

# Water-in-Fluorocarbon Nanoemulsions Stabilized by Phospholipids and Characterized for Pharmaceutical Applications

Kirsten Ullmann,\* Manuel Meier, Carolyn Benner, Gero Leneweit, and Hermann Nirschl

Fluorocarbons are one of the most promising hydrophobic phases for future pharmaceutical production processes and various biomedical applications. Yet, because of their specific characteristics such as high density and refractive index similar to water, analysis of water-in-fluorocarbon (w/fc) nanoemulsions remains a challenge. The present work examines w/fc nanoemulsions stabilized with phospholipids as natural emulsifiers and tackles the measuring problems of photon correlation spectroscopy (PCS) when used for investigation of fluorocarbon nanoemulsions. These emulsions are suitable to form liposomes via centrifugation and thus, are required to meet certain criteria such as stability and size. The results imply a stability of up to 4 weeks with an average size of 180 nm. The intensity mean diameter gained from PCS measurements shows large scattering directly after sonication which is due to gas bubbles from sonication. The number mean is not influenced by gas bubbles and gives a more accurate depiction of the produced nanoemulsions. These findings are supported by small-angle X-ray scattering data, which are additionally applied for liposome analysis measuring a size of approximately 60 nm.

atoms makes fluorocarbons very inert and hydrophobic.<sup>[1]</sup> Because of the addressed characteristics of the fluorocarbon, they have been used for biomedical applications more recently. For example, in recent years they have been used as oxygen carriers in case of blood loss as they are known to dissolve a large amount of gas, and applied in MRI and NMR as a contrast agent.<sup>[2–4]</sup> For in vivo applications, perfluorocarbon nanoemulsions are required, stabilized by emulsifiers that are biocompatible as well. Phospholipids meet these criteria as they play a key role in the food and pharmaceutical industry because of their ubiquity in all organisms and absolute safety. As natural compounds and functional components in cell membranes, phospholipids are endogenous substances. Additionally, their amphiphilic, interfacial characteristics allow to use them as solubilizers, wetting agents or emulsifiers and thus, an appropriate

## 1. Introduction


Perfluorocarbons are one of the most promising hydrophobic phases for future pharmaceutical production processes and biomedical applications. They are characterized by a high electron density, and their chemical structures, either cyclic or linear, as well as a strong bond between the carbon and fluor

alternative to common synthetic, artificial emulsifiers like polysorbates.<sup>[5,6]</sup> These attributes make phospholipids interesting candidates for drug delivery systems such as liposomes. However, conventional laboratory methods for the production of liposomes, for example, the film method<sup>[7,8]</sup> or a homogenizer<sup>[9]</sup> for larger volumes, tend to leave a large part of the active ingredient outside the vesicles unused. The encapsulation efficiency found in literature for high molecular weight molecules varies from 10 up to only 50%.<sup>[10,11]</sup>

A process engineering method may help to tackle these challenges by combining nanoemulsions and PLs for emulsification. Pautot et al. (2003) developed a mechanism based on engineering aspects which had first been introduced by Träuble and Grell (1971).<sup>[12,13]</sup> They propose the centrifugation of a water-in-oil (w/o) emulsion into a second aqueous phase, whereby the interface between the two phases is coated with additional phospholipids of a different composition. As a result, an asymmetric bilayer can be formed. Pautot and other authors used this method to produce large unilamellar vesicles between 1 and 10  $\mu\text{m}$ , which are, however, unsuitable for pharmaceutical applications. Alike conventional methods, the use of solvents during the preparation needs comprehensive removal in the production process and is therefore extremely costly and time-consuming.<sup>[14]</sup> Several limitations of the process such as the stability of w/o nanoemulsions, the control of droplet size,

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and a successful transfer of micelles through the interface due to gel formation still did not lead to an efficient process. Sommerling et al. investigated the needed centrifugal force for a feasible transfer and found that it is impossible to transfer solid particles of nanometer size and low density difference with common centrifuges because of the hindrance by interfacial tension due to wetting or gel formation.<sup>[15]</sup> In addition, the increasingly studied w/o emulsions show a short stability depending on the hydrophobic phase and the chosen phospholipid for stabilization.<sup>[16]</sup> Based on these experiments, the selection of the hydrophobic emulsion phase is a major challenge to overcome. Preliminary work in our research group addressed the mentioned problems systematically from measurements of interfacial tensions to characterization of nanoemulsions as well as the selection of a suitable hydrophobic phase.<sup>[17]</sup> For the latter, a fluorocarbon, perfluoroperhydrophenanthrene (PFPH), was selected as the biocompatibility and inert characteristics make fluorocarbons unique in biomedical use.<sup>[18,19]</sup> While there are short-chain- and long-chain- fluorocarbons commercially available, it was decided to use a cyclic molecule with long chains and an exceptional high density while having a low viscosity ( $\rho = 2.03 \text{ g cm}^{-3}$ ;  $\eta = 28.4 \text{ mPa s}$ ). The interfacial properties of different phospholipid suspensions added to a fluorocarbon in previous studies revealed lower interfacial tensions of phospholipids with shorter chain lengths. According to the Gibbs approach, a concentration of 150 mM is necessary to stabilize nanoemulsions that are used for further processing of liposomes.<sup>[17]</sup>

