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ORIGINAL ARTICLE

Cationic nanoemulsions as potential carriers for intracellular delivery

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Abstract Successful cytosolic delivery enables opportunities for improved treatment of various genetic disorders, infectious diseases and cancer. Cationic nanoemulsions were designed using alternative excipients and evaluated for particle size, charge, effect of sterilization on its stability, DNA condensation potential and cellular uptake efficiency. Various concentrations of non-ionic and ionic stabilizers were evaluated to design formula for colloidally stable cationic nanoemulsion. The nanoemulsion comprised of 5% Capmul MCM, 0.5% didodecyldimethylammonium bromide (DDAB), 1% phospholipid, 1% Poloxamer 188 and 2.25% glycerol and possessed particle size of 81.6 ± 3.56 nm and 137.1 ± 1.57 nm before and after steam sterilization, respectively. DNA condensation studies were carried out at various nanoemulsion: DNA ratios ranging from 1:1 to 10:1. Cell uptake studies were conducted on human embryonic kidney (HEK) cell lines which are widely reported for transfection studies. The nanoemulsions showed excellent cellular uptake as evaluated by fluorescence microscopy and flow cytometry. Overall, a colloidally stable cationic nanoemulsion with good DNA condensation ability was successfully fabricated for efficient cytosolic delivery and potential for *in vivo* effectiveness.

1. Introduction

A myriad of drug delivery systems are being engineered today with the aim to achieve cytosolic delivery which enables the actives to exert its therapeutic action within the cytoplasm, other intracellular organelles or the nucleus. Intracellular delivery systems are the mainstay approach for therapy of diseases associated with defects arising in subcellular structures/biochemical pathways. Novel nanocarrier systems are being

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researched and evaluated for efficacious intracellular delivery of anti-cancer drugs as well as proapoptotic drugs and siRNA (Torchilin, 2006; Chou et al., 2011). However, design of delivery systems for effective cytosolic delivery is challenging. The main barrier to intracellular delivery is the lipophilic plasma membrane which prevents the entry of compounds inside the cell. It is well known that the cell has a negatively charged surface due to the presence of sulfated proteoglycans, namely heparin, heparan sulfate, dermatan sulfate, chondroitin sulfate, and keratan sulfate. Reports have proven that these proteoglycans act as plasma membrane carriers (Belting, 2003) and mediate cellular internalization of cationic delivery systems (Mounkes et al., 1998; Mislick and Baldeschwieler, 1996). Recently, cationic liposomes have demonstrated selectivity for tumor vascular endothelial cells, since the tumor vessels have an over abundance of negatively charged molecules (Campbell et al., 2002). Besides, the drug carrier must also escape the processes that affect the disposal of macromolecules, which include interaction with blood components, vascular endothelial cells and uptake by the reticuloendothelial system. Degradation of therapeutic DNA and RNA by serum nucleases is another significant threat for functional delivery to the target cell (Quong and Neufeld, 1998). Thus, an ideal intracellular carrier system should be able to protect and transport the therapeutic materials to target cells without causing any toxicity and immune responses (Smith et al., 1997). Further, to be therapeutically useful, the delivery system should possess small size to allow internalization into cells and when endocytosed must be able to escape endosome-lysosome processing (Mansouri et al., 2004; Jayakumar et al., 2010) .

Various systems have been designed to overcome these barriers for effective intracellular delivery of drugs and genes. These include polymeric nanoparticles, dendrimers, liposomes, lipopolyplexes, to name a few (Shah and Amiji, 2006; Xiong et al., 2005). Most of these systems are stable at ambient storage conditions, can be produced in large quantities and produce minimum immune response in host. Besides, they can be target specific and have advantages of high drug loading as well as good DNA condensation capacity. Further, efficient cellular internalization is facilitated as a result of electrostatic interaction between the cationic delivery system and the negatively charged cell membrane.

