Polyvinyl Derivatives as Novel Interactive Polymers for Controlled Gene Delivery to Muscle

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Purpose. DNA plasmids (pDNA) can be taken up by and expressed in striated muscle after direct intramuscular injection. We have developed interactive polymeric gene delivery systems that increase pDNA bioavailability to muscle cells by both protecting pDNA from nucleases and controlling the dispersion and retention of pDNA in muscle tissue.
Methods. A DNA plasmid, containing a CMV promoter and a β-galactosidase reporter gene (CMV-β-gal), was injected either in saline or formulated in polyvinyl pyrrolidone (PVP) and polyvinyl alcohol (PVA) solutions. Interactions between PVP and pDNA were assessed by dynamic dialysis, Isothermal Titration Calorimetry (ITC), and Fourier-Transformed Infra Red (FT-IR) spectroscopy. Formulations (50 μl) were injected into rat tibialis muscles after surgical exposure. Immunohistochemistry for β-gal was used to visualize the sites of expression in muscle.
Results. β-gal expression using pDNA in saline reached a plateau while β-gal expression using PVP formulations increased linearly in the dose range studied (12.5–150 μg pDNA injected) and resulted in an increase in the number and distribution of cells expressing β-gal. The interaction between PVP and pDNA was found to be an endothermic process governed largely by hydrogen-bonding and results in protection of pDNA from extracellular nucleases.
Conclusions. Significant enhancement of gene expression using interactive polyvinyl-based delivery systems has been observed. The improved tissue dispersion and cellular uptake of pDNA using polyvinyl-based systems after direct injection into muscle is possibly due to osmotic effects.

KEY WORDS: muscle; DNA plasmid; gene delivery system; polyvinyl pyrrolidone; polyvinyl alcohol; non-viral gene therapy; gene expression system.

INTRODUCTION

Wolff et al. demonstrated first that genes can be transferred into skeletal muscle cells of rodents and primates after the intramuscular (i.m.) injection of a DNA plasmid in saline (1). Resulting gene expression has been reported to last for up to several months and may be due to persistence of pDNA caused by the slow turnover rate of myofiber nuclei (2). The expression of genes in muscle may provide a safe and cost-effective treatment for a variety of diseases by producing therapeutic proteins for local or systemic effect.

The potential clinical usefulness of direct gene transfer to muscle of pDNA in saline is limited by low and highly variable levels of gene expression (2–3). Intramuscular injection of pDNA in saline results in a very small amount of the injected pDNA being taken up by cells and expressed, while the majority of the pDNA is rapidly degraded or removed from the muscle (e.g., via the lymphatic system) (3). The uptake of pDNA into the muscle fibers is a saturable process. For example, Levy et al. have observed that gene expression can be inhibited by a 20-fold excess of non-encoding pDNA (4).

We have developed polymeric gene delivery systems that are designed to: (*i*) disperse pDNA throughout the muscle, (*ii*) protect pDNA from nuclease degradation, (*iii*) retain pDNA in muscle, and (*iv*) facilitate uptake of pDNA into muscle cells. We describe results obtained with two interactive polymers, polyvinyl pyrrolidone (PVP) and polyvinyl alcohol (PVA).

MATERIALS

Injectable grade polyvinyl pyrrolidone (PVP) (Plasdone-C15, Mw 10 kDa and Plasdone-C30, Mw 50 kDa) was from ISP Technologies (Wayne, NJ) and the BASF Corp. (Mount Olive, NJ) (Kollidon K17PF; Mw 10 kDa). Polyvinyl alcohol (PVA; 87–89% hydrolyzed) of molecular weight average 18 kDa and 40 kDa was from Aldrich Chemicals (Milwaukee, WI). DNA plasmids, containing a CMV promoter and either chloramphenicol acetyltransferase (CMV-CAT) or β-galactosidase reporter gene (CMV-β-gal), were prepared and purified at GENEMEDICINE, INC. Spectra/Por CE (cellulose ester) membranes with a Mw cut-off of 25 kDa were from Spectrum (Houston, TX). A chemiluminescence detection system for β-galactosidase (Galacto-Light™) was from Tropix, Inc. (Bedford, MA). ^{14}C-chloramphenicol was from NEN Dupont Co. (Doralville, GA) and butyryl CoA was from Sigma (St. Louis, MO).

METHODS

Preparation of pDNA Formulations

Concentrated pDNA stock solutions were made by lyophilizing and rehydrating pDNA with water to a final pDNA concentration of 3–5 mg/ml. Formulations were made by aliquoting appropriate volumes of sterile stock solutions of pDNA, 5M NaCl, and polymer to obtain a final pDNA concentration in an isotonic polymer solution. The osmotic pressure of selected formulations was measured (n = 3) using a Fiske One-Ten Micro-Sample Osmometer (Fiske Associates; Norwood, MA).

Dynamic Dialysis

Dynamic dialysis was used with various interactive polymer formulations to measure binding between PVP and pDNA. One ml of formulations and corresponding controls were placed in prewashed dialysis sacs. The dialysis sacs were closed and suspended in stirred saline solutions (100 ml) at 25°C. One ml aliquots were taken from the acceptor compartment over time and replaced with fresh media. The concentration of PVP in the diffused samples collected over time was measured spectroscopically at 220 nm.

