Controlled DNA Delivery Systems

Dan Luo,[1] Kim Woodrow-Mumford,[1] Nadya Belcheva,[1] and W. Mark Saltzman[1,2]

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Purpose. Genes are of increasing interest as pharmaceuticals, but current methods for long-term gene delivery are inadequate. Controlled release systems using biocompatible and/or biodegradable polymers offer many advantages over conventional gene delivery approaches. We have characterized systems for controlled delivery of DNA from implantable polymer matrices (EVAc: poly (ethylene-co-vinyl acetate)) and injectable microspheres (PLGA and PLA: poly (D, L-lactide-co-glycolide) copolymer and poly (L-lactide), respectively).

Methods. Herring sperm DNA and bacteria phage λ DNA were encapsulated as a model system. Released DNA concentration was determined by fluoroassays. Agarose electrophoresis was used to determine the dependence of release rate on DNA size. The Green Fluorescent Protein (GFP) gene was used to determine the integrity and functionality of released DNA.

Results. Both small and large DNA molecules (herring sperm DNA, 0.1–0.6 kb; GFP, 1.9 kb; λ DNA, 48.5 kb) were successfully encapsulated and released from EVAc matrices, and PLGA or PLA microspheres. The release from DNA-EVAc systems was diffusion-controlled. When co-encapsulated in the same matrix, the larger λ DNA was released more slowly than herring sperm; the rate of release scaled with the DNA diffusion coefficient in water. The chemical and biological integrity of released DNA was not changed.

Conclusions. These low cost, and adjustable, controlled DNA delivery systems, using FDA-approved biocompatible/biodegradable and implantable/injectable materials, could be useful for *in vivo* gene delivery, such as DNA vaccination and gene therapy.

KEY WORDS: DNA; controlled release; microsphere; EVAc; PLGA; PLA.

INTRODUCTION

Directly injected DNA can express its encoded proteins and elicit specific immune responses in animals (1–2). Within a short period, DNA will be available as a pharmaceutical for gene delivery, vaccination, and other applications in molecular medicine. Most of the DNA delivery technologies reported so far have been focused on naked DNA delivery (as in DNA vaccination) and non-viral or viral vector mediated systems (see review 3). Clinical applications of viral-mediated systems have been delayed by safety issues such as mutagenic potential and immunogenicity (4–5). The generally poor efficiency of delivery and expression by non-viral systems remains one of the main limitations to the development of gene therapy and DNA vaccination (6–7). Much attention is therefore being paid to the design of new formulations of DNA with various substances, such as lipid (8), polycation/polysaccharide (9), peptide (10), peptoid (11), gold particles (12), protein (13), polymers (14) and other complexes (15). All these systems deliver DNA as a bolus, without long-term sustained release. Since interstitial administration of a single dose of naked DNA can lead to gene expression, it is reasonable to speculate that prolonged, continuous DNA delivery to tissues will enhance gene expression.

Controlled release systems using biocompatible and/or biodegradable polymers provide an attractive alternative for long-term delivery of therapeutic agents (including DNA). There are many advantages of polymer-mediated controlled release systems over conventional delivery systems (see review 16): (i) agents can be delivered to tissues in a sustained, continuous and predictable fashion; (ii) they are well protected before being released; (iii) site specific delivery (such as in brain) can be achieved by simple implantation or direct injection; and (iv) repeated drug administration is not necessary. Despite the fact that in recent years controlled release systems have been successfully employed to deliver proteins and other macromolecules (17–19), polymer-based DNA controlled release systems have not been fully explored. The few previous reports of DNA delivery using synthetic polymers have important limitations, such as limited range of DNA sizes and DNA dosages, reliance on non-FDA approved materials, difficulty in control of release rate (20–24).

In this study, we examined several biocompatible polymers (including EVAc and PLGA, which are approved by the FDA) for their ability to encapsulate and release DNA. We followed the short- and long-term release of DNA from these controlled release systems and evaluated factors relevant to delivery of DNA in humans. DNA was released in a controlled and sustained fashion from a variety of polymer formulations. Although DNA was released for up to 1 month, the integrity and function of released DNA was maintained, which is a necessary requirement for clinical application for this novel approach to DNA delivery.

MATERIALS AND METHODS

Fabrication of DNA-Encapsulated EVAc Matrices

One hundred mg of HS-DNA (10 mg/ml) were lyophilized overnight. The dry powder was ground to a size <100 μm before incorporation into EVAc matrices. The encapsulation procedures were modified based on a previously published protein encapsulation method (25). Briefly, 100 mg of EVAc (Dupont, Wilmington, DE) was dissolved in 1 ml of methylene chloride. After adding DNA powder, the mixture was vortexed briefly and immediately poured into a dry-ice chilled mold. The mixture in the mold was then quickly chilled and was removed and placed in the −20°C freezer. Methylene chloride was evaporated for 2 days and then for another 2 days under vacuum at room temperature. The resulting DNA-EVAc slabs were cut into 4 blocks weighing about 40 mg. No detectable degradation of pure, non-encapsulated HS-DNA was found during continuous incubation in PBS at 37°C for up to 35 days (data not shown).

