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Characterization of a pH-sensitive surfactant, dodecyl-2-(1′-imidazolyl) propionate (DIP), and preliminary studies in liposome mediated gene transfer

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Abstract

The inefficiency of non-viral gene delivery systems, relative to viral systems, is likely due, in part, to the failure of endosomes to release DNA before reaching degradative lysosomes. A solution is to incorporate compounds in a delivery vector that will selectively increase the release of endosomally encapsulated DNA. To meet the above criteria, we designed, synthesized, and characterized the physicochemical and biological properties of such a compound, dodecyl-2-(1′-imidazolyl) propionate (DIP) to enhance cationic liposome mediated gene delivery. Several surface active techniques were used to characterize DIP lysing membranes. The critical micelle concentration of DIP was between 0.10–0.18 mM and the effective release and solubilization ratios were 1.0 and 4.0, respectively. DIP facilitated membrane disruption in both a pH and concentration dependent manner. In the presence of esterase at pH 7.0, the hydrolysis rate increased 32-fold indicating DIP can be degraded in the biological milieu. Toxicity of DIP by MTT assay in the SKnSH cell line demonstrated an ID_{50} of 1.2 mM, which is 30-fold higher than the concentration of DIP used to enhance gene transfection. When incorporated into cationic-liposomes, DIP enhanced transgene expression in vitro by 5-fold. The results of the study indicate that DIP may be a useful adjuvant to increase non-viral gene delivery to cells. © 1998 Elsevier Science B.V.

Keywords: Surfactant; Liposome; pH-sensitivity; Endocytosis; Gene delivery

1. Introduction

The therapeutic promise of using nucleic acids such as plasmid DNA (pDNA) and oligodeoxynuceotides as drugs is attractive. Several technical problems including stability and delivery must be overcome. An impediment to the therapeutic use of nucleic acids is their inefficient transfer to the cytoplasm and nucleus.

A number of strategies have been employed to facilitate cell transfection with pDNA. The ideas behind these strategies are either to increase the amount of pDNA that associates with cells or to

Abbreviations: BPS(s), biodegradable pH-sensitive surfactant(s); CMC, critical micelle concentration; CTAB, hexadecyltrimethylammonium bromide; DIP, dodecyl 2-(1′-imidazolyl) propionate; DMPG, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; DOPE, dioleoylphosphatidyl ethanolamine; DOSPA, 2,3 dioleyloxy-*N*-[(spermine carboxamino) ethyl]-*N*,*N*-dimethyl-1-propanaminium; DOTAP, dioleoyltrimethyl phosphatidyl phosphate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; pDNA, plasmid DNA; R, molar ratio of DIP/total lipid; Re, effective release ratio; SDS, sodium dodecyl sulfate; Se, effective solubilization ratio

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increase the amount of nucleic acids that reach the cytoplasm by transiently disrupting endosomal membranes. The most common non-viral vectors used in gene delivery are cationic-liposomes [1]. In addition to protecting pDNA from enzymatic degradation [2], cationic-liposomes offer the potential to target genes via attached ligands. Other nucleic acid carriers include cationic polymers such as poly-lysine [3] and polyamido starburst dendrimers [4]. Systems which have been shown to disrupt cellular membranes include virosomes [5,6], pH-sensitive liposomes [7], viral peptides [8] and α-helical forming peptides [9,10]. However, each method has its disadvantages. pH-sensitive, anionic liposomes have low capacity to entrap pDNA since both have a negative charge. Cationic polymers and cationic-liposomes eventually become toxic to cells as their concentrations are increased more so than neutral counterparts. Fusogenic peptides are expensive to produce and pose the problem of immunogenicity on repeat administration.

Although nucleic acid uptake by cultured cells is inefficient [11], the use of cationic-liposomes has been shown to increase cellular nucleic acid delivery [12]. Evidence exists that pDNA/cationic-liposome complexes enter the cell via endocytosis in vitro [13] and initially accumulate in endosomes, with a majority of the pDNA later being degraded by enzymes within lysosomes. Because nucleic acids must first escape the endocytotic pathway to have an effect in the nucleus or cytoplasm, the endosomal membrane presents an additional barrier to an efficient delivery of nucleic acids to their subcellular targets. A manner to increase the amount of pDNA reaching the cytoplasm is by increasing the amount escaping from the endocytotic pathway.

The use of detergents to disrupt phospholipid bilayers [14] is efficient, but most detergents are indiscriminate of membrane type and attack the first cellular membrane they contact. One class of detergents which may offer some degree of site specific membrane disruption are the lysosomotropic detergents. This family of detergents was first described by Firestone et al. [15] and exhibited the ability to concentrate within intracellular acidic compartments and cause membrane rupture. Lysosomotropic detergents are particular lipophilic amines with pK_a values between 5 and 9, becoming protonated at acidic intralysosomal pH [15,16]. Upon protonation the surface activity of the lipophilic amine is enhanced leading to endosomal membrane destabilization. Early attempts at lysing late-stage endosomes used pH-sensitive lysosomotropic detergents [17]. At neutral pH in the cytosol or intercellular fluid, a largely unionized lysosomotropic amine bearing a hydrocarbon chain has less surface active properties as compared to the ionized species. Upon ionization within an endosome due to a pH gradient, the surface active properties of the substance increase with its hydrophobic tail buried in the hydrocarbon zone of the lipid bilayer and its hydrophillic protonated head group facing the aqueous interior of the lysosome. The surfactant then leads to the formation of mixed micelles within the endosomal membrane. Although later abandoned due to problems with nonspecific lysosomal cellular destruction, the theory behind this approach has provided the basis for the development of biodegradable pH-sensitive surfactants (BPSs).

