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# OBSERVATIONS ON THE IMPACT OF AEROSOLIZATION ON MACROMOLECULAR THERAPEUTICS

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**ABSTRACT**
Aerosol therapeutics are becoming a practical option for treating chronic lung diseases and illness. This is largely due to ease of delivery of therapeutics to the lungs. The type of therapeutics required can range from small molecules to large macromolecules (such as DNA and proteins). The current trend is toward using macromolecules for use in gene and protein delivery. As for techniques, there are many types of atomization devices currently used for aersolizing therapeutics. However, not all the methods are equal in their ability to delivery structurally active molecules. Our interests focused in finding the correct atomization method for any type of therapeutic molecule. We have explored the effects of many types of nebulizers and other atomization devices on the molecules DNA, RNA, and proteins. From these findings, we recommend that electro hydrodynamic atomizers as the best method for the atomization for the entire range of macromolecular therapeutics. Additionally, we have found that single pass devices perform better than multi pass systems and altering the turbulent length scale or macromolecule hydrodynamic size can mitigate stability issues. We hypothesize that protein stability could be evaluated using melting temperatures from DSC measurements. Finally, we observed that siRNA appear to be stable regardless of aerosolization condition.

## INTRODUCTION

Aerosol therapeutics are becoming a practical option for treating chronic lung diseases and illnesses as well as vaccines for infectious diseases. This is largely due to ease of delivery of therapeutics to the lungs. Macromolecular delivery to the lung is especially intriguing as it circumvents first pass metabolism issues. Macromolecular vaccine options range from small interfering RNA (siRNA) to proteins to large plasmid DNA. The literature indicates a wide range of aerosolization methods are being employed to deliver these therapeutics to the lung with mixed results. We hypothesis much of the variability observed in the literature results from molecular degradation during the aerosolization process. Our objective is to develop recommendations for macromolecule – aerosolization device pairings to ensure structural stability.

## MATRIALS AND METHODS

### Atomization Devices

The mechanism of atomization for aerosol devices ranges from pressurized gas to ultrasound to high voltage. Physicians most commonly prescribed jet nebulizer, ultrasonic nebulizer, meter dose inhaler (MDI), and with more frequency a vibrating mesh nebulizer. The jet nebulizer uses pressurized gas to break columns of liquid into drops that are fractured further into smaller drops upon impaction on a baffle. The ultrasonic nebulizer generates standing pressure waves within a fluid reservoir to eject droplets from the pool surface. An MDI uses a large pressure difference across a small circular orifice to generate a polydisperse spray plume. A vibrating mesh nebulizer takes a very fine mesh and pushes fluid through it to produce droplets. Finally, the electrohydrodynamic (EHD) atomizer is a new comer to the use in pharmaceutical, but it has been successful applied in industries such as combustion engines and inkjet printers. EHD atomizers apply large enough voltages to a fluid in order to over come surface tension and form monodispersed drops (Figure 1).

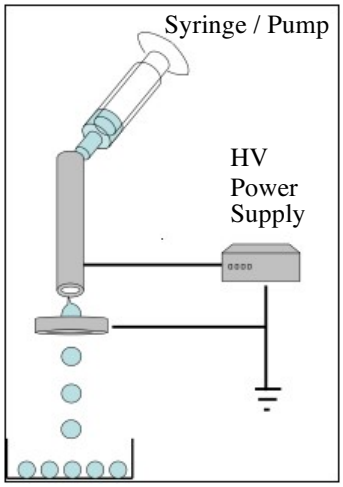


Figure 1 - Diagram of the Electrohydrodynamic

Numerous mechanical and chemical stresses (e.g. acid-catalyzed hydrolysis, enzymatic degradation by DNases, oxidation by free radicals or molecular oxygen, temperature effects due to evaporation, surface tension effects, and hydrodynamic shear stress) are known to lead to macromolecular degradation. We investigated the presence and importance of each of these mechanisms to the different atomization techniques.

### Macromolecules

There have been tremendous research efforts in the area of macromolecular therapeutics. Disease causing genes have been discovered as well as missing or misshapen proteins that cause illness. In addition, the newest area of RNA research, siRNA, shows great promise for use as a therapeutic. In order

to fully understand the aerosolization of therapeutics, all three types of molecules were explored. Many configurations and sizes exist for DNA, the DNA used in the experiments ranged from large cosmids (37 kbp) to plasmids (9 and 5.9 kbp) to small, linear genomic DNA. The structural integrity of the DNA was observed by gel electrophoresis. Not only do proteins have many configurations and sizes like DNA, they also have different jobs with inherently different stability. Two different proteins were picked with opposite features in size and stability. Chymotrypsin (purchased from Sigma), an enzyme of the stomach, is a very stable protein with a size of 25 kDa, a melting temperature of 77°C, an isoelectric point from 8.3-8.8, and is 46% hydrophobic (equation obtained from [1]). Citrate synthase (purchased from Sigma), an enzyme important in the citric acid cycle, is a less stable protein with a size of 52 kDa, a melting temperature of 48°C, an isoelectric point from 6.1-6.6, and is 50% hydrophobic. Enzymatic assays were used to determine the structural stability and activity of each of the proteins during atomization. The RNA used was a double stranded siRNA of 22 base pairs (purchased from Transgenomics). The structure of the siRNA was monitored with a fluorescent dye (Pico Green) that binds more tightly with double stranded RNA than single stranded.

