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Robust biopolymer based ionic-covalent entanglement hydrogels with reversible mechanical behaviour.

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Emerging applications of hydrogels such as soft robotics and cartilage tissue scaffolds require hydrogels with enhanced mechanical performance. We report the development of a robust biopolymer based ionic-covalent entanglement network hydrogel made from calcium cross-linked gellan gum and genipin cross-linked gelatin. The ratio of the two polymers and the cross-linker concentrations significantly affected the mechanical characteristics of the hydrogels. Hydrogels with optimized composition exhibited compressive fracture stress and work of extension values of up to 1.1 ± 0.2 MPa and 230 ± 40 kJ.m^{-3} for swelling ratios of 37.4 ± 0.6 and 19 ± 1, respectively. The compressive and tensile mechanical properties, swelling behavior (including leachage), pH sensitivity and homogeneity are discussed in detail. Fully swollen hydrogels (swelling ratio of 37.4 ± 0.6) were able to recover 95 ± 2% and 82 ± 7% of their energy dissipation (hysteresis) at 37 °C after reloading to either constant stress (150 kPa) or constant strain (50%), respectively.

Introduction

Hydrogels are highly swollen, materials prepared from hydrophilic polymers that can absorb up to a thousand times their dry weight in water[1]. As a result of their high water content, most hydrogels are soft and weak materials compared to other polymeric materials such as rubbers. For this reason, hydrogels are typically utilised for applications which do not require them to be particularly strong or resilient (for example, in foods, ointments and creams)[1–3]. As soft and wet materials, hydrogels are a substance that is reminiscent of soft biological tissue and have been investigated over the past 30 years as candidate materials for soft tissue engineering scaffolds[1,2,4,5]. However, new applications of hydrogels such as soft robotics[6–8] and cartilage tissue scaffolds[9] require hydrogels with enhanced mechanical performance which has stimulated an investigation into how hydrogels may be made tougher and more enduring[10–13].

Tough hydrogels have been prepared using methods such as slip-ring hydrogel synthesis, nano-composite hydrogels, and double network hydrogels[11,13–17]. Of all the tough hydrogel synthetic strategies, the double network approach affords the highest versatility in terms of composition and resulting properties[17]. Double network (DN) hydrogels are interpenetrating polymer networks (IPN) which are formed in a two-step synthesis where a highly cross-linked, rigid and brittle polyelectrolyte is swollen in a monomer solution of a ductile, neutral polymer that it is subsequently polymerised[12,17]. This two-step synthesis process can be limiting for those types of applications (e.g. tissue engineering) requiring *in situ* fabrication through additive manufacturing techniques such as extrusion printing.

A relatively recent innovation is the advent of ionic-covalent entanglement (ICE) network hydrogels which can be prepared in a "one-pot" synthetic approach[14,16,18–20]. ICE hydrogels consist of a tough and self-recovering, interpenetrating network of an ionotropic polymer and a chemically cross-linkable polymer and have demonstrated some impressive mechanical properties[10,21,22]. In particular, it has been demonstrated that the "one-pot" synthetic approach allows for the fabrication of hydrogel structures using extrusion

printing[23]. This ability to print these tough hydrogels using additive manufacturing is a current advantage of ICE gels over DN gels.

ICE network hydrogels prepared from gellan gum and PAAm possessed compressive strain energy to failure of 44 kJ.m^{-3} and were able to recover 53% of their hysteresis (within 1 hour) when compressed to a constant stress (25 kPa) at 21 °C [21]. ICE network hydrogels made from alginate and PAAm were able to be stretched a phenomenal 23 times their original length and resulting in fracture energies of 9000 J.m^{-2} [10]. They were able to recover up to 74% of their hysteresis (upon stretching to a constant strain) when rested for 1 day at 80 °C. A DN hydrogel based on a combination of six arm star-shaped poly(ethylene oxide-stat-propylene oxide) and PAAm possessed high compressive failure stress (several MPa) and was also able to recover from ~1 MPa compressive stress[24].

The ICE hydrogels described in this article are based on the readily available (and edible) biopolymers gellan gum and gelatin. They are versatile (and edible) ingredients in well-known food products such as the commercially available product Aeroplane Jelly. Moreover, the combination of ionically cross-linked gellan gum and covalently cross-linked gelatin networks is compatible with the "one-pot" synthetic approach for ICE hydrogels.

Gellan gum is an anionic polysaccharide biopolymer derived from the bacteria *Pseudomonas elodea*[25] that gels ionotropically in the presence of calcium cations when its temperature is reduced below the coil-helix transition temperature (ca. 40˚C)[26]. Recently, gellan gum has been used as cartilage tissue surrogate materials and as an injectable, *in situ* forming hydrogel polymer for cellular delivery[26–28].

Gelatin is a highly versatile biopolymer which can be obtained at a range of isoelectric points, molecular weights and gel strengths[29] and has been used in a plethora of biomedical devices, pharmaceuticals and tissue engineering applications for over fifty years[30–42]. Without cross-linking, gelatin hydrogels are very weak and readily dissolve at temperatures above 29˚C which would prohibit their use in tissue engineering[43,44]. However, covalent cross-linking with genipin significantly improves the mechanical performance and stability of gelatin hydrogels[47,48].

