Swelling and Mechanical Properties of Biopolymer Hydrogels Containing Chitosan and Bovine Serum Albumin

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Experimental and theoretical investigations of the swelling and mechanical properties of hydrogels formed from chitosan, bovine serum albumin (BSA), and chitosan/BSA mixtures cross-linked with genipin were performed. The properties of cross-linked chitosan hydrogels were explained in terms of its polyelectrolyte behavior, which led to a gradual increase in swelling ratio below the p*K* value, but whereby its swelling ability was eliminated by the presence of salt that screened the charges. Comparison of theoretical and experimental calculations of the swelling ratio, however, indicated that complications arising from wastage of cross-links, and formation of polymerized genipin cross-links must be considered before quantitative prediction can be achieved. Cross-linked BSA hydrogels swelled even in the presence of salt, and a marked increase in swelling was observed below pH = 3 that was explained as the result of an acid induced denaturation of the protein that led to unfolding of the molecule. Swollen BSA hydrogels were mechanically weak, however. Composite gels made from a cross-linked mixture of chitosan and BSA exhibited the swelling behavior of BSA combined with the mechanical properties of chitosan and were therefore considered most suitable for use in a gastric environment.

Introduction

Materials. Hydrogels, which are formed from cross-linked water soluble polymers, are a widely studied class of material, and interest in both natural and synthetic hydrogel-forming polymers is driven by practical aspects of their use as, for example, superabsorbent materials, and the theoretical aspects of polyelectrolyte network formation and behavior. In this study the main interest was in covalently cross-linked biopolymer networks based on the polysaccharide chitosan, the protein bovine serum albumin (BSA), and mixtures of the two, cross-linked using the natural cross-linking agent genipin. These biopolymers, genipin, and the cross-linking phenomenon have been described previously.[1−3]

The interest in such systems, including the cross-linking and swelling phenomena, arises from their potential use as edible systems designed to achieve a level of satiety or for the use of hydrogel systems for controlled release of pharmaceuticals and other functional ingredients in gastric conditions. It has been shown that cross-linked synthetic hydrogel particles can swell to several times their original volume in the acid environment of the gut, resulting in a slowing of gastric emptying.[4,5] Biopolymers such as chitosan and proteins contain basic amine groups that become protonated and charged in acid media. This forms the basis of an acid-induced swelling. Physical mechanisms for enhancing satiety also include increasing the viscosity of the stomach chyme, to delay gastric emptying, and increasing the volume of the stomach contents to increase internal pressure and induce a feeling of fullness. The role of chyme viscosity on gastric emptying has been previously investigated,[6,7] showing a reduced emptying rate with increasing viscosity. In addition, the control of food breakdown in the gut is a critical issue in the management and prevention of conditions such as diabetes. Many methods and devices for controlled release of active agents have been described previously,[8−11] including the use of swellable hydrogels for site specific delivery.[11]

Theory. The swelling of covalently cross-linked polymer gels in excess solvent is a much studied phenomenon, the theoretical basis of which was originally described by Flory[12] and later elaborated by, among others, Tanaka and co-workers.[13−16] The theory of gel swelling includes both kinetic and equilibrium aspects. In this study, only the latter will be considered and only data of this kind described and analyzed. A small gel particle (ideally spherical) prepared by cross-linking an appropriate biopolymer with a certain amount of genipin is the subject of the theoretical study. This particle is placed in excess solvent and allowed to swell continuously until an equilibrium degree of swelling is achieved. In the present work, this external solution contained either HCl or NaOH adjusted in concentration to achieve a range of constant external pH values, and in some cases, NaCl was included as an additional electrolyte.

The swelling gel particle reaches equilibrium when certain thermodynamic conditions are met. These include (1) that the chemical potential of water should be the same inside the swollen gel as outside in the excess swelling medium, (2) that the chemical potentials of all freely diffusible electrolyte components such as HCl, NaOH or NaCl should also be in equilibrium in the same sense, and (3) that the same should be true of the electrochemical potentials of the individual Na^+, Cl^-, OH^-, and H_3O^+ ions. This last condition has to take into account electrostatic contributions to the individual ion chemical potentials.

Condition (1) is essentially that of osmotic balance, and conditions (2) and (3) are those normally defining the Donnan equilibrium. The osmotic equilibrium is dependent on a number of contributions to free energy that must come into balance (sum to zero) when equilibrium swelling is achieved. First, there is the osmotic pressure associated with a polymer solution at a given concentration as described, for example, by Flory−Huggins theory.[12] This will generally cause gel swelling, but collapse is also possible. Mathematically, this takes the form

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$$\Pi_{mix} = RT\{\ln(1 - \phi_2) + \phi_2 + \chi(\phi_2)^2\}/V_s \quad (1)$$

Here R and T are the gas constant and absolute temperature, ϕ_2 is the polymer volume fraction in the gel at any stage of swelling, χ is the polymer−solvent Flory−Huggins parameter, and V_s is the molar volume of the solvent (approximated here as the figure appropriate to pure water).

Opposed to the osmotic swelling of the gel is the increase in free energy associated with the overall expansion of the cross-linked network (elastic free energy). Here, it is assumed that the gel is in a relaxed cross-linked reference state when it is cured at the initial polymer concentration (using a certain amount of genipin for a certain time) and that, on swelling, elastic elements are deformed leading to an energetically higher state. The implications of this for the solvent chemical potential, and hence the osmotic pressure, have been described by Flory[12] and are summarized in the equation

$$\Pi_{elastic} = -Gb \quad (2)$$

where G is the shear modulus of the gel. For ideal networks, this is often expressed in terms of the number of elastically active chains per unit volume, a, multiplied by RT. In Flory's formulation, the quantity b was originally written as $(1/2r - 1/r^{1/3})$ for network chains that follow simple Gaussian statistics. In this last expression, r is the gel swelling ratio at any stage of swelling and is equal to the current gel volume divided by the initial volume. For real networks based on less flexible polymer chains (limited number of statistical segments), a more complex form[17] has been proposed for b and has been used in swelling work by some authors.[18] This is

$$b = \{1/2r - (1 - 1/N + 0.4/N^2 + 0.32/N^3)/r^{1/3} + (-1/N + 2.6/N^2 - 1.72/N^3)r^{1/3} + (-2.2/N^2 + 8.84/N^3)r - (6.84/N^3)r^{5/3}\} \quad (3)$$

where N is the number of statistical segment lengths (on average) per elastically active network chain. N would be very large for ideal Gaussian network chains but could be quite small for the polysaccharide and protein gels considered here. For the latter, in particular, the network chains are expected to be substantially nonideal as they will take the form of a "strings of beads". In fact, eq 3 is only one of a number of formulations that could be applied to real systems, but it is used here as a starting point in calculations.

