Control and Prediction of Gelation Kinetics in Enzymatically Cross-Linked Poly(ethylene glycol) Hydrogels

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ABSTRACT: We demonstrate control of gelation kinetics in formation of poly(ethylene glycol) (PEG) hydrogels by enzymatic cross-linking. A predictive model for gelation kinetics based on macromer structure and composition, stoichiometric ratios of reactants, cross-linking enzyme concentration, and the underlying kinetics of enzyme reaction was developed on the basis of classical Flory−Stockmayer theory. Experiments with substrate-functionalized multiarm comb PEG showed good agreement with theory upon variation of the enzyme concentration, with a slight overprediction in the time to gelation. Experiments with a substrate-functionalized difunctional PEG in conjunction with a polypeptide where macromer concentrations were varied were also consistent with theory, but with a slight underprediction of gelation time.

Introduction

We have previously demonstrated the feasibility of forming hydrogels using a naturally occurring tissue enzyme to cross-link peptide-modified poly(ethylene glycol) (PEG) macromers.[11] This mode of gel formation may be particularly useful in tissue engineering and drug delivery applications because it can be carried out under mild, physiological conditions with macromers that may include biological moieties susceptible to cross-linking by standard chemical means. An additional advantage is afforded by the mechanism of enzymatic cross-linking whereas kinetic control allows minimally invasive delivery to an in vivo target site following the initiation of the cross-linking process by addition of enzyme to the pre-gel sol.

The specific enzyme we use for cross-linking belongs to the family of transglutaminases (EC 2.3.2.13), enzymes that catalyze a calcium-dependent amine−γ-glutaminyl acyl-transfer reaction in the extracellular environment.[1] In vivo substrates are typically peptide-bound glutamine residues (amine acceptors) and primary amines such as lysine (amine donors). From the perspective of forming synthetic gels, an attractive feature of transglutaminase is that it can effectively cross-link very small substrates (1−2 amino acids),[7] thus simplifying synthesis and reducing the possibility of immunogenicity.

In developing a practical in vivo gelation system, it is essential to tune the kinetics to allow sufficient time for delivery while limiting diffusion and loss of the pre-gel macromers. We explore here the various means of achieving predictable kinetic control in gels formed by enzymatic cross-linking of peptide-modified PEG macromers. We first present a model that predicts gelation time on the basis of the kinetics of the enzymatic cross-linking process as well as other controllable parameters. Model predictions are compared with measured gelation times for two types of enzymatically cross-linked PEG gels to demonstrate the robustness of this model.

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Materials and Methods

Materials. Glutaminamide (Q_a) was purchased from Bachem. Triethylamine from Fisher was purified prior to use by distillation. Guinea pig liver transglutaminase and poly(lysine: phenylalanine)·HBr ($D_{p,vis}$ = 185; 54 mol % lysine; MW_{vis} = 33.2 kDa) were purchased from Sigma.

PEG Characterization. An eight-arm, 40 kDa (nominal) poly(ethylene glycol) comb configuration (cPEG) was purchased from Shearwater Polymers. This molecule consisted of a poly(glycerol) backbone with multiple PEG arms attached. Size exclusion chromatography with refractive index and light scattering detection was used to determine molecular weights and polydispersity (M_w = 38 kDa, M_n = 33 kDa, D_p = 1.16). The number-average arms per molecule (7.76) was calculated from elemental analysis for sulfur following reaction with trifluoroethanesulfonyl chloride as described below.

A nominally tetrafunctional PEG (PEG-OH) was purchased from Polysciences. Previously published analysis showed this molecule is polydisperse in molecular weight.[11] PEG-OH consisted of linear 10 kDa PEG chains joined by reaction with bisphenol A diglycidyl ether. As purchased, the product was found to contain 68 wt % and 94% number-averaged single chains by gel permeation chromatography.