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DNase I Degradation

The protection of pDNA from DNase I degradation by 5% PVP (50 kDa) was studied. 100 μL solutions of pDNA in saline or pDNA in 5% PVP (50 kDa) in 150 mM NaCl were mixed with 100 μL of various amounts of DNase I in 2X-Activity Buffer and allowed to incubate at 37°C for 15 minutes (2X-Activity Buffer = 100 mM sodium acetate, pH 6.5 with 20 mM $MgCl_2$ and 4 mM $CaCl_2$). The final concentration of pDNA was 100 μg/ml of 1X-Activity Buffer. For the DNase I used, a 1:10,000 DNase I to pDNA ratio entirely degraded pDNA in saline at 37°C in 15 minutes. pDNA stability and topology were assessed by 1% agarose gel electrophoresis using 1% tris-acetate-EDTA (TAE) buffer and ethidium bromide staining.

Isothermal Titration Calorimetry

Calorimetric measurements were performed using a microtitration calorimeter (Hart Scientific; Calorimetry Sciences Corp.; Pleasant Grove, UT) by titrating pDNA into 15% PVP (50 kDa) in 150 mM NaCl. All titrations of pDNA into PVP solutions were corrected for heats of mixing by subtracting a titration profile of an equal concentration of pDNA mixed with 150 mM NaCl.

Fourier-Transformed Infra Red (FT-IR) Studies

Three different solution samples (250 μl) were loaded onto the attenuated total reflectance (ATR) cell, which was placed on the sample chamber of a Magna IR™ 550 spectrometer (Nicolet Analytical Instruments; Madison, WI). A 1% solution of CH_3CN was added to each sample as an internal reference. Approximately 2,000 scans were collected at 25°C and 8 cm^{-1} resolution using ATR cell correction. Different spectra were obtained by subtracting a solution spectrum of 150 mM NaCl and by correcting for CN stretch vibration at 2260 cm^{-1}.

Intramuscular Administration of pDNA to Rats

Five to six week old male rats (Fischer 344 strain, 120–130 g) from Harlan Sprague-Dawley laboratories were used for all animal studies. NIH guidelines for the care and use of laboratory animals were observed. The rats were anesthetized by intraperitoneal administration of a mixture of ketamine (42.8 mg/ml), xylazine (8.6 mg/ml) and acepromazine (1.4 mg/ml) at a dose of 0.5–0.7 ml/kg. A 2–4 mm incision in the skin was made aseptically and 50 μl of the formulation was injected into the tibialis muscle of both legs using a 28G needle. Saline injections were used as controls. At various time intervals after injection, animals were sacrificed by CO_2-asphyxiation and the tibialis muscle was harvested and stored on dry ice at −70°C until assayed for β-galactosidase or chloramphenicol acetyltransferase (CAT). Expressed β-galactosidase or CAT was extracted from the muscle with 1.5 ml of Tris-EDTA-NaCl buffer containing the protease inhibitors leupeptin (1 μM), pepstatin (1 μM) and PMSF (0.25 mM). The extract was centrifuged at 13,000 rpm for 15 min at 4°C. Relative Light Units (RLU) were determined according to the Galacto-Light™ protocol from Tropix, Inc. Results are expressed as mean ± SEM of RLU, as indicative of β-galactosidase activity, per 100 μg muscle protein. Muscle protein was measured with a Coomassie Blue G250-based assay (Bio-Rad; Hercules, CA). CAT activity was assayed according to a modified method of Gorman et al. (5) and expressed as ^{14}C-butyryl chloramphenicol (disintegrations per minute; DPM) per 10 μg muscle protein.

Immunohistochemistry for β-galactosidase

Rat tibialis muscle samples were collected at 7 days after injection of pDNA in saline or formulated in 5% PVP (50 kDa) in 150 mM NaCl. Paraffin sections (5 μm) were placed on Plus slides (Fisher Scientific; Pittsburgh, PA). Deparaffinized sections were treated with 10% normal goat serum after an antigen retrieval treatment (Signet; Dedham, MA). Sections were then incubated with mouse anti-β-galactosidase (Sigma Chemicals) in 10% normal goat serum. The antibody binding sites were visualized using a streptavidin/biotin immunostain system (Kirkegaard & Perry Laboratories, Inc.; Gaithersburg, MD) and Vectastain Red (Vector; Burlingame, CA). After counterstaining with Mayer's hematoxylin, β-galactosidase immunostaining was digitalized using an integrated image analysis system (Meyer Instruments; Houston, TX) attached to an Olympus BX60.

RESULTS AND DISCUSSION

Polyvinyl pyrrolidone (PVP) has been widely used in pharmaceutical products. PVP has been complexed with various drugs to achieve their slow release in the muscle due to restricted diffusion (6). PVP is used as an excipient for oral dosage forms due to its low toxicity and biological and chemical inertness. PVP has also been used as a blood-plasma substitute and expander (6). In addition, PVP has been shown to stabilize protein drugs (6–7). Importantly for this study, PVP has been found to induce a macromolecular crowding effect on Escherichia coli DNA (8).

We found in initial *in vivo* experiments in rats (Figure 1A), using a chloramphenicol acetyltransferase (CAT) reporter gene injected i.m. in isotonic solutions of up to 20% PVP (50 kDa), that a 5% PVP formulation resulted in the highest β-gal expression, with a 10-fold enhancement in the levels of β-gal expression over pDNA injected in saline.

