# A DNA Controlled-Release Coating for Gene Transfer: Transfection in Skeletal and Cardiac Muscle

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Received March 13, 1998. Accepted for publication April 28, 1998.

**Abstract** □ In this paper we report a novel technique of DNA-polymer coating for gene transfer. A proprietary DNA polymer solution was used for thin-layer coating on a chromic gut suture as a model study. The coated sutures were characterized for physical properties such as coating thickness, mass of the DNA deposited on the suture, surface characteristics as determined by scanning electron microscopy, and in vitro DNA release characteristics under simulated physiologic conditions. The in vivo gene transfection using DNA-coated sutures was demonstrated in rat skeletal muscle and in canine atrial myocardium. A heat-stable human placental alkaline phosphatase (AP) plasmid was used as a marker gene. Incisions of 1 to 1.5 cm were made in the rat skeletal muscles or the canine atrial myocardium. The sites were closed with either the DNA-coated sutures or control sutures. Two weeks after the surgery, the tissue samples adjacent to the suture lines were retrieved and analyzed for AP activity. The DNA-coated sutures demonstrated a sustained release of the DNA under in vitro conditions, with an ~84% cumulative DNA release occurring in 26 days. An agarose gel electrophoresis of the DNA samples released from the suture demonstrated two bands, with the lower band corresponding to the input DNA (supercoiled). It seems that there was a partial transformation of the DNA from a supercoiled to an open circular form due to the polymer coating. The tissue sites, which received the DNA-coated sutures, demonstrated a significantly higher AP activity compared with the tissue sites that received control sutures. In the rat studies, the mean AP activity (square root of cpm/$\mu$g protein) was 43.6 ± 3.3 vs 20.6 ± 2.1 ($p = 0.001$) at the control sites. Similarly, in the canine studies, the AP activity was 73.6 ± 7.4 Vs 21.6 ± 1.4 ($p = 0.0009$) at the control sites. Thus, our studies demonstrated a successful gene transfer using our DNA-polymer coating technique. This technique could be useful for coating sutures used in vascular and general surgery, and also for coating medical devices, such as stents, catheters, or orthopedic devices, to achieve a site-specific gene delivery.

## Introduction

The concerns associated with viral vectors for gene delivery,[1−3] such as immunogenicity with the adenoviral vectors or the risk of mutation with the retroviral vectors, have led to a renewed interest in investigating nonviral methods of gene delivery.[4−9] A variety of nonviral methods of gene delivery including liposomes,[10,11] cationic lipid−DNA complexes,[12,13] and cationic polymer−DNA condensates[14−16] are under investigation. However, the important limiting factor with the nonviral methods of gene delivery is their comparative inefficiency to facilitate cellular uptake of DNA and gene expression.[17,18]

Sustained-release drug delivery systems are currently being investigated for DNA. These formulations include microspheres, nanospheres, and polymer matrixes. The DNA is either entrapped or dispersed into the polymer matrix or adsorbed onto the surface. Biodegradable (polyesters, polyanhydrides) and nonbiodegradable polymers (ethylene vinyl acetate copolymers), either synthetic or natural (collagen, gelatin), have been investigated for formulating sustained-release delivery systems.[19] These systems have demonstrated their efficacy for the sustained delivery of proteins and peptides, which are comparatively unstable in biological systems and require multiple dosing to maintain a prolonged therapeutic effect.[20−22]

Sustained-release DNA delivery systems, in addition to providing a controlled release of DNA, could also protect the entrapped DNA from degradation due to nucleases. Prolonged availability of the DNA released slowly from the sustained-release DNA delivery system at the site of an implant could increase the efficiency of cellular DNA uptake. This prolonged availability could also lead to a prolong duration of gene expression. In this paper, we have described a novel DNA-polymer coating method for the sustained delivery of DNA. A heat-stable human placental alkaline phosphatase (AP) plasmid was used as a marker gene. The goals of the studies were (1) to formulate a sustained-release DNA coating; (2) to characterize the DNA coating, study in vitro release characteristics of the DNA, and characterize the DNA released from the coating; and (3) to demonstrate in vivo gene expression in animal model studies.

