Controlled Release of Plasmid DNA from a Genetically Engineered Silk-Elastinlike Hydrogel

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Received March 15, 2002; accepted March 26, 2002

Purpose. The purpose of this study was to evaluate the potential of a genetically engineered silk-elastinlike polymer (SELP) as a matrix for the controlled release of plasmid DNA.

Methods. The influences of SELP concentration, DNA concentration, SELP cure time, and buffer ionic strength on the release of DNA from SELP hydrogels were investigated. To calculate the average effective diffusivity of DNA within the hydrogels, the release data were fitted to a known equation.

Results. DNA was released from SELP hydrogels by an ion-exchange mechanism. Under the conditions studied, the release rate was influenced by buffer ionic strength, SELP concentration, and SELP cure time but not DNA concentration. The apparent diffusivity of pRL-CMV plasmid DNA in SELP hydrogels ranged from $3.78 \pm 0.37 \times 10^{-10}$ cm^2/s (for hydrogels containing 12% w/w SELP and cured for 4 h) to $4.69 \pm 2.81 \times 10^{-9}$ cm^2/s (for hydrogels containing 8% w/w SELP and cured for 1 h).

Conclusions. The ability to precisely customize the structure and physicochemical properties of SELPs using recombinant techniques, coupled with their ability to form injectable, *in situ* hydrogel depots that release DNA, renders this class of polymers an interesting candidate for further evaluation in controlled gene delivery.

KEY WORDS: genetically engineered polymers; controlled release; gene delivery; silk-elastinlike polymers; hydrogels.

INTRODUCTION

Nonviral gene delivery research has focused, in part, on the synthesis and characterization of new delivery vehicles, including polymers, lipids, and peptides (1–3). Although the current generation of nonviral vectors has shown an ability to transfect cells, transient gene expression and low transfection efficiency often limit their clinical utility. Viral vectors can overcome these shortcomings, but their use has been limited by safety issues and manufacturing difficulties. Nonviral vectors generally are considered safer but thus far have exhibited transfection efficiencies that are considerably lower than their viral counterparts. Both types of vectors typically have been administered as a bolus dose, imposing limitations on the duration and location of gene expression, depending on the rate at which the vector is cleared from the site of administration.

As a consequence, attention to the controlled delivery of genes, using polymeric matrices, has increased (4–7). This interest is motivated largely by the fact that controlled gene delivery systems potentially provide the means to manipulate the exact location and time course of transgene expression. Polymeric matrix-controlled gene delivery systems provide many advantages over the bolus approach typically used in gene delivery. Chief among these are the following: 1) the ability to manipulate the DNA release profile, delivering DNA to tissue in a sustained and predictable manner and possibly increasing the effectiveness of the therapy; 2) the ability to localize delivery to a specific tissue, either by injection or implantation; and 3) the possible protection of DNA from endogenous nucleases when encapsulated in a polymer matrix.

Controlled delivery of plasmid DNA has been reported with chemically synthesized polymers such as poly (vinyl pyrrolidone), poly (vinyl alcohol), poly (ethylene-co-vinyl acetate), and poly (D,L-lactide-co-glycolide) (4–8). Although these polymers have shown utility, there is a need to develop novel biomaterials in which the physicochemical characteristics and biological fate of the matrix can be precisely controlled. Recent research has focused on the development of new polymers using genetic engineering techniques (9–11). Recombinant technology allows for the design and synthesis of polymers with precise composition and sequence, where it is possible to control the physicochemical properties important for controlled drug delivery, such as biodegradation (12), stimuli-sensitivity (13), and biorecognition (10).

One class of genetically engineered biomaterials is the family of silk-elastinlike polymers (SELPs), which consist of alternating blocks of silk-like (Gly-Ala-Gly-Ala-Gly-Ser) and elastin-like (Gly-Val-Gly-Val-Pro) blocks. By combining the silk-like and elastin-like blocks in various ratios and sequences, it is possible to produce an assortment of biomaterials with diverse material properties. Previous reports on silk-elastinlike polymers have shown that the solubility, material strength, immunogenicity, and *in vivo* degradation profile of SELPs can be controlled by varying the composition and sequence of the polymers (12). The utility of these polymers in controlled gene and oligonucleotide delivery is unexplored. Their versatile structure and the ability to control sequence and, therefore, properties, at the molecular level, provides a promising approach for the design of gene delivery matrices tailor-made for specific therapeutic needs.