It is worth noticing, that various researchers have evaluated cationic nanoemulsions as an alternative for drug/gene delivery and it has demonstrated significant potential to do so (de Araújo et al., 2007; Liu and Yu, 2010). Cationic emulsions designed to date use excipients like DOTAP and DOTMA.[1] It is thought worthwhile to formulate cationic nanoemulsions with alternative excipients which display good condensation, efficient cytosolic delivery and lower cytotoxicity. It is reported, that the cationic surfactant, when used in conjunction with the non ionic surfactant demonstrates better colloidal stability. It can be thus hypothesized, that a cationic nanoemulsion can be formulated by optimizing the ratio of the non ionic and the cationic surfactant rendering the fabrication process economic. The objective of the present investigation is to formulate a cationic nanoemulsion with regular excipients for effective intracellular delivery.

[1] Abbreviations: DDAB, didodecyldimethyl ammonium bromide; CTAB, cetyltrimethylammonium bromide; DOTAP, N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate; DOTMA, N-[1-(2, 3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride; PCS, photon correlation spectroscopy.

2. Materials and methods

Capmul MCM was a kind gift from Abitec Corporation, USA. Miglyol 812 and Miglyol 840 were obtained from Sasol, Germany. Capryol 90 and Labrafac FCC were kind gifts from Gattefosse Pvt. Ltd., Mumbai, India. Phospholipids were obtained as kind gift samples from Lipoid, Germany. Tween 20, Tween 80 and stearylamine were obtained from S.D. Fine Chem. Ltd., Mumbai, India. Solutol HS 15 and Poloxamer 188 were obtained from BASF, Mumbai, India. MYS 40 was obtained from Nikko Chemicals Co. Ltd., Tokyo, Japan. Didodecyldimethylammonium bromide (DDAB) and Cetyltrimethylammonium bromide (CTAB) were purchased from Fluka Chemicals (New Jersey, USA). Ethanol was purchased from S.D.Fine Chem. Ltd., Mumbai, India and Glycerol from Qualigens, Mumbai, India.

2.1. Formulation and optimization of cationic nanoemulsions

Cationic nanoemulsion was fabricated by the probe sonication method (Fig. 1). The components of the nanoemulsions included an oil, non-ionic and/or ionic surfactant, osmotic agent, phospholipids and 0.2 μm double filtered distilled water. Briefly, the aqueous phase and the oil phase were separately heated on a water bath at 70 °C. The aqueous phase was then added to the oily phase under vortex (REMI CM101) to spontaneously produce coarse emulsions. The coarse emulsion was then transferred to a vial preheated at 70 °C. The coarse emulsion was probe sonicated for 5 minutes at an output of 35 W to yield the final nanoemulsion formulation. Nanoemulsions were prepared with varying compositions and concentrations of surfactants and oils. The prepared nanoemulsions were evaluated for colloidal stability after being autoclaved.

2.2. Characterization of cationic nanoemulsions

2.2.1. Particle size analysis and zeta potential measurements

Particle size analysis was carried out on a photon correlation spectroscopy (PCS) equipment (Beckman Coulter, N5 submicron particle size analyzer) after appropriate dilution with 0.2 μm double filtered distilled water. Zeta Potential was measured using the Malvern Zetasizer, nanoseries.

2.2.2. Freeze drying of cationic nanoemulsions

The final optimized nanoemulsion was freeze dried with condenser temperature of −60 °C and 200 torr pressure. The freeze dried product was then reconstituted with 0.2 μm double filtered distilled water to evaluate the effect of lyophilization on particle size and polydispersity of nanoemulsion as shown by PCS evaluation.

