

Aerosol Delivery of Mammalian Cells for Tissue Engineering

by

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1.0 Introduction.....	4
2.0 Materials and Methods.....	14
2.2 Cell Lines	14
2.2.1 Bovine Chondrocytes.....	14
2.2.2 Porcine Epithelial Cells.....	16
2.3 The Gelling Agent.....	17
2.4 The Sprayer.....	18
2.5 The Model Trachea.....	19
2.6 Trachea Coating Experiments.....	20
2.7 Cell Viability.....	20
2.8 Cell Spraying Experiments	21
2.9 Culturing Sprayed Cells	22
2.9.1 Bovine Chondrocytes.....	22
2.9.2 Culturing Epithelial Cells	24
3.0 Results and Discussion	26
3.1 Spray Coating Experiments	26
3.2 Cell Spraying Experiments	36
3.2.1 Chondrocyte Experiments.....	36
3.2.2 Epithelial Experiments.....	38
3.3 Culturability Experiments.....	40
4.0 Summary, Conclusions, and Future Work	62
4.1 Bovine Chondrocytes.....	62
4.2 Porcine Epithelium.....	62
4.3 Chondrocytes Versus Epithelium	63
4.4 Phenotype Testing.....	63
5.0 Bibliography	64

1.0 Introduction

Every year over 20,000 [3] people die as a result of being in a fire.

Although flames have the biggest visual impact, it is usually the smoke produced by the combustion of natural and synthetic materials that causes more damage and claims more lives. The main constituents of smoke, both the particulate matter as well as the hot and toxic gasses, are devastating to the tracheal and lung tissues. The damage caused to the lung and trachea by inhaling this smoke can increase a fire victim's susceptibility to infectious disease significantly [1]. Between 20% and 50% of people who suffer inhalation injury contract pneumonia due to the weakened status of their body's defenses [2] and between 4,800 and 6,400 [1] people die from either pneumonia or other complications. Despite the importance of the inner-lining of the trachea to a burn victim's health and survival, current treatments consist of keeping the patient in a clean environment, supplying fresh oxygen, keeping the airways open, and letting the patient's body heal itself [1]. This treatment is not so much an active healing mechanism; rather it is a passive means of allowing the body to repair itself.

The main goal of this work is to develop a minimally invasive technique that will replace lost cells on the inside surface of the trachea as efficiently as possible, actively healing the patient's injury. Ideally, the patient would receive a single treatment and then make a complete recovery on his or her own. The main challenge lies in delivering an even layer of intact cells to the inner-surface of the trachea in such a manner that they will stay in place and will replace the damaged or missing tissue. The overall approach is to spray a suspension, composed of epithelial cells in an aqueous solution of Pluronic F-127 polymer, onto the trachea using a jet atomizer. Because Pluronic F-127 solutions can

be liquids at room temperature but gels at body temperature, the role of the polymer will be to immobilize the cells onto the tracheal surface long enough for them to attach and grow.

The trachea is a long, hard tube of cartilaginous rings that starts at the throat, in front of the esophageal opening, and runs down into the bronchi which open into the lungs (Figure 1.1). The trachea is 1.90cm to 2.54cm in diameter and approximately 10.16 cm long, and is generally larger in the male than female. The outside of the trachea is ridged while the inside of the trachea is smooth and lined with ciliated epithelium.

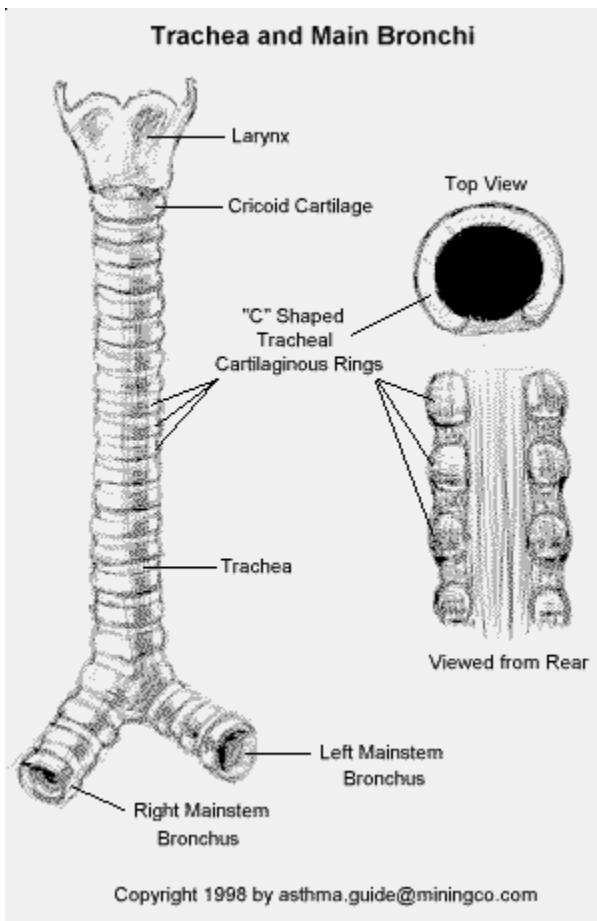


Figure 1.1: A diagram of the male trachea showing the dimensions of the opening and branches for the bronchi. [4]



Figure 1.2: A cross-section of columnar epithelium showing the features of the inner-trachea surface. [5]

The ciliated epithelial cells illustrated in Fig. 1.2, fulfill two main roles in the body's defense system. First, they provide a direct, physical barrier against bacteria and other foreign objects that enter the trachea with the air inhaled in normal respiration. In addition, the cells are endowed with cilia, small hairs that can move. Thus, the cilia can transport mucous from the lungs, together with encapsulated foreign particles and bacteria, up to the mouth [18] where it can be swallowed or expectorated. Without a functioning tracheal lining the body's defense against airborne disease and contaminants is severely hindered, making it more susceptible to infection.

The key biotechnological advance that makes the work in thesis possible is *in vitro* culture of mammalian cells. *In vitro* culture of mammalian cells only became common 20 years ago, and not until about 10 years ago did actual application of tissue engineering get a real start with the pioneering work of doctors Joseph and Charles Vacanti [19]. One of the reasons for this is the difficulty of duplicating the environment of the body for the cells to grow in. In addition to keeping the cells warm and supplied with air there is also the difficulty of preventing infection by bacteria or molds and the difficulty of feeding them the right combination of nutrients and growth factors to make them behave as they would within the body. A number of studies have investigated optimal culture conditions and media compositions for tracheal epithelial cells [23, 24].

A second important enabling technology is the development of block copolymers such as Pluronic F-127. This polymer, developed by BASF, is biocompatible [8, 9, 10, 14] and has had extensive experimental trials performed on it in the field of tissue engineering. The basic make-up of Pluronic copolymers is a three block structure of ethylene oxide, propylene oxide and ethylene oxide as shown in figure 1.5

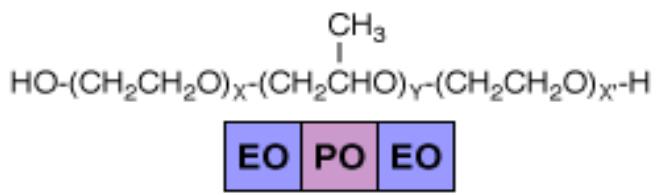


Figure 1.5: The block structure of a typical Pluronic co-polymer showing the building blocks of the structure [16].

As illustrated in the phase diagram in Fig. 1.6, Pluronic F-127 is a liquid at low temperatures and concentrations and gels as the temperature and concentration increase.

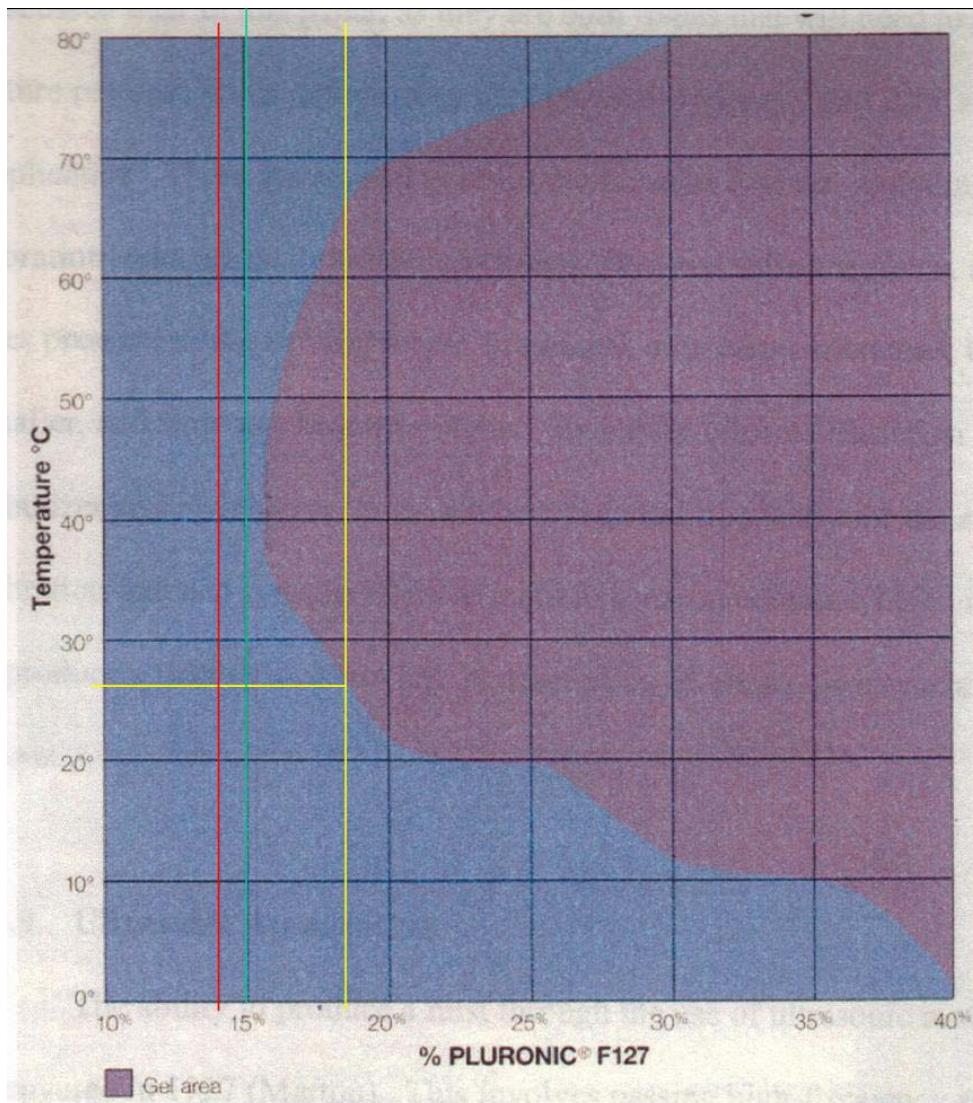


Figure 1.6: The phase diagram for Pluronic F-127 copolymer in water showing gellation at body temperature and above at a concentration of a little more than 15%wt. and above. Lines are drawn at 12%wt. and 15%wt. to indicate solutions used in this work as well as a line at 18% to show where a known gel boundary occurs [16].

For the work in this project, however, the standard phase diagram is not appropriate since cells mixed in a water solution die of osmotic shock. Therefore, all Pluronic F-127 mixtures made for this work were mixed in PBS or Phosphate-Buffered

Saline. The salts present in PBS directly affect the gellation phase boundary of the polymer. Although experiments were not carried out to determine the precise shift in the phase diagram, in general, Pluronic dissolved in PBS gelled at lower concentrations and cooler temperatures than Pluronic dissolved in water. These observations are supported by the work done by Susan Roberts and her group at UMass Amherst who studied the effect of various solutes on the phase-boundary of Pluronic F-127 [20]. The phase diagram for Pluronic mixed in MEM (Modified Eagle Medium, Gibco) is given in figure 1.7.

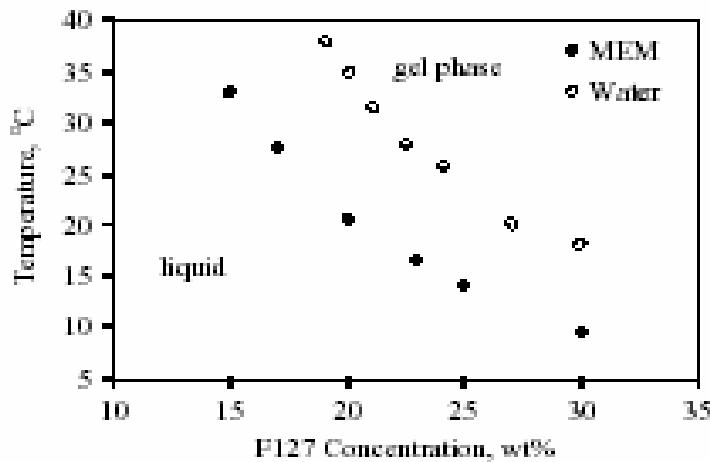


Figure 1.7: Phase diagram for Pluronic F-127 mixed in modified eagle medium showing a shift of the gellation boundary to lower temperatures and lower concentrations [20].

Pluronic F-127 has been used as a hydrogel scaffold for various applications in tissue engineering, most notably as a way to design cartilage formations such as ears or nipples *in vivo* in plastic surgery [8, 9]. Experiments have also been done with epithelial cells in a Pluronic scaffold [17] *in vivo*. *In vitro* experiments have been performed using fibroblast cell-lines with an aerosol-generating device [15]. Now experiments have been

performed on chondrocyte and epithelial cell lines in combination with an atomizer and Pluronic F-127.

Current medical technology allows doctors limited access to the trachea through the throat. Without cutting the patient open, the trachea can be sealed from the lungs with an endotracheal tube and an operation within the trachea can be observed and lit with a laryngoscope. Given the space limitations it is difficult to fit both a laryngoscope in and a cell-deposition device into the trachea. It would, therefore, be beneficial to find a device that can reliably deposit cells on the inner-surface of the trachea without need for observation during the procedure.