Initial studies have shown that SELP copolymers with appropriate sequence and composition undergo an irreversible sol-to-gel transition when transferred from room temperature to body temperature (11). The polymers, with appropriate amino acid sequences, can easily form an injectable, *in situ,* gel-forming depot and be localized by injection through a needle, avoiding more invasive surgical implantation procedures. Once localized, the DNA can be released while the polymer matrix degrades to relatively nontoxic amino acids.

In this article, we present the initial characterization of a controlled gene delivery system based on a model genetically engineered silk-elastinlike hydrogel. We studied the influence of ionic strength, hydrogel cure time, polymer concentration, and DNA concentration on the release of plasmid DNA from the hydrogel matrix. The apparent diffusivity of plasmid

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DNA within the hydrogel matrices has been calculated by fitting to a known equation.

MATERIALS AND METHODS

Silk-Elastinlike Polymers and Plasmid DNA

Model silk-elastinlike copolymer, namely Polymer 47K (Protein Polymer Technologies, Inc., San Diego, California structure shown in Fig. 1) was synthesized by recombinant DNA techniques and characterized by previously described methods (11,12). Solutions of 12% Polymer 47K were aliquoted into 3-mL syringes and frozen at –80°C until use. A lyophilized form of Polymer 47K stored at –20°C was used for turbidity experiments. Plasmid pRL-CMV (Promega, Madison, Wisconsin) was used to chemically transform *Escherichia coli* strain NovaBlue (Novagen, Madison, Wisconsin). Plasmid DNA was isolated with a GigaPrep kit according to the manufacturer's instructions (Qiagen, Valencia, California). The pRL-CMV plasmid contains the *Renilla* luciferase gene under the control of a cytomegalovirus promoter. The molecular weight of the plasmid, based on an average of 635 Da per base pair (14), is approximately 2.59 MDa. The concentration and purity of the plasmid were determined by measuring absorbance at 260 and 280 nm with a UV-Vis spectrophotometer (Amersham Pharmacia Biotech, Piscataway, New Jersey). The ratio of A_{260} to A_{280} was in the range of 1.8 to 2.0 for all of the plasmids used in the studies.

Preparation of the DNA-Containing Silk-Elastinlike Polymer Hydrogels

Before the incorporation of DNA, Polymer 47K solutions were defrosted by placing one syringe in a beaker containing 500 mL of room temperature MilliQ water for 5 min at room temperature. After 5 min, the required volume of concentrated plasmid solution was added to the syringe to obtain a final concentration of either 50 μg/mL or 250 μg/mL. The plasmid was mixed with the polymer solution by inverting the syringe several times, allowing an air bubble to traverse the length of the solution. The liquid polymer-DNA solution was then injected, through a 21-gauge needle into molds made from silanized glass microscope slides separated by a 0.16-cm thick rubber gasket. The molds were placed in a 37°C incubator for the desired cure time. After curing at 37°C, the DNA-containing polymers formed a hydrogel. Cylindrical discs were cut from each hydrogel slab using a cork borer with a diameter of 0.58 cm. For gels containing 8% polymer, a chilled diluent composed of 71% Dulbecco's phosphate-buffered saline (PBS; Invitrogen, Carlsbad, California) and 29% MilliQ water was used to dilute the 12% polymer solution before the plasmid was incorporated.

MDPVVLQRRDWENPGVTQLNRLAAHPPFASDPM
GAGSGAGAGS[(GVGVP)$_4$GKGVP(GVGVP)$_3$(GAGAGS)$_4$]$_{12}$
(GVGVP)$_4$GKGVP(GVGVP)$_3$ (GAGAGS)$_2$GAGA
MDPGRYQDLRSHHHHHH

Fig. 1. The 884-amino acid Polymer 47K sequence has a molecular weight of 69,814 Da. It is composed of a head and tail sequence and a series of silk-like (GAGAGS) and elastin-like (GVGVP) repeats. Residues that are predominantly positively charged at pH 7.4 are boxed. Abbreviation Key: A = alanine; D = aspartic acid; E = glutamic acid; F = phenylalanine; G = glycine; H = histidine; K = lysine; L = leucine; M = methionine; N = asparagine; R = arginine; P = proline; Q = glutamine; S = serine; T = threonine; V = valine; W = tryptophan; Y = tyrosine.

