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*Original Article*

# Factors affecting liposomes particle size prepared by ethanol injection method

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## Abstract

Ethanol injection is one of the techniques frequently used to produce liposomes which favors both simplicity and safety. In this process, an ethanolic solution of lipids is rapidly injected into an aqueous medium through a needle, dispersing the phospholipids throughout the medium and promoting the vesicle formation. Being a critical parameter that determines the fate of liposome and its distribution, we studied different factors affecting the particle size of liposomes including different phospholipid (Phosal® 53 MCT) and cholesterol concentrations and the use of different types of non-ionic surfactants at fixed Phosal® 53 MCT concentration of 50 mg per formulation. Both Phosal® 53 MCT and cholesterol concentration had direct effect on liposomes particle size. Non-ionic surfactants produced liposomes of smaller particle size when compared to conventional liposomes formed using Phosal® 53 MCT 300 mg per formulation only, whereas this effect was diminished when higher Phosal® 53 MCT to cholesterol ratios were used that obviously increased liposomes size. Smaller liposomes sizes were obtained upon using non-ionic surfactants of lower hydrophilic/hydrophobic balance (HLB) as both Tween 80 and Cremophor RH 40 produced liposomes of smaller particle size compared to Poloxamer 407. The smallest liposomes particle size was successfully obtained in the formulation comprising 300 mg Phosal® MCT, 150 mg cholesterol and 50 mg Tween 80.

***Keywords:*** Phosal® 53 MCT; Cholesterol; Nonionic surfactants; Liposomes

## INTRODUCTION

Over the last decades, liposomes have been thoroughly investigated as drug delivery systems for variety of drugs and routes of administration. Liposomes are formed when amphiphilic lipids organize themselves spontaneously in bilayer vesicles as a result of interactions between phospholipids and water (1). As these lipid vesicles possess lipophilic and hydrophilic portions, they can entrap substances with different polarities either in the phospholipid bilayer or the aqueous compartment or at the bilayer interfaces (2), which can modify physicochemical properties and can enhance biological activity of entrapped compounds (3).

Several techniques were reported in literature for the preparation of liposome suspensions. Ethanol injection method was first reported in the early 1970s by Batzri and Korn (4). It involves the dissolution of phospholipids into an organic solvent and further dispersion of the lipids into water. This immediate dilution of ethanol in the aqueous phase causes the lipid molecules to precipitate and form bilayer planar fragments which further transform into liposomal system (5). This method is simple, rapid and easy to scale-up.

Liposomes particle size is one such property that affects the half-life of liposomes in the vascular system and may range from few minutes to many hours. The particle size has an influence on the vesicles removal from circulation by the reticuloendothelial system, phagocytosis by kupffer cells, distribution and accumulation in specific organs (6). Smaller liposomes (0.1-1 µm) are located principally in the liver and spleen whereas liposomes larger than 3 µm are deposited in the lungs.

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Effect of surfactant on liposomes particle size was previously studied using only one type of surfactant (Tween 80) with various phospholipid/cholesterol ratios (7). Another study was also performed using different types of surfactants at fixed phospholipid/cholesterol ratio (8). Therefore, the purpose of this work was to demonstrate the effects of Phosal® 53 MCT and cholesterol concentrations as well as the type of nonionic surfactants on the liposomes particle size. In this study we produced liposomes of desirable size from an optimized formulation comprising Phosal® 53 MCT, cholesterol and a surfactant.

## MATERIALS AND METHODS

### *Materials*

Phosal® 53 MCT was provided by Phospholipid GmbH (Germany), which is composed of phosphatidylcholine 53% w/w, lysophosphatidylcholine 6% w/w, medium chain triglycerides (caprylic/capric triglycerides), alcohol 3-6% w/w, glyceryl stearate, oleic acid and ascorbyl palmitate (9). Cholesterol, Cremophor RH40, poloxamer 407 and Tween 80, ethanol 96% were obtained from Sigma-Aldrich (St. Louis, USA). Purified water was obtained by double distillation.

### *Liposome preparation*

Liposomes were prepared by ethanol injection method (8). Phosal® 53 MCT, cholesterol and surfactant were dissolved in ethanol 96%. The resultant organic phase was gently heated at 40 ± 2 °C to improve the solubility of cholesterol and miscibility of Phosal® 53 MCT. The volume of organic phase was adjusted to 15 mL to dissolve the highest amount of cholesterol (150 mg) used in this study, without any precipitation in the needle during injection. The ethanolic solution was then injected into 85 mL preheated water at 60 ± 5 °C. The injection rate was fixed to 500 µL/min. Liposomes formed spontaneously with continuous stirring at 500 rpm using a magnetic stirrer. Ethanol was evaporated by means of a rotary evaporator (Heidolph, Germany). The effects of various concentrations of Phosal® 53 MCT, cholesterol, and surfactant types including Tween 80, Cremophor RH 40, and Poloxamer 407 were evaluated on the liposome particle size.

