The effects of hydration media on the characteristics of non-ionic surfactant vesicles (NISV) prepared by microfluidics

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Abbreviations

NISV: Non-ionic surfactant vesicles; PBS: Phosphate buffered saline; HEPES: (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); NS: Normal saline; DW: Distilled water; MPG: Monopalmitin glycerol; Chol: Cholesterol, DCP: Dicetyl phosphate; HPLC: High Performance Liquid Chromatography, SEM: Scanning electron microscope, RT: Relative Turbidity; PDI: Polydispersity index; ZP: Zeta potential.

Abstract

Non-ionic surfactant vesicles (NISV) are colloidal particles that provide a useful delivery system for drugs and vaccines. One of the methods that is used for NISV preparation is microfluidics in which the lipid components dissolved in organic phase are mixed with an aqueous medium to prepare the particles through self-assembly of the lipids. In this work, we examined the effect of using different types of aqueous media on the characteristics of the NISV prepared by microfluidics. Five aqueous media were tested: phosphate buffered saline, HEPES buffer, Tris buffer, normal saline and distilled water. The resulting particles were tested for their physical characteristics and cytotoxicity. The aqueous media were found to have significant effects on the physical characteristics of the particles, as well as their overall stability under different conditions and their cytotoxicity to different human cell lines. Careful consideration should be taken when choosing the aqueous media for preparing NISV through microfluidics. This is an important factor that will also have implications with respect to the entrapped material, but which in addition may help to design vesicles for different uses based on changing the preparation medium.

Key Words

Non-ionic surfactant vesicles, Microfluidics, Hydration media, Drug delivery

1. Introduction:

Non-ionic surfactant vesicles (NISV) are synthetic vesicles constructed through the self-assembly of hydrated non-ionic surfactants with cholesterol and other additives, into a bilayer structure enclosing an aqueous core. In terms of physical properties and structure, NISV are similar to liposomes, which are the most commonly used lipid particles as drug delivery systems [1]. NISV were first reported by the cosmetic company L'Oréal in the 1980s and since then they have gained in interest as a drug delivery system, as they offer more advantages

compared with liposomes in terms of lipid cost and stability [1, 2]. Non-ionic surfactants are the basic components of NISV. These surfactants are amphiphilic molecules with both a hydrophilic (water soluble) head and hydrophobic (organic soluble) tail with no charged groups in their hydrophilic heads [3]. The bilayer structure of the NISV makes them capable of encapsulating both hydrophilic and hydrophobic substances. Hydrophilic substances are thought to be encapsulated in their aqueous core or adsorbed on the bilayer surface, while hydrophobic substances are embedded into the lipophilic domain of the bilayer [3]. Surfactants commonly used to prepare NISV include polyoxyethylene fatty acid esters (Tweens), sorbitan fatty acid esters (Spans), alkyl ethers, and alkyl glyceryl ethers (Brijs) [4]. The most common additive in a NISV formulation is cholesterol, which affects the membrane structure and the physical properties of the vesicles [5] and its most important effect is the modulation of the mechanical strength of the bilayer structure and water permeability [6, 7]. Moreover, cholesterol incorporation tends to enhance drug entrapment efficiency, vesicle stability, and can modulate drug release in the NISV formulations [8, 9]. Other additives include charged molecules to enhance the stability of the NISV formulations during storage and prevent vesicle aggregation by electrostatic repulsion. Dicetyl phosphate (DCP) and phosphatidic acids are used to impart a negative charge on the surface of the NISV, while cationic molecules such as stearylamine and cetylpyridinium chloride are used to provide a positive charge on the vesicles [1, 3]. Due to their potential to carry and encapsulate a variety of drugs, NISV have been widely used to deliver drugs to specific target sites, to control drug release and enhance permeation. They have been investigated as a potential drug delivery system for anticancer [10, 11], anti-inflammatory and anti-infective drugs [12, 13], peptides [14, 15], transdermal drug delivery [16, 17] and gene delivery [18].

Numerous methods for NISV preparation are reported. The thin-film hydration method (TFH) is simple and widely used. The surfactants and other additives are dissolved in organic solvent in a round-bottomed flask. The organic solvent is then removed using a rotary vacuum evaporator to form a thin film of lipids on the wall of the flask, which is then hydrated by the addition of an aqueous solution with or without drug to form multi-lamellar vesicles [3, 19]. In the reverse-phase evaporation (REV) method, after evaporating the organic solvent as in the TFH method, the dried lipid film is purged with nitrogen and the lipids are re-dissolved with a second organic phase of diethyl ether and/or isopropyl ether followed by the addition of aqueous mixture to form large multilamellar vesicles and then the organic solvent is removed under reduced pressure by rotary evaporation [20, 21]. In the organic injection method, the organic solvent containing the dissolved surfactants and other additives is slowly injected through a needle in an aqueous solution to form NISV of various sizes [22]. Other methods include ''bubble'', sonication and freeze-thaw methods [3]. However, in most of these, the local chemical and/or mechanical environments are not well controlled and the vesicles that are formed are large with considerable size polydispersity, which requires a suitable post-preparation size reduction step e.g. by sonication or extrusion, to obtain small and homogeneous vesicles [23]. More recently, a microfluidic method has been employed for the preparation of lipid-based nanoparticles that uses microfluidic hydrodynamic focusing and has been shown to produce small sized nanoparticles for drug encapsulation [24]. In this method, the surfactants and other additives dissolved in an organic phase, are mixed with an aqueous phase at high flow rates and passed through a precisely defined microchannel at a temperature above the phase transition of the lipids. Factors such as flow rate ratios (FRR) between the aqueous and organic phases and the total flow rates (TFR) of both phases can be

94 controlled during the mixing process to prepare homogeneous small particles in a single step
95 [25, 26].

