JPP Journal of Pharmacy and Pharmacology

JPP 2005, 57: 23–30

Received June 18, 2004
Accepted September 22, 2004
DOI 10.1211/0022357055092
ISSN 0022-3573

Kanamycin incorporation in lipid vesicles prepared by ethanol injection designed for tuberculosis treatment

Oselys Rodriguez Justo and Angela Maria Moraes

Abstract

The primary goal of this study was the production of liposomes encapsulating kanamycin for drug administration by inhalation. The selected drug is indicated for multiresistant tuberculosis, and administration through inhalation allows both local delivery of the drug to the lungs and systemic therapy. The ethanol injection method used for the liposome production is easily scaled up and is characterized by simplicity and low cost. Vesicles were prepared using different lipid compositions, including hydrogenated soybean phosphatidylcholine and cholesterol (SPC/Chol), egg phosphatidylcholine and cholesterol (EPC/Chol), distearoyl phosphatidylcholine and cholesterol (DSPC/Chol), distearoyl phosphatidylcholine, dimyristoyl phosphatidylethanolamine and cholesterol (DSPC/DMPE/Chol), dipalmitoyl phosphatidylcholine and cholesterol (DPPC/Chol) and dipalmitoyl phosphatidylcholine, dipalmitoyl phosphatidylglycerol and cholesterol (DPPC/DPPG/Chol). The effects of different operational conditions for vesicle production and drug encapsulation were evaluated, aiming at a compromise between final process cost and suitable vesicle characteristics. The best performance concerning drug incorporation was achieved with the DSPC/Chol system, although its production cost was considerably larger than that of the natural lipids formulations. Encapsulation efficiencies up to 63% and final drug to lipid molar ratios up to 0.1 were obtained for SPC/Chol vesicles presenting mean diameters of 132 nm incubated at 60°C with the drug for 60 min at an initial drug-to-lipid molar ratio of 0.16.

Introduction

The world has experienced, during recent years, the explosive occurrence of infectious diseases, such as tuberculosis, AIDS, hantavirus pulmonary syndrome and cholera, among others. Despite being under control during the 1980s, recently the number of tuberculosis cases has increased significantly due to the deterioration of public health services mostly in developing countries, in association with the advent of HIV infection. Nowadays it is considered one of the diseases that kills the most adults, numbers of deaths worldwide reaching as high as 3 million yearly. This has led to the search for new strategies for tuberculosis therapy. It is estimated that one in every three people is infected with *Mycobacterium tuberculosis* (Corless et al 2002).

A combination of antibiotics can be successfully used for tuberculosis therapy, including isoniazid, rifampicin (rifampin), pyrazinamide, streptomycin, ethambutol and thioacetazone (WHO 2003). The therapy, however, is characterized by side effects, such as toxicity to normal cells, and extended treatment periods as well as the need for frequent doses to provide adequate drug concentration in the compartments where the *M. tuberculosis* lodges. If the patient withdraws from the therapy, the microorganism can become resistant to the drugs. For the management of chronic or multidrug-resistant tuberculosis cases, drugs such as kanamycin should be employed, mostly if the microorganism is resistant to streptomycin (WHO 2003).

Kanamycin is an aminoglycosidic antibiotic, normally employed as a sulfate salt, and its mechanism of action is based on microorganism protein synthesis inhibition. Its chemical structure is shown in Figure 1, and since the molecule is a cation, it is poorly absorbed orally. The optimal dose is 15 mg kg^{-1} body weight and the drug is normally administered daily or on 5 days per week by deep intramuscular injection. Its main

Department of Biotechnological Processes, Chemical Engineering School, State University of Campinas (UNICAMP), CP 6066, Campinas – SP, 13083-970, Brazil

Oselys Rodriguez Justo, Angela Maria Moraes

Correspondence: A. M. Moraes, Department of Biotechnological Processes, Chemical Engineering School, State University of Campinas (UNICAMP), CP 6066, Campinas - SP, 13083-970, Brazil. E-mail: ammoraes@feq.unicamp.br

Funding: the authors are grateful to the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), from Brazil, for the financial support to this research.

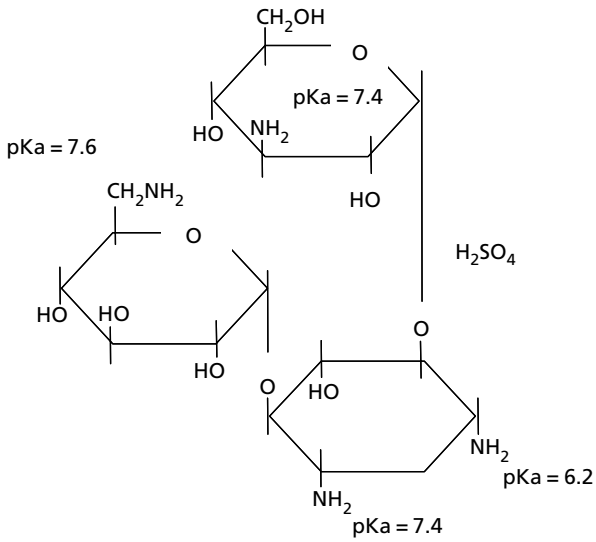

Figure 1 Kanamycin A sulfate chemical structure and pK_a values for the distinct amino groups (adapted from Walter et al 1999).

possible adverse reactions are ototoxicity, deafness, vertigo and reversible nephrotoxicity (WHO 2003).

