Efficient Synthesis and Cell-Transfection Properties of a New Multivalent Cationic Lipid for Nonviral Gene Delivery

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Lipid-mediated delivery of DNA into cells holds great promise both for gene therapy and basic research applications. This paper describes the efficient and facile synthesis and the characterization of a new multivalent cationic lipid with a double-branched headgroup structure for gene delivery applications. The synthetic scheme can be extended to give cationic lipids of different charge, spacer, or lipid chain length. The chemical and physical properties of self-assembled complexes of the cationic liposomes (CLs) with DNA give indications of why multivalent cationic lipids possess superior transfection properties. The lipid bears a headgroup with five charges in the fully protonated state, which is attached to an unsaturated double-chain hydrophobic moiety based on 3,4-dihydroxybenzoic acid. Liposomes consisting of the new multivalent lipid and the neutral lipid 1,2-dioleoyl-*sn*-glycerophosphatidylcholine (DOPC) were used to prepare complexes with DNA. Investigations of the structures of these complexes by optical microscopy and small-angle X-ray scattering reveal a lamellar L_α^C phase of CL−DNA complexes with the DNA molecules sandwiched between bilayers of the lipids. Experiments using plasmid DNA containing the firefly luciferase reporter gene show that these complexes efficiently transfect mammalian cells. When compared to the monovalent cationic lipid 2,3-dioleyloxypropyltrimethylammonium chloride (DOTAP), the higher charge density of the membranes of CL−DNA complexes achievable with the new multivalent lipid greatly increases transfection efficiency in the regime of small molar ratios of cationic to neutral lipid. This is desired to minimize the known toxicity effects of cationic lipids.

Introduction

Somatic gene therapy holds great promise for future medical applications, for example, as new treatment for various inherited diseases as well as cancers.[1,2] This has spurred large research efforts directed toward developing and fundamentally understanding efficient DNA carriers, i.e., vectors. Viral vectors have been studied and used extensively,[3,4] but synthetic nonviral vectors have attracted increasing interest over the past years because of their inherent advantages.[5,6] These include their easy and variable preparation, unlimited length of the transported DNA, and lack of immunogenicity. In nonviral vectors, the negatively charged DNA is complexed with cationic liposomes[7−10] or cationic polyelectrolytes.[11−13] Cationic liposome−DNA (CL−DNA) complexes have already been taken to the stage of clinical trials[14] and are used in other applications of DNA delivery.[15] For them to become widely useful for gene therapeutical purposes, however, the efficiency of synthetic vectors still needs to be improved.

To improve the efficiency of CL−DNA complexes, a large number of cationic lipids have been synthesized. The early results of this research have been reviewed in detail,[16−18] and several more recent reviews with less broader scope have also appeared.[19−21] Among the various chemical approaches that have been taken to improve CL−DNA complexes, the use of cationic lipids with multivalent headgroups has been especially promising. High transfection efficiencies have been reported for multivalent lipids such as DOGS,[22] DOSPA,[23] and RPR120535[9] and several of its structural analogues.

A large amount of work has also been performed in order to understand formation of CL−DNA complexes, their structures, and their mechanism of action.[24] For example, CL−DNA complexes were shown to assume an inverted hexagonal (H_{II}^C) or lamellar (L_α^C) liquid crystal (LC) phase, depending on the neutral lipid used and the composition.[25] Significant differences in the transfection properties of hexagonal and lamellar complexes have been reported.[26] The majority of the complexes studied so far display the lamellar L_α^C phase.

As part of our ongoing investigations of structure−property relationships of CL−DNA complexes and the mechanism of their action, we were in need of sufficient quantities of multivalent cationic lipids to examine the behavior of their DNA complexes using small-angle X-ray diffraction and other methods. The scales on which the synthesis of RPR120535 was described[9,27] and on which DOGS is commercially available were insufficient to meet our needs. Moreover, the synthesis of DOSPA has not been published[16] and that of DOGS has not been described in sufficient detail to allow for a straightforward synthesis.[28] This prompted us to investigate efficient synthetic procedures for multivalent cationic building blocks of variable charge. We report

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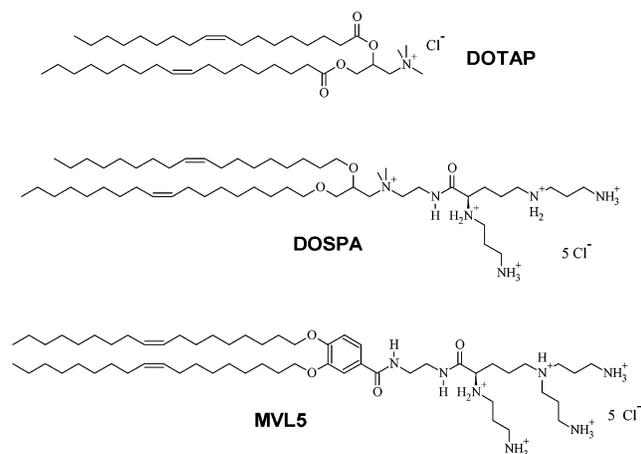† University of California.
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Figure 1. Structures of the common monovalent lipid DOTAP,[31] the multivalent cationic lipid DOSPA,[23] and the new lipid MVL5.
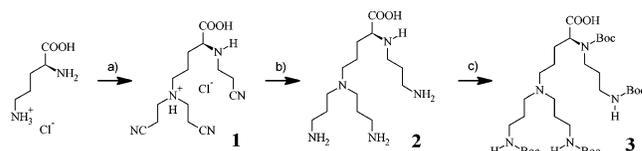

Figure 2. Synthesis of the pentavalent headgroup building block **3** starting from ornithine hydrochloride: (a) 4 equiv of acrylonitrile, NaOH, 60 °C; (b) Raney Ni, 60 psi of H_2; (c) Boc$_2$O, NaOH.

