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Cytotoxicity evaluation of anionic nanoliposomes and nanolipoplexes prepared by the heating method without employing volatile solvents and detergents

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Submicron lipid vesicles (nanoliposomes) are being used as carriers of bioactive compounds. In addition, complexes of nanoliposomes and nucleic acids (nanolipoplexes) are promising tools for the treatment of cancer, and viral and genetic disorders. Toxicity of some of these formulations, however, still remains a concern in their clinical utilisation. To address this problem, anionic liposomes were prepared by two different techniques, the conventional thin-film method, and the heating method (HM), in which no volatile organic solvent or detergent is used. An anionic nanolipoplex was constructed by incorporating plasmid DNA (pcDNA3.1/His B/lacZ) into the HM-nanoliposomes by the mediation of calcium. The toxicity of the nanoliposomes, with and without plasmid and Ca^{2+}, was assessed using a human bronchial epithelial cell line (16HBE14o-) in the presence of serum. Cytotoxicity evaluations performed by two different assays (i.e. NRU and MTT) indicated that HM-nanoliposomes were completely non-toxic in the cell-line tested, whereas conventional liposomes revealed significant levels of toxicity. This may be due to the presence of trace amounts of chloroform and/or methanol applied during their preparation. Similar results were obtained for different sizes of lipid vesicles (prepared by 100 nm and 400 nm pore-size filters). In addition, it was observed that incorporation of DNA (15 μg/285 μg lipid) and Ca^{2+} (50 mM) to the nanoliposomes did not have any effect on their cytotoxicities. These findings indicate that the HM-liposomes have great potential as non-toxic delivery vehicles in human gene therapy and drug delivery applications while liposomes made using organic solvents should be used with caution.

1. Introduction

Nucleic acid therapeutics, including antisense nucleotides and functional genes, are under investigation for the treatment of viral, neoplastic and inherited diseases. Viral based gene delivery systems are the most widely used nucleic acid transfer vectors. The major problems with the use of viral vectors are their generally invasive route of administration and the immune response of the host against viral proteins. Liposomal gene therapy approaches, in contrast, possess many advantages including lower production costs and fewer safety concerns. Research in the field of liposome-derived DNA delivery has been attracting numerous scientific groups since 1978 (Hoffman et al. 1978). However, the toxicity of some liposomal formulations, particularly those incorporating cationic agents (Campbell 1983; Filion and Phillips 1997; Dokka et al. 2000; Nagahiro et al. 2000; Omidi et al. 2005), is the major drawback in their clinical application. The origin of the toxicity caused by cationic liposomes has not been completely elucidated. It has been suggested that intermixing of the cationic lipids and the anionic lipids of cell organelle membranes, such as mitochondrial membranes, is responsible for cytotoxicity (Xu and Szoka 1996). Another postulated mechanism for cationic lipid-mediated toxicity in the lung is the involvement of reactive oxygen intermediates (Dokka et al. 2000). An alternative to cationic liposome protocols is the employment of anionic lipid vesicles as gene transfer vectors. A method of incorporating polynucleotides, by the mediation of divalent cations, to anionic liposomes has been first reported by our group (Kahveci et al. 1994) and the structure of the ternary complexes of liposome/Ca^{2+}/DNA has been characterised morphologically using scanning probe and other microscopes (Zareie et al. 1997; Mozafari et al. 1998). In addition, the mechanism of calcium induced DNA interaction with liposomes containing zwitterionic lipids, as well as those containing anionic lipids, has been studied (Mozafari and Hasirci 1998). However, there has been no evaluation of the toxicity of these formulations to date.

The main concern regarding the toxicity of these anionic liposomal formulations is the utilisation of volatile organic solvents such as chloroform and methanol during their preparation. The majority of liposome preparation meth-

ods involve the application of organic solvents or detergents to solubilise the lipids. Residues of these solvents may remain in the final liposome preparation and contribute to potential toxicity and influence the stability of the vesicles (Vemuri and Rhodes 1995; Cortesi et al. 1999).

