In quest of effect directed analysis at the smart laboratory: Automated system for flow-through evaluation of membranotropic effects of emerging contaminants

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Abstract

The rate-determining step of the human exposome workflow is the acquisition of physiologically relevant data (e.g., effect direct analysis), which can be performed retrospectively or with ad hoc experiments. In this contribution, an automated system is proposed for evaluating potential interaction mechanisms of xenobiotics across cell membranes, the so-called membranotropic effects, using liposomes as a mimicry of biological membranes, and fluorescent membrane probes. The smart fluidic method features real-time acquisition of fluorescence readouts, data processing and feedback in a fully unsupervised mode. As a proof of concept applicability, the behavior of newly synthesized cholesterol-laden biomimetic liposomes, and the *in-vitro* potential toxicant action of bisphenol A and diclofenac as model of emerging contaminants on cell membrane surrogates were investigated in a flow-through format. Unattended operation resulted in excellent intermediate precision (<1.5%) and unveiled that diclofenac affected the liposomal bilayer order very slightly, regardless of the cholesterol concentration, because it accumulates at a superficial level, while the membranotropic effect of bisphenol A was more pronounced at low concentration levels of cholesterol because at increased levels, the membrane reduces its permeability.

Keywords: exposomics; emerging contaminants; membrane effects; smart system; fluidics

1. Introduction

Exposome evaluation determines or fingerprints the overall chemical composition of complex samples by resorting to a plethora of analytical techniques that yield huge amounts of data in a holistic format under increasingly unattended workflows [1,2]. The other side of the exposomic coin, that is, the biological assays applied to fractionated complex samples still remains a cumbersome task that is normally carried out manually [3]. Because of current regulations that pose concerns and limitations of the experimentation with living beings [4], the number of assays encompassing superior

animals is minimized in favor of cell or surrogates testing, which constitute a unique opportunity for developing miniaturized and automatic methods due to the ease of manipulation of cellular entities by e.g. flow approaches. In this context, toxicologic effects that aim for the cell membrane as the biological target can be studied *in-vitro* using artificial membrane surrogates, such as liposomes [5,6], that is, nanovesicles with chemical composition and structure that mimics the phospholipid bilayer of eukaryotic living cells. While liposomes are easily synthesized with pure phospholipids, such as phosphatidylcholine, the variation of the precursor composition with the introduction of different phospholipid classes extracted from biological sources [7–9], surfactants or other additives can tune the physicochemical properties of the liposomes and endow them with varied flexibility, permeability, surface charge or affinity to different tissues to name a few. For example, human cells can contain up to 40% in weight of cholesterol, which changes or controls the order and fluidity of the membrane, thus altering the morphology, lipid packing and permeability of the bilayer [10]. Among the different analytical techniques for studying the effects of selected xenobiotics or mixtures into the liposomal membrane, fluorometric measurements using polarity-sensitive membrane probes constitute a gold standard methodology that benefits of low-cost, non-invasiveness and simplicity. The fluorescent membrane probe used in this work, Laurdan, is a hydrophobic molecule with a lauryl chain and naphthalene fluorescent moiety that locates at the level of the glycerol backbone of the lipid bilayer [11]. The emission spectrum of Laurdan exhibits a continuous red shift from 440 to 490 nm when changing from a hydrophobic (e.g., lipid phase) to polar medium due to the so-called dipolar relaxation phenomenon. The reorientation of solvent's dipoles in the surrounding of the probe requires energy and hence, decreasing the energy of Laurdan's excited state. The higher the number of molecules of water (or another polar molecule) around the probe, the higher the red shift of the emission spectrum of the fluorescent dye. To quantify this shift, the so-called generalized polarization (GP) [12] is often exploited (Eq. 1):

$$GP = \frac{I_B - I_R}{I_B + I_R} \qquad \text{(Eq. 1)}$$

Where I_B and I_R are the fluorescence emission intensities at the blue and red spectral components of the emission spectrum respectively, that is, at 440 nm and 490 nm. The

returned value is related to the lipid order and packing, hydration and fluidity, and thus with the membrane phase state: When the membrane is composed of well packed phospholipids, GP values are positive, when the membrane is more disordered and hydrated, GP becomes negative [13].