here in detail the gram-scale synthesis of a new multivalent cationic headgroup and lipid. CL−DNA complexes were prepared using mixtures of this lipid, DOPC, and DNA. The structure of the lipid−DNA complexes was probed on length scales from subnanometer to micrometer by X-ray diffraction and optical microscopy. The CL−DNA complexes appear as condensed globules in solution, which aggregate in the presence of salts. X-ray diffraction shows that the complexes are in the L_α^C phase as found for complexes of DNA with other, simpler lipids (e.g., DOTAP/DOPC[29]). The DNA chains form a one-dimensional lattice in which they are highly condensed even in the presence of cell culture medium (DMEM[30]), i.e., salts, owing to the high membrane charge density. When compared with an optimized DOTAP/DOPC system, complexes with the new lipid exhibit improved transfection efficiency, and the difference between the two systems increases dramatically from 1 to 3 orders of magnitude as the amount of cationic lipid is reduced from 50 to 20 mol %. As we describe, this very large difference in transfection efficiency between the univalent and multivalent cationic lipids at high mole percents of neutral lipids results from the much larger charge density of the membranes of the CL−DNA complexes of multivalent cationic lipids. For the new lipid, the transfection efficiency remains essentially unchanged, while it drops quickly for DOTAP. This is of great importance for potential applications, since the cationic lipid is the more expensive compound and the toxicity of cationic lipids is a known problem in clinical applications. Our in vitro studies should apply primarily to the optimization of transfection efficiencies in ex vivo cell transfection studies, where cells are removed and returned to patients after transfection.

Results and Discussion

Lipid Design and Synthesis. The chemical structure of our new lipid MVL5 is shown in Figure 1, together with that of the multivalent cationic lipid DOSPA and the monovalent lipid DOTAP,[31] which is frequently used for comparison. MVL5 is based on the basic hydrophobic moiety reported in a previous paper.[32] The lipid has unsaturated hydrophobic chains similar to DOSPA and DOTAP to prevent side chain crystal-

lization and a headgroup that is structurally similar to that of DOGS but bears five charges in the fully protonated state to realize a high membrane charge density.

The synthesis of MVL5 makes use of carboxy-functionalized lipid and headgroup building blocks, which are linked by an ethylenediamine spacer. This synthetic scheme can easily be extended to give lipids of different charge, spacer, or lipid chain length. As depicted in Figure 2, the first step in the synthesis of the multivalent building block **3** is the Michael addition of acrylonitrile to ornithine. This method has been used by others in the synthesis of carboxyspermine[28,33] where an addition of one molecule of acrylonitrile to each amino group of ornithine took place. A similar reaction has been used to synthesize propylenimine dendrimers,[34,35] yielding two additions on every amino group. Unexpectedly, we found that in the reaction with ornithine conditions can be tuned to achieve a total of three additions, two on the δ-nitrogen and one on the α-nitrogen to yield compound **1**. Following hydrogenation of the nitrile moieties and Boc-protection of all amino groups, compound **3** was obtained. To allow coupling with this headgroup unit, the lipid building block **4**[32] was reacted with excess ethylenediamine. The resulting compound **5** was then coupled with **3** to yield the Boc-protected multivalent lipid **6** as shown in Figure 3. Deprotection of the amino groups yielded the lipid MVL5.

Characterization of CL−DNA Complexes. CL−DNA complexes were formed by combining DNA and cationic liposomes consisting of mixtures of DOPC and MVL5 at different ratios. In addition to transfection experiments, we studied the complexes in situ using optical microscopy and small-angle X-ray scattering (SAXS) to probe their structures on multiple length-scales. All measurements were performed in DMEM and at the same lipid to DNA ratio as in our transfection experiments.

Optical Microscopy. Direct imaging of the complexes was performed using DIC and double-fluorescence microscopy. In Figure 4, complexes prepared in water (A) and DMEM (B) are shown, imaged in DIC mode (left), DNA fluorescence mode (middle), and lipid fluorescence mode (right). The complexes were made from DOPC/MVL5 lipid mixtures containing 40 wt % MVL5 at a cationic lipid to DNA weight ratio of 2.5, corresponding to a charge ratio of 2.85. They form distinct globules of about 1 μm diameter. In DMEM, these globules aggregate to form larger particles because their charge is screened by the relatively high salt concentration (Figure 4B). The observation of colocalization of lipid and DNA by fluorescence microscopy indicates the formation of complexes.
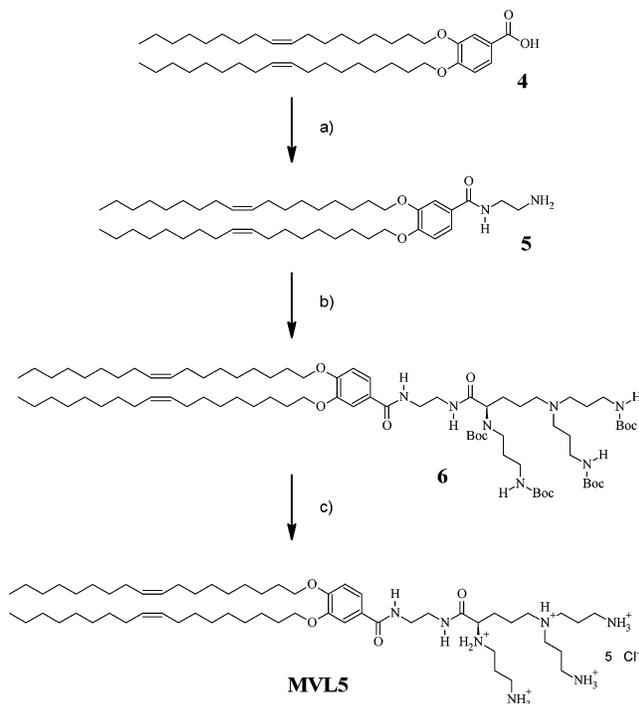
Figure 3. Synthesis of the pentavalent cationic lipid MVL5: (a) (1) *N*-hydroxysuccinimide, *N,N*-dicyclohexylcarbodiimide (DCC), (2) excess ethylenediamine; (b) **3**, DCC, 1-hydroxybenzotriazole; (c) HCl in EtOAc.
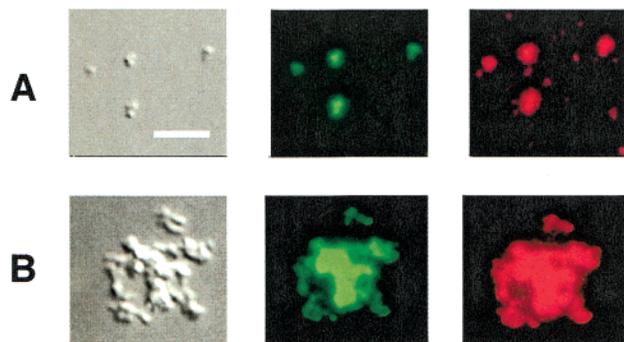

Figure 4. Typical images of CL−DNA complexes prepared in water (A) and DMEM (B) from DOPC, MVL5, and plasmid−DNA in high-resolution DIC mode (left), DNA fluorescence mode (middle), and lipid fluorescence mode (right). The DNA and the lipid were stained using YOYO dye (green fluorescence) and DHPE-Texas Red dye (red fluorescence), respectively. The size of the scale bar is 5 μm. The observed double fluorescence of the globules indicates the presence of both DNA and lipid in the complex particles. The complexes in these pictures were made with 31 mol % MVL5 at a cationic lipid to DNA charge ratio of 2.85.