[1] School of Chemical Engineering, Cornell University, Ithaca, New York 14850.

[2] To whom correspondence should be addressed. (e-mail: saltzman@cheme.cornell.edu)

ABBREVIATIONS: EVAc, poly(ethylene-co-vinyl acetate); GFP, green fluorescent protein; HS-DNA, herring sperm DNA; λ DNA, bacteria phage λ DNA; PLA, poly(L-lactide); PLCL, poly(D, L-lactide-co-(ε) caprolactone); PLGA, poly(D, L-lactide-co-glycolide); BSA, bovine serum albumin.

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Fabrication of DNA-Encapsulated Biodegradable Microspheres

The manufacturing procedures were a modification of the double emulsion (water/oil/water) solvent evaporation technique (26–27). Briefly, 200 mg of PLGA (M_n = 54,100, Birmingham Polymers, Birmingham, Alabama) or PLA (M_n = 2000, and 300,000, Polysciences Inc. Warrington, PA) were dissolved in 2 ml of methylene chloride in a short glass test tube (5.8 × 1.4 cm). One mg of HS-DNA (10 mg/ml) was added drop-wise into the polymer solution while vortexing. Sonication was performed in crushed ice for 10 s (Tekmar Soni Disrupter model TM300, 40% duty cycle, microtip #4) to achieve a homogeneous milky mixture. Four ml of aqueous 1.0% PVA (poly (vinyl alcohol), 25000 M_w, 88 mol% hydrolyzed, Polysciences) was then slowly added to the milky 1st emulsion in ice. Sonication was repeated for another 10 s to form the second emulsion. Finally, the 2nd emulsion was added to 100 ml of vigorously stirring 0.3% PVA solution, and the mixture was kept under continuous stirring at room temperature for 3 h to form microspheres. Centrifugation was performed at 3000 rpm at 4°C for 10 min to collect microspheres. The collected microspheres were washed 3 times with milli-Q water before freezing in the −70°C freezer. The microspheres were then lyophilized for 24 hours.

Microsphere Entrapment Efficiency Analysis

Microspheres were evaluated for DNA content using an extraction procedure developed in this lab. Briefly, 30 mg of microspheres were dissolved in a glass scintillation vial with 1 ml of methylene chloride. To extract the DNA from the organic solution, Milli-Q water was added to the oil phase and vortexed vigorously for 1 minute before centrifuge at 1000 rpm for 10 minutes. The aqueous phase was collected carefully. Three extractions were performed with a total collection of 1.5 ml of aqueous fraction. Each microsphere formulation was analyzed in duplicate. Blank, DNA-free microspheres and pure DNA were also subjected to the same extraction procedure as controls. The extracted DNA content was analyzed by a DNA-specific fluoroassay.

DNA Assays

DNA concentration was determined using a PicoGreen (E_x = 480 nm, E_m = 520 nm) dsDNA quantitation Kit (Molecular Probes, Inc., Eugene, Oregon). A standard curve of λ DNA was constructed at the same time, and DNA concentrations were determined from unknown samples by comparison to the standard curve. The limit of detection for this assay was 50 pg/ml.

Scanning Electron Microscopy (SEM)

Microsphere morphology was analyzed by scanning electron microscopy (Steroscan 440, Leica Cambridge, Ltd.). A monolayer of dry microspheres was mounted on an aluminum stub using double-sided carbon tape. The sample was coated with a 10 nm thick palladium/gold (60:40) film using a sputter coater (Desk II, Denton Vacuum, Inc.). The coated samples were examined using an electron acceleration voltage of 5–10 keV. Size distribution and average particle diameter were determined by analyzing 5–10 images, representing >2000 particles, using the freeware program NIH-Image (NIH-Image, which was written by Wayne Rasband, is available by anonymous FTP from zippy.nimh.nih.gov).

Electrophoresis

Gel electrophoresis was performed at a constant 100 volts. DNA was separated in 1% of agarose gel and stained for 0.5 hr in SYBR Green II solution (Molecular Probes, Inc., Eugene, Oregon). DNA bands were quantified using the Multi- Analysis program (Bio-Rad Laboratory, Hercules, CA).

Controlled Release of DNA into PBS

EVAc matrices (quadruplicates) were incubated in 4 ml of phosphate-buffered saline (PBS) containing 0.02% gentamicin sulfate for up to 30 days at 37°C with gentle shaking. At a predetermined time point, the entire PBS solution was replaced with fresh PBS. An aliquot of pure non-encapsulated DNA was also incubated in PBS as controls; samples were removed for evaluation of DNA degradation at the same time intervals.

Microspheres were incubated in 0.3 ml of PBS containing 0.02% gentamicin sulfate for up to 30 days at 37°C with gentle shaking. Periodically, 0.2 ml of buffer was collected after centrifugation and replaced with 0.2 ml of fresh buffer. Blank microspheres, treated identically, were used as controls.

