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Degradation Behavior of Ionic-Covalent Entanglement Hydrogels

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ABSTRACT: Ionic-covalent entanglement (ICE) hydrogels are a recently introduced new type of robust materials for potential future application in the fields of tissue engineering and soft robotics. Here the degradation behavior of gellan gum/polyacrylamide ICE hydrogels immersed in PBS or enzyme solutions is presented. It is demonstrated that ICE gels immersed in enzymes became stiffer, whereas under cyclic testing their mechanical responses stabilize after 10 loading/unloading cycles whether immersed in PBS or enzyme solutions. The leachates of the ICE hydrogels were found to be non-cytotoxic for the growth of L929 and PC12 cells. These findings will be of benefit to the future development of tissue engineering applications based on these gel materials. © 2014 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2015**, *132*, 41216.

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INTRODUCTION

The popularity of hydrogels in regenerative medicine is the result of their desirable characteristics such as cytocompatibility, soft rubbery consistency, satisfactory *in vivo* performance and ability to be fabricated into a wide range of morphologies.[1,2] Moreover, diffusion in hydrogels is often quite high, and thus fulfills some of the most sought after requirements in tissue engineering.[1,2]

Ionic-covalent entanglement (ICE) hydrogels are a new class of interpenetrating polymer network (IPN) hydrogels,[3–5] targeting tissue engineering and soft robotics applications. ICE hydrogels consist of two polymer networks that are independently cross-linked. The first network is an ionically cross-linked biopolymer (e.g., alginate) and the second network consists of a covalently cross-linked synthetic polymer (e.g., poly(acrylamide)). It has been demonstrated that these ICE gels exhibit remarkable mechanical performance where the ionic cross links in the biopolymer network act as sacrificial bonds that dissipate energy under stress,[3,4] which is advantageous for numerous tissue engineering applications. For example, initial stretching (loading) of ICE gels results in an unzipping of the ionically cross-linked polymer network, which limits the damage to the covalently cross-linked polymer network.[4] As only the ionic cross-links are broken, the polymer chains themselves remain intact and the ionic cross-links can re-form (during unloading), thereby restoring the network and its mechanical characteristics.[3,4] Understanding of the degradation behavior is one of the key considerations in the development of hydrogels for tissue engineering applications. In particular, investigating the effect of degradation on the mechanical properties is of critical importance in the development of these materials. In this work, we investigate the degradation behavior of an ICE hydrogel based on the biopolymer gellan gum (GG) and the synthetic polymer polyacrylamide (PAAm). GG is a linear, anionic extracellular polysaccharide produced by fermentation of *Sphingomonas elodea*,[6] whereas PAAm is a water-soluble polymer formed by exothermic copolymerization of acrylamide and bis-acrylamide ("bis," N,N′-methylene-bisacrylamide). Gellan gum is FDA and European Union (E418) approved as a food additive, and has found wide application as a multifunctional gelling, stabilizing and suspending agent.[7–11] The ability to synthesize GG into structures (e.g., particles, fibers, membranes) under mild processing conditions has made gellan gum an attractive candidate for tissue engineering applications.[12–16] For example, gellan gum has been proposed or used in scaffold materials for tissue engineering applications[16,17] and in drug delivery (as a tablet binder, disintegrant, gelling agent and controlled release polymer).[18–20]

PAAm hydrogels are widely used in horticulture, the cosmetics industry, gel electrophoresis,[21] bacterial cell culture,[22] ophthalmic operations, drug treatment and food packaging.[23] The gels' inherent characteristics of chemical inertness, pH and temperature stability, mechanical strength, ability to form gradient gels, unchanged surface chemistry with varying mechanical properties and anti-fouling characteristics result in negligible non-specific cell-receptor binding.[24] In tissue engineering, PAAm gels are successfully being applied as soft tissue fillers[23,25] and in cardiac tissue regeneration.[26] The peptide/protein mimicking amide structure of PAAm is regarded as an added advantage over other synthetic polymers in cardiac tissue engineering.[26]

Although GG and PAAm are being studied as separate gel systems in tissue engineering applications, only limited reports have been presented in the literature detailing interpenetrating polymer network hydrogel systems.[3–5,27] In particular, mechanical characterization of ICE gels in the presence of enzymes remains unexplored to date. Here, the behavior of a GG/PAAm ICE hydrogel immersed in phosphate-buffered saline, lysozyme and trypsin at 37°C and pH 7.4 is reported. The resulting hydrogels are characterized in detail in terms of mass loss analysis, swelling characteristics, mechanical compression and cyclic testing, and the effect of hydrogel leachates on L929 and PC12 cell growth.

EXPERIMENTAL

Materials

Endotoxin-free GG (low-acyl, molecular weight range 2–3 $\times$ 10^5 Da, Gelzan CM, lot 9K6969A) was a gift from CP Kelco (USA). Acrylamide (molecular weight 71.08 g mol^{-1}, lot BCB4267V) was purchased from Fluka. Potassium persulphate (KPS, molecular weight 270.32 g mol^{-1}, lot MKBC7654V), *N,N,N′,N′*-tetraethylmethylenediamine (TEMED, molecular weight 158.28 g mol^{-1}, lot SHBB4240V), *N,N′*-methylenebisacrylamide (MBAAm, molecular weight 154.17 g mol^{-1}, lot MKBF1194V), lysozyme from chicken egg white (activity $\geq$4.0 $\times$ 10^4 units. mg of $protein^{-1}$), trypsin from porcine pancreas (activity 1.0–2.0 $\times$ 10^3 BAEE units. mg of $solid^{-1}$), $CaCl_2{\cdot}2H_2O$ and salts required for phosphate-buffered saline (PBS) solution preparation were purchased from Sigma–Aldrich. Eagle's minimum essential medium (EMEM), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin (PS), and Dulbecco's phosphate-buffered saline (DPBS) were purchased from Invitrogen. PBS solutions (pH 7.4, temperature 37°C) were prepared using NaCl (137 m*M*), KCl (2.7 m*M*), anhydrous Na_2HPO_4 (10.1 m*M*), and anhydrous KH_2PO_4 (1.7 m*M*) in Milli-Q water (resistivity 18.2 MΩ cm).

