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Efficient and safe delivery of siRNA using anionic lipids: Formulation optimization studies

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ABSTRACT

Novel formulations based on physiologically occurring anionic lipids have been designed to achieve safe and efficient siRNA delivery. Anionic liposomes (DOPG/DOPE) were complexed with siRNA using calcium ion bridges to prepare anionic lipoplexes. Various formulation parameters (liposome composition, lipid and calcium concentration) were evaluated and optimized to achieve efficient silencing and high cell viability in breast cancer cells. The optimal anionic lipoplexes composed of 1 μg/mL lipid (40:60 (DOPG/DOPE m/m)), 2.4 mM calcium and 10 nM siRNA, showed maximum silencing (~70% knockdown) without being cytotoxic. These lipoplexes also showed stability and high efficiency in the presence of serum. Additionally, optimal anionic lipoplexes showed efficient intracellular uptake and endosomal escape. Characterization studies indicated the optimal anionic formulations were 324.2 ± 19.6 nm with a surface charge of (−22.9 ± 0.1) mV and 98.5 ± 1.4% encapsulation efficiency. Control cationic lipoplexes (Lipofectamine 2000) showed silencing comparable to the anionic lipoplexes but were highly cytotoxic as indicated by IC50 values (cationic – 22.9 μg/mL, compared to anionic – greater than 10^7 μg/mL). Calcium–siRNA complexes (without liposomes) showed low efficiency (~50% silencing), and highly variable results. The optimized anionic formulations may offer a safer alternative to conventional cationic based systems for efficient *in vitro* as well as *in vivo* delivery of therapeutic siRNAs.

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1. Introduction

The discovery of RNA interference (1998) has accelerated the development of treatments for several genetic disorders such as hyperlipoproteinemia (Soutschek et al., 2004), respiratory syncytial virus (Bumcrot et al., 2006), Huntington's disease (DiFiglia et al., 2007) and cancer (Oh and Park, 2009). Unlike DNA, RNA therapeutics do not require nuclear entry, thereby simplifying their cellular delivery. In addition, siRNA has several advantages over other nucleic acid therapeutics that are functional in the cytoplasm. For example, siRNA is more potent compared to antisense oligonucleotides. Unlike antisense oligonucleotides, a single siRNA molecule can interfere with the translation of several mRNA molecules of the same kind (Haley and Zamore, 2004). When compared to microRNA therapy, siRNA is more specific and consequently encounters lower risk for off-target effects (Alvarez et al., 2006). However, there are challenges associated with the delivery of siRNA such as, enzymatic instability, low cellular uptake and inability to escape from the endosomes. To address these issues, several delivery vectors have been utilized (Kapoor et al., 2012; Ozpolat et al., 2010; Wang et al., 2010). Non-viral vectors have been more commonly used than viral vectors since the latter can be highly immunogenic and toxic (Yang et al., 1994, 1995).

Among the non-viral vectors, cationic liposomes are by far the most common for siRNA delivery. Cationic liposomes protect siRNA against enzymatic degradation as well as facilitate cellular uptake and efficient endosomal escape leading to effective cytoplasmic delivery (Tseng et al., 2009). However due to their positive charge, cationic liposomes may undergo non-specific interaction with negatively charged cellular components (such as opsonins, serum protein and enzymes). This results in interference with the activity of ion channels, reduction in cellular adhesion (Burger et al., 1992; Litzinger and Huang, 1992), interference with mitosis, formation of vacuoles in the cytoplasm (Lappalainen et al., 1994) and hemolysis (Senior et al., 1991). Further, recent reports have indicated that systemic administration of cationic siRNA nanoparticles resulted in hepatotoxicity and significant weight loss in mice (Kedmi et al., 2010). Ikebe et al. reported that the administration of cationic lipid–siRNA nanoparticles triggered the release of pro-inflammatory cytokines (such as IL-2 and TNF-α) thereby causing increased risk of angiogenesis and cancer progression (Ikebe et al., 2009). Besides being toxic, some cationic lipoplexes have also been shown to be inactive in the presence of serum due to charge neutralization thereby adversely affecting their efficiency (Audouy and Hoekstra, 2001; Boukhnikachvili et al., 1997; Liu et al., 1997;

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Yang and Huang, 1997; Zelphati et al., 1998). Although cationic lipoplexes have been shown to be effective *in vitro* and *in vivo* (reviewed in reference, Kapoor et al., 2012), alternate delivery vectors with lesser toxicity and improved serum stability are desirable.

Anionic liposomes offer a safer alternative to cationic liposome based formulations due to the absence of positive charge. However, siRNA delivery using anionic liposomes is challenging due to electrostatic repulsion between negatively charged liposomes and anionic siRNA resulting in poor encapsulation efficiency. For example Foged et al. attempted to prepare siRNA associated anionic liposomes that showed encapsulation efficiency of only 7–9% and no activity in HeLa cells (Foged et al., 2007). Therefore, there is a need for a third agent (bridging agent) that can associate the siRNA with the anionic liposomes. In some reports, cationic polymer (Lee and Huang, 1996) or lipid (Mignet et al., 2008; Shi et al., 2002) has been utilized for the purpose. However, these are themselves toxic at effective concentrations. Thus, physiological ions such as calcium (Ca^{2+}) are relatively more desirable to prepare safe anionic lipid–siRNA complexes. For many years, calcium phosphate nanoparticles have been utilized as transfection reagents for nucleic acid delivery (Dudek et al., 2001; Kovtun et al., 2009; Kulkarni et al., 2006). Although these are effective because of the ability of calcium to promote cellular uptake and endosomal escape, they often result in irreproducible results due to the uncontrolled complexation between calcium and nucleic acids (Patil et al., 2004). Previously, formulations composed of anionic liposomes, Ca^{2+} and nucleic acid, were optimized to reduce variability in results and achieve effective delivery (Patil et al., 2004, 2005; Srinivasan and Burgess, 2009). However, these formulations were prepared and optimized specifically for DNA delivery. Although both DNA and siRNA are nucleic acids in nature, they differ significantly in their molecular properties (size, molecular weight), conformation and transfection mechanisms, consequently requiring different formulations for effective delivery of each of these molecules (Spagnou et al., 2004; Zhang et al., 2010).

In the present work, novel anionic lipoplexes were prepared for siRNA delivery using anionic liposomes (DOPG/DOPE), Ca^{2+} ions and siRNA. Different formulation parameters (liposome composition, lipid and calcium concentrations) were evaluated to optimize anionic lipoplexes for safe and efficient siRNA delivery. The optimized anionic lipoplexes showed silencing efficiency equivalent to cationic (LF2000) formulations with negligible cytotoxicity and better serum stability in a breast cancer cell line. The optimized anionic formulation was a more effective and robust formulation than the Ca^{2+}–siRNA control and showed proficient intracellular uptake and endosomal escape.

2. Materials and methods

2.1. Preparation of anionic liposomes and lipoplexes

Anionic liposomes were prepared with different DOPG/DOPE molar ratios using the film hydration method (Cullis et al., 1987). For this purpose, the lipids, DOPG (anionic) and DOPE (zwitterion) (Avanti Polar Lipids Inc., Alabama, USA) were dissolved in chloroform and dried into a thin film using nitrogen flux. This was followed by overnight vacuum-desiccation to remove any residual organic solvent. The dried lipid films were hydrated using 10 mM HEPES buffer, pH 7.4 for 1 h in 37 °C shaker water bath. Formed multilamellar vesicles were vortexed for 30 sec and extruded using a Lipex™ extruder with polycarbonate membranes of sequential sizes (400, 200, 100, and 100 nm) at 200–300 psi pressure, to form small unilamellar vesicles. The formed vesicles were filtered using 0.22 μm sterile filters, prior to cell studies.

Anionic lipoplexes were prepared with various lipid/Ca^{2+}/siRNA ratios in 10 mM HEPES buffer, pH 7.4. For the purpose, siRNA concentration was kept constant at 10 nM (unless otherwise stated) and different lipid (0.05–32 μg/mL) or calcium (0.6–153.6 mM) concentrations were utilized to obtain the desired component ratio. For lipoplexes, appropriate volume of anti-eGFP siRNA (50 μM stock) was mixed with Ca^{2+} ions (from 4.3 M $CaCl_2$ stock) for 5 min. This was followed by addition of pre-formulated anionic liposomes (5 mg/mL stock) for 10 min, to obtain anionic lipoplexes with desired lipid/Ca^{2+}/siRNA ratio. Ca^{2+}–siRNA complexes were similarly prepared except for the addition of anionic liposomes. The anionic lipid concentration is in μg/mL, and not μM, since the results were compared with LF2000 control whose molecular weight is not known. RNAiMax was not selected as a control since its lipid stock concentration is unknown).

