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# Dual Centrifugation - A Novel “in-vial” Liposome Processing Technique

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Additional information is available at the end of the chapter

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## Abstract

Conventional liposome preparation methods bear many limitations, such as poor entrapment efficiencies for hydrophilic drugs, batch size limitations, and limited options for aseptic manufacturing. Liposome preparation by dual centrifugation (DC) is able to overcome most of these limitations. DC differs from normal centrifugation by an additional rotation of the samples during the centrifugation process. Thus, the direction of the centrifugal forces changes continuously in the sample vials. The consequential powerful sample movements inside the vials result in powerful homogenization of the sample. Since this “in-vial” homogenization is optimal for viscous samples, semisolid “vesicular phospholipid gels” (VPGs) are preferred intermediates in the liposome manufacturing by DC. The DC method easily enables aseptic preparation and is gentler as compared to other methods, such as high-pressure homogenization. The method allows very small samples to be prepared, and VPG batches down to 1–5 mg scale have been prepared successfully. VPGs have several applications; they are attractive as depot formulations, or as stable storage intermediates, and can be easily transferred into conventional liposomal formulations by simple dilution. Here, we aim to present the novel DC-liposome technique; the concept, advantages, and limitations; and provide an overview of the experiences of liposome preparation by DC so far.

**Keywords:** dual centrifugation, dual asymmetric centrifugation, liposomes, vesicular phospholipid gels, homogenization, aseptic manufacturing

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## 1. Introduction

Liposomes as a drug delivery system are considered to be one of the most successful developments regarding the transfer from laboratory research to actual products on the market.

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From the first liposome drug delivery system approved for human use by the Food and Drug Administration (FDA) in 1995 (Doxil®), the field has expanded and, currently, 15 liposome- or lipid-based drug formulations are approved for human use [1]. Moreover, in 2013, there were 589 interventional drug studies focusing on liposomal drug formulations, of which at least 107 were active (ClinicalTrials.gov). The development in the field of liposome technology for extensive clinical studies requires industrial scale production not only due to the larger quantities but also under quality assurance and Good Manufacturing Practice guidelines [2].

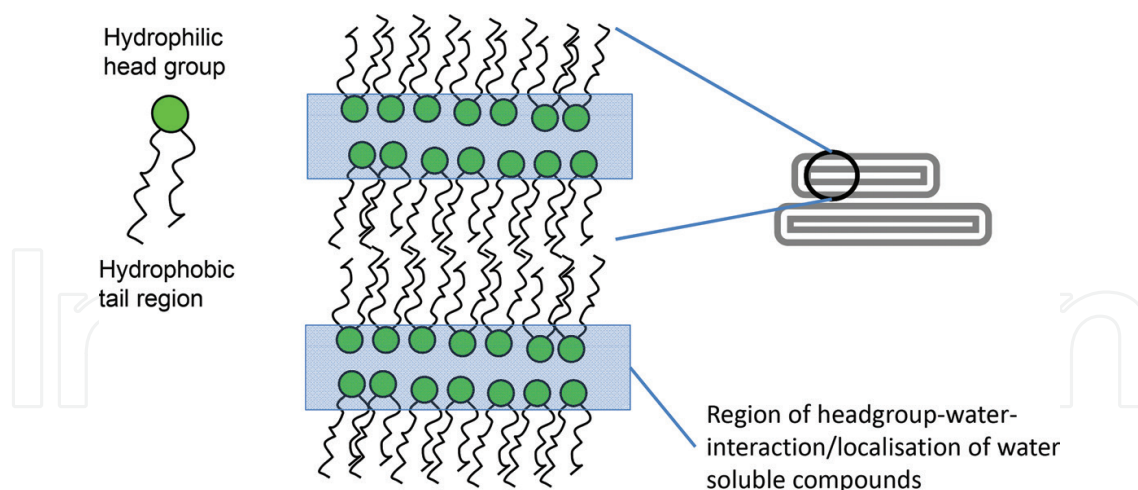
The aim of this chapter is to provide an overview over the state-of-the-art of a novel liposome preparation technique: “in-vial” homogenization using dual centrifugation (DC). First, we explain the concept of the new method and why highly concentrated lipid dispersions, usually named vesicular phospholipid gels (VPGs), offer several advantages to this method. Second, we comment on the potential of this method and on our own experiences when applying DC for the preparation of VPGs or liposome dispersions. Finally, we compare the two dual centrifuges that have been used for liposome production so far: the Speedmixer DAC 150 FVZ (Hauschild GmbH & Co KG, Hamm, Germany), used in the first liposome manufacturing by this technique, as well as the recently constructed ZentriMix 380 R (Andreas Hettich GmbH & Co KG, Tuttlingen, Germany).

## 2. Liposome preparation by dual centrifugation: concept and mode of action

One of the most important and easy ways to prepare liposomes is to homogenize membrane-forming lipids together with an aqueous phase like a buffer and the drug substance to be entrapped. As homogenization tools, high-pressure homogenizers [3, 4], the “French press” [5, 6], microfluidizers [7, 8], and related devices can be used. For entrapping water-soluble drugs into liposomal vesicles with the highest possible entrapping efficiency (EE), it is of advantage to homogenize highly concentrated lipid-water-drug mixtures. Since this is also the preferred way to make liposomes by DC, the characteristics of this strategy will be discussed first, followed by the presentation of the concept of DC and its use to make liposomes.

### 2.1. Background: preparation of liposomes from highly concentrated lipid-water mixtures by homogenization

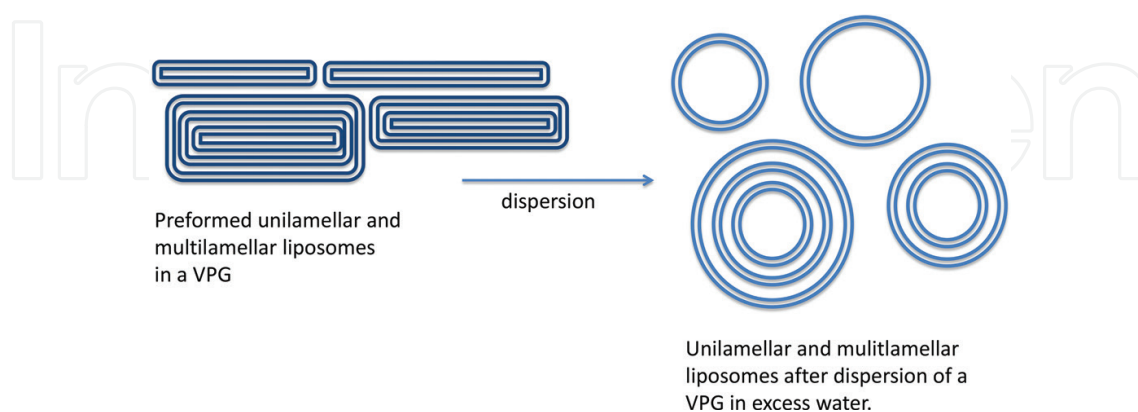
Homogenization of membrane-forming lipids together with an amount of water that is only sufficient to hydrate the polar head groups of the lipids (typically 50–70% water) results in highly concentrated, vesicular phospholipid gels (VPG) [3, 9]. Since the water used for VPG production is not sufficient to fill the inner core of the liposomes, it can be suggested that VPGs does not contain vesicular liposomes, but preliposomes that are expected to be flat structures without an aqueous core (**Figure 1**). The amount of water, which is sufficient only to hydrate the polar head groups, depends on the lipids used for VPG production. If bulky water-binding



**Figure 1.** Schematic view of a VPG consisting of preliposomes without an aqueous core. Roughly, all water molecules—together with water-soluble drug compounds—are in use for hydrating the polar head groups of the membrane forming lipids.

structures like PEG-chains are part of the lipid membranes, more water is needed in contrast to lipids carrying only phosphocholine head groups [10].

VPGs have a creamy consistency [11] and can directly be used as depot formulations, e.g., as subcutaneous injectable depots [12, 13] or can be dispersed in an excess of water to get normal aqueous liposomal formulations [14]. Dispersion of the VPGs allows the formation of normal liposomes by filling the preliposomes with additional water molecules, which can easily diffuse across the lipid membranes (**Figure 2**). Since in preliposomes the water molecules together with the water-soluble drug molecules were only distributed over the surfaces of the polar membranes, and since the surface area inside the liposomes is nearly as large as outside, it becomes clear that EE values up to 50% can be reached in the dispersed liposomal formulations [10, 15–17].