The expression for this ionic contribution to swelling is given by

$$\Pi_{ionic} = RT\Sigma_i(C^i_{out} - C^i_{in}) \quad (4)$$

where the summation is made (over all labile ion types) of the differences in concentration of the ions inside and outside the network. This includes polymer counterions, provided that they are unbound in either a chemical or a Manning sense. When using (4) the ionic concentrations in the external medium can be assumed fixed and equal to the values set up initially in experiments, whereas the ion concentrations inside the gel require a separate calculation based on the ideas of Donnan equilibrium. Various conditions are used to establish this equilibrium including (1) that there is zero net charge in the gel and in the external phase at all times, (2) the polymer ionization based on a protonation equilibrium is consistent with a known equilibrium constant (or, for example, for a protein, a set of constants), (3) the ionic product for water K_w is always satisfied, and (4) all salt/acid/base component chemical potentials are balanced inside and outside the gel through ion product equalities such as: $[Na^+]_{in}[Cl^-]_{in} = [Na^+]_{out}[Cl^-]_{out}$, $[H^+]_{in}[Cl^-]_{in} = [H^+]_{out}[Cl^-]_{out}$, etc. In these expressions, as an initial approximation, activity coefficients are. assumed equal to unity.

Finally, the equilibrium value for the gel swelling (r_{eq}) is determined by the condition

$$\Pi_{mix} + \Pi_{elastic} + \Pi_{ionic} = 0 \quad (5)$$

In terms of parameters G, the shear modulus, can be measured for the initial gel and Flory−Huggins values are often available from literature, as are appropriate dissociation constants for acids and bases. Therefore, equilibrium swelling should be a predictable quantity from measurement coupled to literature information and structural information about the number of charged (or potentially charged) residues on the network. This is particularly true if N can be assumed to be very large (ideal rubber assumption), although it is much less certain what the best value for N is for the biopolymer gels of interest in the current study. The fact that genipin cross-links biopolymers[1−3] by reacting with sites that themselves are chargeable (amine groups) presents a further complication. These matters will be discussed later.

Experimental Section

Materials and Sample Preparation. The materials under investigation in this study were chitosan ("Chitoclear", 90% deacetylated, supplied by Primex), bovine serum albumin (BSA, supplied by Aldrich), covalently cross-linked with genipin (supplied by Challenge Bioproducts Ltd, Taiwan). The details of the cross-linking reaction are given elsewhere.[1−3]

To make the samples for the swelling measurements that were compared with theory, chitosan was dissolved in 1% (v/v) acetic acid by stirring at room temperature. A solution of genipin was made in the same solvent by stirring and heating to 60 °C to ensure complete dissolution. The two stock solutions and solvent were cooled to room temperature and added in the required amounts at room temperature to achieve a final concentration of 1.5% (w/v) chitosan, 5 mM genipin, and 1% (v/v) acetic acid. The use of 5 mM cross-linker provided samples whose swelling ratio could be readily measured. A 20 mL sample was made by stirring the reaction mixture in a glass vial and allowing to stand at room temperature for 16 h before swelling measurements were made. Stock solutions of BSA and genipin were made up in distilled water and mixed in appropriate quantities to achieve a final concentration of 15% (w/v) BSA and 5 mM genipin. A 20 mL sample was made by gently stirring the reaction mixture in a glass vial to avoid generating foam and allowing to stand at room temperature for 16 h before swelling measurements were made.

To make the samples for the mechanical properties measurements, chitosan was dissolved in 1% (v/v) acetic acid and 0.05% (w/v) sodium azide (used as a preservative, at a low enough concentration to have no effect on the properties of the gels) by stirring at room temperature. A solution of genipin was made in the same solvent by stirring and heating to 60 °C. The two stock solutions and solvent were cooled to room temperature and added in the required amounts in borosilicate glass vials at room temperature to achieve a final concentration of 1.5% (w/v) chitosan, 10 mM genipin, 1% (v/v) acetic acid, and 0.05% (w/v) sodium azide. A higher cross-linker concentration of 10 mM was used for the mechanical properties samples because the 5 mM samples were too weak to measure accurately. The sample was mixed by shaking, and the vials were sealed and allowed to stand at room temperature for over 100 h to let the reaction proceed. Stock solutions of BSA and genipin were made up in 0.05% (w/v) sodium azide and mixed in borosilicate glass vials to achieve a final concentration of 15% (w/v)

BSA, 10 mM genipin, and 0.05% (w/v) sodium azide. The mixtures were then gently shaken, to avoid generating foam, and allowed to react for over 100 h. Where composite gels of chitosan and BSA were used, stock solutions of chitosan, genipin, and BSA were mixed to achieve a final concentration of 1.5% (w/v) chitosan, 15% (w/v) BSA, 10 mM genipin, 0.5% (v/v) acetic acid, and 0.05% (w/v) sodium azide, in borosilicate glass vials, and the reaction was allowed to proceed for over 100 h.

Swelling Measurement. For the swelling ratio samples, small portions (of dimensions approximately 5 × 5 × 5 mm) were cut from the 20 mL bulk gels, made as described above, weighed, and placed in at least 50 mL of a range of hydrochloric acid and sodium hydroxide solutions, whose concentrations were adjusted to give a range of pH values between 1 and 12. After immersion for 24 h, by which time the gels were judged visually to have completed swelling, the final pH value of the solvent containing the gel piece was measured (this value was used in plots of swelling ratio versus pH) and the swollen gel portions were removed from the solvents. Excess solvent was removed carefully using paper tissue and the gel portions were then weighed again. At least three gel portions were measured for each initial pH condition.

The swelling ratio, r, was calculated from the change in mass of the samples before and after introduction of the solvent, using eq 6

$$r = 100\%\left(\frac{m_f - m_i}{m_i}\right) \tag{6}$$

where m_f was the final mass of the sample after swelling and m_i was the initial mass of the gel sample.

For the mechanical test samples, the degree of swelling was given by the swelling factor, q, that was related to the swelling ratio, r, through eq 7. The swelling factor, q, is always positive, and therefore allowed a measure of the swelling to be plotted on a logarithmic axis

$$q = q_0\left(\frac{r}{100} + 1\right) \tag{7}$$

where q_0 is the effective swelling factor of the samples as made.

Different swelling ratios were achieved for the chitosan mechanical properties samples by immersing the glass vials containing the cross-linked gel into sodium chloride solutions ranging in concentration from 10 to 320 mM. Six sample vials (a total of ≈20 mL of sample) were immersed in not less than 3 L of each solvent. The volume of solvent was far in excess of the sample volume to limit the effect of ions already present in the sample on the swelling behavior. The pH of each solvent bath was measured at the end of the soak period (∼80 h) to be pH 4.3. For the BSA and BSA/chitosan composite samples, the vials were placed into 3 L containers of HCl solution at different pH, ranging from pH 1.0 to pH 4.8, for ∼150 h before being removed for measurement.

Swelling Computation. The computational approach that was adopted used expressions (1), (2), and (4) for the contributions to osmotic pressure at any given level of swelling, or deswelling, and sought to establish the value (r_{eq}) of the swelling ratio r at which these terms summed to zero, to a required accuracy (maximum discrepancy usually 10^{-15}). To achieve this, the final gel polymer volume fraction was scanned from zero (infinite swelling) to unity, and for each trial value of r, the osmotic balance was calculated. For each trial swelling ratio, ion concentrations within the gel were calculated using the ionization equilibria and Donnan conditions set out in the Introduction. As zero was passed, the increment used in the scan was subtracted, and the scan resumed with a 10-times smaller increment. This was continued until the balance was achieved to the required accuracy.

