

Characterization of Interaction between Cationic Lipid-Oligonucleotide Complexes and Cellular Membrane Lipids Using Confocal Imaging and Fluorescence Correlation Spectroscopy

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ABSTRACT Complexes formed by cationic liposomes and single-strand oligodeoxynucleotides (CL-ODN) are promising delivery systems for antisense therapy. ODN release from the complexes is an essential step for inhibiting activity of antisense drugs. We applied fluorescence correlation spectroscopy and confocal laser scanning microscopy to monitor CL-ODN complex interaction with membrane lipids leading to ODN release. To model cellular membranes we used giant unilamellar vesicles and investigated the transport of Cy-5-labeled ODNs across DiO-labeled membranes. For the first time, we directly observed that ODN molecules are transferred across the lipid bilayers and are kept inside the giant unilamellar vesicles after release from the carriers. ODN dissociation from the carrier was assessed by comparing diffusion constants of CL-ODN complexes and ODNs before complexation and after release. Freely diffusing Cy-5-labeled ODN (16-nt) has diffusion constant $D_{\text{ODN}} = 1.3 \pm 0.1 \times 10^{-6} \text{ cm}^2/\text{s}$. Fluorescence correlation spectroscopy curves for CL-ODN complexes were fitted with two components, which both have significantly slower diffusion in the range of $D_{\text{CL-ODN}} = \sim 1.5 \times 10^{-8} \text{ cm}^2/\text{s}$. Released ODN has the mean diffusion constant $D = 1.1 \pm 0.2 \times 10^{-6} \text{ cm}^2/\text{s}$, which signifies that ODN is dissociated from cationic lipids. In contrast to earlier studies, we report that phosphatidylethanolamine can trigger ODN release from the carrier in the full absence of anionic phosphatidylserine in the target membrane and that phosphatidylethanolamine-mediated release is as extensive as in the case of phosphatidylserine. The presented methodology provides an effective tool for probing a delivery potential of newly created lipid formulations of CL-ODN complexes for optimal design of carriers.

INTRODUCTION

Cationic lipid-based carriers have been widely used as biocompatible transporters of foreign genetic DNA, antisense oligodeoxynucleotides (ODN) or ribozymes, drugs, peptides, and proteins, for therapeutical, pharmaceutical, and biotechnological applications (Ulrich, 2002; Scherer and Rossi, 2003; Marshall, 2000, 2002). Reduced immunogenicity and safety of cationic lipid-based delivery systems compared to viral vectors motivate significant efforts focused on overcoming the notoriously low efficiency of these carriers. A huge potential in adaptability of the biophysical parameters of the active lipid formulation offers promising prospects for creating an individually designed nonviral carrier, optimized to a specific purpose. Toward this goal, the structure, morphology, and functions of the cationic lipid-based complexes have been extensively investigated by various experimental and theoretical methodologies in an

attempt to discover correlation between the physical and chemical properties of the complex and its delivery potential. In particular, it has been shown that internal nanostructure of the cationic lipid-DNA (CL-DNA) particle (Koltover et al., 1998, 2000), its total surface charge (Koltover et al., 1999; Xu et al., 1999) and lipid charge density (Lin et al., 2003), and the DNA accessibility to enzymatic degradation after assembling of the complex (Zhang et al., 1997; Ferrari et al., 2001) are important parameters that affect transfection capability of the carrier.

A current understanding of the cellular machinery of DNA/ODN delivery by the cationic complexes suggests that the particles are internalized via endocytosis (Zelphati and Szoka, 1996a; Zuhorn et al., 2002a,b; Zuhorn and Hoekstra, 2002), and cellular membrane lipids, primarily anionic phosphatidylserine (PS), mediate DNA/ODN dissociation from the cationic lipids and escape from endosomes (Zelphati and Szoka, 1996a,b; Nakanishi and Noguchi, 2001; Noguchi et al., 1998). Given that DNA/ODN should dissociate from the carrier and be released into the cell cytoplasm for carrying out their functions, comprehensive knowledge of the release mechanisms, which has not been attained yet, is essential for designing efficient carriers.

Although extensive research has been focused on the characterization of CL-DNA complexes, only a few studies have investigated biophysical and biochemical properties of cationic lipid-ODN (CL-ODN) particles and their

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interactions with cellular components (Jääskeläinen et al., 1998; Meidan et al., 2000; Zelfhati and Szoka, 1996b; Zelfhati and Szoka, 1997; Weisman et al., 2004). The similarities and differences between the DNA and ODN complexes are not well identified, providing no evidence that lipid formulation effective for DNA delivery will give the same results when used for ODN transport. Indeed, in their recent cryo-transmission electron microscopy and small angle x-ray scattering studies, Weisman and coauthors (2004) have shown that the internal nanostructure of CL-ODN complexes differs from the structure of CL-DNA particles. Specifically, for lipid compositions forming lamellar structure of the complexes, which represent one type of possible arrangement of these particles, a CL-DNA complex was shown to exhibit a monolayer lattice of parallel helices, whereas a CL-ODN complex shows no ordering of intercalated ODN molecules, oriented fairly randomly between the lipid bilayers (Weisman et al., 2004). This finding indicates that the short, flexible ODN molecules presumably bind cationic lipids of the carrier differently than do the more rigidly structured DNA helices. The differences in binding of DNA and ODN within the complex might result in different behavior of ODN molecules while interacting with cellular membrane lipids, possibly leading to altering barriers for ODN release from the carrier.

In this work, we aim to gain deeper insights into the mechanisms controlling ODN dissociation from the carrier and release across endosomal membranes. Toward this end, we use laser scanning confocal microscopy (LSM) and fluorescence correlation spectroscopy (FCS) to characterize interaction between fluorescently labeled CL-ODN complexes and cellular membrane lipids, resulting in ODN release. As accurate manipulation of lipid membranes in intact cells is difficult to achieve, we use giant unilamellar vesicles (GUVs) to model cellular membranes and monitor ODN release across the target membranes of controllable lipid compositions. The efficiency of ODN release by specific membrane lipids is assessed via LSM analysis of labeled ODN transport inside the corresponding GUVs. Using FCS provides a tool for quantitative estimation of ODN dissociation from the cationic lipids upon the transfer

across the target membrane and accumulation within the GUVs.