DNA Release and Detection Protocol

Hydrogel discs were weighed into 4-mL glass vials containing 2 mL of the appropriate release buffer. PBS (10 mM, pH 7.4, 0.01% w/v NaN_3) with total ionic strength (μ), adjusted with NaCl, of 0.03 M, 0.10 M, 0.17 M, 0.25 M, and 0.50 M, was used as a release medium. Vials containing hydrogels submerged in 2 mL of buffer were incubated at 37°C in a shaking (120 rpm) incubator for 28 days. At predetermined time points, the buffer was sampled and replaced with fresh buffer. DNA concentration was determined by the PicoGreen assay (Molecular Probes, Eugene, Oregon). A standard curve was constructed with pRL-CMV in each buffer during each assay. All data points represent hydrogels prepared in triplicate.

Turbidity Assay

Solutions containing 50 μg of DNA were prepared in PBS with ionic strengths of 0.03 M and 0.17 M. A solution of Polymer 47K was prepared in the corresponding buffer. Polymer and buffer solutions were added to DNA solutions, in an amount appropriate to achieve the desired charge ratio and final volume (137 μL), assuming full ionization of the polymer primary amines, and DNA phosphates, at a pH of 7.4. Immediately after vortexing the mixture, the transmittance at 400 nm was determined using an UV-Visible spectrophotometer (Amersham Pharmacia Biotech, Piscataway, New Jersey). Relative turbidity was determined by calculating $(100 - T) / (100 - T_{min})$, where T is the transmittance of the sample and T_{min} is the transmittance of the sample with minimum transmittance, or greatest turbidity. All measurements were performed in triplicate.

Calculation of Apparent Diffusivity

For each formulation, graphs of fraction released vs. time were constructed. The average effective diffusivity of the plasmid within the hydrogels was determined using the following equation, which describes diffusion from a cylinder, in both the radial and axial directions (15,16):

$$\frac{M_t}{M_\infty} = 1 - \frac{32}{\pi^2}\sum_{i=1}^{\infty}\frac{1}{\alpha_i^2}\exp\left(-\frac{\alpha_i^2}{r^2}D_e t\right)\sum_{j=0}^{\infty}\frac{1}{(2j+1)^2}\exp\left(-\frac{(2j+1)^2\pi^2}{h^2}D_e t\right) \quad (1)$$

In this equation, r is the radius of the cylinder, h is the height of the cylinder, M_t is the cumulative amount of solute released at time t, M_∞ is the amount released as $t\to\infty$, D_e is the average effective intra-gel diffusivity of the solute, and α_i are the roots of the zero-order Bessel function, $J_0(\alpha_i) = 0$. D_e was estimated from a nonlinear fit of Eq. (1) to the experimental release data using the software program Mathematica (Wolfram Research, Champaign, Illinois v. 4.1). The R^2 values for each fit were determined by computing the ratio of the

difference between the corrected total sum of squares and the residual sum of squares to the corrected total sum of squares. Equation (1) assumes that the diffusion coefficient is independent of solute concentration, that the concentration of solute at the surface of the hydrogel is effectively zero, that no convection occurs, and that there are no polymer–solute interactions.

RESULTS

Preparation of Silk-Elastinlike Polymer–DNA Hydrogels

Several SELP-based controlled gene delivery systems were prepared with varying polymer concentrations, DNA concentrations, and hydrogel cure times. The release of DNA from these hydrogels was evaluated in buffers with ionic strengths of 0.03 M, 0.10 M, 0.17 M, 0.25 M, and 0.50 M. The characteristics of each hydrogel are summarized in Table I. Hydrogels containing 8 or 12% Polymer 47K, cured for 1 or 4 h, and containing 50 or 250 μg/mL of pRL-CMV were prepared. At an ionic strength of 0.17 M, the time for release of 50% (T_{50}) of the loaded dose varied between 1 and >28 days for the hydrogels, depending on the polymer concentration and cure time.

