



DNA delivery from photocrosslinked PEG hydrogels: encapsulation efficiency, release profiles, and DNA quality

Deborah J. Quick^a, Kristi S. Anseth^{a,b,*}

^aDepartment of Chemical and Biological Engineering, University of Colorado, Boulder, CO 80309, USA

^bHoward Hughes Medical Institute, University of Colorado, Boulder, CO 80309, USA

Received 3 November 2003; accepted 30 January 2004

Abstract

Sustained DNA delivery from polymer matrices provides a means for enhanced and prolonged gene therapy; however, limitations exist with respect to tailoring the DNA release profiles and maintaining the quality of the encapsulated DNA over time. To address these issues, PEG-based macromolecular monomers were photopolymerized to produce hydrogels with various degradation rates to control the DNA release profiles. Photocrosslinked PEG-based hydrogels were designed that released DNA for periods of 6–100 days with either nearly linear or delayed burst release profiles. Plasmid DNA was released primarily in the relaxed and supercoiled forms, and the released DNA showed high biological activity in plated cell cultures. The addition of both chemical and physical protective agents helped preserve the supercoiled form of the plasmid DNA during photoencapsulation (up to 75% compared to non-encapsulated plasmid controls), thereby enhancing the biological activity of the released DNA.

© 2004 Elsevier B.V. All rights reserved.

Keywords: Controlled release; Gene delivery; Photopolymerization

1. Introduction

Many approaches are being explored to improve the efficiency of current gene therapy methods. One area of research is focused on identifying polymer matrices to deliver DNA to cells *in vivo*, where they have been shown to increase DNA resistance to degradation by nucleases [1–4], increase plasmid DNA uptake compared to bolus delivery methods

[2,5,6], and provide controlled dosing. In addition, sustained DNA release can prolong foreign gene expression [5–9], thereby reducing the need for repeated dosing, which is a significant advantage for long-term gene therapy.

Several types of polymer matrices have been explored for DNA delivery, with poly(lactic-co-glycolic acid) (PLGA) systems being the most common. However, with PLGA delivery vehicles, DNA encapsulation efficiencies can be low; and thus, recent efforts have improved encapsulation often by introducing amphiphilic molecules to increase the association of DNA with the hydrophobic polymers [10–14]. More important, though, is the DNA damage that has been observed as result of PLGA degradation [5,15].

* Corresponding author. Department of Chemical and Biological Engineering, University of Colorado, ECCH 111, CB 424, Boulder, CO 80309, USA. Tel.: +1-303-492-3147; fax: +1-303-735-0095.

E-mail address: kristi.anseth@colorado.edu (K.S. Anseth).

Degrading PLGA produces acidic degradation products that accumulate and are thought to damage encapsulated DNA. Researchers are investigating more porous PLGA structures to reduce accumulation of degradation products in the implant interior, thereby mitigating damage to pH-sensitive molecules like DNA [16].

An alternative to PLGA-based delivery systems is to encapsulate DNA in more hydrophilic polymer matrices, such as hydrogels. In general, hydrogels have high encapsulation efficiencies and are much less damaging to DNA than PLGA. Hydrogels based on naturally occurring biopolymers, including alginate [17–19], chitosan [20,21], collagen [22], and gelatin [9], have shown transfection of cells *in vitro* and/or *in vivo* with released DNA. However, the biopolymer hydrogels release DNA by ionic exchange or enzymatic degradation, which can make it difficult to control the DNA dosing and/or can reduce the ability to sustain release for long periods.

The limited control of DNA release with biopolymer-based hydrogels has spurred research with hydrogels based on synthetic polymers. Synthetic hydrogels can offer broader control of the release characteristics, but the gelation conditions and the chemical environment must be carefully selected to limit DNA damage. Cell transfection has been observed with DNA released by hydrolytic degradation of succinimidyl-crosslinked poly(ethylene glycol) (PEG) gels [6], hydrogels formed by emulsion with triblock copolymers of PEG and poly(lactic acid) (PLA) [23], and hydrogels formed by the temperature transition of triblock copolymers of PEG and poly(propylene oxide) (PPO) [24]. Self-associating hydrogels, such as the last two systems, tend to rely on diffusion and disruption of weak interactions to release DNA. In contrast, chemically crosslinked gels provide a robust platform to systematically control gel degradation, but the degradation kinetics must be well tuned to provide the desired DNA release profiles.

Covalently crosslinked hydrogels can provide a wide range of release profiles through the polymerization of multifunctional monomers that contain hydrolytically cleavable blocks. Gels crosslinked through photopolymerization have been used for cell encapsulation [25–27] and protein delivery [27–29], but they have been relatively unexplored for DNA encapsulation and delivery [30,31]. One group has

developed multifunctional, styrenated gelatin as a photocrosslinked hydrogel for DNA delivery [30]. Cell transfection was observed *in vivo* with DNA released from stents coated with the photopolymerized gelatin, demonstrating that released DNA retained at least a portion of its initial activity during photoencapsulation. However, by basing the hydrogel on gelatin, DNA release was controlled by enzymatic degradation.

In this work, the suitability of photocrosslinked hydrogels formed from multifunctional PEG monomers to serve as DNA delivery systems is addressed. Ultimately, the goal is to develop a degradable polymer gel that forms under mild conditions enabling encapsulation of DNA with minimal damage and a matrix that can be tailored to provide a wide range of DNA release profiles. Despite the detrimental effects of photoinitiator radicals on DNA in the absence of monomer, methods have been identified for protecting DNA [31]. Here, the effects of photoencapsulation, both in the presence and absence of these protective additives, on the efficiency of DNA encapsulation and the activity of the released DNA are addressed. The DNA release profiles from photocrosslinked hydrogels formed from several monomer chemistries were characterized, and the quantity and quality of the released DNA were evaluated. The effects of protective additives on DNA recovery and the integrity of released DNA are also described.

