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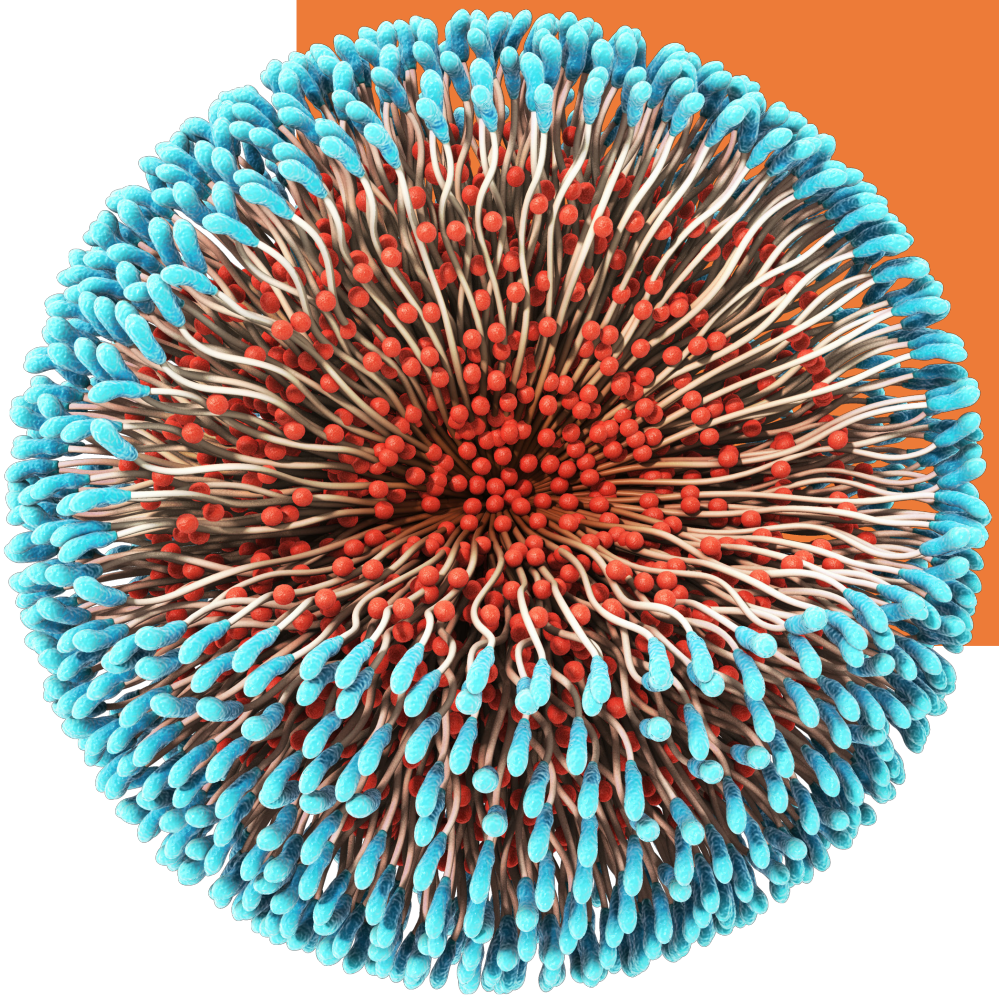
Formulating Polymer Nanoparticles



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Introduction

Polymeric nanoparticles are excellent carriers for a variety of drugs and are being explored for applications that include:

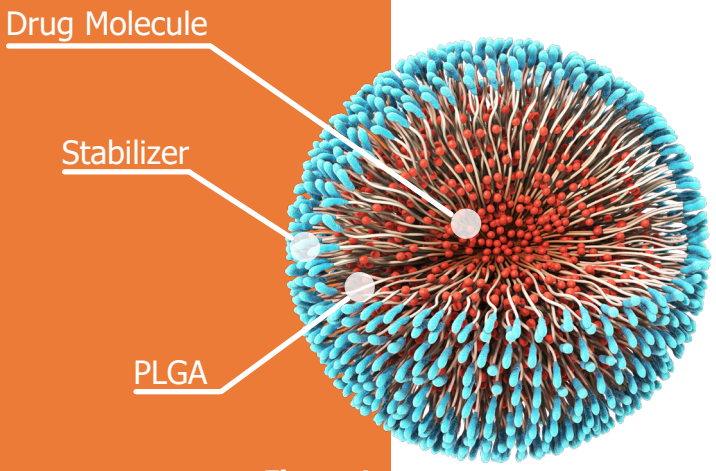


Figure 1

- Selective biodistribution of chemotherapeutic agents in tumors to reduce off-target toxicity and widen the therapeutic window
- Encapsulation and delivery of biomolecules for genetic medicine, gene editing and immunotherapy
- Encapsulation and co-delivery of multiple APIs and/or image contrast agents for combination drug therapy or theranostics

A polymer matrix that encapsulates drug molecules can offer sustained release features. Hydrophobic biodegradable polymers are typically used, such as poly(lactide-co-glycolide) (PLGA) or amphiphilic block-copolymers of poly(ethylene glycol) and PLGA (PEG-b-PLGA). PLGA degrades in the body to its monomeric biocompatible components, lactic acid and glycolic acid, thus releasing the drug from the polymer matrix.

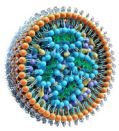
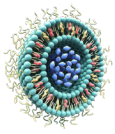
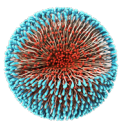
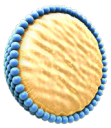
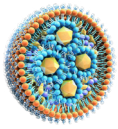
To formulate polymeric nanoparticles with the NanoAssemblr® technology, the polymers and hydrophobic drug molecules are dissolved together in an organic solvent and mixed rapidly with an aqueous phase where the increase in polarity creates a momentary supersaturation of the polymer and drug triggering rapid precipitation into particles. Homogeneous mixing ensures particles are formed under consistent conditions resulting in a narrow size distribution. Computer-controlled independent injection of both liquids allows mixing speed and mixing ratio to be easily dialed-in to systematically optimize particle formation parameters and reduce batch-to-batch variability.

Amphiphilic block copolymers assemble into micelles with a hydrophobic core protected by the hydrophilic shell. Hydrophobic polymers are typically formulated with a surfactant dissolved in the aqueous phase, which assembles on the surface of nascent particles to stabilize them (**Figure 1**). However in some cases, a surfactant is not needed or much lower surfactant concentrations can be used compared to conventional methods (such as emulsification solvent diffusion).

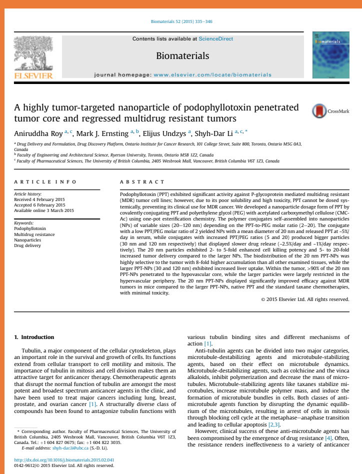
This collection highlights applications of NanoAssemblr technology for producing polymeric nanoparticles. Several examples from peer-reviewed literature as well as some application notes with original results are presented below. These illustrate the multitude of applications of NanoAssemblr technology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

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

Versatile Applications

PARTICLE TYPE		ACTIVE INGREDIENT				EXAMPLE APPLICATION	CARRIER MATERIALS
Nucleic acid Lipid Nanoparticles (LNP)		Nucleic Acids	Peptides and Proteins			• 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 Polymer Publications



Biomaterials 52 (2015) 335–346

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

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ABSTRACT

Podophyllotoxin (PPT) exhibited significant activity against P-glycoprotein mediated multidrug resistant (MDR) tumor cell lines; however, due to its poor solubility and high toxicity, PPT cannot be dosed systemically, preventing its clinical use for MDR cancer. We developed a nanoparticle dosage form of PPT by covalently conjugating PPT and polyethylene glycol (PEG) with acetylated carboxymethyl cellulose (CMC-Ac) using one-pot esterification chemistry. The polymer conjugates self-assembled into nanoparticles (NPs) of variable sizes (20–120 nm) depending on the PPT-to-PEG molar ratio (2–20). The conjugate with a low PPT/PEG molar ratio of 2 yielded NPs with a mean diameter of 20 nm and released PPT at ~5%/day in serum, while conjugates with increased PPT/PEG ratios (5 and 20) produced bigger particles (30 nm and 120 nm respectively) that displayed slower drug release (~2.5%/day and ~1%/day respectively). The 20 nm particles exhibited 2- to 5-fold enhanced cell killing potency and 5- to 20-fold increased tumor delivery compared to the larger NPs. The biodistribution of the 20 nm PPT-NPs was highly selective to the tumor with 8-fold higher accumulation than all other examined tissues, while the larger PPT-NPs (30 and 120 nm) exhibited increased liver uptake. Within the tumor, >90% of the 20 nm PPT-NPs penetrated to the hypovascular core, while the larger particles were largely restricted in the hypervascular periphery. The 20 nm PPT-NPs displayed significantly improved efficacy against MDR tumors in mice compared to the larger PPT-NPs, native PPT and the standard taxane chemotherapies, with minimal toxicity.