Tuberculosis was declared a world emergency in 1993 by the WHO and multidrug resistance related to poor treatment management is becoming a serious concern in several countries. Therefore, there is increased interest in the development of new therapeutic modalities that could increase the ratio between the effectiveness and the side effects of classic tuberculostatic drugs. In this sense, the modification of drug pharmacokinetics and biodistribution through the use of alternative drug administration routes, such as by inhalation, and by drug incorporation in liposomes could improve therapeutic results (Justo & Moraes 2003).

The administration of drugs through the lungs is particularly interesting in the case of tuberculosis therapy, since local and systemic drug delivery can be simultaneously performed. In addition, if the antibiotics are administered incorporated in liposomes, which can be can be prepared using materials endogenous to the lungs, sustained drug release could be achieved, provided that vesicles with appropriate diameters are employed. Particles larger than 15 μm are retained in the throat and swallowed, while particles with diameters of 2–15 μm can be deposited throughout the airway system. For targeting the alveoli, particles with diameters in the range of 0.1–2 μm should be employed (Taylor & Newton 1992; Bridges & Taylor 2000). If both pulmonary and systemic delivery are desired, vesicles should present diameters around 100 nm to be able both to be deposited in the alveoli and to present reduced clearance from the blood stream by the reticuloendothelial system.

Due to the potential benefits of antibiotics encapsulated in liposomes for the therapy of tuberculosis, during the last decade many liposomal systems have been developed for this particular application (Agarwal et al 1994; Gangadharam et al 1995; Kurunov et al 1995; Wong et al 1995; Deol & Khuller 1997; Deol et al 1997; Adams et al 1999; Labana et al 2002; Justo & Moraes 2003).

Specifically concerning the efficacy of nebulized liposomal formulations, promising results are reported in the literature against chronic pulmonary (Beulac et al 1999) and respiratory-tract infections (Conley et al 1997). The recently published work of Vyas et al (2004) also indicates that the delivery of rifampicin in liposomal formulations to albino Wistar rats through aerosol improves chemotherapy against pulmonary tuberculosis.

In most studies, assessment of in-vitro and in-vivo performance is focused upon, while the production and characterization of the liposomal systems are not described in detail. Also, process scale-up is normally not a concern, and liposomes are prepared by time consuming and cumbersome methods, such as lipid hydration followed by extrusion through polycarbonate membranes.

This report details the production of liposomes designed to increase the effectiveness of kanamycin through its controlled release by inhalation. This compound should be a fairly good option for encapsulation into liposomes by active loading, since it presents low molecular weight and adequate pK_as, in spite of its low partition coefficient in octanol/water systems. Phosphatidylcholine and cholesterol were used as the main components of lipid vesicles, aiming for adequate vesicle storage stability in aqueous solution. The ethanol injection approach was employed to produce the vesicles mainly due to its simplicity and low cost requirements, characteristics that render it easily scaled up.

Materials and Methods

Materials

Soyabean phosphatidylcholine (SPC) was from Lucas Meyer Co. (Hamburg, Germany). Egg phosphatidylcholine (EPC), dipalmitoyl phosphatidylcholine (DPPC), distearoyl phosphatidylcholine (DSPC), dimyristoyl phosphatidylethanolamine (DMPE), dipalmitoyl phosphatidylglycerol (DPPG), cholesterol (Chol) and kanamycin A monosulfate were obtained from Sigma Chemical Co. (St Louis, MO). All reagents used were of at least analytical grade.

Evaluation of apparent kanamycin partition coefficient in 1-octanol-aqueous phase systems

The interactions between kanamycin and liposomal membranes were roughly characterized by the evaluation of drug partition in systems containing 1-octanol and aqueous solutions of different compositions essentially as described by Moraes et al (1999). Briefly, kanamycin was dissolved at final concentrations of 0.2 mM in the following aqueous solvents: deionized water, 300 mM citric acid solution, pH 4.0 and 7.4, and 10 mM HEPES, pH 7.4. Three millilitres of each solution were mixed with 3 mL of 1-octanol pre-saturated with deionized water. The samples, pre-equilibrated at 30°C, were mixed by repeated inversion (30 times), and then the phases were allowed to

separate for 30 min at the same temperature. Samples from the bottom phase were collected and analysed for kanamycin concentration.

The apparent partition coefficient was defined as the top phase (1-octanol) kanamycin concentration, estimated as being the difference between the amount originally added and the quantity detected in the aqueous phase, divided by the kanamycin concentration in the bottom aqueous phase. No correction has been applied to include material adsorbed to the tube walls or air/octanol and octanol/water interfaces.

