**Title:** **Investigating the role of cholesterol in the formation of non-ionic surfactant based bilayer vesicles: thermal analysis and molecular dynamics**

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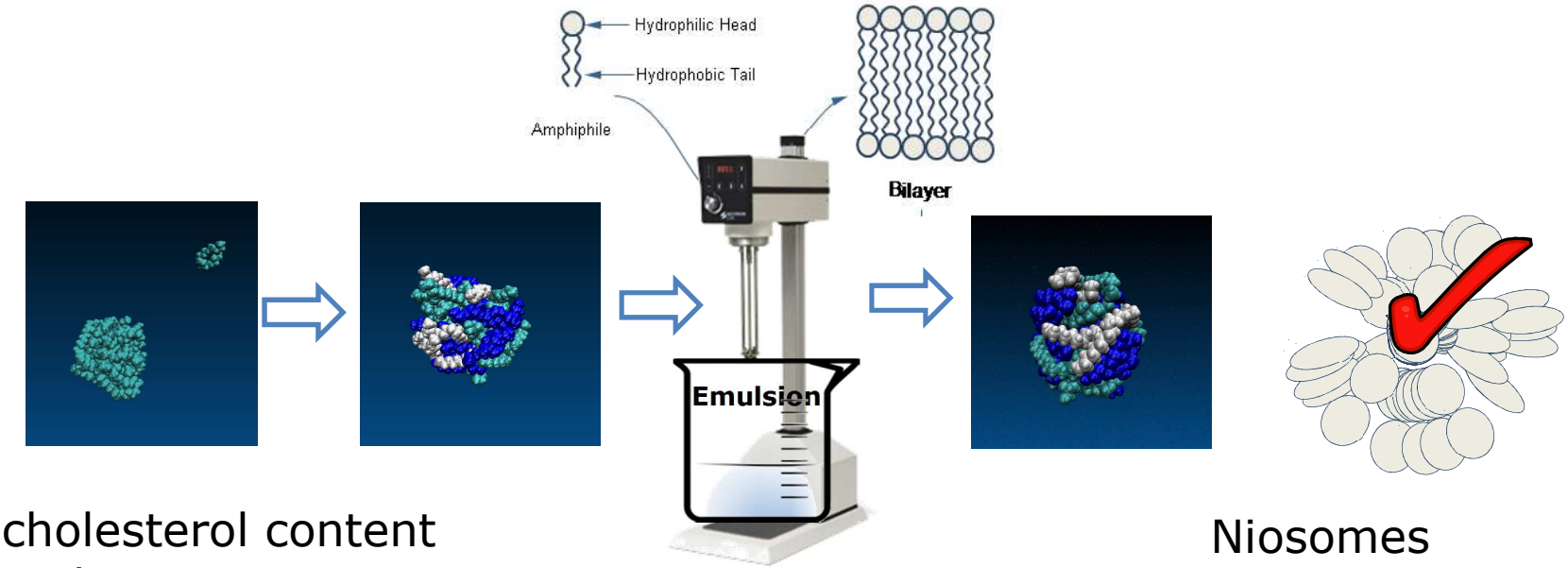
**Abstract**

The aim of this research was to investigate the molecular interactions occurring in the formulation of non-ionic surfactant based vesicles composed Monopalmitoyl glycerol (MPG), Cholesterol (Chol) and Dicetyl Phosphate (DCP). In the formulation of these vesicles, the thermodynamic attributes and surfactant interactions based on molecular dynamics, Langmuir monolayer studies, differential scanning calorimetry (DSC), hot stage microscopy and thermogravimetric analysis (TGA) were investigated. Initially the melting points of the components individually, and combined at a 5:4:1 MPG:Chol:DCP weight ratio, were investigated; the results show that lower (90 °C) than previously reported (120 to 140 °C) temperatures could be adopted to produce molten surfactants for the production of niosomes. This was advantageous for surfactant stability; whilst TGA studies show that the individual components were stable to above 200 °C, the 5:4:1 MPG:Chol:DCP mixture show ~2 % surfactant degradation at 140 °C, compared to 0.01 % was measured at 90 °C. Niosomes formed at this lower temperature offered comparable characteristics to vesicles prepared using higher temperatures commonly reported in literature. In the formation of niosome vesicles, cholesterol also played a key role. Langmuir monolayer studies deomonstrated that intercalation of cholesterol in the monolayer did not occur in the MPG:Chol:DCP (5:4:1 weight ratio) mixture. This suggests cholesterol may support bilayer assembly, with molecular simulation studies also demonstrating that vesicles cannot be built without the addition of cholesterol, with higher concentrations of cholesterol (5:4:1 vs 5:2:1, MPG:Chol:DCP) decreasing the time required for niosome assembly.

Graphical abstract




↑cholesterol content
↓melting temperatures
↑Increase vesicle formation rate

Niosomes formed

## 1. Introduction

Non-ionic surfactant vesicles (NISV) or niosomes are generally prepared from non-ionic surfactants such as e.g. monopalmitoyl glycerol, sorbitan esters or ethoxylated sorbitan esters, combined with cholesterol and often charged lipids such as dicetyl phosphate or stearylamine (Uchegbu and Florence, 1995, Brewer and Alexander, 1992, Carafa et al., 1998, Manosroi et al., 2003). Niosomes, like liposomes, have been investigated to enhance the delivery of a range of drugs and vaccines (Uchegbu et al., 1995, Uchegbu and Florence, 1995, Wilkhu et al., 2013b). For example, work from our laboratories have investigated their ability to enhance vaccine efficacy for antigens such as subunit influenza H1N1, H3N2 (Wilkhu et al., 2013a, Wilkhu et al., 2013c) and malarial antigens Merozoite surface protein 1 (MSP1) and glutamate rich protein (GLURP) (Vangala et al., 2006, Wilkhu et al., 2013a, Wilkhu et al., 2013c). We have recently considered the *in vivo* fate of such antigen-loaded niosomes and our results demonstrate that incorporation of antigen within vesicles enhanced delivery and targeting of the antigen to the Peyer's Patch compared to antigen alone. Delivery to both the Peyer's patches and mesentery lymphatics was shown to be dose dependent at lower concentrations, with saturation kinetics applying at higher concentrations(Wilkhu et al., 2013a).

In the production of niosomes, melting of the surfactants and subsequently homogenising them into an aqueous phase can be used (Uchegbu, 2000); however, quoted melting temperatures used in many of these protocols is high and based on the individual melting points of each of the surfactants used. For example, in the preparation of niosomes composed of monopalmitoyl glycerol (MPG), cholesterol and dicetyl phosphate (DCP) temperatures of 120 to 140 °C have been used (Mann et al., 2004, Mann et al., 2009). Yet, high temperatures may cause surfactant degradation; therefore, consideration of the required temperatures employed in such protocols is a vital component in niosome formulation studies.

To understand further the properties of surfactants used to build niosomes, a range of methods can be adopted. For example, Differential Scanning Calorimetry (DSC) has been widely used in its application in understanding the thermal characteristics of materials (Bouzidi et al., 2005) where an insight into a range of thermal properties including melting temperatures, phase transitions and heat capacity changes can be obtained. Thermogravimetric analysis (TGA) can also be employed to understand thermal degradation and lipid/surfactant decomposition when elevating the temperature of the sample (Skala et al., 1997).

To consider the organisation of surfactants into niosomes, molecular modelling techniques can also be used to mimic the behaviour of molecules on an atomic level (Cai et al., 2011). Currently there are numerous reports in the field of lipid simulations and progress in this area has been summarised in recent reviews (Bennett and Tieleman, 2013, Rabinovich and Lyubartsev, 2013, Schneck and Netz, 2011, Lyubartsev and Rabinovich, 2011, Notman and Anwar, 2013). All-atomic molecular dynamics (MD) simulations are capable of providing the detailed information of each atom in the simulated lipid systems based on an empirical force field (e.g. GROMOS, CHARMM, AMBER), which is hard to obtain otherwise (Cai et al., 2011). Currently, all-atomic MD simulations are able to simulate these systems with hundreds of lipids and water from the order of several nanoseconds to hundreds of nanoseconds (Bennett and Tieleman, 2013, Notman and Anwar, 2013).