2.2. siRNA

A combination pack of anti-eGFP siRNA (Silencer® GFP siRNA) and negative (scrambled) siRNA control, was purchased from Ambion (AM4626, Austin, TX). Dose response studies were performed with 0.1–100 nM anti-eGFP siRNA using LF2000 tranfection reagent. Accordingly, maximum knockdown was achieved at 10 nM siRNA followed by saturation. An siRNA concentration of 10 nM was therefore utilized to carry out most of the experiments. siRNA concentrations greater than 100 nM were not evaluated in order to avoid possible off-target effects.

Sequence specificity of anti eGFP-siRNA was established by comparing its silencing efficiency (dose dependent knockdown) with the negative siRNA control (no silencing at any dose investigated). For flow cytometry and confocal studies, alexa-488 labeled siRNA (gifted by Alnylam Pharmaceuticals, Cambridge, MA) was utilized.

2.3. Particle size and surface charge

Liposomes and lipoplexes were characterized for particle size and surface charge using a Malvern Zetasizer ZS90 with Zetasizer software. For particle size, percentage intensity was used. Measurements were performed in triplicate following appropriate dilution in nuclease-free water.

2.4. Encapsulation efficiency

siRNA encapsulation was determined using Quant-IT Ribogreen assay (Invitrogen) using the manufacturer's protocol. The lipoplexes were prepared in HEPES buffer as per the method described in Section 2.1. Ribogreen fluorescence was measured before and after addition of 0.5% Triton X-100 (TX-100) and 2.5 mM disodium EDTA to the lipoplexes, using Spectramax Gemini XPS spectrofluorimeter (Molecular Probes) at λ_{ex} = 490 nm, λ_{em} = 520 nm. Percentage encapsulation efficiency was calculated using the formula:

$$\%\,\text{Encapsulation efficiency} = 100 - \left(\frac{(\text{free siRNA concentration}) \times 100}{\text{initial siRNA concentration}}\right)$$

Free siRNA concentration was obtained from sample (lipoplex) fluorescence in the absence of 0.5% TX-100 and 2.5 mM disodium EDTA while initial siRNA concentration was determined from the fluorescence obtained when the lipoplexes were treated with a mixture of 0.5% TX-100 and 2.5 mM disodium EDTA. This value matched with the siRNA concentration used for sample preparation. Concentrations were obtained from the fluorescence using a

standard curve prepared with standard siRNA solution. The results were obtained from three independent experiments ($n = 3$).

2.5. Cell culture

Breast cancer cells (MDA-MB-231) stably transfected with enhanced green fluorescence protein (eGFP) (Cell Biolabs Inc., CA) were cultured in Dulbecco' modified Eagle's medium (DMEM) with 10% heat inactivated fetal bovine serum (FBS), 0.1 mM MEM non-essential amino acids (NEAA), 2 mM L-glutamine and 1% antibiotics (penicillin–streptomycin). The cells were incubated at 37 °C in 5% CO_2 atmosphere. For flow cytometry and confocal studies, breast cancer cells without eGFP (ATCC) were utilized.

2.6. Transfection studies

One day before transfection, the cells were trypsinized with 0.25% trypsin–0.53 mM EDTA (ATCC), centrifuged and resuspended in media without antibiotics. 5×10^3 cells/well in 100 μL were plated in black 96-well plates (BD Optilux, Perkin Elmer) and grown overnight. On the day of the experiment, the cells were treated with 50 μL of various anionic lipoplexes (prepared with different liposome composition, calcium and lipid concentrations in Opti-MEM I media (Invitrogen, CA, USA)). The transfected cells were incubated at 37 °C in 5% CO_2 atmosphere and assayed for silencing efficiency and cell viability after 48 h. Untreated cells, anti-eGFP siRNA alone, scrambled siRNA (negative control siRNA provided with anti-eGFP siRNA from Ambion), and lipid + siRNA mixture were used as the negative controls. The activity of anionic lipoplexes was also compared to Ca^{2+}–siRNA complexes and cationic (LF2000) lipoplexes. For experiments without serum, the procedure was the same as before with the following exception. Before transfection, the media was aspirated, cells were washed with 1× HBSS (Hank's Balanced Salt Solution) and resuspended in media without serum (no antibiotics). 4 h post-transfection, media was replaced with the original media containing serum. The cells were incubated for another 44 h (total 48 h) and assayed for silencing efficiency and cell viability. For all transfections, the concentrations of Ca^{2+} and siRNA in the controls (siRNA alone, lipid + siRNA mixture, Ca^{2+}–siRNA complexes and cationic (Lipofectamine™ 2000 lipoplexes or LF2000 lipoplexes) were the same as in case of the anionic lipoplexes. LF2000 lipoplexes were prepared with 2 μg/mL lipid concentration for 10 nM siRNA.

2.6.1. Silencing efficiency studies

The silencing efficiency of anionic lipoplexes prepared with different lipid/Ca^{2+}/siRNA ratios was evaluated. For this purpose, media was aspirated followed by washing of the cells twice with 1× HBSS buffer, 48 h post-transfection. The buffer was aspirated and replaced with 100 μL fresh 1× HBSS buffer (low background fluorescence). The readout for GFP expression was obtained in terms of relative fluorescence units (RFU) using microplate spectrofluorimeter (Spectramax Gemini XPS, Molecular Probes, with $\lambda_{ex} = 488$ nm, $\lambda_{em} = 525$ nm). The resulting fluorescence activity was normalized for the amount of protein (μg) using the BCA assay (Pierce) to obtain normalized GFP expression. Percentage silencing efficiency was obtained using the formula:

$$\%\,\text{Silencing efficiency} = 100 - \left(\frac{\text{GFP expression from sample} \times 100}{\text{GFP expression from untreated cells}}\right)$$

The results were obtained from two independent experiments performed on different days each time in triplicate ($n = 6$).

2.6.2. Cell viability studies

Cell-Titer Blue assay (Promega) was utilized to investigate the viability of the cells transfected with anionic lipoplexes prepared with different lipid/Ca^{2+}/siRNA ratios. This assay is based on the live cell's ability to reduce resazurin (non-fluorescent) to resorufin (fluorescent). 48 h post-transfection, the media was aspirated and the cells were washed twice with 1× HBSS buffer. The buffer was aspirated and an appropriate volume of Cell Titer Blue™ reagent (as per the manufacturer's protocol) diluted with Opti-MEM I media was added. The cells were then incubated for 2 h at 37 °C. The readout was obtained using microplate spectrofluorimeter (Spectramax Gemini XPS, Molecular Probes, with $\lambda_{ex} = 544$ nm, $\lambda_{em} = 590$ nm). The percentage cell viability was obtained after normalizing the data by untreated cells. IC50 values were obtained from cell viability studies using anionic lipoplexes prepared with different lipid/siRNA ratios, using GraphPad Prism version 5.0 software. The results were obtained from two independent experiments performed on different days each time in triplicate ($n = 6$).

2.7. Flow cytometry

Quantitative cellular uptake studies were performed using FACS Calibur flow cytometer (Becton-Dickinson, San Jose, CA) with Cell Quest software. MDA-MB-231 cells (without eGFP) were seeded at 0.25×10^6 cells/well. The cells were grown overnight at 37 °C in 24-well plates (Corning) in 500 μL media with 10% serum (without antibiotics). The following day, the cells were transfected with 200 μL anionic lipoplexes prepared with alexa-488 tagged siRNA, in Opti-MEM I media. 4 h post-transfection, the cells were gently washed twice with 1× HBSS buffer, trypsinized with 200 μL 0.25% trypsin–0.53 mM EDTA and diluted to 1 mL with fresh media. Finally the cell suspension was filtered into culture tubes for flow cytometry analysis. Gating was performed on forward scatter-side scatter plots (FSC-SSC) to eliminate the dead cells and cell debris. 10,000 cells were collected for each sample with each sample prepared and tested in triplicate. Data analysis was performed using Flow Jo software (version 9.0). Untreated cells, siRNA alone, lipid + siRNA mixture, Ca^{2+}–siRNA complexes and cationic (LF2000) lipoplexes, were used as controls. The results were reported as fold-uptake that was obtained by comparing with untreated cells. The samples were prepared with 40 nM siRNA.

