

# New multivalent cationic lipids reveal bell curve for transfection efficiency versus membrane charge density: lipid–DNA complexes for gene delivery

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## Abstract

**Background** Gene carriers based on lipids or polymers – rather than on engineered viruses – constitute the latest technique for delivering genes into cells for gene therapy. Cationic liposome–DNA (CL–DNA) complexes have emerged as leading nonviral vectors in worldwide gene therapy clinical trials. To arrive at therapeutic dosages, however, their efficiency requires substantial further improvement.

**Methods** Newly synthesized multivalent lipids (MVLs) enable control of headgroup charge and size. Complexes comprised of MVLs and DNA have been characterized by X-ray diffraction and ethidium bromide displacement assays. Their transfection efficiency (TE) in L-cells was measured with a luciferase assay.

**Results** Plots of TE versus the membrane charge density ( $\sigma_M$ , average charge/unit area of membrane) for the MVLs and monovalent 2,3-dioleyloxypropyltrimethylammonium chloride (DOTAP) merge onto a universal, bell-shaped curve. This bell curve leads to the identification of three distinct regimes, related to interactions between complexes and cells: at low  $\sigma_M$ , TE increases with increasing  $\sigma_M$ ; at intermediate  $\sigma_M$ , TE exhibits saturated behavior; and unexpectedly, at high  $\sigma_M$ , TE decreases with increasing  $\sigma_M$ .

**Conclusions** Complexes with low  $\sigma_M$  remain trapped in the endosome. In the high  $\sigma_M$  regime, accessible for the first time with the new MVLs, complexes escape by overcoming a kinetic barrier to fusion with the endosomal membrane (activated fusion), yet they exhibit a reduced level of efficiency, presumably due to the inability of the DNA to dissociate from the highly charged membranes in the cytosol. The intermediate, optimal regime reflects a compromise between the opposing demands on  $\sigma_M$  for endosomal escape and dissociation in the cytosol. Copyright © 2005 John Wiley & Sons, Ltd.

**Keywords** gene therapy; cationic lipids; transfection efficiency; membrane charge density

## Introduction

Cationic liposome–DNA (CL–DNA) complexes are attracting considerable attention as gene vectors due to their safety and other inherent advantages over viral delivery methods [1,2]. These advantages

Received: 22 July 2004  
Revised: 9 September 2004  
Accepted: 20 September 2004

include ease and variability of preparation, lack of immunogenicity, and a capacity for DNA of unlimited size, allowing for delivery of human artificial chromosomes [3]. Recent setbacks in clinical trials with viral vectors, in particular a fatality induced by a severe inflammatory response [4] and insertional mutagenesis caused by retroviral vectors [5], have further promoted a diligent effort in developing efficient nonviral methods.

Currently, lipofection is a prevalent nonviral gene transfer technology used in clinical trials worldwide [6]. CLs for transfection typically consist of a mixture of cationic and neutral (helper) lipid. Numerous lipids with varied chemical and physical properties have been synthesized [7–9] to improve the transfection efficiencies of CL-DNA complexes to the level of viral vectors. These include multivalent lipids, which have been described as superior to their monovalent counterparts [10,11].

Despite this abundance of different cationic lipids, unifying themes and a comprehensive understanding of the interactions between CL-DNA complexes and mammalian cells are lacking. In order to rationally design and improve lipid-based delivery systems, however, such an understanding is essential. In particular, it is necessary to identify the interactions between the CL-DNA complexes and the cells along the transfection pathway to overcome the biological impediments to optimal transfection by directed alteration and optimization of CL-DNA complex formulations.

In part, the lack of mechanistic understanding of gene delivery by CL-DNA complexes is due to the large number of parameters involved. Few investigations to date include a complete examination of lipid performance as a function of lipid-bilayer composition and lipid/DNA charge ratio ( $\rho_{\text{chg}}$ ). Even in comparative studies [12], typically only one or two data points per lipid are evaluated, allowing the ideal lipid composition (the ratio of neutral to cationic lipid) or cationic lipid/DNA ratio to be overlooked [10,11].