96 All of the above mentioned methods involve the hydration of the surfactant and lipid
97 mixtures with an aqueous phase at elevated temperature, followed by an optional size
98 reduction with some preparation methods [27]. Phosphate buffered saline (PBS) is a
99 common buffer used for NISV preparation. It is an ionic buffer composed of sodium chloride,
100 sodium phosphate, and (in some formulations) potassium chloride and potassium phosphate
101 and has a pH range from 5.8-8.0 at 25°C [28]. PBS is the preferred buffer for particle formation
102 because the osmolarity and ion concentrations match those of human body fluids such as blood
103 [29]. Other buffers such as (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES),
104 Tris, citrate and carbonate can also be used [30, 31]. NISV can also be prepared using distilled
105 water (DW) as an aqueous media. However, it is imperative to select an optimal buffer system
106 for drug encapsulation in NISV. For example, phosphate and citrate buffers are not
107 recommended for components that contain calcium ions, as phosphate forms an insoluble
108 calcium phosphate precipitate, while citric acid chelates calcium [32]. Tris buffer is used for
109 the storage of nucleic acids and is suitable for formulating NISV where nucleic acids are being
110 encapsulated. However, Tris can interfere in vivo and in vitro with copper by chelation and can
111 act as a competitive inhibitor to some enzymes [32, 33].

112 In the present work, we investigated the effect of five different aqueous media on the
113 characteristics of empty NISV. Given that previous work from our lab has investigated the use
114 of NISV for vaccine delivery using a surfactant combination of monopalmitin glycerol
115 (MPG):cholesterol (Chol): dicetyl phosphate (DCP) at a molar ratio of 50:40:10 [34, 35], we
116 have used this formula as a model to examine the various physicochemical aspects of vesicles
117 composed with these lipid components, but prepared using five different aqueous media and
118 using a microfluidic mixing method of preparation. In vitro cytotoxicity experiments were

subsequently performed to evaluate the effect of the different formulations resulting from the use of the different hydrating media on human A375 (skin malignant melanoma), A2780 (ovarian carcinoma) and PNT2 (normal prostate epithelium) cells.

2. Materials and methods

2.1 Materials

MPG was purchased from Larodan Fine Chemicals AB (Sweden). Chol, DCP, resazurin powder, PBS tablets, HEPES buffer solution, Tris buffer solution, sodium hydrochloride (NaCl), serum-free and antibiotic-free medium Roswell Park Memorial Institute medium (RPMI 1640), L-glutamine, penicillin–streptomycin, and foetal bovine serum (FBS) were purchased from Sigma-Aldrich (UK). The human cell lines A375, A2780, and PNT2 were purchased from American Type Culture Collection (ATCC®) and kindly provided by Mrs Louise Young, (University of Strathclyde).

2.2 NISV preparation by microfluidics with different hydration media

NISV were prepared by employing a microfluidic micromixer as described elsewhere [24]. The hydration media used to prepare the vesicles were PBS (10 mM, pH 7.4), HEPES buffer (10 mM, pH 7.4), Tris buffer (10 mM, pH 7.4), 0.9% (w/v) normal saline (NS) and DW. An ethanol solution containing MPG, Chol, and DCP at a molar ratio of 50:40:10 was prepared at a concentration of 10 mg/ml total lipids. The microfluidic apparatus used was the NanoAssemblr™ Benchtop™ (Precision NanoSystems Inc., Vancouver, Canada) which enables a controlled nanoprecipitation process by hydrodynamic flow, focused through a two-channel microfluidic system.

For the preparation of empty vesicles, a specific volume of each of the tested hydration media was mixed with the lipid phase in ethanol at a volumetric flow rate of 3:1 (aqueous: lipid) in the microfluidic micromixer at a total flow rate of 12 mL/minute (9 mL/minute for the aqueous

phase and 3 mL/minute for the lipid phase) at 50ºC. The mixed materials, upon leaving the micromixer outlet, was diluted into an equal volume of the aqueous media used in the preparation in order to reduce the ethanol content in the final preparation to 12.5%. The NISV mixture was then dialysed overnight against 1000 volumes of aqueous media used in the vesicle preparation using SnakeSkin™ Dialysis Tubing (10,000 Da molecular weight cut off; Thermofisher Scientific, UK) at 25°C.

2.3 Particle size, polydispersity and charge of NISV prepared with different hydration media

Particle size, polydispersity index (PDI) and zeta potential (ZP) were measured with a Zetasizer Nano-ZS (Malvern Instruments, UK). The measurements were carried out for NISV prepared in each hydration media at 25°C at a 1/20 dilution. All samples were prepared in triplicate and the $Z_{Average}$, PDI, and ZP reported.

2.4 Stability of NISV at different temperatures

Stability of the NISV was evaluated over two months at 4, 25, 37, and 50 °C in controlled temperature rooms over the duration of the study. Size, PDI and zeta potential were measured at different time points (0, 1, 2, 3, 4, 6 and 8 weeks).

2.5 Morphological analysis of NISV using scanning electron microscopy

Morphological analysis of the NISV was carried out using a FEI Quanta 250 field emission variable pressure scanning electron microscope (SEM) (FEI, Oregon, USA) equipped with an Everhart–Thornley type detector and running FEI software. Each sample of NISV was diluted 1:50 with the media used in the formulation and 2µl of each diluted sample was dried on a silicon substrate and placed under vacuum. An accelerating voltage of 5 kV was applied to each sample in high vacuum mode and secondary electron images were collected.