2.2.3. DNA condensation study (Hung et al., 2005)

The nanoemulsion was appropriately diluted to yield the final DDAB concentration of 1 μg/μl in the final dilution. The concentration of the DNA solution was 1 μg/μl in tris-EDTA buffer (20 mM Tris-Cl and 1 mM EDTA pH 7.5). This DNA

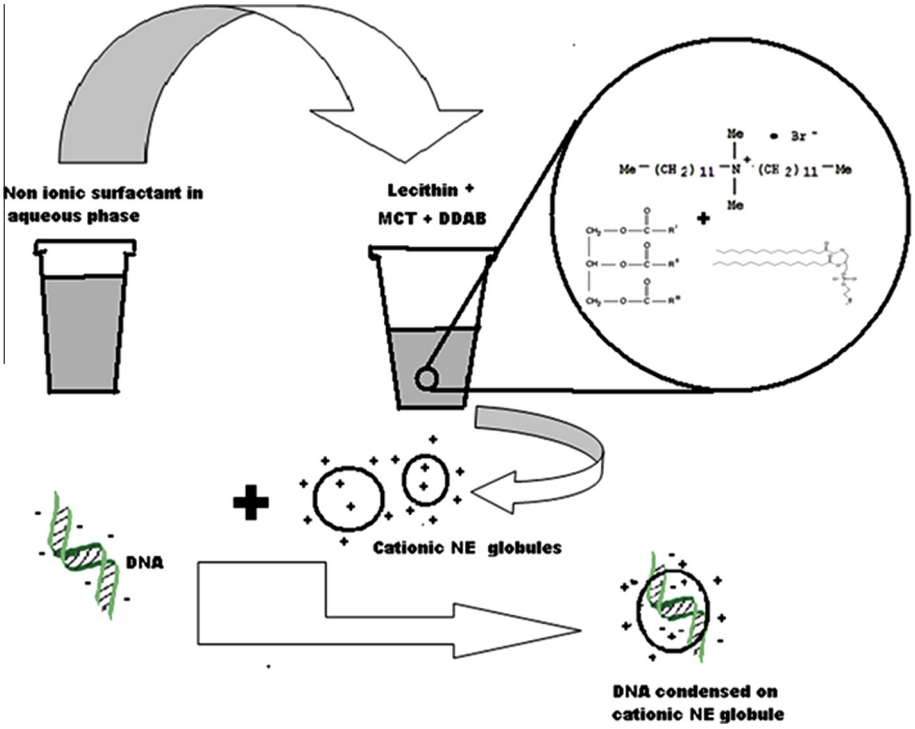

Figure 1 Schematic of cationic nanoemulsion formulation and condensation with DNA.

solution was mixed with increasing amounts of the diluted cationic nanoemulsion to a fixed total reaction volume. Four ratios of carrier: DNA viz. 1:1; 3:1; 5:1; 10:1 were evaluated for complexation. The solutions were incubated for 1 h and 10 μL aliquots of the complexes were mixed with 10 μL of the loading buffer (0.25% bromophenol blue and 30% glycerol) and samples were loaded onto 0.8% w/v agarose gel. The samples were loaded into five lanes as follows: Lane 1: Naked DNA; Lane 2: DNA: Carrier (1:1); Lane 3: DNA: Carrier (1:3); Lane 4: DNA: Carrier (1:5); Lane 5: DNA: Carrier (1:10). Electrophoresis was carried out with a current of 70 V for about 2 h in tris-borate-EDTA running buffer (100 mM Tris-Cl, 100 mM boric acid and 2 mM EDTA pH 7.5). After 1 h of staining with ethidium bromide, gel photographs were taken under UV light. Naked DNA was also allowed to run on the gel as control.

Table 1 Formula of cationic nanoemulsion.

Ingredients	Proposed use	%w/v (prototype formula)	%w/v (optimized final formula)
Capmul MCM	Oil phase	5	5
Poloxamer 188	Impart better stabilization	1	1
DDAB	Impart positive charge	0.5	0.5
PL 90 H	Impart Thermal stability	0.5	1
Glycerol	Impart isoosmolarity	2.25	2.25
Water	Vehicle	q.s. 10 gms	q.s. 10 gms

Table 2 Compositions of batches for optimization of prototype formula.

