# Chapter 4

Chapter 4 contains the manuscript of a technical note to be submitted to AAPS PharmSciTech. The technical note contains the summary, experimental methods, results and discussion of the determination of the entrapment efficacy of lipid drug delivery systems (No introduction is given as stipulated in the guidelines for author). The technical note is prepared according to the author guidelines (http://www.aapspharmscitech.org/auth_resources/default.asp). Some formatting has been changed to help with ease of reading. Language and grammar is consistent with US English. References style used in Vancouver style (No more than 20 reference may be used according to the guidelines for authors). Some of this data was also presented at the 7th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology from 8 – 11 March 2010, Valletta, Malta. A copy of the abstract and the poster as presented, are included in Annexure A.

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# Quantitative and qualitative determination of the entrapment ability of two lipid drug delivery systems to entrap hydrophilic and lipophilic compounds

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- Pheroid™ Technology
- Liposomes
- Mefloquine
- UV-spectrophotometry
- Sephadex column

## List of Abbreviations

| | |
|---|---|
| %EE | Percentage entrapment efficacy |
| CLSM | Confocal laser scanning microscopy |
| DDS | Drug delivery system |
| MQ | Mefloquine |
| PBS | Phosphate buffer solution |
| UV | Ultra violet |

## 1. Summary

Research into drug delivery systems (DDS) has increased mainly because it can improve bioavailability and decrease side effects of poorly soluble drugs (1,2). Lipid DDS like the novel Pheroid™ vesicles consisting of essential fatty acids and the widely used liposome formulation are two examples. They have the ability to entrap both hydrophilic and lipophilic compounds (3-5). Because 100% entrapment efficacy of a selected drug is difficult, it is essential to separate the unentrapped drug from the DDS. Different methods can be utilized including ion exchange, Millipore filtration, gel filtration and dialysis (6-9). Labelling of proteins with a fluorescent probe makes it possible to qualitatively determine the entrapment ability of lipid DDS. Mefloquine (MQ), an antimalarial drug is highly lipophilic and can be analysed by HPLC, UV-Spectrophotometry, gas chromatography and thin layer chromatography (10,11). The aim of this paper was to determine the ability to entrap hydrophilic or lipophillic compounds. Method development to separate and determine the entrapment efficacy of MQ in lipid DDS utilizing UV-spectrophotometry and Sephadex® columns was also done.

## 2. Methods

### 2.1 Materials

Purified egg yolk phosphatidyl choline, cholesterol, Sephadex® G50 and Nile Red were purchased from Sigma-Aldrich® (St. Louis, MO, USA). Vitamin F ethyl ester from CLR (Berlin, Germany), Cremophor® RH40 from BASF (Germany), DL-α-tocopherol from DSM (Basel, Switzerland) and mefloquine hydrochloride from Sifavito S.p.A (Mairano, Italy). Alexa Fluor 430 was purchased from Molecular Probes (Invitrogen™, Breda, The Netherlands). All reagents were of analytical grade.

### 2.2 Pheroid™ vesicles preparation

Pheroid™ vesicles consist of three phases. An oil phase and a water phase saturated with a nitrous oxide gas phase. Vitamin F, Cremophor® EL and DL-α-tocopherol in a 15:5:1 ratio was heated in various steps, added to preheated water phase and

homogenized at 13500 rpm. Formulation was continuously shaken and left to cool to room temperature (3).

### 2.3 Liposome preparation

The oil phase of liposomes consisted of a 3:2 ratio of phosphatidyl choline and cholesterol. The oil phase was dissolved in a sufficient amount of a 2:1 (v/v) chloroform:methanol solution. The organic phase was removed under reduced pressure using a rotary evaporator at 40°C. When all the organic solvent was completely removed, PBS (pH 7.4) was added to give a final concentration of 2.5%. The film hydration method was used with the addition of glass beads to remove the lipid from the side of the flask during the hydration with PBS (5,12).

### 2.4 Entrapment and labelling of proteins

Proteins were added at time zero to the Pheroid™ vesicles. Samples were taken and measured after 15 minutes incubation period with Alexa Fluor 430 and measured at time intervals of 1, 3, 9, 12 and 24 hours. Proteins were added to the PBS of the liposomes before hydration and analysed.

### 2.5 Analysis of protein labelling

Samples, with entrapped labelled proteins, were analysed by confocal laser scanning microscopy (CLSM) and flow cytometry. Analysis was done by capturing images on a CLSM equipped with a He/Ne red laser and an argon green laser. EZ-C1 software was used for the qualitative analysis of samples in duplicate. Lipids were stained with Nile Red giving a contrast to the green labelled proteins. Quantitatively, the entrapment ability was analysed using a FACSCalibur™ equipped with a 488 nm argon ion laser linked to CellQuest Pro software for analysis (Becton & Dickson, Mountain view, CA, USA). Duplicate samples were diluted with PBS (pH 7.4) and a total of 100000 events were counted for each sample.