In previous experiments with commercially available lipids, Lin *et al.* identified the membrane charge density,  $\sigma_M$ , as a universal parameter for transfection by lamellar CL-DNA complexes, but the scope of these investigations was limited by the lipids used [13]. The membrane charge density is the average charge per unit area of the membrane. It is controlled by the ratio of neutral to cationic lipid in the liposome formulation. On the other hand, the lipid/DNA charge ratio,  $\rho_{\text{chg}}$ , is the number of charges on the cationic lipid divided by the number of charges on the DNA. In our experiments, we keep both the amount of DNA and  $\rho_{\text{chg}}$  (and thus the number of charges contributed by the cationic lipid) constant. Thus,  $\sigma_M$  is varied solely by changing the amount of neutral lipid per transfection assay, “diluting” the cationic lipid in the membrane.

The work reported here presents both a confirmation and a significant extension of the earlier findings. We have synthesized a set of new multivalent lipids (MVLs) through methodical variation of headgroup size and charge and have examined the dependence of

transfection efficiency (TE) on two key parameters, lipid composition and lipid/DNA charge ratio,  $\rho_{\text{chg}}$ . The MVLs [14], which form lamellar DNA complexes alone and when mixed with neutral 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC), enabled us to systematically probe very high membrane charge densities for the first time. For all DNA complexes of the MVLs as well as monovalent 2,3-dioleoyloxypropyltrimethylammonium chloride (DOTAP), TE plotted versus  $\sigma_M$  fits the same, bell-shaped curve, confirming  $\sigma_M$  as a universal parameter. The curve shows three distinct regimes of TE and a clear maximum of TE at an optimal charge density,  $\sigma_M^*$ . Here, the TE rivals that of hexagonal CL-DNA complexes. The optimal  $\sigma_M^*$  of the universal curve shifts systematically with  $\rho_{\text{chg}}$ , with an increase in  $\rho_{\text{chg}}$  resulting in higher values for  $\sigma_M^*$ . Only the new, highly charged lipids investigated here have permitted unambiguous identification of the universal maximum in TE and its shift with  $\rho_{\text{chg}}$ , as well as the discovery of a third regime of TE, where TE decreases (not saturates) with increasing  $\sigma_M$ .

## Materials and methods

### Materials

The multivalent lipids (MVLs), MVL2 (molecular weight (MW) = 884.2 g/mol), MVL3 (MW = 977.8 g/mol), MVL5 (MW = 1552.7 g/mol), and TMVL5 (MW = 1253.0 g/mol) (Table 1), were synthesized according to the procedure previously described [14].  $N_\alpha, N_\delta$ -Bis(Boc)-ornithine and  $N_\delta$ -Boc-ornithine (Novabiochem) were used as the starting materials for MVL2 and MVL3, respectively. 2,2'-(Ethylenedioxy)diethylamine for TMVL5 was purchased from Aldrich. For all lipids except MVL2, the final deprotection was performed by dissolving of the protected lipid in trifluoroacetic acid (Fisher), incubating at room temperature for 30 min and drying in vacuum, in deviation from the published protocol [14]. As described below, cationic liposomes were prepared containing these lipids as well as the cationic lipid 2,3-dioleoyloxypropyltrimethylammonium chloride (DOTAP, MW = 698.55 g/mol), in combination with the neutral lipids DOPC (MW = 786.13 g/mol) and 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (DOPE, MW = 744.05 g/mol), all from Avanti Polar Lipids. CL-DNA complexes were formed from these cationic liposomes and the appropriate DNA. For X-ray samples, ethidium bromide (EtBr) experiments, and transfection assays, highly purified  $\lambda$ -phage DNA (New England Biolabs), highly polymerized calf thymus DNA (Amersham Life Sciences) and pGL3 plasmid DNA containing the luciferase gene (Promega Corp.) were used, respectively.

### Liposomes

Lipid mixtures were prepared volumetrically by combining chloroform/methanol (4 : 1) solutions of cationic and

Table 1.

Name (max. chg.)	Structure
MVL2 (+2)	
MVL3 (+3)	
MVL5 (+5)	
TMVL5 (+5)	

neutral lipids. The solvents were evaporated, first under a stream of nitrogen and subsequently in a high vacuum to ensure complete removal of the solvents. The dried lipid mixtures were hydrated at 37 °C for at least 6 h with the appropriate amount of deionized water of 18.2 MΩ (final concentration of 20 mg/ml for X-ray samples; final concentration of 0.5 mg/ml for transfection, and EtBr assay samples), sonicated to clarity with a VibraCell from Sonics and Materials Inc., and filtered through a 0.2 μm Teflon filter (Whatman). The obtained liposome solutions were stored at 4 °C.