TG Substrate Peptide Synthesis. Transglutaminase substrate peptides (Table 1) were synthesized on a Rainin PS2000 peptide synthesizer by standard Fmoc methods in dimethylformamide (DMF). Reagents for the peptide synthesizer were purchased from Rainin. Peptide resin (Wang) and all Fmoc amino acids were purchased from American Peptide (Fmoc-glycine, Fmoc-proline, Fmoc-leucine, Fmoc-isoleucine, Fmoc-alanine, Fmoc-cysteine (trityl), Fmoc-serine (*tert*-butyl), and Fmoc-histidine (trityl) with the exception of Fmoc-glutamine (4-methyltrityl) from Bachem). Redistilled piperidine from Aldrich was used at a concentration of 20% (v/v) in DMF as the Fmoc deprotectant. Peptides were cleaved from the resin and deprotected with aliquots (100 mL total) of 2.5% ethanedithiol (Aldrich) and 5% water in trifluoroacetic acid (TFA) (J.T. Baker). Excess TFA was removed by rotary evaporation. The peptide was precipitated by pouring into diethyl ether. The precipitate was isolated by centrifugation, redissolved in a minimum of TFA, and reprecipitated three times. The final product was washed with diethyl ether. Residual ether was removed in vacuo. Purity was confirmed by reverse phase high-performance chromatography.

PEG−Peptide Coupling. Peptide substrates were covalently linked to cPEG by tresyl (trifluoroethanesulfonyl) chemistry.[14] Briefly, cPEG tresylation (tresyl chloride, Aldrich) was performed in a dried solution (over molecular sieves

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Table 1. Amine Acceptor Substrates

peptide	composition	$k_{cat}/K_{m,app}$ (mM^{-1} s^{-1})
A	Gln-NH_2	0.022
B	Gly-Gln-Gly	0.020
C	Gly-Gln-Leu-Gly	0.53
D	Gly-Gln-Leu-Lys-Gly	0.27
E	Gly-Pro-Leu-Gly-Ile-Ala-Gly-Gln-Leu-Gly	not detectable
F	Gly-Pro-Leu-Gly-Ile-Ala-Gly-Gln-His-Ser-Gly	0.75
Q1	Gly-Cys-Pro-Leu-Gly-Ile-Ala-Gly-Gln-His-Ser-Gly	0.78
Q2	Gly-Cys-Gly-Gln-His-Ser-Gly	0.59

previous to tresylation) of cPEG in methylene chloride (10 mL/g of PEG) under argon with triethylamine as an acid acceptor. Purified cPEG-Tr was obtained by successive reprecipitations from acidified methanol, using decreasing concentrations of hydrochloric 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 triethylamine was found to be 0.02 wt % by ^{1}H NMR.

Peptides Q1, Q2, and K (100 mM) were coupled to cPEG-Tr (20 mM in Tr) at pH 7.5 and room temperature where coupling via the sulhydryl group of cysteine is favored. Coupling via the cysteine sulfhydryl was confirmed by the retention of free primary amines as detected by *o*-phthaldialdehyde,[10] a primary-amine-sensitive fluorophore. The number of peptides per cPEG molecule was quantified on the basis of the ratio of peptide to PEG by ^{1}H NMR.

Coupling of peptides A−F (Table 1) was accomplished by tresyl-mediated chemistry.[14]

Enzyme Preparation and Storage. Enzyme aliquotting was performed in a 4 °C cold room. Lyophilized transglutaminase was dissolved in a buffer of 10 mM Tris-Cl, pH 7.1, 160 mM KCl, and 1 mM EDTA. Enzyme solution (10 μM TG) was stored at −70 °C and thawed immediately prior to use.

Kinetic Measurement of PEG-Bound Substrates. Kinetic measurements were made for cPEG-peptide (amine acceptor) by a standard monodansyl cadaverine (mdc) transglutaminase (TG) activity assay. Kinetic experiments were conducted in a solution of 5.0 μM TG, 8 mM $CaCl_2$, 0.2 mM EDTA, 50 mM 2-(*N*-morpholino)ethanesulfonic acid, and 5 mM Tris, pH 6.0 at 37 °C. Reactions were initiated by addition of TG to a solution of the other components. After 2 min the extent of reaction was assessed by injection onto a size exclusion column (TSK G4000PW with a mobile phase of 2.0 mM H_2KPO_4 with 5.0 ppm TFA running at 4.0 mL/min). The amount of PEG-linked mdc relative to free mdc was quantified by integration of absorbances at 247 nm. Experiments (amine acceptor concentration: 5.0, 2.5, 1.67, and 1.25 mM) were conducted such that no more than 10% of the substrate was depleted. Values for k_{cat} and $K_{m,app}$ were regressed separately for each substrate, peptides Q1 and Q2, with eq 4 by minimizing the sum of the squares of the deviation from the measured data (d[cross-link]/dt).

