An Electrospray Solution to the Pulmonary Delivery Problem

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Glossary

Name	Comment
Antibody	A blood protein produced in response to and counteracting a specific antigen. Antibodies combine chemically with substances that the body recognizes as alien, such as bacteria, viruses, and foreign substances in the blood.
CF	Cystic Fibrosis
Comminution	To reduce to fine particles
COPD	Chronic Obstructive Pulmonary Disease
DNA	Deoxyribonucleic acid, a self-replicating material present in nearly all living organisms as the main constituent of chromosomes. It is the carrier of genetic information
E-cadherin	E-cadherins (named for "calcium-dependent adhesion") are a class of type-1 transmembrane proteins that adhere to epithelial cells. They play a role in cell adhesion, ensuring that cells within tissues are bound together.
Ex vivo	Taking place in a tissue taken from or out of the living organism
GFP	Green Fluorescent Protein
In Vitro	Taking place in a test tube, culture dish, or elsewhere outside a living organism
In Vivo	Taking place in a living organism.
Lipid	Any of a class of organic compounds that are fatty acids or their derivatives and are insoluble in water but soluble in organic solvents. They include many natural oils, waxes, and steroids.
Methylene Blue	A dye used in cancer diagnosis
PBS	Phosphate buffered saline is a buffer solution commonly used in biological research. It is isotonic and non-toxic to cells
PDT	Photodynamic Therapy; a compound is introduced systemically and accumulates in a tumour. The drug is activated by light and becomes toxic to cancer cells locally
Protein	Any of a class of nitrogenous organic compounds that consist of large molecules composed of one or more long chains of amino acids and are an essential part of all living organisms, esp. as structural components of body tissues such as muscle, hair, collagen, etc., and as enzymes and antibodies.
Rabbit-Anti Mouse	Antibody used to identify and locate intracellular and extracellular proteins and cancer research
RNA	Ribonucleic acid, a nucleic acid present in all living cells. Its principal role is to act as a messenger carrying instructions from DNA for controlling the synthesis of proteins, although in some viruses RNA rather than DNA carries the genetic information.
siRNA	Small interfering ribonucleic acid; involved in the RNA interference (RNAi) pathway, where it interferes with the expression of a specific gene
TE Buffer	TE (TRIS EDTA) buffer is used to protect DNA or RNA from degradation
TRIS	Tris(hydroxymethyl)aminomethane, A buffer, pH range (7-9) coincides with the typical physiological pH of most living organisms
Virus	An infective agent that typically consists of a nucleic acid molecule in a protein coat, is too small to be seen by light microscopy, and is able to multiply only within the living cells of a host

Nomenclature

Symbol	Parameter
h	Capillary to plane height
k	Thickness of moving liquid layer
E_θ	Electric field normal to cone surface
E_r	Electric field along cone surface
σ	Liquid conductivity
I	Atomization Current
Δp	Internal pressure in the cone
F	Liquid flow rate
q	Charge density at the surface
R	Radial distance from apex of cone
R_c	Radial distance to capillary edge
R_f	Radial distance to ligament edge
r_c	Radius of capillary
T	Surface tension of liquid
V	Electric Potential
V_0	Electrospray onset potential
ρ	Liquid Density
ε_0	Permittivity of free space
ε	Liquid permittivity
θ	Angle from cone apex
θ_0	Cone half angle
n	Unit normal to elemental volume in radial direction
r_0	The radius of a fluid colloid

Abstract

This thesis addresses the critically poor efficacy of existing strategies to treat lung disease. A new device is developed using electrospray technology to deliver molecules into respiratory tissues via a bronchoscope. Novel diagnostic and therapeutic interventions are now possible by delivering appropriate molecules to target sites. The impact of this thesis is to facilitate earlier diagnosis of lung disease and enable local delivery of therapeutics to the lungs.
Delivering molecules into the lung and airway tissues is challenging. The methodology is to design and implement an electrospray device for delivery of molecules to the respiratory tissues and to analyse its performance relative to existing delivery methodologies.

A critical analysis of current electrospray methodologies of introducing molecules into tissues is presented. An electrospray device was fabricated for bronchoscopic clinical use based on a set of working parameters deduced from a rig and trialled in a large animal model.

Molecules including DNA, siRNA, proteins, chemotherapeutics and marker dyes were delivered to tissues. The device's performance was analysed for consistency, targeted delivery, safety and efficacy. DNA and siRNA were delivered to human epithelial cell lines with up to 90% transfection. Proteins were delivered. Cisplatin, a chemotherapeutic, was delivered to cancer cells in a controlled experiment and killed >90% the cancer cells targeted. Methylene blue, the basis of an early stage cancer diagnostic test, was delivered to pigs, in vivo, using the device.

It is concluded that an electrospray device will have a role in lung cancer diagnosis and therapy. The research outputs will ultimately be applicable to other disease types and to a wide range of therapeutic and diagnostic molecules.

Chapter 1

Introduction

This thesis endeavours to address lung disease through the design, development and testing of a novel device for delivery of diagnostic and therapeutic molecules to the lungs. The device uses the phenomenon of electrospray, a form of electrical liquid atomisation. Electrospray might be described as a solution in search of a problem since it was observed and described by Zeleny (1917). In the ensuing decades, engineers have applied electrospray to activities as diverse as crop spraying and mass spectrometry (Fenn 2002) whilst scientists have refined numerical models describing the phenomena (Wilm and Mann 1994). Recently, the analytical treatment of electrospray has facilitated an increasing number of drug delivery and biological applications (Coffey 1999; Sahoo, Lee et al. 2010).

Lung disease is a major global problem. Pneumonia, COPD (chronic obstructive pulmonary disease), tuberculosis and lung cancer rank among the ten leading causes of death worldwide. Asthma is the most common chronic disease affecting children (Lodderkemper 2003). The lungs and airways are uniquely challenging to diagnose and treat. Diagnosis and treatment frequently takes the form of delivery of genes, dyes, chemotherapeutics and other agents collectively known as molecules to the lungs. The mechanism by which these agents enter the lung cells to diagnose or treat them is called the vector. It is generally accepted that for clinical application, current vectors have not worked well and better vectors are needed (Davies 2005; Lu, Xie et al. 2005). Among the main challenges are navigating the airway and lung geometry, lung humidity, lung clearance mechanisms and the presence of lung disease (Labiris and Dolovich 2003a). In this work, the challenge of accessing the disease site and delivering appropriate therapeutics locally is entitled the pulmonary delivery problem. The principal objective of this work is to design, implement and trial a new

device capable of addressing this problem (Desai, Tai et al. 1997). The research progression is presented in Figure 1-1.

In Section 1.1 the physiological background to lung disease is reviewed and the pulmonary delivery problem is introduced. In Section 1.2 electrospray is introduced. In Section 1.3 the pulmonary delivery problem and electrospray are drawn together by examining electrospray as a potential solution. The significance of realising such a solution is discussed. Section 1.4 sets out the contributions of this thesis. The chapter concludes with an outline of the thesis in Section 1.5.

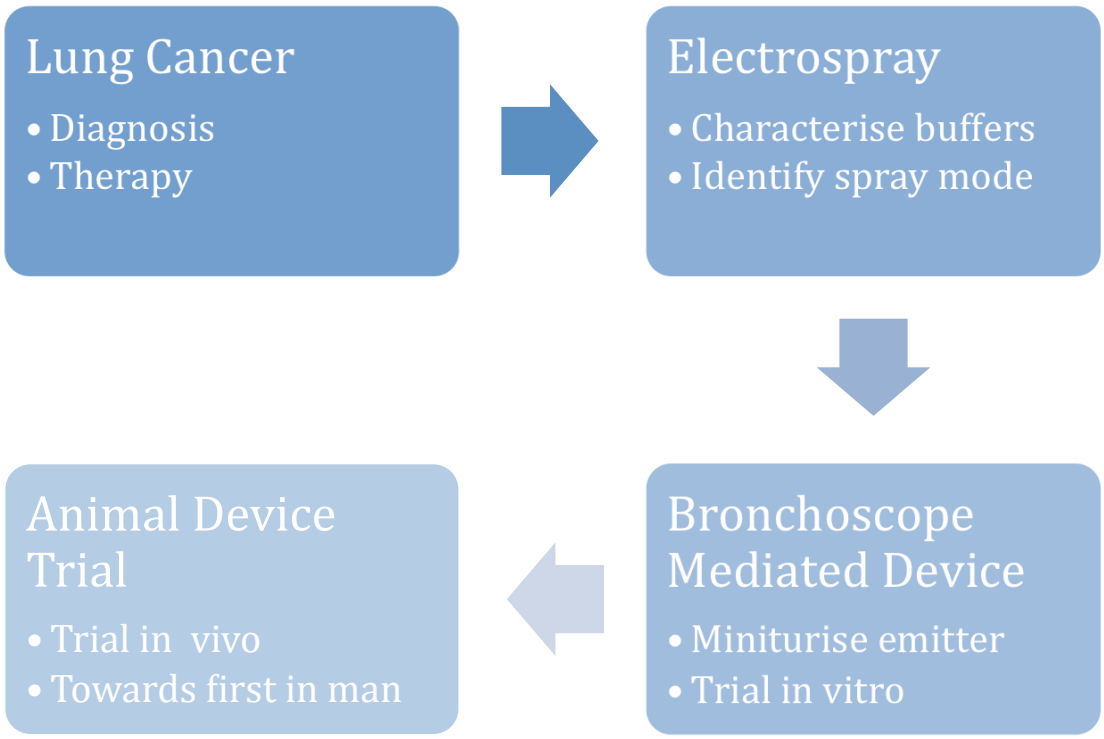


Figure 1-1 Research progression

1.1 Motivation for addressing the Pulmonary Delivery Problem

Lung disease has been a poor relation to positive developments elsewhere in medicine. Within the major disease groups in lung medicine; lung cancer, chronic obstructive pulmonary disease (COPD), tuberculosis and asthma, patients' prognosis has improved little. Lung cancer remains the leading cause of cancer death in the western world. Advances in the treatment of breast, cervical, and prostate cancer have led to improved survival rates, whereas mortality from lung cancer has remained largely unchanged (Birring and Peake 2005). New chemotherapeutics and gene therapy hold substantial promise for improvements in patient care in these areas, however, delivery of treatments remains a significant problem. The focus of this work is on a novel method of delivering chemotherapeutics, genes and dyes to lung cells towards diagnosing and treating lung cancer.

1.1.1 Lung cancer research

Lung cancer research is carried on in many centres globally and might be described as a continuum from basic research in the laboratory to applied research conducted within hospitals. Much of the work for this thesis has been carried out in the Epithelial Immunology laboratory in NUI Maynooth with Dr. Shirley O'Dea who has extensive experience in the lung epithelium and the signalling networks that influence its functions in health and disease. In basic research, cell lines are cultured and used in vitro. In vitro refers to work carried out on the bench, from the Latin word *in vitro* or *in glass*. In the applied research setting, clinical research attempts to validate candidate drugs and devices in clinical trials. This type of work is referred to as *in vivo* or *in the living organism*. In vivo work also refers to work on animals in the laboratory setting. *Ex vivo* or *from the living organism* work occurs in recently harvested organs, usually of animal models. Animal models are animals used in research for a particular defined research purpose. These may be small animal models such as mouse or rat. Large animal models such as sheep or pig are a good analogue for the human lung in respiratory research. Similarities in size and structure between sheep, pig and human lungs allow testing of new treatments using the same equipment and procedures used in human medicine, thereby facilitating translation of findings into the clinical setting (Meeusen, Snibson et al. 2010). These animals are used under tightly controlled ethical and regulatory licence.

1.1.2 Diagnosis of lung disease

The key factors in poor outcomes from lung cancer are late diagnosis and patient history. Despite the many diagnostic tools available to the physician, late presentation of the patient will limit the therapeutic options and survival rate. The diagnostic tools available to the physician include x-ray, sputum cytometry, spirometry, and bronchoscopy (Hirsch, Franklin et al. 2001). Recently, advanced imaging techniques have been added to bronchoscopes manufactured by Olympus, however, these are very expensive and their clinical value is yet to be proven (Herth 2010).

The flexible bronchoscope is the key interventional device available to the physician. It enables the physician to observe features within the airways and

the lungs. It is fed through the mouth, under sedation, into the airway and the lobes within the lung (See Figure 2-4, Chapter 2). A bronchoscope has a light source, camera lens and a narrow instrument and suction channel. The device proposed in this thesis uses the instrument channel of the bronchoscope to convey the electrospray emitter to a local target in the lung. In the diagnostic aspect of this work, a dye is administered that stains tumour tissue differently from healthy tissue on very early stage cancers known as carcinoma in situ (Mathelin, Croce et al. 2009). This may be the basis of a novel early stage cancer diagnostic test.

1.1.3 Therapeutic strategies

In treating lung cancer, physicians have a range of therapeutic options. Chemotherapeutics are delivered intravenously or orally. Being highly toxic, chemotherapeutics are not generally aerosolised because of the risk of secondary exposure or 'blowback' (Brooks, Tong et al. 2000). Steroids and antibiotics can be aerosolised and administered by inhaler or nebuliser. More recently, devices and device-drug combinations have been developed for respiratory disease.

In basic research, groups are looking at therapies based on DNA, siRNA, antibodies and proteins. In applied research, researchers are looking at improving the way current therapies such as chemotherapeutics and photo-activated drugs are delivered. Each of these therapies holds substantial promise for treating lung cancer through different modalities. The vector, however, via which they are introduced into the target cells remains problematic for example, DNA has been delivered by incorporation into viruses (viral vectors) which are then used to infect (transfect) the patient. In the case of gene therapy at least two drug trials were discontinued in the 1990s and early 2000s. The trials were ceased because of the death of a healthy subject in one case (Fox 2000) and instances of leukaemia in another. It emerged in both cases that the vector rather than the therapeutic caused the problem.

1.1.4 The pulmonary delivery problem

In basic research, a routine way to introduce biological material into cells is lipofection. A cationic lipid is a synthetic molecule that facilitates transfection. Lipids can be toxic to cells in vivo. This prevents the use of lipids to treat chronic disease. Viruses are important vectors in vitro as they have evolved to be excellent at introducing DNA into cells in nature. They do, however, require special skills and handling arrangements and are expensive. In vivo, viruses are immunogenic meaning that they elicit a response from the immune system. This has proved challenging to researchers in gene therapy. Physical force vectors are being explored that are potentially neither toxic nor immunogenic in vivo. Such vectors could be invaluable for basic research in vitro and be translated to in vivo work without significant modification.

Each of the current disease therapy routes has some limitations. Oral and intravenous drug administration involves systemic availability of the drug and resulting side effects. Aerosolised and nebulised administration involve only a fraction of the drug finding the intended target. Dosage and targeting become difficult to predict. The geometry of the lung anatomy and the narrowing of the distal bronchiole, tapering and branching tubular structures within the lung, make access too difficult.

Drug companies continue to develop increasingly sophisticated drugs including chemotherapeutics and very recently gene therapies. However, delivery remains the problem. The device proposed in this thesis addresses some of the challenges outlined here by using the bronchoscope to locate the target and electrospray to deliver diagnostic and therapeutic molecules directly to the lung tissues in a targeted and controllable manner. The rationale underlying this approach is that a targeted, physical mode of delivery will empower clinicians to enhance patient care by delivering agents that cannot easily be delivered by existing delivery modes, circumventing problems and limitations associated with aerosol mediated, intravenous, viral and liposome vector delivery and enabling novel diagnostic approaches.

1.2 The Challenges of Electrospray Liquid Atomisation

The term electrospray refers to a process where fluid is subjected to an electric field as it passes through a capillary in order to generate a very fine spray of the fluid. When subjected to an intense electric field, the fluid forms a Taylor cone at the top of the capillary (Taylor 1964). The cone is born of equilibrium between electrostatic and hydrostatic forces stretching the meniscus into a cone like feature. A thin fluid ligament or jet elutes from the apex of the cone. Fluid instabilities break up the emitted jet into a spray of charged droplets. Droplet evaporation and field-ion emission result in a fine spray of charged droplets that fly towards a counter electrode. The total assembly is known as an emitter. The fluid is usually an electrolyte and its chemical and mechanical character play a role in producing the electrospray. Electrospray differs from aerosol because it is driven by internal electrostatic forces rather than mechanical external forces. In electrospray, fluid colloids of similar charge repel each other and achieve high speeds and tiny volumes relative to those of an aerosol of a similar fluid. Electrospray can be finer, more controllable and can travel faster than aerosol. This high speed and small volume enables the propulsion of molecules into cells marking, modifying or medicating them.

There are many applications for electrospray technology including mass spectrometry, chemical deposition and satellite colloid thrusters. More recently, the process has been refined and applied to areas of biology such as protein mass spectrometry and tissue engineering. To date, the fundamental processes underlying electrospray formation remain incompletely understood, limiting full exploitation of the potential of this phenomenon. In this work, a novel electrospray system is developed and used in vitro to deliver biological molecules into cells and in vivo to deliver diagnostic dyes to a large animal model. This technology holds potential as a novel drug delivery method.

1.3 Addressing the Pulmonary Delivery Problem with Electrospray

Like many research projects, the genesis of this work was a conversation between engineering and biology researchers about a device called the Gene Gun. This device was developed at Stanford University, USA and is used widely in plant biology. It propels biological material into plant cells by coating gold particulate with a biologic and then firing it at the target plant cells by way of blasts of helium gas. The question posed was whether an equivalent device existed for the mammalian cell. A brief review of the literature indicated at least one group working on electrospray (Chen, Wendt et al. 2000). Other contemporary approaches were inkjet technology, ultrasound technology and related technologies such as corona discharge.

The typical electrospray bench configuration reported by researchers involves syringe pumps, high-voltage power supplies, mechanical stages, retort stands, glass capillaries and instrumentation. Naturally such as set-up could not be used in a clinical setting. Further, the use of high-voltage direct current, albeit at currents in the nano to microampere range, requires careful design for safety and regulatory requirements (Underwriters_Laboratories 2004). Electrospray was chosen as the basis for this work and has emerged as a fascinating cross-disciplinary research subject. The technical challenge was to engineer a device that performs the function of the bench set-up described but in an optimised and miniaturised form and to validate the design by conducting in vitro and in vivo experiments. The resulting device will be used in conjunction with a bronchoscope to deliver biological material to lungs in vivo. There is clearly an unmet need for such a device to address the pulmonary delivery problem. It is the principal objective of this work to address this need with a novel bronchoscopically mediated electrospray emitter.

1.4 Thesis Contributions

1.4.1 Novel bronchoscopically mediated electrospray device

A novel bronchoscopically mediated electrospray device was designed and fabricated in this work and has been tested in vivo in a large animal model (pig).

Consultant Respiratory Physician, Professor James Egan of the Mater Hospital Dublin and Veterinary Surgeon, Dr. Peter Nolan carried out the procedure in Trinity College Dublin in May 2010.

1.4.2 Early-stage cancer diagnostic test

The role of dyes in medical diagnoses is widely reported. Methylene blue is used in cancer diagnosis to stain suspected tumours in mouth and in sentinel node mapping in breast cancer(Mathelin, Croce et al. 2009). In the case of lung cancer, dye has not been used to date because of the difficulty in targeting the area of interest. Methylene blue dye was delivered to the lungs of a pig in vivo and to pig trachea ex vivo. This is a novel procedure that is the basis of an early stage lung cancer diagnostic test.

1.4.3 Non-toxic electrospray mediated in vitro delivery of DNA encoding green fluorescing protein (GFP) in human epithelial cells

BEAS-2B (transformed human airway cells) cells were electrosprayed with GFP plasmid and underwent a transfection rate of up to 60%. This transfection method is cheaper, has fewer steps and lends itself to automation more readily than virus or lipid work.

1.4.4 Non-toxic, electrospray mediated in vitro delivery of siRNA tagged with $Alexa_{488}$ to human epithelial cells

BEAS-2B (transformed human airway cells) cells were electrosprayed with scrambled RNA bound to $Alexa_{488}$ fluorophore and underwent a transfection rate of up to 90%. This transfection method is cheaper, has fewer steps and lends itself to automation more readily than virus or lipid work.

1.4.5 Non-toxic electrospray mediated delivery of DNA encoding GFP in mouse epithelial cells

Normal primary mouse airway epithelial cells were electrosprayed with green fluorescing protein (GFP) plasmid and underwent a transfection rate of up to 30%. This transfection method is cheaper, has fewer steps and lends itself to automation more readily than virus or lipid work.

1.4.6 Mechanism for delivering proteins and antibodies

It is potentially useful to deliver antibodies directly into cells. This is not currently done and the delivery is the difficulty. In this work, it was demonstrated that an antibody could be electrosprayed without affecting its function. Rabbit anti-mouse antibody was electrosprayed and collected. It was tested for viability subsequent to electrospraying on a known robust assay and found to be viable. This is a new result.

1.4.7 Novel delivery of cisplatin to human lung cancer cells

DLKP (human lung cancer cell line) cells were electrosprayed with cisplatin, a common chemotherapeutic. Cells within a circular area apparently targeted were killed completely whilst those outside remained viable. This demonstrates an apparent targeting effect and confirms that electrospray mediated delivery of cisplatin will induce cell death.

1.4.8 Novel electrospray mediated in vitro delivery of DNA encoding E-cadherin in human lung cancer cells

E-cadherin is a protein that is not expressed in DLVP lung cancer cells. Consequently, when it is detected with an assay it must be as a result of foreign DNA entering the cell and producing it within the cell. E-cadherin is of interest because it is a tumour suppressor protein and a potential gene therapy for cancer. DNA was electrosprayed into human cancer cells DLKP (human lung cancer cell line) in vitro and E-cadherin was detected in the cells at time points after 24 and 48 hours. This is a novel result.

1.4.9 Novel electrospray mediated delivery of methylene blue dye to pig trachea ex vivo

Methylene blue dye was electrosprayed onto pig trachea ex vivo. The dye, a common marker, entered the cells in the epithelium and stained them such that the mark could not be washed off. Conversely, a similar amount of dye dropped onto a neighbouring piece of tissue was washed off easily with PBS. This result shows the ability of the electrospray to penetrate the epithelial cell membrane compared to dropped on application where the dye is not taken up.

1.4.10 Novel delivery of diagnostic methylene blue dye to pig lungs in vivo

The device developed in this work has been tested in pig bronchus in vivo and should have broader applicability to other disease sites in the body that can be accessed by endoscopy or with minimally invasive surgery. Examples of these are colorectal cancer, cervical cancer and oesophageal cancer.

1.4.11 An ionic current feedback mechanism to control electrospray

Biological material is delivered into cells most consistently with the cone-jet mode of electrospray, although other modes have been proposed. The cone-jet occurs over a range of emitter–counter electrode potentials. Consequently, a novel closed-loop feedback system is demonstrated based on current feedback to stabilise the electrospray mode. This increases the consistency of delivery when compared to the open-loop system. This is a novel application for an ionic current feedback system.

1.4.12 Patent application No. PCT/EP2009/054680

A patent relating to the electrospray device developed during this work has been filed and examined with a significant number of claims upheld. The nationalisation and grant phase will take place in September 2010. International Patent Application No. PCT/EP2009/054680 was lodged in the UK patent office. The patent search yielded no blocking IP.

1.4.13 Contributions to the field of research

Biological buffers are aqueous solutions that resist changes in pH. They are used to dissolve DNA, siRNA and other molecules such that they can be delivered to cells. As a consequence of this work, biological buffers have been characterised for the first time for use with electrospray. In particular, PBS, TRIS and TE buffers were characterised. Separately, methylene blue dye was characterised. The characterisation involved the measurement of conductivity, viscosity and surface tension. These measurements are presented and should be useful to others working in the field.

1.4.14 Research validation through funding awards

As a result of this work, research funding has been obtained to further the project. The research group in the human epithelial lab have been successful in securing a grant from the EU FP7 Marie Curie research framework. The grant is to support basic scientific research into electrospray as it pertains to biological applications. The group are hopeful of further funding from SFI-HRB translational fund and potentially an Enterprise Ireland C+ grant to realise a commercial embodiment of the device described.

1.5 Thesis Structure

In this chapter the background and motivation for this work has been introduced. The problem of diagnosing and treating lung cancer is reviewed and the thesis contributions are detailed. The physiology of the lung and the epithelium is introduced in Chapter 2. The motivation for delivering DNA, siRNA, chemotherapeutics and diagnostic dyes, known here collectively as molecules, is introduced. The challenge of introducing molecules to the lungs is discussed and framed as the pulmonary delivery problem. Current methods of introducing molecules into cells, known as the vector, are introduced and different methodologies are reviewed. Electrospray is introduced as a potential physical force vector. Chapter 3 contains a review of electrospray commencing with a historical perspective and concluding with current biological and drug delivery applications for electrospray. The mechanical and electrical parameters that contribute to electrospray are introduced and models reviewed. In Chapter 4 the problem statement is developed. The problem domain is that of delivering molecules to the lungs with an electrospray device to address the pulmonary delivery problem. The physiological background and electrospray phenomenon related in Chapters 2 and 3 are drawn together to propose a bronchoscopically mediated electrospray device. In Chapter 5 this solution is designed and implemented. In Chapter 6 the performance of this device is tested in vitro and in vivo. Chapter 7 contains a discussion of the results and conclusions drawn from the work and avenues for new work are suggested. The thesis concludes with the bibliography and appendices.

Chapter 2

Delivering Molecules to the Lungs, a Literature Review

In this chapter, the physiological background of the thesis is introduced. The anatomy and function of the pulmonary system and the epithelium are reviewed. Terminology relating to the different research settings; in vivo, ex vivo and in vitro is introduced. Research into lung disease depends on differing methods for introducing molecules into cells. These methods, called vectors, are reviewed and compared indicating their strengths, weaknesses and applicability to the delivery challenge concerned. Electrospray is introduced as a potential vector that can be used in vitro or in a novel in vivo setting.

In Section 2.1 lung physiology is reviewed. In Section 2.2 the epithelium is reviewed with particular reference to its highly specialised cells and to its structure. The cell lines that are used later in the work are introduced. In Section 2.3, lung disease is reviewed briefly and in Section 2.4 the reason for focusing on lung cancer in this work is explained. In Section 2.5 the implications of intravenous versus local delivery of molecules are reviewed. In Section 2.6 the common approaches to researching diagnostics and therapy are introduced. For these and other biological approaches, it is desirable to transfect cells by introducing some new biological material or, more generally, molecules into the cell. In Section 2.7 current methods of introducing material to cells are reviewed. The chapter concludes with a summary in Section 2.8.

2.1 Lung Physiology

The lungs are concerned with the vital function of respiration. The lungs lie in the thoracic cavity on each side of the heart. They rest on the diaphragm and extend upward from this to the clavicle. The lungs comprise lobes covered in membrane known as the pleura. The right lung has three lobes or sections. The left lung has two lobes and is characterised by a cardiac notch where the heart

is accommodated in the anatomy. Air is breathed through the mouth and nose and is conducted by the trachea, composed of several rings of cartilage that keep its structure ridged, to the bronchus (Figure 2-1). The exchange of gases, absorbing oxygen from the air and excretion of carbon dioxide takes place at the alveoli located at the ends of bronchioles deep in the lung (Bird 1995).

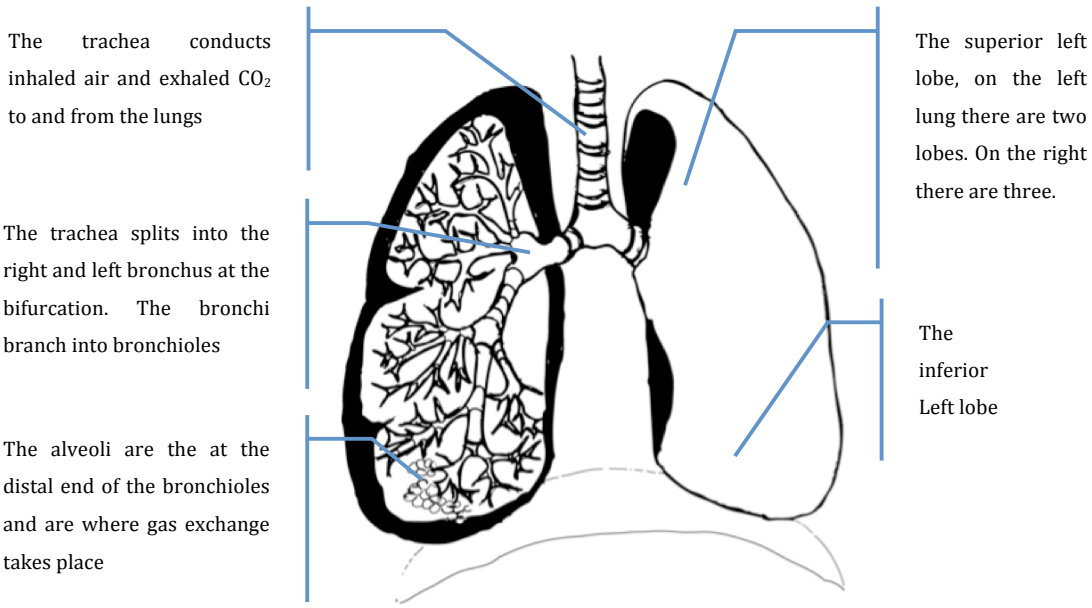


Figure 2-1 The lungs

It is noteworthy that each lobe within the lung is complete within itself. It has blood vessels, elastic connective tissue, bronchioles and alveoli. In the case of disease, this gives surgeons therapeutic options to remove part of the lung whilst conserving the patient's ability to breathe. Human lungs are over-designed mechanically affording more breathing capacity than that which is usually required. This may be an evolutionary artefact. Consequently, patients can and often do, survive with part or all of a lung removed.

During inspiration, the chest cavity expands, the diaphragm falls and air is drawn into the lungs. Gas exchange occurs at the alveoli. During expiration, the diaphragm and intercostal muscles relax shrinking the chest cavity and forcing

out the exchanged gases. Respiration rate varies from individual to individual but is generally 6 to 9 breaths per minute in an adult. Gas exchange takes place in the alveoli by diffusion over the membrane. Net diffusion of oxygen occurs between the alveoli and the blood where a partial pressure gradient is produced by the continuous use of oxygen by the cells in the body. Oxygen is both dissolved in the blood and carried in combination with haemoglobin (oxyhaemoglobin) within the blood. Conversely, CO_2 builds up in the blood stream as a by-product of cellular activity. This creates a partial gas pressure gradient from the blood to the alveoli. The resulting CO_2 is exhaled. Neither CO_2 nor O_2 is dissolved well in the blood and consequently the process of gas exchange requires a catalyst. The catalyst is an erythrocyte enzyme, carbonic anhydrase (Sherwood 2007).

The connective tissue between the airways within the lung is elastic in nature. As breathing occurs, the lung is stretched and then returns to its resting size. The full lung volume of both lungs in an adult is about 5.5 Litres. The empty lungs retain about a litre of fluid. The lungs are over designed so in normal breathing the lungs do not fill beyond about 3 litres. The volume excursions due to breathing tend to be around 0.5 litres centred around ~2 litres of capacity in normal breathing. In lung disease, the interconnective tissue may lose its elasticity rendering breathing progressively more difficult. In emphysema and bronchitis, diseases that frequently co-exist and are collectively termed chronic obstructive pulmonary disease (COPD), the patient finds it progressively more difficult to breathe due to a loss of elasticity in the lung and a build up of mucus in the airways. In lung cancer, a tumour may occupy and render useless whole sections of the lung. Asthma is an immune response disease causing constriction of the bronchiole leading to difficulty with breathing for the sufferer. Cystic fibrosis is an inherited disease, most prevalent in Ireland (Brennan 2008) where the cells in the airway secrete a thick sticky mucus creating difficulty in breathing. In the warm, moist environment in the lung, sufferers are prone to repeated infections. Respiration is vital to our health and lung disease is critically debilitating for the sufferer. Research into therapies, diagnosis and devices that restore or ameliorate lung function are important and warranted.

2.2 The Epithelium

The lung epithelium, depicted in Figure 2-2, is a surface of highly specialised cells lining the airways and the lungs. These cells have adapted as an interface between fluid and tissue compartments in the body. Epithelial cells line other parts of the body where fluid and tissue come into contact such as the intestine and the urinary tract. The functions of epithelial cells are mainly concerned with ion transport. The ion transport channels or pores facing the fluid interface are different to those in contact with the tissue interface (Palmer 2007). Whereas the ion channels in nerve or muscle tissue carry mainly information, the epithelial cell ion channels carry materials across the membrane. Where lung function is impaired, frequently the epithelium can be part of the problem. It may be inflamed, damaged, occluded by a tumour or secreting abnormal mucus. The ability to diagnose and deliver therapeutic molecules to the epithelium to restore correct function is core to some lung therapies.

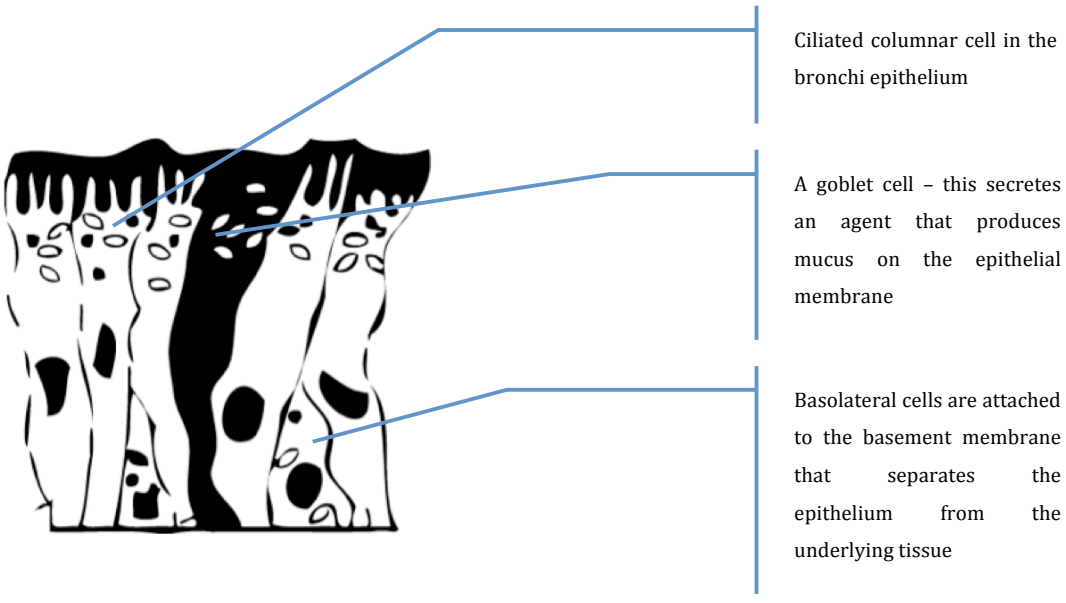


Figure 2-2 The epithelium

The epithelium has apical cell domains and basolateral cell domains. Apical cells are in contact with the fluid compartment or the outside world and are characterised by tight junctions filled with caulking material. This is to keep the membrane selectively integral. The basolateral cells are in contact with the

underlying body tissues and are not tightly packed (Lewin 2007). Transporters on the basolateral side carry nutrients out to the blood. The epithelial cells have other features adapted to specific areas in the body. In the intestine, the epithelium has special folds called villi and on each villi perpendicular microvilli increase surface area for nutrition. In the airways, the epithelial cells have hair like features called cila. Cila is from the Latin *cilum* meaning eyelash. Cila conduct fluid movement across the surface membrane by oscillating forth and back carrying mucus and trapped particles away from the lung. There are additionally epithelial cells with features called flagellum. These are longer than cila, 40 μm Vs 10 μm and exhibit a whipping motion. In cila, the fluid is moved perpendicular to the epithelial cell while in flagellated cells the fluid movement is parallel to the cell axes. In the human airway and lung there may the 0.5m^2 of ciliated airway surface comprising ~10^{12} cila (Bolsover 2004). It is noteworthy that in smokers cila are paralysed making mechanical movement the only way to move mucus hence the smoker's cough.

In cystic fibrosis, the epithelium ion transport function is responsible for the debilitating symptoms of this disease. Cystic fibrosis is a disease of the lungs, the pancreas and the reproductive organs. Sticky mucus that is inadequately hydrated is produced in these organs. In the lungs this leads to difficulty breathing and the requirement for daily physiotherapy and nebulised surfactants. In the pancreas, supplementary digestive enzymes are required for nutrition. The body becomes colonised by the bacteria, pseudomonas aeruginosa, that gives rise to recurring infections. The chloride ion channel in the epithelial cells is defective in this condition due to a genetic mutation. It is postulated that correcting 10% of the affected cells could make the disease asymptomatic (Johnson, Olsen et al. 1992). Although it is 20 years since the cystic fibrosis gene was identified, unfortunately a cure or therapy is still elusive. One of the main reasons for this is an inability to deliver a corrected copy of the gene to the affected cells. In the case of the lungs, this is an example of the pulmonary delivery problem. In this work, therapeutic and diagnostic molecules will be delivered mainly to epithelial cells. Researchers are hopefull

that gene therapy will provide alleviation and even a cure for cystic fibrosis in time.

In vitro, continuous cell lines are used as research tools. Epithelial cells are seeded out into well plates and incubated. In this way layers of cells can be grown and transfections performed. An important difference between in vitro cell membranes and those in vivo is the action of the immune system. A 24 well plate has no immune system so a result in vitro may not be directly applicable to the in vivo setting. The cell lines and tissues used in this work in vitro are DLKP (human lung cancer cell line), normal primary mouse airway epithelial cells and BEAS-2B (transformed human airway cells) cells.

2.3 Respiratory Disease

Respiratory disease is a particular problem in Ireland and the UK, both ranking in the top four countries for the highest prevalence rates of asthma in the world. Ireland also has the second highest death rate from respiratory disease in Europe and the highest incidence of cystic fibrosis in the world. Deaths from respiratory disease in Ireland exceed those from coronary artery disease. Respiratory disease is the most commonly reported long-term illness in young adults (Brennan 2008). Respiratory diseases are the most common reason to visit a GP and the third most common reason for acute admission to hospital. Significant social and economic consequences follow. The cost of respiratory disease in Ireland was estimated to be € 946.5 million in 2006 and 6,034.4 working years were lost due to mortality and production losses. Increased understanding of respiratory disease processes and novel approaches to treating these diseases are urgently required.

The major respiratory disease groupings, cancer, COPD, pneumonia, tuberculosis, asthma and cystic fibrosis, have multiple underlying causes and frequently complications such as infection or immune reaction. The development of a novel in vivo means of local delivery of molecules is likely to be most useful initially in lung cancer diagnosis and treatment. This view was formed in light of multiple considerations including medical ethics, regulatory

affairs and clinical trials through discussions with Professor James Egan, Consultant Respiratory Physician, The Mater Hospital Dublin when presented with the new electrospray device. Accordingly, the focus will be to apply the electrospray device to lung cancer diagnosis and therapy.

2.4 Lung Cancer Diagnosis

Lung cancer is a leading cause of cancer related death globally, and it accounts for more deaths than breast, colon, and prostate cancer combined in the United States (Greenlee, Murray et al. 2000). From a historical perspective, the premise behind early lung cancer detection strategy is that early detection of lung cancer is justified if early treatment improves the outcome. The prognosis of early stage lung cancer is superior to that of late stages. Smoking is by far the most common cause of lung cancer accounting for 85-90% of cases. The age adjusted relative risk of developing lung cancer indicates that those who smoke more than 20 cigarettes a day have a 2000% increased risk compared to life long non-smokers. Many studies have shown that women who smoke are more likely to develop lung cancer than male cancers (Akopyan 2006).

Lung cancers are classified into two main categories, small cell lung cancer (SCLC) which accounts for approximately 20% of cases and non-small cell lung cancer (NSCLC) which accounts for the other 80%. Non-small cell lung cancer includes squamous cell (35%), adenocarcinoma (27%) and large cell (10%)(Le Chevalier, Brisgand et al. 1994). In practice, however, not all patients receive histological confirmation of the cell type of their disease. Registry basis population studies indicate that 55% of patients have a confirmed diagnosis of NSCLC, 11% as SCLC and 34% have no histological confirmation of cell type. Lung cancer is the leading cause of cancer mortality in Ireland representing approximately 20% of all death due to cancer . There are 2474 new cases per year of which 933 are female and 1541 male. This leads to a significant burden on the general population with 2286 deaths per year of which 862 are women and 1424 are men. The incidence of lung cancer is projected to increase by 141% in women and 61% in men between 2010 and 2030. Compared to other EU countries, incidence rates for men are below average but for women are

almost twice the EU average. The 5-year survival rates in Ireland are 8% for men and 10% for women. These compare to rates of 13% and 16% in France and 15% and 18.5% in the United States. Currently >75% of patients present with locally advanced or disseminated disease. Therefore there is a major clinical need to enhance simple diagnostic strategies. The total financial burden of lung disease in Europe amounts to nearly 102 billion Euro. This is equivalent to the total GDP of Ireland. The European lung foundation (www.European-lung-foundation.org) has called for improved early diagnosis and targeted new therapies to deal with the major clinical challenge which lung cancer presents for national economies. In 2001 the average cost of treating a patient with lung cancer was 17,000 Euro, which equates to 42 million euro per annum in Ireland. Hospitalisation accounts for 50% of the costs (Lodderkemper 2003).

2.4.1 Diagnostic approaches

Lung cancer diagnosis has multiple aspects including patient history, physical exam, x-ray, spirometry, blood tests, sputum cytology and bronchoscopy (Hirsch, Franklin et al. 2001). The symptoms of early lung cancer include chest pain, shortness of breath, weight loss and bone pain. The diagnosis is likely to be lung cancer in patients presenting with these symptoms if the symptoms are persistent, occur in smokers (and ex-smokers) over 50 and with chronic airflow obstruction. Early lung cancer is largely asymptomatic and the internal nature of tumours means patients do not notice physical changes as they might with other diseases. The symptoms can be hard to detect if there is co-existing respiratory disease such as COPD (Birring and Peake 2005).

The bronchoscopy procedure is relevant because it is the standard method by which any device is introduced to the lung. A device is inserted into the lungs through the mouth or nose under mild sedation. Respiratory physicians are expert in the use of the bronchoscope and can perform the procedure in matter of minutes. A bronchoscope is a diagnostic tool and to a more limited extent a therapeutic interventional medical device. The bronchoscope is designed to view the lung and airway. Bronchoscopes were originally developed as a straight, rigid medical device used by a physician to inspect the airways and

larger bronchi in an anaesthetised patient. The rigid bronchoscope (Figure 2-3) is still used as it has a large working channel in which instruments can be inserted during a procedure.

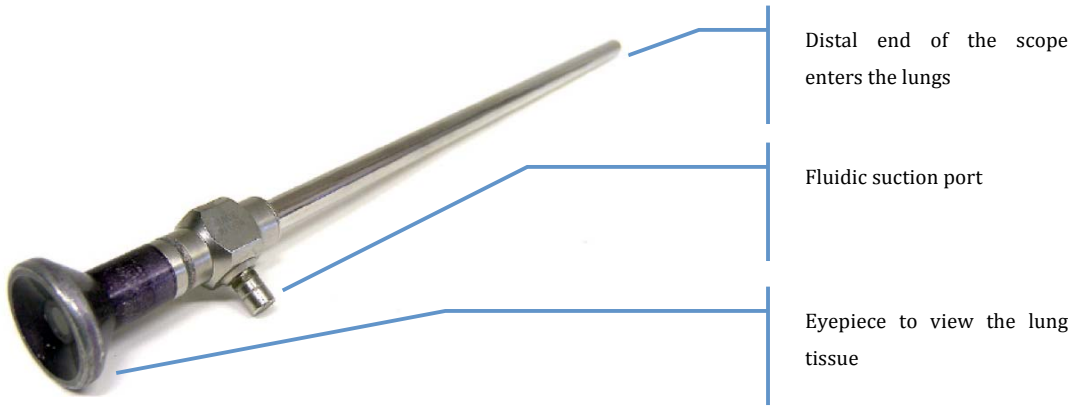


Figure 2-3 A rigid bronchoscope

It has an eyepiece on one end and a lens on the other. These are connected by an inflexible lens system. A light source is connected to illuminate the area under inspection. In the last 15 years, the rigid bronchoscope has been replaced largely by the flexible bronchoscope (Figure 2-4). Companies such as Pentax, Olympus, and Stortz manufacture these devices. The flexible bronchoscope is about 65cm long with an articulating tip. The articulation of the tip is controlled by a series of mechanical wires called Bowden wires connected to a hand-held control (Prutchi and Norris 2005). Bowden was a nineteenth century Irish engineer resident in London and filed English patent No. 25,325 and U.S. patent No. 609,570 for the mechanism he envisaged as a replacement for the rods and eyelets in bicycle brakes. The output of the flexible bronchoscope can be viewed through a video monitor or through the eyepiece of the device. A light source of near white light is attached to the bronchoscope and this illuminates the area under inspection. The image of the target is conveyed back through a fibre optic bundle to a lens system in the handle. Most recently, bronchoscopes have developed further as diagnostic instruments by using their optics to interpret the nature of the tissue they are seeing (Zeng, McWilliams et al. 2004). This capacity has a few forms and is known as autofluorescence bronchoscopy (AFB) and narrow band imaging (NBI).

