Large scale Manufacturing of Calcium Phosphate Nanoparticles for Medical Applications

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ABSTRACT

Calcium phosphate (CaP) nanoparticles find medical applications in a number of areas, including delivery of nucleic acid therapeutics and calcium-based tissue engineering. CaP is well-studied biocompatible, bioactive, and bioabsorbable material and is used extensively in laboratory settings. However, current methods to produce CaP nanoparticles are laboratory based, inefficient, often require harsh organic solvents, and result in particles that readily agglomerate. Delphi Scientific, LLC has employed a continuous, GMP compatible, process and unique formulations to produce CaP-RNA particles. Batches of uniform particles below 200 nm and as low as 35 nm were produced. The particles were readily filter sterilazable, which is a requirement for many drug delivery applications and were fairly stable for a few days. SAXS analysis revealed particles with a core at about 35 nm and corroborated these findings. The particles were stable for at least 24-48 hrs.

Keywords: calcium phosphate, nanoprecipitation, DNA, RNA, continuous manufacturing.

1 INTRODUCTION

CaP nanoparticles have been successfully used for delivery of nucleic acid therapeutics, antibiotics and anti-inflammatory compounds, in bone regeneration, and as vaccine adjuvants [1-6]. With respect to nucleic acid therapeutics, CaP nanoparticles have been used as carriers of RNA or DNA in cell transfection processes for decades. Additionally, CaP is a biomaterial found in the body, therefore non-toxic. Carefully designed delivery systems may replace current delivery systems such as lipid nanoparticles, polymer conjugates and polyplexes [7-9].

However, CaP particles are known to be unstable and grow over time. Additionally, the current manufacturing methods employ low yield and inefficient processes [4], or use large amounts of flammable organic solvents [3], making them unsuitable for implementation in common pharmaceutical manufacturing environments. For example, reference [4] reports DNA concentrations in the final formulation of about 10 µg/ml, while in many applications the concentration requirement is several times higher.

Additionally, since aseptic processing is a key requirement for injectable/implantable formulations, including those for nucleic acid therapeutics delivery, filter sterilization is typically used to produce a sterile formulation. In particle-based formulations, particle size of 220 nm is the upper limit for filter sterilization, i.e., for the particles to pass through the pores of the membrane used.

Several technologies and methodologies to produce nanomaterials efficiently, and on a large scale, have previously been developed by our group [10-12]. Those included the formation of nano-emulsions [10] and nano-crystalline drugs [11]. These methodologies identify key parameters to (a) determine the significant process operating variables that affect the production of a specific nano-formulation, (b) develop control and scale up strategies that optimize these variables, and (c) design equipment that is suitable for large scale manufacturing. This methodology has been successfully applied to several emulsion formulations. Results indicate that stable nano-emulsions with an average droplet size less than 60 nm can be produced successfully [10]. The energy requirements were reduced by a factor of 5-7 when compared to requirements of conventional methods, while scale-up to several liters per minute was attainable.

The compatibility of RNA therapeutics with high pressure homogenization technology has been demonstrated by Delphi Scientific, LLC in the past, with several formulations. In this current work at Delphi Scientific, LLC, we have employed the principals listed above to produce various formulations based on CaP nano-particles. The objective of this work was to create uniform particles that can carry active pharmaceutical ingredients (APIs) and specifically RNA, increase the process efficiency and yield, and produce formulations that can be filtered sterilized.

To achieve this goal, we carefully selected the ingredients and the relative proportions used in the production of the particles. This was coupled with scalable manufacturing processes that allow for the production of the nanoparticles under conditions spacially and temporary uniform.

2 MATERIALS AND METHODS

CaP nanoparticles were produced via a precipitation reaction of calcium and phosphate salts, by mixing those at at pH ≈ 9. The mole ratio of Ca^{2+} to HPO_4^{2-} ions was 1.3-1.67. Under these conditions the calcium phosphate is expected to be amorphous [6] with slight water solubility.

Sodium phosphate dibasic anhydrous and calcium chloride were used, both from Millipore-Sigma, Burlington, MA, USA. RNA from torula yeast, type VI was used, also from Millipore-Sigma, Burlington, MA, USA. Table 1 shows the parameters of the formulations used in these experiments. NS indicates that no surfactant was used, while wS idicates the use of a biocompatible, proprietary surfactant. The RNA loading varied in the range of 50-250 µg/ml based on the experimental conditions.

High pressure homogenization was used to form the particles. The homogenizer used in these tests is the HP350-30 ShearJet® from DyHydromatics®, LLC, located in Maynard, MA, USA. The homogenizer was equipped with a 75.1T followed by a 200.2L Reaction Chamber®, which are the processing modules, inside which the energy is dissipated. Pressures of 138 MPa (20kpsi) were used in these tests. The flowrates of the homogenizer were about 320 ml/min.

A control batch was manufactured as a reference by mixing the reactant streams in a beaker using a magnetic stirrer. No surfactant was used in this test.

