

Transferrin Receptor-Targeted Liposomes Encapsulating anti-*BCR-ABL* siRNA or asODN for Chronic Myeloid Leukemia Treatment

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The present work aimed at the development and application of transferrin receptor (TrfR)-targeted sterically stabilized liposomes encapsulating anti-*BCR-ABL* siRNA or asODN. Transferrin was coupled to the surface of liposomes encapsulating siRNA or asODN through the postinsertion method. Cell association and internalization were assessed by flow cytometry and confocal microscopy, respectively. *BCR-ABL* mRNA and Bcr-Abl protein levels were evaluated by qRT-PCR and Western blot, respectively. Cell viability was assessed using the resazurin reduction method. The amount of coupled transferrin and the size and stability over time of the liposomes were very satisfactory and reproducible. The siRNA encapsulation yield was dependent on the concentration of the encapsulation buffer used (20 or 300 mM), as opposed to asODN encapsulation yield which was high for both concentrations tested. Cell association and internalization studies were performed in leukemia cell lines treated with liposomes coupled to Trf (Trf-liposomes) or albumin (BSA-liposomes) or with nontargeted liposomes (NT-liposomes) encapsulating fluorescently labeled siRNA (Cy3-siRNA). These experiments clearly indicated that BSA- and NT-liposomes have no ability to promote the delivery of the encapsulated nucleic acids and that the Trf-liposomes deliver the nucleic acids by a Trf receptor-dependent mechanism. The Trf-liposomes encapsulating siRNA or asODN promote sequence-specific down-regulation of the *BCR-ABL* mRNA, although a certain extent of nonspecific sequence effects at the protein and cell viability level were observed. Overall, our results indicate that Trf-liposomes encapsulating gene silencing tools allow combining molecular and cellular targeting, which is a valuable approach for cancer treatment.

INTRODUCTION

Chronic myeloid leukemia (CML) is a hematopoietic stem cell disease caused by a t(9;22)(q34;q11) reciprocal translocation between chromosomes 9 and 22, leading to the *BCR-ABL* oncogene, that encodes a constitutively active tyrosine kinase, the Bcr-Abl protein, which is responsible for the malignant transformation in the disease (1–3).

Antisense oligonucleotides (asODN) are short, single-stranded molecules of DNA or RNA (typically 18–25 nucleotides in length) promoting targeted mRNA cleavage or blocking its translation (4, 5). Small interfering RNA (siRNA) are short, double-stranded RNA molecules normally with 21–23 nucleotides in each strand that promote the cleavage of complementary mRNA by activation of the RNA interference mechanism (6–9).

AsODN and siRNA are powerful gene silencing tools, although there are many obstacles to their *in vitro* and *in vivo* use, namely, enzymatic degradation mediated by nucleases present in the serum (6, 10, 11), low cellular uptake caused by the high molecular weight and the net negative charge (10–12), as well as renal excretion (6, 10). All these features contribute to poor *in vivo* pharmacokinetic properties of asODN and siRNA molecules. To overcome these limitations, different approaches have been tried in the past years, like chemical modifications of siRNA (8, 10) and asODN (13, 14), in an attempt to generate

molecules more resistant to nuclease degradation and in some cases less prone to off-target effects (8, 10).

The use of liposomes to deliver siRNA or asODN allows protection of nucleic acids from nuclease degradation and can be engineered in order to confer sustained plasma concentration and targeted delivery, thus leading to more specific nucleic acid accumulation in tumor cells.

The transferrin receptor (TrfR) is a cell membrane-associated glycoprotein (15–17) that has been explored as a target to deliver liposomes into cancer cells (18, 19), due to its overexpression on tumor cells (15, 16, 20), accessibility on the cell surface, and constitutive endocytosis (20). Transferrin (Trf), an 80 kDa glycoprotein, is the natural ligand for TrfR and undergoes internalization mediated by this receptor (17, 20).

When considering intravenous administration, which is relevant for implementing a therapeutic approach against CML, it is important to confer long circulating times to the liposomes. This feature can be achieved by “insertion” of hydrophilic polymers in the liposome surface, with poly(ethylene glycol) (PEG) being one of the most extensively studied for this purpose. The main aim is to obtain an aqueous shield around the liposomes that prevents their aggregation during preparation and opsonization by serum components, which can further lead to blood elimination by macrophages of the mononuclear phagocytic system, thus improving biodistribution and consequently pharmacokinetics (21–23).

This work aimed at efficiently encapsulating asODN or siRNA (designed to specifically silence the *BCR-ABL* oncogene) in TrfR-targeted sterically stabilized liposomes. These liposomes were characterized in terms of their size, siRNA or asODN encapsulation yield, the amount of coupled Trf protein, and size stability over time. Our results clearly indicate that cellular

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Table 1. Nucleic Acid Sequences

specific anti- <i>BCR-ABL</i> siRNA	5'AAGGGCUUUUGAACUCUGCdTdT3'
scrambled anti- <i>BCR-ABL</i> siRNA	5'GUCUCAAGUUUUCGGGAAGdTdT3'
specific anti- <i>BCR-ABL</i> asODN	5'G*A*A*G*G*G*C*T*T*T*G*A*A*C*T*C*T3'
scrambled anti- <i>BCR-ABL</i> asODN	5'G*G*T*A*T*G*T*G*A*T*G*T*A*T*C*A*C*C3'

* asODN exhibit phosphorothioate modifications. The siRNA sequences indicated are the antisense strands; the complementary sense strands exhibit dTdT overhangs.

internalization of the targeted liposomes occurs through TrfR-mediated endocytosis. In addition, it was demonstrated that the developed TrfR-targeted formulation allowed to achieve the desired gene silencing effect mediated by either siRNA or asODN. A correlation between the reduction of the levels of *BCR-ABL* mRNA and decrease of the corresponding Bcr-Abl protein and cytotoxicity was observed.

EXPERIMENTAL PROCEDURES

Chemicals. Cholesterol (Chol) was obtained from Sigma (St Louis, MO, USA); all the other lipids, *N*-palmitoyl-sphingosine-1-[succinyl(methoxypolyethylene glycol) 2000] (C16 mPEG 2000 Ceramide), 1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine (DSPC), 1,2-dioleoyl-3-dimethylammonium-propane (DODAP); 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-[maleimide (polyethylene glycol)2000] ammonium salt (DSPE-PEG-MAL), were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Human holo-transferrin (Trf) and bovine serum albumin (BSA) were purchased from Sigma.

Small interfering RNA molecules were purchased from Dharmacon RNA Technologies (Lafayette, CO, USA). The Cy3-labeled anti-*BCR-ABL* siRNA (Cy3-siRNA) (custom synthesis) and asODN molecules were purchased from Ambion (Austin, TX, USA) and Microsynth AG (Switzerland), respectively. The sequences of the used nucleic acids are presented in Table 1. All the other chemicals were obtained from Sigma unless stated otherwise.

Preparation of Trf or BSA-Coupled PEG2000-DSPE Micelles. Trf and BSA proteins were modified with the addition of thiol groups through reaction with 2-iminothiolane hydrochloride (2-IT). For this purpose, Trf or BSA and 2-IT freshly dissolved in HEPES buffer (20 mM HEPES, 145 mM NaCl, pH 8) were mixed in a protein/2-IT molar ratio of 1:10 and gently stirred for 1 h, in the dark at room temperature.

A lipid film of DSPE-PEG-MAL was prepared by solvent evaporation under a mild stream of N₂ and further dried under vacuum for 2 h. This dried lipid film was then hydrated with MES buffer (20 mM HEPES, 20 mM MES, pH 6.5), at a concentration above 2.3 μM, the critical micellar concentration of the lipid (24). Micelles were formed by strong vortex followed by 15 s heating in a water bath at 38 °C and a second vortex shaking. Then, the freshly thiolated proteins were coupled to the freshly prepared DSPE-PEG-MAL micelles by a thioether linkage (protein to micelles molar ratio of 1:1). The coupling reaction was performed overnight, in the dark at room temperature with gentle stirring. Micelles without conjugated ligand were prepared, as controls for Trf-micelles, by adding HEPES buffer to the micelles.

The remaining free MAL groups in the micelles were quenched by the addition of β mercaptoethanol at a maleimide/β mercaptoethanol molar ratio of 1:5, under stirring for 30 min at room temperature.