**Figure 2.** Schematic drawing of preliposomes in a VPG made by homogenization of a highly concentrated lipid-water mixture. The amount of water is sufficient to hydrate the phospholipid head groups, but is not sufficient to form vesicular, water-filled liposomes. The flat preliposomes became vesicular after dispersion of the preliposomes in an excess of water.

However, EE values higher than 50% for VPG-derived liposomes have been reported. This “overload” of water-soluble drugs is most probably due to the formation of multilamellar preliposomes and —after dispersion— of multilamellar liposomes (MLVs) (**Figure 2**). In MLVs, more of the membrane-associated, water-soluble drug molecules are entrapped due to the “entrapment” of additional membranes. Unfortunately, only a few articles reporting EE values >50% present also data on lamellarity —e.g., EM pictures [10, 17] or NMR studies.

Liposomes made by homogenization via VPG intermediates tend to have a higher lamellarity as seen by EM pictures [10, 17]. The lamellarity can somewhat be influenced by the type of lipids used [10]. Using lipids that ease the interactions between the surfaces of two membranes like PEG-carrying phospholipids leads to a higher lamellarity [17], whereby this interaction is further supported by the close vicinity of the membranes due to the low amount of water in the VPGs. In addition, also compounds to be entrapped may have an influence. Especially charged or very polar compounds that help to enable membrane-surface interactions like the multivalent-charged DNA molecules or divalent cations result preferably in the formation of MLVs during VPG production [16], and thus in very high EE-values.

In summary, the formation of liposomes with high EE values for water-soluble drug molecules as well as the formation of MLVs depends largely on the high lipid/water ratio used. Using an excess of water, EE as well as lamellarity goes down. Vice versa, using only the minimal necessary amount of water, EE is at its maximum, but lamellarity will be high as well.

Theoretically, reduction of the liposome diameter might help to reduce lamellarity, simply because in small liposomes there is only limited space to accommodate additional membranes. However, the average sizes of liposomes made by homogenization of highly concentrated lipid mixtures are small, but seldom below 100 nm with PI values > 0.2 (PCS (Photon Correlation Spectroscopy), intensity weighted<sup>1</sup>) [10, 16–21]. At a first glance, this appears somewhat confusing, since it is known that the liposome diameters clearly depend on the energy by which the highly concentrated lipid-water mixture has been treated and thus, liposomes made by HPH would have been expected to be very small.

The limited possibility of making very small liposomes via a VPG intermediate can be discussed as an intrinsic property of its high lipid concentration, typically >40% (w/v). During homogenization, the bigger membrane vesicles, which resulted from the initial hydration of the lipids, are broken into smaller membrane fragments, which spontaneously form smaller vesicles by self-organization. These smaller vesicles are disrupted again, forming even smaller vesicles. However, below a certain size, these lipid fragments not only form smaller vesicles by self-organization but also start to recombine into bigger vesicles. This process of recombination is more probable at an immediate vicinity of these fragments— or, in other words— at higher lipid concentrations as used for VPG-preparation.

To sum up, homogenization of highly concentrated lipid blends has been the preferred way to make liposomes with high EE for water-soluble drugs. The liposome sizes are still small enough even for parenteral use as well as for tumor accumulation via the so-called enhanced

<sup>1</sup>Many articles about liposomes made by HPH or DC present number-weighted PCS results, which values are much lower than the respective intensity-weighted PCS values.

permeability and retention effect (EPR-effect) [22]. The rather high content of multilamellar liposomes contributes to reach EE values of >50%. It has to be mentioned that multilamellarity of liposomes has never been reported as a problem in preclinical studies, e.g., in mice [14]. Furthermore, a high lipid load is sometimes of advantage. It has been found that liposomes made from hydrogenated phospholipids (PL) have anti-metastatic properties [23, 24] and that this effect is due to the high load of hydrogenated PL [25].

Despite the above-described advantages of liposomes made by homogenization, the homogenization tools currently in use have its limitations, especially for making liposomes in small batch sizes, under gentle and aseptic conditions. There is, therefore, a need for an alternative method that might overcome these limitations, which are listed below:

#### *2.1.1. Batch sizes*

Most of the homogenizers that are in use for liposome preparation have been developed to process bigger batches or at least batch sizes of several grams and very small batches are not possible due to the relatively high dead volumes of the devices. These "high" minimum batch sizes are a problem when only small amounts of liposomes are needed, i.e., for cell culture or animal experiments, and especially when the raw material applied is expensive or rare, like siDNA.

#### *2.1.2. Harsh conditions*

Since the samples in the normal homogenizers are in direct contact with the homogenizing unit (e.g., the homogenizing valve in HPH) for only a very short period of time (milliseconds), a large quantity of energy is needed to successfully homogenize the sample in that moment (VPG production with HPH: typically >700 bar, 10 cycles). Thus, the samples will heat up, which might damage the samples.

#### *2.1.3. Cleaning/metal contamination*

During homogenization, the liposomes are in contact with various parts of the homogenizers. Thus, a careful device cleaning prior to the liposome preparation is necessary. Furthermore, since the liposomes are squeezed through pumps and valves during homogenization, there is always the danger of product contamination with metal abrasion.

#### *2.1.4. Number of samples*

None of the known homogenizers is able to process more than one sample at once, which makes screening approaches burdensome, e.g., the screening for an optimal lipid composition for a new liposomal formulation.

#### *2.1.5. Sterile formulations*

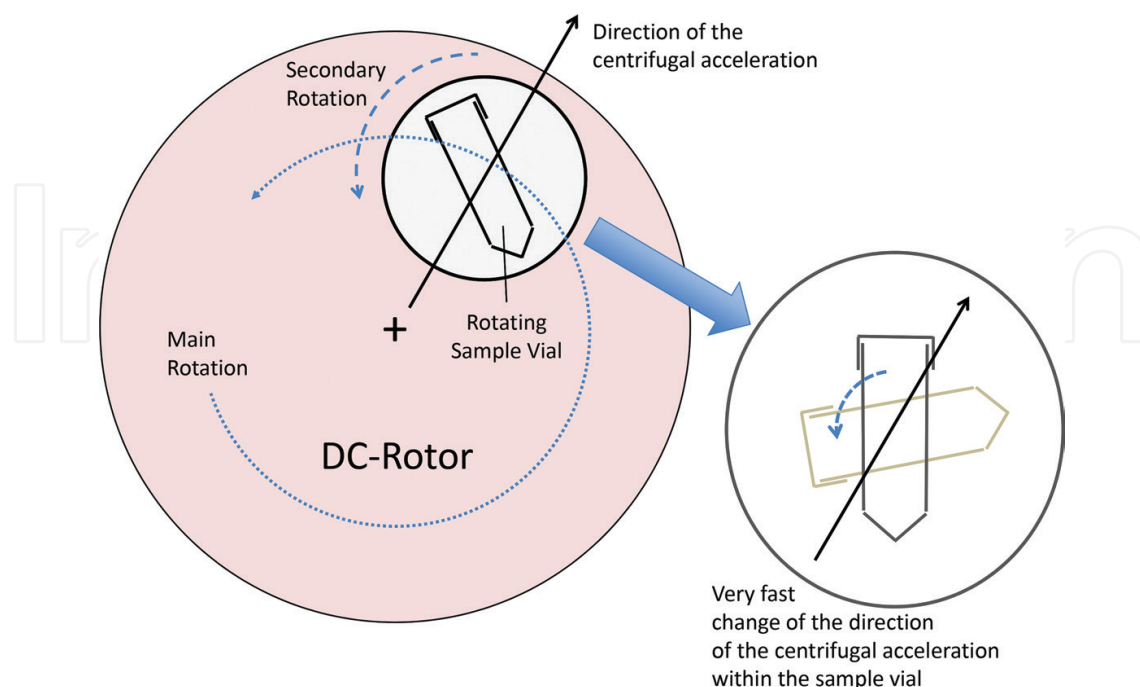
The dimensions of the known homogenizers are rather big, only a few of them can be placed into a sterile bench. Thus, the production of sterile liposome formulations that are needed for cell culture, animal experiments, or for human use is difficult.

