

Lipoplexes Targeting the CD44 Hyaluronic Acid Receptor for Efficient Transfection of Breast Cancer Cells

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Abstract: Lipoplexes containing a hyaluronic acid–dioleoylphosphatidylethanolamine (HA–DOPE) conjugate were designed to target the CD44 receptor on breast cancer cells. Cationic liposomes composed of a mixture of [2-(2,3-didodecyloxypropyl)hydroxyethyl]ammonium bromide (DE) and dioleoylphosphatidylethanolamine (DOPE) with or without HA–DOPE were prepared, characterized, and used to form a complex with plasmid DNA pCMV-luc. Lipoplexes displayed a negative zeta potential and a mean diameter between 250–300 nm. Cytotoxicity and transfection efficiency of the lipoplexes were determined on the MDA-MB-231 and MCF-7 breast cancer cell lines. Cytotoxicity was not modified by the presence of HA–DOPE. However HA–DOPE increased the level of transfection on CD44-expressing MDA-MB-231 cells compared to the MCF-7 line, which expresses very low levels of CD44. The transfection on the MDA-MB-231 cells was highly inhibited by anti-CD44 Hermes-1 antibody but not by the nonspecific anti-ErbB2 antibody. In conclusion, cationic liposomes containing the HA–DOPE conjugate mediated good transfection on CD44 expressing cell lines in culture.

Keywords: Cationic liposomes; CD44; hyaluronic acid; MCF-7; MDA-MB-231; targeting

Introduction

The design of highly efficient carriers for gene delivery has been approached by many research groups. These nonviral vectors should be able not only to cure diseases with genetic defects but also to treat and prevent major chronic diseases such as cancer, cardiovascular diseases and rheumatoid arthritis. Among the nonviral gene delivery systems, cationic liposomes have been widely investigated.

Their main advantages include low immunogenicity¹ and toxicity^{2,3} compared to viral vectors^{4,5} and their potential to transfect diverse tissue and cell types.^{3,6} Nevertheless, the low transfection efficiency, particularly *in vivo*, still remains

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an issue for an extended use of cationic liposomes. Mostly, interactions with plasma proteins and poor targeted delivery are strong issues to circumvent. There is therefore a need for developing a new generation of DNA carriers. One strategy consists of designing cationic liposomes that are able to bind specifically to target cells avoiding uptake by nonrelevant cells. For this purpose, the alternative consists of the insertion into cationic liposomes of a modified lipid bearing a targeting moiety. Hyaluronic acid (HA) was described by several authors to be an interesting ligand (for review see ref 7). It is a naturally occurring high molecular weight (10^6 Da) glycosaminoglycan. It exists in living systems, and it is a major component of the extracellular matrix. The hyaluronic acid receptor CD44 is found at low levels on the surface of epithelial, hematopoietic and neuronal cells, and is overexpressed in many cancer cells.^{8,9} CD44 appears to regulate lymphocyte adhesion to cells of the high endothelial venules during lymphocyte migration,¹⁰ a process that has many similarities to the metastatic dissemination of solid tumors.¹¹ It is also implicated in the regulation of the proliferation of cancer cells.¹² Recently, the cancer stem cell theory¹³ has proposed that CD44 represents a marker of breast cancer stem cells.^{14,15} The relationship between tumor cells and hyaluronic acid receptors indicates that it may be possible to recruit hyaluronic acid for active targeting to

tumor cells bearing this receptor. If located on the surface of lipoplexes, hyaluronic acid can successfully bind to CD44 receptors resulting in attachment and internalization of the loaded carrier. The discovery that many cancer types overexpress this receptor led to the development of HA–drug conjugates^{16–18} and HA-modified particulate drug carrier systems^{19–24} that were able to target these tumors very specifically *in vitro* and *in vivo*. Among the carrier systems mentioned above, HA-modified liposomes were reported to be very efficient in the delivery of anticancer drugs like doxorubicin or mitomycin C.^{19–21} In mouse tumor models, these liposomes showed a much higher efficacy compared to nonmodified liposomes and also a longer systemic circulation time. Prolonged circulation time was attributed to a “hydrophilic coat effect” of the HA, and this occurs using high molecular weight HA²¹ despite the fact that, when present in the vascular compartment, high molecular weight HA were shown to be cleared from the circulation by the monocyte phagocytic system.²⁵ In terms of cytotoxicity, CD44-expressing cells were much more sensitive to the cytotoxic drugs encapsulated in HA-modified liposomes while toxicity for CD44 deficient cells was unchanged. Despite all these advances made, there was no attempt

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described in the literature to attach hyaluronic acid to cationic liposomes to deliver DNA to cancer cells. In this paper, the transfection efficiency of lipoplexes containing a HA conjugate to dioleoylphosphatidylethanolamine (DOPE) was tested in human breast cancer lines (MCF-7 and MDA-MB-231) and compared to plain lipoplexes. Lipoplexes containing the conjugate displayed a greater transfection efficacy to cells expressing a high level of CD44 receptor with a very clear selectivity.

Experimental Section

Chemicals. High molecular weight hyaluronic acid (HA) (1500 KDa) was purchased from Fluka (Saint-Quentin-Fallavier, France), L- α -dioleoylphosphatidylethanolamine (DOPE), 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDAC), DNase I and molybdenum blue spray reagent 1.3% were obtained from Sigma-Aldrich (Saint-Quentin-Fallavier, France) and ethidium bromide (EtBr) from Bio-Rad (Marne la coquette, France). Dichloromethane and chloroform were provided by Carlo Erba Reagenti (Milano, Italy). Dulbecco's modified Eagle's medium (DMEM), PennStrep and phosphate buffer saline (PBS) were from Gibco (Eragny, France). Fetal calf serum (FCS) was purchased from Eurobio (Courtaboeuf, France). 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) [Cell Titer 96AQueous One Solution Reagent] was from Promega (Charbonnières-les-Bains, France). The Hermes-1 supernatant hybridoma antibody was provided by the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA), and the primary rabbit polyclonal anti-HER-2/*neu* antibody Neu(C-18) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Vectastain kit (antirabbit ABC peroxidase pk6104) was purchased from Vector Laboratories (Biovalley, Conches, France).

