nPort™ Platform Nanotechnology for Liposome Manufacturing: Excellence in Particle Size Control and Scalability

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ABSTRACT

Liposome-based drug delivery is one of the advanced technologies for the treatment of human cancer and severe infectious disease. The complex manufacturing process, poor reproducibility, and low scalability of the traditional liposome making methods represent the huge obstacles in liposome manufacturing that dramatically limit the development and commercialization of liposomal drugs. To surmount those obstacles we recently invented nPort™ platform nanotechnology for liposome manufacturing. In the nPort™ process, lipids solution(s) in a water-miscible organic solvent is mixed with aqueous solutions in a specially designed multi-port mixing chamber. The flowrate of the solutions is adjustable in the range from microliters to multi-liters per minute. The liposome is spontaneously assembled during the mixing. The particle size, morphology, and product scales are precisely controlled by multiple parameters including the temperature, concentration, pH, the flowrate, the number and the geometry of the inlet/outlet ports. In summary, the nPort™ technology brings unparalleled advantages to liposome manufacturing including: 1) robust scalability from micrograms for lab research to kilograms for commercialization; 2) continuous particle size control from 20 to 200 nm; 3) reliable reproducibility; 4) a platform for varieties of liposomal formulations, payloads and morphologies; 5) dramatic time and cost-saving.

INTRODUCTION

Liposome-based drug delivery is a clinically demonstrated technology for the treatment of cancer and infectious disease[1]. It improves pharmacokinetics, reduces toxicity, and enhances efficacy of the drugs. Traditionally, lipids extrusion at high pressure and high temperature is used for liposome manufacturing. However, some liposomes such as DNA/cationic lipid liposomes are very difficult to be made by extrusion, particularly in a large scale due to the solid-like multilamellar or inverted hexagonal morphology of the liposome[2] formed with strong charge-charge interactions between the payload and the lipid molecules. The poor reproducibility and scalability of the traditional liposome making methods have been the bottleneck of liposomal drug development in the past decades[3]. It is commonly accepted that the particle size of liposome is a critical factor affecting the in vivo distribution, toxicity, and efficacy of liposomal drugs[4,5]. The experimental data in the literature, however, is very limited due to the extreme difficulty to control the particle size of liposome with the traditional methods. Therefore, a simple, robust, scalable, particle size controllable liposome manufacturing platform technology is definitely essential for promoting the research and development of liposomal therapeutics. We recently invented the nPort™ platform nanotechnology that satisfies the essential requirements for liposome preparation mentioned above. In this process, lipids solution(s) in a water-miscible organic solvent is mixed with aqueous solutions in a specially designed multi-port mixing chamber. The flowrate of the solutions is adjustable in the range from microliters to multi-liters per minute. Liposome is spontaneously assembled in the mixed medium. The particle size in the range of 20-200 nm is controlled by altering the processing parameters such as the flowrate of the solution and the geometry of the mixing chamber. This platform technology is suitable for varieties of lipids formulations, small molecules and biological payloads, and various of morphologic liposomes. In this report, we discuss the effects of the flowrate and geometry of the mixing chamber on the size distribution and morphology of the liposomes.

MATERIALS AND METHODS

Materials 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), Hydrogenated Soy L-α-phosphatidylcholine (HSPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (mPEG2000-DSPE), and cholesterol were purchased from Avanti Polar lipids (Alabaster, Alabama); 1,2-dilinoleyloxy-N,N-dimethyl-3-aminopropane (DLinDMA) and APOB-specific siRNA was synthesized by CRO companies for research use. Doxorubicine was purchased from MicroBiopharm Japan (Tokyo, Japan).

nPort™ technology for liposome preparation The nPort™ process of liposome preparation is illustrated by the flow diagram in Figure 1. The lipids are dissolved in a

water-miscible organic solvent, such as anhydrous ethanol. The lipids solution is then mixed with aqueous solutions specifically designed for a specific liposome and payload to form liposome spontaneously. Depending on the physical properties, the payload can be dissolved either in the lipid solution or in the aqueous solution. Alternatively, the payload can be encapsulated by remote loading.

An instant homogeneous mixing of the lipid solution with the aqueous solutions and the percentage of the organic solvent in the mixture are two key parameters dominating liposome particle size, morphology, payload encapsulation, and homogeneity. We designed nPort™ flow/collide mixing device to systemically control these parameters and manipulate liposome size, morphology and homogeneity. An exemplar nPort™ device is shown in Figure 2. The numbers of the ports is typically in the range of 4-20. Usually multiple ports are used as the inlets of an aqueous solution, a single or multiple ports is (are) used as the inlet(s) of the lipid solution or as the outlet(s) of the liposome. The number of inlets/outlets and the geometry of the device play important roles in manipulating the liposome size and morphology.