Given that previous work from our laboratories has investigated using niosomes for the delivery of vaccines using a surfactant combination of MPG:Chol:DCP at a 5:4:1 wt ratio (Wilkhu et al., 2013c, Wilkhu et al., 2013a), the aim of this current work was to investigate the thermal conditions required for the production of non-ionic based vesicles prepared via hot-melt homogenisation and to consider the molecular interactions in the system, via MD simulations, with particular consideration of the role of cholesterol within the system.

## 2. Materials and Methods

### 2.1 Surfactants

The surfactants used in the study were Monopalmitoyl glycerol (MPG) (Larodan Labs, Sweden), Cholesterol (Synthecol) (Sigma Aldrich, UK) and Dicetyl Phosphate (DCP) (Sigma Aldrich, UK).

### 2.2 Differential scanning calorimetry investigations of surfactants and surfactant blends

The surfactants were analysed in the solid state using a TA Instruments Q200 Thermal Analysis DSC. The DSC was calibrated using sapphire and indium for a cell constant and temperature calibration based on the heat flow and type of cooler in place on the system. The individual surfactants were weighed into T-Zero aluminium pans and then hermetically sealed. All experimental runs started at an initial temperature of 20 °C, purged under nitrogen gas, with a scan rate of 10 °C /minute to 120 °C.

After the individual surfactants were analysed for melting, different blends of the surfactant mixtures were further analysed based on a design of experiments template created where the ratio of surfactants was changed. The surfactants were mixed at the appropriate ratios and a sample of this blend was placed into the pan and investigated.

### 2.3 Thermogravimetric analysis of surfactant blends

The three surfactants (MPG, Chol and DCP), either individually or at a 5:4:1 ratio, were placed onto the Perkin Elmer TGA and analysed for their stability across a temperature range of 20 to 250 °C. Samples were also heated to 90 °C, 120 °C or 140 °C and held isothermally for 10 minutes at each of the three temperatures respectively to consider the stability at melting commonly reported (Mann et al., 2004, Mann et al., 2009). All samples were run in triplicate to determine degradation and reproducibility, and all formulations were carried out using nitrogen gas and air.

### 2.4 Design of Experiments

To afford better understanding of the temperatures required to melt the surfactants employed in the preparation of MPG:Cholesterol:DCP niosomes, further studies adopting Design of Experiments using a D-optimal design were employed. The MPG ratio was fixed at a wt ratio of 5 whilst cholesterol varied from a wt ratio of 2-4 and the DCP wt ratio from 0-3, hence effects of the individual components could be analysed for melting onset temperature, melting enthalpy, crystallisation enthalpy and reheat enthalpy.

### 2.5 Investigation of surfactant packing in monolayers

Langmuir monolayer studies have been used widely to understand the packaging of surfactants when mixed together by spreading insoluble amphiphilic molecules in chloroform onto an aqueous water subphase (Gopal and Lee, 2006). Monolayer studies of the individual surfactants (MPG, Chol and DCP), and a mixture of surfactants in the ratio 5:4:1 of MPG:Chol:DCP respectively, were carried out using a KSV mini trough Langmuir system (KSV Instruments Ltd, Helsinki, Finland) equipped with a platinum Wilhelmy plate in an isolated area. Ultrapure water 18 Ω (Milipore, UK) formed the subphase within these studies and the temperature of the trough was 20 ± 1 °C. Stock solutions of the individual surfactants were prepared at a 0.5 mg/mL in chloroform and a mixture was also prepared in chloroform at the set ratio. The method used was adapted from Ali et al, (2010) where 20 µL of the surfactant stock solutions

was spread onto the air/water interface using a glass Hamilton syringe precise to ± 0.2 µL (Ali et al., 2010). Upon spreading of the samples onto the interface, the chloroform was left to evaporate and the hydrophilic barriers were set to close at a speed of 10 mm/min to form monolayer isotherms. Each sample ran until it reached its collapse pressure and triplicate samples were tested.

### 2.6 Simulation of Molecular Dynamics

The molecular dynamics (MD) simulations utilized the AMBER11 software package (Case et al., 2005) with the general AMBER force field (gaff) (Wang et al., 2005) for all molecules. All molecules and models were built by Discovery Studio Visualizer 3.1. For simulation of the melt process, the simulated annealing method was used to mimic the hot melt preparation method of solid dispersion in the experiments (Ouyang, 2012). In the minimisation procedure, the structures were subjected to 1000 steps of steepest descent minimisation followed by 1000 steps of conjugate gradient minimization. After minimisation, 1 ns simulated annealing simulation was performed. Langevin dynamics was used with a time step of 2 fs and a cut off of 12 Å for non-bonded interactions. Firstly, the system was gradually heated from 0 to 400 K in 200 ps, and then kept at a temperature of 400 K for 300 ps to equilibrate the systems. Next, the system was cooled from 400 to 300 K in 100 ps and finally the systems were kept at a temperature of 300 K for 400 ps for equilibration. The simulated annealing procedure was repeated 10 times (10 ns) for complete convergence of the systems.

For the simulation of lipid molecules in water, a classical MD simulation was performed. The electrostatic interactions were calculated with the particle mesh Ewald method (Darden et al., 1993, Essmann et al., 1995, Crowley et al., 1997, Sagui and Darden, 1999, Toukmaji et al., 2000, Sagui et al., 2004) and the cut off was 10 Å. Using the LEAP module in AmberTools 1.5, the lipid structure was immersed in a truncated octahedral water box with a solvation shell of 10 Å thickness using TIP3P model for water (Jorgensen et al., 1983). This procedure resulted in solvated water structures containing approximately 30,000 atoms which included the above lipid molecules, with the remainder being water molecules. The simulation protocol with 10 ns was the same as previous publications (Ouyang et al., 2010a, Ouyang et al., 2010b, Ouyang et al., 2011).

### 2.7 Preparation of non-ionic surfactant based vesicles

To prepare the bilayer vesicles, the surfactants in the powder form (307.5 mg) were mixed at the appropriate ratio (5:4:1 of MPG:Chol:DCP respectively), melted in an oil bath at the appropriate temperature (90, 120 or 140 °C depending on the experiment) for 10 minutes. While maintaining the molten lipid mix, vesicles were created by the addition of 5.45 mL of 25 mM sodium bicarbonate (Sigma Aldrich, UK) buffer pH 7.6 (60 °C) and homogenised for 5 minutes using a Silverson machines homogeniser model #L4RT. After 5 minutes, a pre-incubated (60 °C) solution of OVA (1 mg/mL; 0.55 mL) was added to the vesicles and further homogenised for 5 minutes. Upon cooling, the niosome formulation was incubated for 2 hours in an incubating mini-shaker (VWR) with gentle shaking at 220 rpm.

### 2.8 Determination of vesicle size and zeta potential

The vesicle size distribution was determined using laser diffraction on a sympatec 2005 (Helos/BF) cuvette analyser. 20 µL of the niosome suspension was diluted into the cuvette with 40 mL double distilled water. The zeta potential, which is an indirect measurement of the vesicle surface charge, was measured in 1.5 mL double distilled water at 25 °C on a Zeta Plus Brookhaven Instrument. 15 µL of the niosome suspension was mixed in 1.5 mL double distilled water and then analysed.

### 2.9 Determination of OVA entrapment

The OVA loading was determined in the vesicles after separation via centrifugation, produced by the addition of 300 µL of the niosome sample in a 3.9 mL Beckman centrifuge tube filled with double distilled water for a period of 40 minutes at 354,000 X g (TLN-100 rotor). The protein content of niosomes was measured directly using the Ninhydrin assay at a wavelength of 560 nM (Brewer et al., 1995).

## 3. Results

### 3.1 Cholesterol dissolution in the lipid melts promotes lower melting temperature requirements for niosomes production.

