

Research Article

Genipin Cross-Linked Chitosan-Polyvinylpyrrolidone Hydrogels: Influence of Composition and Postsynthesis Treatment on pH Responsive Behaviour

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Understanding the factors that influence the pH responsive behaviour of biocompatible cross-linked hydrogel networks is essential when aiming to synthesise a mechanically stable and yet stimuli responsive material suitable for various applications including drug delivery and tissue engineering. In this study the behaviour of intelligent chitosan-polyvinylpyrrolidone-genipin cross-linked hydrogels is examined as a function of their composition and postsynthesis treatment. Hydrogels are synthesised with varying amounts of each component (chitosan, polyvinylpyrrolidone, and genipin) and their response in a pH 2 buffer is measured optically. The influence of postsynthesis treatment on stability and smart characteristics is assessed using selected hydrogel samples synthesised at 30, 40, and 50°C. After synthesis, samples are exposed to either continuous freezing or three freeze-thaw cycles resulting in increased mechanical stability for all samples. Further morphological and mechanical characterisations have aided the understanding of how postsynthesis continual freezing or freeze-thaw manipulation affects network attributes.

1. Introduction

Smart polymers, also termed “intelligent” or “stimuli responsive” polymers, are a class of materials that can adjust themselves in a particular way in response to external stimuli, for example, pH, temperature, electric field, mechanical stress, chemical/biological agents, magnetic field, radiation, and ultrasound [1]. Smart polymeric materials have been in existence for some time and a large number of studies of possible applications of these polymers in areas such as pharmaceuticals, oil recovery, fluidics, and tissue engineering have been reported [2–6]. Predominantly, research has focused on smart polymer gels, in particular smart hydrogels that are responsive to pH (e.g., chitosan) or temperature (e.g., poly(*N*-isopropylacrylamide)) [1, 4]. Hydrogels are of interest as it is possible to adjust their response with respect to both the rate and the extent of swelling by varying their processing conditions [7].

In this work the behaviour of a chitosan-polyvinylpyrrolidone hydrogel chemically cross-linked with genipin

(Cht-PVP-Gen hydrogel) is studied. In addition to its smart response to variation in pH, this hydrogel is also known for its biocompatibility, which makes it a good candidate for *in vivo* applications such as drug delivery [2–4]. However, effective applications can only be achieved if the hydrogel can be synthesised as a stable smart material, with an adjustable response. This paper aims to understand the variables that influence these desirable characteristics.

Chitosan (Cht) (Figure 1) is a linear, natural polysaccharide, derived from the deacetylation of chitin, an abundant natural polymer, obtained from crustacean, and fungal mycelia [8–11]. Cht is a copolymer consisting of (1,4)-2-acetamido-2-deoxy- β -D-glucan (N-acetyl D-glucosamine) and (1,4)-2-amino-2-deoxy- β -D-glucan (D-glucosamine) units [8, 9].

The properties of Cht depend largely on the degree of deacetylation and the resulting molecular weight [11, 12]. The decision on which properties to preserve is hence dependent upon the desired application. For example, high solubility requires a high degree of deacetylation whilst good

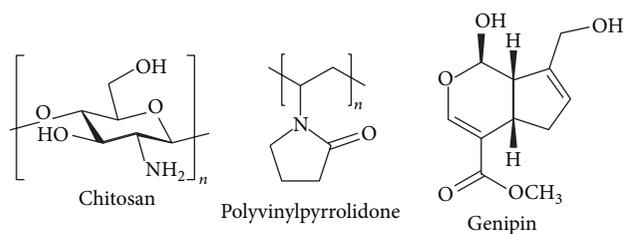


FIGURE 1: Chemical structure of chitosan, polyvinylpyrrolidone and genipin.

biocompatibility is achieved with lower deacetylation [11, 13]. Cht is a polyelectrolyte with both amino and hydroxyl groups [1, 5, 12, 14]. At low pH values, (below \sim pH 6) the Cht amino groups are protonated, forming quaternary ammonium salts possessing positive charges, making it a water-soluble, cationic polyelectrolyte [11, 13]. At higher pH the amino groups become deprotonated and Cht becomes insoluble. The transition between the soluble and insoluble forms of Cht following a change in pH accounts for its smart nature. The exact pKa, which determines the transition pH value, is largely dependent on the degree of deacetylation during production [11, 12, 15].

Polyvinylpyrrolidone (PVP) is a nonionic, synthetic polymer usually obtained by free radical polymerisation of N-vinylpyrrolidone in water with hydrogen peroxide as an initiator [16, 17]. PVP can also be produced in organic solvents, such as 2-propanol, with an organic peroxide as the initiator [17]. The structure of PVP is illustrated in Figure 1. Due to its nonionic nature, swelling in PVP hydrogels is independent of pH and is controlled by the forces of interaction between PVP and the liquid medium [16]. PVP is soluble in both highly hydrophilic and hydrophobic solvents [17]. In aqueous solutions, the viscosity of PVP is dependent on its average molecular weight and concentration. PVP is amphiphilic in nature, hence exhibiting good adhesive and cohesive properties. Due to its structure, high polarity, and ability to accept protons, PVP is cross-linkable and forms chemical complexes. PVP is a superdisintegrant and continues to swell until maximum swelling power is attained [16]. Consequently, it is used in tablet preparation to enhance moisture penetration and improve dispersion allowing for enhanced matrix formation. At the same time, PVP is physiologically inert and hemocompatible [17, 18].