In the light of the above, this study aimed to evaluate the influence of the preparation method, and particularly utilisation of volatile organic solvents, on the safety profile of nanolipoplexes. For this, the cytotoxicity of anionic nanoliposomes prepared without employing any volatile solvent or detergent by the heating method (HM-nanoliposomes) was evaluated in the presence and absence of DNA and Ca^{2+}. *In vitro* toxicity was assessed in a human bronchial epithelial cell line using the neutral red uptake (NRU) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. The cytotoxicity results obtained were compared with those of nanoliposomes, with similar composition, prepared by the conventional thin-film hydration method using volatile organic solvents.

2. Investigations, results and discussion

A new method for the preparation of lipid-based carrier systems, without utilisation of any harmful solvent or detergent, has been developed in our laboratory (Mozafari 2005). To further assess the suitability of these carriers for clinical applications we evaluated their cytotoxicity, in comparison with conventionally produced carriers, in the cultured HBE cells. This cell line was used as a relevant *in vitro* model for pulmonary gene therapy challenges such as cystic fibrosis. Cell viability was evaluated using two cytotoxicity assays instead of just one. This is because certain chemicals may give divergent results in different toxicity tests including the NRU and MTT assays (Olivier et al. 1995; Chiba et al. 1998) used in this study. Besides, Evans et al. (2001) have recently found that in some cases there is a difference in the sensitivity of these two assays in detecting the toxicity of non-viral transfection reagents.

Due to several reports on the toxicity and other limitations in the application of cationic lipid vesicles (Campbell 1983; Filion and Phillips 1997; Dokka et al. 2000; Nagahiro et al. 2000; Omidi et al. 2005), anionic liposomes were utilised in this study. It has been postulated that negatively charged liposomes, in general, associate more effectively and are taken up more readily by the cells compared with neutral liposomes (Monkkonen et al. 1994). In addition, anionic liposomes are reported to be less cytotoxic than cationic delivery systems (Campbell 1983; Welz et al. 2000). In terms of size two types of nanoliposomes were prepared using filters of 100 nm or 400 nm pore sizes. The mean particle size of HM-nanoliposomes prepared using 100 nm and 400 nm filters were 159.6 +/− 2.3 nm and 510.4 +/− 15.8 nm, respectively. Observation that DPPC-containing vesicles are larger than the filter pores through which they were extruded has also been reported (Nayar et al. 1989; MacDonald et al. 1991). The phase transition temperature (T_C) of DPPC (the major constituent of the vesicles prepared in this study) is 41 °C. Hence these vesicles are at gel phase and inflexible at room temperatures. The explanation of how vesicles which are solid at ambient temperatures can pass through pore sizes smaller than their diameters relies on the fact that the extrusion process was carried out at temperatures above the T_C, when vesicles are flexible, while size determinations were carried out at room temperature when they are at gel state. Incorporation of DNA molecules to the anionic nanoliposomes was achieved by the employment of 50 mM calcium. This concentration was selected based on our previous investigations (Zareie et al. 1997; Mozafari et al. 1998; Mozafari and Hasirci 1998). The Table shows the cytotoxicity of different concentrations of Ca^{2+} determined by the NRU and MTT assays. Both assays confirm that 50 mM calcium (the concentration used in the preparation of liposome/Ca^{2+}/DNA complex) is well tolerated by the HBE cells.

The cytotoxicity evaluation of the two populations of nanoliposomes prepared using 100 nm filters is shown in

Table: Cell viability (%) of HBE cells in the presence of calcium

Ca^{2+} concentration (mM)	Percent viability NRU assay	Percent viability MTT assay
5	99.7 ± 3.2	98.7 ± 2.6
10	97.3 ± 4.4	97.1 ± 2.7
25	97.2 ± 0.8	94.8 ± 2.4
50	94.4 ± 2.8	93.3 ± 2.4
100	89.9 ± 7.1	90.6 ± 8.2
150	43.2 ± 2.0	56.7 ± 3.9
200	23.6 ± 2.5	43.2 ± 2.8
250	21.3 ± 2.1	23.6 ± 2.6

