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# Flash NanoPrecipitation for the Encapsulation of Hydrophobic and Hydrophilic Compounds in Polymeric Nanoparticles

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We are submitting this manuscript, entitled "Flash NanoPrecipitation for the Encapsulation of Hydrophobic and Hydrophilic Compounds in Polymeric Nanoparticles," to the Journal of Visualized Experiments in response to your initial invitation from 2017 (Manuscript JoVE56575). We have prepared a manuscript presenting the Flash NanoPrecipitation method as applied to the encapsulation of both hydrophobic and hydrophilic compounds. The work in our lab has expanded the slate of compounds that can be encapsulated by this method significantly in the last few years. Additionally, we have described the use of two mixing geometries: a confined impinging jet mixer and a multi-inlet vortex mixer.

We seek to enable the use of this technology widely. To this end, we are including CAD drawings of the mixers to allow other research groups to fabricate their own mixers. The visualized format of JoVE is ideal for the widespread dissemination of the best practices we have developed in our lab. Thank you for your consideration.

The authors have contributed in the following specific manners: C. Markwalter was the primary author of the text, prepared the figures, and generated the data not otherwise attributed below. R. Pagels assisted in editing, figure design, and generated the data in Figure 4. B. Wilson and K. Ristroph generated data for the protocols described in the supplemental information for TGA analysis of concentration and assisted in editing. R. Prud'homme is the principal investigator for this work.

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Yours truly,

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**TITLE:**

# Flash NanoPrecipitation for the Encapsulation of Hydrophobic and Hydrophilic Compounds in Polymeric Nanoparticles

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**KEYWORDS:**

Nanoparticle, micromixing, block copolymer, drug delivery, peptide, protein, process scale-up

**SUMMARY:**

Flash NanoPrecipitation (FNP) is a scalable approach to produce polymeric core-shell nanoparticles. Lab-scale formulations for the encapsulation of hydrophobic or hydrophilic therapeutics are described.

**ABSTRACT:**

The formulation of a therapeutic compound into nanoparticles (NPs) can impart unique properties. For poorly water-soluble drugs, NP formulations can improve bioavailability and modify drug distribution within the body. For hydrophilic drugs like peptides or proteins, encapsulation within NPs can also provide protection from natural clearance mechanisms. There are few techniques for the production of polymeric NPs that are scalable. Flash NanoPrecipitation (FNP) is a process that uses engineered mixing geometries to produce NPs with narrow size distributions and tunable sizes between 30 and 400 nm. This protocol provides instructions on the laboratory-scale production of core-shell polymeric nanoparticles of a target size using FNP. The protocol can be implemented to encapsulate either hydrophilic or hydrophobic compounds with only minor modifications. The technique can be readily employed in the laboratory at milligram scale to screen formulations. Lead hits can directly be scaled up to gram- and kilogram-scale. As a continuous process, scale-up involves longer mixing process run time rather than translation to new process vessels. NPs produced by FNP are highly loaded with therapeutic, feature a dense stabilizing polymer brush, and have a size reproducibility of ± 6%.

**INTRODUCTION:**

Since the late 1990s, there has been a steady increase in the number of clinical trials employing nanomaterials[1,2]. The rising interest reflects the promise of nanomaterials to improve the bioavailability of hydrophobic drugs and to enable preferential targeting within the body[3]. Polymeric nanoparticles (referred to as nanoparticles or NPs here) represent a growing proportion of this class of materials[2]. NPs have garnered interest because they have highly tunable properties such as size, composition, and surface functionalization[4]. When applied to the administration of poorly soluble drugs, NPs frequently have a core-shell structure where the therapeutic is encapsulated in the hydrophobic core and the shell consists of a hydrophilic polymer brush. A simple way to generate this structure employs an amphiphilic diblock copolymer (BCP) consisting of a degradable hydrophobic block, which forms part of the particle core, and a hydrophilic poly(ethylene glycol) (PEG) block, which forms the polymer brush and imparts steric stabilization[4,5].

Nanoprecipitation is a common fabrication technique for polymeric nanoparticles because it is simple and not energy intensive[6]. In its simplest form, nanoprecipitation involves addition by pipette of NP components in an organic solvent like acetone to an excess volume of stirred water. The change in solvent to a dilute aqueous solution results in the precipitation of the insoluble core component. The stabilizer assembles on this growing particle surface, directed by adsorption of the collapsed hydrophobic block[7-10]. A uniform particle size distribution is obtained when the solvent and water rapidly mix to form a homogeneous solution. Mixing that is slower than the nucleation and assembly of the components results in a larger, more polydisperse particle population. Though readily accessible for a simple test, the stirred batch approach results in wide variability due to mixing inconsistency and is not amenable to scale-up[6,11]. Microfluidics have emerged as another avenue to NP production that can be run continuously. This means of production has been recently reviewed by Ding *et al.*[11]. A common approach uses laminar flow focusing to reduce the solvent length scale to sub-micron values. Mixing of the antisolvent occurs by diffusion, so small flow dimensions are crucial to ensure uniform particles[11,12]. Parallelization of multiple microfluidic chambers for scale-up is problematic for large production volumes.

The rapid mixing conditions that favor uniform nanoprecipitation in microfluidics alternately can be produced in confined, turbulent flows. Flash NanoPrecipitation (FNP) employs special mixing geometries to achieve these conditions under higher volumetric flowrates than possible with microfluidics. Inlet streams enter a mixing chamber under turbulent conditions that lead to the generation of vortices, so that solvent/anti-solvent lamellae form on the length scale of diffusion[11,13]. Thus, uniform mixing on a time scale shorter than nucleation and growth of the therapeutic is achieved. The confined geometry of the mixer does not permit stream bypassing of the region where turbulent energy dissipation occurs and the entire system experiences the same process history[13]. Nucleation occurs uniformly in the mixing chamber and particle growth proceeds until halted by the assembly of the BCP onto the surface[9,14]. The mixed stream containing stable particles may then be diluted with additional antisolvent to suppress Ostwald ripening of the particles[15-17].

A confined impinging jet (CIJ) mixer is the simplest mixer design for FNP and permits mixing of two streams in a scalable and continuous fashion, as shown in **Figure 1A**[13]. A multi-inlet vortex

mixer (MIVM) was developed to enable up to four different stream inputs while still achieving the rapid micromixing required for uniform particle formation, as shown in **Figure 1B**[18]. FNP enables simple formulation screening that can readily be translated to commercial-scale production. Due to the continuous nature of the process, larger batch sizes do not require new vessels but rather longer run times, enabling simple translation to kilogram-scale production in the same equipment train.

Hydrophilic compounds such as peptides and proteins ('biologics') can also be encapsulated in a process termed inverse Flash NanoPrecipitation (iFNP). The technique requires an amphiphilic BCP where one block is hydrophobic and the other is a polyacid[19]. The initial step involves rapid mixing of a dimethyl sulfoxide (DMSO) stream containing the biologic and the BCP against a lipophilic solvent such as dichloromethane or chloroform. This results in the formation of particles stabilized with the hydrophobic block brush. Here, such an architecture will be termed an 'inverted' NP. The core contains the polyacid, which is then ionically crosslinked using a multivalent cation. This stabilizes the particles for processing into an aqueous environment in the form of microparticles or PEG-coated nanoparticles by techniques that have been reported in the literature[19-21].

This protocol can be employed for the lab-scale production of polymeric core-shell nanoparticles encapsulating either hydrophobic or hydrophilic compounds. The subsections of the protocol provide instructions on the use of both mixer classes – the CIJ and the MIVM. The reader should be able to adapt the protocol for novel core components and reproducibly generate nanoparticles of a desired size using the appropriate mixer for the stream inputs. Three example formulations using FNP and iFNP are presented below. Two employ the CIJ mixer and one requires the MIVM[15,22]. The first formulation demonstrates encapsulation of a model *hydrophobic* compound by FNP. The second formulation demonstrates encapsulation of a model *hydrophilic* compound by iFNP in a CIJ mixer. The final formulation provides an example of protein encapsulation by iFNP using a MIVM. The protocol for this third formulation describes the use of a small-scale, handheld MIVM termed the 'µMIVM.' The mixer design is smaller to allow for simplified formulation screening, but the scaling behavior is well understood and the mixer is not a microfluidic device[22]. The final section of the protocol includes some notes on scale-up of lead formulations identified in screening. These formulations are intended to provide access points for learning the process and consequently use non-degradable poly(styrene)-based polymers. Alternative stabilizers have been described in the literature, with a number of biocompatible commercial options available[14,23,24].

**PROTOCOL:**

## 1. Encapsulation of Hydrophobic Compounds in Polymeric NPs Using a CIJ Mixer

### 1.1. Prepare and clean equipment.

1.1.1. Procure and validate a CIJ mixer.

Note: See **Supplemental Information** section 1 for construction guidance. CAD files are available as **Supplemental Information** as well.

1.1.2. Before each use, ensure that all fittings on the CIJ mixer are snug and the outlet tubing is not bent or pinched.

1.1.3. In a fume hood, attach a 5 mL luer lock syringe containing 2-3 mL of solvent to each inlet adapter. Select a solvent (*e.g.*, acetone) that will clean any compounds recently used in the mixer.

Note: Typical selections are acetone or tetrahydrofuran (THF). Only use polypropylene syringes to avoid solvent compatibility problems such as leaching. Do not use syringes with rubber O-ring seal plungers.

1.1.4. Set the CIJ assembly over a waste container.

Note: A flask with an opening smaller than the CIJ body works well as this supports the mixer and allows easy operation of the syringes.

1.1.5. Steadily depress the syringe plungers to empty the contents through the mixing chamber over a few seconds. Remove the syringes.

Note: Syringes can be retained and reused for multiple rounds of cleaning between FNP runs.

1.1.6. Dry the CIJ mixer internals using a $N_2$ stream. A male luer adapter on the end of a $N_2$ line is effective.

Note: If the cleaning solvent is not volatile (*e.g.,* DMSO), repeat steps 1.1.3-1.1.5 with acetone or THF before proceeding to step 1.1.6. It is crucial to remove residual solvent for run-to-run consistency.

1.2. Prepare solvent and antisolvent streams at target compositions.

1.2.1. Dissolve the hydrophobic compound (*i.e.*, vitamin E) in unstabilized THF at 10 mg/mL in sufficient amount to complete the desired number of FNP runs. Prepare slightly more than needed per run.

Note: Other solvents can be used in these steps, subject to the constraints in the Discussion section. If employing THF, stabilizer-free solvent is recommended because butylated hydroxytoluene has low aqueous solubility. Use caution to avoid peroxide buildup (including peroxide testing) and be aware that low levels of peroxides may interfere with certain NP applications (*e.g.,* bleaching of dyes).

1.2.2. Mix the vitamin E solution on a vortex mixer until dissolved.

Note: For some compounds, bath sonication for 1-2 min may assist in generating a dissolved solution. It is important that all NP components are molecularly dissolved.

1.2.3. Dissolve the block copolymer stabilizer (*i.e.*, poly(styrene)-*b*-poly(ethylene glycol), $PS_{1.6k}$-*b*-$PEG_{5k}$) in THF at 10 mg/mL at approximately the same volume as in step 1.2.1 to form the polymer solution.

Note: Other solvents can be used, subject to the constraints detailed in the Discussion section.

1.2.4. Mix the polymer solution with a vortex mixer until dissolved. If necessary, place the solution in a sonication bath for 1-2 min to aid in solids dissolution.