## RESULTS

*DNA* - Plasmid DNA processed though the jet nebulizer was found to structurally break into linear pieces. Of all the possible degradation methods, hydrodynamic shear stress caused by the resulting turbulent length scales was the most significant factor. Investigations, in our lab, on the affect of turbulence on macromolecular structural integrity using small diameter pipes demonstrated that when the Kolmogorov length scale is below the molecular length molecular degradation occurs rapidly (figure 2). Combining this effective fragmentation process with the multi pass nature of the device, the jet nebulizer preformed the worst but is the most often used in literature. We found that a small change in formulation, which also frequently improves transfection, can diminish degradation rates by complexing the DNA with a cationic agent (such as PEI or PLL). By complexing the DNA with such an agent, the resulting molecule is condensed by a factor of at least 10 times smaller its original size placing it below the size of the turbulent length scale of the nebulizer.

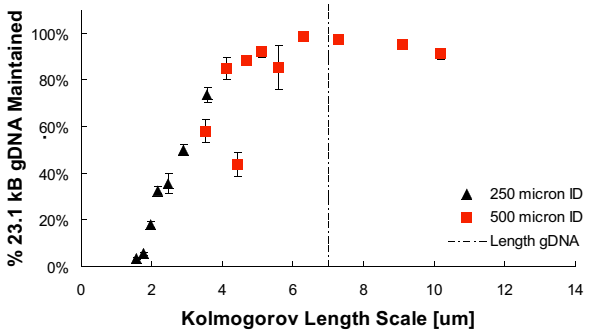


Figure 2 - Kolmogorov Length Scale. This graph shows that a turbulent length of of 7 microns or above is need to retain DNA structure.

In the ultrasonic nebulizer, we found that DNA enhanced transient cavitation more specifically cavitation increases proportionally with increasing size of the DNA. Upon the collapse of bubbles formed by transient cavitation, shock waves are created that yield areas of extreme temperature and pressure. These areas lead to the formation of hydroxyl radicals that are known to damage and break DNA.

In addition, two other atomization methods, the vibrating mesh nebulizer, and electrohydrodynamic atomizer, were tested with DNA. We found the turbulent length scales in the vibrating mesh to be an issue (Figure 2). Even though the orifices should promote laminar flow they are dominated by entrance effect where turbulence is sustained.

It was found that only the EHD atomizer was capable of processing all the DNA sizes without any significant lose in the DNA structure. The EHD atomizers proved to be the best choice for atomizing DNA while the jet nebulizer proved to be one of the worst (Figure 3).

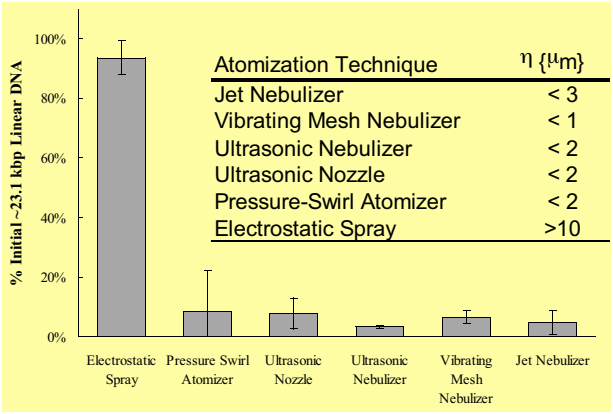


Figure 3 - Percent of DNA structure maintained after atomization in different devices. The inset table shows the smallest turbulent length for each device.

*Protein* - Referring to the results of the DNA experiments, only the best (EHD atomizer) and worst (jet nebulizer) atomizers were used for the protein experiments. The results from the protein experiments were not as clear-cut as the

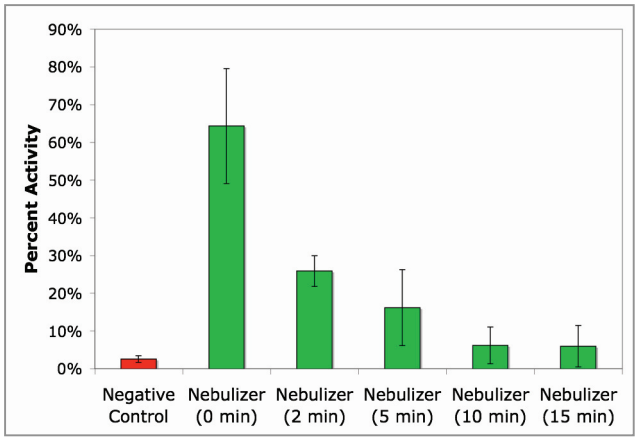


Figure 4 - Percent activity of citrate synthase during nebulization. As resonance time in the nebulizer increases the activity of the protein decreases.