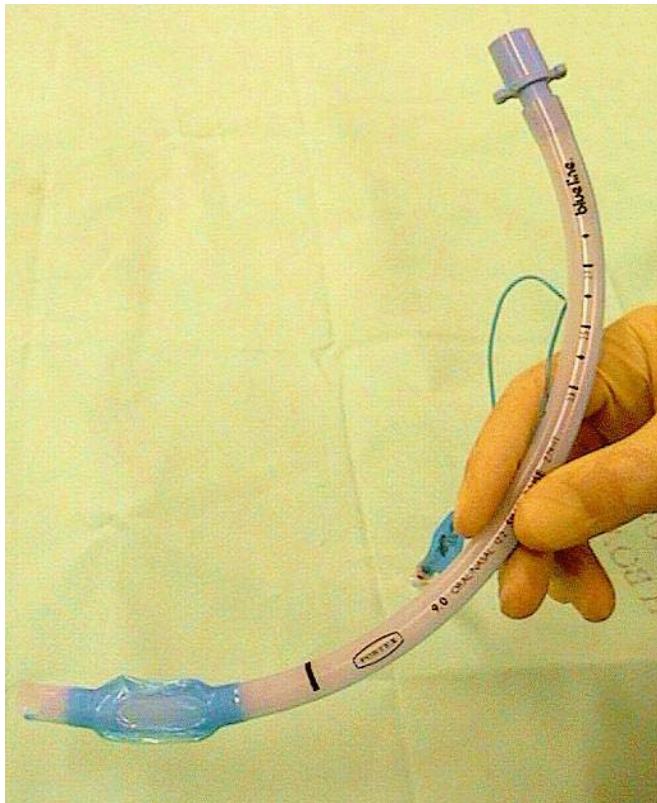


Figure 1.3: A picture of a typical endotracheal tube showing the inner-tube that allows respiration while the blue balloon is inflated to close off the lungs. This prevents respiration through the trachea while the operation is occurring. [6]

Most cells are too large to be transported any distance in a small tube by a gas stream. Thus, to deliver cells to the trachea efficiently as a spray, one must produce the aerosol at the entrance to the trachea, just beyond the vocal chords. An ideal sprayer would transport a liquid stream with cells and an air stream to the deposition site, keeping both streams separate until the point of aerosolization.

This work first developed methods for working with Pluronic and an aerosolizing nozzle so that flowrates for both a liquid and air flow rate could be determined that would coat a model trachea. Various model tracheas were experimented with, giving a final

model that optimally fit the parameters. Testing then began on the bovine chondrocyte cell line with both viability and in-vitro culturability assays followed by a test on the target cell line, porcine epithelium. The methods of the experiments are presented in section 2.0, the results of those experiments are presented in 3.0 and the final conclusions from the work as well as avenues of future research are presented in section 4.0. Section 5.0 lists the resources used in this paper.

2.0 Materials and Methods

2.1 Medium formulation

Two medium formulations were used in these experiments. The base medium was prepared by mixing 500 mL of Ham's F-12 media, 5 mL antibiotics/antimycotics (AB/AM, 100 X), and 50 mL Fetal Bovine Serum (FBS, 10X). All of these components were ordered from GIBCO. Both the FBS and the AB/AM were stored in a -20°C freezer until they were thawed and added to the F-12 media. This was the medium used to culture bovine chondrocytes. To culture porcine epithelial cells, three additional components were added to the base medium. Insulin 500 μ g/mL (Sigma-Aldrich) was stored in the refrigerator at 4°C and 5 mL of the stock solution were added to 500 mL of F-12 media giving a 2X final concentration of insulin. Two other components, transferrin (Sigma) and epidermal growth factor (EGF, Peprotech) were stored in the -20°C freezer. On the day the cells were fed, the last two components were thawed and added to the medium at levels of 1mL at 100X per 100 mL of medium to make a final concentration of 1X within the medium. Medium preparation was carried out under sterile conditions

2.2 Cell Lines

2.2.1 Bovine Chondrocytes

Bovine chondrocytes were harvested from the joint cartilage of a fresh calf shoulder (Research 87, Hopkinton, NY) that was refrigerated until use. The person collecting cartilage donned sterile surgical gloves and a facemask. The shoulder was placed on a sterile cloth on a countertop that had been disinfected by spraying with 70%

ethanol. The joints of the shoulder were covered in iodine and the muscles and tendons that hold the joints together were severed with a size 10 scalpel. With the joint exposed, slices of cartilage less than 0.3 cm thick and 0.6 cm in diameter were cut with a new disposable scalpel and placed into a vial containing a sterile solution of PBS, antibiotics, and antimycotics. The AB/AM was at 1X concentration in PBS (one 5mL 100X aliquot in 500mL of PBS) the vial was prepared in a Biosafety 2 laminar flow hood to ensure sterility.

After evacuating the first joint of all cartilage (the surface was red indicating no more cartilage existed and bone was being scraped), a second joint was harvested using a new scalpel and a second vial. Once both vials were filled with the appropriate amount of cartilage they were each washed 3 times with a sterile PBS solution under a laminar flow hood.

To wash the cartilage samples the vials were shaken vigorously for 20-30 seconds. The solution was then aspirated using a Pasteur pipette. If cartilage clogged the pipette, removing the suction often cleared the blockage. Tapping the pipette against the inner wall of the vial to shake the flake loose also helped otherwise a new and sterile Pasteur pipette was used. Once the original PBS was removed from the vial, 40mL of fresh sterile PBS was added to each vial and the cleaning procedure was repeated. If the cells were not immediately digested and used, ~ 40mL of F-12 media with antibiotic/antimycotic solution was added to the vials and they were then stored in a refrigerator at 4°C.

To digest the cartilage ~20 mL of F-12 media (no FBS or AB/AM) and 0.06g collagenase were added to the sample vials to yield a solution containing ~ 0.3%

collagenase. Collagenase dissolves the matrix that binds the cells together and yields the free chondrocyte cells. Digestion was done in sterile conditions. The Falcon Tubes containing the cells and collagenase were tightly capped and covered in parafilm [22]. A shake flask incubator at 37°C was used for the digestion. The time required to digest the cartilage depended on the thickness of the cartilage slices. Approximately 10 – 12 hours was enough to digest most of the large pieces 0.6cm by 0.3cm, while 8 – 10 hours was required to digest the thinner 0.4cm by 0.2mm pieces. Digestion times that are too long will kill the cells, while digestion times that are too short will not yield enough free cells. In the current experiments, 10 hours of digestion resulted in close to 100% free cells with 90 – 100% viability.

The digested cartilage was spun down in a centrifuge for 5 – 7 minutes at 2000rpm to produce a large cell pellet at the bottom of the Falcon Tube. The collagenase solution was removed and 10 mL of the base medium were added. The suspension was mixed on a vortex mixer and 90 µL of the cell suspension was placed in a centrifuge tube and mixed with 10 µL of Trypan blue stain (obtained from Fisher). The capsule was sealed, mixed using a vortex mixer and, finally, 10 µL of the cell suspension was placed in a haemocytometer to both count the number of cells and determine cell viability. Trypan Blue is an exclusion dye that does not stain living cells. It can, however, penetrate the cellular membrane of dead cells. A batch of cells was only used if at least 90% of the cells were viable.

2.2.2 Porcine Epithelial Cells

Porcine epithelial cells were harvested from a pig trachea using sterile techniques similar to those described above and placed in the appropriate growth medium (Sec. 2.1). The cells were grown for several weeks in T-175 or T-225 flasks (Fisher), and passaged at weekly intervals to ensure that the cells maintained the epithelial phenotype and that they had enough room to grow. To passage the cells, 10mL (20 mL) of trypsin was added to each T-175 (T-225 flask) flask, the flasks were placed on a rotary shaker at a speed of 100rpm at 37°C for 10 minutes, and the degree to which cells were freed from the bottom of the flask was monitored with a microscope. The free cells and trypsin from up to 4 flasks were aspirated into a 50 mL Falcon Tube and sterile PBS was added to bring the total volume up to 50mL. The tube was centrifuged for 7 minutes to form a cell pellet, and the trypsin – PBS solution was aspirated leaving the cell pellet intact. F-12 media (5 mL) was added to the cell pellet, and the solution and cells were mixed by aspirating both into the pipette, and then depositing them back into the tube several times. The content of all of the Falcon tubes was combined, diluted with an equal volume of F-12 medium and counted in a haemocytometer to determine the cell concentration. New flasks were seeded at a level 560,000 cells/225cm².

2.3 The Gelling Agent

Pluronic F-127 was used as the gelling agent in the spray experiments. To produce a solution with the desired concentration, a known weight of Pluronic powder was dissolved in a known volume of PBS. The density of PBS was taken as 1 g cm⁻³. All solutions sat over night in the lab refrigerator at 2-8°C. For the culturing experiments the

Pluronic powder was weighed onto a weight boat and placed in a laminar flow hood for five minutes before being mixed with sterile PBS under the hood.

2.4 The Sprayer

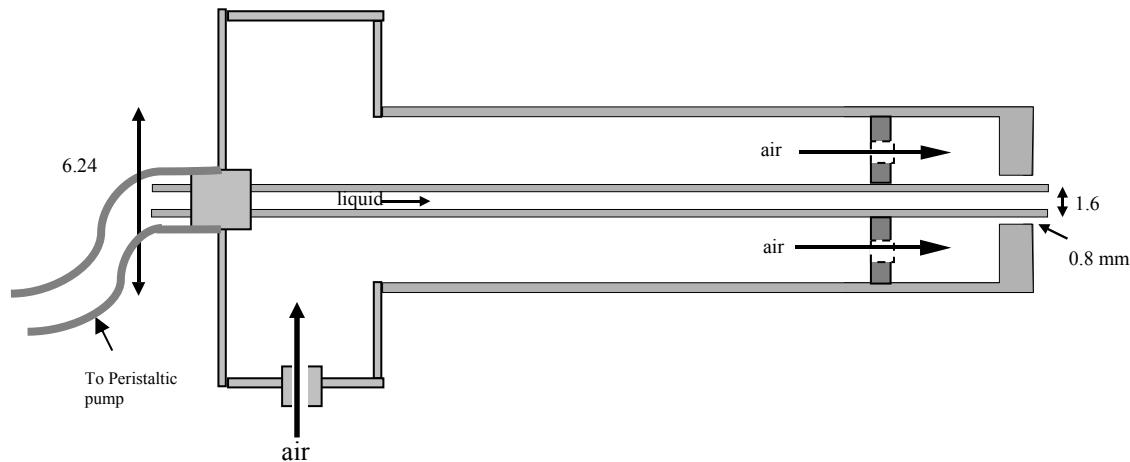


Figure 2.1: Schematic of Liquid and Air Flows in Nozzle

The sprayer, illustrated schematically in Fig. 2.1, was a simple concentric tube design. The liquid flowed through the inner 1.6 mm (1/16in) diameter stainless steel tube and the gas flows through the outer $\frac{1}{4}$ inch steel pipe. One end of the outer pipe was capped and the inner tube passed through the 0.126 in hole drilled through the center of the cap leaving only a 0.08 cm gap between the outer wall of the inner tube and the inside of the hole in the cap. The centering piece, close to the cap, kept the inner tube centered while still allowing the gas to flow freely. A brass manifold at the other end of the sprayer connected the air supply to the outer tube and centered the other end of the inner tube.

Figure 2.2 illustrates the complete sprayer setup. The peristaltic pump on the left feeds the liquid to the inner tube through 0.8 mm Viton tubing. The brass manifold is in the middle of Fig. 2.2. Model trachea A, described below, is to the right. A rotameter

controlled the flow of air that came from a house air supply or a N₂ tank. A 0.2 µm filter located upstream of the nozzle, prevented particles from entering the sprayer. Most spray experiments used a liquid flow-rate of 2mL/min and an air flow-rate of 6Lpm.

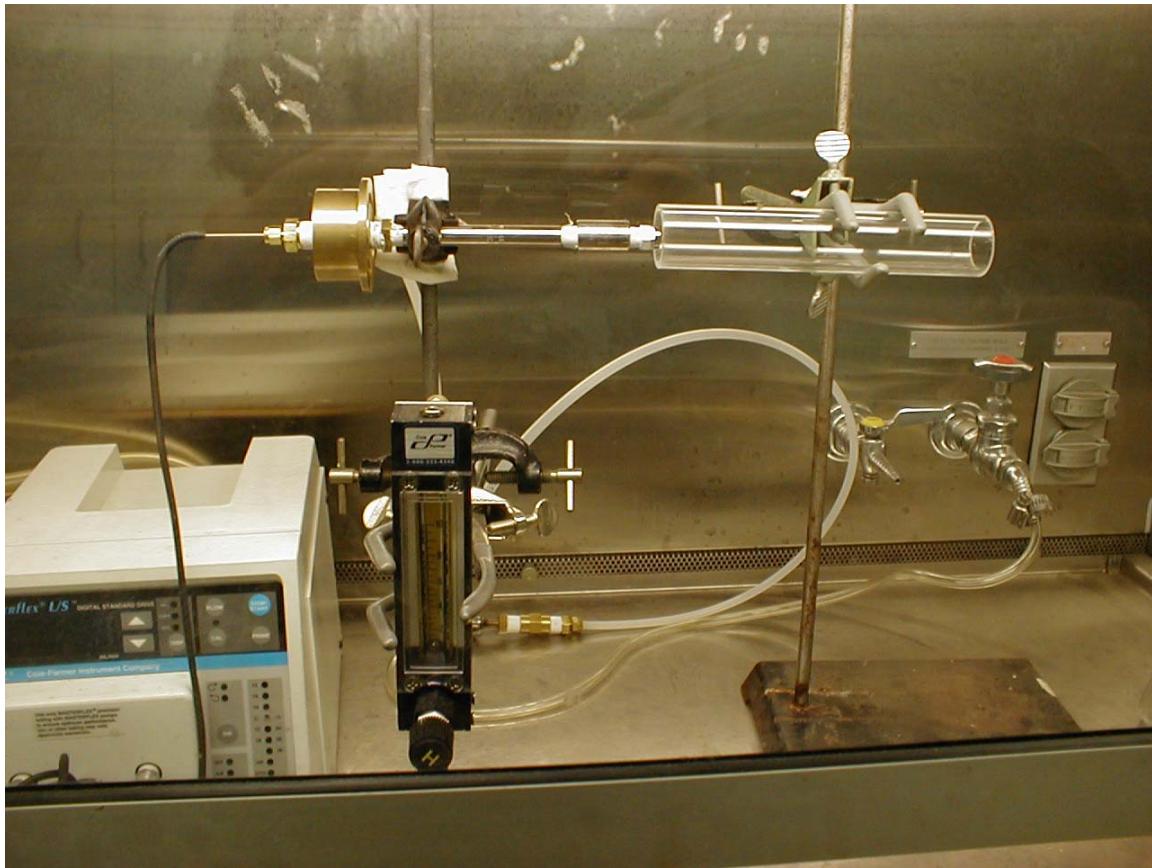


Figure 2.2 A photo of the typical sprayer set up showing the pump, nozzle and air and liquid lines.

