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Macromolecular diffusion and release from self-assembled β -hairpin peptide hydrogels

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Abstract

Self-assembling peptide hydrogels are used to directly encapsulate and controllably release model FITC–dextran macromolecules of varying size and hydrodynamic diameters. MAX1 and MAX8 are two peptide sequences with different charge states that have been designed to intramolecularly fold and self assemble into hydrogels at physiological buffer conditions (pH 7.4, 150 mM NaCl). When self-assembly is initiated in the presence of dextran or protein probes, these macromolecules are directly encapsulated in the gel. Self-diffusion studies using fluorescence recovery after photobleaching (FRAP) and bulk release studies indicate that macromolecule mobility within, and release out of, these gels can be modulated by varying the hydrogel mesh size. The average mesh size can be modulated by simply varying the concentration of a given peptide used to construct the gel or by altering the peptide sequence. In addition, results suggest that electrostatic interactions between the macromolecules and the peptide network influence mobility and release. Depending on probe size, release half-lives can be varied from 8 h to over a month.

Keywords

Hydrogel; Delivery; Self-assembly; Peptide

1. Introduction

Advances in genomics, proteomics, and cell biology have led to the rapid development of small protein and antibody therapeutics [1–5]. Growth factors, hormones, enzymes, cytokines, and monoclonal antibodies have been developed for a range of ailments such as cancer, autoimmune diseases, and metabolic disorders [1,3,6–8]. Although the number of biopharmaceuticals approved and in advanced clinical testing continues to expand, several limitations remain in the preparation and administration of these drugs [9–11]. For example, the susceptibility of proteins to physical and chemical degradations during storage and proteolytic degradation upon administration can severely limit the therapeutic efficacy; the half-lives, $t_{1/2}$, of many protein-based therapeutics range from only 2 to 100 min [12]. To address this problem, hydrogel materials have been used as carriers and delivery devices for

protein-based drugs [13-16]. Some hydrophilic gels provide a suitable environment to enhance macromolecule stability and help limit enzymatic degradation during delivery. Encapsulation of the protein therapeutic within the hydrogel can also enable prolonged activity if the rate of release of the drug can be controlled.

Hydrogel matrices for protein drug delivery have been prepared from both naturally derived (collagen, hyaluronic acid, chitosan, alginate) and synthetic (polyethylene oxide, polyacrylamide, polyvinyl alcohol) polymers [17-19]. A promising approach for the design of hydrogels is the use of self-assembling materials in which non-covalent interactions between molecules drive the assembly and formation of supramolecular networks that define the material [20,21]. Using peptides as building blocks for self-assembly allows one to make sequence specific modifications at the molecular level that ultimately influence the bulk properties of the self-assembled hydrogel. Therefore, changes to the peptide sequence, which are accomplished easily by solid phase methodology, can be used to quickly fine tune material properties, such as crosslink density, mesh size, hydrophilicity/electrostatics, and degradation rate [22-24]. All of these factors influence the release profile of a therapeutic that has been encapsulated into the gel. An additional benefit of using self-assembled gels is realized during the formulation of the drug-loaded material. In principle, precise concentrations of therapeutics can be directly encapsulated into the gel network during the self-assembly process. This is in contrast to methods that diffuse the drug into a preformed gel where the ultimate concentration of encapsulated therapeutic may be difficult to control. Herein, we report the application of self-assembled peptide hydrogels to encapsulate and controllably release model bio-macromolecules. Experiments indicate that macromolecule mobility within, and release out, of these gels can be modulated by varying the hydrogel mesh size. The average mesh size can be modulated by simply varying the concentration of peptide used to construct the gel or by making changes to the peptide sequence. In addition to mesh size, results show that electrostatic interactions between the macromolecule and the peptide network influence the release profile.

We have designed a class of self-assembling peptides that undergo triggered hydrogelation in response to physiological pH and salt conditions (pH 7.4, 150 mM NaCl) to form mechanically rigid, viscoelastic gels [25-28]. The parent peptide, MAX1, consists of 20 amino acids, eight of which are lysine residues. When dissolved in low ionic strength (≤ 10 mM NaCl) aqueous solutions at pH 7.4, MAX1 remains unfolded and soluble mainly due to electrostatic repulsion between the positively charged lysine side chains. However, when a physiological relevant concentration of NaCl (150 mM) is added, this charge is effectively screened and the peptide folds into an amphiphilic β -hairpin. The hairpin is composed of two β -strands of alternating lysine and valine residues connected by a four residue type II' β -turn, [25,29,30] Fig. 1. The lysine-rich face of the hairpin is hydrophilic and the valine-rich face is hydrophobic. The folded hairpin is highly amenable to self-assembly, ultimately forming a network of β -sheet rich fibrils. MAX1 self-assembles laterally forming a network of hydrogen bonds along the long axis of a given fibril. The peptide also assembles facially, burying the hydrophobic valine-rich face from water, forming a bilayer. Thus, each fibril is composed of a bilayer of hairpins where the surface of the fibril displays positively charged lysine side chains. Transmission electron microscopy (TEM) and small angle neutron scattering (SANS) show that in the long portions of the fibrils, hairpins facially assemble in a manner in which all of their β -strands are in register with each other and the maximal amount of valine side chain surface area is shielded from water [27,29,31]. However, imperfections in this mechanism where the face of one hairpin is rotated relative to its partner in the bilayer can occur. This results in a site for nascent fibril growth in a new three-dimensional direction and constitutes the formation of an interfibril crosslink, Fig. 1. In addition to the interfibril crosslinks, at sufficient peptide concentrations, entanglements of fibrils also contribute to the mechanical rigidity of the gel.

