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Dual asymmetric centrifugation (DAC)—A new technique for liposome preparation

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Abstract

This is the first report on the use of a "dual asymmetric centrifuge (DAC)" for preparing liposomes. DAC differs from conventional centrifugation by an additional rotation of the sample around its own vertical axis: While the conventional centrifugation constantly pushes the sample material outwards, this additional rotation constantly forces the sample material towards the center of the centrifuge. This unique combination of two contra rotating movements results in shear forces and thus, in efficient homogenization. We demonstrated that it is possible to prepare liposomes by DAC, by homogenizing a rather concentrated blend of hydrogenated phosphatidylcholine and cholesterol (55:45 mol%) and 0.9% NaCl-solution, which results in a viscous vesicular phospholipid gel (VPG). The resulting VPG can subsequently be diluted to a conventional liposome dispersion. Since DAC is intended to make sterile preparations of liposomes, or to entrap toxic/radioactive compounds, the process was performed within a sealed vial. It could be shown that the DAC speed, the lipid concentration, the homogenization time and the addition of a mixing aid (glass beads) are all critical for the size of the liposomes. Optimized conditions resulted in liposomes of 60±5 nm and a trapping efficacy of 56±3.3% for the model compound calcein.

Keywords: Liposome; Dual asymmetric centrifuge; Vesicular phospholipid gel; Preparation; Sterility; Bedside preparation

1. Introduction

Dual asymmetric centrifugation (DAC) is a special kind of centrifugation in which—as usual—a vial is turned around the main rotation axis with a defined distance and at a defined speed. The main difference of DAC to normal centrifugation is that the vial is turned around its own center (vertical axis) during the normal centrifugation process. This results in two overlaying movements of the sample material in the centrifugation vial: While the main rotation pushes the sample material in an outward direction in response to centrifugal forces, the rotation of the centrifugation vial around its own center pushes the sample in the opposite direction due to adhesion between the sample material and the rotating vial. The latter movement, the inward transport of the sample material, is effective if sufficient adhesion of the sample material on the vial material is given and if the sample material is sufficiently viscous, since both influence the amount of energy which can be transferred into the sample material.

DAC has been known since the 1970s as a convenient technology for the rapid mixing of viscous components and is widely used to rapidly mix two-component composites [1,2]. Since mixing by DAC is astoundingly fast, the DAC-technology is also named "speed-mix"-technology and the DAC-apparatus "Speedmixer".

The DAC used in this study is shown in Fig. 1 (Counter weight is located inside the machine). It is suitable to process samples up to 150 g. Fig. 2 shows schematic drawings of such a DAC with the main rotating arm and the vial holder at its distal end. The main rotation arm of the DAC forms an angle of about 40° with the rotation plane. At this angle, the rotating arm forces the content of the vial into the corner between the bottom and the vial wall. The DAC (shown in Fig. 1) allows a maximum speed of 3540 rpm, and reaches a maximum acceleration of the

Abbreviations: DAC, dual asymmetric centrifuge; VPG, vesicular phospholipid gel; EPC3, hydrogenated phosphatidyl choline from egg; Chol, cholesterol; HPH, high-pressure homogenization.

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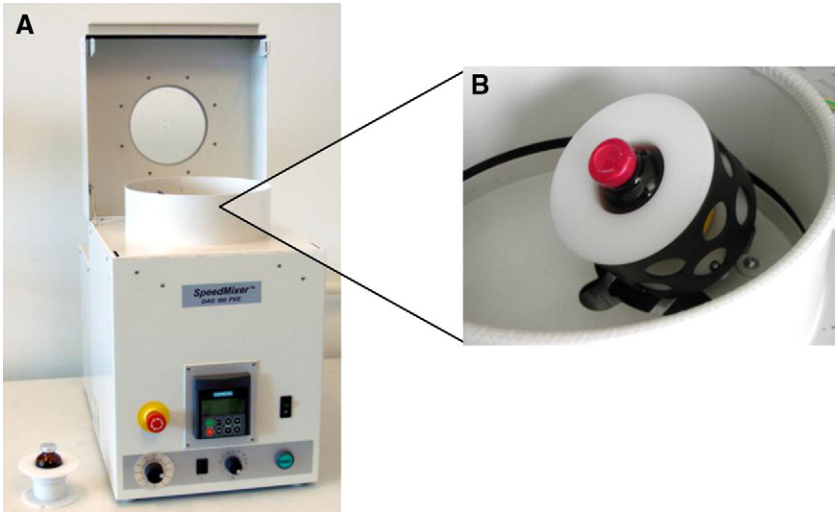

Fig. 1. Dual asymmetric centrifuge (DAC) to process samples up to 150 g (A. DAC with open lid; B. view into the rotation chamber).

sample of about 911 × g. The vial holder rotates in the opposite direction with approximately one fourth of the rotating arm's frequency. For the experiments presented here, a glass injection vial with a diameter of 36 mm was used which resulted in a counterclockwise movement of its glass walls of about 1.5 m/s at maximum speed.

