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RESEARCH ARTICLE

Optimizing cationic and neutral lipids for efficient gene delivery at high serum content

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Abstract

Background Cationic liposome (CL)–DNA complexes are promising gene delivery vectors with potential application in gene therapy. A key challenge in creating CL–DNA complexes for application is that their transfection efficiency (TE) is adversely affected by serum. In particular, little is known about the effects of a high serum content on TE, even though this may provide design guidelines for application *in vivo*.

Methods We prepared CL–DNA complexes in which we varied the neutral lipid [1,2-dioleoyl-*sn*-glycerophosphatidylcholine, glycerol-monooleate (GMO), cholesterol], the headgroup charge and chemical structure of the cationic lipid, and the ratio of neutral to cationic lipid; we then measured the TE of these complexes as a function of serum content and assessed their cytotoxicity. We tested selected formulations in two human cancer cell lines (M21/melanoma and PC-3/prostate cancer).

Results In the absence of serum, all CL–DNA complexes of custom-synthesized multivalent lipids show high TE. Certain combinations of multivalent lipids and neutral lipids, such as MVL5(5+)/GMO–DNA complexes or complexes based on the dendritic-headgroup lipid TMVLG3(8+) exhibited high TE both in the absence and presence of serum. Although their TE still dropped to a small extent in the presence of serum, it reached or surpassed that of benchmark commercial transfection reagents, particularly at a high serum content.

Conclusions Two-component vectors (one multivalent cationic lipid and one neutral lipid) can rival or surpass benchmark reagents at low and high serum contents (up to 50%, v/v). We propose guidelines for optimizing the serum resistance of CL–DNA complexes based on a given cationic lipid.

Keywords cationic liposomes; gene delivery; glycerol monooleate; multivalent cationic lipid; serum

Introduction

Cationic liposome (CL)–DNA complexes are one of the most promising nonviral vectors for both *in vitro* and *in vivo* therapeutic application [1–8]. However, their efficiency still lags behind that of viral vectors, especially *in vivo*. It is highly desirable to develop more efficient nonviral vectors because

of the safety concerns associated with engineered viruses [9–12]. Further advantages of nonviral vectors include facile and variable preparation, low potential immunogenicity, and the ability to transfer very large pieces of nucleic acids [13].

For *in vitro* application, progress in the development of nonviral vectors has yielded multivalent cationic lipid vectors that exhibit transfection efficiencies (TEs; the ability to transfer DNA into cells followed by expression) competitive with those of viral vectors. Beyond the chemical structure of the lipids, the properties of the self-assembled CL–NA complexes profoundly impact transfection [14–16]. Salient examples are the nanoscale structures of CL–DNA and CL–small interfering (si)RNA complexes (lamellar L_{α}^{C}; inverted and regular hexagonal H_{II}^{C} and H_{I}^{C}; gyroid cubic $Q_{II}^{G,\ siRNA}$) (Figures 1A to 1D) [17–21] and the lipid membrane charge density [22,23]. Custom synthesis of multivalent lipids enabled the discovery that membrane charge density is a predictive chemical parameter for transfection by L_{α}^{C} CL–DNA complexes [23,24], whereas H_{I}^{C} complexes from a dendritic MVL (+16*e* charge) significantly improved TE in hard-to-transfect mouse embryonic fibroblast cells [19]. Bicontinuous gyroid cubic CL–siRNA complexes (Figure 1D) exhibit high silencing efficiency because the cubic phase facilitates fusion of the membranes of complexes and endosomes, leading to efficient cytosol delivery [20,25].

The TE of nonviral vectors is reduced, often drastically, by the presence of serum in cell culture media [1,26–28]. Vectors that maintain high TE in the presence of low amounts of serum (5–10% of culture medium, v/v) are desirable for ease of use and because serum starvation can affect the cell cycle of cultured cells. More importantly, high TE in the presence of high serum content may help predict high TE *in vivo* [27–29], which is the main current challenge for nonviral vectors.

Numerous attempts have been made to determine how serum decreases the TE of CL–DNA complexes, with limited success. Serum is a complex mixture of components, and several processes, some of them affecting TE in opposing ways, take place simultaneously when complexes are exposed to serum. For example, negatively-charged serum components bind to the positively-charged CL–DNA complexes. This can reduce the interactions between the complexes and the cell membrane, leading to reduced uptake and inefficient endosomal escape. The binding of serum components can also cause structural reorganization of the complexes, colloidal instability (e.g. aggregation and dissociation of the complexes) and rapid clearance by the reticuloendothelial system [27–32].

A variety of strategies to improve the serum resistance of CL–DNA complexes have been investigated. Steric stabilization of CL–DNA complexes by incorporation of poly(ethylene glycol) (PEG)-lipids (PEGylation) increases their circulation time [33–35] and may improve their stability in serum, although it also reduces TE [36–38]. Additional strategies include the design of novel cationic lipids [39,40], the development of new formulations [30,31,40–42] and the use of mixtures of cationic polymers with liposomes [43–45]. Multivalent cationic lipids, such as those used in the present study, typically yield DNA complexes with high TE and lower toxicity compared to univalent lipids [46–48]. The addition of a neutral ('helper') lipid can affect TE by controlling the membrane charge density (σ_M) of CL–DNA complexes [23], the structure of lipid self-assemblies [17,18,20,25], the thickness of the hydration layer, and the nucleic acid secondary and tertiary structures [49,50]. For example, the inclusion of fusogenic 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) can increase TE *in vitro* [2,22,51,52] and cholesterol can enhance the colloidal stability and TE of CL–DNA complexes in the absence or the presence of serum [43,53–56].

We are pursuing strategies to increase the efficiency of PEGylated complexes [57,68], but the present study aimed to find formulation strategies for serum-stable complexes based solely on cationic and neutral lipids. A number of commercially available reagents have achieved this goal

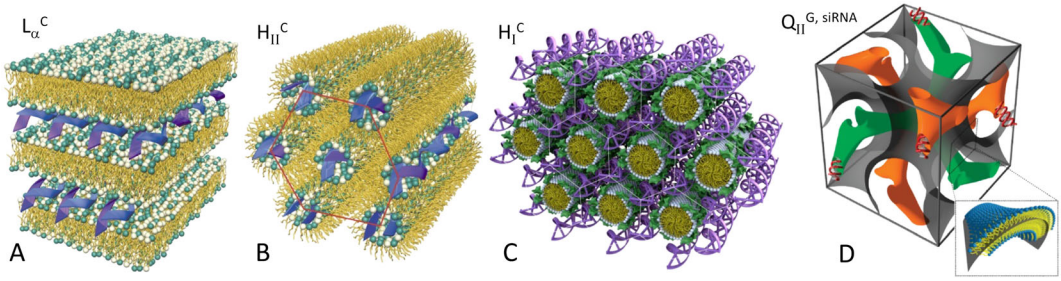

Figure 1. Schematics showing the local nanoscale structures of CL–NA complexes, which result spontaneously when CLs are mixed with nucleic acids. Structures were derived from synchrotron X-ray scattering data. (A) Lamellar (L_{α}^{C}), (B) inverted hexagonal (H_{II}^{C}) and (C) hexagonal (H_{I}^{C}) CL–DNA complex structure. (D) Gyroid cubic CL–siRNA complex structure ($Q_{II}^{G,\ siRNA}$) with siRNA incorporated in the water channels. (A, B) Reprinted with permission [18]. (C) Reprinted with permission [19] (© 2006 American Chemical Society). (D) Reprinted with permission [20] (© 2010 American Chemical Society).