Formulation	Capmul MCM	Poloxamer 188	DDAB	PL 90H
Prototype formula (S1)	5	1	0.5	0.5
S2	5	2	0.5	0.5
S3	5	3	0.5	0.5
S4	5	4	0.5	0.5
S5	5	5	0.5	0.5
S6	2.5	1	0.5	1
S7	2.5	2	0.5	1
S8	2.5	1	1	1
Final optimized Formula (S9)	5	1	0.5	1

Table 3 Particle size analysis of batches S1-S9.

Formulation	Before sterilization		After autoclaving		Other observations
	M.P.S.	P.I.	M.P.S.	P.I.	
Prototype formula (S1)	73.2 ± 9.21	0.709 ± 0.053	111.7 ± 3.28	0.524 ± 0.126	Not stable for long after autoclaving
S2	108.3 ± 2.07	0.963 ± 0.046	NS	NS	Oil globules seen under microscope
S3	101.0 ± 1.67	0.615 ± 0.002	NS	NS	Same as S2
S4	95.3 ± 1.15	0.951 ± 0.004	NS	NS	Same as S2
S5	65.5 ± 1.54	0.839 ± 0.048	NS	NS	Same as S2
S6	70.2 ± 1.94	NS	NS	NS	Same as S2
S7	87.0 ± 3.83	NS	NS	NS	Same as S2
S8	72.3 ± 5.69	NS	NS	NS	Same as S2
S9	81.6 ± 3.56	0.767 ± 0.18	137.1 ± 1.57	0.210 ± 0.042	M.P.S. 6 h after autoclaving: 143.1 ± 5.11; P.I.: 0.271 ± 0.166

M.P.S.: Mean particle size; P.I.: Polydispersity index; NS: Not satisfactory.

2.2.4. Fluorescence microscopy and flow cytometry

HEK (Human embryonic kidney) cells were seeded (2×10^5/well) in 35 mm plate with 2 mL of growth medium, and incubated at 37 °C, in a CO_2 incubator for 24 h prior to addition of formulations. Cells were treated with cationic nanoemulsions containing nile red dye (100 μg/mL) or vehicle alone at 37 °C. After 48 h, the plates were observed under a fluorescence microscope. Representative fields were visualized and photographed with an Axiovert 200 M (Inverted Microscope) Carl Zeiss, Germany. Exposure times were 300–500 milliseconds. Treated cells and their corresponding untreated control cells were then harvested for flow cytofluorometry. Cells were washed with 1 × PBS and spun at 2000 rpm for 10 min at room temperature. Cells were suspended in 1 × PBS (500 μL). Cells were acquired on a Flow Cytometer (FACS Calibur BD) to quantify the red fluorescence (FL2) and the results were analyzed using the CellQuestPro software. The study was carried out at two concentrations i.e., 0.3 μL and 0.6 μL of nanoemulsion.

3. Results and discussion

Various oils were evaluated to fabricate colloidally stable nanoemulsions that included Miglyol 812 and 840, Labrafac FCC, Capryol 90 and Capmul MCM at concentrations of 2.5% w/v and 5% w/v. It was observed that all oils evaluated except Capmul MCM, at concentrations of 2.5% w/v and 5% w/v in combination with various stabilizers at varying concentrations could yield nanoemulsion with size ranging from 120 to 240 nm and poor colloidal stability (data not shown). However, Capmul MCM at both concentrations yielded cationic nanoemulsion with size less than 85 nm. Formulae were prepared using only non-ionic surfactants and combination of both, ionic and non-ionic surfactants, to enable better understanding about stabilizing potential of ionic surfactants. Non ionic stabilizers evaluated include Polysorbate 80, Solutol HS 15, Mys 40 and Poloxamer 188, whereas CTAB and DDAB were ionic surfactants employed. 2.25% w/v glycerol was a part of all formulations to impart iso-osmolarity to formulations.

