RESEARCH PAPER

Residual Solvents in Nanomedicine and Lipid-Based Drug Delivery Systems: a Case Study to Better Understand Processes

Amrita Dikpati [1,2] • Farzad Mohammadi [2,3] • Karine Greffard [2] • Caroline Quéant [2] •
Philippe Arnaud [4] • Guillaume Bastiat [5] • Iwona Rudkowska [2,3] • Nicolas Bertrand [1,2]

ABSTRACT

Purpose Complexities surrounding the manufacture and quality control of nanomedicines become increasingly apparent. This research article offers a case study to investigate how, at the laboratory scale, various stages of liposome and nanoparticle synthesis affect the amount of residual solvent found in the formulations. The objective is to bring insights on the reliability of each of these processes to provide final products which meet regulatory standards and facilitate identifying possible bottleneck early during the development process.

Methods The residual solvent at various stages of preparation and purification was measured by headspace gas chromatography. Liposomes were prepared by two different methods with and without solvent. Polymer nanoparticles prepared via nanoprecipitation and purified by ultrafiltration were studied. The effects of purification by size exclusion chromatography and dialysis were also investigated.

Results The complete removal of residual solvent requires processes which go beyond usual preparation methods.

Conclusions This work might prove valuable as a reference for scientists of different fields to compare their own practices and streamline the translation of nanomedicines into efficacious and safe drug products.

Amrita Dikpati, Farzad Mohammadi and Karine Greffard contributed equally to this work.

✉ Nicolas Bertrand
nicolas.bertrand@pha.ulaval.ca

[1] Faculty of Pharmacy, Université Laval, Pavillon Ferdinand-Vandry, 1050, avenue de la Médecine, Québec G1V 0A6, Canada

[2] Endocrinology and Nephrology Unit, CHU de Québec - Université Laval Research Center, 2705 Laurier Blvd, Québec City G1V 4G2, Canada

[3] Department of Kinesiology, Faculty of Medicine, Université Laval, 2300 rue de la Terrasse, Québec G1V 0A6, Canada

[4] Faculté de Pharmacie de Paris, Université de Paris, 4 avenue de l'Observatoire, 75006 Paris, France

[5] Micro et Nanomédecines Translationnelles, MINT, UNIV Angers, UMR INSERM 1066, UMR CNRS 6021, 4 rue Larrey, 49933 Angers, Cedex 9, France

Pharmacopoeias define residual solvents as organic volatile impurities found in final drug products [1]. Solvents can originate from the manufacturing and purification of active drug substances and excipients, but also from the cleaning and maintenance of equipment. Importantly, in complex drug products like nanomedicines, organic solvents can also be used during the formulation processes which substantiate their unique properties.

Residual solvents have no therapeutic value and, in some cases, may be toxic. Besides health considerations, residual solvents can also affect physicochemical properties of drugs, notably particle size, dissolution and wettability [2, 3]. Irregular and unregulated concentrations of these impurities can therefore result in risks for patients and/or inconsistent product quality. For these reasons, the quantity of residual solvent tolerated in final drug products is well-described in pharmacopeias and closely monitored by regulatory agencies.

In 1997, the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) released a guidance document to orient manufacturers in their manufacturing practices [4]. These guidelines distribute existing solvents in three classes, based on their individual health and environmental hazards. Class 1 solvents are known

to cause the most severe toxicities and/or the most danger for the environment. Their use in manufacturing must be avoided at all costs. Examples of Class 1 solvents include benzene and carbon tetrachloride. Class 2 lists solvents which must be regulated because of documented toxicities. These limitations ensure that patients do not receive doses beyond a certain permitted daily exposure (PDE), in mg per day. The limit in concentration tolerated in a pharmaceutical product is calculated by dividing the PDE by the quantity of product a patient would take in one day. When the daily dose is unknown, the ICH assumes 10 g as the maximum quantity of drug product that could be taken in one day. In other words, the PDE is divided by a high, hypothetical dose which ensures that all patients taking less than 10 g of the drug would have safe and acceptable exposure to solvents. Examples of Class 2 solvents include methanol, tetrahydrofuran, dichloromethane, chloroform and acetonitrile. Finally, the Class 3 solvents are more innocuous and their quantities in final drug products must be limited as per the best possible manufacturing practices. Residual Class 3 solvents are tolerated without justification in drug products as long as the expected daily exposure does not exceed 50 mg. Ethanol, dimethylsulfoxide (DMSO), acetone and ethyl acetate are examples of Class 3 solvents.

Given the higher leniency toward Class 3, most literature surrounding residual solvents in nanomedicines focuses on Class 2 solvents. Various efforts are channeled toward the development of elegant synthesis or purification techniques to complement existing methodologies. For example, the use of supercritical fluid has recently gained traction to synthesize liposomes [5]. Using this technology, dried liposomes with low residual concentrations of methanol and dichloromethane were prepared by precipitating lipids and extracting solvents in supercritical carbon dioxide [6]. A similar approach was also employed for the synthesis of polymeric nanoparticles, including poly(lactic-co-glycolic acid) (PLGA) particles with low residual dichloromethane [7]. Microfluidics chips, where small channels are fabricated to enable the precise mixing of fluids, are another technology proposed to limit residual solvents in nanomedicines [8]. For example, a microfluidic system was designed to prepare polymer nanoparticles with low residual tetrahydrofuran [9]. Similarly, a sophisticated microfluidic device with in-line synthesis and purification was proposed to fabricate pure liposomes in continuous flow [10]. Yet, despite these very commendable efforts, these technologies are not broadly available to most laboratories. It therefore seems important to characterize common, accessible processes, and better understand how to control residual solvents while fabricating drug delivery systems.

