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Physicochemical characterization of anionic lipid-based ternary siRNA complexes

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ABSTRACT

Physicochemical characterization is a useful tool in understanding lipoplex assemblies and their correlation to biological activity. Anionic lipid-based ternary siRNA complexes composed of anionic liposomes (DOPG/DOPE), calcium ions and siRNA, have recently been shown to be safe and efficient in a breast cancer cell culture model. In the present work, the effects of various formulation parameters such as liposome composition (DOPG/DOPE ratio) and anionic lipid/Ca^{2+}/siRNA molar charge ratio, on the physicochemical attributes (particle size, surface charge, siRNA loading efficiency and serum stability) of these ternary anionic lipoplexes were evaluated. Particle size, siRNA loading efficiency and serum stability correlated with the *in vitro* silencing efficiency of these lipoplexes. For example, large lipoplex particles (5/2.5/1 anionic lipid/Ca^{2+}/siRNA molar charge ratio) showed less efficient silencing while absolute serum stability and high siRNA loading (1.3/2.5/1 anionic lipid/Ca^{2+}/siRNA molar charge ratio), exhibited maximum silencing in breast cancer cells. The physicochemical properties also indicated that the siRNA exists in the complexed and/or encapsulated form within the lipoplexes, depending on the anionic lipid/siRNA charge ratio. Based on these studies a model representing lipid–siRNA association within the anionic lipoplexes prepared under various formulation conditions is proposed. Physicochemical attributes can be utilized to estimate *in vitro* activity of lipid–siRNA complexes and understand their morphology.

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1. Introduction

Small interfering RNA (siRNA) therapeutics show great promise for treatment of several 'undruggable' targets such as Huntington's disease [1], TTR amyloidosis [2] and respiratory synctial virus [3]. The rapid development in this field is evident by the number of potential products in clinical trials within only a decade of siRNA discovery [4]. siRNA has several advantages over other nucleic acid therapies such as its high potency, high specificity and no constraint for nuclear delivery. Unfortunately, like other nucleic acids, effective delivery of siRNA is a critical issue. This is due to its susceptibility to enzymatic degradation, low cellular uptake and endosomal escape [5]. Several non-viral delivery vectors have been utilized to resolve these issues [6,7], such as liposomes, which facilitate efficient cellular uptake, effective endosomal release of siRNA and protect siRNA against enzymatic attack [8]. There are several lipid based delivery systems currently employed in clinical trials for siRNA molecules [4,9]. A number of cationic liposomes that electrostatically interact with siRNA, have been employed to achieve efficient siRNA delivery both *in vitro* and *in vivo* [4]. However, cationic lipid formulations are inactive in serum and result in severe cytotoxicity [10–14]. For these reasons, anionic lipid formulations are preferred [15,16]. Anionic lipid based systems, composed of anionic liposomes, Ca^{2+} ions and siRNA, which show safe and efficient delivery of siRNA have recently been developed [17].

Despite the extensive use of liposomes for nucleic acid delivery, there are no official FDA guidelines (only a draft form exists [18]) that can ensure robust product development strategies for such liposomal products. With Quality by Design principles gaining popularity, development of well characterized liposome based siRNA delivery systems is imperative in order to facilitate rapid product development and ensure product reproducibility. Consistent product performance is critical specifically for siRNA products in clinical trials. Due to the increasing number of liposome siRNA formulations entering into clinical trials, there is a need for methodological evaluation of physicochemical attributes of lipid-based siRNA formulations. Such systematic studies can help elucidate the relationship between physicochemical properties and biological activity [4] since it has been observed that formulation parameters of lipoplex assemblies indirectly affect biological activity due to their impact on the physicochemical properties of the formulations [19–22]. Accordingly, there can be faster optimization of formulations for effective delivery, consequently saving both time and resources. This is important for cost-effective production of siRNA formulations on a commercial scale. Physicochemical characterization also facilitates thorough understanding of factors affecting the association of lipoplex formulation components. This can help in building a formulation and processing design space. Additionally, a deeper understanding can be obtained regarding the

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morphology of lipoplex assemblies prepared using various process or formulation parameters.

Relative to DNA lipoplexes [16,21–33], there are very few reports on comprehensive characterization of siRNA lipoplexes [34,35]. Additionally, these studies are based on two-component systems (lipids and nucleic acids). In the present work, detailed physicochemical characterization of a three-component system (anionic lipid-based ternary complexes) was performed. The effects of various formulation parameters (liposome composition and anionic lipid/Ca^{2+}/siRNA molar charge ratios) on particle properties were evaluated. These results were utilized to understand the morphology of the anionic siRNA lipoplexes as well as correlation of particle properties to the silencing efficiency. A model has been proposed describing the association of formulation components (lipid, Ca^{2+}, siRNA) within the novel anionic-siRNA lipoplex assemblies and how this changes with formulation composition.

2. Materials and methods

2.1. Materials

The lipids DOPG (1,2-dioleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) and DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine) were purchased from Avanti polar lipids Inc. (Alabama, USA). Anti-eGFP (enhanced green fluorescent protein) siRNA (Silencer® GFP siRNA) and nuclease free water were obtained from Ambion (Austin, TX). Calcium chloride, disodium EDTA (ethylenediamine tetra-acetic acid), triton X-100 (TX-100) and HEPES buffer were purchased from Sigma. For cell culture, MDA-MB-231 cells (breast cancer) stably transfected with eGFP were purchased from Cell Biolabs (Cell Biolabs Inc., CA). Dulbecco' Modified Eagle's medium (DMEM), Modified Eagle's medium–non essential amino acids (MEM–NEAA), Hank's balanced salt solution (HBSS), Opti-MEM I, L-glutamine, heat inactivated fetal bovine serum (FBS) and Quant-IT Ribogreen intercalation assay kit were obtained from Invitrogen (Carlsbag, CA, USA). Penicillin–streptomycin solution was purchased from ATCC (American Type Culture Collection).

2.2. Liposome and lipoplex preparation

For preparation of anionic liposomes, with various anionic to fusogenic lipid ratios (DOPG/DOPE molar ratio), appropriate volume of lipids (25 mg/mL stock solution in chloroform), was added to clean scintillation vials and dried using nitrogen flux to form a thin film. Residual solvent was removed by overnight vacuum-desiccation. The formed lipid film was hydrated using 10 mM HEPES buffer, pH 7.4 for 1 h at 37 °C in water bath. This was followed by 30 s vortexing and extrusion (Lipex™ extruder) to form unilamellar vesicles of size *ca.* 100 nm. The prepared anionic liposomes (5 mg/mL stock solution) were mixed appropriately with siRNA (50 μM stock solution) and Ca^{2+} ions (4.3 M $CaCl_2$ stock solution), for 15 min to obtain lipoplexes with desired anionic lipid/Ca^{2+}/siRNA molar charge ratio. For the purpose of lipoplex preparation, the siRNA concentration was fixed and the concentrations of lipid and calcium were varied. The controls, Ca^{2+}-siRNA complexes and lipid + siRNA mixtures, were prepared similarly except for the addition of lipid and calcium, respectively.

2.3. Particle size analysis

The hydrodynamic size of the particles was measured by dynamic light scattering using a Malvern Zetasizer ZS90 with Zetasizer software at 25 °C. For these studies, lipoplexes were prepared with 50 nM siRNA (100 μL volume) in 10 mM HEPES buffer, pH 7.4. The particle size of the lipoplexes and liposomes was measured in triplicate with at least 12 runs for each measurement. Results were reported in terms of the intensity distribution.

2.4. Zeta potential studies

The zeta potential of the anionic liposomes and the lipoplexes was measured by Laser Doppler Velocimetry (LDV) using a Malvern Zetasizer ZS90. The lipoplexes were prepared in 10 mM HEPES buffer with 50 nM siRNA and the net surface charge was measured using a universal 'dip' cell (Malvern, volume 1 mL). Samples were prepared and evaluated in triplicate.

