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THE CROSSFLOW INJECTION TECHNIQUE: AN IMPROVEMENT OF THE ETHANOL INJECTION METHOD

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ABSTRACT

A novel scalable liposome preparation technique for pharmaceutical application is presented. Previous experiments have shown that the concept of continuous crossflow injection is a promising approach. For the characterization of the process, we focus on the influencing parameters like the lipid concentration, the injection hole diameter, the injection pressure, the buffer flow rate, and system performance. These experiments demonstrate that the injection hole diameter and the system performance do not influence the vesicle forming process and that a minimum of buffer flow rate is required to affect batch homogeneity. In contrast, strongly influencing parameters are lipid concentration in combination with increasing injection pressures. After exceeding the upper pressure limit of the linear range, where injection velocities remain constant, the vesicle batches are narrowly distributed, also when injecting higher lipid concentrations. Reproducibility and scalability data show similar results with respect to vesicle size and size distribution and demonstrate the stability and robustness of the novel continuous liposome preparation technique.

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Key Words: Modified ethanol injection technique; Scalability; Defined process parameters

INTRODUCTION

Many different liposome preparation techniques have been established since liposomes were first published in 1965.[1] The choice of a convenient preparation technique depends on the substance to be entrapped, the lipid composition and the pharmaceutical dosage form. Keeping in mind that the liposomal product should be produced in an industrial scale for medical application, further aspects, such as scalability, product stability, and sterility play an important role.

For many reasons, one feasible preparation technique is the injection of a lipid containing solution, which is miscible with water (e.g., ethanol), into an aqueous phase. This was one of the earliest alternatives for the preparation of unilamellar vesicles without sonication, published by Batzri and Korn.[2] During the injection of lipid solutions into an aqueous buffer system, lipids tend to form ''precipitates,'' followed by self arrangement in vesicles. The obtained vesicle size depends on factors such as lipid concentration, stirring rate, injection rate, and the choice of lipids. Subsequently Kremer et al.[3] optimized this method, varying preparation conditions. In conclusion, they postulated that the lipid concentration in the injected ethanol mainly influences the liposome size. Furthermore, the ethanol concentration in the aqueous phase is limited to approximately 7–10% (v/v),[4] which in the case limits high encapsulation capacities. The advantage of this method is its simplicity, the absence of potentially harmful chemicals, and physical treatments, and furthermore, the possibility of scale-up. But with the limitation of operating at classical stirred batch scale it is difficult to achieve reproducibility. Because of low solubility of certain lipids in ethanol, only low concentration of vesicles can be produced. At higher concentrations, however, the liposomes are rather large and heterogeneous which, in turn requires additional treatments for pharmaceutical purposes.

The aim of our study was to develop a novel preparation strategy, based on the principles of the ethanol injection technique. This novel technology should avoid the disadvantages, observed by other published methods. The simplicity and safety of the injection technique should be supplemented by a minimum of technical requirement.

Here we show an advancement of the ethanol injection technique. Data of liposome suspensions prepared with the invented crossflow injection module are presented. Many experiments were performed to characterize the influencing parameters for the vesicle forming process. Therefore, the influence of injection pressure and the velocity respectively, buffer flow rate and lipid concentration for optimized preparation were studied.

Additionally, we examined the influence of already formed liposomes on the formation of further vesicles. Therefore, we pumped the aqueous suspension, including the formed vesicles, either back to the buffer reservoir—continuous mode—, or into a second vessel—batch mode.

MATERIALS AND METHODS

Materials

Dipalmitoyl-phosphatidyl-cholin (DPPC) was purchased by Genzyme pharmaceuticals (Switzerland) and cholesterol was obtained from Avanti Polar Lipids (Alabaster, AL). Stearylamine was purchased from Sigma (St. Louis, MA). DPPC, cholesterol, and stearylamine were used for vesicle preparation in a molar ratio of 7 : 2 : 1. Usually a lipid concentration of 10 μmol DPPC/mL aqueous phase has been used. Some experiments were performed using 20 μmol DPPC/mL buffer solution. Phosphate buffered saline (PBS, pH = 7.2–7.4) was used as hydration buffer. All these reagents were purchased with analysis certificates.

Preparation of Liposomes

Liposomes were produced by the crossflow injection technique. As shown in Fig. 1, the preparation system consists of the crossflow injection module, vessels for the polar phase (PBS-buffer solution), an ethanol/lipid solution vessel and a nitrogen pressure device. The crossflow injection modules used for these studies are made of two stainless steel tubes welded together forming a cross. At the connecting point the modules are adapted with an injection hole drilled by spark erosion (150 and 250 μm drill holes).