MATERIALS AND METHODS

Composition and preparation of GUVs

All lipids were purchased from Avanti Polar Lipids (Alabaster, AL) except for GM1, which was purchased from Calbiochem (San Diego, CA). Data on the lipid compositions used are given in Table 1 in molar ratio. The lipid analog dyes 3,3'-dihexadecyloxycarbocyanine perchlorate (DiO) and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) were purchased from Molecular Probes (Eugene, Oregon). GUVs were prepared by the electroformation method using indium tin oxide-coated coverslips as described in detail elsewhere (Angelova and Dimitrov, 1986; Dimitrov and Angelova, 1988). This technique has been proven to yield a high percentage of unilamellar vesicles with negligible lipid oxidation (Kahya et al., 2003; Scherfeld et al., 2003). GUVs were formed in water medium using a 400- μ l flow chamber, which allowed for injecting solutions into the medium surrounding the GUVs after their formation. For GUV formation, an alternative voltage of 1.2-V amplitude and a 10-Hz frequency was applied for 1 h at 60°C. For visualization by LSM, 0.1 mol % of DiO or DiI lipid analog dye was added to each lipid mixture. For modeling the exoplasmic leaflet of the plasma membrane, GUVs were prepared using equimolar fractions of dioleoyl-phosphatidylcholine (DOPC), sphingomyelin, cholesterol, and ganglioside GM1, added in a small amount, similar to previous studies by others (Dietrich et al., 2001; Kahya et al., 2003). For the experiments, aimed to assess a role of aminophospholipids in the ODN release, GUVs were composed of PS, phosphatidylethanolamine (PE), and phosphatidylcholine (PC) in the molar fractions 0.15/0.25/0.6, respectively, as relevant to the phospholipid composition of endosomal membranes (Urade et al., 1988; Kobayashi et al., 2002). Although PE and PC lipids in these mixtures were always used as unsaturated species—dioleoyl-phosphatidylethanolamine (DOPE) and DOPC, respectively—PS was used both as dipalmitoyl-phosphatidylserine (DPPS) and as 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (DOPS), which reproduces the distribution of saturated and unsaturated phospholipid molecular species in cells for these specific types of lipids (Blom et al., 2001; Urade et al., 1988). In addition, using PS in two different modifications allows for probing the influence of the acyl group on PS lipid activity in the ODN release. GUVs composed of variable amounts of DOPC, DPPS (DOPS), and/or DOPE were used for investigating the role of each of these cellular lipid species in the ODN release.

Liposomes and CL-ODN complexes

The CL-ODN complexes were prepared by mixing equimolar dioleoyl-trimethylammoniumpropane (DOTAP)/DOPE liposomes with the appropri-

TABLE 1 Lipid composition of GUVs and efficiency of ODN release

Sample	Lipid composition of GUV membranes in mole fraction	Lipid charge	DNA transfer efficiency ($\pm 2.5\%$, four experiments)
1	0.33 sphingomyelin/0.33 DOPC/0.33 cholesterol/0.1 GM1	All neutral	<5%
2	0.15 DPPS/0.25 DOPE/0.6 DOPC 0.15 DOPS/0.25 DOPE/0.6 DOPC	Neutral/ anionic/ neutral	35% 35%
3	a) 0.15 DOPE/0.85 DOPC b) 0.25 DOPE/0.75 DOPC c) 0.4 DOPE/0.6 DOPC	All neutral	10% 20% 35%
4	0.15 DPPS/0.85 DOPC 0.15 DOPS/0.85 DOPC	Anionic/neutral	10% 10%
5	DOPC	Neutral	<5%

ate amount of ODN. We used two different high-performance liquid chromatography-grade labeled ODNs (IBA Co., Goettingen, Germany), a 16-nt oligonucleotide labeled 3' with Cy-5, CTG TTG AAT TCG GAT C-Cy-5, and a 66-nt oligonucleotide labeled 5' with rhodamine green identical to that described elsewhere (Ketting et al., 1998). DOTAP/DOPE lipid mixtures were prepared at 5 mM lipid concentration by dissolving the lipids in chloroform. Liposomes were formed by a hydration method similar to that described by others (Kahya et al., 2001). The final solution was stored at 4°C until use under argon atmosphere. For preparation of CL-ODN complexes, labeled ODNs (100 pmol for LSM experiments and 1 pmol for FCS measurements) were added to the unlabeled liposome solution at 2:1 cationic/anionic molar charge ratio and incubated for 20 min. After 20 min, the preparation was diluted to the total volume of ~340 μ l, injected into the GUV flow chamber, and allowed to diffuse evenly throughout the sample at room temperature.

Laser scanning confocal fluorescence microscopy

LSM imaging was performed using a Zeiss 510 Meta laser scanning microscope (Zeiss, Jena, Germany) with argon ion (488 nm, 30 mW, at 40% of maximum power output) and helium-neon (633 nm, 5.0 mW, used at full power) lasers. A water immersion objective 40 \times /1.2W (Zeiss) was used in the epidetection configuration with an adjustable pinhole set at 76 μ m, corresponding to a <0.7- μ m-thick confocal image slice of the sample. A band-pass filter transmitting 505–550 nm (Zeiss) was used in the experiments with DiO, while a long-pass filter at 650 nm (Zeiss) was applied to separate Cy-5 fluorescence.

Evaluation of ODN release using confocal LSM images

For evaluation of ODN release from the carriers upon interaction with the target membrane lipids, we analyzed the confocal images and estimated the number of GUVs filled with labeled ODNs inside their lumen. Once ODN molecules were transported inside the GUVs due to interaction of CL-ODN complexes with the membrane lipids, released ODNs were not able to cross the membrane bilayers in the opposite direction and remained accumulated inside GUVs for extensive periods of time. This allows for convenient comparison of the extent of ODN release for various compositions of the target membranes via counting a fraction of the ODN-filled GUVs in the corresponding set of the confocal images. Each GUV sample typically contained ~1000 vesicles ranging in size from 10 to 100 μ m, which provides a reliable basis for statistics. All GUV samples were evaluated after incubation with CL-ODN complexes for 2 h. Due to this, a bias in counting the fractions of filled GUVs, caused by possible vesicle-to-vesicle fusion events during the interaction with the complexes, was excluded. After 2 h incubation with CL-ODN complexes, the microscope objective was positioned on a randomly selected group of vesicles using 488 nm illumination. After that, a single snapshot image of the selected region was taken with the 633-nm laser line (5 mW power). The procedure was repeated several times to provide an image set (usually 15–20 images) containing ~1000 vesicles for the given sample. Each experiment was repeated four times, corresponding to evaluation of ~4000 vesicles in total for every studied lipid mixture. Thereafter, each set of images was processed by the following algorithm. First, a total number of vesicles in the image were calculated. Next, an intensity threshold was set at 100 ADU above the outside background to separate and count ODN-filled GUVs. The given threshold value was determined based on analysis of the fluorescent signals inside the GUVs for all images. The fraction of ODN-filled GUVs was determined for each membrane composition by calculating a percentage ratio of ODN-filled vesicles versus the total number of the vesicles in the set of images (as counted after the sample incubation with the complexes). Although not strictly exact for absolute evaluation of ODN transfer, this

criterion allows for robust comparison of the relative efficiency of ODN release for different lipid compositions. We note that in control experiments with an equivalent amount of 100-pmol free labeled ODNs, some vesicles (<5%) were permeable to free ODNs. However, in contrast to the active ODN transport by CL-ODN complexes, a steep increase of ODN concentration inside the GUV lumen compared to the outside solution was never built up by free diffusion of ODN into leaky vesicles. For CL-ODN complexes used here, a fraction of free ODN molecules, present in the solution after formation of the complexes, was undetectable in LSM images. Therefore, the effect of the small proportion of leaky vesicles on quantifying GUVs containing high levels of accumulated ODNs was negligible because leaky vesicles were discarded during application of the intensity threshold.

