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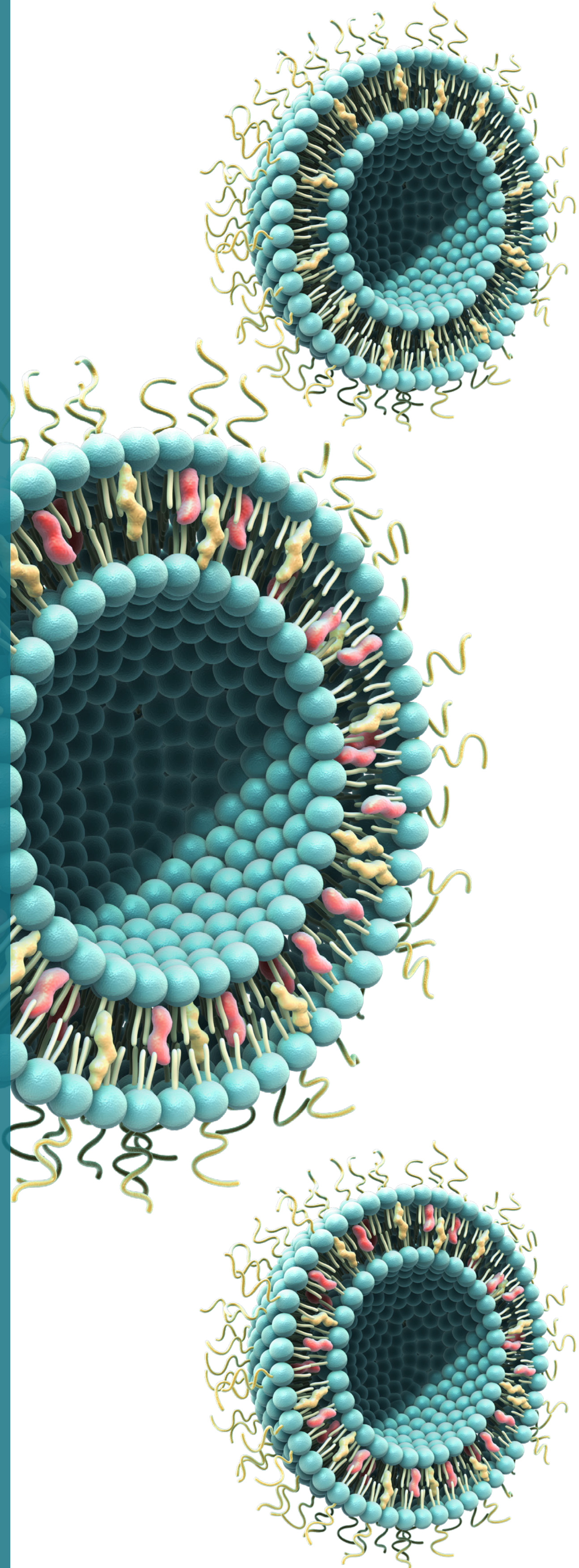
Liposomes

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



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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 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.

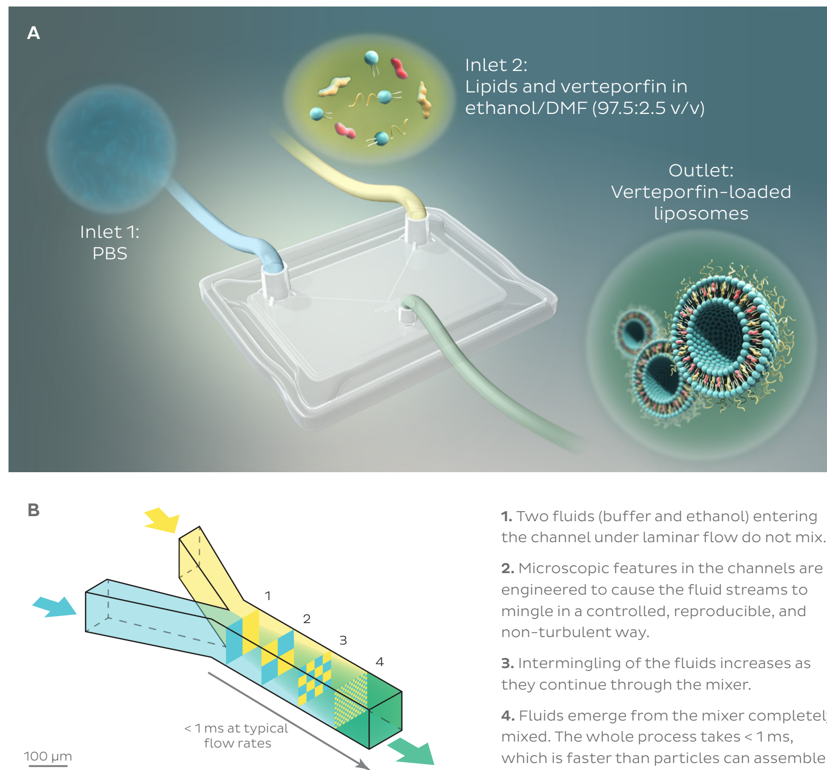


Figure 1. A) *PBS buffer is injected into the left inlet while an organic solvent with dissolved lipids and the drug verteporfin is injected into the right inlet of the NanoAssemblr microfluidic cartridge. Following controlled mixing in microfluidic channels, liposomes are spontaneously formed. Computer 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.* ***B)*** *Diagram depicting two fluids mixing under laminar flow in a microfluidic mixer. The two fluids enter the channel as adjacent streams. Specially engineered microscopic features within the channels cause intermingling of the two fluid streams, effectively increasing the surface area between them, increasing the interface between the two liquids across which diffusion can take place, and reducing the diffusion distance.*