Cell Transfection. For transfection studies, we compared CL−DNA complexes prepared with DOTAP or MVL5 as the cationic lipid. While the ratio of neutral to cationic lipid was varied, all complexes were prepared at a lipid to DNA charge ratio of 2.85, which was previously found to be optimal for complexes made from DOTAP and DOPC.[36] The only partial protonation of the MVL5 headgroup at neutral pH was taken into account by assigning 4 unit charges to it. The results of transfection experiments using mouse fibroblast L cells and a luciferase reporter assay are displayed in Figure 5. The new lipid MVL5 gives higher transfection efficiencies for all ratios of neutral to cationic lipid.
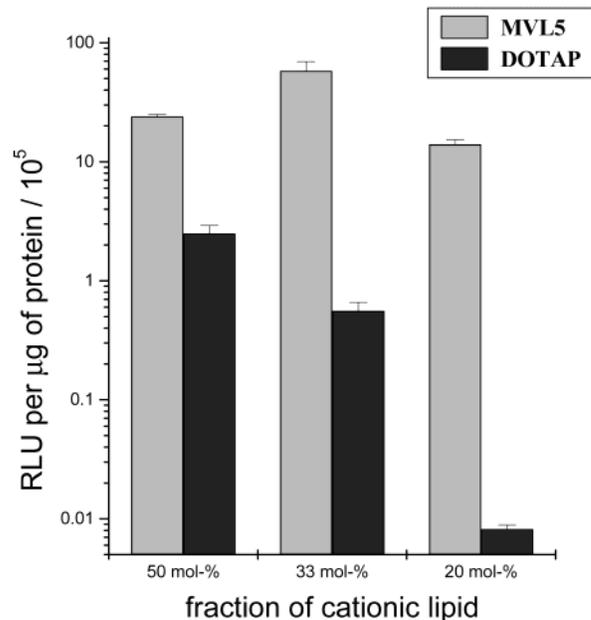

Figure 5. Comparison of transfection efficiencies in mouse fibroblast L cells for cationic lipids DOTAP and MVL5 in mixtures with DOPC. Data are shown for molar ratios 1:1, 1:2, and 1:4 neutral to cationic lipid. The lipid to DNA charge ratio was kept at 2.85 in all experiments.

However, the difference in transfection efficiencies increases dramatically from 1 to 3 orders of magnitude as the amount of cationic lipid is reduced from 50 to 20 mol %. For MVL5, the transfection efficiency remains essentially unchanged while it drops quickly for DOTAP. This is important for potential applications, since the cationic lipid is the more expensive compound and the toxicity associated with large doses of cationic lipids is a problem for clinical applications.

X-ray Diffraction. The structures of the CL−DNA complexes on the scale of 1−100 nm were investigated by SAXS. In Figure 6, high-resolution X-ray scans are shown for complexes with weight fractions of cationic lipid of 40% (MVL5) and 30% (DOTAP), corresponding to equivalent molar fractions (31% and 33%, respectively) of cationic lipid in the membrane. At this composition, the transfection efficiency of the MVL5 complexes is approximately 100-fold higher than that of the DOTAP complexes. The lipid to DNA charge ratio was again kept at 2.85 for all complexes. Both CL−DNA complexes are in the lamellar L_α^C phase. As with DOTAP, the L_α^C phase is observed throughout the lipid composition range for MVL5/DOPC/DNA complexes. For the MVL5 complexes in Figure 6, three relatively sharp peaks at $q = 0.088$, 0.175, and 0.261 Å^{-1} are observed, corresponding to the (00h) peaks of the layered structure with an interlayer spacing $\delta = 2\pi/q_{001}$ of 71.2 Å (Figure 6B). The broad peak at $q_{DNA} = 0.201$ Å^{-1} arises from correlations between DNA molecules within a water gap and gives their interaxial spacing $d_{DNA} = 2\pi/q_{DNA} = 31.2$ Å. This spacing is much larger ($q_{DNA} = 0.106$ Å^{-1}, i.e., $d_{DNA} = 59.5$ Å) for the complexes prepared with an equivalent molar ratio of the monovalent lipid DOTAP. As was shown previously, at the isoelectric point of L_α^C CL−DNA complexes, the average spacing d_{DNA} between the DNA molecules can be expressed in terms of the average distance per anionic charge along the DNA backbone (l_0) and the charge density (σ_M) of the mem-
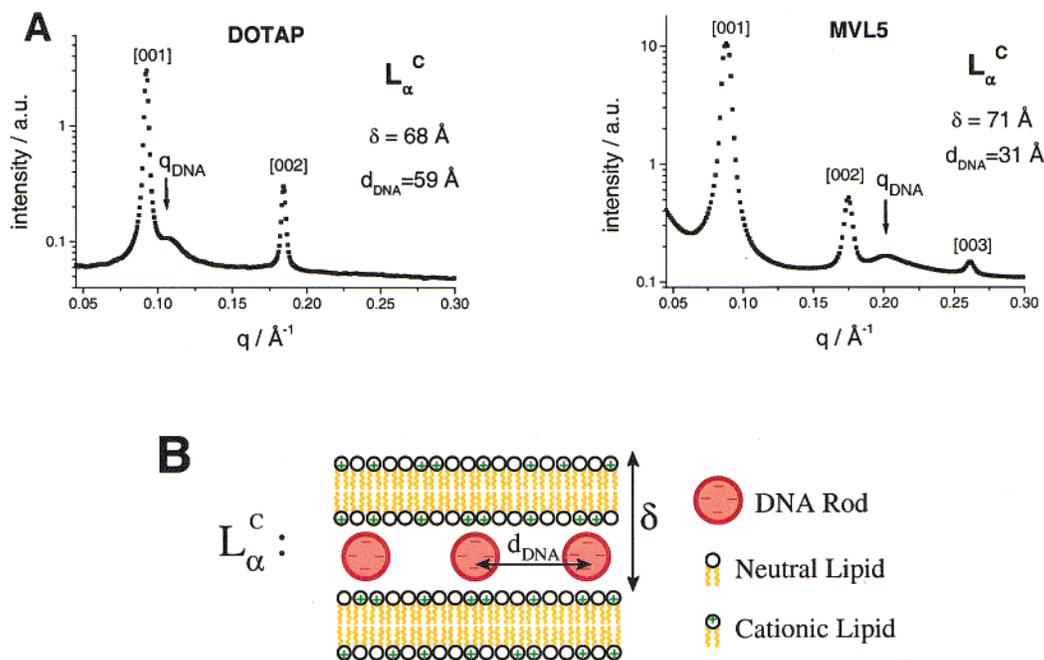
Figure 6. (A) SAXS scans of CL−DNA complexes in DMEM made from DOPC/DOTAP (left) and DOPC/MVL5 (right) lipid mixtures at the same molar ratios. The complexes are in the lamellar L_α^C phase, as evident from the [00h] diffraction peaks. The lipid mixtures contained 30 wt % DOTAP and 40 wt % MVL5, respectively, corresponding to a molar ratio of cationic to neutral lipid of 31:69 (A) and 33:67 (B). The cationic lipid to DNA charge ratio was 2.85 for both complexes. The interlamellar spacing is 68 Å for DOTAP and 71 Å for MVL5. At the same molar ratio of cationic to neutral lipid, the DNA interaxial spacing is reduced from 59 to 31 Å because of the higher membrane charge density in the complexes with MVL5. (B) Schematic of a CL−DNA complex in the L_α^C phase, displaying a condensed multilamellar structure with DNA rods intercalated between lipid bilayers. The key length scales are the bilayer spacing δ and the interaxial DNA spacing d_{DNA}.