Fluorescence correlation spectroscopy

FCS analysis was performed according to the procedure outlined in detail elsewhere (Rigler et al., 1993; Schwille et al., 1997; Haustein and Schwille, 2003). In FCS, experimentally obtained temporal correlations of fluorescence signal from minute molecule ensembles are evaluated by mathematical modeling and a numerical curve fitting provides the so-called diffusion time of molecules, τ_d , from which the diffusion coefficient D can be calculated. Here, the data-fitting was performed using a three-dimensional Brownian diffusion model (Schwille et al., 1997; Haustein and Schwille, 2003):

$$G(\tau) = \frac{1}{CV_{\text{eff}}} \times \frac{1}{(1 + \tau/\tau_d)} \times \frac{1}{\sqrt{1 + (\omega_0/z_0)^2 \times (\tau/\tau_d)}} \times (1 - T + T \times e^{-\tau/\tau_T}) \quad (1)$$

where C denotes the particle concentration in the effective measurement volume V_{eff} , τ is time, τ_d is the diffusion time, and z_0 and ω_0 determine effective focal volume size in axial and lateral directions, respectively. The last factor in Eq. 1 characterizes the photophysical dynamics of the used dye (Cy-5), with dark fractions T and characteristic flickering times τ_T (Widengren et al., 1995). V_{eff} is determined in calibration measurements. The diffusion coefficient is calculated using the relationship $D = \omega_0^2/4\tau_d$.

The FCS measurements were performed using a commercial microscope ConfoCor2 (Zeiss) and a home-built FCS setup. The commercial ConfoCor2 microscope was used in the configuration described elsewhere (Kahya et al., 2003). The home-built setup was assembled in the epifluorescence configuration using the inverted Olympus IX70 microscope (Olympus, Tokyo, Japan). A helium-neon laser (633 nm, 14mW; Research Electro-Optics, Boulder, CO) was coupled onto the back aperture of the 40 \times /1.2W water-immersed objective (UPlanApo, Olympus, Hamburg, Germany) by the dichroic mirror Q 645 LP (AHF, Tübingen, Germany). The fluorescence emission was filtered by a band-pass filter (AHF, model 675DF45) and projected onto a spatially adjustable 100- μ m core diameter optical fiber (Thorlabs, Newton, NJ), which core dimension provides a pinhole. The light collected by the optical fiber is directed to an avalanche photodiode (SPCMCD3017, Perkin Elmer Optoelectronics, Vaudreuil, Quebec, Canada) and analyzed using a commercial correlator card (ALV, Langen, Germany).

In all FCS experiments, GUVs were unlabeled and visualized using a halogen lamp with differential interference contrast optics. Positioning in lateral and axial directions inside the GUV lumen was achieved such that the measurement volume was just below the widest cross section of the vesicle.

FCS analysis of CL-ODN complex and free ODN diffusion

Due to significant differences in their molecular weights, CL-ODN complexes and free ODN molecules are characterized by largely different diffusion constants. (A diffusion coefficient of a molecule species in solution in the first approximation is proportional to the third root of the molecular

weight (Schwille et al., 1997)). Based on this fact, they can be discriminated by FCS analysis, which has been recognized as a reliable method for measuring diffusion constants of molecular species with high precision (Rigler et al., 1993; Schwille et al., 1997; Haustein and Schwille, 2003). Therefore, FCS is used here to assess the release of ODN from the complexes upon interaction with the target membranes by measuring the diffusion constants of the molecular species inside lumens of ODN-filled GUVs and comparing the obtained values to the diffusion constants of free ODNs and of the CL-ODN complexes in solution. Importantly, as the effective measurement volume in the FCS experiments is ~ 2 orders of magnitude smaller than the average GUV volume, the FCS measurements are strictly confined within the GUV lumen, thereby assessing exclusively the diffusion of fluorescent species accumulated inside the GUV. Additionally, the amplitude value of the correlation curve $G(0)$, which is inversely proportional to the particle concentration, gives an estimate of ODN concentration in the measurement volume. For each lipid composition of the GUV membranes, mean diffusion coefficients for ODN within GUVs were determined from a series of at least 10 independent FCS measurements in different vesicles, and the error was calculated as a half of the difference between the largest and smallest measured value. The diffusion coefficient of free ODN in water solution was calculated by taking the mean of eight independent measurements.

RESULTS

LSM evaluation of ODN release inside GUVs

The evaluation of ODN release from the CL-ODN complexes via confocal imaging of labeled ODN accumulation inside the GUV lumen revealed a striking difference in the extent of ODN release depending on the lipid composition of the interacting GUV membrane. In the first series of experiments, ODN transfer was investigated for the model membranes, replicating a lipid composition of the exoplasmic leaflet of the plasma membrane. The plasma membrane is the first biological barrier encountered by the ODN carriers on their way into the cell cytoplasm. Therefore, it is important to know whether the lipid contents of the exoplasmic leaflet supports fusion with the CL-ODN complexes and ODN release. The GUV sample was prepared using 0.33 sphingomyelin/0.33 DOPC/0.33 cholesterol/0.1 GM1, similar to the exoplasmic leaflet mixtures used by others (Dietrich et al., 2001; Kahya et al., 2003). For visualization, the GUV membrane bilayer was labeled with the fluorescent lipid analog DiO dye (emitting green fluorescence) and the CL-ODN complexes contained ODNs covalently labeled with Cy-5 (emitting a red light signal). In control experiments, a water solution of free ODN molecules, not complexed with the cationic lipid carrier, was injected into the GUV medium. Fig. 1 A shows a representative confocal image, collected 2 h after injection of CL-ODN complexes to the GUVs of the given lipid composition. As seen in Fig. 1 A by the distribution of red fluorescence, no significant amount of free ODNs is observable in the GUV surrounding medium due to the packaging of ODNs into discrete particles, which are visible as red dots in solution or yellow dots when attached to the green GUV membrane. After adding CL-ODN complexes to the GUV sample, some vesicles fused together over time, altering a distribution of

the initially evenly spaced GUVs into a network, as seen in overview (Fig. 1 B). Due to this observation, no estimation of a number of vesicles in the confocal images was done before the injection of the complexes, and image processing was always performed for the images, collected 2 h after the injection of the CL-ODN solution. As demonstrated in Fig. 1 B, for the given lipid composition only a small number of GUVs accumulated a clearly distinguishable concentration difference of labeled ODN within their lumen with respect to the outside solution, accounting for $<5\%$ of a total number of analyzed GUVs. In all images, GUVs were counted as ODN-filled if they had an averaged fluorescent signal of 100 ADU above the background, or higher throughout their lumen, similar to the three vesicles in Fig. 1 A. In contrast, the two remaining vesicles in Fig. 1 A were discharged by the applied intensity threshold (<100 ADU). Additionally, the diffuse nature of fluorescence inside the GUV lumen in Fig. 1 A indicates that ODN molecules are likely no longer condensed within the CL-ODN complexes. Also, the accumulation in one GUV was notably independent of the accumulation in the neighboring GUVs.

The results of control experiments (data not shown for this lipid composition; instead, a similar negative control is presented for the next studied lipid formulation (see Fig. 2 C)) with injection of the same amount of free Cy-5-labeled ODNs showed no active accumulation of ODNs inside the GUV lumen for an extensive period of time (>5 h). Although a low percentage of the GUVs ($<5\%$) were leaky, and ODNs could penetrate inside such vesicles, a fluorescence signal inside those vesicles was either similar to or lower than that outside the vesicle, indicating that ODNs could not be accumulated within the leaky GUVs. Correspondingly, a steep concentration increase of labeled ODNs within the leaky GUVs was never observed.