2. Materials and methods

2.1. Materials

DNA plasmids that encode reporter proteins were purchased from commercial vendors (pSEAP2-Control: Clontech, Palo, Alto, CA and pCMV-SPORT-Bgal: Gibco/Invitoren, Carlsbad, CA) and subsequently amplified by a commercial vendor (Aldevron, Fargo, ND). D,L-Lactide (Polysciences, Warrington, PA), ϵ -caprolactone (Aldrich, St. Louis, MO), poly(ethylene glycol), 4000 and 4600 g/mol (Fluka, Milwaukee, WI), stannous 2-ethyl hexanoate (Sigma, St. Louis, MO), triethylamine (Aldrich), and methacryloyl chloride (Aldrich, 97%) were used as received without further purification. Solvents were of reagent grade.

2.2. Monomer synthesis

Dimethacrylated poly(lactic acid)-*b*-poly(ethylene glycol)-*b*-poly(lactic acid) triblock copolymers (PEG–PLA–DM) and dimethacrylated poly(caprolactone)-*b*-PEG-*b*-poly(caprolactone) triblock copolymers (PEG–PCL–DM) were synthesized according to procedures reported previously [32]. Monomers were synthesized with the same PEG core molecule (4000 g/mol) but with different numbers of lactic acid units ranging from 2.5 LA/PLA block (lactic acid units per poly(lactic acid) block) to 8.5 LA/PLA block (see structure in Fig. 1). In addition, monomers with the same 5 LA/PLA block but differing PEG core molecular weights, 4000 and 4600 g/mol, were synthesized. Finally, monomers were synthesized with PEG 4000 and caprolactone following the same reaction scheme to obtain monomer with 3 CL/PCL block (caprolactone units per poly(caprolactone) block). Numbers of lactic acids and caprolactones per molecule were determined by ¹H NMR as the ratio of the integrals of peak areas (ratio of ester

proton of PEG to the backbone proton of PLA or the acetate proton of PCL). Methacrylation efficiency ranged from 70% to 100%, as determined by ¹H NMR.

2.3. DNA photoencapsulation

Selected PEG-based monomers (described above) were dissolved in deionized water to give a final concentration of 10 wt.% monomer. A photoinitiator ((4-(2-hydroxyethoxy)phenyl)-(2-propyl)ketone, I-2959, Ciba-Geigy, Tarrytown, NY) was added to the monomer solution to give a final concentration of 0.05 wt.% I-2959. Plasmid DNA was added to the monomer/initiator solution at a loading of 1–10 μg of DNA per mg of monomer. Where noted, plasmids were complexed to protamine sulfate (~ 4 kDa) at ratios of ~ 0.15 μg protamine sulfate (Sigma) per μg DNA for 15–20 minutes prior to being mixed into the monomer solution. The monomer/DNA mixtures were transferred to lids of microcentrifuge tubes and exposed to 5–11 mW/cm² of 365 nm light for 10 min to

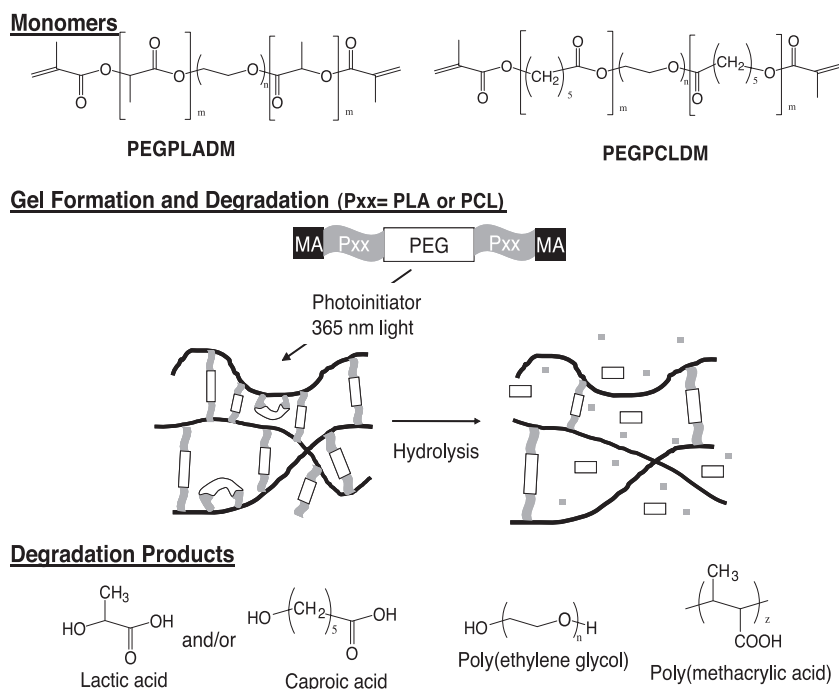


Fig. 1. Schematic representation of the polymerization of PEG–PLA–DM monomers to form degradable gels, along with the degradation process and chemical structures of the starting monomer and final degradation products.

produce the degradable hydrogels. The hydrogels were approximately 8 mm in diameter and 3 mm in thickness.

2.4. Gel degradation and DNA release studies

Polymerized gels were degraded in 2–5 ml phosphate-buffered saline (PBS, pH = 7.4) at 37 °C on an orbital shaker. Buffer was removed periodically, and the DNA concentration in solution was determined by the PicoGreen assay (Molecular Probes, Eugene, OR), which fluoresces upon interaction with double-stranded DNA. Control gels that contained no DNA were degraded in parallel to account for the effects of the polymer degradation products on the PicoGreen assay. Fresh buffer was added to the gels, which were then returned to the shaker. Mass loss was determined by freeze-drying disks on selected days throughout the degradation process and comparing the dry mass to the original dry polymer mass.

2.5. Quality of released DNA

Quality of the released DNA was examined with both agarose gel electrophoresis and cell transfections. Agarose gel electrophoresis was conducted by loading 20–50 ng of DNA per lane on a 0.8 wt.% agarose gel (high melting temperature agarose, Fisher). Electrophoresis was conducted at 100 V for 45 min. Gel photographs were analyzed with Kodak 1D software (Eastman Kodak, Rochester, NY).

