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Article

A Fresh Look at the Potential of Cyclodextrins for Improving the Delivery of siRNA Encapsulated in Liposome Nanocarriers

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Cite This: *ACS Omega* 2022, 7, 3731−3737

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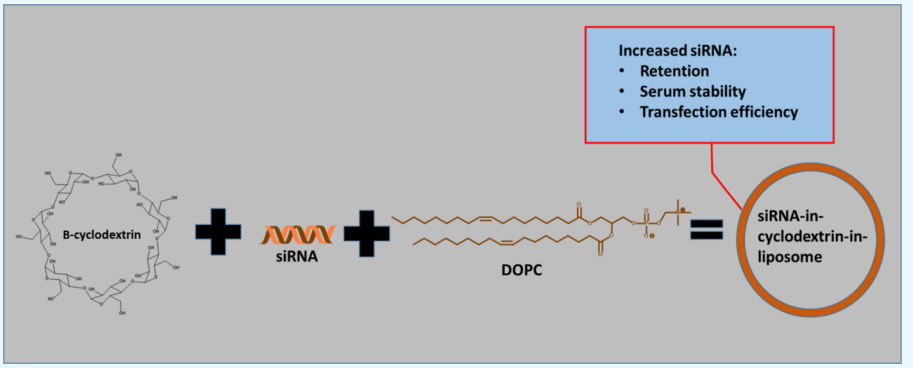
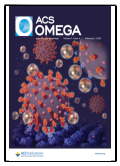


ABSTRACT: Liposomes are among the most effective vehicles to deliver siRNAs to cells, both *in vitro* and *in vivo*. However, despite numerous efforts to improve the potential of liposomes, siRNAs begin to leach out of liposomes as soon as they are formulated. This decreases the value of liposomes for drug delivery purposes significantly, masking their true potential. In this study, we examine the effect of β-cyclodextrins on the retention time and transfection efficiency of siRNAs formulated in a liposome. Cyclodextrins have been widely studied as solvating agents and drug delivery vectors mainly because these cyclic nontoxic glucose structures can bind several molecules of different physicochemical characteristics, through H-bonding or by forming inclusion complexes. These properties, although beneficial for most applications, have resulted in some contradictory results published in the literature, whereas cyclodextrins have been found to destabilize a liposome's membrane. Here, we present a systematic study, which shows that β-cyclodextrin binds, possibly via hydrogen bonding, with siRNA and DOPC liposomes, resulting in increased siRNA serum stability and in vitro siRNA's transfection efficiency when formulated together.

INTRODUCTION

Short interfering RNA (siRNA) therapy for treating diseases such as cancer has shown an increased potential over the last two decades, in part because siRNAs can be tailor-synthetized to target virtually any gene. Delivering siRNAs or any other oligonucleotide to a specific tissue, however, remains a challenge. siRNAs are quickly digested by nucleases in serum, and they cannot cross the cell wall to reach the cytoplasm.[1−3] To overcome these obstacles and send siRNAs specifically to the target tissue, researchers have used a variety of delivering vehicles that range from solid nanoparticles to polymers and liposomes. Liposomes can encapsulate siRNA molecules, and they offer several advantages over other delivering vehicles: they are easy to prepare, they exhibit low cytotoxicity, and they are inexpensive, among several other positive factors. The phospholipids and additives used to make liposomes vary, and as a result, their physical properties such as their size, surface charge distribution, rigidity, and membrane fluidity vary greatly.[4] However, molecules (such as siRNAs) inside liposomes can permeate this membrane and escape to the exterior environment.[5,6] On average, more than 50% of siRNA molecules escape their liposome host during the first 24 h, and in cell culture medium, siRNAs are quickly digested.[7] This poses a challenge in the drug delivery field because immediately after formulating and administering the siRNA/liposome dose, the amount of siRNA molecules in the liposome decreases exponentially. In this initial study, we report a simple method to improve siRNA retention inside a liposome in solution. It involves formulating the liposome/

Received: November 15, 2021
Accepted: December 31, 2021
Published: January 14, 2022



https://doi.org/10.1021/acsomega.1c06436
ACS Omega 2022, 7, 3731−3737

Table 1. Thermodynamics of Binding of βCD[a]

