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Novel Method for Concentrating and Drying Polymeric Nanoparticles: Hydrogen Bonding Coacervate Precipitation

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Abstract: Nanoparticles have significant potential in therapeutic applications to improve the bioavailability and efficacy of active drug compounds. However, the retention of nanometer sizes during concentrating or drying steps presents a significant problem. We report on a new concentrating and drying process for poly(ethylene glycol) (PEG) stabilized nanoparticles, which relies upon the unique pH sensitive hydrogen bonding interaction between PEG and polyacid species. In the hydrogen bonding coacervate precipitation (HBCP) process, PEG protected nanoparticles rapidly aggregate into an easily filterable precipitate upon the addition various polyacids. When the resulting solid is neutralized, the ionization of the acid groups eliminates the hydrogen bonded structure and the ~100 nm particles redisperse back to within 10% of their original size when poly(acrylic acid) and citric acid are used and 45% when poly(aspartic acid) is used. While polyacid concentrations of 1−5 wt % were used to form the precipitates, the incorporation of the acid into the PEG layer is approximately 1:1 (acid residue):(ethylene oxide unit) in the final dried precipitate. The redispersion of dried β-carotene nanoparticles protected with PEG-*b*-poly(lactide-co-glycolide) polymers dried by HBCP was compared with the redispersion of particles dried by freeze-drying with sucrose as a cryprotectant, spray freeze-drying, and normal drying. Freeze-drying with 0, 2, and 12 wt % sucrose solutions resulted in size increases of 350%, 50%, and 6%, respectively. Spray freeze-drying resulted in particles with increased sizes of 50%, but no cryoprotectant and only moderate redispersion energy was required. Conventional drying resulted in solids that could not be redispersed back to nanometer size. The new HBCP process offers a promising and efficient way to concentrate or convert nanoparticle dispersions into a stable dry powder form.

Keywords: Polymeric nanoparticles; nanoparticle; drying; hydrogen bonding; coacervate; poly(acrylic acid); poly(aspartic acid); citric acid; lyophilization; redispersion; aggregation

Introduction

Increased attention has been given to polymeric nanoparticles (NPs) as drug delivery vehicles because of their ability to minimize drug degradation, target delivery, and increase drug bioavailability.[1−5] The preparation of NP formulations requires purification, concentration, and often drying. The

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nanometer size scale makes ultrafiltration or dialysis the most practical processes for purification or concentration in the liquid state. While ultrafiltration and dialysis are easily employed in a laboratory setting, they become both costly and time-consuming at larger industrial scales. Furthermore, to make the final form stable, it is most often necessary to completely dry the sample. The drying of NPs is a significant challenge because aggregation during the drying process usually results in the inability to retain the desired nanometer particle size. Processes that would enable production of concentrated or dried NP dispersions in a simple and cost-effective manner would be highly desirable.

In this paper we present hydrogen bonding coacervate precipitation (HBCP), a novel technique to purify and concentrate polymer protected NPs using a specific hydrogen bonding interaction between polyethylene oxide groups on the NP surface and poly-carboxylic acid compounds. The strong interaction leads to rapid but reversible precipitation of a coacervate. The precipitate is easily recovered using standard filtration or centrifugation. In this step impurities are easily removed. The coacervate is reversed by neutralizing the solution to pH 7 to produce concentrated dispersions. The coacervate is dried either by conventional drying or freeze-drying to produce biocompatible, stable dry powder formulations of NPs. This provides a novel and rapid route to purify, concentrate, and prepare dry NP solids that can be redispersed back to their original NP size.

Conventional drying processes irreversibly aggregate NPs as they are forced together during the drying or freeze-drying process. Diluents (i.e., cryoprotectants) can be added to prevent particle−particle contacts, but the concentrations of these agents can be problematic. Chasteigner et al. have explored various cryoprotectants, determining that carbohydrates could provide partial protection against permanent aggregation during the drying step at concentrations of 10 wt %, and also found that crystallization of the PEG coating provided by Pluronic polymers destabilized itraconazole nanospheres, an effect that was notably not observed for SDS stabilized spheres.[6] Recent work by Johnston has shown that rapid precipitation of hydroxypropyl(methylcelluslose) (HPMC)-protected NPs using high concentrations of salt (sodium sulfate) produced open aggregates that could be filtered by conventional filtration processes and which could be redispersed back to nanometer size.[7] The Johnston approach relies upon having a dense polymer layer on the particle that (i) can be collapsed onto the NP surface during salt addition, (ii) has the mechanical integrity to not rearrange during the drying step, and (iii) provides sufficient osmotic force to separate the NPs past London−van der Waals attractive distances during rehydration. Our work follows the strategy of Johnston but invokes the specific polyethylene oxide−polyacid hydrogen bonding interaction to initiate particle flocculation and redispersion, rather than salt-induced polymer precipitation. Polymeric NPs are most commonly encapsulated with an amphiphilic biocompatible block copolymer.[1−5] Most of the amphiphilic block copolymers used in drug delivery contain poly(ethylene glycol), PEG [also known as poly(ethylene oxide), PEO], to provide NPs with long circulation times that are protected against clearance by the reticuloendothelial system;[8,9] therefore, the process presented here has wide applicability.