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(high TE at low serum), although their compositions are proprietary. More importantly, CL–DNA complexes are often claimed to be serum-resistant without examination of their TE at the high serum concentrations (≥ 50%) potentially relevant to gene delivery *in vivo*.

We measured TE in the presence of varied amounts of serum for CL–DNA complexes prepared with a variety of cationic and neutral lipids, using a luciferase assay in mouse L-cells. We employed five different cationic lipids with varied headgroup charge and structure: DOTAP(1+) (2,3-dioleoyloxy-propyl-trimethylammonium chloride) and the custom synthesized lipids (Figure 2) MVL5(5+) [24], TMVLG3(8+), TMVLBG1(8+) and TMVLBG2(16+) [19,58,59]. TMVLG3, TMVLBG1 and TMVLBG2 are lipids with dendritic headgroups (DLs). As the neutral lipid (NL), we employed 1,2-dioleoyl-*sn*-glycerophosphatidylcholine (DOPC), cholesterol or glycerol monooleate (GMO), at a number of different cationic lipid to neutral lipid molar ratios. Varying this ratio (1:0, 3:1, 1:1 and 1:3 mol/mol) allowed us to assess the effect of the membrane charge density (σ_M, the average charge per unit area of membrane) [23]. For selected efficient lipid formulations, we further measured TE in the human PC-3 (prostate cancer) and M21 (melanoma) cell lines. We also assessed the cytotoxicity of the complexes. We compare our results with the benchmark commercial transfection reagent, Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Serum-stable combinations of a single multivalent cationic and neutral lipid were able to rival or outperform Lipofectamine 2000, especially at a high serum content.

Materials and methods

Lipids and liposome preparation

Lipofectamine 2000 was purchased from Invitrogen and used in accordance with the manufacturer's instructions. DOTAP and DOPC were purchased as solutions in chloroform from Avanti Polar Lipids (Alabaster, AL, USA). Cholesterol and GMO were purchased from Sigma-Aldrich (St Louis, MO, USA) and Nu-Check-Prep (Elysian, MN, USA), respectively, and dissolved in chloroform to prepare stock solutions. MVL5, TMVLBisG1, TMVLBisG2 and TMVLG3 (Figure 2) were synthesized as described previously [19,24,58] and dissolved in chloroform/methanol (9:1, v/v) to prepare stock solutions. Measurements of the headgroup charge of these cationic lipids in complexes with DNA via an ethidium bromide displacement assay indicated complete protonation of the headgroups [i.e. headgroup charges of +5*e* (MVL5), +8*e* (TMVLBisG1 and TMVLG3) and + 16*e* (TMVLBisG2)] [58]. Liposomes were prepared at varied mol fraction of neutral lipid, Φ_{NL}, where $\Phi_{NL} = N_{NL}/(N_{CL} + N_{NL})$ [with N_{CL} and N_{NL} representing the amount (in mol) of cationic and neutral lipid, respectively]. The relationship between the membrane charge density, σ_M, and Φ_{NL} is $\sigma_M = eZN_{CL}/(N_{CL}A_{CL} + N_{NL}A_{NL}) = [1 - \Phi_{NL}/(\Phi_{NL} + r\Phi_{CL})]\sigma_{CL}$. Here, $r = A_{CL}/A_{NL}$ is the ratio of the headgroup areas of the cationic and the neutral lipid; $\sigma_{CL} = eZ/A_{CL}$ is the charge density of the cationic lipid with valence Z; and $\Phi_{CL} = 1 - \Phi_{NL}$ is the mol fraction of the cationic lipid. To prepare liposomes, lipid

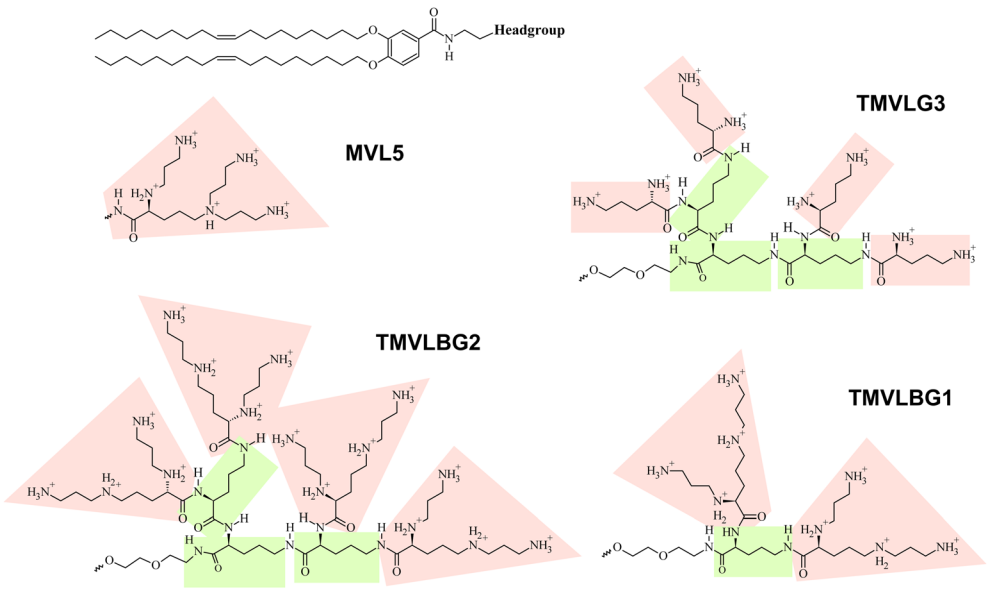

Figure 2. The chemical structures of the multivalent cationic lipid MVL5 and the multivalent lipids with dendritic headgroups used in the present study [19,24,58]. All lipids are based on a dioleoyl-tail moiety (top). The branching ornithine moieties in the headgroups of TMVLG3, TMVLBG1 and TMVLBG2 are highlighted in green, and the charge-bearing groups are highlighted in pink (ornithine for TMVLG3, carboxyspermine for TMVLBG1 and TMVLBG2, and aminopropylated carboxyspermine for MVL5).

