

Continuous Suspension of Lipids in Oil by the Selective Removal of Chloroform via Microfluidic Membrane Separation

Simon Berger, Joanna Stawikowska, Dirk van Swaay, and Andrew deMello*

Department of Chemistry and Applied Biosciences, Institute of Chemical and Bioengineering, ETH Zürich, Wolfgang Pauli Strasse 10, 8093 Zürich, Switzerland

S Supporting Information

ABSTRACT: A continuous flow method for the suspension of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) lipids in oil using a microfluidic platform is presented. The system consists of a microfluidic device housing a semipermeable membrane, a vacuum pump, and a syringe pump. Separation is achieved using a counter current flow of chloroform and a lipid containing oil stream, driven by the syringe pump and vacuum. Using such a system, a high efficiency extraction method was realized through the use of a semipermeable polydimethylsiloxane (PDMS) membrane on an anodized aluminum oxide (AAO) support. For a liquid flow rate of 5 $\mu\text{L}/\text{min}$, an air flow rate of $\sim 100 \text{ mL}/\text{min}$, and initial chloroform concentrations between 0.245 and 1.619 M, extraction rates of 93.5% to 97.9% and a retentate stream purity of between 99.79% and 99.29% were achieved.

INTRODUCTION

Lipid molecules are a fundamental component of cell membranes and a range of other cellular structures.^{1–3} As such, their use in forming model membrane structures such as micelles, liposomes, supported lipid bilayers, and droplet interface bilayers has greatly enhanced our understanding of the functions and properties of living cells.^{4–7} Commercially available lipids are commonly dissolved in chloroform or provided in dry form (which must be dissolved in chloroform or another solvent before use). Unfortunately, for most applications, lipid molecules must be suspended in an aqueous or oleic medium prior to the formation of the desired structure.⁸ Accordingly, a variety of different protocols has been established to perform medium transfer, with particular attention being paid to solvent removal. Solvent removal is typically achieved by evaporating the chloroform solvent under vacuum for several hours. This generates a dry film of lipids at the bottom of the holding vessel, which is subsequently resuspended into the desired medium for use.^{9–12} Due to solubility differences, it is considerably more difficult and time-consuming to resuspend dry lipids in an oil rather than aqueous medium. Depending on the lipid and oil combination, this may be done by stirring, heating, or sonication over a period of several hours.^{11,13,14} While the first step guarantees complete removal of any traces of chloroform, the second step can cause appreciable damage to the lipid molecules, since large amounts of energy must be applied.^{15–18} Additionally, since each step may take several hours (depending on the lipid and oil in question), the entire process represents a bottleneck in many experimental procedures and, more significantly, a source of error due to the varying state of the lipids extracted and the varying concentration of intact lipids obtained.¹⁸

A direct solution to this problem is to adopt a selective separation method that is able to remove chloroform from oil and can be performed in continuous flow at room temperature. Membrane separation methods have long been used in large-scale industrial applications for the selective and continuous

removal of one or more components from both gas and liquid phase mixtures. In most situations, the membrane allows for selective removal of components from a mixture based on differential diffusion rates through the membrane material. In simple terms, membranes are categorized as being either *porous* or *dense*, with each offering specific advantages with respect to throughput, selectivity, or operational lifetime. In order to facilitate separation, a range of driving forces can be applied across the membrane. These include concentration gradients, pressure gradients, temperature gradients, gravity, electrostatic potential, or combinations therein.^{19,20}

In recent years, membrane separations have been successfully transferred to chip-based microfluidic formats,²¹ with applications primarily focused on sample purification or concentration prior to downstream analysis. In general terms, microfluidic systems offer numerous advantages over conventional instrumental platforms. These include faster processing times, superior analytical performance, reduced sample/reagent consumption, minimized instrument footprint, enhanced portability, and facile monolithic integration of functional components.^{22–31} In the current context, in addition to the facile handling of small sample volumes, a key advantage offered by microfluidic methods for membrane separation is the dramatic reduction in diffusional length scales, which essentially eliminates bulk diffusion and limits the system to film and membrane diffusion, thus enhancing the efficiency of the separation process.^{19,20} Unfortunately, the integration of membranes into microfluidic devices comes with a number of challenges. First the membrane must be integrated within the chip-based structure, either over the entire chip surface or in a spatially localized region. Regardless of the approach taken,