Vesicle preparation

Liposomes with different lipid compositions were prepared by ethanol injection (Batzri & Korn 1973), in few experiments at small scale dispersed with bath sonication, and at larger scale through the use of a pump and a mechanical impeller. The following lipid compositions were employed: SPC/Chol (60:40 mol%); EPC/Chol (60:40 mol%); EPC/Chol (70:30 mol%); DSPC/Chol (60:40 mol%); DSPC/DMPE/Chol (40:20:40 mol%); DPPC/DPPG/Chol (55:5:40 mol%) and DPPC/Chol (60:40 mol%).

For small-scale (up to 10 mL of liposome dispersion final volume) preparation, the method consisted of injecting the lipids solubilized in ethanol at appropriate proportions into a 300 mM citrate buffer at pH 4.0, using a 4-μm (in diameter) stainless-steel needle coupled to a 1-mL glass syringe, while sonicating for 3 min in a bench sonicator at 65°C. The final ethanol concentration in the citrate buffer was kept constant at 5% (v/v). Before the injection, the lipids suspended in ethanol were heated at 50°C for 5 min to improve the solubility of the preparations.

For larger-scale preparation, an automated system was used. The vesicles were obtained by the injection of the lipids solubilized in ethanol (also pre-heated at 50°C for 5 min) through a syringe pump (model ST-670T, Samtronic) coupled to a 4-μm (in diameter) stainless-steel needle into the citrate buffer, at the ratio of 4 volumes of organic solution to 76 volumes of aqueous solution. While injecting the alcoholic solution and 3 min after, the mixture, kept at different temperatures, was homogenized through an impeller (model Q-251D2, Quimis) at distinct mixing rates.

Kanamycin incorporation in liposomes

The drug was actively loaded into liposomes presenting a transmembrane pH gradient as follows. The vesicles, originally prepared at pH 4.0, had their external media titrated to pH 7.4 with 10 M NaOH. The liposomal samples, now presenting an internal pH equal to 4.0 and external pH of 7.4, were diluted and mixed with appropriate amounts of kanamycin dissolved in 10 mM HEPES buffer at pH 7.4. The resulting suspensions were incubated at several temperatures for different periods at 200 rev min^{-1}.

For comparison purposes, the drug was passively loaded in liposomes prepared in small scale as follows. An SPC and Chol lipid suspension at 100 mM in ethanol previously heated at 50°C was injected in an aqueous solution at 65°C containing 100 mM of kanamycin dissolved in 10 mM HEPES, pH 7.4, using a 4-μm (in diameter) stainless-steel needle coupled to a 1-mL glass syringe, while sonicating for 3 min in a bench sonicator, at an initial drug-to-lipid ratio of 20. As previously described, the final ethanol concentration in the drug aqueous solution was 5% (v/v).

After the encapsulation of kanamycin, unentrapped drug was removed by gel permeation chromatography using 10 mM HEPES buffer, pH 7.4, as eluent. A Sephadex G-50 column equilibrated with the same buffer was used.

Statistical analysis

A 2^3 experimental design with duplicates at the central point was employed to study the effects of different operational conditions during drug incorporation in liposomes. In this set of tests, the influence of the initial drug-to-lipid molar ratio (0.16–1.6), incubation temperature (25–60°C) and period (10–60 min) on final drug-to-lipid molar ratio and drug encapsulation efficiency was evaluated. Analysis of variance of the obtained data was performed through the software Statistica version 5.0 (StatSoft Co.). Significance levels of $P < 0.1$ and $P < 0.05$ for the final drug-to-lipid molar ratios and the drug active encapsulation efficiency, respectively, were assumed to be statistically significant in this study.

In the set of experiments performed in larger scale for SPC/Chol (60:40 mol%) liposomes, a second 2^3 experimental design with duplicates at the central point was employed to determine the effects of initial lipid concentration, mixing rate and organic solution injection flow rate on mean vesicle diameter and kanamycin active encapsulation efficiency. The experimental ranges selected (initial lipid concentration 50–100 mM, stirring rate 150–650 rev min^{-1} and organic solution injection flow rate 60–300 mL h^{-1}) were based on the data reported by Szoka (1996). As before, data analysis of variance was performed through the software Statistica 5.0. A significance level of $P < 0.1$ was assumed to be statistically significant in this study.

Vesicle characterization

The hydrodynamic radii of the vesicles before and after antibiotic entrapment were determined by quasi-elastic light scattering (QLS), based on the measurement of time-dependent fluctuations of scattered light intensity to determine the particle diffusion coefficient in diluted solutions. Measurements were performed at a 90° scattering angle, using an Autosizer 4700 equipment (Malvern Instruments).

Determination of the phospholipid concentration of liposomal samples using modifications of a total phosphate spectrophotometric assay technique (Chen et al 1956) was performed using a Beckman DU 640 UV-visible spectrophotometer. The molar concentration of total lipid was calculated dividing the phospholipid concentration

determined using the phosphate assay by the mole fraction of phospholipid in the vesicle preparation (equal to 0.6), to account for the presence of cholesterol.