Encapsulation of asODN or siRNA into Liposomes and Subsequent Postinsertion of Trf-conjugates. A solution containing 13 μmol of total lipid composed of Chol/DSPC/

DODAP/C16 mPEG 2000 Ceramide (45:22:25:8, mol %) in 200 μL of absolute ethanol, and a solution of 0.041 μmol of siRNA or 0.096 μmol of asODN in 300 μL of 20 mM or 300 mM citrate buffer, pH 4, were heated at 60 °C. The lipids were then slowly added under strong vortex to the siRNA or asODN solution. Upon their formation, the liposomes were extruded, 21 times, in a LipoFast mini extruder (Lipofast, Avestin, Toronto, Canada) through 100-nm-diameter polycarbonate filters (Avestin, Toronto, Canada). Then, dialysis was performed in HBS, pH 7.4, through a regenerated cellulose tubular membrane with MWCO 6000–8000 (Cellu Sep T2, Membrane Filtration Products, Inc., Seguin, TX, USA) for 3 h at room temperature to remove ethanol and raise the external pH. Subsequently, the total lipid concentration was assessed by cholesterol quantification. For this purpose, samples were added to absolute ethanol (1:6, v:v) and Infinity Cholesterol Liquid Stable Reagent (Thermo Electron; Melbourne, Australia). Absorbance was measured at 500 nm in a spectrophotometer and the concentration assessed against a cholesterol standard curve. The cholesterol quantification allowed the determination of the total lipid that remained at this stage and consequently the determination of the amount of micelles to be added.

Finally, 2 or 4 mol % (relative to the total lipid concentration) of Trf/BSA-coupled PEG-DSPE micelles or PEG-DSPE micelles without ligand was added to the liposomes. The insertion of protein-conjugates onto the liposomes was performed upon incubation in a water bath at 38 °C for 17 or 24 h, in the dark.

Purification of Liposomes. After incubation with micelles, Trf/BSA-liposomes or NT-liposomes (nontargeted liposomes) were purified by size exclusion chromatography on a Sepharose CL-4B column, using HBS, pH 7.4, as running buffer to remove external siRNA or asODN, as well as nonconjugated micelles and chemical reagents used during the liposomal preparation.

Liposome Characterization. Particle size distribution was assessed by photon correlation spectroscopy (N5 submicrometer particle size analyzer, Beckman Coulter, Miami, FL, USA). For this purpose, liposomes were diluted in filtered HBS, pH 7.4, at room temperature, so that the required signal ((1–10) × 10⁵ counts/s) was achieved. Measurements were made at a 90° angle and at 20 °C. The quantification of Trf or BSA associated with the liposomes was made with the BCA Protein Assay Kit (Pierce, Rochford, IL, USA) against a BSA standard curve at 570 nm in a microplate reader, Multiskan Ex (Thermo Lab-systems, Vantaa, Finland).

The amount of siRNA and asODN entrapped inside liposomes was assessed by the Quant-iT RiboGreen RNA assay (Molecular Probes, Invitrogen, Karlsruhe, Germany) against a siRNA/asODN standard curve. Liposomes were dissolved upon addition of 0.6 mM of octaethylene glycol monododecyl ether (C₁₂E₈) and the RiboGreen fluorescence (λ_{ex} 485 nm, λ_{em} 530 nm, cut-off 515 nm) was measured using a Spectra Max Gemini EM plate reader fluorimeter (Molecular Devices, Sunnyvale, CA).

Cell Lines. K562 and LAMA-84 cells purchased from DSMZ (Braunschweig, Germany) and BJ cells, human fibroblasts, kindly provided by Paulo J. Oliveira (Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal) were maintained in culture at 37 °C, 5% CO₂, under humidified atmosphere, in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Gibco, invitrogen, California, USA), penicillin (100 U/mL), and streptomycin (100 μg/mL) (Cambrex, NJ, USA).

Cell Transfection. K562 and LAMA-84 cells (20 000 cells/well) in RPMI-1640 culture medium supplemented with 10% FBS and antibiotics were seeded in 96 round well plates. BJ cells (8000 cells/well) also in RPMI-1640 culture medium supplemented with 10% FBS and antibiotics were seeded in 96 flat well plates. Cells were transfected with Trf- or BSA-

associated liposomes or with nontargeted liposomes at 37 °C for 4 h. After incubation with liposomes, the medium was replaced with fresh medium and cells further incubated for 44 h. Following this first transfection, cell culture medium was replaced with fresh medium, and a second transfection protocol was initiated (similar formulations and same concentrations of asODN or siRNA as used in the first transfection protocol). Concerning K562 and LAMA-84 cells, immediately after adding liposomes to cells, the resulting suspension was transferred to 48-well plates. After 4 h of incubation, 800 μ L of culture medium were added to each well and cells were kept in culture.

Cell Viability. Cell viability was evaluated 48 h after the second transfection, by the resazurin reduction assay (25). The assay measures the chemical reduction of the resazurin dye resulting from cellular metabolic activity and allows the determination of viability over the culture period without harming the cells. Briefly, 200 μ L of cell suspension, double transfected as described in the previous section, was transferred to 96-well plates, and the culture medium was replaced with 10% (v/v) resazurin dye in RPMI-1640 medium without serum and antibiotics. After 2.5 h of incubation at 37 °C, the absorbance at 540 nm (reduced form) and 630 nm (oxidized form) was measured in a microplate reader Multiskan Ex (Thermo Labsystems, Vantaa, Finland). Cell viability was calculated as percentage of control cells using the equation

$$[(A_{540} - A_{630})_{\text{treated cells}} \times 100] / (A_{540} - A_{630})_{\text{control cells}}$$

Western Blot. Forty-eight hours after the second transfection, cells were collected, washed twice with cold PBS, and lysed with lysis buffer [25 mM HEPES, 1 mM EGTA, 1 mM EDTA \cdot Na₂, 2 mM MgCl₂, 50 mM NaF, pH 7.5, containing freshly added 200 mM PMSF, 2 mM DTT, 1 mM NaVO₄, 0.1% (v/v) Triton X-100 and protease inhibitors (Chymostatin, Pepstatin A, Leupeptin L, Antipain Hydrochloride) (Sigma Chemical Co., St. Louis, MO, USA)]. Concentration of protein lysates was estimated through the Bradford method (Bio-Rad Protein Assay, Bio-Rad), and equal amounts (30 μ g) of protein were loaded in a 4.5% stacking, 6% resolving SDS-polyacrylamide gel. Electrophoresis was carried out at 80 V for 15 min and 110 V for approximately 30 min. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences, UK) and incubated under stirring for 1 h at room temperature in blocking solution (TBS containing 0.1% Tween 20 and 5% dry milk). Bcr-Abl protein and actin were detected with anti c-Abl monoclonal antibody (Santa Cruz, CA, USA) and mouse antiactin monoclonal antibody (Immuno, MP Bio-medicals, Ohio, USA), respectively.

Proteins were detected by chemifluorescence using an alkaline phosphatase labeled secondary antibody, antimouse IgG + IgM alkaline phosphatase linked antibody (Amersham Biosciences, UK), and the enzyme substrate ECF (Amersham Biosciences, UK) to yield a highly fluorescent product detected in a VersaDoc imaging system (Bio-Rad). The analysis of band intensity was performed using the *Quantity One* software (Bio-Rad).

qRT-PCR. Cells (20 000 cells/well) were doubled-transfected as described above. Twenty-four hours after the second transfection (72 h after beginning the treatment), cells were collected and kept at -80 °C until RNA extraction.

Frozen cellular pellets were slightly thawed at room temperature and RNA extraction performed with RNeasy Mini Kit (Quiagen, Hilden, Germany), according to the manufacturer's recommendations. RNA concentration was determined by the Quant-iT RiboGreen Assay Kit (Molecular Probes, Invitrogen, Karlsruhe, Germany) using a rRNA standard curve.

cDNA synthesis was performed with SuperScript III First-Strand Synthesis SuperMix Kit for qRT-PCR (Invitrogen, Karlsruhe, Germany). For each sample, cDNA was produced

Table 2. Sequences of Primers Used in qRT-PCR

<i>BCR-ABL</i>	forward primer	5'TCCGCTGACCATCAATAAGGA3'
	reverse primer	5'CACTCAGACCCTGAGGCTCAA3'
<i>B2M</i>	forward primer	5'GAGTATGCCTGCCGTGTG3'
	reverse primer	5'AATCCAAATGCCGCATCT3'

from 1 μ g of total RNA and the reaction was performed according to the manufacturer's instructions and the following protocol: first step, 10 min at 25 °C; second step, 30 min at 50 °C; third step, 5 min at 85 °C. Then, samples were chilled on ice and incubated with RNase H at 37 °C for 20 min in order to remove any RNA contamination.