However, many questions remain as the material system of phospholipids and fluorocarbons have not been investigated in detail. Additionally, the problems described above – the partial transfer of droplets, the low encapsulation efficiency, droplet size, and stability of w/o emulsions – have not been examined for fluorocarbons. Although both water-in-fluorocarbon (w/fc) and fluorocarbon-in-water (fc/w) emulsions have been investigated, control of droplet size and stability remain difficult. Moreover, w/fc emulsions have not been reported yet using phospholipids exclusively as emulsifiers. fc/w emulsions show, depending on the used fluorocarbon, stable droplet sizes for a couple of weeks<sup>[20,21]</sup> and month<sup>[22]</sup> while w/fc emulsions reveal large droplets in the micrometer range.<sup>[23,24]</sup> Hence, for these nanoemulsions, a better investigation is necessary. This work shows the characterization of w/fc nanoemulsions prepared with PFPH as the hydrophobic phase, as many fluorocarbons have not been extensively described for the preparation of nanoemulsions yet. The influence of different chain lengths, head groups, and various concentrations of phospholipids were investigated and tested for their ability to stabilize water droplets in PFPH. Additionally, the material system was examined with regard to the measurement technology and the difficulties that arise between a fluorocarbon and a water phase. Following these results is the introduction of a novel centrifugation process to produce liposomes from fluorocarbon nanoemulsions, which makes the use of solvents unnecessary and holds the ability for a high encapsulation efficiency.

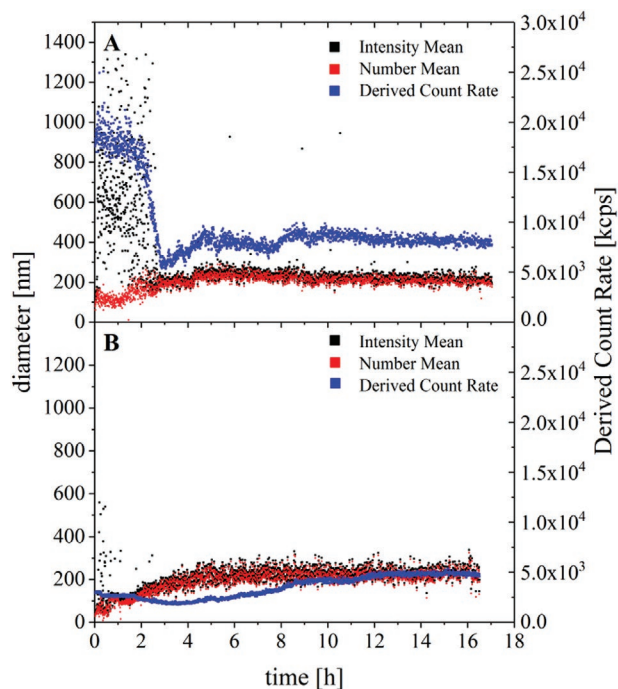
The aim is to answer the question whether the material system of PFPH and phospholipids is applicable for preparation of w/fc nanoemulsions and the centrifugation process

to transfer droplets, respectively. These findings include the question whether common measurement systems such as photon correlation spectroscopy (PCS) are suitable for characterization and which artefacts during measurements can be found. Eventually, w/fc nanoemulsions are planned to be processed as liposomes by centrifugation of droplets.

## 2. Results and Discussion

### 2.1. Short Term Stability of Water/Fluorocarbon Nanoemulsions Stabilized with DPPC

For a better understanding of the fluorocarbon nanoemulsions, the stability of fluorocarbon nanoemulsions was monitored at short term to address effects that appear in the beginning after emulsification and later over a period of several weeks. Thus, for a nanoemulsion stabilized with DPPC, a detailed development of droplet size directly after sonication was monitored by PCS (Figure 1). According to the intensity mean diameter, large droplets of up to 1300 nm appear in the very beginning after emulsification of 1 vol% of a 150 mM stock suspension. After 3 h, the mean size of the droplets remains stable at 200 nm. As a decrease in size is not possible without any energy input, gas bubbles are likely to be the cause and the phenomenon, which is discussed in the following section. Furthermore, based on the values for the number mean diameter, there ought to be a larger number of small droplets in comparison to large droplets. This finding is supported by the course of the derived



**Figure 1.** Monitoring of w/fc nanoemulsion droplet size for 17 h after emulsification, stabilized with DPPC at a concentration of A) 150 mM and B) 300 mM in the stock suspension. Figured are the mean diameter based on intensity (black) and number (red) as well as the derived count rate (blue).