branes of CL−DNA complexes, $d_{DNA} = e/(l_0\sigma_M)$.[37,29] Thus, as σ_M increases, the DNA interaxial spacing d_{DNA} decreases, and σ_M is the main parameter that determines d_{DNA}. Therefore, our data prove that because of the higher headgroup charge of MVL5, σ_M is much higher for an MVL5-containing membrane than for one containing DOTAP at an identical molar ratio. This corresponds to a higher density of the DNA and stabilizes the complexes at moderate ionic strength. X-ray experiments show that with MVL5, stable CL−DNA complexes can be prepared in the presence of up to 500 mM monovalent salt.[38] Most importantly, our experiments provide a rationale for the generally superior efficiency of multivalent lipids. The membrane charge density in CL−DNA complexes in the L_α^C phase has recently been identified as a universal parameter enhancing their transfection efficiency,[39] with the transfection efficiency increasing exponentially for a linear increase of σ_M. Thus, the higher headgroup charge of multivalent lipids, which increases the complexes' membrane charge density, leads to improved transfection.

Experimental Section

General Methods. NMR spectroscopy was carried out on a Bruker Avance 200 MHz spectrometer. MALDI-TOF mass spectrometry was done on a Dynamo spectrometer from Thermal BioAnalysis Ltd. using 2,5-dihydroxybenzoic acid as the matrix and 70 vol % acetonitrile in water as the solvent. Thin-layer chromatography was performed using silica-covered plastic sheets with fluorescence indicator (Macherey-Nagel). Detection of spots was achieved using UV light and ninhydrin reagent (300 mg in 95 mL of 2-propanol and 5 mL of acetic acid). By addition of methanol to a 4:2:1 (v/v/v) mixture of chloroform, methanol, and 25% ammonia until the mixture became homogeneous, solvent mixture A was obtained. Silica

gel from Fisher Scientific with a mesh size of 200−425 was used for flash chromatography.[40]

Liposome Preparation. Preparation of liposome solutions was as described previously.[37] In brief, lipid solutions (DOTAP, DOPC from Avanti Lipids) in chloroform/methanol were prepared at the desired ratio of lipids and dried, first by a flow of nitrogen and subsequently in a vacuum for 8−12 h. The residue was suspended in high-conductivity (18.2 MΩ) water, incubated for 12 h, sonicated to clarity, and filtered through 0.2 μm filters to give solutions of the desired concentration.

Transfection. Mouse fibroblast L cells were cultured and transfected as reported elsewhere.[36] Briefly, cells seeded at a number of approximately 300 000 in 6-well plates were transfected with CL−DNA complexes containing 2 μg of luciferase plasmid DNA (pGL3 Control Vector, Promega). Complexes were diluted to a final volume of 1 mL in DMEM (Gibco BRL) and transferred onto the cells. After 6 h, the cells were washed free of complexes with phosphate-buffered saline, followed by a 24 h expression period in culture medium. Results were quantified using the Promega Luciferase assay system (E4030) and a Perkin-Elmer AutoLumat luminometer. The BioRad protein assay (500-0001) was used to determine the amount of protein for normalization.

Optical Microscopy. A Nikon Diaphot 300 equipped for epifluorescence and differential interference contrast (DIC) and a Nikon Coolpix 990 digital camera were used. For fluorescence microscopy, liposome stock solutions were prepared at 0.5 mg/mL with 0.2 mol % DHPE-Texas Red dye (Molecular Probes). The DNA (0.1 mg/mL) was stained using 3.3 mol % (calculated per base pair) YOYO dye (Molecular Probes). Samples were prepared by diluting the stock solutions 6-fold with water or DMEM and combining appropriate volumes of the resulting liposome and DNA solutions. The complexes were imaged 20 min after preparation.