DNA Release Studies

Effect of Ionic Strength of the Medium on DNA Release

The effect of the ionic strength of the medium on the release of DNA from the hydrogels was evaluated by performing release studies in PBS with different ionic strengths (Fig. 2A). At low ionic strength (μ = 0.03 M, 0.10 M), virtually no DNA was released from the hydrogels, whereas nearly identical release profiles were obtained for hydrogels placed in PBS with higher ionic strengths (μ = 0.17 M, 0.25 M, 0.50 M). At the higher ionic strengths (μ = 0.17 M, 0.25 M, 0.50 M), T_{50} was approximately 1 week for the 12% Polymer 47K hydrogels cured for 1 h at 37°C.

Table I. Composition and Properties of DNA-Containing SELP Hydrogels

Sample	Polymer conc. (%)[a]	Cure time (h)[b]	DNA Conc. (μg/mL)	D_e (cm^2/s)[c]	T_{50} (days)[d]
1	12	1	50	$1.03 \pm 0.19 \times 10^{-9}$	7
2	12	1	250	$1.07 \pm 0.20 \times 10^{-9}$	7
3	12	4	50	$3.77 \pm 0.37 \times 10^{-10}$	>28
4	12	4	250	$3.78 \pm 0.72 \times 10^{-10}$	>28
5	8	1	50	$4.69 \pm 2.81 \times 10^{-9}$	1
6	8	4	50	$2.52 \pm 0.80 \times 10^{-9}$	2
7	8	4	250	$2.16 \pm 0.31 \times 10^{-9}$	2

[a] (w/w).
[b] Time incubated at 37°C.
[c] Average effective diffusivity of DNA in hydrogel, determined by nonlinear fit of Equation 1 to release data in PBS with μ = 0.17 M. Values are mean ± standard deviation (n = 3).
[d] Approximate time for 50% release of the loaded DNA in PBS with μ = 0.17 M. Plasmid pRL-CMV was used in release studies. For polymer structure, see Figure 1.

Turbidity Assay

To further examine the effect of the ionic strength of the medium on the interaction between Polymer 47K and DNA, turbidity studies were performed in PBS with ionic strengths of 0.03 M and 0.17 M (Fig. 2B). The transmittance of light is one method of determining the turbidity of liquids containing insoluble interpolyelectrolyte complexes (17). This method permits the detection of insoluble IPECs formed by electrostatic interaction between Polymer 47K and DNA. The relative turbidity of the mixtures showed a substantial increase when complexes were prepared in PBS with low ionic strength (μ = 0.03 M), indicating that, at this ionic strength, insoluble complexes form between Polymer 47K and DNA. No increase in turbidity was observed in the buffer with an ionic strength of 0.17 M. Zeta potential measurements (Zetasizer 3000, Malvern Instruments, Inc., Southborough, Massachusetts, data not shown) supported the turbidity measurements by showing that the polymer exhibited an ability to neutralize DNA in low ionic strength buffer (μ = 0.03) but not higher ionic strength buffer (μ = 0.17).

Effect of Cure Time and Polymer Concentration on DNA Release

The cure time and polymer concentration of the hydrogels were both found to significantly impact the rate at which DNA was released from the systems (Fig. 3). The effect of cure time was more significant in gels containing 12% polymer vs. those containing 8% polymer. In the gels containing 12% polymer, increasing the cure time from 1 to 4 h increased T_{50} from 7 days to more than 28 days. For 8% gels, cured for 1 or 4 h, T_{50} values were approximately 1 to 2 days. As expected, the hydrogels containing 12% polymer released DNA at a slower rate than gels containing 8% polymer.

Effect of DNA Concentration on DNA Release

The effect of DNA concentration on the release profile was evaluated by increasing the incorporated DNA dose from 50 to 250 μg/mL (Fig. 4). Results indicate that within this range, the rate of release is unaffected by the DNA concentration. The data also indicate that despite the change in DNA concentration, the effect of cure time on the release profile remains the same. A similar lack of dependence on DNA concentration, within the 50 to 250 μg/mL range, was also observed for hydrogels containing 8% polymer (data not shown).

