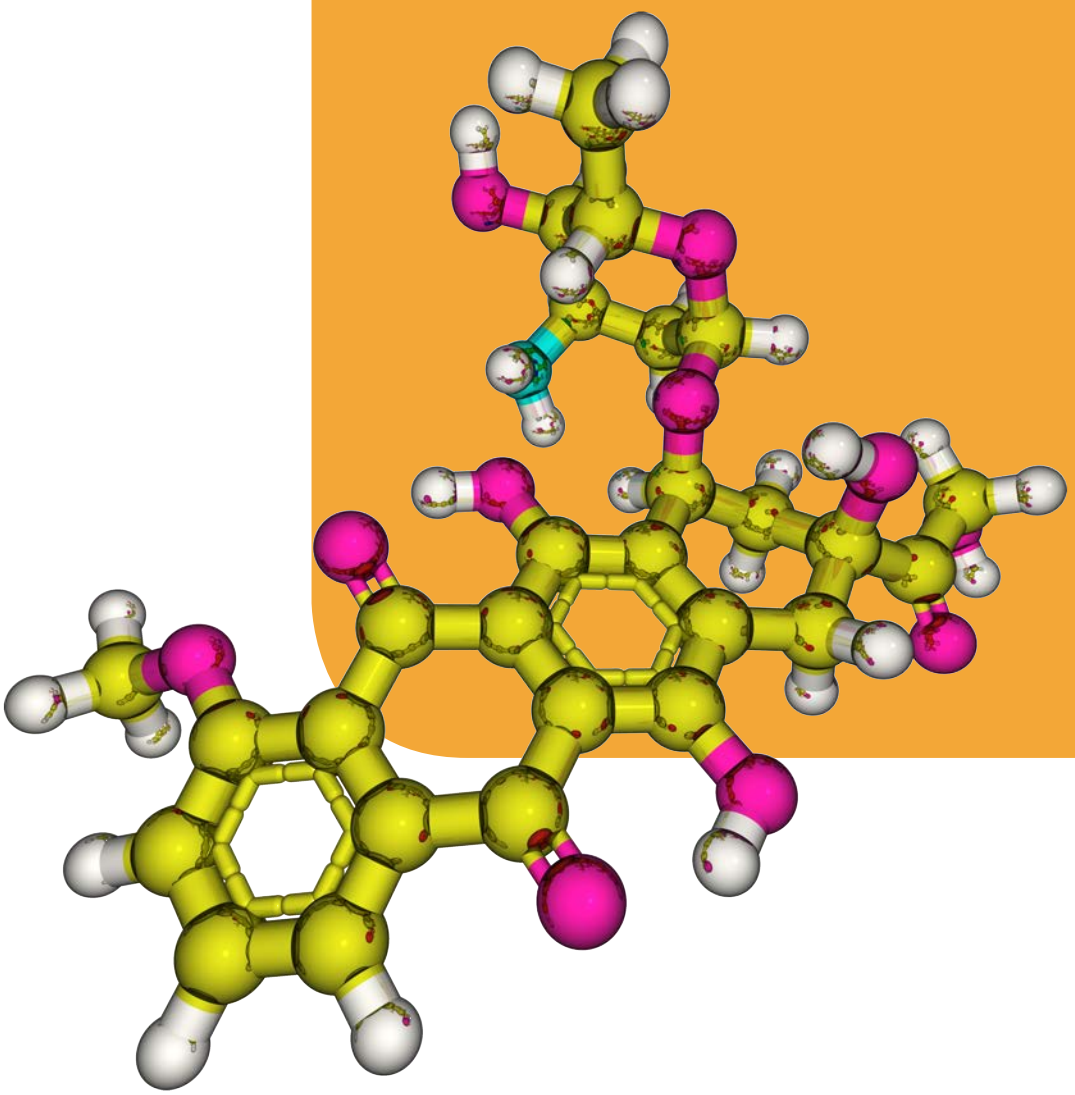
PRECISION NANOSYSTEMS

Create Transformative Medicines™

Small Molecule Delivery Systems



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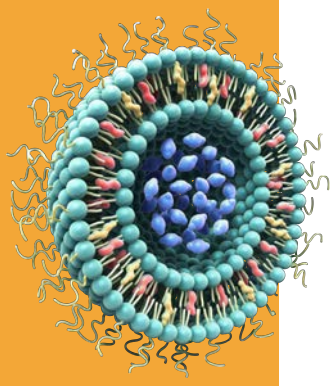
Introduction

Small molecules make up the majority of current pharmaceuticals and are used to treat and manage symptoms of cancer, infections, and depression, among others.

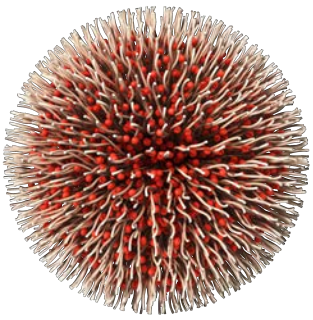
Applications of nanomedicine for small molecule drugs include:

- Drug targeting
- Controlled release
- Improving solubility
- Combination therapy

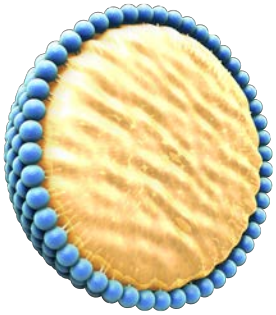
Small molecule drugs can be formulated into numerous nanoparticle materials. On the market, there are several liposomal formulations of small molecules including Doxil and Visudyne.



Liposomes



Polymer Nanoparticles and Micelles



Nano-emulsions

There are however several challenges to producing small molecule-loaded nanoparticles that are addressed by NanoAssemblr technology.

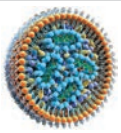
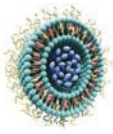
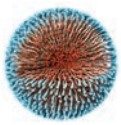
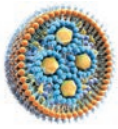
Challenges with Conventional Method	Benefits of NanoAssemblr® Technology
Inconsistent production conditions	Time-invariant formulation conditions
Production is complex and time-consuming	Rapid formulation development
Difficult to scale-up production	Inherently scalable
Different formulations require different process and equipment	Produce a variety of formulations

This collection highlights applications of NanoAssemblr technology for producing nanoparticles for small molecule delivery. Several examples from peer-reviewed literature as well as some application notes with original results are presented below. These illustrate the multitude of applications of NanoAssemblr technology for developing and optimizing drug delivery systems.

Precision NanoSystems' technology is trusted by world-leading biopharma and academic researchers to drive development of diverse nanomedicines

Vaccines | Cell Therapy & Regenerative Medicine | Immuno-Oncology | Targeted Therapeutics | Small Molecule Delivery | RNA & DNA Therapeutics | CRISPR & Gene Editing

Versatile Applications

PARTICLE TYPE		ACTIVE INGREDIENT				EXAMPLE APPLICATION	CARRIER MATERIALS
Nucleic acid Lipid Nanoparticles (LNP)		Nucleic Acids	Peptides and Proteins			• 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						• 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						• Cancer chemotherapy • Drug formulation • Controlled release/ biodistribution	• Triolein/POPC • Oil/Surfactant
Organic/ Inorganic NPs						• Theranostics • Imaging	• Lipids • Noble metal NPs • Rare Earth Metals • III-V semiconductors

Featured Small Molecule-loaded Nanoparticle Publications


Nanotheranostics 2018, Vol. 2 387

IVYSPRING INTERNATIONAL PUBLISHER

Nanotheranostics

2018; 2(4): 387-402. doi: 10.7150/ntno.26969

Research Paper

A Scalable Method for Squalenoylation and Assembly of Multifunctional ^{64}Cu-Labeled Squalenoylated Gemcitabine Nanoparticles

Samantha T. Tucci[1], Jai W. Seo[2], Hamilton Kakwere[1], Azadeh Kheirolomoom[2], Elizabeth S. Ingham[2], Lisa M. Mahakian[2], Sarah Tam[2], Spencer Tumbale[2], Mo Bailoghbi[2], Holland Cheng[3], Katherine W. Ferrara[2]✉

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Received: 2018.04.30; Accepted: 2018.09.02; Published: 2018.09.05

Abstract

Squalenoylation of gemcitabine, a front-line therapy for pancreatic cancer, allows for improved cellular-level and system-wide drug delivery. The established methods to conjugate squalene to gemcitabine and co-form nanoparticles (NPs) with the squalenoylated gemcitabine (SqGem) conjugate are cumbersome, time-consuming and can be difficult to reliably replicate. Further, the creation of multi-functional SqGem-based NP theranostics would facilitate characterization of *in vivo* pharmacokinetics and efficacy.

Methods: Squalenoylation conjugation chemistry was enhanced to improve reliability and scalability using *tert*-butyldimethylsilyl (TBDMS) protecting groups. We then optimized a scalable microfluidic mixing platform to produce SqGem-based NPs and evaluated the stability and morphology of select NP formulations using dynamic light scattering (DLS) and transmission electron microscopy (TEM). Cytotoxicity was evaluated in both PANC-1 and KPC ($K_{RAS^{G12D/+}}$; $Trp53^{R172H/+}$; $Pdx\text{-}Cre$) pancreatic cancer cell lines. A ^{64}Cu chelator (2-S-(4-aminobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid, NOTA) was squalenoylated and used with positron emission tomography (PET) imaging to monitor the *in vivo* fate of SqGem-based NPs.

Results: Squalenoylation yields of gemcitabine increased from 15% to 63%. Cholesterol-PEG-2k inclusion was required to form SqGem-based NPs using our technique, and additional cholesterol inclusion increased particle stability at room temperature: after 1 week the PDI of SqGem NPs with cholesterol was ~ 0.2 while the PDI of SqGem NPs lacking cholesterol was ~ 0.5. Similar or superior cytotoxicity was achieved for SqGem-based NPs compared to gemcitabine or Abraxane® when evaluated at a concentration of 10 μM. Squalenoylation of NOTA enabled *in vivo* monitoring of SqGem-based NP pharmacokinetics and biodistribution.

Conclusion: We present a scalable technique for fabricating efficacious squalenoylated-gemcitabine nanoparticles and confirm their pharmacokinetic profile using a novel multifunctional ^{64}Cu-SqNOTA-SqGem NP.

Key words: Nanoparticles, gemcitabine, squalene, radiolabeling, ^{64}Cu, pancreatic cancer

Introduction

Squalenoylation is a promising, novel technique that chemically modifies drug molecules, altering their systemic and cellular transport properties [1-6]. Squalenoylation requires a multistep organic synthesis to prepare squalene, a ubiquitous hydrocarbon and cholesterol precursor, for conjugation to a chemotherapeutic, siRNA, antibiotic, or other therapeutic moiety [1, 2, 7-13].

http://www.ntno.org

Nanotheranostics, 2018

A Scalable Method for Squalenoylation and Assembly of Multifunctional 64Cu-Labeled Squalenoylated Gemcitabine Nanoparticles

Tucci S, Seo J, Kakwere H, Kheirolomoom A, Ingham E, Mahakian L et al. Nanotheranostics 2018; 2: 387-402.