However, since this unique combination of two very fast movements of a viscous sample material should result in constant

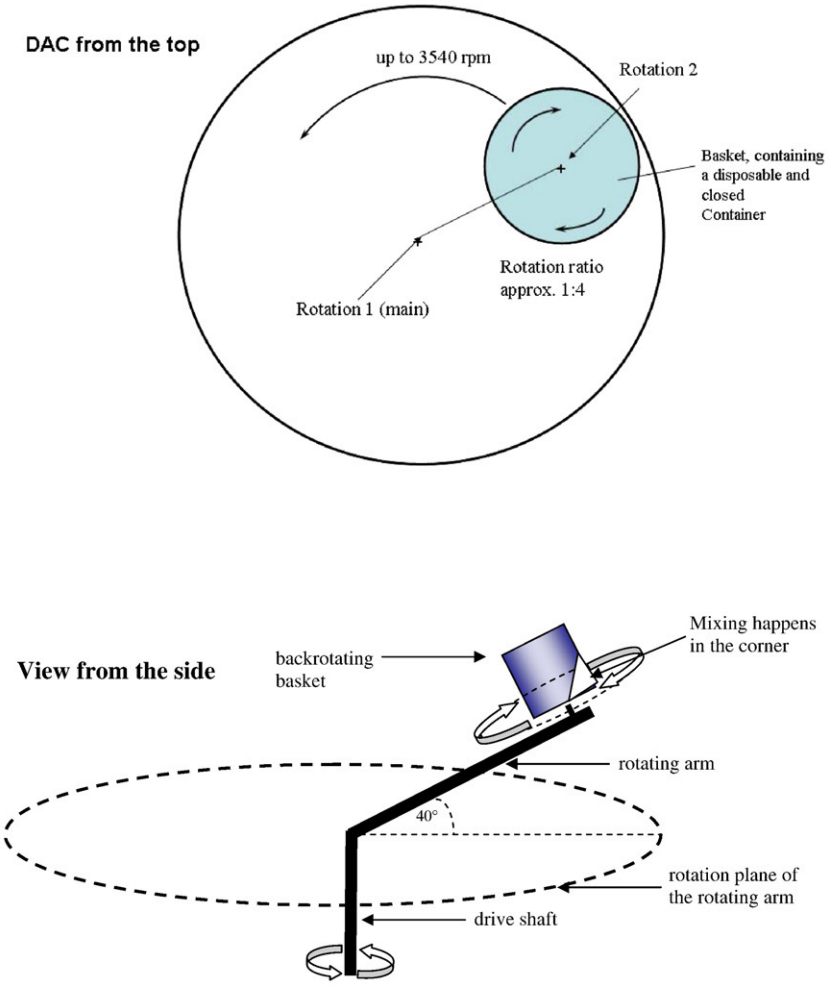

Fig. 2. Schematic drawings of the principle of dual asymmetric centrifuging.

and strong shear forces within the material, we concluded that DAC might also be used for homogenization purposes and thus for the production of liposomes, which are often made by the homogenization of a phospholipid/water blend using different homogenization machines. Viscous liposome formulations seemed especially suitable to be produced by DAC.

VPGs are highly concentrated liposomal dispersions where the liposomes form a three-dimensional network [3–5]. VPGs are viscous due to the high lipid concentration [6]. The amount of aqueous media which is entrapped within the liposomal vesicles reaches the same magnitude as the amount of aqueous medium located between the vesicles [3,4]. The same amount of water outside and inside the liposomes results in high trapping efficiencies for water-soluble compounds, which is usually around 50%. VPGs can be diluted to conventional liposomes (redispersion) [6] and then used for parenteral application and diverse other applications. Examples of liposomal formulations which base on VPG are liposomal Gemcitabine (GemLip) [7–9] and liposomal Vincristine (VCRlip) [10].

Today, VPGs are usually made by high-pressure homogenization (HPH) [11,12], which has advantages when batch sizes of tens to hundreds of grams of VPG or subsequently liposomes have to be produced. Furthermore, due to the high energy intake into lipid dispersions, HPH is especially useful for the production of very small liposomes as they are especially desired for intravenous applications. However, in the early stages of liposome development there is a clear need to produce batch sizes of about a gram or even less. Therefore, the aim of the current study was to investigate whether DAC would satisfy such a need.

We decided to use a blend of hydrogenated phosphatidylcholine and cholesterol (hydrated egg-PC/Chol (55:45 mole/mole) for studying the process of VPG/liposomes-preparation by DAC, since lipid mixtures containing fully hydrogenated phosphatidylcholine are rather common for parenteral (i.v.) application. Examples are liposomal Doxorubicin (Caelyx®), liposomal Daunorubicin (Daunoxome®) [13] or liposomal Gemcitabine (GemLip) [8,14].

2. Materials and methods

2.1. Preparation of vesicular phospholipid gels (VPG) and liposomes by DAC

In all experiments a molecularly dispersed lipid blend consisting of hydrogenated egg phosphatidylcholine (EPC3) and cholesterol of a ratio of 55:45 (mole/mole) was used. The blend was prepared by Lipoid GmbH (Ludwigshafen, Germany). The aqueous compound was a 0.9% sodium chloride solution and the lipid amount was 40% of the VPG (650 mM lipid) unless otherwise noted. All experiments were performed using brown injection vials (according to ISO 8362-4-3-OH, Ø 36 mm, height 62.8 mm, capacity 30 ml). Glass beads of different diameters were purchased from Sartorius (Göttingen, Germany) and Merck (Darmstadt, Germany), the beads of stainless steel were from Nanoball (Wermelskirchen, Germany).