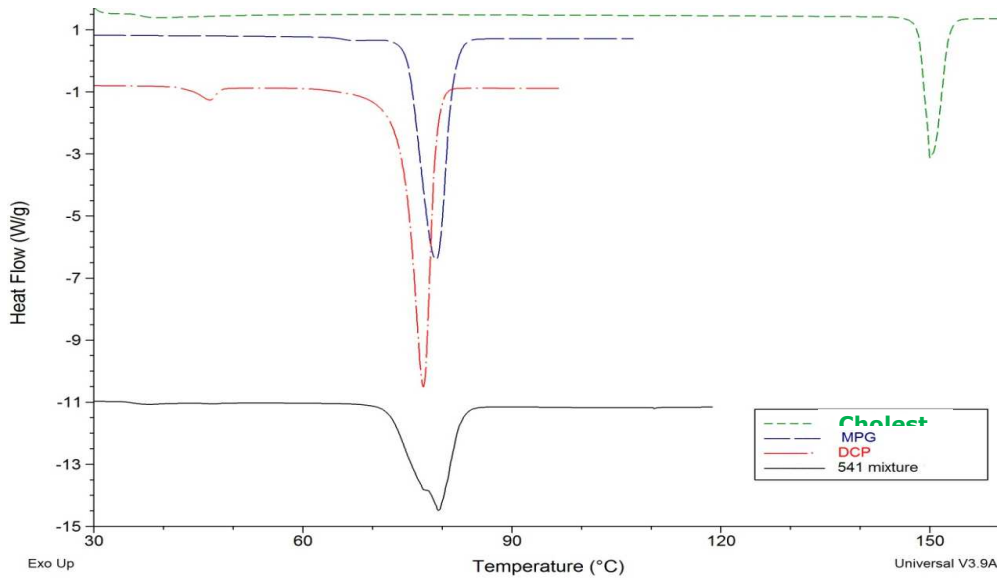
To investigate the thermal characteristics of the surfactant mixtures used in the production of niosomes, the individual surfactants and their physical mix blend were investigated by differential scanning calorimetry to determine their melting properties both independently and

as a blend. Figure 1A, represents the thermograms of the individual components, and a mixture blend of MPG:Chol:DCP at a 5:4:1 wt ratio of respectively, all in the solid state. From figure 1A, DCP has a melting onset temperature of 74.2 ± 0.3 °C with a Tmax of 77.3 °C, followed MPG (melting onset of 74.9 ± 0.2 °C with a Tmax of 79.1 °C), and finally cholesterol which had the highest melting point of 148.7 ± 0.07 °C with a Tmax at 150.1 °C. However, when these three lipids were mixed at a 5:4:1 weight ratio (MPG:Chol:DCP), the mixture has a melting onset temperature of 72.3 ± 0.7 °C with a complete melt (Tmax) at 79.4 °C (Figure 1A) with no further thermal events occurred after this temperature (data not shown).

Hot stage microscopy was used to further investigate the melting of the surfactant blend. The hot stage was set with start and end temperatures the same as those adopted in the DSC; however, a slightly slower scan rate (6 °C/ min) were employed due to the resolution and accuracy of the recording software. Figure 1A shows two image reels, one of cholesterol alone and one of a MPG:Cholesterol:DCP (5:4:1 wt ratio) powder blend with cholesterol in contact with MPG and DCP. As can be seen, cholesterol on its own does not melt until 148-150 °C. However, the mixture represented in Figure 1A shows that just before the complete melt (Tmax) at 80 °C there are still crystals present and that complete melting occurs at 81 °C which corresponds to the DSC thermogram of the mixture (Figure 1A). These results suggest that cholesterol may be dissolving into the molten MPG/DCP mixture.

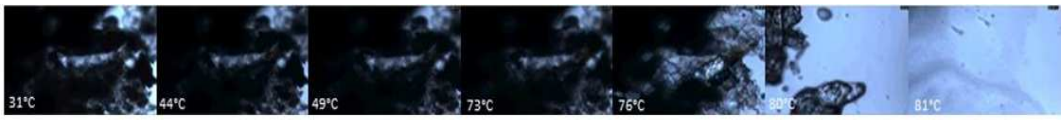
To further investigate if cholesterol melted or solubilised within the solid state lipid mixture upon heating, hyper DSC was used on the surfactant mixture MPG:Chol:DCP (5:4:1 wt ratio) to try to separate the thermodynamic events from any kinetic events (Figure 1B). Faster heating rates are employed to provide insufficient time for dissolution/solubilisation to take place, therefore should represent clear endotherm peaks at the melting points of the individual components (Gramaglia et al., 2005). The results from the hyper DSC give rise to a large broad melting peak which contains multiple peaks representing the surfactant mixture, with no presence of a melting peak at the cholesterol melting point temperature (149 °C; Figure 1B), indicating that cholesterol integration has taken place prior to its melt. Unfortunately, figure 1B indicates that a scan rate of 500 °C/min was not sufficient to circumvent dissolution of cholesterol occurring in the mixture.

A




| Surfactant | Melting Onset Temperature (°C) | Melting enthalpy (J/g) |
|---|---|---|
| MPG | 74.9 ± 0.20 | 194 ± 18.8 |
| Chol | 148.7 ± 0.07 | 72 ± 10.2 |
| DCP | 74.2 ± 0.34 | 222 ± 32 |
| MPG:CHOl:DCP (5:4:1) | 72.3 ± 0.75 | 135 ± 6.2 |

Cholesterol




Mixture


B

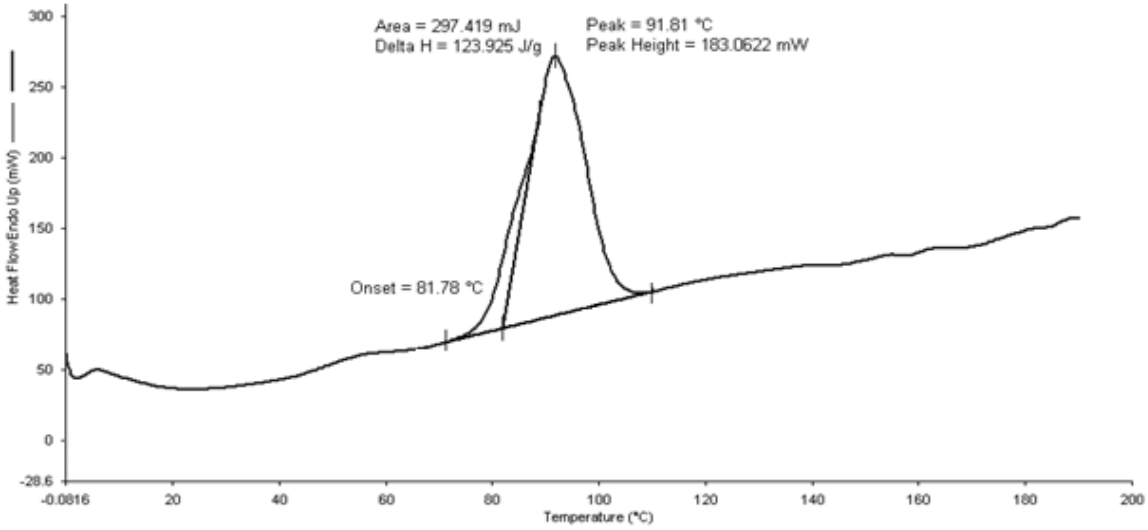



Fig. 1. (A) Melting ofmixture andindividual surfactants overlaidonto a single scanandmelting onsettemperatures andcorrespondingmelting enthalpies for the individual surfactants and their mixtures (region of 25–160◦C with a scan rate 10◦C/min). Hot stage microscopy images of cholesterol and the 5:4:1 MPG:Chol:DCP mixture are also shown (scan rate of 6◦C/min). (B) Hyper DSC thermogram of the surfactant mixture in the region of 0–200◦C at a scan rate of 500◦C/min.

