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Formation of nanoparticles for the oral delivery of small molecules by Flash Nanoprecipitation

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Abstract

Nanoparticles have shown considerable potential in many biological applications including drug delivery, bio-imaging, and medical diagnostics. Specifically, the development of nanoparticle-based drug formulations holds opportunities to improve the dissolution rate and oral availability of poorly water-soluble drugs. The goal of this project is to improve oral bioavailability of a small molecule drug ("G-1") through the formation of nanoparticles using the flash nanoprecipitation (FNP) process. Interestingly, "G-1" formed ~80 nm particles that are electrostatically stabilized without the use of stabilizing polymers and concentration as high as 160 mg/mL of "G-1" formed nanoparticles. Additionally, trehalose was found to be an effective cryoprotectant for lyophilization of "G-1" nanoparticles suspension into stable dried powders. Unexpectedly, the release kinetics of "G-1" in its free powder form exhibit rapid dissolution rate in the modified biorelevant media (FaSSIF with 1.5% Tween 20). Further formulations with "G-1" were conducted to generate ~300 nm particles with PS-b-PEG as stabilizing polymer. Through the use of a tangential flow filtration (TFF) system, drug loading (wt%) of the lyophilized "G-1" nanoparticles were increased by nearly 2-fold (30.5% *versus* 16.7%). These lyophilized nanoparticles were introduced to Genentech's in vivo and in vitro studies and have provided more insight in the bioavailability and pharmacokinetics properties of this drug.

Keywords

nanoparticles, formulations, pharmaceutical

Disciplines

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Andrew Tsourkas, Academic Advisor

Nanoparticles have shown considerable potential in many biological applications including drug delivery, bio-imaging, and medical diagnostics. Specifically, the development of nanoparticle-based drug formulations holds opportunities to improve the dissolution rate and oral availability of poorly water-soluble drugs. The goal of this project is to improve oral bioavailability of a small molecule drug ("G-1") through the formation of nanoparticles using the flash nanoprecipitation (FNP) process. Interestingly, "G-1" formed ~80 nm particles that are electrostatically stabilized without the use of stabilizing polymers and concentration as high as 160 mg/mL of "G-1" formed nanoparticles. Additionally, trehalose was found to be an effective cryoprotectant for lyophilization of "G-1" nanoparticles suspension into stable dried powders. Unexpectedly, the release kinetics of "G-1" in its free powder form exhibit rapid dissolution rate in the modified biorelevant media (FaSSIF with 1.5% Tween 20). Further formulations with "G-1" were conducted to generate ~300 nm particles with PS-b-PEG as stabilizing polymer. Through the use of a tangential flow filtration (TFF) system, drug loading (wt%) of the lyophilized "G-1" nanoparticles were increased by nearly 2-fold (30.5% *versus* 16.7%). These lyophilized nanoparticles were introduced to Genentech's in vivo and in vitro studies and have provided more insight in the bioavailability and pharmacokinetics properties of this drug.

Formation of nanoparticles for the oral delivery of small molecules by Flash Nanoprecipitation

by

Jing Zhi Lo

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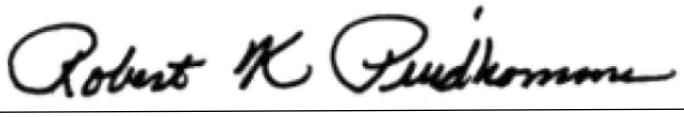
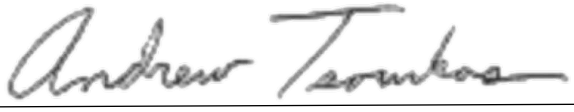


Robert K. Prud'homme, representing Organic Chemistry



Andrew Tsourkas, representing Organic Chemistry

I understand that my Capstone Report will become part of the permanent collection of the University of Pennsylvania Master of Chemical Sciences Program. My signature below authorizes the release of my final report to any reader upon request.

Jing Zhi Lo, Author

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Introduction

Nanotechnology offers substantial potential to revolutionize the drug delivery system. In the last few decades, there has been tremendous progress in the development of therapeutic nanoparticles.[1] Size of nanoparticles range from 1 to 100 nm and these dimensions allows the particles to interact with the biological systems in interesting ways.[2] Several advantages of nanoparticles include drug targeting to specific sites, the ability to cross biological barriers, protection of the drug from degradation, and prolonged circulation times.[3] In addition, nanoparticles can be used for various route of drug administration such as oral, ocular, and parenteral.[4] These advantages of nanoparticles provide for promotion in drug bioavailability and reduction in toxicity.

Flash Nanoprecipitation (FNP) is a technique used to construct drug-loaded polymeric nanoparticles. In the technique of FNP, hydrophobic molecules and polymer are dissolved in an organic solvent and rapidly mixed with the aqueous antisolvent. Upon rapid mixing of the organic solvent and antisolvent, a condition of high supersaturation is attained which leads to the nucleation and growth of particles. The block copolymer stabilized the surface of the particle and arrest particle growth, resulting in precipitation of specific size distribution of nanoparticles.[5] Most importantly, FNP is an inexpensive and scalable process that can generate stable nanoparticles with high drug loading and encapsulation efficiency.[6]

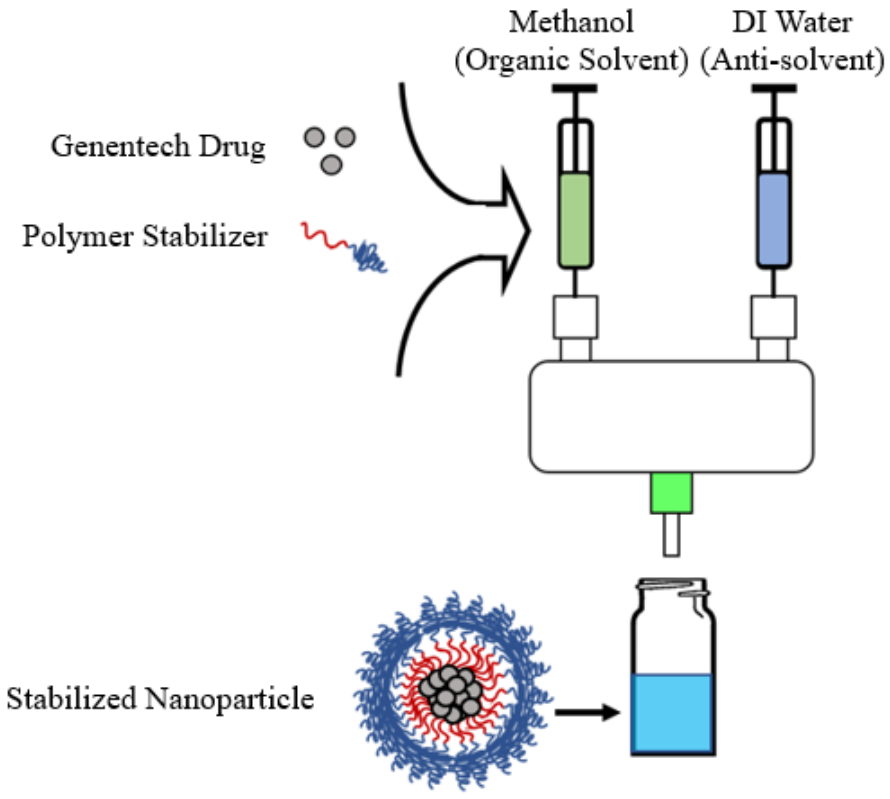

Figure 1. Schematic representation of the Flash Nanoprecipitation process in a Confined Impingement Jets mixer.[7]

In FNP, mixing of organic solvent and antisolvent occurs in a confined mixing chamber which can vary in geometry and size. In a Confined Impingement Jets (CIJ) mixer, the organic solute and stabilizing polymer are dissolved in the organic stream. The organic stream and the antisolvent stream collide each other at equal ratio of solvent to anti-solvent (**Figure 1**).[7] Higher supersaturations levels are limited through the use of CIJ. The Multi-Inlet Vortex Mixer (MIVM) design allows unequal ratio of solvent to anti-solvent mixing which overcomes the limitation of the CIJ (**Figure 2**).[8] Moreover, the CIJ has a two-inlet design compared to the MIVM which has a four-inlet design that allows additional streams of MQ water and increase supersaturation in the rapid mixing process.[9,10] The additional volume of water in the mixing process, decrease the solubility of the drug and result in higher precipitation of nanoparticles. **Figure 2** shows the arrangement of the four inlet streams on the MIVM and an example stream composition for FNP.

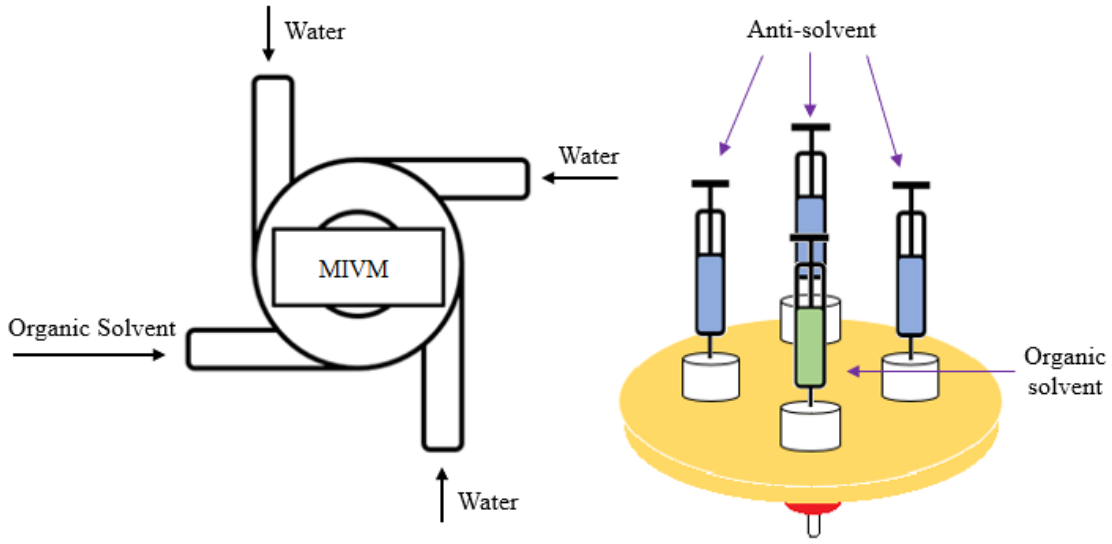

Figure 2. Schematic representation of the Flash Nanoprecipitation process in the Multi-Inlet Vortex Mixer.[8]

It is estimated that 40% of active ingredients developed in pharmaceutical industry are poorly water-soluble drugs.[11] Oral drug delivery is the most convenient and preferred route of administration based on low cost-effectiveness and high patient compliance.[12] Administration of poorly-water soluble drugs provided the motivation for oral delivery through nanoparticle-based therapeutics.[13] Hydrophobic small molecule drugs are difficult to absorb in the body due to poor water solubility and low dissolution rate in water. Formulation of a poorly water-soluble compound using a nanoparticle approach can enhance dissolution rate, drug solubility, and bioavailability.[14] In comparison of nanoparticles to conventional crude suspension, nanoparticles can minimize variation in bioavailability of fed vs. fasted state.[15] In addition, many poorly water-soluble molecules are not dose proportional. Nanoparticle formulations of these molecules can improve or implement dose proportionality.[16] Moreover, drug nanoparticles have higher surface area and surface interaction than particulates greater than 1 micron. The increased surface area

of nano-size particles can enhance dissolution rate and maximize the amount of drug absorbed at the duodenal–jejunal area of the gastrointestinal tract.[17] However, the enormous increase in surface area can cause nanoparticles to aggregate or agglomerate into a more thermodynamic stable state. For that reason, addition of stabilizing polymer is needed to dampen or sensitize the surface energy of the nanoparticles through steric and/or ionic interaction.[18]

In formulating nanoparticles for oral delivery of small molecule drug, a weakly hydrophobic Genentech drug named "G-1" is used for nanoparticle formulation. "G-1" has a log *P* of 6.18 and pK_a of 4.3. The chemical structure of the drug has two notable functional groups: carboxylic acid and indazole. The carboxylic acid can be deprotonated with a base to generate carboxylate anion for nanoparticle formation. Various excipients such as Hydroxypropyl Methylcellulose Acetate Succinate (HPMCAS), Hydroxypropyl Methylcellulose E3 (HPMC E3), polyethylene glycol (PEG), polyvinyl alcohol (PVA), Vitamin E TPGS and polystyrene-*b*-poly(ethylene glycol) (PS-*b*-PEG) were tested as stabilizing polymer to encapsulate "G-1" into nanoparticles. Numerous nanoparticle formulations are investigated through the FNP process via CIJ and MIVM. These formulations include changes in drug concentration, percent of drug, percent of stabilizing polymer, type of stabilizing polymer, equivalents of base, pH of antisolvent, choice of organic solvent, volume of organic and antisolvents.

