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Characterization of a new class of DNA delivery complexes formed by the local anesthetic bupivacaine

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Abstract

Bupivacaine, a local anesthetic and cationic amphiphile, forms stable liposomal-like structures upon direct mixing with plasmid DNA in aqueous solutions. These structures are on the order of 50–70 nm as determined by scanning electron microscopy, and are homogeneous populations as analyzed by density gradient centrifugation. The DNA within these structures is protected from nuclease degradation and UV-induced damage in vitro. Bupivacaine:DNA complexes have a negative zeta potential (surface charge), homogeneous nature, and an ability to rapidly assemble in aqueous solutions. Bupivacaine:DNA complexes, as well as similar complexes of DNA with other local anesthetics, have the potential to be a novel class of DNA delivery agents for gene therapy and DNA vaccines.

Keywords: Bupivacaine; DNA delivery; Local anesthetic; DNA complex; Genetic immunization

1. Introduction

DNA immunization and gene therapy using non-viral vectors require efficient methods for delivery of plasmid DNA to target cells in vivo. Complexes of plasmid DNA and synthetic cationic lipids, such as DOTMA and DOTAP, have been successful in enhancing transfection, in cell culture. However, in general, these cationic lipid:DNA complexes do not result in increased muscle cell transfection after in vivo intramuscular injection, nor immune responses against antigens encoded by the DNA, when compared to naked DNA [1].

Although naked DNA can transfect muscle cells in vivo, pretreatment of muscle with the local anesthetic bupivacaine several days prior to injection of DNA, results in increased DNA uptake, as evidenced by increased DNA expression at the injection site [2,3]. Increased expression of DNA is also associated with increased immune responses to antigens encoded by the injected DNA [4] (unpublished data Higgins, Shroff). Increase in muscle transfection by pretreat-

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ment with bupivacaine is thought to be due to the myogenic activity of bupivacaine. Treatment of muscle with bupivacaine causes muscle fiber degeneration, and subsequent recruitment of myoblasts into muscle regeneration [2]. Dividing myoblasts are postulated to be more likely to be transfected with DNA than the non-dividing, differentiated muscle cells, and are presumably responsible for the observed increase in muscle transfection. In addition, the recruitment of inflammatory cells to the site of bupivacaine injection [2,3,5,6] may allow for transfection of immune cells, as well. Due to the temporal nature of muscle cell degeneration and regeneration, the window of opportunity to transfect regenerating muscle fibers is 1 to 7 days post-bupivacaine treatment.

We have shown that mixtures of DNA and bupivacaine delivered simultaneously also result in increased transfection and expression in muscle cells (unpublished data Higgins, Shroff). However, free bupivacaine concentrations in mixtures of DNA and bupivacaine are orders of magnitude below the threshold concentrations required to cause muscle degeneration. Furthermore, both intramuscular and intradermal administration of mixtures of DNA and bupivacaine generates a high and uniform immune response when compared to the use of DNA alone (unpublished data Higgins, Shroff). The mechanism of muscle transfection by mixtures of DNA and bupivacaine is not predicted to be related to the process of bupivacaine pretreatment, due to the temporal nature of muscle cell degeneration and regeneration, and the demonstrated lack of muscle damage by the DNA and bupivacaine mixture.

We have discovered interactions between bupivacaine and DNA that may explain the observed increase of in vivo transfection when mixtures of bupivacaine and DNA are used. Aqueous solutions of bupivacaine form uniform sized complexes, upon direct mixing with DNA. Although these complexes share some physical and biochemical features with the synthetic cationic DNA liposomes that have been shown to transfect cells in vitro, bupivacaine:DNA complexes are dissimilar in that they are formed as uniform sized particles of negative zeta potential, upon both direct mixing in aqueous solution, as well as when prepared from pre-made bupivacaine vesicles.

2. Materials and methods

2.1. Preparation of complexes

2.1.1. Rapid mixing

Bupivacaine hydrochloride (Sigma, St. Louis, MO, USA) (2.5% in water, pH 6.4) was added to aqueous DNA solutions such that the final bupivacaine concentration was 0.25%. The experiment was also performed in 10 mM citrate buffer at pH 6.7. The mixtures were inverted by hand to mix. For density gradient analysis, complexes were made with 500 ug/ml DNA (charge ratio, +:−::5.3:1) that contained 2×10^5 cpm ^{33}P-labeled DNA. For DNA protection studies, complexes were made using 500 ug/ml DNA. For the gold labeling experiment, complexes were made in the presence of gold-labeled DNA and unlabeled DNA. Gold-labeled plasmid DNA was mixed with a 10-fold excess of unlabeled plasmid DNA to a final DNA concentration of 500 ug/ml.

2.1.2. Slow mixing by dialysis

Bupivacaine was slowly added to DNA by dialysis of a 0.25% bupivacaine hydrochloride solution across a 10 kDa membrane over a 3 h period. DNA concentrations ranged from 10 ug/ml (+:−::264:1) to 800 ug/ml (+:−::3.3:1) in the experiments presented here.

2.1.3. Bupivacaine and bupivacaine:DNA vesicles prepared from dried films

An ethanolic solution of bupivacaine hydrochloride (10 ml) was dried to a thin film on a rotary evaporator at 45°C, and placed under high vacuum for a minimum of 1 h. The film was resuspended in a volume of either water or aqueous DNA solution (1 mg/ml) for a final concentration of bupivacaine of 2.5 mg/ml (+:−::2.6:1), and vortexed moderately for 1 min. Films resuspended in water were subsequently treated with aqueous DNA solution for a final DNA concentration of 1 mg/ml, and a final bupivacaine concentration of 2.5 mg/ml. The resulting suspensions were processed for transmission and scanning electron microscopy by drying over EM grids under vacuum. The structures were visualized by carbon shadowing (angle of carbon spray = 20°). Scanning EM was carried out on gold plated grids to

achieve maximal reflections. All EM experiments were carried out at Micron, Wilmington, DE, USA, on a JEOL TEM and a Phillips TEM.

