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Determination of the Size Distribution of Liposomes by SEC Fractionation, and PCS Analysis and Enzymatic Assay of Lipid Content

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ABSTRACT In this study, small liposomes obtained by high-pressure homogenization were fractionated according to their particle sizes by size exclusion chromatography (SEC). The subfractions were analyzed by photon correlation spectroscopy (PCS) as well as enzymatic phosphatidylcholine (PC) assay for their particle sizes and lipid contents, respectively. For small egg PC-liposomes, a size range of 15 nm to 60 nm was found, with 80% of the vesicles being smaller than 30 nm in size. This is in contradiction to a mean size of 85 ± 32 nm as indicated by PCS without fractionation. The PCS technique appears to underestimate very small particles below 30 nm if (few) bigger particles are present. The PCS particle size analysis of unfractionated hydrogenated egg PC/cholesterol-liposomes (2:1, mole/mole) by PCS did not yield any significant results. On fractionation, however, a particle size range of 40 nm to 120 nm was determined in a reproducible manner. Our results indicate that the combination of size exclusion fractionation with subsequent photon correlation spectroscopic particle size analysis and enzymatic PC assay can give both more detailed and more reliable insight into the particle size distribution of small liposomes than PCS alone.

Key Words: phospholipid, vesicle, size distribution, size exclusion chromatography, photon correlation spectroscopy

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INTRODUCTION

Phospholipid vesicles (liposomes) are under investigation both as models for biological membranes and as carriers for various bioactive agents such as drugs, diagnostic and genetic materials, and vaccines [1]. A rigorous control of vesicle size and lamellarity is stringent for achieving the desired pharmacokinetic behavior and thus performance of liposomal drug carriers in terms of drug targeting and/or controlled release [2] as well as for achieving meaningful results during model membrane studies [3]. In most laboratories routine liposome size analysis is carried out by photon correlation spectroscopy (PCS) using commercial instruments. This technique gives a measure for the mean size of the vesicles. Although PCS allows in principle the determination of particle size distributions, the reproducibility and reliability of the method for calculation is insufficient. Quantitative determination of the whole liposome size distribution, thus, is still difficult. Although a number of powerful approaches like electron microscopy [4], ultracentrifugation [5], analytical size exclusion chromatography [6-9], and field-flow fractionation [10] have been suggested, none of these approaches has found widespread use due to various reasons, such as the need for advanced and expensive instrumentation. The approach used here is to fractionate liposomes by size exclusion chromatography and analyze the subfractions in terms of particle size and vesicle content by a commercial PCS instrument and an enzymatic

phosphatidylcholine (PC) assay, respectively. Our aim was to gain a complete and quantitative overview over the particle size distribution of vesicle dispersions.

MATERIALS AND METHODS

Vesicle preparation

PC-rich natural and hydrogenated egg-phospholipids, E 80 and E 80-3, respectively, were a kind gift of Lipoid GmbH (Ludwigshafen, Germany). Cholesterol (5-Cholesten-3β-ol, 95%-98%) was purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany) and recrystallized as described by Tardi [11]. In brief, cholesterol was dissolved in methanol under reflux (60°C water bath) and vacuum-filtered through a paper filter using a prewarmed porcelain filter holder. The filtered solution was kept at 2°C to 8°C overnight and the precipitate collected on a filter and washed twice with ice-cold methanol. The process was repeated until a white, or almost white, odorless powder was obtained. To remove traces of methanol, the cholesterol was kept under 20-hPa vacuum overnight. Isotonic, 22 mM phosphate-buffered saline (PBS), pH 7.4 [11], was filtered through a 0.22 μm filter (cellulose acetate, Sartorius AG, Göttingen, Germany) to remove any particulate contaminants. PBS was used for preparation and dilution of the vesicular phospholipid gels (VPGs) as well as size-exclusion chromatography (SEC). In the latter case it was degassed under vacuum prior to use.

For preparation of VPGs [12] the 1-step method [13] was used. In brief, E 80 was mixed with PBS, allowed to swell for some minutes, and high-pressure homogenized 10 times at 70 MPa using an APV MicronLab homogenizer (APV Deutschland GmbH, Unna, Germany). When preparing VPGs of E 80-3 and CH (2:1, mole/mole), it is necessary to dissolve the lipids in a blend of chloroform and methanol (approximately 2:1) and remove the solvents by rotary evaporation to ensure a homogeneous blend of the lipids [14]. An alternative approach for solvent removal is to freeze-dry the lipid solution [15]. Otherwise, hydration and homogenization is carried out as above. All VPGs contained a total of 400 mg lipids per gram of preparation.