## Materials and Methods

**Materials**—A proprietary biodegradable DNA-polymer coating preparation was obtained from Matrigen, Inc. (Ann Arbor, MI). Trizama-HCl (Tris(hydroxymethyl)aminomethane) and ethylenediaminetetraacetic acid (EDTA) sodium salt were obtained from Sigma Chemicals (St. Louis, MO). The following assay kits were purchased: Alkaline Phosphatase Enzyme Kit (Phospho-Light) from Tropiz, Inc. (Bedford, MA) and BCA Protein Assay Kit from Pierce (Rockford, IL). Potassium phosphate, urea, and potassium chloride were obtained from Mallinckrodt Specialty Chemical Company (Paris, KY). All organic solvents and reagents were either of HPLC grade or American Chemical Society analytical grade reagents.

**Plasmid DNA Sustained-Release Preparation**—The plasmid pcDNA3 (Invitrogen, Carlsbad, CA), containing a human placental alkaline phosphatase gene under the control of human cytomegalovirus (CMV) promoter, was propagated in *Eschericia coli* and was isolated using a Qiagen Ultrapure column (Qiagen, Inc., Chatswirth, CA). The structure and purity of the plasmid preparation was assessed by agarose gel (0.8%) electrophoresis in the presence of ethidium bromide to detect DNA bands. The concentration of DNA in the plasmid preparation was determined by ultraviolet (UV) spectrophotometric absorption at 260/280 nm wavelengths. The plasmid DNA was incorporated into the Matri-

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10.1021/js980077+ CCC: $15.00
Published on Web 09/04/1998

gen coating system and applied to a 3−0 Chromic gut suture (Ethicon, Inc. Somerville, NJ).

*DNA-Polymer Coating*—Two proprietary formulations of coating, varying in DNA dose, were investigated. The high-dose formulation contained 3.0 mg of DNA and the other formulation contained 1.0 mg of DNA per 70-cm length of suture. A multilayer solvent-casting procedure was used for the DNA-polymer coating. The coating was dried in air first for 3−4 h and then under desiccation for 48 h.

*Characterization of DNA Suture*—The DNA-coated sutures were characterized for the mass weight increase, for surface characteristics by scanning electron microscopy, and for in vitro release of the DNA under physiologic conditions.

*Scanning Electron Microscopy*—A piece of coated and uncoated suture was sputtered with carbon and was observed under a scanning electron microscope (Amray, Model 1810, Bedford, MA) at 150X magnification.

*In Vitro Release of DNA*—A DNA-coated suture (total length = 70 cm) was cut into pieces ~7 cm in length. The pieces were incubated with a Tris-EDTA buffer (pH 7.3, 50 mM, 5 mL) at 37 °C on a rotary shaker at 110 rpm. The buffer was replaced at regular intervals of time. The experiment was performed in triplicate. The samples were assayed for the DNA concentration by high-pressure liquid chromatography (HPLC) as described next. The samples were also analyzed by agarose gel electrophoresis (0.8% agarose, 100 V, 40 min) to test the integrity of the released DNA.

*HPLC Analysis of DNA*—The HPLC system (Waters Company, Milford, MA) consisted of two 501 pumps, an automated gradient controller, a 712 WISP autosampler, a 481 Lambda-Max UV detector, and a 756 Data module. A Nucleogen DEAE 4000−7 (Alltech, San Jose, CA) column was used for the DNA separations using a mobile phase consisting of 5 M urea and 0.2 M potassium phosphate adjusted to pH 6.5. The gradient was created with potassium chloride (0.2−1.5 M).[23] The flow rate was set at 2 mL/min, and separations were monitored at 260 nm. A 100-$\mu$L sample was injected for the purpose of DNA quantitation. Standard curves of the input DNA were created for quantitation.

**In Vivo Gene Expression**—*Rat Skeletal Muscle Wound Closure*—Six-week-old Sprague−Dawley rats (Charles River Laboratories, Burlington, MA) were used for this study. Animals were anesthetized with an intramuscular injection (0.001 mL/kg) of a combination (4:3 v/v) of ketamine (100 mg/mL) and xyalazine (20 mg/mL). Under sterile conditions, the skin in the area of the hindlimb was opened and 1-cm incisions were made with a scalpel in the femoral extensor musculature. The wounds were closed with either control or DNA-coated sutures (low-dose DNA suture, 260 $\mu$g of DNA per site). Two weeks after the surgery, the tissue from the incision sites adjacent to the suture lines were retrieved and stored frozen (−70 °C) until taken for the determination of the AP activity.

