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# FORMATION AND PURIFCATION OF TAILORED LIPOSOMES FOR DRUG DELIVERY USING A MODULE-BASED MICRO CONTINUOUS-FOW SYSTEM

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**ABSTRACT:**

Liposomes are lipid based bilayer vesicles that can encapsulate, deliver and release low-soluble drugs and small molecules to a specifc target site in the body. They are currently exploited in several nanomedicine formulations. However, their development and application is still limited by expensive and time-consuming process development and production methods. Therefore, to exploit these systems more efectively and support the rapid translation of new liposomal nanomedicines from bench to bedside, new cost-efective and scalable production methods are needed. We present a continuous process fow system for the preparation, modifcation and purifcation of liposomes which ofers lab-onchip scale production. The system was evaluated for a range of small vesicles (below 300nm) varying in lipid composition, size and charge; it ofers efective and rapid nanomedicine purifcation with high lipid recovery (>98%) combined with efective removal of non-entrapped drug (propofol >95% reduction of non-entrapped drug present) or protein (ovalbumin >90% reduction of OVA present) and organic solvent (ethanol >95% reduction) in less than 4minutes. The key advantages of using this bench-top, rapid, process development tool are the fexible operating conditions, interchangeable membranes and scalable high-throughput yields, thereby ofering simultaneous manufacturing and purifcation of nanoparticles with tailored surface attributes

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## INTRODUCTION:

Liposomes are a well-established formulation strategy to improve drug delivery and enhance therapeutic outcomes for a range of drugs, such as pharmaceuticals, biopharmaceuticals, and vaccines. Due to their bilayer vesicle structure, which is akin to natural cells, liposomes are able to incorporate drugs both within their aqueous core and their lipidic bilayers. Trough such means, the pharmacokinetics of a drug can be controlled and dictated by the liposomal delivery system rather than the drug attributes. Tis has allowed the development of a range of clinically approved liposome-based medicines including DOXIL/Caelyx® (doxorubicin), AmBisome® (amphotericin B) and Daunoxome® (daunorubicin), which when combined have an annual market revenue of approximately $100 million. However, despite these advantages, their wider application is limited by their complex and costly production requirements. Currently, manufacturing methods include the use of solvent injection, reverse-phase evaporation and emulsifcation methods1 . Such methods have the disadvantage of involving multi-step processes, ofen adopt large amounts of organic solvents and are limited to batch-release processes. Furthermore, a crucial attribute to an efective liposomal drug system is the vesicle size range, which can be controlled the production method, e.g. sonication (20–40 nm)2 , extrusion (70–415 nm)3 or high-pressure homogenisation (20–140

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nm)4 ; and, more recently, microfuidic mixing (20–80nm)5,6 or fow focusing (50–150nm)7 . Upon administration, the pharmacokinetic profle and fate of liposomes is dictated by their size and therefore controlling particle size and polydispersity (PDI) is a key issue in their manufacturing and a key parameter in the product specifcations. To produce liposomes in a controlled size range, downsizing through extrusion or homogenisation is ofen adopted. Tis adds further steps to the manufacturing process and exposes the liposomes and drug constituents to harsh

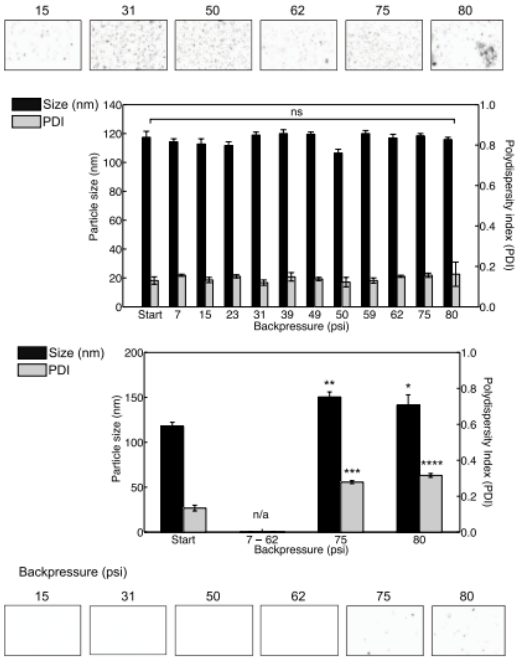


Figure 1. Particle size and polydispersity as a function of increasing backpressures in the TFF system as collected on the retentate side of the membrane. Images from NTA analysis, verifying particles in permeate (top) and retentate (bottom) stream at increasing backpressures. Particles were found in the permeate at backpressures exceeding 75psi. All experimental datasets are presented as mean and standard deviation (mean±s.d.) resulting from three independent runs (n=3).

and potentially detrimental processing conditions. To address these issues, and allow the wider adoption of liposomal systems to improve health-care, new methods in liposome manufacture are therefore required.

Microfuidic devices operate with small volumes, ofer exquisite control over the fuid fow8,9 , and make efcient use of materials, reagents and energy10. Tese advantages have been applied for the reproducible formation of liposomes with uniform size distribution1,6,11. Typically, liposome formation occurs at the interface of an aqueous and a solvent phase, containing lipid molecules12, and microfuidic devices are well suited to establish and fnely control such interfaces. In hydrodynamic fow focusing (HFF) devices, for example, where the solvent phase is microinjected in between two co-fows of aqueous bufer, liposomes with well-controlled size distributions can be assembled 6,13. Furthermore, by changing the ratio between the fow rates of the aqueous bufer and

| Lipid | Application | Reference |
|---|---|---|
| Dimethyldioctadecyl ammonium bromide(DDA) | Vaccine adjuvant,cationic head group, uptake of vaccine antigens to antigen presenting cells | Smith Korsholm *et al.*[34] Christensen *et al.*[35] |
| Trehalose 6,6-dibehenate(TDB) | Synthetic immunstimmulator derived from the membrane of mycobacterium | |
| 1,2-dioleoyl-sn-glycero-3-phsphoethanolamine(DOPE) | Fusogenic helper lipid, available in the commercial Lipofectin™ transfection reagent | Henriksen-Lacey *et al.*[36] |
| 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) | Cationic lipid often used in transfection | |
| Egg Phosphatidylcholine(PC) | Neutral head group, drug delivery | Senior and Gregoriadis[37] Gregoriadis and Senior[38] |
| 1,2-Dipalmitoyl-sn-glycero-3-phospho-rac-(1-glycerol)(DPPG) | Negative charged head group, drug delivery | Oku *et al.*[39] Kirby *et al.*[40] |
| 1,2- Dipalmitoyl-*sn*-glycero-3-phosphocholine(DPPC) | Neutral head group, drug delivery | |
| Cholesterol (Chol) | Added for membrane stabilization, known to effect drug encapsulation efficiency in bilayer and aqueous core | Senior and Gregoriadis[37] Kirby *et al.*[40] |