When performing these calculations the external pH was used to establish the equilibrium degree of ionization of network groups, despite the fact that the internal pH was not usually equal to the external value. This was because counterions inside the gel experience an electrical potential from the charged nework that, in turn, influences their chemical potential: this being the quantity actually balanced between the inside and outside. If the normal dissociation constant of the polymer ionizable groups is used, the ionization equilibrium is better represented using the external pH. Where the ionic product for water was being used inside the gel, correct results are obtained only when internal concentrations are used. This is because both mobile hydrogen and hydroxide ions are involved, and for these, the electrostatic contributions to chemical potential are equal and opposite and cancel out.

In the computations, the calculation of the elastic contribution to osmotic pressure also needs further comment. Equations 2 and 3 are self-explanatory, and one option is simply to use a measured value for the shear modulus G of the unswollen starting gel at a known pH or to treat G as a variable parameter. In the present work a slightly more elaborate approach was taken in which G was itself expressed in terms of the degree of cross-linking of the network, and the free energy contribution (aRT) of the elastically active chains. A cascade network theory[19] was used to do this in which

$$G = Cf\alpha(1 - \upsilon)^2(1 - \beta)\{aRT\}/2 \tag{8}$$

Here C = initial polymer concentration (mol/mL), f = potential number of cross-linking sites per molecule (so-called functionality), α = degree of cross-linking, υ = so-called extinction probability from recurrence relationship $\upsilon = (1 - \alpha + \alpha\upsilon)^{f-1}$, $\beta = (f - 1)\alpha\upsilon/(1 - \alpha + \alpha\upsilon)$, and a = number of RT units contributed by each mole of network chains (nonideality factor: $a = 1$ for ideal rubber).

An explanation of the cascade approach and explanations of terms such as the extinction probability have been given elsewhere.[19,20] Suffice it to say that if a value of α is available from some form of experiment, and a can be estimated, G can be calculated. This would allow testing not only of the swelling theory itself but also of the cascade approach and would allow a more molecular route to predicting swelling since one could directly estimate the likely influence of changing the degree of cross-linking on the degree of swelling. However, this step is not absolutely necessary, and the computer calculations can, in principle, be run purely with insertion of trial (and/or) experimental values of the unswollen G as starting data. In the present work, although values of a and α were input to achieve a particular value of G, these parameters were not available experimentally. In the present work they effectively collapsed into the single quantity G which could be compared with experiment. These parameters were therefore highly correlated, and used merely for convenience in the present work, to set up a value for G (which was the experimental value at a known pH).

To apply the computer model described in the Experimental Section, the following input quantities were required. Some of these simply refer to the experimental conditions, some could be obtained independently from literature, and some remained the essentially variable parameters of the analysis. These quantities were as follows: (a) temperature, taken to be 20 °C in all experiments; (b) chitosan initial concentration, taken to be 1.5%(w/w); (c) chitosan molecular weight, estimated at 500 000; (d) chitosan functionality (i.e., the number of residues per molecule carrying amino groups) was fixed at 2727 based on an average residue weight of 165 and the estimate that 90% of the polymer residues are deacetylated to give free amino groups; (e) chitosan partial specific volume, taken as the standard value for polysaccharides of 0.61 mL g^{-1}; (f) chitosan average residue volume taken as 110 mL mol^{-1} from the average residue weight and partial specific volume; (g) polymer−solvent Flory−Huggins χ value [no value is available for chitosan but an initial estimate of 0.48 was chosen based on values for other polysaccharides]; (h) chitosan amine group proton dissociation constant pK_a, taken as 6.3 from literature;[21,22] (i) external salt concentration, as defined in experiments; (j) salt cation charge, taken as 1 as would apply to NaCl, for example; (k) external pH, as defined in experiments; (l) maximum error allowed in osmotic balance, 10^{-15}; (m) fraction of functionalities reacted to produce active cross-links, varied to give appropriate value for G; (n) assigned front factor a to make active chain contribution to modulus aRT, fixed at 1 to combine with α to give a

desired value of G; (o) N, the average number of statistical segment lengths per elastically active chain, treated as a variable parameter but indefinitely large for an ideal network model; and (p) the fraction of amine groups capable of becoming charged, treated as a variable parameter because an unknown amount of the amine residues may have reacted with genipin without an increase in modulus being involved; examples are blocked groups or wasted cross-links.

Although the above may seem like a formidable collection of quantities, in fact only (m−p) are essentially unknown, and (m) and (n) collapse to one quantity, the shear modulus G. In cases where G is available experimentally (true in the present case) only (o) and (p) remain. For a lightly cross-linked ideal polymer network, N could be assumed indefinitely large, leaving only (p) as the true single parameter of the model. In the present case, because of the mode of genipin action, this quantity is uncertain, and its incorrect value may cause quantitative differences between the model and experimental values of swelling ratio; the underlying qualitative predictions should remain valid, however.

A starting point in the analysis of the current chitosan swelling data was to assume a value of 73 Pa for the modulus G of the unswollen gel from experiment, fix a at 1, and vary α to give this value for G. Previous studies on hydrogels of chitosan, cross-linked with 5mM genipin, gave a modulus of 73 Pa for gels that had been allowed to react for the amount of time used in the swelling experiments performed in this study.[3] In the first instance N was assumed very large (actually fixed at 81.3 based on published chitosan persistence length data[23]), and the fraction of charged residues varied, as the principal parameter of the model. In practice χ was also varied (from an initial value of 0.48) to achieve a good fit to swelling data at high pH where the network was uncharged, but its value was not crucial to results where swelling was significant.

A modified version of the chitosan computer program was written to deal with gel swelling involving proteins. The calculation proceeded in a similar way to that for chitosan, with the main difference being that information was obtained not only about the equilibrium swelling but also about the states of charge of the various amino acid residues, and the concentrations of ions inside and outside the network. Again, most of the quantities required for modeling are obtainable from experimental conditions, or from the literature. These quantities were the following: (a) temperature (20 °C); (b) BSA concentration in initial gel (15% w/w); (c) BSA molecular weight (66 296 from literature); (d) BSA functionality (59, based on number of lysine residues per molecule); (e) BSA partial specific volume (0.71 mL/gm typical of protein); (f) amino acid residue volume (80.9 mL/mol from average residue weight 113.9 and partial specific volume); (g) BSA Flory−Huggins χ value (unknown: 0.48 was assumed based on a polypeptide such as gelatin); (h) Fractions of ionizable residues of different types (arg 0.039519, asp 0.0652921, cys 0.0017182, glu 0.101375, his 0.0292096, lys 0.101375 but set to 0 here, tyr 0.0326460); (i) pK_a's for the ionizable groups (arg 12.1, asp 3.5, cys 8.14, glu 3.5, his 6.04, lys 10.67, tyr 10.1); (j) External salt concentration; (k) Salt cation charge (1, e.g., for NaCl); (l) appropriate external pH (defined by experiment); (m) maximum error allowed in osmotic balance (10^{-15}); (n) modulus of initial gel (taken from experiment; α and a used simply to calculate G as for chitosan); (o) N (parameter of model, but expected to be low for nonideal BSA network); and (p) Fraction of residues capable of being charged (calculated from numbers of ionizable groups of different types with (see below) all lysine groups assumed fully reacted with genipin)