Genipin is natural product from the gardenia plant, *Gardenia jasmindides Ellis* and is a non-cytotoxic cross-linker and anti-inflammatory cross-linking agent[48,50–52]. It forms covalent cross-links between the primary amino groups present in ε-amino groups of lysine and hydroxylysine residues and the guanidinium group of arginine residues in gelatin[45,46,49].

In this paper, we report the preparation of a new type of ICE network hydrogel based on the biopolymers gellan gum (calcium cross-linked) and gelatin (genipin cross-linked). We investigated the mechanical properties in compression and tension as well as in "as prepared" state and equilibrium swollen state. The behaviour of the hydrogels when immersed in simulated body fluid was also investigated with respect to polymer leaching, cation migration, pH and dimensional changes. Finally, the ability of these hydrogels to dissipate energy after repeated compressions with different length resting periods in ambient conditions and simulated body fluid at 37 °C was examined.

Experimental section

Materials

All reagents used were AR grade unless otherwise stated and deionised (DI) water was prepared using a combined ion exchange and osmosis filtration system (Millipore, Australia) to a resistivity 18.2 MΩ cm. Low acyl gellan gum (Lot #1/1443A, Gelzan-CM, CP Kelco, Singapore) and type A, porcine gelatin (Bloom number 300, molecular weight 87.5 kDa, pI 7.0-9.0, Sigma Aldrich, USA) were used to prepared hydrogels. A 20.3% (w/v) genipin (Challenge Bioproducts, Taiwan) solution in 60% (v/v) ethanol (Ajax Finechem, Australia) was used for cross-linking gelatin and a 1 M $CaCl_2$ (Sigma Aldrich, Australia) solution was used for cross-linking gellan gum.

Simulated body fluid (SBF) was prepared with DI water and contained 0.035% (w/v) $NaHCO_3$; 0.0548% (w/v) $MgCl_2.6H_2O$; 2% (v/v) HCl; 0.02% (w/v) NaN_3; 0.80% (w/v) NaCl; 0.0224% (w/v) KCl; 0.0174% (w/v) KH_2PO_4; 0.0368% (w/v) $CaCl_2.2H_2O$; 0.0071% (w/v) Na_2SO_4; and 0.606% (w/v) tris(hydroxymethyl)aminomethane (Chem-Supply, Ajax Finechem, and Sigma Aldrich, Australia). The pH of SBF was adjusted to 7.4 ± 0.2 at 37˚C using 1 M NaOH solution and pH meter (826 pH Mobile, Metrohm, Australia).

Hydrogel preparation

Hydrogels samples with varying compositions (0.495-1.98% (w/v) gellan gum, 0.88-3.52% (w/v) gelatin, 0-20% (w/w) Ca^{2+} and 0-20% (w/w) genipin) were prepared using the following general method: Gellan gum was first dissolved in 80˚C DI water with rapid stirring on a hot plate/stirrer (CB162, Stuart, UK). Gelatin was then added and dissolved under the same conditions. Next, sufficient 1 M $CaCl_2$ solution and sufficient 20.3% (w/v) genipin solution were added to the solution in order to reach the desired calcium and gelatin concentrations. The solution was then stirred for 3 minutes before being poured into glass petri dish moulds (60 mm diameter x 15 mm height, Schott, Australia) and left to cure, covered, for 24 hours at 21 ± 5˚C.

Mechanical analysis

Mechanical analyses were performed using a universal mechanical testing apparatus (EZ-S, Shimadzu, Japan). For compressive mechanical analysis, samples were cut from slabs of hydrogel into rectangular prisms 10 mm x 10 mm x 7 mm, and subsequently compressed at a rate of 1 mm.min^{-1} at 21˚C. The resulting stress-strain data was used to determine the compressive failure strain (ε_c), compressive secant modulus over 20%-30% strain (E_c), compressive failure stress (σ_c) and compressive strain energy to failure (U).

For tensile mechanical analysis, samples were cut with a "dog-bone" shaped cutter (conforming with JIS – K6250[60])

with a thickness of 1.7 mm, neck width of 4 mm and gauge length of 50 mm, and subsequently pulled at a rate of 4 mm.min^{-1} at 21˚C. The resulting stress-strain data was used to determine the elongation to failure (ε_t), Young's modulus (E_t), tensile fracture stress (σ_t) and work of extension (W).

Trouser tear tests based on the Japanese Industrial Standard method were used for fracture analysis[61]. Trouser shaped samples were cut with a steel cutter 1.7 mm thick (T), 50 mm long and 4 mm wide with a 25 mm split length. The legs of the trousers were pulled in tension perpendicular to the direction of crack propagation (mode III tearing) at a rate of 4 mm.min^{-1} and at 21˚C. The critical fracture energy (G_c) was calculated as follows:

$$G_c = \frac{2F}{T}, \quad (1)$$

where F was the force require to propagate the crack in a hydrogel.