Note: The polymer cannot be in a micellar form. Dynamic light scattering (DLS) can be a useful tool to determine whether a new stream composition meets this criterion.

1.2.5. Create the solvent input stream containing 5 mg/mL of both vitamin E and the stabilizer (50% vitamin E loading) by first pipetting 0.25 mL of the vitamin E solution into a 1.5 mL centrifuge tube. Then pipette 0.25 mL of the polymer solution into the same tube.

Note: Volumes larger than 0.5 mL per run are feasible with different syringe sizes. Above 10 mL input volume, it is practical to use a syringe pump.

1.2.6. Mix well on a vortex mixer for 5-10 s. Optionally, centrifuge the tube at 1000 x g for 5-10 s to recover any liquid stuck to the cap, which improves reproducibility between CIJ runs.

1.2.7. Pipette 0.525 mL of deionized water into a second 1.5 mL centrifuge tube as the antisolvent stream.

Note: It is better to have excess antisolvent, which ensures that the solvent stream never enters the mixing chamber without antisolvent present. In some cases where salt solubility in the solvent/antisolvent mixture is not limiting, buffered aqueous systems can be used.

1.2.8. Pipette 4 mL of deionized water into a 20 mL scintillation vial or other suitable container as a quench bath. Place a small magnetic stir bar in the vial.

Note: The quench bath reduces the Ostwald ripening by lowering the final solvent content to 10% by volume[15,17]. This volume may be adjusted to address process constraints and can be directly scaled with input stream volume.

1.3. Produce NPs by FNP using the CIJ mixer.

1.3.1. Position the open quench bath vial below the cleaned CIJ mixer on a stir plate in a fume hood. A practical configuration uses a 50 mL test tube rack block to support the CIJ mixer with the vial below and the outlet tubing directed into the vial. See **Figure 1A** for orientation.

1.3.2. Begin stirring the quench bath *via* the magnetic stir bar at around 75% max speed.

1.3.3. Using a 1 mL polypropylene syringe fitted with a blunt-tip needle, draw the full volume from the antisolvent tube.

Note: Do not use syringes that contain a rubber O-ring seal to avoid compatibility concerns. For larger inlet volumes, use an appropriately sized luer lock syringe. The syringe outlet must be centered on the syringe axis or it will be unstable during depression.

1.3.4. Carefully remove all air bubbles from the syringe and remove the blunt tip needle, disposing in a sharps container.

1.3.5. Prime the plunger so that the stream comes just to the syringe opening. Attach the syringe to one of the CIJ inlet fittings.

1.3.6. Repeat steps 1.3.3-1.3.5 for the solvent solution.

1.3.7. Rapidly, smoothly, and uniformly depress the syringes at the same time by placing the ball of the hand, the palm of the hand, or one thumb each on the tops of the plungers depending on personal preference. Collect the effluent in the quench bath vial.

Note: A 0.5 mL input should be depressed in less than 0.5 s.

1.3.8. Set aside the CIJ mixer *with the syringes still attached*. Remove the stir bar and cap the vial, which now contains the NP dispersion with a core-shell particle structure (**Figure 1C**).

1.3.9. Hold the mixer over a waste solution container and remove the syringes. The hold-up volume (about 0.25 mL) will then drain out. Dispose of the used syringes and repeat the cleaning step 1.1 before the next FNP trial.

Note: Do not allow the hold-up volume to empty into the vial containing the NPs as this will negatively impact sample uniformity.

1.4. Perform analysis and post-processing of NP dispersion.

1.4.1. To characterize the NP size using DLS, pipette 100 µL of the NP dispersion into a plastic cuvette and add 900 µL of the quench bath solvent (*e.g.,* water).

Note: Smaller volumes may be used for low-volume cuvettes. A 10-fold dilution is generally sufficient.

1.4.2. Mix well by pipetting up and down or by mild shaking. Follow the instrument-specific instructions to analyze the sample.

Note: Alternative characterization techniques such as zeta potential analysis or electron microscopy may be carried out as required. The NP dispersion can be processed further as dictated by the application and reviewed in the Discussion section.

## 2. Encapsulation of Hydrophilic Compounds in Inverted NPs Using a CIJ Mixer

2.1. Prepare solution in a fume hood.

2.1.1. Complete the cleaning and preparation procedures described in step 1.1, using DMSO as a cleaning solvent and adhering to the note in step 1.1.6 to complete a second rinse with THF.

2.1.2. Dissolve the hydrophilic compound (*i.e.*, maltodextrin (MD) with dextrose equivalent (DE) of 4-7, average molecular weight = 3,275 g/mol, "3k MD") in DMSO at 10 mg/mL in sufficient volume to complete the desired number of FNP runs.

Note: Other solvents can be used, subject to the constraints outlined in the Discussion section.

2.1.3. Mix the maltodextrin solution with a vortex mixer until dissolved. If necessary, place the solution in a sonication bath for 1-2 min to aid in solids dissolution.

2.1.4. Create a block copolymer stabilizer (*i.e.*, poly(styrene)-*b*-poly(acrylic acid), $PS_{5k}$-*b*-$PAA_{4.8k}$) stock solution in THF at 11.1 mg/mL at approximately the same volume as in step 2.1.2 to form the polymer solution.

Note: Other solvents and stabilizer concentrations can be used. DMSO can readily be used as a solvent in place of THF.

2.1.5. Mix the polymer solution with a vortex mixer until dissolved. If necessary, place the solution in a sonication bath for 1-2 min to aid in solids dissolution.

Note: The polymer input cannot be in a micellar form. DLS can be used to determine whether a new stream composition meets this criterion.

2.1.6. Prepare the solvent stream input (0.5 mL) by combining the following, in order, in a 1.5 mL centrifuge tube: 0.250 mL of the 3k MD solution, 0.225 mL of polymer solution, and 0.025 mL deionized water.

Note: The water content of this stream has a strong impact on NP size and polydispersity. Generally it is best to operate in the 2.5-10 vol% range[20]. Values on the high end of the range may help encapsulation of larger molecular weight compounds.

2.1.7. Mix well on a vortex mixer for 5-10 s.

2.1.8. Optionally, centrifuge the tube at 1000 x g for 5-10 s to recover any liquid stuck to the cap, which improves reproducibility between CIJ runs.

2.1.9. Prepare a crosslinker solution of calcium chloride ($CaCl_2$) dihydrate in methanol at 25.0 mg/mL.

Note: The crosslinker will be added at a 1:1 charge ratio to the acid groups in the PAA block. Adjust the concentration accordingly if a different crosslinker is used or if a different PAA block size or polymer concentration is used[20,21].

2.1.10. Prepare the antisolvent stream by pipetting 0.5 mL of chloroform and 0.05 mL of the crosslinker solution (0.55 mL total) into a microcentrifuge tube.

Note: Other acceptable antisolvents are dictated by the block copolymer choice and typically include dichloromethane or acetone. The crosslinker may instead be added to the quench bath, with additional aging of the NP dispersion to allow for crosslink formation[20].

2.1.11. Mix well on a vortex mixer for 5-10 s.

2.1.12. Optionally, centrifuge the tube at 1000 x g for 5-10 s to recover any liquid stuck to the cap, which improves reproducibility between CIJ runs.

2.1.13. Add 4 mL of the antisolvent (*i.e.*, chloroform) to a 20 mL scintillation vial to form the quench bath. Place a small magnetic stir bar in the vial.

Note: This volume may be adjusted to address process constraints.

2.2. Complete the protocol for NP formation as described in step 1.3.

2.3. Perform analysis and post-processing of NP dispersion.

2.3.1. To characterize the NP size using DLS, pipette 100 µL of the NP dispersion into a glass cuvette and add 900 µL of the solvent used for the quench bath.

2.3.2. Mix well by pipetting up and down or by light agitation of the cuvette. Follow the software instructions to analyze the sample.

Note**:** Crosslinking of the NPs can be qualitatively assessed by DLS using a good solvent like DMSO or dimethylformamide (DMF) as DLS diluent[20]. Particles which are stably crosslinked will exhibit an autocorrelation function in the solvent with minimal change in particle size. Poorly crosslinked particles swell and exhibit a weak autocorrelation function and scattering strength[21].

2.3.3. Optionally, add a base, such as ammonia, to drive ionic complexation and strengthen crosslinking in the particle core.

2.3.3.1. Optionally, prepare a 3.48 mg/mL solution of ammonia in methanol gravimetrically using ammonium hydroxide solution (typically, 30 wt% ammonia). Add 50 µL (*i.e.*, 0.6 equivalents with respect to the acid groups on the polymer) dropwise with stirring.

Note: The equivalents can be adjusted if desired by varying either the concentration or the volume added[25].

2.3.3.2. Optionally, age no less than 30 min with mild stirring for crosslinking to occur.

2.3.4. Process the NP dispersion to produce either microparticles or coated NPs as described in the literature[19-21].

## 3. Encapsulation of Ovalbumin in Inverted NPs Using a µMIVM

3.1. Prepare solvent and antisolvent solutions.

3.1.1. Prepare a 50 mg/mL solution of ovalbumin in deionized water ("OVA").

3.1.2. Prepare 0.75 mL of Solution A in a 1.5 mL centrifuge tube by diluting 75 µL of the OVA solution with 0.675 mL of DMSO to generate a 5 mg/mL solution of OVA in DMSO containing 10% water by volume. Mix well and centrifuge briefly as described previously.

Note: See step 2.1.6 regarding water effects. As in previous sections, the solution volumes can be scaled up or down to fit material needs.

3.1.3. Prepare solution B by dissolving the block copolymer stabilizer (*i.e.*, poly(styrene)-*b*-poly(acrylic acid), $PS_{5k}$-*b*-$PAA_{4.8k}$) in DMSO at 6 mg/mL. Mix well and sonicate to dissolve if needed. Pipette 0.75 mL into a 1.5 mL centrifuge tube.

3.1.4. Pipette 0.75 mL of THF (Solution C) into a 1.5 mL centrifuge tube.

3.1.5. Pipette 1.85 mL of chloroform (Solution D) into a glass scintillation vial.

3.1.6. Prepare a 60.0 mg/mL calcium chloride dihydrate crosslinker solution in methanol. Mix using a vortex mixer.

3.1.7. Prepare a 4.17 mg/mL ammonia solution in methanol as described in step 2.3.4.

3.1.8. Add 5.25 mL of chloroform to a 15 mL centrifuge tube as the quench bath.

3.2. Prepare mixer assembly and stand.

3.2.1. Gather the bottom receiver, mixing geometry disk, the top disk, the spanner wrench, and an O-ring. See **Figure 2** for schematic of components and mixer stand terminology.

Note: Details on MIVM construction may be found in **Supplemental Information** (Section 1) and in the literature[22]. CAD files are available as **Supplemental Information** as well.

3.2.2. Place the O-ring into the groove, ensuring that it fits well and that there are no signs of wear or damage.

Note: Normal operation will lead to worn or solvent-swollen O-rings. If the O-ring appears stretched or deformed, allow it to air dry overnight before use. If the shape does not recover overnight, dispose of the O-ring. Keep a large stock, as this is a consumable part.

3.2.3. Carefully align the mixing disk holes with the pegs on the top disk and push together. Ensure that the O-ring does not become displaced by checking the two pieces sit flush.

3.2.4. Invert the two pieces and manually assemble them with the bottom receiver. Ensure that the outlet tubing fitting has been loosened so that it does not interfere with complete tightening of the disk.