The impact of the surfactant mixture composition on the resulting melting points required to form niosomes was considered in a Design of Experiments study; results (Figure 2) showed that when the ratio of cholesterol was increased relative to DCP, the onset of melting point decreases (Figure 2A). This trend correlates with a decrease in enthalpy, which takes place when cholesterol content increases within the mixture (Figure 2B). Similarly, at fixed cholesterol content, increasing DCP content increased the melting enthalpy (Figure 2B). When the cholesterol content increases, the heat enthalpy required to break the bonds and allow for melting of the mixture decreases, as further represented in figure 2C. Figure 2C demonstrates that the cholesterol lowers the melting onset, which can be due to its effect on lowering the heat energy required to melt the mixture. When cholesterol content is increased, the heat enthalpy required (which is used to break the bonds and allow for melting to occur) decreases. Again, this suggests that the cholesterol is not melting, but is dissolving into the molten mixture, therefore allowing the mixture to melt at a lower temperature as visualised by the hot stage microscopy (Figure 1A).

### 3.2 Reducing surfactant degradation through reduced preparation temperatures

Given the results from DSC analysis (Figures 1 to 3) suggest that lower temperatures than previously reported for the formulation of MPG-based niosomes (e.g. Skala et al., 1997) may be employed, thermal gravimetric analysis of the components was conducted to consider the stability (in terms of weight loss) of the surfactants at different temperatures. TGA isotherms of the individual surfactants (MPG, cholesterol and DCP) from 30 °C to 250 °C are shown in Figure 3A. The results show that for the three individual surfactants, there is no measured weight loss until temperatures are in excess of 180 °C (Figure 3A) which exceeds the working temperature range commonly reported in the manufacture to produce niosomes. However, when MPG, Cholesterol and DCP are combined into a 5:4:1 wt ratio mixture (Figure 3A; 5:4:1 mix), the surfactant mixture shows degradation at temperatures lower than the individual components, with changes in weight being measured at around 120 °C (Figure 3A). To further investigate this, the 5:4:1 blend of MPG:CHO:DCP was held isothermally for 10 minutes at three melting range temperatures 90, 120 and 140 °C and weight loss recorded (Figure 3B). The results show that when the surfactant blend was held at 120 and 140 °C (temperatures adopted in previously reported niosome formulation protocols e.g. Mann et al., 2004, Mann et al., 2009) there was measured weight loss of 0.6 ± 0.3 % and 2.3 ± 0.9 % respectively (Figure 3B). However, by adopting a temperature of 90 °C, which was shown to promote the melting

of the surfactant powder mix (Figure 1), notably lower weight loss was measured (0.01 ± 0.0015 %; Figure 3B).

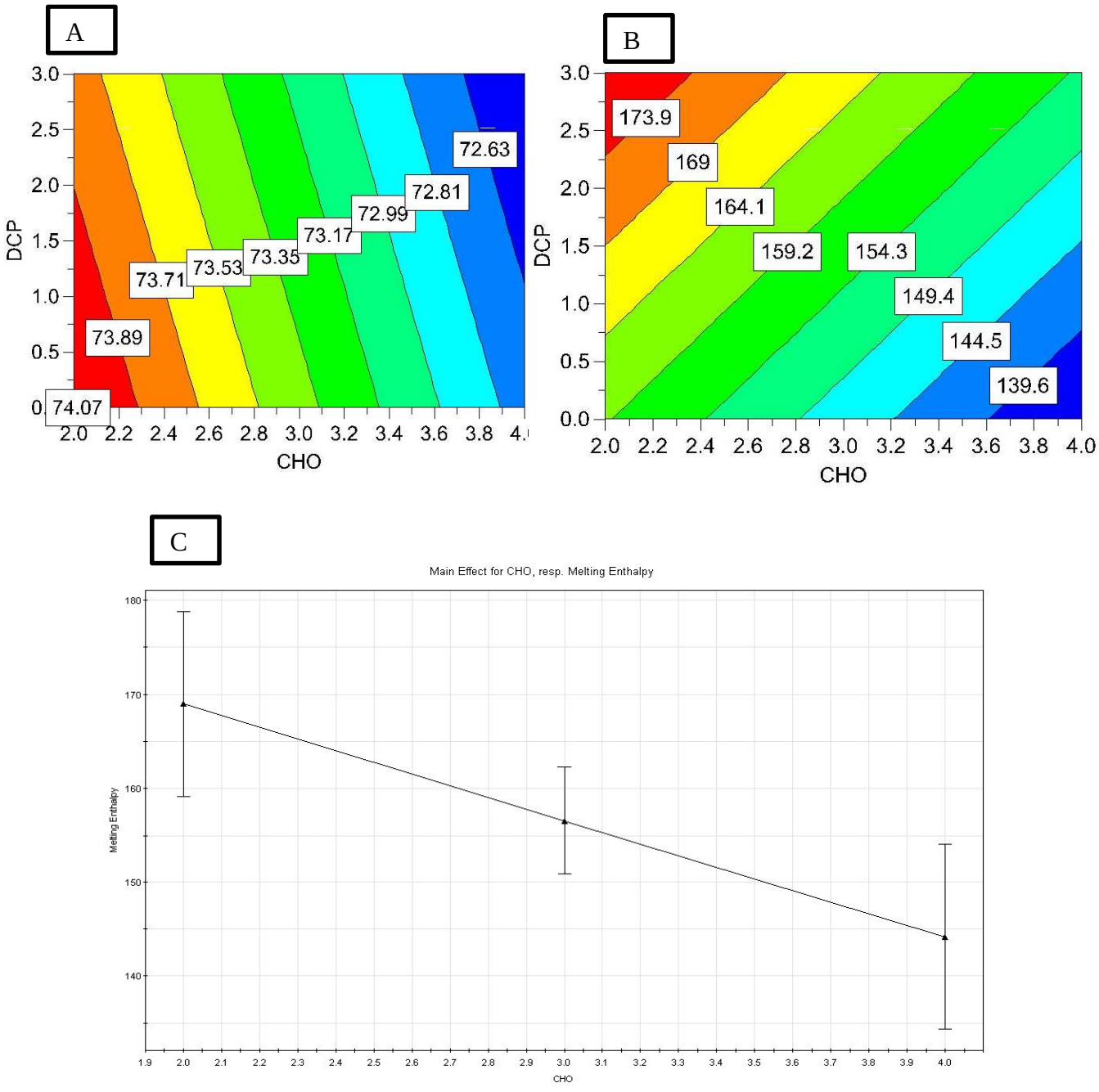



Fig 2. Contourplots showingthe thermodynamic effects of DCP and cholesterol upon melting.(A)The effectof cholesterol and DCP on the onset of melting(◦C), (B) the effect of cholesterol and DCP on the melting enthalpy (J/g) of the mixture. MPG surfactant kept constant ratio of 5 for each ratio of DCP and cholesterol varied (scan rate = 10◦C/min) and (C) Main effects plot for the effect of cholesterol content upon melting enthalpy of the system (J/g).