In those assays, the fluorescent membrane probe is incubated with the liposome until a constant emission spectrum is obtained. Then, the xenobiotics are added to the mixture, and again the analyst waits until the fluorescence spectrum stabilizes. The spectrum shifts, and ΔGP values calculated from the values obtained after and before the addition of the target compound account for membranotropic effects. The time required for the probe to stabilize varies greatly with the composition of the liposomes, the target species to be studied or the temperature and thus, experimental conditions must be optimized whenever a single parameter of the batch analysis is altered. Some combinations of factors may yield incubation times of more than a working day but setting extreme long incubation delays compromises the sample throughput since other combinations may stabilize very fast. To the best of our knowledge the GP assays have until now being always developed in a supervised batch format, even if the procedure is simple and does not resort to biological entities. This manual operation jeopardizes the required throughput in the exposomics field.

Among the various techniques available for automating those analysis, fluidic methods, that is, those which resort to the pressure driven manipulation of liquids in closed manifolds through the use of pumps and valves are a very appealing candidate to automate the GP measurements[14,15] because of the simple components required, and the possibility of assembling dedicated manifolds. Those systems usually work in a mechanized regime: computer-controlled pumps and valves manipulate the fluids in a reproducible way for every sample without analyst intervention. A performance enhancement is achieved through the use of the so called ‘automated methods’ according to IUPAC definition, where the computer does not only control the mechanical part of the system, but also receives an analytical feedback allowing it to take decisions at real time.

In this contribution, we present a fluidic system capable of developing GP tests for investigation of membranotropic effects in an unattended manner by resorting to a

smart method of data acquisition and treatment. The smart system provides continuous feedback and thus, this should be regarded as a unique example of liposome-based *automated* system. As a proof of concept, our fluidic automated system has been applied to liposomes containing different cholesterol levels aiming at simulating varied membrane cells, using Laurdan as a fluorescent probe, and the plastic additive bisphenol A and the nonsteroidal anti-inflammatory drug (NSAID) diclofenac as models of potentially emerging contaminants, the latter included in the First EU Watch list of emerging water pollutants.

2. Experimental

2.1. Reagents

Natural soybean L-α-phosphatidylcholine (PC), LIPOID S100, was purchased from LIPOID GmbH (Ludwigshafen, Germany) with a concentration of L-α-phosphatidylcholine not less than 94% and a lipid tail distribution of linoleic acid (C18:2, (9Z,12Z)-octadeca-9,12-dienoic acid) as the main fatty acid, followed by palmitic acid (C16:0, n-hexadecanoic acid) and oleic acid (C18:1, cis-9-octadecenoic acid) with percentages of ca. 63, 15 and 11%, respectively. Cholesterol and the target xenobiotics in this work, *viz.*, diclofenac sodium salt (DCF), and bisphenol A (BPA) were purchased from Sigma-Aldrich/Merck KGaA (Darmstadt, Germany). The fluorescent membrane probe 6-dodecanoyl-N,N-dimethyl-2-naphthylamine (Laurdan) was also obtained from Sigma-Aldrich/Merck KGaA.

Stock solutions of pollutants, BPA and DCF, were prepared in methanol at a final concentration of 50 mM. The fluorescent probe Laurdan, 1 mM, was prepared in DMSO and cholesterol was dissolved in chloroform to a final stock solution of 12 mM.

The phosphate-buffered saline (PBS) 10X was prepared by dissolving 1.2 g of potassium phosphate monobasic, 7.2 g of disodium phosphate, 40 g of sodium chloride and 1 g of potassium chloride in 500 mL of water. Working solution (PBS 1X, pH 7.4) was prepared by a 10-fold dilution of the stock solution in water.

2.2. Synthesis of liposomes

Liposomes were prepared by lipid film hydration [16] followed by extrusion for unilamellar liposome formation [17]. To this end, 50 mg of soybean phosphatidylcholine (PC, LIPOID S100, average molecular weight of 787 g/mol) was dissolved in chloroform in a round bottom flask without or with cholesterol. Chloroform was then removed in a rotary evaporator under low pressure (290 mbar) and 30 ºC for 2 h followed by vacuum pump at room temperature for at least 2 h to obtain a uniform dried lipid film on the flask bottom wall without any organic solvent traces. Next, the lipid was hydrated with appropriate given volume of PBS (pH 7.4) at room temperature to afford a fixed lipid concentration of 12.7 mM for PC, while that of cholesterol was 10, 20 or 30% mol of cholesterol per mol of PC. The solution was then vortexed for 1 h (1 min every 5 min) to obtain multilamellar vesicles (MLVs). The milky suspension was stored at 4 ºC overnight for stabilization. Later, large unilamellar vesicles (LUVs) were obtained by extruding the MLVs solution through a 100 nm pore size polycarbonate filter for 29 times [18]. Liposomal quality was evaluated by dynamic light scattering (DLS) using Zetasizer Nano ZS90 (Malvern Panalytical, Malvern, UK). The hydrodynamic diameter (Z-Average) was in all cases about 130 nm and Polydispersity Index (PdI) no more than 0.08.