Small-Angle X-ray Scattering (SAXS). High-resolution X-ray experiments were done at the Stanford Synchrotron Radiation Laboratory (SSRL). For typical powder samples, θ−2θ scans were obtained using an image plate detector (Mar

instruments). Samples were prepared from 10 mg/mL liposome solutions and 5 mg/mL DNA solutions, using 100 μg of λ-phage DNA (New England Biolabs) per sample. The CL−DNA complexes were formed by combining the solutions, diluting 2-fold in DMEM, and incubating them at 4 °C for 2−3 days following extensive centrifugation in an Eppendorf tube. Samples were then transferred to 1.5 mm quartz capillaries and flame-sealed. Typically the CL−DNA complexes formed a white precipitate.

Synthesis. Chemicals were from Fisher Scientific and at least of analytical grade unless otherwise indicated.

***N*$_\alpha$,*N*$_\delta$,*N*$_\delta$-Tris(2-cyanoethyl)ornithine (1).** To a solution of 4.00 g (100 mmol) of sodium hydroxide in 120 mL of methanol, a total of 16.9 g (100 mmol) of L-ornithine hydrochloride (Sigma) was added with stirring. After 2 min, a total of 26.3 mL (21.2 g, 400 mmol) of acrylonitrile (Fluka) was added in one portion. Stirring was continued for 24 h at room temperature, and a total of 100 mL of methanol was added. The reaction mixture was then acidified slightly by the addition of concentrated hydrochloric acid (about 13 mL) and was filtered immediately. The pH of the filtrate was adjusted to 6 using 25% sodium hydroxide solution. After standing at 4 °C for 16 h, the mixture was filtered to yield 20.3 g (62 mmol, 62%) of **1** (hydrochloride form) as white crystals. $R_f = 0.79$ (solvent mixture A). ^{1}H NMR (D$_2$O, 200 MHz): δ 1.5−2.3 (2 m, 4 H, C**H**$_2$−C**H**$_2$−CH), 2.6−3.4 (2 m, 12 H, C**H**$_2$−CH$_2$−CN), 3.4−4.0 (2 m, 3 H, C**H**$_2$−CH$_2$−CH$_2$−C**H**). ^{13}C NMR (D$_2$O): δ 15.4, 15.8 (**C**H$_2$−CN), 22.1 (**C**H$_2$−CH$_2$−CH), 27.7 (**C**H$_2$−CH), 42.4, 48.6 (**C**H$_2$−CH$_2$−CN), 52.4 (**C**H$_2$−CH$_2$−CH$_2$−CH), 63.0 (**C**H), 118.0, 121.2 (**C**N), 173.5 (**C**O$_2$H).

***N*$_\alpha$,*N*$_\delta$,*N*$_\delta$-Tris(3-aminopropyl)ornithine (2).** To a mixture of 9.6 g (36 mmol) of **1** and 80 mL of 1.4 M sodium hydroxide in 95% ethanol in a Parr hydrogenator flask, a total of 5.00 g of a supension of Raney nickel in water (Fluka) was added. The mixture was agitated for 16 h under 60 psi (4 bar) of hydrogen pressure at room temperature. The Raney nickel was removed by filtration, and the solvent was evaporated. The resulting oil (15.5 g) was used for the next step without further purification. Pure hydrochloride for analytical purposes was obtained by adding concentrated hydrochloric acid to the reaction solution and collecting and drying the precipitate. ^{1}H NMR (D$_2$O, 200 MHz): δ 1.65−2.30 (m, 10 H, C−C**H**$_2$−C), 2.95−3.50, (m, 14 H, C**H**$_2$−N), 3.65−3.8 (m, 1 H, C**H**). ^{13}C NMR (D$_2$O): δ 20.0, 22.2 (2 C), 24.3, 27.0 (C−**C**H$_2$−C), 37.1 (3 C, **C**H$_2$−NH$_3^+$), 44.4 (**C**H$_2$−NH$_2^+$), 50.4 (2 C), 52.6 (**C**H$_2$−NH$^+$), 62.3 (**C**H), 173.3 (**C**O$_2$H).

***N*$_\alpha$-*tert*-Butylcarbamoyl-*N*$_\alpha$,*N*$_\delta$,*N*$_\delta$-tris(3-[*tert*-butylcarbamoylamino]propyl)ornithine (3).** The raw product from the previous step (15.5 g; assay calculated for 36 mmol = 100% yield from previous step) was dissolved in water/THF (1:1, v/v). Over a period of 1 h, a solution of 34.5 g (158 mmol) of Boc$_2$O (Novabiochem) in 70 mL of THF and 40 mL of 4 M sodium hydroxide in water were added in four portions with stirring. The reaction mixture was kept at room temperature by cooling with a water bath, and stirring was continued for 6 h. Most of the THF was then evaporated. After addition of 250 mL of diethyl ether, the mixture was acidified using half-concentrated hydrochloric acid. The phases were separated, and the aqueous phase was extracted two more times with 150 mL of ether. The combined organic phases were washed twice with 250 mL of water, dried (Na$_2$SO$_4$), and evaporated. The residue was purified by flash chromatography on 800 g of silica gel using chloroform/methanol (19:1) as the eluent[41] to yield 22.5 g (32.0 mmol, 89%) of **3** as a colorless solid. $R_f = 0.40$ (6:1 CHCl$_3$/MeOH). ^{1}H NMR (CDCl$_3$, 200 MHz): δ 1.39 (s, 36 H, C**H**$_3$), 0.7−2.2 (2 m, 10 H, C−C**H**$_2$−C), 2.8−3.4 (2 m, 14 H, C**H**$_2$−N), 4.0−4.2 (m, 1 H, C**H**), 5.0−5.6 (b, 2 H, N**H**), 5.9−6.3 (b, 1 H, N**H**). ^{13}C NMR (CDCl$_3$): δ 21.2 (−**C**H$_2$−CH$_2$−CH), 24.3 (−**C**H$_2$−CH$_2$−N−(CH$_2$)$_3$−CH), 27.4 (CH−N(Boc)−CH$_2$−**C**H$_2$), 28.8 (C**C**H$_3$), 30.1 (−**C**H$_2$−CH), 38.0 (**C**H$_2$−N(H)Boc), 45.7 (**C**H$_2$−N(Boc)−CH), 50.6, (**C**H$_2$−N−(CH$_2$)$_3$−CH), 52.9 (**C**H$_2$−CH$_2$−CH$_2$−CH), 59.7 (**C**H), 79.9, 81.2 (**C**CH$_3$), 156.9 (O−**C**(O)−N), 173.9 (**C**O$_2$H). MALDI-MS: *m/z* 705.8 (M + H$^+$).