Summary

- Gemcitabine is a front-line chemotherapeutic for pancreatic cancer, which is the fourth leading cause of cancer deaths with a 5-year survival rate of just 9%
- Like most chemotherapy drugs, gemcitabine is toxic, formulating it into nanoparticles to alter its biodistribution and pharmacokinetics is being explored to reduce off-target toxicity and improve clinical outcomes
- This can be achieved by conjugating the drug to squalene to create a nanoparticle pre-cursor, which can then be formed into nanoparticles by precipitating it from organic solvent, however, both the conjugation and nanoprecipitation processes are challenging to reproduce consistently and to scale up for clinical testing
- Researchers led by Dr. Katherine Ferrara of Stanford University and the University of California at Davis report an improved squalene conjugation method to address the challenges of drug conjugation, and they have chosen NanoAssemblr technology to address the remaining challenges with nanoprecipitation
- Their squalenoylation method involves protecting the drug prior to conjugation, improving yield from 15% to 63%, and NanoAssemblr technology allowed efficient nanoparticle formulation optimization leading to the discovery that the including cholesterol-PEG2k and cholesterol improved particle stability at room temperature
- Being a group focused on image-guided drug delivery, the Ferrara group also created a formulation whose biodistribution an pharmacokinetics can be tracked by PET scan; they conjugated squalene to a chelator of 64Cu and included those in the formulation
- The authors reported squalene conjugated gemcitabine NPs had similar or improved cytotoxicity compared to free drug and the clinically available nanoparticle drug Abraxane
- These findings demonstrate improved practicality of synthesizing squalene-drug conjugates as nanoparticle precursors, and how NanoAssemblr Technology has allowed efficient optimization of lead formulations that can be readily scaled to bring their improved squalenoylation technology closer to the clinic

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, 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

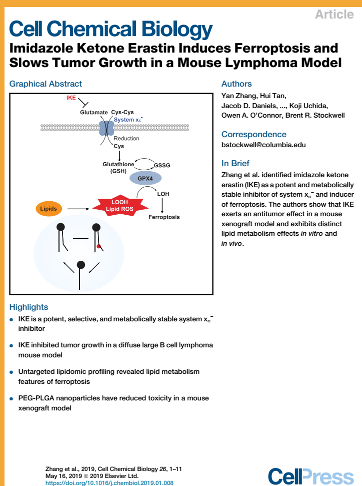
Journal of Liposome Research, 2015

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


Article

Cell Chemical Biology

Imidazole Ketone Erastin Induces Ferroptosis and Slows Tumor Growth in a Mouse Lymphoma Model

Graphical Abstract

Authors

Yan Zhang, Hui Tan, Jacob D. Daniels, ..., Koji Uchida, Owen A. O'Connor, Brent R. Stockwell

Correspondence

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In Brief

Zhang et al. identified imidazole ketone erastin (IKE) as a potent and metabolically stable inhibitor of system x_c^- and inducer of ferroptosis. The authors show that IKE exerts an antitumor effect in a mouse xenograft model and exhibits distinct lipid metabolism effects *in vitro* and *in vivo*.

Highlights

- IKE is a potent, selective, and metabolically stable system x_c^- inhibitor
- IKE inhibited tumor growth in a diffuse large B cell lymphoma mouse model
- Untargeted lipidomic profiling revealed lipid metabolism features of ferroptosis
- PEG-PLGA nanoparticles have reduced toxicity in a mouse xenograft model

Zhang et al., 2019, Cell Chemical Biology 26, 1–11
May 16, 2019 © 2019 Elsevier Ltd.
https://doi.org/10.1016/j.chembiol.2019.01.008

CellPress

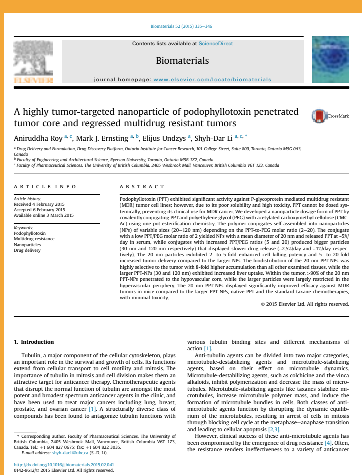
Cell Chemical Biology, 2019

Imidazole Ketone Erastin Induces Ferroptosis and Slows Tumor Growth in a Mouse Lymphoma Model

Zhang Y, Tan H, Daniels J, Zandkarimi F, Liu H, Brown L et al. Cell Chemical Biology 2019; 26: 623-633.e9.

Summary

- Ferroptosis is a form of programmed cell death being explored for treating diffuse large B-cell lymphoma (DLBCL) but most ferroptosis inducers are unusable in vivo due to lack of potency, specificity, and/or metabolic stability
- Researchers at Columbia University led by Dr. Brian Stockwell - a recipient of a Howard Hughes Medical Institute Early Career Scientist Award, identified imidazole ketone erastin (IKE) as a viable, potent drug candidate with high metabolic stability but solubility and possible toxicity may limit its application
- They formulated IKE into PEG-PLGA nanoparticles using NanoAssemblr technology to improve solubility and reduce off-target toxicity
- Repeat dose studies of IKE-loaded nanoparticles confirmed induction of ferroptosis in tumour tissue along with reduced toxicity in mice compared to free drug
- These findings demonstrate how encapsulating IKE transforms it into a promising drug candidate for treatment of DLBCL and other ferroptosis-sensitive cancers while reducing adverse side effects.


Biomaterials

A highly tumor-targeted nanoparticle of podophyllotoxin penetrated tumor core and regressed multidrug resistant tumors

Biomaterials, 2015

A highly tumor-targeted nanoparticle of podophyllotoxin penetrated tumor core and regressed multidrug resistant tumors

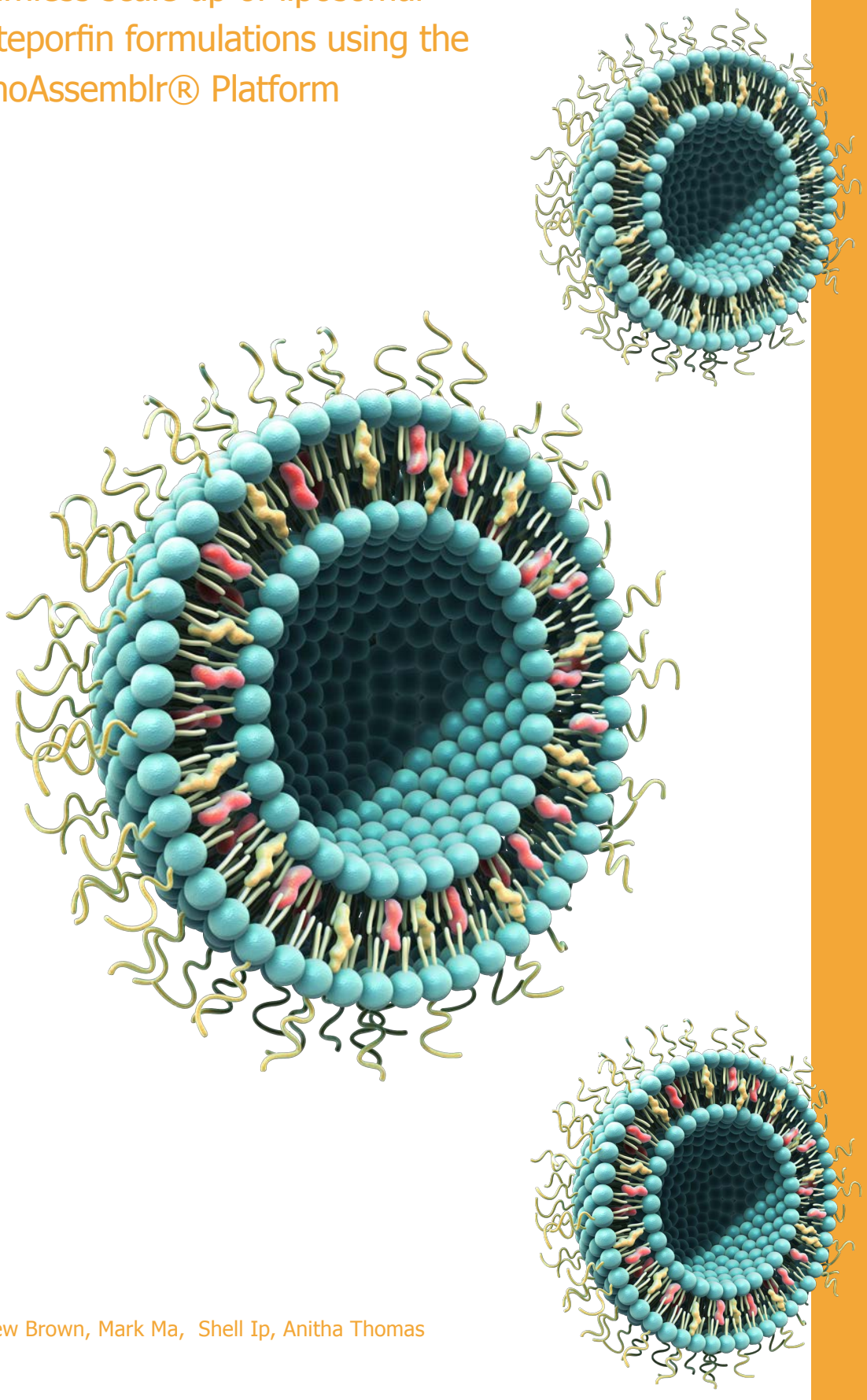
Roy A, Ernsting M, Undzys E, Li S. Biomaterials 2015; 52: 335-346.

Summary

- Multi-drug resistance (MDR) frequently arises after first-line treatment of cancer, leading to few treatment options and high mortality rates
- Podophyllotoxin (PDT) is a drug known to circumvent a major mechanism of cancer drug resistance, but its high cytotoxicity means people cannot tolerate high enough doses to be practical as a treatment
- In this paper Dr. Shyh-Dar Li's group at the University of British Columbia demonstrated a means of incorporating PDT into nanoparticles that selectively accumulate in tumors and avoid accumulation in liver and other healthy organs to widen the therapeutic window
- They formulated PDT into nanoaprticles by conjugating it along with polyethylene glycol to a modified cellulose backbone and used NanoAssemblr technology to control nanoprecipitation from organic solvent
- In mice bearing a metastatic tumor xenograft, particles less than 20 nm in diameter were highly selective to the tumor with 8-fold higher accumulation than all other examined tissues, leading to improved efficacy against MDR tumors with minimal toxicity, compared to the larger nanoparticles that accumulated in off-target tissues such as liver, native PPT and the standard taxane chemotherapies
- Conjugating drug molecules to polymers and formulating into nanoparticles can transform a toxic drug into a practical treatment, and precise control over particle size is critically important to controlling drug accumulation in tumor tissue

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 on the NanoAssemblr® Benchtop, 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

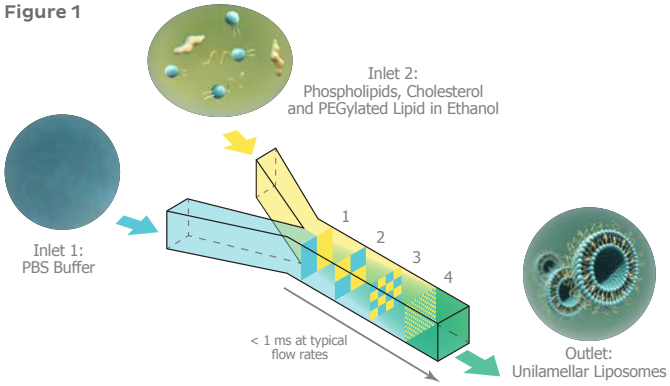
Over 75% of new chemical entities emerging from drug discovery programs are low solubility biopharmaceuticals. Their insolubility makes dosing and administration difficult. Hence, drug delivery systems such as liposomes are increasingly important. An example of a commercially available liposomal formulation 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 vision1. 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 reduced1. This mechanism is also being investigated to treat solid tumours2. Verteporfin is hydrophobic; contact with water causes verteporfin to aggregate, which inhibits its activity by reducing its ability to form singlet oxygen upon photoexcitation3. 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 technology4-7. 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 FRR8. These studies employed the NanoAssemblr Benchtop instrument, which uses common and disposable syringes and collection tubes for fluid handling. While this format makes the Benchtop 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 Benchtop and Blaze systems allows parameters optimized on the Benchtop 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

