The Impact of Improved Materials in Poly (vinyl chloride)-Based Endotracheal Tubes

By

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B.S., Materials Science & Engineering
Massachusetts Institute of Technology, 2003

SUBMITTED TO THE DEPARTMENT OF MATERIALS SCIENCE AND ENGINEERING IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTERS OF ENGINEERING
at the
MASSACHUSETTS INSTITUTE OF TECHNOLOGY

September 2004

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Abstract

Endotracheal tubes (ETs) are used to aid artificial ventilation in millions of medical patients every year and are known to invoke the proliferative phase in the cell linings. The technical objective of this work was to investigate *in vitro* the interaction between epithelial cells and current poly(vinyl chloride)-based ET materials, as well as some ET samples embedded with materials intended to improve biocompatibility properties of the tubes. Cells were grown in wells with small samples of ETs and proliferation and migration were observed using phase microscopy. ETs appeared to increase cell growth wherever cells came into contact with the material. The cell morphology altered once in contact with the ET sample. Cell growth on and around the ETs with embedded material appeared to slow, but had significant visible changes in cell morphology. The need for continued research in this area of research and development and future steps are addressed. A proposal for starting a company around a safer material for use in endotracheal tubes was developed and showed significant barriers to entry for a small medical device company with a single product. Subsequently, the most appropriate approach for bringing a new ET to the market would be by way of a licensing with an existing manufacturer.

Submitted on: June 21, 2004

Thesis Supervisor: Professor Christine Ortiz
Title: Assistant Professor of Materials Science & Engineering

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Acknowledgements

I would like to extend my great appreciation to Prof. Christine Ortiz and members of the Ortiz Laboratory for their support and encouragement over the past few years. I would like to thank my UROP, Yiqun Bai, for both her friendship and for her assistance and dedication in growing and testing our epithelial cells. I would like to thank Prof. James Sherley and his laboratory for their assistance with epithelial cell culturing. In particular, I want to extend thanks to Sumati Ram-Mohan who has been exceptionally giving of her time and assistance in cell culturing and testing. I would also like to thank Dr. Seth Dailey of Brigham & Women's Hospital and Dr. Victor Pacheco-Fowler of Columbia University Medical School for their support. In particular, I want to thank Dr. Pacheco-Fowler for his assistance in providing additional endotracheal tube materials for bio-adhesion analysis. I want to thank the M.I.T. Department of Materials Science & Engineering, in particular Prof. Eugene Fitzgerald and Prof. Carl Thompson, for their support of the Masters of Engineering program during the past year. I would like to thank the friends I made in the Masters of Engineering program who helped lighten my load by adding humor wherever possible. I'd especially like to thank my wonderful family for all their support over my past five years at MIT. Without them, I wouldn't be who I am today. I would finally and especially like to thank my wonderful husband, Reuben Domike, for the support, love and care provided and truly helping me keep things in perspective.

1. INTRODUCTION

1.1. Current Endotracheal Tube Design

The endotracheal tube (ET) is a polymeric conduit between the lungs and a mechanical ventilator that is used to form a closed system necessary to maintain artificial respiration for patients unable to breathe on their own. The primary function of an ET is to create the necessary pressure seal and protect the lungs from any foreign material that may be aspirated into the trachea. All of the designs currently in use consist of a plastic tube and an inflatable balloon cuff. The tube is inserted into the mouth, passes through the vocal cord region and then into the patient's trachea as shown in Figure 1. The cuff is then inflated to form a tight seal in the trachea and holds the tube in place to prevent fluids from dripping into the lungs. The ovoid cuff geometry allows the ET to create a pressure seal necessary for positive air-flow to be pumped into and then extracted from the lungs.[1]

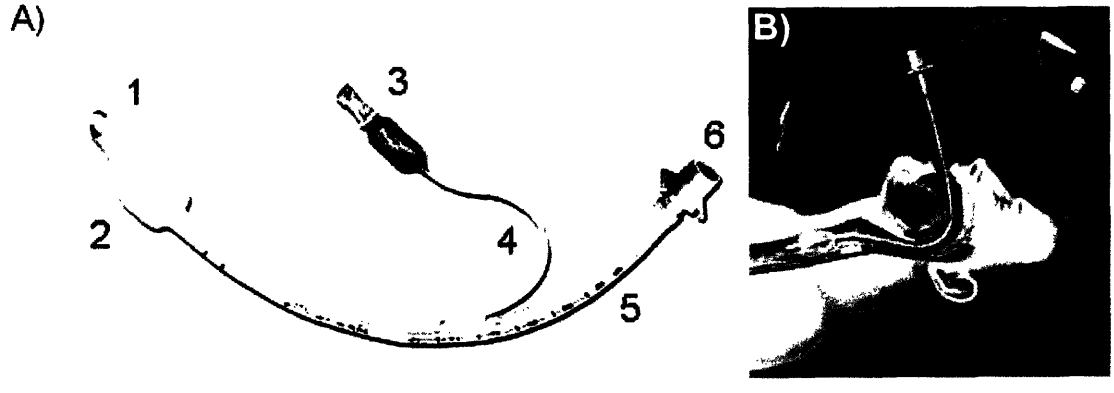

Figure 1 (A) Components of an Endotracheal Tube: 1.tapered tip, 2.balloon cuff, 3.valve for syringe tip, 4. secondary tube, 5. primary tube, 6. connection to ventilator[2] and (B) Illustration of an ET In-Patient[3]

[1] Cox, Everard, M.D. Personal Discussion. Fall 2003.
[2] Lin, J., *Tracheal Tube*, Hangzhou Jinlin Medical Appliances Co., Ltd.
[3] http://www.mallinckrodt.com/Respiratory/resp/Product/HiLoEvac/HiLoMini.html

1.2. Current Endotracheal Tube Materials and Structure

The endotracheal tubes currently used in humans are all composed of poly(vinyl chloride) (PVC) combined with about 30-40 weight % of low molecular weight plasticizer.[4] The molecular weight range for medical grade PVC is generally very high, ranging from 120-200 kg/mol.[5] The most common plasticizer combined with PVC in the construction of endotracheal tubes is di-ethylhexyl phthalate (DEHP). The chemical structures of PVC and DEHP are given in Figure 2.

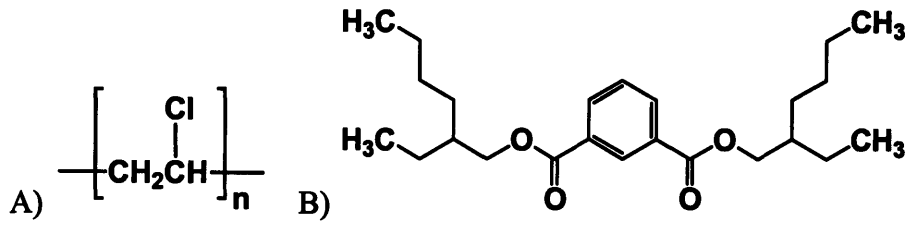

Figure 2 Chemical Structures of A) Poly(vinyl chloride) and B) Di-ethylhexyl Phthalate

Medical grade PVC is an atactic, semicrystalline polymer, with crystallinity typically between 10-15%.[9] For a typical, unplasticized PVC, the glass transition temperature, T_g, above which polymer chain segments in the amorphous regions exhibit increased motion, is ~ 100°C and the melting point, T_m is ~ 170°C.[6] The crystalline regions of medical grade PVC exhibit a fringe-micellar structure, which act as "tie points" or mechanical crosslinks for interconnected amorphous regions. When the amorphous regions are plasticized with DEHP, their T_g is lowered to a value below room temperature, making the material macroscopically flexible resulting in a tough, rubbery mechanical behavior, as detailed in the next Section 1.3. Heating above the T_m results in flow of the entire material.[6]

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[4] Tickner J, Hunt P, Rossi M, Haiarna N, Lappe Ma. 1999. *The Use of Di-2-Ethylhexyl Phthalate in PVC Medical Devices: Exposure, Toxicity, and Alternatives.* Lowell: Lowell Center for Sustainable Production, University of Massachusetts Lowell. Webpage: www.noharm.org.

[5] Roscom, Inc. Personal Discussion, Fall 2003.

[6] Hammond, Paula. MIT Polymer Laboratory Course 10.467 Notes, Fall 2002.

1.3. Endotracheal Tube Processing and Mechanical Properties

PVC is usually synthesized by one of two methods. Emulsion polymerization is used to produce "paste grade" PVC, one with very fine particles. The second method is suspension polymerization, which produces "dry blending" PVC that contains larger size particles. The paste grade, which is the emulsion produced, PVC is particularly useful for "plastisols," which are dispersions of PVC in plasticizing liquids like DEHP. The rheological behavior of the formed plastisols is of particular interest in the PVC processing industry. When plastisols are heated above the glass transition temperature, T_g, of PVC, the diffusion coefficient of plasticizer into polymer increases. Because of this, the emulsion processed PVC absorbs the suspending solvent, and a PVC paste with plasticizer inside it is formed. This paste can then be molded into products at temperatures above the melting temperature of PVC. The paste is often formed into plasticized PVC pellets, which can then be used in extrusion molding, blow molding and injection molding processes. The resulting polymer product is viscoelastic and hence shows the characteristic property of hysteresis. At higher levels of plasticizer, the T_g of the resulting PVC is lowered.[6]

As demonstrated in Figure 3, the shear modulus changes with temperature for various concentrations of plasticized PVC. T_f is the temperature corresponding to a flexural modulus of 800 MPa for each various plasticizer concentration. T_4 is the temperature corresponding to a modulus of 70 MPa. For typical percentages of plasticizer (35-40 wt. %) and at body temperature (37°C), the stiffness would be approximately 6 MPa. Endotracheal tubes must have strength and flexibility. The ET cuff will be inflated, but it cannot leak or break. The tube must be flexible, but strong enough never to buckle, which could cut of air flow to the patient. PVC plasticized to 35% has desirable strength and flexibility qualities.

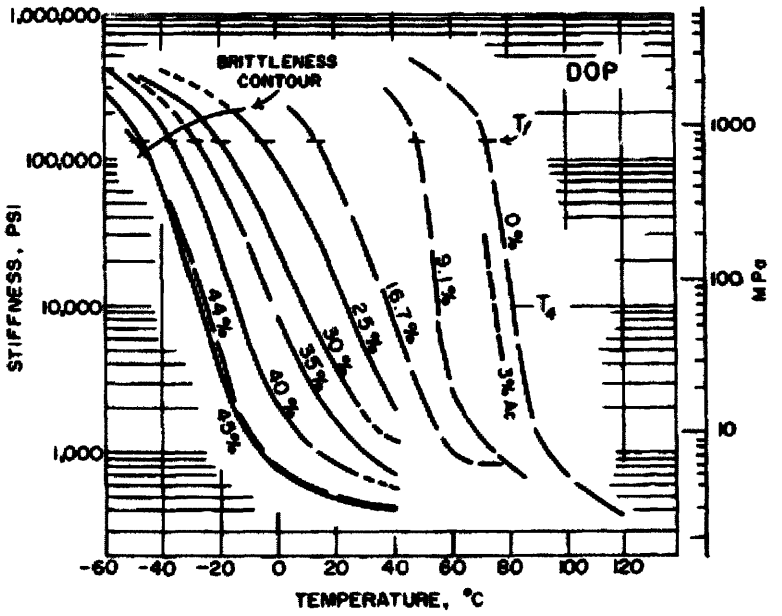

Figure 3 Shear Modulus of PVC Plasticized with Various Concentrations of DOP as a Function of Temperature[7]

Typically, the strains the material experiences *in vivo* are very small for the tube. The balloon cuff is inflated no greater than 40 cm H_2O, reasons discussed in sections 1.4. Inflating the balloon cuff stretches the plasticized PVC material, but since studies have shown that the balloon holds its shape over days in the body, the strains and stresses acting on the material are such that no weakening or necking of the material will occur. A summary of some of the material properties of ETs are discussed in section 2.2.

1.4. Endotracheal Tube Problems: Motivation for Cell-ET Interaction Studies

Plasticized PVC, including DEHP, is FDA approved for use in the body, despite the fact that DEHP is a potential carcinogen.[8] Plasticized PVC is hydrophobic (contact angle of 81.5° +/-

[7] Sears, J. Kerns and Darby, Joseph R. The Technology of Plasticizers. 1982.

6.5°),[9] thereby attracting proteins, bacteria, and mucosal secretions which adhere and, in the case of bacteria, travel along the walls of the endotracheal tubes, potentially causing severe illness to the patient.[10]

Endotracheal tube intubations (insertions of the ET into patient) are performed in over 30 million patients in the U.S. each year.[11] The highest risk patients are those intubated for prolonged periods. A prospective study on laryngotracheal sequelae in long-term intubation showed that patients intubated for lengths greater than 11 days are at a 12% risk of getting narrowing of the tracheal opening.[12] Patients suffering from heart attack, stroke, and trauma victims are commonly intubated for greater lengths of time (days to weeks). Lower risk patients are those intubated for a short time and are typically out-patient cases, such as those undergoing surgery for sinus, gallbladder, or breast surgery.

Endotracheal tubes connect a ventilator to the lungs by passing through the mouth, laryngeal and tracheal areas. The passage from mouth to lungs is lined with epithelial cells, creating a layer called the epithelium. The primary areas of concern are anywhere that this epithelial lining may be compromised by the presence of a foreign object, namely the endotracheal tube. An endotracheal tube can exert a wide range of pressure on the epithelium lining. It has been shown that tube cuff pressures necessary for sealing the trachea, greater than 40 cm H_2O[13], compromises capillary blood flow and injures the trachea wall. Damage to the tracheal tissues begins as soon as the balloon cuff is inflated. The extent of injury suffered from intubation depends on factors such as the duration of intubation, the individual patient cell

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[8] U.S. EPA Document. Technical Factsheet on: DI (2-ETHYLHEXYL) PHTHALATE (DEHP). January 27, 1998.
[9] Brodie, Kristin. MIT Undergraduate Thesis, 2003.
[10] Bauer, T.T. Biofilm formation in endotracheal tubes. Monaldi Arch Chest Dis 2002; 57: 1, 84-87
[11] In Vivo: The Business & Medicine Report, January 2000, pg. 42 (www.windhover.com)
[12] Whited, RE. A prospective study of laryngotracheal sequelae in long-term intubation. Laryngoscope 1984; 94:367-377
[13] Braz, Jose Reinaldo Cerqueira, et. al. Endotracheal tube cuff pressure: need for precise measurement. Sao Paulo Med. J., Nov. 1999, vol. 117, no. 6, p.243-247.

proliferation response, and the extent of infection and scarring from the inflammation response. The basic mechanism of damage is circumferential scarring within the trachea which narrows the airway and can cause suffocation. In some cases, injury can be so serious as to result in death.[14]

1.5. Physiological Considerations

The tracheal passageway from the mouth to the lungs has many small, subtle components. It is important to understand where the ET lies when it is inserted in the patient, and what areas may come into contact with the device. Sections 1.5.1 and 1.5.2 discuss the biology of the airway and some potential areas of injury resulting from endotracheal tube intubation. In Sections 1.5.3 and 1.5.4, respectively, the airway environment and tracheal cell lining properties are reviewed in detail. This discussion is meant to provide a more complete understanding of the human tracheal airway environment and the variables anesthesiologists encounter during patient intubation.

1.5.1. Biology of the Healthy Airway

The trachea connects the passageways from the larynx to the principal bronchi.[15] Figure 4 is a schematic of the cross-section of a human head. Notable are the areas where an ET comes into contact with a patient. The ET first enters the mouth, passes the soft palate, and is put through the oropharynx, commonly known as the larynx or vocal cords. The tube then passes through the regions containing the epiglottis and then enters the trachea.