Polymerase chain reaction was performed in real time (qPCR) with the iQ SYBR Green Supermix Kit (Bio-Rad). The endogenous gene (housekeeping gene, hk) β -2-microglobulin (*B2M*) was used as endogenous control. The housekeeping gene and respective primers used in this work were tested and selected by the Europe Against Cancer program (26, 27). The sequences are presented in Table 2, and the concentrations were optimized to 150 nM for *BCR-ABL* and *B2M*.

Briefly, 5 μ L of the cDNA (2.5 \times diluted with DNase free deionized water) obtained in the reverse transcription reaction was used to evaluate *BCR-ABL* and housekeeping genes levels. Analysis was done in 96-well PCR plates (iCycler iQ, Bio-Rad), and qPCR was performed as follows: one single cycle at 95 °C for 1.30 min, followed by 40 cycles of two steps, first step of 15 s at 95 °C, second step of 30 s at 60 °C. A melting curve was always built to demonstrate a single amplification product reaction per well. The melting curve protocol started immediately after the qPCR and consisted of 10 s heating at 55 °C, followed by 80 steps of 10 s, with a 0.5 °C increase in temperature at each step. Nontemplate controls (cDNA was replaced with water to evaluate whether qPCR reagents were contaminated with the cDNA) were always evaluated. In addition, a negative control with a *BCR-ABL* negative cell line, HL-60, was performed. The threshold cycle (*C_t*) values were generated automatically by the iQ5 Optical System Software. To each gene, and in each experiment, a standard curve was performed. The reaction efficiencies of all genes were between 90% and 110%, and $r^2 > 0.99$, thus allowing the use of the comparative method for mRNA level quantification, according to the following formula: $2^{-\Delta\Delta C_t \times 100}$ (where *C_t* is the threshold cycle and $\Delta\Delta C_t$ stands for ΔC_t sample - ΔC_t control \leftrightarrow (Ct *BCR-ABL* - Ct hk) sample - (Ct *BCR-ABL* - Ct hk) control).

Flow Cytometry Studies. To evaluate the extent of cell association of Trf- or BSA-associated liposomes or NT-liposomes, cells were transfected with fluorescently labeled siRNA (Cy3-siRNA) encapsulated in each one of the mentioned liposomes. Cells were seeded in RPMI-1640 culture medium (200 000 cells/well) in 48-well plates. To demonstrate that the receptor-mediated internalization is temperature-dependent, cells were incubated 1 h at 4 °C or at 37 °C, prior to liposome addition. To demonstrate that the internalization of Trf-liposomes is mediated by TrfR, this receptor was saturated by incubating the cells for 30 min with 125 μ M of free holotransferrin (dissolved in HBS, pH 7.4), as performed by others (19, 28). After these pretreatments, cells were incubated with the formulations under study for 4 h at 37 or 4 °C. Subsequently, cells were collected in conic tubes (BD, Biosciences), washed twice with cold PBS, and resuspended in 500 μ L of cold PBS. All samples were immediately analyzed in a FACS Calibur flow cytometer (BD, Biosciences). Cy3 fluorescence was evaluated in the FL-2 channel. The data were then analyzed by *Cell Quest* software (BD). Cell association was only assessed in viable cells, these being gated based on the morphological features and on Trypan Blue staining (FL-3 channel). The cell association efficiency of each formulation was assessed by comparing the relative fluorescence units (RFU)

increase relative to nontreated cells (RFU of cells treated with the formulation under study/RFU of nontreated cells).

Confocal Microscopy Studies. In order to assess the extent of cellular internalization of the developed liposomes, confocal microscopy studies on transfected cells were performed. Cells were transfected with the formulations under study as described in the previous section. Then, cells were collected in conic tubes (BD, Biosciences), washed with cold PBS, fixed with 4% paraformaldehyde for 20 min in the dark, at room temperature, washed with cold PBS, stained with the fluorescent DNA-binding dye Hoechst 33342 (Molecular Probes, Oregon, USA) (1 $\mu\text{g}/\text{mL}$) for 5 min, in the dark, washed with cold PBS, and mounted in Mowiol mounting medium (Fluka, Sigma). Confocal images were acquired in a point scanning confocal microscope Zeiss LSM 510 Meta (Zeiss, Germany), with a 40 \times EC Plan-Neofluar or with a 63 \times Plan-Apochromat oil immersion objectives, and argon (488 nm), DPSS (561 nm), diode (405 nm), and helium–neon (633 nm) lasers. Preparations were excited at 561 nm for the Cy3 (em: >560 nm) and at 405 nm for Hoechst 33342 (em: 420–480 nm). Differential interference contrast (DIC) images were obtained using the helium–neon (633 nm) laser. Digital images were acquired using the LSM 510 Meta software. All instrumental parameters pertaining to fluorescence detection and image analyses were held constant to allow sample comparison.

Statistical Analysis. All data are presented as mean \pm standard deviation (SD), and are the result of at least three independent experiments. One-way ANOVA analysis of variance combined with Tukey post-test or regular two-way ANOVA analysis of variance combined with Bonferroni post-test were used for multiple comparisons. Statistical differences are presented at probability levels of $p > 0.05$, $p < 0.05$, $p < 0.01$, and $p < 0.001$.

RESULTS

Development of Trf-Liposomes Encapsulating siRNA or asODN. Stabilized nucleic acids lipid particles (SNALP) were prepared on the basis of previous work developed by Semple et al. (29). SNALP is the adopted designation for siRNA encapsulation mediated by electrostatic interaction with stabilized lipid particles, whereas the ODN-containing counterpart is designated by stabilized antisense lipid particles (SALP). However, in this paper the SNALP designation was adopted for liposomes encapsulating either siRNA or asODN.

In this study, liposomes composed of Chol/DSPC/DODAP/C16 mPEG 2000 Ceramide (45:22:25:8, mol %) were used. The inclusion of 25% of the ionizable aminolipid DODAP, which has a positive net charge at pH 4, and becomes neutral at physiological pH, allows to achieve high nucleic acid encapsulation efficiencies and simultaneous obtainment of liposomes with neutral net charge (30). Thus, the encapsulation step is performed at pH 4, under which conditions the majority of the DODAP lipid is positively charged, forming complexes with the negatively charged asODN or siRNA at a 2/1 (\pm) charge ratio, resulting in high encapsulation yields. The liposome external pH is then raised to 7.4, leading to neutralization of the DODAP and consequent release of the nucleic acids bound to the liposomal external membrane surface.

The encapsulation yields obtained for both asODN and siRNA were very high (Figure 1A). Remarkably, our results demonstrated that the buffer concentration is extremely important for siRNA encapsulation, since at 20 mM of citrate buffer a $92.17 \pm 1.39\%$ encapsulation yield was achieved, whereas at 300 mM a drastic reduction of the encapsulation yield to $7.72 \pm 5.29\%$ was observed. In contrast, asODN encapsulation was independent of the buffer concentration, reaching values of approximately 100% for both 20 and 300 mM citrate buffer.