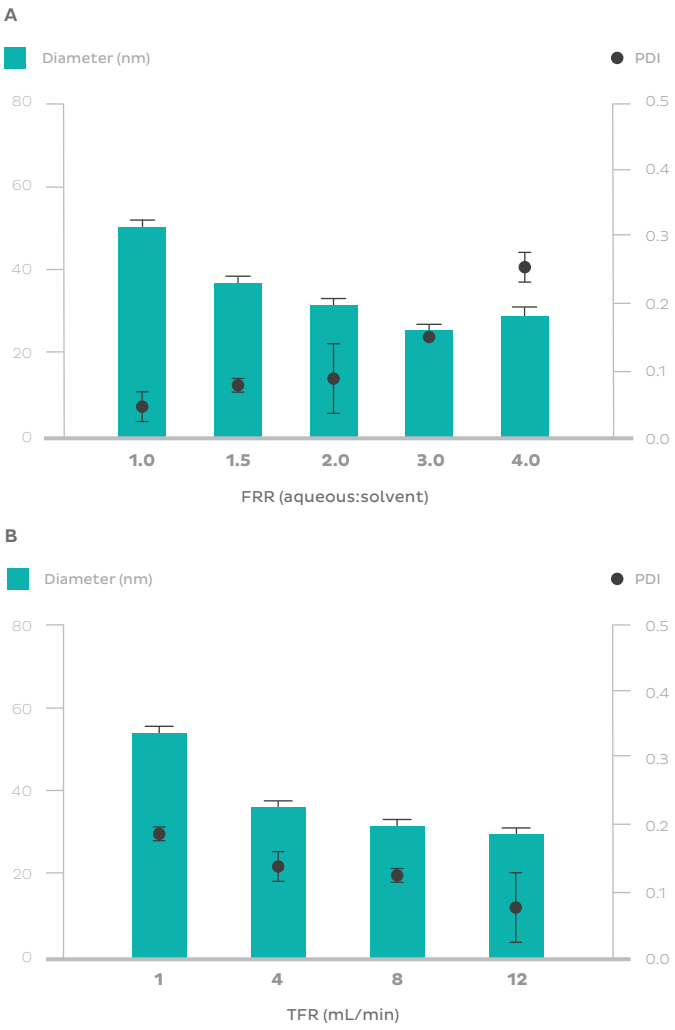


1. Two fluids (Buffer and ethanol) entering the channel under laminar flow do not mix.
2. Microscopic features in the channels are engineered to cause the fluid streams to mingle in a controlled, reproducible, and non-turbulent way.
3. Intermingling of the fluids increases as they continue through the 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 NanoAssemblr Benchtop 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 Benchtop or Blaze 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.

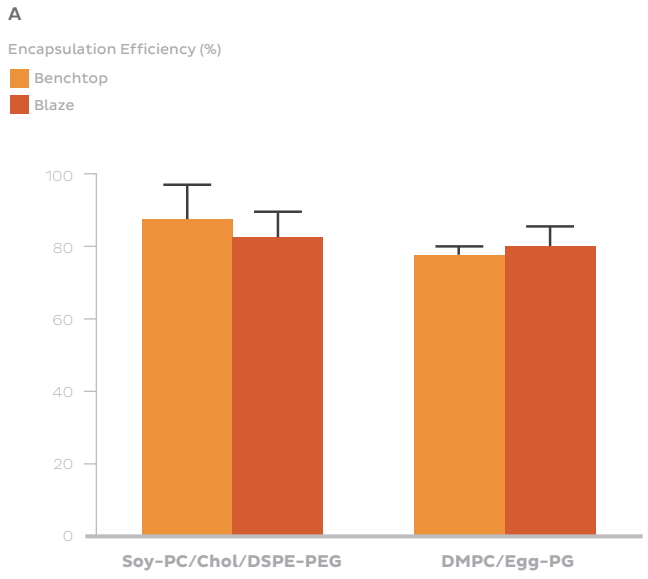
Soy-PC based formulations were previously optimized on the NanoAssemblr Benchtop by systematically varying TFR and FRR and measuring size and PDI. These findings are summarized in Figure 2. Liposome size was found to decrease asymptotically with increasing FRR and TFR, which is consistent with previous results reported in the literature4,5. This asymptote is understood the be the "limit-size" - the smallest possible size calculated based in ideal packing of constituent molecules4. 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. Samples were prepared in triplicate and values represent mean and error bars represent standard deviation of the mean.



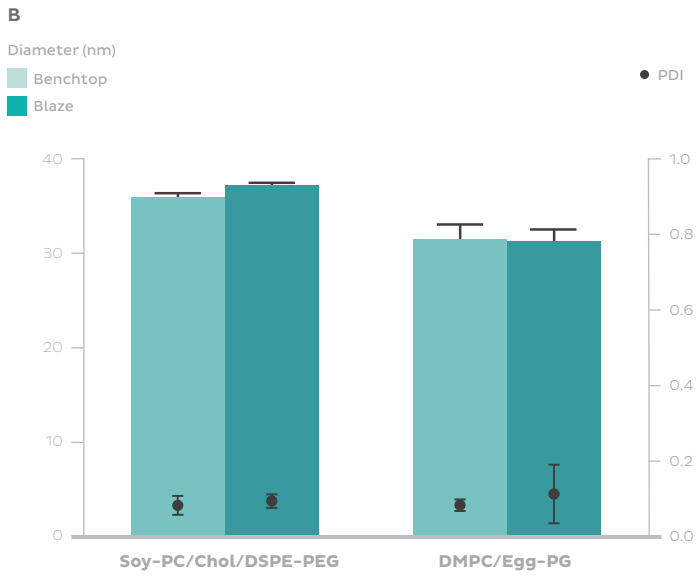
Lipid composition	Soy-PC:Chol:DSPE-PEG (52:45:3 mol%)
Total lipid concentration in organic phase	10 mg/mL
Organic solvent	Ethanol
Aqueous solvent	PBS pH 7.4
Total Flow Rate	A) 12 mL/min B) As indicated on horizontal axis
Flow Rate Ratio (aqueous:organic)	A) n:1 - n indicated on horizontal axis B) 2:1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

Figure 3. *Liposomal verteporfin formulations made with the Benchtop* and 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.*



* 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

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	Benchtop: 2 mL Blaze: 20 mL
Solvent Removal	Benchtop: 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 the NanoAssemblr Benchtop 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 on the Benchtop 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 instruments, 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 Scale-Up 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 Benchtop 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 Benchtop 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 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

Related Material

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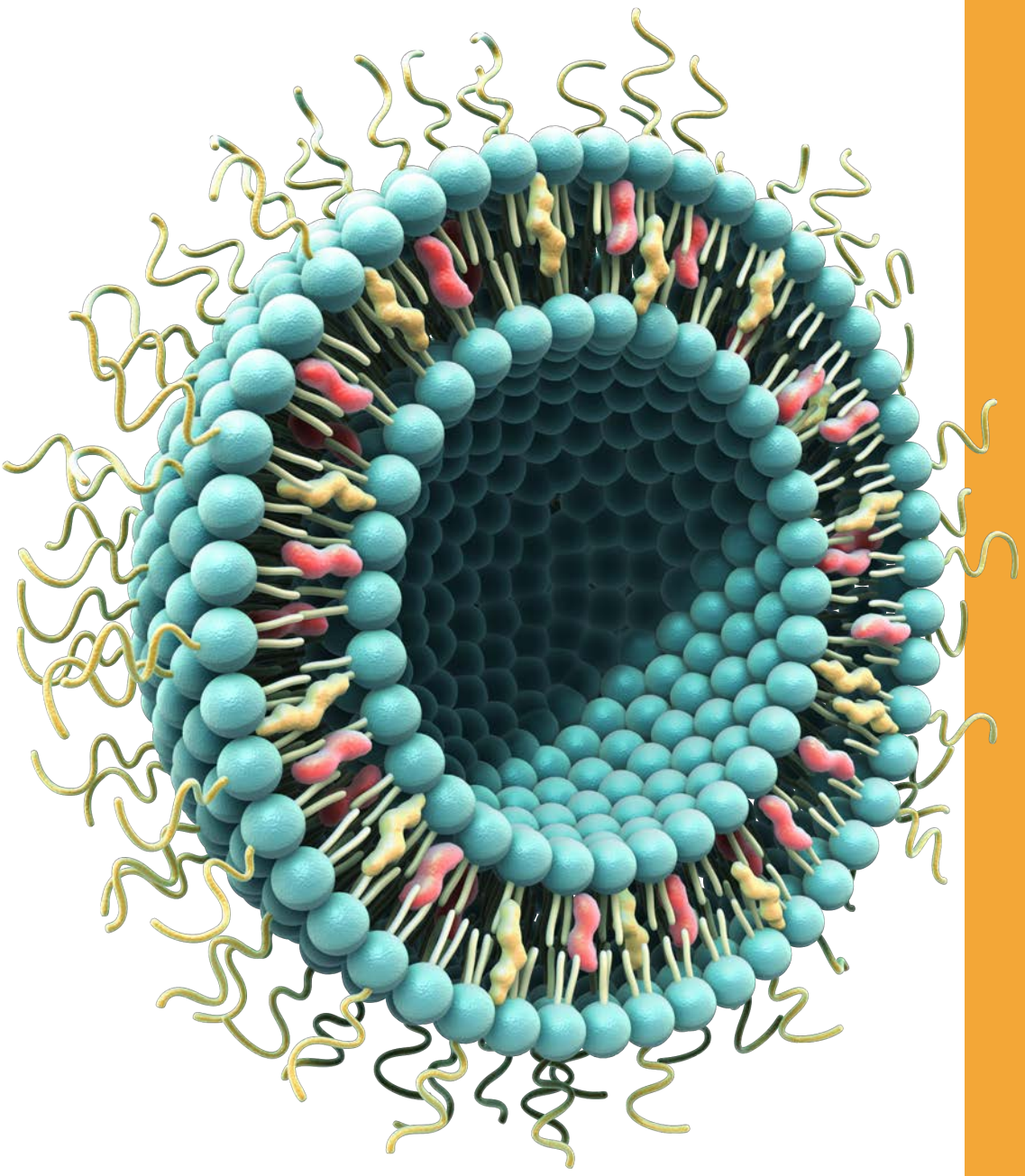
Liposomes

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 tissues1 . Prior research has demonstrated the utility of the NanoAssemblr for formulating unilamellar liposomes2 for remote loading of chemotherapeutics3, in situ loading with hydrophobic and hydrophilic small molecules 4,5, nucleic acids6, and vaccine adjuvants7. 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

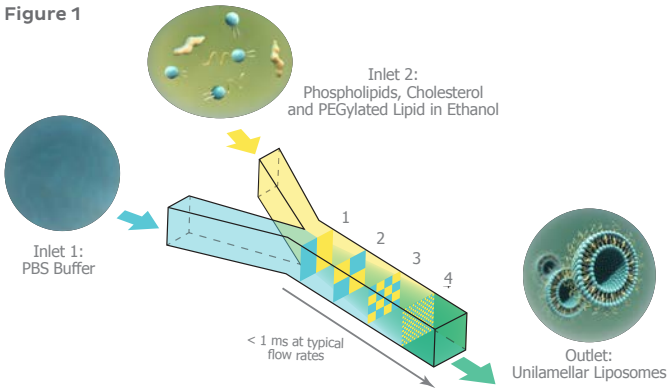


Figure 1. PBS buffer is injected into the left inlet while an organic solvent with dissolved lipids is injected into the right inlet of the NanoAssemblr microfluidic cartridge. *Following controlled mixing in microfluidic channels, unilamellar liposomes are spontaneously formed. Computer controlled injection 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 THE NANOASSEMBLR

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 RATIO

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.