Mechanical Measurement. Reported measurements of the mechanical strength of swollen hydrogels frequently utilize simple uniaxial compression and small scale sinusoidal uniaxial compression by applying the force through an acoustic diaphragm.[24,25] The use of small scale deformations applied in this way allows for small sample sizes but requires very sensitive force measurement. As this technique was unavailable, a compressive large-scale deformation technique was used. In this test, a texture analyzer (T.A.XTplus, Stable Microsystems (TA), with a 5 kg load cell) was used to compress samples prepared and swollen in glass vials, in situ. The force was measured as a flat probe was pushed, at constant speed, into a sample contained in the vial in which it was made. A standard Instron test procedure could not be used for the hydrogel samples in the current study because they were too weak for the sample preparation procedures used for making compression specimens in this equipment. Because the TA test was a nonstandard one, however, standard cylindrical plugs of agar and gellan gels were tested using in a parallel plate compression test an Instron (model 4502, with a 5N load cell at a constant displacement rate of 10 mm min^{-1}) and in the texture analyzer, to assess whether quantitative information about the gel strength could be obtained from the texture analyzer. The results of these comparisons indicated that the conversion factor between the Instron measure and the TA measure was consistent over the range of concentrations of samples used and for different materials, within the limits of experimental uncertainty.

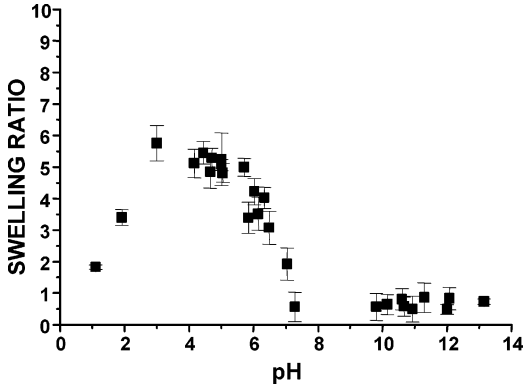

Figure 1. Swelling data for 1.5% chitosan gels cross-linked with 5 mM genipin.

Results and Discussion

Swelling Behavior of Chitosan Hydrogels. Swelling data for cross-linked chitosan using 5 mM genipin, in the absence of salt, and at a range of pH values, is shown in Figure 1. Above pH values of about 6.5, no swelling was observed because the chitosan amine groups were un-protonated and therefore uncharged. Below pH 6.5, near the pK_a of chitosan where the amine groups become protonated and therefore charged, swelling occurred and reached a maximum around pH 3. This behavior is consistent with standard polyelectrolyte charge-induced swelling.

The initial fit to this experimental data, using the experimentally measured[3] modulus of 73 Pa in the unswollen case (for chitosan cross-linked with 5 mM genipin) and a large number of elastically active residues consistent with the chitosan persistence length, is shown in Figure 2. This is good from around pH 12 to 5.5 but deviates strongly from the experiment after this (i.e., at low pH). The optimum value for the fraction of charged residues is also remarkably small at 0.0014 though presumably this is not impossible. Overall, however, the model shown in Figure 2 seems intrinsically incorrect, and in view of this, a further approach was followed. This was to introduce N as a second variable. When this was done and the experimental modulus value of 73 Pa was retained, the best fit achieved is that shown in Figure 3. This requires the lowest possible value of N (i.e., a value of unity). Physically, such a value implies that the chitosan network is very stiff indeed and deviates very strongly from the ideal rubber situation, even this fit is not particularly good, however, deviating significantly in the pH region 7−10, and to some extent also between pH's 2−5. Shifting the charged amine dissociation constant to a value close

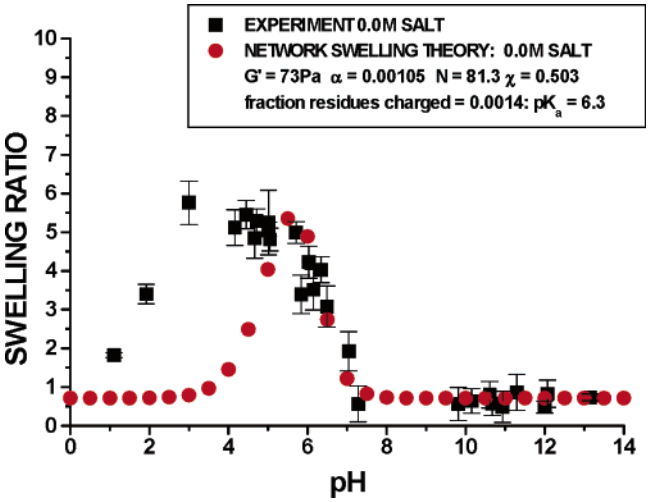

Figure 2. Modeling cross-linked chitosan swelling data using experimental *G* and large N consistent with chitosan persistence length.

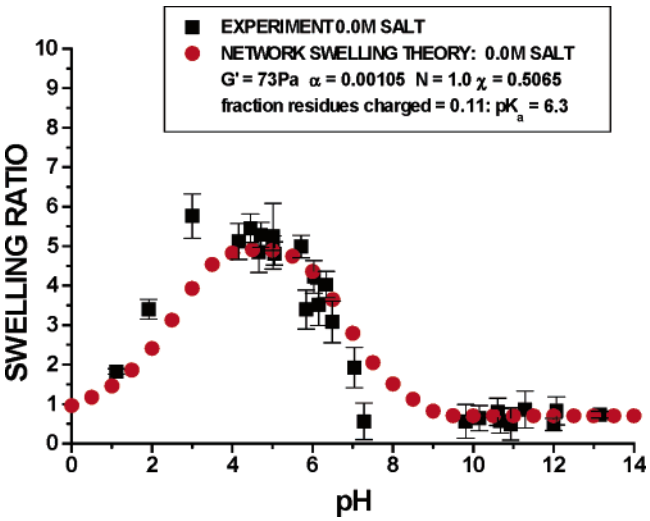

Figure 3. Modeling cross-linked chitosan swelling data using experimental *G* and $N = 1$ (highly stiffened network model).

to 6.1 improved the fit, but as the current experimental average value[21,22] is slightly higher at 6.3, such a change may not be justified. Even the value of 6.3 is very much lower than would normally be expected for a secondary amine, but presumably, in the chitosan polymer, a polyelectrolyte electrostatic effect prevails. A further objection to the model of Figure 3 arises from persistence length data for chitosan which (at maximum) implies only about 20 sugar residues to a statistical segment.[23] On the basis of this one would not have expected the value of *N* that is proposed in Figure 4, as a low modulus chitosan network should be reasonably flexible. The results may indicate, however, that the quite high reaction with genipin implied by the low fraction of amine residues which remain (0.11) has produced cycles and loops (wasted cross links) and network strands with a high degree of substitution. This, in turn, might produce a much stiffer, and less ideal, network than expected, but this remains to be proved. The wastage of cross-links may be related to the polymerization of genipin that can occur as an additional reaction during the cross-linking process.[1,3] It is possible, also, that some cross-links may be formed from not just one genipin molecule but dimers, trimers, oligomers, and possibly polymers of genipin, that is not accounted for in the current study.