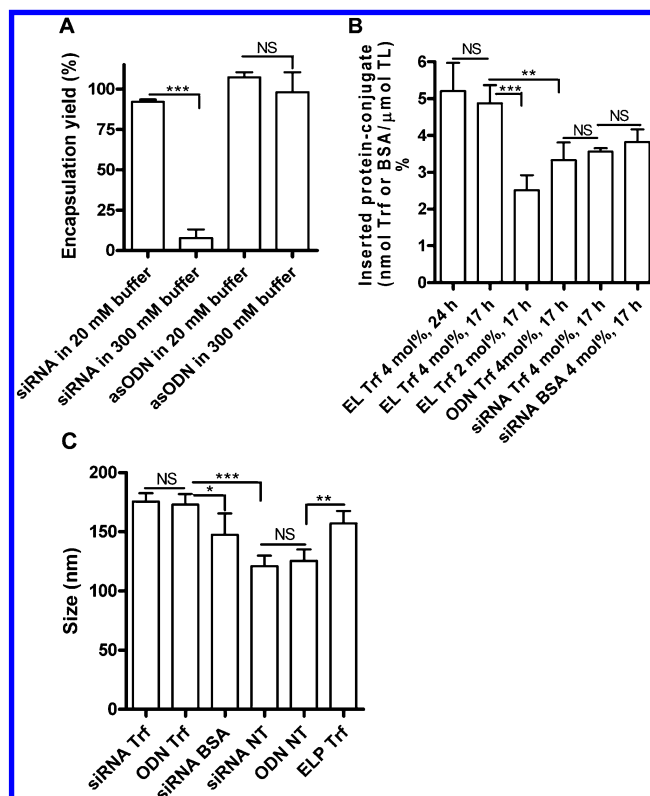


Figure 1. Physicochemical characterization of the developed liposomes. (A) Encapsulation yields: siRNA or asODN dissolved in 20 mM or 300 mM citrate buffer, pH 4, were encapsulated, as described in Experimental Procedures. The encapsulation yield was assessed by the RiboGreen assay. (B) Inserted protein–conjugate: the amount of protein–conjugate (Trf- or BSA-PEG-DSPE) inserted onto the liposomes was assessed by the BCA assay. The influence of using either 2 or 4 mol % of micelles and 17 or 24 h of incubation time, in the presence or absence of nucleic acids, on the extent of insertion of protein–conjugates onto preformed liposomes was evaluated. The tested conditions were as follows: empty liposomes incubated with 4 mol % Trf-micelles for 24 h (EL Trf 4 mol %, 24 h) or 17 h (EL Trf 4 mol %, 17 h); empty liposomes incubated with 2 mol % of Trf-micelles for 17 h (EL Trf 2 mol %, 17 h); liposomes encapsulating siRNA incubated for 17 h with 4 mol % BSA-micelles (siRNA BSA 4 mol %, 17 h) or with 4 mol % Trf-micelles (siRNA Trf 4 mol %, 17 h); and liposomes entrapping asODN incubated for 17 h with 4 mol % of Trf-micelles (ODN Trf 4 mol %, 17 h). (C) Liposome particle size: Particle size distribution of the Trf- or BSA-coupled liposomes encapsulating siRNA (siRNA Trf and siRNA BSA, respectively), Trf-coupled liposomes encapsulating asODN (ODN Trf), nontargeted liposomes encapsulating siRNA or asODN (siRNA NT and ODN NT, respectively), or Trf-coupled liposomes without nucleic acids (ELP Trf) were analyzed by photon correlation spectroscopy. One-way ANOVA analysis of variance combined with Tukey post-test was performed. NS $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

The attachment of Trf or BSA to the liposomal surface was performed by postinsertion of Trf/BSA-coupled PEG₂₀₀₀-DSPE conjugates (in a micellar form) into preformed liposomes, as described in Experimental Procedures. The influence of the incubation time and the percentage of micelles added to the liposomes on the amount of inserted protein–conjugate onto the liposomes was evaluated. As can be observed in Figure 1B, there is no advantage to prolonging the incubation time beyond 17 h, as no enhancement on the amount of inserted protein–conjugate is achieved (for 4 mol % of micelles). In contrast, the percentage of micelles incubated with the liposomes significantly affects the amount of inserted protein–conjugate. In fact, when preformed liposomes were incubated with 4 mol % of micelles, a value of 4.88 ± 0.49 nmol Trf/ μmol total lipid (TL) was obtained, whereas incubation of preformed liposomes

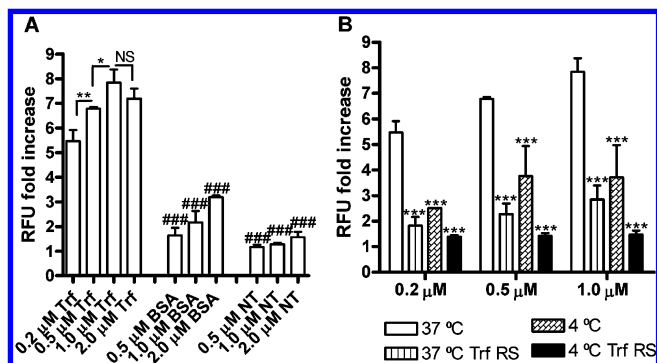


Figure 2. Extent of association of Trf-coupled liposomes loaded with Cy3-siRNA to LAMA-84 cells. (A) Cells were incubated at 37 °C for 4 h with different concentrations of Trf-coupled liposomes encapsulating 0.2–2.0 μM Cy3-siRNA (0.2–2.0 μM Trf), BSA-coupled liposomes encapsulating 0.5–2.0 μM Cy3-siRNA (0.5–2.0 μM BSA), or nontargeted liposomes encapsulating 0.5–2.0 μM Cy3-siRNA (0.5–2.0 μM NT). (B) Cells were incubated with different concentrations of Trf-liposomes loading Cy3-siRNA at 37 °C in the presence (37 °C Trf RS) or absence (37 °C) of an excess of free Trf or incubated at 4 °C in the presence (4 °C Trf RS) or absence (4 °C) of free Trf. The fluorescence of the Cy3-siRNA associated to LAMA-84 cells was assessed by flow cytometry. (A) One-way ANOVA analysis of variance combined with Tukey post-test and (B) regular two-way ANOVA analysis of variance combined with Bonferroni post-test for multiple comparisons were used. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, and NS $p > 0.05$ when the comparison was established between the conditions indicated by the lines (A) or when comparison was established with cells treated at 37 °C in the absence of free Trf (B). #### $p < 0.001$ when the comparison was established with the corresponding concentration of siRNA encapsulated in Trf-liposomes (A).

with 2 mol % resulted only in 2.51 ± 0.41 nmol Trf/ μ mol TL. It was also observed that the postinsertion of Trf or BSA micelles is more efficient in empty liposomes (4.88 ± 0.49 nmol Trf/ μ mol TL) than in liposomes containing nucleic acids (3.55 ± 0.10 nmol Trf/ μ mol TL) and that Trf- and BSA-PEG-DSPE conjugates are transferred with the same efficiency by postinsertion.

Results obtained from photon correlation spectroscopy revealed that the size of all developed formulations is under 200 nm (Figure 1C) with a polydispersion index below 0.3 (data not shown), which indicates that liposomes exhibit a narrow particle size distribution. We also observed that, six months after liposome preparation, the size and polydispersion index did not change (data not shown), indicating a good stability of the formulations over time. As expected, the insertion of protein-conjugates onto the liposome surface slightly enhances liposomal size: Trf- (siRNA Trf) or BSA- (siRNA BSA) coupled liposomes encapsulating siRNA exhibit 175.5 ± 7.28 nm and 147.65 ± 17.81 nm, respectively, and NT-liposomes containing siRNA (siRNA NT) present 121.03 ± 8.83 nm. No differences in the particle size of liposome encapsulating siRNA or asODN were observed. The extent of protection conferred by the liposomal formulations to the encapsulated nucleic acids against the nuclease degradation was assessed. For this purpose, liposomes encapsulating siRNA or asODN were incubated with Quant-iT RiboGreen RNA reagent in the presence or absence of the surfactant C₁₂E₈. In the absence of the surfactant, the extent of the interaction between the probe and the nucleic acids is reduced to approximately 90%, indicating that these formulations confer a high degree of protection against nuclease degradation (data not shown).

Cell Association of Trf-Liposomes Is Specifically Mediated by Trf Receptor. The extent of association of liposomes bearing Trf or BSA attached at the end of PEG chains or of NT-liposomes, encapsulating Cy3-labeled siRNA, to LAMA-84 cells was assessed by flow cytometry. As can be observed in Figure 2A, the presence of Trf attached to the end

of PEG-grafted lipids is the main thing responsible for the observed extent of liposome–cell association. Cells incubated with BSA-liposomes or NT-liposomes revealed no significant cell association, illustrated by the low increase of the fluorescence intensity of the transfected cells as compared to nontreated cells. As can be observed, the RFU fold increase for cells treated with 2.0 μM siRNA encapsulated in Trf-liposomes (2.0 μM siRNA Trf) was 7.20 ± 0.40 , in contrast to 3.20 ± 0.07 and 1.57 ± 0.22 fold increase observed upon treatment of the cells with BSA-liposomes and NT-liposomes, respectively. Our results also indicate that the extent of cell association of Trf-coupled liposomes containing Cy3-siRNA is dependent on the lipid concentration incubated with the cells up to 0.34 mM of total lipid, which corresponds to 1.0 μM siRNA, since no significant difference on the RFU of cells treated with 1.0 μM or 2.0 μM siRNA encapsulated in Trf-liposomes was observed.

