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Journal

Journal of Biological Chemistry, 273(25)

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Publication Date

1998-06-01

DOI

10.1074/jbc.273.25.15621

Peer reviewed

Cationic Liposomes Coated with Polyethylene Glycol As Carriers for Oligonucleotides*

(Received for publication, August 18, 1997, and in revised form March 2, 1998)

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Modification of liposome surface with polyethylene glycol was used to improve oligodeoxyribonucleotide (ODN) loading, stability of the resulting complexes, and specificity of cellular delivery of ODN by cationic liposomes. Liposomes composed of a cationic lipid (DOTAP, DOGS, DDAB), a neutral lipid (DOPE), and a phospholipid derivative of polyethylene glycol (PEG-PE) formed a complex with 18-mer phosphorothioate up to ODN/lipid molar ratio of 0.25. The complexes showed intact vesicular structures similar to original liposomes and their size (100–130 nm) was unchanged after several weeks of storage, whereas complexes lacking PEG-PE showed progressive aggregation and/or precipitation. After exposure to human plasma, PEG-modified cationic liposomes retained over 60% of the originally bound ODN. PEG-coated complexes resulted in 4–13-fold enhancement of the ODN uptake by human breast cancer cells in serum-supplemented growth medium, relative to free ODN. Complexes containing conjugated anti-HER2 F(ab') fragments at the distal termini of PEG chains efficiently delivered ODN primarily into the cytoplasm and nuclei of HER2 overexpressing cancer cells and greatly enhanced the biological activity of antisense ODN. The development of PEG-modified cationic liposomes may lead to improved ODN potency *in vivo*.

Oligodeoxyribonucleotides (ODN)¹ can be designed to specifically inhibit gene expression by blocking translation, splicing, or transcription process, thereby providing potentially powerful therapeutic tools against viral diseases and cancer. However, the biological efficacy of ODN is restrained by their poor

cellular uptake and availability to hybridize to their RNA or DNA targets present in the cytoplasm and/or nucleus (1). Cellular uptake of ODN, as well as their activity in cell cultures, can be improved by cationic liposomes (2–4), first used to improve cell transfection with plasmid DNA (5) and RNA (6). Such formulations, however, require substantial excess of cationic lipid over ODN (4) and are unsuitable for *in vivo* use because of inadequate particle size stability and increased risk of toxicity associated with the high dose of cationic lipid co-administered with the therapeutic dose of ODN. Furthermore, positively charged particulates are prone to nonspecific interactions with plasma proteins and negatively charged cell surfaces. This may lead to destabilization, dissociation, and rapid clearance of the liposome-ODN complexes by the mononuclear phagocyte system before reaching the diseased tissue.

PEG-PE, a phospholipid derivative of the hydrophilic polymer polyethylene glycol ($M_r = 2,000$), increases particle stability of small unilamellar phosphatidylcholine liposomes in plasma and reduces their interaction with biological macromolecules and/or cell surfaces (7). PEG-PE containing sterically stabilized liposomes have long circulation lifetimes, show high accumulation in pathological foci, such as tumors (7, 8), and are successfully being used as a delivery system for the cytotoxic drug doxorubicin in human patients (9, 10). More recently, studies have shown accumulation in mice tumor of encapsulated antisense ODN using sterically stabilized liposomes (11). Specific delivery of sterically stabilized liposomes to target cells can be achieved by attachment of target-seeking molecules, such as antibodies or their fragments, preferably at the distal ends of liposome-associated polyethylene glycol chains (12). However, the use of this system for the delivery of ODN may be limited by its low entrapment efficiency and difficulty for the entrapped ODN to leave the liposome interior.

Recently, it was found that incorporation of PEG-PE can stabilize the particles of cationic liposome-plasmid DNA complexes (13). Therefore, we hypothesized that incorporation of PEG-PE into cationic liposomes will increase particle stability of liposome-ODN complexes and make them more attractive for medical use. Using an 18-mer phosphorothioate as a model for any type of biologically active ODN, we show here that modification with PEG-PE not only prevents aggregation and dramatically increases particle stability of cationic liposome-ODN complexes but also allows extraordinarily high loading of ODN, resulting in a particle with overall neutral or negative charge. We further demonstrate that complexes of ODN with PEG-modified cationic liposomes have good stability in human plasma, and retain ultrastructural characteristics of original liposomes. We also show that PEG-coated cationic liposomes enhance the cellular uptake of ODN in serum-supplemented cell growth medium and that conjugation of antibody frag-

* This work was supported by the Bay Area Breast Cancer SPORE Program (P50CA58207), the California Breast Cancer Research Program (2CB-0250), and by a grant from Genta, Inc. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: ODN, oligodeoxyribonucleotide; C₁₂E₈, octaethylene glycol dodecyl ether; DDAB, dimethyldioctadecylammonium bromide; DOGS, N,N-dioctadecylamidoglycylspermine; DOTAP, 1,2-dioleoyl-3-(trimethylammonium)propane; DOPE, 1,2-dioleoyl-3-sn-phosphatidylethanolamine; DSPE, 1,2-distearoyl-3-sn-phosphatidylethanolamine; FITC, fluorescein isothiocyanate; HBS, HEPES-buffered saline; MES, 2-(N-morpholino)ethanesulfonic acid; MP-PEG-PE, ω-(3-(N-maleimido)propionylamido)-PEG-DSPE; PEG, polyethylene glycol; PEG-PE, N-(ω-methoxypoly(oxyethylene)oxycarbonyl)-DSPE; Rh-PE, N-lissamine-rhodamine B dihexadecanoylphosphatidylethanolamine; FBS, fetal bovine serum; ELISA, enzyme-linked immunosorbent assay.

ments recognizing the HER2 receptor on such cationic liposome-ODN complexes triggers efficient delivery of ODN to their sites of action (cytoplasm/nucleus) resulting in enhanced biological activity of antisense ODN in a target-specific manner.

