A glance into factors affecting the possible combined entrapment of curcumin and methylene blue into niosomal formulations as a potential anticancer therapy

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Abstract

Niosomes are non-ionic surfactant vesicles that has successfully attracted a great deal of attention over the last few decades. The best ratio between main components is the key to creating the optimum formulations with substantial loading capacity. Therefore, this study investigated formulation factors that could affect the entrapment of two compounds with different solubilities, namely Methylene blue (MB) and curcumin (CUR) as model hydrophilic and hydrophobic compounds, respectively. The aim was to propose an optimum formula to encapsulate both compounds as a potential hybrid anticancer therapy. The factors with potential influence on the entrapment efficiency (EE) included the type of the non-ionic surfactants (Span 60 versus Span 65), cholesterol to surfactant ratio, and the concentrations of the co-surfactant (Cremophor RH40). Niosomes were prepared by thin film hydration technique and downsized using probe sonicator. The morphology, vesicle size and polydispersity index (PDI) of the prepared vesicles were inspected. The data showed the superiority of Span 65 over Span 60 in terms of higher encapsulation efficiency for MB and CUR. The results reflected positive impact of cholesterol and Cremophor RH40 concentration on EE of the two compounds. The optimum composition of Span 65:cholesterol:Ccremophor RH40 was at the mol% ratio of 45:45:10, respectively, with EE of 91.19% for CUR alone, 51.70% for MB alone, and 45.32%/84.40% for combined MB/CUR loaded formulations. The vesicle size of the formulations after probe sonication, fall into the range of 100-200 nm, ideal for parenteral delivery to the target sites.

Key words: Niosomes, non-ionic surfactants, Cremophor RH40, Curcumin, methylene blue, Span 65, Span 60

1. Introduction

Niosomes are non-ionic surfactant-based vesicles. There are multiple factors influencing the properties of niosomes, including the vesicles composition, surface charge, physicochemical properties of encapsulated drug(s), vesicles size, and vesicles lamellarity (Ruckmani and Sankar, 2010; Yeo *et al.*, 2018).

The versatile and biocompatible structure of niosomes has been employed in various fields, such as drug and gene delivery, cosmetic industry, targeted therapy, wound healing, diagnostic imaging, and food industry. Niosomes can be introduced to the body via different routes of administration including oral, parenteral, ocular, and topical (Junyaprasert *et al.*, 2012). Niosomes structure provides a great opportunity to accommodate drugs with a wide range of hydrophilicity, along with releasing the encapsulated drug in a controlled and targeted manner. Additionally, this nanocarrier protects the enclosed drug from biological environment and increases its therapeutic impact via delayed clearance from the circulation. These characteristics of niosomes had led into a significant potential in multiple aspects of cancer research and cancer therapy, including cancer diagnosis, radiotherapy enhancement, chemotherapy drug delivery, targeted and combination therapy (Aparajay and Dev, 2022).

There are four major categories of non-ionic surfactants used in the production of niosomes, including alkyl amides, alkyl esters, alkyl ethers, and esters of fatty acids. Span 60 (sorbitan monostearate) and Span 65 (sorbitan tristearate) are esters of fatty acids. The number in their names indicates the average number of ethylene oxide moles being added to the sorbitan molecule during ethoxylation process. They both have hydrophobic properties with similar hydrocarbon chain lengths of C18.

The choice of non-ionic surfactants is influenced by factors, including hydrophilic lipophilic balance (HLB) value of the surfactant, critical packing parameter (CPP), and gel-liquid transition temperature (Tc). It was reported that the ideal HLB value for preparation of niosomes with spherical bilayers falls in the range of 4 to 8. This means that Span 60 with HLB value of 4.7 falls into this group. Having said that, other surfactants like Span 65 with HLB value of 2.1 could also be an ideal candidate for niosomes preparation with the addition of a co-surfactant and cholesterol to stabilize the formulation (Rad *et al.*, 2022). The CPP enables the prediction of vesicles geometry and is represented as '$CPP = V / (I_c \times a_0)$', in which, 'V' represents the volume of non-polar hydrophobic group, 'a_0' represents the area of polar hydrophilic head group, and 'I_c' represents the critical non-polar group length (I_c). Based on CPP value, $CPP \leq 1/3$ increases the chance of micelles formation, while $1/3 \leq CPP \leq 1/2$, increases

the chance of non-spherical micelles formation. Additionally, having the CPP value of 1/2≤CPP≤1, could result in the formation of bilayer vesicles, while CPP≥1, could result in the formation of inverted micelles (Thabet et al., 2022).

On the other side, Tc is also considered to have a critical influence on the niosomes formation. The surfactants with a saturated alkyl chain and higher number of carbons have a higher gel to liquid phase transition temperature, resulting in niosomes with reduced membrane permeability and enhanced entrapment efficiency (Thabet et al., 2022).

Methylene Blue (MB) is a salt used as a dye and medication. Lately, MB was explored for its potential as anticancer agent because of its phenothiazine chromophore introducing what is called photodynamic therapy for cancer treatment (Cecatto et al., 2020). This chromophore is photosensitized via absorbing light at the wavelength of 630-680 nm leading to the production of reactive oxygen species following by cell death (Lim, 2021). MB was previously explored for its anticancer activity and a promising results were obtained against breast cancer (Dos Santos et al., 2017) and lung cancer (Sanchala et al., 2018). Additionally, nanocarrier systems were employed to deliver MB for photodynamic therapy (Lim, 2021; De Leo et al., 2024).

