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Mechanical Characteristics of Swollen Gellan Gum Hydrogels

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ABSTRACT: The behavior of swollen gellan gum hydrogels in terms of mechanical properties, weight loss, and cell growth inhibition of leachates is presented. Low-acyl gellan gum (LAGG), high-acyl gellan gum (HAGG), and a HAGG–LAGG blend were soaked in phosphate-buffered saline (PBS) at pH 7.4 and 37°C for up to 168 days. The gels exhibited their maximum mass loss and swelling after 28 days of immersion in PBS. LAGG gels exhibited lower value for mass loss and the chain-release diffusion coefficient than gels consisting of HAGG and the HAGG–LAGG blend. The change in mechanical and rheological characteristics during soaking of the three hydrogels was attributed to mass loss, while LAGG hydrogels also showed evidence of effects because of cation exchange with the surrounding medium. The mechanical characteristics of the LAGG, HAGG, and blend hydrogels relative to each other did not change during swelling (although the magnitude changed). L929 fibroblasts growth inhibition tests showed that the leachate products of the three gels can be considered noncytotoxic, which is important for their future application in tissue engineering. © 2013 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 130: 3374–3383, 2013

KEYWORDS: gels; mechanical properties; polysaccharides; swelling

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INTRODUCTION

Hydrogels are hydrophilic polymer networks which contain up to thousands of times their dry weight in water. A range of chemical and physical cross-linking approaches have been used to form chemically stable or degradable materials.[1–3] They have long received attention because of their innate structural similarities to the extracellular matrix and their capacity for facilitating cellular proliferation and survival.[4] Systematic study of the degradation behavior is one of the key considerations in the development of biomimetic 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 article, we investigate the mechanical characteristics of hydrogels based on the biopolymer gellan gum (GG), which is a linear, anionic extracellular polysaccharide produced by fermentation of *Sphingomonas elodea*.[5] In its native form, usually referred to as high-acyl gellan gum (HAGG) the tetrasaccharide repeat unit consists of residues of β-D-glucose, β-D-glucuronate, and α-L-glucose with two acyl substituents on one of the glucose residues (Figure 1). The low-acyl (LAGG) form is produced by removing these substituents from the native high-acyl form by strong alkali treatment.[6,7] Gellan molecules form a threefold double-helical structure under an appropriate aqueous environment and the aggregation of these helical segments leads to the formation of a so-called true gel network.[8] The presence of monovalent (Na^+) and/or divalent (Ca^{2+}) cations enhances this aggregation and increases the mechanical properties of the gels.[9–11] However, to reach optimum gel strength requires an order of magnitude larger concentration of monovalent ions than divalent ions.[12]

The presence of the acyl substituents in HAGG does not change the overall helical structure, but changes the binding (cross-linking) sites for the cations.[9,11,13–15] It has been suggested that this change is responsible for the loss of so-called "cation-mediated aggregation" between the HAGG helices.[11] The result of this difference in aggregation is that LAGG forms hard (nonelastic) and brittle gels, whereas HAGG gels are soft (elastic) and nonbrittle.[11,13] As such mixtures of LAGG and HAGG can be used to tune the gel characteristics depending on ratio of mixture.

GG is USFDA and European Union (E418) approved food additive, and has found wide application as a multifunctional gelling, stabilizing, and suspending agent.[11,16] There has been significant interest in GG as materials for tissue engineering

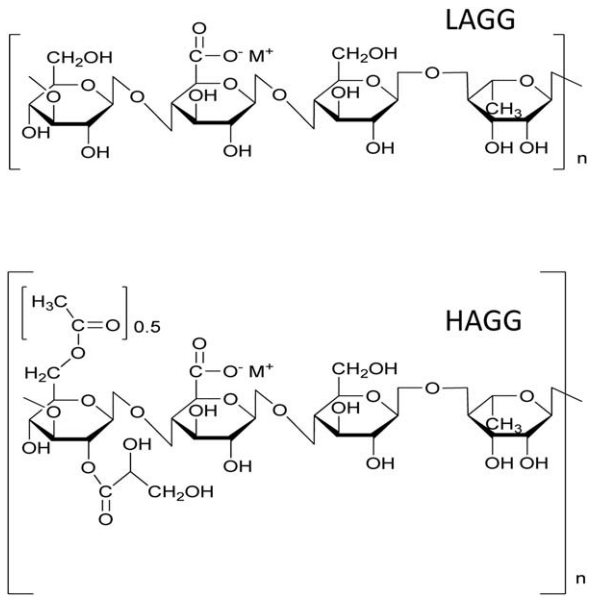

Figure 1. Tetrasaccharide repeating unit of LAGG and HAGG.

applications because of its capacity to form hydrogels under mild processing conditions.[17–21] For example, the gelation temperature of GG can be tuned to 37°C which led to suggestions that it can be used in injectable approaches to tissue engineering.[17] In other recent work, GG hydrogels have been proposed as materials to improve intervertebral disc regeneration and as a cell support for cartilage regeneration.[18,20] It has also been demonstrated that LAGG can be made photo-cross-linkable which dramatically improves its mechanical properties, thereby opening up tissue engineering applications requiring stiffer hydrogels.[22]

Although GG's chemical structure, gelation mechanisms,[8,9,13,23,24] rheological behavior, and mechanical characteristics[6,17,19,20,25] have been characterized in detail, only limited evidence has been presented in the literature detailing its swelling behavior at 37°C and pH 7.4.[18–20,22,26] In particular, the effect of weight loss on the mechanical characteristics (of GG hydrogels) remains unexplored, which is of critical importance for the future application of GG hydrogels in tissue engineering.

Here, the behavior of Ca^{2+} cross-linked LAGG, HAGG, and LAGG–HAGG blend hydrogels immersed in phosphate-buffered saline (PBS) at 37°C and pH 7.4 is reported. The resulting hydrogels are characterized in detail using mass loss analysis, volumetric swelling, circular dichroism spectroscopy (CD), rheological testing, mechanical compression testing, and L929 fibroblasts cell growth inhibition (CGI) on hydrogel leachate products. Establishing these characteristics is important for the future application of GG materials in tissue engineering.

EXPERIMENTAL

Materials

Endotoxin-free LAGG (molecular weight range as specified by manufacturer 2–3 × 10^5 Da, Gelzan CM, Lot #9K6969A) and HAGG (molecular weight range as specified by manufacturer 1.5–2.5 × 10^6 Da, Kelcogel LT 100, Lot #9K6878A) were a gift from CP Kelco. Note, the molecular weight ranges should be seen as indicative values as determining the molecular weight of GG is not straightforward.[27] Eagle's minimum essential medium (EMEM), fetal bovine serum (FBS), penicillin/streptomycin (PS), and Dulbecco's PBS (DPBS), salts required for PBS solution preparation and $CaCl_2{\cdot}2H_2O$ were purchased from Sigma-Aldrich. 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).