The goal of this capstone project is to improve the oral bioavailability of a small molecule hydrophobic drug ("G-1") by formation of nanoparticles through the FNP process. The objective is to formulate nanoparticles of different sizes suitable for oral delivery and process nanoparticles into dried powder form which can demonstrate re-dispersibility. HPMCAS is a widely used polymer for spray-dried dispersion and hot melt extrusion in the pharmaceutical industry.[19,20] HPMCAS can enhance the solubility and increase bioavailability of poorly soluble active pharmaceutical ingredients. Genentech generated amorphous solid dispersion of "G-1" with HPMCAS by spray drying.[21] The drug dissociates from the HPMCAS polymer in vivo and precipitate to form nanoparticle in situ. In this work, the usage of HPMCAS as a stabilizing polymer to encapsulate "G-1" into nanoparticles was investigated for comparison to Genentech's spray-dried dispersion of "G-1" with HPMCAS. Formulation of different size nanoparticles can provide size-dependent dissolution profile and better understanding in the pharmacokinetics of this drug. Nanoparticles in dry stable form are desirable for transportation and long-term storage. Cryoprotectants are added into the nanoparticle suspensions before lyophilization to stabilized against aggregation during freezing.[22] Testing of various selection of cryoprotectant is vital for re-dispersion of dried powders into nanometer-sized particles when placed in water or an alternative water-based environment. It is critical that these dried powders are capable of re-dispersing into non-aggregated/non-agglomerated nanoparticulate dispersion for the development of solid dosage form.[23] The re-dispersed nanoparticles will be screened for their end application criteria: size, polydispersity index (PDI), and stability. Formulations are optimized based on the re-dispersity of the nanoparticles. Samples of the final formulations are sent to Genentech for dissolution testing and pharmacokinetic studies.

Materials and Method

Materials

Affinisol HPMC-AS 126 G (>94% purity), Affinisol HPMC-AS 716 G (>94% purity), Affinisol HPMC-AS 912 G (>94% purity), and Methocel E3 Premium LV Hydroxypropyl Methylcellulose (HPMC E3) were purchased from Dow Chemicals. Tetrahydrofuran (HPLC grade, 99.9%), methanol (HPLC grade, 99.9% purity), dimethyl sulfoxide (HPLC grade, 99.9% purity), acetone (HPLC grade, 99.9% purity), Tween 80, Tween 20, and sodium hydroxide pellets were purchased from Fisher Chemicals. Phosphate buffered saline (10X) was purchased from Lonza. Hydrochloric acid and potassium chloride were purchased from EMD Millipore. Pluronic F-127 was purchased from BASF Corporation. Vitamin E-TPGS were purchased from Peboc. Hydroxypropyl-β-cyclodextrin were purchased from Acros Organics. Poly(vinyl alcohol), polyethylene glycol and mannitol (>98% purity) were purchased from Sigma Aldrich. Fasted-state simulated intestinal fluid (FaSSIF) and fedstate simulated intestinal fluid (FeSSIF) were purchased from Biorelevant.com. "G-1" was supplied by Genentech. Trehalose (>99% purity) was purchased from Fluka. Poly(styrene)$_{1.6kDa}$-*block*-poly(ethylene glycol)$_{5kDa}$ were purchased from were purchased from Polymer Source Inc. DI (DI) water (18.2 MΩ·cm) was prepared by a NANOpure Diamond UV ultrapure water system (Barnstead International, Dubuque, IA).

Solubility Profile of "G-1" and HPMCAS-126

An excess of different organic solvent (methanol, tetrahydrofuran, dimethyl sulfoxide, and acetone) was added separately to glass vials containing 15.0 mg of "G-1". Solutions of HPMCAS-126 in different organic solvent (methanol, tetrahydrofuran, dimethyl sulfoxide, and ethanol) at a concentration of 20 mg/mL were prepared. Visual observation on the solubility of "G-1" and HPMCAS-126 was conducted.

Precipitation and Solubility Studies of "G-1"

A solution of 1x PBS was prepared through a tenfold dilution with 10x PBS. "G-1" in different organic solvent (methanol, tetrahydrofuran, and dimethyl sulfoxide) at a concentration of 20 mg/mL were generated and were mixed with antisolvent (water or PBS) in a final volume of 1.5 mL at different percentages, ranging from 90 % of the organic solvent and 10 % of antisolvent to 10 % of the organic solvent and 90 % of the antisolvent. The mixed solvent with precipitation were centrifuged at 14800 rpm for 15 minutes. A sample was aliquot out of the supernatant of the solution. The sample was diluted up to 1000-fold and analyzed by UV-vis spectrometry.

Preparation of "G-1" Nanoparticles.

"G-1" with HPMCAS

A pellet of sodium hydroxide was added to methanol to prepare a stock solution at a concentration of 10 mg/mL through sonication. A stock solution of HPMCAS-126 in methanol at a concentration of 20 mg/mL with 0.50 and 0.75 equivalents of sodium hydroxide were prepared. A stock solution of "G-1"in methanol at a concentration of 20 mg/mL were prepared. Attempts to generated "G-1" nanoparticles with HPMCAS were prepared via CIJ and MIVM. The organic stream consisted of "G-1"and HPMCAS-126

dissolved in 0.5 mL of methanol were prepared with the stock solutions. The rapid mixing of organic stream and antisolvent DI water streams was conducted via CIJ and MIVM. The mixture was subsequently dispersed in 9.0 mL (CIJ) or 6.0 mL (MIVM) of DI water which decrease the organic solvent to 10 vol%.

"G-1" with various excipients (HPMC E3, F-127, PVA, Tween 80, and Vitamin E TPGS)
"G-1" nanoparticles with various excipients to act as stabilizing polymers were prepared via MIVM. The antisolvent 10 mM HCl streams were prepared by dilution of 1M HCl with DI water. The three antisolvent stream consisted of 1.0 mL of 10 mM HCl or DI water were rapidly mixed with the organic stream which consisted of "G-1" (20 mg/mL) and stabilizing polymer (2 mg/mL) in 0.5 mL of methanol via MIVM. The mixture was dispersed into a quench bath of DI water (6.0 mL), resulting in a final organic concentration of 10 vol%.

"G-1" with no stabilizing polymers
"G-1" nanoparticles in suspension without stabilizing polymers were generated by FNP using the four-stream MIVM. Different concentration of "G-1"in methanol (20 mg/mL, 40 mg/mL, 80 mg/mL, and 160 mg/mL) was prepared with different equivalents of sodium hydroxide (0.00 eq, 0.05 eq, 0.10 eq, 0.20 eq, and 0.40 eq) for the organic stream (1.0 mL) in the MIVM. Rapid mixing of the organic stream and the three antisolvent DI water stream (1.0 mL each) was conducted via MIVM. The suspension mixture was dispersed as previously described.

"G-1" with PS-b-PEG
PS-b-PEG "G-1" nanoparticles in suspension were generated by FNP using the four-stream MIVM. Different amount of % drug (25, 50, 75% and etc.) and % PS-b-PEG (75, 50, 25% and etc.) were dissolved in 0.5 mL of methanol (total mass concentration: 40 mg/mL or 20 mg/mL). The rapid mixing of the organic stream (0.5 mL of methanol) and antisolvent stream (4.5 mL of DI water) resulted in a mixture with a final organic concentration of 10 vol% which was dispersed into a 20 mL vial.

"G-1" with increased drug loading (wt%)
"G-1" nanoparticles (80 mg/mL of "G-1" with 0.05 eq of NaOH) in suspension were generated as previously described. The rapid mixing of the organic stream (4.0 mL of methanol) and antisolvent stream (12.0 mL of DI water) was conducted via MIVM. The mixture was dispersed into a quench bath of DI water (24.0 mL), resulting in a final organic concentration of 10 vol%. The nanoparticle suspension (40.0 mL) underwent filtration using a MicroKros® Module (MWCO: 100 kD) and removed water (32.0 mL) from solution, concentrating the nanoparticle suspension by 5-fold.

Nanoparticle Characterization
Nanoparticle size and PDI were assessed by dynamic light scattering using a Zetasizer Nano-ZS at 25 °C with a detection angle of 173° in triplicate. To avoid multiple light scattering, the samples were diluted 10-fold prior to DLS. The size is determined through a series of light scattering correlation function. The PDI is obtained through the Taylor

series expansion of the correlation function from the Malvern Nanosizer data analysis software.

Nanoparticle Lyophilization

Nanoparticle suspensions were lyophilized into dry powders using a benchtop VirTis Advantage without and with cryoprotectants (i.e. PVA, PEG, Trehalose, Mannitol, F-127, and HPMC E3). 200 mg of cryoprotectant were added to 5 mL of nanoparticle solutions to afford NP:cryoprotectant weight ratios of 1:5. The mixture solution were flash frozen in a container of dry ice. The frozen samples were transferred to the freeze-dryer at -20 °C under vacuum to remove the water and organic solvents from the nanoparticles. After 24 hours, dried powders were obtained and stored at -20 °C. Samples were re-disperse with 5 mL of DI water for DLS analysis.

"G-1" Solubility and Release Kinetics

FeSSIF (Fed State Simulated Intestinal Fluid) and FaSSIF (Fasted State Simulated Intestinal Fluid) buffer consisted of different percentage of Tween 20 (0.5% and 1.5%) were prepared. An excess of "G-1" powder was added to FeSSIF and FaSSIF buffer, respectively, followed by a slow rotation on the Glas-Col rotator for 96 h to allow maximum saturation of the drug in solution. The solution was aliquot into Amicon and Pall filters to remove the undissolved drug from the solution. The solution was centrifuged at 5000 rcf for 10 minutes. The supernatant of the solution was analyzed by UV-vis spectrometry. The concentration of "G-1" was calculated using a calibration curve with known standard solution. In the dissolution testing procedures, approximately 5.0 mg of "G-1" is added to 20.0 mL of FaSSIF with 1.5% Tween. Solution was aliquot into Pall filters and the supernatant of the solution was analyzed by UV-Vis spectrometry at 15-minute and 30-minute timepoint. The concentration of "G-1" at each time point was calculated using a standard curve.