The above-listed limitations can be overcome by using dual centrifugation (DC) as a homogenization technique [15]. DC easily allows the gentle preparation of liposomes and other lipid nanoparticles in very small batch sizes. The use of closed and disposable vials avoids metal contamination as well as cleaning of the homogenization device (“in-vial” homogenization). Using sterile vials allows production of sterile formulations. Furthermore, since the vials are tightly closed during DC, safe handling of particles with toxic compounds is possible. Many samples can be produced in parallel, which allows effective formulation screening.

## 2.2. Principles of dual centrifugation (DC)

DC is based on centrifugation. But in contrast to normal centrifugation where samples are turned around a central axis, during DC, the sample vials are additionally turned around a second rotation axis (**Figure 3**). Due to that the direction of high centrifugal acceleration in the sample vials changes continuously, which results in high frequent and very powerful sample movements. In contrast to normal centrifugation, DC is not a separation technique. DC is simply the opposite, a process which can be used for extremely powerful sample mixing, milling, extraction, dissolution, and homogenization, which is the central theme of this chapter.

While using DC for mixing purposes is well known for decades (e.g., for the mixing of two-component dental filling materials), its usefulness for homogenization and for the preparation of lipid nanoparticles was first described in 2008 [15]. Homogenization power of a DC depends on the rotating speed and the diameter of the dual rotor (→ centrifugal acceleration) as well as the rotating speed around the second axis (→ frequency of changing the direction of centrifugal acceleration in the sample vial). Furthermore, the shape and the orientation of the sample vial placed in the dual rotor strongly influence the homogenization power.



**Figure 3.** Principles of dual centrifugation (DC). Left: Dual rotor with a vial turning around its own axis (only one vial is shown). Right: Visualization of the changing direction of the centrifugal forces in a sample vial.

Homogenization power can also be increased by adding homogenization aids to the samples like ceramic beads.

#### 2.2.1. Speed of main and secondary rotation

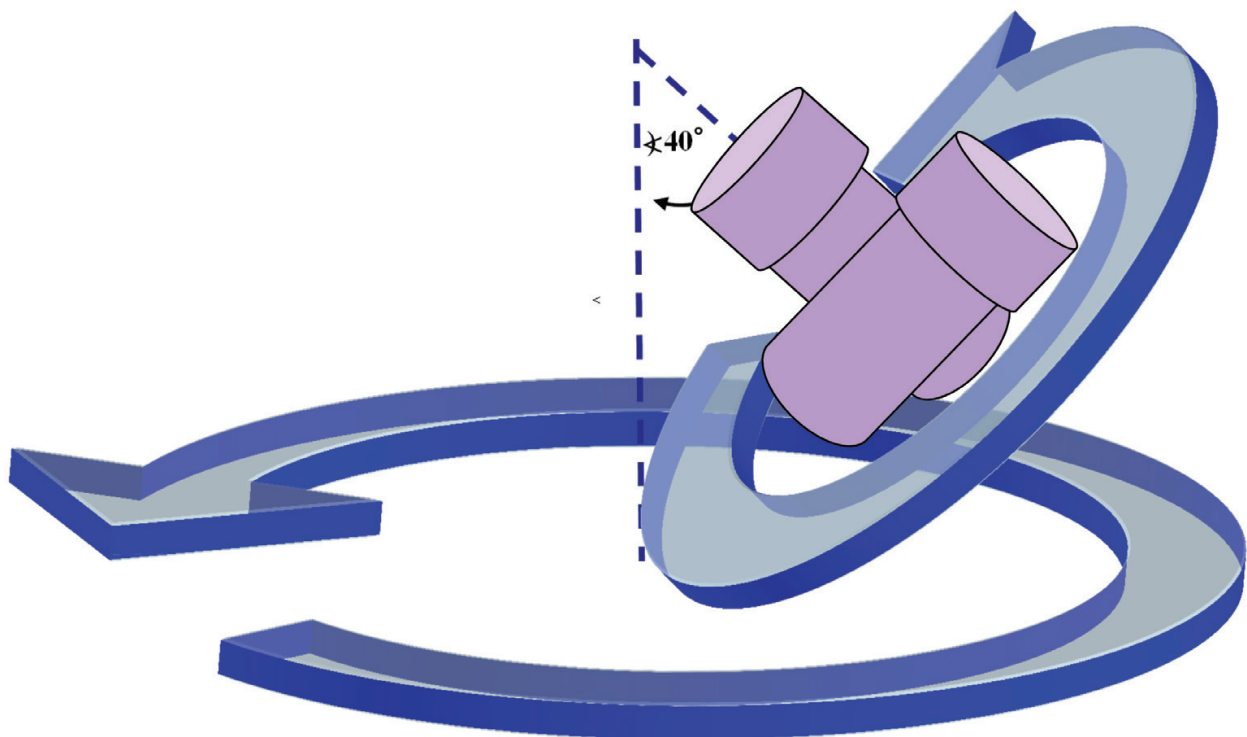
These parameters strictly depend on the type of dual centrifuge used for liposome preparation. Until now, two different dual centrifuges have been successfully used for this purpose, the ZentriMix 380R and the Speedmixer DAC 150. Despite being different in many aspects (the technical features of both DC devices are compared in Section 5), both devices are able to reach roughly the same maximum acceleration (about  $600\text{--}700 \times g$ ) and have about the same ratio between the main and secondary rotation (about 3-4:1), which is sufficient for making liposomes from highly concentrated lipid dispersions.

#### 2.2.2. Vial orientation during DC

Vials with a rather longish shape can be placed into the dual rotor in two different orientations—vertical or horizontal in relation to the plane of the second rotation unit (**Figure 4**). These different orientations result in two different homogenization processes.

##### 2.2.2.1. Vertical vial orientation

Using the vertical vial orientation, the rotating vessel walls are always equally distant from the secondary rotation axis. Processing highly concentrated lipid dispersions, the viscous and sticky material adheres to the rotating vessel wall, whereby it is transported against the



**Figure 4.** Vertical and horizontal orientation of long-shaped vials into a dual rotor.

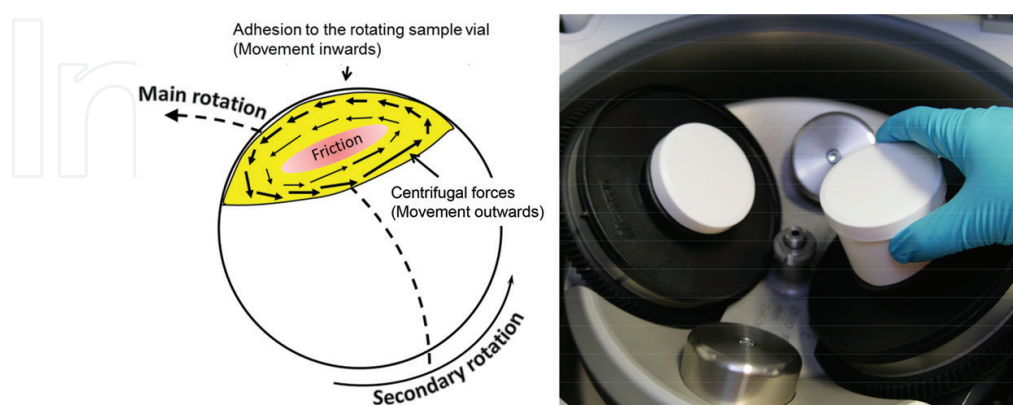
centrifugal forces (inward movement). At the same time, the centrifugal force pushes the sample material that is not in close contact to the vessel wall in the opposite direction (outward movements). Both contrary movements results in strong friction and shear forces within the viscous material and thus, in homogenization (**Figure 5**, left). Homogenization using vertical vial orientation is especially effective in vials with a rather large diameter, e.g., 150 ml disposable PP beakers for the homogenization of bigger batches (**Figure 5**, right).