BPSs are similar to the originally described lysosomotropic detergents in that they become ionized at acidic pH. However, the novelty of the BPS delivery system stems from the biodegradability of the surfactant and the exploitation of a naturally occurring transport mechanism with a pH gradient. By developing BPSs which can be protonated at the endosome (early lysosome) stage, they may be able to disrupt these endosomal membranes and liberate endosomal contents such as pDNA. Unlike the first described lysosomotropic detergents, BPSs can be easily degraded because of the introduction cleavable connector within the structure. Depending on the chemical characteristics of the connector, metabolism can occur by a variety of mechanisms.

In this report, we have characterized one member of the BPS family, dodecyl 2-(1-imidazolyl) propionate (DIP), by measuring its surface active properties, cellular toxicity and stability. Using a model tissue culture system, we also investigated the use of DIP to enhance cationic-liposome mediated gene delivery.

2. Material and methods

2.1. Preparation of reagents

Calcein, rhodamine-6-G, ferric chloride and ammonium thiocyanate were purchased from Aldrich

(Milwaukee, WI). L-α-lecithin, dioleoylphosphatidyl ethanolamine (DOPE), dioleoyltrimethyl phosphatidyl phosphate (DOTAP) and 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPG) were purchased from Avanti Polar Lipids (Alabaster, AL). Porcine esterase (300 U/mg protein) and cholesterol were purchased from Sigma (St. Louis, MO). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Amresco (Solon, OH). The SKnSH (HTB-11) cell line was purchased from ATCC (Rockville, MD).

DIP was synthesized as previously reported [18], purified through flash chromatography and its structure confirmed through ^{1}H-NMR, mass spectroscopy and elemental analysis. Ammonium ferrothiocyanate (0.1 M) was prepared by dissolving 8.1 g ferric chloride and 15.2 g ammonium thiocyanate in 500 ml of distilled water.

All pH buffers were adjusted with NaCl to 0.5 ionic strength. The pH of the buffers (and their chemical compositions) used were as follows: pH 1.4 (420 mM KCl and 80 mM HCl), pH 2.8 (123 mM citric acid and 60 mM NaOH), pH 3.0 (120 mM citric acid and 60 mM NaOH), pH 4.3 (150 mM sodium acetate and 350 mM glacial acetic acid), pH 5.0 (300 mM KH_2PO_4 and 50 mM Na_2HPO_4), pH 5.5 (300 mM KH_2PO_4 and 56 mM Na_2HPO_4), pH 6.0 (150 mM KH_2PO_4 and 100 mM Na_2HPO_4), pH 6.5 (240 mM KH_2PO_4 and 56 mM Na_2HPO_4), pH 7.0 (80 mM KH_2PO_4 and 120 mM Na_2HPO_4), pH 7.5 (70 mM KH_2PO_4 and 150 mM Na_2HPO_4) and pH 8.0 (200 mM KH_2PO_4 and 188 mM NaOH).

Liposomes (L-α-lecithin: DMPG: cholesterol; weight ratio 5:1:4) were used as a model membrane system. In this case, the reverse-phase evaporation vesicle method [19] was used to produce unilamellar vesicles. Liposomes (600 nm) were produced with polycarbonate membranes (Poretics; Livermore, CA) through a high pressure extruder (Lipex Biomembrane; Vancouver, Canada). Calcein (100 mM) was entrapped within the liposomes as a fluorescent marker to monitor membrane lysis events.

The concentration of phospholipid in each experiment was measured by a modification of a spectrophotometric technique [20]. Briefly, varying amounts of L-α-lecithin (0–50 μg/ml) were added to test tubes containing 2 ml of 0.1 M ammonium ferrothiocyanate and 2 ml of chloroform. The contents were mixed vigorously for 1 min and centrifuged at 6,000 rpm (Safeguard Centrifuge, Clay–Adams) for 5 min to fully separate the two phases. The aqueous phase was removed and the absorbance of the remaining organic phase was measured at 488 nm with a spectrophotometer (UV/Vis Perkin–Elmer spectrophotometer Lamda 3) to establish a calibration curve. The concentrations of unknown samples were then determined from the calibration curve.

2.2. CMC determination

To determine the critical micelle concentration (CMC) of ionized DIP, rhodamine 6-G was mixed with increasing concentrations of DIP in solution at pH 3.0 (0.001 M HCl). The solution was excited at a wavelength of 480 nm and observed at 550 nm in a Perkin–Elmer luminescence spectrophotometer LS-50B. The fluorescence intensity of the solution was plotted against concentration of ionized DIP, with 100% intensity defined as the rhodamine 6-G intensity with no surfactant added. The inflection point of the curve was reported as the CMC.

A second method to determine CMC was performed using the relationship of ionized DIP concentration and surface tension. Surface tension measurements were performed using a CRC-DuNoüy interfacial tensiometer [21]. The pH of the solution was adjusted to pH 3.0 in distilled H_2O with hydrochloric acid with a constant temperature of 22°C.

2.3. Effective release ratio (Re) and effective solubilization ratio (Se) determination

Unilamellar liposomes (600 nm; 25 mM) containing 100 mM calcein (> self-quenching concentration) were suspended in pH 4.3 acetate buffer with increasing DIP. Equilibration was allowed to occur for 30 min at room temperature. The released calcein was excited at 496 nm and observed at 517 nm at 22°C. The percentage of released calcein was calculated by the equation $I(\%) = (I_a - I_b)/(I_x - I_b) \times 100$ [22], where I_x is the 100% fluorescence intensity value when adding excess Triton X-100 (10 mM) and I_a and I_b are the fluorescence intensities after incubation with and without DIP, respectively. The effective molar ratio (Re) of surfactant to total lipid including L-α-lecithin, DMPG and cholesterol was defined as

the point when 50% of calcein was released. During this process, the surfactant must come in contact with the lipid bilayer and partition into the hydrophobic environment. A substantial portion of the effect of most detergents causing membrane lysis has taken place after 30 min incubation with liposomes [23]. Complete equilibrium between surfactants and lipids can take several hours [24]; however, after this time, detergent-induced release of liposomal contents could be masked by the concomitant spontaneous diffusion of solutes out of the vesicles. This fact has to be taken into account when using tracer molecules to study fusion or leakage events.