Note: If the threading catches during assembly, carefully disassemble and apply a food- or pharmaceutical-grade anti-seize to the threading to prevent galling.

3.2.5. After manual tightening, fit the spanner wrench to the top disk pegs and snugly tighten the assembly. Then tighten the outlet tubing fitting so that it sits firmly against the bottom face of the mixing geometry. Ensure that the syringe fittings on the top disk are snug.

3.2.6. Place the assembled mixer onto the mixer stand so that the outlet tubing extends below the support plate. Support the mobile plate so that it is suspended out of the way of the work space.

3.2.7. Optionally, to check mechanical stop alignment, first attach the empty glass syringes to the mixer inlets.

Note: Volumetric flowrates are varied using syringes of different barrel diameters, since the syringes are depressed simultaneously at the same linear velocity. The initial and final vertical heights must be the same for all syringes and can be adjusted using set screws tapped into the plunger shaft[22]. The mechanical stops ensure that excessive damage to the glass syringes does not occur.

3.2.7.1. Optionally, lower the mobile plate so that is comes to rest on the mechanical stops. Ensure that these are aligned so that the plate also comes to rest immediately before contacting the empty syringes (as seen in **Figure 2**).

3.2.7.2. Optionally, loosen the mechanical stops and reposition, if needed. Remove the glass syringes and reset the mobile plate out of the way.

Note: For operation with plastic syringes, the mechanical stops are not required.

3.2.8. Place the open quench bath below the outlet tubing to collect the effluent.

3.2.9. Draw Solution A into a 1 mL gas-tight syringe using a blunt tip needle. Remove all air bubbles and dispose of the needle. Prime the solution to the end of the syringe luer fitting. Repeat this process for Solutions B and C.

3.2.10. Draw Solution D into a 2.5 mL gas-tight syringe using a blunt tip needle. Remove all air bubbles and dispose of the needle. Prime the solution to the end of the syringe luer fitting.

Note: These volumes have been selected so that the initial syringe plunger heights are the same. If volumes are changed, they still must meet this height requirement.

3.2.11. Assemble the four syringes onto the mixer in a clockwise fashion in alphabetical order. See **Figure 1B** for final appearance and syringe orientation schematic.

Note: Check that no syringe height is significantly different from the others and troubleshoot as needed.

3.3. Perform mixer operation and cleaning.

3.3.1. Grip the bearing housing on both sides of the mobile plate. Do not place fingers on the bottom face of the housing because this is a pinch hazard against the mechanical stops. Slowly lower the mobile plate so that it is resting evenly but barely touching the syringes.

3.3.2. Steadily and smoothly depress the plate, aiming to complete the operation in about 0.5-1 s for these stream volumes[22].

3.3.2.1. Remove and cap the quench bath tube which now contains the NP dispersion.

3.3.3. Take the mixer with the syringes still attached and hold over a waste container. Remove the syringes, allowing the hold-up volume to drain into the container. Hold the mixer assembly upside and disassemble the mixer using the spanner wrench.

3.3.4. Using a spray bottle, rinse the outlet tubing with several milliliters of solvent (*e.g.,* acetone) and dry with air or nitrogen.

3.3.5. Rinse the mixing geometry with a good solvent (*e.g.,* deionized water or DMSO) and then rinse with acetone using several milliliters from a spray bottle. Dry with an air or nitrogen stream.

3.3.6. Rinse the O-ring in a stream of deionized water and blot dry.

3.3.7. Rinse the top disk thoroughly with several milliliters of acetone using a solvent bottle until visually clean. Dry with an air or nitrogen stream both the surface and the syringe fittings.

3.3.8. Rinse each syringe with several milliliters of a good solvent (*e.g.,* deionized water or acetone) from a solvent bottle. Apply a final rinse of several milliliters of acetone and air dry before next use.

3.4. Perform post-processing and analysis.

3.4.1. Add 50 µL of the calcium chloride dihydrate crosslinker solution dropwise while stirring at about 75% maximum speed.

3.4.2. Add 50 µL of the ammonia solution dropwise while stirring at 75% maximum speed. Age for at least 30 min.

3.4.3. Characterize the NP size as described in steps 2.3.1 and 2.3.2.

3.4.4. Process the NP dispersion to produce either microparticles or coated NPs as described in the literature[19-21].

## 4. Modifications for Formulation Scale-up

4.1. Prepare the solvent and antisolvent solutions as described in steps 1, 2, or 3 at the desired composition and at sufficient volume for the required formulation size.

4.2. Optionally, if needed, clean and sterilize the mixer in place using a suitable protocol prior to NP formation.

Note: Sequential rinses of CIP 100, water (to neutral pH), CIP 200, water (to neutral pH), and a suitable solvent have been employed in the past. Additionally, sterile filters can be attached to the inlets of the mixer in instances where final particle size precludes sterilization by filtration.

4.3. Load the solutions into gas-tight syringes of suitable volume and attach polytetrafluoroethylene (PTFE) tubing with a luer adapter fitted on the end. Manually prime the solutions to the end of the tubing.

4.3.1. Load the syringes into a syringe pump and attach the syringes to the mixer inlets on either the CIJ or the MIVM, as required.

Note: Alternatively, flow controllers can be used at lab or pilot scale to provide larger volume capabilities than a syringe pump. Successful operation requires steady flow and sufficient

pressure drop, which means that pressurized vessels with flow metering on the outlet are the most appropriate selection for large scale production.

4.4. Place a collection vessel containing a quench bath of sufficient volume, if required, beneath the outlet tubing.

4.5. Set the volumetric flow rates to match those achieved manually (*e.g.,* about 30-60 mL/min per stream).

Note: If using the CIJ, the pump flow rates must be identical. If using the MIVM, different inlets can have different flow rates.

4.6. Simultaneously begin the pumps. Collect about 5-10 mL of effluent as waste in a small vial (this is a "start-up volume") and then begin collecting in the quench bath.

4.7. Characterize and process as described in the corresponding formulation section above.

**REPRESENTATIVE RESULTS:**

Screening of NP formulations with FNP is rapid and requires small quantities of material (on the order of 1-10 mg). The FNP protocol to encapsulate hydrophobic compounds such as vitamin E (step 1) results in a stable, clear or lightly opalescent NP dispersion. Dynamic light scattering (DLS) provides a robust means to characterize the particle size. As shown in **Figure 3**, the process produces NPs with a low polydispersity in a reproducible fashion. The typical polydispersity index (PDI) is less than 0.20, indicating a relatively monodisperse population. The PDI is obtained from the autocorrelation function and is often implemented into instrument software. It is a ratio of the second to the first moment, where values of 0.1 are generally obtained for monodisperse particles[26]. For the four vitamin E/PS-*b*-PEG formulation replicates reported, the value was 0.12 ± 0.02 and the average diameter was 107 ± 7 nm. A typical "misfire" due to either uneven depression of the syringes or slower depression speed is also reported in **Figure 3**. The polydispersity was unaffected, but the size was slightly larger (135 nm). Including this sample, the new metrics for particle size are 113 ± 14 nm. A misfire results in periods of time where the chamber contains only a single stream type. It is important that the entire stream experiences the same process history and relative volumes of the organic and aqueous streams within the mixer. Without a stabilizer, an opaque solution with visible aggregates is produced. The DLS autocorrelation function for this sample is non-monotonic and does not decay smoothly, as seen in the **Figure 3** inset.

Particle size control by FNP is demonstrated in **Figure 4**, where varying the relative amounts of core material – poly(styrene)$_{1.8k}$ in this case – and PS-*b*-PEG stabilizer resulted in particles sizes that ranged from 49-152 nm. These particle sizes were generated with THF streams containing a total mass concentration of core and stabilizer of 20 mg/mL, where 25%, 50%, or 75% of the mass was the poly(styrene) core material. The polydispersity of the nanoparticles was always less than 0.15. Extensive discussion of parameter effects on particle size produced by FNP may be found in the literature[10]. The loading can be tuned by holding the solvent volume constant and varying

the relative volumes of the core and stabilizer stock solutions. Similarly, the total mass concentration can be varied by preparing stock solutions at values other than 10 mg/mL. Under certain conditions, it is possible to observe an empty micelle population by DLS[27]. This does not have any detrimental effect other than broadening the measured particle size distribution. When the sizes are similar, this may manifest as a single broad peak rather than two separate peaks.

The same CIJ mixer can also be used to encapsulate hydrophilic compounds by iFNP, as exemplified in step 2 of the Protocol. The particles produced in the reported formulation are around 65 nm with a low polydispersity of 0.08. The size distribution can be seen in **Figure 5A** (dashed lines). The effect of crosslinking the PAA carboxylic acid residues on particle stability is demonstrated by DLS analysis in a strong solvent such as DMSO, as shown in **Figure 5B**. The autocorrelation function for well-crosslinked particles should start near a value of 1 and drop off sharply to 0 at a characteristic time that is related to the particle size (solid line). Particles that swell extensively or dissolve are not crosslinked and show minimal autocorrelation signal (dotted line). For iFNP, failed trials manifest in similar ways as described for FNP above. Visible aggregates may be seen or poor DLS autocorrelation function shape may be observed. The MIVM can be used for FNP or iFNP when more than two inlet streams are required due to system constraints such as solubility or chemical incompatibility. A small-scale version of the MIVM (the μMIVM) with its mixer stand is shown in **Figure 2**. As with the CIJ, this mixer can be used to encapsulate hydrophobic or hydrophilic compounds[22]. In step 3, a protocol for the encapsulation of a hydrophilic protein, OVA, by iFNP was described. The particle size distribution is shown in **Figure 5A** (solid line). The size is around 125 nm with a PDI of 0.16. A general protocol for syringe pump operation at larger scales is provided in step 4.

**FIGURE LEGENDS:**

**Figure 1: Mixer assembly and internal flow pattern schematics.** (**A**) The confined impinging jets (CIJ) mixer with attached syringes is positioned above the quench bath. Not pictured are a stir bar in the quench bath vial and a stir plate. The mixing geometry is depicted in the expanded view showing the two stream inlets that impinge in the center of the chamber. (**B**) A multi-inlet vortex mixer (the μMIVM) is shown with glass syringes and positioned in the stand above a quench bath. The mobile plate and the mechanical stops have been cropped from the picture. The expanded view shows the vortex chamber and the inlet channels schematically. (**C**) A schematic representation of core-shell NPs produced by FNP. Red spheres represent the therapeutic which, combined with the blue collapsed polymer block, comprise the NP core. The yellow polymer block forms the brush layer imparting steric stabilization to the NPs.

**Figure 2: μMIVM terminology and components for assembly.** The μMIVM requires a mixer stand to enable uniform depression of the four syringes. In this case, the syringe plunger heights must all be uniform to ensure consistent mixing. It can alternatively be operated using syringe pumps. The mixer stand with labeled components is shown at left of the figure. On the right is the disassembled mixer with the O-ring in place on the mixing geometry disk.

**Figure 3: Particle size distribution of polymeric nanoparticles containing a core of vitamin E and stabilized by PS-*b*-PEG.** Dynamic light scattering (DLS) provides intensity-weighted size

distributions that indicate the NP diameter distribution. Curves are the average of triplicate analyses for each trial and have been rescaled to produce identical maximum peak heights. The four replicates (solid lines) indicate the high reproducibility of the method (standard deviation = 7 nm). Also included is a representative misfire (dashed line), such as slower syringe speed or uneven depression of the two syringes, which results in larger particle diameter. The standard deviation of the NP size including the misfire was 14 nm. **(Inset)** Without the PS-*b*-PEG stabilizer, large micron-scale aggregates (or droplets, in the case of an oil like vitamin E) are formed. The DLS autocorrelation function of a run without the stabilizer (dotted line) is shown along with a representative autocorrelation from a nanoparticle replicate (solid line). The autocorrelation function shows a number of characteristic timescales for the control sample, indicating a polydisperse population.