As a result, we have determined the dose-response for pDNA in saline and formulated in 5% PVP (50 kDa) using i.m. injection in rats (Figure 1B). It was found that the enhancement of β-gal expression using the 5% PVP (50 kDa) formulation increased linearly with dose over the range studied (12.5 −150 μg CMV-β-gal injected), whereas expression with pDNA in saline reached maximal expression between 50 μg and 100 μg dose. The plateau in β-gal expression observed with pDNA in saline is consistent with previous reported results and may be due to a saturable limiting step in transfer of pDNA into muscle cells (3, 4). Figure 1C shows the time-course of β-gal expression in rat muscle after i.m. injection of pDNA in saline and formulated in 5% PVP (50 kDa). It was found that the enhancement in β-gal expression using the PVP formulation was maintained throughout the time of the study (3 weeks).

A lower molecular weight PVP (10 kDa) was also examined for i.m. administration since it has a faster elimination rate from the body and has general regulatory approval for both i.v. and i.m. administration (6). Figure 2A shows β-gal

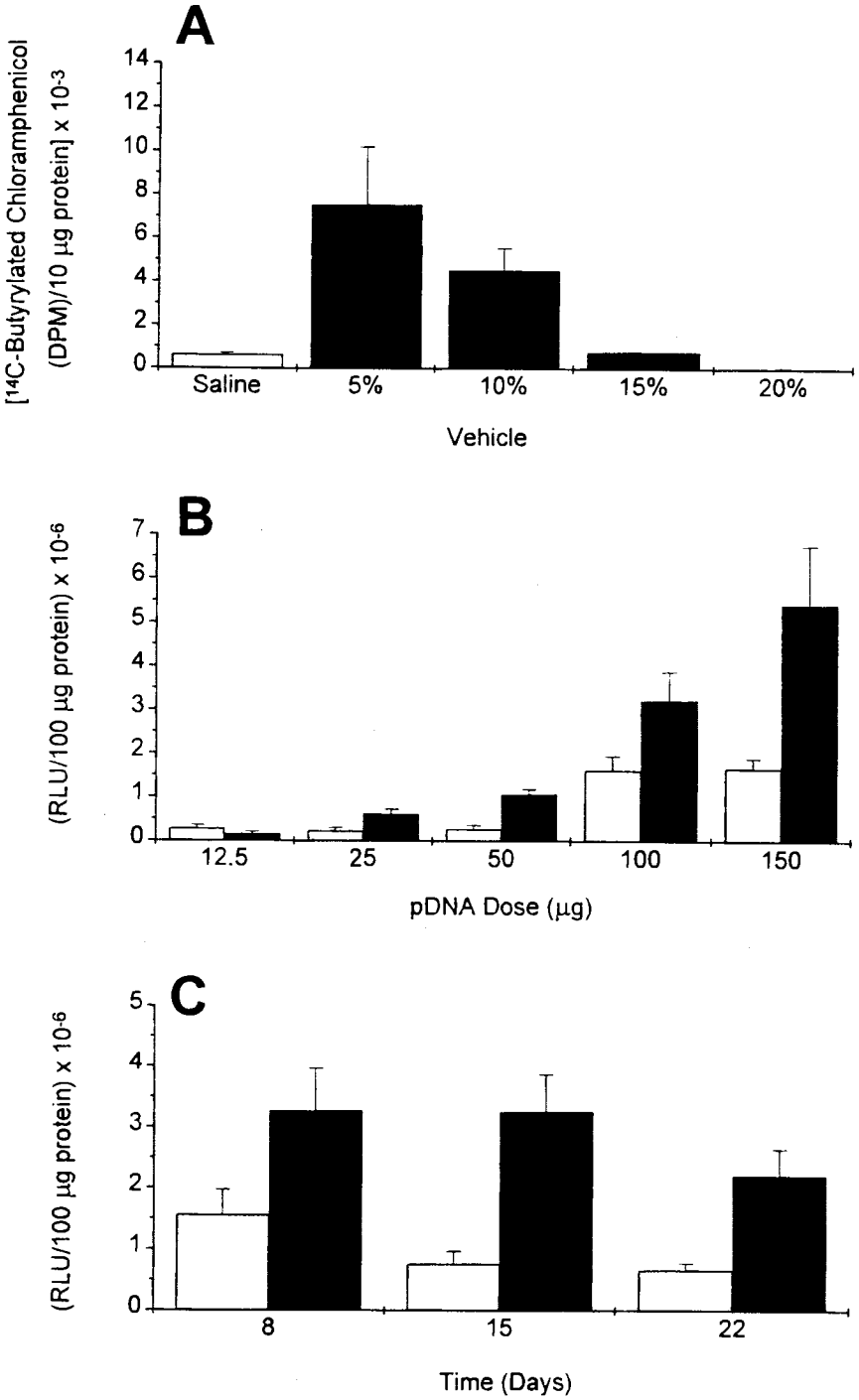


Fig. 1. Reporter gene expression in rat tibialis muscle after i.m. injection of pDNA in saline (□) or formulated in PVP (50 kDa) in 150 mM NaCl. (■). A) Magnitude of CAT expression at 7 days after injection of CMV-CAT (50 µg/muscle); B) Dose-response of β-gal expression at 8 days; C) Time-course of β-gal expression after injection of CMV-β-gal (100 µg/muscle). Results reported as mean ± SEM (n = 9 − 12).