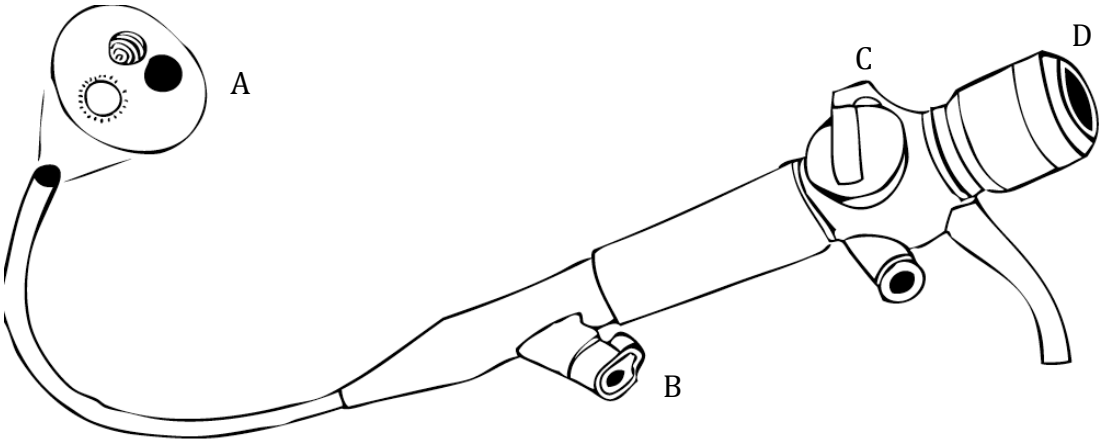


Figure 2-4 A flexible bronchoscope. (A)the head comprising light source, lens and working channel (B)the tool channel port (C)the control (D)the eye piece

In current work in Japan, the red, green, blue (RGB) filter arrangement in a flexible scope has been replaced with an alternative spectral regime. This can pick out blood vessels and particularly, heightened vascularisation of tumours (Shibuya, Nakajima et al. 2010). Notwithstanding these considerable advances in bronchoscope technology questions still remain about the value of these developments in clinical practice. In technological push there is a danger that the availability of technology could lead to over diagnosis and overtreatment (Lee and Sutedja 2007). This introduces the wider question of screening and the ethics of subjecting people in various risk groups to invasive procedures (Henschke, Shaham et al. 2006; Read, Janes et al. 2006). Whilst the new bronchoscope technologies described are extremely expensive and currently beyond the reach of endoscopy suites in public hospitals, these technologies will eventually fall in price. In this work, we will use the standard bronchoscope to access the lungs and use the working channel of the bronchoscope to deploy the electrospray device using the optical feedback to observe its performance. Bronchoscopes still have limitations in reaching or seeing beyond the first, second or third bronchi. After this, the bronchi become too small. Clearly, disease can and frequently does exist in areas of the lung beyond the range of the bronchoscope.

2.4.2 Diagnostic dyes and markers

A developing methodology for early cancer diagnosis is the use of diagnostic dyes. These are used in breast cancer (Mathelin, Croce et al. 2009) and mouth cancer (Chen, Lin et al. 2007a; Chen, Lin et al. 2007b). The potential role of dyes in early detection of lung cancer has been evaluated by Vignaud, Menard et al (2006). This involves the deposition of a diagnostic dye on an area of tissue for the purposes of bringing about some observable optical demarcation between healthy tissue and tumour tissue. Methylene blue is the most common dye but other variants namely patent blue and isosulfan blue have also been evaluated (Thevarajah, Huston et al. 2005; Barthelmes, Goyal et al. 2010). The cell walls of epithelial cells maintain their integrity through the existence of fluid tight junctions (Bolsover 2004). This means that methylene blue dye will not stain healthy epithelial tissue. Conversely, in cancerous tissue the cell wall lacks integrity because of the presence of disease. The methylene blue dye stains the tumour and this stain is clear to the observer. This test can be brought about, in the case of oral cancer by mouth rinsing with a solution of the dye (Chen, Lin et al. 2007a). In the lung, however, the problem of delivering the dye to the local target remains and is complicate by the lack of intrinsic drainage. In this work, the proposed electrospray device will be used to electrospray methylene blue.

The toxicity of methylene blue (Alford, Simpson et al. 2009) needs to be addressed ahead of establishing a test based on it. It is unclear currently how regulatory authorities will classify a diagnostic dye (Te Velde, Veerman et al. 2010). Notwithstanding this, diagnostic dyes can potentially yield information comparable to that of higher price technologies.

2.4.3 Diagnostic interventional bronchoscopy

Sputum cytology has been used as an initial screening method followed by autofluorescence bronchoscopy to detect early stage lung cancer in the central airway. In two studies, the technique detected early cancers where thoracic computed tomography (CT) scan was used as a control. It was concluded that this was a practical way of detecting early stage lung cancer in central airway (Lam, Lam et al. 2009). Current developments in interventional bronchoscopy include the ability to magnify airway structures in real time, explore vascular

flow and view bronchogenic carcinogenesis at a cellular level (Colt and Murgu 2010).

2.5 Lung Cancer Therapies

2.5.1 Chemotherapy

Chemotherapy in lung cancer involves the administration of cytotoxic drugs to destroy cancer cells. Chemotherapy is used successfully for small cell lung cancer and for non-small-cell lung cancer. Chemotherapeutics destroy cancer cells by disrupting uncontrolled cell division (Schwartz and Shah 2005). The drug circulates the bloodstream and can reach cancer cells throughout the body. Typical cytotoxic drugs used in lung cancer include cisplatin and bleomycin. Chemotherapy drugs are usually administered intravenously or in oral form and typical treatment times are 3-6 months. Side effects including tiredness, sickness, suppressed appetite and low resistance to infection occur because the whole body is treated with the chemotherapeutic. With certain drugs body hair may fall out.

In the case of small cell lung cancer that has frequently spread from the lung to other parts of the body by the time it is diagnosed, it is clear that a systemic treatment is appropriate to prevent secondary cancers from occurring. However in other lung cancers, with earlier detection, the disease may be highly localised. In these circumstances it may be more desirable to treat the cancer in a localised fashion thereby avoiding treating the whole body. Local administration of chemotherapeutics becomes the challenge.

2.5.2 Brachytherapy

Brachytherapy is a form of internal radiotherapy where the radiation source is placed within the body within centimetres of the tumour. Brachytherapy is used for palliation and more recently proposed as curative treatment (Hennequin, Bleichner et al. 2005) for patients for whom other forms of therapy are unsuitable. These include patients who have already had surgery or have

respiratory insufficiency. Therapy is administered with the assistance of a bronchoscope to the lungs.

2.5.3 Photodynamic Therapy (PDT)

Photodynamic therapy has been evolving since the mid 1970s. The therapeutic concept involves the administration of a photosensitive agent, known as a photosensitiser, systemically followed by activation through local illumination. When the agent is activated, it kills cancer cells through multiple modalities. Depending on the agent, the cell's internal structures are damaged triggering cell death. Unlike chemotherapy, the cell's DNA and RNA is not usually impacted. PDT also creates local hypoxia in the area of the tumour, starving it of oxygen. PDT agents can be chlorophylls, porphyrins or dyes (Folkes and Wardman 2003). The agent is carried in the bloodstream and accumulates in the tumour. Some time after administration, the tumour area is illuminated by a light source and the agent becomes active. Unlike chemotherapy, PDT is a local treatment however the agent currently needs to be administered systemically. Some PDT agents tend to accumulate in particular tissue types. PDT is currently expensive and not practiced widely outside the US (Morgan 2009). Limitation of PDT include photosensitisation of the subject during the period of treatment and the need to stay out of direct sunlight and wear protective clothing and eyewear. Commercially available PDT agents include Photofrin and Foscan. PDT is routinely used in cervical cancer therapy. In a trial in Japan, 71% of a group of 48 subjects having squamous cell carcinoma where the lesion could be seen bronchoscopically and was <1cm diameter went into complete remission after PDT (Hayata, Kato et al. 1988; Hayata, Yamamoto et al. 1990; Hayata, Kato et al. 1993). The prospect of delivering the PDT agent to the lung locally, followed by bronchoscopic illumination would represent an advance in this therapy. The proposed electrospray device designed in this work could deliver PDT agents to tissues within the lung.

2.5.4 Gene therapy for lung cancer

Overall survival rates in lung cancer have remained stubbornly low. This can be attributed to the frequently advanced state of disease at the time of diagnosis. Earlier diagnosis and developments in chemotherapy, PDT, surgery and radiotherapy have not yet yielded significant increases in overall survival rate. Further, strategies using combinations of conventional therapies only slightly improve survival rates even in the early stages of lung cancer. It has been suggested that a therapeutic plateau has been reached in traditional approaches to lung cancer therapeutic strategies.

Developments in molecular biology and immunology have yielded a better understanding of the processes underlying lung cancer at a cellular level namely, the blocking of tumour promoting genes and replacement of tumour suppressing genes (Gutierrez, Lemoine et al. 1992). Gene therapy strategies that address the underlying mechanisms of lung cancer, specifically tumour suppressor gene replacement, suicide gene expression, cytokine-based therapy and vaccination approaches have been evaluated in clinical trials over two decades and generally virus or lipids have been used as vectors (Vachani, Moon et al. 2010). In many of these trials, the vector is core to the trial outcome and in these trials, as elsewhere in gene therapy, the vector has been problematic.
One of the proteins produced by DNA delivery, in this work, is E-cadherin. This is a common tumour suppressor protein. The electrospray device proposed in this work could deliver DNA producing the E-cadherin protein in targeted cells. This physical method of delivery in vivo would have the advantage of no immune response from the body and low toxicity. This would address the need for novel, more specific and less toxic therapeutic strategies. The therapeutic aim of this molecular approach to bronchogenic carcinomas is to significantly improve survival (Toloza 2006).

2.5.5 Interventional Bronchoscopy

An excellent example of interventional bronchoscopy is that of bronchoscope mediated electrocautery. A trial reports treating patients with radiographically occult lung cancer (cancer that cannot be seen on an x-ray or CT scan) by

electrocauterising it through a bronchoscope (van Boxem, Venmans et al. 1998). This is a surgical procedure occurring in a diagnostic setting. The technique requires an insulating bronchoscope to carry an electrocautery device to the lungs and view the procedure. The relevance for this work is that the proposed electrospray device will be used with a bronchoscope and this trial establishes a reference point for similar bronchoscopy and intervention or 'see and treat'.

2.6 Research Methods in Lung Disease

Basic research into lung disease is conducted through a combination of in vitro and in vivo work. New therapies are conceived and tested by working with continuous cell lines. A continuous cell line is a source of cells that are immortalised. Specialised cell lines, for instance of lung cancer cells, can be continually cultured for many years. The process of transfection is depicted in Figure 2-5.

In vitro, identification of genes involved in the disease process has stimulated intensive research into the potential of gene therapy approaches involving both the replacement of defective genes and the introduction of therapeutic genes. Gene therapy approaches have been attempted for several lung diseases including lung cancer and cystic fibrosis (CF) (Riordan, Rommens et al. 1989; O'Dea and Harrison 2002). However, it has not been possible to deliver DNA to cells in the lungs to date, creating an unmet clinical need for such a capability. The introduction of foreign biological material into cells is known as 'transfection'. The material may be DNA, siRNA or protein. DNA genes and siRNA are the most commonly transfected molecules.

In vivo, molecules must be transfected directly into cells to avoid destruction by the body's immune system defences. In comparison to other organs, the lung offers seemingly straightforward access for gene delivery via the trachea. However, accessing specific parts in the lung remains a challenge. Viruses and cationic lipid or liposomes are the most widely used vectors for gene delivery in vivo and have been delivered to the lungs in liquid and aerosol form (Pickles 2004). Many viruses naturally target lung cells and inject their contents into the

host cells. Liposomes merge with the lipid membrane that surrounds cells and deliver their contents to the interior of the cell. To date, however, these vectors have had limited success in the field of gene therapy (O'Dea and Harrison 2002; Pickles 2004; Griesenbach and Alton 2009). Vector-related problems such as lack of target specificity, toxicity, severe immune responses and limitations with vector production have proven difficult to solve. In 1999, a patient on a clinical trial in the United States died as a result of side effects of the viral vector used. This damaged scientific and public belief in these strategies (Fox 2000). Gene delivery problems have become so significant that many pharmaceutical companies have now down-sized or abandoned their gene therapy research programmes as they wait for academic research groups to solve the delivery problems (Branca 2007; Gao X 2007). Because of the problems associated with existing delivery methods, only one gene therapy drug has been approved for human use. The treatment, approved in China, consists of an adenovirus designed to insert a gene called p53 to treat head and neck squamous cell carcinoma (Peng 2005). Thus, current technologies for delivering DNA to cells and tissues in vitro and in vivo utilise biological or physical methods. However, problems associated with biological vectors, viruses and liposomes, have severely limited success to date. So while potential therapeutics exist, in the form of DNA and genes, the delivery method has so far been elusive.

2.7 Current Approaches to Introducing Molecules into Cells

Direct delivery of therapeutic and diagnostic molecules into lung cells in vivo using physical methods is desirable for reasons including reduction of off-target effects, systemic dosage effects and efficacy. However, delivery of therapeutic molecules directly into cells has remained a challenge. In particular, research activity towards the development of novel gene therapy strategies has slowed because of problems associated with the safe and effective delivery of DNA-based molecules into cells. Current systems for delivering DNA by physical methods include biolistic methods, electroporation, magnetofection and sonoporation. The latter two methods are relatively specialised and will not be discussed in this work. The proposed electrospray device in this work can be assessed in relation to other physical targeted delivery methods.

Aerosolisation is widely used in lung therapy and to a lesser extent involved in gene delivery methods. The lung is a drug target itself but is also used as an access point to deliver drugs systemically by aerosolising them and designing the drug to cross the lung blood barrier. Characterisation of drug delivery to the lungs is a complex task involving the determination of delivered, deposited and absorbed dose (Cryan, Sivadas et al. 2007). The problems of access to the distal airways, mucus coverage of tissues, diseased tissues and lack of receptors within airways frustrate the delivery of biologics and compound the pulmonary delivery problem. Further, methods of introducing biological materials involving viruses or lipids have problems of immunogenicity and toxicity.

2.7.1 Lipids

Lipids are fat compounds that can coalesce with the lipid cell membrane of cells. If the lipid carries some DNA or other biological matter this will be transported over the cell membrane and will transfect the cell. Lipids are widely used in vitro and to a lesser extent in vivo. Cationic lipids have high transfection efficacy in lipofection. Transfection rates in the region of 70%-80% are reported routinely depending on the cell line and the biologic involved. Lipids are, however, extremely toxic. Histopathological changes following administration of DNA by cationic lipid in sheep were characterised by inflammation of the airways. The severity of the inflammatory response appeared to correlate with the administered dose of DNA (Emerson, Renwick et al. 2003). As with any conventional drug, the body's response to cationic lipid-DNA complexes is dependent on the dose administered and the route of delivery. Acute inflammation and tissue damage can occur that is sometimes quite profound. Consequently, other strategies are required to improve the therapeutic potential of lipid vectors, such as transient immune suppression (Yew and Scheule 2005) or their transfection consistency needs to be reproduced in vivo but without the toxicity.

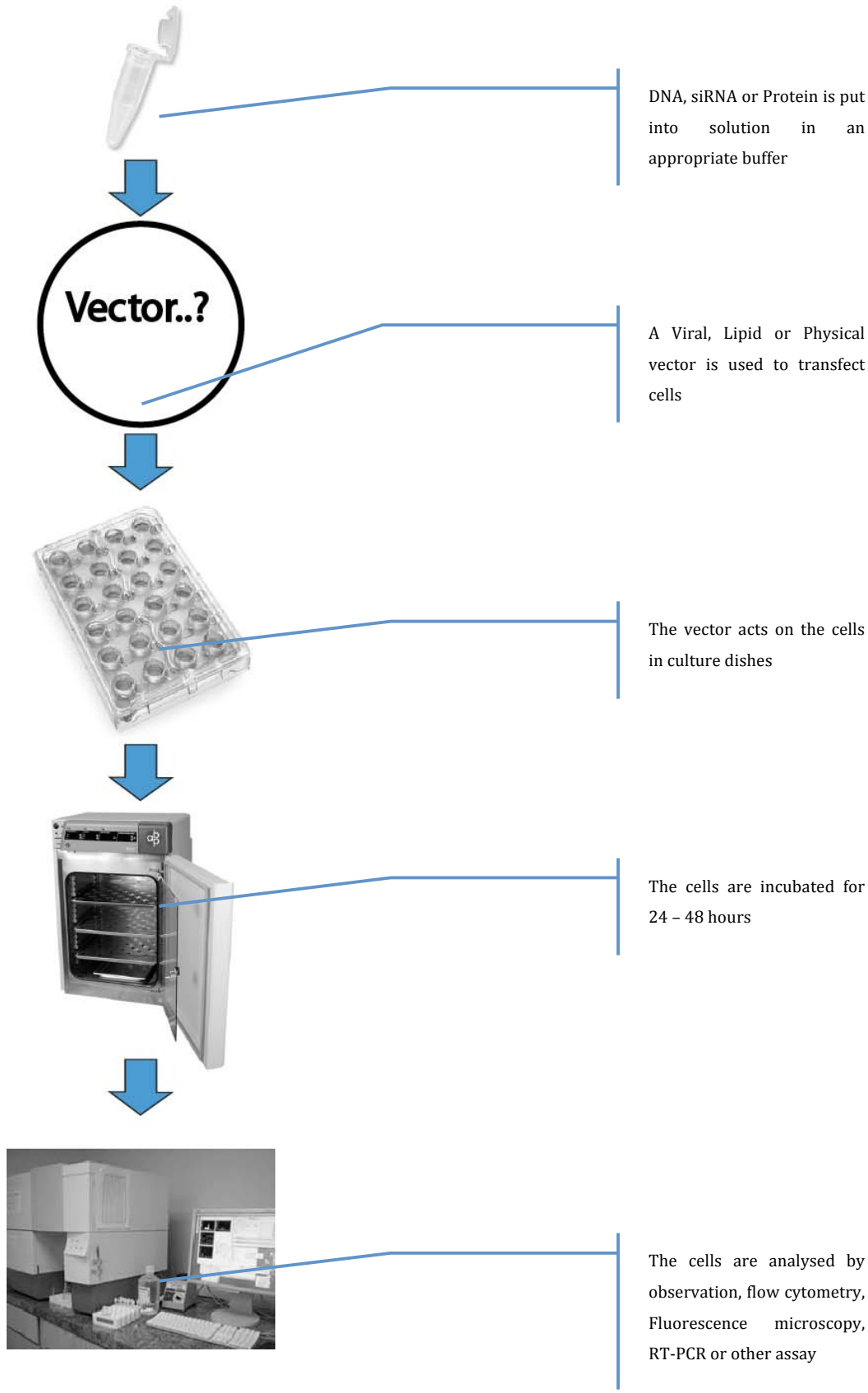


Figure 2-5 Transfecting cells

2.7.2 Viruses

Viruses are small, dependent entities that comprise a genetic core surrounded by a protein coat called a capsid. Viruses insert their genome into the host cell and transfect it. Viruses such as adenovirus are used as a vehicle for transporting material into human cells in gene therapy research. Although expensive, highly specialised and slow, viral vectors work well in vitro. A culture dish, however, does not have an immune system and in vivo, problems associated with immune responses are reported. An immune response occurs where the body recognises and rejects the virus. This limits the potential for administrating a therapeutic and for repeated treatments in chronic disease.

The lungs and airways have evolved to provide limited sites for viruses to attach to and to inject their load. Viruses have caused situations where gene therapy medications have gone badly wrong. In two cases of note, a gene therapy was developed to address SIDS also known as bubble boy syndrome. Unfortunately, the vector gave rise to incidence of leukaemia in some subjects on the clinical trial. Subsequent to this, the gene therapy trial was discontinued and there has been considerable difficulty in getting trials approved since. In a second case a young adult Jessy Gelsinger died during an American gene therapy trial. Once again, the vector was at fault rather than the biologic (Fox 2000). Non-viral gene transfer agents are generally less efficient than viral vectors in transfecting lung epithelium, however, if the virus is free of non-human protein components, it is less likely to induce an immune response, thereby allowing for repeat administration. This is critical for the treatment of CF and other chronic diseases that will require life-long treatment (Griesenbach and Alton 2009).

2.7.3 Physical force vectors; the Bio-Rad gene gun

The BioRad Gene Gun (Figure 2-6) was developed at Stanford University USA is used widely in plant biology (Beetham, Kipp et al. 1999). It propels biological material into plant cells by coating gold particulate with the biologic and then firing it at the target plant cells by way of a blast of helium gas. A plant cell is different from a human cell in that it has a cellulose cell wall whereas the mammalian cell does not have a cell wall (Bolsover 2004). Whilst the gene gun is widely used in plant biology labs, it does not readily translate to human

biology. The gun cannot be placed in the lungs or airways and the system releases one shot at a time with reloading required between shots. It has an inaccurate delivery system. It was developed for plant biology and is unsuitable for human in vivo work. Agglomeration of nanoparticles causes pitting damage to cells.

Figure 2-6 The Gene Gun Licensed and manufactured by Bio-Rad Inc.

2.7.4 Electroporation

Electroporation is a process where cells, normally in vitro, are subjected to a brief, strong electric field pulse. This process has the effect of opening pores in the cell membrane allowing transport of biological material into the cell. From here the cell can be transfected. When the electric field strength across a cell membrane is sufficiently large, channels in the cell membrane open. The effect brings about an overall increase in the membrane permeability (Wolf, Rols et al. 1994; Palmer 2007). Electroporation of the cell membrane can become irreversible if one or more channels or pores become too large or where many pores fuse. The membrane potential is proportional to the pore size until an irreversible state is reached. This occurs when the pore size reaches a diameter of one half of the thickness of the membrane. The exact mechanism of electrical membrane failure has not been determined. It is thought to be initiated by molecular defects in the bilateral component of the cellular membrane (Lee 2006). Electroporation can be detected in culture as a sudden reduction of electrical impedance across the cellular membrane. The membrane voltage

required for electroporation depends on the duration of the stimulus. Reversible electroporation is used as a physical vector in biology in vitro.

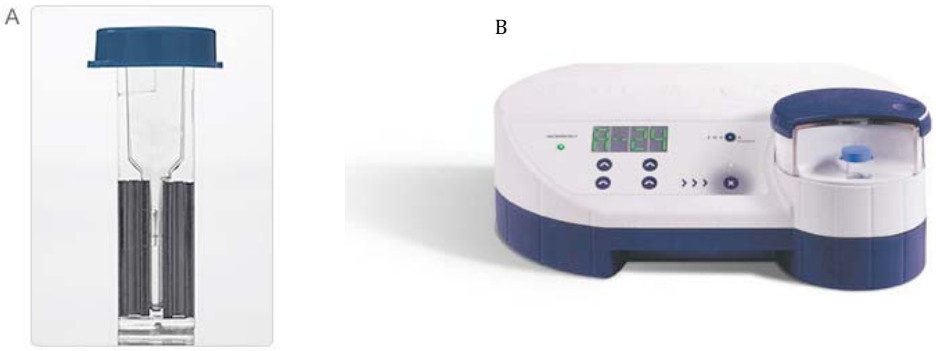


Figure 2-7 (A) Nucleofector Cuvette (B) Nucleofector Device, by Amaxa Inc

A commercial electroporator, the Nucleofector is shown in Figure 2-7. The transfection takes place in a proprietary cuvette (A) into which are placed the cells, the biologic and a proprietary buffer. The device (B) controls the duration of the transfection and permits selection of other transfection types. Recently, electroporation has been proposed as an in vivo method of transfecting cells within tissues. A sample of tissue is held within 2 electrodes. A brief pulse of electricity, typically $300Vcm^{-1}$ -$1000Vcm^{-1}$ is applied across the tissue. A drug has been administered systemically prior to the procedure. As the drug circulates through the body, the area of electroporated tissue has a greater susceptibility to uptake of the drug than the surrounding tissues (Weaver James, Langer et al. 1995).

One of the potential disadvantages of this technique is the risk of non-specific uptake. The cell membrane is designed to keep foreign material out of the cell so cells that have had their membrane compromised in this manner are vulnerable to whatever may be circulating in the bloodstream at that time. In contrast, a cell subjected to physical ballistic vector will only admit material at the point it reaches the cell membrane. Benefits of electrospray over existing technologies include delivery of genes without complications experienced by other vectors such as immunogenicity, risk of viruses causing cancer, toxicity and low efficiency associated with liposomes.

2.7.5 Nanoparticle generator

The nanoparticle generator (Figure 2-8) is a laboratory device. It was licensed to its manufacturers by researchers (Chen, Wendt et al. 2000). It is essentially a mass spectrometry frontend in its own enclosure. It operates in a vacuum or with the assistance of a carbon dioxide sheath gas. The working fluid for which it is designed is deionised water and nanoparticles in solution. The device runs only in negative ion phase, that is a counter electrode is raised to high potential whilst a metal capillary is grounded. Its form factor is that of a large laboratory instrument. It cannot be placed in the lungs or airways and requires an electrode behind target. It requires a mechanism to strip excess charge from the electrospray and is restricted to one working fluid.

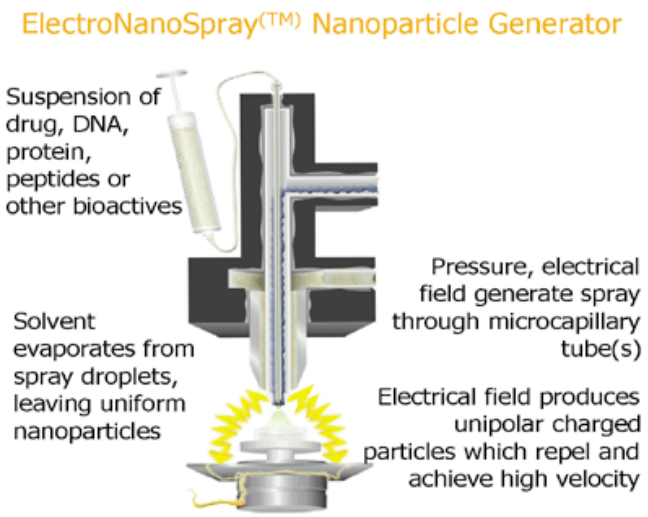


Figure 2-8 Nanoparticle generator by Nanocopoeia Inc

2.7.6 Endoscopic spray catheter

A novel device for creating a localised aerosol in the lungs is a bronchoscopic spray catheter depicted in Figure 2-9. The catheter is fed down the working channel of an endoscope until the physician can see its tip within the lungs or airways. A high pressure is applied to fluid within the catheter and a mechanically produced spray issues from the tip (Coates, Koehler et al. 2004). Depending on the manufacturer, Trudell Inc being one example, the tip may

comprise multiple channels. The spray that issues from the catheter is typically 5-10μm in diameter. Practical experience with such catheters indicates that they frequently block (McLachlan 2010). They also require a high backpressure to operate effectively. Lastly, their efficacy is dependent on the viscosity of the working fluid (Coates, Koehler et al. 2004; McLachlan, Baker et al. 2010). The normal mode of operation is to produce a spray of gold nanoparticles in solution. The biologic is precipitated onto the particles and enters the cell in a 'shotgun' fashion. Cells can experience pitting damage when bombarded with metal particulate.

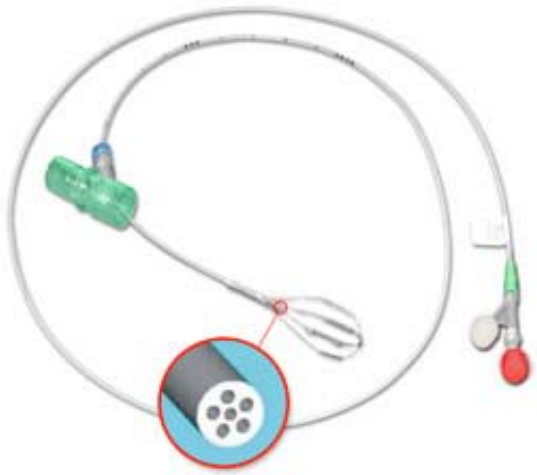


Figure 2-9 Endoscopic spray catheter by Trudell Inc

2.7.7 Aerosols, inhalers and nebulizers

The continuing development of aerosolisation devices perhaps best typifies the pulmonary delivery problem. Devices can be categorised as inhalers, nebulisers, aerosols, dry powder inhalers or as sprays. A solid or a liquid is generally nebulised and a liquid is aerosolised. An inhaler generally gives short bursts of aerosol whereas a nebuliser may be left running for a protracted period and connected to a facemask or part of an endotrachial tube. The anatomy of the mouth, trachea and lung mean that a given particle has a long way to travel before arriving at a particular target. Geometry, humidity, lung clearance mechanisms and presence of lung disease influence the deposition of aerosols

and therefore influence the therapeutic effectiveness of inhaled medications (Labiris and Dolovich 2003a; Labiris and Dolovich 2003b). In the case of lung cancer a potential therapeutic might have to travel through the entire lung structure before arriving at a potential target. Generally, much of the active drug does not reach the intended target and to varying degrees is dissipated en route. Aerosol particle size characteristics can play an important role in avoiding the physiological barriers of the lung, as well as targeting the drug to the appropriate lung region. Mechanically produced aerosol has a droplet size in the region of 5-10 μm. Colloid particles of this size are too big to penetrate an epithelial cell (Pilcer and Amighi 2010). Lately, the research focus within the field of respiratory drug delivery has broadened to include a wide range of potential applications for inhalation by delivering drugs not just onto the lung mucosa but across it (Cryan, Sivadas et al. 2007). In this work, a clear motivation is to treat the diseased lung, not to use it as an anatomical drug delivery device. Placing an electrospray source within the lung will permit finer control of the delivery of therapeutic and diagnostic molecules to identified tissues. Conventional spraying devices have also been used experimentally to attempt to deliver DNA. These have been used to coat the airways, which is highly inefficient for drug delivery, especially in diseased lungs which are usually lined by large volumes of viscous mucus, rather than deliver therapeutics directly into cells (Sakagami 2006).

2.8 Summary

This thesis addresses the critically poor efficacy of existing treatment strategies for lung disease, a major health problem in the EU. In vitro, non-physical vectors are expensive, specialised and not ideal in all circumstances. In vivo, the human anatomy makes delivery of therapeutic and diagnostic molecules challenging and current methods are toxic and immunogenic to varying extents. Physical vectors have been successfully used in plant biology and to an extent in human biology research. A novel electrospray device is proposed to deliver therapeutic and diagnostic molecules into cells. Aimed primarily at lung diseases, the technology will ultimately be applicable to address other disease types and to deliver a wide range of therapeutic and diagnostic molecules.

Table 2-2 Summary of vectors for delivering DNA, siRNA and Protein

Methodology	Toxicity	In Vivo	In Vitro	Targeted Delivery	Safety	Efficacy
Lipids	High	Yes	Yes	No	Poor	High
Viruses	Immunogenic in vivo	Yes	Yes	No	Poor	High
Aerosols	Low	Yes	No	No	Good	Low
Gene Gun (Bio Rad)	Potentially (gold particulate)	No	Yes	Poor	Poor, pitting damage to cells	Low
Electroporation	Non-specific take up	Recently	Yes	Yes	Unknown	High
Nanoparticle generator	Low	No	Yes	Yes	Good	High
Endoscopic spray catheter	Low	Yes	Yes	Yes	Good	Low
Electrospray	Low	Yes	Yes	Yes	Good	Low

Chapter 3

Electrospray in Biology, a Literature Review

There has been a steady increase in interest in electrospray since about 1990. The term electrospray refers to a process where fluid is subjected to an electric field as it passes through a capillary in order to generate a very fine spray of the fluid. When subjected to an intense electric field E_0, the fluid forms a Taylor cone at the top of the capillary (Taylor 1964). The cone is born of equilibrium between electrostatic and hydrostatic forces stretching the meniscus into a cone like a fine spray of charged droplets that fly towards a counter electrode. The total assembly is known as an emitter. Figure 3-1a shows a typical electrospray configuration.

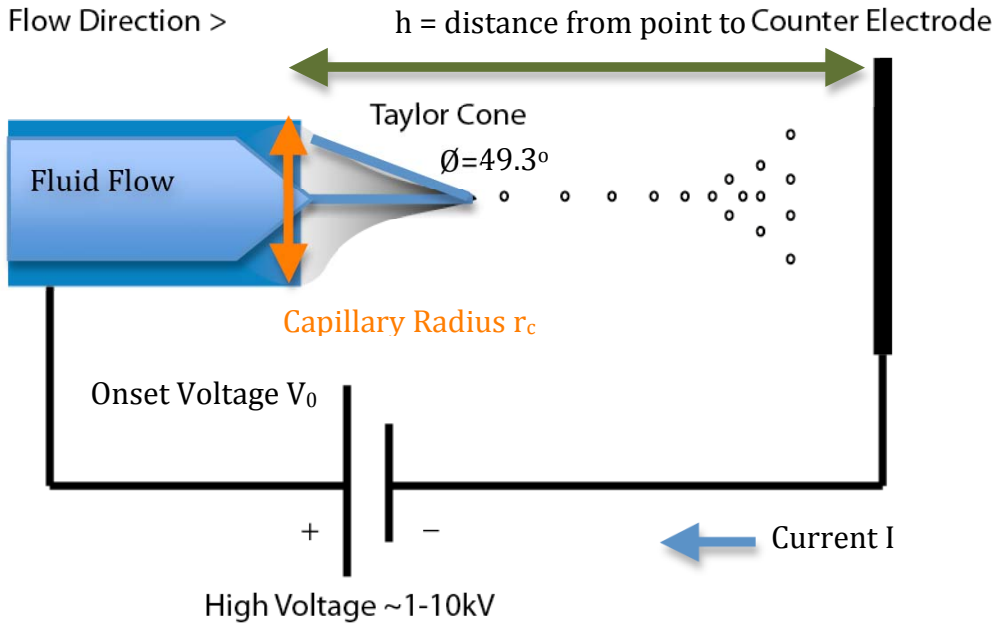


Figure 3-1a Typical electrospray configuration. Fluid has surface tension T and conductivity σ

Figure 3-1b shows a graph of cited papers by year from 1970 to the present containing the term electrospray in the title. Rayleigh (1878) first described the

interaction of electricity and water droplets. Until the 1960s, the phenomenon of electrospray was reported as a series of observations with no obvious application. Since the then, however, applications for electrospray have developed and include crop spraying, paint spraying, mass spectrometry and drug delivery.

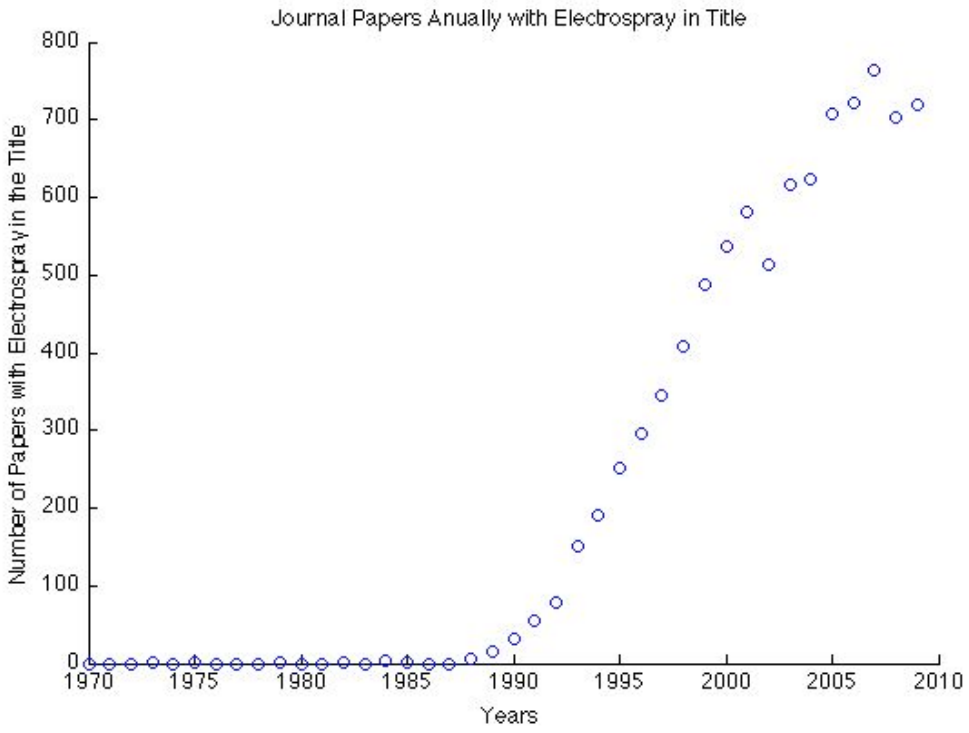


Figure 3-1b A graph showing journal papers annually with electrospray in the title

In Section 3.1 the historical context of electrospray is reviewed. The Taylor cone (Taylor 1964) is central to the production of electrospray. This is reviewed in Section 3.2. There are up to six identifiable electrospray modes (Cloupeau and Prunet-Foch 1994). These are reviewed in Section 3.3 with reference to choosing an appropriate mode with which to deliver molecules to cells. In Sections 3.4 and 3.5, the important mechanical and electrical parameters that influence electrospray are discussed and numerical models of electrospray are reviewed. In Section 3.6 the working fluid is discussed with reference to the different chemical formulations and types of fluids that have been sprayed. In Section 3.7 current work pertaining to biological and drug delivery work in the area is reviewed and discussed. In Sections 3.8 and 3.9 electrospray emitters and counter electrode topologies are reviewed. A review of closed loop control

in electrospray systems is related in Section 3.10. Safety and efficacy are reviewed in Section 3.11 with a view to regulatory classification and in vivo use of an electrospray device. Conclusions on the review are presented in Section 3.12.

3.1 Electrospray Historical Context

The interactions between water and electricity have been of interest to researchers since the nineteenth century. Rayleigh describes the effect of electricity on colliding drops (1878) and on the equilibrium of liquid conducting masses charged with electricity (Rayleigh 1882a). Equilibrium between hydrostatic and electrostatic forces is at the core of all models of electrospray. Electrospray, a spray or jet of charged droplets, was first reported in the literature by Zeleny (1917). Reporting on observations from the Sloane Laboratory at Yale, Zeleny describes and photographs ethyl alcohol (ethylene) eluting from a capillary as the potential between the capillary and a metal plate is varied. The resulting plume exhibits the main modes of electrospray. The presentation style was one of observation rather than analysis.

In the 1920s JJ Nolan, Professor of Experimental Physics at University College, Dublin reports on the breaking of water drops by electric fields (Nolan 1926). It is of historical interest that he and his brother PJ Nolan formed the Atmospheric Physics Group that gained international recognition as the 'Nolan School'. Wilson and Taylor (1925) report a fascinating experiment where a soap bubble is placed on a metal plate forming a hemisphere. A parallel plate is positioned close to it. A potential is applied across the plates. The bubble is seen to deform into a peak pointing towards the opposing plate. A short-lived fluid jet is seen to emanate from the soap bubble peak prior to it bursting. The importance of this experiment is that it presents the bubble as a surface in physical equilibrium until an electrical force disturbs it. The fact that Taylor went on to model his eponymous cone on this experiment, marks the soap bubble experiments as the beginning of the analytical treatment of electrospray.

In the General Electric Laboratory in the 1950s there was interest in the interaction of water droplets on high voltage (HV) conductors. Vonnegut and Neubauer (1952) describe experiments in which a drop of water is placed on a wire and the voltage increased. Beyond 5000VDC the drop develops a 'tiny sharp bump' from which a mist is ejected. The description continues, describing smoke emanating from the droplet. From the 1960s, due mainly to the work of Taylor (Taylor 1964; Taylor 1969) electrospray was described in analytical terms by separating out the component forces at work and describing their interrelationships. It was known that the jet or electrospray carried a charge and that a small current flowed from emitter to the ground plane. Basic relationships emerged beginning with Taylor's scalar (Taylor 1964)

$$I \sim F^{1/2} \tag{1}$$

where I is current carried on the droplets in the electrospray and F is the fluid flow rate. Recent models make excellent predictions of observed features under many conditions of electrical potential, fluid flow and fluid character (Fernández de la Mora 2007).

A closely related process, electrospray ionisation (ESI) is also termed 'soft' ionisation. It was first proposed that ions could be made of macromolecules such as proteins and DNA in the late 1960s (Dole, Mack et al. 1968) but it took another 15 years to produce convincing experimental results. ESI is the process of generating a gas phase ion from a chemical solution and is called 'soft' because the molecule being ionized is not broken up during the process. This capability is now widely used in biology, analytical chemistry, pharmacology and medical research. Fenn won the Nobel prize in Chemistry in 2002 (Fenn 2002) for work based on reporting the first experimental evidence for ESI of macromolecules (Yamashita and Fenn 1984). ESI is an important reference point for this work because it is a more developed application based on the same technology and it is apparent that DNA, proteins and other biological molecules can survive the physical and electrical stresses and shearing forces involved in electrospray.

Smith's study (1986) on the onset parameters of electrohydrodynamic (EHD) atomisation of liquids presents a qualitative model including capillary radius, liquid conductivity and current – voltage characteristics. One significant finding is the relationship between electrospray onset electric field strength, E_0, and the capillary – counter electrode plane as depicted in Figure 3-1a. This relationship is given by Equation (2) that will be derived in Section 3.5 in this Chapter. Where r_c is the capillary radius, T is the fluid surface tension, θ_0 is the Taylor Angle or the Half angle of the apex of the cone and ε_0 is the permittivity of the ambient medium.

$$E_0(r_c) = \left[\frac{2T\cos\theta_0}{\varepsilon_0 r_c} \right]^{1/2} \tag{2}$$

In the 1990s interest developed in electrospray as a method of forming aerosols for drug inhalation. The most significant finding was that colloids forming electrosprays are monodisperse or of a similar size and that this size can be predicted as a function of flow rate (Gomez 1993). In practice, the aerosol volume output of one emitter would limit the effectiveness of such devices. When compared to an inhaler or nebuliser device, however, the possibility of arrays of emitters would overcome this problem. In the field of jet propulsion, arrays of emitters are routinely used (Krpoun, Raber et al. 2007).

Research examining electrospray as a physical vector for delivery of therapeutic molecules has successfully demonstrated proof-of-principle (Chen, Wendt et al. 2000) but is constrained to the in vitro environment due to the form factor of the equipment. Additionally, results remain inefficient and inconsistent. The inefficiency and inconsistency may be due to limited understanding of the impact of the process of electrospray on cellular targets. In addition, current work is limited to DNA-based molecules with no reference to other therapeutic or diagnostic molecules such as proteins, chemotherapeutics, antibiotics, radioisotopes and dyes.

3.2 The Taylor Cone

The pointed fluidic feature that forms on the top of a fluid filled capillary under electrical stress is called the Taylor Cone. In a typical setup, an electric field is present at the open end of the capillary. The capillary generally has an outside diameter < 100μm. Electrostatic and fluidic forces are balanced on the surface of the liquid interface. Under circumstances where no flow and no electric field exist, the fluid interface with the surrounding atmosphere, usually air, is a meniscus (Figure 3-2a). When an electric field is present (Figure 3-2b), like the soap bubble experiments described in Section 3.2, the meniscus becomes elongated. The fluid surface comes under the influence of the electric field. Under electric stress the curvature of the surface about the axis tends towards zero radius (Figure 3-2c) and the meniscus tends towards a cone (Taylor 1964).