**Figure 4: Particle size control by FNP through varying relative ratios of core material to stabilizer.** The intensity-weighted size distributions of three formulations with a poly(styrene) core stabilized by PS-*b*-PEG are depicted. The total mass concentration in THF was 20 mg/mL and the antisolvent was water. The formulations were prepared in a CIJ mixer. The fraction of the mass composed of the core material is listed in the legend. For example, the 25% core sample contained 5 mg/mL poly(styrene) and 15 mg/mL PS-*b*-PEG. The average sizes for the 25% (solid line), 50% (dashed line), and 75% (mixed dash line) core loadings were 49 nm, 96 nm, and 152 nm, respectively. All PDI values were less than 0.15.

**Figure 5: Characterization of inverted NPs made in a CIJ mixer or µMIVM.** (**A**) DLS curves are the average of triplicate analyses for each formulation. The dashed line indicates the size distribution of 3k MD particles made in the CIJ mixer while the solid line is the size distribution of OVA particles made in the µMIVM. (**B**) The strength of crosslinking can be assessed by DLS using DMSO as the diluent. The DLS autocorrelation function indicates the strength of crosslinking through the initial autocorrelation value and the observation of a clean transition to a value of zero. The dashed line depicts the autocorrelation function for a particle with no crosslinker showing a weak initial signal and a broad decay time. The solid line depicts the autocorrelation after addition of a strong crosslinker (in this case, tetraethylenepentamine), which shows a strong initial signal and a defined decay timescale.

**Figure 6: Supersaturation, S, as a function of the relative mixing ratios of organic solvent to water.** (**A**) Comparison of highest attainable supersaturation for (○) boscalid, a pesticide, and (■) peptide B, a seven-residue model peptide. The organic stream contains boscalid at a concentration of 230 mg/mL and peptide B at 200 mg/mL, their saturation concentrations. There is a maximum supersaturation that depends on each active pharmaceutical ingredient (API)/solvent system. (**B**) When the concentration of boscalid in the organic stream is decreased 20-fold, the conditions at which supersaturation and nanoprecipitation are achieved become limited. This figure is reprinted with permission from Elsevier[9].

**DISCUSSION:**

The encapsulation of hydrophobic compounds such as vitamin E, as in step 1 of the Protocol, has been extensively described[9,14,28]. Relatively monodisperse particles are produced because the

time scale for mixing is shorter than the time scale for the aggregation and growth of the particles. Specifically, the mixed solvent/antisolvent solution rapidly becomes homogeneous, which enables nucleation to occur uniformly. Assembly of the block copolymer to the particle surface then provides steric stabilization that halts particle growth[5]. Since mixing time in the chamber (turbulence) is a function of the inlet flow rates to the CIJ or the MIVM, there is an inlet rate, which occurs after the transition to turbulent mixing, where the particle size is essentially constant[13]. This provides additional robustness to the process as some batch-to-batch variation in inlet flowrate (*i.e.*, syringe depression speed) can be tolerated without significant impact to the final NP size as seen from **Figure 3**. Slower or uneven inlet speeds can result in larger particles or more polydisperse distributions, as seen for the misfire example. FNP has also been extended to encapsulate hydrophilic compounds in nanoparticles by inverse Flash NanoPrecipitation. These inverted nanoparticles can then be used to create microparticles or be coated with PEG to create water-dispersible nanoparticles[25]. The underlying assembly principles remain the same, though there is the added complexity of crosslinking the particle core. This is necessary for stabilization of the particle in an aqueous environment. In general, a 1:1 charge ratio compared to the polyacid block is sufficient, though the ionic interactions can be promoted by pH adjustment through the addition of a base[19]. In this protocol, only the first process step to form inverted NPs has been described.

In addition to fast mixing, successful formulation by FNP or iFNP is limited to instances where several conditions can be met[9,14]. First, all stream inputs must be miscible. While emulsions have been used to produce NPs, FNP requires a uniform solution phase in the mixer. Second, the core component must be nearly insoluble at the solvent conditions in the mixer (for the CIJ, a 50/50 mixture by volume) to drive rapid nucleation. Otherwise, a significant portion will remain unencapsulated or will precipitate after further dilution with antisolvent. The MIVM can enable higher antisolvent content in the mixing chamber to address core material solubility limitations. It is often useful to generate supersaturation curves from solubility data as a function of solvent composition to guide process design[9]. **Figure 6** shows representative curves for two compounds. Low supersaturation at the mixing chamber conditions merits operating at different compositions, typically using the MIVM. Higher supersaturation favors the nucleation of the core component over particle growth but a mismatch in assembly time of the core material and the stabilizer can result in large aggregates of the therapeutic. D'Addio and Prud'homme have reviewed the application of such supersaturation curves in detail[9]. Finally, the BCP must be molecularly dissolved in the solvent stream and the antisolvent stream must be selective for one block. The BCP must be sufficiently amphiphilic to provide both a solvophobic driving force from the collapsed block to anchor the stabilizer on the particle surface and for the solvated block to impart steric stability to the particle. Solvents other than those described in the protocol may be used as long as they meet these constraints.

Practice with manual syringe operation can improve the success rate during screening. As noted above, operation above the transition to homogeneous, turbulent mixing conditions means that small variations in flow rate are tolerated in the process[28]. Scale-up to pump-driven, computer-controlled flows results in even greater gains in consistency due to the reproducible inlet flow rates. At any point during post-processing of the particles, visual inspection or DLS analysis may

indicate the presence of large aggregates which can be due to incidental dust or particle instability. When necessary, the stream can be filtered with an appropriate filter pore size. In the absence of aggregates, we have found that less than 5% mass is typically lost when filtering PEG-coated nanoparticles if the nominal filter size is larger than the particle size distribution. When filtering aggregates, experimental determination of mass lost during the process is necessary. Quantification of the mass loss can be carried out in one of two ways. The total solids mass in a given volume can be determined by thermogravimetric analysis before and after filtration to identify the extent of change (see **Supplemental Information** Section 2). Alternatively, the particles can be recovered (*e.g.*, by lyophilization) and dissolved in a good solvent. The concentration of the core material can then directly be measured by an appropriate technique such as ultraviolet-visible spectrophotometry or chromatography.

For FNP, the residual 10 vol% organic solvent (*e.g.*, THF) must be removed from the aqueous dispersion. This can be done by evaporative distillation[14,29], dialysis[30], or tangential flow filtration[31,32]. Practical considerations for each processing step are described in the citations provided. For dialysis, typical membranes are 3.5 kDa or 6-8 kDa cutoffs, though larger options are available. This molecular weight cutoff is sufficient for solvent removal when dialyzed for 24 h using several bath changes. The use of tangential flow filtration entails some process development as care must be taken to avoid inducing aggregation due to concentration polarization at the membrane surface. We have found that reducing the organic solvent composition below a system-dependent value, usually 2-10 vol%, eliminates aggregation at the membrane surface. After processing, the concentration of nanoparticles is readily determined by thermogravimetric analysis (see **Supplemental Information** Section 2). It is often desirable to transport or store particles in a highly stable form. Aqueous dispersions can simply be frozen rapidly using a dry ice/acetone mixture and then stored at -80 °C. Alternatively, dry powders can be obtained by lyophilization[33,34] or spray drying[24]. Frequently, a cryoprotectant must be added to reduce nanoparticle aggregation during freezing or drying. Sugars (sucrose, trehalose, *etc.*), poly(ethylene glycol), or cyclodextrins can be screened for effectiveness over a range of concentrations by monitoring size by DLS[35-38]. Common NP stability problems during processing are often related to solubility or phase separation in the core resulting in rearrangement towards a lower energy state under conditions where mobility is increased. Use of co-core materials, alternative stabilizers, or modified external solution composition can help improve stability[14,16,17,39-41].

As noted above, the MIVM enables higher antisolvent content in the mixing chamber when required to achieved high supersaturation. It can also allow for the physical segregation of species into more than two streams when reactivity or solubility constraints demand it. An example is the formation of zein protein-stabilized nanoparticles of the antibiotic clofazimine[24]. The hydrophobic clofazimine is introduced in an acetone stream; zein is introduced in a 60% ethanolic aqueous stream; casein, which complexes with zein, is brought in with an aqueous buffer stream, and the fourth stream is additional buffer to increase the ratio of water to acetone and ethanol. Two solvent streams are required since clofazimine and zein are not soluble in a common solvent. This process could not be accomplished in a two-jet CIJ mixer. This protein-stabilized formulation also demonstrates that FNP is not limited to BCP stabilizers. Janus particles

have been produced without stabilizer[42] and a range of low-cost stabilizers have been demonstrated for oral applications[24]. Notably, copolymers such as hydroxypropyl methylcellulose can be used in lieu of block copolymers[24]. Core materials can be made more hydrophobic by a number of techniques. Hydrophobic ion pairing has been applied to encapsulate a wide range of compounds that have intermediate solubility[43-45]. Extremely hydrophobic prodrugs have been generated and then encapsulated[46]. Nucleic acids have been encapsulated through complexation with cationic lipids[47]. Importantly, these studies have shown that FNP can produce a range of particle surface chemistries. Further, mixed stabilizers containing a fraction of BCP that has been modified with a targeting ligand on the chain end have been used. This enables precise control over ligand content on the surface since particle composition reflects the input stream composition[23,48]. Similarly, it is possible to incorporate multiple core components as well, including dyes and inorganic nanoparticles[3,8].

Flash NanoPrecipitation is a scalable approach to polymeric nanoparticles comprised of either a hydrophobic or a hydrophilic core. If the criteria enumerated above are met, generally over 95% of the core material is encapsulated at high mass fraction in the particle. The three examples presented here were carried out at bench scale, requiring a few milligrams of material and about 0.5 mL in each inlet stream. This allows for rapid screening of particle conditions for formulation optimization. Scale-up of lead formulations to larger batch sizes is a matter of running the process for longer, which can readily be accomplished through the use of syringe pumps or flow controllers. By contrast, the scale-up of bulk addition nanoprecipitation faces well-documented challenges in maintaining sufficient micromixing at the point of addition and accounting for the effect of changing vessel geometry[49]. This is a major barrier, since it is crucial to manufacture particles in a consistent manner to meet FDA requirements[50]. Microfluidics techniques can also produce uniform, reproducible nanoparticles, but only enable production in the milligram range. For example, Karnik *et al.* reported production rates of 0.25 mg/min for a drug release study[51]. Further scale-up typically entails parallelization at high capital cost[12]. With FNP, it is straightforward to produce 1 gram of nanoparticles at 600 mg/min with a syringe pump and a few fittings to connect to the mixer inlets. Consequently, FNP represents both an accessible lab-scale screening tool as well as a scalable approach to NP production for translational work.

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**DISCLOSURES:**

The authors have nothing to disclose.