In this work, we will look at residual solvents resulting from common laboratory-scale fabrication and purification procedures for two classes of drug delivery systems: liposomes and polymer nanoparticles. Liposomes represent the nanomedicine technology with the largest number of approved drug products [11], while polymer nanoparticles sustain pharmaceutical and scientific interest due to their differentiated physicochemical characteristics [12]. Finally, a third drug delivery system, lipid nanocapsules [13], will be used to validate our findings on some purification procedures.

In this case study, two Class 2 solvents will be investigated: chloroform and acetonitrile. The objective of the study is not to provide an extensive representation of all possible methods and solvent combinations, but simply to provide examples for what can happen when using both water-miscible and immiscible solvents. Chloroform is a chlorinated and volatile solvent which is not miscible with water. It is believed to be carcinogenic and hepatoxic [14]. Its acute oral median lethal dose (LD_{50}) is around 690 mg/kg. The PDE of chloroform is 0.6 mg/day which results in a maximum tolerated concentration in drug products of 60 ppm [4]. Acetonitrile is a water-miscible solvent that can be teratogenic and cause toxicities of the respiratory and central nervous systems [15]. Its oral LD_{50} is around 450–800 mg/kg in rats. According to ICH guidelines, acetonitrile has a PDE of 4.1 mg/day and a maximum acceptable concentration in drug products of 410 ppm [16].

Herein, residual solvents will be quantified by headspace-gas chromatography coupled to mass spectrometry (HS-GC/MS). HS-GC is the analytical method recommended by the United States Pharmacopeia [1, 17]. At each step of fabrication, the influence of some critical parameters on the residual levels of solvents will be evaluated. The objective is to inform on how better prepare nanomedicines which are free of solvent, without the necessity for sophisticated or experimental technologies.

MATERIALS AND METHODS

Materials

Soy lecithin Ultralec® F (97%, Product no: 2516, CAS# 8030-76-0) was a gracious gift from Medisca Inc. (Montréal, QC, Canada). Elaidic acid was purchased from Nu-check Prep, Inc. (Elysian, MN, USA). Poly(ethylene glycol)-b-poly(-lactic-co-glycolic acid) (PEG_{5k}-$PLGA_{27k}$, Mw/Mn = 1.47) copolymers were synthesized as described previously [18, 19]. Carboxy-terminated $PLGA_{30K}$ polymer (Lactel product # B6013–2, Mw/Mn = 1.88) was purchased from Durect Corporation (Birmingham, AL, USA). Acetonitrile (HPLC grade) and sodium chloride were purchased from Sigma Aldrich (Oakville, Ontario, Canada). Kolliphor® HS 15 and Labrafac™ lipophile WL 1349 were obtained from BASF (Ludwigshafen, Germany) and Gattefossé (Saint-Priest, France), respectively. Sorbitan monooleate (Span® 80) was acquired from Alfa Aesar (Fisher Scientific, Tewksbury, MA, USA) Ultrafiltration 100 k Microsep™ filters were purchased from Pall Corporation (Port Washington, NY, USA) and

Preparation of Coarse Lipid Suspensions

Method 1: Hydration of Lipid Films Formed by Solvent Evaporation

Lipid films were prepared from organic solutions as per common procedures [20, 21]. Briefly, to prepare lecithin liposomes, 500 or 1000 µL of a 40 mg/mL chloroform solution of lecithin were added to a 10-mL round bottom flask. In formulations containing elaidic acid, 14 wt% of the trans fatty acid (5.5 mol%) were also added as a 40 mg/mL chloroform solution. Lipid films were formed by evaporation under reduced pressure on a Büchi R-100 Rotary Evaporator equipped with a I-100 pressure control interface (Büchi, Switzerland), using regular bump trap without drain holes. The heating bath was equilibrated at 50°C and the vacuum pressure was set at 3.3 kPa. Evaporation times of 10, 20, 40 and 60 min were predetermined. Separate batches were evaporated for 60 min as described above, followed by overnight drying on a ramp under vacuum (< 100 Pa). After evaporation (with or without overnight drying), the lipid film was hydrated by addition of 2 mL of ultrapure water and briefly sonicated until a homogeneous suspension was obtained.

Method 2: Hydration of Powders at 60°C

The solvent-free hydration method was adapted from literature [22]. Briefly, oxygen was removed from 4 mL of ultrapure water in a 20-mL glass vial by bubbling nitrogen for 10 min. Lecithin powder (with or without 14 wt% elaidic acid) was then added to reach a total lipid concentration of 40 mg/mL. Hydration was conducted under magnetic stirring at 60°C for 6 h.

Preparation of Large Unilamellar Liposomes

Coarse lipid suspensions prepared by both aforementioned methods were extruded using a LiposoFast manual extruder (Avestin, Ottawa, ON, Canada) to prepare large unilamellar vesicles [20]. Polycarbonate membranes with pores of 200, 100 and 50 nm were used sequentially. Liposomes were characterized by dynamic light scattering (DLS) at room temperature on a Malvern Nanosizer S (Malvern Panalytical, Westborough, MA).

Preparation of Polymeric Nanoparticles

Nanoparticles were prepared by nanoprecipitation of acetonitrile solutions of PEG_{5k}-$PLGA_{27k}$ (Mw/Mn = 1.47) and $PLGA_{30k}$ (Mw/Mn = 1.88) polymers in water [18]. Briefly, 600 µL of a 10 mg/mL solution of PEG_{5k}-$PLGA_{27k}$ in acetonitrile were combined with 400 µL of a 10 mg/mL solution of $PLGA_{30k}$. This mixture was added dropwise to 10 mL of water under stirring at a speed of 1600 rpm. Nanoparticles were characterized by DLS. In all samples, nanoparticle concentration was determined by gravimetry.