Unit definitions of enzymes are as follows, Lysozyme: one unit produces a change in A_{450} of 0.001 per minute at pH 6.24 at 25°C, using a suspension of *Micrococcus lysodeikticus* as substrate, in a 2.6 mL reaction mixture (1 cm light path). Trypsin: one BAEE unit produces a change in A_{253} of 0.001 per min at pH 7.6 at 25°C using BAEE as substrate, in a 3.2 mL reaction mixture (1 cm light path). All materials were used as received.

Hydrogel Preparation

GG/PAAm hydrogels were synthetized using a simultaneous network formation technique,[3] i.e., GG/PAAm hydrogel with 2% (w v^{-1}) GG (cross-linked with 5 m*M* $CaCl_2$) and 4% (w v^{-1}) PAAm (cross-linked at 5 mol % MBAAm) was prepared by combining hot (80°C) solutions of MBAAm/AAm and GG, followed by drop-wise addition of hot (80°C) solutions of TEMED, $CaCl_2$ and KPS in the given order. The resulting hot solution was rapidly transferred to a mold placed in a desiccator, which was then evacuated (to 100 mbar) and held for 2 h. The evacuation was disturbed a couple of times at the beginning of the evacuation cycle for deaeration. The concentrations of the reactants (cross-linker, initiator, and accelerator) were selected such that polymerization of PAAm completed at room temperature (21°C) within 2 h in the presence of GG and $CaCl_2$. GG hydrogels (without addition of poly(arylamide) were prepared as described above by cross-linking with $CaCl_2$. Gel discs (diameter 17 mm, height 5–10 mm) were cut out using a razor blade prior to characterization.

Mass Loss and Swelling Ratio

The initial weights (m_i) of all hydrogels discs were obtained prior to transfer to permeable tissue cassettes (embedding M516-2, Simport). Hydrogels in tissue cassettes were immersed in PBS (pH 7.4 and 37°C) and enzymatic (37°C) solutions for up to 42 days. Microbial contamination and preservation of continuous enzymatic activity during these tests was ensured as follows. The immersion media (PBS and enzyme solutions) were prepared with sodium azide (0.2%). All starting materials, media, and glassware/labware were either sterilized with ethylene oxide, or autoclaved. All experiments were performed in sterile hoods.

At each time point, three tissue cassettes were removed from the medium (either PBS, lysozyme or trypsin), blotted dry and weighed to obtain the swollen weight of the hydrogels (m_s). The hydrogels were lyophilized (Labconco, Feezone 4.5) and weighed to get the dry weight of the polymer in the hydrogel (m_d). Mass loss (M_L) was evaluated using:

$$M_L = (m_{id} - m_d)/m_{id}, \quad (1)$$

where the initial dry polymer mass (m_{id}) is obtained by multiplying m_i by the polymer weight fraction. The swelling ratio (q) of the hydrogel was calculated according to:

$$q = m_s/m_d, \quad (2)$$

where m_s and m_d are the mass of the swollen hydrogel and the mass of the dry polymer, respectively.

Circular Dichroism Spectroscopy

Circular dichroism spectroscopy (CD) measurements were performed using a Spectropolarimeter (Jasco, J-810) equipped with a temperature peltier (Jasco, CDF-426S). CD intensity was measured as a function of wavelength (190–250 nm) at a scanning rate of 100 nm/min using CD-matched cuvettes (path lengths of 1–5 mm). A standard curve was constructed by diluting a 5 mg mL^{-1} GG solution and measuring the CD intensity at 201 nm at 37°C. CD analysis was used to detect gellan gum in extracts of the medium after 7, 14, 21, and 28 days of immersion of the GG/PAAm and GG hydrogels.

Ultraviolet Visible Spectroscopy

Ultraviolet–visible (UV–Vis) spectroscopy of solutions and hydrogel extracts were performed using a spectrophotometer (Varian, Cary 500 UV-Vis NIR) with data interval = 0.5 nm, scan speed = 300 nm min^{-1} and wavelength range 200–800 nm. Quartz cuvettes with path length 5–10 mm were used. A standard curve was constructed by measuring the UV intensity at 198 nm (at 37°C) of a set of standard solutions of acrylamide (AAm). UV analysis was used to detect acrylamide in extracts of the medium after 3, 7, 14, 21, and 28 days of immersion of GG/PAAm hydrogels.

Mechanical Characterization

Compressive stress–strain measurements were carried out using an universal mechanical testing apparatus (EZ-S, Shimadzu, Japan) at a compression rate of 2 mm/min. Hydrogel discs (diameter 17 mm, height 5 mm) were submerged in PBS (pH 7.4, 37°C) or enzymatic (37°C) solutions up to 42 days prior to testing at each time point. The temperature of the samples was maintained using a water bath (37°C). Measurements were conducted in quadruplicate for each hydrogel composition at each time point on samples which had not been tested previously. From the resulting compressive stress–strain plots, the tangent modulus (E_t), stress-at-failure (σ_f), and strain-at-failure (ε_f) values were determined.

Cyclic Testing

Compressive cyclic testing was carried out using the universal mechanical testing apparatus at a compression rate of 50 mm min^{-1}. Hydrogel discs were prepared and immersed as described above, and tests were carried out at 37°C. Hydrogels were exposed to a cyclic testing regime between 15% and 25% compressive strains at a compression rate of 50 mm min^{-1}. The gels were held for 2 s between loading and unloading cycles, 20 consecutive loading/unloading cycles were performed for each hydrogel sample.

Cell Growth Inhibition Assay

Hydrogel samples (diameter 17 mm, height 6 mm) weighing approximately 1 g each were prepared under sterile conditions, and extracted in 3.75 mL of DPBS in 35 mm well plates. Five extraction blanks (for each time point) of 3.75 mL of DPBS in empty well plates were used as control. All the samples were placed in a 5% CO_2 humidified atmosphere at 37°C (Thermo Scientific, Heraeus BB 15). At relevant time points, 1 mL of DPBS extract was sampled, and diluted with EMEM (supplemented with 10% FBS and 1% PS) to a ratio of 1 : 3.