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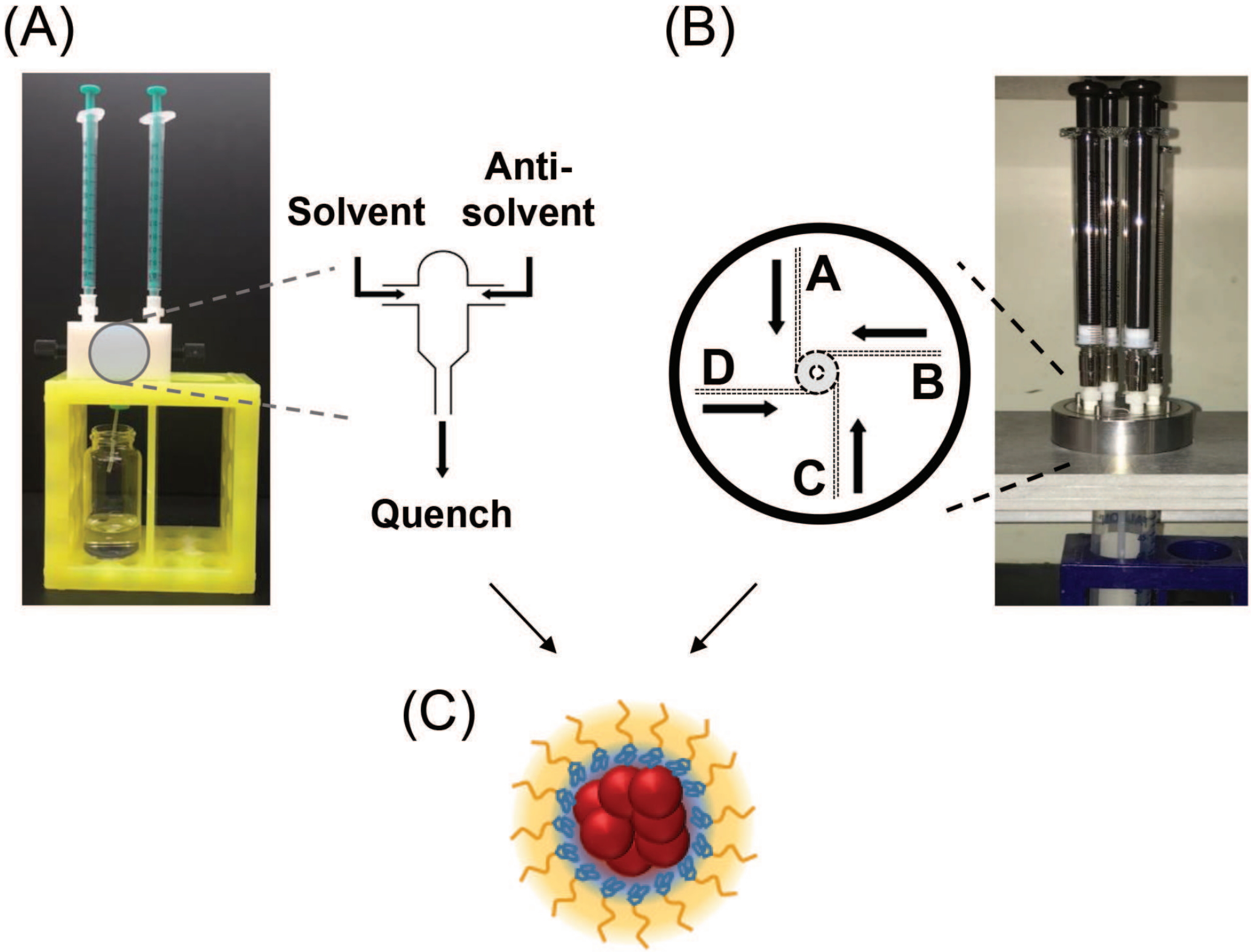

Figure 2

Figure 3

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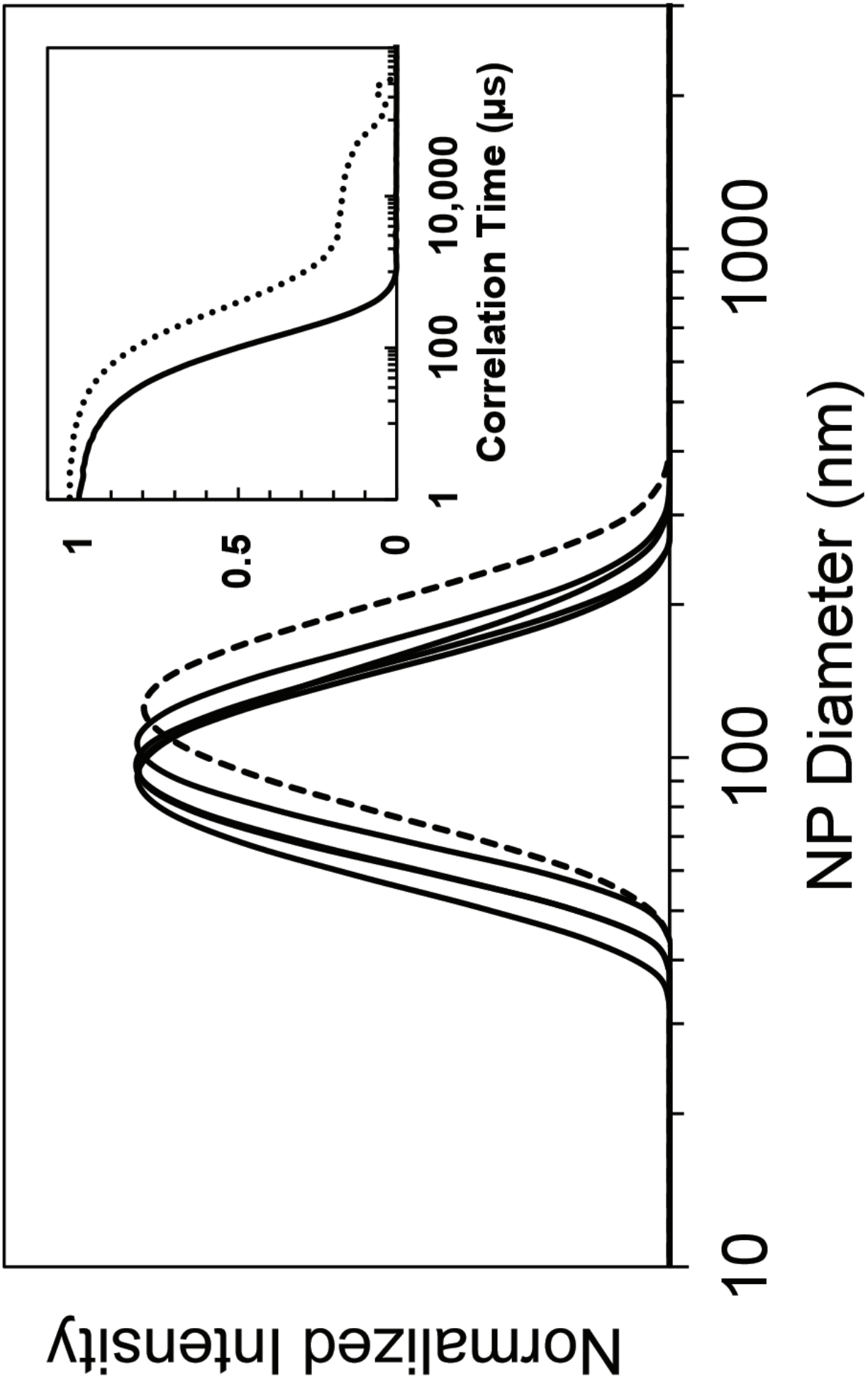

Figure 4

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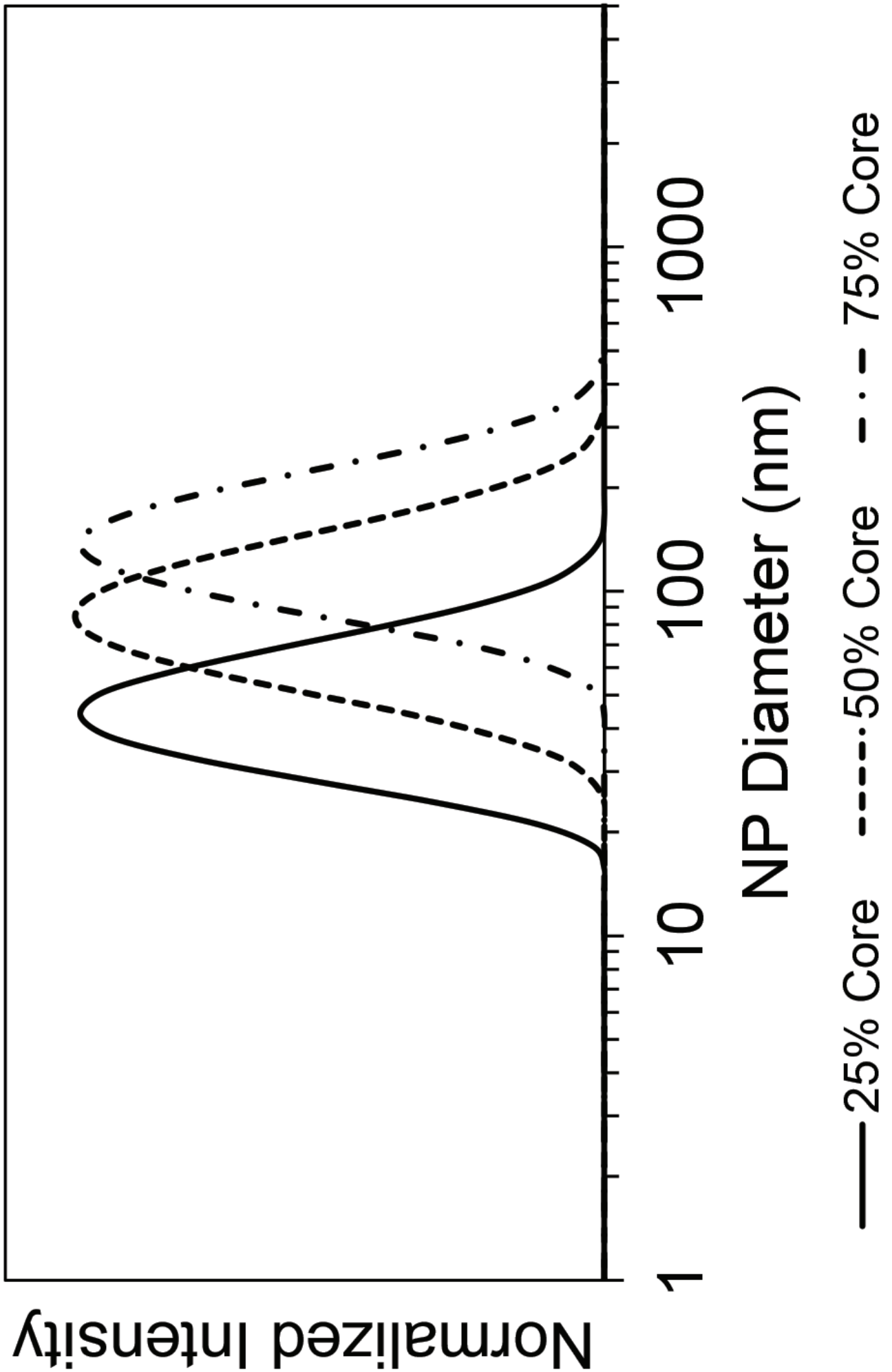