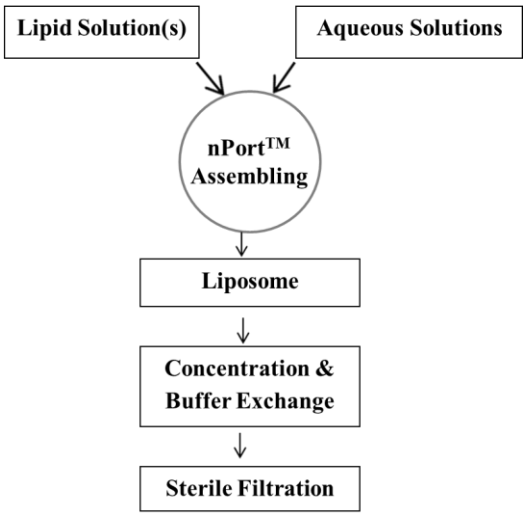

Figure 1. Flowchart of liposome prepared by nPort™ technology.

Figure 2. A typical design of an nPort™ mixing device

Doxorubicin Liposome Preparation The composition of the liposome encapsulating doxorubicin is identical to Doxil, the FDA approved liposomal doxorubicin. In brief, HSPC, cholesterol, and mPEG2000-DSPE were dissolved in anhydrous ethanol at 17.24 mg/ml, 5.75 mg/ml, and 5.75 mg/ml respectively. Ammonium sulfate was dissolved in distilled water at 250 mM, pH 6.5. The lipid solution and the solution of ammonium sulfate were loaded into syringes and were pumped by a syringe pump into the mixing chamber of a 5-ports nPort™ device as illustrated in **Figure 3.** The pore size (diameter) of the inlet port was 1.0 mm, or 1.6 mm. The flowrate was set in the range of 5-50 ml/min. The mixed solution containing the self-assembled liposomes exited through the outlet port and was collected into a glass vial. The liposome was then dialyzed against a buffer of 10 mM histidine buffered 9.2% (w/v) sucrose, pH 6.5. After the lipid concentration determination by HPLC, doxorubicin was dissolved in the liposome suspension at lipids : doxorubicin = 8 : 1 (w/w). The solution was then incubated in a 42°C water bath for 2 hr to reach 99.5% of doxorubicin encapsulation (the encapsulation method is not discussed here.)

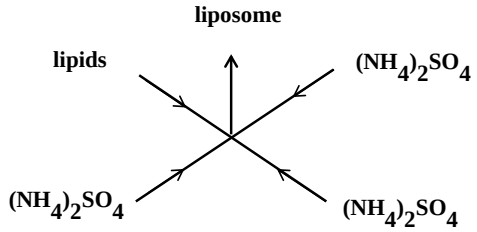

Figure 3. The configuration of doxorubicin liposome preparation by nPort™. The four inlet ports are in the same plane, and the outlet port of the liposome is perpendicular to the inlets.

siRNA liposome preparation Three solutions were prepared for siRNA liposome preparation: 1) the lipids solution in anhydrous ethanol contained 2.78 mg/ml of DLinDMA, 2.10 mg/ml of cholesterol, 1.20 mg/ml of DSPC, and 0.57mg/ml of mPEG2000-DSPE; 2) siRNA solution in citrate buffer (20 mM, pH 5.0) contained 0.5 mg/ml ApoB siRNA, and 3) 20 mM citrate duffer with 100 mM NaCl, pH 5.0. One milliliter of lipid, siRNA, and citrate buffer were loaded into separate syringes and were pumped by a syringe pump into the mixing chamber of a 5-ports manifolds as illustrated in **Figure 2**. The pore size of the 5-port manifold mixer was, 1 mm, or 1.6 mm. The flowrate was in the range from 5 to 50 ml/min.

Characterization of liposome The particle size and polydispersity index liposome was determined by Malvern Zetasizer Nano ZS in HEPES buffered saline (10 mM HEPES, pH 7.4, 138 mM NaCl). The encapsulation percentage of doxorubicin and siRNA were determined by filtration and spectrometry and RiboGreen, respectively. The details of the methods are not presented here due to the defined page constraint. The morphology of liposome was imaged by Cryo-TEM.