L929 Cell Growth Inhibition Assay

Murine dermal fibroblasts (L929) were seeded at 1×10^5 cells/plate in EMEM (supplemented with 10% FBS and 1% PS) in tissue culture dishes (diameter 35 mm), and incubated in a 5% CO_2 humidified atmosphere at 37°C for 1 day to establish a sub-confluent cell monolayer of adherent fibroblasts. The media in each plate was replaced by gel extracts, positive control (both 7.5% ethanol and latex extracts) and negative control solutions (1 : 3 EMEM to DPBS) and incubated for another 2 days. At the end of the test period, cells were harvested, counted using a Cell Viability Analyzer (Vi-cell XR, Beckman coulter) and compared with cell numbers in negative control (EMEM/DPBS) plates. Positive controls were expected to show greater than 70% inhibition of cell viability. Three independent samples of each degradation product were tested. The percentage cell growth inhibition (CGI) was obtained by:

$$\mathrm{CGI} = (n_c - n_s)/n_c, \tag{3}$$

where n_s and n_c are the number of cells in the sample and media control dishes, respectively.

PC12 Cell Growth Inhibition Assay

The same procedure as in L929 cell growth inhibition assay was followed. PC12 cells exist both as adherent and suspended populations. Therefore, during the cell growth inhibition study, at day 2 (where EMEM was replaced with test solutions) and day 4 (where cells were suspended for cell count), the media was centrifuged and free-floating cells were added back to the cell suspensions. At the end of the test period (day 4), 1 mL of lysis buffer (0.1% sodium dodecyl sulfate (SDS) in Tris–EDTA buffer (TE)) was added to each plate on an orbital shaker for 5 min. The number of cells in each plate was estimated using PicoGreen reagent, an asymmetrical cyanine dye that exhibits a >1000-fold fluorescence enhancement, upon binding to DNA.[28] A linear standard curve was constructed for up to 4×10^5 PC12 cells. Duplicate 10 μL volumes of cell suspension were added to the wells of a black 96-well plate followed by addition of 90 μL of TE buffer. Hundred microliters of PicoGreen reagent (Quant-IT PicoGreen dsDNA, Life Technologies) was added to each well after 1 : 200 dilution with TE assay buffer. DNA binding proceeded for 5 min at room temperature and fluorescence measurements ($\lambda_{ex} = 485$ nm and $\lambda_{em} = 520$ nm) were taken using a fluorescence plate reader (Fluostar Omega, BMG Labtech). The percentage cell growth inhibition (CGI) was calculated using eq. (3).

RESULTS AND DISCUSSION

Mass Loss

The degradation behavior of GG/PAAm ICE hydrogels immersed in PBS with or without enzymes (lysozyme and trypsin) was followed for a period of up to 42 days. The mass loss profiles of all ICE gels showed a small mass gain (<5%) after 4 h followed by a steady mass loss until the gel stabilized by 7 days [Figure 1(a)]. The mass loss profiles suggest that the gels reached their equilibrium state by 7 days, and then maintained a constant mass without further degradation during the remainder of the test period between days 7 and 42 [Figure 1(a)].

Over the course of the entire immersion period (42 days), the ICE gels showed, on average a higher mass loss when immersed in enzymes (lysozyme or trypsin), compared to gels immersed in PBS. The highest mass loss for ICE gels was observed when immersed in trypsin [Figure 1(a), Table I]. For example, ICE gels immersed in lysozyme or trypsin exhibited average mass losses in the equilibrium (stable) period (i.e., 7–42 days) of 5.4 ± 1.0 % or 9.5 ± 1.1 %, respectively (Table I). The mass loss values for the corresponding GG gels were similar except for gels immersed in lysozyme or trypsin [10 ± 3 % or 9 ± 2 %, respectively, Figure 1(b), Table I].