2.8. Confocal microscopy

Andor spinning disc confocal microscope with Andor-iQ2 software was used to visualize cellular uptake and endosomal escape of siRNA (alexa-488 labeled) delivered using anionic lipoplexes and controls. The cells were incubated overnight (in cell culture media with serum but no antibiotics) at 0.1×10^6 cells/well in 8-well chamber slide (Lab-Tek II, Thermo Scientific). The following day, cells were transfected with anionic lipoplexes and controls in Opti-MEM I media for 4 h at 37 °C. The cells were then washed twice with 1× HBSS buffer followed by media replacement with Opti-MEM I containing organelle staining dyes. DRAQ5 (Biostatus, UK), a far-red dye, was used to counterstain the nucleus at 5 μM concentration. For time-lapse studies, Hoescht 33342 (Invitrogen) was used to stain the nucleus (5 μg/mL) and lysotracker red DND-99 (Invitrogen) was used for lysosomes (50 nM). Confocal images were obtained with a 40× oil objective using 405 nm, 488 nm, 561 nm and 640 nm excitation lasers for Hoescht 33342, alexa-488, lysotracker red DND-99 and DRAQ5, respectively. During imaging, the cells were maintained at 37 °C, 5% CO_2 and 75% relative humidity atmosphere. The transmitted image was obtained using DIC (differential interference contrast). Five fields of view were selected for each sample and Z-sectioning was performed for each of these fields ($n = 5$). Image quantification was performed using CIDIQ

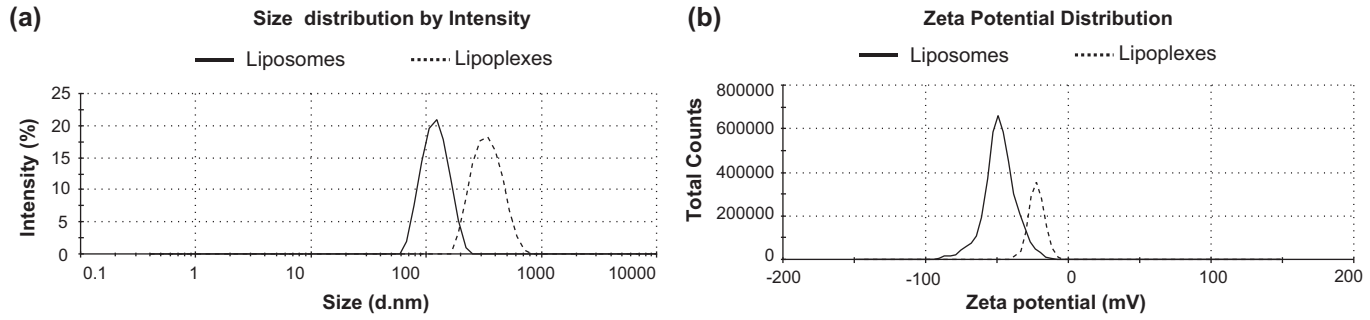

Fig. 1. (a) Particle size, and (b) surface charge of anionic liposomes (solid line) and anionic lipoplexes (dotted line), determined using the Malvern Zetasizer. These anionic lipoplexes are composed of 40:60 DOPG/DOPE, 1 μg/mL lipid, 2.4 mM Ca^{2+} and 10 nM siRNA. The samples were prepared in 10 mM HEPES buffer, pH 7.4 and the measurements were taken at room temperature. Mean ± SD (n = 3).

(confocal image-assisted three-dimensionally integrated quantification) method with slight modifications (Akita et al., 2004, 2010). Using the modified method (see Supplementary data 1), average punctate to diffused staining ratio (P/D ratio) was obtained for each sample. One of the five confocal images was used to represent each sample. Untreated cells, siRNA alone, lipid + siRNA mixture, Ca^{2+}–siRNA complexes and cationic (LF2000) lipoplexes, were used as controls. For confocal studies, the samples were prepared with 14.2 nM siRNA.

2.9. Serum stability studies

The physical stability of lipoplexes in the presence of serum was evaluated using Quant-IT Ribogreen fluorescence assay. Anionic lipoplexes prepared in HEPES buffer, as described previously, were incubated in DMEM (no phenol red) + 10% FBS, for various time points (0, 1, 2, 3, and 4 h) at 37 °C. After each time point, an aliquot of lipoplexes (in DMEM + 10% FBS) was transferred to black 96-well plates and diluted with 1× TE (Tris–EDTA, pH 7.8) buffer. The dissociated (uncomplexed) siRNA was estimated at each time point, using Ribogreen fluorescence assay. Concentrations were obtained from fluorescence using a standard curve prepared with standard siRNA solution. Percentage dissociated siRNA was calculated by considering the initial siRNA concentration as 100%. Ca^{2+}–siRNA complexes, lipid + siRNA mixture, siRNA alone and LF2000 lipoplexes (2 μg/mL lipid) were used as controls. The samples were prepared with 10 nM siRNA and evaluated in triplicate in two independent experiments (n = 6).

2.10. Statistics

For comparison of several groups, one-way ANOVA with Dunnett's post-test was performed using GraphPad Prism version 5.0 software. The values are reported as mean ± SD. For comparison of two samples at a time, the Student t-test was used.

3. Results

3.1. Formulation characterization

Liposomes prepared using the film hydration followed by extrusion had a mean particle size of 111.2 ± 2.0 nm (PDI < 0.1). However, after complexation with Ca^{2+} and siRNA, the size of the resulting lipoplexes (40:60 (DOPG/DOPE), 1 μg/mL lipid/2.4 mM Ca^{2+}/10 nM siRNA) was 324.2 ± 19.6 nm with a unimodel distribution (PDI 0.07 ± 0.02) (Fig. 1a). This mean size was almost three times to that of the liposomes and is due to the electrostatic interaction between the three components (anionic liposomes, Ca^{2+} and siRNA). Further increase in the calcium concentration (Ca^{2+} > 4.8 mM) increased the mean particle size to 389.7 ± 17.4 nm. High lipid concentrations (4–32 μg/mL) also resulted in the formation of larger particles with mean size of 806.3 ± 75.5 nm.

Addition of Ca^{2+} (2.4 mM) and siRNA (10 nM) to liposomes (1 μg/mL) significantly reduced the charge from (−47.7 ± 0.9) mV for the liposomes, to (−22.9 ± 0.1) mV for the anionic lipoplexes. This may be due to partial neutralization of anionic lipid and siRNA by Ca^{2+} ions (Fig. 1b). With increase in lipid concentration (lipid ≥4 μg/mL), the surface charge of the lipoplexes increased to (−30) mV due to increase in the anionic lipid concentration. Conversely, higher Ca^{2+} (Ca^{2+} > 2.4 mM) reduced the charge, reaching near-neutral values at 76.8 mM concentration.

The encapsulation efficiency of anionic lipoplexes prepared with 1 μg/mL lipid and 10 nM siRNA, increased with increase in Ca^{2+} from less than 10% at 0.6 mM to 82.9 ± 3.0% at 0.9 mM and 98.5 ± 1.4% at 2.4 mM. No encapsulation was observed in the absence of Ca^{2+} at this lipid concentration.

3.2. Silencing efficiency studies

3.2.1. Effect of liposome composition on silencing efficiency

Anionic lipoplexes prepared with different DOPG/DOPE molar ratios showed (Fig. 2a) an increase in silencing efficiency with increase in DOPG content from 10 mol% (10:90 DOPG/DOPE ratio) to 40 mol% (40:60 ratio) (2 μg/mL lipid, 2.4–3.3 mM Ca^{2+}, 10 nM siRNA). This increase was significant for lipoplexes prepared with liposomes composed of a 20:80 DOPG/DOPE molar ratio, and highly significant for liposomes composed of 40:60 molar ratio, when compared to liposomes composed of 10:90 molar ratio. Further increase in DOPG content from 40 to 80 mol% (40:60 to 80:20 molar ratio of DOPG/DOPE) did not improve silencing efficiency at all calcium concentrations investigated, indicating that saturation has been reached. Since maximum silencing was obtained at a 40:60 molar ratio (DOPG/DOPE), this was considered as optimal.