2.6 High Performance Liquid Chromatography (HPLC) analysis of cholesterol content of NISV

In order to assess the concentration of the NISV produced and to determine the yield and preparation efficacy, NISV were analysed using HPLC to measure the quantity of cholesterol present post-preparation. HPLC was performed using an Agilent Technologies 1260 Series Liquid Chromatography system controlled by Clarity Chromatography software. The conditions of the run were as follows: mobile phase acetonitrile:methanol:2-propanol; (7:3:1, v/v/v), flow rate 1 mL/min, total run time 10 min; column YMCbasic C18, 250 X 3.0 mm, column temperature 60ºC, injection volume 20 µL, detection 205 nm, retention time 1.55 min. A standard curve of Chol (31.25 – 1000 µg/ml) was constructed by measuring the area under the curve (AUC). NISV prepared were lysed with isopropyl alcohol (50%, v/v) and then analysed by HPLC as previously described [36]. The Chol concentration was determined by measuring the AUC and calculating the concentration using the equation generated from the standard curve.

2.7 Turbidity assay

To understand NISV behaviour under physiological conditions, the aggregation tendency of the NISV was studied using a turbidity assay [37]. FBS was added to each NISV formulation to a final concentration of 10% (v/v) in each hydration medium. This concentration of FBS was chosen as it is generally used for in vitro studies. Turbidity was determined by measuring the absorbance at 298 nm using a HELIOS ALPHA ThermoSpectronic spectrophotometer using serum alone as a background [37]. NISV (625 µg/ml) were incubated at 37 °C and analysed over a 2 h time period. Relative turbidity was calculated by dividing sample absorbance at a specific time by the time zero value incubated in the corresponding hydration buffer used for NISV preparation.

2.8 Cytotoxicity of NISV evaluated using a number of human cell lines

NISV were assessed for cytotoxicity on three different cell lines (A375, A2780, and PNT2). Each cell line was seeded in a 96-well plate at a density of 1×10^4 per well in 100µl and incubated for 24 h at 37°C, 5% CO_2 and 100% humidity in RPMI 1640 medium supplemented with 10% (v/v) FBS, 1% (v/v) L-glutamine and 1% (v/v) penicillin-streptomycin. After 24 h, cells were treated with different concentrations of NISV (9.77-1250 µg/mL). Dimethyl sulphoxide (DMSO) was used as a positive kill control and one column per plate contained untreated cells and medium. Each of the hydration buffers without the particles was also included to ensure that the media itself are not toxic. The plates were then incubated for a further 24 h and then 20 µl of resazurin (0.1 mg/ml) was added to each well and incubated for a further 24 h. Resazurin is bio-reduced by viable cells from blue into a pink resorufin product, which indicates the presence of metabolically active cells and results in both a colorimetric and fluorometric change. After 24 h, the quantity of resorufin was measured on a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA, USA) at 560 nm - 590 nm. The absorbance reading at this wavelength is directly proportional to the number of metabolising cells in the medium. In this study, cell viability was calculated and expressed as a percentage of the positive control (i.e., untreated cells):

$$\%\ \text{Cell viability} = \left(\frac{\text{Absorbance of cells treated with NISV at } \lambda ex = 560\ nm, \lambda em = 590\ nm}{\text{Absorbance of untreated cells } \lambda ex = 560\ nm, \lambda em = 590\ nm}\right) \text{x } 100$$

2.9 statistical analysis

All experiments were performed in triplicate and one way analysis of variance (ANOVA) was used to assess statistical significance. Tukey's multiple comparison test and t-test was 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.

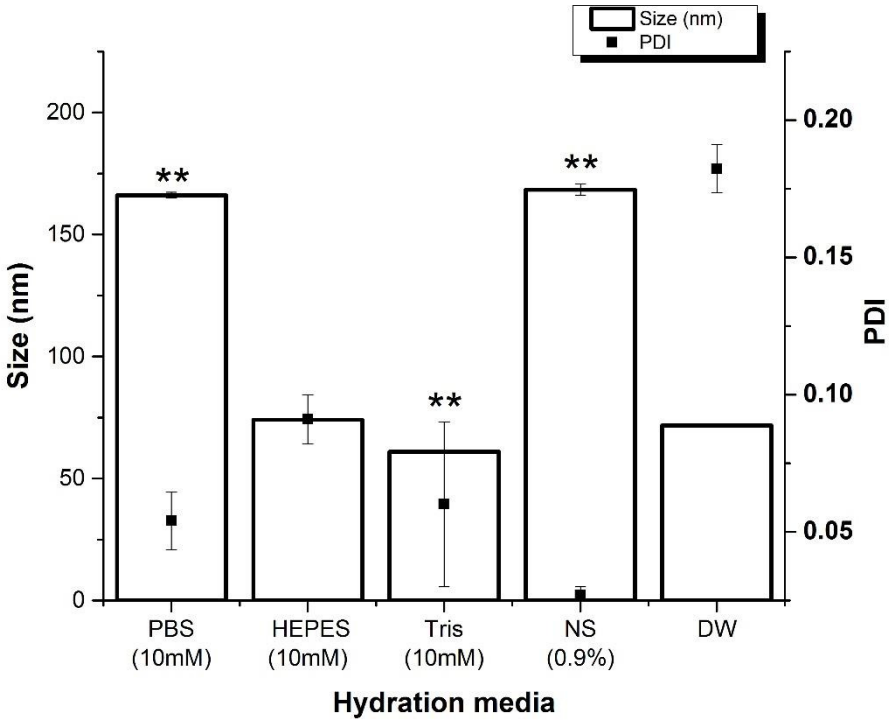
3. Results and Discussion

NISV composed of MPG:Chol:DCP at a molar ratio of 50:40:10 were prepared using microfluidic mixing by changing the aqueous media used in formulating the particles. The production of NISV through microfluidic mixing is based on rapid and controlled mixing of two miscible fluids (aqueous and solvent) in a microchannel [25]. The objective of this work was to assess the effects of the aqueous media on the physicochemical properties of the resultant particles. Five different hydration media were studied.