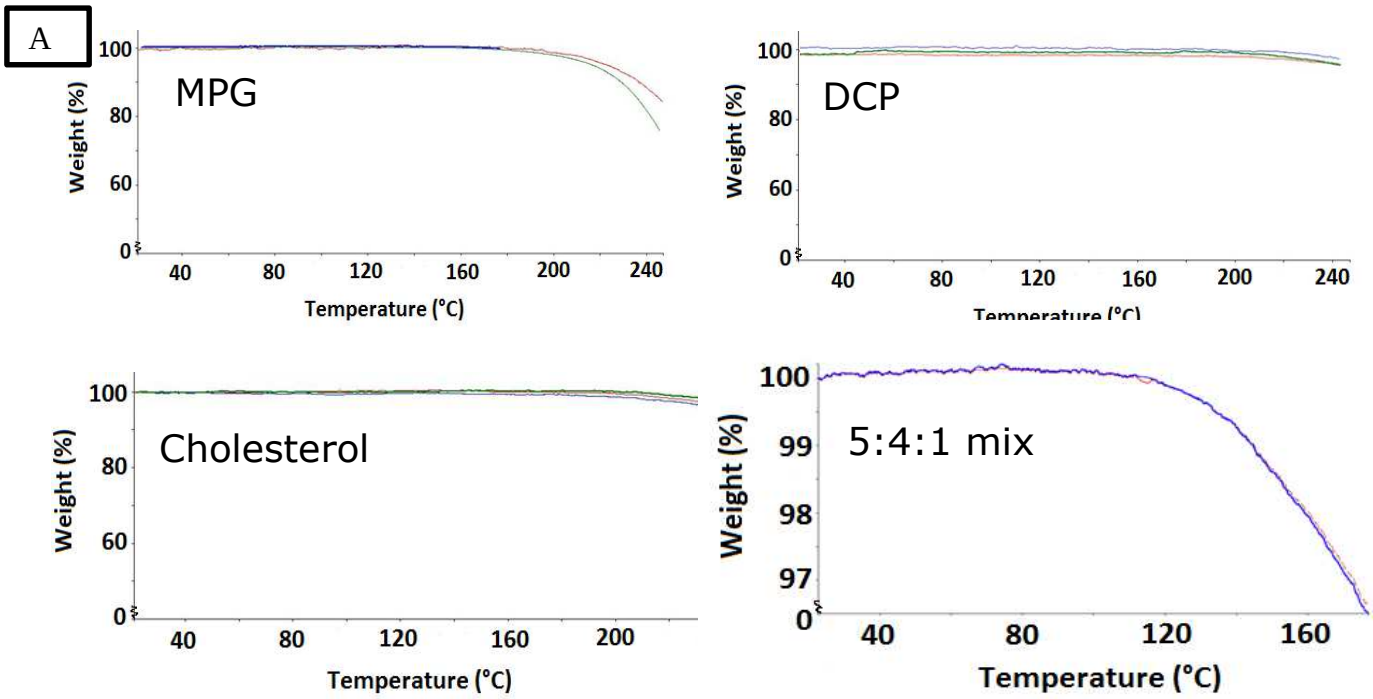
### 3.3 Adopting lower temperatures for the production of niosomes

Based on the information obtained about the surfactant mixtures in Figures 1 to 3, vesicles were produced at two temperature ranges with an oil bath either set between 90-95 °C or 120-125 °C and upon manufacture, the niosomes were investigated to elucidate if lower temperatures could be adopted in their manufacture. Two sets of formulations were prepared: 'empty' niosomes alongside niosomes formulated with the model antigen, OVA, as these vesicles as commonly investigated as vaccine delivery systems. The average vesicle size, zeta potential and entrapment for formulations are presented in Table 1. An average vesicle size of ~ 6 µm was produced irrespective of the temperature used to melt the surfactants (Table 1). Similarly, there is no notable difference in zeta potential or OVA loading ( ~23 % of initial amount added; 550 µg) between the two methods of preparation suggesting that the lower temperature production method can be used in the hot-melt homogenisation production of niosomes.

### 3.4 Surfactant packing studies: the impact of cholesterol

To consider how cholesterol may influence the packing of the surfactants in the bilayer vesicles, monolayer isotherms for the individual surfactants and the mixture of the surfactants were investigated, and the experimental and ideal extrapolated mean molecular area and surface area compression pressure of mixed and pure monolayers at the air/water interface measured (Figure 4).

The scan of MPG (Figure 4) shows that the surfactant isotherm exhibits several phase changes prior to collapsing when the surface pressure is increased. At 65 - 50 $A^2$/molecule there is a gaseous phase, followed by a liquid expanded and gaseous phase from 50 - 35 $A^2$/molecule. At 35 - 22 $A^2$/molecule a liquid condensed/ liquid expanded phase is present followed by a short liquid condensed phase to 20 $A^2$/molecule where a sharp solid phase up to 16 $A^2$/molecule and the surface collapse pressure of 51.6 ± 1.5 mN/m is reached. In terms of cholesterol (Figure 4) the phase changes upon increasing surface pressure differ to those of the MPG surfactant; cholesterol starts in a gaseous phase from 40 - 35 $A^2$/molecule followed by liquid condensed phase from 35 - 30 $A^2$/molecule. After 30 $A^2$/molecule a sharp solid phase occurs up to 27 $A^2$/molecule at a surface collapse pressure of 46.6 ± 0.5 mN/m. The DCP isotherm is very similar to that of cholesterol, where a gaseous phase exists between 40 - 50 $A^2$/molecule until a liquid condensed phase is achieved between 36 - 40 $A^2$/molecule until a final collapse pressure is reached of 53.8 ± 0.9 mN/m at 34 $A^2$/molecule.




| Temperature | 90 °C | 120 °C | 140 °C |
|---|---|---|---|
| % degradation after 10 min | 0.01 ± 0.0015 | 0.6 ± 0.3 | 2.3 ± 0.9 |