While other procedures have been described for incorporating of the fluorescent probes during the synthesis of liposomes[19–21], the herein described procedure was chosen because of the simplicity for mixing of the liposomes and Laurdan. In fact, when the combination of more than one kind of liposome, as is the case in this work, and more than one probe must be evaluated, our fluidic system does necessitate a smaller number of ports of the selection valve (see the fluidic system section).

2.3. Fluidic System

The fluidic system and components thereof are depicted in Fig. 1. It consists of a bi-directional Cavro XCalibur (Männedorf, Switzerland) syringe pump furnished with a 50 µL-syringe (Hamilton, Bonaduz, Switzerland) and a 3-position ceramic stream selector, a 14-port C25Z-31814EMH Cheminert stream selector (VICI AG International, Schenkon, Switzerland) and a Minipuls 3 bidirectional peristaltic pump (Gilson Incorporated,

Middleton, USA) furnished with 1.3 mm i.d. Tygon tube. The mixing chamber is a glass barrel from a Ruthe syringe of 5 mL covered with aluminum foil for preventing the photodegradation of the labile fluorescent probe. The flow-through fluorimeter used as a detector is a Jasco FP-4025, configured to excite at 360 nm and monitor the emission at 440 nm and 490 nm simultaneously using the double wavelength mode. Data acquisition was carried out with a Taylor made 12-bit ADC converter with USB data transmission based on the Lopy4 chip (Pycom, London, UK). The internal reference voltage for the ADC is not calibrated from the factory and may vary with e.g. temperature changes. For this reason, a two-point calibration was implemented in the firmware based on the measurement of regulated voltage dividers and executed before every biochemical measurement. Additional information on the wiring, as well as the firmware can be found in the ESI and Fig. S1. All the tubing was 1/16" o.d., 1/32" i.d. fluorinated ethylene propylene (FEP) (IDEX Health and Science, Lake forest, Illinois, USA), except the tubes that connected the VICI stream selector to the reagents reservoir, that were 1/64" i.d. to minimize void volumes. The total volume of the recirculation loop was 1000 µL including the tube of the peristaltic pump, tube from the peristaltic pump to the valve, valve rotor, tube towards the fluorimeter, flow-through cell and tube towards the mixing chamber. The syringe pump and the stream selector were controlled in a multipoint RS232 bus (the selector in simplex), while the contact closure outputs of the syringe pump were used to control the activation/stop as well as the flow direction of the peristaltic pump. The flowrate of the syringe pump was set to 50 µL/min in all analytical cycles, and to 600 µL/min for priming. The flowrate of the peristaltic pump was fixed to 1750 µL/min, and thus, the volume aspirated or dispensed is proportional to the time the pump is activated. The ambient temperature was set to 23 °C throughout in order to minimize variations in GP values through batches of experiments.

A smart automated method was executed through the CocoSoft 4.5 freeware [22] for (i) controlling the entire fluidic instrumentation, (ii) acquiring and processing the bioanalytical data at real time and (iii) ultimately to modify the execution according to the data treatment results, as described in the next section. Figure 2 shows the CocoSoft window in experimental runtime. The instrumental method is running in the right hand

of the screen, while emission at 440 and 490 nm are acquired through the Lopy4 adapter and plotted at real time in the top left of the screen. The GP value is calculated and appears in the bottom left part of the screen. In this window, x axis units are the number of measurements, that will be converted *a posteriori* to minutes.