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stock solutions were mixed at the appropriate ratios in glass vials and then dried, first under a stream of nitrogen and then in a vacuum (rotary vane pump) for 8–12 h. To the resulting thin lipid film, sterile high-resistivity (18.2 MΩ cm) water was added, and the mixture was incubated at 37°C for at least 12 h. The final total lipid concentration was 1 mM. All aqueous lipid solutions were sonicated (tip sonicator) prior to use and stored at 4°C.

Cell culture and transfection

Luciferase plasmid DNA (pGL3 Control Vector; Promega, Madison, WI, USA) was propagated in *Escherichia coli* and isolated using a Qiagen Giga Kit (Qiagen, Valencia, CA, USA). Mouse fibroblast L-cells (ATCC number: CCL-1), M21 cells (human melanoma; a gift from the group of E. Ruoslahti) and PC-3 cells (ATCC number: CRL-1435; human prostate cancer) were maintained in supplemented culture medium (Dulbecco's modified Eagle's medium, containing 1% penicillin (Invitrogen) and 5% or 10% fetal bovine serum (Thermo Scientific, Waltham, MA, USA) for mouse and human cell lines, respectively) at 37°C in a humidified atmosphere with 5% CO_2. Cells were reseeded approximately every 72 h to maintain subconfluency. For transfection, approximately 80 000 cells/well were seeded in 24-well plates and incubated for 18–24 h prior to transfection. A solution of pGL3 plasmid DNA [4 μg/ml in Opti-MEM (Invitrogen)] was prepared from a DNA stock solution at 1.0 mg/ml. Appropriate volumes of liposome solutions (to yield the desired lipid to DNA charge ratio, ρ) were diluted with Opti-MEM. The charge ratio $\rho = N_+/N_- = ZN_{CL}/N_{nt}$, where N_+ and N_- are the numbers of positive (lipid) and negative (DNA) charges, Z is the valence of the cationic lipid, and N_{CL} and N_{nt} are the amounts (in mol) of cationic lipids and nucleotides, respectively. Equal volumes of liposome and DNA solutions were combined and, after incubation for 20 min at room temperature, 200 μL of this mixture (containing 0.4 μg of DNA) were added per well. Thus, the amount of DNA added was identical for all TE measurements. For transfection in serum, complexes were first prepared as described above. To the complex suspension in Opti-MEM, the appropriate volume of serum to achieve the desired final serum concentration of 0%, 10%, 20% or 50% (v/v) was then added, and the total volume of the mixture was made up to 400 μL with Opti-MEM. The resulting mixture was added to the cells. After 6 h of incubation at 37°C, the transfection medium was removed, each well was washed once with phosphate-buffered saline (PBS) and fresh culture medium was added to each well. Cells were harvested in 150 μL of Passive Lysis Buffer (Promega) after another 18–20 h (mouse fibroblasts) or 40–48 h (human cell lines). Luciferase expression was measured in accordance with manufacturer's instructions (Promega). A multilabel counter (Perkin-Elmer 1420 Victor3 V; Perkin Elmer, Waltham, MA, USA) was used to measure the relative light units (RLU) from the luminescence assay. Data points shown are the average of duplicate (and in some cases quadruplicate) measurements, with error bars showing the SD. All experiments were repeated two or three times to ensure reproducibility.

Cytotoxicity

Cytoxicity was assessed using a commercial, tetrazolium-salt-based assay (CellTiter 96 Aqueous One assay; Promega) for cell viability. Mouse fibroblast L-cells were seeded in 96-well plates (15 000 cells/well) in maintenance medium. After 18–20 h of incubation, the cells were washed once with PBS, and 40 μL of a mixture containing medium and complexes were added (prepared as for transfection; 0.08 μg of DNA per well; i.e. transfection concentration). After 6 h of incubation, the complex-containing medium was replaced by a mixture of 60 μL of Opti-MEM and 20 μL of the cell proliferation assay. Following 3 h of incubation, absorbance at 490 nm was measured using a scanning multi-well spectrophotometer (Perkin-Elmer 1420 Victor3 V). The experiment was performed simultaneously for all lipids and the results were normalized to control wells, which differed from the experimental wells only in that they were treated with Opti-MEM instead of the medium/complexes mixture. Each data point represents the average of at least quadruplicate measurements, with error bars showing the SD.

Results

The TE of CL–DNA complexes *in vitro* typically decreases, often drastically, in the presence of serum. Our goal was to better understand this phenomenon by looking for correlations between TE and lipid structure or composition. We used a luciferase reporter gene expression assay in mouse fibroblast L-cells to evaluate the TE of a variety of lipid formulations in the presence of increasing amounts of serum. We systematically varied the neutral lipid, the membrane charge density (via the ratio of neutral to cationic lipid), the headgroup charge of cationic lipids and the chemical structure of those headgroups. We also evaluated the TE of selected formulations in two human cell lines: M21 (melanoma) and PC-3 (prostate cancer). Figure 2 shows the chemical structures of the investigated multivalent cationic lipids, MVL5(5+) [24], TMVLBisG1(8+), TMVLG3(8+) and TMVLBisG2 (16+) [19,58]. These lipids have identical hydrophobic moieties of two oleyl tails attached to a spacer based on 3,4-dihydroxybenzoic acid. The TMVL lipids have a

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slightly longer spacer and dendritic headgroups with ornithine cores and ornithine or carboxyspermine endgroups. The headgroup of MVL5 is an aminopropylated carboxyspermine. In serum-free transfection medium, DNA complexes of mixtures of all of the investigated multivalent lipids with neutral DOPC efficiently transfect cells over a broad range of composition [19,23,24,58]. As a univalent cationic lipid to compare with the multivalent lipids, we chose the commercially available univalent lipid DOTAP (1,2-dioleoyl-3-trimethyammonim propane) (for the structures of DOTAP and DOPC, see Supporting information, Figure S1).

The effect of neutral lipids for univalent DOTAP and multivalent MVL5

The inclusion of a neutral lipid in CL–DNA complexes frequently increases the TE over that of cationic lipid alone in serum-free medium [2,51,52], for example, by tuning the membrane charge density [22,23] or CL–NA complex structure [7,19,20,22,25,59]. To assess how the choice of neutral lipid affects TE in the presence of serum, we investigated CL–DNA complexes based on the commercially available cationic lipids DOTAP (univalent) and MVL5 (multivalent), as well as the neutral lipids DOPC, cholesterol (Chol) and GMO. Although much of our previous work has used DOPC, Chol has been employed by others to confer serum resistance and high TE *in vivo* [43,53–55] and GMO displays intriguing phase behavior (including gyroid cubic structures) and high silencing efficiency in CL–siRNA complexes [20,25]. The chosen formulations [with a charge ratio (ρ) of 3 and neutral lipid mol fraction (Φ_{NL}) of 0.25 for DOTAP-based complexes and $\rho = 10$ and $\Phi_{NL} = 0.5$ for MVL5-based complexes] exhibit high TE in the absence of serum. The TE of DOTAP-based complexes containing DOPC and cholesterol is almost unaffected by 10% serum (Figure 3A). By contrast, the efficiency of complexes containing GMO, as well as of those without neutral lipid, moderately drops at this low serum content. As the serum content increases, the TE of all DOTAP-based complexes drops strongly. Their TE at a serum content of 50% (TE $\sim 10^6$ RLU/mg protein) is as low as that of uncomplexed DNA in serum-free medium and, in some cases, even lower (for DOTAP/GMO).