We also observed that a number of GUVs of exoplasmic leaflet composition accumulated ODNs and/or CL-ODN complexes bound to the membrane surface. As shown in Fig. 1 C, the homogenous distribution of ODN fluorescence on the GUV membrane indicates that bound complexes have significantly decondensed, but failed to transfer integrated ODNs into the GUV lumen. This is confirmed by quantitative analysis of the distribution of fluorescence signal on the GUV membrane and through the GUV lumen. Cy-5 fluorescence signal, and therefore corresponding ODN concentration, is more than five times higher on the GUV membrane, compared to the inside of the GUV lumen. The fluorescence peaks for Cy-5-labeled ODN (channel 1, red) are clearly distributed outside of the DiO peaks, which identify the membrane (channel 2, green). The intensity in the Cy-5 channel drops sharply toward the center of the plot, decreasing to nearly the background level within the GUV. Taken together, a described series of experiments clearly demonstrated that lipid membrane, enriched in cholesterol, DOPC, and sphingomyelin, is largely unresponsive to the CL-ODN complex fusion and ODN release.

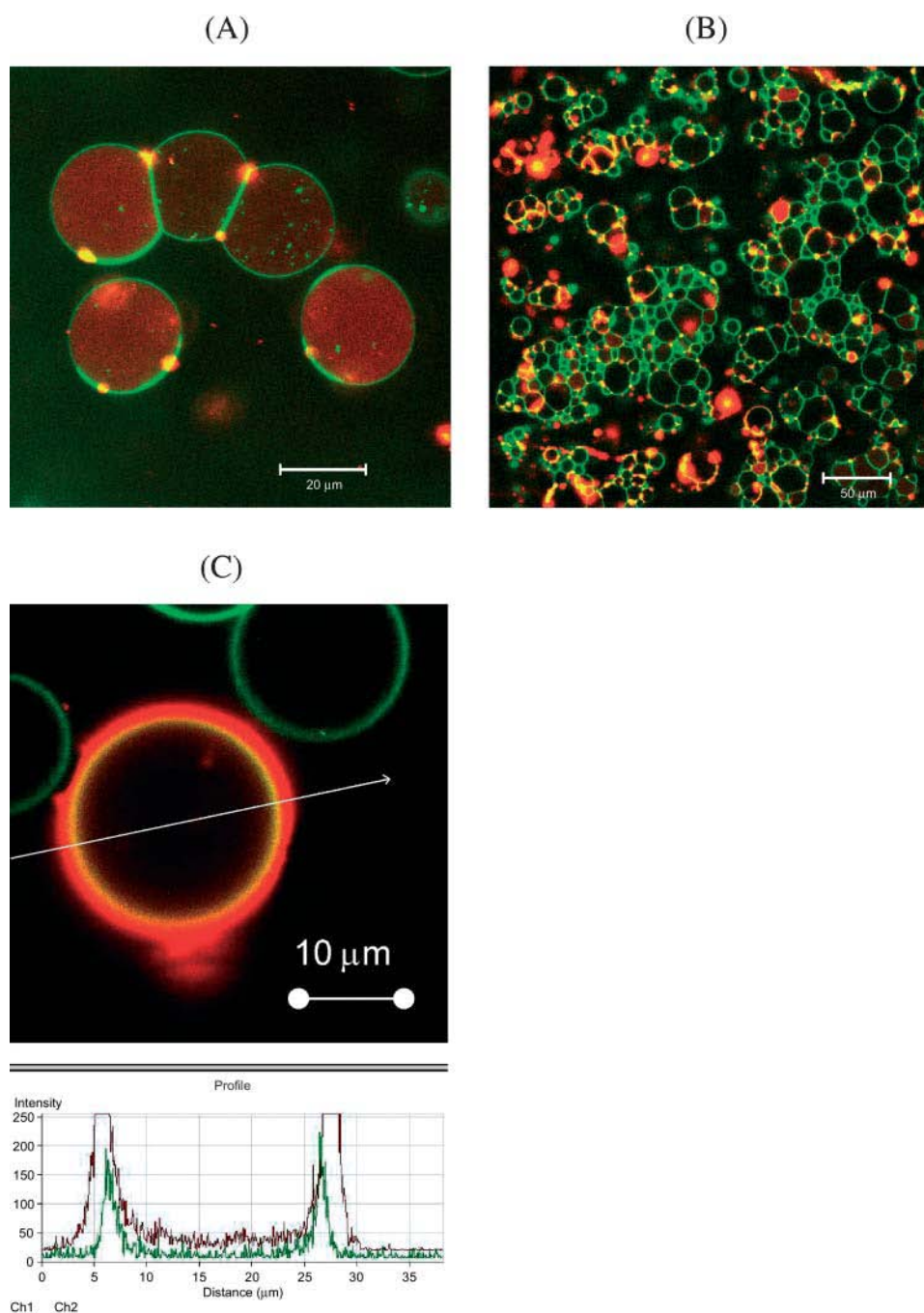


FIGURE 1 ODN transport into GUVs modeling the exoplasmic leaflet of the plasma membrane is low. The confocal images show GUVs composed of DOPC/sphingomyelin/cholesterol/GM1 (at the mole fractions 0.33/0.33/0.33/0.1) labeled with DiO lipid analog dye (*green*) after 2 h incubation with the CL-ODN complexes, containing Cy-5-labeled ODN (*red*). Image A demonstrates a clear accumulation of ODN within vesicles, marked by arrows. The vesicles were considered as ODN-filled if the fluorescence signal inside was at 100 ADU over the background level, or higher. The complexes are seen as yellow dots of intense fluorescence on the GUV surface. Overview picture (B) illustrates that ODN transfer is low and is distinguishable in <5% of the vesicles. Image C shows the accumulation of ODN or fused CL-ODN complexes on the GUV membrane, frequently observed for this type of membrane composition. The associated intensity profile of fluorescence signal across the representative GUV, marked by a white line in the image, shows that Cy-5-labeled ODN (channel 1, *red*) is clearly localized on the external side of the GUV membrane (channel 2, *green*), with negligible amounts of ODN within the lumen of the vesicle. In the control experiments, adding a solution of free Cy-5-labeled ODNs (100 pmol) to the GUV sample exhibits no active transfer of ODN into the GUV lumen during extensive observation time, >5 h (data not shown; see the similar control in Fig. 2 C). The white scale bars indicate 20, 50, and 10 μm, respectively.

In the next series of experiments we aimed to investigate interaction of the CL-ODN complexes with model endosomal membranes. According to the existing hypothesis, endocytosis is a prevailing route for cellular internalization of the cationic complexes (Zelphati and Szoka, 1996a; Zuhorn et al., 2002a,b; Zuhorn and Hoekstra, 2002). Moreover, the experiments described above provide indication that the CL-ODN complexes do not fuse with the external leaflet of the plasma membrane, giving additional indirect evidence that the particles rather need to be enveloped into the endosomes to gain the cellular entry.

Therefore, CL-ODN complex interaction with the lipids present in the endosomal membrane is of key importance for ODN releasing from endosomal compartments.

