Synthesis and Characterization of Enzymatically-Cross-Linked Poly(ethylene glycol) Hydrogels

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ABSTRACT: We demonstrate formation of a hydrogel network by cross-linking functionalized poly(ethylene glycol) (PEG) and a lysine-containing polypeptide through the action of a natural tissue enzyme, transglutaminase. The enzyme reaction rate using a PEG-modified peptide substrate is the same as the reaction rate for free substrate. Both the ratio and total concentration of the two macromers determine whether gelation will occur and the nature of the gel which forms. Under suitable conditions, clear gels form and swell to give a final composition which is 90% water. Diffusion coefficients of small proteins and albumin in the gel are comparable to those in free solution. Gelation proceeds under mild conditions and thus these gels hold potential for forming highly hydrated networks around living cells.

Introduction

New applications of biomaterials have created a compelling need for new synthetic or semisynthetic polymers which can form hydrogels under mild, physiological conditions. The need is particularly acute in the areas of cell-based therapeutics and tissue engineering. Cells in the body normally interact with highly complex gels of proteins and charged polysaccharides which serve as mechanical supports, transport barriers, and as sources of specific molecular signals to govern cell behavior.[1] Hydrogels which mimic certain features of natural extracellular matrix (ECM) are thus envisioned as a means to control the migration, growth, and organization of cells during tissue regeneration and wound healing *in vivo* or *in vitro*, or for stabilization of encapsulated and transplanted cells.

Many applications require the formation of a gel in the presence of cells, and it is often desirable to form a gel *in situ*, e.g., at an injection site. Biologically-derived gel-forming polymers—including polysaccharides, such as alginate,[2,3] and proteins, such as Type I collagen[4−7]—have been used with some success but have limitations with respect to control of degradation and molecular interactions with cells. Synthetic and semisynthetic hydrogels based on physical,[8−11] chemical,[12] or photochemical cross-linking[13] have been developed which offer improvements in the barrier and degradation properties of the gels. Gels incorporating cell adhesion moieties have also been developed.[14]

Another potential route to gel formation is through enzymatic cross-linking of synthetic macromolecular precursors. Enzyme cross-linking is attractive for several reasons. Enzymes can form covalent bonds between substrates, yielding bonds generally stronger and more permanent than physical cross-links. Further, enzymes often exhibit a high degree of substrate specificity, potentially allowing pendant peptide or polysaccharide moieties meant for interactions with cell surface receptors to be spared during the cross-linking reaction. Because the amount of enzyme present is one of the key determinates of the overall cross-linking rate, enzymatic cross-linking offers the potential for kinetic control of gel formation and thus for formation of homogeneous gels *in situ* via simple control of enzyme concentration. This type of kinetic control of gel formation may greatly aid the ability to deliver cell-based therapies in a noninvasive fashion—a solution of the gel precursor mixed with cells can potentially be injected through a needle to fill a defect many times larger in dimension than the injection needle itself.

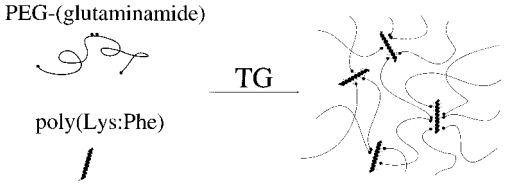

Figure 1. Transglutaminase (TG) cross-linking reaction with glutaminamide-functionalized PEG and poly(lysine:phenylalanine) to form a network. Each poly(KF) molecule is considered a cross-link node for the purposes of equilibrium swelling calculations.

Here, we describe the formation and properties of hydrogels obtained by enzymatically cross-linking end-modified poly(ethylene glycol) (PEG) macromers and a synthetic polypeptide as shown in Figure 1. PEG was chosen as the structural gel-forming synthetic polymer because it is hydrophilic, is relatively inert in body fluids, and is a widely used and accepted biomaterial.[15] Cross-linking is achieved by the action of a well-characterized and readily-available enzyme isolated from pig liver, transglutaminase. Transglutaminase is a family of enzymes whose function is to form an amide linkage between the γ-carboxamide group of certain peptidyl glutamine residues and primary amines such as the ϵ-amino group of lysine.[16] These calcium-dependent enzymes are ubiquitous in fluids and ECM throughout the body, forming cross-links in skin, liver and blood clots. Thus, the procedure could be adapted to human therapeutic use by choice of a human-derived enzyme.

The presence of transglutaminase throughout the body implies an equally broad distribution of naturally occurring substrates. This could allow intimal integration of the host tissue with the gel formed *in situ*, in the same manner that many cells and natural matrix molecules are held together.[17]

Experimental Section

Materials. A polyfunctional precursor, nominally "Tetrahydroxy PEG" ("PEG-$(OH)_4$") was purchased from Poly-

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sciences. Poly(ethylene glycol) (PEG) standards were from Scientific Polymer Products (90.4K M_w, 1.03 M_w/M_n; 35.2K M_w, 1.04 M_w/M_n; 10.9K M_w, 1.18 M_w/M_n; 3.07K M_w, 1.06 M_w/M_n). Glutaminamide (Q_a) was purchased from Bachem. Tresyl chloride was obtained from Aldrich. Triethylamine from Fisher was purified prior to use by distillation. Poly(lysine: phenylalanine)·HBr (Dp_{vis} = 185; 54 mol % lysine; MW_{vis} = 33.2 kDa), poly(lysine)·HBr (25.7 kDa,MW (LALLS) = 25.0 kDa; Dp(vis) = 123, Dp(LALLS) = 120; M_w/M_n = 1.20 by SEC-LALLS), and poly(lysine)·HBr (1 kDa, Dp(vis) = 6) were purchased from Sigma. Phosphate-buffered saline (PBS) (0.20 g/L KCl, 0.20 g/L KH_2PO_4, 8.00 g/L NaCl, 1.15 g/L Na_2HPO_4, 2.16 g/L $Na_2HPO_4{\cdot}7H_2O$, pH = 7.1) was from Gibco. Monodansylcadaverine was purchased from Aldrich. Transglutaminase and the diffusion test proteins—hen egg lysozyme, bovine erythrocyte carbonic anhydrase, and bovine serum albumin—were purchased from Sigma.

Transglutaminase Storage and Activity Assay. Enzyme aliquoting was performed in a 4 °C cold room. Lyophilized transglutaminase was dissolved in a transglutaminase storage buffer[18] of 10mM Tris−Acetate at pH = 6.0 with 160 mM KCl, 1 mM EDTA, and 2 mM DTT. The aliquoted enzyme solution was stored at −70 °C until use.

Transglutaminase activity was determined by the method of Folk and Chung.[18] Briefly, the incorporation of hydroxylamine into carbobenzyloxy-glutaminylglycine is measured by a colorimetric assay of the complex formed between the reaction product and acidic ferric chloride. The activity of the aliquoted enzyme was found to be 0.010 U/μL where one transglutaminase unit is defined by the amount necessary to form 1 μmol of hydroxamate per minute from *N*-α-benzyloxycarbonyl-glutamine-glycine and hydroxylamine at pH 6.0 at 37 °C.

Transglutaminase Activity with PEG-Bound Glutamine. To compare transglutaminase's activity toward peptidyl and PEG-bound glutamine residues, a standard transglutaminase activity assay based on the incorporation of monodansylcadaverine (mDC) was carried out on PEG-glycine-glutamine-glycinamide (PEG-GQG_a). PEG-GQG_a was synthesized by tresyl-mediated coupling (described below) in a solvent of dimethylformamide with methylene chloride. The assay reaction was carried out at room temperature with 9.56 mg/mL PEG-GQG_a and 1.64 mg/mL mDC in 100 mM Tris/chloride buffer containing 30 mM NaCl, 1 mM EDTA, and 50 mM $CaCl_2$. At various times during the reaction, aliquots were separated by gel permeation chromatography (cf. next section) to quantify the amount of PEG-linked mDC versus unlinked mDC.