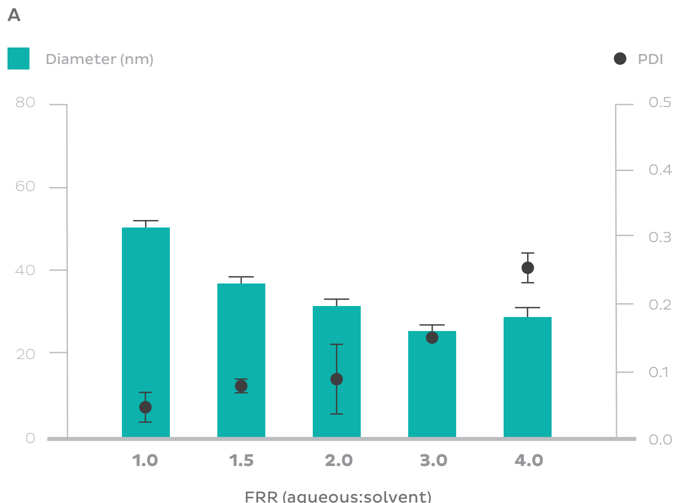
Several groups have demonstrated development of liposomal drug delivery systems using NanoAssemblr technology[4-7]. Additionally, a prior application note has reported *in situ* loading of verteporfin into soy- and egg-PC liposomes and optimization of size by systematic exploration of TFR and FRR[8]. These studies employed 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.

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.

Result

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 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.

A



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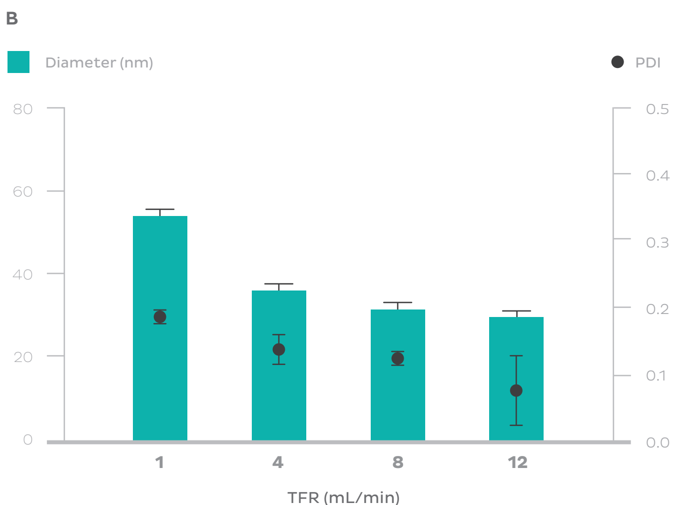


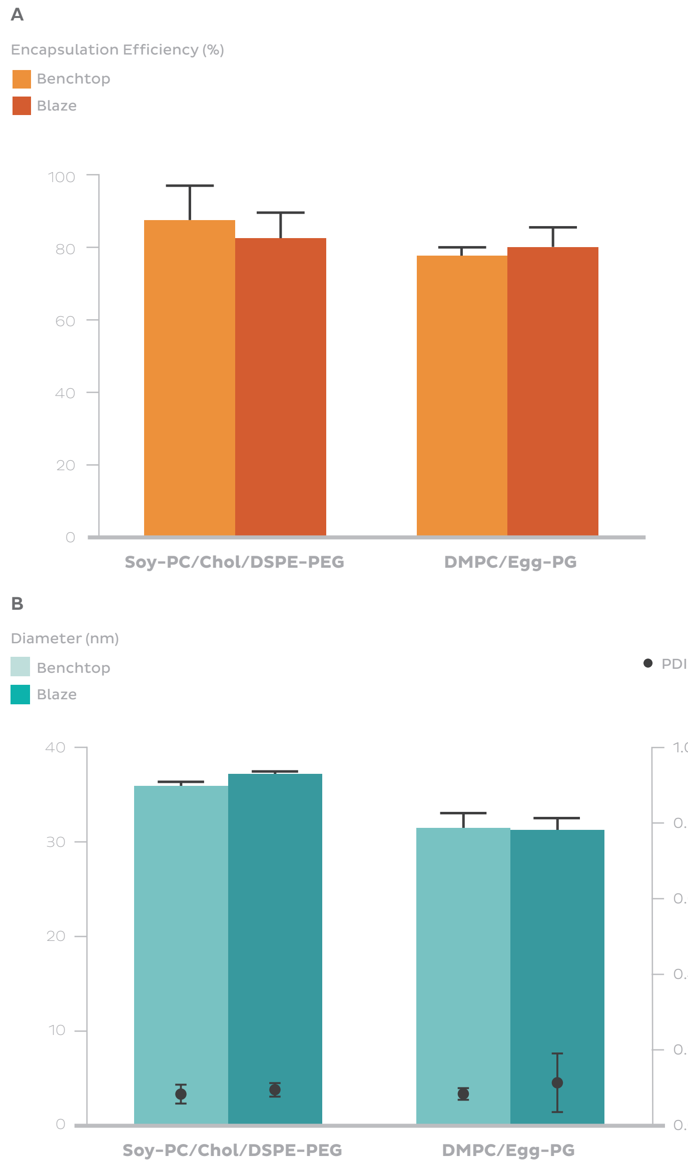
Figure 2. Soy PC liposome size decreases asymptotically with increasing FRR and TFR. *Hydrodynamic size and particle size distribution (expressed as polydispersity index, PDI) (both determined by dynamic light scattering) of soy-PC liposomes formulated at various flow rate ratios (A) and various total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