EXPERIMENTAL PROCEDURES

Preparation of PEG-modified Cationic Liposomes and Liposome-ODN Complexes—DOPE, DDAB, and DOTAP were from Avanti Polar Lipids (Alabaster, AL). DOGS was a gift from Dr. Francis Szoka, Jr., UCSF). PEG-PE was a gift from Sequus Pharmaceuticals, Inc. (Menlo Park, CA), and all phosphorothioate 18-mer bcl-2 antisense ODN (5'-tct-ccc-agc-gtg-cgc-cat-3') and corresponding FITC-labeled and ^{35}S -labeled ODN were provided by Genta, Inc. (San Diego, CA). Other reagents were from Sigma unless indicated otherwise. Liposomes containing cationic lipid, DOPE, PEG-PE, and Rh-PE (molar ratio 25:25:3:0.1) were prepared by repeated freeze-thawing followed by extrusion through 0.05- μm pore membranes (14). Liposome-ODN complexes were produced by overnight incubation of the liposomes with ODN in HBS (20 mM HEPES-Na, 144 mM NaCl, pH 7.2) at 4 °C with gentle stirring. Unbound ODN was removed by gel-exclusion chromatography on Sepharose 4B (Amersham Pharmacia Biotech). For cell culture studies, the preparations were filter sterilized through 0.45- μm pore membrane (Corning, NY). Particle size was determined by dynamic laser light scattering using unimodal and differential size distribution processor analysis (Coulter Model N4 particle size analyzer).

Preparation of HER2-targeted PEG-modified Cationic Liposome-ODN Complexes—PEG-modified cationic liposomes bearing anti-HER2 F(ab') at the distal ends of PEG chains were prepared by a modification of our earlier method (15). DOTAP/DOPE/PEG-PE/MP-PEG-PE/Rh-PE liposomes (25:25:2:1:0.1 molar ratio) were prepared as above except that all procedures were performed in MES-buffer (20 mM MES, 2 mM EDTA, 144 mM NaCl, pH 6). F(ab') fragments of the recombinant anti-HER2 monoclonal antibody (rhuMabHER2) (Genentech, Inc, South San Francisco, CA), obtained as reported previously (16), were added to the liposomes at 0.13 mg of protein per each 10 μmol of lipid, and incubated at pH 7.3–7.4 (NaOH) for 2 h at room temperature under inert atmosphere. The reaction was stopped by blocking excess maleimido groups with 2 mM β -mercaptoethanol. Unconjugated protein was removed by gel-exclusion chromatography on Sepharose 4B, and liposomes with conjugated F(ab') fragments were loaded with ODN as above.

Quantitation of Liposome Lipids, ODN, and Liposome-conjugated F(ab')—Liposome concentration in stock preparations and ODN concentration in stock solutions were determined by phosphorus assay (17) and spectrophotometry ($\epsilon_{260}^{260} = 159, 160 \text{ M}^{-1}\text{cm}^{-1}$), respectively. The amount of liposome-conjugated F(ab') fragments per liposome was determined after separation of the conjugates by SDS-polyacrylamide gel electrophoresis followed by Coomassie Blue staining. The bands (46 kDa for intact F(ab') and 48–49 kDa for PEG-conjugated F(ab')) were cut out and extracted with 50% dimethylformamide, and the amount of protein per band was determined by spectrophotometry using simultaneously run F(ab') standards. In diluted samples containing octaethylene glycol dodecyl ether (C_{12}E_8) (0.05% (w/v)), liposome lipids and ODN were assayed by fluorescence spectroscopy using Rh-PE and FITC-ODN, respectively.

Zeta Potentials—Electrophoretic mobility of liposomes and liposome-ODN complexes were measured in 2 mM Tris-HCl, pH 8, containing 10% sucrose (w/v), using a Zetasizer 4 (Malvern Instruments, Ltd., UK). Zeta potentials were calculated from the mean of three runs after adjustment to a negatively charged standard (AZ55, Malvern).

Freeze-fracture Electron Microscopy—Specimens were quenched rapidly for freeze-fracture electron microscopy using the sandwich technique and liquid propane (cooling rate $>10^4$ K/s). Cryo-fixed samples were fractured and shadowed in a Balzers BAF 400D freeze-fracture device at -120 °C and 2×10^{-6} torr. The cleaned replicas were examined in a Zeiss CEM 902A transmission electron microscope (18).

Treatment with Plasma—Liposome-ODN complexes were incubated for 4 h at 37 °C in citrate phosphate adenine-preserved recovered human plasma (Blood Transfusion Center, Moffit Hospital, UCSF) diluted with an equal volume of HBS. The samples were then chromatographed on a Sepharose 4B column, and collected fractions were assayed by fluorometry for liposome lipids and ODN. Proteins were determined by dye binding assay (Bio-Rad).