Intra-Gel Diffusivity of the Plasmid

Experimental data (mean ± standard deviation; n = 3) obtained for the 12% Polymer 47K hydrogels containing 50 μg/mL pRL-CMV fit fairly well with the theoretical predictions of Eq. (1). For 12% gels cured for 1 h, the calculated effective diffusivity was $1.03 \pm 0.19 \times 10^{-9}$ cm^2/s, and the R^2 value was 0.829. The 12% gels cured for 4 h exhibited a stronger adherence to the equation with an effective diffusivity of $3.77 \pm 0.37 \times 10^{-10}$ cm^2/s and R^2 value of 0.987. Release of DNA from hydrogels containing 8% Polymer 47K deviated considerably from the predictions of Eq. (1), with R^2 values of 0.335 and 0.781 for the 1 and 4 h cure times, respectively.

The calculated values for the effective diffusivity of DNA

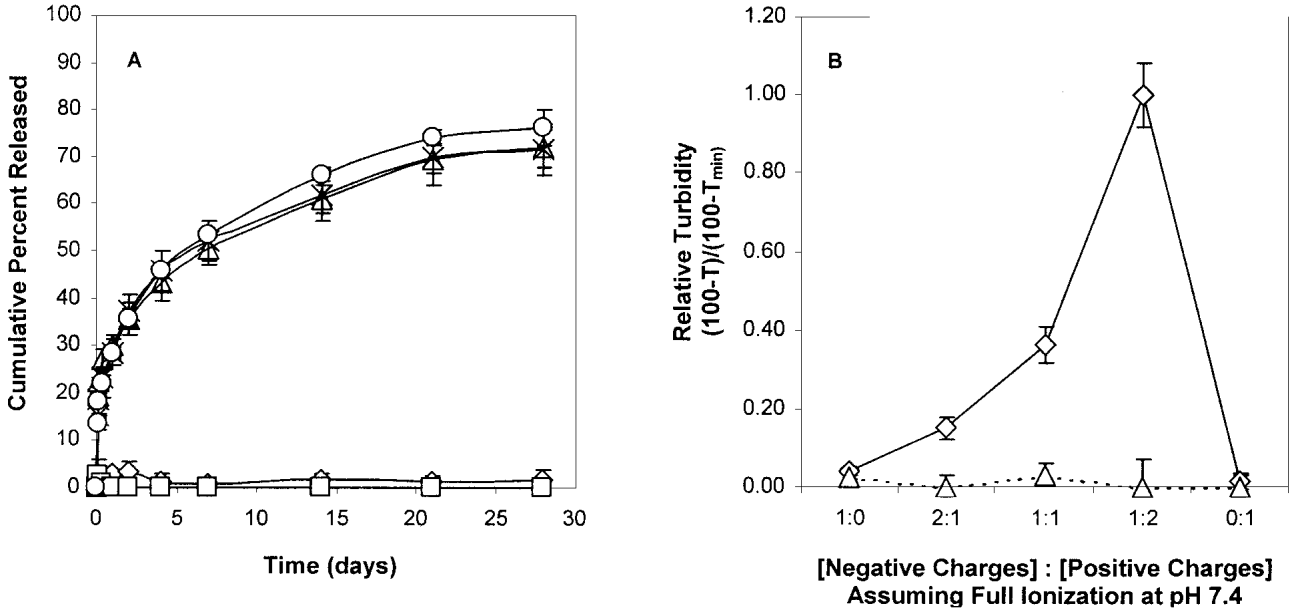

Fig. 2. (A) Cumulative release of pRL-CMV from 12% (w/w) Polymer 47K hydrogels in PBS with μ = 0.03 M (◇), 0.10 M (□), 0.17 M (△), 0.25 M (X), and 0.50 M (◯). Hydrogels were cured for 1 h at 37°C before placement in the appropriate buffer. Each point represents average ± standard deviation (n = 3). (B) Effect of ionic strength on the formation of insoluble complexes between Polymer 47K and pRL-CMV, in PBS with μ = 0.03 M (◇, solid line) and μ = 0.17 M (△, dashed line). Ratios on the x-axis indicate the molar ratio of negative (DNA) charges to positive (polymer) charges, assuming 100% ionization of each at pH 7.4. The y-axis represents relative turbidity (100 – T)/(100 – T_{min}). Data points represent average ± standard deviation (n = 3).

at various polymer concentration and cure time combinations are reported in Table I. At 12% polymer concentration, a higher cure time resulted in slower diffusion of DNA in the matrix. A similar but less pronounced effect was observed for the 8% polymer concentration. The effective diffusivity of DNA remained constant at initial DNA concentrations of 50 μg/mL and 250 μg/mL. The values obtained from the calculation of diffusion coefficients should be regarded as preliminary because, particularly in the case of lower polymer concentrations (8%), the equation did not seem to adequately describe the experimental data.