[14] Dailey, Seth, M.D. Personal Interviews at Brigham & Women's Hospital. Jan.-June 2004.
[15] Crowther, Damien, M.D. General Practitioner's Notebook. The Cambridge Institute for Medical Research

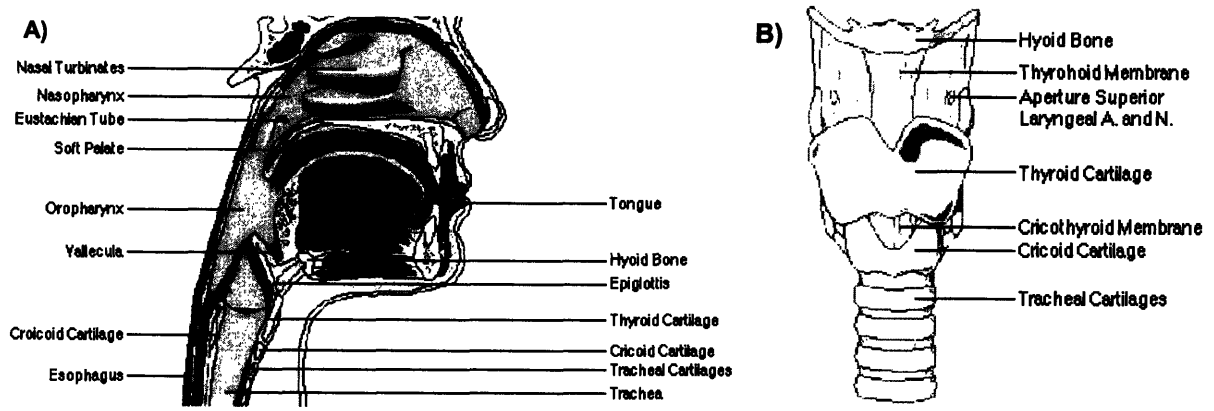

Figure 4 Labeled Schematics of Upper Airway Properties

A) Side Cross-sectional View of Respiratory Pathway, B) Front View of Larynx and Trachea[16]

The trachea is approximately 2 cm in diameter in an average adult and is comprised of multiple cartilage rings. The largest ring is the cricoid cartilage, located at the top of the trachea. There are a series of additional tracheal cartilages equally spaced down the tracheal passage. Figure 5 is an image from an office examination of the larynx and proximal trachea taken using a flexible fiberoptic scope. The image was taken of a healthy patient, who had not been intubated and had no laryngeal or tracheal complications. At the opening to the trachea, the equally-spaced tracheal rings that were described can be observed.

[16] Engel, Thomas, M.D., et al. Management of the Difficult Airway Published by Cook Incorporated and its licensors. Copyright 1995-2001

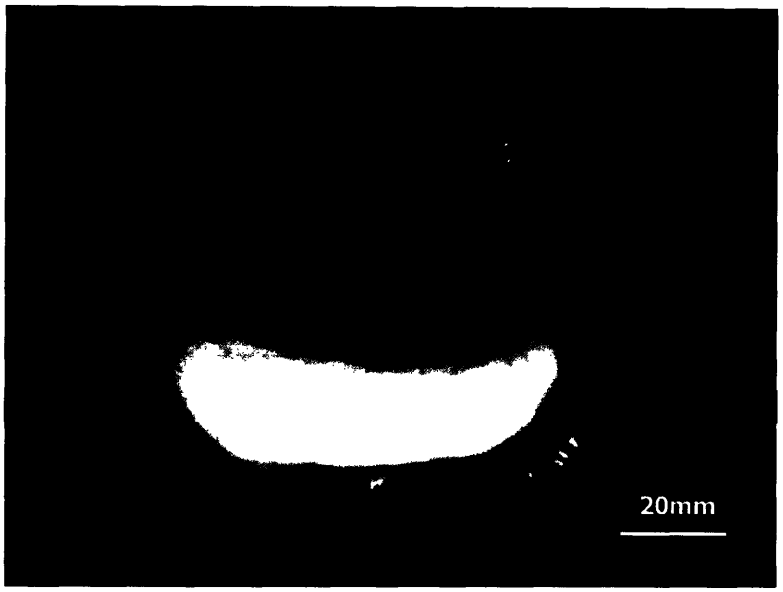

Figure 5 Image Looking Down Healthy Patient's Airway[17]

The vocal chords are also an area where a great deal of damage occurs from ET intubation. Vocal cords are essentially flaps of tissue that vibrate when a human speaks. In order for proper breathing and speaking to occur the vocal cords must be able to open and close propertly. Healthy vocal cords are V-shaped and appear smooth along the length of the cartilage. Looking at the vocal cords from an upside-down perspective, Figure 6 shows healthy vocal cords with an unobstructed range of motion.

Figure 6 Image of a Healthy Larynx[18]

[17] Provided by Dr. Seth Dailey from Brigham & Women's Hospital
[18] Eynon-Lewis, N.J., FRCS. Lecture Notes on Phonosurgery. St. Bartholomew's Hospital, London. http://www.city.ac.uk/lcs/lecturenotes/phonosurgery.pdf

1.5.2. Biology of an Injured Airway

It is not uncommon to see airway complications in patients following an ET intubation. While it is more common for more serious complication to occur following lengthy intubations, complications have arisen in patients intubated less than 72 hours. Figure 7 is an image from an office examination of the larynx and proximal trachea taken using a flexible fiberoptic scope. The patient was intubated for only 72 hours. Tracheal narrowing, referred to as stenosis, occurred so terribly that her trachea was one fourth the size of a healthy trachea. This patient was taken to the operating room and a tracheotomy was performed. This patient now lives permanently on a ventilator system. Ventilator systems are discussed in Section 1.5.3.

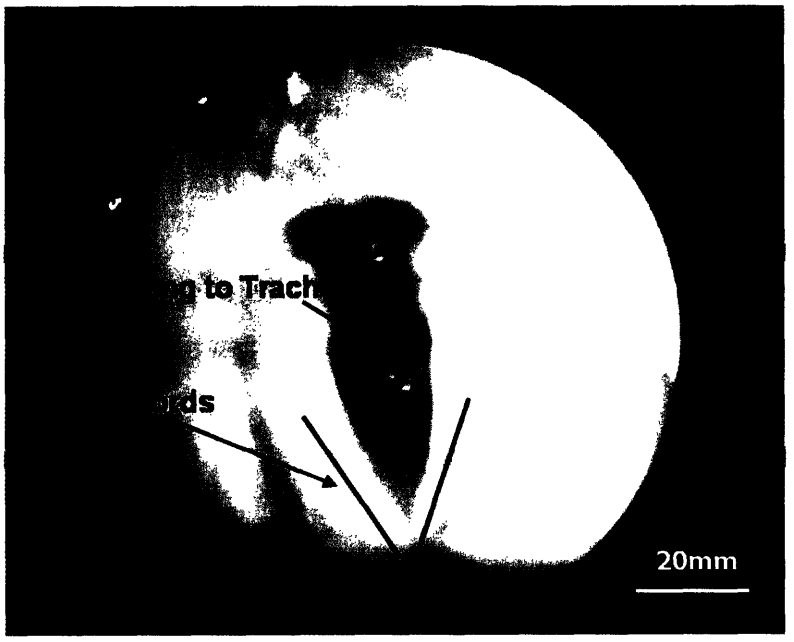

Figure 7 Patient Suffering from Narrowed Trachea[17]

Whereas cell proliferation and overgrowth leading to narrowing of the trachea always obstructs breathing, injury that occurs in the vocal cord region can cause a wide range of problems. Some common problems are when growth of cells around the vocal cords inhibits the opening and closing of the vocal cords. When a patient has an ET in their body for a lengthy

period of time, cells frequently begin to grow around the ET, and, in some cases, fuse together at the ends completely surrounding the tube. Figure 8 A shows some nodes forming on the vocal cords where an ET was located. The letter I indicates the location where the tube was lying. A visible circular shape appears to have been formed because of the ET. Speech in the patient was inhibited because of these nodes. Figure 8 B, in which the letter I also indicates where the ET lay, is an image from a different patient in whom tissue grew completely around the ET. The patient has lost mobility in their vocal cords to open and close and can no longer speak properly. This patient must undergo surgery which may or may not result in the patient regaining full use of their vocal cords.

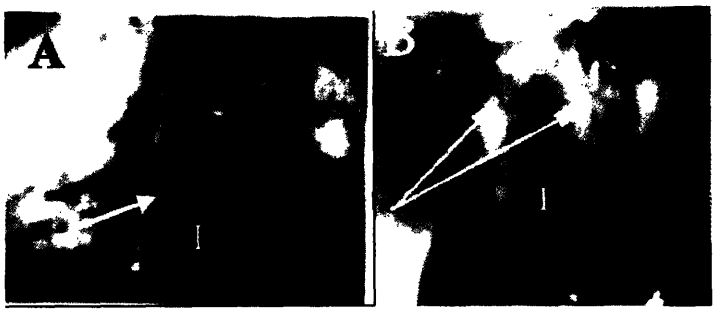

Figure 8 Severe Cases of Injury from Prolonged Intubation
A) Nodes Forming on Vocal Cords from Intubation, B) Nodes that Connected around ET during Intubation[19]

Apart from loss of vocal cord movement and tracheal narrowing seen in patients following ET intubation, narrowing can also occur in the epiglottis, the region between the vocal cords and trachea. The tube often comes into contact with several areas along the airway path. Additionally, bacterial infections are also commonly seen in patients who have been intubated. Pneumonia often occurs among intubated patients, and it has been hypothesized that the bacteria are attracted to the hydrophobic plasticized PVC. Complications from both narrowing of the

[19] Belafsky, Peter, M.D. Ph.D. M.P.H Director, San Diego Center for Voice & Swallowing Disorders, http://www.sandiegovoice.org

trachea, glottis or laryngeal areas (vocal cords) and infection have all lead to death in some more extreme cases of patient intubation.[14]

To correct problems that may occur following endotracheal tube intubation, surgery must be performed. The surgery procedures are complicated and difficult. Often, patients must undergo multiple surgeries to relieve problems of airway narrowing. To illustrate the difficult of this procedure, an example of endoscopic surgery that was performed to alleviate circumferential subglottic stenosis will be discussed.

In this case, the patient was put under general anesthesia, and a laryngoscope was used to visualize the area of stenosis. An operating microscope was brought into position and a carbon dioxide (CO_2) laser was use to make several linear cuts in the area. Special laryngeal dilators were then used in an attempt to dilate the stenosis and open up the airway.

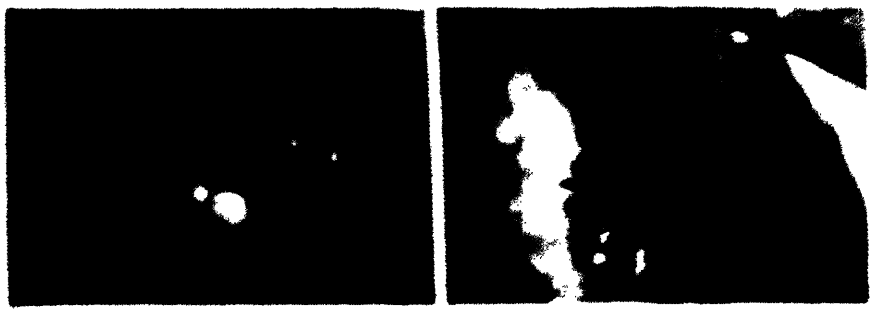

Figure 9 An Endoscopic View of a Patient with Stenosis
A) Prior to Surgery, B) After Linear Incisions and Dilations with a CO2 Laser

Several of these endoscopic procedures may be necessary to control the recurrence of the stenosis. Recurrence occurs depending on the hardness or softness of the tissue and patient cell response. Surgeons cannot say whether a surgery of this sort will be successful prior to testing because so many variables are completely dependent on individual response.

1.5.3. Properties of the Tracheal Airway Environment and Respiratory Ventilator Protocol

Inside the airway, the environment is in constant flux. During inhalation (inspiration) the diaphragm contracts, drawing air downward, and during exhalation (expiration) these processes are reversed and the natural elasticity of the lungs returns them to their normal volume. At rest, humans breathe an average of 15-18 times a minute. Humans exchange about 500ml of air each cycle of inhalation and exhalation. In more vigorous expiration, the wall of the abdomen contracts and pushes the stomach and liver upward. During vigorous expiration, an average adult (male) can flush his lungs with about 4 liters of air per breath. This is known as the vital capacity, although even with maximum expiration about 1200ml of residual air remains in the lungs. Table 1 shows the composition of atmospheric air that is inhaled and exhaled by a typical adult. This provides insight into the main types of gases that are taken into the lungs.[20]

Table 1 Composition of Atmospheric Air and Expired Air in a Typical Healthy Human[20]

Note that only a fraction of the oxygen inhaled is taken up by the lungs.		
Component	**Atmospheric Air (%)**	**Expired Air (%)**
N_2 (plus inert gases)	78.62	74.9
O_2	20.85	15.3
CO_2	0.03	3.6
H_2O	0.5	6.2
	100.0%	100.0%

In order to appreciate the delicacy of the environment for patients using ventilatory support, it is important to note comfortable breathing conditions for the human body. Humidity and other local environmental conditions affect the air taken into the body. A study performed

[20] Kimball, John W. Biology. Published 1994 by Wm. C. Brown.

by the Austrian Federal Ministry of Environment analyzed the levels of optimal comfort for a human. The ideal air conditions determined suggest that a relative humidity between 40-60% and temperature between 19-22°C (66-71°F) are the most comfortable. The following graph displays human comfort as a function of temperature and relative humidity. During patient ventilation, the goal is to keep humidity, air concentrations and breathing rate within the very comfortable ranges.

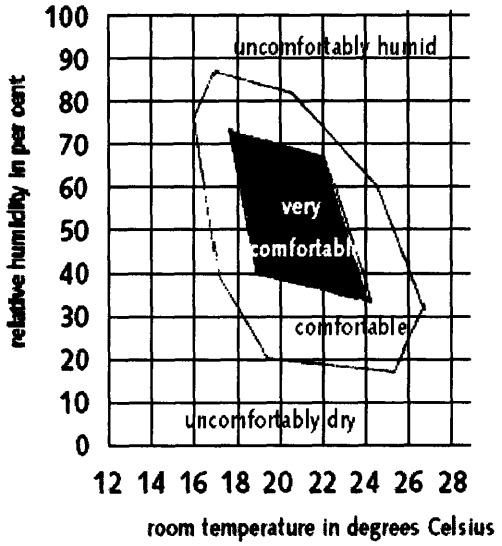

Figure 10 Humidity Comfort Range for the Human Airway[21]

In a ventilatory situation, the air pressures and concentrations are monitored. The adult respiratory ventilator protocol has several parameters and goals. Ventilation may be controlled by volume for the majority of patients, but if peak pressures rise over 40 cm H_2O or plateau pressures rise >30 cm H_2O, pressure ventilation (PV) should be considered. The tidal volume is kept within a reasonable range, generally 4 to 12 mL/Kg of ideal body weight (IBW). The tidal volume refers to the amount of air inspired and expired with each breath. Minute ventilation, the liters per minute of air flowed through the body, is calculated by the following: 4.0 x BSA (Body

[21] The Healthy Indoor Air Guide, http://www.ibo.at/wegweiserE.pdf

Surface Area) = V_E (L/min) for males and 3.5 x BSA = V_E (L/min) for females. The rate of breathing, anywhere from 8 to 26 breaths per minute, is adjusted to achieve optimum total cycle time and maintain desired minute ventilation. The fraction of inspired oxygen (FiO_2) is initially set between 0.6-0.9 and is then adjusted by the respiratory therapist. The PEEP (positive end-expiratory pressure) is set from 5 to 15 cm H_2O. Higher PEEPs may be required with acute lung injury or acute respiratory distress syndrome. Lastly, the pressure support (PS) should be between 8 and 20 cm H_2O and adjusted to reduce work of breathing and patient fatigue and support effective ventilation.[22]

1.5.4. Biology of the Tracheal Wall

The lining of the trachea walls are comprised of four layers: the mucous membrane, submucosa, cartilaginous smooth muscle layer, and the adventitia (associated with blood vessels, nerves, and sometimes fat).[23] The mucous membrane is the lining with the most contact with the ET surface.