Kanamycin concentration was determined, after vesicle disruption with Triton X-100, using 2,4,6-trinitrobenzenesulfonic acid hydrate (TNBS), which reacts with primary amines to give a color change that can be monitored at 410 nm, according to Moraes et al (1999). As the lipid DMPE also reacts with the TNBS, the effect of its presence was considered when necessary.

Percentual encapsulation efficiency was calculated as the drug-to-lipid molar ratio after removal of unentrapped compound divided by the initial kanamycin-to-lipid molar ratio, before free compound removal.

Evaluation of liposome storage stability

Vesicle stability concerning storage at 5°C was evaluated for 8 weeks by monitoring changes in vesicle mean diameter.

Results and Discussion

Partitioning of kanamycin in 1-octanol/aqueous solutions systems

The results of the experimental determination of the partitioning of kanamycin in systems containing 1-octanol and different aqueous solutions focusing the external and internal environments of liposomes presenting a pH gradient are illustrated in Table 1.

The low partition coefficients observed indicate, as expected, preferential accumulation of the therapeutic compound in the aqueous phase and dominating hydrophilic character. From the analysis of the chemical structure of this compound and pK_a data for its four amino groups, at $pH < 6.2$, the molecule would predominantly present four positive charges, while at pH 6.2–7.4, three positive charges would be expected. At pH values of 7.4–7.6, only one amino group would be preferentially ionized, and at pH values > 7.6, the molecule would be uncharged. Therefore, low apparent partition coefficients were expected at all the pH values evaluated, although these coefficients could be influenced by the nature of the aqueous solution.

Table 1 Partition coefficients for kanamycin between 1-octanol and different aqueous solutions

Aqueous phase composition	Apparent partition coefficient
Deionized water	0.013 ± 0.010
Citric acid, 300 mM, pH 4.0	0.313 ± 0.021
Citric acid, 300 mM, pH 7.4	0.290 ± 0.161
HEPES, 10 mM, pH 7.4	0.000 ± 0.000

Aqueous solutions: 300 mM citric acid at pH 4 or 7.4; deionized water; and 10 mM HEPES at pH 7.4 at 30°C. All tests performed in duplicate. Values are presented as mean ± s.d.

According to the results for the citric acid systems both at pH 4.0 and pH 7.4, positively charged drug molecules were able to discretely partition as an ion-pair formed by the charged kanamycin and the citrate anion provided by the buffer. However, the ion species suffers partition only while it can still be effectively neutralized by the citrate and solvated by the slightly polar oil phase. In the remaining systems (deionized water and HEPES buffer at pH 7.4), however, no counter-ions could be provided by the aqueous phase, and as a consequence, kanamycin remains positively charged, not being able to significantly accumulate in the organic phase.

Since results from the partition coefficient analysis can not, by themselves, explain the behaviour observed for the active loading of a given compound in liposomes (Madden et al 1990), kanamycin active loading in liposomes through pH gradients, despite being possible due to the four primary amino groups present in the drug structure, would still be limited, in all solvents tested, by the high hydrophilic character of the drug. On the other hand, once in the aqueous core of the vesicles, the ionized drug would remain effectively trapped.

Effect of operational conditions during incorporation in liposomes

The incorporation of kanamycin in SPC/Chol liposomes was evaluated for different operational conditions through a 2^3 experimental design, aiming to improve drug encapsulation, despite its strong hydrophilic character. The lipids dissolved in ethanol were, in this isolated experiment, manually injected in the citrate buffer heated at 65°C, resulting in vesicles with average diameters of 132–152 nm. The results obtained are illustrated in Table 2.

It is clear that the incorporation efficiency and the final drug to lipid molar ratio are strongly affected by the operational conditions employed. The best encapsulation efficiency was achieved with a low initial drug-to-lipid molar ratio and high incubation temperature and period. The best final drug-to-lipid molar ratio, on the other hand, was obtained for vesicles incubated at 42.5°C for 35 min at an initial drug-to-lipid molar ratio of 0.88 (tests 9A and 10A). These results are relevant to the final product administration in man, since they are associated with lower lipid dosage requirements. Considering that the therapeutic efficacy of liposomal kanamycin would be at least equivalent to that observed for the drug in its free form, it is possible to estimate the volume of liposomal preparation required for drug in-vivo administration. For a preparation presenting a final drug-to-lipid molar ratio around 0.1 mM and total lipid concentration of 2 mM, a total volume of approximately 30 mL would be required daily, and this value seems to be acceptable.