2.4. Automated flow method

The working principle of the proposed smart fluidic system is to prepare *in-situ* an appropriate composite solution of liposomes with Laurdan in PBS buffer into the mixing chamber. To this end, large volumes of PBS (ca. 2.5 ml) will be aspirated by the peristaltic pump from the multiposition valve. Reagents that are needed in much smaller amounts, such as the liposomes (20 µL), probe (2.54 µL) and xenobiotic (2.54 µL) will be added by the syringe pump because of its enhanced resolution (16 nL/step) by aspirating the required amount of a given solution or target species towards the holding coil, dispensing it to the mixing chamber via the flow-cell by reversed flow, and activating the peristaltic pump for bringing the composite liposome/probe/xenobiotic plug to the mixing chamber where it will be diluted with PBS and homogenized by the recirculation in the fluorimeter loop.

The fluorescence signal is captured by CocoSoft 4.5 at regular times through the Lopy4 adapter and the GP calculated and stored at real time. Then, two time-windows are defined, and the average GP is calculated in each window: the first one was the average GP [from now to now-x minutes], and the second one [from now-x minutes to now-2x minutes]. Figure 3 illustrates a graphical explanation of this algorithm.

If the absolute difference of both GP time-averaged values is smaller than a preset tolerance the GP is deemed constant, which indicates that the probe is stabilized within the lipid bilayer, and thus, the circulation is halted. At this time and without analyst intervention, the syringe pump adds a minute volume of xenobiotic (viz., 2.54 µL) to the mixture of Laurdan and PC liposomes and the monitoring procedure is repeated unsupervised. In case that the absolute difference is higher than the tolerance, the circulation continues because this indicates that the GP value has varied significantly during the last two time-windows (2x minutes) and thus the probe is not yet stabilized

into the lipid structure (Figure 2). At the end of this protocol, the steady GP values, before and after the xenobiotic addition, are exported and the whole procedure is repeated with the next combination of liposomes, probe and xenobiotic after a cleaning program. Any combination of factors can be programed at will, and the 14-port count of the VICI selector allows the variation of several parameters through the biochemical assay without human intervention.

The length of the time windows and the tolerance allowed for the comparison of average GP values were optimized for a high-performance method with minimum dead time and no type II errors in the identification of steady state conditions of the incubation mixture.

The CocoSoft method is available in the SI, including the detailed control program, with description of flowrates and volumes, as well as the real-time GP calculations of the smart system.

2.5. Study of the cholesterol influence in lipid membrane packing

To demonstrate the unattended capabilities of the designed fluidic system, the effect of modifying experimental conditions by addition of cholesterol to the LUVs was undertaken. Liposomes were synthesized according to the above described procedure. However, their composition was modified with 0, 10, 20 and 30% mol of cholesterol per mol of PC. In the automatic system, 20 µL of modified liposomes (12.7 mM PC) were diluted in 2.47 mL of PBS buffer (final concentration of 100 µM PC), incubated with 2.54 µL of Laurdan stock (final concentration of 1 µM) and the GP values were recorded and reported continuously by the smart method. The differences of GP values across different cholesterol concentrations will be related to the effects of cholesterol on the membrane packing, hydration of the bilayer and lipid ordering, and ultimately, on the facility of the xenobiotic to penetrate across the lipidic vesicle. After the GP reached a constant level, the fluidic system was automatically flushed by discarding the content of the recirculation loop through the waste port and cleaned 5 times with 1.3 mL of PBS buffer for 1 min. All this procedure was carried out in quadruplicate for every cholesterol level.

2.6. Study of membranotropic effects of selected xenobiotics

In a fully automatic mode, 20 µL of the synthesized liposomes with varying concentrations of cholesterol (0-30% mol of cholesterol per mol of PC) were diluted into 2.47 mL of PBS buffer (final concentration 100 µM PC) and incubated with 2.54 µL of Laurdan (final concentration 1 µM). When the smart method identified a steady GP value, a 2.54 µL aliquot of bisphenol A or diclofenac stock solution was added to a final concentration in the mixture of 50 µM (in the range of the used in those studies[23–27]), and the GP monitored again until reaching the next constant value. The used concentrations are in the same range as those reported elsewhere[23–27] for this kind of assays, as a tradeoff between sensitivity and representativity of expected concentrations. The GP variation before and after addition of the xenobiotic was calculated by subtraction, and this value along with the individual GPs and the entire temporal profiles for kinetic exploration were exported in a .txt file as plain text. The fluidic system was finally flushed and rinsed according to the same procedure described in the previous section. All experiments were carried out in quadruplicate (programmed by the user-friendly software).