Turbidity measurements of a membrane preparation as a function of added detergent were analyzed in terms of percent solubilization [25]. The lipid concentration was 180 μM and the turbidity of the 600 nm liposome preparation was determined with a spectrophotometer (340 nm) in pH 4.3 acetate buffer at 22°C. The turbidity was plotted against the molar ratio of surfactant to lipid to calculate its effective solubilization ratio, Se (molar ratio of surfactant to total lipid), defined as the point at which 50% of lipids were solubilized.

2.4. pH Sensitivity of DIP and its effect on calcein release from liposomes

To determine the ability of DIP to cause membrane lysis/leakage, studies were conducted which varied either the pH or DIP concentration in a solution of calcein containing liposomes (described above). Increasing DIP concentrations (0.01–0.8 mM) were added into three phosphate buffer systems (pH 5.5, 6.5, and 7.5) containing liposomes with calcein. The suspensions were incubated for 30 min at 22°C and the percentage of calcein release was calculated by the equation $I(\%) = (I_a - I_b)/(I_x - I_b) \times 100$ as previously described [22].

2.5. Membrane lysis profile of DIP when incorporated into liposomes

Liposomes were prepared with calcein and different molar ratios of DIP. The various DIP-liposome preparations were incubated with phosphate buffers (pH 5–8) for 30 min to determine the release characteristics. The residual fluorescence of the liposomes at pH 8.0 was considered to be 0% and 100% release was set by lysing the liposomes with Triton X-100 at each pH [26].

2.6. Chemical and biological stability of DIP

The aqueous stability of DIP was determined by incubating different concentrations of DIP in pH buffers (pH 2–8) with 5% DMSO (dimethyl sulfoxide) as a co-solvent at 37°C. Periodic samples were removed and DIP concentration was quantified using an HPLC method. The HPLC system consisted of a Milton Roy CM 4000 pump, an LDC Analytical 3200 absorbance detector, a Hewlett Packard 3395 integrator and a Spectra Physics SP 8780 autosampler. A 3.9 × 75 mm C8 column (Nova-Pak) along with a mobile phase consisting of 55% acetonitrile and 45% 10 mM pH 8.0 Na_2HPO_4 solution was used to separate and determine intact DIP from its degradation products at 210 nm. The flow rate was set at 1.0 ml/min. The degradation rate constants were plotted against pH to create the pH hydrolysis profile of DIP.

To determine the hydrolysis rate of DIP in biological systems, a porcine esterase was used to hydrolyze the ester linkage. Varying concentrations of DIP in pH 7.0 phosphate buffer solution with 5% DMSO were incubated at 37°C with increasing concentrations of the esterase. Aliquots were removed at periodic intervals and DIP concentration was measured with HPLC as described above. Rate constants of DIP were calculated to predict its biodegradability.

2.7. Cellular toxicity

The cellular toxicity of DIP was monitored in a human neuroblastoma cell line, SKnSH, using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [27]. Briefly, subconfluent monolayered cultures were incubated in a 96-well plate (10^4 cells/well) with 200 μl of RPMI 1640 growth medium (100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum) at 37°C, 5% CO_2 and 100% humidity environment for three days. The growth medium was removed and DIP added from 100 ng/ml to 10 mg/ml in 200 μl fresh growth medium. The cells were maintained for an additional 48 h. The incubation medium was removed, cells were fed with 200 μl of fresh medium

at the end of the growth period and 50 μg of MTT was added to each well. The plates were wrapped with aluminum foil and incubated for 6 h at 37°C. After the incubation, the medium was removed and the remaining MTT-formazan crystals were dissolved in 200 μl of DMSO. Glycine buffer (25 μl, pH 10.5; 0.1 M glycine and 0.1 M NaCl) was added to all wells, the plates shaken for 2 min and the absorbance at 490 nm was obtained immediately. The ID_{50} was defined as the drug concentration required to reduce by 50% the absorbance of the MTT-formazan crystals, indicating 50% cell death.

2.8. Ability of DIP to facilitate cationic lipid mediated gene transfer

Cationic-liposomes were prepared from DOTAP and DOPE with or without DIP. All liposomes were prepared by hydration of a dried lipid film with sterile water [12]. The liposome suspensions were sonicated with a probe sonicator until clear. Two liposome formulations were tested: DOTAP/DOPE (1:1, molar ratio) and DOTAP/DOPE/DIP (1:1:1, molar ratio). The cationic lipids (15 μg of DOTAP) were mixed with 3 μg of pDNA (pGL3, Promega) which had been isolated using a Megaprep Kit (Promega). The pGL3 plasmid codes for the production of a truncated cytoplasmic compartmentalized luciferase under the control of a SV40 promoter and enhancer. The lipid/plasmid suspension was added to SKnSH cells (2×10^5 cells/well) in 500 μl serum-free medium (RPMI 1640). After 5 h, the medium was removed and replaced with medium containing 10% fetal bovine serum (1 ml) and the cells were incubated for an additional 24 h. The cells were lysed and the luciferase enzyme expression measured by luminescence as described [28]. Luciferase content was corrected by total cellular protein using a BCA (Pierce) protein assay.

3. Results

3.1. CMC determination of DIP

An important parameter in characterizing surfactants is the concentration at which micelles form. All experiments were conducted at pH 3.0 to ensure that DIP was in an ionized state (> 99.9%). Rhodamine 6-G is a fluorophore that has been shown to be useful in determining the CMC of cationic surfactants [29] such as ionized DIP. We measured the decrease of fluorescence intensity in an ionized DIP solution as the fluorophore (rhodamine 6-G) was quenched within micelles. As the ionized DIP concentration increased, fluorescent intensity showed a sharp decrease indicating micelle formation.