It was observed that, nanoemulsions containing only non-ionic stabilizers did not possess adequate colloidal stability. Non-ionic surfactants were thought to be inefficient alone to control Ostwald ripening phenomenon at concentrations employed, resulting in rapid aggregation of oil globules and destabilization (Jain et al., 2011). In combination with ionic surfactants, however, scenario changed and nanoemulsions with better colloidal stability were formed. This depicts better stabilizing potential of ionic surfactants, probable mechanism being the positive charge imparted on globule surface causing them to repel each other and minimizing aggregation. Nanoemulsions with and without phospholipids were prepared and autoclaved. Those without phospholipids could not maintain their colloidal stability on being autoclaved, whereas ones containing phospholipids showed slight increase in particle size with retention of colloidal stability. It was evident that phospholipids conferred sufficient thermal stability to cationic nanoemulsions to be conveniently autoclaved without drastic increase in particle size and polydispersity. On autoclaving, it was observed that cationic nanoemulsions containing DDAB demonstrated less increase in particle size as compared to CTAB, which on combination with any surfactant could not control enormous growth in globule size, hence DDAB incorporated nanoemulsion was utilized for further studies.

Nanoemulsion comprising of Capmul MCM, DDAB, phospholipid and Poloxamer 188 was finally selected for further studies as it demonstrated adequate colloidal stability before and after autoclave sterilization. The formula of prototype cationic nanoemulsion is mentioned in Table 1. Further optimization studies were conducted, by varying

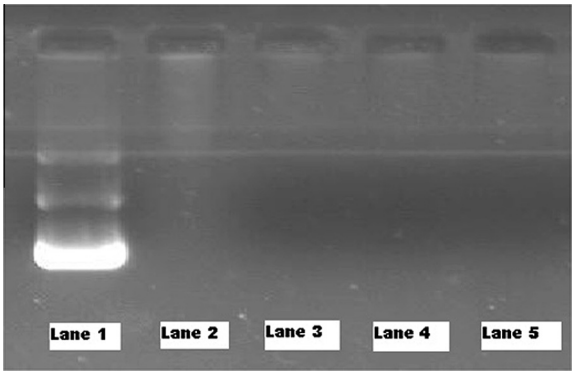

Figure 2 DNA condensation results. Lane 1 shows naked DNA whereas absence of bands on the gel in Lane 2, 3 and 4 indicates complete condensation between the DNA and the delivery system.