On the other side, curcumin (CUR) is a yellow polyphenolic compound derived from the Curcuma Ionga plant species. CUR has a wide range of useful pharmacological activities, such as antioxidant, anti-inflammatory, chemo-preventive and chemo-therapeutic effects (Hatcher et al., 2008; Tomeh et al., 2019). However, its oral use is very limited due to poor bioavailability arising, mainly, from its poor aqueous solubility (Liu et al., 2020).

The aim of this research was to investigate the effect of formulation variables on the entrapment efficiency of drug(s) within niosomes. The explored factors involved the type of the non-ionic surfactant, (Span 60 versus Span 65), surfactant to cholesterol ratio, cosurfactant (Cremophor RH40) concentrations, and the physicochemical characterization of the drug. The later factor was completed by using the hydrophilic MB against the hydrophobic CUR, alone or in combination. These two model drugs were selected based on their reported potential anticancer properties (Taldaev et al., 2023; Rad *et al.*, 2022). To the best of our knowledge, combined MB and CUR in the same vesicular system has not been investigated yet. As the extent of drug entrapment is a key factor in the success of any vesicular delivery system, especially in the cancer therapy, this study is expected to pave the way towards better understanding of how to optimize drug loading within vesicular nanocarriers.

2. Materials and Methods

2.1. Materials

Curcumin (CUR) was purchased from Sigma-Aldrich (UK), Methylene Blue (MB) from Pro-Lab Diagnostics, UK. Sephadex G50, Span 60, Span 65, and Cholesterol were purchased from Sigma-Aldrich (UK). Cremophor RH40 was from BASF, Germany. All other materials used were of analytical grade.

2.2. Preparation of niosomes

The niosomes were prepared using the thin film hydration technique, refer to Table 1 for compositions of the prepared niosomes. In brief, a 300-micromoles stoichiometric mixture' contents of Span 60 or Span 65, cholesterol, and Cremophor RH40 were weighed (after conversion of %molar content into mg) and then dissolved in chloroform (12mL) and transferred into a round-bottomed flask. For formulations containing curcumin, the accurately weighed CUR (0.5mg) was also added to the mixture at this stage. Utilizing rotary evaporator, the solvent was evaporated at reduced pressure of 470 mbar, 100 rpm, and 60°C. The deposited film layer in the bottom of the flask was left in the fume cupboard for further 30 minutes, to ensure absence of solvent traces, before hydration step (Figure 1). Trizma buffer (pH 7.4), 5mL, was added at this stage to hydrate the film. For formulations containing MB, the MB (volume of concentrate equivalent to 0.5mg MB) was added at this step after addition of the trizma buffer. The round-bottomed flask was placed in a water bath shaker at 150 rpm at 60°C (above phase transition temperature) for 30 minutes, followed by further 40 minutes rest in the dark. Based on the preliminary niosomal formulations with Span 60 as a main non-ionic surfactant with MB, it was proved that Span 60 led to low % EE of MB, hence it was used only with CUR in F15 (Table 1). F15 confirmed low %EE of CUR with Span 60.

Downsizing of the vesicular system was performed using probe sonication (Active Motif, Singapore) that was set at amplitude of 40%, five cycles of two minutes each with one minute rest between the cycles was employed. During the process, an ice-bath was used to prevent sample overheating. To remove the potential impurities, the samples were centrifuged (Hettich Mikro 200R, Germany) at 5000 rpm for 5 minutes.

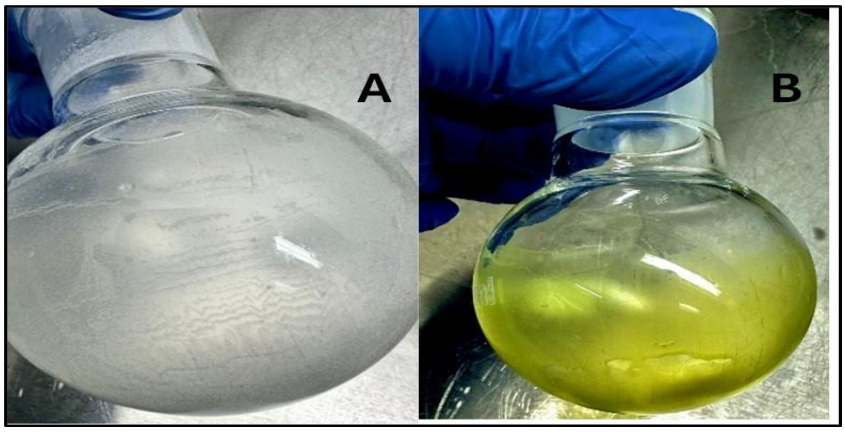

Figure 1. Image (A) shows the thin film layer formed, in the absence of drugs, after solvent evaporation. Image (B) is for the thin film-incorporating curcumin.

2.3. Morphology of niosomes

The morphology of selected niosomal vesicular formulations containing either MB or CUR, were characterized, directly after preparation and before probe sonication and sephadex G50 gel filtration, utilizing optical microscope (Bioblue Lab Binocular, UK) and Transmission Electron Microscopy, TEM, (Hitachi HT7800, Japan). TEM employed with an Emsis Xarosa camera and Radius software; a negative staining procedure was performed using a 2% uranyl acetate reagent.

2.4. Determination of entrapment efficiency (EE%)

The size exclusion chromatographic technique was reported to be highly appropriate for controlling loaded liposome systems, whatever the encapsulated substances (water soluble, hydrophobic, charged or uncharged) (Grabielle-Madelmont *et al.*, 2003). Accordingly, Sephadex G50 gel filtration "size exclusion" chromatography column was utilized in this study to separate the unentrapped drug from the niosomal vesicles, in order to determine the direct encapsulation efficiency inside the eluted niosomes. The unentrapped drug was bonded to the gel while vesicles traversed down through the gel and were collected. Trizma buffer (pH 7.4) was used to wash the niosomal formulations down the column and the obtained eluted niosomes were disrupted by isopropanol, then 1:1 ratio of isopropanol: Trizma buffer (pH 7.4) was used for dilution if needed, to determine the concentration of methylene blue and/or curcumin within niosomes, utilising UV-Vis spectrophotometer (M501, Spectronic Camspec, UK) at wavelengths of 665nm and 425nm, respectively, after construction of calibration curves.