Quantitation of ODN Uptake by Cells—Human breast cancer cells (SK-BR-3 and MCF-7) were provided by Dr. Christopher Benz, UCSF. The cells were cultured in McCoy's 5A or Dulbecco's modified Eagle's

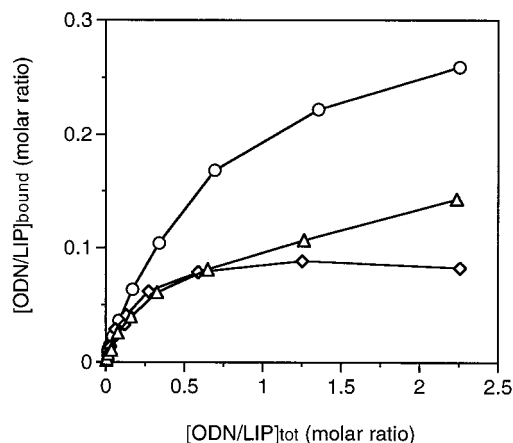


FIG. 1. **Binding of ODN to PEG-modified cationic liposomes.** Small unilamellar liposomes composed of DOTAP (○), DDAB (△), or DOGS (◇) in the mixture with DOPE, PEG-PE, and Rh-PE (25:25:3:0.1 molar ratio) were incubated with ODN overnight at 4 °C at the ODN/lipid molar ratio equal to $[\text{ODN/LIP}]_{\text{tot}}$, and the complexes were separated by gel chromatography. The amount of bound ODN is expressed as ODN/lipid molar ratio in the resulting complexes ($[\text{ODN/LIP}]_{\text{bound}}$).

H-21 medium (Life Technologies, Inc.), respectively, supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin (100 units/mg of each). Cells were plated in 12-well plates at 3×10^5 cells/well and allowed to attach overnight. The cells were incubated in 1 ml of complete (10% FBS-supplemented) growth medium containing 1 μM ^{35}S -labeled ODN alone or complexed with Rh-PE-labeled cationic liposomes at a final ODN to lipid molar ratio ($[\text{ODN/LIP}]_{\text{bound}}$) of 0.007 (corresponding to a $[\text{+/-}]$ net charge ratio of 4:1) for 4 h at 37 °C. The cells were then rinsed four times with cold PBS and solubilized with 1 ml of 0.1% (w/v) aqueous C_{12}E_8 , pH 7. ODN, liposome lipid, and protein in cell lysates were determined by radioactivity counting, fluorometry, and Bio-Rad dye protein assay, respectively.

Intracellular Distribution of ODN and Liposome Lipids—Cells were grown to subconfluency in Permax[®] chamber slides (Nunc, Inc. Naperville, IL). Liposome-ODN complexes ($[\text{ODN/LIP}]_{\text{bound}} = 0.007$) containing fluorescent lipid tracer Rh-PE and fluorescent ODN tracer FITC-ODN were diluted to a final concentration of 1 μM ODN into 0.5 ml of 10% FBS-supplemented cell culture medium and added to the cells. After 4 h of incubation at 37 °C, cells were washed four times with PBS and postincubated for 20 h in fresh FBS-supplemented medium. Cells were washed again with cold PBS and fixed for 30 min at 4 °C in PBS containing 2% (w/v) paraformaldehyde. Fixed cells were mounted in Gelvatol[®] (Monsato Chemicals, MI) and examined by epifluorescence microscopy.

Biological Activity of bcl-2 Antisense—Cellular expression of the bcl-2 protein was determined by ELISA (Endogen, Inc., Woburn, MA). Human breast carcinoma BT-474 cells were grown to subconfluency in 8-well plates and treated for 4 h with bcl-2 antisense either free or complexed with PEG-coated cationic liposomes, in 10% FBS-supplemented cell growth media, at a final antisense concentration of 2 μM . At the end of incubation time, cells were washed, fresh medium was added, and cells were allowed to grow for an additional 44 h before bcl-2 expression determination.

RESULTS

Characterization of ODN Complexes with PEG-modified Cationic Liposomes—PEG-modified cationic liposomes consisted of homogeneous populations of vesicles with mean sizes in the range of 100–130 nm. After incubation of liposomes with ODN, the ODN was co-eluted from the Sepharose 4B column with the liposome peak (void volume fraction), indicating binding of ODN to liposomes. Liposome-ODN binding profiles obtained by plotting the molar amount of liposome-bound ODN per mole of lipid ($[\text{ODN/LIP}]_{\text{bound}}$) versus the molar ODN to lipid ratio ($[\text{ODN/LIP}]_{\text{tot}}$) in the initial incubation mixture are presented in Fig. 1. On a Scatchard plot (not shown), the binding curve of ODN to DOGS/DOPE/PEG-PE liposomes fits well into a

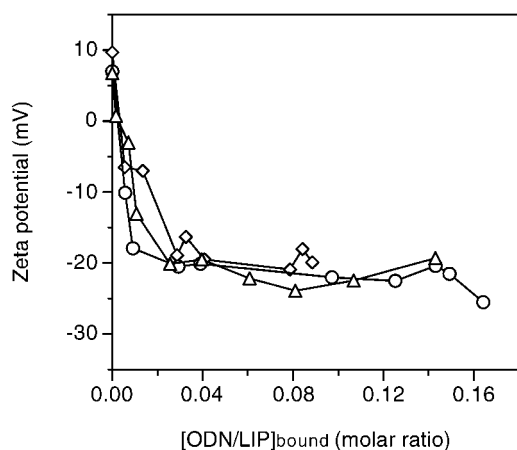


FIG. 2. Zeta potentials of PEG-modified cationic liposome-ODN complexes at various ODN to lipid ratios. Liposome composition: DOTAP/DOPE (\circ), DDAB/DOPE (\triangle), or DOGS/DOPE (\diamond), 1:1 molar ratio, all contain 5.7 mol% PEG-PE. Data are the means of three parallel runs. S.D. is within ± 4 –5 mV.

straight line, consistent with the model of simple saturation binding with independent binding sites, apparent $K_d = 0.103 \pm 0.02$ mol of ODN/mol of lipid, and maximum binding corresponding to charge equivalent complex. In the case of DOTAP- and DDAB-containing liposomes, the slope decreased with increasing $[\text{ODN/LIP}]_{\text{bound}}$, indicating the possibility of negative cooperativity of the binding or of multiple binding sites. Analysis of the curves according to Hunston (19) showed good agreement with the mechanism of negative cooperativity ($K_{d,2} = 0.51$ – 0.56 mol of ODN/mol of total lipid, cooperativity parameter $\mu = 4$ – 19) with maximum binding equivalent to approximately one molecule of ODN per molecule of a cationic lipid. Using DOTAP, the complexes with $[\text{ODN/LIP}]_{\text{bound}} = 0.25$ could be produced, which is equivalent to ODN content of approximately 70% by mass. Conjugation of antibody F(ab') fragments to PEG-modified cationic liposomes had no effect on the ODN binding.