General Procedures. *Synthesis and Characterization of HA-DOPE Conjugate.* HA-DOPE conjugate was synthesized by a modified reaction described by Yerushalmi and Margalit.²⁶ Briefly, 14 mg of HA was dissolved in 5 mL of distilled water and preactivated for 2 h at 37 °C by incubation with 6 mg of EDAC at pH 4 adjusted by titration with HCl 0.1 N. Subsequently, a suspension of DOPE (360 μ g) was added to the HA solution and the pH was adjusted at 8.6 with a 0.1 M borate buffer pH 9.4. The reaction proceeded for 24 h at 37 °C. The solution containing the HA-DOPE conjugate was purified by ultrafiltration (MWCO 100 kDa) (Amicon Ultrafiltration, Millipore Corporate, Saint Quentin en Yvelines, France). Through this process, the HA-DOPE conjugate was completely retained over the filter, while EDAC and all other byproducts of the reaction were washed away. DOPE that did not react with HA was present as very small traces and was also eliminated during ultrafiltration. To ensure total elimination of DOPE (as demonstrated by

thin layer chromatography) the mixture was further dialyzed. Dialysis was carried out using a Spectra/Por regenerated cellulose membrane with a molecular cutoff of 12,000–14,000. Sample volume was 1.5 mL while the volume of the dialysis fluid was 3 L. The dialysis bag was changed 5 times every 10 h ensuring elimination of the last traces of DOPE. Although DOPE is known to self-aggregate, the dilution was such that it allows total removal of DOPE. These purification methods do not allow removal of free HA which remained in the solution containing the HA-DOPE conjugate. The final product was then lyophilized and the amount of DOPE linked to HA was quantified after resuspension in distilled water by a phosphate assay using the method of Bartlett.²⁷ The reactions were monitored by TLC using F₂₅₄ silica gel precoated sheets (Saint-Quentin-Fallavier, France). After migration of the mobile phase, sheets were exposed to iodine vapors, solutions of molybdenum blue and ninhydrin (2,2-dihydroxyindene-1,3-dione) solution (100 mg/100 mL ethanol).

Preparation and Characterization of Liposomes and Lipoplexes. The cationic lipid [2-(2,3-didodecyloxypropyl)-hydroxyethyl] ammonium bromide (DE) was synthesized as previously described,²⁸ dissolved in chloroform and mixed in 1:1 ratio (w/w) with a solution of DOPE in chloroform. The mixture was then dried under vacuum. For the preparation numbered 1, the lipid film was hydrated with Milli-Q water to a final concentration of 1 mg/mL of lipids and vortexed. In order to prepare HA-DOPE containing liposomes (2 to 5) the conjugate was dissolved in Milli-Q water and added in increasing amounts to the lipid suspensions. The different preparations contained the following amounts of HA-DOPE: 0.01 mg (preparation numbered 2); 0.04 mg (preparation numbered 3); 0.10 mg (preparation numbered 4) and 0.15 mg (preparation numbered 5). Lipid concentration was adjusted to a final concentration of 1 mg/mL. Finally, the aqueous dispersions were vortexed for 1 min at room temperature. Liposomes containing HA alone instead of HA-DOPE conjugate were prepared as described above.

Lipoplexes were prepared at a 2:1 lipid/DNA ratio (w/w). Plasmid DNA pCMV-luc (1 μ g) was complexed with plain cationic liposomes (preparation 1) or cationic liposomes containing increasing amounts of HA-DOPE as described above (preparations 2, 3, 4 and 5). After formation, lipoplexes were incubated at room temperature for 30 min. The main physicochemical characteristics of both liposomes and lipoplexes were then evaluated through size and zeta potential measurements (Malvern-Zetasizer-nano ZS, Malvern Worches-tre, U.K.).

Cryogenic Transmission Electron Microscopy (Cryo-TEM). DE:DOPE HA-DOPE 0.1 mg liposomes and lipoplex containing pCMV-luc plasmid DNA at a 2:1 w/w

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lipid/DNA ratio (preparation 4) were prepared and observed using the cryogenic transmission electron microscopy technique (cryo-TEM). Samples preparation was performed as follows: 5 μ L of each formulation was placed on 300 mesh Lacey Formvar/Carbon coated copper grids (Ted Pella Inc. Redding, CA). The excess amount of liquid was then blotted with a Whatman No.5 filter paper, and the grids were immediately plunged into a liquid ethane bath cooled with liquid nitrogen using a Leica EM CPC Cryoworkstation (Leica Microsystems SAS, Rueil Malmaison, France). Afterward, grids were constantly maintained in liquid nitrogen and carefully transferred in a cryo-holder (626 DH Gatan). Preparations were investigated at -170°C on a Cryo-JEM 2100 JEOL electron microscope operating at an accelerating voltage of 200 kV under low electron dose.

DNase I Sensitivity Assay. In order to obtain lipoplexes at a ratio ranging from 1:1 to 5:1 lipid:DNA (w/w), plasmid DNA pCMV-luc (1 μ g) was complexed with cationic liposomes (preparation 1) and cationic liposomes containing increasing amounts of HA-DOPE (preparations 2, 3, 4 and 5). Subsequently, they were treated with 10 μ L of DNase I (at a final concentration of 1 μ g/mL) in the presence of 20 mM of MgCl_2 and incubated for 10 min at 37°C . The reaction was then halted by adding EDTA (to a final concentration of 200 mM) and incubating at 60°C for 20 min in a water bath. Lipids were then extracted from the aqueous DNA sample with 30 μ L of a chloroform saturated solution of phenol and centrifuged at 10000g for 5 min. The aqueous DNA containing supernatants were separated, and 15 μ L was loaded on a 1% agarose gel (prestained with ethidium bromide); 6 $\times$ loading buffer made of 0.25% bromophenol blue in 30% w/v glycerol in H_2O was added to each complex, and gel electrophoresis was carried out at 110 V for 1 h in TAE buffer pH 8 (20 mM tris-acetate, 1 mM EDTA). As a control, the same preparations, treated identically but without DNase I, were submitted to an agarose gel electrophoresis in the same conditions as treated samples.

Cell Culture. Human breast cancer MDA-MB-231 and MCF-7 cells were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), PennStrep 1% and glutamine 1%. Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 .

CD44 Reverse Transcription-Polymerase Chain Reaction (RT-PCR). The mRNA level of CD44 standard and isoforms was examined by reverse transcription PCR (RT-PCR). Total RNA was extracted from MCF-7 and MDA-MB-231 cell lines with Trizol (Invitrogen, Cergy-Pontoise, France) according to the manufacturer's instructions, and the yield was quantified by UV analysis at 260/280 nm. Reverse transcription was performed with 5 μ g of total mRNA in the presence of 5 μ L of random primers (40 ng/mL) (Invitrogen, Cergy-Pontoise, France) and 1 μ L of dNTP (containing a mixture of every nucleotide at 1 mM (Invitrogen, Cergy-Pontoise, France) in a total volume of 12 μ L of H_2O . The mixture was incubated at 65°C for 5 min and kept in ice. Subsequently, 4 μ L of the first strand buffer 5 $\times$