Directly after weighing of the constituents (lipids, 0.9% NaCl-solution and glass beads), the homogenization was performed in the dual asymmetric centrifuge (DAC 150 FVZ, Hauschild GmbH & Co KG, Hamm, Germany) in multiples of 5-minute runs (since 5 min is the maximum runtime of the DAC that can be set up, the instrument was immediately restarted after every 5-minute run until the total mixing time was reached). After production, the VPGs were redispersed with a double volume of 0.9% sodium chloride solution to produce a liposome dispersion.

2.2. Preparation of vesicular phospholipid gels and liposomes by HPH

Lipid composition was the same as stated above for the DAC preparations of VPG and liposomes by DAC. Homogenization was performed 10 times at 700 bars (Micron Lab 40 from APV, Lübeck, Germany) and the VPG was then aliquoted in the brown injection vials. After production the VPGs were redispersed with a double volume of 0.9% sodium chloride solution to produce a liposome dispersion.

2.3. Entrapping efficiencies

A calcein solution (50 mM, pH 8.0, containing 50 mM PBS) was used as an aqueous phase instead of the 0.9% sodium chloride solution for VPG preparation by DAC and HPH and subsequently redispersed to liposomal formulations (as described above). The non entrapped calcein was removed by ion exchange chromatography (Dowex 1×8 from Sigma Aldrich). The remaining liposomes were further diluted to a concentration of 3.3 μmol/L lipid and disintegrated by the addition of Triton X-100 (resulting conc. 1%) prior to calcein measurement. For the calculation of the encapsulation efficiency, the total amount of calcein in the sample was measured prior to ion exchange chromatography (diluted to 3.3 μmol/L lipid which corresponded to 150 nmol/L calcein): Encapsulation efficiency [%] = (calcein inside the liposome / total amount of calcein in the sample) *100%.

Calcein-fluorescence intensity measurements were performed by using a fluorescence spectrometer LS 50 B from Perkin Elmer (excitation wavelength: 490 nm, emission wavelength: 520 nm, slits: 5 nm, calibration range: 5–200 nmol/L ($r^2 = 0.9986 \pm 0.0016$)).

2.4. Photon correlation spectroscopy

The vesicle dispersions were diluted with 0.9% sodium chloride solution until a count rate of 300 ± 50 kHz was reached (recommended by the Photon Correlation Spectroscope manufacturer, Nicomp submicron particle analyzer model 380, Nicomp Inst Corp, Santa Barbara, CA, USA). All samples were allowed to equilibrate to 23 °C.

Each measurement was performed as a five-minute measurement and each sample was measured twice with a fixed channel width of 5 μs unless stated otherwise. The other machine parameters were: temperature 23 °C, liquid viscosity 0.933 (water), liquid index of refraction 1.333 (water). The correlation was evaluated using a Gaussian distribution model and only measurements with a good fit, as expressed as Chi-square lower 3.0, were taken into account.

2.5. Lyso-phosphatidylcholine (Lyso-PC) determination

Lyso-PC was measured by using a validated HPTLC method, as recently published [15]. In brief: Liposomes were redispersed with 0.9% NaCl solution and extracted 3 times with $HCCl_3$/MeOH 2:1 (v/v). The collected organic phases were dried under a stream of nitrogen. The dried samples were redissolved with the mobile phase of the HPTLC ($CHCl_3$/MeOH/H_2O/NH_3 (65/25/4/0.4 v/v/v/v), spotted on the HPTLC-plate (silicagel 60, Merck, Darmstadt, Germany) by using a HPTLC-autosampler (Automatic TLC Sampler III, Camag, Switzerland) and developed in a glass tank (run distance 10 cm, mobile phase: see above). The plates were dried at 180 °C on a heating plate (Desaga, Darmstadt, Germany) and stained by dipping (about 7 cm) the plate into a copper sulphate/ phosphoric acid solution (14.7% w/v; 10% v/v) (Chromatogram Immersion Device III, Camag, Switzerland) and heating the plate in a oven at 180 °C for 6 min. The spots were quantified by UV absorption at 530 nm using a HPTLC-scanner (TLC Scanner II, Camag, Switzerland). 1-O-Palmitoyl-lyso-PC was used as standard (calibration range: 0.1–2.0% lyso-PC, related to EPC (r^2: 0.9972).

2.6. Measurement of glass fragments

To test if the use of glass beads during the production of liposomes by the DAC-protocol might generate glass particles in the formulations, the usual vials were loaded with particle free water (0.5 ml) and glass beads (Ø 1 mm, 0.5 g) and processed by DAC for 30 min at 3540 rpm, which are the conditions we found to be optimal for liposome preparation. Afterwards, the vials were filled up to 30 ml with particle free water. The water was further diluted 1:6 and particles were measured by using an AccuSizerTM Optical Particle Sizer (PSS Nicomp particle sizing system, Santa Barbara, CA, USA).

3. Results

In preliminary experiments, we tested DAC for homogenization of lipid water blends with a high lipid content of more than 300 mg/g. After 20 min of DAC it was found that a homogeneous cream-like paste similar to VPGs produced by HPH had been produced. Upon the addition of the excess of aqueous medium, the paste was converted into a liposome dispersion which entrapped calcein at an efficiency of about 50%. In the following experiments, all parameters that might influence the product were systematically varied.