Results and Discussion

Solubility Profile of "G-1" and HPMCAS

In the FNP process, the drug and the stabilizing polymer are dissolved in the organic solvent and rapidly mixed with the antisolvent to form nanoparticles in suspension. Thus, several common organic solvents (e.g., methanol, THF, DMSO and acetone) were screened as candidates for the FNP process. Solubility of "G-1" and HPMCAS in the organic solvents were qualitative measured by visual representations. The solubility of "G-1" was tested in methanol, THF, DMSO, and acetone. "G-1" is found to be soluble in all four solvents. The solubility of HPMCAS was tested in acetone, ethanol, methanol, and THF. HMPCAS is found to be soluble in all solvents except ethanol (**Table 1**). Methanol, THF, DMSO, and acetone were selected as the organic solvent for the FNP process as "G-1" and HPMCAS were both soluble in those solvents.

Table 1. Solubility profiling of "G-1" and HPMCAS in different solvents.

	Methanol	THF	DMSO	Acetone	Ethanol
"G-1"	Soluble	Soluble	Soluble	Soluble	N/A
HPMCAS	Soluble	Soluble	Soluble	Soluble	Insoluble

"G-1" Detection by UV-Vis

Ultraviolet–visible spectroscopy (UV-Vis) can be used to detect the presence of "G-1" and determine the concentration of "G-1" in an organic solvent. UV-Vis was used to analyzed "G-1" in methanol, THF, DMSO and acetone, respectively. Analysis of "G-1" in methanol (50 μg/mL) by UV-Vis gave corresponding absorption peaks at the wavelength of 255 nm and 305 nm (**Figure 3**). Absorption spectrum of the blank methanol solution (blue line) and "G-1" methanol solution (orange line) is shown in **Figure 3**. "G-1" in methanol absorbed most strongly at 255 nm and 305 nm wavelength, creating two maxima in the absorption spectrum. In addition, "G-1" can be detected in THF and DMSO by UV-Vis as absorption spectrum can be generated from the drug in either solvents. However, UV-Vis cannot be used for the detection of "G-1" in acetone due to the overlap absorption peak of blank acetone and the absorption peak of the drug at 305 nm.

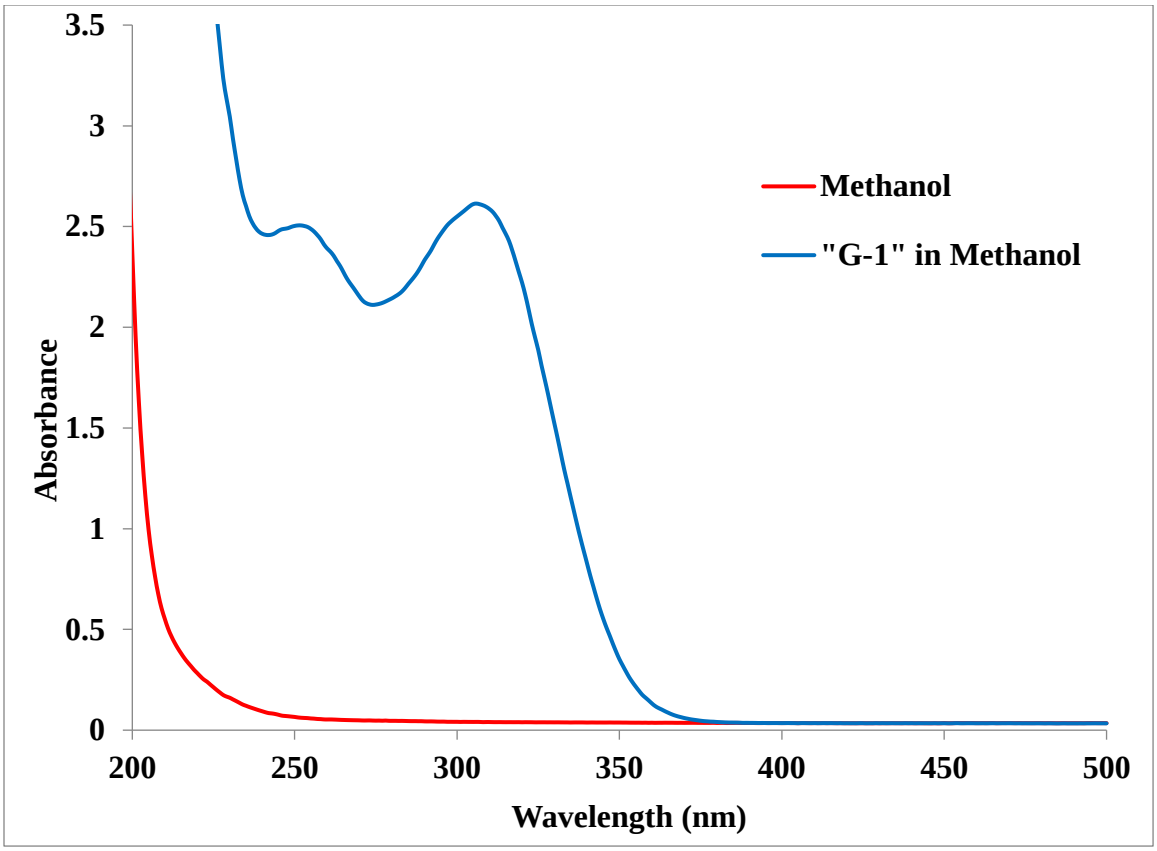

Figure 3. "G-1" dissolved in methanol and its corresponding absorption peak at 305 nm shown from the UV-VIS.

Precipitation and Solubility Studies of "G-1"

High "G-1" supersaturation must be achieved for formation of nanoparticles when the organic stream and antisolvent stream collide in FNP. For that reason, precipitation and solubility studies of "G-1" in methanol, THF, and DMSO were performed to investigate potential organic solvent for the FNP process. "G-1" in different organic solvents (methanol, THF, and DMSO) were separately mixed with antisolvent deionized (DI) water at different percentage to generate three series of mixtures (Series A, Series B, and Series C) and were observed for precipitation (**Table 2**). Each series of mixture were prepared with different organic solvents: Series A (methanol), Series B (THF), and Series C (DMSO). In series B, precipitation of the drug occurs at 40% THF and 60% DI water. In series A and C, precipitation of the drug occurs at 60% methanol or DMSO and 40% DI water. Calibration curves were constructed in methanol by preparing a series of concentrations of the drug (1, 5, 10, 25, 50, 75 μg/mL). Solubility curves of "G-1" were generated from four final mixed solvents (**Figure 4**). Solubility curve of "G-1" in four final mixed solvents (methanol with DI water, methanol with PBS, THF with DI water, and DMSO with DI water) are shown in **Figure 4**. The replacement of DI water with PBS was expected to decrease the solubility of "G-1" in methanol. However, "G-1" in methanol with PBS had a higher solubility curve compare to "G-1" in methanol with DI water. Similarly, the solubility curve of "G-1" in THF with DI water was higher than the solubility curve of "G-1" in methanol with DI water. The solubility curve of "G-1" in DMSO with DI water displayed a similar trend to the solubility curve of "G-1" in methanol with DI water.

Although, supernatant of the solution and the precipitation of "G-1" at 20% DMSO could not be separated through centrifugation. Most importantly, solubility of "G-1" is lowest at 20% methanol. In other words, vast amount of "G-1" precipitate at 20% methanol and high supersaturation is desired for the FNP process. Thus, methanol was selected for the organic stream in the FNP process due to the low solubility of "G-1" in the final mixed solvent.

Table 2. Precipitation studies of "G-1" in different solvents. P = Precipitation and NP = No Precipitation.

Organic Solvent %	Antisolvent %	Series A Methanol:Water	Series B THF:Water	Series C DMSO:Water
100 %	0 %	NP	NP	NP
90 %	10 %	NP	NP	NP
80 %	20 %	NP	NP	NP
70 %	30 %	NP	NP	NP
60 %	40 %	P	NP	P
50 %	50 %	P	NP	P
40 %	60 %	P	P	P
30 %	70 %	P	P	P
20 %	80 %	P	P	P
10 %	90 %	P	P	P
0 %	100 %	P	P	P

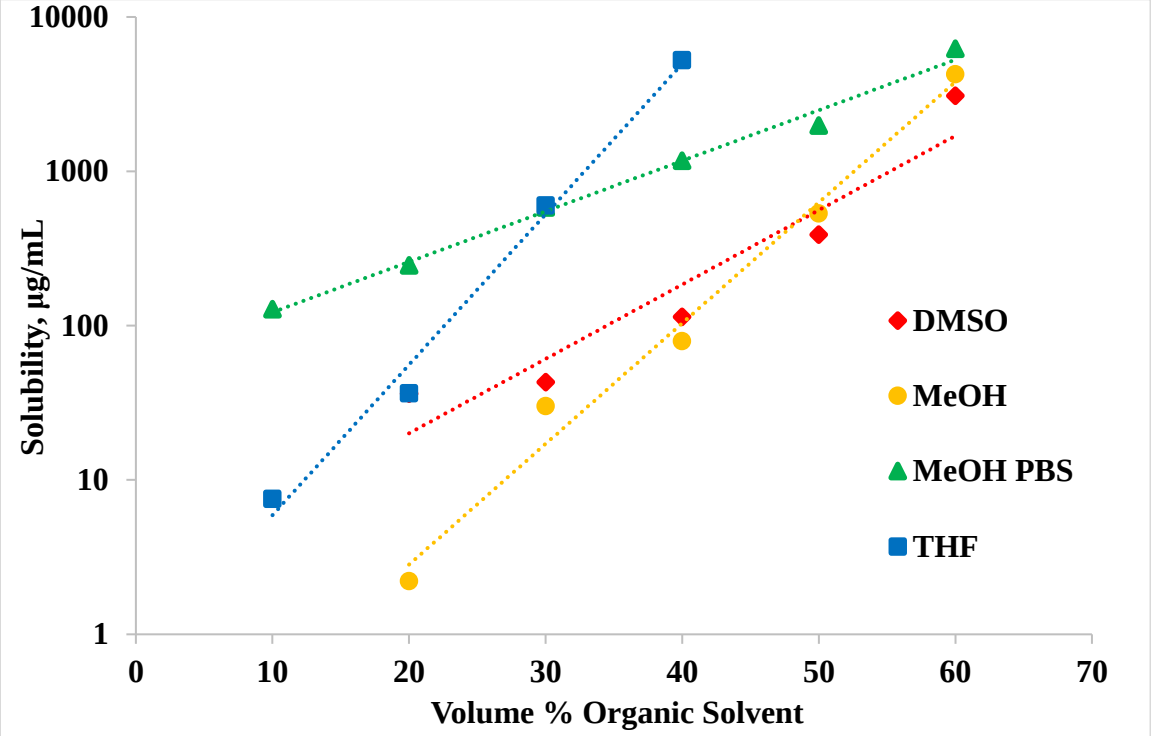

Figure 4. Solubility of "G-1" in mixtures of organic solvents to antisolvent in different percentages (Methanol:Water, Methanol:PBS, DMSO:Water, and THF:Water).

