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Liposome Size Analysis by Dynamic/Static Light Scattering upon Size Exclusion-/Field Flow-Fractionation

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The aim of the current study was to analyse the particle size distribution of a liposome dispersion, which contained small egg phosphatidylcholine vesicles and had been prepared by high-pressure homogenisation, by various size analysis techniques. Such liposomes were chosen since they can be looked at as a prototype of drug nano-carriers. Three sub-micron particle size analysis techniques were employed: (1) fixed-angle quasi-elastic laser light scattering or photon correlation spectroscopy (PCS), (2) size exclusion chromatographic (SEC) fractionation with subsequent (off-line) PCS size-analysis and quantification of the amount of particles present in the sub-fractions, and (3) field-flow-fractionation coupled on-line with a static light scattering and a refractive index (RI)-detector. When designing liposome-based drug carrier systems, a reliable and reproducible analysis of their size and size distribution is of paramount importance: Not only does liposome size influence the nanocarrier's *in-vitro* characteristics such as drug loading capacity, aggregation and sedimentation but also it is generally acknowledged that the pharmacokinetic behaviour and biodistribution of the carrier is strongly size-dependent. All three approaches of liposome size analysis used here were found to yield useful results, although they were not fully congruent. PCS indicated either a broad, mono-modal, log-normal size distribution in the range of below 20 to over 200 nm in diameter, or alternatively, a bimodal distribution with two discrete peaks at 30 to 70 nm and 100 to over 200 nm. Which of the two distribution models represented the best fit depended primarily on the data collection times used. In contrast, both fractionating techniques revealed a size distribution with a large, narrow peak well below 50 nm and a minor, broad, overlapping peak or tail extending to over 100 nm in diameter. The observed differences in liposome size distribution may be explained by the inherent limitations of the different size analysis techniques, such as the detection limit and the fact that PCS is overemphasizing bigger particle sizes.

Keywords: Liposome, Drug Carrier, Particle Size, Photon Correlation Spectroscopy, Quasi-Elastic Light Scattering, Static Light Scattering, Flow Field Flow Fractionation, Size Exclusion Chromatography.

RESEARCH ARTICLE

1. INTRODUCTION

Liposomes or phospholipid vesicles emerged during the past 25 years as versatile and potent carriers for drugs and diagnostics, both, low-molecular weight compounds and peptide-/protein-drugs and genetic material (Fig. 1). The size distribution of liposomal drug carriers is of key interest because size not only affects the vesicle's *in-vitro* characteristics such as the amount of drug that can be accommodated, aggregation and sedimentation behaviour.[1] but also its *in vivo* behaviour such as circulation time in the blood-stream upon i.v.-injection, and consequently also biodistribution, especially their targeting to solid tumors. The disappearance of liposomes from blood circulation is primarily due to uptake by the mononuclear phagocytic system (MPS), which is mediated by various blood proteins, among others complement. A decrease in liposome size reduces complement recognition.[2] This does not only hold true for plain phospholipid vesicles but also advanced lipid compositions. As reported by Liu et al.:[3] ganglioside GM1-containining liposomes avoid the MPS, but only if liposome size is between 70 and 200 nm. The same holds

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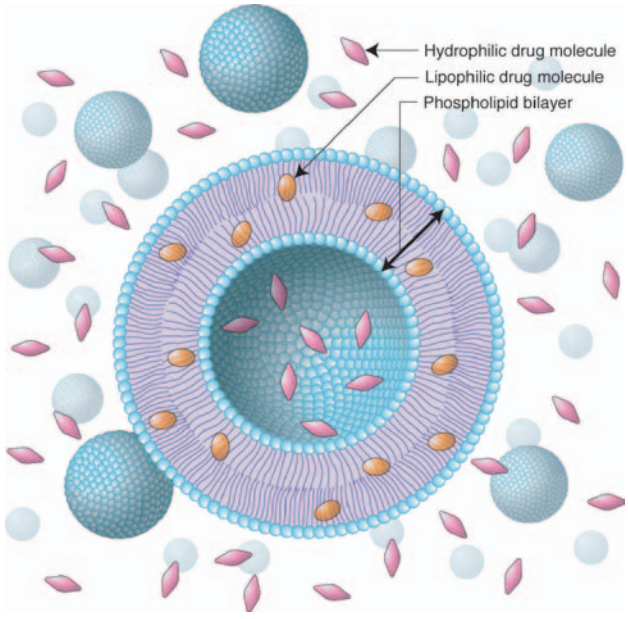