(Invitrogen, Cergy-Pontoise, France), 2 μ L of DTT 0.1 M, 1 μ L of RNase out (40 U/L) (Invitrogen, Cergy-Pontoise, France) and 1 μ L of M-MLV reverse transcriptase (200 U/ μ L) (Invitrogen, Cergy-Pontoise, France) were added and incubated for 10 min at 25°C , followed by 50 min at 37°C and finally 15 min at 60°C in order to inactivate the enzyme. A human CD44 cDNA was amplified by PCR using the following primers: CD44^{S5}, 5' GCA GCA CTT CAG GAG GTT ACA T 3'; CD44^{S11}, 5' CAA GAG GGA TGC CAA GAT GAT 3'. The relative expression of actin was used as reference using the following primers: 5' TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA 3' and 5' CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG 3'. Briefly, 2 μ L of cDNA was added to a mixture of 2.5 μ L of amplification buffer (Invitrogen, Cergy-Pontoise, France), 0.5 μ L of dNTP 10 mM, 0.75 μ L of MgCl_2 50 mM, 0.2 μ L of Taq platinum polymerase (Invitrogen, Cergy-Pontoise, France), 1 μ L of CD44 primers 10 μ M (Invitrogen, Cergy-Pontoise, France) and 0.15 μ L of actin primers 10 μ M (Invitrogen, Cergy-Pontoise, France) in a total volume of 25 μ L of H_2O . The PCR reactions were carried out as follows: 95°C for 1 min, 60°C for 1 min and 72°C for 1 min over 35 cycles, followed by 72°C for 10 min in a PCR Crocodile III vector (AppliGene Oncor, Illkirch, France). PCR products were then analyzed on a 2% agarose gel.

Quantitative PCR. ARN samples were prepared and reverse-transcribed as previously described in RT PCR above. The Q PCR was performed on Roche LightCycler version 3.5 during 40 cycles of amplification at 62°C (Tm). The sequences of the primers (Eurogentec, Liège, Belgium) used in this study are 5' CA-GCA-CTT-CAG-GAG-GTT-ACA-T 3' (sense), 5' CAA-GAG-GGA-TGC-CAA-GAT-GAT 3' (antisense). The cycle at which the fluorescence exceeds a detection threshold, called crossing point, Cp, was determined since it correlates to the number of target cDNA molecules present in the added cDNA.

Fluorescence-Activated Cell Sorting (FACS). Analyses of MCF-7 and MDA-MB-231 by cell sorting were performed in a flow cytometer FACS calibur Becton Dickinson (Franklin Lakes, NJ). After rinsing with PBS-FCS 1%, 10^5 cells were incubated 25 min at 4°C with a CD44-labeled FITC antibody (Clone J.173, Beckman Coulter, California) and with the viability dye 7-AAD (Beckman Coulter, California) according to manufacturer's instructions, diluted in PBS-FCS 1%. Then, the cells were washed twice in PBS-FCS 1% before being recovered in DMEM-FCS 1%.

Cell Viability. MCF-7 and MDA-MB-231 were seeded in DMEM with 10% FCS in 96 well plates (1×10^4 cells/well) and incubated one day prior to the experiment in a humidified atmosphere containing 5% CO_2 . Preparations 1, 2, 3, 4, and 5 were incubated with plasmid pCMV-Luc for 30 min at room temperature in order to obtain a lipid/DNA charge ratio of 2:1 w/w. Cell viability was evaluated for three different concentrations of lipoplexes maintaining the same lipid/DNA ratio [15 μ g/mL (10 μ g of lipid:5 μ g of DNA), 7.5 μ g/mL (5 μ g of lipid:2.5 μ g of DNA), 3.75

$\mu\text{g/mL}$ (2.50 μg of lipid: 1.25 μg of DNA)]. Lipoplexes were then incubated with cells in serum free medium. Medium was replaced after 12 h with fresh medium containing 10% FCS, and incubation was continued in the same conditions. Cell viability was quantified after 24, 48, and 72 h using the Cell Titer 96AQueous One Solution cell proliferation assay (MTS salt)²⁹ and monitoring the absorbance at 490 nm (Elisa Microplate Reader, Merteck Inc., Korea) after suitable color development (1 h). Cell viability was determined by comparing the amount of MTS reduced by cells incubated with lipoplexes to the amount of MTS reduced in control cells.

Transfection Experiments. Cells (1×10^5) were seeded in 6 well-plates one day before the transfection experiments and grown in the appropriate medium with 10% FCS. The cell lines reached 40–50% confluence at the time of transfection, prepared in the same manner as described above, and were incubated with cells in serum free medium at a concentration of 7.5 $\mu\text{g/mL}$. The same assay was carried out also with lipoplexes containing HA instead of HA–DOPE conjugate. Transfection was performed in serum-free medium by adding the complexes dropwise to the cells followed by incubation in a humidified atmosphere containing 5% CO_2 for 12 h. Medium was then replaced with fresh medium containing 10% FCS and incubation continued for an additional 42 h.

Luciferase Assay. Transfected cells were rinsed with phosphate buffer saline (PBS) and lysed in 250 μL of the luciferase buffer (Tris 25 mM, H_3PO_4 pH 7.8, MgCl_2 10 mM, Triton X100 1%, glycerol 15%, EDTA 1 mM, DTT 1 mM). Cell lysates were cleared by centrifugation (10 min, 14000g, 4 $^\circ\text{C}$). Quantification of the luciferase activity was performed in a luminometer (Lumat LB 9507, Berthold, Bad Wildbad, Germany) after addition of ATP plus luciferin. Results were normalized to the protein content measured by the Bradford assay,³⁰ and the data are expressed as relative light units (RLU) per mg of protein. Luciferase assays were performed in triplicate from at least three independent experiments. Comparison of relative induction between plain liposomes and liposomes containing HA–DOPE conjugate was determined by nonparametric Student *t* test. A *p* value less than 0.05 was considered statistically significant.

Transfection Assay in the Presence of Anti-CD44 Hermes-1 and Anti-ErbB2 Antibodies. A transfection assay in the presence of an anti-CD44 antibody was carried out. MCF-7 and MDA-MB-231 cells were treated with increasing amounts (0.01, 0.1, 0.5, and 1 $\mu\text{g/mL}$) of the supernatant anti-CD44 primary antibody Hermes-1 or Anti-ErbB2 antibody (0.5 and 1 $\mu\text{g/mL}$) for 2 h at 37 $^\circ\text{C}$ before adding the

lipoplexes (preparations 1 and 4). Luciferase activity assay was carried out as described above.

Results

Synthesis of HA–DOPE Conjugate. Hyaluronic acid is a polymer composed of D-glucuronic acid and D-N-acetylglucosamine, linked together via alternating β -1,4 and β -1,3 glycosidic bonds.³¹ In our procedure, HA was preactivated and added to a DOPE solution in order to obtain an HA–DOPE conjugate which can be introduced during the lipid film hydration step in liposome preparation procedure. The HA–DOPE conjugate was prepared using a reaction in which the DOPE amino group is randomly linked to the carboxylic residues of HA. Through the condensation reaction between HA and DOPE, an amidic bond between the carboxylic group of HA and amino group of DOPE was formed. This linkage was obtained by using a soluble carbodiimide (EDAC) that activated the carboxylic residues of HA making them available for the reaction with the DOPE amino group.