Genipin (Gen) is a small molecule chemical cross-linker obtained from natural sources such as *Genipa americana* and *Gardenia jasminoides Ellis* [19, 20]. Production of Gen from *Gardenia jasminoides Ellis* requires extraction of glucose from geniposide by hydrolysis. The structure of Gen is illustrated in Figure 1. Gen possesses several characteristics which makes it useful in a diverse range of applications. Gen is a unique cross-linker due to its low cytotoxicity and has been used in traditional Chinese medicine to treat jaundice, skin ulcers, and pyrogenic infections [19, 20]. Other studies show that Gen acts as an anti-inflammatory and antiangiogenesis agent, while inhibiting lipid peroxidation [19]. Gen has potential to replace toxic cross-linkers such as glutaraldehyde, especially

in biomedical applications such as production of bioadhesives for surgery [19]. In native form Gen is colourless; however reaction with amino acids generates blue pigments, a property that is being exploited in food colouration in parts of Asia [19]. Gen is known as an effective cross-linking agent in polymers and molecules that comprise amines. The proposed mechanism of cross-linking involves a nucleophilic attack of primary amines on the C3 carbon of Gen, resulting in the opening of the dihydropyran ring and formation of an aldehyde. The aldehyde group is subsequently attacked by the secondary amine group followed by dimerisation formed by a radical reaction [19]. Following successful cross-linking, genipin-containing networks exhibit a high degree of autofluorescence [21], making such materials promising candidates for imaging and use as tracers.

With the increasing need for biocompatible smart hydrogels for *in vivo* applications, understanding the behaviour of smart Cht-PVP-Gen hydrogels is of interest. Studies involving Cht-PVP hydrogels with other cross-linking agents such as acrolein [22] and glutaraldehyde [23–26] have been reported previously, as well as studies focusing on hydrogels with only Cht polymers cross-linked with Gen and tripolyphosphate [20]. Research in the area of Cht-PVP-Gen hydrogels is, however, more limited. Khurma et al. [27] used gravimetric analysis to explore the effects of varying concentration of PVP and polymerisation temperature on swelling. By varying the molar ratios of PVP (0–3.84) and keeping that of Gen and Cht constant (Cht at 1.00 and Gen at 0.025), they showed that the degree of hydrogel swelling and equilibrium water content was dependent on the temperature and pH of the swelling medium. The study demonstrated that the response achieved was enhanced with increasing PVP content in the hydrogel which was concluded to be due to the gel structure becoming less compact and more inhomogeneous. Maximum swelling was observed at low pH and high temperature. Hurst and Novakovic [18] investigated the morphology and smart characteristics of a single formulation of Cht-PVP-Gen hydrogel. The evaluation of pore structures by morphological characterisation was attempted using scanning electron microscopy (SEM), environmental scanning electron microscopy, and *in situ* confocal laser scanning microscopy (CLSM) applied in both reflectance and fluorescence modes. In particular, CLSM studies allowed *in situ* observation of pore size changes during swelling providing useful information on variations in internal structure.

The focus of this study is to enhance understanding of the factors that influence pH responsive behaviour in Cht-PVP-Gen hydrogels, aiming to assist the tailored design of mechanically stable and yet stimuli responsive materials suitable for various applications including drug delivery. Factors assessed were amount of Cht, Gen, and PVP in a hydrogels sample. Furthermore the stability and, inherently, the mechanical properties of smart hydrogels are reported to be affected by freezing and freeze-thaw cycles following their synthesis [28]. At the same time, it is recognized that polymerisation temperature is an important parameter [29]. Therefore in this study the effects of freezing time and polymerisation temperature on Cht-PVP-Gen hydrogels were investigated. In addition, morphological and mechanical

TABLE 1: The code and composition of hydrogels obtained using a full factorial method of experimental design. Three factors (solutions of Cht, PVP, and Gen) at two levels (low (L) and high (H) concentrations) were employed. Samples were polymerised at 50°C in an oven for 24 h and subsequently stored at 5°C in a fridge.

Gel code	Independent factors						Average molar ratio Cht:PVP:Gen
	Cht		PVP		Gen		
	Level	Volume ^α (mL)	Level	Volume ^ψ (mL)	Level	Volume ^ξ (mL)	
HHH	H	0.5	H	0.5	H	0.100	1:19:69
HHL	H	0.5	H	0.5	L	0.004	1:19:3
HLL	H	0.5	L	0.2	L	0.004	1:8:3
LLL	L	0.2	L	0.2	L	0.004	1:19:7
LLH	L	0.2	L	0.2	H	0.100	1:19:171
LHL	L	0.2	H	0.5	L	0.004	1:49:7
HLH	H	0.5	L	0.2	H	0.100	1:8:69
LHH	L	0.2	H	0.5	H	0.100	1:49:171

^α1.5% (w/v) Cht in an aqueous 1% (v/v) acetic acid solution; ^ψ5% (w/v) PVP aqueous solution; ^ξ0.5% (w/v) Gen aqueous solution.

characterisation studies were performed to aid the understanding of how postsynthesis continual freezing or freeze-thaw manipulation affects network attributes.

2. Materials and Methods

PVP (approximate molecular weight 40,000 g/mol; product number PVP40, lot number BCBG0598V), Cht (molecular weight 190,000–300,000 g/mol with 80% deacetylation; product number 448877, lot number MKBF1336V), Gen ($\geq 98\%$; product number G4796, lot number 063M4705V), and glacial acetic acid were purchased from Sigma Aldrich. Glycine (pH 2) and phosphate (pH 7) buffers were obtained from Fisher Scientific. All chemicals were used as received.