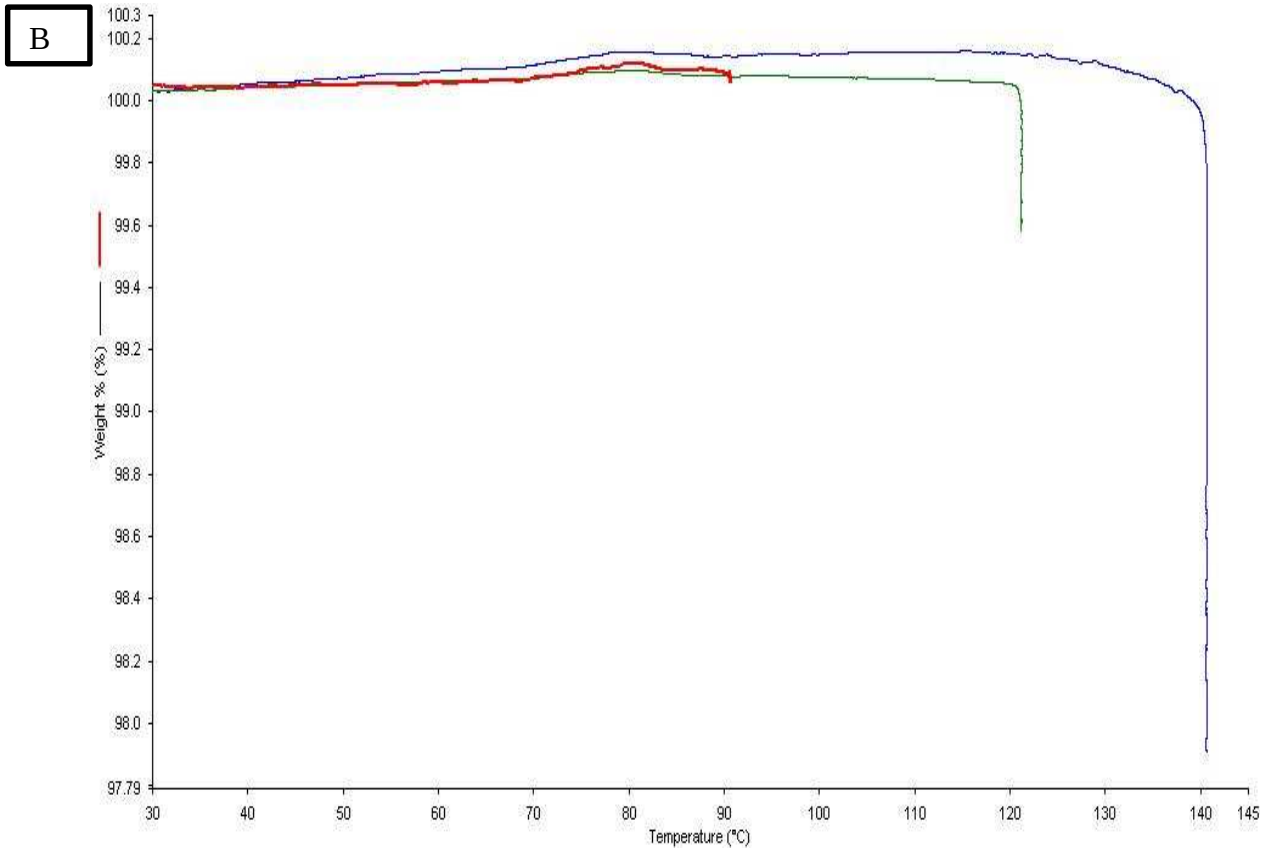
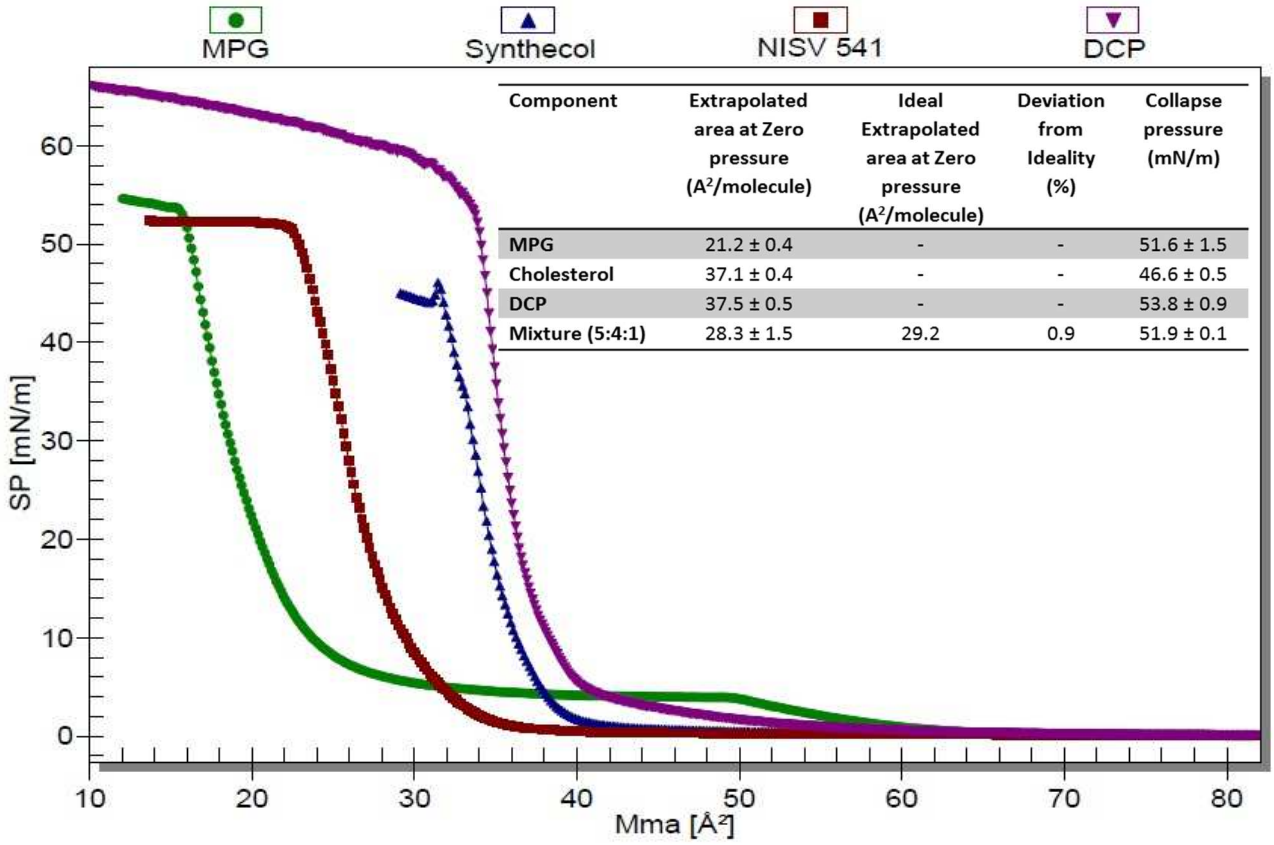



Fig. 3. (A) Thermogravimetric analysis scans ofthe individual surfactants and of a 5:4:1 blend of MPG:Chol:DCP respectively from 20 to 250◦C and (B) thermogravimetric scans of the 5:4:1 MPG:Chol:DCP blend at three melting range temperatures held isothermally for 10 min all at a scan rate of 10◦C/min.

The MPG:Chol:DCP mixture (5:4:1 weight ratio) was also investigated (Figure 4); the mixture exhibits a gaseous phase from 31 - 38 $A^2$/molecule where a liquid condensed phase occurs at 28 - 31 $A^2$/molecule followed by a sharp solid condensed phase at 24 $A^2$/molecule with a collapse pressure of 51.9 ± 0.1 mN/m (Figure 4). When extrapolating the mean molecular area at zero surface pressure for the mixture, there was no significant difference between the calculated ideal extrapolated molecular area at zero pressure and that measured experimentally (28.3 ± 1.5 $A^2$/molecule compared with 29.2 ± 0.2 $A^2$/molecule respectively; Figure 4).

Table 1 Initial vesicle size, zeta potential and antigen entrapment for the stability study

| | Temperature (Ova NISV) | | Temperature (empty NISV) | |
|---|---|---|---|---|
| | 95 °C | 120 °C | 95 °C | 120 °C |
| Size (μm) | 6.15 ± 0.1 | 6.52 ± 0.5 | 6.25 ± 0.04 | 6.58 ± 0.25 |
| Span | 1.73 ± 0.08 | 1.73 ± 0.05 | 1.81 ± 0.02 | 1.76 ± 0.01 |
| Zeta potential (mV) | −63.2 ± 9.5 | −61.7 ± 8.4 | −81.5 ± 9.4 | −80.2 ± 9.5 |
| Ova entrapment (%) | 23.5 ± 2.1 | 23.3 ± 1.2 | | |




| Component | Extrapolated area at Zero pressure ($A^2$/molecule) | Ideal Extrapolated area at Zero pressure ($A^2$/molecule) | Deviation from Ideality (%) | Collapse pressure (mN/m) |
|---|---|---|---|---|
| MPG | 21.2 ± 0.4 | - | - | 51.6 ± 1.5 |
| Cholesterol | 37.1 ± 0.4 | - | - | 46.6 ± 0.5 |
| DCP | 37.5 ± 0.5 | - | - | 53.8 ± 0.9 |
| Mixture (5:4:1) | 28.3 ± 1.5 | 29.2 | 0.9 | 51.9 ± 0.1 |

Fig. 4. Langmuir monolayer isotherms showing surface pressure as a function of mean molecular area representing individual surfactants and MPG:Chol:DCP (5:4:1 weight ratio) including the experimental and ideal extrapolated mean molecular area and surface area compression pressure of mixed and pure monolayers at the air/water interface (at 20◦C).

### 3.5 Simulation of surfactant interactions using molecular dynamics

Molecular modelling techniques are able to describe the complex systems on an atomic level and there are a number of studies on lipid structures by molecular simulation techniques (Lyubartsev and Rabinovich, 2011, Berkowitz, 2009). Within the preparation of niosomes, the lipid mixture was heated and melted in an oil bath. Thus, a simulated annealing technique in the modelling was used to investigate the molecular interaction by simulating the melting procedure. The simulated annealing procedure (Figure 5A) can represent the temperature change (heat to high temperature, maintained high temperature, and cool to room temperature) of the niosome production process used. After completing 10 simulated annealing cycles for the complete convergence of the systems, Figure 5B demonstrates that under heat, MPG molecules cannot bind together without the presence of cholesterol, whilst MPG with cholesterol and DCP can effectively mix and interact together in the melting process. Moreover, in the simulation, the MPG:Chol:DCP mixture at the ratio of 5:4:1 reaches equilibrium more easily than that of 5:2:1 (data not shown).

Similar results are also observed from the simulations in aqueous environment; Figure 6 shows that MPG:Chol:DCP at the ratio of 5:4:1 only needs about 500 ps to reach equilibrium, whilst reducing the cholesterol content to a ratio of 5:2:1 needs four-fold longer (about 2 000 ps) to acquire the equilibrated state (Figure 6), again demonstrating the supporting role of cholesterol in the formulation and construction of these niosome systems. Furthermore, as Figure 7 indicates, that the MPG alone cannot form a stable system even after 10 ns.