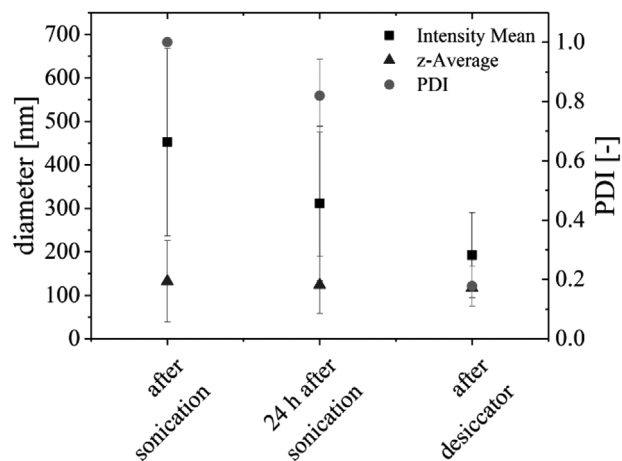
count rate (DCR): in the beginning more scattering centers are detected, after 3 h, the count rate decreases to a stable number. The number mean drop size increases from 100 to 200 nm after 3 h leading to a decrease in the total number of droplets. That leads to the question whether a higher concentration of the stock suspension shows the same phenomenon in the beginning. Indeed, a 300 nm stock suspension of DPPC shows almost no scattering of the intensity-based diameter in the beginning, but starts with a size of 100 nm and increases to 200 nm after 3 h, resulting in the same course as the one observed for the number mean diameter.

These findings lead to the conclusion, that the nanoemulsions are apparently not yet ready for the centrifugation process directly after preparation. Instead, a period of at least 3 h seems to be necessary between preparation and centrifugation of droplets. An increase of the phospholipid concentration to 300 nm leads to smaller droplets in the beginning. However, the PCS is known to be prone to error as the measurement is based on the refractive index (RI). The fluorocarbon PFPH and water have almost the same RI which may lead to an inaccuracy of the results. The consequence is that the z-average, the value usually taken for comparison, is also prone to error and may not be suitable for the fluorocarbon system. A high intensity mean implies a polydisperse sample and thus increases the overall mean value as well. The number mean, however, depicts the course of the droplet stability quite well for this system. Therefore, the data were analyzed under the perspective of measuring errors and specific characteristics of the fluorocarbon system in the following section.

## 2.2. Effects of Gas Bubbles on PCS Measurements

As fluorocarbons absorb a high amount of oxygen,<sup>[25]</sup> gas bubbles could as well affect the large scattering after sonication and lead to measuring errors. Further research revealed that indeed that is the case – gas bubbles float to the surface and thus, are not monitored after 3 h anymore. For this reason, only pure PFPH was sonicated and measured with the PCS afterward, which revealed droplets of up to 800 nm in size. As fluorocarbons accumulate high amounts of oxygen, it is likely that ultrasonic sound releases the dissolved oxygen due to cavitation. In order to remove gas bubbles, the pure fluorocarbon was placed in a vacuum desiccator for different periods of time to detect the changes of bubbles. After 30 min, no significant change of DCR could be detected (data not shown). Hence, the w/fc nanoemulsions were tested for gas removal as well. Experiments were carried out with gas removal 24 h after sonication. In **Figure 2**, the change of the intensity mean diameter, z-average, and PDI are depicted. After sonication, the diameter of the droplets is measured with 450 nm (intensity mean) and 132 nm with a standard deviation of 98 nm for the z-average. The intensity mean decreases to 300 nm after 24 h as does the z-average ( $123 \pm 66$  nm). After vacuum treatment, the size decreases to less than 200 and 117 nm, respectively, which reduces the polydispersity within the sample and explains well the phenomenon of scattering directly after sonication.

These results imply that directly after preparation of nanoemulsions, gas bubbles interfere with the PCS measurement