The entrapment efficiency (expressed as the percentage CUR or MB encapsulated within the vesicles) of different formulations were determined prior and after probe sonication. Direct encapsulation efficiency of the noisome formulations was determined by the application of the following equation:

Encapsulation efficiency (%) = (Encapsulated amount/total initial amount) X 100

2.5. Determination of vesicle size and polydispersity index (PDI)

Dynamic light scattering technique was employed to determine the vesicular size, and PDI before and after probe sonication, in triplicates, employing Zetasizer-Nano (Malvern, UK), at 25°C.

Table 1. Composition of the prepared niosomal formulations, along with their total calculated HLB value* and % entrapment efficiency (EE), vesicle size and polydispersity index (PDI) prior to probe sonication. All excipients are represented at % molar ratios. Methylene blue (MB) and curcumin (CUR) were added at a constant total amount of 0.5 mg to each 5 mL formulation.

Formulations	Span 60 (%)	Cremophor RH40 (%)	Cholesterol (%)	Span 65 (%)	MB (mg)	CUR (mg)	*Total HLB Value	EE (%)	Vesicle Size (nm)	PDI
F1	**45**	10	45	-----	0.5	-----	6.6	21.6± 2.6	743.6 ± 25.9	0.82 ± 0.26
F2	**55**	10	35	-----	0.5	-----	6.3	20.7± 2.5	NA	NA
F3	**75**	10	15	-----	0.5	-----	5.9	7.4 ± 1.3	NA	NA
F4	**45**	20	35	-----	0.5	-----	7.9	10.8 ± 2.2	NA	NA
F5	-----	10	45	45	0.5	-----	4.5	51.7 ± 1.6	206.7 ± 7.9	0.50 ± 0.07
F6	-----	10	35	55	0.5	-----	4.1	50.8 ± 2.4	224.7 ± 4.9	0.54 ± 0.07
F7	-----	10	30	60	0.5	-----	3.9	24.4 ± 3.0	NA	NA
F8	-----	10	15	75	0.5	-----	3.6	15.6 ± 2.2	NA	NA
F9	-----	20	35	45	0.5	-----	6.1	46.5 ± 1.6	NA	NA
F10	-----	25	30	45	0.5	-----	6.7	23.2 ±0.6	211.4 ± 35.4	0.44 ± 0.12
F11	-----	10	45	45	-----	0.5	4.5	91.2	197.4	0.46

								± 1.5	± 3.0	± 0.02
F12	-----	10	35	55	-----	0.5	4.1	78.1 ± 0.7	160.9 ± 0.9	0.34 ± 0.02
F13	-----	10	30	60	-----	0.5	3.9	73.6 ± 0.2	160.8 ± 1.3	0.34 ± 0.01
F14	-----	25	30	45	-----	0.5	6.7	58.8 ± 1.5	174.8 ± 2.5	0.49 ± 0.03
F15	**45**	10	45	----	-----	0.5	6.6	41.8 ± 2.8	403.5 ± 9.8	0.61 ± 0.08
F16	-----	10	45	45	0.5	0.5	4.5	MB 45.3 ± 2.5 CUR 84.4 ± 0.4	326.4 ± 12.8	0.56 ± 0.12

NA (not applicable)

* Equation used for calculation of the total HLB value:

A=100(X – HLB of B)/(HLB of A - HLB of B) (Billany, 2002)

where X is the total HLB value, A is the weight percentage of the main non-ionic surfactant, B is the co-surfactant

2.6. Statistical analysis

Independent student's t-test was employed to determine the statistical difference in %EE, at $P < 0.05$ value indicating significant differences.

3. Results and Discussion

The obtained entrapment efficiencies, EE, of different formulations are presented in Table 1. The obtained drug encapsulation efficiencies indicated the overall superiority of niosomes prepared using Span 65, in comparison to those prepared using Span 60 in entrapping both drugs. The highest entrapment efficiency was obtained at equimolar ratio of cholesterol and Span 65 for both MB (formula F5) and CUR (formula F11) loaded formulations with EE values of 51.7% and 91.2%, respectively (Table 1). These results were considered satisfactory for the hydrophilic and hydrophobic nature of MB and CUR, respectively. Consequently, formulation containing Span 65 with 1:1 mol% ratio to cholesterol, was selected to be loaded with both MB and CUR (formula F16), Table 1.

Optical microscopy was previously employed to provide information about the morphology of nanoparticles (Asthana et al., 2016). The theory is based on applying visible light to magnify the images of the tested samples. Therefore, selected formulations were examined under an optical microscope employing 400x magnification power and the obtained images are presented in Figure 2 A and B. The images reflect the spherical nature of the vesicles, indicating the formation of niosomes. This was also confirmed by TEM photomicrographs (Figure 2 C and D) that reflected the spherical shape of the vesicular systems. It is worth noting that vesicles containing the lipophilic CUR are bigger in size compared to those for MB. This coincides with the reported data indicating that incorporation of lipophilic molecules with high entrapment efficiency usually leads to increased vesicle size and reduced dispersion homogeneity (Essa, 2010). This is reflected, in the current study, in the TEM images (Figure 2C and D) where CUR vesicles are less homogenous in size compared to those containing MB.