PEG-PE, as well as ODN loading substantially affected the surface charge of cationic liposomes. Incorporation of 6 mol% PEG-PE reduced the positive zeta potential of cationic liposomes from 25–30 mV to 7–10 mV. Increased loading of liposomes with ODN further decreased and eventually changed their zeta potential to negative values (Fig. 2). When $[\text{ODN/LIP}]_{\text{bound}}$ increased from 0 to 0.03–0.04, zeta potentials decreased, passed neutrality, and reached a plateau at about -20 mV.

Freeze-fracture electron micrographs of DOTAP/DOPE/PEG-PE liposomes (Figs. 3A and 4A) showed spherical vesicles with smooth fracture faces. About 80% of the vesicles were less than 100 nm in diameter while 15% were larger than 100 nm and smaller than 300 nm, with the rest slightly larger. The presence of conjugated F(ab') fragments had no effect on the structure and size of PEG-modified cationic liposomes or their complexes with ODN (Fig. 3, B and D). Vesicular structures and size were well preserved after the complex formation with ODN both at lower ODN load (Fig. 3C) or higher load (Fig. 4B). In all PEG-PE containing preparations, particles of the complexes were much smaller than in similar preparations without PEG-PE (data not shown). At low ODN loads, the presence of ODN seemed to transform small liposomes into an involuted shape (two of such structures are marked by arrowheads in Fig. 3C). In the preparations containing conjugated F(ab') (Fig. 3D) as well as at higher ODN/lipid ratio (Fig. 4B), some of the vesicles were aggregated (seldom more than three), forming slightly larger particles (up to 300 nm in diameter). However,

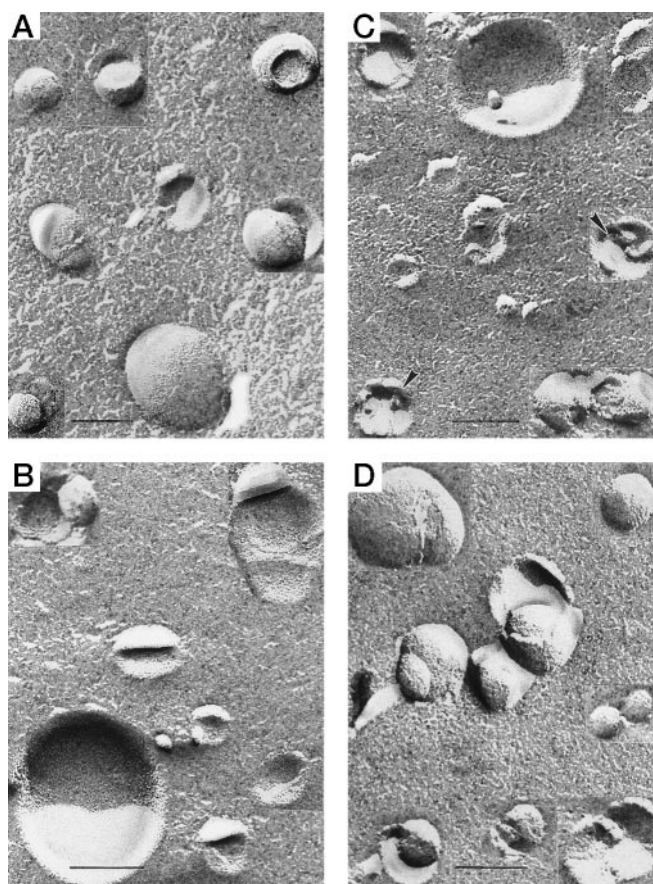


FIG. 3. Freeze-fracture electron micrographs of PEG-modified cationic liposomes and their complexes with ODN at $[\text{ODN/LIP}]_{\text{bound}} = 0.007$, $[+/-] = 4:1$. A, DOTAP/DOPE/PEG-PE liposomes; B, DOTAP/DOPE/PEG-PE/F(ab')-MP-PEG-PE immunoliposomes; C, DOTAP/DOPE/PEG-PE liposome-ODN complexes; D, DOTAP/DOPE/PEG-PE/F(ab')-MP-PEG-PE immunoliposome-ODN complexes. "Involved" structures are marked by an arrowhead. In all electron micrographs, the bar represents 100 nm, and the shadow direction is running from bottom to top.