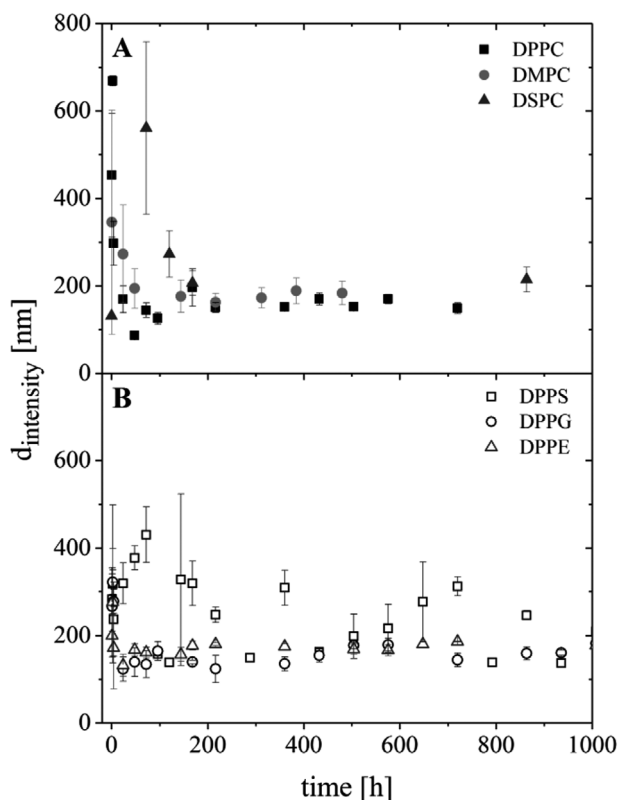


**Figure 2.** Change of the intensity mean diameter (square), z-average (triangle) and polydispersity index (PDI, circle) of a 1% w/fc nanoemulsion stabilized with a stock suspension of 150 mM DPPC without and with treatment in a desiccator. Depicted are the diameters and the PDI directly after sonication, 24 h later, and after 30 min in the desiccator.

leading to artefact data. During the first 24 h, an autonomous degassing effect takes place because the phospholipids do not only stabilize the water droplets but the air bubbles as well. This effect is rather slow and only accelerated by vacuum. Here it is clear to see that the z-average is influenced by the intensity mean diameter and overestimates the droplet size, which makes a comparison directly after sonication inaccurate. To examine a possible effect of gas bubbles on the long-term stability of nanoemulsions and the accompanying detected problems with the PCS data, measurements were carried out for several days.

## 2.3. Long-Term Stability of Water/Fluorocarbon Nanoemulsions Stabilized with Phospholipids of Different Chain Lengths and Head Groups

As the air bubbles only showed an effect directly after sonication, long-term measurements of nanoemulsions stabilized with different phospholipids were investigated as well. Nanoemulsions were stabilized with phospholipids of different chain lengths and head groups (**Figure 3**). Depicted is the intensity mean to compare the results to short-term measurements and see a possible effect of gas bubbles. Droplets of a w/fc nanoemulsion prepared with DMPC, DPPC, and DSPC (with saturated chains with 14:0, 16:0, or 18:0 carbon atoms) all show long-term stability of up to four weeks with an average size based on the intensity mean diameter of 180 nm. The results show that in terms of the evaluation of different head groups, DPPS leads to a larger scattering of the size over a longer period of time while DPPG and DPPE show a constant size of 150 nm for the investigated period of time. This is in accordance with previously published data where nanoemulsions prepared with a high molecular weight fluorocarbon are more stable than others.<sup>[21]</sup> However, directly after the emulsifying process, the water droplets show a size of 450 nm (DMPC, DPPE, DPPG) and up to 700 nm (DPPC, DSPC) indicating an inhomogeneous



**Figure 3.** Long term stability of w/fc nanoemulsion droplets stabilized with phospholipids of different chain lengths and head groups. A) DMPC, DPPC, and DSPC. B) DPPS, DPPG, and DPPE. Each nanoemulsion containing 1 vol% is stabilized with 150 mM phospholipid in the aqueous phase (1.5 mM phospholipids in the final emulsion). Depicted is the mean diameter based on intensity.

nanoemulsion. The previous investigated measuring artefacts due to air bubbles in the beginning are likely to be the cause here as well. Additionally, a homogenous size for DSPC is first measured after 200 h indicating that the autonomous degassing effect takes place before.

In terms of stability, the zeta potential is often used to explain different phenomena of nanoemulsions and their droplet profiles.<sup>[26,27]</sup> Electrostatic interactions, when repulsive, prevent the droplets from coalescence and thus, stabilize the nanoemulsions. Additionally, Pal et al.<sup>[28]</sup> describe the phenomenon of steric hindrance that overcomes electrostatic forces and therefore explain the stability of the studied o/w nanoemulsions even though the zeta potential decreases. Fluorocarbons are non-conductive and thus, measurements are demanding and often lead to inconclusive results. In addition, zwitterionic molecules (phospholipids) show a very weak signal in general, increasing the difficulty to receive an adequate zeta potential. In fc/w emulsions stabilized with 150 mM DPPC, a zeta potential of  $8.29 \pm 0.557$  mV was measured. However, in w/fc emulsions, the zeta potentials are significantly smaller as the charges are partially shielded by the monolayer. Thus, it is assumed that the repulsive electrostatic forces are insignificant. However, the entropic stabilization is likely to be an influencing factor. Oswald ripening, a destabilization effect in the beginning and observed during many emulsification processes,

cannot be studied here to completely explain the increase in droplet size in the beginning as the gas bubbles interfere with the measurement values (cf. Figure 1). No significant influence of Oswald ripening on the droplet size and destabilization is assumed because of the characteristics of the material system. It is known that entrapped insoluble species lower the Oswald ripening rate because of the build-up of osmotic pressure in comparison to soluble compartments.<sup>[29]</sup> Additionally, Delmas et al.<sup>[30]</sup> observed that the similar insoluble compartments when present in membranes, prevent the disappearance of small droplets. As PFPH is very inert and insoluble for both lipophilic and hydrophilic compartments, the same stabilizing effect is expected here: the insoluble lipids do not interfere with the fluorocarbon phase or the hydrophilic water phase and thus, contribute to the long-term stabilization of the nanoemulsion.