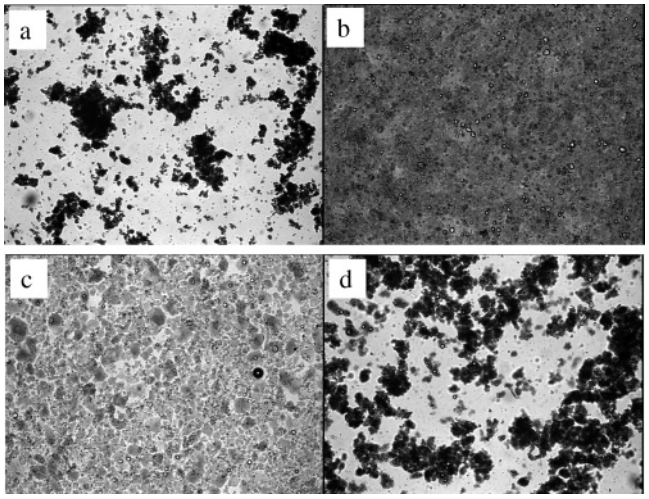

Figure 4. Optical micrographs of suspensions of beads of 1.5% chitosan gels cross-linked with 10mM genipin, (a) pH 7, (b) pH 5, (c) pH 2, and (d) pH 5 with added salt. The image width is 650 μm.

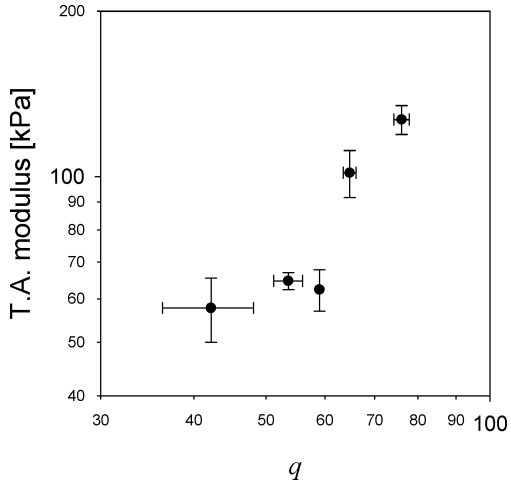

Figure 5. Plot of the apparent modulus of swollen chitosan 1.5% gels cross-linked with 10 mM genipin, as measured by TA analysis, as a function of the swelling factor. The as-made sample has $q = 59$.

The theory itself also needs further examination. It is not clear how realistic the expression involving *N* is in describing the free energy increases accompanying significant swelling of networks based on polysaccharides, nor is the Flory−Huggins mixing theory perfect for such systems. Some further development of the theory will almost certainly be needed. What is clearly explained, however, is the tendency for swelling to occur as pH falls into the range of the pK_a of the amine groups (6.3). A sigmoidal transition occurs in this region, followed by a collapse in swelling after pH 4, which the theory explains as a salt effect generated by the increasing levels of HCl required to lower pH. This collapse phenomenon becomes inhibited as the effective network charge increases (cf Figures 2 and 3). At the very least, therefore, the model achieves a qualitative agreement with the principal features of the phenomenon at the expense of two variable parameters, one potentially measurable, and related to the degree of residual free amine groups on the network after genipin action, and one less easy to establish, based on network structure and flexibility.

Figure 5 shows micrographs of suspensions of gelled chitosan particles at different pH, showing the substantial swelling of chitosan hydrogels at low pH, in the absence of salt. The effect

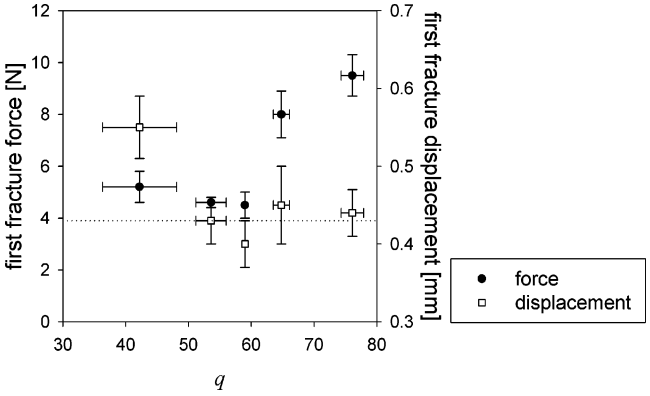

Figure 6. Fracture properties of 1.5% chitosan gels cross-linked with 10 mM genipin, swollen in salt solution as a function of the swelling factor, *q*.

of salt is also shown, with the particles in the sample containing salt much less swollen than those in the solution that was only acidified.

Mechanical Behavior of Chitosan Hydrogels. When the force−displacement curves were measured for different swelling ratios using the TA, by measuring samples in the presence of different amounts of salt at pH 4.3, roughly linear regions were found in the deformation range of 0−0.4 mm. The slopes of the curves in this region were measured and the conversion factor of 7.7×10^3 was applied to give an approximate measure of the modulus. Figure 5 shows the dependence of this modulus on the swelling factor for chitosan hydrogels in the presence of salt. The data are plotted in terms of the swelling factor *q*, rather than the ratio *r*, since *q* is always a positive number and therefore allows for the presentation of the data in double logarithmic format.

The modulus of the gels increased with increasing swelling factor, indicating that the chains between the cross-links were becoming stressed as the average distance between junction points increased. For the range of swelling ratios measured here, no rise in modulus was observed as the gels de-swelled, although the data do appear to show some evidence of leveling off at low swelling. This observation is consistent with data for hydrogels of hydroxyethyl cellulose (HEC) /carboxymethyl cellulose (CMC),[24] in which a similar increase in modulus was measured as the gels were swollen in solvent from the as-made state. Upon decreasing the water content of the gels, by dehydration rather than de-swelling in strong salt, the modulus of many of the HEC/CMC samples continued to decrease. Only at rather high polymer concentration did the modulus decrease with increased swelling. The solid content of the gels presented here, even at the lowest swelling ratio, is still less than 2.4 wt %, so it is not unexpected that the minimum in the curve is not encountered within the experimental range above.