2.5. siRNA loading efficiency

Ribogreen assay was used to determine the siRNA loading efficiency using the manufacturer's protocol (Invitrogen). This assay is based on the strong fluorescence of ribogreen upon intercalation with siRNA. For this assay, anionic lipoplexes were prepared with various anionic lipid/Ca^{2+}/siRNA molar charge ratios (10 nM siRNA, 100 μL volume) in black 96 well plates (BD Optilux, Perkin Elmer). This was followed by addition of either disodium EDTA (final concentration 2.5 mM) alone or a mixture of disodium EDTA and TX-100 (final concentration 0.5% v/v) to the lipoplexes. (EDTA chelates divalent ions *e.g.* Ca^{2+}, TX-100 is a nonionic surfactant that solubilizes the liposomes). After 5 min incubation, ribogreen was added to the samples (1:2 v/v) for another 2–4 min at room temperature. Following this, the ribogreen fluorescence was measured. Fluorescence was also determined for anionic lipoplexes that were not treated with either one (disodium EDTA) or both the reagents (disodium EDTA and TX-100). A Spectramax Gemini XPS spectrofluorimeter (Molecular Probes) at $\lambda_{ex} = 490$ nm, $\lambda_{em} = 520$ nm, was used to conduct the measurements. The free siRNA concentration was obtained from sample fluorescence in the absence of both reagents, whereas the total siRNA concentration was determined from sample fluorescence in the presence of both reagents. siRNA concentrations were obtained from the fluorescence using a standard curve prepared with standard siRNA solution. Liposomes alone, siRNA alone, Ca^{2+}–siRNA complexes, lipid + siRNA mixtures and blanks (no lipid, Ca^{2+} or siRNA) were used as controls. The fluorescence of 'liposomes alone' was subtracted from that of anionic lipoplexes and lipid + siRNA mixtures (at corresponding lipid concentration) to account for any probable contribution from the liposomes. The results were obtained from three independent experiments (n = 3). The percentage siRNA loading efficiency was calculated using the following formula:

$$\%\ \text{siRNA loading efficiency} = 100 - [(\text{Free siRNA concentration}) * 100/(\text{Total siRNA concentration})].$$

The percentage siRNA released from lipoplexes in the presence of either disodium EDTA alone or both the reagents was also evaluated. For these studies, the initial siRNA concentration was considered as 100%.

2.6. Serum stability studies

The stability of the lipoplexes prepared with various anionic lipid/Ca^{2+}/siRNA molar charge ratios, was evaluated in the presence of 10% serum using Ribogreen assay (Invitrogen). The lipoplexes were incubated in DMEM (no phenol red) with 10% FBS for 4 h at 37 °C. At various time points (0–4 h) an aliquot of the lipoplexes in serum was withdrawn, diluted with 1× TE (Tris-EDTA, pH 7.8) buffer and analyzed with ribogreen reagent. Fluorescence from ribogreen intercalation was measured using a spectrofluorimeter ($\lambda_{ex} = 490$ nm, $\lambda_{em} = 520$ nm). Uncomplexed siRNA in the lipoplexes at 'time 0' was subtracted from the siRNA released at 'time t' to account solely for released siRNA due to serum. The percentage siRNA released was obtained by considering the concentration of 'siRNA only' as 100%. Ca^{2+}-siRNA complexes, lipid + siRNA mixtures, siRNA only

and blank media were used as controls. The samples were prepared with 10 nM siRNA and evaluated in triplicate in two independent experiments (n = 6).

2.7. Cell culture

Breast cancer cells (MDA-MB-231) stably transfected with eGFP were utilized. The cells were cultured in DMEM with 10% FBS, 0.1 mM MEM-NEAA, 2 m L-glutamine and 1% antibiotic mixture (penicillin–streptomycin). The cells were incubated at 37 °C in 5% CO_2 atmosphere. Media was changed once every 2–3 days.

2.8. Silencing efficiency studies

One day before transfection, the cells were plated at 5×10^3 cells per well in 100 μL culture media without antibiotics in black 96 well plates. On the following day, the cells were transfected with 50 μL anionic lipoplexes prepared with 10 nM siRNA in Opti-MEM I media. 48 h post-transfection, media was aspirated and the cells were washed twice with 1× HBSS buffer. The buffer was aspirated and replaced with 100 μL fresh HBSS buffer. GFP expression readout was obtained using a microplate spectrofluorimeter at $\lambda_{ex}=488$ nm, $\lambda_{em}=525$ nm. The readout was normalized for protein content (μg) using a standard BCA assay (Pierce). The percentage GFP expression was obtained by considering untreated cells as 100%. Silencing efficiency was obtained by subtracting the % GFP expression of treated cells from the % GFP expression from the untreated cells (100%). The results were obtained from two independent experiments performed on different days and each day the experiment was performed in triplicate (n = 6). Untreated cells, siRNA only, lipid + siRNA mixtures and Ca^{2+}–siRNA complexes were used as controls.

2.9. Statistics

For comparison between many groups, one-way ANOVA (analysis of variance) with Tukey's post-test was performed using GraphPad Prism software (version 5.0). For comparison of several groups with a control group, one-way ANOVA with Dunnett's post-test was used. For comparison of two samples at a time, student *t*-test was utilized.

3. Results

3.1. Particle size analysis

3.1.1. Effect of liposome composition on particle size

Liposomes were prepared with 10:90 DOPG/DOPE molar ratio (10 mol% DOPG) to 80:20 DOPG/DOPE molar ratio (80 mol% DOPG). The particle size of the anionic liposomes decreased with increase in mol% DOPG (Fig. 1a). On the other hand, the particle size of the anionic lipoplexes (anionic lipid/Ca^{2+}/siRNA molar charge ratio of 1.3/2.5/1) was not affected by change in the liposome composition (Fig. 1b). The average particle size of the lipoplexes (344.0 ± 9.8 nm) was approximately three times that of the liposomes (106.9 ± 3.7 nm).

3.1.2. Effect of Ca^{2+}/siRNA molar charge ratio on particle size

Based on the Ca^{2+}/siRNA molar charge ratio, the particle size of the lipoplexes (40 mol% DOPG, 1.3/1 lipid to siRNA molar charge ratio) varied from 100 nm to 400 nm. The particles were *ca.* 100 nm at the Ca^{2+}/siRNA molar charge ratio of 0.6/1. However, the size increased significantly at higher charge ratios (Fig. 1c). Lipoplexes were *ca.* 300 nm in size at a charge ratio of 2.5/1 and 400 nm in size at a charge ratio of 5/1. Beyond this charge ratio there was no further increase in particle size even up to a Ca^{2+}/siRNA molar charge ratio of 160/1.