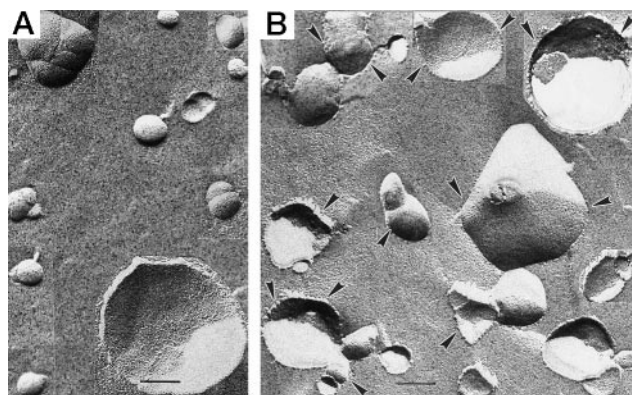


FIG. 4. Freeze-fracture electron micrographs of PEG-modified cationic liposomes and their complexes with ODN at $[\text{ODN/LIP}]_{\text{bound}} = 0.16$, $[+/-] = 1:6$. A, DOTAP/DOPE/PEG-PE liposomes; B, DOTAP/DOPE/PEG-PE liposome-ODN complexes. The boundaries of some of the complexes are dotted, and some of the dotted areas are marked by arrowheads. In all electron micrographs, the bar represents 100 nm, and the shadow direction is running from bottom to top.

the particle size of complexes was similar to that of the corresponding control liposomes within each preparation. For high ODN to lipid ratio, the size distribution of complexes seen on electron micrographs corresponds to the values of particle size obtained by differential (multimodal) dynamic light scattering analysis (Table I, footnote d). Furthermore, at lower ODN

TABLE I
Stability of ODN-cationic liposome complexes against aggregation in HBS buffer

Lipid composition ^a	ODN/lipid	Day 1	Day 4	Day 7	Day 21	Day 30
DOTAP/DOPE (1:1)	0.007	4093 ± 250 ^b	A/P ^c	A/P	A/P	A/P
DOTAP/DOPE (1:1)	0.2	215 ± 64	288 ± 85	330 ± 90	367 ± 100	A/P
DOTAP/DOPE/PEG-PE (1:1:0.12)	0.007	102 ± 30	102 ± 28	102 ± 28	107 ± 31	105 ± 30
DOTAP/DOPE/PEG-PE (1:1:0.12) ^d	0.2	123 ± 42	115 ± 37	113 ± 37	117 ± 38	116 ± 39

^a Molar ratio.

^b Mean ± S.D. by unimodal cumulant analysis.

^c Aggregation and/or precipitation. Presented data are the average of three measurements.

^d Differential size distribution processor analysis based on particle number, particle weight, and light intensity gave two major distinct size populations of 65–80 and 200–250 nm in diameter.

TABLE II
Uptake of ODN, PEG-modified cationic liposome-ODN complexes, and corresponding HER2-targeted complexes by human breast cancer cells *in vitro*

Cell type	SK-BR-3 ^a ODN ^c	Lipids ^d	MCF-7 ^b ODN ^c	Lipids ^d
ODN alone	15.6 ± 5.9 ^e	NA ^f	14.5 ± 1.6	NA ^f
Non-targeted complexes	76.1 ± 14.3	22.5 ± 2.3	65.7 ± 14.6	18.9 ± 2.8
Targeted complexes	200.8 ± 22.7	48.1 ± 1.4	152.6 ± 10.1	34.9 ± 4.3
Targeted complexes + free F(ab)	153.5 ± 27.5	32.4 ± 4.2	ND ^f	ND ^f

^a HER2-overexpressing breast cancer cells.

^b Breast cancer cells with low expression of HER2.

^c Pmol of ODN/mg of cell protein.

^d nmol of total lipid/mg of cell protein.

^e Mean ± S.D. (*N* = 6 for ODN, *N* = 3 for lipids).

^f NA, not applicable; ND, not done.

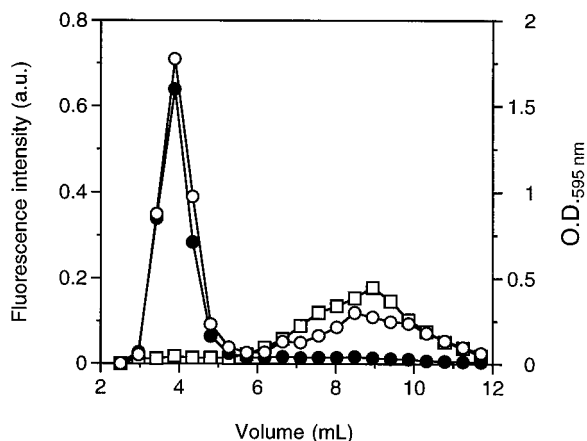


FIG. 5. Stability of PEG-modified cationic liposome-ODN complexes in human plasma. DOTAP/DOPE/PEG-PE liposome-ODN complexes ($[\text{ODN}/\text{LIP}]_{\text{bound}} = 0.2$) were incubated in 50% (v/v) human plasma/HBS at 37 °C for 4 h and chromatographed on Sepharose 4B (eluent HBS). Elution profiles are for liposome lipids (●), ODN (○), and plasma proteins (□).

loads, the boundaries of the vesicles were smooth, continuous, and intact (Fig. 3, C and D), whereas at high ODN loads, the boundaries were often dotted and discontinuous (some of these areas are marked with arrowheads on Fig. 4B). Thus, the high amount of interacting ODN seemed to distort the normally smooth boundaries of the vesicles. These distortions resemble micellar structures attached to the surface of the lipid bilayer. However, the individual ODN strands are not visible on freeze-fracture electron micrographs because of the resolution limit (>2 nm) (18).

Liposomal vehicles for drug delivery must be sufficiently stable against the loss of drug load and particle aggregation. The stability against dissociation was defined as the ability of PEG-modified cationic liposomes to retain ODN molecules in a physiological medium, such as blood plasma. After incubation with 50% human plasma, the complex of ODN with PEG-modified DOTAP/DOPE-containing liposomes was chromato-

graphed on Sepharose 4B. About 65% of the ODN and 100% of the lipid were co-eluted in the void volume fraction (as determined by the peak area), whereas the rest of ODN was co-eluted with the broad peak of plasma proteins (Fig. 5).

