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Short Communication

**CONSTRUCTION OF STABLE ANIONIC LIPOSOME-PLASMID  
PARTICLES USING THE HEATING METHOD: A PRELIMINARY  
INVESTIGATION**

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**Abstract:** Recent advances in liposome technology have resulted in the production of effective drug delivery formulations, although toxicity concerns remain. In order to overcome this problem we prepared anionic liposomes without using any volatile organic solvent or detergent. Liposomes prepared by this heating method (HM-liposomes) were characterised in terms of morphology, stability and DNA incorporation efficiency. Scanning tunnelling microscopy (STM) and optical microscopy were used to study the morphological characteristics and size distribution of HM-liposomes. Microscopic studies revealed formation of spherical bilayered structures with stabilities of at least eight months and also enabled measuring the diameter and the bilayer thickness of the vesicles. Plasmid DNA encapsulation efficiencies of up to 70.3% were determined for HM-liposomes.

**Key words:** Anionic Liposomes, Plasmid DNA, Heating Method, Scanning Tunnelling Microscopy

**INTRODUCTION**

As a promising delivery system liposomes are becoming more and more favourable in drug administration to the human body. This is due to many distinct advantages of these lipid vesicles which include biocompatibility, targetability, ideal specific gravity, and possibility of producing them in different

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size ranges. Before any liposomal system could be utilised for drug delivery some critical points should be addressed: toxicity, stability, drug entrapment efficiency and size variation of the liposomal formulation [1]. Toxicity of some liposomes seems to be caused by the application of organic solvents or detergents during their manufacture, or employing cationic agents in their structure. The origin of toxicity caused by cationic liposomes has not been completely elucidated. It has been suggested that the interaction between the cationic liposomes and the anionic lipids of cell organelle membranes is responsible for cytotoxicity [2]. Another postulated mechanism for cationic lipid-mediated toxicity in lung is the involvement of reactive oxygen intermediates [3]. On the other hand, organic solvent residues, remaining in the lipid and/or aqueous phases of the liposomes during their preparation, could result in toxicity [4]. Recent developments have made it possible to prepare liposomes without using any organic solvent or detergent, examples of which are the polyol dilution method [5], the bubble method [6], and the heating method [7-8]. Here we present the characterisation of anionic liposomes prepared by the heating method in terms of their morphology, entrapment efficiency, and structural stability.

## MATERIALS AND METHODS

Lipids and solvents were of high purity grade obtained from Sigma Chem. Co. (Dorset, UK). Osmium tetroxide ( $\text{OsO}_4$ ) was purchased from Agar Scientific Ltd, Essex, UK. All other chemicals were of commercial analytical grade. Liposomes composed of dipalmitoylphosphatidylcholine (DPPC), dicetylphosphate (DCP), and cholesterol (in a 7:2:1 molar ratio) were prepared either by the conventional thin-film hydration method, or the heating method [7-8]. In the heating method the lipids are hydrated each during 1-2h at room temperature, separately, and then mixed at high temperatures (up to  $120^\circ\text{C}$ ) in the presence of glycerol (3% v/v) in a silicone oil bath. To obtain vesicles with smaller diameters liposomes were extruded, using an extruder (LiposoFast<sup>TM</sup>-Basic, Glen Creston Ltd, UK), 11 times, above the phase transition temperature ( $T_c$ ) of the lipids, through polycarbonate filters of either 100 or 400nm pore sizes. The effect of heating on the lipids was checked by thin layer chromatography (TLC) on silica gel using  $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}$  (65:25:4, by volume). In order to prepare the ternary complexes of liposome/ $\text{Ca}^{2+}$ /DNA, as a gene transfer vector, plasmid DNA (pcDNA3.1/His B/*lacZ* 15 $\mu\text{g}$ /285 $\mu\text{g}$  liposome) and then calcium (50mM) were introduced to the liposomal suspension and the complexes formed by the incubation of the mixture for 30min at room temperature. Formation of anionic liposome-DNA complexes in the presence of 50mM  $\text{Ca}^{2+}$  was well documented in our previous works [9-11]. Separation of free DNA was carried out by centrifugation at  $100 \times 10^3 \text{g}$  for 60min at  $4^\circ\text{C}$ . DNA concentration was measured spectrophotometrically, by multiplying the absorbance at 260nm by a factor of 50. Association of DNA to

HM-liposomes was confirmed by light scattering measurements, as well as through agarose gel electrophoresis studies [Mozafari et al., manuscript in preparation].