3.1.3. Effect of anionic lipid/siRNA molar charge ratio on particle size

The particle size of lipoplexes prepared with various anionic lipid/siRNA molar charge ratios (40 mol% DOPG, 2.5/1 Ca^{2+}/siRNA molar charge ratio) was evaluated. As shown in Fig. 1d, at low anionic lipid/siRNA molar charge ratios (0.6/1 to 2.5/1), the lipoplexes were *ca.* 350 nm in size. Further increase in anionic lipid/siRNA molar charge ratios consistently increased the particle size to *ca.* 600 nm at a charge ratio of 10/1, and even higher to *ca.* 1200 nm at a charge

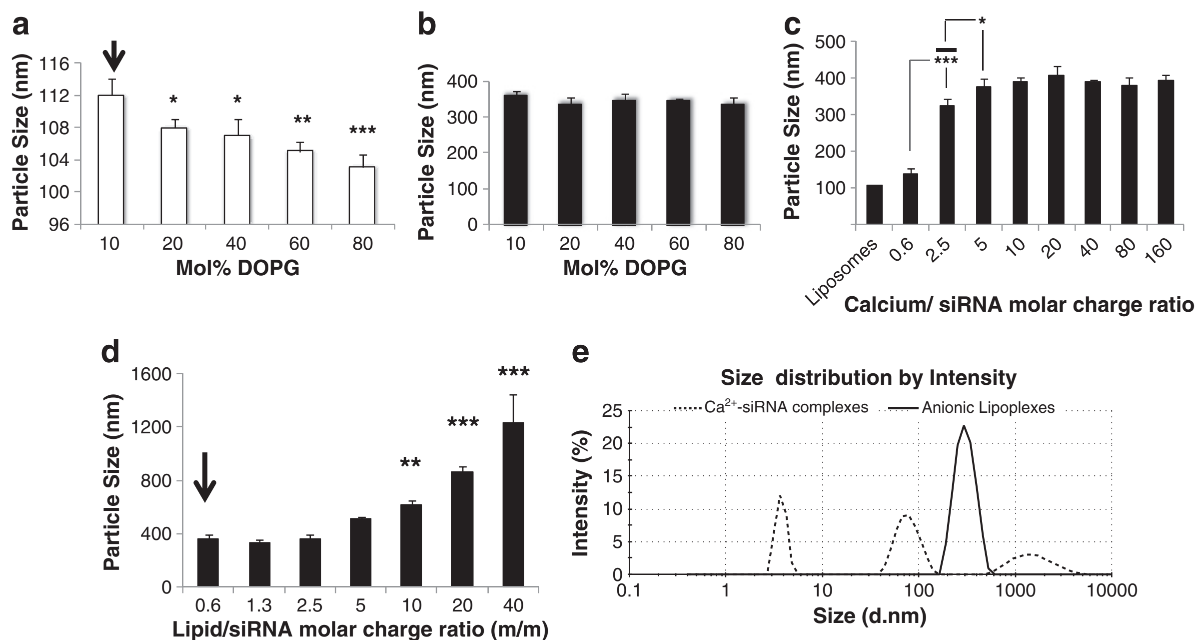

Fig. 1. Particle size analysis of: (a) anionic liposomes prepared with different liposome compositions (mol% DOPG); (b) anionic lipoplexes prepared with various liposome compositions at anionic lipid/Ca^{2+}/siRNA molar charge ratio of 1.3/2.5/1; (c) anionic lipoplexes composed of different Ca^{2+}/siRNA molar charge ratios (anionic lipid/siRNA molar charge ratio of 1.3/1); (d) anionic lipoplexes prepared with different anionic lipid/siRNA molar charge ratios (Ca^{2+}/siRNA molar charge ratio of 2.5/1); and (e) Ca^{2+}–siRNA complexes (dotted line) (Ca^{2+}/siRNA molar charge ratio of 2.5/1) and anionic lipoplexes (solid line) (anionic lipid/Ca^{2+}/siRNA molar charge ratio of 1.3/2.5/1). (n = 3). Arrows indicate the control groups. *p < 0.05, **p < 0.01, and ***p < 0.001. Mean ± SD.

ratio of 40/1. In the absence of lipids, Ca^{2+}–siRNA complexes formed aggregates with a polydispersity index of 0.9 (Fig. 1e).

3.2. Zeta potential studies

3.2.1. Effect of liposome composition on zeta potential

The zeta potential of the liposomes prepared with various anionic lipid/fusogenic lipid (DOPG/DOPE) ratios (or various mol% DOPG) was evaluated. As expected, the anionic liposomes had a negative surface charge (average −49.9 ± 6.8 mV). The liposome surface charge increased with increase in mol% of the anionic lipid, DOPG (Fig. 2a). This trend was also evident in the case of lipoplexes that were formed after the addition of Ca^{2+} ions and siRNA to anionic liposomes (anionic lipid/Ca^{2+}/siRNA molar charge ratio of 1.3/2.5/1) (Fig. 2b). The addition of Ca^{2+} and siRNA reduced the surface charge from −49.9 ± 6.8 mV to −22.0 ± 5.0 mV (average) indicating partial charge neutralization of the liposomes upon lipoplex formation.

3.2.2. Effect of Ca^{2+}/siRNA molar charge ratio on zeta potential

At a Ca^{2+}/siRNA molar charge ratio of 0.6/1 (40 mol% DOPG, anionic lipid/siRNA molar charge ratio of 1.3/1), the zeta potential measurements indicated a reduction in the liposome surface charge (40 mol% DOPG) from −47.7 ± 0.9 mV (no calcium) to *ca.* −15 mV (Fig. 2c). Further increase in the Ca^{2+}/siRNA molar charge ratio to 2.5/1, increased the charge to −22.9 ± 0.6 mV. Additional increase in the Ca^{2+}/siRNA molar charge ratio continuously reduced the charge towards zero and at a 160/1 Ca^{2+}/siRNA molar charge ratio the lipoplexes had a positive surface charge.

3.2.3. Effect of anionic lipid/siRNA molar charge ratio on zeta potential

Increase in the anionic lipid/siRNA molar charge ratio from 0.6/1 to 5/1 (40 mol% DOPG, Ca^{2+}/siRNA molar charge ratio of 2.5/1), increased the zeta potential values from −18.3 ± 0.8 mV to −28.7 ± 0.7 mV (Fig. 2d). Any further increase in the anionic lipid/siRNA molar charge ratio did not significantly affect the surface charge.

3.3. siRNA loading efficiency

3.3.1. Effect of Ca^{2+}/siRNA molar charge ratio on loading efficiency

In the absence of lipids (Ca^{2+}–siRNA complexes), siRNA loading was negligible up to a Ca^{2+}/siRNA molar charge ratio of 0.6/1 (Fig. 3a). However, at higher Ca^{2+}/siRNA molar charge ratios, the loading efficiency increased significantly to 80% at a charge ratio of 0.9/1 and 100% at a charge ratio of 2.5/1. These results indicated that complete loading was achieved at a Ca^{2+}/siRNA molar charge ratio of 2.5/1.

3.3.2. Effect of anionic lipid/siRNA molar charge ratio on loading efficiency

At a low anionic lipid/siRNA molar charge ratio (up to 5/1), minimal siRNA was loaded into the lipoplexes (40 mol% DOPG) with Ca^{2+}/siRNA < 0.6/1 (Fig. 3a). However, within the same range of Ca^{2+}/siRNA molar charge ratios, the loading efficiency increased with increase in the anionic lipid/siRNA molar charge ratio. There was 20% loading at a 10/1 ratio that increased to 60% at a 40/1 anionic lipid/siRNA molar charge ratio. This increase in loading efficiency was also evident in the case of lipid + siRNA mixtures (no calcium) (Fig. 3a, Table 1). However, even at a very high anionic lipid/siRNA molar charge ratio (40/1), complete loading was not achieved at Ca^{2+}/siRNA molar charge ratios ≤ 0.6/1.

