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A comparison of carboxylate salts as liposomal cryoprotectants

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Abstract

The use of liposome suspension for the delivery of water soluble drugs is limited by drug leakage on long term storage. This problem may be overcome by freezing of the liposome suspension if the damage caused by the freeze-thaw process could be overcome. This paper describes an investigation into the use of various carboxylates as cryoprotectants for the frozen storage of liposomes. Although all the compounds investigated reduced the leakage of an entrapped marker from the liposomes, the choice of carboxylate salt was shown to be important in achieving maximal cryoprotection. Acetates were shown to be more effective than formates, propionates and hexanoates and the tetramethylammonium salts were found to be more effective than the sodium, potassium or ammonium salts.

Keywords: Carboxylate salts; Liposome cryoprotectants; Freezing and thawing; Betaines; Cryopreservatives; MLVs

1. Introduction

Liposomes have recently become more widely accepted as carriers for drug substances. These systems have a wide range of applications including targeted anticancer chemotherapy (Allen, 1994), drug delivery to the lung (Taylor and Farr, 1993) and the delivery of biotherapeutic peptides and proteins (Storm et al., 1991). However, their application is limited for water soluble drugs by the limited long term stability of the liposomal formulation (Ausborn et al., 1992). The leakage of water soluble drugs from liposomal suspensions can be particularly problematic as the free drug may exhibit toxicity which is normally restricted by the use of the liposomal carrier. It may however be possible to overcome the problems of stability by using frozen liposomal suspensions if the problems of osmotic changes and ice crystal formation on cooling and warming can be overcome. Previous work has suggested that the naturally occurring non-toxic compound glycinebetaine and its derivatives may be used to reduce leakage from liposomes following freezing and thawing (Higgins et al., 1986, 1987; Lloyd et al., 1992b, 1994). Glycinebetaine is a zwitterionic inner salt which has been shown to be effective at reducing salt stress in plants (Storey and Wyn Jones, 1977) and is structurally related to the tetramethylammonium carboxylates (Fig. 1). This paper describes a study of a series of structurally related compounds in an attempt to identify the

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features which dictate the ability of this compound to reduce freeze-thaw damage to liposomes.

2. Materials and methods

Grade IIb egg lecithin (Lipid Products, Redhill) was used as supplied. The phosphate buffer was prepared from sodium dihydrogen orthophosphate (Analar Grade, BDH, Dorset) and dipotassium hydrogen orthophosphate (Analar Grade, BDH, Dorset). All water was deionised and double distilled in glass. All other reagents were reagent grade commercial products and used without further purification.

2.1. Preparation of tetramethylammonium carboxylates

Tetramethylammonium formate, acetate, propionate and hexanoate were prepared by potentiometric titration of the appropriate acid (Aldrich Chemical Company) with tetramethylammonium hydroxide (5 M in 0.04 M phosphate buffer) and diluting the solutions obtained to the correct additive and buffer concentrations.

2.2. Preparation of N,N,N′,N′-tetramethylethylenediammonio-N,N′-diacetate

N,N′-Tetramethylethylenediamine (0.1 mol, 15 cm^3), ethylchloroacetate (0.3 mol, 30 cm^3) and ethanol (50 cm^3) were refluxed for 1 h. The ethanol was removed by reduced pressure rotary evaporation. Water (100 cm^3) and conc. HCl (10 cm^3) were added to the residue and the mixture refluxed for 3 h. Removal of water under reduced pressure gave a gelatinous solid which formed a white precipitate on addition of ethanol (100 cm^3). The white solid was collected by filtration using a sintered glass funnel and was recrystallised from ethanol/water to give *N,N′*-dicarboxymethyl-*N,N′*-tetramethylethylene diammonium dichloride. Yield: 12.9 g (42%); m.p.: 204–205°C; analysis (wt. %) carbon: 38.8 (39.3), hydrogen: 7.4 (7.2), nitrogen: 9.0 (9.2).

