Comparison of the Physical Characteristics of Monodisperse Non-Ionic Surfactant Vesicles (NISV) Prepared Using Different Manufacturing Methods

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Abbreviations

NISV: Non-ionic surfactant vesicles; TFH: Thin-film hydration; PBS: Phosphate buffered saline; MPG: Monopalmitin glycerol; Chol: Cholesterol, DCP: Dicetyl phosphate; AFM: Atomic force microscope; PDI: Polydispersity index; ZP: Zeta potential; FRR: flow rate ratios; TFR: total flow rates.

Abstract

Non-ionic surfactant vesicles (NISV) are synthetic membrane vesicles formed by self-assembly of a non-ionic surfactant, often in a mixture with cholesterol and a charged chemical species. Different methods can be used to manufacture NISV, with the majority of these requiring bulk mixing of two phases. This mixing process is time-consuming and leads to the preparation of large and highly dispersed vesicles, which affects the consistency of the final product and could hinder subsequent regulatory approval. In this study, we have compared the physical characteristics of NISV prepared using two conventional methods (thin-film hydration method and heating method) with a recently introduced microfluidic method. The resulting particles from these methods were assessed for their physical characteristics and *in vitro* cytotoxicity. Through microfluidics, nano-sized NISV were prepared in seconds, through rapid and controlled mixing of two miscible phases (lipids dissolved in alcohol and an aqueous medium) in a microchannel, without the need of a size reduction step, as required for the conventional methods. Stability studies over two months showed the particles were stable regardless of the method of preparation and there were no differences in terms of EC50 on A375 and A2780 cell lines. However, this work demonstrates the flexibility and ease of applying lab-on-chip microfluidics for the preparation of NISV that could be used to significantly improve formulation research and development, by enabling the rapid manufacture of a consistent end-product, under controlled conditions.

Key words: Non-ionic surfactant vesicles, microfluidics, thin-film hydration, heating method, drug delivery, cytotoxicity.

1. Introduction

Non-ionic surfactant vesicles (NISV) or "niosomes", are synthetic bilayer vesicles typically formed by the self-assembly of non-ionic surfactants [1], cholesterol and the addition of a charged species. The self-assembly of non-ionic surfactants into bilayer vesicles, first reported in the 1980s by a group of cosmetic researchers from L'Oréal industries [2], have since been applied extensively as drug delivery systems. NISV exhibit more advantages over liposomes, in terms of cost and stability, and constituent surfactants have a wider range of chemistries that can be selected to provide greater potential for innovation related to vesicle composition [1, 3]. Surfactants commonly employed include polyoxyethylene fatty acid esters (Tweens), sorbitan fatty acid esters (Spans), alkyl ethers, and alkyl glyceryl ethers (Brijs) [4], while other additives include cholesterol, which affects the mechanical strength and permeability of the bilayer structure [5, 6], and charged molecules such as dicetyl phosphate (negative) and stearylamine (positive) [3], which prevent particle aggregation through electrostatic repulsion mechanisms.

NISV have been used to deliver hydrophilic drugs that are encapsulated in the interior aqueous compartment or adsorbed on the bilayer surface, and hydrophobic drugs that are localised within the lipid bilayer of the NISV [7]. NISV have also been used to improve solubility and subsequently bioavailability of poorly soluble drugs, as exemplified by aciclovir and griseofulvin [8, 9]. Moreover, these particles can also improve the stability of peptide drugs, e.g. they have been shown to protect encapsulated insulin in the gastrointestinal tract from degradation by proteolytic enzymes and exhibit good stability in the presence of bile acid salts such as sodium deoxycholate [10]. Other applications of NISV have been in the area of transdermal delivery of different drugs such as oestradiol,

enoxacin and minoxidil [11-13] and in gene delivery of topical DNA vaccines [14, 15]. In recent years, NISV have also been used as carriers for contrast agents for clinical imaging applications in medical diagnostic tools [16].

Various conventional bulk methods have been used in the preparation of NISV (e.g. thin-film hydration, reversed phase evaporation, and heating methods), which utilise mixing of two liquid phases on a bench scale at elevated temperature, in order to facilitate spontaneous self-assembly of the lipid components into bilayer vesicles [1, 17, 18]. The hydration of a thin lipid film (Bangham method) is a simple and widely used process, in which a mixture of lipids are dispersed in an organic solvent (such as chloroform) followed by evaporation of the solvent using a rotary evaporator to form a dry lipid film on the flask wall. NISV are then spontaneously self-assembled by hydrating the lipid film with an aqueous buffer at a temperature above the phase transition temperature of lipids [19]. Hydrophilic drugs can be encapsulated in the formed vesicles by adding the drug in the aqueous buffer when hydrating the lipid film, while hydrophobic drugs can be dissolved with the lipid components before forming the lipid film [20]. Another method reported by Mozafari *et al*. is the heating method [21], in which NISV can be prepared without the use of organic solvents, where the various components are hydrated in aqueous media at room temperature followed by heating at 120°C with mechanical stirring [22]. However, the methods described above, result in the production of large particles, with high polydispersity, as a result of inadequate control of chemical and mechanical environments. These methods necessitate the use of post-production size-altering steps, such as extrusion or sonication, in order to obtain smaller and more homogeneous vesicle dispersions [1, 18].