The cooperative hydrogen bonds formed between PEG and polyacids have been studied extensively as a means to controlling polymer phase behavior. The hydrogen bonding interaction is reversible with a shift in pH: below pH ∼4, where the carboxylic acid is protonated, the interaction is turned on and the hydrophobic complex precipitates, and at pH > 7 the ionized acrylate groups cannot hydrogen bond, the complex dissociates, and the species become water-soluble. In essence, in the complexed form, the groups that make the PEGs and the poly(acids) water-soluble (i.e., the ether oxygens and acid residues, respectively) are associated with each other and not with water and therefore the complex is hydrophobic. In a classic review by Tsushida, the nature of the hydrogen bond interaction between the oxygen on PEG and the proton on the caroboxylic acid group is presented in detail.[10] The complementary spacing between the groups on PEG and poly(acrylic acid) (PAA) chains causes strong interactions when the "critical molecular weight of PEG is above 8.8K."[10] Klier, Scranton, and Peppas created novel comb graft poly(methacrylic acid) (PMAA)-*comb*-PEG polymers where the attachment of the PEG chain to the polymer backbone reduced the entropic penalty for complexation and resulted in association at lower molecular weights of the PEG chain.[11−13] Guerrin, et al. used the PEG−PAA interaction to form coacervate shells around

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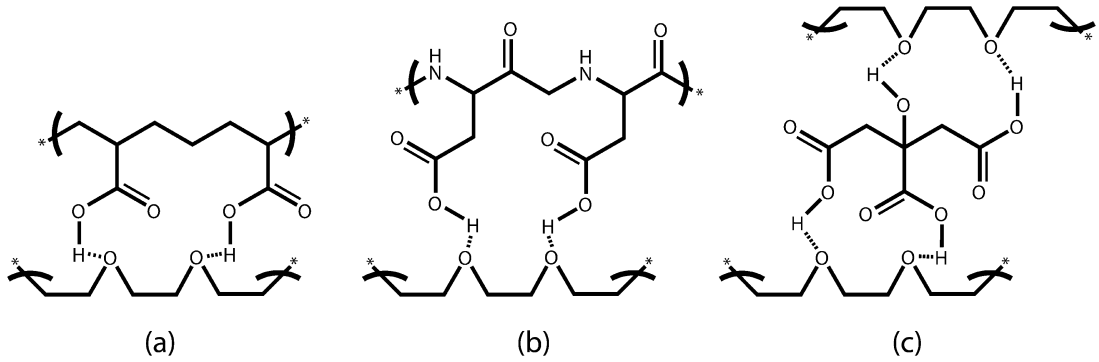

Figure 1. A schematic representation of the hydrogen bonding interactions between PEG chains and (a) poly(acrylic acid), (b) poly(aspartic acid), (c) citric acid.

PEG-surfactant-coated emulsion droplets.[14] The coacervate coating enabled drying of the emulsion and redispersion with a pH shift. Interestingly, there are no systematic studies of the critical molecular weight required for small polyacids to complex with larger PEG chains to provide guidance on the minimum number of acid groups for cooperative complexation.

In this work, we demonstrate complexion and phase separation of NPs coated with a dense layer of self-assembled PEG chains when mixed with poly(acrylic acid), poly(aspartic acid), and citric acid (Figure 1). The last two compounds are chosen because of their biocompatibility. Cytotoxicity assays are performed with the polyacid compounds to determine the acceptability of these compounds for drug delivery applications. The drying and redispersion of PEG coated NPs prepared by this HBCP is compared against conventional drying, freeze-drying, and spray freeze-drying techniques.

Experimental Section

Experimental Reagents. Tetrahydrofuran (99.9%), citric acid (99.5%), and poly(acrylic acid) (M_w ~1800) were purchased from Sigma-Aldrich and used as received. Poly(ethylene glycol)-*b*-poly(lactide-*co*-glycolide) (PEG-*b*-PLGA) (5K-*b*-7K) was synthesized by ring-opening polymerization of DL-lactide and glycolide using methoxy poly(ethylene glycol) (5K, Aldrich) as macroinitiatior and stannous octoate as catalyst according to published procedures.[15,16] β-Carotene (99.9%) was received from BASF (Ludwigshaven, Germany). Poly(aspartic acid) sodium salt (M_w ~2000) was purchased from Alamada Polymers, Inc. (Madison, AL) and converted to the acid form using an Amberlite IR-120 resin (4.4 mequiv/g by dry weight). Ten milliliters of a 5 wt % sodium poly(aspartate) solution was passed through a bed of 0.811 g of resin, for a 10-fold excess of resin, to convert the poly(aspartate) to the acid form.