HM-liposomes were imaged by scanning tunnelling microscopy (STM) and by optical microscopy. For STM unilamellar vesicles were prepared using 100nm pore-size filters by the heating method and visualised as previously described [10-12]. Briefly, the sample was deposited on HOPG (highly oriented pyrolytic graphite) and was then dried at room temperature under atmospheric pressure in a clean room with a relatively slow drying rate. The STM was operated in air at atmospheric pressure with a tip-to-substrate bias of 800 mV-1.5 V (sample positive) and tunnelling currents of 20-50 pA. Liposomal samples used for the optical microscopy studies were prepared by the heating method, using 400nm pore-size filters, stained with 1% OsO<sub>4</sub> and visualised by Nikon Eclipse E600 microscope (Japan).

## RESULTS AND DISCUSSION

It was confirmed by TLC that no degradation of the lipids occurred at the above-mentioned temperatures. As it is essential to prove the formation of closed, continuous bilayered structures before proper application of the term liposome for any new phospho/lipid formulation, we performed STM and optical microscopic studies. The high-resolution imaging technique of STM, previously employed to visualise DNA [10-12], tRNA [13] and liposomes [10-12, 14], has been applied for two and three dimensional morphological investigation of the HM-liposomes and to study the effect of storage on their structure. STM also enabled measurement of the diameter and the bilayer thickness of the lipid vesicles. The STM of HM-liposomes, prepared by using 100nm filters, is shown in figure 1. Three vesicles are indicated inside circles with diameters of 92.3, 83.5, and 42.1nm, and mean bilayer thickness of around 10nm. Similar structures were observed for the HM-liposomes after eight months of their preparation and storage under N<sub>2</sub> at 4°C. Figure 2 demonstrates HM-liposomes visualised by optical microscopy. A total number of 16 vesicles can be observed in Fig. 2 which were prepared using filters with 400nm pore size. The mean diameter of these liposomes was measured to be 373.9nm which is less than the pore sizes of the filters used in their preparation. This has been encountered by other groups as well (for example see [5]). However, the mean particle sizes are close to the pore sizes of the filters used in their preparation and the particles exhibit a homogeneous size distribution. In addition, HM-liposomes showed no signs of aggregation as confirmed by the two microscopic techniques. An average plasmid entrapment efficiency of 70.3% ( $\pm 1.5$ ) was obtained for HM-liposomes which is higher than the value obtained for conventional liposomes (i.e. 50%).

In summary, the liposome preparation method described above is a mild technique capable of producing with ease, liposomes exhibiting good

reproducibility, long-term stability and with the potential of liposome production in large quantities. Another important point is that due to the employment of heat (and filtration when smaller sized vesicles required), in the manufacture of HM-liposomes, there is no need to carry out further sterilisation steps which consequently reduces the time and cost of liposome production by the heating method.

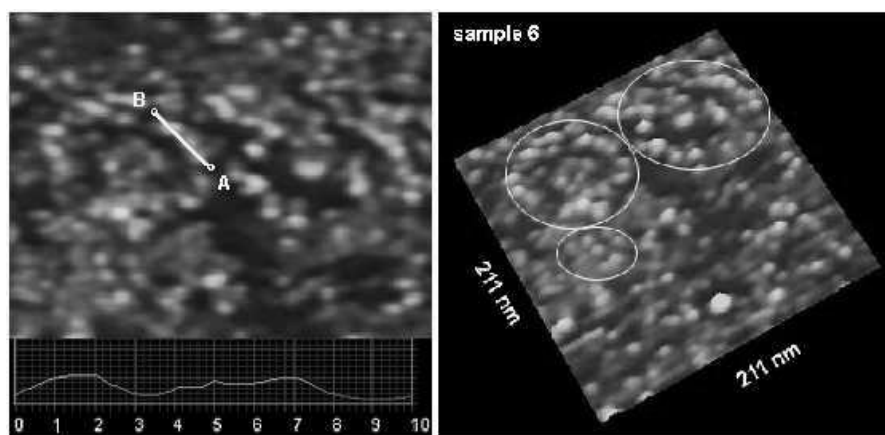


Fig. 1. STM image of six months old unilamellar vesicles. A profile of the surface corrugation is taken along the line AB. Scan area 211X211 nm.