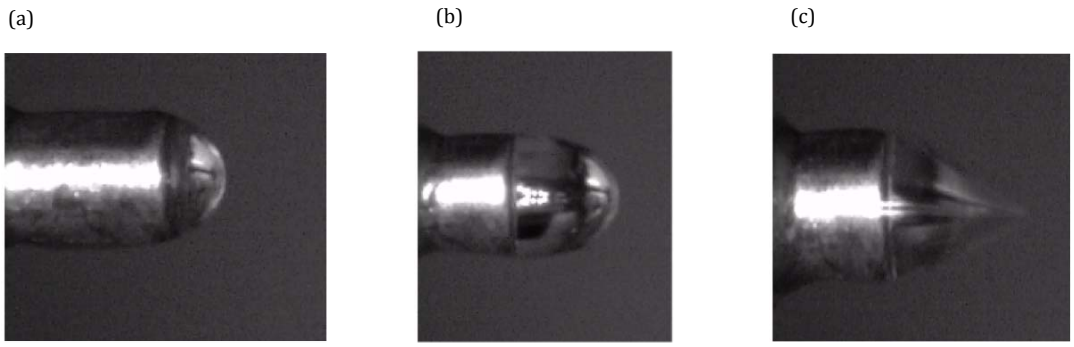


Figure 3-2 (a) The end of the capillary under no flow or electric field. (b) No flow, electric potential applied $V < V_{onset}$ of electrospray. (c) Curvature of the surface about the axis tends towards zero radius. Photographed by the author

As the potential is increased to $V > V_{onset}$ the fluid forms a sharp point. At the tip of the cone the fluid interface ruptures and the fluid changes shape into a fine jet . Within the jet, a point is reached when the liquid can hold no more electrical charge (Rayleigh 1882). At this point the jet becomes unstable and explodes into a mist of fine highly charged droplets of less than 5μm in diameter (Rosell-Llompart and Fernandez de la Mora 1994). The droplets all carry the same electrical charge and so repel each other very strongly, moving apart from each other to cover a relatively wide surface area. The droplets fly out towards the plane opposite in charge to their own whilst rapidly shrinking as molecules

evaporate from their surface. As droplets are produced, another process termed coulombic fission is occurring (Fernandez de la Mora 1996; Fernández de la Mora 2007). In essence the droplets produced break into smaller satellite drops. This is due to a droplet reaching its Rayleigh limit where it cannot sustain any more charge. The Rayleigh limit is calculated as a limiting charge, C or voltage V that an isolated droplet of radius r_0 can hold before becoming unstable (Rayleigh 1882a).

$$C^2 > 4\pi r_0^3 T(n+2) \tag{3}$$

The droplet becomes unstable for a displacement proportional to the Legendre function $P_n(\cos\theta)$ provided $n \geq 2$. n denotes the index of the Legendre polynomial $P_n(\cos\theta)$. When $C^2 < 16\pi r_0^3 T$ or $V < (16\pi r_0 T)^{1/2}$, the droplet is stable for all displacements and when V first exceeds this value the droplet becomes unstable only for the disturbance $P_2(\cos\theta)$ for which it becomes slightly ellipsoidal whilst the displacement is small.

As charge escapes from the cone rupture, it is replaced at the cone (Higuera 2003). Thus a current flows that can be measured and used as the basis of a feedback system (Gamero-Casta, Ntilde et al. 2002). In Taylor's equilibrium model (Taylor 1964) the angle made between the central axis and the cone generatrix is calculated to be 49.3 degrees and equilibrium can only exist at this angle. It is assumed that the generatrix is straight. In other models and experimentally, it has been demonstrated that there may be a number of angles for given flow rates at which this equilibrium exists (Joffre, Prunet-Foch et al. 1982). This deviation from the ideal, in air, is attributed to corona discharges in the area immediately around the capillary relaxing the electric field and altering the hydrostatic – electrostatic balance. In current models, the geometry of the observed cone is predicted. Whereas the cone is described usually as having convex generatrix, frequently it is observed with convex-concave generatrix. This is attributed to the flow rate (Cherney and Leonid 1999; Chiarot, Gubarenko et al. 2008).

Electrospray is a phenomenon that occurs under specific conditions of voltage, flow rate, fluidic properties, emitter geometry and atmospheric conditions. Maintaining and controlling electrospray is challenging and its underlying mechanism is not yet completely understood.

3.3 Important Parameters of Electrospray

With reference to Figure 3-1a depicting a point to plane configuration electrospray source, Table 3-1 lists the significant parameters that contribute to the production of electrospray.

Table 3-1 Electrospray parameters

Parameter	Symbol	Comment	Units	Reference
Electrospray voltage onset	V_0	Potential rises until a cone is formed.	Volts	(Taylor 1964)
Fluid Flow rate	F	Flow rate increases in inverse proportion to the conductivity: Increases flow rate	µLitres min^{-1}	(Higuera 2003)
Conductivity of fluid	σ	As conductivity increases, flow rate, and droplet size decrease	Siemens m^{-1}	(Fernández de la Mora 2007)
Electric field at emitter tip	E	At its greatest at cone apex	Volts m^{-1}	(Smith 1986)
Fluid viscosity	η	Drop size increases with viscosity	Pascale.Seconds Pa S	(Smith 1986)
Radius of the emitter capillary	r	V_0 increases with increasing radius	meters	(Smith 1986)
Distance between emitter and earthed plane	h	V_0 decreases continually with decreasing h	meters	(Grace and Marijnissen 1994)
Current carried by charges on the droplets	I	Current is proportional to the root of Flow rate	Amps	(Gomez 1993)
Liquid Surface tension	T	Effects the V_0 only. High T fluids cannot be electrosprayed as medium breaks down before V onset is reached	Newtons m^{-1}	(Fernández de la Mora 2007)
Dielectric strength of the ambient medium	ε_0	Impacts the droplet velocity and V_0 for high T fluids	farads per meter	(Aguirre-de-Carcer and de la Mora 1995)

3.4 Electrospray Modes

3.4.1 Dripping mode

In dripping mode, depicted in Figure 3-3a, fluid eluting from the capillary, where no electric potential is applied, tends to form a droplet larger than the radius of the capillary and clings to the outside radius. As the fluid flow continues, where fluid flow is typically in the order of unitary μLs per minute, the droplet forming at the base of the capillary is drawn down by gravity until it hangs down and eventually falls. As the fluid flow continues the droplet expands, becoming heavy, and eventually drops off the capillary. Thereafter, a new droplet replaces it. As the DC potential is increased for a constant fluid flow rate, the creation of droplets increases to a frequency of up to 500 droplets per second (Cloupeau and Prunet-Foch 1990). Beyond this point other identifiable modes commence. Characteristically the current recorded is in the form of peaks occurring at intervals matched to droplet formation (Evans and Hendricks 1972). In general, in dripping mode the size of the drops is greater than the size of the capillary.

3.4.2 Micro-dripping mode

This mode, depicted in Figure 3-3b, has been described in the literature, however was not observed in this work. Micro-dripping mode tends to occur in fluids having very low conductivities. The micro-dripping mode is characterised by a long (relative to the length of the Taylor cone) thin jet, extending from the apex of the Taylor cone. The jet may terminate in a transient droplet at its end. The length of the jet is variable and it forms and disintegrates at regular intervals. These intervals range from tens of cycles per second to thousands of cycles per second (Cloupeau and Prunet-Foch 1994).

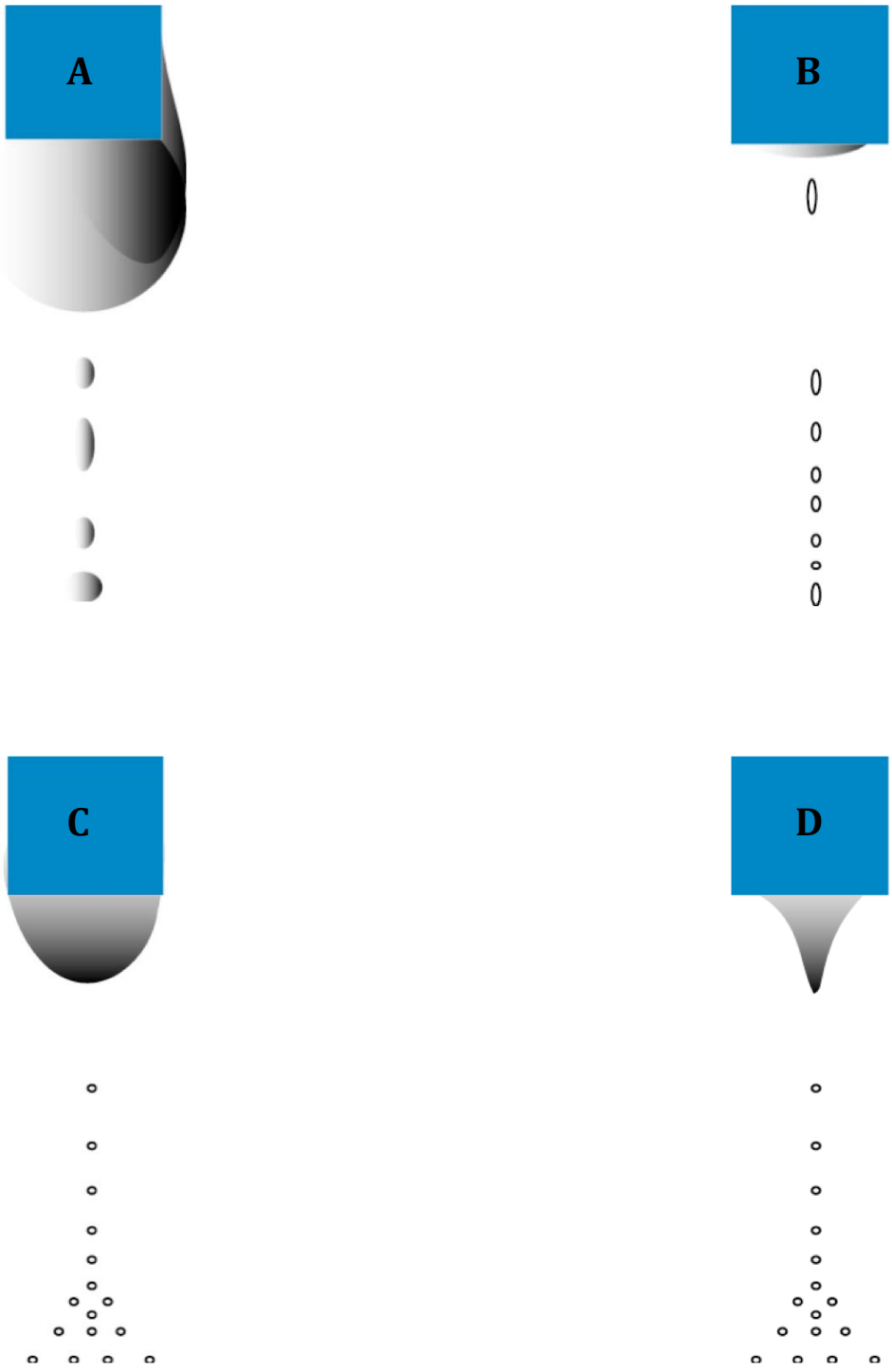


Figure 3-3 (a) Dripping Mode (b) Micro dripping mode (c) Pulse-jet mode (d) Cone-jet mode

3.4.3 Cone-jet mode

As DC potential is raised beyond the dripping mode and for a constant flow rate, the next functioning mode that occurs, depicted in Figure 3-3d, is the cone-jet mode. The term, a compound word, describes the emergence of a permanent jet from the apex of the Taylor cone. Cone-jet mode occurs most easily in liquids of relatively high conductivity and the jet that emerges gives rise to a spray of droplets. The jet is very narrow relative to the radius of the capillary. This mode is very widely reported as giving rise to a spray of monodisperse droplets that have potential for applications in drug delivery (Gomez 1993; Tang 1994; Gomez 2002).

In this mode, the surface of the Taylor cone is equipotential except at the apex of the cone. This is the only point from which the jet can issue. This mode occurs through a wide range of higher conductivity fluids from 10^{-1} Sm^{-1} to 10^{-9} Sm^{-1} (Cloupeau and Prunet-Foch 1989). The work in this thesis is done in air rather that under experimental conditions of vacuum or in the presence of sheath gases. At higher potentials in air, corona discharge accompanies the electrospray increasing the current readings (Aguirre-de-Carcer and de la Mora 1995; Fernandez de la Mora, de Juan et al. 1997).

3.4.4 Pulse-jet mode

Intermittent cone-jet mode, also known as pulse-jet (Chen, Pui et al. 1995), depicted in Figure 3-3c, is a variant of the cone-jet mode. The principal difference between the modes is the discontinuity of the electrospray. Pulse-jet occurs at higher potentials for a given flow rate cone jet mode. It precedes further chaotic modes variously known as multi-jet, spindle and ramified jet. Pulse-jet can be identified using an oscilloscope where the electrospray current is measured and takes on a characteristic of current waves in the frequency range 1kHz to 10kHz. The transition from steady cone-jet to pulsating mode is qualitatively understood to be due to surface liquid momentum. As the potential is increased, liquid flows faster to and along the surface towards the apex of the cone. The greater volume of fluid at the apex alters current flow. This causes the potential to drop near the cone apex and causes the meniscus to relax back. The

resulting reduction in fluid flow to the apex permits the apex to form a sharp point again and so the system oscillates through phases of cone and meniscus formation and resulting discontinuous electrospray production (Smith 1986). Pulse-jet mode was not used in this work. It is also known to create droplets that are not monodisperse but vary in size as the mode oscillates between jet formation and disappearance.

3.4.5 Transition from mode to mode

It is of experimental interest to observe the transition between modes. In a given setup, where the flow rate is kept constant and the potential gradually increased, the transition between dripping mode and cone-jet mode is signalled by the appearance of a steady ionic current, measured with an electrometer and an oscilloscope, flowing between the capillary and the counter electrode. Beyond this mode, a higher frequency oscillation is observed on the oscilloscope signalling the commencement of intermittent cone-jet or pulse-jet mode. Similarly, for a given potential, variations in the flow rate to the capillary will bring about changes in the electrospray mode. Lastly, for a given setup the mode of electrospray is heavily influenced by the conductivity of the fluid (Smith 1986). The conductivity is normally assumed to remain constant throughout the experiment. This assumption may give rise to future work. Reducing the potential or flow rate in any given setup manifestly indicates significant hysteresis in the system.

3.5 Models of Electrospray

3.5.1 Nomenclature

Symbol	Parameter
h	Capillary to plane height
k	Thickness of moving liquid layer
E_θ	Electric field normal to cone surface
E_r	Electric field along cone surface
σ	Liquid conductivity
I	Atomization Current
Δp	Internal pressure in the cone
F	Liquid flow rate
q	Charge density at the surface
R	Radial distance from apex of cone
R_c	Radial distance to capillary edge
R_f	Radial distance to ligament edge
r_c	Radius of capillary
T	Surface tension of liquid
V	Electric Potential
V_0	Electrospray onset potential
ρ	Liquid Density
ε_0	Permittivity of free space
ε	Liquid permittivity
θ	Angle from cone apex
θ_0	Cone half angle
n	Unit normal to elemental volume in radial direction
r_0	The radius of a fluid colloid

Models describing electrospray production derived since Taylor's model (1964) are generally based on the balance between hydrostatic and electrostatic forces. These are generally presented in the case of the field between a point and a plate (Figure 3-4). In this work, the mode of interest is the cone-jet mode

because it is indicated that this mode gives rise to monodisperse colloids and may be suitable for drug delivery. Fortunately, this mode has been widely modelled. A useful starting point is Smith's (1986) model of the cone-jet steady state. This model is widely cited in the literature(Grace and Marijnissen 1994; Fernández de la Mora 2007; Chiarot, Gubarenko et al. 2008). The model assumes constant current flow and linear cone generatrix and an infinitely conducting liquid. The general case for the electric field strength between a point and a plane is presented from the work of MD Van Dyke in Appendix 1 (Taylor 1969).

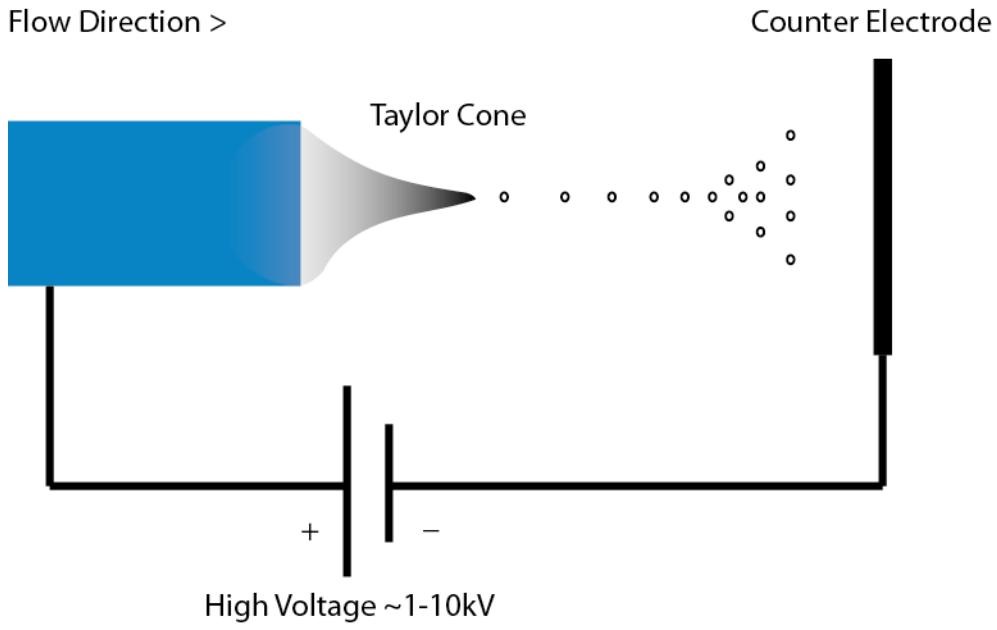


Figure 3-4 Typical point to plane geometry of electrospray setup

3.5.2 Model solution for V_0 the electrospray onset voltage

It is useful to estimate the voltage value of the onset of electrospray, V_0, when evaluating a potential new buffer or solution to electrospray. It is assumed that the potential drop between the fluid jet and the cone is negligible and the cone is equipotential. It is further assumed that that the same field electric conditions are required to establish the cone of a given surface tension regardless of the geometric setup. Then V_0 is related to the electric field required to establish the cone from the liquid surface at the emitter's capillary edge $E_0(r_c)$ by (4)

$$E_0 = \frac{V_0}{f(r_c, h)} \tag{4}$$

Where $f(r_c, h)$ is a geometrical function of the capillary radius r_c and the emitter to plane distance h. The function has been derived by (Jones and Thong 1971) and is derived in Appendix 1 and A_1 is a constant arrived at experimentally. Values for A_1 are presented in Table 3-2.

$$f(r_c, h) = A_1 r_c \ln(4h/r_c) \tag{5}$$

Table 3-2 Values for Constant A_1

Reference	Comments	Value
Van Dyke (1969)		0.707
Jones and Thong (1971)		0.5
Experimentally Measured Value		0.91
(Pantano, Gaón-Calvo et al. 1994)	1994 Expression for the value	$A_1 = \frac{\sin\theta_0 (y_1 \tan\theta_0)^{1/2}}{\ln(4hr_c)}$ $y_1 = \frac{(\varepsilon_0 V_0^2)}{2\sigma r_c}$

At V_0 at the time that the cone is formed, the voltage is such that it gives rise to a field that balances the fluidic pressure due to surface tension T. This is of the form

$$E_0(r_c) = \left[\frac{2T\cos\theta_0}{\varepsilon_0 r_c} \right]^{1/2} \tag{6}$$

Where θ_0 is the half angle of the cone. This form was assumed by Taylor (1969), and the effect of hysteresis is ignored Resulting from (4) and (6) the voltage at the point of establishment of the cone is

$$V_0 = A_1 \left[\frac{2T \cos\theta_0}{\varepsilon_0} \right]^{1/2} \ln(4h/r_c) \quad (7)$$

3.5.3 Electrohydrodynamics of the cone-jet mode

When an electrospray is established it is fed with fluid from the apex of the Taylor cone. It is proposed that this fluid is eluted from the conical meniscus due to a radial electric field component. When the potential on the fluid meniscus is raised beyond the critical instability value, the meniscus tends towards zero radius of curvature on the axis (Miksis 1981). A very high field is created and charge is emitted on the jet and resulting droplets. To conserve charge, a current flows compensating for the loss in charge and the cone presents a resistance to this current according to its bulk conductivity σ. As such a radial electric field E_R is created which gives rise to surface stress in the radial direction as described by the Maxwell stress tensor. This causes fluid near the surface to move towards the apex. The convection current moving fluid and charge towards the surface is maintained by a conduction current. Steady state is reached when the voltage reaches a level that brings about a field creating a cone. The assumptions for the model are listed in Table 3-3.

Table 3-3 Model assumptions

Assumptions	Reference
Relaxation time (ε / σ) of the charge carriers is short relative to the other times in the system	(Taylor 1964)
the liquid is inviscid (has no viscosity)	(Taylor 1964)
The emitter capillary potential is constant	(Taylor 1964)
System is operated at 0 hydrostatic pressure	(Taylor 1964)

With reference to Figure 3-5, spherical polar coordinates are used in the analysis, R denotes radial direction and θ in the surface normal direction. The model is symmetrical so Φ is ignored. The Maxwell stress tensor describes the

stresses on the surface. In the radial direction the stress is $\varepsilon_0 E_R E_\theta$ where E_θ is the value of the normal component of the field. In Equation (8), in the surface

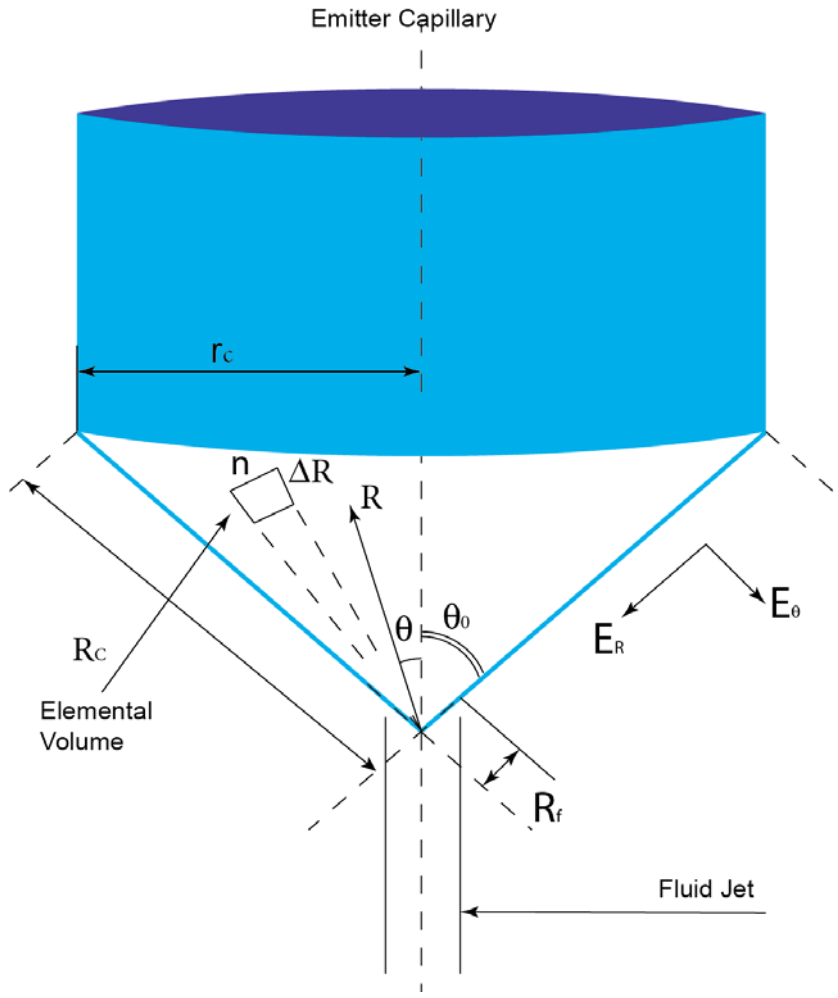


Figure 3-5 Electrospray model diagram

normal direction a pressure balance exists between the internal pressure Δp, surface tension pressure and the normal component of the stress tensor

$$\Delta p = \frac{T \cot\theta_0}{R} - \left[\frac{\varepsilon_0 E_\theta^2}{2} - \frac{(\varepsilon_0 - \varepsilon)}{2} E_R^2 \right] \tag{8}$$

where ε is the liquid permittivity. The radial field E_R can be found by deriving an expression for the impedance Z presented by a cone to a current.

$$Z = \int_R^{R_c} \frac{dR}{2\pi\sigma(1-\cos\theta_0)} \tag{9}$$

$$\therefore V_c - V(R) = \frac{I}{2\pi\sigma(1-\cos\theta_{0)}} \cdot \left[\frac{1}{R} - \frac{1}{R_c}\right] \tag{10}$$

where the subscript *c* means evaluation at the capillary edge and $V(R)$ is the voltage at R. The radial field is therefore given by Equation (11)

$$E_R = \frac{I}{2\pi\sigma R^2(1-\cos\theta_{0)}} \tag{11}$$

For values found experimentally, $1/2(\varepsilon_0 - \varepsilon)E_R \ll T\cot\theta_0 / R$ normally from (8)

$$E_{\theta=}\left[\frac{2T\cot\theta_0}{\varepsilon_0 R}\right]^{1/2} \tag{12}$$

Conservation of mass requires from the flow (in steady state so that $\partial / \partial t \equiv 0$)

$$\int_A \rho v \bullet n \, dA = 0 \tag{13}$$

where A is the surface area of an elemental section of the cone. However for an inviscid liquid the flow will be confined to the surface, as there are no internal forces. A very thin layer of liquid will be assumed to move with a thickness k that is constant with R. Evaluating over a surface element of the cone yields

$$\left\| \rho 2\pi R \sin\theta_0 k v \right\| = 0 \tag{14}$$

where $\| \ \|$ indicates evaluation between R and $R + \Delta R$ similar to Melcher and Warren (Melcher and Warren 1971) and v is the velocity on the surface.

$$\therefore 2\pi R \sin\theta_0 k v = F \tag{15}$$

where F is the flow rate which is constant. For conservation of charge, again $\partial / \partial t \equiv 0$

$$\int_A (q\upsilon + \sigma E) \bullet n dA = 0 \tag{16}$$

where q is the charge density and convection current is assumed to be due solely to movement of surface charge density $\varepsilon_0 E_\theta$. The conduction current I as used in (10) and (11) is $\int \sigma E \bullet n\, dA$ so (16) becomes

$$2\pi R \sin\theta_0 \varepsilon_0 E_\theta \upsilon + I = 0 \tag{17}$$

This is the current that flows to compensate for the charge loss. However for conservation of mass condition (15) and the expression for E_θ (12)

$$I = -1\left[\frac{2\varepsilon_0 \cot\theta_0}{k^2}\right]^{1/2}\left[\frac{T}{R}\right]^{1/2} F \tag{18}$$

This means that the amount of charge that is caused to flow because of the surface stress increases with decreasing R. The equation of motion appropriate for this is given by Equation 19

$$\int_A \rho v (v \bullet n)\, dA = -\oint_A pn\, dA + \oint_A T \bullet n dA \tag{19}$$

The pressure and gradient of pressure are assumed to be insignificant compared to the EHD forces and the radial component of the stress tensor T is reduced to $\varepsilon_0 E_R E_\theta$. Evaluating between R and $R + \Delta R$ yields Equation (20)

$$\left\| 2\pi R \sin\theta_0 p\upsilon^2 \right\| = 2\pi R \sin\theta_0 \Delta R \varepsilon_0 E_R E_\theta \tag{20}$$

And from (11), (12), (15) and (18)

$$\left\| \frac{\rho F^2}{2\pi R \sin\theta_0 k^2} \right\| = \frac{2\varepsilon_0 \cos\theta_0}{k(1-\cos\theta_0)} \cdot \frac{TF}{\sigma R^2} \Delta R \tag{21}$$

$$\therefore F = \frac{4\pi\varepsilon_0 \sin\theta_0 \cos\theta_0 k}{(1-\cos\theta_0)} \cdot \frac{T}{\rho\sigma} \tag{22}$$

Meaning flow rate increases in inverse proportion to the conductivity of the fluid. From (18) at $R = R_f$

$$I \sim \frac{T^{3/2}}{\rho\sigma R_f^{1/2}} \tag{23}$$

This means current is expected to be higher for fluids of higher surface tension and lower density.

3.6 The Working Fluid; Buffers, Reagents and Solutions

The terms buffer and solution are used interchangeably in the literature. A buffer resists changes in pH and is generally used in biology to dissolve DNA, siRNA and other molecules. A reagent is that which is added to a solution or a buffer. In the literature, to date, such biological buffers have not been characterised for electrospray. On the materials specification data sheet (MSDS) for commercial buffers it is common to see the chemical compound and some chemical characterisation such as pH and molarity. Figures for electrical conductivity, surface tension and viscosity are not generally given. Consequently, in this work, these measurements were made empirically.

It is nonetheless useful to reference other fluids that are generally used in electrospray for the purposes of reference. For instance, methanol and ethanol at various dilutions and with modifiers such as acetic acid are commonly reported. In Table 3-1, the character of common fluids are listed from the literature. These are also useful in carrying out ranging calculations and test setups for the electrospray experimental rig.

Table 3-4 Electrospray Reported Parameters

Fluid	Conductivity (S m^{-1})	Surface Tension (N m^{-1})	Absolute Viscosity (mPa s)
dH_2O	1.2×10^{-4}	.073	0.98 (@22^{0}C)
Water	2.5×10^{-4}	.03	-
Water 0.01%NaCl	6×10^{-2}	.044	-
Water 0.05%NaCl	2.9×10^{-1}	.044	-
Water 0.1%NaCl	5×10^{-1}	.044	-
Methanol 1% acetic acid	2.16×10^{-3}	.016	-
Methanol	2.3×10^{-3}	0.021	0.817
Ethanol	1.1×10^{-5}	0.023	1.2

It is noteworthy that there are often variations in the values reported for similar fluids. This is generally put down to the fact that fluids are used off the shelf and there may be variations in preparation, temporal changes in character or impurities in the formulation (Grace and Marijnissen 1994).

3.6.1 Electrical and physical fluid character

The effects of surface tension and conductivity on the electrospray process are reported experimentally. They can also be predicted from the model in Section 3.5. From Equation (4), V_o potential is expected to be proportional to the surface tension T of the liquid. From Equation (23) the electrospray current I should be proportional to $T^{3/2}$. Experimentally and from data presented by Smith (1986) $V_0^2 \propto T$. The onset of EHD was observed to be dependent only on the surface tension for constant capillary plane parameters. Fluid conductivity has a significant effect on flow rate. From Equation (22) flow rate is inversely proportional to conductivity. The effect of viscosity on electrospray production is not predicted by the model but is experimentally reported to increase droplet size (Tang and Gomez 1996).

3.7 Electrospray in Biology and Drug Delivery Applications

Aerosolisation is an established science and has known and reported limitations in drug delivery (Labiris and Dolovich 2003a). Electrospray is relatively new and has qualities that present significant possibilities for research and clinical application. Electrospray differs from aerosol as internal electrostatic forces

rather than mechanical external forces drive it. In electrospray, fluid colloids of similar charge repel each other and achieve high speeds and tiny volumes relative to those of an aerosol of a similar fluid.

3.7.1 Creating sprays with predictable and consistent droplet size

Tang (1994) established that the stability of electrospray depends on conductivity and that flow rate was the most significant contributor to droplet size. The application of his work was an inhaler to deliver consistent droplets to the airways. He reports achieving a droplet size between 2 and 10 μm consistently. He concludes that such a device would be suitable for drug inhalation.

In the case of delivery applications in biology, water is frequently the solvent. Water creates unique problems for electrospray because it has very high surface tension in the region of 73×10^{-3} Nm^{-1} and low conductivity of 1.2×10^{-4} Sm^{-1} (Tang and Gomez 1994). This means high potential differences between the emitter and the counter electrode that can result in corona discharge in atmospheric conditions. Corona discharge is a flow of ions from a conductor to a surrounding fluid and creates instability in the Taylor cone. This necessitates the use of a sheath gas when using water. The sheath gas travels coaxially to the direction of the electrospray and prevents corona discharge from occurring. It is noteworthy that corona discharge can be used elsewhere within a system to neutralise any residual charge on an electrified aerosol. A variation on the electrospray aerosol was patented by Electrosols UK Ltd (Coffey 1999). Among the claims of this patent is the use of positive and negative electrospray emitters. As depicted in Figure 3-6, these are targeted at each other such that the charge on the droplets is neutralised.

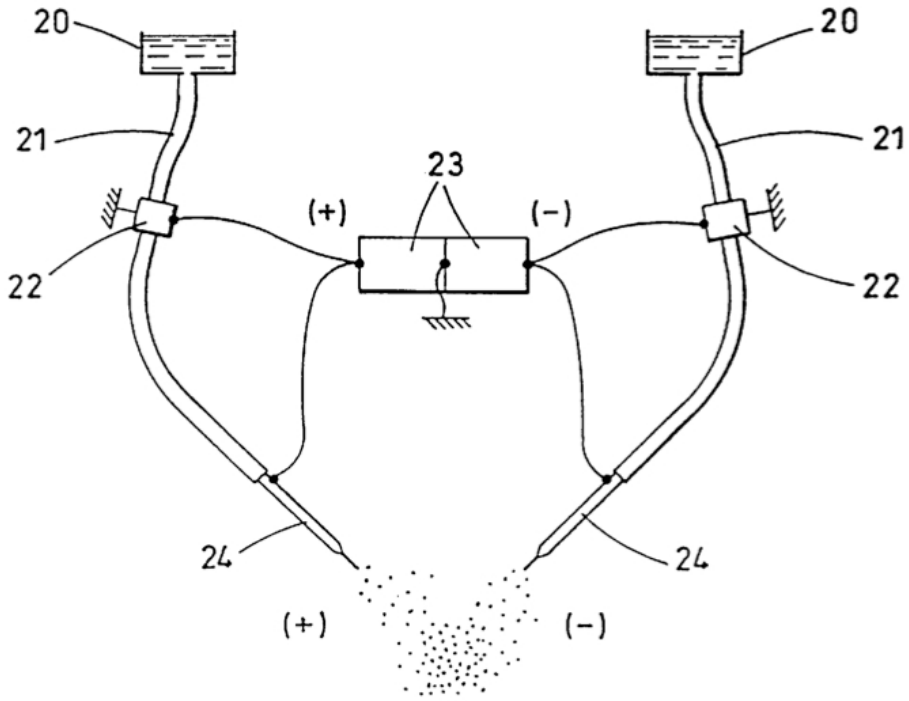


Figure 3-6 Arrangement of two electrospray sources of opposite polarity to neutralise the charge on the electrospray for drug delivery from US Patent 5915377. Reservoir (20) Tubing (21) Fluidic earth connection (22) HV supply+/- (23) Glass capillary (24)

3.7.2 Electrospraying DNA material into cells – electrospray scheme 1

A general electrospray aerosol generator was developed in the 1990s and licensed to a US manufacturing company TSI Inc. (Figure 3-7). The purpose of the device was to generate sub-5µm aerosol monodisperse colloids for aerosol research. The device includes a polonium 210 charge neutraliser (Chen, Pui et al. 1995). The prospect of putting DNA into cells is proposed by (Chen, Wendt et al. 2000) where they develop a bench top in vitro device for device for spraying DNA onto cells. The cells used were fibroblast cells and the DNA was enhanced green fluorescing protein. A cell target dish was place inside the device chamber and sprayed with a combination of electrospray and CO_2. The ambient media was an air and CO_2 mixture. A dual coaxial capillary arrangement was used with the sheath gas intended to prevent the onset of corona discharge. The ambient atmosphere could be evacuated in their system allowing for rapid velocities of the fluid colloids.

It is noteworthy that they report using distilled water as a DNA buffer. This is an unusual choice as it has high surface tension and normally requires a high V onset to commence spraying. Additionally, DNA will decay in distilled water over time. The DNA was in the form of gold particulate with DNA precipitated onto it. The gold would facilitate the transport through the cell membrane but pitting damage due to gold particle agglomeration can occur. The device presented in this paper appears to be commercially available however it is not widely reported in use and the paper was not followed by subsequent material from the authors. It is clear that such a device could not operate in vivo. The necessity to use gold particulate as in the case of the gene gun raises the question of toxicity. Gold is clearly inert and biocompatible but in chronic disease it is not desirable to introduce gold into the system (Bar-llan, Albrecht 2009).

Figure 3-7 The TSI electrospray generator based on Pui, Chen et al (1995)

3.7.3 Electrohydrodynamic comminution – electrospray scheme 2

Electrohydrodynamic comminution (Davies 2005) is a novel technique presented for the aerosolisation of plasmid DNA where a naked plasmid DNA could not be delivered as a potential gene therapy by conventional nebulisation devices. Figure 3-8 shows the basic setup of their apparatus. A variable resistor

controls a DC-DC converter. HV is applied to a 40mm, 1mm ID stainless steel needle. A liquid supply pumps fluid and four discharging needles strip charge from the resulting colloid shower.

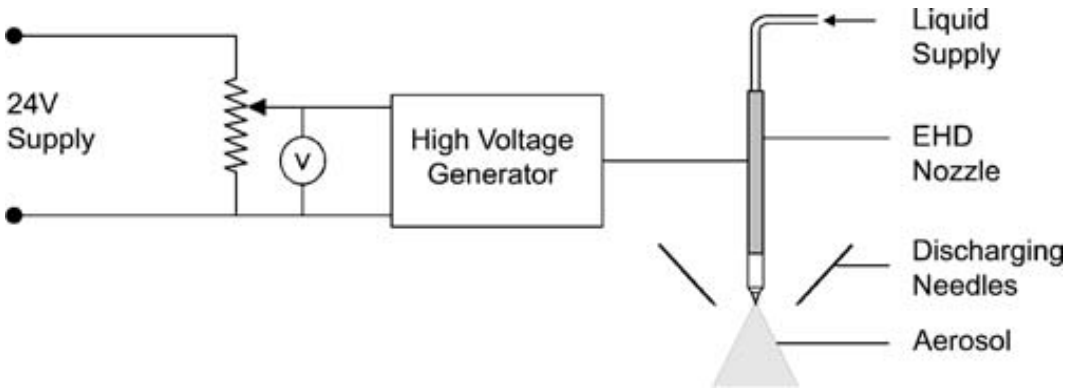


Figure 3-8 Diagram of liquid comminution device (Davies 2005)

They report that the formulation of the electrospray solution is critical to the performance of the electrospray. In this case they are using naked DNA (no gold) and water. The authors find difficulty is spraying water, indicating conductivity, surface tension and viscosity making water particularly difficult to aerosolise using EHD comminution. They note that the high electric field strengths required to generate a Taylor cone in water generally exceed the ionisation potential of the surrounding air and consequently regard this approach as impractical for small animal aerosol delivery applications but could be applied to a larger animal model where a low volume sheath of inert gas could be applied immediately around the generated Taylor cone. Nonetheless, they achieved good transfection rates of 60%+. It is noteworthy that their needle internal diameter was relatively large. This is to increase the volume throughput to levels suitable for comparison with conventional aerosolisation and nebulisation techniques. If the emitter diameter had been reduced to 0.02 mm ID and the number of emitters increased, as per their comments in their discussion, V_0 and the flow rate could have been significantly reduced and problems with corona discharge overcome. A good deal of this paper is given to testing the viability of DNA after the electrospray process. Naked DNA is extremely sensitive to applied shear forces (Levy, Collins et al. 1999). It is

degraded when subjected to shear forces such as those used to generate breathable aerosols from bulk liquids in conventional nebulisation and aerosolisation devices (Eastman, J. et al. 1997; Pillai, Petrak et al. 1998). A further requirement of a naked DNA delivery system is that it should not have its form changed by the aerosolisation process, as this will create regulatory hurdles if a drug or medicine was approved for use in humans but the delivery process changed the form of the drug. They conclude that several major obstacles will need to be overcome if a practical aerosol device is to be developed for use in the clinic.

3.7.4 Bio-electrospraying – electrospray scheme 3

Researchers Sahoo, Lee et al (2010) have reported an interesting and novel application of electrospray into tissue culture depicted in Figure 3-9. They use the term Bio-electrospraying to describe where progenitor cells are sprayed onto scaffolds in tissue engineering. The key differences here are that the cells are sprayed rather than being the target and the capillary used was larger (> 1mm) than commonly used for electrospray. Their major finding was that 88% of the cells sprayed were viable subsequent to the process. Stable electrospray was achieved at voltages between 7.5kV and 15kV. They use the term linearly directed which suggests the jet does not spread out. No figure is given for the viscosity of the solution of cells or indeed the diameter of the needle, although it is described as a large bore. They report a spraying distance if 15cm between tip and plane which may account for the high V_0. In some useful comments on cell viability they observed damage from electroporation and attributed electric field strength of 0.5–2.0 $kVcm^{-1}$ to this damage.

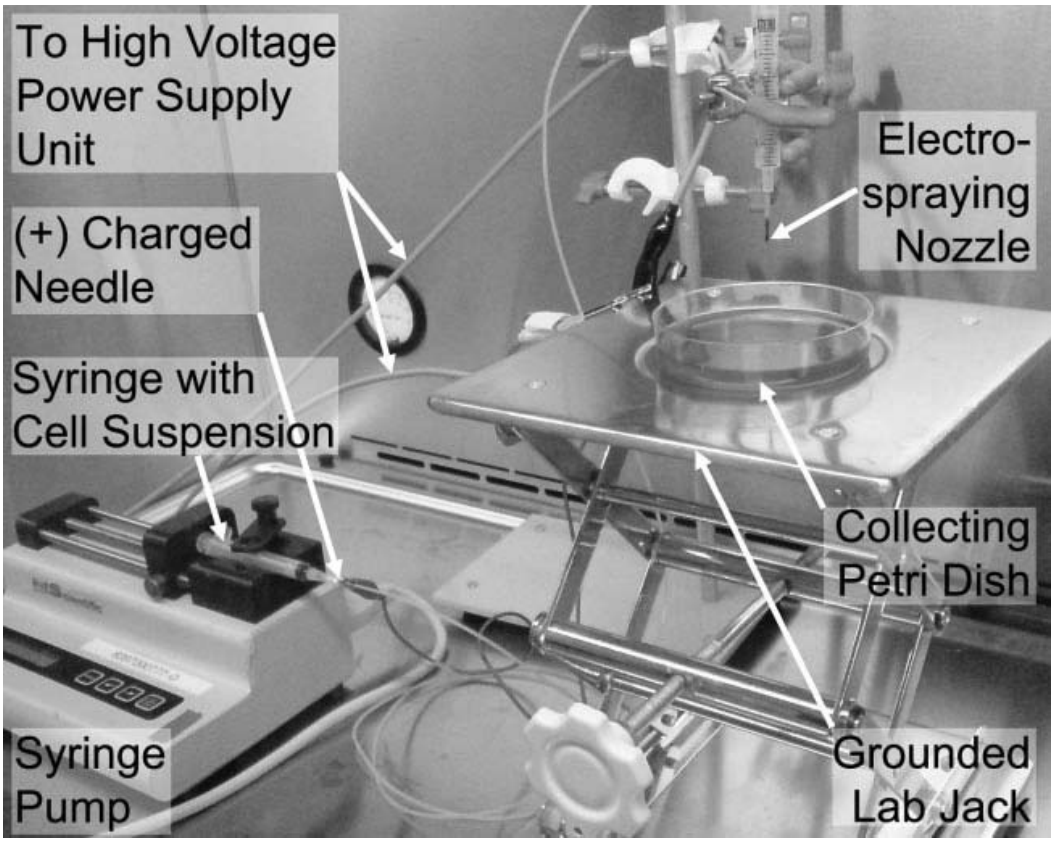


Figure 3-9 Cell spraying setup (Sahoo, Lee et al. 2010)

3.8 The Emitter

3.8.1 Fluid feed considerations

The fluid regime can be complex to bring about. Many setups operate at zero hydrostatic head. This, in theory, involves setting up a fluid reservoir close to the apparatus with the base at the same height as the capillary. In practice, this is rarely reported. It is more general now to see the use of a syringe pump for steady flow conditions. Another issue reported as being troublesome is the various fluidic dead spaces within the system. These appear inside valves, tubes and connectors. A problem peculiar to biological applications of electrospray is that the apparatus may need to be confined within a fume cupboard or laminar flow unit. Additionally, the fluid volumes often used are in the μL scale. At these volumes fluids can exhibit strong hydrophilic or hydrophobic behaviour. The implications of this are that with small biological volumes the entire sample could wet a material junction and be unavailable to the electrospray process.

3.8.2 Emitter types

There are three emitter types in common usage. Namely, internally fed, externally fed and bulk porous. Externally fed emitters and bulk porous, or 'felt-tip' systems, have significantly fewer models describing them. Clearly. this will change over time. It is possible that they present advantages over the conducting capillary systems most frequently reported but this has yet to be established. In this work we will focus entirely on the internally fed emitter type (Gassend 2007). The design of the emitter device will be crucial to the application of generating electrospray within the lung.

3.8.3 Emitter material

Capillaries are generally made of fine gauge stainless steel and range from outside diameter 0.2mm to 0.6mm, inside diameter 0.06mm to 0.15mm. They are generally 30-60mm in length and may come with a Luer for connection to a syringe or some other fluidic connection. Borosilicate glass capillaries can be pulled to micron or nano scale diameters. These can be adapted for use in electrospray by deposition of gold leaf or silver loaded paint. Many researchers make their own capillaries but they can be purchased from Harvard Instruments Inc., World Precision Instruments or New Objective Inc.