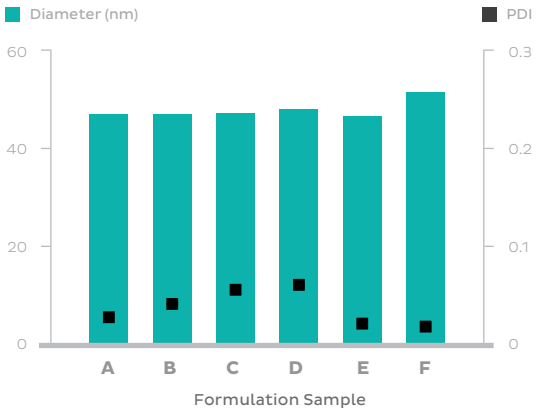
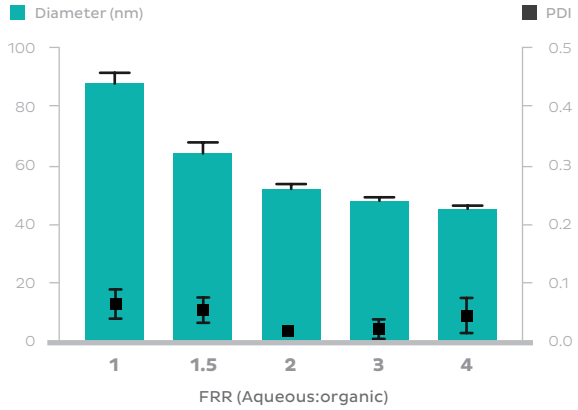


Figure 2. Liposome size and polydispersity were consistent across batches prepared by different operators. *Six independent batches of liposomes, with identical lipid composition and NanoAssemblr process parameters were generated by different operators. Liposomes generated had a size range of 46-54 nm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

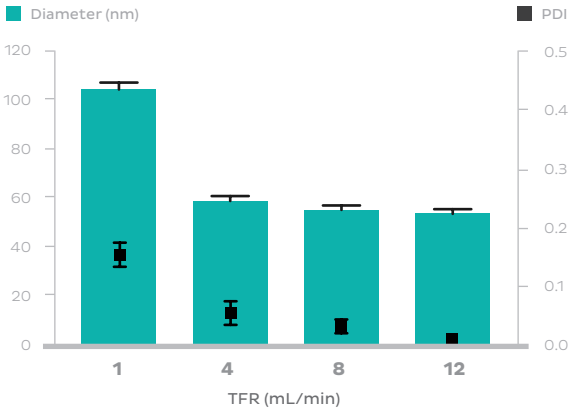
Composition	POPC/Chol/DSPE-PEG_{2000} (52:45:3 mol%)
Lipid concentration in organic solvent	10 mg/mL
Organic solvent	Ethanol
Aqueous solvent	PBS pH 7.4
Total Flow Rate (TFR)	12 mL/min
Flow Rate Ratio (FRR)	5:1

Figure 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)

Discussion

Liposome size and polydispersity are crucial factors affecting efficacy. Hence, size control and size uniformity within a batch and from batch-to-batch 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).

Related Material

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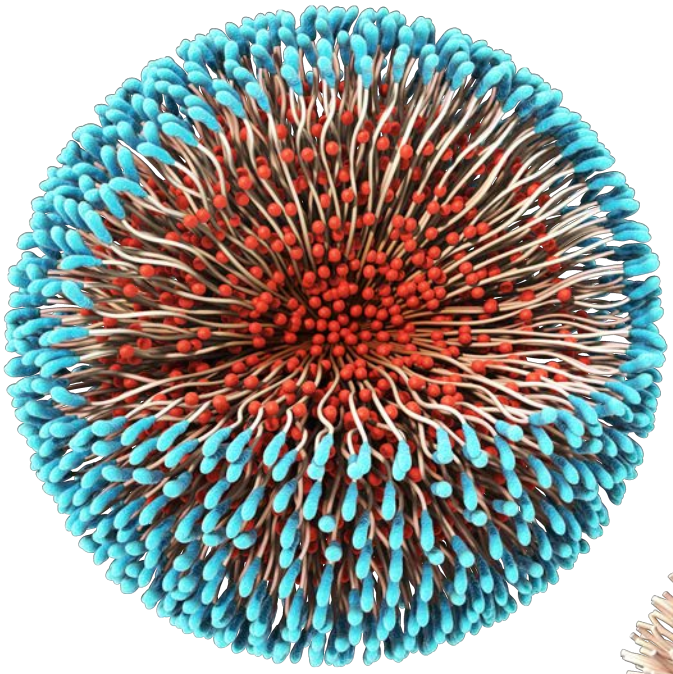
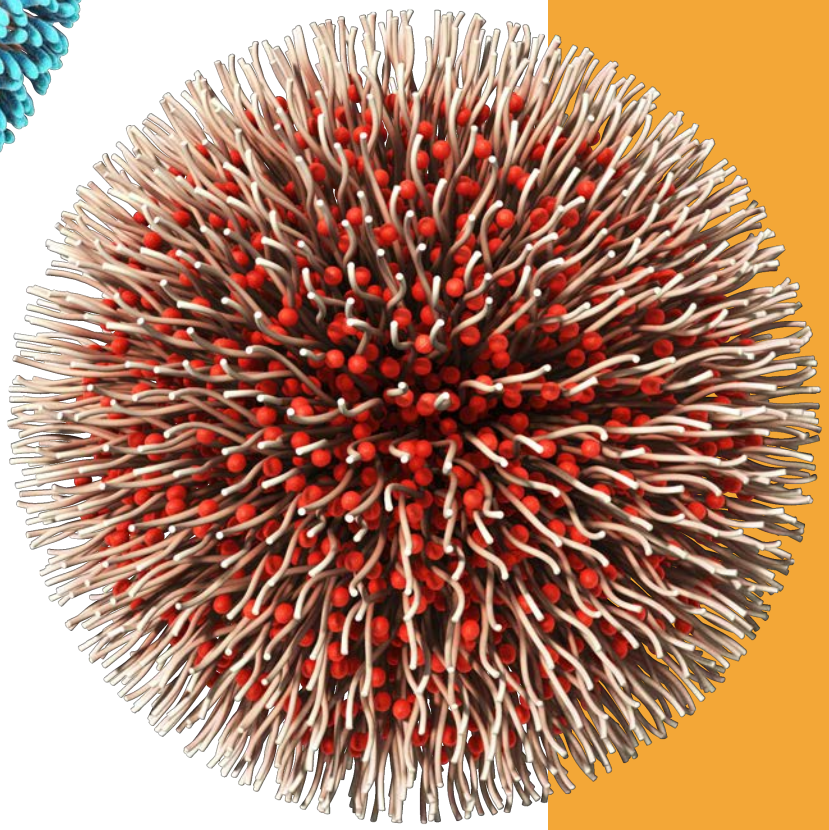
* The formulations described in this work were produced using the discontinued NanoAssemblr Benchtop. The NanoAssemblr Ignite can be used in place of its predecessor, the NanoAssemblr Benchtop

PLGA Nano-particles

Production and In Situ Drug Loading Using the NanoAssemblr® and the Impact of Solvent Removal Methods





S.M. Garg, A. Thomas, G. Heuck, A. Armstead, J. Singh, T.J. Leaver, A.W. Wild, S. Ip, R.J. Taylor, E.C. Ramsay

Abstract

There is extensive interest in using polymer-based nanoparticles as drug delivery agents due to the range of suitable payloads, extended release characteristics, and high biocompatibility of these polymeric systems. One of the challenges associated with nano-based drug carriers is ensuring that the nanoparticles (NPs) are of a desired size (at or below 100nm) and have high drug encapsulation efficiency, to ensure that the nanoparticle delivers sufficient amounts of drug to its intended target to achieve the desired therapeutic effect. Here, we present a novel approach for manufacturing poly(lactide-co-glycolide) or PLGA nanoparticles encapsulating coumarin-6 as a model hydrophobic drug using the NanoAssemblr®. Two methods of removing the solvent, namely dialysis and centrifugal filtration, were compared to determine the effects on particle size, uniformity (polydispersity index, PDI), and drug encapsulation efficiency. An encapsulation efficiency of 75% was achieved, which is higher than reported in literature using traditional production methods. Additionally, the choice of solvent removal method was found to influence PDI and encapsulation efficiency.

Introduction

Polymeric nanoparticles (NPs) are gaining major interest in the field of nanomedicine, because polymeric carriers are suitable for delivering a range of payloads (i.e. small molecules, nucleic acids, peptides, etc.) and offer sustained release properties, high biocompatibility and low toxicity.1-3 In particular, poly(lactide-co-glycolide) or PLGA is a top candidate for NP-based drug delivery applications and has already been approved by the US Food and Drug Administration (FDA) for a number of therapeutic applications.2 While a number of methods are currently used to manufacture PLGA NPs for drug delivery, these NPs are large (> 100 nm), and one of the challenges is to manufacture drug-loaded NPs smaller than 100 nm4 as these have more desirable biodistribution profiles.5 Microfluidic methods for NP manufacture have recently gained momentum as a suitable manufacturing method for producing sub-100 nm NPs.6

Encapsulation of drug molecules with a polymeric carrier can change the pharmacokinetic profile of the drug, enabling it to reach its target site. For these drug delivery applications, it is critical that the NPs are loaded with sufficient amounts of the payload (e.g. hydrophobic small molecules) so that the intended dose is delivered and the therapeutic action is attained.7 However, high drug encapsulation efficiencies may be difficult to achieve with current techniques, depending on the limitations of the production method as well as the formulation of the NP itself and how the components interact with the intended payload.

Herein, we describe the production of sub-100 nm PLGA NP encapsulating Coumarin-6 (C6) as a model hydrophobic drug molecule using the NanoAssemblr. C6 is a low molecular weight (MW: 350.43 g/mol) fluorescent probe that, like many drug molecules, is insoluble in water but soluble in water-miscible organic solvents such as ethanol, methanol, N,N-dimethylformamide, and acetonitrile. The effects of NanoAssemblr system parameters, formulation parameters, drug loading and choice of solvent removal methods are explored for this model PLGA-C6 NP system.

Result

PLGA NPs were formulated on the NanoAssemblr benchtop using the microfluidic mixing approach, illustrated in Figure 1. This process promotes rapid and even mixing of the aqueous and solvent phases, driving controlled precipitation of the PLGA NP at the desired size.

Figure 1

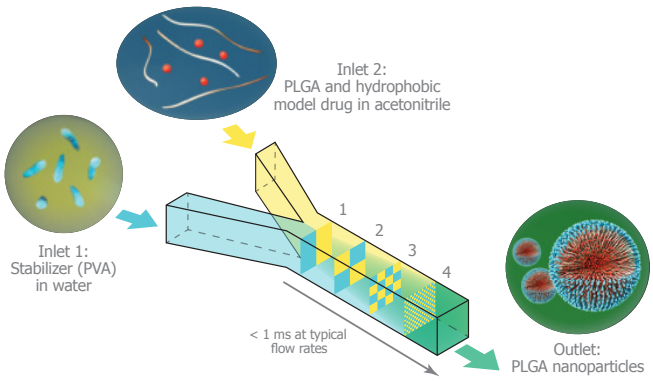


Figure 1. *Microfluidic mixing process employed by the NanoAssemblr provides rapid, homogeneous mixing and controls the nanoprecipitation of PLGA NPs with in situ drug loading.*

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.