An independent measurement of CMC using surface tension measurements of ionized DIP in aqueous solution corroborated the results from the fluorescence studies. As DIP concentration increased, the surface tension of the solution sharply decreased until the formation of micelles occurred. From these two independent experiments, we determined the CMC of DIP to be 0.10–0.18 mM (Table 1).

3.2. Effective release ratio (Re) and effective solubilization ratio (Se) determination of DIP

While the CMC value is useful to describe surfactants, it does not necessarily describe how a surfactant may release materials from lipid vesicles [23,30]. Re describes the molar ratio of a surfactant to total lipid required to release the liposomal contents to the outer environment. The Re was determined by fitting a curve of calcein release from liposomes at increasing molar ratios of DIP to lipid. A computer program, Scientist (Micromath; Salt Lake City, Utah), was used to fit a curve to the data. The Re was then determined to be 1.0 (Fig. 1).

Table 1
The CMCs of different surfactants and ionized DIP determined through rhodamine 6-G or through CRC-DuNoüy interfacial tensiometer

Compound	Literature CMC (mM)	CMC through tensiometer (mM)	CMC through rhodamine 6-G (mM)
Triton X-100	0.2–0.4	0.15–0.3	0.2–0.5
SDS	8.2	7.5–8.5	N/A[a]
CTAB	0.7–0.92	N/A[a]	0.6–1.0
Ionized DIP	N/A[a]	0.1–0.18	0.1–0.25

[a] Not applicable.

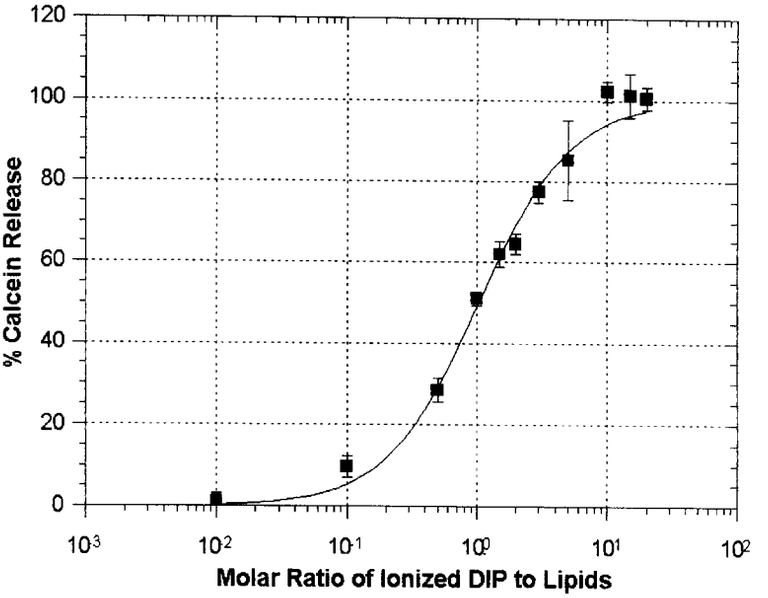

Fig. 1. The ability of DIP to induce calcein release at increasing molar ratios of DIP to lipid when incubated in a pH 3.0 citrate buffer for 30 min ($n = 3$). The effective release ratio was determined to be 1.04 ± 0.09 (mean $\pm$ SD).

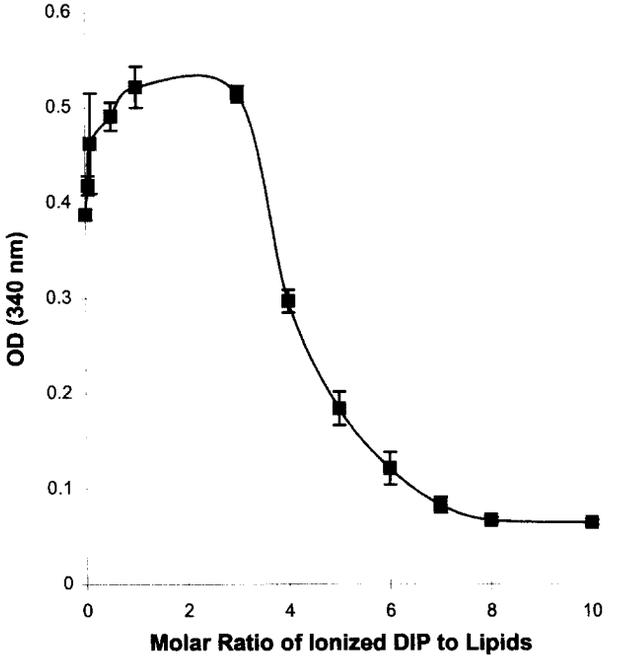

Fig. 2. The effective solubilization ratio determined by turbidity analysis ($n = 3$). Liposomes were incubated with increasing DIP in a pH 3.0 citrate buffer while the optical densities (mean $\pm$ SD) were recorded. The effective solubilization ratio (Se) was determined to be approximately 4.0.

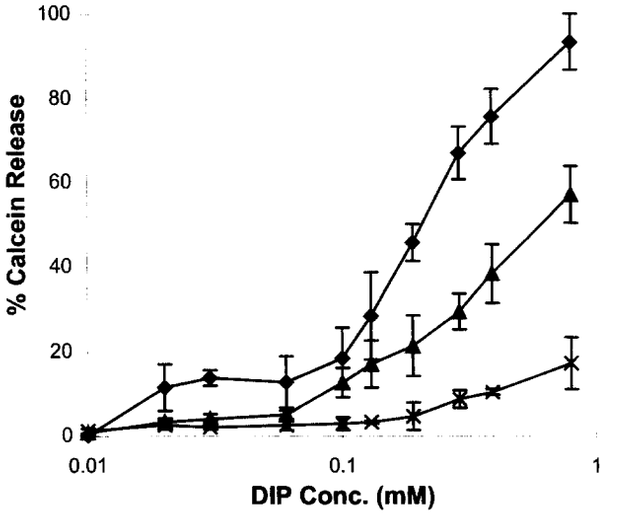

Fig. 3. The percent (mean $\pm$ SD) DIP-induced calcein release from liposomes after 30 min in three phosphate buffer solutions: pH 5.5 (◆), 6.5 (▲), and 7.5 (×) ($n = 3$). Significant differences ($p < 0.01$; ANOVA) were observed at all pH groups after DIP reached 0.19 mM.