3.8.4 The HV connection

Common to all electrospray setups is a HV supply. This can be positive or negative, whilst generally a DC-DC converter, HV amplifiers do exist with reasonable bandwidth for example the TREK Model 20/20C with output 20kV and bandwidth 7.5kHz. Corona discharge can take place between the emitter tip and the surrounding air and indeed the counter electrode. When this happens, the appearance of the cone will change. In some configurations an electron curtain gas such as oxygen or SF6 can be used to prevent discharge. Any sharp edges can give rise to points of high electric field density and can result in corona discharge where air is the ambient.

3.9 The counter electrode

There is relatively little mention in the literature of the counter electrode in electrospray. From several reviews, (Cloupeau and Prunet-Foch 1990; Grace and Marijnissen 1994; Fernández de la Mora 2007) it is clear that the standard geometric setup for electrospray involves a point to plane geometry. Here the V onset increases with increasing distance. The electrospray emitter is considered to be a point and the counter electrode considered to be an infinite plane.

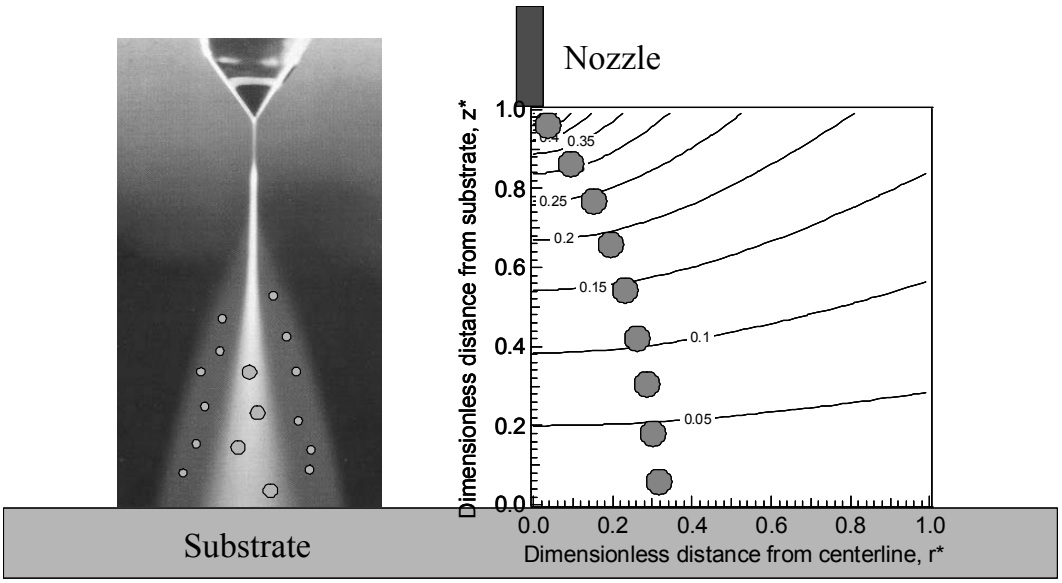


Figure 3-10 Electrospray path (Wilhelm 2004)

Unlike some of the standard applications for electrospray such as spray painting and material deposition, in the biological applications frequently the counter electrode is a tissue membrane. The arrangements reported are those using the target placed on a metal plate, the target placed behind some form of metal gauze (Jones and Thong 1971) or those using a planer electrode with a hole in it also described in the literature as an accelerator, extractor or intermediate electrode (Gassend 2007).

In Figure 3-10, charged droplets follow the external electric field that is applied between the emitter capillary and the counter electrode (Gamero-Casta, Ntilde et al. 2002). The results are compared to experimental droplet size and velocity values obtained with a phase Doppler anemometer (Wilhelm 2004). He reports

the axial droplet velocity close to the capillary exit at ~10ms^{-1} and decreasing towards the ground plane following the decreasing electric field gradient. Due to the nature of the breakup, the electrospray is very narrow close to the capillary exit but spreads towards the plate due to the mutual repulsion of the charged droplets.

An unusual counter electrode arrangement is that described by Gassend (2007) where the application is a colloid thruster and the approach is termed neutralisation. With ionic liquids or doped polar solvents, the electrospray process can emit both positive and negative particles. This eliminates the need for a cathode. Two thrusters that are operated simultaneously, one in the positive polarity and one in the negative polarity will require no cathode. This observation is close to that patented by Coffey (1999) and reproduced in Figure 3-6.

3.10 Stability in electrospray systems

The most common model of electrospray is that of the single emitter directed at an earthed plane. A distance of a few millimetres separates the emitter and plane and the ambient environment is air, air+CO_2 or vacuum (Grace and Marijnissen 1994). The stability of the cone that is produced is usually described in observational terms rather than analytical terms. There is relatively little analysis of the stability of the electrospray. Researchers frequently report a stable cone, referring to cone jet mode (Cloupeau and Prunet-Foch 1989). There are other modes that have different characteristics such as dripping, spinning and multi jet modes. These are described observationally and reported by researchers (Cloupeau and Prunet-Foch 1994) but are not analysed in terms of stability, as the term would be used of a control system. Modes other than cone jet are characterised by discontinuities and some chaotic behaviour. The following reviews will be referred to later as electrospray schemes 4-9 respectively.

In a deposition application reported by Deng, Waits et al. (2010) a matrix of 61 emitters is fabricated forming a 'digital' array. Individual emitters can be addressed to deposit minute amounts of solutions onto a metal substrate with a resolution of 675μm and a response frequency up to 100 Hz. The array of fabricated emitters was reported to operate with stability.

Reported instability in larger emitters is addressed with the novel approach of cutting notches into the capillary edge (Duby, Deng et al. 2006) as depicted in Figure 3-11. A research application requiring multi jet mode is described as being unstable. Sharp features were machined on the face of the tube and the electric field was locally raised dramatically enhancing the stability of the multi-jet mode. A comparison of the behaviour of the multi-jet mode with the cone-jet mode could be carried out systematically. Each cone-jet was found to obey, on average, the same power laws that had been observed for single cone-jet mode, both in terms of current and droplet size dependence on flow rate. This mode of operation could be stabilized over a broad range of liquid flow rates.

Figure 3-11 Multi spray stability emitter (Duby, Deng et al. 2006)

Researchers Gapeev, Berton et al (2009) hypothesise that controlling the current emitted by electrospray could bring about a more stable nano-spray. Their application is mass spectrometry and on-line gradient chromatography

where the objective is to generate a stable stream of ions of a chemical species for detection. The term nano-spray is used in mass spectrometry as the droplets produced are almost always < 1 micron. In this work, this hypothesis was independently arrived at prior to their publication date for the application of generating a stable electrospray to transfect cells and tissues with biologics. This work further proposes that stability of cone-jet electrospray reduces dynamic stresses and shearing forces normally experienced at the capillary tip thus making DNA and other biologic delivery more viable.

Their apparatus comprises a current sensing device, analogue to digital converter, digital proportional, integral and derivative (PID) controller algorithm and a digital to analogue converter producing a control voltage. PID tuning methodology or stability criteria are not reported. It is reported that chemical species normally considered difficult to nano-spray such as 2.5 M ammonium acetate were easily sprayed. They conclude that deeper understanding of current – voltage relationships is required to further improve spraying regimes. Researchers Marginean, Kelly et al. (2009) adopt a similar arrangement although with an analogue controller in a liquid chromatography application.

Marsh, Nunn et al. (1988) report a proportional integral (PI) controller to stabilise an electrospray of ethanol for a thin film deposition application. Only voltage was controlled based on current feedback. Changes in emitter – plane distance and fluid flow rate were considered unmeasured disturbances to the system. The regime was reported to stabilise the electrospray mode given disturbances in hydrostatic head and emitter – plane separation. The authors describe periodic discontinuities and a method for establishing steady electrospray subsequent to these. The paper concludes that a regime that includes flow control would be desirable.

A study into pulsations in current read at an earthing plane are reported by researchers Paine, Alexander et al. (2007a) using a 1 MHz oscilloscope. The frequency of the current pulsations was found to be proportional to the fluid

conductivity and inversely proportional to the emitter radius. Pulsations were in the ranges 1-1000Hz and 100kHz-1MHz. Water solutions were observed undergoing high frequency pulsations, with these pulsations often occurring in lower frequency bursts. This suggests a degree of modulation. A zero flow rate electrospray of water was also identified as being in the steady cone-jet mode by observing consistency in the pulsation frequency with a higher degree of confidence than by observation. Although no reference is made to it in the paper the scale, frequency and morphology of the waveforms reported bear close resemblance to pulses in point to plane corona reported in 1941 (Loeb, Kip et al. 1941). Comparing Figures 3-12 and 3-13, it can be seen that both waveforms occur in the range of 100kHz to 1Mhz. The researcher Trichel is reported to have observed these eponymous pulsations when ionising dry, dust free air in the early part of the 20th century. They are attributed to ionic saturation and relaxation in charge occurring at the point of a conductor and modulating the current flowing from the point to the plane. It is clear that the same saturation – relaxation oscillatory behaviour may be exhibited at the apex of a charged fluid cone.

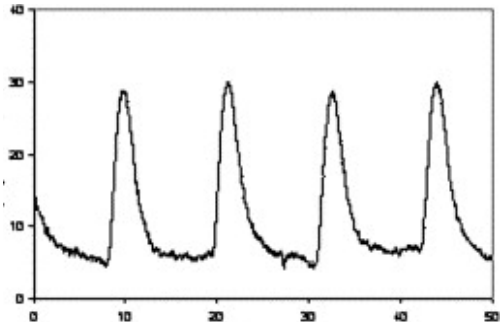


Figure 3-12 Current pulsations X-axes in μseconds, y-axes in nA (Paine, Alexander et al. (2007a)

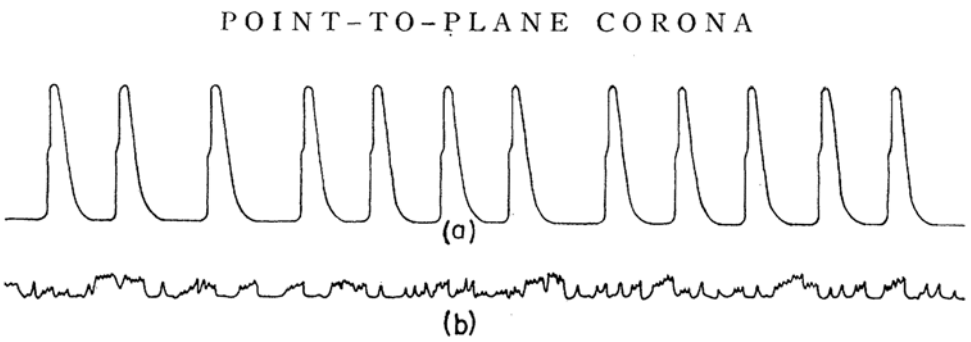


FIG. 1(a). Regular Trichel pulsations obtained with points having radius of curvature greater than about 0.002 centimeter. (The break shown in the steep wave front is probably instrumental in character. Pulses observed by Kip and by Hudson did not show this break, but were of the same form as photographed by Trichel, reference 1; (b) irregular forms of pulses obtained with points having radius of curvature of about 0.0004 centimeter. The jagged form is very similar to the irregularities in current obtained in cold emission from unconditioned points during "breakdown."

Figure 3-13 Trichel pulsations caused by oscillations in ionised air (Loeb, Kip et al. 1941)

Researchers Valaskovic, Murphy et al (2004) in an application note for New Objective Inc. report a fascinating electrospray system with a feedback loop (Figure 3-14) based on image processing. The system is reported to be self-starting and self-tuning. The application is nanobore gradient elution chromatography, a derivative of liquid chromatography analysis. The system seeks 'optimal Taylor cone mode' given deviations in flow rate or emitter voltage. There is no stability analysis reported.

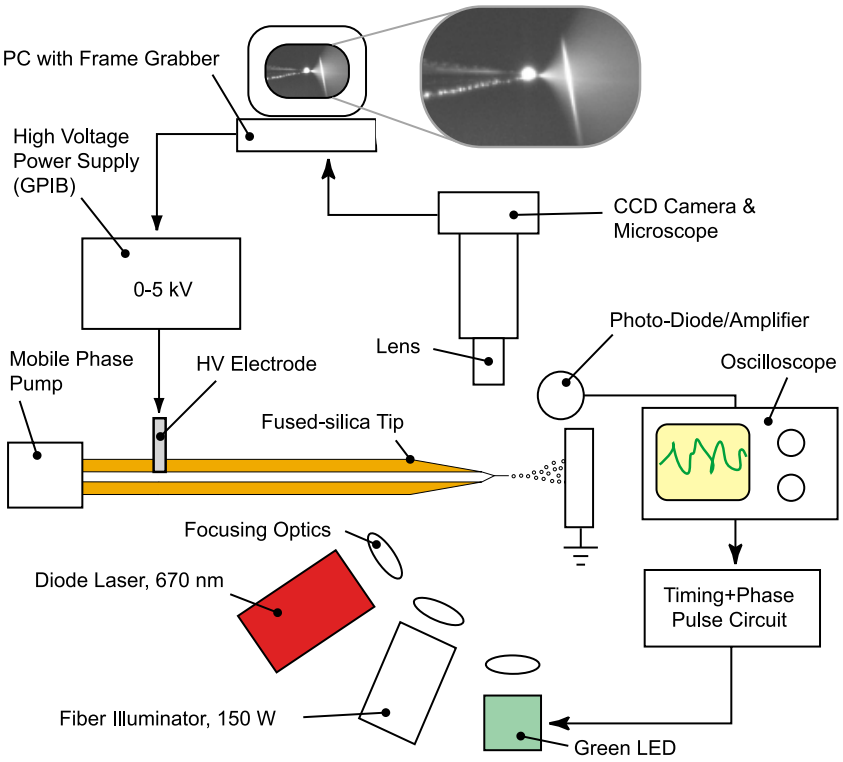


Figure 3-14 Self starting and self tuning electrospray system

3.11 Safety and Efficacy In Vivo

Safety and efficacy are the basis of European and US regulatory approval and medical device classification (Kimmel and Gerke 1995). Accordingly, the regulatory pathway needs to be considered in research into a new diagnosis or therapy based on a device, drug or drug-device combination. Although regulation draws together diverse issues such as product packaging, labelling and electromagnetic compatibility, the central point is to establish safety and efficacy.

3.11.1 Efficacy

Efficacy is generally understood to how well an objective is achieved. In our case measuring the efficacy of transfection can be done easily using two methods. The first is flow cytometry in which cells from the target dish are trypsinized and a sample fed to the flow cytometer. A base number of cells, for instance

10,000, are sampled by the flow cytometer and the number of these cells that have become florescent is presented as a shift in a graph. The greater the shift the greater the number of fluorescing cells and the more efficient the reaction was. The second is fluorescence microscopy.

3.11.2 Safety

The internal fluids in a cell are composed largely of water with ions of different species. The concentration of ions inside and outside the cell is different leading to electrochemical forces across the cell membrane. The membrane is described as a dielectric insulator that allows some ionic interchange (Reilly 1998).

Nerve and muscle cells have membranes that are excitable, such that exposure to a current that alters the cell's resting potential can trigger a sudden change of membrane conductance. The resulting membrane voltage change will effect an action potential in muscle tissue and the propagation of a nerve impulse in nerve tissue. In epithelial cells, the membrane does not exhibit this all or nothing quality due to the nature of its ion channels. This distinguishes excitable cell membrane from ordinary cellular membrane. The relevance of this is that we need not expect a nerve impulse or action potential response to the use of electrospray within the lung.

Charts are widely published in electrical safety standards that tend to focus on 50Hz-60Hz AC current, however, DC thresholds are also available (Bikson 2005). They normally indicate a lower threshold of perception of electric current in the body at ~2mA. Typical values for the resistance of the body and skin range from 20KΩ to 100KΩ and the point of application of potential as well as the earthing point all play a role in electrical safety. The threats to the body from electric current are fibrillation, burns, respiratory arrest and electroporation. Electrical fibrillation, which is still used intentionally in heart surgery to stop the heart in the absence of cardioplegia, occurs when 2-20mA of current are applied directly to the myocardium. The effect depends on disrupting the depolarising wave on the myocardium by a strong AC current. Fibrillation can occur over a broad range of frequencies and voltages but

generally frequencies in the 20Hz – 500Hz range are reported as being effective at inducing fibrillation. The electrospray emitter produces about 1μA of current and the cellular membrane is an effective low pass filter due to its cellular composition. Given the separation of the heart and lungs and the fact that the lung tissue is an effective earth to this current, fibrillation could not be expected to occur. There is too little energy to cause burns or respiratory arrest so electroporation is the remaining risk. In serious electric shock and lightening strikes, entire areas of the body may be irreversibly electroporated with devastating effects on the anatomy. In the proposed electrospray emitter device, a facility for stripping charge from the electrospray may be adopted, however, electroporation has not been reported elsewhere or observed during cell potential measurements in this work.

3.12 Summary

Electrospray refers to the process where electrical charges are provided to a fluid in order to generate a very fine electrospray. Electrospray is used widely in industry diverse applications from spray-painting and crop dusting. There has been increasing interest in electrospray since 1990 and some developments in it as a process for introducing biological material into cells in vitro. The electrospray emerges from a Taylor cone and carries a small current between an emitter and a counter electrode. The cone forms on an emitter that can be a capillary made of glass or metal. Models exist for the cone and describe the interrelationships of the parameters involved. The fluid being electrosprayed is a key component in the model. Six identifiable electrospray modes exist and from these, the cone-jet mode produces monodisperse colloids of fluid < 5μm in diameter. These are potentially useful for delivery of therapeutic and diagnostic molecules to the lungs. Nine schemes are reviewed in which the researchers attempt to stabilise the electrospray including schemes involving optical and current feedback. Of these, three schemes employ electrospray to introduce biological material into cells. Electrospray is a suitable basis to address the problem of introducing diagnostic and therapeutic molecules locally to the lungs. Table 3-5 summarises some sensible values of electrospray parameters.

Table 3-5 Summary of sensible values for independent electrospray variables

	V_0	Flow Rate	Distance	Electrospray Current	Capillary Radius	Capillary Material
Higher values	5.7kV	6 µLmin^{-1}	100mm	2.5 µA	0.03mm	Stainless Steel
Lower Values	1.9kV	0.5 µLmin^{-1}	4mm	100nA	.005mm	Borosilicate Glass Pipette

	Conductivity	Viscosity	Surface Tension	Fluid Examples	Ambient environment
Higher Values	0.000015 Sm^{-1}	1.2 mPa s	0.007 Nm^{-1}	Ethanol (70%)	Air
Lower Values	0.00025 Sm^{-1}	1 mPa s	0.03 Nm^{-1}	Deionised Water	Air/ CO2

Chapter 4

The Pulmonary Delivery Problem

This Chapter develops the pulmonary delivery problem and describes the methods by which it will be addressed. In Chapter 2, it was concluded that the anatomy of the airway and the lung makes delivery of molecules challenging. It was additionally concluded that each of the vectors reviewed is not ideal for in vivo use in the lung. It is noteworthy that no commercial device, to date, has been approved by the FDA or any European medical device agency for bronchoscopically mediated in vivo drug delivery to the lungs. In Chapter 3 it was concluded that electrospray seems to be a promising process for introducing biological material into cells in vitro. As the overarching objective of this thesis is to address the poor efficacy of existing strategies to treat lung disease, an approach is proposed in which electrospray is used in vivo through the design and implementation of a bronchoscopically mediated electrospray device.

The methodology is to design, implement and trial a device that is capable of use with a bronchoscope to deliver molecules locally to diagnose lung cancer. In vitro, therapeutic molecules including 1) chemotherapeutics 2) genes and 3) antibodies will be delivered to cells to treat them. The methodology necessitates the construction of a test rig on which most of the electrospray parameters can be varied and the results measured. Additionally, the rig can be used to investigate the models developed in Chapter 3. The development challenges emerge in detail in Section 4.1 from an analysis of the electrospray methodologies reviewed. The results will inform the design specifications for an electrospray emitter. The emitter device needs to be safe and efficacious. The availability of such a device would represent a significant benefit to physicians. It would offer them, for the first time, the ability to deliver diagnostic and therapeutic molecules locally to lung tissues. Using a bronchoscope, the device can be positioned at the point of a tumour or a lesion. Using the optics of the

bronchoscope, the tumour can be targeted and electrosprayed with a chemotherapeutic, gene or dye.

A critical analysis of existing electrospray methods and the physiological challenges are drawn together in Section 4.2. Here, a novel bronchoscope mediated electrospray device is proposed and the manner in which it will be used during the bronchoscopy procedure is explored. Dr. Shirley O'Dea and the author attended a human bronchoscopy procedure in April 2010 in the Mater Hospital Dublin by invitation of Professor James Egan and with the consent of the patient. At this procedure, an appreciation of the needs of the patient and the physician was gained. Section 4.3 describes an electrospray test rig apparatus that was built for the purposes of making measurements, varying parameters and observing the electrospray. The apparatus was used to inform the design decisions for the proposed in vivo device. In Section 4.4 the problem of identifying fluids that can be electrosprayed with stability and without degrading molecules and dyes is detailed. The problem of selecting appropriate emitter geometry and material is discussed in Section 4.5. In Sections 4.6 and 4.7 the issue of selecting an electrospray mode emerges and the use of a controller is explored. In Section 4.8 the stability of electrospray is considered and visualisation of the electrospray process is presented in Section 4.9. Target cells are described for the in vivo and in vitro setting in Section 4.10 and the impact that electrospray has on the cells is presented in Section 4.11. The Chapter concludes with a summary in Section 4.12.

4.1 Analysis of Existing Electrospray Methodologies

The electrospray schemes presented by other authors are invaluable for eliciting sensible values for parameters, dependencies of one parameter on another and any anecdotal reporting of what worked and what did not. The electrospray technologies reviewed in Chapter 3 are comparatively analysed to inform the operational parameters and design considerations.

4.1.1 Analysis of reported electrospray schemes

Of the nine systems reviewed in Chapter 3, none was reported as designed for in vivo use. However, three of them anticipate in vivo use in some ways. The co-axial electrospray generator of Pui, Wendt et al. (2000) involves a bench-top instrument, their working fluid is distilled water and they use a sheath gas to achieve higher potentials without corona discharge. The electrical setup of their system is a grounded capillary with the target plane at a negative potential. In their discussion they indicate the possibility of automation and scaling up the system to transfect cells on an industrial scale.

The EHD comminution system reported by Davies et al (2005) similarly involved an in vitro setup, however, the purpose here is to develop a basis for an electro-aerosolised delivery system. The researchers make reference to EHD aerosols generated over liquid delivery rates from 0.4 μls^{-1} to 1.4 μls^{-1}. These delivery rates are up to 30 times greater than those proposed in the device in this thesis. The disparity is due to the difference between proposed local delivery to a single area measuring perhaps $2cm^2$ and an electro-aerosol approach that would deliver a monodisperse aerosol to be inhaled and delivered onto the full area of the lungs. Aerosols with suitable size characteristics for respiratory delivery to humans are defined as having a colloid with median diameter of less than 5µm (Coates, L. et al. 1998). An author on the Davies paper (2005), Ronald Coffey, patented an earlier EHD inhaler mechanism (Coffey 1999). This device was licensed by Battelle Inc. and is manufactured and sold by them. This validates the EHD approach to aerosolisation and demonstrates that EHD solutions can be approved through the regulatory pathway.

The bio-electrospraying system reported by researchers Sahoo, Lee et al (2010) to electrospray progenitor cells onto scaffolds in tissue engineering uses higher voltages, a larger capillary and greater electrode separation than the other setups described. However, their aim of spraying cells rather than therapeutics has potential application in lung and airway regeneration. None of these setups could be directly translated into an in vivo model. The remaining schemes

employ standard point to plane geometry but employ some other controlling subsystem or method. Table 4-1 shows the operational and the design parameters of the in vivo device proposed in this thesis. Features of these and other setups for the purposes of reference and comparison are included.

Table 4-1 Comparative analysis of operational parameters of reported electrospray schemes

V_0(kV)	Flow Rate	Distance	Current (μA)	Radius (mm)	Control	Sheath Gas	Fluid	Ambient	Application	Reference
-5	40-60μL/min	-	not measured	1	no	no	dH_2O	Air	DNA Delivery	(Davies 2005)
4.3	1μL/min	25 mm	not measured	0.08	no	yes	dH_2O	Air CO_2	DNA Delivery	(Chen, Wendt et al. 2000)
7.5 - 15	6mL/hr	100-150 mm	not measured	1	no	no	dH_2O	Air	Cell Delivery	(Sahoo, Lee et al. 2010)
3.8 - 5.46	-	25 mm	.245-2micro amps	.08 -.35	-	No	Ethelyne Glycol	Air	Model Development	(Smith 1986)
3.65 – 4.15	1-4μL/min	30 mm		0.5	No	No	kerosene	Vacuum	Fuel Atomisation	(Jones and Thong 1971)
1 - 2	4μL/min	1 mm	5.3nA	100	Yes	No	Ethanol/ H_2O mix	Vacuum	Mass Spectrometry	(Gapeev, Berton et al. 2009)
3.7 - 6.7	-	19-35 mm	25-200nA	0.2	No	Yes	ethanol	Air	Particle Production	(Marsh, Nunn et al. 1988)
3	500 nL/min	4 mm	25 – 200 micro amps	.05	Yes	Yes	Methanol	Air	Model Development	(Valaskovic, Murphy et al. 2004)
0.81 - 1.26	-	2-10 mm	0.7micro amps	0.1 externally fed	No	No	Ethanol	Vacuum	MEMS emitter	(Deng, Waits et al. 2010)
2.64	-	-	-	.02	No	No	Ethanol	Air	Drug Delivery	(Tang and Gomez 1996)
2.4	nL/min	460 μm	<20nA	.1	No	No	EMI-BF Propellant	Air	General	(Wang, Tan et al. 2010)
2.8-4.2	**3uL/Min**	**10mm**	**0.8 micro A**	**.08mm**	**Yes**	**No**	**TRIS**	**Air**	**Drug Delivery**	**Maguire 2010**

4.1.2 Designing for the regulatory process

The regulatory process is a necessary, ethical and welcome safeguard on medical devices being sold in the market place. In Europe and America, the regulatory process places demands on designers and manufacturers to document the validation and verification of their designs. Regulatory issues are as much a part of the design specification as any other design parameter (Kimmel and Gerke 1995). For the proposed device, the responsible regulatory authorities in the EU are the country Medical Device Agency implementing the EU Medical Device Directive. In Ireland this is the Irish Medicines Board. In the US, the responsible regulatory authority is the FDA. Because the device proposed is both a device and a medicine, it needs to be considered as a drug-device combination. Fortunately, there is precedent here in the form of drug-eluting stents, implantable pain management pumps and certain nebulisers. The main issues that need to be considered at the design stage are electromagnetic compatibility, biocompatibility and potentially scintigraphy studies. In scintigraphy studies (Kim 2000), the question of how much drug was deposited in the lung is addressed. Finally, it is important to demonstrate that the delivery process does not modify the therapeutic.

4.1.3 Working Fluid

The working fluids in the systems reviewed in Chapter 3 vary from fuels such as kerosene to distilled water. The schemes that deliver biological material (Chen, Wendt et al. 2000; Davies 2005; Sahoo, Lee et al. 2010) use distilled water as the working fluid. Distilled water has the advantage of being widely reported in electrospray schemes. Distilled water has, however, a very high surface tension and this means that higher potentials between the emitter and the counter electrode are required to establish electrospray. In a scheme where the conductivity of water was increased by the addition of NaCl (Smith 1986), it was found that as the conductivity rose the V_0 voltage remained the same at about 5.2kV. To reduce the V_0 the surface tension must be reduced. The desire to reduce the V_0 is driven by the need to avoid corona discharge. Corona discharge disrupts the electrospray by collapsing the electric field locally. Further, it produces ozone, which is harmful in the lung as it reacts with the epithelium

and causes it to leak (Lippmann 1989). The reduction of V_0 can be achieved by selecting a buffer that has inherently lower surface tension. Modifiers such as non-polar solvents can additionally reduce surface tension without markedly changing conductivity. In the development of this device, fluids that have lower surface tension and higher conductivity than that of water will be utilised. If the physical or electrical parameters are not ideal, there are a number of options for doping the fluids to lower surface tension or increasing conductivity. In the therapeutic case, it will be necessary to work with buffers rather than water. This is because a buffer resists changes of pH and biological material is not degraded over time when dissolved in buffers. Buffers will be sought with surface tension lower than that of water and conductivity higher than that of water.

4.1.4 Lung topology

The topology of the lung is complex and less than ideal for positioning a bronchoscopically mediated device. Its dendritic nature, depicted in Figure 4-1, means that with each progression into the lung the physician is presented with branching bronchioles. It is reported that in the human lung there are 27,994 terminal bronchioles (Horsfield and Cumming 1968). It is only possible for a bronchoscope to get to the larger airways within the lung. Clearly the use of dyes is limited to the areas of the lung that can be seen, however, in therapy, monodisperse colloids in the micron size range may reach the distal airways if they are produced within the lung (McLachlan, Baker et al. 2010).

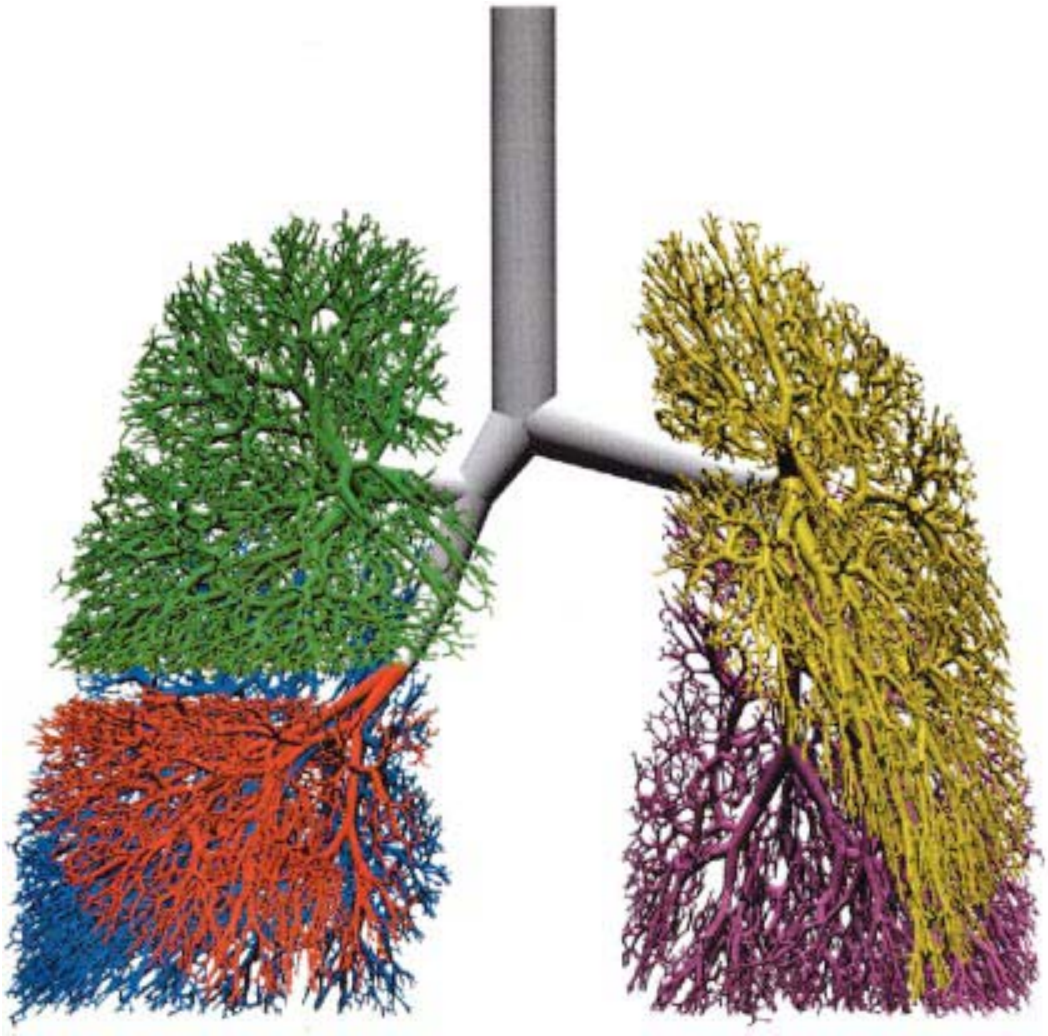


Figure 4-1 A model of the lungs illustrating the challenge in treating them (Tawhai, Pullan et al. 2000)

4.1.5 Toxicity of the device and the buffer

The biocompatibility of the materials used in the proposed device needs to be considered. In addition to the primary materials of fabrication, any glues sealants or inks on device markings will need to be checked for biocompatibility. Up to 1% of the general population and estimates of 8% of the hospital population are intolerant to latex (Chowdhury and Maibach 2005). As such, the plastics of construction will need to be latex free. The form factor of the device, as it is to be used internally, will need to avoid sharp edges or anything that could cause trauma to the patient internally. Consideration needs to be given to the remote possibility that some part of the device could detach during the procedure and to how such a part could be identified in or retrieved from the body.

In the case of dyes, the material safety data sheet (MSDS) for the dye needs to be available and the dye used at an indicated safe concentration. The MSDS for methylene blue is in Appendix 5. In the case of a therapeutic, the buffer needs to be non-toxic to the body at the dosage indicated. If the treatment were for a chronic condition that required some repeated therapeutic sessions, consideration would need to be given to the accumulation of any residual by-products of the electrospray process. The collection and establishment of this type of information is important for regulatory approval of the device.

4.1.6 Use of HV within the body

High potentials are inherent in the production of electrospray. Fortunately, these potentials give rise to small ionic currents in the order of 0.5μA – 2μA. Small currents can, in certain circumstances, cause injury within the body. The four main threats to the body from electricity are fibrillation, burns, respiratory arrest and electroporation (Bikson 2005). The energy levels in electrospray are not high enough to produce burns or respiratory arrest. Fibrillation requires a current of 1 to 2 mA applied directly to the myocardium and with frequency component >2 Hz, to prevent depolarisation of the heart muscle. Lastly, electroporation requires an electric field of $\sim$1000Vcm^{-1} to open the pores in epithelial cells. This effect will be explored when the device is trialled.

EMC testing involves the testing of a device's emissivity and susceptibility to electromagnetic interference. Careful consideration needs to be given to the design of this device and to the contraindications for its use. For example, a patient with a pacemaker may not be suitable for treatment with an electrospray device.

4.1.7 Stability of electrospray

To deliver therapeutic and diagnostic molecules, the electrospray device must operate in stable cone jet mode (Rosell-Llompart and Fernandez de la Mora 1994). Maintaining stability presents two challenges. The first challenge is maintaining stability within the cone jet mode and the second is recognising when the electrospray emitter is operating outside of the chosen mode. In designing the device for use in vivo, consideration needs to be given to the

appropriateness of using one of the many control solutions available. These include PID or PI controllers. The controlled parameter here is the current passed between the electrospray emitter and the counter electrode. In an idealised laboratory setup, this can be measured effectively using a pico-ammeter connected between the counter electrode and earth. However with the device within the body, the current measurement would need to take place upstream of the emitter. It may be that with many of the variables fixed in the in vivo solution, feedback control is not necessary. However this question needs to be answered through the use of a test apparatus. In one potential configuration, the target tissue is itself used as a counter electrode.

4.1.8 Singe dose or continuous use

According to whether a dye or a therapeutic is being delivered, it may be appropriate to operate the electrospray emitter continually from a reservoir of fluid or with a fixed quantity of fluid located in a small local reservoir close to the emitter tip. In the case of continual operation, perhaps a dye being sprayed over a large area, a supply reservoir of fluid must exist up to 2.5m from the emitter device. The bronchoscope is up to 65 cm in length. The additional length is to allow the device's electrical and fluidic tether to route over the shoulder of the physician (Figure 4-2) and into the control unit located on the bronchoscope trolley. In the case of a fixed quantity of fluid, perhaps a metered dose of therapeutic, this could be placed in a small local reservoir within the device, close to the emitter. This would avoid the need to pump a small amount of fluid from a control unit at some distance. It is noteworthy that the dead space inside the tether even with very small internal diameter tubing can equal or exceed the fixed dose volume. A further consideration is that movements of the physician during the procedure (Figure 4-2) can raise the hydrostatic head above or below the height of the reservoir. This introduces variable hydrostatic pressure that can create instability in the electrospray mode through flow rate variability. If enough fluid containing dye or therapeutic can be accommodated at or near the emitter, the process will be less prone to hydrostatic head variations and consequently inclined to be more stable. It is possible for the device to operate at zero hydrostatic head, in other words the emitter draws as

much fluid from the source or reservoir as it elutes. With the very low volume rates required in this application, this is an attractive option in that that no pumping mechanism is required. If, in addition to a small local reservoir, a fluidic connection is made back to a control unit, this can exert a gas backpressure on the reservoir and increase the flow rate from zero. With a low-pressure gas controller, flow rate can be controlled effectively. This type of arrangement will be tested on the apparatus.

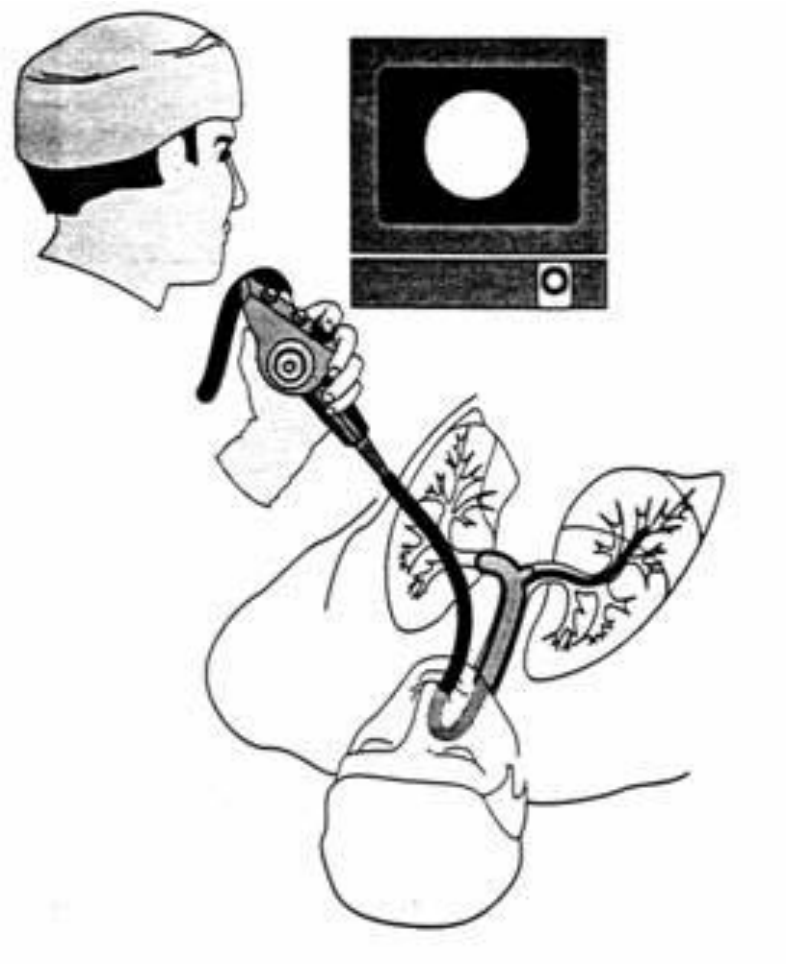


Figure 4-2 Hydrostatic pressure in the proposed device can rise and fall.

4.2 A Novel Bronchoscope Mediated Device and Procedure

There are intrinsic and extrinsic design factors to consider in the proposed device. Intrinsic factors are influenced by the required performance of the

device. Extrinsic design factors are influenced by the therapeutic or diagnostic procedure, regulatory affairs and the limitations of operating in conjunction with a bronchoscope. In the intrinsic aspect of the design, the sensible values presented in Section 4.1 give some guidance on the types of parameters that might be expected in the design. Of the extrinsic design factors, the most limiting factor is the width of the working channel in the bronchoscope. An early concept sketch in Figure 4-3 indicates the diameter parameter.

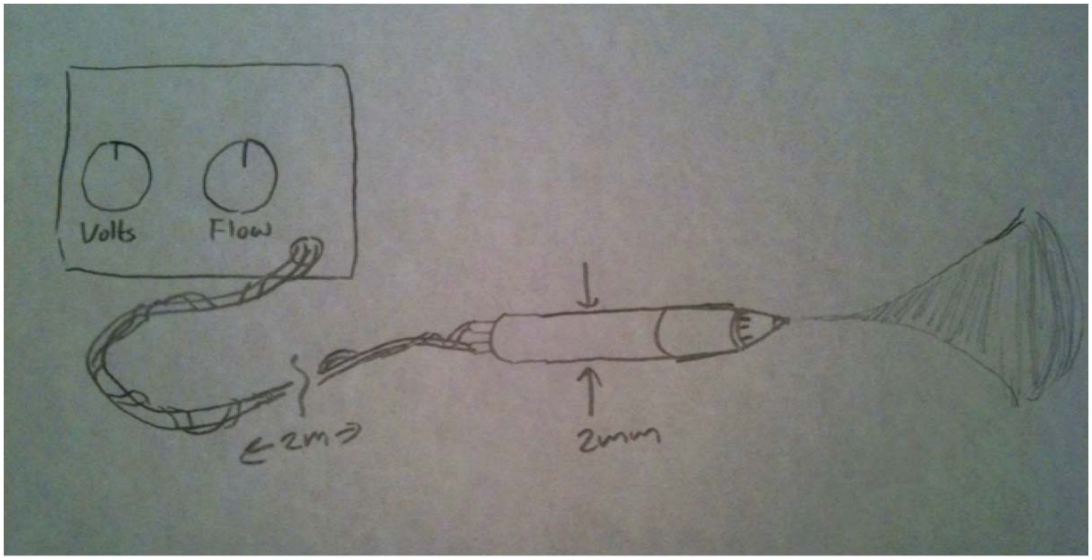


Figure 4-3 An early concept sketch of the electrospray device (M.Maguire)

4.2.1 Ascertaining parameters from a rig

To establish a set of working parameters that will produce stable electrospray emission for a given set of biologic and buffer combinations, an empirical route is taken. With reference to other designs from Section 4.1, some electrospray schemes are tested on a rig developed for the purpose. The operational parameters are then refined and modified for schemes that match the design requirements and the design of suitable counter-electrodes is presented. Such parameter sets should envisage in vitro, ex vivo or in vivo delivery of molecules to targets. The contribution of parameters is not always obvious experimentally, as such, the models derived in Chapter 3 will help provide the basis for determining the electrospray operational parameters.

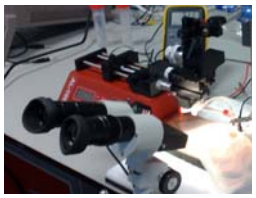
4.2.2 Known device design parameters

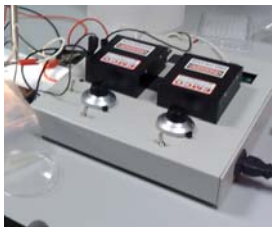
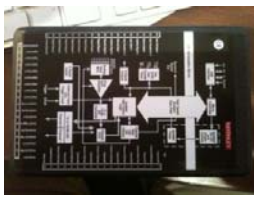
The bronchoscope channel is between 2.2 mm and 2.8 mm depending on the model. The volume of the reservoir within the device has to be adequate to contain a useful dosage of the therapeutic or dye to be delivered to the pulmonary tissue. There is a rigid aspect of the device where the emitter, reservoir, fluidic and electrical connections are located. This must not prevent the bronchoscope from articulating properly. The working channel will only accommodate one fluidic and electrical conduit, therefore they will need to run coaxially. Further, the electrical insulation properties of the conduit are important as it is placed inside the body. Biocompatibility of all materials, glues, plastics as well as metals need critical attention. The design of counter electrodes at which to target electrospray is also important.

4.3 Electrospray Test Rig

An electrospray test rig was constructed that allows variation of parameters and interchange of components such as capillaries. The equipment in the rig is tabulated in Table 4-2.

