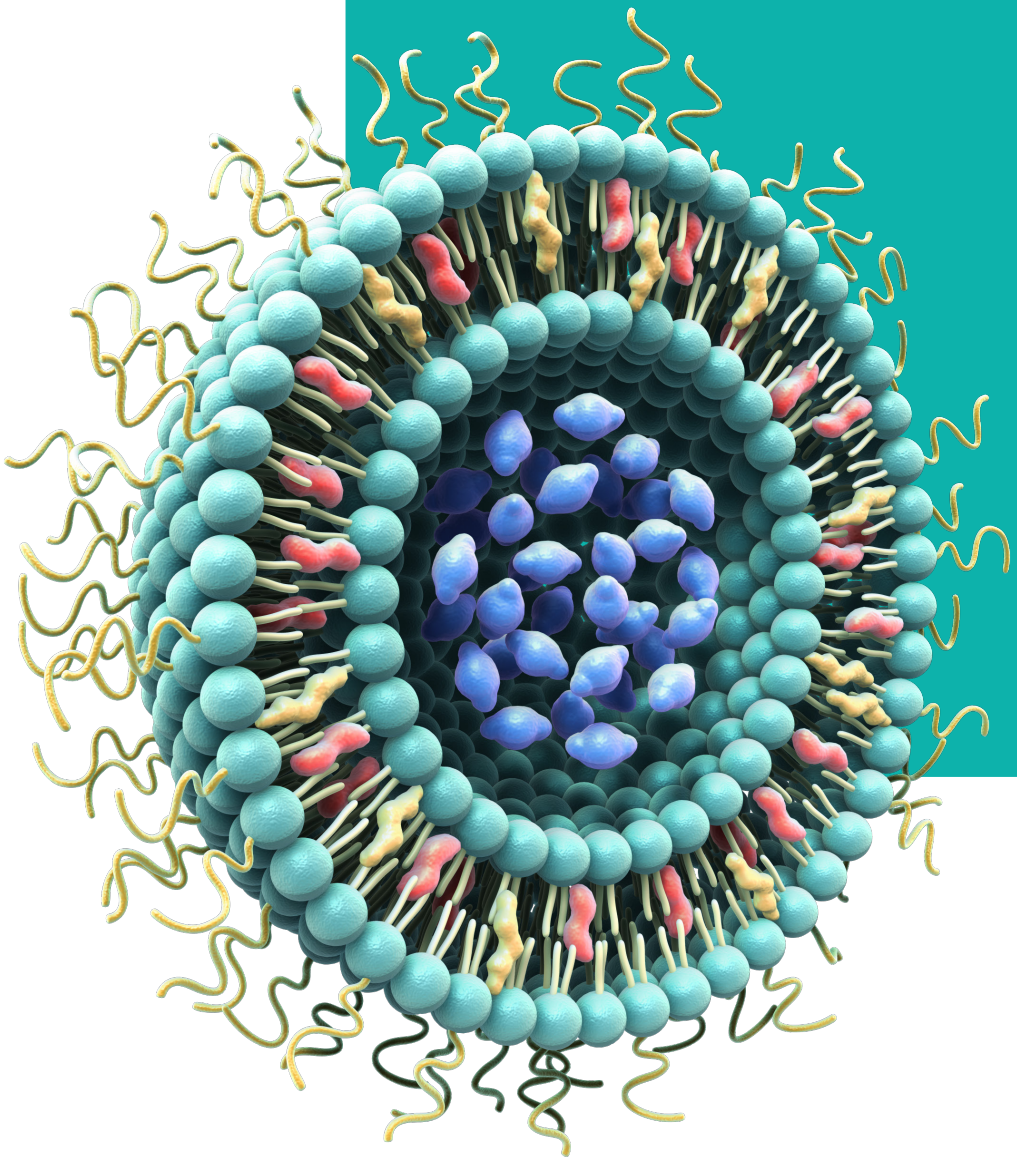
PRECISION NANOSYSTEMS

Create Transformative Medicines™

Formulating Liposomes



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Introduction

Liposomes are particles composed of a lipid bilayer containing an interior aqueous core (illustrated in **Figure 1A**). Liposomes can be formulated using a variety of lipids and are generally composed of mixtures containing 3-5 different lipids for greater stability. With an aqueous core, liposomes can be used to encapsulate small hydrophilic drug molecules or can be used to encapsulate other hydrophilic payloads such as peptides or proteins. Hydrophobic drug molecules may be incorporated within the lipid bilayer itself (illustrated in **Figure 1B**), leaving an 'empty' interior compartment. Additionally, complex liposomal formulations can incorporate both hydrophilic and hydrophobic payloads (**Figure 1C**). Any of these formulations can incorporate lipids chemically conjugated to polyethylene glycol (PEG) to extend the circulation half-life while in the body.

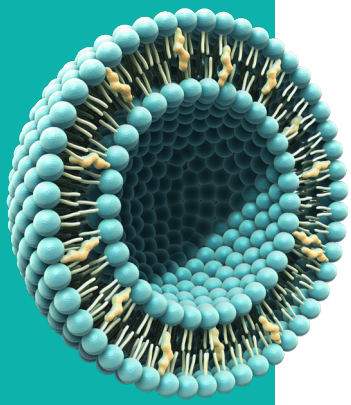


Figure 1A.
Liposome with single bilayer and interior aqueous core for hydrophilic payloads.

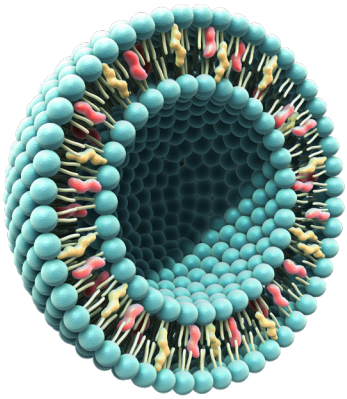


Figure 1B.
Liposome with single bilayer containing encapsulated hydrophobic drug molecules (red).

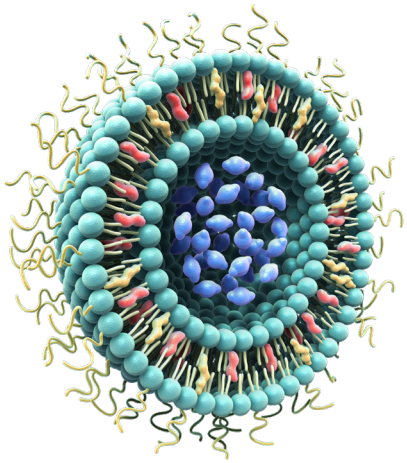
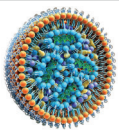
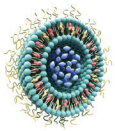
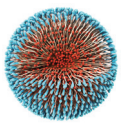
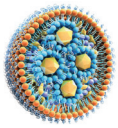


Figure 1C.
Liposome containing hydrophilic payload in the core (blue), hydrophobic payload in the bilayer and bearing surface PEG coating.

NanoAssemblr® Technology has been used to formulate liposomes composed of a variety of lipids loaded with hydrophobic drugs, hydrophilic drugs, proteins, peptides or nucleic acids. Microfluidic preparation of liposomes using NanoAssemblr technology allows for rapid production and optimization of formulations compared to thin-film hydration. Consistent process conditions afforded by non-turbulent mixing of solvent and aqueous phases ensures consistent results, removing user-to-user and batch-to-batch variability. Additionally, NanoAssemblr technology allows production of so-called "limit-sized" liposomes, which are the smallest liposomes compatible with the molecular packing of constituents. Several examples from peer-reviewed literature as well as some application notes with original results are presented here. These illustrate the multitude of applications of NanoAssemblr technology for developing and optimizing drug delivery liposomes.

Precision NanoSystems' technology solutions are world-leading biopharma and academic researchers to drive development of diverse nanomedicines

Versatile Applications

PARTICLE TYPE	ACTIVE INGREDIENT	EXAMPLE APPLICATION	CARRIER MATERIALS
Nucleic acid Lipid Nanoparticles (LNP) 	Nucleic Acids, Peptides and Proteins, Imaging Contrast Agents	• Rare genetic diseases • mRNA protein replacement • mRNA vaccines • Gene and cell therapy	• Ionizable lipids • Phospholipids • Cholesterol • PEG-Lipids
Liposomes 	Small Molecules, Imaging Contrast Agents	• Vaccine adjuvants • Antimicrobials • Cancer chemotherapy • Diabetes combination therapy	• Phospholipids • Cholesterol • PEG-Lipids
Polymer NPs 	Small Molecules, Imaging Contrast Agents	• Cancer chemotherapy • Targeted protein delivery • Controlled release/ biodistribution • Immuno-oncology	• Poly-lactides (ex: PLGA) • Block copolymers (ex: PEG-b-PLGA) • Polysaccharides (ex: chitosan, cellulose)
Emulsions 	Small Molecules, Imaging Contrast Agents	• Cancer chemotherapy • Drug formulation • Controlled release/ biodistribution	• Triolein/POPC • Oil/Surfactant
Organic/ Inorganic NPs 	Imaging Contrast Agents	• Theranostics • Imaging	• Lipids • Noble metal NPs • Rare Earth Metals • III-V semiconductors

Featured Liposome Publications

COMMUNICATION

ADVANCED MATERIALS

Biomimetic Nanovesicles

www.advmat.de

Design and Development of Biomimetic Nanovesicles Using a Microfluidic Approach

Roberto Molinaro, Michael Evangelopoulos, Jessica R. Hoffman, Claudia Corbo, Francesca Taraballi, Jonathan O. Martinez, Kelly A. Hartman, Donato Cosco, Grasiele* Costa, Isabella Romeo, Michael Sherman, Donatella Paolino, Stefano Alcaro, and Ennio Tasciotti**

The advancement of nanotechnology toward more sophisticated bioinspired approaches has highlighted the gap between the advantages of biomimetic and biohybrid platforms and the availability of manufacturing processes to scale up their production. Though the advantages of transferring biological features from cells to synthetic nanoparticles for drug delivery purposes have recently been reported, a standardizable, batch-to-batch consistent, scalable, and high-throughput assembly method is required to further develop these platforms. Microfluidics has offered a robust tool for the controlled synthesis of nanoparticles in a versatile and reproducible approach. In this study, the incorporation of membrane proteins within the bilayer of biomimetic nanovesicles (leukosomes) using a microfluidic-based platform is demonstrated. The physical, pharmaceutical, and biological properties of microfluidic-formulated leukosomes (called NA-Leuko) are characterized. NA-Leuko show extended shelf life and retention of the biological functions of donor cells (i.e., macrophage avoidance and targeting of inflamed vasculature). The NA approach represents a universal, versatile, robust, and scalable tool, which is extensively used for the assembly of lipid nanoparticles and adapted here for the manufacturing of biomimetic nanovesicles.

Recently, advances in biomimicry, i.e., the biologically inspired design of materials,[1] has spurred the development of novel strategies to bestow nano- and microparticles with multiple functionalities necessary to negotiate biological barriers.[2] Current approaches for drug delivery carriers include mimicking of leukocytes,[3] red blood cells (RBCs),[4] platelets,[5] and cancer cells[6] to achieve superior delivery of therapeutics compared to conventional nanoparticles. These hybrid biomimetic carriers showed advantageous pharmaceutical properties (i.e., defined size and shape, physical stability, ability to load and release chemically different therapeutics) resulting from the synthetic backbone materials (mesoporous silicon,[4a] phospholipids,[3a] and polylactic-co-glycolic acid[7]) they were derived from. Furthermore, these biomimetic strategies demonstrated innate biological features and intrinsic functionalities (long circulation, selective targeting toward

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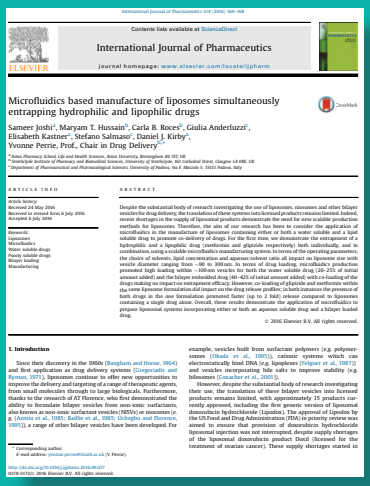
Advanced Materials, 2018

Design and Development of Biomimetic Nanovesicles Using a Microfluidic Approach

Molinaro R, Evangelopoulos M, Hoffman J, Corbo C, Taraballi F, Martinez J et al. Advanced Materials 2018; 30: 1702749.

Summary

- Biomimetic and biohybrid nanoparticles consisting of liposomes with membrane-bound proteins are potentially advantageous in drug delivery applications, for instance by mimicking leukocytes, nanoparticles take on their innate biological features including long circulation and selective targeting to biological compartments, but clinical translation of these technologies is hindered by challenges in manufacturing and scale-up production
- The Tasciotti group at Houston Methodist Research Institute have leveraged Nanoassemblr technology to make liposomes and embed membrane proteins from human leukocytes within the liposome bilayer in a single continuous-flow step
- Their process involved injecting membrane protein extracts in aqueous suspension in one inlet of the NanoAssemblr mixer and lipids dissolved in ethanol in the other inlet, where rapid mixing triggers self-assembly of liposomes incorporating the membrane protein into the bilayer
- Compared to conventional methods, NanoAssemblr technology simplified particle optimization in terms of size, homogeneity, zeta-potential and protein incorporation into the bilayer, with 90% of membrane proteins initially added being associated with he final formulation compared to 63% for conventional thin-film methods
- The NanoAssemblr Platform offers a robust and scalable path towards clinical development and manufacturing of biomimetic nanovesicles that are advantageous for a spectrum of drug delivery applications



International Journal of Pharmaceutics, 2016

Microfluidics based manufacture of liposomes simultaneously entrapping hydrophilic and lipophilic drugs

Joshi S, Hussain M, Roces C, Anderluzzi G, Kastner E, Salmaso S et al. International Journal of Pharmaceutics 2016; 514: 160-168.

Summary

- In this study, the Dr. Yvonne Perrie's group at Strathclyde University use the NanoAssemblr platform to optimize the simultaneous encapsulation of both hydrophobic and hydrophilic drugs into liposomes to create new combination therapies
- In microfluidic mixing, many variables such as solvent choice, total flow rate (TFR) and flow rate ratio (FRR) are important parameters that affect the liposome size and characteristics
- NanoAssemblr technology enabled the group to efficiently analyze the effect of total and relative flow rates of reagents on liposome size
- Reproducible conditions enabled systematic exploration of formulation parameters such as phospholipid composition, organic solvents and aqueous buffers
- This work demonstrates how NanoAssemblr technology is a convenient, flexible and scalable system for manufacturing of liposomes for both hydrophobic and hydrophilic drug encapsulation and delivery applications

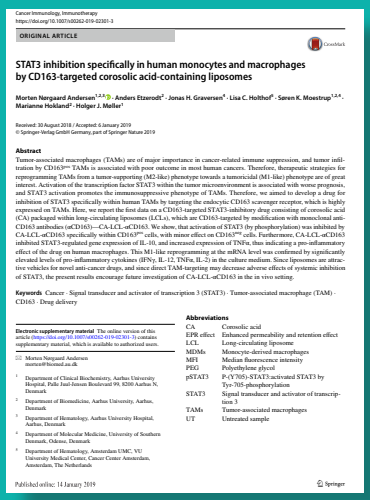
Journal of Liposome Research, 2015

Production of limit size nanoliposomal systems with potential utility as ultra-small drug delivery agents

Zhigaltsev I, Tam Y, Leung A, Cullis P. Journal of Liposome Research 2015; : 1-7.