2.1. Hydrogel Synthesis for Smart Response Studies. A design of experiments was used to select the composition of hydrogels to be synthesised. Three factors were chosen (i.e., amount of Cht, Gen, and PVP) and each factor was considered at two levels (low (L) and high (H) concentration). A full factorial method of experimental design [30] was used giving a total of (2)³ hydrogels as detailed in Table 1.

Regarding *Cht solution*, Cht was dissolved in an aqueous 1% (v/v) acetic acid solution, made using deionised water, with the aid of stirring for 24 h at room temperature to attain a 1.5% (w/v) viscous solution with a pale yellow colour. Regarding *PVP solution*, a 5% (w/v) homogeneous transparent PVP solution was obtained by dissolving PVP in deionised water at room temperature with constant stirring for 24 h. Regarding *Gen solution*, a transparent Gen solution was obtained by dissolving Gen powder in deionised water for 15 min to obtain a 0.5% (w/v) solution. To ensure a homogeneous solution was obtained, the dissolution of genipin was carried out in an ultrasonic bath at room temperature.

Based on the volumes and concentrations of the Cht, PVP, and Gen solutions used, the molar concentration of Cht, PVP, and Gen was calculated for each sample and converted to molar ratio. To acknowledge that the molecular weight of Cht is a range, the average number of moles of Cht was used to calculate an average molar ratio (Table 1). Hydrogels were

prepared using the quantities of Cht, PVP, and Gen solutions specified in Table 1. Cht and PVP were combined in a 5 mL, 1 cm diameter polyethylene vial and continuously stirred at ambient temperature for 10 min. Gen solution was then added in two portions at 5 min intervals with constant stirring. The stirrer bar was removed and sample vials were closed and sealed with parafilm to prevent opening during polymerisation. Samples were polymerised at 50°C for 24 h in an oven. Following polymerisation hydrogels were stored in a fridge at 5°C. Prior to use, hydrogel samples were extracted from the vials by removal of the vial base and gentle pushing of the gel from the top of the vial using a cork borer. Hydrogel samples were washed with deionised water (24 h at ambient temperature) and the wash water sample was analysed using Fourier transform infrared spectroscopic analysis. Method confirmed absence of unreacted Cht, PVP, and Gen.

2.2. Hydrogel Preparation for Postsynthesis Freezing and Freeze-Thaw Studies. The effects of freezing time and polymerisation temperature on Cht-PVP-Gen hydrogels were investigated using a smart hydrogel with the code LHL (Table 1). Selection was based on results recorded in optical evaluation of gel response to pH change. Six LHL hydrogel samples were synthesised, this time at three different oven temperatures: 30°C (LHL1 and LHL2), 40°C (LHL3 and LHL4), and 50°C (LHL5 and LHL6). The polymerisation time for all samples was 24 h. The gels were then either frozen continuously for 72 h at -10°C (LHL1, LHL3, and LHL5) or subjected to three freeze-thaw cycles each comprising 24 h freezing at -10°C and 3 h thawing at room temperature (LHL2, LHL4, and LHL6). Subsequently, the gels were stored at 5°C. Prior to use, the samples were extracted from the vials and washed with deionised water. Experimental conditions and treatments are summarized in Table 2.

2.3. Smart Characteristics Studies. Due to a large variation in the stability of samples, the swelling response of the hydrogels prepared (Tables 1 and 2) was followed optically using cameras, rather than gravimetrically. Washed hydrogels were contracted in phosphate buffer solution (pH 7) for 24 h.

TABLE 2: Process parameters used to study the effect of polymerisation temperature and postsynthesis treatment (freezing time and number of freeze-thaw cycles) on the responsiveness of LHL Cht-PVP-Gen hydrogels.

Gel code ^a	Polymerisation temperature (°C)	Freezing time at -10° C (h)
LHL1	30	72
LHL2	30	3 freeze-thaw cycles: 24 h freezing and 3 h thawing each
LHL3	40	72
LHL4	40	3 freeze-thaw cycles: 24 h freezing and 3 h thawing each
LHL5	50	72
LHL6	50	3 freeze-thaw cycles: 24 h freezing and 3 h thawing each

^aSample composition: 0.2 mL Cht solution, 0.5 mL PVP solution, and 0.004 mL Gen solution; average molar ratio of Cht : PVP : Gen is 1 : 49 : 7.

After that, gels were removed from the solution and their surface blotted to remove any excess liquid. Gels were then fixed to the base of a petri dish using a minimal amount of fluid resistant glue. The petri dish was placed on a custom-made stand on an optical bench. Two cameras, Nikon D7000 and D5100, with AF-S micro-Nikon 40 mm DX 1:2.8 G lenses were set to take shots of the top surface area and height of the gel. This allowed the overall volume change of the gel to be assessed as a function of time. The interval timers on both cameras were set at 10 min over a period of 7 h. After the timers were set off simultaneously and the first shots taken, glycine buffer (pH 2) was added to the petri dish and images were obtained. Optical studies were performed at room temperature. The images were analysed using ImageJ software (<http://rsbweb.nih.gov/ij/download.html>) and the top surface area and height of the hydrogels were quantified. The relative volumetric swelling ratio was calculated as

$$\Psi_V = \left[\frac{V - V_0}{V_0} \right] * 100, \quad (1)$$

$$V = A * H, \quad (2)$$

$$V_0 = A_0 * H_0, \quad (3)$$

where Ψ_V is relative volumetric swelling ratio; V is relative volume of the hydrogel at a given time following glycine buffer addition; V_0 is relative volume of the hydrogel before glycine buffer was added (time zero); A is surface area of the hydrogel calculated following immersion of the gel in glycine buffer; H is height of the hydrogel measured following immersion of the gel in glycine buffer; A_0 is surface area of the hydrogel calculated before glycine buffer was added (time zero); H_0 is height of the hydrogel measured before glycine buffer was added (time zero).