NRU and MTT assays performed in 96-well plates after 24 h incubation of cells with the indicated amounts of calcium. Data are expressed as mean ± S. D. of three or more independent experiments

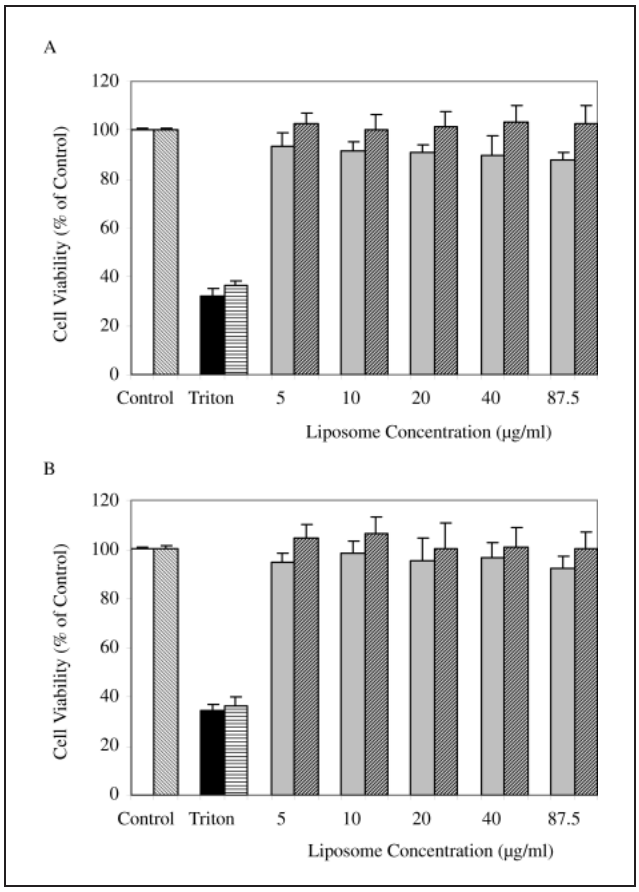

Fig. 1: Cytotoxicity of liposomes according to their preparation method. Liposomes were prepared by extrusion through 100 nm filters. Cell viability was determined after 24 h by (A) neutral red, and (B) MTT assays. Normal bars: liposomes prepared by the conventional method; slashed bars: liposomes prepared by the heating method. Triton X-100 (78 ppm) was used as positive control. Results represent means ± S.D. of data obtained from at least three independent experiments and the two liposome types differed significantly ($P < 0.001$)