2.2. Analysis of complexes

2.2.1. Octanol extraction

The mixtures were extracted with an equal volume of octanol, by vigorous vortex mixing for 1 min and centrifugation at 12 000 rpm. for 10 min. One hundred microliter of the octanol and water phases were used to determine bupivacaine concentrations. Bupivacaine concentrations were determined at 260 nm by reverse phase chromatography, using a C18 HPLC column (Waters, Milford, MA, USA) using a linear acetonitrile gradient, in 10 mM phosphate, at pH 6.5.

In order to detect DNA in complexes extracted into the octanol phase, 500 μl of the octanol phase was removed and dried under vacuum. The dried pellet was resuspended in 0.02 ml of water, and electrophoresed in 1×Tris–acetate–EDTA (Sambrook et al.) on 1% agarose gels. DNA was visualized by staining with cyber-green (Molecular Probes), a DNA intercalating fluorescent dye. As control, uncomplexed DNA was also extracted with octanol, and analyzed as described above.

2.2.2. Density gradient analysis

2.2.2.1. Preparation of ^{33}P plasmid DNA. A plasmid transformed, *Escherichia coli* strain DH10b (Life Technologies, Grand island, NY, USA) was grown to stationary phase in 10 ml minimal media containing limiting PO_4^{-2} (Neidhardt's MOPS medium), 5 mCi ^{33}P-orthophosphoric acid (NEN), isoleucine, leucine and 40 ug/ml kanamycin. Plasmid DNA was purified using a hydrophobic interaction matrix (Sigma, St. Louis, MO, USA) followed by chromatography on a DEAE-Silica matrix (Qiagen, Valencia, CA, USA). The eluant was precipitated with 0.7 v/v isopropanol. DNA precipitate was collected by centrifugation at 12 000 rpm for 10 min, washed with 70% ethanol and dried under vacuum. The DNA pellet was dissolved in water. Specific activity of the plasmid preparation was calculated by determining the concentration of DNA, and by scintillation counting of a fraction of the preparation. Purity of the plasmid DNA was ascertained by the ratio of its absorbance at 260 and 280 nm, and by electrophoresis on agarose gels. Gels were soaked in 10% TCA for 10 min, vacuum dried, and exposed to phosphorimager to visualize radioactive ^{33}P material in the preparation.

2.2.2.2. Ficoll density gradients. Discontinuous gradients were prepared using 3.5 ml of 40% Ficoll and 6.5 ml of 20% Ficoll. Bupivacaine complexes: labeled plasmid DNA was mixed with unlabeled plasmid DNA to achieve a final specific activity of 2×10^5 cpm per 500 ug of DNA. Bupivacaine (2.5% solution) was added to the mixture to a final concentration of 0.25%, in 1 ml (+:−::5.3:1). Lipofectamine (Life Technologies, Grand Island, NY, USA) complexes: labeled plasmid DNA was mixed with unlabeled plasmid DNA to achieve a final specific activity of 2×10^5 cpm per 2.5 ug DNA. Lipofectamine:DNA complexes were made according to the manufacturer's directions and at a +:−::5.3:1. Complex volume brought up to 1 ml in water. Replicate gradients were prepared. Complexed or free DNA (1 ml) was applied to the gradients. The gradients were topped off with 1 ml of water, and centrifuged in an SW41 rotor at 35 000 rpm for 24 h. Following centrifugation, fractions (0.5 ml) were collected from the gradient, and the associated radioactivity measured by scintillation counting.

2.2.2.3. Sucrose density gradients. 3–30% sucrose density gradients were prepared by repeated freezing and thawing (four times) of 11 ml of a 15% sucrose solution. One milliliter of uncomplexed and bupivacaine complexed DNA were layered onto the gradient as described for the Ficoll gradients. The gradients were centrifuged in an SW41 rotor at 35 000 rpm for 24 h. 0.5 ml fractions were collected and analyzed for refractive index determination and scintillation counting.

2.2.2.4. Protection studies with restriction enzyme SacI. Ten microgram of complexed DNA and uncomplexed DNA were subjected to enzymatic digestion. Replicate samples were digested using the restriction enzyme *Sac*I (Promega, Madison, WI, USA), at 0.25 U/μg DNA. *Sac*I reactions were performed in 200 μl, and were incubated at 25°C. *Sac*I

was chosen because it cleaves the plasmid molecule once, thus linearizing the plasmid. Time course of the restriction enzyme digestion was generated by quenching 40 μl of the reaction mixture with EDTA (25 mM) at various time intervals. Reaction products were separated on agarose gels, electrophoresed in TBE buffer, in the presence of ethidium bromide (0.5 μg/ml), following incubation at 55°C in the presence of 10 μg Proteinase-K (Worthington Biochemical Corp., Freehold, NJ, USA) and 0.5% SDS. Band intensities were quantified using a video gel scanner. Specific intensity of the singularly digested plasmid band was determined by normalizing the fluorescence intensity of product with the total intensity in that lane. A time course was plotted to determine a linear window for the reaction.

2.2.2.5. Protection studies with DNAase. DNAase digestions were carried out under conditions in which only 10% of uncomplexed DNA was digested. Digestions were carried out at 25°C, in 25 μl, using 1 μg complexed or uncomplexed plasmid DNA, and using 0.05 U of DNAase I (Worthington Biochemical Corp., Freehold, NJ, USA). The reactions, treated with 0.5% SDS and 10 μg Proteinase-K, were electrophoresed on agarose gels as described previously. The DNA was stained and analyzed as described above.