2.5 The Model Trachea

Two model tracheas were constructed. The first, model trachea A, was a clear acrylic tube 16.5 cm long with an inner diameter of 2.54 cm and a smooth inner surface. The initial 6.35 cm of the tube represented the length of the mouth and the opening into the trachea. During a medical procedure, a balloon may be placed at the end of the

trachea to prevent cells from entering the lung. To simulate the effect of the balloon, a rubber stopper wrapped in Teflon tape was placed into the end of the tube.

The second model trachea, model trachea B illustrated in Fig. 3.1.2, had the same inner dimensions as trachea A but also had a heat exchanger shell on the outside. Water circulated through the gap between in the two tubes and maintained the inner surface of trachea B at 37°C.

2.6 Trachea Coating Experiments

The distribution of Pluronic deposited on the inside of the model tracheas was examined in two ways. The first was to examine the deposited film by eye. In these experiments the solution was dyed to improve the contrast. In the second method, a polypropylene folder (Staples office supply) was cut to the correct size, weighed, and placed tightly against the inside wall of the model trachea. The polymer solution was sprayed, and then the plastic was removed, weighed, and cut into 2.54cm wide axial segments. Each segment was weighed and the sum of the individual weights was compared to the total weight determined earlier. Some of the samples were also freeze-dried after spraying to eliminate the effect of water evaporating from the samples after spraying but before they were weighed.

2.7 Cell Viability

Cell viability was determined using the L-3224: LIVE/DEAD® Viability/Cytotoxicity Kit for animal cells (Molecular Probes in Eugene OR, <http://www.molecularprobes.com>). Each kit contained two 40µL vials of Calcein AM

4mM in anhydrous DMSO and two 150 μ L vials of Ethidium Homodimer-1 2mM in DMSO/H₂O 1:4 (v/v). The chemicals were stored in sealed containers at -20°C to prevent degradation of the reagents. Since Calcein AM is susceptible to hydrolysis, contact with the atmosphere was minimized as much as possible. Given the limited sample sizes in the current experiments, only 0.5mL of dye was mixed for one day of testing.

For each reagent a concentration of 1 μ M was effective for staining the bovine chondrocytes. Mixing 0.25 μ L of Ethidium Homodimer and 1 μ L Calcein AM into 0.5mL of PBS yielded the desired concentration. Only 10 μ L of the dye was required per microscope slide and 20 minutes of incubation at room temperature ensured that the cells absorbed enough dye to determine which cells were dead or alive under a fluorescent microscope. The fluorescent microscope used here could not simultaneously make both the both the LIVE and DEAD dyes fluoresce, thus composite images were analyzed in Photoshop (versions 6 and 7). Dye concentrations were increased with time as the reagents degraded.

If a cell fluoresces red it was assumed to be dead even if it also fluoresced green. A section of each microscope slide (upon which cells had been sprayed and subsequently stained) was placed under the fluorescent microscope and exposed to the fluorescent light at wavelengths of between 494-517nm and 528-617nm (to make the cells fluoresce green and red respectively). Photos were taken at 100X magnification.

2.8 Cell Spraying Experiments

The sprayer was not sterilized to conduct the cell spraying experiments. A suspension containing 2 million cells/ mL was made using 5 mL of a polymer solution. The cell suspension was placed in a 50mL Falcon Tube on a Styrofoam tray to the left of the sprayer. The tube from the peristaltic pump was placed in the Falcon tube at least one cm below the surface but not touching the bottom of the vial. The apparatus was run at 6 L/min air and 2 ml/min liquid. After completing a spray experiment, the system was flushed with water to prevent the tube and sprayer from clogging. If the inner tube of the sprayer became a sterile hypodermic needle was used to clear it.

2.9 Culturing Sprayed Cells

For either cell line, Pluronic solutions were filter-sterilized using a $0.22\mu\text{m}$ syringe filter (Millipore) before being mixed with the sterile cell pellet. To sterilize the sprayer, each component was gas sterilized in a separate bag. The bags were sprayed with 70% ethanol in water and the parts withdrawn using latex gloves. The sprayer was, however, reassembled in the lab rather than in a laminar flow hood or other sterile environment. After assembly, the sprayer was run with dry air to maintain a filtered, sterile air flow past the nozzle tip and prevent any airborne contaminants depositing on the tip.

2.9.1 Bovine Chondrocytes

Bovine chondrocyte cells were sprayed onto 6-well plates and each well was sprayed between 0.5 and 3 seconds. The wells are covered with 5mL of the appropriate medium, the plate was covered, and the covered plates were incubated at 37°C with 5%

CO₂. During the first set of experiments, medium was changed and photos were taken upon spraying, after 7 days and after 11 days. In later experiments the medium was changed more frequently (2 or 4 days). Controls consisted of cells sprayed in PBS and unsprayed (pipetted) samples of cells in Pluronic solution or PBS. The volume of sample pipetted on to the plates was calculated by measuring the weight of the sprayed samples and assuming that the density of the sprayed samples was 1 g cm⁻³. Typically, 7 plates were prepared for each concentration of Pluronic and for the PBS control. The three top wells of each 6 well plate were sprayed with the chondrocytes suspension for one second, while and the bottom three wells were seeded by pipetting the corresponding suspension.

Every two days the plates to be harvested were visualized via phase contrast microscopy (Nikon TE 200, Microvideo Instruments, Milford, MA) and photomicrographs were recorded at 100X using a digital camera (Spot Jr., Microvideo Instruments, Milford, MA) The media was aspirated from the plates that were not to be harvested, and a fresh 5 mL of media was added to each of the wells, and the plates were returned to the incubator. The media was then aspirated from the plates to be harvested, 1.5mL of trypsin was added to each well, and the plates were put in a shaking flask incubator for 10 minutes. The plates were banged fifteen times after five minutes and an additional fifteen times after ten minutes to help release the cells. Cell release was confirmed by observing the samples under the microscope. Any cells that were still attached were freed by scouring each well with a sterile cell-scraper for ten seconds by hand. The cell and trypsin suspension from each well was then pipetted into a labeled microcentrifuge tube and placed in a micro-centrifuge for 4 minutes with the hinges of the tubes facing out. The tubes were then removed and the trypsin removed from the cell

pellet via a two-stage micro-pipette aspiration. A 1000 μ L micro-pipette was used to remove most of the trypsin followed by a 90 μ L pipette to remove the trypsin close to the cell pellet. For very low cell concentrations the cell pellet was invisible and so the only guideline to prevent removing the pellet was to aspirate from the opposite side of the hinge. Enough PBS (\geq 90 μ l) was added to each micro-centrifuge tubes produce a cell concentration low enough to measure with the haemocytometer.

2.9.2 Culturing Epithelial Cells

Porcine tracheal epithelial cells were detached from the surface of the T-225 flasks by use of 20mL of 0.05% Trypsin and at least 10 minutes of incubation in the shake flask incubator. They were then collected into a single, sterile, 50mL Falcon tube. After a small sample (100 μ L) was diluted and placed in a microcentrifuge tube it was stained with 10% Trypan blue and the cells were counted using a haemocytometer using the average of four large squares. The 14mL of cells in suspension in F-12 media were then either re-suspended in PBS and Pluronic and used in the spray culturing experiment or were used to seed 10 new T-225 flasks or they were discarded. Each new flask was seeded with 560,000 cells (slightly higher than a typical cell culture such as for chondrocytes [7]) and 30mL of media (Gibco/Invitrogen guidelines).

The cell suspension for the spray experiments was evenly divided between two Falcon tubes, under sterile conditions, and spun down into pellets. The media was aspirated and the cell pellets were resuspended into 7 milliliters of Pluronic or sterile PBS to form concentrations of 2 million cells/mL. The cell suspensions were then either sprayed or pipetted onto 6-well plates as described in the bovine chondrocyte experiments,

with the exception that twice as many plates were sprayed. Each well was covered with 5 mL of the appropriate medium, covered and placed in a 37°C incubator. Every two days, 4 plates were photographed and harvested, while the remaining plates were fed with fresh media. The harvested cells were then counted using the method outlined in section 2.9.1 with the exception that the cells were placed in the microcentrifuge for 7 minutes instead of 4.

3.0 Results and Discussion

3.1 Spray Coating Experiments

A fundamental assumption in this work is that the distribution of sprayed cells will mirror that of the Polymer. Thus, before conducting experiments with cells, it was important to conduct initial spraying experiments in order to (1) demonstrate that the sprayer can deposit an even coating Pluronic on the inner surface of the model trachea, (2) demonstrate that the gel forms quickly enough to avoid excessive draining from the top of the model trachea to the bottom, and (3) to determine which operating parameters are most critical for producing an even spray coating. These experiments also investigated the effect that the presence or absence of a “balloon” at the end of the trachea had on the spray pattern. Finally, some tests included a drying step to determine if this would help lower Pluronic concentrations gel adequately. Tables 3.1.1 and 3.1.2 summarize the experiments that were conducted and the method of analysis that was used to characterize the coating. The parameters that were varied included the Pluronic concentration, the spraying procedure, and the method used to evaluate the spray coating quality.

Table 3.1.1: A summary of the spray coating experiments conducted with model trachea

A. For these experiments, the spray appeared to favor the top middle of the tube, therefore two changes in the spraying technique were implemented. First, the model trachea was rotated 180° about the z-axis between sprays. Secondly, the nozzle tip was inserted half-way down the model so that the back-end of the model would receive more Pluronic. The end of the trachea was open to the atmosphere for all of these experiments and some Pluronic spray could be seen leaving the end of the model.

Model Trachea A

Test	Pluronic Concentration	Spray Procedure	Method	Observations
A-1	12% wt. in water	30 seconds at entrance	visual	Pluronic stuck to the middle top of the model and ran down after several minutes
A-2	12% wt. in water	15 seconds at entrance, 15 seconds rotated 180° at entrance	visual	Pluronic appeared to more evenly coat the first two-thirds of the model
A-3	12% wt. in water	7 seconds at entrance, rotated 180° sprayed for about 7 seconds, inserted half-way down and repeat	visual	Pluronic appeared to coat the inside of the tube evenly
A-4	12% wt. in water	30 seconds at entrance	weight	Pluronic weight too light, could not get firm, quantitative results

Table 3.1.2: A summary of the spray coating experiments conducted with model trachea

B. The end of the trachea was plugged for all of these experiments, changing the flow pattern enough that rotating the nozzle was no longer necessary.

Model Trachea B

Test	Pluronic Concentration	Spray Procedure	Method	Observations
B-1	12% wt. in water	15 seconds at entrance, 15 seconds inserted half-way down the model	visual w/dye	Pluronic appeared to evenly coat the inner-surface with some puddling at the bottom
B-2	15% wt. in water	15 seconds at entrance, 15 seconds inserted half-way down the model	visual w/dye	Pluronic appeared to evenly coat the inner-surface with less puddling at the bottom
B-3	15% wt. in PBS	15 seconds at entrance, 15 seconds inserted half-way down the model	weight	Pluronic weight lost in transport to scale. Ratios used, however visual evidence differed
B-4	15% wt. in PBS	15 seconds at entrance, 15 seconds inserted half-way down the model	weight	Pluronic freeze-dried, too light, could not weigh accurately compared to polyethylene folder

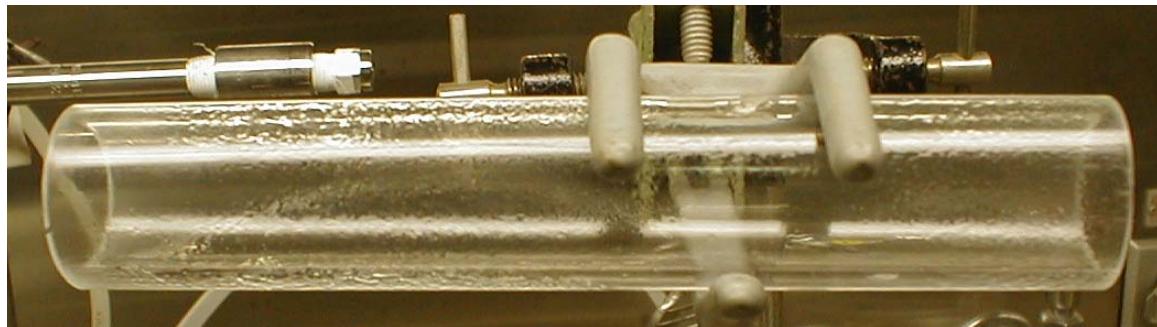


Figure 3.1.1: Model trachea A showing a layer of 12%wt. Pluronic through the inner-surface with greater amounts in the middle than the two ends.

Figure 3.1.1 illustrates the results of spray experiment A-3. Initial experiments showed that Pluronic was sticking and gelling to the inside walls of model trachea A, but over the course of several minutes about a 20% of the Pluronic drained from the top and

the walls of the trachea and formed a puddle on the bottom. The spraying procedure necessary to get an even coat in trachea A over the course of 30 seconds required a 180° rotation of the trachea after approximately 7 seconds, an insertion of the tip of the nozzle to the midway point of the trachea after 15 seconds, and a final rotation after about 22 seconds.

To better model deposition onto a human trachea and the presence of a balloon at the end of the trachea, several experiments were conducted using model trachea B. Adding dye to polymer solution also made it easier to see the polymer through the heat exchange shell. Figures 3.1.2 and 3.1.3 illustrate two of the experiments completed using Trachea B.

As expected, the warmer surface helped gel the Pluronic shortly after it contacted the surface. An additional, and unexpected, improvement was due to the presence of the stopper at the exit of the trachea. Because the aerosol was now forced to return via the entrance of the tube, the droplets flowed back closer to the walls and resulted in a more even coverage that had been possible without the stopper. It was, therefore, no longer necessary to rotate the trachea to ensure an even coating. As illustrated in Fig. 3.1.2, puddling still occurred for a 12%wt. Pluronic solution, but was less severe for trachea A.