#### 2.2.2.2. Horizontal vial orientation

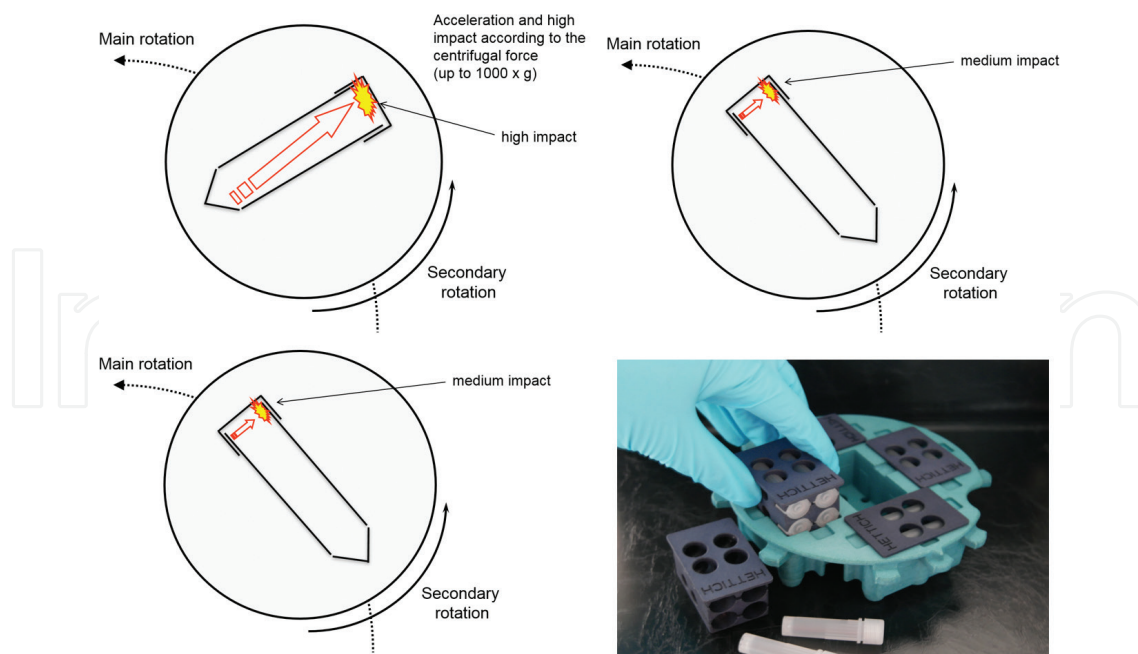
Using the horizontal vial orientation, the position of the vial in relation to the centrifugal acceleration changes continuously while turning around the second axis (**Figure 6**). Having the lengthy vial parallel to the acceleration vector allows the material to gain speed and kinetic energy over a rather long distance, thus clashing to the top or the bottom of the vial with high impact. Within the next forth of rotation, nothing happens until the vial turns into an angel, which allows the movement of the sample material again. “From the vials point of view,” the sample material constantly moves from the bottom to the top of the vial with high acceleration, including a soft transition in between. This type of movement is similar to that in a horizontal ball mill, with the difference that the acceleration of the sample material (and the balls) in DC is much higher and always constant (up to  $1.000 \times g$ ). Especially when very small amounts of sample material will be homogenized in small vials (e.g., PCR tubes or 2 ml PP vials), the horizontal orientation is much more effective than the vertical orientation.

#### 2.2.3. Homogenization aids

Despite liposome preparation by DC-homogenization is possible without any homogenization aids, the outcome gets better and the process faster if homogenization aids, in the form of heavy beads, are added. Those beads work in two ways: on the one side, the beads help to bring the sticky and viscous lipid blends rapidly in motion—simply by increasing the density



**Figure 5.** Left: Schematic view into a rotating vial in a dual rotor. At the same time, the viscous sample material (e.g., VPGs)—is transported against the centrifugal forces due to adhesion to the vessel wall, and in the opposite direction due to centrifugal acceleration of sample material rather distant to the vessel wall. Both contradictory movements result in shear forces and inner friction of the viscous sample material and thus, homogenization and formation of small liposomes. Right: Using standard PP beakers in a dual centrifuge placed in vertical orientation (view from top).

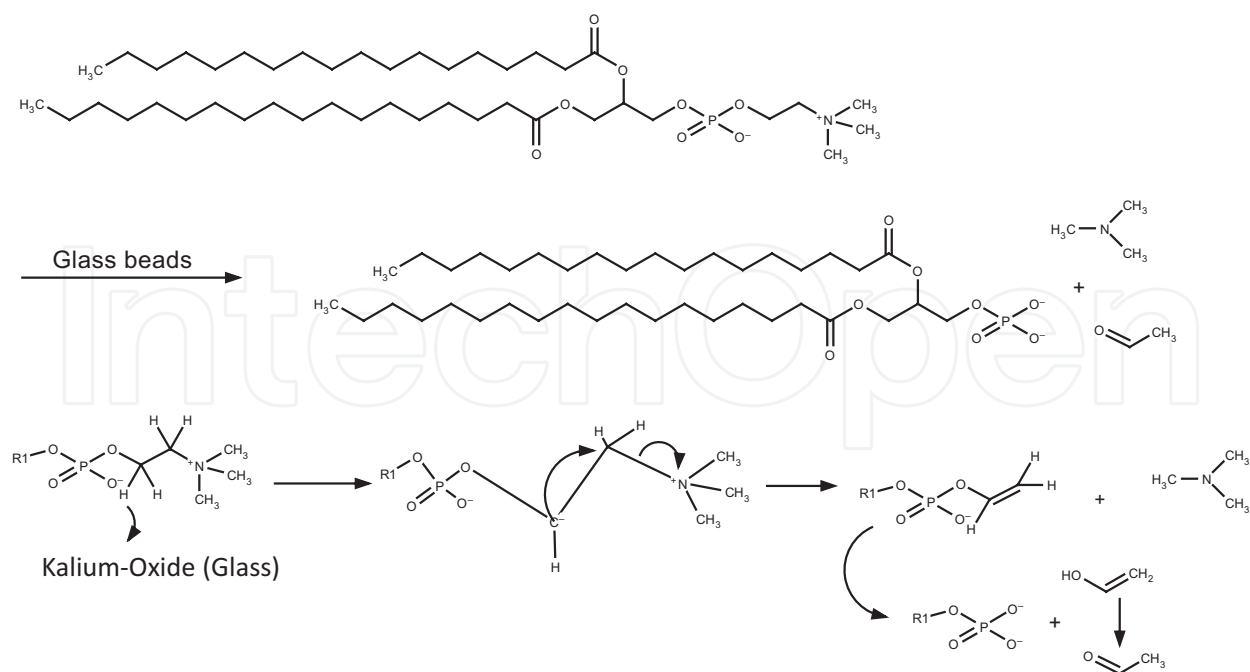


**Figure 6.** Schematic view into a lengthy vial in a dual rotor in "horizontal orientation," showing the movement of sample material (for detailed description of the process, see text). Below and left: Adapter for placing 20 × 2 ml vials in a dual centrifuge in horizontal position (only one of the two possible adapters is shown).

of the bead-lipid-water mixture and thus its potential energy which is not only a function of the centrifugal force but also of the weight of the accelerating material. On the other side, DC-homogenization is more effective due to the collision between the beads—with the viscous lipid mixture in between. That the bead-bead interactions play an important role for effective homogenization is supported by the finding that an increase in the number of beads while keeping the bead mass constant (smaller beads) results in the best DC-homogenization (more beads—more bead-bead interactions). Possible bead materials are glass, stainless steel, or ceramic.

#### 2.2.3.1. Glass beads

At the onset of the development of DC-homogenization, glass beads which are cheap, easy to sterilize, and believed to be inert have been used. However, it turned out that glass beads get rapidly smaller during DC, which can be explained by their rather soft surface and the very frequent bead-bead interactions. In addition, depending on the intensity of the DC process and the processing time, samples developed an unpleasant smell of fish, a phenomenon which unfortunately was reproducible. We concluded that the frequent interactions between the glass beads results in micro- and nano-sized glass particles (dust) with a huge and, of course, fresh basic surface, e.g., due to the potassium oxide within the glass. We propose that due to these polar and basic particles, a so-called Hoffmann elimination of phosphatidylcholines takes place (**Figure 7**), resulting in phosphatidic acid, acetaldehyde, and trimethylamine, explaining the fishy smell. The reaction proposed here belongs to the field of heterogenic catalysis and has not been seen so far with phospholipids. Beside chemically of interest, even a little degradation of phospholipids due to the use of glass beads might be a problem. Thus, after a few initial studies, we completely avoided glass beads in DC for making liposomes.