2.2.2.6. UV protection. One microgram each of complexed and uncomplexed DNA were subjected to 2 min of UV at a distance of 15 cm from the source. The source was a UV scan box, with a broad range band width output of 280–320 nm. In control studies with uncomplexed DNA, nearly 100% cross linking (UV dimer formation) was achieved in 15 min of UV exposure. DNA in complexes was purified by ethanol precipitation, and extensive washes of the precipitate with 70% ethanol. UV exposed complexed and uncomplexed DNA were subjected to 1 U of the bacteriophage T4 endonuclease V (Epicentre, Madison, WI, USA) digestion. The samples were treated with 0.5% SDS and 10 μg of Proteinase-K prior to agarose gel electrophoresis. Analysis was carried out as described for the *Sac*I protection experiment.

2.2.2.7. Scanning electron microscopy. All microscopy was performed by Micron, located in Wilmington, DE, USA.

2.2.2.8. Transmission electron microscopy. Bupivacaine:DNA complexes were placed on a collodium/carbon coated 200 mesh copper grid and stained for 4 min with 0.1% OsO_4 and for 30 s with 1% uranyl acetate. Micrographs were taken with a Philips 420 electron microscope at 60 kV using a 50 μm objective aperture at 51 000× magnification.

2.2.2.9. Gold labeling of DNA. Fifty microgram of nicked open circular DNA was subjected to a strand displacement reaction using Klenow (Promega, Madison, WI, USA), 50 uM dATP, dGTP and dCTP, 20 uM dUTP and 2 uM biotinylated dUTP (Boehringer Mannheim, Indianapolis, IN, USA). The plasmid was purified from free nucleotides by two rounds of ethanol precipitation, and two 70% ethanol washes. The amount of biotin incorporated into the plasmid was determined by a kinetic ELISA, by reacting dilutions of the plasmid with streptavidin-HRP (Boehringer Mannheim, Indianapolis, IN, USA). Three molecules of biotin were incorporated per plasmid molecule. One microgram of biotinylated plasmid was reacted with 10^{11} molecules of gold conjugated streptavidin (Boehringer Mannheim, Indianapolis, IN, USA). (One microgram of a 5 kbp plasmid DNA corresponds to 1.25×10^{14} molecules and 3.75×10^{13} conjugated biotin molecules.) The 100-fold excess of conjugated biotin ensures the absence of free streptavidin-gold. The labeled DNA was then mixed with unlabeled DNA to achieve a final DNA concentration of 500 ug/ml. A 2.5% solution of bupivacaine was added to 0.25% to form complexes. The mixture was analyzed by transmission electron microscopy.

3. Results and discussion

3.1. Bupivacaine imparts hydrophobicity to interacting DNA molecules

The local anesthetic bupivacaine is a cationic amphiphile comprised of a protonated cyclic tertiary amine attached to a hydrophobic dimethyl phenyl ring via an amide linkage (Fig. 1). The uncharged

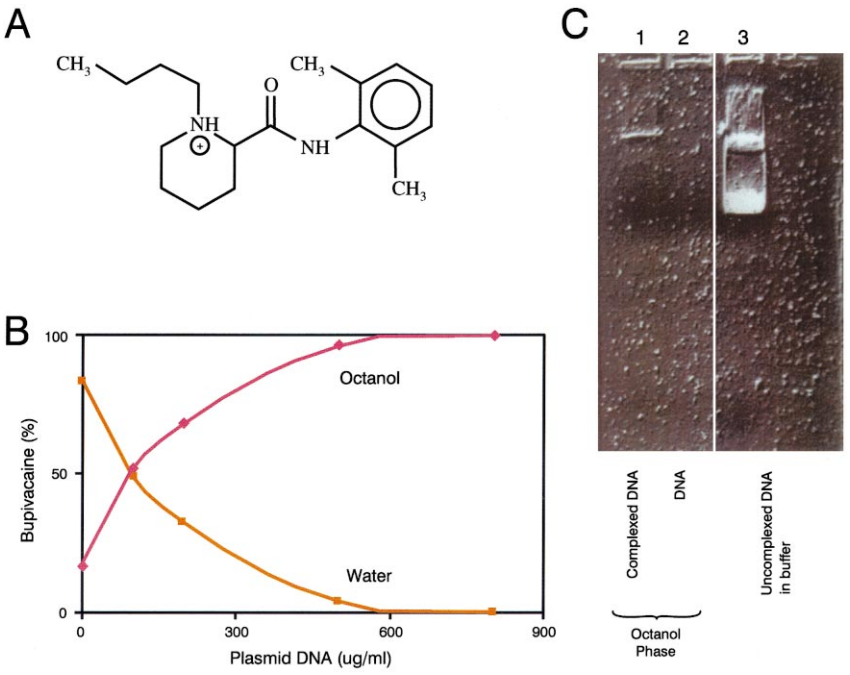

Fig. 1. (A) Structure of bupivacaine. The molecular structure of bupivacaine is depicted. Note that the cyclic tertiary amine is protonated and positively charged. (B) and (C) Partitioning of DNA:bupivacaine complexes into octanol. (B) Bupivacaine:DNA mixtures were made using the indicated concentrations of supercoiled plasmid DNA. The mixtures were extracted with octanol. The amount of bupivacaine in the octanol and water phase was determined and is expressed as the percentage of bupivacaine extracted into either octanol or water. (C) A bupivacaine:DNA mixture made using plasmid DNA at a concentration of 300 ug/ml (lane 1) or 300 ug/ml plasmid DNA alone (lane 2) that was octanol extracted. A portion of the octanol phase was dried under vacuum and resuspended in water. The samples were electrophoresed in 1× Tris–acetate EDTA on a 1% agarose gel. DNA was visualized by staining with cybergreen. Plasmid DNA that was not octanol extracted (lane 3) was run as a control.

species of bupivacaine partitions readily into nonpolar solvents such as *n*-octanol. At pH values below its pK_a (pH 8.1), the nitrogen of bupivacaine is protonated and the bupivacaine molecule is positively charged. The presence of a positive charge renders the bupivacaine molecule soluble in water and only sparingly soluble in *n*-octanol. We initially observed that the water solubility of bupivacaine was compromised in the presence of high concentrations of DNA (>1 mg/ml) at pH values approaching the pK_a of bupivacaine, suggesting the presence of ionic interactions between the polyanionic DNA and the positively charged bupivacaine, which result in charge neutralized complexes.