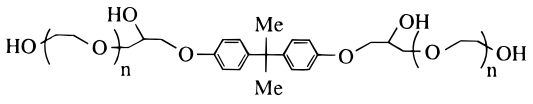
Gel Permeation Chromatography. Aqueous gel permeation chromatography (GPC) for analysis of PEG-OH and PEG-Q_a compounds was performed using two columns in series (TSK G4000PW and TSK G6000PW) with water/0.05% sodium azide as the mobile phase. Detection was by refractive index and light scattering. To calculate absolute molecular weights from light scattering data, the d*n*/d*c* of pure poly(ethylene oxide) was used.

Aqueous GPC for the transglutaminase activity assay was performed on a TSK G2000PW column with aqueous 0.05% sodium azide at 1.00 mL/min using UV photodiode array (200−350 nm range) detection.

Aqueous GPC for analysis of gel leachates was performed on three GPC columns in series (2 × TSKG4000PW and 1 × TSKG3000PW). The mobile phase was 50 mM Tris·HCl with 50 mM Tris base at 1.50 mL/min. Detection was at 266 nm.

Organic GPC for PEG-OH and PEG-Q_a characterization was run on two columns in series (Phenomenex linear and 1000 Å styrene−divinylbenzene) with chloroform at 1.00 mL/min at room temperature. Detection was by refractive index. For molecular weight determination, a calibration curve was generated using monodisperse linear PEG's.

PEG-OH and PEG-Q_a Characterization. The PEG precursor is described by the manufacturer as a predominately tetrafunctional reaction product of 10K PEG with Bisphenol A diglycidyl ether (structure **I**, "PEG-OH"). The product contains a mixture of single 10K chains, double PEG chains (structure **I**), and molecules containing three or more chains. On the basis of UV spectral analysis, the product was found



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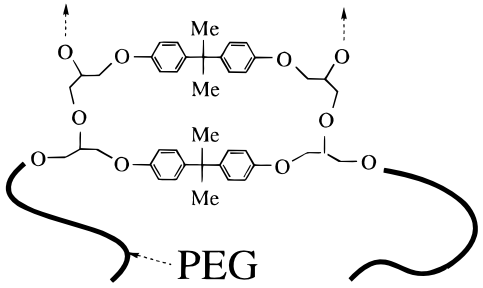

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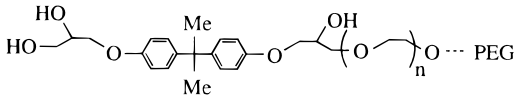

III

to contain approximately one Bisphenol unit per PEG chain. This could be explained either by some degree of stacking of Bisphenol units (structure **II**) due to aggregation during the synthesis process or by an incomplete synthesis reaction, resulting in molecules which terminate in Bisphenol units (structure **III**). It is presumed that the synthesis reaction is run to completion and that structure **II** accounts for the concentration of Bisphenol units in the product, implying that "PEG-OH" will have two hydroxyl groups for each PEG chain. The presumption of structure **II** is supported by evidence of branched species discussed below.

To determine the molecular weight distribution, PEG-OH was separated by both organic and aqueous gel permeation chromatography (GPC) under the conditions described above. Whereas PEG-Q_a in chloroform formed only unfilterable aggregates, PEG-Q_a was analyzed by aqueous GPC only. Using organic GPC, PEG-OH single chains (at 10.2K by linear PEG standards) were baseline separated from double chains (at 22.3K by linear PEG standards) and higher multiples, confirming the molecular weight of a single chain. It is possible that the presence of relatively stiff Bisphenol A in the double PEG chain enlarges the PEG random coil somewhat, causing it to elute at a slightly higher molecular weight than twice the single chain molecular weight. Peak integration gives 68 wt % single chains, 7.3 wt % double chains, and 25 wt % of triplets or higher. Species beyond 50K were beyond the linear range of the organic column.

Aqueous GPC with light scattering and refractive index detection was used to better resolve the higher molecular weight species. The highest molecular weight species was found to be 7.6×10^6 by LS, equivalent to approximately 700 chains. By comparison with the molecular weight of this species based on the elution volume of high molecular weight linear poly(ethylene oxide) standards (1.0×10^6), it was concluded that high molecular weight PEG-OH has some degree of branching, possibly by Bisphenol self-reaction, shown in structure **III**.

Pregel Macromer Synthesis. A multifunctional glutaminyl-PEG adduct, PEG-Q_a, was synthesized by tresyl-mediated coupling. PEG-OH (40 g) was dried by incubation at 4 °C overnight in 300 mL of dry methylene chloride over molecular sieves. Just prior to use the PEG/methylene chloride solution was decanted from the sieves. The sieves were rinsed with two 25 mL portions of dry methylene chloride to yield a total

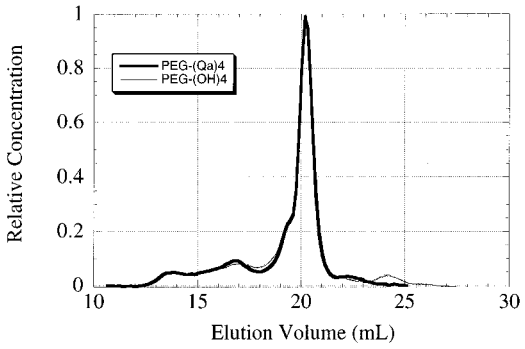

Figure 2. Gel permeation chromatogram for synthesis starting material (PEG-OH) and pregel macromer (PEG-Q_a).

of 350 mL of the PEG/methylene chloride solution. To this was added 2.64 mL of triethylamine with stirring. To initiate the tresylation, 2.0 g of tresyl chloride was added dropwise. The reaction flask was filled with dry argon and stirred for 2 h. The resulting PEG-Tr was purified by successive reprecipitations from acidified methanol using decreasing amounts of acid (37% aqueous HCl in MeOH: 1 × 1.7 μL/mL, 1 × 0.67 μL/mL, 2 × 0.33 μL/mL, 2 × pure MeOH). Residual amounts of methanol were removed *in vacuo*. Dry PEG-Tr was aliquoted into vials and stored at −70 °C until use. Based on the manufacturers stated molecular weight of 18.5K for PEG-OH, tresylation as found to be 90% ± 2% based on sulfur elemental analysis [53.72% C, 8.61% H, 0.54% S; average of two samples].

For the PEG/Q_a coupling reaction, 2.15 g of PEG-Tr was added to 19.6 mL of a solution of 100 mM Q_a·HCl and 200 mM imidazoyl acetate buffer at pH 7.0. The coupling proceeded overnight at 4 °C. Under these conditions in a separate experiment the apparent second-order rate constant for the displacement of the tresyl group by Q_a was measured to be 0.13 M^{-1} min^{-1} by monitoring the loss of primary amines of Q_a by the *o*-phthaldialdehyde fluorometric assay for primary amines.[19] The solution was frozen and dried by lyophilization. Purification was by successive reprecipitations from methanol. Residual amounts of methanol were removed *in vacuo*. The absence of residual uncoupled glutaminamine was verified by the *o*-phthaldialdehyde fluorometric assay for primary amines. Elemental analysis showed a substitution level of 0.126 ± 0.012 mmol Q_a per gram of polymer based on nitrogen elemental analysis [55.05% C, 8.83% H, 0.53% N; average of two samples].

PEG-OH and PEG-Q_a have identical molecular weight distributions (Figure 2), indicating that no detectable scission or cross-linking of PEG chains occurred during the synthesis process.