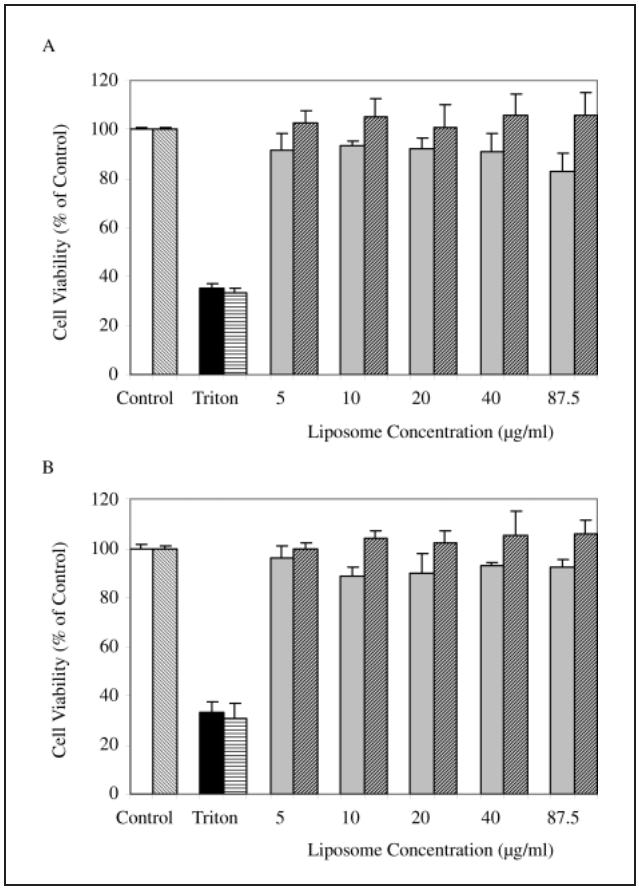

Fig. 2: Cytotoxicity of liposomes according to their preparation method. Liposomes were prepared by extrusion through 400 nm filters. Cell viability was determined after 24 h by (A) neutral red, and (B) MTT assays. Normal bars: liposomes prepared by the conventional method; slashed bars: liposomes prepared by the heating method. Triton X-100 (78 ppm) was used as positive control. Results represent means ± S.D. of data obtained from at least three independent experiments and the two liposome types differed significantly ($P < 0.001$)

Fig. 1. Both NRU (Fig. 1A) and MTT (Fig. 1B) assays indicate that the nanoliposomes prepared by the conventional technique exhibit statistically significant ($P < 0.001$) levels of toxicity when compared with the nanoliposomes prepared by the heating method. Fig. 2 demonstrates cell viability studies of the cultured cells incubated with the conventional nanoliposomes and HM-nanoliposomes prepared using 400 nm filters. Again both NRU (Fig. 2A) and MTT (Fig. 2B) assays attest that the conventional nanoliposomes display statistically significant ($P < 0.001$) levels of toxicity when compared with the HM-nanoliposomes. It seems that the toxicity of the two nanoliposome types is similar for 100 nm and 400 nm filtered vesicles. The chemical composition of the conventional nanoliposomes and HM-nanoliposomes is the same and the difference between them, apart from the presence/absence of the organic solvents, is the employment of glycerol in the manufacture of HM-nanoliposomes. Glycerol is a water-soluble and physiologically acceptable chemical with the ability to increase the stability of lipid vesicles (Kikuchi et al. 1994). On the other hand, chloroform and methanol are well known toxic agents (Timbrell 2000; Dwivedi 2002) which exert cytotoxicity through different mechanisms including destabilisation of membrane proteins (Ivanov 2001). The toxicity observed for the nanoliposomes prepared by the conventional method is therefore believed to be caused by the presence of chloroform and/or methanol employed in their manufacture. Trace amounts of these potentially toxic agents remain in the liposomal formulation, no matter how much effort is undertaken to remove them (Deamer and Uster 1983; Cortesi et al. 1999). We propose that as a result of liposome interaction with cells, which can be in the form of lipid exchange, adsorption, fusion, or endocytosis, small amounts of the organic solvents, entrapped in the lipid vesicles, can be released into the cells and cause cytotoxicity. It is possible that even if these vesicles are not taken up by the cells (through endocytosis or fusion) the organic solvents present in their structure, and initially those present in their lipid phase, can interact with the cells after being released from the vesicles by the mechanism of lipid exchange. A first adverse effect of these organic solvents would be on the cell membrane proteins as observed by Ivanov (2001).

The next step was to evaluate whether the incorporation of plasmid DNA into the anionic nanoliposomes can alter their toxicity towards the HBE cells. Results indicated that incorporation of plasmid, by the mediation of Ca^{2+}, into the two populations of nanoliposomes used in this study did not significantly change their cytotoxicity. This was the same for 100 nm (Fig. 3) and 400 nm (Fig. 4) filtered lipid vesicles as confirmed by both NRU (Fig. 3A and 4A) and MTT (Fig. 3B and 4B) assays. These findings suggest that presence of DNA as a plasmid vector does