3.1 Effect of hydration buffer on the particles size and PDI

Changing the hydration media altered the size of the NISV significantly (Figure 1). The smallest particles were formed using Tris, followed by DW and HEPES with particle sizes of 60.96 ±0.36 nm ($p < 0.05$), 71.83 ± 0.44 nm ($p < 0.05$), and 74.10 ± 0.51 nm ($p < 0.05$), respectively. The largest particle size was obtained with NS (168.40 ± 2.26 nm, $p < 0.05$) followed by PBS (166.10 ± 1.23 nm, $p < 0.05$). The PDI of these particles showed that all the formulations, had a narrow size distribution with values of 0.027 ± 0.003 (NS), 0.054 ± 0.010 (PBS), 0.060 ± 0.030 (Tris), 0.091 ± 0.010 (HEPES) and 0.180 ± 0.010 for DW with the value of PDI for particles prepared with DW that was significantly different than the others ($p < 0.05$). This difference in the particle sizes could be attributed to the ion components of each media. NS and PBS showed similar sizes, while DW, HEPES and Tris were grouped together. The similarity between PBS and NS could be attributed to the NaCl ions, which are the major component in both buffers [38]. HEPES and Tris buffers and DW resulted in smaller particles, but within the same range, so the effects of the ionic components of the HEPES and Tris might have no significant effects as the sizes from both buffers were close to the particles prepared with DW. In drug delivery, small particle sizes (<200 nm) are preferred for drug permeability

238 and tumour targeting as nanoparticles in this size range tend to accumulate passively in tissues
239 with leaky or abnormal architecture blood vessels (i.e. tumour and inflamed tissues) after
240 intravenous administration in a phenomenon known as enhanced permeability and retention
241 (EPR) [39]. Moreover, it has been reported that larger particles are more rapidly removed from
242 the circulation than smaller particles due to the lower uptake by the reticuloendothelial system
243 (RES) of smaller particles [40]. He et al. reported that in vitro macrophage uptake of larger
244 particles was higher compared with smaller counterparts [41]. These factors directly affect the
245 biodistribution and circulation time of NISV [42]. Therefore, the size of the NISV has
246 significant implications on their efficacy when used as a drug delivery system. Here, although
247 the aqueous buffers resulted in different sizes, the formulations were all below 200 nm, which
248 makes them suitable for tumour-targeted drug delivery.



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250 Figure 1: Size and PDI of NISV prepared using microfluidics with five different hydration
251 media. The data represents the mean ± SD (n=3) as measured by DLS, $^{**}p < 0.05$ indicates
252 significant difference in size compared with the DW formulation.

3.2. The effect of the hydration media on the charge of the resultant NISV

Particles prepared with DW had the highest absolute value of zeta potential (-76.83 ± 0.81 mV) followed by the particles prepared with Tris (-57.4 ± 3.33 mV), HEPES (-51.87 ± 1.18 mV), NS (-33.2 ± 2.46 mV) and PBS (-30.63 ± 2.06 mV) as shown in Figure 2. The effect of the hydration media on the total charge of the resultant particles could also be attributed to the ions present in the buffers. The surface charge of NISV gives rise to electrostatic repulsion among the nanoparticles, improving the stability of the dispersion system [43]. Zeta potential is an important factor that confers stability on the nanoparticles and higher values ensure that the particles will repel each other and resist aggregation [44]. Particles with zeta potential values that are < -30 mV or $>+30$ mV would both be stable dispersions as these values are considered high enough to prevent particle aggregation [43]. Although each of the media examined resulted in particles with different charge, all of them had a zeta potential < -30 mV, which means that they would be stable regardless of the type of the aqueous media used in their preparation.

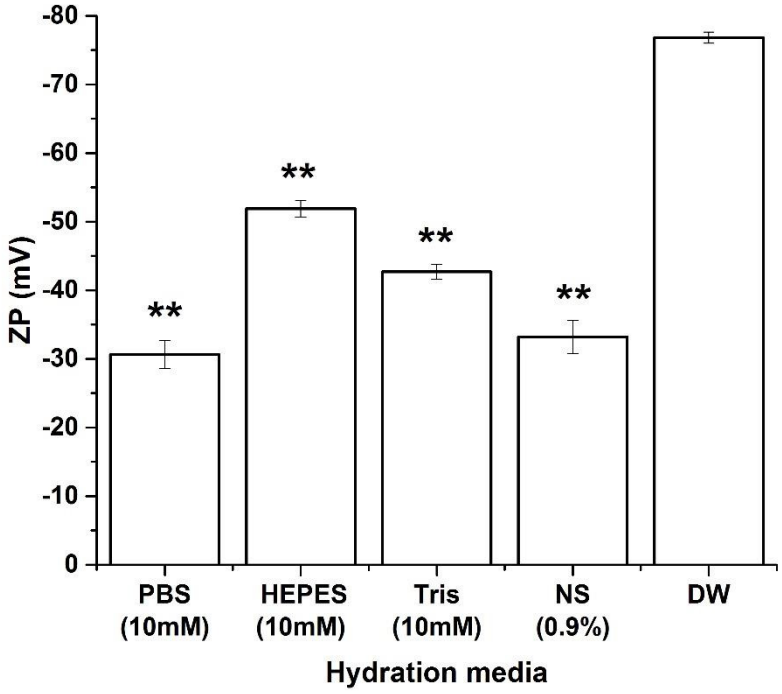

Figure 2: ZP for NISV prepared with microfluidics using five different aqueous media. The data represents the mean ± SD (n=3) measured by DLS, $^{**}p < 0.05$ indicates significant difference in size compared with the DW formulation.