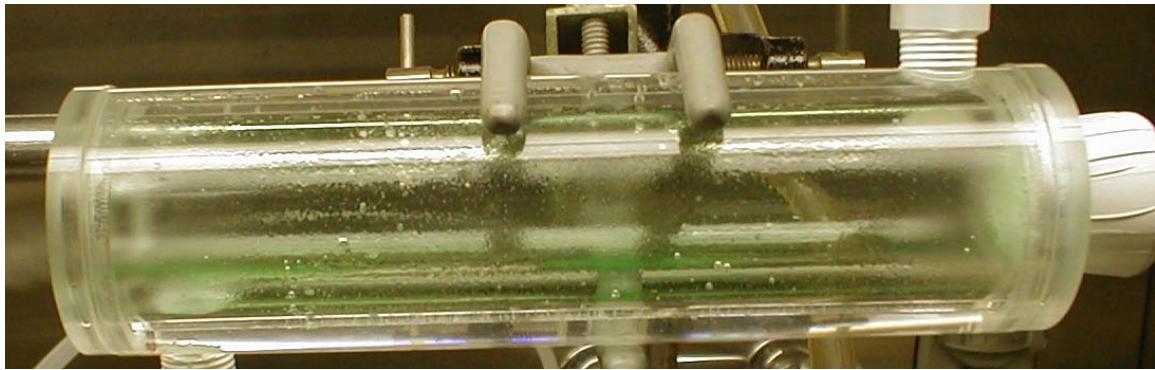


Figure 3.1.2: Model trachea B sprayed was sprayed with a 12% solution of Pluronic and the coverage appeared good despite some puddling which can be seen as a dark green line within the model above.

Several experiments were therefore conducted with 15%wt. polymer solutions. Although the 15%wt. solutions dried more quickly, puddling was still similar to the 12%wt. solution. Higher concentrations were not tested as they became increasingly viscous and more difficult to work with. Up to 14 minutes of drying (running the sprayer with no liquid flow) was found to be very helpful in gelling the Pluronic. Both 12% and 15% wt. solutions of Pluronic were entirely gelled after the drying procedure.

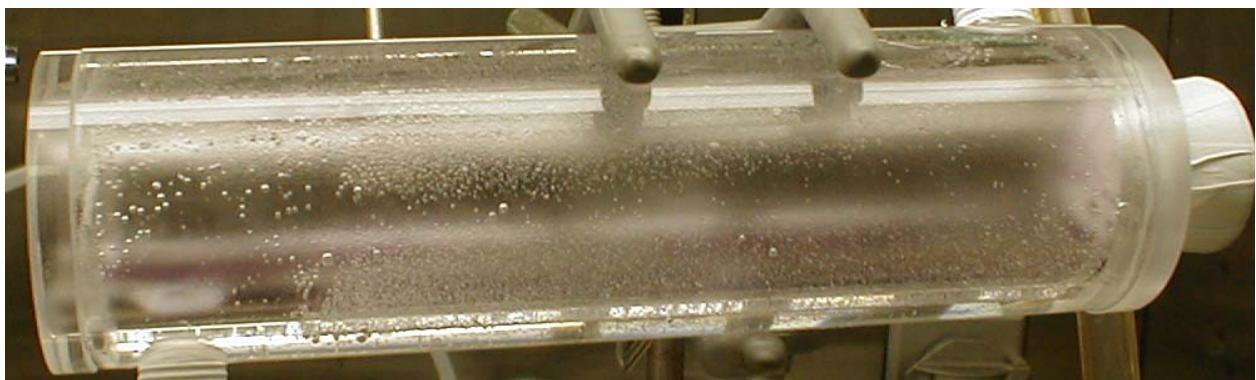


Figure 3.1.3: The model was also sprayed with a 15%wt. solution of Pluronic which was found to gel somewhat faster, although the tell-tale dark-purple line shows that some puddling still occurred.

In an attempt to quantify polymer deposition, experiments were conducted to weigh the polymer that was deposited on a polypropylene folder using the techniques outlined in section 2.6. In most cases these experiments were inconclusive. When the film was freeze dried to remove water, there was so little Pluronic on each section of film that slight deviations in the size of the pieces changed the weight more than the mass of Pluronic on the section of film. Alternatively, when the Pluronic layer was weighed while wet, the sum of the weights of the individual pieces of film did not agree with the initial weight of the coated film due to continued evaporation of water. In summary, although Pluronic was not deposited solely on a single part of the tube, it was not possible to quantify deposition more accurately than by visual inspection.

To investigate whether the cell distribution was uniform with respect to the sprayed polymer, cells were suspended in a 12% Polymer solution, dyed with the Live/Dead Kit assay, sprayed onto a thin polypropylene folder, and photographed under a fluorescent microscope as well as under room light. Typical results are illustrated in Figs 3.1.4-3.1.7. By comparing the fluorescent and phase photographs, it is clear that the regions with high concentrations of green cells correspond to areas with higher concentrations of Pluronic.



Figure 3.1.4: A 100X fluorescent photo of epithelial cells demonstrates that most of the cells survive spraying.

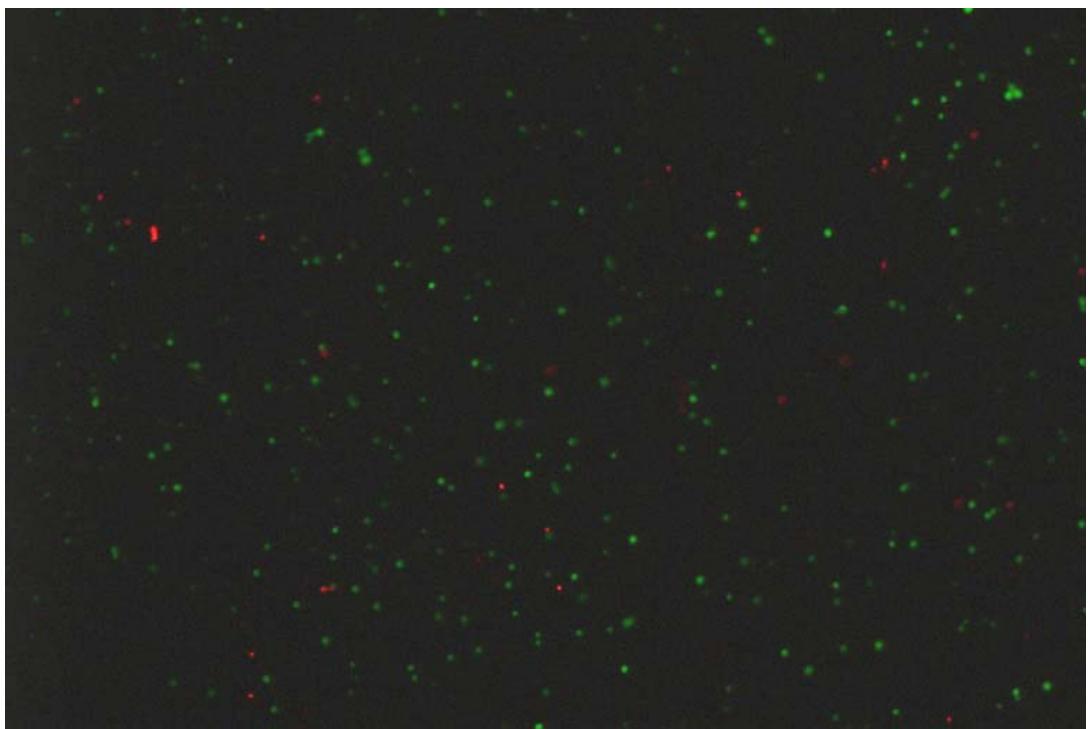


Figure 3.1.5: A 100 X fluorescent photo of chondrocyte cells after spraying demonstrates that most of the cells are alive.

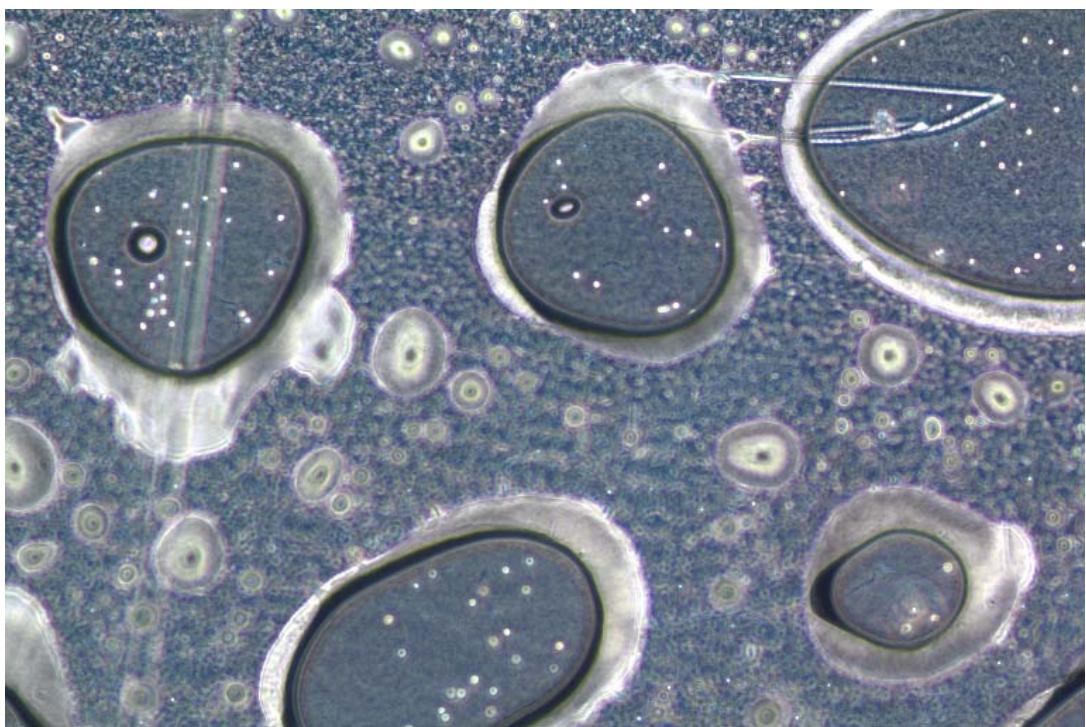


Figure 3.1.6: A phase I photo of chondrocytes in Pluronic showing the cells within the Pluronic droplets (100X).

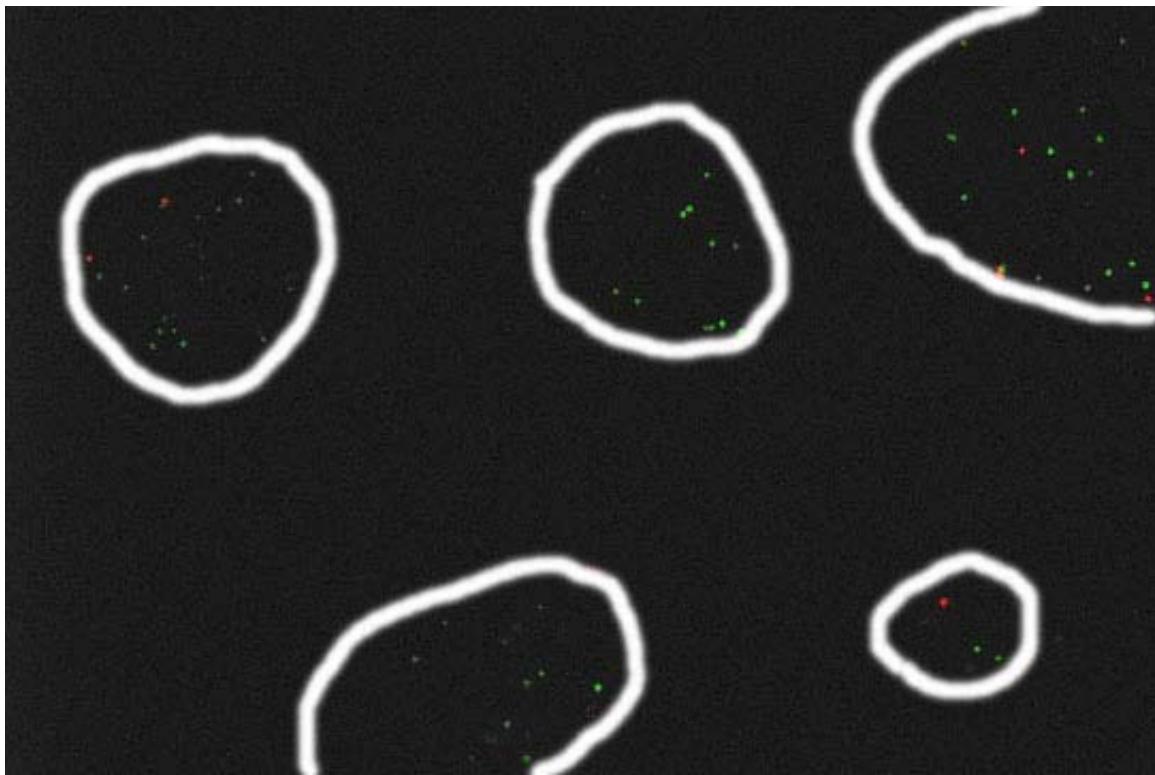


Figure 3.1.7: Fluorescent photo of the cells within the droplet showing the locations of the living and dead cells in comparison to the phase 1 picture (100X).

3.2 Cell Spraying Experiments

Although Fig 3.1.5 illustrates that most of the chondrocytes survived spraying, additional experiments were conducted to better quantify the results and to determine whether cell viability depended on the air flowrate through the sprayer. Increasing the flow rate of air through the nozzle should increase the shear at the liquid gas interface and could decrease cell viability.

3.2.1 Chondrocyte Experiments

Chondrocytes suspended in a 15% wt. Pluronic solution were sprayed onto uncoated microscope slides at a constant liquid flowrate of 2mL/min and air flowrates that varied between 4 and 9Lpm. The lower flowrate corresponded to the minimum level required to produce a stable spray. Flow rates that are too high could reduce the deposition of the droplets, and could also lead to higher pressures inside the trachea.

Figure 3.2.1 illustrates a LIVE/DEAD composite photo for chondrocytes that had been sprayed with a liquid flowrate of 2mL/min, an air flow-rate of 6Lpm.