Figure 5

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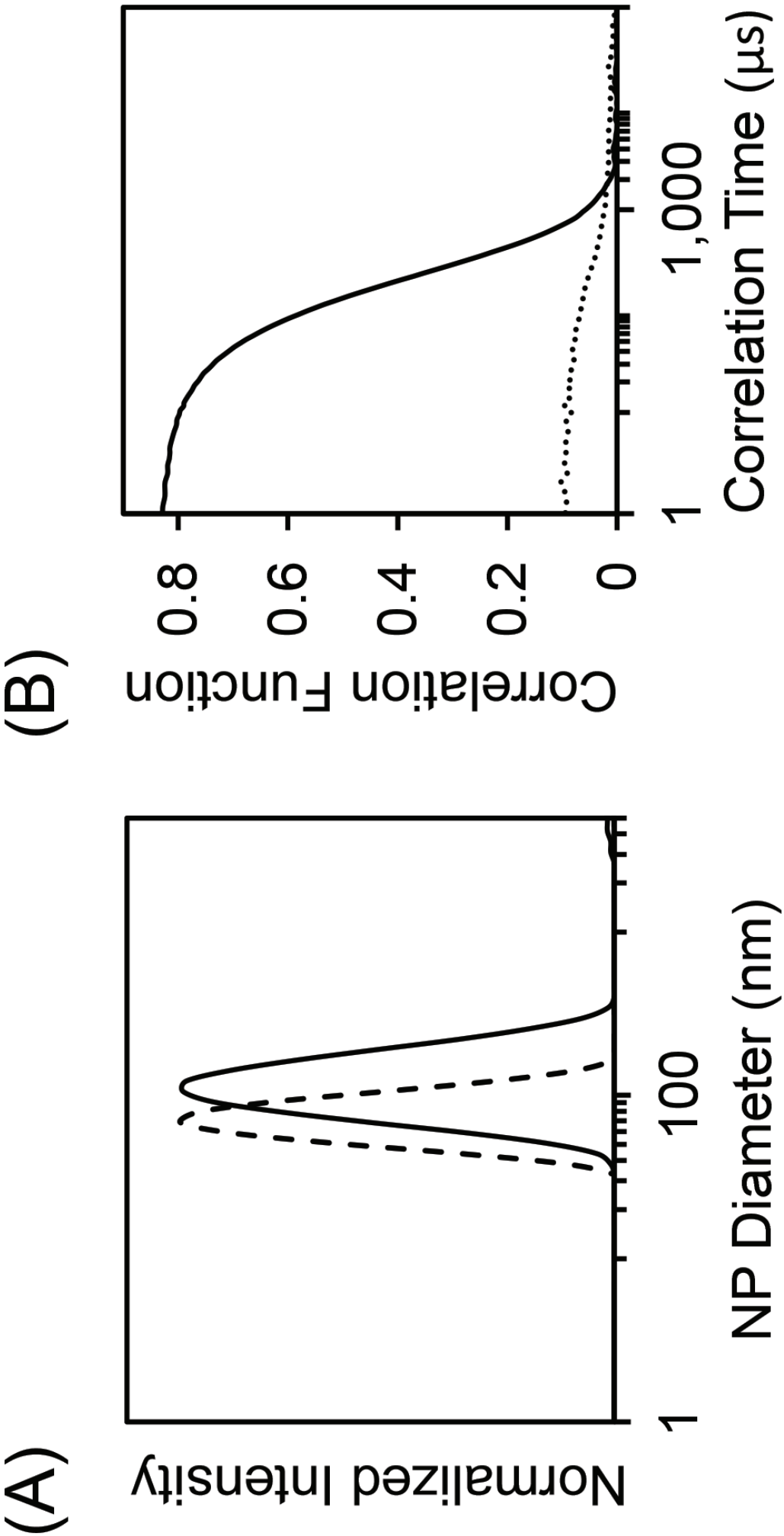
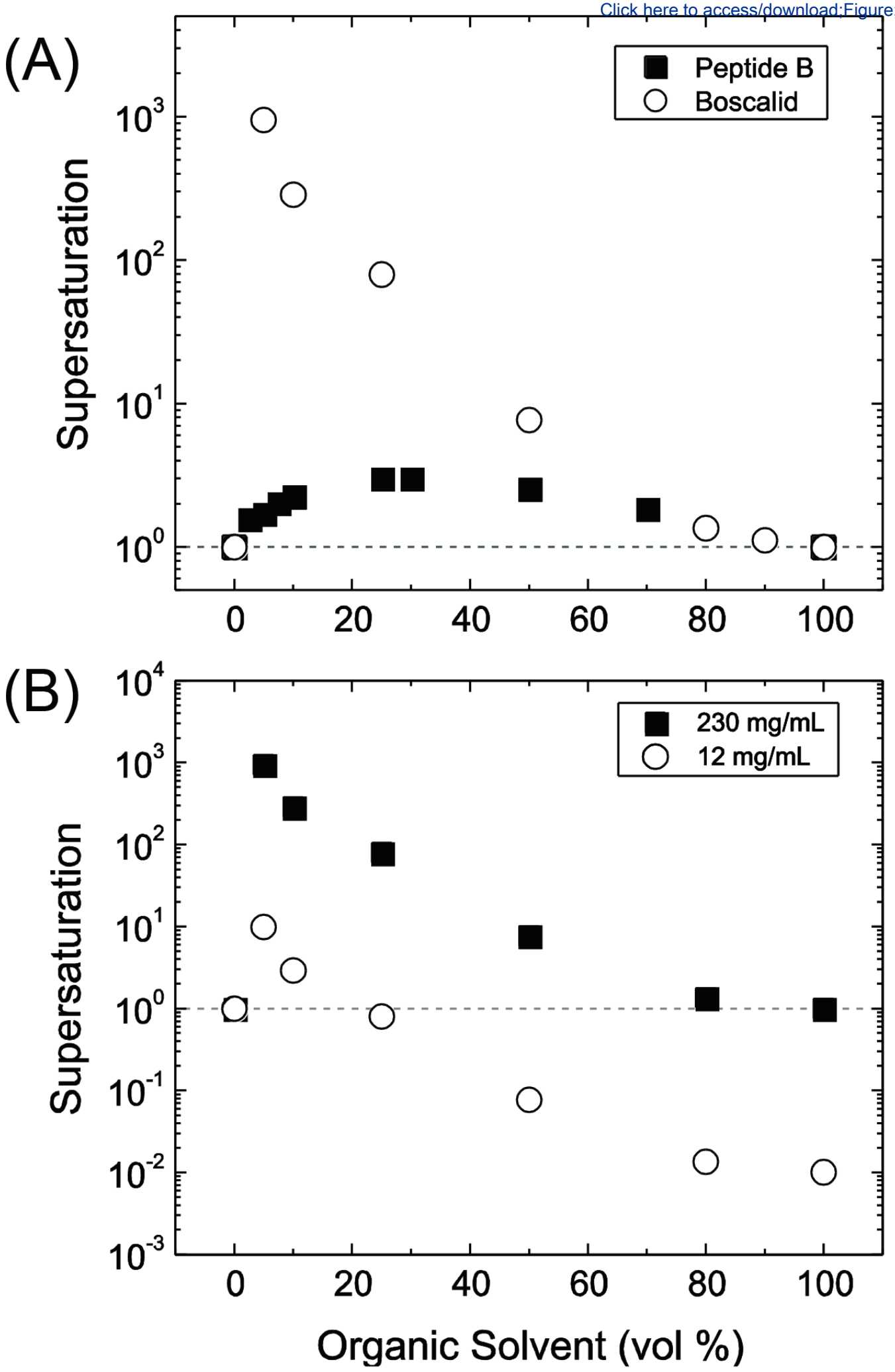


Figure 6





| Name of reagent/equipment | Company | Catalog number |
|---|---|---|
| Confined Impinging Jets Mixer | NA | NA |
| Luer fitting | Idex Health & Science | P-604 |
| Plug fitting | Idex Health & Science | P-309 |
| Outlet fitting - CIJ | Idex Health & Science | P-205 |
| Outlet ferrule - CIJ | Idex Health & Science | P-200 |
| Outlet tubing - CIJ | Idex Health & Science | 1517 |
| Tetrahydrofuran (THF) | Fisher Scientific | T425-4 |
| Norm-ject syringe (3 ml) | VWR | 53548-017 |
| Vitamin E (α-tocopherol) | Sigma-Aldrich | 90669-50G-F |
| poly(styrene-*b*-ethylene glycol), $PS_{1.6k}$-*b*-$PEG_{5k}$ | Polymer Source | P13141-SEO |
| $poly(styrene)_{1.8k}$ | Polymer Source | P2275-S |
| Scintillation vial | DWK Lifesciences | 74504-20 |
| Luer-slip plastic syringes, 1ml (100 pk) | National | S7510-1 |
| Maltodextrin DE 4-7 | Sigma-Aldrich | 419672-100G |
| poly(styrene-*b*-acrylic acid), $PS_{5k}$-b-$PAA_{4.8k}$ | Polymer Source | P5917-SAA |
| Dimethyl Sulfoxide (DMSO) | Fisher Scientific | D159-4 |
| Calcium chloride dihdyrate | Sigma-Aldrich | 223506-25G |
| Methanol | Fisher Scientific | A452-4 |
| Ammonium Hydroxide | Fisher Scientific | AC423300250 |
| Albumin from chicken egg white (Ovalbumin, OVA) | Sigma-Aldrich | A5503-1G |
| Multi-Inlet Vortex Mixer | NA | NA |
| Outlet fitting - MIVM | Idex Health & Science | P-942 |
| Outlet tubing - MIVM | NA | NA |
| O-ring (MIVM) | C.E. Conover | MM1.5 35.50 V75 |
| Mixer stand | NA | NA |

| Comments/description |
| --- |
| See supplemental information for engineering drawings. Review text for new mixer validation |
| Assemble on CIJ or MIVM mixer inlet with corresponding threads |
| Assemble on CIJ mixer sides (seal access point from drilling) |
| Assemble with ferrule and tubing on CIJ chamber outlet |
| Assemble with outlet fitting (large end flush with tubing) |
| Use tubing cutter for clean ends. Ensure extra tubing doesn't protrodue into mixing chamber |
| Use stabilizer-free THF to avoid solubility limits of BHT. Peroxides may interfere in some applications. |
| |
| Store cold |
| Other block sizes acceptable depending on application |
| Example hydrophobic core material |
| |
| |
| Other block sizes acceptable depending on application |
| |
| Hygroscopic. |
| |
| |
| See supplemental information for engineering drawings. Review text for new mixer validation |
| Combination with ferrule |
| Fit to ferrule ID. |
| Order bulk - consumable part. Ensure solvent compatibility if using an alternative source. |
| See Markwalter & Prud'homme for design.[17] |

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Author(s): MarkWalker, Pagels, Wilson, Ristroph, Prud'homme

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**CORRESPONDING AUTHOR:**

Name: Robert K Prud'homme

Department: Chemical & Biological Engineering

Institution: Princeton University

Article Title: Flash NanoPrecipitation for the encapsulation ...

Signature: Robert K Prud'homme Date: July 9, 2018

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Dear Xiaoyan Cao,

We would like to thank the editorial staff for their care in reviewing this manuscript. We also appreciate comments from the peer reviewers and thank them for their time. We have addressed all comments. Please see replies in red next to each, below.

Best,

Markwalter et al.

