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# The roadmap to micro: Generation of micron-sized polymeric particles using a commercial microfluidic system

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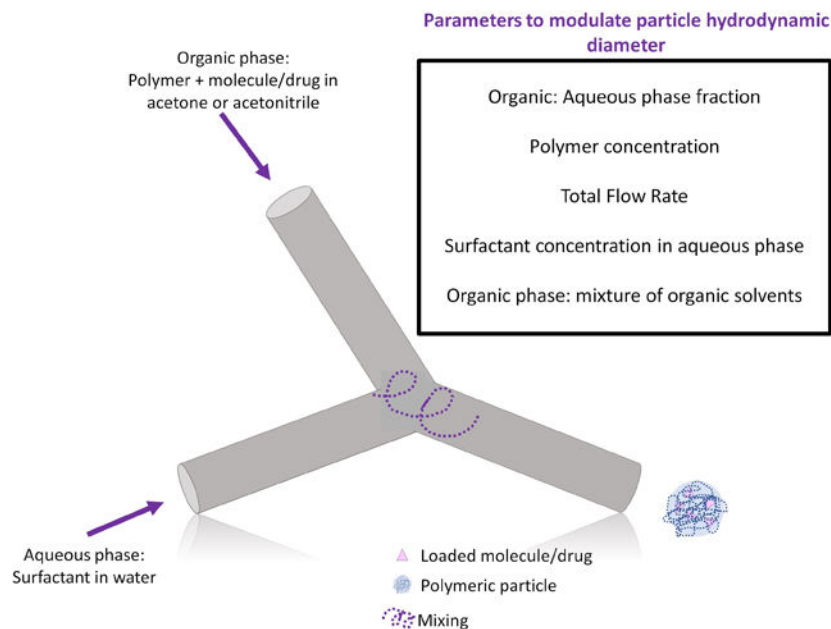
## Abstract

Microfluidic-assisted particle fabrication provides a route to circumvent the disadvantages associated with traditional methods of polymeric particle generation, such as low drug loading efficiency, challenges in controlling encapsulated drug release rates, batch-to-batch variability in particle physical properties and formulation instability. However, this approach primarily produces particles with nanometer size dimensions, which limits drug delivery modalities. Herein, we systematically studied parameters for the generation of micron-sized poly(lactic-co-glycolic) acid (PLGA) particles using a microfluidic system, the NanoAssemblr benchtop. Initially, we used two organic solvents that have been reported suitable for the fabrication of PLGA nanoparticles - acetone and acetonitrile. Subsequently, we methodically manipulated polymer concentration, organic:aqueous flow rates, total flow rate, organic phase composition, and surfactant concentration to develop a route for the fabrication of micron-sized PLGA particles. Further, we incorporated hydroxychloroquine (HCQ), a clinically approved drug to for malaria and lymphoma, and measured how its incorporation impacted particle physicochemical properties. Briefly, altering the organic phase composition by including ethyl acetate (less polar solvent), resulted in micron-scale particles, as well as increased polydispersity indexes(PDIs). Adjusting the surfactant concentration of poly vinyl alcohol (PVA) after the addition of these solvent mixtures rendered large particles with lower PDI variability. Moreover, encapsulation of HCQ influenced particle hydrodynamic diameter and PDI in a PVA concentration dependent manner. Finally, we demonstrated that unloaded and HCQ-loaded particles did not affect the viability of RAW 264.7 macrophages. This study provides an itinerary for fabricating biocompatible, drug-loaded, micron-sized polymeric particles, particularly when the drug of interest is not readily soluble in conventional organic solvents.

## Graphical Abstract

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Parameters to modulate particle hydrodynamic diameter

Organic: Aqueous phase fraction

Polymer concentration

Total Flow Rate

Surfactant concentration in aqueous phase

Organic phase: mixture of organic solvents

Organic phase: Polymer + molecule/drug in acetone or acetonitrile

Aqueous phase: Surfactant in water

△ Loaded molecule/drug

⬡ Polymeric particle

Mixing

## Keywords

NanoAssemblr; microparticles; polymeric; poly(lactic-co-glycolic) acid; microfluidics

## Introduction

Polymer-based drug delivery systems allow for safe, non- or minimally-invasive treatment procedures in the clinic, and exhibit enhanced stability in the biological environment compared to lipid-based particles (e.g. liposomes) (1). Their formulation methods allow for the encapsulation of hydrophilic and hydrophobic drugs, which provides a wide range of therapeutic applications (2–7). Furthermore, the release of drugs from degradable, polymeric particles show several benefits compared to the conventional drug administration methods, including control over the rate of drug release for extended times and reduction of drug toxicity (2–4, 8). The numerous benefits of administering drugs loaded into polymeric particles serve as the foundation for present and future medical endeavors.

One material that has gained significant traction in the drug delivery realm is poly(lactic-co-glycolic acid) (PLGA). This polyester has been approved for use in various controlled and sustained drug release strategies by the United States Food and Drug Administration (FDA) and European Medicines Agency (EMA) (4, 5). It is a biocompatible and biodegradable polymer, composed of lactic acid and glycolic acid, that hydrolyzes into biocompatible monomers (10). Moreover, it has been demonstrated that PLGA can efficiently encapsulate a wide range of agents including nucleic acids, hydrophilic and hydrophobic small molecules, and proteins (11, 12). The degradation and erosion of the polymer matrix triggers the complete release of encapsulated molecules (13). However, factors limiting wider pharmaceutical use of PLGA products include low drug loading efficiency, challenges in controlling encapsulated drug release rates, batch-to-batch variability in particle physical

properties and formulation instability (14). Evidently, the manufacture of PLGA particle-based medicines at scale is a major limitation that must be overcome if these products are to ever be used in the clinic.

Despite many successes at the laboratory level, scale-up of polymeric particles using conventional particle generation equipment has often proven difficult. Unsurprisingly, the scale-up stage of polymeric particle-based drug carrier often results in inferior particle quality, such as size heterogeneity, which can induce Ostwald ripening (the dissolution of smaller particles into larger particles) further exacerbating batch-to-batch uniformity and reproducibility (15). Ostensibly, manufacturing approaches that efficiently optimize PLGA particle formulations in a reproducible manner and at scale are required to improve the translational outlook of these polymeric particle formulations. In this direction, the use of microfluidic, high-throughput instruments is now under investigation. The fabrication of polymeric particles using microfluidic devices allows for a controlled emulsion process which renders homogeneous particle properties. Microfluidic systems add control over the mixing time by varying solvent flow rates or channel geometry. Additionally, improved heat transfer due to large surface areas allows for efficient temperature control by preventing large temperature gradients. Finally, as the channel length is directly proportional to the time taken by the organic and aqueous phases to mix and flow through the microchannels, the supersaturation, nucleation and particle growth or reaction time can be controlled by tuning the channel length (16). The advantages of microfluidic devices over conventional equipment, such as homogenizers, include improved efficiency in generation time, particle quality, reproducibility and after-production handling. Particle hydrodynamic size control and increased encapsulation efficiencies are the major draws of microfluidic system use for potential clinical applications (14, 17).

Particle hydrodynamic diameter is particularly relevant due to its role in determining drug delivery mechanism, biodistribution and final fate of the material and drugs in the body (18–26). Some of the parameters that govern final hydrodynamic diameter of hydrophobic polymer-based particle fabrication via microfluidic device are polymer concentration, organic:aqueous flow rates, total flow rate, miscibility of aqueous and organic phases, and surfactant concentration (14, 27–33). Of consequence, is also the polydispersity index which is a strong indicator of hydrodynamic diameter homogeneity (or heterogeneity). The uniformity of generated particles can impact their function as drug delivery vehicles. Due to its small dimensions, the flow of a fluid through a microfluidic system is within the laminar regime. Therefore, the governing mass transfer phenomenon between two streams is diffusion within the convergence area. The formation of the particles take place upon a supersaturation of hydrophobic molecules (due to aqueous and organic solvents mixture), which leads to nucleation of particles, and growth of the formed nuclei (34–36). The diffusion process produces local supersaturation, and the turbulences at the interface of streams (diffusion layer) result in efficient mixing that drive nucleation and particle growth (35, 36). A higher level of super saturation leads to increasing the nucleation rate compared to growth rate and causes fabrication of smaller sized particles.

In this study, we focused on determining routes for the preparation of micron-sized PLGA particles using a microfluidic system - the NanoAssemblr benchtop. The NanoAssemblr

benchtop is a microchannel-based system for the fabrication of nano- and micrometer delivery systems. The microchannels of the NanoAssemblr system are built in a platform called cartridge. Once organic and aqueous phases are introduced into the cartridge, they mix within the system. The staggered herringbone structure in the microchannels (**Graphical Abstract**) stirs the mixture with chaotic advection, contributing to a homogeneous and reproducible self-assembling emulsion-based nanoparticle production (27, 29, 30, 37). Although this system is efficient in the fabrication of homogenous particles, the microchannels are compatible with a limited number of solvents, such as ethanol, acetone, acetonitrile, ethyl acetate, 1-propanol, isopropanol, methanol and dimethyl sulfoxide. Since the solvents selected play a role in the mixing dynamics of the organic and aqueous phases and final emulsion, the limited solvent compatibility adds challenges to generating particles with specific characteristics. Our goal was to determine the parameters of fabrication for consistent generation of PLGA particles with an average hydrodynamic diameter of ~1 μm. We were interested in loading an organic soluble (unsalted) form of hydroxychloroquine (HCQ), a clinically-approved drug to treat several pathologies including malaria and lymphoma, as a model drug.