***N*1-(2-aminoethyl)-3,4-di(oleyloxy)-benzamide (5).** To a solution of 4.00 g (6.11 mmol) of **4** in 10 mL of dichloromethane, 0.77 g (6.72 mmol) of *N*-hydroxysuccinimide (Bachem) and, after stirring for 5 min, 1.39 g (6.72 mmol) of *N,N*-dicyclohexylcarbodiimide (DCC, Sigma) in 2 mL of dichloromethane were added. The reaction mixture was stirred for 2 h and then filtered. To the filtrate, a total of 4.1 mL (3.7 g, 61 mmol) of ethylenediamine was added quickly with stirring. After being stirred for 3 h, the reaction mixture was filtered and 200 mL of chloroform, 50 mL of methanol, and 100 mL of water were added. The phases were separated after thorough mixing. The organic phase was then extracted two more times with water to which NaCl and some NaOH had been added, dried (Na$_2$SO$_4$), and evaporated. The resulting residue was purified by flash chromatography on 250 g of silica gel using chloroform/methanol (9:1) as the eluent to yield 3.66 g (5.25 mmol, 86%) of **5** as an off-white, waxy solid. $R_f = 0.22$ (9:1 CHCl$_3$/MeOH). ^{1}H NMR (CDCl$_3$, 200 MHz): δ 0.84 ("t", $J = 6.7$ Hz, 6 H, CH$_2$−C**H**$_3$), 1.0−2.5 (4 m, 56 H, alkyl−C**H**$_2$), 2.90 (t, $^3J = 6.1$ Hz, 2 H, H$_2$N−C**H**$_2$), 3.46 (q, $^3J = 5.9$ Hz, 2 H, C(O)−NH−C**H**$_2$), 3.8−4.2 (m, 4 H, O−C**H**$_2$), 5.2−5.5 (m, 4 H, =C**H**−), 6.61 (bt, 1 H, C(O)−N**H**), 6.81 (d, $^3J = 8.4$ Hz, 1 H, **H**$_{ar}$ *m*-C(O)), 7.25 (dd, $^3J = 8.4$ Hz, $^4J = 2.1$ Hz, 1 H, **H**$_{ar}$ *p*-OR), 7.38 (d, $^4J = 2.1$ Hz, 1 H, **H**$_{ar}$ *o*-C(O), *o*-OR). ^{13}C NMR (CDCl$_3$): δ 14.5 (**C**H$_3$), 23.1 (**C**H$_2$−CH$_3$), 26.5, 27.6, 29.7, 29.8, 30.0, 30.1, 30.2, 30.3, 32.3 (**C**H$_2$−**C**H$_2$−C), 40.6, 40.1 (**C**H$_2$−N), 69.8, 69.3 (**C**H$_2$−O), 112.4, 113.3, 121.3, (aromatic **C**H), 125.9 (**C**−C(O)N), 130.2, 130.3 (=**C**H−CH$_2$), 149.0, 152.6 (**C**−O−CH$_2$), 168.8 (**C**(O)NH).

***N*1-[2-((1*S*)-1-[*tert*-Butylcarbamoylamino(3-[*tert*-butylcarbamoylamino]propyl)amino]-4-[di(3-[*tert*-butylcarbamoylamino]propyl)amino]butylcarboxamido)ethyl]-3,4-di[oleyloxy]benzamide (6).** To a solution of 615 mg (0.88 mmol) of **5**, 619 mg (0.88 mmol) of **3**, and 135 mg (0.88 mmol) of 1-hydroxybenzotriazole (HOBt, Peptides International) in 5 mL of dichloromethane, a total of 197 mg (0.955 mmol) of DCC in 0.5 mL of dichloromethane was added. After being stirred for 6 h, the reaction mixture was filtered and evaporated. The resulting residue was dissolved in 40 mL of diethyl ether, and the solution was washed with 10% citric acid, twice with saturated NaHCO$_3$, and with water. The ether solution was dried (Na$_2$SO$_4$) and evaporated, and the residue was purified by flash chromatography on 60 g of silica gel using chloroform/methanol (15:1) as the eluent[41] to yield 1.095 g (0.792 mmol, 90%) of **6** as a colorless solid. $Rf = 0.32$ (9:1 CHCl$_3$/MeOH). ^{1}H NMR (D$_2$O, 200 MHz): δ 0.85 ("t", $J = 6.7$ Hz, 6 H, CH$_2$−C**H**$_3$), 1.40 (s, 36 H, C**H**$_3$), 1.0−2.6 (several b/m, 66 H, C−C**H**$_2$−C), 2.8−3.3 (m, 14 H, C**H**$_2$−N), 3.3−3.6 (b, 4 H, N−C**H**$_2$−C**H**$_2$−N), 3.8−4.2 (m, 4 H, O−C**H**$_2$), 5.1−5.3 (b, 1 H, C**H**), 5.2−5.4 (m, 4 H, =C**H**−), 6.61 (bt, 1 H, C(O)−N**H**), 6.80 (d, $^3J = 8.3$ Hz, 1 H, **H**$_{ar}$ *m*-C(O)), 7.0−7.2 (b, N**H**), 7.33 (dd, $^3J = 8.3$ Hz, $^4J = 2$ Hz, 1 H, **H**$_{ar}$ *p*-OR), 7.44 (d, $^4J = 2$ Hz, 1 H, **H**$_{ar}$ *o*-C(O), *o*-OR). ^{13}C NMR (D$_2$O): δ 14.5 (**C**H$_2$−**C**H$_3$), 23.1 (**C**H$_2$−CH$_3$), 26.5, 27.3, 27.6, 29.6, 29.7, 29.8, 29.9, 30.2 (**C**H$_2$−**C**H$_2$−CH$_2$, =CH−**C**H$_2$), 32.3 (CH$_3$−**C**H$_2$−CH$_2$), 28.7, 28.9 (C(**C**H$_3$)$_3$), 38.6, 39.5, 41.7 (**C**H$_2$−N(H)−C(O)), 43.9 (**C**H$_2$−N(Boc)−CH), 52.0, 53.7, (**C**H$_2$−N−**C**H$_2$−(CH$_2$)$_2$−CH), 60.2 (**C**H), 69.5, 69.6 (**C**H$_2$−O), 79.4, 81.3 (**C**(CH$_3$)$_3$), 112.6, 113.3, 120.3, (aromatic **C**H), 127.0 (**C**$_{ar}$−C(O)), 130.2, 130.4 (=**C**H−CH$_2$), 149.2, 152.2 (**C**$_{ar}$−O−CH$_2$), 156.5 (O−**C**(O)−N), 167.8 (**C**(O)−C$_{ar}$), 173.3 (**C**(O)−CH). MALDI-MS: *m/z* 1382.6 (M + H$^+$).