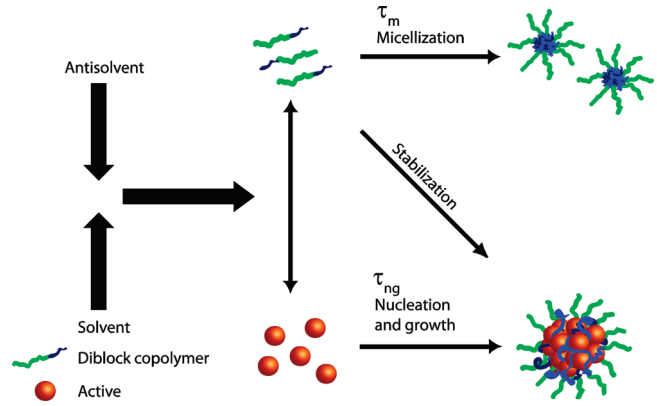

Figure 2. A schematic representation of the FNP method. An active (β-carotene) and an amphiphilic diblock copolymer (PEG-*b*-PLGA) are molecularly dissolved in organic phase and mixed rapidly with a miscible antisolvent for the active and one block of the copolymer. When water is the antisolvent, a hydrophobic active and hydrophobic portion of the copolymer are precipitated simultaneously to form NPs. The mixing time must be short compared with the composite process to obtain homogeneous kinetics and a narrow size distribution.

Nanoparticle Formation. The NPs were produced using the Flash NanoPrecipitation (FNP) method.[17,18] In FNP, a hydrophobic or hydrophobically modified active and an amphiphilic diblock copolymer are molecularly dissolved in a water-miscible organic phase and rapidly impinged against an aqueous antisolvent stream to precipitate the active with a tunable, narrow size distribution from 30 nm to 400 nm (Figure 2). The details of the experimental apparatus and process have been described previously.[19,20] In this study,

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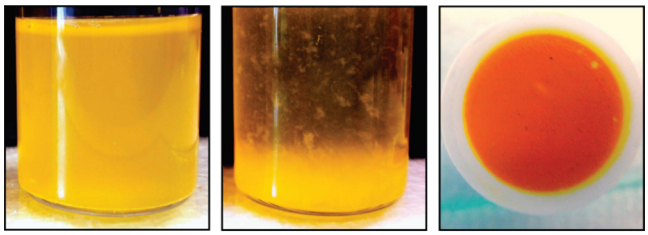

Figure 3. (a) The colloidally stable 2 mg/mL PEG-*b*-PLGA β-carotene NP dispersion. (b) After addition of 3 wt % poly(acrylic acid) to the NP dispersion, aggregates form and settle in the vial. (c) The aggregates are easily filtered and captured as a filter cake when filtered with a 1.2 μm filter.

Table 1. Compositions of Nanoparticle and Polyacid Solutions

acid solution	nanoparticles/ polyacid (w/w)	[EO]/[COOH] (mol/mol)
0.5 wt % poly(acrylic acid)	0.4	1.23×10^{-2}
1 wt % poly(acrylic acid)	0.2	6.19×10^{-3}
3 wt % poly(acrylic acid)	0.067	2.06×10^{-3}
5 wt % poly(acrylic acid)	0.041	1.28×10^{-3}
1 wt % poly(aspartic acid)	0.2	1.08×10^{-2}
1 wt % citric acid	0.2	5.51×10^{-3}

β-carotene was selected as the active and PEG-*b*-PLGA (5K-*b*-7K) was used as the stabilizing amphiphilic polymer.

β-Carotene NPs were prepared by mixing a stream of THF containing 10 mg/mL β-carotene and 10 mg/mL PEG-*b*-PLGA with a stream of Milli-Q H_2O at a 1:10 mixing ratio. The NP suspension was collected at the outlet stream and dialyzed against 4 L of Milli-Q H_2O for 24 h. This formulation gave rise to an intensity averaged particle size of 110−140 nm with a narrow particle size distribution.

Characterization of Nanoparticle Dispersions. The particle size distributions were measured using dynamic light scattering (ZetaSizer Nano ZS, Malvern Instruments, Worcestershire, U.K.). Each measurement was repeated at least 3 times per sample. The particle size as reported is the intensity weighted average.

Methods of Precipitating and Drying Nanoparticle Dispersions. Hydrogen bonding coacervate precipitation (HBCP) involves complex formation and filtration: Polyacids in dry powder form were added to a 2 mg/mL NP dispersion (Figure 3a) and were allowed to precipitate for 30 min. Table 1 shows the ratios of NPs to acid by weight as well as the ratios of the concentration of ethylene oxide (EO) groups to the concentration of carboxylic acid (COOH) groups.