Preparation of Lipid Nanocapsules

Lipid nanocapsules were prepared according to a previously published phase-inversion thermal cycling procedure [23], without the use of solvent. Labrafac™ lipophile WL 1349 (260 mg), and Span® 80 (70 mg) Kolliphor® HS 15 (250 mg), sodium chloride (15 mg) and ultrapure water (222 mg) were weighed and mixed in a 20-mL glass vial containing a magnetic stir bar. The biphasic mixture was stirred at room temperature for 2 min before successively heating to 75°C and cooling to 45°C at a rate of 5°C/min for 3 cycles. At the end of the cycling procedure, 370 µL of cold water was added to the solution when it reached a temperature of 60°C. The resulting lipid nanocapsules were characterized by DLS. The formulation was diluted 10-fold before use. The concentration of lipid nanocapsules in all samples was determined by gravimetry.

Measurement of Phospholipid Concentrations

The phospholipid concentrations in coarse lipid suspensions and liposome preparations were determined by the phosphate assay, using a calibration curve of lecithin in chloroform/methanol (8:2) ranging from 0 to 4 mg/mL [24, 25]. Briefly, samples were diluted 10- to 20-fold in ultrapure water, and 10 µL of these dilutions or calibration standards were deposited in 12x75mm glass tubes. Sixty µL of concentrated sulfuric acid (95 vol%) and 10 µL of hydrogen peroxide (30 vol%) were added, vortexed and heated at 200°C for 10 min. Samples were removed from the heating block and 670 µL of ultrapure water was added, followed by 20 µL of sodium metabisulfite (100 mg/mL). Samples were vortexed and heated at 100°C for 5 min. In the last step, 200 µL of a 20 mg/mL solution of ammonium molybdate and 20 µL of ascorbic acid (100 mg/mL) were added and vortexed. Samples were heated for 10 min at 100°C to reveal a blue color. Concentration was determined by measuring absorbance at 820 nm and reporting to the calibration curve.

Addition of External Solvent to Formulations

To ensure reproducible quantities of solvent in some samples, a DMSO solution containing 10 mg/mL of chloroform was prepared. This solution (10 µL) was added to 1.2 mL of coarse lipid suspensions of lecithin hydrated by the solvent-free method or liposome formulations resulting from such lipid suspensions. The phospholipid concentration of these formulations was around 37 to 39 mg/mL. Lipid nanocapsules (1 mL at 46–48 mg/mL) were spiked with 10 µL of a DMSO solution containing 10 mg/mL of chloroform and 10 mg/mL of acetonitrile.

Purification of Nanoparticle by Ultrafiltration

Nanoparticles were purified on ultrafiltration filters (Pall Microsep®, MWCO 100 kDa). Prior to washing, the whole formulation (ca. 11 mL) was concentrated on the filter to a volume of 1 mL and the volume was made up to 5 mL with ultrapure water. One cycle of washing consisted of reducing the content of the filter to 1 mL and diluting it back to 5 mL with ultrapure water. Individual batches of nanoparticles were prepared to investigate the effect of 2, 3, 4, 5, 6 and 12 washing cycles (n = 3–6).

Purification by Size Exclusion Chromatography

Independent batches of liposomes and nanoparticles were purified by size exclusion chromatography [26]. Polymer nanoparticles were purified by 6 cycles of ultrafiltration as described above, while liposomes and lipid nanocapsules were prepared by solvent-free methods and supplemented with solvent. Separation was conducted on a 1 × 10 cm gravity flow column (Kimble-Chase gravity Flex-column, #420401–1010, Vineland, NJ, USA) packed with Sephadex G-75 hydrated in water. Briefly, 500 µL of formulation were loaded on the top of the gel bed and ultrapure water was flowed using a peristaltic pump at a rate of approximately 3 mL/min. Fractions of 1 mL were collected using a fraction collector (Bio-Rad Model 2110, Berkeley, CA, USA). Volumes containing nanoparticles were identified by adding 10 µL of a methanol solution of Nile Red (0.1 mM) to 100-µL aliquots of each fraction and measuring fluorescence in a plate reader ($\lambda_{ex}/\lambda_{em}$: 549/628 nm).

Purification by Dialysis

Slide-A-Lyzer™ Dialysis Cassette (Cat# 66003, Thermo Scientific, Tewksbury, MA, USA) with a MWCO of 20 kDa were hydrated in ultrapure water for 30 min. Lipid nanocapsules were supplemented with acetonitrile and chloroform as described above, and one sample was kept for analysis of initial solvent concentration (T = 0 h). Three mL of formulation were introduced to the dialysis cassette using a syringe as per the manufacturer's recommendations, and the samples were immersed in a beaker covered with an aluminium foil containing 1000 mL of ultrapure water (dialysis media). Dialysis was carried under constant stirring at room temperature for 24 h. After 1, 4, 8 and 24 h, aliquots of 500 µL were collected from the cassette using a new disposable syringe for each sample and timepoint. Dialysis media was fully replaced with fresh media at each sampling time. The concentration of each sample was determined by gravimetry and analyzed by HS-GC/MS.

Residual Solvent Analysis by HS-GC/MS

Residual solvent in formulations were quantified by headspace-gas chromatography on an Agilent gas chromatographer 7890B coupled to a single quadrupole mass-spectrometer 5977B and equipped with a HP-Innowax column (30 m × 0.25 mm × 0.25 m, from Agilent). Briefly, calibration solutions and samples (100–3000 µL) were added to a 20 mL headspace crimp cap vial (Agilent, Santa Clara, CA, USA) and the volume was adjusted to 6 mL by addition of ultrapure water. Equilibration of headspace gases was achieved by incubation at 95°C for 40 min by the automated Pal 3 injection system (CTC Analytics AG, Zwingen, Switzerland). One mL of the gas phase was injected with the pulsed split mode (10:1) into a 1 mL/min flow of helium carrier gas. The temperature program consisted of 8 min at 40°C followed by a 15°C/min ramp to 260°C (total run of 22.7 min). The mass detector scanned from 29 to 200 amu, with an electron impact ion source set to 70 eV of collision energy.