**Editorial comments:**

Changes to be made by the Author(s) regarding the written manuscript:

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3. Figure 6: Please use capitalized letters for panel labels as in other figures.- We have corrected the figure to have capital letter labels.
4. Please rephrase the Long Abstract to more clearly state the goal of the protocol.- We added two sentences and slightly rearranged the abstract to clarify the goals.
5. Please rephrase the Introduction to include a clear statement of the overall goal of this method. – We provided clarification starting at line 130.
6. Please revise the protocol (e.g., Lines 147-148, 255-271, 326-332, 365-368, etc.) to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. - We removed many details from 1.1.1 that did not fit the imperative tense and placed them in Section 1 of the SI. Section 1.5.2 was modified to the imperative, with details provided as a note. The changes at 326-332 were made by combining it as a note to the previous instruction (and later moving it to the discussion as noted below). Portions of the 365 section and others in that vicinity were reworded or moved to the SI. All instructions are now be in the imperative tense.
7. In the JoVE Protocol format, "Notes" should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section. – We have reduced extensive notes by moving to the SI, results, or discussion. In some cases, the wording was simplified or removed. In particular, we simplified the note in 1.3.1 and 1.3.4 but have kept it in the protocol because it relates directly to the step. We moved 1.4.7 to the results section. Some notes (e.g. what was originally 1.5.2 and 1.5.3 as submitted) were moved to the discussion section. We believe that

these are important to include and so we have not placed them in the SI.
8. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary. Please move the discussion about the protocol to the Discussion. Some examples:
1.1.1: Please break up into sub-steps. – We addressed this as noted above.
1.4.7, 1.5.4, 3.1.6: Please consider moving it to Discussion.- We moved 1.4.7 to the results section where it fit nicely. We have reorganized 1.5.4 into the discussion. We have moved 3.1.6 to a different location and shortened the explanation. However, we want to keep it in the protocol section because it aids in understanding why the mechanical stops are necessary and how to align them properly – this is an important safety consideration to avoid shattered glass. Other changes have been made to other portions of the protocol that we felt needed addressing based on the guidance provided here.

9. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. – We addressed the specific examples below as well as modifications to a number of sections to meet these requirements.

Some examples:
1.3.7: Please specify centrifugation parameters (force and time). – We added this information in each of the relevant steps.
1.5.1: Since this step is highlighted for filming, please describe the action in more details so it can be replicated. – We added additional details. However, the instrument analysis will vary depending on if it is supplied by a company or custom built, so we have maintained that as being more vague. It is not central to the protocol's success.
3.3.5, etc.: Please specify the type and volume of solvent used in the step. – We added additional details to clarify the cleaning steps.
Lines 440-441: As this is highlighted for filming, please describe the specific actions. – We added additional details or references to the section that is being replicated here.
10. Please include single-line spaces between all paragraphs, headings, steps, etc. – This has been implemented.
11. After you have made all the recommended changes to your protocol (listed above), please re-evaluate your protocol length. There is a 10 page limit for the Protocol. Please revise the protocol section to meet this page limit. – We are under this limit after addressing some of the brevity comments for the notes.
12. There is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. – We have modified the highlighted portions based on the changes. We would like to include some videography of Section 4 but do not need to show many details. Just general operation.
13. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. – We believe that everything selected fits this criteria.
14. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are

given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted. – We believe the highlights capture all of the important steps.
15. Discussion: Please mention any limitations of the technique.- We rephrased the beginning of the second discussion paragraph to more clearly indicate that we are addressing the limitations of the technique at that point. The process is constrained by solubility and solvent interactions.
16. Somewhere in the manuscript, please mention that CAD files for the CIJ and MIVM mixers are available in supplemental files. – This has been added where “procurement” is mentioned in the protocol.

**Reviewers' comments:**

**Reviewer #1:**
Manuscript Summary:
This manuscript describes a versatile and facile preparation of nano emulations that encapsulate therapeutics. The manuscript is well written, with a clear background introduction, easy-to-follow operation protocol and thorough discussion. I recommend publish this manuscript with some minor revisions. Please see my comments below. – We thank the reviewer for their time and care in reviewing.

Major Concerns:
No.

Minor Concerns:
-Line 189, should it be 'copolymer stabilizer' or 'copolymer'? It seems like the author defines the copolymer as the stabilizing factor for therapeutics, but reading 'copolymer stabilizer' terminology made some thing it's a stabilizer for copolymer. If possible, I suggest change the description. – We understand the reviewer’s concern but feel that including the additional descriptor helps to indicate what the function of the block copolymer is in the process. Further, since the potentially confusing descriptor is then followed by naming the block copolymer, we feel that it has been sufficiently clarified.
-Line 234, section 1.4.6. I just feel that it probably needs a lot of practice for one who can depress both solutions at a same rate. I do not know how crucial it is to control the solvent dispensing rate, is there a possible improvement to increase solvent depression consistency and minimize human error? – The reviewer brings up a good point, but we feel that we have addressed this sufficiently in the discussion. Since we are constrained by space, we feel that we are limited to providing references (of which we give many) and the short discussion already included. However, we have added a more explicit statement clarifying that the process is insensitive to flow rate above the transition to turbulent mixing.
-A general comment, since the procedure uses stabilizer-free THF, I suggest the authors add a THF peroxide test step for a work safety practice.- We added a note where BHT is mentioned regarding peroxide testing.
-Another general comment, does this method encapsulate all therapeutics into nanoparticle emulations? If not, I suggest adding a purification step or discussion either in the protocol section or in the discussion section. – We agree that noting purification methods and other characterization is important. We have included a number of references describing potential

work that could be done with TFF or dialysis to purify solvents or unencapsulated therapeutic away. However, with correct solvent selection, we achieve very high encapsulation rates.

**Reviewer #2:**

Manuscript Summary:

This manuscript is very detailed and clear to understand with careful reading. The combination of pictures and schematics for the equipment required is very informative for the user in implementing the approach. – We thank the reviewer for their time and care in reviewing.

Major Concerns:

I have no major concerns.

Minor Concerns:

Wouldn't using DMSO as the solvent for biologics limit the range of materials that could survive that environment and be encapsulated? - The reviewer brings up an important point. This is an area of focus in our laboratory currently but is far beyond the scope of this protocol as it is a complex subject in and of itself. The protocol is focused on achieving encapsulation of a range of therapeutics and we do not address stabilization techniques purposefully. We can provide patent references if desired.

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**Supplemental Information:**

Flash NanoPrecipitation for the Encapsulation of Hydrophobic and Hydrophilic Compounds in Polymeric Nanoparticles

## Section 1: Drawings and files for FNP mixers

A CIJ mixer can be produced from polyacetal thermoplastic according to the details provided in Han et al. [15] Required parts are enumerated in the materials list for this manuscript. The μMIVM can be fabricated as described by Markwalter & Prud'homme.[22] The CAD files for the CIJ and μMIVM mixers are available as supplemental files with this article. Engineering drawings for the MIVM are below (Figure S1-S4) for ease of reference. The authors can provide drawings for a two-part MIVM rather than the three-part option included here. This design may be more straight-forward to design.

*CIJ validation before use*

Validation of proper machining is crucial for consistent performance. First, check chamber inlet alignment with a 26S gauge needle, which should pass without strong resistance or bending across the mixing chamber and into the second bore. Inspect the mixing chamber and inlets for burrs from machining. Carefully work to remove these with the drill bit or a fine scalpel. Residual burrs or threads of polymer can interfere with jet alignment and lead to inconsistent results in replicates. A stereoscopic microscope is useful in this inspection. Assemble plugs onto mixer sides and Luer adapters onto the two inlets. Assemble the outlet tubing fitting such that no tubing extends past the fitting and into the mixing chamber itself. **Note**: One source of high polydispersity is tubing that has been pushed up into the mixing chamber.

*MIVM validation before use*

New MIVMs should be prepared by first polishing the face of the mixing disk with a 15μm silicon carbide paper. If produced from all stainless steel parts, we suggest electropolishing of the faces. Proper alignment of the top disk and the mixing geometry is best confirmed using a fine syringe and a dye solution in the following manner. Assemble the two disks together. Transfer a small amount of the dye solution to the tip of the needle. Pass the needle through each inlet hole in the top disk to leave a light colored mark on the mixing geometry where the stream is aligned. Then disassemble the disks and check to ensure that each mark falls in the corresponding channel.

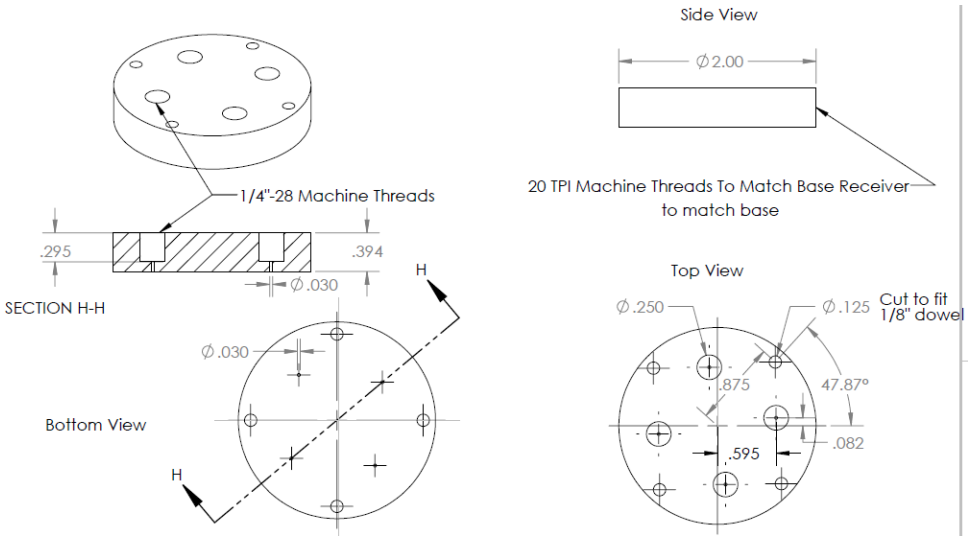


*Figure S1: Engineering drawing for the top disk of the μMIVM mixer, with the dowels not included for clarity (see Figure 2). The mixer is not functional without the dowels because they ensure alignment of the inlets with the mixer channels. The outer edge of the disk is threaded to fit the base receiver. Dimensions in inches.*

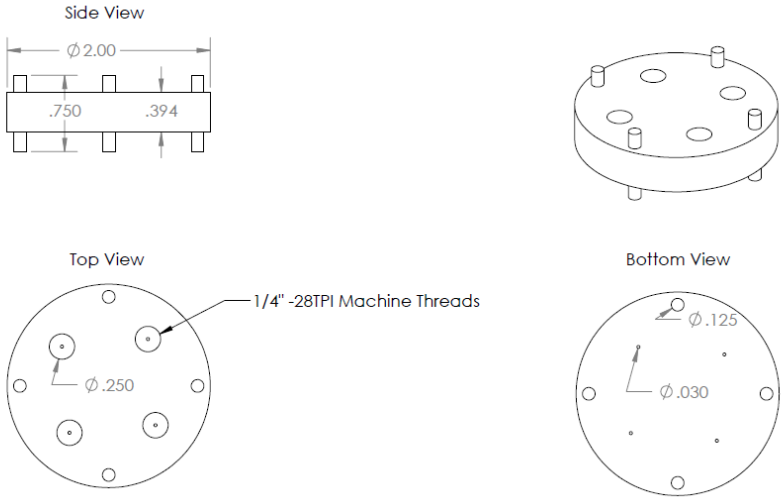


*Figure S2: Engineering drawing for the top disk of the μMIVM mixer, with the dowels included. Dimensions in inches.*