Test	$CaCl_2$ Conc.	Na_2HPO_4 Conc.	Ca^{2+}/HPO_4^{2}	$CaCl_2$/RNA	Surfactant	pH
	mg/ml	mg/ml	mol/mol	g/g	mg/ml	
Cntr.	2.5	2.5	1.30			7.3
1A	5.0	5.0	1.30			7.3
2-NS	2.5	2.5	1.50	4.40		7.3
2-wS	2.5	2.5	1.50	4.40	1.50	7.3
8	1.0	1.0	1.67	3.00	2.88	9
10	2.5	2.5	1.67	3.41	0.96	9

Table 1. Key parameters of formulations tested.

Selected batches were filtered through a 0.22 micron filter to determine if aseptic filtration is feasible. Nylon syringe filtrers from Pall were used in these experiments.

The particle sizes were measured using a Dynamic Light Scattering (DLS) instrument, Model Litesizer 500, from Anton Paar GmbH, Graz, Austria. The same instrument was used to measure the zeta potential of selected tests. The particle sizes were also measured using SEM for comparison. Finally, Small Angle X-ray Scattering (SAXS) was used to determine the structure of the particles and veryfy their sizes. SAXSPoint 2.0 from Anton Paar was used for the analysis.

3 RESULTS

Particle size analysis results using DLS are shown in Table 2. Hydrodynamic Diameter, "Hydro. Dia." as depicted in the Table 2 refers to the average particle size of the distribution, while Polydispersity Index, "PDI" refers to the spread of the distribution. Finally, the "Peaks" and "Areas" refer to the various peaks and respective areas under the peaks of the ditribution. All particle size analysis parameters refer to distributions by intensity.

The particles of the Control sample agglomerated quickly during production and formed fairly stable aggomerates with sizes 7-10 microns (7,000-10,000 nm). Efforts to deagglomerate those using high pressure homogenization were not sucessful, since particle agglomeration took place within seconds, after homogenization.

Sample 1A was produced using similar composition as the Control sample, but with twice the salt concentration in each in the reactant streams and it was homogenized during production. Agglomeration was also evident, but the particle size was smaller than that of the Control, and stayed stable for a longer duration, even though the CaP concentration was twice as much as that of the control.

Sample 2 contained RNA and was processed with and without surfactant. Incorporation of RNA reduced the apparent particle size, as can be seen by comparing tests 1A and 2-NS (No Surfactant). The average particle size of 2-NS was 338 nm as opposed to the particle size of 1A that was 998 nm. However, sample 2-NS also contained large agglomerates with a peak at 10,000 nm. Addition of surfactant reduced the average particle size to 208 nm as can be seen in the 2-wS (with Surfactant) sample. It also shifted the location of the main and the agglomerate peak to smaller particle sizes, 194 nm and 4900 nm, respectivelly. Finally, it reduced the area associated with the agglomerate peak from 8.26% to 2.44% for the 2-NS and 2-wS samples, respectivelly.

Samples 8 and 10 resulted in a unimodal particle size distributions, with average particle sizes of about 160 nm. These samples were stable for at least 24-48 hrs and were readily filterable through a 0.22 micron filter.

Test	Hydro. Dia.	PDI	Peak 1	Area 1	Peak 2	Area 2
	nm	%	nm	%	nm	%
Cntr.	7340	34.2	1665	69.29	10528	30.71
1A	998	40.3	1741	1000		
2-NS	338	24.2	277	91.74	10000	8.26
2-wS	208	14.2	194	97.56	4900	2.44
8	161	10.9	161	100		
8- Filtered	162	9.0	155	100		
10	160	18.4	170	100		
10-Filtered	166	18.7	160	100		

Table 2. Particle size analysis results using DLS.

Figure 1 shows the particle size distribution of Sample 8, superimposed by the particle size distribution of Sample 2-wS for comparison.

Figure 2 is the SEM picture of sample 1A; which shows uniform spheroidal particles with sizes 35-60 nm. Comparison between the DLS measurements and the SEM pictures of sample 1A indicate that while the primary size of the CaP particles is around 50 nm, the particles are highly agglomerated and form agglomerates of about 1000 nm.

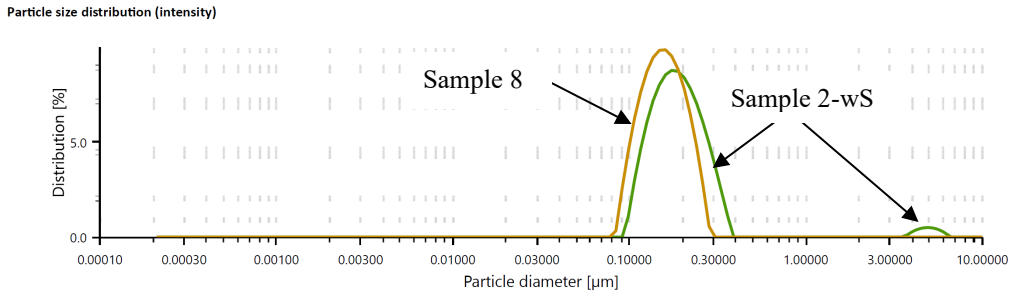

Figure 1. Particle size distributions of Samples 8 and 2-wS.