Fig. 1. Schematic drawing of small unilamellar liposome-drug carrier with hydrophilic drug in the aqueous compartments and lipophilic drug incorporated into the phospholipid bilayer.

true for PEG-covered (sterically shielded) liposomes.[4, 5] Liposomes smaller than 70 nm can escape through the fenestrae of the liver sinus.[3] For liposomes bigger than 200 nm, the steric shield appears to be less efficient. Smaller sizes on the other hand are desired when it comes to passive targeting due to the so-called enhanced permeability and retention effect[6] (EPR, review in Ref. [7]), where extravasation of liposomes into solid tumors is essential. Liu et al.[8] demonstrated that not only mean liposome size but also size distribution matters. The aim of the current study was to investigate and compare the potential of different techniques for resolving the particle size distribution of nano-scaled drug carriers (liposomes).

(1) Photon correlation spectroscopy (PCS),

(2) size exclusion chromatographic (SEC) fractionation followed by PCS-analysis and quantitative enzymatic phosphatidylcholine (PC) assay and

(3) flow field flow fractionation (FFFF) coupled with RI- and static light scattering (LS) detection were applied.

2. MATERIALS AND METHODS

2.1. Materials

Egg phosphatidylcholine (egg PC, E-80, egg phospholipid with at least 80% phosphatidyl choline (PC)) was delivered by Lipoid GmbH, Germany. Phospholipids B test kit (Wako Chemicals USA, Inc., Richmound, VA, USA) was used for PC quantification. Triton X-100 was obtained from Sigma-Aldrich Chemie (Steinheim, Germany). Water was freshly distilled and media filtrated through 0.22 μm pore size filters before use.

2.2. Liposome Preparation

The 100 mg/g liposome dispersion was produced by dispersion of 4.0 g phospholipids (Lipoid E-80 from Lipoid GmbH, Ludwigshafen, Germany) in freshly filtrated (0.22 μm filter) 10 mM $NaNO_3$-solution as described earlier.[9] In brief: the lipid was blended with medium and left to swell for 30 min at room temperature before homogenisation using a lab-scale high-pressure homogeniser (APV Gaulin, micron lab 40 from APS Homogeniser Group, Lübeck, Germany) at intermediate pressures of 70 MPa (700 bar) for 10 repetitive cycles. The liposome dispersions were autoclaved in 10-ml injection vials for 15 min at 121 °C in a steam sterilizer (CertoClave Typ CV-EL 10L/12L, CertoClav Sterilizer GmbH, Traun, Austria), and stored at 4 to 8 °C until analysis.

2.3. Photon Correlation Spectroscopy (PCS)

The intensity of light scattered by dispersed particles tends to fluctuate with time, and PCS measures the scattered light intensity at a given, in our case 90°, angle. These fluctuations in the intensity versus time profile are caused by the constant changing of particle positions brought about by Brownian motion. In PCS, also called dynamic light scattering (DLS), a correlation function is calculated from the intensity versus time profile. This exponentially decaying correlation function is analysed for characteristic decay times, which are related to diffusion coefficients and then by the Stokes-Einstein equation to a particle radius. PCS is generally regarded suitable to obtain the particle size distribution for samples with particles ranging from a few nm to several microns. We used a NicompTM model 380 particle sizing system (Santa Barbara, CA, USA) with software version C-370 V-1.51a, and equipped with a fixed 90° external fiber angle and a 632.8 nm, 5 mW He–Ne laser. According to the user manual[10] the following quality parameters may be indicative for the quality of the fit: For distributions fitted by using the Gaussian distribution model, the parameter χ^2, describes the quality of the fit and should ideally range between 0 and 2; a value >3 indicates a poor fit. The factor *"baseline adjustment"* is used to adjust for baseline noise caused by aggregates or dust particles, and should be <0.03 for the Gaussian distribution model. For the so-called "Nicomp-model" the fit error should be not more than 1.5 and the residual should ideally range between 0 and 3, residuals over 10 indicate a significant amount of off-scale particles.