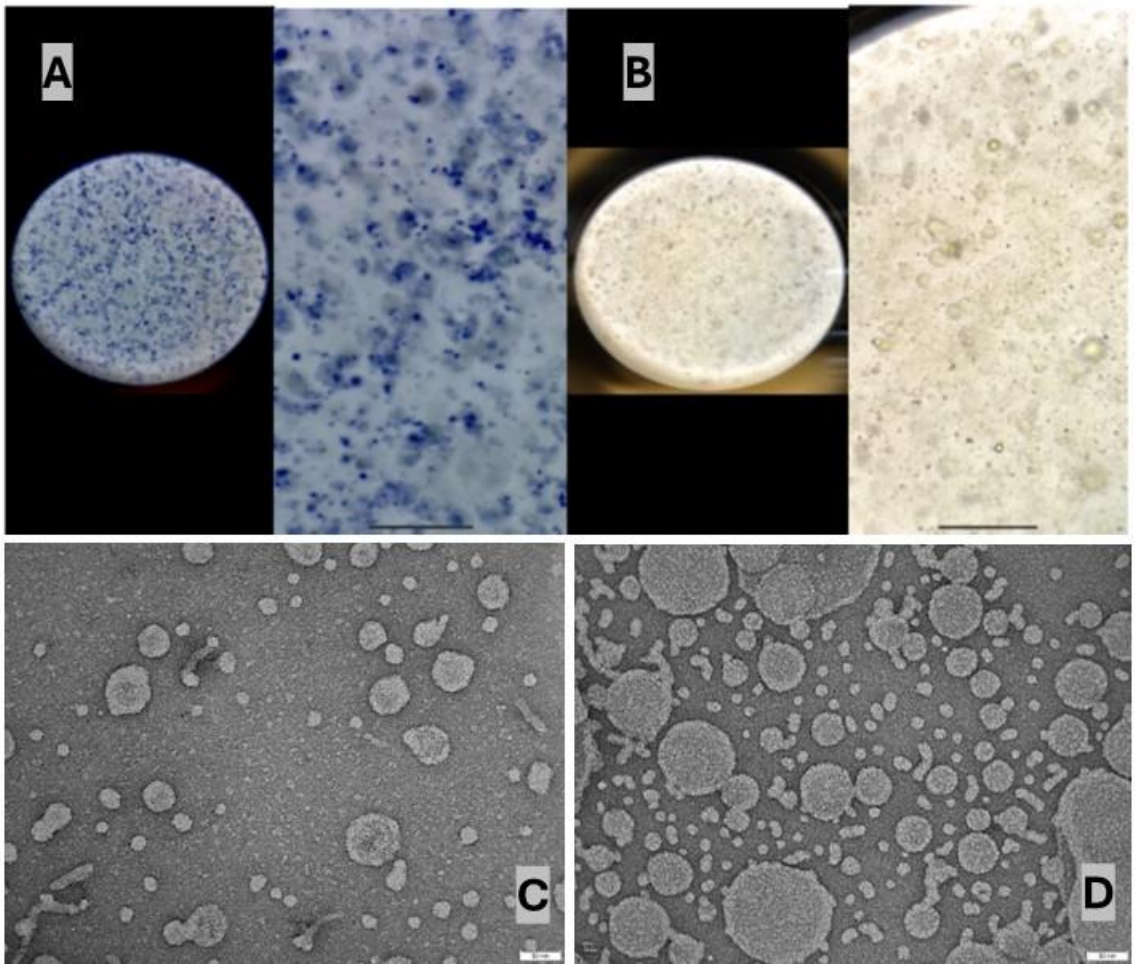

Figure 2: Optical microscope images of niosomes (before probe sonication) entrapped A) methylene blue or B) curcumin (Total magnification was 400X). TEM photomicrographs for

vesicles (before probe sonication) containing C) methylene blue or D) curcumin (Total magnification was 100000X).

3.1. Factors influencing entrapment efficiency of the niosomes

3.1.1 Effect of type of non-ionic surfactants

To elucidate the effect of surfactant type on the entrapment efficiency of MB, 8 formulations were selected with identical composition and concentrations, but differ in the used main non-ionic surfactant. Based on the niosomal formulation details shown in Table 1, formulations F1, F2, F3 and F4 (prepared using Span 60) were compared to formulations F5, F6, F8 and F9 (prepared using Span 65). For clarity, the entrapment efficiency values of the 8 selected formulations are presented as histogram in Figure 3.

There was a significant increase ($P < 0.0049$) in the % EE for the niosomal formulations containing span 65 at all cholesterol: main non-ionic surfactant ratios. This could be explained by taking the structures of Span 60 and Span 65 into account. Incorporation of Span 65 (tristearate) comprising three hydrocarbon tails into the formulations could result in a more packed lipid bilayer with reduced permeability, compared to the incorporation of Span 60 (monostearate) with one hydrocarbon tail. Additionally, Span 65 has lower HLB value of 2.1 compared to 4.7 of Span 60 (Needs, 1976), comprising a bigger portion of hydrophobic tail in comparison to the hydrophilic head group, which could further lead to a less leaky bilayer with better containment of the hydrophilic MB in the aqueous compartment(s) of the vesicles. This also confirms more entrapment of curcumin, hydrophobic in nature, with the same formula compared to MB.

Span 65 containing niosomes also demonstrated a superiority in encapsulating CUR, over span 60 containing formulations (Figure 3). Comparison of CUR formulations F11 and F15 containing span 65 and span 60 respectively, the CUR % EE is more than doubled in formulation F11 containing span 65 than with F15. This can also be justified considering the structure of span 65 with greater hydrophobic tail portion (tristearate) compared to span 60 (monostearate); this provides more space in the bilayer, for hydrophobic CUR to be entrapped, as well as formation of less leaky niosomes with more stable bilayer (Miao et al., 2020).