The ability to control vesicle size and polydispersity is a crucial factor in the success of any manufacturing method as the particle size of the delivery system influences *in vivo* performance [23]. Microfluidic mixing is a recently developed method used to prepare liposomes, which results in the production of small vesicles with efficient encapsulation of a therapeutic agent [24]. In microfluidics, lipids are dissolved in an organic phase and the aqueous phase is introduced from different inlets into a precisely defined microchannel that allows for fast mixing between the two phases at high flow rates and at a temperature above the phase transition of the lipids. By controlling flow rate ratios (FRR) between the aqueous and organic phase and total flow rates (TFR) of both phases, homogeneous small vesicles can be prepared in a single step [23, 25].

The main objective of this work was to compare the characteristics of NISV prepared by these different manufacturing methods. Previous work from our lab has successfully investigated the development of NISV for vaccine delivery composed of monopalmitin glycerol (MPG), cholesterol (Chol) and dicetyl phosphate (DCP) at a molar ratio of 5:4:1 of MPG:Chol:DCP [26] so this was used to prepare the NISV. Moreover, in previous work, we have demonstrated that the type of the aqueous media can significantly affect vesicle characteristics prepared by microfluidics [27], so we chose phosphate buffered saline (PBS) to prepare the NISV in all the methods of preparation. The prepared particles were then compared for their physical characteristics, stability over time and *in vitro* cytotoxicity.

Materials and methods

2.1. Materials

MPG was purchased from Larodan Fine Chemicals AB (Sweden). Chol, DCP, PBS tablets, resazurin powder, serum-free and antibiotic-free Roswell Park Memorial Institute medium (RPMI 1640), L-glutamine, penicillin–streptomycin, and foetal bovine serum (FBS) were purchased from Sigma–Aldrich (UK) (all at cell culture grade). The human cell lines skin malignant melanoma (A375) and ovarian carcinoma (A2780) were purchased from the American Type Culture Collection (ATCC®).

2.2. Preparation of NISV by the thin-film hydration (TFH) method

NISV were prepared using the thin-film hydration (TFH) method as described elsewhere [28]. Briefly, MPG, Chol and DCP were mixed at a molar ratio of 5:4:1 with a total weight of 22.5 mg (MPG: 9.96mg, Chol: 9.27mg, DCP: 3.27mg). The mixture was placed in a round bottomed flask and dissolved in 9 ml chloroform. Chloroform was then evaporated using a rotary evaporator (Rotavapor R-3, BTECH, Switzerland) operated at 50rpm under vacuum at 50°C until complete solvent evaporation and a thin lipid film formed on the flask wall. The thin-film was hydrated with 9 ml of PBS (pH 7.4) at 50°C by rotating the flask at 50 rpm until the lipid film was completely hydrated and a milky suspension was formed with a final concentration of 2.5 mg/ml.

2.3. Preparation of NISV by the heating method

NISV were prepared by the heating method as described elsewhere with modifications [22]. Briefly, MPG, Chol and DCP at a molar ratio of 5:4:1 were hydrated at room temperature with PBS (10 mM, pH 7.4). The mixture was then heated to 140°C with continuous stirring for two min to form the NISV with a final concentration of 2.5 mg/ml.

138 **2.4. NISV particle size reduction**

139 NISV suspensions prepared by the TFH and heating methods were manually extruded 21
140 times using an Avanti miniextruder containing a 100 nm pore diameter polycarbonate (PC)
141 membrane (Avanti polar lipids, Alabaster, AL, USA) at 50°C to reduce the particle size
142 and distribution.

143 **2.5. Preparation of NISV by microfluidics**

144 NISV were prepared using a NanoAssemblr^{TM} (Benchtop, Precision NanoSystems Inc.,
145 Vancouver, Canada) as described by Obeid *et al*. [27]. The mixing of the two phases
146 allowed formation of controlled sized NISV [29]. To prepare NISV at a final concentration
147 of 2.5 mg/ml, MPG, Chol and DCP were dissolved in ethanol to prepare a stock solution
148 of 20 mg/ml for each of the components. Specific volumes from each stock solution were
149 mixed together to prepare the lipid phase of MPG, Chol and DCP in a molar ratio of 5:4:1.
150 The lipid phase was injected into the first inlet and the aqueous phase into the second inlet
151 of the microfluidic micromixer, with the mixing temperature set at 50°C. The FRR of
152 aqueous phase to lipid phase was set at 3:1 and the TFR was set at 12 ml/min. Dispersions
153 were collected from the outlet stream and immediately diluted with PBS in order to reduce
154 the final ethanol content in the preparation to 6.25% (v/v).

155 **2.6 Particle size, polydispersity and charge of NISV**

156 Particle size, poly dispersity index (PDI) and Zeta potential (ZP) were measured with a
157 Zetasizer Nano-ZS (Malvern Instruments, UK). The measurements were performed for
158 NISV prepared by each method at 25°C at a 1 in 20 dilution in PBS. All samples were
159 prepared in triplicate and the $Z_{Average}$, PDI, and ZP reported.

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2.7 Stability studies of NISV prepared by different methods

NISV prepared by all methods were tested for their stability over a two month period at either 4, 25, 37 or 50°C storage in controlled temperature rooms. Size, PDI, and ZP were measured at 0, 10, 20, 30, 40, 50, and 60 days.

2.8 Morphological analysis of NISV using atomic force microscopy (AFM)

Morphological examination of the NISV was performed by atomic force microscopy (AFM). Five µL of each formulation was deposited onto freshly cleaved mica surfaces (G250-2 Mica sheets 1" x 1" x 0.006"; Agar Scientific Ltd., Essex, UK), and air dried for ~1 h before AFM imaging. The images were obtained by scanning the mica surface in air under ambient conditions using a Dimension FastScan BioAFM (Bruker, CA, USA) operated on Peak Force QNM mode. The AFM measurements were obtained using ScanAsyst-air probes; the spring constant was calibrated by thermal tune (0.52 N m^{-1}; Nominal 0.4 N m^{-1}) and the deflection sensitivity calibrated using a silica wafer. AFM images were collected by random spot surface sampling (at least three areas). The analyses were performed using the Nanoscope Analysis v1.4 (Bruker, USA).