To clearly demonstrate that the ligand Trf coupled at the surface of the liposomes triggers their cell internalization through the TrfR, competitive inhibition studies were performed. Cells were incubated with 125 μM of free human holo-Trf to block the TrfR. As illustrated in Figure 2B, a drastic decrease of cell association of Trf-liposomes for all tested concentrations was observed. At 1.0 μM siRNA and incubation at 37 °C, the observed RFU was 7.84 ± 0.54 , whereas with presaturation of the Trf receptor, the RFU decreased to 2.85 ± 0.54 . The effect of the temperature on the extent of liposome–cell association was examined through the incubation of tumor cells with Trf-liposomes at 4 and 37 °C. As expected, incubation at 4 °C resulted in a significant reduction of the extent of liposome–cell association as compared to the incubation at 37 °C (at 1.0 μM siRNA, the RFU was reduced from 7.84 ± 0.54 to 3.72 ± 1.25). It was also observed that Trf receptor saturation performed at 4 °C resulted in the highest reduction observed for the extent of cellular association, with a RFU of 1.48 ± 0.15 , indicating that binding of the liposomes to the cell surface is also affected by the saturation of TrfR.

The results of liposome–cell association obtained with Trf-, BSA-, or NT-liposomes encapsulating FAM-asODN were very similar to those obtained with correspondent counterparts containing Cy3-siRNA, suggesting that the association process is not dependent on the nature of the encapsulated nucleic acid (data not shown).

Cell Internalization of Trf-Liposomes Is Specifically Mediated by Trf Receptor. In order to gain further insights into the pathway of cell internalization of the developed formulations, confocal microscopy studies were performed (Figure 3). Upon incubation of the cells with liposomes, at 37 °C, cell internalization of Cy3-siRNA was only detected for the cells incubated with Trf-liposomes (Figure 3A,F). For BSA- (Figure 3B) or NT-liposomes (Figure 3C), no Cy3-siRNA inside the cells was observed. This same observation was registered upon saturation of the Trf receptor at 37 °C (Figure 3D) or upon treatment of the cells at 4 °C (Figure 3E).

The results obtained for cell internalization of liposomes encapsulating FAM-asODN were very similar to those obtained for Cy3-siRNA encapsulated into liposomes (data not shown). Therefore, altogether our results indicate that cell association and internalization of the developed formulations occur via temperature- and Trf receptor-dependent mechanisms.

Cytotoxicity Mediated by TrfR-Targeted Liposomes Encapsulating anti-BCR-ABL siRNA or asODN. In order to assess the biological activity of the developed formulations, toxicity in two leukemia cells lines, LAMA-84 and K562 cells, and in a nonmalignant cell line, BJ cells (human fibroblasts), was evaluated by the resazurin reduction method (Figure 4). As can be observed in Figure 4A, for LAMA-84 cells the nontargeted liposomes and the BSA-coupled liposomes do not

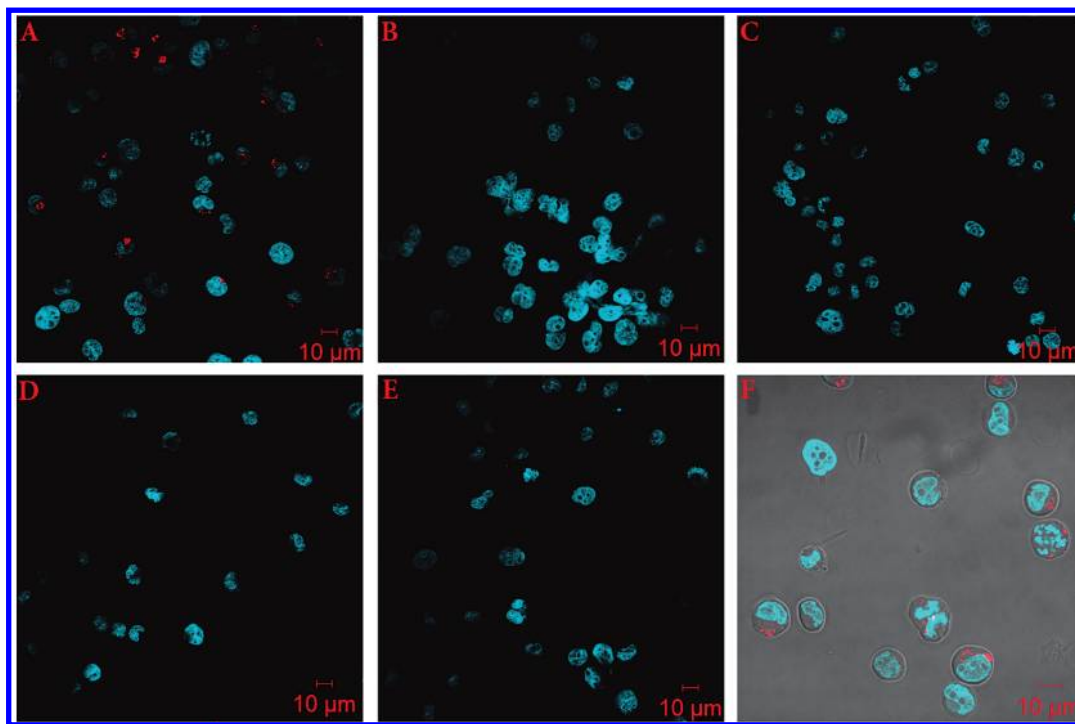


Figure 3. Confocal microscopy images from studies performed in LAMA-84 cells incubated with Trf-coupled liposomes loaded with Cy3-siRNA. Cells were incubated for 4 h, at 37 °C, with 1 μM of Cy3-siRNA encapsulated in (A,F) Trf-coupled liposomes, (B) BSA-coupled liposomes, (C) NT-liposomes, or (D) Trf-coupled liposomes in the presence of an excess of free Trf. (E) LAMA-84 cells incubated with Trf-coupled liposomes at 4 °C. Confocal images were acquired in a point scanning confocal microscope Zeiss LSM 510 Meta (Zeiss, Germany), (A–E) with a 40 \times Plan-Neofluar. (F) Differential interference contrast (DIC) images were taken with a 63 \times Plan-Apochromat oil immersion objectives.

cause significant effects on the cell viability. In contrast, Trf-liposomes encapsulating specific siRNA anti-*BCR-ABL* led to dose-dependent cytotoxicity, reaching, for example, a cell viability of $46.97 \pm 2.73\%$, upon incubation with 2 μM siRNA. Sequence specificity was not proven for the entire range of concentrations tested, since the scrambled one also significantly decreased cell viability ($57.93 \pm 9.73\%$ at 2 μM).

In order to validate the results obtained with LAMA-84 cells, Trf-liposomes and BSA-liposomes were tested in K562 cells, whose *BCR-ABL* mRNA levels are 1.72-fold higher than LAMA-84 cells, as assessed by qRT-PCR (data not shown). In addition, TrfR levels of LAMA-84 cells are 15.68-fold higher than K562 cells, as assessed by CD71 measurement using flow cytometry (data not shown). As observed for LAMA-84 cells, the BSA-liposomes promote much lower toxicity than Trf-liposomes on K562 cells (Figure 4B). Treatment of K562 cells with Trf-liposomes resulted in higher extent of cytotoxicity as compared to LAMA-84 cells. For K562 cells, the off-target effects, evaluated by the cytotoxicity promoted by the scrambled siRNA, are lower than those observed for LAMA-84 cells. Concerning the nonmalignant BJ cells, which have no *BCR-ABL* oncogene, Trf- or BSA-liposomes encapsulating anti-*BCR-ABL* siRNA at the tested concentrations led to very low cytotoxicity.

As illustrated in Figure 4C, the observed toxicity effect of liposomes loaded with asODN against LAMA-84 cells reinforced the results obtained with siRNA-loaded liposomes. As can be observed, the cytotoxicity promoted by Trf-liposomes is dependent on the asODN dose. However, off-target cytotoxic effects mediated by the scrambled asODN were observed at a certain extent (70.38 ± 3.077 of cell viability for 2.0 μM). In addition, nontargeted liposomes (2.0 μM specific NT) or empty TrfR-targeted liposomes (2.0 μM ELP Trf) at the same lipid concentration as the one used to treat cells with 2.0 μM asODN-containing Trf-liposomes do not induce cytotoxicity.