Network Formation. All gels were formed with 2.5 U/mL (2.3 μM) transglutaminase and 6 mM calcium chloride in a buffer of 50% transglutaminase storage buffer and 50% reaction buffer (100 mM Tris−HCl, 1 mM EDTA, pH 8.3) at 37 °C. The mixture of the enzyme plus the buffers has a pH of 7.5 at 37 °C. Gels for the diffusion assay were made with 20 wt % PEG-Q_a (25 mM Q_a) and 5.0 wt % poly(KF)·HBr (149 mM Lys). For swelling measurements, gels were made with 20%/5%, 10%/5%, and 20%/2.5% PEG-Q_a/poly(KF)·HBr concentrations. Gel components were mixed at 4 °C and then warmed to 37 °C for gelation.

To measure the amount of unreacted PEG-Q_a and poly(KF), gels were cast in the bottom of a 12.0 mm diameter glass vial and allowed to equilibrate in a series of three PBS wash solutions over a time span of 13 days (well beyond the observed equilibrium as well as the theoretical equilibrium based on analysis of the measured diffusion behavior of several solutes). The wash solutions were analyzed by aqueous GPC with detection at 266 nm.

Swelling Measurements. Experiments were conducted in a uniaxial swelling mode. Gels were formed in the bottom of a 12.0 mm diameter glass vial as described above and allowed to swell to equilibrium by addition of excess PBS on top of the formed gel. The swelling ratio, α, defined as the ratio of the swollen gel volume to the initial volume, is the ratio of the final (equilibrium) gel height to the initial gel height for the case of uniaxial swelling.

Diffusion Assay. A two half-cell diffusion apparatus was used to measure the diffusion constant of macromolecules in PEG-poly(KF) gels. The gel was formed and swollen in a $^3/_4$ in. inner diameter stainless steel ferrule secured to a glass slide. After swelling, the gel was further incubated for 4 days in multiple volumes of PBS to remove trace unreacted polymers and enzyme. To assemble the apparatus, gel and ferrule were inverted onto 0.45 μm Millipore PVDF (type HVLP04700) filter paper. The ferrule was then loaded into the washer housing and backed by another section of filter paper.

At the start of each experiment, the donor cell was filled with 3 mL of a PBS solution of a single test protein (1−2 mg/mL), and the acceptor cell was filled with 3 mL of pure PBS. The concentration of the acceptor cell was monitored over time by absorbance at 280 nm. Experiments were conducted at 22 ± 1 °C.

For partition coefficient determination, a gel was cast in the bottom of a 12.0 mm diameter glass vial, allowed to swell to an equilibrium height (12.5 mm), and cleared of unreacted poly(KF) and PEG-Q_a by multiple PBS washes over 4 days. A mixture of the test proteins (2.0 mg/mL BSA, 1.0 mg/mL carbonic anhydrase, and 3.0 mg/mL lysozyme in PBS) was added to the top of the gel, allowing the proteins to diffuse into the gel over 13 days. Samples of the protein solution above the gel were taken periodically and separated by aqueous GPC with detection at 280 nm to quantify the concentration of each protein individually. To compensate for the possibility of protein adsorption to the glass vial and protein degradation, a control vial containing the protein mixture but no gel was also monitored by aqueous GPC with detection at 280 nm. According to mathematical analysis of the time required to reach equilibrium, using measured diffusion coefficients, all proteins were expected to be in equilibrium after 13 days. These predictions were supported by constancy of protein concentration in the supernate by 13 days and thus partition coefficients were determined at the 13 day time point.

Results and Discussion

Activity of Transglutaminase toward PEG-Modified Substrates. Modification of enzymes and other therapeutic proteins with PEG chains is a common approach to enhancing the half life of these molecules in the bloodstream.[15] Such modifications typically do not substantially alter protein activity, presumably because small substrates can readily diffuse to the active sites without hindrance from the PEG chains. The situation here is the inverse of the therapeutic one—here, a small substrate is being modified with a large PEG chain—and we were concerned that the swollen, randomly-coiled PEG chain might impair the substrate's ability to reach the enzyme's active site. We thus compared the enzyme activity of PEG-bound substrates to free substrate by measuring the coupling of a standard, easily-detected donor amine, mDC, to PEG-glycine-glutamine-glycinamide (PEG-GQG$_a$), an analog of the macromolecular gel precursor, PEG-Q_a.

Based on the initial rate and the maximal mDC coupling achieved, an apparent rate constant of 1.2 ± 0.4 mM^{-1} min^{-1} (mean ± S. E. for a reaction profile) was calculated. This compares well with a literature value for benzyloxycarbonyl-glycine-glycine-glutamine-glycine of 1.3 mM^{-1} min^{-1} for mDC incorporation under the same buffer conditions.[20] It is not entirely surprising that the activity of the enzyme is unaffected by the presence of the large PEG chain. PEG is highly mobile in aqueous solution and recent measurements using the surface force apparatus have demonstrated that PEG-tethered ligands can bind to receptors under conditions where the PEG chain is stretched to its fully-extended length to bind.[21]

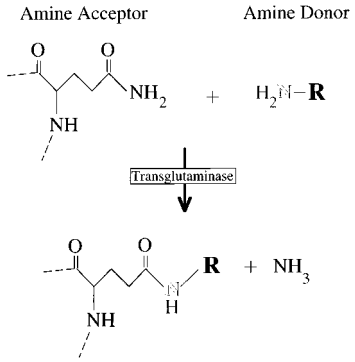

Figure 3. Typical substrates for the transglutaminase cross-linking reaction. *In vivo*, amine acceptors are generally peptidyl glutamines, and amine donors are a variety of primary amines.

An estimate of the gelation time can be derived using the measured enzyme activity along with other system parameters. Assuming equal reactivity of all substrates, Flory's theory for the critical point of gelation[22] predicts a critical conversion of Q_a residues as

$$X_{Q_a,\mathrm{crit}} = \sqrt{\left(\frac{C_{K,o}}{C_{Q_a,o}}\right)\frac{1}{(n_K - 1)}} \qquad (1)$$

where n_K is the number of lysine residues per molecule of poly(KF) (100) and $C_{K,o}/C_{Q_a,o}$ is the ratio of the initial concentrations of lysines and glutamines, respectively. This expression for the case of 100% difunctional PEG differs only slightly from the population of PEG-Q_a where 94% of the molecules are single PEG chains. For reaction mixtures comprising 20/5, 10/5, and 20/2.5 wt % PEG-Q_a/wt % polyKF, $X_{Q_a,\mathrm{crit.}}$ are 0.23, 0.33, and 0.16, respectively.

For a homogeneous solution, the excess of lysine residues results in a reaction which is pseudo first order in Q_a concentration. At a reaction rate of 1.2 mM^{-1} min^{-1}, the time to critical conversion is calculated to be 1.6, 2.4, and 1.1 h for 20/5, 10/5, and 20/2.5 gels, respectively. This represents a useful approximation but may differ from actual gelation times due to differences in activity between mDC and poly(KF) as well as differences in reactivity of individual lysine residues.[23] This calculation represents the minimum time to complete gelation, as potential transport limitations of the reactants and enzyme during the significant viscosity increases during the reaction are ignored. On the basis of these calculations, the enzyme concentration (2.3 μM) was chosen to allow transfer and manipulation of the "activated" (with transglutaminase) pregel solution before gelation is complete.