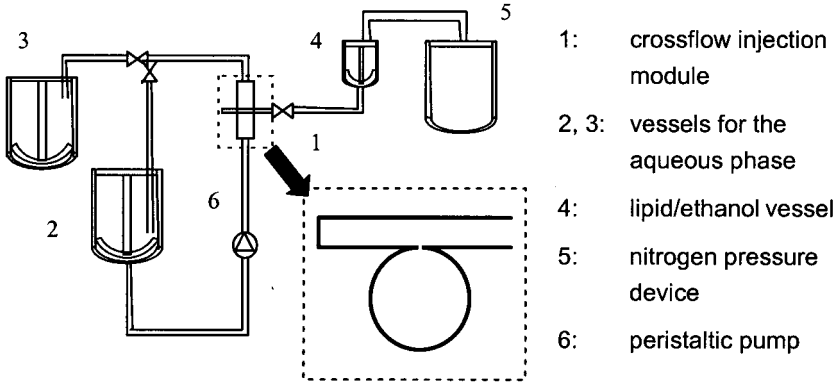
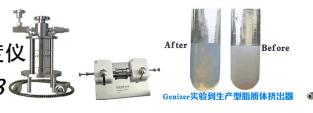


Figure 1. Schematic sketch of the crossflow injection system.



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The lipid mixture is dissolved in stirred 95% ethanol at 55°C. The buffer/protein solution is also tempered at 55°C. Preparation volumes varied from 100 to 2400 mL.

The aqueous solution is pumped with a peristaltic pump from vessel 2 to vessel 3 (continuous mode) or in the batch mode back to vessel 2. While pumping the polar phase through the crossflow injection module the ethanol/lipid solution is injected into the polar phase with varying pressures applied by a nitrogen pressure device.

Liposome Size Distribution

Liposome size and size distribution were measured by a novel technique using flow cytometry.[5] The size determination was performed using a FACS Vantage (Becton Dickinson, San Jose, CA), equipped with a 5 W Argon Laser (Coherent Innova 305, St. Clara, CA). The laser was tuned to 488 nm and an output of 500 mW. Fl1 at linear amplification was used as threshold parameter (700 V, Gain 16). Forward scatter height (FSC-H, scattering angle 10°) and side scatter height (SSC-H, scattering angle 90°) were set to a logarithmic amplification to accommodate the entire size range of 26 nm to 1 μm. In each sample 10,000 particles were measured.

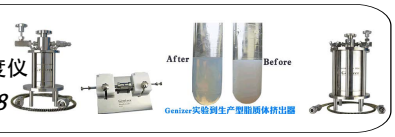
Observation of the Liposomes

Liposome conformational behavior was measured by electron microscopy studies using the negative stain technique[6] or freeze fracture technique.[7–9] Microscopical studies were done using a Phillips CM 100 electron microscope.

RESULTS

Comparison of 150 and 250 μm Crossflow Modules with Constant Injection Pressures

Liposome suspensions were prepared at 150 mL scale with a constant lipid concentration. The experiments were performed with 150 and 250 μm injection hole modules at 1.2 bar injection pressure. Buffer flow velocities remained constant at 2700 mL/min. The liposome suspensions were prepared at 55°C, quite above the transition temperature of the lipid mixture. Data in Fig. 2 demonstrate that the diameter of the injection hole did not effect vesicle size and size distribution, even when the injection pressure and buffer flow rates were kept constant.



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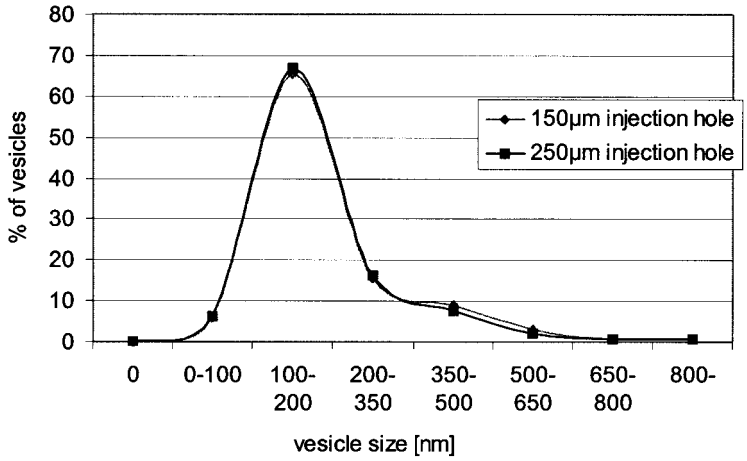


Figure 2. Comparison of lipid vesicles produced with 150 and 250 µm crossflow injection modules prepared with 1.2 bar.