The statistic analysis of these data indicates that increases in all tested variables generally resulted in improvement of the final drug-to-lipid molar ratios. However, for the final drug-to-lipid molar ratio, only the effect of the incubation temperature was statistically

Table 2 Experimental design data for the incorporation of kanamycin at different conditions in SPC/Chol liposomes prepared in bench scale by injection at 65°C

Test	Initial drug-to-lipid molar ratio	Incubation temp. (°C)	Incubation period (min)	Final drug-to-lipid molar ratio	Encapsulation efficiency (%)
1A	0.16	25	10	0.0368 ± 0.0008	23.0 ± 0.5
2A	1.60	25	10	0.0352 ± 0.0015	2.2 ± 0.1
3A	0.16	60	10	0.0387 ± 0.0040	24.2 ± 2.5
4A	1.60	60	10	0.0816 ± 0.0043	5.1 ± 0.3
5A	0.16	25	60	0.0397 ± 0.0004	24.8 ± 0.3
6A	1.60	25	60	0.0448 ± 0.0054	2.8 ± 0.3
7A	0.16	60	60	0.1006 ± 0.0031	62.9 ± 1.9
8A	1.60	60	60	0.0704 ± 0.0013	4.4 ± 0.1
9A	0.88	42.5	35	0.1206 ± 0.0047	13.7 ± 0.5
10A	0.88	42.5	35	0.1135 ± 0.0008	12.9 ± 0.1

Initial mean vesicle diameters = 132 nm. Central points (9A and 10A) were performed in duplicate to estimate the experimental error.

significant, while for the encapsulation efficiency, all independent variables and their interactions were statistically significant. The analysis of variance for the final drug-to-lipid molar ratio and kanamycin incorporation efficiency showed that the correlation coefficient was above 0.9 only for the drug incorporation efficiency; however, for this dependent variable, the calculated F value was only 2.3 times that of the listed value. Therefore, statistically significant mathematical models cannot be proposed for the tested system at the stipulated confidence levels.

The results showed that the variable that presents the strongest effects on system performance is the initial drug-to-lipid molar ratio when considering encapsulation efficiency. Thus, low values of initial drug-to-lipid molar ratio were selected to be used in the following experiments. Since long incubation periods at high temperatures could result in drug thermal degradation, incubation at low initial drug-to-lipid molar ratios at 60°C was subsequently performed for a period of only 10 min.

Influence of operational conditions during liposome preparation on kanamycin incorporation

Different operational conditions employed during vesicle production could result in distinct lipid packing patterns, affecting drug incorporation and retention. The results of the active loading of kanamycin in SPC/Chol liposomes prepared in larger scale are shown in Table 3, for vesicles prepared at room temperature varying stirring rate, initial lipid concentration and injection rate.

The mean vesicle diameters before the chromatographic process varied from 390 to around 2000 nm, possibly as a result of the poor solubility of the lipids in ethanol at high initial lipid concentrations, the difficult dispersion of these solutions in the aqueous phase and the low temperature of the aqueous phase. Drug encapsulation efficiency varied from 20 to 100%, with the best results obtained for vesicles prepared at an agitation rate of 150 rev min^{-1}, an initial

Table 3 Complete experimental design (2^3) for the evaluation of the effects of initial lipid concentration, lipid in ethanol solution flow rate and mixing rate on mean SPC/Chol vesicle diameter and on kanamycin incorporation efficiency

Test	Stirring rate (rev min^{-1})	Lipid solution flow rate (mL h^{-1})	Initial lipid concn (mM)	Mean vesicle diameter (nm)*	Encapsulation efficiency (%)
1B	150	60	50	432.2 ± 2.1	57.2 ± 18.9
2B	650	60	50	293.8 ± 4.8	52.9 ± 1.7
3B	150	300	50	571.4 ± 9.3	59.7 ± 20.8
4B	650	300	50	216.0 ± 7.5	25.2 ± 7.7
5B	150	60	100	457.7 ± 5.2	100.0 ± 0.0
6B	650	60	100	290.0 ± 4.4	66.9 ± 10.2
7B	150	300	100	604.3 ± 2.0	100.0 ± 0.0
8B	650	300	100	333.6 ± 5.4	74.7 ± 1.1
9B	400	180	75	228.0 ± 6.9	25.1 ± 5.7
10B	400	180	75	264.8 ± 2.0	20.0 ± 8.6

*Samples submitted to gel permeation chromatography. Aqueous phase temperature during lipid injection: 30°C. Operational conditions for drug incorporation: initial drug-to-lipid molar ratio of 0.2, incubation temperature of 60°C, incubation time of 10 min, agitation at 200 rev min^{-1}. Central points (9B and 10B) were performed in duplicate to estimate the experimental error.

lipid concentration of 100 mM and a lipid solution injection flow rate of 60 mL h^{-1} (test 5B) or 300 mL h^{-1} (test 7B).

Statistical analysis showed that the variable that most affected vesicle diameter was the stirring rate. An increase in this variable resulted in a significant decrease in the response variable, and was statistically significant at a confidence level of 90%. It should be noticed, however, that if drug removal by gel permeation chromatography were not performed, some of the vesicles obtained in this set of experiments would be too large and, therefore, not appropriate for liposomal administration through inhalation. Since the mixing rate intensity determines the movement and the organization of the molecules in the aqueous phase, strong agitation results in increased system homogeneity, preventing aggregation of lipid molecules and the formation of large particles, as pointed out by Szoka (1996). An inverse effect can be associated to the lipid solution flow rate and concentration; an increase in these variables produces large vesicles, possibly due to local supersaturation in lipids.