2.9 In vitro cytotoxicity studies

NISV were assessed for cytotoxicity on two different cell lines (A375 and A2780). Each cell line was seeded in a 96-well plate at a density of 1×10^4 per well in RPMI 1640 medium supplemented with 10% (v/v) FBS, 1% (v/v) L-glutamine and 1% (v/v) penicillin-streptomycin and incubated at 37 °C, 5 % CO_2 and 100% humidity for 24 h. The cells were treated with a range of concentrations of NISV (9.77-1250 µg/ml) prepared by each method. Dimethyl sulphoxide (DMSO) was used as a positive kill control and one column per plate contained untreated cells and medium. PBS alone without the particles was also

included to ensure that the media itself used to prepare the particles was not toxic. The plates were then incubated for 24h and then treated with 20 μL of resazurin (0.1 mg/ml) to each well and incubated for a further 24 h. The transformation of resazurin into resorufin by the live cells was then detected by measuring the absorbance at 560 – 590 nm using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA, USA). The cell viability was calculated as a percentage of the absorbance from the treated cells with NISV to the absorbance of the untreated cells.

2.10 The effects of TFR and FRR on NISV prepared by microfluidics

The effects of TFT and TFR on the characteristics of the NISV prepared by microfluidics were also investigated. The TFR of aqueous buffer and lipid phase was varied from 0.5 ml/min to 12 ml/min and the FRR of the aqueous to lipid phases was varied from 1:1 to 5:1 and the particle size, charge and PDI measured.

2.11 Statistical analysis

All experiments were performed in triplicate and one way analysis of variance (ANOVA) was used to assess statistical significance. Tukey's multiple comparisons test and a t-test were performed for paired comparisons. The statistical analysis was performed using Minitab software version 17. A value of $p < 0.05$ was considered to be statistically significant. Graphs were produced using OriginPro 2015.

207 ## 3. Results

208 ### 3.1 The effect of the manufacturing method on the particles size, PDI and ZP

209 Table 1 shows the characteristics of NISV, prepared by the TFH and heating methods
210 (before and after extrusion) and those prepared by microfluidics.

211 Dynamic light scattering revealed that the particle size of the extruded NISV prepared by
212 the TFH method and heating method were small and monodisperse (124.7 ± 0.72 nm and
213 152.34 ± 1.76 nm, respectively) while the non-extruded particles were large and
214 polydisperse (Table 1). However, particles prepared by microfluidic mixing were small
215 with a narrow particle distribution (165.90 ± 0.92 nm). Microfluidics can prepare small
216 and monodisperse particles in minutes. However, the preparation of these particles with
217 the other methods took hours to get the same results of microfluidics. The PDI values of
218 the extruded NISV prepared by the TFH and heating methods were low (0.12 ± 0.01 and
219 0.10 ± 0.02 respectivly) and comparable to the PDI value of the particles prepared by
220 microfluidics (0.08 ± 0.02) with no significant difference ($p>0.05$). Moreover, since all the
221 particles prepared by the three methods used the same lipid compositions, the ZP values
222 for the extruded particles prepared by the TFH and the heating methods and by
223 microfluidics were the same with no significiant difference ($p>0.05$) (Table 1).

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Table 1. Comparison of particle characteristics prepared by the TFH method, heating method, and microfluidic mixing in terms of size, PDI and ZP. n=3 ± SD

Method of preparation	Size (nm)	PDI	ZP (mV)
TFH (before extrusion)	1027.17 ± 75.79	0.83 ± 0.03	-12.30 ± 3.22
TFH (after extrusion)	124.70 ± 0.72	0.12 ± 0.01	-28.70 ± 1.39
Heating method (before extrusion)	3938.00 ± 95.25	0.85 ± 0.04	-14.50 ± 1.25
Heating method (after extrusion)	152.34 ± 1.76	0.10 ± 0.02	-36.67 ± 3.14
Microfluidic mixing	165.90 ± 0.92	0.08 ± 0.02	-31.38 ± 1.80

3.2. The effects of the manufacturing method on overall NISV stability

Figure 1 shows the stability in term of particles size of the NISV prepared by the three methods when stored at four different temperatures over two months. Samples were characterised immediately after preparations and again at each time point. The method of preparation was shown to have no effects on the particles stability as the particles prepared by the three methods exhibited nearly identical size distribution as the original samples at all the tested temperatures.