Influence of Macromolecular Precursor Structure and Concentration on Gel Formation. The design of gel precursors was driven by the substrate specificity requirements of the cross-linking enzyme transglutaminase. As a class of enzymes, transglutaminases exhibit a common set of substrate specificity requirements. Guinea pig liver transglutaminase was chosen for use here because it is the best characterized, is the most readily available and exhibits the most broad substrate specificity requirements. Numerous reviews describe the details of different transglutaminase catalyzed reactions.[16,24,25] The basic cross-linking chemistry carried out by the enzyme is illustrated in Figure 3. For the amine-donor substrate, absence of charge near the glutamine residue is necessary presumably due to a hydrophobic pocket at the active site.[23] For this reason glutaminamide was used rather than glutamine. For the amine donor substrate, the requirements are less stringent. Many primary amines are known to be substrates for transglutaminase at various levels of activity. The highest reaction rates are obtained for lysine when a hydrophobic amino acid immediately follows the lysine residue, reading N to C.[26] For this reason a random copolymer of lysine and phenylalanine (poly(KF)) was used to ensure a population of the preferred substrate. It is likely that the hydrophobic portion of the mDC probe used in the transglutaminase activity assay toward PEG-(GQG_a) accounts for its high level of activity.[27] Poly(lysine) was also attempted in the hope that lysine lacking an adjacent hydrophobic residue would be sufficiently active, yet these failed to gel. We therefore compared gels made from various amounts of PEG-Q_a and poly(KF). All gels were formed with a concentration of transglutaminase feasible for use in an *in vivo* setting, 0.017 wt %.

The concentrations of precursors can potentially affect gel formation in several ways, but a key lower bound on each precursor concentration can be identified. Poly(KF) is assumed to act as a rigid cross-link point for the mobile PEG chains; thus, its concentration determines the average distance between cross-links. The poly(KF) concentration must then be at least great enough that the average distance between cross-links, $(C_{\mathrm{poly(KF)}}N_A)^{-1/3}$, is less than the fully extended length of the PEG chains (60 nm) in the PEG-Q_a precursor. To achieve network formation with the minimum structural functionality (three) at all cross-links, the concentration of PEG chains functionalized on each end with Q_a must be at least $^3/_2$ the concentration of poly(KF). Effects of absolute and relative concentrations of each of the precursors are discussed below in the context of gelation results.

The concentrations of poly(KF), poly(K), and PEG-Q_a used to form gels in this study, and the resulting gel properties, are shown in Table 1 and gels will be referred to by the designations listed there. The average distances between cross-links for the concentrations of poly(KF) shown in Table 1—10 nm for 5 wt % and 13 nm for 2.5 wt %—are comparable to the RMS end-to-end distance of the PEG chains (10 nm) or slightly greater. The concentrations of PEG-Q_a used were 6−12-fold greater than the minimum (lower bound) concentration and are well above the minimal overlap concentration (1.4 wt %) for 10K PEG chains. All gels were formed with at least a 6-fold stoichiometric excess of lysine:Q_a residues, corresponding to on average 8−17 PEG chains cross-linked to a single poly(KF) chain, assuming all Q_a residues and poly(KF) react. The concentration of enzyme was approximately 3 orders of magnitude lower than the concentration of poly(KF) in all cases.

Mixtures (20/5.0) formed clear colorless gels with significant viscosity increases within 30 min. The gelation time is thus comparable to the estimate, obtained above, based on negligible diffusion resistance of the enzyme during gel formation. Based on analysis of components washed from the gel, 90% of the initial poly(KF) and 91% of the initial PEG-Q_a were covalently linked in the gel. The majority of leached PEG material comprised single chains. Reducing either the poly(KF) or the PEG-Q_a by half (20/2.5 and 10/5.0 gels) also allowed formation of clear gels, but small amounts of opacity concentrated at the top of both types of the gel were observed. The 10/2.5 combination formed only a viscous, opaque solution. No evidence of gelation was observed for either poly(K) precursor, despite compa-

Table 1. Gelation Behavior of Macromers

initial [poly(*x*)], wt % (mM)	initial [PEG-Q_a], wt % (mM)	designation %PEG/%KF	gel appearance	% precursor retained in final gel	
				PEG(Q_a)	poly(KF)
poly(KF), 33K					
5 (1.4)	20 (12.5)	20/5	clear, colorless gel	91	90
5 (1.4)	10 (6.3)	10/5	clear gel with thin opaque layer on the top	86	60
2.5 (0.7)	20 (12.5)	20/2.5	clear gel with thin opaque layer on the top	84	31
2.5 (0.7)	10 (6.3)	10/2.5	no gel; viscous, opaque solution		
poly(K), 1K					
5 (50)	20 (12.5)		no gel; clear solution		
poly(K), 25K					
5 (2.0)	20 (12.5)		no gel; clear viscous solution		

Table 2. Swelling and Network Behavior of PEG-Poly(KF) Gels

gel composition	swelling ratio α	entanglement ratio Φ
20/5	2.1	1.2
20/2.5	1.5	2.5
10/5	1.8	0.93
10/2.5	N/A	N/A

rable concentrations of available lysines, confirming the structural specificity of the enzyme.

The presence of opacity most likely is indicative of microgels that did not become cross-linked into the bulk of the gel. Excess fluid added to the top of the 20/2.5 and 10/5 dispersed the opaque portion, indicating incomplete gel formation in the upper opaque region. The clear regions of all gels remained entirely intact over several weeks in water.

Several explanations are possible for the observed incomplete gel formation. A tendency toward phase separation may play a role. PEG solutions with high concentrations of salt are known to phase separate into PEG-rich and PEG-poor phases.[28] This phenomenon is typically observed for small (2−3K) PEG at salt concentrations 1 order of magnitude higher than those found in the present study, yet at longer chain lengths, this effect is enhanced. Another possibility is a reaction driven inhomogeneity. During the process of gel formation, it is possible that a slight density increase in cross-linked PEG's could cause the more fully cross-linked species to sink, increasing the likelihood that a continuous gel layer would form below the discontinuous microgel layer. In the case of the 20/2.5 gel, it is possible that a transglutaminase-catalyzed termination side reaction may play a role. It has been shown that the rate of the competing hydrolysis reaction of PEG-glutamine → PEG-glutamic acid is enhanced as the concentration of the lysine substrate is reduced.[29,30] This reduction of the number of cross-linkable moieties could lead to the observed incomplete reaction.

Equilibrium Swelling Behavior. Swelling behavior is an important parameter for many of the intended applications of the gels and was used to assess features of the resulting network structure. Gels were swollen to equilibrium in cylindrical glass vials and exhibited swelling ratios in the range 1.5−2.1 (Table 2).

We can use these swelling data to estimate the efficiency of linking both ends of a PEG-Q_a macromer into the gel under the different gelation conditions by modifying Flory's equilibrium swelling theory to account for the types of cross-links in the PEG-Q_a/poly(KF) gels. Analysis of swelling data provides the number of elastically effective chains in the gel, and this number can be compared to the total number of macromer chains present in the gel. Elastically effective chains may arise from both chemical cross-links and physical cross-links (entanglements), and thus separate information on the relative numbers of physical and chemical cross-links is needed to estimate the fraction of free chain ends.