PCR and Transfection Assay

PCR reactions were performed using the GeneAmp PCR System 2400 (Perkin Elmer Applied Biosystems) according to standard PCR protocols. Briefly, 3 ng of template DNA pGFP-C2 (Promega, Madison, WI) was amplified with 0.5 μM of primers (BGFPUP and BGFPDOWN, CTGATTCTGTGGATAACCGTATT and TGGAACAACACTCAACCCTATCT, respectively). An expected single 1.9 kb band was detected. CHO cells (American Type Culture Collection, Rockville, MD) were transfected by FUGENE 6 (Boehringer Mannheim, Indianapolis, IN) with 1 μg of DNA. GFP expressions were visualized by fluorescence microscopy 24 hours post-transfection.

RESULTS

EVAc System

Herring Sperm DNA (HS-DNA) was employed as a model system for evaluating delivery of linear, double-stranded DNA of small size (600 bp). DNA was continuously released from all DNA-EVAc matrices over the duration of the experiment (>1 month) (Fig. 1). Among different EVAc matrices, we observed a similar bi-phasic behavior of DNA release: an initial burst of release (phase I) and a period of slow, but continuous, release (phase II). Release rates during phase I increased as the percentage of DNA initially in the matrix increased, whereas release rates during phase II were similar among different loaded matrices (Fig. 1a inset). The cumulative percentage of DNA released was also affected by the EVAc loading capacity (Fig. 1b). After two weeks of incubation in the buffered solution, about 1/2 of the encapsulated DNA was released from 50% loaded EVAc while only 20%, 10%, and 5% of the payload was released from 40%, 30%, and 20% loaded matrices, respectively.

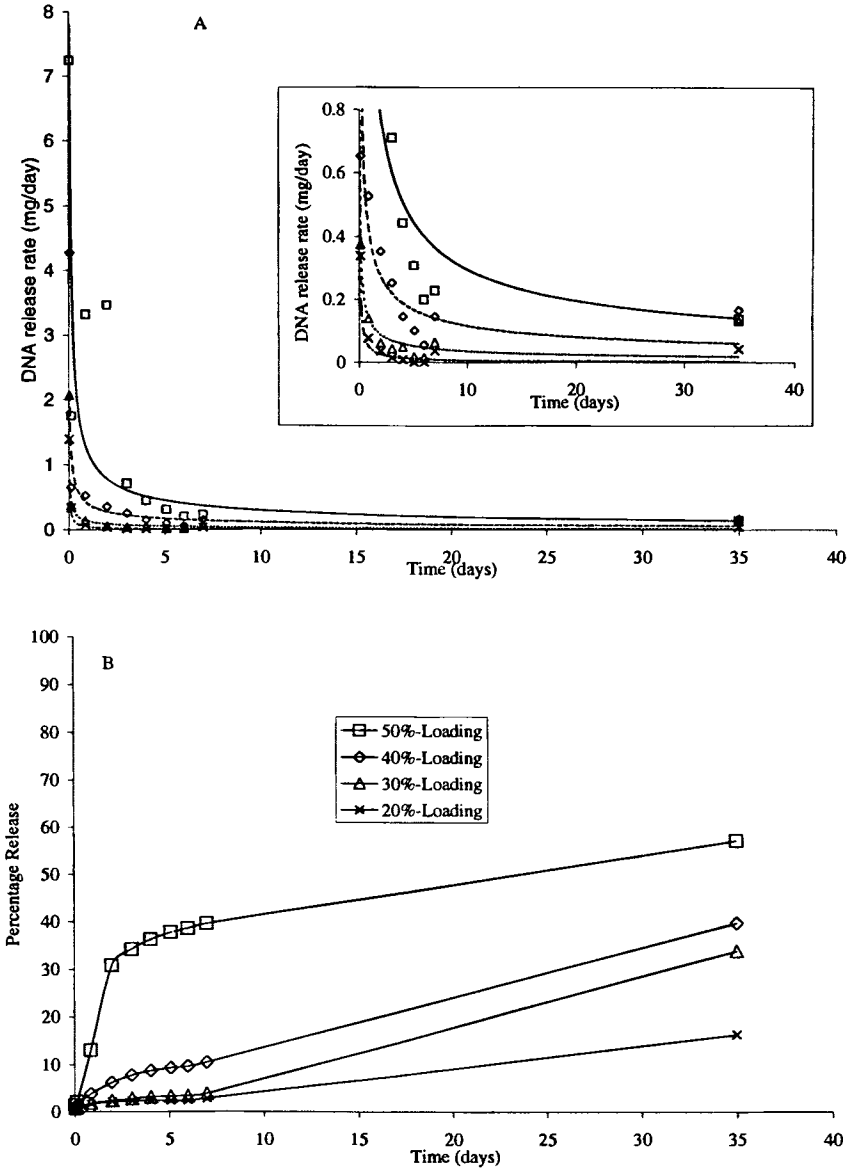

Fig. 1. Controlled DNA release from EVAc matrices. Each data point is the average of quadruplicate EVAc discs. The symbols represent different amounts of DNA (weight percentage) encapsulated in EVAc matrices: 50% (□), 40% (◇), 30% (△) and 20% (×). (A) DNA release rate (mg/day), obtained from dividing net amount of released DNA by the length of two time points, is plotted at the time point 2. (B) Cumulative DNA release in percentage. Error bars represent the standard error.