MVL5-based CL–DNA complexes are as efficient (TE $\sim 10^9$ RLU/mg protein) as the benchmark commercial reagent Lipofectamine 2000 in the absence of serum (Figure 3B). MVL5/GMO–DNA complexes continue to rival Lipofectamine 2000 at 10% and 20% serum and even surpass it at 50% serum. Their TE remains very high after an initial small drop at 10% serum. The TE of the three other MVL5-based complexes (MVL5/DOPC, MVL5/Chol and MVL5 only) drops strongly at 10% serum but then stays constant (MVL5/Chol) or only drops slightly for 20% and 50% serum. This behavior is markedly different from that of DOTAP-based complexes. As a function of serum content, TE declines gradually for complexes of monovalent DOTAP. However, for complexes of multivalent MVL5, TE shows an initial step drop and then remains almost constant as serum content increases. As a result, all MVL5-based complexes surpass the DOTAP-based complexes in efficiency at no serum and 50% serum, whereas the TE of MVL5- and DOTAP-based complexes is comparable at lower (10% and 20%) serum contents. The notable exception are the MVL5/GMO–DNA complexes, which show higher TE throughout.

To assess the effect of membrane charge density (σ_M), we varied Φ_{NL} for the MVL5-based complexes and the DOTAP/Chol–DNA complexes (again using $\rho = 3$ for

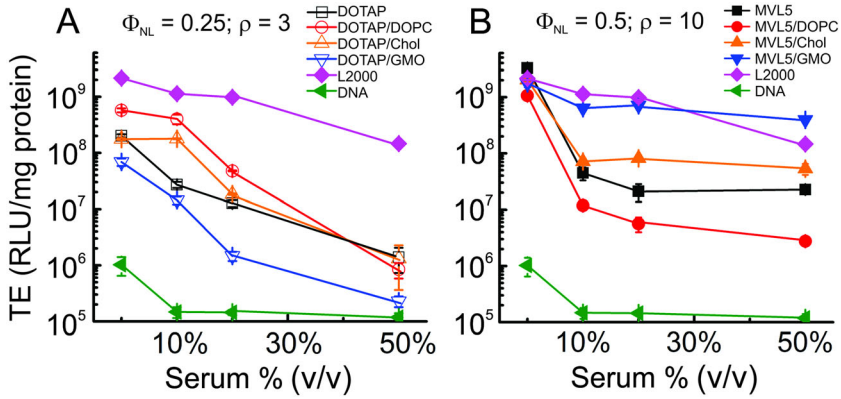

Figure 3. TE as a function of serum content for CL–DNA complexes based on DOTAP and MVL5 in combination with different neutral lipids (NLs). (A) TE of DOTAP/NL–DNA complexes at $\rho = 3$, without NL and with DOPC, cholesterol, or GMO at $\Phi_{NL} = 0.25$. (B) TE of MVL5/NL–DNA complexes at $\rho = 10$, without NL and with DOPC, cholesterol or GMO at $\Phi_{NL} = 0.5$. Also shown are the TE of the benchmark commercial reagent Lipofectamine 2000 and uncomplexed DNA. Note the difference in shape of the curves for monovalent DOTAP (gradual decline) and multivalent MVL5 (initial step drop to nearly flat slope). For further discussion, see text.

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DOTAP-based complexes and $\rho = 10$ for MVL5-based complexes). With the one exception of $\Phi_{Chol} = 0.75$, MVL5-based complexes exhibit very high TE (TE $> 10^9$ RLU/mg protein) in the absence of serum, independent of Φ_{NL} (and equivalently σ_M) (Figures 4A to 4C). As a function of increasing serum content, the MVL5-based complexes exhibit the behavior already seen in Figure 3B: the largest drop in TE occurs at 10% serum, with little to no change (especially compared to the initial drop) at higher serum contents (Figures 4A to 4C) [the largest changes in TE at high serum content (10% serum to 50% serum) are seen for complexes with $\Phi_{DOPC} = 0.75$ and $\Phi_{GMO} = 0.25$].

Differences between the neutral lipids are evident when comparing the TE of MVL5-based complexes as a function of Φ_{NL}. The TE of MVL5/GMO–DNA complexes at $\Phi_{GMO} = 0.5$ is the least sensitive to the addition of serum (Figure 4C) and thus remains exceptionally high even at high serum contents (as already seen in Figure 3B). Similarly, the initial drop in TE (0–10% serum) for complexes at $\Phi_{GMO} = 0.25$ and 0.75 is smaller than for most other MVL5-based complexes (all but MVL5/Chol–DNA complexes at $\Phi_{Chol} = 0.75$, which, however, start at a lower TE). The TE of MVL5/DOPC–DNA complexes (Figure 4A) decreases slightly with increasing Φ_{NL}, both in the absence and in the presence of serum. The TE of MVL5/Chol–DNA complexes (Figure 4C) in the absence of serum also decreases with increasing Φ_{NL}, although the drop in TE from $\Phi_{Chol} = 0$ to $\Phi_{Chol} = 0.25$ and 0.5 is very small, whereas that from $\Phi_{Chol} = 0.5$ to $\Phi_{Chol} = 0.75$ is larger than for DOPC. However, in the presence of serum, TE is remarkably unaffected by Φ_{Chol}. This reflects a comparatively small initial drop in TE (when moving from no serum to 10% serum) at $\Phi_{Chol} = 0.75$, where TE in the absence of serum is lower than at the other values of Φ_{Chol}. In other words, increasing cholesterol content reduces the serum sensitivity of TE for MVL5/Chol–DNA complexes. This effect is even more pronounced for DOTAP/Chol–DNA complexes (Figure 4D). Although the TE of these complexes in serum-free medium decreases with Φ_{Chol}, it stays essentially constant (10% serum) or even increases (20% and in particular 50% serum) with Φ_{Chol} in the presence of serum. As a consequence, the lipid composition with the highest TE in the absence of serum exhibits the lowest TE at higher serum content (20% and 50%) and vice versa (there is little change in TE with Φ_{Chol} at 10% serum). Other studies have observed previously that complexes optimized for *in vitro* transfection in serum-free medium performed poorly at high serum contents or *in vivo*, and that complexes which transfected efficiently *in vivo* were sub-optimal for transfection *in vitro* [27,43,54].