## 2.6 Entrapment of mefloquine

MQ was entrapped in Pheroid™ vesicles and liposomes during the formulation process. MQ, in various concentrations ranging from 0.1% - 0.5%, was dded to the oil phase of both lipid drug delivery systems. The ability of Pheroid™ vesicles to entrap MQ when added after formulation was also evaluated. The drug was added to the formulation, placed on a shaker and left for 14 days. Formulations were left for 24 hours before analysis.

## 2.7 Separation of unentrapped mefloquine from the DDS

A method, with slight modification as described by Fry *et al*. (6), was used. Sephadex® G50 was left for 24 hours to swell in PBS (pH 7.4). Non-absorbent cotton wool and polyethylene filter paper were placed in the bottom of the barrel of a 2 ml syringe. Sephadex® was added to the column and centrifuged to remove the water. A sample volume of 200 µl Pheroid™ vesicles or liposomes was carefully added to the top of the column. After centrifuging, the column was washed twice with PBS to remove any remaining DDS. Any unentrapped MQ was present in the column. Methanol was added to the column to dissolve the MQ and removed by centrifuging. Samples were filtered before spectrophotometric evaluation.

## 2.8 Preparation of calibration curve

Serial dilutions from a stock solution of 0.5 mg/ml in methanol were prepared and filtered. Absorbance was measured by a UV-1800 ultra violet (UV) spectrophotometer linked to UV Probe 2.32 software (Shimadzu Corporation, Kyoto, Japan). Peak absorbance values at 283 nm of the concentration range were plotted against the concentration. The graph was fitted with linear regression yielding an equation of $y = mx + c$ (11). Samples were run in triplicate and accuracy was determined by analyzing samples of know concentrations.

## 2.9 Evaluation of entrapment efficacy of liposomes and Pheroid™ vesicles

Samples, as obtained from separation of the unentrapped drug and DDS, were analyzed as described previously (13). Mass in mg/ml was determined by substitution of the

absorbance value into the linear equation. The percentage entrapment efficacy (%EE) was calculated as follows:

$$\%EE = \frac{\text{Initial drug load} - \text{unentrapped drug}}{\text{Initial drug load}} \times 100$$

## 3. Results

### 3.1 Protein labeling

The entrapment ability of liposomes and Pheroid™ formulations were determined qualitatively and quantitatively. CLSM results showed the presence of labeled proteins in the Pheroid™ formulations with an increases over time. Figure 1 illustrates the protein entrapped Pheroid™ vesicles at three time intervals. Liposomes also showed entrapment of proteins to a lesser extent (Figure 1).

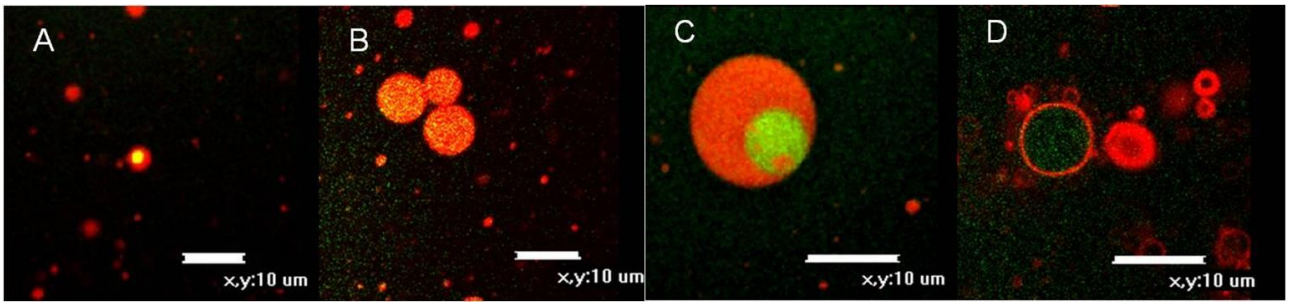


Figure 1 Micrographs of Pheroid™ vesicles at time intervals of 1 hour (A), 6 hours (B) and 12 hours (C) and liposomes (D) with entrapped labeled protein. The green represents the labeled proteins and the Pheroid™ vesicles and liposomes are red in color.

The entrapment capability of liposomes and Pheroid™ were analyzed qualitatively by flow cytometry using a FSC/FL1 plot. All samples were compared to the control of unstained liposomes and Pheroid™ respectively. Results showed a higher entrapment ability of Pheroid™ over time with a 59% trapping efficacy of Pheroid™ vesicles after 24 hours (Figure 2A). Unilaminer liposomes gave an entrapment efficacy of 33%. The liposomes measured before sonication showed higher entrapment ability of 59% (Figure 2B). This could be due to the size difference between the two liposome samples.