EFFECT OF SOLVENT REMOVAL METHODS ON THE SIZE AND PDI OF EMPTY PLGA NP

After formulation of PLGA NPs on the NanoAssemblr, the solvent (acetonitrile) was removed using either dialysis or centrifugal filtration. As shown in Figure 2, NP that underwent dialysis had a small increase in the PDI as compared to the NPs before dialysis (Student's t-test, $P < 0.05$). For NPs that underwent centrifugal filtration, no change in the PDI after solvent exchange can be seen. In the case of nanoparticle size, both dialysis and centrifugal filtration caused no change in the size of the particles.

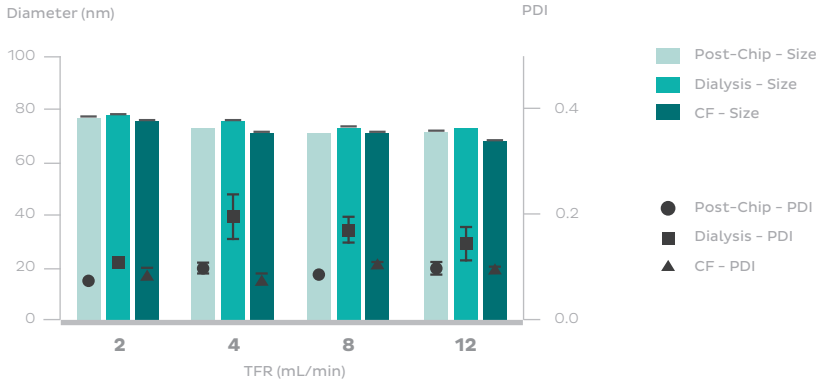


Figure 2. *Effect of solvent removal method on the size and PDI of PLGA nanoparticles at polymer concentrations of 5 mg/mL (CF, centrifugal filtration). Each bar/plot represents the mean ± SEM for 3 independent size/PDI measurements on three independent samples (n = 3)*

Polymer (PLGA) concentration	5 mg/mL
Stabilizer (PVA) concentration	2.0 % w/v
Total flow rate (TFR)	2 - 12 mL/min
Aqueous: solvent flow rate ratio (FRR)	1:1

IN SITU ENCAPSULATION OF THE MODEL DRUG COUMARIN-6 WITHIN PLGA NANOPARTICLES

Encapsulation of C6 within PLGA NPs was achieved by dissolving the C6 in the solvent phase along with the PLGA polymer and formulating the NPs with poly(vinyl alcohol) (PVA) as a stabilizer in the aqueous phase. The effect of total flow rate (TFR) on the encapsulation efficiency of C6 in PLGA NPs is shown in Figure 3. C6 was successfully encapsulated at high efficiencies (> 50%) at varying total flow rate (TFR). The maximum encapsulation was 75% w/w when formulated at a TFR of 12 mL/min.

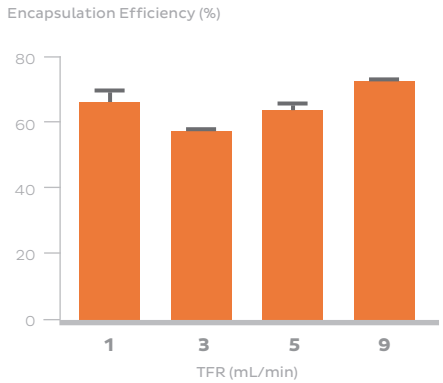


Figure 2. *Encapsulation of coumarin-6 in PLGA NPs as a function of total flow rate (TFR). Each bar represents the mean ± SEM for 3 independent measurements on three independent samples (n = 3).*

EFFECT OF COUMARIN-6 ENCAPSULATION AND SOLVENT REMOVAL METHODS ON THE SIZE AND PDI OF C6-LOADED PLGA NP

The effects of drug encapsulation and downstream processing on the size and PDI of the PLGA NP is shown in Figure 4. The encapsulation of C6 into PLGA NPs did not significantly increase the size when compared to empty PLGA (polymer only) NPs formulated under identical conditions. At the same time, the buffer exchange method did not significantly change the size of the loaded nanoparticles (Student's t-test, $P > 0.05$), but significantly reduced the PDI in case of nanoparticles processed using CF for most flow rates (Student's t-test, $P < 0.05$).

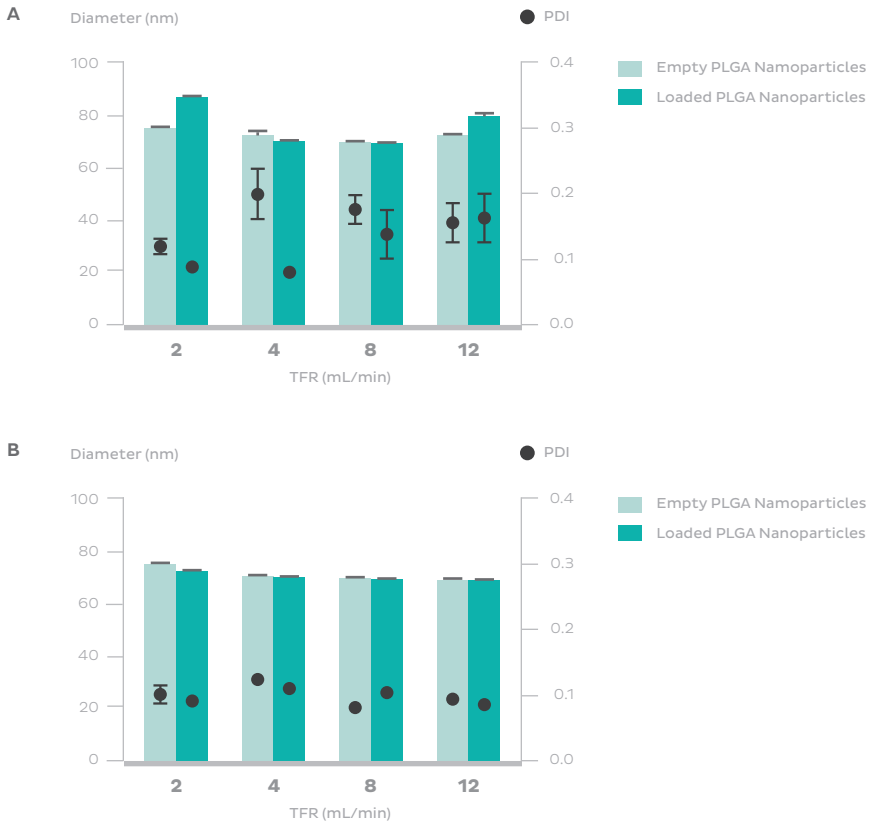


Figure 4. *The effect of coumarin-6 encapsulation on the size and PDI of PLGA NP at polymer concentrations of 5 mg/mL buffer-exchanged by either A) dialysis or B) centrifugal filtration. Each bar/plot represents the mean ± SEM for three independent size/PDI measurements on three independent samples (n = 3).*

Polymer (PLGA) concentration	5 mg/mL
Stabilizer (PVA) concentration	2.0 % w/v
Total flow rate (TFR)	2 - 12 mL/min
Aqueous: solvent flow rate ratio (FRR)	1:1

EFFECT OF THE SOLVENT REMOVAL METHOD ON THE ENCAPSULATION OF COUMARIN-6

The effect of the solvent removal method on the final encapsulation of C6 is shown in Figure 5. NPs processed using centrifugal filtration showed lower encapsulation efficiencies as compared to the NPs processed using dialysis. Maximum C6 encapsulation of 75% was achieved at a TFR of 12 mL/min using dialysis for solvent removal.

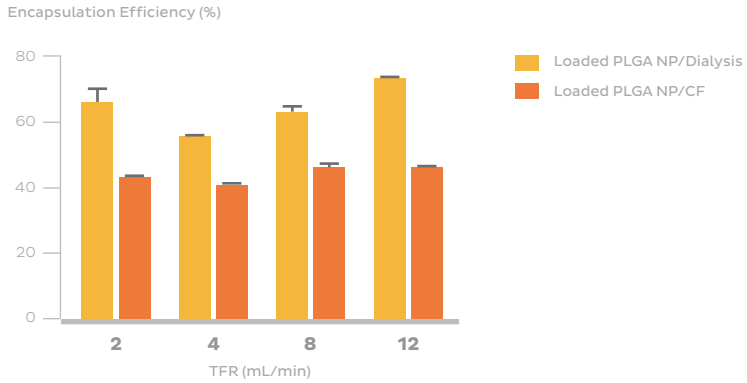


Figure 5. *Encapsulation of C6 (0.25 % w/w of the polymer) in PLGA nanoparticles at polymer concentrations of 5 mg/mL either dialyzed or buffer exchanged by centrifugal filtration (CF). Each bar represents the mean ± SEM for 3 independent measurements on three independent samples (n = 3).*

Polymer (PLGA) concentration	5 mg/mL
Stabilizer (PVA) concentration	2.0 % w/v
Total flow rate (TFR)	2 - 12 mL/min
Aqueous: solvent flow rate ratio (FRR)	1:1

Discussion

In addition to size, particle stability is also an important consideration when developing NP based drugs for the clinic. During the manufacturing process, certain solvents are required in order to successfully formulate the NP (i.e. acetonitrile, ethanol, methanol, etc.), but these solvents must be removed for downstream in vitro and in vivo testing.[8] This process not only increases the long-term stability of the NP, but is also required to minimize toxicity due to residual solvent content in the final NP product. For the PLGA NPs formulated as described here, we employed two different solvent removal methods: dialysis and centrifugal filtration (CF).

The solvent removal method employed can influence the size, PDI, and the total drug encapsulation of the final formulation. As shown in Figures 2, dialysis led to a slight increase in the PDI of the PLGA NPs whereas centrifugal filtration showed no such change in the PDI. This could be due to differences in the speed at which the solvent removal takes place during centrifugal filtration and during dialysis. It is possible that the slow removal of solvent during dialysis facilitates an uneven amount of particle growth and increase in PDI during the acetonitrile removal process.

Coumarin-6, selected as a model hydrophobic small molecule, was successfully encapsulated within PLGA NPs at high efficiencies, shown in Figure 3. The maximum C6 encapsulation obtained under these conditions on the NanoAssemblr was 75% w/w, with an initial drug loading of 0.25% w/w of the PLGA polymer. This value was considerably higher than that reported in literature (60% w/w C6 encapsulation) for a similar PLGA polymer and stabilizer system, using a similar theoretical drug loading and a single-emulsion method to formulate the NPs.[9] In addition to achieving a higher C6 encapsulation, the size of the PLGA NP produced on the NanoAssemblr were much smaller than those reported in the literature, 80 nm vs. 177 nm, respectively.[9] This indicates the ability of the NanoAssemblr microfluidic mixing platform[6] to produce smaller NP with higher encapsulation efficiencies, a promising find for the field of nanomedicine. [4, 6]

However, we found significant differences in the final C6 NP encapsulation between the two methods employed for removal of acetonitrile, shown in Figure 5. C6 NPs that were processed using CF had a lower final encapsulation of C6 compared to NPs processed using dialysis. This could be due to the increased stress and force that the PLGA NPs undergo during the CF process itself, leading to lower encapsulations when compared to NP processed via dialysis. Alternatively, solvent exchange by dialysis is a slower process than CF, allowing solvent exchange to occur under equilibrium conditions where structural relaxation of the PLGA core can occur, trapping more drug molecules. These findings highlight the importance of selecting a suitable solvent removal method for a given NP application, depending on the final goals for the NP drug product.