N,N′-Dicarboxymethyl-*N,N′*-tetramethylethylenediammonium dichloride was converted to the corresponding inner salt using ion exchange chromatography with a 300 mm × 25 mm glass column packed with 100 g (10 mequiv. excess) of an anionic exchange resin (Dowex-1, 1X8-50, 8% crosslinked). The column was equilibrated by passing NaOH (2 M, 100 cm^3) through at a rate of 2 cm^3 min^{-1}. The column was then rinsed with distilled water until the eluent was no longer alkaline to litmus (ca. 500 cm^3). The *N,N′*-dicarboxymethyl-*N,N′*-tetramethylethylene diammonium dichloride (5 g) was dissolved in water (5 cm^3) and pipetted onto the column. A reservoir of distilled water was attached to the head of the column and the dibetaine eluted from the column at a rate of 2 cm^3 min^{-1}; 200 cm^3 of eluent was collected and the water removed by reduced pressure evaporation. Final traces of water were removed by freeze drying the sample over night in an Edwards Modulo freeze dryer. The dried *N,N,N′,N′*-tetramethylethylenediammonio-*N,N′*-diacetate was stored in a vacuum desiccator over phosphorus pentoxide until required. The structure of the *N,N,N′,N′*-tetramethylethylenediammonio-*N,N′*-diacetate was confirmed using infra-red and NMR spectroscopy.

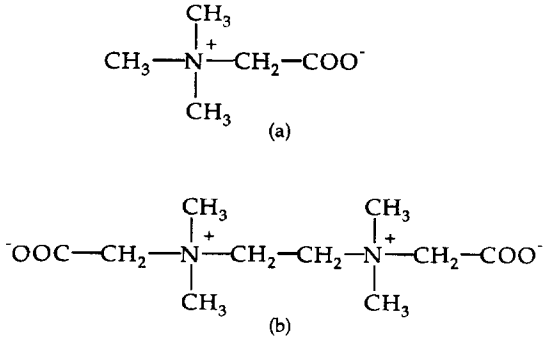

Fig. 1. Chemical structures of (a) glycinebetaine and (b) *N,N,N′,N′*-tetramethylethylenediammonio-*N,N′*-diacetate.

2.3. Liposome preparation

Multilamellar Large Vesicles (MLVs) were prepared as described previously (Lloyd et al., 1992b). In brief, a thin film of egg lecithin (Lipid Products) was deposited onto the walls of a 250 ml round bottom flask by reduced pressure rotary evaporation from a chloroform solution. Final traces of the solvent were removed by rotating the flask in a stream of oxygen-free nitrogen. The lipid was hydrated with a 1% w/v solution of a water soluble dye, amaranth (BDH), in 0.02 M pH 7.0 phosphate buffer (5.0 ml). The liposome suspension was transferred to a 10 ml polypropylene tube and stored at −20°C until required. After thawing, the liposome suspension was freeze-thawed three times by direct immersion of the tube in liquid nitrogen followed by immersion in a waterbath at 50°C, to improve the entrapment. The resultant liposome suspension was maintained at 50°C for 1 h to anneal the liposome structure. The suspension was then diluted with 0.02 M pH 7 phosphate buffer to give a lipid concentration of 2 mg ml^{-1}. The liposomes were washed twice to remove the unentrapped amaranth, before resuspension in 0.02 M phosphate buffer to give a stock solution containing 10 mg ml^{-1} lipid for use in freeze-thaw experiments.

2.4. Freeze-thaw protocol and determination of entrapped amaranth

The liposomes were diluted with cryoprotectant solutions and aliquots of the liposome suspensions (200 μl) were placed in 1.5 ml polypropylene Eppendorf centrifuge tubes. Samples were cooled in a circular rack by immersion into liquid nitrogen (2 min) and then warmed in a waterbath at 50°C (2 min). Phosphate buffer (1.0 ml) was added to each tube and the liposomes pelleted by centrifugation at 15 000 rev./min (15 600 × g) in an Eppendorf centrifuge (model 5414S). The supernatant was discarded and the liposome pellet washed by resuspension in buffer and further centrifugation. The liposome pellets were finally solubilised in 50% propan-1-ol (1.2 ml). The amaranth remaining entrapped was determined by measuring the absorbance of each solution at 522 nm using a Perkin-Elmer Lamda 2 spectrophotometer.