Cell transfections were conducted with NIH 3T3 cells (ATCC, Manassas, VA). Cells were seeded in 12-well plates and allowed to reach ~75% confluency. DNA samples (released from gels and unencapsulated controls) were complexed with Lipofectamine (Gibco), using 3 μ l Lipofectamine per μ g of DNA in the sample. After allowing the DNA complexes to form for 20 min, the plated cells were washed with PBS; 1 ml Opti-MEM (Gibco) media was added to each well; and 2 μ g of DNA in complexed form was added to each well. After 5 h, the transfection media was removed; cells were rinsed with PBS; and 1 ml of complete media (DMEM with 10% calf serum, Invitrogen) was added to each well. After 2 days, evidence of transfection was measured. To detect transfection by plasmids that encode secreted alkaline phosphatase (SEAP), media samples were removed and assayed for SEAP activity using a SEAP assay kit (Clontech) and a Wallac Victor 2 fluorometer (Perkin-Elmer, Wellesley, MA).

tase (SEAP), media samples were removed and assayed for SEAP activity using a SEAP assay kit (Clontech) and a Wallac Victor 2 fluorometer (Perkin-Elmer, Wellesley, MA).

3. Results and discussion

3.1. Mechanism of DNA release from photocrosslinked PEG-based gels

Hydrogels formed from PLA-*b*-PEG-*b*-PLA or PCL-*b*-PEG-*b*-PCL monomers degrade via a relatively homogeneous, bulk hydrolysis mechanism. Over time, the PLA or PCL blocks degrade, leading to a decrease in the gel crosslinking density, which influences mass loss and DNA release. Initially, mass loss is due to the release of PEG from the degrading crosslinks. As more of the crosslinks degrade, the gel begins to release the backbone kinetic chains, which is rapidly followed by the complete dissolution of the gel. Basically, the gel becomes soluble when there are less than two crosslinks per kinetic chain. Metters et al. [33] have characterized this behavior extensively, and Fig. 2 shows a typical mass loss profile.

In this work, degradable PEG-based hydrogels formed from different monomer chemistries to control DNA release were investigated. DNA release from

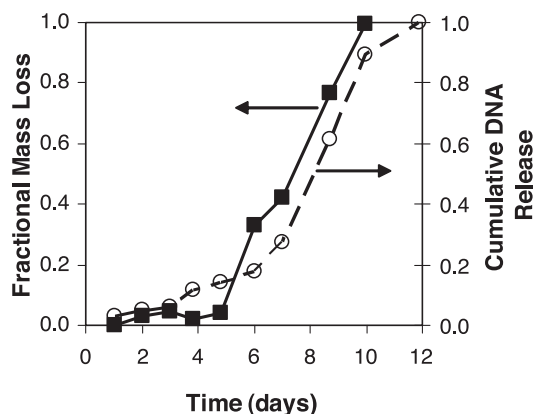


Fig. 2. Mass loss (—■—) from photocrosslinked PEG-PLA-DM hydrogels (PEG = 4600 g/mol, 5 LA/PLA block) with concurrent DNA release (---○---).

these networks depends on the rate of hydrolysis of the crosslinks and the microstructure of the gel (e.g., number of crosslinks per kinetic chain, length of the kinetic chains, relative amounts of cyclization versus crosslinking). In these systems, the polymer mesh expands as the crosslinks degrade, allowing DNA to be released from the gel. Since the diameter of a DNA helix is approximately 2 nm [19], DNA does not readily diffuse through these networks, and release is strongly coupled to the degradation rate. Since the gel degradation dictates the release of encapsulated DNA, DNA release profiles closely resemble the mass loss profile (Fig. 2).

To vary and control the degradation rate, PEG–PLA–DM macromers were synthesized with varying PLA block lengths and copolymerized macromers with PEG–PCL–DM. Macromer chemistry affects gel degradation through the molecular weight of the PEG core, the length of the PLA or PCL blocks, and the choice of PLA or PCL degradable blocks. The size of the PEG core mainly affects the gel mesh size, whereas the size and type of PLA or PCL blocks affect the rate of hydrolysis. For large molecules like plasmid DNA, increasing the PEG core molecular weight is insufficient to increase the mesh size of the hydrogels and allow DNA diffusion through the network. Instead, the mesh size is controlled and increased by hydrolysis of the crosslinks. PLA blocks are more susceptible to hydrolysis than PCL blocks, and thus, DNA is released more quickly from PEG–PLA–DM gels than PEG–PCL–DM gels. Since each lactic acid unit (LA) or caprolactone unit (CL) in these PLA or PCL blocks contains a hydrolytically unstable ester bond, with more LA or CL in the blocks, the probability that a crosslink will be degraded is increased, and they are cleaved more quickly. Thus, with more LA per PLA block or CL per PCL block, DNA will be released more quickly, up to a point. PLA and PCL are hydrophobic, and thus, with enough LA or CL per block, the block hydrophobicity will overcome the hydrophilicity of the PEG core, leading to reduced hydrolysis of the crosslinks and slower degradation despite the presence of more sites for hydrolysis.

Here, plasmid DNA was encapsulated in copolymer and homopolymer PEG-based gels of varying chemistries to evaluate the DNA release profiles, the encapsulation efficiency, and the quality of the re-

leased DNA. The following sections detail how the gel degradation influences each of these factors using either naked or protected DNA.