A convenient way to control mesh size of the gel is to simply increase the peptide concentration used to prepare the gel. The additional peptide leads to the formation of more fibrils that entangle and crosslink into the network, providing gels with smaller mesh sizes.

A second method to control mesh size involves modulating the rate of gel formation. In general, the kinetics of peptide folding and self-assembly dictates the number of crosslinks that are formed during the material formation process [25,27]. In turn, the number of crosslinks directly influences the mechanical rigidity and mesh size of the gel. Slow kinetics of assembly lead to less rigid gels with fewer crosslinks and a larger average mesh size. Fast kinetics lead to a more rigid gel containing more crosslinks and a smaller average mesh sizes. Smaller mesh sizes will more effectively hinder macromolecular diffusion during delivery.

For the β -hairpin peptides described herein, it is possible to control the folding and self-assembly kinetics to form hydrogels with different mesh sizes. For example, making changes to the peptide sequence can directly influence the rate of peptide folding and self-assembly. MAX8, a design descendent of MAX1, contains a point substitution on the hydrophilic face of the hairpin where one lysine at position 15 is replaced with a glutamic acid [27]. This simple substitution reduces the overall charge state of the peptide to about +7 at physiological pH. For comparison, MAX1 contains eight lysine residues and an N-terminal ammonium group leading to a charge state of about +9 at pH 7.4. Since MAX8 has a lower amount of positive charge to be screened, it folds and self-assembles much faster than MAX1 in response to similar buffer conditions [27]. Therefore, at identical peptide concentrations and solution conditions, MAX8 will form more rigid gels that contain more crosslinks, resulting in smaller mesh sizes. As will be shown, one can vary either peptide sequence or peptide concentration or a combination of both to modulate hydrogel mesh size, which directly influences the release profiles of encapsulated therapeutics.

This study focuses on correlating the mesh sizes of gels formed from two self-assembling peptide sequences, MAX1 and MAX8, to the diffusion and mass transport properties of model dextran and protein probes. First, dynamic oscillatory rheology is used to characterize the mesh sizes of MAX1 and MAX8 hydrogels of varying weight percents. Next, well characterized probe molecules, either FITC-labeled dextrans or the protein lactoferrin, are encapsulated within networks of varying mesh sizes and their diffusion within and from the gel are studied. In this study, the dextran probes range in molecular weights of 20 kDa, 70 kDa, and 150 kDa and have hydrodynamic diameters of 6.2 nm, 9.1 nm, and 12.1 nm, respectively. Self-diffusion coefficients of these dextrans within the hydrogel networks are determined using the non-invasive technique, fluorescence recovery after photobleaching (FRAP). These studies are most conveniently carried out by allowing the dextran probes to first diffuse into pre-set gels and then measuring their diffusion within the networks. These self-diffusion coefficients are then compared with measurements of the bulk release kinetics of the dextran probes out of the hydrogels, which is monitored over a month time period. For these bulk release studies, probes are added into the solution during peptide folding and self-assembly, yielding gels with macromolecules directly encapsulated into the network. Lactoferrin, a 77 kDa protein having a hydrodynamic diameter of 7.2 nm was also studied to investigate the effect of macromolecular charge on release. We find that self-diffusion and bulk release are dependent not only on hydrogel mesh size but also on electrostatic interactions that take place between the gel network and macromolecule.

2. Experimental

2.1. Materials

Bis-tris propane (BTP), sodium chloride (NaCl), fluorescein isothiocyanate dextran (FITC-dextran) probes, and lactoferrin were purchased from Sigma-Aldrich. The FITC-dextrans

probes had molecular weights of 19,400 Da (commercially available as 20 kDa), 75,090 Da (70 kDa), and 170,000 Da (150 kDa). MAX1 and MAX8 peptides were synthesized according to a previously published protocol [29]. Briefly, peptides were synthesized on RINK amide resin using standard Fmoc-protocol with HCTU activation on an automated ABI 433A peptide synthesizer. Cleavage and side-chain deprotection of the dry resin-bound peptides were performed with a trifluoroacetic acid (TFA):thioanisole:ethanedithiol:anisole (90:5:3:2) cocktail for 2 h under N₂ atmosphere. Filtration followed by ether precipitation yielded crude peptides. Purification of the crude peptides was performed by RP-HPLC using a preparative Vydac C18 peptide column with a flow rate of 8 mL/min. Peptide is injected onto the column under isocratic conditions. MAX1 gradient: 0% B for 2 min, then a linear gradient from 0 to 18% B over 4 min, then 18–100% B over 164 min. Peptide elutes at 29 min. MS (ESI) m/z : 1115.4 [(M + 2H)²⁺, calculated: 1115.9]. MAX8 gradient: 0% B for 2 min, then a linear gradient from 0 to 24% B over 4 min, then 24–100% B over 152 min. Peptide elutes at 30 min. MS (ESI) m/z : 1115.9 [(M + 2H)²⁺, calculated: 1116.5]. Elutants for RP-HPLC consisted of Standard A (0.1% TFA in water) and Standard B (90% acetonitrile, 9.9% water, 0.1% TFA). Analytical HPLC chromatographs and mass spectra ESI (+) for the pure peptides are located in Supporting information. Lyophilized purified peptides were dissolved in water at 1 mg/mL and re-lyophilized twice before using for hydrogelation.