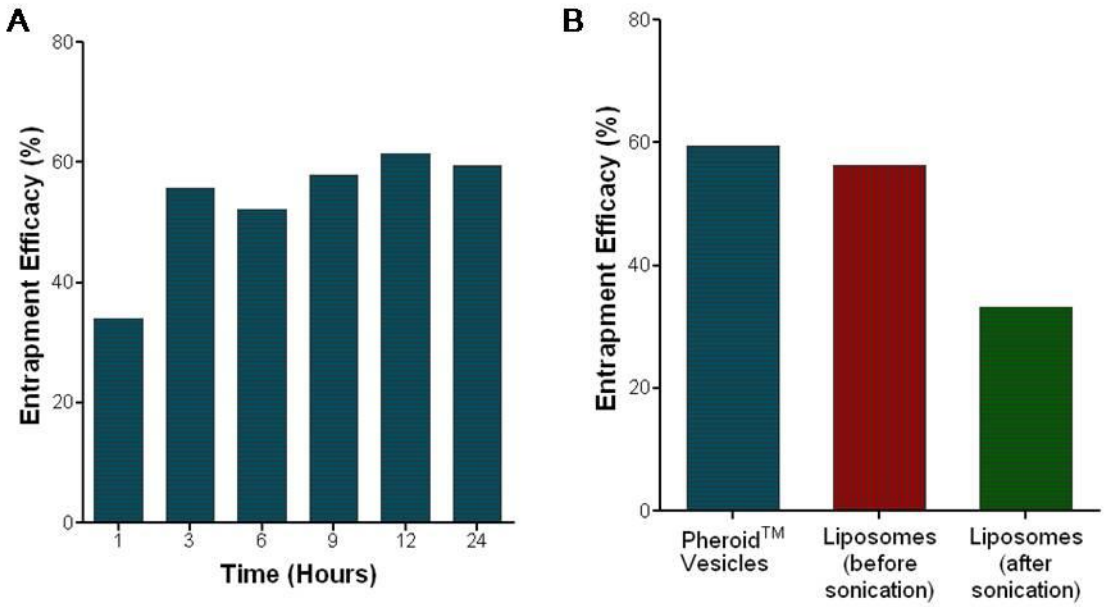


Figure 2 Entrapped proteins in Pheroid™ vesicles over time (A) and entrapped proteins after 24 hours in Pheroid™ vesicles and liposomes before and after sonication (B).

### 3.2 Mefloquine entrapment efficacy

Different concentrations of MQ dissolved in methanol gave distinct absorbance curves (Figure 3A) with a peak absorbance at 283 nm. A linear relationship existed between the absorbance and concentration with correlation coefficients of $r^2 = 0.9975$ (Figure 3B). The Sephadex® column with little dilution successfully separated the unentrapped MQ. The amount of unentrapped MQ was analyzed by UV-spectrometry with no shift in peak (data not shown). Results obtained from the Pheroid™ vesicle and liposome formulations when MQ was added in different concentrations to the oil phase during manufacturing are illustrated in Figure 4A. A concentration dependent increase in %EE was observed with both formulations. The highest entrapment efficacy is seen at 0.5% for Pheroid™ vesicles and 0.4% for liposomes. The addition of MQ to the already formulated Pheroid™ vesicles showed entrapment efficacy of around 40% from day 1 to 10, with the exception of day 2 (Figure 4B). Day 11 to 14 resulted in %EE of above 50%. After 14 days of shaking, only a 1% difference is seen between the two Pheroid™ vesicle formulations.