Modification of cationic liposomes with PEG-PE remarkably increased the stability of ODN-liposome complexes against aggregation (Table I). Without PEG-PE, cationic liposomes mixed with ODN in HBS at ODN/lipid molar ratio of 0.007 formed large particles which further aggregated and completely precipitated within 4 days at 4 °C. When ODN was in substantial excess over the cationic lipid ($[\text{ODN}/\text{LIP}]_{\text{bound}} = 0.2$), the particles were smaller and more stable to aggregation, although ultimately there was complete precipitation of the complex in 4 weeks. In contrast, the size of complexes of ODN with PEG-modified cationic liposomes did not change from its initial value of 110–125 nm for at least 4 weeks at both low and high ODN/lipid ratios, according to unimodal cumulant results from dynamic light scattering measurements.

Interaction of Liposome-ODN Complexes with Cancer Cells—

The effect of PEG-modified cationic liposomes on the cellular uptake of ODN was studied in two human breast cancer cell lines: SK-BR-3, which overexpresses the HER2 oncoprotein (approx. 10^6 copies/cell), and MCF-7, a line with low expression of HER2 (approx. 10^4 copies/cell) (20). The uptake data are summarized in Table II. Free ODN was poorly taken up by either cell line (about 15 pmol of ODN/mg of cell protein). However, when ODN was complexed with PEG-modified cationic liposomes ($[\text{ODN}/\text{LIP}]_{\text{bound}} = 0.007$), the amount of cell-associated ODN increased 4.2–4.5 fold. Conjugation of anti-HER2 F(ab') fragments at the terminal segment of liposome-associated PEG chains in the complexes (12 μg protein/ μmol of total lipid, or 1 F(ab') per 3, 800 lipid molecules) further increased the amount of cell-associated ODN in both cell lines by the factor of 2.3–2.6, to about 40–60% of the amount of ODN initially present in the incubation mixture. These data suggest that in addition to a HER2-specific enhancement of ODN uptake, conjugation with F(ab') fragments also led to a marked nonspecific enhancement. When the specific targeting effect was eliminated by preincubation of SK-BR-3 cells with 10-fold

FIG. 6. Intracellular distribution of PEG-modified cationic immunoliposome-ODN complexes studied by fluorescence microscopy. Liposomes of DOTAP, DOPE, PEG-PE, and maleimido-PEG-PE were conjugated to F(ab') fragments of rhuMabHER2 antibody and complexed with FITC-ODN ($[\text{ODN}/\text{LIP}]_{\text{bound}} = 0.007$, $[\text{+/-}] = 4:1$). The complexes were incubated with SK-BR-3 cells in 10% fetal bovine serum-supplemented growth medium as described under "Experimental Procedures." *Top panels*, localization of the lipid marker; *bottom panels*, localization of the ODN marker. Incubation conditions were: without free anti-HER2 F(ab') (*left*) or in the presence of a 10-fold excess of free F(ab') (*right*).

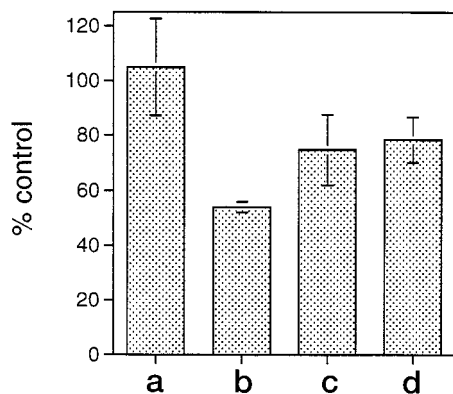
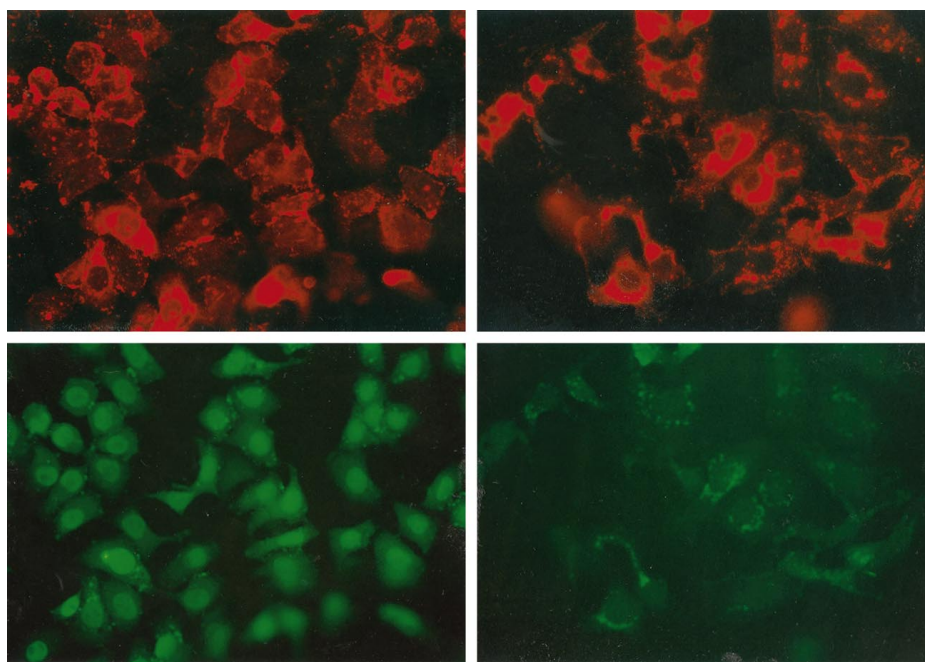


