Plasmid DNA Purification and Formulation for Vaccine Applications

Gareth M. Forde [a,], James Friend [b], Michael Danquah[b], Ying Han [a], Tom Williamson [b], Anthony D. Coomes [a], Fiona K. Giliam [a], and Miranda J. Horsfall [a]*

[a] Bio Engineering Laboratory (BEL), Department of Chemical Engineering, Monash University, Clayton VIC 3800, Melbourne, Australia.

[b] Department of Mechanical Engineering, Monash University, Clayton VIC 3800, Melbourne, Australia.

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* Corresponding author: Tel.: +61 3 9905 1995, Email: gareth.forde@eng.monash.edu.au

Abstract

Plasmid DNA offers the promise of a new generation of pharmaceuticals that will address the often overlooked issue of vaccine production by offering a simple and reproducible method for producing a vaccine. Through reverse engineering, production could be reduced from up to 9 months to as little as 1 month. Simplified development and faster turn-around times means that DNA offers a solution to the vaccine crisis and will help to contain future viral outbreaks by enabling the production of a vaccine against new viral strains in the shortest possible time. Work currently being completed in the area of plasmid DNA production, purification and encapsulation will be presented.

Introduction

According to a WHO 'Epidemic Alert and Response' report from November 2004[1], the present world capacity to produce a pandemic vaccine is woefully inadequate. It states that 'a world adequately prepared for an influenza pandemic would have governments in all countries engaged, according to their ability, in preparedness activities, including vaccine development' and having as much vaccine available as early as possible. A range of ideas were put forward by WHO such as the exchange of clinical trial data, antigen-sparing, increased funding for a pandemic vaccine, encouraging industry, waiving regulatory licensing fees, and tax incentives, but the long-term solution is a lot more complex than this. As the report briefly eludes to, the greatest need of all in addressing a pandemic is vaccine production, which leads to a demand for forward-looking innovations to address production issues. DNA vaccines offers a solution to the current world vaccine crisis.

The bulk of the world's vaccine production comes from a very small number of companies compared to the number of companies producing drugs. How has this occurred? Vaccines have long development and processing times, and are difficult, hence expensive, to produce. Technological issues, stemming from production difficulties, are crippling the vaccine industry and leading to vaccine shortages and financial woes for companies. For example, on the 5th October 2004, the FDA was caught off guard by a shortage of flu vaccine, caused by a contamination in a UK plant of one of only two suppliers of U.S. flu vaccines.

In the case of an influenza pandemic, vaccination will be one of the key interventions. A crucial stage in the control of a pandemic will be to provide as many vaccine doses to the new viral strain in the shortest possible time, however the demand for a pandemic vaccine will significantly surpass the available supply from existing manufacturing capacities. To this end, new production and purification technologies should assist in the rapid production of DNA by using unit operations that enable fast processing, display high time yields and are part of a whole systems approach to reduce the total number of unit operations required. Technologies currently being researched at Monash University for DNA production and purification include monolithic solid phase adsorbents, affinity ligands, and ultrasonic atomization for encapsulation. Exploration and improvement of the constituent parts of the production process then lead to a systematic, integrated, commercially viable solution for the production of DNA vaccines from fermentation to down-stream purification and formulation.

How Was the Production Crisis Permitted to Occur?

The world's vaccine market is very small at approximately 1.6 % of sales by value in the pharmaceutical market[2]. This, in addition to the barriers of entry into the vaccine production market, leads to few suppliers and few producers wishing to break into the market. The traditional barriers of entry include:

- the huge fixed costs of research and development, quality control and assurance, distribution, and construction of production facilities. These reach up to 90 % of the total fixed costs.
- a need for economies of scale and productivity gains, both of which are obtained over time, in order to off-set the high fixed costs; and
- barriers to technology transfer due to the low numbers of vaccine producers and the competitive nature of the industry[3].

The nature of the industry leads to an ugly circle (Figure 1) where any technological or production problems leads to financial issues, hence fewer companies, hence a reduction in know-how, technology transfer and further technological short comings, which compounds the problem. The bottom line: it is hard and is becoming increasingly harder to make a profit out of vaccines. This is a sorry state of affairs when one considers the low cost of a vaccine dose versus the massive health benefits of vaccines.