To achieve "classical" liposome dispersion, VPGs were diluted with excess buffer by mechanical agitation as first described by Brandl et al [16]. For a detailed protocol see Brandl and Massing [15]. Aliquots of the VPGs and PBS (pH 7.4) were transferred to a 2-mL vial to make up a final lipid concentration of 100 mg/g. Three glass beads with a diameter of 1 mm to 2 mm were added. The vial was shaken using a ball mill Retsch MM 200 (Retsch GmbH&Co KG, Haan, Germany) at a frequency of 30 Hz for 3 minutes.

Size exclusion chromatography

Sephacryl S-500 or alternatively Sephacryl S-1000 (Amersham Biosciences, Uppsala, Sweden) was packed into XK 16/70 columns as recommended by the manufacturer [17] to obtain bed heights of 54.7 cm and 47.2 cm, respectively. To minimize loss of lipid due to adsorption to the gel material, the columns were presaturated by running a sample of the diluted VPG that was to be analyzed [18]. Diluted VPGs in 500-μL aliquots were injected and eluted at a flow rate of 1 mL/minute. The elution was followed by optical density measurements at λ = 254 nm and 5-mL fractions were collected (ÄKTA Prime, Amersham Biosciences, Uppsala, Sweden).

Photon correlation spectroscopy

The vesicle dispersions were diluted with freshly filtrated PBS (0.22-μm Minisart, Sartorius AG, Göttingen, Germany) in order to reach a count rate of 250 kHz to 350 kHz, which is recommended by the manufacturer of the PCS instrument (Nicomp submicron particle analyzer model 380, Nicomp Inst Corp, Santa Barbara, CA). The sample preparation procedure was carried out in accordance with annex E of the ISO 13321 guideline (ie, in a laminar airflow [LAF] bench, using single-use syringes, needles, and pipette tips). All other glassware was bath-sonicated (Branson, Danbury,

CT) in filtrated buffer prior to use (see above). All samples were placed in the machine for 5 minutes prior to start of measurements to eliminate temperature differences between the sample and the machine. ISO guidelines were followed [19]. Three cycles of 30 minutes of data collection time each were run. The instrument parameters were set as follows: automatic choice of channel width; vesicle mode; number weighting; and automatic change from Gaussian distribution mode to multimodal mode (so-called Nicomp distribution mode) if the value for Chi-squared exceeded 3.00.

Quantitation of phosphatidylcholine

For quantitation of phosphatidylcholine (PC), an enzymatic assay [20] was used in the form of a "Phospholipids B" kit (Wako Chemicals GmbH, Neuss, Germany). The kit is meant for quantitative determination of the phospholipid content of serum and plasma, but it was found applicable to liposomes containing PC. A liposome sample (10-500 mL) and 1.0 mL of the color reagent solution were vortexed for 5 seconds and incubated at 37°C ± 2°C for 10 minutes. After cooling to room temperature, the absorbance at 505 nm was read on a Hitachi U-2001 UV-spectrophotometer (Hitachi Scientific Instruments, Merck Eurolab AS, Oslo, Norway). If extinction values outside the calibration range were obtained, the samples were diluted with PBS up to 200-fold prior to assay. Standard curves were established, both from the choline standard included in the set and samples with known concentrations of E 80 and E 80-3 in the range 150 mg to 600 mg /100 mL, respectively. The calibration values were fitted using a linear model in all cases ($R > 0.999$). The response of E 80 samples compared with choline standard was within 98% to 107% of the expected value (choline content of the batch of E 80 in use as certified by manufacturer). Blank and standards were included with each set of samples. The repeatability (CV) was 2.7% ($n = 9$).

RESULTS

PCS analysis of unfractionated vesicle dispersions

Two different vesicle dispersions were chosen for this study: a dispersion made of E 80 and a dispersion made of E 80-3 and cholesterol in a 2:1 (mole/mole) ratio. Both dispersions were obtained from VPGs containing a total of 400 mg/g of lipid by appropriate dilution. Each vesicle dispersion was analyzed 3 times by PCS using data collection times of 30 minutes each. The results are given as mean and standard deviation of 3 independent measurements of 1 batch of diluted VPG.

The analysis of the E 80-containing vesicle dispersion yielded Gaussian distributions throughout, with an average of the mean diameter of 84.5 ± 2.9 nm and an average of the size range (SD) of 31.6 ± 0.0 nm, which indicates intermediate vesicle sizes and a moderate degree of polydispersity. The variability between the parallels, both in terms of mean diameter and range was extraordinarily low. The figures for Chi-squared (0.47 ± 0.41) were small and the amount of data collected in channel number 1 as the basis for the autocorrelation function (1591.1 ± 67.3 k) were high compared with the manufacturer's recommendation (1000 k).