2.2. Dynamic oscillatory rheology

Oscillatory rheology experiments were performed on a Paar Physica MCR 500 rheometer using 25 mm diameter stainless steel parallel plate geometry. For the rheological measurements, gels were prepared directly on the rheometer plate in the following manner. For both MAX1 and MAX8 gels of varying weight percents, peptide stock solutions were first prepared in glass vials by dissolving 2, 4, and 8 mg of each peptide in 200 μ L of sterile, chilled water resulting in three stock solutions per peptide. To each stock, 200 μ L of chilled BTP buffer (100 mM, pH 7.4) containing 300 mM NaCl was added. To prepare gels that directly encapsulate the dextran molecules, 200 μ L of chilled BTP buffer (100 mM, pH 7.4) containing 300 mM NaCl and 1.2 mg/mL of the respective FITC–dextran were added to the stock solution. This results in 0.5, 1.0, and 2.0 wt% gels, respectively, of a final total volume of 400 μ L. Then 300 μ L of the resulting solution was quickly added to the rheometer plate, which was pre-equilibrated at 5 °C. The parallel plate tool was then lowered to a gap height of 0.5 mm and the temperature was ramped linearly to 25 °C to initiate gelation. Initially, a dynamic time sweep was performed to measure the storage (G') and loss (G'') modulus at a frequency of 6 rad/s and 0.2% strain as a function of time until a plateau modulus was reached. A dynamic frequency sweep (0.1–100 rad/s at constant 0.2% strain) was then performed, followed by a dynamic strain sweep (0.1–1000% strain at constant 6 rad/s), which was used to verify that the former measurements were within the linear visco-elastic regime.

2.3. FRAP diffusion measurements

For the FRAP experiments, 1, 2, or 4 mg of the appropriate peptide was fully dissolved in 100 μ L of sterile, chilled water to prepare separate stock solutions. Then, 75 μ L of each stock was added to a separate well of a Lab-Tek II Chamber 8 well plate. To each well, 75 μ L of BTP buffer (100 mM, pH 7.4) containing 300 mM NaCl was added. Samples were carefully agitated to cover the bottom of the well and to initiate hydrogelation, resulting in 0.5, 1.0, and 2.0 wt % gels, respectively, of a final total volume of 150 μ L. Stock solutions of 20, 70, and 150 kDa FITC–dextran probes were prepared fresh at a concentration of 1.05 mg/mL of FITC–dextran in pH 7.4, 50 mM BTP, 150 mM NaCl prior to each FRAP experiment. 24 h after gel preparation, 200 μ L of this solution was added to the top of the hydrogels to yield a final dextran concentration of 0.6 mg/mL (37 mM with respect to the fluorescein groups for all probes). This concentration was determined to display an optimal fluorescence within the linear region of fluorescence intensity for the FRAP experiments (see Supporting information).

The diffusion coefficients of the FITC–dextran probes were measured using the FRAP method with laser scanning confocal microscopy (LSCM) [32,33]. Measurements were taken on a Zeiss 510 LCM confocal microscope with a 20× magnification objective and an argon ion laser set at 488 nm with 50% power. The 20× objective lens has a cylindrical bleaching volume in which diffusion is avoided in the third dimension. Briefly, for a typical FRAP experiment, the confocal microscope scans an image along the *xy* plane of the sample. The center of a 512× 512 pixel image (30 μm above the bottom of the plate) was chosen as the *z*-component of the confocal image. A circle of a diameter of 115 pixels (pixel 0.9 μm) was selected as the bleaching area. The area was monitored by 15 pre-bleach scanned images at low laser intensity (2%), then bleached with 50 iterations (about 10 s total) at 100% laser intensity, and followed by detection of the fluorescence recovery again at low intensity. A total of about 500 image scans (<1 s each) were collected for each sample. FRAP experiments of the hydrogels were performed after 24 h of incubation with the 20 kDa and 70 kDa dextran solutions, and after 48 h of incubation with the 150 kDa dextran solution to ensure that the respective probes had diffused homogeneously throughout the gel. Homogeneity was verified visually by monitoring the color change of the gel as the probes diffused into the matrix. All experiments were performed at 25 °C.

2.4. FRAP data analysis

Two-dimensional lateral self-diffusion coefficients (*D*) are determined by first collecting normalized fluorescence recovery curves for each probe in both MAX1 and MAX8 gels of differing weight percents. For each experiment, a normalized fluorescence recovery curve, *f* (*t*), is obtained according to Equation (1) where *F*(*t*) is the observed fluorescence after photobleaching and *F*_{in} is the background fluorescence intensity obtained prior to photobleaching [34,35].

$$f(t) = 1 + \frac{1}{R} \left(\frac{F(t) - F_{in}}{F_{in}} \right) \quad (1)$$

The parameter, *R*, is the mobile fraction of the probes in the network. Due to interactions between the probes and the hydrogel network, a portion of the fluorescent probes remain immobile, and this immobile fraction has to be considered in the calculation of the normalized fluorescence values. *R* is determined by Equation (2) where *F*_∞ is the fluorescence intensity of the bleached spot at the end of the experiment and *F*₀ is the fluorescence intensity of the bleached spot immediately after bleaching.