Quantification of residual solvents was obtained by building a calibration curve from mixtures of standards for USP 467 Class 2 residual solvent analysis (Agilent, Santa Clara, CA, USA). Briefly, Class 2A (product # 5190–0492) and 2B standards (product # 5190–0513) were diluted 100-fold in ultrapure water. Calibration curves were obtained by diluting equal proportions of these dilutions (100–600 µL) in sufficient quantity of ultrapure water to obtain a final volume of 6 mL. The calibration curves were freshly prepared and re-injected at the beginning of each analysis. Analysis of unknown formulations and determination of concentrations in parts per million (ppm) were calculated using volumes equivalent to 10 mg of material and completing the volume to 6 mL using ultrapure water. The limit of quantification (LLOQ) was determined to be around 12.5 ppm for chloroform (m/z = 85) and 20 ppm for acetonitrile (m/z = 41).

RESULTS AND DISCUSSION

Vacuum Drying Removes Chloroform from Lipid Films Unpredictably

The experimental conditions used for the preparation of coarse lipid suspensions were representative of research and development, laboratory-scale production of liposomes [21, 27]. The round-bottom flasks used were small (10 mL), and

20 to 40 mg of phospholipids, dissolved in 500 or 100 µL of chloroform were used for each preparation. Importantly, lecithin is a natural phospholipid composed of multiple chains of fatty acids: linoleic (C18:2, 56 wt%), palmitic (C16:0, 20 wt%), oleic (C18:1, 9 wt%), alpha linoleic (C18:3, 6.5 wt%) and stearic acid (C18:0, 5 wt%) [25]. The presence of multiple varieties of unsaturated fatty acid chains results in a very low phase transition temperature (Tm). Evaporation was carried under a combination of vacuum and temperature facilitating the removal of chloroform (i.e., 3.3 kPa, 50°C) [28]. Altogether these conditions were chosen to represent a "best-case" scenario: small quantities of fluid lipids and low volumes of chloroform, under conditions expediting evaporation.

Drying times as short as 10 min resulted in visually dry lipid films, without remaining liquid chloroform. Upon addition of water, lipid films were hydrated within minutes to homogeneous coarse lipid suspensions without discrete aggregates, consistent with multilamellar vesicles suspensions and compatible with further extrusion and processing toward liposome preparation.

These suspensions were analysed by HS-GC to measure the residual amounts of chloroform (Fig. 1A). Films dried between 10 and 40 min consistently resulted in suspensions with residual amounts of chloroform 15- to 200-fold higher than the 60-ppm limit prescribed by the ICH guidelines. When the drying time was extended to 60 min, the average solvent concentration remained around 7100 ± 7700 ppm. Further investigation to explain the high variability of the results highlighted that the evaporator could act as a source of contamination, likely upon re-equilibration to atmospheric pressure (Supplementary Fig. S1 and supplementary discussion). Altogether, these observations suggest that evaporation on the rotary evaporator alone is ill-suited to consistently prepare formulations without residual chloroform.

In contrast, overnight drying on a vacuum ramp (< 100 Pa) resulted in 7 out of 9 lipid suspensions with acceptable levels of residual solvent. In this instrument, the contamination observed with the rotary evaporator is not present, presumably because the air flowing during re-equilibration is not in contact with the condensed solvent. This drying procedure therefore appears to be more compatible with the preparation of formulations without remaining chloroform. Nevertheless, the presence of two samples with levels of chloroform exceeding the acceptable threshold despite overnight evaporation (by 12- and 42-fold, respectively) draws caution on the reproducibility of this drying process.

Suspensions of Lecithin Can Be Obtained without the Preparation of Lipid Films

Given the difficulty of getting rid of chloroform using vacuum drying, an alternative method was investigated to hydrate the lipids directly, without prior solubilization in organic solvents. Colas *et al.* have shown that lecithin powder can be dispersed in water by stirring at 60°C for 6 h [22]. In this process, the low Tm of the phospholipid allows it to effortlessly self-assemble into bilayers. Figure 1B shows the residual solvent measured in coarse lipid suspensions prepared by this approach. As expected, only minimal quantities of chloroform are detected in these formulations, and all of them were below the acceptable threshold. The addition of 14 wt% of elaidic acid, a model hydrophobic free fatty acid, shows that similar dispersions can be obtained even when encapsulating bioactive molecules.

