Release of Fluorescent Markers from Phase-Separated Gelatin-Maltodextrin Hydrogels

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ABSTRACT: Genipin-crosslinked gelatin-maltodextrin phase-separated hydrogels consisting of gelatin-continuous or bicontinuous microstructures were developed to regulate swelling and release behavior of four fluorescent markers of varying molecular weights [(fluorescein (332 Da) and FITC-dextrans (FD) (4000–250,000 Da)]. Bicontinuous hydrogels showed significantly greater swelling than gelatin-continuous hydrogels under all conditions (at pH 1.5 and 7.4 and three genipin/gelatin crosslinking ratios) ($P < 0.05$). With both microstructures, fluorescein showed the largest release rate and total release followed by FD 4000 Da, FD 40,000 Da, and FD 250,000 Da ($P < 0.05$). Marker molecular weight, pH, and crosslink ratio all affected the rate and amount of release. The mode of transport for the solvent and all markers was Fickian or slightly anomalous, with diffusional exponent (n) values ranging from 0.35 to 0.64. These results demonstrated that with the proper combination of crosslink density, solvent pH, and microstructure, hydrogels with a specified swelling behavior may be developed. This, coupled with a marker of appropriate size, can lead to controllable levels and rates of release. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 121: 2662–2673, 2011

Key words: hydrogel; phase separation; gelatin; maltodextrin; genipin; controlled release

INTRODUCTION

Controlled release matrices are defined as devices that deliver an entrapped therapeutic agent to a desired body location and/or provide its timely release. The controlled delivery of compounds such as drugs and nutraceuticals from natural or synthetic hydrogels occurs when triggered by external stimuli including changes in pH, ionic strength, or temperature. Such release depends on numerous factors including the matrix design and target application.[1–4]

Composition and microstructure can be used to regulate hydrogel swelling as well as the diffusion of incorporated bioactive compounds, offering the possibility of improved therapeutic efficacy by controlling the rate at which entrapped compounds enter the bloodstream.[5–7] A novel means of generating hydrogel microstructures is via the phase separation of thermodynamically incompatible binary biopolymer mixtures (e.g., some proteins and polysaccharides). When the environmental conditions of such a mixture are altered (e.g., temperature quench, shear), an initially homogeneous blend is brought into a metastable or unstable state leading to phase separation. This instability results in a continuous phase rich in one component and a dispersed phase rich in the other.[8–13]

Phase separation in biopolymers occurs via one of two mechanisms: nucleation and growth (NG) or spinodal decomposition (SD). With NG, the resulting structure consists of randomly distributed spherical droplets varying in size, with a fixed chemical composition. With SD, two types of structures can be obtained: interconnected (bicontinuous) or droplet type.[14–18] In the droplet-type morphology, droplets are distributed randomly in a continuous matrix and are fairly uniform in size and shape. Under a narrow range of conditions, however, a bicontinuous network can be obtained, where both components are continuous. Microstructure and phase distribution depend on several factors including the biopolymer type, concentration and molecular weight (MW) distribution, solvent type, pH, ionic strength, thermal history and treatment, pressure as well as temperature.[13,19] In this context, numerous researchers have investigated the microstructure of gelatin-maltodextrin hydrogels, noting the impact of cooling rate, holding time, holding temperature and gelatin type on final phase-separated morphology.[8–10]

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Control over phase separation, and thus microstructure, and its influence on therapeutic compound release has shown promising results. Shen et al.[20] showed that tailored microstructures generated via microphase separation could be used in controlling the release profiles of *p*-nitroaniline and disperse yellow 3 from bioerodible polyanhydride systems, with release driven by gel microstructure, drug-polymer phase partitioning and drug solubility. Using gelatin and glycidyl methacrylated (GMA) dextran hydrogel, Aso et al.[4] found that release of β-galactosidase and bovine serum albumin was affected by the degree of GMA substitution and gelatin concentration. Zhou et al.[21] found that a matrix based on microcrystalline waxes and starch derivatives provided a flexible drug delivery system, whereby drug release depended on the type and the concentration of the hydrophobic and hydrophilic components. Finally, Kurisawa and Yui[22,23] explored gelatin/dextran interpenetrating networks as dual stimuli-responsive biodegradable hydrogels for controlled release applications.

Gelatin is a well-known biocompatible biopolymer that exhibits a sol-gel transition in response to temperature changes.[4] Its uses in the biomedical field include hard and soft capsules, sealants for vascular prostheses, wound dressing and adsorbent pads for surgical use, and tissue regeneration.[24] Gelatin-based biomedical materials must be crosslinked as gelatin is water-soluble. Genipin is a promising naturally occurring crosslinking agent obtained from gardenia fruits *Gardenia jasminoides Ellis* (Asia) or *Genipa americana* (South America).[24] It has been used in herbal medicine, and the dark blue pigments obtained by its spontaneous reaction with amino acids have been used in the fabrication of food dyes. Studies carried out on porcine pericardia crosslinked with genipin have indicated that it can form stable crosslinked products, with significantly lower cytotoxicity than glutaraldehyde, formaldehyde, or epoxy compounds.[25–30] As with other crosslinkers, crosslinking gelatin with genipin will alter the structural integrity, insolubility, and elasticity of a gelatin-based hydrogel, and as a result, its swelling and diffusive properties.

Solvent and solute diffusion in a hydrogel is complex with diffusivities lying between those of liquids and solids. Alfrey et al.[31] proposed a classification relating solvent diffusion rate to polymer relaxation rate, namely Fickian and non-Fickian (Case II and anomalous) diffusion:

$$\frac{M_t}{M_\infty} = kt^n \tag{1}$$

where M_t/M_∞ is the fractional solute release or solvent uptake normalized to equilibrium conditions, k is the kinetic constant related to the diffusion coefficient with units t^{-n}, and n is the diffusional exponent describing the mode of transport. This model has been popular given its ease of use and utility in describing the importance of Fickian ($n = 0.5$) and Case II ($n = 1.0$) transport.[32,33] With values of n between 0.5 and 1.0, diffusion is termed anomalous[34] whereas with $n < 0.5$, the approach to final equilibrium is slower.[35]

Peppas and Sahlin[36] developed a heuristic model based on the equation of Alfrey et al.[31] for solvent transport through a gel where the two phenomena that control release from a polymer matrix are additive:

$$\frac{M_t}{M_\infty} = k_d t^m + k_r t^{2m} \tag{2}$$

where k_d represents the Fickian contribution and k_r constitutes the Case II or relaxational contribution to release. The exponent m is the Fickian exponent for any system that displays controlled release. The value of m varies with the aspect ratio of the controlled release device ($2a/l$), where $2a$ is diameter and l is height.