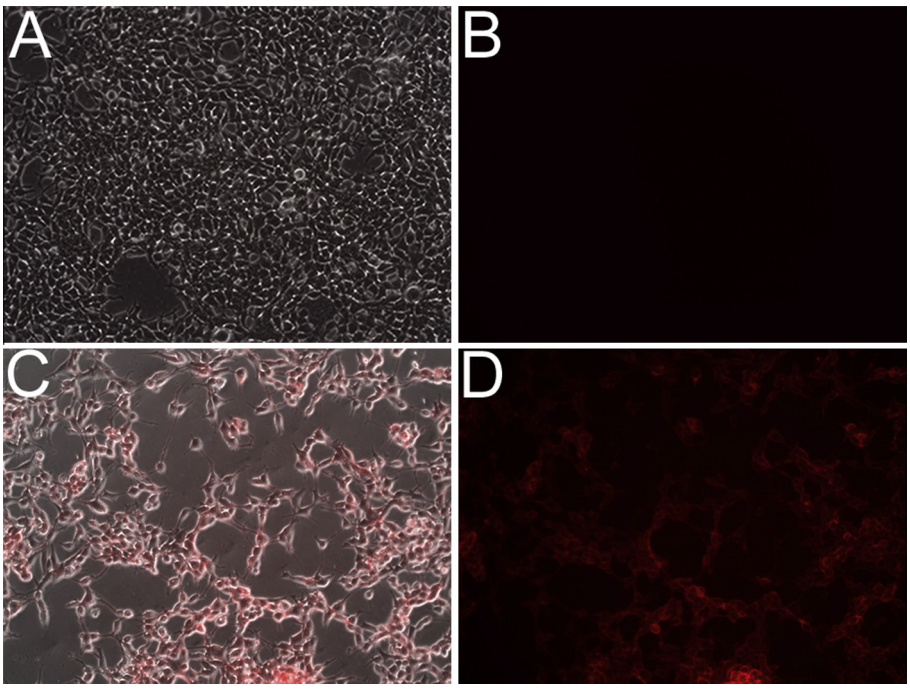

Figure 3 Fluorescence images of HEK cells (A) and (B) represent merged and fluorescence images of untreated control whereas (C) and (D) represent the merged and fluorescence images of nanoemulsion treated cells, respectively.

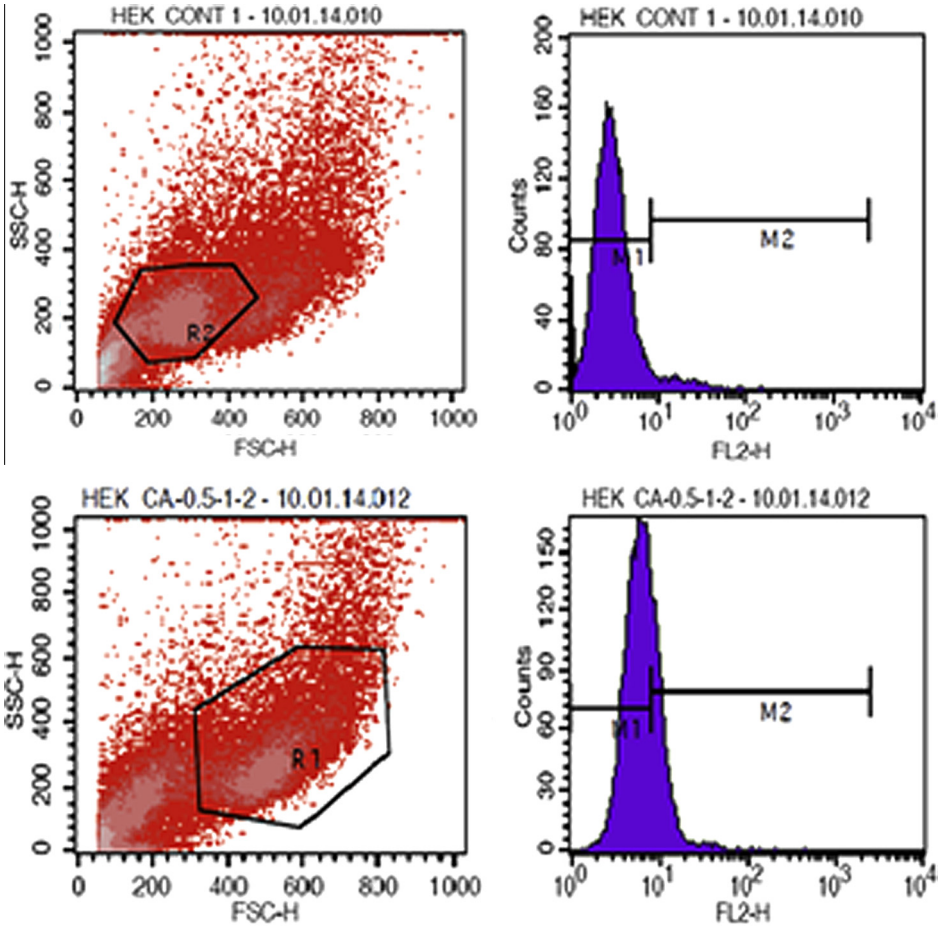

Figure 4 Flow cytometry histograms of untreated control and nanoemulsion treated cells.