Conclusion

These data indicate that the microfluidic approach utilized by the NanoAssemblr successfully generates PLGA NPs less than 100 nm in size, with higher encapsulation efficiencies of Coumarin-6. The encapsulation of Coumarin-6 did not change the NP size, however, the solvent removal method used was found to influence the PDI and the encapsulation efficiency of drugs in PLGA NPs. In conclusion, the NanoAssemblr platform offers an attractive solution for manufacturing drug-loaded nanoparticles with small size and high encapsulation efficiencies.

Materials & Methods

MATERIALS

PLGA ester-terminated (lactide to glycolide ratio 50:50, molecular weight 45,000 - 55,000) was obtained from Akina, Inc (West Lafayette, IN). Poly(vinyl alcohol) (PVA) (molecular weight ~31,000, 87-89 mol% hydrolyzed) was purchased from Sigma-Aldrich (St Louis, MO). All other materials were reagent grade.

MICROFLUIDIC MANUFACTURE OF PLGA NANOPARTICLES.

PLGA nanoparticles were prepared using a microfluidic mixer with the NanoAssemblr Benchtop* instrument (Precision NanoSystems Inc., Vancouver, BC) by mixing appropriate volumes of PLGA solutions in acetonitrile with aqueous solutions containing PVA. Briefly, PLGA was dissolved in acetonitrile at concentration of 5 mg/mL, whereas, PVA was dissolved in deionized water at a concentration of 2% w/v. For synthesis of PLGA nanoparticles, the PLGA solution in acetonitrile was injected through one inlet of the microfluidic mixer whereas, the PVA solution was injected through the other inlet of the microfluidic mixer. The NP formulations were prepared at aqueous:solvent FRR of 1:1 and various TFR from 2-12 mL/min. The PLGA nanoparticles were collected in the sample collection tube by discarding an initial and final waste volume of 0.25 and 0.05 mL, respectively. The sample collected was then further processed using one of two methods to remove the organic solvent. For dialysis, samples were dialyzed against 1L deionized water using a dialysis bag (MWCO - 10,000) for 12 h replacing the dialysis medium twice in the first 4 h. For solvent exchange using centrifugal filtration, samples were diluted 3 times followed by centrifugation at 1500 - 1600× g for 30 min using Amicon® Ultrafiltration tubes (MWCO – 10,000). The samples were washed 3 times during this process until the original formulation volume was reached.

* 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

For encapsulation of Coumarin-6, the drug was dissolved in a stock solution of PLGA in acetonitrile such that the concentration of PLGA is 5 mg/mL and the drug/polymer w/w ratio is 1/400. The Coumarin-6 - PLGA solution in acetonitrile was then injected through one inlet of the microfluidic mixer whereas, the 2% w/v PVA solution was injected through the other inlet of the microfluidic mixer to form nanoparticles using the same parameters as listed above at different flow rates from 2 - 12 mL/min. The samples were dialyzed as mentioned above, followed by centrifugation at 8000 × g for 5 minutes to remove and/or precipitate the free drug.

CHARACTERIZATION OF PLGA NANOPARTICLES.

PLGA nanoparticles prepared using the NanoAssemblr were characterized for their size (Z-Average size) and polydispersity index (PDI) by dynamic light scattering using a Malvern Zetasizer 3000 (Malvern, UK) by diluting 50 µL of the prepared nanoparticle formulation with 300 µL of deionized water and measured at 25 ºC.

Encapsulation of C6 was measured in PLGA nanoparticles by first adding ethanol to solubilize the drug followed by precipitation of the solution at 15000 × g for 10 minutes. The solution was then measured against a standard curve of C6 in ethanol using fluorescence spectroscopy at an excitation and emission wavelength of 450 nm and 505 nm, respectively.

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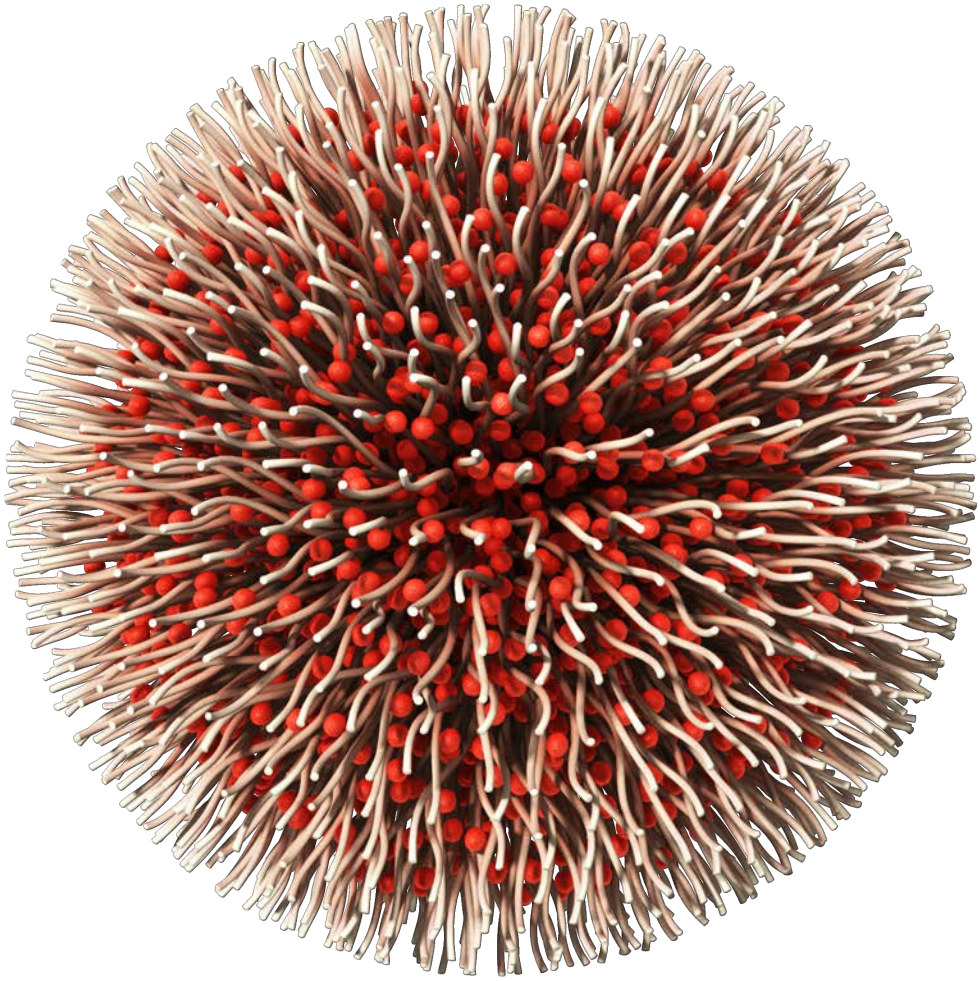
PLGA Nano-particles

Tuning Particle Size Using The NanoAssemblr®



S.M. Garg, A. Thomas, G. Heuck, A. Armstead, J. Singh,
T.J. Leaver, A.W. Wild, S. Ip, R.J. Taylor, E.C. Ramsay

Abstract

Emerging evidence from a growing number of studies about nanoparticle-based drugs indicates that the size of these drug-loaded nanoparticles (NPs) plays a critical role in drug efficacy and ultimate clinical success. It is therefore important that manufacturing processes enable precise NP size control during the production process. Current methods for producing NP drugs offer a range of achievable NP sizes but lack the ability to specifically control NP size while maintaining high drug encapsulation efficiency and low polydispersity. Here, we present a novel solution for NP production using the NanoAssemblr, which utilizes microfluidic mixing driven by computer controlled pumps to offer a high degree of control over the solvent/antisolvent nanoprecipitation process and hence the ability to rationally optimize NP properties. Instrument and formulation parameters were systematically explored to tune NP size on the NanoAssemblr® using a representative polymeric poly(lactide-co-glycolide) (PLGA) NP system. Higher flow rates of reagents through the microfluidic mixer resulted in smaller particles, while higher aqueous-to-solvent mixing ratios increased particle size. Additionally, increasing the polymer concentration in the solvent phase led to increased particle size. Through examination of 4 parameters, particle sizes were tuned between 70 and 200 nm with PDIs < 0.2.

Introduction

With the increasing amount of research and development centered on nanoparticle (NP) based therapeutics and drug products, it is evident that NP size plays an important role in drug efficacy. NP size may impact the drug's tissue adsorption and bio-distribution,1-3 so the ability to reproducibly tune the size of drug-loaded NPs is extremely important for clinical success. Current NP production methods lack precise size control and are difficult to scale up to large clinical production batches. The NanoAssemblr microfluidic platform addresses these shortcomings by enabling users to control the environment during NP precipitation through computer controlled parameters. This leads to precise NP size control, a high degree of particle uniformity, and batch-to-batch reproducibility. Furthermore, formulations can be scaled up by increasing the quantities of materials pumped through the system or by employing multiple microfluidic mixers in parallel. These features, significantly reduce time and cost associated with developing NP-based drug candidates.4

Optimization of NP size on the NanoAssemblr platform is achieved via modulation of built-in instrument parameters that control fluid mixing within the microfluidic cartridge and/or by altering the formulation parameters. The first instrument parameter is the total flow rate (TFR), which controls the speed at which the aqueous and solvent phases are mixed together within the microfluidic cartridge. The second instrument parameter is the flow rate ratio (FRR), which controls the mixing ratio of the aqueous and solvent phases. By systematically varying one or both of these instrument parameters, one can achieve a variety of reproducible nanoparticle sizes that can be optimized for a given application.

Additionally, NP size can be tuned on the NanoAssemblr by altering formulation parameters, such as the concentration of the starting materials dissolved in the aqueous and solvent phases (i.e. increase/decrease the concentration of polymers in the solvent phase and/or changing the concentration of stabilizers in the aqueous phase). Here, we present a case study on how these different parameters can be used to tune the size of a NP composed of poly (lacticco-glycolic) acid (PLGA) core and a poly (vinyl alcohol) (PVA) coating using the NanoAssemblr microfluidic mixing platform. The tested parameters are summarized in Table 1.

Instrument Parameters	
Total flow rate (TFR) 2 - 12 mL/min	POPC/Chol/DSPE-PEG_{2000} (52:45:3 mol%)
Flow rate ratio (FRR) 1:1 - 9:1 (aqueous:solvent)	10 mg/mL

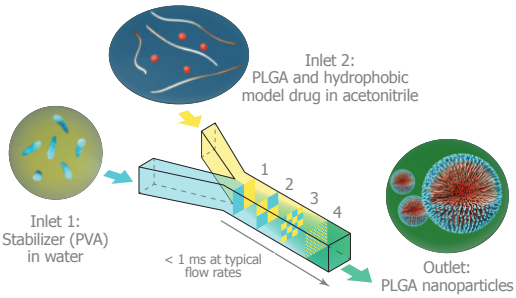
Formulation Parameters	
Polymer (PLGA) concentration 5 - 40 mg/mL	POPC/Chol/DSPE-PEG_{2000} (52:45:3 mol%)
Stabilizer (PVA) concentration 0.5 - 4.0 % w/v	10 mg/mL

Table 1. *Parameters Tested on the NanoAssemblr*

Result

PLGA NPs were manufactured on the NanoAssemblr via microfluidic mixing, illustrated in Figure 1. This approach achieves rapid and uniform mixing of the PLGA (solvent phase) with the stabilizer poly(vinyl alcohol) (PVA, aqueous phase) which drives controlled precipitation of the PLGA NP.