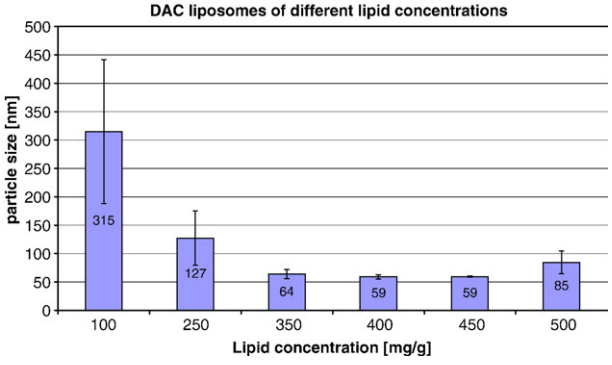

Fig. 3. Mean liposome sizes (mean ± SD, $n=4$) vs phospholipid contents of DAC liposomes; batch size 0.5 g; glass beads (ø 1 mm) 0.5 g; agitation time 30 min, agitation speed 3540 rpm.

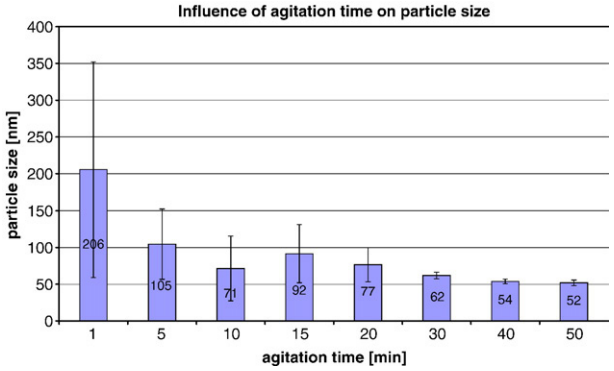

Fig. 4. Mean liposome sizes (mean ± SD, $n=4$) vs agitation time of DAC liposomes; batch size 0.5 g, lipid content 400 mg/g, glass beads (ø 1 mm) 0.5 g; agitation speed 3540 rpm.

In order to compare PCS-data, the channel width was held constant at 5 µs. For comparison, 10 and 20 µs results are given for one series of experiments (Fig. 9 see below).

3.1. Influence of lipid concentration

As shown in Fig. 3, liposome size is influenced by the lipid concentration of the lipid/buffer dispersion used for DAC-homogenization. With increasing lipid concentrations up to 350 mg/g, a significant decrease in both mean liposome size and variability between the replicates was observed. Lipid concentrations of 350, 400 and 450 mg/g result in the smallest liposomes (around 60 nm) which statistically did not differ. The variability between the repeated experiments of one lipid concentration was very low. An even higher lipid concentration of 500 mg/g resulted in a slightly higher mean liposome size and a significantly higher variability. With the exception of the 100 mg/g lipid dispersion all preparations had a cream-like consistency and are addressed as vesicular phospholipid gels (VPG).

3.2. Influence of homogenization time and DAC-speed

Mean liposome size and variability within a series of equal batches were influenced by the DAC-homogenization time (Fig. 4). A minimum of 10 min is necessary to get the desired small liposomes and a duration of 20 to 30 min is necessary to get small liposomes with only minimal size differences between the batches. With even longer homogenization times neither mean liposome size nor the variability between the repeated preparations seem to decrease.

In the following experiment, the influence of the DAC speed was investigated (Fig. 5). The smallest liposomes with low variability between repeated experiments were produced at the maximum speed of 3540 rpm. Stepwise reduction of the speed

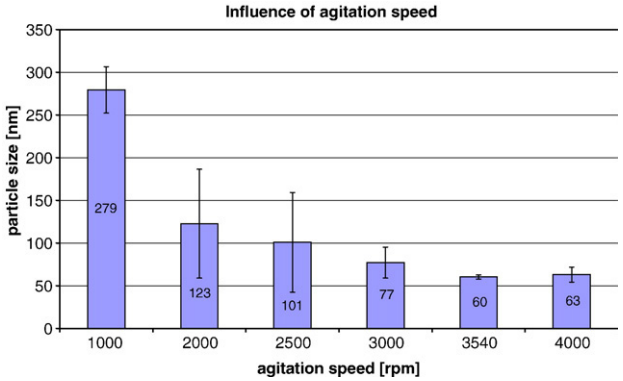

Fig. 5. Mean liposome sizes (mean ± SD, $n=3$) vs agitation speed of DAC liposomes; batch size 0.5 g; glass beads (ø 1 mm) 0.5 g; lipid content 400 mg/g, agitation time 30 min.