DNA results. The chymotrypsin protein remained 100% active in both the jet nebulizer and EHD atomizer when compared to the non-atomized protein. However the same

| Protein | Size kDa | PI | Type | pH in Study | Tm °C | Conc. mg/ml | % Hydro | Results |
|---|---|---|---|---|---|---|---|---|
| Chymo-trypsin | 25 | 8.3, 8.8 | enzyme | 7.0 | 77 | 1 | 46% | 100% |
| Insulin (Bovine) | 5.7 | 5.3, 5.7 | hormone | 3-3.35 | 70 | 10 | 57% | 100% |
| BSA | 66 | 4.7-4.9 | globular | 7 | 62 | 5 | 47% | Fair |
| | | | | | | 20 | | Good |
| | | | | | | 50 | | Didn't work |
| Candida Cylindracea Lipase | 67 | 4.5-5.7 | enzyme | 7.5 | 58 | 0.1 | 53% | 42% – 93% |
| Citrate Synthase | 52 | 6.1-6.6 | enzyme | 4.7 | 48 | 0.001 | 50% | < 20% |
| | | | | | | 0.5 | 50% | ~ 98% |

Table 1 - Protein properties and the final activity after atomization. The correlating parameters appear to be concentration and melting temperature.

was not true for the citrate synthase. The citrate synthase retained only 20% of its activity during EHD atomization. When processed with the jet nebulizer, the activity of the protein decreased (Figure 4) with increasing resonance time in the nebulizer's reservoir (dropping from 60% a time zero to 0% at 15 minutes). It was unclear as to why the jet nebulizer's zero time point began at 60%. To understand this, a thin film assay was attempted. A small drop of the protein solution was placed on a slide sitting at room temperature for 5 and 10 minutes, then the solution was assayed for protein activity. The resulting activity of the thin film assay was 60%. Therefore, the citrate synthase protein is so unstable that it denatures sitting out upon the bench top. Correlating these finding with other published atomization studies [2-4], a trend relating protein properties to atomization stability was found (Table 1). It appears that the protein's melting temperature and its concentration in solution are good indicators of how well the protein can be processed in atomization devices. Further work on this topic is exploring the relationship between surface or Gibbs Free Energy and the enthalpy of melting of the protein.

*siRNA* - The experiments concerning siRNA only used the jet nebulizer as the sole method of atomization. It was assumed that because siRNA is so small it would have no problem being processed through the EHD atomizer. The siRNA was subjected to atomization for 15 minutes in the jet nebulizer. No structural degradation could be found, which is consistent with previous results.

Our on going work concentrates on spraying atomized macromolecules directly onto living cells. Preliminary results using Epiairway epithelial cells show that EHD atomized macromolecules do not harm the cells. This observation was made by monitoring the cells for lactose dehydrogenase (cellular toxicity – Figure 5) and IL-8 (inflammation). It appears that the cells no release of LDH and very little IL-8.

## CONCLUSIONS

From these findings, we recommend that EHD atomizers as the best method for the atomization for the entire range of

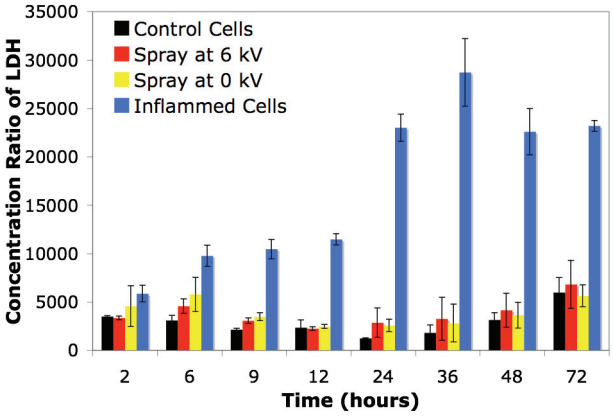


Figure 5 - Release of extracellular LDH from lungs cells after applicatino of electrosprayed DNA.

macromolecular therapeutics. However, single pass devices are superior to multi pass systems and altering the turbulent length scale or macromolecule hydrodynamic size can mitigate stability issues. Proteins should be evaluated using melting temperatures – if available and siRNA appear to be stable regardless of aerosolization condition.

## REFERENCES

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