Micron-sized PLGA particles have been generated using the NanoAssemblr benchtop with ethyl acetate as the solvent in the organic phase of the emulsion process (16, 18). However, these studies were not comprehensive, particularly with respect to the nature of the drugs loaded. For instance, our drug of interest, HCQ, is only partially soluble in ethyl acetate but readily soluble in acetone or acetonitrile. Moreover, reports using acetone or acetonitrile in the organic phase for PLGA particle fabrication using the NanoAssemblr benchtop only show successful fabrication of nanospheres (38, 39). To determine the appropriate parameters for the generation of particles with hydrodynamic diameter in the micron scale, we systematically changed the particle preparation parameters following a decision-making workflow, which is shown in Figure 1. We also studied particle characteristics upon the encapsulation of HCQ, including size, polydispersity index (PDI), loading efficiency and HCQ release kinetics. Finally, blank and HCQ-loaded particles were co-incubated with RAW 264.7 cells and their effect on cell viability was evaluated.

## Materials and Methods

Poly(lactic-co-glycolic acid) (~22 kDa), acid terminated, with differing ratios of lactic to glycolic acid 50:50 (PURAC 5004A) was kindly donated by Corbion (Amsterdam, Noord-Holland). Polyvinyl alcohol (PVA), and hydroxychloroquine sulfate were purchased from Thermo Fisher Scientific (New Jersey, USA). All other chemicals were reagent grade and used without purification. Poly(lactic-co-glycolic acid) particles were prepared using a NanoAssemblr Benchtop Instrument (Precision NanoSystems, Vancouver, BC, Canada) as specified. RAW 264.7 macrophage cells were obtained from ATCC (Manassas, VA). Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham media, heat inactivated American grade fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Millipore Sigma (Burlington, MA).

### Desalting of HCQ

For improved solubility in organic solvents, sulfate was removed from HCQ. Hydroxychloroquine sulfate (dispersed in methanol) was converted to a salt-free form by reaction with an aqueous solution of sodium hydroxide (2-fold molar excess) for 1 h at 25 °C. The salts were then removed by filtration (7 mm filter) and centrifugation at 8500 rpm. Methanol in the filtrate was evaporated using a rotary evaporation. Lastly, concentrated unsalted HCQ was stored at 4 °C.

### Particle Fabrication

Briefly, a syringe of acetone or acetonitrile solutions containing either PLGA or PLGA and a quantity of unsalted HCQ (concentrations varied), and another syringe of aqueous solution containing a defined concentration of PVA were attached to the instrument cartridge. The solutions were dispensed into this microfluidic cartridge and mixed in the staggered herringbone structure. Total flow rate was controlled by software linked to the system. The product mixture containing PLGA particles or hydroxychloroquine-loaded, PLGA particles was collected in a collection tube. This collection tube had PVA % (w/v) solutions equal to the aqueous phase used in the corresponding particle fabrication procedure sufficient for a 6-fold dilution of the sample. Particles were washed 3 times with DI water followed by centrifugation (8500 rpm, 10 min, using Benchtop Centrifuge, Sorvall) to remove excess of PVA, organic solvent and free HCQ (if applicable). The samples were lyophilized and stored at −20°C until use. The mean hydrodynamic diameter and PDI of the particles were subsequently measured using a Zetasizer (Malvern, UK).

### Determination of Hydroxychloroquine Loading Efficiency

Hydroxychloroquine loading was determined by solubilizing HCQ particles in methylene chloride and extracting HCQ with acidified water (sulfuric acid solution). These aqueous solutions containing HCQ were centrifuged to remove excess of salts, water was rotary evaporated, and samples were diluted in known amounts of DI water. The absorbances of these samples in DI water was measured at 343 nm using a NanoDrop microvolume spectrophotometer (Thermo Fisher, NJ, USA). The encapsulation efficiency of hydroxychloroquine in the PLGA nanoparticles was calculated from the concentration of hydroxychloroquine measured in the known particle mass and based on the initial quantity of HCQ added for the particle fabrication (**Equation shown below**).

$$\text{Loading efficiency} = \frac{Measured\ HCQ\ Concentration\ on\ Sample}{Theorectical\ HCQ\ Concentration} \times 100$$

### In Vitro Drug Release

Hydroxychloroquine release from PLGA particles was measured as previously reported with some modifications (5). Briefly, 10 mg PLGA particle samples (in triplicate) were dispersed in 200 μL of 1% Tween-20 solution in PBS. The samples were incubated at 37°C. At different time points (1 h, 24 h, 48 h, 72 h), samples were centrifuged at 8500 rpm for

10 minutes and supernatants collected. The absorbance of these collected solutions was measured at 343 nm using the NanoDrop microvolume spectrophotometer.

### Scanning Electron Microscopy (SEM)

Solutions of blank and hydroxychloroquine-loaded PLGA particles were dried by air on a stub and their appearance and shape was observed using a scanning electron microscope (FIB-Scios Dual Beam SEM, Thermo Fisher, NJ, USA).

### Cytotoxicity Study

A macrophage cell line, RAW 264.7, was cultured at 37°C, 5% carbon dioxide and using Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham media, supplemented with 10% FBS and 5% penicillin/streptomycin. Selected particle batches were co-incubated with RAW.264.7 cells for 2 hours (40, 41). Cells were then washed twice with phosphate buffered saline, and the viability of these cells was measured using an LDH assay (Abcam, USA) 24 hours after co-incubation via a Safire Fluorescence Plate Reader (Tecan).

### Statistical Analysis

The data are presented as mean ± S.E.M. and analyzed using one-way ANOVA, followed by a Tukey's test. Values of P  0.05 were considered statistically significant.

## Results

### Acetone-mediated PLGA Particle Fabrication – Varying Polymer Concentration

The fabrication parameters described in Morikawa et al. were used as starting point in our study to determine the conditions for our desired particle hydrodynamic diameter (1 μm) with a low PDI. In this study, a 3:1 organic to aqueous flow rate ratio, 1% (w/v) PVA as surfactant in the aqueous phase, and 3 ml/min total flow rate resulted in nanoparticles with an average hydrodynamic diameter smaller than 300 nm. We kept these parameters and focused on manipulating the starting PLGA concentration - results are summarized in Figure 2A. We observed that 30 mg/ml PLGA samples had the largest average hydrodynamic diameter ($409 \pm 42$ nm). However, it was not statistically different to the smallest hydrodynamic diameter obtained using a starting PLGA concentration of 10 mg/ml ($271 \pm 31$ nm). Also, some PLGA clogging was observed in the cartridge at the highest concentration of PLGA (50 mg/ml PLGA). Larger PDIs, 0.360 and 0.313, were obtained on higher PLGA concentration, for 30 and 50 mg/ml, respectively (Figure 2A).

### Acetone-mediated PLGA Particle Fabrication – Varying Organic:Aqueous Phase Flow Rate Ratio

We fixed the PLGA concentration to 30 mg/ml (due to the largest diameter obtained) and investigated whether a change in organic to aqueous flow rate ratio, at a total flow rate of 3 ml/min would affect final particle diameter and PDI (Figure 2B). We observed a statistically significant decrease in particle hydrodynamic diameter on the organic:aqueous flow rate of 2:1 ($282 \pm 40$ nm) in comparison to the 3:1 ratio ($409 \pm 42$ nm), which also had the largest PDI (Figure 2B). The hydrodynamic diameter of the particles slightly increased on 1:1

organic:aqueous flow rate ratio ($350 \pm 79$ nm). We also ran 20 mg particle syntheses at 1:2 and 1:3 organic:aqueous flow rates. However, we could not collect any particle samples after centrifugation during particle washes. Probably, supersaturation of PLGA and nucleation was significantly reduced when increasing aqueous flow rate relative to organic flow rate in this combination of parameters.

### Acetone-mediated PLGA Particle Fabrication – Varying Total Flow Rate

At the next stage, we investigated the effect of the total flow rate on final particle size while keeping the organic:aqueous flow rate ratio at 3:1 (Figure 2C). We observed nanoparticle average hydrodynamic diameters of $409 \pm 67$ nm, $314 \pm 72$ nm and $345 \pm 61$ nm at total flow rates of 3, 8 and 13 ml/min, respectively (Figure 2C). The PDI of particle preparations were 0.360, 0.222, and 0.213, respectively (Figure 2C). There was no statistical difference between particle hydrodynamic diameters nor PDIs.