Table 4-2 Electrospray rig system components

Item	Description	Manufacturer	Part number	Image
1	HV module 0 to +10kV & HV module 0 to -10kV	EMCO inc.	4100 & 4100N	
2	long objective USB microscope & Olympus Phase contrast stereo microscope X 200	Dino Lite	AM411T	
3	Aladdin Programmable Syringe Driver	World Precision Instruments	Aladdin-220	

4	3 axes micromanipulator	World Precision Instruments	M3301	
5	µTip Metal coated glass capillary OD 0.2mm, ID 0.05mm	World Precision Instruments		
6	Control Unit – Bespoke Built by Author			
7	Electrometer / pico/nanoAmmeter	Keithley	6517A	
8	16 bit A-D / D-A module	Keithley	KUSB-3108	
9	33 Gauge (~100 micron) Stainless Blunt Capillary Needles	Harvard Instruments	725510	
10	Counter Electrodes- Bespoke designed by the Author – built by J Malocco/D Buckley			
11	PTFE & Silicon tubing	WPI/ Harvard		

12	Assorted fluidic connectors/filters	CPC / Milipore		
13	Oscilloscope	Tektronix	TDS 3054	
14	Frequency Analyser	Tektronix	RSA3303A	
15	Tuneable PID process controller	Omega	Cni32	

The electrospray rig comprises an assembly of the equipment listed in Table 4-1. The fluid sample to be electrosprayed is introduced into a syringe. This is placed in the syringe driver. The syringe driver is programmed to deliver fluid at a fixed rate. The syringe is connected to 1.2mm OD PTFE tubing via a Luer© lock assembly. The tubing is a fluidic conduit between the syringe driver and the electrospray capillary. The tubing is connected to the capillary by different means. Some commercial connectors were used that provide a compression fit between the capillary and the tube and some bespoke connectors were used where it was deemed to be necessary. The fluidic connector incorporates an electrical connector (Figure 4-4). It is at this point that an electrical HV connection is made with the capillary. The capillary can be made of glass or stainless steel. In the case of glass capillaries, metallic coating is precipitated on to the borosilicate glass and this conducts the current to the tip of the capillary.

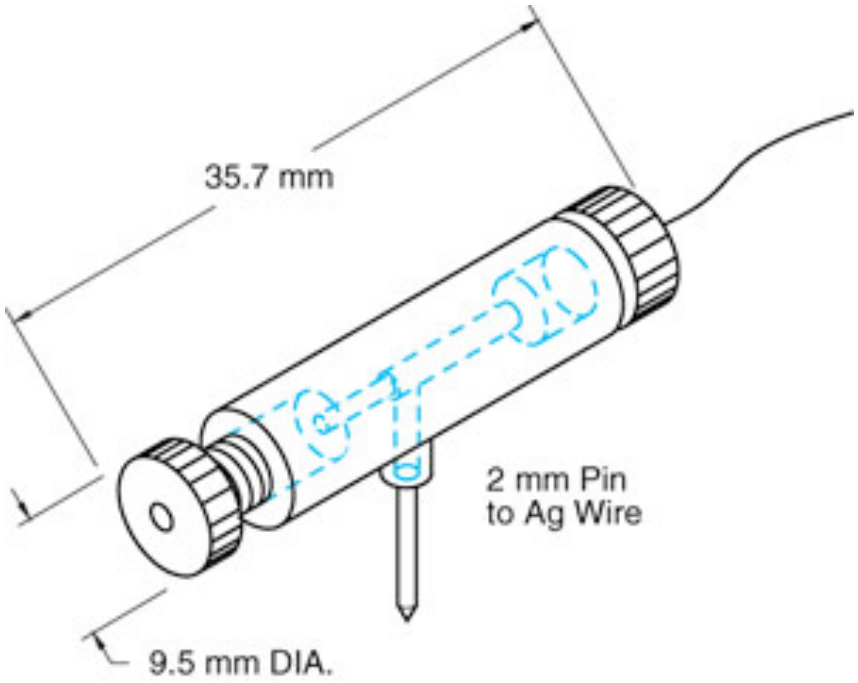


Figure 4-4 Diagram of a glass capillary holder. The Ag wire can make a pressure fit with the outside diameter of the capillary. For highly conducting fluids, it can be inserted into the feed end of the capillary. (World Precision Instruments Catalogue, 2010)

The capillary is held in the micromanipulator. This device permits accurate and repeatable movement in 3 dimensions. The micromanipulator has millimetre scales of measurement on each axis and 0.1mm fine adjustment on the height adjustment axes. This permits accurate distances to be set and reset between point and plane (Figure 4-5a). A HV supply generates potentials of +/-10,000 V. A voltage of 0-5V controls the HV module output. The module also has an enable control and a voltage out sense. The modules, one for each polarity, were incorporated into a bench unit constructed by the author. The unit included HV lock out controls, digital displays, additional inputs and outputs for a control loop, HV 'on' indication, foot switch control and ten turn dials controlling precision potentiometers.

Various counter electrodes were used. Initially a piece of copper printed circuit board (PCB) served as an earthing plane. When current measurements were taken, by placing the Keithley electrometer in the current loop, a gold plated PCB was used. This was fabricated from a piece of RF grade circuit board with a gold plated earth connector. Additionally, several alternative counter electrodes

were fabricated specifically to fit inside cell culture dishes. These were fabricated with the 3D printer in NUI Maynooth and involved various ring electrodes attached to a plastic substrate that would fit the 24 well cell culture dishes generally used. Each of the system components, the syringe driver, the HV modules and the electrometer can be interfaced with a PC affording the possibility of digital feedback control. A series of additional links in the rig enable analogue control to be implemented as required. The Keithley electrometer 6705 detects current and has a +\-2V voltage output proportional to full-scale deflection. This was used as a feedback signal. A Tektronix oscilloscope and frequency spectrum analyser were used to look at waveforms within the electrospray current modes. An Olympus phase contrast stereomicroscope was used to look at and photograph Taylor cones and a long objective Dino-Lite USB microscope was used to record video of Taylor cones. The rig has evolved during the period of research to be capable of use within a laminar flow unit or fume cupboard. It is worthy of mention that good earthing practices were observed and current shielding arrangements were put in place when small current measurements were being taken. Finally, the rig was employed to test the new electrospray devices that were fabricated for in vivo use, replacing the glass and stainless steel capillaries. Figure 4-5b shows the rig operating inside a laminar flow cabinet.

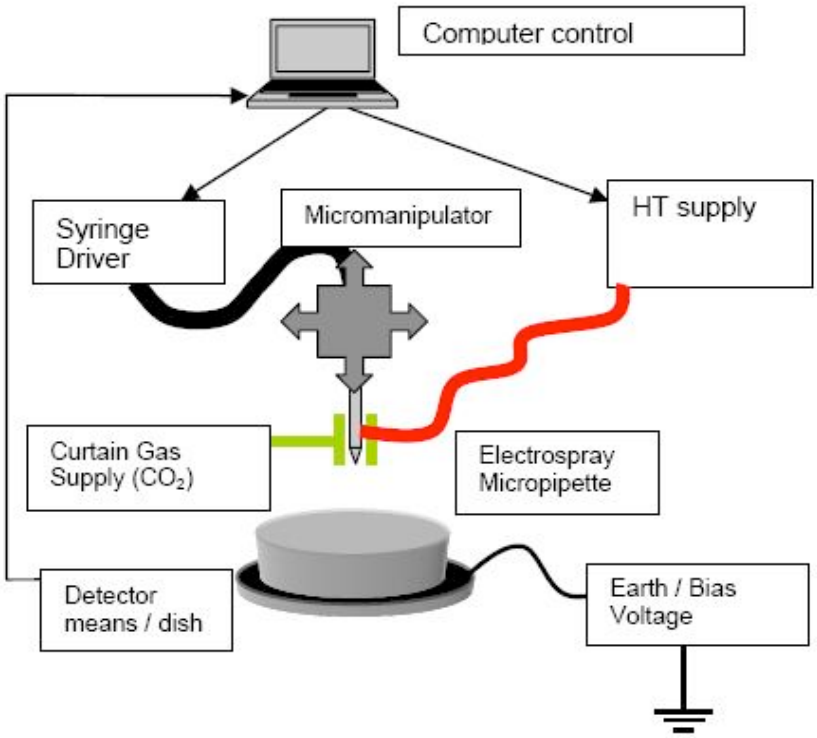


Figure 4-5a System diagram of rig

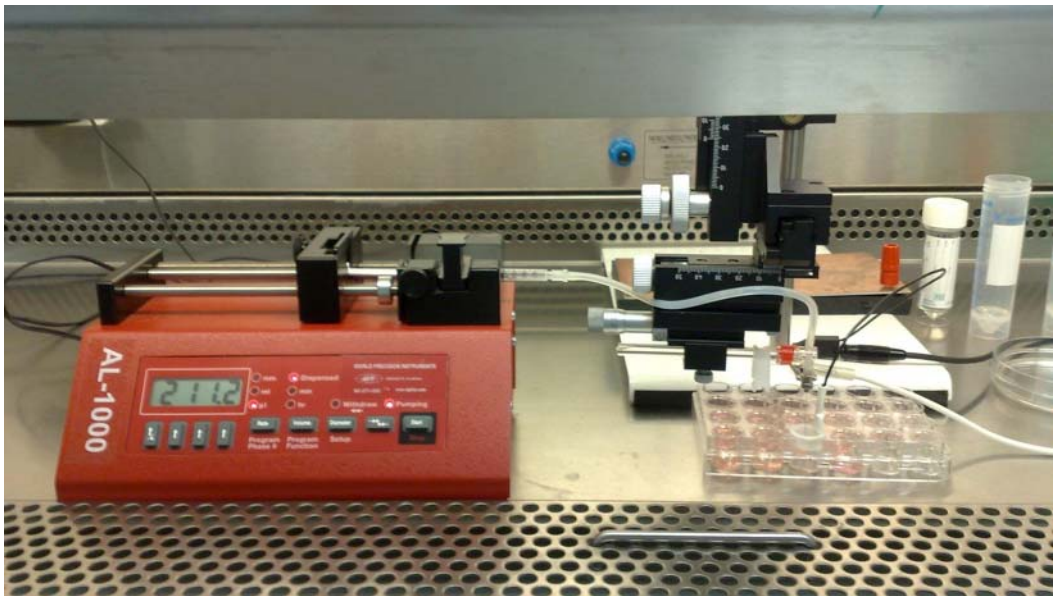


Figure 4-5b Photograph of rig in laminar flow unit

4.4 Dyes and Therapeutics in Solution

There are three forms of therapeutic and diagnostic molecules characterised for use in solution, biologics (DNA, siRNA), chemotherapeutics and dyes. In each case, there are benefits to electrospraying the molecule. In Table 4-3 the molecules chosen are presented.

Table 4-3 Biological electrospray solutions

Molecule	Solution Concentration	Molecule Type	Purpose	Reference
Methylene blue	2% Dye in PBS	Dye	Diagnostic dye	(Chen, Lin et al. 2007a)
Cisplatin	0.083 $\mu g l^{-1}$	Chemical Molecule	Chemotherapeutic	(Arriagada, Dunant et al. 2010)
DNA encoding GFP	0.5 $\mu g l^{-1}$	Biological Molecule	Gene transfection	(Davies 2005)
DNA encoding E-cadherin	0.5 $\mu g l^{-1}$	Biological Molecule	Gene Delivery for tumour suppression	Sigma Inc.
siRNA tagged with $Alexa_{488}$	40mM	Biological Molecule	Potential to Inhibit protein expression	Sigma Inc.
Rabbit – Anti mouse	1/1000 dilution in TRIS	Antibody	To negate the effect of a protein that is having a harmful effect	Sigma Inc.

Each molecule needs a dilutant. The molecule and dilutant are together characterised chemically and physically. This is done with a battery of test equipment. Typical parameters measured are 1) conductivity, 2) surface tension, 3) viscosity and 4) pH. The physical characterisation of the first two parameters is most important to establishing electrosprays. The last parameter is to ensure that the molecule, especially in the case of DNA, is within a pH range that will not degrade it. Measurements were with Hanna HI 2210 pH meter and Hanna conductivity meter HI 993310. Surface tension was measured using the Du Noüy ring method with an Attension tensiometer.

4.4.1 Methylene Blue

2% Methylene blue was made up in a solution with PBS. PBS is isotonic and non-toxic to cells so is a good choice for making up the dye. This yielded higher conductivity and lower surface tension than water. The characteristics of the solution are tabulated in Table 4-4.

4.4.2 Cisplatin

Cisplatin is a chemotherapeutic. This compound is highly toxic so safety precautions were taken to ensure it was properly handled and prepared. 0.083µg cisplatin was made up in a solution of PBS. A table of the characteristics of the solution are tabulated in Table 4-4.

4.4.3 DNA encoding GFP

DNA encoding GFP is important to deliver as gene therapy involves the delivery of a gene to replace the function of a damaged cell. DNA was made up in TRIS with a concentration of $0.5\mu gl^{-1}$. TRIS was used, as DNA requires a pH of 8 to remain stable. The characteristics of the solution are tabulated in Table 4-4.

4.4.4 DNA encoding E-cadherin

DNA encoding E-cadherin is a tumour suppressor. DNA was made up in TRIS with a concentration of $0.5\mu gl^{-1}$. TRIS was used as DNA requires a pH of 8 to remain stable. The characteristics of the solution are tabulated in Table 4-4.

4.4.5 siRNA tagged with $Alexa_{488}$

40mMol solution of siRNA tagged with $Alexa_{488}$ was prepared. $Alexa_{488}$ is a fluorophore that is not naturally occurring in cells. It blocks the action of proteins and is of enormous potential value in gene therapy. If the siRNA is delivered to cells and remains functional, it will fluoresce and can be viewed with a fluorescence microscope. The characteristics of the solution are tabulated in Table 4-4.

4.4.6 Antibody

Rabbit anti-mouse antibody (A5060) Sigma Inc. was made diluted to 1 in 1000 concentration in TRIS. It was collected in a 24 well plate and tested on an antibody assay (Appendix 3).

Table 4-4 Solution Characteristics

Solution	Conductivity (Sm^{-1})	Surface tension (Nm^{-1})	pH
De-ionised water	0.0002	0.0686	7.4
PBS	0.00126	0.023	8
Tris 100mM	0.478	0.0381	7.7
PBS + acetic acid 1% +siRNA	0.49	0.028	8
PBS + acetic acid 1% +cisplatin	0.504	0.0339	8
Tris + acetic acid 0.01% +GFP	0.116	0.0312	7.9
TE Buffer (maxiprep)	0.002	0.0554	-
Methylene blue +PBS	0.167	0.05148	-
Ethanol (70%)	0.000015	0.021	-

4.5 Electrospray Emitters

One or more emitters will be fabricated to facilitate the assessment of different sprays. The rig can support different emitters and will serve as a measurement and visualisation platform. Some specialised measurement equipment to process high potentials and to visualise electrospray is required. Electrospray emitters will be fabricated for each of the biologic and buffer combinations. The test rig will facilitate the electrospray characterisation activity.

4.6 Selection of an Appropriate Electrospray Mode

It was necessary to observe and describe the different electrospray modes observed or reviewed on the rig. The purpose of this was to identify which electrospray mode yielded the greatest efficacy in cell transfection whilst being cognisant of any potential damage to cells. Different modes have different current characteristics, voltage characteristics and morphology. By a combination of measurement and observation the optimum electrospray mode was determined to be cone-jet mode.

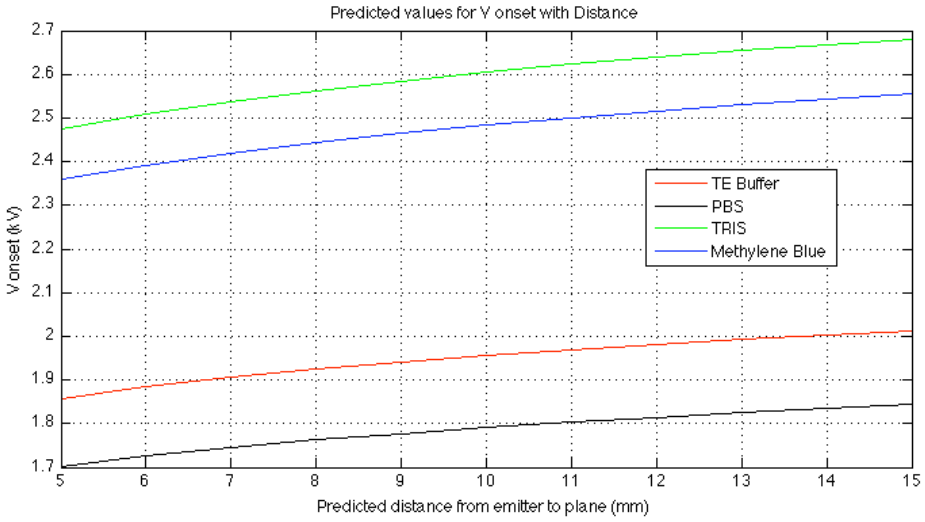


Figure 4-6 Predicted relationship of emitter-plane distance and V_0

It is useful to present some of the important interrelationships between electrospray parameters graphically. The V_0 of electrospray, as predicted by the model in Chapter 3, is presented in Figure 4-6. This is useful, as only the Surface Tension value needs to be changed for a given scheme with a given capillary radius to get a good approximation of what V_0 is likely to be. This is useful for ranging calculations with new buffers and compares very well with actual values. The model assumes no hydrostatic head ~zero flow so the V_0 voltages are lower than those at even modest flow rates of $3\mu l^{-1}$. Measured values for V_0 are presented in Chapter 5. The measured values of current and voltage in cone-jet mode at a separation of 10mm are presented in Figure 4-7. These values were measured for different buffers used at a flow rate of $3\mu lmin^{-1}$. The monotonic relationship between voltage and current is apparent in these graphs and they contain useful information of where the cone-jet mode starts and stops.

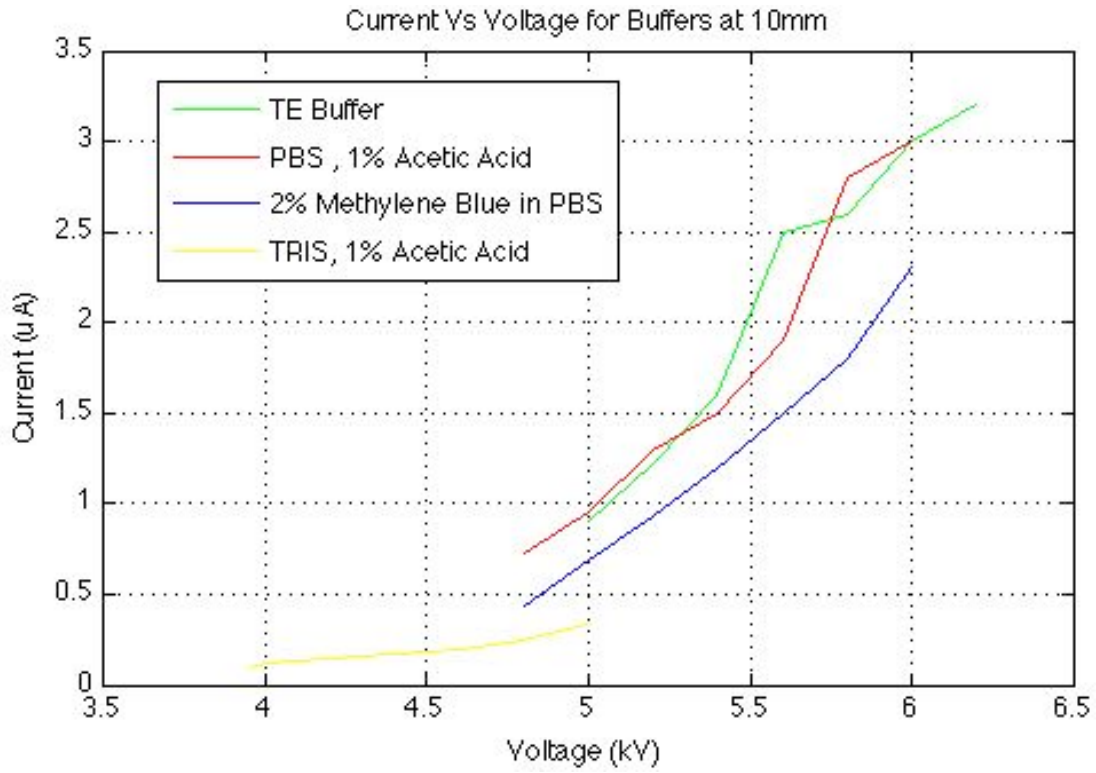


Figure 4-7 Voltage – Current relationship for buffers at h=10mm

In Figure 4.8, the inverse relationship between counter electrode distance and current is presented for different buffers. For values of current > 2μA in this scheme, the likelihood is that corona discharge was taking place. It is notable that current increases rapidly when electrode separation is < 7mm.

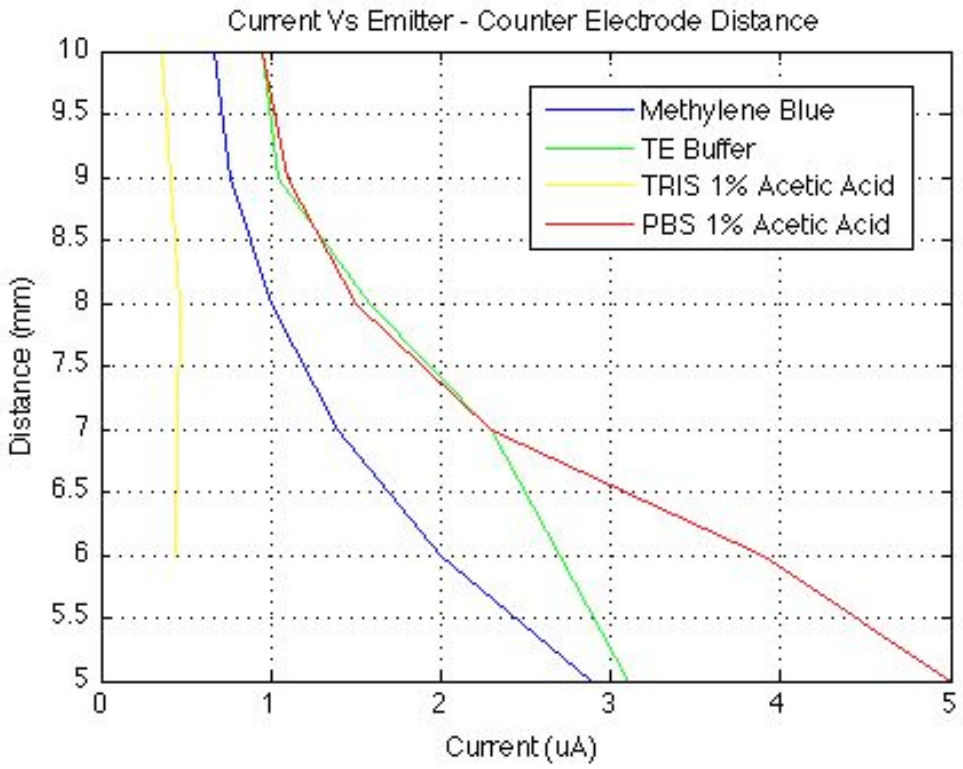


Figure 4-8 Current and emitter - Counter electrode distance relationship

In Figure 4-9, current, V_0 and distance are presented in a 3d graph. These are the main parameters that need to be considered in an electrospray scheme for the delivery of molecules. Flow rate, in this application, is generally kept as low as possible. Current $I \sim F^{1/2}$.

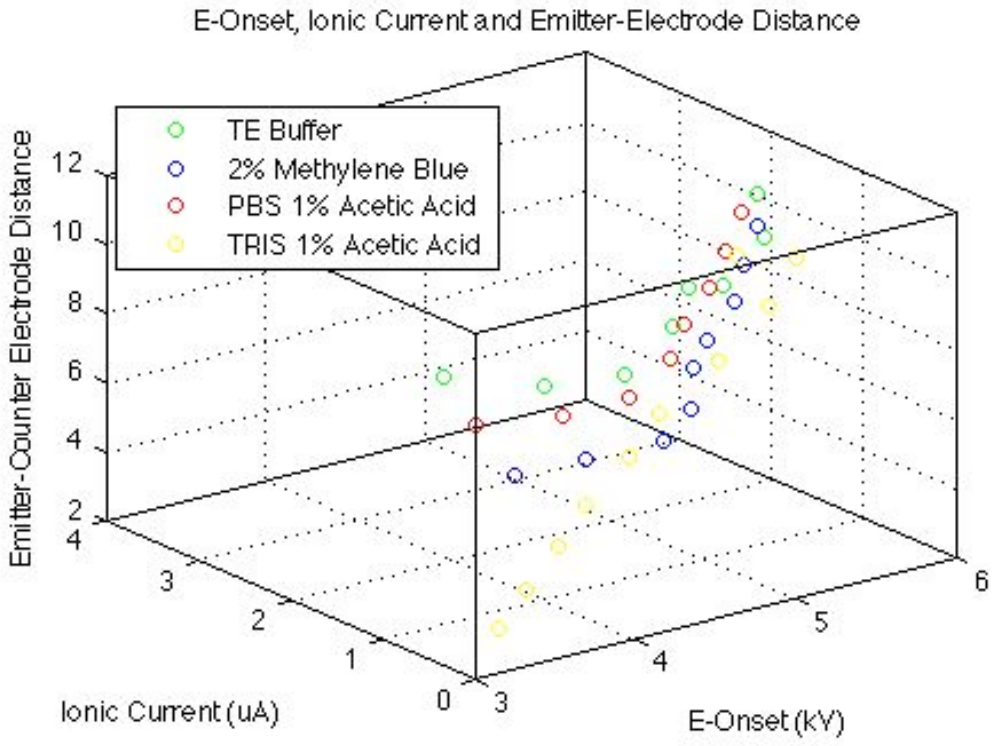


Figure 4-9 Interrelationships of Current, V_0 and distance

4.7 Use of a Constant Current Control System

4.7.1 Selection of the control parameters

It was observed that the electrospray current varied during the electrospray process within cone-jet mode and that this may have impacted on the efficacy of transfection. The possibility of controlling the electrospray current through manipulation of the electrospray voltage was explored. The control parameters are HV potential and Electrospray current.

4.7.2 Selection of controller type

A PID process controller was chosen, as this controller type is robust, well understood and universally used in industry. The functional diagram of the control system is depicted in Figure 4-10.

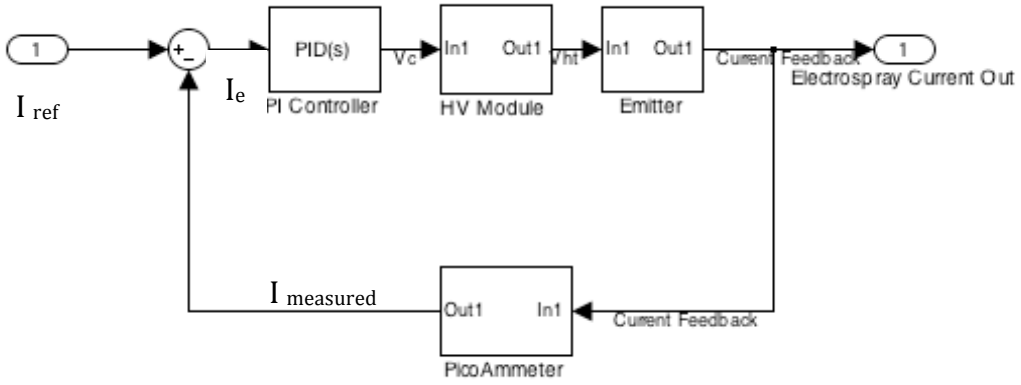


Figure 4-10 Functional diagram of controller

4.8 Achieving Stability within the Cone-jet Phase

It is widely reported that the variation of electrospray current varies linearly with voltage (Smith 1986; Saville 1997; Gamero-Casta, Ntilde et al. 2002; Smith, L. et al. 2006) in the stable cone-jet mode. It is also reported that colloids produced in this mode are monodisperse (Jones and Thong 1971; Tang and Gomez 1994; Chen, Pui et al. 1995; Tang and Gomez 1996). Consequently, a process controller was proposed to maintain the electrospray in cone-jet mode in the face of disturbances such as small variations in flow rate or in the emitter to counter electrode distance.

To explore the area of feedback, a PID process controller (Omega) was employed using current from the Keithley pico-ammeter as feedback. The choice of compensator implementation is a proportional, integral (PI) controller because the electrospray process can be electrically noisy. It was decided not to have a derivative term in the controller as this would amplify noise and potentially render the system unstable. The function of the proportional control is to change the output, in proportion to the error, towards the set point. From Figure 4-10, the equation for such a PI controller is given in equation 1. The constants K_p and K_I are constants based on the tuning of the system.

PI Controller

$$V_c = K_p I_e + K_I \int I_e dt \qquad (1)$$

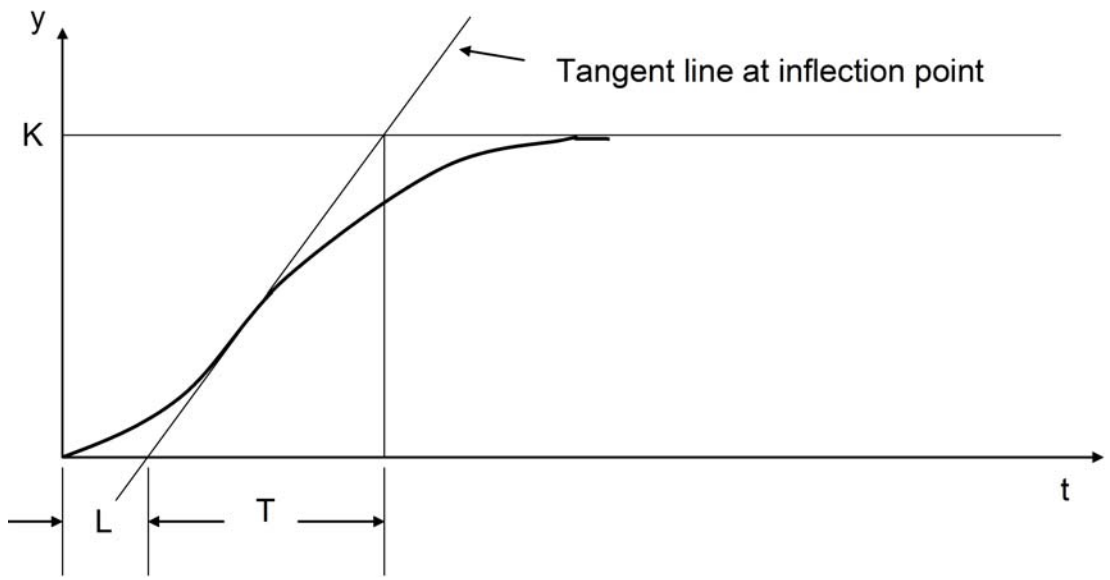


Figure 4-11 Reaction curve.

The open loop step response of the system shows a first order system with some dead time. As such, the Ziegler Nichols tuning method is appropriate and with reference to Figure 4-11, gives a methodology for determining values of K_p and K_I. The reaction curve in this method, is characterised by two constants, delay time L and time constant T, that are determined by drawing a tangent line at the inflection point of the curve and finding the intersections of the tangent line with the time axis and the steady-state level line.

Table 4-5 PI controller tuning constants

Controller	**K_P**	**K_I**	**K_D**	**Reference**
PI Controller	0.9 T/L	0.27 T/L^2	0	(Ziegler and Nichols 1942)

The step response curve of the electrospray current, I, is filtered in Figure 4-12 to remove high frequency noise. The digital filter was implemented in Matlab using the *filtfilt* function. This ensured no phase delay was introduced. The filter was a 5th order low pass Butterworth design. The cut off frequency was 5Hz.

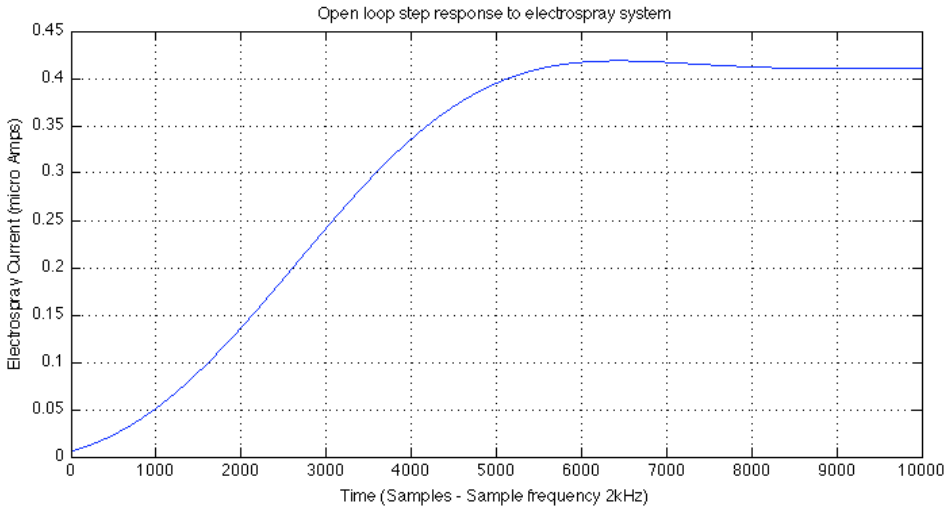


Figure 4-12 Open loop response curve for electrospray system

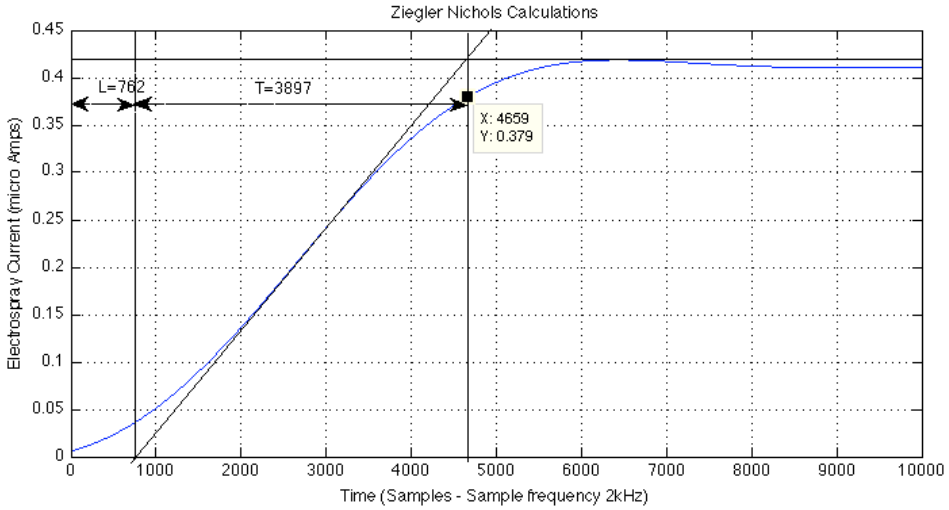


Figure 4-13 Ziegler Nichols tuning parameters calculated from the graph

Converting to the parameters L and T to seconds, we can set the values of K_P, K_I according to the formula shown in the table above.

L=0.381 s T=1.9485 s K_P=4.602 K_I=3.624

It is clear that there is some overshoot in the characteristic in Figure 4-13 and this impacts the tuning rules, however, the exercise was one of exploration rather than refinement and the tuning parameters had a positive effect on transfection consistency. Hence the controller is described by Equation 2.

PI Controller $$V_c = 4.602 I_e + 3.624 \int I_e dt \quad (2)$$

4.9 Electrospray Visualisation

Visualisation of the Taylor Cone was of particular interest during this work. Prior to observing the electrospray current characteristic to indicate what phase was occurring, there was a heavy reliance on adjusting the system whilst viewing the cone and jet to achieve stability. The rig included a long objective microscope and white light source (Dino-Lite Pro, USB) system. This enabled magnified images of the Taylor Cone and jet in its various electrospray modes where this was visible. The optical character of the spray can tell much about its physical character. In Figure 4-14 an image of a Taylor cone issuing from an electrospray with a coloured spectrum is termed a higher order Tyndall scattering spectra. This optical phenomenon occurs only when the colloids are monodisperse. This method of identifying monodispersity is largely superseded now but is worthy of mention.

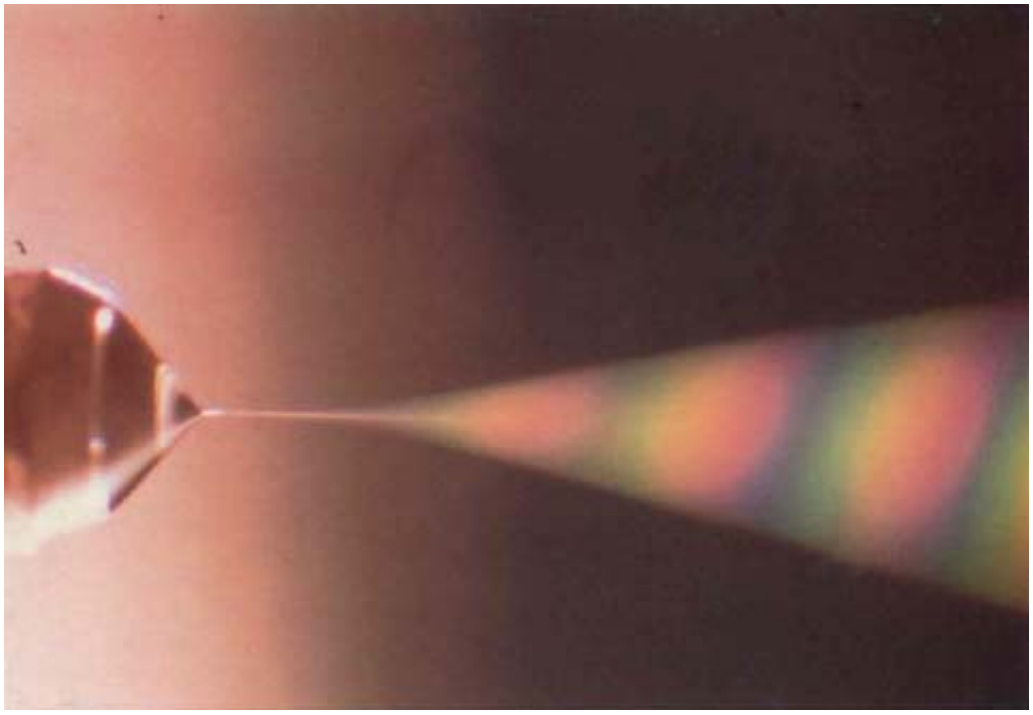


Figure 4-14 Image of electrospray in cone-jet mode. The colour spectrum within the spray indicates its monodisperse nature (Pantano, Gaón-Calvo et al. 1994).

4.10 Targets Cells

Information collected on targets after electrospraying of therapeutic molecules will form the basis of a better understanding of how physical vectors can effect transfections. Biological molecules are generally delivered into cells in vitro using viral and lipid vectors. For the purposes of comparative analysis with the proposed method, chemical delivery of the same molecules will be carried out as controls. Biological techniques to determine specific mechanisms of cell toxicity and cell death will be deployed in the event that these occur.

4.11 Impact of Charged Spray on Cells

There are probably many interactions between the cell membrane and the incoming colloid. It is believed, on the basis of review, that there is insignificant charge on the colloids to bring about electroporation at the cell membrane. In cell culture and tissues, little significant damage to the cells was observed. It is believed that the action of transfection is brought about by a colloid containing DNA measuring ~<0.5 μm impacting the epithelial cell measuring >10μm with a cell membrane thickness of 15nm (Bolsover 2004)

4.11.1 Toxicity of Electrospray

The number of cells that become unviable after the electrospray process determines toxicity. The cells are assessed at 24 and 48 hours. Viability is determined by observation and also the use of assays that can detect and quantify signature material from dead cells.

4.11.2 Immunogenicity of Electrospray in vivo

In vitro, a cell culture has no immune system so immunogenicity is not an issue, however in vivo, the human immune system can and does present an immune response to viral material in the body. An additional concern is the combining of a viral vector with a wild type virus and producing unpredictable results. As electrospray used naked genes, and other molecules and the buffers are isotonic, there is no basis for an immune response.

4.11.3 Consistency of Electrospray

One of the goals of this work is to make the transfection rate consistent. Although factors extrinsic to the operation of electrospray emitter can reduce its effectiveness such as 1) cell confluence and 2) cell type, in general if the electrospray operates consistently, the transfection results should be consistent.

4.11.4 Targeted Delivery of Electrospray

The spray pattern that the electrospray produces is connected with its ability to target cells. In general, the electrospray targets a circular area normal to the emitter. It is unclear whether the spray acceleration is uniform over this area.

4.11.5 Safety of Electrospray

Electrospray occurs at low current values in the order of 1-2 μA. These levels of current in the body are below those reported to be either sensible or harmful (Bikson 2005).

4.11.6 Efficacy of Electrospray

The efficacy with which transfections take place needs to be an improvement on what is conventionally available. One solution component concerns the quantification of evaporation within the system. In this case, fixed volumes of solution were supplied to the emitter. The solution was sprayed for time intervals ranging from 1-4 minutes. The solution was collected using a micropipette and measured. The difference in the volumes delivered on average was determined to be the evaporation rate.

4.12 Summary

In this chapter, the pulmonary delivery problem has been developed. The constituent aspects of the problem have been explored. Other electrospray solutions have been comparatively analysed under the headings of 1) physical and electrical character 2) working fluids 3) lung topology 4) toxicity 5) safety of HV in the body and 6) stability of electrospray. A concept for a bronchoscopically mediated electrospray device is introduced and a test rig to discover operational parameters for buffers is described. The mode of electrospray that is best for cell transfection is discussed and a series of

interrelationships of electrospray parameters is presented. Stability within the cone-jet mode is discussed, its impact on cell transfection and a feedback system introduced. In Chapter 5 a solution to the pulmonary delivery problem is presented.

Chapter 5

Design and Implementation of an Electrospray Solution

An endoscopically mediated electrospray device is proposed in this chapter as a putative solution to the pulmonary delivery problem.

5.1 Design Concept and Considerations

The electrospray emitter and a control unit should be connected by a fluidic and electrical connector (Figure 5-1). The electrospray device should pass through a bronchoscope and emerge into the trachea or lung lobes where the molecule will be delivered. The therapeutic molecule or dye is in solution and contained in the reservoir integral to the device. It must not leak out of the device. The device must pass through the bronchoscope's instrument channel. The channel width is 2.0 mm-2.8 mm in diameter depending on the model of the scope. The body length of the electrospray emitter device is limited to about 15mm in order to permit full articulation of the bronchoscope.

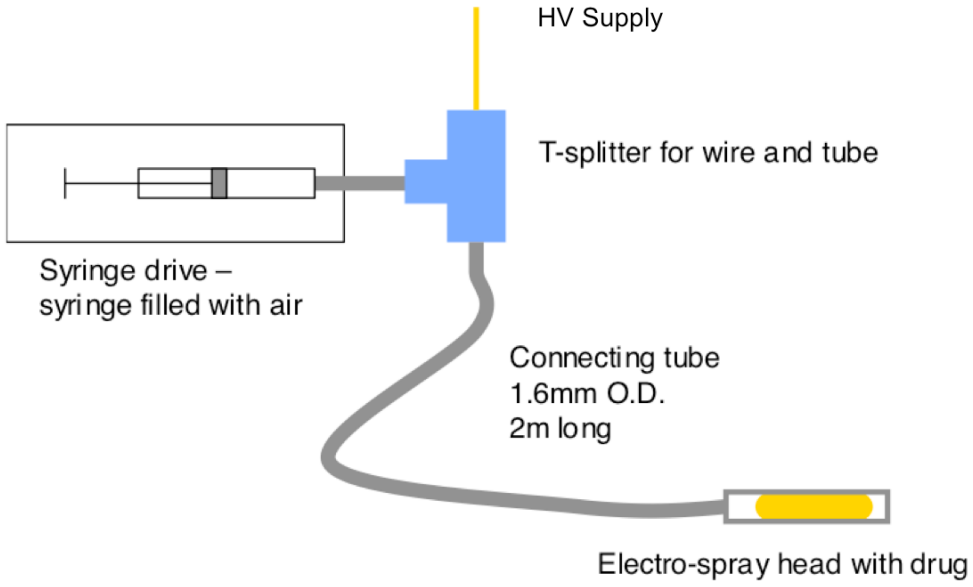


Figure 5-1 Design concept for bronchoscopically mediated electrospray

The device should accommodate a minimum of 10 µl of solution stored within it. The device should have an 80 µm I.D. metal capillary or emitter. The emitter should be circular in cross section and should ideally have smooth surfaces to avoid localised corona discharge. The emitter should ideally be made of stainless steel. The emitter assembly should be connected to the fluid supply and the HV supply by a fluidic and electrical connection. Ideally these should be co-axial such that only one tether connects the emitter assembly to the control unit, up to 2.2 m away.