3.3 Stability of NISV at different storage temperatures

Vesicle stability was assessed by monitoring changes in size (Figure 3) and PDI (data not shown) of the particles over time, to predict their swelling, aggregation or precipitation characteristics. For NISV prepared with DW, the particle size showed a slight decrease in the first two weeks and then remained stable throughout the study when stored at 4, 25, and 37 °C with no significant change in the particle size ($p > 0.05$). However, for the particles stored at 50°C, there was a significant ($p < 0.05$) increase in the particle size during the study, which increased from 71.8 ± 0.4 nm at time zero to 101.1 ± 0.4 nm at the end of the study. NISV prepared with HEPES buffer was stable at the four different temperatures with no significant ($p > 0.05$) increase in particle size during the study. NISV prepared with NS were stable in terms of size and PDI when stored at 4°C with no significant ($p > 0.05$) change. When these

283 particles were stored at 25 and 37°C, they showed an increase in size during the first week and
284 then remained stable for the rest of the storage duration. However, NISV prepared with NS and
285 stored at 50°C increased significantly ($p < 0.05$) in size from 168.4 ± 2.26 to 208.77 ± 1.89 nm
286 at the end of the study with no significant ($p > 0.05$) increase in the PDI. Particles prepared with
287 PBS remained stable with no significant ($p > 0.05$) change in the particle size regardless of the
288 storage temperature. For NISV prepared using Tris buffer, the particles remained stable at 4
289 and 25°C with no significant ($p > 0.05$) change in particles size. When these particles were
290 stored at 37 °C, the size increased significantly ($p < 0.05$) in the first week from 60.69 ±0.36
291 nm to 66.84 ± 0.14 nm and then remained stable for the rest of the storage duration. For
292 particles prepared with Tris and stored at 50 °C, there was a significant ($p < 0.05$) increase in
293 particles size from 60.96 ±0.36 to 76.18 ± 0.39 nm at the end of the storage. These stability
294 results of the formed particles showed that the type of the hydration media used to prepare the
295 NISV might have an effect on particle stability. This effect has been shown to be more obvious
296 at elevated temperatures. All the formulations were stable at 4, 25, and 37 °C with no change
297 in the particles size and PDI. At 50°C storage conditions, the particles prepared with DW, NS,
298 and Tris increased significantly in terms of size. At elevated temperatures, lipid vesicles
299 undergo a phase transition which affects their permeability and increase the fluidity of the lipid
300 bilayers [30]. Different studies have reported the effects of the temperature and the dispersion
301 media on nanoparticle stability. Some consider the increase in the temperature as an energy
302 input, attributing this effect to the change in the crystalline structure on the particles'
303 components or zeta potential which might affect the particle size during storage [45, 46].

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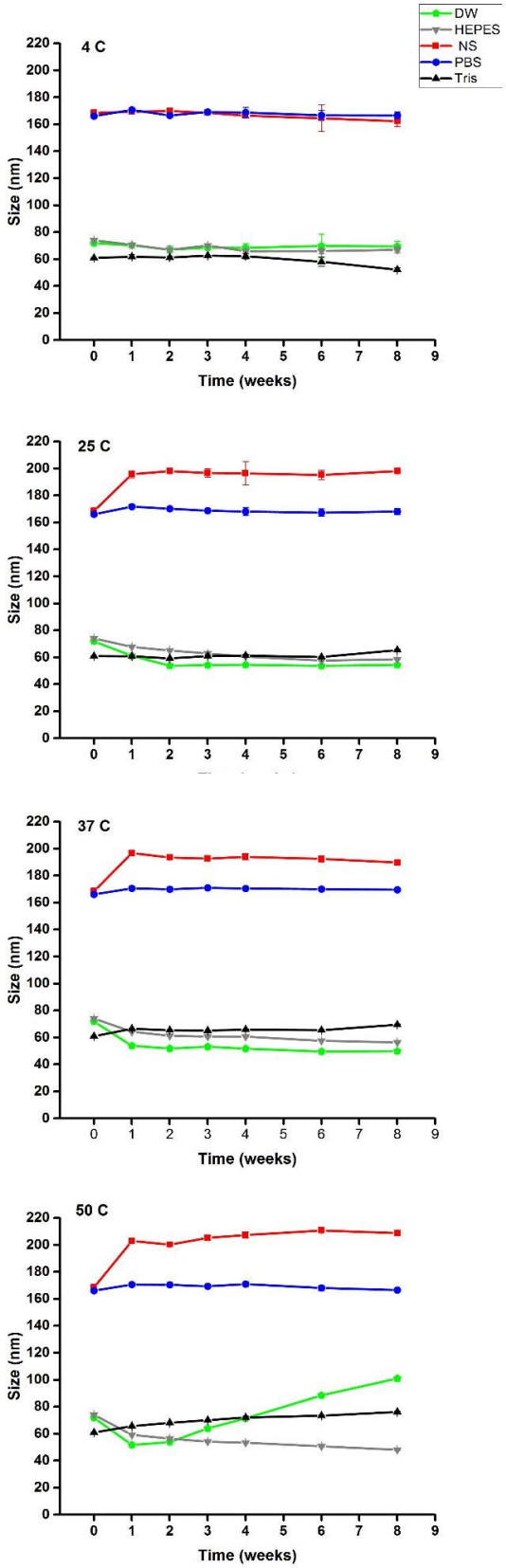

Figure 3: Stability of NISV prepared with microfluidics using five different hydration media, stored at 4, 25, 37 and 50 °C. The data represents the mean ± SD (n=3) measured by DLS.