**Figure 7.** Proposed mechanism of the degradation of phosphatidylcholine during DC of phospholipid blends with glass beads as homogenization aids (top: Overview of the Hoffmann elimination of phosphatidylcholine. Below: Mechanism of the Hoffmann elimination of the phosphocholine unit).

#### 2.2.3.2. Stainless steel beads

The biggest advantage of steel is its high density of typically  $> 7.5$  kg/l, which would be ideal to support the process of the “in-vial” homogenization. Unfortunately, steel has a rather soft surface, resulting in steel abrasion that is not acceptable for formulations used in cell culture, animal experiments, or in men. Generation of lipophilic steel abrasion can easily be seen by the resulting yellowish-colored liposome preparations.

#### 2.2.3.3. Ceramic beads

Ceramic is the material of choice, and there is a lot of experience for beads made of zircon oxide stabilized with yttrium (ZY-beads). ZY-beads have a density of  $> 6$  kg/l, which is about three times higher than glass. In contrast to glass and steel, the surface is extremely hard (microhardness: 1.200 HV [hardness according to Vickers, corresponding to a Mohrs-hardness between 7 and 8]). ZY-beads are commercially available, even in food and pharmacy grade. Finally, using ZY-beads resulted in the best DC-homogenization so far (unpublished data). A typical procedure for making liposomes is to add the same weight of beads, as the weight of the lipid-water mixture in the respective vial. A typical diameter of ZY-beads used for liposome preparation is 1.5 mm which is big enough to subsequently remove the beads by filtration, or—after dispersion of the resulting VPG—by simply removing the liquid dispersion by a standard pipette, the beads are too big to be sucked into the pipette.

It has to be mentioned that zirconia-containing particles are in use to remove traces of phospholipids from extracts of biological samples to reduce the matrix effects during MS-analytics.

Thus, it can be assumed that the ZY-beads used for DC-homogenization also interact with the phospholipids during VPG production, an effect—if real—which obviously has no negative effect on liposome production.

### 3. Potential of the DC-liposome technique

Manufacturing of liposomal dispersions destined for human use and biomedical research should fulfill some important criteria, and the resulting liposomes should (i) be uniform/homogeneous, (ii) have a reproducible particle size range, (iii) be sterile, and (iv) be free of any traces of harmful substances such as organic solvents or glass residues. Not related to the manufacturing process, but very helpful for its acceptance would be an adequate shelf life of the liposomes [1]. In addition, the cost- and time-effectiveness of the manufacturing process should be considered. Based on these criteria, we discuss the potential of the newly developed DC-homogenization in liposome production in relation to

- I. its reproducibility and robustness (Section 3.1),
- II. the possible batch sizes and the sample capacity (Section 3.2),
- III. the gentleness of the DC method as compared to other liposome preparation methods (Section 3.3) and
- IV. the cost-effectiveness relative to other methods (Section 3.4)

In addition to these issues, we also discuss the potential of applying DC for the dilution of VPGs to normal liposomal dispersions (Section 3.5).

#### 3.1. Reproducibility and robustness of the DC method (liposome size, PI, and EE)

##### 3.1.1. Lipid concentration and composition

As already stated in Section 2, efficient homogenization and size reduction by DC demand concentrated and thus viscous lipid dispersions. With increasing lipid concentrations from 10% (w/v) up to 35% (w/v), an improved efficiency of DC-homogenization was observed, which was illustrated by decreasing vesicle sizes [15]. The reproducibility of the process was also improved with higher lipid contents, resulting in a lower variability of the liposome sizes between the batches. However, there is a threshold lipid concentration, and VPGs made from 50% (w/v) lipids showed a small increase in size and a poorer reproducibility [15]. Thus, a lipid concentration range between 35 and 45% (w/v) seemed advisable regarding the applied DC processing conditions; EPC3/cholesterol (55:45 mole/mole; 0.5 g batches in 30 ml injection vials plus 0.5 g glass beads ( $\varnothing = 1$  mm); DC for 30 minutes at 3540 rpm (Speedmixer DAC 150 FVZ). However, different threshold values have been reported with different lipids applied. When a phospholipid mix with 80% phosphatidylcholine (Lipoid E-80) was the applied lipid raw material, VPGs with up to 50% lipids were successfully prepared [26]. Here, the 50% (w/v) VPGs enabled smaller liposomes ( $163.4 \pm 58.2$  nm) with lower standard deviation and

batch-to-batch variations, as compared to VPGs containing less lipids (30% (w/v):  $486.2 \pm 92.8$  nm; 40% (w/v):  $216.5 \pm 76.3$  nm). In addition, the more concentrated VPGs gave higher EE values and a lower polydispersity index (PI), with a PI of  $0.287 \pm 0.065$  and  $0.186 \pm 0.044$ , in the 30 and the 50% (w/v) VPGs, respectively [26].

The effect of lipid composition on the liposome size has also been demonstrated for VPGs with the same lipid concentration and processing conditions. Using a more rigid lipid blend containing saturated phosphatidylcholine (HSPC/CHOL/DSPE-PEG) resulted in bigger liposomes than a more fluid mixture containing an unsaturated phosphatidylcholine (POPC/DDAB/DSPE-PEG) in 35% (w/v) VPGs [10]. Similarly, the morphology of the liposome vesicles was affected by the lipid composition, with more lamellas formed in more rigid lipid formulations [10]. The same effect was observed when propylene glycol was added to the lipid mixtures (PG-Lip) [18]. PG obviously reduces the lipid interactions in the lipid bilayers and makes the membranes more flexible than the conventional liposome formulation without PG (C-Lip). This gave a significant shorter DC processing time required to reach the aimed liposome sizes of 200–300 nm, the optimal liposome size for topical drug deposition [27], with a processing time of 3 minutes with PG vs. 50 minutes without PG [18]. In these experiments, the standard deviation of the vesicle sizes, and the PI values were higher for the PG-Lip formulation because of the reduced processing time. However, the reproducibility of the processing method was still found acceptable;  $PI = 0.31 \pm 0.03$  (PG-Lip) and  $PI = 0.13 \pm 0.02$  (C-Lip), mean liposome sizes and standard deviation ( $n \geq 3$ ) =  $278 \pm 66$  nm (PG-Lip) and  $282 \pm 30$  nm (C-Lip), respectively [19].

### 3.1.2. Processing time

As expected, but only up to a certain extent, the DC processing time affects the vesicle size [15]. When using a rather rigid lipid blend consisting of saturated phosphatidylcholine and cholesterol (55:45 [mole/mole]), 5 minutes of DC-homogenization at maximum speed gave a mean liposome size of 105 nm (measured by PCS, number weighted), and after 30 minutes of DC processing, the size was further reduced to 62 nm with a reduced standard deviation. Since longer DC processing (up to 60 minutes) gave no further size reduction, a processing time of 30 minutes was recommended as optimal [15].

### 3.1.3. Mixing aids

In the first DC-experiments, the addition of glass beads as a mixing aid has been demonstrated to increase the efficiency of the particle size reduction by DC-homogenization [15]. Regarding the amount of glass beads ( $\varnothing = 1$  mm), the addition of 50–100% (w/w), relative to the total weight of the VPG, enabled the most efficient particle size reduction, as well as a more homogeneous size distribution [15].

Recently, alternative materials to glass as mixing aids have been tested and found more appropriate (see also Section 2.2.3). Even if glass beads have been the most applied mixing aid in DC [10, 15–19, 21, 28–32], and the minor amounts of glass particles found in the preparation postprocessing was judged acceptable [15]; new findings reported in Section 2.2.3 show that even a small amount of glass abrasion will catalyze the degradation of PC. Thus, ceramic beads

made of zircon oxide stabilized with yttrium (ZY-beads) seem to be the material of choice. The higher density of the ZY-beads, as compared to glass beads, results in a smaller volume of ZY-beads necessary to support DC-homogenization. In addition, since the ZY-beads are heavier and available in very small diameters, the number of beads can be increased to gain the aimed bead-bead interactions needed for efficient size reduction, whereas keeping the bead volume constant. However, successful preparation of liposomes by DC was also demonstrated without any mixing aid present [13, 33, 34].