"G-1" Nanoparticles

HPMCAS is a cellulosic derivative polymer with acetyl and succinyl substitutions along its backbone (**Figure 5**). In addition, HPMCAS is a polymer that can provide stable amorphous solids dispersions with poorly soluble drugs. Hypothetically, the succinate groups on HPMCAS can be deprotonated with a base which allows the anionic succinate group to act as the surface stabilizing polymer to encapsulate "G-1" into nanoparticles. Various formulations consisted of different percentage of drug and HPMCAS-126 in 0.50 and 0.75 equivalents of sodium hydroxide was conducted through via CIJ and MIVM (**Table 3**). The formulations that underwent the FNP process in an attempt to form "G-1" nanoparticles with HPMCAS are listed in **Table 3**. Initial formulations were conducted via CIJ and later formulations were conducted via MIVM, in order to increase precipitation of "G-1" nanoparticles by introducing higher volume of DI water during the FNP process to decrease the solubility of "G-1" in methanol. Several variables of the formulations were modified such as increasing equivalents of sodium hydroxide and changing the percentage of the drug and HPMCAS-126. attempts to form "G-1" nanoparticles with HPMCAS. For all formulations tested in **Table 3**, no formulations were successful in forming "G-1" nanoparticles with HPMCAS-126. It is hypothesized that "G-1" form electrostatically stabilized nanoparticles which have an anionic surface that repels the anionic succinate groups of HPMCAS, preventing HPMCAS to act as a stabilizing polymer with the drug to form nanoparticles.

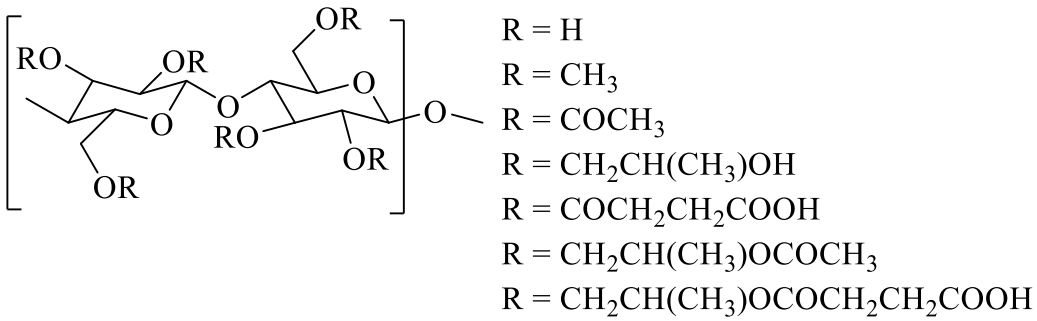

Figure 5. Chemical structure of Hydroxypropyl Methylcellulose Acetate Succinate.

To investigate whether "G-1" and its free acid form can participate in nanoparticle formation, a series of formulation consisted of "G-1" and various excipients (HPMC E3, F-127, PVA, Tween 80, and Vitamin E TPGS) dissolved in methanol (1.0 mL) were rapidly mixed with 10 mM HCl or DI water through the FNP process via MIVM (**Table 4**). The series of formulations were observed for nanoparticle formation and aggregation in the quench bath. The list of different formulations tested through the FNP process in an attempt to form "G-1" nanoparticles with a stabilizing polymer are shown in **Table 4**. Through visual observation, no aggregation was found in the formulations with DI water as the antisolvent. In addition, aggregation was found in all formulations with 10 mM HCl as the antisolvent except when F-127 was used as stabilizing polymer. In particular, three formulations resulted in forming well defined nanoparticles with "G-1" (**Figure 6**). Particle size distribution of the three successful nanoparticle formulation (PVA in DI water, vitamin E TPGS in DI water, and F-127 in 10 mM HCl) are shown in **Figure 6**. The size of the nanoparticles produced from these formulations were less than 200 nm with narrow size

distribution (PDI > 0.25). “G-1” nanoparticles can be formulated with specific stabilizing polymer and antisolvents.

Table 3. Formulations to prepare "G-1" nanoparticles with HPMCAS.[a]

Type of Mixer	Total mass concentration	Sodium Hydroxide Equivalents to HPMCAS	% Drug	% HPMCAS-126	Nanoparticle formation with HPMCAS-126
CIJ	20 mg/mL	0.50 eq	100%	0%	No
CIJ	20 mg/mL	0.50 eq	75%	25%	No
CIJ	20 mg/mL	0.50 eq	50%	50%	No
MIVM	20 mg/mL	0.50 eq	100%	0%	No
MIVM	20 mg/mL	0.50 eq	90%	10%	No
MIVM	20 mg/mL	0.50 eq	50%	50%	No
MIVM	20 mg/mL	0.75 eq	90%	10%	No
MIVM	20 mg/mL	0.75 eq	50%	50%	No

[a] Methanol (0.5 mL) was used as the organic stream.

Table 4. Formulations consisted of “G-1” with different stabilizing polymers in combination with 10 mM HCl or DI water as antisolvent.[a]

“G-1” Concentration	Stabilizing Polymer Concentration	Stabilizing Polymers	Antisolvent	NP Formation (Size >200 nm) (PDI >0.25)
20 mg/mL	2 mg/mL	HPMC E3	10 mM HCl	No
20 mg/mL	2 mg/mL	F-127	10 mM HCl	Yes
20 mg/mL	2 mg/mL	PVA	10 mM HCl	No
20 mg/mL	2 mg/mL	Tween 80	10 mM HCl	No
20 mg/mL	2 mg/mL	Vitamin E TPGS	10 mM HCl	No
20 mg/mL	2 mg/mL	HPMC E3	DI water	No
20 mg/mL	2 mg/mL	F-127	DI water	No
20 mg/mL	2 mg/mL	PVA	DI water	Yes
20 mg/mL	2 mg/mL	Tween 80	DI water	No
20 mg/mL	2 mg/mL	Vitamin E TPGS	DI water	Yes

[a] Methanol (1.0 mL) was used as the organic stream.

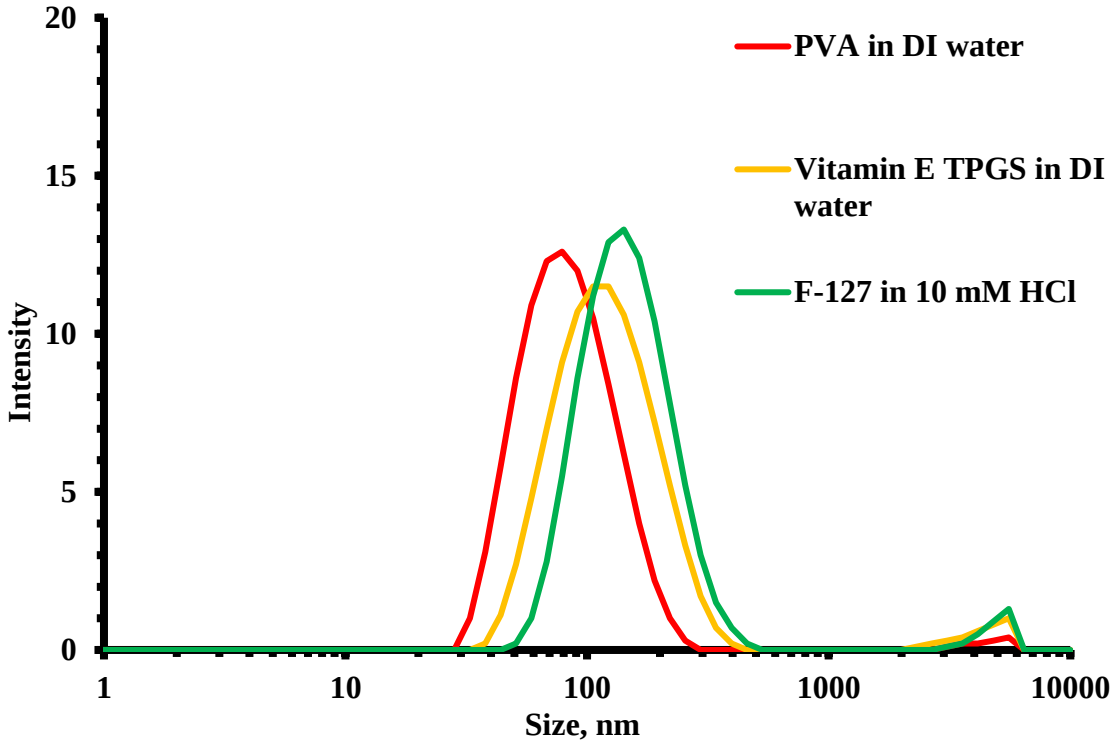

Figure 6. Particle size distribution of "G-1" nanoparticles with different stabilizing polymer and antisolvents.

Table 5. Formulations consisted of "G-1" with different equivalents of sodium hydroxide.[a]

"G-1" Concentration	Sodium Hydroxide Equivalents (eq)	Antisolvent	NP Formation	Size	Size Distribution (PDI)
20 mg/mL	0.05	DI water	Yes	80	0.23
20 mg/mL	0.10	DI water	Yes	60	0.23
20 mg/mL	0.20	DI water	Yes	50	0.28
20 mg/mL	0.40	DI water	Yes	40	0.26

[a] Methanol (1.0 mL) was used as the organic stream.

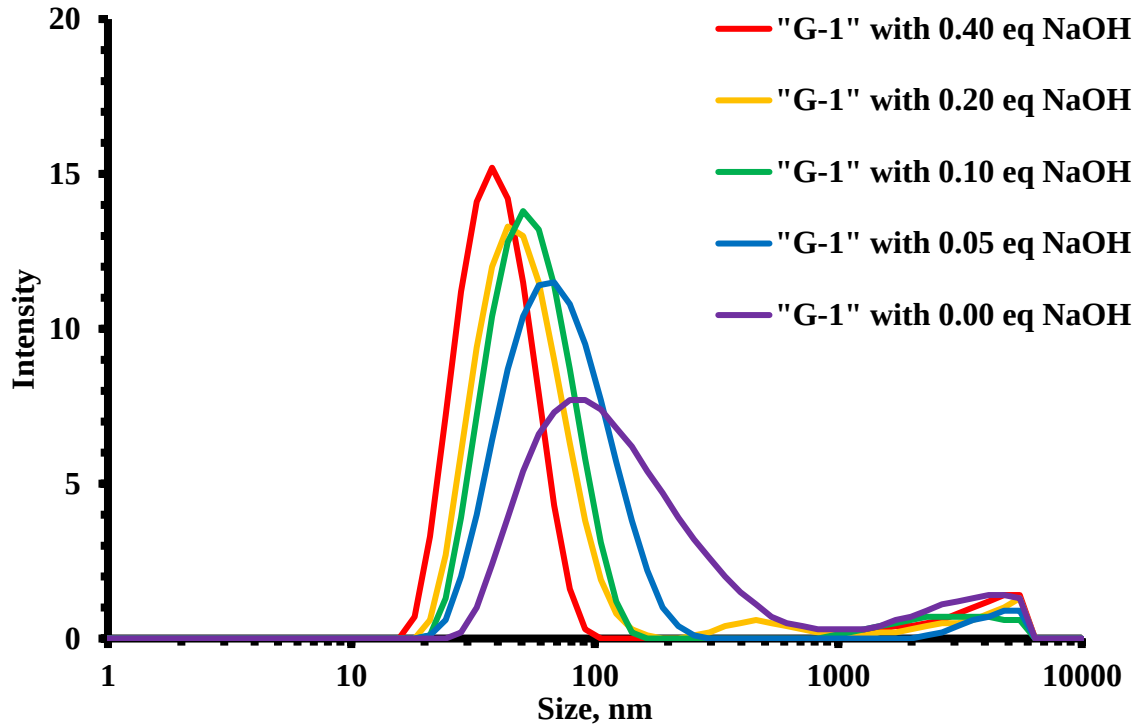

Figure 7. Particle size distribution of "G-1" nanoparticles formed under different equivalents (0.00 eq, 0.05 eq, 0.10 eq, 0.20 eq and 0.40 eq) of NaOH.