During swelling, a compatible solvent will penetrate the hydrogel, causing it to swell. To accommodate this stress, the biopolymer chains "stretch," expanding the mesh size and thus hydrogel volume. Swelling behavior, which strongly depends on the type and extent of crosslinking applied to a hydrogel, can be explained by the Flory-Rehner theory.[37,38] A modified version of this theory was proposed by Peppas and Merrill[39] for hydrogels prepared in water. Water alters the change in chemical potential due to elastic forces and must be accounted for in the expression. In addition, the chemical potential strongly depends on the local ionic environment. The following equation was derived for the swelling of an anionic hydrogel prepared in the presence of a solvent[40]:

$$\begin{aligned}\frac{V_1}{4I}\left(\frac{v_{2,s}}{\bar{v}}\right)^2\left(\frac{K_a}{10^{-\mathrm{pH}}-K_a}\right)^2 &= \left[\ln(1-v_{2,s})+v_{2,s}+\chi_1 v_{2,s}^2\right] \\ &+ v_{2,r}\left(\frac{V_1}{\bar{v}\bar{M}_c}\right)\left(1-\frac{2\bar{M}_c}{\bar{M}_n}\right)\left[\left(\frac{v_{2,s}}{v_{2,r}}\right)^{1/3}-\left(\frac{v_{2,s}}{2v_{2,r}}\right)\right]\end{aligned} \tag{3}$$

and for the swelling of a cationic hydrogel:

$$\begin{aligned}\frac{V_1}{4I}\left(\frac{v_{2,s}}{\bar{v}}\right)^2\left(\frac{K_b}{10^{\mathrm{pOH}}-K_a}\right)^2 &= \left[\ln(1-v_{2,s})+v_{2,s}+\chi_1 v_{2,s}^2\right] \\ &+ v_{2,r}\left(\frac{V_1}{\bar{v}\bar{M}_c}\right)\left(1-\frac{2\bar{M}_c}{\bar{M}_n}\right)\left[\left(\frac{v_{2,s}}{v_{2,r}}\right)^{1/3}-\left(\frac{v_{2,s}}{2v_{2,r}}\right)\right]\end{aligned} \tag{4}$$

where $\overline{M}_n$ is the MW of the polymer without crosslinking, $\overline{M}_c$ is the number-average polymer MW between two adjacent crosslinks, $\overline{v}$ is the specific volume of the hydrogel prior to swelling, V_1 is the molar volume of the solvent water (18 mL mol^{-1}), $v_{2,s}$ is the

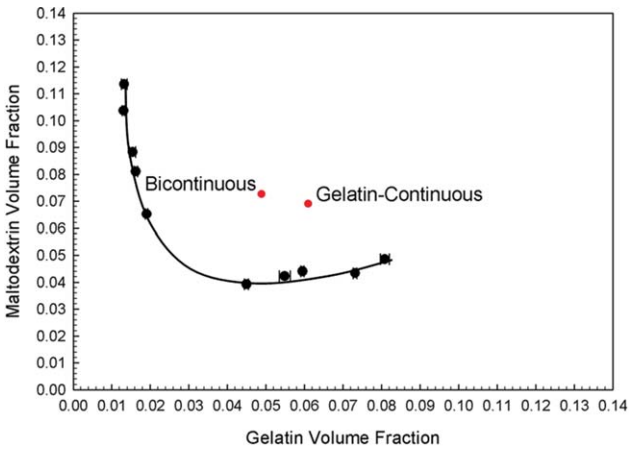

Figure 1 Experimentally determined gelatin-maltodextrin phase diagram, where points along the binodal represent phase compositions resulting from phase separation at 70°C.[46] The compositions of the gelatin-continuous and bicontinuous hydrogels used in this study are also shown. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

polymer volume fraction in the swollen state determined as roughly the inverse of the equilibrium swelling ratio, $v_{2,r}$ is the polymer volume fraction in the relaxed state (the state of the polymer immediately after crosslinking but before swelling), I is the ionic strength, K_a and K_b are the dissociation constants for the acidic and basic moieties on the polymer, and χ_1 is the Flory-Huggins parameter describing the polymer-solvent interaction.[6,41] Using $\overline{M}_c$, the crosslink density, q, can be determined from[42]:

$$q = \frac{\bar{M}_n}{\bar{M}_c} \tag{5}$$

The parameter $v_{2,s}$ is determined from the volume-swelling ratio, q_v[43]:

$$\nu_{2,s} = \frac{1}{q_v} \tag{6}$$

The volume-swelling ratio is calculated as[43]:

$$q_v = 1 + \frac{(q_w - 1) \times \rho_2}{\rho_1} \tag{7}$$

where ρ_2 and ρ_1 are the densities of the polymer network and solvent, respectively. The weight-swelling ratio, q_w, is determined from[43]:

$$q_w = \frac{m_s}{m_o} \tag{8}$$

where m_o and m_s are the mass of the unswollen gel and the mass of the swollen gel at equilibrium, respectively.

In this research, the composition and microstructure of chemically crosslinked phase-separated gelatin-maltodextrin hydrogels were used to control the diffusion of four fluorescent markers of varying MWs: fluorescein (MW 332 Da), and three FITC (fluorescein isothiocyanate)-dextran markers with MWs of 4000 Da, 40,000 Da, and 250,000 Da. Two hydrogel microstructures were developed: gelatin-continuous networks where the dispersed phase consisted of maltodextrin inclusions, and biocontinuous networks where both gelatin and maltodextrin were continuous. The systems were swelled in pH 7.4 and 1.5 buffers and the hydrogels were crosslinked at three genipin/gelatin (w/w) ratios (0.025, 0.0125, and 0.00625).

MATERIALS AND METHODS

Hydrogel preparation

A gelatin (Type A, Bloom 300, Sigma–Aldrich Co., Oakville, ON, Canada) stock solution was prepared by dispersing the gelatin powder in distilled deionized water (DDW) at 50°C for 30 min with continual stirring. Maltodextrin (Star-dri, DE 10, Staley Manufacturing Co., Decatur, IL) was dissolved at room temperature under continuous stirring, heated to 98°C for 30 min, and then cooled to 50°C. Evaporative losses were corrected for with the addition of DDW at 50°C. A 200 m*M* genipin stock solution (Challenge Bioproducts Co., PRC) was prepared by dissolving the genipin powder in 60% (v/v) ethanol.[44] Stock solutions (1 mg mL^{-1}) of FITC-dextran markers of varying MWs (4000, 40,000, and 250,000 Da) (Sigma–Aldrich Co., Oakville, ON, Canada) were prepared by dissolving the fluorophores in DDW. A fluorescein (Sigma–Aldrich Co., Oakville, ON, Canada) stock solution [0.08% (w/w)] was made in a similar fashion. All marker stock solutions were stored in the dark at 5°C prior to use. To prepare the hydrogels, the