expression at 8 days after i.m. injection of pDNA in either PVP (50 kDa) or PVP (10 kDa) formulations. The highest β-gal expression was observed using the 5% PVP (50 kDa), which was approximately 7-fold greater than using pDNA in saline. With PVP (50 kDa), the level of β-gal expression decreased proportionally to the concentration of PVP over the range 1–5%. β-gal expression with PVP (10 kDa) was greater than that found using pDNA in saline, with no statistical difference in β-gal expression using concentrations of PVP (10 kDa) ranging from between 2.5 to 10%. However, the levels of gene expression using PVP (10 kDa) were approximately 2-fold lower than using 5% PVP (50 kDa) formulations. For reference, 10% PVP

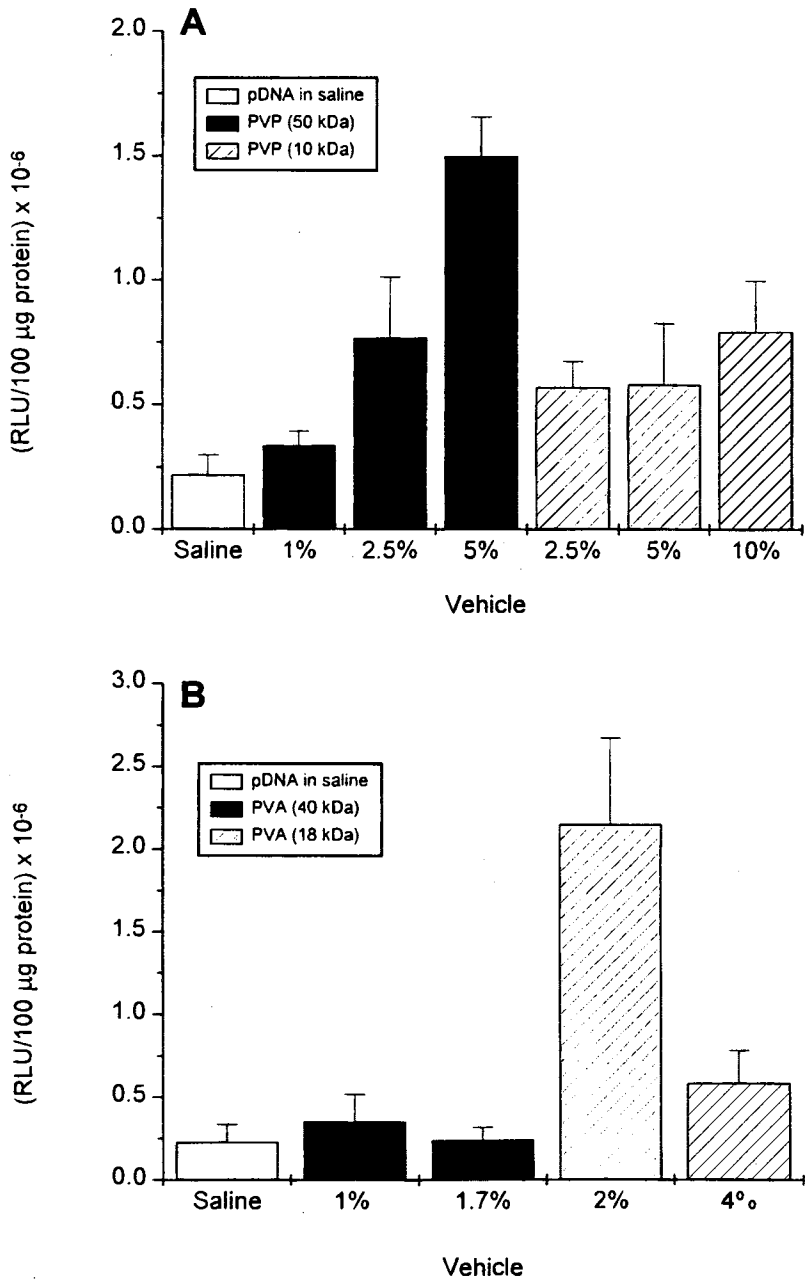


Fig. 2. β-gal expression in rat tibialis muscle at 8 days after i.m. injection of pDNA in saline or formulated in the following polyvinyl-based delivery systems. A) 5% PVP (10 or 50 kDa) in 150 mM NaCl. CMV-β-gal injected per muscle was 150 µg. Results reported as mean ± SEM (n = 10 − 12); or B) PVA (18 or 40 kDa) in 150 mM NaCl. CMV-β-gal injected per muscle was 50 µg. Results reported as mean ± SEM (n = 9 − 12).

(10 kDa) has a similar viscosity as 5% PVP (50 kDa). The ability of PVP to bind drugs is related to the molecular weight of PVP, with higher molecular weight polymer having the stronger binding (6). This may explain why greater gene expression was observed in muscle for the 5% PVP (50 kDa) formulation as compared to the 5% PVP (10 kDa) formulation, although the weight ratios of PVP to pDNA for both formulations were kept constant at 50:1 w/w.

Other amphiphilic polymers, with structures similar to PVP, have also been examined. One such amphiphilic polymer is polyvinyl alcohol (PVA) which is able to form hydrogen-bonds through its alcohol moieties. For example, PVA interacts with Cibacron Blue, although to a lesser extent than does PVP (9). However, PVA functions as a hydrogen-bond donor while PVP is a hydrogen-bond acceptor (6). Nevertheless, hydrogen-bonding to pDNA is possible with both polymers, since pDNA can function as both a hydrogen-bond donor or hydrogen-bond acceptor molecule.

β-gal expression at 8 days after i.m. injection of pDNA formulated in isotonic PVA solutions are presented in Figure 2B.