A simpler to develop vaccine based on DNA that harness identical purification procedures could assist to overcome the traditional barriers of entry to vaccine production by empowering more companies and/or governments to produce their own vaccines rather than relying on a handful of large bio-manufacturers.

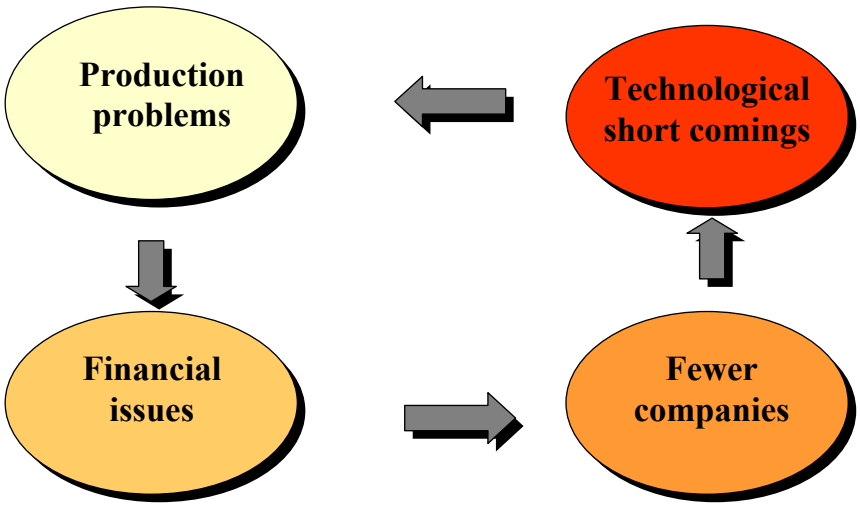

Figure 1: The ugly circle of the vaccine production industry which is partly responsible for the current vaccine production crisis.

Solving the Production Crisis: What does DNA offer?

The current time scale for the vaccine development process from raw ingredient to the vaccine actually being in the surgery fridge can take almost 20 months (according to the UK Vaccine Industry Group). This includes 4-9 months for production, 3 months for quality control, 1-2 months for product finishing, up to 2 months for control agency testing and up to three months for distribution. It has been shown that a DNA vaccine could reduce the initial production period to 1 month, reducing the total process time by up to 8 months, or 40 %. How many lives could this save in the first 8 months of a pandemic?

Current vaccine production methods are based on 50 year old technology, which can confront such production issues such as requiring 100 % inactivation or attenuation of the vaccine and waiting for the delivery of hen's eggs. The often overlooked issue of vaccine production will be solved by the introduction of smarter, faster response purification procedures. DNA has the following advantages over current vaccines (Boyle et al., 1998):

- An excellent safety profile, free from specific safety concerns associated with viruses.

- Generally simpler to develop

- Easier to manufacture than an inactivated pathogen, subcellular fraction or recombinant protein vaccine.

- Different DNA encoding different antigens can be prepared in identical ways, which enables technology transfer and reduced production times; and

- DNA is very stable and resistant to extremes of temperature; thus facilitating the storage, transportation and distribution of DNA-based vaccines to remote areas.

Developments in genomics and structural biology are enabling scientists and engineers to develop new and better purification processes for DNA such as affinity chromatography. Affinity chromatography enables the capture and purification of vastly different types of DNA in one chromatographic step without the co-purification of contaminants such as gDNA, RNA, protein and endotoxins, thus potentially eliminating the need for further purification or polishing stages.

Research Topics and Results

Monolithic Solid Phase Adsorbents

Current biomolecule production methods are generally directed towards the purification of small (less than 10 nm) molecules such as protein. Increasing effort is being made to optimize unit operations for larger biomolecules such as pDNA (over 100 nm). Hence, most commercially available adsorbents have been designed for the purification of relatively small biomolecules rather than the larger pDNA molecules. The use of adsorbents with non-

optimized pore sizes has been shown to greatly reduce the binding capacity of these adsorbents for pDNA. For example, Sepharose™ and Fractogel™ adsorbents exhibit binding capacities for pDNA in the range of 0.2-7.5 % of the binding capacity for protein on a mg/ml adsorbent basis[5, 6, 7, 8, 9]. It has previously been found that the solid support has an effect on the protein affinity adsorption kinetics and protein equilibrium binding capacity[10]. This may be a result of non-covalent interactions between the target and the solid phase (steric hindrance, electrostatic, hydrophobic interactions) and the way in which the ligand is presented to the adsorbate. To further understand the effect of the solid phase adsorbent (pore size, material, adsorbent-ligand-buffer-adsorbate interaction) on the equilibrium binding capacity and elution yield, monoliths will be assembled in-house and comparisons made with commercially available adsorbents[11, 12, 13, 14].