1. Introduction

Tubulin, a major component of the cellular cytoskeleton, plays an important role in the survival and growth of cells. Its functions extend from cellular transport to cell motility and mitosis. The importance of tubulin in mitosis and cell division makes them an attractive target for anticancer therapy. Chemotherapeutic agents that disrupt the normal function of tubulin are amongst the most potent and broadest spectrum anticancer agents in the clinic, and have been used to treat major cancers including lung, breast, prostate, and ovarian cancer [1]. A structurally diverse class of compounds has been found to antagonize tubulin functions with various tubulin binding sites and different mechanisms of action [1].

Anti-tubulin agents can be divided into two major categories, microtubule-destabilizing agents and microtubule-stabilizing agents, based on their effect on microtubule dynamics. Microtubule-destabilizing agents, such as colchicine and the vinca alkaloids, inhibit polymerization and decrease the mass of microtubules. Microtubule-stabilizing agents like taxanes stabilize microtubules, increase microtubule polymer mass, and induce the formation of microtubule bundles in cells. Both classes of anti-microtubule agents function by disrupting the dynamic equilibrium of the microtubules, resulting in arrest of cells in mitosis through blocking cell cycle at the metaphase–anaphase transition and leading to cellular apoptosis [2,3].

However, clinical success of these anti-microtubule agents has been compromised by the emergence of drug resistance [4]. Often, the resistance renders ineffectiveness to a variety of anticancer

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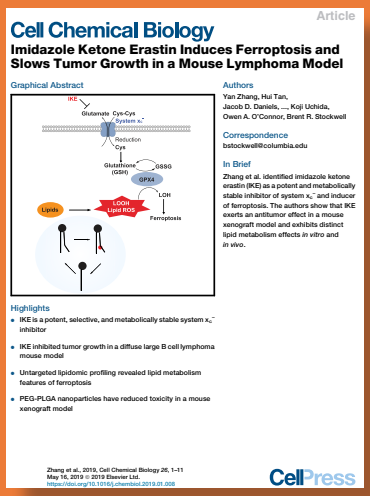
Biomaterials, 2015

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 fewer treatment options and higher mortality rates
- Podophyllotoxin (PDT) is a drug known to circumvent a major mechanism of cancer drug resistance, but its high cytotoxicity cannot be tolerated in high enough doses to be practical as a treatment
- In this paper 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
- The PDT-incorporated nanoparticles targeting of tumors and avoidance of healthy organ accumulation widens the therapeutic window of PDT
- They formulated the nanoparticles by conjugating PDT 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 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


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

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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
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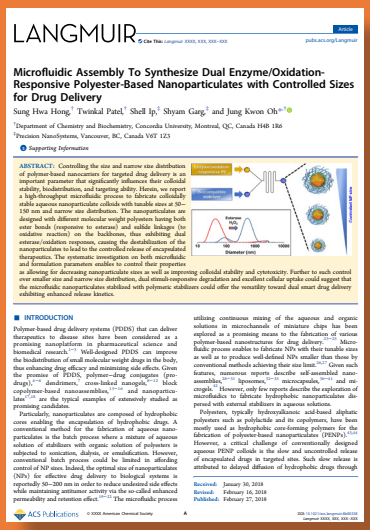
https://doi.org/10.1016/j.chembiol.2019.01.008

CellPress

Cell Chemical Biology, 2019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Microfluidic Assembly To Synthesize Dual Enzyme/Oxidation-Responsive Polyester-Based Nanoparticulates with Controlled Sizes for Drug Delivery

Sung Hwa Hong,[†] Twinkal Patel,[†] Shell Ip,[†] Shyam Garg,[†] and Jung Kwon Oh*[,†]

[†]Department of Chemistry and Biochemistry, Concordia University, Montreal, QC, Canada H4B 1R6
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Supporting Information

ABSTRACT: Controlling the size and narrow size distribution of polymer-based nanocarriers for targeted drug delivery is an important parameter that significantly influences their colloidal stability, biodistribution, and targeting ability. Herein, we report a high-throughput microfluidic process to fabricate colloidally stable aqueous nanoparticulate colloids with tunable sizes at 50–150 nm and narrow size distribution. The nanoparticulates are designed with different molecular weight polyesters having both ester bonds (responsive to esterase) and sulfide linkages (to oxidative reaction) on the backbone, thus exhibiting dual esterase/oxidation responses, causing the destabilization of the nanoparticulates to lead to the controlled release of encapsulated therapeutics. The systematic investigation on both microfluidic and formulation parameters enables to control their properties as allowing for decreasing nanoparticulate sizes as well as improving colloidal stability and cytotoxicity. Further to such control over smaller size and narrow size distribution, dual stimuli-responsive degradation and excellent cellular uptake could suggest that the microfluidic nanoparticulates stabilized with polymeric stabilizers could offer the versatility toward dual smart drug delivery exhibiting enhanced release kinetics.

■ INTRODUCTION

Polymer-based drug delivery systems (PDDS) that can deliver therapeutics to disease sites have been considered as a promising nanoplatform in pharmaceutical science and biomedical research.[1,2] Well-designed PDDS can improve the biodistribution of small molecular weight drugs in the body, thus enhancing drug efficacy and minimizing side effects. Given the promise of PDDS, polymer–drug conjugates (pro-drugs),[3,4] dendrimers, cross-linked nanogels,[5–13] block copolymer-based nanoassemblies,[14–19] and nanoparticulates[17,20] are the typical examples of extensively studied as promising candidates.

Particularly, nanoparticulates are composed of hydrophobic cores enabling the encapsulation of hydrophobic drugs. A conventional method for the fabrication of aqueous nanoparticulates is the batch process where a mixture of aqueous solution of stabilizers with organic solution of polymers is subjected to sonication, dialysis, or emulsification. However, conventional batch process could be limited in affording control of NP sizes. Indeed, the optimal size of nanoparticulates (NPs) for effective drug delivery to biological systems is reportedly 50–200 nm in order to reduce undesired side effects while maintaining antitumor activity via the so-called enhanced permeability and retention effect.[20−22] The microfluidic process utilizing continuous mixing of the aqueous and organic solutions in microchannels of miniature chips has been explored as a promising means to the fabrication of various polymer-based nanostructures for drug delivery.[23−30] Microfluidic process enables to fabricate NPs with their tunable sizes as well as to produce well-defined NPs smaller than those by conventional methods achieving their size limit.[30,31] Given such features, numerous reports describe self-assembled nanoassemblies,[30−32] liposomes,[30,33] microcapsules,[30,34] and microgels.[35] However, only few reports describe the exploration of microfluidics to fabricate hydrophobic nanoparticulates dispersed with external stabilizers in aqueous solutions.