The leaflets of the plasma membrane are known to differ substantially in lipid composition (Devaux, 1991). Specifically, the exoplasmic leaflet of the plasma membrane is enriched in cholesterol, glycosphingolipids, and PC, but is generally devoid of both negatively charged PS and overall neutral PE. In contrast, the cytoplasmic leaflet does not include large amounts of sphingolipid and cholesterol, but in addition to PC contains high fractions of aminophospholipids

PS and PE. Although the asymmetric lipid distribution in the leaflets of the plasma membrane is tightly regulated, this asymmetry is likely disrupted during endocytosis, with significant amounts of PS and PE entering the luminal leaflet of the endosome (Devaux, 1991; Xu, and Szoka, 1996; Devaux, 2000). Also, cationic drugs have been shown to cause the disruption of lipid asymmetries in the cells, and for in vitro liposome systems (Moreau et al., 1997). Therefore, an exact understanding of the roles of cellular aminophospholipids, PS and PE, in disruption of the CL-ODN complexes and ODN release is highly significant for engineering of the effective ODN carriers.

To characterize specific roles of major endosomal lipids, coming into a close contact with the CL-ODN complexes (i.e., PC, PS, and PE) in ODN release, we designed a series of experiments where these lipid species were subsequently excluded and/or exchanged in the cellular relevant proportions in model membranes. First, GUVs were prepared from pure DOPC lipids. For this sample, we observed low ODN transfer, <5%, similar to the endoplasmic leaflet composition GUVs. Next, GUVs composed of DPPS/DOPE/DOPC and of DOPS/DOPE/DOPC at the mole fractions 0.15/0.25/0.6 were incubated with the CL-ODN complexes. The particular selection of the lipid species and their relative amounts in these compositions is a carefully reasoned choice. Particularly, the molar proportion and the selection of acyl groups of PS and PE lipids reflects a distribution found in the endosomal membranes in cells (Blom et al., 2001; Urade et al., 1988). The additional rationale for investigating two otherwise analogous compositions with a substitution of DPPS by DOPS is to verify whether varying the length and saturation of the hydrophobic tails may have a significant effect on the activity of PS in the ODN release. As shown in Fig. 2 A, after 2 h ~35% of DPPS/DOPE/DOPC GUVs accumulated significant amounts of labeled ODNs within their lumen, which exceeded the 100-ADU intensity threshold across the lumen of the vesicle. GUVs with extensive amounts of membrane-bound, yet not fused, complexes (as observed in the experiments with the model plasma membrane exoplasmic leaflet GUVs) were not detected. The qualitative visual assessment of the confocal images, showing the distribution of labeled ODNs in the GUV lumen as opposed to ODN concentration on the GUV membrane and in the outside solution, is enhanced by quantitative analysis of the fluorescence intensity profiles across selected individual GUVs. Fig. 2 A clearly demonstrates that the intensity of Cy-5-labeled ODNs within the GUV lumen of the less bright vesicle significantly exceeds the outside signal (~150 ADU on average), indicating that concentration of ODNs within the vesicle is considerably higher. The same results were obtained for the analogous lipid composition, DOPS/DOPE/DOPC, where the level of ODN transfer across the membrane stayed about the same, ~35% (data not shown).

To probe the specific roles of PS and PE in ODN release, we prepared membrane compositions containing various

amounts of either PS or PE lipids, keeping the proportion of DOPC constant. Correspondingly, the fraction of the anionic PS lipids in the initial composition was fully replaced by the neutral PE lipids in a DOPE/DOPC mixture at the mole fraction 0.4/0.6. We observed that the ODN transfer across the membrane remains at about the same high level as in the previous case, ~35% (Fig. 2 B). Considering that this finding is in contrast with the earlier results of others for ODN release by DOPE/DOPC liposomes (Zelphati and Szoka, 1996b), the given observation was examined in more detail. Specifically, we investigated GUVs of only neutral lipids DOPE/DOPC with a variable DOPE fraction (exact data are given in line 2 of Table 1). In general, we observed that an increase in DOPE fraction results in a rise of ODN release. The role of PS lipids in ODN release was verified by preparing membranes, devoid of PE but containing PS, DPPS/DOPC and DOPS/DOPC compositions at 0.15/0.85 molar fractions. Again, in these mixtures PS was included in two modifications, with varying acyl groups. For both compositions we observed about the same degree of ODN transfer, ~10%.

Negative control experiments, with injection of solution of naked, nonlipid-enveloped, Cy-5-labeled ODNs to each of the samples, clearly demonstrated that ODNs remained largely outside vesicles. Fig. 2 C shows a representative image for the negative control experiment for the DPPS/DOPE/DOPC mixture. The fluorescence signal in the leaky vesicles was always at the same level or lower compared to the outside solution, as seen for the vesicle marked by the arrow in Fig. 2 C.

An additional interesting observation, which we report here, is that during CL-ODN complex interaction with GUV samples containing PS and/or PE lipids, a binding of the complex to the membrane induced formation of budding vesicles in the contacting membrane. Usually, the vesiculation was more strongly expressed for PE-enriched membranes. The induced vesicles often were large enough to resolve the details of their internal structure. Fig. 3 shows that ODN can be accumulated within a vesicle, budding from a site of the complex merger to the GUV membrane. This likely indicates that released ODN remained trapped within the membrane of the associated vesicle, giving indirect evidence that free ODNs cannot cross the membrane bilayer.

FCS assessment of ODN release from the carrier lipids

To verify whether ODN dissociates from the cationic lipids of the carrier after the transfer across the contacting membrane, we measured the diffusion coefficient of fluorescent species inside the filled vesicles and compared obtained values to the diffusion constants of CL-ODN complexes and free ODNs in the solution. Fig. 4 A shows normalized autocorrelation curves for freely diffusing ODNs (control ODN) and for the CL-ODN complexes. From the