Methacrylate based monolithic columns have been synthesized using varying ratios of ethylene diamethacrylate, butyl methacrylate, and glycidyl methacrylate along with different solvent compositions to produce monoliths with a range of physico-chemical characteristics. To date, these have been analyzed via scanning electron microscopy (see Figures 2 and 3), mercury porosimetry (see Figure 4) and nitrogen porosimetry (Figure 5).

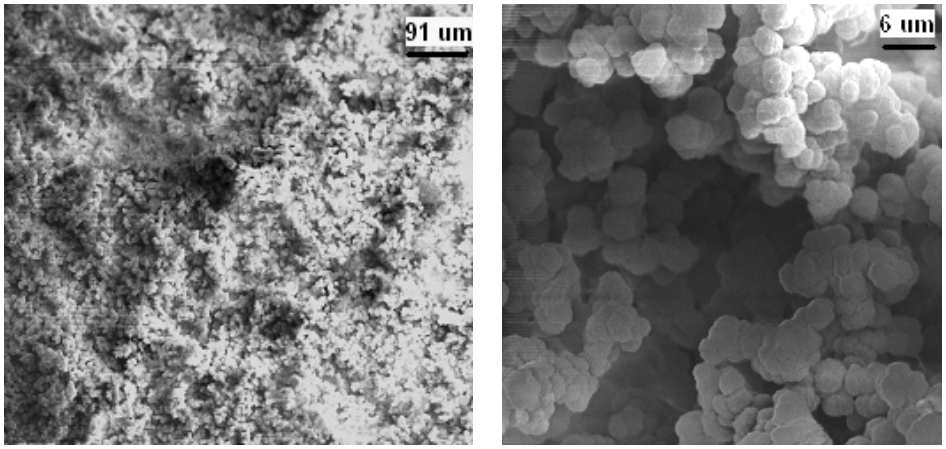

Figure 2: Scanning electron microscope image of monolith solid phase adsorbent prepared from an initial monomer solution of 33 % EDMA, 63 % BMA, 4 % AMPS (v/v).

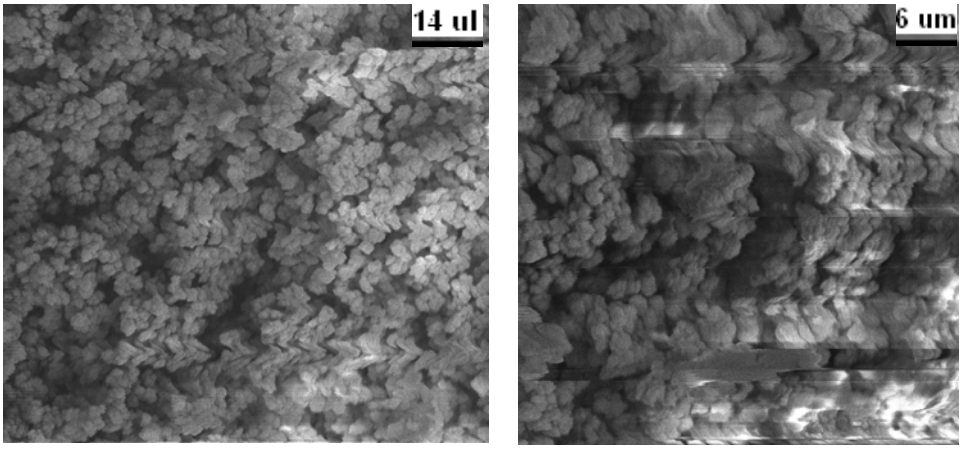

Figure 3: Scanning electron microscope image of monolith solid phase adsorbent prepared from an initial monomer solution of 32 % EDMA, 58 % BMA, 7 % AMPS, 3 % Lys-OMe (v/v).