Table 1. Lipids investigated in this study.

| Backpressure (psi) | 7 | 15 | 23 | 31 | 39 | 49 | 50 | 59 | 62 | 75 | 80 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Flow rate (mL $min^{-1}$) | 0.01 | 0.02 | 0.03 | 0.1 | 0.05 | 1 | 0.3 | 2 | 2.5 | 0.5 | 0.1 |
| Capillary I.D. (μm) | 50 | 50 | 50 | 63 | 50 | 100 | 63 | 100 | 100 | 63 | 50 |
| Capillary length (mm) | 50 | 50 | 50 | 50 | 50 | 50 | 25 | 30 | 25 | 25 | 50 |

Table 2. Backpressures and fow rates through the Tangential Flow Filter (TFF) that were investigated in this study. Liposomes in solution were fed into the TFF device at fow rates ranging between 0.01 and 2.5mLmin−1 . Backpressure was attained by connecting a restrictive

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capillary with selected (I.D.) and/or length on the retentate side of the TFF outlet.

organic phase, the concentration of lipid molecules in the organic phase, or by adapting the channel geometry, the size of the liposome can be fnely prescribed14,15. Scaling up of HFF devices, however, is difcult which constrains the amount of liposomes that can be produced per unit time16. In contrast, devices based on chaotic advection micromixing11,17 are more suitable for high throughput production of liposomes. Lipid nanoparticles with sizes between 20 and 50 nm were reported using a staggered herringbone mixer (SMH) by varying triglyceride ratios5 . More recently, Kastner et al. used the same SMH to prepare liposomes encapsulating propofol18, a poorly water-soluble drug. Tese works demonstrate the potential application of microfuidics for the rapid, reproducible and size-controlled formation of drug-loaded liposomes

Purifcation remains a signifcant hurdle in the development of liposomal products. Irrespective of which production method is adopted, non-entrapped contaminant molecules, small molecule drugs or proteins must be removed from the fnal liposome product. Separation is typically achieved by fltration19,20 or ultra-centrifugation, which can be challenging for the large-scale purifcation. Other possible routes for removal of non-encapsulated material include dialysis, gel-permeation chromatography, ion-exchange chromatography, and size exclusion chromatography. However, these processes are time-intensive and can furthermore diminish product yield by column equilibration, which dilutes the fnal liposomal product, even with size exclusion chromatography.

To address these issues of post assembly refnement, here we investigate a ‘lab-on-chip’ module-based microfuidic manufacturing and purifcation system for the production of liposomes. In contrast to previously reported on-chip devices by other groups and by us5,18,22,23, where lengthy dialysis procedures for removal of non-entrapped drug and solvent residues were required, we present a novel continuous microfuidic liposome production and purifcation process train which generates purifed liposomal products in less than 4 minutes. Furthermore, the purifcation step is based on a tangential fow-fltration device with an easily exchangeable membrane, allowing therefore the purifcation of a broad variety of liposome formulations. Tis robust process train facilitates the identifcation of prospective formulations, optimal operating conditions and scale-up parameters, whilst signifcantly reducing the time required for developing versatile adjuvant and drug delivering systems.

## RESULTS

In order to obtain a continuous microfuidic liposome production and purifcation process train, we frst characterised a tangential fow fltration (TFF) device24 and its purifcation capabilities for a variety of liposome formulations using lipids as outlined in Table 1.

**On-chip purifcation of liposomes for determination of the range of operational transmembrane pressures.** To identify the operational backpressure which yields the maximal recovery, we introduced in to the TFF device a commonly used formulation of neutral liposomes (PC:Chol; 1:1 molar ratio; Table 1). By varying the fow rates, and by implementing capillaries with diferent inner diameters and lengths downstream of

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the TFF device, we investigated backpressures from 7 to 80 psi (Table 2). To assess the liposome retention on the retentate side of the membrane (the volume of the liquid, which does not pass through the membrane), we

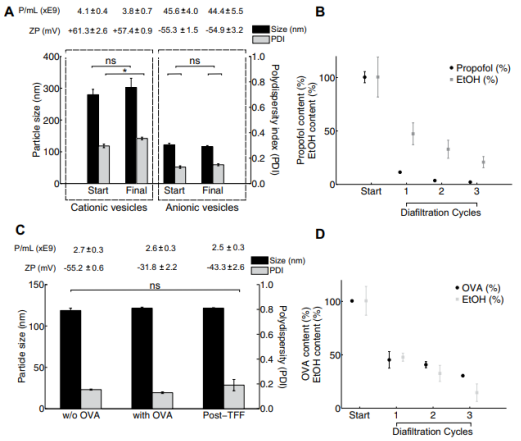


Figure 2. (A) Vesicle size, polydispersity (PDI), zeta potential (ZP) and particle concentration (P/mL) for cationic (DDA:TDB) and anionic (DPPG:DPPC:Chol) liposomes before and afer the TFF purifcation. (B) Images from NTA show vesicles present in the retentate side only. (C) Propofol and ethanol removal achieved over three diafltration cycles for anionic liposomes (DPPG:DPPC:Chol), expressed as a percentage of the initial amount of contaminants present.

collected samples from both the retentate and permeate (the volume which passes through the membrane), and measured the liposomal size and polydispersity index (PDI) at each of the backpressure conditions. Te results (Fig. 1) show that there was no signifcant change in the size (approximately 115nm) and PDI (0.15) of the liposome suspension in the retentate across the pressure range tested. However, at backpressures of 75 psi and 80psi, particles were detected in the permeate as confrmed by qualitative image-based nanoparticle tracking analysis NTA (Fig. 1). To confrm membrane integrity and exclude membrane damage as a possible cause for the liposome transferred into permeate, a leak-test applying pressures higher than 80psi was run and confrmed the membrane was intact. Tis suggests that at pressures above 75psi, the TFF device was unable to retain liposomes and these were being pushed across the membrane into the retentate.