In order to understand the nature of the association between siRNA and the other components of the lipoplexes (lipid and Ca^{2+}), the percentage siRNA released from the anionic lipoplexes, when treated with one or both reagents (2.5 mM disodium EDTA, 0.5% TX-100), was determined. As indicated in Fig. 3b, when lipoplexes were treated with EDTA alone, the % siRNA release decreased with increase in the anionic lipid/siRNA molar charge ratio (Ca^{2+}/siRNA ratio of 2.5/1). On the contrary, 100% siRNA was released when the lipoplexes were treated with both reagents (disodium EDTA + TX-100), irrespective of the anionic lipid/siRNA molar charge ratio. For example, at an anionic lipid/siRNA molar charge ratio of 40/1, 40% siRNA was released with disodium EDTA alone, whereas it was 100% in the presence of both disodium EDTA and TX-100. It must be noted that in calculating % siRNA release, we have considered initial siRNA concentration (used for sample preparation) as 100% (see

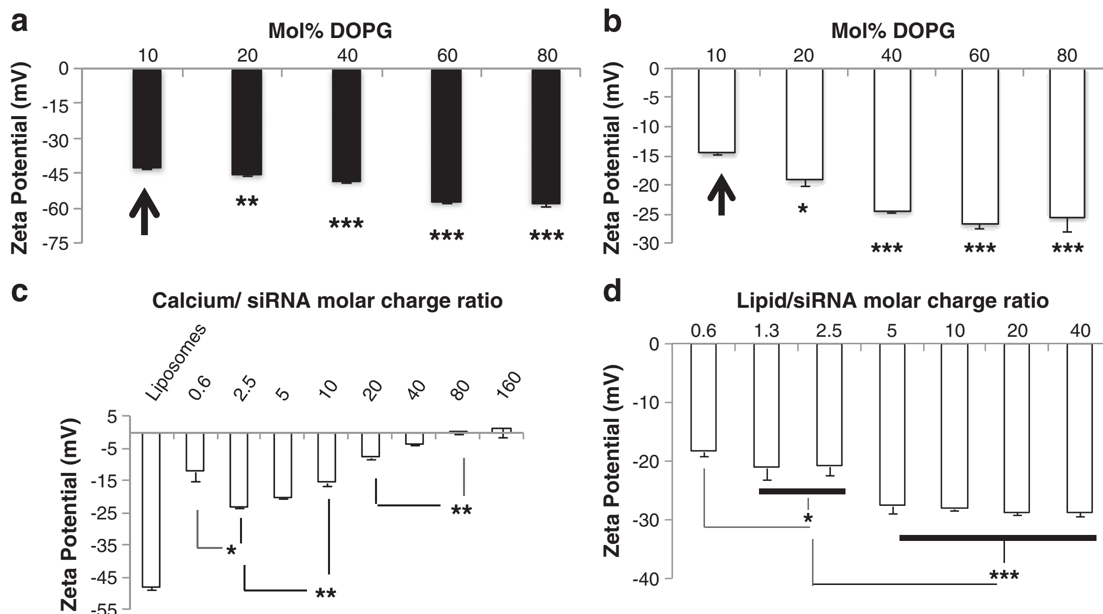

Fig. 2. Zeta potential studies of: (a) anionic liposomes prepared with different liposome compositions (mol% DOPG); (b) anionic lipoplexes composed of various liposome compositions at an anionic lipid/Ca^{2+}/siRNA molar charge ratio of 1.3/2.5/1; (c) lipoplexes prepared with different Ca^{2+}/siRNA molar charge ratios (anionic lipid/siRNA molar charge ratio of 1.3/1); and (d) lipoplexes prepared with different anionic lipid/siRNA molar charge ratios (Ca^{2+}/siRNA molar charge ratio of 2.5/1). Arrows indicate the control groups. (n = 3). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. Mean ± SD.

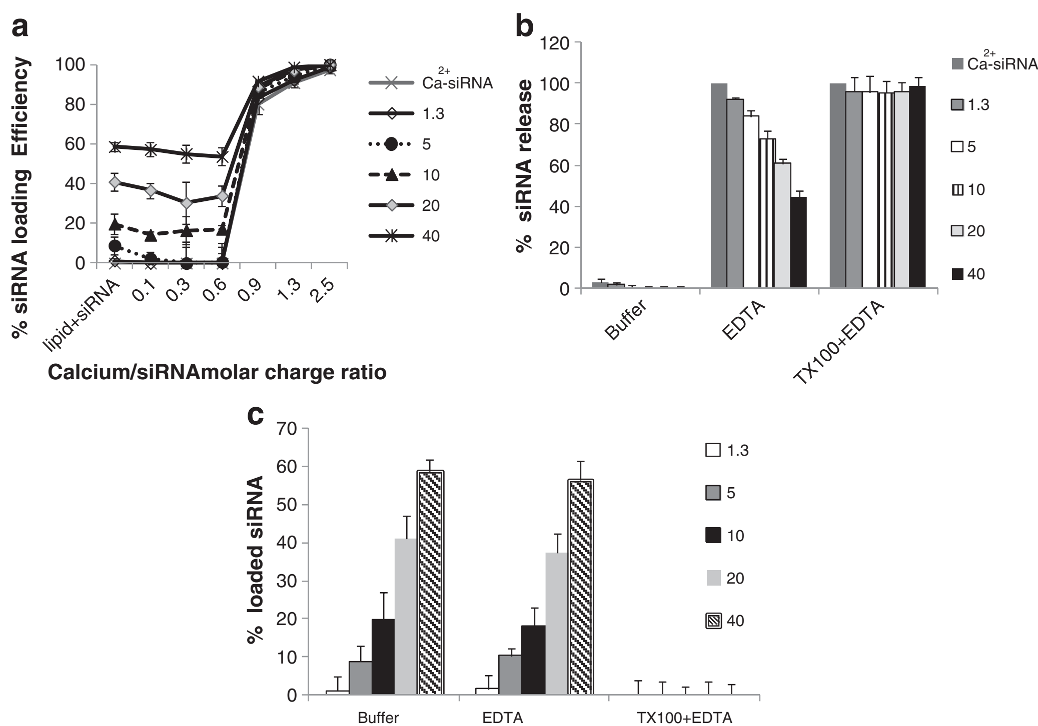

Fig. 3. (a) Percentage siRNA loading efficiency of anionic lipoplexes prepared at various anionic lipid/Ca^{2+}/siRNA molar charge ratios in 10 mM HEPES buffer pH 7.4. (b) Percentage siRNA released (recovered) from lipoplexes prepared with different anionic lipid/siRNA molar charge ratios and treated with either 2.5 mM disodium EDTA alone or a combination of 2.5 mM disodium EDTA and 0.5% TX-100 (Ca^{2+}/siRNA ratio of 2.5/1). (c) Percentage loaded siRNA in lipid + siRNA mixtures (no Ca^{2+}), prepared with various anionic lipid/siRNA molar charge ratios, when treated with disodium EDTA alone or a combination of disodium EDTA and TX-100. The legends indicate charge ratios where the denominator is 1 in each case. *e.g.* 1.3 is 1.3/1 charge ratio. (n = 3). Mean ± SD.

Materials and methods Section 2.5). The fact that we obtained 100% siRNA release from anionic lipoplexes treated with TX100 + EDTA (at all lipid/siRNA ratios) indicates that complete siRNA recovery was achieved with all the anionic formulations.

In the case of the controls, complete siRNA was released when the Ca^{2+}–siRNA complexes were treated with disodium EDTA alone. Conversely, complete siRNA was released (no loaded siRNA) when lipid + siRNA mixtures (no calcium) were treated with a combination of reagents (TX-100 + disodium EDTA) but not with disodium EDTA alone (Fig. 3c).

3.4. Serum stability

3.4.1. Effect of Ca^{2+}/siRNA molar charge ratio on serum stability

Complex stability of the Ca^{2+}–siRNA complexes was evaluated in media containing serum for 4 h at 37 °C. As shown in Fig. 4a, at a low Ca^{2+}/siRNA molar charge ratio of 0.9/1, 40% siRNA was released in 1 h and this increased to 75% in 3 h followed by no further release. However, at higher Ca^{2+}/siRNA molar charge ratios (1.3/1) both the rate and extent of released siRNA decreased significantly. There was only 20% release in the initial two hours followed by saturation. There was no release of siRNA at a Ca^{2+}/siRNA molar charge ratio of 2.5/1 indicating complete serum stability at this ratio. siRNA alone did not degrade in 4 h.

Table 1
Encapsulated and complexed siRNA in anionic lipoplexes (Ca^{2+}/siRNA molar charge ratio of 2.5/1) and lipid + siRNA mixtures (no calcium) prepared at various anionic lipid/siRNA molar charge ratios.