Recovery of hysteresis behaviour of swollen hydrogels was examined in compression to either a specific stress, or to a specific strain value. Samples of the hydrogels were prepared in disc moulds (17.5 mm diameter, 5 mm height) and then immersed in SBF for 3 days at 37˚C to allow them to reach their equilibrium swollen state. The hydrogels were then loaded in compression at a rate of 1 mm.min^{-1} until they reached either a stress of 150 kPa, or strain of 50% and then unloaded at a rate of 1mm.min^{-1} to the original height. Samples were then subjected to up to 4 subsequent loading and unloading cycles after a period of resting in SBF at 37˚C (wet recovery) or wrapped in plastic wrap (Gladwrap, Clorox Australia Pty Limited, Australia) at 37˚C (dry recovery). The energy dissipated during a cycle (hysteresis, U_i) was calculated using

$$U_i = \int_{loading} \sigma d\varepsilon - \int_{unloading} \sigma d\varepsilon \, , \quad (2)$$

where σ and ε are the compressive stress and strain during cycle i.

Optical microscopy

The homogeneity of the hydrogels was examined using light microscopy (Z16, Leica, Germany) of hydrogels which had been paraffin embedded, sectioned and stained using periodic acid-Schiff (PAS) staining[62]. The hydrogels were first embedded using automated tissue processing and embedding stations (ASP200S, EG1150C and EG1150H, Leica, Germany) which dehydrated the hydrogels using a solvent gradient of 70% (v/v) ethanol, 90% (v/v) ethanol, absolute ethanol and xylene before embedding in paraffin. The gels were then sectioned using a microtome (RM2255, Leica, Germany) to a thickness of 10 µm and collected on glass microscopy slides (Knittel, Germany). The hydrogel sections were then re-hydrated using the reverse of the abovementioned solvent gradient; oxidised with 5% (w/v) periodic acid (BDH, England) for 5 minutes; and stained with Schiff's Reagent (Merck, USA) for 15 minutes before being dehydrated using an automated staining station (ST4020, Lecia, Germany).

FTIR spectroscopy

The connectivity between gellan gum and gelatin polymer networks in the hydrogels was examined using Fourier transform infrared spectrometry (IRAffinity-1, Shimadzu, Australia). Hydrogel samples were oven dried (FD, Binder, USA) at 80 ± 5˚C ˚C for 4 hours prior to analysis with a diamond ATR accessory. The spectra were processed using Happ-Genzel apodisation, ATR correction, smoothing and baseline correction algorithms.

Immersion studies

Gels were immersed in SBF at 37 ± 1˚C in a temperature controlled chamber (Thermoline, Australia) for 148 hours. The swelling ratio of the hydrogels as well as the pH and gellan gum, gelatin, sodium, magnesium, potassium and calcium concentrations in the immersion solutions were measured at 0, 3, 6, 12, 24, 48, 72, 96, 120 and 144 hours.

Gelatin and gellan gum concentrations were measured with a Coomassie Plus assay (Thermo Scientific, Australia) and a Total Carbohydrates assay (Biovision Incorporated, USA), respectively, in microplate format using a plate reading spectrophotometer (Polarstar, BMG Labtech, Germany).

The sodium, magnesium, potassium and calcium concentration of the immersion solutions was measured using inductively coupled plasma mass spectrometry (7500CE, Agilent Technologies, Japan). The element concentrations were determined using standard curves based on ^{23}Na, ^{25}Mg, ^{39}K and ^{48}Ca isotopes. Samples were prepared for analysis by diluting to the working ranges of the standard curves with high purity DI water containing 2% (v/v) HNO_3 (Suprapur, Merck Millipore, Australia). Calibration standards were prepared from a certified multi-element standard (Lot# A2-MEB236019, Inorganic Ventures, Australia) in 2% (v/v) HNO_3. High purity argon was used as the plasma/carrier gas and helium was used as the collision/reaction gas.

The swelling ratio (SW) was calculated as the mass of the swollen hydrogels (m_s) divided by the dry mass (m_d). Swollen hydrogel mass measurements were taken on a top-loading balance (PB3002/-S/FACT, Metler-Toledo, Australia) after the gels were extricated from the immersion solutions and blotted dry with filter paper (165 Hardened and Ashless papers, Filtech, Australia).

The effect of pH on the SW of hydrogel was studies by immersing the hydrogels in 0.1 M phosphate buffered saline (PBS) solution for 24 hours. The pH of all immersion solutions was measured with an electrode based pH meter (826 pH Mobile, Metrohm, Australia).

Statistical treatment of data

Dixon's Q-test (95% confidence) was used to confirm and justify the removal of spurious data. Unless otherwise stated, the data presented in this manuscript are the mean ± one standard deviation (SD).

Results

Optimisation of hydrogel composition

Ionic-covalent entanglement network hydrogels (Figure 1) were prepared from calcium cross-linked gellan gum and genipin cross-linked gelatin. The values of the mechanical properties exhibited by the ICE gels is better than the sum of its constituent gel materials, i.e. hydrogels comprising of only calcium cross-linked gellan gum, or only genipin cross-linked gelatin (Figure 2, Table 1).