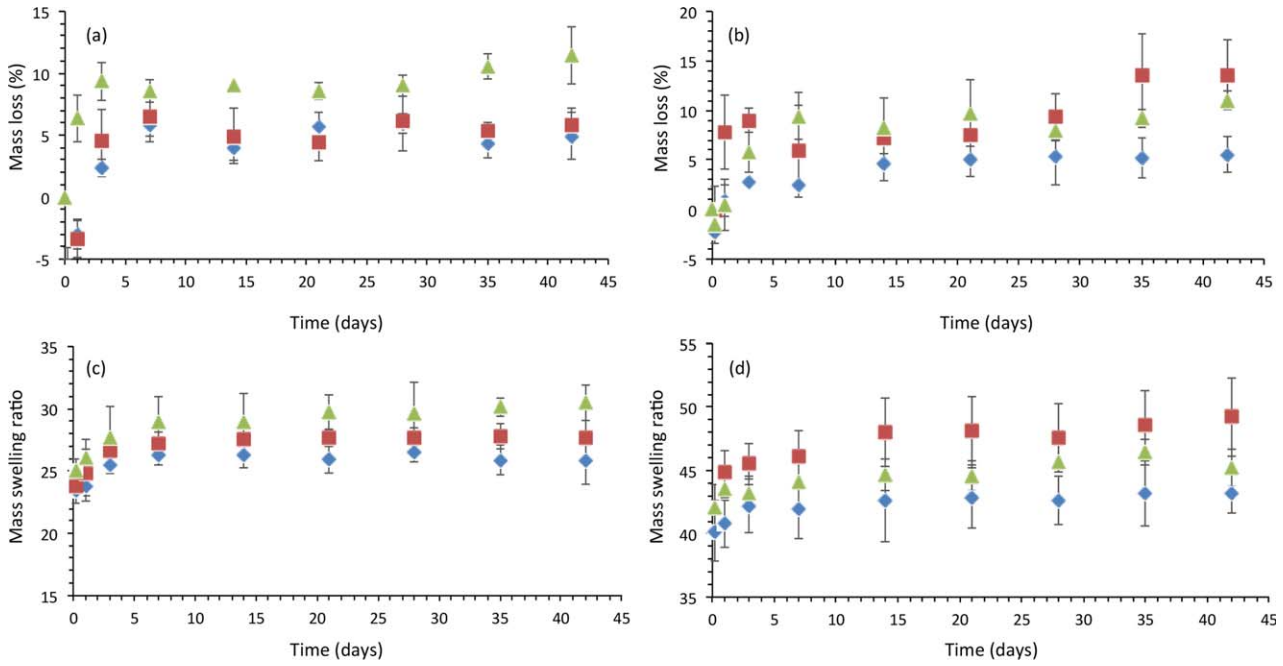

Figure 1. Mass loss (%) and mass swelling ratio (q) of GG/PAAm ICE (a and c) and GG (b and d) hydrogels immersed in PBS (diamonds), lysozyme (squares), or trypsin (triangles) at 37°C for up to 42 days. Error bars represent one standard deviation ($n = 3$). Mass loss (%) and mass swelling ratio calculated using eqs. (1) and (2), respectively. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The steady mass loss behavior of these gellan gum-containing ICE gels can be explained by their chain release behavior. It has been proposed in our earlier studies[29] that free gellan gum chains (those not associated with the gel network) are released first, while the network-associated chains are released over a longer time frame. Therefore, it is suggested that when ICE gels are immersed in enzymes (lysozyme, trypsin), un-associated GG and polyacrylamide chains are released during the rapid mass loss period (0–3 days), whereas the gel network-associated chains are released during the remainder of the study (3–42 days). The observed mass loss profile for ICE hydrogels is distinctly different from the expected "classical" behavior for hydrogels formed via ionic or covalent cross-links,[30,31] which generally involves a time period during which mass loss increases due to loss/breakage of the ionic or covalent cross-links until there are no cross-links left to support the gel network.

Swelling

The swelling characteristics of a hydrogel are crucial in designing hydrogel materials for tissue engineering, i.e., the amount of water imbibed within the hydrogel controls the diffusion properties of hydrogel, which in turn determines the micro-architecture and mechanical properties of these gels.[32]

The mass swelling profile of ICE gels was found to be proportional to the mass loss profile, i.e., with increasing mass loss, the swelling ratio increased until the gel swelling stabilized by 7 days [Figure 1(c)]. The relative amounts of average swelling (after the gels have reached equilibrium) between the ICE hydrogels mirrors that observed for the mass loss profiles, i.e., PBS < Lysozyme < Trypsin (see Table I). The corresponding swelling ratios for the GG hydrogels were considerably higher as is evident from Figure 1(d) and Table I. This could indicate that the interpenetrating polymer network structure counteracts the swelling behavior of gellan gum.

Gellan Gum Leaching

CD analysis (37°C) of extracts from the PBS, lysozyme, and trypsin media, confirmed that gellan gum leached out of the ICE hydrogels over the first 28 days of immersion (data not shown). The CD intensity of the gellan gum characteristic band around 201 nm was used to construct a standard curve. The resulting proportionality constant was used to determine the gellan gum concentration in the surrounding medium (PBS, lysozyme, or trypsin) and the mass fraction remaining in the gel (Figure 2). This data correlates well with the mass loss

Table I. Summary of Average Swelling Ratio (q_{stable}), Average Mass Loss ($M_{L,stable}$) and Diffusion Coefficients (D) Values for GG/PAAm ICE Hydrogels and GG Hydrogels Immersed in PBS and Enzyme Solutions. q_{stable} and $M_{L,stable}$ Values are the Average Values of the Gels in the Steady Mass Loss Period (i.e., 7–42 Days) when Immersed in PBS, Lysozyme, or Trypsin at 37°C

Sample	q_{stable} (%)	$M_{L,stable}$ (%)	D ($\times 10^{-13}$ m^2 s^{-1})
ICE-PBS	26 ± 2	4.7 ± 1.3	1.3 ± 0.6
ICE-lysozyme	27 ± 3	5.4 ± 1.0	1.5 ± 0.4
ICE-trypsin	29 ± 2	9.5 ± 1.1	2.2 ± 0.6
GG-PBS	43 ± 3	5 ± 2	1.1 ± 0.6
GG-lysozyme	48 ± 2	10 ± 3	2.5 ± 0.4
GG-trypsin	45 ± 3	9 ± 2	2.3 ± 0.6

Values for D calculated based on mass loss data over 42 days immersed in PBS, lysozyme, or trypsin at 37°C.

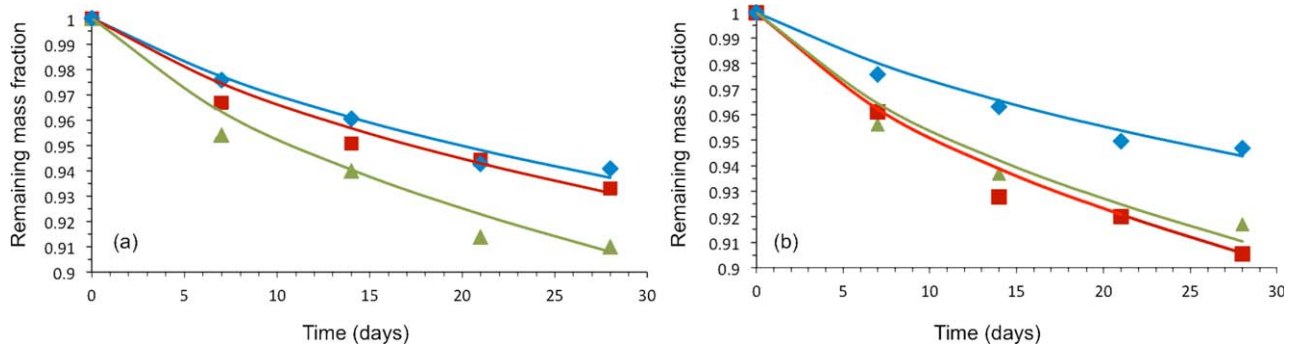

Figure 2. Remaining mass fraction of (a) GG/PAAm ICE and (b) GG gels calculated using mass loss as a function of immersion time (up to 28 days) in PBS (diamonds), lysozyme (squares), or trypsin (triangles) at 37°C. Solid lines indicate theoretical predictions of mass loss assuming the diffusion coefficients as detailed in Table I. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

trends described above. For example, the maximum gellan gum concentration was 0.18 ± 0.009 mg mL^{-1} for the PBS extracts collected at 7 days, which is equivalent to a mass loss of $6.1 \pm 0.4\%$.

