Chapter 3

Chapter 3 is a short communication for publication in the Journal of Pharmaceutical and Biomedical Analysis. It consists of an introduction, methods, resuls and discussion for the size characterization of Pheroid™ formulations and liposomes. The communication is written according to the author guidelines as well as the reference style used as stipulated (http://www.elsevier.com/wps/find/journaldescription.cws_home/525434/authorinstruction).
Some formatting has been changed to help with ease of reading. Language and grammar is consistent with UK English. The results of this study were 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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Size characterization of Pheroid™ formulations and liposomes

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Keywords

- Pheroid™ Technology
- Size determination
- Liposomes
- Flow cytometry
- Dynamic light scattering

List of Abbreviations

CH	Cholesterol
FSC	Forward light scatter
PC	Phosphatidyl choline
PG	Phosphatidyl glycerol

1. Introduction

Size determination of drug delivery systems is an important part of characterization and determination of stability (1,2). Changes in the size of particles over a period of time are an indication of instability (3). Particle size also has an influence on degradation, flow properties, clearance, uptake and distribution (4). Dosage forms and route of administration influence the choice of the size of particles to be manufactured (5).

Different methods exist to determine the size of particles. These include light scattering used to determine the mean size and distribution of particles based on the equivalent spherical principle (4). The angle of light defraction is an indication of size for particles of 1 µm and bigger in size (6). Light defraction include analysis utilizing flow cytometry (7). Microscopy, analytical ultracentrifugation and electrophoresis can also be used (4).

Pheroid™ technology formulations consist of essential fatty acid dispersed in a liquid and nitrous oxide gas phase. Fatty acids are natural ingredient of the body compared to artificial polymer of other lipid based delivery systems. Pheroid™ formulations can entrap both water and lipid soluble compounds with high efficacy. Low toxicity to the ingredients, drug protection, higher volume of distribution and increased efficacy are some of the advantages of Pheroid™ technology. Size is dependent on the formulation, ranging from 200 nm to 50 µm in diameter (8). Limited determination of size analysis has been conducted on Pheroid™ technology. This includes light scattering utilizing a Malvern Mastersizer and microscopically by confocal laser scanning microscopy (8,9).

Liposomes are reputable as a membrane system valuable for the delivering of a vast amount of materials. Liposome formulations have the advantage of larger dose loads, slower release, reduced toxicity and target specific delivery with increased efficacy. This colloidal drug delivery system, dependent on the manufacturing procedure, varies in diameter size from 80 nm to 100 µm (10). Disadvantages include the toxicity of some formulations, low entrapment ability and high cost (11). Determination of liposome particle size distribution is done as standard characterization property mostly utilizing dynamic light scattering. Light scattering size determination of liposomes has been illustrated as well as microscopic evaluation (10,12-14).

Particle size, internal aqueous volume and chemical composition are important parameters affecting the biochemical properties of drug delivery systems (12). Particle size is a key characteristic indicating stability and influencing among others uptake and degradation. Knowledge of the size distribution is important for formulation dosage forms intended for specific routes of administration. It is therefore imperative to accurately determine the size of particles to determine the best formulation for the intended use. Size of the different Pheroid™ and liposome formulations were determined by flow cytometry and with a Malvern Mastersizer.

2. Methods

2.1 Pheroid™ preparation

Pheroid™ vesicles and Pheroid™ microsponges were prepared as reported earlier (9). In short, Pheroid™ consists of an oil, water and gas phase. Vitamin F ethyl ester (CLR, Berlin, Germany) and Cremophor® EL (BASF, Germany) was heated to 75°C. It was left to cool after which DL-α-tocopherol was added to the oil phase. After heating to 55°C, it was added to preheated nitrous oxide saturated water phase and homogenized at 13500 rpm until room temperature was reached. Pheroid™ microsponges consist of the same ingredients, except for the addition of fatty acids with longer aliphatic tails, Incromega E7010 and E3322 to the Vitamin F and Cremophor® (9).