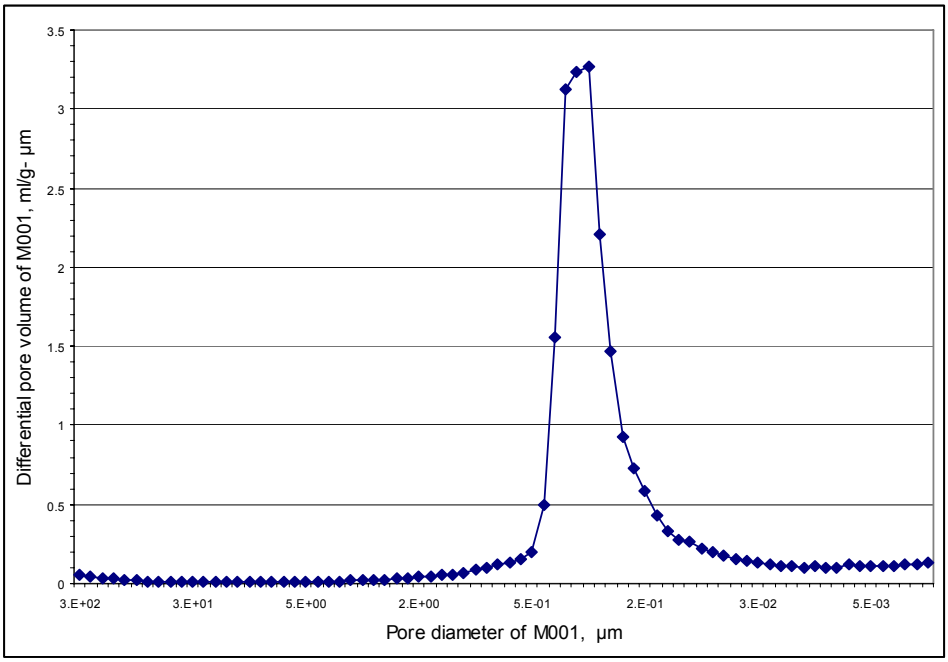

Figure 4: Differential pore volume against pore diameter of monolith composed of 50 % GMA and 50 % EDMA (v/v). The plot shows a predominantly modal pore diameter of 350 nm.

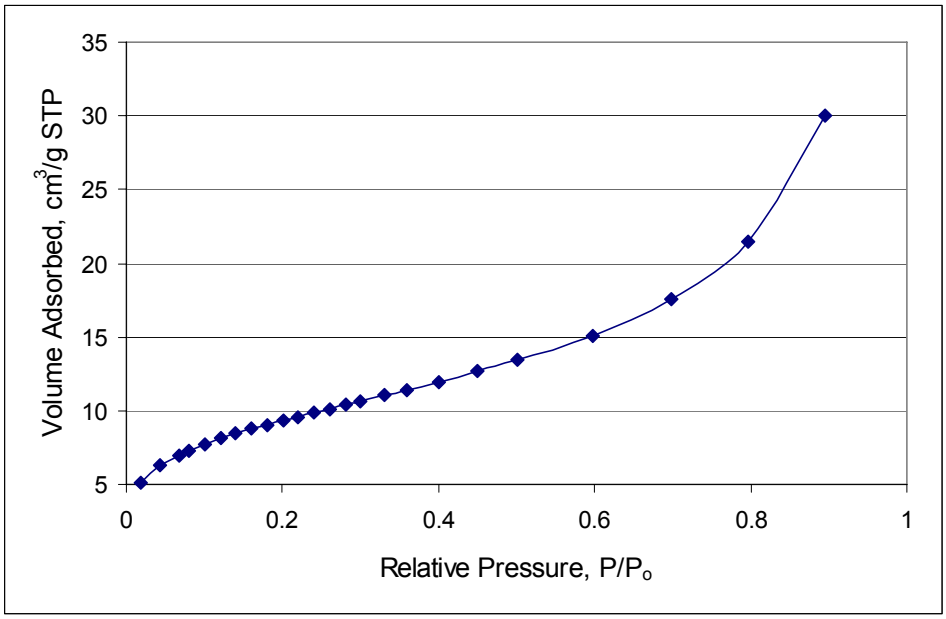

Figure 5: Nitrogen adsorption isotherm (volume adsorbed versus relative pressure) of monolith composed of 50 % GMA and 50 % EDMA (v/v). The BET surface area was calculated to be 19 m^2/g.