3.2.2. Effect of calcium concentration on silencing efficiency

When anionic lipoplexes (40:60 DOPG/DOPE, 1 μg/mL lipid, 10 nM siRNA) prepared with different Ca^{2+} concentrations were evaluated, silencing efficiency increased with increase in calcium concentration from 27.7 ± 16.7% at 0.6 mM to 71.7 ± 3.9% at 2.4 mM Ca^{2+} (Fig. 2b). Further increase in calcium (Ca^{2+} > 2.4 mM) reduced the silencing efficiency. Turbidity was also observed in the anionic formulations with high Ca^{2+} levels (Ca^{2+} ≥ 4.8 mM). From the results, 2.4 mM Ca^{2+} indicated maximum silencing and therefore was considered as the optimal Ca^{2+} concentration.

Ca^{2+}–siRNA complexes also showed increase in silencing efficiency with increase in calcium concentration from 0.6 mM to 2.4 mM. At 4.8 mM Ca^{2+} however, Ca^{2+}–siRNA complexes showed a significant improvement in silencing efficiency (62.5 ± 15.7%)

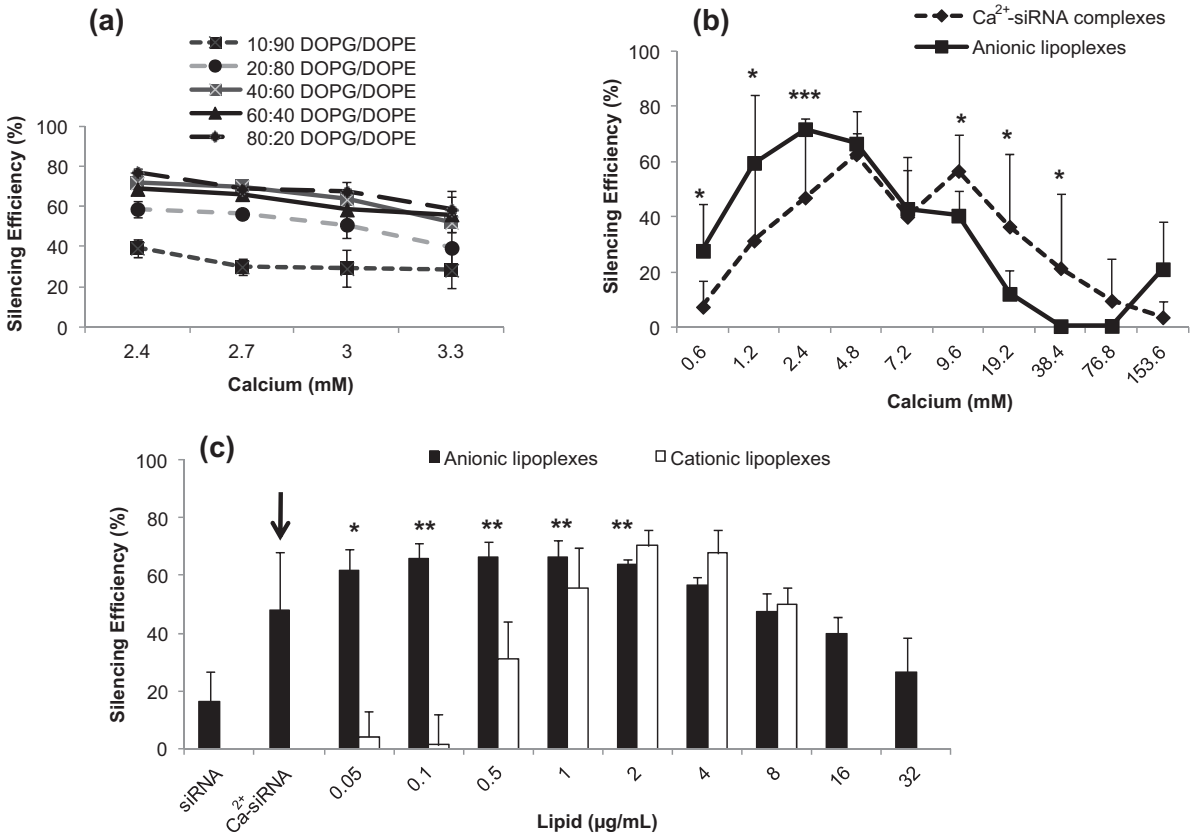

Fig. 2. Effect of formulation parameters: (a) liposome composition (DOPG/DOPE m/m) ($n = 3$), (b) calcium concentration ($n = 6$), and (c) lipid concentration ($n = 6$) on silencing efficiency of anionic lipoplexes in breast cancer cell line model. siRNA alone, Ca^{2+}–siRNA complexes and cationic (LF2000) lipoplexes were used as controls. Mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. p values were obtained by comparing anionic lipoplexes to Ca^{2+}–siRNA complexes. Arrow indicates the control group used for comparison. Mean ± SD.

followed by a reduction at higher calcium concentrations ($Ca^{2+} > 4.8$ mM).

Anionic lipoplexes were significantly more effective compared to Ca^{2+}–siRNA complexes at $Ca^{2+} \leq 2.4$ mM. On the contrary, Ca^{2+}–siRNA complexes showed higher efficiency at $Ca^{2+} > 4.8$ mM. However, the media was extremely turbid due to a large amount of precipitation at $Ca^{2+} \geq 4.8$ mM. There was also considerable variability in the data with Ca^{2+}–siRNA complexes at all Ca^{2+} concentrations investigated.

3.2.3. Effect of lipid concentration on silencing efficiency

Anionic lipoplexes (40:60 DOPG/DOPE, 2.4 mM Ca^{2+}, 10 nM siRNA) prepared with different lipid concentrations (0.05–32 μg/mL) showed significantly higher efficiency of anionic lipoplexes compared to Ca^{2+}–siRNA complexes even at a lipid concentration of 0.05 μg/mL (Fig. 2c). With further increase in lipid concentration, silencing efficiency increased reaching a maximum at 1 μg/mL. Any further increase in lipid concentration reduced the silencing efficiency. Although there was no significant difference in efficiency from 0.1 and 1 μg/mL lipid, highly unstable lipoplexes were obtained at lipid concentrations less than 1 μg/mL. Accordingly, 1 μg/mL lipid concentration was considered to be optimal.

Cationic (LF2000) lipoplexes showed negligible activity until a lipid concentration of 0.5 μg/mL was reached (Fig. 2c). Further increase in lipid concentration significantly improved silencing efficiency reaching a maximum (70.2 ± 5.9%) at 2 μg/mL. Higher lipid concentrations reduced the silencing efficiency to 50 ± 5.8% at 8 μg/mL. Further increase in concentration caused severe cytotoxicity leading to termination of the experiments. Since the maximum efficiency with LF2000 lipoplexes was achieved at 2 μg/mL (for 10 nM siRNA), LF2000 was used at this concentration in all the following experiments.

Comparing the anionic and cationic liposomes, the former were more potent as evident from their higher efficiency at low lipid concentrations (lipid concentration <0.5 μg/mL) (Fig. 2c). However, equivalent efficiency of anionic lipoplexes was achieved with cationic lipoplexes at 2 μg/mL cationic lipid concentration.

From the above results, anionic lipoplexes composed of 1 μg/mL lipid (40:60 DOPG/DOPE), 2.4 mM Ca^{2+} and 10 nM siRNA showed the maximum efficiency (~70%) and therefore were considered to be the optimal formulation. The silencing efficiency of these lipoplexes in comparison with other siRNA delivery systems is shown in Fig. 3. As expected, anti-eGFP siRNA alone, scrambled siRNA, lipid + siRNA mixture and scrambled siRNA lipoplexes showed negligible GFP knockdown. However, the anionic lipoplexes showed significantly high silencing efficiency and less variability when compared to Ca^{2+}–siRNA complexes. The silencing efficiency of the anionic lipoplexes was comparable to cationic (LF2000) lipoplexes (2 μg/mL lipid).