To conclude the performed experiments, they reveal that a statement about the size of droplets gained with the PCS directly after preparation is difficult and inaccurate. Typical stability analysis such as zeta potential or rheological behavior are inconclusive for the used material system. However, long-term stability is possible and shows stable, reproducible, and coherent results due to entropic stabilisation based on the insolubility of lipids and the aqueous phase, excluding Ostwald ripening. A transfer of droplets is, based on these data at this point, not favorable with freshly prepared nanoemulsions. Moreover, the refractive index remains a problem with PCS measurements: gas bubbles especially influence the intensity mean, changing the z-average for the worse and only leaving the number mean as a possible value for comparison. Therefore, an additional measuring system was chosen that is independent of gas bubbles and the refractive index. Using an X-ray beam, gas bubbles are not detected but only the droplets due to their density difference to the surrounding media.

#### 2.4. Influence of Chain Lengths and Phospholipid Concentration on Liposomes

For SAXS measurements of a w/fc emulsion, the water droplets are detected as perforations of the continuous surrounding phase of higher density fluorocarbons which are more intensive scattering centers. Thus, the w/fc emulsion does not describe a suspension of nanoscale scattering centers as would be the case for an o/w emulsion, but rather a continuum of more electron dense material with nanoscale pores making a measurement rather difficult. Liposomes, however, can be examined as with phospholipids stabilized water droplet in a surrounding aqueous phase. Because of a thin oil film between the layers, placing itself around during the centrifugation step, the electron density difference is high enough for a significant scattering behavior. Liposomes were produced via centrifugation according to the method described below; see Figure 6. Several advantages regarding the centrifugation process were achieved by using a fluorocarbon: a) hindrance by interfacial tension during phase transfer is compensated by the much stronger density difference between the hydrophobic and aqueous phase ( $\Delta\rho \approx 1 \text{ g cm}^{-3}$ ,  $\rho_{\text{water}} = 0.998 \text{ g cm}^{-3}$ ,  $\rho_{\text{fluorocarbon}} = 2.03 \text{ g cm}^{-3}$ ) enabling transfer; b) gel formation is no longer detectable. Because of the heavier

**Table 1.** PCS measurement data and SAXS results for liposomes. Listed are the z-average, the number and intensity mean in nm of the formed liposomes, produced with phospholipids of different chain lengths, and a concentration of 150 mM in the stock suspension as well as different concentrations of DPPC. The last column shows the results measured by means of SAXS.

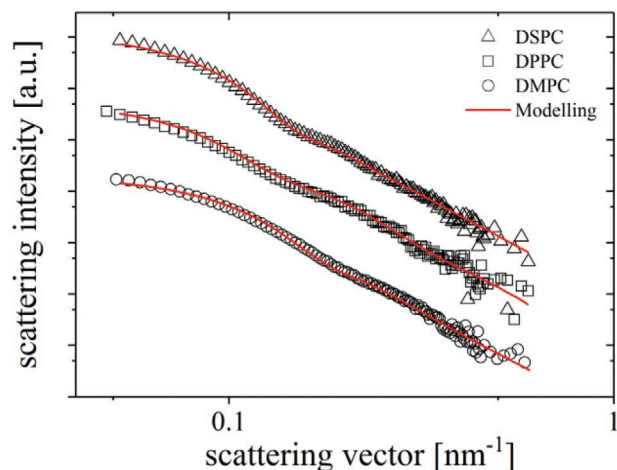
Phospholipid [150 mM]	Z-average [nm] ± standard deviation	Number mean [nm] ± standard deviation	Intensity mean [nm] ± standard deviation	Mean diameter (SAXS) [nm]
DMPC	158.3 ± 1.5	62.7 ± 11.3	392.8 ± 53.2	47.8 ± 10.9
DPPC	132.9 ± 0.3	64.2 ± 10.7	260.5 ± 74.9	60.9 ± 15.8
DSPC	538.8 ± 60.2	55.6 ± 10.2	2342 ± 385.4	57.8 ± 11.9
DPPC concentration [mM]				
25	407.0 ± 18.3	59.6 ± 4.8	1599.6 ± 144.9	–
150	132.9 ± 0.3	64.2 ± 10.7	260.5 ± 74.9	60.9 ± 15.8
300	201.7 ± 4.9	65.5 ± 4.1	1345.0 ± 195.2	64.1 ± 16.1

hydrophobic phase, the aqueous droplets experience buoyancy instead of sedimentation during centrifugation which c) enables to easily harvest the liposomal suspension from the top.