Summary

- Generating lipid nanoparticle drug carrier systems in the 10-50 nm size range is highly desirable to optimize the biodistribution following IV injection, but generating sizes this small is often limited by the manufacturing process
- Most methods available to generate particles in this size range have limitations, including time of manufacture and scalability
- Lipid nanoparticle pioneer Dr. Pieter Cullis from the University of British Columbia demonstrated how NanoAssemblr Technology enabled liposomes to be produced at the smallest possible or "limit-size" dimensions compatible with the physical properties of the lipid constituents
- Rapid and controlled nanoprecipitation using NanoAssemblr technology enabled production of numerous liposome formulations between 30-40 nm which were remote-loaded with doxorubicin and tested for *in vivo* circulation and drug retention to determine the most ideal formulations
- This paper offers guidance for producing limit-size liposomes and how to tune the limit-size using different lipid mixes to optimize drug delivery


Cancer Immunology, Immunotherapy
https://doi.org/10.1007/s00262-019-02301-3

ORIGINAL ARTICLE

STAT3 inhibition specifically in human monocytes and macrophages by CD163-targeted corosolic acid-containing liposomes

Morten Nørgaard Andersen[1,2,3] · Anders Etzerodt[2] · Jonas H. Graversen[4] · Lisa C. Holthof[4] · Søren K. Moestrup[1,2,4] · Marianne Hokland[3] · Holger J. Møller[1]

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Abstract

Tumor-associated macrophages (TAMs) are of major importance in cancer-related immune suppression, and tumor infiltration by CD163[pos] TAMs is associated with poor outcome in most human cancers. Therefore, therapeutic strategies for reprogramming TAMs from a tumor-supportive (M2-like) phenotype towards a tumoricidal (M1-like) phenotype are of great interest. Activation of the transcription factor STAT3 within the tumor microenvironment is associated with worse prognosis, and STAT3 activation promotes the immunosuppressive phenotype of TAMs. Therefore, we aimed to develop a drug for inhibition of STAT3 specifically within human TAMs by targeting the endocytic CD163 scavenger receptor, which is highly expressed on TAMs. Here, we report the first data on a CD163-targeted STAT3-inhibitory drug consisting of corosolic acid (CA) packaged within long-circulating liposomes (LCLs), which are CD163-targeted by modification with monoclonal anti-CD163 antibodies (αCD163)—CA-LCL-αCD163. We show, that activation of STAT3 (by phosphorylation) was inhibited by CA-LCL-αCD163 specifically within CD163[pos] cells, with minor effect on CD163[neg] cells. Furthermore, CA-LCL-αCD163 inhibited STAT3-regulated gene expression of IL-10, and increased expression of TNFα, thus indicating a pro-inflammatory effect of the drug on human macrophages. This M1-like reprogramming at the mRNA level was confirmed by significantly elevated levels of pro-inflammatory cytokines (IFNγ, IL-12, TNFα, IL-2) in the culture medium. Since liposomes are attractive vehicles for novel anti-cancer drugs, and since direct TAM-targeting may decrease adverse effects of systemic inhibition of STAT3, the present results encourage future investigation of CA-LCL-αCD163 in the in vivo setting.

Keywords Cancer · Signal transducer and activator of transcription 3 (STAT3) · Tumor-associated macrophage (TAM) · CD163 · Drug delivery

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00262-019-02301-3) contains supplementary material, which is available to authorized users.

Abbreviations
CA Corosolic acid
EPR effect Enhanced permeability and retention effect
LCL Long-circulating liposome
MDMs Monocyte-derived macrophages
MFI Median fluorescence intensity
PEG Polyethylene glycol
pSTAT3 P-(Y705)-STAT3-activated STAT3 by Tyr-705-phosphorylation
STAT3 Signal transducer and activator of transcription 3
TAMs Tumor-associated macrophages
UT Untreated sample

Published online: 14 January 2019

Cancer Immunology, Immunotherapy 2019

STAT3 inhibition specifically in human monocytes and macrophages by CD163-targeted corosolic acid-containing liposomes

Andersen M, Etzerodt A, Graversen J, Holthof L, Moestrup S, Hokland M et al. Cancer Immunology, Immunotherapy 2019; 68: 489-502.

Summary

- An important strategy for cancer-related immune suppression is reprogramming Tumor-associated macrophages (TAMs) to aid in fighting cancer
- Dr. Møller's research group at Aarhus University were able to successfully formulate a STAT3 (transcription factor) inhibitory drug, which works by promoting antitumor phenotype of TAMs
- The user-friendly and versatile nature of NanoAssemblr platform helped in formulating nanoparticles that contained the small molecule drug within long-circulating liposomes
- These nanoparticles were able to inhibit the activation of STAT3 *in vitro*, leading to significant elevated levels of pro-inflammatory cytokines resulted in the tumor reprogramming
- The promise of liposomes as vehicles for anti-cancer drugs along with the involvement of STAT3 as an important signaling network to target cancer immunotherapy opens various paths for future anticancer drug development


Contents lists available at ScienceDirect

International Journal of Pharmaceutics

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Rapid and scale-independent microfluidic manufacture of liposomes entrapping protein incorporating in-line purification and at-line size monitoring

Neil Forbes[a,1], Maryam T. Hussain[a,1], Maria L. Briuglia[b], Darren P. Edwards[c], Joop H. ter Horst[c], Nicolas Szita[a], Yvonne Perrie[a,*]

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ABSTRACT

1. Introduction

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E-mail address: yvonne.perrie@strath.ac.uk (Y. Perrie).
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International Journal of Pharmaceutics, 2019

Rapid and scale-independent microfluidic manufacture of liposomes entrapping protein incorporating in-line purification and at-line size monitoring

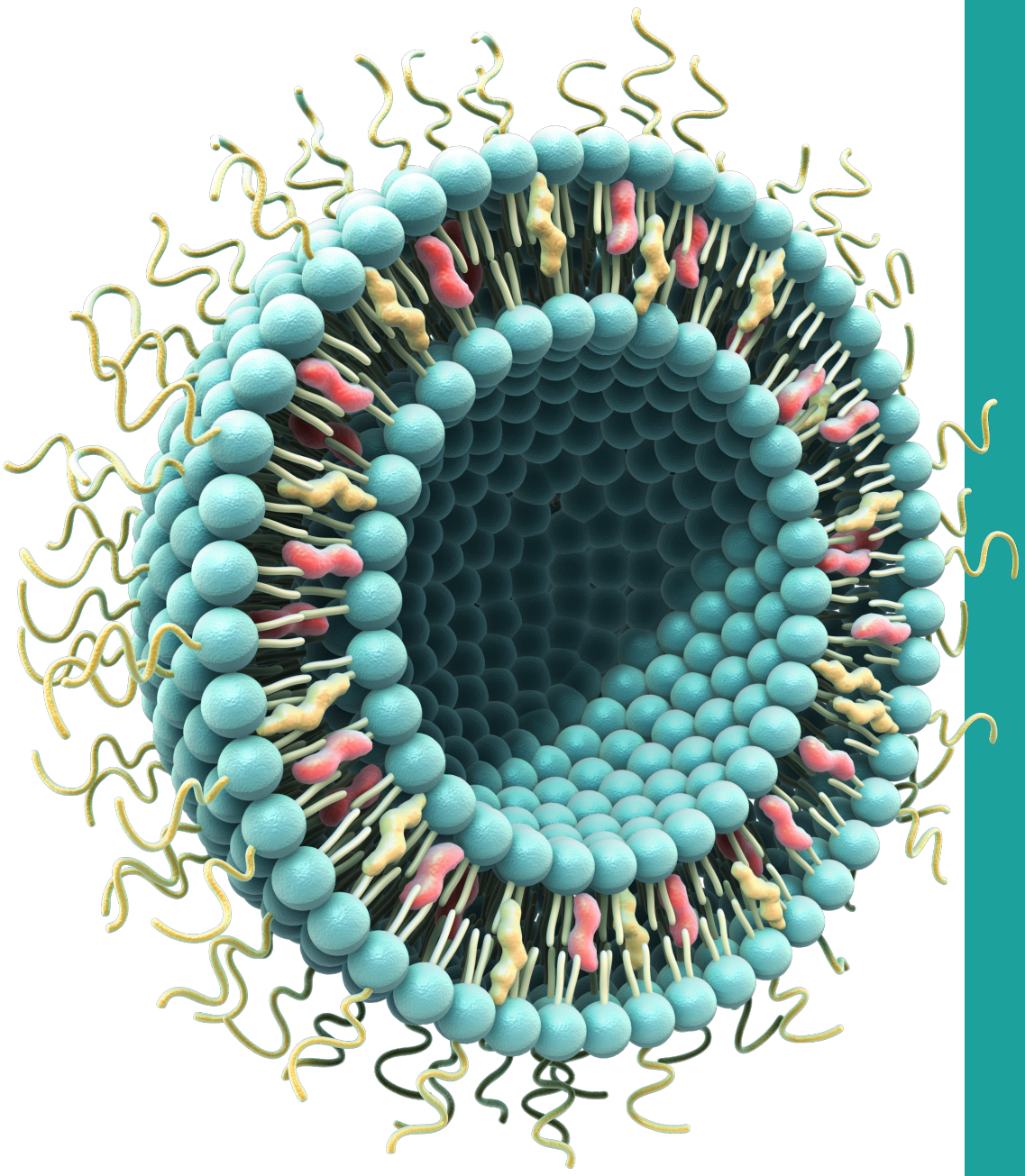
Forbes N, Hussain M, Briuglia M, Edwards D, Horst J, Szita N et al. International Journal of Pharmaceutics 2019; 556: 68-81.

Summary

- Dr. Yvonne Perrie's lab at Strathclyde University, part of Precision Nanosystems' Nanomedicine Innovation Network, have demonstrated a robust, optimized and scalable method for the production of protein-loaded liposomes
- They demonstrated effective production of size-controlled, homogenous high-load liposomes in a scale independent manner using the NanoAssemblr platform
- This is the first time that a simple and scale-independent method to manufacture, purify and monitor the production of liposomes encapsulating proteins has been developed and used
- This process has potential to offer a reliable and quick method to translate production of protein-loaded liposomes from the lab bench to a manufacturing process for nanomedicines

Liposomes

Using the NanoAssemblr® process parameters to reproducibly tune size



Andrew Brown, Anitha Thomas, Mina Ordobadi, Shell Ip, Gesine Heuck, Euan Ramsay.

Abstract

Liposomes are used as drug carriers to deliver a variety of therapeutic molecules such as small molecules, proteins, and nucleic acids. Liposome size and homogeneity are crucial factors that affect efficacy of liposomal drugs. Hence, a liposome production method that streamlines the optimization of these characteristics will expedite formulation development. Here we demonstrated the utility of the NanoAssemblr® microfluidic technology for rapidly and reproducibly generating homogeneous liposome populations with the ability to fine-tune liposome size through computer-controlled parameters. Bench-scale batches of liposomes were produced at sizes ranging from 40 nm to 120 nm with exceptional uniformity (polydispersity index, PDI as low as 0.05). Additionally, the robustness of the NanoAssemblr process was demonstrated by comparing the size and PDI of 6 independent formulations conducted by different instrument operators. These data demonstrate how the technology can be used to expedite development of liposome formulations.

Introduction

Traditional methods of liposome formulation include sonication and extrusion. These methods are laborious and offer limited control over size and polydispersity of liposomes, both of which can affect liposome circulation time, drug retention or its ability to penetrate different tissues.[1] Prior research has demonstrated the utility of the NanoAssemblr® instrument for formulating unilamellar liposomes[2] for remote loading of chemotherapeutics,[3] *in situ* loading with hydrophobic and hydrophilic small molecules [4,5,] nucleic acids,[6] and vaccine adjuvants.[7] The NanoAssemblr platform works through controlled nanoprecipitation of liposomes (**Figure 1**). When lipid molecules dissolved in a low-polarity organic solvents are mixed with water or buffer, the resulting change in polarity triggers the spontaneous self-assembly of the lipids into unilamellar vesicles. The NanoAssemblr platform combines microfluidic mixing with independent computer-controlled injection of both fluids to achieve control over precipitation conditions that directly influence liposome size. This method also ensures consistency among liposomes within any given batch, from batch to batch, and from operator to operator. With a reproducible process, effects of altering the formulation can be isolated (see Related Materials, below, for additional resources). Additionally, the NanoAssemblr platform offers a straightforward path towards clinical development by streamlining scale-up through continuous flow and multiple parallel mixers. Here, we demonstrated that the NanoAssemblr consistently formulates homogenous liposomes by comparing the size and polydispersity (PDI) of 6 independent formulations. Next, we demonstrated how precise computer control over injection speed and the ratio between aqueous and organic phases is used to finely tune liposome size and minimize heterogeneity.

Figure 1

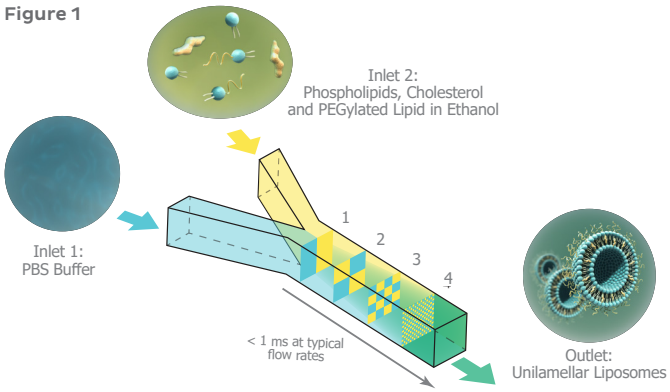


Figure 1. PBS buffer is injected into the left inlet while an organic solvent with dissolved lipids is injected into the right inlet of the NanoAssemblr microfluidic cartridge. *Following controlled mixing in microfluidic channels, unilamellar liposomes are spontaneously formed. Computer controlled injection allows specification of the total flow rate and the flow rate ratio of aqueous to organic solvents to control liposome size.*

1. Two fluids (buffer and ethanol) entering the channel under laminar flow do not mix.
2. Microscopic features in the channels are engineered to cause the fluid streams to mingle in a controlled, reproducible, and non-turbulent way.
3. Intermingling of the fluids increases as they continue through the mixer.
4. Fluids emerge from the mixer completely mixed. The process takes < 1 ms; faster than molecules can assemble into particles.