TABLE I
Parameters Used for the Determination of the Molecular Weight between Polymer Crosslinks and Crosslink Density

Parameter	Value
$\overline{M}_n$ (polymer average MW)	75,000 g mol^{-1} [45]
$\overline{v}$ (hydrogel specific volume)	0.910 cm^3 g^{-1}
V_1 (molar volume of water)	18 mL mol^{-1}
$v_{2,s}$ (polymer volume fraction postswelling)	determined experimentally
$v_{2,r}$ (polymer volume fraction preswelling)	2.121 cm^3
I (ionic strength)	2.20 × 10^{-5} mol cm^{-3}
K_a (gelatin acid dissociation constant)	3.981 × 10^{-4} [45]
K_b (gelatin base dissociation constant)	2.512 × 10^{-7} [45]
ρ_2 (density of hydrogel)	1.098 g cm^{-3}
ρ_1 (density of water)	0.998 g cm^{-3}
χ_1 (Flory-Huggins parameter)	0.49518[41]

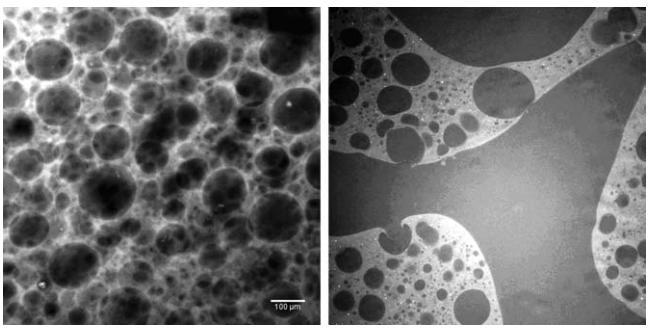

Figure 2 CLSM micrographs of gelatin-continuous (left) and bicontinuous (right) hydrogels. The brighter regions are gelatin and the darker regions are maltodextrin. The scale bar denotes 100 μm.

biopolymers, markers, and genipin were mixed at 80°C and adjusted to pH 7 using either NaOH or HCl. Hot mixtures were poured into cylindrical plastic molds (0.3 cm height, 3.0 cm diameter) prelubricated with a thin layer of cooking spray, and then allowed to set for 24 h at room temperature. Gelatin-continuous hydrogels contained 6.1% (w/w) gelatin and 6.9% (w/w) maltodextrin whereas bicontinuous hydrogels contained 4.9% (w/w) gelatin and 7.3% (w/w) maltodextrin. The phase diagram used to establish these compositions was based on our earlier work (Fig. 1).[45] All hydrogels were crosslinked at genipin/gelatin ratios of 0.025, 0.0125, and 0.00625 (w/w). Hydrogels were loaded with 0.1 mg mL^{-1} of the FITC-dextran fluorophores and 0.008% (w/w) fluorescein. All gels were prepared in triplicate. These genipin/gelatin ratios were chosen based on initial studies that showed negligible hydrogel swelling at a genipin/gelatin ratio of 0.025.

Release experiments

Release experiments were carried out in triplicate in plastic cups containing 15 mL of buffer at pH 7.4 or 1.5 at 25°C for 5 h, which allowed sufficient time for the readings to tend towards a plateau. Throughout the experiment, the cups were stirred before sampling to ensure homogeneity of the buffer solution. Prior to immersion, gel cylinders were gently blotted to remove excess moisture and weighed to determine their initial weight. During the diffusion experiment, 5 mL of buffer were withdrawn from each cup at set intervals and replaced with fresh buffer. A spectrofluorometer (LS50B luminescence spectrometer, Perkin–Elmer Inc., Waltham, MA) was used for fluorophore detection at an excitation wavelength of 490 nm for all markers. Emission scans were taken between 500 and 530 nm to determine the fluorescence at maximum peak intensity. The spectrofluorometer was calibrated using standard solutions of the four fluorophores at pH 7.4. To maintain the fluorescence intensity of the fluorophores, release samples collected at pH 1.5 were first adjusted to pH 7.4 before fluorescence measurements were taken. Potential hydrolysis of the gelatin or maltodextrin hydrolysis at low pH was not evaluated, as this would normally

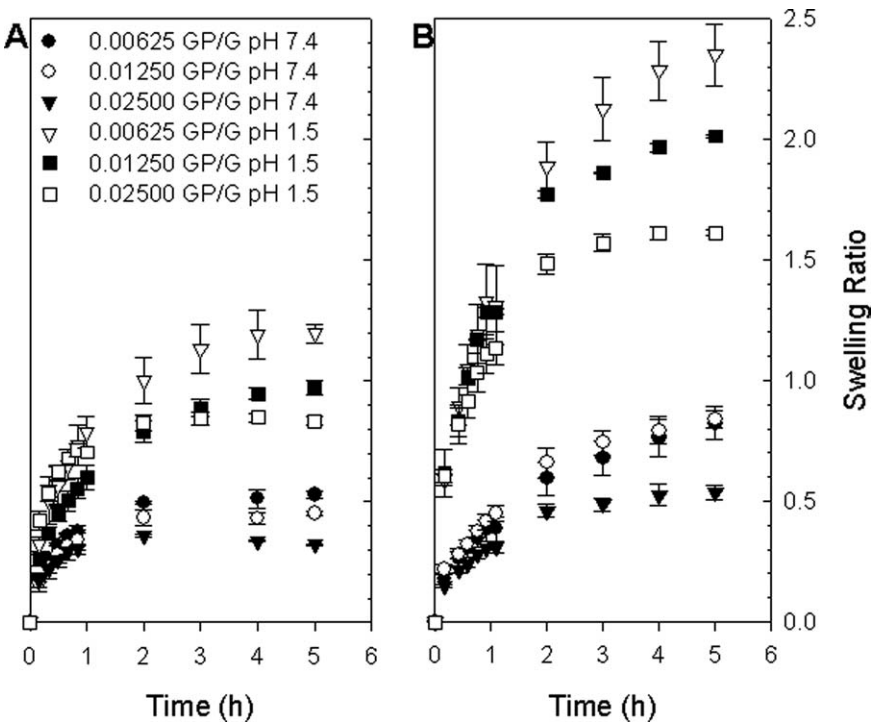

Figure 3 Swelling ratios of gelatin-continuous (A) and bicontinuous hydrogels (B) at pH 7.4 and 1.5 crosslinked at three genipin/gelatin (GP/G) ratios. Values are shown as arithmetic means ± their standard deviations (SD) ($n = 3$).