Hydrogel Preparation

Solutions of LAGG in Milli-Q water were prepared at a concentration of 2% w v^{-1} under continuous stirring at 200 rpm (IKA RW 20 digital) for 2 h at 80°C. Solutions of HAGG (2% w v^{-1}) were prepared under continuous stirring for 2.5 h at 85°C. The LAGG:HAGG blend (2% w v^{-1}) was obtained by combining the LAGG and HAGG aqueous solutions at a ratio of 1:1 (v v^{-1}). Hydrogel discs were prepared by addition of hot (≈80°C) $CaCl_2$ solution to the hot GG solution (final added Ca^{2+} concentration ≈5 m*M*) followed by transfer into polystyrene petri dishes (Sigma-Aldrich). The gels were formed by cooling the solutions to 37°C under controlled conditions (relative humidity 50%, Thermoline Scientific, TRH-150-SD) and gel discs (diameter 17 mm; height 5 and 10 mm) were punched out using a custom-built puncher.

Mass Loss and Volumetric Swelling Ratio

The initial weights (m_i) of all hydrogel 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) for up to 168 days. At each time point, three tissue cassettes were removed from the degrading medium, 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[28] the following equation:

$$M_L = \frac{(m_{id} - m_d)}{m_{id}} \times 100, \quad (1)$$

where the initial dry polymer mass (m_{id}) is obtained by multiplying m_i with the initial polymer weight fraction. The volumetric swelling ratio (Q) of the polymer volume in the swollen state (V_{swollen}) over the volume of the dry polymer (V_{dry}) was obtained as follows[28,29]:

$$Q = 1 + \frac{\rho_{\text{polymer}}}{\rho_{\text{solvent}}}\left(\frac{m_s}{m_d} - 1\right) \quad (2)$$

where ρ_{polymer} and ρ_{solvent} are the biopolymer and solvent densities, respectively. The volume in the swollen state was approximate by the sum of the volumes of the polymer ($m_d/\rho_{\text{polymer}}$) and the solvent [$(m_s - m_d)/\rho_{\text{solvent}}$]. The polymer density of LAGG (1.52 ± 0.08 g mL^{-1}) and HAGG (1.52 ± 0.08 g mL^{-1}) were experimentally determined for this work by adding biopolymer powder to an alcohol solution to a known volume. The

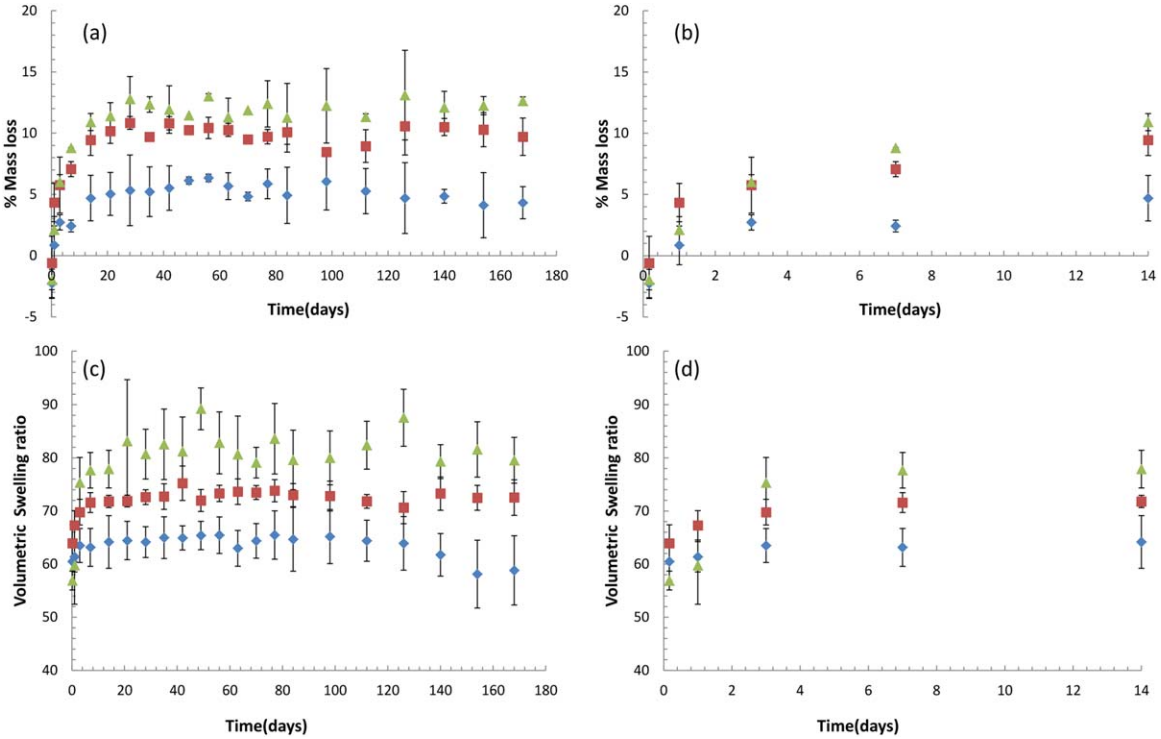

Figure 2. (a and b) Mass loss (%) and (c and d) volumetric swelling ratio of LAGG (diamonds), HAGG (triangles), and blend (squares) hydrogels immersed in PBS at 37°C for 168 days. Error bars represent one standard deviation (n =3). Mass loss (%) and volumetric swelling ratio calculated using eqs. (1) and (2), respectively. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

density of the blend (1.52 ± 0.16 g mL^{-1}) was calculated using the approximation of densities method.[30]

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^{-1} using CD-matched cuvettes (path lengths of 1–5 mm). A standard curve was constructed from a dilution series of LAGG in PBS at 37°C and measuring the CD intensity at 201 nm. CD analysis was used to detect GG in extracts of the medium (PBS-containing hydrogel discs) after 7, 14, 21, and 28 days of immersion in PBS at 37°C.

Rheology

Oscillatory shear rheological measurements were performed at 37°C on a controlled strain rheometer (Anton Paar, Physica MCR 301) with a parallel plate configuration and a heat controlled sample stage (Julabo Compact Recirculating Cooler AWC 100). Hydrogel cubes (20 × 20 × 5 mm^3) were submerged in PBS (pH7.4, 37°C) for up to 28 days and punched out into gel discs (diameter 17 mm; height 5 mm) at appropriate time points. Strain sweeps (up to 100%) were conducted at 5 Hz, while frequency sweeps (up to 500 Hz) were conducted by applying a constant strain of 0.1%. Storage (G') and loss (G'') moduli were determined in the linear viscoelastic (LVE) regions of frequency sweeps. Maximum shear strain (τ_{max}) and maximum shear stress (γ_{max}) were determined from the LVE regions in amplitude sweeps. All tests were run in triplicate on samples, which had not been tested previously.

Mechanical Characterization

Compressive stress–strain measurements were carried out using an universal testing machine (EZ-S, Shimadzu, Japan) at 2 mm min^{-1}. Hydrogel cubes (20 × 20 × 10 mm^3) were submerged in PBS (pH 7.4, 37°C) up to 28 days prior to punching out into gel discs (diameter 17 mm; height 10 mm) for 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.