2.2 Liposome preparation

Six different liposome formulations (L01 to L06) were manufactured using different methods or ingredients (see Table 1). Liposome formulations (L01 to L05) were prepared by the film hydration method. The combination of cholesterol (CH), phosphatidylcholine (PC) and/or phosphatidyl glycerol (PG) (Sigma-Aldrich®) were dissolved in a chloroform:methanol (2:1 v/v) mixture. The organic phase was removed under reduced pressure to form a thin film on the flask. The film was dispersed in phosphate buffer solution. Liposome formulations were attained by rotation with glass beads, sonication or a combination of both. Reverse-phase evaporation vesicles (L06) were manufactured by dissolving the lipids in a mixture of organic and aqueous phase. The organic phase was slowly removed by evaporation to form a gel (10,11).

Table 1 Manufacturing of different liposome formulations using different methods. Main ingredient of all the formulations were cholesterol (CH) and phosphatidylcholine (PC) with addition of phosphatidyl glycerol (PG) to two formulations.

Nr	Method	Ingredient		
		CH	PC	PG
L01	Film hydration, no sonication	X	X	
L02	Film hydration, sonication (bath)	X	X	
L03	Film hydration, sonication (rod)	X	X	
L04	Film hydration, sonication (rod)	X	X	X
L05	Film hydration, no sonication	X	X	X
L06	Reverse phase liposomes	X	X	

2.3 Size determination – Flow cytometry analysis

Forward laser light scatter (FSC) analysis of the formulations was performed using a FACSCalibur™ equipped with a 488 nm Argon ion laser linked to CellQuest Pro (Becton & Dickson, Mountain View, CA, USA). Data were collected by acquiring 100000 events and analysed with FCS Express V3 software (De Novo Software, CA, USA). Size calibration beads ranging from 0.5 μm to 15 μm were analysed alone and in combination as standards yielding distinctive peaks on a FSC histogram. Plotting the geometric mean of each bead size relative to size and calculating the linear regression yielded an equation $y=mx+b$. Gates were set up to determine the size distribution of the different formulations (7,12).

2.4 Size determination – Malvern Mastersizer

The mean particle size, distribution and uniformity of the formulations were measured by dynamic light scattering analysis using a Malvern Mastersizer 2000 equipped with a blue solid light laser. Samples was analysed at an obscuration rate of 10% to 20% at a

scattering angle of 90° in duplicate. Samples were stirred continuously to obtain a homogenous dispersion of the samples.

3. Results and discussion

Forward scatter analysis of the size calibration beads gave distinct population as seen in Figure 1. Correlation between the geometric mean values and size were analysed to give a correlation graph (Figure 1A) with an equation of y=1.607x+0.4496 and a correlation value of r^2=0.9781 (Figure 1B). This mathematical equation was use to divide the forward scatter values into ranges or gates representing different size ranges. Evaluation of dot plots showed a distinct difference in the population of the Pheroid™ vesicles and liposome formulation (Figure 2). Size distribution was determined quantitatively and is displayed in Table 2. Data indicated size range up to 40 µm and 12 µm for liposomes and Pheroid™ formulations respectively. Dynamic laser light scattering was used to evaluate the size range of the formulations and is shown in Table 2. Data illustrated liposomes size ranging from 0.34 µm to 80 µm compared to Pheroid™ formulation ranging between 0.16 µm and 12.7 µm. Figure 3 shows the difference in size distribution evaluation between the Pheroid™ vesicles and L04 liposome formulation as determined by light scattering. Correlation between the flow cytometric evaluation and Malvern analysis was low. This could be because of the irregular shape and structure of the liposomes (data not shown). Pheroid™ vesicles showed a correlation of r^2=0.851 between the different analysis method (Figure 4). A more accurate reading was obtained with FACS analysis.