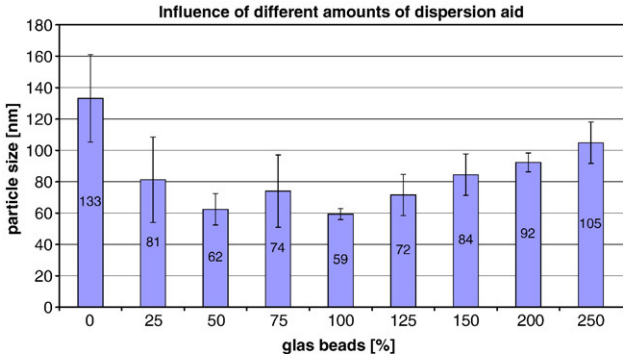

Fig. 7. Mean liposome sizes (mean ± SD, $n=4$) of DAC liposomes vs amount of glass beads (ø 1 mm) added as dispersion aid; batch size 0.5 g; lipid content 400 mg/g, agitation time 30 min; agitation speed 3540 rpm.

resulted in increasing liposome sizes and broader size distributions.

To test if a higher speed would result in smaller liposomes, a new DAC-prototype was developed by the DAC manufacturer that allowed a DAC speed of approximately 4000 rpm. However, liposomes produced at this speed were found not significantly different in size and standard deviation from those produced at 3540 rpm (Fig. 5).

3.3. Choice of beads as dispersion aid

Various sizes of glass beads ranging from 0.3 to 3 mm were tested in terms of supporting the mixing efficiency (Fig. 6). In all cases, the same amount (weight fraction) of glass beads was added. Although there is a slight tendency towards smaller particle sizes with bigger glass beads, the differences are not significant. It was observed however, that the biggest glass beads may damage the glass containers. It was thus concluded that glass beads of 1 mm diameter are well suited and there is no need to use other sizes.

Testing different amounts of glass beads (Fig. 7) showed that all samples homogenized with glass beads are significantly smaller and particle sizes are more homogeneous than the samples agitated without glass beads. With increasing amounts of glass beads, particle sizes were smaller until a plateau was reached at about 50% glass beads (weight fraction). From 125% on increasing the amount of glass beads always led to bigger mean particle sizes.

The particle sizes obtained from the heavier stainless steel beads were not significantly different other than a slight yellowish discoloration of the samples upon agitation.

3.4. Variation of batch size

All experiments described so far have been performed with batch sizes of 0.5 g (lipid plus aqueous phase). Processing lower (0.25 g) as well as bigger batch sizes up to 3.7 g had no statistically significant influence on the liposome sizes (Fig. 8).

3.5. Further characterisation of the optimized DAC-procedure

Summarizing the previous results, optimum process parameters for DAC-preparation of small liposomes from hydrogenated egg-PC/cholesterol in a 30 ml injection vial are as follows (feasible range is given in brackets).

- Initial lipid concentration: 400 mg/g (350–450 mg/g total lipid (581–747 mM))
- Batch size: 0.5 g (0.25 g–3.7 g)
- Duration of DAC: 30 min (or longer)

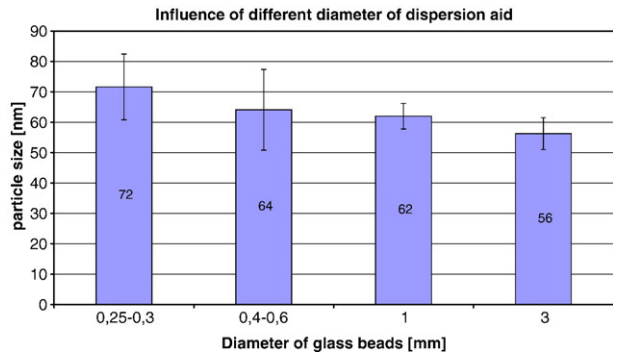

Fig. 6. Mean liposome sizes (mean ± SD, $n=4$) of DAC liposomes vs size of glass beads added as dispersion aid; batch size 0.5 g; glass beads 0.5 g; lipid content 400 mg/g, agitation time 30 min, agitation speed 3540 rpm.

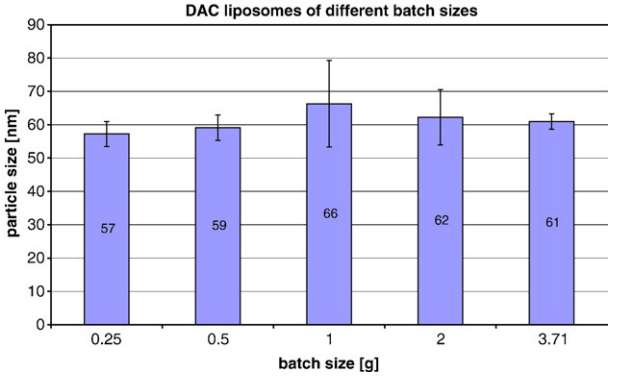

Fig. 8. Mean liposome sizes (mean ± SD, $n=3$) of DAC liposomes vs batch size; 0.5 g glass beads (ø 1 mm) added as dispersion aid; lipid content 400 mg/g, agitation time 30 min; agitation speed 3540 rpm.