Variation of Injection Pressure

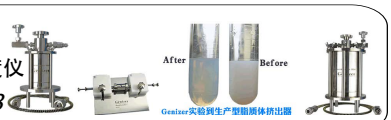
The injection pressures were varied in the range of 0.3–2.6 bar. All vesicle batches consisted of 10 µmol lipid/mL buffer solution and 7.5% ethanol (v/v). The experiments were performed with the 150 µm crossflow injection module and buffer velocities remained constant at 2700 mL/min.

As demonstrated in Fig. 3, vesicle size and homogeneity increased in dependence of increasing injection pressure. By injection of the lipid/ethanol solution into the aqueous phase with a pressure higher than 1 bar, 85% of vesicles were smaller than 350 nm. Liposome suspensions, prepared at 2.6 bar, showed the closest distribution. More than 70% of the formulated vesicles were in the size range around 200 nm, just 5% were larger than 350 nm.

Variation of Buffer Flow Rates

The flow rates of the circulating buffer streams were controlled by a peristaltic pump. Flow rates varied from 500 to 2700 mL/min. The studies were performed with the 250 µm crossflow injection module. Lipid concentration and injection pressure remained constant.

Figure 4 shows that the liposome suspension prepared with the lowest buffer flow rate was rather inhomogeneous and broad distributed. However, liposomes prepared with 1500 and 2700 mL/min buffer flow rate resulted in comparable size distributions. The main population of the lipid vesicles was around 200 nm, but by increasing buffer flow rates the percentage amount of 200 nm vesicles increased, meaning that more homogeneous vesicles were formed.



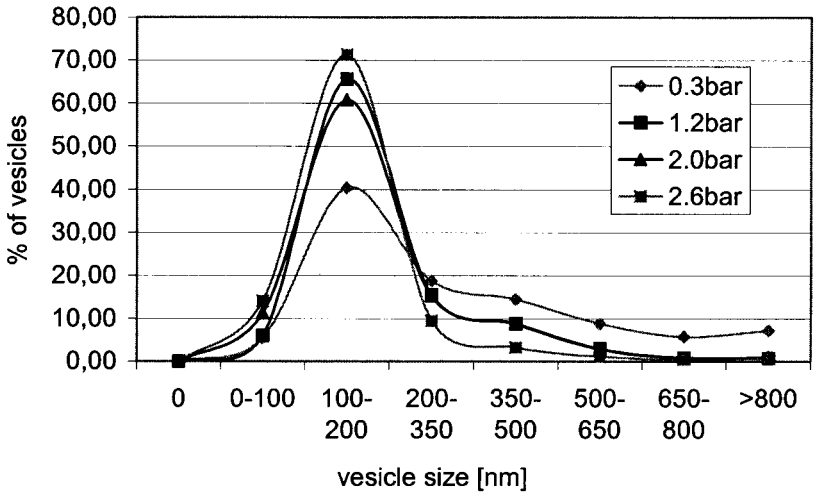


Figure 3. Vesicle preparation with 150 µm crossflow injection module in dependence of injection pressure. The injection pressure varied between 0.3 and 2.6 bar.