Lipid/siRNA ratio (m/m)	% siRNA			
	Anionic lipoplexes		(Lipid + siRNA) mixtures	
	Encapsulated	Complexed	Encapsulated	Complexed
1.3/1	0	100	0	0
5/1	10	90	10	0
10/1	20	80	20	0
20/1	40	60	40	0
40/1	60	40	60	0

3.4.2. Effect of anionic lipid/siRNA molar charge ratio on serum stability

At an anionic lipid/siRNA molar charge ratio of 1.3/1 (40 mol% DOPG), lipoplexes showed a reduced rate and extent of siRNA release when compared to Ca^{2+}–siRNA complexes (Ca^{2+}/siRNA molar charge ratio < 2.5/1) (Fig. 4b). This protection of the complexes against serum dissociation increased with increase in the anionic lipid/siRNA molar charge ratio as indicated in Fig. 4c. However, complete serum stability was not achieved even at anionic lipid/siRNA molar charge ratios of 10/1 until the Ca^{2+}/siRNA molar charge ratio was increased to 2.5/1.

In case of the lipid + siRNA mixtures (no calcium), as shown in Table 1, the siRNA loading was 10% and 20% in mixtures prepared with anionic lipid/siRNA molar charge ratios of 5/1 and 10/1, respectively. When these mixtures were placed in media with serum, the siRNA was entirely released within 4 h (Fig. 4d).

3.5. Silencing efficiency

3.5.1. Effect of liposome composition on silencing efficiency

Anionic lipoplexes (anionic lipid/Ca^{2+}/siRNA molar charge ratio of 5/2.5/1) prepared with different liposome compositions (mol% DOPG) were evaluated for silencing efficiency in breast cancer cells. As shown in Fig. 5a, the silencing efficiency significantly increased with increase in DOPG from 10 to 40 mol% DOPG. Thereafter, there was no further increase in the knockdown efficiency.

3.5.2. Effect of Ca^{2+}/siRNA molar charge ratio on silencing efficiency

When anionic lipoplexes (40 mol% DOPG, anionic lipid/siRNA molar charge ratio of 1.3/1) prepared with different Ca^{2+}/siRNA molar charge

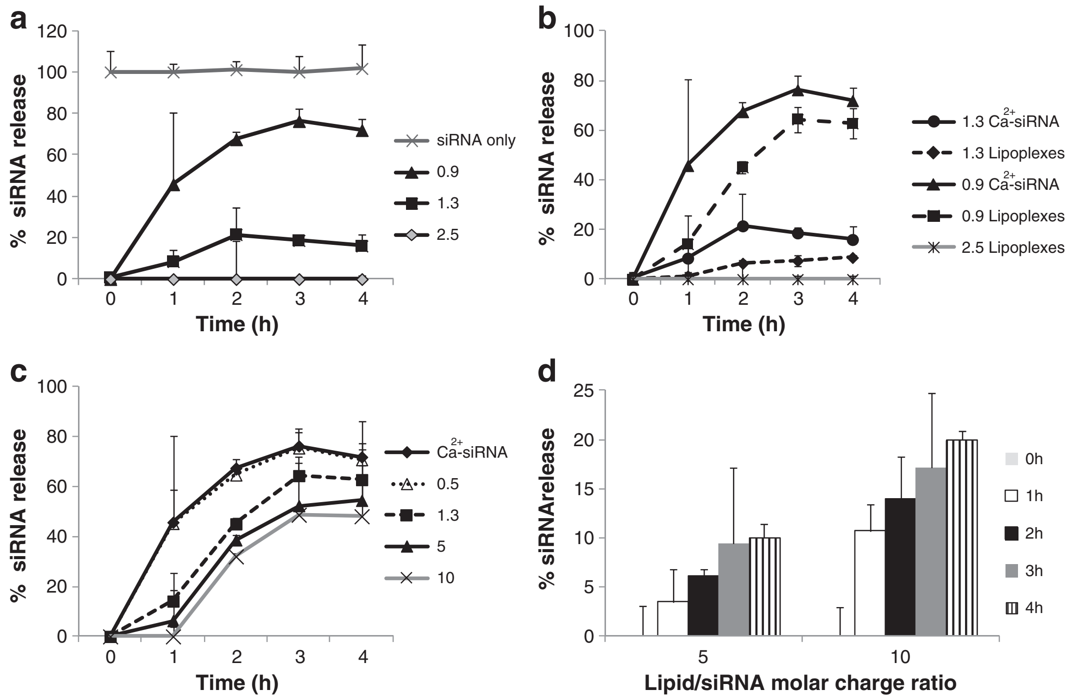

Fig. 4. Serum stability of complexes incubated for 4 h in DMEM + 10% FBS: (a) percentage siRNA released with time, from Ca^{2+}–siRNA complexes prepared at various Ca^{2+}/siRNA molar charge ratios; (b) percentage siRNA released with time, from Ca^{2+}–siRNA complexes and anionic lipoplexes prepared at different Ca^{2+}/siRNA molar charge ratios (anionic lipid/siRNA molar charge ratio of 1.3/1); (c) siRNA released with time from anionic lipoplexes prepared with different anionic lipid/siRNA molar charge ratios (Ca^{2+}/siRNA molar charge ratio of 0.9/1); and (d) percentage siRNA released with time, from lipid + siRNA mixtures (no calcium) at anionic lipid/siRNA molar charge ratios of 5/1 and 10/1. (n = 6). Mean ± SD.

ratios were evaluated, their silencing efficiency increased with increase in molar charge ratio from 0.6/1 to 2.5/1 (Fig. 5b). Further increase in the Ca^{2+}/siRNA molar charge ratio, reduced the silencing efficiency. In the case of the Ca^{2+}–siRNA complexes the knockdown efficiency increased with increase in the Ca^{2+}/siRNA molar charge ratio from 0.6/1 to 5/1 followed by reduction at higher ratios (Fig. 5b). At Ca^{2+}/