A pumping mechanism is required to push the solution out of the emitter at a flow rate of approximately 3 $\mu l min^{-1}$, ideally a range 1 $\mu l min^{-1}$ – 20 $\mu l min^{-1}$. A high potential DC voltage needs to be connected to the emitter assembly by a wire of approximately 0.5mm diameter inside the fluidic connector. The device and connecting wire should be insulated from the internal surface of the body. The insulation should not break down with a HV potential of up to 7.5kV. This includes a safety margin of 2 over the highest expected voltage of 3.5kV. A 100MΩ resistor is placed in series with the HV supply limiting the current to 35 µA at 3,500V. In addition, the electrospray process behaves like a high ohm value resistor (Smith, L. et al. 2006) further reducing the electrospray current to 1 or 2 μA. This is safe to use in the body. 2.2m of suitable tubing are required to connect the emitter to the fluid pump and HV supply.

5.1.1 In vivo mode of use

The device is passed through the bronchoscope (Figure 5-2) using a HV cable and fluid conduit. The therapeutic solution or dye is pumped from an external syringe driver through a connecting tube into the device. The device is passed through the bronchoscope using the electrical and fluidic tether cable. The HV supply is switched on, using a foot switch at which point an electrospray is established using the patient's body as an electrical earth. A foot switch is used in this configuration as the physician was observed to have not had a free hand

to commence the delivery. The procedure is observed using the bronchoscope optics.

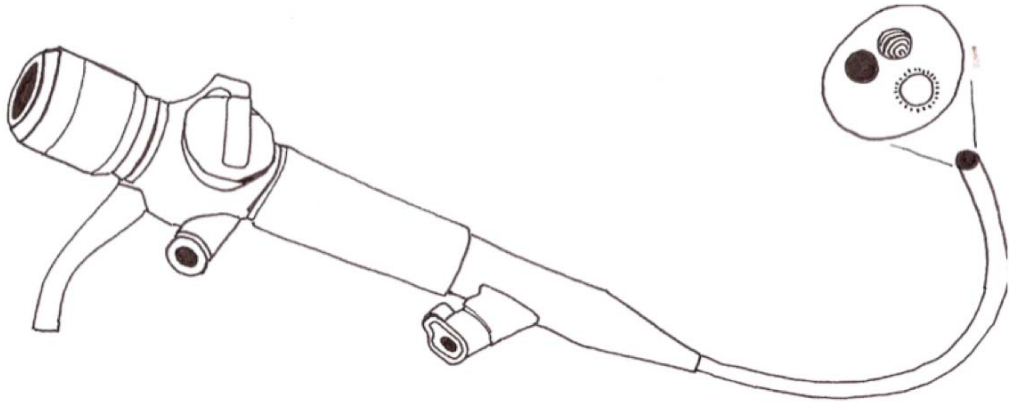


Figure 5-2 Drawing of a bronchoscope

5.1.2 In vitro mode of use

The device is filled with the solution through the tether conduit or through a filling hole depending on whether a continuous electrospray or one limited to the volume of solution in the reservoir is required. If the device has been front-filled, the filling hole is sealed with a pressure sensitive tape. Air pressure is applied via the connecting tube to push the solution out of the emitter. The HV supply is switched on, at which point an electrospray is established using a counter electrode in the 24 well plate as the target.

Table 5-1 Fluidic pumping schemes

Scheme	Flow Range	Max Volume	Air Pressure	Comment
Device and conduit fully filled	0-20μLmin^{-1}	1mL+	N/A	Good fluid control, ~continuous flow Susceptible to conduit movements
Device Only Front Filled – Air Pressure	0-6μLmin^{-1}	15μL	Air pressure provided by syringe pump	Less susceptible to conduit movements but less responsive to changes in flow rate
Device Only Front Filled Zero Hydrostatic Head	0-2μLmin^{-1}	15μL	At atmosphere, second filling hole open to atmosphere	No susceptibility conduit movements Good spray cones observed.

5.2 Key features of the design

The stainless steel 316 metal housing shown in grey in Figure 5-3 includes the electrospray nozzle and has an internal volume of 14ul. There are two fluid ports for injecting the solution.

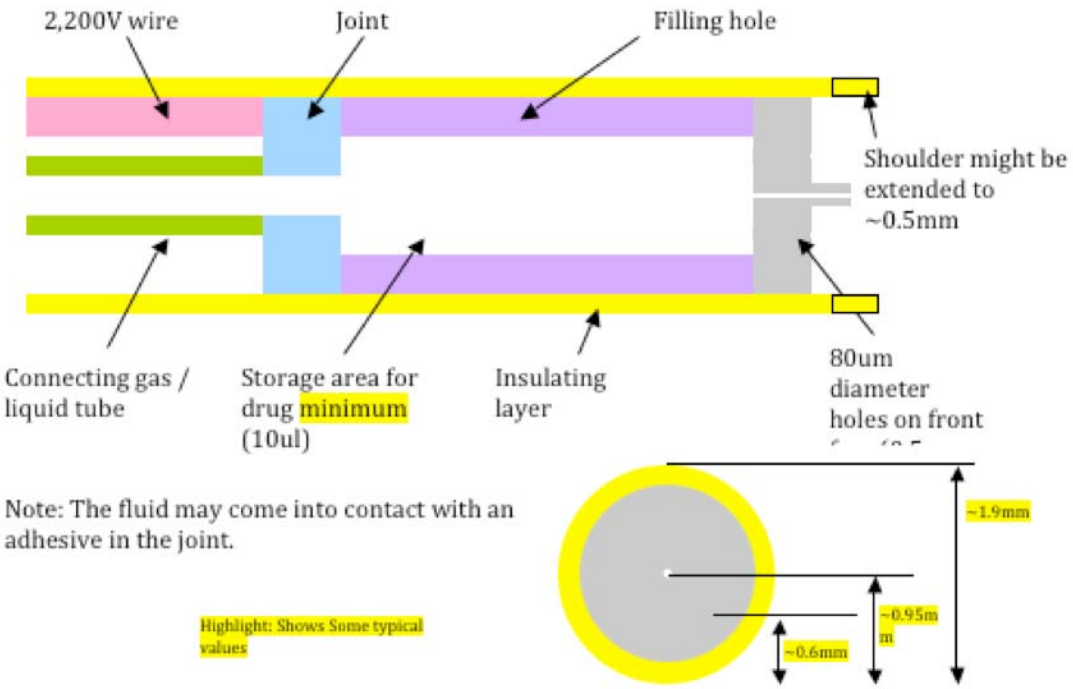


Figure 5-3 The schematic drawing shows some of the different components that will be required in the design

A stainless steel crimp makes electrical contact between the inside surface of the housing and a 0.5 mm O.D. copper wire. The crimping process will not restrict the flow of air from the PTFE tube into the stainless steel housing. There are two crimping processes - crimp to wire and stainless steel housing to crimp. The 1.6mm O.D. PTFE tube will fit inside the inside diameter of the stainless steel housing although, this will not be a gas tight fit. The gas tight seal is generated by the FEP heat-shrink which makes a tight seal on the outside diameter of the tube and the outside diameter of the stainless steel housing. If problems occur with this seal, the option exists to assemble it with a structural

adhesive layer between the outside surface of the PTFE tube and the inside surface of the stainless steel housing.

The bend radius of the 1.6mm O.D. PTFE tube with copper wire is ~5mm. This is higher in the region covered with heat shrink. The outer heat-shrink layer will be assembled last. This provides an electrical insulating layer and also a seal between the stainless steel housing and the PTFE gas feed tube. A T-adaptor (Figure 5-1) is also required. This is connected to the other end of the PTFE tube and will allow the wire and tubing to be split out to facilitate connection to gas pressure and voltage separately.

5.3 Flow rate considerations

In the table below the pressure required to pump the solution through the nozzle at a total rate of 3 $\mu l min^{-1}$ has been estimated using the Bernoulli equation.

Table 5-2 Pressure to flow parameters

Parameter	Value	Unit
Fluid flow rate	.6	uL/m
Fluid viscosity	2.5E-03	Pa.s
Fluid density	1000	Kg/m cubed
Pipe diameter	80	microns
Pipe length	0.5	mm
Volume of channel	0.0025	uL
Flow velocity in channel	.0020	m/s
Back Pressure Required	**12**	**Pa**

The resulting pressure is very low and would be difficult to control using an off-the-shelf pressure regulator. Hence, the following arrangement to control the dispense rate of the solution is suggested. In the set-up shown in Figure 5-1, the syringe volume should be as low as possible, ideally around 100ul. The flow rate on the syringe drive should be set at the desired flow rate for dispensing the solution. As the drive starts moving, the pressure in the syringe and tube will rise to around 0.00012Bar. The solution will then start moving and will be dispensed at the desired flow rate. In the in vitro work completed to date for

electrospray, the fluid has been pumped continuously by filling a 1mL syringe and purging the system of air. The dead space of the fluid conduit and the reservoir are together 185 µL. The absence of compressible media or air within the system results in the flow rate selected on the syringe driver being the rate at which fluid eludes from the system.

5.4 Design Practicalities and Compromises

The internal volume of the housing has been set at 14ul. This allows for some air voids inside the housing after loading the solution. The outside diameter of the head is 2.5mm, which is inside the range 2.0 mm- 2.8 mm. There is a risk that some of the solution will wick up between the crimp O.D. and the housing I.D. This can be avoided by inserting a polytetrafluoroethylene (PTFE) frit in front of the crimp. PTFE frits allow gas flow but prevent liquid flow. Adding the PTFE frit should not require a design change but could potentially cause some problems with the flow control system described above. The copper wire with tin coating might come into contact with solution. If this becomes problematic, the crimp can be redesigned to avoid this. Alternatively a stainless steel wire could be used instead of the copper wire. A stainless steel wire will be less flexible in comparison to the copper wire so it may not be ideal for the application.

5.5 Implementation details

After completing the design specification, Dolomite UK Ltd, a specialist microelectromechanical systems (MEMS) and micro fluidics company near Cambridge, UK was engaged to fabricate 22 emitter devices based on a design specification presented to them. The devices are fabricated as a stainless steel inner reservoir connected by a 2.2m X1.6 mm DuPont™ PTFE tube containing a conductor. PTFE was chosen as it is hydrophobic and does not contain latex. The device is externally finished in TexLoc™ Fluorinated Ethylene Propylene (FEP) heat shrinkable fluoropolymer tubing. This is a high quality tube that has

been expanded mechanically to slide over a part and is then heated to shrink down to a tight fit that will not fall off.

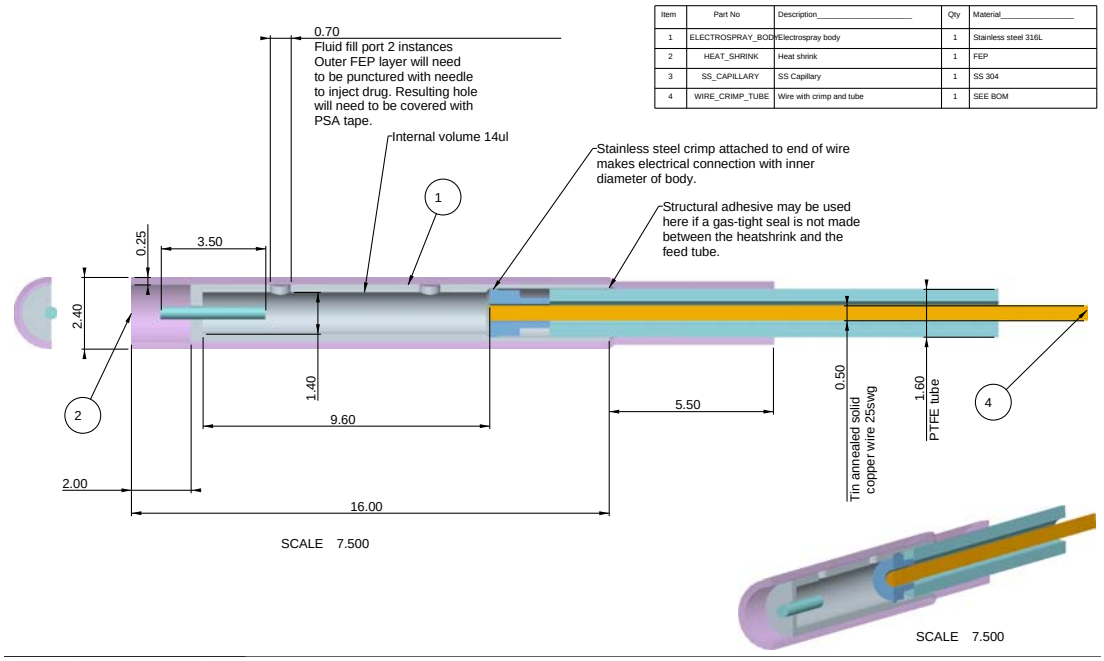


Figure 5-4 Engineering drawing of the proposed device

After a series of discussions and two visits to the fabrication facility, the devices entered production and were delivered 10 weeks later. The progression through Figure 5-4, Figure 5-5 and Figure 5-6 shows the evolution from concept to realisation.

Figure 5-5 3D Rendering of the finished electrospray device design

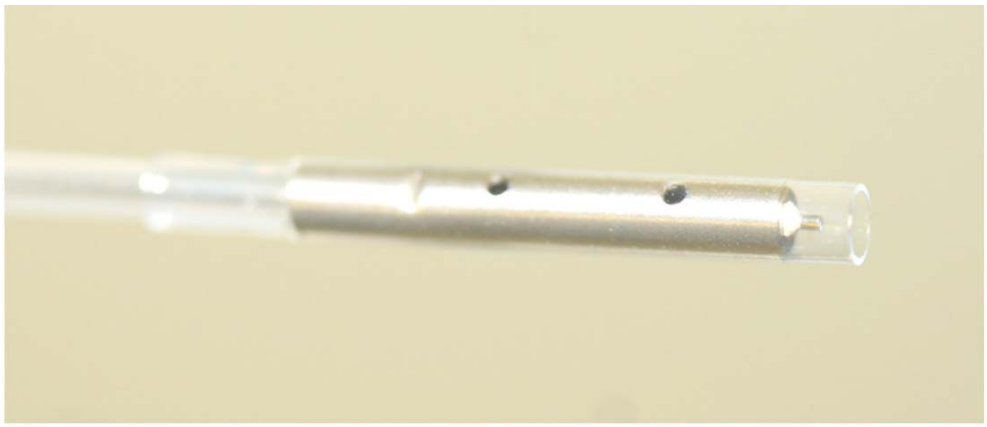


Figure 5-6 Photograph of the fabricated device

Two issues arose during the fabrication: 1) the 2mm overhang of FEP heat-shrink at the end of the nozzle has a length tolerance of 2mm +/-0.3mm. This was because shrinkage of the FEP in the lengthways direction was difficult to control. On the first four emitters fabricated, the FEP overhang shrank back to around 1.7mm. The tooling was then changed and on the remaining eighteen, the overhang was between 2.0 – 2.3mm 2) the conductive epoxy used to bond the capillary into the head can be seen in Figure 5-7. The epoxy fillet protrudes from the front surface of the nozzle between 0.1 mm – 0.5 mm. It is possible to further heat the FEP heat-shrink to reduce the diameter at the end of the overhang, forming a conical shape. This might have some benefits for the application.

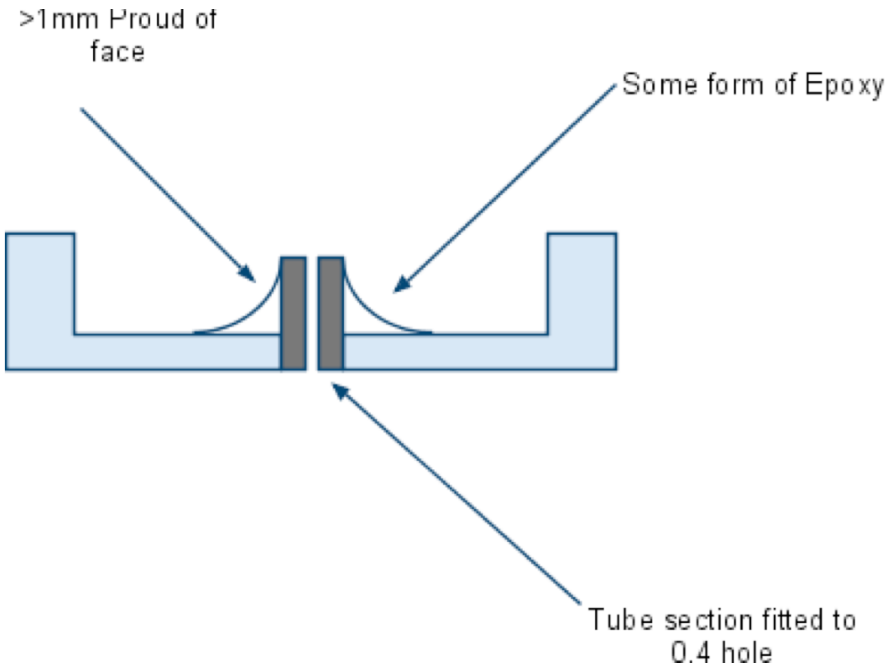
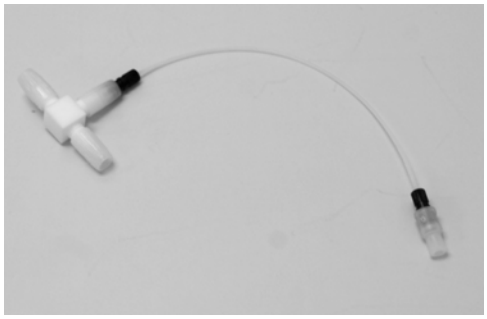
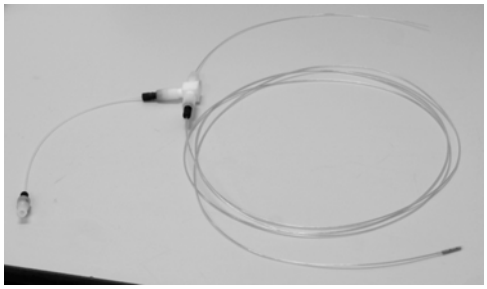
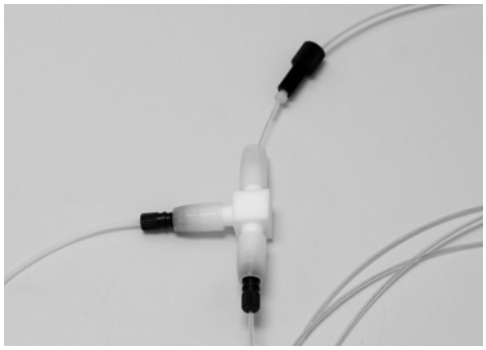
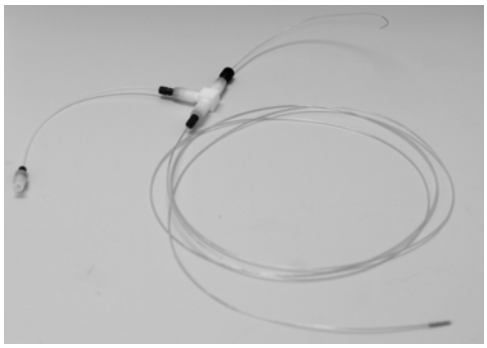
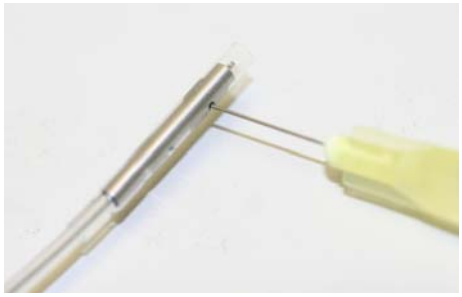


Figure 5-7 Drawing of the capillary or nozzle supported by electrical epoxy

Table 5-3 Device Assembly Steps

1	The T-splitter is connected to a feed tube with Luer adaptor for connection to a syringe or gas supply.	
2	The wire from the electrospray head with 2.2m loom is fed through the T-splitter as shown. Once this is done, the black fitting is screwed into the T-splitter to make a gas tight seal.	
3	Take the length of 0.5mm I.D. PTFE tube with threaded fitting and ferrule and slide this over the wire. Tighten the fitting into the t-splitter to make a gas tight seal. A length of wire should protrude from the end of the tube for connection to the HV supply.	
4	The assembled T-splitter and electrospray head is shown.	
5	The solution is injected into the nozzle. The front port seems to work well. The Luer adaptor was disconnected from the syringe during the filling. A small amount of liquid wicks between the FEP heat-shrink and the outer surface of the Stainless steel body. This does not seem to affect the operation of the nozzle.	
6	The electrical connection between the nozzle and the T-splitter connection was measured. The connection was good with a resistance of around 15-20 ohms. This is the resistance of the conductive epoxy joint. The resistance of the other electrical connections was < 0.5 ohms.	

5.5 Performance of Electrospray Device

a  b  c 

Figure 5-8 Counter electrode configurations

A device was assembled as per the process shown in Table 5-3 and affixed to the micromanipulator on the rig. The device was filled with a solution of 0.01 molar TRIS buffer with 0.1% acetic acid. Acetic acid is a non-polar solvent and has the effect of lowering the surface tension of the solution. In the case of TRIS this lowers its surface tension from 0.0381 Nm^{-1} to 0.0312 Nm^{-1} (see Table 4-4). This has the effect of lowering V_0 and greater spray stability in cone-jet mode (Fernández de la Mora 2007).

The emitter was directed vertically downward and at a separation, initially of 10mm, from a cell culture dish, termed a 24 well plate. A counter electrode ring shown in Figure 5-8a was designed to fit into the 24 well plate shown in Figure 5-8b. A covering of paraffin film is used to cover the adjacent wells.

The V_0 was predicted using the model and the V_C control was set to 25% below this value. The syringe driver was configured and switched on. The HV supply was enabled. The electrospray commenced and observed using the microscope. Current and voltage measurements were observed and recorded. The electrospray commenced dripping and the V_C control was advanced until a cone was observed. The cone remained stable for 8 minutes. Subsequent to the initial test with TRIS, other solutions were trialled. These are presented in Table 5-4.

Table 5-4 An initial shortlist of solutions that can be used with the proposed system

Solution	Chemical Name	Full Name	Solution Type	Comment
De ionised Water	dH2O		Potential naked DNA solution	Hard to spray as high dielectric constant + high surface tension
PBS	NaCl 0.138 M; KCl - 0.0027 M	phosphate buffered saline	DNA, siRNA Buffer	Good and stable spray due to low dielectric constant- if narrow band for cone-jet
TRIS	$(HOCH_2)_3CNH_2$	tris(hydroxymethyl)methylamine	DNA, siRNA Buffer	Good and stable electrospray due to low dielectric constant- if narrow band for cone-jet
PBS+ Acetic Acid	NaCl 0.138 M; KCl - 0.0027 M+ 1% CH_3COOH	phosphate buffered saline + 1% ethanoic acid	DNA, siRNA Buffer	Stable electrospray due to low dielectric constant of PBS- Polar Solvent Acetic acid reduces surface tension.
TRIS+ Acetic Acid	$(HOCH_2)_3CNH_2$+ 1% CH_3COOH	tris(hydroxymethyl)methylamine + 1% ethanoic acid	DNA, siRNA Buffer	Stable electrospray due to low dielectric constant of TRIS- Polar Solvent Acetic acid reduces surface tension.
Methylene Blue	$C_{16}H_{18}ClN_3S$, $3H_2O$	3,7-bis(Dimethylamino)-phenothiazin-5-ium chloride	Clinical Diagnostic Dye	Excellent wide band cone jet
Methanol	CH_3OH	Methanol	Test Solution	Excellent wide band cone jet
Ethanol	C_2H_5OH	Ethanol	Test Solution	Excellent wide band cone jet

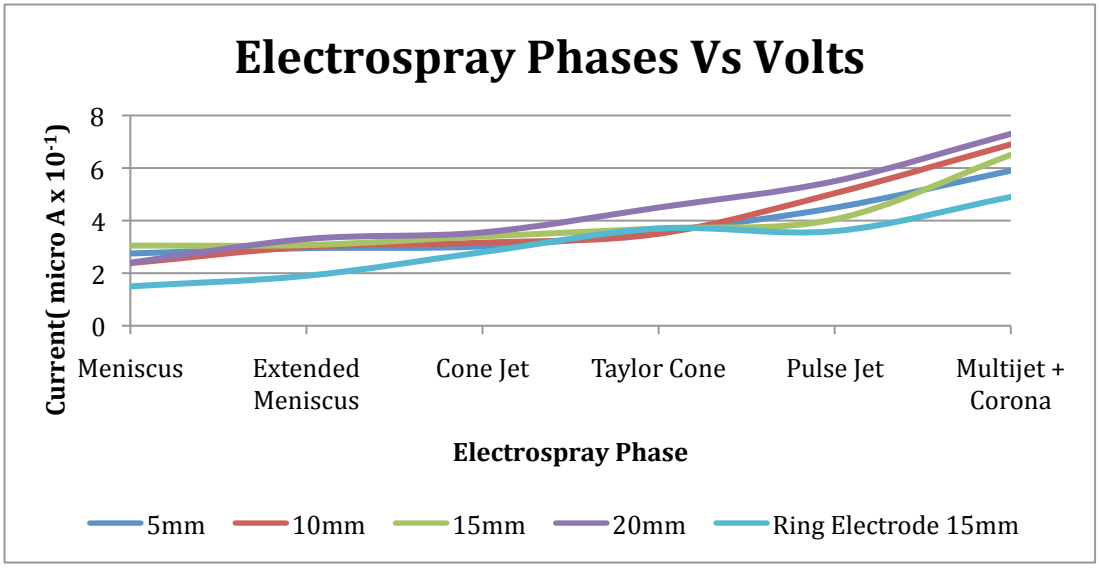
In Table 5-5, the new emitter was compared to the developmental glass and stainless steel capillaries used in Chapter 4 to explore the phenomenon.

Table 5-5 Comparison of new device with existing glass and steel capillaries

V_0 for emitter diameters (Flow rate 3µl min^{-1})

Solution	Emitter Inside Diameter = 120 microns (Harvard Instruments Stainless Needle)	Emitter Inside Diameter = 100 microns (Harvard Instruments Stainless Needle)	Emitter Inside Diameter = 80 microns (NEW Device)	Emitter Inside Diameter = 50 microns (Picotip Micropipette)
Deionised Water	5.81kV	5.94kV	4.19kV	2.84kV
PBS	4.8kV	2.25kV	3.41kV	2.77kV
TRIS	4.25kV	3.95kV	3.34kV	2.4kV
PBS+ Acetic Acid	3.96kV	3.6kV	2.65kV	2.25kV
TRIS+ Acetic Acid	4.6kV	2.11kV	3.11kV	2.44kV
Methylene Blue	5.25kV	4.25kV	3.87kV	2.25kV
Methanol	3.43kV	3.1kV	2.25kV	1.91kV
Ethanol	3.69kV	2.1kV	1.95kV	1.79kV

In Figure 5-9 the progression through the phases of electrospray is presented for different emitter – counter electrode distances. The solution is 0.01M TRIS 1% acetic acid. Current is observed to be in a monotonic relationship with voltage in the region of the cone–jet mode over the range of electrode separation distances. Current was measured by connecting the counter electrode through the Keithley electrometer to earth.



Phase	Current in uA @ 5mm	Current in uA @ 10mm	Current in uA @ 15mm	Current in uA @ 20mm	Current in uA with Ring Electrode
Meniscus	2.75	2.38	3.05	2.4	1.5
Extended Meniscus	2.95	3	3.07	3.3	1.9
Cone Jet	3.02	3.16	3.4	3.55	2.8
Taylor Cone	3.58	3.5	3.7	4.5	3.7
Pulse-jet	4.49	5.04	4.05	5.5	3.6
Multijet + Corona	5.9	6.9	6.5	7.3	4.9

Figure 5-9 Electrospray phase with electrospray current

In Table 5-6, visual observation of electrospray stages for sample buffer TRIS, 0.01% Acetic Acid are presented. Electrospray has a number of phases that it cycles through and finding the one that gives rise to the greatest transfection is the ultimate objective of this exercise.

Table 5-6 Electrospray phases

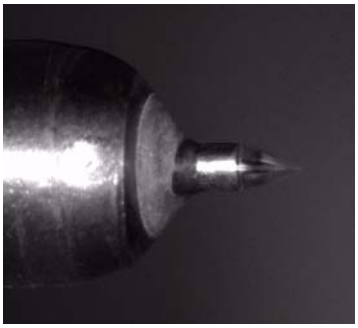
$V < V_0$

Emitter – Target Distance (m)	Volts (kV)	Comments	Electrospray Phase Image
5×10^{-3}	2.75		
10×10^{-3}	2.38		
15×10^{-3}	1.5	Ring Electrode into Well	
15×10^{-3}	3.05		
20×10^{-3}	2.4		

$V \sim V_0$

Emitter – Target Distance (m)	Volts (kV)	Comments	Electrospray Phase Image
5×10^{-3}	2.95		
10×10^{-3}	3.0		
15×10^{-3}	1.9	Ring Electrode into Well	
15×10^{-3}	3.07		
20×10^{-3}			

$V > V_0$ Cone Overfed with fluid. Taylor Angle $\theta << 49.3^0$

Emitter – Target Distance (m)	Volts (kV)	Comments	Electrospray Phase Image
5×10^{-3}	3.02		
10×10^{-3}	3.16		
15×10^{-3}	2.8	Ring Electrode into Well	
15×10^{-3}	3.40		
20×10^{-3}	3.55		

$V > V_0$ Cone. Cone-jet mode. Taylor Angle $\theta \sim 49.3^0$

Emitter – Target Distance (m)	Volts (kV)	Comments	Electrospray Phase Image
5×10^{-3}	3.58		
10×10^{-3}	3.5		
15×10^{-3}	3.7	Ring Electrode into Well	
15×10^{-3}	3.7		
20×10^{-3}	4.5		

$V >> V_0$, cone generatrix becomes convex and cone begins to pulse

Emitter – Target Distance (m)	Volts (kV)	Comments	Electrospray Phase Image

5×10^{-3}	4.49	Pulse Frequency 1.46Hz	
10×10^{-3}	5.04		
15×10^{-3}	3.6	Ring Electrode into Well	
15×10^{-3}	4.05		
20×10^{-3}	5.5		

$V >>> V_0$, cone disappears and fluid elution becomes chaotic

Emitter – Target Distance (m)	Volts (kV)	Comments	Electrospray Phase Image
5×10^{-3}	5.9		
10×10^{-3}	6.9		
15×10^{-3}	4.9	Ring Electrode into Well	
15×10^{-3}	6.9		
20×10^{-3}	7.3		

The Taylor angle was measured for 5 solutions at stable cone jet mode using Imagej™ image processing software. As can be seen from Figure 5-10, the cones emerging from the emitter devices are close to ideal.

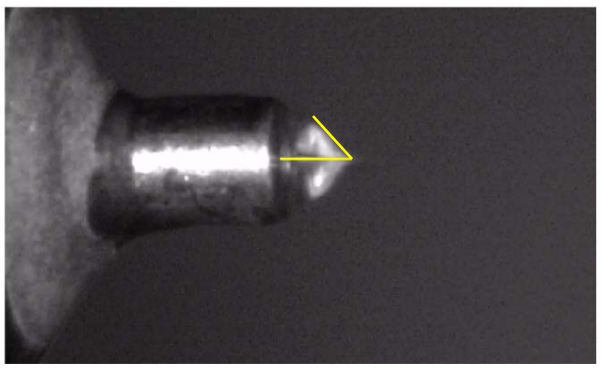


Figure 5-10 Taylor cone angle measured for TRIS, 1% Acetic acid

Table 5-7 Taylor cone angles measured in cone-jet mode for different solutions

Solution	Taylor Cone Angle
TRIS, 1% Acetic acid	47.121
Methylene blue	51.340
Ethane	49.086
PBS, 1% acetic acid	47.121
TE Buffer	49.399

5.6 Performance Irregularities

5.6.1 Over-wet phase

In the period before electrospray commences, the capillary or emitter frequently has excess fluid about it. This has the effect of delaying the onset of stable cone-jet mode until the excess fluid has been 'burned' off. The use of hydrophilic coatings on future devices may assist with this problem. In biological applications, fluid volumes are frequently small so losing fluid in this way is not ideal.

5.6.2 Steady phase

Steady cone-jet phase was occasionally seen to be disrupted and fail to re establish itself. This is due to disturbances to the system such as air bubbles, a sudden fall or rise in fluid flow and the presence of any impurity in the fluid. There is a suggestion that chemistry and ion availability can cause similar disturbances but this has not been investigated.

5.6.3 Depletion of fluid phase

When the fluid runs out in an experiment, the emitter can 'splutter' to a halt. It is unclear what the impact of this is on the cells beneath. Consequently some means of detecting the presence of adequate fluid to electrospray would be ideal. The HV supply might be cut by such a mechanism.

5.6.4 Air locks and dead space

Dead space and air locks in the device, the tubing and the connectors have been a continual problem. The emitter device has minimal dead space and behaves well under steady conditions but other components such as filters and

connectors should be designed for the application to avoid fluctuations in fluid supply.

5.6.5 Leakage current effects

On occasion, where a fluid such as water is being electrosprayed, the observed currents are 2-3 times higher than those expected. Here, current is leaking from the process through corona discharge. Corona discharge begins to appear as a feature at potentials of a~4kV for a electrode separation of 10mm. Efforts have been made through doping of the buffer solutions with acetic acid, a non-polar solvent, to reduce the fluid surface tension and reduce the V onset voltage, V_0. This means the potentials that are likely to give rise to corona discharge do not arise.

5.7 Summary

An endoscopically mediated electrospray device was proposed in this chapter as a putative solution to the pulmonary delivery problem. The electrospray emitter was fabricated and shown to be capable of producing an electrospray. Suitable counter electrodes were designed and fabricated. The emitter design was tested involving 1) comparative analysis with existing capillaries 2) visualisation and comparison of cone shapes with those reported in the literature and 3) measurements of current, voltage at onset and electrode separation distances. Initially sprays from a standard range of buffer and therapeutic combinations were tested. Factors such as electromagnetic compatibility and biocompatibility were considered in the device design. The device is now ready for trials in vitro and in vivo.

Chapter 6

Results and Discussion

In Chapter 5 the electrospray emitter device was designed and implemented. In this chapter the device is trialled in relevant in vitro and in vivo configurations. The purpose of this device is to deliver therapeutic molecules and diagnostic dyes for the purposes of addressing the pulmonary delivery problem developed in Chapter 4. Molecules including DNA, siRNA, proteins, chemotherapeutics and marker dies were delivered to cells and tissues. The device's performance was analysed for consistency, targeted delivery, safety and efficacy. The in vitro and ex vivo experiments were carried out in the Human Epithelial Laboratory in NUI Maynooth. The in vivo device trial was carried out in Trinity College Dublin in May 2010. Respiratory Physician, Professor James Egan and Veterinary Surgeon, Dr. Peter Nolan conducted the trial in the presence of the author and Dr. ODea.

Toxicity and cell viability were assessed in vitro by a process of growing cells on the base of cell culture dishes. These were placed under the electrospray for various time intervals of 1 to 4 minutes. After the process was completed, the cells were incubated for 24 and 48 hours. At this point the cells were counted and compared with a control sample that were not sprayed. Cell viability and toxicity were assessed. Performing the experiments repeatedly assessed consistency of the process. In each case the parameter, in the case of GFP transfection was measured by flow cytometry repeatedly and the results recorded. These proved, at the start, to be generally inconsistent. After observation, of the cone and knowledge of different phases of the electrospray, experiments were done in cone-jet phase. This is the phase that has typically lead to the highest transfection in the past.

Targeted delivery was measured and assessed by observation of the cells after the electrospray process. It was generally quite easy to see where the process had impacted the cell and those areas that were apparently unaffected.

Summary of Molecules Electrosprayed

siRNA (RNA)

GFP plasmid (DNA)

Cisplatin (chemotherapeutic drug)

Methylene blue (biological dye)

Antibody

Summary of cells electrosprayed

In vitro

BEAS-2B (bronchial epithelial cell line)

DLKP-SQ (human lung cancer cell line)

Ex vivo

Lung tissue (pig)

In vivo

Pig Bronchus

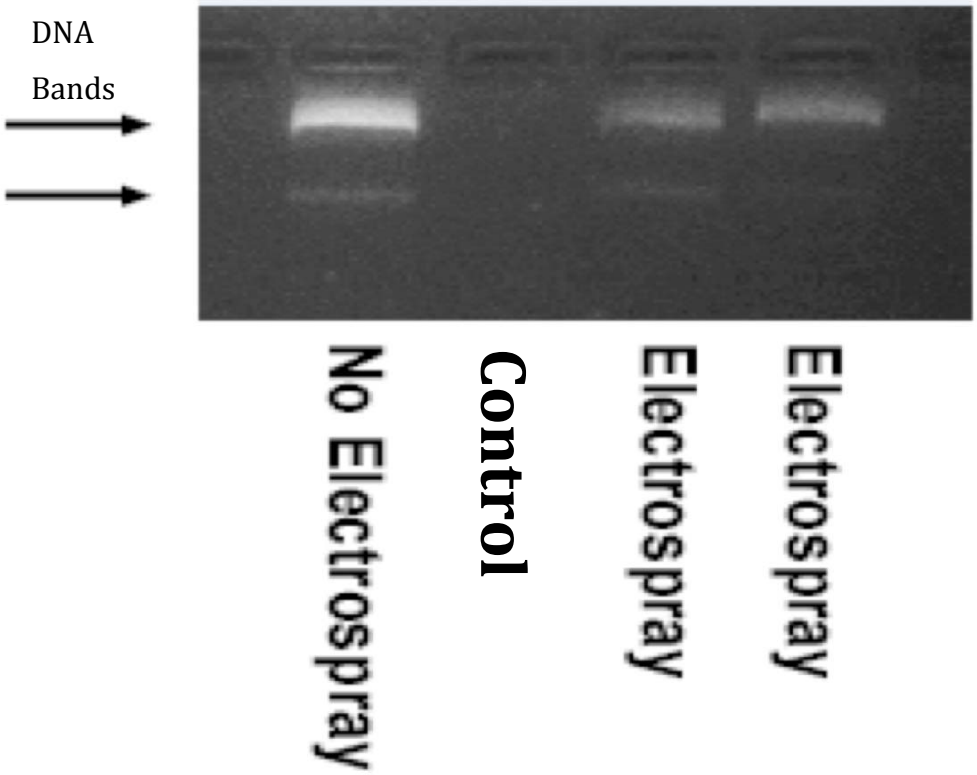


Figure 6-1 DNA retains integrity after spraying

6.1 In vitro tests

6.1.1 Effect of electrospray process on DNA integrity

The electrospray process was examined for any deleterious effect on integrity of plasmid DNA. GFP DNA was delivered by electrospray into empty tissue culture plates. DNA is analysed on an agarose gel to examine integrity. Distinct bands marked by the arrows in Figure 6-1 reveal that DNA molecules have not been degraded during the electrospray process. The resulting solution was retrieved from the plate and subjected to gel electrophoresis. DNA remained intact following the electrospray process.

6.1.2 Non-toxic electrospray mediated in vitro delivery of DNA encoding GFP in human epithelial cells

BEAS-2B (transformed human airway cells) cells were electrosprayed with green fluorescing protein (GFP) plasmid and underwent a transfection rate of

up to 67.5% (Figure 6-2). This transfection method is cheaper, has fewer steps and lends itself to automation more readily than virus or lipid work.

Human lung cells were seeded into tissue culture plates. A solution containing plasmid DNA encoding for green fluorescent protein (GFP) was electrosprayed. Protein expression of green fluorescent protein was examined in cells electrosprayed with GFP DNA. Expression of fluorescent protein was determined by flow cytometry.

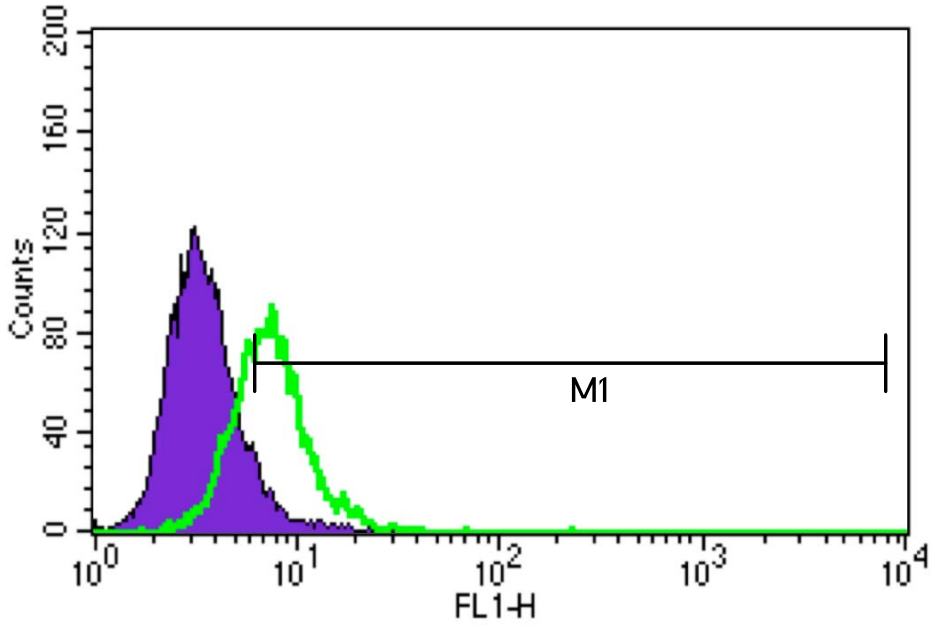


Figure 6-2 Flow Cytometry results. Purple graph represents control untransfected cells, Green represents positively transfected cells with increased fluorescence (GFP) as indicated on X-axis, corresponding to 67.5 % cells transfected (Reading in accompanying printout from the machine).

In Table 6-1 transfection rates for the emitter running in open loop mode are presented. They show some wide variation in consistency and a low mean transfection rate of 19% and 22% respectively. The two columns represent GFP transfection and siRNA transfections as both of these are assessed using the flow cytometer.

siRNA (% increase)	GFP Plasmid (% increase)
15.7	67.5
6.2	37.8
35.3	3.5
18.9	16.7
23.2	30.5
94.1	70.2
13.2	8.5
6.8	12.2
7.2	13.8
15.7	12.6
10.3	10.9
11.7	8.7
7.9	13.0
10.4	12.6
7.9	11.2
Mean **19%**	**22%**

Table 6-1 Transfection rates with open loop control

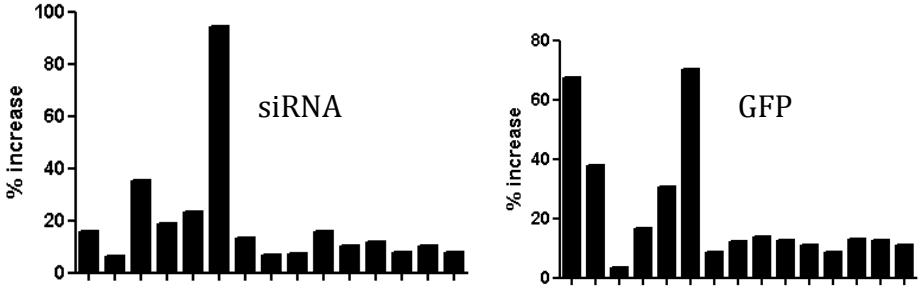


Figure 6-3 Graph of transfection rates with the emitter in open loop mode. 15 transfections are shown

In Figure 6.4 the PI controller is employed and the 16 GFP transfections are shown. The mean transfection has increased from 22% to 33% and the results seem generally to be more consistent. As depicted in Figure 6-5 the viability of cells was not significantly affected by electrospray. In Figure 6-6 toxicity was assessed through the use of Annexin V. The presence of the cell population in the bottom left quadrant of diagram (B) indicates no significant cell death in the population versus the control population (A).