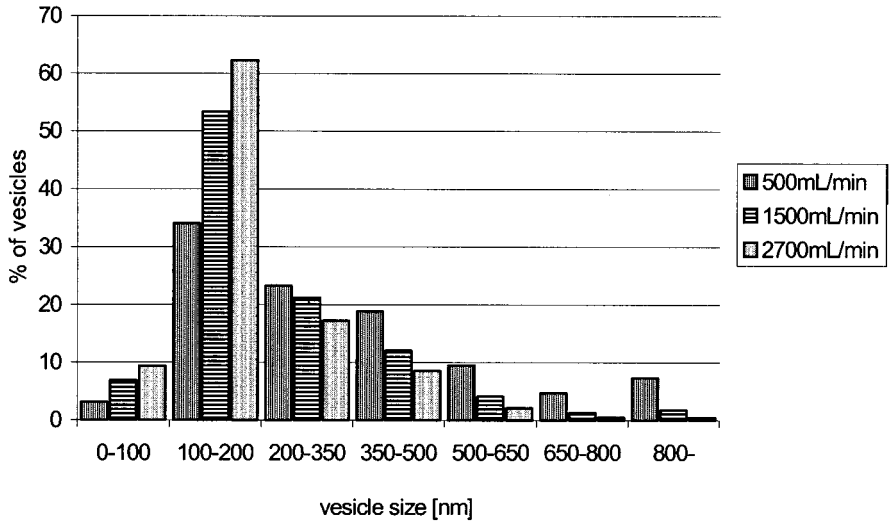


Figure 4. Comparison of liposome batches produced with 250 µm Injector at the same pressure but different PBS flows.

Variation of System Performance

All previous experiments were performed in the batch mode. Evaluation of the influence of preformed vesicles on further liposome formulation was done by comparing the batch mode to the continuous mode. Therefore, the buffer flow rate in both experiments was 900 mL/min. Both preparations

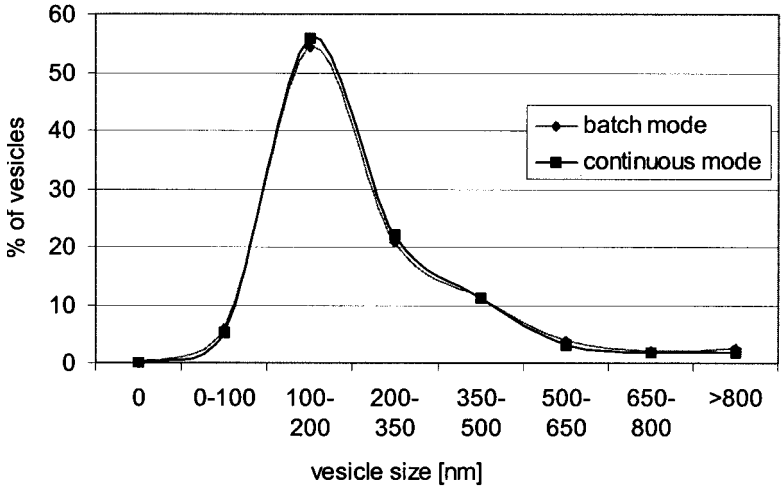


Figure 5. Liposome batches prepared with variation of buffer flow conditions.

were carried out by injecting the lipid ethanol solution with 1.6 bar through the 250 μm injection hole.

As demonstrated in Fig. 5, a change in the system performance is not influencing the vesicle formation procedure significantly. Both experiments resulted in narrowly distributed vesicles with 90% of vesicles smaller than 500 nm.

Variation of Lipid Concentration and Injection Pressure

The lipid concentration is another variable parameter in the optimization procedure working with the 250 μm crossflow injection module. The vesicles were either produced with 10 or 20 μmol DPPC/mL aqueous phase. The injection pressure varied within 1.2–4.8 bar. All other parameters were kept constant. Data of Fig. 6 show impressively that particularly higher concentrated lipid solutions tend to form broadly distributed liposome solutions. But with increasing injection pressure liposomes become narrowly distributed and more homogeneous.

Reproducibility

Demonstration of reproducibility was performed by injecting the lipid/ethanol solution with a concentration of 10 μmol DPPC/mL at 2.4 bar in the buffer system. The 250 μm crossflow injection module was used in all three experiments and the buffer flow rate was kept constant at 2700 mL/min.

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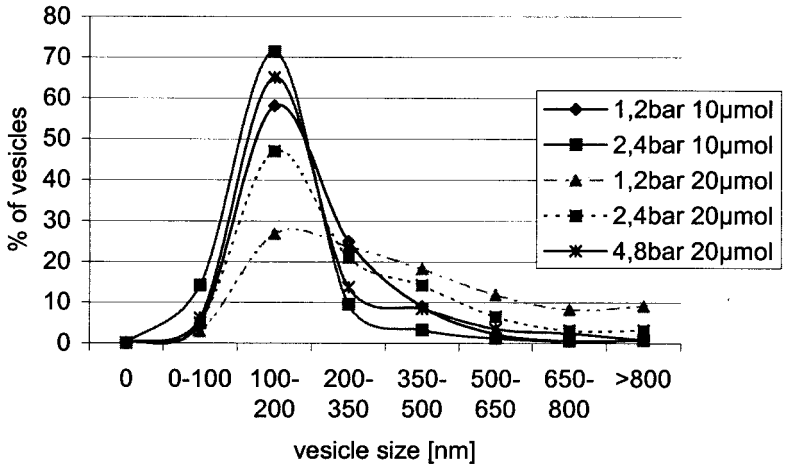

Figure 6. Comparative experiments of vesicles consisting of 10 and 20 µmol DPPC/mL buffer solution injected with 1.2 and 2.4 bar pressure.