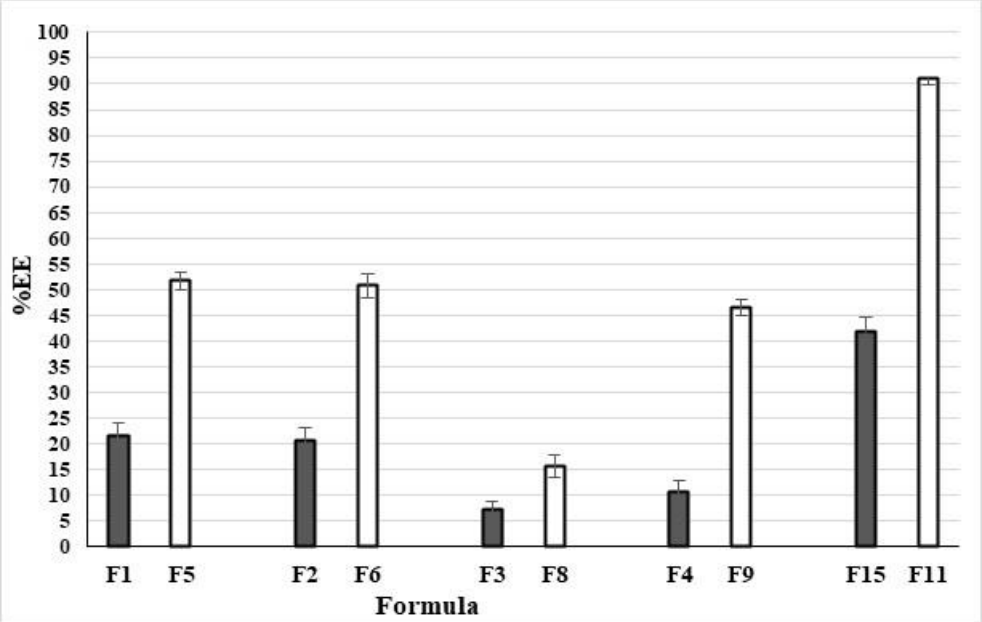

Figure 3: Illustration of the impact of using Span 60 (dark bars) or Span 65 (white bars) as the main non-ionic surfactant on the percentage entrapment efficiency (%EE) of the paired MB and CUR niosomes having the same compositions, apart from the type of the non-ionic surfactant. For formulations' composition refer to Table 1.

3.1.2. Effect of cholesterol to lipid ratio

Cholesterol plays a crucial role in the physical characteristics of lipid bilayers such as, for example, fluidity (de Meyer and Smit, 2009), the permeability of small molecules or drug(s) through the bilayer membranes and entrapment efficiency (Shinoda, 2016).

Therefore, cholesterol to lipid ratio is considered an important factor that can affect drug entrapment within the vesicles (Nasseri, 2005; Yeo et al., 2018). To investigate this factor, number of formulations with increasing cholesterol to surfactant mol% ratio were prepared using Span 60 and Span 65 (Table 1).

For comparison, the selected formulations were those having constant Cremophor RH40 concentrations but with different cholesterol to surfactant ratio. In terms of formulations encapsulating MB and containing either Span 60 (F1-F3) or Span 65 (F5-F8), increasing cholesterol concentration led to increasing MB entrapment efficiency (Figure 4). Inclusion of cholesterol in vesicular membrane is known to increase the rigidity of the bilayer structure and decrease the leakage of water-soluble compounds through membranes (Essa, 2010). The same performance was noticed for niosomes containing CUR (F11-F13). These findings are in good agreement with previously published research work that reported increased drug entrapment by increasing cholesterol content in vesicular systems (Agarwal et al., 2004; Mokhtar et al., 2008).

The obtained results can be justified by considering the impact of cholesterol on membrane integrity. Cholesterol increases the rigidity of the vesicular bilayer membrane that protects against leakage of the encapsulated compound. In terms of Span 65 containing formulations encapsulating CUR, increasing cholesterol concentration could improve solubilizing and incorporation of CUR into the bilayer membrane, resulting in enhanced drug loading. Increased cholesterol also could improve CUR distribution within the vesicle bilayer and more uniform dispersion of CUR in the bilayer, resulting in enhanced encapsulation efficiency.

It is important to mention that a high concentration of cholesterol can result in excessive rigidity of the bilayer membrane, with negative impact on the entrapment efficiency, with the maximum beneficial effect of about 45% (mol%), for which cholesterol is in 1:1 ratio with non-ionic surfactant. This could be explained considering the chemical structure of cholesterol, being able to have only one hydrogen bonding group with the oxygen functionalities of the surfactant, putting the arrangement of the hydrocarbon chains orientation in the optimal position, resulting in an enhanced membrane cohesion (Nasseri, 2005).