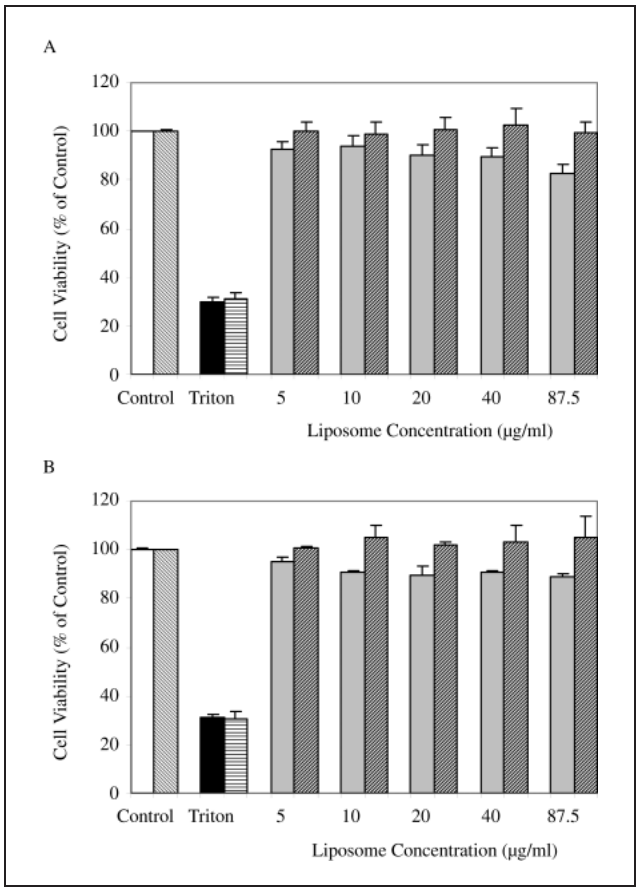

Fig. 3: Cytotoxicity of liposome/Ca^{2+}/DNA complexes according to their preparation method. Liposomes were prepared by extrusion through 100 nm filters. Cell viability was determined after 24 h by (A) neutral red, and (B) MTT assays. Normal bars: liposomes prepared by the conventional method; slashed bars: liposomes prepared by the heating method. Triton X-100 (78 ppm) was used as positive control. Results represent means ± S.D. of data obtained from three or more independent experiments and the two liposome types differed significantly ($P < 0.001$)

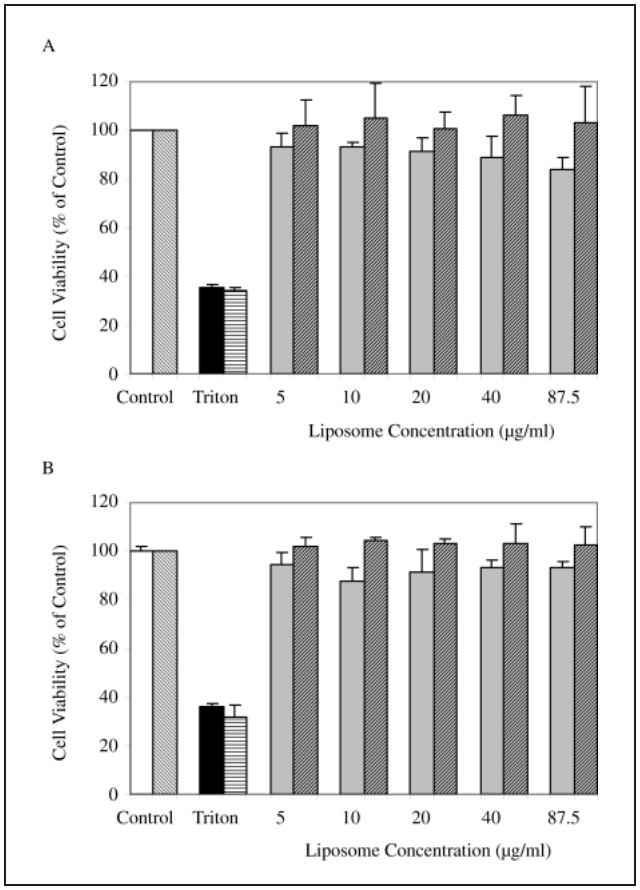

Fig. 4: Cytotoxicity of liposome/Ca^{2+}/DNA complexes according to their preparation method. Liposomes were prepared by extrusion through 400 nm filters. Cell viability was determined after 24 h by (A) neutral red, and (B) MTT assays. Normal bars: liposomes prepared by the conventional method; slashed bars: liposomes prepared by the heating method. Triton X-100 (78 ppm) was used as positive control. Results represent means ± S.D. of data obtained from three or more independent experiments and the two liposome types differed significantly ($P < 0.001$)