	K_a (M^{-1})	ΔG (KJ/mol)	ΔH (KJ/mol)	ΔS (J/molT)	n
βCD + siRNA	$1.0 \times 10^3 \pm 1 \times 10^2$	−17 ± 0	−33 ± 2	−54	10 ± 0
βCD + $ADCH_2COOH$	$1.17 \times 10^5 \pm 5 \times 10^3$	−29.0 ± 0.1	−26 ± 2	10	0.5 ± 0.2
βCD-$ADCH_2COOH$ + siRNA	$1.0 \times 10^3 \pm 1 \times 10^2$	−17 ± 0	−13 ± 3	13	10 ± 0
βCD + Liposome	$2.0 \times 10^3 \pm 6 \times 10^2$	−19.3 ± 0.6	−2 ± 2	60	0.2 ± 0.1

[a]The thermodynamic data were analyzed using the NanoAnalyzeTM software, and the independent variables (ΔH, K_a, and n) were calculated using the independent binding sites model described by Bistri et al. ΔS and ΔG were calculated using the equation $\Delta G = \Delta H - T\Delta S = -RT \ln K_a$.

siRNA in the presence of β-cyclodextrins. β-Cyclodextrins (βCD) belongs to a family of cyclic oligosaccharide molecules composed of seven α-D-glucopyranoside units linked together (in a 1−4 fashion). They have been widely used as catalysts, as enzyme-structure stabilizers during lyophilization and in biomedicine because of their high solubility in water and their ability to incorporate small hydrophobic molecules inside their cavity.[8−12] Cyclodextrins have been extensively used alone or in combination with other molecules or vehicles to solubilize and transport hydrophobic pharmaceuticals to target tissues, hence changing the pharmacokinetic profile of the drug.[13,14] McCormack and Gregoriadis were the first to encapsulate a cyclodextrin in a liposome to transport hydrophobic molecules.[15] They called this system "drug-in-cyclodextrin-in-liposome." Subsequent studies by various groups revealed that the stability of these liposome−cyclodextrin complexes depends on the type of phospholipid and cyclodextrins, as well as the concentrations of each used to make the liposome.[16−18] Cyclodxetrins could also destabilize a liposome by removing cholesterol from its membrane (a common additive used to increase liposome's membrane fluidity),[16] nevertheless, DSPC-base (distearoyl-glycero-phosphocholine) liposomes seemed to be the best suited for incorporating βCD inside their cavities.[16] Interestingly, a recent molecular dynamics study shows that βCD molecules cannot cross the phospholipid bilayer, but rather remain on the surface of the liposome-forming hydrogen bonds with the phosphate groups,[19] which is perhaps the mechanism by which cyclodextrins slow down the bleaching-out of some molecules from inside a liposome, in a drug-in-cyclodextrin-in-liposome complex as that reported by McCormack and Gregoriadis. In addition to delivering organic compounds, βCDs have been used as carriers for siRNAs. Davis et al. introduced a polymer modified with βCD to deliver siRNAs *in vitro* and *in vivo*, and later, Singh et al. reported a cationic βCD complex for siRNA delivery. Although studies have shown that βCD forms inclusion complexes with some of the nucleotide bases, in particular with adenine,[20] suggesting that βCD could promote unwinding of the siRNA double helix, in a molecular dynamics study, Singh et al. demonstrated that βCD interacts, thruH-bonding, with the surface phosphate groups of the siRNA double helix. In view of these last findings, we decided to study if βCD could help stabilize siRNA molecules inside a DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine)-base liposome by forming a H-bond barrier between the siRNAs and the inner-liposome membrane. To answer this question, we completed a series of thermodynamic, stability, and transfection studies of siRNA-in-cyclodextrin-in-liposome complexes. More specifically, we studied the thermodynamics of binding between βCD and siRNA and DOPC liposomes by isothermal titration calorimetry (ITC); the serum stability of siRNA-in-cyclodextrin-in-liposome complexes; the difference of siRNA liposomal encapsulation in the presence and absence of βCD; the *in vitro* transfection efficiency of the siRNA-in-cyclodextrin-in-liposome complexes; and the size and charge distribution, and we also studied the cell proliferation and cell invasion of these complexes. As a proof-or-principle, we target c-MYC in ovarian cancer cells. c-MYC is an oncogene aberrantly abundant in many cancer types, including ovarian cancer.[21,22] Although DSPC liposomes were found to be best suited for βCD encapsulation,[10] in our laboratory, we routinely use DOPC liposomes to deliver oligonucleotides with good success,[21] and because the only difference between these two phospholipids is a double bond in position 9 (cis configuration) in DOPCs (whereas DSPCs are the saturated version of DOPC), we decided to use DOPC liposomes for this study.