**The efect of fltration on particle characteristics of cationic and anionic liposomes.** Afer proving that neutral PC:Chol liposomes were retained, the application of the TFF to purify cationic (DDA:TDB; 8:1 molar ratio; Table 1) and anionic liposomes (DPPC:Chol:DPPG; 4:4:1 molar ratio; Table 1) was assessed (Fig. 2). Te TFF device was frst challenged with a batch-formulated cationic liposomal adjuvant (DDA:TDB) in three diafltration cycles with bufer replenishment afer each cycle, compensating for the volume of liquid passing into permeate. At a fow rate of 1 mL min−1 , the backpressure was 49 psi (capillary length 50 mm, and I.D. 100 µm, Table 2), yielding a calculated fow rate of water through the cellulose membrane, Qtransmemb, of 0.25 mL·min−1 (based on a linear extrapolation from supplier data; 16mLmin−1 cm−2 for 14.5psi).

With the cationic liposomes, the particle concentration of liposomes was 4.1×109P/mL, which reduced to 3.8×109P/mL at the end of the third cycle (Fig. 2A) confrming the yield from the fltration process was 93% for the cationic liposomes. With these systems the liposomal size (approximately 300 nm) and cationic nature (approximately 60mV) were not notably infuenced by the fltration process (Fig. 2A). Furthermore, NTA analysis showed that there were no

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cationic liposomes detected in the permeate.

Similar results were demonstrated with anionic liposomes; fltration of batch-formulated anionic liposomes (DPPC:Chol:DPPG) using three diafltration cycles produced no notable changes in terms of vesicle size (approximately 120nm), PDI (0.14 to 0.15), ZP (−55mV) and particle concentration (4.4 to 4.6×1010P/mL) (Fig. 2). As

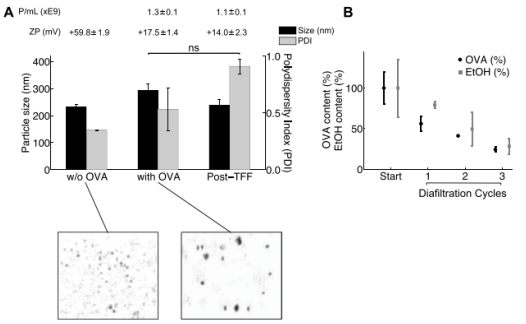


Figure 3. Vesicle size, polydispersity (PDI), zeta potential (ZP) and particle concentration (P/mL) for (A) anionic liposomes (DPPG:DPPC:Chol) and (B) cationic liposomes (DDA:TDB) prior and post OVA-addition (ovalbumin, 100µgmL−1 ), and particle characteristics afer the TFF purifcation. Protein (ovalbumin) and ethanol removal achieved over three diafltration cycles for (C) anionic and (D) cationic liposomes, expressed as a percentage of the initial amount of contaminants present. All experimental datasets are presented as mean and standard deviation (mean±s.d.) average of three independent runs (n=3).

with the neutral and cationic liposomes, NTA analysis in each diafltration cycle verifed that no liposomes were present in the permeate.

**Purifcation of non-incorporated drugs from liposome formulations.** Having successfully demonstrated the capability of the TFF to retain a wide range of diferent liposome systems, we then focused on investigating the efciency of the TFF to purify the liposomal nanomedicines and remove non-incorporated drug (RC membrane, 10 kDa cutof). To study drug removal, propofol (1 mg mL−1 ) was added to a suspension of negatively charged liposomes (DPPC:Chol:DPPG) in aqueous solution containing 20% (v/v) ethanol residual solvent levels found afer liposome production by microfuidics prior to purifcation. Propofol, was employed as it has previously been studied as a low-solubility drug solubilised within liposomes. Te TFF was shown to efectively purify the liposomes by removing both the solvent and non-incorporated drug with 90% of the non-incorporated propofol being removed in the frst diafltration cycle, with a further removal of 80% in the second diafltration run, and a further 60% afer the third diafltration cycle (Fig. 2B). Tus, afer three cycles only 1% of the ‘free’ non-incorporated drug remained within the formulation (Fig. 2B). Simultaneously, the TFF system removed the ethanol which had been used for the liposome formulation (Fig. 2B). Ethanol was reduced by approximately 50% in the frst diafltration cycle, and afer three diafltration cycles the residual ethanol concentration was 3% (v/v) (Fig. 2B).

**Purification of ‘free’ protein from liposome formulations.** To investigate the removal of non-entrapped protein from liposome formulations, both cationic (DDA:TDB) and anionic liposomes (DPPC:Chol:DPPG) were considered given that electrostatic interactions between cationic liposomes and anionic proteins is exploited in the loading of antigens to liposomal adjuvant systems. Terefore both liposome systems were mixed with ovalbumin (OVA; 100µgmL−1

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). At a fow rate for the retentate of 2.5mLmin−1 , the backpressure was 62psi (capillary length 25 mm, and I.D. 100µm, Table 2), yielding a calculated fow rate of water through the PES membrane (MWCO 300 kDa), Qtransmemb, of 1.69 mL·min−1 (based on a linear extrapolation from supplier data; 58mLmin−1 cm−2 for 10psi). Tus, from 2.5mL initial sample only 0.81mL remain in the retentate fraction, and 1.69 mL pass through the membrane. Te theoretical volume of permeate accounted for 67% of the initial liquid.

Given the anionic nature of OVA at the pH range used, electrostatic interactions with the cationic but not the anionic liposomes occurred. Indeed, anionic liposomes maintained a similar size (approximately 120nm) afer the addition of OVA (Fig. 2C); however, for the cationic liposomes, the electrostatic interactions with the anionic OVA resulted in aggregation and in an increased vesicle size from around 220nm to 300nm, and a drop in their cationic nature from 59.8±1.9mV to 17.5±1.4mV (Fig. 3A).