2.4. *Morphological Characterisation via Scanning Electron Microscopy (SEM)*. Hydrogel samples listed in Table 2 (LHL1-LHL6) were synthesised in duplicate. Following freezing/freeze-thawing manipulations, samples were rapidly frozen in liquid nitrogen and dried in a freeze dryer (Lab-conco, Freezone 1) with the ice condenser temperature at -55° C for 48 h until all the solvent sublimed. Each sample was then mounted on a carbon disk supported by an aluminium stub and coated with a 15 nm layer of gold (Polaron SEM, Coating Unit). For comparison, the same procedure was applied to additional LHL hydrogel samples polymerised at 30, 40, and 50° C without postmanipulation. Representative images of the hydrogel morphology were collected on an SEM (Cambridge Stereoscan 240).

2.5. *Mechanical Characterisation via Uniaxial Compression*. Duplicate hydrogel samples were synthesised (LHL1-LHL6) according to Table 2. The diameter and thickness of all specimens were accurately determined using a digital calliper. A Tinius Olsen H25KS mechanical test frame equipped with a 100 N load cell was used to compress specimens to 50 percent of their thickness at a rate of 0.5 mm min⁻¹. A preload of 0.05 N was applied to each sample to ensure an evenly distributed contact between the compression platen and the hydrogel. Stress-strain curves were subsequently recorded and used to compare the mechanical behaviour of individual samples.

3. Results and Discussion

Prior to polymerisation all samples were transparent, viscous, and pale pink. Visual observations following synthesis of the hydrogels listed in Table 1 are summarized in Table 3.

The stability of the hydrogels appears to correlate with the depth of the blue colour observed in the samples. Gen is known to react with amino groups to form blue pigments [19, 31] as a result of oxygen radical induced polymerisation initiated by a ring opening reaction [26, 32]. Based on the observations given in Table 3 and the absence of amine functional groups in PVP, it can be postulated that the key factor in the stability of the hydrogel is the Gen-cross-linked-Cht component of the network. In visually stable samples (HHH, HLH, LLL, and LLH), high cross-linking levels are likely to be present as the average molar ratio of Gen to Cht in the sample is 7 : 1 and higher. At the same time, PVP content is of importance and its increase above certain amount results in decrease in stability (Table 3). Relatively stable samples (HLL, LHH) have either a low PVP content (average molar ratio Cht : PVP : Gen = 1 : 8 : 3) inducing increased stability regardless of the low Gen to chitosan ratio or high molarity of PVP compensated with a high Gen to Cht ratio (average molar ratio Cht : PVP : Gen = 1 : 49 : 171) enabling a higher level of cross-linking. Samples containing higher volumes of PVP and average molar ratio of Gen to Cht of 7 : 1 and lower (HLL, LHL; Table 3) are unstable. PVP is known to exist in a linear form suggesting that while the reaction occurs between Cht and Gen, PVP assembly within the hydrogel network is likely to be in the form of a semi-interpenetrating network structure [33, 34].

TABLE 3: Visual observations of hydrogels. Samples were polymerised at 50°C for 24 h.

Gel code	Average molar ratio Cht : PVP : Gen	Stability judged by the sample's ability to retain the shape of the vial on extraction from the vial	Hydrogel colour
HHH	1 : 19 : 69	Stable	Very deep blue
HHL	1 : 19 : 3	Unstable	Light blue
HLL	1 : 8 : 3	Relatively stable	Light blue
LLL	1 : 19 : 7	Stable	Very deep blue
LLH	1 : 19 : 171	Stable	Very deep blue
LHL	1 : 49 : 7	Unstable	Light blue (lightest shade)
HLH	1 : 8 : 69	Stable	Deep blue
LHH	1 : 49 : 171	Relatively stable	Light blue

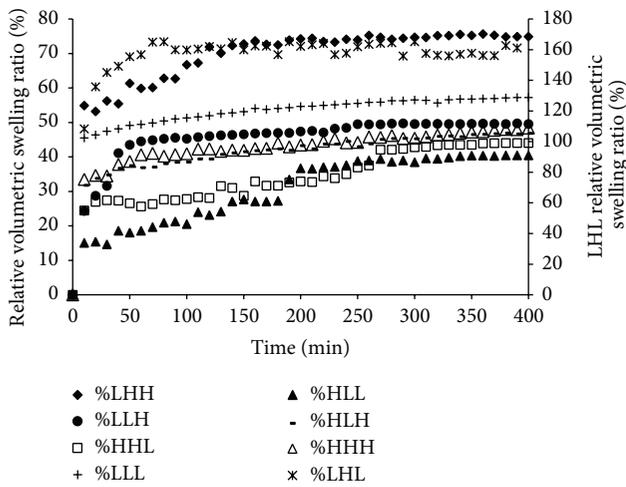


FIGURE 2: Relative volumetric swelling of samples listed in Table 1 screened optically in pH 2 (glycine) buffer. Washed gels were initially contracted with pH 7 phosphate buffer solution. Gels were then removed from solution (time zero on the graph) and pH 2 buffer was added.