TABLE II
Swelling Ratios (SR) After 5 h and Diffusional Exponent *n* at pH 7.4 and 1.5 for Gelatin-Continuous Hydrogels Crosslinked at Three Genipin/Gelatin Ratios

Genipin/ Gelatin (GP/G)	SR ± SD		Diffusional exponent (*n*) ± CI	
	pH 1.5	pH 7.4	pH 1.5	pH 7.4
0.00625	1.217 ± 0.075	0.528 ± 0.018	0.509 ± 0.014	0.474 ± 0.110
0.01250	0.986 ± 0.024	0.451 ± 0.009	0.474 ± 0.051	0.484 ± 0.068
0.02500	0.847 ± 0.017	0.358 ± 0.005	0.351 ± 0.042	0.347±0.036

Values for SR are shown as arithmetic means ± their standard deviations (SD) (n =3). Confidence intervals (CI) are shown for values of n.

occur in the presence of strong acids and at temperatures well above room temperature.

Swelling ratio

Cylindrical samples of gelatin-maltodextrin were initially weighed (W_1) then immersed in 15 mL of phosphate buffered saline (PBS) (0.022*M*) at pH 7.4 or 1.5 at ambient temperature for 5 h. Samples were blotted dry and weighed (W_2) after each time interval. The swelling ratio (SR) was determined as described by[44]:

$$\mathrm{SR} = \frac{(W_2 - W_1)}{W_1} \tag{9}$$

All measurements were performed in triplicate.

Crosslinking characteristics

The parameters used to establish the MW between crosslinks and the crosslink density for the hydrogels are shown in Table I. These parameters, determined from the swelling ratios, were utilized in eqs. (4)–(8) to determine $\overline{M}_c$ and q values.

Microscopy

The microstructure of the gelatin-continuous and bicontinuous hydrogels was ascertained using confocal laser scanning microscopy (CLSM), with fluorescein (Sigma-Aldrich, Oakville, ON, Canada) added to the gels at 0.008% (w/w). After having set for 24 h, thin gels (h = 1 mm) were placed on glass slides and covered with cover slips. The microscope used was a Zeiss Axioplan-2 with a 10× objective and a LSM 510 confocal module (Zeiss Instruments, Toronto, ON, Canada). Excitation of the dye was achieved at 488 nm using an argon laser. Emitted light was detected using a KP 540 filter. Three samples were prepared for each gel type analyzed, with representative images shown.

Statistical analyses

Swelling and release results were analyzed using Systat v.15 (SPSS Inc, Chicago, IL). Oakdale Engineering software (Datafit, Ver. 8.2, Oakdale, PA, USA) was used for fitting data to eqs. (1) and (2) to determine the exponential factor n, release constant k, diffusional release rate k_d, and relaxational release rate k_r. The Levenberg-Marquardt method with double precision was used to perform nonlinear regression. The goodness of fit was determined by calculating adjusted coefficients of multiple determinations (Ra^2) and the 95% confidence interval (CI) was used to describe the reliability of the estimate of the factor n. Distributions of n, k, k_d, k_r and total release after 5 h were tested for deviations from normality using the distribution kurtosis and Shapiro-Wilks W test, and homoscedasticity was tested using Levene's test of homogeneity of variance. Variables with distributions that significantly deviated from normality and were heteroscedastic were analyzed using nonparametric tests. Systat software was used

TABLE III
Swelling Ratios (SR) and Diffusional Exponent *n* at pH 7.4 and 1.5 for Bicontinuous Hydrogels Crosslinked at Three Genipin/Gelatin Ratios

Genipin/ Gelatin (GP/G)	SR ± SD		Diffusional exponent (*n*) ± CI	
	pH 1.5	pH 7.4	pH 1.5	pH 7.4
0.00625	2.349 ± 0.130	0.822 ± 0.069	0.471 ± 0.021	0.481 ± 0.069
0.01250	2.012 ± 0.005	0.838 ± 0.034	0.449 ± 0.052	0.490 ± 0.001
0.02500	1.612 ± 0.013	0.537 ± 0.031	0.357 ± 0.008	0.411 ± 0.030

Values are shown as arithmetic means ± their standard deviations (SD) (n =3). Confidence intervals (CI) are shown for values of n.

TABLE IV
Initial Swelling Rates (k) of Gelatin-Continuous (6.1% G and 6.9% MD) and Bicontinuous (4.9% G and 7.3% MD) Genipin-Crosslinked Hydrogels

Genipin/ Gelatin	Gelatin-continuous k (h^{-n}) ± SD		Bicontinuous k (h^{-n}) ± SD	
	pH 1.5	pH 7.4	pH 1.5	pH 7.4
0.00625	0.100 ±0.005	0.063 ± 0.024	0.201 ± 0.007	0.070 ± 0.010
0.0125	0.090 ± 0.011	0.056 ± 0.003	0.234 ± 0.017	0.055 ± 0.015
0.025	0.187 ± 0.060	0.068 ± 0.018	0.260 ± 0.016	0.056 ± 0.007

Values are shown as arithmetic means ± their standard deviations (SD) ($n = 3$).

for nonparametric analyses (median and Jonckeere-Terpstra) to assess statistically significant differences between medians. Statistical analyses were deemed significant at $P < 0.05$.

RESULTS AND DISCUSSION

Microstructure

Preliminary experiments with maltodextrin-only and maltodextrin-continuous hydrogels showed that these were not rigid as they collapsed under their own weight. Conversely, gelatin-only, gelatin-continuous and gelatin-maltodextrin bicontinuous hydrogels were self-supporting, with the latter two studied for swelling and release characteristics. These hydrogels did not exhibit visible maltodextrin exudation during the experimental timeframe. The gelatin-continuous hydrogel showed discrete inclusions of maltodextrin ranging in diameter from ~ 10 μm to over 100 μm whereas interspersed channels of both gelatin and maltodextrin dominated the bicontinuous microstructure, with a limited number of maltodextrin inclusions also present (Fig. 2). These images also suggested the possibility of secondary phase separation, perhaps as a result of kinetic trapping, where gelling led to the development of inclusions within inclusions. Previous efforts have shown that there remains ~ 1–2% gelatin solubilized within maltodextrin under these experimental conditions.[46] The variety in dispersed domain size suggested that NG was the mechanism by which the gelatin-continuous phase-separated network formed whereas SD was the mechanism that led to the formation of the bicontinuous network. As discussed later, these differences in hydrogel morphology played a role in swelling behavior and thus the release kinetics of the fluorescent markers from the two systems.