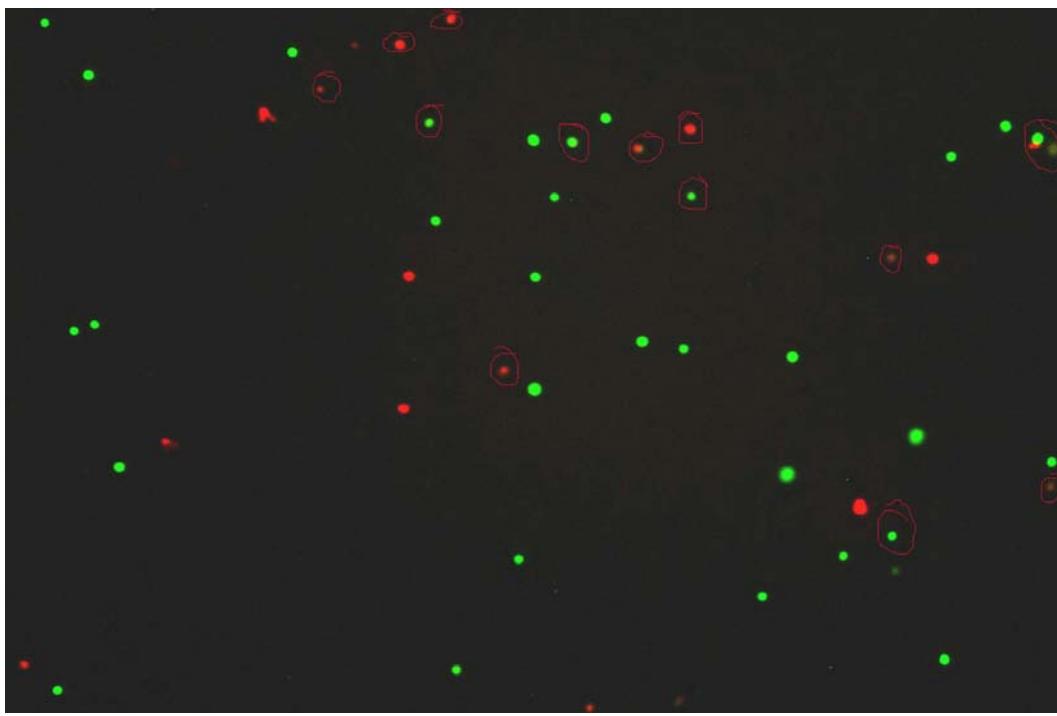


Figure 3.2.1: An example of a composite image of live and dead cells created in Adobe Photoshop showing living and dead cells (100X).

As always, cells that fluoresced red were assumed dead even if they also fluoresced green, since cells will only fluoresce red if the cellular membrane is broken. Because cells fluoresce green as long as esterase is produced (Molecular Probes Assay Product Description), cells with broken membranes can occasionally fluoresce green even though they are no longer viable. The red or green dots that are also outlined in red in Fig. 3.2.1, correspond to cells that fluoresced both red and green. Photos were typically taken at 100X magnification and cells were counted to determine the percentage of viable cells in each photo. Table 3.2.1 summarizes the results of all of the tests that were conducted.

Table 3.2.1. The percentage of viable cells on single photos of 15% wt. Pluronic sprayed with chondrocytes

Air Flowrates	5/23/2002	6/6/2002	6/14/2002	6/21/2002
9L/min		(undetermined)	71%	(undetermined)
8L/min			66%	76%
7L/min			75%	80%
6L/min	32%-36%	42%	71%	76%
5L/min			77%	83%
4L/min		78%		83%

A preliminary experiment on 6/6/2002 suggested that chondrocytes had much higher viability when the air flowrate was reduced from 6Lpm to 4Lpm. However, when more extensive experiments were conducted, 6/14/2002 and 6/21/2002, using chondrocytes harvested independently from two different calf shoulders, cell viabilities were essentially independent of the air flowrate and very similar to each other. One reason for the difference between experiments may be the quality of the harvested cells. For example, if cells are digested for longer than necessary, the action of the collagenase may weaken the cell membrane. The important conclusion that can be drawn from the data in Table 3.2.1 is that chondrocytes are able to survive spraying quite well. Even if only 30% survived, it should still be possible to deliver enough viable cells to a damaged trachea by increasing the overall cell concentration. Further experiments could be conducted to determine whether there is a key step in the cell preparation process that influences the viability of the cells to spraying, but this was not considered an important point in this stage of the research.

3.2.2 Epithelial Experiments

Spray viability experiments were conducted with two different concentrations of epithelial cells by spraying cells previously incubated in LIVE/DEAD stain onto plastic folders. Experiments with epithelial cell concentrations of 166,666 cells per mL and 2 million cells per mL both demonstrated that most of the cells survived spraying. In these experiments the Pluronic concentration was lowered to 12% wt. to attempt to reduce shear stress. Photos of the cells corresponding to these experiments are illustrated in Figs. 3.2.2 and 3.2.3.

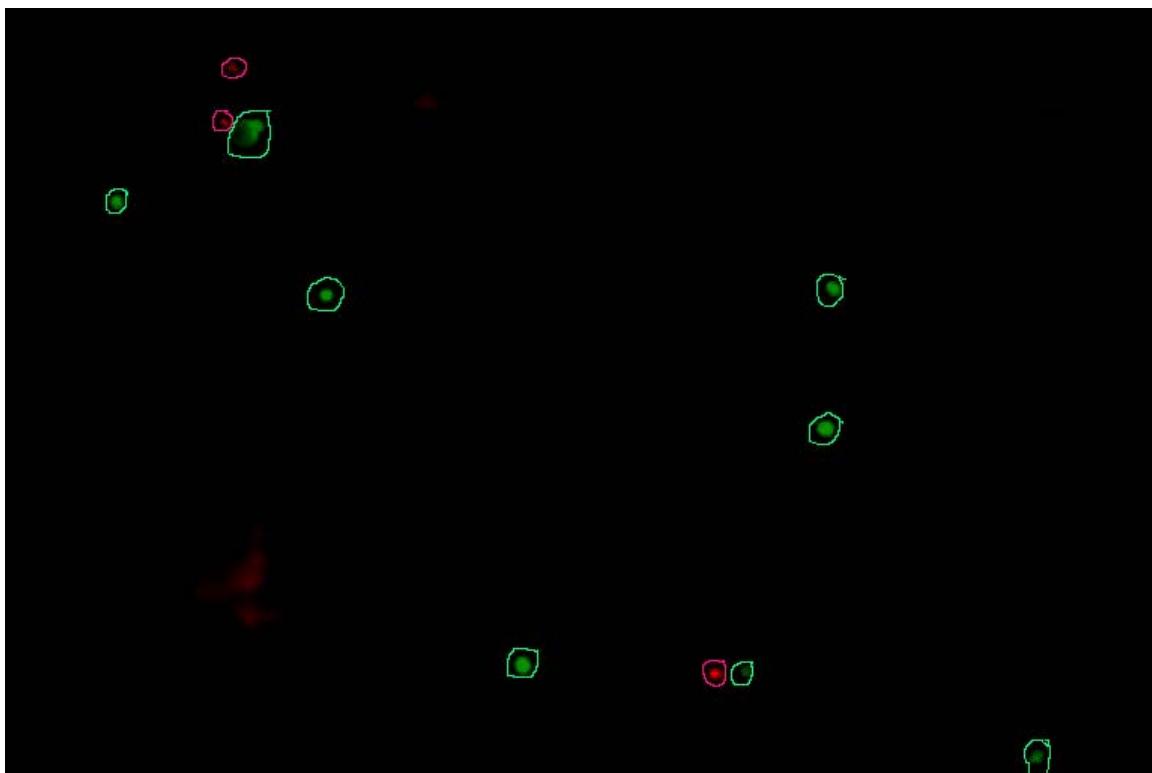


Figure 3.2.2: A LIVE/DEAD epifluorescence photo showing a majority of living cells at a low concentration of epithelial cells in Pluronic 12% wt. (500X)

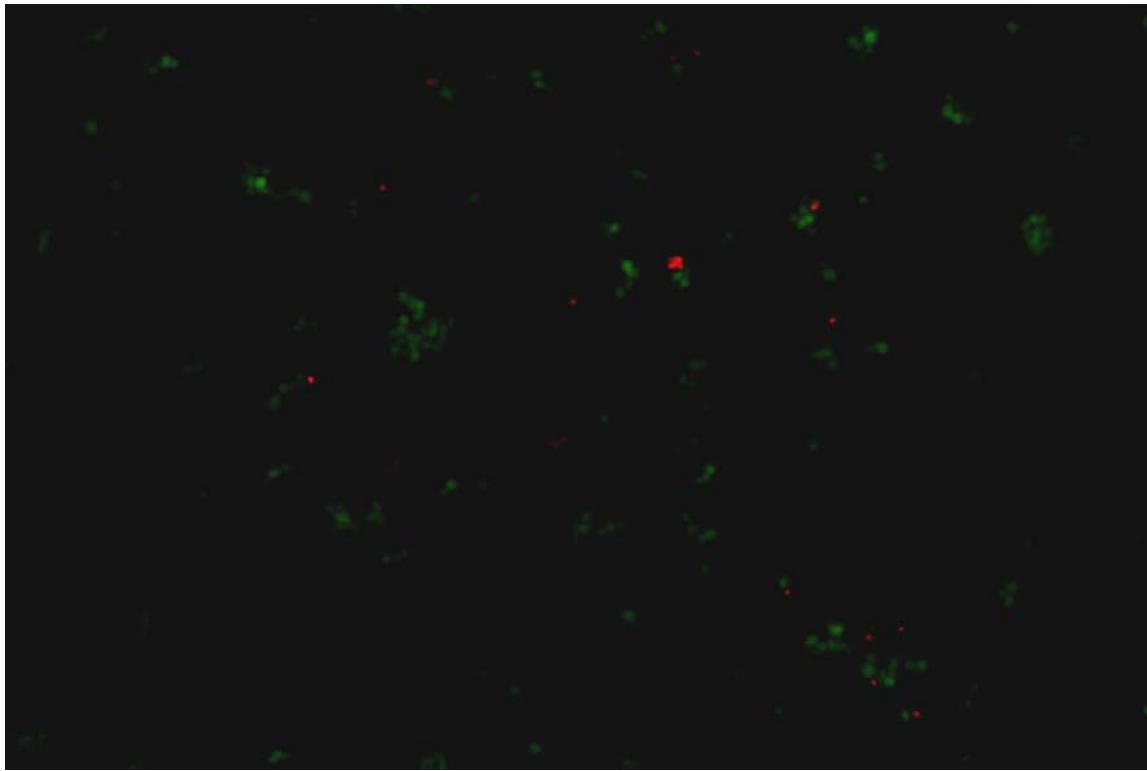


Figure 3.2.3: A LIVE/DEAD epifluorescence photo showing a majority of living cells at a high concentration of epithelial cells in Pluronic 12% wt. (100X)

3.3 Culturability Experiments

Although the cells survived the initial spraying, this was no guarantee that the cells would reproduce and maintain their normal phenotype. The next step, therefore, was to conduct cell culturing experiments with cells that had been sprayed. A typical test for culturability is to place the cells to be cultured in a 6-well plate. For mammalian cell culture the plates must be coated with proteins to aid in the adhesion of the cells to the plate. Once cells adhere to a plate they grow to confluence with nutrient medium changes every few days.

In total, 5 culturability experiments were conducted with chondrocytes and 2 with epithelial cells. Table 3.3.1 and table 3.3.2 summarize the experimental conditions

including the number of plates harvested each time, the feeding and observation schedule, and comments. The goals of these experiments were to ensure cells maintained phenotype, to determine the average growth rates and doubling times, and to ensure that cells grew to confluence.

Table 3.3.1 The results of the 5 main chondrocyte experiments showing the Pluronic concentrations in each experiment, the days on which media was changed, and the observations. Through these tests the best growth was discovered when the least amount of Pluronic was used, as shown in C-5.

Bovine Chondrocyte Experiments

Test	Pluronic Concentration	Media Changing Frequency	Method	Observations
C-1	15% wt. in PBS	Day 7 and day 11	visual	Cells from both the 1-2 and 2-3 second groups grew to confluence by day 11
C-2	15% wt. in PBS	Every 4 days	counting	Phenotype appeared consistent, cells grew to confluence, but more slowly than in C-1
C-3	15% wt. in PBS	Every 2 days	counting	Results were similar to those of C-2
C-4	12% wt. in PBS	Every 2 days	counting	Results were similar to those of C-3 but the doubling times were faster
C-5	Pure PBS, 10%wt., 12% wt., 15% in PBS	Every 2 days	counting	The lower the Pluronic concentration, the better the initial seeding density, growth was similar to C-2&3

Table 3.3.2 The results of the 2 epithelial cell experiments showing the Pluronic concentration used, the media changing frequency and the observations in both experiments. The only classical exponential growth was found with the pipetted cells in PBS.

Porcine Epithelium Experiments

Test	Pluronic Concentration	Media Changing Frequency	Method	Observations
E-1	12% wt. in PBS	Every 2 days	counting	Cells in PBS both sprayed and pipetted appeared to grow well, those in Pluronic did not
E-2	12% wt. in PBS	Every 2 days	counting	Cells pipetted in PBS grew well, cells sprayed in PBS appeared to grow near the end, cells in Pluronic did not grow

In the preliminary qualitative test, experiment C-1, cells were sprayed onto 6-well plates and left to grow with media changes on days 7 and 11 (after initial seeding). Figure 3.3.1 illustrates the changes in cell density of the cultures as a function of time. Each well was fully confluent by the end of 11 days of culturing. Although the wells sprayed for 2-3 seconds had more Pluronic and, therefore, more cells deposited in them than the wells sprayed for 0.5-1 seconds both reached confluence in the same number of days. All future experiments, therefore, used a standard spraying time of one second per well. No infections were present and the shape of the cells suggested that the cells retained their phenotype.