## Cell Transfection

Mouse fibroblast L-cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) supplemented with 1% (v/v) penicillin-streptomycin (Gibco BRL) and 5% (v/v) fetal bovine serum (Gibco BRL) at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub>, reseeding the cells every 2–4 days to maintain subconfluency. The cells were transfected at 60–80% confluency in 24-well plates (7 mm diameter per well). Liposome (0.5 mg/ml) and DNA (1 mg/ml) stock solutions were diluted with DMEM to a final volume of 0.1 ml and complexes, containing 0.4 μg of pGL3-DNA per well, were prepared at the desired cationic-to-anionic charge ratio ( $\rho_{\text{chg}}$ ). The cells were incubated with the complexes for 6 h, rinsed three times with phosphate-buffered saline (PBS, Gibco BRL), and incubated in supplemented DMEM for an additional 24 h (sufficient for a complete cell cycle) to allow expression of the luciferase gene. Luciferase gene expression was measured with the luciferase assay system from Promega Corp., and light output readings were performed on a Berthold AutoLumat luminometer. Transfection efficiency, measured as relative light units (RLU), was normalized to the weight of total cellular protein using the Bio-Rad protein assay dye reagent. For experiments with chloroquine, complexes were formed in the

same manner as above, but, prior to transfer to cells, the complexes were mixed with 1.8 μl of a 6 mg/ml chloroquine solution and incubated for 10 min. The normal transfection protocol was then resumed. To compensate for the variation in cell behavior over time, the data for DOTAP/DOPC complexes was normalized using data taken at the same time as TE data for MVL5.

## X-ray diffraction (XRD)

CL-DNA complexes were prepared by mixing 75 μg of λ-phage DNA at 5 mg/ml with liposome solutions (20 mg/ml) in an Eppendorf centrifuge for approximately 3 h. Samples were prepared at  $\rho_{\text{chg}} = 2.8$ . After storage for 3 days at 4 °C, allowing the samples to reach equilibrium, they were transferred to 1.5 mm diameter quartz X-ray capillaries (Hilgenberg, Germany). The high-resolution XRD experiments were carried out at the Stanford Synchrotron Radiation Laboratory. Two-dimensional powder diffraction images were obtained using an image plate detector (Mar Instruments).

## EtBr displacement assay

Samples were prepared in a 96-well plate. Each well contained 2.4 μg of DNA, 0.28 μg of EtBr and the appropriate amount of cationic lipid. Water was added to each well, achieving a final volume of 200 μl per well. Fluorescence was measured on a Cary Eclipse fluorescence spectrophotometer.

## Results and discussion

### MVL design and structures

Table 1 shows the chemical structures and maximum charges of the MVLs used in this study. These lipids were designed to achieve a systematic variation of the headgroup charge with minimal change in the chemical structure. Only a single aminopropyl unit per cationic charge was added to the headgroup, starting from the ornithine unit of MVL2. The oleyl chains provide strong anchoring in the membrane and miscibility with DOPC. TMVL5 has a slightly longer (triethylene glycol) spacer than the other MVLs.

### X-ray characterization of MVL-DNA complexes shows a lamellar phase

We used XRD to determine the structure of MVL-DNA complexes. For all MVLs, at all investigated cationic/neutral lipid compositions of 0–90% DOPC, MVL-DNA complexes form the lamellar ( $L_{\alpha}^C$ ) phase, the highly prevalent of the two known complex structures [15–17]. Moreover, XRD shows no evidence of phase separation, indicating that the complexes contain both