$$R = \frac{F_{\infty} - F_0}{F_{in} - F_0} \quad (2)$$

The self-diffusion coefficient (*D*) of a given FITC–dextran probe is obtained by fitting the normalized fluorescence recovery curve (*f*(*t*)) with Equation (3) [36].

$$f(t) = \sum_{n=0}^{n=\infty} \frac{(-b)^n}{n!} \frac{1}{1+n \left[1 + (2t/\tau_D) \right]} \quad (3)$$

The bleaching parameter, *b*, is fit and describes the efficiency of bleaching. It is dependent on the intensity of the laser beam, the bleach time, and the sensitivity of the fluorescent molecule for bleaching [34]. Importantly, *τ*_D is also fit and is the characteristic diffusion time. The 2D lateral self-diffusion coefficient, *D*, is then calculated from *τ*_D according to Equation (4).

$$D = \frac{\omega^2}{4\tau_D} \quad (4)$$

where ω is the radius of the Gaussian bleached spot (set constant at 51.75 μm) [33].

2.5. Determination of probe hydrodynamic radius

Hydrodynamic diameters (d_H) were determined for each of the commercially available FITC–dextran probes according to Equation (5).

$$d_H = \frac{k_B T}{3\pi\eta D} \quad (5)$$

where k_B is the Boltzmann's constant, T is the temperature (25 °C, 298 K), η is the zero-shear viscosity of the aqueous solution (0.001 Pa s), and D is the diffusion coefficient of each probe measured in BTP buffer (50 mM, pH 7.4, 150 mM NaCl) in the absence of gel. The diffusion coefficient is measured according to the experimental protocol given in Sections 2.3 and 2.4. Probe diameters are 6.2, 9.3, and 12.1 nm for the 20, 70, and 150 kDa FITC–dextran probes, respectively. Our values of d_H are in good agreement with those reported in the literature using FRAP and other established techniques [34–39].

2.6. Release studies

For the bulk release studies, MAX1 and MAX8 peptide stock solutions were first prepared in glass vials by dissolving 1.5 mg or 6 mg of each peptide in 150 μL of sterile, chilled water resulting in two stock solutions for each peptide. Then, 100 μL of each stock was added to a separate glass vial. 100 μL of chilled, fresh BTP buffer (100 mM, pH 7.4) containing 300 mM NaCl and 1.2 mg/mL of the respective FITC–dextran or lactoferrin was added to the stock solution. Samples were carefully agitated to initiate hydrogelation, resulting in 0.5 and 2.0 wt % gels, respectively, of a final total volume of 200 μL containing 0.6 mg/mL FITC–dextran or protein. Hydrogels were made in cylindrical glass vials with only the top surface exposed for release and had approximate heights of 0.35 cm from the bottom of the vial. After 24 h, 1 mL of pH 7.4, 50 mM BTP, 150 mM NaCl buffer was added to the top of the gel. At scheduled time points, the entire volume of buffer above the gel was removed and replaced with fresh buffer. Probe concentration was determined for each removed aliquot as a function of time. Each time point was performed in triplicate and experiments were carried out for 28 days. The concentration of dextran in the stock solutions and in the supernatant was determined by UV–vis spectroscopy from the area under the absorbance curve between the wavelengths of 415 and 535 nm (FITC has Abs_{max} at 492 nm). Calculated areas were converted to concentration values via calibration curves, which are located in Supporting information. Release profiles for the protein, lactoferrin, were determined in a similar manner except that protein concentration was assessed by absorbance at 280 nm which was compared to a calibration curve.

3. Results and discussion

3.1. Modulation of mesh size

In general, the diffusion of a macromolecule within a non-covalently crosslinked semiflexible network is governed by both the mesh size and any specific or non-specific interactions between the probe and the matrix [38,40–45]. When the size of macromolecule is much less than the mesh size, interactions between the macromolecule and the matrix do not contribute greatly to

the diffusion. When the size of macromolecule approaches the mesh size, mobility is influenced by both diffusional constraints imposed by the mesh size and any physical interactions that take place between the macromolecule and the network.

The mesh sizes of MAX1 gels can be modulated by simply adjusting the weight percent of peptide used to prepare the gel. Since these gels do not appreciably swell, the increased peptide concentration results in the formation of more fibrils that entangle and crosslink into the network. Thus, higher weight percent gels are more mechanically rigid and have smaller mesh sizes than gels of the same volume prepared with lower concentrations of peptide. For example, Fig. 2A shows rheological data that monitors the onset of the storage modulus, a measure of the material's mechanical rigidity, as a function of time for MAX1 gels prepared at different weight percents. Here, gelation is triggered by the addition of a solution of sodium chloride to an aqueous solution of unfolded peptide. It is clear that as the weight percent increases, the resultant storage modulus of the gels increase.

Rheology can also be used to approximate the mesh sizes of viscoelastic gels. These peptide hydrogels have been previously shown to display characteristics of semiflexible biopolymers [31]. The Mackintosh theory can be used to relate the plateau storage modulus of a gel to its mesh size according to Equation (6) [46,47].

$$G'_p k_B T \frac{l_p^2}{\xi^5} \quad (6)$$

The plateau modulus, G'_p , is determined from dynamic frequency sweep data and is defined as the storage modulus at the frequency where the loss modulus is a minimum. G'_p is nearly identical in value to the final storage modulus in Fig. 2A; however, frequency sweep data is provided in Supporting information, along with strain sweep data. In Equation (6), k_B is the Boltzmann's constant, T is the temperature at which the hydrogel is formed (25 °C, 298 K), l_p is the persistence length of the fibrils (~55 nm) [31], and ξ is the characteristic mesh size of the network. Fig. 2B shows the calculated mesh sizes for MAX1 gels as a function of weight percent. It can be seen from the data that increasing the concentration by a factor of four results in about a twofold decrease in the mesh size; the 0.5 wt% gel has a mesh size of 49 nm as compared to 22 nm for the 2 wt% gel.