concentrations of each of the above mentioned components to evaluate their effect on the stability of nanoemulsion. Concentration of Poloxamer was varied from 1% w/v to 5% w/v. It was observed that as concentration of Poloxamer increased, globule size decreased. However, the formulation did not show adequate duration of stability with coagulation of oil globules observed within few minutes. The probable reason could be attributed to an increase in concentration of Poloxamer 188 which reduced the amount of DDAB at globule surface thus reducing colloidal stability. Thus, concentration of Poloxamer 188 was fixed to 1% w/v in the final formula. Surprisingly, it was observed that inclusion of Capmul MCM at concentration of 5% w/v could confer better colloidal stability as compared to 2.5% w/v. Increase in phospholipid concentration from 0.5% w/v to 1% w/v was found to improve stability after autoclaving without significant effect on mean particle size. The final optimized formula employed for DNA condensation studies and intracellular delivery possessing adequate stability before and after autoclaving is mentioned in Table 1. Tables 2 and 3 depict optimization of prototype formula with effect of autoclaving on different batches. Zeta potential measurement demonstrated that optimized cationic nanoemulsion possessed surface charge of +40 mV explaining its good colloidal stability due to repulsion between oil globules and reduced aggregation. Autoclaving resulted in slight increase in particle size. However, even after sterilization, optimized nanoemulsion possessed adequate colloidal stability as mentioned in Table 3. Cationic nanoemulsions were freeze dried to improve their stability upon storage, thus aiding their potential for commercialization. Trehalose was employed as cryoprotectant at concentration of 10% based on earlier reports about its potential of being effective cryoprotectant in most cases. The particle size of the nanoemulsion after freeze drying and reconstitution was ~120 nm with a polydispersity of ~0.5.

Gel electrophoresis was performed to evaluate the complexation between the cationic carrier and the DNA. As observed in Fig. 2, effective complexation was observed at carrier: DNA ratios of 1:1; 3:1; 5:1; 10:1. It was interesting to note that effective complexation was achieved at a ratio as low as 1:1. At higher carrier concentrations, neither free nor retarded DNA bands were observed in the sample. The condensation of the DNA by the cationic nanoemulsion prevented ethidium bromide from intercalating with the base pairs of the DNA thereby accounting for disappearance of bands. Cell uptake studies were performed using fluorescence microscopy and fluorescence was quantified using flow cytometry. The phase contrast and fluorescence micrographs of control and treated HEK cells are shown in Fig. 3. When cell uptake was studied at 1:1 ratio of DNA: carrier, cell cytotoxicity was observed. Therefore cell uptake assay was performed using 1:0.5 ratio of DNA: cationic agent. It is seen from the figures, that most of the cells are fluorescent with negligible background fluorescence. Nile red is a hydrophobic dye and has been reported to determine cellular lipid content since it is strongly fluorescent in the presence of hydrophobic environment. The dye is associated with the oily part of nanoemulsions and its presence inside the cells can therefore be anticipated due to internalization of the nanoemulsion droplets inside the cells thereby rendering the cells fluorescent. Cells were examined for red fluorescence by flow cytofluorometry. The histograms indicate that cells loaded with the nanoemulsions have increased fluorescence intensity relative to control cells. Unstained cells displayed weak fluorescence with partial overlap with the fluorescence of Nile red stained cells. By subtraction analysis, around 23–25% cells treated with nanoemulsions were found to have greater fluorescence intensity than control cells, thus confirming the internalization of the nanoemulsions (Fig. 4).

4. Conclusion

Colloidally stable cationic nanoemulsions were successfully fabricated using DDAB to render positive charge on surface of globules. The properties of the cationic nanoemulsions were influenced by selection and concentration of an oil, non ionic and the cationic surfactant. Inclusion of phospholipids enabled cationic nanoemulsions to maintain sufficient colloidal stability even after steam sterilization. The cationic nanoemulsions showed improved DNA condensation even at lower DNA: carrier ratio of 1:1. Future studies which evaluate higher carrier: DNA ratios and transfection efficiency will be useful. *In vivo* study can give further insight and validation of effective gene delivery.

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