At the beginning of the reaction, the condensing agent (EDAC) was added to the hyaluronic acid solution in water at pH 4 because carbodiimide reacts with carboxylic groups to give an intermediate *O*-acylisourea in acid buffer. Then DOPE was introduced in the mixture, and because the reaction between an amino group and an activated carboxylic residue is particularly favored in a water and basic medium, the pH was adjusted to 8.6. The reaction was monitored by TLC and stopped after 24 h. After purification and lyophilization, the conjugate was again characterized by TLC. Spots were ninhydrin negative and phosphate and iodine positive. The single spot of HA–DOPE on TLC has $R_f = 0$ while DOPE has $R_f = 0.7$. This latest spot did not appear on the TLC, meaning that all free DOPE was removed by the purification procedure. The quantitative lipid assay revealed that in the conjugate the final ratio was 3 μg of DOPE/mg of HA.

Characterization of Liposomes and Lipoplexes. Lipid films made of DE:DOPE 1:1 w/w were hydrated with Milli-Q water (preparation numbered 1) and with increasing amounts of a HA–DOPE solution [0.01 mg (preparation numbered 2); 0.04 mg (preparation numbered 3); 0.10 mg (preparation numbered 4); 0.15 mg (preparation numbered 5)] to obtain a final lipid concentration of 1 mg/mL for each sample. The physicochemical characteristics of liposomes containing different amounts of HA–DOPE conjugate were then evaluated by size and zeta potential measurements (Table 1). The liposomes containing only DE/DOPE showed a mean diameter around 450 nm, but as the amount of HA–DOPE conjugate concentration was increased in the preparation, the size increased up to around 900 nm, and the polydispersity index (PDI) increased in the same way. The diameter and the PDI raised similarly when HA was physically attached

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Table 1. Size and Zeta Potential Values of Liposomes and Lipoplexes^a

preparations	liposome size (nm)	PDI	liposome zeta potential (mV)	lipoplex size (nm)	PDI	lipoplexes zeta potential (mV)
DE	450 ± 47	0.53	91.8 ± 2.1	356 ± 18	0.20	−15.7 ± 0.4
DE HA-DOPE						
0.01	548 ± 53	0.59	89.5 ± 0.5	350 ± 28	0.19	−22.9 ± 0.7
0.04	574 ± 25	0.55	80.1 ± 2.0	367 ± 28	0.24	−30.6 ± 0.7
0.10	659 ± 36	0.59	64.6 ± 2.1	310 ± 12	0.21	−33.1 ± 0.8
0.15	894 ± 59	0.58	56.9 ± 1.6	256 ± 27	0.25	−46.1 ± 0.9

^a Liposomes were prepared as 1:1 (w/w) of DE:DOPE and increasing amounts of HA-DOPE and then mixed with DNA at 2:1 lipid:DNA ratio. Results are expressed as means ± SD of three independent experiments.

Table 2. Size and Zeta Potential Values of Liposomes and Lipoplexes^a

preparations	liposome size (nm)	PDI	liposome zeta potential (mV)	lipoplex size (nm)	PDI	lipoplexes zeta potential (mV)
DE	531 ± 12	0.61	87.2 ± 1.5	403 ± 45	0.47	−21.9 ± 1.8
DE HA						
0.01	607 ± 72	0.59	84.5 ± 1.0	353 ± 13	0.43	−26.3 ± 0.5
0.04	641 ± 16	0.63	67.0 ± 1.0	334 ± 13	0.40	−27.9 ± 0.3
0.10	868 ± 20	0.56	57.6 ± 1.2	574 ± 42	0.63	−32.4 ± 0.4
0.15	888 ± 68	0.55	60.1 ± 0.4	520 ± 39	0.53	−40.1 ± 0.5

^a Liposomes were prepared as 1:1 (w/w) of DE:DOPE and increasing amounts of unconjugated HA and then mixed with DNA at 2:1 lipid:DNA ratio. Results are expressed as means ± SD of three independent experiments.

to liposomes (Table 1). Cationic liposomes displayed a net positive zeta potential value decreasing from +91.8 mV to +56.9 mV as the amount of HA-DOPE conjugate increased. Liposomes containing HA alone instead of HA-DOPE conjugate were also prepared. Size and zeta potential values were measured to evaluate whether the presence of HA linked to DOPE could change either the size or the zeta potential of the liposomes. The results showed that liposomes containing HA are in the same range as those containing the HA-DOPE conjugate (Tables 1 and 2). The same observation could be made for zeta potential (Tables 1 and 2).

The size and zeta potential measurements of lipoplexes prepared at a 2:1 lipid/DNA ratio (w/w) were also performed. Lipoplexes showed very slight size reductions according to the amount of conjugate (from 350 to 300 nm) reaching 250 nm for the largest amount of HA-DOPE (Table 1). As opposed to empty liposomes, the polydispersity index was low and DNA was shown to compact liposomes and decreases their size. This was not the case, particularly for the PDI, when lipoplexes contained physically attached HA (Table 2). Zeta potential of lipoplexes turned out to be negative for all samples particularly when increasing the amounts of the conjugate or free HA; it decreased proportionally to the quantity of conjugate or free HA added to the lipoplexes.

Cryo-Tem micrographs of cationic liposomes indicate the coexistence of spherical unilamellar, bilamellar and invaginated vesicles with some tendency to aggregation (Figure 1A). The heterogeneity of vesicle population correlated well with the data obtained from size measurements where plain liposomes containing HA showed a very high polydispersity index. After addition of pCMV-luc plasmid DNA, a drastic

change in vesicle morphology occurred (Figure 1B). Lipoplexes display a reduction of size heterogeneity and a structure characterized by a dense spherical core with homogeneous shapes, and it is no more possible to observe the unilamellar structure as in the initial liposomal suspension.

Sensitivity of Lipoplexes to DNase I. Tight association of cationic liposomes with DNA protects plasmid from DNase I digestion. The protection is diminished when the complex dissociates or if the DNA is accessible to enzyme degradation. Thus the sensitivity of DNA to DNase I digestion was used to assess the degree of DNA exposure within the lipid-DNA complex. DNase I protection experiments were carried out across the entire range of the lipid/DNA charge ratios (Figure 2A,B). Following DNA digestion, the remaining DNA was separated from the lipid and DNase I and loaded on a 1% agarose gel. When plasmid DNA was incubated with DNase I, it was completely digested within 10 min (band intensity of naked DNA was no longer visible). Band intensities of inaccessible and therefore undigested DNA associated with the cationic liposomes (preparations 1 and 4) across the range of 2:1 to 5:1 (lipid: DNA w/w) were present for all samples demonstrating a protection of DNA versus DNase I digestion (Figure 2A,B). However, at low lipid:DNA ratio (1:1) a stronger band was observed for HA-DOPE conjugate containing liposomes, suggesting a better protection of DNA in these latest cases (Figure 2B). Controls on both types of lipoplexes that did not undergo DNase treatment (Figure 2C,D) showed qualitatively that some of the DNA was degraded particularly at low lipid:DNA ratios.