To characterize the size dependency of DNA release from EVAc, 0.02 mg (1%) of λ DNA (48.5 kb) was co-dispersed with 1.98 mg of HS-DNA (0.1–0.6 kb) in an EVAc matrix (2 mg). One percent of total released DNA (determined by fluoroassays) was calculated as "theoretical released λ DNA". To determine the amount of actual λ DNA released, samples of released DNA were subjected to electrophoresis along with standard solutions containing an amount of λ DNA equal to 1% of DNA. The ratio of actual to theoretical λ DNA released was determined from these gels. On the average, large λ DNA molecules were released much slower than the smaller HS-DNA (Fig. 2), suggesting that DNA molecular weight is another important factor in determining the kinetics of encapsulated DNA release.

To quantify the difference in rate of release at early time, the apparent diffusion coefficient (D_{app}) for DNA release was determined by comparing data to a model for release from a disc (18):

$$\frac{M_t}{M_0} = 4\sqrt{\frac{D_{app}t}{L^2\pi}} \qquad (1)$$

where M_t is the cumulative mass of DNA released, M_0 is the mass initially in the matrix, D_{app} is the apparent diffusion coefficient, and L is the thickness of the disc. The apparent diffusion coefficients (D_{app}) for DNA from EVAc systems depend on both DNA molecular weight and loading. The D_{app} of the smaller HS-DNA is about 22 times higher than that of the larger λ DNA.