*Canine Atrial Myocardium Wound Closure*—This experiment was performed with a male mongrel dog weighing 30 kg. The animal was anesthetized with an injection of sodium pentobarbital (35 mg/kg), and anesthesia was maintained with halothane. Under sterile conditions, the chest was opened and the heart was exposed. A total of four incisions, each ~1.5 cm, were made in the right atrium of the heart. The wounds at two of the sites were closed with the DNA-coated sutures (high-dose DNA suture, 780 $\mu$g per site) and at the other two sites with control sutures. The DNA sites were ~8−10 cm away from the control sites. After 2 weeks, the animal was euthanized with an overdose of pentobarbital, and the tissue samples from the atrium adjacent to the suture linings were retrieved for analysis of AP activity.

**Assay for Alkaline Phosphatase (AP) Activity**—The frozen samples were thawed, homogenized in TMNC buffer [50 mM Tris pH 7.4, 5 mM $MgCl_2$, 100 mM NaCl, and 4% CHAPS (3-[(3−cholamidopropyl)-dimethylammonium]-1-propene sulfonate; 1 g tissue/2.5 mL buffer] and centrifuged at 4000 × *g* for 15 min. The supernatants were heated at 65 °C for 30 min to inactivate the intrinsic cellular AP. A 20-$\mu$L aliquot of the supernatant was mixed with a 100 $\mu$L of the assay solution and incubated at room temperature for 5 min. The substrate (100 $\mu$L) was added to the aforementioned mixture and again incubated at room temperature for 20 min. The light output (cpm) was measured with a liquid scintillation counter (Beckman LS-100, Fullerton, CA) on the $^3$H-channel with the coincidence circuit on. The assay results were normalized to the tissue protein concentration as determined by the standard bicinchoninic acid (BCA) protein assay using bovine serum albumin as a standard. The results were calculated as a square root of the activity (cpm) per amount ($\mu$g) of protein.

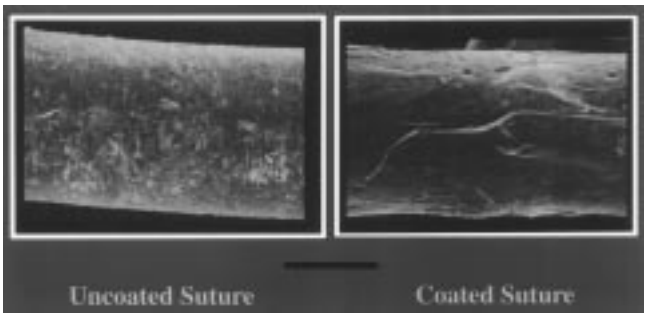


**Figure 1**—Scanning electron micrograph of an uncoated and the DNA-polymer-coated suture. The bar represents 280 $\mu$m.

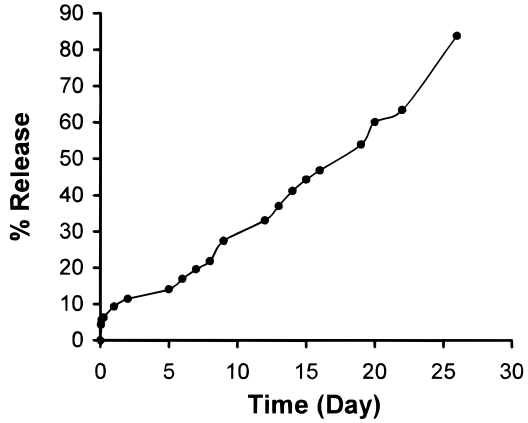


**Figure 2**—In vitro release of DNA from the DNA-coated suture under simulated physiologic conditions.

**Statistical Analysis**—Values were expressed as mean ± standard error of mean (SEM). Comparisons were made using a paired *t*-test as appropriate. A probability value (p) of $<0.05$ was considered significant.