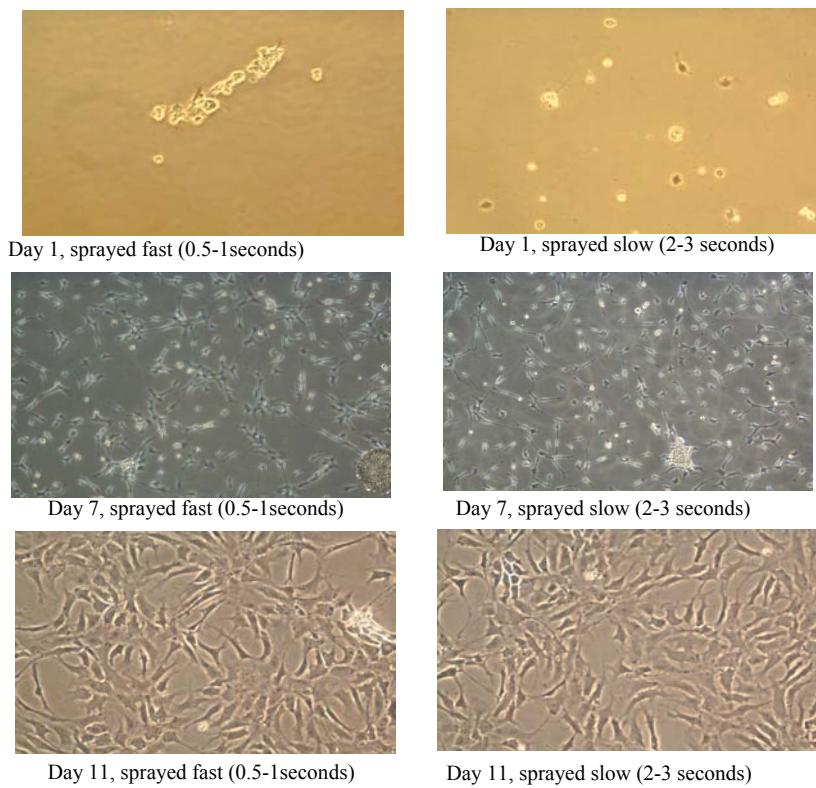


Figure 3.3.1: A series of photos demonstrating the cell density and phenotype of bovine chondrocytes sprayed in a 15%wt. solution of Pluronic for 0.5-1 or 2-3 seconds over the period of 11 days. All wells reached confluence by day 11.

Figure 3.3.2 shows the growth trends of the first quantitative experiment, experiment C-2. This experiment was conducted as a “proof-of-concept” in order to test the cell harvesting and counting methods outlined in section 2.9.1. Here, medium was changed every four days and a 15% solution of Pluronic was used to suspend the cells in to be sprayed. Cells were counted as described in section 2.9.1.

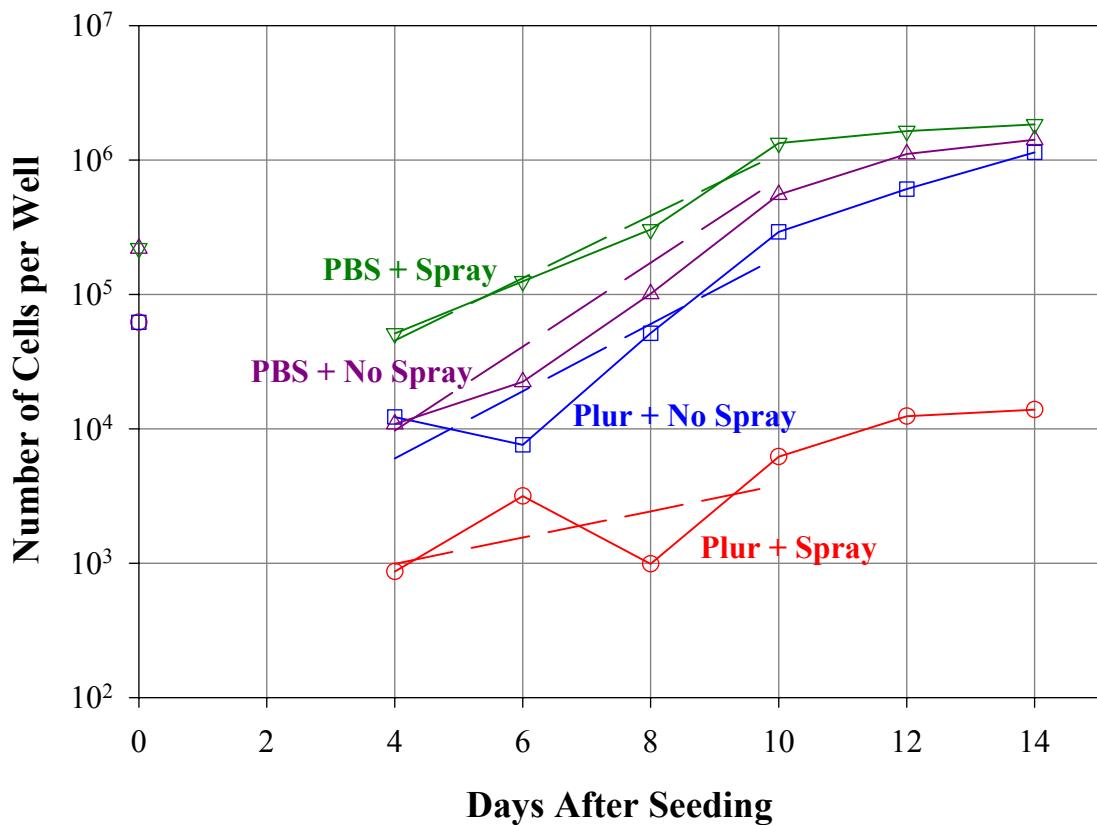


Figure 3.3.2: Cell growth of bovine chondrocyte during experiment C-2. Cells were both sprayed and pipetted in a 15%wt. solution of Pluronic onto 6-well plates. The numbers for the PBS controls sprayed and pipetted are also shown. Strong growth can be seen in all conditions but cells sprayed in Pluronic. The dashed lines are growth curves fit to the log-linear plot.

Figure 3.3.2 shows the data taken from each day that the cells were harvested and counted. The dashed lines in Fig. 3.3.2 are the growth curves calculated by fitting a straight line to the data on a log-linear plot. The x-axis represents the number of days since the cells were seeded onto the 6-well plates and the y-axis is a logarithmic scale of the number of cells per well. The data points on the graph are the average of the two

wells that were counted for each condition on each day. The initial concentration, day 0, is estimated to be 220,000 cells/well for the PBS conditions and 62,000 cells/well for the Pluronic conditions. After the first 4 days, the number of cells/well decreased by as much as a factor of 100 from the initial number of seeded cells and cells seeded from the 15% Pluronic solution appeared to fare worse than those seeded from PBS. The initial decrease in cell number is typical for mammalian cell cultures where cells are unable to multiply unless they are attached to a surface. Although bovine chondrocytes are robust enough to survive in floating in the media for several days without adhering to a substrate and producing an extra-cellular matrix or ECM [21] even these cells will eventually die or be removed from the well when old media is aspirated off and replaced by fresh media. Photos confirmed that the initial adhesion of cells was low for the first several days. By day 8, the strong upward trend of the data demonstrates that cell growth occurred under each of the conditions tested. Since cells did eventually grow under all of the conditions, it was postulated that the biggest effect of Pluronic was to inhibit the cells from adhering to the plate surface.

In the exponential growth region, the slope of the growth curve can be used to calculate the doubling times for cell growth, and in this experiment doubling times ranged from 23 to 79 hours. The doubling times for all of the experiments are summarized in Table 3.3.3. The r^2 values are at least 0.36 for the growth curves, with the lowest value corresponding to the sprayed Pluronic experiment. One reason for this low r^2 value could be that the average number of cells per well was calculated using the data from only two of the three wells, and there can be significant differences from one well to the next. Nevertheless, experiment C-2 demonstrated that bovine chondrocytes

grow after being sprayed, and that our cell counting methods gave growth results that appeared reasonable.

Table 3.3.3 A summary of the bovine chondrocyte doubling times indicating some variation, but typically between 20 and 30 hours, corresponding well with literature. Experiments C-4 and C-5, at low Pluronic concentrations, had the fastest doubling times, indicating the fastest growth curves.

Bovine Chondrocyte Growth Times

Test	Pluronic Concentration	Deposition Method	Doubling Time
C-2	15% wt. in PBS	Sprayed	74 hours
		Pipetted	29 hours
	PBS	Sprayed	23 hours
		Pipetted	31 hours
C-3	15% wt. in PBS	Sprayed	29 hours
		Pipetted	33 hours
	PBS	Sprayed	28 hours
		Pipetted	37 hours
C-4	12% wt. in PBS	Sprayed	19 hours
		Pipetted	16 hours
	PBS	Sprayed	20 hours
		Pipetted	18 hours

In experiment C-2, media was changed only every 4 days. The standard cell culturing procedure is to change the media in the 6-well plates every two days [10] (or 3 in some literature [11, 12, 13]) and, therefore, for the remaining experiments media was changed every two days. More frequent media changes ensured fresh antibiotics/antimycotics were in the media to prevent infection and to provide a fresh batch of nutrients to the cells. Figure 3.3.3 illustrates the results for experiment C-3, the first experiment where media was changed every 2 days.

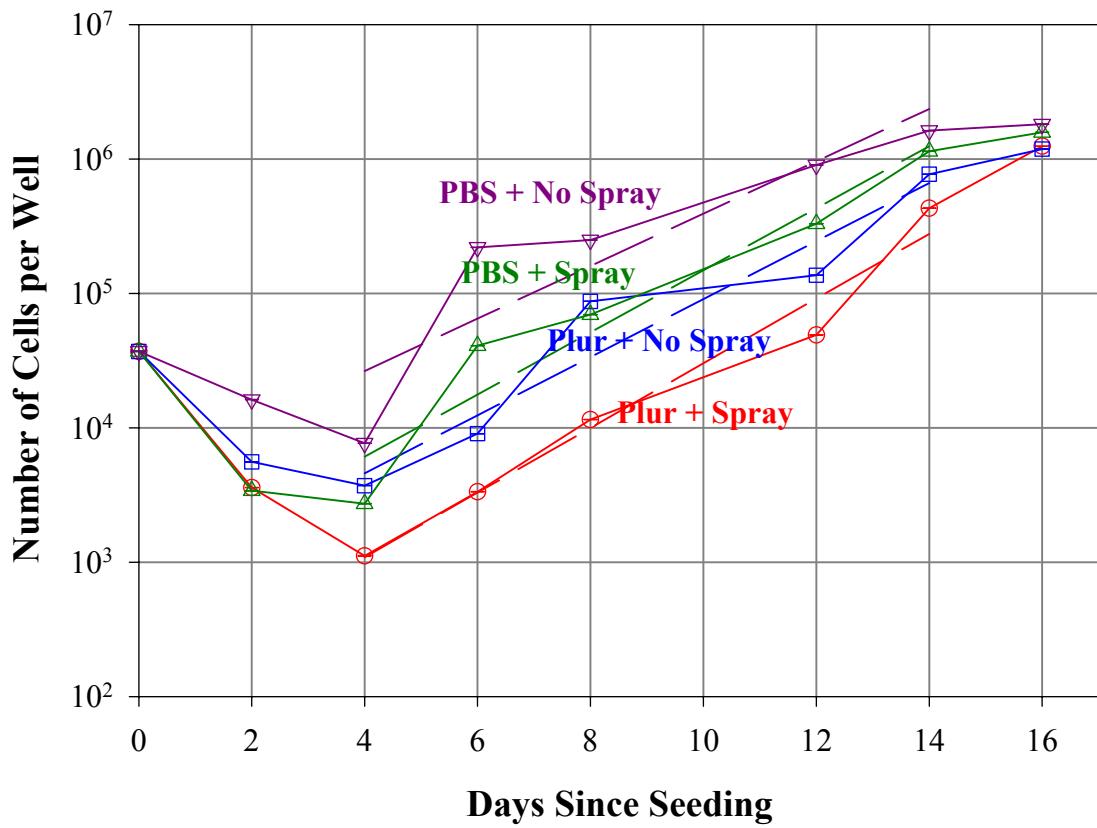


Figure 3.3.3 A 15%wt. solution of Pluronic was used to spray cells in suspension onto plates which were then counted every two days. Media was changed in the remaining plates on those days. Growth trends appear to be very similar between the 4 different sets of conditions; the doubling times range from 28 days to 37 days giving a range of only 9 days difference between the slowest growth (sprayed cells in Pluronic) and the fastest (sprayed cells in PBS).

Experiments C-2 and C-3 are excellent examples of the difference a change in the cell culturing procedure can make. The main difference between these experiments was the feeding time, and increasing the feeding frequency changed some of the curves dramatically. In C-2 the cells that were sprayed from the 15% Pluronic

solution were unable to grow as fast as the other cells. In C-3, the data from the cells sprayed from Pluronic solution lie just below the data from cells that were pipetted from Pluronic solution. Furthermore, once the cells began to grow, the slopes calculated in the exponential growth region are quite similar and the doubling times all lie between 28 and 37 hours. The main difference appears to be rate at which cells are initially lost from the wells between the initial seeding and day 4, and this is most likely due to initial adherence of the cells to the plate. Comparing the experiments conducted with Pluronic and those conducted with PBS, it appears that Pluronic inhibits cell adherence.

To test this theory, the Pluronic concentration in experiment C-4 was reduced to 12%wt. In addition, to improve the statistical accuracy of the data, the cells from all 3 wells were counted each day.