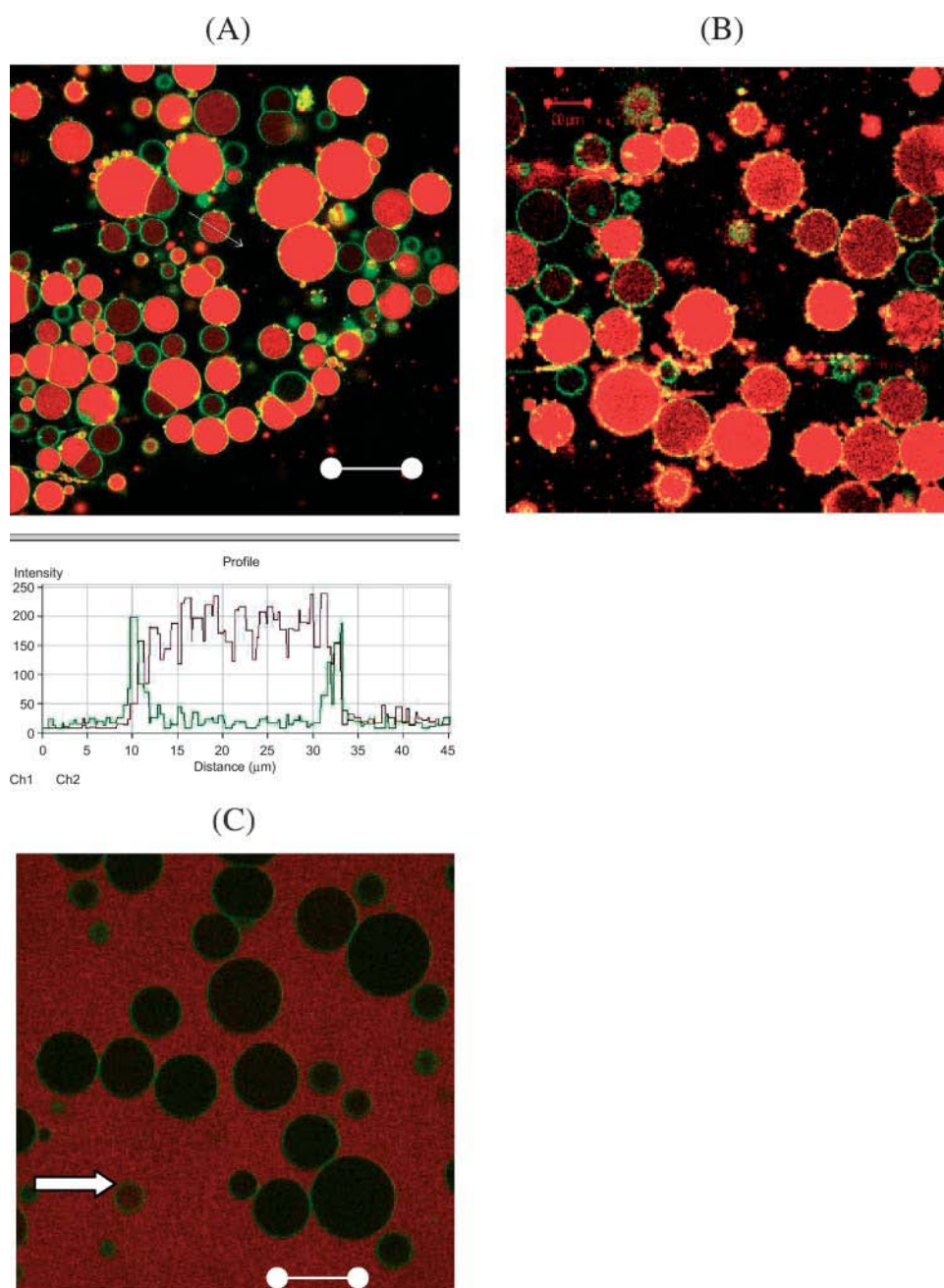


FIGURE 2 Image *A* demonstrates efficient ODN transfer across the membranes, containing aminophospholipids in the amounts as found in the endosomal membranes, DPPS/DOPE/DOPC at the mole fractions 0.15/0.25/0.6, with $\sim 35\%$ of GUVs accumulating Cy-5-labeled ODNs inside. The associated fluorescence intensity profile shows that Cy-5-ODN (channel 1, red) is clearly localized within the lumen of the GUV. The average fluorescence intensity for the selected vesicle is at ~ 150 ADU. A green line shows the corresponding DiO fluorescence signal (channel 2), marking the GUV membrane. Image *B* illustrates that the same efficient transfer, $\sim 35\%$, occurs across the membrane, fully devoid of PS but enriched in PE, DOPE/DOPC at mole fractions 0.4/0.6. Image *C* provides representative results of the negative control experiments for the DPPS/DOPE/DOPC GUV sample, injected with a solution of free Cy-5-labeled ODNs (100 pmol). It shows that free ODN molecules in large part do not transfer inside GUVs, even after 5 h incubation. In addition, for the small-percent of vesicles permeable to free ODNs, a higher concentration of labeled ODNs inside the GUVs with respect to the outside medium was never built up, as seen for the leaky vesicle, marked by the arrow. The white scale bar corresponds to $50 \mu\text{m}$ in *A* and $20 \mu\text{m}$ in *B* and *C*.

plots in Fig. 4 *A* it is clearly observable that the diffusion of the complexes is strongly slowed, which is reflected in the increase of the diffusion time, as they stay longer in the observation volume. The FCS curves for CL-ODN complexes were usually fitted with two components, both of which had diffusion constants in the range of approximately $D_{\text{CL-ODN}} = \sim 1.5 \times 10^{-8} \text{ cm}^2/\text{s}$. The measured value for the diffusion coefficient of free ODN in solution is $1.3 \pm 0.1 \times 10^{-6} \text{ cm}^2/\text{s}$. Because CL-ODN particles form rather inhomogeneous aggregates, varying in molecular weights, data-averaging over measurement series would not be statistically applicable for providing data errors in such

experiments. However, we report that the diffusion of CL-ODN complexes was ~ 100 -fold slower compared to free ODN molecules.

The diffusion coefficients of ODN molecules, released inside the GUV lumen due to CL-ODN complex interaction with the membrane lipids, were measured for different membrane compositions. In FCS experiments GUVs containing significant amounts of ODNs were identifiable by their high fluorescence signal. Fig. 4 *B* shows fluorescence signals inside the ODN-filled vesicle and inside the ODN-devoid vesicle, which differ significantly. In addition, fluorescence fluctuations inside the ODN-devoid vesicle

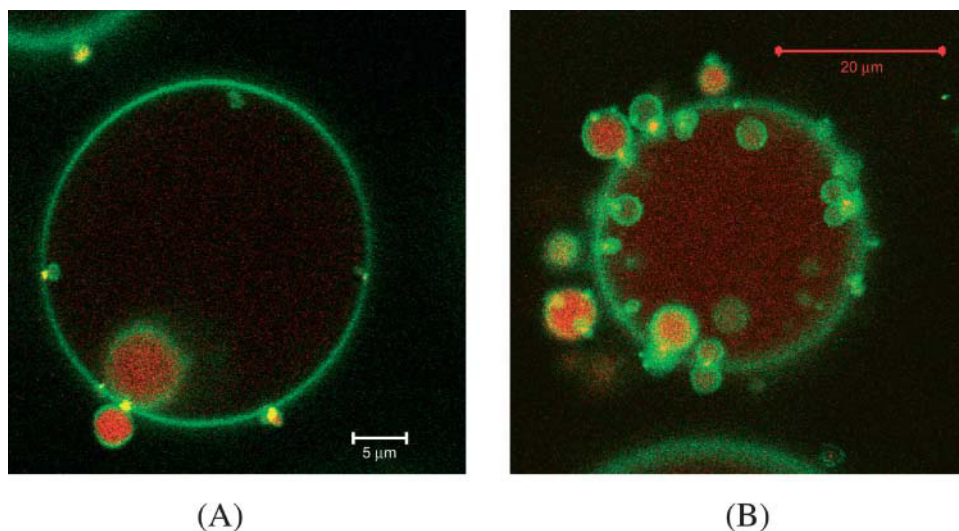


FIGURE 3 CL-ODN complex binding to the membranes, containing PS and/or PE, induces formation of abundant exo- and endocytic vesicle-like structures budding from the membrane. Image *A* shows an enlarged picture of DPPS/DOPC vesicle at mole fraction 0.15/0.85. Image *B* provides an enlarged view of the vesicle with DOPE/DOPC composition at 0.4/0.6 mole fraction. ODN is released from the complex during its interaction with the GUV membrane. The released ODN is trapped within the induced vesicles, indicating that free ODN molecules cannot cross the barrier membrane. The white scale bar corresponds to 5 μm (*A*) and 20 μm (*B*), respectively.