Swelling behavior

Swelling of the gelatin-continuous hydrogels varied with pH and level of crosslinking [Fig. 3(A)]. The maximum swelling, taken after 5 h, increased with decreasing genipin/gelatin ratios. Hydrogels immersed in pH 1.5 buffer showed significantly greater swelling ratios than at pH 7.4 ($P < 0.05$) (Table II). Hydrogels based on biopolymers with pH-sensitive ionizable groups swell to a greater extent in environments that cause the ionization of the biopolymer chains.[47,48] As gelatin is ionizable, with a $pK_a = 3.4$ and $pK_b = 6.6$,[45] a pH of 1.5 caused the biopolymer chains to repel, increasing mesh size and thus solvent uptake. Decreasing the crosslinking ratio increased the swelling ratio at both pHs ($P < 0.05$). The values of n (Table II) showed that solvent diffusion was Fickian at genipin/gelatin ratios of 0.00625 and 0.0125, implying that polymer relaxation was faster than solvent diffusion. At a genipin/gelatin ratio of 0.025, pseudo-Fickian diffusion was observed, with $n \sim 0.35$ at both pHs.[35] This higher crosslinking ratio thus slowed solvent ingress and swelling.

The swelling profile of the crosslinked phase-separated bicontinuous hydrogels showed a similar trend to the gelatin-continuous systems [Fig. 3(B)]. Swelling ratios after 5 h (Table III) differed significantly

TABLE V
Flory-Rehner Crosslink Density q and the Number-Average Molecular Weight between Crosslinks $\overline{M}_c$ According to the Equilibrium Swelling Ratios of Gelatin-Continuous and Bicontinuous Genipin-Crosslinked Hydrogels

Genipin/ Gelatin	q		$\overline{M}_c$ (g/mole)	
	Gelatin-continuous ± SD	Bicontinuous ± SD	Gelatin-continuous ± SD	Bicontinuous ± SD
0.00625	596 ± 24	284 ± 31	126 ± 5	266 ± 29
0.0125	742 ± 20	318 ± 48	101 ± 3	239 ± 34
0.025	997 ± 17	584 ± 50	75 ± 1	129 ± 10

Values are shown as arithmetic means ± their standard deviations (SD) (n =3).

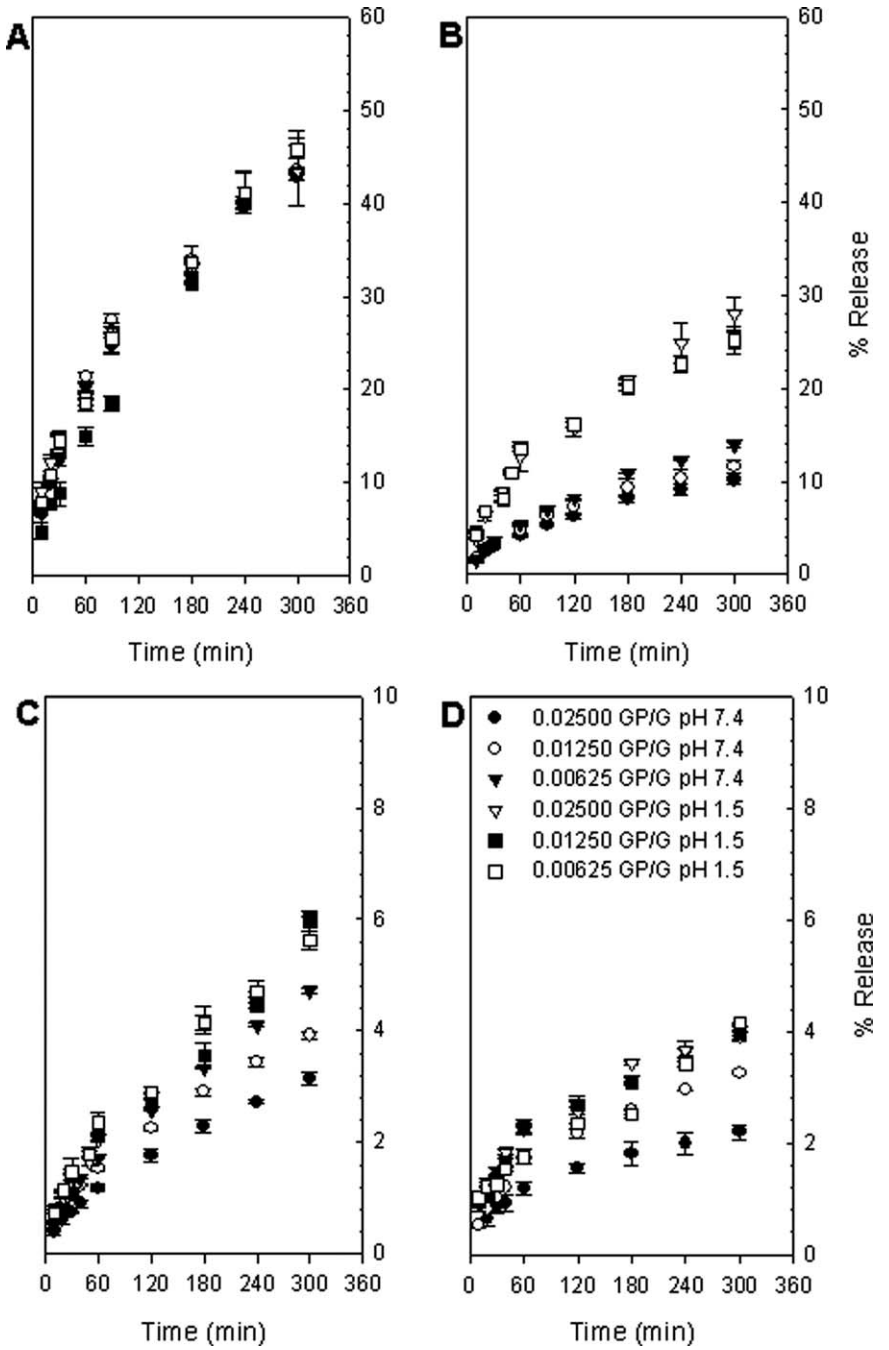

Figure 4 Release (%) of fluorescein (332 Da) (A), FD 4000 Da (B), FD 40,000 Da (C), and FD 250,000 Da (D) at pH 7.4 and 1.5 from gelatin-continuous hydrogels crosslinked at three genipin/gelatin ratios. Note the different *y*-axis scales. Values are shown as arithmetic means ± their standard deviations (SD) ($n = 3$).