Affinity Ligands

The contaminants that pose a particular problem in the production of purified pDNA are anionic polymers of a similar structure, charge and physical behavior to pDNA. These contaminating anionic polymers include genomic DNA, RNA and lipopolysaccharides. Affinity chromatography is a highly specific chromatographic technique that utilizes ligands based on biological systems to obtain highly purified plasmid DNA in one unit operation. By the use of affinity ligands and solid phase adsorbent design, the stages of capture, concentration, and purification can be performed in a single unit operation. Approaches to the development of affinity ligands will be presented and results from scaleable unit operations employing affinity ligands will be shown.

Improving Delivery: A Novel Encapsulation Approach

The aim of this body of work is to develop a particle for drug delivery that is physiologically acceptable, made of a polymer that biodegrades into non-harmful products such as natural metabolites, or materials that can be cleared readily from the body on a time scale suitable for it's proposed use, and utilizes chemical and/or biological approaches to harness the cellular transport mechanism in order to obtain increased transfection efficiencies.

Particles for the delivery of drugs via the nasal route should have a mass median diameter between 10 to 20 μm in order to increase nasal deposition and minimise deposition in the lungs and gastro-intestinal tract[15]. Increased pulmonary deposition occurs below this range which attributes to reduced naso-pharyngeal deposition. However, Vila et al. (2005)[16] found that ca. 200 nm PLA-PEG particles were able to most efficiently transport a tetanus toxoid through the nasal mucosa of rats, ahead of free toxid and toxid encapsulated in 1, 5 and 10 μm particles.

It appears that although there is an optimal physical size of particles for nasal delivery, smaller particles are more efficient at transporting drugs through the nasal mucosa. Hence, it is proposed that an optimal particle for delivery of drugs (such as pDNA) via the nasal route will be 10 to 20 μm agglomerations (to maximise nasal deposition) of smaller particles that are around 200 nm or less in diameter. To this end, a novel strategy was developed that uses ultrasonic sound to produce nanosized (sub 200 nm) particles, then by altering the processing parameters, can also produce particles that of 10 – 20 μm.

The literature shows that the frequency of the atomizing ultrasonic sound affects the diameter of the particles that are produced[17]. A polymeric solution was prepared by dissolving poly-ε-caprolactone (PCL, M_W 65 000, Sigma-Aldrich) in acetone (99.5 %, LabScan) to create a solution of 0.5 % PCL w/v. Under a range of operating parameters (as outlined in Table 1) the polymer solution feedstock was pumped onto the surface of an inverted ultrasonic atomizing diaphragm run at a piezoelectric frequency of 1.645 MHz and atomized into a hardening agent of sodium dodecyl sulfate (SDS, Sigma-Aldrich) in water. Unless otherwise stated, the conditions of 0.56 m/s vibration velocity, 28 ml/min flow rate, 8 cm atomizer height, and SDS concentration of 1 mM was used. The nanoparticle containing hardening agent was centrifuged at 2500 rpm for 10 minutes in a swinging bucket rotor to remove any large agglomerates that may have formed, due predominantly to accumulation of the hydrophobic polymer at the air-liquid interface. The average (Z-average) particle size of each sample is the diameter of the sphere that diffuses at the same speed as the particle being measured. The particle size was determined using a Zetasizer (Malvern Instruments Ltd., UK) which employed Dispersion Technology Software 4.10b1. The results for the nanoparticle size analysis obtained using the Zetasizer are presented in Table 1.

Table I: Results for the ultrasonic atomization of 0.5 % poly-ε-caprolactone in acetone under a range of process parameters. Feed stocks were pumped at a specified flow rate (20, 28, 36 ml/h) onto the surface of an inverted ultrasonic atomizing diaphragm run at a vibration velocity on the atomization surface of 0.48, 0.56 or 0.64 m/s (as measured using a laser Doppler vibrometer) at a frequency of 1.645 MHz into a hardening agent of SDS (1mM, 10 mM or 100 mM) in water from an atomizing surface height of 8, 12, or 16 cm.