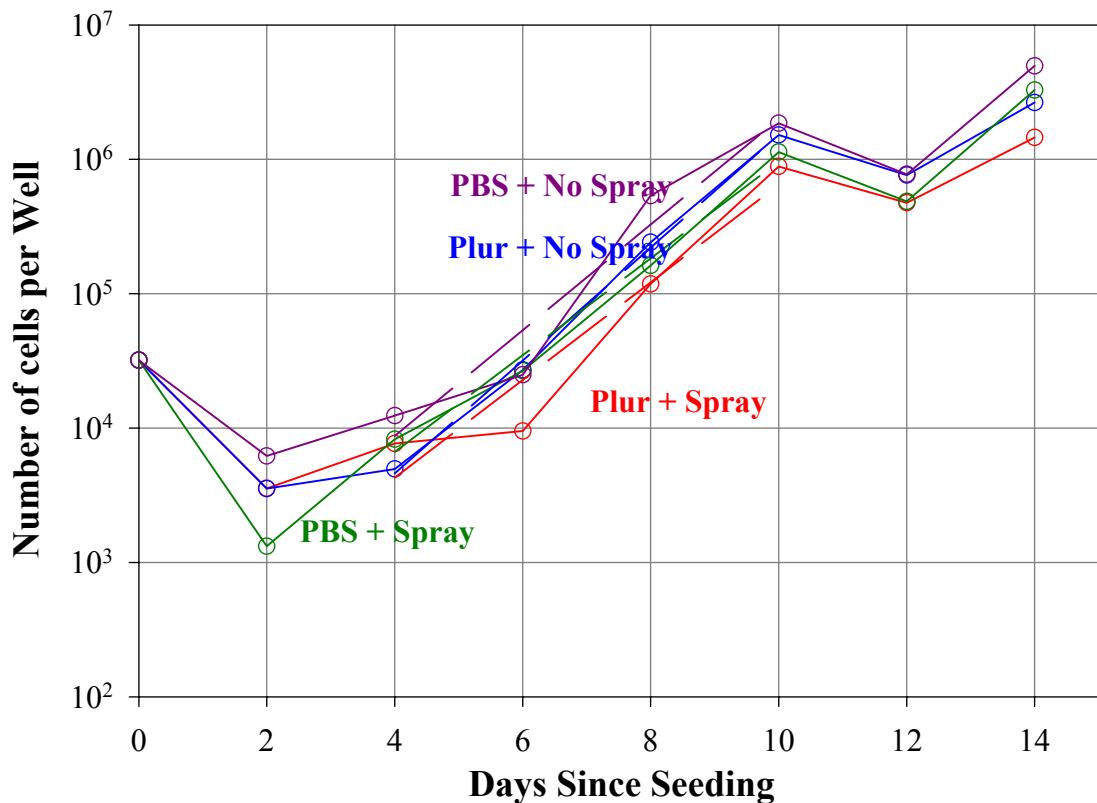


Figure 3.3.4: Cell-growth data for experiment C-4 in which a 12%wt. Pluronic solution was used. The data are very close to each other, showing the points for those wells sprayed and pipetted with Pluronic have nearly merged with those of the wells sprayed and pipetted with PBS.

Figure 3.3.4 summarizes the results from experiment C-4. Although a peculiar data point was discovered on day 12, the data from day 4 through day 10 showed the expected rapid increase in cell number, and, on a log-linear plot, the correlation lines all of the experiments were linear with an r^2 value of 0.83 or higher. As expected, based on the results from experiment C-3 reducing the Pluronic concentration reduced the difference between the cell growth curves to the point that by day 4 the cell numbers for

experiments conducted with Pluronic were only slightly beneath those using PBS. The photographs in Figures 3.3.5 and 3.3.6 clearly illustrate the difference in cell growth achieved by day 6 that accompanied the change in Pluronic concentration.

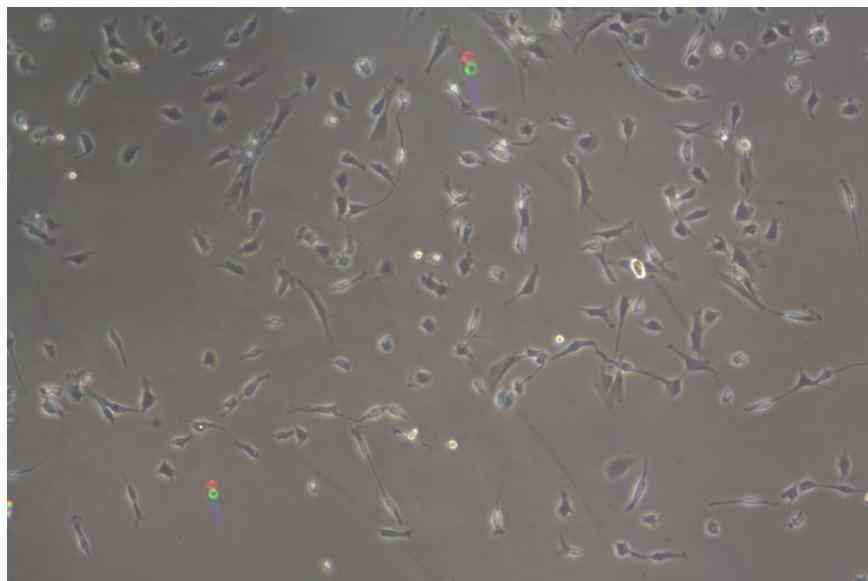


Figure 3.3.5: A sample photograph taken in phase 1 light of sprayed chondrocytes in a 12%wt. solution of Pluronic at their 6th day after seeding showing a large number of adhered cells. Cells that appear darker have begun to generate extra-cellular matrix (a pre-cursor to growth)



Figure 3.3.6: A sample photograph taken in phase I light of sprayed chondrocytes in a 15%wt. solution of Pluronic at their 6th day after seeding showing only a few cells adhered to the plate. The light color and circular shape of the cells indicates that they have barely adhered to the surface and have not yet begun to produce extra-cellular matrix (a precursor to cell-growth).

Although tests were done with both 15wt% and 12 wt% solutions of Pluronic, experiments C-2 – C-4 were conducted with cells harvested from different calf shoulders. It was, therefore, important to conduct a series of experiments using the same batch of bovine chondrocytes and multiple Pluronic concentrations to verify the observed trends. Since the growth trends appear to stabilize by day 8, there was no need to run the experiment longer. Experiment C-5 used followed the same procedures as experiments C-3 and C-4, with four concentrations of Pluronic (0%wt., 10%wt., 12%wt., and 15%wt.) and sprayed or pipetted cells. All Pluronic solutions were made at least a day in advance of the experiment to ensure that the Pluronic powder had completely gone into solution

[14]. The Pluronic solutions were then filter-sterilized and added to the cells. Cell densities remained at 2 million cells per mL of either PBS or Pluronic. Spraying began with the lowest concentration of Pluronic (0%wt. Pluronic F-127) and after the top 3 wells of each of 4 plates were sprayed the next lowest concentration of Pluronic was used (10%wt. Pluronic F-127). Spraying continued in this manner until all of the top wells on each of the 16 6-well plates had been sprayed. The bottom wells on each plate contained the pipetted samples. The cell counts of all three wells were averaged for each condition.

Figures 3.3.7 and 3.3.8 summarize the results of experiment C-5. Data from day 6 was discarded for the 10%wt. Pluronic sprayed and pipetted wells because these data seemed flawed. No infection was observed, but the cell numbers were too low to be considered representative. Because all of the samples came from a single 6-well plate it was considered that the plate itself caused the problem. On day 4 the media was not changed on four of the 10%wt. Pluronic wells that would be counted on day 8 and the cells in the wells containing the pipetted samples and the left well containing a sprayed sample died. The cells in the remaining two wells that contained the sprayed samples grew normally and these data were used.

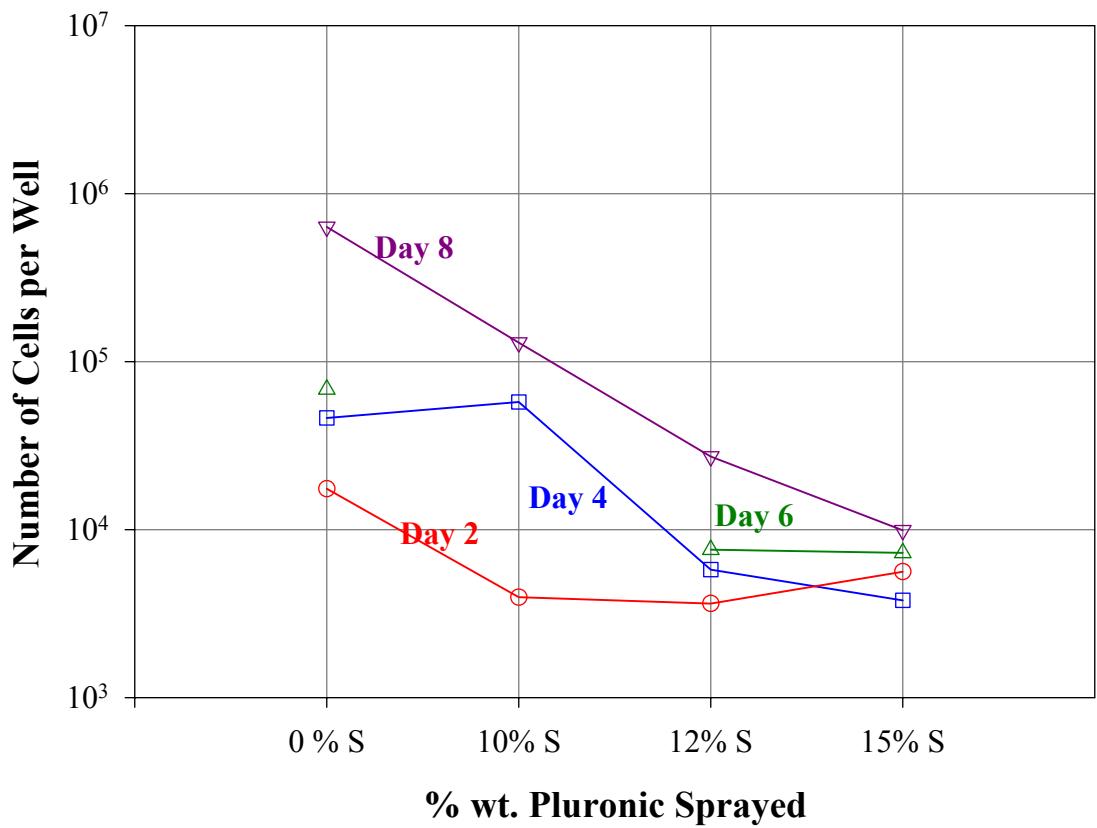


Figure 3.3.7: A comparison of the various sprayed Pluronic concentrations and their growth over a period of 8 days. Growth trends are in line with expectations as the lower Pluronic concentrations grow faster and reach a higher total number of cells by day 8.

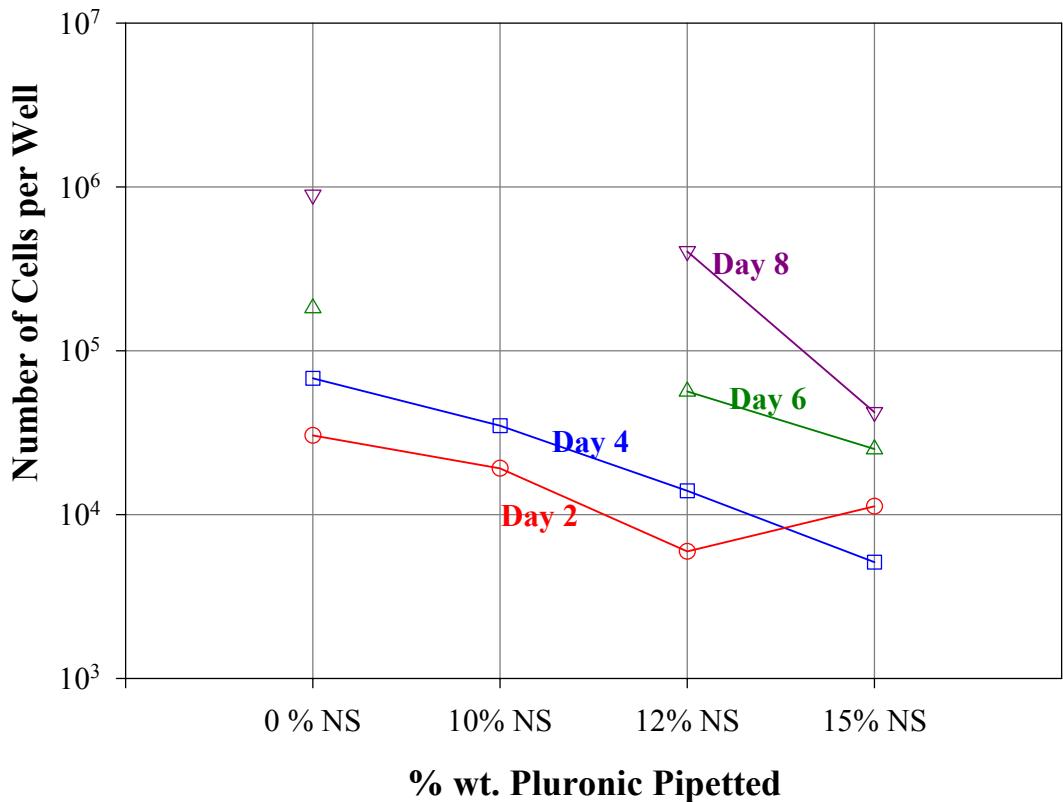


Figure 3.3.8: A comparison of the various pipette-seeded Pluronic concentrations and their growth over a period of 8 days. Growth trends were in-line with expectations; the lower Pluronic concentrations had faster growth and larger numbers of cells by day 8.

The growth curves as a function of Pluronic concentration confirmed the same trends observed in experiments C-2, C-3, and C-4. Because the experiments did not go beyond 8 days, there was not enough data to calculate reliable doubling times. Because the coating experiments presented in Section 3.1 gave satisfactory results with a 12%wt. solution of Pluronic and because the bovine chondrocytes reached confluence more quickly when sprayed in a 12%wt. solution of Pluronic, this concentration was chosen for epithelial cell culture experiments.

Porcine epithelial cell spraying experiments built upon the success of the bovine chondrocyte work. Thus, these experiments used one-second spray time, two-day medium exchanges, 12%wt. Pluronic solution, and the same counting and photographing techniques. Figure 3.3.9 summarizes the results for the first epithelial cell spray experiments. Unfortunately, mold infected many of the plates due to an improperly prepared aliquot of AB/AM. The plates that were not infected with mold, continued to grow and the results were recorded.