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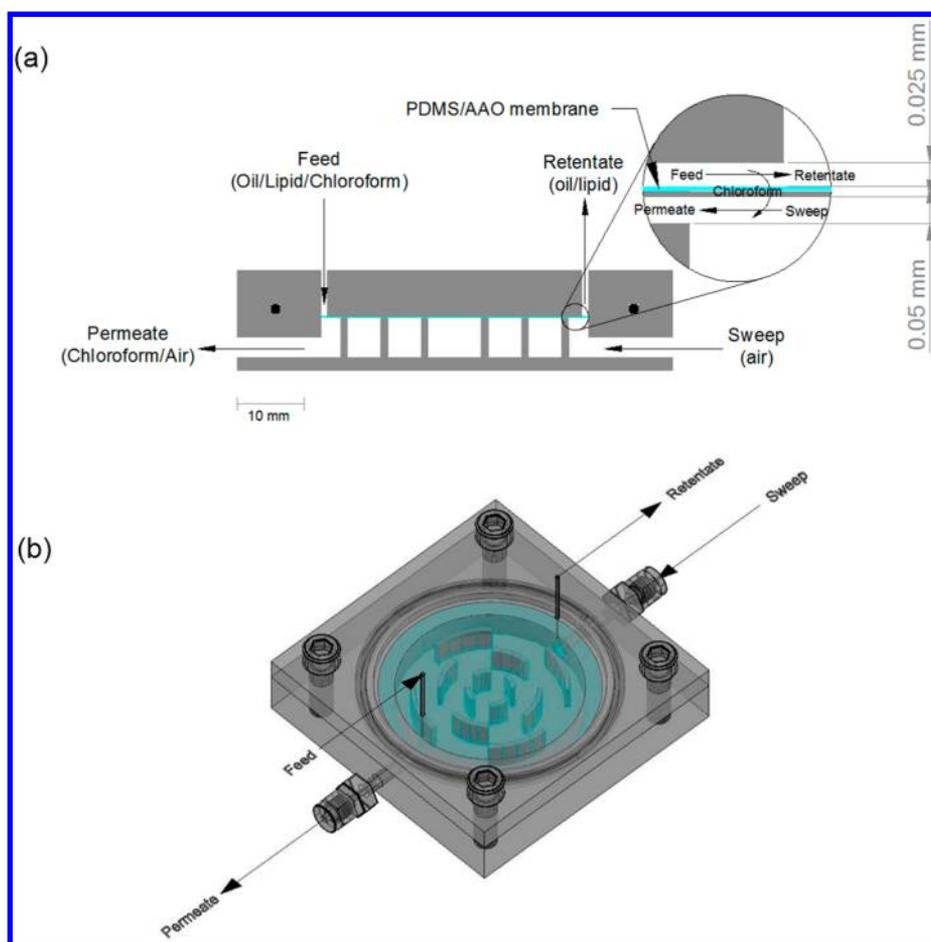


Figure 1. Schematic of the membrane separation device: (a) Cross sectional view of the membrane separation device. The top half rests on the bottom half and is separated by the PDMS/AAO membrane (Turquoise). A liquid mixture of oil, lipid, and chloroform is fed through the “Feed” port into the 25 μm high liquid flow chamber. The 50 μm gap between the support structure and the membrane reduces the risk of deformation of the support into the membrane. (b) Assembled membrane separation device.