### 3.1.4. Drug entrapment efficiency

In general, entrapping water-soluble drugs into VPGs does not seem to affect the liposome vesicle sizes [10, 15, 18]. The water-soluble fluorescence dye calcein has been used in several studies to investigate the entrapment efficiency of water-soluble drugs into DC-manufactured liposomes [15, 16, 21]. These studies, as well as a study where the fluorescence dye 5,6-carboxyfluorescein (CF) was used [35], demonstrate that the DC-VPGs enable a high EE of hydrophilic drugs compared to what is achievable with other techniques, and typically > 50% EE is reported. From the previous experience with VPGs made by HPH [36], the high EE of water-soluble drugs in the VPGs made by DC is somewhat expected. However, EE of calcein into VPGs made by HPH was surprisingly lower than into VPGs made by DC-homogenization ( $56.0 \pm 3.3\%$  vs.  $36.0 \pm 3.2\%$ ) [15]. This difference might be explained by the bigger size of the liposomes produced by DC ( $60 \pm 5$  nm [DC] vs.  $36 \pm 4$  nm [HPH], both number weighted), and the suggested advantages of having liposomes with higher number of lipid lamellar bilayers (see Section 2.1.). Also in accordance with the suggestions made in Section 2.1, a higher drug entrapment of water-soluble drugs with increased lipid content has been demonstrated in several studies. As an example, EE values for the anticancer drug cytarabine (Ara-C) were  $31.7 \pm 0.31\%$  in 30% (w/v) VPGs and  $72.1 \pm 0.25\%$  in 50% (w/v) VPGs, respectively [6].

Also more lipophilic drugs, such as chloramphenicol (CAM), have been entrapped into liposomes by DC-homogenization and EE values close to 50% were found [18, 19]. When comparing this value with EE values obtained with the conventional liposome production techniques, probe sonication and filter extrusion, when using the same lipids and CAM-to-lipid ratio, a 70% increase in EE was achieved by using DC, even though the resulting liposomes were smaller ( $282 \pm 30$  nm) compared to those getting from sonication (836 nm) or extrusion (667 nm). Thus, the higher EE values were suggested to be due to the increased lipid concentration and the less available aqueous media for the drug to diffuse into, as CAM will be in an equilibrium between the dissolved portion of the drug present in the aqueous phase and the portion located in the lipid bilayer [18]. This is in agreement with the previous suggestions, claiming that VPGs that contain drugs that tend to diffuse through and from the liposome bilayers into the aqueous phase of the liposomal dispersion must be stored as VPGs to achieve an acceptable shelf life. This is because liposomal dispersions are characterized by a huge extravesicular water phase where these drugs will participate, and thus the EE of the drug will be reduced as soon as the VPGs are diluted into liposome dispersions [36].

To summarize, the most important factors affecting the size distribution for liposomes made by DC-homogenization are processing time, type, and size of the mixing aid, lipid concentration, and lipid composition. The polydispersity index (PI) obtained for liposomes made

by DC is rather low compared to that of HPH, reflecting a homogeneous size distribution, and PI values  $<0.2$  are frequently reported [19–21, 28, 30, 35]. However, PI increases when bigger liposomes are obtained due to the formulation and/or processing conditions selected [15, 18, 26]. Regarding the entrapping efficiency of drugs into liposomes, DC seems to be advantageous over other manufacturing techniques, independently of the charge or solubility characteristics of the drug, and the DC method turned out to be robust and highly reproducible.

### 3.2. Batch sizes/sample capacity in DC

The batch sizes processed by DC homogenization depend on the capacity of the DC instrument used. So far, the DC technology in liposome production has been demonstrated at the laboratory scale, and the development of this technology for industrial scale production of liposomes remains to be confirmed. However, some progress has been made lately, with the new DC prototype ZentriMix 380 R that has a higher capacity compared to the Speedmixer DAC 150 FVZ which has been used for the basic developments of DC-homogenization (compared in Section 5 of this chapter). With the new ZentriMix 380 R, the maximum cargo has been substantially increased from 150 to 1000 g, but maybe more important, the number of the typically used 2 ml vials has been increased from 4 to 40.

The possibility of processing up to 40 samples with different compositions in the same run is a big advantage when conducting screening experiments, e.g., for comparing different liposome formulations. But also when using bigger vials, e.g., 10 ml injection vials for preparing liposomes for potential human use, 10 vials can be prepared in parallel (see **Table 1**, Section 5).

Beside the advantages of DC processing of many small sample vials in one run, the possibility to prepare very small batches significantly helps to save costs, e.g., when doing experiments with expensive materials such as siRNA [10, 16], or when compounds to be entrapped or special lipids are only available in small amounts [35, 37]. Thus, the typical batch sizes applied in the DC-homogenization are usually  $< 500$  mg (2 ml vials), and batch sizes down to 20 mg [16] and 1–5 mg [37] have been reported as well. These small batch sizes significantly differ from the minimal batch sizes when using HPH. Even with one of the smallest HPH devices, the APV Micron Lab 40 lab-scale homogenizer, relatively huge amounts of lipids and drugs were necessary. Producing a VPG with a final lipid concentration of 40% would demand 16 g lipids and 24 g buffer to fill the 40 ml homogenization vessel [38]. Thus, with the sample material for one HPH experiment, 400 DC experiments (à 100 mg) can be performed, which allows efficient screening of the optimal conditions.

However, one can discuss that small batch sizes will reduce EE values for water-soluble compounds since such an effect was observed when siRNA was entrapped in conventional liposomes. Reduction of the batch size from 60 to 20 mg VPG resulted in a reduction of siRNA entrapment from 71 to 55% [16]. The same effect was observed for siRNA in sterically stabilized liposomes. Since it is known that nucleic acids have an affinity to polar glass surfaces, this batch size effect was explained by a possible unspecific binding of the siRNA to the vials surface [16]. Therefore, absorption of active ingredients onto the vial surface or to the surface

of the mixing aids (glass beads) might reduce EE values, especially when very small batches are processed. However, in the same study, entrapment of calcein, which has virtually no affinity to polar glass surfaces, was found not to be affected by the samples size [16].

Small batch sizes are also advantages when toxic materials, such as cytostatic or radioactive compounds, are used as ingredients in liposomes formulation. Since the DC method also facilitates handling samples in the closed container, it is advantageous to the environmental and personnel safety concerns. Thus, DC was the method of choice and enabled successful preparation of Ara-C containing VPGs for a local injection into the brain tissue, to provide a sustained drug release in treatment of gliomas, and *in vivo* studies in rat and mice models [26].

### 3.3. Gentleness of the DC method and advantages of closed container in DC

#### 3.3.1. Temperature

All homogenization methods suitable for liposome size reduction are based on bringing energy into the sample through applying shear forces, which also contribute to a temperature increase of the sample. Rise in temperature will accelerate any hydrolytic degradation processes and should be avoided to protect phospholipids and other constituents from degradation. Since the Speedmixer DAC 150 FVZ has no cooling unit, the temperature and gentleness of the processing method were investigated carefully [15]. For comparison, VPGs were also prepared by the HPH performing 10 homogenization cycles at 700 bars. Both, DC- and HPH-liposomes were analyzed for phospholipid hydrolysis by measuring the content of the hydrolysis product, lyso-phosphatidylcholine (lyso-PC). The results showed that there was only a slight increase in lyso-PC during the DC processing (<0.1%), whereas the liposomes processed by HPH had a lyso-PC increase of approximately 1% when autoclaved, which is necessary to get sterile formulations [15]. The temperature of the sample during the DC was also monitored and found not to exceed  $50 \pm 1^\circ\text{C}$  during the 30 minutes processing time ( $6 \times 5$  minutes, interrupted by mandatory breaks for downcooling) [15].

When 40% (w/w) DC-VPGs, containing Ara-C and added sodium sulfite as a preservative, were autoclaved, no chemical degradation was noticed after autoclaving, as no degradation products neither for the Ara-C drug nor for the E80 lipids applied in the formulation were found. The only change observed was an improved viscosity [26]. These results are in accordance with the earlier studies on autoclaving of VPGs made by HPH that was also proven stable during autoclaving [39].