### Modification of Organic Phase Composition: Addition of Ethyl Acetate to Acetone-mediated PLGA Particle Fabrication

We investigated the use of a mixture of acetone (75% or 50% v/v) and ethyl acetate (25% or 50% v/v), as organic phases in which we dissolved PLGA (30 mg/ml). We set the total flow rate to 13 ml/min, organic:aqueous flow rate at 3:1, and varied the PVA concentration in the aqueous phase to 0.1%, 0.5% and 1% (w/v). (Figure S2 shows resulting particles at a total flow rate of 3 ml/min. Since largest particle diameter at this condition also had the highest PDI, we continued our experiments at a total flow rate of 13 ml/min). The resulting particle average hydrodynamic diameters and PDIs when using a mixture of 25% ethyl acetate and 75% acetone (v/v) are displayed in Figure 3. Average hydrodynamic diameters were $574 \pm 43$ nm, $654 \pm 160$ nm, and $446 \pm 248$ nm at 0.1, 0.5% and 1% (w/v) PVA, respectively. Using 1% PVA (w/v) resulted in a PDI of 0.186, while 0.5% and 0.1 % PVA (w/v) resulted in PDIs of 0.173 and 0.082, respectively. Particle fabrication using a mixture of 50% (v/v) acetone and 50% (v/v) ethyl acetate was unsuccessful due to formation of large clumps and adherence of the material to the walls of the collection tubes. The resultant polarity of the organic solvent mixture was probably not suitable for the particle formulation. The addition of 25% (v/v) ethyl acetate in the organic phase effectively promoted an increase in nanoparticle hydrodynamic diameter. However, the particles generated still fell short of the desired 1 μm diameter.

### Acetonitrile-mediated PLGA Particle Fabrication – Varying Polymer Concentration

We also investigated using acetonitrile as the organic phase solvent. Acetonitrile is miscible with water, has a relative polarity to water of 0.420 (50) and readily dissolves unsalted HCQ. In Meikle et al., the fabrication of 200 nm acetonitrile-mediated, rifampicin-loaded PLGA nanoparticles was explored using the Nanoassemblr benchtop system by fixing PLGA concentration to 20 mg/ml, organic:aqueous ratio to 2:1, total flow rate to 8 ml/min, and 2% PVA (w/v) as aqueous phase (39). We selected these initial parameters for our study and proceeded to investigate the effect of PLGA concentration on particle hydrodynamic diameter. We ran 30 mg batches, changing the concentration of PLGA in the organic phase (10, 20, 30, 40, and 50 mg/ml). We broadened the mass concentration range to observe more resolution in PDI change. The resulting mean hydrodynamic diameters had no statistical

difference between them and ranged between 319 – 436 nm (Figure 4A). Fabrication using 10 mg/ml PLGA had the lowest average PDI of 0.089. Notably, the PDI increased with the escalating PLGA concentration to an average of 0.517 for 50 mg/ml of PLGA (Figure 4A). The samples obtained using 10 mg/ml PLGA had an average yield of $22 \pm 7\%$, whereas in the 20 mg/ml samples the average yield was $57 \pm 12\%$. Given the emphasis on deciphering a potential method for mass production of these types of particles, we selected a mass concentration of 20 mg/ml for the next parameter test.

### Acetonitrile-mediated PLGA Particle Fabrication – Varying Organic:Aqueous Phase Flow Rate Ratio

The next parameter we manipulated was the organic:aqueous flow rate ratio (3:1, 2:1, 1:1, 1:2, 1:3). Figure 4B shows that the lowest organic:aqueous ratio (1:3) rendered the largest average particle diameter, $385 \pm 27$ nm but it was not statistically different compared to the other flow rate ratios mixtures 1:2, 1:1, 2:1, 3:1 with average hydrodynamic diameters of $244 \pm 57$ nm, $248 \pm 9$ nm, $311 \pm 18$ nm, $280 \pm 8$ nm, respectively. Average PDI of this particular parameter was 0.200.

### Acetonitrile-mediated PLGA Particle Fabrication – Varying Total Flow Rate

Varying the total flow rate rendered particles within the 294–370 nm range. Further, no significant differences were observed in their average hydrodynamic diameter or PDI (Figure 4C).

### Addition of Ethyl Acetate to Acetonitrile-mediated PLGA Particle Fabrication

Thus, we proceeded to test whether the addition of ethyl acetate to the organic phase mixture promoted an increase of the hydrodynamic diameter of particles. We investigated the use of a mixture of acetonitrile (75% or 50% v/v) with ethyl acetate (25% or 50% v/v) as the organic phase in which we dissolved PLGA (20 mg/ml). We kept the total flow rate at 13 ml/min, the organic:aqueous flow rate at 1:3, and PVA concentration in the aqueous phase to 2% (w/v). Resulting particle average hydrodynamic diameters are summarized in Figure 5A.

We obtained a statistically significant increase in particle hydrodynamic diameter: $723 \pm 109$ nm and $916 \pm 173$ nm, with 25% (v/v) ethyl acetate and 50% (v/v) ethyl acetate, respectively. The addition of ethyl acetate to the organic phase successfully promoted the fabrication of microparticles. However, the PDIs for both conditions were higher than 0.2, indicative of highly polydisperse microparticles (Figure 5A). Interestingly, a mixture of PLGA in acetonitrile (50% v/v) and ethyl acetate (50% v/v) with the same particle fabrication parameters, but at a 3:1 organic:aqueous flow rate ratio, rendered highly polydisperse particles with an average volume weighted hydrodynamic diameter of 118 um (Fig. S4). This particle size was beyond our desired goal and as such, we did not further investigate this observation.

### Acetonitrile-ethyl acetate mediated PLGA Particle Fabrication – Varying Surfactant Content

Subsequently, we fixed the mixture of 50% (v/v) acetonitrile and 50% (v/v) ethyl acetate as the organic phase solvent, but changed the surfactant concentration in the aqueous phase to 1 – 4% PVA (w/v). We hypothesized this increase in surfactant concentration would promote

a homogeneous diameter in the microparticle batch, indicated by a lower PDI. This increase in surfactant concentration in the aqueous phase did not statistically affect the PDI, or hydrodynamic diameter of generated particles. However, we observed a 1.35-fold reduction of the average hydrodynamic diameter of particles fabricated at 4% PVA compared to particles fabricated at 1% PVA (Figure 5B). In Figure 5C, SEM micrographs show the variation in physical diameter of our microparticles at different PVA % concentrations, as well as their shape and morphologies which were spherical and smooth regardless of the generation conditions.

### Addition of Hydroxychloroquine to Acetone-ethyl acetate and Acetonitrile-ethyl acetate mediated PLGA Particle Fabrications

We investigated whether the addition of HCQ to the fabrication processes, had any effect on particle hydrodynamic diameter and PDI. In this experiment, we also varied the % PVA (w/v) in the aqueous phase. These fabrications were performed running solutions containing 100 mg of PLGA and an equal amount of unsalted HCQ in the organic phase through the NanoAssemblr benchtop system. The resulting hydrodynamic diameters and PDIs are summarized in Figure 6.

Particle fabrications using the mixture of acetone (75% v/v) and ethyl acetate (25% v/v) were performed in batches of 100 mg PLGA at a total flow rate of 13 ml/min, 3:1 aqueous:organic flow rate and varying PVA concentration in the aqueous phase. Particle yields were relatively low, not enough for both loading efficiency quantification and HCQ release kinetics (Table 1), but enough to characterize their hydrodynamic diameter and PDI on the DLS. At 0.1 % PVA (w/v), the mean hydrodynamic diameter was $946 \pm 82$ nm with a PDI of 0.469 (Figure 6A). At 0.5% and 1% PVA (w/v) mean hydrodynamic diameters were $358 \pm 176$ and $299 \pm 67$ nm with PDIs of 0.343 and 0.251, respectively. The incorporation of HCQ reduced the hydrodynamic size of nanoparticles compared to unloaded/blank formulation, except at the lowest PVA % concentration (w/v) in the aqueous phase. That same sample hydrodynamic diameter was statistically larger than its corresponding blank particle formulation ($574 \pm 43$ nm) and HCQ-loaded PLGA particle fabrications at 0.5% and 1% PVA concentrations. However, its high PDI could indicate the presence of particle aggregates. Blank particles fabricated at 100 mg batches did not show any statistical difference in resulting hydrodynamic diameter (Figure S3) compared to 30 mg blank particle fabrications.

We also loaded HCQ in particle fabrications mediated by acetonitrile (50% v/v) and ethyl acetate (50% v/v), while varying % PVA (w/v) in the aqueous phase. We found that there was a statistically significant difference between HCQ-loaded PLGA particle hydrodynamic diameter at 1% PVA and 3% PVA concentrations, and between fabrications at 1% PVA and 4% PVA. The PDIs, which were 0.235, 0.223, 0.184, and 0.212 for 1%, 2%, 3%, and 4% PVA (w/v), respectively, had no statistically significant difference between them (Figure 6B). The particle yields of HCQ-loaded PLGA particles are summarized in Table 1.

Figure 6C shows the calculated loading efficiency and HCQ release kinetics on these acetonitrile-ethyl acetate-mediated particle fabrications. Fabrications using 1% PVA (w/v) had the greatest HCQ loading efficiency. In general, loading efficiency fell precipitously

when surfactant concentrations were increased above 1% (w/v). We also looked at the release kinetics for these microparticles produced in varying PVA concentrations. Incidentally, the 1% PVA MPs had the slowest release rates, particularly in comparison to the 3% PVA (w/v) sample at 24 and 48 hours. SEM micrographs show the variation in physical diameter of HCQ-loaded MPs at different PVA concentrations (Figure 6D).