For comparing the absolute value of GP obtained by the proposed method with the standard manual methodology, this last one was also applied [12], and the values obtained were compared with a *t*-test. In brief, in the manual method, the same proportion between buffer, probe, liposomes and contaminants were maintained, but carried out in a semimicro quartz cuvette format with a benchtop fluorescence spectrophotometer (Cary Eclipse, Varian). 992 µL of buffer were mixed in the cuvette with 8 µL of liposomes and 1 µL of Laurdan probe and incubated for 1 h protected from ambient light. Then the cuvette was inserted in the spectrofluorimeter, the temperature was stabilized for 10 min to 23 °C, and the emission spectrum was acquired at excitation = 360 nm, 10 nm/s , with PMT voltage = 600 V, both slits set to 10 nm and the cuvette facing the excitation source with the 4 mm side, and the 10 mm side facing the detector system. 1.4 µL of contaminant were added, and emission spectra were acquired every minute until they were constant. This required in average ca. 15 min, which is in good agreement with the online obtained data.

3. Results and discussion

3.1. Investigation of the crucial parameters of the smart method

Data acquisition rate, tolerance and length of time windows are key parameters for the method to perform properly. Data acquisition rate was set to 30 seconds and maintained throughout the remaining study. Higher time resolution was not necessary because of the hour-scale of these incubation-based assays. Tolerance was set to 0.001 for being this the maximum resolution of the fluorimeter. Decreasing this number had no physical sense, and increasing it only boosted the false detection of stability (type II error), unless unpractically high time windows are to set. The time window length should be as minimum as possible yet preventing false stability detection. The time window was increased from the minimum value (equal to the data acquisition rate) and increased in 1-minute intervals between trial and error experiments. This value was finally set to 7.5 minutes because this was the minimum value that did not afford false negatives. Smaller values detected stability prematurely because the noise associated to the signal was not effectively dampened with the averaging procedure. Figure 3 shows the overlap of 3 consecutive replicates of the assay with liposomes (0% cholesterol), probe (Laurdan) and xenobiotic (diclofenac) along with the fundamental principles of the smart algorithm.

It should be considered that the human analyst also requires some time after the stabilization in order to detect steady-state regime, so as can be seen in Fig. 3, the smart algorithm detects the stabilization of the incubating probe and LUV mixture approximately in a similar time than the human analyst would. The time per assay is about 15 minutes longer than that explicitly required for the incubating mixture (Fig. 3) to stabilize in best case for tests involving a single GP measurement (as in the case of liposome/probe characterization), and 30 minutes for tests requiring two GP measurements (effect of xenobiotics), yet all the steps of the assays are performed fully unattended and the experimental results are in-situ obtained. It should be stressed that the incubation time of probe with liposomes is a priori unknown and thus in the

supervised counterpart, the analyst must either monitor the fluorometric data continuously or at least at a given time, probably every 5 to 10 min, and this period must be summed to the time required for detecting the steady state conditions by naked eye.

As per Eq. 1, the GP range is [-1,1], making the Coefficient of Variation unsuitable for assessing repeatability and intermediate precision of measurements. Instead, the following dispersion statistics (Eq.2) was used for every quadruplicates of a given assay:

$$Dispersion = \frac{\max(x)-\min(x)}{dynamic\ range} = \frac{range\ (x)}{(1-(-1))} = \frac{1}{2} range(x) \qquad \text{(Eq.2)}$$

The relative dispersion parameter was in all cases <1.5% for up to 28 different assays in different days (each one in quadruplicate), showing the excellent precision of the designed smart method algorithm that allowed the unattended performance over weekend of up to 8 different membranotropic tests (each one quadruplicated).

In order to compare not only the time required for detecting the stabilization of the signals before and after addition of the xenobiotic, but also the absolute value obtained by the flow method, a *t*-test of comparison of means was performed between the ΔGP obtained by the herein proposed smart setup, and that of the standard manual method in order to assess the trueness of the proposed method [12]. The obtained p value of 0.060 ($p>0.05$, $N=3$) indicates that the results obtained with the proposed method do not differ statistically from those of the manual counterpart and thus, the fluidic setup does not introduce a significant bias onto the biochemical assays.