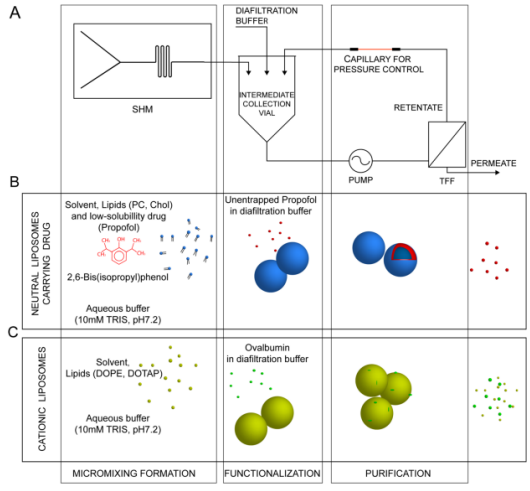


Figure 4. (A) Schematic overview of the module-based microfuidic system. Liposomes were manufactured with a Staggered Herringbone Mixer (SHM) upstream and fowed through the Tangential Flow Filtration (TFF) device for consecutive purifcation. (B) Schematic overview of the formation of liposomes loaded with a lowsolubility model drug, i.e. propofol. Vesicle assembly and drug loading are performed with a SHM, and nonentrapped (free) drug is removed from the mixture by consecutive fltration inside the TFF system. (C) Schematic overview of the formation of liposomes loaded with a model protein, ovalbumin (OVA). Vesicle assembly is performed with a SHM, with post-assembly protein addition; non-entrapped (free) protein is removed by consecutive diafltration cycles inside the TFF system.

Afer the addition of protein, both systems were subjected to three diafltration cycles. Te size and PDI of both the anionic (Fig. 2C) and cationic (Fig. 3A) liposomes were not signifcantly altered through the course of the diafltration cycles and particle recovery was 87% and 96%, respectively. Protein (OVA) and residual ethanol was removed into the permeate stream over the three diafltration cycles, with a fnal removal of 70% of the free protein and 95% of the solvent with the anionic liposomes (Fig. 2D). Similar results were achieved with the cationic liposome systems (Fig. 3B); by using TFF purifcation, ethanol residues were reduced to 4% (v/v) of the starting value and 75% protein was removed (Fig. 3B) afer three diafltration cycles. Te reduced levels of protein removed from the cationic system were a result of protein loading onto the surface of the liposomes due to electrostatic interactions (but not related to the fltration process in the TFF).

**Micro continuous-flow system for production, modification and purification of liposomes.** Having established the efcacy of the TFF purifcation system, the next stage was to

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develop a continuous manufacturing and purifcation process for liposomes. To achieve this, a staggered herringbone micromixer (SHM) was employed to generate the liposomal systems and directly feed the TFF device with the liposomes. To optimise the throughput for each of the two devices separately, and to independently control the fow rates, an intermediate collection vial was used (Fig. 4). Furthermore, the intermediate collection vial allowed purifcation of the liposomes in diafltration mode. Afer each diafltration cycle, fresh bufer was added manually into the intermediate vial to compensate for the volume passing through the membrane into the permeate. Continuous production (Fig. 4) was demonstrated by using 1) neutral liposomes (PC:Chol, 4:1 molar ratio) with propofol and 2) cationic liposomes (DOPE:DOTAP, 1:1 molar ratio; Table 1) loaded with surface-complexed protein. Lipid

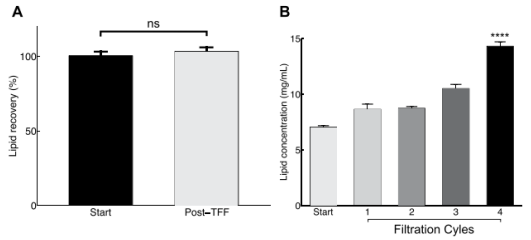


Figure 5. Lipid recovery in the continuous liposome factory-on-a-bench for (A) lipid recovery afer four diafltration cycles. (B) Lipid concentration in four concentration cycles, related to the initial amount of lipids present prior to the concentration cycles. All experimental datasets are presented as mean and standard deviation (mean±s.d.) average of three independent runs (n=3).

| | Liposome with drug after SHM | Liposome with drug after three passes through the TFF* |
|---|---|---|
| Size (nm) | 51.4 ± 2.1 | 61.2 ± 13.2 |
| Polydispersity | 0.29 ± 0.013 | 0.33 ± 0.09 |
| Loading (mol%) | N/A | 51.0 ± 4.0 |
| Effec. ethanol (% v/v) | 16.1 ± 3.9 | 3.1 ± 1.5 |

Table 3. Continuous purifcation of PC:Chol liposomes loaded with propofol. Propofol and lipids were included in the ethanol stream. Liposome formation and drug encapsulation was performed in a staggered herringbone mixer (SHM), operated with a total fow rate of 2mLmin−1 and a ratio of 1:3 ethanol:aqueous solution. Te results are presented as mean and standard deviation (mean±s.d.) resulting from three independent runs (n=3), N/A=not applicable. * Afer each pass a volume of pure bufer was added to compensate for permate and maintain constant volume of retentate.

recovery afer 4 diafltration cycles remained at 100%, matching the initial amount of lipids present prior to TFF (Fig. 5A). Without bufer replenishment, the system performed concentration cycles for formulations (Fig. 5B). Within four cycles, the concentration of lipids measured in the retentate doubled (Fig. 5B). Tis was due to a 50% reduction in volume as the overall quantity of lipids in the retentate remained constant, matching the lipid content afer the SHM (but before the TFF).

**Continuous manufacture and purifcation of liposomes with bilayer loaded drug.** Tis system was then applied for the continuous manufacture and purifcation of liposomes incorporating propofol (Fig. 4B). Liposome (PC:Chol) production and drug encapsulation were performed in a staggered herringbone mixer (SHM), operated with a volumetric fow rate of 2mLmin−1 and a 1:3 solvent:aqueous bufer ratio. Te resulting liposomes were 50nm in size with a PDI of 0.3 (Table 3) in line with previously reported studies25. Using the continuous manufacturing set up (with three diaflitrations), liposomes were therefore

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both manufactured and purifed. Tis continuous system was able to produce a purifed liposome product incorporating 51mol% propofol (in line with previously reported drug loading achieved using a 2 step manufacture and purifcation process based on dialysis18), with clinically acceptable ethanol levels (3% (v/v); Table 3). Furthermore, liposomes manufactured and purifed in this continuous systems retained their physico-chemical attributes and were not signifcantly diferent in size, nor PDI from those not subjected to TFF purifcation (Table 3). Examples of electron microscopy images of liposomes are shown in Supplementary Figures S1 and S2.