Quantification of CD44-mRNA. The mRNA level of CD44 standard and isoforms was examined first by RT-PCR and found to be expressed in both MDA-MB-231 and MCF-7

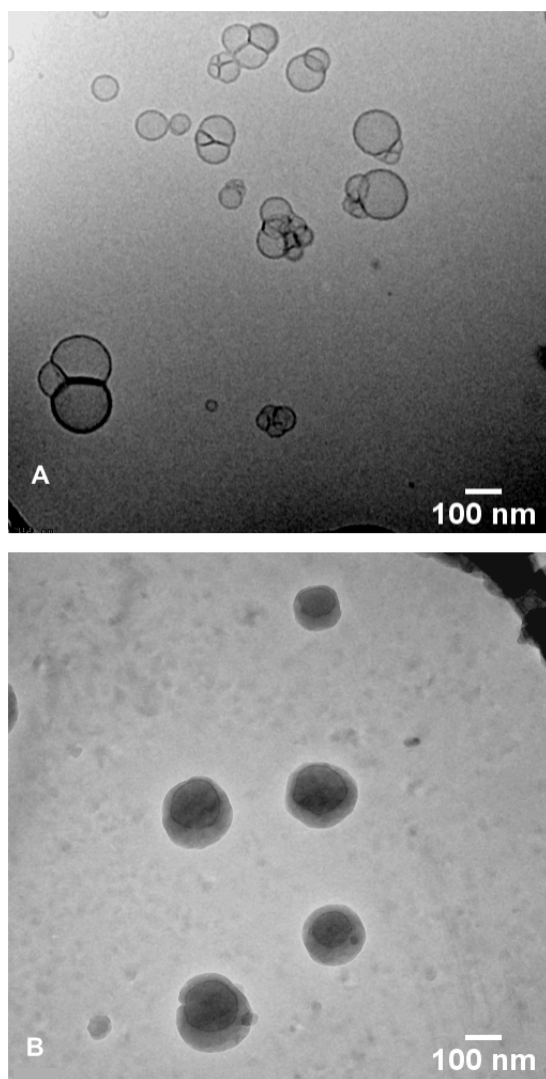


Figure 1. CryoTEM observation of cationic liposomes (A) and lipoplexes (B) both containing HA–DOPE conjugate (preparation 4 HA–DOPE 0.10). Lipoplexes were obtained by mixing cationic liposomes with DNA at 2:1 lipid:DNA ratio. Bar = 100 nm.

breast cell lines (Figure 3A). The expression of actin was used as an internal control. After amplification, the actin fragment size is 610 bp while for CD44 the size is 260 bp (Figure 3A). The levels of CD44-mRNA relative to actin are consistently higher in MDA-MB-231 cells as compared to MCF-7 cells, even though there is a weak band in MCF-7 which suggests a very low expression of CD44 receptor on this cell line. Further the analysis of CD44-mRNA expression was carried out by Q PCR showing that MDA-MB-231 cells ($C_p = 18.1$) contain more CD44-mRNA than MCF-7 cells ($C_p = 26.25$, in gray), indicating a 284-fold increase of CD44-mRNA in MDA-MB-231 compared to MCF-7. In final, FACS analyses revealed that both MCF-7 and MDA-MB-231 breast cancer cells express CD44 but at different levels since the MFI (median fluorescence intensity) are respectively 36.58 (MCF-7) (Figure 3B) and 614.07 (MDA-

MB-231) (Figure 3C) indicating a 17-fold increase of CD44 expression at the surface of MDA-MB-231 compared to MCF-7 cells, and in agreement with Q PCR data.

Cell Viability. Three different concentrations of lipoplexes (15 $\mu\text{g/mL}$, 7.5 $\mu\text{g/mL}$ and 3.75 $\mu\text{g/mL}$) maintaining the same lipid/DNA ratio (2:1 w/w) were assessed for cytotoxicity, but only the results concerning the concentration of 7.5 $\mu\text{g/mL}$ are displayed in Figure 4. Actually, using 15 μg of lipoplexes (10 μg of liposome + 5 μg of DNA) was shown to be toxic both for MDA-MB-231 and MCF-7 cells in particular; after 72 h cell viability was less than 60%. Under these conditions, the presence of the conjugate did not modify the cytotoxicity compared to the plain cationic lipoplexes. Transfection assays were then performed at a concentration in lipoplexes of 7.5 $\mu\text{g/mL}$ as this was shown to be the most efficient and least toxic amount of lipoplexes that could be used (Figure 4).

Transfection Experiments. Transfection efficiency using lipoplexes either containing the HA–DOPE conjugate or not was evaluated on MCF-7 and MDA-MB-231 cells. Cationic liposomes containing HA–DOPE were found to be more efficient than the ones containing only DE/DOPE 1:1 in both MCF-7 and MDA-MB-231 cells. In particular, the transfection efficiency was shown to be higher when increasing the amount of HA–DOPE conjugate in liposomes. Lipoplexes containing 0.1 mg of HA–DOPE (preparation 4, relative induction = 257.69 ± 50.57 corresponding to 7.77×10^7 RLU/mg of protein) are 2–3 times more efficient than lipoplexes without conjugate in MCF-7 (preparation 1 corresponding to 3.33×10^7 RLU/mg) ($p < 0.05$) (Figure 5A) and 6–7 times more efficient in MDA-MB-231 cells (preparation 4, relative induction = 814 ± 47 corresponding to 1.40×10^8 RLU/mg of protein compared to 2.1×10^7 RLU/mg for preparation 1, $p < 0.05$) (Figure 5B). Since preparation 4 had the highest transfection rate among lipoplexes containing different amounts of HA–DOPE conjugate, this formulation was selected for further testing and the results were compared to lipoplexes that did not contain the conjugate (preparation 1). In order to assess whether the presence of the DOPE linked to HA could improve the transfection efficiency, the same assay was then performed with liposomes containing HA alone instead of HA–DOPE conjugate. Lipoplexes associated physically with HA were found to be less efficient than HA–DOPE lipoplexes in both MCF-7 ($p < 0.05$) and MDA-MB-231 cells ($p < 0.05$) (Figure 5A,B).