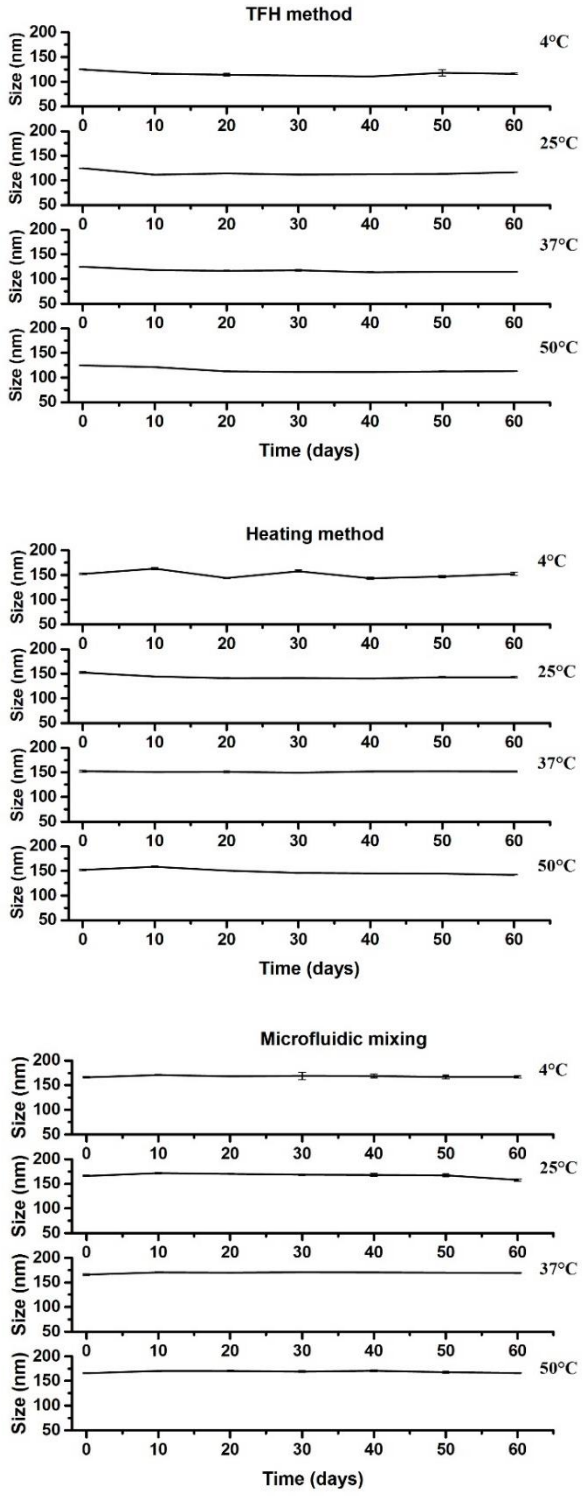

Figure 1. Size of NISV prepared by the TFH method, heating method, and microfluidic mixing and stored over 60 days at 4°C, 25°C, 37°C and 50°C. The data represents the mean ± SD (n=3).

3.3. Morphological analysis of NISV prepared by different methods

Figure 2 shows the morphology of NISV prepared by the TFH and heating methods after extrusion and by microfluidics. All the particles were spherical in shape regardless of the method of preparation. Some images showed large particle aggregates, which are due to the high concentration of these particles in the tested samples which formed upon drying the sample on the mica surface.