***BCR-ABL* mRNA Knockdown Mediated by TrfR-Targeted Liposomes Encapsulating anti-*BCR-ABL* siRNA or asODN.** To evaluate the *BCR-ABL* knockdown efficiency of the liposomes encapsulating anti-*BCR-ABL* siRNA or anti-*BCR-ABL* asODN, *BCR-ABL* mRNA levels were analyzed in LAMA-84 cells double-transfected with the formulations under study. It was observed that only the liposomes encapsulating asODN or siRNA targeted to the TrfR were able to reduce the *BCR-ABL* mRNA levels. Regarding the Trf-liposomes encapsulating siRNA (Figure 5A), the observed silencing effect is dependent on the siRNA concentration up to 1 μM ($59.81 \pm 2.90\%$) without significant differences between 1 and 2 μM . For the Trf-liposomes loading asODN (Figure 5B), a higher dose was required (double) to obtain the same *BCR-ABL* knockdown achieved by Trf-liposomes loading siRNA molecules, where a significant reduction on the *BCR-ABL* mRNA levels ($64.32 \pm 9.0\%$) was only observed at 2.0 μM asODN. The observed *BCR-ABL* mRNA knockdown can be considered sequence-specific for both gene silencing molecules, since no significant reduction in the mRNA levels was observed with the scrambled sequences at the tested concentrations.

Bcr-Abl Protein Levels upon Treatment with TrfR-Targeted Liposomes Encapsulating anti-*BCR-ABL* siRNA or asODN. To evaluate the impact of *BCR-ABL* mRNA knockdown mediated by anti-*BCR-ABL* siRNA and by anti-*BCR-ABL* asODN on the oncoprotein levels, Western blot of LAMA-84 cells double-transfected with liposomes encapsulating siRNA (Figure 6A) or asODN (Figure 6B,C) was performed. As expected, no effect on the oncoprotein levels was observed upon treatment of the cells with nontargeted liposomes. On the other hand, Trf-liposomes encapsulating specific siRNA promoted a reduction in the oncoprotein levels. This effect was dose-dependent up to 1 μM (60.54 ± 12.35), since no further reduction was observed when 2 μM siRNA encapsulated in Trf-liposomes was added to the cells (Figure 6A). Our results also indicate that the protein knockdown

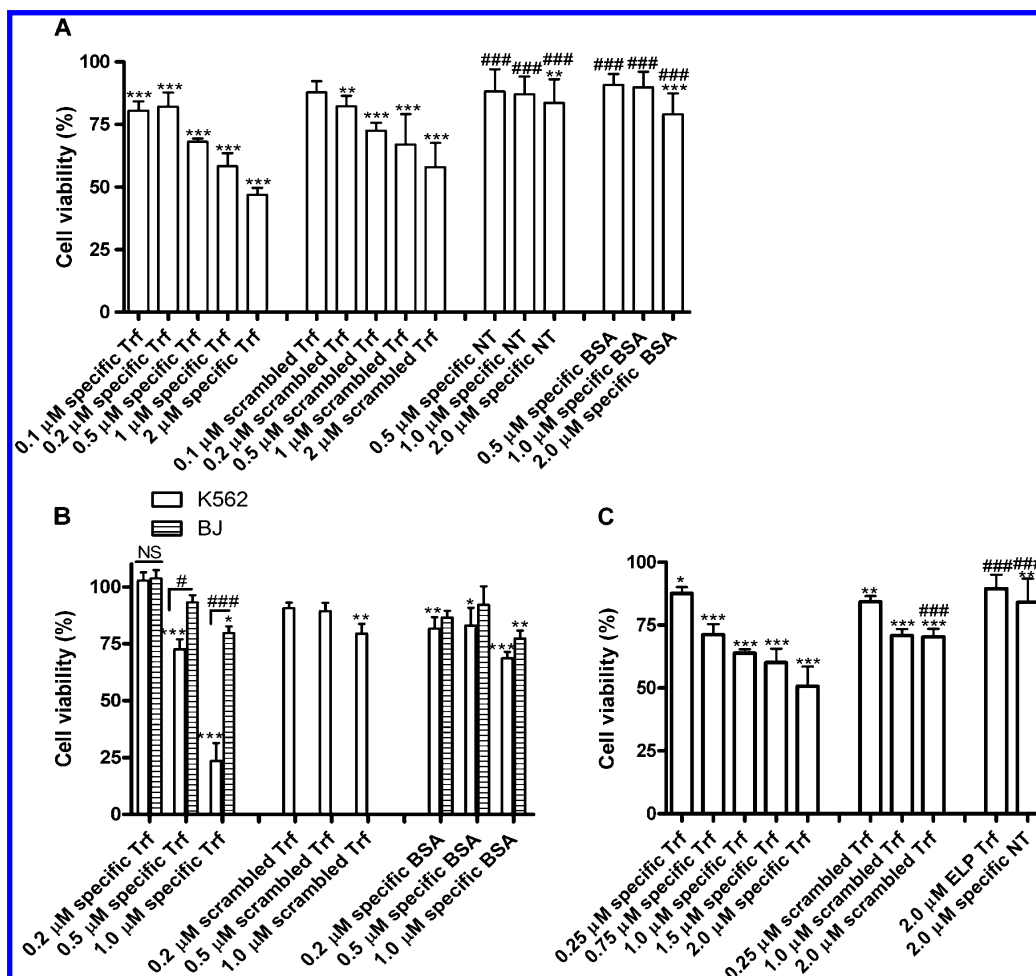


Figure 4. Cell viability upon two transfections with Trf-coupled liposomes loading anti-*BCR-ABL* siRNA or anti-*BCR-ABL* asODN. (A) LAMA-84, (B) K562, and BJ cells were incubated with different concentrations of specific or scrambled anti-*BCR-ABL* siRNA encapsulated in Trf-liposomes, BSA-liposomes, or NT-liposomes. (C) LAMA-84 cells were double-treated with specific or scrambled anti-*BCR-ABL* asODN encapsulated in Trf-liposomes or in NT-liposomes, as well as with empty Trf-liposomes (ELP Trf). Cell viability was assessed 48 h after the second transfection by the resazurin reduction method. (A,C) One-way ANOVA analysis of variance combined with Tukey post-test and (B) regular two-way ANOVA analysis of variance combined with Bonferroni post-test were used for multiple comparisons. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$, and no symbol $p > 0.05$ when comparison was established with nontreated cells; ### $p < 0.001$; # $p < 0.05$, and NS $p > 0.05$ comparison established with the corresponding concentration of specific siRNA/asODN encapsulated in Trf-liposomes (A,C) or comparison established between the conditions indicated by the lines (B).

is sequence-specific up to 1 μM , since the scrambled sequence did not promote any effect up to that concentration. However, for the highest concentration tested (2 μM), a significant reduction in the oncoprotein levels was observed ($66.78 \pm 13.13\%$) when cells were tested with scrambled sequence encapsulated in TrfR-targeted liposomes. Thus, at this concentration the protein knockdown cannot be considered sequence-specific. Concerning Trf-liposomes loaded with asODN (Figure 6B,C), the promoted effect by the specific asODN is dose-dependent for all tested concentrations. However, similarly to what was observed for siRNA, also off-target effects were observed upon treatment of the cells with 2 μM scrambled asODN loaded in TrfR-targeted liposomes.

Nevertheless, it should be emphasized that, independently of the sequence specificity of the siRNA/asODN molecules, reduction in the levels of Bcr-Abl protein is dependent on the cellular targeting specificity. As a matter of fact, in contrast to what was observed to the TrfR-targeted liposomes, no effect on the levels of protein was observed when cells were treated with 2 μM of specific siRNA or asODN encapsulated in BSA-coupled liposomes or nontargeted liposomes.

DISCUSSION

Gene silencing tools, such as siRNA and asODN, play an important role in therapeutic and fundamental research. How-

ever, these molecules have important limitations related to stability and pharmacokinetics. To circumvent these limitations, many approaches such as the use of delivery systems and chemical modifications of the nucleic acids have been tested. In fact, several strategies have been described referencing the design of chemically modified siRNA or asODN molecules less susceptible to nuclease degradation and less prone to immune system activation (8, 10, 13, 31). However, some of the major problems of these molecules are not overcome with these modifications, such as the poor pharmacokinetics, the unfavorable biodistribution throughout the body, and the inability to target specific diseased tissues or cells, like cancer cells. Therefore, the successful clinical application of gene silencing approaches is dependent on the development of effective delivery systems such as liposomes, capable of solving these issues by protecting the encapsulated molecules from nuclease degradation, conferring long circulation times in the bloodstream and with the ability to deliver the carried molecules to target cells through interaction with cell surface receptors.