In order to avoid any contamination with dust, sample preparation was carried out in a clean area using particle-free equipment as described in Ref. [11]. In brief: all handling was done in a laminar air-flow bench, test tubes were submersed in particle-free water and sonicated for 15 min in an ultrasonic bath and rinsed with freshly filtered (0.2 μm pore size syringe filter) water prior to use. The liposome-dispersion was diluted empirically with

freshly filtrated medium until an intensity of 250–350 kHz was achieved. For the non-fractionated samples the PCS-analysis approach that had earlier been found useful when studying binary model blends of latex particles[11, 12] was used. In brief: ten parallels were run, the fit model that appeared most frequently was identified and parallels with other distribution models and/or poor fit parameters were discarded. Longer run times and/or different channel widths were tried in order to improve the fit quality. For the fractionated samples a run time of 30 min was used and the channel width was set to auto. The analyses were run in vesicle mode and the number-based and the mass-(or volume-) distribution, which were calculated from the intensity reading are given for comparison. For all analyses the average from three runs was used, unless indicated otherwise.

2.4. Size Exclusion Chromatographic (SEC) Fractionation

Size exclusion chromatographic (SEC) fractionation was carried out as described in Ref. [12]. In brief: Sephacryl S-1000 (Amersham Biosciences, Uppsala, Sweden) was packed into XK 16/70 columns as recommended by the manufacturer to obtain a bed height of 50 cm and a bed volume of 95 ml. In order to minimize loss of lipid due to adsorption to the gel material, the columns were pre-saturated by running a liposome sample of the kind to be analysed.[13] 2 ml aliquots of the liposome dispersion were injected and eluted at a flow rate of 0.4 ml/minute. The progress of elution was followed by optical density measurements at $\lambda = 254$ nm and 3.0 ml fractions were collected (ÄKTA Prime, Amersham Biosciences, Uppsala, Sweden).

2.5. Quantitative Enzymatic Phosphatidylcholine Assay

The amount of liposomes was determined in terms of quantification of the phosphatidylcholine (PC) content using a Phospholipids B test kit (Wako Chemicals USA, Inc., Richmond, VA, USA) as described by Grohganz et al.[14] with modifications. In brief: 50 μl each of the standards, samples and controls were dissolved in Tris buffer with 10% Triton X-100® to make up a final PC-concentration within the concentration range of 100 to 800 μg/ml. Samples and standards were incubated in a water bath (Certomat® WR, Braun Biotech International GmbH, Melsungen, Germany) at 80 °C and shaking at 80 rpm for 3 min. Samples were thereafter cooled to room temperature. 50 μl of standards, samples and control solutions were mixed with 250 μl Phospholipid B reagent-solution in a Microplate Costar 96 well. Samples were analysed in triplicate. The plate was incubated at 37 °C for 45 min at 200 rpm. A reddish colour appeared, due to formation of a phenole-4-aminoantipyridine-complex (Wako Phospholipids B user manual). Absorbance was measured (Dynatech MR 7000 (Software; Biolinx version 2.0), Dynatech Laboratories Inc., VA, USA) using dual read mode, test filter 492 nm and reference filter 690 nm.

2.6. Flow Field Flow Fractionation Coupled with RI- and Static Light Scattering Detection

Analyses were performed using the Eclipse 2 Instrument system (Wyatt Technology Europe GmbH, Dernbach, Germany) with a DAWN EOS 18 angle detector ranging from ~10° to ~160°, with laser wavelength of 690.0 nm, and an Optilab rEX, differential refractometer (RI detector). The mobile phase used was 10 mM $NaNO_3$-solution. A 350-μm spacer was applied and a main flow of 1.0 ml/min. A cross flow gradient was applied: the cross flow was reduced from 0.8 to 0.15 ml/min during the first 40 minutes and kept at this level for the last 10 minutes. All measurements were done without sample dilution; 10 μl liposome samples containing 100 mg egg PC/ml medium were injected using an injection flow of 0.2 ml/min. The focus-flow was 3 ml/min with one minute post-focusing. Software from Wyatt Technologies (ASTRA 5.1.5 and Eclipse) was used to analyze the data. Light scattering data were evaluated using the Zimm-method.[15]