The important physical aspect of the gel to be captured in equilibrium swelling analysis is the stretching of Gaussian chains. We define a cross-link as a non-Gaussian, or stiff region, that joins together two or more Gaussian chains. Due to the long persistence length of peptides in general and the expected rodlike structure of poly(KF) in ionic solutions,[31,32] it is most appropriate to define each poly(KF) molecule as a node or cross-link. The presumed network structure is depicted in Figure 1**.** The functionality, *f*, of any particular cross-link is the number of PEG chains attached to a particular poly(KF) molecule. The theoretical mean cross-link functionality is determined from the stoichiometry of the reaction solution; *e.g.*, a 20/5 gel would lead to a theoretical cross-link functionality of 17, or 17 PEG chains attached to each poly(KF) molecule. The cross-link functionality distribution of poly(KF) is not known *a priori*; however, the theoretical value is a maximum which may not be reached, and we presume a distribution of cross-link functionalities will exist in the gel. Following the original development of Flory, we derived an expression for the number of elastically effective chains in the gel, ν_e, as a function of α for the case of polydisperse network functionality, and defined a parameter Φ which represents the salient features of the network structure:

$$\Phi = \frac{\text{number of elastically effective chains}}{\text{number of macromer chains}}$$

Details of the derivation and data analysis are provided in the Appendix. Values of Φ from eq A17 for various proportions of PEG to poly(KF) are given in Table 2 and can be compared to gain insight into the process of PEG-poly(KF) gel formation. Note that Φ can be greater than 1, since physical entanglements lead to elastically effective chains. In an ideal gel it would be possible to quantify the number of dangling chain ends based on the Miller−Macosko theory,[33,34] thus making Φ a measure of entanglements for the case of negligible chain loops. However in PEG-poly(KF) gels, the reactivity of each lysine is dependent on adjacent amino acid residues to an unknown degree, precluding the use of this type of probabilistic analysis.

Values of Φ(20/5) and Φ(10/5) are comparable and significantly lower than Φ(20/2.5). Thus, the poly(KF) concentration appears to exert a dominant effect. It is unlikely this effect arises from stoichiometric reaction considerations; lysine residues are present in excess relative to Q_a residues and free lysine concentrations change only slightly throughout the gelation reaction (17% for the 20/5 gel and 8.5% for the 10/5 gel). Furthermore, since the extent of completion of the transglutaminase reaction is a function of lysine concentration, in the absence of diffusion limitations on transglutaminase (affirmed below), the proportion of dangling ends in the 20/5 should be slightly higher than in the 10/5 gel and Φ(20/5) should be slightly less than

Table 3. Properties of Proteins Used in Diffusion Experiments

property	lysozyme	carbonic anhydrase	BSA
M_w(kDa)	14.3	29	66
$10^{-6}D_{\text{free solution}}$ (cm^2/s) (ref)	1.1 (51)	1.0 (52)	0.58 (53)
$K_{pg,apparent} \pm$ S. E.	0.46 ± 0.02	0.46 ± 0.06	3.9 ± 0.2
isoelectric point	11	5.9	4.7
$C_{1\infty}$ (mg/mL) $\pm$ S. E.	0.850 ± 0.004	1.808 ± 0.009	2.06 ± 0.02

Φ(10/5). The opposite is observed—Φ(20/5) is slightly greater than Φ(10/5).

It is probable that the concentration of physical entanglements contributes significantly to the differences in Φ. The average molecular weight between entanglements M_{en}, is 2200 in bulk PEG,[35] and is greater for PEG in solution. Gnanou and co-workers[36] determined the fraction of physical entanglements in end-linked PEG gels with tetrafunctional cross-links as a function of polymer volume fraction in the gel and reported a decreasing fraction of entanglements with decreasing polymer volume fraction and chain length. At a pre-gel polymer volume fraction of 0.32, Gnanou et al. reported that 30% of the chains were entangled for gels with 5.6K PEG chains; we expect a similar amount of overlap for 10K PEG at a volume fraction of 0.20. In the PEG/poly(KF) gels, it is expected that the localization of many chains at PEG-poly(KF) cross-links (17−34) would increase entanglements over the case of the Gnanou study—we expect more physical entanglements in gels with higher average PEG concentrations *or* higher concentrations of PEG at the poly(KF) cross-link.

This interpretation is consistent with the data. With half the number of poly(KF) molecules in the 20/2.5 gel as in the 20/5 gel, the mean cross-link functionality doubles from 17 to 34—effectively doubling the concentration of PEG chains in the vicinity of the cross-link and increasing the probability of entanglements. Likewise, Φ(20/5) is somewhat greater than Φ(10/5), indicating that the bulk PEG concentration exerts a similar effect, but less profound.

In applications of these gels as cellular scaffolds, it may be desirable to reduce the swelling ratio as much as possible. The data in Table 2 imply a means of controlling swelling behavior of PEG-poly(KF) gels by variations in PEG-Q_a and poly(KF) concentrations, yet the formation of a microgel layer is indicative of lower limits of both constituents. Still, there are alternative means of achieving the effects of a higher degree of entanglements and higher cross-link densities. Fractionation of the PEG-OH starting material into a pool of higher molecular weight species could reduce the likelihood of microgel formation, allowing for lower concentrations of PEG-Q_a to be used. Reducing the molecular weight of the PEG chains would also tend to reduce the degree of swelling. Manipulation of the poly(KF) molecule could influence swelling effects as well. By increasing the amount of lysine in poly(KF) by either increasing the proportion of lysine or increasing the molecular weight, a higher cross-link density could be achieved. Any of these manipulations may affect other gel properties in a positive or negative way.

Protein Partitioning and Diffusion in Gels. Diffusion of proteins in the gels is important both for gel formation, where restrictions on diffusion of the enzyme may impair gel formation, and for the function, since many cellular functions are governed by macromolecular nutrients and cytokines released by adjacent cells or transported from blood. Diffusion and partition coefficients were determined by a combination of steady state and transient flux measurements in a standard two-cell diffusion chamber and by equilibrium measurements of partitioning. The partition coefficients for the gel is defined as $K_{pg} = c_{gel}/c_{bulk}$. The apparent values of K_{pg} for lysozyme, carbonic anhydrase, and albumin are shown in Table 3 along with the physicochemical properties of the proteins. The small proteins exhibit comparable exclusion from the gel while albumin shows an apparent partitioning into the gel. Albumin has an isoelectric point (IE) of 4.7[37] and is thus negatively charged at pH 7.1, which makes it attractive to the positively charged lysine residues of poly(KF) in the gel. Thus the apparent partition coefficient for albumin is the product of the true partition coefficient (due to excluded volume) and a binding term. Carbonic anhydrase is slightly acidic (IE = 5.9[38]) yet appears to behave in a neutral fashion with respect to partitioning.

The steady-state flux across the gel, $N_{protein}$, is written in terms of the overall concentration difference between the bulk concentration in the donor chamber, $c_{1\infty}$, and that in the receiver chamber, $c_{2\infty}$, as

$$N_{protein} = (c_{1\infty} - c_{1\infty})/R_{TOT} \tag{2}$$

The overall resistance to mass transfer, R_{TOT}, is the sum of the diffusion resistance of the gel, the diffusion resistances of each supporting membrane (presumed identical), and the convection resistances of each stirred cell (presumed identical):

$$R_{TOT} = \frac{\delta_g}{K_{pg}D_g} + \frac{2\delta_m}{K_{pm}D_m} + \frac{2}{k_c} \tag{3}$$

where δ_g and δ_m are the thicknesses of the gel sample and the PDVF support membrane, respectively, D_g and D_m are the diffusion coefficients in the gel and membrane, and k_c is the convective mass transfer coefficient.