The mucous membrane layer and the submucosa form the lining of the trachea known as the tracheal epithelium. This epithelium contains primarily goblet, columnar ciliated and basal cells, and the surface is coated with a layer of ciliated cells.[24] A cross-sectional histological view of the tracheal epithelium is presented in Figure 11 A. The image was taken using a Hematoxylin and Eosin stain commonly used in epithelium imaging. Hematoxylin is known to stain the nuclei blue, while the eosin is known to stain the cytoplasm pink.

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[22] Adult Respiratory Ventilator Protocol – Guidelines for General Practice. From AARC Protocol Committee; Subcommittee Adult Critical Care Version 1.0a (Sept. 2003).
[23] Atlas of Normal Histology. Mariano S.H. di Fiore. 6th edition. Edited by Victor P. Eroschenko
[24] Epithelial Cell Culture. Edited by Ann Harris. Cambridge University Press 1996

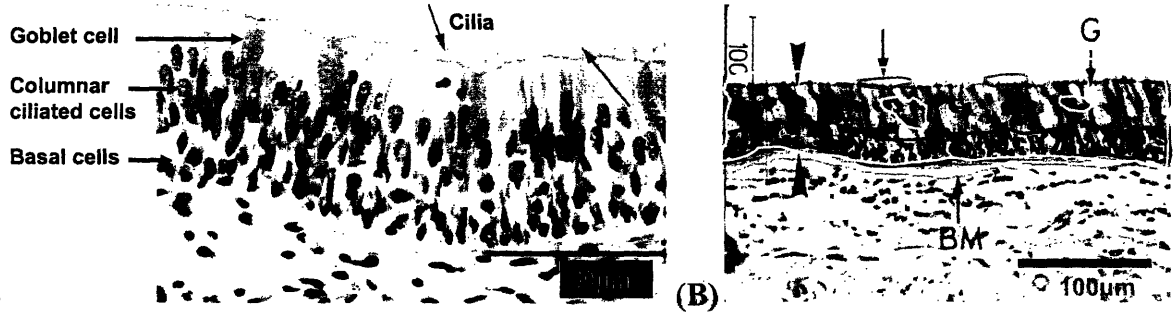

Figure 11 A) Cross-sectional Image of Trachea Pseudostratified Epithelial Membrane,[25] and B) Labeled Image of Tracheal Epithelium[26] labeled as follows: red – cilia, blue – basal cells, green – basement membrane, yellow – nuclei of columnar cells, white – ciliated pseudostratified columnar epithelium, G – goblet cells

All cells in the epithelial membrane are in contact with the basement membrane, the layers of muscle, blood vessels and fat that lie beneath the epithelial lining. But, due to differences in shape and height, the nuclei of the cells appear in different levels and not all cells reach the surface of the epithelium. However, it is important to note that while not all cells reach the surface of the epithelium, most do. While the nuclei may appear to be located at different levels, the cells themselves actually stretch up to the surface and down to the basement membrane.[27] Because of the variation in cell shape and nuclei height, the tissue lining of the airway is said to be a pseudostratified epithelium.[25] An additional image of the epithelium lining, with colored labels of the various cell types, is presented in Figure 11 B.

Pseudostratified, as it applies to epithelium tissue, means that the cell lining appears in a series of integrated layers, but in fact, there is really only one type of cell layer above the basal membrane. In the case of the airway epithelial membrane, it appears pseudostratified because of the random heights at which the nuclei of the goblet and columnar cells are arranged within the epithelium lining. While the nuclei lie at different locations, the body of each cell extends from

[25] http://cal.vet.upenn.edu/histo/epithelium/pseudostrat.html
[26] http://ect.downstate.edu/courseware/histomanual/epithelia.html
[27] http://www.siumed.edu/~dking2/intro/CR006c.htm

the basal membrane to the surface where the cells form cilia. Goblet cells in the lining synthesize and secrete airway mucous. The basal cells act as "stem cells," serving as the progenitors to other types of cells.[27] The outer surface of the trachea mostly contains ciliated cells, many of whom have nuclei that are located within the middle of the lining and have membranes that stretch from the epithelium surface to the basement membrane.

The location of the columnar ciliated cell nuclei is outlined in Figure 11 part B. Ciliated cells have anywhere from a single to hundreds of cilia projecting from the apical portion of the cell. The cilia are short, fine structures which contain two central microtubules surrounded by nine microtubule doublets. Dynein arms within the cilia form bridges between adjacent doublets.[28]

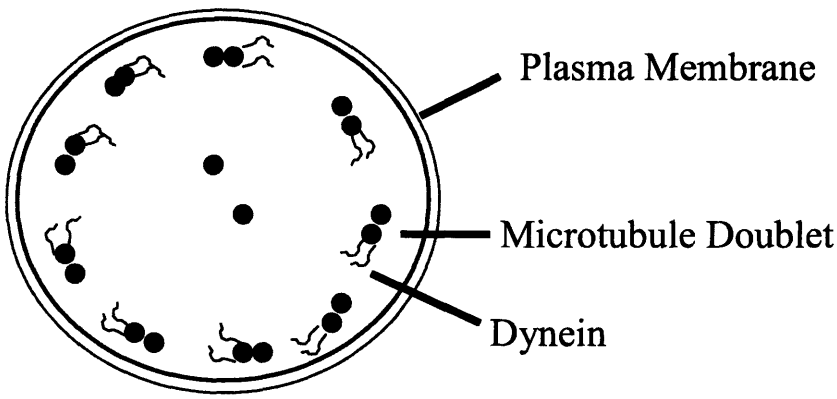

Figure 12 Schematic of Individual Cilia Structure[28]

Figure 12 is a schematic of the cross-section of a single cilia that would be found on the apical portion of a epithelial cell in the trachea. The cilia are each about 0.3μm in diameter and 3-7μm in length. They undergo regular synchronous undulating movements, essentially creating a wave across the epithelium. Cilia on the epithelial cells aid in the movement of airway mucous up the tracheal walls and near the epiglottis so it can be swallowed.[26]

[28] University of Southern California School of Medicine Lecture, http://www.med.sc.edu:89/PPT/40

Because of the pseudostratified arrangement of the epithelial cells in the tracheal lining, it is not only the cilia on the ends of the columnar cells that come in contact with ETs. Some columnar cells have as few as one cilia structure on the apical portion of the cell, so the outer membranes of those cells may also come in contact with an ET. The large goblet cells, whose nuclei are randomly arranged amongst the columnar cells, have areas where they are at the surface of the epithelial lining. Hence, columnar cells, their ciliated regions and goblet cells together compose what is referred to as the outermost mucous membrane.

2. LABORATORY RESEARCH: MATERIALS AND METHODS

The objective of this research was to gain a more complete understanding of the interactions between endotracheal tube materials and epithelial cells, which are the type of cells that line the trachea in contact with the ET material. As described above, when endotracheal tubes come into contact with the human body, they trigger cell proliferation and growth often resulting in tracheal, subglottic, and laryngeal stenosis (narrowing) in the patient.[29] To begin to understand epithelial cell response, it is important to observe and quantify the interactions between epithelial cells and ET material.

2.1. Epithelial Cell Culturing

An *in vitro* model system of human esophageal epithelial cells was chosen based on; 1. ease, stability, and robustness of culturing the cell line, and 2. physiological relevance of the cell line. Since human tracheal epithelial cells were not readily available, the culture was done with *homo sapien* esophagial epithelial cells from the American Type Culture Collection (ATCC) that had been SV40 large T-antigen transfected.[30] This cell line is appropriate for investigating

[29] Wilson, Deborah, M.D., Friedman, Norman, M.D. Subglottic Stenosis, Grand Rounds Presentation. Basic Science Review, Wound Healing Conference. April 14, 1999.
[30] ATCC Number: CRL-2692

putative esophageal carcinogens,[31] suggesting appropriateness for material-cell interaction analysis. While *in vivo* animal models (intubating dogs or sheep for varying lengths of time) and histological studies would likely yield more physiologically relevant results, the funding (an estimated $250,000 for 25 dogs) and facilities for this type of study were not available at this time.[14]

Bronchial epithelial cell medium with BEGM Bullkits additives (BioPharmaLink) was used as propagation medium. The freezing medium had five components; 4.025 ml Leibovitz's L-15 medium (ATCC), 0.5 ml fetal bovine serum (ATCC), and 0.375 ml dimethyl sulfoxide (DMSO) (ATCC), 0.05 ml polyvinyl pyrollidone (Sigma Aldrich) and 0.05 ml HEPES solution (Sigma Aldrich). The cells were grown in T75 flasks and tested in 24-well plates (VWR), both made of polystyrene. The cells were grown in a 37°C incubator. The gas phase in the incubator was composed of laboratory air with a steady 5% concentration of CO_2 added. Coating solution was prepared using 0.01 mg/ml fibronectin and 0.03 mg/ml bovine serum albumin (Sigma Aldrich) and 0.03 mg/ml vitrogen (Cohesion Technologies). To release cells from the surface of the T75 flasks, 2-3 ml of trypsin-EDTA (VWR), was used. The culturing of the epithelial cells was carried out using the splitting protocol, presented in the Appendix Exhibit A, which has been modified slightly from typical culturing procedures for this particular cell line.[31]

A fraction of the cells were frozen every 1-2 months and could be thawed in the case that contamination or other problems occurred that compromised the state of the working cells. The frozen cells were kept at -80°C in order to assure no cell transformation occurred. The cells were frozen according to the protocol stated in the Appendix Exhibit B. The vials remained in the -80°C freezer as long as necessary. The frozen vials of cells were thawed, if necessary, by using the protocol in the Appendix Exhibit C.

[31] Sherley, Prof. James. Personal discussions, Fall 2003 – Spring 2004.

2.1.1. Cell Splitting

The cells were regularly split into multiple flasks once they reached two-thirds confluency (a monolayer of cells) to avoid having overgrowth that would result in cell death. This was empirically determined to require 5-8 days to reach from a 1:3 or 1:4 scale splitting. Figure 13 is a flow chart of the steps taken during the epithelial cell culture procedure during each splitting.

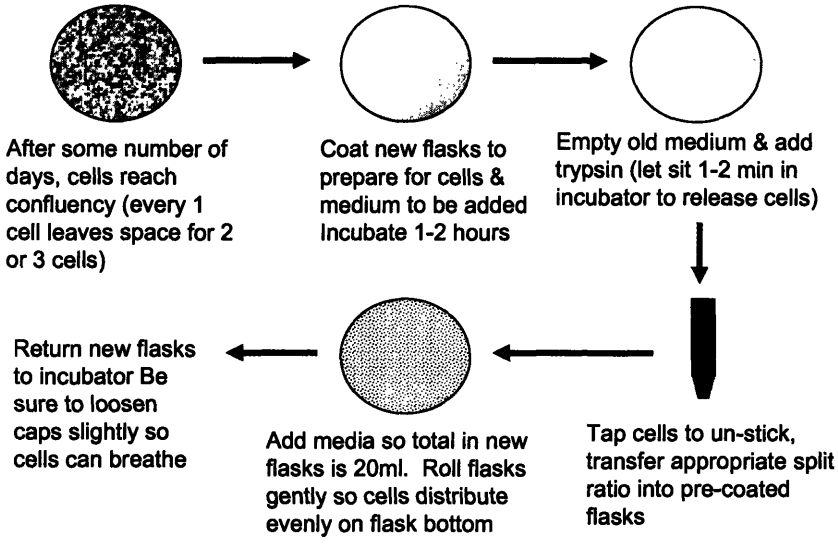

Figure 13 Diagram of Epithelial Cells Sub-culturing Procedure

2.1.2. Cell Counting

In some cases, cells within a sample of the culture were counted at different times to determine their density and rate of growth. Cells were counted using a 4 -quadrant, 100 mm long brightline hemacytometer (Fisher Scientific, Figure 14). A Pasteur pipette and bulb were used to remove about 0.3 ml of the trypsin/cell/media mixture and place it into a small opening in the side of the hemacytometer. The trypsinized cells were small and rounded and did not interact with the hemacytometer. The hemacytometer used a specialized glass coverslip that lay on top of the slide and resulted in a fixed volume of cells mixture entering the counter. The

center of the hemacytometer looks like the schematic shown in Figure 14. Only the cells that lay in one of the four quadrants were to be counted. The hemacytometer was observed using a Nikon Diaphot phase microscope at 10x magnification, and the cells in the four designated quadrants were counted.[32] Once the cells were counted, the hemacytometer was cleaned with ethanol and stored for later use.

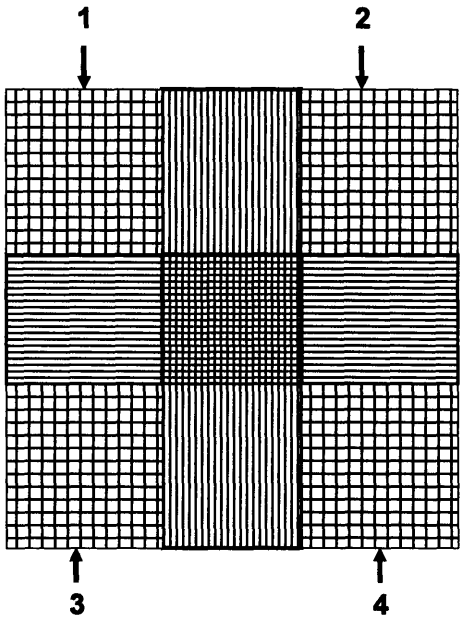

Figure 14 Schematic of Hemacytometer: the four quadrants labeled 1, 2, 3 and 4 are the areas where cells are counted

Cells similar to the ones used in this research are known to grow exponentially in their cultures for at least the first 16 days of growth.[31,33] Data from cell counting in control wells with no ET material was used to estimate the "doubling time" as follows:

$$A_t = A_o e^{kt} \quad (1)$$

[32] Meditech Technical Information, Determination of Cell Numbers. www.cellgro.com
[33] Shen, Zhong-Ying, et. al. Immortal phenotype of the esophageal epithelial cells in the process of immortalization. International Journal of Molecular Medicine, 10: 641-646, 2002.

where: A_t = final number of cells, A_o = number of cells at time = 0, k = growth factor (1/t), t = time of experiment (hours), kt = ln(2) when t is the doubling time.

2.1.3. Quantifying Cell Morphology

To analyze cell morphology changes, images taken with the Nikon Diaphot phase microscope at 10x magnification were recorded following one day incubation periods of cells grown in 24-well plates with each type of ET sample. An image was chosen from each type of sample (control, Mallinckrodt, Rusch, Columbia) to have the cell morphology analyzed. A 200x200 μm^2 area in each well was observed. The images were calibrated using a Petroff-Hausser cell counter with square sides each 50μm in length. The cells within the area had their maximum dimension measured, recorded, and analyzed statistically.