Result

LIPOSOME BATCH REPRODUCIBILITY USING NANOASSEMBLR TECHNOLOGY

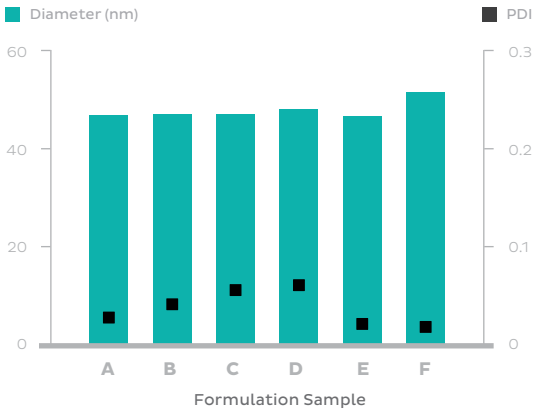
To assess batch-to-batch variation of liposome formulations prepared using the NanoAssemblr, multiple batches of liposomes with the same lipid composition were formulated under identical instrument parameters by independent operators. This led to comparable liposome sizes and PDIs demonstrating consistency across batches and operators **(Figure 2)**.

TUNING LIPOSOME SIZE BY ALTERING FLOW RATE RATIO AND TOTAL FLOW RATE

The aqueous:organic Flow Rate Ratio (FRR) and Total Flow Rate (TFR) are the primary process parameters that impact nanoparticle characteristics. FRR is the volumetric ratio of the organic and aqueous phases being mixed through the microfluidic cartridge. TFR is the total speed in mL/min at which both fluid streams are being pumped through the two separate inlets of the microfluidic cartridge.

Figure 3 indicates the effect of FRR on liposome size. At a constant liposome composition, particle size decreased from 90 to 50 nm as the FRR increased from 1:1 to 3:1. At very high FRRs, size plateaus as liposomes form the smallest thermodynamically possible structures defined as their "limit-size"[2].

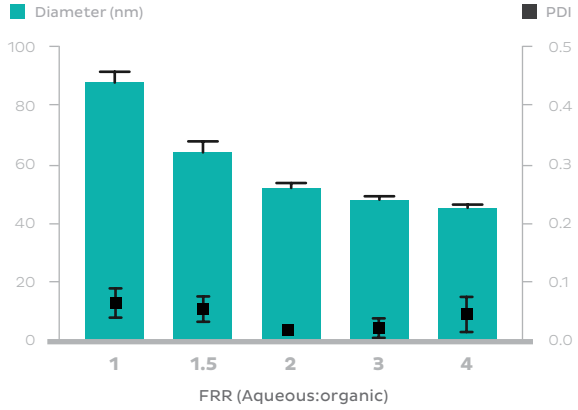
The effect of TFR on liposome size can be observed in **Figure 4**. Liposome size decreased from 100 to about 50 nm when the TFR was increased from 1 to 12 mL/min. The FRR, lipid composition, and total lipid concentration were kept constant throughout the experiment.



Composition	POPC/Chol/DSPE-PEG$_{2000}$ (52:45:3 mol%)
Lipid concentration in organic solvent	10 mg/mL
Organic solvent	Ethanol
Aqueous solvent	PBS pH 7.4
Total Flow Rate (TFR)	12 mL/min
Flow Rate Ratio (FRR)	5:1

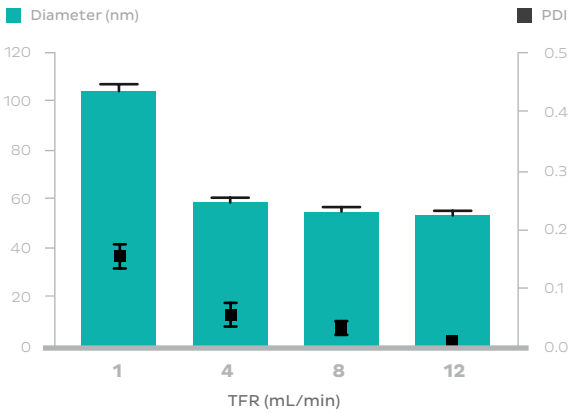
Figure 2. Liposome size and polydispersity were consistent across batches prepared by different operators. *Six independent batches of liposomes, with identical lipid composition and NanoAssemblr process parameters were generated by different operators. Liposomes generated had a size range of 46-54 nm and consistently low polydispersity indices (PDI).*

Figure 3. Liposome size tuning by Flow Rate Ratio. *Higher aqueous:organic Flow Rate Ratio (FRR) reduces liposome size asymptotically. Samples were prepared in triplicate. Measurements represent the mean and error bars represent standard deviation.*



Composition	POPC/Chol/DSPE-PEG$_{2000}$ (52:45:3 mol%)
Lipid concentration in organic solvent	10 mg/mL
Organic solvent	Ethanol
Aqueous solvent	PBS pH 7.4
Total Flow Rate (TFR)	12 mL/min
Flow Rate Ratio (FRR)	n:1, n as indicated

Figure 4. Liposome size tuning by Total Flow Rate. *Increasing Total Flow Rate (TFR) increases mixing speed and reduces liposome size. Samples were prepared in triplicate. Measurements represent the mean and error bars represent standard deviation.*



Composition	POPC/Chol/DSPE-PEG$_{2000}$ (52:45:3 mol%)
Lipid concentration in organic solvent	10 mg/mL
Organic solvent	Ethanol
Aqueous solvent	PBS pH 7.4
Total Flow Rate (TFR)	As indicated (mL/min)
Flow Rate Ratio (FRR)	2:1

Discussion

Liposome size and polydispersity are crucial factors affecting efficacy. Hence, size control and size uniformity within and between batches are crucial factors to optimize in liposomal drug formulation development. The NanoAssemblr process is highly reproducible, as shown in **Figure 2**, where multiple independent operators produced liposome batches with comparable sizes and PDIs. This is achieved through computer control of parameters that affect liposome size. Understanding the factors that affect liposome size provides insight into factors that affect uniformity of size and reproducibility. During nanoprecipitation, lipid molecules start off fully solubilized in a water-miscible organic solvent. When this solution is mixed with water, the polarity of the resulting solution increases, which causes the lipid molecules to self-assemble into unilamellar liposomes. Given a fixed lipid composition, two factors that affect the resulting liposome size are the magnitude of polarity change and the speed of mixing. On the NanoAssemblr platform, the former is controlled by the Flow Rate Ratio (FRR) while the latter is controlled by the Total Flow Rate (TFR). **Figure 3** illustrates that increasing the aqueous-to-organic FRR decreases the size of the resulting liposomes asymptotically. This is because increasing the relative amount of the aqueous phase increases the magnitude of the polarity change upon mixing, which increases the driving force for self-assembly of lipids into liposomes. Additionally faster mixing, achieved by increasing TFR through the NanoAssemblr microfluidic mixer, leads to smaller liposomes, as illustrated in **Figure 4**. This behavior is understood by considering the rate of polarity change compared to the rate of self-assembly. If the rate of polarity change exceeds the rate of self-assembly, lipid molecules experience an immediate super-saturation in the new solvent environment. Rates of diffusion and reorganization then limit the local supply of lipid molecules that can come together into a single liposome. This leads to smaller liposomes in greater abundance. From this understanding, the asymptotic behavior observed in both **Figures 3** and **4** can also be understood. As the driving force and rate of mixing are increased, they meet the limits of how small a particle can be, given the volume and packing of the constituent molecules.

With this understanding of how the solvent environment affects liposome size, it is clear that precise control over the mixing ratio and the mixing rate is necessary to achieve uniform liposome size. Within a given batch, the homogeneity of the population of liposomes is measured by the polydispersity index, where values below 0.2 are preferred for *in vivo* applications. Homogeneity of the solvent environment throughout the volume of the batch is required in order to obtain a homogeneous population of liposomes. PDIs achieved on the NanoAssemblr were below 0.2, with many formulations below 0.1, which is difficult to achieve with conventional methods. In the microfluidic channels, the organic and aqueous phases are spatially confined, which results in laminar, as opposed to turbulent flow. Laminar mixing is gentle and highly consistent. This ensures that, under continuous flow and at steady state, each unit volume flowing through the mixer is experiencing nearly identical mixing conditions. Furthermore, the "Autoswitch" feature of the NanoAssemblr automatically separates microliter volumes from the head and tail of the process in order to isolate the particles produced under steady state. To achieve batch-to-batch consistency the conditions of liposome precipitation must be highly reproducible. In addition to laminar flow mixing, precise computer-controlled injection of the organic and aqueous phases ensures consistent flow rates and mixing ratios between batches, and between independent instrument operators. Additionally, computer controlled injection allows more precise control over TFR and FRR and thus enables rational optimization of these two parameters.

Conclusion

We established that the NanoAssemblr can rapidly tune the size of liposomes by adjusting instrument factors (TFR and FRR) independent of liposome composition. This is a convenient and powerful means of optimizing size and PDI of liposomal drug formulations. Additionally, it was confirmed that liposomes formulated by NanoAssemblr maintained exceptional batch-to-batch consistency and minimal population heterogeneity. Taken together, these results demonstrate how the NanoAssemblr platform facilitates rapid optimization of liposomal drug formulations. Furthermore, the process offers a straightforward path to scaling production to clinically relevant quantities by increasing lipid concentration and implementing continuous flow and multiple parallel microfluidic mixers.

Materials & Methods

Liposomes were composed of cholesterol, POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine), and DSPE-PEG_{2000} (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000]). Lipids were dissolved in ethanol as the organic solvent and Calcium- (Ca^{2+}) and magnesium- (Mg^{2+}) free PBS buffer at neutral pH was used as the aqueous phase. The organic and aqueous phases were rapidly mixed using the NanoAssemblr Benchtop* microfluidic instrument at aqueous to organic Flow Rate Ratios between 1:1 and 4:1 and Total Flow Rates between 2 mL/min and 12 mL/min. Formulations were then dialyzed against PBS to remove ethanol. At Flow Rate Ratios 1 and 1.5, formulations were diluted down to 10% ethanol immediately following the mixing process and before dialysis, since high amounts of ethanol can destabilize liposomes. Particle size and integrity were measured before and after dialysis using a Dynamic Light Scattering technique Particle size and integrity was then investigated using Dynamic Light Scattering (Zetasizer, Malvern Instruments, UK). Where indicated, formulations were prepared in triplicate and size and polydispersity index (PDI) are represented as the mean of 3 samples, and error bars represent standard deviation (SD).

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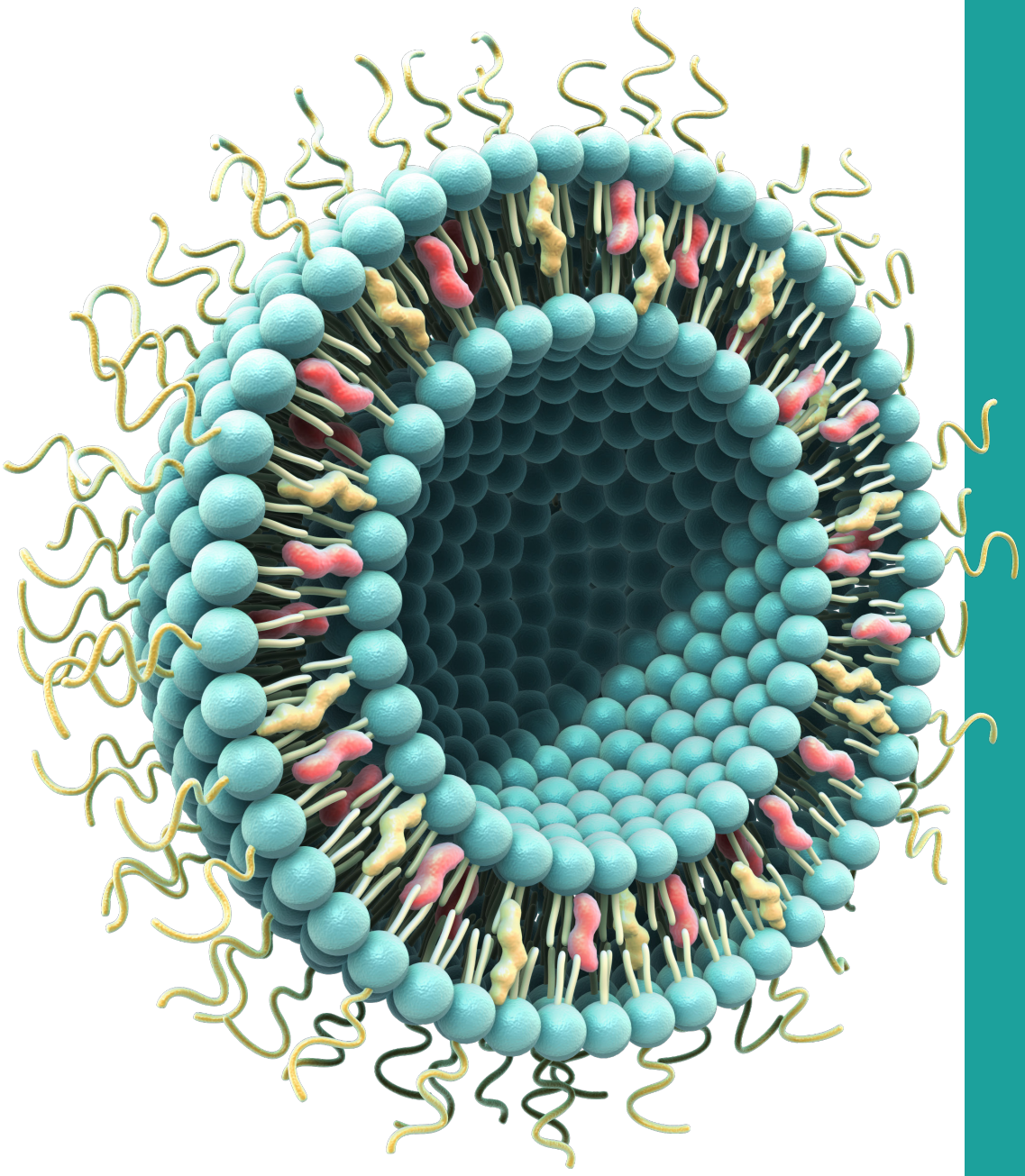
* The formulations described in this work were produced using the discontinued NanoAssemblr Benchtop. The NanoAssemblr Ignite can be used in place of its predecessor, the NanoAssemblr Benchtop

Liposomes

Using formulation parameters to tune size on the NanoAssemblr®



Andrew Brown, Anitha Thomas, Mina Ordobadi, Shell Ip, Gesine Heuck, Euan Ramsay.

Abstract

Liposomes are used as drug carriers to deliver a variety of Liposome encapsulation is a technique that has been used to enhance drug circulation time and reduce toxicity of hydrophilic molecules, or improve solubility of hydrophobic molecules. Depending on the type of drug molecule or target tissue, different liposome compositions are often needed. Additionally, size and homogeneity of the liposome population can impact liposome behavior in vivo. In this application note, we examine the effect of lipid composition and concentration on the size and size distribution of liposomes generated using the NanoAssemblr®. The process reproducibility afforded by the NanoAssemblr allows observed changes in liposome size to be attributed to formulation changes. Increasing cholesterol content in liposome formulations was found to increase the size of resulting liposomes.