leakage around the membrane may occur, resulting in appreciable analyte loss and a significant reduction in filtration efficiency.³² Second, due to the small size of typical microfluidic channels, only a limited surface area of the membrane may be available for the separation process. Finally, it is often challenging to ensure membrane stability within the device, since commonly used membranes (such as aluminum oxide or titanium oxide) are relatively brittle and need a rigid support. Indeed, this is a particularly demanding issue when working with elastomeric-based microfluidic devices.²¹ To this end, we demonstrate herein a microfluidic platform for the continuous suspension of lipids in oil by the continuous extraction of the initial solvent via separation across a polydimethylsiloxane (PDMS)/anodized aluminum oxide (AAO) membrane. Extraction is performed after the solvent, and lipid mixture is added directly to the oil at the desired concentration and without prior desiccation. Stable separation of chloroform from the oil stream is then realized by a counter current flow of liquid and air across the membrane.

EXPERIMENTAL SECTION

Membrane Selection and Fabrication. A two component membrane, consisting of a 50 μm thick PDMS elastomer layer (Sylgard 184 Silicone Elastomer Kit, Dow Corning, mixed at a 5:1 ratio) and a 60 μm thick, 47 mm diameter anodized aluminum oxide support (Whatman, Anopore Inorganic

Membranes, Anodisc 47, GE Healthcare), was used for separation. The support was chosen due to its rigidity and highly porous structure (having an average pore diameter of 200 nm) which provides a stable yet permeable base for the elastomeric membrane. PDMS was chosen as the membrane material due to its high permeability for organic solvents (such as chloroform)^{33–36} and relative low permeability for viscous oils (such as mineral oil). It should also be noted that both PDMS and AAO have in general been shown to exhibit high levels of biocompatibility when in contact with lipids and other biological components and are therefore unlikely to have any adverse effects on the lipids.

The two-component membrane was fabricated by spin-coating an untreated AAO membrane with PDMS at 1500 rpm for 100 s, resulting in a flat, 50 μm thick PDMS layer. A prepolymer-to-curing agent ratio of 5:1 was chosen to provide for enhanced cross-linking and thus a higher resistance to physical stress and potential swelling (induced by chloroform). It should be noted that use of a 10:1 ratio of reagents yielded membranes that showed minimal separation efficiency (results not shown). This was attributed to the ease of membrane blockage caused by PDMS swelling within a confined volume (defined by the membrane separation chamber and support pores).

Microchip Design. The microfluidic chip was manufactured from stainless steel using a conventional computer