In contrast did the PCS analysis of the E 80-3/CH (2:1)-containing vesicle dispersion not result in monomodal (Gaussian-type), but in more complex, multimodal size distribution fits throughout. The results are summarized in Table 1.

A bimodal size distribution was automatically chosen for 2 of the parallels, whereas the fit of the third cycle indicated a distribution with 3 subpopulations. In consequence, major differences were observed for the fitted peak diameters among the parallels. The measurements did not yield reproducible results although the fit error has to be regarded as acceptable and the amount of data collected in the correlator large enough to achieve a reasonable degree of statistical accuracy according to the user manual of the instrument [21].

SEC fractionation of vesicle dispersions

Both the above-mentioned vesicle dispersions were fractionated by SEC and the fractions were analyzed in terms of vesicle size and PC content by PCS and enzymatic PC assay, respectively. Two different gel materials were employed for SEC: Sephacryl S-500 and S-1000. The results are plotted as relative PC contents versus mean particle sizes in Figures 1 and 2, respectively.

In general it was observed that the PCS instrument fitted Gaussian-type (monomodal) distributions for all fractions, which contained quantifiable amounts of lipid. The PCS mean diameters were well reproducible as indicated by low values for the SD of 3 consecutively measured mean diameters (error bars in the figures). The size ranges around the mean diameter, given by the PCS instrument as SD, varied between 20% and 50%. Altogether this indicates a fractionation of the vesicles into classes of well-defined, relatively homogeneous particle sizes.

The fractionation of the E 80–containing vesicle dispersions resulted in fractions of mean particle sizes ranging from approximately 15 nm to 65 nm, no matter whether Sephacryl S-500 or S-1000 was used. The peak of the size distributions was at 20 nm to 25 nm.

The E 80-3/CH (2:1)–containing vesicle dispersions resulted in fractions with mean diameters ranging from 40 nm to 120 nm, again with just 1 peak, at about 60 nm. Again the 2 SEC gel materials showed quite similar results except from the biggest particles. When using S-500, the biggest particles eluted in the void volume, resulting in a fraction of 107-nm mean size, whereas with S-1000 further fractionation was achieved.

DISCUSSION

When comparing the mean diameter and range of the unfractionated E-80 vesicle dispersion with the mean diameters measured for the subfractions, a major discrepancy was observed. The PCS results of the fractionated sample point toward much smaller diameters (15 nm to 65 nm) than the mean diameter and range found for the unfractionated sample would indicate (84.5 ± 31.6 nm). This was surprising since the excellent reproducibility of the PCS results for the unfractionated material, along with a small figure for Chi-squared (0.47 ± 0.41) and a high number of data that were collected in channel number 1 as the basis for the autocorrelation function (1591.1 ± 67.3 k), seemed to indicate a well-founded result. Further results of particle size analyses of diluted VPGs prepared in the same manner and of comparable lipids from earlier studies [16] seem to confirm this observation. When analyzed by negative staining electron microscopy (NS-EM) vesicle sizes ranging to more than 95% below 50 nm had been found, which compares well with the PCS results upon fractionation here. On the other hand, a z-average value had been measured by Malvern Zetamaster, which is comparable with the (unfractionated) mean PCS diameter obtained here. Taken together these results seem to point toward a systematic discrepancy in the measured particle sizes of such vesicle dispersions between PCS analysis (without fractionation) and alternative sizing approaches, such as electron microscopy or a combination of SEC fractionation, PCS analysis, and PC quantitation. PCS appears to underestimate or, more precisely, neglect particles at the lower end of the size spectrum (15-30 nm) unless any bigger particles are removed from the dispersion. It has to be taken into account that this is the result of a straightforward experimental design using a commercial PCS instrument. This type of experimental setting, on the other hand, is to our experience being used for routine analyses.