Polyesters, typically hydroxyalkanoic acid-based aliphatic polyesters such as polylactide and its copolymers, have been mostly used as hydrophobic core-forming polymers for the fabrication of polyester-based nanoparticulates (PENPs).[17,18] However, a critical challenge of conventionally designed aqueous PENP colloids is the slow and uncontrolled release of encapsulated drugs in targeted sites. Such slow release is attributed to delayed diffusion of hydrophobic drugs through

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ACS Publications

Langmuir, 2018

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 Brian Stockwell, 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

Microfluidic Assembly To Synthesize Dual Enzyme/Oxidation-Responsive Polyester-Based Nanoparticulates with Controlled Sizes for Drug Delivery

Hong S, Patel T, Ip S, Garg S, Oh J. Langmuir 2018; 34: 3316-3325.

Summary

- Conventional polymers used for drug delivery degrade by hydrolysis which is not tissue specific
- Professor Jung Kwon Oh from Concordia University and Canada Research Chair in Nanobioscience has developed drug delivery polymers that degrade faster in response to two hallmark characteristics of the tumour environment — reactive oxygen species and esterases — to improve drug release specifically within the tumor
- Since size and size distribution of nanoparticles is important for effective tumour accumulation, the Oh group collaborated with researchers from Precision NanoSystems to optimize and control size and size distribution with NanoAssemblr Technology
- Formulations were rapidly optimized to achieve the desired particle size between 50-150 nm with narrow size distribution (PDI < 0.2) and comparisons with conventional formulation processes show the NanoAssemblr process produced significantly smaller particle size and improved colloidal stability with the same compositions
- Drug release was tested and the presence of the two stimuli — reactive oxygen species and esterases — was found to accelerate drug release
- These findings demonstrate the advantages of using NanoAssemblr technology to rapidly optimize innovative new materials for cancer drug delivery

Journal of Drug Delivery Science and Technology

Microfluidic manufacturing improves polydispersity of multicomponent polymeric nanoparticles

Kathrin Abstiens, Achim M. Goepferich*

Journal of Drug Delivery Science and Technology 2019

Microfluidic Manufacturing Improves Polydispersity of Multicomponent Polymeric Nanoparticles

Abstiens K, Göpferich A. Journal of Drug Delivery Science and Technology 2019; 49: 433-439.

Summary

- Biocompatible, biodegradable nanoparticles composed of poly(lactic acid)-poly(ethylene glycol) (PLA-PEG) surrounding a poly(lactic-co-glycolic acid) (PLGA) core combine desirable features for drug delivery such as loading of hydrophobic drugs, controlled drug release with tunable release profiles and receptor targeting
- However, bulk nanoprecipitation typically results in batch-to-batch inconsistency, unequal distribution of PLGA in the particle core and high polydispersity
- Researchers at Regensburg University led by Dr. Achim Göpferich used NanoAssemblr technology to achieve well defined particle characteristics and batch-to-batch consistency with this formulation
- Particles produced with NanoAssemblr technology were found to be significantly smaller and monodisperse compared to bulk nanoprecipitation, demonstrating that the technology is valuable for reproducible and scalable manufacture of multi-component polymer nanoparticles for drug delivery

Regular Article

The Use of an Efficient Microfluidic Mixing System for Generating Stabilized Polymeric Nanoparticles for Controlled Drug Release

Yoshinori Morikawa, Tatsuaki Tagami, Akihiro Hoshikawa, and Tetsuya Ozeki*

Biological Pharmaceutical Bulletin 2018

The Use of an Efficient Microfluidic Mixing System for Generating Stabilized Polymeric Nanoparticles for Controlled Drug Release

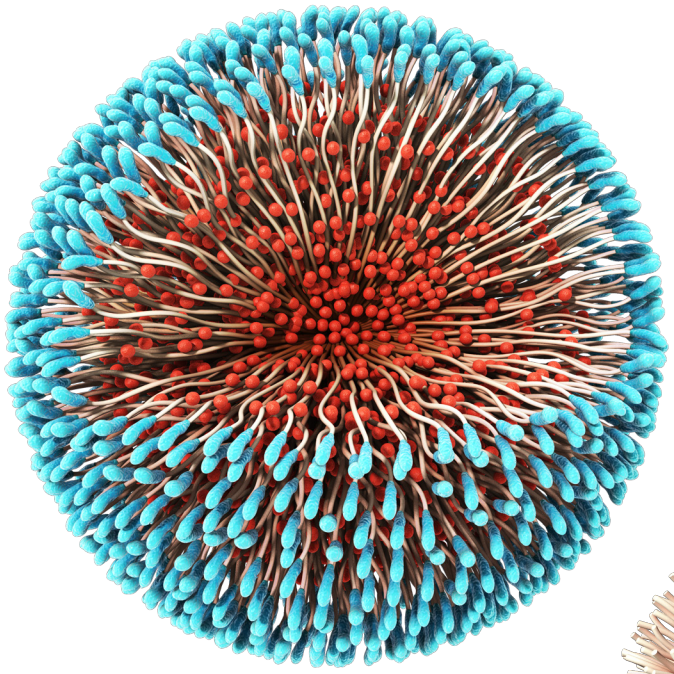
A Morikawa Y, Tagami T, Hoshikawa A, Ozeki T. Biological and Pharmaceutical Bulletin 2018; 41: 899-907.

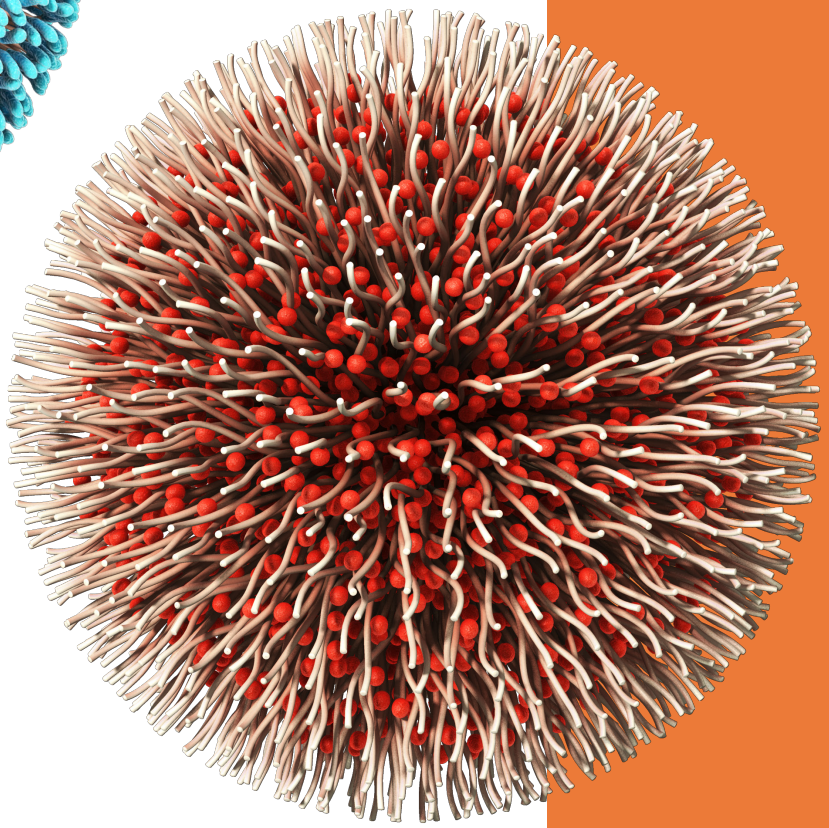
Summary

- Polymeric nanoparticles based on PLGA show promise as carriers for the controlled release of drugs, but development is limited by poor reproducibility and scalability
- The Ozeki group at Nagoya University in Japan is looking to overcome this limitation using NanoAssemblr technology to fine-tune the production of PLGA nanoparticles encapsulating curcumin, making use of the instrument's ability to control key formulation parameters
- By being able to identify and optimise key experimental settings they produced small, homogenous polymeric nanoparticles which efficiently encapsulated curcumin
- These authors demonstrated the utility of NanoAssemblr technology for the production of polymeric nanomedicines, and its potential for use in settings requiring scale-up or high throughput

PLGA Nanoparticles

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 nm[4] 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, the formulation of the NP, and the interaction of the components 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 microfluidic instrument. 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 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.