with pH ($P < 0.05$), although the level of crosslinking did not show an overall significant impact ($P > 0.05$). However, at pH 1.5, a decrease in the crosslinking ratio significantly increased the swelling ratio ($P < 0.05$). Compared with the gelatin-continuous systems, bicontinuous hydrogels showed greater swelling under all conditions ($P < 0.001$). Gabrielii and Gatenholm[49] showed that an increase in the content of chitosan (a glucose polymer) increased the swelling ratio of phase-separated xylan hydrogels. It is likely that maltodextrin's compatibility with water and the overall increase in polymer content led to an increase in water uptake. As maltodextrin gels can imbibe up to 9 g water/g dry mass,[50] its presence effectively augmented the water-holding capacity of the hydrogel compared to its gelatin-continuous counterparts.[51–53] As with the gelatin-continuous hydrogels, solvent diffusion was Fickian at genipin/gelatin ratios of 0.00625 and 0.0125 and pseudo-Fickian at a genipin/gelatin ratio of

TABLE VI
Release Constant k, Diffusional Exponent n, Rate of Diffusional Release k_d, Rate of Relaxational Release k_r, and Total Release of Various Fluorescent Markers at pH 7.4 or 1.5 from Gelatin-Continuous Hydrogels Crosslinked at Three Genipin/Gelatin Ratios (all $Ra^2 > 0.99$)

Fluorescent Marker	GP/G	pH	$k(h^{-n})$	$n \pm$ CI	$k_d(h^{-0.48})$	$k_r(h^{-0.96})$	% release after 5 h ± SD
Fluorescein	0.00625	7.4	2.042	0.537 ± 0.029	2.298	0.034	43.20 ± 0.07
	0.0125	7.4	2.238	0.520 ± 0.018	2.439	0.025	43.56 ± 0.10
	0.025	7.4	1.844	0.559 ± 0.018	2.080	0.055	42.84 ± 0.24
	0.00625	1.5	2.463	0.493 ± 0.049	2.501	0.014	45.75 ± 2.07
	0.0125	1.5	2.341	0.493 ± 0.096	2.410	0.028	45.55 ± 0.69
	0.025	1.5	3.027	0.472 ± 0.013	2.990	−0.008	43.47 ± 3.67
FD 4000 Da	0.00625	7.4	0.407	0.634 ± 0.015	0.540	0.030	13.96 ± 0.22
	0.0125	7.4	0.497	0.555 ± 0.009	0.567	0.013	11.53 ± 0.65
	0.025	7.4	0.471	0.539 ± 0.060	0.515	0.012	10.08 ± 0.41
	0.00625	1.5	1.600	0.484 ± 0.024	1.613	0.002	25.15 ± 1.50
	0.0125	1.5	1.607	0.483 ± 0.004	1.617	0.002	24.87 ± 1.10
	0.025	1.5	1.045	0.576 ± 0.016	1.233	0.042	28.05 ± 1.81
FD 40,000 Da	0.00625	7.4	0.143	0.606 ± 0.008	0.182	0.008	4.72 ± 0.06
	0.0125	7.4	0.148	0.572 ± 0.006	0.172	0.001	3.91 ± 0.08
	0.025	7.4	0.093	0.616 ± 0.008	0.120	0.006	3.14 ± 0.13
	0.00625	1.5	0.169	0.641 ± 0.059	0.176	0.022	5.63 ± 0.16
	0.0125	1.5	0.220	0.546 ± 0.030	0.244	0.006	5.96 ± 0.18
	0.025	1.5	0.179	0.617 ± 0.048	0.215	0.011	6.05 ± 0.00
FD 250,000 Da	0.00625	7.4	0.357	0.421 ± 0.019	0.313	-4×10^{-5}	4.03 ± 0.10
	0.0125	7.4	0.241	0.457 ± 0.020	0.225	-9×10^{-4}	3.26 ± 0.03
	0.025	7.4	0.209	0.415 ± 0.015	0.183	−0.003	2.19 ± 0.13
	0.00625	1.5	0.406	0.368 ± 0.008	0.328	−0.009	4.16 ± 0.01
	0.0125	1.5	0.297	0.449 ± 0.006	0.277	−0.002	3.98 ± 0.11
	0.025	1.5	0.296	0.456 ± 0.030	0.279	−0.001	3.93 ± 0.09

Values are shown as arithmetic means ± their standard deviations (SD) ($n = 3$). Confidence intervals (CI) are shown for values of n.

0.025.The initial rates of swelling (k) for the gelatin-continuous and bicontinuous networks (Table IV) were not significantly different at pH 7.4 ($P > 0.05$) whereas at pH 1.5, the rate of swelling for the latter was greater ($P < 0.001$). At pH 7.4, crosslinking retarded volume expansion by lowering the volume the solvent is able to occupy. However, at pH 1.5 polymer chain repulsion and loss of genipin crosslinks increased the swelling rate. A higher concentration of maltodextrin and the presence of maltodextrin channels in the bicontinuous hydrogels may have also complemented the swelling.

From the swelling data, there were significant differences ($P < 0.05$) in the calculated q and $\overline{M}_c$ values between the gelatin-continuous and bicontinuous hydrogels (Table V). As the rate of chemical crosslinking is typically slower than the rate of phase separation and gelation in protein-polysaccharide hydrogels,[52,53] gel microstructure will be essentially set prior to crosslinking completion. Since genipin does not crosslink, but is soluble in, maltodextrin, q and $\overline{M}_c$ should be the same at similar genipin/gelatin ratios for a maltodextrin content. Not surprisingly, an increase in the genipin/gelatin ratio resulted in a concomitant decrease in the MW between crosslinks $\overline{M}_c$ and an increase in crosslink density q. However, as maltodextrin has a greater capacity for water than gelatin,[54,55] an increase in its concentration shifted swelling equilibrium towards larger $\overline{M}_c$ values. This in turn resulted in a lower Flory-Rehner crosslink density for the bicontinuous systems versus their gelatin-continuous counterparts. Thus, use of the modified Flory-Rehner theory to determine crosslink density from the equilibrium swelling data of a phase-separated hydrogel where one of the polymers is not crosslinked is unrepresentative of the true extent of crosslinking, and only an apparent Flory-Rehner crosslink density is determined.