not affect the toxicity of anionic vesicles while Filion and Phillips (1997) reported that incorporation of DNA reduced the toxicity of cationic vesicles in their study. Once more it seems that the toxicity of both types of nanolipoplexes is similar for 100 nm and 400 nm filtered vesicles.

The observation that HM-nanoliposomes (with and without plasmid) are completely non-toxic towards the cultured HBE cells is based on the fact that no hazardous chemical or process is involved in their preparation. Although some groups, including Adams et al. (1977) and Campbell (1983), detected toxicity for some anionic liposomes (to a lesser degree than the cationic vesicles they studied) they overlooked the application of the volatile solvents in their liposome preparations and ignored the contribution of these solvents to the toxicities they observed. This should be noted in the future toxicity evaluations of liposomal formulations.

In conclusion, the results of this study indicate that nanoliposomes prepared by the heating method, complexed or not complexed with plasmid DNA, are completely non-toxic to HBE cells, in the concentration range tested. In contrast, nanoliposomes prepared by the conventional method using volatile organic solvents are significantly ($P < 0.001$) toxic towards the same cells and hence should be utilised with caution. The two sizes of vesicles tested exhibited similar toxicity profiles. This investigation proved that the second generation of the liposome/Ca^{2+}/DNA lipoplexes prepared by the heating method are superior to the first generation which required volatile solvents for their preparation. In general terms, the heating method was detected to be a fast liposome preparation technique, with the potential for mass production of non-toxic liposomes and nanoliposomes to be utilised in gene and drug delivery.

3. Experimental

3.1. Materials

Dipalmitoylphosphatidylcholine (DPPC), dicetylphosphate (DCP), cholesterol (CHOL), 0.25% trypsin-EDTA solution, neutral red solution (NR, 3-amino-7-dimethylamino-2-methyl phenazine hydrochloride (3.3 g/L)), and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were purchased from Sigma Chemical Co (Dorset, UK). Minimum essential medium (EAGLE) containing Glutamax-1, fetal calf serum, and penicillin/streptomycin (10000 U/mL, 10000 μg/mL) were obtained from GibcoBRL® Life Technologies Ltd (Paisley, UK). The plasmid (pcDNA3.1/His B/lacZ, 8578 nucleotides) was supplied by Invitrogen (Netherlands). All solvents (chloroform, methanol, Analar grade) were obtained from Sigma Chemical Co (Dorset, UK). All other chemicals were of commercial analytical grade.

3.2. Liposome preparation

Multilamellar vesicles (MLV) were prepared by the conventional thin-film hydration method (Mozafari and Hasirci 1998). Briefly, a 3 mL chloroform/methanol (2 : 1, v/v) solution of DPPC : DCP : CHOL (7 : 2 : 1 molar ratio) was evaporated to dryness in a round bottomed flask. To remove traces of the solvents the flask was flushed with nitrogen gas for 1 h and also placed under high vacuum for a time period of at least 1 h. The lipid film was then hydrated, above the phase transition temperature (T_C) of the lipids, with 2 mL sterile phosphate buffered saline (PBS, pH: 7.4), and MLV formed by vortex agitation. In order to prepare conventional nanoliposomes the MLV suspension was extruded 11 times, using a mini-extruder (LiposoFast™-Basic, Glen Creston Ltd, UK), above T_C through two-stacked polycarbonate filters of either 100 or 400 nm pore size.