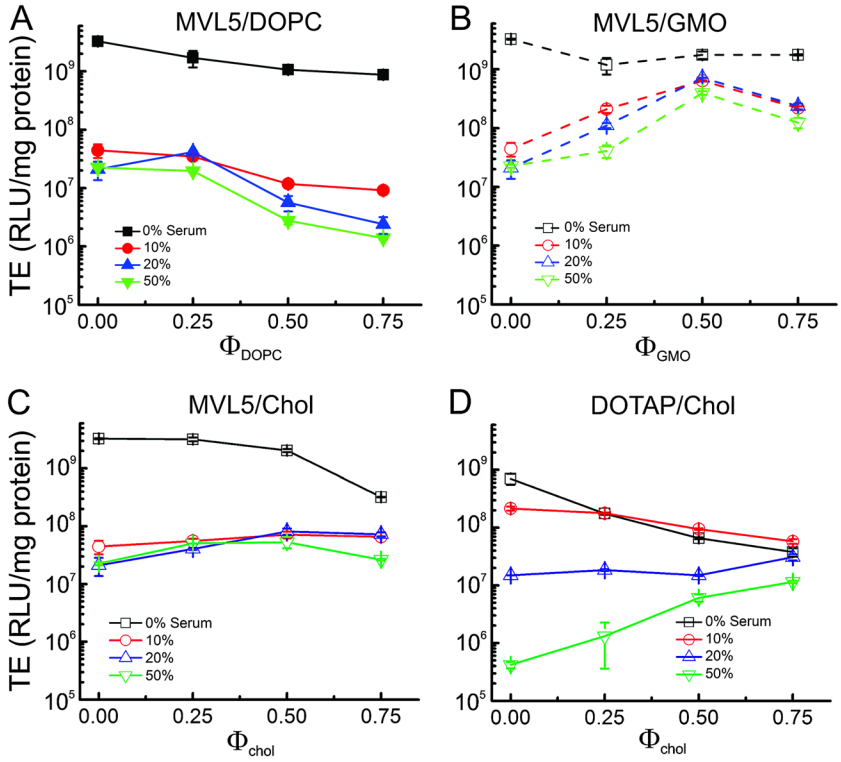

Figure 4. TE as a function of mol fraction of neutral lipid (Φ_{NL}) for CL–DNA complexes based on MVL5 and DOTAP in the presence of varying amounts of serum (0%, 10%, 20% and 50%). (A, B, C) TE for MVL5-based complexes containing DOPC, cholesterol and GMO, respectively. All these complexes were prepared at $\rho = 10$. (D) TE of DOTAP/Chol–DNA complexes at $\rho = 3$.

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Highly-charged cationic lipids with dendritic headgroups

The data displayed in Figures 3 and 4 show that multivalent MVL5 consistently led to higher TE than univalent DOTAP and that Chol and GMO but not DOPC are NLs that can improve serum resistance. Thus, we investigated the TE of complexes of other multivalent lipids with Chol and GMO as a function of serum content. We previously prepared a series of multivalent cationic lipids with dendritic headgroups (DLs; headgroup charges of $+4e$ to $+16e$). In the absence of serum, DNA complexes of these lipids exhibited high TE over a broad range of composition and nonlamellar structures at higher content of DLs (7,19,58,59). For the present study with serum, we investigated three of these DLs (Figure 2): the highly charged TMVLBG2(16+) and two lipids (TMVLBG1 and TMVLG3) with eight charges in their headgroup but different headgroup architectures.

Figure 5 shows the TE for DNA complexes of TMVLG3, TMVLBG1, or TMVLBG2 mixed with cholesterol or GMO as a function of serum content and Φ_{NL} (at fixed $\rho = 10$). In the absence of serum, all of these complexes transfect very efficiently (TE $\sim 10^9$ RLU/mg protein, comparable to Lipofectamine 2000) over the investigated range of membrane charge density.

As seen for MVL5, increasing the serum content from 0% to 10% causes a steep drop in the TE of the DL-based complexes. After that initial drop, the TE drops only slightly or even remains constant (for serum contents of 20% and 50%). The initial drop is a little over one order of magnitude for most complexes containing TMVLG3(8+) (Figures 5A and 5B). Because serum contents of 20% and 50% do not reduce their TE further and because their TE in the absence of serum is very high, these complexes are efficient (TE $\sim 10^8$ RLU/mg protein) even at high serum content. By contrast, the initial drop in TE is much larger for complexes based on the other two DLs, TMVLBG1(8+) and TMVLBG2 (16+) (Figures 5C to 5F). For most of these complexes, there is also a further but much smaller drop as the serum content is increased to 20% and 50%.

Neither the choice of neutral lipid, nor the mol fraction of neutral lipid (which controls σ_M) has a large effect on the serum resistance of DL-based complexes. Indeed, complexes with and without neutral lipid behave essentially the same. This is unlike what we observed for MVL5, particularly for MVL5/GMO–DNA complexes. Whether this is because of differences in σ_M or because the complexes have differing nanostructures is the subject of ongoing investigation.

Transfection of human cell lines

TE, including the relative efficiency of transfection agents, can vary widely between cell lines. To determine whether our findings on serum-resistant formulations are broadly applicable, we measured the TE of selected vectors in two human cell lines, M21 (melanoma) and PC-3 (prostate cancer). As vectors based on multivalent lipids, we chose MVL5/GMO–DNA complexes and TMVLG3/GMO–DNA complexes, both at $\Phi_{GMO} = 0.5$ and $\rho = 10$ (Figure 3B and Figure 5B). As commercially available benchmarks, we used DOTAP/DOPC–DNA complexes, and Lipofectamine 2000.

In the human cell lines, the absolute levels of luciferase expression are low: TE never exceeded 2×10^7 RLU/mg protein, even in the absence of serum (Figure 6). Most notable, however, is the high performance of TMVLG3/GMO–DNA complexes. Their TE in the absence of serum is as high as that of Lipofectamine 2000 in PC-3 cells (Figure 6A) and then only drops slighly with increasing contents of serum, even as the TE of Lipofectamine 2000 drops drastically at 50% serum. In M21 cells, the TE of TMVLG3/GMO–DNA complexes in the absence of serum is higher than that of DOTAP/DOPC–DNA complexes and MVL5/GMO–DNA complexes but approximately one order of magnitude below that of Lipofectamine 2000 (Figure 6B). Nonetheless, because their TE stays almost constant with increasing serum content, it exceeds that of all other vectors, including Lipofectamine 2000, at 20% and 50% serum. Lipofectamine 2000 exhibits the highest TE in both human cell lines in the absence of serum (together with TMVLG3/GMO–DNA complexes in PC-3 cells). However, it also shows the steepest drop with increasing serum content. By contrast, the TE of both vectors based on multivalent lipids exhibits only a small drop with increasing serum content. Because of this, the TE of MVL5/GMO–DNA complexes rivals that of Lipofectamine 2000 at high (20% and 50%, M21 cells) or very high (50%, PC-3 cells) serum content, despite its comparably low value in the absence of serum. The TE of the DOTAP/DOPC–DNA complexes is the lowest of all vectors for all data points. It starts low in the absence of serum and then drops moderately with increasing serum content.