The fracture properties of the gel are of significance to its survival in the stomach. To survive intact, the gel must be able to withstand around 7 kPa pressure,[26] which would equate to forces of the order of 1 N in the current experiments. In the current test, the gels were observed to undergo several small fractures and the first fracture event is used in the analysis below. The force at first fracture and the displacement at first fracture are shown as a function of swelling factor in Figure 6. The fracture force followed the same pattern as the modulus, with a decrease from high to low swelling ratios. The values of the fracture force are comparable to the levels of force which are expected in the stomach. This indicates that at a swelling ratio of 30%, which is roughly the value expected for the pH of the

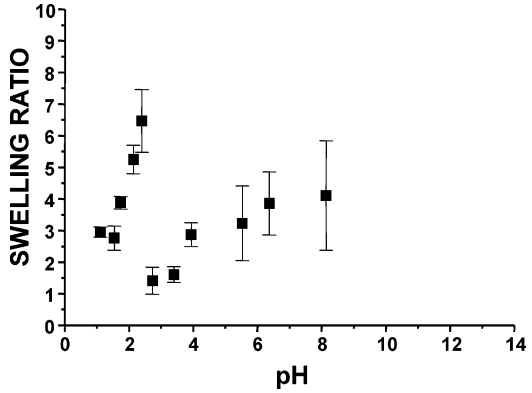

Figure 7. Swelling data for 15% BSA cross-linked with 5 mM genipin.

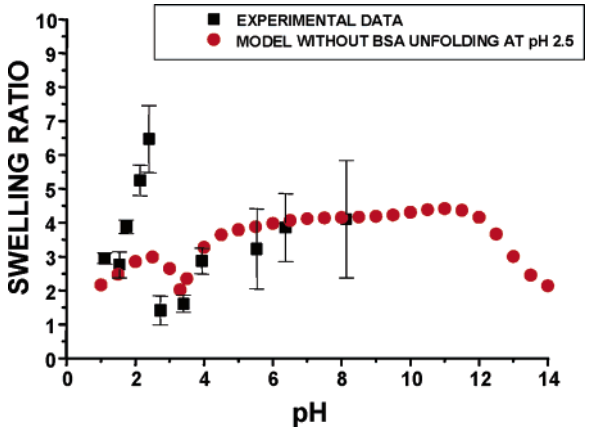

Figure 8. Modeling cross-linked BSA swelling data using electrolyte theory.

gastric environment, these gels would survive intact. The actual degree of swelling would, however, be limited by the presence of other ions present in the gastric environment.

Swelling Behavior of BSA Hydrogels. The experimental swelling data for BSA cross-linked with genipin are shown in Figure 7. The minimum at roughly pH 3 was initially surprising as the isoelectric pH (p*I*) of BSA is around 5.1. Minimum swelling would be expected to occur at the isoelectric condition. However, if all lysine amine residues are assumed to have been removed through reaction with genipin, the isoelectric value becomes much lower. This removal of free lysine was assumed in all of the BSA calculations, and produced the desired result. Also, because of the expected inflexible character of the initial BSA network ("string of beads" model) a value of $N = 1$, the lowest possible value for *N*, was also assumed. This left only one variable parameter to fit the swelling data, the shear modulus *G*. The experimental value for this was around 300 Pa, but when this value was adopted, the calculated swelling was much larger than found. A good fit to the experimental data was only achieved when the modulus was increased to 2281 Pa, the comparison with experiment being shown in Figure 9. As Figure 8 shows, the model now fits the experiment reasonably well down to pH 3 but fails to reproduce the swelling "spike" below this pH that is actually found. An alternative mechanism that was believed to be the acid-induced denaturation leading to unfolding of the BSA molecules below pH 3.0 was therefore considered to explain this excess swelling via a volume change of the BSA molecule.[27,28]

Although this unfolding cannot be modeled precisely using the current swelling program, its implications for swelling can be explored to some extent by increasing *N* to 2.5, at, and below, pH 2.5, to take into account increased peptide chain flexibility.

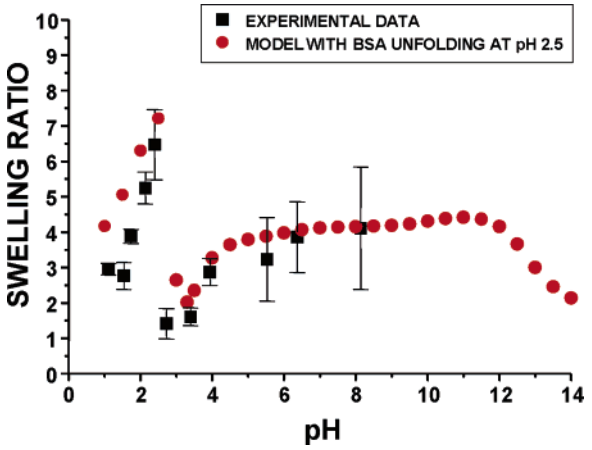

Figure 9. Modeling the BSA swelling data taking into account the denaturation of BSA at pH 3.0.

The shear modulus must also be changed to 1369 Pa to account for the accompanying decrease in the contribution of the more flexible chains to the modulus (i.e., fall in *a*). The physical interpretation of these changes is that the unfolding produces a network of more flexible strands, but with the same points of cross-linking. Shown in Figure 9, as a result of these changes, the swelling spike at low pH is quite well reproduced. As for chitosan, the rapid falloff in swelling at pH's lower than 2.5 was explained through a salt effect generated by added HCl.

The overall result for BSA is, therefore, reasonably convincing but, as in the chitosan case, there remains the problem as to why the modulus required for the best model fit is so significantly higher than the value suggested by experiment (300 Pa for BSA). In the BSA case, this discrepancy is particularly significant, as no satisfactory fit could be obtained assuming the experimental modulus and the BSA swelling data. Again, one can only appeal to problems with the theory related to the character of the protein network. The "string of beads" structure at pH's above 2.5 makes it unlikely that the expressions assumed when formulating the elastic and the mixing contributions to the osmotic pressure are completely realistic.

The swelling behavior of BSA was much less sensitive to salt. Upon increasing the salt concentration by a factor from zero added salt to 100 mM, the swelling of the chitosan gels was reduced by more than 100%, whereas for the BSA gels, the swelling was reduced by only 10%.

Mechanical Behavior of BSA Hydrogels. pH was used to control the swelling of the BSA samples in the vials subjected to the TA mechanical measurement technique. The swelling of the BSA hydrogels used for mechanical testing was less homogeneous than for the chitosan hydrogels, and they possessed an upper layer which appeared to hold more solvent than the gel near the base of the vial. Some dissolution of the gel also occurred, since the solvent removed from the gel was slightly blue in color.

The force−displacement curves for cross-linked BSA gels showed pronounced initial curvature. This may be due to some inhomogeneity within the sample causing the upper layer, which was richer in solvent, to be softer than the main body of the sample. The initial part of the curve was therefore ignored in the data analysis and moduli were obtained from the region of the curve in the deformation range 1−2 mm. The dependence of the modulus on the swelling factor, *q*, is shown in Figure 10 and shows a trend for decreasing modulus with increasing swelling ratio. Swollen BSA samples had much lower moduli than swollen chitosan samples. The un-swollen BSA sample

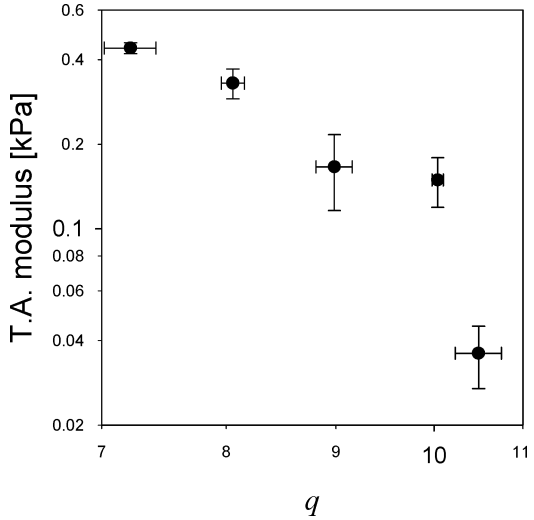

Figure 10. Apparent modulus of swollen 15% BSA gels cross-linked with 10 mM genipin.

possessed a modulus around 10 kPa, which was much greater than those for the swollen samples, however, and was comparable to the swollen chitosan samples.