### *Size distribution analysis*

The mean size and polydispersity index of the liposomal preparations were measured by laser diffraction analyzer (Malvern Inc., Worcestershire, UK). Each sample was diluted with water until the appropriate concentration of particles was achieved and measured. All measurements were performed in triplicate at 25 °C.

### *Statistical analysis*

All experiments were repeated three times and expressed as the mean ± standard deviation. One way analysis of variance (ANOVA) was used to substantiate statistical differences between groups in each series of experiment. Results with $P < 0.05$ were considered significant.

## RESULTS

### *The effect of different Phosal® 53 MCT concentrations on liposome particle size*

In this study, three different liposome dispersions of Phosal® 53 MCT at 300, 600 and 900 mg, but without cholesterol or surfactants were prepared according to the method already described. The average particle sizes ± SD were found to be 268 ± 15.2, 453 ± 11.5 and 913 ± 35 nm, respectively. The polydispersity indices ± SD were found to be 0.671 ± 0.027, 0.864 ± 0.024 and 0.502 ± 0.052, respectively.

### *The effect of different cholesterol concentrations on liposome particle size*

Amounts of 50, 100 and 150 mg of cholesterol were used with Phosal® 53 MCT in the preparation of liposome dispersion without any surfactants. Cholesterol caused an increase in the liposomes particle size compared to blank liposomes. It was found that increasing cholesterol concentrations had direct effect on increasing liposomes particle size significantly. However, the addition of 50 mg cholesterol to 300 and 900 mg Phosal® 53 MCT and 100 mg cholesterol to 300 mg Phosal® 53 MCT, did not significantly increase liposomes particle size compared to their respective control in each series (Fig. 1).

As seen, higher concentrations of Phosal® 53 MCT also produced liposomes of larger particle size.

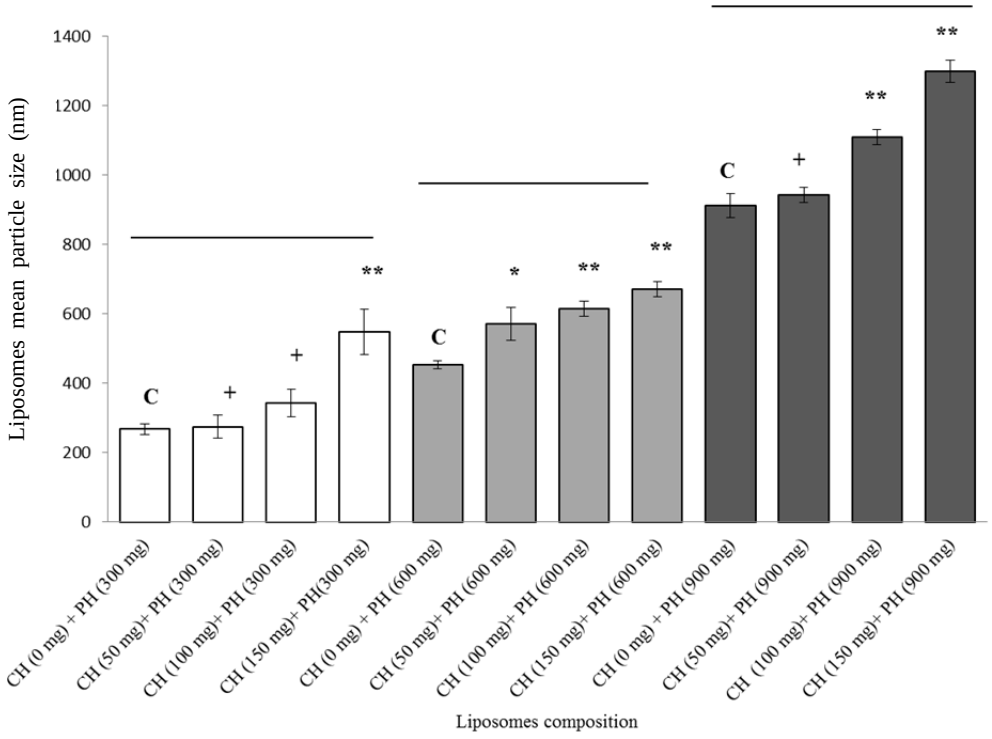


**Fig. 1.** Effect of different cholesterol (CH) concentrations on the mean particle size of liposomes comprising various Phosal® 53 MCT (PH) concentrations without surfactant. C denotes the control liposomes. *Means are significant at $P < 0.05$; **means are significant at $P < 0.01$; $^{+}$means are not significant ($P > 0.05$).