Parameter	Run	Z-Average Particle Diameter	Standard Deviation	Number of runs
Vibration velocity	1. 0.48 m/s	208	5.7	6
	2. 0.56 m/s	186	5.7	6
	3. 0.64 m/s	5641	6474.4	6
Flow rate	4. 20 ml/hr	187	13.9	6
	5. 28 ml/hr	186	5.7	6
	6. 36 ml/hr	181	6.5	6
Atomizer height	7. 8 cm	186	8.4	3
	8. 12 cm	202	3.5	3
	9. 16 cm	215	4.6	3
Detergent concentration	10. 1 mM	186	5.7	6
	11. 10 mM	226	21.4	6
	12. 100 mM	3467	3692.7	6

The particle size frequency distribution on a volume basis of the conditions which resulted in the smallest Z-average particle size (Run 6, 181 nm) is shown in Figure 6, which displayed a monodispersed, symmetrical frequency distribution (post-centrifugation). A small amount of larger agglomerations (ca. 500 – 700 nm) which were not removed by the centrifugation stage are visible in the figure.

Analysis of variance (ANOVA) studies were performed for each of the process parameters of vibration velocity, flow rate, atomizer height, and detergent concentration in the hardening agent. The results of the ANOVA analysis show that, for the conditions listed in Table I, the parameters of vibration velocity, atomizing surface height, and surfactant concentration in the hardening agent have statistically significant effects on the Z-average particle diameter of the PCL nanoparticles (p = 0.05). Variations in the flow rate did not have a statistically significant effect on the Z-average particle diameter (p = 0.05). Hence, the optimal conditions for obtaining a sub-200 nm particles are: 0.56 m/s vibration velocity, a hardening agent containing 1mM SDS in water from an atomizing surface height of 8 cm. For the flow rates shown in Table 1, there was no statistically significant effect on the particle diameter, however the flow rates of 28 ml/hr and 36 ml/hr showed a much smaller standard deviation in the Z-average particle diameter, hence these flow rates will be employed in future work.