2.1.4. Cell Death

Trypan blue dye (VWR) was used in conjunction with a phase microscope to assess which cells were alive and which had died. Trypan blue dye intercalates into the nucleus and membrane of dead cells and results in dead cells being completely dyed blue. The live cells remain unstained. Trypan blue was made into a 0.4% solution in phosphate buffer solution (PBS) warmed to 37°C. Prior to imaging the cells, they were first grown 3-4 days with and without material samples in 24-well plates. The media was then aspirated out of the well, and then 1 ml of the trypan blue solution was added. The cells sat with dye for 5-10 minutes and then the dye was aspirated. The cells were immediately imaged in the phase microscope.

Another live-dead assay using a fluorescent dye, dihydrochloride: hydrate (DAPI), was employed to view cells that were living on the surface of the ET materials and cells that were nearby or underneath parts of the samples. DAPI is a cell-permeating dye that stains the nucleus

of live cells.[34] DAPI dye was made into a 1 mg/ml solution in PBS and used immediately or stored in the dark (wrapped in aluminum foil) at 0-4°C. The cells grown in 24 well-plates were stained by aspirating the media, adding 0.25 ml of DAPI solution, and incubating at 37°C for 5 min. The Nikon Diaphot phase microscope was used, and fluorescence lighting powered by a Nikon Super High Pressure Mercury Lamp was supplied to the microscope. The cells could then be imaged under fluorescence lighting. Live cells glowed bright green under the microscope.

2.2. Endotracheal Tubes

Commercially available ETs chosen as control samples were produced by Mallinckrodt® (Endotrol 8 mm, cuffed) and Rusch® (8 mm, cuffed).[35] Both of these ETs were manufactured via extrusion molding while the balloon cuffs were fabricated by blow-molding, and then each cuff was hand-glued to the tube.[36,37] The ETs were manufactured out of medical grade plasticized PVC plasticized with DEHP or di-octyl acetate (DOA);[38] mechanical and chemical characterization of the tube materials was determined previously and is shown in Table 2.[9]

[34] Molecular Probes, Invitrogen Detection Technologies. Gallery Photo ID: G000651. http://www.probes.com
[35] Mallinckrodt® 8mm Endotrol tubes and Rusch 8mm cuffed endotracheal tubes were purchased from Emergency Medical Products. http://www.buyemp.com
[36] Personal discussions with Mr. Ray Pellerin, Sales Manager at Advanced Polymers, Inc. Fall 2003-Spring 2004.
[37] Personal discussion with Brown Rudnick Berlack Israels Intellectual Property (IP) Practice Group. May 5, 2004.
[38] Brodie, Kristin. MIT undergraduate thesis, "Studies of Poly (vinyl chloride) Based Endotracheal Tubes From the Nano- to Macroscopic Scale." May 2003.

Table 2 Mechanical and Chemical Characterization of ETs[9]

Properties	Method	Mallinckrodt	Rusch
Weight % PVC (approx.)	Gas Chromatography	63.4	64.8
Weight % DEHP (approx.)	Gas Chromatography	36.6	32
Weight % DOA (approx.)	Gas Chromatography	0	3
Fracture stress (MPa)	Uniaxial Tension	30	37
Fracture strain (%)	Uniaxial Tension	350	310
Contact Angle (°)	DI Water	81.5	76
C-H bonds Ester linkages C-C bonds C-O bonds	FTIR	visible	visible
% PVC crystallinity	X-ray diffraction	12	not tested
PVC molecular weight	Gel Permeation Chromatography	140000	not tested

One alternative material, with potentially improved surface properties. was analyzed in addition to the two commercially available ET materials. This alternative material was samples of Mallinckrodt ET tube section (8 mm, cuffed) embedded with chlorhexidine. The samples contained a separate antibacterial agent, silver, as well. Analysis of these tube samples have been performed as a blind study in collaboration with Dr. Victor Pacheco-Fowler, an anesthesiologist at Columbia University. Full details of surface qualities of the alternative material samples will not be discussed in depth due to the proprietary coating procedure that was used in their construction. This alternative material is hereafter referred to as "Columbia sample."

2.3. Experimental Procedures for Assessing Cell-ET Interactions

Each well in the 24-well plate was coated in the same manner as the T75 flasks; two hours in advance using the coating solution, incubated for two hours, then had 2 ml of fresh media added to it. Depending on the test being performed, either material samples were added first and cells later, or cells were added first and material added later. 0.5x0.5 cm pieces of ET samples, used as received, were added to wells. During the splitting process, when cells had been

removed from the flask and were suspended in solution, 0.2 ml of the cell suspension was added to each well. For each 24 well plate, 5 different material samples were tested in four wells each and one set of four wells were used as controls, meaning no material was added to those wells. The following observations were made:

1. Qualitatively observing whether or not cell growth, or overgrowth, occurs on the ET material.
2. Measuring cell length using the Nikon Diaphot phase microscope, at 10x magnification.
3. Qualitatively assessing cell death using dihydrochloride: hydrate (DAPI) and trypan blue fluorescent dye assay in conjunction with the Nikon Diaphot phase microscope at 10x magnification.

3. RESULTS AND DISCUSSION

3.1. Cells in Culture

The growth and density of the cells growing the culture flasks were monitored by microscope. The growth rate and cell density were calculated.

3.1.1. Exponential Growth

Epithelial cells have been seen to experience exponential growth in cell cultures by previous researchers.[39] An estimation of the cell growth doubling time was completed by estimating the total number of cells in a culture flask after 4 hours (essentially time = 0), and again after 92 hours. The estimation showed that the cells increased 418% over the 86 hours. Using this information in conjunction with Equation 1, the exponential growth rate factor (k) was

found to be 0.017 hr^{-1} and the doubling time was found to be 42 hr. This doubling time is within the range of doubling times measured by previous researchers on similar cells.[39]

3.1.2. Cell Density

Cell density analysis was performed a couple of times during the cell splitting procedure. A T75 flask that appeared quite confluent, estimated between ¾ to near full, was measured in the hemacytometer. The test showed 12, 11, 14 and 19 cells per quadrant in the hemacytometer. The cell density was determined to be 140,000 cells/ml. A separate test performed when the cells appeared 2/3 confluent resulted in 3, 5, 7, and 8 cells per quadrant. In this case, the cell density was 60,000 cells/ml. The cells were typically split when flasks contained between 2/3 to near full confluency.

3.2. The Cells in the Control Wells

Cells with no added material were grown in 24-well plates as control wells.

3.2.1. Qualitative Appearance of Cells

The control wells imaged showed healthy epithelial cells in every test performed.[31] Representative images of cells after 1 and 4 days in the control wells are shown in Figure 15 A and B, respectively.

[39] Castro-Munozlezo, F., Development of a spontaneous permanent cell line of rabbit corneal epithelial cells that undergoes sequential stages of differentiation in cell culture. Journal of Cell Science 107, 2343-2351 (1994).

Figure 15 Control Wells After Incubation

A) After 1-Day Incubation, B) After 4-Day Incubation

3.2.2. Cell Morphology

An image of a control well after one day of incubation is presented in Figure 16. The blue lines mark the longest dimension of each cell and were used to measure that dimension. For this particular sample, an average length of 22.6 +/- 11.6 μm, n=22, was calculated.

Figure 16 Marking the Size of Control Cells after 1-Day Incubation

3.3. Cells with ET Material Added

The cells in the wells to which material was added were viewed and measured in a manner similar to the control wells.

3.3.1. Qualitative Appearance of Cells

Images of the cells around the endotracheal tube materials were taken by looking at different locations in a well and imaging multiple focal distances at each location. By doing this, the surface of the sample and bottom of the well could be imaged. This alleviated the concern that cells that may migrate and grow underneath the material were not thought to be on the material.

3.3.1.1. Mallinckrodt ET Samples

The sample in Figure 17 is from a Mallinckrodt Endotrol endotracheal tube cuff. The cuff sample is approximately 0.15 μm thick[9]. In Figure 17 A, the bottom of the well is focused on. The cells appeared to be healthy and similar to those seen in the control wells.[31] In Figure 17 B, the surface of the sample is focused on. Clusters of cells are seen on the edges, and the cells no longer appear healthy and moving about as readily as those on the surface. The cells on the sample edges are smaller and more round than the cells on the bottom of the well.

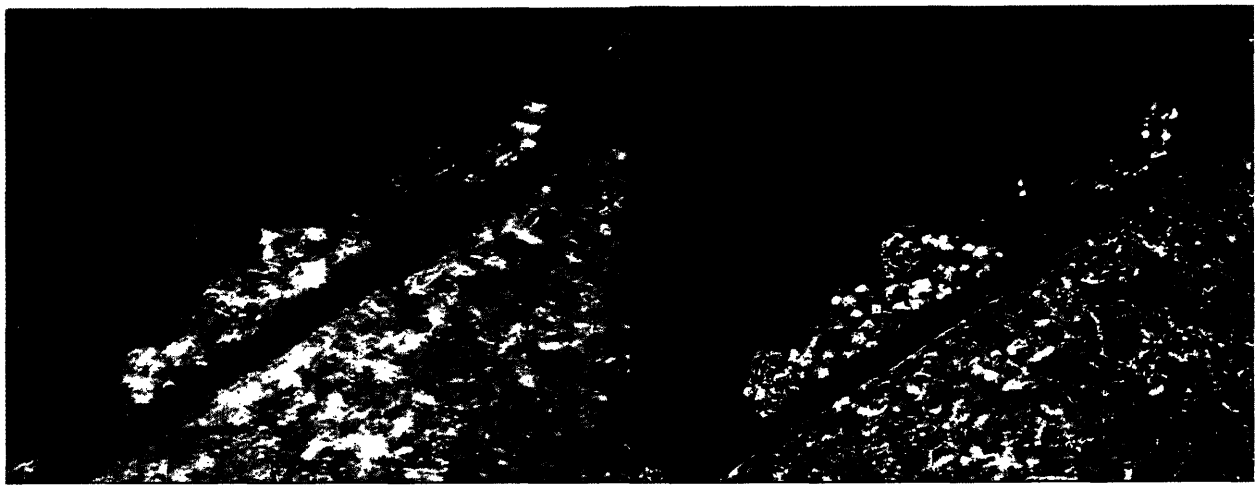

Figure 17 Mallinckrodt Sample After 1-Day Incubation

A) Focus on Bottom of Well, B) Focus on Surface of Sample

A closer look of Figure 17 B is shown in Figure 18. There appear to be round cells on the surface of the sample as well as on the edges.

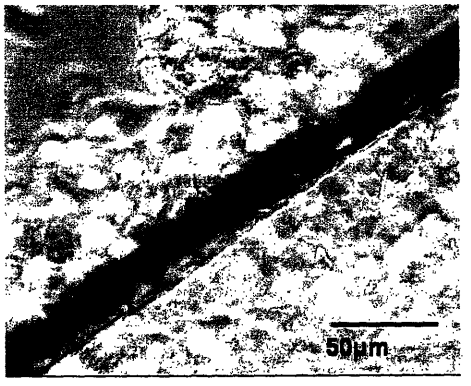

Figure 18 Close-up of the Edge of Mallinckrodt Sample

The clustering of the cells along the edges of the sample continues over time. In Figure 19, a Mallinckrodt sample after four days of incubation is shown and cells growing on the edges of the sample are clearly visible. The cells on the bottom of the well are similar in appearance to the control cells incubated for the same period of time.

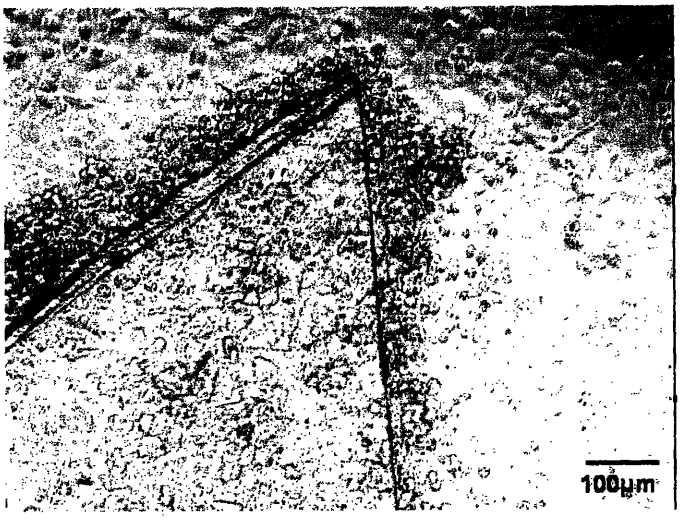

Figure 19 Mallinckrodt Sample after 4-Day Incubation

3.3.1.2. Rusch ET Samples

Similarly to what was seen with the Mallinckrodt cells, the cells not in contact with the sample appear to be healthy and normal, as shown in Figure 20 A. The cells on the edge of the

Rusch surface form large clusters of rounded cells, and those lying on the surface all appear to be small and rounded. The clustering and small rounded cells can be seen in Figure 20 B.

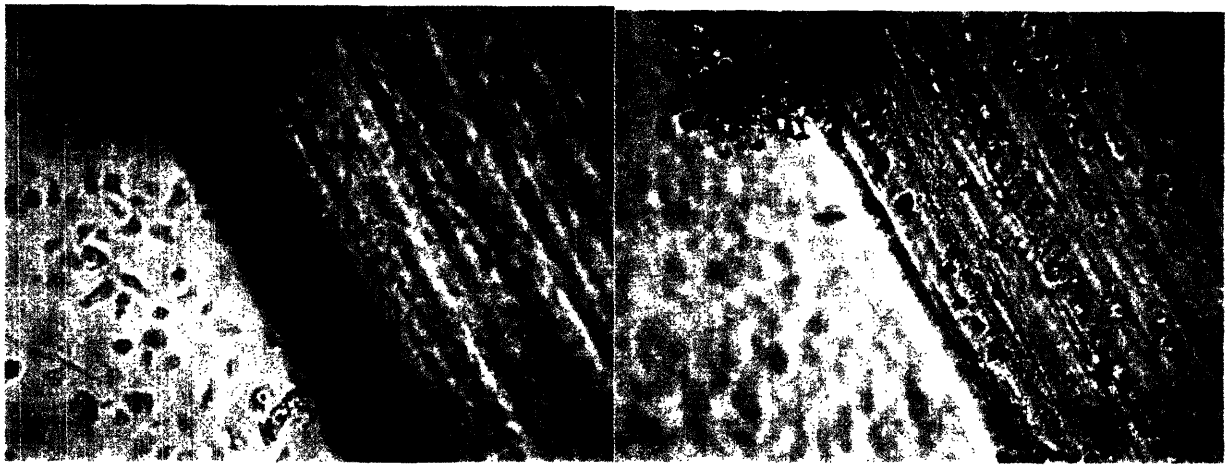

Figure 20 Rusch Sample After 1-Day Incubation

A) Focus on Bottom of Well, B) Focus on Sample Surface

The Rusch samples were observed after several days of incubation. The cells that were growing in the bottom of the well near the sample were similar in appearance to those seen in the control wells. The cells near the samples were clustering along the edges. The cells on the surface of the samples were rounded in shape. Figure 21 is an image of a Rusch sample after four days of incubation. The image shows clustering of cells along the edges of the sample.