For 3 and 5 wt % poly(acrylic acid) and for 1 wt % citric acid, in about 10 min, a precipitate formed and settled to the bottom of the vial as shown in Figure 3b. The precipitate was either vacuum filtered through a 1.2 μm Millipore filter to produce a filter cake as shown in Figure 3c or centrifuged at 250*g* for five minutes. The filter cake contained approximately 92% of the original β-carotene as determined by a UV absorbance assay and a mass balance on the β-carotene in the filtrate. It should be noted that the original 100−140 nm NP dispersion will pass entirely through a 1.2 μm filter, and will completely block a 0.1 μm filter. Similarly, at 250*g*, the force is insufficient to settle nonaggregated 100 nm particles. The 0.5 and 1.0 wt % poly(acrylic acid) dispersion and the 1 wt % poly(aspartic acid) dispersion gave flocs that did not settle as readily, but could be filtered and recovered through a 1.2 μm filter. It is known that the ethylene oxide:acid interaction has a 1:1 stoichiometry.[10] To drive the kinetics of flocculation we used much higher concentrations of acid (Table 1), but excess acid is removed with the filtrate or supernatant.

Freeze-drying (FD) consists of rapid freezing and subsequent lyophilization of the suspension. To achieve rapid freezing, a 5 mL vial of the suspension was placed in an acetone/dry ice bath at −78 °C. Lyophilization was performed in a Benchtop 3.3/Vacu-Freeze (VirTis, Gardiner, NY) under vacuum (< 50 mTorr), with a condenser temperature of −78 °C. After 2 days the dried powders were removed from the lyophilizer and redispersed. These experiments were performed with and without sucrose to investigate the effect of cryoprotectants on aggregation of these PEG protected nanoparticles.

Spray freeze-drying (SFD) is similar to freeze-drying in that both processes consist of freezing and lyophilization.[21,22] However, in the first step of SFD, NP suspensions are fed at 1 mL/min *via* a syringe pump to a 20 kHz ultrasonic nozzle (Sono-Tek Corp., Milton, NY). The atomized droplets fall *via* gravity into liquid nitrogen (LN_2) in a custom chamber, described previously.[22] The advantage of ultrasonic atomization rather than air pressure driven atomization is that the process occurs without air convection currents. Therefore the collection efficiency for small particles is much higher than for conventional atomization processes.[22] After spraying is completed, and excess LN_2 is allowed to boil off, the frozen droplets are lyophilized at −78 °C for the first 36 h and at 25 °C for the next 36 h. The dried powder is then recovered and redispersed.

Vacuum rotary evaporation (rotovap) for drying permits liquid solvents to be removed without excessive heating. NP solutions of β-carotene are evaporated at 55 °C and under 2−5 kPa vacuum. This process occurs over several hours.

Methods of Redispersing the Dried Nanoparticles. To test for aggregation during drying, various dispersion protocols were studied with increasingly vigorous dispersion

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conditions. They involved a combination of sonication with and without heat, for varying amounts of time. For the case of the HBCP drying, 0.1 N sodium hydroxide is added to neutralize the solution to pH ~7, disrupting the hydrogen bonding between NPs and acids.[10] For rotovap drying, FD, and SFD, the pH was not shifted during the drying process.

The first protocol involved dispersing the dried NPs in water with manual shaking. The second protocol was to sonicate the NPs in a bath sonicator (Fisher Scientific FS6) for 15 min at room temperature. The third protocol was to sonicate the NPs for 30 min at 55 °C in a temperature-controlled bath sonicator (VWR Scientific, Aquasonic, model 150D, power level 9). The temperature was chosen to be high enough to disrupt PEG crystallization between NP coronas that might have occurred during drying.[23] At the conclusion of each approach, particle size measurements were taken to determine the effectiveness of the redispersion method.

Cytotoxicity Assay. *In vitro* cytotoxicity was determined for poly(acrylic acid), poly(aspartic acid), and the citric acid using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay kit (Promega Corporation, Madison, WI), in which the soluble tetrazolium salt [3-[4,5-dimethylthiazol-2-yl]-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium] (MTS) is reduced to a purple formazan product. The absorbance is therefore proportional to the number of viable cells.

Solutions of 5 wt % poly(acrylic acid) and citric acid in water were prepared and neutralized by titrating 1 M NaOH until a pH of 7.2 was achieved. A neutral 5 wt % solution of poly(aspartic acid) was prepared directly from the sodium salt. All three solutions were sterilized through a 0.2 μm filter.

MDA-MB-231 human breast cancer cells, received from Winston Soboyejo (Princeton University, Princeton, NJ), were cultured in DMEM supplemented with L-glutamine, 10% fetal bovine serum, penicillin (50 IU/mL), and streptomycin (50 μg/mL) (ATCC, Manassas, VA), hereafter referred to as complete medium, and incubated at 37 °C and 5% CO_2. Cells were seeded in a 96 well plate at 20,000 cells/well and incubated for 24 h. The medium was aspirated and replaced with 50 μL of fresh 2× concentrated complete medium and 50 μL of dilutions of either poly(acrylic acid), to poly(aspartic acid), or citric acid. The plate was incubated for an additional 48 h, after which the medium was aspirated and 100 μL of complete medium and 20 μL of the MTS reagent were added. After a 2 h incubation period, the absorbance was read at a wavelength of 490 nm with a reference wavelength of 650 nm.