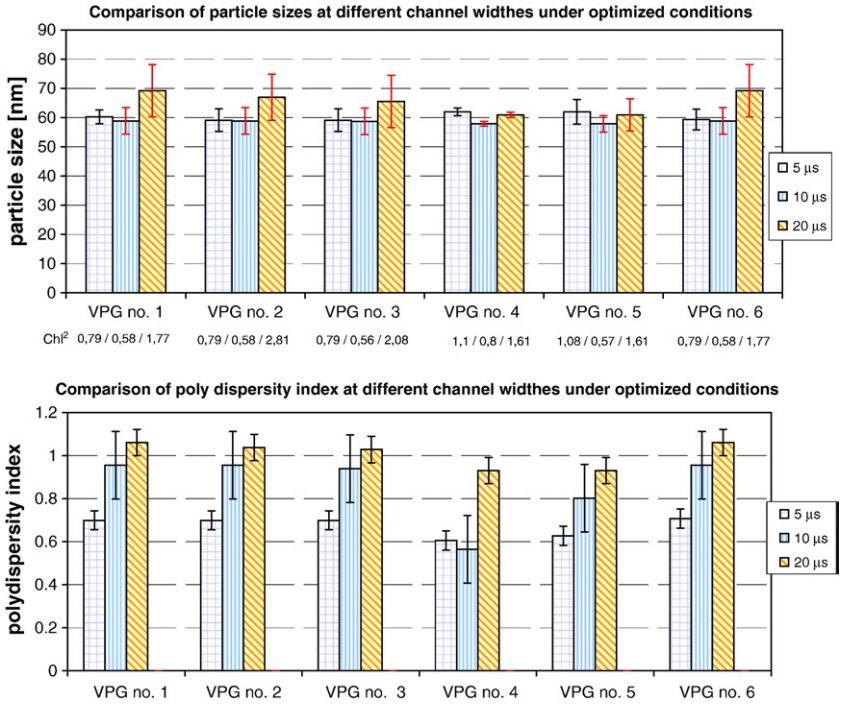

Fig. 9. Particle sizes and polydispersity (P.I.) indices of 6 batches of VPG produced under the optimum process conditions measured at 5, 10 and 20 µs.

- DAC-speed: 3540 rpm (maximum)
- Homogenization aid: glass beads ∅ 1 mm, amount: 100% (equal amount as compared to batch amount)

To further characterize the optimized DAC-process, we investigated the reproducibility of the process as well as the possible occurrence of glass particles. Furthermore, we investigated the temperature of the lipid mixture at the end of the DAC-homogenization, the phospholipid hydrolysis (appearance of lyso-phosphatidylcholine (lyso-PC)) and the entrapping efficiency of the model compound calcein. For comparison, the same lipid mixture was homogenized by HPH and the resulting liposomes were compared with the DAC liposomes in terms of trapping efficiency, size and phospholipid hydrolysis.

3.5.1. Reproducibility of the DAC-process

Six batches were produced under the above conditions and analyzed for their mean particle sizes and polydispersity indices (P.I.). The results are given in Fig. 9. As can be seen, the mean particle sizes for all six batches were similar, irrespective of whether the results were gained at a channel width of 5 µs, 10 µs or 20 µs. In a similar manner, P. I. increased with longer channel widths for all six batches.

3.5.2. Glass particle contamination

Since it is not possible to identify small glass particles within a highly concentrated and viscous phospholipid dispersion, the generation of glass particles from the glass beads, which were added as a mixing aid to the lipid mixtures, was investigated by processing the glass beads in particle free water. We expected that the use of water (low viscosity) instead of highly concentrated vesicular phospholipid gels (high viscosity, cream-like consistency) would result in more glass particles since (i) the cream-like VPGs reduce bead-bead interactions and (ii) when it comes to bead collisions, the impact is much higher. We performed three experiments in water (optimal process conditions: 30 min, 3540 rpm (see above)) and found only a very low number of particles. The particle count was 212±168 (max: 348) particles equal or bigger than 10 µm and 16±18 (max: 36) particles equal or bigger than 25 µm within one vial (n=3).

3.5.3. Lipid degradation and process temperature

To detect phospholipid hydrolysis induced by the DAC-process, the amount of lyso-PC in the liposomal formulations was investigated by using *high performance thin layer chromatography* (HPTLC). Even under optimal conditions, the phospholipids didn't undergo notable degradation: The lyso-PC content of the PC-fraction after DAC-homogenization of 30 min was only 0.23%±0.03%(n=3) (Fig. 10), which was only slightly higher than the lyso-PC content prior to the DAC-procedure (<0.1%, as indicated by the manufacturer of the lipid blend). This high stability of the phospholipids towards hydrolytic degradation wasn't surprising since the temperature of the VPGs didn't exceed 50±1 °C during the DAC-process (as measured by an infrared thermometer immediately after the DAC-process). In contrast, the lyso-PC content of the same formulation was 1.2%±0.3% when made by HPH and subsequent autoclaving.

3.5.4. Encapsulation efficiency and particle size

The fluorescent dye calcein was used as a model compound for studying the encapsulation efficiencies of the DAC- or HPH-made liposomes. The encapsulation efficiency of the DAC-

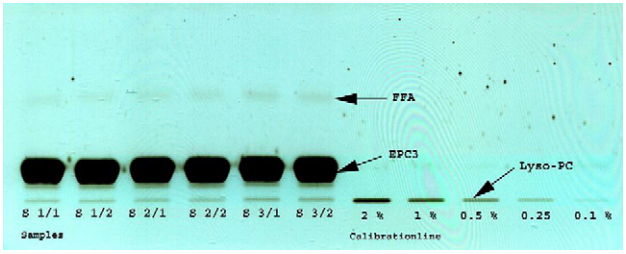

Fig. 10. HPTLC-analysis of the lyso-phosphatidylcholine (lyso-PC) content in liposomes made of hydrogenated egg phosphatidylcholine (EPC-3)/cholesterol (55:45 molar ratio) after 30 min of DAC-homogenisation in 0.9% NaCl-solution at 3450 rpm. Three samples (S1–S3) were analysed in duplicate. 1-Palmitoyl-lyso-phosphatidylcholine was used as standard and applied in concentrations of 0.1–2.0% related to EPC-3. Cholesterol spots are not visible since they are located above the stained area of the HPTLC-plate. (FFA: free fatty acid).