Preparation volume varied within 150–200 mL. Resulting data shows congruently distributed vesicle size distributions in all three batches.

Scale Up

Batch 1 consisted of a 300 mL buffer solution. For batch 2 the volumes were doubled and for batch 3 the volumes were eightfold. The buffer flow rate in all experiments was adjusted to 400 mL/min. Injection pressure in all three experiments was 2.4 bar. The results shown in Fig. 8 demonstrate the reproducibility, independent of the batch scale of the established preparation procedure.

DISCUSSION

Many investigations have been performed to establish scalable liposome preparation techniques.[10–12] In our studies we focused on the establishment of a novel liposome preparation procedure based on the principles of the ethanol injection technique. Therefore, a novel preparation equipment had to be designed, where several aspects such as preparation of liposome suspensions under defined and reproducible conditions had to be considered. Furthermore, the preparation equipment should facilitate scale unrelated production of sterile vesicle suspensions. After the establishment of the hygienic equipment, feasible for sterilization procedures, we focused on the characterization of the parameters, which influence the vesicle forming process.

Therefore, we focused on (1) the injection hole diameter, (2) the buffer flow rate, (3) the injection pressure, (4) the lipid concentration in the ethanolic phase, and (5) the system performance.

The injection modules, the principal items of the equipment, were adapted with 150 and 250 μm injection holes. To estimate the influence of the injection hole diameter, comparable preparations with the 150 and 250 μm modules were performed. In these experiments, the buffer flow rate and the injection pressure were kept constant, whereas the injection velocity increased with the increasing diameter. Similar results in size and size distribution of generated vesicle suspensions were obtained (Fig. 2). Based on these observations which indicate that the injection hole diameter is not the influencing parameter responsible for size and size distribution of prepared vesicle suspensions, further experiments with varying buffer flow rates and injection velocities were carried out. It became obvious that a minimum value of buffer flow rate is required to produce small and homogeneously distributed lipid vesicles (Fig. 4). This presumption could be verified by several experiments. This fact might be of interest, because in the conventional ethanol injection technique, there's a velocity gradient from the center of the vortex to the vessel side wall. This might influence the vesicle formation process. The novel crossflow injection technique permits working at defined buffer flow rates, which can be controlled by peristaltic pumps. This is of particular importance, when scale up operations should be performed.

However, the injection pressure is probably the parameter, which affects the vesicle forming process in case of homogeneity more. Many liposome suspensions were prepared to estimate the influence of injection pressure on liposome formulation. These experiments showed that a minimum pressure for the lipid/ethanol injection is required for the preparation of homogeneously distributed liposome suspensions. Working below a lower limit (0.8 bar) resulted in extremely heterogeneous vesicle populations (Fig. 3). Exceeding higher pressure ranges, the vesicle batches were narrow distributed. This is of particular importance, especially when injecting higher lipid concentrations as demonstrated in Fig. 6. Furthermore, electron microscopy studies demonstrated formation of unilamellar vesicles.

This effect can be explained by the bilayer planar fragment (BPF)-theory established by Lasic.[13–15] According to this concept, small disk-like micelles act as common intermediate structures during all vesicle forming processes. In case of the ethanol injection technique the planar bilayer fragments are formed by changing the solubility conditions. The phospholipid, which is dissolved in the organic phase precipitates at the phase boundary water/gas bubble and while the bubble rises, the precipitated bilayers peel of and form BPFs, which vesiculate.

We think that increasing injection pressure influence the bubble forming process. Higher injection pressure induce formation of smaller uniform bubbles and additional formation of smaller planar bilayer fragments.