As amphiphiles, bupivacaine and other local anesthetics not only react with cellular membranes, but also with ion channel proteins [7]. An ionic interaction between bupivacaine and DNA in aqueous solutions at pH $<$ pK_a, would be expected to result in the charge neutralization of both bupivacaine and DNA. In addition, the interaction between bupivacaine and DNA would be expected to impart a hydrophobic nature to the bupivacaine:DNA complex. Bupivacaine may therefore be expected to confer a membrane reactive property to complexes with DNA.

n-Octanol partitioning experiments were used to partially characterize the nature of the interactions between bupivacaine and DNA at pH values less than the pK_a of bupivacaine. Both bupivacaine and DNA are predicted to become more soluble in and partition into *n*-octanol, if they form a stable, charge neutralized complex. Partitioning experiments demonstrate that the partitioning of bupivacaine into *n*-octanol is DNA dependent, and that the amount of bupivacaine extracted into the octanol phase is proportional to the concentration of DNA in the mixture (Fig. 1B). An increase in the amount of bupivacaine extracted into octanol corresponds with a concomitant decrease of bupivacaine in water. One

hundred percent extraction of bupivacaine into octanol from a 0.25% bupivacaine solution was observed at a DNA concentration of 300 ug/ml (+:−::8.8:1).

The polyanionic DNA also partitioned into the octanol phase when the bupivacaine:DNA mixtures were octanol extracted. The DNA that was extracted into the octanol phase was isolated and migrated with a slower mobility during agarose gel electrophoresis when compared to uncomplexed DNA (Fig. 1C, lanes 1 and 3). The decrease in relative mobility is likely due to changes in both the mass and the charge of complexed DNA. In the absence of bupivacaine, DNA did not partition into octanol (Fig. 1C, lane 2).

The results of the octanol partitioning experiment support the predicted interactions between bupivacaine and DNA. DNA is therefore physically associated with bupivacaine, and some aspects of this interaction must involve the formation of salt bridges, that result in the charge neutralization of the DNA.

The octanol partitioning data also suggested an unusual stoichiometry of the interaction. Eight-fold more molar charge equivalents of bupivacaine were extracted into the octanol phase than there were phosphoryl groups present in the DNA. These data suggest that non-ionic interactions may also play a role in the formation of complexes. However, ionic interactions are a prerequisite for complex formation as evidenced by slower complexation rates in the presence of competing ions: M^{2+}, Na^{+} and PO_4^{-}, or at pH values that preclude electrostatic interactions (data not shown).

3.2. Density gradient analysis of DNA:bupivacaine complexes

Complexing of bupivacaine and DNA is also predicted to result in measurable increases in the density of the complexed DNA. To determine if there were differences in density between complexed and uncomplexed DNA, and to determine if free DNA was present in mixtures of bupivacaine and DNA, the complexes were subjected to analyses by Ficoll and sucrose density gradient centrifugation. Lipofectamine (a DOSPA/DOPE formulation):DNA complexes were used as a control, as these cationic lipid DNA complexes were shown to generate particles having a density different from that of DNA (Fig. 2, top panel). However, it should be noted that lipofectamine:DNA complexes are generated by mixing preformed cationic liposomes with DNA, and can not be formed by direct mixing with DNA in aqueous solution.

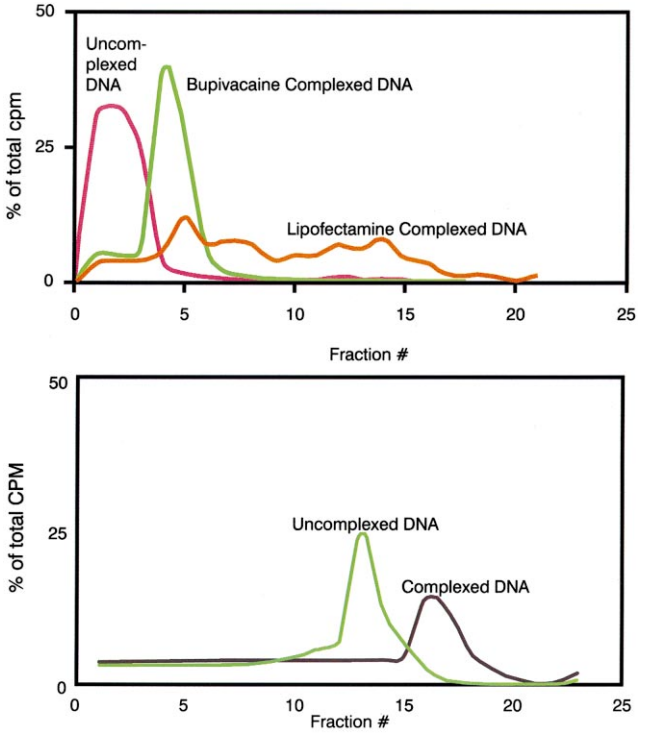

Fig. 2. Density gradient profiles of complexed and uncomplexed DNA. (A) Ficoll gradient analysis. Uncomplexed radiolabeled DNA and bupivacaine and lipofectamine complexed DNA made with radiolabeled DNA were centrifuged through a 20–40% Ficoll gradient. 0.5 ml fractions were collected and the associated radioactivity measured by scintillation counting. Relative migrations of the uncomplexed and complexed DNA are indicated. (B) Sucrose density gradient analysis. Uncomplexed radiolabeled DNA and radiolabeled DNA complexed with bupivacaine were centrifuged through a 3–30% sucrose gradient. 0.5 ml fractions were collected, and the associated radioactivity measured by scintillation counting. The relative migration of uncomplexed and complexed DNA is indicated.