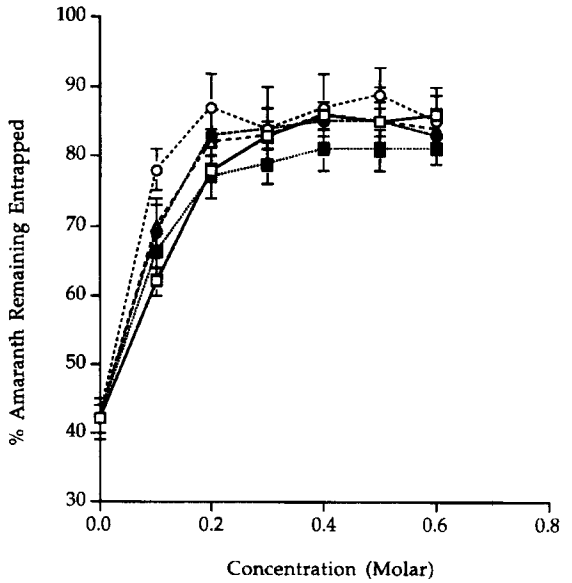

Fig. 2. The effect of glycinebetaine (□), sodium formate (■), sodium acetate (○), sodium propionate (●) and sodium butyrate (△) on amaranth leakage from MLVs on freeze-thaw. Bars indicate ranges (n = 3).

The cryoprotective activity was expressed as the percentage of amaranth remaining entrapped after the sample had been freeze-thawed.

3. Results and discussion

Triplicates of amaranth-containing liposomes suspended in buffer alone or buffer with various concentrations of the various additives were freeze-thawed as outlined above. In control experiments in which the liposomes were not frozen, there were no differences in leakage over the duration of the experiment unless otherwise indicated. Fig. 2 shows the effect of freezing liposome samples in the presence of various concentrations of glycinebetaine, sodium formate, sodium acetate, sodium propionate and sodium butyrate. The amaranth leakage decreased with additive concentration in each case and the carboxylates appeared to be more effective than glycinebetaine at lower additive concentrations. Fig. 3 shows the

effect of freezing the liposomes in the presence of glycinebetaine, ammonium acetate, potassium acetate and tetramethylammonium acetate using a different batch of liposomes which were less susceptible to freeze-thaw damage. However, the inclusion of glycinebetaine as a control permits the comparison of Figs. 2 and 3. Fig. 3 would suggest that tetramethylammonium acetate is more effective at reducing leakage than the other compounds particularly at lower additive concentrations. The increased leakage in the presence of the ammonium acetate is reflected in the increase in leakage observed in the control samples which were maintained at room temperature. This probably reflects the ability of ammonium acetate to penetrate the liposome resulting in leakage due to osmotic shock on dilution of the samples post-thawing prior to centrifugation. Similar effects have been reported for ammonium salts previously (Brearley et al., 1992). Magnesium acetate, not shown, also increased the leakage of entrapped amaranth from the MLVs on freeze-thaw at additive concentrations of 0.1–0.5 M. At concentrations above 0.5 M, the MLVs formed a

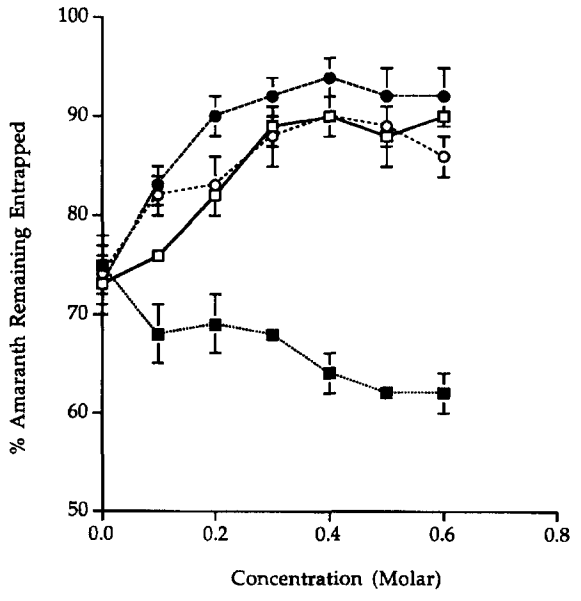

Fig. 3. The effect of glycinebetaine (□), ammonium acetate (■), potassium acetate (○), and tetramethylammonium acetate (●) on amaranth leakage from MLVs on freeze-thaw. Bars indicate ranges (n = 3).