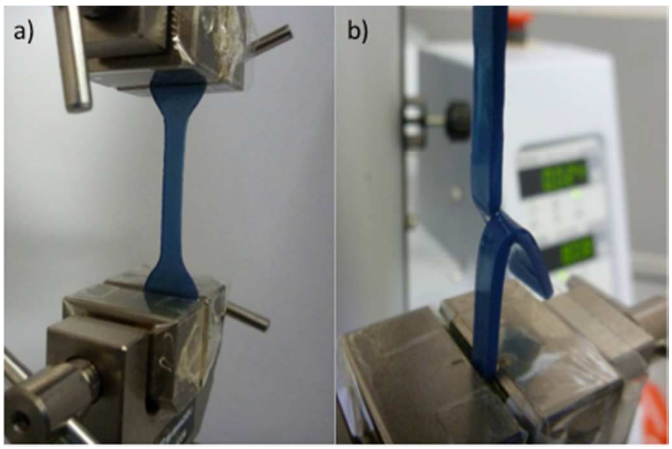

Figure 1. Photographs of typical a typical ICE network hydrogel (2.75% w/v polymer) subjected to tensile (a) and trouser tear (b) tests.

Table 1. Mechanical properties of ICE network, gellan gum and gelatin hydrogels with varying polymer concentrations (± SD). ε_c compressive failure strain; E_c compressive secant modulus; σ_c compressive failure stress; U compressive strain energy to failure; ε_t elongation to failure; E_t Young's modulus; σ_t tensile fracture stress; W work of extension; G_c critical fracture energy; SW swelling ratio. Properties marked with "*" were unable to be determined because the hydrogel samples were too fragile to undergo testing.

	ICE network (2.75% wt)	ICE network (4.12% wt)	ICE network (5.50% wt)	Gellan gum (1% wt)	Gelatin (1.75% wt)
σ_c (kPa)	1100 ± 200	1000 ± 200	1000 ± 200	360 ± 80	20 ± 10
ε_c (%)	85 ± 1	81 ± 5	72 ± 3	82 ± 2	74 ± 5
E_c (kPa)	120 ± 20	260 ± 30	490 ± 30	70 ± 10	4.5 ± 0.6
U (kJ.m^{-3})	147 ± 9	200 ± 40	200 ± 40	57 ± 7	3 ± 1
σ_t (kPa)	270 ± 20	510 ± 30	620 ± 60	70 ± 10	*
ε_t (%)	64 ± 4	66 ± 6	69 ± 3	16 ± 2	*
E_t (kPa)	420 ± 20	780 ± 30	890 ± 50	460 ± 30	*
W(kJ.m^{-3})	80 ± 10	170 ± 30	230 ± 40	6 ± 2	*
G_c (J.m^{-2})	40 ± 10	98 ± 4	126 ± 6	*	*
SW	40 ± 5	25 ± 5	23 ± 5	101 ± 5	58 ± 3

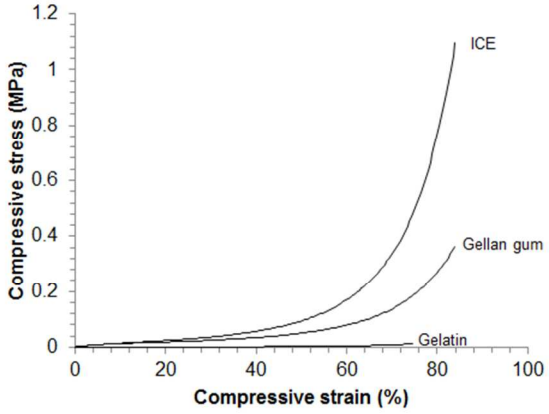

Figure 2. Compressive stress-strain of a typical ICE network hydrogel (2.75% w/v polymer) and its constituent gellan gum and gelatin hydrogel components.

Gels of various compositions were examined to determine the optimum polymer and cross-linker ratios in terms of mechanical characteristics. For example, the polymer ratio was changed by increasing the gelatin content while keeping the amount of gellan gum constant. The compressive stress at failure reached a maximum of 1.1 ± 0.2 MPa for ICE network consisting of 36% (w/w) gellan gum to 64% (w/w) gelatin (Figure 3a). A similar trend was observed for compressive strain energy to failure (see Figure S1, Table S1, Electronic Supplementary Information), while secant modulus increased with gelatin content (Figure 3b). Similar trends were observed for the ratio between Ca^{2+} cross-linker and gellan gum (Figure 3c-d, and Figure S2, Table S2, Electronic Supplementary Information). In contrast, changing the ratio of the genipin to gelatin did not result in a maximum (Figure S3, Table S3, Electronic Supplementary Information). Rather, compressive stress to failure and compressive strain energy increase, while the compressive secant modulus decreases with increasing genipin to gelatin ratio. This is unusual because a higher cross-linker concentration should result in a higher modulus and it is not clear at present why the opposite has occurred. This result suggests that the gellan gum network may have impeded the formation of covalent cross-links in the gelatin network and we speculate that this may be due to a molecular shielding/hindrance effect, reduced molecular mobility, or some other effect.

The distribution of gellan gum and gelatin throughout the hydrogels was confirmed to be homogenous based on microscopic investigations of stained hydrogel sections. Gelatin was stained blue from the genipin[63], and gellan gum was stained pink using periodic acid-Schiff (PAS) staining. There were no distinct blue (gelatin rich) regions or pink (gellan gum rich) regions in the micrographs (data not shown).