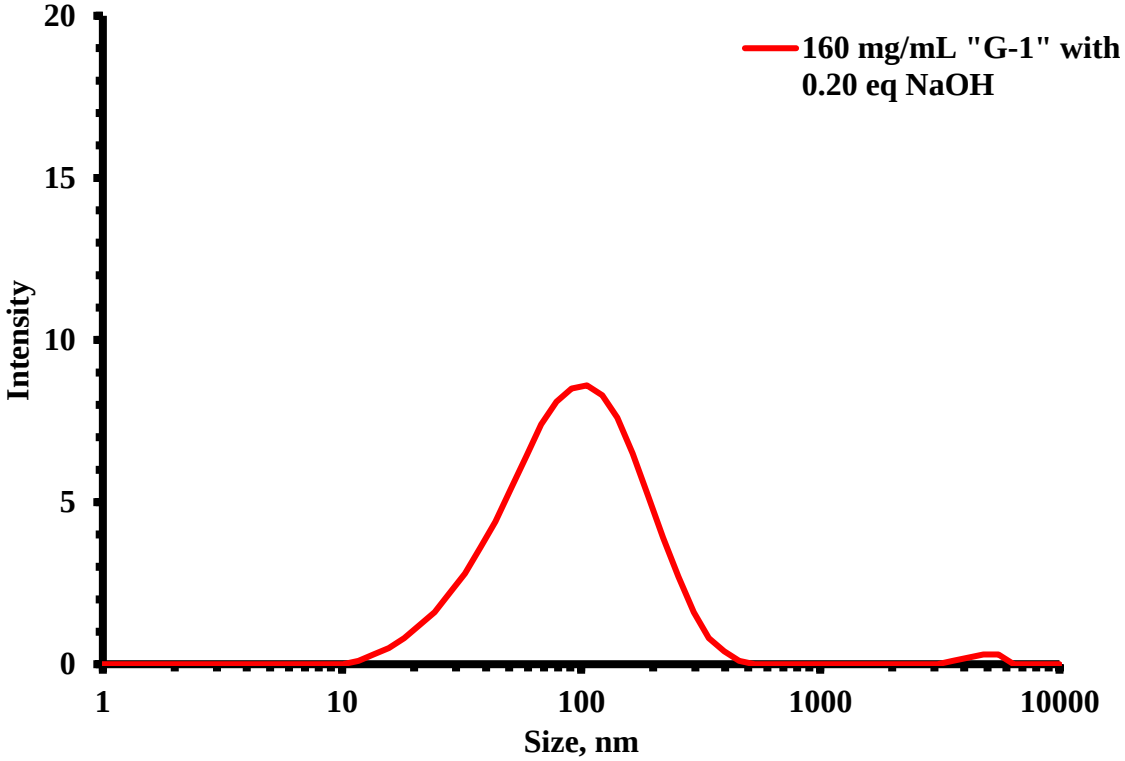

Figure 8. Particle size distribution of "G-1" nanoparticles formed with 160 mg/mL of "G-1" and 0.20 eq of NaOH.

Table 6. Formulations consisted of "G-1" with various grade of HPMCAS in different equivalents of sodium hydroxide.[a]

"G-1" Concentration	Sodium Hydroxide Equivalents (eq)	Stabilizing Polymer Concentration	Stabilizing Polymers	Antisolvent	NP Formation
40 mg/mL	0.00	20 mg/mL	HPMCAS-126	DI water	No
40 mg/mL	0.50	20 mg/mL	HPMCAS-126	DI water	No
40 mg/mL	0.75	20 mg/mL	HPMCAS-126	DI water	No
40 mg/mL	0.00	20 mg/mL	HPMCAS-712	DI water	No
40 mg/mL	0.50	20 mg/mL	HPMCAS-712	DI water	No
40 mg/mL	0.75	20 mg/mL	HPMCAS-712	DI water	No
40 mg/mL	0.00	20 mg/mL	HPMCAS-912	DI water	No
40 mg/mL	0.50	20 mg/mL	HPMCAS-912	DI water	No
40 mg/mL	0.75	20 mg/mL	HPMCAS-912	DI water	No

[a] Methanol (1.0 mL) was used as the organic stream.

"G-1" (20 mg/mL) with different equivalents of sodium hydroxide (0.00 eq, 0.05 eq, 0.10 eq, 0.20 eq, and 0.40 eq) generate nanoparticles through FNP via MIVM (**Table 5**). The list of different formulations tested through the FNP process in an attempt to form "G-1" nanoparticles with different equivalents of sodium hydroxide are tabulated in **Table 5**. Particle size distribution of "G-1" nanoparticles formulated with different equivalents of sodium hydroxide were measured by DLS **(Figure 7**). **Figure 7** shows an inverse correlation between the size of "G-1" nanoparticles and the equivalents of sodium hydroxide. The size of "G-1" nanoparticles decrease as higher equivalents of sodium hydroxide was used in the formulation for the FNP process. The size of nanoparticles can be controlled from ~40 to ~80 nm based on the equivalent amount of sodium hydroxide used in the formulation process. The formulation was optimized for further downstream processing, higher concentrations of "G-1" was tested for nanoparticle formation. "G-1" at concentrations of 40 mg/mL, 80 mg/mL, and 160 mg/mL formed nanoparticles. Particle size distribution of "G-1" nanoparticles formulated with 160 mg/mL of "G-1" and 0.20 eq of NaOH was measured by DLS (**Figure 8**). "G-1" at a concentration of 160 mg/mL were able to form nanoparticles with sizes around ~100 nm, and slightly narrow size distribution (PDI 0.31). In addition, various grade of HPMCAS (HPMCAS-126, HPMCAS-712, and HPMCAS-912) in different equivalents of sodium hydroxide (0.00 eq, 0.50 eq, 0.75 eq) with "G-1" (40 mg/mL) was formulated for nanoparticle formation (**Table 6**). "G-1" did

not form nanoparticle with the different grades of HPMCAS and aggregation was visually observed for all formulations tested. In summary, HPMCAS failed to act as a stabilizing polymer with "G-1" to form nanoparticles.

Freeze-drying of "G-1" Nanoparticles and Redispersion

Lyophilization is a process widely used in pharmaceuticals to dry and improve the stability of pharmaceutical products.[24] "G-1" with 0.05 equivalents and 0.20 equivalents of sodium hydroxide generated nanoparticles that were desirable for lyophilization. Formulations for these nanoparticles were pursued for further testing with various stabilizing polymer and cryoprotectants for freeze-drying. "G-1" with 0.05 equivalents of sodium hydroxide nanoparticles and "G-1" with 0.20 equivalents of sodium hydroxide nanoparticles with different stabilizing polymers were lyophilized without any cryoprotectants (**Table 7**). Formulations with different equivalents of sodium hydroxide and stabilizing polymers conducted through the FNP process via MIVM are listed in **Table 7**. The dry powder form of "G-1" were re-dispersed with DI water and analyzed through DLS. The "G-1" powder failed to re-dispersed back to nanoscale and form aggregation upon re-dispersion with DI water.

The process of freeze-drying can induce mechanical stress that could destabilize colloidal suspension of nanoparticles and cause nanoparticles to aggregate. For that reason, cryoprotectants are added to the nanoparticle suspension before freezing to protect the nanoparticles from freezing stress and preserve re-dispersibility of the nanoparticles.[25] HPMC E3 is a water-soluble HPMC polymer and serves as a cryoprotectant.[26] Three formulations were tested with HPMC E3 to determine if HPMC E3 is an effective cryoprotectant for "G-1" nanoparticles. "G-1" with different equivalents of sodium hydroxide (0.00 eq, 0.05 eq, and 0.20 eq) nanoparticles with HPMC E3 were lyophilized (**Table 8**). The formulations tested with HPMC E3 as cryoprotectant were conducted through the FNP process via MIVM are listed in **Table 8**. The dry powder form of "G-1" were re-dispersed with DI water and analyzed through DLS. In conclusion, HPMC E3 was not an effective cryoprotectant and failed to form powder that can re-disperse back to nanoscale.

"G-1" nanoparticles with 0.05 equivalents of sodium hydroxide and "G-1" nanoparticles with 0.20 equivalents of sodium hydroxide were lyophilized into dried powder form. Seven excipients (PVA, PEG, trehalose, Mannitol, F-127, cyclodextrin, and propylvinylpyridone) were tested to determine which excipient is the most effective cryoprotectant for re-dispersion of freeze-dried "G-1" powder. The lyophilized powder of "G-1" nanoparticles with good redispersity retains similar size and size distribution as nanoparticles before lyophilization. Freeze dried powder of "G-1" with 0.05 equivalents of sodium hydroxide re-dispersed into nanoparticles in DI water when PEG or trehalose is selected as the cryoprotectant for lyophilization (**Figure 9**). Particle size distribution of the re-dispersed "G-1" with 0.05 equivalents of sodium hydroxide nanoparticles with trehalose and "G-1" with 0.05 equivalents of sodium hydroxide nanoparticles with PEG are shown in **Figure 9**. These nanoparticles redispersed back to nanoscale when PEG or trehalose is used as a cryoprotectant. Trehalose was the most effective cryoprotectant in redispersion of "G-1" nanoparticles formulated with 0.05 equivalents of sodium hydroxide as size and size

distribution were similar to nanoparticles before lyophilization. In addition, freeze dried powder of "G-1" with 0.20 equivalents of sodium hydroxide re-dispersed into nanoparticles in DI water when PEG is selected as the cryoprotectant for lyophilization (**Figure 10**). The particle size distribution of re-dispersed "G-1" with 0.20 equivalents of sodium hydroxide nanoparticles with PEG are shown in **Figure 10**. The dried powder did not re-disperse back to nanoscale when trehalose was used as a cryoprotectant.

Table 7. Formulations consisted of "G-1" with and without stabilizing polymers in different sodium hydroxide equivalents.[a]

"G-1" Concentration	Sodium Hydroxide Equivalents (eq)	Stabilizing Polymer Concentration	Stabilizing Polymers	Antisolvent	Dispersion of NP
80 mg/mL	0.05	N/A	N/A	DI water	No
80 mg/mL	0.20	N/A	N/A	DI water	No
80 mg/mL	0.05	2 mg/mL	PVA	DI water	No
80 mg/mL	0.20	2 mg/mL	PVA	DI water	No
80 mg/mL	0.05	2 mg/mL	Vitamin E TPGS	DI water	No
80 mg/mL	0.20	2 mg/mL	Vitamin E TPGS	DI water	No

[a] Methanol (1 mL) was used as the organic stream.