Effect of Anti-CD44 Hermes-1 Antibody on Lipoplex Transfection Efficiency. As shown by CD44 RT-PCR Q PCR and FACS assays, high CD44 expression was detected in MDA-MB-231 cells, while poor expression was seen in MCF-7. Hermes-1 antibody is a supernatant rat IgG2 which recognizes the N-terminal hyaluronate binding domain of CD44 receptor (both CD44s and its

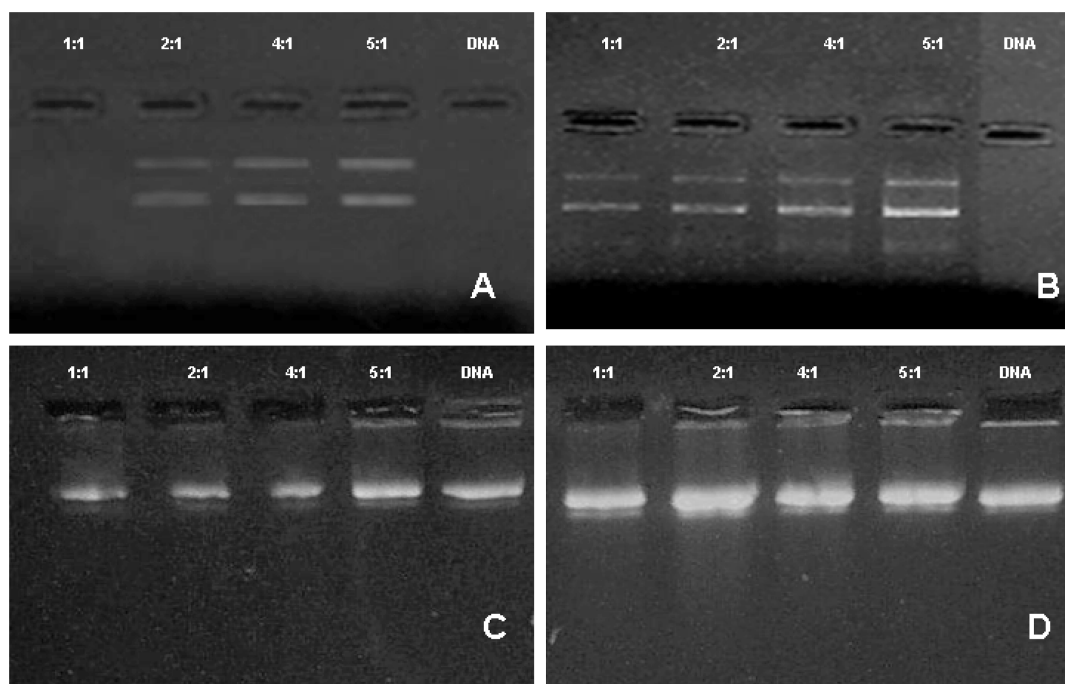


Figure 2. DNase I sensitivity assays of DE:DOPE 1:1 liposomes (A) and DE:DOPE 1:1 HA–DOPE 0.10 liposomes (B) complexed to DNA at increasing lipid:DNA ratios. Lipoplexes and naked DNA were incubated with DNase I, extracted and separated on a 1% agarose gel. Similar experiment was carried out on DE:DOPE 1:1 liposomes (C) and DE:DOPE 1:1 HA–DOPE 0.10 liposomes (D) complexed to DNA at increasing lipid:DNA ratios without treatment with DNase. I.

isoforms CD44v)³² and inhibits hyaluronic acid binding.³³ Transfection assay was performed in both cell lines previously treated with increasing amounts of supernatant antibody Hermes-1 in order to saturate and block the CD44 receptors and were then transfected with DE/DOPE 1:1 (preparation 1) (Figure 6A,B) and 0.1 mg HA–DOPE DE/DOPE 1:1 lipoplexes (preparation 4) (Figure 6A,B). MCF-7 cells did not show appreciable inhibition of luciferase activity whether they were transfected in the presence of Hermes-1 antibody or not ($p = 0.07$) (Figure 6A). However, a clear reduction of transfection was shown in MDA-MB-231 cells by increasing the amount of antibody (Figure 6B). In particular an inhibition of the efficiency of 70% in comparison with the control was seen when cells were treated with 1 $\mu\text{g/mL}$ of anti-CD44 Hermes-1 and then transfected with preparation 4 ($p < 0.05$) (Figure 6B). In both cases, transfection was not inhibited by anti-ErbB2 antibody used as control (Figure 6A,B).

Discussion

The aim of the present study was to design a specific nonviral delivery system that is able to target the hyaluronan receptor CD44 present on breast cancer cells. To achieve this goal, a HA–DOPE conjugate was synthesized and introduced into cationic lipids during liposome formation. Using this approach, it was possible to control and optimize the amount of conjugate present onto liposome surface, and therefore to assess the most efficient and least toxic quantity necessary for optimal cell transfection. While others' attempts to attach HA to liposomes consisted of binding HA to the surface of preformed liposomes with poor control of the density of attachment of HA,^{19,21,26} our method permitted introduction of the desired amount and variation of the density of hyaluronic acid that is necessary for optimal efficiency of lipoplexes.

Liposomes containing or not the HA–DOPE conjugate were thus prepared and their physicochemical properties assessed. Size measurements as well as cryomicroscopy observations evidence a change in the organization of liposomes before and after lipoplex formation. Indeed, the addition of DNA to cationic liposomes, containing or not HA–DOPE, induced a reduction of the mean size and polydispersity index that probably results from the reorganization of liposomes. This reorganization was also confirmed by cryomicroscopy that showed upon addition of DNA to liposomes, a transformation of the vesicular structure into small homogeneous aggregates with a very

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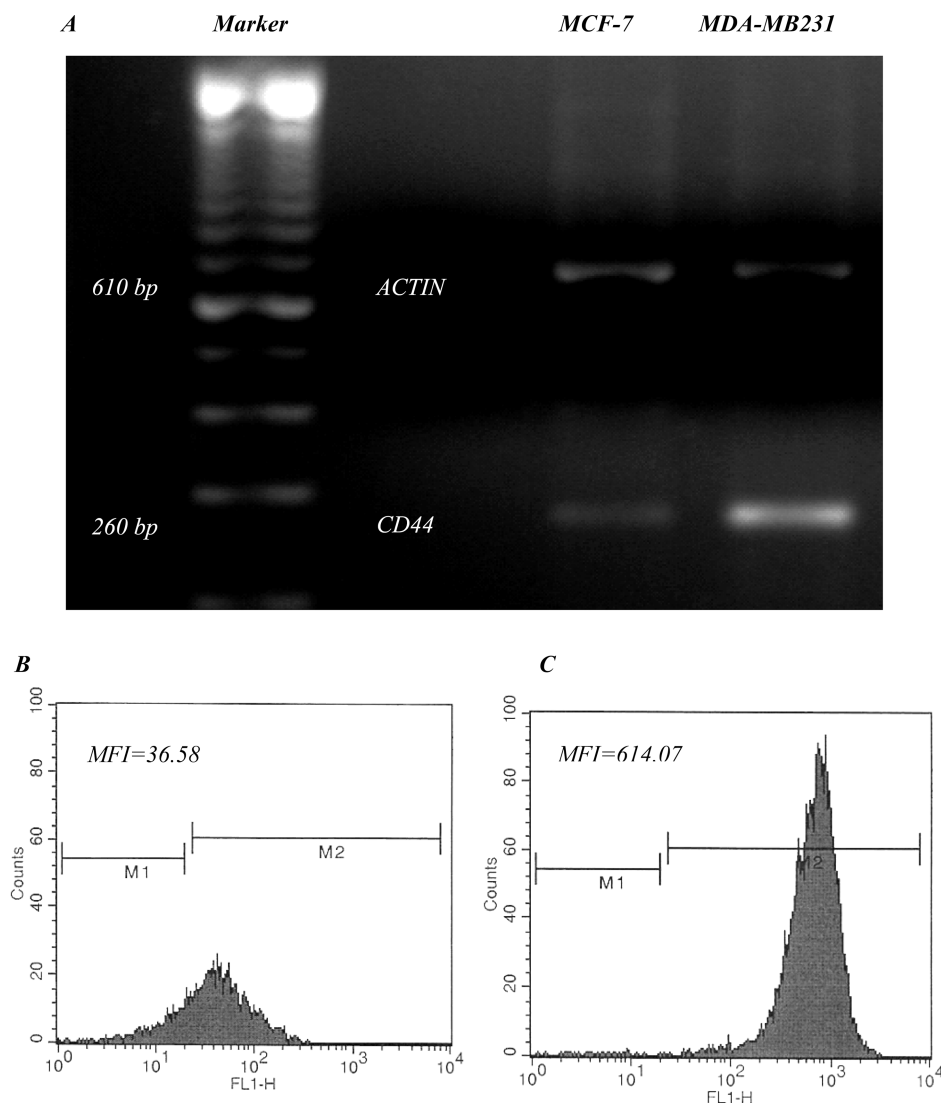