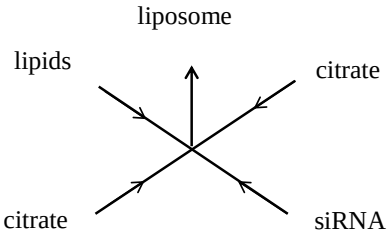

Figure 4. The configuration of siRNA liposome preparation by nPort™ .

RESULTS AND DISCUSSIONS

The effects of the pore size and the flowrate on liposome size We found that the liposome particle size of and morphology of liposome was determined by the jet-flow speed (m/s) of the lipid and aqueous solutions defined by the inlet pore size and the flowrate (ml/min). In order to address these parameters, we used two pore sizes of the 5-ports of nPort™ device: 1.0 mm and 1.6 mm, to make doxorubicin liposome and siRNA liposome. The flowrates of the lipid and aqueous solutions were set in the range of 5-50 ml/min, as described in the Materials and Methods. At the same flowrate (ml/min), the jet-flow speed of a fluid in the 1mm inlet port was about 2.8 fold of that in the 1.6 mm port. The Z-average particle size and PDI were shown in Figures 5-6. The data indicate that at flowrate < 10 ml/ml, the particle size was about 150-200 nm with significantly higher dispersity. The particle size and PDI became smaller along with the increase of the flowrate and reached a stable size at about 20 ml/min. Interestingly, in the flowrate range tested, the particle size of the liposome made by 1.0 mm port of device is about 40 nm smaller than that made by the 1.6 mm port device, and the PDI of the former was also noticeably lower that the later. The capability of making liposomes in different sizes by the nPort™ technology through altering the port numbers, pore sizes, and flowrates provides scientists with a wide selection of particle sizes to optimize liposomal the formulation.

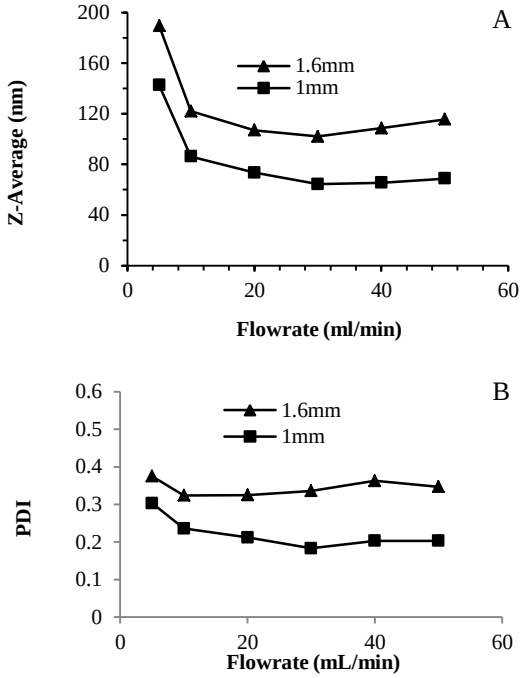

Figure 5. The effects of nPort™ device pore size and the flowrate on doxorubicin liposome particle size and PDI. A) Z-average particle size; B) PDI

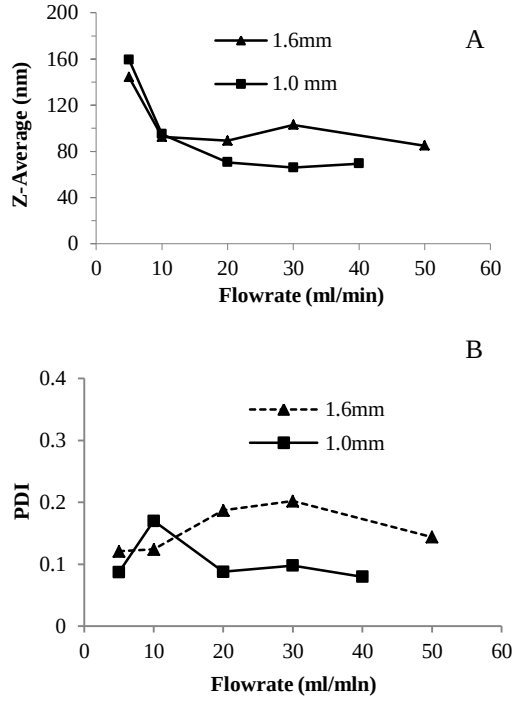

Figure 6. The effects of nPort™ device pore size and the flowrate on siRNA liposome particle size and PDI. A) Z-average particle size; B)PDI