3.2. Encapsulation and release of naked plasmid DNA from PEG-based gels

3.2.1. Recovery of DNA released from photocrosslinked hydrogels

When DNA was photoencapsulated in hydrogels with no protective agent present, up to 70% of the loaded DNA was recovered. Previous results [31,32] demonstrated that exposure of DNA to photoinitiator radicals in the absence of monomer, using the same photoinitiation conditions, led to recovery of only ~ 2% of the naked plasmid. The higher recovery during photoencapsulation may be due to the high reactivity of the double bonds in the methacrylate endgroups of the monomer, which should leave fewer radicals to react with DNA in solutions containing monomer at high concentrations relative to the DNA. Once radicals react with methacrylated monomer to form propagating chain radicals, they may still react with and damage DNA, but the increased molecular weight of the propagating radicals likely reduces their reaction with DNA.

Experiments with monomethacrylated PEG monomer were conducted to elucidate the relative reactivity of radicals with methacrylated monomer compared to DNA and the ability of propagating radicals to damage DNA [31]. With monomethacrylated monomer, no crosslinking occurs and a linear, soluble polymer forms in the presence of radicals, instead of a crosslinked gel, as is formed with dimethacrylated monomers. Soluble polymers allow easy assay of DNA damage by radicals. With 10 wt.% monomethacrylated PEG monomer (5000 g/mol), ~ 80% of naked DNA was recovered after exposure to photoinitiator radicals using the same photoinitiation conditions as before. With PEG alone (4000 g/mol), DNA recovery was not increased over naked DNA (~ 2% recovery). Thus, the methacrylate groups in the monomer provide a competing reaction site for the radicals, thereby mitigating DNA damage. It is important to note, however, that overall radical concentrations will generally be lower in polymerizations that produce linear polymers compared to those forming crosslinked polymers. Even so, the 50–70% DNA recovery from

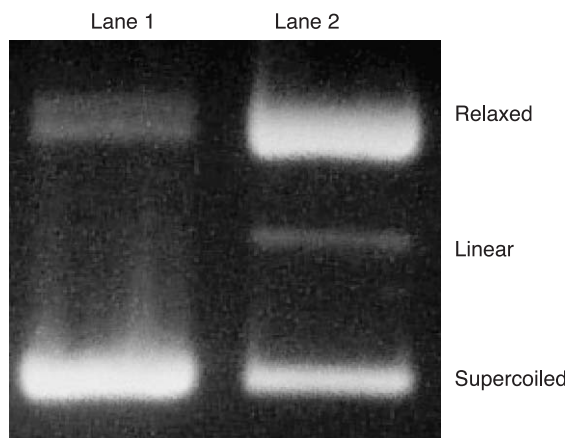


Fig. 3. Photograph of agarose gel electrophoresis showing conformations of plasmid DNA released from PEG–PLA–DM with 8.5 LA/PLA block hydrogels in the absence of protective additives. First lane is non-encapsulated DNA in a solution of degraded polymer, second lane is the released, encapsulated DNA.

photopolymerizations obtained with 10 wt.% dimethacrylated monomer is comparable to the DNA recovery obtained in the presence of ≥ 10 wt.% monomethacrylated PEG. Thus, despite the significant amount of DNA damage incurred by photo-initiation in the absence of monomer, when methacrylated monomer is present in solution, DNA damage is significantly reduced.

3.2.2. Integrity of released, unprotected DNA

High encapsulation efficiency and recovery of encapsulated DNA are just one goal of a gene delivery system; probably more important is the integrity and activity of the released DNA. Fig. 3 shows that the photoencapsulated DNA was released from the PEG-based hydrogels primarily in the relaxed and supercoiled forms with minimal occurrence of the inactive, linear form. The first lane shows non-encapsulated, control DNA in a solution of degraded polymer, while the second lane shows the encapsulated DNA that was released 1–2 days immediately prior to complete degradation of the PEG–PLA–DM containing 8.5 LA/PLA block gels. According to image analysis (Kodak 1D software), the released plasmid DNA contained $\sim 62\%$ relaxed form, $\sim 6\%$ linear form, and $\sim 32\%$ supercoiled form. When compared to the non-encapsulated plasmid, $\sim 40\%$ of the supercoiled form was retained

during encapsulation and subsequent polymer degradation. Similar results were observed for DNA encapsulated and released from gels containing 2.5 and 5 LA/PLA blocks. DNA encapsulated in PEG–PCL–DM, however, when run on an agarose gel resulted in a long smear indicating the DNA released from the gels was cleaved into many fragments. PEG–PCL–DM monomer impurities may have played a role in DNA damage, and the long duration of the polymer degradation could have also been harmful to the DNA. PEG–PCL–DM gels completely degraded after approximately 100 days, while PEG–PLA–DM gels degraded completely after approximately 9–27 days.

3.2.3. Effects of hydrogel chemistry on DNA release profiles

Another goal of a gene delivery device is to provide a wide range of release profiles in order to be adaptable to several applications. Photocrosslinked PEG-based hydrogels show great versatility in their release profiles (Figs. 4 and 5). To vary the gel degradation and DNA release rate, gels were formed with monomers containing different numbers

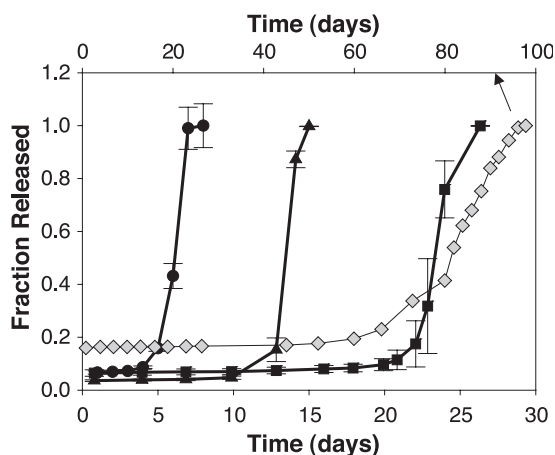


Fig. 4. DNA release from degradable PEG–PLA–DM and PEG–PCL–DM hydrogels (PEG=4000 g/mol) over time. Data are normalized to the total amount of DNA released from the gels. (●) 8.5 LA/PLA block, (▲) 5 LA/PLA block, (■) 2.5 LA/PLA block, all three polymerized with light intensity of 5 mW/cm², (◆) PEG–PCL–DM polymerized with a light intensity of 11 mW/cm² and plotted on upper x-axis, error bars = 1 standard deviation, n = 3–4, except PEGPCL, where n = 1.