Introduction

Liposomal drug delivery can greatly improve drug performance by altering pharmacokinetic and biodistribution profiles. Liposome composition can be tailored to optimize the encapsulation of drug molecules with a range of chemical properties. Additionally, certain lipids can significantly impact liposome properties that influence their efficacy as nanomedicines, such as drug retention, size, and surface charge.

Liposome size is one parameter that can influence circulation half-life and tissue penetration.[1] The NanoAssemblr® is a scalable microfluidic-based manufacturing platform that allows researchers to reproducibly fine tune liposome size. It has been shown that NanoAssemblr technology enables the formation of the smallest thermodynamically stable liposomes, so-called "limit" sized liposomes, based on the packing density of molecules participating in the nanoparticle structure.[2] The platform produces unilamellar liposomes through controlled nanopreciptitation (see **Figure 1**). Lipids dissolved in an organic solvent, typically ethanol, are injected into one inlet of the NanoAssemblr cartridge, while water or a buffer is injected into the other inlet in laminar flow (i.e. the fluids do not mix immediately). Microscopic features within the channels cause controlled mixing at time scales more rapid than diffusion of molecules. The sudden change in solvent polarity due to mixing triggers assembly of lipids into unilamellar liposomes. Liposome size can be tuned by one or more strategies: (i) computer controlled process parameters; (ii) liposome composition; and, (iii) total lipid concentration. The instrument process parameters Total Flow Rate (TFR) and Flow Rate Ratio (FRR) are used to control the conditions of liposome self-assembly by solvent displacement (see Related Material, below, for more details). Here, we focus on the effect of liposome composition, specifically, cholesterol content, and total lipid concentration, on liposome "limit" size. We further determine the parameters required to achieve liposomes of a specific size using the NanoAssemblr.

Figure 1

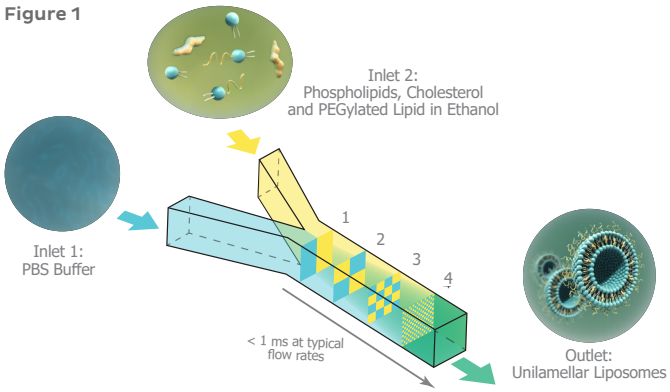


Figure 1. PBS buffer is injected into the left inlet while an organic solvent with dissolved lipids is injected into the right inlet of the NanoAssemblr microfluidic cartridge. *Following controlled mixing in microfluidic channels, unilamellar liposomes are spontaneously formed. Computer controlled injection allows specification of the total flow rate and the flow rate ratio of aqueous to organic solvents to control liposome size.*

1. Two fluids (buffer and ethanol) entering the channel under laminar flow do not mix.
2. Microscopic features in the channels are engineered to cause the fluid streams to mingle in a controlled, reproducible, and non-turbulent way.
3. Intermingling of the fluids increases as they continue through the mixer.
4. Fluids emerge from the mixer completely mixed. The process takes < 1 ms; faster than molecules can assemble into particles.

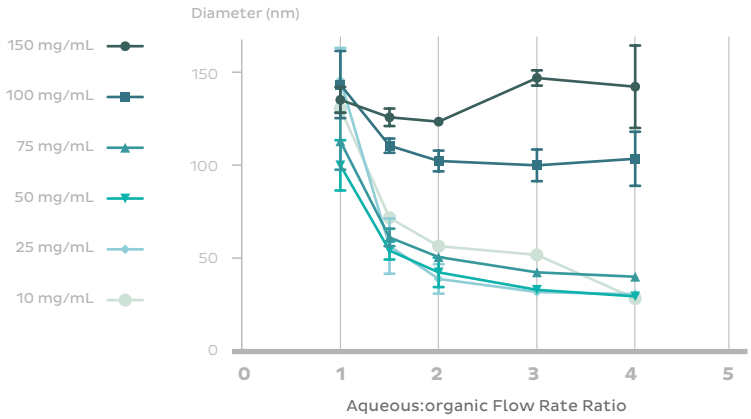
Result

First, we examined the effect of increasing Flow Rate Ratio (FRR), which is the ratio between the aqueous (antisolvent) phase and the organic (solvent) phase being mixed in the microfluidic cartridge on liposome size as a function of total lipid concentration. The TFR was held constant at 12 mL/min. The liposome composition tested comprised the high transition temperature lipid DSPC (Tm = 55°C), cholesterol and DSPE-PEG2000. Liposomes with a high DSPC content should be formulated at temperatures above the lipid's transition temperature. Having the lipids in a fluid state facilitates the formation of liposomal structures. **Figure 2** shows that at total lipid concentrations of ≤ 75 mg/mL, increasing the FRR results in a decrease in liposome size until a plateau is reached at FRR > 2:1 where the liposomes reach limit size (~ 60 nm). At a 1:1 FRR, liposome size was comparable and independent of total lipid concentration. However, at total lipid concentrations ≥ 100 mg/mL, increasing the FRR did not result in a decrease in liposome size.

Liposome concentration can also be increased following formulation by using centrifugal filtration (CF). CF is routinely employed in formulation development to remove unencapsulated drug, exchange buffer and increase the concentration of liposomes irrespective of the method used to form the liposomes. POPC/Chol/DSPE-PEG2000 liposomes were formulated with a starting concentration of 10 mg/mL in ethanol. Following liposome formulation on the NanoAssemblr under limit size conditions, liposomes were dialyzed against PBS to remove ethanol, and thereafter concentrated more than 12-fold using centrifugal filtration. The final lipid concentration was determined to be 25 mg/mL using a cholesterol assay kit. Hydrodynamic size and PDI measurements before and after the concentration step are compared in **Figure 3**. These data indicate that limit size liposomes produced on the NanoAssemblr are robust and retain their integrity following downstream processing.

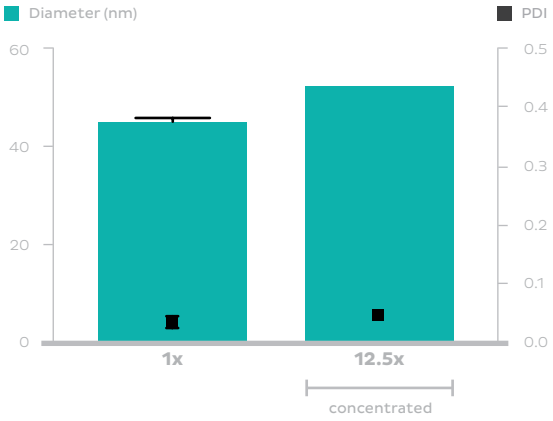
Next, we examined how the lipid composition affects liposome size when operating at instrument process parameters shown to promote limit size. Cholesterol is an integral part of biological membranes and plays an important role in the physical properties of liposomes3. Additionally, cholesterol is known to influence drug release profiles in liposomal formulations.[4] To examine the role of cholesterol on the liposome limit size, the cholesterol content was systematically varied in a series of POPC/cholesterol/DSPE-PEG2000 formulations prepared at a FRR of 4:1 and a TFR of 12 mL/min. **Figure 4** shows that liposome limit size increased from 20 to 42 nm as the cholesterol content was increased from 0 to 45 mol%. Additionally, we observed that ethanol removal by dialysis did not affect liposome size.

Figure 2. *The effect of lipid concentration and Flow Rate Ratio (FRR) on liposome size. Liposomes with identical lipid composition were generated at different total lipid concentrations and at different FRRs. The Heating Controller was set to 65˚C. Liposomes were dialyzed against PBS pH 7.4 to remove residual solvent prior to measuring hydrodynamic size by DLS. Data represents the mean of 3 samples and error bars represent standard deviation.*



Composition	DSPC/cholesterol/DSPE-PEG$_{2000}$ (52:45:3 mol%)
Initial lipid concentration in organic solvent	As indicated
Organic solvent	Ethanol
Aqueous solvent	PBS pH 7.4
Total Flow Rate (TFR)	12 mL/min
Flow Rate Ratio (FRR)	n:1 as indicated on horizontal axis

Figure 3. *Liposomes were concentrated using centrifugal filtration with minimal effect on size. Six samples were prepared with a final concentration of 2 mg/mL and mean size and PDI with SD is shown (1X). Samples were then pooled together and concentrated to a final concentration of 25 mg/mL (12.5X) in PBS pH 7.4 by centrifugal filtration.*



Composition	POPC/Chol/DSPE-PEG$_{2000}$ (52:45:3 mol%)
Initial lipid concentration in organic solvent	10 mg/mL
Organic solvent	Ethanol
Aqueous solvent	PBS pH 7.4
Total Flow Rate (TFR)	12 mL/min
Flow Rate Ratio (FRR)	4:1

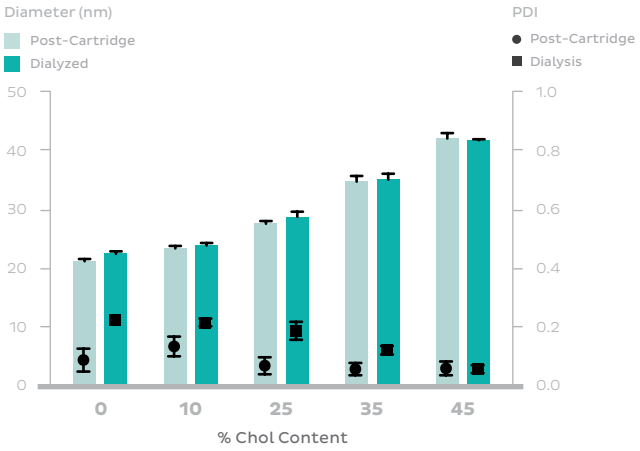


Figure 4. *Liposome limit-size increases with cholesterol content. Addition of cholesterol led to a size increase in liposomes. Liposomes contained 3 mol% DSPE-PEG and varying amounts of POPC and cholesterol. Size was measured soon after formulation and after dialysis. Samples were prepared in triplicate. Measurements represent the mean and error bars represent standard deviation.*

Composition	POPC/Chol/DSPE-PEG_{2000} ((97-x):x:3 mol%) x as indicated
Initial lipid concentration in organic solvent	10 mg/mL
Organic solvent	Ethanol
Aqueous solvent	PBS pH 7.4
Total Flow Rate (TFR)	12 mL/min
Flow Rate Ratio (FRR)	4:1

Discussion

Self-assembly of lipids into liposomes occurs when lipids dissolved in a good solvent are mixed with a polar aqueous antisolvent. Rapid, homogenous mixing creates a sudden change in the polarity of the environment of the lipid molecules, and the magnitude of this polarity change is proportional to the driving force for self-assembly into bilayers and liposomes. The magnitude of the polarity change can be dictated by the aqueous-to-solvent flow rate ratio (FRR), which is a software-controlled parameter on the NanoAssemblr instrument. Higher FRRs lead to greater polarity changes and, typically, smaller liposomes. At sufficiently high FRR, the self-assembly process becomes kinetically limited which leads to smaller liposomes. The lower limit of liposome size is dictated by the mechanical packing density of lipid molecules, leading to the asymptotic behavior observed in **Figure 2**. Additionally, at sufficiently high lipid concentrations, the concentration gradient between free lipids and those assembling into nascent liposomes is great enough to overcome kinetic barriers. This leads to changes in the profiles observed at initial lipid concentrations of 100 mg/mL and greater.

The trends observed in **Figure 2** can be used to tune particle size. First, these data can be used as a look-up-table to determine the FRR, required to achieve a specific size for this formulation. Second, increasing the starting lipid concentration is a means by which to scale up liposome production, hence the change in self-assembly behavior of the lipids at higher concentrations provides guidance on which concentration range to choose in order to achieve the highest "tunability" of liposome size. The total throughput can be calculated by considering the lipid concentration in the organic phase as well as the

FRR and TFR. For instance at an initial lipid concentration of 75 mg/mL, a FRR of 2:1 and a TFR of 12 mL/min, total liposome output through the NanoAssemblr cartridge is 300 mg/min. At a TFR of 18 mL/min, total liposome output would be 450 mg/min. Additionally, these findings provide a means to achieve large liposomes by increasing solvent-phase lipid concentrations above 75 mg/mL with this formulation. Identical studies can be easily performed on the NanoAssemblr to understand the behavior of different lipid compositions.

If desired, liposome concentration can be adjusted as desired by dilution or by centrifugal filtration following NanoAssemblr formulation. **Figure 3** demonstrates that liposomes can be formulated at a particular concentration and concentrated to a final concentration as required with minimal effect on liposome size and identical PDI.

From **Figure 4**, it is clear that liposome limit size increases with the proportion of cholesterol in the formulation. This can be understood by considering the effect of cholesterol on the properties of lipid bilayers. It has been demonstrated that the presence of cholesterol in lipid bilayers consisting of phospholipids with at least one saturated tail tends to increase its mechanical rigidity.[3,4] This rigidity limits the curvature of a bilayer, leading to larger equilibrium liposome size. Hence, as cholesterol content rises, this stiffens the mechanical properties of the bilayer, leading to larger liposomes with larger radii of curvature.

Having a reproducible process is crucial both to observing the differences in liposome size and attributing these differences to formulation parameters such as initial lipid concentration and lipid composition. Poor reproducibility in the preparation method makes it difficult to separate the influence of random process variations from the influence of formulation parameters. This would make the trends observed in the present study difficult to discern. Given that process parameters influence liposome size and dispersity, it is easy to see that an extremely well controlled process achieved with the NanoAssemblr is necessary to fine-tune liposome formulations.

Conclusion

The size of liposomes generated with the NanoAssemblr can be controlled by instrument process parameters as well as lipid concentration and composition. In this application note, we demonstrated that varying lipid concentration can affect the relationship between liposome size and FRR. Over a wide range of initial lipid concentrations, this trend remained unchanged. This offers a means of scaling quantities and throughput of liposome formulations by increasing the concentration of lipids in the organic phase. At initial lipid concentrations of 75 mg/mL, limit size liposomes were produced at a rate of 300 mg/min. Alternatively, liposomes can be concentrated after NanoAssemblr production by centrifugal filtration without appreciably affecting size. Additionally, the amount of cholesterol in a liposome formulation was found to significantly alter the limit size of vesicles. In the case of POPC/chol/DSPE-PEG2000 formulations, a greater proportion of cholesterol increases the stiffness of the bilayer, favoring the formation of larger liposomes, allowing for less strain during bending of the bilayer into a sphere. Ultimately, the reproducibility of the NanoAssemblr microfluidic process was crucial to attributing changes in lipid concentration and composition to the observed differences in size. Hence, the NanoAssemblr is the ideal tool to develop and tune liposomal drug formulations.