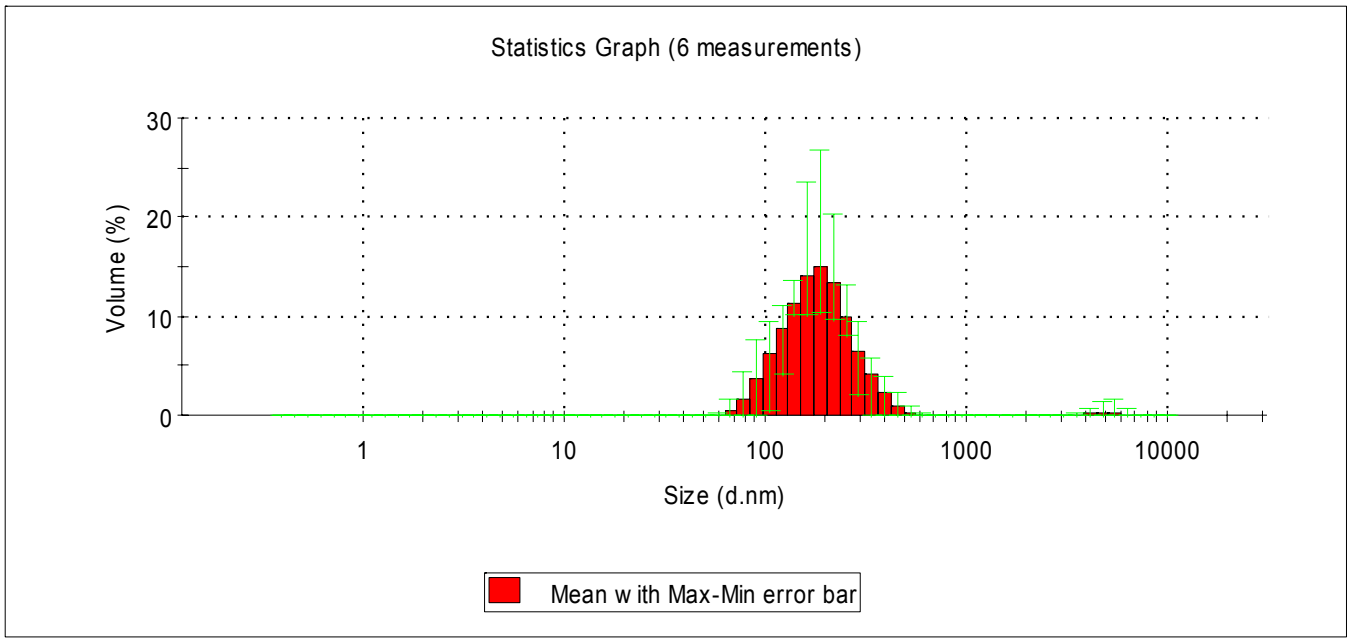

Figure 6: Particle size frequency distribution for atomization of 0.5 % w/v PCL in acetone pumped at a flow rate of 36 ml/h onto the surface of an inverted ultrasonic atomizing diaphragm run at a vibration velocity of 0.56 m/s and a frequency of 1.645 MHz into a hardening agent of 1mM SDS in water from an atomizing surface height of 8 cm.

In order to create the larger 10 – 20 μm particles, a 40 kHz atomization system was used with a 6 mm full wave atomization probe. The effect of solids percent, feed flow rate, volumetric ratio of the polymer stock to the protein stock, and protein concentration in the protein stock on particle size characteristics were determined. It was shown that feed stocks containing 100 parts of 0.5 or 1.0 % w/v PCL in acetone with 1 part 100 mg/ml BSA and 15 mg/ml PVA produced particles with a mass moment diameter (D[4,3]) of 13.17 μm and 9.10 μm respectively in addition to displaying high protein encapsulation efficiencies of 93 and 95 % respectively.

The next body of work shall involve how to incorporate these two different operating conditions (high frequency atomization at 1.645 MHz and low frequency atomization at 0.040 MHz) in order to create a 10 – 20 μm agglomeration of nanoparticle in order to maximize the delivery of drugs via the nasal mucosa.

The Future of Plasmid DNA Vaccines

It is likely that the most immediate use for pDNA will be in specialist, high value vaccine applications as displayed by the West Nile Virus pDNA vaccine for condors[18] and for the immunization of domestic pets. Over time, the body of evidence supporting the use of pDNA as a vaccine will most likely lead to the acceptance of pDNA for time critical applications such a developing an influenza vaccine for high risk groups.

Wider use of DNA as a pharmaceutical in other applications such as gene therapy and research will lead to processing advances and technology transfer which will bring the cost per dose of a DNA vaccine down to levels that are economically competitive. Such advances may include the development of a process to create much shorter fragments of DNA that elicit the same response as the much larger pDNA, leading to higher transformation efficiencies and better time yields from processing equipment. At this point in time, the barriers of entry into the vaccine development market will hopefully be broken down and a wider range of countries will be able to produce their own vaccines, rather than the world needing to rely on a small number of producers. For this to happen, it becomes painfully clear that research centers, public health institutions, governments and vaccine manufacturers throughout the world and particularly in countries that have strengths in vaccine production, will need to collaborate in taking action towards solving the current vaccine production problem in order to confront the looming pandemic. The countries and areas named by WHO in January of this year as having manufacturing capacity for influenza vaccines were Australia, Europe, Japan and North America. It is up to the countries with strengths in vaccine production to lead the way in developing forward-looking innovations to address woefully inadequate production capacities.

The massive capabilities that DNA has as a drug may lead to a new generation of personalized medicine. In the future, cost, time, size and energy efficient devices for producing high purity, encapsulated DNA may eventually lead to GPs being able to diagnose genetic based diseases then being able to prescribe a personalized, DNA based medicine in almost the same time that current medicines can be obtained.

Conclusion

Plasmid DNA offers the promise of a new generation of pharmaceuticals that will address the often overlooked issue of vaccine production by offering a simple and reproducible method for producing a vaccine. Through reverse engineering, production could be reduced from up to 9 months to as little as 1 month. Simplified development and faster turn-around times means that DNA offers a solution to the vaccine crisis and will help to contain future viral outbreaks as a vaccine against new viral strains will be able to be prepared in the shortest possible time. Research into both purification and improving transformation efficiencies will decrease the cost per dose of DNA medicines and may lead to DNA becoming a financially viable pharmaceutical option. Groups with strengths in vaccine production need to play an active role in the development of new technologies, such as pDNA, to safeguard global human health against current and future viral pandemics.

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