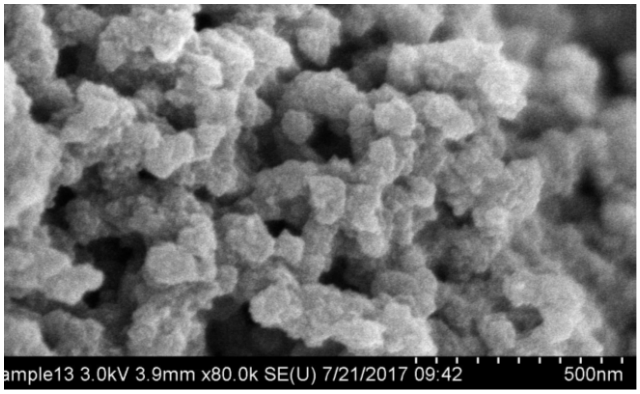

Figure 2. SEM micrograph of calcium phosphate nanoparticles.

Figure 3 shows the signal from the SAXS measurements of Sample 8. The signal indicates that the particles have a core of about 35 nm, surrounded by a halo of at least 150 nm in diameter. The particles appear to be somewhat elongated, as is inferred by the non-symetrical shape of the signal. There is no indication of crystaline structure in the particles.

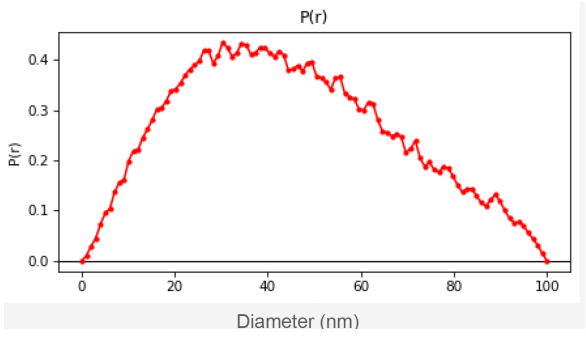

Figure 3. SAXS signal of Sample 8.

Table 3 shows the results from zeta potential measurements of various samples. It can be seen that lack of RNA and surfactant in the Control Sample resulted in a sample with low zeta potential, -4mV, which is consistent with the low stability of the sample. Presence of RNA and surfactant changes the value of zeta potential to (-13.1)-(-19.6) mV, which indicates a more stable formulation.

Test	Zeta Potential (mV)
Cntr.	-4.0
2-wS	-13.1
8	-19.4
10	-19.6

Table 3. Zeta Potential of selected samples.

4 DISCUSSION

Although agglomeration is a known, major problem in CaP nanoparticle based formulations, the combination of RNA, surfactant and high energy processing appears to result in small, uniform and quasi-stable particles. However, once such particles aggomerate, it appears that it is very difficult to deagglomerate and stabilize them properly, even when high energy is used. A better approach would be that stabilization of the particles takes place during their formation.

The analytical techniques employed in this work to characterize the particles gave consistent results.The SEM pictures and SAXS analysis support a model of the CaP particles with a CaP core of about 40-50 nm, surround by a halo, presumably of RNA and surfactant, extending to over 150 nm. The overall particle size is in agreement with the sizes measured by DLS, in which the average size was found to be about 160 nm.

Based on the current production conditions, a lab scale high pressure homogenizer can produce solutions containing at least 10 g of loaded CaP particles per hour, which is enough material for many animal and clinical studies. The concentration of the RNA may be in the range of 50-250 µg/ml, which is within the desirable range for most applications. Additionally, the particles are readily filter sterilizable. Finally, it has been demonstrated in previous studies of this group that our process is compatible with RNA and it does not affect its integrity and efficacy. Based on all these attributes, the process can be used in GMP settings for production of formulations that are used for development or clinical studies. Greater production rates are possible using larger scale homogenizers, which are

readily available. Moving forward, we will concentrate on determining the efficacy of the formulation by performing tranfection studies. We will also work to further stabilize the particles within the formulation and to increase the RNA loading of the formulation.

5 SUMMARRY/CONCLUSIONS

Formulations of calcium phosphate (CaP) nanoparticles loaded with RNA were produced using a high pressure homogenization technique. The RNA concentrations in the formulation were in the range of 50-250 µg/ml and the loaded particles were about 160 nm in diameter. The particles were stable in size for 24-48 hrs, which is much higher than the stability of CaP particles reported in the literature. The formulations were filtered using a 220 nm flter, suitable for aseptic filtration as currently used in the manufacturing of many pharmaceuticals. The production scale using a lab high pressure homogenizer was about 10g of loaded nanoparticles per hour. This scale combined with the ability to filter sterilize the particles, makes the process GMP compatible and appropriate for pharmaceutical manufacturing. Additional work is needed to determine the transfection efficacy of the formulation and improve the stability of the nanoparticles.

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