Table 8. Formulations consisted of "G-1" with HPMC E3 in different sodium hydroxide equivalents.[a]

"G-1" Concentration	Sodium Hydroxide Equivalents (eq)	Stabilizing Polymer Concentration	Stabilizing Polymers	Antisolvent	Dispersion of NP
20 mg/mL	0.00	20 mg/mL	HPMC E3	DI water	No
20 mg/mL	0.05	20 mg/mL	HPMC E3	DI water	No
20 mg/mL	0.20	20 mg/mL	HPMC E3	DI water	No

[a] Methanol (1 mL) was used as the organic stream.

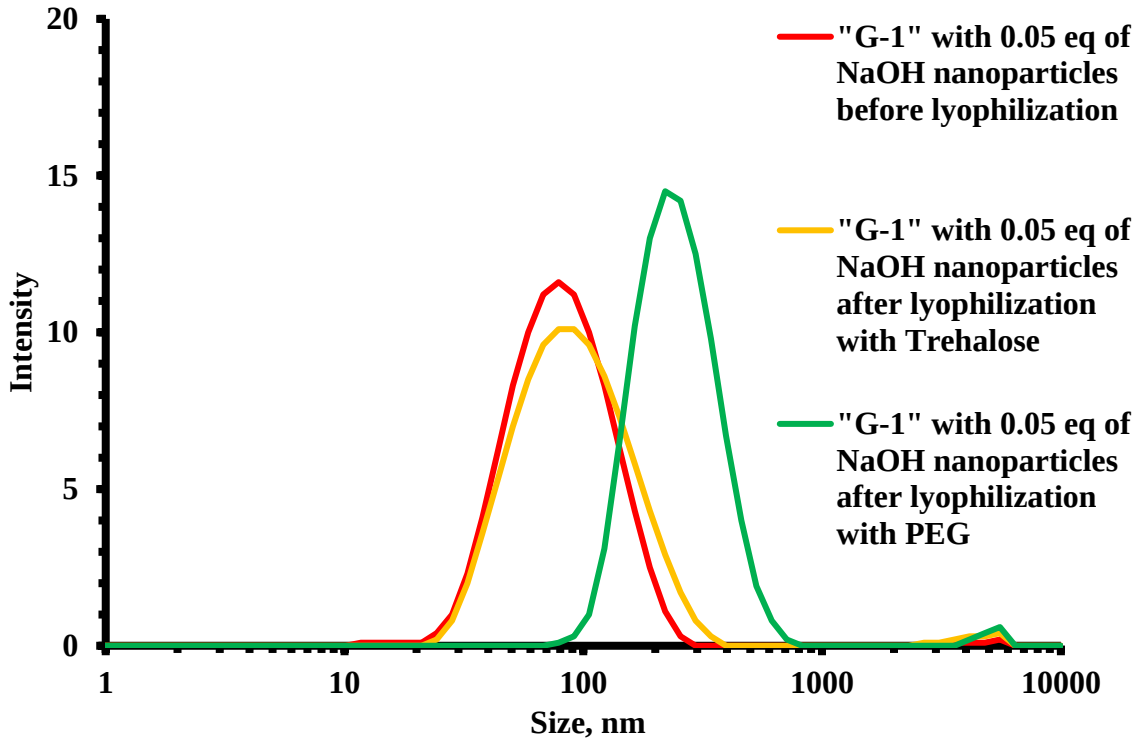

Figure 9. Particle size distribution of "G-1" with 0.05 equivalents of sodium hydroxide nanoparticles before and after lyophilization with selected cryoprotectants (PEG and trehalose).

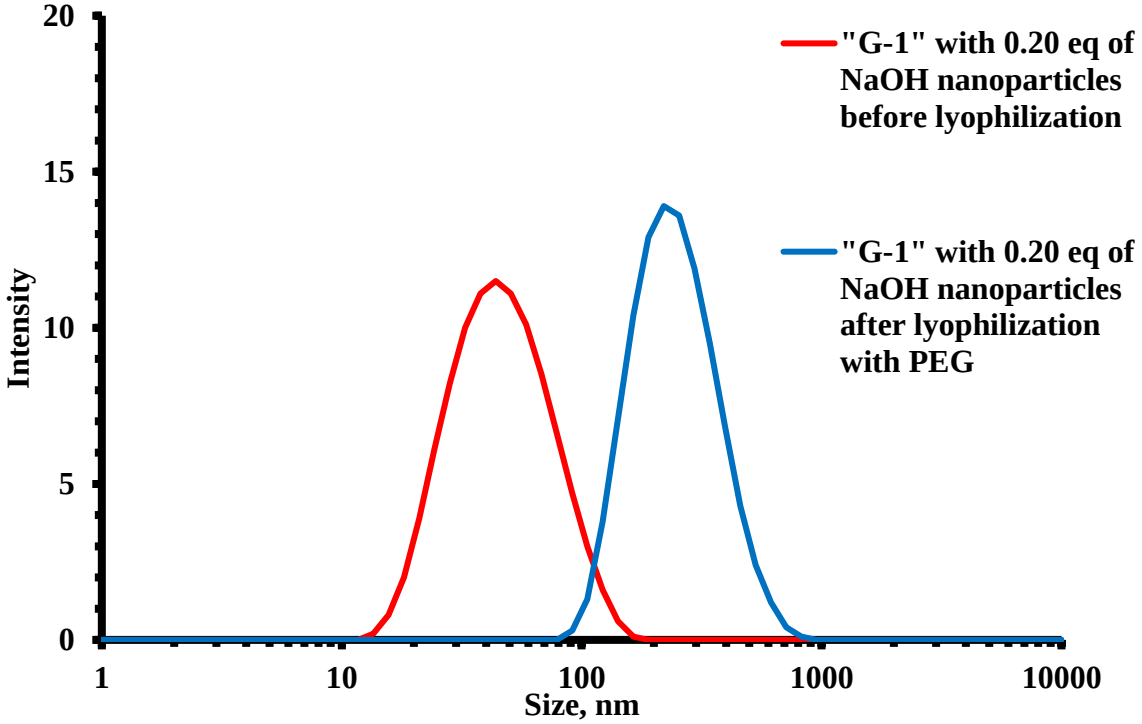

Figure 10. Particle size distribution of "G-1" with 0.20 equivalents of sodium hydroxide nanoparticles before and after lyophilization with PEG as the selected cryoprotectant.

Trehalose was the most effective cryoprotectant for lyophilization of “G-1” nanoparticles. The average size of the re-disperse “G-1” nanoparticles increased by 10 nm with similar PDI. Different mass ratios of trehalose to nanoparticles (5:1, 4:1, 3:1, 2:1, and 1:1) was tested to determine the minimum amount of trehalose required to behave as a cryoprotectant. The re-dispersed “G-1” nanoparticles with different mass equivalents of trehalose (5:1, 4:1, and 3:1) was analyzed through DLS (**Figure 11**). Particle size distribution of different mass equivalents of trehalose to “G-1” nanoparticles (5:1, 4:1, and 3:1) are shown in **Figure 11.** High amount of aggregation was observed for formulations with 2:1 and 1:1 ratio, and thus particle size distribution could not be analyzed by DLS. The formulation (80 mg/mL of “G-1” with 0.05 equivalents of NaOH in MeOH) with 5 mass equivalents of trehalose was repeated and the nanosuspension was lyophilized. The dried powder was sent to Genentech for pharmacokinetics studies. The results from Genentech conclude that the “G-1” nanoparticles with trehalose have faster dissolution rate than the nanoparticles formed in vivo from Genentech's spray dried dispersion.

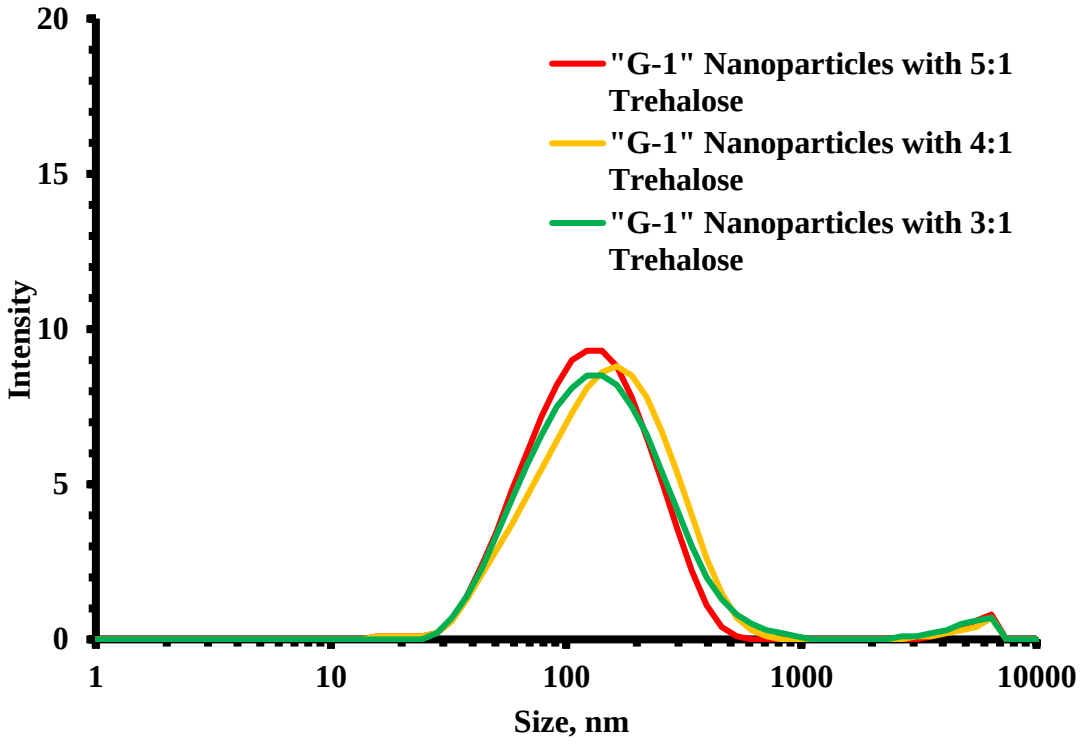

Figure 11. Particle size distribution of “G-1” nanoparticles with different amount of trehalose.

Release Kinetics

“G-1” is insoluble in water (log *P* = 6.18). For oral delivery, rapid dissolution rate (complete dissolution of drug in less than one hour) of the drug is desirable for high bioavailability. A rapid dissolution rate of the drug results in high solubility of the drug in a short amount of time. In this objective, solubility of “G-1” in biorelevant media was measured to determine the released "G-1" concentration and supersaturation level of "G-1". It is hypothesized that free “G-1” powder would have a slower dissolution rate than “G-1” nanoparticles. Slower dissolution rate of free “G-1” powder result in lower solubility of

"G-1" in solution compare to "G-1" nanoparticles. The release experiments were performed on the free "G-1" powder to compare with the release kinetics of "G-1" nanoparticles. In dissolution testing procedures, sink condition is described as a dissolution system that has volume of solvent which is five to ten times greater than the volume of solvent present in a saturated solution.[27] In other words, a "sink" is required to prevent saturation in a dissolution assay. In order to mimic the oral administration of "G-1", FeSSIF and FaSSIF were selected as biorelevant media for in vitro dissolution tests which stimulate the physiological condition in the gastrointestinal tract. FeSSIF and FaSSIF contains biological lipids such as sodium taurocholate and lecithin which can act as a lipid sink for the drug. However, the undissolved nanoparticles cannot be separated from the large lipid globules through centrifugation or filtration due to similar size of the nanoparticles and lipid globules, thus an alternative media is necessary for the dissolution testing.