The mesh size of the β -hairpin gels can also be modulated by varying the peptide sequence. The replacement of a lysine residue of MAX1 at position 15 with glutamic acid results in MAX8. This peptide folds and self-assembles faster than MAX1 under identical solution conditions and peptide weight percent. Fig. 2C shows the onset of gelation as a function of time for 1 wt% gels formed by MAX1 and MAX8. Both peptides form moderately rigid viscoelastic gels. However, MAX8 forms a more rigid hydrogel more quickly than MAX1. After 60 min, the storage modulus of MAX8 is more than an order of magnitude larger than that of the MAX1 hydrogel. Correspondingly, the mesh size of the 1 wt% MAX8 gel (23 ± 0.4 nm) is smaller than the 1 wt% MAX1 gel (34 ± 0.2 nm).

Similar to MAX1 gels, the mesh size of MAX8 gels can be varied by adjusting the peptide concentration. Fig. 2D shows the mesh sizes of MAX8 gels as a function of peptide weight percent; mesh sizes vary from 30 to 18 nm. In addition, comparing Fig. 2B–D, the mesh sizes of MAX8 are smaller than the mesh sizes of MAX1 at a given peptide weight percent, again indicating that peptide sequence can be used to modulate mesh size. The data in Fig. 2 taken together, therefore, demonstrates that hydrogels of varying mesh sizes can be prepared by adjusting either the peptide concentration or the peptide sequence or both.

3.2. Diffusion of probes within hydrogels

Because the mesh sizes (18–49 nm) of these gels are on the order of the hydrodynamic diameters of the probes being used in this study (6–12 nm), one would expect that both mesh size and any physical interactions between the probe and the fibrillar network would influence diffusion [38,40–45]. Thus, before bulk release studies were performed, the diffusivity of macromolecules within the peptide matrices was first studied to ascertain whether or not the peptide matrix could interact with incorporated macromolecules. Diffusion coefficients (D) of FITC-labeled dextran probes within MAX1 and MAX8 of differing weight percents were determined via FRAP experiments employing laser scanning confocal microscopy. In this experiment, fluorescent probes are homogeneously incorporated within the network and a high intensity laser is used to photobleach them within a defined area of the gel. The recovery of fluorescence after photobleaching, due to neighboring fluorescent probes diffusing into the bleached area, is measured as a function of time. The resultant data can be fit to determine the diffusion coefficient (D) of the probes within the network (Equations (3) and (4)). For example, Fig. 3 shows a typical FRAP experiment for the 70 kDa FITC–dextran incorporated into a 2 wt% MAX1 hydrogel. The inset shows the corresponding fluorescence recovery in real time of the probe in the gel. In addition, a recovery curve is also shown for the same probe in buffer alone. It can be seen that the fluorescence recovery of the probe in the hydrogel is slower than in the buffer solution, indicating that the mobility of the probe within the gel is more restricted than in buffer. Recovery plots for every probe, buffer, and gel combination are provided in Supporting information; 21 sets of experiments are included with $n = 5$ –14 for each set.

The self-diffusion coefficients (D) for the FITC–dextran probes within the hydrogels are summarized in Table 1 for MAX1 and MAX8 gels prepared at 25 °C. Comparing the diffusion coefficients of each of the probes in 0.5 wt% and 2.0 wt% (the two weight percent extremes), macromolecular mobility is restricted by a factor of 2 in both MAX1 and MAX8 gels. In Fig. 4, these diffusion coefficients are normalized with respect to the diffusion coefficients of the probes in buffer alone (D_0) and plotted as a function of peptide weight percent. Fig. 4A shows diffusion trends observed for probes incorporated into MAX1 gels. Interestingly, when probes are incorporated in the most dilute gel, 0.5 wt%, the gel network is capable of strongly influencing macromolecular mobility. For example, the 20 kDa probe diffusion is only 60% of what is observed in buffer solution alone; the mobility of the largest probe, 150 kDa, is reduced to 25%. With increasing gel weight percent, it is evident that the mobility of all the probes becomes further restricted; this is expected since the mesh size is smaller at higher weight percents. Also evident is that at a particular gel weight percent, the largest probe, 150 kDa ($d_H = 12.1$ nm) diffuses at a slower relative rate than those of smaller hydrodynamic diameters, and there is little discrimination between the two smaller probes at all weight percents. This indicates that as the probe hydrodynamic diameter approaches the mesh size, macromolecular mobility is significantly hindered. Similar trends are seen for probes incorporated within MAX8 hydrogels, Fig. 4B.