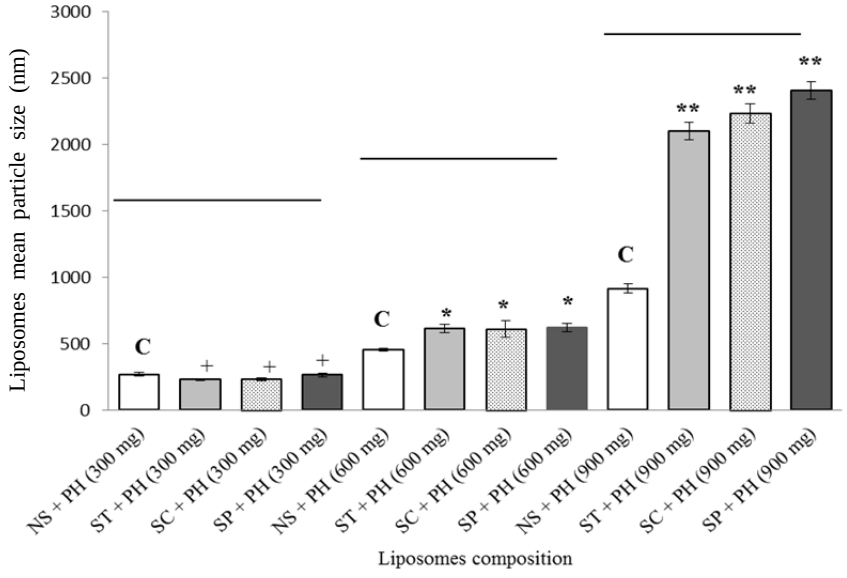


**Fig. 2**. The effect of surfactant type Tween 80 (ST), Cremophor RH 40 (SC), Poloxamer 407 (SP) and No surfactant (NS) on the mean particle size of liposomes comprising different Phosal® 53 MCT (PH) concentrations without cholesterol. C indicates control liposomes.*Means are significant at $P < 0.05$; **means are significant at $P < 0.01$; $^{+}$means are not significant ($P > 0.05$).