3.2.4. Effect of serum on silencing efficiency

Various anionic lipoplexes (40:60 DOPG/DOPE, prepared with different lipid/Ca^{2+}/siRNA ratios) were transfected in the presence and absence of serum. As shown in Fig. 4a, anionic lipoplexes (1 μg/mL lipid) prepared with different calcium concentrations were significantly more effective in serum compared to non-serum conditions. Similar results were obtained for anionic lipoplexes (2.4 mM Ca^{2+}) prepared with different lipid concentrations (Fig. 4b). However at very low and very high calcium (0.6 mM and 9.6 mM,

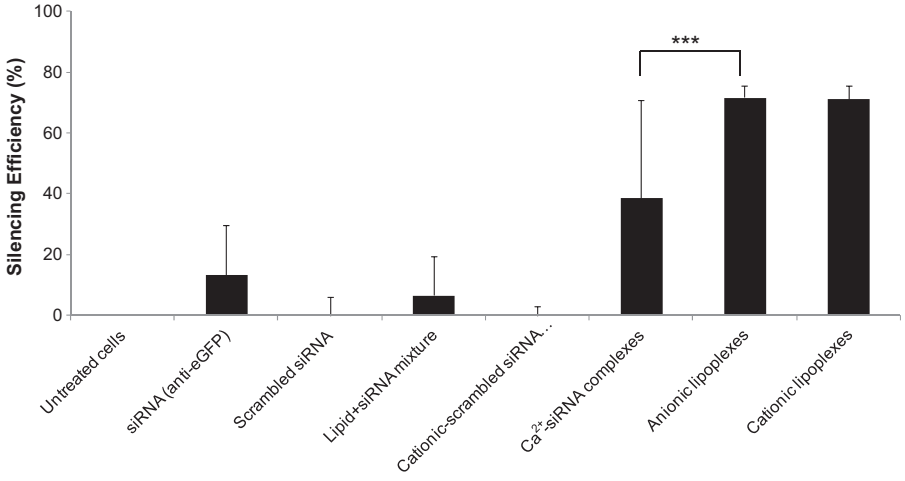

Fig. 3. Percentage silencing efficiency of various siRNA delivery systems in breast cancer cells, 48 h post-transfection. Anionic lipoplexes were composed of 40:60 DOPG/DOPE, 1 μg/mL lipid, 2.4 mM Ca^{2+} and 10 nM siRNA. siRNA (anti-eGFP) alone, scrambled siRNA, lipid + siRNA mixture, cationic scrambled siRNA lipoplexes, Ca^{2+}–siRNA complexes and cationic (LF2000) lipoplexes were used as controls. ***$p < 0.001$. Results were obtained from two independent experiments in triplicate ($n = 6$). Mean ± SD.

respectively) or lipid (0.125 μg/mL and 16 μg/mL, respectively) concentrations, serum did not have any effect on the silencing efficiency (Fig. 4a and b).

Contrary results were obtained for Ca^{2+}–siRNA complexes that showed lower silencing in the presence of serum than in the absence (Fig. 4c). This effect was significant only at low Ca^{2+} levels (0.6–1.2 mM). However, at higher Ca^{2+} concentrations serum did not have any effect on the activity of Ca^{2+}–siRNA complexes. Silencing efficiency of cationic (LF2000) lipoplexes was unaffected by serum (Fig. 4d). These results are in agreement with other published reports (Dalby et al., 2004).

3.3. Cell viability studies

3.3.1. Effect of lipid concentration on cell viability

The viability of breast cancer cells treated with anionic lipoplexes (40:60 DOPG/DOPE, 2.4 mM Ca^{2+}) prepared with different lipid concentrations (0.05–32 μg/mL) was evaluated using the Cell-Titer Blue assay. As shown in Fig. 5a, anionic lipoplexes showed 100% cell viability, throughout the lipid range investigated. On the contrary, LF2000 lipoplexes showed reduced cell viability with increase in lipid concentration. Cytotoxicity was significant at lipid concentrations >4 μg/mL. Cationic (LF2000) lipids had an

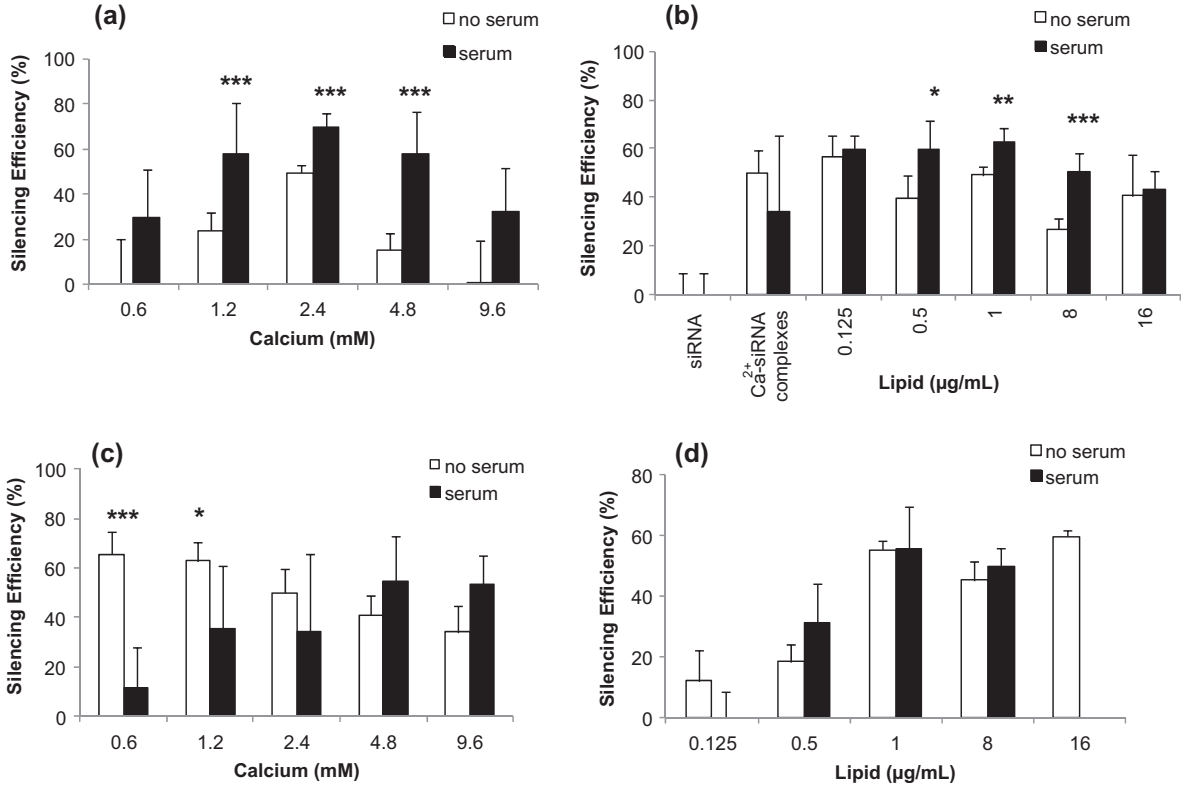

Fig. 4. Effect of serum on silencing efficiency of: (a) anionic lipoplexes prepared with various Ca^{2+} concentrations; (b) anionic lipoplexes prepared with various lipid concentrations; (c) Ca^{2+}–siRNA complexes prepared with different Ca^{2+} concentrations; and (d) cationic (LF2000) lipoplexes prepared at different lipid concentrations. *$p < 0.05$, **$p < 0.01$, the ***$p < 0.001$. p values were obtained from the Student t-test on comparing non-serum conditions to serum conditions. Results were obtained from two independent experiments in triplicate ($n = 6$). Mean ± SD.