L929 Cell Growth Inhibition Assay

Hydrogel samples (diameter 17 mm; height 6 mm) weighing approximately 1 g each were prepared under sterile conditions for all three compositions, 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 pipetted out from each of the well plates and diluted with EMEM (supplemented with 10% FBS and 1% PS) to a ratio of 1 : 3.

Murine dermal fibroblasts (L929) were seeded at 1×10^5 cells per plate in EMEM (supplemented with 10% FBS and 1% PS) in tissue culture dishes (diameter 35 mm), and then incubated in a 5% CO_2-humidified atmosphere at 37°C for 1

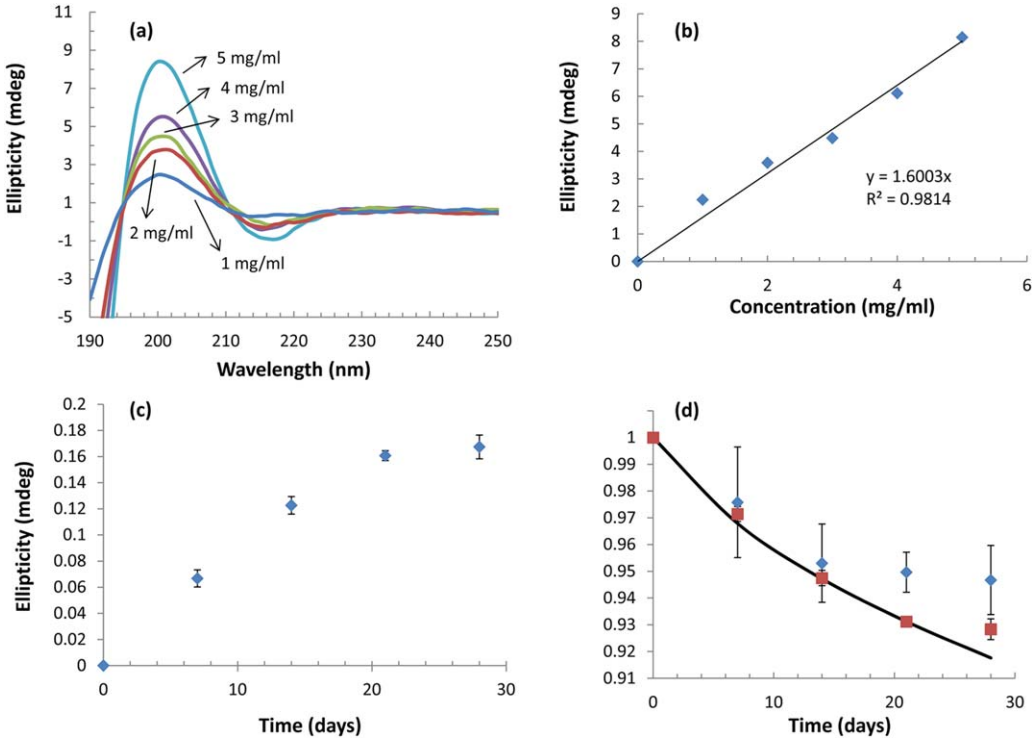

Figure 3. (a) CD spectra of GG as a function of concentration in PBS at 37°C, (b) CD intensity (ellipticity) at 201 nm as a function of GG concentration, (c) ellipticity at 201 nm vs. incubation time in PBS at 37°C, and (d) remaining mass fraction calculated using mass loss (diamonds) and CD analysis (squares) as a function of immersion time in PBS at 37°C. Solid line indicates theoretical prediction of calculated loss assuming diffusion coefficient of 1.1×10^{-13} m^2 s^{-1}. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

day to establish a subconfluent cell monolayer of adherent fibroblasts. The media in each plate were replaced by test extracts (LAGG, HAGG, and blend), positive control (7.5% ethanol and latex extracts), and negative control solutions (EMEM and DPBS) and incubated for another 2 days. Latex extracts were prepared by immersing pieces of latex gloves (Ansell, Thailand) in DBPS and incubated in a 5% CO_2-humidified atmosphere at 37°C. 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) plates. Positive controls were expected to show greater than 70% inhibition to indicate the assay was valid. Three independent samples of each degradation product were tested. The percentage CGI was obtained by the following equation[29]:

$$\mathrm{CGI} = \frac{n_c - n_s}{n_c}, \tag{3}$$

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

Statistical Analysis

The reported results are averages of the values obtained. Reported numerical errors and graphical error bars are given as ±1 standard deviation (SD). Data and outliers were rejected either when instrumental error was known to have occurred, or if data failed a Q-test with a confidence interval ≥95%. Statistical analysis was performed on CGI results using Student's *t*-test. A confidence level of 0.001 was considered significant. All values are reported as the mean (±1 SD).

RESULTS AND DISCUSSION

Mass Loss and Chain Release

The behavior of GG (LAGG, HAGG, and blend) hydrogels in PBS (pH 7.4, 37°C) was followed for a period of up to 168 days. The mass loss profiles [Figure 2(a and b)] showed a small mass gain (2%) after 4 h, a steady mass loss up to 14 days, which was followed by a slow decrease in the mass loss until the gel stabilized after 28 days. This suggests that the gels reached their equilibrium after 28 days and then maintained constant composition without further mass loss during the remainder of the test period between 28 and 164 days. The HAGG and blend hydrogels exhibited a faster mass loss rate than the LAGG gels. For example, in the steady mass loss period (1–14 days) the

Table I. Average Mass Loss ($M_{L,\mathrm{stable}}$) and Average Volumetric Swelling Ratio (Q_{stable}) During the Stable Immersion Period (28–168 days) in PBS at 37°C

Hydrogel	$M_{L,\mathrm{stable}}$ (%)	Q_{stable} (%)
LAGG	5.3 ± 0.7	64 ± 2
HAGG	12.1 ± 0.6	83 ± 3
Blend	10.0 ± 0.7	73 ± 1

HAGG, high-acyl gellan gum; LAGG, low-acyl gellan gum; $M_{L,\mathrm{stable}}$, average mass loss; Q_{stable}, average volumetric swelling ratio.

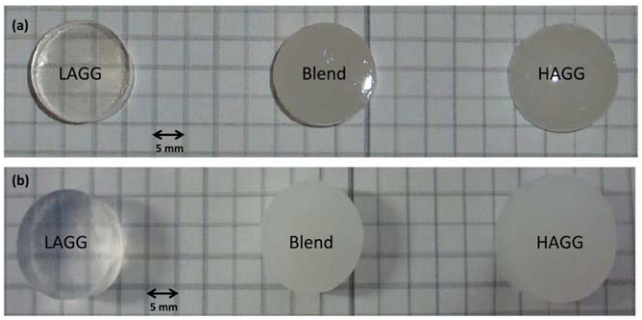

Figure 4. Photographs of typical hydrogels at (a) before immersion ($t = 0$) in PBS and (b) swollen hydrogels (after 28 days in PBS). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

mass loss rates for LAGG, HAGG, and blend hydrogels were $0.25 \pm 0.08\%$ day^{-1}, $0.37 \pm 0.04\%$ day^{-1}, and $0.6 \pm 0.2\%$ day^{-1}, respectively. Over the course of the swelling period LAGG hydrogels exhibits the lowest mass loss (average value of $5.3 \pm 0.7\%$ during days 28–168), compared to the corresponding values of $12.1 \pm 0.6\%$ and $10.0 \pm 0.7\%$ for the HAGG and LAGG–HAGG blend hydrogels, respectively.