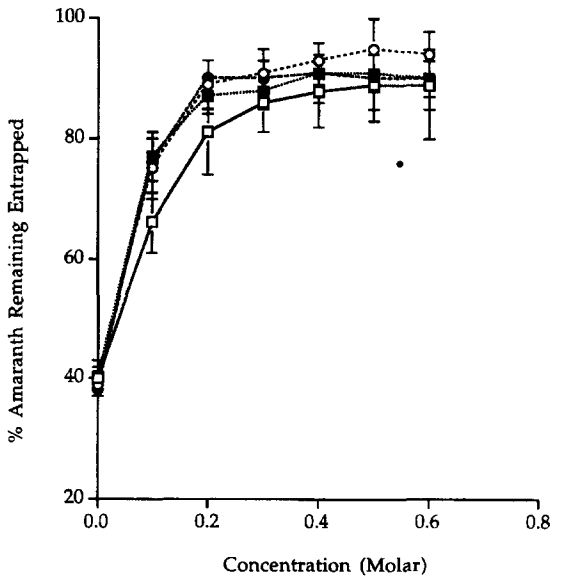

Fig. 4. The effect of glycinebetaine (□), tetramethylammonium formate (■), tetramethylammonium acetate (○) and tetramethylammonium propionate (●) on amaranth leakage from MLVs on freeze-thaw. Bars indicate ranges (n = 3).

solid gel on the side of the Eppendorf tube on centrifugation which could not be solubilised. Magnesium acetate was also found to increase leakage from the control samples of MLVs which were maintained at room temperature with 0.8 M magnesium acetate causing 85% leakage of entrapped amaranth over the course of the experiments.

Fig. 4 shows the reduction in leakage observed in the presence of various tetramethylammonium carboxylates. In general, the tetramethylammonium acetate appeared to be a more effective cryoprotectant than glycinebetaine, tetramethylammonium propionate and tetramethylammonium formate. Comparison with Fig. 2 suggests that the tetramethylammonium carboxylates are generally more effective that the sodium carboxylates at reducing leakage of water soluble markers from liposomes on freezing and thawing under the conditions detailed above. Tetramethylammonium hexanoate, not shown, was found to increase leakage from both the freeze-thawed MLVs and the control samples which were maintained at room temperature. Fig. 5 shows the liposomal

cryoprotection afforded by glycinebetaine, sodium chloride, choline chloride and tetramethylammonium chloride. Although this batch of liposomes were particularly sensitive to leakage on freezing and thawing, the graph clearly demonstrated that sodium chloride is much less effective than glycinebetaine, choline chloride and tetramethylammonium chloride at reducing leakage from frozen liposomes. Glycinebetaine is also clearly the most effective of this group of compounds which may reflect the fact that this compound has a free carboxylate group. Fig. 6 shows a comparison between glycinebetaine and *N,N,N′,N′*-tetramethylethylenediammonio-*N,N′*-diacetate which is essentially a dibetaine. There appears to be no difference between the ability of these two compounds to reduce freeze-thaw damage to MLVs suggesting that the absolute number of molecular species dictates the cryoprotective activity of the solution rather than the number of carboxylate groups.

In summary, these studies suggest that optimal cryoprotection is achieved in the presence of the carboxylate anion and the tetramethylammonium cation. Acetates appear to offer the maximal degree of protection against leakage on freezing and thawing. The longer chain carboxylates are probably less effective as these will have a tendency to penetrate the liposomal membrane and disrupt the bilayer structure. Previous studies have demonstrated that betaines which have a greater number of methylene groups between the quaternary ammonium group and the carboxylate group are more effective at reducing leakage from frozen liposomes (Lloyd et al., 1994). The results of the present study would suggest that increasing the distance between carboxylate and tetramethylammonium groups within the betaine molecules allows them to act independently of each other.