FTIR spectroscopy suggested that the gellan gum and gelatin polymer networks were not covalently cross-linked. FTIR spectra of the gellan gum-gelatin ICE network hydrogels were observed to be a simple combination of the spectra of gellan gum hydrogels and gelatin hydrogels spectra (Figure 4). This indicates that no new covalent bonds were formed or existing covalent bonds were broken during the preparation of ICE network hydrogels. This strongly suggests that the two polymer networks are covalently independent of one another.

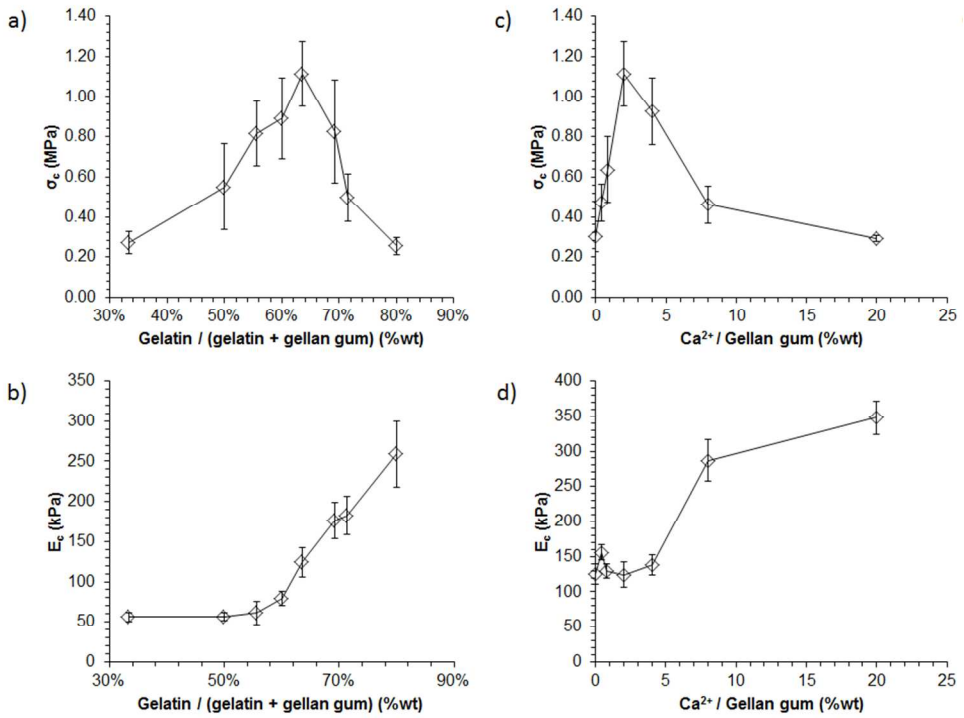

Figure 3. Compressive mechanical stress at failure and compressive secant modulus of gellan gum-gelatin ICE network hydrogels with: a, b) varying polymer ratios (changed by increasing gelatin content while keeping gellan gum content constant) with constant cross-linker concentrations; c-d) Varying Ca^{2+} concentration with constant polymer and genipin concentrations.

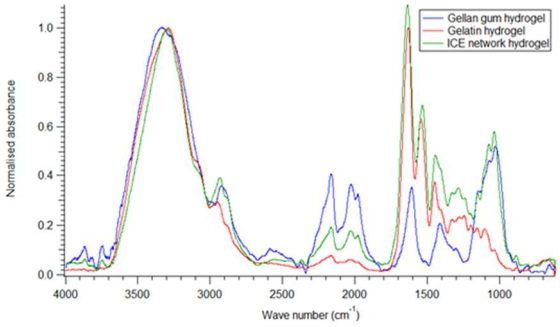

Figure 3. FTIR spectra of gellan gum, gelatin and gellan gum-gelatin ICE network hydrogels.

The mechanical properties were also investigated by testing the most robust hydrogels in tension (Table 1). It is well known that mechanical properties such as fracture energy (G_c) and Young's modulus (E) values increase with decreasing swelling ratio. Figure 5a shows that the G_c and E values of our gels are smaller than those of DN gels and rubbers, but larger compared to conventional gels. It is likely that the difference in G_c values between DN and our ICE gels can be (partially) attributed to the difference in swelling ratio. Our ICE gels have swelling ratios of 20-40, which is larger than the corresponding ratios for DN gels (< 10). However, comparing gels at similar swelling ratios (20-40) reveals that the work of extension values (W) of our gels is better than those of conventional gels, but not as good as NC hydrogels (Figure 5b).

Immersion studies

ICE network hydrogels were immersed in simulated body fluid (SBF) for up to 144 hours. The gellan gum, gelatin, sodium, magnesium, potassium and calcium concentrations and the pH of the SBF was measured at regular intervals in addition to the swelling ratio of the hydrogel samples.

The concentrations of gellan gum and gelatin in the SBF reached a plateau value after 48 hours of immersion (Table S4). It is suggested that this is release of free polymer chains (those unassociated with the gel network)[65,66]. The relative amount of gellan gum leached was observed to increase in proportion to the total polymer concentration to a maximum of 0.042 ± 0.009% (w/w) for 5.500% (w/v) ICE network hydrogels (Figure S4a, Electronic Supporting Information). The amount of gelatin leached as a percentage of the gelatin used to prepare the hydrogels was observed to fluctuate around an average value of 2 ± 2% (w/w), irrespective of the total polymer concentration in the hydrogels (Figure S4b, Electronic Supporting Information). This suggests that gellan gum and gelatin release is small and not likely to have a large impact on gel properties.