It is tempting to rationalize the observed trends in Fig. 4A and B based on mesh size alone. However, because the sizes of the probes are approaching the mesh size of the gels used in this study, it is likely that interactions between the probes and the network also influence macromolecular mobility [38,40–45]. Probe–matrix interactions can be investigated by comparing the diffusion coefficients of a distinct probe incorporated into networks formed from two different peptide sequences. For example, 0.5 wt% MAX1 hydrogels have a mesh size that is about 60% larger than 0.5 wt% MAX8 (49 nm versus 30 nm). One would expect that the diffusion coefficient of a given probe in the 0.5 wt% MAX1 gel should be significantly larger than in the MAX8 gel if mesh size alone dictated macromolecular diffusion. However, the diffusion coefficients of any given probe within the two peptide networks are the same within error. This data suggests that in addition to the mesh size, there are possible probe–

matrix interactions that affect macro-molecular mobility for these peptide-based gels. The nature of these interactions is such that probe mobility within MAX1 gels is more restricted than MAX8 gels for the FITC-labeled dextran probes used in this study.

Previous scattering and microscopy studies of the gels reveal that the local fibrillar morphology of MAX1 and MAX8 networks are similar [27]. Both peptides form fibrils of homogeneous diameter (~ 3 nm) with similar persistence lengths. Also, on the molecular level, CD and FTIR spectroscopies reveal that both peptides adopt well-defined β -hairpins indicating that the secondary structure of peptides assembled into fibrils are similar. The most apparent difference between the two peptide gels lies in the composition of amino acids that comprise each of the peptides, Fig. 1C. MAX1 contains eight positively charged lysine side chains and an N-terminal ammonium group giving it an overall charge of about +9 at neutral pH. MAX8 consists of a sequence in which one of the lysines is replaced by a glutamic acid, giving it an overall charge of about +7, as well as a negatively charged carboxylate side chain displayed from each peptide assembled along a given fibril. Therefore, at similar peptide weight percents, MAX1 hydrogels are more electropositive than MAX8 gels. In addition, the fibrils that constitute MAX8 gels contain point negative charges distributed along their long axes. The dextran probes used in this study are labeled with fluorescein isothiocyanate (FITC), which contains a free carboxylate with a pK_a of 6.5, and therefore, is anionic at neutral pH [41]. The concentration of fluorescein (37 μ M), regardless of which dextran probe is used, is always constant in these studies. In the FRAP experiments, it is observed that diffusion is more hindered in MAX1 versus MAX8 gels. Therefore, this is consistent with the negatively charged FITC-dextran interacting more favorably with the more electropositive MAX1 gels. In addition to these differences between overall charge state between the two peptides (MAX1: $\sim +9$, MAX8: $\sim +7$), the charged glutamic acid side chain of MAX8 + negatively could further actively repulse the negatively charged FITC labels from interacting with the peptide fibrillar network. The FRAP data shown thus far suggests that both mesh size and electrostatics influence macromolecular mobility within the gel network. As will be shown, electrostatic interactions between a given macromolecule and the gel network also strongly influence macromolecular release from the gel network.

3.3. Bulk release studies

One benefit of using a self-assembling system for therapeutic delivery is that the therapeutic can be incorporated during material formation. This allows precise control over the concentration of the incorporated drugs. The bulk release of the same dextran probes used in the FRAP studies was assessed from gels that were formed in the presence of the probe. For example, a 2 wt% gel that encapsulates 0.6 mg/mL of a particular probe can be prepared by first dissolving the peptide in aqueous solution and adding this to a solution of probe in pH 7.4 buffer containing salt. The 1:1 addition of these two solutions initiates hydrogelation and results in the homogeneous incorporation of the probe. This process is shown pictorially in Fig. 5 where a saline solution of the 150 kDa probe (yellow)¹ is added to a 4 wt% solution of unfolded MAX1. The saline solution initiates peptide folding and self-assembly, resulting in hydrogelation and the incorporation of the probe. The encapsulation of these probes does not affect the rheological properties of the hydrogels. Equivalent values of plateau moduli are realized for gels of equivalent weight percent that encapsulate probes compared to gels that contain no probes (see Supporting information).

The 20, 70, and 150 kDa probes were encapsulated into 0.5 and 2.0 wt% MAX1 and MAX8 gels. Gels are allowed to cure for 24 h after which 1 mL of buffer is added to top of the gel. Fig. 6A and B shows the amount of labeled dextran released from the gels (M_t) divided by the

¹For interpretation of the references to colour in this text, the reader is referred to the web version of this article.

initial encapsulated mass (M_0) over a 30 day period. At each allotted time point, the buffer is removed from the top of the gel, the released probe is quantified by UV-vis absorbance, and fresh buffer is added for the next time point. It should be noted that upon the addition of buffer to the top of the gels, the volume of gel remains constant, and no swelling is observed. Also, during the release studies, there is no detectable change in hydrogel height, or no evidence of surface erosion and degradation. Therefore, the release of dextrans encapsulated in these nondegradable hydrogels is solely diffusion controlled and dependent on the interactions between the probe and the peptide network.