Cytotoxicity

Vector toxicity remains a concern for most cationic transfection agents both *in vitro* and *in vivo*. Thus, we measured cell viability after incubation with DNA complexes of the investigated cationic lipids alone and of 1:1 mixtures with the investigated neutral lipids. We used the charge ratios employed in the transfection experiments ($\rho = 10$ for multivalent lipids, $\rho = 3$ for DOTAP). As shown in Figure 7, cell viability remained high at 75% or higher for all data points, with the lowest viabilities observed for Lipofectamine 2000, as well as TMVLG3 and TMVLBisG2 alone and in combination with cholesterol.

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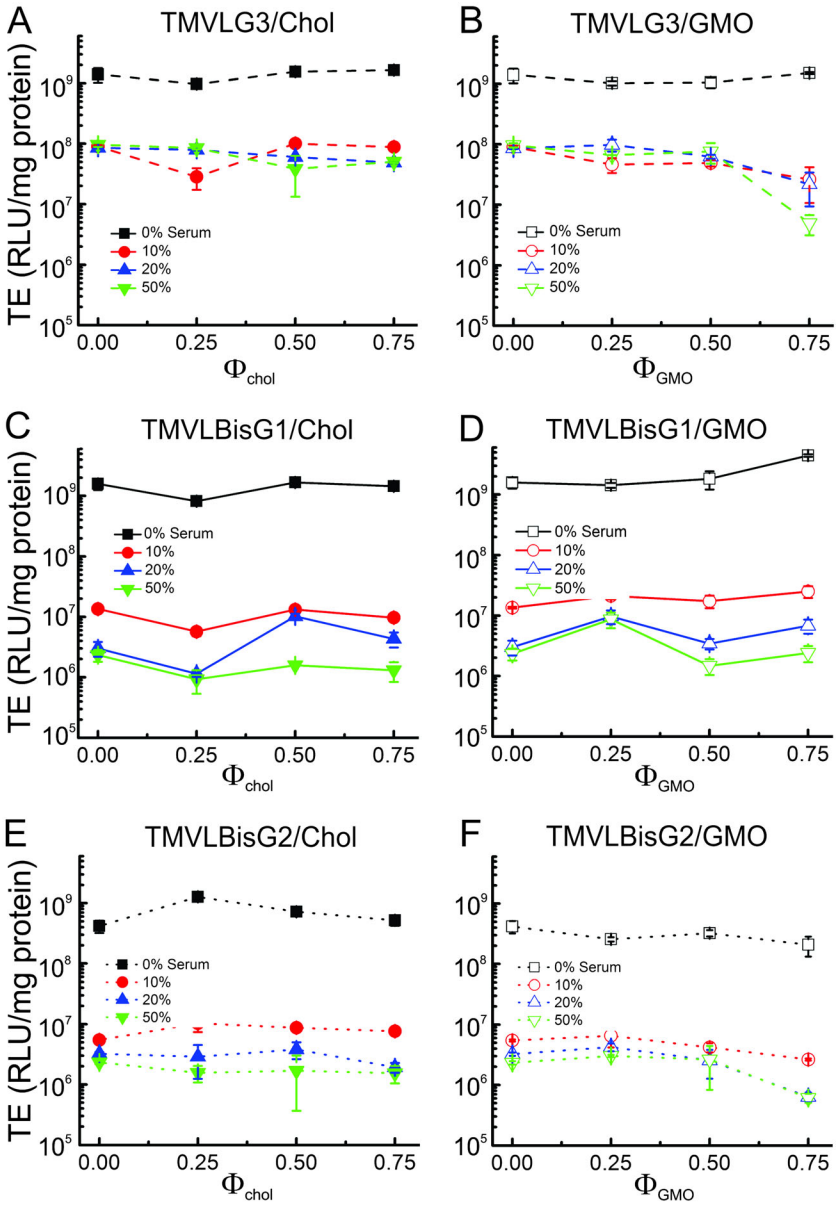

Figure 5. TE of CL–DNA complexes based on the highly charged DLs TMVLG3(8+), TMVLBG1(8+) and TMVLBG2(16+) in the presence of varying amounts of serum (0%, 10%, 20% and 50%). All complexes were prepared at ρ = 10. The plots show the effect of different mol fractions of neutral lipid (Φ_{NL}). The first column [(A) (TMVLG3), (C) (TMVLBG1), (E) (TMVLBG2)] shows the TE of DL/Chol–DNA complexes. The second column [(B) (TMVLG3), (D) (TMVLBG1) and (F) (TMVLBG2)] shows the TE of DL/GMO–DNA complexes.

Discussion

The TE of many synthetic vectors drops in the presence of serum. Although some formulations remain efficient at the level of serum content typically used in cell culture (≤ 10%), the effect of higher serum levels has been investigated less thoroughly in prior studies, despite its possible relevance to choosing viable formulations for transfection *in vivo*. Varying the lipid structure and composition in CL–DNA complexes, we sought to establish guidelines for developing serum-resistant vectors from trends in TE. Below, we refer to the TE in the absence of serum as the 'starting TE' and define serum resistance as a weak dependence of TE on the content of serum (i.e. complexes are called 'serum resistant' if their TE remains close to the starting TE as serum content increases).

Neutral lipids

DOPC as the neutral lipid yields complexes with very poor serum resistance in combination with monovalent lipid

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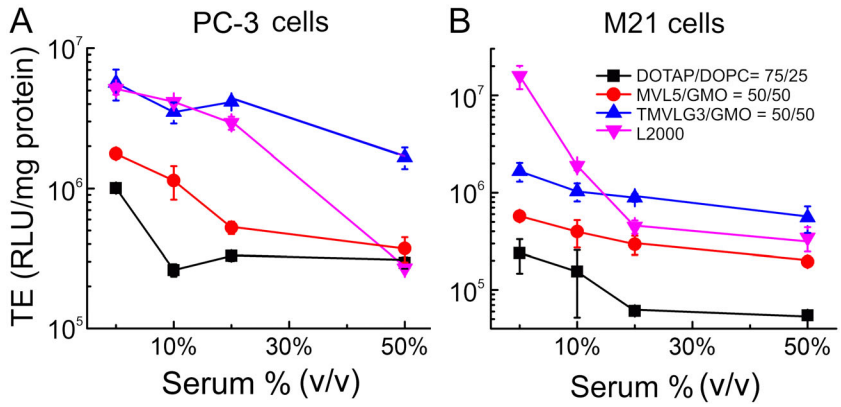

Figure 6. TEs of selected serum-resistant multivalent lipid/GMO–DNA complexes ($\Phi_{GMO} = 0.5$; $\rho = 10$) in two human cell lines [(A) PC-3 (prostate cancer) cells; (B) M21 (melanoma) cells] as a function of serum content. Also shown are the TEs of DOTAP/DOPC–DNA complexes ($\rho = 3$, $\Phi_{DOPC} = 0.25$) and the benchmark commercial transfecting agent Lipofectamine 2000.