3.4 SEM imaging of NISV

The morphology of the NISV was analysed by scanning electron microscopy (Figure 4). NISV were shown to have an almost spherical shape as seen in some of the images and apparent smooth surface regardless of the media used in their preparation. The SEM images confirmed the differences in the sizes between the particles (Figure 4). Figure 4E showed some non-spherical large aggregates as a result of the high concentration of the particles being examined.

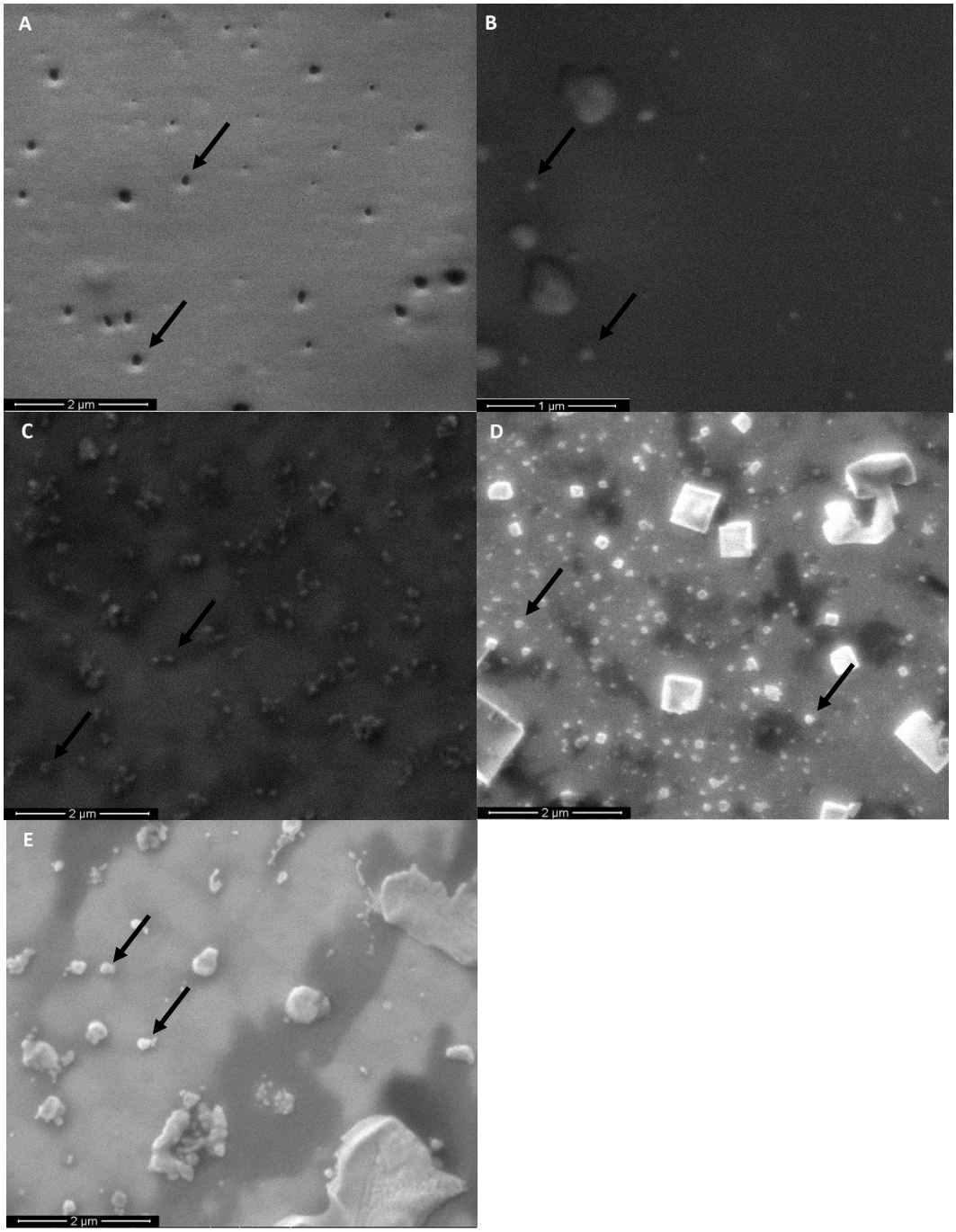

Figure 4. Representative scanning electron micrographs of NISV prepared with (A) HEPES, (B) Tris, (C) DW, (D) NS and (E) PBS (Magnification ×40,000). Salt crystals were observed in the NS micrograph as a cuboid structures (figure 4 D). Figure 4 (E) showed some large non spherical aggregates as a result of the high concentration of the particles examined.

3.5 HPLC analysis of NISVS prepared with different hydration media

The total Chol content in the NISV formulations was measured using HPLC. Figure 5 shows a typical standard curve and the total Chol concentration calculated from it for each formulation shown in Figure 6. The theoretical Chol concentration was 129.05 µg/ml and it was expected that the concentration post-preparation to be close to this. However, after preparation using different media, the calculated concentrations were significantly ($p < 0.05$) lower than the theoretical one for particles prepared with HEPES, PBS, NS and DW. Only particles prepared with Tris buffer had a cholesterol concentration that was not significantly ($p > 0.05$) different from the theoretical one. For particles prepared with PBS and NS, the Chol concentrations were close to each other with no significant ($p > 0.05$) difference in the calculated concentration. Moreover, the concentration for NISV prepared with HEPES and DW was almost the same for both formulations with no significant ($p > 0.05$) difference and this is the same for the particle size for these two formulations. This indicates that the type of hydration media had a significant effect on the apparent Chol concentration recovered. It is worth noting that the Chol concentration was calculated based on the AUC at the retention time of 1.55 min, but there were some peaks just before and after this time (data not shown) and this might explain the difference between the theoretical and actual concentration after preparation as we expect that some interaction occurred between cholesterol and the ions in the buffers that resulted in different separation times. We are currently studying these effects and the possible interaction between the NISV components and the different ions that form each hydration media.