3.1. *Optical Evaluation of Gel Response to pH Change.* After washing, gels were contracted with pH 7 phosphate buffer solution for 24 h and subsequently their ability to respond to a pH change was evaluated optically in a pH 2 (glycine) buffer. The change in hydrogel volume was recorded via changes in sample height and top surface area using ImageJ image processing software and calculated as defined by (1) (Figure 2).

The highest degree of swelling (Figure 2) is recorded in the LHL sample with average molar ratio of Cht : PVP : Gen = 1 : 49 : 7 (165%, secondary vertical axis), followed by the LHH sample with average molar ratio of Cht : PVP : Gen = 1 : 49 : 171 (75%) and the LLL with sample average molar ratio of Cht : PVP : Gen = 1 : 19 : 7 (57%). Subsequently, the LLH (average molar ratio of Cht : PVP : Gen = 1 : 19 : 171), HHH (average molar ratio of Cht : PVP : Gen = 1 : 19 : 69), and HLH (average molar ratio of Cht : PVP : Gen = 1 : 8 : 69) samples achieved a relative volumetric swelling ratio of 50%, followed

by the HHL with average molar ratio of Cht : PVP : Gen = 1 : 19 : 3 (44%) and the HLL with average molar ratio of Cht : PVP : Gen = 1 : 8 : 3 (40%).

It can be noted that the highest degree of swelling is achieved for the sample containing a high PVP content combined with low Gen to chitosan ratio (LHL). The relevance of high PVP content is further demonstrated in the LHH sample that also showed a considerable swelling response, this time in the presence of high Gen to chitosan molar ratio. Both samples were light blue (Table 3) which further indicates that increasing presence of PVP above certain level reduces level of cross-linking present. Similarly Khurma et al. [27] reported the highest swelling ratio in a hydrogel sample with the highest amount of PVP.

In samples with lower PVP to Cht ratio (19 : 1 and lower) and higher Gen to Cht molar ratio (69 : 1 and above) (samples LLH (average molar ratio of Cht : PVP : Gen = 1 : 19 : 171), HHH (average molar ratio of Cht : PVP : Gen = 1 : 19 : 69), and HLH (average molar ratio of Cht : PVP : Gen = 1 : 8 : 69)), the swelling recorded is similar (approximately 50% relative volumetric swelling ratio). All three samples were visually stable and deep blue (Table 3), indicating that reduced swelling response is governed by lower PVP presence.

The lowest degree of swelling is noted in HHL (average molar ratio of Cht : PVP : Gen = 1 : 19 : 3) and HLL (average molar ratio of Cht : PVP : Gen = 1 : 8 : 3) samples. In both cases ratio of Gen to Cht is low (3 : 1) with ratio of PVP to Cht being 19 : 1 and lower. Both samples were light blue (Table 3) indicating low level of cross-linking. While reduced content of PVP results in lower swelling [27], when comparing these with same PVP but higher Gen to Cht molar ratio samples (LLL, LLH, HHH, and HLH), it may be concluded that reduced cross-linking also results in a reduced swelling response. Furthermore, it can be observed (Figure 2) that the rate of swelling in these hydrogel samples is uneven and increases in an approximately stepwise manner. The observed behaviour may be explained by changes in chemical potential difference between the solvent (pH 2 in this case) and the polymer as the solvent travels inwards from the surface of the hydrogel. Hezaveh and Muhammad [35] describe this process in their report on swelling studies of kappa carrageenan/hydroxyethyl cellulose pH-sensitive hydrogels.

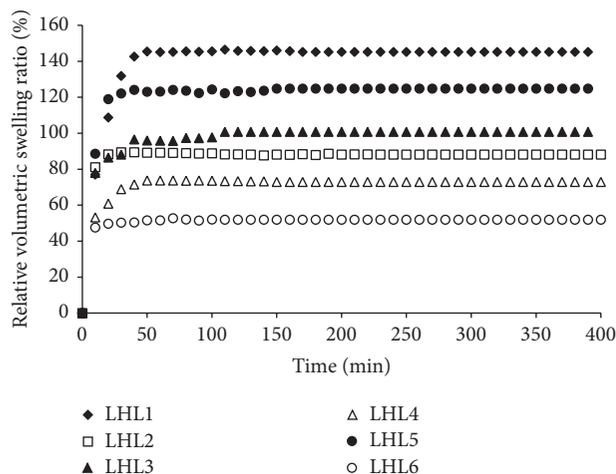


FIGURE 3: Relative volumetric swelling of LHL hydrogels listed in Table 2 following freezing and freeze-thaw treatment. Sample composition: 0.2 mL Cht solution, 0.5 mL PVP solution, and 0.004 mL Gen solution. Average molar ratio of Cht : PVP : Gen = 1 : 49 : 7.