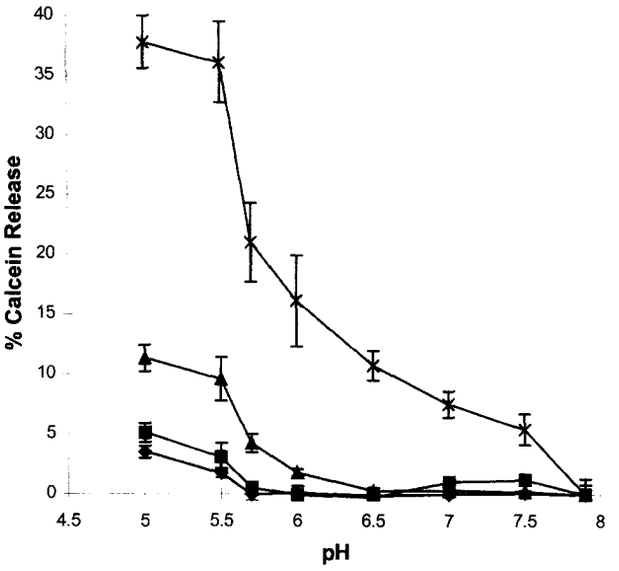

Fig. 4. The effects of pH and DIP/liposome ratios ($R = 0$ (◆), 0.1 (■), 0.2 (▲), and 0.4 (×)) on DIP-induced calcein release (mean ± SD) from liposomes after 30 min ($n = 3$). There were significant differences ($p < 0.01$; ANOVA) among $R = 0.1$, $R = 0.2$ and $R = 0.4$ groups.

Se describes the concentration of a surfactant required to solubilize a given amount of lipid. To determine Se, we measured the turbidity of a DIP/liposome suspension. Complete solubilization was defined as the point at which all membranes were transformed into mixed micelles yielding a transparent solution. The turbidity of the liposome suspension initially increased with the addition of DIP, indicating an interaction with liposomes causing the transition from bilayers to mixed micelles. Further addition of DIP resulted in a decrease of turbidity until complete solubilization was obtained. After this point, additional detergent had only a slight effect on the dispersion's turbidity. The Se of the system was determined to be approximately 4 (Fig. 2).

3.3. pH Sensitivity of liposomal-calcein release by DIP

To determine whether DIP becomes effective in acidic environments but has limited effect at extracellular biological pH, calcein-containing liposomes were incubated with increasing amounts of DIP at three pHs (5.5, 6.5, and 7.5). An increase in fluorescence intensity indicates membrane lysis and calcein release. Calcein was released sigmoidally at pH 5.5 as DIP concentration increased (Fig. 3). However, with the lower surface active properties of DIP at pH 7.5, calcein release was slightly increased at higher DIP concentration. This release was most likely due to saturation of the space between the lipid bilayers with increasing amount of DIP. Since distribution between the aqueous environment and lipid bilayer must occur for DIP to elicit membrane lysis, no significant differences (ANOVA) between calcein re-

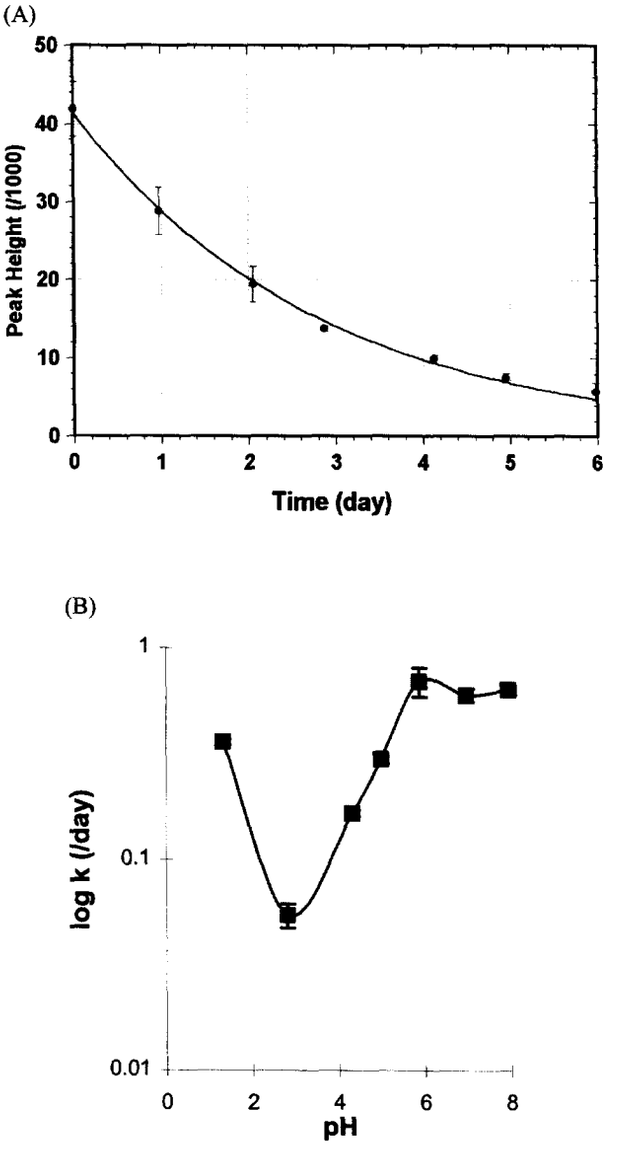

Fig. 5. (A) The chemical degradation profile of DIP at pH 1.4 and 37°C over time ($n = 3$). (B) The overall pH rate profile of DIP at 37°C ($n = 3$). Data are expressed as mean ± SD.

lease and pH were observed until 0.19 mM was reached ($p < 0.01$).