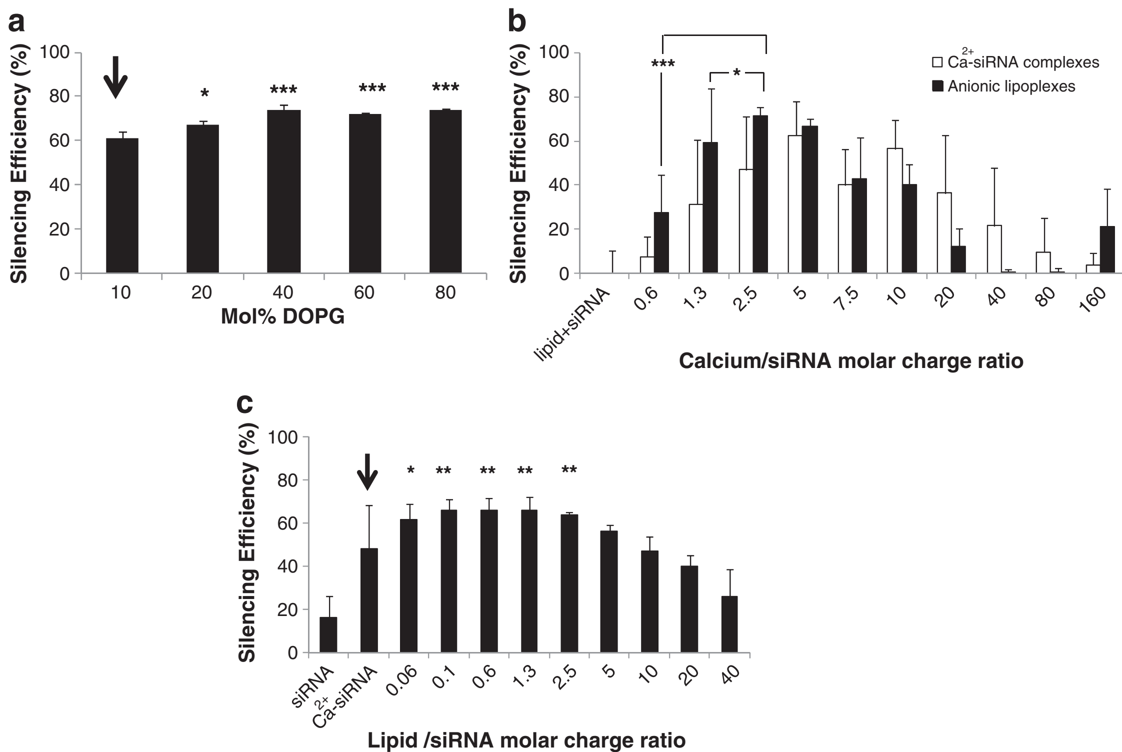

Fig. 5. (a) Silencing efficiency of anionic lipoplexes prepared with different liposome compositions (mol% DOPG) at an anionic lipid/Ca^{2+}/siRNA molar charge ratio of 5/2.5/1, (b) silencing efficiency of lipoplexes prepared with different Ca^{2+}/siRNA molar charge ratios (anionic lipid/siRNA molar charge ratio of 1.3/1), (c) silencing efficiency of lipoplexes prepared at different anionic lipid/siRNA molar charge ratios (Ca^{2+}/siRNA molar charge ratio of 2.5/1). siRNA alone, Ca^{2+}–siRNA and lipid + siRNA mixtures were used as the controls (n = 6). Arrows indicate the control groups used for comparison. *$p<0.05$, **$p<0.01$, and ***$p<0.001$. Mean ± SD.

siRNA molar charge ratios ≤ 2.5/1, the anionic lipoplexes were significantly more effective compared to Ca^{2+}–siRNA complexes. Turbidity was observed in media containing complexes prepared at Ca^{2+}/siRNA molar charge ratios ≥ 5/1. Highly variable results were obtained with Ca^{2+}–siRNA complexes at all Ca^{2+}/siRNA molar charge ratios investigated. As per the results, maximum silencing was obtained with anionic lipoplexes prepared with a Ca^{2+}/siRNA molar charge ratio of 2.5/1.

3.5.3. Effect of anionic lipid/siRNA molar charge ratio on silencing efficiency

Anionic lipoplexes (40 mol% DOPG, Ca^{2+}/siRNA molar charge ratio of 2.5/1) prepared with various anionic lipid/siRNA molar charge ratios showed significantly higher silencing with anionic lipoplexes when compared to Ca^{2+}–siRNA complexes from anionic lipid/siRNA molar charge ratios of 0.06/1 to 1.3/1 (Fig. 5c). Further increase in anionic lipid/siRNA molar charge ratios reduced the silencing efficiency. Highly unstable lipoplexes were obtained at anionic lipid/siRNA molar charge ratios < 1.3/1.

4. Discussion

Anionic lipoplexes prepared with various liposome compositions and anionic lipid/Ca^{2+}/siRNA molar charge ratios were evaluated for their physicochemical properties and silencing efficiency. It was determined that the formulation parameters impacted the physicochemical attributes as well as the biological activity of anionic lipoplexes. Further, physicochemical studies facilitated interpretation of structures formed by lipid–siRNA complexes prepared under various formulation conditions.

4.1. Effect of formulation parameters on physiochemical characteristics of anionic lipoplexes

Formulation parameters such as liposome composition noticeably affected the physicochemical attributes of liposomes and lipoplexes. For example, increase in the mol% DOPG increased the negative charge of the liposomes due to increase in the proportion of the anionic lipid (DOPG) (Fig. 2a). This increase in charge was accompanied by a decrease in particle size (Fig. 1a). This is attributed to the greater repulsion between the charged lipid head groups, which increases the radius of curvature of the lipid bilayer resulting in smaller particles. Although the lipoplex surface charge did increase with the mol% DOPG (Fig. 2b), there was no significant difference in their particle size (Fig. 1b). This was probably due to the multiple events that occur during lipoplex formation: (i) electrostatic interaction of the anionic lipid (DOPG) with the siRNA *via* the calcium ions; and (ii) lipid scrambling due to calcium induced fusion [36,37]. This series of events results in large size particles and would obscure the relatively small change in particle size of the participating liposomes. This hypothesis is supported by the fact that the anionic lipoplexes are approximately three times the size of the anionic liposomes (Fig. 1a and b). Another reason for the lack of difference in lipoplex particle size, irrespective of liposome composition, is the complimentary role of the two lipids (DOPG, DOPE) in the process of lipoplex formation.

Besides liposome composition, formulation parameters such as the calcium/siRNA molar charge ratio also influenced the lipoplex properties. For example, at a low Ca^{2+}/siRNA molar charge ratio (0.6/1), the lipoplex sizes were close to that of the liposomes (*ca.* 100 nm) (Fig. 1c). However, the size of the lipoplexes increased with increase in the Ca^{2+}/siRNA molar charge ratio (≥ 2.5/1). This may be due to the presence of sub-optimal concentrations of calcium at lower ratios, which may lead to inefficient lipoplex formation. This hypothesis is corroborated by the fact that these lipoplexes showed poor siRNA loading (Fig. 3a) and the siRNA loading increased with increase in the Ca^{2+}/siRNA molar charge ratio reaching a maximum at 2.5/1. In support of this, the particles had less negative charge at a low Ca^{2+}/siRNA molar charge ratio of 0.6/1 probably due to the presence of the Ca^{2+} ions on the liposomal surfaces. At higher molar charge ratios (2.5/1), calcium is sandwiched between the anionic lipids and siRNA forming efficient lipoplexes with higher negative charge. At Ca^{2+}/siRNA molar charge ratios > 2.5/1, the surface charge was neutralized to zero and further increased to a positive charge, due to the extremely high concentrations of cationic Ca^{2+} ions (Fig. 2c). Lipoplexes with Ca^{2+}/siRNA molar charge ratios > 2.5/1, all had similar average size probably due to the limited availability of lipid and siRNA to form larger aggregates.

The colloidal properties of the lipoplexes (particle size and surface charge) were also affected by anionic lipid/siRNA molar charge ratios. For example, at high lipid/siRNA molar charge ratios (≥ 5/1), large particles (> 500 nm) were formed (Fig. 1d). This increase in size was probably due to aggregation mediated by excess lipid upon interaction with accessible Ca^{2+} ions that were not completely neutralized during lipoplex formation. Interestingly, these particles (with anionic lipid/siRNA molar charge ratios of 5/1 to 40/1) showed no significant change in their zeta potential values (Fig. 2d). Theoretically, surface charge should have increased with increase in the concentration of the anionic lipid, DOPG, and this held true for lipoplexes with lower anionic lipid/siRNA molar charge ratios (< 5/1). At higher ratios (≥ 5/1), however, the lipoplexes aggregate in the presence of calcium leading to partial surface charge neutralization. This aggregation was confirmed by particle size analysis (Fig. 1d). Very low lipid/siRNA ratios (< 1.3/1) formed unstable complexes due to the imbalance between the charge ratios of the formulation components. An anionic lipid/siRNA ratio of 1.3/1 was therefore optimal.

In ternary anionic lipoplexes, calcium is the primary component that complexes with the siRNA and the lipids. Therefore, as expected, siRNA loading efficiency in the lipoplexes increased with increase in concentration of calcium (Ca^{2+}/siRNA molar charge ratio) (Fig. 3a). Interestingly, it was determined that lipids alone (no calcium) also contributed to loading of the siRNA into the lipoplexes (Fig. 3c, Table 1). This could be due to Van der Waal's attraction between the lipid (DOPE) and siRNA as has been reported by others [30,38]. However, even at the maximum anionic lipid/siRNA molar charge ratio investigated, complete siRNA loading was not achieved probably due to saturation of the lipid groups interacting with the siRNA. 100% siRNA loading was achieved only with lipoplexes composed of a Ca^{2+}/siRNA molar charge ratio of 2.5/1, irrespective of the anionic lipid/siRNA molar charge ratio. This indicates that lipids can only facilitate partial loading and that calcium is required to achieve complete loading.