3.2. Effects of Freezing and Freeze-Thaw Cycles on the Smart Properties and Stability of Cht-PVP-Gen Hydrogels. Applications of unstable materials are limited. This study aimed at assessing if sample stability can be enhanced without changing its chemical composition. The hydrogel with the code LHL (average molar ratio of Cht : PVP : Gen = 1 : 49 : 7) that was the least stable (Table 3) and the most responsive (Figure 2) was selected for this study. Experimental treatment of the gel is summarized in Table 2. All samples obtained following freezing and freeze-thaw cycles were more stable when visually compared with the LHL sample that was not subjected to freezing after synthesis. It was also noted that hydrogel samples polymerised at 30°C were a lighter shade of blue than those polymerised at 40°C and 50°C. The variation in colour suggests that the reduction in temperature decreases the rate of cross-linking between Gen and Cht and thereby reduces the degree of cross-linking which is reflected in the lighter blue pigmentation.

The smart behaviour of hydrogels listed in Table 2 was evaluated optically in a pH 2 (glycine) buffer in the same manner as described previously. The relative volumetric swelling ratio, indicating a change in sample volume, is shown in Figure 3.

All samples tested responded promptly (within 10 min) to the pH 2 environment and displayed a significant response (50–145%). In all cases samples swelled less than the untreated LHL sample (165%, Figure 2). It can be noted that, for all polymerisation temperatures, samples which were continuously frozen swelled more than those that underwent freeze-thaw treatment, with the highest swelling (145%) being observed in the sample polymerised at 30°C (LHL1, Figure 3). Differences between the swelling ratios of continuously frozen and freeze-thawed samples are significant. For hydrogel samples polymerised at 50°C (LHL5 and LHL6), the difference in swelling between the frozen and freeze-thawed samples was approximately 75%, while the differences for samples polymerised

at 30°C (LHL1 and LHL2) and 40°C (LHL3 and LHL4) were approximately 55% and 30%, respectively, as shown in Figure 3. As polymerisation temperature increases, the degree of swelling reduces for samples which were freeze-thawed (Figure 3). This observation is aligned with the expected increase in the level of cross-linking (resulting from increased polymerisation temperature) that leads to restricted swelling. For samples that were continuously frozen for 72 h this dependence was not observed; 30°C samples (LHL1) displayed the highest swelling ratio and 40°C (LHL3) the lowest. To further study how postsynthesis continual freezing or freeze-thaw manipulation affects hydrogel network attributes, morphological and mechanical characterisations are performed.

3.3. Effects of Freezing and Freeze-Thaw Cycles on the Morphology of Cht-PVP-Gen Hydrogels. The variation in network morphology of LHL hydrogels as a function of freezing/thawing including the polymerisation temperature dependence was visually characterized via SEM (Figure 4). Samples without postsynthesis treatment (Figures 4(a)–4(c)) have noticeable differences compared to those which were either continuously frozen (Figures 4(d)–4(f)) or subjected to freeze-thaw cycles (Figures 4(g)–4(i)). The hydrogel polymerised at 30°C without postsynthesis treatment (Figure 4(a)) shows a compact structure and pores of approximately 10–20 μm in diameter. In contrast, the sample polymerised at 40°C (Figure 4(b)) is more porous (approximate pore diameter of 40–50 μm) with a striated morphology. Upon polymerisation at 50°C (Figure 4(c)), the bulk structure recorded shows increased order and porosity with pores ranging between 40 and 80 μm in diameter. Hence, as polymerisation temperature is increased, up to 50°C, the size and order of the pores within the network also increase.

Samples that were continually frozen (Figures 4(d)–4(f)) show distinct differences compared to those not subject to postsynthesis treatment (Figures 4(a)–4(c)). The specimen polymerised at 30°C and continuously frozen (Figure 4(d)) has far larger pores within the bulk structure (approximately 100 μm) than an analogous specimen without postsynthesis treatment (Figure 4(a)) in which the pore diameter is approximately 10–20 μm. The large increase in pore diameter could be due to freezing of liquids within the hydrogel network, leading to crystallization in the pores to create an ordered three-dimensional network [36]. No significant variation in the number, size, or order of the pores was observed upon increasing the polymerisation temperature to 40 or 50°C (Figures 4(e) and 4(f), resp.); hence, for LHL hydrogel specimens, the effect of freezing exerts greater influence over gel morphology than the effect of polymerisation temperature.

Gels that were subject to three freeze-thaw cycles (Figures 4(g)–4(i)) also have a higher porosity than those without postsynthesis treatment (Figures 4(a)–4(c)) and display very similar morphologies to samples that were continuously frozen (Figures 4(d)–4(f)). Freeze-thawed specimens have an even higher average pore diameter of 150 μm. This could be due to liquid expansion within the hydrogel during each freeze cycle, pushing the polymer chains within close proximity of one another, facilitating hydrogen bonding and a greater