Concerning drug incorporation efficiency, lipid concentration and stirring rate were the variables that presented the strongest effect on this dependent variable. Increases in lipid concentration and decreases in stirring rate resulted in increases of drug incorporation efficiency, and these effects were statistically significant ($P < 0.1$). The higher drug encapsulation efficiencies were obtained for large vesicles probably due to their larger citric acid capture volume in the vesicle aqueous core. This results in enhanced intravesicular buffering capacity, allowing increased drug incorporation (Mayer et al 1990).

Therefore, to reduce vesicle diameters, one should consider augmenting the stirring rate and decreasing the lipid concentration in the ethanolic solution as well as the injection flow rate. On the other hand, to optimize kanamycin encapsulation efficiency, it would be necessary to increase the lipid concentration in the ethanolic solution and reduce the injection flow rate and the stirring rate. This is a clear compromise situation, in which the choice of operational conditions should, ideally, satisfy the largest possible number of requirements. Increases in lipid concentration, for instance, could not be effectively achieved in this system due to the limited solubility of SPC and Chol in ethanol at room temperature. The addition of dimethyl sulfoxide (DMSO), as suggested by Szoka (1996), did not improve lipid solubility, possibly because SPC is not as soluble in this solvent mixture as the lipid tested by the author (egg phosphatidylcholine).

The analysis of variance for mean vesicle diameters and kanamycin encapsulation efficiency indicated that also in this case no statistically significant mathematical models can be proposed to efficiently describe the behaviour of the evaluated system at the stipulated confidence levels. For both dependent variables, low correlation coefficients were obtained and the ratios between the calculated F values and the listed ones were lower than 4.

The passive incorporation of kanamycin in the liposomes prepared in small scale at 65°C led to an average encapsulation efficiency of 1.45%, equivalent to a final drug-to-lipid ratio of 0.29 in 157-nm vesicles. This result is clearly superior concerning the final drug-to-lipid molar ratio, although its cost is much higher than any of the tested systems, since up to 20 times more drug is used per mole of lipid.

Effect of lipid composition on the incorporation of kanamycin in liposomes

Since lipid composition can affect the permeability of the liposomal bilayer to the drug for which encapsulation is desired, seven different formulations were tested in the larger-scale experimental apparatus for the incorporation of kanamycin. The different liposome compositions were formulated to determine if, when comparing with the less expensive SPC/Chol formulation, improved drug incorporation and vesicle stability could be achieved. Cholesterol was included in all formulations to improve lipid packing and membrane permeability characteristics.

While the natural phospholipid soybean lecithin presents high stability and biocompatibility, allowing the production of vesicles more resistant to oxidation processes, egg phosphatidylcholine presents lower transition temperature from gel to liquid crystal (Tc) and is more soluble in ethanol, thus resulting in liposomal preparations with larger lipid concentrations.

Since phospholipids obtained from natural sources are normally a mixture of different lipids that can vary from lot to lot, synthetic lipids with high Tc values were also evaluated as the major components of the liposomal membranes. According to the producer, the major component of the SPC employed is dipalmitoyl phosphatidylcholine. However, data from differential scanning microcalorimetry analysis and Langmuir–Blodgett isotherms (data not shown) for this particular lipid indicates behaviour more similar to DSPC than to DPPC. Therefore, both synthetic lipids were also evaluated as major components of the vesicles.

Previously obtained results (Moraes et al 1999) showed that vesicles composed of DSPC/DMPE/Chol containing a boronated compound encapsulated by active loading present high stability during storage in aqueous suspension. Therefore, this formulation was also tested for the incorporation of kanamycin. Finally, the last type of liposome preparation based on DPPC/DPPG/Chol was employed due to the similarity of this formulation to lung surfactant, which contains 85% of lipids, mainly dipalmitoyl phosphatidylcholine followed by phosphatidylglycerol, cholesterol, proteins and carbohydrates (Taylor & Newton 1992).

Taking into account the analysis of the previous results achieved for SPC/Chol vesicles, the liposomes with different compositions were prepared by injection of the different lipid solutions in ethanol at a flow rate of 60 mL h^{-1} in citrate solution at 50°C and stirred at 650 rev min^{-1}. The temperature of the aqueous phase was increased, aiming to reduce mean vesicle diameters and to improve lipid packing and distribution in the liposomal bilayer. Drug active incorporation at an initial kanamycin-to-lipid molar ratio of 0.16 was performed at 60°C for 10 min at 200 rev min^{-1} and the results are shown in Table 4.