RESULTS AND DISCUSSION

Thermodynamic Studies. Our analysis shows strong binding between βCD and siRNA molecules with a K_a of 1.0×10^3 M^{-1}, and favorable ΔG and ΔH (−17 and −33 kJ/mol, respectively) (Table 1). The unfavorable ΔS observed (−56 J/molT) could be attributed to the excess of βCD needed to reach saturation thermodynamics ($n = 10$), which could lead to major water reorganization. However, additional studies are needed to determine the source of the negative entropy observed. These results validate previous reports that cyclodextrins bind to siRNAs; however, from these data, it is still unclear if βCD forms inclusion complexes with some parts of the siRNA molecules or if the binding only involves H-bonding between βCD's primary and secondary hydroxyl groups to, perhaps, the siRNA phosphate groups exposed on the surface in a double helix structure. To answer this question, we blocked βCD's cavity by mixing it with the guest molecule 1-adamantaneacetic acid ($ADCH_2COOH$), which our thermodynamic data show it forms strong inclusion complexes with βCDs ($K_a = 1.17 \times 10^5 \pm 5 \times 10^3$ M^{-1}, ΔH, and ΔS are also favorable for this process). Mixing adamantane-blocked-βCDs with siRNAs in the ITC instrument showed $K_a = 1.0 \times 10^3 \pm 1 \times 10^2$ M^{-1}, suggesting that βCDs bind onto the siRNA surface mostly via H-bonds (because the βCD cavities were blocked). Next, we studied the binding thermodynamics between βCD and DOPC liposomes prepared, as described in the Experimental section, and here, again, we observed strong binding ($K_a = 2.0 \times 10^3$ $M^{-1} \pm 6 \times 10^2$), suggesting that βCD binds to the outer liposome surface. Because phospholipids are found both on the outer and inner surfaces of the liposome, we suggest that during the liposome preparation (in the presence of siRNA and βCD), a % of βCD molecules will be found inside the liposome, binding both siRNA molecules and phospholipids on the inner liposome surface. These results combined hint that βCDs bind to naked siRNAs and to liposomes most likely by forming H-bonds to surface phosphate groups. In all cases ΔG, ΔH, and ΔS were found to be favorable (except for the ΔS obtained for the binding

https://doi.org/10.1021/acsomega.1c06436
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between βCD to siRNA); however, more studies are needed to understand the drastic changes in ΔS observed (Table 1).

Effect of βCD on the siRNA Serum Stability and Liposome Encapsulation Efficiency. To test if βCDs help retain siRNAs inside DOPC liposomes, we incubated regular liposomes or βCD liposomes in 50% fetal bovine serum (FBS). Our results, shown in Figure 1a, revealed that siRNA molecules

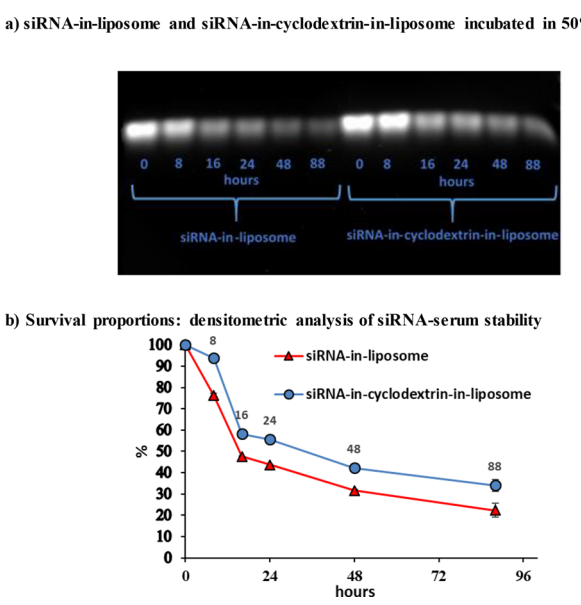

Figure 1. SiRNA serum stability. Incubation of siRNA in liposomes with and without βCD for a total of 88 h in 50% FBS/PBS solution. (a) Polyacrylamide gel of siRNA-cyclodextrin-in-liposome and siRNA-in-liposome after different incubation times. (b) Densitometry analysis of the gel: % siRNA remaining inside the liposomes (in 50% FBS, siRNA molecules outside the liposomes are quickly hydrolyzed and therefore do not show up in the gel). Red triangles: siRNA-in-liposome; blue circles: siRNA-in-cyclodextrin-in-liposome.

encapsulated in liposomes with βCDs invest more time inside their liposomes as compared to siRNAs inside regular liposomes. A densitometric analysis (Figure 1b, Table 2)