Gelation Time Measurements. For comb PEG (cPEG) experiments, in a typical experiment 15−20 μL of a solution containing 10 mM of both PEG-linked substrates (cPEG-$(Q1)_{n_{Q1}}$ and cPEG-$(K)_{n_K}$ or cPEG-$(Q2)_{n_{Q2}}$ and cPEG-$(K)_{n_K}$) was mixed with an equal volume of enzyme at the desired concentration in a microcentrifuge tube. These concentrations correspond to approximately 10 wt % PEG. The mixture was brought to 37 °C in a constant temperature water bath. To initiate the cross-linking reaction, calcium was added to 8 mM from a 300 mM stock solution. Gelation times were measured for transglutaminase concentrations of 2.0, 3.0, and 5.0 μM.

For PEG/polylysine gels, gelation time experiments were conducted substantially as above but with varying weight percentages of polylysine and PEG: 5/20, 5/10, and 2.5/20 wt % poly(KF)/wt % PEG-Q_a . This corresponds to initial ratios of lysine to glutamine ($r_{Lys/Gln} \equiv C_{K,0}/C_{Qa,0}$) of 3, 6, and 12, respectively. Enzyme concentrations for these experiments were in all cases 8.2 μM TG.

Because of the limited amount of macromers available, small gel volumes (30−40 μL) were employed. With these limited volumes the most reproducible assessment of gel formation was found to be by probing with a paper clip. Gelation was assessed by a combination of stirring and sweeping the solution up the sides of the microcentrifuge tube. The time at which the mixture seized to the paper clip was taken as the gel point.

Results and Discussion

We have previously introduced a PEG gel system that is cross-linked by a natural tissue enzyme, transglutaminase.[11] Gradual cross-linking of a liquid preparation of functionalized PEG pre-gel macromers is initiated by the addition of an aliquot of the enzyme. Following initiation, a window of time is desired in order to shape the liquid into its final form by introduction into a mold, coating a surface, or injection into the body. This time window must be regulated and predictable to make the gel practical for use.

The time required for a gelling system to undergo the transformation from a liquid to a semisolid state is a function of the criteria by which the gel point is defined as well as the rate of cross-link formation and the functionality of the cross-linking moieties. A useful convention defines the gel point as the transformation from viscous liquid to elastic gel. This definition provides a convenient observable to measure the gelation while at the same time implying a mathematical criteria for gel formation first developed by Flory and Stockmayer.[5,15]

The conversion of macromer at which gel formation will occur is a function of the pre-gel macromer structure as well as the kinetics of the cross-linking reaction. As the functionality (number of arms) of the macromer increases, the fraction of arms that must become cross-linked in order to produce a gel decreases. Thus, macromer structure sets the fractional conversion of end groups that must be achieved to reach the gel point. The kinetics of cross-link formation determines the rate at which this critical conversion is approached.

Structural Criteria. A mathematical description of the critical point of gelation is constructed around the degree of branching found in the system at any given time. This is most naturally formulated via probabilistic arguments.[15,5] The formulation starts with a number of necessary assumptions about certain aspects of the gel that would be extremely difficult to measure. We assume no loops (i.e., polymer unit connected to itself through a small number of chains) and no entanglements that would act as virtual cross-links.

Consider the present system of PEG-$Gln_{n_{Gln}}$ and PEG-$Lys_{n_{Lys}}$, where n_{Gln} and n_{Lys} are the number-average functionality of glutamines and lysines, respectively. The probability of any particular glutamine *residue* being covalently linked to a PEG-$Lys_{n_{Lys}}$ molecule is given by the fractional conversion to ε-(γ-glutaminyl)-lysine for glutamine, X_{Gln}, assuming equal reactivity of all glutamine residues. To find the expected number of *additional* PEG-$Gln_{n_{Gln}}$ molecules connected to this macromeric multifunctional aPEG-$Lys_{n_{Lys}}$ molecule, the fractional conversion of lysine, X_{Lys}, must be multiplied by the number of opportunities for branching, (n_{Lys} − 1). Therefore, the expected number of PEG-$Gln_{n_{Gln}}$ molecules connected to any particular glutamine residue via PEG-$Lys_{n_{Lys}}$ is given by

$$\alpha = X_{Glu}X_{Lys}(n_{Lys} - 1) = \frac{X_{Gln}^2(n_{Lys} - 1)}{r_{Lys/Gln}} \quad (1)$$

where $r_{Lys/Gln}$ is defined as the ratio of lysine residues to glutamine residues initially present.