Table 4 Effect of lipid composition on the incorporation of kanamycin in liposomes

Liposomal composition	Final drug-to-lipid molar ratio	Encapsulation efficiency (%)	Mean vesicle diameter (nm)
SPC/Chol (60:40 mol%)	0.033 ± 0.006	20.3 ± 4.3	107.9 ± 3.3
EPC/Chol (60:40 mol%)	0.018 ± 0.009	11.3 ± 6.0	116.8 ± 2.8
EPC/Chol (70:30 mol%)	0.010 ± 0.002	6.3 ± 1.1	128.3 ± 0.1
DSPC/Chol (60:40 mol%)	0.093 ± 0.001	57.8 ± 0.6	108.4 ± 2.9
DSPC/DMPE/Chol (40:20:40 mol%)	0.032 ± 0.035	19.7 ± 22.0	145.2 ± 2.4
DPPC/DPPG/Chol (55:5:40 mol%)	0.043 ± 0.005	26.6 ± 2.7	122.7 ± 0.2
DPPC/Chol (60:40 mol%)	0.022 ± 0.013	13.5 ± 9.2	136.5 ± 0.0

Vesicles were prepared in the automatic system, at 50°C, 650 rev min^{-1} and 60 mL h^{-1}, and were incubated with kanamycin at an initial drug-to-lipid molar ratio of 0.16, at 200 rev min^{-1} for 10 min at 60°C. All tests were performed in duplicate. Data are reported as mean values ± s.d.

Vesicles with mean diameters < 150 nm were obtained for all formulations, and the highest encapsulation was achieved for the synthetic DSPC-based composition, closely followed by DSPC/DMPE/Chol, DPPC/DPPG/Chol and SPC/Chol formulations. However, the cost of the last mentioned formulation was much smaller than the others (commercial SPC can be around 1000 times less expensive than the synthetic phospholipids DSPC, DPPC and DPPG tested), and for this reason, this formulation is recommended.

The results of the storage at 5°C of the liposomes prepared with different lipid formulations and actively encapsulating kanamycin, monitored during 8 weeks, are illustrated in Figure 2. The mean vesicle diameters did not present abrupt changes, although a moderate tendency to increase was observed in the majority of the evaluated systems. Since no aggregation or fusion was observed, these vesicles can be considered as fairly stable.

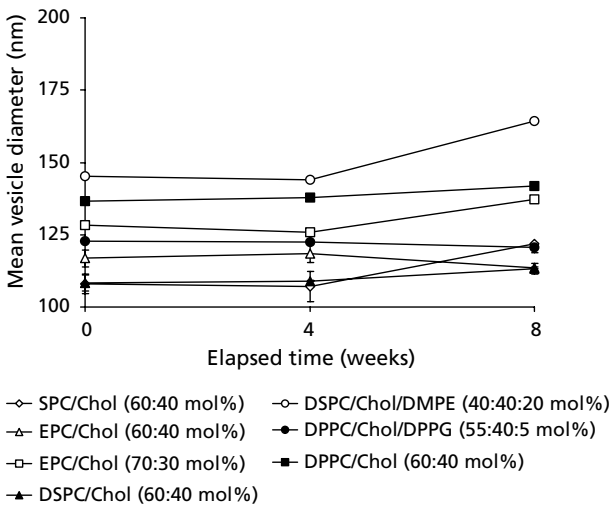

Figure 2 Variation in mean vesicle diameters, over 8 weeks at 5°C, of liposomes presenting different lipid compositions and encapsulating kanamycin at an initial drug-to-lipid ratio of 0.16. All tests were performed in duplicate. Values are presented as mean ± s.d.

Conclusions

This aim of this work was the preparation and characterization of lipid vesicles for the controlled release of kanamycin, to improve the therapeutic index of this tuberculostatic agent. A methodology that allows easy process scale-up (ethanol injection) and formulations based on phosphatidylcholine and cholesterol adequate for administration by inhalation were employed. Kanamycin active loading in liposomes was evaluated, considering both the possible favourable effects of its four amino groups on drug accumulation in the vesicle aqueous core and the limitations related to its predominant hydrophilic character. Different initial active agent-to-lipid molar ratios and distinct preparation conditions strongly affect kanamycin encapsulation efficiency. The best performance concerning drug incorporation was achieved for a synthetic phosphatidylcholine-based system, although its production cost was considerably larger than that of the natural SPC used. Encapsulation efficiencies up to 63% and final drug-to-lipid molar ratios up to 0.1 were obtained for SPC/Chol vesicles presenting mean diameters of 132 nm incubated at 60°C with the drug for 60 min at an initial drug-to-lipid molar ratio of 0.16. Therefore, the preparation of the vesicles with the desired diameter and containing kanamycin at adequate concentration was essentially fulfilled, despite the difficulty of the drug crossing the lipidic membranes.