The aim of this work was to encapsulate siRNA or asODN molecules, designed to specifically silence *BCR-ABL* mRNA, in liposomes targeted to the Trf receptor of leukemia cells. The encapsulation of anionic siRNA and asODN in the aqueous space of lipid vesicles by a passive encapsulation method leads

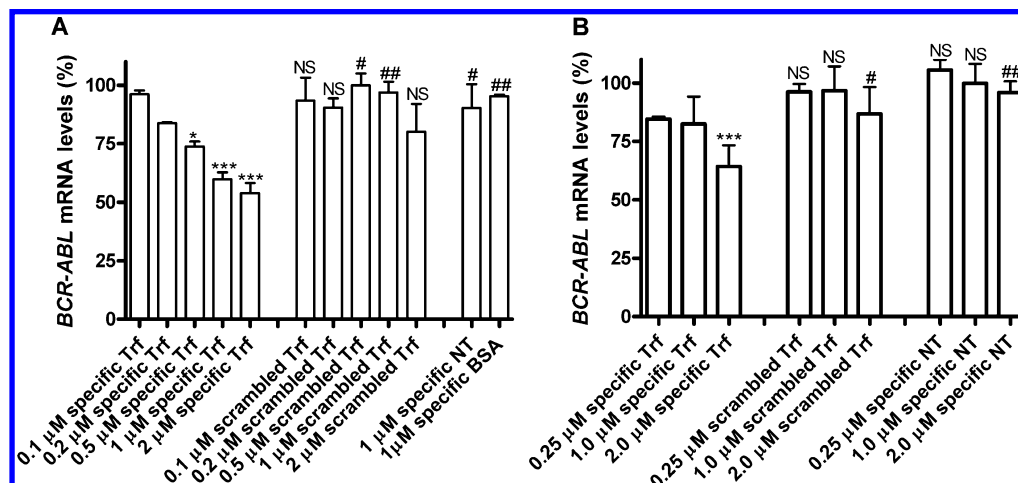


Figure 5. *BCR-ABL* mRNA levels in LAMA-84 cells upon treatment with anti-*BCR-ABL* siRNA or anti-*BCR-ABL* asODN. (A) LAMA-84 cells were double-transfected with different concentrations of specific or scrambled anti-*BCR-ABL* siRNA or (B) anti-*BCR-ABL* asODN, encapsulated in Trf-liposomes (Trf), BSA-liposomes (BSA), or NT-liposomes (NT). *BCR-ABL* mRNA levels of treated samples were evaluated as compared to those of nontreated cells, and were normalized against *B2M* housekeeping gene. Detection was performed by qRT-PCR, as described in Experimental Procedures. One-way ANOVA analysis of variance combined with Tukey post-test was used for multiple comparisons. *** $p < 0.001$, * $p < 0.05$, and no symbol $p > 0.05$ when comparison was established with nontreated cells; # $p < 0.05$, ## $p < 0.01$, and NS $p > 0.05$ when comparison was established with the corresponding concentration of specific siRNA/asODN encapsulated in Trf-liposomes.

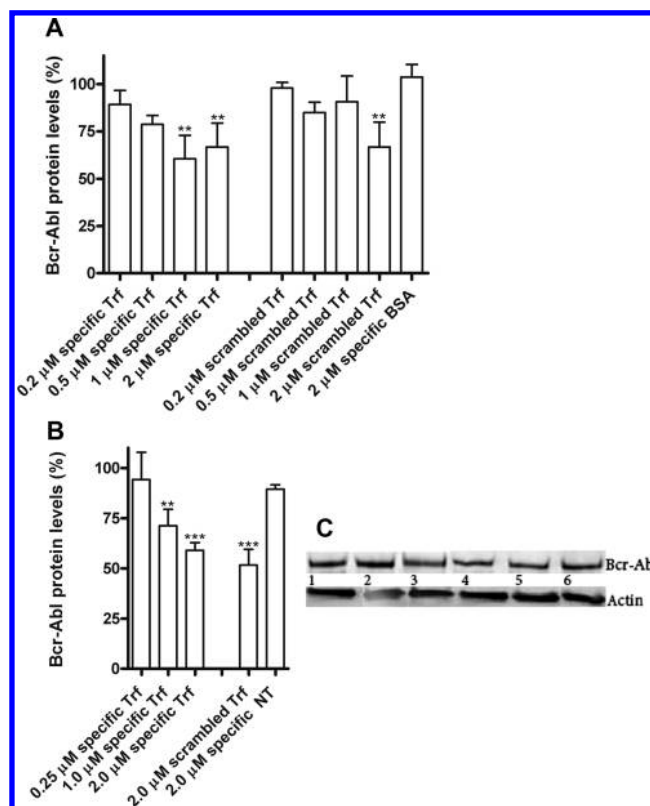


Figure 6. Bcr-Abl protein levels in LAMA-84 cells double-transfected with anti-*BCR-ABL* siRNA or anti-*BCR-ABL* asODN encapsulated in Trf-targeted liposomes. (A) LAMA-84 cells were double-transfected with different concentrations of specific or scrambled anti-*BCR-ABL* siRNA or (B,C) different concentrations of specific or scrambled anti-*BCR-ABL* asODN encapsulated in Trf-liposomes, BSA-liposomes, or NT-liposomes. Cells were collected 48 h after the second transfection for evaluation of Bcr-Abl protein levels by Western blot. One-way ANOVA analysis of variance combined with Tukey post-test was used for multiple comparisons. *** $p < 0.001$, ** $p < 0.01$, and no symbol $p > 0.05$ when comparison was established with nontreated cells. (C) is a representative Western blot result of LAMA-84 cells treated with anti-*BCR-ABL* asODN; lane 1, sample of nontreated cells; lane 2, cells treated with 2.0 μM of specific asODN encapsulated in NT-liposomes; lane 3, cells treated with 2.0 μM scrambled asODN encapsulated in Trf-liposomes; lanes 4, 5, and 6, cells treated with 2.0, 1.0, and 0.25 μM specific asODN encapsulated in Trf-liposomes, respectively.

to very low encapsulation efficiencies and consequently to a tremendous waste of nucleic acids. Cationic liposomes, which form lipoplexes with the nucleic acids, can mediate reasonable levels of transfection *in vitro* without wasting nucleic acids during lipoplex preparation. However, their *in vivo* application

is limited by their large size and net positive charge, which triggers rapid clearance from the blood circulation. In addition, lipoplexes are not tissue-specific, being mainly distributed into lung, liver, and spleen, and very often exhibit toxicity (32–34). Moreover, from the formulation point of view, lipoplexes are

thermodynamically unstable and display a tendency to form larger aggregates over time (14, 35, 36).

Semple et al. developed a new method to encapsulate asODN, leading to the so-called stabilized antisense lipid particles (SALP) (29). These authors demonstrated that the addition of lipids dissolved in ethanol to an aqueous solution of asODN resulted in the formation of small multilamellar liposomes encapsulating the asODN with a very good encapsulation yield. Thus, in this work we have explored this method to carry out asODN encapsulation and assessed whether by performing some modifications it could be applied as well to siRNA encapsulation. This nucleic acid entrapment process is based on the spontaneous electrostatic interaction with cationic liposomes together with the membrane destabilizing effect of ethanol and with the inclusion of PEG-derivatized lipids, which allows control of liposome size to avoid aggregation.

Thus, the high encapsulation yields achieved in this work, for both asODN and siRNA, are mainly attributed to the inclusion of the ionizable cationic lipid DODAP, which is positively charged at pH 4 and consequently able to complex anionic nucleic acids through electrostatic interactions. In our hands, yields of encapsulations of close to 100% were obtained. These high encapsulation yields were also reported by others using SALP for asODN encapsulation in a liposomal formulation similar to that used in this work (29) or for siRNA encapsulation in a different liposomal formulation (37).

Concerning the encapsulation process, our results clearly demonstrate that the major difference between siRNA and asODN encapsulation efficiency is in the significant impact of the citrate buffer concentration. As previously described, very low encapsulation yields of siRNA were observed when using 300 mM citrate buffer as opposed to 20 mM. In contrast, for asODN molecules both 300 mM and 20 mM allowed high encapsulation yields. These encapsulation yields can be explained by the different role of salt and buffer concentrations in the encapsulation of nucleic acid molecules, depending on their chemical characteristics. Indeed, as reported previously, concerning the phosphodiester, as the siRNA molecules used in this work, the use of citrate buffers at 20 mM will be required to facilitate efficient charge interactions between DODAP and the nucleic acid, since at higher buffer concentration, these charge interactions are shielded, resulting in very low encapsulation yields (38–40). On the other hand, phosphorothioate nucleic acids, as the asODN molecules used in this work, bind strongly to cationic molecules, and therefore, the encapsulation efficiency is much less sensitive to the differences in the buffer and salt concentrations.