The steady mass loss behavior of our gels can be explained using the previously observed chain release behavior of GG hydrogels.[31] It should be noted that these studies were carried out using LAGG hydrogels and immersion conditions which differ from our gels, that is, these gels were prepared at 10°C, with the only cross-linking provided by the cations inherently present in the LAGG, that is, mainly K^+ (5.03% w w^{-1}), although significantly smaller amounts of Na^+ (0.42% w w^{-1}), Ca^{2+} (0.37% w w^{-1}), and Mg^{2+} (0.09% w w^{-1}) were also present. Chain release was monitored by immersion of these gels in water (neutral pH) and salt solutions (KCl and tetrylammonium chloride, TMAC) all at 10°C. These gels prepared without addition of $CaCl_2$ (as used in our work) are hereafter referred to as "LAGG-without." The LAGG-without gels (2% w w^{-1}) immersed in water exhibited a steady mass loss for up to 7 h followed by rapid mass loss in a short period of time leading to the collapse of the gel.[31] The authors concluded that free GG chains (those unassociated with the gel network) are released first, while the network (associated) chains are released over a longer time frame.[31] The latter is a result of the release of cross-linking ions from the gel into the surrounding solution. Because these ions are responsible for holding the GG network together, release of these ions results in dissociation of the network leading to gel collapse. Immersion of LAGG-without gels (2% w w^{-1}) in KCl or TMAC solutions (10°C) slowed down the release of chains and did not result in gel collapse over the duration of the immersion experiment (8 h).[31] This lack of erosion was attributed to the gel's uptake of K^+ ions, which facilitates the association of unassociated GG chains into the network resulting in a more stable gel structure.[31]

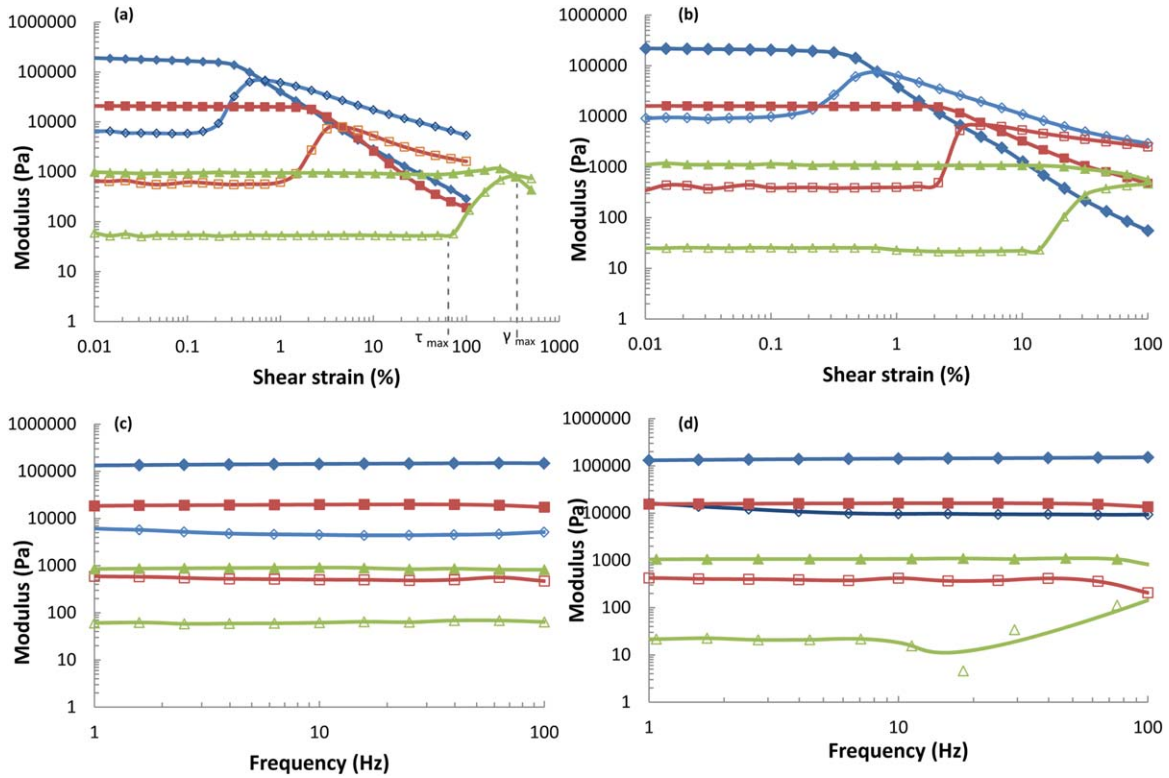

Figure 5. Amplitude sweeps of LAGG (diamonds), HAGG (triangles), and blend (squares) hydrogels at a frequency of 5 Hz at 37°C (a) prior to immersion and (b) after 28 days of immersion in PBS at 37°C. Frequency sweeps of LAGG (diamonds), HAGG (triangles), and blend (squares) hydrogels at a strain of 0.1% at 37°C (c) prior to immersion and (d) after 28 days of immersion in PBS at 37°C. Storage and loss modulus for the respective gels are indicated by filled and open symbols, respectively. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table II. Comparison of Rheological and Compression Results of Hydrogels Immersed in PBS at 37°C