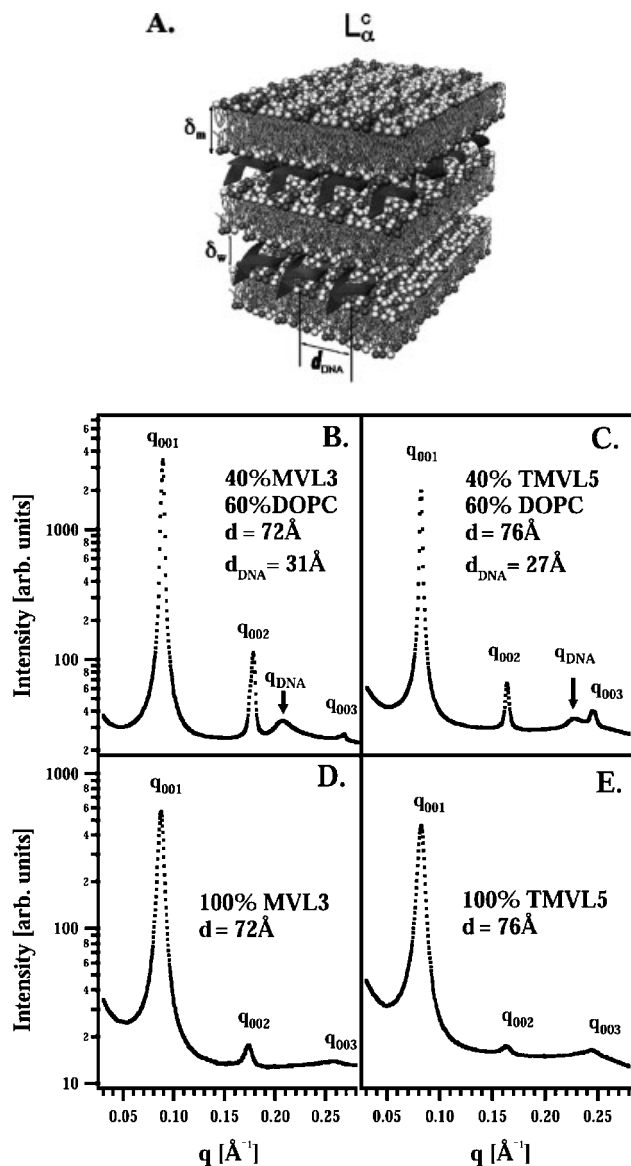


Figure 1. (A) Schematic of the lamellar phase indicating the characteristic dimensions. Reprinted with permission from [15]. (B, C) Typical X-ray diffraction (XRD) scans from lamellar ( $L_{\alpha}^C$ ) CL-DNA complexes, containing 40 mol% MVL ((B) MVL3; (C) TMVL5) and 60 mol% DOPC, at a lipid/DNA charge ratio  $\rho_{\text{chg}} = 2.8$  in the presence of DMEM. (D, E) Typical XRD scans from  $L_{\alpha}^C$  CL-DNA complexes containing 100 mol% MVL ((D) MVL3; (E) TMVL5), prepared at  $\rho_{\text{chg}} = 2.8$  in the presence of DMEM (Reproduced in part with permission from reference 15)

MVL and DOPC, as intended. A schematic of the lamellar phase ( $L_{\alpha}^C$ ) and its characteristic dimensions is shown in Figure 1A. Figures 1B and 1C shows typical XRD patterns of complexes containing 40 mol% MVL (MVL3 (B); TMVL5 (C)) and 60 mol% DOPC, at a lipid/DNA charge ratio of 2.8, prepared in the presence of DMEM. The sharp peaks, labelled  $q_{001}$ ,  $q_{002}$ ,  $q_{003}$ , respectively, give the lamellar repeat distance,  $d$ , which is the sum of the membrane thickness ( $\delta_m$ ) and the thickness of a water/DNA layer ( $\delta_w$ ):  $d = \delta_m + \delta_w = 2\pi/q_{001}$ . The diffuse weaker peak, labeled  $q_{\text{DNA}}$ , results from one-dimensional ordering of the DNA sandwiched between

the lipid bilayers and corresponds to a DNA interhelical spacing  $d_{\text{DNA}} = 2\pi/q_{\text{DNA}}$  [17]. The experimental values are  $d_{\text{DNA}} = 31 \text{ \AA}$  for MVL3 and  $d_{\text{DNA}} = 27 \text{ \AA}$  for TMVL5. In Figures 1D and 1E, XRD patterns of complexes containing 100% MVL3 (D) and TMVL5 (E) under otherwise identical conditions are shown. The narrow peaks and multiple harmonics show that at the salt concentrations present in DMEM, i.e. under conditions as in the transfection experiments, stable and well-defined complexes form even from membranes containing exclusively the highly charged multivalent lipids. The salt present in DMEM screens the electrostatic repulsions between the headgroups and enables formation of stable complexes. However, when prepared in deionized water, complexes without neutral lipids exhibit broadening of the first lamellar peak (at  $q_{001}$ ) and do not show higher harmonics of the first lamellar peak, indicative of smaller size multilamellar assemblies (results not shown).