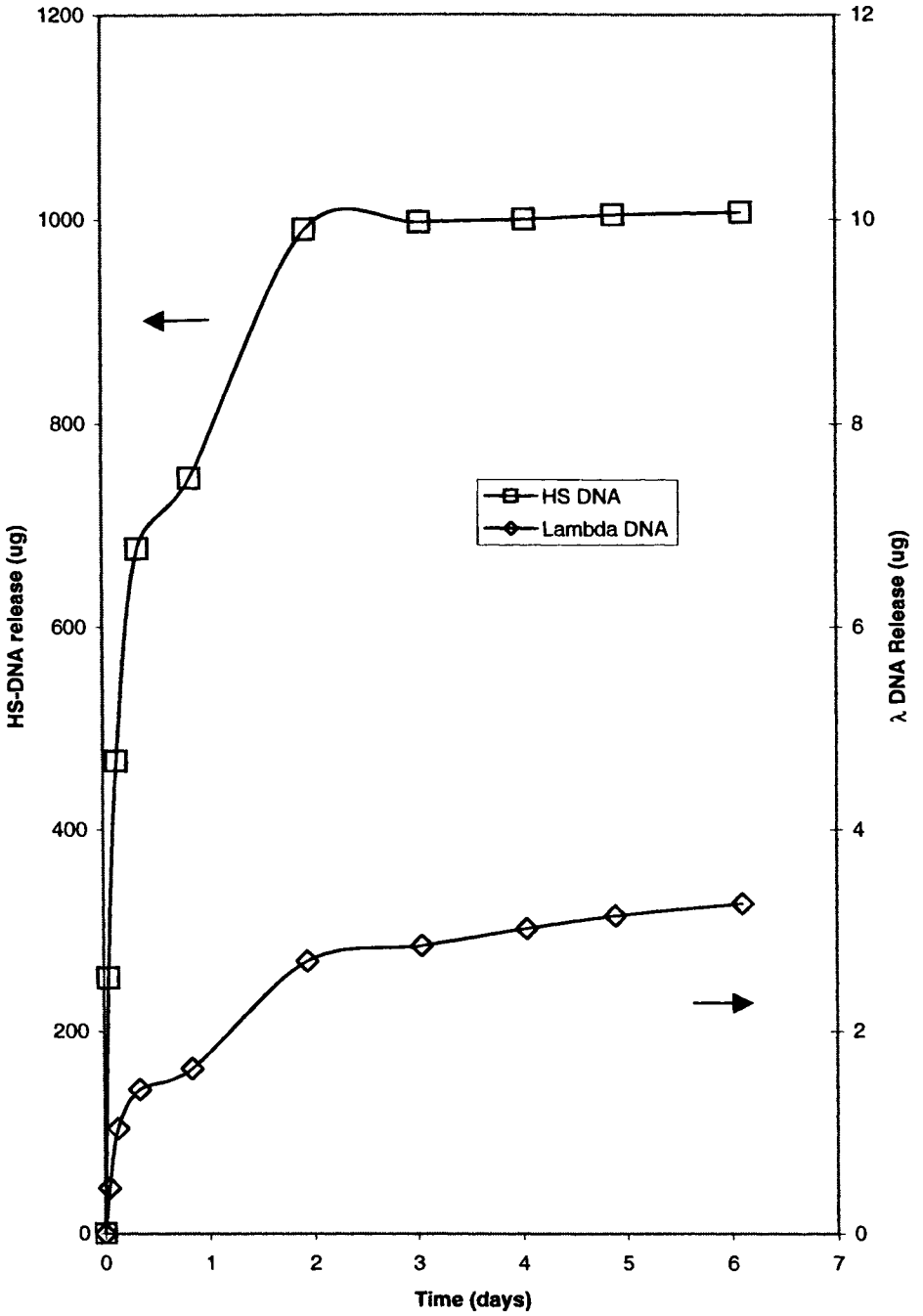

Fig. 2. DNA (HS *vs.* λ) release from smaller EVAc matrices. Two mg of DNA (19.98 mg of HS-DNA (□) plus 0.02 mg of λ DNA (◇)) were encapsulated in EVAc matrices. Actual amounts of released λ DNA were determined by digitized agarose gel images. Notice that the secondary y-axis (right) was deliberately scaled to 1% of primary y-axis (left), so that a graphical direct comparison between theoretical released λ DNA (which is 1% of total released DNA) and actual released λ DNA can be made.

Among different DNA matrices, the D_{app} of 50% loaded EVAc is about 28, 210, and 360 fold higher than that of 40%, 30%, and 20% loaded EVAc matrices, respectively (Fig. 3).

Microsphere System

Biodegradable synthetic polymers were used to produce DNA-loaded microspheres. Most of the formulations tested resulted in particles greater than 100 μm in diameter with non-spherical morphology (Table 1). By decreasing the amount of DNA, formulations based on PLA and PLGA produced the most consistent results. Despite the differences in polymer characteristics, the particles from each of these three formulations had similar size distributions and morphology (Fig. 4).

DNA release was similar among all three polymer systems: an initial burst of release was followed by a period of slow,

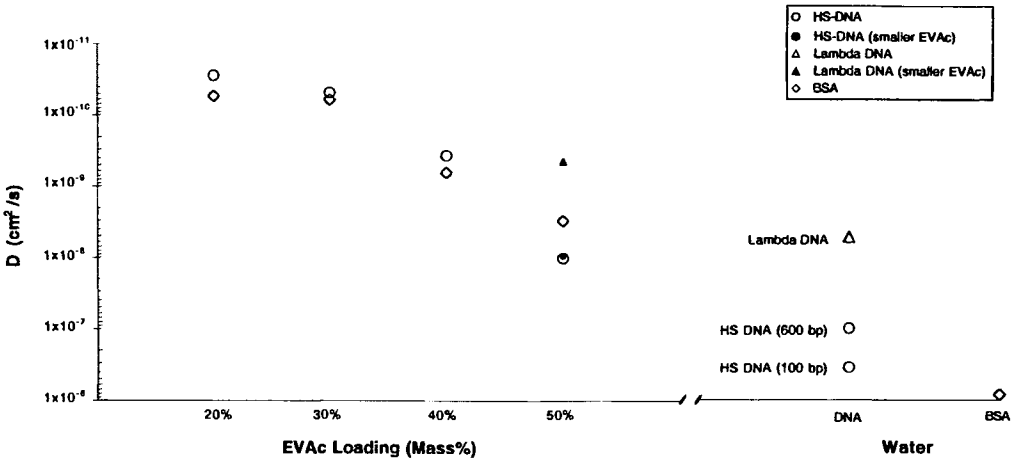

Fig. 3. Diffusion coefficients. D_{app} was calculated based on formula (1). Previously published data (30) were used to establish a standard curve, and $D_{water, DNA}$ was obtained by interpolation. $D_{water, BSA}$ and $D_{app, BSA}$ were obtained from earlier studies (18). Symbols represent: ○: HS-DNA, ●: HS-DNA from smaller EVAc matrices, △: λ DNA, ▲: λ DNA from smailler EVAc, and ◇: BSA.

but continuous, release (Fig. 5a). The cumulative percentage release differed for each formulation (Fig. 5a), despite the fact that these three polymers had similar sizes, size distributions, and surface morphology. Microspheres based on the lower molecular weight PLA-2k released >50% of their payload in the first few hours whereas PLA-300k released only 20% of its total DNA. In general, DNA encapsulated microspheres released most of their loaded DNA in about three weeks, somewhat faster than EVAc systems. PLGA microspheres showed faster DNA release than PLA 2k and PLA 300K; >90% of their DNA payload was released after only two days (Fig. 5a, inset).

To investigate the effect of loading capacity on DNA delivery, PLGA microspheres loaded with different amounts of DNA (from 0.01% to 0.5%) were formulated (Fig. 5b). The time needed to release most of the encapsulated DNA depended on loading. Microspheres loaded with 0.5% DNA released 95% of its DNA after 48 hours incubation in PBS buffer, whereas microspheres loaded with 0.15% and 0.01% DNA released all DNA after only a few hours (Fig. 5b, inset).