Fig. 6A shows that the fastest release is obtained for the smallest probe (20 kDa, d_H % 6.2 nm) in the 0.5 wt% MAX1 gel. This is expected since this gel has the largest mesh size of those studied. The 20 kDa probe and the 70 kDa probe are released with nearly identical rates from 2.0 wt% to 0.5 wt% MAX1 gels, respectively. This is consistent with the FRAP analysis which shows that these two probes have similar diffusion coefficients when incorporated into the respective gels (Table 1: $D_{20 \text{ kDa}-2 \text{ wt\% MAX1}} = 25.9 \pm 4.1$, $D_{70 \text{ kDa}-0.5 \text{ wt\% MAX1}} = 30.0 \pm 4.0$). The experiment also shows that the 70 kDa probe is released more slowly from a 2 wt% gel as compared to 0.5 wt% gel. After 28 days, only 80% has been released. The 150 kDa FITC-dextran, which is the largest probe in the study and similar in size to many therapeutic antibodies, is released the slowest from the 2 wt% gel. Data is shown only for the 2 wt% gel because solutions of 0.5 wt% MAX1 with 150 kDa FITC-dextran did not form hydrogels. (The highly negatively charged dextran prevents low concentrations of MAX1 from self-assembling. However, neutral, unlabeled 150 kDa dextran can be encapsulated into 0.5 wt% MAX1 gels.) Fig. 6B shows similar trends for the release of probes from the MAX8 gels, but with a notable difference that the 150 kDa probe can be encapsulated in 0.5 wt% MAX8 gels. Nearly 70% of this large probe is retained within the matrix even after 28 days. The release of the 20, 70, and 150 kDa probes from a 2 wt% MAX1 gel is visualized in Fig. 6C as a decrease in the FITC-based yellow color over time. Here, a picture of each probe encapsulated in separate gels is shown at time 0 and then after 2 weeks of release. The retention of the 150 kDa probe is clearly seen and is consistent with the bulk release of Fig. 6A.

The characteristic time for release of 50% of the initial mass ($t_{50\% \text{ released}}$) is given in Table 2 for the 20 and 70 kDa probes. Since less than 50% of the 150 kDa is released over the time course of the study, the $t_{25\% \text{ released}}$ value for this probe is reported for both the MAX1 and MAX8 gels. The $t_{50\% \text{ released}}$ values range from about 8, 11, 24, and 28 h for the smallest 20 kDa probe depending on peptide sequence and gel weight percent. The $t_{50\% \text{ released}}$ values for the 70 kDa probe range from about 31 to 67 h. The $t_{25\% \text{ released}}$ values for the largest 150 kDa probe ranges from 14 to greater than 28 days.

The characteristic release times in Table 2 are consistent with the FRAP-derived diffusion coefficients in Table 1. Table 2 shows that for any given dextran probe, the release rate is nearly twice as fast from the 0.5 wt% gel as compared to the 2 wt% gel; Table 1 shows the same trend in diffusion coefficients. This demonstrates that the release rate scales with the diffusion coefficients determined from FRAP. This is of value in formulating hydrogels because relatively rapid FRAP measurements can be used to guide formulation for a desired release rate.

The FRAP data from the dextran probes suggests that electro-statics could influence the bulk release of a given macromolecule from the gel network. This possibility was further investigated by measuring the bulk release of two differently charged macromolecules that have similar molecular weights and hydrodynamic diameters from 2 wt% MAX1 gels. Fig. 7 shows the release of the negatively charged 70 kDa FITC-dextran ($pI \sim 6.5$) compared to the release of a positively charged protein, lactoferrin, of similar weight (77 kDa, $pI \sim 8.4-9.0$) at pH 7.4. The hydrodynamic diameters of the dextran and protein are similar, 9.3 nm and 7.2

nm [48], respectively, but their charge state varies considerably. 2 wt% MAX1 gels were used since these gels have small mesh sizes, the peptide networks are highly positively charged and, thus, electrostatic effects should be pronounced if present. The data shows that the positively charged protein is released more quickly than the negatively charged dextran. After 2 days, ~70% of the lactoferrin has been released compared to ~40% of the FITC-dextran. This suggests that the charge state of the macromolecule affects the release from the peptide hydrogel. For example, attractive electrostatic interactions between the negatively charged dextran and the peptide matrix may retard release. Also, repulsive electrostatic interactions between the positively charged protein and the peptide network could enhance release.

4. Conclusion

Hydrogels prepared from the self-assembling peptides, MAX1 and MAX8, are shown to encapsulate and controllably release model biomacromolecules. Rheological measurements indicate that the hydrogels are mechanically rigid, semiflexible network materials with mesh sizes, ranging from 18 to 50 nm. FRAP measurements of diffusion coefficients for a series of FITC-labeled dextrans within gel networks of differing weight percents show that probe diffusion depends on probe size, peptide sequence, and the mesh size of the gel. The bulk release of the probes from 0.5 to 2.0 wt% MAX1 and MAX8 gels was also assessed; the bulk release rates of the probes mirror the trends observed for the diffusion coefficients measured by FRAP. The full release of the dextran probes and lactoferrin from the gels ranges from days to over a month. Release studies using both dextran and protein probes suggest that the main factors contributing to the release rates are the hydrogel mesh size and the electrostatic interactions between a given macromolecule and the gel network. The mesh size can be controlled by changing the peptide weight percent and/or peptide sequence. This study suggests that the release of various sized and charged macromolecules from these self-assembling β -hairpin peptide hydrogels can be controlled and fine-tuned with simple modifications in the amino acid sequence and the peptide weight percent.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. (A) Proposed mechanism for the folding and self-assembly of MAX hydrogels. (B) Images of 1 wt% MAX8 hydrogels. (C) Peptide sequences of MAX1 and MAX8.

Fig. 2.