Release from gelatin-continuous hydrogels

Fluorescent marker release data (Fig. 4) were fitted to eqs. (1) and (2) to determine the rate and mechanism of release and the diffusion-related coefficients (Table VI). Overall, the MW of the fluorophore was the only factor that had a significant impact on the release constant k ($P < 0.001$), rate of diffusional release k_d ($P < 0.001$), rate of relaxational release k_r ($P < 0.001$), and the total amount of marker released ($P < 0.001$). Fluorescein showed the largest k, k_d, k_r, and total release followed by FD 4000 Da, FD 40,000 Da, and FD 250,000 Da, respectively. The pH significantly affected the total release of the three larger markers after 5 h ($P < 0.05$), but not fluorescein ($P > 0.05$), which suggested that fluorescein's small MW permitted similar total diffusive properties

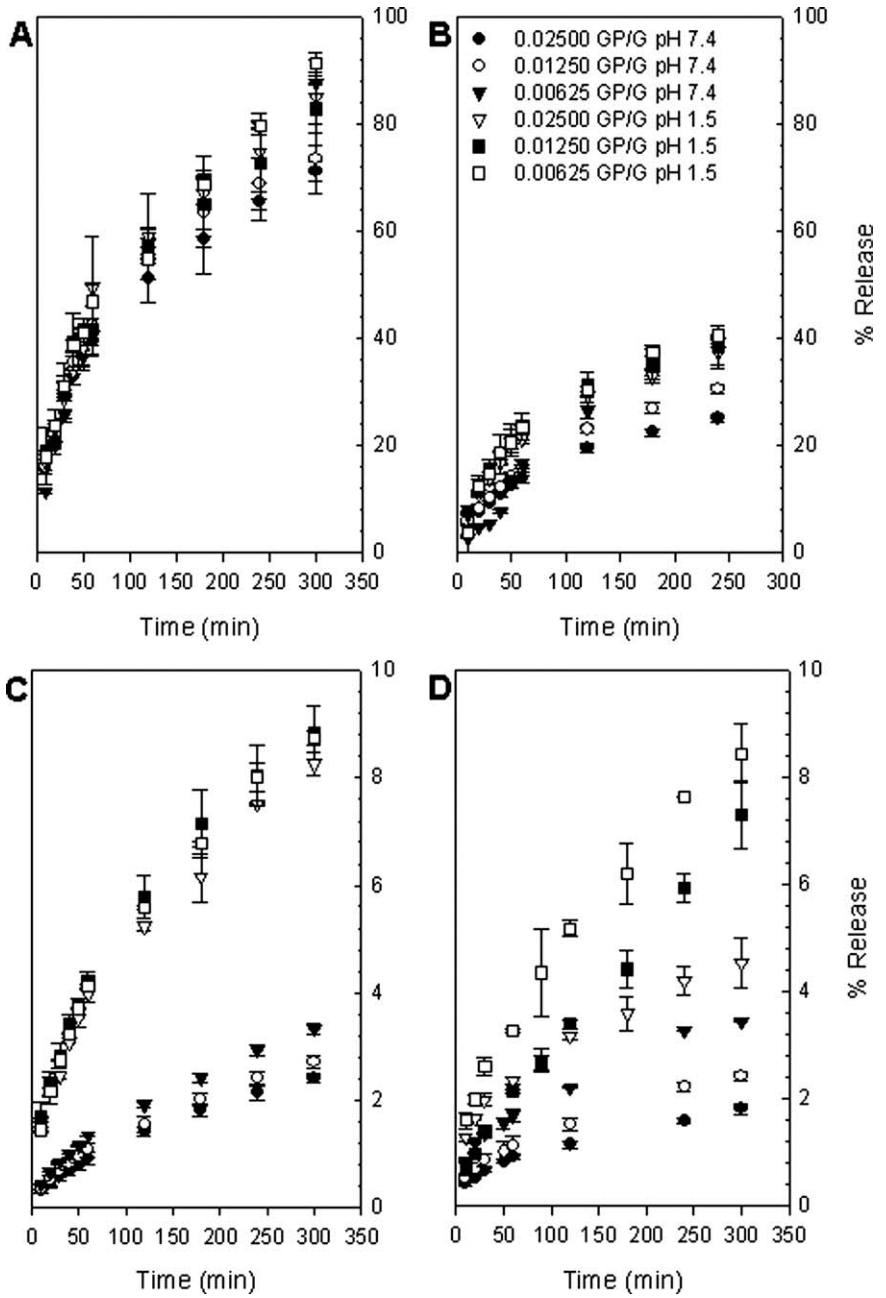

Figure 5 Release (%) of fluorescein (332 Da) (A), FD 4000 Da (B), FD 40,000 Da (C), and FD 250,000 Da (D) at pH 7.4 and 1.5 from bicontinuous hydrogels crosslinked at three genipin/gelatin ratios. Note the different *y*-axis scales. Values are shown as arithmetic means ± their standard deviations (SD) ($n = 3$).

irrespective of gelatin mesh size. Reduced swelling and consequent decreased free volume at pH of 7.4 compared to pH 1.5 reduced the mobility of the larger diffusants retarding their total release.

At pH 7.4, the total release of markers FD 4000 Da, FD 40,000 Da, and FD 250,000 Da was significantly greater with a decrease in genipin/gelatin ratio ($0.025 < 0.0125 < 0.00625$) ($P < 0.05$) whereas at pH 1.5, crosslinking had no significant impact on release ($P > 0.05$). This was likely related to the hydrolysis of the genipin crosslinks at low pH.[56] With the amide linkages between gelatin and genipin hydrolyzed, the gelatin chains were reprotonated, leading to increased polymer–polymer repulsion and consequently hydrogel swelling.

Based on the diffusional exponent n, all markers showed Fickian or slightly anomalous release behavior (Table VI). As expected, the rates of diffusional release k_d values were similar to the release constant k values, with the relaxational contributions (k_r) very small or slightly negative. Ford et al.[57] and Ferrero et al.[58] also obtained negative values for k_r and postulated that the relaxational component was

TABLE VII
Release Constant k, Diffusional Exponent n, Rate of Diffusional Release k_d, Rate of Relaxational Release k_r, and Total Release of Various Fluorescent Markers at pH 7.4 or 1.5 from Bicontinuous Hydrogels Crosslinked at Three Genipin/Gelatin Ratios (all $Ra^2 > 0.98$)