The convective resistances are negligible in comparison to the diffusion resistances in this system, and we can thus write the resistance in the support membranes as a fraction of the total resistance as

$$\frac{2\delta_m/(K_{pm}D_m)}{2\delta_m/(K_{pm}D_m) + \delta_g/(K_{pg}D_g)} = \frac{1}{1 + \left(\frac{\delta_g}{2\delta_m}\right)\left(\frac{K_{pm}}{K_g}\right)\left(\frac{D_m}{D_g}\right)} \tag{4}$$

For this experimental arrangement $\delta_g \approx 300\delta_m$ (i.e., gel = 3 mm and membrane = 0.01 mm), and the partition coefficients, which are governed by excluded volume effects, should be of comparable values for the membrane and gel. We also expect that the diffusion coefficients will be comparable in magnitude or that the gel diffusion coefficient will be less than that in the membrane due to the large, open pores in the membrane compared to the gel. Thus, the resistance of the support membranes is estimated to be <10% of the gel and can be neglected for our purposes here. These assumptions are supported by the values of the measured diffusion coefficients for small solutes in the gel, which are equivalent to those in free solution. Steady state flux measurements, achieved when $t \approx 0.5\delta_g^2/D_g$, thus allow the product K_gD_g to be determined.[39]

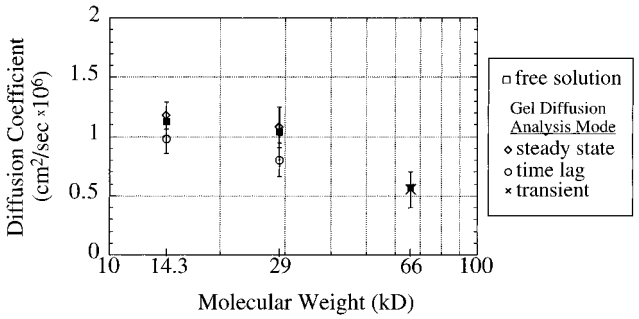

Figure 4. Diffusion coefficients for solutes of various molecular weights in 20/5 gel: lysozyme (14.3 kDa), carbonic anhydrase (29 kDa), and bovine serum albumin (66 kDa).

Independent values of the diffusion coefficients may be extracted from analysis of the transient regime. In the absence of binding to the matrix, the species balance for protein diffusion in the gel is

$$\frac{\partial c_g}{\partial t} = D_g \frac{\partial^2 c_g}{\partial x^2} \tag{5}$$

Under our experimental conditions, the initial concentration of protein in the gel is zero, $c_{1\infty} >> c_{2\infty}$ and $c_{1\infty} \cong 0$ throughout the experiment, and thus

$$\frac{Q_t}{\delta_m c_g} = \frac{D_g t}{\delta_g{}^2} - \frac{1}{6} - \frac{2}{\pi^2}\sum_1^\infty \frac{(-1)^n}{n^2} \exp(-D_g n^2 \pi^2 t/\delta_g{}^2) \tag{6}$$

where Q_t is the total amount of material which has passed through the gel at time t. As $t \rightarrow \infty$, this solution approaches a line which has an intercept on the t-axis given by

$$\tau_{lag} = \delta_g{}^2/6D_g \tag{7}$$

The diffusion coefficients for carbonic anhydrase and lysozyme were determined by both steady state and time lag analysis, and the two analyses led to values that were not statistically different from each other and comparable to the values for diffusion in free solution (Figure 4).

The apparent partition coefficient for albumin is greater than 1 and thus albumin binds to the gel, probably to the positively-charged poly(KF). While rapid, reversible binding does not affect the steady-state flux, the binding sites act as a sink during transient diffusion and this results in a lower apparent value of the diffusion coefficient. For rapid, reversible binding, the transient species balance becomes

$$\frac{\partial c_g}{\partial t} = \frac{D_g}{1 + \dfrac{N_t K_d}{(K_d + c_g)^2}} \frac{\partial^2 c_g}{\partial x^2} \tag{8}$$

where N_t is the concentration of binding sites in the gel, c_g is the concentration of free (unbound) ligand in the aqueous phase in the gel, and the dissociation constant, K_d, relates the concentration of unoccupied binding sites, N, and the concentration of bound protein-site complexes, $N - p$, to the concentration of protein in free solution within the gel:

$$K_d = \frac{N c_g}{N - p} \tag{9}$$

At very high protein concentrations, the diffusion coefficient approaches the true value, and at very low protein concentrations, the apparent diffusivity becomes $D_g/(1 + N_t/K_d)$. The concentration of poly(KF) in the gel is about 1 mM and the size of poly(KF) (~28 nm × 1−2 nm, based on 1.5A rise per residue in an α helix) suggests it can bind 1−2 albumin molecules (13 × 4 nm) each, along with the cross-linked PEG chains; thus $N_t \sim 1-2$ mM. An albumin concentration of ~0.01 mM was used in the partitioning experiments; i.e., $c_g << N_t$ and thus $N \sim N_t$. The total concentration in the gel, $c_{g,tot}$ was 3.9 times greater than that in free solution, c_∞. Since $c_g \sim 0.5c_\infty$, $N - p \cong 7c_g$ and $K_d \cong N_t/7$. Under the experimental conditions used, $c_g \sim 0.01$ mM $<< K_d$ and thus it follows $D_{g,apparent} = D_g/(1 + 7)$.

The apparent BSA diffusivity was calculated from the time-dependent solution and is a factor of 8 less than diffusion in free solution, which indicates that the true diffusivity of BSA in the gel is the same as that in free solution (Figure 4). PEG gels formed from 10K linear diacrylates show similar behavior with the BSA diffusion coefficient in the gel on the order of the value in free solution as calculated from protein release data.[40] PEG gels formed from the acrylated form of the same PEG precursor used in this study are also highly permeable to BSA.[41] However, PEG gels formed by radiation-cross-linking linear PEG to obtain a molecular weight between cross-links (M_c) comparable to the size of the PEG macromer chains used here (10K) significantly inhibit diffusion of albumin compared to free solution.[42] For radiation-cross-linked gels, which have a uniform gel structure comprising tetrafunctional cross-links, $K_{pg} \sim 1$, whereas for the enzymatically cross-linked gels, $K_{pg} \sim 0.5$, suggesting that the gels possess different structures.

From this analysis, we can infer the diffusion characteristics of the enzyme in the gel, which we expect to be comparable to the diffusion behavior in free solution based on the rapidity of gel formation. The diffusion coefficient of transglutaminase in free solution is 0.68 × 10^{-6} cm²/s at 20 °C,[43] a value greater than that for BSA (0.576 cm²/s at 20 °C). Transglutaminase has a greater weight than BSA (77K vs 67K) but is more spherical, resulting in a lower Stokes−Einstein radius for transglutaminase (32 Å vs BSA of 36 Å). Thus, the gel should present negligible diffusional resistance to the enzyme during or after gel formation.

Free diffusion of transglutaminase could be inhibited if the enzyme is covalently cross-linked into the gel. Self-reaction is know to occur,[44] but only in the absence of the glutaminyl substrate. Thus, transglutaminase may cross-link itself onto poly(KF) but this outcome is likely only after gel formation—i.e., consumption of the preferred substrate—is substantially complete. The kinetics for gel formation are comparable to those expected for reaction of the enzyme in free solution, which suggests that such self-cross-linking of the enzyme, if it occurs, happens after the gel is formed.

Conclusions

We have demonstrated the feasibility of forming highly hydrated synthetic PEG-based hydrogels via enzymatic cross-linking using simple macromers. These

gels offer the potential for kinetic control of the gelation process and may thus be useful where formation of a gel in situ, e.g., by extrusion or injection, is desired. In the future, use of multifunctional PEG macromers should enable formation of gels containing pendant ligands for cell interactions, and for formation of gels based solely on modified PEGs.

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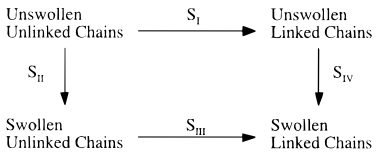
Appendix: Number of Effective Chains from Equilibrium Swelling

Equilibrium swelling data can be used to estimate the number of effective chains in the PEG-poly(KF) network. The number of effective chains may differ from the actual number of chains due to entanglements, loops, and dangling ends. The actual number of chains can be calculated from the chain molecular weight distribution and compared with the calculated number of effective network chains to assess the degree of entanglements versus loops and dangling ends.