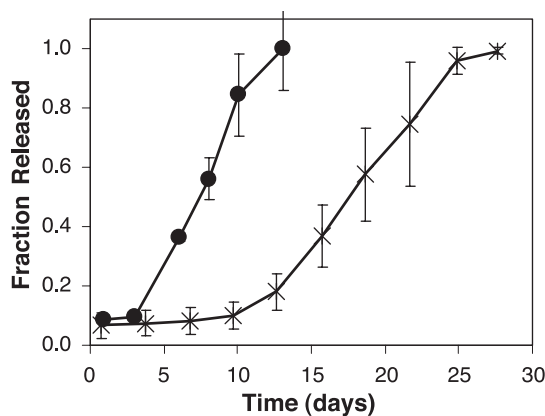


Fig. 5. DNA release profiles from hydrogels formed by copolymerizing PEG–PLA–DM and PEG–PCL–DM monomers. Data are normalized to the total amount of DNA released from the gels. On a weight percent basis, copolymer gels were composed of: (●) 7% 8.5 LA, 2% 5 LA, 1% 2.5 LA; (×) 5% 8.5 LA, 1.5% 5 LA, 1.5% 2.5 LA, 2% PEGPCL; error bars=1 standard deviation, $n=3-4$. LA refers to the number of lactic acid repeat units in the PLA block of the particular monomer.

of lactic acid repeat units in the PLA blocks (LA/PLA block) flanking the PEG core (PEG=4000 g/mol), as well as monomers containing caproic acid instead of lactic acid. The DNA release profiles demonstrate that gels with higher molecular weight PLA blocks degraded and released the entrapped DNA faster (Fig. 4). The data also demonstrate that hydrogels formed with PEG–PCL–DM degraded and released DNA more slowly than gels formed with PEG–PLA–DM.

In general, DNA release profiles from these homopolymer gels followed a characteristic curve, with a small initial burst in DNA release (5–20% of the total). The initial release is likely plasmid DNA at the gel surface that was not encapsulated in the network, possibly as a result of oxygen inhibiting polymerization at the surface. The variability in this initial release correlates with the variability in the total DNA recovery. When the total DNA recovery was higher, the fraction of DNA released in the initial burst was lower (the absolute concentrations on day 1 were similar—ranging from 4% to 10% of the loaded DNA). After the initial burst, the DNA release rate was negligible for an extended period of time, until a significant fraction of the gel crosslinks were cleaved. At this stage, the release rate increased steeply as the gel

completely eroded. As demonstrated in Fig. 4, the time-scale for this delayed release was controlled by the gel chemistry (PEG–PCL–DM >2.5 LA/PLA block >5 LA/PLA block >8.5 LA/PLA block) and varied from 7 to 100 days.

To modify the shape of the DNA release profile, these monomers are readily co-polymerized to form hydrogels with fast and slow degrading crosslinks. When DNA was released from polymers composed of particular combinations of monomers containing various molecular weight PLA blocks or combinations of monomers containing PLA and PCL blocks, the release profile was altered to approximate a more linear release profile with time (Fig. 5). A high percentage (>60%) of fast degrading monomer was needed to create copolymers with the more linear DNA release profiles. When the fast degrading crosslinks cleaved, the remaining network swelled to roughly twice the original disk volume, as would be expected based on the correlation between the swelling ratio and the crosslinking density [34]. DNA was released from this highly swollen mesh as the remaining crosslinks slowly degraded. These data show that the combination of fast and slow degrading crosslinks can significantly alter DNA release profiles, which implies the ability to precisely tailor the gel for a given application.

3.3. Encapsulation and release of protected plasmid DNA

Despite the successful encapsulation and sustained release of naked DNA in the supercoiled form, previously reported protection methods were exploited [31] to increase the recovery and structural integrity of the encapsulated DNA. Many DNA protection methods were explored to minimize radical damage by reducing the activity of radicals, by providing a competing reaction site for the radicals, and/or by forming a physical barrier to radical attack of DNA. When radical activity is reduced or when radicals are consumed by a competing reaction during polymerization, the network structure may be altered, with shorter kinetic chains or lower initial crosslinking densities. Both of these results can produce hydrogels with shorter times to complete degradation. Thus, when adding protective agents to the polymerization, it is important to consider the concentration and type

of chemical added, since these factors will impact the polymerization process.

3.3.1. Release of protected DNA from photocrosslinked hydrogels

When protective agents were added to photopolymerizations of PEG-based hydrogels, slight changes in the DNA release rate were observed. Fig. 6 shows the effects of two protective additives, vitamin C and protamine sulfate, on the DNA release rate from PEG–PLA–DM hydrogels containing 5 LA/PLA block. At the concentration tested (0.85 mM), vitamin C increased the rate of DNA release slightly, as would be expected with its radical scavenging abilities. At higher concentrations of vitamin C, polymer gel formation was completely inhibited during the typical exposure time, and thus higher concentrations of vitamin C could not be used to protect DNA during photoencapsulation. Complexing the DNA to protamine sulfate prior to encapsulation did not affect the release rate but did reduce the initial burst in DNA release. Combining the two protective agents resulted in $\sim 30\%$ faster release of DNA, which suggests that the initial structure of the polymer network was probably affected.