Cryo-TEM imaging Cryo-TEM was employed to imaging and visualize the morphology of the liposomes prepared. The Cryo-TEM imaging of doxorubicin liposome is shown in **Figure** 7A in comparison with respect to theimaging of the clinically used Doxil purchased from a pharmacy (**Figure** 7B). The circle of the particle is the unilamellar lipid bilayer of the liposome, and the bar inside the circle is the crystal of doxorubicin formed with ammonium sulfate. The Cryo-TEM image data demonstrated that both the particle size and the unilamellar morphology of the liposome are highly homogeneous. It should be pointed out that, Doxil was made by the traditional extrusion method through a 100 nm pore size of membrane resulting in ~90 nm liposome. With nPort™ parameter adjustment, we are able to make a generic version of Doxil that is identical to Doxil in every physicochemical properties characterized following FDA's guidelines (data are not shown). Our consecutive preparations at scales from milliliters to tens of liters have repetitively proved the high reproducibility and robust scalability of nPort™ technology (data not shown).

Interestingly, the cryo-TEM imaging of **Figure** 7D showed that this same lipids formed lipid discs at the flowrate of 5ml/min in the 1.0 mm 5-ports nPort™ device.

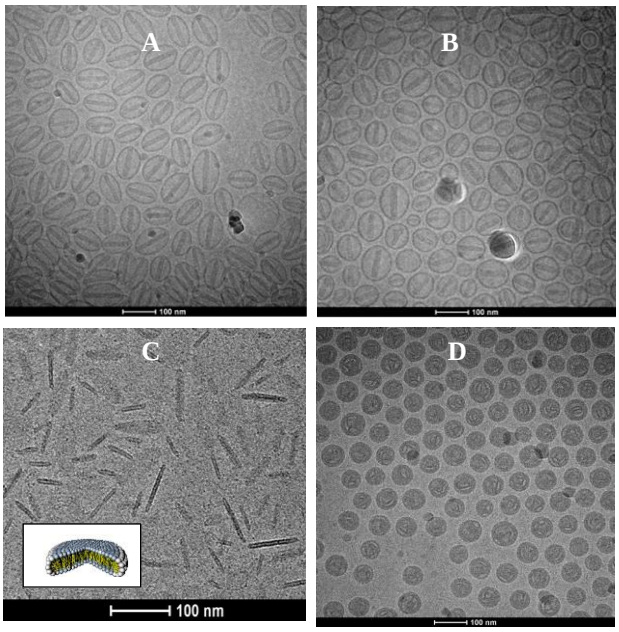

Figure 7. Cryo-TEM imaging of A) doxorubicin liposome. made by nPort™; B) Doxil purchased from a pharmacy. C) lipid discs made by nPort™. Insert, cartoon of lipid disc. D) siRNA liposome made by nPort™; The scale bar represents 100 nm.

Due to the unique properties of lipid discs in drug delivery[6], it will be of scientific, technologic and pharmaceutical interests to further investigate the assembly mechanism of lipid discs in the nPort™ process.

The Cryo-TEM imaging of siRNA liposome made by nPort™ technology is shown in **Figure** 7D. Again, excellent homogeneity in the size and morphology was observed from the siRNA liposomes. Liposome is a powerful delivery vehicle for DNA, mRNA, and siRNA. The heavily negative charged backbone of DNA/RNA interacts with cationic lipids to form multilamellar or inverted hexagonal liposomes. It has been a great challenge to make DNA/RNA liposomes with extrusion due to the hardness of the particles. The nPort™ technology overcomes these challenges and has been employed in our lab to make varieties of DNA, mRNA, and siRNA liposomes.

With the nPort™ technology, we have repetitively prepared 20 nm liposomes encapsulating one or more active pharmaceutical ingredients. Moreover, the nPort™ technology has enabled us to make liposome with continuously escalated particle size from 20 to 200 nm for the same formulation.

In summary, nPort™ technology brings unparalleled advantages to liposome production including: 1) robust scalability from micrograms for lab research to kilograms for commercialization; 2) continuous particle size control from 20 to 200 nm; 3) reliable reproducibility; 4) a platform for varieties of liposomal formulations, payloads and morphologies; 5) fast and cost-saving. We expect that nPort™ technology will promote the scientific research of liposome, and strongly boost liposomal medicine development in for the patients.

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