3. RESULTS

3.1. Liposome Sizes Measured by Photon Correlation Spectroscopy (PCS)

The size distribution of E-80 liposome dispersions was analysed upon empirical dilution of the samples down to suitable concentrations with medium, i.e., until count-rates of approx. 250 to 350 kHz were obtained. In a first attempt "routine conditions" i.e., a data collection time of 5 minutes and a fixed channel width of 5 μs were chosen. The fit quality parameters of these measurements are summarised in Table I. The very low Chi-squared values of

Table I. PCS-analysis of egg PC liposomes with data collection times of 5 minutes and channel widths of 5 μs; fit parameters.

	Counts in channel	Gaussian fit		Nicomp fit	
Cycle no	no. 1 (K)	Chi squared	Baseline adjust (%)	Fit error	Residual
1	110.8	0.64	0.06	2.81	27.35
2	106.3	0.75	0.03	2.57	23.32
3	105.5	0.63	0.01	2.51	0.00
4	111.8	1.23	0.00	2.81	14.08
5	105.1	0.74	0.00	2.63	0.00
6	104.7	0.50	0.01	2.13	0.00
7	104.9	0.53	0.07	2.62	0.00
8	104.9	0.54	0.00	2.41	0.00
9	105.6	0.94	0.00	2.05	21.28
10	104.4	0.87	0.03	2.95	0.00
Mean	106.4	0.74	0.02	2.55	8.60
Std dev	2.6	0.23	0.03	0.29	11.56

Table II. PCS-analysis of egg PC liposomes with data collection times of 5 minutes, mean diameters and distribution widths (mean ± SD, $n = 8$, and 4, respectively).

Channel width	Number Weighted Dn (nm)	Number Weighted Distr. width (nm)	Volume Weighted Dv (nm)	Volume Weighted Distr. width (nm)	Intensity Weighted Dz (nm)	Intensity Weighted Distr. width (nm)	Polydispersity index Dn/Dv
5 μs	32.1 ± 0.8	16.1 ± 0.2	76.9 ± 0.5	38.5 ± 0.5	85.0 ± 0.4	42.6 ± 0.6	0.42
9.7 μs	36.6 ± 2.0	17.4 ± 0.4	80.4 ± 0.4	38.1 ± 1.4	87.5 ± 0.5	41.5 ± 1.5	0.46

0.74 ± 0.23 ($n = 10$) as well as acceptable baseline adjust values (eight of ten parallels below limit) indicated good fits when using the mono-modal (Gaussian) distribution model. The Nicomp fits, in contrast, resulted in unacceptably high fit errors and/or high residuals. Therefore, the Gaussian fit appeared preferable over the Nicomp fit and was used to evaluate the data. The derived volume-(mass-) weighted distribution followed a log-normal distribution model and ranged from below 20 to above 200 nm with a peak at 70 nm. The calculated mean diameters and distribution widths of the eight valid parallels are summarised in Table II.

In a second attempt the data collection time of five minutes was maintained, but the instrument was allowed to select the channel width automatically, which resulted in a channel width of 9.7 μs. The fit parameters of the runs are summarised in Table III. Under these conditions the baseline-adjust values always were 0.00, but six of the ten runs had unacceptably high Chi squared values. Nevertheless appeared the Gaussian fit still more appropriate than the Nicomp fit because for the latter all ten runs showed unacceptably high fit errors. The calculated mean diameters and distribution widths of the four valid parallels are summarised in Table II.

Finally the data collection time was prolonged until a minimum of 1000 k (one million counts) in channel no. 1 was achieved, which resulted in a data collection time of 35 minutes. Again, a channel width of 9.7 μs was selected automatically. The fit quality parameters of the runs are summarised in Table IV. Obviously, with prolonged data collection time, a multimodal Nicomp distribution model becomes preferable over the Gaussian distribution model as indicated by very high Chi-squared values for all ten parallels, but acceptable fit errors (<1.5) for seven of the parallels. Even when discarding parallels no. 2, 5, and 10 because of too high fit errors and/or residuals and parallels no. 4 and 8 because they yielded tri-modal rather than bimodal results, still five out of ten parallels are regarded valid and they consistently indicate a bimodal distribution model. The size distributions of these parallels are presented in Figure 2.