Figure 3. CD44s expression in MCF-7 and MDA-MB231 determined by RT-PCR (A). Expression of actin mRNA was used as control, and PCR products were loaded and separated on a 2% agarose gel or by fluorescence activated cell sorting. MCF-7 (B) or MDA-MB231 (C) cells were stained with CD44-labeled FITC antibody. FL1-H: fluorescence channel 1 height. MFI: median fluorescence intensity.

dense core. The formation of such aggregates was shown to result from a simultaneous rearrangement of the associated lipid via fusion of the cationic liposomes and DNA collapse.³⁴ It is thus interesting to note that the addition of HA-DOPE does not prevent this rearrangement. It was however evidenced that the addition of HA-DOPE led to a decrease of the total charge of cationic liposomes demonstrating that HA is mostly present on vesicle surface, partly shielding the positive charge of the cationic liposomes. This effect can be attributed to the carboxylic negative residues of HA. Moreover when adding DNA to these liposomes, a complete reorganization of charge repartition appears. Indeed, DNA turned the

system into negatively charged particles. Moreover, the zeta potential of such particles decreases proportionally to the density of HA-DOPE. The negative charge of lipoplexes with or without HA-DOPE might be attributed to a particular organization in which a lipid core is surrounded by DNA protruding at the surface.^{35,36} The presence of high MW HA at lipoplexes surface also contributes to shield their positive charge. However, despite being present at the surface, DNA was shown to be significantly protected from degradation against DNase I probably because the high MW HA and cationic lipids prevent access of DNase I to the whole colloidal system. Indeed, lipoplex DNA content analysis after DNase I

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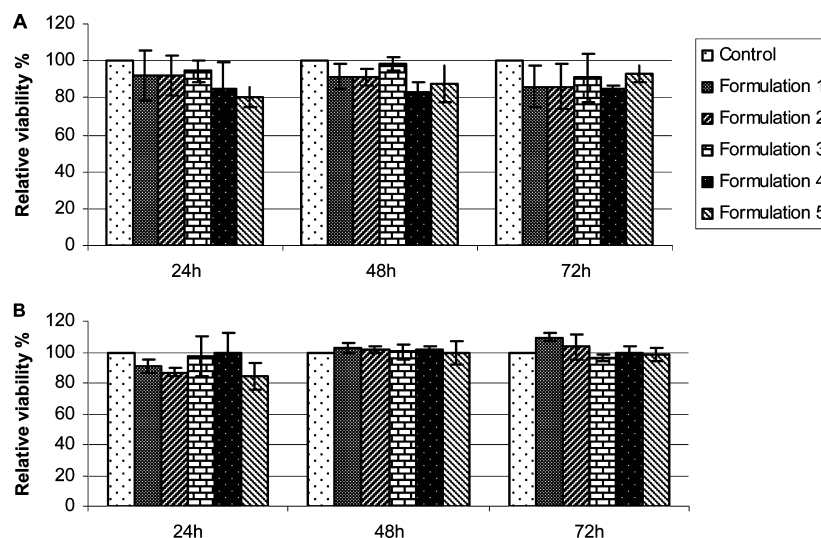


Figure 4. Cell viability of MCF-7 (A) and MDA-MB 231(B) in the presence of lipoplexes. Cells were treated with lipoplexes at a concentration of 7.5 $\mu\text{g/mL}$ (5 μg of lipid:2.5 μg of DNA), and cytotoxicity of preparations containing and not containing the conjugate HA-DOPE (1, 2, 3, 4 and 5) was evaluated through MTS assay after 24, 48, and 72 h.

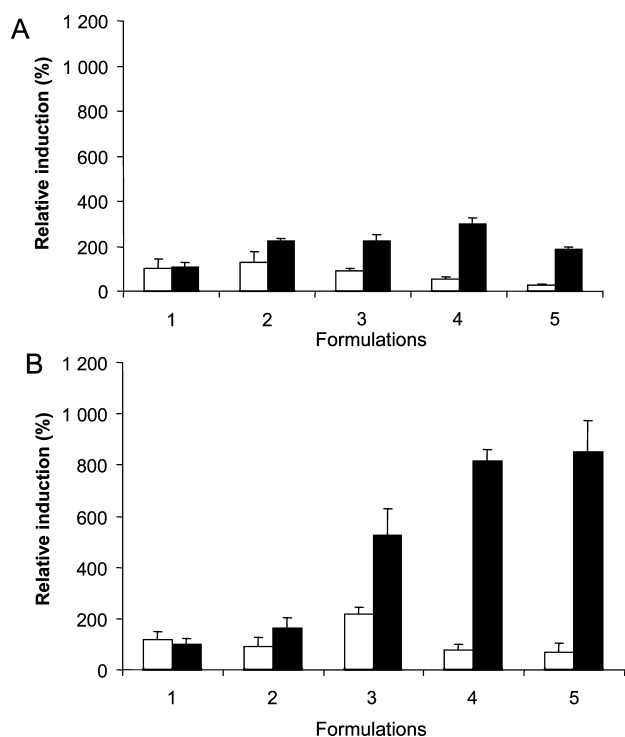


Figure 5. Comparative transfection efficiency in MCF-7 (A) and MDA-MB231 (B) cells treated with lipoplexes containing increasing amounts of HA (white) and lipoplexes containing increasing amounts of the HA-DOPE conjugate (black). (1) DE:DOPE lipoplexes. 2–5 refer to preparations containing either HA or HA-DOPE in different amounts: (2) 0.01 mg/mL of lipid; (3) 0.04 mg/mL of lipid; (4) 0.10 mg/mL of lipid; (5) 0.15 mg/mL of lipid.

digestion show a higher efficient protection of the DNA in the presence than in the absence of HA.