The gentleness of the DC method opens new applications for VPGs and liposomes, namely, to incorporate highly sensitive drugs such as proteins, i.e., References [13, 28, 34]. As regards to the lack of a cooling unit, the short-run cycles are a common way to control the temperature rise, and Tian et al. [13] made 1.5 minutes of centrifugation steps interrupted by a cooling at  $2\text{--}8^\circ\text{C}$  every 6–8 runs, which gave a successful incorporation of model protein erythropoietin (EPO) into the VPGs. However, the new DC device ZentriMix 380 R is equipped with a powerful cooling unit, which allows also longer runtimes without cooling down breaks.

### 3.3.2. Closed container

Since DC homogenization is carried out in closed container(s), the sample vial(s) can be flushed with inert gas to reduce oxidation of sensitive components. This is however not the normal praxis, but sealing of the vials under nitrogen prior to autoclaving of VPGs has been reported [26]. Others have stored DC vials prefilled with beads and lipids (lipid film) under argon at RT until use [17]. The concept of making a homogeneous lipid film (molecular dispersed lipid mixture) directly in the DC vials for subsequent hydration and DC-homogenization has been demonstrated and found highly useful to optimize the “in-vial” performance of the method, avoiding material transfer and possible material loss [18, 19]. This is contrary to other liposome processing methods such as the HPH, membrane extrusion, or sonication. The high material recovery has been demonstrated by phosphorous determination in the liposomes after DC-homogenization and removal of the nonentrapped drug by filtration and or/or dialysis [19]. These results show a lipid recovery of about 100% after the DC-homogenization, and a lipid loss of approximately 13% after dialysis and filtration (0.22  $\mu\text{m}$  filter). Thus, if acceptable EE values are obtained and a removal of nonentrapped drug is avoided, a close to 100% recovery is achievable.

The protection of light-sensitive drugs from light during liposome preparation is also possible by DC. In addition to the dark conditions provided in the locked DC machine, brown injection vials have been applied for extra protection during handling [15, 16, 18]. For comparison, when liposomes are prepared by the HPH, open handling is necessary to move the sample content from the receiver reservoir to the feeding reservoir in between every homogenization cycles (typically 10 cycles). Thus, to protect the sample from light during the processing, one would have to operate the instrument in the dark. Closed containers are also of advantage when toxic materials, such as cytostatic or radioactive compounds, are used as ingredients in liposomes formulation (environmental and personnel safety).

### 3.3.3. Bedside preparation

Since liposomal formulations contain water and since most low molecular weight drug molecules (e.g., conventional cytostatics) are able to diffuse through liposomal membranes from one aqueous compartment into the other, the liposome shelf life is limited to hours, days, or sometimes weeks. Furthermore, drug molecules sensitive to hydrolysis, like alkylants, might also hamper stability. Some drug molecules are also shown to promote phospholipid hydrolysis, like gemcitabine [40] and camptothecin [41]. Taken together, shelf life is a major problem when developing liposomal formulations. Since DC-homogenization allows fast and straightforward preparation of liposomes in sterile containers, bedside liposome preparations for patient treatments might be one potential application of DC in the future [15]. Applying readymade kits—containing the dry lipid mixture, the drug molecule, and mixing aid—would make the liposome preparation easy, as only adding the aqueous media before the subsequent DC processing to form VPGs, remains. This strategy might facilitate personalized medicine and bedside preparation to take place without any need for dedicated production rooms, as the DC method is performed with locked containers. Subsequent dilution (dispersion) of the VPGs and dosing of the resulting liposomes into an infusion bag would be one potential way of administering the drug, where the rapid infusion into the patient would complete the bedside preparations process. DC instruments are bench-top

machines, which fit into any hospital pharmacy, where the bedside preparation and the subsequent dosing can be performed. However, not only liposomes with a limited shelf life can be made assessable for the patients, also liposomal formulations carrying short living isotopes, e.g., PET tracers—for a better tumor visualization due to an improved tumor accumulation of the liposomes due to the EPR effect—might be a promising application for DC-homogenization. If necessary, the DC instruments are small enough to be placed into a sterile bench [15].

### **3.4. Cost-effectiveness of the DC-liposome technique**

#### *3.4.1. Easy and fast DC-homogenization*

Many of the already mentioned advantages of the DC in liposome processing, such as high material recovery [19], small sample sizes [35], and straightforward and fast procedure [19], will also save material cost and personnel expenses in the laboratory. As compared to the more time-consuming techniques such as the filter extrusion [42], HPH homogenization [36], and ultrasound [43], the relatively higher drug EE obtained with concentrated lipid dispersions (VPGs) may make the time-consuming separation step, removing nonentrapped drug from the liposome drug carrier, unnecessary before the administration [18]. Especially when drugs are entrapped into liposomes to be protected from degradation in the blood stream, one might argue that the resulting liposome dispersions with high entrapment might be administered without removing the nonentrapped drug, as the free drug will be destroyed immediately after administration into the blood stream [11]. An example is liposomal gemcitabine, where the free gemcitabine has a half-life of 9 minutes, while the liposomal gemcitabine has a half-life of 13 hours [40].

#### *3.4.2. Device cleaning*

It is not only the fast homogenization procedure that is saving time to the operator as compared to other liposome techniques. With disposable and closed containers, the cleaning of the machine is avoided. This eliminates also the risk of cross contamination, which might happen during other methods resulting from a nonsufficient cleaning of the instrument (i.e., the probe applied in probe sonication—and instrumentation applied during extrusion—or HPH).

### **3.5. Mixing of liposomes into secondary vehicles**

As DC is not only an "in-vial" homogenization tool but also an "in-vial" mixing or dispersion tool, DC machines are also suitable for dispersing the highly concentrated VPGs to normal liposomal dispersions. VPGs-dispersion in an aqueous media like a buffer can easily be performed by simply adding the required volumes of aqueous media to the VPGs, using the same vials already used for DC-homogenization, and continuing the DC process for typically 1–5 minutes [17, 28].

However, if topical liposome administration is the target application, there might be a need to disperse liposomes into more semisolid, viscous vehicles, such as hydrogels, to gain the wanted qualities of the product, such as texture, stability, drug release profile, and bioadhesion [44–47]. The DC technique has been proven suitable for both liposome processing and further

mixing of the liposomes into hydrogels, and the topical preparations as liposome-in-hydrogels was reported to be homogeneous after DC for 5 minutes at 3500 rpm in the Speedmixer [18]. The DC technique has also been applied to prepare liposomes for further embedding into gelatin [21]. Here, the DC technique was useful for both the size reduction of the liposomes forming VPGs and for the direct dispersion of liposomes into gelatin. Solid matrix liposomes in gels are obtained from the gelatin-VPG mass after the cooling [21].

#### 4. Dual centrifugation: current application in liposome research

Since DC-homogenization was first introduced as an innovative method for liposome preparation in 2008 [15], a number of publications have appeared reporting on this technology for different liposome applications. However, the most appreciated advantages of the method seem to be its gentleness, the unusual high EE values for water-soluble drug molecules, and the small batch sizes. Moreover, the majority of the publications concern the entrapment of heat labile peptides and proteins. These proteins and peptides have been entrapped into VPGs and liposomes for different routes of administration: oral [21, 30, 31], nasal [33], and iv injection [17, 20, 28, 29], or as VPG-depot formulations [13, 34, 48]. Various organs were targeted as therapeutic sites: cancerous tissue [10, 26, 35], the central nervous system (CNS) and brain [28, 32, 33], liver [31], and skin [18, 19].

The DC “in-vial” liposome technique has facilitated a straight forward preparation method for entrapment of siRNA under sterile and RNase-free conditions, with EE close to 50% EE [16, 49]. Targeting of the siRNA-liposomes to neuroblastoma cells through applying DC in a combination with a sterol-based postinsertion technique was demonstrated *in vitro* [10].