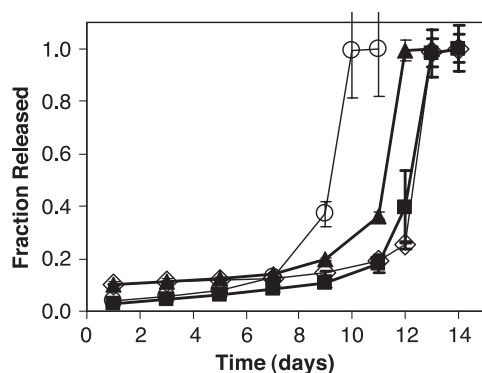


Fig. 6. Fractional DNA release from PEG–PLA–DM hydrogels formed in the presence of protective additives as a function of time. Data are normalized to the total amount of DNA released from the gels. (◇) no protective additives; (▲) vitamin C present during polymerization (0.85 mM); (■) protamine sulfate complexed to DNA prior to photoencapsulation, (○) protamine sulfate complexed to DNA prior to photoencapsulation and vitamin C present (0.85 mM); error bars = 1 standard deviation, $n=3-4$ (365 nm light at ~ 5 mW/cm², 0.05 wt.% I-2959, 10 min).

3.3.2. Recovery of released, protected DNA from photocrosslinked hydrogels

Without protective additives, 50–70% of photoencapsulated DNA was recovered upon complete degradation of the gels; with the remainder presumably denatured by radicals during polymerization. One would expect that the addition of protective additives would increase DNA recovery, but not all of the additives that previously protected DNA from initiator radicals [31] provided as much protection in the presence of polymerizing monomer.

For example, using PEG–PLA–DM containing 5 LA/PLA block, naked DNA was photoencapsulated with no protective additives, and $52 \pm 3\%$ of the DNA was recovered after complete polymer degradation. Using the same hydrogel chemistry, DNA recovery was increased to $61 \pm 5\%$ by complexing the DNA to protamine sulfate prior to encapsulation, but the improvement was not statistically significant. Surprisingly, vitamin C (at 0.85 mM) did not improve DNA recovery from the hydrogels ($47 \pm 2\%$) despite protecting roughly 30% of the DNA from photoinitiator radicals in the absence of monomer. Combining protamine sulfate with vitamin C slightly increased the average recovery ($69 \pm 12\%$), but again the results were not statistically different.

The inability of vitamin C and protamine sulfate to increase DNA recovery was somewhat surprising, given their ability to protect DNA from initiator radicals in the absence of monomer. One hypothesis as to why these protective agents did not improve DNA recovery is based on the relative concentrations of the molecules in solution. With the high concentration of dimethacrylated monomer (22 mM) relative to vitamin C (0.85 mM), radicals were more likely to react with a monomer molecule than vitamin C, thereby diminishing the protective effects of vitamin C. Since protamine sulfate theoretically protects DNA by a different mechanism than methacrylated monomer, by physically blocking radical attack of the DNA rather than chemically altering the nature of the radicals in solution, it might be expected to improve DNA recovery during photoencapsulation. Protamine sulfate alone increased the average DNA recovery by $\sim 17\%$, while the combination of protamine sulfate and vitamin C increased the average DNA recovery by $\sim 33\%$. However, these differences were not statistically significant, and more experiments are

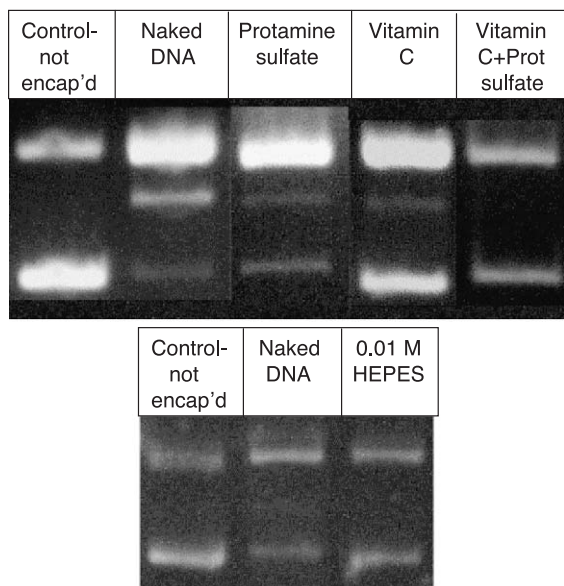


Fig. 7. Photographs of agarose gel electrophoresis showing conformation of released DNA and effects of protective additives using monomer with 5 LA/PLA block and $\sim 5 \text{ mW/cm}^2$ light, lanes in order from left to right: non-encapsulated DNA, released DNA with: no protection, complexed to protamine sulfate, vitamin C present (0.15 g/l), protamine sulfate complexed and vitamin C present (0.15 g/l). Bottom photograph shows conformation of non-encapsulated DNA and DNA released from polymer formed with monomer containing 8.5 LA/PLA block in the absence and presence of 0.01 M HEPES, respectively ($\sim 11 \text{ mW/cm}^2$ light).

needed to establish the amount of DNA protected by protamine sulfate during photoencapsulation.

Unlike vitamin C and protamine sulfate, the common biological buffer known as HEPES (4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid) had a greater effect on DNA recovery from the hydrogels. On average, roughly 80% of DNA photoencapsulated in the presence of 0.01 M HEPES was recovered after polymer degradation. Although the recovery of DNA increased with the use of HEPES, the variability also increased. With polymerizations conducted in PBS or water, DNA recovery ranged from 50% to 70%; in the presence of 0.01 M HEPES, DNA recovery ranged from 50% to 100%. HEPES contains tertiary amines and can scavenge radicals like vitamin C, yet it forms an active radical species that can propagate through the monomer. Thus, high concentrations of HEPES can be used without significantly inhibiting polymerization and gel formation.

3.3.3. Integrity of released, protected DNA

Although some of the protective additives did not significantly improve DNA recovery, striking effects were observed with respect to the integrity of the released DNA (Fig. 7, Table 1). Interestingly, complexing the DNA with protamine sulfate prior to photoencapsulation had little effect on the quality of the released DNA compared to unprotected DNA released from PEG-PLA-DM hydrogels containing 5 LA/PLA block. Use of vitamin C at 0.15 g/l, on the other hand, more effectively preserved the supercoiled form of the plasmid DNA. The combined protection by protamine sulfate and vitamin C prevented the damage leading to the linear form of the DNA and greatly reduced the loss of the supercoiled form, which was quite similar to the non-encapsulated DNA. Thus, although these protective additives did not significantly increase the quantity of released DNA, as discussed in the previous section, they did improve the DNA quality.