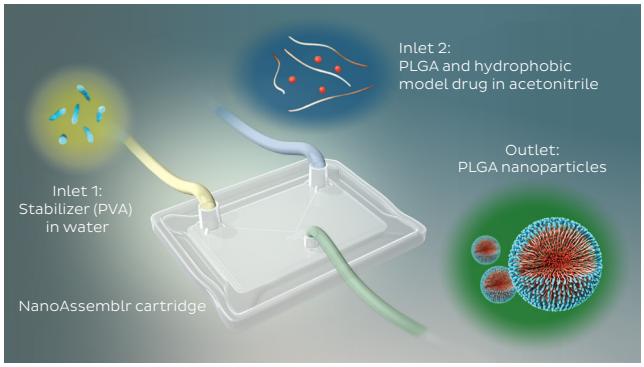


Figure 1. *Microfluidic mixing process employed by the NanoAssemblr provides rapid, homogeneous mixing and controls the nanoprecipitation of PLGA NPs with in situ drug loading.*

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.

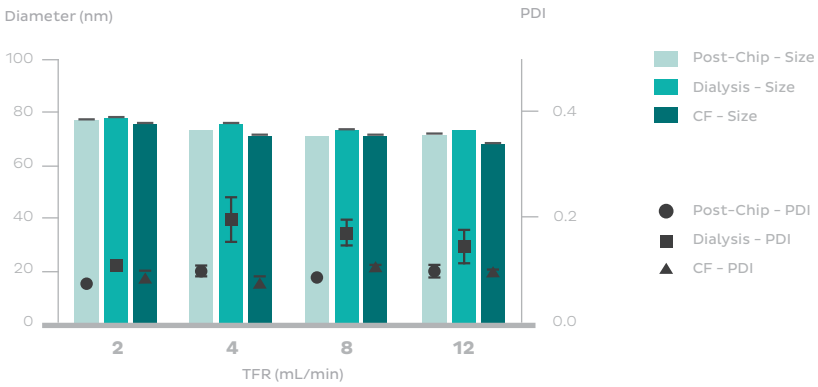


Figure 2. *Effect of solvent removal method on the size and PDI of PLGA nanoparticles at polymer concentrations of 5 mg/mL (CF, centrifugal filtration). Each bar/plot represents the mean ± SEM for 3 independent size/PDI measurements on three independent samples (n = 3).*

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.

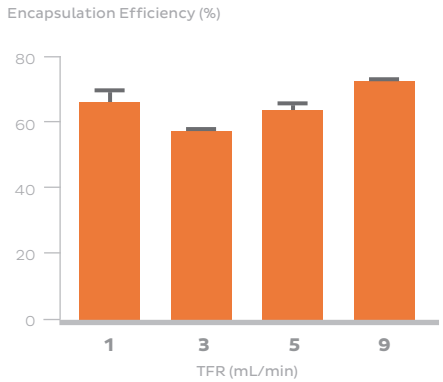


Figure 2. *Encapsulation of coumarin-6 in PLGA NPs as a function of total flow rate (TFR). Each bar represents the mean ± SEM for 3 independent measurements on three independent samples (n = 3).*

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, $P < 0.05$).

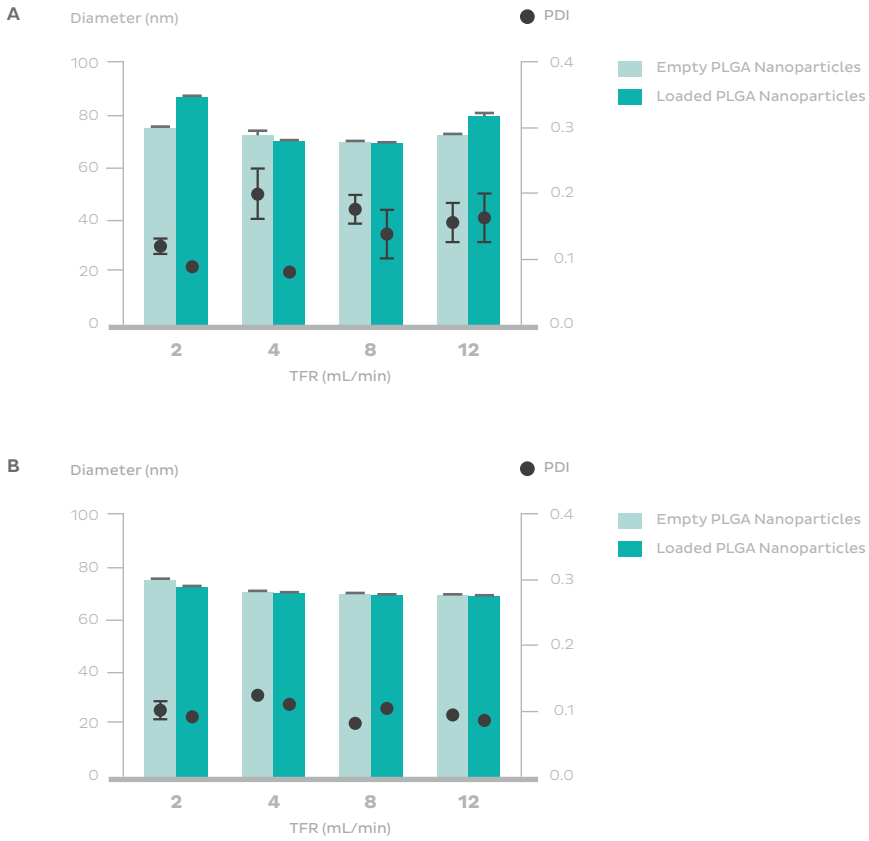


Figure 4. *The effect of coumarin-6 encapsulation on the size and PDI of PLGA NP at polymer concentrations of 5 mg/mL buffer — exchanged by either A) dialysis or B) centrifugal filtration. Each bar/plot represents the mean ± SEM for three independent size/PDI measurements on three independent samples (n = 3). Error bars not shown if smaller than symbol.*

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.

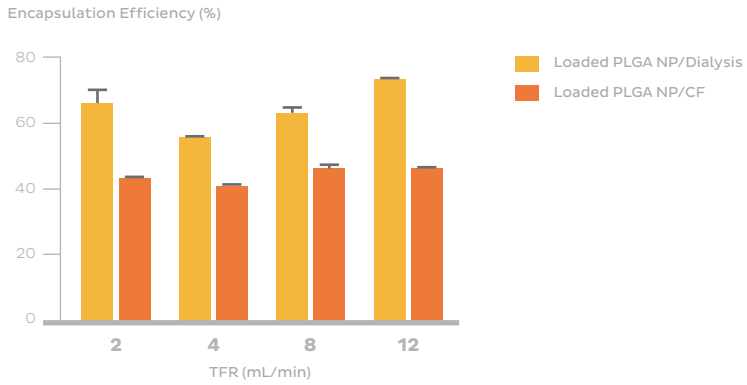


Figure 5. *Encapsulation of C6 (0.25 % w/w of the polymer) in PLGA nanoparticles at polymer concentrations of 5 mg/mL either dialyzed or buffer exchanged by centrifugal filtration (CF). Each bar represents the mean ± SEM for 3 independent measurements on three independent samples (n = 3).*