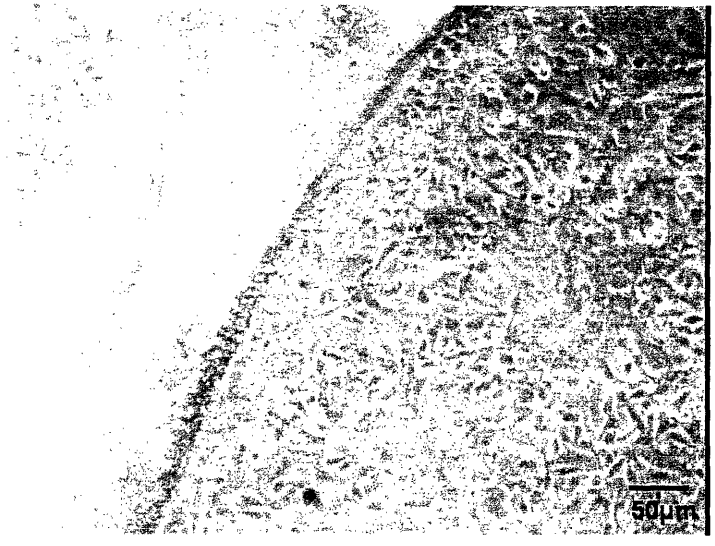

Figure 21 Rusch Sample After 4-Day Incubation

3.3.1.3. Columbia ET Samples

The samples from Columbia were grown in the same 24-well plates alongside control wells, Mallinckrodt, and Rusch samples. Figure 22 A is an image of a Columbia sample with the focus on the bottom of the well. The cells on the bottom of the well-plate that were grown with these samples did not appear similar to those seen in the control wells. The cells were small and rounded in appearance. The cells on the surface of the sample, shown in Figure 22 B, were also rounded in appearance. The cells, however, did not form clusters on the sample edges. The samples were observed several days after plating, and the cells still appeared small and rounded as they do in these images.

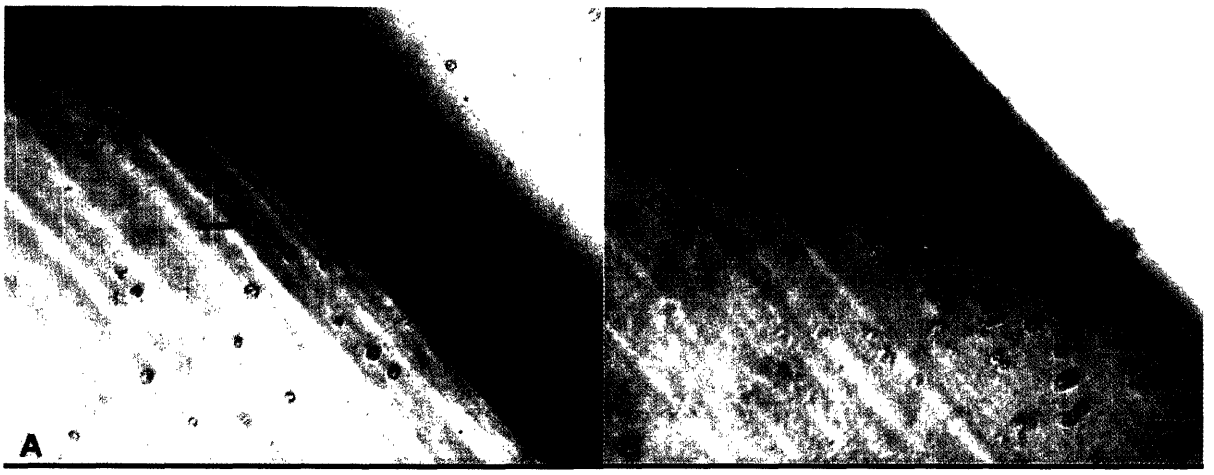

Figure 22 Columbia Sample 1-Day after Incubation

A) Focus on Bottom of Well, B) Focus on Surface of Sample

The Columbia samples were imaged after a four day incubation period. The cells on the bottom of the wells, shown in Figure 23 A, are small and circular as seen before. They do, however, appear to show some contamination amongst the cells.[31] The cause for the contamination may be due to the processing techniques used at Columbia University to embed the samples. Figure 23 B is an image of the surface of the sample. The surface did not appear to have any clustering of cells along the edges, but did appear to have cells scattered on the surface.

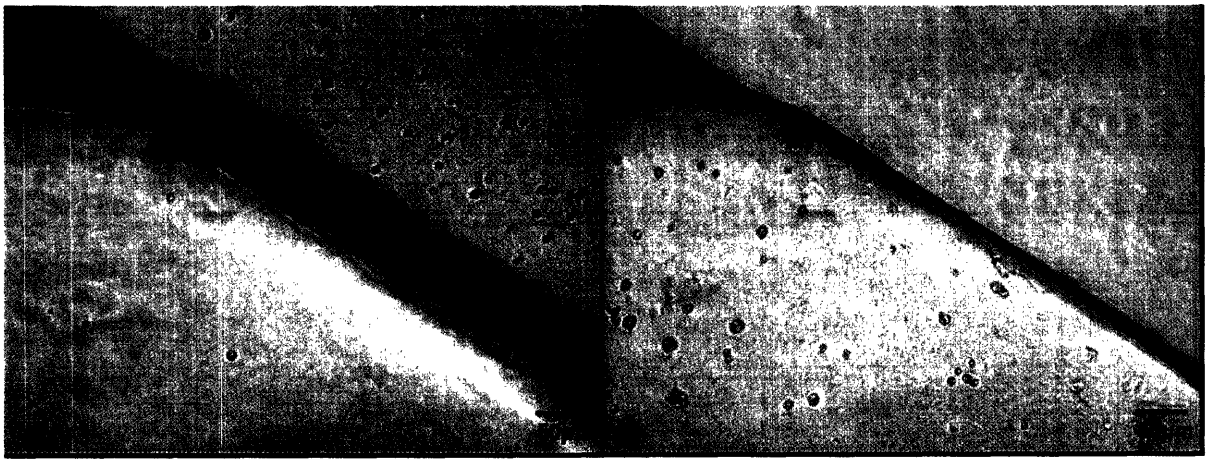

Figure 23 Columbia Sample 4-Days after Incubation

A) Focus on Bottom of Well, B) Focus on Surface of Sample

3.3.2. Cell Morphology

In order to stay consistent during measurements, a 200x200 μm area with partial ET sample visible was chosen for each type of ET used. For example, the Mallinckrodt sample area used for determining the average cell size after one day of incubation is shown below in Figure 24. The 200x200 μm areas chosen to calculate the average cell size for all other ET samples looked similar to the Mallinckrodt sample area.

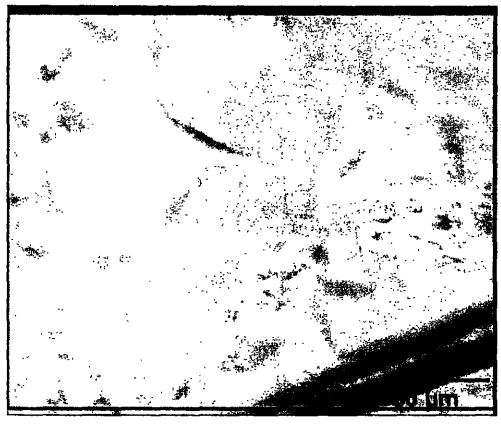

Figure 24 Sample of Mallinckrodt after 1-Day Incubation

Images from control, Mallinckrodt, Rusch, and Columbia samples were analyzed and the average cell length determined for the samples. The results of these measurements and calculations are summarized in Table 3, where the average cell lengths determined for each type of ET sample are listed. The cells from control wells had the largest average length, 23 μm. The Mallinckrodt and Rusch samples had similar average cell lengths, giving measurements of 18 μm and 17 μm, respectively. The cells in Columbia wells had the smallest average length, with a length of only 11 μm.

Table 3 Average Cell Length for ET Samples

Sample	Cells Counted	Average length [μm]	StDev length [μm]
Control	23	23	12
Mallinckrodt	38	18	11
Rusch	22	17	10
Columbia	22	11	1.5

To test the statistical significance of these differences in cell length, unpaired Student t tests were performed between the different data sets. The resulting p values from these tests are presented in Table 4. The p value is the probability that the data sets are members of the same population. From the p values, the length of the cells in the Columbia sample wells is significantly (with greater than 99% confidence, in this case) different from that in the other wells. The cell length populations in the other wells are not significantly different from one another.

Table 4 p Values from Student t Test

	Control	Mallinckrodt	Rusch	Columbia
Control		0.106	0.112	<0.0001
Mallinckrodt			0.92	0.0076
Rusch				0.0049
Columbia				

A graph showing the longest cell dimension for the four different samples is shown in Figure 25.

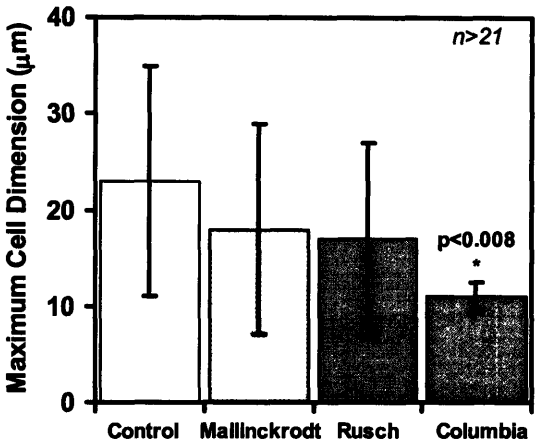

Figure 25 Comparison of Longest Cell Dimension in the Different Sample Wells

3.3.3. Qualitative Analysis of Cell Death

Two different dyes were used to determine the relative health of the cells in the different wells. A trypan blue staining dye was added to some wells to mark those cells that were dead. A fluorescent dye, DAPI, was added to some wells to illuminate those cells that were alive.

3.3.3.1. Mallinckrodt ET Samples

Trypan blue staining was performed on a sample well containing material from a Mallinckrodt endotracheal tube cuff. The images taken focus on both the bottom of the well and the sample surface. Figure 26 A focuses on the bottom of the well near the sample. The cells that were completely dyed blue, both nucleus and membrane, are dead cells. The epithelial cells have bluish nuclei, making it difficult to discern between completely dead cells and live cells. Figure 26 B focuses on the surface of the sample. Blue cell-like forms were visible on the surface of the material. The clustering of cells along the edges of the surface appeared to contain primarily dead cells, with a few visible cells along the edge that are still alive. Several cells

appeared on the material itself, although most seemed to be balled up similarly to what was observed in Figure 17.

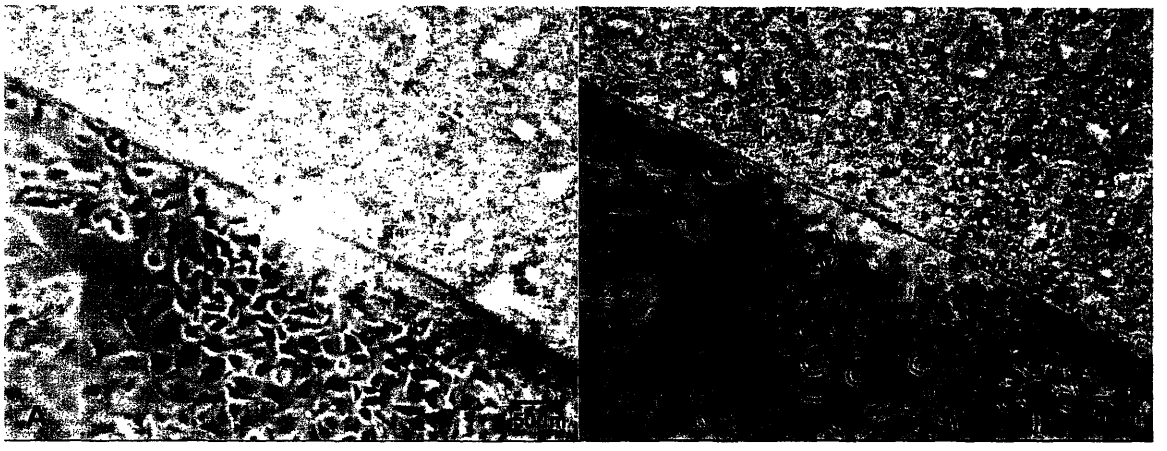

Figure 26 Trypan Blue Staining of a Mallinckrodt Sample

A) Focus on Bottom of Well, B) Focus on Surface of Sample

Fluorescent dye, DAPI, enters cell membranes and causes the live cells to fluoresce. Images taken of a Mallinckrodt tube sample focused on the surface of the ET sample under normal phase microscope lighting and under fluorescence light. Figure 27 are images which focus on the sample itself, clearly showing live, dyed cells on the surface of the material. The cells on the material surface are mostly rounded in shape.

Figure 27 Mallinkrodt Sample with DAPI Dye

A) Focus on Sample Surface without Fluorescence, B) Focus on Sample with Fluorescence

3.3.3.2. Rusch ET Samples

Trypan blue staining was performed to qualitatively observe the amount of dead versus live cells growing on the Rusch sample. Figure 28 A is a phase microscopic image of a Rusch sample, which takes up the bottom ¾ of the image. The focus in this image is on the surface of the sample, and it is evident that there are some dead cells growing on the surface, but many seem alive. The cells do appear rounded in shape, and clusters of cells are evidently lining the edges of the sample. The clusters of cells appear to be mostly dyed completely blue, meaning the cells that are in these clusters are dying. Figure 28 B is an image taken approximately 400 μm from the Rusch sample, and while some clustering of cells appears, the cells that compose the cluster are not dead. In fact, the cells look similar to those seen in the control well images.

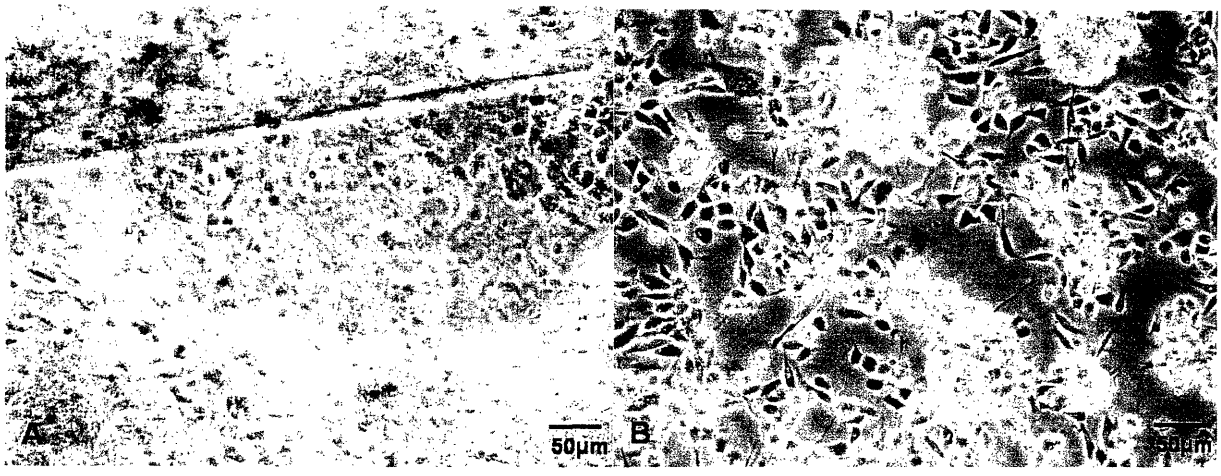

Figure 28 Trypan Blue Staining of a Rusch Sample

A) Focus on Sample, B) Focus on Bottom 400µm from Sample

Rusch samples were dyed with DAPI to get a better understanding of live versus dead cells on the sample. In the analysis performed, it was very apparent that the cells along the edges of the sample were mostly alive. Figure 29 A and B show a Rusch sample in a well that was dyed with DAPI. The images are shown without and with fluorescence lighting, respectively. The green fluorescence was pieces of fluorescent dye that had not dissolved fully. Quantitative values cannot be determined from these tests, but an understanding that cells adhere and are attracted to the material is visible. It appears that cell proliferation and growth is affected by the presence of the ET material, since cells appear to form clusters on and around the sample.