3.2. Liposome Sizes Measured by Photon Correlation Spectroscopy (PCS) upon Fractionation by Size Exclusion Chromatography

When fractionating the egg PC vesicle dispersion by size exclusion chromatography and analysing each sub-fraction for its liposome content and particle size, as first described in Ingebrigtsen et al.,[11] size distribution plots as shown in Figure 3 were obtained. The size distribution represents a surface-weighted distribution. It showed just one peak, which was rather steep to the lower size end and very narrow, but showed a broad tail (shoulder) towards the upper size end. It covered in total a diameter range from as small

Table III. PCS-analysis of egg PC liposomes with data collection times of 5 minutes and channel widths of 9.7 μs; fit parameters.

Cycle no	Counts in channel no. 1 (K)	Gaussian fit Chi squared	Gaussian fit Baseline adjust (%)	Nicomp fit Fit error	Nicomp fit Residual
1	184.5	3.10	0.00	3.68	0.00
2	192.5	2.91	0.00	4.25	0.00
3	194.1	5.22	0.00	3.76	0.00
4	191.2	2.29	0.00	3.41	0.00
5	193.8	3.63	0.00	3.63	0.00
6	195.4	4.20	0.00	3.60	0.00
7	197.2	1.59	0.00	2.92	15.78
8	196.7	1.10	0.00	3.12	0.00
9	197.2	4.96	0.00	3.51	0.00
10	205.9	6.16	0.00	3.22	0.00
Mean	194.9	3.52	0.00	3.51	1.58
Std dev	5.4	1.63	0.00	0.37	4.99

Table IV. PCS-analysis of egg PC liposomes with data collection times of 35 minutes and channel widths of 9.7 μs; fit parameters.

Cycle no	Counts in channel no. 1 (K)	Gaussian fit Chi squared	Gaussian fit Baseline adjust (%)	Nicomp fit Fit error	Nicomp fit Residual
1	1111.9	19.70	0.00	1.14	0.24
2	1112.2	21.77	0.00	1.57	8.38
3	1144.7	18.36	0.00	1.27	0.00
4	1121.8	16.33	0.00	1.31	0.00
5	1122.9	13.73	0.00	1.64	0.00
6	1126.4	17.90	0.01	1.52	0.00
7	1110.1	15.51	0.00	1.34	0.00
8	1099.1	7.97	0.00	1.44	0.00
9	1078.8	13.55	0.00	1.50	0.00
10	1067.6	17.76	0.00	1.62	15.43
Mean	1109.6	16.26	0.00	1.44	2.41
Std dev	22.8	3.87	0.00	0.16	5.28

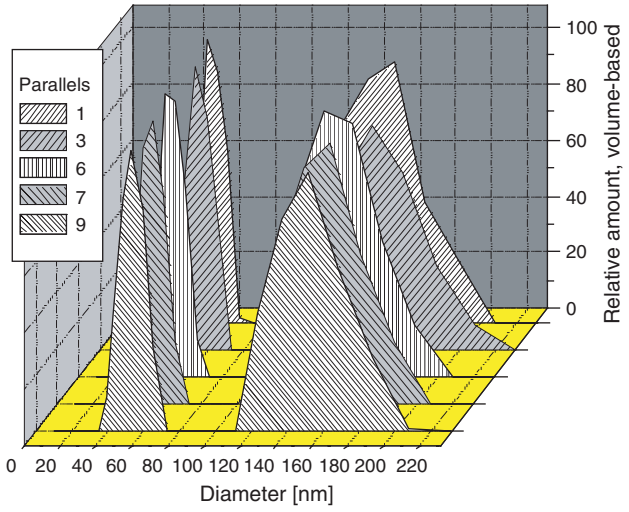

Fig. 2. Size-distributions of egg PC liposome dispersion as obtained by photon correlation spectroscopy (PCS) using data collection times of 35 minutes and channel widths of 9.7 μs, given as volume-weighted distributions (five independent measurements).

as 7 nm to over 130 nm. More than 80% of the vesicles were detected in the fractions with mean particle sizes below 30 nm and the peak of the distribution typically was found below 20 nm. Results from earlier studies[11] of particle size analyses of diluted vesicular phospholipids gels (VPGs) prepared in a similar manner and of the same lipids appear to support this observation.