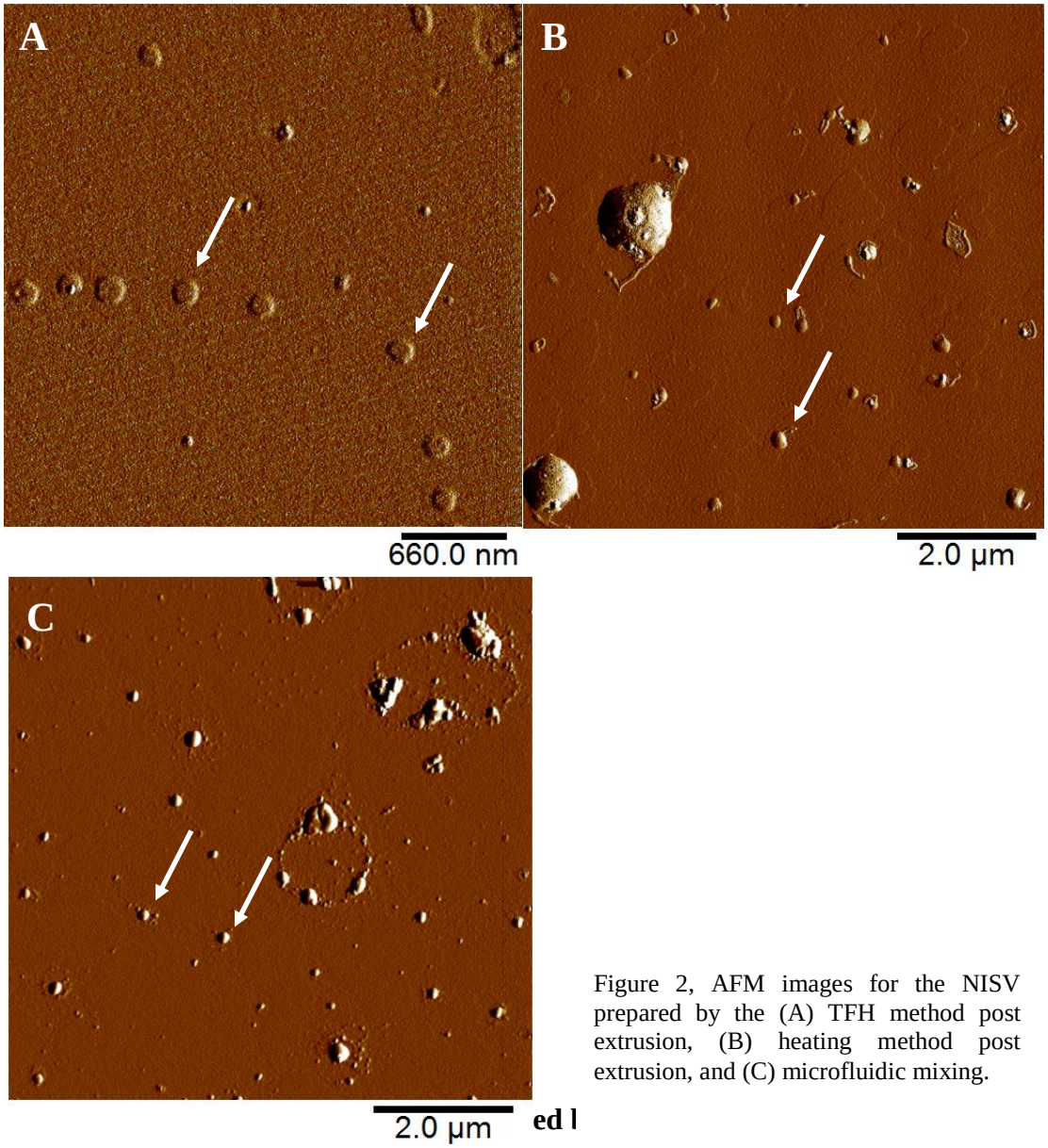

Figure 2, AFM images for the NISV prepared by the (A) TFH method post extrusion, (B) heating method post extrusion, and (C) microfluidic mixing.

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Figure 3 shows the cytotoxicity of the NISV prepared by the three methods on A375 and A2780 cell lines and Table 2 shows the calculated EC50. All three formulations show the same cytotoxicity profile as the difference in the EC50 between the particles on both cell lines was not significant ($p>0.05$). NISV with a concentration ≤ 150 µg/ml found to be non-toxic where 100 % cell viability was detected on both cell lines regardless of the method of manufacturing. The buffer alone used in the vesicle preparation was not toxic and the cells were 100% viable (data not shown).

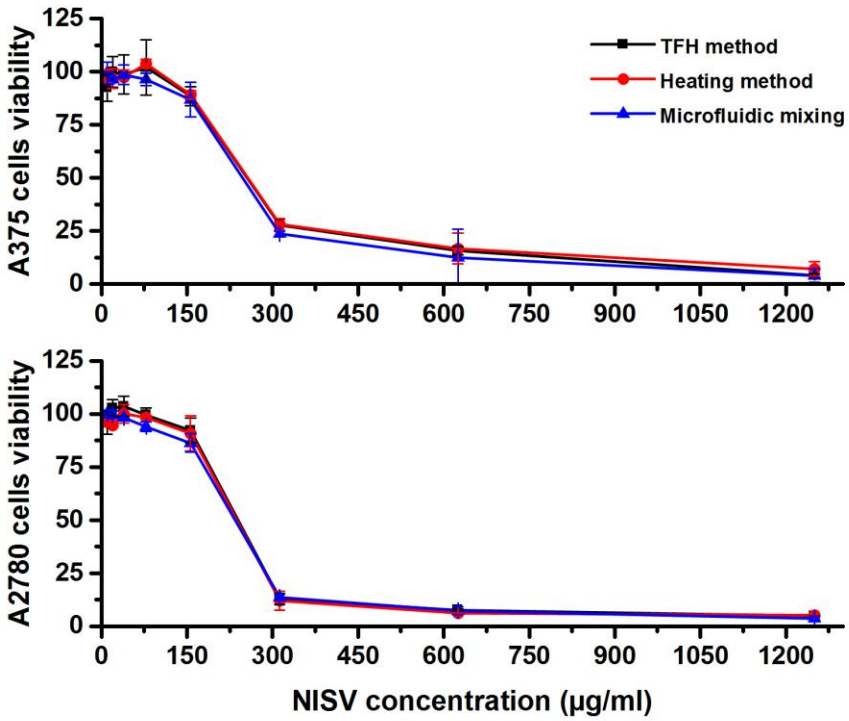

Figure 3. Cytotoxicity of the NISV prepared by three methods on A375 and A2780 cell lines. The data represents the mean ± SD (n=3).

Table 2. EC50 values in (µg/ml) of NISV, prepared using three different manufacturing methods, on A375 and A2780 cells. The data represents the mean ± SD (n=3).

Method of preparation	EC50 (µg/ml)	
	A375 cell line	A2780 cell line
TFH method	254.7 ± 11.5	229.9 ± 14.43
Heating method	258.9 ± 19.53	224.6 ± 28.32
Microfluidic mixing	240.1 ± 13.81	228.9 ± 5.651

3.5. The effects of TFR and FRR on NISV prepared by microfluidics

Figure 4 shows the changes of the particles size by changing the FRR from 1:1 to 5:1 (aqueous: lipid phases) and the TFR from 0.5-12 ml/min. As can be seen in Figure 4, as the aqueous/ethanol FRR increased from 1:1 to 5:1, a significant ($p<0.05$) reduction in NISV size was observed and found to be TFR dependant. At a TFR < 3 ml/min, the difference between the particles prepared at FRR of 3:1 and 5:1 was not significant ($p>0.05$). However, at higher TFR (> 3 ml/min), the difference between these two FRRs was significant ($p<0.05$). For example, at a TFR of 0.5 ml/min, the particle size prepared at FRR of 1:1, 3:1 and 5:1 were 219.71 ± 15.69 nm, 181.14 ± 6.65 nm, and 183.32 ± 4.88 nm, respectively while at a TFR of 12 ml/min, the particle size for NISV was 177.73 ± 5.26 nm at FRR 1:1, 165.90 ± 0.92 at FRR 3:1 and particles prepared at FRR 5:1 was 145.25 ± 4.64 nm. The TFR was shown to have a significant ($p<0.05$) effect on particle size where the increase in the TFR from 0.5 ml/min to 9 ml/min resulted in an overall reduction in particle size at all the FRR. However, further increase in the TFR above 9 ml/min was not associated with a significant decrease in particle size at all the FRR (Figure 4).