HM-nanoliposomes with the same lipid composition as the conventional nanoliposomes were prepared as follows: the lipids, DPPC, DCP and CHOL, were hydrated each in 2 mL sterile PBS (pH: 7.4) for 2 h under N_2 at room temperature. The lipid dispersions were then mixed together, 0.3 mL glycerol added and the volume made up to 10 mL with PBS. The mixture was heated to 120 °C while stirring until all the lipids dissolved. Formulations containing cholesterol, including those utilised in the present work, generally require 20–30 min to dissolve and form lipid vesicles. The suspension was then extruded, as described above, through two-stacked polycarbonate filters of either 100 or 400 nm pore size.

3.3. Particle size analysis

The mean particle size of the lipid vesicles were determined by dynamic light scattering at room temperature with a Brookhaven ZetaPlus Particle Sizing instrument (BI-MAS Option, Brookhaven Instruments Corporation, Holtsville, New York, USA), at the wavelength of 677 nm, using a 15 mW solid state laser as the light source and the MAS OPTION software supplied with the instrument. The scattered light was detected at a scattering angle of 90°. Samples for light scattering measurements were prepared in filtered distilled H_2O using 0.22 μm filters (Millipore, S.A., France). Particle size measurements were performed at least in triplicate.

3.4. Incorporation of DNA into anionic nanoliposomes

In order to prepare ternary complexes of nanoliposome/Ca^{2+}/DNA, as gene transfer vehicles, plasmid (15 μg/285 μg lipid) and then calcium ($CaCl_2$, 50 mM) were introduced to the nanoliposome suspensions and the mixtures were incubated for 30 min under nitrogen at room temperature. Nanolipoplexes were formed by using both types of the nanoliposomes used in this study (i.e. conventional vesicles and those prepared by the heating method). Formation of similar complexes of anionic liposome/Ca^{2+}/DNA has been confirmed by light scattering and UV spectrophotometry (Mozafari and Hasirci 1998) and by different microscopic studies (Zareie et al. 1997; Mozafari et al. 1998).

3.5. Cells

Immortal human respiratory epithelial cells (16HBE14o-), kindly donated by Dr. A. R. Evans (Liverpool John Moores University, England, UK), were maintained at 37 °C, 5% CO_2 and 95% humidity in T75 tissue culture

flasks (Starstedt, Leicester, UK). Complete growth media (cMEM) consisted of minimum essential medium (MEM) supplemented with fetal calf serum (10%), and penicillin/streptomycin (10000 U/mL, 10000 μg/mL). The cells were passaged when almost 80% confluent and plated in fresh cMEM.

3.6. Toxicity assays

HBE cells were plated in 96-well plates (BDH, Leicester, UK) and used for toxicity tests at 50–60% confluency. The cells were treated with the reagents, diluted with cMEM to provide the required concentration, and incubated for 24 h prior to toxicity assessment. *In vitro* toxicity was evaluated using the neutral red uptake (NRU) method of Borenfreund and Puerner (1985) and the MTT method of Mosmann (1983) except that both methods were performed simultaneously in the same microplate and the incubation time with NR and MTT was 2 h. Absorbance of each well was determined at 540 nm using a microplate reader (Titertek Multiskan® MCC/340 MKII Labsystems, Finland). Triton X-100 (78 ppm) was used as positive control. Viability was expressed as percentage to the control untreated cells.

3.7. Statistical analysis

Data are expressed as mean ± standard deviation of the mean from three or more experiments. A two way analysis of variance of the viability data, taking account of liposome concentration and method of preparation was performed. A two sample t-test taking account only of the method of liposome preparation was also performed. All analyses were carried out using the Minitab statistical package (v 13.1, MINITAB Inc. PA, USA). Statistical analysis showed no significant evidence that (within the range used) liposome concentration influenced the outcome ($P > 0.05$), but method of preparation was significant ($P < 0.001$). As there was no evidence that liposome concentration was a significant factor, a t-test was also performed and it confirmed that method of preparation was a significant factor ($P < 0.001$).

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