Table 2. Densitometry Analysis of siRNA Serum Stability

hours of incubation	% siRNA remaining in the liposome: liposomes without βCD	liposomes with βCD	difference
0	100 ± 1	100 ± 1	0
8	76 ± 1	94 ± 0	18
16	48 ± 0	58 ± 1	10
24	44 ± 0	56 ± 1	12
48	32 ± 1	42 ± 2	10
88	22 ± 3	34 ± 3	12

showed that after 8 h of incubation, 94% of the siRNA molecules in the siRNA−βCD−liposome complex remain intact, while only 76% remain intact when βCD is not present in the regular liposomes (18% more than in the siRNA-in-liposomes without βCD, Table 2). After 88 h of incubation, there were 34% intact siRNA molecules in the siRNA-βCD liposome complex, while only 22% in regular liposomes without βCD (a 12% increase).

To determine if these results were due to differences in the encapsulation efficiencies of the two liposome formulations (siRNA−βCD−liposome or and siRNA-regular liposomes), we measured the siRNA concentration in each liposome formulation spectrophotometrically using two different methods. We found that the siRNA concentrations on both formulations were similar with less than 1.0% difference between the two. Therefore, we concluded that the presence of βCDs affects neither the capacity of the liposomes to encapsulate siRNAs nor the number of siRNAs encapsulated. Furthermore, the size and charge distribution of both formulations were similar (about 130 nm in diameter, and positive Z potential) (Table 3). The encapsulation of βCD

Table 3. Dynamic Light Scattering Results of the Liposomes in PBS[a]

	d (nm)	r (nm)	% pd	Z potential (mV)
siRNA-in-liposome	128 ± 10	64 ± 5	63 ± 16	0.365 ± 0.165
siRNA-in-βCD-in-liposome	126 ± 31	63 ± 16	17 ± 5	0.14 ± 0.14
βCD-in-liposome	182 ± 37	91 ± 18	82 ± 21	1.1 ± 0.2

[a]The errors were calculated from three measurements.

only (no siRNA) appears to yield a slightly larger liposome, but more studies are needed to understand the reason. We also measured the size and charge of both formulations (siRNA-regular liposomes and siRNA-βCD liposomes) at 1, 2, and 4 h after the liposomes were constituted (to imitate drug-liposome reconstitution before drug administration) and found no change in size or charge distribution (Figure 2a,b), suggesting that the liposomes are stable during this initial time frame.

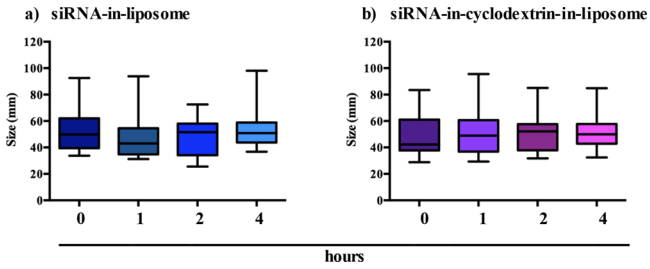

Figure 2. Liposomal size at 0, 1, 2, and 4 h. Size of liposomes (siRNA-cyclodextrin-in-liposome and siRNA-in-liposome) in PBS buffer (pH 7.2) measured by DLS (Mobius, Wyatt Technology) at different incubation times (0, 1, 2, and 4 h). The samples were incubated at 37 °C with shaking. Error bars indicate the range of measurements. $p > 0.05$.

These results of the encapsulation efficiency, size and charge preservation indicate that βCD does not affect the physical properties of the liposomes (size and charge) or the amount of siRNA encapsulated in the liposomes. The serum stability results demonstrate that βCD does not destabilize the liposomes as suggested,[16] but in fact, it helps reduce the amount of siRNA leaching out of the liposomes.

Transfection Efficiency of siRNA-Containing Liposomes in Ovarian Cancer Cells. The purpose of a liposome nanocarrier is to deliver a drug or an oligonucleotide to a target tissue, and the final obstacle any nanocarrier must overcome is to cross the cell membrane and release their cargo to the cell's cytoplasm. Therefore, to assess the ability of both formulations to downregulate a target, we transfected the

https://doi.org/10.1021/acsomega.1c06436
ACS Omega 2022, 7, 3731−3737

A2780CP20 ovarian cancer cells with siRNA-regular liposomes or siRNA-βCD-liposomes. We used a siRNA to target c-MYC. c-MYC is an oncogene highly upregulated in ovarian cancer patients and in cisplatin-resistant ovarian cancer cells.[21,22] In a previous study, we showed that this siRNA effectively reduces c-MYC protein levels in these cells.[21,22] The c-MYC-targeted siRNA inside βCD-liposomes reduces at higher extension the c-MYC protein levels as compared with c-MYC-targeted siRNA inside regular liposomes or with the HiPerfect transfection reagent (Figure 3a). A densitometric analysis of