The diffusion coefficient values (Table I) for gellan gum release from the gels were obtained by fitting the mass loss data (Figure 2) to the expected profile for Fickian diffusion.[33] These estimated diffusion coefficients give a reasonable indication of the release of gellan gum, i.e., the diffusion coefficients of ICE gels followed the order of PBS < lysozyme < trypsin. The diffusion coefficient values were similar to the corresponding values for GG hydrogels (Table I), except that for GG gels immersed in lysozyme and trypsin the values were essentially the same. This could suggest that degradation for ICE gels is dependent on the enzyme, whereas for GG gels the degradation is the same for both enzymes.

Acrylamide Leaching

The leaching of acrylamide from the ICE gels was analyzed using UV–Visible spectroscopy (Figure 3). The UV intensity of the characteristic acrylamide band around 198 nm [Figure 3(a)] was used to construct a standard curve [Figure 3(b)]. The resulting proportionality constant was used to determine the acrylamide concentration in the surrounding medium (PBS). For example, UV analysis (37°C) of extracts from the PBS

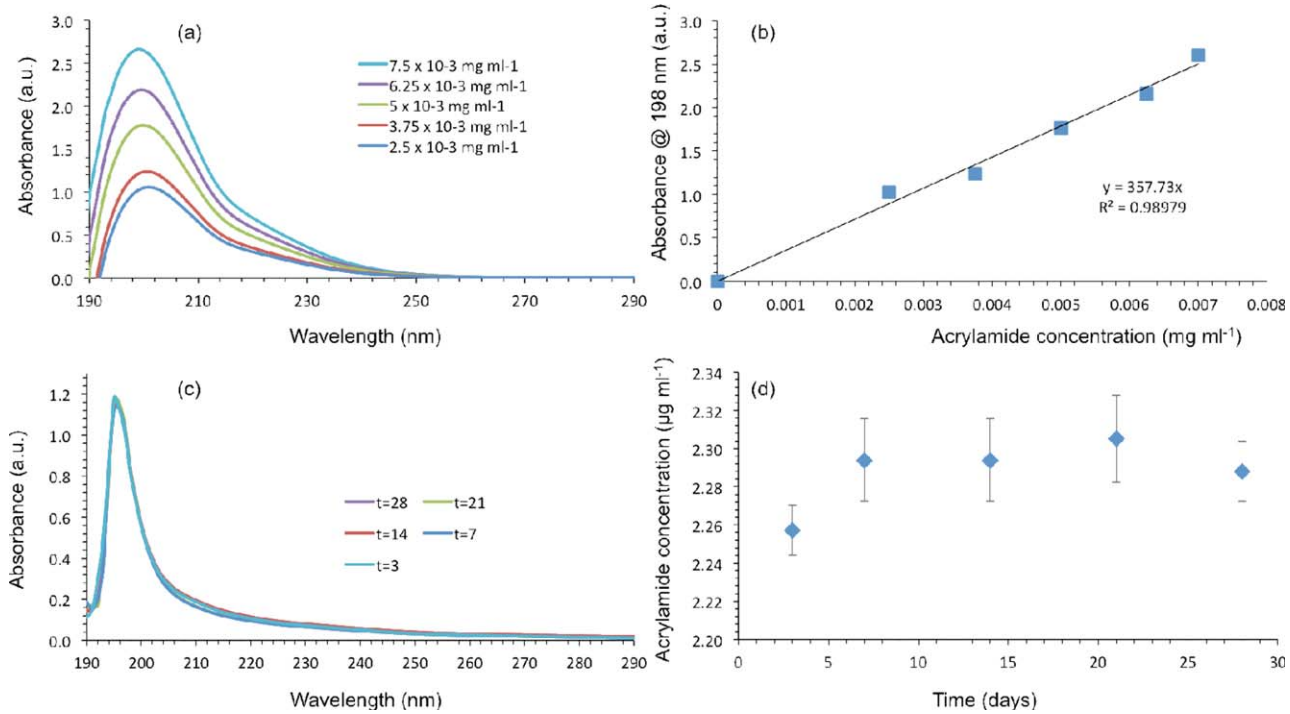

Figure 3. (a) UV–Visible spectrum of acrylamide, (b) absorbance at 198 nm versus acrylamide concentration (straight line is a linear fit for the absorbance data which was used to calculate the proportionality constant) (c) UV–Visible spectra of extracts from GG/PAAm ICE hydrogels with increasing incubation times and (d) acrylamide concentration (μg mL^{-1}) in extracts from GG/PAAm ICE hydrogels with increasing incubation times in PBS. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table II. Summary of Compression Data for GG/PAAm ICE Hydrogels Before Immersion and After Immersion in PBS, Lysozyme, and Trypsin at 37°C for 28 Days

Sample	σ_f (kPa)	ε_f (%)	E_t (kPa)	q_{stable} (%)
As-prepared	196 ± 73	50 ± 5	441 ± 50	24 ± 1
PBS	155 ± 4	49 ± 2	375 ± 32	26 ± 2
lysozyme	159 ± 8	39 ± 3	465 ± 59	27 ± 2
trypsin	182 ± 7	35 ± 4	618 ± 54	29 ± 2

σ_f, ε_f, E_t, and q_{stable} indicate compressive stress-at-failure, strain-at-failure, tangent modulus, and average swelling ratio, respectively.

medium showed a maximum concentration of 2.3 ± 0.02 μg mL^{-1} after 7 days of incubation and remained relatively constant between 7 and 28 days. The maximum acrylamide concentration detected [2.3 ± 0.007 μg mL^{-1} at 7–28 days; Figure 3(c, d)] under our experimental conditions was 300 times lower than the cytotoxicity limit of acrylamide (10 m*M* or 700 μg mL^{-1}) on rabbit synovial fibroblasts exposed for 24 h.[34]