This is probably caused by the fact that injection with higher pressures disperse the lipid/ethanol solution into smaller bubbles. When the bubbles get smaller, the lipid-precipitates get smaller as well and subsequently arrange to bilayer fragments. Vesiculation of these fragments led to the formation of narrowly distributed vesicles. The results of 20 μmol DPPC/mL liposome suspensions with increasing injection pressure demonstrated that high injection pressures lead to the formation of more homogeneously distributed lipid vesicles (Fig. 6). This effect was supported by high buffer flow rates, because the faster these bilayer planar fragments are transported from the injection point, the lower becomes the possibility that they aggregate into larger BPFs, forming larger vesicles. To understand the conformational behavior of membranes, one has to introduce a number of theoretical concepts such as bending elasticity and curvature.[16] Although this is a quite interesting aspect it was not subject of these studies.

We have to point out that the lipid concentration in the experiments presented here are 5–10 fold higher than the highest lipid concentrations of published ethanol-injection data by Kremer et al.[3] As lipid concentration is the parameter, which is most effecting the vesicle forming process, our results are hardly comparable to previously published data.

Further experiments focused on the system performance. The results showed no difference in liposome size between continuous and batch mode (Fig. 5). With respect to the definition of Lasic,[17] where continuous processes are defined as operations where one regulates the influx of reactants and on the other end, one has a pipe with liposomal product, the continuous mode production is an interesting tool for industrial liposome production.

Several experiments were performed in order to prove reproducibility and scale up of the established preparation procedure. Figure 7 shows

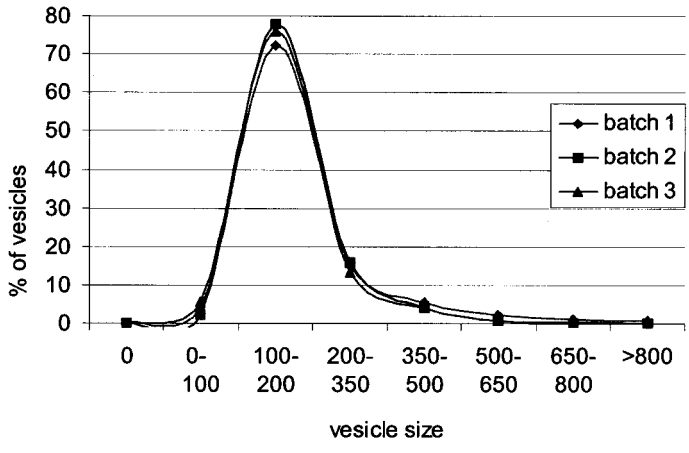

Figure 7. Reproducibility data of liposome suspensions produced at 2.4 bar with maximum buffer flow rate.

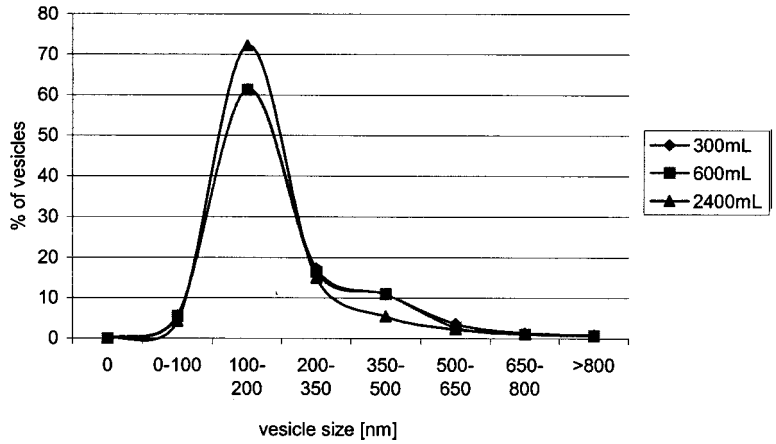

Figure 8. Vesicle size distribution of liposome suspensions with increasing preparation scale.

reproducibility data of three liposome batches prepared under identical conditions. All three preparations show similar results in case of vesicle size and size distribution. Similar results were obtained with scale up experiments and demonstrate, once more, the stability and robustness of this novel, established liposome preparation technique.

CONCLUSIONS

With this investigation, we have presented a novel liposome preparation technique suitable for the continuous and scalable manufacturing of well defined liposome suspensions, provided that the chosen lipid components are soluble in an appropriate solvent and the production parameters are well defined. In contrast to the published batch process, our novel production procedure seems to be more efficient for the reproducible production of sterile liposome suspensions at large scale. Entrapment efficiency studies with the human recombinant superoxide-dismutase will be presented soon.

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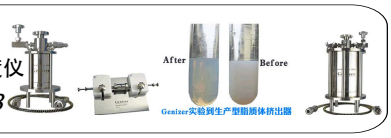

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