Results and Discussion

Hydrogen Bonding Coacervate Precipitation. In this work, addition of polyacids to a suspension of NPs stabilized by a PEG corona results in reversible flocculation. This is beneficial in the process of concentrating and drying NPs due to the fact that the NP flocs enable efficient solvent removal via filtration. Flow through a porous bed is described by Darcy's Law as $Q = (k)(\Delta P)(\mu)^{-1}(L)^{-1}$, where k is the Darcy permeability, ΔP is the pressure drop, μ is the solvent viscosity, and L is the depth of the bed. The Darcy permeability relates to the size of flocs comprising the bed as $k \sim d^2$.[24] For example, at a constant bed thickness if the NPs with a diameter $d = 100$ nm aggregate into flocs with a size $d_f \sim 1000$ μm, as shown schematically in Figure 4, the flux would be enhanced 100-fold. Also, we find that these flocks are mechanically rigid enough not to collapse during filtration at low pressure (ca. 14 psi). The combination of these two factors allows for the rapid solvent flux through the bed.

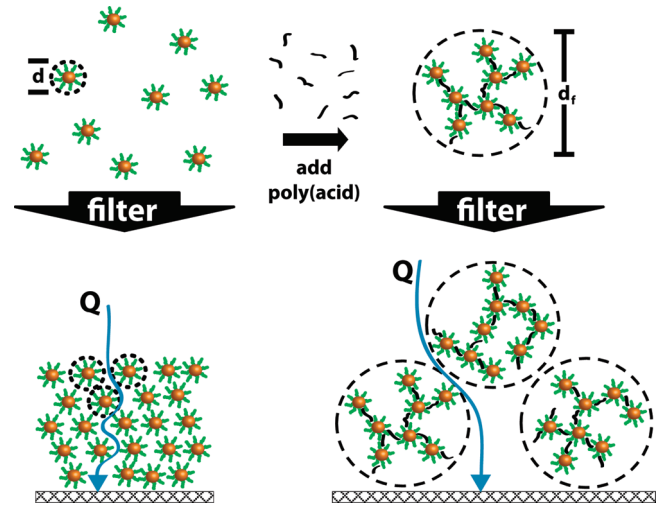

Figure 4. Coacervation of PEG coated NPs and filtration. Suspended 100 nm β-carotene NPs, when filtered, form a porous bed with a low permeability. However, through coacervation, the 100 nm particles form larger flocks that have an open, rigid structure. Therefore, during filtration, solvent removal is improved by the increased permeability of the bed that is formed by the flocs.

Table 2. Initial and Redispersed Particle Sizes for NPs Dried with 1 wt % Acid in the HBCP Precipitation Method and after Redispersion Using Sonication and 30 min of Heating at 55 °C

	poly(acrylic acid)	poly(aspartic acid)	citric acid
initial size (nm)	130 ± 10	140 ± 10	140 ± 10
redispersed size (nm)	140	200 ± 20	150 ± 10

Three different types of acids capable of hydrogen bonding were investigated as network forming groups between PEG-*b*-PLGA coated NPs, listed in Table 2. The 1 wt % poly(acrylic acid) and citric acid coacervates yielded NPs that showed only an 8% increase in size upon redispersion,

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but even these increases were within the standard deviation of repeated measurements. Using 1 wt % poly(aspartic acid) gave rise to a 40% increase in size, an increase from 140 to 200 nm; however, this size is still within the desired range for the parenteral applications we target.

The weight percent of acid bound to the NP, defined as ratio of the mass of bound acid to the mass of acid added (1 wt %), was determined by pH titration of the filtrate. For poly(acrylic acid) the weight of bound poly(acrylic acid) to NP was 7.6%, while for citric acid the value was 21%. A 1:1 stoichiometric ratio for the ethylene oxide monomers to acrylic acid monomers would produce a NP to poly(acrylic acid) ratio of 33%. Therefore, the complex is essentially the expected stoichiometric ratio.

The effect of acid concentration on redispersion was studied using poly(acrylic acid). The initial NP size was 125 nm and was stable. The redispersed sizes after 30 min of sonication at 55 °C were 130, 140, 160, and 150 nm for the 0.5, 1, 3, and 5 wt % poly(acrylic acid) solutions, respectively. Repeated samples showed variability of $\pm 10-20$ nm, and therefore the sizes are essentially independent of polymer concentration. The increased concentration does, however, result in faster particle agglomeration, which is a benefit that outweighs the possible slight increase in NP size upon redispersion. The unbound acid is recoverable in the removed solvent and may be recycled.