Sample	τ_{max} (Pa)	γ_{max} (%)	G' (kPa)	G'' (kPa)	E_t (kPa)	σ_f [kPa]	ε_f [%]
LAGG-0	153 ± 19	0.15 ± 0.002	184 ± 25	6.7 ± 1.1	535 ± 18	130 ± 5	29 ± 1
LAGG-1	154 ± 73	0.15 ± 0.05	158 ± 38	5.9 ± 0.4	295 ± 54	108 ± 6	42 ± 1
LAGG-3	224 ± 18	0.12 ± 0.03	241 ± 7	10.1 ± 0.2	234 ± 5.1	124 ± 28	52 ± 4
LAGG-7	274 ± 02	0.16 ± 0.08	267 ± 44	10.4 ± 0.8	324 ± 55	126 ± 12	40 ± 9
LAGG-14	297 ± 153	0.23 ± 0.12	233 ± 52	10.2 ± 0.7	315 ± 31	130 ± 35	36 ± 8
LAGG-21	173 ± 24	0.17 ± 0.07	207 ± 24	9.1 ± 2.2	235 ± 1.7	172 ± 32	53 ± 7
LAGG-28	178 ± 39	0.24 ± 0.04	211 ± 8	11.1 ± 1.7	277 ± 13	164 ± 32	47 ± 4
HAGG-0	451 ± 42	58.5 ± 13.4	1.0 ± 0.07	0.05 ± 0.003	87 ± 2.7	50 ± 8	68 ± 6
HAGG-1	189 ± 57	19.1 ± 5.1	1.0 ± 0.01	0.03 ± 0.005	6.9 ± 1.1	58 ± 12	73 ± 4
HAGG-3	164 ± 21	13.0 ± 3.5	1.3 ± 0.1	0.03 ± 0.004	18.1 ± 2.2	40 ± 12	73 ± 4
HAGG-7	94.4 ± 13	8.8 ± 2.4	1.0 ± 0.1	0.04± 0.008	21.3 ± 1.9	46 ± 6	61 ± 3
HAGG-14	76.7 ± 8	8.9 ± 2.5	1.1 ± 0.06	0.02 ± 0.001	9 ± 1.2	73 ± 32	66 ± 1
HAGG-21	108 ± 6	9.9 ± 0.007	1.1 ± 0.07	0.02 ± 0.006	10.5 ± 0.4	48 ± 1	63 ± 1
HAGG-28	15.1 ± 1	13.5 ± 0.3	1.1 ± 0.1	0.07 ± 0.006	11.4 ± 3.1	48 ± 10	66 ± 14 14
Blend-0	231 ± 45	1.23 ± 0.33	19.3 ± 1.5	0.5 ± 0.07	110.5 ± 1.7	71 ± 11	48 ± 3
Blend-1	208 ± 4	2.16 ± 0.007	14.4 ± 0.2	0.4 ± 0.02	68.7 ± 5.1	69 ± 1	55 ± 2
Blend-3	244 ± 46	2.66 ± 0.71	14.1 ± 0.9	0.4 ± 0.03	71.6 ± 2.6	68 ± 3	56 ± 2
Blend-7	196 ± 37	1.91 ± 0.38	15.3 ± 0.9	0.4 ± 0.03	57.8 ± 4.8	76 ± 3	60 ± 1
Blend-14	189 ±41	2.03 ± 0.17	15.7 ± 0.8	0.4 ± 0.03	71.3 ± 7.1	92 ± 2	60 ± 2
Blend-21	154 ± 0.4	0.70 ± 0.2	17.5 ± 0.1	0.4 ± 0.06	84.8 ± 7.3	85 ± 4	56 ± 2
Blend-28	135 ± 61	1.57 ± 0.78	14.6 ± 1.1	0.4 ± 0.01	94.7 ± 6.2	76 ± 12	53 ± 2

ε_f, strain-at-failure; E_t, tangent modulus calculated using the slope of a linear fit of the stress–strain plot at 15%–25% strain; G', storage modulus; G'', loss modulus; γ_{max}, maximum shear strain; σ_f, stress-at-failure; τ_{max}, the maximum shear stress.
The naming convention for the sample name is "hydrogel type-days immersed." For example, LAGG-28 indicates a LAGG hydrogel immersed for 28 days. The height of gels used in rheological and compression testing was 5 and 10 mm, respectively.

The mass loss profile for our GG hydrogels (immersed in PBS at 37°C) is very different compared to that reported in Ref. 31. First, the mass profile shows a small increase at short time period and second, the steady mass loss period occurs over a much longer time scale, that is, 28 days rather than 7 h despite the higher temperature (37°C) of the surrounding medium used in our work. As such, the slower time period on which the mass loss occurs for our Ca^{2+} cross-linked hydrogels suggest that there is smaller amount of unassociated GG chains in our gels compared to that of the LAGG-without gels.

The release of GG from our gels was quantified using a spectroscopic method. CD analysis (37°C) of extracts from the PBS medium, confirmed that LAGG leached out of the hydrogels over the first 28 days of immersion (Figure 3). The CD intensity of the GG characteristic band around 201 nm was used to construct a standard curve. The resulting proportionality constant was used to determine the LAGG concentration in the surrounding medium (PBS) and the mass fraction remaining in the gel. The concentration of LAGG leached out at 28 days was 0.100 ± 0.005 mg mL^{-1}, equivalent to a mass loss of $7.2 \pm 0.4\%$. It is suggested that the difference with the measured mass loss by weight [5.3 ± 0.7 %, Figure 3(d)] is because of the diffusion of ions into the gels as a result of the cation exchange. PBS contains 137 m*M* Na^+ compared to only 5 m*M* Ca^{2+} in the as-prepared gels. It is well known that the amount of divalent cations required to form true gels is two orders of magnitude lower than the equivalent amount of monovalent cations.[11] Therefore, it is reasonable to expect that the gel's weight gain because of cation exchange will partially offset the mass loss because of GG chain release.

The diffusion constant for LAGG release from the film was obtained by fitting the data from mass loss data and CD analysis to the expected profile for Fickian diffusion.[32] Figure 3(d) shows that these loss profiles fit diffusion coefficients of $(1.1 \pm 0.2) \times 10^{-13}$ m^2 s^{-1} (CD data) and $(0.8 \pm 0.1) \times 10^{-13}$ m^2 s^{-1} (mass loss data), respectively. This suggests that diffusion coefficients calculated based on mass loss data give a reasonable indication of the release of GG. A similar analysis using the mass loss data for HAGG and the blend results in diffusions coefficients of $(3.8 \pm 0.4) \times 10^{-13}$ m^2 s^{-1} and $(2.8 \pm 0.3) \times 10^{-13}$ m^2 s^{-1}, respectively. The calculated diffusion coefficient for LAGG release from gel to PBS (37°C) is two orders of magnitude slower compared to the self-diffusion coefficient of GG in NaCl (25 m*M*, 40°C).[33] Hence, it is clear that GG mobility is retarded because of the effect of the GG network.

Swelling

The volumetric swelling ratio [Figure 2(c and d)] shows similar characteristics over time as the mass loss profile. Within the first 4 h of immersion, all three types of gels undergo a