Formulation details

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

Both formulations retained their physical characteristics (encapsulation efficiency and size) between 2 mL batches made using the NanoAssemblr Benchtop and 20 mL batches made using the NanoAssemblr Blaze (Figure 3). Formulation parameters such as concentrations of reagents, TFR, and FRR developed using the Benchtop, were held constant when scaling up on the Blaze. Over 80% encapsulation efficiency was observed for all formulations. Using dynamic light scattering, liposome size was determined to be 35 nm with polydispersity index (PDI) less than 0.2, indicating monodisperse populations. Importantly, encapsulation efficiencies and average liposome size were statistically indistinguishable between 2 mL Benchtop batches and 20 mL Blaze batches, demonstrating that parameters optimized using the Benchtop instrument can be transferred to the NanoAssemblr Blaze for larger scale production with identical results and little to no process redevelopment.

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.



Formulation details

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

Discussion

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.

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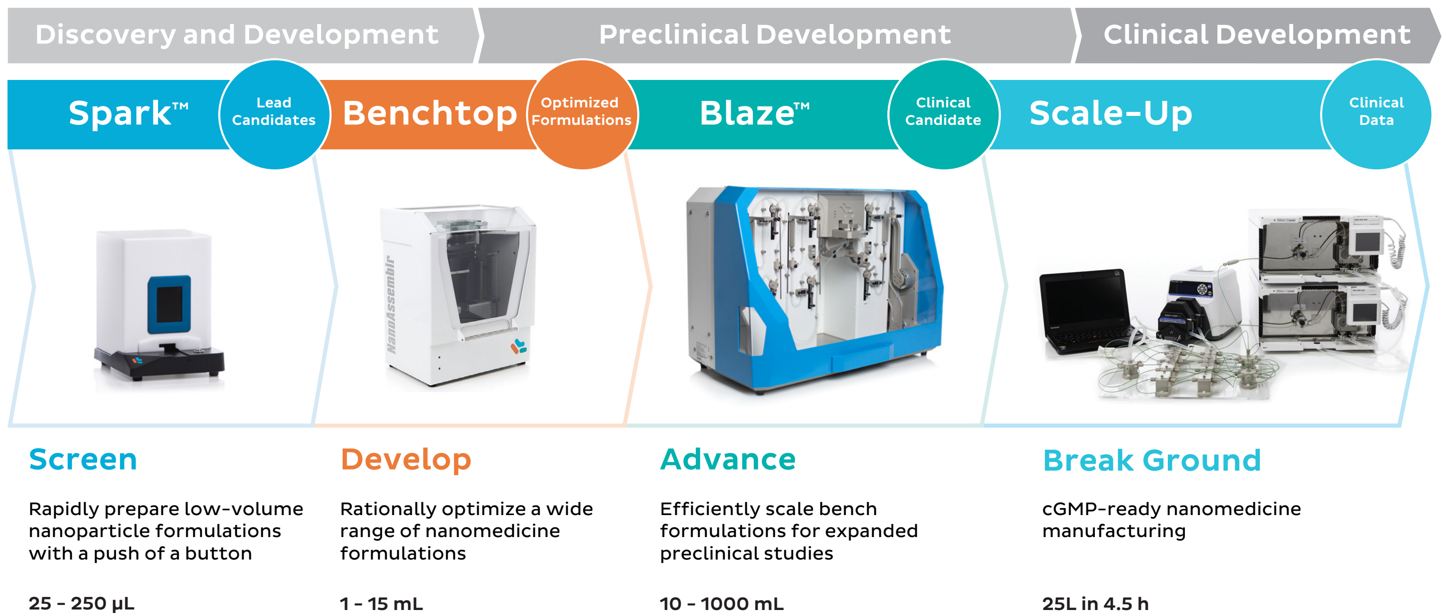

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