For a full comparison of HBCP versus the other more traditional drying techniques, the effect of different redispersion techniques on the NP size after neutralization was investigated. After filtration of 10 mL samples precipitated with either 1 wt % poly(acrylic acid) or 1 wt % citric acid, the filter cake was either freeze-dried for 2 h, vacuum-dried for 2 h at 35 °C, or allowed to remain wet (a total of 6 conditions). Then, a few drops of 0.1 N NaOH and 10 mL of water were added to the solids. A probe sonicator (Fisher Scientific, model 100) was used for 2 min at 4 W to distribute the aggregates in suspension and the size was measured. Then, a progression of sonication for 15 min at RT, 30 min at RT, and 30 min at 55 °C was performed. The summary of intensity average particle size for each sample is plotted in Figure 5. The wet filter cakes resuspend readily to the original average size of 100 nm within 15 min of sonication at room temperature for either poly(acrylic acid) or citric acid complexing agents. The fully dried samples are all resuspended to a size below 175 nm.

The HBCP process can also be used to concentrate nanoparticle dispersions in a way we find is more rapid and simpler than ultrafiltration or ultracentrifugation. We have demonstrated the potential to achieve a 100-fold increase in NP concentration in the following example. To a 200 mL NP suspension, we add 2 g of poly(acrylic acid) (i.e., 1 wt %). The suspension is then centrifuged, and the supernatant decanted, yielding a wet cake with a NP concentration of 560 mg/mL, for a 280-fold increase in concentration. The flocs are redispersed directly by adding 1.5 mL of 1 N NaOH and probe sonicating for 3 min. The final dispersion had a NP concentration of 168 ± 15 mg/mL NPs (of which 50% was the model active compound β-carotene), with a pH of 6.8, an intensity average size of 142 ± 2 nm, and an 84-fold increase over the initial concentration.

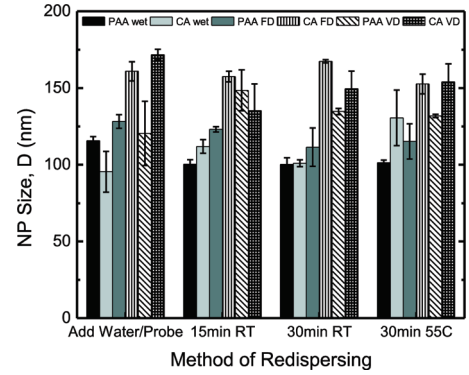

Figure 5. Intensity average particle sizes for redispersed nanoparticles precipitated via addition of poly(acrylic acid) (PAA) or citric acid (CA) and resuspended wet, or after freeze-drying or vacuum drying for two hours. The original average particle size was 100 nm. All data are the average of three measurements. The precipitated material was resuspended by adding a few drops of 0.1 N NaOH and 10 mL of water and then performing the indicated resuspension step: first, probe sonication at 4 W for 2 min, and then bath sonication for 15 or 30 min at room temperature or 55 °C.

Freeze-Drying. Both HBCP and freeze-drying require the addition of excipients to the NP suspension to aid in the drying process. As such, we have evaluated the effectiveness of sucrose as a cryoprotectant for freeze-dried NPs, and the results are shown in Figure 6. The weight ratio of NP:sucrose in suspension ranged from 1:0.1 to 1:60. Freeze-drying at low NP:sucrose ratios resulted in some degree of aggregation. The FD solution with no additional sucrose showed a particle size of 530 nm which decreased to 380 nm after sonication. At a weight ratio of 1:0.1, sucrose had essentially no effect on the reconstituted NP size (560 and 380 nm with gentle agitation and sonication, respectively). When the weight ratios were 1:1 and 1:10, it is observed that sonication results in a decrease in aggregate size, yet a 30% increase in size persists. When sucrose is used at concentrations 60 times higher than NPs, the NPs are protected against irreversible aggregation and the original 110 nm size is restored upon rehydration, whether the sample was gently agitated or sonicated. Therefore, at low NP:sucrose ratios, sonication facilitates improved dispersion of the dried NP powder, while at high NP:sucrose ratios, sonication was not required to redisperse the dried NPs. To prevent irreversible aggregation, high concentrations of cryoprotectant must be added, which will limit the nanoparticle concentration that can be administered while keeping the osmolality of the solution isotonic with the in vivo or in vitro application. Unlike freeze-drying, in HBCP, excess polyacid in solution is removed with the solvent, and therefore the amount of poly(acid) in the powder is fixed by the amount bound to the PEG chains, comprising approximately 10−25% of the final powder mass. This is a