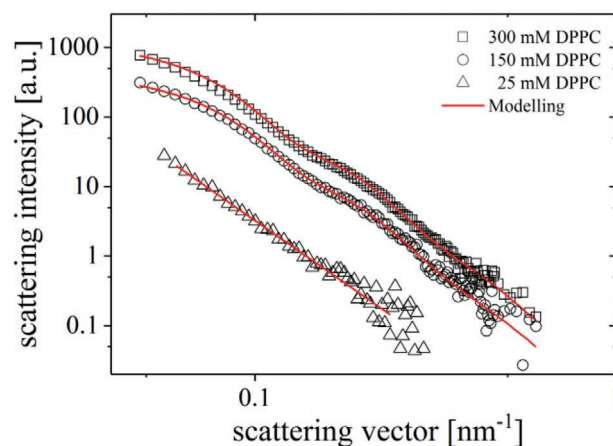
As gas bubbles influence the intensity mean, hence, the z-average; SAXS measurements were performed in order to examine the suitable comparison of the number mean value (cf. Table 1). Additionally, the experiments allow to examine whether liposomes can be formed at all even though previous experiments do not suggest a production with freshly prepared nanoemulsions (cf. Figures 1 and 3). Liposomes stabilized with DMPC, DPPC, and DSPC were measured by means of SAXS. Furthermore, different concentrations of DPPC were examined.

As the particle number concentration and electron density difference is assumed to be the same for all samples and the scattering intensity is additionally proportional to the particle volume (cf. Equation (2)), it is likely that the size of droplets increase with longer chain lengths as well. Hence, the influence of chain lengths was additionally investigated on the formation of liposomes by means of SAXS (Figure 4). The results show a similar behavior in their change of intensity with an increasing chain length. At the same time, the size of the measured liposomes is enlarged. Liposomes formed by DMPC

reveal a size of 48 nm, DPPC a size of 61 nm, and DSPC of 59 nm. All scattering curves can be modeled by a high quality of the fit. The fractal dimension of the surface  $D_{sf}$  is slightly smaller than two, indicating a weak diffusive interface. While DMPC stabilized liposomes are much smaller, DPPC and DSPC do not change the liposome size. As the concentration of phospholipids stabilizing the nanoemulsions showed an effect (cf. Figure 1), three different concentrations were tested on their influence on liposome production (Figure 5). A change of concentration shows that a 25 mM stock suspension leads to liposomes bigger than 130 nm, the detection limit of the SAXS, as the amount of phospholipids are not enough to stabilize smaller droplets.  $D_{sf}$  describes a rough surface with bigger structures which leads to the conclusion, that the thin and smooth fluorocarbon film is not present here. A concentration of 150 mM of phospholipids results in liposomes of 61 nm and the twofold amount of phospholipids in 64 nm. Furthermore, the scattering intensity for liposomes from 300 mM is larger than that of the lower concentrations. It can be concluded that with 300 mM DPPC as a stock suspension, it might be possible to form more liposomes during the transfer while the size is process-specific.



**Figure 4.** Scattering intensity over scattering vector from SAXS measurements of liposomes consisting of phospholipids with different chain lengths, depicted with an offset for clarity. A modeling tool for polydisperse hard spheres was applied to determine the liposome size.



**Figure 5.** Scattering intensity over scattering vector of different liposomes prepared with different concentrations of DPPC in the stock suspension. A modeling tool for polydisperse hard spheres was applied for the measurable concentrations of 150 and 300 mM to determine the liposome size; a Porod fit was applied for the lowest concentration of 25 mM.

These results show that the formation of liposomes via centrifugation needs a certain amount of phospholipids for a stable product. This finding is in accordance with data gained from previous tensiometry experiments (see Ref. [17]) specifically performed between the used fluorocarbon and a phospholipid suspension. SAXS measurements reveal the small enlargement of liposomes the longer the chain lengths (from DMPC to DPPC). Similar to the tensiometric influence, the chain length changes the size as observed here. Furthermore, SAXS measurements support the finding that gas bubbles do not have an influence on the production of liposomes in general. However, PCS data need to be looked at with caution as the intensity mean and z-average falsify the actual size due to measurement artefacts (cf. Table 1). The droplet size determination with the PCS can be misleading regarding a complete determination of the size distribution of complex systems and that the z-average or intensity mean are not comparable values.<sup>[31]</sup> The number mean, however, is in a similar size range for the produced liposomes (50–70 nm). The larger sizes in comparison to SAXS data is due to the measuring system as the PCS gives the hydrodynamic radius.