### Cytotoxic Evaluation of 1um HCQ-loaded PLGA Particles

Finally, we chose the particle formulation with the greatest HCQ loading efficiency and tested its effect on the viability of mammalian cells. In this experiment, acetonitrile-ethyl acetate-mediated HCQ-loaded PLGA particles fabricated at 1% PVA (v/v) in aqueous phase were co-incubated with RAW 264.7 cells for two hours. Cells were washed and cell viability was measured 24 hours after treatment via an LDH assay. Figure 7 shows that none of the particulate treatments had a cytotoxic effect.

## Discussion

Microfluidic devices provide means to circumvent polymeric particle fabrication challenges, such as batch-to-batch variability in particle physical properties and formulation instability. Contrary to most of the polymeric particle fabrication studies using microfluidic devices, which focus on the preparation of nano scale particle formulations, this report shows the route to the fabrication of 1 μm hydrodynamic diameter particles. These microparticles were loaded with unsalted HCQ, a drug not readily soluble in the typical solvents compatible with the commercially available microfluidic system Nanoassemblr benchtop® for the fabrication of micro-size particles. Acetone and acetonitrile are solvents compatible with this system to which our drug of interest, unsalted HCQ, is also soluble in. However, these solvents typically render nano scale particulate in any microfluidic device-mediated emulsion process due to their miscibility with the aqueous phase. To date, no study has explored the fabrication of PLGA microparticle preparations via microfluidic systems using acetone as the organic solvent (43–46). In the case of acetonitrile, only one study has shown the fabrication of acetonitrile-mediated micron-sized PLGA particles via microfluidic-assisted nanoprecipitation. Although their system used the microfluidic cartridges provided by Precision Nanosystems Inc. (to our knowledge, the same used in this study), they included a customized dispensing system and implemented an overnight solvent evaporation step after particle dispensing through a customized 0.8 mm polytetrafluoroethylene (PFTE) tubing system, which may alter final particle characteristics (26). We engineered the methods to sufficiently alter the diffusion dynamics between the organic and aqueous phases leading to the generation of unloaded and HCQ-loaded 1 μm PLGA particles using these solvents.

In the case of acetone, Morikawa et al. explored how different parameters affected the fabrication of curcumin-loaded PLGA nanoparticles in the NanoAssemblr benchtop using acetone as the organic solvent (38). They used 3:1 organic to aqueous ratio, 1% (w/v) PVA as surfactant in the aqueous phase, and 3 ml/min total flow rate, resulting nanoparticles had an average hydrodynamic diameter smaller than 300 nm. We used these parameters as our starting point for our acetone-mediated particle fabrications while varying the polymer concentration in the organic phase.

Previous studies have reported larger particle diameters at higher polymer concentrations (47, 48). We observed a trend in hydrodynamic diameter increase as increasing polymer concentration (and noticed macroscopic aggregates at the highest concentration). However, the difference between effective particle suspensions' hydrodynamic diameters was not statistically significant at the combination of specified set of parameters for this experiment. Therefore, increasing polymer concentration did not have a significant effective impact in the supersaturation of the polymer, subsequent nucleation and growth of PLGA particles at tested conditions. Particle hydrodynamic diameter was not significantly affected during the trajectory inside the microfluidic cartridge by the increase in polymer concentration compared to the formation of more particles via nucleation. Our results indicate that PLGA chains were mainly forming numerous nuclei on the effective conditions tested, thus generating more particles instead of growing into larger particles. This separation between nucleation and growth is suggested as one of the benefits of particle synthesis experimental designs in microfluidic systems (17). There was an increase in PDI as polymer concentration increased, but these changes in mass concentration were not enough to obtain a statistically significant difference in final particle hydrodynamic diameter under the effective conditions. We attribute this to the invariant polymer solution viscosity. Its variation would have affected both the speed and diffusion of the aqueous and organic phases to form the particles. It should be noted that testing higher concentrations was not possible due to clogging of microfluidic channels. Therefore, we proceeded to test the organic:aqueous flow rate ratios in our acetone-mediated fabrications.

Higher flow rates of antisolvent phase promote nucleation because of polymer supersaturation (47). Therefore, at appropriate conditions, smaller particle diameters are expected when aqueous phase flow rate ratio is larger compared to organic flow rate. This has been observed in relatively high total flow rates and depends on the interfacial tension/diffusion between the organic and aqueous phases in the microfluidic system (47, 49). Acetone is miscible in water, whereas PLGA is readily soluble in acetone but not in water. Although the organic:aqueous flow rate ratio of 2:1 rendered particles with an average hydrodynamic diameter statistically smaller than 3:1 organic:aqueous flow rate ratio, particle average hydrodynamic diameter when testing 1:1 organic:aqueous flow rate ratio was not statistically different from 3:1 organic:aqueous flow rate ratio. There was also a higher hydrodynamic diameter standard deviation at 1:1 organic:aqueous flow rate ratio, which suggests this flow rate ratio in combination with the other parameters established for this experiment produced high variability. This is probably due to the diffusion dynamic during the mixing of the phases at that particular total flow rate. The largest PDI at 3:1 organic:aqueous flow rate ratio suggests there was not a homogeneous nucleation time in this particle fabrication. These observations lead to exploring the next parameter, total flow rate.

Generally, smaller particle hydrodynamic diameters are expected at higher total flow rate, at the appropriate conditions (47, 49). Time reduction within channels due to high total flow rates reduces nucleation time and growth. Although the conditions of our experiment did not statistically affect the nucleation time, the highest PDI at the slowest total flow rate tested (3 ml/min) suggests that there was not a homogeneous nucleation time at this total flow rate.

Changing PLGA concentration, organic:aqueous flow rate ratios, and total flow rate did not render particles with hydrodynamic diameters larger than 0.5 μm in our acetone-mediated PLGA particle fabrications. Since our experimental design testing these parameters did not significantly alter the supersaturation point of the polymer, we tested changing the organic phase composition. Previous studies have shown that the polarity of the organic phase, which affects miscibility with aqueous phase, plays a role in the resultant liposome, micelle, protein and polymeric particle diameter fabricated via microfluidic systems (27–29, 31–35, 47, 49). Tuning polarity affects supersaturation of the system since the diffusion of organic and aqueous phases is modified. Acetone is miscible in water and has a relative polarity of 0.355 (50). Reports show that fabrication of PLGA particles using ethyl acetate, which has a lower relative polarity of 0.228 and a miscibility of 8.7 g/100 g of water, results in micron-sized particles due its decreased diffusion with antisolvent and effect in supersaturation process (50). Therefore, we investigated the use of a mixture of acetone (75% or 50% v/v) and ethyl acetate (25% or 50% v/v), as organic phases in which we dissolved PLGA. The low PDIs and larger particle mean hydrodynamic diameters obtained by changing the organic solvent composition show a more homogenous nucleation time within the microfluidic channel and an increase in the dynamic surface tension between the organic and aqueous fluids due to the presence of ethyl acetate.

We also investigated using acetonitrile as the organic phase solvent. Acetonitrile is miscible with water, has a relative polarity to water of 0.420 (50) and readily dissolves unsalted HCQ. In Meikle et al., the fabrication of 200 nm acetonitrile-mediated, rifampicin-loaded PLGA nanoparticles was explored using the Nanoassemblr benchtop system by fixing PLGA concentration to 20 mg/ml, organic:aqueous ratio to 2:1, total flow rate to 8 ml/min, and 2% PVA (w/v) as aqueous phase (39). We started our acetonitrile-mediated PLGA particle fabrications with these parameters while varying PLGA concentration. Our results, again, indicate that selected PLGA concentrations under the specified conditions did not significantly affect PLGA supersaturation to render particles with a hydrodynamic diameter of 1 μm. Obtained PDIs suggest there was a homogeneous nucleation time in the system at 10 mg/ml PLGA when compared to the other concentrations. The organic:aqueous phase flow rate ratio was tested next.

In the case of acetonitrile-mediated fabrications, studies have shown conflicting evidence in how organic:aqueous flow rates affect particle hydrodynamic diameter in microfluidic systems. (26, 27, 47, 48, 51). For instance, in Chiesa et al., is shown that there is a statistically significant increase in hydrodynamic diameter when fabricating nanoparticles in the Nanoassemblr benchtop system when using 10 mg/ml of acid-terminated PLGA (lactic to glycolide ratio of 75:25, 25,000 Da) in acetonitrile and increasing the organic:aqueous flow rate ratios from 1:5 – 1:1 (37). This same behavior was seen at all total flow rates tested of 5, 10 and 15 ml/min in the study. Conversely, Gdowski et al. demonstrated that at a concentration of 10 mg/ml of PLGA (ester-terminated, lactide to glycolide ratio 50:50, 45,000–55,000 Da), in the presence of a PEG-based polymer coating in the aqueous phase and at a total flow rate ratio of 12 ml/min, there was no statistical difference in the hydrodynamic diameter of resulting particles when using organic:aqueous flow rates of 1:1 – 1:5. However, a maximum in average PDI was observed at the 1:5 ratio (48). At the lowest organic:aqueous flow rate ratio tested, 1:9, the hydrodynamic diameter

was the largest (less than 150 nm) and the PDI was relatively lower compared to some greater organic:aqueous flow rates tested. Seemingly, it is difficult to predict the influence of organic:aqueous flow rate ratios on the final particle hydrodynamic diameter as the unique diffusion dynamics present in the specific particle fabrication formulation has greater influence. In our experimental conditions, no statistical difference in hydrodynamic diameter nor PDI was observed when varying organic:aqueous phase flow rate ratios. Moreover, no statistical difference in hydrodynamic diameter nor PDI was observed when varying total flow rate. Polymer concentration had a greater effect in nucleation process of our acetonitrile-mediated fabrications as noted by an increase in PDI at 30 and 40 mg/ml.