(A) Time sweep rheological data measuring the storage moduli of 0.5, 1.0, and 2.0 wt% MAX1 hydrogels as a function of time. (B) Mesh sizes (ξ) of MAX1 hydrogels as a function of peptide weight percent. Inset is a table of MAX1 peptide weight percent, plateau moduli from frequency sweep data (see Supporting information), and the respective mesh size. (C) Time sweep rheological data measuring the storage moduli of 1 wt% MAX1 and MAX8 hydrogels as a function of time. (D) Mesh sizes (ξ) of MAX8 hydrogels as a function of peptide weight percent. Inset is a table of MAX8 peptide weight percent, plateau moduli from frequency sweep data (see Supporting information), and the respective mesh size. Hydrogels formed at pH 7.4, 50 mM BTP, 150 mM NaCl buffer conditions at 25 °C. Error bars are less than symbol height in Panels B and D. All experiments were performed in triplicate.

Fig. 3.

Normalized fluorescence recovery ($f(t)$) of 70 kDa FITC-dextran (0.6 mg/mL) in pH 7.4, 50 mM BTP, 150 mM NaCl buffer (○) and 2 wt% MAX1 gel (□). Data was fit to Equation (3) for each curve. Inset is confocal microscopic images corresponding to the FRAP experiment for 2 wt% MAX1 gel. F_{in} is the time regime that corresponds to the initial fluorescence before bleaching; F_0 is the fluorescence measurement immediately after photobleaching; $F_{t>0}$ corresponds to the recovery of fluorescence after photo-bleaching; F_{∞} corresponds to maximal recovery of fluorescence at the end of the experiment. Scale bar = 100 μ m.

Fig. 4. Normalized diffusion coefficients for FITC-dextran as a function of hydrogel weight percent of (A) MAX1 and (B) MAX8 hydrogels formed at 25 °C. D_0 is the diffusion coefficient of FITC-dextran in buffer alone. Lines represent stretched exponential fits and are included to aid the eye.

Fig. 5.

Image of the 1:1 addition of (A) a 4 wt% MAX1 aqueous solution and (B) a buffer solution (pH 7.4, 100 mM BTP, 300 mM NaCl) containing 1.2 mg/mL 150 kDa FITC-dextran to form (C) a 2 wt% MAX1 gel (pH 7.4, 50 mM BTP, 150 mM NaCl) with the 0.6 mg/mL dextran homogeneously encapsulated within the network. The vial has been inverted in (C) to show the self-supporting nature of the gel.

Fig. 6.

Cumulative bulk release of 20 kDa (■), 70 kDa (▲), and 150 kDa (●) FITC-dextran from 0.5 wt% (closed symbols) and 2.0 wt% (open symbols) from (A) MAX1 and (B) MAX8 hydrogels. Hydrogels formed at pH 7.4, 50 μ M BTP, 150 mM NaCl buffer conditions at 25 $^{\circ}$ C. Release studies were collected at 25 $^{\circ}$ C. Lines are included to guide the eye. Error bars are smaller than the symbol where they are not evident. (C) Images of 2 wt% MAX1 hydrogels encapsulated with 20, 70, and 150 kDa FITC-dextran at the start of the release experiments and at the two week time point. The yellow coloration is due to the fluorescein labels of the dextran.

Fig. 7. Cumulative bulk release of 70 kDa FITC-dextran (■) and lactoferrin (●) from 2.0 wt% MAX1 hydrogels. Hydrogels formed at pH 7.4, 50 mM BTP, 150 mM NaCl buffer conditions at 25 °C. Release studies were collected at 25 °C. Lines are included to guide the eye. Error bars are smaller than the symbol where they are not evident.

Table 1
Hydrodynamic diameters (nm) and diffusion coefficients (10^{-8} cm²/s) of FITC–dextrans in MAX1 and MAX8 hydrogels prepared at 25 °C. Diffusion coefficients measured at 25 °C by FRAP.

Probe MW (kDa)	Probe diameter d_H (nm)	MAX1 diffusion coefficient, D (10^{-8} cm ² /s)			MAX8 diffusion coefficient, D (10^{-8} cm ² /s)		
		0.5 wt%	1.0 wt%	2.0 wt%	0.5 wt%	1.0 wt%	2.0 wt%
20	6.2	43.4 ± 5.3	34.5 ± 5.8	25.9 ± 4.1	41.0 ± 5.8	31.8 ± 5.1	19.2 ± 2.6
70	9.3	30.0 ± 4.0	22.2 ± 4.5	15.5 ± 3.0	23.3 ± 5.6	18.9 ± 3.9	11.3 ± 3.5
150	12.1	10.1 ± 2.0	8.2 ± 1.3	4.6 ± 1.3	7.3 ± 2.1	5.4 ± 1.8	4.0 ± 0.9

Table 2

Time at which 50% ($t_{50\% \text{ released}}$) of the 20 and 70 kDa FITC–dextran have been released from MAX1 and MAX8 hydrogels formed at pH 7.4, 50 mM BTP, 150 mM NaCl buffer conditions at 25 °C. Time at which 25% ($t_{25\% \text{ released}}$) of the 150 kDa FITC–dextran has been released from MAX1 and MAX8 hydrogels.

Peptide	$t_{50\% \text{ released}}$ (h)		$t_{50\% \text{ released}}$ (days)
	20 kDa FITC–dextran (h)	70 kDa FITC–dextran (h)	150 kD FITC–dextran (days)
0.5 wt% Max1	8 ± 3	32 ± 3	–
2.0 wt% Max1	28 ± 3	67 ± 4	>28
0.5 wt% Max8	11 ± 1	31 ± 2	14 ± 3
2.0 wt% Max8	24 ± 2	63 ± 5	>28