Materials & Methods

Liposomes were composed of cholesterol and POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) or DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine) and DSPE-PEG (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000]). Lipids were dissolved in absolute ethanol as the organic solvent phase. Calcium- (Ca^{2+}) and magnesium- (Mg^{2+}) free PBS buffer at neutral pH was used as the aqueous phase. The organic and aqueous phases were rapidly mixed using the NanoAssemblr Benchtop* microfluidic instrument at various Flow Rate Ratios at a Total Flow Rate of 12 mL/min. Formulations with high DSPC content were heated at 65˚C during the mixing process using the NanoAssemblr Heating Controller accessory. The Heating Controller accessory for the NanoAssemblr Benchtop can adjust the formulation process temperature with an accuracy of ±1˚C. Formulations were then dialyzed against PBS for ethanol removal. At Flow Rate Ratios 1 and 1.5, formulations were diluted down to 10% ethanol immediately following the mixing process and before dialysis, since high amounts of ethanol can destabilize liposomes. Particle size and integrity were measured before and after dialysis using Dynamic Light Scattering (Zetasizer, Malvern Instruments, UK). Where indicated, formulations were prepared in triplicate, unless otherwise noted, and size and polydispersity index (PDI) were represented as the mean of 3 samples, and error bars represent standard deviation of the mean.

Conclusion

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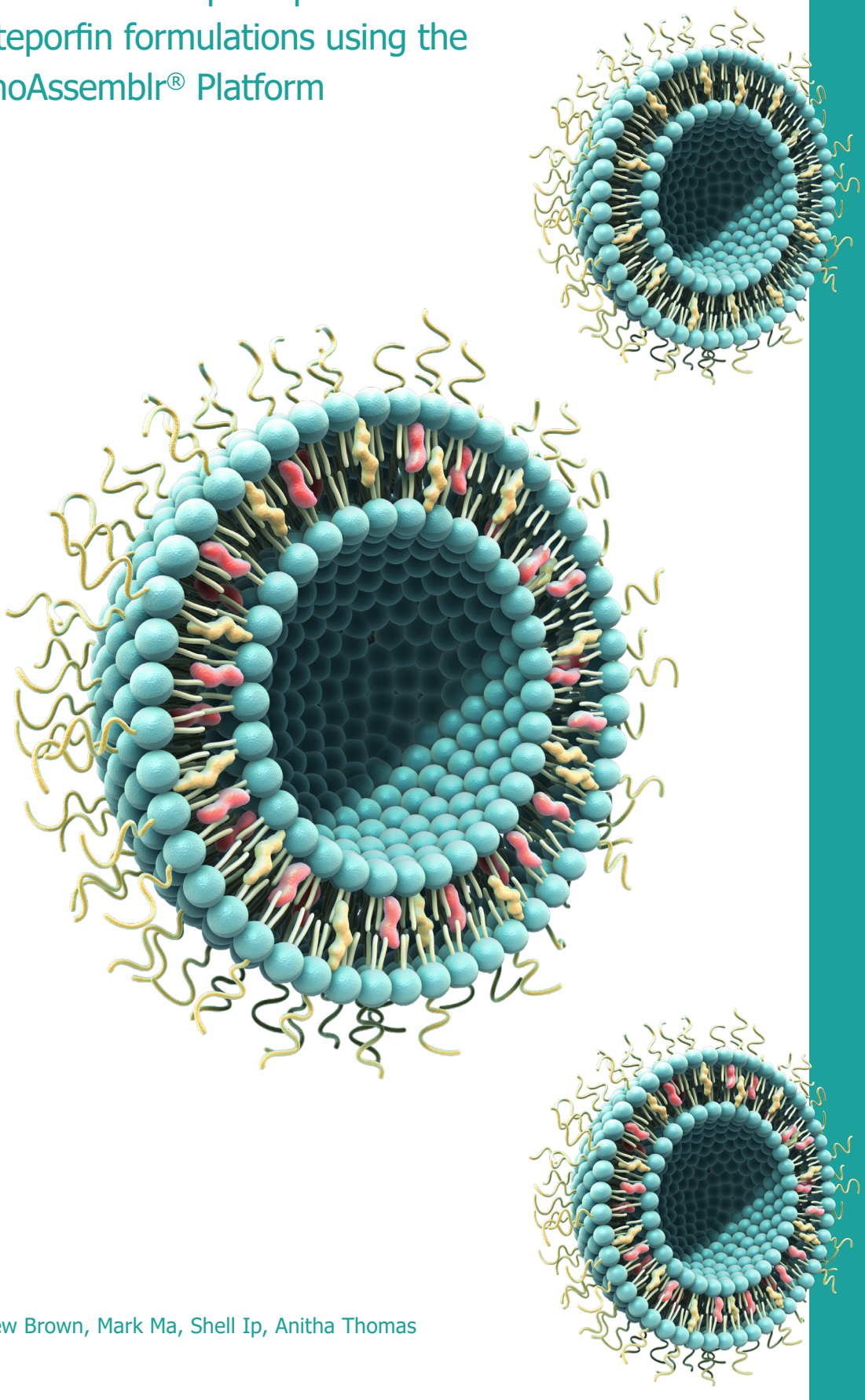
* The formulations described in this work were produced using the discontinued NanoAssemblr Benchtop. The NanoAssemblr Ignite can be used in place of its predecessor, the NanoAssemblr Benchtop

Liposomes

Seamless scale up of liposomal verteporfin formulations using the NanoAssemblr® Platform



Andrew Brown, Mark Ma, Shell Ip, Anitha Thomas

Abstract

Liposomes are attractive drug delivery systems for formulating low solubility drugs. While several liposomal drugs are presently marketed, liposome production is commonly a multi-step process that requires significant process development to scale up production through preclinical and clinical development.

In this application note, we leverage the reproducible, continuous flow nature of the NanoAssemblr® microfluidic platform to reduce scale up process development. Two liposome formulations of the hydrophobic photosensitizer verteporfin were produced as model systems and scaled up in batch volume by an order of magnitude. A process for liposome formation and simultaneous drug loading was initially developed at the bench scale on the NanoAssemblr, designed for rapid formulation optimization at volumes between 1 mL and 15 mL. Optimized formulation parameters were transferred directly to the NanoAssemblr Blaze™, designed for producing preclinical batches between 10 mL and 1000 mL.

As a consequence of conserved microfluidic geometry between the two systems, formulation conditions were replicated exactly. Hence, the physical characteristics and encapsulation efficiency were found to be identical between formulations produced on the two systems. This capability for seamless process transfer reduces the burden of process development commonly encountered when scaling up traditional liposome production methods.

Introduction

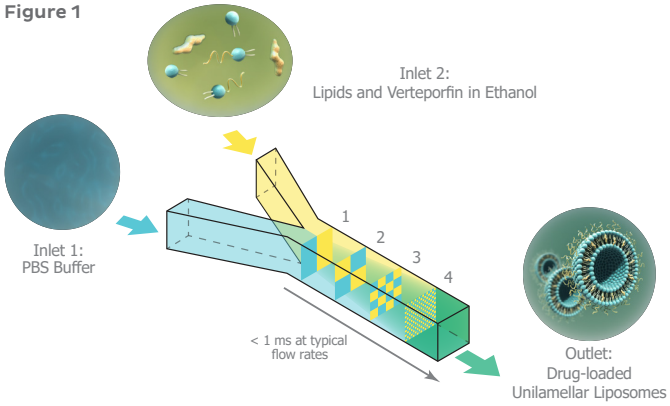
Over 75% of new chemical entities emerging from drug discovery programs are low solubility biopharmaceuticals. Their insolubility makes dosing and administration difficult. Hence, drug delivery systems such as liposomes are increasingly important. An example of a commercially available liposomal formulation of a hydrophobic drug is Visudyne®, used in photodynamic therapy for wet macular degeneration – a condition characterized by abnormal vascularization behind the retina that leads to irreversible loss of central vision.[1] The active pharmaceutical ingredient is the photosensitizer verteporfin, whose primary mechanism of action is photoinduced generation of singlet oxygen species. Systemic injection followed by localized application of light allows the abnormal vasculature to be selectively reduced.[1] This mechanism is also being investigated to treat solid tumours.[2] Verteporfin is hydrophobic; contact with water causes verteporfin to aggregate, which inhibits its activity by reducing its ability to form singlet oxygen upon photoexcitation.[3] Hence, formulation into liposomes is desirable.

Liposome production by thin film hydration and extrusion is a laborious multi-step process that is difficult to scale up. NanoAssemblr technology uses microfluidics to control the nanoprecipitation of unilamellar liposomes from solvent, greatly reducing the number and duration of processing steps. Confinement of fluids in microchannels creates conditions of laminar flow that allows homogeneous, time-invariant mixing. This leads to exceptional reproducibility and fine control over particle size using parameters such as the Total Flow Rate (TFR) and Flow Rate Ratio (FRR). Employing NanoAssemblr microfluidic reactors in different pumping arrangements allows formulation volumes to be scaled over several orders of magnitude using the same parameters. The process is illustrated in **Figure 1**.

Several groups have demonstrated development of liposomal drug delivery systems using NanoAssemblr technology.[4-7] Additionally, a prior application note has reported in situ loading of verteporfin into soy- and egg-PC liposomes and optimization of size by systematic exploration of TFR and FRR.[8] These studies employed a bench-scale NanoAssemblr system, which uses common and disposable syringes and collection tubes for fluid handling. While this format makes the bench-scale system ideal for rapid formulation development, these disposables limit batch sizes to 15 mL. Here, we demonstrate the seamless scale up of liposomal verteporfin formulations beyond this limit using the NanoAssemblr Blaze™ which employs continuous flow pumps to enable scale up of formulations to batch volumes up to 1 L (see the back cover for an overview of NanoAssemblr systems). Conserved microfluidic architecture between the bench-scale and Blaze systems allows parameters optimized at low volumes to be transferred directly to the Blaze, thus allowing scale up of formulations with minimal process development.

Figure 1. A) *PBS buffer is injected into the left inlet while an organic solvent with dissolved lipids and the drug verteporfin is injected into the right inlet of the NanoAssemblr microfluidic cartridge. Following controlled mixing in microfluidic channels, liposomes are spontaneously formed. Computer-controlled injection allows specification of the Total Flow Rate and the Flow Rate Ratio of aqueous to organic solvents to control liposome size. Hydrophobic verteporfin is integrated into the hydrophobic region of the lipid membrane.*

Figure 1

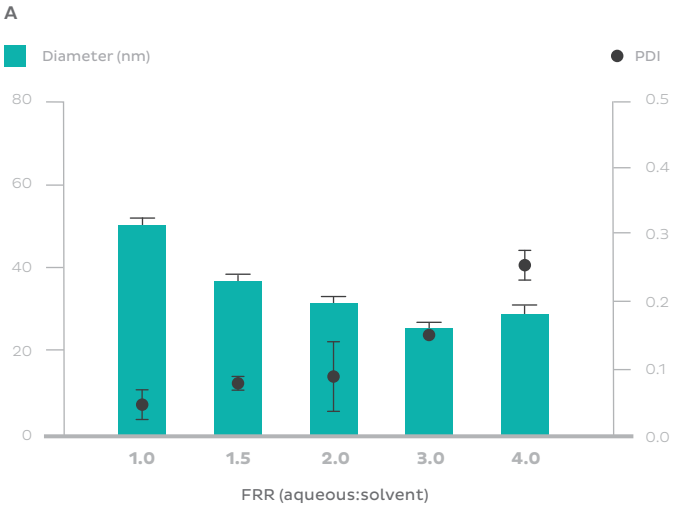


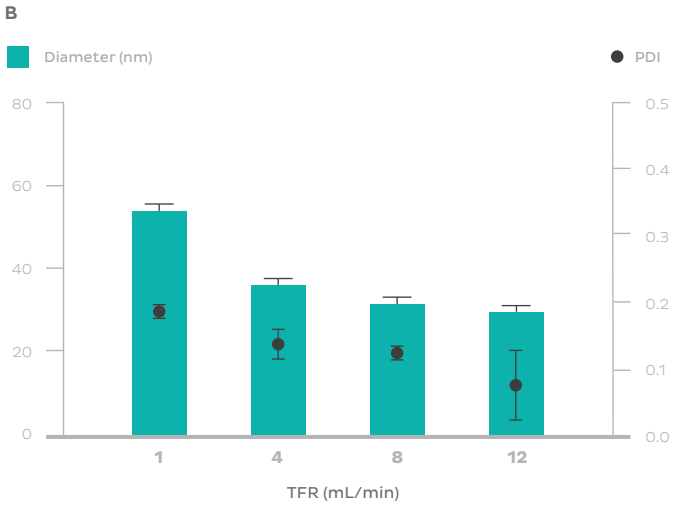
1. Two fluids (buffer and ethanol) entering the channel under laminar flow do not mix.
2. Microscopic features in the channels are engineered to cause the fluid streams to mingle in a controlled, reproducible, and non-turbulent way.
3. Intermingling of the fluids increases as they continue through the mixer.
4. Fluids emerge from the mixer completely mixed. The process takes < 1 ms; faster than molecules can assemble into particles.

Scale up was demonstrated for two different liposome formulations. Process parameters were determined using 2 mL batch sizes with the bench-scale system to conserve materials, then scaled up ten-fold with the Blaze (with volumes up to 1 L possible) using the same parameters. The first formulation consisted of soy-PC/cholesterol/DSPE-PEG (52/45/3 mol%). The second formulation more closely resembles Visudyne, and consisted of DMPC/Egg-PG (67/33 mol%). In both cases, lipids and verteporfin were dissolved in the organic phase and then mixed with PBS buffer within the NanoAssemblr microfluidic cartridges to produce liposomes. Verteporfin was loaded into the hydrophobic lipid bilayer during liposome formation in a single step. Experimental details are provided in the Materials and Methods section.

Soy-PC-based formulations were previously optimized at the bench scale by systematically varying TFR and FRR and measuring size and PDI. These findings are summarized in **Figure 2**. Liposome size was found to decrease asymptotically with increasing FRR and TFR, which is consistent with previous results reported in the literature.[4,5] This asymptote is understood the be the "limit-size" - the smallest possible size calculated based in ideal packing of constituent molecules.[4] Based on this, a FRR of 2:1 and TFR of 12 mL/min were chosen for upscaling both formulations.

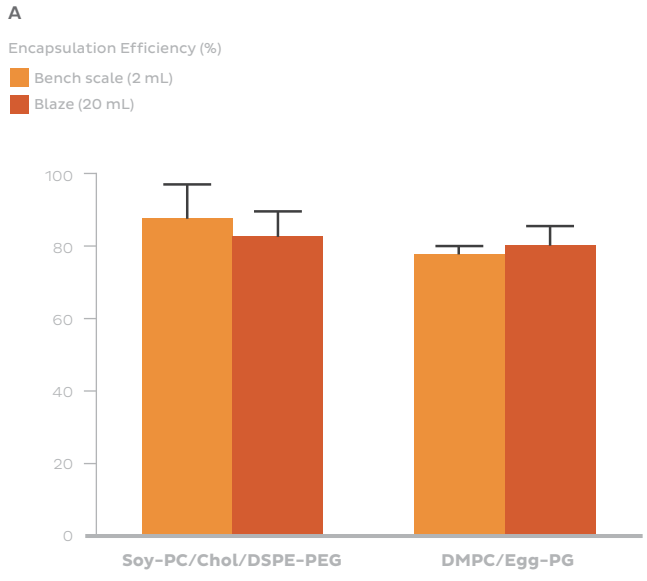
Figure 2. Soy PC liposome size decreases asymptotically with increasing FRR and TFR. Hydrodynamic size and particle size distribution (expressed as polydispersity index, PDI) (both determined by dynamic light scattering) of soy-PC liposomes formulated at various flow rate ratios (A) and various total flow rates (B) on the NanoAssemblr instrument. Samples were prepared in triplicate and values represent mean and error bars represent standard deviation of the mean.