To compare the characteristics and drug loading of PC:Chol liposomes loaded with propofol, the same formulation was prepared using hand-held extrusion (10 passes through a 400nm, 200nm, 100nm and fnal 50nm pore size flters). Whilst this method of liposome manufacture was not the main focus of this study and could be further optimized, again these liposomes were efectively purifed to remove free drug using TFF (with drug loading of 3.6±0.38mol %; data not shown) and the liposome size and PDI remained unchanged by TFF purifcation (107.9±14.1 nm and 109.9±19.0 nm with PDI values of 0.17±0.10 and 0.34±0.06 pre and post TFF purifcation respectively, results not shown).

| | Liposome w/o OVA after SHM | Liposome with OVA in collection vial | Liposome with OVA after three passes through TFF* |
|---|---|---|---|
| Size (nm) | 62.8 ± 1.9 | 88.5 ± 5.7 | 89.3 ± 10.9 |
| Polydispersity | 0.44 ± 0.02 | 0.45 ± 0.01 | 0.42 ± 0.02 |
| Zeta potential (mV) | 83.9 ± 3.5 | 43.6 ± 1.6 | 69.2 ± 6.1 |
| Loading (%) | N/A | N/A | 23.9 ± 0.8 |
| Effec. ethanol (% v/v) | N/A | 15.0 ± 6.9 | 4.1 ± 1.5 |

Table 4. Continuous purifcation of DOPE:DOTAP liposomes loaded with protein (ovalbumin). Te lipids were included in the ethanol stream and liposome formation was performed in a SHM, operated at 2mLmin−1 and a ratio of 1:3 solvent:aqueous solution. Protein was added post-liposome formation. OVA=ovalbumin, N/A=not applicable. * Afer each pass a volume of pure bufer was added to the retentate, keeping the level of liquid constant.

**Continuous manufacture and purifcation of cationic liposomes with adsorbed protein.** Te lab-on-chip micro continuous-fow system was next challenged with the production of cationic (DOPE:DOTAP) liposomes, which were modifed with added ovalbumin in the intermediate connection vial and fnally subjected to purifcation (Fig. 4C). Lipids were included in the ethanol stream and liposome formation was performed using the SHM, which operated at 2 mL min−1 and a 1:3 solvent:aqueous bufer ratio. Te outfowing liposome solvent mixture was collected in the intermediate collection vial afer 1minute of SHM operation, and analysed (size, PDI, ZP). Te resulting liposomes had a size of 62.8±1.9 nm, PDI of 0.4±0.02 and ZP of 84±3.5 mV prior to addition of OVA. Ten ovalbumin was added to the intermediate vial, resulting in vesicles with a larger size (88.5±5.7nm), unaltered polydispersity (0.45±0.01), and reduced ZP (43.6±1.6 mV) (Table 4), again as a result of interactions between the cationic liposomes and the anionic protein. Afer manufacture and purifcation on the system, liposomes were unaltered in size (89.3±10.9nm; PDI 0.42±0.02) and had an increased ZP (69.2±6.1 mV) (Table 4), presumably through the purifcation and removal of 74% ‘free’ protein from the system. Residual solvent levels were also reduced to clinically acceptable levels (4%; Table 4). Examples of electron microscopy images of liposomes are shown in Supplementary Figure S1.

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## Discussion

We successfully investigated the region of operation for the Tangential Flow Filtration (TFF) device with various liposome formulations and confrmed the upper limit of operational pressure for the presented purifcation system to be 75 psi. A pressure range between 5 and 80 psi is a common backpressure implemented in industrial fltrations26 which is virtually identical to our TFF. During pressure tests, the membrane remained intact throughout, and therefore it can be considered that the measured backpressure equaled the transmembrane pressure inside the TFF. Tis transmembrane pressure could be adjusted accordingly using the data available in Table 2; alternatively, it could be calculated from Hagen Poiseuille's equation. Based on these fndings we determined the optimal operational transmembrane pressure of 62psi, which corresponded to a maximum fow rate of 2.5mLmin−1 through a restrictive capillary with an internal diameter of 100µm and a length of 25 mm. At this fow rate, our sample (2 mL) took less than a minute (~48 s) to run through the system, which shows a distinct advantage over the current methods that require lengthy bench-top, post-synthetic dialysis22. At high shear rates, drug release from liposomes can be a problem. However, the calculated average shear rate at the maximum fow rate of 2.5mLmin−1 inside the retentate channel is approximately 590 s−1 (Supplementary Information S3). Tis value is lower than previously reported shear rates27 of 800 s−1 for which no infuence on the permeability or integrity of the liposome membranes was found. Furthermore, the fow rates matched those previously applied for liposome manufacturing using a device with a SHM5,25. Tus, we proved that a SHM can be coupled directly with the TFF, and that we could generate and purify liposomes in a continuous mode without any losses into the permeate. Overall our results show that our fltration system can be implemented for multistage purifcation of a broad range of liposomal products.

Backpressures of 75psi and higher, however, led to losses of liposomes through the intact cellulose membrane into the permeate. One possible explanation could be that of particle extrusion across the membrane at these high pressures. It is well known that liposomes can undergo extrusion through cylindrical pores in membranes. Industrial scale extrusion tends to use higher lipid concentration than in our current study and adopts higher pressures ranging between 100–700 psi. However, extrusion of liposomes is system dependent; polycarbonate flters are used at pressures less than 100 psi, and low lipid concentrations require lower pressure28. Terefore, to avoid extrusion, a backpressure of 75psi was adopted as the critical cut of value. Membrane characteristics also play an important role for liposome recovery as they infuence the fux from the retentate to the permeate. Te calculated transmembrane fow was 0.32mLmin−1 (or 12.8% of the total fow rate, TFR) for a hydrophilic membrane with a pore size of 0.22 µm, at a backpressure of 62psi and nominal fow rate of 2.50mLmin−1 (retentate). In contrast, for the same backpressure and same nominal fow rate, a membrane with a 0.45µm pore size resulted in a transmembrane fux of 1.69 mL min−1 , corresponding to 67.6% of the retentate infow. Furthermore, the presented setup demonstrates that a range of capillaries with varying inner diameter and length can be applied to control the backpressure and the dilution or concentration rates of the system, allowing to tailor resulting fow rates and to adjust throughputs.