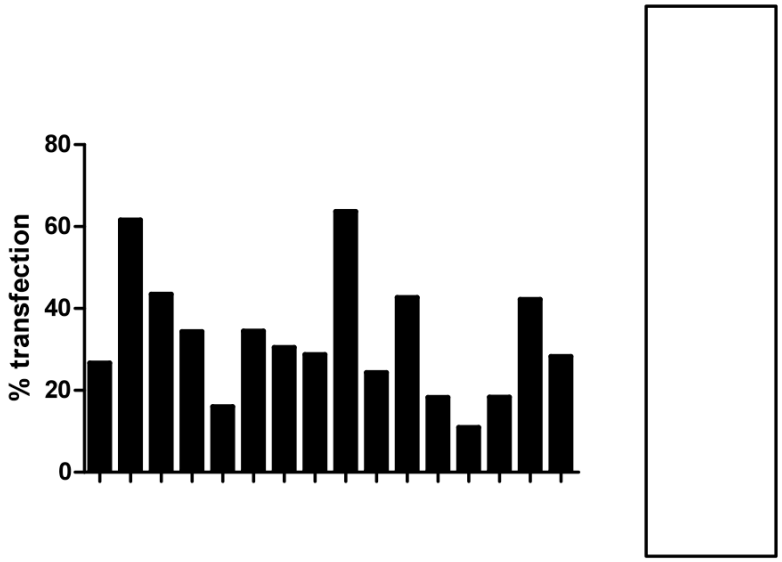
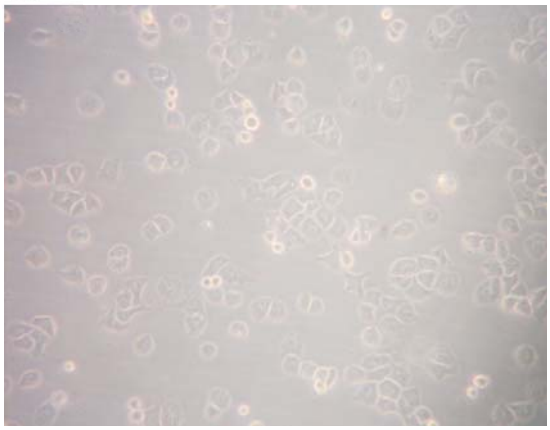


Figure 6-4 Graph of transfection rates of GFP into Human Cells Mean value is 33% GFP transfection 48h following controlled electrospray

A



B

Group	***% Loss of viability***
No	*n/a*
electrospray	*1%*
DNA plasmid	*1%*

Figure 6-5 Viability of cells was not significantly affected by electrospray. DLKP-SQ retained their capacity to adhere to plastic following electrospray (A). No significant loss of viable cells was observed (B). Viability was assessed by counting with ethidium bromdie acridine orange (EB/AO). Results are representative of three experiments.

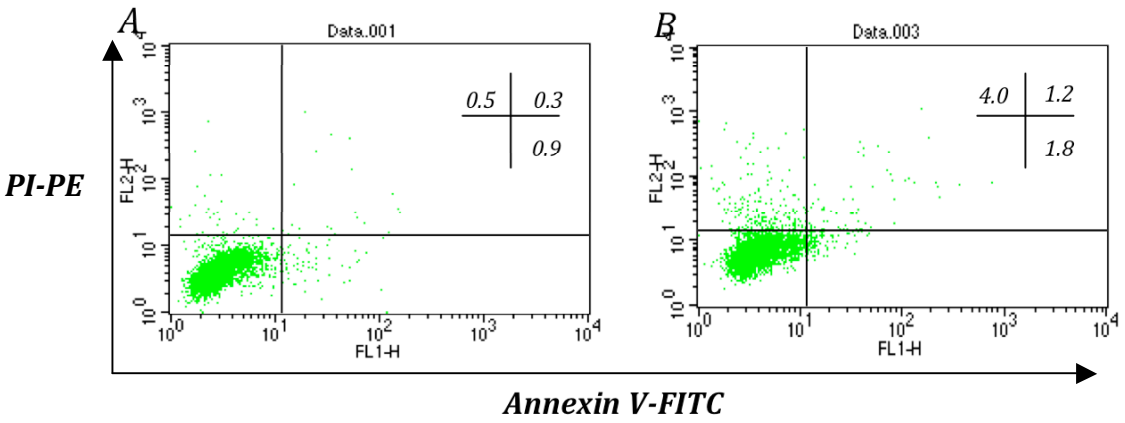


Figure 6-6 (A) DLKP-SQ were stained with Annexin V and propidium iodide to assess apoptotic/necrotic cells. (B) No significant increase in apoptotic cells was observed following electrospray.

6.1.3 Non-toxic electrospray mediated delivery of DNA producing GFP in mouse epithelial cells

Normal primary mouse airway epithelial cells were electrosprayed with green fluorescing protein (GFP) plasmid and underwent a transfection rate of up to 30%. This is important as much research work is done with mouse cells and DNA. This transfection method is cheaper, has fewer steps and lends itself to automation more readily than virus or lipid work.

6.1.4 Non-toxic, electrospray mediated in vitro delivery of siRNA tagged with $Alexa_{488}$ to human epithelial cells

BEAS-2B (transformed human airway cells) cells were electrosprayed with scrambled RNA bound to Alexa488 fluorophore and underwent a transfection rate of up to 90%. With reference to Figure 6-7, the top left photograph marked TRIS Spray indicates buffer only electrosprayed onto the cells. No fluorescence is observed. The panel marked lipofection shows a control where the vector was a lipid. The bottom panels show siRNA electrosprayed at 24 and 48 hours. The overall fluorescence is low but electrospray is clearly comparable with lipofection in this test. This transfection method is cheaper, has fewer steps and lends itself to automation more readily than virus or lipid work.

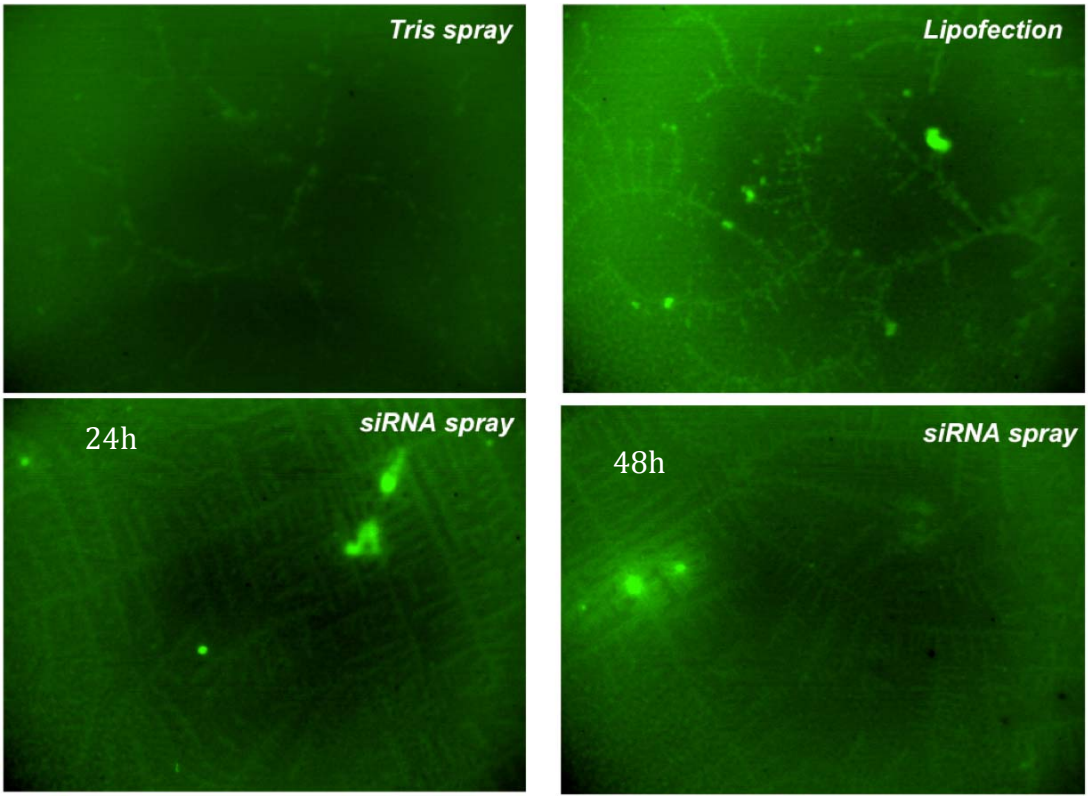


Figure 6-7 Images of fluorescence of cells transfected with electrospray. Images taken with the fluorencence microscope in the epithelial immunology laboratory, NUI Maynooth.

6.1.5 Mechanism for delivering proteins and antibodies

It is potentially useful to deliver antibodies directly into cells. This is not currently done, and the delivery is the difficulty. In this work, it was demonstrated that an antibody could be electrosprayed without effecting its function. Rabbit anti-mouse antibody was electrosprayed and collected. It was tested for viability subsequent to spraying on a known robust assay and found to be viable. This is a new result.

6.1.6 Novel delivery of cisplatin to human lung cancer cells

DLKP (human lung cancer cell line) cells were electrosprayed with cisplatin, a chemotherapeutic, in a solution of PBS. Cells within a circular area apparently targeted were killed completely whilst those outside remained viable. This demonstrates an apparent targeting effect and confirms that electrospray mediated delivery of cisplatin will induce cell death. In Figure 6-8, The PBS spray only (control) has no effect on the cells. The cisplatin spray at low concentration (0.5µg total) and the cisplatin incubation at a high concentration (16µg total), incubation being where the cisplatin is dropped onto the cells, both have a dramatic effect in killing the cells.

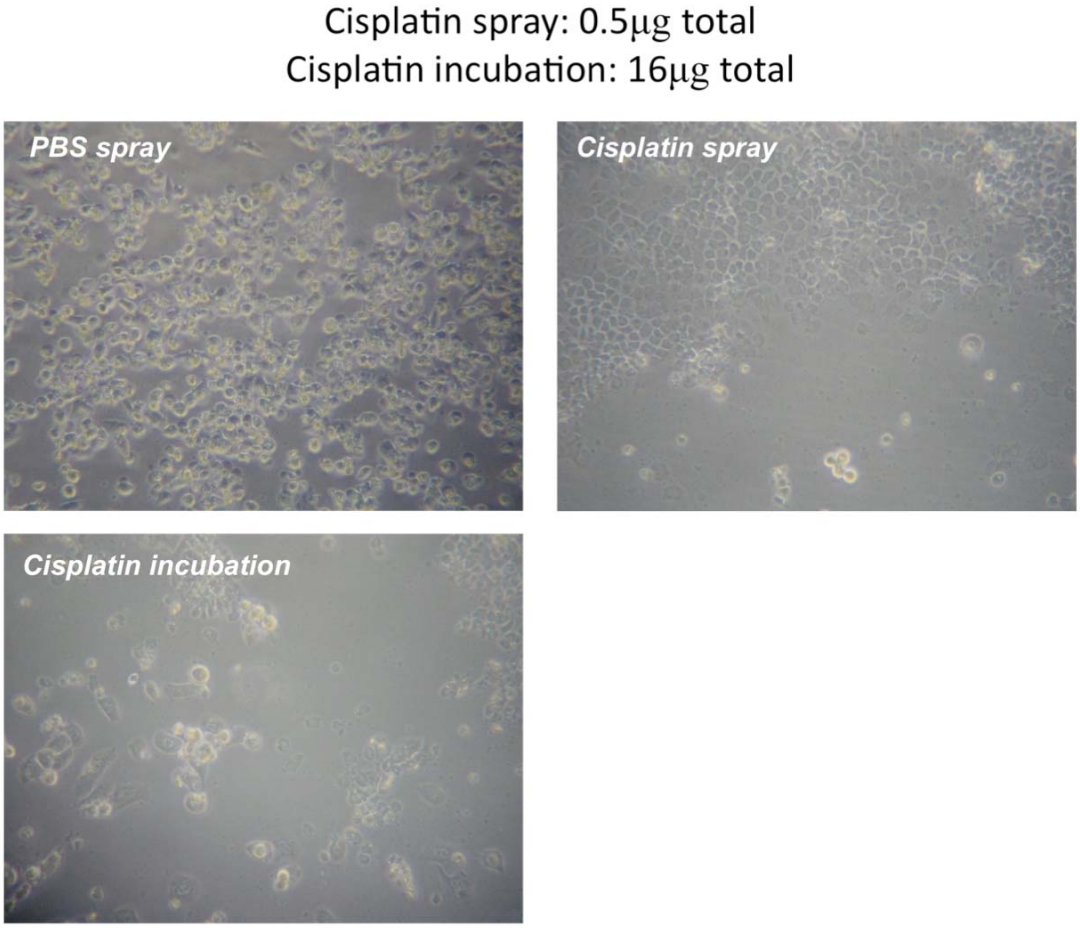


Figure 6-8 Images of cisplatin delivered with electrospray device

A high performance liquid chromatography (HPLC) test is then done on cells recovered from the wells to detect uptake of cisplatin. The purpose of this test is to prove that it was cisplatin that killed the cancer cells. The HPLC results are

shown in Figure 6-9 and a small peak exists at the characteristic signature of cisplatin at 13.7 minutes.

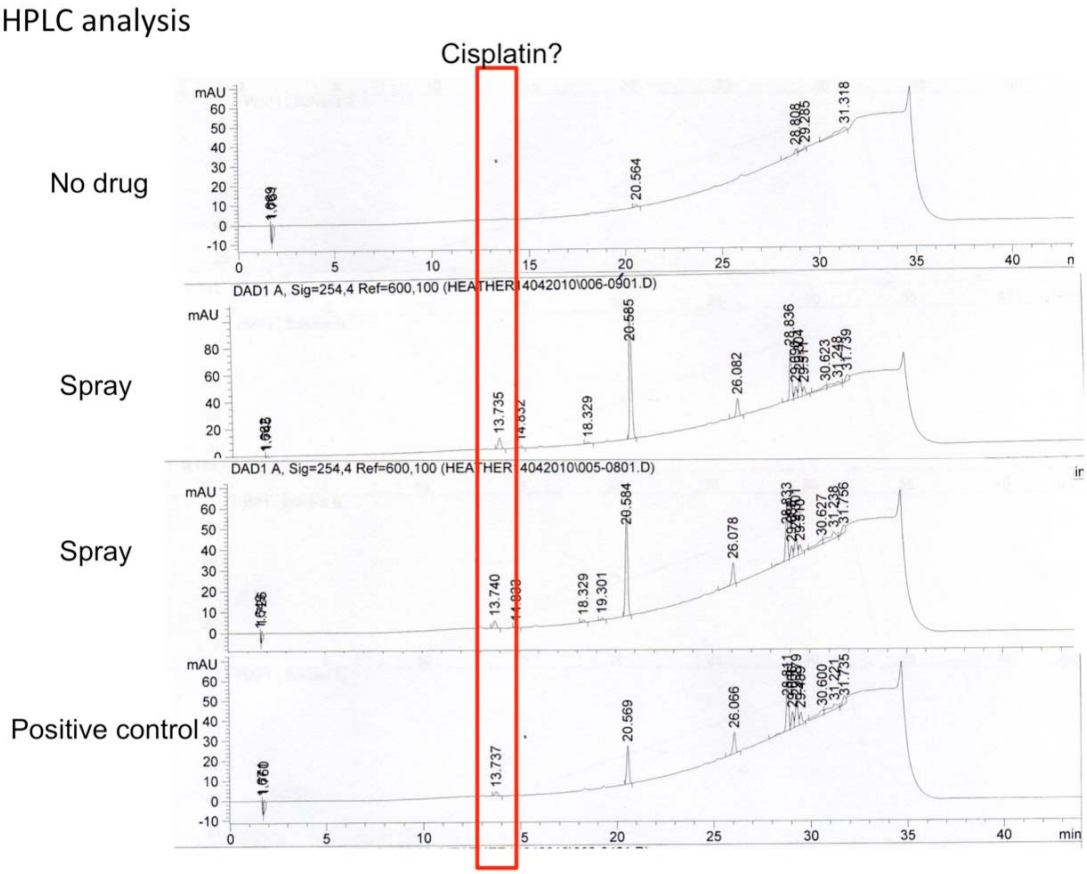


Figure 6-9 HPLC demonstrating that cisplatin entered the cells

In a second test, cisplatin incubation concentration is reduced to (0.5µg total) equal to the spray concentration. Cancer cells are sprayed and assessed at 24 and 48 hours. Cells are also incubated with dropped on cisplatin and assessed at the same time points. With reference to Figure 6-10, it can be seen that at both 24 hour and 48 hour time points, the electrosprayed cisplatin has killed the cancer cells. The low concentration incubation has not killed the cancer cells. This demonstrates that with the same concentrations of cisplatin chemotherapeutic electrospraying is more effective than incubation at killing cancer cells. This is a new result.

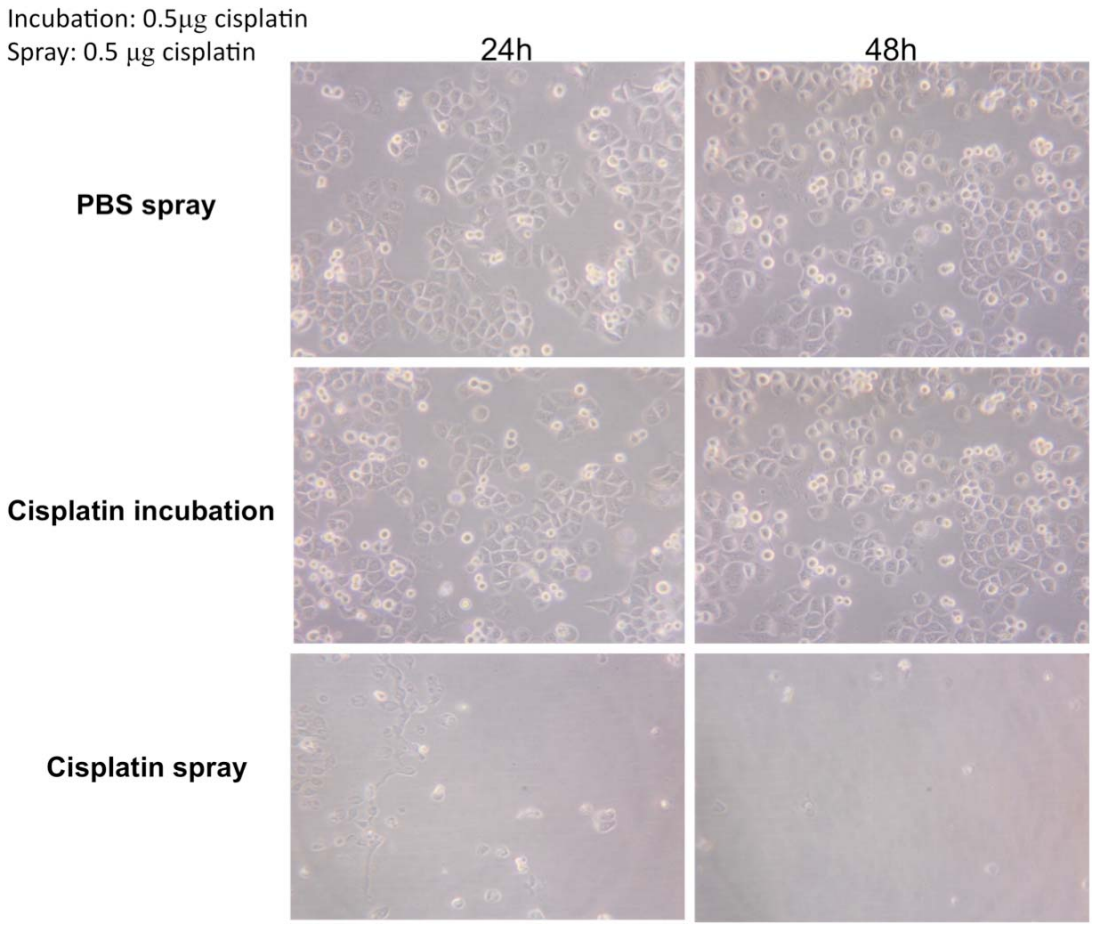
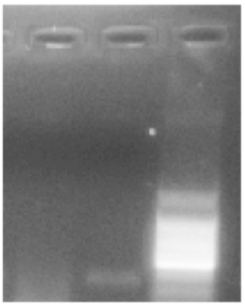


Figure 6-10 Cisplatin at low concentration is effective at killing cancer cells.

6.1.7 Novel electrospray mediated in vitro delivery of DNA producing E-cadherin in human lung cancer cells

E-cadherin is a protein that is not naturally found in human epithelial cells. Consequently, when it is detected with an assay it must be as a result of foreign DNA entering the cell and producing it within the cell. E-cadherin is of interest because it is a tumour suppressor protein and a potential gene therapy for cancer. DNA was electrosprayed into human cancer cells DLKP (human lung cancer cell line) in vitro. Human lung cells were seeded into tissue culture plates. A solution containing plasmid DNA coding for E-cadherin was sprayed onto cells. RNA was extracted from cells following electrospraying with E-cadherin DNA. Real time poly chain reaction (RT-PCR) analysis was carried out, shown in Figure 6-11, to determine E-cadherin mRNA expression in these cells

following electrospray. E-cadherin was detected in the cells at time points after 24 and 48 hours. This is a novel result



A B C

Figure 6-11 (A) E-cadherin is not expressed in control cells. (B) E-cadherin is detected in cells following electrospray. (C) Molecular weight ladder.

Ex Vivo Results

6.1.8 Novel electrospray mediated delivery of methylene blue dye to pig trachea ex vivo

Methylene blue dye was electrosprayed onto pig trachea ex vivo. The dye, a common marker, entered the cells in the epithelium and stained them such that the mark could not be washed off. Conversely, a similar amount of dye dropped onto a neighbouring piece of tissue was washed off easily with PBS. This result shows the ability of the electrospray to penetrate the epithelial cell membrane compared to dropped-on application where the dye is not taken up. With reference to Figure 6-12, methylene blue stains when electrosprayed but washes off when dropped on. The dye solution does not normally penetrate the epithelium and so washes off. However when it is sprayed or the epithelium is damaged or cancerous, the dye stains. This may be the basis for an early diagnostic cancer test.

Methylene blue colour dye may have issues of toxicity and this is the subject of current research. The lung does not have natural drainage so care must be taken to limit the quantity of dye administered. Dye that does not stain tissue can be irrigated out of the lung with the bronchoscope's suction channel.

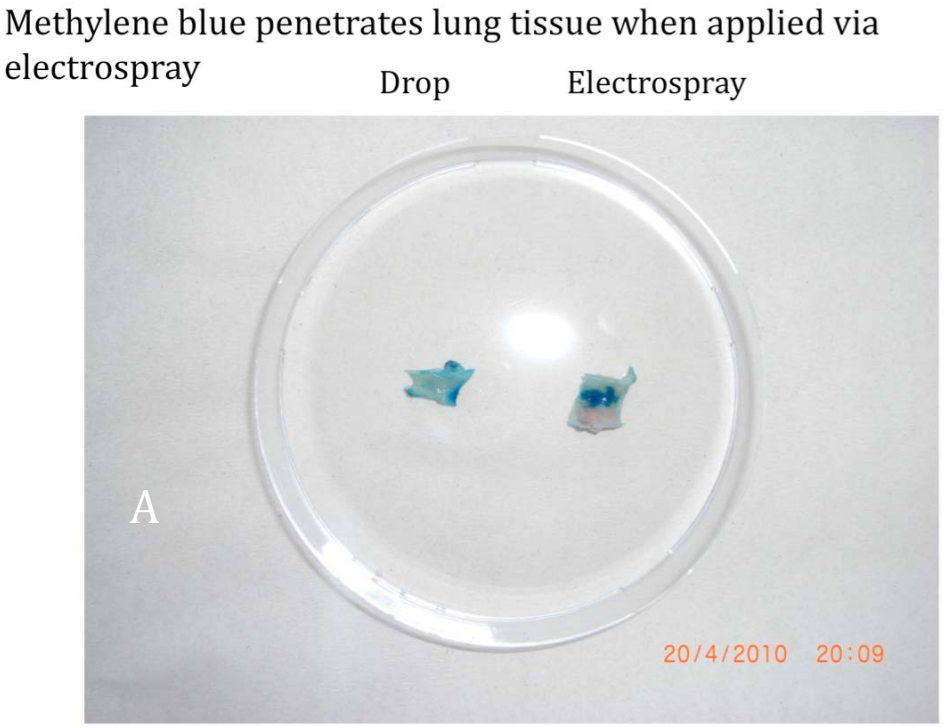


Figure 6-12 a) Samples of pig trachea in PBS. The electrosprayed sample on the right is stained. b) Dropped on methelyne blue dye washes off easily c)Electrosprayed methylene blue stains pig trachea ex vivo

In Vivo Results

6.1.9 Novel delivery of diagnostic methylene blue dye to pig lungs in vivo

The device was trialled in a large animal model, pig, in a trial in Trinity College Dublin. Professor James Egan carried out the procedure with veterinary surgeon Dr. Peter Nolan. The purpose of the trial, which was ethically approved, was to establish how the device would work in vivo for the application of delivering a diagnostic dye as the basis of an early lung cancer test.

With reference to Figures 6-13a to 6-13d, the device was inserted during a bronchoscopy of the animal. The bronchoscope was an Olympus BF-1T160 with a 2.8 mm working channel. Suction was provided and a light box from MDB. The procedure was recorded on video. Methylene blue dye was delivered to the lung. The animal suffered small trauma in one of the upper lobes as the device was pushed through the bronchoscope channel into the airways. Dye was successfully delivered to two lobes and the device was withdrawn.

The device developed in this work has been tested in pig bronchus in vivo and should have broader applicability to other disease sites in the body that can be accessed by endoscopy or with minimally invasive surgery. Examples of these are colorectal cancer, cervical cancer and oesophageal cancer. This is the first time such a procedure has been conducted and may for the basis for a first in man trial in the future.

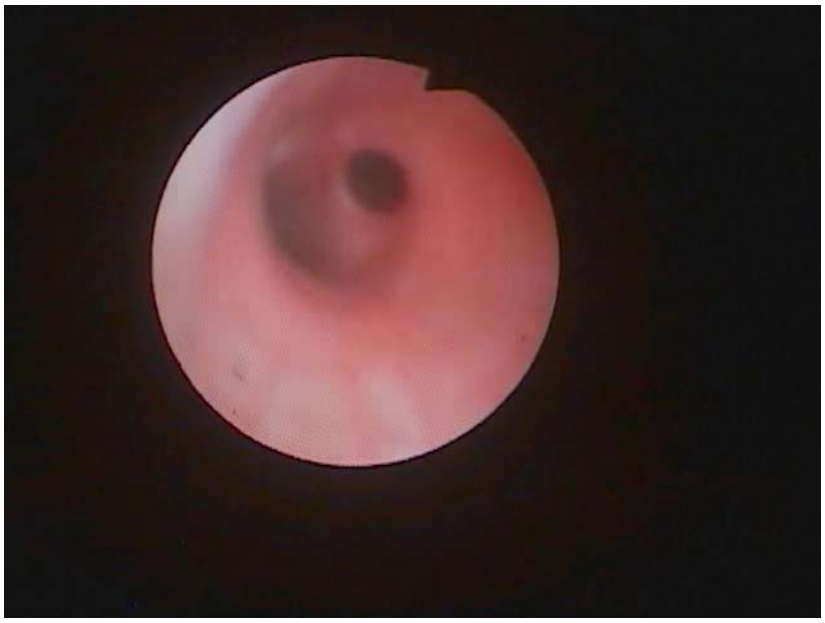


Figure 6-13a Bronchoscope is placed in lobe.

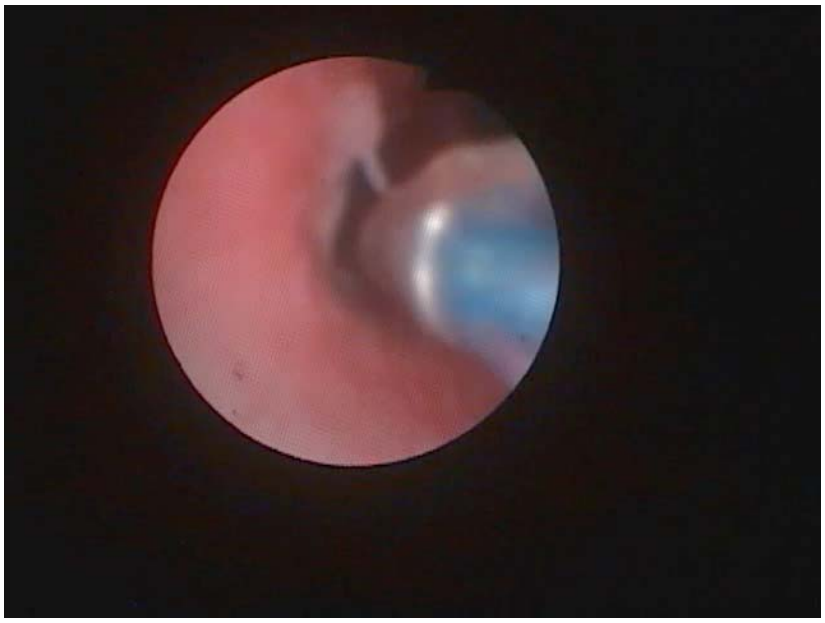


Figure 6-13b Electrospray emitter is introduced through working channel.

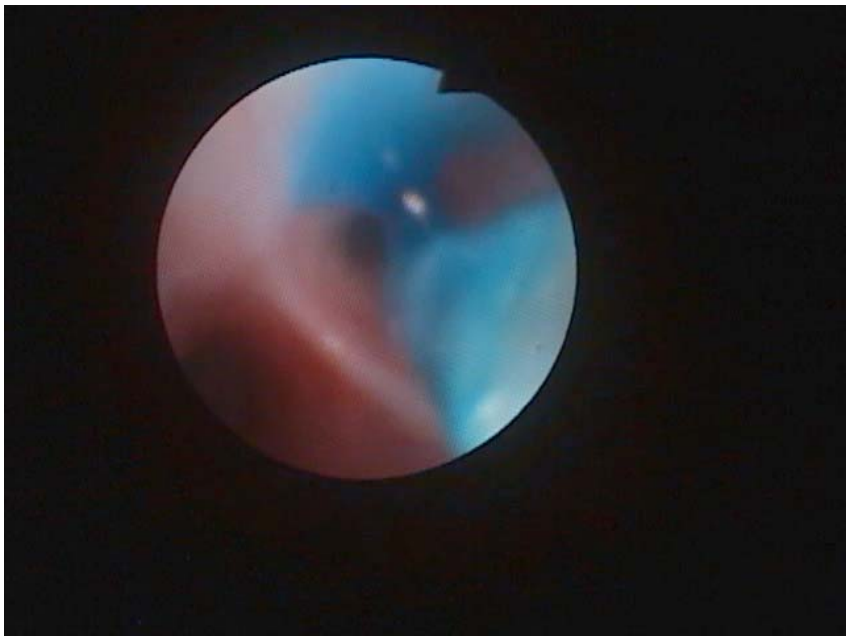


Figure 6-13c Electrospray process spraying the upper surface of the lobe

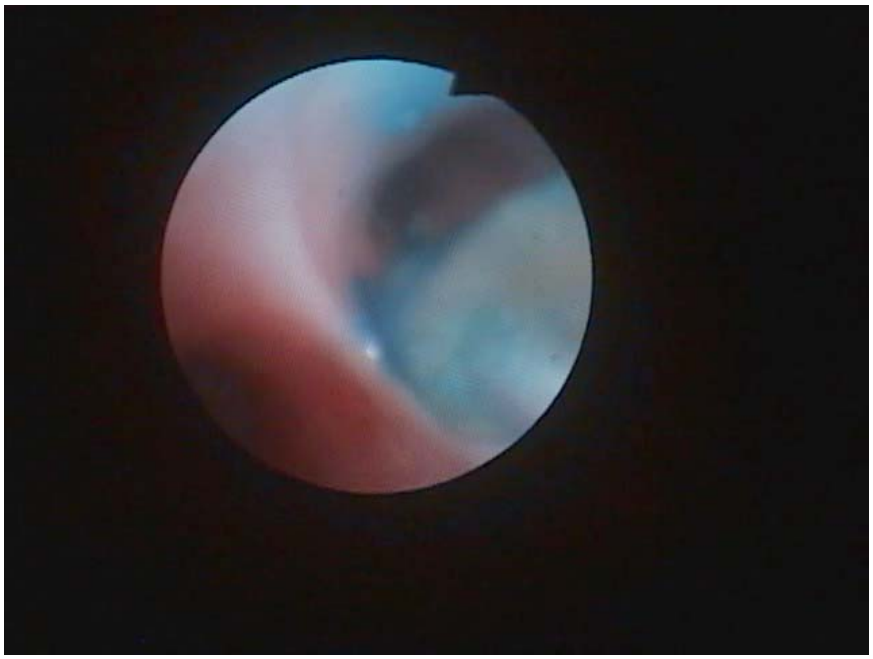


Figure 6-13d The electrospray head is withdrawn and the methylene blue remains on the upper surface of the lobe.

6.2 Discussion of Results

Electrospray appears to be a viable vector based on the results obtained in vitro and in vivo. A range of cells have been transfected with apparent ease and some consistency. The use of a control feedback loop appears to increase the consistency of the process. The application of appropriate statistical measures of consistency and transfection rate would aid development of the technology and allow comparisons with other vectors.

As a consequence of this work, biological buffers have been characterised for the first time for use with electrospray. In particular, methylene blue, PBS and TRIS. The characterisation involved the measurement of conductivity and surface tension. These measurements are presented and should be useful to others working in the field.

The extent to which the spray reaches or acts on tissue has been considered empirically by visualising the target cells after electrospraying. It is still not clear exactly what the spread of the electrospray plume is and whether it exerts a homogenous force on the target cells. Some early experimentation indicated an apparently stronger effect on cells directly under the plume and a reduced effect on cells located towards the edge of the wells. A deeper understanding of this solution component will be necessary.

In vitro, the cell viability tests presented are encouraging. The consequence here is that the electrospray process does not appear to degrade cells. This means that transfections can be carried out with low or no toxicity. This is very attractive as current options are toxic to some extent. In the in vivo testing, being cognisant of the classification requirements of medical devices, safety was a prime concern in the design. The general guiding principle was that electrical current to the tissues of the body should be minimised.

The efficacy of the process is dependent on the therapeutic or diagnostic. In the case of DNA and siRNA the efficacy is measured as the degree to which cells are

transfected. In the case of a chemotherapeutic, such as cisplatin, the measure of efficacy is the cell kill. The transfection and kill rates are very respectable when compared to other vectors. Whilst much development needs to be done on the system as a means of transfection, the operating principle seems to be sound.

The role of dyes in medical diagnoses is widely reported. Methylene blue is used in cancer diagnosis to stain suspected tumours in mouth and breast cancer. In the case of lung cancer dye has not been used to date because of the difficulty in targeting the area of interest. Methylene blue dye was delivered to the lungs of a pig in vivo and to pig trachea ex vivo. This is a novel procedure and a very important result that is the basis of an early stage lung cancer diagnostic test. The animal trial ran smoothly and a first in man trial of the device and technique is expected to take place within 2 years.

6.3 Summary

DNA and siRNA were delivered to human epithelial cell lines with up to 90% transfection. Proteins were delivered, collected and seen to be viable. Cisplatin, a chemotherapeutic, was delivered to cancer cells in a controlled experiment and killed 90% the cancer cells targeted. Methylene blue, the basis of an early stage cancer diagnostic test, was delivered to pigs, in vivo, using the device.

The bronchoscopic delivery system worked well in the animal trial and a human trial is expected within 2 years. In continuing laboratory work, better fluid handling systems are needed as dead space and air bubbles continue to frustrate some experiments. Further work on control, electrical safety and current minimisation as well as some fluid handling modifications to the device will improve its performance. The pulmonary delivery problem has been addressed to the extent that a dye has been delivered for the first time locally to the lungs. The possibility of progressing to treat the lungs with a therapeutic will give rise to a class of 'see and treat' type devices. In Chapter 7 conclusions and further work will be discussed.

Chapter 7

Conclusions and Future Work

7.1 A Solution to the Pulmonary Delivery Problem?

This thesis concerns an electrospray solution to the pulmonary delivery problem. In the preceding chapters, the pulmonary delivery problem was developed and a solution proposed in the form of a novel electrospray emitter device. The delivery device designed, implemented and trialled in this thesis is comparable with an increasing number of drug delivery and biological applications for electrospray (Tang 1994; Coffey 1999; Chen, Wendt et al. 2000; Davies 2005; Sahoo, Lee et al. 2010). As previously noted, the genesis of this work was a conversation between engineering and biology researchers about whether a mammalian equivalent to the Bio-Rad Gene Gun existed. It can be concluded that the development in this thesis serves as a new biolistic device capable of transfecting cells in vitro and in vivo.

7.1.1 In Vitro

In basic research, the most common way to introduce biological material into cells is lipofection. Lipids can be toxic to cells in vivo and this prevents their use to treat chronic disease. Viruses are important vectors in vitro, however, they require special skills and handling arrangements and are expensive. Such vectors are potentially invaluable for basic research and can be translated to in vivo work.

The electrospray device has been demonstrated to be effective at performing transfections without toxicity and with the advantages of lower costs, specialist techniques and with the possibility of automating the process. A rig was constructed where electrospray could be produced. Many of the parameters on the rig are readily adjustable. Experiments were carried out and recorded to

electrospray cells with DNA -GFP and E-cadherin and Protein. A device integrating all the system components has been designed and fabricated using MEMS techniques to ease the experimental practicalities. A controllable electrospray system has been developed. DNA molecules retain activity following the electrospray process. The electrospray is capable of delivering DNA, including potentially therapeutic DNA, into cells in vitro in a consistent reproducible manner. Transfected DNA is capable of being expressed as both RNA and protein. Toxicity was found to be minimal.

7.1.2 In vivo

In the device animal trial it proved, on occasion, difficult to know where the bronchoscope was located in the lung and access to the bronchioles was not possible. Whilst lung geometry was a problem, lung humidity did not appear to inhibit the action of the device in delivering methylene blue dye. Lung clearance mechanisms and the presence of lung disease (Labiris and Dolovich 2003a) were not significant problems in the trial as the pig lungs were healthy. However, in a first in man study, it is expected that lung disease may influence the operation of the device.

In vivo, viruses are immunogenic meaning that they elicit a response from the immune system. The electrospray device developed here is neither toxic nor anticipated to be immunogenic in vivo. It is not clear how the immune system would develop a response to a physical vector. In vivo and ex vivo work is done with animal models. Large animal models such as sheep or pig are considered a good analogue for the human lung in respiratory research, thereby facilitating the translation of laboratory findings into the clinical setting (Meeusen, Snibson et al. 2010).

7.1.3 Ex Vivo

The ex vivo work proved very useful in preparing for the in vivo trial. It was possible to identify the target site in pig trachea or lung and prepare and test the electrospray on the target site in question. The two advantages of this were

1) a programmed approach could be taken to ex vivo work in the laboratory where there was enough time to perform an electrospray procedure, observe the result and then modify some parameter. This cannot happen in an in vivo setting as it could cause distress to the animal. 2) When the animal trial occurs, the procedure has been tried in the laboratory numerous time and those involved are clear about what is being done and what result is expected.

7.1.4 Diagnosis

Late lung cancer diagnosis is the key factor in poor outcomes from lung cancer. The diagnostic tools available to the physician currently include x-ray, sputum cytometry, spirometry, and bronchoscopy (Hirsch, Franklin et al. 2001). The flexible bronchoscope is the key interventional device available to the physician. The device developed in this thesis was seen to work well in conveying the electrospray emitter to a local target in the lung. The device did not inhibit the procedure or the operation of the bronchoscope in the trial. Dye was administered to the lung tissue in vivo and ex vivo. From the outcome of the animal trial, this device has been demonstrated to be the basis of a novel early stage cancer diagnostic test based on the delivery of methylene blue dye to the lungs. The device developed in this thesis addresses some of the challenges outlined by using the bronchoscope to locate the target and electrospray to deliver diagnostic and therapeutic molecules directly to the lung tissues in a targeted and controllable manner.

7.1.5 Treatment

The lungs and airways are challenging to diagnose and treat (Labiris and Dolovich 2003a). This device goes some way towards addressing this statement. Lung disease is a major global problem (Lodderkemper 2003) and mortality from lung cancer has remained largely unchanged (Birring and Peake 2005). New chemotherapeutics and gene therapy hold substantial promise for improvements in patient care in these areas however, delivery of treatments remains a significant problem. The device presented in this work holds substantial promise of addressing the delivery problems through electrospray.

In treating lung cancer, chemotherapeutics are currently delivered intravenously or orally. Being highly toxic, chemotherapeutics are not generally aerosolised because of the risk of secondary exposure or 'blowback' (Brooks, Tong et al. 2000). Cisplatin, a chemotherapeutic was delivered by electrospray to DLPK human lung cancer cells and was shown to kill the cells where the electrospray was applied. This experiment was not trialled in vivo as the risk of secondary exposure was too great in an untested delivery means. A possible solution to this risk is to use an endotrachial (ET) tube such as that used in anaesthesia (Figure 7-1), with the cuff inflated to prevent expiration of drug, except through the tube. The air out can then be filtered. A number 6 ET tube will easily accommodate the bronchoscope.

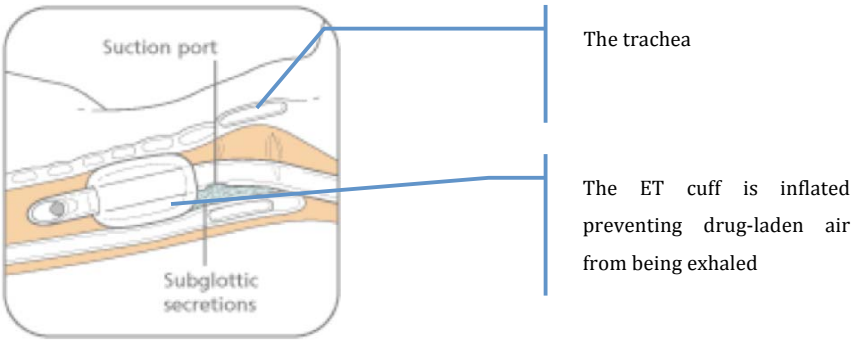


Figure 7-1 An endotrachial cuff to prevent blowback (Adapted from Covidien Respiratory Catalogue 2010)

7.1.6 Electrospray Stability

Electrospray refers to a process where a very fine spray of a fluid is produced from a Taylor cone at the top of a capillary (Taylor 1964). The cone is born of equilibrium between electrostatic and hydrostatic forces. A thin fluid ligament or jet elutes from the apex of the cone. It was found to be relatively easy to generate Taylor cones matching those in the literature, provided the conductivity of the solution was >2.5 x $10^{-4}Sm^{-1}$, the flow rate < $5\mu lm^{-1}$ and the surface tension was <$0.07Nm^{-1}$.

7.1.7 Fluids

The pulmonary delivery problem and electrospray technology were drawn together by exploring electrospray as a potential solution. This exploration involved the characterisation of solutions that were electrosprayed in vitro and in the case of dye, in vivo. The electrospray emitter appears to work well with the solutions chosen given the results presented in Chapter 6. In electrospray, fluid colloids of similar charge repel each other and achieve high speeds and tiny volumes relative to those of an aerosol of a similar fluid. Electrospray was found to be stable and to have propelled molecules into cells marking, modifying or medicating them. The fundamental processes underlying electrospray formation remain incompletely understood however the behaviour biological molecules in electrospray have been informed by this work.

7.1.8 Cells

Four types of cells were targeted in this thesis.

In vitro

BEAS-2B (bronchial epithelial cell line)

DLKP-SQ (human lung cancer cell line)

Mouse epithelial cells

Ex vivo and in vivo

Pig Bronchus

7.1.8 Safety

The use of high voltage direct current, albeit at currents in the nano to microampere range, requires careful design for safety and regulatory requirements(Underwriters_Laboratories 2004). The epithelium is not electrically excitable tissue so the procedure should be safe. The HV supply has a 100MΩ resistor in series limiting the current. The electrospray process within the cone-jet mode acts as a high ohm value resistor. The toxicity of methylene blue needs further investigation as the literature is divided, the dye is used very regularly but may have toxic properties.

7.1.9 Unmet need

The rationale underlying this device solution is that a targeted, physical mode of delivery will empower clinicians to enhance patient care by delivering agents that cannot easily be delivered by existing delivery modes, circumventing problems and limitations associated with aerosol mediated, intravenous, viral and liposome vector delivery and enabling novel diagnostic approaches.

Typical existing electrospray bench configurations reported by researchers involve syringe pumps, high-voltage power supplies, mechanical stages, retort stands, glass capillaries and instrumentation. Naturally such set-ups could not be used in a clinical setting. Electrospray has emerged as a fascinating cross-disciplinary research subject. The technical challenge was to engineer a device that performs the function of the bench set-up described but in an optimised and miniaturised form. The resulting device was used in conjunction with a bronchoscope to deliver biological material to lungs in vivo. There is clearly an unmet need for such a device to address the pulmonary delivery problem.

7.2 Solution Components and Contributions

Solution components developed in this work include 1) a scheme for controlling cone jet stability based on current feedback. 2) New electrical and physical characterisations for common biological buffers PBS +1% Acetic Acid, TRIS +1% Acetic Acid, dH_2O Methylene blue and Cisplatin. 3) An electrospray emitter that can operate within a bronchoscope or any endoscope addressing disease sites in other parts of the body. 4) The basis for a new diagnostic test for early stage cancer.

7.2.1 Novel bronchoscopically mediated electrospray device

A novel bronchoscopically mediated electrospray device was designed and fabricated in this work and has been tested in vivo in a large animal model (pig). Consultant Respiratory Physician, Professor James Egan of the Mater Hospital Dublin and Veterinary Surgeon, Dr. Peter Nolan carried out the procedure in Trinity College Dublin in May 2010.