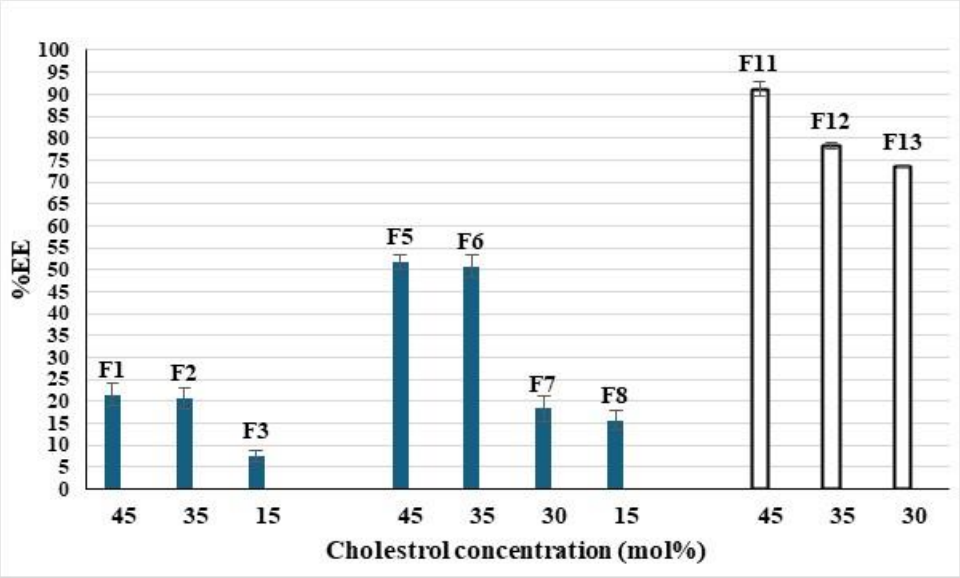

Figure 4: The impact of cholesterol concentration (mol%) on the percentage entrapment efficiency (%EE) of the prepared niosomal dispersions, F1-F3 and F5-F8 niosomes with methylene blue (dark bars); F11-F13 niosomes with curcumin (white bars). For detailed formulation composition refer to Table 1.

3.1.3. Effect of Cremophor RH40 as a co-surfactant

Cremophor RH40 was used as a co-surfactant during niosomes preparation. To investigate the impact of co-surfactant on the entrapment efficiency of MB and CUR in niosomes, Cremophor RH40 was used at three concentrations 10, 20 and 25 mol% of the total composition of niosomal preparations, Table 1. Span 60-vesicles will be compared to Span 65-vesicles.
For formulations prepared using Span 65, the effect of co-surfactant on the extent of entrapment depended on the hydrophilicity of the entrapped molecules. For example, formulations F13 containing 10% Cremophor RH40 showed an entrapment of 73.5% of the lipophilic CUR (Figure 5). Increasing co-surfactant concentration to 25% with the same cholesterol content in formula F14 markedly reduced CUR entrapment to 58.7%. However, for the hydrophilic MB there was no significant ($P>0.05$) difference in the entrapment efficiency with changing Cremophor RH40 concentration and constant cholesterol concentration, this can be seen by comparing EE% of formulations F7 (24.4%) with F10 (23.2%), Table 1 and Figure 5.

For noisome prepared using same mol% of span 60, F1 will be compared to F4, Table 1. Both formulations have 45mol% of span 60 with 10 and 20mol% of the co-surfactant (Cremophor RH40), respectively. Increasing the co-surfactant concentration reduced MB entrapment efficiency from 21.6% (F1) to 10.8% (F4). This can be explained by considering the hydrophilic chemical structure of Cremophor RH40. The HLB value of Cremophor RH40 is between 14 and 16, meaning that increasing the mol% ratio of the Cremophor RH40 to Span 65 (being hydrophobic with relatively much lower HLB value), increases the total HLB value of the formulation (Smail et al., 2021), along with decreasing the membrane resistance to permeability, and decreasing the membrane ability to encapsulate MB and CUR (refer to Table 1).

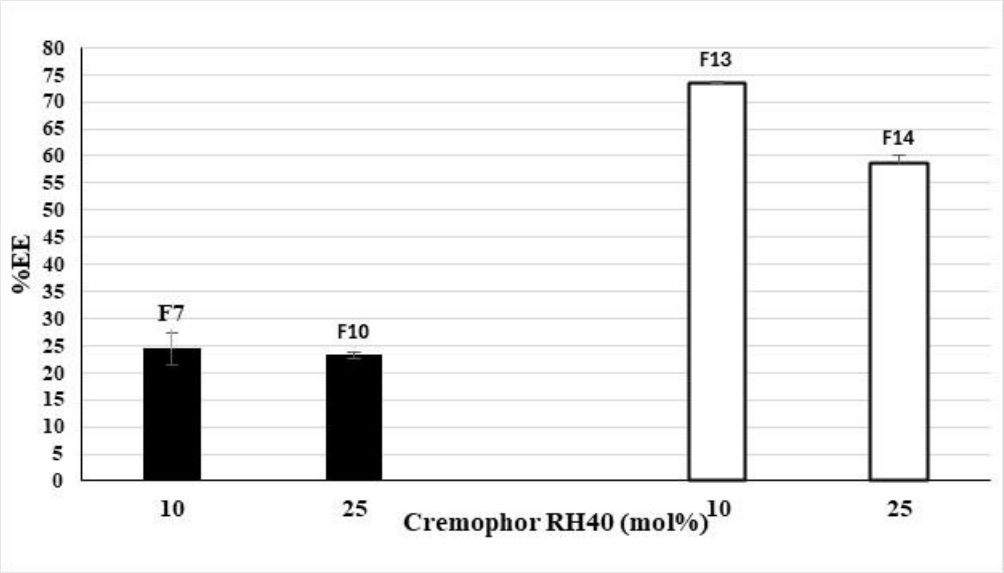

Figure 5: The impact of Cremophor RH40 (mol%) concentration on the percentage entrapment efficiency (%EE) of the prepared niosomes prepared using Span 65 entrapping either methylene blue (F7 and F10) (dark bars) or curcumin (F13 and F14) (white bars). Detailed formulations are in Table 1.

3.1.4. Physicochemical properties of the loaded drugs

The physicochemical property of the drug is an important factor that can affect the extent entrapment in the vesicles based on its partition coefficient (Chrai et al., 2002). It is well accepted that lipophilic compounds are preferentially encapsulated by niosomes relative to hydrophilic ones.