Recently, the antitumor effect of anticancer drug cytarabin (Ara-C) loaded into VPGs was evaluated *in vivo* in nude mice bearing U87-MG glioma cells, as compared to empty VPGs and free drug in solution. The formulations were injected subcutaneously around the tumor. For the same Ara-C-VPG formulation, the *in vivo* drug release was studied in rat plasma and the *in vivo* biodistribution in rat brain evaluated for 28 days using the UPLC-ESI-MS/MS quantification assay [26]. Here, a sustained release of Ara-C from the VPGs was confirmed as the drug (Ara-C) administered as free drug in solution could be detected in rat plasma already after 7 hours postinjection into the brain, whereas no drug was detected after 7 days from the Ara-C-VPG formulation. Moreover, the therapeutic effect lasted at least for 28 days for the Ara-C loaded VPGs, and VPGs were thus suggested as a promising local delivery system for postsurgical sustained chemotherapy of gliomas [26].

The DC liposome technique has also been applied to explore new polymeric amphiphiles, so-called polyether lipids or polymer-lipid conjugates, for obtaining multifunctional liposomes. Here, DC was offering the benefit of testing the rationally designed polymer architectures in a microscale [35, 50].

In a different study, a simple and reproducible method for preparing immune-magneto-liposomes (ML-liposomes), with iron oxide particles (SPIOs) was developed applying the DC liposome “in-vial” technique. After the liposomes were formed, antigen binding to the surface of the

liposomes was successfully carried out applying a postinsertion technique [17]. The antigen should facilitate targeting of the activated platelets in atherothrombosis, and thus form a contrast agent for timely detection and diagnosis [17].

The DC technique has also been proven useful in preparing so-called liposomes-in-hydrogel formulations intended for topical application for local deposition of active ingredients and for a sustained release into the skin [18, 19]. In this production procedure, not only the homogenization and size reduction of the liposomes but also the mixing of the liposomes into the hydrogel vehicle was facilitated by the DC [18].

## 5. Description and comparison of the dual centrifuges used for liposome preparation

Until now, two different DC devices have been used for liposome production (compared in **Table 1** and **Figure 8**). The process of DC-homogenization was first discovered using the

	Speedmixer DAC 150	ZentriMix 380 R
Number of rotation units	1	2
Diameter of the rotation unit/usable area of the rotation unit	7.5 cm/44 cm <sup>2</sup>	14.7 cm/170 cm <sup>2</sup>
Distance between turning-point rotor and turning-point rotation unit	4.5 cm	10.0 cm
Primary rotation (minimum–maximum)	300–3450 rpm	50–2500 rpm
Ratio of primary and secondary rotation	~4:1	~3:1
Maximum centrifugal acceleration at the turning point of the rotation unit	600 × g	700 × g
Time to maximum speed (empty)	ca. 5 seconds	ca. 1 minutes
Maximum cargo (sample plus adapter)	150 g	1000 g (2 × 500 g)
Cooling unit (temperature range)	Nonexistent	Yes (–20 to 40°C)
Runtime	5 seconds–5 minutes	1 minutes–99 hours (30 minutes recommended)
Dimension (width × depth × height; weight); d	27.5 × 49.5 × 41.0 cm; 25 kg	47.0 × 76.0 × 40.0 cm; 82 kg
Maximum number of samples		
Twist top vials, 2 ml	4	40
Injection vials, 10 ml	1	10
Beakers, 150 ml	1	2
Centrifuge tubes, 15 or 50 ml	0*	6
*Tubes are too long for the diameter of the rotation unit.		

**Table 1.** Properties of the dual centrifuges already used for liposome preparation.



**Figure 8.** Pictures of the two dual centrifuges: the Speedmixer DAC 150 FVZ (left) and the ZentriMix 380 R (right). Their respective rotors with typical vial holders are shown in the small pictures.

Speedmixer DAC 150 FVZ (Hauschild GmbH & Co KG, Hamm, Germany), which was predominantly constructed for the effective mixing of two-component dental filling materials as well as for other mixing tasks, e.g., in the field of printing inks. To better meet the requirements of DC-homogenization for the production of liposomes—which in particular are longer processing times and the need for efficient temperature control—a new dual centrifuge has been developed, the ZentriMix 380 R (Andreas Hettich GmbH & Co KG, Tuttlingen, Germany).

### 5.1. Speedmixer DAC 150 FVZ

The Speedmixer is a so-called “dual asymmetric centrifuge” (DAC), meaning that the dual rotor is asymmetrically built and carries only one rotation unit. The resulting imbalance during operation is compensated by a fix or by variable counter weights. Thus, the allowed total cargo is fixed at 5–150 g in predefined steps. In earlier versions of the Speedmixer, for each different sample type a special sample vial adapter had to be used which own weight plus the sample weight has to be 100–150 g. This type of construction is of advantage if the number of samples to be processed is only one. The diameter of the vial holder is approximately 7 cm which limits the number of 2 ml vials that can be processed in parallel to 4. Typical lab PP beakers with a volume of 150 ml can be processed easily, but not vials longer than 7 cm (e.g., 15 or 50 ml disposable centrifuge tubes).

Due to its powerful engine and the rather low weight of the dual rotor, the Speedmixer needs only seconds to reach its maximum speed. The second rotation for the DC process and the

main rotation are mechanically coupled by a v-belt, which has advantages in compensating the forces during acceleration, but also contributes to the rapid contamination of the rotor unit with rubbed-off parts of the v-belt.

The Speedmixer is constructed for rather short runs, and the effective mixing of the mentioned dental filling materials needs only 15 seconds. Longer runtimes resulted in a significant and rapid development of heat caused by the powerful engine and the used mechanic elements (v-belt mechanics and the bearings are deeply embedded in the rotor case). Since the heat production is intense, and no cooling unit is implemented, the maximum allowed and adjustable runtime is 5 minutes. Thus, reaching a runtime of 20–30 minutes needed for making liposomes by DC-homogenization, the Speedmixer has to be restarted at least 4–6 times. If temperature-sensitive samples have to be processed, it is necessary to patiently allow the Speedmixer as well as the samples to cool down between the 5-minute runs. For very sensitive samples, shorter runs have to be performed allowing intermediate downcooling of the Speedmixer and—more important—the samples. Running the Speedmixer in a cool place (cooling chamber) is of advantage.

## 5.2. ZentriMix 380 R

To improve the in-vial homogenization process in terms of temperature control, longer processing times and flexibility in the number, shape, and size of the sample vials, a new type of dual centrifuge (ZentriMix 380 R) was developed. In contrast to the Speedmixer, the ZentriMix is a symmetric dual centrifuge, meaning that the dual rotor carries two symmetrically placed rotation units. However, the abbreviation of this type of dual centrifugation is simply DC, but one might discuss if DSC would be more appropriate—as the opposite of the abbreviation DAC.

The dual rotor of the ZentriMix is rather heavy ( $\approx 15$  kg), very robust, and can process cargos from a few milligrams up to 1 kg ( $2 \times 500$  g). Due to its higher weight, acceleration of the ZentriMix rotor is comparable to that of a normal centrifuge.

The ZentriMix is superior for long runtimes, the technical limit being 99 hours. This was not only achieved through the robust rotor case but also by a very robust mechanical coupling between the rotation units and the main rotation. This coupling has been realized through cogwheels made of polyamide, which also keeps the operation noise in an acceptable range. Furthermore, accumulation of heat is almost entirely prevented by a powerful cooling unit. For an optimal heat transport, sample vial holders made of aluminum are available (sample vial holders are usually made of polyamide), which further helps to keep the sample temperatures in acceptable ranges for most applications, even when 40 samples were processed at once.

## 6. Conclusion

DC has proven to be a straightforward and reproducible method for the production of various types of liposomes carrying a broad variety of drugs/active compounds. The method allows the preparation of different batch sizes. Especially interesting is the possibility to produce sterile liposomes; it is attractive for the immediate preparation of liposomes for *in vitro* cell culture or

*in vivo* animal experiments. The new dual centrifuges with improved manufacturing features and ability to control the in-vial homogenization process (e.g., by temperature control) will further contribute to the success of DC in the field of liposome preparation.

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## Abbreviations

Chol	Cholesterol
DC	Dual centrifugation
DAC	Dual asymmetric centrifugation
EE	Entrapping efficiency
EPR effect	Enhanced permeability and retention effect
EPC3	Hydrogenated phosphatidylcholine
HPH	High-pressure homogenization
MLV	Multilamellar liposome
P.I.	Polydispersity index
VPG	Vesicular phospholipid gel

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