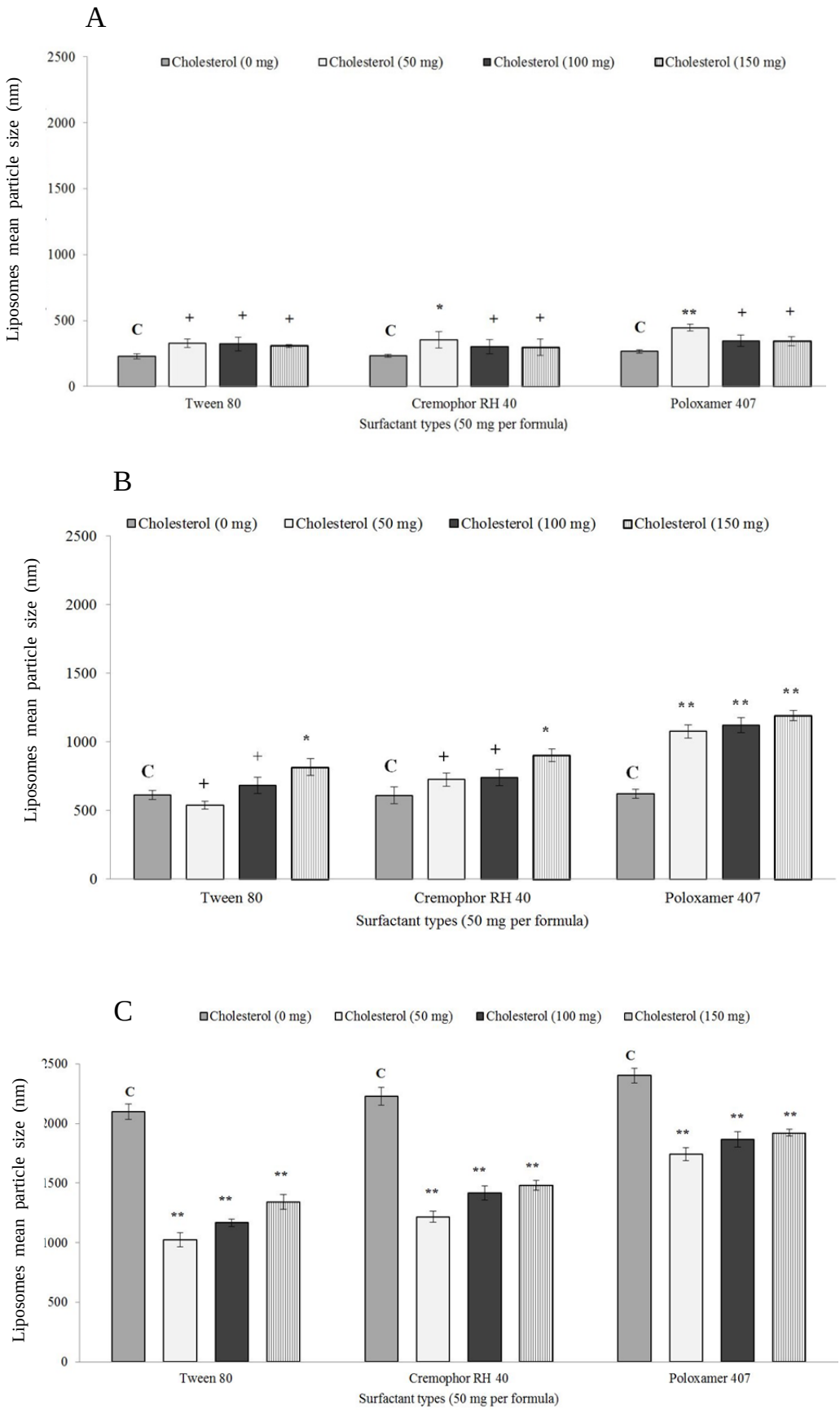


**Fig. 3**. Effect of different cholesterol concentrations and surfactant types on the mean particle size of liposomes comprising (A) 300 mg Phosal® 53 MCT, (B) 600 mg Phosal® 53 MCT and (C) 900 mg Phosal® 53 MCT. C denotes the control liposomes.*Means are significant at $P < 0.05$; **means are significant at $P < 0.01$; $^{+}$means are not significant ($P > 0.05$).

***The effect of surfactant types on liposomes particle size***

In our study, we investigated the effects of three nonionic surfactants Cremophor RH 40, Poloxamer 407 and Tween 80 on liposomes size. They were used at a fixed amount of 50 mg with different Phosal® 53 MCT concentrations in the absence of cholesterol as shown in Fig. 2. Nonionic surfactants were only able to reduce the size of liposomes of Phosal® 53 MCT at 300 mg level. However addition of surfacants to higher concentrations of Phosal® 53 MCT increased the size of liposomes significantly. The liposomes size increased on increasing Phosal® 53 MCT concentrations at 600 and 900 mg. The order of liposomes particle size prepared from various surfactants was found to be for Tween 80 < Cremophor RH 40 < Poloxamer 407 at all different Phosal® 53 MCT concentrations.

***The effect of different cholesterol concentrations and surfactant types on liposomes particle size***

The influence of cholesterol concentration and the type of surfactant on the mean particle size of Phosal® 53 MCT liposomes is depicted in Fig. 3A. As seen in this figure, the addition of cholesterol to the formulations increased the size of liposome. This increase was significant only when 50 mg cholesterol was combined with Cremophor RH 40 and Poloxamer 407. As seen in Fig. 3B, the addition of various cholesterol concentrations significantly increased the size of liposomes composed of Poloxamer 407. On the contrary, the addition of cholesterol 50 and 100 mg to Tween 80 and Cremophor RH 40 did not significantly increase the size of liposomes.

Liposomes comprising Phosal® 53 MCT, surfactant and 50 mg cholesterol had larger particle size than counterpart liposome formulations. However, cholesterol addition to liposome formulations comprising no surfactant increased the particle size in a concentration-dependent manner. It was observed that at higher ratio of Phosal® 53 MCT to cholesterol liposomes of larger sizes were obtained (7). Addition of Poloxamer 407 had a significant effect on increasing liposomes mean particle size at higher Phosal® 53 MCT to cholesterol ratios. Poloxamer 407 increased liposomes particle size, as compared to Tween 80 and Cremophor RH 40. On the other hand, Tween 80 was able to produce liposomes of smaller particle sizes when compared to Cremophor RH 40 at all Phosal® 53 MCT concentrations despite their close HLB values (Figs. 3B and 3C).