To proceed with such calculations, the number functionality of each cross-link must be known. In a two-component gel such as PEG-poly(KF) the question arises as to where to define the cross-link. Realizing that the important physical aspect of the gel to be captured in our analysis is the stretching of Gaussian chains, it is necessary to define a "cross-link" as a non-Gaussian or stiff region that joins together two or more Gaussian chains. As mentioned in the discussion, it is most appropriate to define each poly(KF) molecule as a node or "cross-link", as in Figure 1. Thus the functionality of any particular cross-link will be the number of PEG chains attached to that particular poly(KF) molecule. The stoichiometry determines the mean cross-link functionality. For example a gel of 20 wt % PEG-Q_a and 5.0 wt % poly(KF)·HBr gives a mean cross-link functionality of 17, or 17 PEG chains attached to each poly(KF) molecule if all chains are linked into the network. However, it is not initially obvious how the phenomenon of equilibrium swelling will depend on the distribution of cross-link functionalities. Because the cross-link functionality distribution of poly(KF) is not known *a priori*, the treatment of equilibrium swelling must be reformulated with consideration for a distribution of cross-link functionalities for poly(KF). To do this, we can follow the method of Flory's original derivation for equilibrium swelling of multifunctional cross-links.[45] The total entropy of the swelling process can be described in three steps:

$S_{IV} = S_{II} + S_{III} - S_I$



To describe this process appropriately, it is necessary to describe each PEG-Q_a as consisting of two PEG chains attached at the ends, as shown in Figure 1, because the moiety joining the two chains can itself become covalently linked to poly(KF). Also note that it is probable that a single chain will be involved in cross-links of different functionalities at each end. Therefore, it is best to define a variable, $\epsilon_{PEG,f}$, to denote the number of effective chain ends of a given functionality, f.

The first process, S_I, describes the entropy of joining all chains into the network. To form a network, all chain ends must be localized with the volume of their respective cross-links. Ignoring, for the moment, that PEG chains are joined into pairs, the probability of the first poly(KF) molecule in a cross-link of degree f being colocalized in a volume of a cross-link, δV, with fPEG ends is given by

$$\epsilon_{PEG,f}(\epsilon_{PEG,f} - 1)(\epsilon_{PEG,f} - 2)\cdots(\epsilon_{PEG,f} - f + 1)(\delta V/V_o)^f \quad \text{(A1)}$$

The probability of the second cross-link forming is then

$$(\epsilon_{PEG,f} - f)(\epsilon_{PEG,f} - f - 1)\cdots(\epsilon_{PEG,f} - 2f + 1)(\delta V/V_o)^f \quad \text{(A2)}$$

and so on. The total probability of forming all ($\epsilon_{PEG,f}/f$) cross-links of degree f is the product of all these factors, or

$$\Omega_{I,f} = \epsilon_{PEG,f}! \sum_{i=1}^{\epsilon_{PEG,f}/f} [\delta V/V_0]^f = \epsilon_{PEG,f}!(\delta V/V_0)^{\epsilon_{PEG,f}} \quad \text{(A3)}$$

Until this point the PEG chains have been considered to be singlets. To account for the presence of doublets and higher multiplets, the probability of joining the chains into the distribution of PEG multiplets found in PEG-Q_a can similarly be constructed as

$$\Omega_{I,join} = [(1)^{f_1\nu_{PEG}}][(f_2\nu_{PEG}/4)!(2\partial V'/V_o)^{f_2\nu_{PEG}/4}]\cdots [g(f_i\nu_{PEG})(\partial V'/V_o)^{f_i\nu_{PEG}(i-1)/2i}]$$

$$\Omega_{I,join} = (\partial V'/V_o)^{\kappa\nu_{PEG}}\prod_i g_i(f_i\nu_{PEG}) \quad \text{with} \quad \kappa = \sum_i \frac{f_i(i-1)}{2i} \quad \text{(A4)}$$

where f_i is the weight fraction of PEG-Q_a molecules with i PEG chains. The factor κ can vary from $\kappa = 0$ for the case of solely single chains to $\kappa = {}^1/_2$ at the limit of very large multiplets.

Taking into account that the PEG-poly(KF) system has a distribution of cross-link functionalities, the total probability of forming all network cross-links is given by the product of the probabilities for each f divided by the probability of pairing chains into PEG-Q_a molecules. But we must also take into account that each poly(KF) molecules was randomly assigned a value of f by multiplying by the possible permutations. The total

probability then becomes

$$\Omega_I = \frac{\nu_{poly(KF),e}!}{\prod_{f=2}^{D_p} \nu_{poly(KF),e,f}!}\left(\frac{\prod_{f=2}^{D_p}\Omega_{I,f}}{\Omega_{I,join}}\right) = \frac{\nu_{poly(KF),e}!\prod_{f=2}^{D_p}\left[\left(\frac{\epsilon_{PEG,f}!}{\nu_{poly(KF),e,f}!}\right)\left(\frac{\delta V}{V_0}\right)^{\epsilon_{PEG,f}}\right]}{(^{\partial V'}/_{V_o})^{\kappa\nu_{PEG}}\prod_i g_i(f_i\nu_{PEG})} \quad (A5)$$

where D_p is the number of lysines per poly(KF) molecule and $\nu_{poly(KF),e}$ is the number of effective poly(KF) chains. Employing Stirling's approximation (for n large, $\ln(n!) = n\ln(n) - n$), S_I is given by

$$S_I = k \ln \Omega_I$$

$$S_I = k\left\{\nu_{poly(KF),e}\ln\left(\frac{\nu_{poly(KF),e}}{e}\right) - \kappa\nu_{PEG}\ln\left(\frac{\partial V'}{V_o}\right) - \sum_i \ln(g_i(f_i\nu_{PEG})) + \sum_{f=2}^{D_p}\left[\epsilon_{PEG,f}\ln\left[\frac{\epsilon_{PEG,f}\delta V}{V_0 e}\right] - \nu_{poly(KF),e,f}\ln\left(\frac{\nu_{poly(KF),e,f}}{e}\right)\right]\right\} \quad (A6)$$

For the second mixing step, using the subscript 0 to denote the unswollen state, the entropy can be expressed as

$$S_{II} = -k\left[n_{solv}\ln\nu_{solv} - n_{solv,0}\ln\nu_{solv,0} + \nu_{PEG}\ln\left(\frac{\nu_{PEG}}{\nu_{PEG,0}}\right) + \nu_{poly(KF)}\ln\left(\frac{\nu_{poly(KF)}}{\nu_{poly(KF),0}}\right)\right] \quad (A7)$$

where ν_i, ν_i, and n_1 are volume fractions, number of polymer molecules, and the number of solvent molecules, respectively. This can be reexpressed in terms of the swelling ratio, α, as

$$S_{II} = -k[n_{solv}\ln\nu_{solv} - n_{solv,0}\ln\nu_{solv,0} - (\nu_{PEG} + \nu_{poly(KF)})\ln\alpha] \quad \text{where} \quad \alpha = {}^{V}/_{V_0} \quad (A8)$$

Values for ν_{PEG} and $\nu_{poly(KF)}$ can be calculated from

$$\nu_i = \frac{w_i N_A}{\beta_i M_i} \quad (A9)$$

where w_i is the weight of species i in the network, N_A is Avogadro's number, and M_i is the molecular weight of species i. The parameter β_{PEG} is the number average number of PEG chains per PEG-Q_a molecule, while $\beta_{poly(KF)}$ has a value of unity.