As the reaction proceeds, PEG molecules are linked together to form chains with varying amounts of branching. The value of α defined above can be found to assess the likelihood that a repeat unit (PEG-Gln- -Lys-PEG-Lys- -Gln-PEG) will be completed. The expected number (Φ) of PEG-$Gln_{n_{Gln}}$ molecules connected via a PEG-$Lys_{n_{Lys}}$ molecule to another PEG-$Gln_{n_{Gln}}$ *molecule* is given by the product of α and the number of opportunities for branching, $(n_{Lys} - 1)$, or

$$\Phi = \alpha(n_{Gln} - 1) = \frac{X_{Gln}^2(n_{Lys} - 1)(n_{Gln} - 1)}{r_{Lys/Gln}} \quad (2)$$

When Φ exceeds unity, infinite extension of network chains becomes possible. Thus, the critical condition for gelation based on $\Phi_c = 1$ in terms of X_{Gln} can be expressed as

$$X_{Gln,c} = \sqrt{\frac{r_{Lys/Gln}}{(n_{Lys} - 1)(n_{Gln} - 1)}} \quad (3)$$

The above treatment is equally valid if the roles of glutamine and lysine are reversed. This reversal is only allowed when considering the structural requirements for gelation. When kinetics are considered to derive the time required to reach $X_{Gln,c}$, differences in concentrations of glutamines and lysines are significant.

Kinetic Criteria. To predict the time at which gelation will occur, it is necessary to define a relationship between $X_{Gln,c}$ and time through equations of the form $dX_{Gln,c}/dt = f([Lys], [Gln], [TG], k_{1..i})$. In this way the approach to the gel point and rate of cross-link formation is governed by the underlying kinetics of transglutaminase activity.

Transglutaminase-mediated amide formation between an amine donor and an amine acceptor is known to follow a modified double-displacement mechanism.[3,4,6] This reaction occurs in two steps. First, transglutaminase reacts with the amine acceptor, forming a thioester bond at the active site with the release of ammonia. In the second step, the amine donor displaces the amine acceptor from the enzyme, forming an amide bond.

The first catalytic step is generally rate limiting.[3] Therefore, the kinetics of cross-link formation can be written as

$$\frac{d[\text{cross-link}]}{dt} = -\frac{d[Gln]}{dt} = \frac{k_{cat}[TG]_t[Gln]}{K_{m,app} + [Gln]} \quad (4)$$

where $[TG]_t$ is the total enzyme concentration. Integration from initial conditions to to time t yields

$$t = \frac{X_{Gln}([Gln]_0/K_{m,app}) - \ln(1 - X_{Gln})}{(k_{cat}/K_{m,app})[TG]_t} \quad (5)$$

where $[Gln]_0$ is the initial concentration of amine acceptor. This equation defines the time required to reach a particular conversion given the initial amine acceptor substrate concentration, the amine acceptor substrate kinetic parameters, and the concentration of transglutaminase.

Substitution of the structural gelation criteria into the kinetic equation yields an expression for the time to gelation (t_c):

$$t_c = ([Gln]_0/K_{m,app}) \times \frac{\sqrt{\frac{r_{Lys/Gln}}{(n_{Lys} - 1)(n_{Gln} - 1)}} - \ln\left(1 - \sqrt{\frac{r_{Lys/Gln}}{(n_{Lys} - 1)(n_{Gln} - 1)}}\right)}{(k_{cat}/K_{m,app})[TG]_t} \quad (6)$$

By inspection it can be seen that, for substrates where $K_{m,app}$ deviates significantly from the initial concentration of glutamine residues, $[Gln]_0$, eq 6 may reduce to a simpler form:

$$t_c = \frac{-\ln\left(1 - \sqrt{\frac{r_{Lys/Gln}}{(n_{Lys} - 1)(n_{Gln} - 1)}}\right)}{(k_{cat}/K_{m,app})[TG]_t} \quad (7)$$

Gelation Kinetics. Equation 6 predicts a multiparameter dependence for gelation time. The parameters n_{Lys} and n_{Gln} are controllable at the time of macromer synthesis. Similarly, the kinetic parameters are predetermined by the choice of glutaminyl substrate. Other parameters, including $[Gln]_0$, $[TG]_t$, and $r_{Lys/Gln}$, are determined by the initial conditions.