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 **Figure 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 fewer encapsulations when compared to NP processed via dialysis. Alternatively, solvent exchange by dialysis is a slower process than CF, which allows solvent exchange to occur under equilibrium conditions where structural relaxation of the PLGA core can occur, thus 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 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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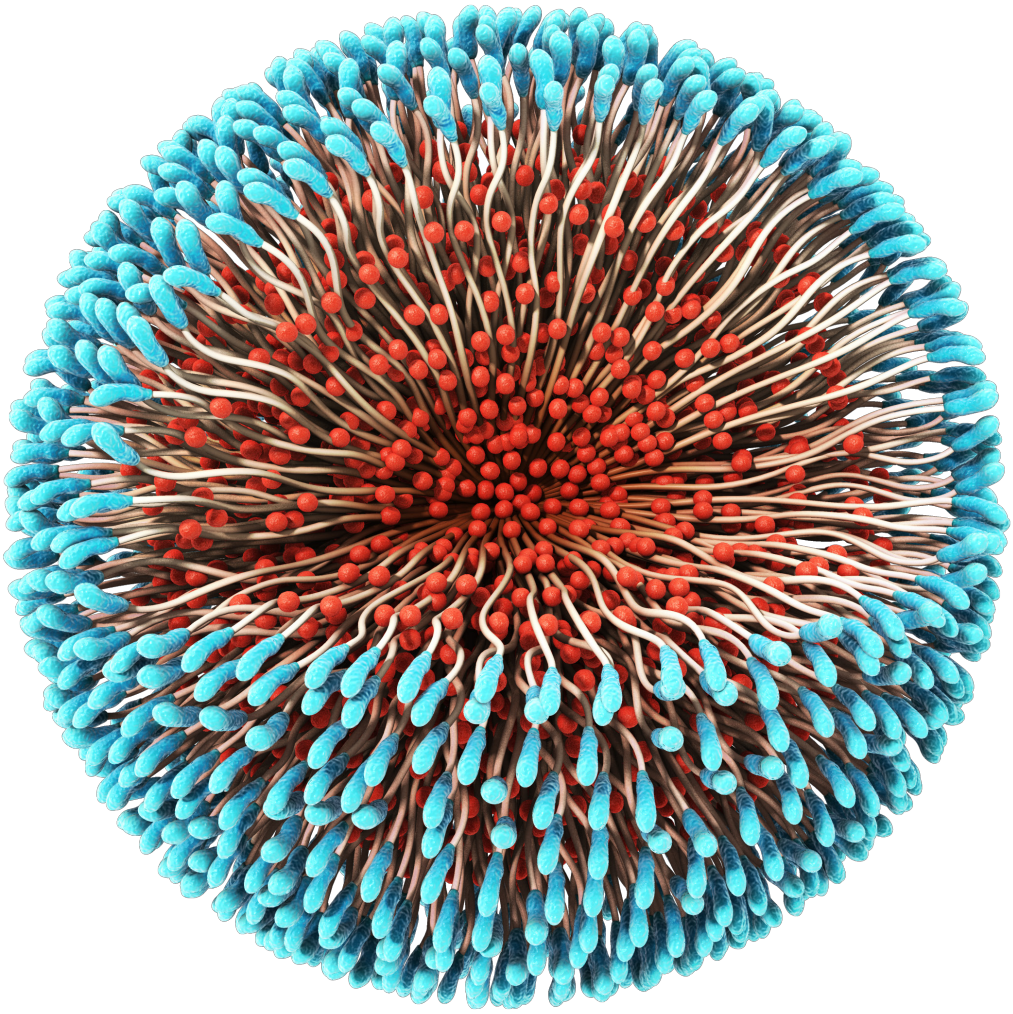
PLGA Nanoparticles

Reproducible Production of Sub-100 nm PLGA Nanoparticles using the NanoAssemblr® Microfluidic Platform



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

Polymeric nanoparticles of biodegradable and biocompatible polymers such as poly(lactic-co-glycolic acid) (PLGA) are emerging as a promising tool for drug delivery applications. However, there are several challenges which exist in translating these technologies from the bench to the clinic. Prominently, current nanoparticle manufacturing methods lack batch-to-batch reproducibility and are unable to generate PLGA nanoparticles below 100 nm in size with narrow particle size distributions. This leads to inconsistencies in nanoparticle quality that hinder the clinical success of the formulation. Here, we present a novel method for the manufacture of polymeric nanoparticles using a microfluidic technology that addresses these key manufacturing concerns.

Introduction

As the field of nanomedicine continues to expand and the number of polymeric nanoparticle based drugs under development increases, the role of the manufacturing process becomes a critical factor to the drug's success. The specific processes used to produce nanoparticles can affect the size, drug encapsulation efficiency and drug release properties. Batch-to-batch consistency across all these characteristics is important, so a high level of manufacturing reproducibility is required. For polymer-based nanoparticles such as poly(lactide-co-glycolide) or poly(lactic-co-glycolic acid) (PLGA), two key challenges for conventional manufacturing are the ability to obtain particle sizes below 100 nm and to maintain batch-to-batch consistency.

Nanoparticle size plays a critical role in tissue penetration, biodistribution, drug release kinetics and drug efficacy.[1] Literature reports suggest that larger size particles tend to be cleared rapidly from the body whereas smaller size particles below 100 nm exhibit reduced clearance and have a greater ability to reach their intended target.[2,3] Nanoparticles below 100 nm also exhibit higher uptake into cells when compared to nanoparticles with sizes > 100 nm of the same composition.[4] Conventional manufacturing methods such as Emulsion Solvent Diffusion (ESD), Emulsion Solvent Evaporation (ESE), and nanoprecipitation can produce PLGA nanoparticles in the range of 100–1000 nm, however, these methods lack the precise control to specifically tune the size of the nanoparticles.[5] Barring a few reports, most conventional methods of manufacturing PLGA nanoparticles are unable to achieve stable sizes below 100 nm.[6]

In addition to nanoparticle size itself, size uniformity is another key factor associated with the manufacturing process. Narrow size distributions (low polydispersity) lead to more consistent results amongst batches which is important for downstream clinical success. Most current methods for manufacturing PLGA nanoparticles operate under heterogeneous mixing environments leading to inconsistency amongst batches and typically yields broad size distributions (high polydispersity). These nanoparticle batches require additional purification and processing steps that lowers the overall yield and contributes to product loss during manufacturing. For PLGA nanoparticles in particular, lack of uniformity and batch-to-batch size variability can cause differences in drug efficacy which highlights the need for a highly reproducible manufacturing process.[5]

Microfluidic methods for manufacturing nanoparticles provide precise control over nanoparticle characteristics such as nanoparticle size and size distribution by providing a rapid and controlled mixing environment at the nanolitre scale.[7] The NanoAssemblr platform (Precision NanoSystems Inc.) is an automated microfluidic system which, in addition to precise control, removes user variability leading to a high degree of reproducibility between batches. Here, the authors present the NanoAssemblr platform for manufacturing PLGA nanoparticles, utilizing a homogenous mixing environment to achieve high batch-to-batch reproducibility, sub-100 nm nanoparticle size and low polydispersity in particle size.

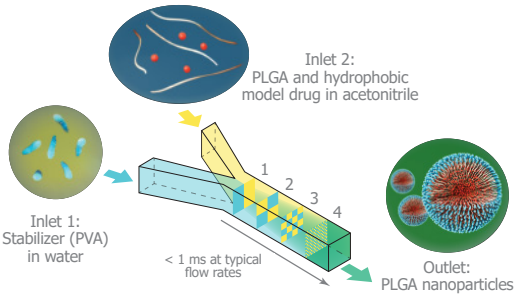
PLGA Nanoparticles were manufactured on the NanoAssemblr as illustrated in **Figure 1**. The platform provided a rapid and controlled mixing environment which favored the formation of PLGA nanoparticles at reproducible sizes below 100 nm when manufactured at different times by different users (**Figure 2**). As seen in the figure, three batches of PLGA nanoparticles prepared by different users exhibit a size of ~75 nm which is not significantly different between each batch ($P > 0.05$). Similarly, the Polydispersity (PDI) was not significantly different between batches ($P > 0.05$) and was at or below 0.2 indicating a narrow size distribution for PLGA nanoparticles.