Tween 20 can be used to mimic biological lipids which can facilitate separation from the undissolved nanoparticle. Most importantly, Tween 20 can act as a hydrophobic sink to increase the solubility of "G-1" in FaSSIF and FeSSIF media.[28] In preparation of the modified biorelevant media, Tween 20 (0.5% and 1.5%) were added to FeSSIF and FaSSIF buffers in substitution of the SIF powder which contains the biological lipids, sodium taurocholate and lecithin. Four different dissolution media (FaSSIF with 0.5% Tween 20, FaSSIF with 1.5% Tween 20, FeSSIF with 0.5% Tween 20, and FeSSIF 1.5% Tween 20) were prepared and free "G-1" powder was added to each media to determine the maximum saturation of the drug in solution and its sink conditions. The pH and concentration of "G-1" in each dissolution media was measured by pH indicator and UV-Vis, respectively (**Table 9**). Among the four different dissolution media, "G-1" in FaSSIF with 1.5% Tween 20 had the highest maximum saturation solubility (2504 μg/mL). FaSSIF with 1.5% Tween 20 was selected for dissolution testing of "G-1" with the sink condition at 250 μg/mL, ten times below saturation limit.

Methods of separating undissolved "G-1" from dissolved "G-1" were investigated for the dissolution test. Undissolved and dissolved "G-1" was separated by Amicon filter (made of regenerated cellulose) and the concentration of "G-1" were measured. High levels of adsorption to the filter membrane were observed. The concentration of filtered "G-1" was substantially lower than the concentration of "G-1" before filtration as shown in **Table 9**. Standard curve of "G-1" before filtration and after Pall filtration (made of modified polyethersulfone) was generated (**Figure 12**). Minor adsorption to the filter membrane were quantitatively observed. The concentration of "G-1" did not change dramatically There are minimal differences between the two different standard curves of "G-1": before filtration (blue line) and after Pall filtration (orange line). In order to achieve the sink condition of the dissolution test (250 μg/mL), 5.0 mg of "G-1" is added to 20.0 mL of FaSSIF with 1.5% Tween. Dissolution testing of "G-1" in FaSSIF with 1.5% Tween 20 was performed and the concentration of "G-1" was measured by UV-Vis (**Figure 13**). In addition, the maximum saturation solubility for the dissolution test (250 μg/mL) was measured by UV-Vis and the absorbance value of the maximum saturation solubility is 1.52 au. In **Figure 13**, about >90% of the free "G-1" powder had dissolved in solution within the first timepoint (15 minutes). Due to rapid dissolution of the free "G-1" powder,

dissolution test for "G-1" nanoparticles were not tested as it would be difficult to differentiate the differences in release kinetics between the free powder drug and the drug nanoparticle.

Table 9. Measurements of pH, maximum saturation solubility, and maximum saturation solubility in FaSSIF and FeSSIF solutions with different amounts of Tween 20 (0.5% or 1.5%)

Dissolution Media	pH	"G-1" Concentration (maximum saturation solubility)	"G-1" Concentration after Amicon filter (maximum saturation solubility)
FaSSIF in 0.5% Tween 20	6.36	644 μg/mL	473 μg/mL
FaSSIF in 1.5% Tween 20	6.17	2504 μg/mL	792 μg/mL
FeSSIF in 0.5% Tween 20	5.00	292 μg/mL	147 μg/mL
FeSSIF in 1.5% Tween 20	5.02	1226 μg/mL	412 μg/mL

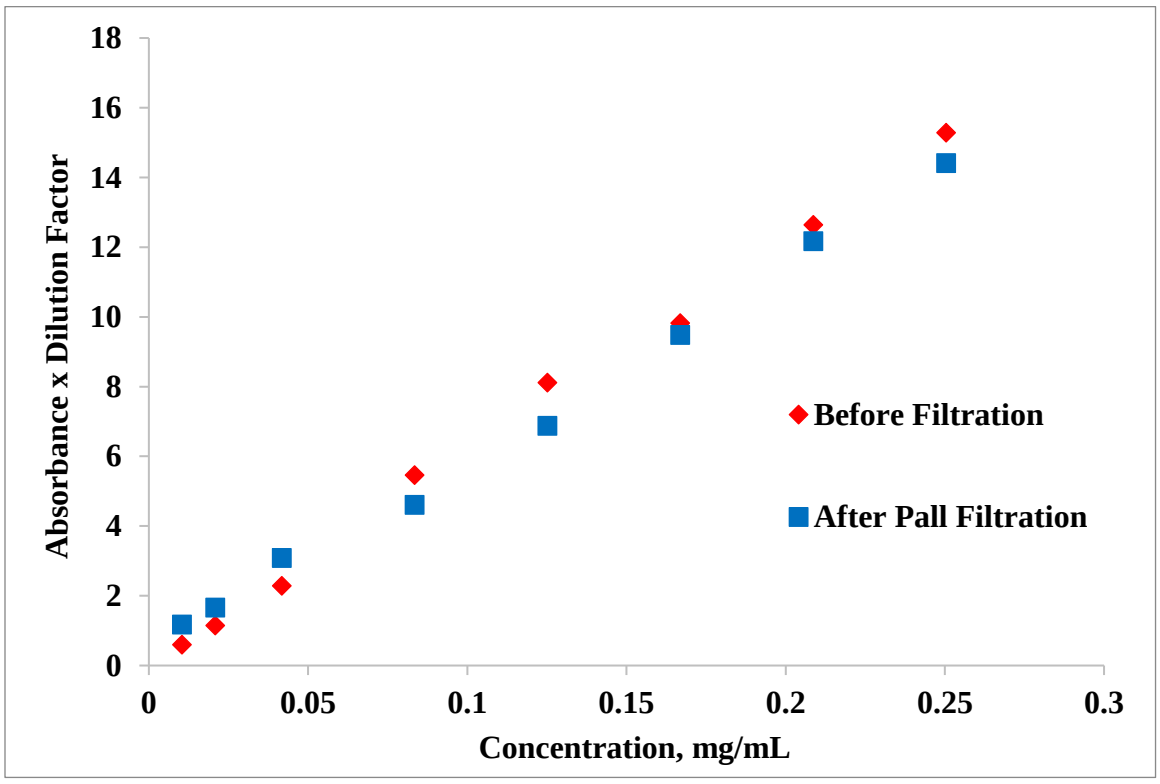

Figure 12. Standard curve of "G-1" before filtration and after Pall filtration.

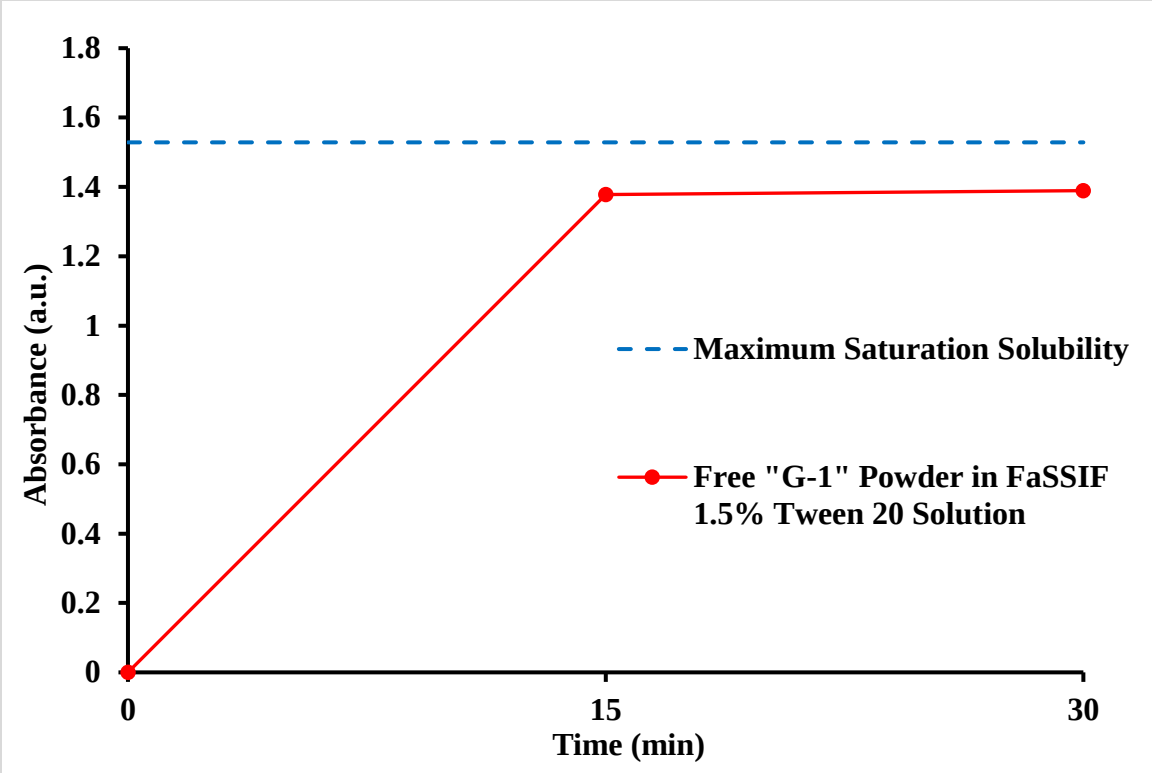

Figure 13. Release kinetic of "G-1" in FaSSIF with 1.5% Tween 20.

Increase Nanoparticle Size Formulation

Various formulations were investigated to increase the size of "G-1" nanoparticles for comparison with Genentech's spray-dried dispersion of "G-1" with HPMCAS which precipitate and formed 200 to 800 nm particles in vivo. Formulation of different size nanoparticles can provide more in-depth knowledge about the dissolution behavior and oral absorption of "G-1". Particle size is often related to the solubility of the drug. The decrease in solubility of the drug result in larger particles due to the particles having less interaction with the solvent.[29] Hydrophobicity of the drug is increased when "G-1" is in its free acid form which allow the polymer to stabilize the drug nanoparticle. By decreasing the solubility and increasing the hydrophobicity of "G-1", larger size nanoparticles can be generated. Concentration of "G-1" in four different solutions was measured by UV-Vis: HCl, KCl, HCl with KCl, and DI water (**Table 10**). Among the four solutions, "G-1" had the lowest solubility in 10 mM HCl. "G-1" is insoluble in 10 mM HCl as concentration of "G-1" in 10 mM HCl was below detection limit. 10 mM HCl was selected as the antisolvent to completely protonate drug and reduce surface charge in the formation of nanoparticles.

Table 10. Solubility of "G-1" in HCl, KCl, HCl with KCl, and DI water.

Solution	"G-1" Concentration
10 mM HCl	Below detection limit
150 mM KCl	4 μg/mL
10 mM HCl and 150 mM KCl	2 μg/mL
DI water	37 μg/mL

Formulation to generate ~200 to 300 nm particles from "G-1" were desired. The DLS analysis of formulations to generate ~200 to 300 nm "G-1" nanoparticle is tabulated in **Table 11.** For all formulations shown in **Table 11**, THF was selected for the organic stream and 10 mM HCl was selected for the antisolvent stream in the FNP process. In addition, all the formulations are conducted via MIVM and the nanoparticle suspension were measured by DLS. Polystyrene-b-poly(ethylene glycol) (PS-b-PEG) is an amphiphilic diblock copolymer which can stabilize hydrophobic drug nanoparticles formed by flash nanoprecipitation (FNP).[30] THF was selected for the organic stream in the FNP process because PS-b-PEG is soluble in THF and not soluble in methanol. PS-b-PEG polymer were able to form "G-1" nanoparticles, with sizes ranging from ~100 to ~500 nm, and narrow size distribution, (PDI 0.07−0.22).

