross-linking. Both hydrogels tend to expel water increasing ionic strength and pH and are inclined to swelling at very low pH values, ranging between 1 and 2. This particular behavior might suggest the use of these systems in applications, in which the pH plays an important role. In particular, these formulations could be used for drug delivery systems in oral administration applications.

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