Table 1. Screening and Characteristics of DNA Microspheres

Polymer	MW	Conc. (w/v%)	DNA (wt%)	Particle size (μm)	Morphology
PLA		5%	1%	>100	Spherical
		10%	0.5%	<10 or >100	Spherical
PLA	2K	5%	1%	1.8 ± 1.4[a]	Spherical
		10%	0.5%	<10	Irregular
		15%	0.25%	<5	Spherical
		20%	0.12%	<5	Spherical
PLA	50K	5%	1%	>100	Spherical
		10%	0.5%	>100	Spherical
PLA	100K	5%	1%	>100	Spherical
		10%	0.5%	<10 or >100	Spherical
PLA	300K	5%	1%	>100	Spherical
		10%	0.5%	2.6 ± 2.1[a]	Spherical
PLGA (50:50)		5%	1%	<10	Irregular
		10%	0.5%	<10	Irregular
PLGA (50:50)		5%	1%	>100	Spherical
		10%	0.5%	0.95 ± 0.46[a]	Spherical
PLGA (75:25)		5%	1%	<10	Irregular
PLCL (75:25)		5%	1%	<10	Irregular
		10%	0.5%	<10	Irregular

Note: Concentration refers to w/v solution of polymers in 2.0 ml methylene chloride. DNA (wt%) refers to theoretical DNA loading weight (DNA wt/Polymer wt). Particle size and morphology was obtained by scanning electron microscopy.

[a] These samples are used in controlled release study.

Function and Integrity of Released DNA

Green fluorescence protein gene (GFP), amplified by PCR, was used to examine the integrity and functionality of DNA released from biodegradable DNA delivery systems. Agarose gel electrophoresis indicated no detectable DNA degradation among released DNA after 1d, and only 20% degradation was detected after 7d (Fig. 6). Expression of GFP genes within CHO cells was followed by fluorescence microscopy (data not shown). No significant differences were observed between non-encapsulated GFP DNA and released GFP DNA, indicating that the released GFP DNA is capable of expression.

DISCUSSION

The release of proteins from EVAc matrices, first demonstrated over 20 years ago (28), is controlled by diffusion. The time course of release can be modified by changing either the polymer or the encapsulants. For example, manipulation of the internal pore structure of a matrix (18), the molecular weight of the polymer within the matrix (29), or addition of different codispersants (27), can change the release profile significantly. A simple mathematical model can be employed to calculate the apparent diffusion coefficient, D_{app}, which can be used to compare quantitatively the release rates among different EVAc systems.

In this study, DNA was encapsulated in different amounts (20%, 30%, 40% and 50%) in DNA-EVAc systems with two different geometries (100 mg and 2 mg of EVAc). The controlled