Regarding the effects of the FRR on the total particle charge, the increase in the solvent concentration at lower FRR (1:1) results in a higher percentage of the charged material (i.e. DCP) in the particles. Therefore, as the FRR increased from 1:1 to 5:1 there was a decrease in the absolute value of the ZP from about -30 mV at 1:1 to about – 20mV at 5:1 regardless of the TFR. This means that the FRR factor also has an effect on the ZP in addition to its effect on particle size. However, this effect on the ZP was not significant ($p>0.05$).

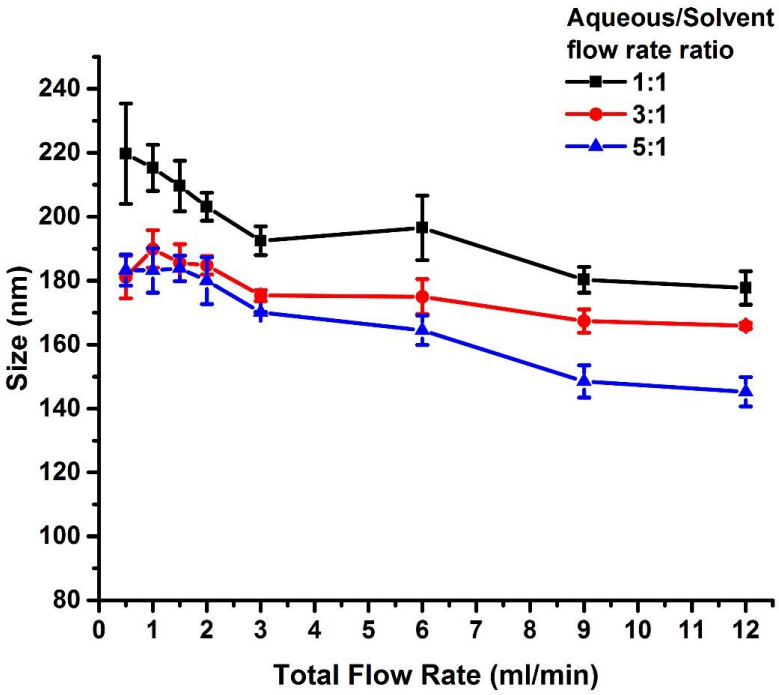

Figure 4. Size changes of NISV prepared at different TFR and FRR of the aqueous and lipid phase. The data represents the mean ± SD (n=3).

4. Discussion

The objective of this work was to assess the physicochemical properties of the NISV produced by three different methods. The TFH and heating methods have previously been reported to produce large multilamilar particles that require a post-manufacturing size reduction step [30], as confirmed by this study. Microfluidic mixing on the other hand was shown to produce small sized nanoparticles with low distribution in a single production step [24].

Traditionally, the production of small and monodisperse particles using the TFH and heating methods were limited by the use of the post-manufacturing size reduction step to produce particles of the required size and to reduce the PDI. This has limited the use of these methods to bench scale since there is a much longer industrial scale process required to produce a consistently size end product. However, microfluidic mixing allows the production of controlled particle size with homogenous distribution in a single step without the need for post-manufacturing size reduction (Table 1). This offers the potential to facilitate the production of NISV at larger scale. Moreover, the production of these small particles by microfluidics can save time as the total preparation time took minutes while the production of small particles by the other methods required several hours.

Next, we evaluated the stability of the vesicles over two months, at different storage temperatures following extended incubation by monitoring any changes in the particles size, PDI, and ZP. As can be seen in Figure 1, TFH and heating methods (post extrusion) and microfluidic mixing produced stable particles with respect to size with no significant change at all storage temperatures. Also, there was no significant change in the particles PDI and ZP at all the tested temperatures regardless of the method of preparation (data not

361 shown). Temperature can have an energy input to the system and can sometimes lead to
362 changes in the crystalline structure of the lipids or might cause changes in the ZP and these
363 changes might affect the stability of the particles [31]. Several researchers have reported
364 the instability of the particles when stored at high temperatures. In two different studies,
365 Feritas *et al.* (1998 and 1999) reported the instability of their solid lipid nanoparticles
366 (SLN) with the introduction of energy to the system. This instability was reported in terms
367 of size increase and reduction of ZP when the particles were stored at 50ºC [32, 33]. At
368 4ºC, this was generally the most favourable storage condition although some reports
369 indicate the instability of the formed particles when stored at low temperatures [31]. In this
370 study, all three methods exhibited excellent stability at four different temperatures with no
371 significant increase in the average particle size, PDI, and ZP ($p>0.05$) when stored for two
372 months even at the higher storage temperatures. These data indicate that microfluidics not
373 only enables rapid, robust, and scalable production of NISV, but also supports the stable
374 formation of these vesicles which is necessary for applications requiring prolonged shelf
375 life such as in pharmaceutical drug delivery. Although there was some residual ethanol in
376 the formulations prepared by microfluidics, this good vesicles stability suggests that the
377 amount of ethanol sequestered in the NISV bilayer is not significant as high ethanol content
378 will promote rapid degradation of the bilayer structure which is not the case in these
379 formulations. However, this residual ethanol can be removed, if necessary, via
380 conventional batch purification techniques such as evaporation, extraction, or dialysis
381 [34].
382 Morphological observations of AFM images confirmed the formation of spherical particles
383 of NISV prepared by the TFH and heating methods after extrusion and by microfluidics

(Figure 2). These results confirmed that the particles prepared by microfluidics in a single step are similar to the extruded particles prepared by more traditional TFH and heating methods.