Enzymatic Degradation

The rate of enzymatic degradation depends on the structure of the network, the amount of cross-linking, the pH, and the temperature of the surrounding medium.[35] Poly(acrylamide) gels are known to have resistance towards enzymatic degradation when immersed in enzymatic solutions at pH range 3.5–7.[36,37] However, it is reported that GG gels undergo degradation in the presence of soil enzymes (e.g., galactomannanase[38]) and microbial gellan lyase.[39] Previous studies on hydrogels consisting of a blend of GG and a glycosaminoglycan (hyaluronic acid) immersed in trypsin showed higher mass loss and swelling ratio compared to gels immersed in lysozyme.[40] Moreover, *in vivo* degradation was reported for methacrylated-GG through the action of hydrolytic enzymes (e.g., lysozyme and amylose).[41]

Previous studies on PAAm hydrogels showed that PAAm single network hydrogels do not undergo enzymatic degradation, whereas hydrogels consisting of PAAm and the polysaccharide chitosan degraded in the presence of soil enzymes, such as *Trichoderma viride*,[35] *Enterobacter agglomerans*, and *Azomonas macrocytogenes*.[42,43] It has been proposed that under the action of some amidases, as the first stage, poly(acrylamide) is hydrolyzed to poly(acrylic acid) and ammonia and as the second step the poly(acrylic acid) is degraded to carbon dioxide, ammonia and water that takes part in biosynthesis of amino acids.[44] Therefore, it is suggested that the mass loss and swelling of our ICE hydrogels immersed in trypsin is due to the hydrolytic cleavage of peptide links in the PAAm network. The ICE gels immersed in lysozyme also showed higher swelling compared to gels in PBS, which suggests that the hydrolysis of the GG network also occurs in the ICE gel. However, the effect of peptidase (trypsin) has a greater effect than amylase (lysozyme) on ICE gels as higher mass loss and swelling were displayed in trypsin compared to lysozyme (Table I). Lysozyme preferentially cleaves the β (1–4) glycosidic linkages of polysaccharides,[45–47] whereas trypsin is known to hydrolyze peptide linkages.[48,49] The ICE gel comprises both glycosidic (GG network) and amide (PAAm network) bonds. Irrespective of the higher enzyme concentration (lysozyme; 1×10^4 U mL^{-1} > trypsin; 1×10^3 U mL^{-1}), ICE gels displayed lower mass loss in lysozyme compared to mass loss in trypsin. However, the true mass loss, swelling and diffusion coefficients in lysozyme may have been affected by the intake (absorption and adsorption) of lysozyme in the media. Lysozyme is a positively charged molecule and this, coupled with its small size, results in its adsorption on to negatively charged substrates (e.g., gellan gum) under physiological body conditions.[50,51] Moreover, increased uptake of lysozyme has been observed with increased anionic polysaccharide concentration.[50] Therefore, it is suggested that the true mass loss, swelling, and diffusion-coefficients for ICE gels in lysozyme could be higher than reported here.

Compression Testing

The mechanical characteristics of the hydrogels were evaluated using stress–strain testing during compression at 37°C. After 28 days of degradation in enzymes (lysozyme or trypsin) ICE gels become stiffer (Table II). In particular, the ICE gels immersed in trypsin showed a significant enhancement in tangent modulus, i.e., from 441 ± 50 kPa to 618 ± 54 kPa. The increase for the gels immersed in lysozyme was more modest, while the modulus for gels immersed in PBS decreased.

In comparison with ICE gels immersed in PBS, the gels immersed in enzymes exhibited lower compressive strain-at-failure after immersion (Table II). The values of compressive strain-at-failure of ICE gels followed the order of PBS > lysozyme > trypsin, whereas the stress-at-failure values followed the opposite trend. This behavior suggests a mechanical reinforcement upon swelling in the presence of enzymes and this can be explained as follows. As the hydrolysis of amide bonds progresses, the PAAm chains break exposing carboxylic groups while ammonia evolves. Thus, the degree of anionicity of the molecule increases. Therefore, it is suggested that this attracts cations from the surrounding medium (PBS), which helps in creating additional cation-mediated junction zones in the gel network. As a result during the immersion in trypsin, the stiffness of ICE gels increases.

The observations for gels immersed in PBS appear to be consistent with the general mechanism governing the mechanical characteristics of gels, i.e., decline in mechanical properties is linked to decrease in density of network strands and/or cross-links.[52] However, it is not clear at present how the observations for gels immersed in the enzyme solutions relate to this. More detailed studies including experiments in tension are needed to provide insights into our observations.

Cyclic Testing

All gels were subjected to the following cyclic loading/unloading regime. First, they were strained to 15% compressive strain, and held at this strain for a finite period of time. Then, the gels were cycled between 15% and 25% compressive strain, while the strain was held constant at the minimum and maximum applied strain [Figure 4(a, b)]. During the cyclic regime all gels exhibited the following characteristic behavior: (1) stress rapidly increased during increase in strain to 25%; (2) stress showed creeping flow during constant strain period; (3) stress rapidly decreased during relaxation to 15% strain; and (4) stress shows