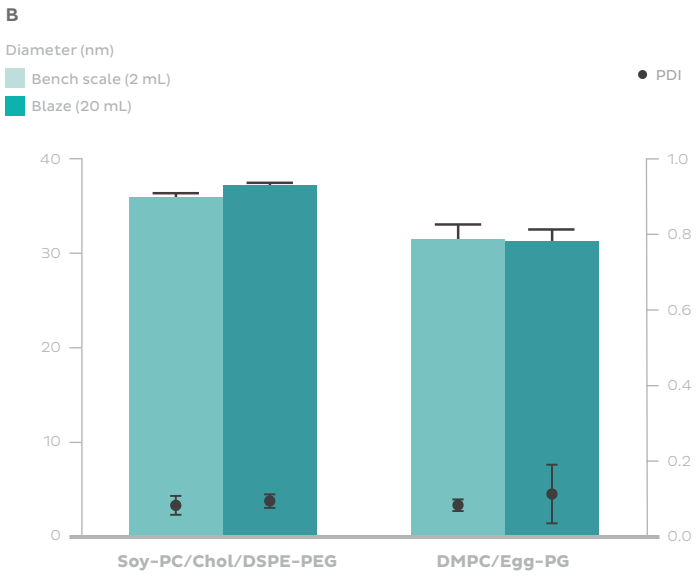


Lipid composition	Soy-PC:Chol:DSPE-PEG (52:45:3 mol%)
Total lipid concentration in organic phase	10 mg/mL
Organic solvent	Ethanol
Aqueous solvent	PBS pH 7.4
Total Flow Rate	A) 12 mL/min B) As indicated on horizontal axis
Flow Rate Ratio (aqueous:organic)	A) n:1 - n indicated on horizontal axis B) 2:1
Solvent Removal	Dialysis

Figure 3. *Liposomal verteporfin formulations made a bench scale and on Blaze are identical in terms of encapsulation efficiency (A) and particle size, Z-Ave (B) for two different lipid compositions. Encapsulation efficiency determined by fluorescence spectroscopy. Hydrodynamic size and particle size distribution (expressed as polydispersity index, PDI) (both determined by dynamic light scattering). Samples were prepared in triplicate and values represent mean and error bars represent standard deviation of the mean.*



Lipid composition	Soy-PC:Chol:DSPE-PEG (52:45:3 mol%) DMPC:Egg-PG (67:33 mol%)
Total lipid concentration in organic phase	10 mg/mL
Drug/lipid ratio	0.1 (wt/wt), 0.1 (mol/mol)
Organic solvent	Ethanol/DMF (97.5/2.5 v/v)
Aqueous solvent	PBS pH 7.4
Total Flow Rate	12 mL/min
Flow Rate Ratio (aqueous:organic)	2:1
Formulation Volume	Bench scale: 2 mL Blaze: 20 mL
Solvent Removal	Bench scale: Dialysis Blaze: Ultrafiltration



Traditionally, liposome preparation is a multistep process involving dissolution in a Class 2 solvent, evaporation of the solvent to form a film, hydration of the film to form large multilamellar vesicles, followed by extrusion, homogenization, or ultrasonication to reduce size. Each step requires optimization when changing scale. Ethanol injection is a simpler, more scalable process but the nature of bulk mixing leads to variations in precipitation conditions over time that results in inhomogeneous product and poor control over size. Microfluidic production offers a solution by employing the physics of laminar flow in confined volumes to achieve homogeneous and time-invariant mixing. This approach ensures each volume of fluid passing through the NanoAssemblr cartridge undergoes the same process. This leads not only to process reproducibility, but also a means of preserving identical conditions of liposome precipitation regardless of the volume being processed. Thus, the conditions of liposome formation at scales between 1 mL and 15 mL on a bench-scale NanoAssemblr system can be replicated at scales between 10 mL and 1 L on the NanoAssemblr Blaze. This eliminates the necessity to re-optimize the process when changing the scale of production thereby saving time and reducing risk.

Additionally, the NanoAssemblr platform offers a means of tuning particle size by adjusting process parameters such as TFR and FRR. Size control by process and formulation parameters have been studied in prior application notes and peer-reviewed publications. These parameters can be optimized at small scales appropriate for process development and small in vivo studies with minimal waste. These parameters translate directly to the Blaze for production up to 1 L. This workflow was demonstrated here, yielding identical results between systems, indicating that the NanoAssemblr process is robust and transferable. It follows that scaling up to 1 L using the Blaze is also straightforward. It also follows that scaling up to even larger volumes can be achieved in less time using multiple NanoAssemblr microfluidic mixers in parallel, such as with the NanoAssemblr GMP System designed for the cGMP environment. This process has been demonstrated with nucleic acid lipid nanoparticle formulations and found to preserve physical characteristics, chemical composition and in vivo efficacy across all systems.[9]

Conclusion

In summary, a proof of concept for scaling production of two liposomal formulations of the hydrophobic photosensitizer verteporfin was demonstrated using the NanoAssemblr platform. A soy-PC-based formulation and a formulation similar to the commercial Visudyne were used. For both, the process was optimized using the NanoAssemblr at 2 mL batch sizes, with volumes up to 15 mL possible. Optimized parameters such as reagent concentrations, TFR and FRR were transferred one-to-one to the Blaze to produce 20 mL batches, thereby reducing the need to redevelop or re-optimize the process when changing batch size. The NanoAssemblr Blaze can formulate volumes between 10 mL and 1 L, suitable for large-scale animal studies. Dynamic light scattering analysis determined the liposomes retained their 35 nm diameter and PDI < 0.2 between bench-scale and Blaze batches. Encapsulation efficiency of > 80 % was also retained. Thus, the NanoAssemblr platform substantially reduces the burden of scale up development, which will accelerate the advancement of liposomal or other nanomedicine formulations through the drug development process.

Materials & Methods

1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG), and egg-phosphatidylglycerol (egg-PG), were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Soy-phosphatidylcholine (soy-PC) was obtained from Lipoid (Germany), and cholesterol (chol) was obtained from MilliporeSigma (St. Louis, MO, USA).

Liposomes were manufactured at 2 mL and 20 mL formulation volumes, using the NanoAssemblr Benchtop* and the NanoAssemblr Blaze (Precision NanoSystems Inc. Vancouver, Canada), respectively. Lipid compositions were based on DMPC/Egg-PG (67:33 mol%) and SoyPC:Chol:DSPE-PEG (52:45:3 mol%). An in situ loading process was employed, wherein the drug verteporfin and lipids were dissolved in Ethanol:DMF (97.5:2.5 v/v) and mixed with calcium- and magnesium-free PBS using NanoAssemblr microfluidic technology at a buffer:solvent Flow Rate Ratio (FRR) of 2:1 and at a Total Flow Rate (TFR) of 12 mL/min. Formulations produced on the Benchtop were dialyzed against PBS to remove ethanol and unencapsulated drug. An aliquot of Blaze formulations were diluted 4X with PBS, then concentrated using Amicon®ultra-15 centrifugal filters.

Particle size (Z-Avg) and PDI were measured after dialysis or centrifugal filtration using Dynamic Light Scattering (Zetasizer, Malvern Instruments, UK). Unless otherwise indicated, formulations were prepared in triplicate. Size and polydispersity index (PDI) were represented as the mean of 3 samples, and error bars represent standard deviation of the mean.

Verteporfin content was quantified by fluorescence spectroscopy (Synergy H1 Hybrid Multi-Mode Reader, BioTek®, USA) . Phospholipid content was measured using the Phospholipids C Assay (Wako life Sciences Inc, Japan). Total lipid content was calculated based on the weight ratios of PC:Chol:PEG-DSPE that were present in the original lipid mix. EE was calculated (wt/wt) as a percentage of the initial drug to lipid ratio.

* The bench-scale formulations described in this work were produced using the discontinued NanoAssemblr Benchtop. The NanoAssemblr Ignite can be used in place of its predecessor, the NanoAssemblr Benchtop

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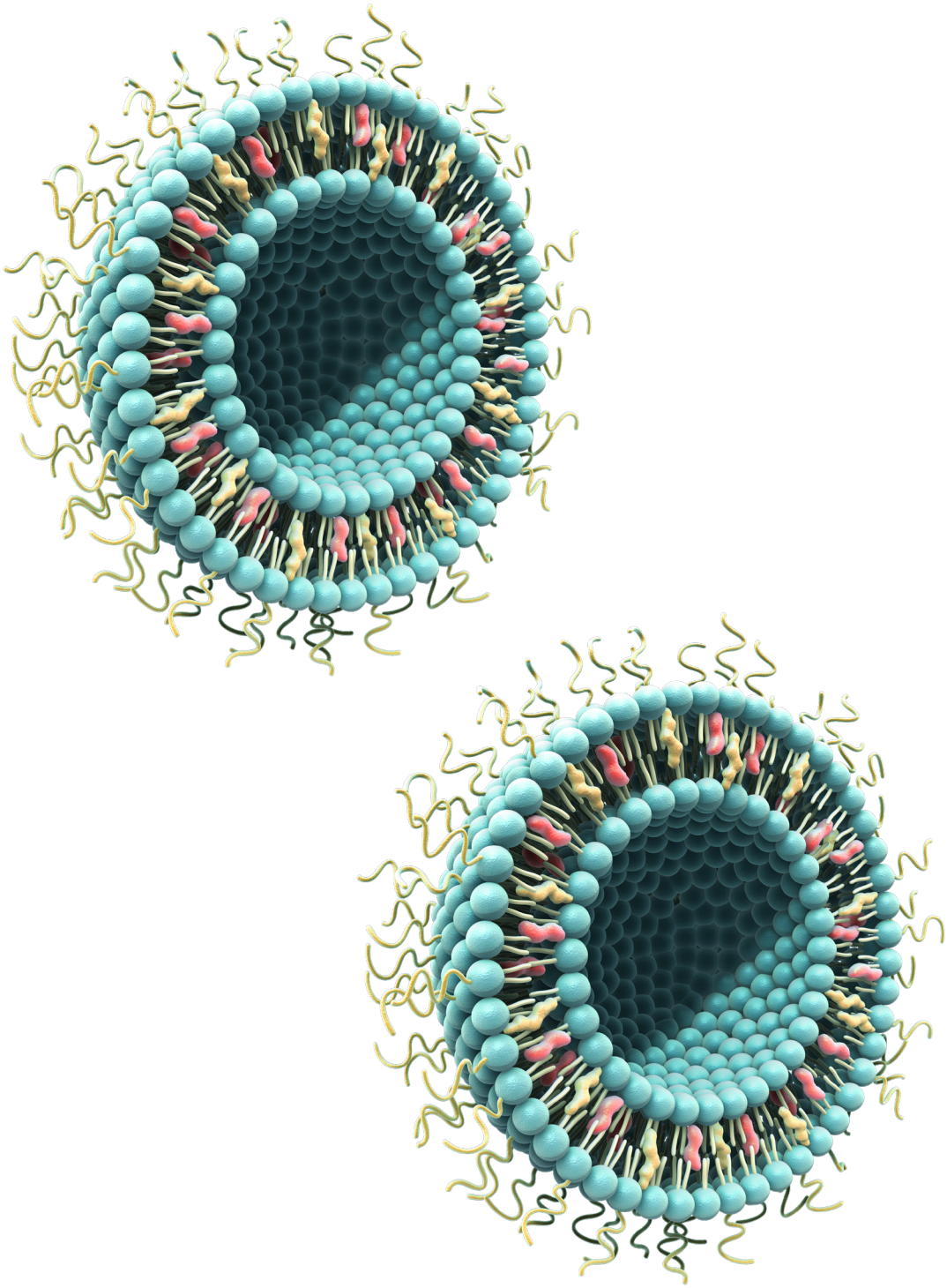
Liposomes

Using formulation parameters to tune size on the NanoAssemblr®



Andrew Brown, Anitha Thomas, Mina Ordobadi, Shell Ip, Gesine Heuck, Euan Ramsay.

Abstract

Both natural and synthetic lipids are utilized in FDA approved liposomal drug products. While synthetic lipids are often favoured for their purity, natural lipids are less costly. In this study, liposomes containing mostly natural lipids with cholesterol a PEGylated lipid were produced using the NanoAssemblr® microfluidic technology. Microfluidic technology was used to achieve exquisite control over liposome size and *in situ* loading of the hydrophobic drug verteporfin. An encapsulation efficiency of 90% and a limit size of 34 ± 1.5 nm was achieved with soy-PC formulations. Egg-PC formulations had >80% encapsulation efficiencies at a size of 39.3 ± 0.9 nm. Interestingly, when substituting a synthetic PC analog for egg-PC, formulations with the synthetic lipid tended to aggregate. A possible explanation considering the differences in molecular organization of saturated and unsaturated PC species is discussed.

Introduction

Phosphatidylcholines (PCs) used to form liposomes can be naturally derived or chemically synthesized. Natural phospholipids such as egg-PC and soy-PC contain a mixture of diverse PCs. Synthetic phospholipids are offered as higher purity reagents consisting of one PC species. However, due to the synthesis process, the cost is also significantly higher. Both natural and synthetic lipids are utilized in FDA approved liposomal drug products1. Microfluidic preparation of unilamellar liposomes comprised of synthetic lipids and loaded with hydrophobic and hydrophilic drug molecules has previously been reported using the NanoAssemblr platform.[2-4] Herein, we report microfluidic assembly of soy-PC and egg-PC liposomes using the NanoAssemblr. For comparison, liposomes were also formulated using a synthetic analog for egg-PC. The photosensitizer verteporfin was used as a model hydrophobic drug molecule, and liposomes were loaded *in situ* during liposome formation. Verteporfin is commercially available in a liposomal formulation (trade name Visudyne®) and approved by the FDA for photodynamic therapy to treat wet macular degeneration[1].

In situ loading of liposomes allowed for encapsulation efficiencies of over 90% while liposome size and size distribution were controlled by process parameters available on the NanoAssemblr. Unexpectedly, verteporfin liposomes prepared with natural phospholipids were significantly more stable than those prepared with synthetic PCs.