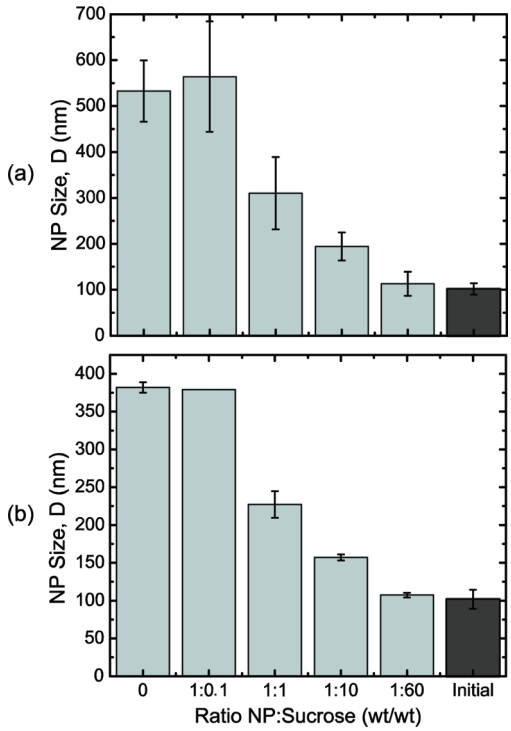

Figure 6. NP sizes (nm) for FD solutions of various NP:sucrose by weight ratios redispersed (a) by gentle agitation and (b) with sonication for 15 min at room temperature.

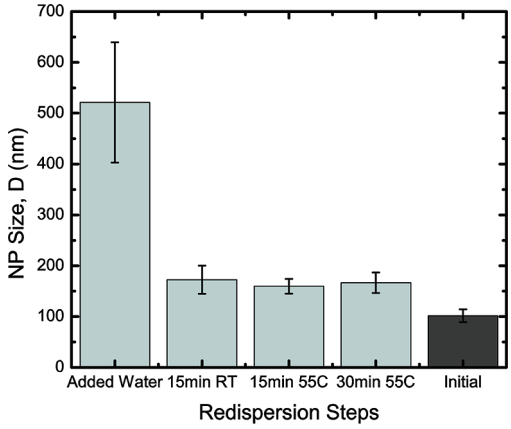

Figure 7. NP sizes (nm) of SFD NPs after redispersion with sonication for 15 or 30 min at either ambient temperature or 55 °C. No sucrose was used in the NP solutions. The "added water" sample is reconstituted with gentle agitation at ambient temperature without sonication. Neither temperature nor sonication time longer than 15 min has a significant effect on particle redispersion.

significant improvement over freeze-drying which produces powders that require over 98% cryoprotectant in the final powder.

Spray Freeze-Drying. Spray freeze-drying is compared to HBCP because pure NP powders may be obtained without the addition of any cryoprotectant. Figure 7 shows the particle sizes of spray freeze-dried NPs after redispersion using various methods. Sonication for 15 or 30 min at ambient temperature or 55 °C results in dispersion to 150 nm NPs, in contrast to 375 nm particles prepared by the conventional freeze-drying process. Similar to what was observed with the redispersion of particles dried via HBCP, sonication beyond 15 min at RT has little effect on further reducing the particle sizes. However, the particles are never redispersed back to the original 100 nm size, indicating some limited, irreversible aggregation.

While this process yields powders that resuspend with sonication, it requires processing times of over 3 days since all liquid is cryogenically processed. On the other hand, HPCP is performed rapidly, completed in less than 4 h since nearly all liquid is removed via filtration.

Vacuum Rotary Evaporation. The drying and resuspension done by rotary evaporation produced particles that were larger than 1 μm. The particles were not able to be redispersed below this μm size even after extensive sonication and heating at 55 °C.

Morphological Comparison of the Dried Nanoparticles. Scanning electron microscopy (SEM) was used to investigate the structural differences of the dried aggregates. No direct correspondence was found between morphology and the dispersibility of the dried powders. Further information may be found in the Supporting Information.

Cytotoxicity. For drug delivery applications, the toxicity of the proposed excipients at relevant dosage concentrations was evaluated. The relevant concentrations of acids were determined based on both the ratio of acid to NP needed to achieve a resuspendable suspension and previously determined IC_{50} values for NP formulations of paclitaxel prodrug conjugates.[25] As noted previously, the weight percent of acid bound to the NPs was approximately 10 and 25%. This analysis establishes that the concentration of acid present in suspension with a redispersed NP solution at normal dosage levels would be on the order of 100 ng/mL. The assays were run with additive levels 10^3 times higher than this value.