## DISCUSSION

Phospholipids are the main constituent of liposomal membrane, thus they can have a direct effect on liposomes particle size (10). In this study, the increase of Phosal® 53 MCT concentrations in the aqueous phase enhanced the viscosity of the dispersion as well as the particle size. It has been reported in the Wu YN, *et al*. study that particle size of papain liposomes decreases with decreasing phosphatidylcholine concentration (10). Liposomes of lower amounts of phospholipid (phosphatidylcholine 50%), were smaller in the size than liposomes of a higher amount of phospholipid (phosphatidylcholine 95%) (11).

Cholesterol is often included in liposomes formulation to give further rigidity to the bilayer and improve the physical stability of liposomes (12). Cholesterol enhances the retention of entrapped solutes and reduces serum-induced instability. An increase in cholesterol content in liposomes results in a dramatic decrease in membrane permeability for solutes (13). Cholesterol doesn't form a bilayer itself but it is dissolved in the phospholipid bilayer (14). As the concentration of cholesterol increases more cholesterol molecules will be distributed in the phospholipid bilayer, causing an increase in the liposome mean diameter at all Phosal® 53 MCT concentrations. It has been reported that higher cholesterol concentrations interferes with the close packing of the phospholipid bilayer by contributing to an increase in membrane fluidity which results in an increased distribution of aqueous phase within the liposomal vesicles (15). This explains the direct increase of liposomes mean diameter observed with cholesterol concentrations up to 150 mg in the formulations.

Surfactants were used at a fixed amount of 50 mg. At low concentrations, the surfactant molecules were taken up into the liposome membrane without breaking up the vesicle. As the concentration increases, the proportion of surfactant molecules in the membrane increases until a critical value. Above this threshold value, rupture of parts of the membrane is induced (10). Therefore, the concentrations of surfactants need to be carefully selected to ascertain vesicle formation (16). Incorporation of tween 80 in liposomes (0 to 0.2 molar ratio, Tween 80: lipids) caused a dramatic decrease in liposomal size due to steric repulsion rendered by surfactant molecules which prevents or minimizes the aggregation of the vesicles (16). The results of our study suggest that at higher Phosal® 53 MCT concentrations the effect of surfactants was counterbalanced by the effect of Phosal® 53 MCT concentrations to increase the liposomal size. By comparing the effect of different surfactants, it seems to exist a rational relationship between lipophilicity (HLB values) of surfactants and mean particle sizes; increasing the HLB value resulted in larger liposomes (17). The HLB values for Tween 80, Cremophor RH 40 and Poloxamer 407 were 15, 14-16 and 18-23 respectively which explains the increasing order of liposomes size. The physical state of surfactants may also partially explain the size difference, where Tween 80 has a liquid nature, Cremophor RH 40 is a paste, and Poloxamer 40 is a solid at 25 °C.

The liposomes particle size was influenced by the surfactant type and the ratio between Phosal® 53 MCT and cholesterol. As with some other studies, it was observed that as the ratio of Phosal® 53 MCT to cholesterol increased the particle size (9).

On using Phosal® 53 MCT 300 mg, it was expected that the particle size increase, as reported in literature, but the surfactant effect to lower particle size was more prominent than cholesterol enlarging effect (9), owing to surfactant steric repulsion among surfactant molecules (16). Moreover, cholesterol might increase the rigidity of the phospholipids bilayer and decrease their fusion to larger vesicles (18).

On the other hand, at higher Phosal® 53 MCT concentrations (600 and 900 mg), the effect of the surfactant to lower liposomes particle size was opposed by the higher Phosal® 53 MCT to cholesterol ratios which obviously tended to increase liposomes size. In addition, increasing cholesterol concentrations might interfere with the close packing of the phospholipid bilayer (19).

The influence of high HLB values of surfactant on increasing the liposome particle size was strongly supported with high Phosal® 53 MCT concentrations (18). Despite close HLB values of Tween 80 and Cremophor RH 40, the latter gave lower liposome sizes. This behavior might be attributed to the branched structure and relative bulkiness of the Cremophor RH 40 molecules leading to increased size of the vesicles (19).

## CONCLUSION

Both Phosal® 53 MCT and cholesterol increased the liposomes particle size in a concentration-dependent manner. However the addition of a surfactant lowered the liposomes particle size at Phosal® 53 MCT 300 mg with either cholesterol 100 or 150 mg per formulation. The type of surfactant had an influence on liposomes particle size; Tween 80 was the surfactant of choice. Liposomes of smaller particle size were obtained with 300 mg Phosal® 53 MCT, 50 mg Tween 80 (non-ionic surfactant) and 150 mg Cholesterol.

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