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.

Figure 1. *Illustration showing the manufacture of PLGA nanoparticles using the NanoAssemblr.*

EXPLOITING INSTRUMENT PARAMETERS TO TUNE THE SIZE OF PLGA NP

The effect of total flow rate (TFR) on the size of PLGA particles using a fixed concentration of PLGA and PVA is demonstrated in **Figure 2**. Shown in **Figure 2A**, NPs produced using 5 mg/mL of PLGA showed a small decrease in size, from ~82 to ~73 nm, as TFR increased. NPs produced using a higher concentration of PLGA at 20 mg/mL (**Figure 2B**) exhibited a larger decrease in the particle size, ranging from ~150 nm down to ~120 nm, as TFR increased from 2 to 12 mL/min. The PDI remained consistent (~0.2) regardless of PLGA concentration or TFR.

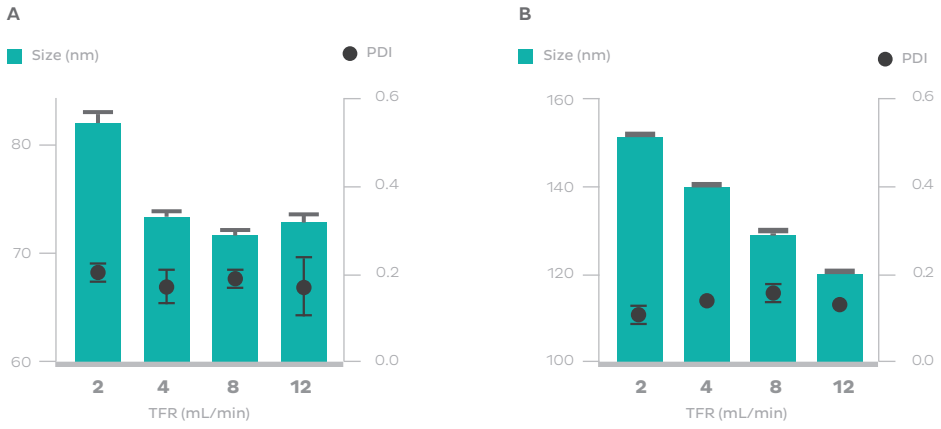
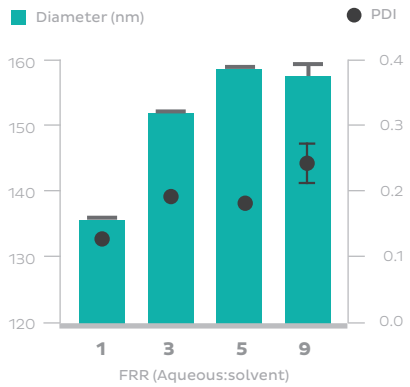


Figure 2. *Effect of total flow rate (TFR) on the size and polydispersity (PDI) of PLGA NP at polymer concentrations of (A) 5 mg/mL and (B) 20 mg/mL using the NanoAssemblr. Each bar/ plot represents the mean ± SEM for three independent size/PDI measurements on three independent samples (n = 3).*

Polymer (PLGA) concentration	5 or 20 mg/mL
Stabilizer (PVA) concentration	2.0 % w/v
Total flow rate (TFR)	2 - 12 mL/min
Aqueous: solvent flow rate ratio (FRR)	1:1

Figure 3 demonstrates the effect of flow rate ratio (FRR) on the size and PDI of PLGA NPs. The aqueous:solvent FRR is the ratio of the two phases that are mixed with each other as they are pumped through the microfluidic device; a ratio of 3:1 indicates that 3 parts aqueous phase (PVA in water) is mixed with 1 part solvent phase (PLGA in acetonitrile). As shown in **Figure 3**, as the FRR increased from 1:1 to 9:1, the PLGA NP size increased from ~135 to ~160 nm.

Figure 3. *Effect of aqueous:solvent flow rate ratio (FRR) on the size and PDI of PLGA nanoparticles at polymer concentrations of 20 mg/mL using the NanoAssemblr. Each bar/plot represents the mean ± SEM for 3 independent size/PDI measurements on three independent samples (n = 3).*

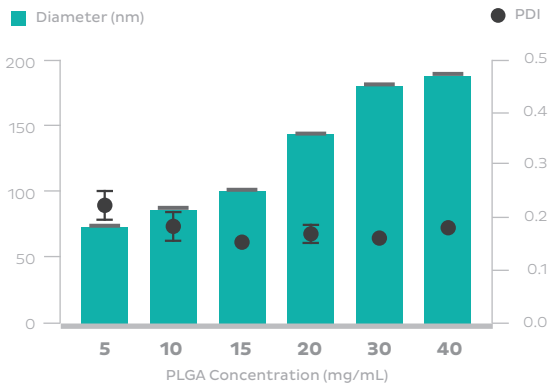


Polymer (PLGA) concentration	20 mg/mL
Stabilizer (PVA) concentration	2.0 % w/v
Total flow rate (TFR)	12 mL/min
Aqueous: solvent flow rate ratio (FRR)	1:1 - 9:1

EXPLOITING INSTRUMENT PARAMETERS TO TUNE THE SIZE OF PLGA NP

The other approach to tune the size of the NPs is to alter the formulation parameters, such as polymer concentration, shown in **Figure 4**. As the concentration of PLGA increased from 5 to 40 mg/mL, NP size also increased from ~70 to ~200 nm while maintaining a PDI around 0.2.

Figure 4. *Effect of PLGA polymer concentration on the size and PDI of PLGA nanoparticles using the NanoAssemblr. Each bar/plot represents the mean ± SEM for 3 independent size/PDI measurements on three independent samples (n = 3).*



Polymer (PLGA) concentration	5 - 40 mg/mL
Stabilizer (PVA) concentration	2.0 % w/v
Total flow rate (TFR)	8 mL/min
Aqueous: solvent flow rate ratio (FRR)	1:1

A PLGA concentration of 5 mg/mL may be too low for certain applications which require a final NP size below 100 nm with high polymer content. To achieve high PLGA content and NP size < 100 nm, a large batch of 5 mg/mL PLGA NPs were produced and subsequently concentrated to 25 mg/mL using centrifugal filtration, shown in **Figure 5**. This approach achieves a final NP size ~90 nm at the final desired PLGA concentration of 25 mg/mL, as opposed to formulating directly at 25 mg/mL which produced NP of ~170 nm as indicated in **Figure 4**.

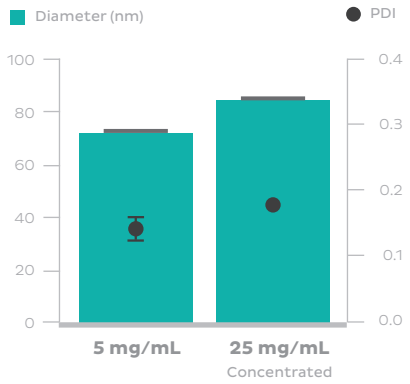


Figure 5. *Change in size of PLGA nanoparticles prepared using the NanoAssemblr at 5 mg/mL compared to the same formulation after concentrating to 25 mg/mL. Each bar/plot represents the mean ± SEM for 3 independent size/PDI measurements on three independent samples (n = 3).*

Polymer (PLGA) concentration	5 mg/mL
Stabilizer (PVA) concentration	2.0 % w/v
Total flow rate (TFR)	8 mL/min
Aqueous: solvent flow rate ratio (FRR)	1:1

The concentration of the stabilizing agent, PVA, can also impact the size of the PLGA NP, which is highlighted in **Figure 6**. As the PVA concentration increased from 0.5 to 2% w/v, the PLGA NPs decreased in size from ~163 to ~147 nm. No further reduction in particle size was observed at PVA concentrations > 2% w/v.

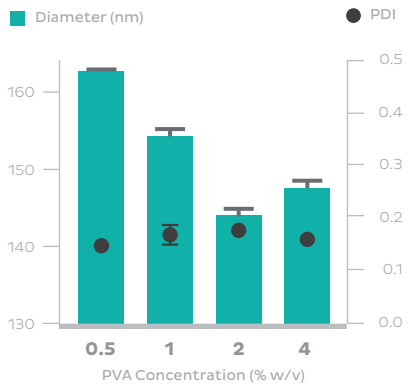


Figure 6. *Effect of PVA stabilizer concentration on the size and PDI of PLGA nanoparticles at polymer concentrations of 20 mg/mL using the NanoAssemblr. Each bar/plot represents the mean ± SEM for 3 independent size/PDI measurements on three independent samples (n = 3).*

Polymer (PLGA) concentration	20 mg/mL
Stabilizer (PVA) concentration	0.5 - 2.0 % w/v
Total flow rate (TFR)	8 mL/min
Aqueous: solvent flow rate ratio (FRR)	1:1

Discussion

Size plays an important role in the biodistribution, tissue penetration, drug release and drug efficacy of NP-based therapeutics,[1, 2] so it is critical to maintain control over NP size during the manufacturing process. Compared to conventional methods,[5] the innovative microfluidic mixing technology employed by the NanoAssemblr platform offers exquisite control over NP size during the formulation process. This is achieved by modulating instrument parameters including the total flow rate (TFR) or flow rate ratio (FRR) and formulation parameters such as concentration. Herein, we presented examples of how these parameters can tune the size of PLGA NPs, selected as a representative biodegradable polymer that is currently approved by the FDA for drug delivery applications.[6]

The TFR in mL/min is the total combined speed at which the two fluids are being pumped into the two inlets of the microfluidic device (illustrated in **Figure 1**). As TFR increases, faster mixing time is achieved which reduces PLGA NP size, as shown in Figure 2. As mixing time becomes much faster than the precipitation time of PLGA, NP size approaches an asymptote with increasing TFR. This asymptote is believed to be the 'limit size', which is the smallest, thermodynamically stable NP size for a given system.[7] For 5 mg/mL PLGA specifically, this limit size was ~70 nm for this particular system under these conditions, shown in **Figure 2A.**

The relative amounts of aqueous and solvent phases being mixed at any given moment are dictated by the FRR. As solvent and antisolvent phases are rapidly mixed, there is a sudden shift in polarity that leads to a transient supersaturation of the molecule in the new solvent environment. This drives the precipitation of dissolved molecules into NPs. Here, larger PLGA particles were obtained with higher FRR, which contrasts with trends observed with amphiphilic molecules such as phospholipids (data not shown). The different trends can be understood through differences in the dynamics of particle assembly with these materials. With amphiphilic molecules which self-stabilize, greater changes in solvent polarity drive nucleation of particles which are rapidly stabilized by the hydrophilic portion of the molecule. With hydrophobic PLGA, rapid increase in polarity leads to rapid precipitation of the PLGA core, but surface passivation is limited by the kinetics of the assembly of the PVA corona. For the PVA, as the proportion of the aqueous phase to the organic phase increases, the magnitude of the polarity reduction experienced by the PVA upon mixing decreases. This lowers the driving force for the PVA to assemble on the surface of nascent PLGA particles. This delayed passivation favors growth of larger PLGA cores.