The covalent attachment of a protein to the liposome surface can be performed by active or passive coupling methods (41). However, as the maleimide group of derivatized lipids used for coupling the protein to the lipid is susceptible to hydrolysis, forming ring-opened maleamic acids which are unreactive (41), the implementation of coupling methods that allow a fast reaction of the maleimide function with the thiol functionalized protein is mandatory. Indeed, it has been demonstrated that the postinsertion method (passive coupling method) allows higher levels of protein coupling as compared to the active method (41). Thus, in this work, the postinsertion coupling method was selected. Trf and BSA coupled at the terminus of MAL-PEG-derivatized lipids, organized in a micellar form, were transferred into preformed liposomes encapsulating nucleic acids. It has been reported that postinsertion occurs in a temperature- and time-dependent manner (24, 42, 43) and frequently is performed close to the phase transition temperature (T_c) of the membrane-forming lipids. However, in this work, the postinsertion was performed for 17 or 24 h at 38 °C, which is below the T_c of the membrane forming lipid DSPC (55 °C), in an attempt to prevent

transferrin denaturation observed at temperatures close to 55 °C. As a consequence of the low temperature used for the postinsertion, the obtained yields of protein–conjugate inserted onto the liposomal surface were very low ($12.18 \pm 1.23\%$ for empty liposomes and $8.9 \pm 0.24\%$ for siRNA-encapsulating liposomes; data not shown). Another possible explanation for the low transfer yield is the high amount of PEG-Cer lipid existing in the preformed liposomes (8 mol %), which was reported to decrease the postinsertion efficacy (41, 43). Under these circumstances, prolonging the transfer time beyond 17 h did not improve the postinsertion yield. In addition, the incubation of 4 mol % instead of 2 mol % of protein-containing micelles led to a higher amount of protein coupled to liposomes, although the postinsertion yield was not enhanced. To the best of our knowledge, this is the first work establishing a correlation between the effect of transfer time (17 and 24 h) and content of Trf-micelles (2 and 4 mol %) added to the liposomes, and the amount of protein–conjugate transferred onto the liposome surface. Our results also indicate that the amount of protein transferred onto the liposomes is similar for both BSA and Trf, and therefore, BSA-liposomes are good control formulations for targeting specificity studies. The yield and the amount of protein–conjugate transferred are significantly higher for empty liposomes as compared to nucleic acid-loaded liposomes. A possible explanation is that loaded liposomes have more rigid lipid membranes and therefore are less prone to the insertion of protein–conjugates into the preformed liposomes, through replacement of pre-existing PEG-lipids (41).

Concerning the size of the liposomes, and similarly to what was previously reported (19), insertion of Trf or BSA protein–conjugates onto liposomes leads to a significant increase of particle size. A higher size enhancement was observed following Trf coupling as compared to BSA, which can be explained by the different molecular weight of these proteins (Trf ~ 80 kDa and BSA ~ 66 kDa). Nevertheless, it should be emphasized that all developed formulations exhibit sizes below 200 nm, with homogeneous particle size distribution (PI smaller than 0.3) and without aggregation during the six month period of observation upon storage at 4 °C. Thus, this liposome preparation method allowed the production of liposomes with reproducible protein/lipid ratio, high levels of nucleic acid encapsulation, and interesting shelf life properties. Therefore, it can be concluded that, from a production point of view, this technology allowed us to produce sophisticated formulations in a quite simple and reproducible way with a very good accuracy.

Results from targeting specificity and competitive inhibition studies clearly indicate that BSA- and NT-liposomes have no ability to promote the delivery of the encapsulated nucleic acids. Additionally, when TrfR was saturated and the temperature was decreased to 4 °C, the extent of cell association and internalization of Trf-liposomes was drastically reduced, clearly demonstrating that internalization of Trf-liposomes is mainly mediated by the TrfR at the cell surface.

The molecular and cellular effects promoted by the developed formulations on leukemia cells (LAMA-84 and K562) and on a nonmalignant cell line (BJ) were assessed. As expected, only Trf-liposomes encapsulating anti-*BCR-ABL* siRNA or asODN were able to promote modifications on *BCR-ABL* mRNA and oncoprotein Bcr-Abl levels, as well as on cell viability. Indeed, the advantage of targeted liposomes as compared to nontargeted liposomes concerning the intracellular delivery and consequently the biological activity of the delivered nucleic acids has been reported (19, 44). A dose-dependent cytotoxicity for both anti-*BCR-ABL* siRNA and asODN when delivered by Trf-liposomes was observed. Nevertheless, off-target effects mediated by scrambled sequences were also observed to a certain extent,

namely, for the highest concentration tested. The Trf-liposomes encapsulating anti-*BCR-ABL* siRNA or asODN were effective in decreasing viability in both leukemia cell lines tested, without significant effect on the nonmalignant cell line BJ that does not present the *BCR-ABL* translocation.

Our results from qRT-PCR indicated that the *BCR-ABL* mRNA downregulation was only possible when siRNA or asODN were delivered by Trf-liposomes. The anti-*BCR-ABL* siRNA led to more efficient reduction of mRNA levels as compared to the anti-*BCR-ABL* asODN, since siRNA promoted the same extent of gene silencing with lower concentrations. Both siRNA- and asODN-mediated *BCR-ABL* mRNA knock-down were sequence-specific at the tested concentrations.

At the protein level, Trf-liposomes encapsulating siRNA led to dose-dependent reduction of Bcr-Abl levels up to 1 μ M, beyond which (at 2 μ M siRNA) significant off-target effects were observed. Therefore, 2 μ M of siRNA had no advantage since the slight enhancement in cytotoxicity, as compared to that observed for 1 μ M concentration, is in part attributed to the enhancement of the off-target effects at the protein level. Thus, as previously reported by others (45–47), siRNA molecules mediate dose-dependent off-target effects. Therefore, it is possible that above a certain concentration (1 μ M) siRNA molecules can knockdown proteins (including Bcr-Abl) by nonspecific mechanisms. This off-target effect observed at the 2 μ M concentration was also observed for asODN molecules. Similarly to what was observed for *BCR-ABL* mRNA, at the Bcr-Abl protein level siRNA are more potent gene silencing tools than asODN, since 2 μ M asODN was required to achieve the same extent of protein knockdown as 1 μ M siRNA. This higher potency of siRNA as compared to asODN has been reported by other authors (44, 48) and is in agreement with our previous results (unpublished data). In contrast to the specificity observed at the mRNA level, off-target effects at 2 μ M were observed for both siRNA and asODN molecules regarding protein knockdown. These findings suggest that the siRNA and asODN molecules are capable of interacting nonspecifically with the Bcr-Abl protein. In fact, it has been previously reported that both siRNA and asODN molecules can promote off-target effects even when the sequences are designed to have no physiological target (49–51). In addition, it was demonstrated that these molecules can act at the protein level without affecting the mRNA levels (45, 51), acting as aptamers (nucleic acids with the ability of interfering directly with protein, further leading to its degradation). Therefore, the cytotoxicity caused by off-target effects mediated by Trf-liposomes encapsulating scrambled siRNA or scrambled asODN, at the 2 μ M concentration, is not caused by interference at the *BCR-ABL* mRNA level, but rather by unspecific interaction with Bcr-Abl protein and probably also with other proteins. Moreover, the possibility of siRNA scrambled sequences acting as a microRNA should not be excluded (52), and consequently, some of the off-target effects observed for the scrambled sequences at 2 μ M concentration may result from off-target mRNA translation inhibition mediated by siRNA through mechanisms similar to those described for microRNA. Therefore, the assessment of the extent of off-target effects triggered by siRNA and asODN molecules is a very difficult task. In fact, it has been reported that some of the observed effects mediated by siRNA are siRNA sequence-specific rather than target-specific (53). Moreover, it has been demonstrated that some of the sequences most widely used as controls for siRNA off-target effects, such as the siRNA against GFP, deregulates endogenous genes (49). Therefore, it seems that off-target effects constitute a well-established limitation of this gene silencing approach, and consequently, caution should be employed in the interpretation of the results.

In conclusion, the developed liposomes exhibited excellent features in terms of asODN and siRNA encapsulating efficiency, particle size, stability over time, and reproducibility of manufacturing. Results from studies in LAMA-84 and K562 cells clearly demonstrated that cell association and internalization of Trf-liposomes are mediated by the TrfR and a notorious *BCR-ABL* mRNA and Bcr-Abl protein down-regulation leading to significant toxicity in leukemia cells. These results illustrate the importance of combining molecular and cellular targeting as a valuable approach for cancer treatment. Further *in vivo* studies are required to assess the clinical potential of the developed formulations for the treatment of CML.

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