To investigate the potential influence of physicochemical properties of the loaded drug(s) on the extent of entrapment, formulations with identical compositions were loaded with either MB or CUR. The hydrophilic MB tends to be encapsulated in the aqueous pockets of the niosomes or adsorbed on the bilayer surface, while the lipophilic CUR has a tendency towards the hydrophobic region of the bilayer membrane (Bashkeran et al., 2023). Comparing formulations F1 with F15 (Figure 6), both have the same compositions but loaded with MB and CUR, respectively. The entrapment efficiency of F15 is nearly doubled compared to that for F1 (Table 1). This indicates the superiority of the formulation to encapsulate hydrophobic CUR over hydrophilic MB.

Comparing formulations F5, F6, F7, and F10 with F11, F12, F13, and F14 with MB, CUR, respectively, also indicate a significant superiority of the formulations containing Span 65 to encapsulate CUR over MB ($P < 0.05$). This could be partially due to the hydrophobic nature of the CUR; in one hand, a high tendency towards adhesion to the round bottom flask

inner surface, could result in a better incorporation into the thin film layer, formed during the production process, refer to Figure 1, and on the other hand, the tendency of CUR to be entrapped into the hydrophobic region of the bilayer membrane (Bashkeran et al., 2023), by just passing polar heads.

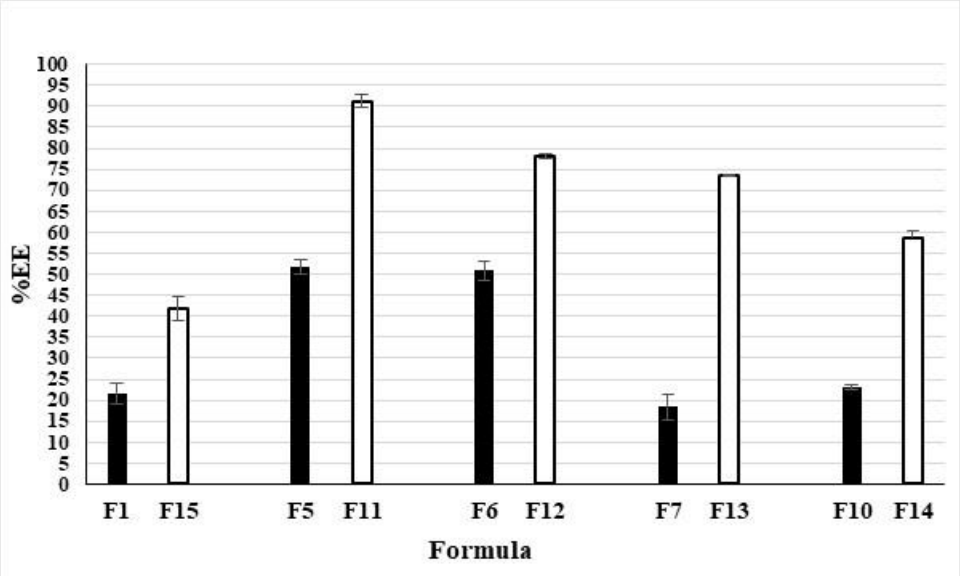

Figure 6: The impact of physicochemical properties of the loaded drug on the percentage entrapment efficiency (%EE) of the grouped niosomal formulations with identical compositions, but of different loaded compounds of either methylene blue (dark bars) or curcumin (white bars). Detailed formulations are in Table 1.

3.1.5. Impact of probe sonication

The probe sonication technique has a potential to increase or decrease the EE profile of the niosomes, considering the intensity and the duration of sonication process (Yeo, et al., 2019). In other words, adjusting the intensity and duration of probe sonication process can improve the uniformity of the vesicles distribution in the suspension media leading to more homogeneous distribution of the drug molecules into the niosomes. It also contributes to the breakdown of aggregates and multilamellar vesicles into the unilamellar structure with a more consistent and higher capacity for encapsulation of drugs. It also enhances the drug loading, particularly in term of hydrophobic drugs such as CUR, due to the generated mechanical energy facilitating drug solubilization within the bilayer membrane.

Nevertheless, probe sonication can impact negatively if applied with too high intensity and excessive duration for the prepared formulation, resulting in drug leakage, disruption of vesicles, and promoting the reformation of aggregates, leading to reduced entrapment efficiencies. There are certain factors to be considered while adjusting the probe sonication

intensity, duration, and temperature. These factors could include the formulation composition, and the physicochemical properties of the loaded drug(s) (Nowroozi et al., 2018; Yeo et al., 2019). In this study, the formulation samples were subjected to 5 cycles of 2 minutes each, with one minute rest in between of the cycles, with an amplitude being set at 40%. The data obtained after probe sonication showed mostly no significant difference in %EE (Table 2) versus data on Table 1.

Table 2: The % entrapment efficiency (EE%), vesicles size and polydispersity index (PDI) subsequent to probe sonication of the optimal niosomal formulations. The data are mean values ± standard deviations (SD) of triplicates (n=3). Detailed formulations are in Table 1.

Formulations	EE (%)	Vesicle size (nm) ± SD	PDI ± SD
F1	21.2	171.8 ± 12.2	0.50 ± 0.06
F5	50.1	177.2 ± 1.4	0.32 ± 0.02
F6	34.1	195.5 ± 3.2	0.35 ± 0.02
F10	13.0	149.5 ± 1.7	0.32 ± 0.01
F11	94.0	186.4 ± 7.1	0.35 ± 0.01
F12	79.0	151.2 ± 1.1	0.33 ± 0.02
F13	74.4	150.8 ± 0.9	0.33 ± 0.01
F14	64.3	165.4 ± 2.1	0.34 ± 0.01
F15	43.0	135.3 ± 2.8	0.40 ± 0.05
F16	MB 64.5 CUR 89.5	207.9 ± 5.3	0.36 ± 0.04

3.2. Factors impacting vesicle size and polydispersity index

The niosome size is influenced by various factors, such as the preparation method, lipid composition, and the type of incorporated non-ionic surfactant (Nowroozi et al., 2018; Bhardwaj et al., 2020). Regarding, the niosomes containing span 60 and span 65, there was a noticeable difference in the vesicle size of the prepared formulations. In particular, the formulations containing span 60 yielded relatively larger vesicles, compared to the

formulations containing span 65, before probe sonication, e.g., formulations F1 and F5, or formulations F11 and F15, Table 1.