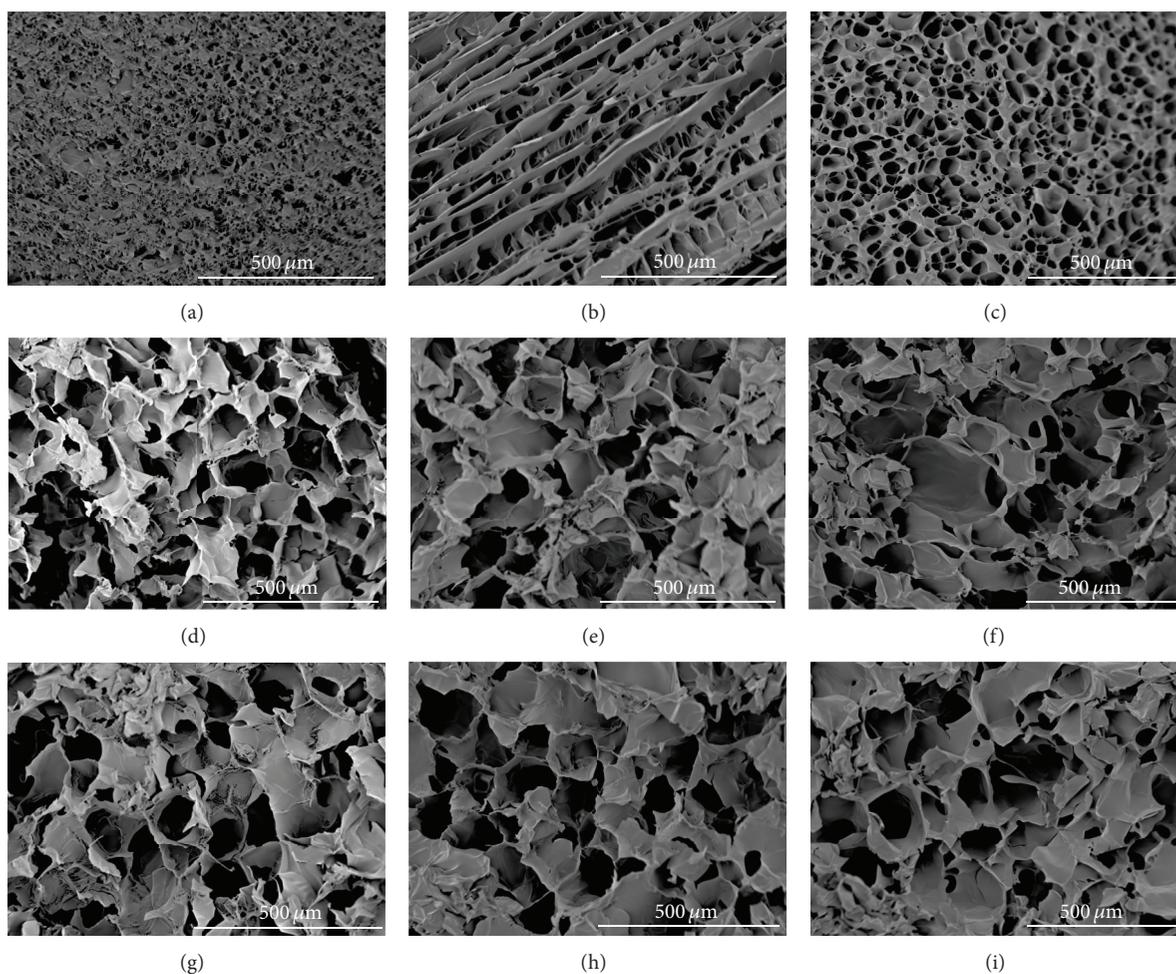


FIGURE 4: (a)–(c) SEM images of samples polymerised at 30, 40, and 50°C, respectively, with no postsynthesis manipulation; (d)–(f) samples polymerised at 30, 40, and 50°C, respectively, and continuously frozen for 72 h; (g)–(i) samples polymerised at 30, 40, and 50°C, respectively, and subjected to three freeze-thaw cycles (24 h freezing and 3 h thawing).

degree of cumulative crystallite formation [36]. As for frozen samples, the effect of increasing polymerisation temperature has a negligible impact upon final gel morphology (Figures 4(g)–4(i)).

3.4. Effects of Freezing and Freeze-Thaw Cycles on the Mechanical Properties of Cht-PVP-Gen Hydrogels. The mechanical properties of LHL hydrogels following postsynthesis continual freezing or freeze-thaw treatment (Table 2) were evaluated via uniaxial compression testing. Samples with no manipulation were too unstable to perform reliable quantitative tests. As polymerisation temperature is increased, the rate of cross-linking increases, as evidenced by the increasingly blue pigmentation. The material also becomes more gel-like, as illustrated by a successful inversion test for the specimen polymerised at 50°C (Figure 5); however the network remains notably unstable.

Uniaxial compression tests of continuously frozen and freeze-thawed LHL hydrogels (Table 2) were conducted (Figure 6). Mechanical properties recorded for samples that were continuously frozen for 72 h after synthesis (Figure 6(a))

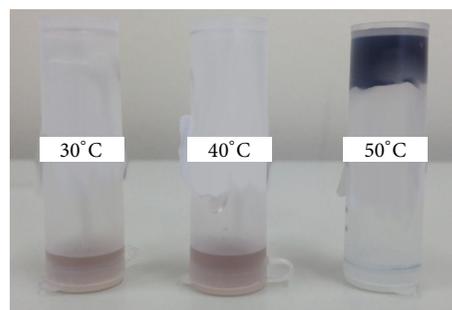


FIGURE 5: Photograph of LHL hydrogels polymerised at 30, 40, and 50°C and stored in a fridge (5°C) without subsequent manipulation. Sample composition: 0.2 mL Cht solution, 0.5 mL PVP solution, and 0.004 mL Gen solution.

do not correlate with their polymerisation temperature. Similarly, a lack of correlation was also noted between polymerisation temperature and extent of swelling in pH 2 buffer (Figure 3). LHL5 (50°C) was recorded to have highest