3.4. Lysis profile of DIP when incorporated into liposomes

We determined the ability of unionized DIP incorporated into liposomes to be protonated at lower pHs,

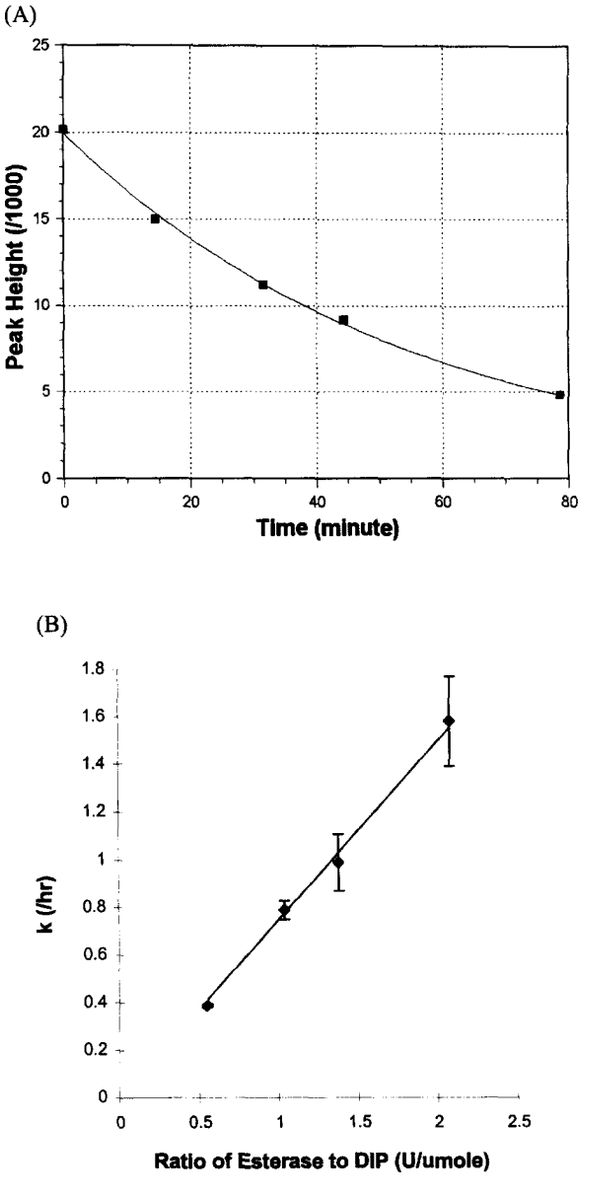

Fig. 6. (A) Biological degradation profile of 0.65 μmole of DIP when incubated with 0.9 U of esterase at pH 7.0 and 37°C. (B) The linear relationship between the degradation rate constant (mean ± SD) of DIP and the ratio of esterase to DIP (U/μmole) at 37°C ($n = 4$, $R^2 = 0.99$).

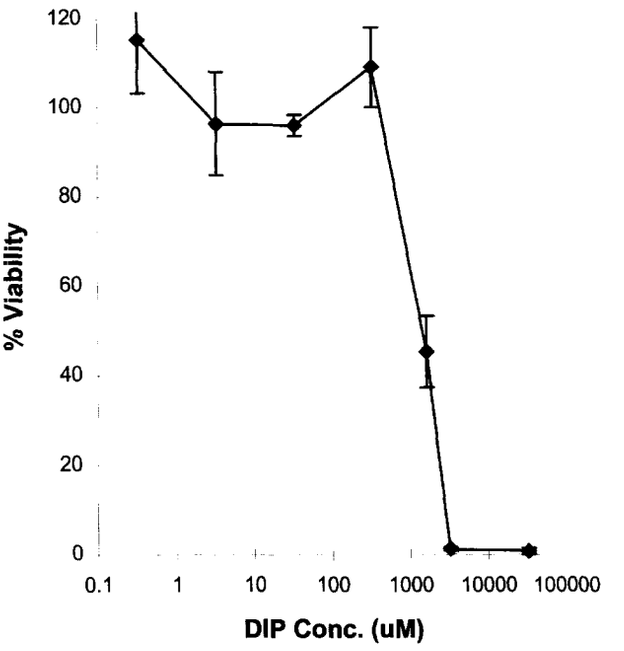

Fig. 7. The effect of DIP concentration on cell (SKnSH) survival measured by MTT assay after 48 h. The ID_{50} of DIP was determined to be 1.2 mM ($n = 4$). Data are expressed as mean ± SD.

facilitating the release of entrapped materials. Liposomes containing calcein were prepared with increasing ratios of DIP and incubated at decreasing pHs. Minimal calcein release was observed at the lower DIP/liposome molar ratio group (0.1) but at the 0.4 ratio group calcein release increased sigmoidally (Fig. 4). As pH decreased, calcein release increased in all groups. After the pH dropped to 6.0, significant differences (ANOVA; $p < 0.01$) were observed among the ratio groups 0.4, 0.2 and 0.1. Compared to other groups, the system at ratio 0.4 is unstable at physiological pH probably due to the alternations in lipid packing.