Experiment

Detailed experimental methods and source of materials are available in the materials and methods section. Briefly, the NanoAssemblr process produces unilamellar liposomes by controlled solvent displacement in a microfluidic mixer as illustrated in **Figure 1**. A water-miscible solvent containing dissolved lipids is mixed with an aqueous buffer under laminar flow conditions. Upon mixing, solvent displacement increases the polarity of the environment, causing the self-assembly of amphiphilic phospholipid molecules into liposomes. Laminar flow and computer-controlled injection enable reproducible fine-tuning of the conditions of liposome formation by specifying in the NanoAssemblr software, the Total Flow Rate (the sum of flow rates between the solvent and aqueous phases) and the Flow Rate Ratio (the volumetric ratio of buffer to solvent being mixed per unit time). The hydrophobic drug verteporfin was loaded *in situ* by dissolving it in the solvent phase along with the lipids prior to injection into the microfluidic channels. Hydrophilic drug molecules can also be loaded by including them in the aqueous phase. A range of Total Flow Rates and Flow Rate Ratios were explored to optimize the size of verteporfin-loaded liposomes.

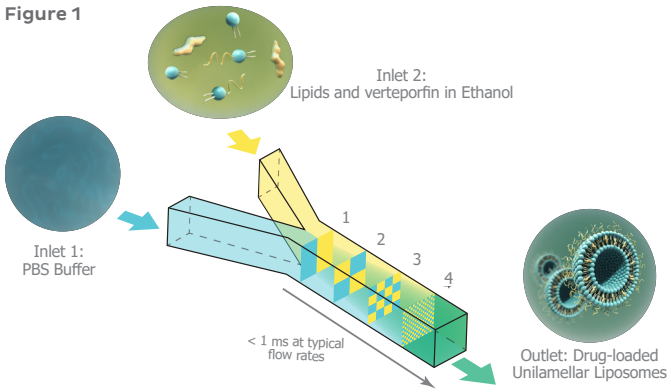


Figure 1. PBS buffer is injected into the left inlet while an organic solvent with dissolved lipids is injected into the right inlet of the NanoAssemblr microfluidic cartridge. *Following controlled mixing in microfluidic channels, unilamellar liposomes are spontaneously formed. Computer-controlled injection allows specification of the total flow rate and the flow rate ratio of aqueous to organic solvents to control liposome size.*

1. Two fluids (buffer and ethanol) entering the channel under laminar flow do not mix.
2. Microscopic features in the channels are engineered to cause the fluid streams to mingle in a controlled, reproducible, and non-turbulent way.
3. Intermingling of the fluids increases as they continue through the mixer.
4. Fluids emerge from the mixer completely mixed. The process takes < 1 ms; faster than molecules can assemble into particles.

Separate liposome formulations containing egg-PC or soy-PC mixed with cholesterol and synthetic PEG-lipid at molar ratios of 52:45:3 were compared for size, size homogeneity (polydispersity index or PDI) and efficiency of verteporfin encapsulation. To compare natural PCs to synthetic PCs, egg-PC in these formulations was replaced with equimolar quantities of the synthetic phospholipid POPC. POPC represents the main component of Egg-PC and contains one saturated 16-carbon acyl chain and one mono-unsaturated 18-carbon acyl chain. To further investigate the role of acyl chain saturation on drug loading, formulations with DSPC, which has two saturated 18-carbon acyl chains, were also made. Structural formulae for DLPC (the main component of Soy PC), POPC and DSPC are illustrated in **Figure 2**.

The behavior of empty soy-PC liposomes with respect to the NanoAssemblr formulation parameters TFR and FRR was determined by holding one parameter constant while exploring a range of values for the other parameter. At a constant TFR of 12 ml/min, FRRs of 1:1 through 4:1 were tested. As seen in **Figure 3A**, liposome size was found to

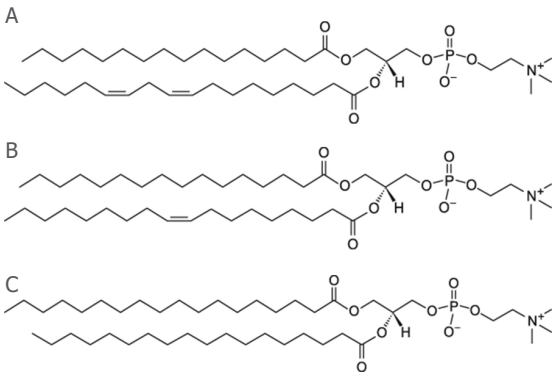


Figure 2. *Structural formulae of A) palmitoyl-linoleoyl-phosphatidylcholine (PLPC), the predominant PC in soy-PC, B) palmitoyl-oleoyl-phosphatidylcholine (POPC), the predominant PC in egg-PC, and C) distearoyl-phosphatidylcholine (DSPC).*

decrease asymptotically with increasing FRR. Likewise, at a constant FRR of 2:1, the size of soy-PC liposomes was found to decrease asymptotically with increasing TFR as seen in **Figure 3B**. This behavior is consistent with previously published trends for synthetic liposome formulations,[2] and the asymptote in size has been found to correspond to the so-called "limit size" representing the smallest possible particle size based on ideal packing of constituent molecules.[2] The limit size of 25 nm was achieved at a TFR of 12 mL/min and FRRs greater than 3:1.

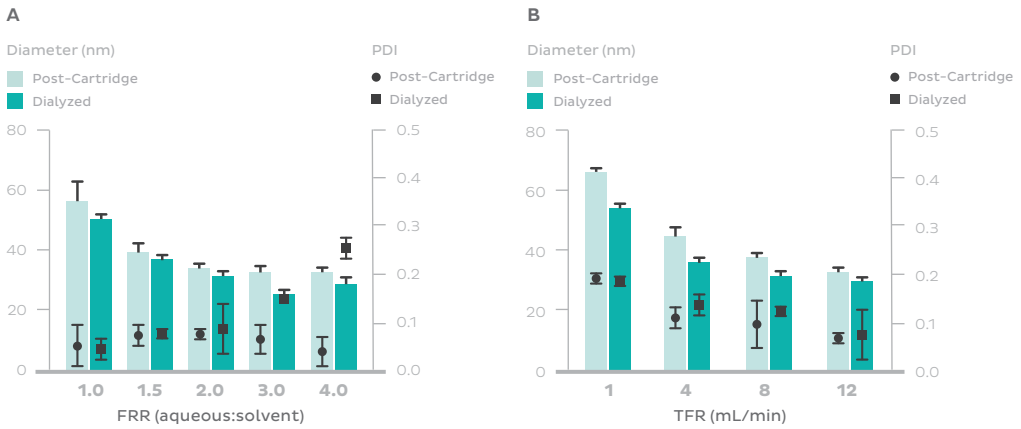


Figure 3. *Soy PC liposome size decreases asymptotically with increasing FRR and TFR. Hydrodynamic size and particle size distribution (expressed as polydispersity index, PDI) (both determined by dynamic light scattering) of soy-PC liposomes formulated at various flow rate ratios (A) and various total flow rates (B) on the NanoAssemblr. Samples were prepared in triplicate and values represent mean and error bars represent standard deviation of the mean.*

Lipid composition	Soy-PC:Chol:DSPE-PEG (52:45:3 mol%)
Total lipid concentration	10 mg/mL
Organic solvent	Ethanol
Aqueous solvent	PBS pH 7.4
Total Flow Rate	**A)** 12 mL/min **B)** As indicated on horizontal axis
Flow Rate Ratio (aqueous:organic)	**A)** n:1 - n indicated on horizontal axis **B)** 2:1
Solvent Removal	Dialysis

Compared to soy-PC, egg-PC formulations followed the same general trends, as shown in **Figure 4**. Egg-PC liposomes, however, were found to be larger than soy-PC liposomes at the same TFR and FRR. The limit size of egg-PC formulations was approximately 40 nm.

In situ loading of soy-PC and egg-PC liposomes was achieved by dissolving verteporfin and the lipid mixture at a 0.09 (mol/mol) ratio in the organic phase. 2.5% (v/v) DMF was added to the organic phase as a co-solvent, to ensure complete dissolution of verteporfin. The sizes of soy- and egg-PC liposomes were not affected by drug loading (**Figure 5**). In contrast, synthetic lipid formulations containing either POPC or DSPC were observed to aggregate in the presence of verteporfin, even though these formulations were stable without the drug. This aggregation is manifested in the larger observed size for the loaded POPC and DSPC liposomes when compared to the empty liposome case.

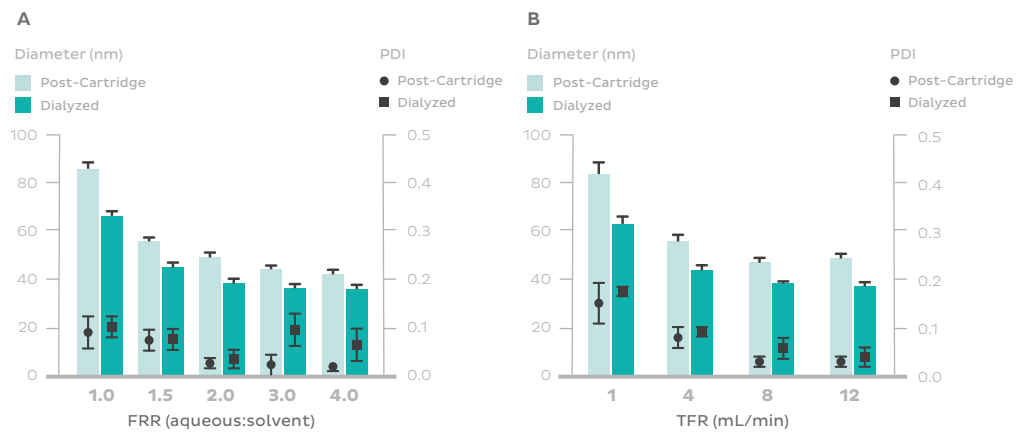


Figure 4. *Egg-PC liposome size decreases asymptotically with increasing FRR and TFR. Hydrodynamic size and particle size distribution (expressed as polydispersity index, PDI) (both determined by dynamic light scattering) of egg-PC liposomes when formulated at various flow rate ratios (A) and various total flow rates (B) on the NanoAssemblr. Samples were prepared in triplicate and values represent mean and error bars represent standard deviation of the mean.*

Lipid composition	Egg-PC:Chol:DSPE-PEG (52:45:3 mol%)
Total lipid concentration	10 mg/mL
Organic solvent	Ethanol
Aqueous solvent	PBS pH 7.4
Total Flow Rate	A) 12 mL/min B) As indicated on horizontal axis
Flow Rate Ratio (aqueous:organic)	A) n:1 - n indicated on horizontal axis B) 2:1
Solvent Removal	Dialysis

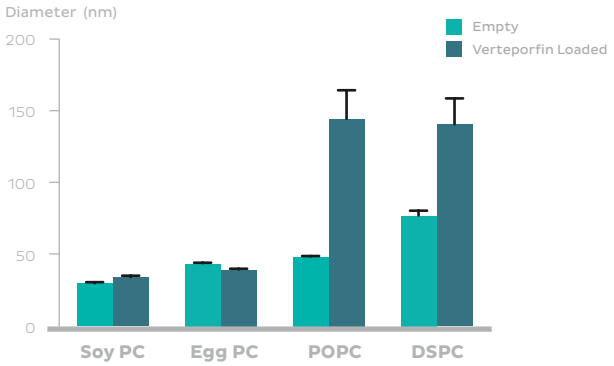


Figure 5. *Verteporfin loading did not affect size of natural-PC liposomes. hydrodynamic size (determined by dynamic light scattering) of empty and verteporfin loaded liposomes prepared with either natural (Egg PC, Soy PC) or synthetic (POPC, DSPC) phospholipids. Synthetic lipids were found to aggregate in the presence of verteporfin. Samples were prepared in triplicate and values represent mean and error bars represent standard deviation of the mean.*

Lipid composition	PC*:Chol:DSPE-PEG (52:45:3 mol%) *PC=EGG PC, Soy PC, POPC, DSPC
Total lipid concentration	10 mg/mL
Verteporfin concentration in lipid mix	1 mg/mL
Drug/Lipid Ratio	0.1 (w/w), 0.09 (mol/mol)
Organic solvent	Ethanol/DMV (97.5:2.5 v/v)
Aqueous solvent	PBS pH 7.4
Total Flow Rate	12 mL/min
Flow Rate Ratio (aqueous:organic)	2:1
Solvent Removal	Dialysis

Verteporfin encapsulation was assessed by fluorescence spectroscopy, and encapsulation efficiencies of over 90% and over 80% were observed for soy-PC and egg-PC formulations, respectively. Significantly lower encapsulation efficiency was observed in DSPC based liposomes, possibly due to the occurrence of large aggregates in presence of verteporfin (**Figure 6**). Furthermore, the aggregation was found to result in approximately a 70% loss of material.

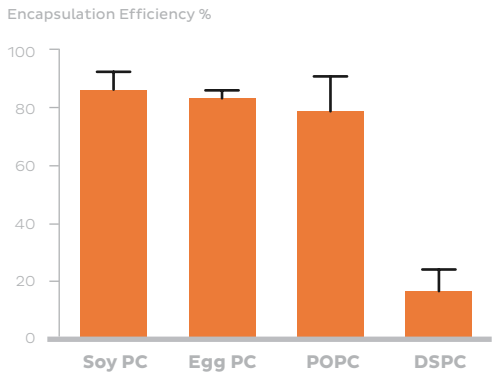


Figure 6. *90% encapsulation efficiency of verteporfin in soy-PC liposomes. Encapsulation efficiency of verteporfin-loaded liposomes containing natural (egg-PC, soy-PC) or synthetic phospholipids (POPC, DSPC) as the primary lipid component. Aggregation with ~70% loss of material was observed in POPC and DSPC formulations. Samples were prepared in triplicate and values represent mean and error bars represent standard deviation of the mean.*

Lipid composition	PC*:Chol:DSPE-PEG (52:45:3 mol%) *PC=EGG PC, Soy PC, POPC, DSPC
Total lipid concentration	10 mg/mL
Verteporfin concentration in lipid mix	1 mg/mL
Drug/Lipid Ratio	0.1 (w/w), 0.09 (mol/mol)
Organic solvent	Ethanol/DMV (97.5:2.5 v/v)
Aqueous solvent	PBS pH 7.4
Total Flow Rate	12 mL/min
Flow Rate Ratio (aqueous:organic)	2:1
Solvent Removal	Dialysis

Discussion

Soy-PC liposomes were found to have a smaller limit-size than egg-PC liposomes, all other parameters being equal. This is consistent with previous reports that confirm that more saturated lipid species tend to form larger liposomes. This can be understood by considering the flexibility (inversely proportional to the bending modulus) of the lipid bilayer. Smaller liposomes require the bilayer to bend more than larger liposomes, and hence more flexible bilayers will form smaller liposomes, and stiffer bilayers will form larger liposomes. Because unsaturated lipids do not pack as densely and have less intermolecular interactions than saturated lipids, greater degrees of unsaturation lead to more flexible lipid bilayers.

Interestingly, liposomes containing the less saturated soy-PC had greater encapsulation efficiency than the more saturated egg-PC. Given that verteporfin is a hydrophobic drug, it is understood that verteporfin would partition into the lipophilic portion of the bilayer. Hence, one might posit that the less densely packed soy-PC bilayer would have more intermolecular spaces in which the verteporfin molecules could reside.