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Having established optimal operational conditions of our TFF device, its purifcation capacity for the removal of non-incorporated hydrophobic drug (propofol) and residual ethanol was studied. Over three diafltration cycles, the quantity and quality of liposomes were preserved afer purifcation for the anionic vesicles. For the cationic liposomes, there was a small increase in polydispersity, but no signifcant increase in liposome size (Fig. 2A). Te propofol content decreases much faster in comparison to the ethanol (Fig. 2B), with the hydrophilic membrane (0.22 µm pore size). Tis could potentially be due to capillary action that channels the separation of lipophilic propofol29 (Log Kow=3.79) through the membrane. Te ethanol content was the critical factor, which determined the required number of diafltration cycles. Afer three diafltration cycles, only 1% of non-incorporated propofol and 3% residual ethanol remained within the liposomal suspension with no changes in liposome physico-chemical attributes or concentration (Fig. 2) demonstrating the ability of this system to provide liposomes purifed to a level as would be expected for a therapeutic product.

In terms of removal of non-associated protein from liposomes, as might be required for liposomal adjuvants or biological therapeutics, protein removal is challenging because high concentrations of protein can lead to membrane fouling due to protein-membrane interactions24. Such protein-membrane interactions occur due to electrostatic repulsion forces. Similar to propofol removal, the dilution by replenishing with fresh bufer, and subsequent fltration facilitates the reduction in concentration of free protein in the retentate. Purifcation therefore occurs as a result of two cumulative efects: one from the separation at the membrane and the other from the dilution of the retentate. As demonstrated, separation can be controlled by adjusting fow rates and restrictive capillary sizes; also by varying the amount of liquid that is replenished afer each diafltration cycle. In our results, the volume amounted to the volume of the permeate, thus maintaining constant the amount of liquid circulating in lab-on-chip purifcation system.

The tolerated levels of free protein depend on the requirements implied by the target application of the liposomes and the number of diafltration cycles can be adjusted accordingly to match those criteria for purity. A particular focus in the delivery of protein antigens is the use of cationic liposomes, with electrostatic attractive forces dominating and ofen leading to a surface-adsorption reaching close to 100% depending on protein concentrations used30 and purifcation can further be complicated by the cross-linking and/or aggregation of cationic liposomes (DDA:TDB) with protein. We have demonstrated the capability of the fltration device to separate non-adsorbed ovalbumin (OVA) from a cationic liposome formulation and residual solvent with high liposome adjuvants recovery (87%) (Fig. 3). Tis presents compelling evidence that our micro continuous-fow purifcation device, i.e. TFF device, is capable of providing an efective post-production purifcation step, with the option to recycle purifed protein for subsequent applications.

The liposome process fow system presented here (Fig. 4) facilitates the complete removal of the free drug, which was previously only achievable by time intensive, bench-top dialysis18,22. Te encapsulation and solubilisation of drug with low aqueous solubility in the bilayer of liposomes has been investigated

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previously using a microfuidics based system18. In that study the assembly of PC:Chol liposomes was performed using a SHM, and the method was established as a robust, reproducible approach for preparing size-controlled liposomes as solubilising agents. Te same SHM is implemented in this herein reported system to investigate the efects of continuous processing on drug encapsulation by measuring amounts of drug encapsulated in the liposome bilayer. Very importantly, the amount of encapsulated drug and physical characteristics (size, PDI) show that continuous processing and the pressures applied in the TFF have no adverse efect on liposome integrity (Table 3). Te presented assembly utilizes the methanol solubilisation as the initial step of liposome production. However, it is possible to replace time-intensive production and dialysis (hours) with a micro continuous-fow system (minute-long process) manufacturing and purifcation to rapidly remove residual solvent. Among the main merits of using the continuous microfuidic process fow are the mild conditions during the assembly of the liposomes and the replacement of long ultracentrifugation steps for protein removal31. It can be concluded that the performance of the process fow system demonstrated (Fig. 4) for liposomes is consistent with: frst, the results from the stand-alone SHM in terms of particle characteristic; second, the results from the stand alone TFF in terms of purifcation.

## CONCLUSIONS

We have successfully demonstrated for the first time the feasibility for on-chip purification of liposomal batches for process development. Liposome manufacture, drug loading and removal of contaminants (such as un-entrapped drug or protein as well as solvent residues) were performed in a continuous mode using two microfuidic devices, allowing for manufacturing, purifcation and concentration of liposomal drug products. Tese devices were successfully challenged with a range of liposomes, varying in lipid composition, surface potential, size and concentration. Te results demonstrate the ability of the on-chip fltration unit to be tailored to a broad diversity of lipid-based nanoparticles by varying the operational parameters. Te microfuidic devices allow for an efcient and quick investigation of several lipid or drug candidates, and meet high throughput requirements of early stage development processes. Te continuous process may permit determination of liposomal characteristics (e.g. size, surface potential, particle number) and encapsulation efciencies of a wide variety of drug molecules, allowing for future integration of process analytical technologies (PAT) to further aid reproducibility. Furthermore, the setup is of considerable interest for cost-intensive drugs or protein encapsulation development, as the process requires micro volumes. Te microfuidic device developed herein can cope with a variety of proteins developed by the biopharmaceutical industry. Te device has the fexibility of integrating diferent types of membranes to cater for a variety of uses; also has the option of scalability through parallelization of the mixer chips and TFF membranes, and thereby can be easily translated to industrial setting.