The effects of PLGA and PVA (stabilizer concentration) were also explored. Increasing the concentration of PLGA led to an increase in NP size (**Figure 4**), which is similar to results reported in the literature.[8] Concentrated polymer solutions are more viscous, so it is thought that this change in viscosity may decrease the speed of diffusion of the solvent phase into the aqueous phase which subsequently leads to formation of larger NPs.[8] The role of the PVA is to stabilize the particles by reducing the interfacial tension between the PLGA polymer and the aqueous phase. Changing the concentration of the stabilizer can thus have an impact on the size and PDI of PLGA nanoparticles, shown in **Figure 6**. These results are similar to that reported in literature and is due to the reduction in interfacial tension as concentration increases.[8] No further reduction in particle size was reported at PVA concentrations of 4% w/v which may be because concentrations of 2% w/v are enough to efficiently stabilize these nanoparticles.

It is important to note, that having a precisely controlled, reproducible process is necessary to isolate the effects of formulation parameters on particle size. Without such control, batch-to-batch variability could result in experimental errors that could obscure the effects of the formulation changes. In particular, the effect of PLGA concentration between 5-15 mg/mL on particle size is subtle. Batch-to-batch reproducibility afforded by the NanoAssemblr platform results in nearly negligible experimental error, which lowers the noise floor for these observations allowing changes in size to be both detected and attributed to changes in formulation.

Conclusion

These data demonstrate how NP size can be rationally tuned on the NanoAssemblr by modulating the instrument parameters (TFR and FRR) and formulation parameters (polymer and stabilizer concentrations). Through a series of optimization experiments, the NanoAssemblr was able to formulate PLGA NPs in a range of sizes (70 - 200 nm) which is difficult to attain by most conventional methods of manufacture. Batch-to-batch reproducibility and the ease with which NP size can be tuned on this platform is promising for the field of nanomedicine.

Materials & Methods

MATERIALS

PLGA ester-terminated (lactide to glycolide ratio 50:50, molecular weight 45,000 - 55,000) was obtained from Akina, Inc (West Lafayette, IN). Poly(vinyl alcohol) (PVA) (molecular weight ~31,000, 87-89 mol% hydrolyzed) was purchased from Sigma-Aldrich (St Louis, MO). All other materials were reagent grade.

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CHARACTERIZATION OF PLGA NANOPARTICLES.

PLGA nanoparticles prepared using the NanoAssemblr Benchtop were characterized for their size (Z-Average size) and polydispersity index (PDI) by dynamic light scattering using a Malvern Zetasizer 3000 (Malvern, UK) by diluting 50 µL of the prepared nanoparticle formulation with 300 µL of deionized water and measured at 25 ºC.

* 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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NxGen Technology

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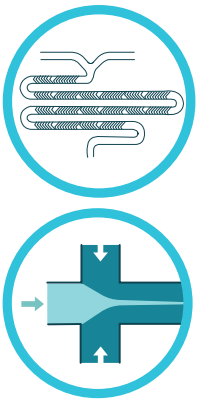
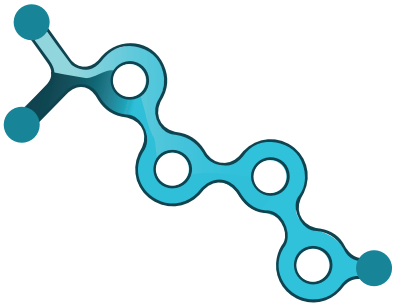
		- Some scaling challenges remain	+ Easy to Scale
		- Mixers are difficult to make	+ Mixers are Easy to make
- Limited applications	- Limited applications	+ Expanded Applications	+ Potential multi-mixer integration opens possibilities
- Difficult to reproduce	- Difficult to reproduce	+ Reproducible	+ 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

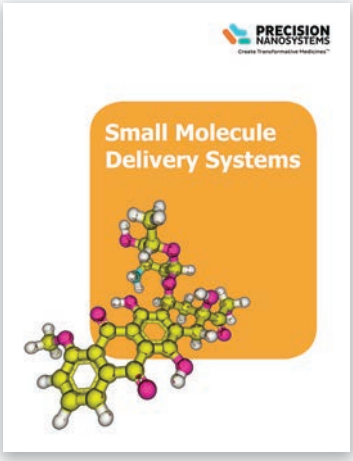
Drug developers of all backgrounds have options to bring solutions into their lab or work with out team based on their needs

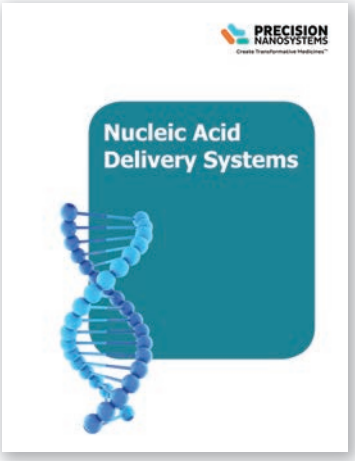
Innovation

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

Other Resources

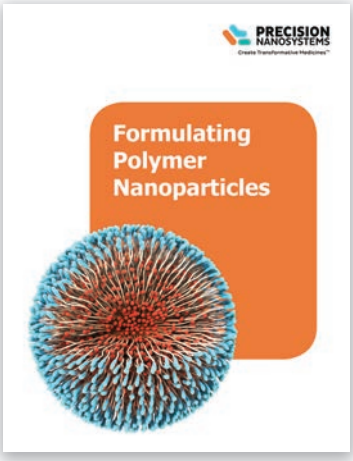
Read other application notes in this series

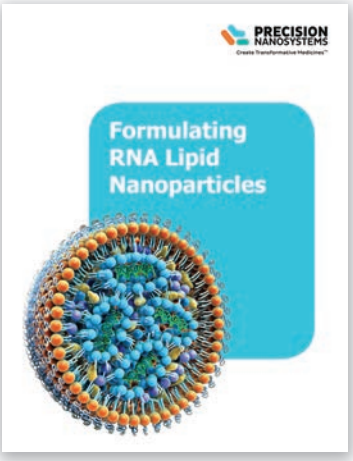


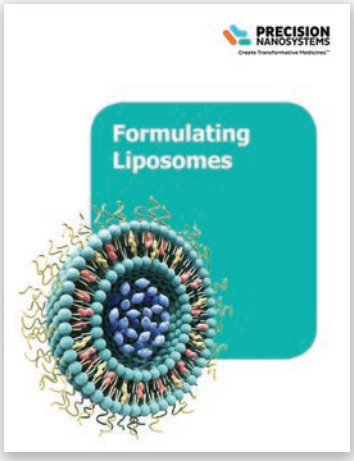


Learn about:

- 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







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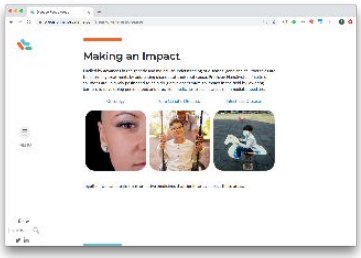
Learn about our 3 disease focus areas



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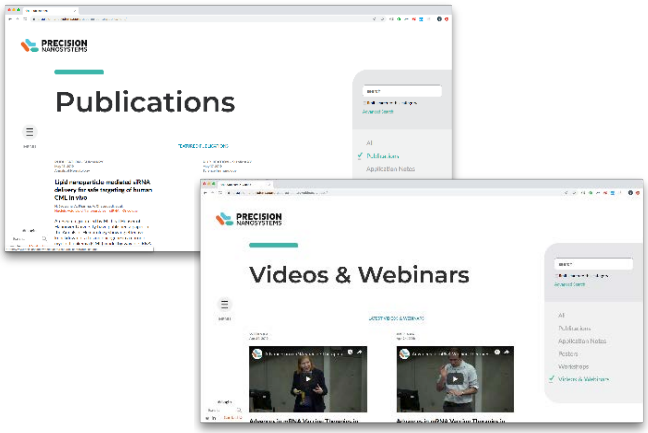

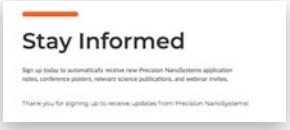

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We have the expertise, flexibility and resources to provide a full range of custom formulation solutions.

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

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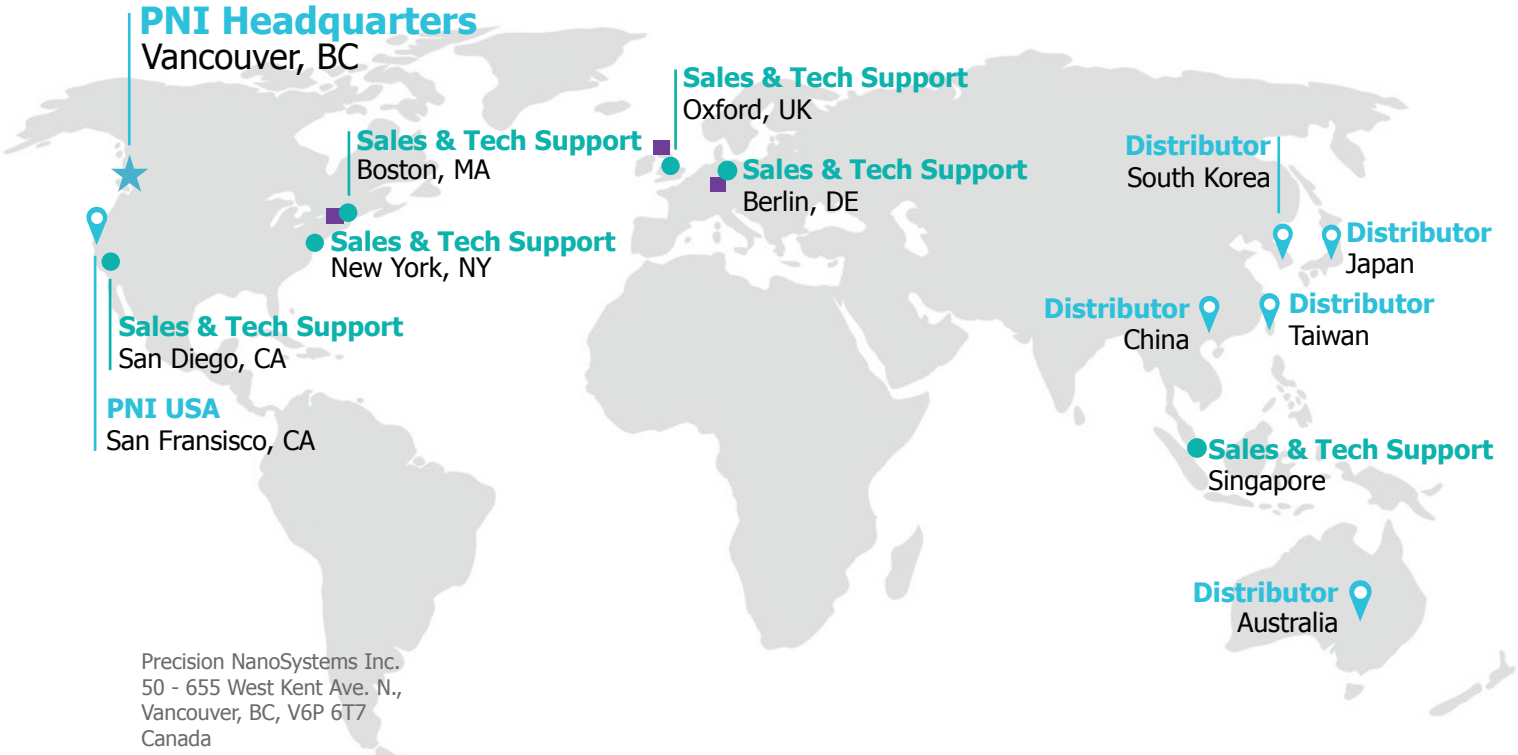

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