Experiments presented here were designed to span the parameter space. Gelation times for two sets of substrate-functionalized multiarm comb PEG (cPEG) were measured at various enzyme concentrations to investigate the dependence on enzyme concentration. Gelation times for substrate-functionalized difunctional PEG in conjunction with a polylysine compound were measured at various lysine/glutamine stoichiometric ratios to test the dependence on initial stoichiometry.

A number of amine acceptor substrates were screened for transglutaminase activity. Kinetics of cross-linking monodansyl cadaverine to each PEG-bound glutaminyl substrate was measured for each peptide in Table 1. The shortest substrates, peptides A and B, showed measurable, yet limited activity. Insertion of a leucine residue adjacent to and on the carboxyl side of the glutamine substrate increases activity by approximately 1 order of magnitude, as was suggested by the literature.[9] Peptides E, F, and Q1 were elongated with a site for enzymatic cleavage to allow for eventual bioabsorption of the gel by enzyme-mediated degradation.[12] As peptide E was inactive, possibly due to disfavorable folding, peptides F, Q1, and Q2 were synthesized with the Gln-His-Ser motif of casein[8] to increase the hydrophilicity of the substrate. Peptides Q1 and Q2 were selected for gel studies as these two peptides have high, yet distinguishable kinetics and possess similar glutamine environments yet different hydrophobicities.

For cPEG experiments, two PEG-peptide amine acceptor substrates, cPEG-$(Q1)_{n_{Q1}}$ and cPEG-$(Q2)_{n_{Q2}}$, and one cPEG-peptide amine donor substrate, cPEG-$(K)_{n_K}$, were selected as model gel systems. For these particular polymers, n_{Q1} and n_{Q2} describe the number-average functionality (n_{Gln}) for the respective derivative. Similarly, $n_K = n_{Lys}$ for cPEG-$(K)_{n_K}$. Values for these parameters are summarized in Table 2 along with the calculated critical conversion, $X_{Gln,c}$, for each amine acceptor substrate based on eq 3.

Table 2. Substrate-Functionalized PEG Properties

species	gram polymer per millimole substrate	no. of functionalized arms per molecule[a]	critical conversion[b] ($X_{Gln,c}$)
cPEG/cPEG gel			
cPEG-(Q1)$_{n_{Q1}}$	6.60	$n_{Q1} = 6.00$	0.27
cPEG-(Q2)$_{n_{Q2}}$	7.64	$n_{Q2} = 4.71$	0.31
cPEG-(K)$_{n_K}$	9.48	$n_K = 3.67$	
PEG/poly(KF) gel			
PEG-(Qa)$_{n_{Qa}}$	7.94	$n_{Qa} = 2.06$	$f(r_{Lys/Gln})$[c]
poly(KF)	336	100	

[a] Number-average value. [b] Conversion based on glutamine conversion by eq 3. [c] $X_{Gln,c} = 0.174$, 0.25, and 0.35 for $r_{Lys/Gln} = 3.0$, 6.0, and 12.0, respectively.

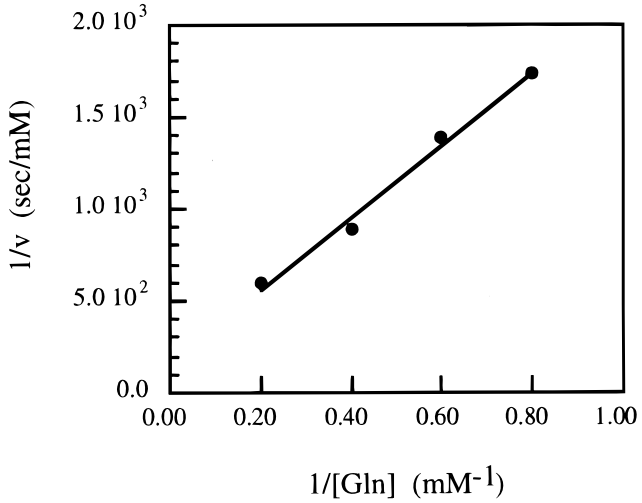

Figure 1. Double-reciprocal plot for cPEG-(Q1)$_{n_{Q1}}$. Chi-squared minimization fit (line) to data (points) of eq 4 yields $K_{m,app} = 7.6$ mM; $k_{cat} = 0.86$ s^{-1} mM^{-1}.