3.5. Stability test and toxicity screen of DIP

To be useful in facilitating pDNA transfer, an ideal surfactant must be degraded in the intercellular milieu, thus limiting potential toxicity. DIP should be degraded by ester hydrolysis either chemically or enzymatically. The hydrolytic stability of DIP was assessed by incubating the compound in pH buffers and monitoring the concentration of the starting material remaining intact. Using the program Scientist to

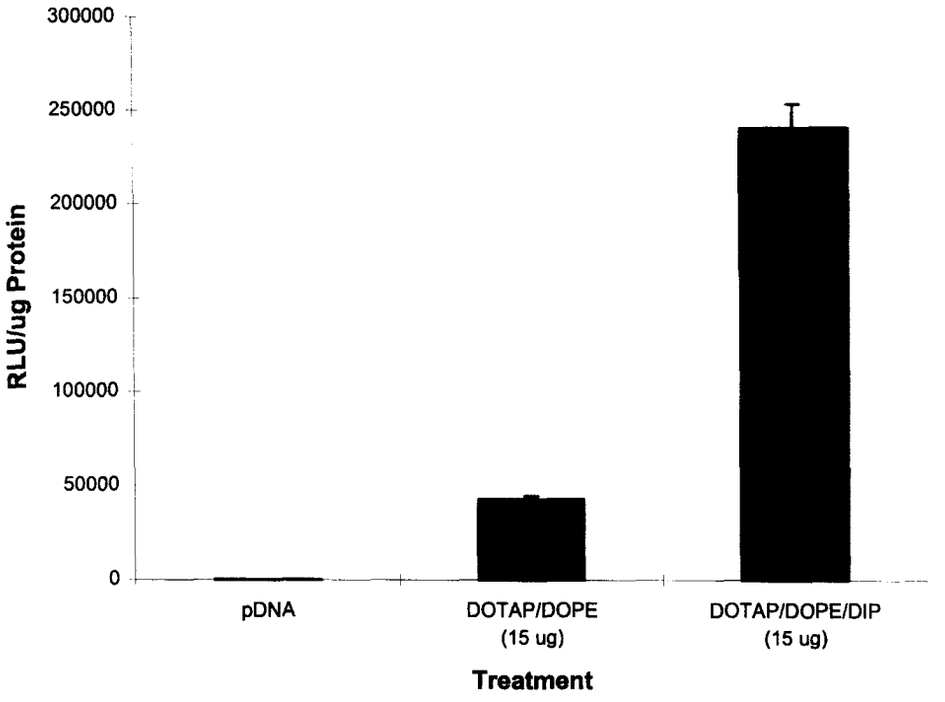

Fig. 8. In vitro liposome mediated gene transfection of SKnSH cells with the pGL3 plasmid in the presence and absence of DIP ($n = 3$). A 5-fold increase in transgene expression was observed when adding DIP to a DOTAP:DOPE formulation (1:1:1 molar ratio). Significant difference was observed between DOTAP/DOPE and DOTAP/DOPE/DIP ($p < 0.01$, ANOVA). Data are expressed as mean ± SD.

fit the degradation curve, we were able to determine the pH dependent pseudo-first order degradation rate constants (k). DIP is at its most stable state ($k = 0.055$ per day) at pH 2.8. The degradation rate constant reached a plateau after pH 5.9 ($k = 0.70$ per day) (Fig. 5(B)).

To assess the enzymatic stability of DIP, we used porcine esterase as a model enzyme. Fig. 6(A) illustrates the biological degradation of DIP when the ratio of DIP to esterase equals 1.4 U/μmole. By comparing the chemical degradation rate constant and biological degradation rate constant, we found that the biological degradation of DIP at pH 7.0 was approximately 32 times faster than that of DIP in buffer solution (1.0 U/μmole), demonstrating the biodegradability of DIP (Figs. 5 and 6).

A more important parameter than biodegradability of DIP is cellular toxicity. A commonly used MTT assay was used to measure the cellular toxicity of DIP. The number of viable cells (measured indirectly by the absorbance of MTT byproduct) started to drop at 100 μg/ml and stopped after 1000 μg/ml (Fig. 7). ID_{50} was then determined at 380 μg/ml (1.2 mM).

3.6. Ability of DIP to facilitate gene transfer

A 5-fold increase in gene transfection over the control cationic-liposome DOTAP/DOPE was achieved by the incorporation of DIP into the lipid system, demonstrating the ability of DIP to increase gene transfer (Fig. 8). This experiment illustrates the efficacy of DIP use in the SKnSH cell line. It should be noted with each particular cationic lipid mixture and each cell line the transfection protocol must be optimized. In this experiment a commonly used ratio of cationic lipid to pDNA (1:5 w/w) was chosen for the transfection protocol. Other results might be expected with a different transfection protocols or with an alternative cell line.

4. Discussion

Gene therapy is a promising approach for the treatment of a variety of disorders. While several approaches are available for cellular delivery of DNA,

the non-viral cationic-liposome approach is attractive due to the ease of production of liposomes, the ability of a non-viral system to transfect a variety of cell types, and the lack of immune toxicity. While non-viral systems are currently somewhat inefficient, they will undoubtedly improve with the production of new non-viral delivery systems and the determination of rate limiting mechanisms which govern non-viral gene delivery. In this report, we investigated the escape of DNA from endosomes as a potential limiting step in cationic-liposome-mediated delivery of genes. The underlying objective was to evaluate the usefulness of an endosome disrupting compound to increase cytoplasmic concentrations of pDNA.

Imidizolyl based lipids have been used successfully for in vitro delivery of pDNA [31] establishing a rational for the use of imidazole based vectors. DIP is an imidazolyl based surfactant in the BPS family which is proposed to facilitate the transport of pDNA through the endosomal pathway. BPSs take advantage of the acidic environment within endosomes to protonate a lysosomotropic amine thus increasing its surface active properties. After the BPS becomes ionized, it can assist the destabilization of the endosomal membrane. To lessen adverse effects of the ionized BPS, an ester bond was introduced into DIP's structure making it biodegradable. DIP, has recently been shown to reduce the concentration of oligonucleotides required to produce a biological effect [18] using a tissue culture system. In this report, DIP was further characterized and evaluated for its usefulness in non-viral gene delivery.