## METHODS

**Chemicals.** Egg Phosphatidylcholine (PC), CAS: 8002–43–5, 1,2-Dipalmitoyl-sn-glycero-3-phosphorac-(1-glycerol) sodium salt (DPPG), CAS: 67232–81–9, 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), CAS: 63–89–8, and Cholesterol (Chol), CAS: 57–88–5,

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were obtained from Sigma-Aldrich Company Ltd. (Poole, UK). 1,2-dioleoyl-sn-glycero-3-phsphoethanolamine (DOPE), CAS: 4004–05–1, 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), CAS: 144189–73–1, dimethyldioctadecylammonium bromide (DDA), CAS: 3700–67–2, and trehalose 6,6-dibehenate (TDB), CAS: 66758–35–8 were purchased from Avanti Polar Lipids, Inc., (Alabaster, AL), purity >99% (Table I). Ethanol, CAS: 64–17–5, and methanol, CAS: 67–56–1, were obtained from Fisher Scientifc (Loughborough, UK). TRIS Ultra Pure, CAS: 77–86–1, was obtained from ICN Biomedicals, Inc., (Aurora, Ohio, US). Propofol (2,6-Bis(isopropyl)phenol), CAS: 2078–54– 8, and ovalbumin (chicken egg), CAS: 9006–59–1, were obtained from Sigma-Aldrich Company Ltd., (Poole, UK). Ultrafltration regenerated cellulose membranes (p\n: U2755–10AE) were obtained from Sigma-Aldrich Company Ltd., (Poole, UK) (10 kDa, pore size 0.22 µm), and Biomax polyethersulfone ultrafltration membrane discs with 300 kDa cutof, pore size 0.45 µm (p\n: PBMK06210) from Merck Milipore (Darmstadt, Germany).

**Liposome batch formulations for characterisation of the Tangential-Flow Filtration (TFF) device.** Multilamellar vesicles (MLV) were prepared using the lipid flm hydration method33. Lipids were weighed and dissolved in a chloroform/methanol (9:1 v/v) mixture. Cationic liposomes comprised DDA:TDB (8:1 molar ratio) and anionic liposomes comprised DPPG, DPPC, Chol (1:1:1.3 molar ratio). Te organic solvent was subsequently removed by rotary evaporation under vacuum (100 RPM, 180 mBar, Rotavapor R-100, BÜCHI Labortechnik AG, Switzerland), followed by fushing with nitrogen for removal of solvent residues (5minutes). Te thin lipid flm on the bottom of a round bottom fask was hydrated with 10mMpH 7.2 TRIS bufer. Small liposomes were formed via probe sonication (Soniprep150plus, MSE, UK; 5 min at amplitude of 5). Ethanol was manually added to the liposome formulation to a fnal concentration of 20% (v/v) to simulate solvent contents commonly resulting from the microfuidics production method. Ovalbumin (100 µg mL−1 ) was used as a model protein, and propofol (1 mg mL−1 ) as a model drug. Tese were added to the liposome formulation post-production to mimic the conditions post liposome manufacturing by microfuidics.

**Device fabrication.** As previously reported, the fltration system was designed to seal membranes in place by means of mechanical clamping24. Two poly(methylmethacrylate) (PMMA) plates, with a straight channel (1 mm width, 1 mm depth, 45 mm length) and a 1 mm hole milled at each end were clamped together using M3 screws along the edges (Torque 10 Ncm). A 1 mm wide and 0.75 mm deep cutting was used to hold the PDMS gasket in place, which was used to secure the membrane in place (Supplementary Figure S4). Diferent commercially available membrane sheets were cut to the required size using a CO2 laser marking head (Synrad Inc., Mukilteo, WA, USA). Te membranes used in this set of experiments had a cut-of of 10 kDa or 300 kDa, for drug or protein fltration, respectively. Te membranes were cleaned afer each experiment by back-fushing with water and stored inside the TFF system in 0.8M saline solution, ready for the next experiment.

Additionally, a clamping system was made from PMMA (two plates held together by screws [M3]) for the staggered herringbone micromixer (SHM) chip using

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a micromilling machine (M3400E, Folken IND, Glendale, USA). Te gasket for the fltration unit was manufactured from poly(dimethylsiloxane) (PDMS, Sylgard 184, Dow Corning, Midland, USA), according to the manufacturer's instructions and cast in PMMA moulds, manufactured as described above. Interconnect ports (milled from 5 mm PMMA), with two holes tapped with an M3 thread were used for connection to the fltration unit; an M6 threaded hole was used for standard connection fttings (P-221, Upchurch Scientifc, Oak Harbor, WA, USA).

**Backpressure regulation.** Backpressures were regulated through capillaries, which were attached to the retentate outlet of the fltration device (see Supplementary Fig. S3). Tese capillaries restricted the fow as they were selected with internal diameters smaller than the polytetrafuoroethylene (PTFE) tubing (1/16 in.×0.031 in., p\n: 58700-U, Sigma-Aldrich Int.) which connected the TFF device with auxiliary pumps and collection vials. Backpressure was calculated using Hagen-Poiseuille's Law

$$\Delta P = \frac{128 \cdot \mu \cdot L \cdot Q}{\pi \cdot D^4} \quad (1)$$

where μ, L, d and Q are the dynamic viscosity of the medium at 25 °C, the length and internal diameter of the restricting capillary, and the volumetric fow rate, respectively. We used Hagen-Poiseuille's equation (1) to select the capillary sizes and the fow rates to attain the backpressure range from 5 to 80 psi. For each backpressure analysis, a capillary was connected to the TFF retentate outlet using PTFE tubing, ferrules (p\n: P-200, IDEX Europe GmbH, Germany) connectors (Flangeless Nuts, p\n: P-247, PEEK, M6 Flat-Bottom, for 1/16 in. OD, IDEX Europe GmbH, Germany) and metric unions (Metric Union, M6 Port, p\n: P-602, IDEX Europe GmbH, Germany). Te inlet of the TFF was connected through a Luer-lock ftting and polytetrafuoroethylene PTFE tubing to a single-use plastic syringe. Water was fed in the TFF device at discrete fow rates ranging from 0.01 to 2.5 mL min−1 attained by a syringe pump (Nemesys, Cetoni GmbH, Germany). Backpressures were measured experimentally with a pressure sensor (40PC100, Honeywell, NJ, USA) connected on the retentate side; the data was logged with a LabVIEW virtual instrument (National Instruments, TX, USA). We compared the theoretical backpressures from equation (1) to the measured backpressures, and the measured values exceeded their calculated counterparts from 20% to 6.25% when increasing the applied backpressure from 5 to 80 psi, respectively (Supplementary Figure S5, and Supplementary Table S6). One of the TFF outlets was intentionally sealed with a fat bottom plug (p\n: P-314, M6, IDEX Europe GmbH, Germany) while a single outlet connected through a ferrule (p\n: P-200), nut (p\n: P-247) and tubing into a collection vial for liquid passing through the membrane.