Serum is known to interfere with the transfection efficiency of the cationic lipoplexes due to neutralization of the lipoplex charge by the mostly anionic serum proteins [10,14,39]. In the case of Ca^{2+}–siRNA complexes, serum proteins compete with siRNA for binding to calcium ions. If interaction between Ca^{2+} and siRNA is not sufficiently strong, serum may cause siRNA to dissociate from the complexes. For this reason, the serum stability of the Ca^{2+}–siRNA complexes was poor at low Ca^{2+}/siRNA molar charge ratios (Fig. 4a) while it improved with increase in calcium (higher Ca^{2+}/siRNA molar charge ratios) due to the presence of optimal amounts of Ca^{2+} that could bind strongly to the siRNA. Additionally, the presence of lipids in the complexes reduced the accessibility of Ca^{2+} to the serum proteins thereby preventing complex destabilization. This effect is evident from Fig. 4b where it is shown that lipoplexes are more stable than Ca^{2+}–siRNA complexes. With increase in lipid, the protection against serum dissociation improved as indicated by Fig. 4c. Although lipids improved serum stability, complete stability was achieved at a Ca^{2+}/siRNA molar charge ratio of 2.5/1 indicating the requirement of an adequate amount of calcium to bind strongly with the siRNA and lipid thereby prohibiting complex dissociation.

Physicochemical characterization of anionic lipoplexes has also been useful in differentiating anionic lipoplexes from liposomes and

the Ca^{2+}–siRNA control. For example, lipoplexes were larger in size and had lower surface charge due to partial charge neutralization (Figs. 1b and 2b), when compared to the anionic liposomes (Figs. 1a and 2a). Also, the lipoplex particles were monodisperse while the Ca^{2+}–siRNA complexes were polydisperse (Fig. 1e).

4.2. Effect of formulation parameters on the biological activity of lipoplexes — correlation with physicochemical properties

The silencing efficiency can be explained on the basis of the physicochemical properties of the complexes. For example, the large variation in the activity of the Ca^{2+}–siRNA complexes (Fig. 5b) could be due to the high polydispersity of these particles (Fig. 1e). In addition, the lipid + siRNA mixtures dissociated in the serum (Fig. 4d) and therefore showed no silencing in the cell culture studies (Fig. 5b). In the case of lipoplexes, the low silencing efficiency at Ca^{2+}/siRNA molar charge ratios < 2.5/1 (Fig. 5b) was probably due to the inefficient siRNA loading as indicated in Fig. 3a. However, when investigated, lipoplexes with a Ca^{2+}/siRNA molar charge ratio of 1.3/1, showed high siRNA loading (80%). The reduced silencing efficiency of these lipoplexes (Fig. 5b) was probably a result of lipoplex dissociation in serum as indicated in Fig. 4b. For lipoplexes prepared with a Ca^{2+}/siRNA molar charge ratio of 0.6/1, the loading efficiency (in HEPES) was negligible (Fig. 3a). However, these complexes did show some knockdown effect (Fig. 5b). In order to understand this, the loading efficiency of these complexes was evaluated in cell culture media (Opti-MEM I) and was determined to be 60% (data not shown). The higher siRNA loading in cell culture media compared to HEPES could be due to the greater ionic strength of the former that may have improved ionic interactions between lipid, Ca^{2+} and siRNA in unstable anionic lipoplexes with unbalanced charge ratios (low Ca^{2+} at Ca^{2+}/siRNA ratio of 0.6/1). Even though the loading was improved, these lipoplexes presumably destabilized in serum (very low serum stability was observed with Ca^{2+}/siRNA ratio of 0.9/1 in Fig. 4b) leading to low silencing efficiency in breast cancer cells (Fig. 5b). At the higher Ca^{2+}/siRNA molar charge ratio of 2.5/1, maximum silencing efficiency of the lipoplexes was achieved (Fig. 5b). This is considered to be due to efficient loading (Fig. 3a) and complete retention of loaded siRNA in the presence of serum (Fig. 4b). Therefore, a Ca^{2+}/siRNA molar charge ratio of 2.5/1 was optimal to prepare stable (in serum) and efficient lipoplexes. For lipoplexes prepared with high Ca^{2+}/siRNA molar charge ratios greater or equal to 5/1 (Fig. 5b) or high anionic lipid/siRNA molar charge ratios greater than 5/1, (Fig. 5c) the silencing efficiency was low as a result of extreme aggregation in the cell culture media. Consequently, the cellular uptake of these complexes was inefficient [40–42]. These results are in contrast to some reports that have indicated high biological activity with large particles (micron range) [43,44].

The silencing efficiency of the lipoplexes prepared with various liposome compositions did not correlate with lipoplex particle size. The lipoplexes prepared with various mol% DOPG were in same size but showed increase in silencing efficiency with increase in mol% DOPG (up to 40 mol%) (Figs. 1b and 5a). This indicates that the silencing efficiency is affected by the proportion of the lipids (DOPG, DOPE). DOPG is an anionic lipid that facilitates lipoplex formation. DOPE is a fusogenic lipid that undergoes phase transition from lamellar to inverted hexagonal phase at low endosomal pH, thereby causing endosome destabilization and releasing the siRNA [45–48]. Consequently, increase in DOPG promotes efficient lipoplex formation thereby increasing siRNA loading and hence increasing the silencing efficiency. On the other hand, increase in DOPE facilitates the endosomal escape of siRNA and thereby increases the silencing efficiency. Therefore, an optimal balance of DOPG and DOPE results in maximum silencing.

In general, the particle properties correlated well with the biological behavior of the anionic lipoplexes. Physicochemical characterization studies also helped in understanding the role of the formulation components on the biological activity. For example, lipids and Ca^{2+} cooperatively contributed to siRNA loading (Fig. 3) and serum stability (Fig. 4) and accordingly played a significant role in enhancing the silencing efficiency of the anionic lipoplexes. Additionally, systematic physicochemical characterization facilitated estimation of the optimal formulation (lipid/Ca^{2+}/siRNA molar charge ratio of 1.3/2.5/1) for maximum silencing efficiency.

4.3. Structure of siRNA-lipoplex assemblies and the proposed model

Within the lipoplexes, siRNA is associated with other formulation components (lipids, Ca^{2+}), based on the anionic lipid/siRNA molar charge ratio. To understand this association, the percentage siRNA released from lipoplexes after the addition of either disodium EDTA or disodium EDTA and TX-100 was determined. TX-100 is a nonionic surfactant that solubilizes anionic liposomes and disodium EDTA is a chelating agent that complexes with Ca^{2+} ions and releases siRNA. The nature of the siRNA association can be determined as follows: (i) complexed siRNA can be determined by the % siRNA released from lipoplexes treated with EDTA alone, and (ii) complexed + encapsulated siRNA can be determined by the % siRNA released from lipoplexes treated with both EDTA and TX-100. The percentage encapsulated siRNA can be calculated by subtraction of (i) from (ii).

As shown in Fig. 3b, in the absence of lipids (Ca^{2+}–siRNA complexes) or at low anionic lipid/siRNA molar charge ratios (≤1.3/1), siRNA was completely (100%) released from the complexes, when treated with EDTA alone (Fig. 3b). In addition, at these anionic lipid/siRNA molar charge ratios, siRNA loading of anionic lipoplexes was equivalent to that obtained with Ca^{2+}–siRNA complexes (Fig. 3a and b) whereas negligible loading was obtained in case of lipid + siRNA mixtures (Fig. 3c). This indicates that at low anionic lipid/siRNA ratios (<1.3/1), siRNA loading is only due to the Ca^{2+} *via* complexation and there is no role of lipids in this process (represented in Fig. 6a). In other words, siRNA is solely in the 'complexed' form within anionic lipoplexes at low anionic lipid/siRNA ratios (<1.3/1) (represented in Fig. 6a).