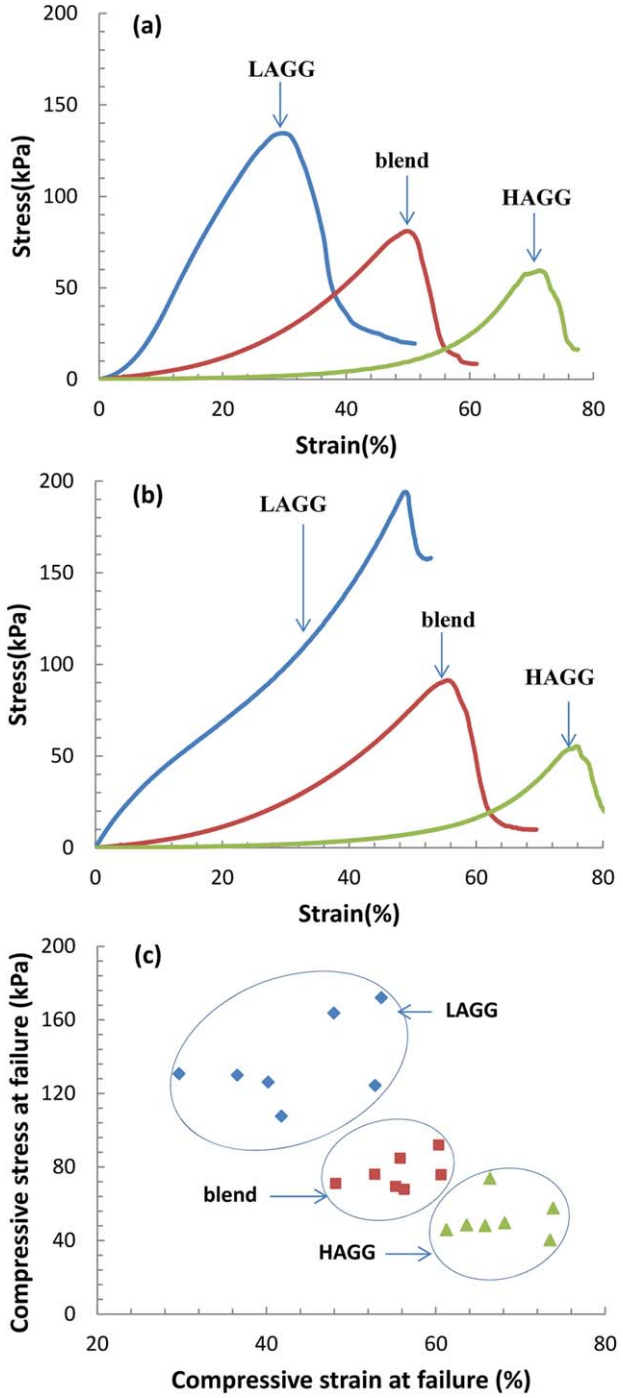

Figure 6. Typical compressive stress–strain curves for LAGG, HAGG, and blend (a) before immersion and (b) after 28 days of immersion. (c) Compressive strain at failure vs. compressive stress at failure (during 28 days of immersion) of LAGG (diamonds), HAGG (triangles), and blend (squares) hydrogels. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

significant amount of swelling. For example, HAGG hydrogels show a rapid increase to $Q = 57 \pm 2$ % over 4 h of immersion, followed by a slower rate of increase (to 83 ± 3 %) in the following 28 days of immersion. Once the hydrogels reached their equilibria in approximate 28 days, the swelling ratio remained constant for the remainder of the immersion period (up to 168 days), see Table I. The relative amounts of swelling between the three types of hydrogels mirrors that observed for the mass loss profiles, that is, largest amount of swelling for HAGG, lowest for LAGG with the blend somewhere in between (Figure 4). LAGG gels are clear in appearance, whereas HAGG gels are cloudy as is the blended gel. According to the manufacturer, the commercial production method results in the generation of cellular debris, which causes LAGG and HAGG to have a cloudy appearance.[34] Both LAGG and HAGG can be clarified, but only LAGG is commercially available in a clarified form. However, HAGG's cloudiness could also be indicative of a phase separation.[35]

The higher swelling of HAGG (compared to LAGG) can be explained using the difference in the proportion of chains associated with the gel network. X-ray diffraction and computer modeling studies have demonstrated that the intrachain hydrogen bonds between the glyceryl groups provide stiffness to the polysaccharide chains whereas the interchain hydrogen bonds between carboxylate groups stabilize the double helix.[8,9,13] In HAGG, the presence of bulky acyl substituents in the interior of the double helix, changes the chain conformation (because of steric hindrance) resulting in HAGG having a lower proportion of chains associated with the gel network compared to LAGG.[9,13] As a result, the HAGG network will not be cross-linked to the same degree as LAGG network resulting in weaker mechanical characteristics (discussed below). Although there are many approaches for explaining the swelling of polyelectrolyte gels (see Ref. 36 for a recent review), it is reasonable to assume that swelling decreases with increasing degree of cross-linking. Hence, HAGG will exhibit a higher degree of swelling than LAGG.

Rheological Testing

The mechanical properties of the hydrogel samples were tested under shear stress using rheology, that is, the effect of the strain rate was tested by increasing the strain amplitude from 0.01% to 100%, while keeping the angular frequency at 5 Hz. All hydrogels exhibited amplitude sweeps with a clear plateau of storage (G') and loss (G'') moduli [Figure 5(a)]. This region is commonly referred to as the LVE region during which the polymer network undergoes reversible deformation.[37] The higher values of G' compared to G'' in this region indicate the presence of a well cross-linked polymer network. At higher strain values (beyond the plateau region), the storage/loss moduli become dependent on strain. Previous studies on Ca^{2+} cross-linked polysaccharide (alignate) hydrogels attributed this to separation of the ionic cross-linking sites.[38,39] As such the end of the LVE region corresponds to the maximum shear stress (τ_{max}) and shear strain (γ_{max}) the hydrogel can be subjected to (under shear conditions) before the gel network starts to break down. Figure 5 and Table II show that the as-prepared gels exhibit the expected mechanical characteristics, that is, LAGG is brittle (low γ_{max}), HAGG is ductile (high γ_{max}), and the blend lies somewhere in between. Similarly, the G', G'', and τ_{max} values are all largest for LAGG and smallest for HAGG.

During the swelling period, LAGG exhibited increases in storage modulus (from 184 ± 25 kPa to 211 ± 8 kPa), γ_{max} (from