The next aspect of the methodology was to explore changing the organic solvent composition. Mixtures of acetonitrile (75% v/v) and ethyl acetate (25% v/v), and acetonitrile (50% v/v) and ethyl acetate (50% v/v) were tested in the organic solvent phase. The addition of ethyl acetate increased the average hydrodynamic diameter of particles. Particularly, the mixture of acetonitrile (50% v/v) and ethyl acetate (50% v/v) significantly increased the hydrodynamic diameter of particles to the micrometer range. Moreover, the combination of appropriate aqueous phase viscosity via the addition of a higher concentration of detergent (4% PVA) may have limited the diffusion of PLGA organic phase within the system, modulating final particle hydrodynamic diameter back to nanometer scale. Interestingly, this is not always the case. For instance, in Chiesa et al., increasing PVA concentration synthesized PLGA nanoparticles of larger hydrodynamic diameters (49).

The next stage was to incorporate HCQ into these microparticles. Although unsalted HCQ is not soluble in ethyl acetate, it is soluble in the mixtures of ethyl acetate and acetone, and ethyl acetate and acetonitrile tested. We investigated whether the addition of HCQ to the fabrication processes, had any effect on particle hydrodynamic diameter and PDI. In this experiment, we also varied the % PVA (w/v) in the aqueous phase. The high PDI at the lowest PVA% of the HCQ-loaded PLGA particle in the acetone-mediated fabrication shows that the addition of the drug affected the diffusion of the organic phase in the aqueous phase at set organic:aqueous flow rate ratios and total flow rate. The increase in surfactant (PVA) in the aqueous phase shifted the diffusion parameters and promoted smaller and homogeneous hydrodynamic diameters. We also observed a significant hydrodynamic diameter reduction as PVA concentration increased in the in acetonitrile-ethyl acetate mediated particle fabrications loaded with HCQ. The HCQ-loaded PLGA particles' hydrodynamic diameters varied as a function of PVA % concentration (v/v) in the aqueous phase in a pattern consistent with blank particles. In the conditions tested, the less miscible the organic phase is with the aqueous phase, the larger the particles. Moreover, as the viscosity of the aqueous phase increases with a higher concentration of PVA as surfactant in the acetonitrile-ethyl acetate mediated fabrication, the smaller unloaded and HCQ-loaded PLGA particles are obtained (having more impact in the HCQ-loaded fabrications). Evidently, HCQ addition impacted the diffusion between the phases in the acetone-mediated system at the conditions tested. We also observed a higher HCQ loading efficiency in the acetonitrile-ethyl acetate particle fabrications at the lowest surfactant concentration, indicative of the influence of PVA in the diffusion dynamics of the organic and aqueous phases. Generally, HCQ release results show lack of an initial burst that is typical of PLGA, which demonstrates that most of this drug was well dispersed in the

polymer matrix. SEM micrographs show the variation in physical diameter of unloaded and HCQ-loaded smooth particulate spheres at different PVA concentrations. Finally, the particles with the highest HCQ loading and largest diameter, the acetonitrile-ethyl acetate mediated HCQ-loaded PLGA MPs fabricated at 1% PVA, and its unloaded MP counterpart, were found not toxic to mammalian cells 24 hours after two-hour co-incubation.

## Conclusion

Here we described a systematic approach for the preparation of PLGA micron-sized particles using a microfluidic system - the NanoAssemblr benchtop. While PLGA concentration, organic to aqueous phase rates, and total flow rates did not significantly increase the nanoparticle diameter, the PDI variability was affected in many instances. Mixing acetone or acetonitrile with a less polar solvent, ethyl acetate, had the major impact on the hydrodynamic size of particles, skewing them towards the micron-scale, as well as significantly increasing associated PDIs. This adjustment in the organic phase composition effectively modulated diffusion dynamics of both organic and aqueous phases, promoting larger hydrodynamic diameters. Adjusting PVA concentrations was key to obtaining microparticles with lower PDIs. However, escalating the concentration of PVA beyond a threshold does contribute to a reduced particle hydrodynamic size.

Loading of HCQ into PLGA particles fabricated using acetone-ethyl acetate in the organic phase had a significant effect on heterogeneous nucleation time or particle aggregation at the lowest PVA concentration tested, compared to the unloaded formulation. For HCQ-loaded PLGA particle fabrications via acetonitrile-ethyl acetate mixtures, the effect on particle hydrodynamic diameter was PVA concentration dependent and similar to corresponding unloaded particle formulations. Moreover, HCQ loading efficiency was higher with lower PVA concentrations in the aqueous phase of particle in acetonitrile-ethyl acetate mediated fabrications. Finally, HCQ-loaded particles, fabricated based on the determined parameters, did not affect the viability of RAW 264.7 cells. Based on our results, modulating polarity via the mixture of solvents in organic phase and surfactant content in aqueous phase are crucial in achieving appropriate diffusion parameters for achieving significant hydrodynamic diameter change of PLGA microspheres using the nanoassemblr system. Using microfluidic systems such as the NanoAssemblr benchtop increases handling efficiency, which is crucial in the scale up manufacturing of PLGA particle-based medicines. Therefore, this work provides an itinerary for developing microparticles that encapsulate complicated drugs at scale and may be critical as modern medicine moves towards using drug delivery technologies in the clinic.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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1. If desired particle characteristics are not obtained, select sample with hydrodynamic diameter closer to 1μm and lowest possible PDI.