Bupivacaine:DNA complexes made by direct mixing, and lipofectamine:DNA complexes made in the presence of ^{33}P-labeled supercoiled plasmid DNA were characterized by Ficoll density gradient rate centrifugation. In experiments with uncomplexed ^{33}P-DNA, fractions 1, 2 and 3 contained the uncomplexed labeled DNA (Fig. 2, top panel). In experiments with bupivacaine complexed DNA, the majority of labeled DNA was found in fractions 4, 5, 6 and 7, indicating that these complexes sediment with a

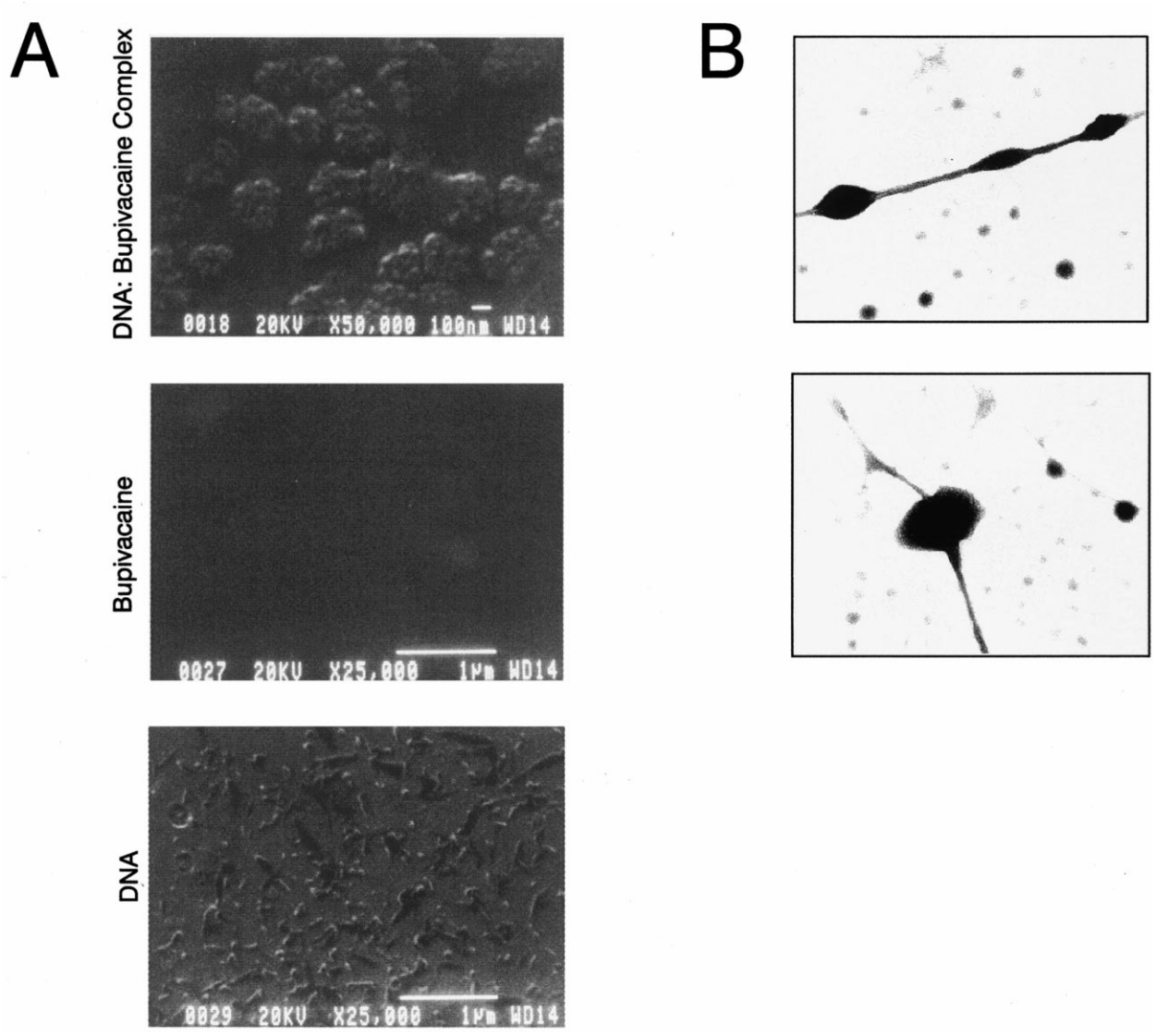

Fig. 3. Scanning and transmission electron micrographs of bupivacaine:DNA complexes. (A) Scanning electron micrographs are presented of DNA:bupivacaine complexes photographed at 50 000× (top), bupivacaine alone, photographed at 25 000× (middle) and DNA alone, photographed at 25 000× (bottom). (B) Transmission electron micrographs of DNA:bupivacaine complexes stained with osmiumtetroxide and uranyl acetate are shown. (Scale: 1.5 cm = 100 nm.)