In comparing natural lipid formulations to synthetic lipid formulations, egg-PC and POPC are directly comparable since POPC is the most abundant PC in egg-PC. It is therefore surprising that these two formulations behaved so differently from each other. While egg-PC formulations remained stable with the addition of verteporfin, POPC formulations, in contrast, aggregated. The reason for this is unclear from the present data. One hypothesis is that at least one lipid component of egg-PC (and likely soy-PC) is significantly unsaturated, and a small quantity of this lipid is sufficient to create much of the intermolecular spaces necessary to accommodate the verteporfin in the lipid bilayer. The observation, that formulations containing DSPC, a fully saturated phospholipid, achieved the lowest encapsulation efficiency, lends to the interpretation that greater intermolecular

organization in the bilayer leads to lower solubility of the drug molecule in the lipid portion of the bilayer. Further tests of this hypothesis with a better understanding of the distribution of different PCs in natural lipids and with a variety of different drug molecules are warranted. Notwithstanding, these observations are powerful in directing the choice of lipids in liposomal formulations of hydrophobic small molecule drugs.

Conclusion

Liposomes containing natural lipids, cholesterol, and a DSPE-PEG_{2000} were formulated and in situ-loaded with verteporfin using the NanoAssemblr. Liposome size was optimized using instrument process parameters to achieve 34 ± 1.5 nm and 39.3 ± 0.9 nm diameter verteporfin-loaded liposomes with soy-PC and egg-PC respectively. Verteporfin encapsulation efficiencies of > 90% for soy-PC and > 80% with egg-PC were obtained. Substituting egg-PC with a synthetic PC representing its most abundant PC, namely POPC, resulted in aggregation and loss of 70% of the material. However in the absence of verteporfin, this POPC formulation assembles into stable 49 nm liposomes. Verteporfin-loaded liposomes featuring the more saturated DSPC resulted in much lower encapsulation efficiency (~15%) and similar aggregation and loss of material as POPC formulations. This suggests that the mixture of different PC species in natural lipids is favourable for the stability and loading of verteporfin. Furthermore, a pattern of lower encapsulation efficiency with higher degrees of lipid-tail saturation was observed. This suggests verteporfin may intercalate more effectively between loosely packed unsaturated lipid tails than into more ordered arrangements of saturated lipid tails, but further research is needed to understand these observations. These findings demonstrate that naturally derived phospholipid formulations can be produced with the NanoAssemblr and that natural phospholipids can be advantageous for encapsulating hydrophobic small molecules.

Materials & Methods

Liposomes were composed of one of either soy-PC (Lipoid, Germany), egg-PC (Lipoid, Germany), POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) or DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine), cholesterol and DSPE-PEG (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000]). Phospholipids, cholesterol and PEGylated lipid a 52:45:3 molar ratio were dissolved in absolute ethanol. Calcium- (Ca^{2+}) and magnesium- (Mg^{2+}) free PBS buffer at neutral pH was used as the aqueous phase. The organic and aqueous phases were rapidly mixed using the NanoAssemblr Benchtop* microfluidic instrument at various Flow Rate Ratios (1:1–4:1) and Total Flow Rates (1 mL/min – 12 mL/min) to form unilamellar liposomes of various size. Verteporfin was loaded by dissolving it in the lipid solution at a concentration of 1 mg/mL (0.09 molar ratio to total lipids). Here, 2.5% by volume DMF was added to ethanol as a co-solvent. Formulations with high DSPC content were heated at 65°C during the mixing process using the NanoAssemblr Heating Controller accessory. The Heating Controller accessory for the NanoAssemblr Benchtop can adjust the formulation process temperature with an accuracy of ±1°C. Formulations were then dialyzed against PBS for ethanol removal. At Flow Rate Ratios 1 and 1.5, formulations were diluted down to 10% ethanol immediately following the mixing process and before dialysis, since high amounts of ethanol can destabilize liposomes. Particle size and integrity were measured before and after dialysis using Dynamic Light Scattering (Zetasizer, Malvern Instruments, UK). Unless otherwise indicated, formulations were prepared in triplicate. Size and polydispersity index (PDI) were represented as the mean of 3 samples, and error bars represent standard deviation of the mean. Verteporfin content was quantified by fluorescence spectroscopy.

* The formulations described in this work were produced using the discontinued NanoAssemblr Benchtop. The NanoAssemblr Ignite can be used in place of its predecessor, the NanoAssemblr Benchtop

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The NanoAssemblr® Ignite™
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Precision NanoSystems is proud to introduce the next stage of evolution in nanomedicine formulation. The Ignite

embodies everything that made the NanoAssemblr Benchtop the workhorse of nanomedicine development for over 100 biopharma companies, while bringing the latest innovations developed for the cGMP system to the bench.

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Develop a robust, scalable, manufacturing process from the earliest stage enabling you to create transformative medicine at the bench

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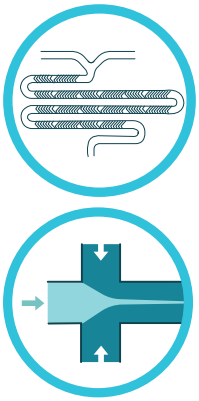
Developed for drug researchers who are targeting delivery of the drug to the disease cells/ tissues/organs enabling the creation of transformative medicines yet to be imagined

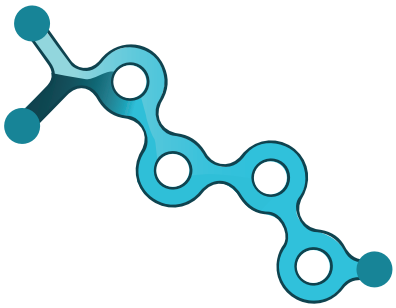
Designed to Maximize Time

Innovative, intuitive technology improves efficiency of nanomedicine development to overcome key challenges and accelerate data generation

NxGen™ Technology

High Energy Techniques	In-Line Macromixing	Microfluidic Approaches	NxGen Microfluidics
		- Some scaling challenges remain	+ Easy to Scale
		- Mixers are difficult to make	+ Mixers are Easy to make
- Limited applications	- Limited applications	+ Expanded Applications	+ Potential multi-mixer integration opens possibilities
- Difficult to reproduce	- Difficult to reproduce	+ Reproducible	+ Reproducible
- Harsh process conditions	+ Gentler process conditions	+ Non-turbulent process conditions	+ Non-turbulent process conditions
- Difficult to scale	+ Improved Scalability		





Freedom to Scale

The only technology that can scale a single mixer from early in-vitro screening to commercial, GMP manufacturing

Process Matters

NxGen technology provides precise, time-invariant control to ensure the best results on each run.

Empowerment

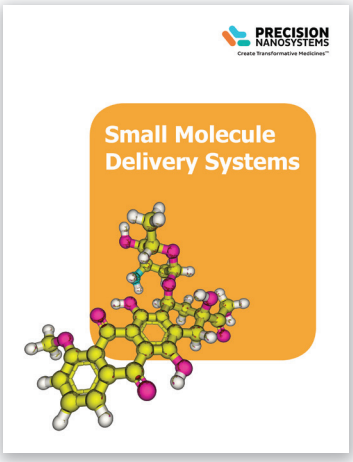
Drug developers of all backgrounds have options to bring solutions into their lab or work with out team based on their needs

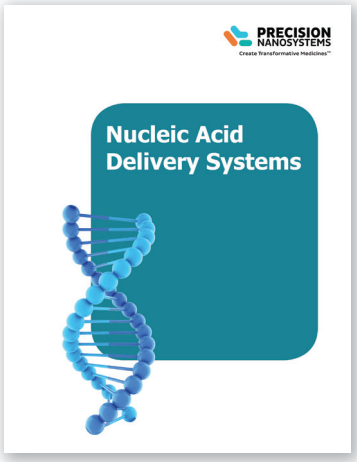
Innovation

NxGen is a unique and innovative technology offering drug developers the solutions they need to bring transformative medicines to patients

Other Resources

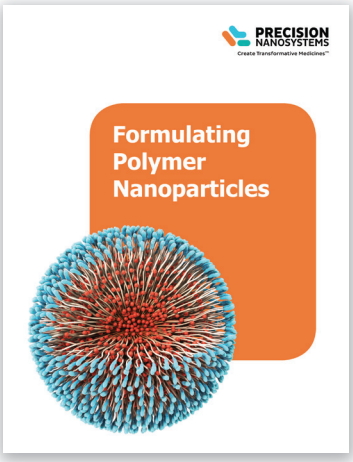
Read other application notes in this series

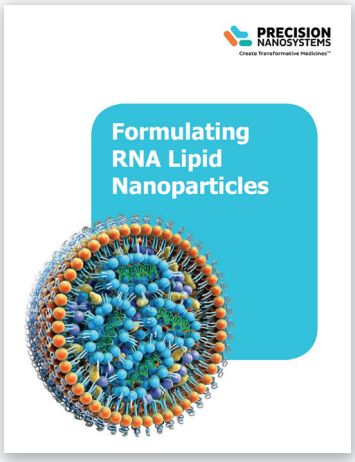




Learn about:

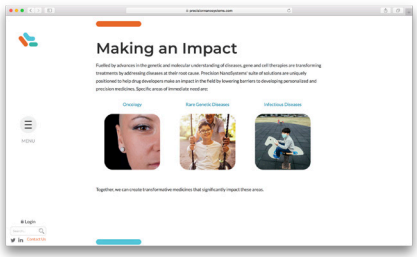
- The delivery of specific active ingredients with different formulation
- Or the versatile applications of different nanoparticle materials
- Exciting papers featuring NanoAssemblr technology published in peer reviewed journals
- Detailed application notes about formulating nanoparticles with the NanoAssemblr platform





Visit us online to:

Learn about our 3 disease focus areas



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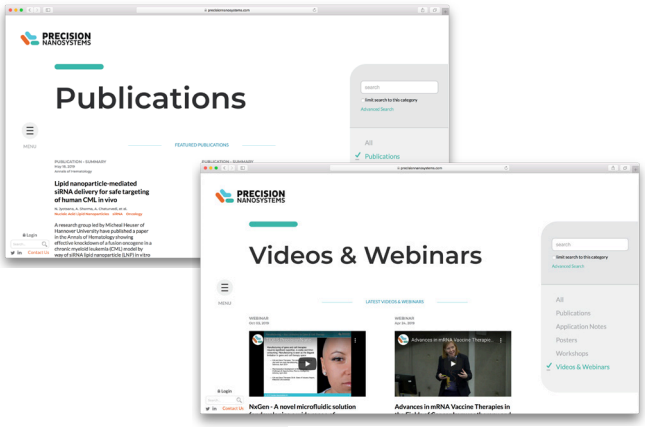

Trusted Globally

The most innovative biopharmaceutical companies and academic research institutes use the NanoAssemblr Platform to accelerate their nanomedicine programs. NanoAssemblr instruments are used in over 20 countries worldwide and supported by a global team of field application scientists.

We have the expertise, flexibility and resources to provide a full range of custom formulation solutions.

Precision NanoSystems scientists apply innovative approaches to advance your drug development needs in an uncomplicated, straightforward process.

Our Locations

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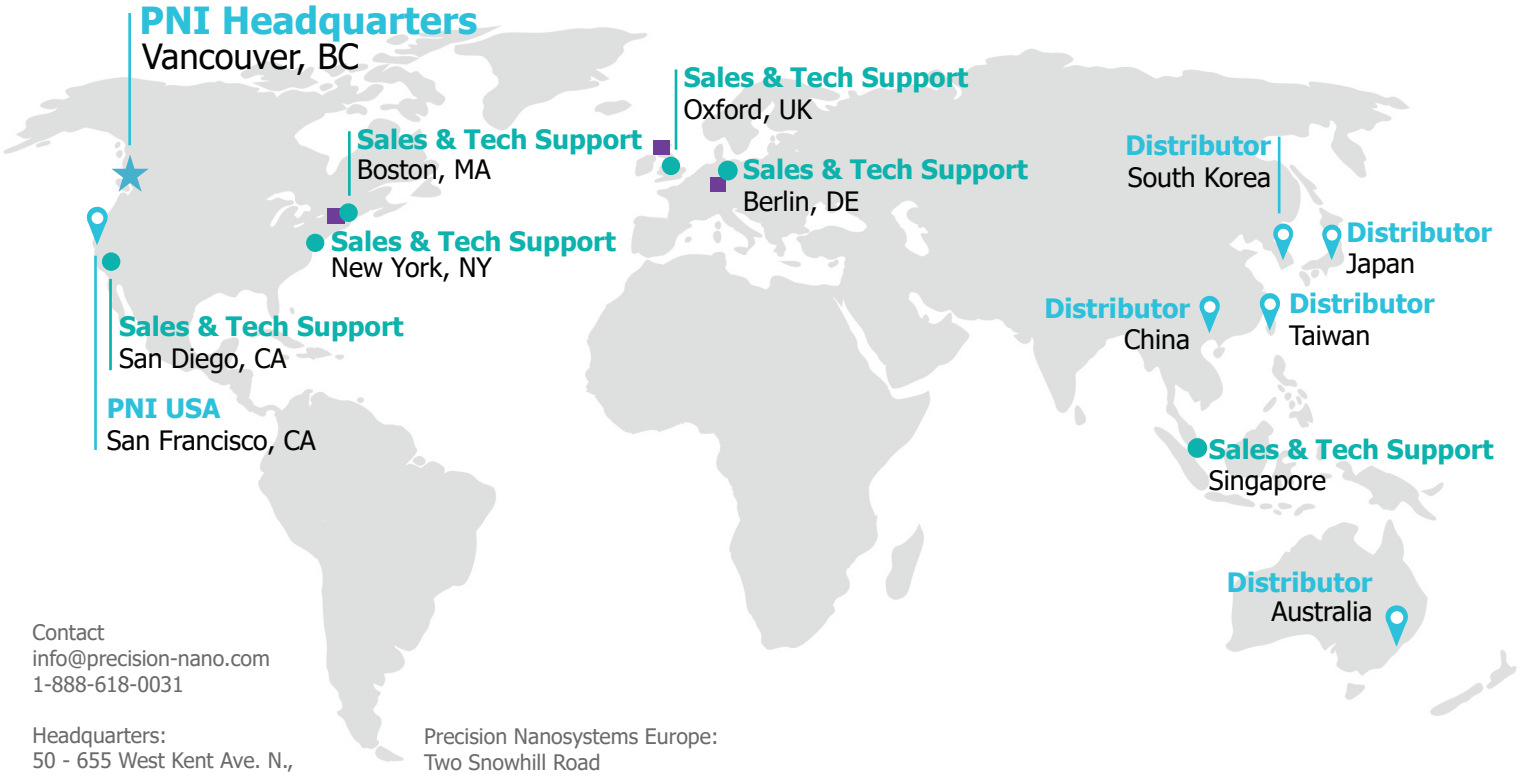
Darmstadt, DE
Boston, MA

CMO Partners Network
Evonik
Ajinomoto bio-Pharma



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