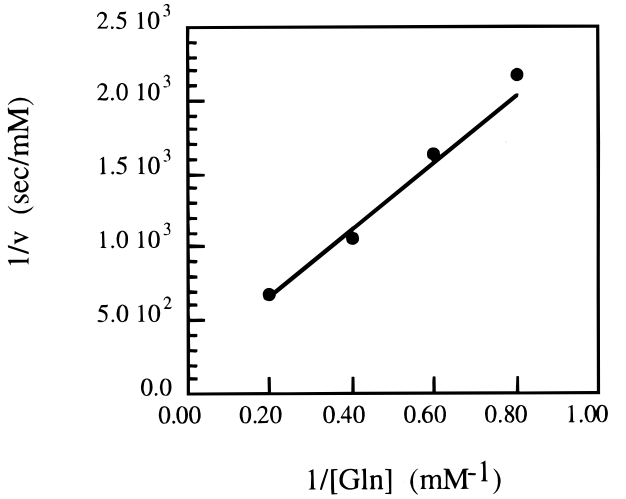

Figure 2. Double-reciprocal plot for cPEG-(Q2)$_{n_{Q2}}$. Chi-squared minimization fit (line) to data (points) of eq 4 yields $K_{m,app} = 11$ mM; $k_{cat} = 0.95$ s^{-1} mM^{-1}.

To predict gelation times from eq 6, it was necessary to measure the kinetic parameters k_c and $K_{m,app}$ for each amine acceptor substrate. Initial rates at a constant enzyme concentration were measured at 37 °C and pH 6.0. Amine acceptor substrate concentrations were chosen to correspond to concentrations used in gelation experiments below. Double-reciprocal plots for substrate concentrations in the range to be used in gel formation are shown in Figures 1 and 2 for cPEG-(Q1)$_{n_{Q1}}$ and cPEG-(Q2)$_{n_{Q2}}$, respectively. Values for k_{cat} were found to be similar, but the value of $K_{m,app}$ for peptide Q1 was found to be somewhat less than for peptide Q2, possibly to enhanced binding to the hydrophobic region absent in peptide K. For peptide concentrations well below the measured $K_{m,app}$ values, the relevant kinetic parameter becomes $k_{cat}/K_{m,app}$ for peptide Q1 and for peptide Q2.

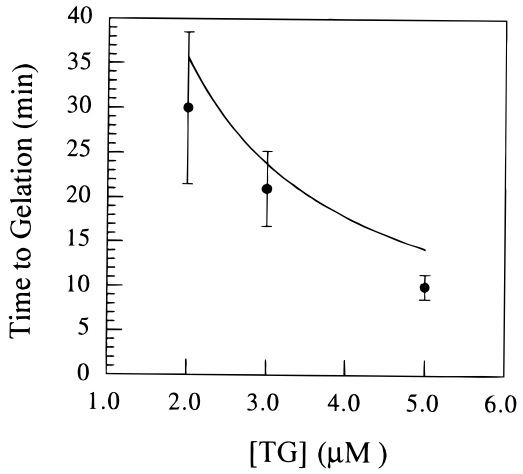

Figure 3. Time to gelation for cPEG-(Q1)$_{n_{Q1}}$/cPEG-(K)$_{n_K}$ gel. Points ($n = 2$) and standard deviation represent measured gelation times as compared to model predictions (line) based on macromer structure and substrate kinetics (see text).