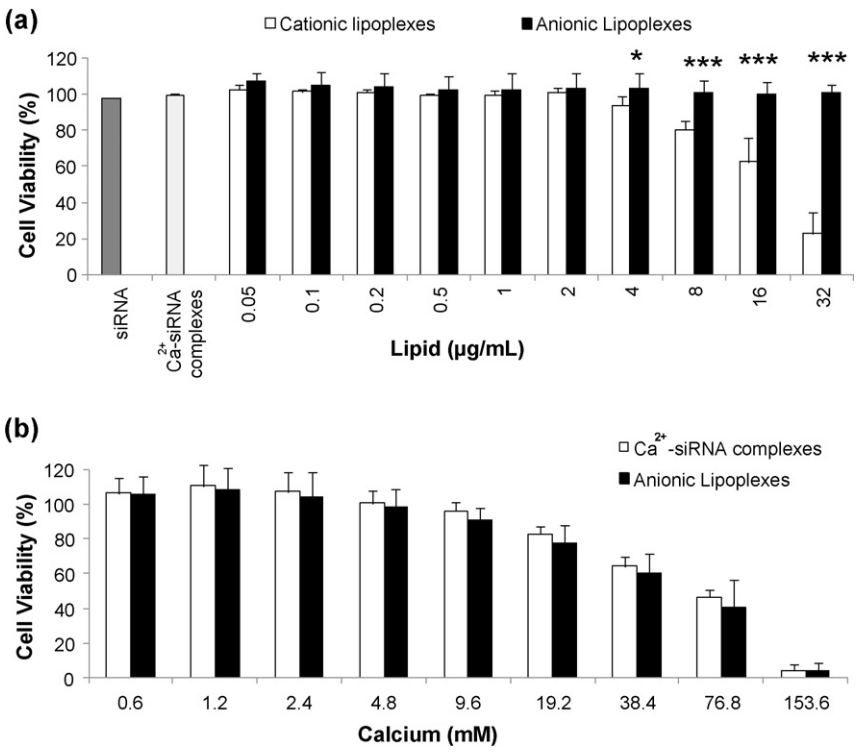

Fig. 5. (a) Cell viability studies of cationic (LF2000) and anionic lipoplexes (40:60 DOPG/DOPE, 2.4 mM Ca^{2+}, 10 nM siRNA) 48 h post-transfection in breast cancer cell line. The data have been normalized to untreated cells which were considered 100% viable. LF2000 showed significantly lower cell viability at lipid concentrations ≥4 μg/mL. $^{*}p < 0.05$ and $^{***}p < 0.001$. p values were obtained by applying the Student t-test between the two formulations at each lipid concentration. (b) Effect of calcium concentration on the cell viability of breast cancer cells treated with anionic lipoplexes (1 μg/mL lipid, 10 nM siRNA) and Ca^{2+}–siRNA complexes. Results were obtained from two independent experiments in triplicate ($n = 6$). Mean ± SD.

IC50 value (for 50% cell viability) of 22.9 μg/mL while for anionic lipids this value was in the order of 10^7 μg/mL. siRNA alone and Ca^{2+}–siRNA complexes (2.4 mM Ca^{2+}) showed no cytotoxicity.

3.3.2. *Effect of calcium concentration on cell viability*

Anionic lipoplexes (1 μg/mL) prepared with various calcium concentrations (0.6–153.6 mM) showed 100% cell viability up to 4.8 mM Ca^{2+} (Fig. 5b). Further increase in Ca^{2+} reduced the cell viability from 90.7% at 9.6 mM to 3.8% at 153.6 mM indicating cytotoxicity at high Ca^{2+} levels. Similarly, cytotoxicity was observed with Ca^{2+}–siRNA complexes at $Ca^{2+} > 4.8$ mM.

3.4. *Flow cytometry*

Intracellular uptake of optimized anionic lipoplexes was quantitatively studied using flow cytometry. Fig. 6a is a representative FSC-SSC plot for untreated cells. Fig. 6b shows a representative histogram of anionic lipoplexes (black) along with that of the untreated cells (gray). The quantified results (Fig. 6c) indicated an 11-fold uptake of anionic lipoplexes (40:60 DOPG/DOPE, 1 μg/mL lipid, 2.4 mM Ca^{2+} for 10 nM siRNA) compared to the untreated cells. siRNA alone and the lipid + siRNA mixture showed no uptake while Ca^{2+}–siRNA complexes and cationic (LF2000) lipoplexes showed uptake comparable to anionic lipoplexes.

3.5. *Confocal microscopy*

Intracellular uptake of anionic lipoplexes and controls was visualized using confocal microscopy. For this purpose, lipoplexes were prepared using alexa-488 siRNA (40:60 DOPG/DOPE, 1 μg/mL lipid, 2.4 mM Ca^{2+} for 10 nM siRNA) and the nucleus was counterstained using a far-red dye (DRAQ5). 4 h post-transfection, there was efficient siRNA uptake from anionic lipoplexes, Ca^{2+}–siRNA and cationic (LF2000) complexes (Fig. 7A). The P/D ratios for the Ca^{2+}–siRNA complexes, the anionic lipoplexes, and the cationic lipoplexes were 0.41, 0.18 and 0.02, respectively. Lower P/D ratios signify lesser punctate staining (compared to diffused) indicating better endosomal escape. Therefore, it appears that the endosomal escape of the cationic lipoplexes was the most efficient followed by the anionic lipoplexes, while the Ca^{2+}–siRNA complexes showed the least effective endosomal release of the siRNA.

To visualize the time dependent uptake process, time-lapse studies of anionic lipoplexes (with alexa-488 siRNA) were performed for up to 4 h in breast cancer cell line incubated at 37 °C. For this purpose, the nucleus was stained with Hoescht 33342 (blue) and the lysosomes were stained with lysotracker red. As seen in Fig. 7B, anionic lipoplexes were shown to be taken up slowly by the cells over the 4 h time period. Additionally, diffused staining was shown to increase with time demonstrating efficient endosomal escape facilitated by the anionic lipoplexes. There was also some co-localization observed with lysosomes indicating endocytosis to be a probable mechanism of internalization.

3.6. *Serum stability studies*

Serum can potentially affect the stability of lipoplex formulations due to electrostatic interaction between the charged lipids and the serum components, leading to siRNA dissociation (Audouy and Hoekstra, 2001; Boukhnikachvili et al., 1997; Liu et al., 1997; Yang and Huang, 1997; Zelphati et al., 1998). This can result in reduced silencing efficiency. With this perspective, anionic lipoplexes (40:60 DOPG/DOPE, 1 μg/mL lipid/2.4 mM Ca^{2+}/10 nM siRNA) were evaluated for stability in serum containing media for 4 h at 37 °C. At time zero, the entire siRNA (almost 100%) was encapsulated within both the anionic and cationic (LF2000) lipoplexes as indicated by the absence of any free siRNA. Conversely, without

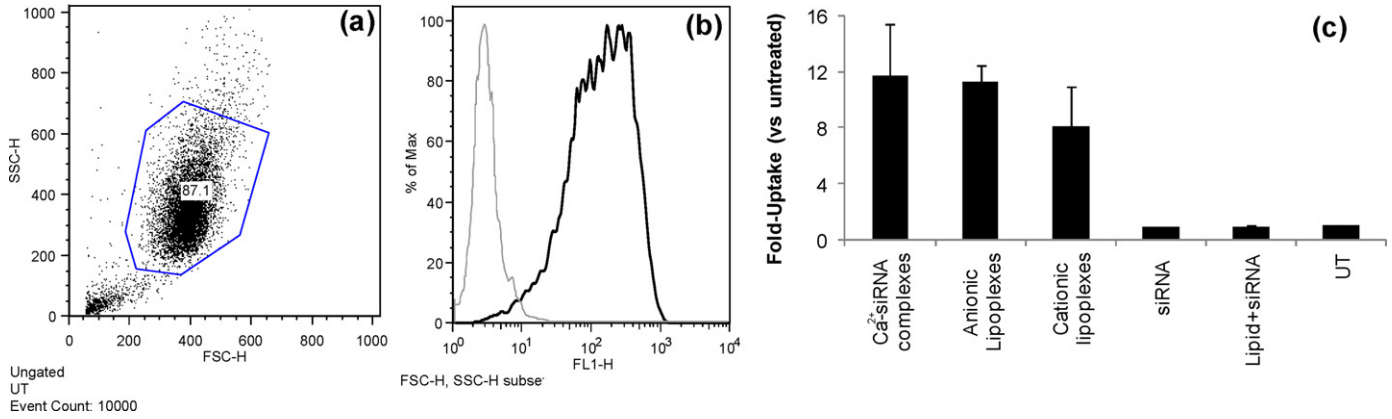

Fig. 6. (a) Typical FSC-SSC plot for untreated breast cancer cells with gating (blue) used for data analysis. (b) Histogram representing uptake of alexa-488 labeled siRNA from anionic lipoplexes (black line) and untreated cells (gray line). (c) Quantified uptake of siRNA from various delivery systems. siRNA alone, lipid + siRNA mixture, Ca^{2+}–siRNA complexes and cationic (LF2000) lipoplexes were used as controls. For these studies anionic lipoplexes were prepared with 4 μg/mL lipid/9.6 mM Ca^{2+}/40 nM siRNA. Controls were prepared using 40 nM siRNA. Mean ± SD ($n = 3$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

calcium (lipid + siRNA mixture), no encapsulation was observed. Anionic lipoplexes (1 μg/mL lipid/2.4 mM Ca^{2+}/10 nM siRNA) did not show any dissociation during the 4 h time period, indicating that these are stable in the presence of serum (Fig. 8). Conversely, LF2000 lipoplexes showed around 15–20% dissociation during the 4 h time period. Therefore, the anionic lipoplexes were more stable in serum compared to the cationic lipoplexes. Interestingly the Ca^{2+}–siRNA complexes (2.4 mM Ca^{2+}) were also stable in serum, indicating the role of calcium in providing stability against serum triggered siRNA dissociation.