Nevertheless, given that chloroform was not present in the raw material (data not shown) and that the processes did not involve solvent, further investigation was necessary to account for the traces detected. Supplementary Fig. S2 depicts the contamination that can occur after sharing the same pipettes to transfer volatile solvents and clean samples. It is therefore posited that the minute contamination in formulations prepared without solvent might come from the use of contaminated equipment. Using disposable measuring devices, dedicated equipment or ensuring thorough washing between solutions could solve this cross-contamination issue.
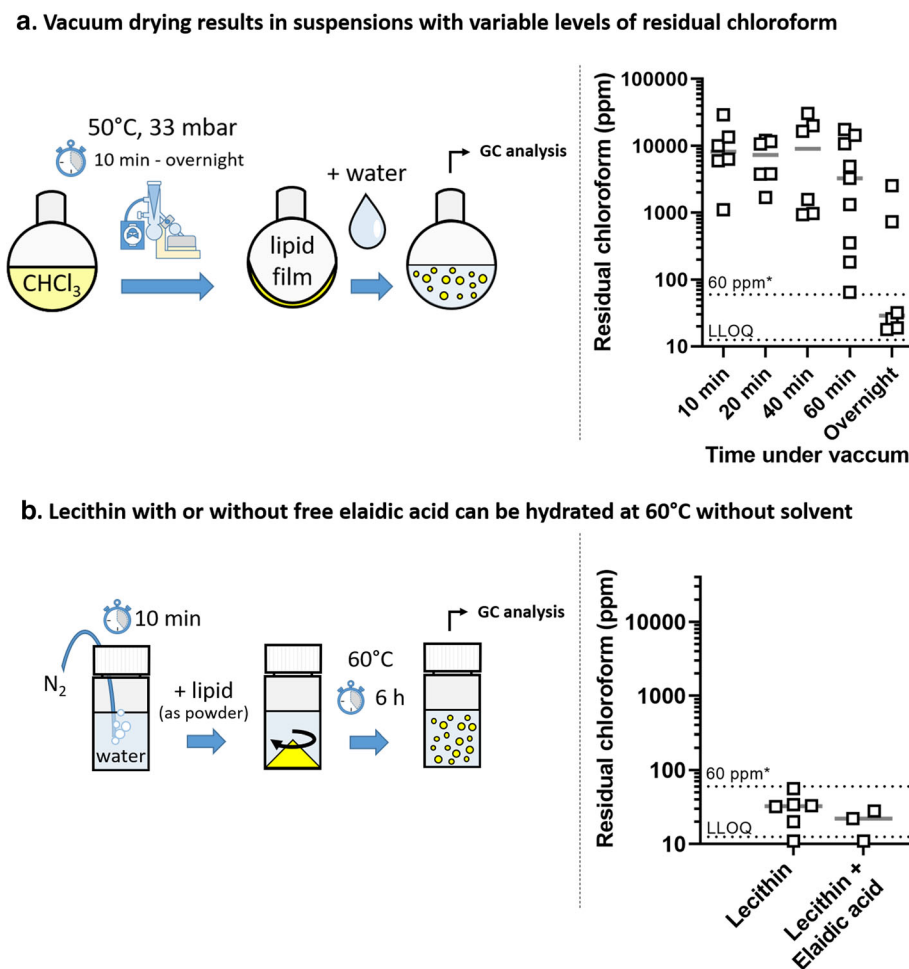
Extrusion of Coarse Lipid Suspensions Does Not Significantly Reduce the Amount of Residual Solvent

The coarse lipid suspensions prepared above represent the first step of liposome fabrication. Hydration of phospholipids creates large multilamellar vesicles which have broad size distributions and are poorly suitable for drug delivery applications. One common approach to reduce their size is to extrude them through polycarbonate filters which have pores of definite size [20]. This leads to unimodal and monodisperse distributions of unilamellar vesicles.

In theory, extrusion is unlikely to significantly affect the concentration of residual solvent found in formulations; extruders have inert components and the process does not notably dilute the samples. Nevertheless, chloroform is a volatile solvent which could potentially evaporate upon handling and aeration of the formulations. It therefore seems important to investigate to which extent extrusion can further reduce the amount of solvent in liposome preparations. In parallel, because laboratory instruments are used with a variety of materials, the possible contamination of solvent-free suspensions with residual solvents from other formulations was also assessed. To do so in a predictable and reproducible manner, solvent-free preparations were supplemented with controlled quantities of chloroform and assessed by HS-GC before and after extrusion.

Figure 2 shows how extrusion affects residual solvent in suspensions supplemented or not with chloroform. With the former, handling of the formulations and passage through the membranes decreased the solvent content by 50 to 70% of its

Fig. 1 The removal of chloroform from lipid film by vacuum drying is unpredictable, but solvent-free suspensions can be prepared by hydrating the lipids while heating at 60°C. **a**. Lipid films were dried under vacuum for different lengths of time. **b**. Hydration of lecithin for 6 hours at 60°C allows consistent preparation of lipid suspensions without residual solvent. Each data point represents one single replicate (n = 3-12), gray bar represents median * is the ICH Q3C(R6) acceptable chloroform concentration, LLOQ is the lower limit of quantification of the HS-GC method.

a. Vacuum drying results in suspensions with variable levels of residual chloroform

b. Lecithin with or without free elaidic acid can be hydrated at 60°C without solvent


initial values, but all samples remained more than 15-fold above the acceptable limits of 60 ppm. In samples prepared by the solvent-free hydration method, four independent batches showed no increase in residual chloroform. This

suggests that proper cleaning and rinsing of extruders can adequately prevent cross-contamination.

Interestingly, the concentration of phospholipid in the coarse suspension formed without solvent was very similar to that

Fig. 2 Extrusion through polycarbonate membranes does not significantly affect the residual solvent found in lipid suspensions. Each data point represents one single replicate (n = 3-4), gray bar represents median, * is the ICH Q3C(R6) acceptable chloroform concentration, LLOQ is the lower limit of quantification of the HS-GC method.
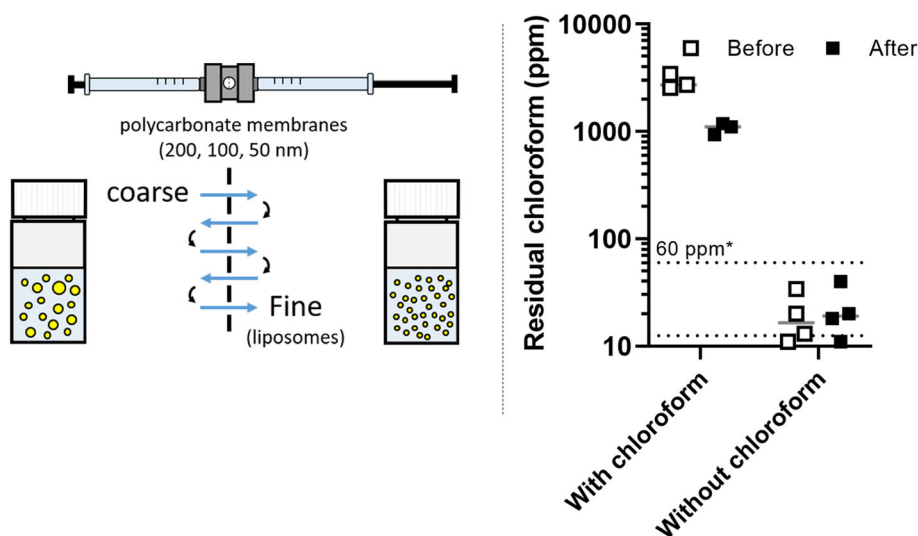
measured in the extruded liposomes. The yield of the extrusion process was ca. 90% (data not shown). These values are comparable to those obtained when suspensions are prepared by the lipid-film hydration method. This suggests that prolonged stirring at 60°C is sufficient to properly hydrate lecithin and that this approach is truly viable to form liposomes, at least for phospholipids with low Tm. The solvent-free hydration process was also compatible with the use of 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC, Tm = −17°C), but not hydrogenated soy phosphatidylcholine (HSPC, Tm = 49°C) (data not shown). For the latter type of phospholipids, the freeze-drying of mixtures of water and tert-butyl alcohol, a class 3 solvent [29], could prove valuable to prepare formulations without using chloroform.