could not be correlated, as seen by the typical plot, presented in Fig. 4 *C*. The mean diffusion coefficient of ODN within the lumen of GUVs, composed of DPPS/DOPE/DOPC (at the mole fractions 0.15/0.25/0.6), is $1.1 \pm 0.2 \times 10^{-6} \text{ cm}^2/\text{s}$ and a representative curve is shown in Fig. 4 *D*. Relative comparison of the $G(0)$ values for FCS measurements inside and outside the filled vesicles (Fig. 4 *E*) shows that ODN concentration inside the vesicle is ~ 40 -fold higher with

respect to the outside solution. The diffusion coefficients measured for ODNs diffusing inside DOPE/DOPC vesicles (at the mole fraction 0.25/0.75) and DOPC vesicles are correspondingly $1.5 \pm 0.4 \times 10^{-6} \text{ cm}^2/\text{s}$ and $1.6 \pm 0.4 \times 10^{-6} \text{ cm}^2/\text{s}$, with representative FCS curves, shown in Fig. 4, *F* and *G*, respectively. The diffusion constants from all measurements inside ODN-filled GUVs, regardless of the lipid composition of the interacting membranes, are in good

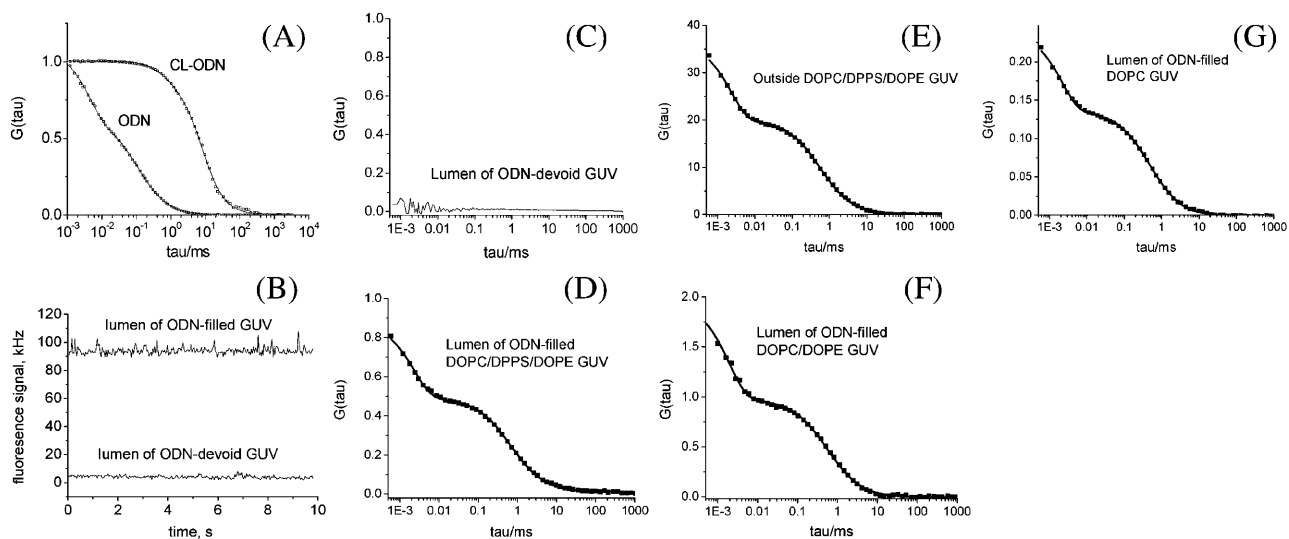


FIGURE 4 Diffusion coefficients, obtained by the FCS technique, indicate that regardless of the lipid composition of the contacting membrane, ODN molecules released inside the GUVs are largely dissociated from the carrier lipids. Graph *A* provides normalized autocorrelation curves for freely diffusing ODN (control ODN) and for CL-ODN complexes. ODN-filled GUVs were identifiable by their high fluorescence signal, compared to a negligible background in ODN-empty GUVs, as is demonstrated in graph *B*. In addition, a low fluorescence signal in ODN-devoid GUVs could not be correlated, as seen from the lack of correlation curve in plot *B*. Graphs *D*, *F*, and *G* provide FCS curves measured inside the ODN-filled GUVs of various lipid compositions, DOPC/DOPE/DPPS at 0.6/0.25/0.15, DOPE/DOPC at 0.25/0.75, and pure DOPC, respectively; and the results of the numerical fitting. Graph *E* gives experimental and fitted correlation curves for the FCS measurements in the outside solution, a few micrometers apart from the membrane of ODN-filled GUV (DOPC/DOPE/DPPS at 0.6/0.25/0.15). Comparison of the amplitude $G(0)$ values of the correlation curves in plots *D* and *E* shows that ODN concentration inside the vesicle lumen is significantly higher than in the outside solution ($\sim 615 \text{ pmol}$ in the lumen compared to 14.8 pmol outside the GUV). In each logarithmic graph, every third experimental data point is given by a square symbol and a solid black line shows the data fit.

agreement with the mean diffusion coefficient of free ODNs in solution.

Therefore, based on the obtained FCS data we report that ODN molecules diffusing in the GUV lumen after release from the CL-ODN complexes are largely dissociated from the cationic lipids of the carrier. Additional important evidence of truly monomerically ODN diffusion in the GUV lumen is also given by the pronounced shoulder in the fast time range of the FCS curves seen in Fig. 4 A (control ODN) and in Fig. 4, D–G. This short fast range decay in the temporal correlation reflects on the specificity of the Cy-5 photophysics related to its isomerization reactions (Widengren and Schwille, 2000). If many fluorescent units diffuse in aggregates, as is the case for the cationic particles (Fig. 4 A, CL-ODN complexes), the pronounced photophysics, which is visualized as fast flickering of single dye molecules, is statistically disguised. Note that the curve in Fig. 4, C–G, cannot be directly compared by τ_d to those in Fig. 4 A, as τ_d is a setup-determined parameter and the measurements were performed on separate setups, with different observation volumes.

DISCUSSION

ODN delivery by the cationic lipid carriers into cells is a complex process that includes several steps. To be released into the cell cytoplasm, ODN has to cross several biological barriers created by the cellular membranes. The existing hypothesis is that the anionic lipids of the endosomal membranes are the primary mediators of ODN release into the cytoplasm (Zelphati and Szoka, 1996b). This conclusion was based on FRET experiments showing that anionic liposomes, when added to the CL-ODN complexes containing labeled ODNs, were able to release ODN into the solution. To investigate this premise in more detail, and to probe the specific roles of other common cellular lipids such as DOPC, DOPE, sphingomyelin, and cholesterol in interaction with CL-ODN complexes, we used giant unilamellar vesicles as a model membrane system, which allows us to directly visualize ODN transport. In contrast to the investigations based on using liposomes to model interaction of CL-ODN complexes with cellular membranes, a methodology that relies on working with GUVs provides a convenient approach not only to probe the mechanisms of action for contacting CL-ODN complexes and membrane lipids, but also to visualize and monitor the release process in real time under the optical microscope.