2. Proceed with the evaluation of next parameter until reaching desired particle characteristics.
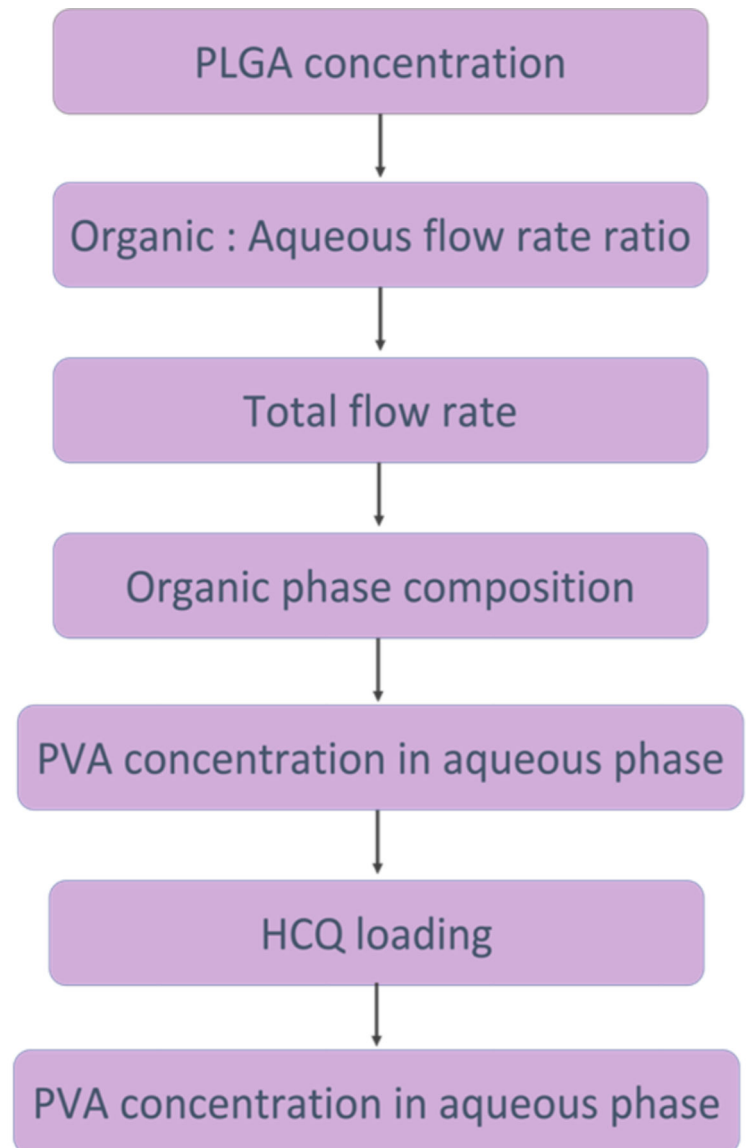
**Figure 1.**
Methods process for experimental design. After establishing the starting parameters for nanoparticle fabrication (based on previous reports), we tested how changing the polymer concentration affects particle hydrodynamic diameter and PDI. Our goal was to obtain particles with a hydrodynamic diameter of 1 μm and the lowest PDI possible. Therefore, we selected a particle sample with characteristics closer to that at each parameter evaluation, proceeded testing the next parameter, until we reached our desired characteristics in particle fabrication.
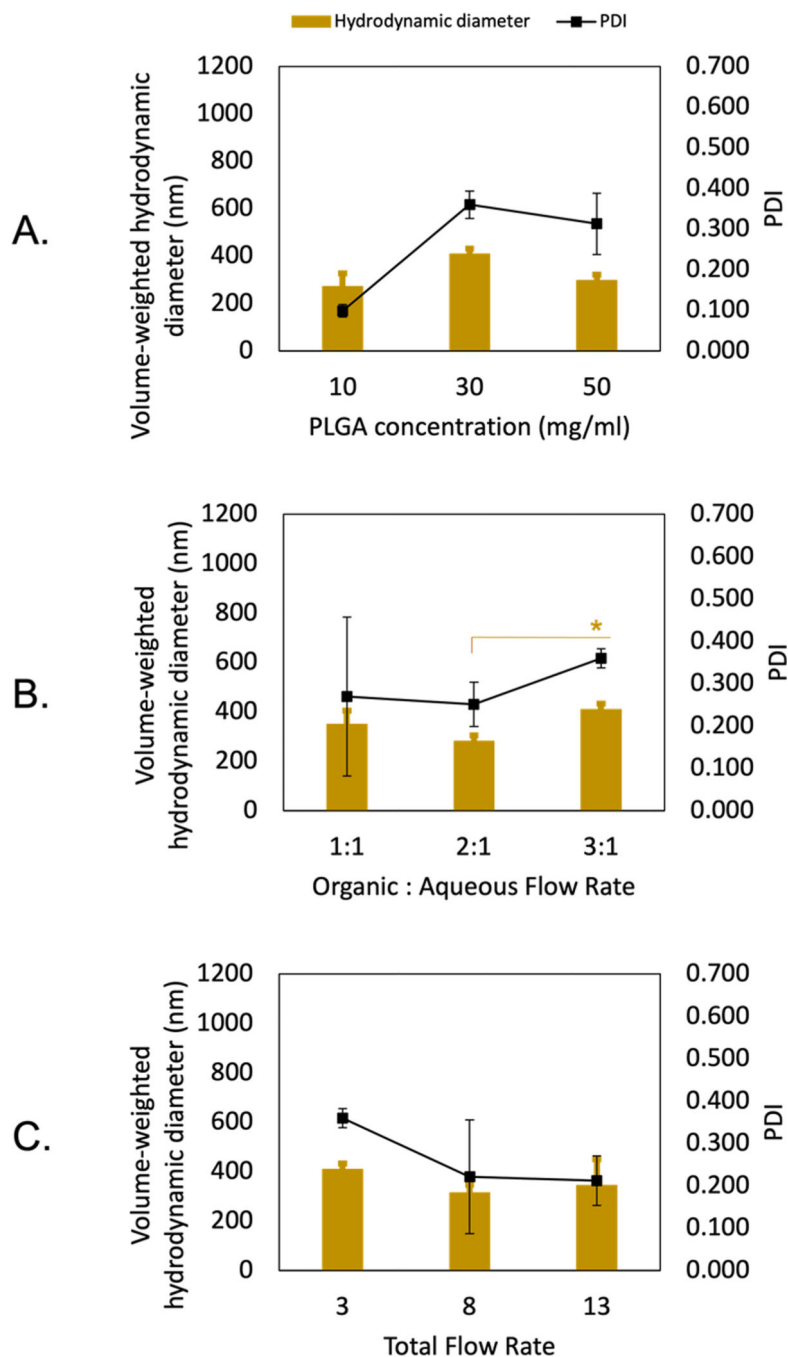
**Figure 2:**
(A) Effect of PLGA concentration on hydrodynamic diameter of particles and PDI
of acetone-mediated particle fabrications. (B) Effect of organic:aqueous flow rate on
hydrodynamic diameter and PDI of acetone-mediated particle preparation. (C) Effect of
total flow rate on hydrodynamic diameter and PDI of acetone-mediated particle preparation.
(n = 3, DLS measurements performed 3 times per sample, statistical difference evaluated via
one-way ANOVA and Tukey's test, $p < 0.05$. Statistical significance highlighted in mustard
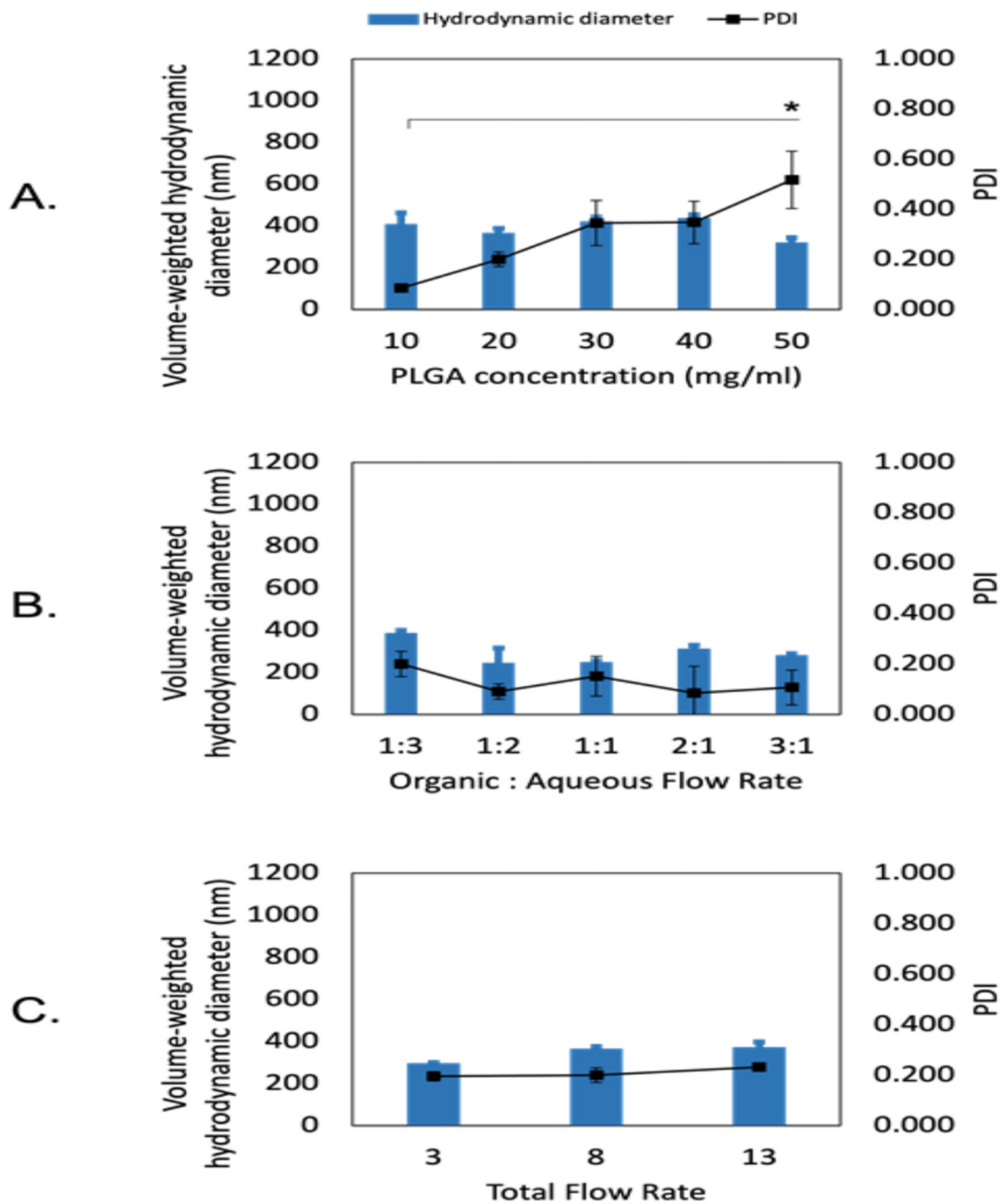for hydrodynamic diameter or black for PDI).
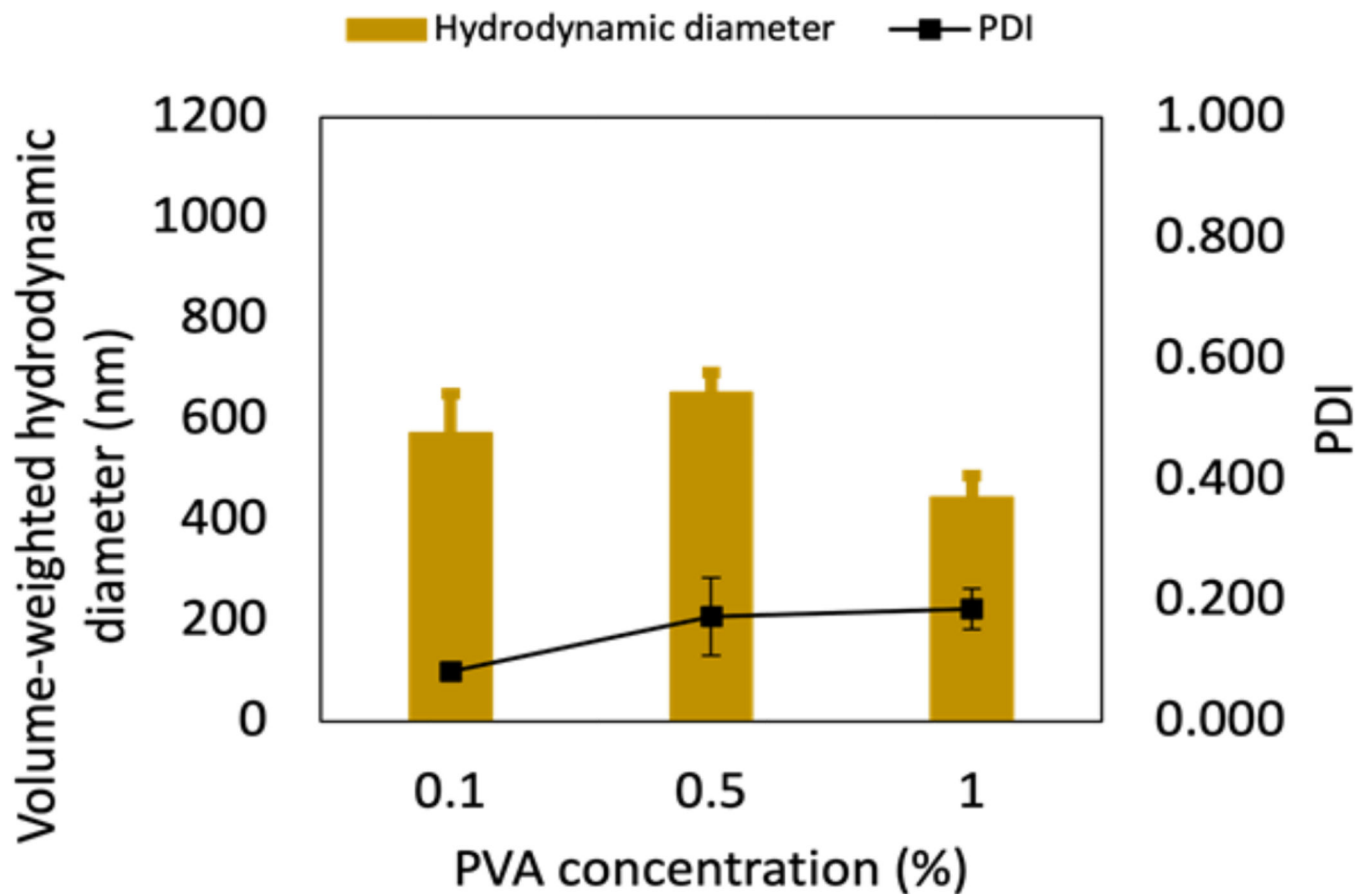
**Figure 3:**
Effect of ethyl acetate addition to the organic phase on hydrodynamic diameter and PDI of acetone-mediated particle preparation while varying PVA % (w/v). (n = 3, DLS measurements performed 3 times per sample, statistical difference evaluated via one-way ANOVA and Tukey's test, $p < 0.05$. Statistical significance highlighted in mustard for hydrodynamic diameter or black for PDI).