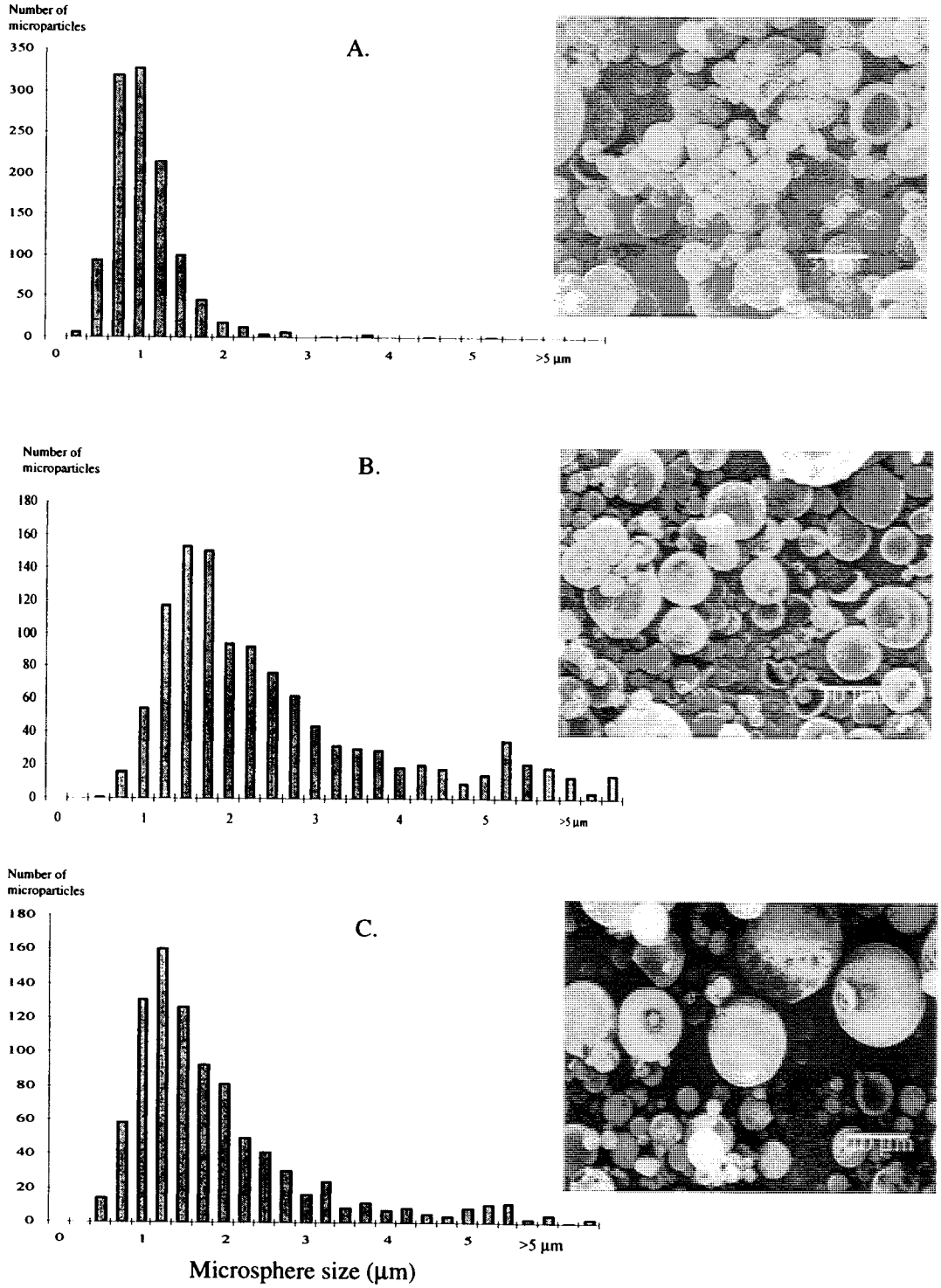

Fig. 4. Size distribution and morphology of DNA encapsulated microspheres. All microspheres were evaluated by scanning electron microscopy. Size distributions were based on more than 1000 particles visualized. Three representative DNA-microspheres based on (A) PLGA 50:50 (B) PLA-300k; and (C) PLA-2k were shown here.

release of DNA was similar in all cases: an initial burst (phase I) was followed by a slow, but continuous, release (phase II). This trend was also seen in protein-EVAc controlled release systems, such as NGF-EVAc (27). The release rate in the first phase of DNA release depended on loading; For example, the D_{app} increased 13 fold from 20% to 40% loaded EVAc (2.77×10^{-11} vs. $3.55 \pm \times 10^{-10}$ cm^2/s). This was comparable to previously reported bovine serum albumin-EVAC (BSA-EVAc) systems, where the rate increased by 12 fold from 20% to 40% loading (18). Furthermore, the overall apparent diffusion

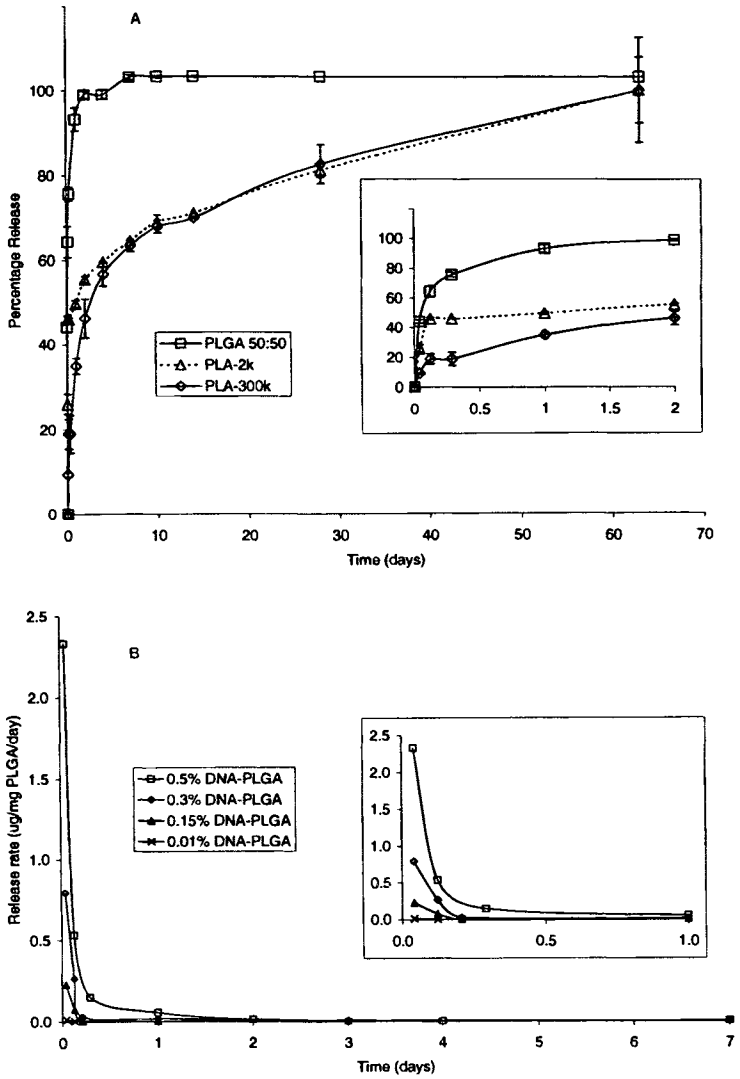

Fig. 5. Controlled DNA release from microspheres. A). Cumulative DNA release in percentage, based on PLGA 50:50 (□), PLA-2k (△), and PLA-300k (◇). Average and standard deviations were calculated from triplicates. B). DNA release rate in μg/mg PLGA/day. PLGA microspheres are loaded with 0.5% (□), 0.3% (◇), 0.15% (▲), and 0.01% (×) DNA (wt/wt). Insets have the same legends as the main figures.

coefficients within a specific loading were comparable between the HS-DNA-EVAc system and the BSA-EVAc system (Fig. 3). The similarity between DNA-EVAc and BSA-EVAc systems supports the notion that DNA release from EVAc polymers, like protein release, is controlled by diffusion.