Regarding the effects of the manufacturing methods on particle cytotoxicity, the viability of A375 and A2780 cells were measured after treatment with a range of NISV concentrations (9.76 -1250 µg/ml) prepared by all three methods. Cell metabolic activity measurements by conversion of resazurin showed no difference in cytotoxicity of the NISV prepared by the three methods as assessed by their EC50 values (Table 2). NISV with lipid concentrations below 150 µg/ml were non-toxic with 100% cell viability retained. Any difference in the physical characteristics of the particles such as size or charge would affect their cellular uptake, which would then affect cell viability [27, 35]. Here, since the particles prepared by the three methods have comparable characteristics in terms of size and charge, there was no difference on cell viability regardless of the method of preparation. This reflects the potential to have significant impact on various drug delivery applications by improving the manufacturing process of currently available NISV-based drugs. This would be achieved by replacing conventional methods of preparation with microfluidics to obtain the same outcomes, while gaining advantages in terms of rapid production of reproducible particles.

For the formation of lipid-based particles through microfluidic mixing, the rate of mixing as well as the ratio of aqueous-to-solvent streams were anticipated to be crucial factors in particle preparation as these factors will affect the ratio of each phase in the mixing process as well as the mixing time between both phases [25, 36]. Therefore, NISV composed of MPG:Chol:DCP (5:4:1 molar ratio) were prepared by microfluidic mixing at different TFR

and FRR. The FRR strongly affected the final solvent concentration. At lower FRR (1:1), the final solvent concentration increased, thus boosting the production of larger particles due to particle fusion and lipid exchange while at higher FRR (5:1), the chance of producing large particles was reduced as a result of reduced solvent concentration. Previous work using hydrodynamic flow-focusing techniques for the preparation of NISV using different types of sorbitan esters surfactant have also been reported to increase NISV size with the decrease in FRR, which is in agreement with results in this study [23].

The effect of the TFR on particle size is still debatable. While some researchers have reported that TFR does not have a significant effect [37], others have reported the contrary [38]. In this study, TFR was shown to have an impact on particle size especially at values < 9 ml/min. This means that these two factors (FRR and TFR) should be optimised when NISV are formulated by microfluidic mixing. In our previous work, we have demonstrated that the aqueous media used also has a significant effect on NISV characteristics when prepared by microfluidics [27]. So microfluidic mixing allows the production of NISV with a tuned particle size by varying the TFR, FRR, and aqueous media.

5. Conclusions

In this work, the characteristics of NISV prepared by microfluidics were compared with those prepared by the conventional TFH and heating methods. Microfluidic mixing enabled preparation of small, monodisperse particles in a single step, without the need of a size reduction step as in the case of the other methods. The method of preparation did not have significant effects on particle stability and toxicity. Using microfluidic mixing, a homogenous NISV suspension was prepared with high reproducibility. FRR and TFR between the two phases of the microfluidic mixing are the factors that have significant

effects on particle characteristics, which can be optimised in order to produce NISV with a defined size which is important in developing an effective drug delivery system. This work suggests that the use of microfluidic mixing in NISV preparation may facilitate the development and optimisation of these dispersions for nanomedicine applications at both bench and industrial scales.