These PVA formulations had viscosities approximately equal to the PVP formulations. The results showed that pDNA formulated in 2% PVA (18 kDa) led to an approximate 10-fold increase in the level of β-gal expression over pDNA formulated in saline. The results also showed that other PVA formulations resulted in similar or only slightly enhanced levels of β-gal expression as compared to pDNA in saline.

Immunohistochemical staining of β-gal in muscle demonstrated that pDNA formulated in 5% PVP (50 kDa) was better dispersed in the muscle tissue as compared to pDNA injected in saline (Figure 3). The staining also showed that the PVP formulation resulted in an increase in the number of cells expressing β-gal and that these cells were distributed over a larger area as compared to pDNA injected in saline. pDNA injected in saline was mostly expressed close to the injection site as reported by others (2). It is suggested that the increased tissue dispersion of pDNA using PVP formulations is due to a hyper-osmotic effect in the muscle (10–12). For example, a formulation consisting of pDNA (3 mg/ml) in 5% PVP (50 kDa) in 150 mM NaCl exerts an osmotic pressure of 341 ± 1 milliosmols/kg H_2O (mOsm/kg H_2O) whereas normal physiological osmotic pressure is ~290 mOsm/kg H_2O. However, the enhancement of β-gal expression observed with PVP (50 kDa) and PVA (18 kDa) in the muscle was likely due not only to osmotic effects, since other polymer formulations investigated, including PVP (10 kDa), exert similar osmotic pressures but only result in modest increases in levels of β-gal expression as compared to pDNA in saline.

Dynamic dialysis data demonstrated that the rate of diffusion of PVP (10 kDa) was greatly reduced in the presence of a fixed amount of pDNA (Figure 4). Possible Donnan effects were considered to be negligible since NaCl was present in both the donor and acceptor compartments. The diffusion rate of PVP through the membrane was observed to be biphasic, with a greater diffusion rate during the first 3 hours (D_{R1}) as compared to the second phase occurring between 3 to 10 hours (D_{R2}). The D_{R1} ratio ($D_{R1}'/D_{R1}*$) for samples without pDNA (D_{R1}') and in the presence of pDNA ($D_{R1}*$) was 1.5, 1.63, 2.18, 2.66, and 3.33 for samples containing 90, 60, 30, 20, and 10 mg PVP, respectively. The reduction in the diffusion rate for PVP in the presence of pDNA was directly proportional to the initial amount of PVP in the dialysis sac. The diffusion rate differences (as given by the D_{R1} ratios) increased with lower amounts of PVP. Further, although the levels of pDNA were fixed, approximately 60–80% of the initial amount of PVP remained in the dialysis sac for all samples, irrespective of the initial amount of PVP.

The major mechanisms of PVP complexation are known to be (*i*) ionic interaction, (*ii*) hydrogen bonding, and (*iii*) hydrophobic interaction (6, 9, 14). It has been reported that at low salt concentrations, PVP binds to Cibacron Blue through ionic interaction (9). However, at higher salt concentrations, PVP binds to Cibacron Blue mainly by hydrogen bonding, with some contribution from hydrophobic interactions (9). Some synthetic polyvinyl cationic derivatives, including polyvinyl pyrridinium salts, are known to interact with DNA both ionically and hydrophobically (15). Such interactions are reported by Kabanov et al. (15) to result in a hydrophobic-coating of pDNA by the polymers which leads to both enhanced stabilization of the pDNA against extracellular nuclease degradation and increased interaction of the polymer-coated pDNA with the hydrophobic components of cell membranes. Also, the enhanced fluorescence of phenprocoumon and warfarin bound to PVP has been shown to result from their incorporation into the hydrophobic environment of PVP (14).

We have also used gel electrophoresis to show that PVP stabilized pDNA against nuclease degradation (Figure 5). The results of a DNase I challenge study showed that higher amounts of DNase I were needed to degrade pDNA in the presence of PVP, than in its absence. Furthermore, longer incubation times of DNase I were needed to degrade pDNA in the presence of PVP.

The average cumulative heats of interaction upon injection of pDNA into a solution of PVP are shown in Figure 6A. The positive heats of reaction are indicative of an endothermic process. In order to generate favorable free energy, PVP:pDNA interactions must be driven by a favorable entropic component. Most likely, the entropic contribution occurs via release of water and/or counterions from both molecules upon their mixing. PVP has also been shown to have an endothermic interaction with several benzodiazepine derivatives (16). Figure 6B shows a comparison of the FT-IR spectra of pDNA, PVP, and pDNA in the presence of PVP. The region 1085-1225 cm^{-1} shows increased intensity and diminished band broadening of the symmetric and antisymmetric phosphodioxy stretch vibrations. A similar phenomenon is observed for the phosphodiester vibrations at 895 cm^{-1} and 970 cm^{-1}. These observations represent an increased ordering of the pDNA backbone in the presence of PVP and demonstrate greater stabilization of the pDNA duplex. In the presence of PVP, perturbations are observed in the ring stretching vibrations of cytosine and/or adenine near 1534 cm^{-1} and 1546 cm^{-1}, respectively, which are indicative of nucleic acid/base perturbations. In addition upon complexation, CH_2 bending vibrations near 1464 cm^{-1} are perturbed significantly for both pDNA and PVP. Alterations in the pDNA backbone are consistent with perturbations of the phosphodiester band at 970 cm^{-1} and the phosphodioxy bands at 1085 cm^{-1} and 1225 cm^{-1}. The region 1600–1800 cm^{-1} is assigned to vibrational modes of the exocyclic base residues of pDNA and carbonyl stretch vibrations of PVP. A comparison of the pDNA spectrum with that for PVP:pDNA reveals perturbations at 1642 (dC), 1657 (dT), and 1741 cm^{-1} (dG), resulting in frequency shifts to 1620 cm^{-1}, 1673 cm^{-1}, 1749 cm^{-1}, and a shoulder near 1706 cm^{-1}. No such bands are observed in the PVP or pDNA spectra alone. These perturbations suggest extensive interactions between hydrogen-bond donor/acceptor groups of pDNA and PVP.