**Filtration.** Filtration was performed in diafltration mode to investigate the liposome behaviour in the established pressure and fow rate range (Table 2). For this experiment, bench-top prepared liposomes in aqueous solution were spiked with drug, protein or solvent, and were introduced into the TFF by means of syringe pumps (Nemesys, Cetoni GmbH, Germany), connectors and capillaries as described earlier. A capillary was connected to the TFF, in cis-confguration (on the same side of the membrane), and closed the loop of the retentate fuidic line (see Supplementary Fig. S3). Retentate from the TFF was collected in an

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intermediate collection vial and could be injected in the device hence allowing for multiple passes, referred in this article as diafltration cycles. Transmembrane pressure was attained by controlling the fow rates in the pump; also by adding a constriction capillary of known geometry, i.e. internal diameter and length. Retentate and permeate fractions were collected in Eppendorf tubes, assessed by weight, and used for further analysis, i.e. zeta potential, size, polydispersity, quantifcation via HPLC. A volume of TRIS bufer, 10mMpH 7.2, was added afer each diafltration cycle to compensate for the amount of liquid passing through the membrane (in permeate) and to sustain steady concentration levels (in retentate) during the continuous purifcation process.

**Continuous process fow confguration.** To test the continuous processing of liposome formation followed by liposome purifcation, a SHM and a TFF device were connected in sequence. Te SHM (Precision Nanosystems Inc., Vancouver, Canada) consisted of two inlets, a bifurcated channel with herringbone structures, and single outlet moulded in PDMS. Te channels were 200 µm in width and 79 µm in height with herringbone features of 50 µm in width, 31 µm in height, 45° angle, asymmetry index 2:1 (according to Precision Nanosystems, Inc.). Luer-lock ftting and polytetrafuoroethylene (PTFE) tubing (1/16 in.×0.031 in., Sigma- Aldrich Int.) were used to link disposable 1mL syringes with the two inlet ports of the chip; fow rates and fow rate ratios were controlled by syringe pumps (Nemesys, Cetoni GmbH, Germany) and the whole system was primed with Tris bufer (10mM, pH 7.2) prior to operation. Organic phase, a weighed amount of lipids in ethanol, was injected into the frst inlet of the SHM device, while in the second inlet aqueous phase (TRIS bufer, 10mM, pH 7.2) was injected. Te micromixer was held in place using a clamping device made out of PMMA. Te micromixer was connected to the tangential fow fltration unit via an intermediate collection vial (2.0 mL Eppendorf) for additional functionality. Tis allows the addition of various components such as of microfuidics-manufactured liposomes prior to the fltration system for purifcation. A bi-directional milliGAT pump (VICI Valco, Valco Instruments Co.) was connected in-line with the retentate loop of the TFF through a capillary at the bottom of that intermediate collection vial. Transmembrane pressures was varied by restricting the fow of the retentate using diferent small diameter capillaries connected in-line with the TFF outlet. Te retentate fowed through the capillary and was collected in the intermediate vial, while permeate passed through the membrane and was gathered in a separate tube. Both fractions were analysed for content of liposomes, propofol, protein, lipid and ethanol.

**Measurement of particle characteristics.** Nanoparticle tracking analysis (NTA) was performed with a Nanosight LM20 (NanoSight, Amesbury, UK), connected to a microscope (with 20×magnifcation). Liposomes were diluted 1:10 to 1:100 in distilled water, to achieve an optimal particle concentration of 107 –109 particles/ mL during measurement. NTA analysis was used to determine the particle concentration per millilitre (P/mL), recording time was 60 seconds and camera settings (shutter and gain) were adjusted manually to maximise resolution. Dynamic light scattering (DLS) (Malvern Zetasizer Nano-ZS, Malvern Instruments, Worcestershire, UK) was used to report the z-average (intensity based mean particle diameter), and to report the polydispersity (PDI), in order to assess the width of the

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particle distribution. Liposomes were diluted 1:10 in distilled water and measurements took place at 25 °C. Zeta potential (ZP) was measured using particle electrophoresis (Malvern NanoZS, Malvern Instruments, Worcestershire, UK).

**Propofol quantifcation.** Quantifcation of propofol was performed by reverse phase HPLC (Luna 5µ C18, Phenomenex, UK, pore size of 100Å, particle size of 5 µm) at 268nm. Te fow rate was constant at 1 mLmin−1 throughout with a gradient elution from 5:95 (Methanol: 0.1% Trifuoroacetic Acid, TFA, in water) to 100:0 (Methanol: 0.1% TFA in water) over 10minutes. HPLC-grade solvents were used, sonicated and fltered. Te column temperature was controlled at 35 °C. All analysis was made with Clarity, DataApex, version 4.0 (DataApex, Prague, Czech Republic). For quantifcation, established calibration curves of propofol were used as reported previously14.

**Protein and lipid quantifcation.** Samples were loaded on a HPLC and elution was performed with a gradient from 5:95 to 100:0 (Methanol: 0.1% TFA in water) over 20 and 40minutes for protein and lipid detection, respectively. Quantifcation was performed by an evaporative light scattering detector (ELSD) (Sedex 90, Sedere, France), set at 52 °C and coupled to the HPLC as described previously18. A calibration curve was established from standards (ovalbumin in TRIS bufer, pH 7.2, lipids in ethanol) in six replicates at concentrations from 5 to 100µgmL−1 (protein) and 0.05 to 1.5mgmL−1 (lipids).

**Statistical analysis.** Unless stated otherwise, results were reported as the mean±one standard deviation (s.d., n = 3). One- or two-way analysis of variance (ANOVA) was used to assess statistical signifcance, followed by Tukey's multiple comparing test (post-hoc analysis). A t-test was performed for paired comparisons. Signifcance was acknowledged for p values lower than 0.05, marked with and asterisk (*). All calculations were made in GraphPad Prism version 6.0 (GraphPad Sofware Inc., La Jolla, CA, US).

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