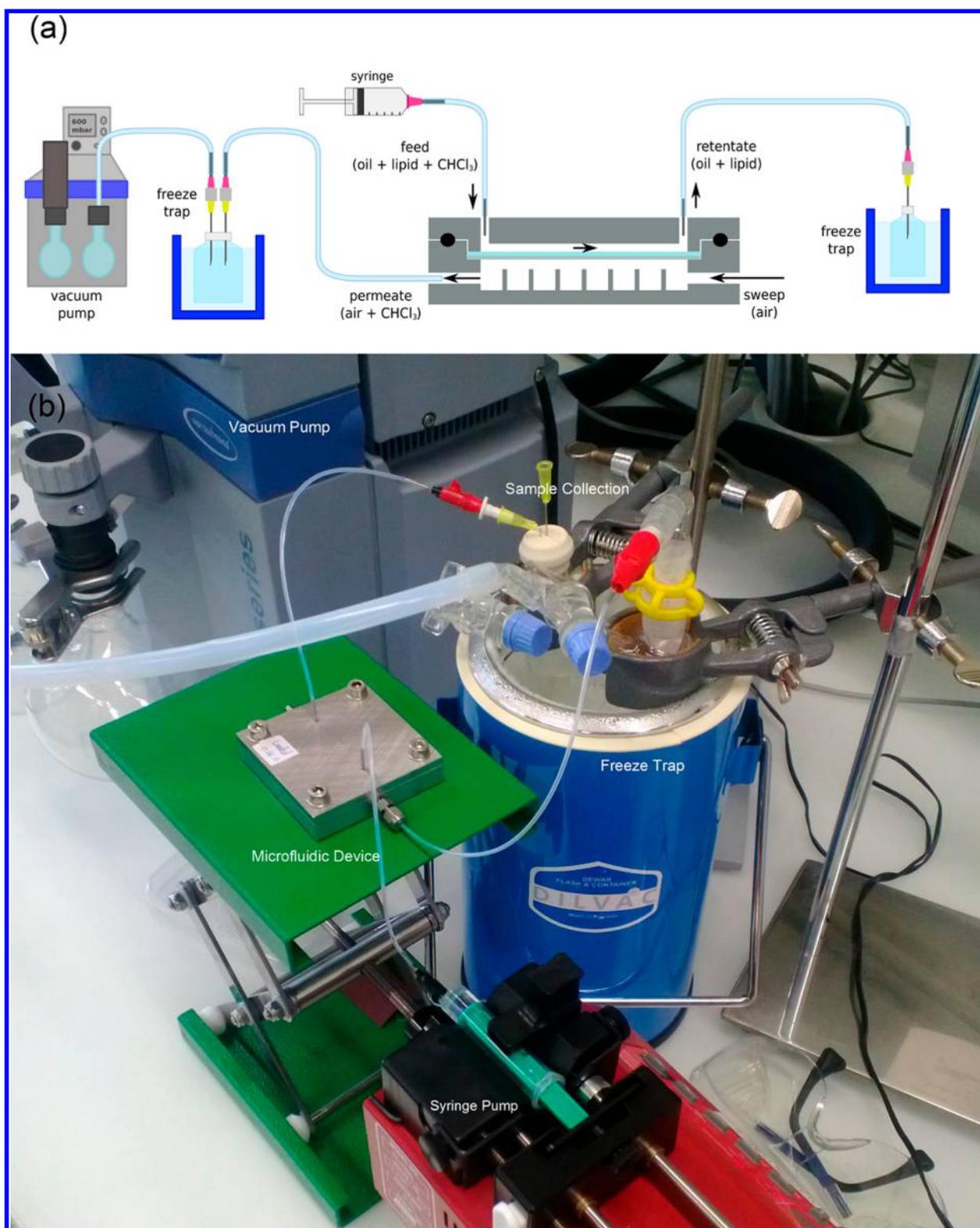


Figure 2. (a) Schematic representation of the complete system: A syringe pump feeds liquid into the separation device, a vacuum pump delivers air from the surroundings, and a freeze trap collects the sample and protects the pump from chloroform vapors. (b) Photograph of the system in operation.

numerical control (CNC) milling machine (DMU 50T Deckel Maho, DMG Mori Seiki AG). Stainless steel was chosen as the substrate material due to its high mechanical strength and durability and its high chemical resistance. The microfluidic device consists of two halves, as shown in Figure 1. The top half

contains a circular liquid chamber having a height of 25 μm , a diameter of 40 mm, and a total exchange area of 12.57 cm^2 . This results in a liquid flow chamber volume of 31.4 μL . A circular chamber was chosen over a serpentine channel to use the entire surface of the membrane available, which otherwise

would be diminished by walls necessary for serpentine, but this results in a wider residence time distribution. An offset of 1.9 mm between the liquid chamber and the remaining part of the top half was incorporated to aid in membrane alignment and sealing of the membrane separation cell. Two stainless steel pins (1.5 cm long and 794 μm OD) were pressed into 780 μm diameter holes drilled into the steel cell, to provide for stable and long-term connections for input and output tubing. Liquid is pumped through PTFE tubing (EW-06407-41, 1.58 mm OD, 0.79 mm ID, Cole-Parmer).

The bottom half of the cell contains the gas flow chamber with a height of 6 mm, a diameter of 40 mm, and a total volume of 7.5 mL. In addition, the bottom chamber contains a concentric support structure that prevents membrane deformation and breakage. The bottom chamber was designed such that high air flow rates are achievable without introducing significant back pressures, which would have a potentially adverse effect on separation performance. The bottom half output (labeled permeate) was connected to a freeze trap via a 1/16 in. Swagelok and 1/16 in. FEP tubing. The freeze trap (a 100 mL volume Schlenk flask) was then connected to a vacuum pump using 6 mm OD FEP tubing, allowing for high air flows. The top and bottom halves were connected using four 4 mm screws, with the PDMS membrane acting as a gasket. The resulting cell has total external dimensions of 65 \times 65 \times 15 mm.