3.2. Influence of the cholesterol concentration on lipid ordering

As seen in Fig. 4 the higher the concentration of cholesterol up to 30% mol of cholesterol per mol of PC in LUVs the greater is the GP value recorded. It is known that the role of cholesterol in a hydrated and disordered lipid membrane is to enhance the lipid order by disturbing the mobility of the phospholipid's tails [28]. Our results agree with previous results in the literature because soy PC liposomes have a transition temperature from gel to liquid-crystalline phase lower than 0 ºC [11] due to the high concentration of 18:2 fatty acids, thereby soy PC liposomes are at room temperature encountered in a highly fluid state. In fact, the degree of unsaturation of lipids affect the

stiffening effect of cholesterol [29]. Phospholipids containing two unsaturated hydrocarbon chains, compared to one or none, as is the case with natural PC, are expected to cause a weakening effect of cholesterol on the membrane bending modulus [30,31]. The increase of the GP values caused by the addition of cholesterol (see Fig. 4) implies a decrease of the membrane phase polarity that could be explained by the extrusion of water outward the liposomal bilayer due to the interaction of cholesterol with the unsaturated phospholipid tails [32].

3.3. Membranotropic effects of diclofenac

The addition of the NSAID diclofenac into the liposomal solution caused a small yet significant ΔGP towards more positive values, thus implying a slight increase of lipid order (See Fig. 5). Similar results were obtained regardless of the concentration of cholesterol, from ΔGP = 0.028 for 0% cholesterol to ΔGP = 0.024 for 30% mol of cholesterol per mol of PC, thus indicating that the sterol did not disturb the effect of diclofenac over the membrane (Table 1). At pH 7.4, diclofenac molecules are mostly ionized (pK_a = 3.97) and thus the anti-inflammatory drug would at physiological pH interact preferably with the polar head of the phospholipids on the surface of the liposomal membrane [33], which would explain the low disturbing effect at the level of glycerol group where Laurdan fluorophore is predominantly located. This hypothesis is in accordance with other studies of molecules whose polarity is pH dependent [34]. Nevertheless, the lack of any enthalpic contribution seen by Manrique-Moreno and coworkers suggests that there might not be strong electrostatic interactions between diclofenac and the choline group of PC, and that the interaction process might be mediated by only entropic processes at the lipid/water interface [35]. Similar results were obtained by Fernandes *et al.* [36] supporting the entropic rather than electrostatic effects.

3.4. Membranotropic effects of bisphenol A

The effect of BPA on the liposomal membrane polarity was very acute (See Fig. 6, Table 1), increasing the value of ΔGP more than two times compared to that of DCF

($\Delta GP_{BPA}/\Delta GP_{DCF}$=4.12 for 0% cholesterol and 2.17 for 30% mol of cholesterol per mol of PC) and thus, causing a high impact on the order of the phospholipids. The difference observed in the GP values of the Laurdan in Fig 5 and Fig 6 in the absence of contaminant but with different concentrations of cholesterol is attributed to the small temperature changes in the laboratory environment. Nevertheless, a paired *t*-test of the Laurdan GP values in the various sets of experiments unveils no significant differences ($p=0.052>0.05$).

The presence of cholesterol at increasing concentrations lessened the effect of this pollutant on the behavior of the liposomal bilayer as indicated by the decrease of ΔGP. This phenomenon could be a consequence of the change of the liposome-water partition coefficient. A highly ordered membrane promoted by cholesterol onto the saturated acyl chains is less permeable to compounds due to the high degree of packing [37] and consequently, minimal interspaces between phospholipids do exist. Thermal data, NMR studies [38], and molecular dynamics simulations [39] agree with this observation and suggested that the preferable location of BPA is close to the lipid head group at the vicinity of the glycerol region wherein Laurdan is located. This fact would explain the strong effect on the medium polarity at the hydrophobic-hydrophilic interface, and thus onto Laurdan's emission fluorescence, caused by BPA, which dehydrates the liposomal bilayer. In fact, BPA is expected to be orientated in such a way that the hydroxyl moieties are directed towards the phosphate group forming hydrogen bonds while the main body of BPA is immersed into the PC tails.

4. Conclusions

This paper reports the first fully automated fluidic system that allows the unattended investigation of membranotropic effects of emerging contaminants on membrane surrogates consisting on liposomes. The flow setup features a high degree of intermediate precision (<1.5%) as a result of the smart algorithm implemented in CocoSoft 4.5 user-friendly freeware. Its performance has been exemplified by characterizing the membrane packing of natural PC liposomes with varying amounts of

cholesterol, and investigation of the interaction of liposomes with two model examples of emerging water contaminants.

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Declaration of interest

None

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