The behavior of the BSA modulus with swelling ratio is expected for the suggested BSA swelling mechanism of an unfolding conformational transition around pH 3, rather than swelling solely due to the polyelectrolyte behavior. In the conformational transition mechanism, instead of the chain between cross-links becoming stretched as the gel swells, the chain simply changes conformation, releasing stress while still allowing the junction zones to move further apart. It is possible that higher swelling ratios would involve stretching of the unfolded chains and may lead to an increase in modulus, although this range was not approached in the current experiment.

The fracture properties of the BSA gels could not be measured. The untreated gel did undergo fracture at a displacement of around 5 mm (the sample depth in the vial was around 10 mm). However, the softer swollen samples showed no clear evidence of fracture within the 8 mm range of the test. This indicates that the samples were highly deformable. With reference to application in the stomach, particles made from material such as this would be likely to pass through the pyloric sphincter with relative ease as they would deform sufficiently to pass through the small opening. BSA alone then would not seem to offer opportunities for gastric retention and prolonged satiety although their ability to swell in gastric conditions (i.e., at low pH in the presence of salt) does still offer opportunities for controlled delivery.

Swelling and Mechanical Behavior of BSA/Chitosan Mixed Hydrogels. From the previous experiments, it was clear that, although possessing suitable mechanic strength, chitosan hydrogels would be of little use in gastric conditions where swelling was necessary because of the adverse effects of gastric salt concentrations on the ability of the hydrogels to swell. Conversely, while remaining swellable in gastric conditions, BSA hydrogels possessed unsuitable mechanical properties. In an attempt to achieve good swelling under gastric conditions and suitable mechanical properties, a composite sample of chitosan and BSA was co-cross-linked with genipin and its behavior assessed. Stock solutions of reagents were mixed, and the reaction was allowed to proceed for >100 h before samples were immersed in 3 L of solvent. The salt content of the solvent was adjusted to 10 mM, to prevent excessive swelling of the

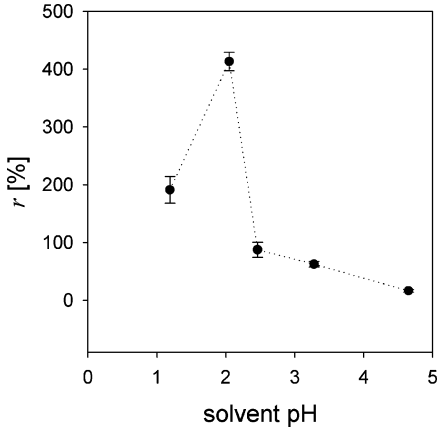

Figure 11. Swelling ratio of 1.5% chitosan/15% BSA composite gels cross-linked with 10 mM genipin, in 10 mM salt, as a function of pH.

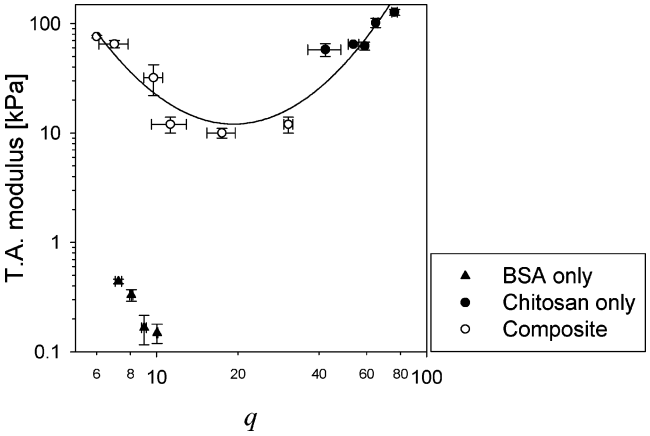

Figure 12. Modulus of 1.5% chitosan/15% BSA composite hydrogels cross-linked with 10 mM genipin, as a function of the swelling factor. The solid line represents a parabolic fit to the chitosan only and composite data as a guide to the eye. Data for chitosan and BSA are shown for comparison.

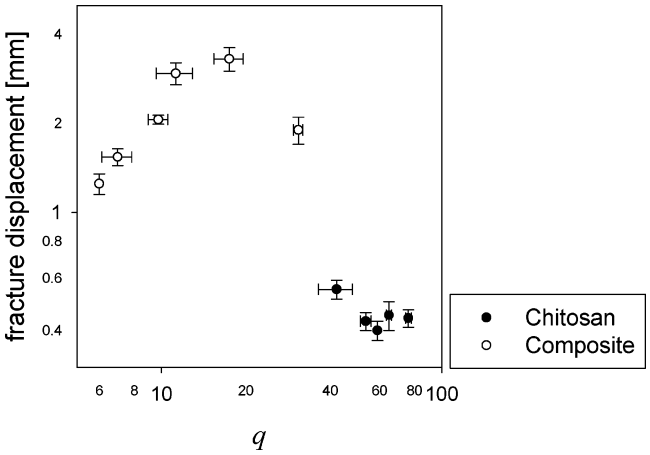

Figure 13. Comparison of the displacement at first fracture for 1.5% chitosan/15% BSA composite hydrogels cross-linked with 10 mM genipin and chitosan-only hydrogels.

chitosan and structural failure of the samples, using 0.05% sodium azide and 0.135% sodium chloride. The salt solutions were then acidified with HCl to give pHs in the range 1−5, and samples were left under solvent for >100 h before being removed and removing the excess water for measurement. Figures 11−14 show the results of the swelling and mechanical testing experiments of these samples.

The swelling behavior that is shown in Figure 11 for the composite hydrogels was similar to that of the BSA hydrogels. This result indicates that, at low pH values, the BSA unfolding mechanism was primarily responsible for the observed swelling. The behavior at higher pH was somewhat different. The increase in swelling ratio seen for BSA hydrogels was not observed for the composite material. It is likely that at higher pH values, above the BSA unfolding transition, the chitosan network prevented the dissolution of the gel and kept the swelling ratio low. The swelling ratio of the composite at pH 4.3 was comparable to the swelling ratio observed for the chitosan hydrogels under comparable conditions of 10 mM salt. At this pH, the swelling should be controlled by the chitosan since the BSA will still be in its folded conformation. The maximum observed swelling of the composites was relatively unaffected by the presence of the salt, again indicating that a conformational transition, rather than the polyelectrolyte behavior, was the controlling effect. Interestingly, the maximum observed swelling ratio for the composites was recorded for pH 2.05 to be greater than 400%. This is significantly higher than the maximum of ∼60% observed at pH 1.8 for the BSA only samples. Some of this difference may be accounted for by the sensitivity of the swelling ratio to pH in this region. However, the cross-link density between BSA chains may also be lower in the composite sample, allowing a greater degree of swelling to be possible. The level of genipin used was constant between the samples, and in the case of the composite, a significant proportion of the genipin will react with the chitosan. The resulting effective decrease in cross-link density for BSA may give rise to the greater degree of swelling observed in the composite hydrogels.