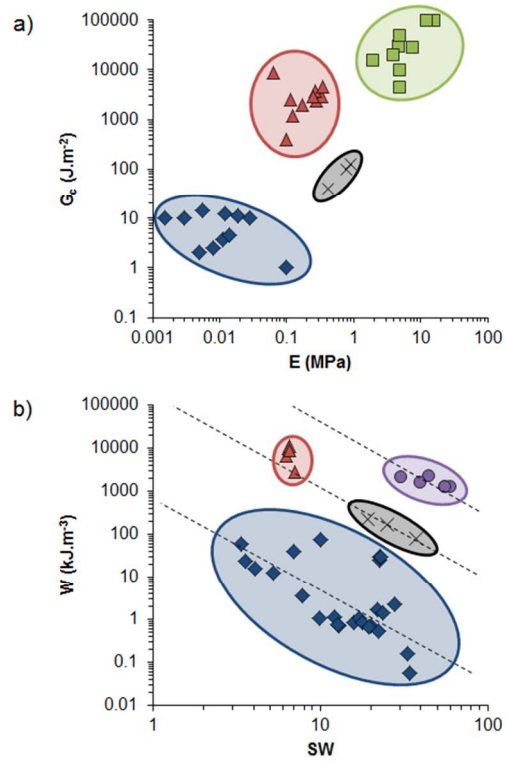

Figure 4. a) Fracture energy (G_c) versus Young's modulus (E) and b) work of extension (W) versus swelling ratio (SW) comparison charts of ICE network hydrogels reported in this work (crosses) and conventional hydrogels (diamonds), DN hydrogels (triangles), NC hydrogels (circles), rubbers (squares) adapted from reference[64].

The concentration of calcium in the SBF surrounding 2.75% (w/w) ICE network hydrogels increased from 0.20 ± 0.05 μg.L^{-1} to 0.5 ± 0.3 μg.L^{-1} over the first 48 hours and did not change significantly afterwards (Figure S4c, Electronic Supporting Information). The concentrations of sodium, magnesium and potassium did not change significantly during the immersion period (Figure S5, Electronic Supporting Information). Calcium ions are directly involved in the cross-linking of gellan gum and it is possible that they were exchanged with other ions such as sodium in the SBF resulting in weaker networks[26].

The pH of SBF was observed to decrease by ~0.4 over the 144 hour study period with the most dramatic change occurring within the first 24 hours for most polymer concentrations (Figure S4d, Electronic Supporting Information). The gellan gum possesses carboxylic acid functional groups and the type of gelatin used to prepare the hydrogels is an acid hydrolysed porcine gelatin so it is unsurprising that leaching of these materials lowered the pH of solutions in which they were immersed.

All of the hydrogels de-swelled to some extent during the immersion study with the majority of the de-swelling occurring within the first 24 hours. The hydrogels with 1.375% (w/v) polymer de-swelled from a swelling ratio of 75 ± 3 to 64 ± 4 over 144 hours, while all of the other hydrogels only changed marginally (Figure S4e, Electronic Supporting Information). The swelling ratio of ICE network hydrogels did not change significantly when immersed in 0.1 M phosphate buffered saline (PBS) solutions at pHs between 4.5 and 8.6 (Figure S4f, Electronic Supporting Information). Ordinarily, proteinaceous polymers such as gelatin change their volume in response to being immersed in solution of different pH. For example, a genipin cross-linked gelatin hydrogel (without gellan gum) de-swelled to 23% of its original volume in a previously reported experiment[37]. This phenomena occurs as a result of ionisation of functional groups in these proteins at pHs above, below and at the isoelectric point of the protein.

Characteristics of swollen hydrogels

In the previous section, the composition was optimised and it was determined that 48 hours immersion in SBF was sufficient time for the hydrogels to reach an equilibrium swelling state. ICE network hydrogels comprising of 36% (w/w) gellan gum, 64% (w/w) gelatin, 2% (w/w) Ca^{2+} and 20% (w/w) genipin were prepared with polymer concentrations between 1.375% (w/v) and 5.500% (w/v), and immersed in SBF.

The compressive mechanical properties of ICE network hydrogels immersed in SBF were compared to analogous as-prepared hydrogels, i.e. gels which had not been immersed in SBF. The compressive failure stress and strain energy to failure increased with increasing polymer concentration between 1.375 and 2.750% (w/v) but plateaued for higher polymer concentrations (Figure 6a-b). As-prepared hydrogels possessed higher failure stresses and strain energies than immersed hydrogels. The compressive secant moduli of both the as-prepared and immersed hydrogels increased with increasing polymer concentration. However, the moduli of immersed hydrogels were slightly lower than the non-immersed hydrogels (Figure 6c). The compressive strain to failure decreased with increasing polymer concentration. Immersed hydrogels fractured at a lower strain than the corresponding as-prepared hydrogels (Figure 6d).