higher density than supercoiled DNA alone. Less than 3% of the total DNA was found in fractions 1–3, where uncomplexed supercoiled DNA migrates in the gradient. Lipofectamine complexed DNA also sedimented at densities higher than DNA alone, and was more dense than the bupivacaine:DNA complexes. Furthermore, the lipofectamine:DNA complexes were broadly distributed throughout the gradient suggesting that the complexes were more heterogeneous than the bupivacaine:DNA complexes. In addition, while lipofectamine:DNA complexes (+:−::5.3:1) have a positive net surface charge of +20, the bupivacaine:DNA complexes (+:−::5.3:1) have a negative zeta potential of −52 (data not shown). The bupivacaine:DNA complexes thus appear to be more similar to the cationic detergent–DNA complexes described by Blessing [8]. Although the primary interactions with DNA are predicted to be similar for both bupivacaine and lipofectamine, and are mediated through their respective cationic headgroups, the secondary and tertiary hydrophobic interactions are likely to be differ-

ent. Lipofectamine has two long aliphatic chains while bupivacaine has a dimethyl phenyl group. These differences are reflected in the solubility differences of the two molecules in both polar and nonpolar solvents. It is therefore tempting to speculate that density and charge differences between bupivacaine and lipofectamine complexes are the result of their secondary and tertiary interactions. In addition, complexes made with lipofectamine and other similar cationic lipids are made by mixing DNA with preassembled liposomes. Liposome formation is primarily mediated through hydrophobic interactions, and addition of DNA acts to effect the reordering of these pre-existing structures. Direct mixing of DNA and bupivacaine, on the other hand, allows electrostatic and hydrophobic interactions to occur simultaneously.

Bupivacaine:DNA complexes were also characterized by sucrose density gradient rate centrifugation. Sucrose density gradients were chosen to improve the resolution of bupivacaine:DNA complexes. Uncomplexed DNA was found in fractions 12, 13 and 14 (15–18% sucrose) while the bupivacaine:DNA complexes were found in fractions 16, 17, 18 and 19 (at 22–29% sucrose) (Fig. 2, bottom panel). Symmetry of the bupivacaine:DNA complex peak suggests the absence of detectable amounts of uncomplexed DNA (less than 1%) suggesting that all of the DNA is complexed with bupivacaine. The narrow symmetrical Gaussian distribution is also consistent with the presence of complexes that are homogeneous in density.

3.3. Electron microscopic characterization of complexes

To further characterize the complexes of DNA and bupivacaine derived from direct mixing of aqueous solutions of these molecules, they were analyzed by both scanning and transmission electron microscopy. Scanning electron microscopy reveals the presence of 50–70 nm particles arranged in clusters (Fig. 3A, top). The uniform size of these particles is consistent with the homogeneous nature of the complexes revealed by density gradient centrifugation. These particles are not seen in the bupivacaine and uncomplexed DNA control (Fig. 3A, middle and bottom); only particles with the size of supercoiled DNA can be seen in the DNA control. The typical 'beads on a string' structures that have been reported for cationic liposome:DNA complexes [9] can also be observed in transmission electron micrographs of these preparations (Fig. 3B).

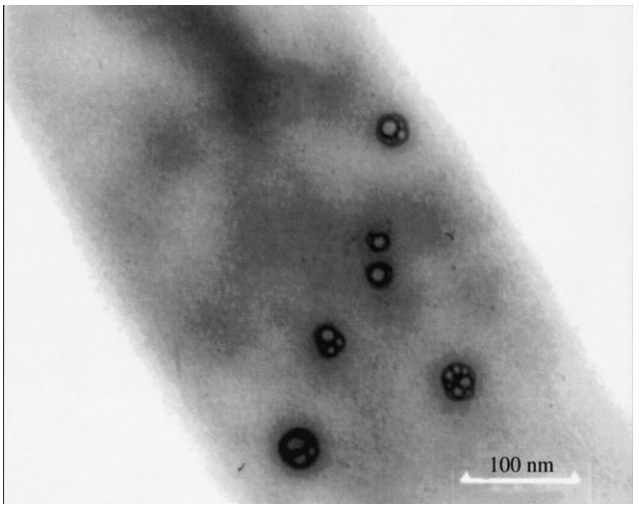

Fig. 4. Complexes made with gold-stained DNA. Transmission electron micrographs of DNA:bupivacaine complexes made with gold-stained DNA.

In order to confirm the presence of DNA in these structures we have employed bupivacaine:DNA complexes made with gold-labeled DNA. Complexes of gold-labeled DNA were analyzed by transmission electron microscopy. Results in Fig. 4 show that the gold label is associated with the complexes. These 70 nm labeled particles appear to be contained within higher order vesicle-like structures.

3.4. Complexed DNA is protected from nucleases and ultraviolet light

To determine if any of the DNA associated with the particle is in the interior of the particle, DNA protection experiments were performed. The extent to which complexed DNA is susceptible to enzymatic digestion has routinely been used as a measure of complexation. Protection of complexed DNA from nucleases (DNAase I or restriction endonucleases) has been previously cited as a measure of complexation [10]. The DNA in bupivacaine:DNA complexes is also protected from nucleases. Over 10-fold more protection has been observed with bupivacaine complexed DNA when compared with the di-