***N*1-[2-((1*S*)-1-[(3-Aminopropyl)amino]-4-[di(3-aminopropyl)amino]butylcarboxamido)ethyl]-3,4-di[oleyloxy]-benzamide (MVL5).** To a concentrated solution of 1.00 g (0.72 mmol) of **6** in ethyl acetate, a total of 10 mL of a freshly prepared saturated solution of HCl in ethyl acetate was added. The mixture was stirred for 30 min at room temperature, during which time a white precipitate formed, and evaporated. To the residue, a total of 5 mL of ethyl acetate was added with stirring and was evaporated. This was repeated to yield 830 mg (0.71 mmol, 99%) of MVL5 as a white powder. $R_f = 0.64$ (2:2:1 CHCl$_3$/MeOH/25% NH$_4$OH). ^{1}H NMR (CDCl$_3$/MeOH-*d*$_4$, 9:1, 200 MHz): δ 0.74 ("t", $J = 6.7$ Hz, 6 H, C**H**$_3$), 0.9−1.5

(b/m, 44 H, C−CH$_2$−C), 1.5−2.5 (3 b/m, 22 H, C−CH$_2$−C), 2.7−3.6 (b/m, N−CH$_2$), 3.6−4.4 (m, 4 H, CH, O−CH$_2$), 5.0−5.4 (m, 4 H, =CH−), 6.6−6.9 (m, 1 H, H$_{ar}$ *m*-C(O)), 7.2−7.5 (m, 2 H, H$_{ar}$ *o*-C(O)), 8.16 (b, 3 H, NH). ^{13}C NMR (CDCl$_3$/MeOH-*d*$_4$, 9:1): δ 13.8 (CH$_2$−CH$_3$), 22.4 (CH$_2$−CH$_3$), 24.6, 25.9, 26.0, 27.0, 27.9, 29.1, 29.2, 29.3, 29.4, 29.5, 29.6, 31.7, 32.3, 32.6 (C−CH$_2$−C), 33.9, 37.2, 38.7, 39.8 (b, CH$_2$−N), 51.3 (CH), 68.9, 69.4 (CH$_2$−O), 112.1, 112.9, 120.7 (aromatic CH), 125.6 (C$_{ar}$−C(O)), 129.5, 129.7 (=CH−CH$_2$), 148.5, 152.1 (C$_{ar}$−O−CH$_2$), 167.3, 168.0 (C(O)−C$_{ar}$). MALDI-MS: *m*/*z* 983.6 (M + H$^+$), 1005.5 (M + Na$^+$).

Conclusions

Following an efficient synthetic scheme, we have prepared the new multivalent lipid MVL5 and examined its behavior in lipid−DNA complexes using microscopy, X-ray diffraction, and transfection assays. Our experiments provide a rationale for the generally superior efficiency of multivalent lipids. The membrane charge density σ$_M$ in CL−DNA complexes has recently been identified as a universal parameter controlling and enhancing their transfection efficiency.[39] In the L$_α^C$ phase of cationic lipid−DNA complexes, transfection efficiency increases exponentially with a linear increase of σ$_M$ [which also results in a linear decrease in the DNA−DNA interaxial distance (*d*$_{DNA}$)]. Our data show that the membrane charge density σ$_M$ is much higher for an MVL5-containing membrane than for one containing monovalent DOTAP at an identical molar ratio. Thus, the higher headgroup charge of multivalent lipids, which increases the complexes' membrane charge density, leads to improved transfection.