In conclusion, we have observed significant enhancement of gene expression over saline for reporter genes using interactive polyvinyl-based delivery systems. We have shown that this polymeric gene delivery system results in extensive dispersion of pDNA in the muscle tissue, most likely due to osmotic effects in the muscle. The system also protects pDNA from nuclease degradation, most likely due to hydrogen-bonding followed by an apparent hydrophobic-coating of pDNA by PVP that leads to enhanced stabilization of the pDNA. We are currently determining if these delivery systems retain pDNA in the muscle as compared to pDNA in saline, and also the mechanism by which these delivery systems facilitate pDNA cellular uptake. Novel interactive co-polymers based upon polyvinyl backbones are now being studied to further enhance gene delivery to muscle cells *in vivo*.

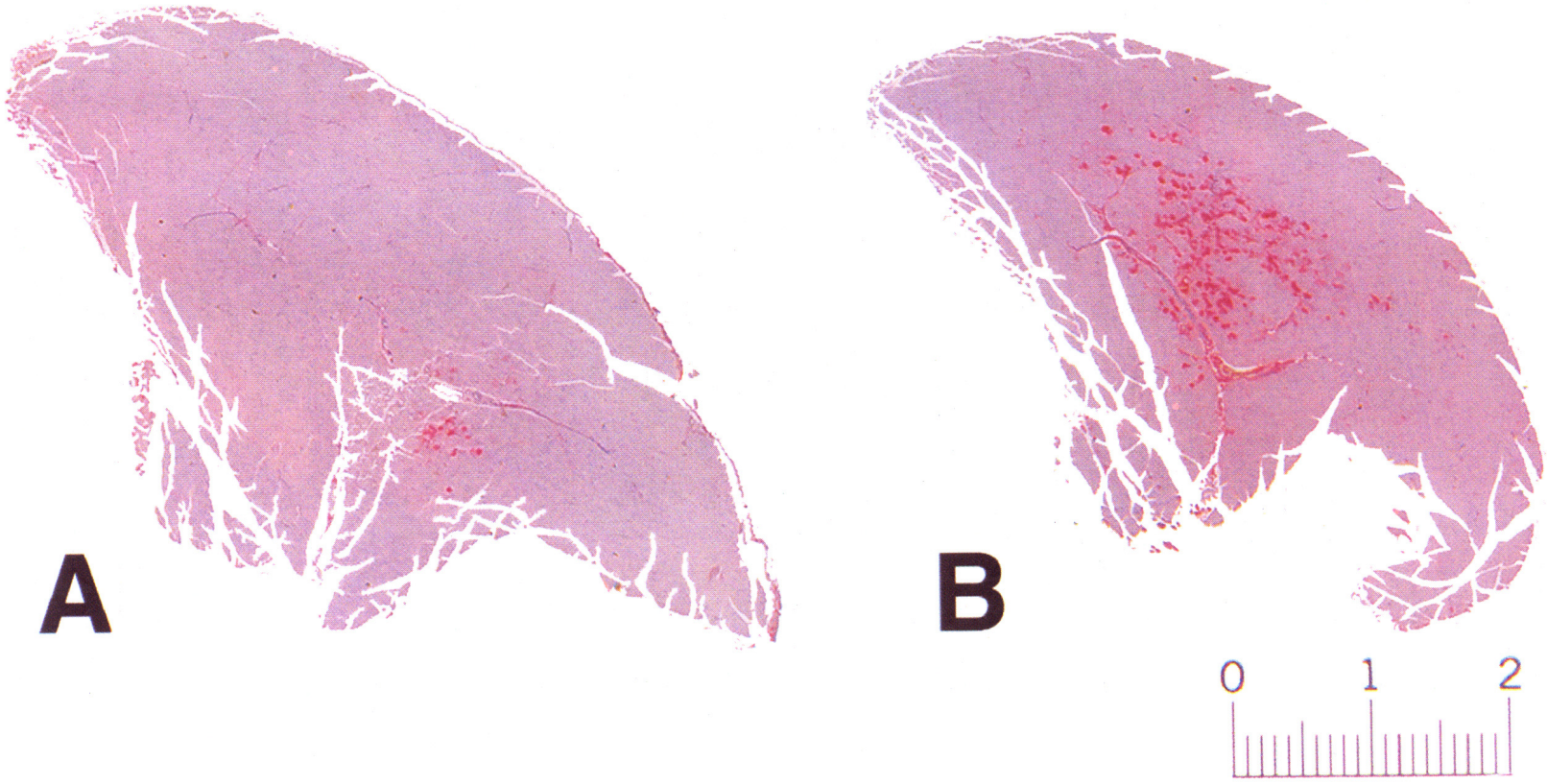


Fig. 3. Immunohistochemical staining of rat tibialis muscle for β-gal at 7 days after i.m. injection of pDNA in: A) saline and B) 5% PVP (50 kDa) in 150 mM NaCl. CMV-β-gal injected per muscle was 150 μg. The scale shown is in millimeters.