In general, our data show that ODN release from the CL-ODN complexes during complex interaction with the membrane lipids strongly depends on the lipid composition of a target membrane, and that the different cellular lipids differently contribute to this effect. The principal novelty of our observations with GUV membrane systems, compared to earlier studies based on using small liposomes, is that we directly observe transfer of ODN molecules across lipid

bilayers. Although modeling interaction with biological membranes by liposomes allows for investigation of ODN release, such an approach fails to assess the state of lipid bilayers during interaction with the CL-ODN complexes and provides no information on interaction between released oligonucleotides and contacting membranes. By using GUVs we demonstrated for the first time that model biological membranes remained intact after interaction with CL-ODN complexes and that the lipid bilayer boundaries were capable of keeping released ODN inside the GUV lumen, with no ODN leakage for an extended time. On the other hand, free ODN molecules, when injected into GUV samples, remained mostly outside GUVs and were not able to cross lipid bilayers. This is an important observation as it unambiguously confirms that cationic lipid carriers are capable of mediating ODN penetration across lipid bilayers, otherwise impermeable to free oligonucleotides.

Specifically, we observe that ODN transfer into the lumen of GUVs composed of an equimolar mixture of DOPC, sphingomyelin, and cholesterol (mimicking the exoplasmic leaflet of the plasma membrane) is low (<5%, Table 1). Consistent with cellular studies by others, suggesting that the complexes are internalized primarily via endocytosis (Zuhorn et al., 2002a,b), our data with the model endoplasmic leaflet membrane might provide indirect indication that the CL-ODN complexes need to be enveloped into endosomes to gain cellular entry, because they do not actively fuse at the external leaflet of the plasma membrane. At the same time, we clearly observed that whereas a pure DOPC lipid composition remains largely unresponsive for ODN transfer (<5%, Table 1), similar to cholesterol- and SM-containing membranes, availability of aminophospholipid species, i.e., PS and/or PE, significantly enhances the ODN transport. In a most general context, aminophospholipids may stimulate the transfer of ODN across the membrane through their tendency to promote membrane fusion with the CL-ODN complexes (Devaux, 1991) and due to the propensity of these lipids (particularly PE) to form nonbilayer structures.

Another important observation, which we report here, is that the degree of ODN transfer across the membranes, which contain either negatively charged PS (DPPS/DOPC and DOPS/DOPC compositions) or overall neutral PE (DOPE/DOPC composition) aminophospholipids, is similar. Additionally, for the membrane containing PE but devoid of PS, an increase of DOPE fraction in the membrane results in a higher level of ODN transfer across the bilayer. In other words, we have observed that PE lipids can mediate ODN release from the cationic carriers without PS interference, and that they can do this as efficiently as anionic PS lipids. This observation and the recent cellular studies of others (Lin et al., 2003), showing that the transfection efficiency of the DOTAP/DOPE lipid-DNA complexes is independent of their membrane charge density, indicate that PE lipid species, being overall neutral aminophospholipids, have an important function in ODN release, other than direct

mediation of electrostatic interactions with the cationic lipids.

Whereas in the main our observations regarding the active promoting role of anionic PS lipids in ODN release from the carriers support existing knowledge (Xu and Szoka, 1996; Zelphati and Szoka, 1996a,b), what we report here about the PE capability to release ODN from the CL-ODN complexes in the absence of anionic lipids is a new finding. In contrast to the earlier data by others (Xu and Szoka, 1996; Zelphati and Szoka, 1996a,b), we show that PE lipids by themselves can induce ODN release from the CL-ODN complexes to the same extent as anionic PS lipids. This is a significant result as it revises the prevailing concept of the DNA/ODN release mechanisms, which suggests that the presence of the anionic lipids in the contacting membrane is a primary prerequisite for ODN release (Xu and Szoka, 1996; Zelphati and Szoka, 1996a,b). The given concept was developed based on FRET measurements of labeled DNA/ODN molecules released from the complexes upon addition of various composition liposomes. Specifically, it was observed that addition of PS/DOPE/DOPC (1:2:1) liposomes to the CL-ODN complexes induced significant release of labeled nucleotides, ~80% (as estimated by fluorescence quenching assay), whereas DOPE/DOPC (2:1) liposomes were releasing only a minor amount of ODNs, ~7% (Zelphati and Szoka, 1996b). Importantly, in the studies of Szoka's group, releasing activity of various anionic lipids (PS, PI, PA) was always probed in triple mixtures of anionic lipid/DOPE/DOPC in the presence of a high fraction of PE in the liposome compositions (1:2:1 fractions, which equate to 50% PE). The double composition of PS/DOPC was not investigated. Therefore, we assume that in thus designed experiments the observed strong release of ODN by the triple-mixture liposomes might be a combined effect of both anionic lipids and PE. However, further investigation is needed to resolve the discrepancy between our data and the earlier observations by others regarding PE releasing activity, considering that we applied assays and measurement methodologies different from those of Szoka's group. So far the vigorous research being focused on understanding of the DNA/ODN-releasing actions is primarily for anionic lipids (Tarahovsky et al., 2004). By reporting our finding that PE can be the same efficient trigger of ODN release as PS, we suggest that more extensive investigation is also desired to comprehend a multifaceted role of membrane PE lipids in this process.

The diffuse nature of the ODN fluorescence in the GUV lumen signifies that released ODN is no longer aggregated into the original complex. However, this visual indication does not give a strict confirmation that ODN inside the vesicle is truly dissociated from the cationic lipids. By measuring the diffusion coefficients of ODN transferred within the GUV lumen and comparing them to the diffusion constant of free ODN in solution, we provide a quantitative confirmation that released ODN molecules dissociate from the carrier lipids.

A mechanism of ODN release from the cationic complexes in the absence of negatively charged anionic lipids in the barrier membrane is not understood and needs further investigation. A pertinent explanation of PE supporting activity in the ODN dissociation from the cationic lipids might be the specificity of its amine group. Given that the positive charge of the amine group in PE is relatively less shielded, it might be rather active in interaction with negatively charged molecular groups, such as phosphate groups in ODN. As a result, the amine group of PE can possibly compete with the cationic lipids of the carrier for the ODN phosphate groups, thus assisting in disassembly of the complex and allowing the ODN to dissociate from the carrier. This proposed mechanism of the PE amine group involvement in interaction with phosphate groups of ODNs also agrees with the observations, reported by others, showing that PE promotes destabilization of the CL-DNA complexes (Wong et al., 1996) and that the CL-DNA complexes prepared with PE helper lipids disassemble more readily than those prepared with PC lipids (Harvie et al., 1998).

Finally, data presented in this study support a growing trend in literature (Devaux, 2000; Cans et al., 2003), indicating that lipid-lipid interactions and lipid self-reorganization play highly active roles in diverse processes, which were previously thought to be governed exclusively by protein action.

CONCLUSIONS

In summary, our goal in this work was to replicate interactions of cationic lipid-ODN complexes with cellular membranes using a giant unilamellar vesicle system, and to study the roles of specific cellular lipids in ODN release from the carriers. Clearly, the use of the model membranes does not include the complex cellular environment, yet it allows for probing certain biophysical aspects of ODN delivery under controllable experimental conditions for well-defined lipid compositions. By comparing ODN transfer across the model membranes, composed of variable amounts of common cellular membrane lipids, we demonstrated that overall uncharged PE aminophospholipids can, alone, release ODN from the carriers equally as well as negatively charged PS. To conclude, the here-presented methodology of using a GUV system for modeling biological membranes and application of confocal imaging enhanced with fluorescence correlation analysis of ODN diffusion provides a novel convenient and robust tool for quick probing of the delivery potential of newly designed formulations of lipid-based carriers.

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