As expected in a diffusion-controlled system, apparent diffusion coefficient decreases with molecular weight of the released DNA. We simultaneously encapsulated two different-sized DNA molecules (HS-DNA, average M_w = 231 kDa (from 66 kDa to 396 kDa) and λ DNA, average M_w = 32010 kDa) and determined their diffusion coefficients (D_{app}). (Since HS-DNA and λ DNA are loaded together, and both molecules are diffusing through exactly the same pore space, only the total loading matters). The ratio of $D_{app,\lambda}$ to $D_{app,HS}$, which was about 22, was very close to the ratio of λ to HS DNA diffusion in water ($D_{water,\lambda}/D_{water,HS}$ = 19 to 67) (30), further evidence that DNA release from EVAc systems is controlled by diffusion. This finding also suggests that the overall rate of gene release can be controlled by adjusting the size of the released DNA molecules.

A clinical situation may allow only certain sizes or geometrics of delivery system. In addition, different molecular medicine procedures require different doses of DNA (e.g., gene therapy vs. DNA vaccination vs. antisense oligonucleotide therapy). We explored two different sizes of DNA-EVAc system: one large scale (100 mg of EVAc) and one small scale (2 mg of EVAc). In both situations, the apparent diffusion coefficients were almost identical: 0.95×10^{-8} cm^2/s vs. 1.01×10^{-8} cm^2/s, confirming that the rate of release depends on the internal structure of the composite material, not overall geometry.

On the other hand, release of DNA from a microsphere delivery system depends on both erosion (subsequent to hydrolysis) and diffusion. A variety of factors can influence DNA release from microspheres, including chemical properties of the polymer, molecular weight, particle size and morphology,

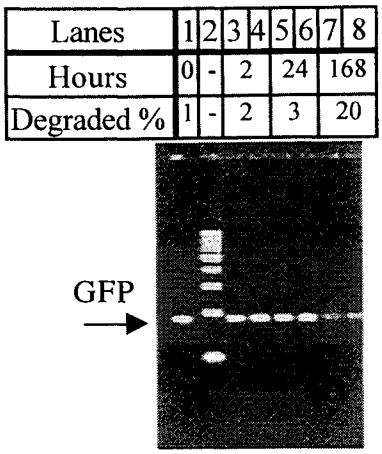

Fig. 6. Integrity of released GFP DNA from PLGA microsphere. PCR amplified GFP DNA was encapsulated in PLGA based microspheres. Controlled release of GFP DNA was subject to 0.8% agarose gel electrophoresis. The gel is stained with SYBR II fluorescence dye. Lane 1: 0.5 μg of GFP DNA without encapsulation (input DNA, controls). Lanes 2: molecular weight marker (1kb ladder). Lanes 3 and 4 (duplicates): 0.5 μg of released DNA after 2 hour incubation. Lanes 5 and 6: 0.5 μg of released DNA after 24 hour incubation. Lanes 7 and 8: 0.5 μg of released DNA after 168 hours (1 week) of incubation.

DNA loading, and DNA solubility. While this finding suggests many approaches for controlling DNA release from microspheres, no simple mathematical model can be applied to predict or quantify release rates in these systems.

In our study, the release profiles of PLA based DNA-microsphere systems exhibited a similar bi-phasic trend: an initial burst followed by a slow release. The microspheres based on the lower molecular weight PLA-2k, however, released a greater amount of loaded DNA than microspheres based on the high molecular weight PLA-300k. This can be attributed to the fact PLA-2k possesses a higher rate of degradation since the polyesters have elevated amounts of end-carboxylic groups. Among all 3 DNA-microsphere delivery systems, the fastest release of DNA was from PLGA based microsphere: 95% of their DNA load was released after only two days. Since copolymers of poly (D, L-lactide) and polyglycolide usually have lower glass transition temperatures (T_g) and lower crystallinity (T_m), PLGA shows a greater susceptibility to hydration than PLA. Therefore, it is expected that PLGA releases DNA at a much faster rate than PLA.

In summary, we have successfully engineered biocompatible polymeric discs and biodegradable microspheres to encapsulate different-sized DNA molecules in different dosages for controlled delivery. These DNA controlled release systems are easy to produce with low cost, are based entirely on materials previously approved by the FDA for drug delivery, are implantable and/or injectable, and can be readily adapted to suit different needs of delivering DNA as a pharmaceutical. We expect that new therapies will arise from DNA controlled release systems.

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