FIG. 7. Effect of free and liposomal bcl-2 antisense ODN on cellular level of protein expression. BT-474 cells in 10% FBS-supplemented growth medium were treated for 4 h with bcl-2 antisense ODN (final $[\text{ODN}] = 2 \mu\text{M}$) either complexed with PEG-coated cationic liposomes (*bar a*), complexed with corresponding HER2-targeted liposomes (*bar b*), complexed with HER2-targeted liposomes after pre-incubation with a 10-fold excess of F(ab') antibody fragments (*bar c*), or free (*bar d*). All liposome-ODN complexes were such that $[\text{ODN}/\text{LIP}]_{\text{bound}} = 0.007$, $[\text{+/-}] = 4:1$. After being washed, cells were incubated an additional 44 h before quantitative bcl-2 protein determination by ELISA. Data are expressed in percent of bcl-2 units/mg of cell protein over untreated cells and represent mean \pm S.D. of two experiments.

excess of free anti-HER2 F(ab'), the uptake of ODN decreased slightly (by 25%) and reached the level of target-negative (MCF-7) cells. The amount of cell-associated liposome lipid followed the same trend as that of ODN.

While immunotargeting of the liposome-ODN complex to HER2-overexpressing cells only slightly affected the total amount of cell-associated ODN, there was a pronounced effect on the intracellular distribution of ODN. Fluorescence microscopy of SK-BR-3 cells incubated with HER2-targeted liposome-ODN complexes revealed a large diffuse cytoplasmic localization of FITC-ODN as well as a high accumulation in the nucleus of every cell, with few additional cytoplasmic punctate patterns (Fig. 6, *bottom left panel*). Lipid distribution was different from that of ODN and was only seen at the cell surface and within intracellular organelles (Fig. 6, *top left panel*). Flu-

orescent punctate cytoplasmic patterns are typical for endocytic vesicles (15, 16). When SK-BR-3 cells incubated with HER2-targeted complexes in the presence of an excess of free F(ab'), FITC-ODN was exclusively concentrated inside perinuclear vesicles (Fig. 6, *bottom right panel*), together with colocalized lipid (Fig. 6, *top right panel*). The same feature was observed after incubation of non-targeted complexes with SK-BR-3 cells or after incubation of HER2-targeted complexes with MCF-7 cells (not shown).

Effect of Liposome-Antisense Complexes on bcl-2 Expression—Fig. 7 shows the effect of cellular treatment with free and formulated bcl-2 antisense on the level of expression of bcl-2 protein in BT-474 cells. No antisense effect could be detected when ODN was associated with PEG-coated cationic liposomes (*bar a*). On the contrary, when the corresponding targeted ODN delivery system was used, a 46% inhibition of bcl-2 expression was observed (*bar b*). In cases where ODN antisense was delivered by HER2-targeted liposomes after pre-incubation with a 10-fold excess of free F(ab') fragments (*bar c*) or where free antisense molecules were used (*bar d*), only a 20% inhibition of bcl-2 expression was obtained.

DISCUSSION

In this study, we explored the possibility of forming PEG-modified cationic liposome-ODN complexes. We studied their physico-chemical characteristics, their stability in biological fluids, and the applicability of the above-cited targeting method *in vitro* in order to develop a suitable ODN delivery system for future *in vivo* applications.

Binding of ODN to cationic liposomes was very efficient even when containing a relatively high amount of PEG-PE (5.7 mol%) (Fig. 1). Evidently, PEG coating does not shield the positively charged liposome surface from interaction with ODN. Furthermore, such liposomes can bind an impressive amount of ODN without the loss of structural stability in a wide range of ODN/lipid charge ratios. When liposomes were composed of DOTAP or DDAB, cationic lipids bearing single positive charge, the amounts of bound ODN exceeded the charge equivalent and, with gradually decreased affinity, the binding continued until the physical limit of one ODN molecule per one positively charged lipid group. This mechanism af-

forded exceptionally high loads of ODN in the complex without aggregation. Thus, after overnight incubation of PEG-coated DOTAP/DOPE liposomes with excess ODN, the resulting complex contained up to 0.25 mol of ODN per mol of total lipid (Fig. 1). Obviously, at such high ODN content the number of negatively charged groups in the complex far exceeded the number of available positive charges. This property of PEG-modified cationic liposomes is remarkable, since it has been shown (21) and confirmed by our present data (Table II) that ODN induce aggregation and fusion of cationic liposomes as a function of ODN/lipid charge ratio. Modification of cationic liposomes with PEG-PE allowed the formation of ODN complexes that are stable in size over prolonged storage (Table II). The stabilizing effect of PEG coating on the cationic liposome-ODN complex is likely to result from preventing cross-linking (and thus aggregation and precipitation) of the vesicles by ODN molecules whose stretched length approximates the extension length of the liposome PEG coating in "brush" regime (5–7 nm at ≥ 4 mol% PEG-PE) (22, 23), as well as avoiding bilayer contact needed for the aggregation to occur. Stabilization of cationic liposomes by PEG coating also contributed to high ODN-binding capacity by allowing the complex to pass through the charge neutralization point at increasing ODN/lipid ratio without aggregation.

Modification of cationic liposomes with PEG-PE not only increased particle stability after complexation with ODN, but also resulted in practically complete preservation of the original vesicles in the complex, as shown by freeze-fracture electron microscopy (Figs. 3 and 4). Preservation of the original small size of the vesicles after complexation with ODN may favor their better extravasation into the target tissue *in vivo* since the size limit for liposome extravasation ranges between 400 and 600 nm (24).