### Estimation of lipid headgroup charge: EtBr displacement assay

Figure 2 shows data from an EtBr displacement assay [18–21], performed to examine the ability of the MVLS to condense DNA within the CL-DNA complexes. The data was acquired by collecting fluorescence measurements at various weight ratios of MVL to DNA (with a fixed weight of DNA and EtBr per point) and normalizing the intensity to the fluorescence of DNA and EtBr in solution. EtBr fluoresces when intercalated between the base pairs of DNA, but self-quenches in solution. As the MVL liposomes are introduced and self-assemble into the  $L_{\alpha}^C$  phase occurs, EtBr is displaced and overall fluorescence decreases until all DNA has been incorporated into the MVL-DNA complexes at the isoelectric point. Thus, this method allows a quick and efficient assessment of the effective charge on the headgroup of these lipids. The

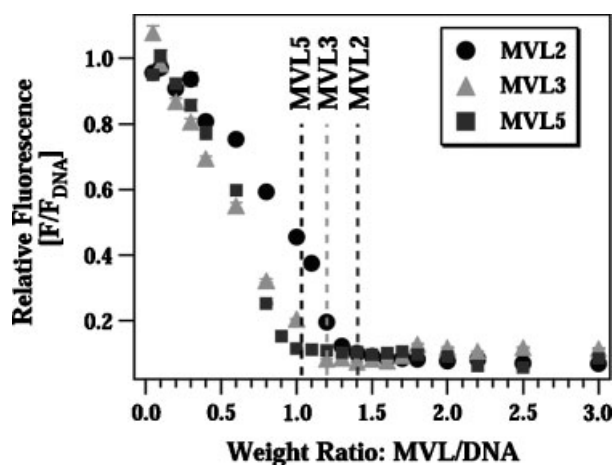


Figure 2. Normalized fluorescence data from the EtBr displacement assay for MVL2, MVL3 and MVL5. The dashed lines are drawn at the isoelectric points, determined as described in the text. They result in valencies of  $Z_{\text{MVL2}} = 2.0 \pm 0.1$ ,  $Z_{\text{MVL3}} = 2.5 \pm 0.1$ ,  $Z_{\text{MVL5}} = Z_{\text{TMVL5}} = 4.5 \pm 0.1$



isoelectric point was determined as the intersection point of linear fits to the data at high and low lipid/DNA ratio [22,23]. This gives headgroup charges of  $Z_{MVL2} = 2.0 \pm 0.1$ ,  $Z_{MVL3} = 2.5 \pm 0.1$ ,  $Z_{MVL5} = 4.5 \pm 0.1$ . The dashed lines in Figure 2 indicate the corresponding isoelectric MVL/DNA weight ratios.

## Transfection efficiency as a function of lipid composition

Figure 3 shows TE results for complexes transfecting mouse fibroblast cells at various MVL/DOPC ratios. Also included is data for the monovalent lipid DOTAP mixed with DOPC, a well-investigated reference system which constituted the starting point of our studies [13]. The complexes were prepared at  $\rho_{\text{chg}} = 2.8$ , which Lin *et al.* have found to be the optimum charge ratio for DOTAP/DOPC complexes [24]. The amount of DNA and cationic lipid per sample was kept constant. Thus, only the amount of neutral lipid varies between data points. All MVL-DNA complexes form globular particles of around 0.2  $\mu\text{m}$  diameter in water, as previously reported for DOTAP [25] and MVL5 [14]. In DMEM, these particles form much larger aggregates due to screening of their electrostatic repulsion by the high salt concentration, as we have shown for MVL5 by optical and epi-fluorescence microscopy [14]. Figure 3A shows the TE data as a function of the molar fraction of cationic lipid. For all cationic lipids, a maximum in TE as a function of lipid composition is observed: at 65 mol% for MVL2, 70 mol% for MVL3, 50 mol% for MVL5, 55 mol% for TMVL5, and 90 mol% for DOTAP. The optimal molar ratio results in a TE that is over two decades higher than that of the lowest transfecting complexes in these systems, and each data set fits a skewed bell-shaped curve.

## Membrane charge density is a universal parameter: three regimes of transfection efficiency

Figure 3B shows the data from Figure 3A plotted versus the membrane charge density,  $\sigma_M$ . A notable simplification occurs and all the data points merge onto a single curve. This identifies  $\sigma_M$  as a universal parameter for transfection by lamellar CL-DNA complexes.