The q dependence of the modulus is compared to that of the chitosan and BSA hydrogels in Figure 12. Again, it would appear that the swelling behavior was dominated by the BSA. At low swelling ratios, a similar decrease in modulus was measured with increased swelling ratio. As the swelling increased further, the slope of the curve reached a minimum value at $q = 20$. Importantly, although the swelling mechanism may be controlled by the BSA component, the mechanical properties of the hydrogel were imparted by the chitosan component. The solid line of Figure 12 represents a quadratic fit to the combined data sets of the composite hydrogels and the chitosan hydrogels. This type of parabolic dependence has been observed previously.[24] At low degrees of swelling, it is believed that the mobility of the extra solvent leads to a decrease in modulus. At higher swelling ratios, the finite extensibility of the polymer chain segments between cross-links leads to an increase in modulus. The position of the minimum of the modulus versus swelling ratio curve depends on the flexibility of the polymer chains. The single curve that fitted both the composite and chitosan data demonstrates that the behavior was consistent for both composite and chitosan hydrogels. This indicates that the chitosan was responsible for the structural strength of the composite hydrogels. The minimum modulus was of the order of 10 kPa which indicates that the composite material would be structurally stable in gastric conditions even when highly swollen (swelling ratio 400%). This value is also consistent with the gel strength required for survival in the stomach. The minimum in the quadratic fit occurred at $q \approx 19$, equivalent to a solids concentration of around 5%.

The fracture properties of the composite hydrogels were intermediate between those of chitosan and BSA hydrogels. The displacement at first fracture is compared for the composite and chitosan-only gels in Figure 13. Whereas the BSA hydrogels continued to deform without fracture throughout the entire test range, the composite gels did have a fracture profile. Fracture

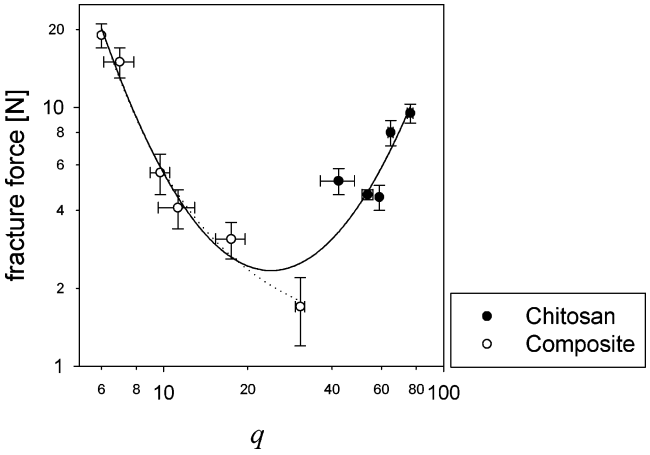

Figure 14. Comparison of the q dependence of the fracture force of 1.5% chitosan/15% BSA composite gels cross-linked with 10 mM genipin and chitosan-only hydrogels. The dotted line represents an exponential fit to the composite data only, to enable a prediction of the minimum fracture force. The solid line represents a parabolic fit all the data as a guide to the eye.

occurred at significantly higher degrees of deformation than seen for the chitosan samples however. The force at fracture is compared in Figure 14. It can be seen from this that the force required to fracture the composite samples decreased with an increase in swelling ratio. There was pronounced curvature in the data, however. Fitting the data to an exponential decay gave a value for the minimum fracture force >1 N, which is greater than the 0.65 N threshold suggested for survival of objects in the stomach.[26] The absolute force required for fracture was also comparable to that required for fracture of the chitosan samples (see solid line as a guide for the eye) again indicating that the mechanical strength of the samples is derived from the chitosan network. Indeed when the two sets of data were compared it was possible to speculatively fit a smooth curve between the two, as indicated by the solid line of Figure 14, and again a minimum was observed at around $q \approx 20$ and $F \approx 2$ N.

Conclusions

The swelling and mechanical properties of swellable hydrogels formed from chitosan, BSA, and mixtures of chitosan and BSA, cross-linked with genipin, were measured, and theoretical calculations were made for the swelling ratio of chitosan and BSA hydrogels as a function of pH.

Chitosan hydrogels possessed good mechanical strength, although they were brittle and fractured at low displacements. Their swelling behavior was dictated by their polyelectrolyte nature, and an increase in swelling was observed below the pK_a of the chitosan amine groups as they became protonated. Similar behavior was predicted by theoretical calculations, although quantitative agreement between theory and experiment was not obtained. It was postulated that this was due to the nature of the genipin cross-linker, which may form wasted cross-links as well as polymerize, leading to cross-links formed from not just one genipin molecule but various dimers, trimers, oligomers, and possibly polymers of genipin. Similar considerations apply for the BSA hydrogels. Owing to the polyelectrolyte nature of chitosan, limited swelling occurred in the presence of salt that screened the charges on the protonated amine groups. The use of chitosan hydrogels as materials to swell in the stomach is therefore limited, as gastric conditions include high ionic concentrations.

Genipin cross-linked BSA hydrogels were weak and highly deformable when swollen and underwent partial dissolution when left for long periods of time under gastric pH conditions. Although these gels were not observed to undergo fracture, even at very high strains, the highly deformable nature would allow the gels to be passed through the stomach with ease, limiting their use as materials that required gastric retention. The swellability of these gels was much less affected by the presence of ionic species, however, and the swelling behavior was markedly different from chitosan. A sharp increase in swelling ratio was measured around pH 3, which was postulated to occur due to an acid induced denaturation of the BSA molecule that caused unfolding of the protein conformation, rather than being due to polyelectrolyte effects.

A composite hydrogel formed from a cross-linked mixture of both chitosan and BSA was found to have both good mechanical and good swelling properties for use in gastric conditions. The swelling was determined, at low pH, by the BSA moiety, whereas the mechanical properties were determined by the stronger chitosan moiety. These composite materials may therefore be suitable for use as swellable materials to enhance gastric retention or for controlled release in gastric conditions. The volume change observed under conditions similar to those found in the stomach was approximately 400%. At this degree of swelling, the gel was still found to possess a modulus of 10 kPa, and from the fracture properties, it was predicted that the composite hydrogel would be able to withstand the 7 kPa pressure inflicted by the grinding action of the stomach. It is believed that for successful gastric retention the diameter of an object must be at least 2 mm. Given the observed swelling ratio, this would indicate that the average particle size on consumption should be at least 1 mm in diameter for effective use of these materials for gastric retention, although significantly smaller particles could be used in controlled release applications.

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