At high lipid/siRNA molar charge ratios (>5/1), loaded siRNA in lipid + siRNA mixtures was released (unloaded) with TX100 + EDTA, but not with EDTA alone (Fig. 3c). This indicates that siRNA is solely in the 'encapsulated form' within these complexes where the encapsulation occurs *via* Van der Waal's interaction between lipid (DOPE) and siRNA. In anionic lipoplexes prepared at high anionic lipid/siRNA ratios, % siRNA release with EDTA decreased with increase in lipid/siRNA ratio, whereas 100% release was obtained with TX100 + EDTA at all lipid/siRNA ratios (Fig. 3b). This means that the proportion of loaded siRNA in the 'complexed' form decreases and 'encapsulated' form increases with increase in lipid/siRNA ratio. Considering that the amount of 'encapsulated siRNA' in anionic lipoplexes is equivalent to the amount in lipid + siRNA mixtures at the same lipid/siRNA ratio (Table 1), it can be concluded that 'encapsulation' is only due to the lipids (DOPE) with no involvement of calcium (see 'encapsulated form' in Fig. 6b). Therefore, it can be inferred that calcium primarily contributes to siRNA complexation whereas lipids contribute to siRNA encapsulation and both of these processes are required to achieve complete siRNA loading. The characterization studies thus revealed the synergistic role of lipids and Ca^{2+} in associating with siRNA and forming the lipoplex assemblies.

It is clear that siRNA association within the lipoplexes depends on the anionic lipid/siRNA molar charge ratios. Accordingly, lipoplexes are in the 'complexed' form at low anionic lipid/siRNA molar charge ratios and in both the 'complexed' and 'encapsulated' forms at higher ratios. Based on this conclusion, a schematic model representing anionic lipoplex structures obtained using various anionic lipid/siRNA molar charge ratios is proposed as shown in Fig. 6. This model describes the arrangement of lipid, Ca^{2+} and siRNA within the lipoplexes at low (0.06/1–1.3/1) or high (≥5/1) anionic lipid/siRNA molar charge ratios. The

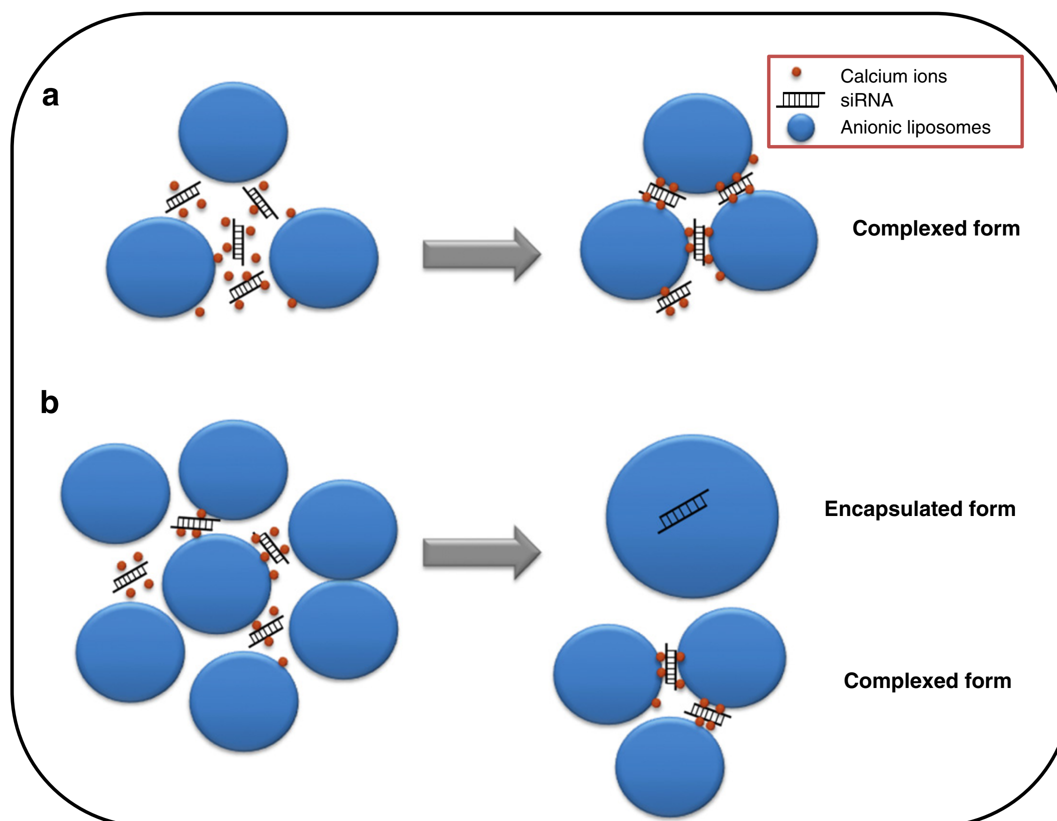

Fig. 6. Schematic representation of ternary anionic siRNA lipoplexes prepared with: (a) low anionic lipid/siRNA molar charge ratios (0.06/1 to 1.3/1) indicating the 'complexed' form; and (b) high anionic lipid/siRNA molar charge ratios (5/1 to 40/1) indicating both 'encapsulated' as well as 'complexed' forms. (fixed Ca^{2+}/siRNA molar charge ratio of 2.5/1).

details of the formation of lipoplex structures at various lipid/siRNA ratios, has been discussed previously in the same Section 4.3. Fig. 6 also shows that the optimal formulation (lipid/Ca^{2+}/siRNA molar charge ratio of 1.3/2.5/1), represented by Fig. 6a, has a slight excess of calcium which is accessible even after complex formation. Calcium is a divalent ion that electrostatically binds to the phosphate groups of the DOPG lipid and of the siRNA. However, the complexation of calcium with lipid and siRNA cannot be controlled stoichiometry. Additionally, the model suggests steric hindrance preventing one to one interaction between lipid, calcium and the siRNA. Another reason could be the requirement of excess calcium for efficient cellular uptake of lipoplexes *via* interaction with cell membrane proteoglycans.

According to Table 1, Figs. 3b and 5c, it can be observed that the efficiency of lipoplexes decreases with increase in the proportion of siRNA in the encapsulated form speculating that lipoplexes in the complexed form are more efficient that the ones in the encapsulated form. Encapsulation mainly occurred at high lipid/siRNA ratios where the size of particles was observed to be large. Low efficiency therefore could be attributed to this large size rather than their morphology. Another reason for low silencing could be reduced availability of Ca^{2+} as a result of the entrapment process. As speculated earlier, Ca^{2+} could facilitate cellular uptake and hence improve the silencing efficiency of the anionic lipoplexes. Encapsulation with excess lipids (high lipid/siRNA ratios) resulted in formation of large particles probably due to the small size of siRNA and limited scope of condensation after electrostatic interaction with other formulation components. Both the complexed and encapsulated forms are represented by Fig. 6.

5. Conclusions

Physicochemical characterization of ternary anionic lipoplexes composed of anionic liposomes, Ca^{2+} and siRNA prepared with various formulation parameters was performed. A good correlation between physicochemical characteristics and biological activity of the anionic lipoplexes was achieved that was utilized to estimate optimized formulations for efficient siRNA delivery. The physicochemical studies also helped in understanding the role of formulation components (lipids, Ca^{2+}) on the biological activity of the anionic lipoplexes. The proposed model has helped understand the contributions of formulation components towards the formation of lipid–siRNA complex assemblies and the influence of various formulation conditions on their morphology. To the best of our knowledge this is the first time that an elaborate systematic characterization study has been conducted on a three-component system for siRNA delivery. Such well-characterized delivery systems are very useful for rapid product optimization as well as product consistency and therefore facilitate economical siRNA product manufacturing on a commercial scale.

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