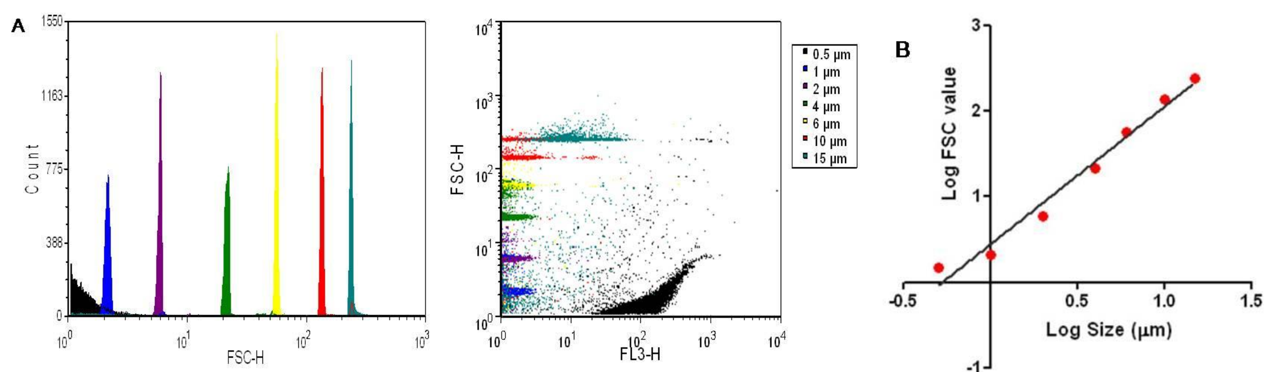

Figure 1 Forward scatter histogram and dot plot of different sized calibration beads as determined by flow cytometry (A). Size calibration curve fitted with linear regression as determined by plotting the geometric mean of each bead size to the corresponding size (B).

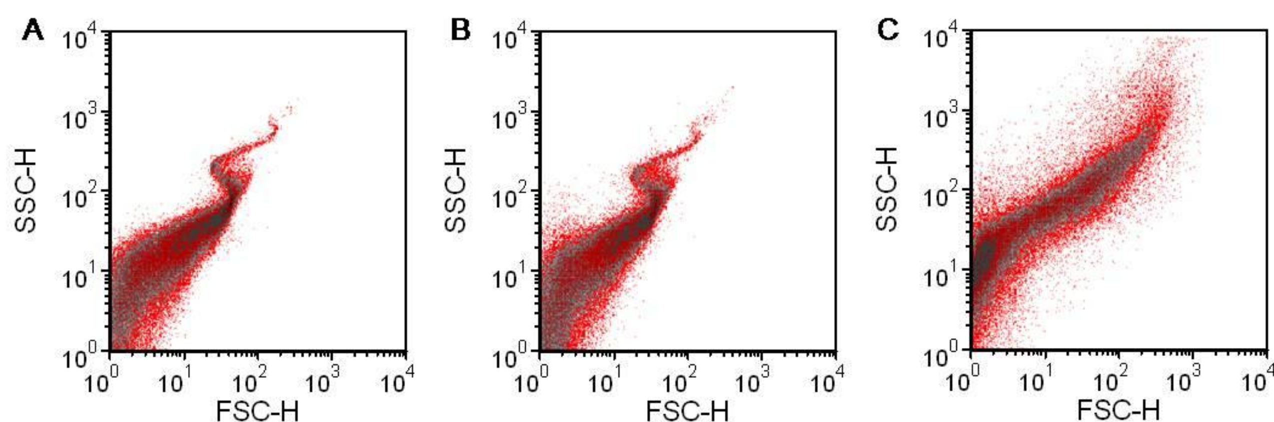

Figure 2 Dot plots of Pheroid™ vesicles (A), Pheroid™ microsponges (B) and liposome formulation L01 (C).

Table 2 Size distribution of the different formulations as determined by flow cytometric analysis and Malvern size distribution.

Formulation	**Size distribution FACS**	**Size distribution Malvern**
L01	0.1 – 38.39 µm	0.399 - 83.11 µm
L02	0.1 – 40.17 µm	0.399 – 82.365 µm
L03	0.1 – 34.19 µm	0.399 – 40.414 µm
L04	0.1 – 25.05 µm	0.399 – 57.347 µm
L05	0.1 – 29.34 µm	0.399 – 44.091 µm
L06	0.1 – 31.40 µm	0.359 – 63.669 µm
Pheroid™ vesicles	0.1 – 12.34 µm	0.356 – 12.619 µm
Pheroid™ microsponges	0.1 – 10.61 µm	0.159 – 12.619 µm