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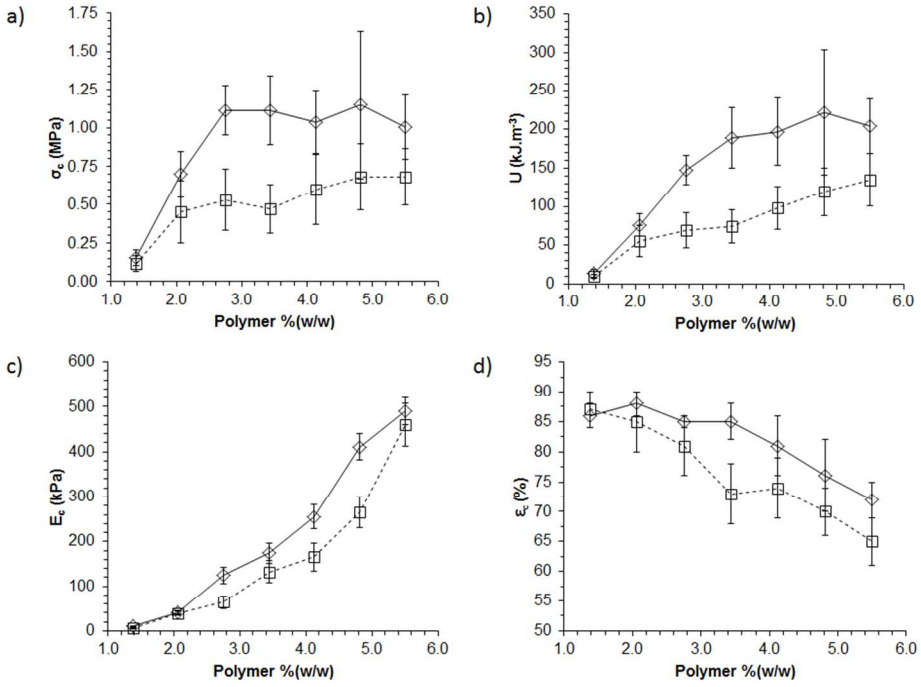

Figure 6. The polymer concentration affects the a) compressive failure stress (σ_c), b) compressive strain to failure (ε_c), c) compressive secant modulus (E_c), and d) strain energy to failure (U), of "as prepared" (diamonds) and equilibrium swollen state (squares) hydrogels (± SD).

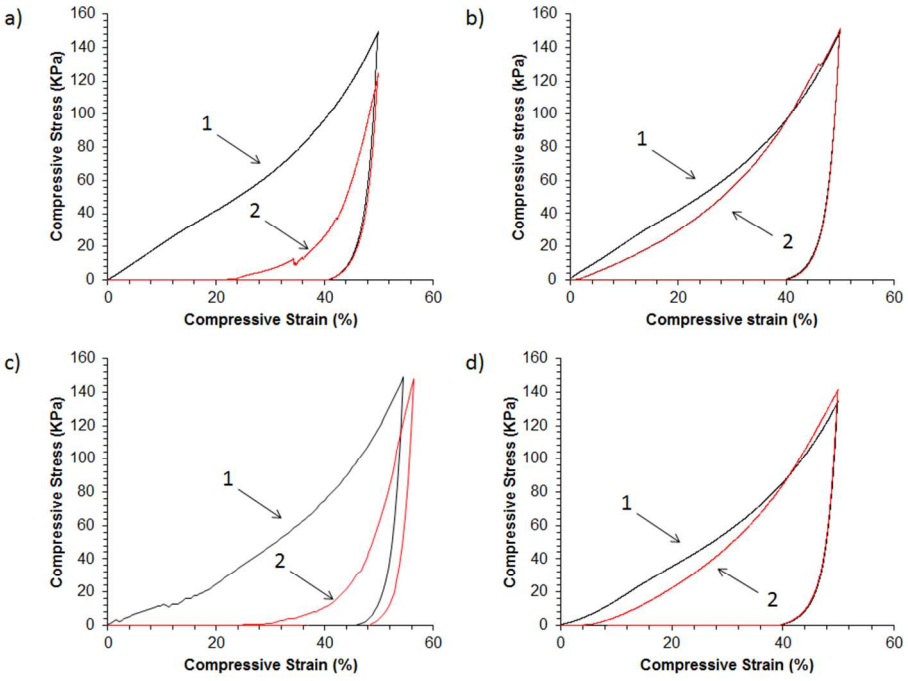

Figure 7. a) and b) Stress-strain curves for loading/unloading cycles 1 and 2 of typical ICE network hydrogel (2.75% w/w) samples compressed to 50% after resting in air or immersed in SBF for 10 min between cycles, respectively. c) and d) Stress-strain curves for loading/unloading cycles 1 and 2 of typical ICE network hydrogel (2.75% w/w) samples compressed to 150 kPa after resting in air or immersed in SBF for 10 min between cycles, respectively.