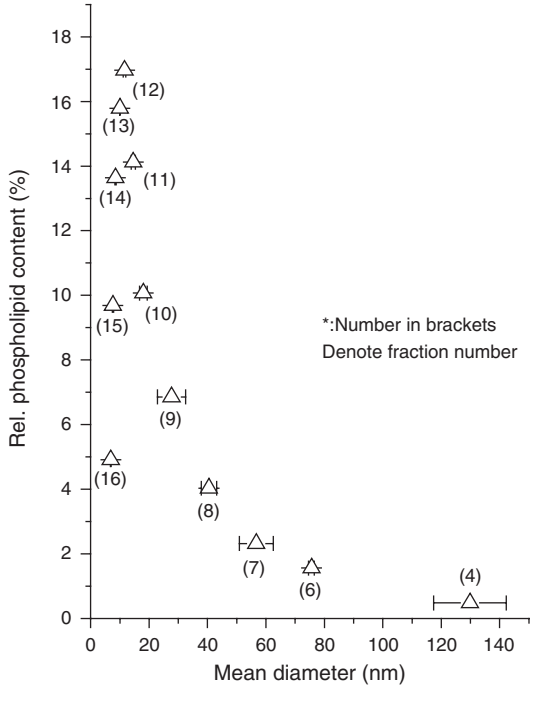

Fig. 3. Size-distribution of egg PC liposome dispersion as obtained by size exclusion chromatography and subsequent photon correlation spectroscopy and quantitation of phosphatidylcholine-content (relative PC content versus diameter in nm (mean ± SD, ($n = 34$)).

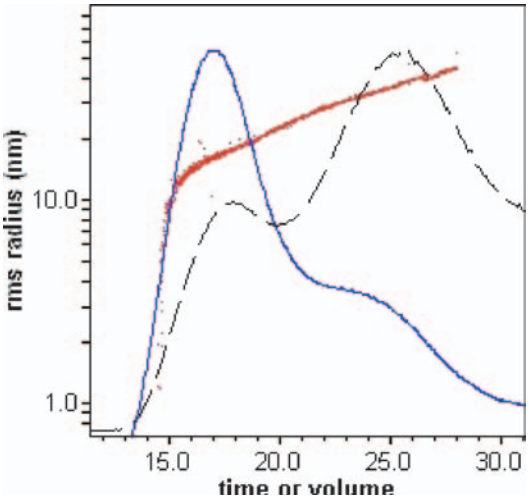

Fig. 4. Elution diagram of egg PC liposomes: differential refractive index (proportional to concentration, blue curve) and light scattering intensity (90 deg angle, black, dashed curve) versus elution volume in ml (equal to elution time in min). In addition the calculated radius (rms) is given (red, dotted curve).

3.3. Flow Field Flow Fractionation Coupled with RI- and Static Light Scattering Detection

Another technique, which is suited to fractionate submicron particles according to size is field flow fractionation. Here, a setup was used, where FFFF was coupled online with a multi-angle static light scattering detector and a refractive index detector. A typical result for egg PC-liposomes is given in Figures 4 and 5. Figure 4 shows the elution diagram, i.e., the time course of the refractive index and light scattering signal at 90° angle along with the calculated radius. The refractive index signal shows one peak with a shoulder towards longer elution times, whereas the light scattering signal shows two peaks, which are not fully separated from each other. The radius is constantly

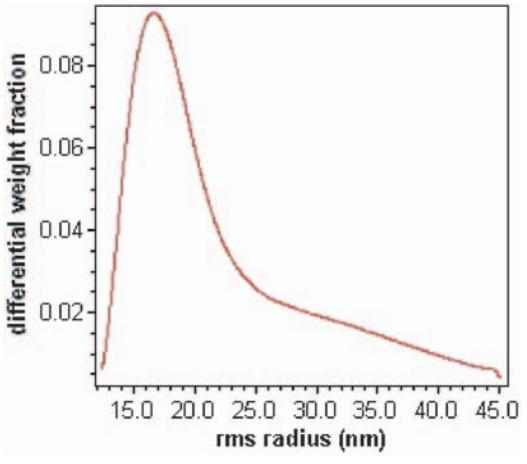

Fig. 5. Differential size distribution of egg PC liposomes as obtained from flow field fractionation and static multi-angle light scattering and refractive index analysis.