In entry #1 to #3, different percentage of drug (25.0, 50.0, and 75.0%) and PS-b-PEG (75.0, 50.0, and 25.0%) were formulated to generate PS-b-PEG nanoparticles. In entry #4, formulation consisted of "G-1" without PS-b-PEG was conducted to investigate whether PS-b-PEG is necessary for the formation of nanoparticles. "G-1" nanoparticles did not form without PS-b-PEG and aggregations were visually observed. In entry #5, formulation without the quench bath was conducted to observe whether the quench bath affect the formation of nanoparticles. Particle size increased from 220 nm to 500 nm when quench bath is removed in the formulation. The water in the quench bath plays a role in the assembly of PS-b-PEG nanoparticles. Formulation consisting 75% antisolvent and 25% organic solvent in mixer, quenched to 10% organic solvent were switched to 90% antisolvent and 10% organic solvent in the mixer, without quench bath. These formulations were modified to observe the effects of higher volume of antisolvent in the mixing chamber and to retain 10% vol of organic solvent in the final solution. Overall, nanoparticle size decrease in entry #6, #7, and #8 compare to entry #1, #2, and #3, respectively.

Size of nanoparticles can be controlled in FNP by two process variables: the percent core (hydrophobic drug) of the formulation and the total mass concentration of solids (hydrophobic drug and stabilizer) in the solvent stream.[31] In entry #9 to #11, formulations with increasing % drug (75.0%, 87.5% and 100.0%) were conducted to determine its size range. The size of nanoparticles increased as % drug increase in the formulations. To increase the size of nanoparticles and concentration of drug in the nanoparticle suspension, the total mass concentration in the formulation were switched from 20 mg/mL to 40 mg/mL in entry #12 and #13. 80.0% drug loading and 85.0% drug loading was selected for testing because the desired size of nanoparticles is between 75.0% drug and 87.5% drug (167 nm and 488 nm). The 85.0% drug loading generated nanoparticles that had a small increase in size compare to 80.0% drug loading. Entry #12 generated PS-b-PEG nanoparticles, with size at 165 nm and narrow size distribution (PDI 0.07) The stability of the 80.0% drug loading nanoparticles was measured by DLS (**Table 12**). Size increase of "G-1" nanoparticles were observed over different timepoints (0, 30, 60 minutes). The particle size started at 185 nm and the size of the nanoparticle increase to 230 nm at 30-minute timepoint which further increase to 320 nm at 60-minute timepoint. The size increase in the stability study can explained by the phenomenon referred to as Ostwald ripening. Ostwald ripening results from uncontrollable precipitation which leads to particle-size growth following

stabilization.[32] The 80% formulation was repeated to test for re-dispersity with various cryoprotectant.

Table 11. Formulations conducted to generate ~200 to 300 nm "G-1" nanoparticles. [a,b]

Entry	Total Mass Concentration	% Drug	% PS-*b*-PEG	Quench Bath	Size (nm)	PDI
#1 [a]	20 mg/mL	25.0%	75.0%	Yes (6.0 mL)	335	0.22
#2 [a]	20 mg/mL	50.0%	50.0%	Yes (6.0 mL)	220	0.10
#3 [a]	20 mg/mL	75.0%	25.0%	Yes (6.0 mL)	215	0.08
#4 [a]	20 mg/mL	100.0%	0.0%	Yes (6.0 mL)	N/A	N/A
#5 [a]	20 mg/mL	50.0%	50.0%	No	505	0.07
#6 [b]	20 mg/mL	25.0%	75.0%	No	205	0.15
#7 [b]	20 mg/mL	50.0%	50.0%	No	130	0.07
#8 [b]	20 mg/mL	75.0%	25.0%	No	160	0.07
#9 [b]	20 mg/mL	75.0%	25.0%	No	170	0.07
#10 [b]	20 mg/mL	87.5%	12.5%	No	490	0.21
#11 [b]	20 mg/mL	100.0%	0.0%	No	785	0.14
#12 [b]	40 mg/mL	80.0%	20.0%	No	165	0.07
#13 [b]	40 mg/mL	85.0%	15.0%	No	225	0.09

[a] For entry #1-5, THF (1.0 mL) was used for the organic stream and HCl (3.0 mL, 10 mM) as an antisolvent.
[b] For entry #6-13, THF (0.5 mL) was used for the organic stream and HCl (4.5 mL, 10 mM) as an antisolvent.

Table 12. Stability study of PS-b-PEG "G-1" nanoparticles (Formulation: 80% Drug + 20% PS-b-PEG in 0.5 mL THF (TMC = 40 mg/ml) and 4.5 mL of 10 mM HCl water).

Timepoint	Size (nm)	PDI
0 min	185	0.05
30 min	230	0.09
60 min	320	0.20

The nanoparticle suspension (Formulation: 80% Drug + 20% PS-b-PEG in 0.5 mL THF (TMC = 40 mg/ml) and 4.5 mL of 10 mM HCl water) was lyophilized without and with 20 mg/mL of cryoprotectants (PEG, cyclodextrin, and trehalose) at the 30-minute timepoint (**Table 13**). The lyophilized nanoparticles were tested for re-dispersity and analyzed

through DLS. The size and PDI of the re-disperse nanoparticles with various cryoprotectant are listed in **Table 13**. Cyclodextrin was the most effective cryoprotectant for lyophilization of PS-b-PEG "G-1" nanoparticles. The PS-b-PEG "G-1" nanoparticles re-dispersed, with size at 325 nm, and narrow size distribution, (PDI 0.26). However, substantial amount of aggregation was found in suspension. The formulation was repeated and lyophilized with 40 mg/mL of cyclodextrin to improve re-dispersion. No aggregation was found in the nanoparticle suspension. Moreover, the stability of the re-dispersed PS-b-PEG "G-1" nanoparticles was measured by the DLS (**Table 14**). The size and size distribution of the re-dispersed nanoparticles are listed in **Table 14**. The re-dispersed nanoparticles were stable as size and PDI remain nearly unchanged.

Table 13. Re-dispersity test of PS-b-PEG "G-1" nanoparticles with different cryoprotectants (Formulation: 80% Drug + 20% PS-b-PEG in 0.5 mL THF (TMC = 40 mg/ml) and 4.5 mL of 10 mM HCl water).

Cryoprotectants	Size (nm)	PDI
No Cryoprotectant	N/A	N/A
PEG	460	0.53
Cyclodextrin	325	0.26
Trehalose	N/A	N/A

Table 14. Stability study of re-dispersed PS-b-PEG "G-1" nanoparticles with cyclodextrin (Formulation: 80% Drug + 20% PS-b-PEG in 0.5 mL THF (TMC = 40 mg/ml) and 4.5 mL of 10 mM HCl water).

Timepoint	Size (nm)	PDI
0 min	365	0.22
15 min	350	0.21
30 min	350	0.22
60 min	360	0.20

Increase Drug Loading (Wt%) Formulation

Nanoparticle formulations with higher drug loading is desired. At a higher drug loading, less non active excipients are used to produce the same quantity of active pharmaceutical ingredient (API) in the nanoparticle formulation and a lower number of nanoparticles need to be manufactured to deliver the same dose of API.[33] The objective is to generate "G-1" nanoparticles with highest drug loading possible and good redispersity. In the original formulation (80 mg/mL of "G-1" in 0.05 eq of NaOH with concentration of trehalose at 40 mg/mL), the drug loading (wt%) of the lyophilized "G-1" nanoparticle is 16.7%. In addition, decreasing the amount of trehalose result in poorer redispersion of nanoparticles. It is hypothesized that a critical concentration of trehalose is required to act as a cryoprotectant. The removal of water can concentrate nanosuspension and thus allows cryoprotectant to interact with the nanoparticles in suspension more effectively. By concentrating nanoparticles before lyophilization, less cryoprotectant compared to drug mass is needed.

In order to increase drug weight % in the lyophilized nanoparticles, methods to increase nanoparticle concentration was investigated. Tangential Flow Filtration (TFF) was used to increase the concentration of nanoparticles by removing the water in the nanoparticle suspension through filtration. "G-1" nanoparticles were formulated through the FNP process via MIVM. The nanosuspension was concentrated by 5-fold (8 mg/mL into 40 mg/mL) through TFF. The concentration of "G-1" nanoparticles suspension before filtration and after filtration was measured by UV-Vis. The concentration of "G-1" nanoparticles suspension before filtration is 8 mg/mL. The concentration of "G-1" nanoparticles suspension after filtration is 35 mg/mL. The concentrated nanosuspension was tested against different concentration of trehalose in the lyophilization process. The dried powder was re-dispersed into nanoparticles and analyzed through DLS (**Table 15**). Particle size distribution of the re-dispersed nanoparticles are tabulated in **Table 15**. Concentrated "G-1" nanoparticles with 80 mg/mL of trehalose (Increased Concentration #2) re-disperse better than all other formulations. The size and the size distribution of the re-disperse nanoparticles were the closest to the original formulation. The drug loading in "Increased Concentration #2" formulation (30.5 wt% drug) is nearly double, compare to the drug loading in the original formulation (16.7 wt% drug).

Table 15. Re-dispersity test of concentrated "G-1" nanoparticles (80 mg/mL of "G-1" in 0.05 eq of NaOH) with different concentration of cryoprotectant (trehalose)

Formulation	Nanoparticle Concentration (mg/mL)	Trehalose Concentration (mg/mL)	% Drug Loading in Dried Powder	Size (nm)	PDI
Original	8	40	16.7%	88	0.21
Increased Concentration #1	35	40	46.6%	151	0.30
Increased Concentration #2	35	60	36.8%	185	0.41
Increased Concentration #3	35	80	30.5%	126	0.25
Increased Concentration #4	35	120	22.5%	137	0.31

Conclusion

The oral bioavailability of “G-1” was improved through the formation of nanoparticles through FNP. “G-1” formed ~80 nm particles which are self-stabilized without additional use of steric copolymers. Nanoparticles can be formed with concentration of “G-1” as high as 160 mg/mL. Lyophilization of “G-1” nanosuspensions with trehalose as cryoprotectant, result in good redispersion of “G-1” nanoparticles. The dried powder of the final formulations was sent to Genentech. The nanoparticles with trehalose show faster dissolution rate than the nanoparticles formed in vivo from Genentech's spray dried dispersion. Due to the rapid dissolution rate of free “G-1” powder, the differences in the release kinetics of the free powder and the nanoparticles could not be discerned. Series of formulations were conducted through the FNP process to generate ~300 nm “G-1” nanoparticles. Nanoparticles with higher drug loading was achieved. Specifically, drug loading of the original formulation was increased from 16.7% to 30.5%. The dried powder of the final formulations (“G-1” nanoparticles with trehalose, ~300 nm “G-1” particles, and 30.5 wt% drug “G-1” nanoparticles) was sent to Genentech for better understanding in the dissolution behavior of “G-1”, contributing to the knowledge of nanomedicine and pharmaceutical sciences at Genentech.

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