The results of the toxicity assay are presented in Figure 8. Percent survival is calculated as the ratio of the absorbance of the test well compared to an acid-free control. It is apparent that, at the concentrations tested, there is no adverse impact on cell survival. At higher concentrations, > 60 ng/mL tested, the results indicate >100% cell survival. Citric acid is an intermediate in the citric acid cycle, which takes place in the mitochondria of cells;[26] therefore, it is possible that cell metabolism is affected, resulting in an apparent increase in cell survival over control cells, without any acid. Repeated tests validated that this is outside the standard errors of the experiment. Based on the fact that there is no significant cell death for the acids tested, suitable polyacid

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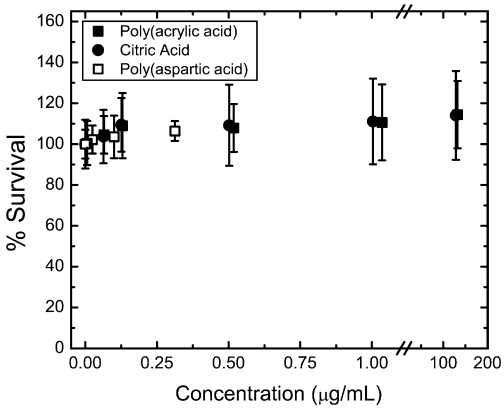

Figure 8. The percent survival of MDA-MB-231 cells incubated for 48 h with ■ poly(acrylic acid), ● citric acid, and □ poly(aspartic acid), which have all been shown to be effective at precipitating resuspendable NP networks. All data shown are the mean of 5 experiments.

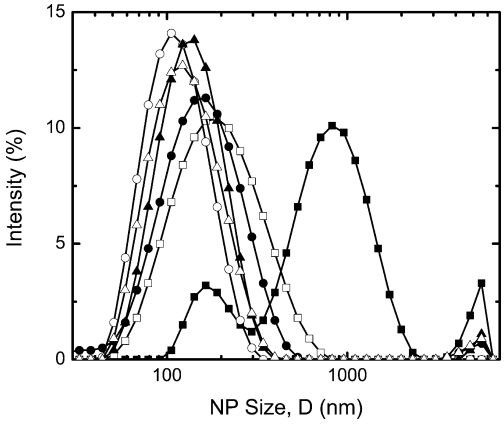

Figure 9. Intensity weighted particle size distributions for redispersed NPs that were dried via various preparations as compared to the initial particle size distribution. Sample preparations: Δ, the initial NP size distribution; ▲, 1 wt % citric acid precipitated; ○, 1 wt % poly(acrylic acid) precipitated; ●, spray freeze-dried with no sucrose; □, freeze-dried with 1:60 NP:sucrose by weight; and ■ rotovap. All samples were redispersed by sonication for 15 min at 55 °C.

excipitents may be chosen based on factors such as biocompatibility and/or cost.

Conclusion

We compare four different methods for concentrating and drying PEG protected polymeric NPs. Rapid drying processes such as spray freeze-drying and freeze-drying give rise to more dispersible particles than slow processes such as rotary evaporation. Particle size distributions for all of the drying methods are shown in Figure 9.

In freeze-drying, adding a cryoprotectant such as sucrose decreases the sizes of the redispersed NPs, but levels of at least 1:10 NP:sucrose had to be added to limit the increase in NP size to below 50% (i.e., 100 to 150 nm), and levels of 60:1 reduced the size increase to less than 10%. The SFD method reduced aggregation to 50% without the addition of sucrose. Redispersion of particles dried by the SFD process also did not require heating to 55 °C, presumably because the rapid freezing process prevented the formation of crystalline, interdigitated PEG domains.

The main contribution of this work is the introduction of hydrogen bonding coacervate precipitation (HBCP) as a novel precipitation technique involving cooperative hydrogen bonding between surface PEG groups on the NP surface and polyacids. We have shown that the coacervates are readily filtered or centrifuged and dried. The hydrogen bonded structure is eliminated by ionizing the acid groups with a pH shift to 7 to redisperse the NPs to within 10% of the original size. Advantages of the HBCP method to concentrate NPs are (i) no need for thermal stress (cold or hot), (ii) ease and speed of the process, (iii) retention of the original the particle size, and (iv) reversible aggregation by a modest pH shift.

The process has been successfully demonstrated using poly(acrylic acid), poly(aspartic acid), and citric acid. We have shown that these are not cytotoxic over the range of concentrations where they would be used in iv drug formulations. For pharmaceutical applications the citric acid would be most desirable since it is ubiquitous *in vivo* and has FDA approval.

While in these studies we have taken the samples to dryness, we have also shown it is possible to use HBCP to concentrate samples for subsequent chemical modification or processing. As shown in Figure 3b, excess solvent may be decanted from the settled flocs and the pH shifted to release the complex. One hundred-fold increases in NP concentration are possible in a single step, and the released polyacid can be removed either by calcium precipitation or dialysis.

Possible extensions of this work include the preparation of dry powder dosage forms from active compounds that are amorphous and "sticky". These materials are often difficult to mix uniformly with other dry ingredients in drug formulations where they wet and agglomerate other components. The glass transition temperature of the PEG-poly(acrylic acid) coacervate can be as high as 95 °C depending on the polymer ratios. The resulting coacervate shell with a high T_g can encapsulate "sticky" low T_g components to allow compounding into dry dosage forms.

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Supporting Information Available: Further details on the morphology of dried powders, including methods and results can be found in the Supplementary Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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