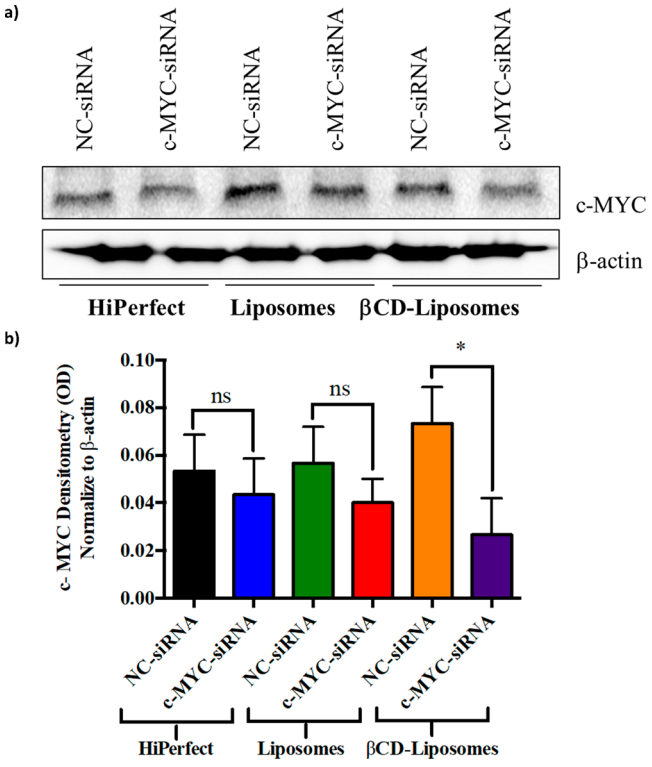

Figure 3. SiRNA transfection efficiency. (a) Western blot of the transfection efficiency study using A2780CP20 cells. c-MYC-siRNA and a negative control (NC-siRNA) were encapsulated in siRNA-cyclodextrin-in-liposome and siRNA-in-liposome. HiPerfect transfection reagent (Qiagen) was used as a control. (b) Statistical analysis of the densitometric data of the gel shown in (a), using the student's t-test in the GraphPad Prism (San Diego, CA) software. *p*-values <0.05 were statistically significant. HiPerfect: siRNAs (c-MYC and NC) were transfected using HiPerfet; Liposomes: siRNAs transfected without βCD (siRNA-in-liposome); βCD-liposome: siRNAs transfected with βCD (siRNA-cyclodextrin-in-liposome). Error bars: triplicates. $*p < 0.05$.

the band's intensity confirmed our observations (Figure 3b). These results indicate that βCD is an effective additive to enhance the transfection efficiency of siRNAs encapsulated in liposomes.

Effect of c-MYC-Targeted siRNA Containing Liposomes on Cell Proliferation and Invasion. Then, we studied the ability of both formulations (siRNA-in-liposome or siRNA-in-cyclodextrin-in-liposome) to reduce cell proliferation and the invasion ability of A2780CP20 ovarian cancer cells. In a colony formation assay, the c-MYC-targeted siRNA inside βCD-liposomes (siRNA-in-cyclodextrin-in-liposome) reduced at higher extension the number of colonies as compared with c-MYC-targeted siRNA inside regular liposomes or with the HiPerfect transfection reagent (Figure 4a). In an invasion assay (Figure 4b), we observed that although both (regular and βCD