7.2.2 Early-stage cancer diagnostic test

The role of dyes in medical diagnoses is widely reported. Methylene blue is used in cancer diagnosis to stain suspected tumours in mouth and breast cancer. In the case of lung cancer dye has not been used to date because of the difficulty in targeting the area of interest. Methylene blue dye was delivered to the lungs of a pig in vivo and to pig trachea ex vivo. This is a novel procedure that is the basis of an early stage lung cancer diagnostic test.

7.2.3 Non-toxic electrospray mediated in vitro delivery of DNA producing GFP in human epithelial cells

BEAS-2B (transformed human airway cells) cells were electrosprayed with green fluorescing protein (GFP) plasmid and underwent a transfection rate of up to 60%. This transfection method is cheaper, has fewer steps and lends itself to automation more readily than virus or lipid work.

7.2.4 Non-toxic, electrospray mediated in vitro delivery of siRNA tagged with $Alexa_{488}$ to human epithelial cells

BEAS-2B (transformed human airway cells) cells were electrosprayed with scrambled RNA tagged with $Alexa_{488}$ fluorophore and underwent a transfection rate of up to 90%. This transfection method is cheaper, has fewer steps and lends itself to automation more readily than virus or lipid work.

7.2.5 Non-toxic electrospray mediated delivery of DNA producing GFP in mouse epithelial cells

Normal primary mouse airway epithelial cells were electrosprayed with green fluorescing protein (GFP) plasmid and underwent a transfection rate of up to 30%. This transfection method is cheaper, has fewer steps and lends itself to automation more readily than virus or lipid work.

7.2.6 Mechanism for delivering proteins and antibodies

It is potentially useful to deliver antibodies directly into cells. This is not currently done, and again, the delivery is the difficulty. In this work, it was demonstrated that an antibody could be electrosprayed without affecting its function. Rabbit anti-mouse antibody was electrosprayed and collected. It was

tested for viability subsequent to spraying on a known robust assay and found to be viable. This is a new result.

7.2.7 Novel delivery of cisplatin to human lung cancer cells

DLKP (human lung cancer cell line) cells were electrosprayed with cisplatin, a common chemotherapeutic. Cells within a circular area apparently targeted were killed completely whilst those outside remained viable. This demonstrates an apparent targeting effect and confirms that electrospray mediated delivery of cisplatin will induce cell death.

7.2.8 Novel electrospray mediated in vitro delivery of DNA producing E-cadherin in human lung cancer cells

E-cadherin is a protein that is not naturally found in human epithelial cells. Consequently, when it is detected with an assay it must be as a result of foreign DNA entering the cell and producing it within the cell. E-cadherin is of interest because it is a tumour suppression protein and a potential gene therapy for cancer. DNA was electrosprayed into human cancer cells DLKP (human lung cancer cell line) in vitro and E-cadherin was detected in the cells at time points after 24 and 48 hours. This is a novel result.

7.2.9 Novel electrospray mediated delivery of methylene blue dye to pig trachea ex vivo

Methylene blue dye was electrosprayed onto pig trachea ex vivo. The dye, a common marker, entered the cells in the epithelium and stained them such that the mark could not be washed off. Conversely, a similar amount of dye dropped onto a neighbouring piece of tissue was washed off easily with PBS. This result shows the ability of the electrosprayed dye to penetrate the epithelial cell membrane compared to a dropped on dye where the dye is not taken up.

7.2.10 Novel delivery of diagnostic methylene blue dye to pig lungs in vivo

The device developed in this work has been tested in pig bronchus in vivo and should have broader applicability to other disease sites in the body that can be accessed by endoscopy or with minimally invasive surgery. Examples of these are colorectal cancer, cervical cancer and oesophageal cancer.

7.2.11 An ionic current feedback mechanism to control electrospray

Biological material is delivered into cells most consistently with the cone-jet mode of electrospray, although other modes have been proposed. The cone-jet occurs over a range of emitter–counter electrode potentials. Consequently, a novel closed-loop feedback system is demonstrated based on current feedback to stabilise the electrospray mode. This increases the consistency of delivery when compared to the open-loop system. This is a novel application for an ionic current feedback system.

7.2.12 Patent application PCT/EP2009/054680

A patent relating to the electrospray device developed during this work has been filed and examined with a significant number of claims upheld. The nationalisation and grant phase will take place in September 2010. International Patent Application No PCT/EP2009/054680 was lodged in the UK patent office. The patent search yielded no blocking IP.

7.2.13 Contributions to the field of research

Biological buffers are aqueous solutions that resist changes in pH. They are used to dissolve DNA, siRNA and other molecules such that they can be delivered to cells. As a consequence of this work, biological buffers have been characterised for the first time for use with electrospray. In particular, methylene blue, PBS, TRIS and TE buffer. The characterisation involved the measurement of conductivity, viscosity and surface tension. These measurements are presented and should be useful to others working in the field.

7.2.14 Research validation through funding awards

As a result of this work, research funding has been obtained to further the project. The research group in the epithelial immunology laboratory in NUI Maynooth have been successful in securing a grant from the EU FP7 Marie Curie research framework. The grant is to support basic scientific research into electrospray as it pertains to biological applications. The group are hopeful of further funding from SFI-HRB translational fund and potentially an Enterprise Ireland C+ grant to realise a commercial embodiment of the device described.

7.3 Future Work

A deepened understanding of the physical and electrical processes involved in electrospray generation is required to continue developing the technology towards achieving its potential to deliver of a wide range of biological molecules into cells. Melcher and Taylor(Taylor 1964; Smith 1986; Gomez 1993; Saville 1997) and subsequent research have provided a comprehensive mathematical and observational treatment of the Taylor cone. However, the plume that emerges from the cone is less well understood and this limits the current field applications. Future work to be done includes visualising the plume with a thermal camera, the use of a particle laser system to measure colloid sizes and further development on the interactions of charged colloids and cells.

Arrays of emitters may be preferable to a single electrospray source depending on the specific target form. It is possible to fabricate arrays of electrospray emitters cost effectively. Determination of how the plumes from arrays of emitters will interact should increase the volumes of solution delivery.

Electrospray is a process that could benefit from the application of a suitable control system. Controlling fluid flow and HV to achieve stable, monodisperse electrospray would lead to a better system that was more immune to disturbances. Identification of the mode of spray could also benefit from a control theory approach. All of the phases except cone-jet exhibit some pulsatile behaviour. This could maintain a system within the cone-jet mode. Within cone-jet mode the colloid size varies with flow rate, conductivity and viscosity. If an ideal colloid size and delivery velocity were determined, this might be a starting point to developing a robust electrospray ‘engine’.

In medical applications, miniaturisation and portability are a concern. Development of a portable electrospray unit would have the advantages of mobile usage and disposability. The main area for advancement in electrospray is medicine and biology. There are myriad possibilities for delivering hard-to-deliver drugs, compounds and new biologics such as antibodies and proteins. Gene therapy is stating to emerge again as an approach to treating disease after

the problems of viral delivery in the 1990s and early 2000s. The vector is central to the success of gene therapy. With refinement, the electrospray could deliver genes to locations in the body that are hard to access. Cystic fibrosis (CF) has particular resonance in Ireland. The CF consortium in Edinburgh has recently started to address CF with endoscopically mediated local delivery (McLachlan, Baker et al. 2010). The group is keen to attempt delivery of compounds using the electrospray device after seeing it on a visit by the author and Dr. O'Dea to Edinburgh in March 2010.

Engendering interest in this fascinating, cross-disciplinary subject is the most important future work. Successful, enduring outcomes in this research towards addressing the pulmonary delivery problem will depend on getting more engineering and biology researchers enthusiastic about problem solving in the electrospray domain. The continuing support and enthusiasm of thought leaders in the clinical community will ensure that the research outputs will ultimately be applicable to other disease types and to a wide range of therapeutic and diagnostic molecules.

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Appendices

Appendices

Appendix 1 Derivation of Emitter-Plane Distance (Taylor 1969)

APPENDIX. THE ELECTROSTATICS OF A NEEDLE NORMAL TO A PLANE

By M. D. Van Dyke

We consider a charged needle normal to a nearby uncharged plane, using the notation shown in figure A 1. We admit the limiting cases of an isolated needle ($h \to \infty$) and a semi-infinite one ($L \to \infty$). Except near a very blunt end, the field can

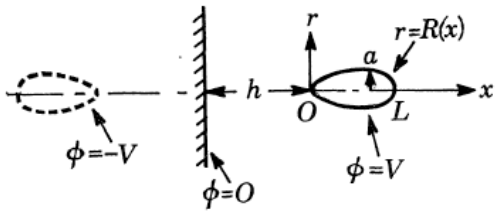


FIGURE A 1

be represented by a continuous distribution $\sigma(x)$ of charge along the axis of the needle. By the method of images this is seen to satisfy the integral equation

$$\int_0^L \sigma(\xi)\left[\frac{1}{\sqrt{\{(x-\xi)^2+R^2(x)\}}}-\frac{1}{\sqrt{\{(2h+x+\xi)^2+R^2(x)\}}}\right]\mathrm{d}\xi = V \quad (0<x<L). \quad (\mathrm{A}\,1)$$

The solutions for an isolated ellipsoid and for a one-parameter family of semi-infinite hyperboloids can be found by separation of variables. Otherwise, aside from inverse solutions, we must approximate on the basis that the needle is slender, using methods familiar from the aerodynamic theory of slender bodies.

We must first manipulate (A 1) to avoid divergent integrals. Adding and subtracting terms and integrating, following the procedure introduced into aerodynamics by Schultz-Piszachich (1951) and used also by Landau & Lifshitz (1960) for a similar electrostatic problem, gives

$$\sigma(x)\left[\sinh^{-1}\frac{x}{R}+\sinh^{-1}\frac{L-x}{R}+\sinh^{-1}\frac{2h+x}{R}-\sinh^{-1}\frac{2h+L+x}{R}\right]$$
$$-\int_0^L[\sigma(x)-\sigma(\xi)]\left[\frac{1}{\sqrt{\{(x-\xi)^2+R^2\}}}-\frac{1}{\sqrt{\{(2h+x+\xi)^2+R^2\}}}\right]\mathrm{d}\xi = V. \quad (\mathrm{A}\,2)$$

We can now approximate formally for small R to obtain

$$\sigma(x)\ln\frac{4x(L-x)(2h+x)}{R^2(x)(2h+L+x)}-V=\int_0^L[\sigma(x)-\sigma(\xi)]\left[\frac{1}{|x-\xi|}-\frac{1}{2h+x+\xi}\right]\mathrm{d}\xi. \quad (\mathrm{A}\,3)$$

The neglected terms are formally of order $(a/L)^2$ or $(a/h)^2$, whichever is the greater, and for a smooth body this indicates the actual order of magnitude of the error. Near blunt ends the error is much greater over a short distance, but integrated effects are in error by only that order. (For example, (A 3) predicts constant charge

distribution along the entire length of an isolated ellipsoid, whereas it should actually extend only between the foci; but the capacity is affected only by a factor of order $(a/L)^2$.)

Approximate charge distribution, capacity, and axial force

To solve (A 3) for a general shape, we must accept a much greater error, of relative order $\{\ln(L/a)\}^{-2}$ or $\{\ln(h/a)\}^{-2}$. To that approximation the right-hand side of (3) is negligible, so that the charge distribution is

$$\sigma(x) = V \Big/ \ln \frac{4x(L-x)(2h+x)}{R^2(x)(2h+L+x)} \left[1 + O\left(\frac{1}{\ln^2(L/a)}, \frac{1}{\ln^2(L/h)}\right)\right]. \tag{A 4}$$

That the error is of the stated order can be verified, for example in the case of the isolated cylinder or isolated pointed parabolic-arc spindle, by recalculating the neglected terms.

The capacity C of the needle is the total charge divided by its potential; in our approximation

$$C = \int_0^L \mathrm{d}x \Big/ \ln \frac{4x(L-x)(2h+x)}{R^2(x)(2h+L+x)}. \tag{A 5}$$

The attractive force between the needle and plane is most easily found from a virtual displacement at constant potential according to (Jeans 1925, p. 104).

$$F = \tfrac{1}{2} V^2 \partial C/\partial h. \tag{A 6}$$

This gives (with the sign reversed to make an attractive force positive)

$$F = V^2 \int_0^L \left(\frac{1}{2h+x} - \frac{1}{2h+L+x}\right) \mathrm{d}x \Big/ \left[\ln \frac{4x(L-x)(2h+x)}{R^2(x)(2h+L+x)}\right]^2. \tag{A 7}$$

(It is tempting to conjecture that for a needle with a long cylindrical midsection the two terms in (A 7) give the forces on the two ends.) These results remain valid, and simplify, in the limits $L \to \infty$ and $h \to \infty$, the force of course vanishing for an isolated needle.

Semi-infinite paraboloid

These equations reproduce known results for the isolated ellipsoid and semi-infinite hyperboloid. Another example for which all the integrations can be given

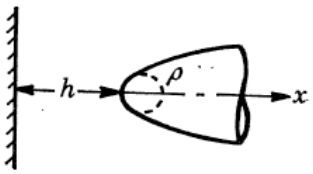


FIGURE A 2

explicitly is the semi-infinite paraboloidal needle (figure A 2). Putting $R(x) = \sqrt{(2\rho x)}$ where ρ is the nose radius, gives the charge distribution

$$\sigma(x) = \frac{V}{\ln\{2(2h+x)/\rho\}}. \tag{A 8}$$

Near the vertex this agrees with the exact result for the hyperboloid of the same nose radius, except that the distribution should start at the focus rather than the vertex. The capacity is infinite, but the force is

$$F = \frac{V^2}{\ln(4h/\rho)}. \tag{A 9}$$

This vanishes as the wall recedes, in agreement with the exact solution for an isolated paraboloid.

Ellipsoid

For an ellipsoidal needle (figure A 3) with $R(x) = 2a\sqrt{\{x(L-x)/L\}}$, the charge distribution is

$$\sigma(x) = V \Big/ \ln \frac{L^2(2h+x)}{a^2(2h+L+x)}. \tag{A 10}$$

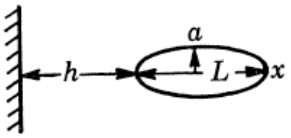


FIGURE A 3

Although the integral for the capacity has not been evaluated, that for the force gives

$$F = V^2 \left[1 \Big/ \ln \frac{2hL^2}{a^2(2h+L)} - 1 \Big/ \ln \frac{L^2(2h+L)}{a^2(2h+2L)} \right]. \tag{A 11}$$

As the length L increases, the second term vanishes, and the first reproduces the result (A 9) for the semi-infinite paraboloid.

Semi-infinite cylinder

For other shapes, one must resort to numerical integration, or to further approximation. For $R = \text{const.} = a$ and $L = \infty$ (figure A 4) the charge distribution is

$$\sigma(x) = V \Big/ \ln \frac{4x(2h+x)}{a^2}. \tag{A 12}$$

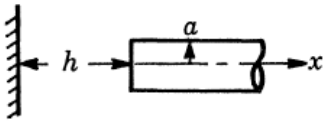


FIGURE A 4

This has violent fluctuations near the end, varying from zero at $x = 0$ to infinity at $x/a = a/8h$ and back to 1.4 V at twice that distance. However, all this detail is spurious, since the slender-body approximation breaks down where $x = O(a)$; and the fluctuations disappear if the end is rounded —for example, as a hemisphere. The capacity is again infinite; the force is given by

$$F = V^2 \int_0^\infty \mathrm{d}x \Big/ (2h+x) \left[\ln \frac{4x(2h+x)}{a^2} \right]^2. \tag{A 13}$$

Using (A 15) in (A 7) gives the second approximation

$$F = \frac{V^2}{\{2\ln(L/a)\}^2}\ln\frac{(2h+L)}{4h(h+L)}\left[1+\frac{1}{2\ln(L/a)}\ln\frac{h+L}{h}+\ldots\right]. \tag{A 18}$$

Expanding the full result (A 11) for large h/L confirms this.

Distant cylinder

For a distant cylinder, the second approximation for the capacity is

$$C = \frac{L}{2\ln(L/a)}+\frac{1}{\{2\ln(L/a)\}^2}[2(1-\ln 2)L+2h\ln 2h + (2h+2L)\ln(2h+2L)-2(2h+L)\ln(2h+L)]. \tag{A 19}$$

In the limit $h/L\to\infty$ this reproduces the first two terms of the expansion for an isolated cylinder due to Hallén (1929),

$$\frac{C}{L} = \frac{1}{2\ln(L/a)}+\frac{2(1-\ln 2)}{\{2\ln(L/a)\}^2}+\frac{1.08676}{\{2\ln(L/a)\}^3}+\frac{5.5180}{\{2\ln(L/a)\}^4}+\ldots.$$

The second approximation for the force is

$$\frac{F}{V^2} = \frac{1}{\{2\ln(L/a)\}^2}\ln\frac{(2h+L)^2}{4h(h+L)}+\frac{1}{\{2\ln(L/a)\}^3}\left[\mathscr{L}_2\left(\frac{L}{2h+2L}\right)-\mathscr{L}_2\left(\frac{L}{2h+L}\right) -\ln 2\ln\frac{(2h+L)^2}{4h(h+L)}+\tfrac{1}{2}\ln^2\frac{2(h+L)}{L}-\ln^2\frac{2h+L}{L}+\tfrac{1}{2}\ln^2\frac{2h}{L}\right]+\ldots, \tag{A 20}$$

where $\mathscr{L}_2$ is the dilogarithm.

The curves shown in figure 2 were calculated from this approximation, but an attempt has been made to increase its accuracy by recasting the series according to

$$\frac{1}{\{2\ln(L/a)\}^2}A(h/L)+\frac{1}{\{2\ln(L/a)\}^3}B(h/L)\to\frac{A}{\{2\ln(L/a)-\frac{1}{2}(B/A)\}^2}. \tag{A 21}$$

This modification has been adopted because it is found to increase the accuracy of the two-term approximation in various special cases—such as the ellipsoid—where more accurate results are known for comparison. It was also used by Landau & Lifshitz (1960) in a similar problem. More generally, it seems to improve the second approximation in any perturbation problem where the solution proceeds in powers of $(\ln 1/\epsilon)^{-1}$; an example from viscous flow theory is a circular cylinder in a uniform stream at low Reynolds number, where Lamb's Oseen approximation for the drag—which implicitly involves the modification of (A 21)—is much more accurate than the straightforward two-term expansion of Proudman & Pearson (1957).

This has not been evaluated, but fairly close bounds are easily found. Except very near the end, the denominator is less than $(2h+x)\,[\ln 4(2h+x)^2/a^2]^2$ and greater than $(h+x)\,[\ln 4(h+x)^2/a^2]^2$, and this gives

$$\frac{V^2}{4\ln(4h/a)} < F < \frac{V^2}{4\ln(2h/a)}. \tag{A 14}$$

These are probably true bounds, because the gap between them is much greater than the error arising from our approximations. They are shown in figure 2, p. 455.

Distant finite needle

For any needle whose distance from the plane is not small compared with its length (including the isolated needle) (figure A 5), the integrals can be approximated

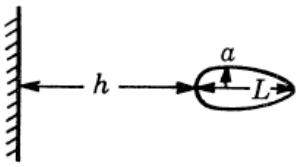


FIGURE A 5

in simple form, but with an increased error—of order $\{\ln(L/a)\}^{-1}$. The logarithm in (A 7) can be written as

$$\ln\frac{4x(L-x)\,(2h+x)}{R^2(x)\,(2h+L+x)} = 2\ln\frac{L}{a}\left[1+\frac{1}{2\ln(L/a)}\ln\frac{4x(L-x)\,(2h+x)\,a^2}{L^2(2h+L+x)\,R^2(x)}\right]. \tag{A 15}$$

The second term can be neglected with an error of relative order $(\ln L/a)^{-1}$ provided that L/h is of order unity (i.e. not large). This gives

$$C = \frac{L}{2\ln(L/a)}\left[1+O\left(\frac{1}{\ln(L/a)}\right)\right], \tag{A 16a}$$

$$F = \frac{V^2}{\{2\ln(L/a)\}^2}\ln\frac{(2h+L)^2}{4h(h+L)}\left[1+O\left(\frac{1}{\ln(L/a)}\right)\right]. \tag{A 16b}$$

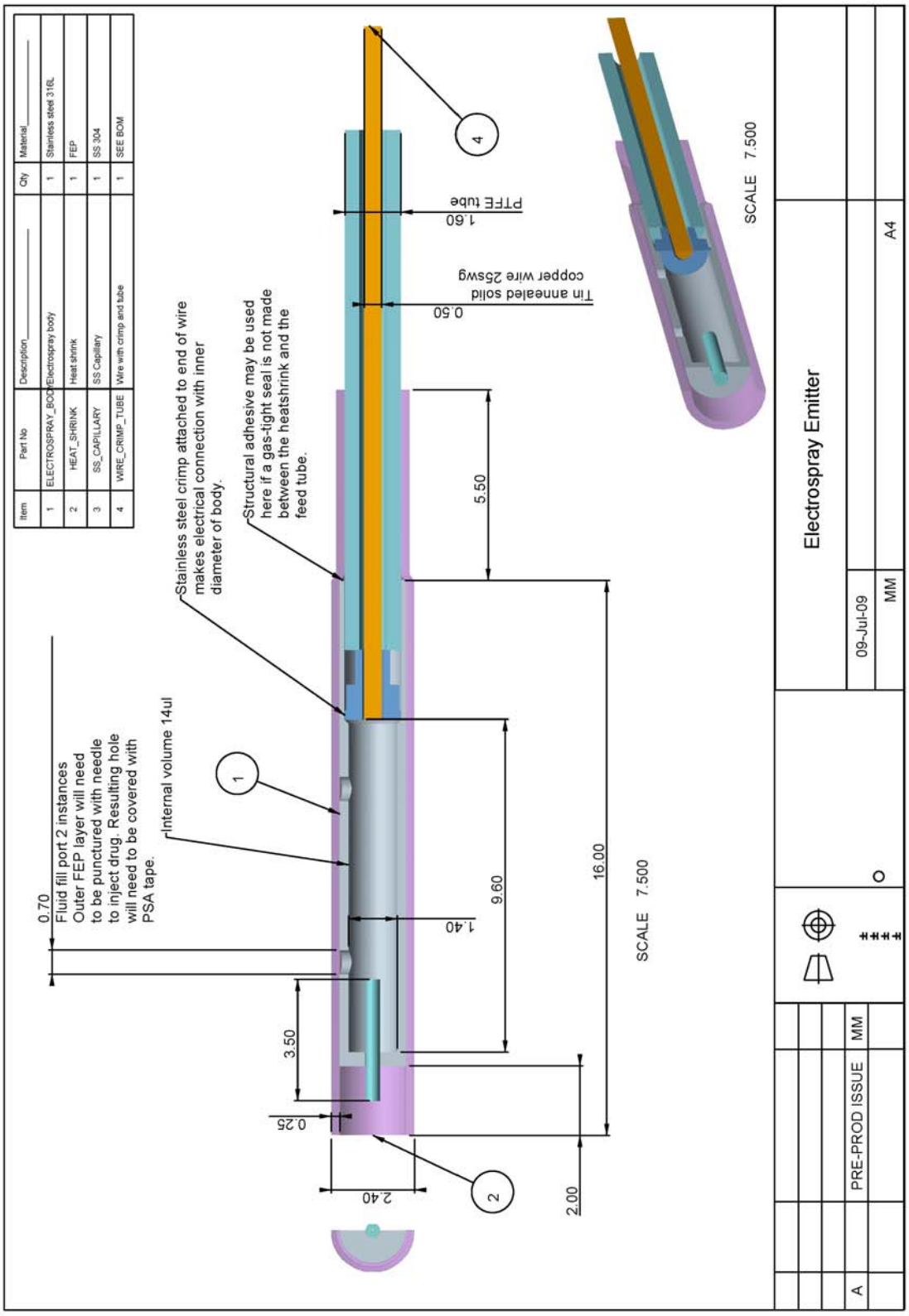
This force vanishes like h^{-2} as the plane recedes, which is the correct behaviour, exemplified by a sphere (Jeans, p. 103). These approximations are easily seen to be true lower bounds for any shape no sharper than an ellipsoid—and hence, in particular, for a cylinder.

Distant ellipsoid

Higher approximations can be calculated for a distant needle of specified shape. Thus for the ellipsoid, using (A15) in (A5) gives

$$C = \frac{L}{2\ln(L/a)} + \frac{1}{\{2\ln(L/a)\}^2}[(2h+2L)\ln(2h+2L) + 2h\ln 2h - 2(2h+L)\ln(2h+L)] + \ldots. \tag{A 17}$$

Differentiating this gives only the previous first approximation (A16*b*) for the force.



Appendix 2 Drawings of Electrospray Emitter Device

Appendix 3 Laboratory SOPs

Standard Operating Procedures (SOPs)

EI 1.1 Culture BEAS-2B

EI 1.2 Culture DLKP-SQ

EI 1.3 Trypsinisation of cells

EI 1.4 Counting cells

EI 1.5 Seeding cells – 24-well

EI 1.6 DNA preparation

EI 2.1 Electrospray

EI 3.1 Harvesting cells for RNA extraction

EI 3.2 Harvesting cells for flow cytometry

SOP: EI 3.2 **03.09.09**

Harvesting Cells for Flow Cytometry

NB: Change pipettes/tips between wells to avoid cross-contamination.

1. Label flow tubes.
2. Make up appropriate volume of 20 % FCS / PBS (eg. 2 ml FCS + 8 ml PBS)
3. Remove medium from cells.
4. For 24-well plates, add 500 ul PBS to each well.
5. Remove PBS from each well.
6. Add 200 ul 1X-trypsin to each well.
7. Incubate at 37°C for 5-10 min.
8. Pipette cells up and down to ensure complete detachment. View under microscope to confirm complete detachment.
9. Add 100 ul FCS/PBS to each well.
10. Transfer samples to appropriate flow tubes.

SOP: EI 3.1

Harvesting Cells for RNA Extraction

NB: Change pipette tips between wells to avoid cross-contamination.

1. Label sterile eppendorfs.
2. Dispense appropriate volume of Trizol into a universal.
3. Remove medium from cells.
4. Add 0.5 ul Trizol to each 24-well (note: volume will differ for different vessels).
5. Scrape bottom of each well with pipette tip to detach cells.
6. Pipette solution up and down several times to mix and lyse cells. View under microscope to ensure complete detachment.
7. Allow solution to incubate at room temperature for 5 min to enable Trizol to fully lyse cells.
8. Transfer samples to appropriate eppendorfs.
9. Store at -20°C prior to RNA extraction.

SOP: EI 1.6

DNA Preparation

0.01 M Tris, pH 7.5

0.121g Tris in 100 ml H_2O

- adjust pH to 7.5
- filter sterilise in laminar flow cabinet
- aliquot 5 x 20 ml
- store at 4°C for 1 month

Resuspend DNA in 250 µl 0.01 M Tris, pH 7.5

SOP: EI 1.5

Seeding BEAS-2B or DLKP-SQ Cells into 24-Well Plates

1. Harvest cells by trypsinisation according to SOP EI 1.3 and count according to SOP EI 1.4.
2. Seed cells at 4 x 104 cells / 500 ul / well.

SOP: EI 1.4

Counting Cells

For a total cell count:

1. Using a pastette, resuspend cell pellet in appropriate volume of medium (eg. 12 ml for cells from a confluent 75 cm2 flask). Resuspend well to ensure a single cell suspension.
2. Remove 30 ul aliquot and count using a haemocytometer (count each of 4 x 16 squares and divide by 4 to get average ('A')).
3. This means you have 'A'x 104 cells per ml of cell suspension.

For a viability count (Trypan blue exclusion):

1. Using a pastette, resuspend cell pellet in appropriate volume of medium (eg. 12 ml for cells from a confluent 75 cm2 flask). Resuspend well to ensure a single cell suspension.
2. Transfer 30 ul of cell suspension to a universal, add 10 ul trypan blue and mix well. Immediately transfer aliquot of this to a haemocytometer and count (count 4 x 16 squares and divide by 4 to get average ('A')).
3. Multiply 'A' by 4/3 dilution factor to get 'B'.
3. This means you have 'B'x 104 cells per ml of cell suspension.

SOP: EI 1.3

Trypsinisation of Cells

1. Remove medium from culture flask.
2. Rinse flask with 5 ml sterile PBS and remove.
3. Add 5 ml 1X-trypsin/EDTA solution to each 75cm2 flask.
   (Add 2.5 ml 1X-trypsin/EDTA solution to each 25 cm2 flask)
4. Incubate at 37°C for 5-10 min. Tap flask occasionally to aid cell detachment and check for detachment with microscope.
5. As soon as all cells have detached, add 5 ml serum-containing culture medium to inactivate trypsin. Cells may be counted at this stage or at step 7 (see SOP EI 1.4).
6. Transfer cell suspension to a universal and centrifuge at 1,000 rpm for 5 min.
7. Resuspend cell pellet in appropriate volume of medium. Cells may be counted at this stage (see SOP EI 1.4).

SOP: EI 1.2

Culture of DLKP-SQ cells

Medium

DMEM:F12

5 % FCS

1 % L-glutamine

Passage numbers

Use between passage numbers 35 and 50.

SOP: EI 1.1

Culture of BEAS-2B cells

Medium

DMEM:F12

5 % FCS

1 % L-glutamine

Passage numbers

Use between passage numbers 43 and 47.

Antibody Assay

IgG, rabbit anti-mouse
Dot Blot with electrosprayed 2° Ab (IgG , rabbit anti-mouse)

Three strips 1-Spray
2 No Spray
3 Tris
with 1ul,2ul of Beas-2B lysate and DLKP SQ lysate.

Let air-dry and then block for 1 hour with marvel/TBST.Wash x2 in TBST.

Primary antibody 1/2000 for 45 mins in TBST anti-actin (20-33) produced in rabbit Sigma (A5060) isotype Rb 42 kDa on all three strips for 45 mins

Wash x3 then secondary anti-rabbit 1/2000 Dako(P0448) for 45 mins in 5% Marvel/TBST
Wash x3 then develop

Appendix 4 EMCO HV Supply Details

General Purpose High Voltage Supply

0 to + or - 1,000 through 0 to + or - 33,000 VDC @ 10 Watts
4000 Series

EMCO HIGH VOLTAGE CORPORATION

The 4000 Series is a line of fully adjustable DC to DC converters providing an economical source of high voltage that is ideal for CRT's, lasers, Q switches, PMT's, detectors, electron and ion guns, capacitor charging, test equipment, and many other applications. These units feature remote voltage programming, resistance programming, or manual adjustability via an externally accessible potentiometer. Short circuit protection, arc protection, and reverse polarity protection are standard as well as remote on/off and a 0 to 10 volt output voltage monitor. The 4000 Series has a built-in 10V precision reference output available. These units also feature excellent EMI/RFI shielding. Two #6-32 studs mount the supply in a three inch mounting pattern. Call, e-mail or fax your requirements for immediate attention.

FEATURES
0 to 100% Adjustability
Voltage Programmable:
high impedance input >100K ohm
Voltage Monitor *2
Remote On/Off Control
EMI/RFI Shielding
Short Circuit Protection
Reverse Polarity Protection
Arc Protection

APPLICATIONS
Cathode Ray Tubes
Lasers and Q Switches
Capacitor Charging
Test Instrumentation
Electrostatic Field Generators
Electrophoresis

OPTIONS
Negative Output Voltage,
put "N" after model(Forexample:4300N)
RoHS(-R' suffix denotes the product is designed to meet RoHS requirements i.e 4010R)
Output Connector (AMP#861753-1): Available for models 4010 thru 4150

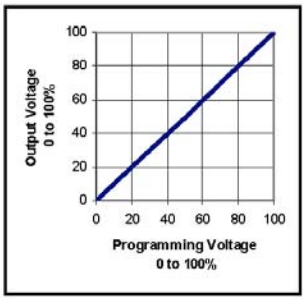
MODEL	OUTPUT VOLTAGE	OUTPUT *1 CURRENT	RIPPLE P-P	INPUT CURRENT NO LOAD	INPUT CURRENT FULL LOAD
4010	0 to 1,000	10 mA	.1%	<100 mA	<1.0 A
4020	0 to 2,000	5 mA	.1%	<100 mA	<1.0 A
4030	0 to 3,000	3.3 mA	.2%	<130 mA	<1.0 A
4050	0 to 5,000	2 mA	.1%	<150 mA	<1.0 A
4070	0 to 7,000	1.4 mA	.1%	<160 mA	<1.0 A
4100	0 to 10,000	1 mA	.1%	<175 mA	<1.0 A
4120	0 to 12,000	.8 mA	.1%	<190 mA	<1.0 A
4150	0 to 15,000	.67 mA	.1%	<190 mA	<1.0 A
4200	0 to 20,000	.5 mA	.2%	<230 mA	<1.0 A
4300*2	0 to 30,000	.33 mA	.4%	<270 mA	<1.0 A
4330*2	0 to 33,000	.30mA	.2%	<400 mA	<1.0 A

*Note: 1. At Maximum Rated Output Voltage
2. No Voltage Monitor

PHYSICAL CHARACTERISTICS
MODELS 4010 thru 4200 • SIZE: 4.1 (104) x 3.1 (78.7) x 1.4 (35.56)
MODELS 4300 thru 4330 • SIZE: 4.1 (104) x 3.6 (91.4) x 1.4 (35.56)
WEIGHT: 1.5 Pound (.675 kg.) Approx.
CASE MATERIAL: Black Anodized Aluminum
INPUT CONNECTOR: Molex Header 09-75-2074
MATING CONNECTOR: Molex Crimp Terminal Housing 09-50-3071 (Supplied)
MOLEX Crimp Terminals: 08-50-0106 (supplied)
MODELS 4010 thru 4200 • HV LEAD: 30KV 22 AWG
MODELS 4300 thru 4330 • HV LEAD: 40KV 20 AWG

ELECTRICAL SPECIFICATIONS
INPUT VOLTAGE: +24 Volts (+20%- 5%)
OUTPUT VOLTAGE: *See Table*
OUTPUT CURRENT: *See Table*
ANALOG CONTROL: 0 to 10 Volts=0 to 100% Output
VOLTAGE MONITOR: 0 to 10 Volts=0 to 100% Output
ON/OFF CONTROL: On=0 Volts or N.C.
Off=5 Volts
REFERENCE OUTPUT: 10 Volts (± 0.5%)
OPERATING TEMP: -10° to +60° C

Programming Voltage vs Output Voltage



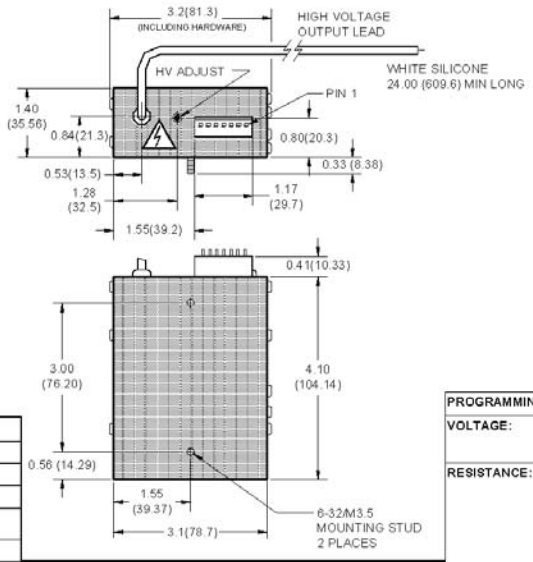
e-mail sales@emcohighvoltage.com
Web site www.emcohighvoltage.com

Phone (209) 267-1630 Fax (209) 267-0282
70 Forest Products Road, Sutter Creek CA 95685

We reserve the right to make changes without notification

4705AE

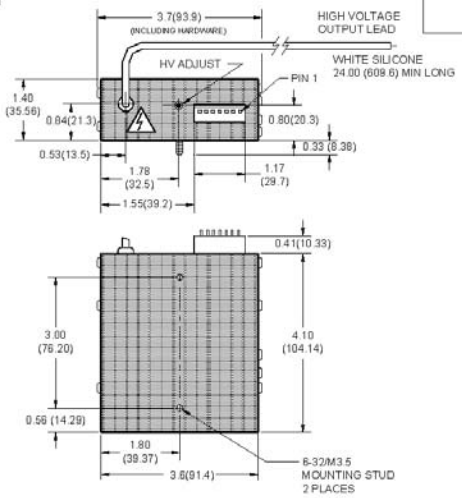
4010-4200



PIN	FUNCTION
1	On-Off Control
2	Ground/Case
3	+24 Volt Input
4	10 Volt Reference Output
5	Programming Voltage
6	Internal Potentiometer
7	Voltage Monitor

PROGRAMMING	
VOLTAGE:	Apply 0-10V to pin 5 for 0-100% output.
RESISTANCE:	Connect potentiometer to pin 4 (reference voltage), connect wiper arm to pin 5, and connect potentiometer to ground.
MANUAL:	Connect pin 6 to pin 5, turn front panel pot for voltage adjust.

4300-4330



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Appendix 5 MSDS for Methylene Blue

SAFETY DATA SHEET

according to Regulation (EC) No. 1907/2006
Version 4.0 Revision Date 27.02.2010
Print Date 22.08.2010
GENERIC EU MSDS - NO COUNTRY SPECIFIC DATA - NO OEL DATA

1. IDENTIFICATION OF THE SUBSTANCE/MIXTURE AND OF THE COMPANY/UNDERTAKING

Product name	:	Methylene blue
Product Number	:	M9140
Brand	:	Sigma-Aldrich
Company	:	Sigma-Aldrich Ireland Ltd. Vale Road ARKLOW Wicklow . IRELAND
Telephone	:	+35340220300
Fax	:	+35340231147
Emergency Phone #	:	
E-mail address	:	EIRProductStewardship@sial.com

2. HAZARDS IDENTIFICATION

Classification of the substance or mixture

According to Regulation (EC) No1272/2008
Acute toxicity, Oral (Category 4)
Skin irritation (Category 2)
Eye irritation (Category 2)
Specific target organ toxicity - single exposure (Category 3)

According to European Directive 67/548/EEC as amended.
Harmful if swallowed. Irritating to eyes, respiratory system and skin.

Label elements

Pictogram

Signal word: Warning

Hazard statement(s)

H302	Harmful if swallowed.
H315	Causes skin irritation.
H319	Causes serious eye irritation.
H335	May cause respiratory irritation.

Precautionary statement(s)

P261	Avoid breathing dust/fume/gas/mist/vapours/spray.
P305 + P351 + P338	IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.

Hazard symbol(s)

Xn	Harmful

R-phrase(s)

R22	Harmful if swallowed.
R36/37/38	Irritating to eyes, respiratory system and skin.

S-phrase(s)

S26	In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.
S36	Wear suitable protective clothing.

Other hazards - none

3. COMPOSITION/INFORMATION ON INGREDIENTS

Synonyms : Tetramethylthionine chloride
3,7-bis(Dimethylamino)phenazathionium chloride
Basic Blue 9

Formula : $C_{16}H_{18}ClN_3S \cdot 3H_2O$
Molecular Weight : 373,9 g/mol

CAS-No.	EC-No.	Index-No.	Classification	Concentration
Methylthioninium chloride				
7220-79-3	200-515-2	-	Acute Tox. 4; Skin Irrit. 2; Eye Irrit. 2; STOT SE 3; H302, H315, H319, H335 Xn, R22 - R36/37/38	-

For the full text of the H-Statements mentioned in this Section, see Section 16.

4. FIRST AID MEASURES

General advice
Consult a physician. Show this safety data sheet to the doctor in attendance.

If inhaled
If breathed in, move person into fresh air. If not breathing give artificial respiration Consult a physician.

In case of skin contact
Wash off with soap and plenty of water. Consult a physician.

In case of eye contact
Rinse thoroughly with plenty of water for at least 15 minutes and consult a physician.

If swallowed
Never give anything by mouth to an unconscious person. Rinse mouth with water. Consult a physician.

5. FIRE-FIGHTING MEASURES

Suitable extinguishing media
Use water spray, alcohol-resistant foam, dry chemical or carbon dioxide.

Special protective equipment for fire-fighters
Wear self contained breathing apparatus for fire fighting if necessary.

6. ACCIDENTAL RELEASE MEASURES

Personal precautions
Use personal protective equipment. Avoid dust formation. Avoid breathing dust. Ensure adequate ventilation.

Environmental precautions
Do not let product enter drains.

Methods and materials for containment and cleaning up
Pick up and arrange disposal without creating dust. Keep in suitable, closed containers for disposal.

7. HANDLING AND STORAGE

Precautions for safe handling
Avoid contact with skin and eyes. Avoid formation of dust and aerosols.

Provide appropriate exhaust ventilation at places where dust is formed. Normal measures for preventive fire protection.

Conditions for safe storage
Store in cool place. Keep container tightly closed in a dry and well-ventilated place.

8. EXPOSURE CONTROLS/PERSONAL PROTECTION

Personal protective equipment

Respiratory protection
Where risk assessment shows air-purifying respirators are appropriate use a dust mask type N95 (US) or type P1 (EN 143) respirator. Use respirators and components tested and approved under appropriate government standards such as NIOSH (US) or CEN (EU).

Hand protection
The selected protective gloves have to satisfy the specifications of EU Directive 89/686/EEC and the standard EN 374 derived from it.

Handle with gloves.

Eye protection
Safety glasses with side-shields conforming to EN166

Skin and body protection
Choose body protection according to the amount and concentration of the dangerous substance at the work place.

Hygiene measures
Handle in accordance with good industrial hygiene and safety practice. Wash hands before breaks and at the end of workday.

9. PHYSICAL AND CHEMICAL PROPERTIES

Appearance

Form	powder
Colour	dark green

Safety data

pH	no data available
Melting point	190 °C
Boiling point	no data available
Flash point	no data available
Ignition temperature	no data available
Lower explosion limit	no data available
Upper explosion limit	no data available
Water solubility	soluble

10. STABILITY AND REACTIVITY

Chemical stability
Stable under recommended storage conditions.

Conditions to avoid
no data available

Materials to avoid
Strong oxidizing agents

Hazardous decomposition products
Hazardous decomposition products formed under fire conditions. - Carbon oxides, nitrogen oxides (NOx), Sulphur oxides, Hydrogen chloride gas

11. TOXICOLOGICAL INFORMATION

Acute toxicity
no data available

Skin corrosion/irritation
no data available

Serious eye damage/eye irritation
no data available

Respiratory or skin sensitization
no data available

Germ cell mutagenicity
no data available

Carcinogenicity

IARC: No component of this product present at levels greater than or equal to 0.1% is identified as probable, possible or confirmed human carcinogen by IARC.

Reproductive toxicity
no data available

Specific target organ toxicity - single exposure
Inhalation - May cause respiratory irritation.

Specific target organ toxicity - repeated exposure
no data available

Aspiration hazard
no data available

Potential health effects

Inhalation	May be harmful if inhaled. Causes respiratory tract irritation.
Ingestion	Harmful if swallowed.
Skin	May be harmful if absorbed through skin. Causes skin irritation.
Eyes	Causes eye irritation.

Signs and Symptoms of Exposure
Absorption into the body leads to the formation of methemoglobin which in sufficient concentration causes cyanosis. Onset may be delayed 2 to 4 hours or longer., Vomiting, Diarrhoea, To the best of our knowledge, the chemical, physical, and toxicological properties have not been thoroughly investigated.

Additional Information
RTECS: SP5740000

12. ECOLOGICAL INFORMATION

Toxicity

no data available

Persistence and degradability
no data available

Bioaccumulative potential
no data available

Mobility in soil
no data available

PBT and vPvB assessment
no data available

Other adverse effects
no data available

13. DISPOSAL CONSIDERATIONS

Product
Observe all federal, state, and local environmental regulations. Contact a licensed professional waste disposal service to dispose of this material. Dissolve or mix the material with a combustible solvent and burn in a chemical incinerator equipped with an afterburner and scrubber.

Contaminated packaging
Dispose of as unused product.

14. TRANSPORT INFORMATION

ADR/RID
Not dangerous goods

IMDG
Not dangerous goods

IATA
Not dangerous goods

15. REGULATORY INFORMATION

This safety datasheet complies with the requirements of Regulation (EC) No. 1907/2006.

16. OTHER INFORMATION

Text of H-code(s) and R-phrase(s) mentioned in Section 3

Acute Tox.	Acute toxicity
Eye Irrit.	Eye irritation
H302	Harmful if swallowed.
H315	Causes skin irritation.
H319	Causes serious eye irritation.
H335	May cause respiratory irritation.
Skin Irrit.	Skin irritation
Xn	Harmful
R22	Harmful if swallowed.
R36/37/38	Irritating to eyes, respiratory system and skin.

Further information
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