## 4. Discussion

In general, the formation of non-ionic surfactants into bilayer vesicles is dependent on many factors including: temperature, surfactant choice and concentration, electrostatic and electrodynamic interactions of the surfactants within the aqueous phase. Interestingly in our studies (Figures 1 – 3) we have demonstrated that the choice of surfactant blend, in particular incorporation of cholesterol, influences the thermal attributes of the surfactant blends, both in the initial melting of the surfactants and in formed vesicle bilayers in the aqueous phase. Our studies demonstrate that in the formulation of niosomes where MPG, Chol and DCP surfactants are melted, the cholesterol itself does not melt; rather, it becomes miscible with the molten MPG/DCP mixture.

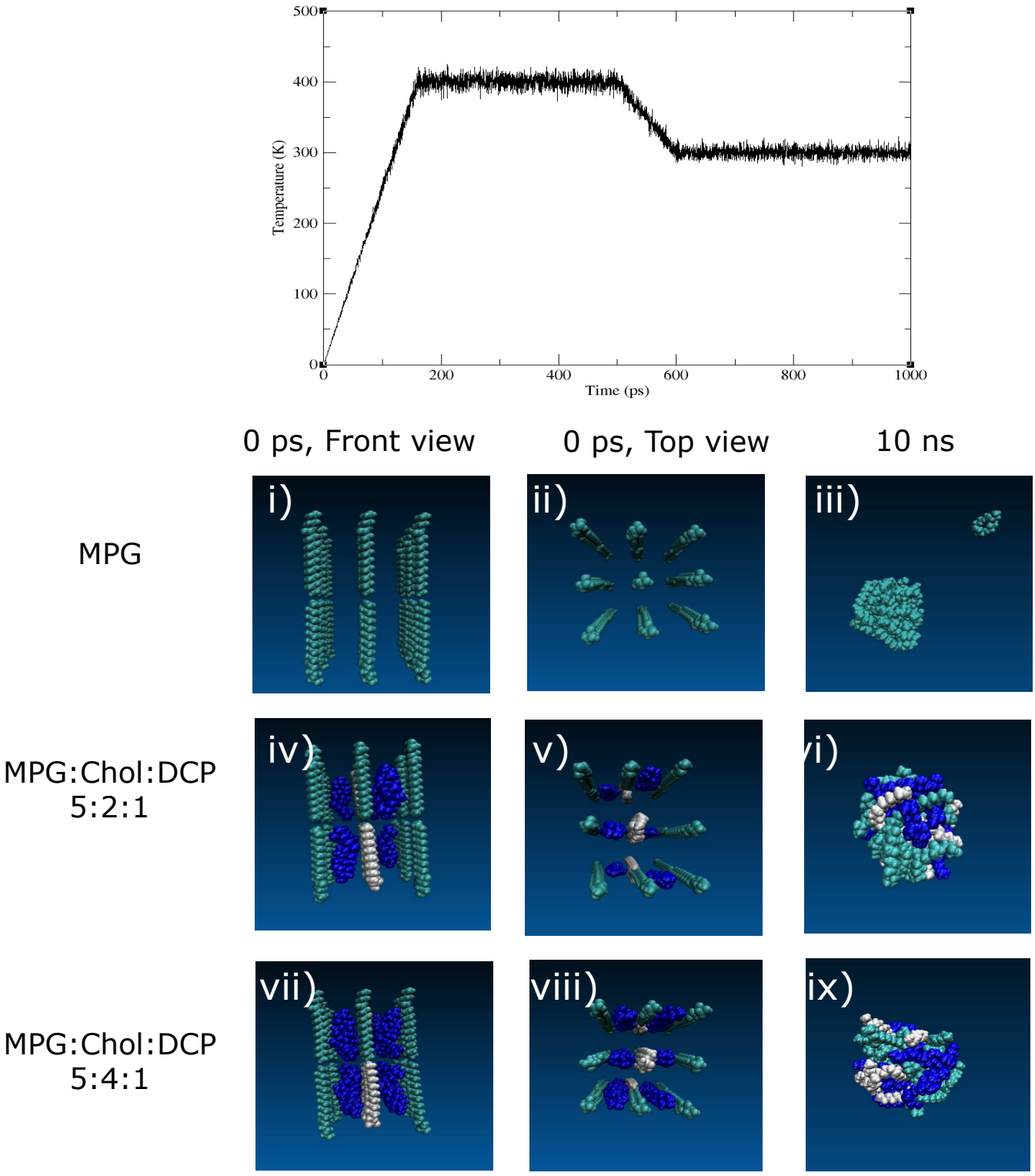



Fig 5(A) Temperature change vs time for the lipid systems in a simulated annealing cycle. (B) Snapshots of lipid systems in the simulated annealing simulations. MPG systems: (i) 0 ps from the front view, (ii) 0 ps from the top view, (iii) 10 ns; lipid system at the ratio of 5/2/1: (iv) 0 ps from the front view, (v) 0 ps from the top view, (vi) 10 ns; lipid system at the ratio of 5/4/1: (vii) 0 ps from the front view, (viii) 0 ps from the top view, (ix) 10 ns. (Light blue colour represents MPG molecule; dark blue colour represents CHO molecule; white colour represents DCP molecule.). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

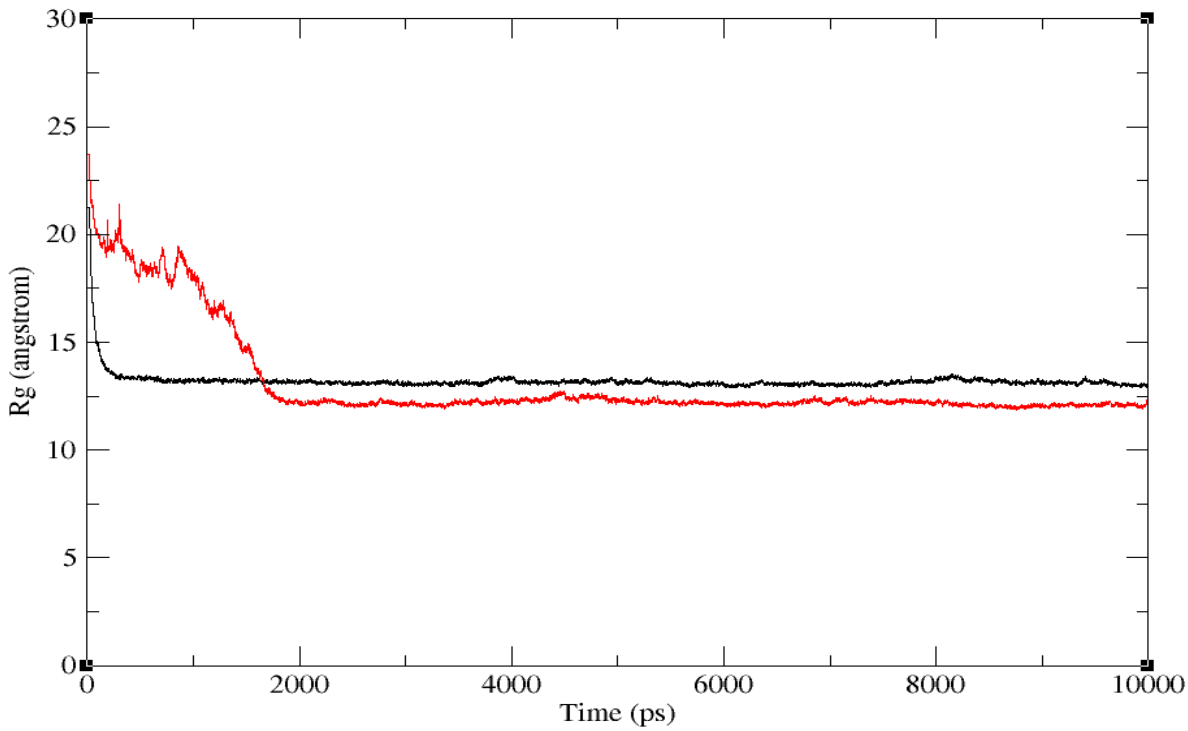



Fig. 6. The radius of gyration, with fluctuation as a function of time for both simulations: red (ratio of 5:2:1; MPG:Chol:DCP) and black (ratio of 5:4:1; MPG:Chol:DCP).(For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