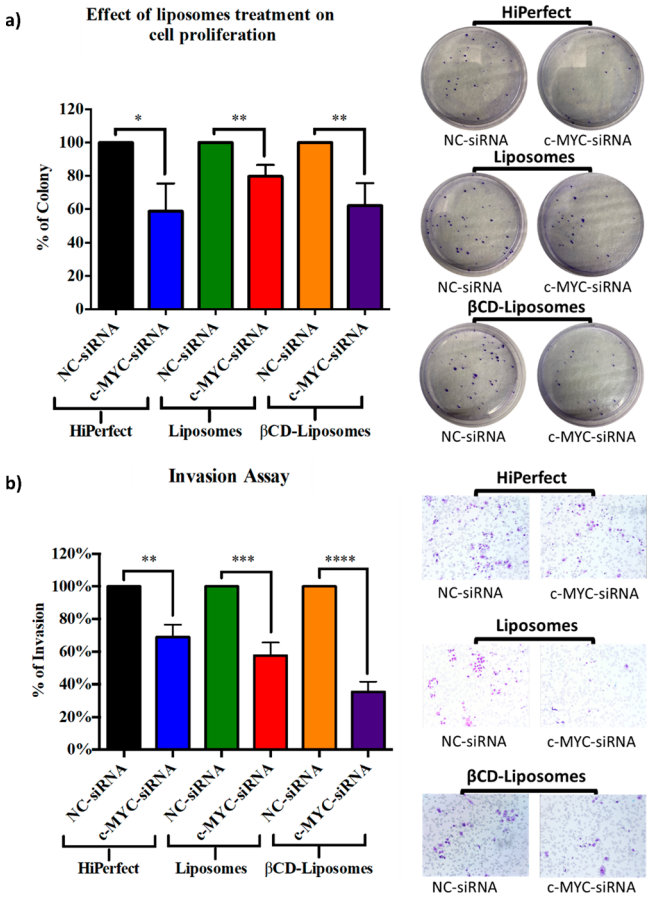

Figure 4. Effect of β-cyclodextrin on cell proliferation and invasion assay. (a) Cell proliferation assay and (b) cell invasion assay. Two types of siRNAs were used in all studies: c-MYC-siRNA and NC-siRNA. HiPerfect: siRNAs transfected using HiPerfet (control); Liposomes: siRNAs transfected without βCD (siRNA-in-liposome); βCD-liposome: siRNAs transfected with βCD (siRNA-cyclodextrin-in-liposome). Error bars: triplicates. $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

liposomes) significantly reduced the invasion ability of A2780CP20 cells, the regular liposomes showed a better effect. Together, these results indicate that siRNA-in-cyclodextrin-in-liposomes had more durable effects (colony formation assays represent the long-term effects on cell growth and proliferation) than regular liposomes.

CONCLUSIONS

Liposomes have been shown to deliver drugs and oligonucleotides to target tissues in vitro and in vivo with good success. However, these formulations need to be improved to boost the pharmacokinetics of their cargo for *in vivo* applications. Here, we present a simple method to enhance their potential to deliver siRNA fragments to cell lines *in vitro*. It consists of adding βCDs to the siRNA-in-liposome formulation, without any chemical modifications of the liposome phospholipids, siRNAs, or βCDs. Our results, obtained with one formulation (one phospholipid type, one siRNA to βCD ratio, and using monomeric βCD), significantly improves the retention of siRNAs inside a liposome, enhancing their transfection

https://doi.org/10.1021/acsomega.1c06436
ACS Omega 2022, 7, 3731−3737

efficiency and stability in the cell culture medium. These results demonstrate that the effectiveness of siRNAs-in-liposomes for treating disease can be enhanced without complex chemical modifications of the siRNA or phospholipid molecules. We believe that the transfection efficiency and tissue delivery of siRNAs encapsulated in liposomes can be improved by varying the ratio of βCD-siRNA and by using different types of βCDs such as CD-polymers and CD-sponges.

EXPERIMENTAL SECTION

Materials. 18:1 (Δ9-Cis) PC (DOPC) 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), cholesterol, and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy-(polyethylene glycol)-2000] ammonium salt (PEG-2000) siRNAs and β-cyclodextrins were purchased from Sigma Aldrich (St. Lois, MO). The c-MYC-targeted siRNA (c-MYC-siRNA) and the negative control siRNA (NC-siRNA were described previously.[21,22]

Cells and Culture Conditions. A2780CP20 cells were kindly gifted by Dr. Anil K. Sood (MD Anderson Cancer Center, Houston, TX). The cells were propagated in vitro in RPMI-1640 medium (Thermo Scientific, UT, USA) supplemented with 10% FBS (Thermo Scientific) and 0.1% antibiotic/antimycotic solution (Thermo Scientific) and maintained at 37 °C in 5%CO_2/95% air. Cells were screened for mycoplasma using the LookOut Mycoplasma PCR detection kit (Sigma) and authenticated by the American Type of Culture Collections (ATCC) using short tandem repeat (STR) analysis. In vitro assays were performed at 70−85% cell density.