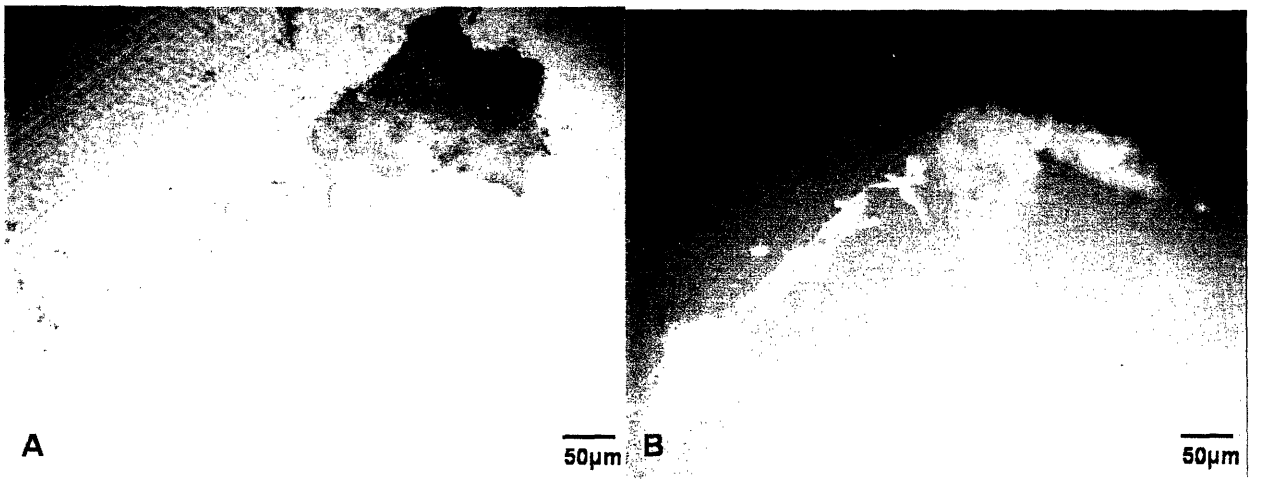

Figure 29 Rusch Sample with DAPI Dye

A) Focus on Sample Surface without Fluorescence, B) Focus on Sample with Fluorescence

3.3.3.3. Columbia Samples

Because of the unusual appearance of the cells in contact with the Columbia sample, a DAPI dye was performed to observe if the cells on the bottom of the well and the cells on the surface of the sample were alive. The microscopic images shown in Figure 30 are of a Columbia sample with the microscope focusing on the bottom of the well. The images are shown with and without the fluorescence lighting.

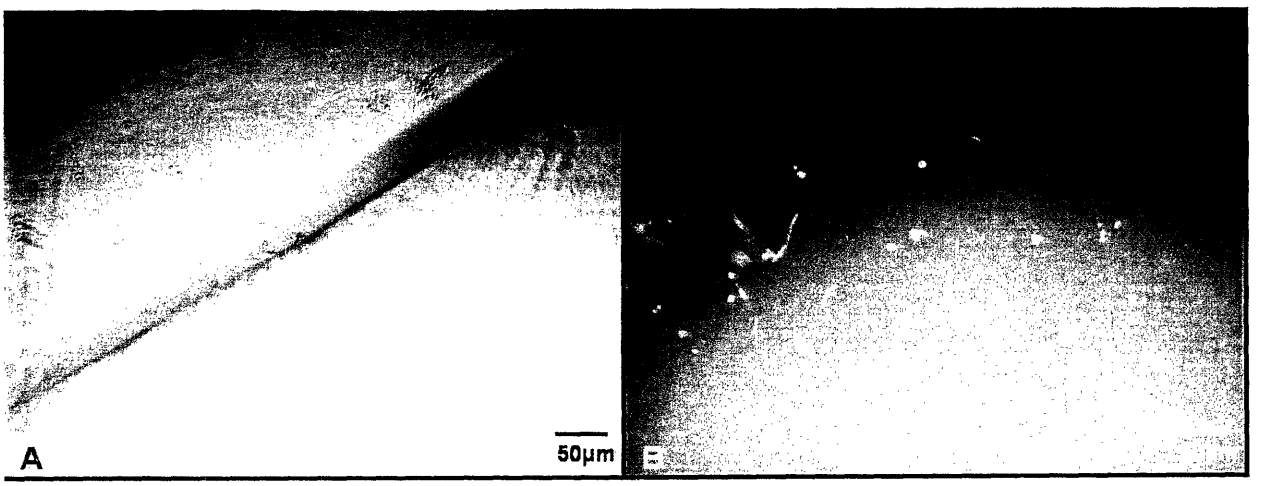

Figure 30 DAPI Dye of Columbia Sample Bottom of Well

A) Image without Fluorescence Lighting, B) Image with Fluorescence

The images of the Columbia sample with and without fluorescence lighting shown in Figure 31 A and B, respectively, clearly show that there were cells alive on the surface of the Columbia sample. There are no clusters of cells on or near the edge of the sample, as was observed on the Mallinckrodt and Rusch sample.

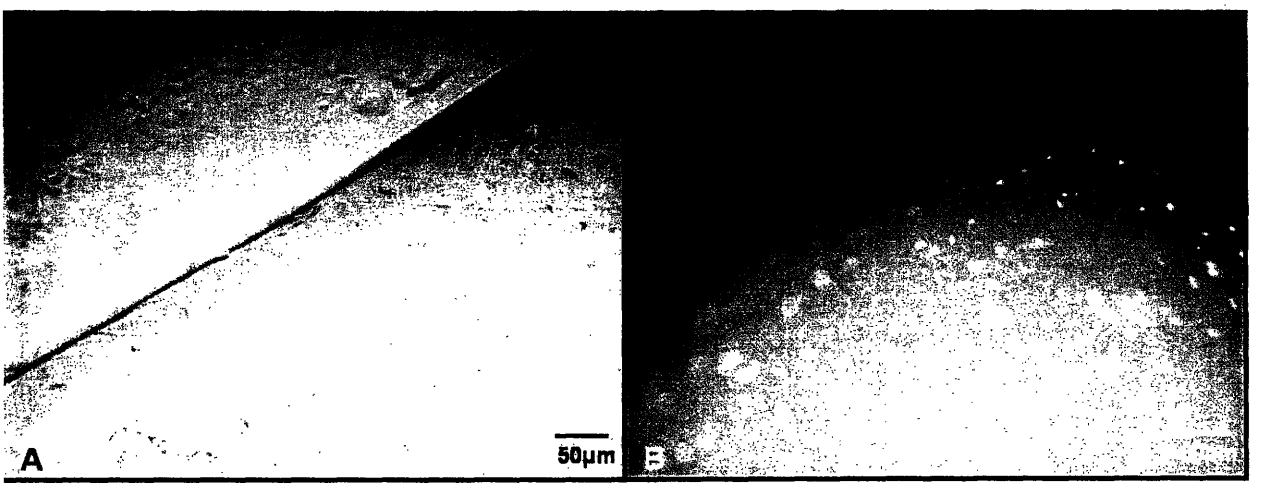

Figure 31 DAPI Dye of Columbia Sample Surface

A) Image without Fluorescence Lighting, B) Image with Fluorescence

3.4. Interpretation of Results

The data from the cell culturing analysis provided insight into the interaction of epithelial cells with the plasticized PVC surface of currently used ETs. The cells proliferate and grow on the surface of the Mallinckrodt and Rusch ET material, as seen via trypan and DAPI testing that was performed. Cells clustered and grew rapidly around the edges of the material where cells on the bottom of the well most readily come into contact with the ET samples. The cells on the bottom of the wells appeared healthy and normal like those seen in the control wells. This suggests that the cells morph only after coming into contact with the ET material. The morphology tests, in which the Rusch and Mallinckrodt average cell length were about the same, suggested that both Rusch and Mallinckrodt samples affect epithelial cells similarly.

The Columbia samples analyzed demonstrated significant effects on both the cells in contact with the material as well as the cells on the bottom of the well. The cells were smaller on average, and did not cluster or appear to move around like the cells seen on and near the Rusch and Mallinckrodt samples. Because little is known about the processing and sterilization performed on the Columbia samples, their qualities and effects on the cells have been noted, but further testing of cell-material interaction will be performed at Columbia University.

The results from the Mallinckrodt and Rusch ET samples suggest that cell growth and proliferation are effected by the presence of endotracheal tube samples. These tests have given insight into the potential hazards of the endotracheal tube material when in contact with the human epithelium. If the material is, indeed, promoting cell proliferation and growth onto ETs, then perhaps much of the scarring and damage caused to the airway passages could be reduced by a significant material advancement in this field.

In addition to biological adhesion testing, the commercialization potential of manufacturing an endotracheal tube with improved material properties has been investigated and is discussed in chapter 4.

4. ECONOMIC ANALYSIS

The commercialization of an improved endotracheal tube material is discussed in the following sections. It discusses the endotracheal tube market and current manufacturers. Some feasible strategies for entry into this medical device market are discussed. This section was completed using the assumption that the material improvement to endotracheal tubes was at the stage where FDA approval testing was eminent.

4.1. The Market

4.1.1. Market Size

Approximately 32 million intubations are performed annually worldwide. Cuffed endotracheal tubes sell within the range of $2 – $15 per unit, with an average price of $5, and thus the current market is $150 million per year[40,41]. Medical device market growth rates are estimated at 5.5% per year[42].

4.1.2. Current ET Manufacturers

The endotracheal tube (ET) manufacturing market is highly consolidated, with the two major players holding approximately 86% of the market share. The major manufacturer is Tyco, having acquired Mallinckrodt Medical Products in 2000. As of 2000, the Mallinckrodt ET accounted for 72% of the market[43]. As a result of the acquisition of Mallinckrodt, Tyco was required by the Federal Trade Commission (FTC) to divest their Sheridan® line of ET tubes to Hudson Respiratory Care Inc. (RCI). At the time of the sale, the Sheridan line accounted for about 14% of the ET market[43]. Thus, these two major players make up 86% of the market. Many other smaller companies make up the remaining 14% of the market, including Concord, Curity, Portex, Rusch, and Sherwood. All of the companies essentially produce the same product, which is based on the now expired Mallinckrodt patent.

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[40] In Vivo: The Business & Medicine Report, January 2000, pg. 42 (www.windhover.com)
[41] http://www.allmed.net/catalog/showitem.php/2126
[42] The World Medical Market Report 2003: Current trends and future prospects, Espicom Business Intelligence (www.espicom.com)
[43] http://www.mallinckrodt.com/news/news_releases/00-09-19TycoSheridan.html

4.1.3. Barriers to Entering the United States Medical Device Distribution Industry

Most hospitals make their purchases of medical devices through Group Purchasing Organizations (GPOs). GPOs negotiate contracts for large groups of hospitals, which allow the hospitals to receive significant discounts. These GPOs typically form sole provider contracts with certain medical device companies that last two to three years. For example, it is not be surprising for a GPO to carry only a single brand of ET. One can understand the barriers to a small company manufacturing and distributing a new medical device by considering Tyco's recent acquisition of the medical supplies company Mallinckrodt. The FTC had concerns regarding this acquisition due to the barriers to entry into the manufacturing and distribution of a device, as shown by the following statement issued by the FTC[44]:

The United States market for Endotracheal Tubes is characterized by significant barriers to entry. Entry into the endotracheal tube market in the relevant geographic area requires the development of a full line of products in a range of sizes and configurations, procurement of manufacturing equipment and establishment of production practices in conformity with FDA regulations, and development of a track record and customer acceptance. Entry into the Endotracheal Tube market in the United States would be relatively costly and is not likely to occur because sales opportunities would likely be too small to justify the costs and risks associated with new entry.

[44] http://www.ftc.gov/os/2000/10/tycocompaint.htm

It is likely that the FTC's concerns are well justified, owing to the nature of the medical products supply chain in the United States. Further, the GPOs favor contracting with medical supply companies capable of offering a wide range of products, as the GPOs can then obtain bundle discounts for buying multiple products. The following schematic demonstrates the current supply chain and the pressures that lead to using manufacturers with a broad portfolio.

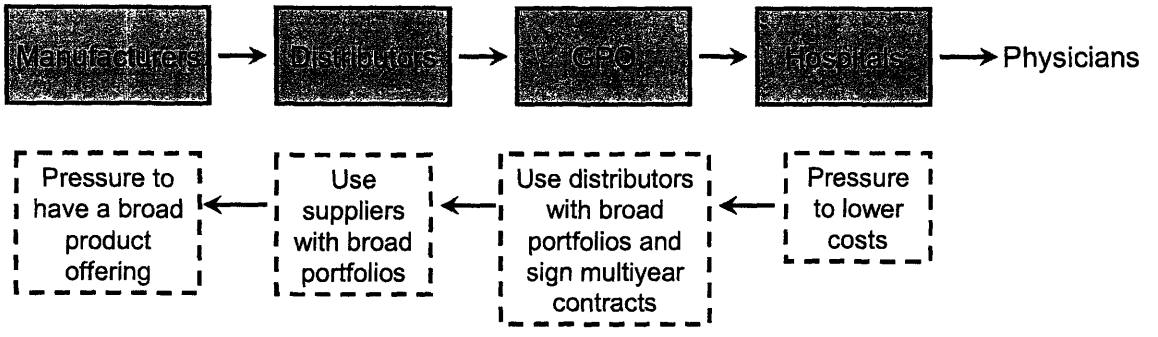

Figure 32 The Current Medical Device Supply Chain

Small medical supply companies have banded together to form the Medical Device Manufacturers Association (MDMA) and are promoting a FTC and Department of Justice (DOJ) investigation into the practices of GPOs[45]. The FTC and DOJ recently held a hearing to focus on the following specific issues: (1) product bundling, (2) lengthy manufacturer / GPO sole source contracts and (3) high hospital / GPO commitment contracts. The FTC and DOJ held these hearings over concerns that the "anticompetitive behavior by some large GPOs has resulted in disincentives to competition in the marketplace, stifled innovation and failure to reduce health care costs overall". Since the outcome of these hearings and moves against the GPOs are still unresolved, it is anticipated that a small device distributor will experience significant barriers to entry in the near term.

[45] http://www.medicaldevices.org/public/

The large suppliers of ET recognize these barriers, as can be deduced from analyzing their financial statements. The 2002 annual report of Hudson RCI[46], the 2nd largest ET supplier, makes the following observations about the competitive landscape and Hudson RCI's positioning within the medical supplies market, which have been listed here because they are pertinent to the business strategy, as will be shown later:

- Cost containment has caused consolidation throughout the health care product supply channel, which has favored reliable manufacturers with large, high quality product offerings and competitive pricing.
- Service providers have consolidated and affiliated with GPOs, which take advantage of group buying power to obtain lower supply prices.
- This, in turn, has led to consolidation among distributors, who seek to provide "one-stop shopping" for these large buying groups.
- Distributors have also sought to concentrate purchases among fewer vendors in an effort to reduce supply costs.
- Since selection as a contracted GPO provider and strong relationships with distributors are critical to many health care manufacturers, they have responded to these trends by providing a broad range of integrated products, combined with reliable delivery and strong after-sales support.
- [Hudson RCI] believes that its broad product offering represents a competitive advantage over suppliers with more limited product offerings, as health care providers seek to reduce medical supply costs and concentrate purchases among fewer vendors.