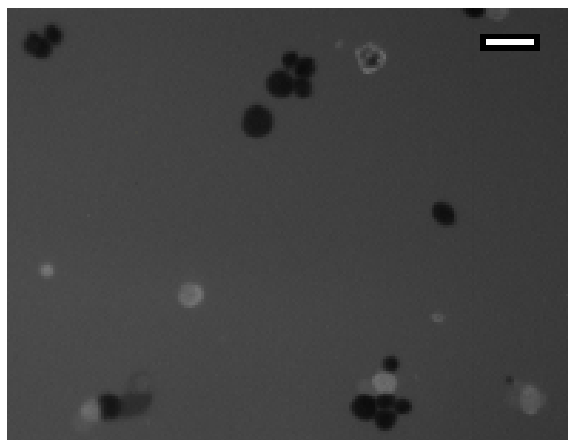


Fig. 2. Nikon Eclipse microscope image of HM-liposomes. Bar = 1 $\mu$ m.

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## REFERENCES

1. Mozafari, M.R., Reed, C.J., Rostron, C., Kocum C. and Piskin, E. Formation and characterisation of non-toxic anionic liposomes for delivery of therapeutic agents to the pulmonary airways. 9. Liposomes. From Models to Applications, Poland. **Cell. Mol. Biol. Lett.** 7 (2002) 243-244.
2. Xu, Y. and Szoka, F.C.Jr. Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection. **Biochemistry.** 35 (1996) 5616-5623.
3. Dokka, S., Toledo, D., Shi, X., Castranova, V. and Rojanasakul, Y. Oxygen radical-mediated pulmonary toxicity induced by some cationic liposomes. **Pharmaceut. Res.** 17 (2000) 521-525.
4. Cortesi, R., Esposito, E., Gambarin, S., Telloli, P., Menegatti, E. and Nastruzzi, C. Preparation of liposomes by reverse-phase evaporation using alternative organic solvents. **J. Microencapsul.** 16 (1999) 251-256.
5. Kikuchi, H., Yamauchi, H. and Hirota, S. A polyol dilution method for mass production of liposomes. **J. Liposome Res.** 4 (1994) 71-91.
6. Talsma, H., van Steenberg, M.J., Borchert, J.C. and Crommelin, D.J. A novel technique for the one-step preparation of liposomes and nonionic surfactant vesicles without the use of organic solvents. Liposome formation in a continuous gas stream: the bubble method. **J. Pharm. Sci.** 83 (1994) 276-280.
7. Mozafari, M.R., Reed, C.J. and Rostron, C. Development of non-toxic liposomal formulations for gene and drug delivery to the lung. **Technol. Health Care** (In Press).
8. Mozafari, M.R., Reed, C.J. and Rostron, C. Reduced cytotoxicity of anionic liposome/calcium/DNA complexes prepared by the heating method. British Pharmaceutical Conference Science Proceedings 2001, pp. 100 (Pharmaceutical Press, London, UK).
9. Mozafari, M.R. and Hasirci, V. Mechanism of calcium ion induced multilamellar vesicle-DNA interaction. **J. Microencapsul.** 15 (1998) 55-65.
10. Mozafari, M.R., Zareie, M.H., Piskin, E. and Hasirci, V. Formation of supramolecular structures by negatively charged liposomes in the presence of nucleic acids and divalent cations. **Drug Deliv.** 5 (1998) 135-141.
11. Zareie, M.H., Mozafari, M.R., Hasirci, V. and Piskin, E. Scanning tunnelling microscopy investigation of liposome-DNA-Ca<sup>2+</sup> complexes. **J. Liposome Res.** 7 (1997) 491-502.
12. Mozafari, M.R., Yamout, N. and Rajabi-Siahboomi, A.R. Factors affecting the liposomal delivery of nucleic acids. **Iran. J. Chem.** 11 (1998) 24-27.
13. Zareie, M.H., Piskin, K., Verimli, R. and Piskin, E. Imaging of tRNA by Scanning Tunnelling Microscopy. **Nanobiology.** 4 (1996) 101-104.
14. Zareie, M.H., Borucu, A., Ozden, M.Y., Hasirci, V. and Piskin, E. Imaging of liposomes by scanning tunneling microscopy. **Artif. Cell Blood Sub.** 24 (1996) 525-531.