The bottom two panels in Fig. 7 show the effects of HEPES on the quality of the DNA released from PEG-PLA-DM hydrogels containing 8.5 LA/PLA block. Interestingly, HEPES did not have a significant effect on the quality of the released DNA. No linear form of the released plasmid was detected when

Table 1

Image analysis of agarose gel electrophoresis of released, photoencapsulated DNA

	Relaxed (%)	Linear (%)	Supercoiled (%)	Fraction in supercoiled form relative to non-encapsulated controls (%)
No protection	81	16	2	3
Protamine sulfate	80	15	5	9
($\sim 0.15 \mu\text{g PS}$: $\mu\text{g DNA}$)				
Vitamin C	66	10	24	40
(0.85 mM)				
Protamine sulfate/ Vitamin C	57	ND	43	70
($\sim 0.15 \mu\text{g PS}$: $\mu\text{g DNA}$, 0.85 mM VitC)				
No protection	67	ND	33	48
HEPES (0.01 M)	49	ND	51	74

Relative forms are calculated relative to the non-encapsulated plasmid in the appropriate degraded polymer solution. ND = none detected.

photoencapsulated in the presence of 0.01 M HEPES, but in this particular experiment, no linear plasmid was detected in the gels polymerized with no HEPES, possibly due to the selected monomer and photo-initiation conditions. Image analysis (Table 1) indicated that there was slightly more plasmid DNA released in the supercoiled form with HEPES present than without HEPES. Thus, despite the improvement in the quantity of DNA recovered from the degrading hydrogels polymerized in the presence of HEPES, HEPES had only a marginal effect on the quality of the released DNA.

3.3.4. Activity of released, protected DNA

To evaluate the activity of photoencapsulated and released DNA, the ability of the plasmid to produce the encoded reporter protein (secreted alkaline phosphatase) in cell transfections was tested. Several DNA samples, taken from the delayed burst portion of the gel release, were tested for their ability to transfect NIH 3T3 cells using Lipofectamine as the transfection agent (Fig. 8). Three DNA samples released from gels formed with 5 LA/PLA block in the presence of protective additives (vitamin C, protamine sulfate,

and both additives) were compared to an unprotected DNA sample released from gels formed with 2.5 LA/PLA block. Unfortunately, there was insufficient unprotected DNA recovered from the hydrogels prepared with 5 LA/PLA block monomer to conduct cell transfections.

All released DNA samples were capable of transfecting cells with at least half the efficiency of non-encapsulated DNA (Fig. 8). With protective agents present during polymerization, up to 80% of the signal of non-encapsulated DNA was achieved with released DNA. As shown previously, each sample contained different amounts of the supercoiled, relaxed, and linear forms, and as expected, the samples with the highest amount of the supercoiled form (70% of non-encapsulated) gave the highest level of transfection (80% of non-encapsulated). While it may be possible for damaged DNA to transfect cells, this trend of increasing transfection efficiency with the supercoiled form of the DNA reiterates its superiority over the other forms for transfection and the importance of releasing plasmid DNA in the supercoiled form. The ability of photocrosslinked, PEG-based hydrogels to release DNA capable of transfecting cells shows promise for their application in gene delivery.

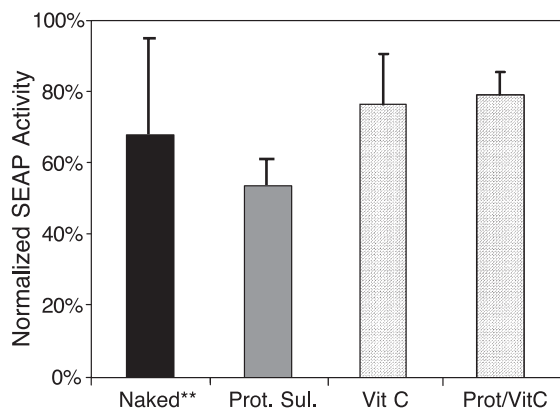


Fig. 8. Normalized secreted alkaline phosphatase (SEAP) activity for NIH 3T3 cells that were transfected with DNA released from gels with different protective agents. SEAP activity was normalized to cells transfected with non-encapsulated DNA in an appropriate degraded polymer solution, Naked**=no protective agent, 5 LA/PLA block monomer; all others 10 LA/PLA block monomer: Prot. Sul. = DNA complexed to protamine sulfate, VitC=0.15 g/l vitamin C present during polymerization, Prot/VitC= DNA complexed to protamine sulfate and 0.15 g/l vitamin C present, *P*-values are relative to the protamine sulfate protected DNA. Error bars = 1 S.D., *n* = 3.

4. Conclusions

Photoencapsulated DNA was released from degradable PEG-based hydrogels in an active, supercoiled form. Protective agents, primarily vitamin C, improved the quality of the released DNA by preserving the supercoiled form of the plasmid DNA. The addition of HEPES buffer increased the amount of DNA recovered from the hydrogels, suggesting an ability to reduce DNA damage by radicals during photoencapsulation. Additional evidence for the suitability of these photocrosslinked hydrogels for gene delivery was the ability of photoencapsulated plasmid DNA to transfect plated NIH 3T3 cells at levels 50–80% of that achieved with non-encapsulated plasmid DNA. DNA release profiles were tailored to provide release over 6 to 100 days by varying the monomer chemistry. By copolymerizing different monomers, the release profile was altered to provide steady DNA release over time. Through photopolymerization, more complex release profiles may be obtained

(e.g., pulsatile or different DNA released at different times), which expands the potential applications of photocrosslinked hydrogels making them a highly recommended system for gene delivery.