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References

1. Marianecci, C., et al., *Niosomes from 80s to present: the state of the art.* Advances in colloid and interface science, 2014. **205**: p. 187-206.
2. HANDJANI-VILA, R., et al., *Dispersions of lamellar phases of non-ionic lipids in cosmetic products.* International journal of cosmetic Science, 1979. **1**(5): p. 303-314.
3. Moghassemi, S. and A. Hadjizadeh, *Nano-niosomes as nanoscale drug delivery systems: an illustrated review.* Journal of Controlled Release, 2014. **185**: p. 22-36.
4. Paecharoenchai, O., et al., *Nonionic surfactant vesicles composed of novel spermine-derivative cationic lipids as an effective gene carrier in vitro.* AAPS PharmSciTech, 2014. **15**(3): p. 722-730.
5. Nasseri, B., *Effect of cholesterol and temperature on the elastic properties of niosomal membranes.* International journal of pharmaceutics, 2005. **300**(1): p. 95-101.
6. Pozzi, D., et al., *Effect of cholesterol on the formation and hydration behavior of solid-supported niosomal membranes.* Langmuir, 2009. **26**(4): p. 2268-2273.
7. Kumar, G.P. and P. Rajeshwarrao, *Nonionic surfactant vesicular systems for effective drug delivery—an overview.* Acta Pharmaceutica Sinica B, 2011. **1**(4): p. 208-219.
8. Attia, I.A., et al., *Influence of a niosomal formulation on the oral bioavailability of acyclovir in rabbits.* AAPS PharmSciTech, 2007. **8**(4): p. 206-212.
9. Jadon, P.S., et al., *Enhanced oral bioavailability of griseofulvin via niosomes.* AAPS PharmSciTech, 2009. **10**(4): p. 1186-1192.
10. Pardakhty, A., J. Varshosaz, and A. Rouholamini, *In vitro study of polyoxyethylene alkyl ether niosomes for delivery of insulin.* International journal of pharmaceutics, 2007. **328**(2): p. 130-141.
11. Fang, J.-Y., et al., *Effect of liposomes and niosomes on skin permeation of enoxacin.* International journal of pharmaceutics, 2001. **219**(1): p. 61-72.
12. Fang, J.-Y., et al., *In vitro skin permeation of estradiol from various proniosome formulations.* International journal of pharmaceutics, 2001. **215**(1): p. 91-99.
13. Balakrishnan, P., et al., *Formulation and in vitro assessment of minoxidil niosomes for enhanced skin delivery.* International journal of pharmaceutics, 2009. **377**(1): p. 1-8.
14. Vyas, S., et al., *Non-ionic surfactant based vesicles (niosomes) for non-invasive topical genetic immunization against hepatitis B.* International journal of pharmaceutics, 2005. **296**(1): p. 80-86.
15. Perrie, Y., et al., *Surfactant vesicle-mediated delivery of DNA vaccines via the subcutaneous route.* International journal of pharmaceutics, 2004. **284**(1): p. 31-41.
16. Luciani, A., et al., *Glucose-Receptor MR Imaging of Tumors: Study in Mice with PEGylated Paramagnetic Niosomes 1.* Radiology, 2004. **231**(1): p. 135-142.
17. Arunothayanun, P., et al., *The effect of processing variables on the physical characteristics of non-ionic surfactant vesicles (niosomes) formed from a hexadecyl diglycerol ether.* International journal of pharmaceutics, 2000. **201**(1): p. 7-14.
18. Sahin, N.O., *Niosomes as nanocarrier systems*, in *Nanomaterials and nanosystems for biomedical applications*. 2007, Springer. p. 67-81.
19. Bangham, A., M.M. Standish, and J. Watkins, *Diffusion of univalent ions across the lamellae of swollen phospholipids.* Journal of molecular biology, 1965. **13**(1): p. 238-IN27.
20. Ranade, V.V. and J.B. Cannon, *Drug delivery systems*. 2011: CRC press.
21. Mozafari, M., *Method and apparatus for producing carrier complexes.* UK Patent No. GB 0404993.8, Int. Appl. No. PCT/GB05/000825 (03/03/2005), 2005. **14**.
22. Mozafari, M.R., et al., *Construction of stable anionic liposome-plasmid particles using the heating method: a preliminary investigation.* Cellular and Molecular Biology Letters, 2002. **7**(3): p. 923-928.
23. Lo, C.T., et al., *Controlled self-assembly of monodisperse niosomes by microfluidic hydrodynamic focusing.* Langmuir, 2010. **26**(11): p. 8559-8566.
24. Belliveau, N.M., et al., *Microfluidic synthesis of highly potent limit-size lipid nanoparticles for in vivo delivery of siRNA.* Molecular Therapy—Nucleic Acids, 2012. **1**(8): p. e37.
25. Carugo, D., et al., *Liposome production by microfluidics: potential and limiting factors.* Scientific reports, 2016. **6**.
26. Gebril, A.M., et al., *Assessment of the antigen-specific antibody response induced by mucosal administration of a GnRH conjugate entrapped in lipid nanoparticles.* Nanomedicine: Nanotechnology, Biology and Medicine, 2014. **10**(5): p. 971-979.

27. Obeid, M.A., et al., *The effects of hydration media on the characteristics of non-ionic surfactant vesicles (NISV) prepared by microfluidics.* International Journal of Pharmaceutics, 2016.
28. Mann, J.F., et al., *Oral delivery of tetanus toxoid using vesicles containing bile salts (bilosomes) induces significant systemic and mucosal immunity.* Methods, 2006. **38**(2): p. 90-95.
29. Stroock, A.D., et al., *Chaotic mixer for microchannels.* Science, 2002. **295**(5555): p. 647-651.
30. Abdelkader, H., A.W. Alani, and R.G. Alany, *Recent advances in non-ionic surfactant vesicles (niosomes): self-assembly, fabrication, characterization, drug delivery applications and limitations.* Drug delivery, 2014. **21**(2): p. 87-100.
31. Heurtault, B., et al., *Physico-chemical stability of colloidal lipid particles.* Biomaterials, 2003. **24**(23): p. 4283-4300.
32. Freitas, C. and R.H. Müller, *Effect of light and temperature on zeta potential and physical stability in solid lipid nanoparticle (SLN™) dispersions.* International journal of pharmaceutics, 1998. **168**(2): p. 221-229.
33. Freitas, C. and R. Müller, *Correlation between long-term stability of solid lipid nanoparticles (SLN™) and crystallinity of the lipid phase.* European Journal of Pharmaceutics and Biopharmaceutics, 1999. **47**(2): p. 125-132.
34. Hood, R.R. and D.L. DeVoe, *High-Throughput Continuous Flow Production of Nanoscale Liposomes by Microfluidic Vertical Flow Focusing.* small, 2015. **11**(43): p. 5790-5799.
35. He, C., et al., *Effects of particle size and surface charge on cellular uptake and biodistribution of polymeric nanoparticles.* Biomaterials, 2010. **31**(13): p. 3657-3666.
36. Bottaro, E. and C. Nastruzzi, *"Off-the-shelf" microfluidic devices for the production of liposomes for drug delivery.* Materials Science and Engineering: C, 2016. **64**: p. 29-33.
37. Jahn, A., et al., *Microfluidic directed formation of liposomes of controlled size.* Langmuir, 2007. **23**(11): p. 6289-6293.
38. Yu, B., R.J. Lee, and L.J. Lee, *Microfluidic methods for production of liposomes.* Methods in enzymology, 2009. **465**: p. 129-141.