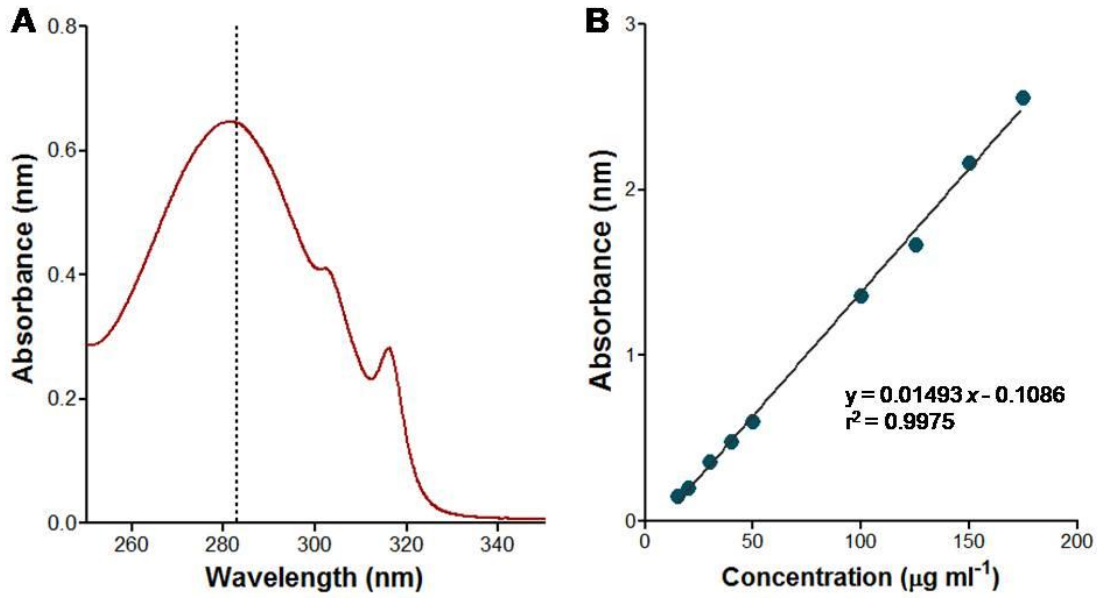


Figure 3 Wavelength curve of MQ hydrochloride in methanol with peak absorbance at 283 nm (A). Peak absorbance values of different concentrations of MQ fitted with linear regression (B).

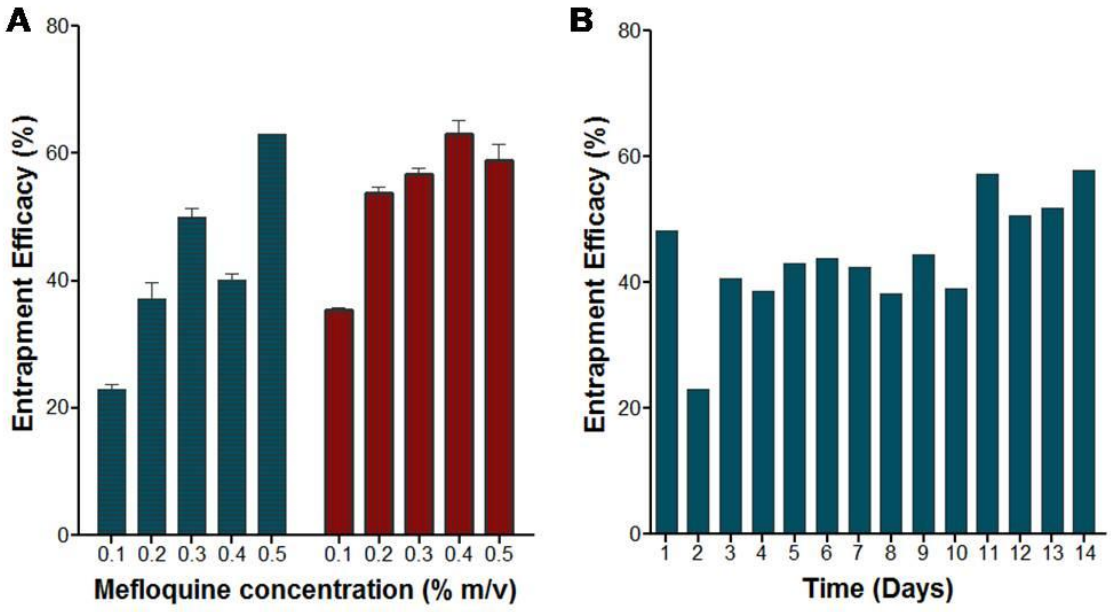


Figure 4 Bar graphs of Pheroid™ vesicles (teal) and liposomes (maroon) of different concentrations MQ entrapped in the oil phase during manufacturing (A). A 14 day entrapment efficacy study of Pheroid™ vesicles with addition of MQ after formulations (B). Each bar represents the mean ± SEM of triplicate values with very small SEM for data in B.

## 4. Discussion

Pheroid™ vesicles' entrapment ability increased over time. Pheroid™ vesicles at 24 hours showed a 26% higher entrapment efficacy compared to liposomes. Pheroid™ formulations have the advantage to entrap proteins after manufacturing. This can be an advantage especially to other compounds with low stability in solution. Separation of the unentrapped MQ from the lipid DDS was adequate with little dilution of the sample. Sephadex® columns are a simple, fast and highly effective method to separate drugs from lipid DDS. The amount of unentrapped MQ could be estimated successfully by dissolving it in methanol and reading it by UV-spectrophotometry at 283 nm. Pheroid™ vesicles and liposomes can entrap both lipophillic and hydrophilic drugs that can be determined qualitatively and quantitatively. In this study the results illustrated that the amount of unentrapped MQ can be estimated after separation from the DDS. The separation of unentrapped drug from the DDS and the determination of MQ by UV-spectrophotometry can be successfully combined. Using the combination of these methods are ideal because of simplicity, reproducibility and accuracy.

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