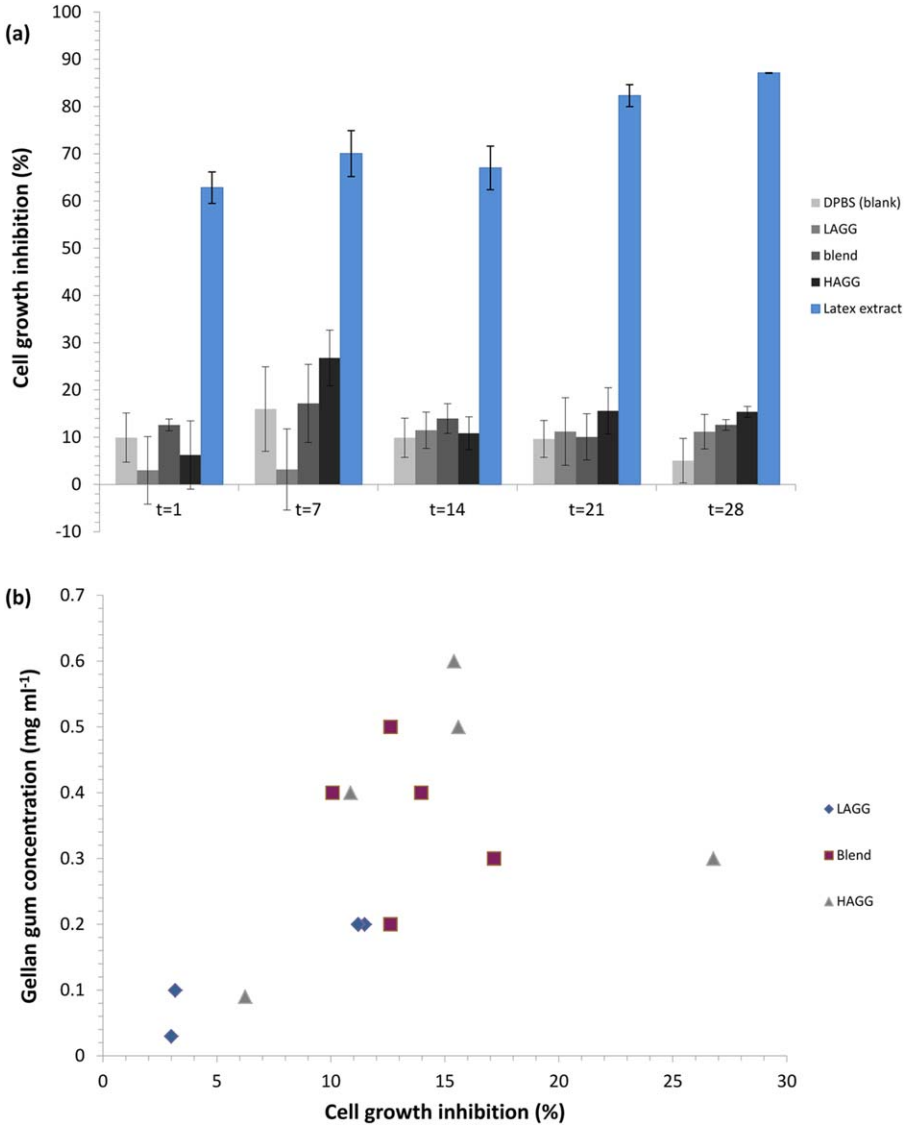

Figure 7. (a) CGI (%) of L929 fibroblasts in LAGG, HAGG, and blend extracts when incubated in DPBS at 37°C. Error bars represent one standard deviation calculated from three independent samples. Statistical analysis through a two-tailed Student's *t*-test showed that LAGG, HAGG, and blend data sets were statistically different (*$P < 0.001$). (b) GG concentration as a function of CGI for LAGG, HAGG, and blend extracts. GG concentration in DPBS extracts is calculated from mass loss data. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

$0.150 \pm 0.002\%$ to $0.240 \pm 0.04\%$), and τ_{max} (from 153 ± 19 Pa to 178 ± 39 Pa). The changes in these properties over the course of the immersion period can be attributed to the loss of polymers to the surrounding medium (polymer leaching) and exchange of cations with the surrounding medium. The observed increases in storage/loss moduli and maximum shear strain/stress may indicate that cross-link density in the swollen gels has been optimized (i.e., increased) compared to the as-prepared gels. In contrast, HAGG is insensitive to changes in the ionic environment, with the observed reductions in τ_{max} (from 451 ± 42 Pa to 15.1 ± 1 Pa) and γ_{max} (from 58.5 ± 13.4 % to 13.5 ± 0.3 %) mainly because of polymer leaching to the surrounding medium.

An assessment of the frequency dependent behavior during immersion [Figure 5(c and d); Table II] showed a linear correlation (up to 100 Hz) for all three gel types, except for HAGG hydrogels at $t = 28$. The moduli of the latter gels became independent of frequency at 10 Hz.

Compression Testing

Hydrogel samples were tested for their mechanical properties under compression at 37°C. From the resulting compressive stress–strain plots [Figure 6(a and b)], the tangent modulus (E_t), stress-at-failure (σ_f), and strain-at-failure (ε_f) values were determined (Table II). After 28 days of degradation, all gels have become more elastic, but only LAGG hydrogels increase their ductility. For example, the strain-at-failure value for LAGG hydrogels increases from $29 \pm 1\%$ to $47 \pm 4\%$ over the course of 28 days of immersion. In contrast, the corresponding values for HAGG hydrogels are unchanged, that is, $68 \pm 6\%$ compared to $66 \pm 14\%$. LAGG, HAGG, and the blended gels exhibited a reduction in tangent modulus values during swelling of

approximately 50%, 85%, and 15%, respectively. The magnitude of the stress-at-failure increased for LAGG hydrogels (from 130 ± 5 to 164 ± 32 kPa), while those of HAGG and blended gels remained virtually the same. For example, the stress-at-failure values for HAGG gels prior to and after swelling are 50 ± 8 and 48 ± 10 kPa. The relative mechanical characteristics have been preserved during swelling [Figure 6(c)], that is, at the end of the mass loss period (28 days of immersion). LAGG hydrogels are stiffer but less ductile than HAGG hydrogels with the blend somewhere in between. The observed increases in strain and stress-at-failure value for LAGG supports the suggestion that the density of the cross-links in the gel immersed for 28 days has been optimized compared to the as-prepared gels.

L929 Cell Inhibition Assay

A L929 CGI assay was carried out for 28 days. CGI was calculated with respect to the negative control (EMEM media) and all the GG extracts were found to be noncytotoxic during the test period [Figure 7(a)]. For example, after 28 days of immersion ($t = 28$ days), the CGI values of the LAGG, HAGG, and blend hydrogels are $11.2 \pm 3.6\%$, $15.4 \pm 2.5\%$, and $12.6 \pm 1.1\%$, respectively. The toxicity of the positive controls on L929 cell growth was clear, given the severe increase in CGI (85% for latex extract) compared to the negative control (<10%).

The CGI of hydrogels was found to be proportional to mass loss, that is, CGI values increased with increasing GG concentration (c_{GG}) in the degradation medium [Figure 7(b)]. The data at $t = 7$ days for HAGG (CGI $= 27 \pm 6\%$, $c_{GG} = 0.30 \pm 0.08$ mg mL^{-1}) and blended hydrogel (CGI $= 17 \pm 8\%$, $c_{GG} = 0.30 \pm 0.16$ mg mL^{-1}) does not appear to fit with the overall trend. However, the overall trend clearly shows that the gels with the highest mass loss (HAGG at $t = 28$ days, $c_{GG} = 0.60 \pm 0.18$ mg mL^{-1}) exhibit the highest CGI value. Whereas, the CGI value is the lowest for the gel with the smallest mass loss (LAGG at $t = 1$ day, $c_{GG} = 0.030 \pm 0.003$ mg mL^{-1}). In others, CGI increases with amount of GG released. This could suggest that small amount of released GG do not have any adverse effect on the cells, but larger amount may influence their behavior. Therefore, it is important for future application of these materials concerning cell interaction (e.g., tissue engineering) to control the amount of GG that is released.

The data shown in Figure 7(b) can also be used to explain the relative differences in CGI between the gels at a particular immersion time. For example, at $t = 28$ days c_{GG} values of LAGG (0.20 ± 0.04 mg mL^{-1}), HAGG (0.60 ± 0.18 mg mL^{-1}), and the blend (0.50 ± 0.07 mg mL^{-1}) are consistent with the corresponding CGI values $11.2 \pm 3.6\%$, $15.4 \pm 2.5\%$, and $12.6 \pm 1.1\%$, respectively.