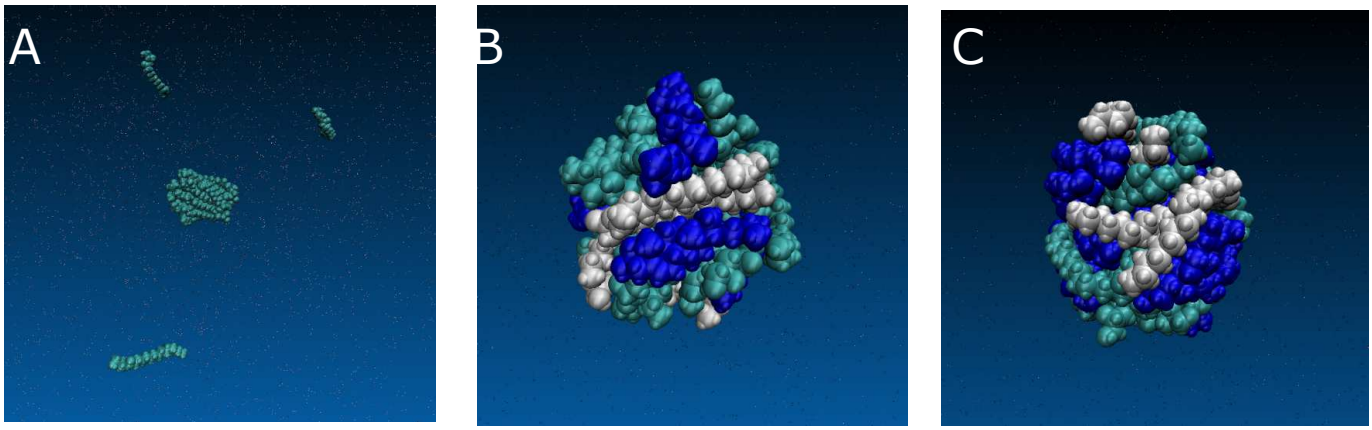



Fig. 7. Snapshots of lipid systems in the aqueous environment: (a) 10 ns of MPG systems; (b) 10 ns of lipid system at the weight ratio of 5:2:1; (c) 10 ns of lipid system at the weight ratio of 5:4:1. Light blue colour represents MPG molecule; dark blue colour represents CHO molecule; white colour represents DCP molecule. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

This is supported by the molecular dynamic studies in Figure 5, which show that cholesterol mixes with the MPG and DCP molecules in the melting process. Studies by Brostow and Datashvili, (2007) show that melting point depression of low density polyethylene occurs upon addition of different ratios of melamine formaldehyde indicating miscibility between the two components (Brostow and Datashvili, 2007). Furthermore, the increase in cholesterol ratio results in a greater surface area of cholesterol within the mixture and interferes with the MPG and DCP lipid matrix which results in interference with the crystalline structures, hence further reducing the enthalpy of melting. DSC, and hot-stage microscopy results confirm that the cholesterol is able to dissolve into the molten MPG/DCP mixture and that this acts as a solvent for the cholesterol to dissolve in. Overall, these findings show that when manufacturing at a large scale, the order of addition of the components could increase melting efficiency by enabling melting to take place at lower temperatures, in turn reducing the risk of thermal degradation of the surfactants. Within this study, we have also demonstrated that lower temperatures can be employed to melt surfactant mixtures and form niosomes (Table 1).

The molecular attributes of the surfactant is also an important parameter in niosome formation, as the type of colloidal or vesicular structure a specific surfactant forms is to a large extent dictated by its molecular shape and the mixture of surfactant combinations used, as this will influence its geometrical packing properties in a given solution environment. The shape of a surfactant may be expressed as its critical packing parameter (CPP), which is a measure of the overall geometry of a surfactant molecule. This can be used a predictor of the resultant structures they can form: a CPP < 0.5 indicates a large contribution from the hydrophilic head group area of the molecule and that such surfactant will form spherical micelles, whilst surfactants with a value between 0.5 < CPP < 1 forms bilayer vesicles, and a CPP > 1 (indicating a large contribution from the hydrophobic group volume) results in inverted micelles (Uchegbu and Vyas, 1998, Israelachvili and Mitchell, 1975, Israelachvili et al., 1977, Wilkhu et al., 2013b).

It is interesting to note that many single chain surfactants have CPP < 0.5 and form micelles, yet these are often used to formulate niosomes; however, not without the addition of additional components. For example, soluble surfactants (such as solulan C-24 and polysorbate 20) with high hydrophilic-lipophilic balance values readily form micelles. Yet with the inclusion of cholesterol into the mixture, niosome vesicle structures are formed (Uchegbu and Florence, 1995, Manosroi et al., 2003, Carafa et al., 1998). The ability of cholesterol to promote the

formation of two-phase bilayer systems, rather than single phase micellar solutions, is related to the overall CPP of the lipid mixture. When a mixture of surfactants and additives (such as cholesterol) are used to prepare vesicles, the operational CPP values will consider the average of the overall components (Kumar, 1991). Indeed, Manosroi et al, (2003) have confirmed that as cholesterol is incorporated within a Tween 61 surfactant mixture, an average CPP value between 0.5 - 1 was obtained, hence the formation of bilayer vesicles rather than micelles (Manosroi et al., 2003). Similarly, Tween 20 has a CPP below 0.5; however, when mixed with cholesterol, unilamellar vesicles were formed (Carafa et al., 1998) as does Span 60, which is widely used in the formulation of niosomes (Ning et al., 2005, Uchegbu et al., 1995). This is due to the average CPP of the mixture moving into the range of 0.5-1, hence showing capabilities of forming bilayer vesicles (Wilkhu et al., 2013b).

Cholesterol is also recognised for its ability to enhance the stability of vesicles (Gregoriadis and Davis, 1979). This has been attributed to the ability of cholesterol to increase the packing density of phospholipid membranes (Semple et al., 1996), which is thought to result from the accommodation of cholesterol within molecular cavities formed by surfactant monomers assembled into vesicles (Devaraj et al., 2002). This was shown in monolayer studies where a decrease in effective area per molecule with increasing cholesterol content was noted (Rogerson et al., 1987, Moghaddam et al., 2011). Unlike the reported intercalation of phospholipids and cholesterol (Ali et al., 2010), no condensing of a MPG:Chol:DCP monolayer was seen in these current studies (Figure 4). This correlates with the proposal that the inclusion of cholesterol in this niosome formulation helps to increase the overall CPP of the system, as intercalation of cholesterol within the system would be counterproductive, reducing the average area per molecule and reducing the overall CPP.

This was supported further by molecular dynamic studies (Figure 5 -7) which demonstrated the beneficial role of cholesterol in the formation of niosomes. These studies show that niosome vesicles cannot be built from a MPG:DCP mixture, and that cholesterol was required with higher concentrations of cholesterol (5:4:1 vs 5:2:1 MPG:Chol:DCP) decreased the time required for niosome assembly.

## 5. Conclusion

Overall, this study demonstrates for the first time that, not only can cholesterol aid the initial melting of surfactants to form niosomes, but also that cholesterol supports the arrangement of

non-ionic surfactants into bilayer vesicles, potentially through cholesterol increasing the overall CPP of the surfactant mixture. This was supported by the use of molecular dynamics, which was able to simulate the behaviour of lipids upon melting, and in an aqueous environment. Based on these studies we were able to successfully formulate niosomes at reduced temperatures thereby reducing thermal degradation of the surfactant and demonstrate the supportive role of cholesterol within building of niosomes.

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