[46] Form 10-K available from www.sec.gov

- [Hudson RCI] also benefits competitively in the United States from its extensive relationships with leading GPOs, as large purchasing organizations play an increasingly important role in hospitals' purchasing decisions.
- In the current US hospital market environment, GPO and Integrated Delivery Network ("IDN") relationships are an essential part of access to [Hudson RCI's] target markets and the Company has entered into preferred supplier arrangements with eight national GPOs.
- [Hudson RCI] is typically positioned as either a sole supplier of respiratory care disposables to the GPO, or as one of two suppliers.
- [GPO] arrangements are terminable at any time, but in practice usually run for two to three years.
- [Hudson RCI] enjoys longer terms with three of its major GPOs.
- [Hudson RCI] utilizes a network of approximately 120 international distributors, typically on an exclusive basis by product category or market/country within each market.

One way that a small start-up could be successful would be to form strong, potentially exclusive, relationships with distributors and purchasers and convince these groups to switch from a competitor's product to the start-up's product. There is precedence in the ET market for a start-up firm entering with a novel tube and retaining control of the production and distribution of their product. Parker Medical introduced the Parker Flex-Tip™ tube to the market a couple of years ago. Their tube is designed to pass more easily through the airway anatomy and cause fewer traumas[47]. This innovation does not compete with material innovation because they have a change in the tube as opposed to a change in the material surface properties.

[47] http://www.devicelink.com/expo/awards02/pr_parker.html

Parker Medical is outsourcing manufacturing to Euromedical Industries Sdn. Bhd. in Malaysia. Parker is operating distribution within the United States and outsourcing overseas distribution, with contracted distributors throughout the globe[48]. Unfortunately, Parker is not a publicly traded company, so it is not feasible to scrutinize their financial statements and determine whether this is a viable business model. Analysis leads me to believe that if Parker continues to distribute its product independently, then Parker will be unable to establish contracts with hospitals because of their inability to offer a wide portfolio of products. Alternatively, a small start-up could license its technology to an existing distributor and avoid having to overcome the barriers.

4.2. Proposed Business Strategy: Licensing

The clear medical advantages of the improved medical device material or coating suggest that with the proper sales and distribution force, the new product will become the dominant ET on the market. There are two options for bringing the product to market. As described previously, one can try to retain control of the manufacturing and distribution process ourselves *or* license the technology to an existing ET supplier.

Based on the barriers to market entry in the current business environment outlined above, the more viable business plan appears to be to license the technology to one of the major ET manufacturers, either Tyco or Hudson RCI. As previously mentioned, the significant medical benefits of the new product over the existing product will result in the rapid capture of market share. Thus, the major ET manufacturers will bid against one another for the license to our material. Part of the initial funding will be used to hire legal experts skilled in licensing contracts and negotiations. With the assistance of licensing professionals, the company can

[48] www.parkermedical.com

expect to license the product for a multi-million dollar royalty contract (see Financial Analysis section for financial calculations and projections).

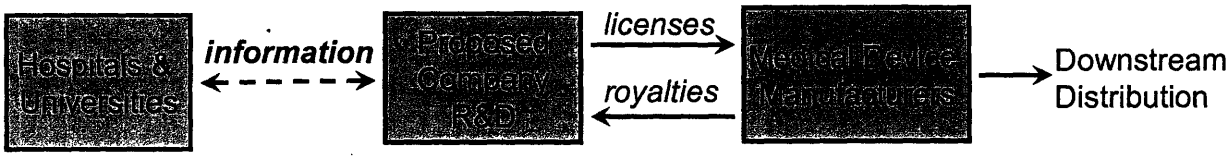

Figure 33 Schematic for Proposed Business Strategy for Success

With the revenue from the licensing agreement, the company will continue to invest substantially in medical device research and development (R&D). The company will work to expand the product portfolio to apply the process to other medical devices. The company will investigate and patent the feasibility of using the improved coating on all polyvinylchloride medical devices. The company will continue to expand by branching into additional medical-device related polymeric-related research.

The core strength of the company would be R&D in medical devices and materials. Because the company will be made primarily of engineers and doctors, it is expected that no one will have any detailed expertise in sales and distribution. The company will plan to license or outsource the technology portfolio to third parties until it reaches a point where the company has a "critical mass" of products necessary to effectively form strong distribution and purchaser contracts in the medical supply value chain. At that point in time, the company would move to manufacture and distribute its own products, which would allow it to capture more of the value in the supply chain.

4.3. Financial Analysis

As discussed previously, the business plan is to license our technology to an existing ET manufacturer. This section outlines the costs associated with bringing the product to the point of

a negotiated license as well as the expected revenue from the license in the next five plus years, assuming all test results are encouraging.

4.3.1. Costs For Bringing Product To The Point Of A Negotiated License

It will require an estimated $1.2 million to complete the research and demonstration of safety and effectiveness to the FDA, as required for a 510(k) pre-market notification. Estimations are based on discussions with doctors, manufacturers, and licensing companies. This amount will cover the manufacture of ETs with the new material for use in testing ($100,000), the personnel and equipment required for *in vitro* and *in vivo* testing ($900,000), and fees associated with negotiating and finalizing our licensing contract with a current ET manufacturer ($200,000). Manufacturing is expected to take 4-6 months. The testing stage is expected to be completed within twelve months. The license negotiations may take up to an additional four months. The funds are expected to come from private investors (including a number of the physicians), venture capitalists, small business loans and small business grants.

4.3.2. License Valuation

The estimation of the value of licensing the innovative technology to an existing ET manufacturer was based on the following points:

- ET tubes are sold for an average of $5 per unit by distributors.

- Assuming that distributors have gross margins ([revenues – cost of goods sold (COGS)]/revenues) of approximately 40%, then the manufacturers are selling the tubes for approximately $3.50 per unit.
- We can estimate the gross margins of the manufacturers from their financial statements. For example, Hudson RCI had gross margins of approximately 40% from 1998-2002.
- This means that every ET unit sold contributes $1.40 of gross profit to the Hudson RCI bottom line.
- We expect that an exclusive licensing contract would result in a royalty payment of 10% of gross profit ($0.14/unit).

The total amount of the royalty payments depend upon which of the ET manufacturers the technology is licensed to. Licensing to other device manufacturers is also under consideration, but the base case financial models presented here, have not included this scenario. An estimation of the total value of royalties paid to the company when the contract is licensed to either Tyco or Hudson RCI is shown in the table below. Conservative estimates show that licensing to Tyco would result in royalty revenues of $3.2 - $5.3 million annually over the next 5 years and licensing with Hudson RCI results in royalty revenues of $0.6 - $1.5 million annually.

Table 5 License Valuation

(Figures based on assumptions outlined in text)

	2006	2007	2008	2009
Total ET Market Size (million units)	33.8	35.6	37.6	39.6
Licensing to Tyco				
Tyco market share[1]	72%	90%	95%	95%
Million units sold	24.3	32.1	35.7	37.7
Royalty paid / unit ($/unit)	0.14	0.14	0.14	0.14
Total royalty to Company ($M)	3.4	4.5	5.0	5.3
Licensing to Hudson RCI				
Hudson RCI market share[1]	14%	18%	22%	27%
Units sold	4.7	6.2	8.2	10.8
Royalty paid / unit ($/unit)	0.14	0.14	0.14	0.14
Total royalty to Company ($M)	0.66	0.87	1.2	1.5

[1]Based on current market share figures given in "The Market" Section and then assuming the superior technology increases market share by 25% per year, with a 95% penetration maximum

4.3.3. Projected Five Year Income Statement

As discussed in the "The Market" section, the proceeds of the contract will be used to invest significantly in research and development in related medical devices and materials, which will be our core expertise. The company plans on investing one million dollars in R&D in 2006 (including the hiring of five additional researchers) and then growing the R&D budget at a rate proportional to the medical device market growth rate, currently 5.5%. It is expected that our R&D investment will produce a significant, innovative product every five years. This product portfolio growth will provide the company with significant long term positive cash flow.

A conservative income statement for the scenario of licensing to Tyco is included as below. To be conservative, it is assumed that the company only has a single licensed product through 2009. As discussed above, the total time required to complete the improved ET material and licensing contract is approximately 16 months. Then, it will take an estimated half year for the licensee to bring the company's product to market. Thus, cash flow becomes positive in the first quarter of 2006 and net income of approximately $2.2 million is expected that year. For 2007-2009, net income is projected to remain at approximately $2 million annually. If R&D efforts yield a new product during this period, the net income will increase significantly.

Table 6 Five Plus Year Income Statement
(Values in $1000s; figures based on assumptions outlined in text)

	2004	2005	2006	2007	2008	2009
Revenue	0	0	3,403	4,488	4,998	5,272
Expenses:						
ET clinical testing[1]	450	500	0	0	0	0
Contract negotiations	0	200	0	0	0	0
SG&A[2]	0	0	340	449	500	527
R&D	50	0	1,000	1,055	1,113	1,174
Total expenses	500	700	1,340	1,504	1,613	1,701
Pretax income	(500)	(700)	2,063	2,984	3,385	3,571
Tax liability	(190)	(266)	784	1,134	1,286	1,357
Net income	(500)	(700)	1,735	1,850	2,099	2,214

[1]Includes the costs for producing prototypes for testing

[2]Assumed as 10% of revenues

4.4. Secondary Business Strategy: Manufacturing

As stated earlier, approximately 32 million intubations are performed annually worldwide. Each tube sells for approximately $5 per unit and thus the current market is in

excess of $150 million[40]. Additionally, the medical device market growth rates are estimated at 5.5% per year[42]. The safety advantages of a product with improved surface characteristics will create a strong demand amongst medical professionals, thus increasing the likelihood that the company could expect to capture a significant portion of the market within the first few years of sales.

The customers will be emergency medical professionals, hospitals and doctors who currently make use of endotracheal tubes. The improved materials have been designed, from collaborations at hospitals and universities, involving highly respected doctors, to specifically address deficiencies of the currently available product. As such, it is expected that the medical community will be receptive towards our product. Our first customers are expected to be the colleagues of doctors at Massachusetts General Hospital (MGH), Brigham & Women's Hospital, and Columbia Medical School who have been involved with the project team in developing the improved device.

4.4.1. Product Development and Sales

The secondary business strategy proposes to construct a private manufacturing site at which the tubes will be coated, sterilized, packaged and distributed. Assuming completion of Phase I trials demonstrating the "proof-of-concept" for our device, and Phase II, refinement of the manufacturing process necessary, the next phase would require significant capital investments. The tubes would qualify as a class II device and would require an FDA 510K application. Phase III testing, which would consist of animal and clinical trials for gaining FDA 510K approval, requires that the devices being tested are manufactured on the machines that will be used for the large-scale manufacturing. For device manufacturing, the machines must conform to the Good Manufacturing Practice (GMP) guidelines written by the FDA. Hence, the

production facility will need to be built and in good running order before any products can be manufactured for animal testing. Once the facility is built and prototypes are made, the Phase III testing, including animal studies, should take an average of 3 months.[49]

Production will be performed by using a blow-molding extrusion process to manufacture the cuffs, which will subsequently be attached to polymer extruded tubes. It was determined that these tubes could potentially be purchased at a price comparable to what it would cost to manufacture the tubes at our site. Regardless of whether the ETs are manufactured in our facility or elsewhere, significant capital expenditures are necessary to purchase the materials, machinery and human labor necessary for coating, packaging and distributing the ETs. This is expected to cost approximately $2.35 million in capital expenditures. A breakdown of expenses necessary for a small scale production facility (capacity of 3.5 million to allow for growth, and an initial production volume starting at 550,000 in year one) is shown in Table 7.

Table 7 Expenditures and Unit Cost for Small Scale Production

VARIABLE COSTS	**per piece**	**per year**	**percent**	
Material Cost	$0.01	$7,503.86	0.52%	
Energy Cost	$0.03	$15,640.94	1.08%	
Labor Cost	$1.02	$605,539.82	41.63%	
Total Variable Cost	**$1.06**	**$628,684.62**	**43.22%**	
FIXED COSTS	**per piece**	**per year**	**percent**	**investment**
Main Machine Cost	$0.30	$177,598.95	12.21%	$1,512,000.00
Auxiliary Equipment Cost	$0.05	$29,599.83	2.03%	$252,000.00
Tooling Cost	$0.45	$264,995.48	18.22%	$840,000.00
Fixed Overhead Cost	$0.32	$189,226.21	13.01%	
Building Cost	$0.23	$134,300.00	9.23%	
Maintenance Cost	$0.05	$30,324.71	2.08%	
Total Fixed Cost	**$1.39**	**$826,045.17**	**56.78%**	**$2,352,000.00**
Total Coating Fabrication Cost	**$2.44**	**$1,454,729.80**	**100.00%**	
Cost of Tube	$5.83	/piece		
Profit Markup	50%			

[49] Scully, Tom, et. al. Health Care Industry Market Update. Medical Devices & Supplies. Oct. 10, 2002. Provided by CMS, Center for Medicare & Medicaid Services.

Obviously, many assumptions were made in order to create this diagram. The cost of machinery necessary was taken as an average value of current blow-molding and extrusion molding machines. The factory location was assumed to be in western Massachusetts, where costs of renting would be significantly less than in Boston, MA. Energy costs were determined based on the amount of power needed to run the machine, air condition the facility, provide lighting in both the factory and accompanying offices, and heat the accompanying offices. The primary cost is labor, followed by tooling, equipment, and fixed overhead costs, shown in a cost breakdown, by element, in Figure 34.

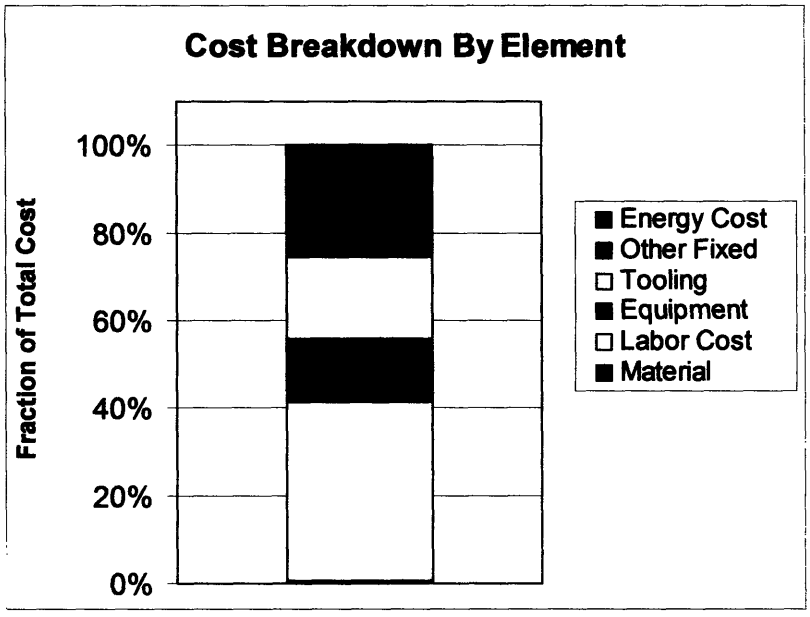

Figure 34 Cost Breakdown by Element of Fabrication Cost

The new tube is assumed to demand a price premium over the current tube and would sell for $12/tube. Multiple prices were analyzed for their potential profit-making abilities depending on the growth rate of the company. The cost for coating the tube (i.e. creating a finished product) was plotted as cost for coating per tube versus the production volume. Figure 35 shows the cost versus production volume. From this plot, a reasonable value for production volume

was determined so the cost of coating would be such that the total cost of the tube, including a 50% markup, would be reasonably close to the commodity ETs on the market. A reasonable production value was determined to be near half a million tubes per year.