For basic amines such as DIP, the intrinsic ionization constant and the local pH environment will determine the percent ionized. The reported pK_a of DIP is 6.8 [18]. When the pH drops to endosomal pH, approximately 6.0–5.0 [32], up to 98% of DIP can be ionized. In this pH range, DIP exhibits increased surface active properties and could presumably lyse the endosomal membrane before it matures into a lysosome. At physiological pH, unionized DIP resides within lipid bilayer. When pH declines, DIP will be protonated and form mixed bilayers with endosomal lipids. When the amount of ionized DIP increases, vesicles will be humpbacked with DIP at regions of high curvature and eventually the mixed micelles will be observed [33]. Throughout the transition from lipid vesicles, mixed bilayer sheets to mixed micelles, pDNA may be released. If passive diffusion is the cellular entry pathway of DIP, the larger pH gradient in the lysosomes would force accumulation in these intracellular compartments, similar to the lysosomal detergent, dodecyl imidazole. Lysosome rupture would then lead to severe toxic effects. In this report, DIP was incorporated into liposomes to force the compound to enter via endocytosis, allowing DIP to encounter the early endosomes before reaching the later stage lysosomes.

The number of possible analogs of the BPS family are immense. In order to determine what physicochemical parameters influence the biological activity of BPSs, we established a series of evaluation tests associated with surfactants. Presently, it is not clear which measured parameter would be useful in the characterization of BPS for gene delivery. The currently established methods have been optimized for studying small molecule transport instead of macromolecules such as pDNA. In later presentations, structure–activity relationships will be established using analogs of DIP and the various parameters (CMC, Re, Se, etc.) will be used to correlate transfection efficiency.

The first parameter determined for DIP was the CMC. While the CMC is a useful parameter in describing surfactants (i.e. hydrophilicity, surface excess), it may not be the best parameter to measure the ability of a surfactant to cause membrane lysis. Therefore, the effective Re and Se were utilized to describe the ability of DIP to lyse membranes. The lower the Se and Re, the less surfactant is required to lyse membranes. The amount of DIP required to reach Se was approximately four times higher than that required to reach Re which is similar to other surfactants [23]. For example, the Re and Se of Triton X-100 are 0.3 and 1.7 and the Re and Se of SDS are 1.2 and 2.5 [23] while for DIP the values are 1.0 and 4.0, respectively. A compound that increases the release rate of an entrapped compound without causing indiscriminate membrane lysis should have reduced toxicity. At present, the values of CMC, Re, and Se for DIP have little biological relevance. As other members of the BPS class are characterized within these parameters, it is expected that particular physicochemical properties will be predictive of biological activity.

The ability of DIP to release liposomally entrapped

molecules was tested as a proof of the principle experiment of the compound's pH sensitivity. As the DIP/liposome molar ratio was increased and pH decreased, more calcein was released from the liposome model system. Calcein release induced by DIP when it was incorporated into the liposome system was slightly higher than that caused by DIP when it was added to a solution containing calcein liposomes. The greater release could be due to the dilution of DIP or may be related to partitioning barriers. This set of experiments demonstrated the ability of DIP to facilitate liposomal release of membrane entrapped materials and may be indicative of events occurring within cellular endosomes.

After releasing of membrane entrapped molecules in a pH-sensitive manner, the biodegradability of DIP had to be confirmed. In the first experiments, the hydrolytic stability of the compound was tested over a range of pHs. DIP was stable to hydrolytic cleavage at physiological pH with a degradation half life of 27 h. The half life of DIP was 55 min in the presence of porcine esterase at a ratio of 1.0 U (esterase)/μmole (DIP) at pH 7.0. The degradation rate of DIP in vivo would be expected to be greater due to the greater number of esterase (e.g. lipase) molecules which are available for DIP metabolism after DIP's release from the liposomal membrane.

A major concern with the use of agents to enhance non-viral gene delivery is that any compound added to a delivery vector might contribute to the toxicity of the system. The toxicity of a given compound is often related to its stability. The biodegradability of DIP may decrease its cellular toxicity. In the MTT screen, the addition of an ester group to the surfactant resulted in a less toxic effect as compared to *N*-dodecyl imidazole, a first generation lysosomotropic detergent [16], by approximately 30-fold. While this study implies that ester containing imidazole based surfactants are less toxic than their straight chain analogs, it is unclear how the toxicity of DIP alone will compare to cationic-liposome mediated delivery. When DIP was incorporated into anionic liposomes, the toxicity was two times higher than that caused by DIP only (data not presented). The toxicity is most likely related to the total amount of DIP delivered to the cell which is increased by the use of liposomes.

In the final set of experiments, the inclusion of DIP into cationic-liposomes increased the amount of transgene expressed using an in vitro tissue culture model. The increase in luciferase expressed might be an accumulation of multiple effects of DIP acting at the membrane level through surface activity and/or serving as a pro-fusogen. The ability of DIP to increase cell transfection has also been demonstrated in primary rat glial cells and transformed human embryonal kidney cells. DIP also has been shown to be effective when used with DOSPA cationic lipid gene delivery systems (data not shown). Interestingly, when DOTAP/DIP (1:1) lipid particles were made, they were as effective in gene transfection as DOTAP/DOPE liposomes. This feature implies that DIP bears fusogenic properties within the cell. It should be pointed out that the increase in endosomal release of pDNA is not the only mechanism which could lead to increased transfection. Other explanations include the possibility that DIP may increase either the association of pDNA with cationic lipid complexes or increase the amount of total lipid–DNA complex which is transported within the cell. Future studies will delineate the mechanism of increased transgene expression. While only preliminary experiments have been conducted with DIP containing cationic-liposomes, these early results appear promising and additional studies are required to understand usefulness in non-viral gene delivery.

The inclusion of pH-sensitive surfactants within non-viral lipid delivery system is another method for increasing transgene expression. It is assumed that the increased efficacy is due to greater transfer of pDNA from the endosome to the cytosol. In this report, we present a starting point to understand the role of DIP in membrane fusion and gene delivery.

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