Acknowledgements

This work has been supported by the Packard Foundation and the Howard Hughes Medical Institute. DJQ is grateful for fellowships sponsored by the Department of Education's Graduate Assistance in Areas of National Need (GAANN) program and a NIH Training in Pharmaceutical Biotechnology Grant. We would also like to thank Tom Anchordoquy (UCHSC) for many helpful discussions and Kelly K. Macdonald for her assistance in the revision of this manuscript.

References

- [1] K.N. Atuah, E. Walter, H.P. Merkle, H.O. Alpar, *Journal of Microencapsulation* 20 (3) (2003) 387–399.
- [2] H. Cohen, R.J. Levy, J. Gao, I. Fishbein, V. Kousaev, S. Sosnowski, S. Slomkowski, G. Golomb, *Gene Therapy* 7 (22) (2000) 1896–1905.
- [3] B.I. Florea, P.G.M. Ravenstijn, H.E. Junginger, G. Borchard, *STP Pharma Sciences* 12 (4) (2002) 243–249.
- [4] D. Quong, R.J. Neufeld, *Biotechnology and Bioengineering* 60 (1) (1998) 124–134.
- [5] L.D. Shea, E. Smiley, J. Bonadio, D.J. Mooney, *Nature Biotechnology* 17 (6) (1999) 551–554.
- [6] K. Roy, D.Q. Wang, M.L. Hedley, S.P. Barman, *Molecular Therapy* 7 (3) (2003) 401–408.
- [7] B. Gander, L. Meinel, E. Walter, H.P. Merkle, *Chimia* 55 (3) (2001) 212–217.
- [8] R.E. Eliaz, F.C. Szoka, *Gene Therapy* 9 (18) (2002) 1230–1237.
- [9] Y. Fukunaka, K. Iwanaga, K. Morimoto, M. Kakemi, Y. Tabata, *Journal of Controlled Release* 80 (1–3) (2002) 333–343.
- [10] M.A. Benoit, C. Ribet, J. Distexhe, D. Hermand, J.J. Letesson, J. Vandenhoute, J. Gillard, *Journal of Drug Targeting* 9 (4) (2001) 253–266.
- [11] S. Hirose, B.G. Muller, R.C. Mulligan, R. Langer, *Journal of Controlled Release* 70 (1–2) (2001) 231–242.
- [12] G.G. del Barrio, F.J. Novo, J.M. Irache, *Journal of Controlled Release* 86 (1) (2003) 123–130.
- [13] Y.Y. Hsu, T. Hao, M.L. Hedley, *Journal of Drug Targeting* 7 (4) (1999) 313–323.
- [14] F.F. Zhuang, R. Liang, C.T. Zou, H. Ma, C.X. Zheng, M.X. Duan, *Journal of Biochemical and Biophysical Methods* 52 (3) (2002) 169–178.
- [15] E. Walter, K. Moelling, J. Pavlovic, H.P. Merkle, *Journal of Controlled Release* 61 (3) (1999) 361–374.
- [16] J.W. Kostanski, B.C. Thanoo, P.P. DeLuca, *Pharmaceutical Development and Technology* 5 (4) (2000) 585–596.
- [17] N. Aggarwal, H. HogenEsch, P.X. Guo, A. North, M. Suckow, S.K. Mittal, *Canadian Journal of Veterinary Research-Revue Canadienne De Recherche Veterinaire* 63 (2) (1999) 148–152.
- [18] K.H. Bouhadir, D.J. Mooney, *Journal of Drug Targeting* 9 (6) (2001) 397–406.
- [19] J. Ho, R.J. Neufeld, *STP Pharma Sciences* 11 (1) (2001) 109–115.
- [20] C. Aral, S. Ozbas-Turan, L. Kabasakal, M. Keyer-Uysal, J. Akbuga, *STP Pharma Sciences* 10 (1) (2000) 83–88.
- [21] H.Q. Mao, K. Roy, V.L. Troung-Le, K.A. Janes, K.Y. Lin, Y. Wang, J.T. August, K.W. Leong, *Journal of Controlled Release* 70 (3) (2001) 399–421.
- [22] J. Bonadio, *Advanced Drug Delivery Reviews* 44 (2–3) (2000) 185–194.
- [23] T. Kissel, Y.X. Li, F. Unger, *Advanced Drug Delivery Reviews* 54 (1) (2002) 99–134.
- [24] J. Liaw, S.F. Chang, F.C. Hsiao, *Gene Therapy* 8 (13) (2001) 999–1004.
- [25] S.J. Bryant, K.S. Anseth, *Journal of Biomedical Materials Research* 59 (1) (2002) 63–72.
- [26] K.T. Nguyen, J.L. West, *Biomaterials* 23 (22) (2002) 4307–4314.
- [27] J. Elisseff, W. McIntosh, K. Fu, T. Blunk, R. Langer, *Journal of Orthopaedic Research* 19 (6) (2001) 1098–1104.
- [28] J.A. Burdick, M.N. Mason, A.D. Hinman, K. Thorne, K.S. Anseth, *Journal of Controlled Release* 83 (1) (2002) 53–63.
- [29] J.A. Hubbell, *Journal of Controlled Release* 39 (2–3) (1996) 305–313.
- [30] Y. Nakayama, J.Y. Kim, S. Nishi, H. Ueno, T. Matsuda, *Journal of Biomedical Materials Research* 57 (4) (2001) 559–566.
- [31] D.J. Quick, K.S. Anseth, *Pharmaceutical Research* 20 (11) (2003) 1730–1737.
- [32] A.S. Sawhney, C.P. Pathak, J.A. Hubbell, *Macromolecules* 26 (4) (1993) 581–587.
- [33] A.T. Metters, K.S. Anseth, C.N. Bowman, *Polymer* 41 (11) (2000) 3993–4004.
- [34] G. Odian, *Principles of Polymerization*, Wiley, New York, 1991.