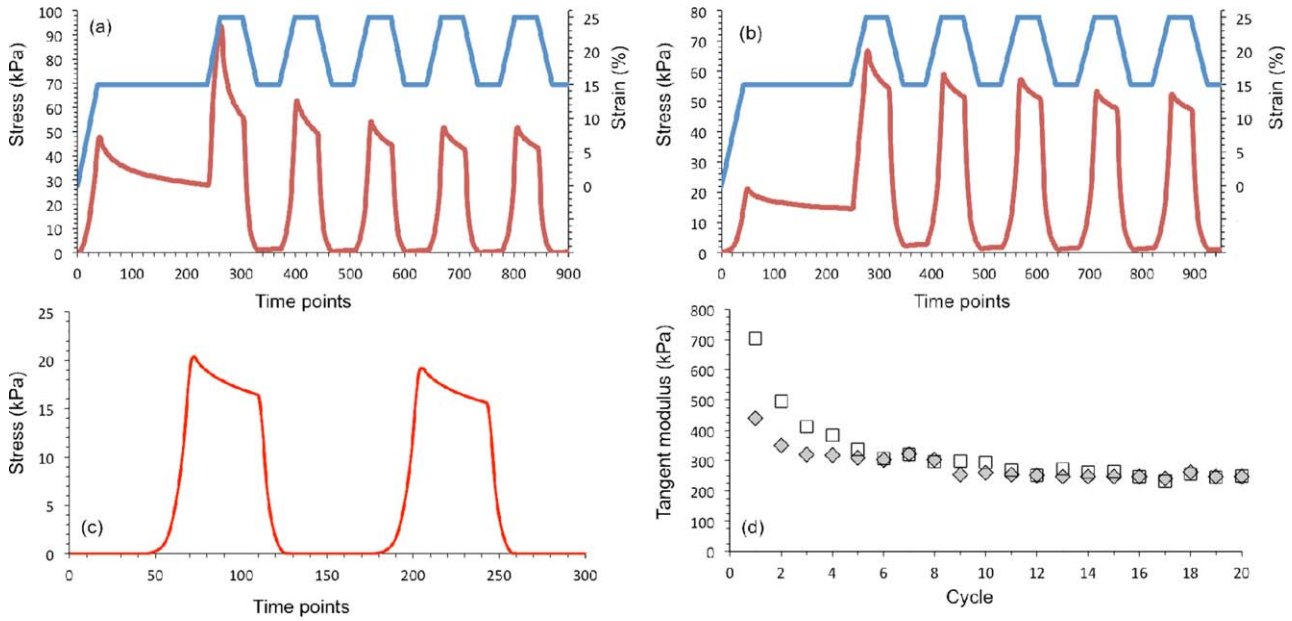

Figure 4. Stress (red lines) and strain (blue lines) as a function of time for typical GG/PAAm ICE hydrogels: (a) before and (b) after immersion in PBS for 28 days at 37°C. (c) Stress as a function of time for 2 cycles of a typical GG hydrogel after immersion in PBS for 28 days at 37°C. (d) Tangent modulus (20–25% strain) during 20 cycles for GG/PAAm ICE hydrogels before (squares) and after (diamonds) immersion for 28 days in PBS. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

amorphous flow. In cyclic region (3) the stress relaxed to a significantly lower value compared to that observed at strain = 15% in the initial straining regime. It is observed that the stress at strain = 15% in the cyclic regime had almost returned to zero. All subsequent cycles show this characteristic behavior regardless of the gels tested, i.e., as-prepared and after immersion in PBS or enzyme solutions. It is likely that the observed behavior is indicative of the melting and reforming of the gellan gum network structure.[53] The following observations are in support of this suggestion: (i) gellan gum hydrogels (prepared without polyacrylamide) exhibited the four characteristic regions [Figure 4(c) and (ii) PAAm hydrogels (prepared without gellan gum) did not (data not shown).

Figure 4(d) shows tangent moduli evaluated over the increase in strain from 15% to 25% of a typical example of an ICE gel before and after immersion for 28 days in PBS. The modulus reduced during the first 10 cycles and remained relatively constant thereafter (10–20 cycles). It is evident that the modulus of the gels immersed for 28 days becomes similar to as-prepared gel (i.e., prior to immersion) after five loading/unloading cycles. Similar results were obtained for the gels immersed in the enzyme solutions. The average values in the plateau region for the ICE gels immersed in PBS and the lysozyme and trypsin solutions are 250 ± 16 kPa, 315 ± 20 kPa, and 317 ± 25 kPa, respectively. In comparison, the value for the GG hydrogel (after immersion for 28 days in PBS) is significantly weaker, i.e., 97 ± 11 kPa.

L929 Cell Inhibition Assay

L929 cell growth inhibition assays were carried out for ICE gel extracts collected in PBS for 28 days. Cell growth inhibition (CGI) was calculated with respect to the negative control (EMEM media). The toxicity of the positive controls on L929 cell growth was clear, given the dramatic increase in cell growth inhibition (85% for latex extract) compared to the negative control [<10%, see Figure 5(a)]. All ICE gel extracts were found to be non-cytotoxic (i.e., less than 20% inhibition) during the test period [Figure 5(b)]. For example, after 28 days of immersion ($t = 28$) in PBS the cell growth inhibition values for ICE hydrogels extracts were 17.6 ± 6.4 %. The cell growth inhibition of ICE hydrogel was found to be proportional to the mass loss, i.e., CGI values increased with increasing leachate concentration (gellan gum and acrylamide) and remained constant after the maximum mass loss [Figure 5(b)]. The CGI increased for samples taken during the first 7 days of gel incubation (CGI = 19.3 ± 2.6 %). This could be related to the leaching of gellan gum (0.18 ± 0.01 mg mL^{-1}) and acrylamide (2.3 ± 0.03 μg mL^{-1}) to the surrounding medium [Figure 5(b)]. For leachate collected during the next 21 days, the CGI remained relatively constant, correlating with the proposed sessation of chain release after 7 days of incubation, i.e., cell growth inhibition increases with amount of gellan gum and acrylamide released. This suggests that small amounts of released gellan gum and acrylamide do not have adverse effects on cell proliferation, but larger amount may adversely affect their proliferation. Therefore, it is important to control the leachate concentration for future application of these materials involving cell interactions (e.g., tissue engineering).