### 3. Conclusion

This work shows the characteristics of w/fc nanoemulsions as a first step to produce liposomes from nanoemulsions. Applying perfluoroperhydrophenanthrene as the hydrophobic phase led to stable nanoemulsions over a period of 4 weeks. Different phospholipid chain lengths showed no influence on the average droplet size determined by PCS over 4 weeks. A variation of head groups revealed larger scattering of the droplet size for the phospholipid DPPS that are likely due to gas bubbles. As fluorocarbons are natural oxygen accumulators, gas bubbles, carried into the fluorocarbon by sonication, influence the measurements with the PCS especially directly after preparation. Evaporation eliminates this effect, however, during centrifugation of water droplets for liposome production, gas bubbles do not influence the final product. The data gained from PCS first led to the conclusion, that the freshly produced nanoemulsions are not suitable for liposomes production in the sense that a post-treatment to remove gas bubbles is needed. However, these experiments also reveal the limits of different measurement systems such as the PCS for complex systems. Here, the refractive index of the fluorocarbon and water are almost identical. As PCS measures particle size based on photon intensity fluctuation for which the RI is crucial, errors are amplified due to the small difference in RI. An overestimation of droplet size, as shown here, is likely, as the calculation is based on the diffusion coefficient and thus, an indirect method. Additionally, the dynamic viscosity plays a key role and the present system of fluorocarbons, phospholipids, and buffer can influence the calculation. The final hydrodynamic radius gained from the PCS measurements is calculated with all of the factors from above and thus, an overestimation is probable. However, the PCS allows a quick overview of the system and is suitable for long-term measurements which is not possible with SAXS as a time resolved measurement requires measuring time and resources. Here, information about the size is given directly

after preparation and shows that the produced nanomemulsions are suitable for a droplet transfer to form liposomes with an average size of 60 nm, and that a certain concentration is needed to form liposomes at all. SAXS experiments revealed that indeed the intensity mean falsifies the mean value of nanoemulsion droplets and liposomes produced from a fluorocarbon system, but the number mean gives equivalent results. This work gives an insight into the measuring difficulties of fluorocarbon nanoemulsions and phospholipids and how these systems can yet be analyzed. What remains to be achieved is a detailed analysis of liposomes: how long are they stable, is sterile filtration of the product possible, how much active pharmaceutical ingredient can be encapsulated with this method? These questions have yet to be answered, however, the method holds the ability for a high encapsulation efficiency.

### 4. Experimental Section

**Materials:** All phospholipids used for the experiments were provided by Lipid (Ludwigshafen, Germany). The synthetic phospholipids 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), and 1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine (DSPC); 1,2-dipalmitoyl-*sn*-glycero-3-phosphorac-glycerol (DPPG), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE), and 1,2-dipalmitoyl-*sn*-glycero-3-phospho-L-serine (DPPS) were received in powder form. Perfluoroperhydrophenanthrene (PFPH) was purchased from F2 Chemicals (Preston, UK). The long-chain and cyclic molecule had a density of 2.03 g cm<sup>-3</sup> and a dynamic viscosity of 28.4 mPa s.

**Preparation of Lipid Suspensions:** Because of the very low solubility of phospholipids in PFPH (beyond detection: minimally <10<sup>-6</sup> mM or lower), stock suspensions were prepared with phosphate buffer (15 mM) if not stated otherwise. Stock suspensions for all utilized phospholipids were prepared in different concentrations depending on the experimental set up (from 25 to 300 mM). The acquired amount of phospholipids was weighed into a 2 mL reaction tube (Eppendorf AG, Hamburg, Germany) and dissolved in 1 mL phosphate buffer (15 mM). For better dissolution of the lipids, ultrasonic sound with a 100% cycle and 10% (40 W) output control of the nominal converter amplitude (with sonotrode Digital Sonifier 450, Branson Ultrasonic Corporation, Danbury, USA, with a nominal power of 400 W) was applied for 10 s as a premixing followed by a 50% sonication cycle and 10% output control for 10 min. The temperature was controlled with an ice bath to compensate the heat input from the sonication procedure and kept at 30 °C during the preparation. Denaturation of phospholipids could be neglected because of the temperature control and the low energy input. As described by Juliano et al.,<sup>[32]</sup> radicals of lipids during sonication were below 2 μM during a sonication frequency of 20 kHz and full power settings for 6 min. Here, only 10% of the nominal power was used with a cycle of 50%.

**Preparation of w/fc Nanoemulsions:** The w/fc nanoemulsion contained 1 vol% of dispersed phase, prepared as stated above. Thus, the viscosity of the nanoemulsion changed slightly to a new viscosity  $\eta_1 = 29.1$  mPa s based on Einstein's equation<sup>[33]</sup> with  $\eta_1 = (1 + 2.5 C) \eta_0$  where C is the dispersed phase's volume fraction. The total volume of the emulsion was 1 mL with PFPH as the continuous phase. As homogenizers operate in a high pressure range and fast mass flows, it did not allow the phospholipids to adsorb at the interface between the aqueous phase and the fluorocarbon because of the short residence time.<sup>[34,35]</sup> Thus, ultrasonic sound was chosen for emulsification because of a short energy input, yet long residence time. Ultrasonic sound (Digital Sonifier 450, Branson) emulsified the dispersed phase with the PFPH; the settings for emulsification were the same as for the preparation of the stock suspension. The concentration of phospholipids needed for

stabilization of water droplets and being able to form a bilayer from phospholipids in excess came from previous tensiometry experiments (see Ref. [17]) and was found to be at least 150 nm. For the most part, experiments were carried out with 150 nm of phospholipids in the stock suspension. Stock suspensions (25 and 300 nm) served as comparison.