Removal of Acetonitrile from Polymer Nanoparticles Requires Thorough Ultrafiltration
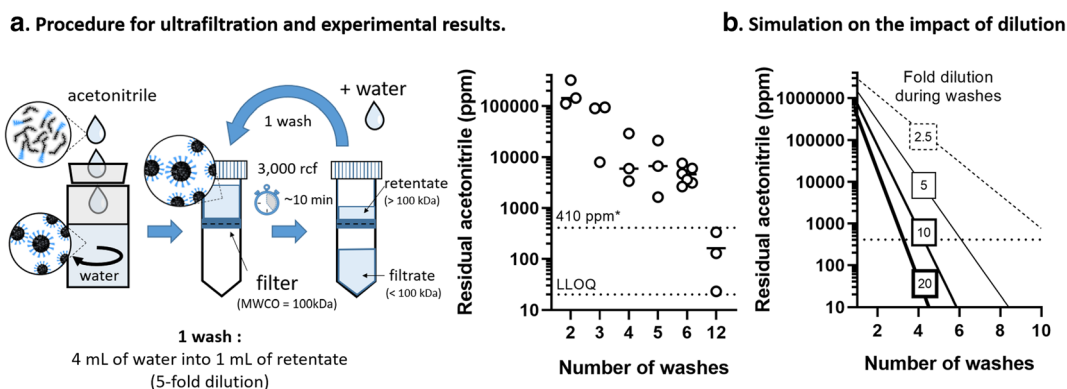
Next, residual acetonitrile in formulations of nanoparticles was investigated. Like other water-miscible solvent, acetonitrile is commonly used for the synthesis of polymer nanoparticles by nanoprecipitation [18, 30]. In this method, amphiphilic copolymers are solubilized in acetonitrile and slowly added to water [30]. After nanoprecipitation, suspensions have acetonitrile concentrations ranging between 5 and 10 vol% [18].

Ultrafiltration relies on semipermeable membranes to separate large molecular weight species in the retentate (e.g., nanoparticles) from smaller solutes in the filtrate. At the laboratory scale, disposable filters can harness centrifugal force to conveniently remove small molecular weight impurities from batches of nanoparticles. At the industrial scale, continuous processes, like tangential flow filtration, are often preferred for their higher throughput. Figure 3 presents the residual acetonitrile concentration detected in polymer nanoparticles prepared by nanoprecipitation and purified by successive ultrafiltration washes using disposable filters. The experimental data suggests that up to 12 washing cycles could be necessary

to obtain nanoparticles preparations with acceptable levels of acetonitrile (Fig. 3A). With centrifugation cycles lasting approximately 10 min, thorough purification could therefore represent a much lengthier process than the relatively short nanoprecipitation synthesis.

In this experiment, each wash was normalized to dilute the residual solvent five-fold. This protocol was chosen based on previous experience, to minimize aggregation of PLGA-PEG nanoparticles during filtration and to increase reproducibility. However, other dilution factors, where nanoparticles would be either more diluted or concentrated in the retentate, could significantly affect the speed at which solvent is removed. Figure 3B simulates the impact of selecting other dilution factors on the number of washes necessary to eliminate solvent. Evidently, larger dilution at each cycle results in faster removal of solvent. These parameters would therefore need to be carefully selected to maximize efficiency and throughput while maintaining the properties of the nanoparticles. When using disposable filters, this means selecting the dilution factor and the volumes of water added to the retentate, but similar optimization would also be required on a larger scale and when using continuous processes.

Other groups have showed that extensive washing is also required to thoroughly remove other class 2 solvents from PLGA nanoparticles. Han *et al.* [31] have looked at removing dichloromethane, a water-immiscible solvent, from nanoparticles with diameters between 300 to 400 nm. Using 1-h centrifugations followed by resuspension in fresh media, they showed that increasing the number of washing cycles from one to three could reduce the residual solvent by half (from 400 to 200 ppm). Other procedures like extraction with carbon dioxide or lyophilisation have also been investigated. Treating particles for 24–48 h with supercritical CO_2, Falk and Randolph were able to reduce the levels of dichloromethane from 50 ppm to 2–3 ppm [32]. Similar supercritical CO_2 extraction has also been optimised to remove ethyl acetate in 20 min,

a. Procedure for ultrafiltration and experimental results.   **b. Simulation on the impact of dilution**

Fig. 3 Extensive washing is necessary to eliminate residual acetonitrile in preparations of polymer nanoparticles. **a**. Each wash consists of concentrating nanoparticles by ultrafiltration to 1 mL and diluting them back to 5 mL with ultrapure water (5-fold dilution). **b**. Other dilution factors would significantly affect the number of steps necessary for complete solvent removal. Each data point represents one single replicate (n = 3-6), * is the ICH Q3C(R6) acceptable chloroform concentration, LLOQ is the lower limit of quantification of the HS-GC method.

yielding formulations with very low residual solvent (< 20 ppm) [33]. Although less efficient, a 24-h exposure to gaseous CO_2 was also found to significantly decrease solvent levels from 25,000 to 5000 ppm [34]. Finally, some reports support that lyophilisation appears to result in formulations with very minimal residual solvent (< 1 ppm) [35]. However, freeze-drying nanoparticles while maintaining their colloidal and physicochemical properties is a complex procedure and often requires the addition of lyo- and cryoprotectants [36].