Setup Design. The entire membrane separation system consists of a vacuum pump (Vacuubrand PC 3004 Vario, Vacuubrand GmbH + Co. KG), a syringe pump (Aladin AL-1000, World Precision Instruments, Inc.), and the microfluidic device (Figure 2). The lipid, oil, and chloroform mixture was delivered at a total volumetric flow rate of 5 $\mu\text{L}/\text{min}$. Concurrently, air was drawn over the opposite side of the membrane by application of a negative pressure of \sim 400 mbar using the vacuum pump. Both to protect the vacuum pump and to conserve chloroform extracted from the sample for later analysis, a dry ice/ethanol cooling trap was installed between the vacuum pump and the microfluidic device. All experiments were carried out at room temperature.

Sample Preparation. Samples were prepared by gently mixing DOPC in chloroform (1,2-dioleoyl-*sn*-glycero-3-phosphocholine, Avanti Polar Lipids Inc., 25 mg/mL DOPC in CHCl_3) with mineral oil (Sigma Aldrich, Mineral oil BioReagent, for molecular biology, light oil) at the desired lipid concentration.

Analytical Method. Chloroform content in the retentate phase was measured using gas chromatography GC-ECD (Trace GC Ultra Gas Chromatograph, Thermo Electron Corporation equipped with a Combi Pal GC Autosampler, CTC Analytics GmbH) with a Restek Rxi-1 ms column (fused silica length 30 m, ID 0.32 mm, and d_f 4 μm , Restek Corporation). In order to protect the GC, samples were taken from the head space of the GC vials. An injection volume of 1 μL was diluted with 9 μL of air, and a split factor of 150 was applied. Analyses were performed at 40 $^\circ\text{C}$. 40 mg samples were collected online, diluted with 360 mg of pure mineral oil, immediately frozen at -72 $^\circ\text{C}$, sealed, and stored at -20 $^\circ\text{C}$. The material content of each sample was confirmed by weight.

Control Experiment. In order to verify that lipids remain in the retentate and do not permeate or adsorb onto the membrane during separation, 3 μL of fluorescently labeled lipids (18:1 PE CF 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(carboxyfluorescein) (ammonium salt), Avanti Polar Lipids Inc., 1 mg/mL in CHCl_3) was added to the usual test

mixture of DOPC, mineral oil, and chloroform. Fluorescence emission was assessed qualitatively using a microscope equipped with a fluorescence camera (Nikon Eclipse Ti-E Microscope equipped with a Hamamatsu C11440-22C Orca Flash 4.0 V2).

Standard Method. The microfluidic method was compared to samples prepared using a standard method. For this, a predefined amount of DOPC in chloroform was dried in a round-bottom flask, by flowing nitrogen over the flask and then placing the flask in a desiccator for 24 h. The dried lipid film was then resuspended in mineral oil by continuously stirring at 40 $^\circ\text{C}$ for an additional 12 h. Samples with the maximum chloroform concentration assessed were measured using GC-ECD.

RESULTS AND DISCUSSION

Initially, the robustness of the seals within the microfluidic chip was determined, by applying pressure to the chamber while sealing all but one connection. All seals were able to withstand 5 bar of pressure without a membrane and 500 mbar on the liquid side with a membrane installed. Further testing of the stability under negative pressure showed that the membrane is capable of withstanding -400 mbar over long periods of time (tests were carried out over a period of 2 months) without mechanical failure or a deterioration in separation performance.