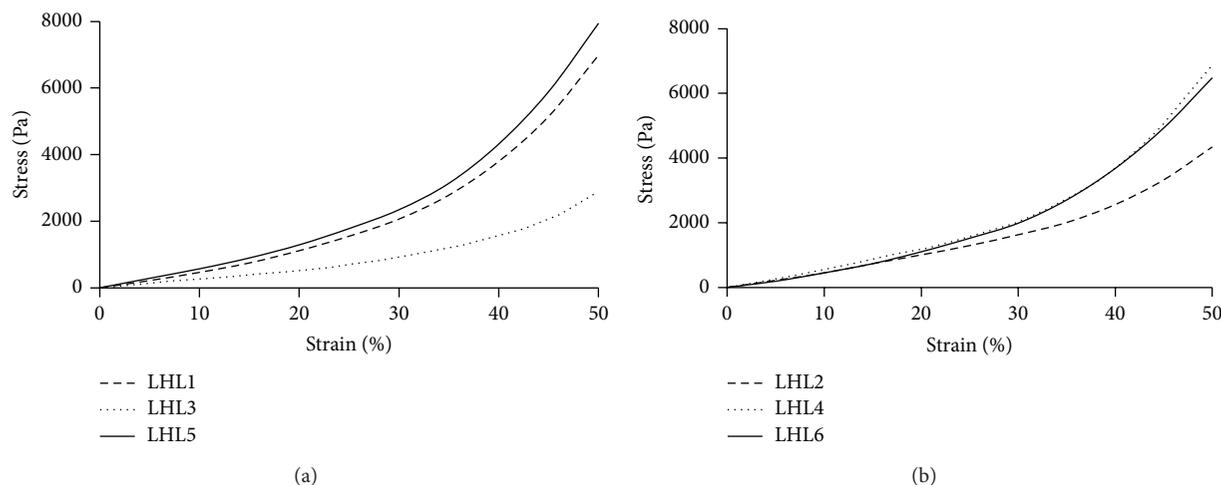


FIGURE 6: Uniaxial compression test of LHL hydrogels that were subject to postsynthesis continuous freezing (a) or freeze-thaw treatment (b).

mechanical properties followed by specimens polymerised at 30°C (LHL1) and 40°C (LHL3). This is despite no apparent variation in pore size between continuously frozen samples (approximately 100 μm as illustrated in Figures 4(d)-4(e)). Samples that were subject to postsynthesis freeze-thaw treatment displayed more predictable behaviour. Upon increasing polymerisation temperature the degree of cross-linking was presumably enhanced, leading to a lower degree of swelling (Figure 3) and improved mechanical properties (Figure 6(b)). Similarly to continuously frozen samples this was not apparent from SEM studies.

4. Conclusions

Experimental design was employed to study the influence of composition of Cht-PVP-Gen hydrogels on their response to pH change (from pH 7 to pH 2). The stability of the hydrogels correlates with the intensity of the blue colour of the gel which is associated with the level of cross-linking between the Gen and Cht components of the network. Results also indicate that stability decreases with the increase in molar ratio of PVP. Therefore, in the hydrogel sample composed of Cht, PVP, and Gen, sample stability may be tailored using the interplay between these three components. It was noted that while the unstable LHL sample (Cht:PVP:Gen = 1:49:7) achieved the highest swelling (165%, Figure 2), the low stability of samples is not directly correlated with a high swelling response. For example, the HHL sample (Cht:PVP:Gen = 1:19:3) was also classed as unstable yet it exhibited the second lowest swelling response. In the case of LHL and HHL samples, low stability is a result of low Gen to Cht molar ratio (7:1 and lower), while the significant difference in swelling is a consequence of the difference in the amount of PVP present, with larger quantities leading to greater swelling.

Stability was shown to be enhanced by postsynthesis treatment via continuous freezing of hydrogel samples or by subjecting the specimens to freeze-thaw cycles. Hydrogels with the LHL composition were studied as they were the least

stable and most responsive. Samples were synthesised at three different temperatures (30, 40, and 50°C). All samples that had been manipulated after synthesis were visually more stable and, to some degree, less responsive than untreated specimens (Figure 3). Samples which were continuously frozen swelled more than those subject to freeze-thaw treatment which may suggest that more physical cross-links are formed following repeated freezing and thawing cycles than when only freezing is applied. Potentially, hydrogen bonds in the water trapped within the gel structure play a role in the process. Whilst freezing and freeze-thaw sample manipulations can be successfully used to improve sample stability, it is important to note that these methods hamper sample responsiveness to some degree. SEM studies of LHL samples polymerised at 30, 40, and 50°C with and without postsynthesis manipulation revealed significant differences. At 30°C specimens manipulated postsynthesis had far more porous bulk structure with an average pore diameter of approximately 100 μm (continuously frozen) and 150 μm (freeze-thaw treatment) compared to the 10–20 μm pores of nontreated specimens. This is likely due to the freezing of liquids within the gel structure leading to crystallization in the pores to create an ordered three-dimensional network. The increased pore diameter of specimens subject to freeze-thaw treatment could be due to liquid expansion during each freezing cycle, increasing the proximity of the polymer chains to one another, facilitating hydrogen bonding and a greater degree of cumulative crystallite formation [36]. Upon increasing polymerisation temperature to 40°C and 50°C, SEM images display no significant variation in the number, size, or order of the pores in the samples manipulated after synthesis. However, studies of the mechanical properties of postsynthesis manipulated samples showed some degree of difference (Figure 6). Being able to improve the stability of smart Cht-PVP-Gen hydrogels without altering their initial chemical composition further confirms their potential for prospective medical applications, for example, in wound dressing and drug delivery.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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