4. Discussion

Anionic lipoplexes (DOPG/DOPE, Ca^{2+}, siRNA) were prepared and optimized to facilitate effective siRNA delivery and also ensure high cell viability. Formulation optimization studies revealed that anionic formulations composed of 40:60 DOPG/DOPE, 1 μg/mL lipid, 2.4 mM Ca^{2+} and 10 nM siRNA showed maximum (~70%) protein knockdown without any cytotoxicity in a breast cancer cell line model. It was observed that various formulation parameters such as liposome composition, lipid and calcium concentration affected the silencing efficiency and cell viability.

The optimized formulation represents the best charge balance between the three components – anionic lipid, Ca^{2+} and siRNA with a slight excess of calcium to facilitate interaction between the lipoplexes and the cellular membrane.

The increase in the silencing efficiency with increase in DOPG content compared to DOPE content (up to 40 mol% DOPG) at a fixed lipid/Ca^{2+}/siRNA ratio (Fig. 2a), is considered to be due to efficient lipoplex formation facilitated by sufficient DOPG to interact with the siRNA *via* the Ca^{2+} ions. At DOPG/DOPE ratios greater than 40 mol% DOPG, there was no further change in silencing efficiency as the concentration of DOPG required to interact with the siRNA *via* the Ca^{2+} was saturated.

The total lipid concentration significantly affected the silencing efficiency of the ternary anionic lipoplexes. Even low concentrations of lipid (0.05 μg/mL) improved the silencing efficiency significantly compared to the control Ca^{2+}–siRNA complexes (Fig. 2c). This is considered to be due to improved endosomal escape facilitated by the DOPE in the lipoplexes. DOPE undergoes phase transition from lamellar (Lα) to inverted hexagonal phase (H_{II}) at low endosomal pH. This results in fusion of the DOPE tails with the endosomal lipids thereby destabilizing the endosomal membrane (Cullis and De Kruijff, 1979; Farhood et al., 1995; Hafez and Cullis, 2001; Hafez et al., 2001; Koltover et al., 1998; Srinivasan and Burgess, 2009; Wong et al., 2003). Increase in lipid concentration from 0.05 μg/mL to 1 μg/mL did not significantly increase the silencing efficiency. It speculated that 0.05 μg/mL of lipid provides sufficient DOPE to facilitate endosomal destabilization. However, at lipid concentrations below 1 μg/mL (with 2.4 mM Ca^{2+}, 10 nM siRNA), the lipoplexes were unstable. This is probably due to insufficient DOPG to stabilize the lipoplexes through DOPG–Ca^{2+}–siRNA bridging. Accordingly, 1 μg/mL lipid concentration was considered as optimal.

Similarly an optimal calcium concentration was determined, where the silencing efficiency was maximum (Fig. 2b). This concentration achieved optimal complexation between the siRNA and the liposomes. The divalent Ca^{2+} ion increases the amount of siRNA complexed to the liposomes and therefore, the amount of siRNA delivered by lipoplexes is increased enhancing the gene knockdown effect.

Ca^{2+}–siRNA complexes were employed as a negative control since it is known that complexation of DNA with calcium can facilitate intracellular delivery of DNA (Kulkarni et al., 2006). However, uncontrolled complexation of the DNA with calcium results in inconsistent data as a consequence of aggregation. Similar results were obtained in the present study using Ca^{2+}–siRNA complexes. Additionally, these complexes have lower silencing efficiency in the breast cancer cell line, compared to the anionic lipoplexes. Silencing efficiency is determined by two rate-limiting steps – cellular uptake and endosomal escape. Since the cellular uptake of Ca^{2+}–siRNA complexes was equivalent to that of anionic lipoplexes (Fig. 6c), the increased silencing efficiency achieved with anionic lipoplexes is a result of more efficient endosomal escape. This is supported by the increased diffused compared to punctuate staining (low P/D ratio), observed with the anionic lipoplexes compared to the Ca^{2+}–siRNA complexes (Fig. 7A and B). The effective endosomal escape achieved by the anionic lipoplexes was probably due to presence of DOPE in these formulations.

There was a dramatic decrease in silencing efficiency of the anionic lipoplexes and the Ca^{2+}–siRNA complexes at high Ca^{2+} concentrations ($Ca^{2+} > 4.8$ mM) (Fig. 2b). This is considered to be a result of a significant increase in particle size at high Ca^{2+} levels. Increase in particle size at high calcium concentration is probably due to interaction of excess calcium (not neutralized by lipoplexes) with anions or serum proteins present in high ionic strength cell culture media, thereby forming large aggregates responsible for media turbidity. As has been reported in the literature, silencing efficiency of large particles is low as a consequence of reduced cellular uptake (Igarashi et al., 2006; Patil et al., 2004; Scarzello et al., 2005;