CONCLUSION

We have established the mechanical characteristics of three types of swollen hydrogels consisting of LAGG, HAGG, and a LAGG–HAGG blend. It was found that these gels exhibited mass loss for 28 days and then remained stable for the remaining period of the study (an additional 140 days). LAGG showed a lower mass loss ($5.3 \pm 0.7\%$) compared to that of HAGG ($12.1 \pm 0.6\%$). The mass loss was attributed to polymer leaching (chain release), which was partially offset by the influx of ion because of cation exchange with the surrounding medium. Rheological and compression testing during the rapid mass loss period (up to 28 days) showed that the LAGG, HAGG, and blend hydrogels retained their mechanical characteristics relative to each other, that is, LAGG gels are stiffer and less ductile than HAGG and the blend gels. The leachates of all three hydrogels were found to be noncytotoxic during the testing period, CGI values were $11.2 \pm 3.6\%$ (LAGG), $15.4 \pm 2.5\%$ (HAGG), and $12.6 \pm 1.1\%$ (blend). This article contributes to understanding of mechanical properties and cytotoxity of fully swollen GG hydrogels, which is an important step towards the future application of GG hydrogels in tissue engineering.

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REFERENCES

1. Hoffman, A. S., Ratner, B. D. *ACS Symp. Ser.* **1976**, *31*, 1.
2. Hoffman, A. S. *Adv. Drug Deliv. Rev.* **2002**, *54*, 3.
3. Lim, D.; Wichterle, O. *Nature* **1960**, *185*, 117.
4. Brandon, V.; Slaughter, S. S. K.; Shahana, S. K.; Omar, Z. F.; Ali, K.; Peppas, N. A. *Adv. Mater.* **2009**, *21*, 3307.
5. Kuo, M. S.; Mort, A. J.; Dell, A. *Carbohyd. Res.* **1986**, *156*, 173.
6. Huang, Y.; Tang, J.; Swanson, B. G.; Rasco, B. A. *Carbohyd. Polym.* **2003**, *54*, 517.
7. Mao, R.; Tang, J.; Swanson, B. G. *Carbohyd. Polym.* **2000**, *41*, 331.
8. Chandrasekaran, R.; Puigjaner, L. C.; Joyce, K. L.; Arnott, S. *Carbohyd. Res.* **1988**, *181*, 23.
9. Chandrasekaran, R.; Thailambalal, V. G. *Carbohyd. Polym.* **1990**, *12*, 431.
10. Noda, S.; Funami, T.; Nakauma, M.; Asai, I.; Takahashi, R.; Al-Assaf, S.; Ikeda, S.; Nishinari, K.; Phillips, G. O. *Food Hydrocolloid.* **2008**, *22*, 1148.
11. Morris, E. R.; Nishinari, K.; Rinaudo, M. *Food Hydrocolloid.* **2012**, *28*, 373.
12. Milas, M.; Rinaudo, M. *Carbohyd. Polym.* **1996**, *30*, 177.
13. Chandrasekaran, R.; Radha, A.; Thailambalal, V. G. *Carbohyd. Res.* **1992**, *224*, 1.
14. Upstill, C.; Atkins, E. D. T.; Attwool, P. T. *Int. J. Biol. Macromol.* **1986**, *8*, 275.
15. Chapman, H. D.; Chilvers, G. R.; Miles, M. J.; Morris, V. J. In Gums and Stabilizers for the Food Industry 4; Philips, G. O., Wedlock, D. J., Williams, P. A. Eds.; IRL Press: Oxford, **1987**; p. 146.

16. Giavasis, I.; Harvey, L. M.; McNeil, B. *Crit. Rev. Biotechnol.* **2000**, *20*, 177.
17. Ferris, C. J.; in het Panhuis, M. *Soft Matter* **2009**, *5*, 3430.
18. Pereira, D. R.; Silva-Correia, J.; Caridade, S. G.; Oliveira, J. T.; Sousa, R. A.; Salgado, A. J.; Oliveira, J. M.; Mano, J. F.; Sousa, N.; Reis, R. L. *Tissue Eng. Part C* **2011**, *17*, 961.
19. Silva-Correia, J.; Oliveira, J.; Caridade, M. S. G.; Oliveira, J. T.; Sousa, R. A.; Mano, J. F.; Reis, R. L. *J. Tissue Eng. Regen. Med.* **2011**, *5*, 97.
20. Oliveira, J. T.; Martins, L.; Picciochi, R.; Malafaya, P. B.; Sousa, R. A.; Neves, N. M.; Mano, J. F.; Reis, R. L. *J. Biomed. Mater. Res. Part A* **2010**, *93* A, 852.
21. Smith, A. M.; Shelton, R. M.; Perrie, Y.; Harris, J. J. *J. Biomater. Appl.* **2007**, *22*, 241.
22. Coutinho, D. F.; Sant, S. V.; Shin, H.; Oliveira, J. T.; Gomes, M. E.; Eves, N. M.; Khademhosseini, A.; Reis, R. L. *Biomaterials* **2010**, *31*, 7494.
23. Jansson, P. E.; Lindberg, B.; Sandford, P. A. *Carbohyd. Res.* **1983**, *124*, 135.
24. Ohtsuka, A.; Watanabe, T. *Carbohyd. Polym.* **1996**, *30*, 135.
25. Camelin, I.; Lacroix, C.; Paquin, C.; Prevost, H.; Cachon, R.; Divies, C. *Biotechnol. Prog.* **1993**, *9*, 291.
26. Lee, H.; Fisher, S.; Kallos, M. S.; Hunter, C. J. *J. Biomed. Mater. Res. Part B Appl. Biomater.* **2011**, *98* B, 238.
27. Taylor, D. L.; Ferris, C. J.; Maniego, A. R.; Castignolles, P.; Panhuis, M. i. h.; Gaborieau, M. *Aust. J. Chem.* **2012**, *65*, 1156.
28. Martens, P.; Holland, T.; Anseth, K. S. *Polymer*, **2002**, *43*, 6093.
29. Mawad, D.; Martens, P. J.; Odell, R. A.; Poole-Warren, L. A. *Biomaterials*, **2007**, *28*, 947.
30. Martens, P. J.; Bryant, S. J.; Anseth, K. S. *Biomacromolecules* **2003**, *4*, 283.
31. Hossain, K. S.; Nishinari, K. *Prog. Colloid. Polym. Sci.***2009**, *136*, 177.
32. Crank, J. The Mathematics of Diffusion; Oxford University Press: New York, **1980**.
33. Takahashi, R.; Tokunou, H.; Kubota, K.; Ogawa, E.; Oida, T.; Kawase, T.; Nishinari, K. *Biomacromolecules* **2004**, *5*, 516.
34. Vialli, R. C.; Miskiel, F. J. in Handbook of Dietary Fiber; Dreher, M. L.; Cho, S. S. Eds.; Marcel Dekker: New York, **2001**; p. 868.
35. Kapasis, S.; Giannouli, P.; Hember, M. W. N.; Evageliou, V.; Poulard, C.; Tort-Bourgeois, B.; Sworn, G.; *Carbohyd. Polym.* **1999**, *38*, 145.
36. Quesada-Perez, M.; Maroto-Centeno, J. A.; Forcada, J.; Hidalgo-Alvarez, R.; *Soft Matter.* **2011**, *7*, 10536.
37. Mezger, T. G. The Rheology Handbook; Vincenz: Hanover, **2011**.
38. Webber, R. E.; Shull, K. R. *Macromolecules* **2004**, *37*, 6153.
39. Kong, H. J.; Wong, E.; Mooney, D. J. *Macromolecules* **2003**, *36*, 4582.