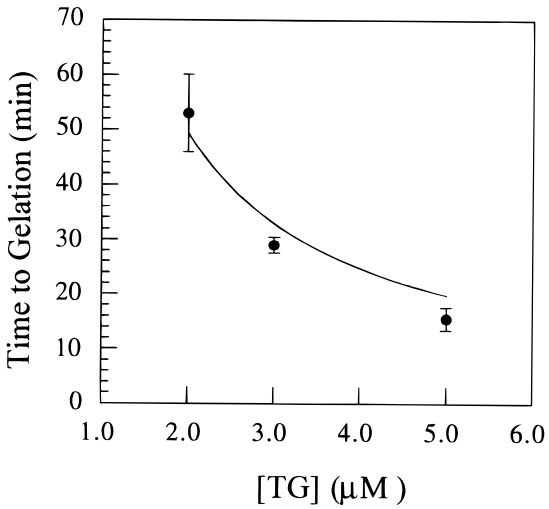

Figure 4. Time to gelation for cPEG-(Q2)$_{n_{Q2}}$/cPEG-(K)$_{n_K}$ gel. Points ($n = 2$) and standard deviation represent measured gelation times as compared to model predictions (line) based on macromer structure and substrate kinetics (see text).

For a given k_{cat}, $K_{m,app}$, $r_{Lys/Gln}$, and $[Gln]_0$ eq 6 predicts gelation times as a function of the concentration of enzyme present, $[TG]_t$. For simplicity, gels were formed at stoichiometric levels of amine acceptor and amine donor substrates, corresponding to a $r_{Lys/Gln}$ of unity. Gels were formed at $[Gln]_0 = 5$ mM, chosen to ensure generous overlap of polymer chains and thereby minimize the effect of self-termination.

Figures 3 and 4 show predicted and measured gelation times for the two macromer pairs examined. In both cases, the measured gelation rates come very close to theory. Spreads in measured gelation rates may be attributed to imperfect initial mixing or more likely due to difficulties in assessing the gel point. A number of counteracting deviations from the assumptions made in the formulation of the structural requirements for

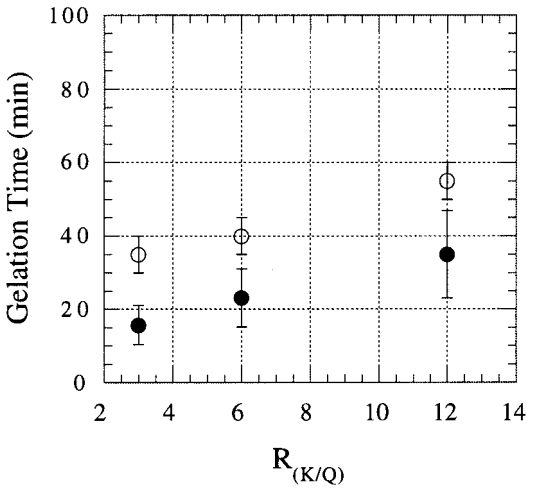

Figure 5. Predicted (line) and experimentally measured (○) time required to reach the gel point following enzyme addition as a function of the ratio of the initial concentration of the glutaminyl substrate to lysyl substrate.

gelation may influence the correlation of eq 6 and experiment. Overlapping chains may cause virtual cross-linking, accelerating the observed gelation time. Cross-links that do not add to the network structure such as loops would tend to delay the onset of gelation. Under certain conditions, gelation may also be delayed by deactivation of self-cross-linking[2] of transglutaminase; however, at pH 6.0, these effects are anticipated to be minor.[13]

For the case of the PEG/polylysine gels, substrate kinetics (high K_m) are such that eq 7 is applicable. Figure 5 shows predicted and measured gelation times for the PEG/polylysine gels. The model captures the positive correlation with increasing $r_{Lys/Gln}$; however, in all cases gelation times were somewhat longer than predicted by eq 7. This may be due to assumptions made in formulating this model. Equation 7 assumes no network imperfections such as loops that do not add to the network structure that would tend to lengthen measured gelation times. Also implicit in this model is an assumption of equal reactivity. However, with such a large number of PEG ends per polyKF (33, 17, and 8 for 20/2.5, 20/5, and 10/5 gels, respectively), it is likely that steric effects will increase observed reaction times, especially for the 20/2.5 gel ($r_{Lys/Gln} = 3$). This may be manifested in a larger deviation from predictions for this gel.

Conclusions

We have found that a relatively simple model for gelation time based on various controllable parameters is able to describe and predict gelation times for enzymatically cross-linked gels across a range of polymer functionalities, initial stoichiometric ratios, and substrate kinetics. In all cases examined for comb PEG gels, the data agree with the model quite well. For the case of the PEG/polylysine system, the model correctly predicts the shape of the dependence on the initial stoichiometry, although steric effects may have shifted actual gelation times to larger values than predicted by polymer functionality and substrate kinetics alone.

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