As mentioned above,  $\sigma_M$  is the average charge per unit area of the lipid membrane; therefore, the headgroup areas of the lipids, their charge, and the molar fractions of cationic and neutral lipid are the parameters required to calculate  $\sigma_M$ . We calculated  $\sigma_M$  as described by Lin *et al.* [13], with  $\sigma_M = \text{total charge}/\text{total membrane area} = eZN_{\text{cl}}/(N_{\text{cl}}A_{\text{cl}} + N_{\text{nl}}A_{\text{nl}}) = [1 - \Phi_{\text{nl}}/(\Phi_{\text{nl}} + r\Phi_{\text{cl}})]\sigma_{\text{cl}}$ , where  $N_{\text{cl}}$  and  $N_{\text{nl}}$  are the number of cationic lipids and neutral lipids in the complexes, respectively;  $r = A_{\text{cl}}/A_{\text{nl}}$  is the ratio of the headgroup areas of the cationic and the neutral lipid;  $\sigma_{\text{cl}} = eZ/A_{\text{cl}}$  is the charge density of the cationic lipid

with valence  $Z$ ; and  $\Phi_{\text{nl}}$  and  $\Phi_{\text{cl}}$  are the molar fractions of the neutral and cationic lipids, respectively. For our data, we used  $A_{\text{nl}} = 72 \text{ \AA}^2$  [26,27],  $r_{\text{DOTAP}} = 1$ ,  $r_{\text{MVL2}} = 1.05 \pm 0.05$ ,  $r_{\text{MVL3}} = 1.30 \pm 0.05$ ,  $r_{\text{MVL5}} = 2.3 \pm 0.1$ ,  $r_{\text{TMVL5}} = 2.5 \pm 0.1$ ,  $Z_{\text{DOTAP}} = 1$ ,  $Z_{\text{MVL2}} = 2.0 \pm 0.1$ ,  $Z_{\text{MVL3}} = 2.5 \pm 0.1$ ,  $Z_{\text{MVL5}} = Z_{\text{TMVL5}} = 4.5 \pm 0.1$ . The values for  $Z$  were obtained by the EtBr displacement assay as described above. The values for  $r$  can be considered as fitting parameters, but they yield physically reasonable values that agree with chemical intuition. (The values of  $r$  were determined based on agreement with the Gaussian fit. For the monovalent lipid DOTAP,  $r$  was assumed to be equal to 1, consistent with previous findings [13].) It is interesting to note that the optimal TE for MVL3 is found at a larger molar fraction of cationic lipid than for MVL2 despite the fact that MVL3 has a higher headgroup charge. This can be attributed to the significantly larger headgroup size of MVL3 and shows the importance of the parameter  $r$ .

The resulting curve of TE vs.  $\sigma_M$  can be described empirically by a simple Gaussian (solid line in Figure 3B):

$$\text{TE} = \text{TE}_0 + A \exp -[(\sigma_M - \sigma_M^*)/w]^2 \quad (1)$$

where  $\text{TE}_0 = -(1.9 \pm 5.6) \times 10^7$  RLU/mg protein;  $A = (9.4 \pm 0.6) \times 10^8$  RLU/mg protein;  $w = (5.8 \pm 0.5) \times 10^{-3} \text{ e/\AA}^2$ . For the optimal charge density, the fit gives  $\sigma_M^* = (17.4 \pm 0.2) \times 10^{-3} \text{ e/\AA}^2$ .

Remarkably, in extension of previous results which showed the increase and a subsequent levelling-off of TE with  $\sigma_M$  [13], we see an entire bell curve of efficiency, including a decrease in TE at higher charge densities. Previously, without the series of new MVLS, Lin *et al.* were not adequately prepared to measure this range of charge densities.

The new universal TE curve of lamellar complexes exhibits three well-defined regimes. Regime I (dark gray), corresponding to low  $\sigma_M$ , features an exponential increase in efficiency over three orders of magnitude. Regime III (light gray), corresponding to high  $\sigma_M$ , is characterized by a decrease in efficiency with increasing  $\sigma_M$ , suggesting that there also is an obstacle of electrostatic nature to successful DNA delivery by lamellar CL-DNA complexes. The competition of the two effects that give rise to regimes I and III leads to the existence of the intermediate regime II (white) as the region of optimal charge density, corresponding to the highest TE. This clearly demonstrates the importance of including neutral lipid in the formulation of CL-DNA complexes, particularly those of newer, multivalent lipids. Due to the universality of the curve, it should also be possible to estimate the optimal composition for any given lipid by performing the simple EtBr displacement assay and estimating the headgroup size of the lipid from its chemical structure. We will address the implications of the universal curve for the mechanism of transfection in more detail below.

In addition to data for the lamellar complexes, Figure 3B shows TE data for the commonly used DOTAP/DOPE lipid system. The inverted hexagonal ( $H_{II}^C$ )

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