Addition of ODN even at low concentrations sharply modified the zeta potential of PEG-modified cationic liposomes to the saturation value of -22 to -25 mV, reached at approximately stoichiometric charge ratio of ODN to cationic lipid. The existence of this plateau suggests shielding effects of PEG on the excess negative charge carried by ODN nucleotides which are not associated with lipid positive charges. Although the pharmacokinetic behavior of PEG-modified cationic liposome-ODN complexes has not been completely characterized as yet (the study is in progress), the shielding effect of PEG on excess negative charges of liposome-bound ODN may have a beneficial effect on their ability to evade mononuclear phagocyte clearance.

PEG-modification increased the stability of cationic liposome-ODN complexes not only against aggregation, but also against dissociation and disintegration in the presence of plasma proteins. After incubation with plasma, practically all of the lipid and the major portion of the originally bound ODN was retained in the complex (Fig. 5). Due to the high initial load of ODN ($[\text{ODN}/\text{LIP}]_{\text{bound}} = 0.2$), the remaining ODN still constituted an impressive proportion of the complex, about 50% by mass.

Complexation of ODN with PEG-modified cationic liposomes enhanced the cellular uptake of ODN severalfold, similar to that observed with nonmodified cationic liposomes (2, 4). In an attempt to increase the cellular ODN uptake in a targeted manner, we modified the complex with a targeting device previously developed for neutral liposomes (15). In the case of PEG-modified complexes, conjugated anti-HER2 F(ab') fragments further increased the cellular uptake of both lipid and ODN by a factor of 2.3–2.6, regardless of the cellular expression level of the target antigen. Since this enhancement was not completely abolished by preincubation of cells with free F(ab')

that blocked the surface antigen, it must be attributed to additional nonspecific interactions which at present are poorly understood. The targeting effect, however, revealed itself in an unexpected way, namely, as dramatically increased diffuse cytoplasmic and nuclear localization of ODN in target-positive (SK-BR-3) cells. The green fluorescence has been attributed exclusively to the presence of ODN since it was shown that fluorescent ODN metabolites are quickly discarded from cells (25). Depending on their mode of action, ODN must either accumulate free within the cytoplasm to hybridize to their target mRNA, or in the nucleus when they are designed to be complementary to sites of transcription factor binding, splicing sites, nascent transcripts or to form a triple helix with DNA (26). Herein, we show that PEG-coated cationic liposome-ODN complexes conjugated to anti-HER2 F(ab') at their surface efficiently delivered ODN diffusely in the cytosol and to a larger extent in the nucleus of HER2-overexpressing breast cancer cells. Because there was no co-localization of ODN and lipid in these regions, ODN was presumably free of lipid, which is a requirement for their biological activity. When the specific interaction between the targeted complex and cell surface p185^{HER2} was blocked or missing, FITC-ODN was exclusively localized in the endosomal/lysosomal compartment, evidence of the importance of antibody-antigen interactions for successful delivery of ODN to their sites of action. We address the dependence of the cellular disposition of ODN upon the targetability of the delivery system used, in terms of the ability for ODN to evade endocytic compartments. It has been shown that after endocytosis of cationic liposome-ODN complexes, the escape of ODN from early endosomes into the cytoplasm is a critical step for their biological activity (4). Therefore, HER2 receptor-mediated endocytosis could direct the targeted complex into early endosomes that may be more permissive for the escape of ODN into the cytoplasm than the early endosomes formed in response to non-target specific interactions of the cell membrane with the complex itself. Confirmation of this hypothesis as well as the determination of the exact mechanism by which HER2-targeted complexes deliver ODN into the cytoplasm and nucleus of target-positive cells require further study.

Bcl-2 proto-oncogene encodes a 26-kDa protein that promotes cell survival by blocking programmed cell death (also termed apoptosis) (27). *Bcl-2* protein has been detected in a wide variety of cancers including breast carcinomas (28). To test the cell specificity of our ODN delivery systems in terms of biological antisense activity, we took advantage of the human breast cancer cell line BT-474, which both expresses the *bcl-2* protein (29) and overexpresses the HER2 receptor (20). Importantly, in a nontargeted complex *bcl-2* antisense did not promote any detectable inhibition of the level of expression of the *bcl-2* protein in BT-474 breast cancer cells, whereas when the targeted system was employed, a 46% inhibition of cellular *bcl-2* level was found over untreated cells. However, in accordance to cell uptake experiments (see Table II), presaturation of the HER2 receptor with free F(ab') fragments did not block entirely the antisense activity of ODN when delivered by the HER2-targeted liposomes, where the level of *bcl-2* expression reached about the same as when free antisense molecules were used. In view of these data, it seems that cell binding/internalization of positively charged particles, which involve both non-specific (electrostatic) and specific (ligand-receptor) interactions (the complexes used had a net positive charge, [+/-] = 4:1) can only be rendered partially tissue-specific. Therefore, despite a drastic enhancement in uptake by target cells, in intracytosolic/intranuclear ODN accumulation, and in antisense activity observed when ODN was delivered by HER2-targeted liposomes compared with non-targeted liposomes, we

believe that there is still a need for additional developments (currently in progress in our laboratory) to further optimize the ODN delivery system in terms of cell specificity.

PEG-modified cationic liposomes described in this study offer a number of advantages as carriers for cellular delivery of ODN. They allow exceptionally high loads of ODN without loss of structural stability. The resulting complexes are stable against aggregation and retain both their ODN load in blood plasma and the ability to enhance cellular delivery of ODN in the presence of serum. The presence of PEG also limits the negative zeta potential observed on the surface of the complexes. Finally, PEG-modified cationic liposome-ODN complexes can be made to incorporate the previously developed liposome targeting technology, resulting in efficient delivery of ODN into the cytoplasm and nuclei of target cells as well as enhanced antisense activity. In conclusion, PEG-modified cationic liposomes may provide a useful step in the development of an efficient pharmaceutical carrier for systemic *in vivo* delivery of ODN.

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