After these pressure limits were established, optimal process conditions were determined. A liquid flow rate of 5 $\mu\text{L}/\text{min}$ was found to be optimal, in terms of separation performance and selectivity. This results in a residence time of 6.28 min, which is sufficient for efficient extraction of chloroform from the liquid stream (see GC results below). At higher flow rates, the residence time, and therefore the extraction performance, decreases. In addition, the increase in backpressure associated with an increase in flow rate leads to a higher loss of mineral oil through the membrane (\sim 15 wt % compared to \sim 5 wt % when operating under optimal process conditions). Oil loss increases by a similar margin when using lower flow rates, as the residence time goes up allowing a higher amount of oil to diffuse through the membrane. The negative pressure, and therefore the air flow rate of the permeate stream, was limited to -400 mbar as this afforded good separation performance without applying excessive stress on the membrane. A higher vacuum did not lead to a higher loss of mineral oil, suggesting that the main driving force for separation is the concentration gradient and not the pressure gradient across the membrane (since the bottom chamber is only slightly below ambient pressure). The primary driving forces for the presented process are concentration and pressure gradients across the membrane. The overall separation process can be considered to consist of three underlying mass transfer processes: bulk, film, and membrane diffusion. Due to the reduced height of the fluidic chamber, bulk diffusion is minimized, with only film and membrane diffusion being significant. In simple terms, film diffusion of chloroform can be represented, assuming a well-mixed steady state case following Fick's first law, as

$$j_i^f = -D_i \frac{\delta c_i}{\delta x} = D_i \frac{(c_i^0 - c_i^d)}{d} \left[\frac{M}{m^2 \cdot s} \right]$$

Here, D_i is the molecular diffusion coefficient, d is the film thickness, c_i^0 is the concentration at the membrane surface, and c_i^d is the concentration at the maximum of the liquid film. Membrane diffusion on the other hand may be dominated by

solution diffusion or pore diffusion, driven by the pressure and concentration gradient across the membrane. Concentration driven diffusion can be understood using Fick's law and the pressure driven contribution represented using Darcy's law, i.e.,

$$j_i^p = -\frac{k}{\mu} \frac{P^p - P^r}{L} \left[\frac{M}{m^2 \cdot s} \right]$$

where k is the intrinsic permeability of the membrane, L is the thickness of the membrane, P^p is the pressure on the permeate side, P^r the pressure on the retentate side, and μ is the dynamic viscosity of the medium. The overall flux across the membrane can therefore be written as

$$j_i^m = -D_i^m \frac{(c_i^0 - c_i^p)}{L} - D_i^p \frac{(P^p - P^r)}{L} \left[\frac{M}{m^2 \cdot s} \right]$$

Here, D_i^m is the diffusion coefficient for the membrane, D_i^p is the diffusion coefficient for diffusion following Darcy's law, c_i^0 is the concentration at the membrane surface, and c_i^p is the concentration at the permeate side. Further simplifications can be made by assuming c_i^p is zero due to the fast removal of any substance permeating through the membrane. Using these equations, it is evident that the amount of chloroform and mineral oil permeating through the membrane depends on two factors: (1) the driving force applied and (2) the residence time within the membrane separation device. Equilibration of these two factors ensures that diffusion of the mineral oil is kept to a minimum and diffusion of chloroform maximized.

Control experiments using fluorescent lipids provide confirmation that lipids do not permeate through the membrane material, as no fluorescence could be detected in the chloroform collected from the permeate stream. A quantitative assessment of time-integrated fluorescence intensities originating from the permeate could not be determined due to photobleaching and a variation in fluorescence caused by solvent effects; however, data indicate that the vast majority of lipids remain in the retentate stream. Minor losses of lipid can be attributed to oil permeating through the membrane and adhesion to the membrane or separation cell itself.

Gas chromatographic analysis of the samples collected showed an extraction rate of between 93.5% and 97.9% and a purity of the oil-lipid mixture of between 99.29% and 99.79% (Table 1). As expected, the lowest extraction rates were obtained for the samples with the lowest initial chloroform content, as in these the driving force for the transfer of chloroform from the feed to the permeate side is lowest. The highest extraction rates were achieved for the samples with the highest chloroform content. Inversely, the highest purity of the retentate stream was achieved for low initial chloroform concentrations. This is due to limitations in driving force and residence time which limit the total amount of chloroform extracted.