increasing over the elution time, as expected. In the mass-weighted distribution plot (Fig. 5), a steep and narrow peak with a shoulder or tail is obtained. The peak represents over 60% of the particles and covers a range of below 10 to about 25 nm in radius, i.e., about 20 to 50 nm in diameter, whereas the tail represents the remaining 35% of the particles extending to over 45 nm in radius, i.e., sizes of up to approximately 100 nm.

4. DISCUSSION

PCS results of the (non-fractionated) egg PC liposome dispersion could be nicely fitted either by a mono-modal, Gaussian model or by a bimodal "Nicomp" model. Which of the two was preferred by the software depended on the measurement run time. The shorter data collection time of five minutes clearly promoted the Gaussian fit, resulting in log normal size distributions ranging from about 15 nm (volume-weighted distribution) up to over 200 nm. In contrast, extended data collection times of 35 minutes promoted bimodal fits with two discrete peaks, one ranging from 30 to 70 nm (volume weighted) and the other from 100 to over 200 nm in diameter. In the volume-weighted distribution the latter peak accounted for 45 to 50% of the overall distribution.

Neither the mono-modal nor the bimodal distribution with two discrete peaks was confirmed by the fractionation methods for the liposome dispersion under investigation here. Both, the combined SEC + PCS-approach and the FFFF + LS-approach revealed distributions composed of a rather narrow peak at the lower size end and an overlapping broader peak or tail of bigger particles. Regarding the size ranges obtained, the PCS diameters were found significantly shifted towards smaller sizes by the combined SEC + PCS-approach. This is attributed to the effect that PCS underestimates smaller particles if bigger particles are present. Such underestimation of smaller particles has been demonstrated earlier for model blends of latex particles.[12] Obviously, PCS is able to resolve smaller particles only if bigger particles are absent or removed. This effect is observed irrespective of whether number- or volume-based distributions are compared although it is more evident in the latter case. In contrast, FFFF + LS again indicated somewhat bigger minimum diameters. This is regarded an effect of the different detection limits of the light scattering techniques. According to the manufacturer, the PCS-instrument used can detect particles down to a few nm in diameter, whereas the multi-angle static light scattering hardly can resolve particles of 10 nm or below in the setting used. Regarding the upper limits, the fractionating techniques indicated maximum particle sizes of around 100 nm (FFFF + LS) and 130 nm (SEC + PCS), whereas the PCS-fits of the non-fractionated liposomes, irrespective of the fit model selected, extended up to sizes of over 200 nm, due to overemphasizing of the bigger particles.

In conclusion, for the liposome sample investigated here, results obtained by either FFFF- or SEC-fractionation allowed for a more reproducible, detailed and correct insight into the size distribution as compared to analysis by PCS alone. It remains to be tested to which extent this holds true for other liposome samples, including those prepared by other preparation methods, from other kinds of phospholipids and those in isotonic medium and body fluids. Having in mind that for drug delivery applications size distribution really matters, as revealed by Liu et al.,[8] fractionation-based size analysis of liposome samples appears strongly recommended. Unfortunately, do most literature reports on the influence of liposome size on pharmacokinetics and biodistribution.[16, 17] still give mean PCS liposome sizes only. This, however, may be misleading. Although PCS alone can give a realistic impression of liposome size distributions, if one takes into account the inherent limitations of PCS, more advanced fractionation-based techniques do appear preferable under the prerequisite that the findings reported here are reproducible and generally applicable to all kinds of liposomal drug carriers, something, which remains to be proven.

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ABBREVIATIONS

FFFF	Flow Field Flow Fractionation
SEC	Size Exclusion Chromatography
PCS	Photon Correlation Spectroscopy
RI	Refractive Index
PC	Phosphatidylcholine

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