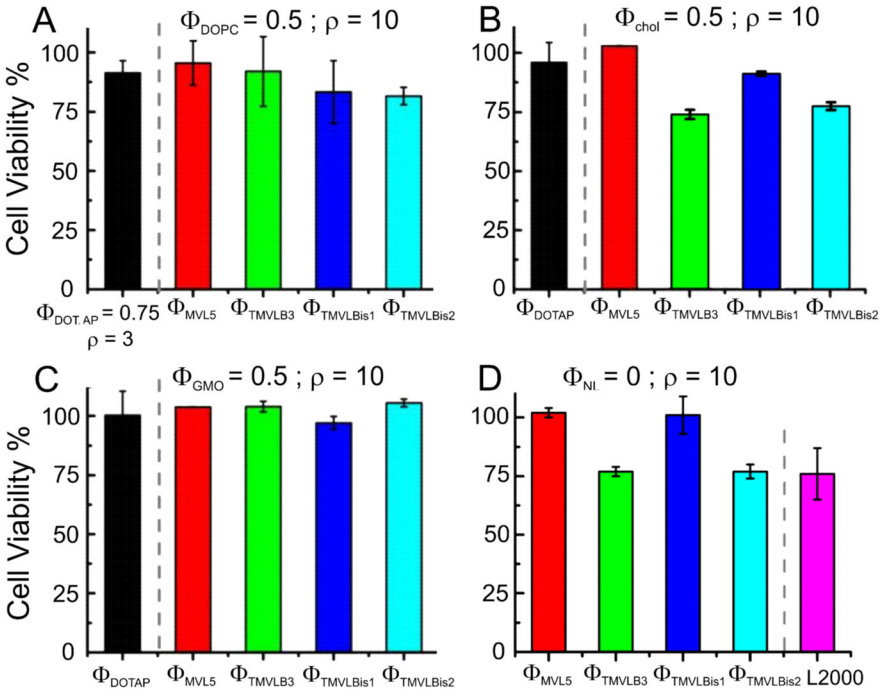

Figure 7. Assessment of the cytotoxicity of representative CL–DNA complexes. The plots show cell viability after treatment with CL–DNA complexes prepared with different neutral lipids: DOPC [(A) $\Phi_{DOPC} = 0.5$, except for DOTAP ($\Phi_{DOPC} = 0.25$)], cholesterol [(B) $\Phi_{Chol} = 0.5$], GMO [(C) $\Phi_{GMO} = 0.5$] and no neutral lipid (D). Complexes were prepared and added to the cells as for transfection ($\rho = 10$ for multivalent lipids, $\rho = 3$ for DOTAP). Toxicity is negligible in many cases, and never worse than that of the commercial benchmark reagent Lipofectamine 2000 (L2000).

(DOTAP; Figures 3A and 6), as well as multivalent (MVL5; Figures 3A and 4A) lipid. Therefore, we did not study mixtures of DOPC with the lipids with dendritic headgroups (DLs; Figure 2) (we did not investigate formulations with DOPE because of their relatively high toxicity and reported poor efficiency *in vivo*) [21,27,43].

Cholesterol (Chol) has been reported to increase the serum resistance of DOTAP-based CL–DNA complexes [53,55]. Our data confirm this: cholesterol was the only neutral lipid (out of the three tested) able to preserve relatively high TE in serum (TE ~ 10^7 RLU/mg protein at 50% serum, TE ~ 10^8 RLU/mg protein at 10% and 20% serum) with univalent DOTAP (Figure 4D).

Chol-based complexes become more serum resistant with decreasing σ_M (increasing Φ_{Chol}) for DOTAP and MVL5 (Figures 4C and 4D), although their starting TE

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drops with σ_M. This is one of the few trends shared by complexes based on monovalent DOTAP and multivalent MVL5. Indeed, our data are consistent with σ_M being a key parameter governing the starting TE and serum resistance of Chol-containing complexes: a drop in starting TE and increase in serum resistance occurs at $\Phi_{Chol} = 0.25$ for DOTAP and at $\Phi_{Chol} = 0.75$ for MVL5, whereas no drop in starting TE and no increased serum resistance is observed for the even more highly charged DLs at the investigated Φ_{Chol}. For the DLs, both starting TE and serum resistance essentially do not change with Φ_{Chol} over the investigated range. Whether σ_M has a direct effect on TE and serum resistance or whether these findings are related to the structure of the complexes is the subject of ongoing investigation.

GMO showed the most diverse and cationic lipid-dependent effects of the investigated neutral lipids. A poor choice in combination with DOTAP, GMO is an excellent helper lipid for MVL5 with a strong effect of Φ_{GMO} on serum resistance (Figure 4B). Serum resistance is maximized at $\Phi_{GMO} = 0.5$, where TE (even at 50% serum) remains nearly as high as in serum-free medium. The serum resistance of MVL5/GMO–DNA complexes with $\Phi_{GMO} = 0.25$ and $\Phi_{GMO} = 0.75$ is also increased compared to $\Phi_{GMO} = 0$.

For the more highly charged DLs, however, Φ_{GMO} scarcely affects starting TE or serum resistance (Figures 5B, 5D and 5F); the biggest effect is seen at $\Phi_{GMO} = 0.75$. Given the success of GMO in combination with the multivalent lipids, the poor TE and serum resistance of DOTAP/GMO–DNA complexes (Figure 3A) was surprising. This highlights once again that monovalent and multivalent lipids can show fundamentally different behavior, as we discuss in more detail below [21,60].

Further work is needed to determine the cause of the uniquely high serum resistance of MVL5/GMO–DNA complexes. The membrane charge densities of MVL5- and DL-based complexes with GMO as the neutral lipid overlap widely. Thus, differences in σ_M are unlikely to be the cause of the differences in serum resistance. Differing complex nanostructures [lamellar (MVL5) [24,25,60] versus nonlamellar (DLs) [7,19,58,59]] or specific interactions between neutral and cationic lipids remain as possible explanations, which are the subject of ongoing investigation.

It is intriguing that, for MVL5/NL–DNA complexes, the serum resistance as a function of Φ_{NL} is distinctly different for the three NLs. It decreases with Φ_{DOPC}, increases with Φ_{Chol} and exhibits a maximum as a function of Φ_{GMO}. This diversity of observed behaviors and their dependence on the cationic lipid makes it challenging to rationalize the differences between neutral lipids based on their structures or properties. Nonetheless, the large, well-hydrated headgroup of DOPC appears to be a feature unfavorable for serum resistance, whereas the small (hydroxy) headgroup of cholesterol (which results in a thinner hydration layer and less permeable membranes) appears favorable. GMO, too, has a fairly small headgroup consisting of two hydroxy groups. Both cholesterol and DOPC have been shown to interact with serum albumin [61–64], the main protein component of serum, although the nature of these interactions may be different and contribute to the difference in serum stability.