**Figure 4:**
(A) Effect of PLGA concentration on hydrodynamic diameter of particles and PDI of acetonitrile-mediated particle fabrications. (B) Effect of organic:aqueous flow rate on hydrodynamic diameter and PDI of acetonitrile-mediated particle preparation. (C) Effect of total flow rate on hydrodynamic diameter and PDI of acetonitrile-mediated particle preparation. (n = 3, DLS measurements performed 3 times per sample, statistical difference against smallest average hydrodynamic diameter or PDI in experiment evaluated via one-

way ANOVA and Tukey's test, p < 0.05. Statistical significance highlighted in blue for
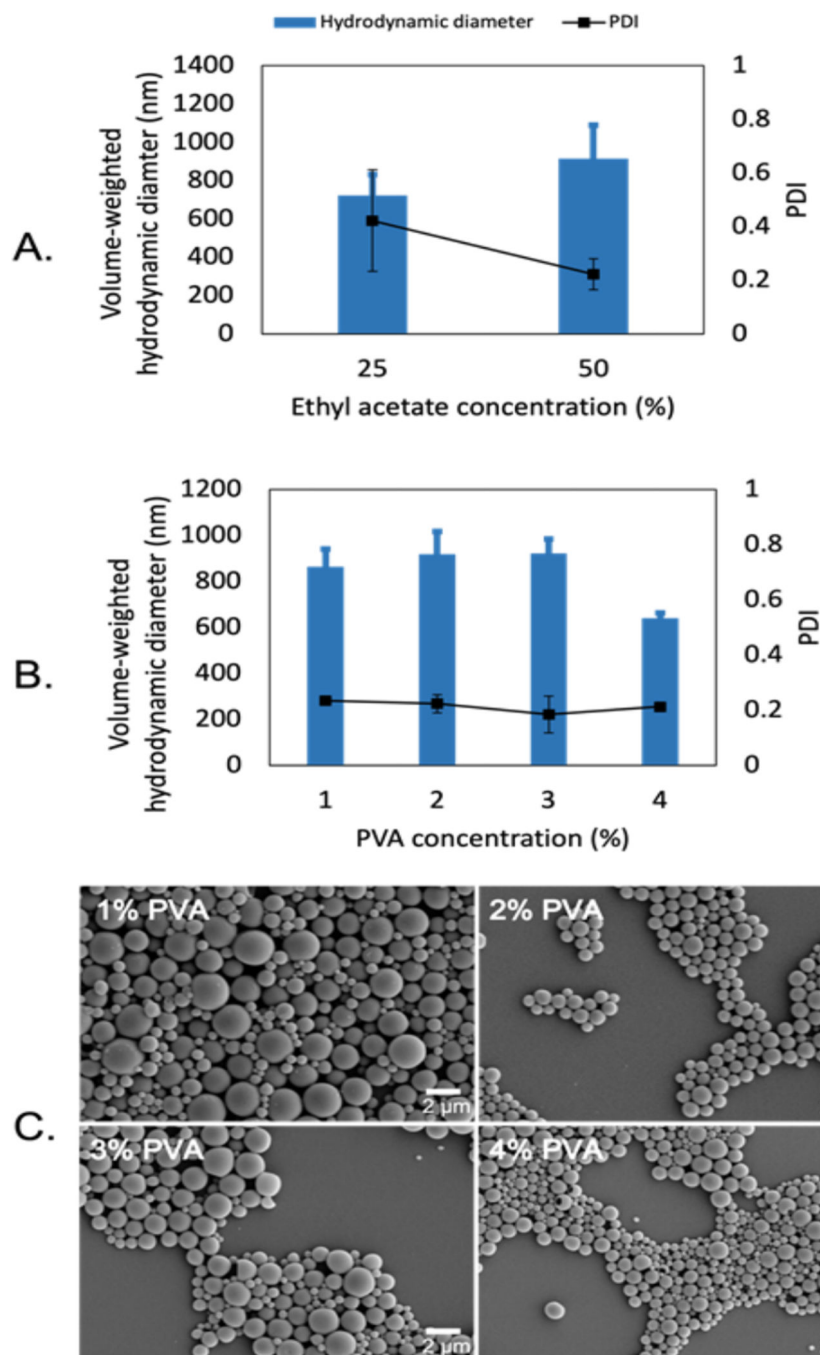hydrodynamic diameter or black for PDI).

**Figure 5:**

(A) Effect of ethyl acetate addition to the organic phase (25 or 50% v/v) on hydrodynamic diameter and PDI of acetonitrile-mediated particle preparation (n =3). (B) Effect of PVA % (w/v) on particle fabrication using a mixture of acetonitrile (50% v/v) and ethyl acetate (50% v/v) in organic phase (n = 3, DLS measurements performed 3 times per sample, statistical difference evaluated via one-way ANOVA and Tukey's test, p < 0.05. Statistical significance highlighted in blue for hydrodynamic diameter or black for PDI). (C) Representative SEM