made liposomes was 56±3.3% whereas it was 36.0±3.2% for the HPH-made liposomes. Correspondingly, the HPH-made liposomes were somewhat smaller than the DAC-made liposomes (36±4 nm vs. 60±5 nm). P.I. of the liposomes made by HPH was 0.6±0.04.

4. Discussion

Liposomes are under investigation as cell membrane models, as carriers for drugs and bioactive molecules [16–18], gene-transfer [19] and as immunological adjuvants in vaccines [20]. While the characteristics of the bilayer itself are mainly determined by the nature of the phospholipid(s) used, lamellarity (uni- or multilamellar), vesicle size and entrapment of water-soluble molecules are primarily a function of the method of preparation [21].

A range of preparation methods have been described so far [22–26]. Industrial scale production of liposomes mainly uses high-pressure homogenizers and filter extruders [27–29,12,30]. The main advantages of such liposomes are their small and homogeneous vesicle size. For the development of a method which produces small unilamellar vesicles (SUVs) from powdered lipid(s) and aqueous drug solution in just one step, a discontinuously working laboratory-scale ultrahigh-pressure homogenizer (Micron Lab 40, APV Gaulin, Germany) was used [11,12]. HPH also allowed the preparation of highly concentrated (300 to 600 mg/g), semisolid phospholipid dispersions, so-called vesicular phospholipid gels (VPGs).

What most of the liposome preparation techniques described in literature have in common are that only a few specialized labs are able to perform them since dedicated equipment and a distinct know-how is needed. So, the aim of the current study was to investigate whether DAC would be suitable for preparing liposomes easily and on a small-scale. The current study clearly demonstrates that DAC is suitable for preparing small batches of VPGs and subsequently, liposome dispersions in a standard injection vial under sterile conditions. The influences of various process parameters have been demonstrated:

(i) Lipid concentration: Variation of the concentration of the lipids which were homogenized by DAC (max. speed of 3540 rpm, 30 min.) showed that there is a broad optimal concentration range from about 350 up to 450 mg/ml at which liposomes of 60±5 nm mean diameter were formed. The observed tendency towards bigger and less homogeneous liposome sizes with lipid contents at 250 mg/g and below can be explained by the lower viscosity and thus to a reduced transfer of energy into the samples by the axial rotation of the sample vial as well as less contact between the lipid aggregates (or less shearing of the lipid aggregates). In contrast, a lipid content of 500 mg/g obviously results in a dispersion that is too viscous which might reduce the outward movement of the sample material and thus negatively affect homogenization efficiency—liposomes become bigger and the size distribution is more heterogeneous. It was earlier observed that VPGs with 500 mg/g lipid prepared by HPH are not fully composed of small and homogeneous vesicles due to packing constraints [4].

(ii) Homogenization time and speed: It could clearly be shown that both the duration of DAC-homogenization as well as DAC-speed influences the size and the size distribution. Homogenization over periods of 30 min or more at maximum speed of 3540 rpm resulted in the smallest liposomes and the lowest variability in mean size. In contrast, the use of HPH (700 bar) resulted in somewhat smaller liposomes by running 10 homogenization cycles, each about 2 s long. But the real homogenization time in which the sample passes the valve of the HPH is much shorter, approximately a few microseconds. This comparison clearly shows that the shear forces generated by DAC are very much lower than those generated by HPH, which on the other hand might be advantageous when sensitive compounds are entrapped within VPGs.
Stepwise increasing of the DAC-speed from 1000 rpm to 3540 rpm reduced the liposome size in a roughly exponential way, clearly showing that the outward movement of the viscous lipid dispersion plays an important role in the homogenization process (centrifugal forces increase with the square of the speed). However, increasing the speed beyond 3540 rpm (maximum speed of the commercial available DAC) up to 4000 rpm (maximum speed of a specifically modified prototype DAC) does not result in significantly smaller particles. This shows that to get smaller liposomes, much more energy is needed and liposomes of about 50–60 nm are the minimum that can be produced by using a DAC of a given geometry and speed, a vial with a diameter of 36 mm and the respective lipid blend. The energy which can be transferred into a lipid mixture by HPH at 700 bars, as discussed above, is an order of magnitude higher and this resulted in somehow smaller liposomes (36 nm± 4 nm) for the same lipid mixture.