DISCUSSION

Effect of Ionic Strength of the Medium on DNA Release

The release of DNA from the hydrogels showed a strong dependence on the ionic strength of the medium. This result

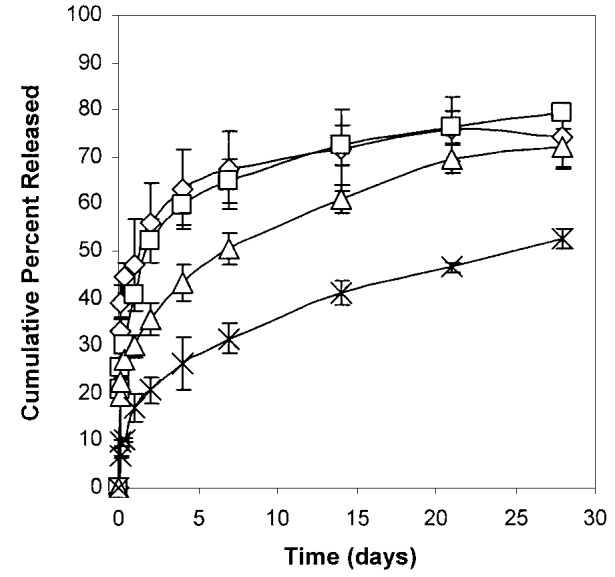

Fig. 3. Effect of polymer concentration and cure time on the release of pRL-CMV from hydrogels: 8% polymer, 1 h cure time (◇), 8% polymer, 4 h cure time (□), 12% polymer, 1 h cure time (△), and 12% polymer, 4 h cure time (×). All hydrogels were placed in PBS with ionic strength adjusted to 0.17 M. Each point represents an average ± standard deviation (n = 3).

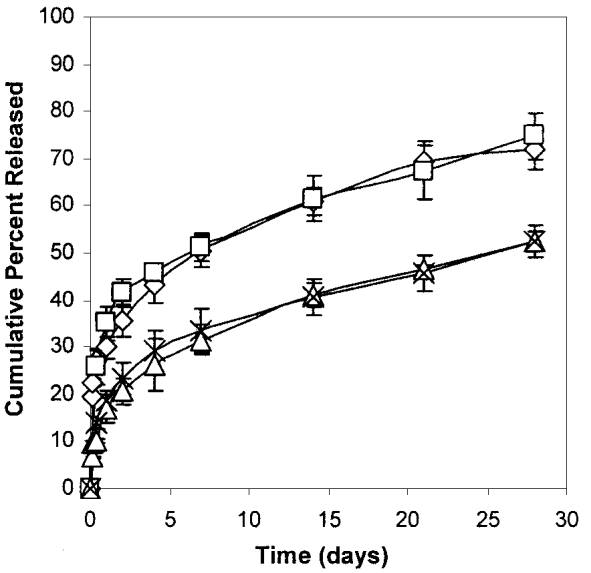

Fig. 4. Effect of DNA concentration on the release of pRL-CMV from 12% Polymer 47K hydrogels: 50 μg/mL, 1 h cure time (◇), 250 μg/mL, 1 h cure time (□), 50 μg/mL, 4 h cure time (△), and 250 μg/mL, 4 h cure time (×). Each point represents an average ± standard deviation (n = 3).

can be explained by the fact that the Polymer 47K sequence contains 13 lysine and 5 arginine residues per molecule (Fig. 1, boxed letters). These amino acids contain primary amines in their side chains, which can be expected to be significantly protonated at pH 7.4, thereby potentially interacting with the negatively charged phosphates of DNA. As the ionic strength of the buffer increases, the concentration of counterions also increases. Counterions screen the primary amines on the polymer and phosphates on the DNA, weakening the interaction between the two and liberating electrostatically bound DNA from the hydrogel matrix. As a consequence, at higher ionic strengths DNA is released at a greater rate (Fig. 2A).