micrographs of these blank particles fabricated at different PVA % (w/v) in the aqueous phase (scale bar: 2 μm).
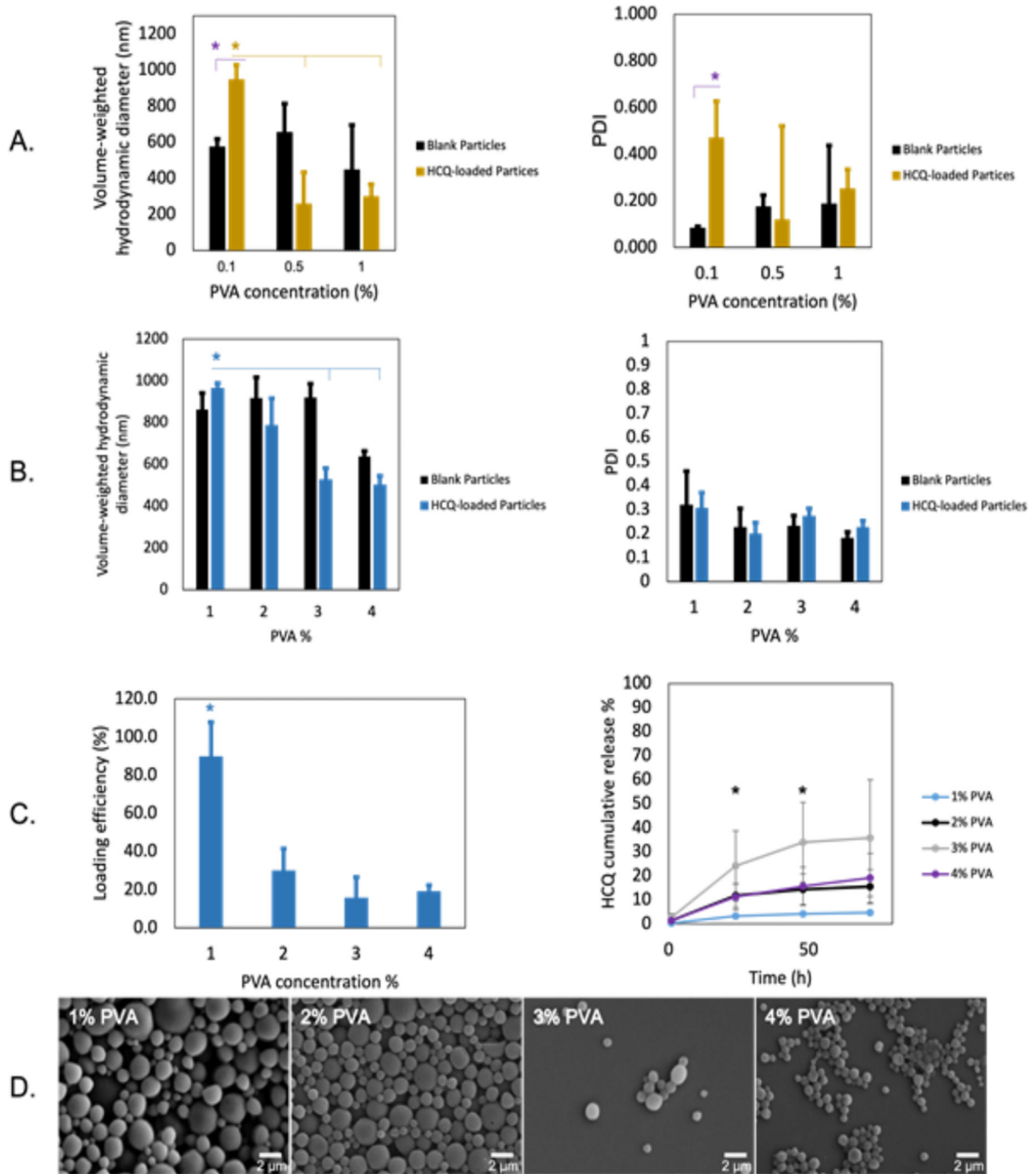
**Figure 6:**
(A) Effect of HCQ addition on hydrodynamic diameter and PDI of PLGA particle fabrications using acetone (75% v/v) and ethyl acetate (25% v/v) in the organic phase, at various PVA % concentrations (w/v). (B) Effect of HCQ addition on hydrodynamic diameter and PDI of PLGA particle fabrications using acetonitrile (50% v/v) and ethyl acetate (50% v/v) in the organic phase, at various PVA % concentrations (w/v) (n = 3, DLS measurements performed 3 times per sample, statistical difference evaluated via one-way ANOVA and Tukey's test, p < 0.05. Statistical significance highlighted in mustard or

blue for comparisons within acetone- ethyl acetate mediated syntheses or acetonitrile-ethyl acetate mediated fabrications, respectively, or purple for comparisons between blank and loaded fabrications). (C) Loading efficiency and HCQ % release kinetics of PLGA particle fabrications using acetonitrile (50% v/v) and ethyl acetate (50% v/v) in the organic phase, at various PVA % concentrations (w/v). (D) Representative SEM micrographs of these HCQ-loaded particles fabricated at different PVA % (w/v) in the aqueous phase (scale bar: 2 μm).
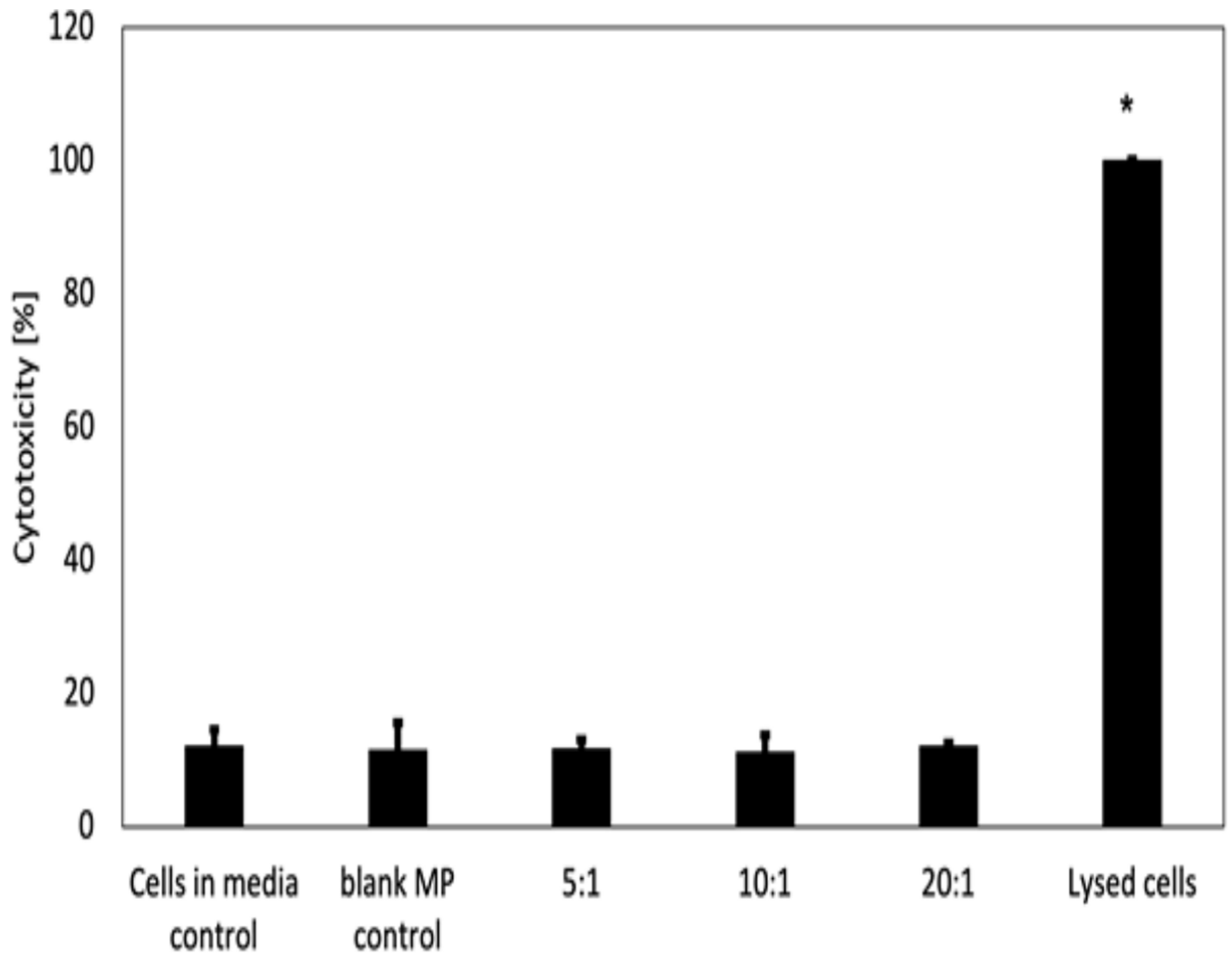
**Figure 7:**
Effect of acetonitrile-ethyl acetate mediated HCQ-loaded particles on viability of RAW 264.7 cells. (n = 3, statistical difference evaluated via one-way ANOVA and Tukey's test, p < 0.05).

**Table 1.**

(Top) HCQ-loaded PLGA particle fabrication average yields quantified from particle syntheses using acetone (25% v/v) and ethyl acetate (75% v/v) on organic phase. Yields quantified from HCQ-loaded PLGA particle fabrications using acetonitrile (50% v/v) and ethyl acetate (50% v/v) on organic phase.

| Acetone-Ethyl Acetate mediated HCQ-loaded PLGA Particle Yield out of 100 mg fabrications | | |
|---|---|---|
| **0.1% PVA** | **0.5% PVA** | **1 % PVA** |
| **Percentage (%)** | **Percentage (%)** | **Percentage (%)** |
| $6.33 \pm 0.03$ | $15.33 \pm 0.11$ | $28.17 \pm 0.04$ |

| Acetonitrile-Ethyl Acetate mediated HCQ-loaded Particle Yield out of 100 mg fabrications | |
|---|---|
| **1 % PVA** | **2% PVA** |
| **Percentage (%)** | **Percentage (%)** |
| $77.30 \pm 0.05$ | $84.07 \pm 0.06$ |
| **3% PVA** | **4% PVA** |
| **Percentage (%)** | **Percentage (%)** |
| $82.13 \pm 0.09$ | $77.60 \pm 0.04$ |