This difference in size could be explained by considering factors related to the type of on-ionic surfactant being incorporated, such as HLB values, self-assembly behavior of surfactants, degree of hydrophobicity, interactions with other components, solubility and dispersion (Nowroozi et al., 2018). Span 60 having higher HLB value, is more hydrophilic compared to span 65, this could impact the arrangement and packing of the lipids in the niosomal bilayer with different bilayer curvature. Higher HLB value also indicates the larger head group compared to the hydrocarbon tail, that could result in the formation of larger vesicles. Additionally, the difference in HLB values can impact vesicles self-assembly behavior during the formation of the niosomes, resulting in a different niosomal vesicle size (Chen et al., 2019; Bhardwaj et al., 2020). Hydrophobicity of the surfactants also plays a role in this, as span 65 (tristearate) comprising three hydrocarbon tails, comparing to span 60 (monostearate) with one hydrocarbon tail, could result in a tighter packing and smaller vesicle formation. Solubility of the surfactants in the solvent (chloroform in this study) or lipid mixture incorporated into the formulation, could impact the degree of surfactant incorporation into the vesicle bilayer, resulting in different niosomal vesicles' size.

Cholesterol content has a significant impact on the vesicle size (Essa, 2010), depending on the factors such as concentration of cholesterol, and the lipid composition of the niosomes (Nowroozi et al., 2018). However, thin film hydration preparation method of niosomes can interact with cholesterol influence on the vesicle size. In one hand, considering formulation F5 and F6, containing span 65 and loaded with MB, the vesicle size in F5 formulation is smaller while its cholesterol content (mol%) is higher. This can be explained considering that the addition of cholesterol amount can reduce the vesicle size by enhancing the tighter packing of the lipids within the bilayer, resulting in the formation of smaller niosomes with more stable and less permeable membrane bilayer. The impact of cholesterol incorporation in niosomes, towards reducing membrane fluidity, can also result in a more stable, more uniform, and a smaller vesicle size (Nowroozi et al., 2018). Cholesterol can also impact the bilayer membrane curvature, influencing the niosomes shape and size. On the other hand, considering formulations F11, F12, and F13, containing span 65 and loaded with CUR, the vesicle size increases as the amount of cholesterol increases. This can be explained by the hydrophobic nature of CUR that tends to be entrapped in the hydrophobic region of the bilayer. As amount of cholesterol increases, solubilization and incorporation and loading of the CUR in the bilayer

membrane increases, resulting in a larger and more stable vesicle. Comparison between formulations F13 and F14 that contain span 65 and are loaded with CUR prior the probe sonication, a vesicle size increase in F14, Table 1, is observed, despite the same cholesterol content (mol%) and lower span 65 content (mol%) in F14 formulation, Table 1. This can be explained by the chemical structure and higher mol% content of co-surfactant, i.e., Cremophor RH40, in these formulations. Cremophor RH40 having relatively high HLB value and being more hydrophilic in nature with larger portion of hydrophilic head to the hydrophobic region, results in the formation of larger vesicles with less convenient environment in bilayer for CUR entrapment, leading to lower CUR loading in the bilayer membrane, as well as formation of larger niosomes (Bhardwaj et al., 2020).

Therefore, reducing the size of vesicles prepared by the thin film hydration method using techniques such as probe sonication is required as it will result in smaller size vesicles with improved PDI profile (Nowroozi et al., 2018). The data obtained after the application of the probe sonication (Table 2) indicate an ideal size range and PDI profile, beneficial for drug delivery for example to tumors, for all formulations containing either span 60 or span 65, loaded with MB and/or CUR (Table 2). It is worth to mention that there are other factors impacting the vesicle size including hydration time and hydration volume during manufacturing process (Yeo et al., 2019), however these factors are out of the scope of this study and for this reason have been kept at constant levels for all formulations.

4. Conclusion and future perspective

This study investigated the influence of multiple compositional variables on the entrapment efficiency of niosomal formulations loaded with methylene blue and/or curcumin (hydrophilic and hydrophobic drugs, respectively). Combined encapsulation of the two compounds in the same nanocarrier is an attractive strategy as each compound is individually reported to have anticancer activity. All prepared formulations successfully yielded spherical vesicles with acceptable average size range. The study reflected the impact of the type of the used non-ionic surfactants (Span 60 or Span 65) with different HLB values on the entrapment efficiency of niosomes. Span 65 was superior to Span 60 regarding entrapping both methylene blue and curcumin. The hydrophobic curcumin showed higher drug encapsulated compared to methylene blue. Cholesterol concentration positively affected entrapment efficiency. The presence of Cremophor RH40 as a co-surfactant increased the encapsulation of the hydrophilic

methylene blue, but inversely affected the encapsulation of curcumin. The optimized niosomes combining both methylene blue and curcumin provides the formulator with nanocarrier dispersion with enhanced entrapment of both compounds with potential use as anticancer therapy. However, other characterizations will be needed for future developing this anticancer intervention to target affected sites, those characterizations include for example drug release from niosomes, storage stability studies and cell biology testing on different types of cancer cells.

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