## Results

The overall results demonstrated a successful DNA-polymer coating methodology, sustained DNA release characteristics, and in vivo gene expression in model studies.

**Characterization of DNA Coated Suture**—The coating resulted in a total deposition of 35 ± 2.5 ($n$ = 6) mg of DNA polymer-coating preparation. Based on the amount of DNA load in the coating formulation, the DNA coated per entire suture length (70 cm) was 777.2 ± 0.05 $\mu$g. The coating also resulted in a 30-$\mu$m increase in the suture thickness. The scanning electron micrograph demonstrates a uniform coating of the DNA-containing polymer (Figure 1), which is distinctly different from the surface of the uncoated suture. The coating seems to have no effect on the physical properties of the suture that would affect its ability to function as a wound closure.

The in vitro release studies demonstrated sustained DNA release characteristics with a 9.3% cumulative release occurring during the first 24 h as a burst phase, followed by a gradual elution with 84% cumulative release occurring in 26 days (Figure 2). The coating was found to detach from the suture after ~3 weeks of the in vitro release study. Thus, the DNA release from the coating seems to be due to degradation of the polymer matrix. The DNA samples released from the sutures demonstrated two bands of an almost equal intensity on the agarose gel electrophoresis, with a lower band corresponding to the original DNA

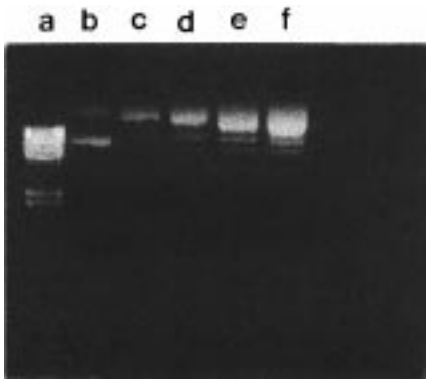


**Figure 3**—Results of agarose gel electrophoresis of the DNA samples released under in vitro conditions from the DNA-coated suture. (a) DNA ladder (75 base pairs to 23 Kilobase pairs; (b) input plasmid DNA, human placental alkaline phosphatase, (c) 5-day controlled-release sample (2 $\mu$L); (d) 5-day controlled-release sample (5 $\mu$L); (e) 8-day controlled-release sample (4 $\mu$L); (f) 8-day controlled-release sample (10 $\mu$L).

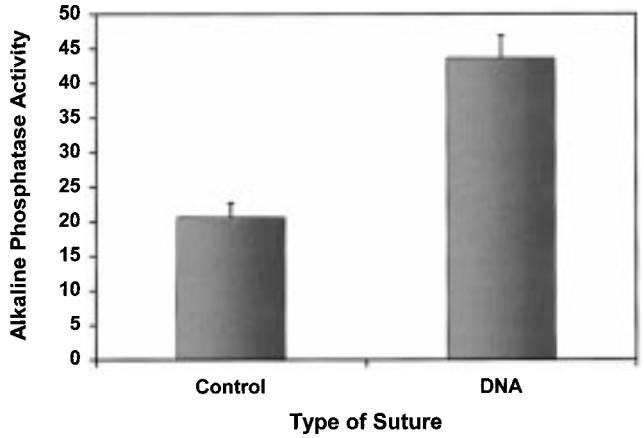


**Figure 4**—Alkaline phosphatase activity in rat skeletal muscles at DNA-coated suture sites ($n = 14$) and control sites ($n = 5$). The AP activity is represented as a square root of the activity (cpm) normalized activity to amount ($\mu$g) of protein. Data as mean ± sem.

(supercoiled) (Figure 3). These data indicate that there was a partial transformation of DNA from a supercoiled to an open circular form. A similar transformation of the DNA was observed in studies by Jong et al.[24] when the DNA was dispersed in the EVAc matrixes. As the solvent from the DNA polymer coating solution evaporates, the DNA is compressed in the polymer matrix. The physical forces encountered during such entrapment could be responsible for a transformation of DNA.[25]

**In Vivo Gene Expression**—Each incision site received 1/3 of the suture length (~23 cm), which represents a total DNA dose of 260 $\mu$g per site (or 7.6 $\mu$g/day) in rat studies and 780 $\mu$g per site (or 22.8 $\mu$g/day) in dog studies. The estimated dose was calculated from the DNA load in the suture coatings and the in vitro DNA release characteristics. There was no apparent inflammatory or untoward tissue reactions at the incision site, indicating that the coated sutures were well tolerated.