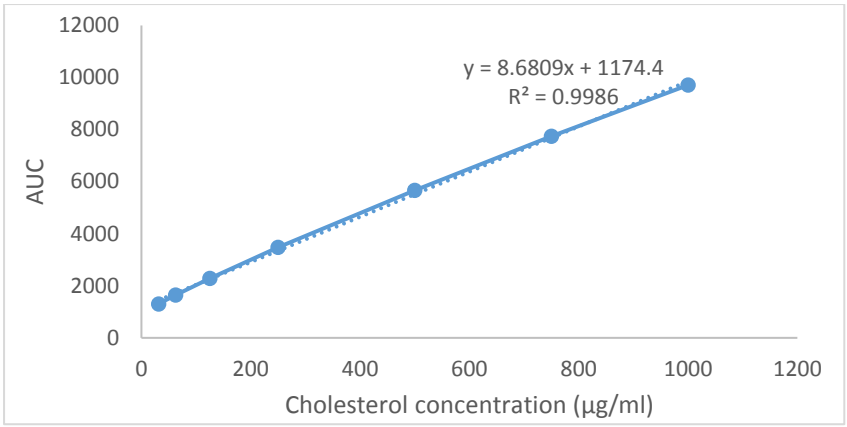

Figure 5. Cholesterol standard curve prepared by measuring the AUC of various cholesterol concentrations as measured by HPLC.

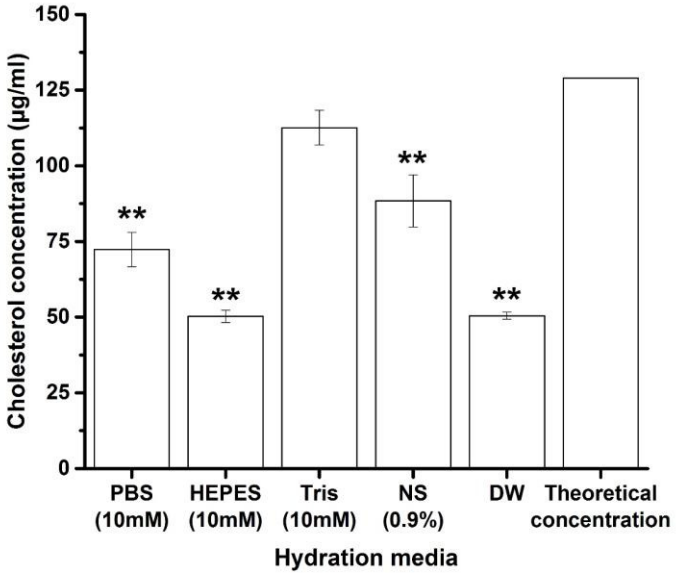

Figure 6. Calculated cholesterol concentrations after preparing NISV with microfluidics using different hydration media compared to the theoretical concentration. The data represents the mean ± SD (n=3) measured by HPLC, $^{**}p < 0.05$ significant decrease in Chol concentration compared with the theoretical concentration.

3.6 Turbidity assay

The interaction of the different NISV with FBS was then examined in an attempt to predict the stability of these particles when exposed to physiological conditions by calculating the RT of each formulation when incubated with 10% (v/v) FBS. The turbidity assay measures the degree of light scattering through a sample with suspended particles. Turbidity depends mainly on the concentration of the suspended particles, the size distribution of the particles in the liquid phase and the difference in the refractive index between the particles and the suspending medium [47, 48]. Microbiological instability or increase in the particle size of the suspended particles as a result of aggregation will result in an increase in the RT of the liquid [46]. All the NISV showed good stability in terms of RT over two hours at 37°C (Figure 7). This can be seen with the minimal increase of the RT for all formulations with time, bearing in mind that this increase was not significant ($p > 0.05$). This result suggests that all the aqueous media used to prepare the NISV were effective in preventing particle aggregation when incubated with 10% (v/v) FBS.

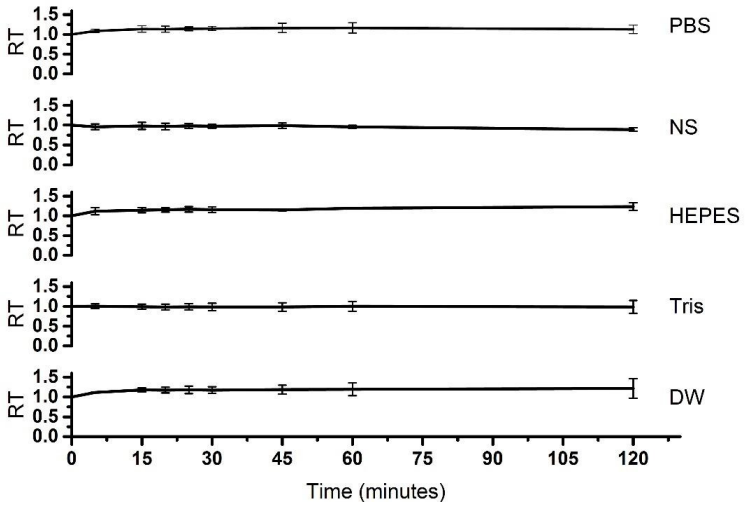

Figure 7. Relative turbidity (RT) of the NISV prepared with PBS, NS, HEPES, Tris and DW and incubated at 37°C with 10% v/v FBS. The data represents the mean ± SD (n=3).