References

(1) Felgner, P. L.; Rhodes, G. Gene Therapeutics. *Nature* **1991**, *349*, 351−352.
(2) Smyth-Templeton, N., Lasic, D. D., Eds. *Gene Therapy. Therapeutic Mechanisms and Strategies*; Marcel Dekker Inc.: New York, 2000.
(3) Smith, A. E. Viral Vectors in Gene Therapy. *Annu. Rev. Microbiol.* **1995**, *49*, 807−838.
(4) Kay, M. A.; Glorioso, J. C.; Naldini, L. Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics. *Nat. Med.* **2001**, *7*, 33−40.
(5) Felgner, P. L., Heller, M. J., Lehn, P., Behr, J.-P., Szoka, F. C., Eds. *Artificial Self-Assembling Systems for Gene Delivery*; American Chemical Society: Washington, DC, 1996.
(6) Mahato, R. I., Kim, S. W., Eds. *Pharmaceutical Perspectives of Nucleic Acid-Based Therapeutics*; Taylor and Francis: London and New York, 2002.
(7) Felgner, P. L.; Gader, T. R.; Holm, M.; Roman, R.; Chan, H. W.; Wenz, M.; Northrop, J. P.; Ringold, G. M.; Danielsen, M. Lipofection: A highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 7413−7417.
(8) Wheeler, C. J.; Felgner, P. L.; Tsai, Y. J.; Marshall, J.; Sukhu, L.; Doh, S. G.; Hartikka, J.; Nietupski, J.; Manthorpe, M.; Nichols, M.; Plewe, M.; Liang, X.; Norman, J.; Smith, A.; Cheng, S. H. A novel cationic lipid greatly enhances plasmid DNA delivery and expression in mouse lung. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 11454−11459.
(9) Byk, G.; Dubertret, C.; Escriou, V.; Frederic, M.; Jaslin, G.; Rangara, R.; Pitard, B.; Crouzet, J.; Wils, P.; Schartz, B.; Scherman, D. Synthesis, Activity, and Structure−Activity Relationship Studies of Novel Cationic Lipids for DNA Transfer. *J. Med. Chem.* **1998**, *41*, 224−235.
(10) Remy, J.-S.; Sirlin, C.; Vierling, P.; Behr, J.-P. Gene Transfer with a Series of Lipophilic DNA-Binding Molecules. *Bioconjugate Chem.* **1994**, *5*, 647−654.
(11) Boussif, O.; Lezoualc'h, F.; Zanta, M. A.; Mergny, M. D.; Scherman, D.; Demeniex, B.; Behr, J.-P. A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: Polyethylenimine. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 7297−7301.
(12) Haensler, J.; Szoka, F. C. Polyamidoamine Cascade Polymers Mediate Efficient Transfection of Cells in Culture. *Bioconjugate Chem.* **1993**, *4*, 372−379.
(13) Tang, M. X.; Redemann, C. T.; Szoka, F. C. In Vitro Gene Delivery by Degraded Polyamidoamine Dendrimers. *Bioconjugate Chem.* **1996**, *7*, 703−714.
(14) Griesenbach, U.; Geddes, D. M.; Alton, E. W. F. W. In ref 6, pp 337−356.
(15) Ziauddin, J.; Sabatini, D. M. Microarrays of cells expressing defined cDNAs. *Nature* **2001**, *411*, 107−110.
(16) Miller, A. D. Cationic Liposomes for Gene Therapy. *Angew. Chem., Int. Ed.* **1998**, *37*, 1768−1785.
(17) Zabner, J. Cationic Lipids Used in Gene Transfer. *Adv. Drug Delivery Rev.* **1997**, *27*, 17−28.
(18) Behr, J.-P. Gene Transfer with Synthetic Cationic Amphiphiles: Prospects for Gene Therapy. *Bioconjugate Chem.* **1994**, *5*, 382−389.
(19) Byk, G.; Scherman, D. Genetic Chemistry: Tools for Gene Therapy Coming from Unexpected Directions. *Drug Dev. Res.* **2000**, *50*, 566−572.
(20) Chesnoy, S.; Huang, L. Structure and function of lipid−DNA complexes for gene delivery. *Annu. Rev. Biophys. Biomol. Struct.* **2000**, *29*, 27−47.
(21) de Lima, M. C. P.; Simões, S.; Pires, P.; Faneca, H.; Düzgüne, N. Cationic lipid−DNA complexes in gene delivery: from biophysics to biological applications. *Adv. Drug Delivery Rev.* **2001**, *47*, 277−294.
(22) Behr, J.-P.; Demeneix, B.; Leffler, J.-P.; Perez-Mutul, J. Efficient gene transfer into mammalian primary endocrine cells with lipopolyamine-coated DNA. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 6982−6986.
(23) 2,3-Dioleyloxy-*N*-[2-(sperminecarboxamido)ethyl]-*N,N*-dimethyl-1-propanammonium chloride.
(24) Safinya, C. R.; Lin, A. J.; Slack, N. L. Koltover, I. In ref 6, pp 190−209.
(25) Koltover, I.; Salditt, T.; Rädler, J. O.; Safinya, C. R. An Inverted Hexagonal Phase of Cationic Liposome−DNA Complexes Related to DNA Release and Delivery. *Science* **1998**, *281*, 78−81.
(26) Lin, A. J.; Slack, N. L.; Ahmad, A.; Koltover, I.; George, C. X.; Samuel, C. E.; Safinya, C. R. Structure and Structure−Function Studies of Lipid/Plasmid DNA Complexes. *J. Drug Targeting* **2000**, *8*, 13−27.
(27) Byk, G.; Wetzer, B.; Frederic, M.; Dubertret, C.; Pitard, B.; Jaslin, G.; Scherman, D. Reduction-Sensitive Lipopolyamines as a Novel Nonviral Gene Delivery System for Modulated Release of DNA with Improved Transgene Expression. *J. Med. Chem.* **2000**, *43*, 4377−4387.
(28) Behr, J.-P. Photohydrolysis of DNA by Polyaminobenzenediazonium Salts. *J. Chem. Soc., Chem. Commun.* **1989**, 101−103.
(29) Rädler, J. O.; Koltover, I.; Salditt, T.; Safinya, C. R. Structure of DNA−cationic liposome complexes: DNA intercalation in multilamellar membranes in distinct interhelical packing regimes. *Science* **1997**, *275*, 810−814.
(30) DMEM: Dubellco's modified eagle's medium.
(31) Leventis, R.; Silvius, J. R. Interactions of mammalian cells with lipid dispersions containing novel metabolizable cationic amphiphiles. *Biochim. Biophys. Acta* **1990**, *1023*, 124−132.
(32) Schulze, U.; Schmidt, H.-W.; Safinya, C. R. Synthesis of novel cationic poly(ethylene glycol) containing lipids. *Bioconjugate Chem.* **1999**, *10*, 548−552.
(33) Huber, M.; Pelletier, J. G.; Torossian, K.; Dionne, P.; Gamache, I.; Charest-Gaudreault, R.; Audette, M.; Poulin, R. 2,2′-Dithiobis-(*N*-ethyl-spermine-5-carboxamide) is a high affinity, membrane-impermeant antagonist of the mammalian polyamine transport system. *J. Biol. Chem.* **1996**, *271*, 27556−27563.
(34) de Brabender-van den Berg, E. M. M.; Meijer, E. W. Poly-(propylene imine) dendrimers−large-scale synthesis by heterogeneously catalyzed hydrogenations. *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 1308−1311.

(35) Wörner, C.; Mülhaupt, R. Polynitrile-functional and polyamine-functional poly(trimethylene imine) dendrimers. *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 1306−1308.

(36) Slack, N. Ph.D. Dissertation, University of California, Santa Barbara, 2000.

(37) Koltover, I.; Salditt, T.; Safinya, C. R. Phase Diagram, Stability, and Overcharging of Lamellar Cationic Lipid−DNA Self-Assembled Complexes. *Biophys. J.* **1999**, *77*, 915−924.

(38) Data not shown.

(39) Lin, A.; Slack, N.; George, C. X.; Samuel, C.; Safinya, C. R. Paper submitted.

(40) Still, W. C.; Kahn, M.; Mitra, A. Rapid chromatographic technique for preparative separations with moderate resolution. *J. Org. Chem.* **1978**, *43*, 2923−2925.

(41) The retention factor of compounds **3** and **5** increases with their concentration. Thus, they elute more quickly than estimated by thin-layer chromatography, especially when applied to the column as a highly concentrated solution.