Cationic lipids

We observed a number of differences between monovalent and multivalent lipids. For example, GMO reduced the starting TE and serum resistance only for complexes based on monovalent DOTAP. An important and consistent observation is that the response to different levels of serum is also distinctly different for monovalent and multivalent cationic lipids: although the decline of the TE of DOTAP-based complexes with increasing serum content is gradual, the TE of complexes based on multivalent lipids shows the biggest drop from 0% to 10% serum but generally remains very stable beyond that. Interestingly, this is the case even when the drop in TE from 0% to 10% serum is small (Figures 4B and 6) and these are the complexes that performed better than the commercial benchmark, Lipofectamine 2000. All DOTAP-based complexes have very low TE at 50% serum and thus are not good candidate vectors for *in vivo* gene delivery application.

The difference in TE behavior between DOTAP and multivalent lipids with increasing serum content may be related to differences in the efficiency of attachment of anionic serum components. The initial drop at 10% serum and following plateau behavior (where TE is almost constant between 10% and 50% serum content) that we observe for multivalent lipids suggests that the main interactions between complexes and serum components (e.g. adhesion of anionic serum components) occur already at a serum content of between 0% and 10% and that additional interactions between serum components and complexes at higher serum contents are minimal (i.e. for multivalent lipids, $\leq$ 10% serum may be the concentration where anionic serum components have essentially neutralized the complexes). In other words, facile neutralization of complexes as a result of stronger binding by serum components to multivalent lipid-based complexes causes the initial drop in TE that is followed by a plateau. By contrast, higher concentrations of serum are required for complex neutralization for univalent lipids, which exhibit weaker binding. Stronger binding of serum components to multivalent lipid-based complexes is expected because cationic membranes containing

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multivalent lipids are more efficient at counterion condensation at the complex–solution interface than those containing univalent lipids [65]. Efficient counterion condensation leads to a large adhesion energy for binding of anionic serum components to the complex because of the gain in solution free energy from the release of counterions upon binding [66,67].

The most striking feature in the data for DL-based complexes is how TE is almost independent of the choice of NL and Φ_{NL} (Chol versus GMO; we did not investigate DOPC-containing complexes of DLs in detail because of the poor serum resistance of both DOTAP/DOPC–DNA and MVL5/DOPC–DNA complexes). This feature of robustness of TE with respect to Φ_{NL} may prove beneficial for applications that require the addition of neutral lipids to perform distinct functions, such as PEG-lipids. This is why we used TMVLG3/GMO–DNA complexes rather than the equally serum-resistant TMVLG3–DNA complexes (Figures 5A and 5B) for the experiments with the human cell lines.

Intriguingly, the TE of TMVLG3(8+)-based CL–DNA complexes in the presence of serum is higher than both that of complexes based on TMVLBG2(16+) and TMVLBG1(8+). This is because the initial drop in TE (from 0% to 10% serum) is very large (approximately two orders of magnitude) for TMVLBG2 and TMVLBG1 but much less (approximately one order of magnitude) for TMVLG3. This finding is especially surprising considering that TMVLG3 and TMVLBisG1 have the same headgroup charge, exhibit the same very high starting TE at all investigated Φ_{NL} (independent of the neutral lipid) and form the same DL/DOPC–DNA complex structures as a function of Φ_{DOPC} [58]. Thus, not only the headgroup charge of the cationic lipid affects the serum compatibility, but also (and to a large extent) the chemical structure of the headgroup. The DL headgroups are based on internal branching units (ornithine; green background in Figure 2). In the case of TMVLG3, ornithine (with two amino groups; i.e. two positive charges) also serves as the charge-bearing unit attached to the branching units. In the case of TMVLBG1 and TMVLBG2, carboxyspermine (with four amino groups ie. four positive charges), is the charge-bearing unit. The branched core of the TMVLG3 headgroup (1+2 ornithine moieties) is thus larger than that of the TMVLBG1 headgroup (a single ornithine), whereas their overall headgroup size and charge density is comparable. The additional ornithine moieties in the TMVLG3 headgroup provide more hydrogen bond donors and acceptors (nine versus three amide bonds in the headgroup). These increased capabilities for hydrogen bonding may stabilize the membrane and complex against the detrimental effects of serum. In addition, it is possible that the very high headgroup charge of TMVLBG2 is unfavorable for serum resistance (i.e. there may be an optimal range of headgroup charge).

In our transfection experiments with human cell lines, the robust and high performance of TMVLG3/GMO–DNA complexes with respect to both serum resistance and level of TE in the different cell lines stands out. They transfect more efficiently than Lipofectamine 2000 at higher serum content (20% and 50%) in both cell lines. At 10% serum (and without serum for PC3 cells), the TE of these complexes is almost identical to that of Lipofectamine 2000. MVL5/GMO–DNA complexes maintained their high serum resistance in human cell lines but had a surprisingly low starting TE. Thus, our data suggest that the serum resistance of complexes based on multivalent cationic lipids and GMO is independent of the transfected cell line. However, the choice of cationic lipid may have to be optimized to achieve high absolute TE.

Conclusions

Our data show that optimized combinations of a multivalent lipid and a neutral lipid can outperform benchmark commercial transfection reagents such as Lipofectamine 2000 (equal TE at low serum content, higher TE at high serum content). Below, we summarize the findings from the present study in the form of guidelines for optimizing the TE of CL–DNA complexes in the presence of serum (with a focus on high serum content).

If designing a novel cationic lipid (a) a multivalent headgroup is desirable (intermediate valency, i.e. +5*e* and +8*e*, gave the most favorable results) and (b) incorporating a large number of hydrogen bonding donor and acceptor groups in the headgroup appears favorable. Synthesizing a small library of structurally diverse headgroups according to these design principles increases the likelihood of finding a lipid that is efficient in a variety of cell lines. If optimizing a formulation starting from a given cationic lipid, the starting TE of a formulation (TE in the absence of serum) will not predict the TE in the presence of serum. If the cationic lipid is monovalent (a) cholesterol should be the first choice of neutral lipid (at medium to high Φ_{Chol}) and (b) all serum contents of interest should be tested. If the cationic lipid is multivalent (a) cholesterol and GMO are the preferred neutral lipids (b) the TE at 10% serum will likely predict the TE at higher serum contents, and (c) even if the starting TE varies little with Φ_{NL}, this parameter should be varied. In our experience, testing a single, appropriate charge ratio ($\rho = 3$ for monovalent and $\rho = 10$ for multivalent lipids) is sufficient for assessing the potential of a given cationic lipid. In general, optimization may have to be performed separately for each cell line,

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although this is not necessarily the case: certain vectors, such as the TMVLG3/GMO–DNA complexes investigated in the present study, perform extremely well in a variety of cell lines, especially at high serum content. A future challenge is to increase the absolute values of TE for human cell lines.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web site.
Figure S1. The chemical structures of DOTAP and the neutral lipids used in the present study.

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