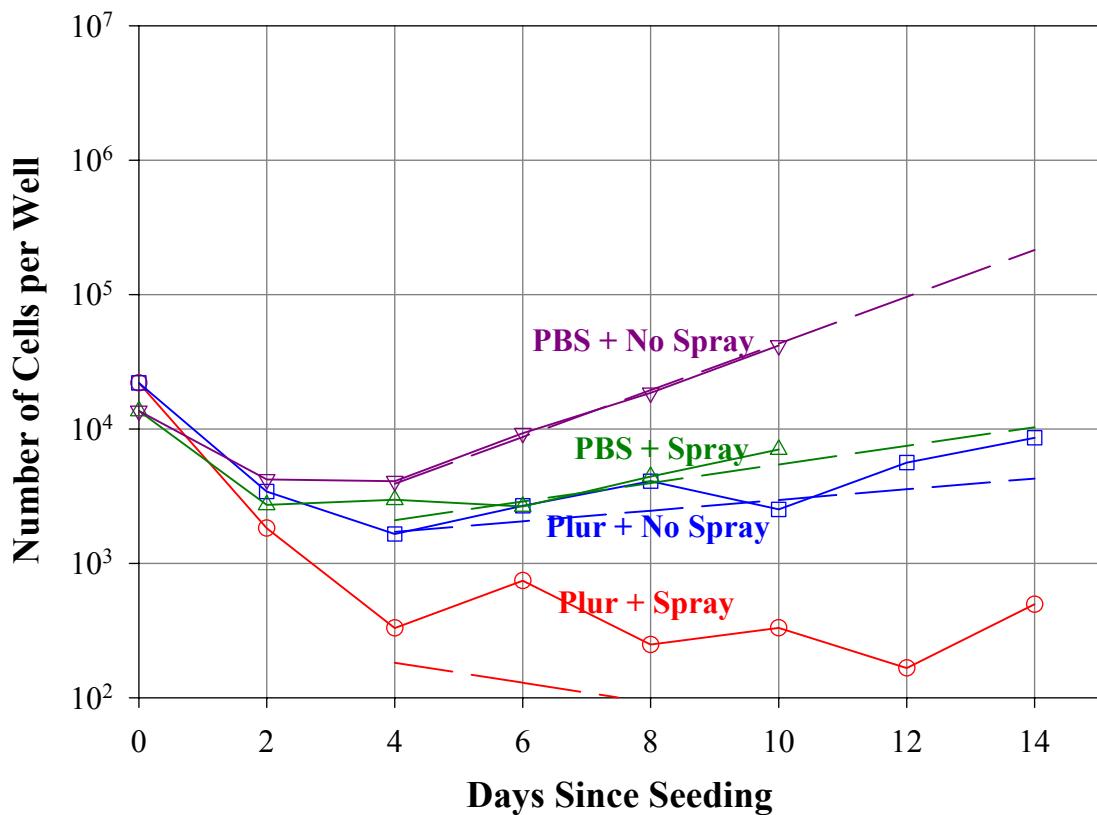


Figure 3.3.9: A graph of the average cell counts for epithelial cell experiment E-1. Cells were seeded at approximately their 11th passage. A definitive exponential growth curve appears for cells in PBS that were pipetted. All other curves represent either weak growth, as with PBS sprayed and Pluronic pipetted, or no growth, as with the Pluronic sprayed.

The growth curves for experiment E-1 were discouraging but not unexpected. Compared with the bovine chondrocytes, the growth of the epithelial cells was poor especially for cells that had been sprayed; even those sprayed in PBS did not grow as well as those that had been pipetted in PBS. Although Sec. 3.2 had shown good viability of sprayed epithelial cells there are two important factors that must be considered. First, epithelial cells are not able to survive unless they adhere to a plate immediately after being seeded and produce ECM. In addition, the epithelial cells used in this experiment were from a much later passage than those used in the two viability tests. Typically cells should not be used if they have been passaged beyond 5 times [17]. The cells in experiment E-1 were from the 11th passage.

Due to time constraints, experiment E-2 used the 13th passage of the same epithelial cells that had been used for all other experiments, including the viability tests and E-1, and many of the culture flasks contained cells that were floating in the media. In an attempt to counteract the poor cell quality of the late-passage epithelial cells, a higher cell density, 3.2 million cells per mL rather than 2 million cells per mL, was used for experiment E-2. Finally, after the cells had been sprayed (on day 0) the growth medium added contained a solution of freshly purchased EGF (epidermal growth factor) from Peprotech. This seemingly minor change altered the phenotype of the cells back to a more epithelial-like state both in the culture flasks and in the wells, a change that is clearly visible in Figs.3.3.10 and 3.3.11:

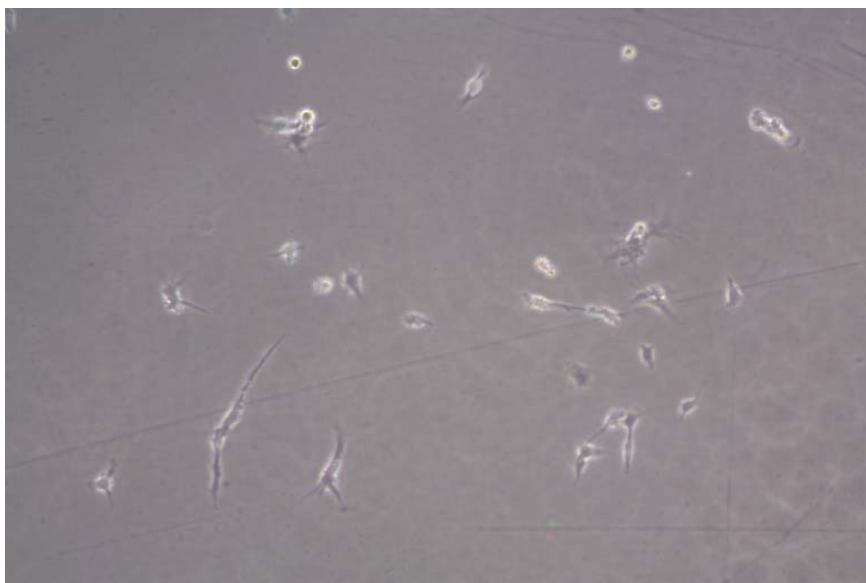


Figure 3.3.10: A photo taken of epithelial cells pipette-seeded in PBS in a 6-well plate during E-1, day 10 after seeding, showing only weak resemblance to the classic epithelial cell phenotype.

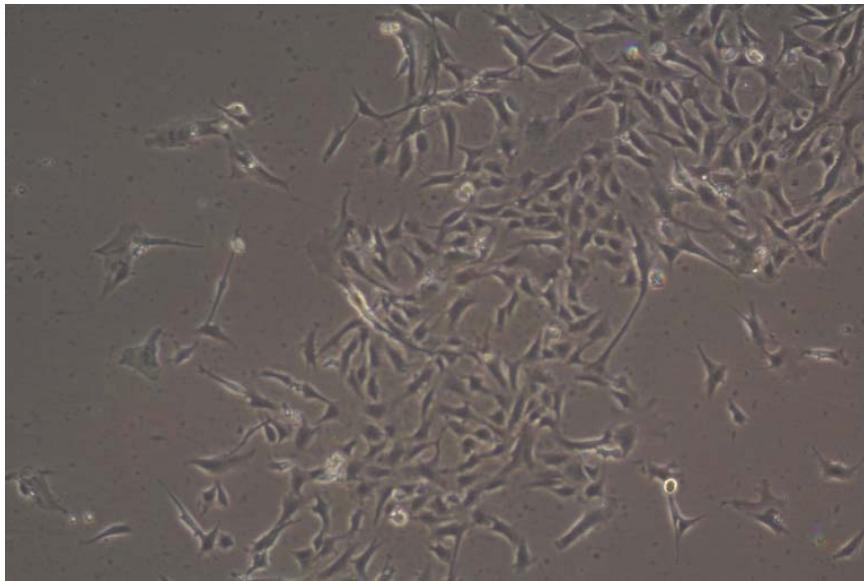


Figure 3.3.11: A photo of epithelial cells pipette-seeded in PBS in a 6-well plate during E-2, day 10 after seeding, showing a much stronger resemblance to the classic epithelial cell phenotype than those in Fig. 3.3.10.

The average cell count as a function of time from experiment E-2 is illustrated in Fig. 3.3.12. Over the length of the experiment, only the cells pipette-seeded from PBS grew well. Near the end of the experiment, the cells exposed to other conditions were starting to increase in number as well. Photographs taken during the last several days confirmed that the cells both pipette-seeded and sprayed in PBS were expressing the epithelial phenotype, similar to the cells shown in Fig. 3.3.11.

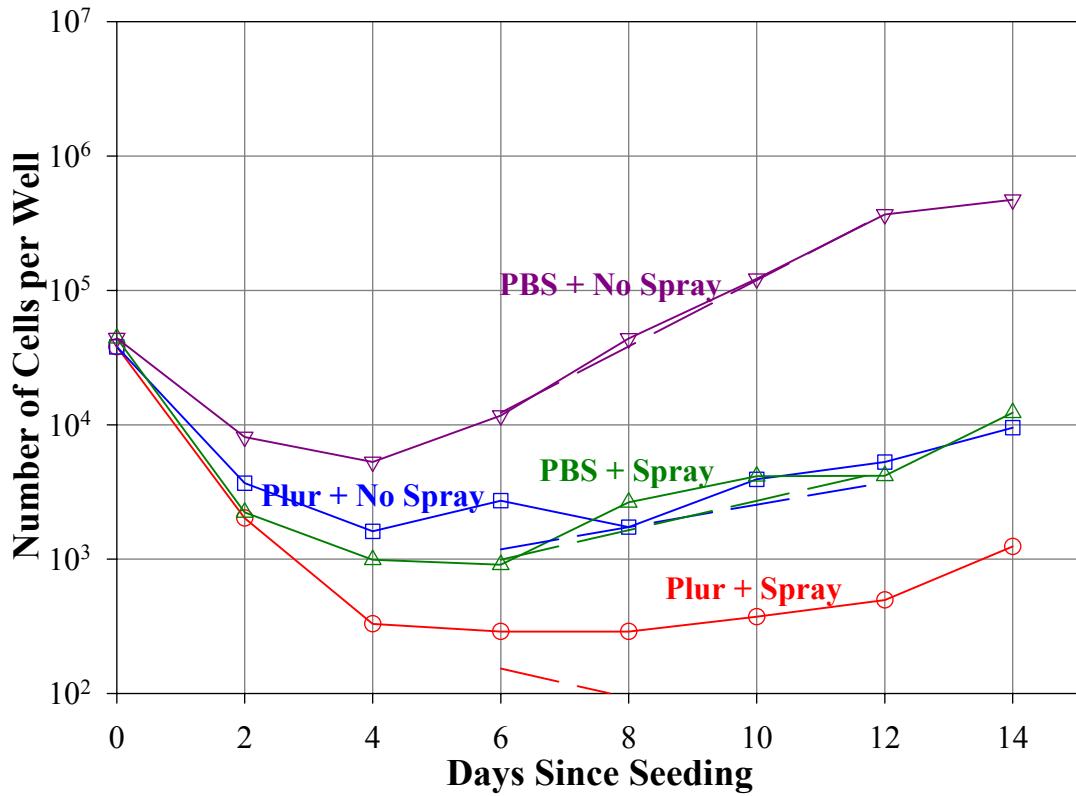


Figure 3.3.12: A graph of cell number vs. days after seeding from E-2 showing a strong growth trend for the cells pipette-seeded in PBS but no other strong growth trends.

As in the chondrocyte experiments, doubling times were calculated from the exponential growth curves and are summarized in Table 3.3.4, however, many of the doubling times do not agree with literature values because growth did not occur or only started after an elongated lag phase. Despite these difficulties, it is still encouraging that sprayed epithelial cells survived, grew and expressed the correct phenotype.

Table 3.3.4 Doubling time of the epithelial cell line with the various conditions of the experiments. DNG stands for “did not grow” and indicates either cell death for that set of conditions or merely a lack of any upward trend.

Porcine Epithelium Growth Times

Test	Pluronic Concentration	Deposition Method	Doubling Time
E-1	12% wt. in PBS	Sprayed	DNG
		Pipetted	182 hours
	PBS	Sprayed	104 hours
		Pipetted	41 hours
E-2	12% wt. in PBS	Sprayed	DNG
		Pipetted	87 hours
	PBS	Sprayed	66 hours
		Pipetted	29 hours

4.0 Summary, Conclusions, and Future Work

4.1 Bovine Chondrocytes

This work demonstrated that the bovine chondrocyte cells can be sprayed onto 6-well plates and cultured *in vitro*. Although Pluronic appears to inhibit the initial growth when compared to cells sprayed in PBS, all cells reached confluence in 10-14 days. This suggests that the next set of experiments for bovine chondrocytes could be *in vivo* animal trials to judge the ability of the cells to grow on actual damaged tissue. A typical trial might be to cause a small defect in a cartilage surface and then attempt to resurface the area using the atomized chondrocytes in a Pluronic suspension. The prime difficulty in this experiment would be to find an area of the body where the cartilage is not being stressed since the everyday stress on articular joints would most likely displace the cells before they had a chance to fix themselves to the bone surface. This is even more of a problem given the difficulties that these experiments have encountered with regards to the reduced adhesive properties of cells in Pluronic.

4.2 Porcine Epithelium

The porcine epithelium used in these experiments came from a late generation passage. Even though the cells were restored to their earlier phenotype when they were fed new EGF, this only occurred after the cells had been sprayed in Pluronic. The porcine epithelial cell culturability experiments should be repeated with cells that have been harvested more recently.

If the cells from these proposed experiments are still difficult to culture in Pluronic, another possible step could be to spray a layer of cells in PBS and then spray a

layer of the Pluronic on top to hold the cells in place. Due to the very thin nature of the film of cells that is being produced over the course of one second of spraying, the Pluronic would still be able to gel, but it should allow the cells to come into direct contact with the surface of the 6-well plate, giving more opportunity for unrestricted bonding to the plate surface.

4.3 Chondrocytes Versus Epithelium

The bovine chondrocytes gave an initial point of success, probably due to their ability to grow in media without bonding to a surface. In addition, the chondrocytes used were always first passage, digested directly from a calf shoulder and used. In light of these two factors it is not surprising that the epithelium did not fare as well when the same set of experiments were performed on them. The inability of epithelium to survive when floating in media for extended periods and the late-generation starting point for the epithelium severely hindered its ability to adhere and grow on the culture dishes in Pluronic.

4.4 Phenotype Testing

Although the phenotype of the two cell lines was visually checked during culturability testing, a more quantitative way to test the bovine chondrocyte cell phenotype is to check the collagen that the cells produce. Although samples of the cells from each culturability experiment were saved and frozen, the collagen test has not yet been performed which would quantitatively confirm what was seen visually in these experiments.

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