Liposome Preparation. siRNAs were mixed with DOPC (1:10 w/w), cholesterol (50% w/w of DOPC), and PEG-2000 (5% mol/mol DOPC/PEG-2000), and or βCD (at a ratio of 1:30 siRNA:βCD), in the presence of excess tert-butyl alcohol. The mixture was frozen in an acetone-dry ice bath and lyophilized. For *in vitro* use, the lyophilized powder was hydrated with Ca^{2+} and Mg^{2+}-free PBS. We have extensively used and characterized these types of liposomes.[21,22] A liposome (sometimes referred to as "lipid nanocarriers)" is a spherical vesicle composed of at least one lipid bilayer. They are usually prepared with phospholipids such as DOPC, DMPC (1,2-dimylristoyl-sn-glycero-3-phosphocholine), DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine), DSPE (1,2-distearoyl-sn-glycero-3-phosphorylethanolamine), and so forth. For example, the mRNA in the RNA COVID-19 vaccine is incorporated in aliposome formulation prepared with DOPC, cholesterol, and DSPE-PEG-2000.[23] Cholesterol is added to increase the liposomal membrane fluidity, and polyethylene glycol (PEG) is added to increase the stability of liposomes in circulation and are commonly known as PEGylated liposomes. PEG protects liposomes from macrophages engulfment (by keeping the macrophages away from the liposomes)[24,25] - by probability 50% of the PEG molecules are inside the liposomes are 50% are outside. In addition, the type of phospholipid used determines the surface-charge of the liposome, and it has been shown that neutral liposomes (such as DOPC) help avoid rapid macrophage engulfment as well.[26,27] Although roto-evaporation is the conventional method used to prepare liposomes, for therapeutic applications, where the liposome formulation must be sterile, the freeze-drying method is preferred.[28]

Thermodynamic Studies. ITC was used to study the binding thermodynamics between βCD and siRNAs (Affinity, TA instruments). In individual titrations, injections of an aqueous solution of βCD (2 mM; 9 μL per injection) were added at intervals of 300 sec to a solution of siRNA placed in the sample cell of the instrument (0.05 mM in water) with stirring at 150 rpm at 25 °C. The reference cell contained water alone without siRNA. The amount of heat produced per injection was calculated by the integration of the area under individual peaks using the instrument software (NanoAnalyzeTM software, TA instruments) after taking into account the heat of dilution. The experimental data were fitted to a theoretical titration curve provided by the NanoAnalyzeTM software, in which the independent variables of interest—ΔH, the enthalpy change in kJ mol^{-1}, K_a, the association constant in M^{-1}, and n, the complex stoichiometry—were calculated using the "independent binding sites" model, as described in the literature.[29] ΔS and ΔG are dependent variables calculated from the equation $\Delta G = \Delta H - T\Delta S = -RT \ln K_a$.

Encapsulation Efficiency of siRNA-Cyclodextrin-in-Liposome and siRNA-in-Liposome. *Filtration Method.* Naked siRNA or siRNA-containing liposomes (regular liposomes and βCD-liposomes) were reconstituted in Ca^{2+} and Mg^{2+}-free PBS (pH 7.2) and sonicated for 15−20 min. Each sample was added to an Amicon 50 K filter (EMD Millipore) and centrifuged at 4500 rpm for 15 min. The eluted fraction was collected to measure the amount of free siRNA using an ultraviolet/visible (UV/vis) spectrophotometer.

Dialysis Method. Naked siRNA and siRNA-containing liposomes were reconstituted as mentioned above, and samples were dialyzed in the same buffer for 3 h. Using a 1.0 mL−50 μm pore size dialysis membrane, dialyzed against 3 × 30 mL fractions of PBS, changed every hour. The dialysis membrane allows free siRNAs (outside the liposome) to pass through, while preventing the 100 ηm (on average) liposomes to escape the dialysis bag. After dialysis, the concentration of siRNAs inside the dialysis membranes corresponding to the two formulations was measured spectrophotometrically at 260 nm. The dilution factor (dialysis membranes swell) was considered measuring the volume change inside the membrane.

Particle Size and Zeta Potential Studies. The size and charge distribution of the liposomes was measured by dynamic light scattering (DLS). SiRNA-containing liposomes (siRNA-cyclodextrin-in-liposome and siRNA-in-liposome) were reconstituted in 300 μL in Ca^{2+} and Mg^{2+}-free PBS (pH 7.2) and sonicated for 15−20 min. After sonication, the particle size and zeta potential were measured (for time zero) at room temperature with a Mobius instrument (Wyatt Technology). The Mobius instrument measures the zeta potential and the particle hydrodiameter simultaneously. It uses a unique design, which allows to draw a current between two electrodes inside the 45 μL cell. As particles migrate from one electrode to the other, a laser beam passes between the electrodes, and its diffraction pattern (from encountering the particles) is captured by 31 detectors arranged at 5 degrees from each other. This arrangement allows to accurately measure the particle size and charge distribution.