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

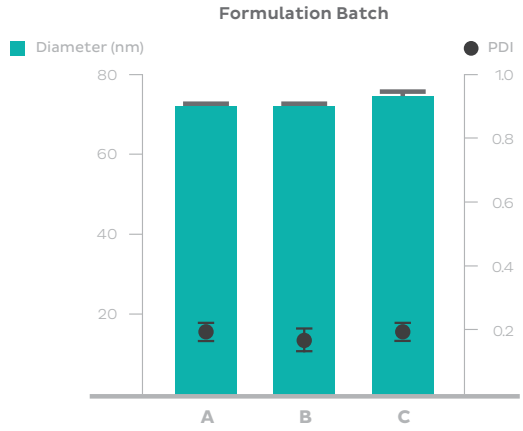
Figure 2. *PLGA nanoparticles of size ~75 nm and PDI ~0.2 were reproducibly manufactured using the NanoAssemblr by three different users. Each bar/plot represents the mean ± SD for 3 independent size measurements on the same sample. *denotes significant difference in size between different users (P < 0.05), #denotes significant difference in PDI between different users (P < 0.05) (One way ANOVA followed by Tukey's post-hoc test, P < 0.05).*

Figure 3*. Effect of Coumarin-6 encapsulation on the size and PDI of PLGA nanoparticles at polymer concentrations of 5 mg/mL prepared using the NanoAssemblr and purified using dialysis. Each bar/plot represents the mean ± SD for 3 independent size measurements on three independent samples (n = 3). *denotes significant difference in size between empty and loaded PLGA nanoparticles (P < 0.05), #denotes significant difference in PDI between empty and loaded PLGA nanoparticles (P < 0.05) (Students' t-test, P < 0.05).*

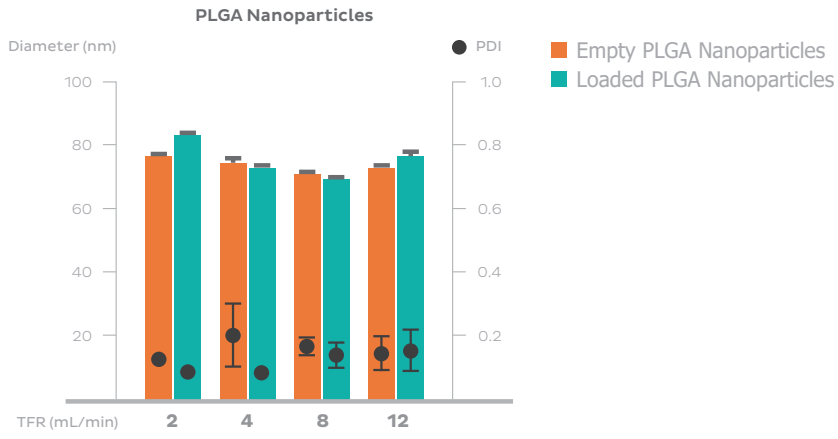
Figure 1



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.



Additionally, the NanoAssemblr platform was assessed for its utility in generating drug-loaded PLGA nanoparticles below 100 nm using Coumarin-6 as a model hydrophobic small-molecule drug. As demonstrated in **Figure 3**, the NanoAssemblr produced PLGA nanoparticles ≤ 100 nm under a variety of conditions. Particle size was not a strong function of flow rate. Additionally, no change in size was observed upon encapsulation of C6. The PDI of the nanoparticles was between 0.1–0.2 indicating a narrow size distribution for drug-loaded PLGA nanoparticles.



Discussion

Research on synthetic biodegradable polymers such as PLGA has recently gained momentum in drug delivery applications due to their biocompatibility and biodegradability. While there are a number of methods that are currently used for the manufacture of PLGA nanoparticles for drug delivery, challenges remain such as achieving sub-100 nm sizes and consistent particle characteristics from batch-to-batch. Here, we described the production of PLGA nanoparticles using a novel microfluidic manufacturing solution, the NanoAssemblr, which addresses these key issues. PLGA was selected as the model polymeric system as it is one of the most attractive polymers for nanoparticle-based drug delivery applications due to its current approval by the United States Food and Drug Administration (US FDA) and European Medical Agency (EMA) for parenteral drug delivery systems.[8]

The results presented here demonstrate the ability of the NanoAssemblr instrument to produce PLGA nanoparticles in a highly reproducible manner, achieving sizes below 100 nm that are difficult to obtain using conventional manufacturing methods. The smaller size of 100 nm expands the possibilities of PLGA nanoparticles for use in drug delivery applications that favor small size particles. As shown in **Figure 2**, the size of the PLGA nanoparticles remain consistent across batches, highlighting the excellent reproducibility of the PLGA nanoparticle formulations developed using the NanoAssemblr platform. We also demonstrated that sub-100 nm nanoparticles are achievable in both the 'empty' state (PLGA only) or when loaded with a model hydrophobic drug such as Coumarin-6, shown in **Figure 3**. Together, these data demonstrate that microfluidic manufacturing of polymer-based nanoparticles using the NanoAssemblr platform is an attractive alternative to conventional manufacturing methods.

Conclusion

The NanoAssemblr can reproducibly achieve sub-100 nm size PLGA nanoparticles, thereby expanding their applications in drug delivery and also eliminating concerns regarding batch-to-batch variability in particle characteristics. In conclusion, the NanoAssemblr platform is an important tool for the efficient manufacture of PLGA nanoparticles to enable the encapsulation and delivery of hydrophobic small-molecule therapeutics.

Materials & Methods

MATERIALS

Ester-terminated PLGA (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) and Coumarin-6 were purchased from Sigma-Aldrich (St Louis, MO). All other materials were reagent grade.

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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

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 2% w/v PVA solution was injected through the other inlet of the microfluidic mixer. The total formulation volume, aqueous:organic flow rate ratio (FRR), and the total flow rate (TFR) was set at 2 mL, 1:1, and 8 mL min, respectively. 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 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 testing the reproducibility between batches, three independent experiments were carried out by three different users by following the above mentioned method.

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.

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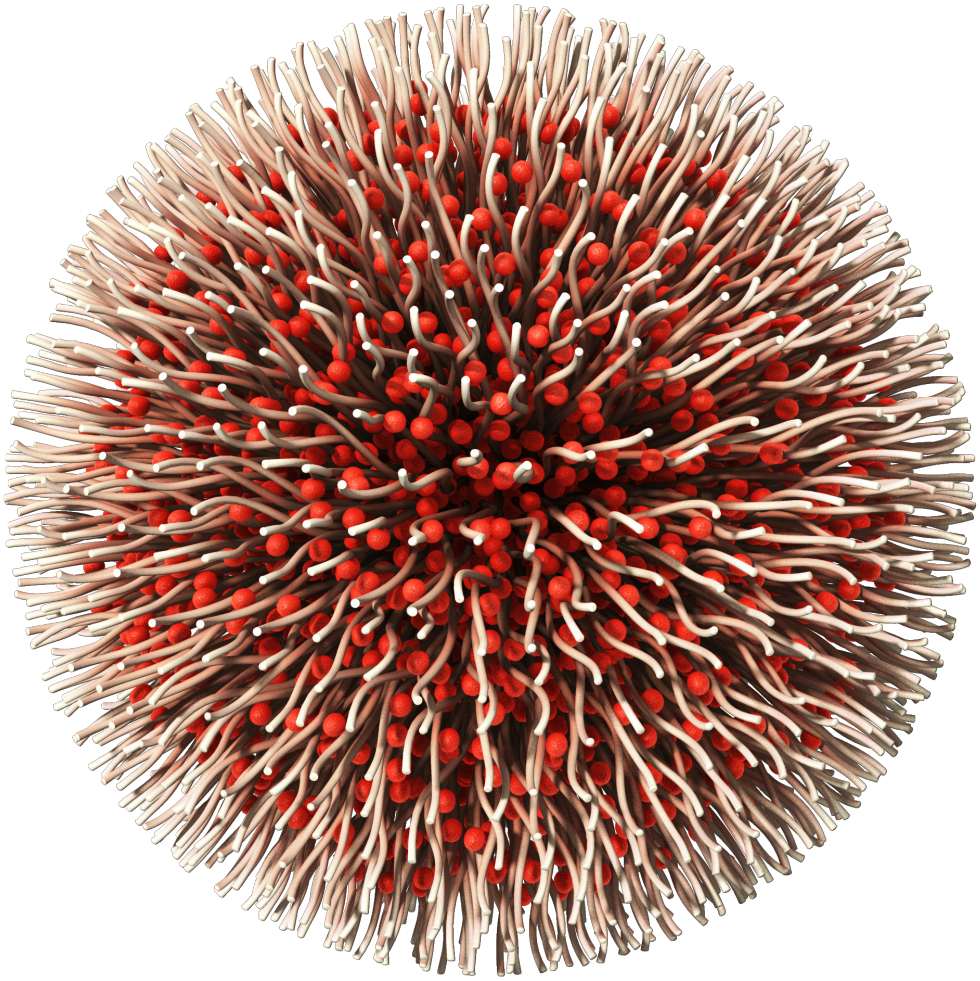
PLGA Nanoparticles