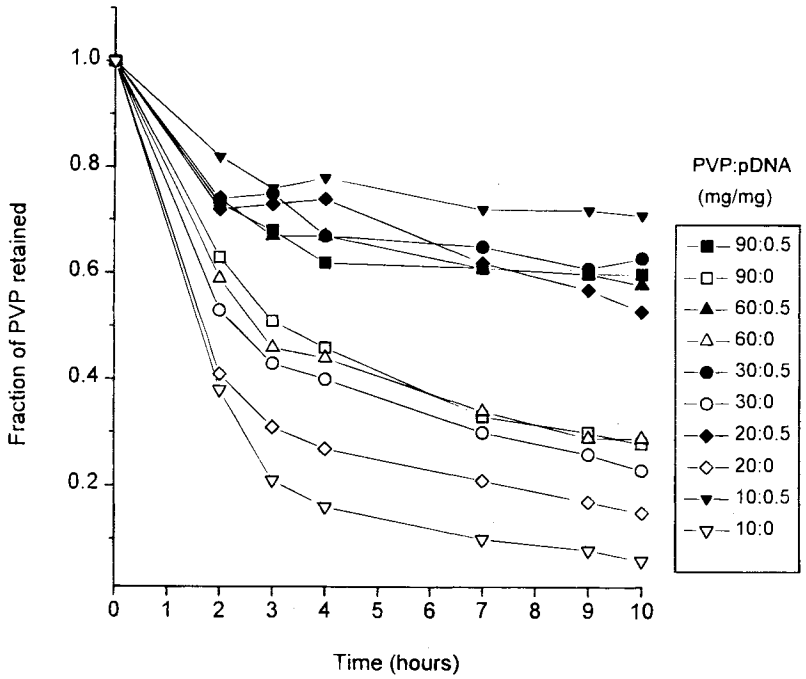


Fig. 4. Dynamic dialysis of various PVP (10 kDa):pDNA ratios at 25°C. Spectra/Por CE membranes with Mw cut-offs of 25 kDa were used. Data are reported as fraction of PVP (10 kDa) remaining in the dialysis sac over time.

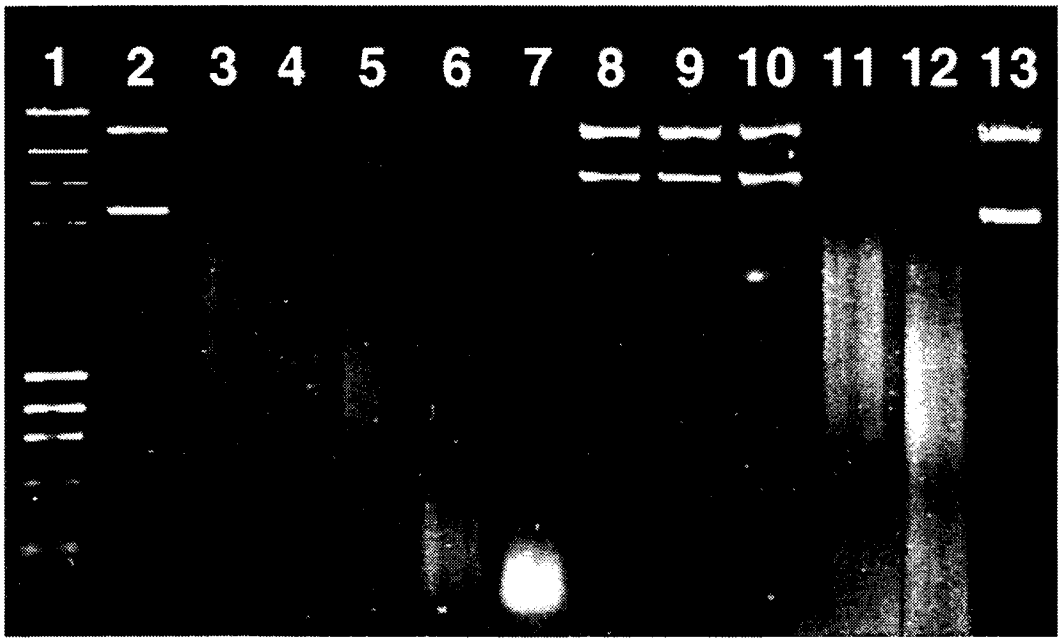
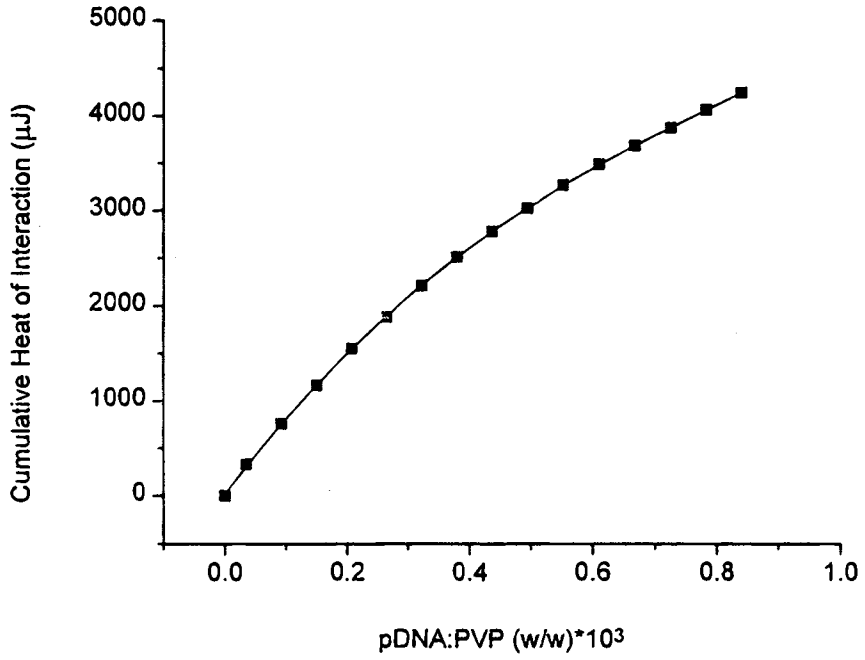