(iii) Influence of homogenization aid: It is not possible to get liposomes smaller than 100 nm without using glass beads as an homogenization aid. Addition of 50 up to 125 weight percent of glass beads (related to the total mass of

lipid dispersion) resulted in the desired small liposomes, showing that additional shear forces, resulting from the collision of the beads with each other or the wall of the glass vial, are necessary to get small liposomes. The diameters of the glass beads (0.25–3 mm) have no influence, which is in accordance with the finding that the addition of the glass beads is important but not the exact amount (as discussed above). However, a slight but not significant trend towards smaller particles with bigger glass beads was seen. Nevertheless, for further experiments we decided for 1 mm glass beads since the liposomes are sufficiently small and there was no danger of the beads destroying the glass vial (which occasionally happens with the 3 mm beads). Parenteral applications are also possible because the 1 mm glass beads themselves don't produce a critical amount of glass splinters under optimized DAC-conditions (see also discussion below). The use of approx. the same number of stainless steel beads (0.5 and 1 mm) resulted in the same liposome sizes as using glass beads but VPG took on a slight yellowish coloring. However, amounts of glass beads higher than 150% resulted in larger liposomes. One explanation for that effect might be the prevention of the inward movement of the lipid dispersion from sticking to the "heavy" beads, which, due to their higher weight and round shape, are generally expected to be affected more by the centrifugal forces and not very effectively by the back rotation of the vial (low adhesion to the vial).

(iv) Influence of batch size: Variation of the amount of lipid dispersion for DAC from 0.25 up to approx. 4 g resulted always in the same liposomes sizes. This can be primarily explained by the fact that the whole load, irrespective if it is higher or lower, is subjected to the centrifugal forces and secondly, if there are increasing amounts of lipid dispersion, there is a larger contact area between the dispersion and the wall of the vial.

In conclusion, under optimum process conditions, small liposomes (60±5 nm, see Results Fig. 9) can be produced in a highly reproducible manner. Under these conditions, the channel width chosen for PCS-measurements (5, 10 or 20 μs) appears to be of minor relevance, which indicates that the redispersed liposome formulations contained only a minor amount of bigger particles. The high P.I. values found for all preparations showed that the particle size distributions were rather broad. However, the mean particle size as well as the P.I. values were similar to those found for liposomes made by HPH, and such small liposomes are suited for any kind of pharmaceutical application including i.v.-administration, as was shown for a new liposomal formulation of Gemcitabine based on VPGs [8,9].

Compared to liposomes made of the same lipid composition and sodium chloride solution by HPH at 700 bars (10 cycles), DAC liposomes are somewhat bigger (60±5 nm vs. 36±4 nm). Comparison of the trapping efficiency of the model compound calcein showed that in respect to the liposome size the trapping efficiency is also higher for the DAC liposomes (56.0±3.3% vs. 36.0±3.2%).

What is of importance, especially for the entrapment of drugs and biological compounds like peptides, proteins or nucleic acids (DNA, RNA, siRNA) is that the temperature of the lipid mixture stays at less than 51 °C for 30 min of DAC at maximum speed. It can therefore be expected that most sensitive drugs as well as the biological compounds (with the exception of some proteins) can be entrapped within liposomes by DAC without significant degradation. If lower temperatures are necessary, DAC-homogenization can be divided into a series of short DAC-runs combined with sample cooling between the runs.

A further proof that the process conditions of DAC are more gentle than HPH is that only minimal phospholipid degradation—as measured by lyso-PC-generation—could be observed, while HPH plus autoclaving (the latter is necessary to get the same sterile product as for the DAC-process) results in significant phospholipid degradation (0.23% vs. 1.2%).

The generation of glass particles from the glass beads during the DAC-process was found to be very low. A maximum of 348 particles equal to or greater than 10 μm and 36 particles equal to or greater than 25 μm were produced. Applying the European Pharmacopoeia criteria for i.v. infusions to evaluate whether the glass particle contamination is acceptable, all three samples passed the test (European Pharmacopoeia 5.1, specified in section 2.9.19 "Particulate contamination: sub-visible particles": 6000 particles equal to or greater than 10 μm and 600 equal to or greater than 25 μm/per vial). Liposomal formulations prepared by DAC are therefore suited for parenteral applications.

Most of the problems all established liposome preparation techniques have in common, such as overly large batch sizes, sterility and safety issues or the harsh conditions which limit the entrapment of sensitive compounds may be overcome by using DAC. DAC can be performed within a hermetically sealed vial/container that shields the product against (microbial) contamination and the apparatus, environment or the operator against contamination by toxic/radioactive material. Furthermore, by using a hermetically sealed container for liposome preparation, the process can easily be performed under an inert gas. Very small batch sizes of well below a gram are possible to process and by using even smaller vials for DAC, even smaller batch sizes are expected to be feasible, which is especially useful for entrapment of expensive materials like biological compounds (e.g. siRNA, Cytokines) for experiments in cell culture or animal experiments. On the other hand, it has to be mentioned that bigger DAC-machines are available on the market and thus, even medium batch sizes for preclinical testing and stability studies might possibly be produced.

The DAC apparatus used in this study is a bench top machine, that is cheaper than most alternative lab-scale machines such as high-pressure homogenization or filter extrusion and can readily be used in any research lab without the need of much extra space. In contrast to the aforementioned techniques, DAC is gentler and might therefore be suitable for entrapping sensitive compounds within liposomes. Furthermore, since the process is fast and can be performed aseptically within sealed sterile containers, it might be possible to even entrap compounds which are short-lived (e.g. isotopes) or chemically instable (e.g. alkylants) in a bedside preparation without the need of a dedicated production environment.

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