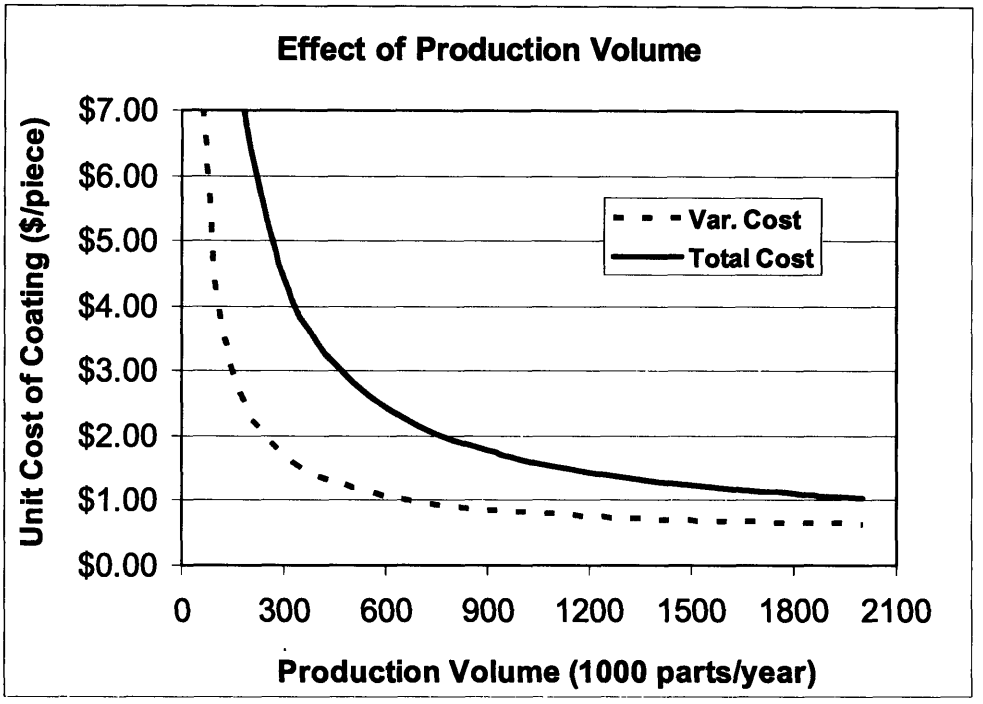

Figure 35 Graph of the Unit Cost of Coating a Tube versus Production Volume

From the evaluation of tube versus production volume, two values, $10.50 and $12.00, were chosen for the cost of the tubes. These values were used in the evaluation of 1-5 year and 5-10 year growth periods for the company. Following analysis for a small, medium and large-scale production facility, it was determined that a small scale manufacturing facility would be better. This choice was based on low, medium and high growth rates of 10, 20 and 30% respectively. Evaluation was performed based on an initial production volume of 550,000 units/year in a plant made for a small capacity of 3.5 million units/year. This size allows for excellent growth in the case that growth is high in both periods one and two. Figure 36 is a tree

diagram comparing the two selling prices ($10.50, $12) for the tube and how much revenue can be expected depending on the consumer demand (i.e. high, medium or low growth).

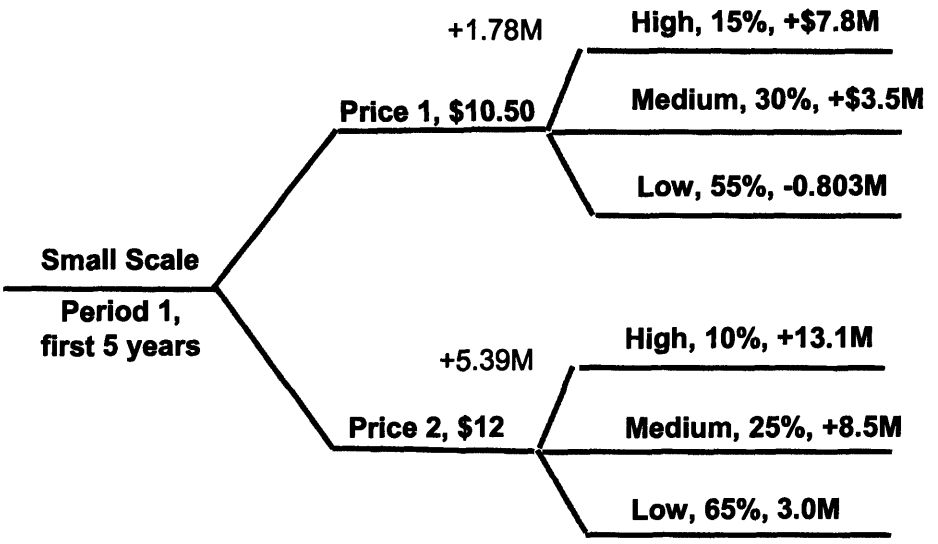

Figure 36 Tree Diagram of Period 1, Small Scale Production

The values located at the end of the branches are first the percent likelihood that high, medium or low growth rate occurs, and the second value is the expected revenue if high or medium or low growth rates occurred. The value written about "Price 1" and "Price 2" is the average expected value based on the likelihood and return gained for all three growth rates. The average value for Price 2 is significantly higher, $5.39 million, versus $1.78 million for Price 1. While both suggest a positive outcome, the higher price has a better return on investment. Whereas the low growth for Price 1 implies loss, the higher price did not lose money in any of the scenarios. Figure 4 then compares the two prices assuming we used the small scale facility and sold the tubes for $12/unit at the lowest growth rate. Again, it shows that the price of the tube should not be lowered, but should remain high in order to ensure monetary gain. Small scale production, selling tubes at $12/unit, will give an expected value of $5.23 million for the first five years.

Analysis for years 5-10 were performed under the assumption that small-scale growth occurred during the initial 5 years. A similar tree-diagram of this growth, for both price quotes, is shown in Figure 37. It shows that if the lower price were demanded over the first five years, the second five years has a negative average expected value. If the higher price ($12) were asked, the study suggests that regardless of a low, medium or high growth rate, the company will make money.

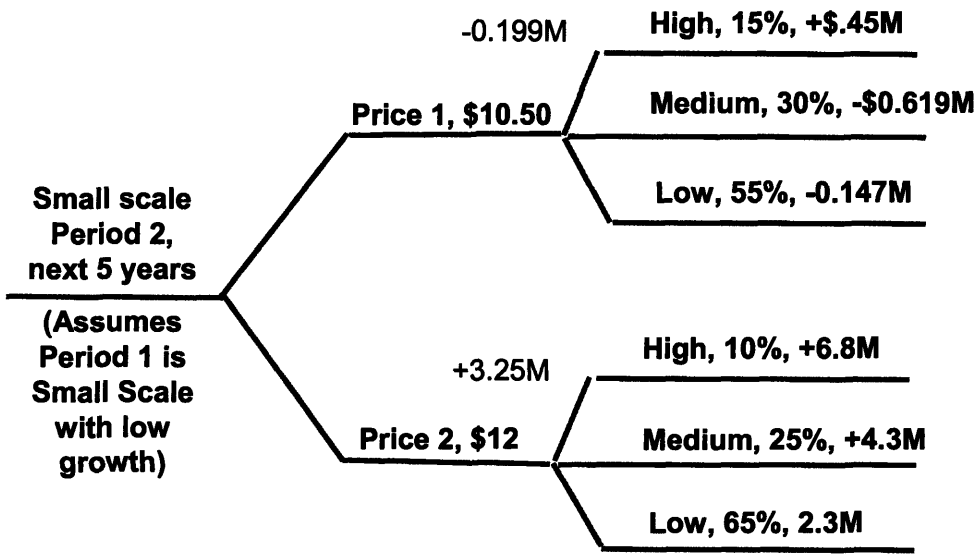

Figure 37 Tree Diagram of Period 2, Small Scale Production

Conservative growth rates were considered for this model in order to take into account the difficulty of entering the medical market as a small company. To ensure success, a small growth rate of 4% was used over the course of 10 years, giving an expected value of $3.5 million. The results for the $12 tube, shown in Figure 38, suggest that at the higher price, the company will gain money even at such a low growth rate, which is very encouraging.

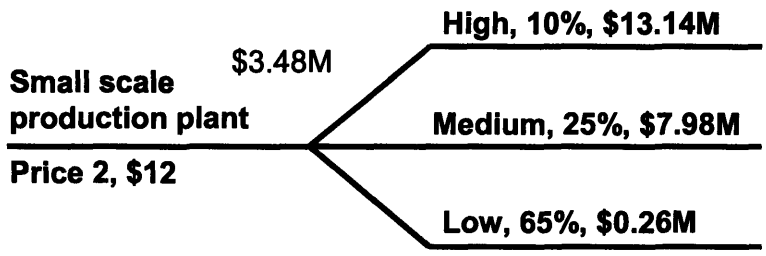

Figure 38 Tree Diagram of Period 1 Small Scale Production at Low 4% Growth Rate

The financial study of building a manufacturing plant suggests that any investment will not be lost as long the market share starts at 1.7% in year one, selling ~550,000 tubes. It does not, however, seem as readily marketable as licensing the technology to an existing manufacturer. Nonetheless, if each hospital uses an average 10,000 units/year, this would mean having our tubes used in 55 hospitals worldwide. Massachusetts, alone, has 70 different hospitals. With strong medical support, which this company will have through high-profile collaborations, infiltration into at least 55 hospitals worldwide is a feasible goal.

4.4.2. Future Business Development

Initial integration into the medical device industry may be difficult, since the company will not be in a strong position to compete with the large manufacturers to distribute these improved products. In addition to material science engineers and doctors, businesspeople with expertise in distribution negotiations will be key to the success of the company. To facilitate successful production of the medical devices and coatings, engineers with manufacturing expertise will be needed either as full-time employees or consultants. Factory laborers will also be needed in order to run the machines to create, package and prepare the devices for distribution.

Over time, significant amounts of revenue would potentially be retained for ongoing research and development were the manufacturing of a facility to be the course taken. One area of research would be to continue working on finding additional applications/uses for improved

materials for biomedical applications. If superior material coatings can be applied to other existing devices, the company will be able to create a large, superior product portfolio.

5. CONCLUSIONS

Endotracheal tubes (ETs) are used daily to ventilate patients for both long and short term periods of time. ETs are important in that they allow anesthesiologists and doctors the ability to provide artificial ventilation during surgery and emergency procedures. Damage in the airway passage as a result of ET intubation occurs frequently. Some studies claim significant damage occurs as frequently as 2-12% of all cases.[29] Patients who suffer from complications following ET intubations may feel the effect for the rest of their lives, particularly if it affects their breathing or speech skills. The improvement of ET properties is an area that has long been ignored. This thesis has provided reason to believe that a re-evaluation of the safety and effectiveness of current ETs should be performed.

The technical objective of this thesis investigated, *in vitro,* the interaction between epithelial cells and current poly(vinyl chloride)-based ET materials. The interaction testing provided reasonable proof that the cells that are in contact with the current ET material do, in fact, change morphology slightly and become more rounded in form. Overall, the size of the cells near the material gets smaller. The cells also grow in clusters on and near the material. The analysis of cell-material interactions performed for this work has provided evidence that epithelial cells do alter when in contact with ET material.

The ET samples from Columbia University demonstrated different cell-material interactions than the Rusch and Mallinckrodt samples. The cells, while alive, did not appear to proliferate over time. The average cell size was about half of the control cells. Whether or not

the Columbia samples are indeed an improvement over the current material will require further testing. Nonetheless, the analysis of this alternative material suggests that the epithelial cells do, in fact, react and grow differently when in contact with dissimilar materials.

The technical studies performed for this thesis suggest that further analysis is necessary to fully understand the cellular interaction with ETs. Additional testing should be performed on current ET materials, in addition to alternative materials that could potentially be used in ET manufacturing. Provided ample funding, dog or sheep studies should be performed using ETs made from both current and "potentially" improved materials. The epithelium lining could then be studied and compared between samples.

Following the discovery of an improved material, the economic analysis of the endotracheal tube market suggests that this is a potential area for success. The U.S. ET market is in excess of 32 million annually. The proposal for starting a company around a safer material for use in endotracheal tubes that was developed shows that there are significant barriers to entry for a small medical device company. However, the most appropriate approach for bringing the product to market, licensing, has the potential to earn enough revenue within the first five years to jump-start a medical device research and development company.

With further research and continual improvement in polymer study, a new ET may be developed—one with significant economic potential, but most importantly, a device with the potential to avoid the patient complications that arise from current endotracheal tube intubations.

6. APPENDIX

Exhibit A

Cell culturing/splitting instructions

1. Clean area with ethanol
2. Make sure all pipettes, flasks, etc. are sterile (autoclaved)
3. To split 1 cell line use 2 T75 flasks with canted lids
4. Coat each flask with 5ml prepared coating solution
5. Incubate 2 hours at 37°C
6. HBSS, BEGM media and trypsin-EDTA warmed in a 37°C H_2O bath
7. Put 15-20 ml new medium into each T75 flask without touching neck
8. Aspirate medium out of flask that contains cells to be split
9. First rinse cells by putting 5ml HBSS directly into bottom of flask
    a. Rock back and forth 10x to rinse
10. Aspirate HBSS wash with a new sterile pipette
11. Put in 1-2ml trypsin-EDTA to release cells
    a. Put on bottom and rock flask 2x
    b. Let sit 1-2 min. in incubator, give 10 knocks w/ hand
    c. Rock, knock, etc. until all cells are off
12. Add 10ml BEGM media to flask w/ trypsin & cells using new pipette
    a. Put liquid in on side of flask quite forcefully
13. Suck up media/cell mixture, wash out, suck up, wash out to break up cell to cell adhesions, avoiding bubbles
14. For a 1/3 split, put 4ml into the prepared flask w/ 15-20ml media.(for 1/4 split, put 3 ml, 1/10 put ~0.8ml)
15. Tighten caps on prepared flasks with cells, roll onto side of flask and shake a bit to allow cells to disperse
16. Put into incubator, loosen cap so CO_2 can get in and helps cells grow

Exhibit B

Freezing Cells

1. A freezing medium mixture is made by putting together the following and leaving in refrigerator:
    a. 4.025ml L-15 medium
    b. 0.05ml 10mM HEPES
    c. 0.05ml 1% PVP
    d. 0.5ml 10% fetal bovine serum
    e. 0.375ml 7.5% DMSE
2. Warm trypsin, HBSS, and BEGM media in 37°C
3. Aspirate media from T75 with cells
4. Rinse cells with 5ml HBSS, aspirate HBSS
5. Add 3ml trypsin-EDTA, coat cells, put in incubator 1-2 minutes
6. Knock flask several times to knock cells off the side of flask
7. Add 7ml BEGM media to make a 10ml total
8. Pipette out all 10 ml, put immediately into a 15 ml conical tube
9. Centrifuge at 4°C and 1500RPM for 5 minutes, a cell pellet should form at the bottom of the tube
10. Aspirate out liquid, careful not to touch the cell pellet
11. Add the 5ml prepared freezing media to tube
12. Vortex tube
13. Pipette 1ml into each freezing vial
14. Put vials in freezer for 2 hours
15. Transfer vials to -60°C freezer overnight
16. Next day: put vials into Liquid Nitrogen Freezer

Exhibit C

Thawing Cells

1. Coat T75 flask for 2 hours at 37°C with normal coating solution, warm BEGM media
2. Place vial with frozen cells in water bath at 37°C

3. Aspirate coating solution, add 10ml of fresh medium to T75 flask
4. Remove vial with cells from the water bath and pipette in and out of vial 2 times
5. Place cells in the T75 flask with medium making sure the tip of the pipette is in the medium when adding the cells
6. Place the flask on its side in the incubator, rock gently back and forth a few times, and then loosen cap
7. One day later, replace the 10ml of BEGM media with 20ml of fresh BEGM media