Fig. 5. DNase I incubation with pDNA in saline (Lanes 2–7) or pDNA formulated in 5% PVP (50 kDa) in 150 mM NaCl (Lanes 8–13). Incubation of DNase I with formulations was for 15 minutes at 37°C: Lane 1) molecular weight marker; Lane 2) pDNA in saline control; Lanes 3–7) DNase I:pDNA (in saline) 1:50,000, 1:40,000, 1:30,000, 1:20,000, and 1:10,000, respectively; Lanes 8–12) DNase I:pDNA (in 5% PVP in 150 mM NaCl); 1:50,000, 1:40,000, 1:30,000, 1:20,000, and 1:10,000, respectively; Lane 13) pDNA in 5% PVP (50 kDa) in 150 mM NaCl control.

(UPPER PANEL)



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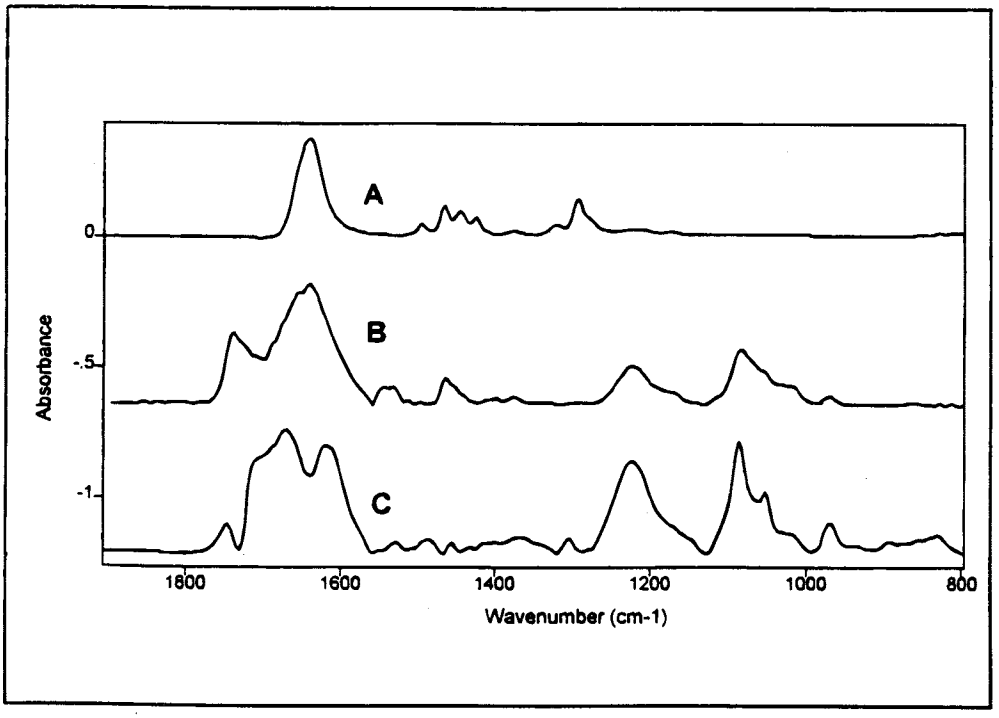

Fig. 6. Evidence for hydrogen-bonding between PVP and pDNA. (Upper panel) Titration calorimetric studies of pDNA:PVP (50 kDa) interactions. pDNA (1.73 mg/ml) in 150 mM NaCl was titrated into a 1 ml stock solution of 15% PVP (50 kDa) in 150 mM NaCl; (Lower panel) FT-IR studies of PVP:pDNA (50 kDa) interactions. A) 5% PVP (50 kDa) in 150 mM NaCl, B) pDNA (1 mg/ml) in 150 mM NaCl, and C) pDNA (1 mg/ml) in 5% PVP (50 kDa) in 150 mM NaCl. Band assignments: pDNA backbone (895 cm^{-1}), DNA backbone (970 cm^{-1}), symmetric PO_2^- (1053 cm^{-1} and 1086 cm^{-1}), antisymmetric PO_2^- (1225 cm^{-1}), purine (1373 cm^{-1}), deoxyribose (1414 cm^{-1}), CH_2 bending (1463 cm^{-1}), ring stretching of the bases (1500–1600 cm^{-1}), ring stretching of cytosine (1534 cm^{-1}), ring stretching of adenine, quanine, and cytosine (1561 cm^{-1} and 1575 cm^{-1}), in plane stretching of carbonyl bond (1600–1750 cm^{-1}), carbonyl bond stretching of thymine (1672 cm^{-1}) and carbonyl bond stretching of guanine (1711 cm^{-1}).

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