Fluorescent marker	GP/G	pH	$k(h^{-n})$	$n \pm$ CI	$k_d(h^{-0.48})$	$k_r(h^{-0.96})$	% release after 5 h ± SD
Fluorescein	0.00625	7.4	4.904	0.506 ± 0.044	5.366	0.021	87.62 ± 0.21
	0.0125	7.4	4.527	0.553 ± 0.037	4.809	0.188	73.67 ± 6.57
	0.025	7.4	4.426	0.545 ± 0.029	4.752	0.149	71.20 ± 1.74
	0.00625	1.5	7.113	0.441 ± 0.023	6.498	−0.054	91.28 ± 2.10
	0.0125	1.5	6.490	0.440 ± 0.016	5.975	−0.046	82.87 ± 6.78
	0.025	1.5	5.792	0.471 ± 0.029	5.719	−0.015	85.14 ± 6.92
FD 4000 Da	0.00625	7.4	2.043	0.513 ± 0.025	2.189	0.018	33.53 ± 0.84
	0.0125	7.4	1.858	0.512 ± 0.022	2.007	0.016	30.57 ± 0.72
	0.025	7.4	1.757	0.490 ± 0.033	1.803	0.004	25.12 ± 0.57
	0.00625	1.5	2.623	0.535 ± 0.124	2.878	−0.058	40.60 ± 0.26
	0.0125	1.5	1.921	0.614 ± 0.022	2.127	0.173	38.73 ± 2.21
	0.025	1.5	1.821	0.602 ± 0.020	1.963	0.152	37.62 ± 2.40
FD 40,000 Da	0.00625	7.4	0.097	0.629 ± 0.030	0.111	0.010	3.33 ± 0.11
	0.0125	7.4	0.088	0.601 ± 0.012	0.113	0.004	2.71 ± 0.10
	0.025	7.4	0.068	0.625 ± 0.017	0.090	0.004	2.39 ± 0.08
	0.00625	1.5	0.506	0.501 ± 0.012	0.530	0.003	8.75 ± 0.14
	0.0125	1.5	0.564	0.486 ± 0.021	0.577	4×10^{-4}	8.85 ± 0.50
	0.025	1.5	0.490	0.498 ± 0.015	0.510	0.002	8.26 ± 0.23
FD 250,000 Da	0.00625	7.4	0.360	0.399 ± 0.025	0.303	−0.005	3.45 ± 0.01
	0.0125	7.4	0.177	0.461 ± 0.029	0.170	-8×10^{-4}	2.43 ± 0.09
	0.025	7.4	0.153	0.429 ± 0.023	0.137	−0.002	1.82 ± 0.09
	0.00625	1.5	0.518	0.481 ± 0.025	0.517	2×10^{-4}	8.45 ± 0.54
	0.0125	1.5	0.125	0.690 ± 0.019	0.153	0.019	7.30 ± 0.63
	0.025	1.5	0.399	0.365 ± 0.045	0.352	−0.003	4.55 ± 0.46

Values are shown as arithmetic means ± their standard deviations (SD) (n =3). Confidence intervals (CI) are shown for values of n.

inhibiting release rather than being additive, i.e., slowing down the rate of release.

Release from bicontinuous hydrogels

Marker release from the bicontinuous systems showed similar trends to the gelatin-continuous hydrogels (Fig. 5 and Table VII). With an increase in marker size, there was a parallel decrease in the rate and total amount of release ($P < 0.001$). Overall differences in release rate were due to marker size and to buffer pH (Table VII). At pH 1.5, the release constant k, diffusional release rate k_d, and total release were significantly greater than at pH 7.4 ($P < 0.001$).

TABLE VIII
Comparison of the Statistically Significant Elements Involved in the Release of Markers from Gelatin-Continuous (G) and Bicontinuous (B) Crosslinked Gelatin-Maltodextrin Hydrogels Based on the Diffusional Exponent n, Release Constant k, Diffusional Release Rate k_d, Relaxational Release Rate k_r, and the Marker Released after 5 h

Factors	n	k	k_d	k_r	Release after 5 h
G vs. B					
Fluorescein	NS	$P < 0.001$	$P < 0.001$	NS	$P < 0.001$
FD 4,000 Da	NS	$P < 0.001$	$P < 0.001$	NS	$P < 0.001$
FD 40,000 Da	NS	NS	NS	$P < 0.001$	NS
FD 250,000 Da	NS	NS	NS	NS	NS
at pH 7.4 (G vs. B)					
Fluorescein	NS	$P < 0.001$	$P < 0.001$	NS	$P < 0.001$
FD 4000 Da	$P < 0.001$	$P < 0.001$	$P < 0.001$	NS	$P < 0.001$
FD 40,000 Da	NS	NS	$P < 0.05$	NS	NS
FD 250,000 Da	NS	NS	NS	NS	NS
at pH 1.5 (G vs. B)					
Fluorescein	$P < 0.05$	$P < 0.001$	$P < 0.001$	$P < 0.05$	$P < 0.001$
FD 4000 Da	NS	$P < 0.001$	$P < 0.001$	$P < 0.05$	$P < 0.001$
FD 40,000 Da	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$
FD 250,000 Da	NS	NS	NS	NS	$P < 0.05$

NS = not significant ($P > 0.05$).

As well, the crosslinking ratio statistically influenced the release of the three larger markers at pH 7.4 ($P < 0.05$), but not fluorescein ($P > 0.05$). As per the gelatin-continuous gels, crosslinking ratio had no impact on release at pH 1.5 ($P > 0.05$).

Comparison of release in gelatin-continuous and bicontinuous hydrogels

Overall, there was little difference in diffusional exponent (n) values between the gelatin-continuous and bicontinuous hydrogels (Table VIII). Fluorescein and FD 4000 Da showed a significant increase in release constant k and amount of release from the bicontinuous compared to the gelatin-continuous hydrogels at both pH 7.4 and 1.5 ($P < 0.001$). For FD 40,000 Da and FD 250,000 Da, at pH 7.4, there was no significant difference in release constant k nor amount of release when comparing the gelatin-continuous and bicontinuous hydrogels ($P > 0.05$) whereas at pH 1.5, both markers showed a significant increase in the amount released from the bicontinuous over the gelatin-continuous hydrogels ($P < 0.05$). Generally speaking, diffusants showed statistically different n, k, k_d, and k_r values in relation to composition and pH.

When comparing hydrogel composition and microstructure, accrued solvent ingress and diffusant transport to the external environment from the bicontinuous hydrogels were due to its higher maltodextrin content and its distinctive microstructure characterized by uncrosslinked maltodextrin channels that may have acted as conduits, thereby reducing tortuosity. Conversely, in the gelatin-continuous gels, solvent and diffusant transport phenomena were more broadly dictated by the extent of gelatin crosslinking as the maltodextrin consisted of discrete inclusions unable to promote diffusion as extensively.

CONCLUSIONS

These results suggest that crosslinked phase-separated gelatin-maltodextrin hydrogels are a viable option as controlled release platforms. From the comparison of release from the gelatin-continuous and bicontinuous hydrogels, it can be concluded that different hydrogel compositions can significantly impact release behavior. Increased marker size, a neutral pH, and increased crosslinking all tended to reduce the rate and amount of release. Conversely, a pH less than the pI of gelatin, reduced crosslinking, smaller marker sizes, and increased maltodextrin all tended to increase the rate and amount of release. As a result, with the proper combination of crosslink density, solvent pH and hydrogel microstructure, swelling behavior can be structured towards a specified swelling ratio. This, coupled with a marker of appropriate size, can lead to controllable levels and rates of release. A means to control release from such biologically compatible matrices can lead to applications in bioengineering, where biodegradable nontoxic devices are desired. These attributes could also be used to tailor the system to function as a drug delivery platform, where a construct capable of carrying a wide variety of therapeutic agents with the ability to controllably release them in varying environments is possible.

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