The third step of interlinking the diluted chains of step 2 is achieved in two steps: stretching followed by linking in a process similar to step 1. The elastic component is identical to Flory's except that in the PEG-poly(KF) system the gel is swollen uniaxially as described in the Swelling Measurements section, yielding

$$S_{III,el} = k\frac{\epsilon_{PEG}}{2}[\ln\alpha - (\alpha^2 - 1)/2] \quad (A10)$$

The stretching component development is identical to step 1 above. The result differs only in that the volume is V instead of V_0.

$$S_{III,stretch} = k\left\{\nu_{poly(KF),e}\ln\left(\frac{\nu_{poly(KF),e}}{e}\right) - \kappa\nu_{PEG}\ln\left(\frac{\partial V'}{V}\right) - \sum_i \ln(g_i(f_i\nu_{PEG})) + \sum_{f=2}^{D_p}\left[\epsilon_{PEG,f}\ln\left[\frac{\epsilon_{PEG,f}\delta V}{Ve}\right] - \nu_{poly(KF),e,f}\ln\left(\frac{\nu_{poly(KF),e,f}}{e}\right)\right]\right\} \quad (A11)$$

Summing eqs A6, A8, A10, and A11 gives

$$S_{IV} = -k[n_{solv}\ln\nu_{solv} - n_{solv,0}\ln\nu_{solv,0} - ((1-\kappa)\nu_{PEG} + \nu_{poly(KF)})\ln\alpha + e_{PEG}(\alpha^2 - 1)/4] \quad (A12)$$

Having derived an expression for the entropy of swelling, we can now focus on the condition where the PEG-poly(KF) gel has swollen to equilibrium. At the condition of equilibrium swelling,

$$N_A\left(\frac{\partial\Delta F_{swell}}{\partial n_{solv}}\right)_{T,P} = 0 \quad (A13)$$

where

$$\Delta F_{swell} = \Delta H_{mix} - T\Delta S_{mix} - T\Delta S_{elastic} = \chi_{solv,PEG}\nu_{PEG}n_{solv} + \chi_{solv,poly(KF)}\nu_{poly(KF)}n_{solv} - TS_{IV} \quad (A14)$$

Using the following relations

$$\left(\frac{\partial\alpha}{\partial n_{solv}}\right) = {}^{\nu_{solv,0}}/_{n_{solv,0}}, \qquad \left(\frac{\partial\nu_{solv}}{\partial n_{solv}}\right)_{T,P} = \frac{\nu_{solv}}{n_{solv}} - \frac{\nu_{solv}^2}{n_{solv}},$$
$$\nu_{solv} = 1 - \frac{\nu_{PEG,0} + \nu_{poly(KF),0}}{\alpha} \quad (A15)$$

evaluation of (5) gives

$$N_A kT\left\{\ln\left[1 - \frac{\nu_{PEG,0} + \nu_{poly(KF),0}}{\alpha}\right] + \frac{1}{\alpha}(\nu_{PEG,0} + \nu_{poly(KF),0})\left(1 + \frac{\chi_{solv,PEG}\nu_{PEG,0} + \chi_{solv,poly(KF)}\nu_{poly(KF),0}}{\alpha}\right) + \frac{\nu_{solv,0}}{n_{solv,0}}\left(\frac{\alpha\epsilon_{PEG}}{2} - \frac{\nu_{PEG}(1-\kappa) + \nu_{poly(KF)}}{\alpha}\right)\right\} = 0$$

or

$$\epsilon_{PEG}/N_A = \frac{2}{\alpha^2}\left\{\frac{\nu_{PEG}(1-\kappa) + \nu_{poly(KF)}}{N_A} - \frac{n_{solv,0}}{N_A\nu_{solv,0}}\left[\alpha\ln\left(1 - \frac{\nu_{PEG,0} + \nu_{poly(KF),0}}{\alpha}\right) + (\nu_{PEG,0} + \nu_{poly(KF),0})\left(1 + \frac{\chi_{solv,PEG}\nu_{PEG,0} + \chi_{solv,poly(KF)}\nu_{poly(KF),0}}{\alpha}\right)\right]\right\} \quad (A16)$$

where $\chi_{solv,PEG}$ is taken as 0.45[46] and $\chi_{solv,poly(KF)} = 0.47$ (assumed to be bounded by the value for poly(lysine), $\chi_{water,poly(KF)} = 0.43$[47] and the theoretical maximum $\chi = 0.5$).

The number of ends participating in effective cross-links, ϵ_{PEG}, can be used to calculate a net entanglement ratio, Φ:

$$\Phi = \frac{\epsilon_{PEG}/2}{\beta_{PEG}\nu_{PEG}} \qquad \text{(A17)}$$

Values of Φ can be used to assess the degree of entanglements versus loops and dangling chain ends. Entanglements will increase Φ above unity, while chain loops and chains attached at only one end will tend to decrease Φ below unity. Therefore, Φ gives a measure of the balance of the two effects.

To calculate Φ from eq A17, it is first necessary to calculate κ of eq A4 and β_{PEG} of eq A9, which are dependent on the molecular weight distribution of PEG-Q_a. Contributions from single-chain and double chain species were taken directly from organic GPC peak integrations ($f_1 = 0.679$, $f_2 = 0.073$). The weight fractions for species of three chains and higher were calculated from aqueous GPC light scattering/refractive index data. For the distribution of PEG-Q_a in Figure 2, $\kappa = 0.13$ and $\beta_{PEG} = 1.4$.

In interpreting the values for Φ of different PEG/poly-(KF) proportions, it is instructive to examine the sensitivity of the Φ to perturbations in the various parameters and variables in eq A16. A straightforward means of doing this is by evaluating $\partial\Omega/\partial m_i$ for every parameter and variable, m_i. This was done by varying a single m_i by 0.1% while holding every other $m_{j\neq i}$ constant and performing the following calculation:

$$\left(\frac{\partial\Omega}{\partial m_i}\right)_{m_j\neq i} \cong \frac{(\Omega|_{m_i+\Delta m_i})_{m_j\neq i} - (\Omega|_{m_i})_{m_j}}{\Delta m_i} \qquad \text{where}$$

$$\Delta m_i = 0.001 m_i \qquad \text{(A18)}$$

It is also useful to define a factor, η as

$$\eta(i) = m_i(\partial\Omega/\partial m_i) \qquad \text{(A19)}$$

The factor $\eta(i)$ is then a measure of the fractional change that would result in Φ for a given fractional change in m_i. For example, $\eta(i) = -2$ would mean that a 1% increase in m_i would cause a 2% decrease in Φ if $(\partial^2\Omega/\partial m_i^2) = 0$. Through this sensitivity analysis, it was found that α ($\eta(\alpha)_{20/5\ gel} = -4.8$) and χ ($\eta(\chi_{solv,PEG})_{20/5\ gel} = -4.0$) had the largest influence on Φ for all gels. The next largest influence was from ρ_2 ($\eta(\rho_2)_{20/5\ gel} = -1.7$). This suggests that the uncertainty in values for Φ are largely a function of the uncertainty in α and $\chi_{solv,PEG}$. The precision of the measurement of α predicts an uncertainty of 1.7%. A survey of literature values for $\chi_{solv,PEG}$ reveals that reported values[42,46,48,49] vary in a range of 0.43 to 0.45, the value of 0.45 giving the best agreement with SANS data.[50] This range could be taken as twice the uncertainty in $\chi_{solv,PEG}$ (2.2%). Using these values for the uncertainties in α and $\chi_{solv,PEG}$ and the values for $\eta(\alpha)$ and $\eta(\chi_{solv,PEG})$ for the 20/5, 10/5, and 20/2.5 gels, uncertainties in Φ were calculated to be 12%, 9.4%, and 28%, respectively.

References and Notes

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