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

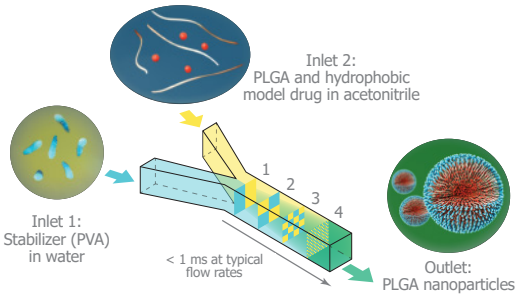
Formulation Parameters	
Polymer (PLGA) concentration 5–40 mg/mL	POPC/Chol/DSPE-PEG_{2000} (52:45:3 mol%)
Stabilizer (PVA) concentration 0.5–4.0 % w/v	10 mg/mL

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.

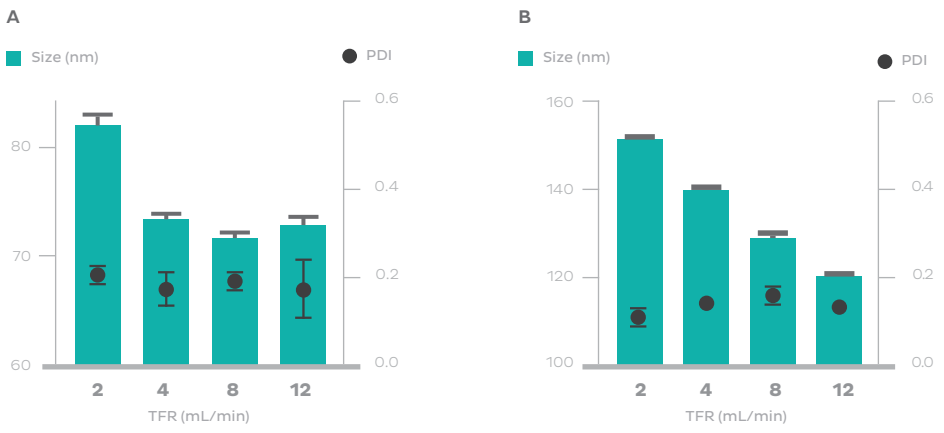


Figure 2. *Effect of total flow rate (TFR) on the size and polydispersity (PDI) of PLGA NP at polymer concentrations of (A) 5 mg/mL and (B) 20 mg/mL using the NanoAssemblr. Each bar/plot represents the mean ± SEM for three independent size/PDI measurements on three independent samples (n = 3). Error bars not shown if smaller than symbol.*

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.

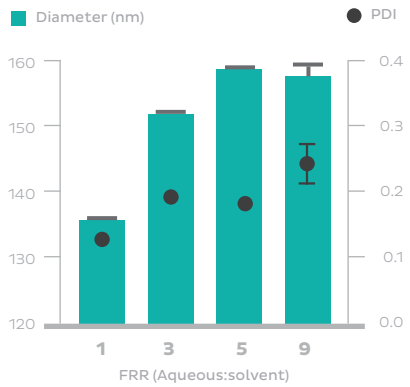


Figure 3. *Effect of aqueous:solvent flow rate ratio (FRR) on the size and PDI of PLGA nanoparticles at polymer concentrations of 20 mg/mL using the NanoAssemblr. Each bar/plot represents the mean ± SEM for 3 independent size/PDI measurements on three independent samples (n = 3). Error bars not shown if smaller than symbol.*

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.

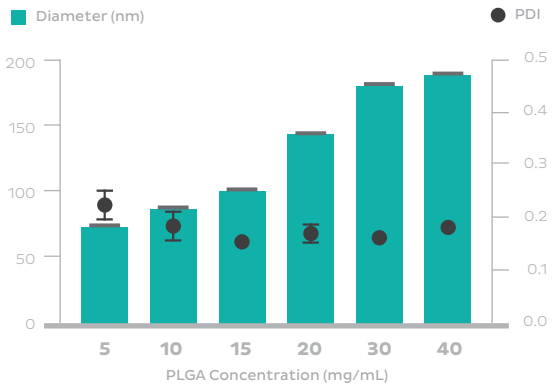


Figure 4. *Effect of PLGA polymer concentration on the size and PDI of PLGA nanoparticles using the NanoAssemblr. Each bar/plot represents the mean ± SEM for 3 independent size/PDI measurements on three independent samples (n = 3). Error bars not shown if smaller than symbol.*

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 was 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**.

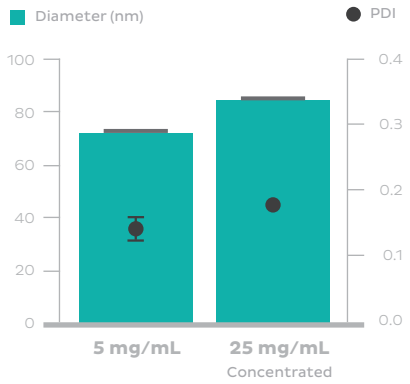


Figure 5. *Change in size of PLGA nanoparticles prepared using the NanoAssemblr at 5 mg/mL compared to the same formulation after concentrating to 25 mg/mL. Each bar/plot represents the mean ± SEM for 3 independent size/PDI measurements on three independent samples (n = 3). Error bars not shown if smaller than symbol.*

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.

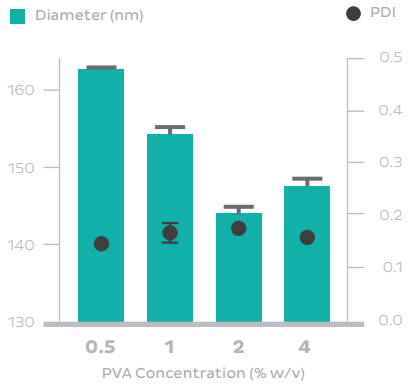


Figure 6. *Effect of PVA stabilizer concentration on the size and PDI of PLGA nanoparticles at polymer concentrations of 20 mg/mL using the NanoAssemblr. Each bar/plot represents the mean ± SEM for 3 independent size/PDI measurements on three independent samples (n = 3). Error bars not shown if smaller than symbol.*

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) as well as 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', 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 that 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**), 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 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.

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 concentrations ranging from 5–40 mg/mL, whereas, PVA was dissolved in deionized water at concentrations of 0.5–4 % 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 various TFR and aqueous:solvent FRR from 2–12 mL/min and 1:1–9:1, respectively. 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 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.

CHARACTERIZATION OF PLGA NANOPARTICLES.