It has been suggested that the principal mechanism by which betaines and other ionic species reduce damage on rapid cooling is through facilitating glass formation thereby circumventing the establishment of large osmotic gradients due to the crystallisation of extracellular ice (Lloyd et al., 1992a,b). The ability of such compounds to facilitate glass formation is probably related to their

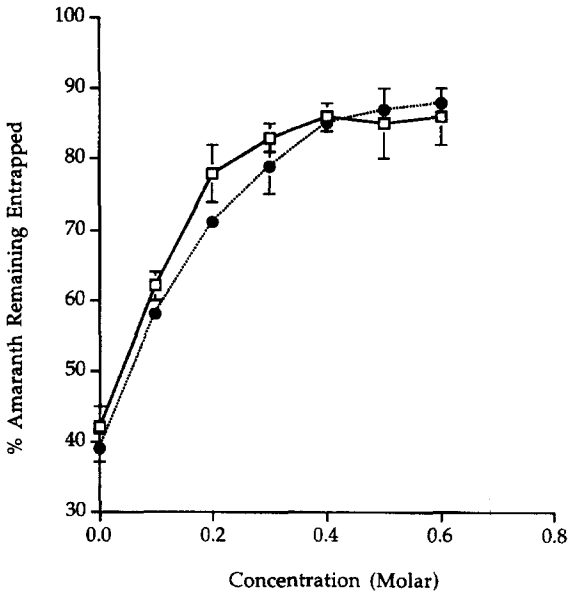

Fig. 6. The effect of glycine betaine (□), and *N,N,N′,N′*-tetramethylethylenediammonio-*N,N′*-diacetate (●) on amaranth leakage from MLVs on freeze-thaw. Bars indicate ranges (n = 3).

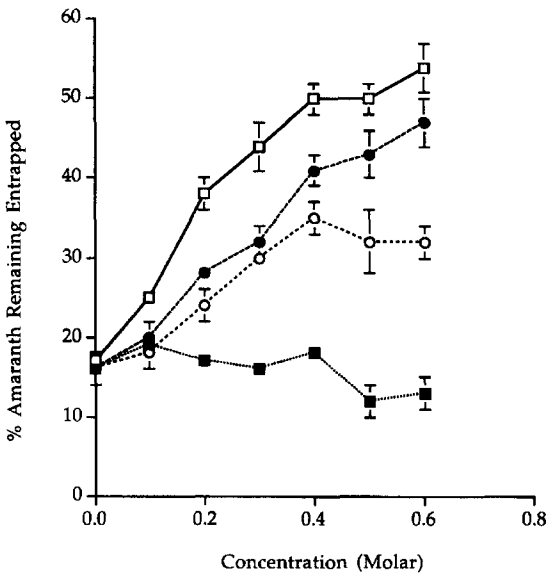

Fig. 5. The effect of glycinebetaine (□), sodium chloride (■), choline chloride (○) and tetramethylammonium chloride (●) on amaranth leakage from MLVs on freeze-thaw. Bars indicate ranges (n = 3).

ability to destructure bulk water limiting the formation of nucleation sites for crystal growth (Macfarlane and Forsyth, 1990). Previous studies have demonstrated that freeze-thaw injury to erythrocytes and erythrocyte ghosts is also strongly cation dependent. For the Group I cations, this dependence is nonmonotonic in nature with injury increasing in the order $Li^+ < Na^+ < Cs^+ < K^+$ and freeze-thaw damage to such systems may be reduced using less injurious cations such as tetramethylammonium acetate (Brearley et al., 1992). Similar nonmonotomic effects have been observed previously for the release of calcein from MLVs on freezing in the presence of various Group I chlorides (Brearley et al., 1991). It has been suggested that these nonmonotonic effects are due to differences in the cooling induced interactions of ions with the membrane/water interface. In contrast to the situation with the cations, the extent of injury to erythrocytes and erythrocyte ghosts induced by anions appears to follow a Hofmeister lyotropic series with injury increasing as the hydrated radius decreases and suggests that water structuring effects are important (Brearley et al., 1992). The results of the present study are consistent with these studies and suggest that minimal damage may be achieved by selecting cations which have minimal interactions at the membrane/water interface and anions which are highly hydrated. Such systems should minimise ion induced membrane fusion and/or disruption of the membrane bilayer and facilitate glass formation on cooling. The fact that the tetramethylammonium carboxylates are more effective than glycinebetaine may reflect the greater number of ionic species present at the same molar concentration which would increase the osmotic shrinkage of the MLVs prior to cooling and warming and reduce the likelihood of ice crystal formation within the vesicles during the cooling process.

Although freezing damage to liposomal drug delivery systems may be reduced by the inclusion of cryoprotective additives such as trehalose, the appropriate selection of buffer ions may also be critical in minimising freezing damage to liposomal formulations.

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