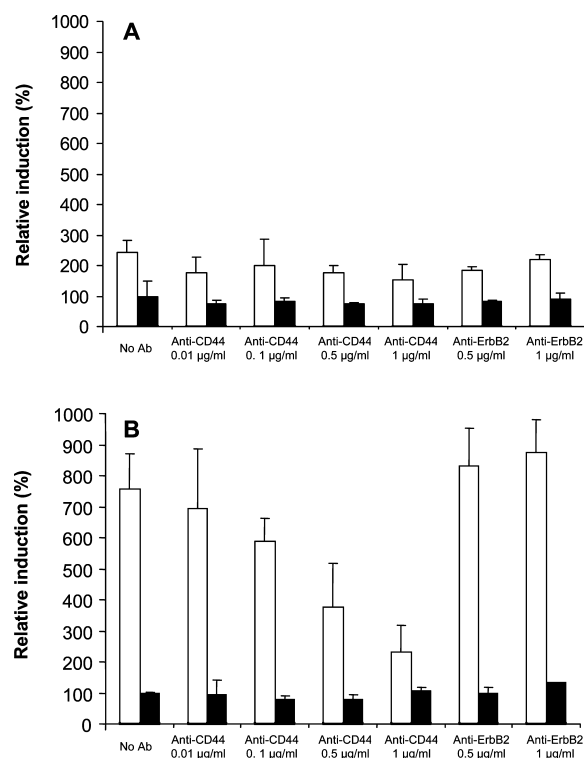


Figure 6. Comparative transfection efficiency in MCF-7 (A) and MDA-MB231 (B) cells treated with DE:DOPE lipoplexes (black) and lipoplexes containing 0.10 mg/mL of HA-DOPE conjugate (white) in the presence of increasing amounts of anti-CD44 antibody Hermes-1 and anti-ErbB2 antibody used as control.

Transfection efficiency was evaluated on both MCF-7 and MDA-MB-231 cells. It was impossible to obtain any trace of the presence of CD44 receptors on MCF-7 cells using Western blot (data not shown) confirming previous

observations.³⁷ However, RT-PCR, Q PCR and FACS revealed that CD44 was expressed in both cell lines although at a much higher extent on MDA-MB-231 cells. On both cell lines, lipoplexes containing or not the HA-DOPE conjugate display a similar low toxicity profile mainly because in all cases the carriers were negatively charged by contrast to other observations describing a high toxicity of cationic lipoplexes.³⁸ This might be the reason why more charge shielding provided by HA does not reduce more the cytotoxicity of lipoplexes. Moreover, in previous experiments, the DE used in cationic liposome formulation to deliver decoy oligonucleotides to macrophages displayed a much lower toxicity than Lipofectamine.³⁹ The reduced toxicity is therefore the combination of both a lipid with a low toxicity and the negative charge of lipoplex formulations. Using a pCMV luciferase plasmid, we have evaluated the potentiality of HA containing lipoplexes to increase transfection of CD44 rich breast cancer cells. Transfection of MDA-MB-231 cells was clearly increased when increasing the amount of conjugate until reaching saturation. A similar profile, but to a lower extent, was observed on MCF-7 cells. The mechanism involved in the augmentation of transfection efficiency was related on one hand to the presence of HA attached on the liposome surface and on the other hand to a large amount of CD44 receptors. The mechanism of transfection involves CD44 recognition, and this explains why negatively charged lipoplexes known to be currently less efficient for transfection, display in the present case around 7-fold increased transfection efficacy compared to plain lipoplexes. As a matter of fact, the positive charge of lipoplexes is important for binding to the cell surface by electrostatic interactions. The cationic complexes were shown to bind to glycosaminoglycans on the cell surfaces by electrostatic interactions.^{40,41} This type of interaction

becomes unnecessary in the present case, the transfection being only due to interactions between HA and the CD44 receptor. A study by Lesley and co-workers⁴² demonstrated that cooperativity is the primary feature of HA binding by cell surface CD44. This cooperativity is the result of multiple binding sites on the repeating disaccharide ligand and multiple closely arrayed receptors on the cell surface. The ligand factor shown to be relevant is the length of the carbohydrate chain, which determines the number of physically connected binding sites. The longer the sugar chain, the more linked binding sites are present, thus reducing the probability of the HA polymer dissociating from the cell surface.⁴² This is the reason why as expected, since MCF-7 was found to express a low amount of the receptors, transfection on this cell line was found to only slightly increase by the addition of HA. Indeed, hyaluronan binding properties characteristic of cells expressing CD44 requires threshold levels of receptor expression and hyaluronan of high molecular mass.^{43,44} However, it was established recently that MCF-7 cell line possesses a subpopulation of CD44 positive cells which does not represent more than 1% of the entire population.⁴⁵ Importantly, this CD44 positive subpopulation in MCF-7 cells, as well as in other breast cell lines such as T47D and in tumors from patients, is thought to be responsible for metastasis. Thus it is conceivable that our HA-targeted lipoplex could serve as an efficient vehicle for bringing genes of interest into dysfunctioning cells.

To validate the specific uptake of lipoplexes through the CD44 receptor, cells were incubated in presence of an anti-CD44 antibody (Hermes-1) that was shown to bind specifically to the CD44 receptor.³² Introducing increasing amounts of Hermes-1 in the culture medium decreased the transfection of MDA-MB-231 cells by 70% compared to untreated cells, and that was not the case of the nonspecific anti-ErbB2 antibody. As expected there was a reduction of transfection only with lipoplexes containing the HA-DOPE conjugate while plain lipoplexes induced similar transfection level in the presence or not of the anti-CD44 antibody (Hermes-1). Other evidence for CD44 mediated uptake of lipoplexes was demonstrated by (i) the poor transfection inhibition induced by anti-CD44 antibody (Hermes-1) on MCF-7 due to the poor level of receptor expression and (ii) by the poor transfection efficiency when free HA is associated physically with plain lipoplexes providing the

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necessity of HA to be linked to DOPE on the surface of lipoplexes in order to see their uptake.

Conclusion

In conclusion, this is the first attempt to modify lipoplexes for targeting the CD44 receptor on cancer cell lines. We were able to demonstrate that a HA–DOPE conjugate can anchor on cationic liposomes surface without changing the complexation properties of the lipids and without affecting the protection of lipoplexes against DNA enzymatic degradation. Moreover, when CD44 is highly present on cell surface, a significant increase of transfection occurs, demonstrating the potentialities of targeting such a receptor for gene delivery.

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