PLGA nanoparticles 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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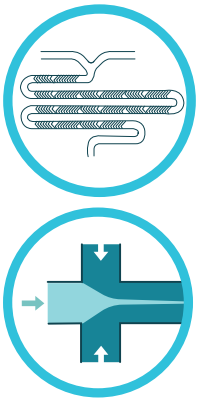
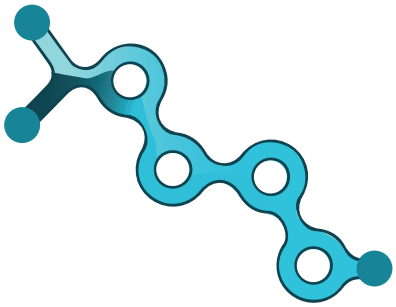
- Difficult to reproduce	- Difficult to reproduce	+ Reproducible	+ Reproducible
- Harsh process conditions	+ Gentler process conditions	+ Non-turbulent process conditions	+ Non-turbulent process conditions
- Difficult to scale	+ Improved Scalability		





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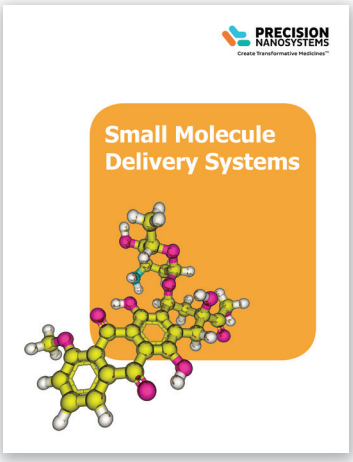

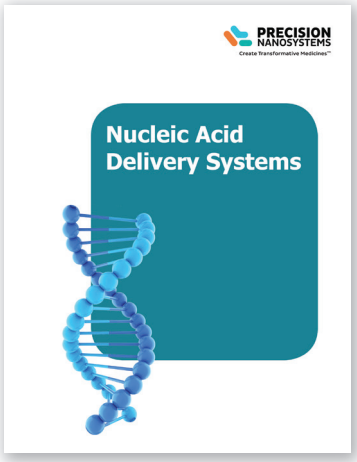

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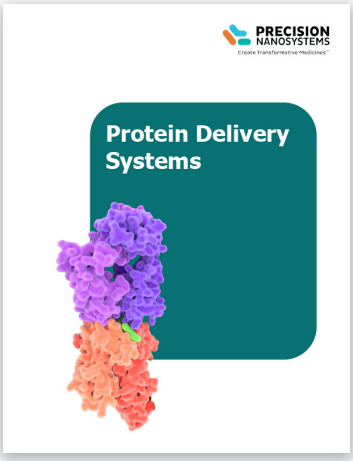

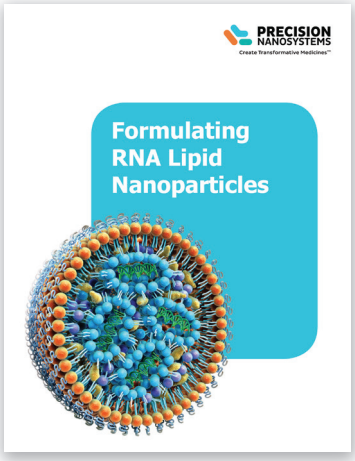

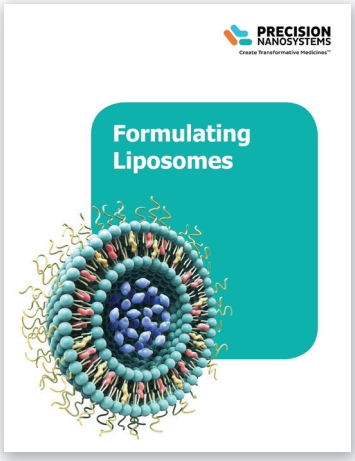

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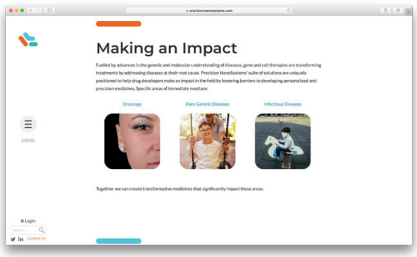

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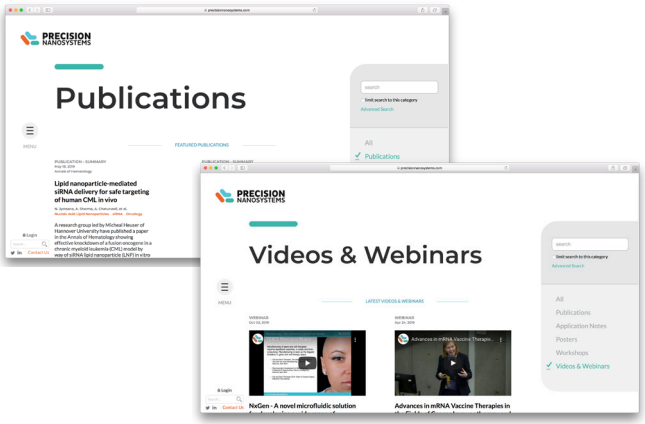

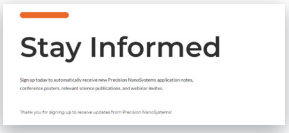

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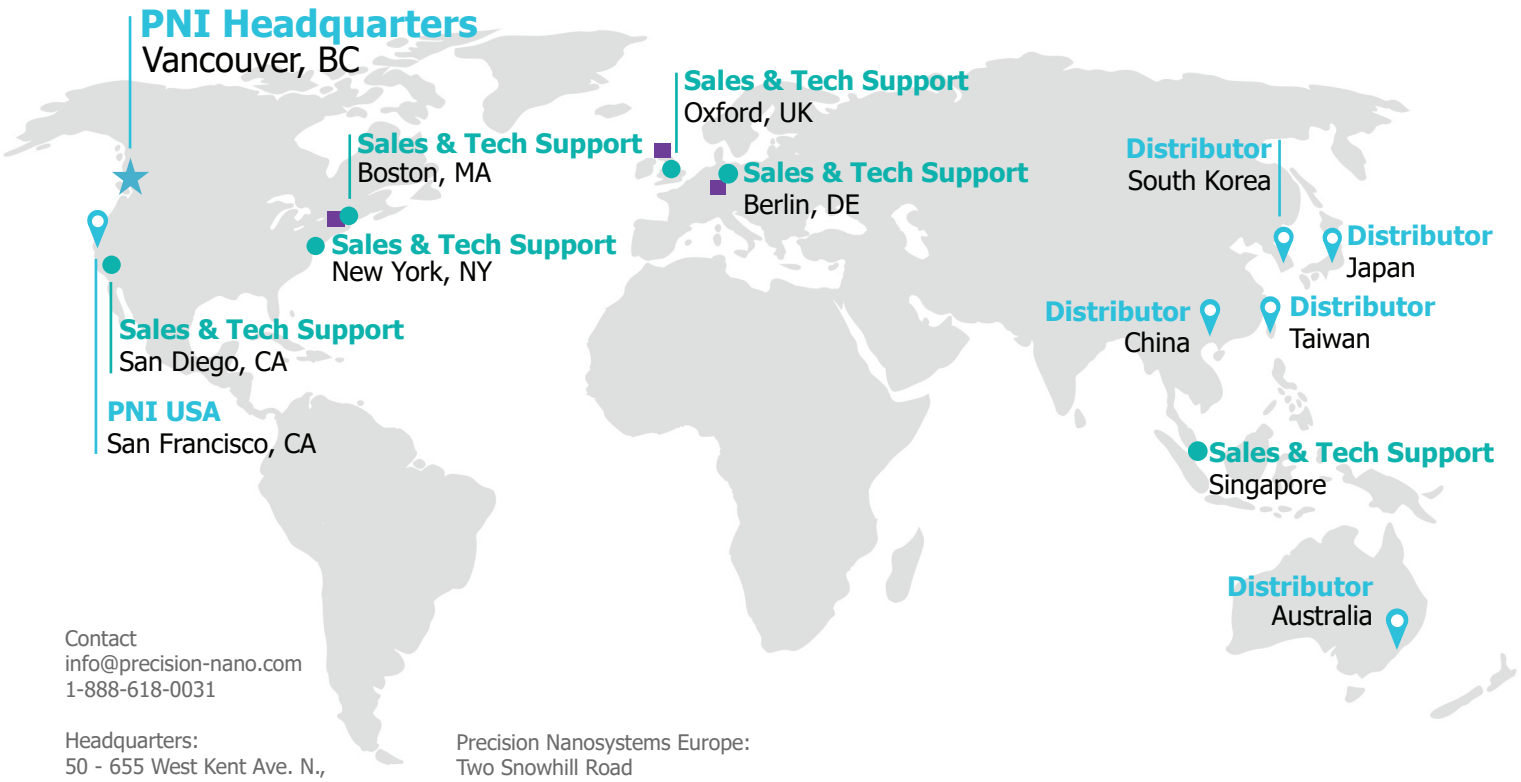

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