PC12 Cell Growth Inhibition Assay

PicoGreen assay (a sensitive assay for detecting double-stranded DNA in solution) was used to estimate the PC12 cell numbers after incubation for 2 days in GG/PAAm ICE hydrogel extracts. A standard curve was constructed using a range of PC12 cell concentrations and the proportionality

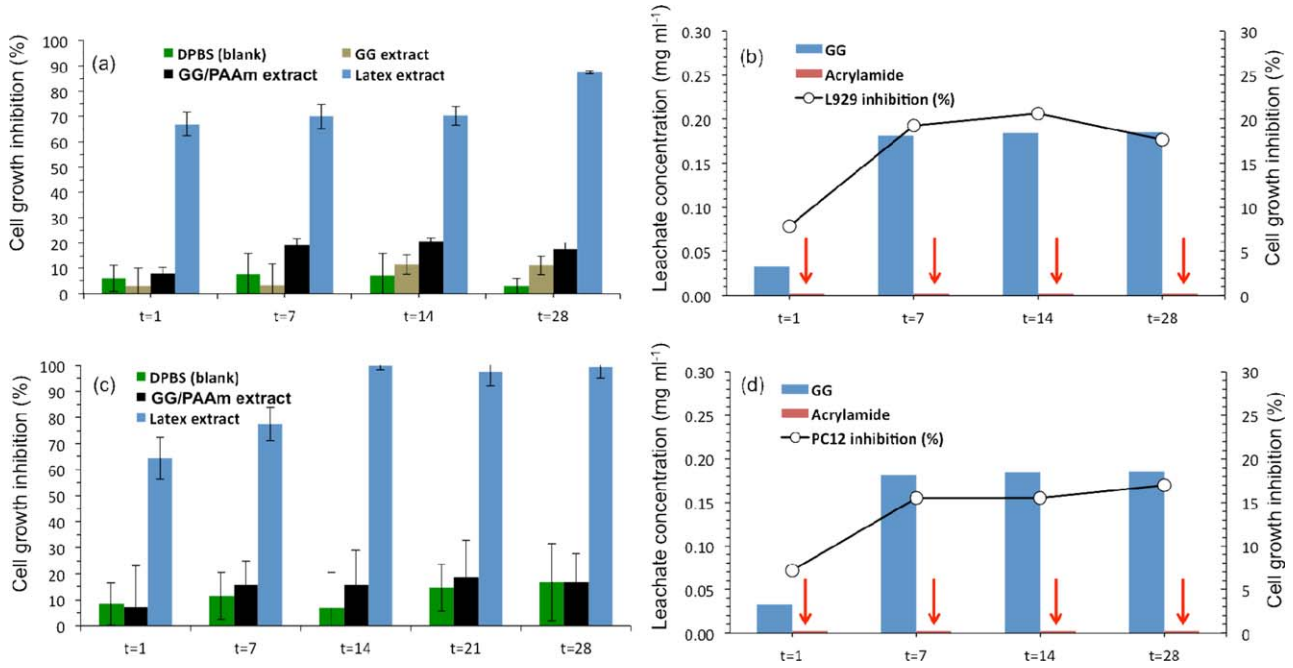

Figure 5. (a) Cell growth inhibition (%) of L929 fibroblasts in GG and GG/PAAm ICE extracts extracted in DPBS at 37°C and diluted in 1 : 3 in EMEM and (b) the relationship between L929 cell growth inhibition (%) and GG (blue) and acrylamide (red, arrows). (c) Cell growth inhibition (%) of PC12 in GG/PAAm ICE extracts extracted in DPBS at 37°C and diluted in 1 : 3 in EMEM and (d) the relationship between PC12 cell growth inhibition (%) and GG (blue) and acrylamide (red, arrows) detected in GG/PAAm ICE gel extracts. Error bars represent one standard deviation calculated from three independent samples. Gellan gum and acrylamide concentrations in DPBS extracts are calculated using CD and UV–Visible data, respectively. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

constant of the linear part (cell concentration up to 5×10^5 cells mL^{-1}) was used to determine the cell density in ICE gel extracts (data not shown). CGI was calculated with respect to the negative control (EMEM media) and all the ICE gel extracts were found to be non-cytotoxic (i.e., CGI less than 20%) for PC12 growth during the test period [Figure 5(c)]. For example, cell growth inhibition values for cells exposed for 2 days to 28 day extracts ($t = 28$) of GG/PAAm hydrogels were $17 \pm 11\%$.

The CGI profile of PC12 cells in ICE gel extracts was similar to that of L929 cells, i.e., CGI proportionally increased with increasing leachate concentration [Figure 5(d)]. The CGI increased for leachates extracted during the first 7 days of incubation ($CGI = 15.5 \pm 9.5$ %), and remained relatively constant for 7–28 day leachates (average $CGI = 15.7 \pm 1.5$ %). This can be explained using the same argument as in the previous section. The CGI obtained for ICE hydrogels incubated in PBS at 37°C shows that the PBS extracts containing a maximum acrylamide concentration of 2.3 ± 0.03 μg mL^{-1} and a maximum gellan gum concentration of 0.18 ± 0.01 mg mL^{-1} were not cytotoxic to the growth of PC12 cells. The results obtained are in agreement with previous cell inhibition studies of acrylamide cytotoxicity on sericin/PAAm IPN hydrogels.[24]

CONCLUSIONS

In this work, the degradation behavior of gellan gum-polyacrylamide ionic-covalent entanglement hydrogels in PBS and enzyme solutions of up to 42 days is presented. ICE gels undergo a modest amount of swelling and mass loss, which stabilizes after 7 days of immersion in the solutions. The compressive modulus of the ICE gels increased as a result of immersion in enzyme solutions, and decreased as a result of immersion in PBS. This stiffening effect was attributed to an increase in cation-mediated junction zones as a result of the enzymes' hydrolyzing interaction with the gel polymer networks. However, cyclic testing revealed that the stiffer gels could be returned to their initial mechanical response (prior to immersion) by subjecting them to at least five loading/unloading cycles. A similar effect was observed for those ICE gels were the modulus was reduced (through immersion in PBS). This demonstrates that the ICE hydrogel mechanical response is not adversely affected by immersion in either PBS or enzyme solutions. It was found that leachates (e.g., acrylamide) of these ICE hydrogels over 28 days of immersion can be considered non-cytotoxic for the growth of L929 and PC12 cells.

Given the toughness and recover-ability of ICE gels,[3,4] we envisage that the findings presented in this paper (e.g., non-cytotoxicity of leachates) could help with the development of these hydrogel materials for their future application in tissue engineering. In addition, it is possible that the observed stability (after 7 days of immersion) may lead to investigation of the applicability of these materials as components in gel devices operating under humid or wet conditions, i.e., as substrates for wet strain gauges. Regardless of the intended application, any

further studies on these ICE hydrogels should include an assessment of the effects of degradation on the internal network structure using spectroscopic methods.[54]

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