3.7 Cytotoxicity studies

Finally, we studied the formulations on the viability of two cancer cell lines (A375, A2780) and a normal PNT2 cell line. Figure 8 shows the cytotoxicity of the formulations on the cells and Table 2 shows the calculated EC_{50}. Cell viability measurements showed that regardless of the media used to prepare the NISV, all the cell lines were 100% viable at a total lipid concentration of 78.1 µg/ml and below. We observed a media-dependent toxicity on the A375 cell line. The type of the media used to prepare the particles had a significant ($p < 0.05$) effect on the viability of these cells as there was a significant difference between the EC_{50} of each formula. When the media alone was tested on these cells, they were not toxic and the cells were 100% viable (data not shown). The media-dependent toxicity on the A375 cells was probably due to the difference in the particle size or surface charge in each formulation which would affect its cellular uptake and the subsequent impact on viability [41]. Moreover, it has been reported that the particle size, shape and surface chemistry all have effects on cellular internalisation and intracellular trafficking [49]. Since each formulation resulted in different particle characteristics in term of size and charge, this might be the reason for the difference in the cell viability for the A375 cells. Different cell types have different sensitivities and nanomaterial interactions with cells depends on the colloidal forces and the dynamic biophysicochemical interactions between the cells and the particles [50]. These effects of the type of the hydration media used for particles preparation on the cellular viability needs to be investigated more extensively and consideration given to this phenomenon by researchers. For the other cell lines (A2780 and PNT2), although there were differences between the EC_{50} for each formulation, they were not significant and the EC_{50} for each formulation was close to the others, taking into consideration that the media alone were not toxic to any of these cell lines.

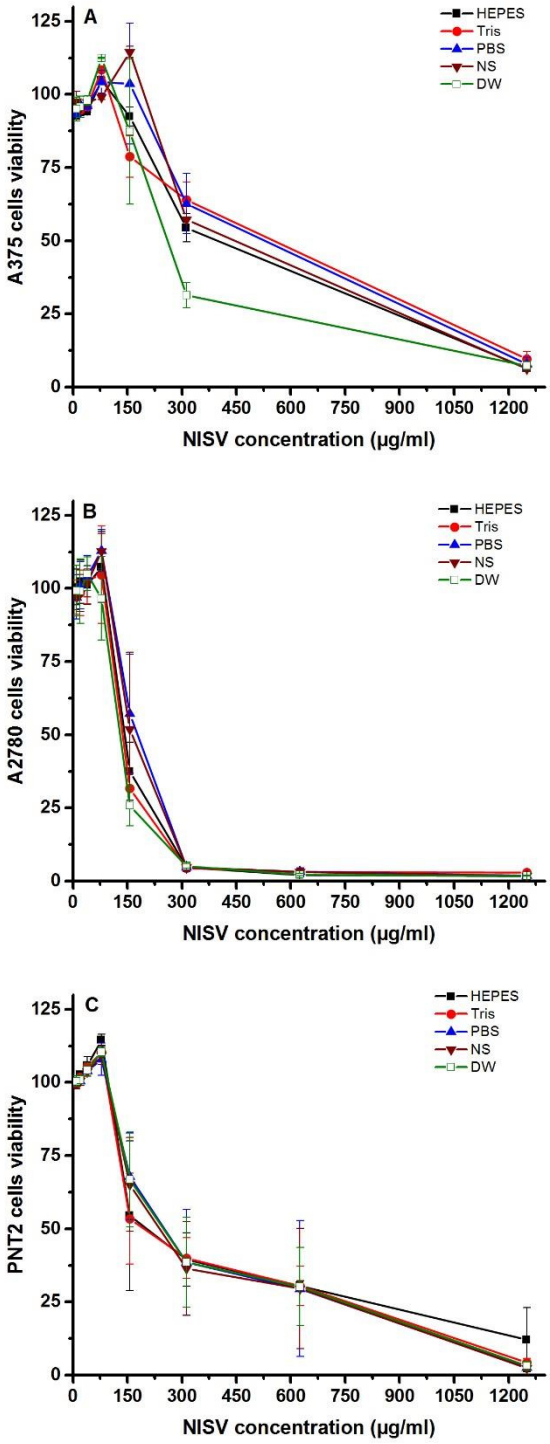

Figure 8. Figure 8 cytotoxicity of the NISV prepared with PBS, NS, HEPES, Tris and DW on (A) A375, (B) A2780 and (C) PNT2 cell lines. The data represents the mean ± SD (n=3).

4. Conclusion

In this paper, we report for the first time that the aqueous media used to prepare NISV by microfluidics had a significant effect on the physiochemical characteristics of the resultant particles. These findings provide strong evidence that the type of the media used to prepare NISV by microfluidics has significant effects on particle size, distribution and surface charge. The type of the media used should be taken into consideration in order to modulate these characteristics of the formed particles. This is an important factor that will also have implications with respect to the entrapped material as the media can be chosen based on the compatibility with the intended drug to be encapsulated which in addition may help to design vesicles for different uses based on changing the preparation medium. Our future aim is to use NISV to therapeutically target cancer cells, therefore, establishing cytotoxicity of the drug delivery system alone and effect of the preparation media on the NISV cytotoxicity, while the media wee not cytotoxic on their own was also an important finding.

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