Shell and Serum Stability Measurements. Liposomes were reconstituted in Ca^{2+} and Mg^{2+}-free PBS and incubated at room temperature by 1, 2, or 4 h. After these periods of time, the size and charge of liposomes were measured by DLS. For serum stability, liposomes (containing 10 μg siRNA) were incubated at 37 °C in 300 μL of 50% FBS in PBS buffer pH 7.4. Aliquots of 50 μL were withdrawn at 0, 8, 16, 24, 48, and

https://doi.org/10.1021/acsomega.1c06436
ACS Omega 2022, 7, 3731−3737

88 h and frozen at −20 °C. The samples (15 μL) were treated with 0.1% Triton X-100 and vortex-mixed for 2 min. The loading dye (5 μL) was added to each tube, and samples were loaded into a 3% tris-borate-ethylenediaminetetraacetic acid (TBE) agarose gel (1% EtBr). Bands were imaged using a gel imager (Gel Doc XR+, Bio Rad).

Transfection Efficiency and Western Blots. c-MYC-siRNA and a negative control (NC-siRNA) were encapsulated in regular liposomes or βCD-liposomes. A2780CP20 cells (2×10^4 cells/mL) were plated in 10 mL Petri dishes, and 24 h liposomes were reconstituted and added to the cells (100 nM siRNA, final concentration). The HiPerfect transfection reagent (Qiagen) was used as a control. The next day, cells were collected, and cell pellets were lysed with ice-cold lysis buffer (1% Triton X, 150 mmol/L NaCl, 25 mmol/L Tris HCl, 0.4 mmol/L $NaVO_4$, 0.4 mmol/L NaF, and protease inhibitor cocktail from Sigma), and the total protein concentration was determined using Bio-Rad DC Protein Assay reagents (Bio-Rad, Hercules, CA). Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto nitrocellulose membranes. The membranes were blocked in either 5% nonfat dry milk (Bio-Rad) or 5% bovine serum albumin (BSA, HyClone) and probed with anti c-MYC primary antibody (Abcam Inc.). Membranes were then incubated with mouse IgG horseradish peroxidase (HRP)-linked secondary antibody (Cell Signaling), followed by enhanced chemiluminescence and autoradiography. Bands were imaged with a gel imager (Gel Doc XR+). Densitometry analysis was completed using the software provided by the instrument.

Clonogenic and Invasion Assays. Cell growth was assessed with clonogenic assays. Briefly, A2780CP20 cells (2×10^4 cells/mL) were seeded into six-well plates, and 24 h later, cells were transfected with siRNAs encapsulated on the HiPerfect transfection reagent, regular liposomes, or βCD-liposomes. The next day, cells were collected, and 1000 transfected cells were seeded in 10 cm Petri dishes. Colonies formed after seven days were stained with 0.5% crystal violet in methanol. Colonies of at least 50 cells were quantified under a light microscope (CKX41; Olympus) at 10× magnification in five random fields. Percentages of clonogenicity were calculated relative to the NC-siRNA. To assess cell invasion, cells (2×10^4 cells/mL) were seeded in 10 cm Petri dishes. Twenty-four hours later, cells were transfected with siRNAs encapsulated on the HiPerfect transfection reagent, regular liposomes, or βCD-liposomes. The next day, 70,000 cells were seeded into matrigel-coated transwells. Forty-eight hours later, cells were fixed and stained using the Fisher HealthCare PROTOCOL Hema 3 Manual Staining System. The invading cells were counted at 20X on an Olympus 1X71 microscope equipped with a digital camera (Olympus DP26). Percentages of invaded cells were calculated, taking the untransfected cell values as 100% of cell invasion.

Statistical Analysis. Graphing and statistical analysis were performed using Student's t-test in the GraphPad Prism (San Diego, CA) software. p-values <0.05 were considered to be statistically significant. All experiments were performed at least in triplicate.

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Author Contributions

G.L.B. and P.E.V.M. conceptualized the project, B.C.C., G.L.B., M.F.C., and R.J.R.-F. performed the experiments, analyzed data, and constructed graphs, and G.L.B. and P.E.V.M. wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported in part by the National Institute on Minority Health and Health Disparities (NIMHD) CCRHD (U54MD007600) (PEVM), institutional seed funds from the UPR Comprehensive Cancer Center (PEVM), the National Institute of General Medical Sciences (NIGMS) RISE (R25GM061838) (MFC) and (5R25GM061151-20) (RJR-F), and the National Institute of Health−SCORE (S03 5SC3GM122622) Programs.

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https://doi.org/10.1021/acsomega.1c06436
ACS Omega 2022, 7, 3731−3737