Size Exclusion Chromatography Can Adequately Remove Residual Solvent from Formulations

Given the shortcomings of the solvent-elimination methods described above, size exclusion chromatography was investigated as an alternative purification approach. Size exclusion is a type of chromatography where solutes with larger hydrodynamic radii are excluded from the pores of the stationary phase and exit the column earlier than smaller molecules [26]. Figure 4A shows the chromatograms obtained when the same column is loaded with liposomes, polymer nanoparticles or lipid nanocapsules. All colloids exit the column at approximately the same retention times (ca. 4 mL), whereas small molecular solutes are retained until later fractions.

Despite the well-known purification capabilities of size exclusion chromatography, it has been shown that hydrophobic and amphiphilic molecules can preferentially interact with the surface of nanoparticles, notably due to their high surface-to-volume ratio [19]. It therefore remains relevant to ascertain whether such interactions could prevent the purification process from getting rid of residual solvents.

Hence, liposomes, polymer nanoparticles and lipid nanocapsules containing respectively chloroform, acetonitrile and both chloroform and acetonitrile were purified in the same manner. To ensure reproducible quantities of residual solvents, polymer nanoparticles were washed only 6 times, while liposomes and lipid nanocapsules were prepared by solvent-free synthetic procedures and spiked with 10 μL of DMSO containing 10 mg/mL of either chloroform or chloroform and acetonitrile.

Figure 4B shows that the residual chloroform in liposomes can be reduced between 15- and 25-fold by passage on the column. Although all three purified replicates remained above the acceptable limits (118, 99 and 78 ppm), the initial chloroform concentration was arbitrarily chosen to be relatively high (>1800 ppm). It is probable that formulations with lower initial quantities of solvent could very well fall below the threshold. The ability of the column to remove solvent can also be observed from the data obtained with acetonitrile in polymer nanoparticles. The column decreases the concentration in water-miscible solvent considerably, between 25- and 160-

fold, resulting in formulations with impurities below the acceptable limit.

Finally, the ability of the column to remove solvents was confirmed with lipid nanocapsules. This type of nanoparticles are prepared with injectable oils and surfactants which are formulated by phase-inversion thermal cycling, without the use of solvent [23]. Because nanocapsules possess a fluid lipid core, they represent a drug delivery platform which significantly differs from both liposomes and polymer nanoparticles. In certain aspects, the hydrophobic, de-hydrated core of these nanocapsules is similar to those of other lipid complexes and nanoparticles. This system therefore provides an opportunity to confirm whether the present observations are applicable beyond vesicular and polymeric systems.

Right after synthesis and before spiking with chloroform and acetonitrile, lipid nanocapsules contain no detectable solvent (Fig. 4B). Addition of an external source of solvent increases the levels of chloroform and acetonitrile to values comparable to those obtained with liposomes and polymer nanoparticles. In the same way as with these systems, passage on the size exclusion column returns the levels back to acceptable values which are below the limit of quantification. That suggests that the ability of size exclusion columns to remove residual solvent holds true for various drug delivery systems.

The Dialysis of Water-Miscible Acetonitrile Is more Efficient than that of Chloroform

Dialysis relies on diffusion through a semi-permeable membrane to separate solutes with different hydrodynamic radii and molecular weight. This procedure is often used to purify colloids and large molecules from small impurities; it was therefore attractive to investigate its efficiency in removing residual solvent.

Again, to facilitate reproducibility, lipid nanocapsules prepared without solvent were supplemented with small quantities of acetonitrile and chloroform. Figure 5 depicts important differences in the speed at which acetonitrile and chloroform diffuse out of the formulation, despite similar initial concentrations of both solvents. Acetonitrile reached concentrations <410 ppm after 1 h (the first sampling point) and was undetectable in all samples thereafter. In contrast, chloroform took as long as 8 h to near acceptable limits, at which time only 1 out of 3 samples had a concentration < 60 ppm (others were 68 and 63 ppm). After 24 h of dialysis, 2 out of 3 samples had chloroform below the LLOQ. This difference of behavior between solvents can be explained by their distinct water solubility, as well as possible differences in their inherent partitioning within the lipid nanocapsules.
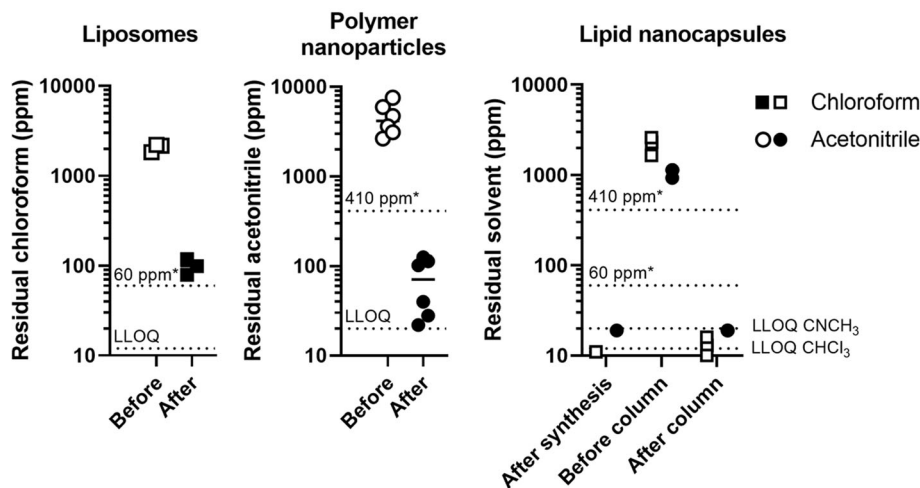
## a. Size exclusion chromatography adequately separates drug delivery systems
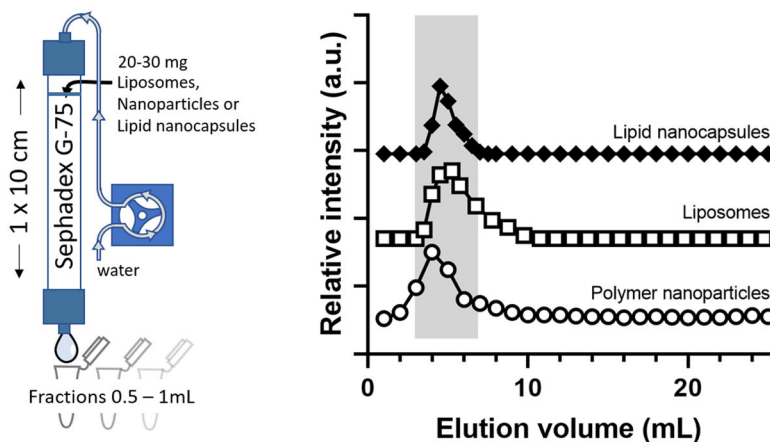
b. Size exclusion chromatography can remove water-miscible and immiscible solvents