**Size Determination of Nanoemulsion Droplets and Liposomes:** The droplet size of the nanoemulsions as well as the liposomes was measured with photon correlation spectroscopy (PCS; Zetasizer nano ZSP, Malvern Instruments, Worcestershire, UK). Each sample was measured 4 times with 11 runs within each measurement at room temperature (20.0 °C). The results of the PCS were depicted as the z-average, the mean diameter based on the change in intensity, the polydispersity index (PDI), and the derived count rate (DCR). The DCR is the scattering intensity, compensated for the signal attenuation during measurements, given in photon kilocounts per second. The z-average and PDI were both calculated from an autocorrelation function, where the PDI gave the size distribution based on the deviation between the autocorrelation function to a unimodal size function.

In addition to the PCS, specific samples were measured with small angle X-ray scattering (SAXS; Xenocs Xeuss 2.0, Sassenage, France). Measurements were performed in a gel-holder with a sample to detector distance of 1750 mm. The collection of scattering data with the 300 K-S detector was set to 1800 s. The intensity  $I(q)$  was measured by a function of the scattering vector  $q$  with

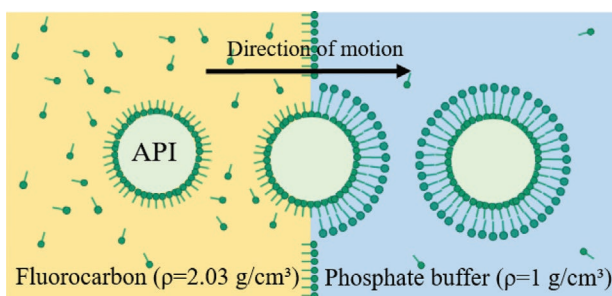
$$q = 4\pi/\lambda \cdot \sin(\theta/2) \quad (1)$$

where  $\theta$  is the scattering angle and  $\lambda$  the wavelength of the Cu-K $\alpha$  X-ray beam ( $\lambda = 0.154$  nm). The intensity is also a function of the particle volume  $V_p^2$  and the particle number concentration  $NV^{-1}$ , where the requirement for a successful measurement is the electron density difference  $\Delta\rho_e^2$  of the two investigated phases within the system:

$$I(q) \propto NV^{-1} \cdot V_p^2 \cdot \Delta\rho_e^2 \quad (2)$$

For data evaluation, a modeling tool for polydisperse, hard spheres by the software IgorPRO (version 8.04) were used. The data evaluation included the analysis of the Guinier-regime to obtain information about the particle size as well as the Porod-regime to describe the outer particle structure. Further information about the sample preparation, measurement techniques, as well as the data evaluation can be found elsewhere.<sup>[36–38]</sup>

**Preparation of Liposomes:** For the production of liposomes, a second aqueous phase, phosphate buffer (15 mM, if not stated otherwise), incorporated the aqueous droplets covered with phospholipids from the w/fc nanoemulsion after centrifugation. Both phases, the nanoemulsion and the aqueous phase, were at a volume ratio of 1:1, via centrifugation (Eppendorf Centrifuge 5430 R, Eppendorf AG, Hamburg, Germany) at 0 °C and 4000 g for 30 min; the aqueous droplets from the w/fc nanoemulsion



**Figure 6.** Schematic illustration of the centrifugation process to form a liposome. A w/fc nanoemulsion contains inverse micelles with the encapsulated model API and freely available phospholipids that adsorb at the interface. The applied centrifugal force directs the droplets toward the lighter phase (= buffer), thereby covering them with an additional monolayer of phospholipids.

start migrating to the upper phase. Due to a large excess of PLs in the nanoemulsion, not all PLs are bound at the interfaces of the inverse micelles forming the w/fc nanoemulsion. The remaining PLs adsorb at the interface between the nanoemulsion and the upper aqueous phase. If an inverse micelle contacts the interface due to the applied centrifugal forces, the interfacially adsorbed PLs form a second monolayer around the droplet. A liposome is formed by the transfer to the lighter aqueous phase (Figure 6). A return to the fluorocarbon phase is impossible due to the density difference. This process holds the capability of encapsulating a high amount of active pharmaceutical ingredients.

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## Conflict of Interest

The authors declare no conflict of interest.

## Keywords

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