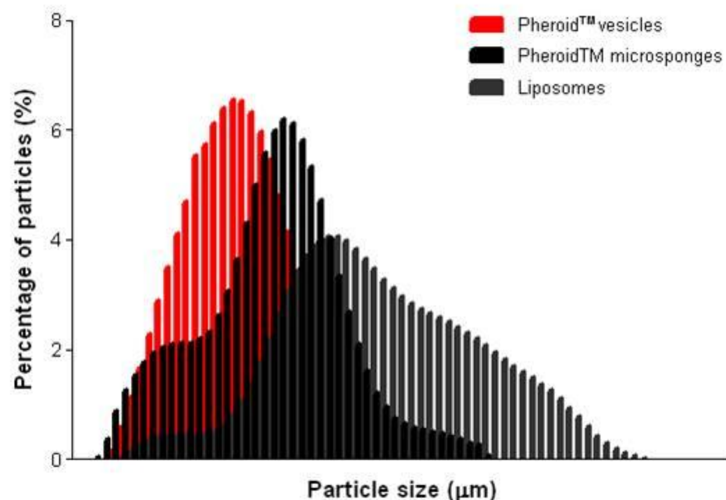

Figure 3 Malvern size evaluation of Pheroid™ vesicles, Pheroid™ microsponges and liposome formulation L04.

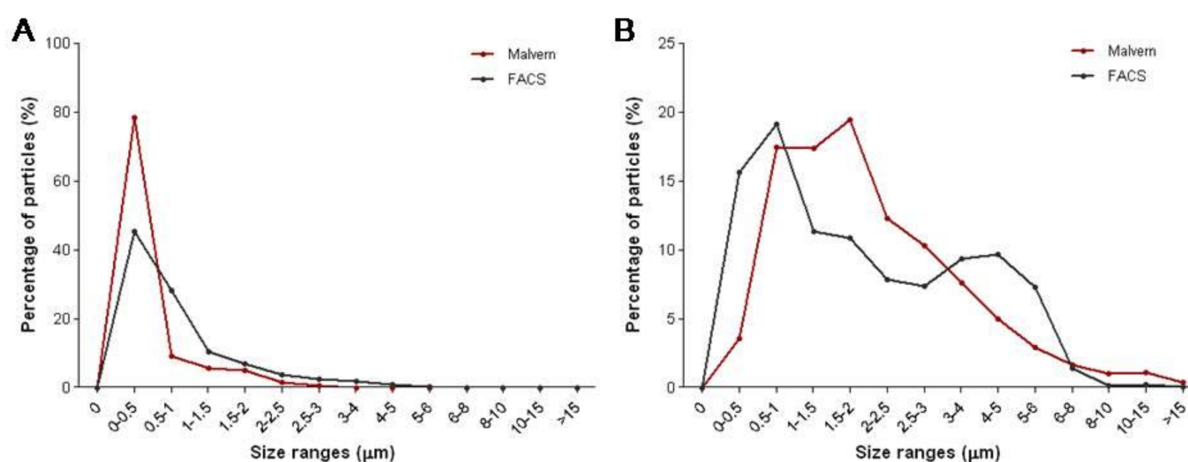

Figure 4 Correlation between Malvern and flow cytometric analysis of Pheroid™ microsponges (A) and Pheroid™ vesicles (B).

4. Conclusion

Flow cytometric analysis of liposome formations showed that the method as well as the ingredients had an influence on the particle size analysis. Preparation of liposomes with the film hydration method utilizing an ultrasonic rod showed the lowest distribution of size with a maximum size of 25 µm. Addition of PG to the formulation lowered the overall particle size distribution to below 30 µm. Liposomes formulations without PG with longer

sonication period showed a decrease in particle size to 10.52 μm (as determined by flow cytometric analysis). Analysis of Pheroid™ vesicles and Pheroid™ microsponges showed similar size distribution. The optimal formulations according to size characterization was liposomes prepared by the film hydration method forming a thin film of cholesterol and phosphatidylcholine with extended period of sonication. No distinctive difference between the Pheroid™ formulations was seen in size. Because Pheroid™ vesicles is more like liposomes in structure (data not shown), more comparative studies can be conducted when using both liposomes and Pheroid™ vesicles.

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