While these results support that dialysis can remove residual solvent from formulations, it highlights that the speed of the process might differ between contaminants. The kinetics at which delivery systems release their drug content, as well as inherent susceptibility to hydrolysis might determine whether extensive dialysis remains compatible with the final use of the formulation.
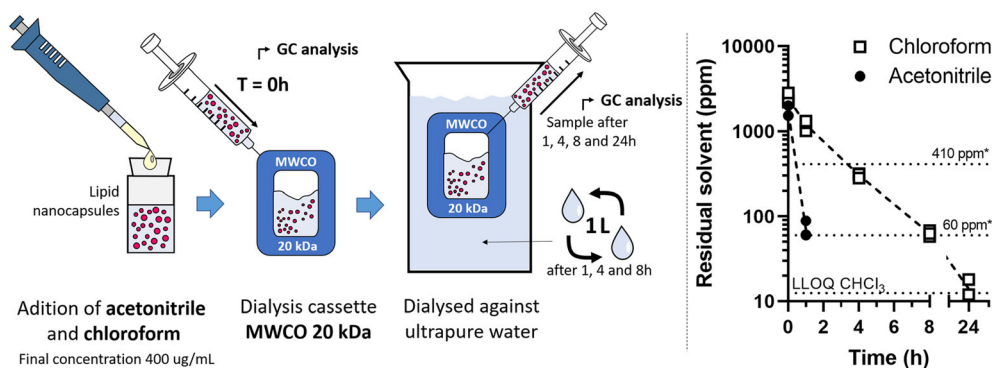
Fig. 5 Dialysis efficiently removes acetonitrile from formulations, while diffusion of chloroform is slower. Using lipid nanocapsules supplemented with both acetonitrile and chloroform, differences between the behavior of both solvents were appreciated. Each data point represents one single replicate (n = 3), * is the ICH Q3C(R6) acceptable concentration for chloroform and acetonitrile, LLOQ is the lower limit of quantification of the HS-GC method.

Concluding Remarks

This work highlights how the preparation of common types of drug delivery systems can result in significant levels of residual solvent if adequate precautions are not taken. The drying procedure necessary to evaporate chloroform from lipid films and the number of washes required to remove acetonitrile from polymer nanoparticles are more strenuous than usual purification procedures used in our laboratory. Important notions on the possible cross-contamination of samples were also evidenced. Although this study focuses specifically on limited numbers of Class 2 solvents and delivery systems, we believe the findings are more generally applicable to other lipid-based and colloidal systems. This work will therefore be significant to refine our protocols, and hopefully help other laboratories adapt their own.

Despite that solvent concentrations detected in some of these experiments exceed the limits required for regulatory approval of drug products, they remain very low. Herein, ppm concentrations are relative to the quantity of nanoparticles. During the preclinical development of nanomedicines, most punctual experiments (e.g., the dosing of animals to study the pharmacokinetics or pharmacodynamics) involve quantities of nanoparticles rarely exceeding the milligram scale. In this scenario, animals would be exposed to very minute quantities of solvent, well below the doses expected to cause acute toxicities. In contrast, more uncertainty could surround the effects of residual solvents in long-term or repeated dosing studies. Additional precautions might therefore be necessary to prepare solvent-free systems in the context of such investigations.

Finally, while the present results cannot replace thorough optimization and validation of synthetic procedures, they highlight general recommendations that can help prepare more reliable drug delivery systems and streamline industrial manufacturing. First, because the removal of solvent is a strenuous process, even at the small laboratory scale, solvent-free preparation methods or the use of Class 3 solvents should be privileged when possible. Second, to avoid the contamination of cleaner samples, dedicated or disposable measuring tools should be used when dispensing solvents. Third, rotary evaporation should be combined with other drying methods to compensate for any solvent which would have been re-introduced upon returning to atmospheric pressure. Fourth, relying on multiple purification procedures when possible will afford lower residual solvents in final formulations, when high degree of purity is required.

Examining current procedures with these aspect in mind might provide researchers with an opportunity to better understand their current protocols and the properties of their drug delivery systems.

AUTHOR CONTRIBUTIONS

NB designed the experiments. AD, FM, KG, CQ, PA and NB conducted the experiments and analyzed the results. NB prepared the figures and wrote the manuscript. GB, IR, NB contributed methods, funding and/or infrastructures. All authors read and commented the manuscript.

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