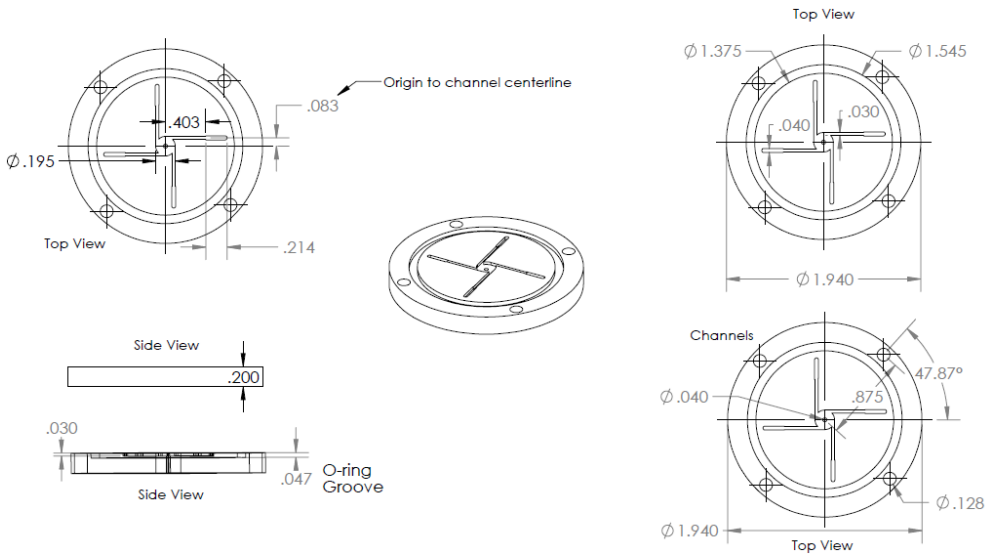


*Figure S3: Engineering drawing for the center disk of the μMIVM mixer showing the mixing geometry. Dimensions in inches.*

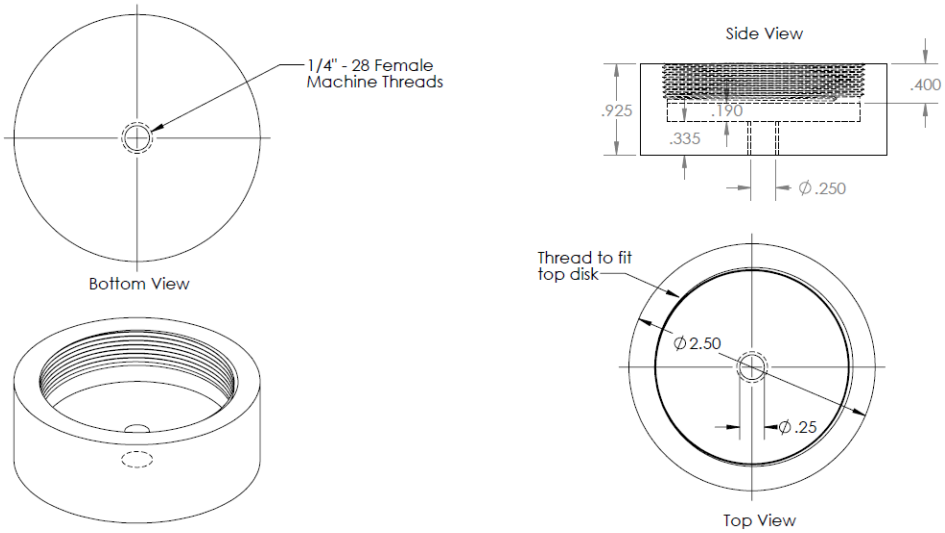


*Figure S4: Engineering drawing for the base receiver of the μMIVM mixer. The inner edge is threaded to fit the top disk. Dimensions in inches.*

## Section 2: Analysis of particle concentration by thermogravimetric analysis (TGA)

Thermogravimetric analysis may be employed to measure residual solids after solvent evaporation from the pan. This is a useful technique for the accurate determination of nanoparticle concentration (yield) after FNP as long as the solvent composition is composed of all volatile components. The presence of salts or buffer components will complicate the analysis. Figure S5 illustrates a typical TGA cycle for a nanoparticle solution. Particle concentration, C, is determined on a mass percent basis according to the formula:

$$C = \frac{N - T}{M - T} * 100$$

where N is the residual nanoparticle solids after solvent evaporation, determined from the average TGA mass in the region 115 – 130 °C; T is the pan tare, determined from the average mass measured from 550-600 °C; and M is the initial sample mass in the pan.

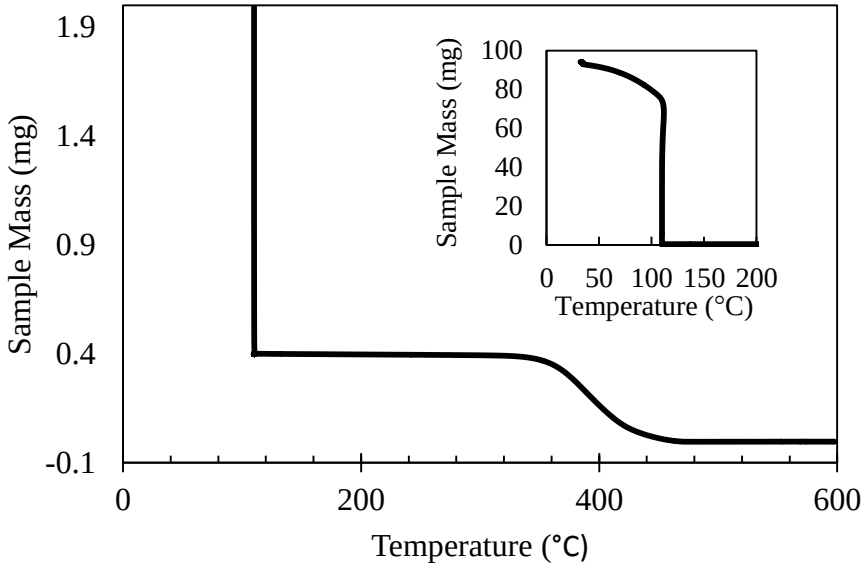


*Figure S5: Model TGA data generated from a nanoparticle solution in THF/Water. The initial steep drop is water evaporation (TGA held at 110 °C) from an initial mass of 94.18 mg. The plateau from 100 to 350 °C represents the nanoparticle mass. Further heating results in removal of organic solids to afford an* in situ *tare of the pan and any non-volatile inorganics. (inset) The full TGA profile showing solvent evaporation.*

Sample loss during filtration can be determined when TGA is conducted on the sample before and after the operation. We have found that minimal sample loss occurs when filtering relatively monodisperse PEG-coated particles produced by FNP, as long as the filter pore size is larger than the DLS size distribution. See Table S1 for representative results for a 140 nm nanoparticle with a poly(styrene) core and a PS-*b*-PEG corona with two different PVDF filter pore sizes. The loss value for each filter type is relative to the initial value. Figure S6 demonstrates the minimal size distribution change upon filtration.

*Table S1: Result of TGA analysis for filtered nanoparticle solutions*

| | **Monodisperse sample** | | **Aggregated sample** | |
|---|---|---|---|---|
| **Initial Value** | 0.428 wt% | | 0.225 wt% | |
| **0.45µm Filter** | 0.423 wt% | 1.2% loss | 0.186 wt% | 17.3% loss |
| **0.22 µm Filter** | 0.406 wt% | 5.3% loss | NT | |

Sample aggregation can occur in the FNP step or in later processing steps. Since DLS analysis results are dominated by light scattering from larger sub-populations, minor aggregation can strongly impact apparent sample quality. To address this, filtration can often be employed with

minimal losses. Table S1 presents the results for a PEG-stabilized ecumicin nanoparticle that had aggregated after lyophilization ("aggregated sample"). It was treated with a 0.45μm filter and characterized by DLS and TGA. Losses were 17.3% and the polydispersity by DLS was reduced from 0.66 to 0.18. Figure S7 illustrates the changes in the DLS analysis that result from removal of this minor aggregate population.

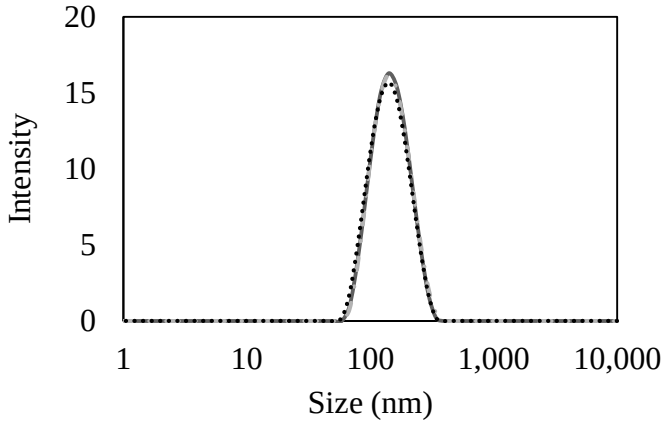


*Figure S6: DLS analysis before and after filtration of a monodisperse nanoparticle solution. Initial sample (solid gray line); after 0.45μm filter (dashed light gray line); after 0.22μm filter (dotted black line). These operations are associated with minimal sample loss by TGA (Table S1).*

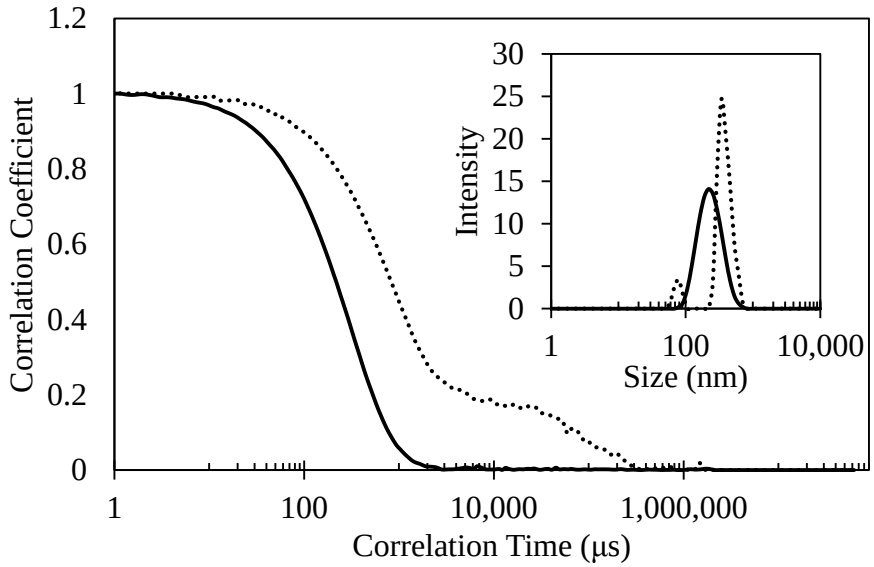


*Figure S7: Correlation function from the DLS analysis of a PEG-stabilized ecumicin nanoparticle solution before (dashed line) and after (solid line) filtration showing the recovery of a low polydispersity sample. (inset) DLS size distribution of the ecumicin nanoparticle solution before (dashed line) and after (solid line) filtration. The peaks that appear prior to filtration were generated from the correlation function but were a poor fit of the data.*

CIJ Mixer CAD file