The results from the turbidity assay are consistent with the release data in that they confirm the formation of insoluble Polymer 47K: DNA interpolyelectrolyte complexes at low ionic strength (μ = 0.03 M) but not at higher ionic strength (μ = 0.17 M; Fig. 2B). Surface charge measurements (data not shown) indicate that Polymer 47K interacts with and neutralizes DNA in low ionic strength buffer (μ = 0.03 M) but not higher ionic strength buffer (μ = 0.17 M). Taken together, these data suggest an ionic interaction between DNA and polymer, which might potentially be used to control the release of DNA. Previous studies have shown that chemically altering the overall charge and charge density of collagen matrices is useful in controlling the release of other polyionic macromolecules, such as polylysine (18).

Effect of Cure Time and Polymer Concentration on DNA Release

Formation of SELP hydrogels occurs through crystallization of the silk-like blocks of the polymer chains, an irreversible, kinetic process (11). It has been shown that as the cure time of these hydrogels increases, their degree of swelling decreases (19). The presumed reason for this phenomenon is that increased polymer-polymer interaction, as a function of time, results in increased cross-linking density and a corresponding decrease in the degree of swelling. Upon extended curing, the more-dense network and the resulting decrease in the degree of swelling may result in a decreased mean pore size, impeding the release of DNA. As observed in Figure 3, at 12% polymer concentration, an increase in cure time results in a decrease in the percentage of DNA released at given time points.

For essentially the same reasons, hydrogels containing 12% polymer release DNA more slowly than hydrogels containing 8% polymer (Fig. 3). An increase in polymer concentration results in an increase in polymer-polymer interaction and cross-linking density, and therefore decreased swelling and mean pore size, leading to slower DNA release.

Intra-Gel Diffusivity of the Plasmid

The results from the release studies can be interpreted in the context of an equation describing two-dimensional diffusion of a solute from a cylinder (15,16). As the percentage of polymer and the cure time increased, the delivery system exhibited a stronger adherence to theoretical predictions. This observation can be explained in terms of two effects: 1) the effect of polymer soluble fraction on DNA release and 2) convection. It is known that an increase in cure time or polymer concentration of SELP copolymers results in a decrease in polymer soluble fraction (19). In hydrogels with a relatively high soluble fraction, DNA release would be controlled not only by diffusion, but also by release of the soluble fraction, which would kinetically increase the pore volume. The rate of new pore formation, caused by release of the soluble fraction over the first 24 h, is not considered in Eq. (1). In addition, particularly in the case of hydrogels containing 8% polymer, the pore size may be sufficiently large to allow convective movement of the release medium into the hydrogel network. Because Eq. (1) assumes no convection, it would not be adequate to describe this type of system.

A more sophisticated approach to modeling the release of DNA from SELP hydrogels would involve applying the effective diffusivities calculated from Eq. (1) to a theoretical model based on hydrogel network structure and solute properties (i.e., free volume theory, hydrodynamic theory, obstruction theory). Amsden (20) has successfully used this approach to analyze the diffusion of bovine serum albumin in calcium alginate hydrogels. However, application of the appropriate model will first require further characterization of the SELP hydrogels to determine whether they are homogeneous or heterogeneous in nature.

Despite the very general nature of the calculated diffusivities, we believe that this simple approach provides a foundation for the rational design of SELP hydrogel : DNA delivery systems under prescribed conditions, namely relatively high polymer concentration or cure time, and a release medium that minimizes the interaction between SELP and DNA (i.e., physiological ionic strength). By designing systems with these criteria in mind, one can achieve a predictable DNA release profile with a time-scale on the order of 1 month or greater.

We conclude that the genetically engineered silk-elastinlike polymer described in this work shows potential for the controlled delivery of plasmid DNA. We are currently conducting studies to assess the *in vitro* and *in vivo* bioactivity of the released DNA. The system offers several advantages over existing matrices, in particular, the ability to precisely and accurately customize the chemical structure, and thus the physicochemical properties and biological fate of the polymer offer many opportunities for gene delivery systems tailormade for specific applications.

ACKNOWLEDGMENTS

This work was partially funded by NIH grant GM61360 (HG) and a National Cancer Center Predoctoral Fellowship (ZM). The authors also thank Adam Dinerman for many thoughtful discussions.

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