When looking at the PCS results of the E 80-3/CH (2:1)–containing vesicle dispersion, it is obvious that by fractionation first sufficiently homogeneous samples were obtained to yield reproducible PCS results. Other than PCS analysis alone the combined SEC fractionation/PCS approach points toward a monomodal, but relatively broad, particle size distribution. The bi- to trimodal distributions, which

Table 1. PCS Analysis of the Unfractionated E 80-3/CH (2:1)-containing Vesicle Dispersion

1st Peak		2nd Peak		3rd Peak		Parameters			
Diameter (nm)	Amount (%)	Diameter (nm)	Amount (%)	Diameter (nm)	Amount (%)	Fit error	Residual	Data (k)	Ch. width (μsec.)
16.0	88.3	100.0	11.6	787.9	0.1	1.72	1.146	1731.4	17.0
48.6	76.4	126.4	23.6	---	---	1.96	40.744	1494.4	17.0
88.7	98.4	461.6	1.6	---	---	1.94	0.000	1214.2	17.0

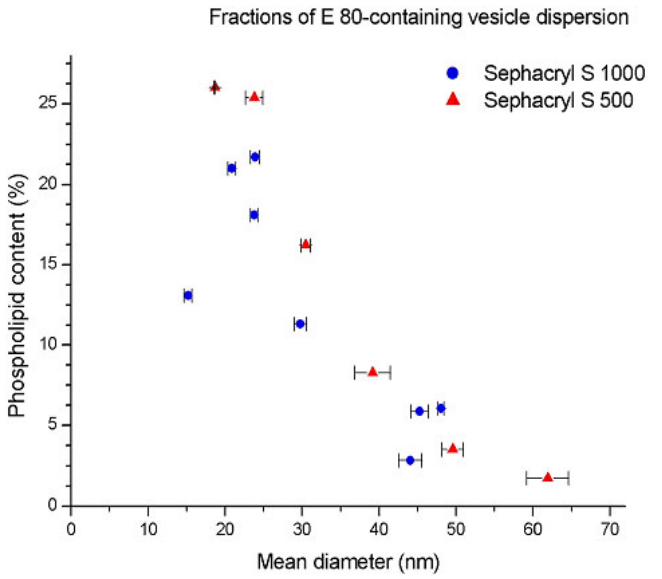

Figure 1. Relative lipid content versus mean particle sizes of E 80 liposomes. Lipid contents given as percent of total lipid. Mean particle sizes given as average and SD of the means of 3 consecutive measurements.

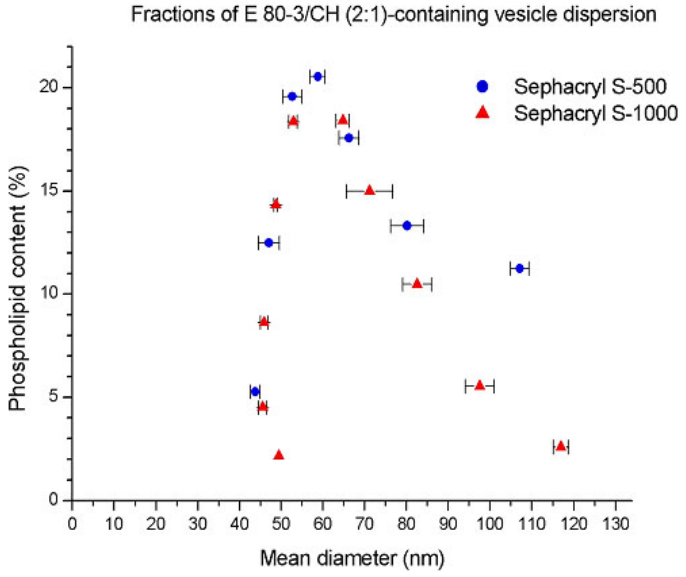

Figure 2. Relative lipid content versus mean particle sizes of E 80-3/CH liposomes. Lipid contents given as percent of total lipid. Mean particle sizes given as average and SD of the means of 3 consecutive measurements.

were fitted when measuring the unfractionated sample, could not be confirmed.

In conclusion, a combination of size exclusion chromatography, photon correlation spectroscopic particle size analysis, and enzymatic PC assay allowed quantification of the polydispersity of 2 small-sized vesicle dispersions, or, in other words to quantitatively determine the particle size distribution in the size range of 15 nm to 150 nm. The fractionation transferred samples, too heterogeneous for direct mean particle size measurement by PCS, into subfractions, which were sizable. Surprisingly, the combination of SEC, PCS, and PC assay revealed a significant underestimation of very small particles (below 30 nm) within a vesicle dispersion of moderate polydispersity when measuring the mean diameter by PCS alone. Such very small vesicles, however, have been reported to show completely different physico-chemical characteristics [14], as well as pharmacokinetic behavior [2].

CONCLUSIONS

To characterize the size distributions of vesicle dispersions in a reliable manner it appears useful to employ a sizing technique that can resolve heterogeneous and/or small-sized particles. For example, the combination of SEC fractionation, PCS, and enzymatic PC quantitation suggested here.

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