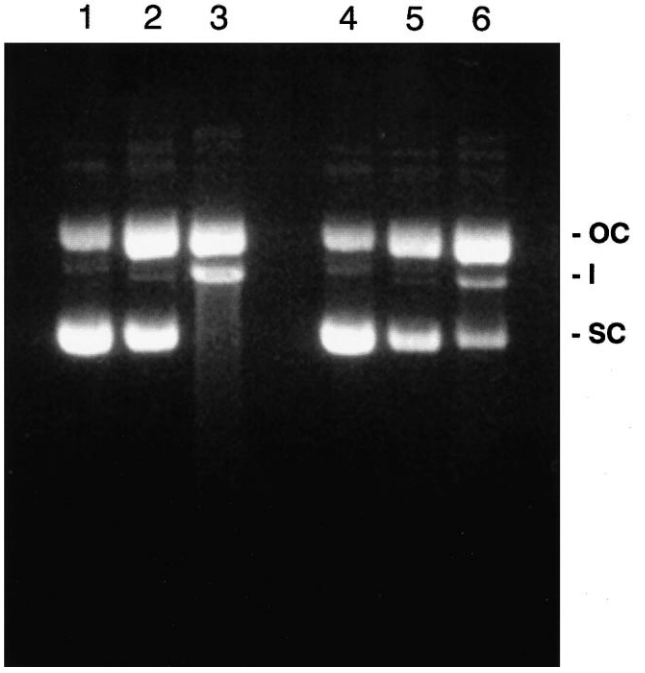

Fig. 5. UV protection analysis. DNA (lanes 1, 2 and 3), or DNA complexed with bupivacaine (lanes 4, 5 and 6) were exposed to UV alone (lanes 2 and 5) or exposed to UV and then incubated with T4 endonuclease V (lanes 3 and 6) as described in Section 2. DNA was then subjected to agarose gel electrophoresis. Control DNA (lane 1) or control complexed DNA (lane 4) was not exposed to UV or incubated with T4 endonuclease V. The positions of supercoiled (sc), linear (l) or open circular (oc) plasmid DNA forms are indicated.

gestion rate of uncomplexed DNA. The basis of DNA protection in all such studies has been attributed to entrapment of DNA into liposome-like vesicles. The premise of these experiments, however, is questionable. Cationic lipid or bupivacaine complexes of DNA may not be a nuclease substrate, the true substrate being DNA with a divalent cation coordinated to the oxygen on the phosphoryl group. Therefore, the protection results are only indicative of an altered substrate, and not necessarily of unavailability due to entrapment. In addition, the unreacted molecules of the cationic reagent may be non-competitive inhibitors of the enzyme.

We have devised a novel approach to determine if DNA is present in the interior of bupivacaine:DNA complexes. The approach is to determine the level of protection from ultraviolet light. Ultraviolet light induces thymidine dimer formation of neighboring thymidines in DNA. If DNA is free or is associated on the outside or is in the outer leaflet of the vesicle-like structures of the bupivacaine:DNA complexes, adjacent thymidines are expected to form dimers upon exposure to ultraviolet light. Since bupivacaine absorbs UV ($\lambda_{max} = 259$ nm), it is expected to protect DNA from UV-induced dimer formation only when the DNA is in the interior of the complexes. Following exposure of the complexes to UV light, DNA was purified from bupivacaine and incubated with the repair enzyme, T4 endonuclease V. This enzyme cleaves the 5′ glycosyl bond and the phosphodiester linkage located 3′ to the thymidine dimer, resulting in removal of the thymidine dimer. Control DNA exposed to UV light and incubated with the repair enzyme was analyzed by agarose gel electrophoresis. As anticipated, the DNA was degraded by the repair endonuclease. Note the absence of supercoiled DNA

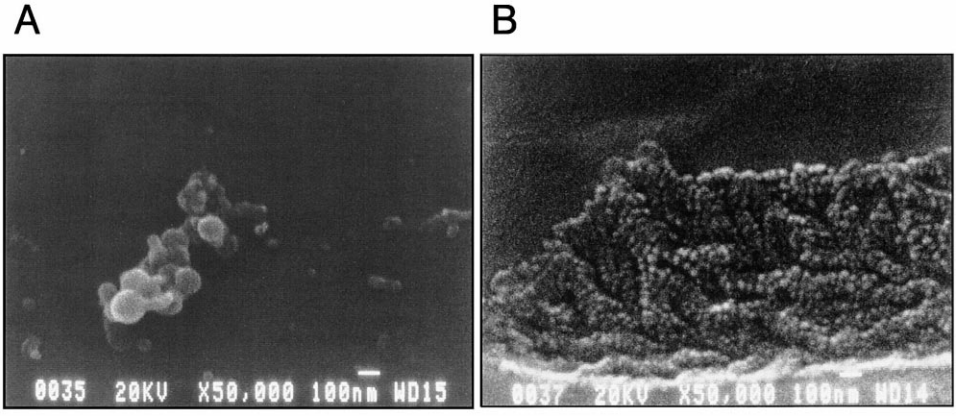

Fig. 6. Scanning electron micrographs of bupivacaine and bupivacaine:DNA vesicles prepared from dry films. (A) Bupivacaine vesicles formed by hydration of a bupivacaine film with water. (B) Bupivacaine:DNA vesicles formed by the addition of aqueous DNA to bupivacaine vesicles prepared as described for A.

in Fig. 5, lane 3. However, DNA isolated from complexes that were exposed to UV light and then subjected to enzymatic digestion were partially protected (Fig. 5, compare lane 6 with lane 3). Protection of complexed DNA indicates the presence of DNA in the interior of the vesicle-like structures. The presence of some unprotected DNA in the complexes, the negative zeta potential of the particles and the absence of free DNA, suggest that some DNA is associated on the particle's exterior surface.

3.5. Rehydration of dried films

Although the addition of aqueous solutions of bupivacaine to DNA results in complex formation, we have also demonstrated that bupivacaine alone can form vesicular liposome-like structures when hydrated from dried films. When observed by scanning electron microscopy, these structures (Fig. 6A) appear to be similar to those vesicles prepared from films of cationic lipid [11]. These vesicles assume collapsed and condensed structures when mixed with DNA (Fig. 6B). The results indicate that preformed bupivacaine vesicles can also interact with DNA, similar to complexes formed with other cationic lipids.