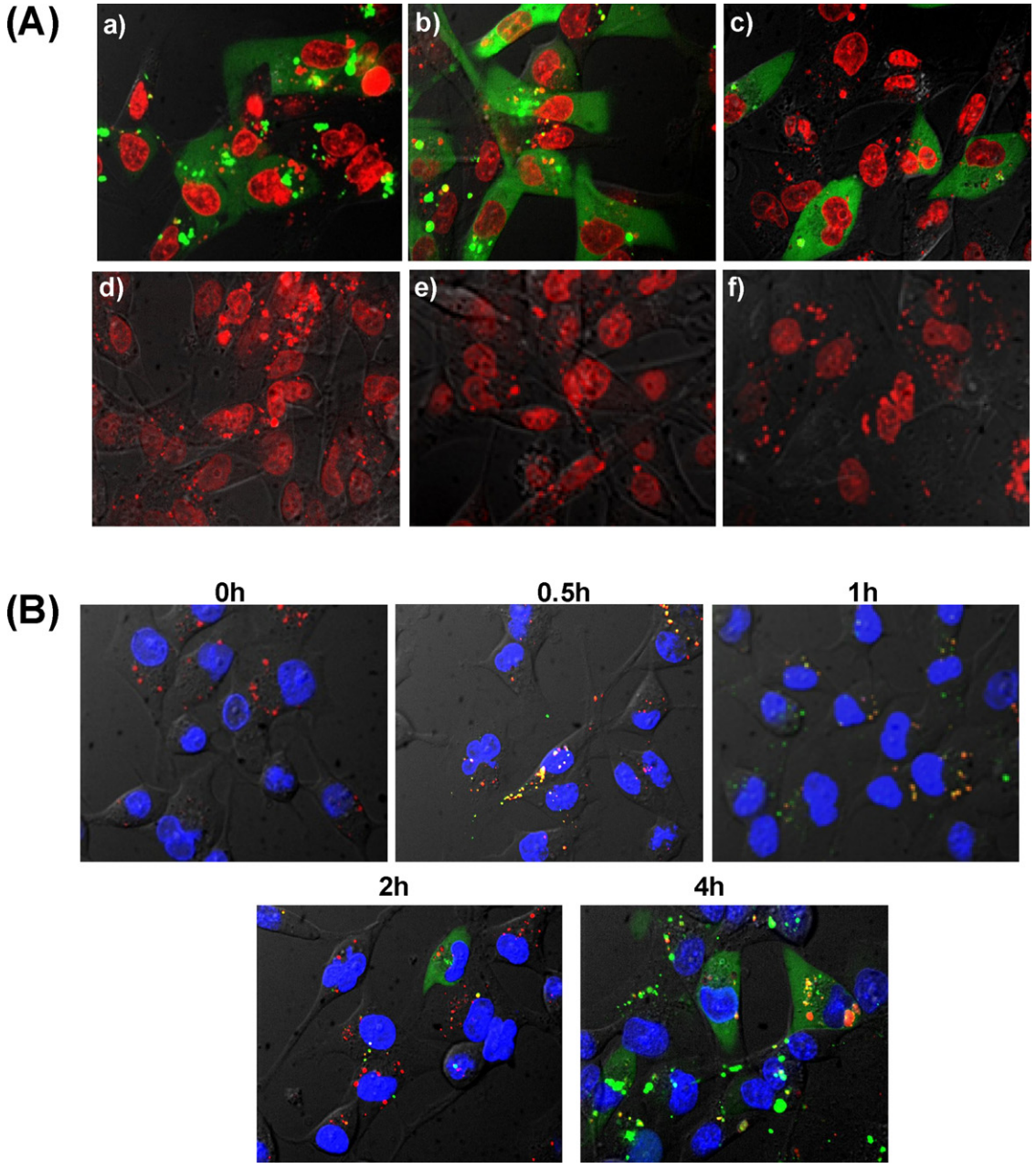

Fig. 7. (A) Confocal images for uptake of various delivery systems prepared with alexa-488 siRNA (green), 4 h post transfection in breast cancer cells. (a) Ca^{2+}–siRNA complexes, (b) anionic lipoplexes, (c) cationic (LF2000) lipoplexes, (d) siRNA only, (e) lipid + siRNA mixture, and (f) untreated cells. The cells were counterstained with DRAQ5 (red), DNA permeant dye staining nucleus (also stains mitochondria – red punctates). Overlay images of green, red and transmitted channels are shown. (B) Time-lapse studies for uptake of anionic lipoplexes over a 4 h time period. Cells were stained with Hoescht 33342 (blue) for nucleus and lysotracker red DND-99 for lysosomes. Overlay images of blue, green, red and transmitted light channels are shown. For confocal studies, anionic lipoplexes were prepared with 1.42 μg/mL lipid, 3.4 mM Ca^{2+} and 14.2 nM siRNA. Controls were prepared using 14.2 nM siRNA ($n = 5$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Wasungu and Hoekstra, 2006). For the same reason, the anionic lipoplexes prepared with high lipid concentration also showed low silencing efficiency (Fig. 2c).

Compared to anionic lipoplexes, the control cationic lipoplexes showed low silencing efficiency at lipid concentrations less than 1 μg/mL. This may be due to reduced cellular uptake and/or less efficient endosomal escape at low charge ratios (cationic lipid/siRNA). As the lipid concentration was increased from 1 to 2 μg/mL, the silencing efficiency increased. It has been reported that cationic lipoplexes (Lipofectamine–siRNA complexes) are taken up by the cells *via* interaction between the cationic lipids and cell membrane proteoglycans (anionic) (Payne et al., 2007). Once these lipoplexes are inside the cells, endosomal release occurs by fusion of anionic endosomal membrane lipids with cationic lipids causing destabilization of the endosome (Hoekstra et al., 2007; Ouahabi et al., 1999; Xu and Szoka, 1996). Increase in lipid concentration to greater than 4 μg/mL, reduced the silencing efficiency of the cationic lipids probably due to increased cytotoxicity (Figs. 2c and 5a). At 16 μg/mL the majority of the cells died due to the high concentration of cationic lipid. The cationic lipids interact with negatively charged cell components, interfere with cell adhesion (Burger et al., 1992; Litzinger and Huang, 1992) as well as cell division and consequently cause cell death (Lappalainen et al., 1994; Senior et al., 1991). This is also supported by the low IC50 values for the cationic lipids (22.9 μg/mL) compared to the anionic lipids (greater than 10^7 μg/mL). Anionic lipids (such as the DOPG) are physiological

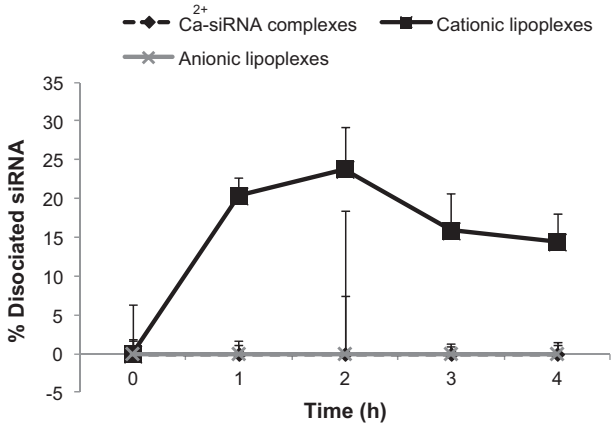

Fig. 8. Serum stability studies of various siRNA delivery systems evaluated for 4 h in DMEM + 10% FBS at 37 °C in incubator. Ca^{2+}–siRNA complexes and cationic (LF2000) lipoplexes were used as controls. Percentage dissociation was obtained by considering initial siRNA concentration (used for complex preparation) as 100%. Results were obtained from two independent experiments in triplicate.($n = 6$). Mean ± SD.

safe lipids that do not obstruct the aforementioned cellular mechanisms.

For anionic lipoplexes prepared with different Ca^{2+} concentrations, no cytotoxicity was observed until 4.8 mM Ca^{2+} indicating that these lipoplexes were safe at the optimal Ca^{2+} concentration (2.4 mM) (Fig. 5b). However, higher Ca^{2+} levels resulted in cell death due to the presence of excess Ca^{2+}. This was also observed with the control Ca^{2+}–siRNA complexes at high Ca^{2+} concentrations. Calcium is known to cause bilayer redistribution that may be responsible for membrane destabilization (Dekkers et al., 2002; Duzgunes et al., 1981; Papahadjopoulos et al., 1978).

Cationic lipoplexes are known to have poor serum stability leading to reduced activity (Audouy and Hoekstra, 2001; Boukhnikachvili et al., 1997; Liu et al., 1997; Yang and Huang, 1997; Zelphati et al., 1998). This is due to charge neutralization by anionic serum proteins and this phenomenon is often responsible for dissociation of siRNA from cationic lipoplexes. For this reason cationic (LF2000) lipoplexes (2 μg/mL lipid) were unstable in the presence of serum. The dissociated siRNA was 20% in the first hour and reduced to 15% after 2 h (Fig. 8). This reduction in dissociated siRNA was probably due to degradation of siRNA in serum or a result of electrostatic interaction of dissociated siRNA with other cationic species in the media. Interestingly, this serum instability of the cationic lipoplexes did not affect their silencing efficiency (Fig. 2c). This was probably due to highly efficient endosomal escape as indicated by their low P/D ratio (0.02) compared to the anionic lipoplexes (P/D ratio 0.18) (Fig. 7A).

Although cationic in nature, Ca^{2+}–siRNA complexes (2.4 mM Ca^{2+}) showed no siRNA dissociation in the presence of serum during the 4 h study (Fig. 8). In addition, serum did not affect the silencing efficiency of the Ca^{2+}–siRNA complexes at 2.4 mM or higher Ca^{2+} concentrations (Fig. 2b). A plausible explanation could be the presence of sufficient Ca^{2+} ions required to bind with siRNA in the presence of other competing species such as the anionic serum proteins. This is supported by the fact that the Ca^{2+}–siRNA complexes were less efficient in the presence of serum, at lower Ca^{2+} concentrations (Fig. 4c). Anionic lipoplexes were not only stable in serum (due to the presence of sufficient calcium at 2.4 mM Ca^{2+}) (Fig. 4a), but also showed more efficient silencing (Fig. 4b) probably due to favorable interactions with serum components.

5. Conclusions

Anionic lipid-based siRNA lipoplexes (anionic liposomes, Ca^{2+} ions and siRNA) were prepared and optimized to achieve high silencing efficiency with negligible cytotoxicity in a breast cancer cell culture model. The silencing efficiency of the optimized anionic lipoplex formulation was similar to that of the control cationic lipoplexes (Lipofectamine 2000), yet the anionic lipoplex formulation was relatively several-fold safer. In addition, the anionic lipoplexes, in contrast to the cationic lipoplexes, were highly stable in the presence of serum. In comparison to control Ca^{2+}–siRNA complexes, the optimized anionic lipoplexes showed advantages of high efficiency with effective endosomal escape and reproducible results. The formulation optimization studies helped in clarifying the contribution of the different formulation components (lipids (DOPG, DOPE) and Ca^{2+} ions) to silencing efficiency and cytotoxicity. To the best of our knowledge, this is the first effective anionic lipid based siRNA formulation prepared without using any potentially toxic moiety such as cationic lipids or polymers. Such anionic formulations show promise as a prospective delivery vector for safe and efficient delivery of therapeutic siRNA *in vitro* as well as *in vivo*.

Conflict of interest

There are no potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpharm.2012.04.058.

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