The tissue retrieved from the DNA suture site had significantly higher AP activity compared with AP activity in the tissue retrieved from the control suture sites. The mean AP activity in the rat studies was 43.6 ± 3.3 in the DNA delivery sites vs 20.6 ± 2.1 ($p = 0.001$) at the control sites (Figure 4). Similarly, in the canine studies, the mean AP activity was 73.6 ± 7.4 in the DNA controlled-release sites vs 21.6 ± 1.4 ($p = 0.0009$) at the control sites. The AP activity was represented as a square root of the activity in cpm normalized to protein content ($\mu$g). The AP activity in canine tissue samples was comparatively higher than that in the rat tissues. This difference could reflect a dose effect, because the sutures used in the dog studies had three-fold higher DNA loading than the sutures used in the rat studies.

## Discussion

Our study demonstrated a novel DNA coating technique for sustained DNA delivery. Successful in vivo gene expression was demonstrated in rat skeletal muscles and in canine myocardium. Sustained DNA delivery systems offer many advantages over plasmid DNA solution administration in which the injected DNA could be rapidly washed out from the site of administration or it could undergo rapid degradation by nucleases. It is also known that the injected DNA (e.g., intramuscular or subcutaneous injections) drains rapidly into the lymphatics in certain tissue,[26,27] which could also significantly limit the availability of DNA. Sustained-release systems could prolong the availability of the DNA, which could also lead to a sustained duration of gene expression.

Other investigators have also demonstrated gene expression using sustained-release DNA delivery systems. Fang et al.[28] demonstrated gene expression in a rat bone fracture model, which was packed with collagen sponge soaked in a DNA solution for several hours before implantation. Their studies demonstrated induction of new bone formation with therapeutic genes (PTH-34 and BMP-4). Collagen is probably serving a twofold function in this experiment: as a scaffold for the migrating cells and sustained delivery of DNA. In another study, gene expression was demonstrated on the serosal side of the intestine in rats that received oral administration of polyanhydride microspheres containing a marker DNA ($\beta$-galactosidase).[29] The bioadhesive characteristics of the polyanhydride microspheres provided sustained release of DNA at localized sites in the intestine. We have also demonstrated gene expression in the rabbit iliac arterial wall following a local infusion of PLGA nanospheres containing AP plasmid DNA.[30]

Our coating technique could be useful for medical devices such as stents, catheters, or orthopedic devices, to deliver therapeutic genes locally at the site of implant. Stents coated with a therapeutic gene could be useful for preventing restenosis. Myocardial gene expression has many therapeutic applications, such as inducing angiogenesis in the damaged myocardium, or expression of a gene that could modulate pathways of electrical conductance to control cardiac arrhythmias.[39–44] In addition, coated sutures with an appropriate therapeutic gene (TGF-$\beta$) could be used to enhance the wound healing process.[45] Coating of scaffolds with genes in tissue engineering is yet another application of our coating technique.[46]

## Conclusions

Our studies have demonstrated a novel method of DNA coating for the sustained release of plasmid DNA. Our rat and canine studies demonstrated in vivo reporter gene expression using DNA-coated sutures. We conclude this DNA delivery system offers the capability of incorporating sustained-release DNA into virtually any implantable or interventional device. Thus, surgical and invasive procedures, as well as wound dressings, could be enhanced through the coincorporation of the DNA delivery system described in these studies. Therapeutic DNA administration could therefore be broadly used in a site-specific manner with this technique.

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## Acknowledgments


This work was supported by funding from Matrigen, Inc., Ann Arbor, MI. Dr. Levy's efforts were partially supported by an Endowed Chair at the Children's Hospital of Philadelphia. It is also disclosed that some of the authors (VL, JB, SG, RJL) may potentially benefit from the results.

JS980077+