References

Adams, L. B., Sinha, I., Franzblau, S. G., Krahenbuhl, J. L., Metha, R. T. (1999) Effective treatment of acute and chronic murine tuberculosis with liposome encapsulated clofazimine. *Antimicrob. Agents Chemother*. **43**: 1638–1643

Agarwal, A., Kandpal, H., Gupta, H. P., Singh, N. B., Gupta, C. M. (1994) Tuftsin-bearing liposomes as rifampin vehicles in treatment of tuberculosis in mice. *Antimicrob. Agents Chemother*. **38**: 588–593

Batzri, S., Korn, E. D. (1973) Single bilayer liposomes prepared without sonication. *Biochim. Biophys. Acta* **298**: 1015–1019

Beaulac, C., Sachetelli, S., Lagace, J. (1999) Aerosolization of low phase transition temperature liposomal tobramycin as a dry

powder in an animal model of chronic pulmonary infection caused by *Pseudomonas aeruginosa*. *J. Drug Target*. **7**: 33–41

Bridges, P. A., Taylor, K. M. G. (2000) An investigation of some of the factors influencing the jet nebulisation of liposomes. *Int. J. Pharm*. **204**: 69–79

Chen, P. S., Toribara, T. Y., Warner, H. (1956) Microdetermination of phosphorus. *Anal. Chem*. **28**: 1756–1758

Conley, J., Yang, H., Wilson, T., Blasetti, K., Dininno, V., Schnell, G., Wong, J. P. (1997) Aerosol delivery of liposome-encapsulated ciprofloxacin: aerosol characterization and efficacy against *Francisella tularensis* infection in mice. *Antimicrob. Agents Chemother*. **41**: 1288–1292

Corless, J. A., Stockton, P. A., Myers, S. B., Davies, P. D. O. (2002) A world-wide internet survey of public knowledge about tuberculosis. *Resp. Med*. **96**: 59–60

Deol, P., Khuller, G. K. (1997) Lung specific stealth liposomes: stability, biodistribution and toxicity of liposomal antitubercular drugs in mice. *Biochim. Biophys. Acta* **1334**: 161–172

Deol, P., Khuller, G. K., Joshi, K. (1997) Therapeutic efficacies of isoniazid and rifampin encapsulated in lung-specific stealth liposomes against *Mycobacterium tuberculosis* infection induced in mice. *Antimicrob. Agents Chemother*. **41**: 1211–1214

Gangadharam, P. R. J., Ashtekar, D. A., Flasher, D. L., Düzgünes, N. (1995) Therapy of *Mycobacterium avium* complex infections in beige mice with streptomycin encapsulated in sterically stabilized liposomes. *Antimicrob. Agents Chemother*. **39**: 725–730

Justo, O. R., Moraes, A. M. (2003) Incorporation of antibiotics in liposomes designed for tuberculosis therapy by inhalation. *Drug Deliv*. **10**: 201–207

Kurunov, I. N., Ursov, I. G., Krasnov, V. A., Petrenko, T. I., Iakovchenko, N. N., Svistel'Nik, A. V., Filimonov, P. A. (1995) Effectiveness of liposomal antibacterial drugs in the inhalation therapy of experimental tuberculosis. *Probl. Tuberk*. **1**: 38–40

Labana, S., Pandey, R., Sharma, S., Khuller, G. K. (2002) Chemotherapeutic activity against murine tuberculosis of once weekly administered drugs (isoniazid and rifampicin) encapsulated in liposomes. *Int. J. Antimicrob. Agents* **20**: 301–304

Madden, T. D., Harrigan, P. R., Tai, L. C., Bally, M. B., Mayer, L. D., Redelmeier, T. E., Loughrey, H. C., Tilcock, C. P. S., Reinish, L. W., Cullis, P. R. (1990) Accumulation of drugs within large unilamellar vesicles exhibiting a proton gradient: a survey. *Chem. Phys. Lipids* **53**: 37–46

Mayer, L. D., Tai, L. C. L., Bally, M. B., Mitilenes, G. N., Ginsberg, R. S., Cullis, P. R. (1990) Characterization of liposomal systems containing doxorubicin entrapped in response to pH gradients. *Biochim. Biophys. Acta* **1025**: 143–151

Moraes, A. M., Santana, M. H. A., Carbonell, R. G. (1999) Preparation and characterization of liposomal systems entrapping the boronated compound o-carboranylpropylamine. *J. Microencapsul*. **16**: 647–664

Szoka, F. C. (1996) Preparation of liposome and lipid complex compositions. USA Patent 5549910

Taylor, K. M. G., Newton, J. M. (1992) Liposomes for controlled delivery of drugs to the lung. *Thorax* **47**: 257–259

Vyas, S. P., Kannan, M. E., Jain, S., Mishra, V., Singh, P. (2004) Design of liposomal aerosols for improved delivery of rifampicin to alveolar macrophages. *Int. J. Pharm*. **269**: 37–49

Walter, F., Vicens, Q., Westhof, E. (1999) Aminoglycoside–RNA interactions. *Curr. Opin. Chem. Biol*. **3**: 694–704

WHO (2003) *Treatment of tuberculosis: guidelines for national programmes*. 3rd edn, (document WHO/CDS/TB 2003.313), World Health Organization, Geneva

Wong, J. P., Di Ninno, V. L., Cherwonogrodzky, J. W., Dela Cruz, R., Saravolac, E. G. (1995) Liposome-encapsulated ciprofloxacina for the prevention and treatment of infections diseases caused by intracellular pathogens. In: Florence, A. T., Gregoriadis, G. (eds) *Liposomes in biomedical applications*. Harwood Academic Press, Amsterdam, The Netherlands, pp 105–120