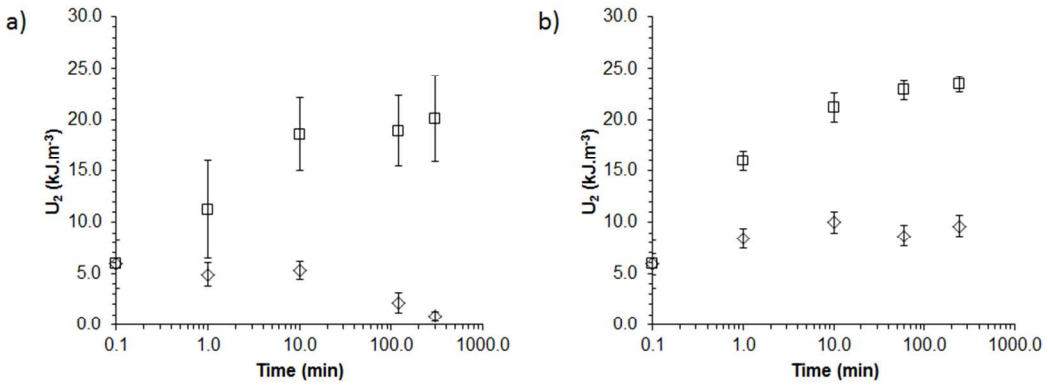

Figure 8. a) Hysteresis of the second loading/unloading cycle (U_2) of a typical ICE network hydrogel (2.75% w/w) samples compressed to 50% of strain after resting in air (diamonds) or immersed in SBF for 10 min (squares) after the initial loading/unloading cycle. b) Hysteresis of the second loading/unloading cycle of a typical ICE network hydrogel (2.75% w/w) samples compressed to 150 kPa of stress after resting in air (diamonds) or immersed in SBF for 10 min (squares) after the initial loading/unloading cycle.

Overall (and as expected), the magnitude of the mechanical properties of hydrogels decreased as a result of immersion (Figure 6 and Table S4, Electronic Supporting Information). It is suggested that the diminution of mechanical properties of immersed hydrogels may be caused by loss of calcium ions from the hydrogels. This suggestion is supported by a previous study where calcium cross-linked gellan gum hydrogels were shown to be degraded via sodium-calcium ion exchange *in vivo*[67].

Recovery of hysteresis

Gellan gum-gelatin ICE network hydrogels comprising 2.75% (w/w) polymer were compressed to either a constant stress of 150 kPa or a constant strain of 50% to determine their ability to recover after a resting period in air (dry recovery) or immersed in SBF (wet recovery). The energy dissipated (hysteresis) during the first loading/unloading cycle to constant stress (150 kPa) or constant strain (50%) was 24 ± 2 kJ.m^{-3} (Figure 7). The hysteresis reduced to 6 ± 2 kJ.m^{-3} when the gels were immediately subjected to a second loading/unloading cycle (Figure 8). This reduction could be partially attributed to observed expelling of water during the initial loading cycle. This could be indicative of a de-swelling effect and contributing to the observed stiffening of the gels. Regardless of the resting time, the gels do not improve the value of their hysteresis in the experiments to either constant strain (Figure 8a), or constant stress (Figure 8b).

However, placing the gels in SBF results in a significant improvement in the hysteresis (Figure 7). Allowing the gels to rest between cycles 1 and 2 for 5 hours in SBF resulted in an increased hysteresis value (to constant strain) during the second cycle of U_2 = 20 ± 4 kJ.m^{-3}, which is 82 ± 7% of the hysteresis of the first cycle (Figure 8a). It is likely that the re-swelling of the gels is responsible of the observed improved recovery in hysteresis. Similar results were obtained for gels subjected to loading/unloading cycles to constant stress (Figure 8b). Resting the gels between cycles 1 and 2 for 4 hours in SBF resulted in U_2 = 23.4 ± 0.8 kJ.m^{-3}, which is equivalent to 95 ± 2% recovery of the hysteresis value of the first cycle.

Furthermore, our hydrogels were subjected to 5 cycles in succession each with 10 min resting in SBF between cycles. The hysteresis value decreased between cycles 1 and 2, but remained constant for subsequent cycles 3-5 (≈ 80% of the hysteresis value of the first cycle). This behavior combined with the observation that the re-swollen gels regain their initial volume (but not swell beyond that volume) appears to suggest that our gels might not be permanently damaged by the first loading/unloading cycle as reported for some microgel reinforced gels[68].

Conclusions

This paper describes the preparation and characterization of a robust biopolymer based ionic-covalent entanglement network hydrogels from gellan gum and gelatin with reversible mechanical behaviour. The optimal concentrations of gellan gum, Ca^{2+}, gelatin and genipin were identified. The compressive fracture stress and work of extension values of the optimized hydrogels were 1.1 ± 0.2 MPa (swelling ratio 37.4 ± 0.6) and 230 ± 40 kJ.m^{-3} (swelling ratio 19 ± 1), respectively. The behaviour of the hydrogels when immersed in simulated body fluid was investigated and it was observed that calcium was leached from the hydrogels over time.

The ICE network hydrogels were able to recover to a significant proportion of their mechanical characteristics (i.e. hysteresis) when rested in simulated body fluid (37 °C) for

more than 10 minutes between compression cycles 1 and 2. We showed that the hysteresis recovery was 95 ± 2% under cyclic compression to a constant stress and 82 ± 7 under cyclic compression to a constant strain.

This paper contributes to the preparation, characterisation and understanding of interpenetrating polymer network hydrogels with (reversible and sacrificial) ionic cross-linkers.

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Notes and references

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Graphical abstract

A robust ionic-covalent entanglement hydrogel from gelatin and gelatin with reversible mechanical characteristics is reported.