3.6. Controlled assembly

In order to control the rate of assembly and the size of the resultant particles, the steps of complexation must be ordered. This can potentially be achieved by slowing the reaction rate. This is expected to allow stabilization of the primary reactions prior to the development of competing secondary and tertiary reactions that consume one or both of the reactants unproductively. We have controlled the rate of bupivacaine and DNA interaction by slowing

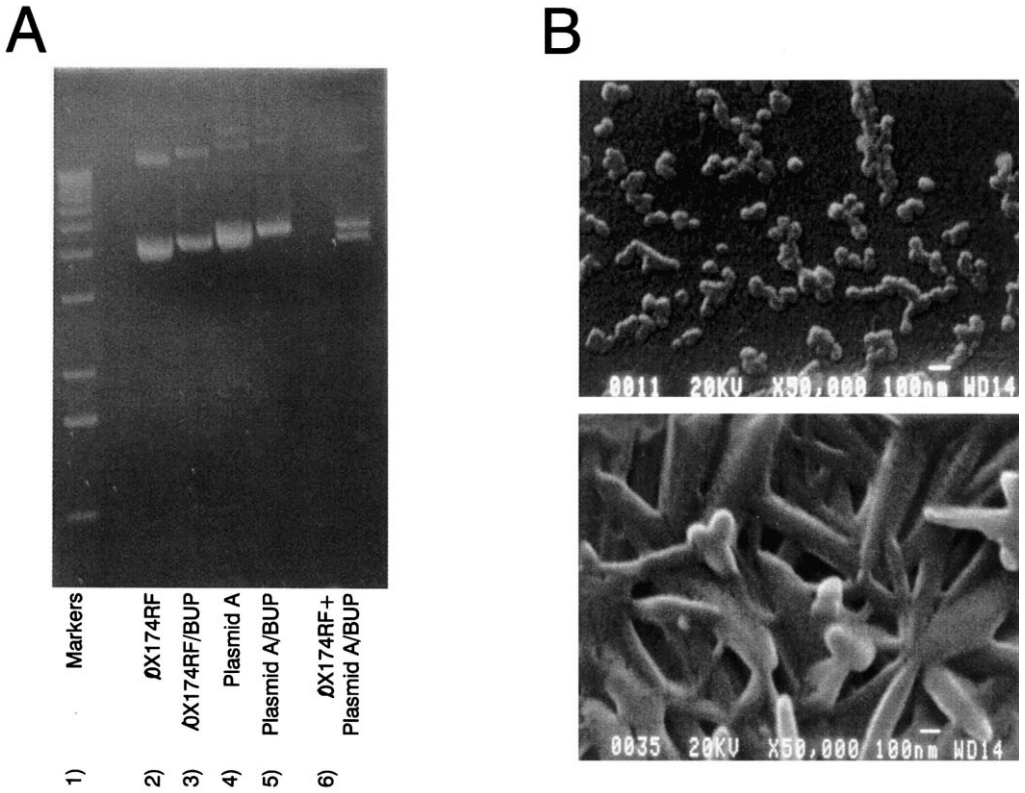

Fig. 7. Generation of unimolecular and multimolecular complexes by controlled assembly. (A) Complexes formed by slow mixing are unimolecular at low DNA concentrations. DNA alone or DNA complexed with bupivacaine was subjected to agarose gel electrophoresis. Lane 1, 1 kbp ladder (Gibco-BRL); lane 2, X174RF DNA; lane 3, X174RF DNA complexed with bupivacaine; lane 4, plasmid A DNA; lane 5, plasmid A DNA complexed with bupivacaine; and lane 6, X174RF DNA and plasmid A DNA complexed with bupivacaine. (B) Scanning electron micrograph of complexes made by slow mixing and at DNA concentrations of 10 ug/ml (top) and transmission electron micrograph of complexes made by slow mixing and at DNA concentrations of 10 ug/ml or 800 ug/ml.

the rate of mixing. Slow mixing was achieved by dialyzing bupivacaine across a 10 kDa membrane, into a solution of DNA. At low DNA concentrations (10–100 ug/ml) (+:−::264:1)–(+:−::26.4:1), unimolecular complexes are made that contain a singular DNA molecule (Fig. 6). At low DNA concentrations (100 ug/ml), complexes made with either supercoiled ox174 DNA (a 2 kbp DNA) or a supercoiled 8 kbp plasmid DNA (plasmid A), demonstrate distinctly different gel shifted bands with respect to each other (Fig. 7A, compare lanes 3 and 5). Multimolecular complexes (containing more than one DNA molecule) made with a mixture of the two DNA molecules are predicted to result in gel shifts that are intermediate to those exhibited in lanes 3 and 5. The absence of intermediate gel shifts (Fig. 7A, lane 6) argues against the formation of multimolecular complexes at low DNA concentrations and suggests the formation of unimolecular complexes. However, at higher DNA concentrations the intermediates of unimolecular complexes undergo tertiary interactions, resulting in larger complexes. Increasing amounts of DNA in the reaction mixture result in the formation of aggregates that are gel shifted to higher positions on the gel (data not shown). Electron micrographs are consistent with this result (Fig. 7B). At all DNA concentrations tested (10 μg–2 mg/ml), all of the DNA was found to be complexed with bupivacaine. These results suggest that DNA contributes to and is a participant in the secondary interactions. However, the secondary and tertiary interactions and the nature of the complexes are expected to vary, and are expected to be dependent upon both the DNA and bupivacaine concentrations.

4. Summary

Bupivacaine, a cationic amphiphile, at $pH < pK_a$, interacts with DNA to form stable complexes. These complexes differ from those described for other cationic lipid:DNA complexes in that they are homogeneous in size and have a negative zeta potential. Furthermore, unlike other cationic lipid mixtures of DNA, these complexes can be made by directly mixing aqueous solutions. In addition, intramuscular immunization with bupivacaine:DNA complexes results in immune responses that are greater than those incurred with 'naked DNA'. In general, complexes made with other cationic lipids do not result in these responses. In one report, however, the use of dehydration–rehydration vesicles (DRVs) comprised of DNA and cationic lipids has been shown to enhance immune responses [12]. DRV particles are smaller and more uniform in size than the previously described cationic lipid:DNA complexes. The smaller size of the DRV particles may be a critical parameter for their ability to generate enhanced immune responses. It will be interesting to determine which biophysical parameters of bupivacaine:DNA complexes are critical for in vivo transfection and subsequent generation of immune responses.

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