Variations in extraction rate and purity for a fixed initial chloroform concentration are sometimes observed. Several factors are likely responsible for such variations. First, due to the volatility of chloroform, such evaporation of chloroform is inevitable. Evaporation was minimized by keeping all samples frozen for as long as possible. A second factor is related to the sampling method. Samples were collected online by allowing the retentate to drip into a 2 mL GC vial which was cooled down to -72 °C. The sampling time was estimated on the basis of the retentate flow rate, and samples of 40 mg were collected.

Table 1. Summary of the Measured Chloroform Concentrations, Extraction Rates, and Retentate Purities Obtained, with the Respective Standard Deviations (Std)

initial CHCl ₃ conc. [M]	measured CHCl ₃ conc. [M]	std. [M]	extraction rate [%]	std. [%]	oil purity [%]	std. [%]
0.243	0.014	0.011	93.696	0.050	99.795	0.002
0.362	0.015	0.038	95.583	0.114	99.783	0.005
0.477	0.016	0.107	96.425	0.240	99.766	0.015
0.591	0.021	0.052	96.366	0.094	99.706	0.007
0.703	0.024	0.245	96.441	0.375	99.658	0.035
0.812	0.028	0.083	96.434	0.109	99.604	0.012
0.919	0.028	0.153	96.915	0.177	99.610	0.022
1.025	0.034	0.881	96.520	0.911	99.513	0.124
1.128	0.045	0.179	95.870	0.172	99.367	0.025
1.230	0.050	0.372	95.755	0.329	99.293	0.052
1.330	0.047	0.470	96.364	0.381	99.341	0.066
1.428	0.037	0.307	97.306	0.227	99.470	0.043
1.524	0.045	1.420	96.921	0.986	99.359	0.200
1.619	0.033	0.522	97.902	0.336	99.529	0.074

The sample size was chosen so as not to oversaturate the ECD (electron capture detector) which is limited by its narrow linear range. As the retentate flow rate varied due to small fluctuation in the supply pumps, the weight of all samples was checked afterward. This limits the sampling precision to the precision of the scale.

Comparison with the standard method showed no chloroform remaining in the samples prepared via the standard method, defining an extraction rate and purity of 100%. This however is achieved at the cost of time and lipid quality, which is most likely affected by the additional energy input needed for suspension of the lipids in the mineral oil.

CONCLUSIONS

A practical continuous flow method for the suspension of DOPC in mineral oil was developed. The method showed excellent separation performance over a wide range of chloroform/lipid concentrations delivering retentate streams of 99%+ purity with respect to mineral oil content. The method was highly suited to the simple preparation of oil-lipid mixtures or the direct incorporation of the membrane separation device with other microfluidic components. Compared to the standard method of suspending lipids in oil, by evaporation of chloroform under vacuum and resuspending the dry lipid film in oil, the method presented in this work allows for the continuous suspension of lipids in oil rather than the batch wise preparation of each sample, allowing for the use of the lipids within 30 min compared to several hours or days required in the standard method, while not affecting the quality of the lipids. Further improvement of the separation performance can be achieved by adjusting the porosity of the membrane material and enlarging the membrane surface available. In the near future, further tests with a variation of lipids and oils will be carried out to assess the universal applicability of the method presented. In addition, the state of lipids postcollection will be analyzed using LCMS, to quantify lipid loss and chemical changes induced by the separation process.

■ ASSOCIATED CONTENT

● Supporting Information

Variation of the purity of the mineral oil-lipid mixture and extraction rate collected as a function of the initial chloroform concentration. This material is available free of charge via the Internet at <http://pubs.acs.org>.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: andrew.demello@chem.ethz.ch.

Notes

The authors declare no competing financial interest.

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