Studying a Temperature Sensitive N-isopropylacrylamide Polymer Coated Scaffold for High Density Mammalian Cell Growth

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Abstract

The objective of this research project is to test the effectiveness of using poly(*N*-isopropylacrylamide) (PIPAAm) coated onto a multilayered scaffold. Fibroblast 3T3 cells normally grow as a monolayer that adheres to the surface of tissue culture plasticware. Since the cells grow as a monolayer, the surface area of the plasticware on which they are growing limits the number of cells that can be harvested. Prior work has shown the applicability of rapid prototyping to design a functional, autoclavable multilayer scaffold, on which high density mammalian cells can be grown[1]. The capability to increase the bioactivity in the scaffold makes this technology an advancement in designing a scaffold because of the increased cell proliferation. Aqueous PIPAAm, used as a coating, has been extensively studied and is known as a temperature sensitive polymer that will allow the cells to detach from the scaffold when the temperature is decreased below the lower critical solution temperature (LCST) of 32° C. Therefore, PIPAAm coated onto scaffolds can be used as an effective method to detach a significantly higher number of healthy cells, thereby making the process enzyme free and more efficient. PIPAAm was grafted onto surfaces using two different methods in this research, both of which are different from the most common grafting method used today. The most common grafting method uses electron beam irradiation to both polymerize and graft the monomer *N*-isopropylacrylamide (IPAAm) onto a surface. Because an electron beam was unavailable, the two methods tested in this research were the adsorption method used by Callewaert's group[2] and the ultraviolet monomer grafting method used by Morra's group[3]. Both methods showed that using PIPAAm to detach the cells greatly increased the number of live cells released.

Keywords: 3T3 Fibroblast, Scaffold, poly(*N*-isopropylacrylamide) (PIPAAm)

1. Introduction:

1.1. background:

In recent years, research in the field of tissue engineering has grown exponentially. The term "tissue engineering" has now been around for more than 30 years and "focuses on regeneration of neotissues from cells with the support of biomaterials and growth factors[4]." The end goal of the tissue engineering process is to be able to culture an organ, bone or other body part that a person has lost function of, typically through disease, a congenital abnormality, or trauma. In this case, research is focusing on the tissue engineering of organs. According to the International Association for Organ Donation, every day in the United States 19 people who are on the organ waiting list die from not receiving an organ transplant. Furthermore, while approximately 27,000 people receive an organ transplant each year in the United States alone, there are over 100,000 people waiting for an organ and that number increases by approximately 4,000 each month[5]. Because the demand for organ transplants far outweighs the supply, scientists, physicians, and engineers have turned to tissue engineering as a viable, and now, very real, possibility for those in need of a new organ.

An important aspect of tissue engineering is the harvesting of cells once they have become confluent. Typically adhered cells are treated with a proteolytic enzyme, such as trypsin, to harvest the cells from culture dishes. However, trypsin, being a protease, often damages cells; in the last decade many researchers have begun to search for an alternative method of cell detachment. One of the methods is to use thermoresponsive polymers to harvest cells[6-8]. These thermoresponsive polymers are mostly based on poly(*N*-isopropylacrylamide) (PIPAAm), which allows cell growth and proliferation and then harvesting of the cells by simply decreasing the temperature below the lower critical solution temperature (LCST) of approximately 32°C.

In this study 3T3 fibroblast cells originally derived from a cell line of embryonic Swiss mice were used. Because fibroblasts 3T3 cells can proliferate and grow continually given the right amount of media and conditions, they are an excellent model to study.

The hypothesis in this research is that by combining the use of PIPAAm with a multilayer scaffold, not only will more cells be able to be grown than on a monolayer surface, but also the cells can be detached without being damaged by trypsin in the process. Also, based on previous work done by Burton[9], this research will look further into varying the volume ratios of the scaffolds themselves, hypothesizing that the scaffold with the highest volume ratio will have the most amount of cell growth. In this case the term “volume ratio” means the percent of the volume of the scaffold out of the same object if it was solid.

2. Methodology:

2.1. cell culture:

The process of designing a scaffold for high density cell mammalian growth is one that involves many systematic techniques. The first procedure requires culturing 3T3 mouse fibroblast cells. Culturing is the process where cells are cultivated in a laboratory under conditions that are controlled in *ex vivo* and the specific idea to maintain cells separate from their original environment. The media is composed of 880 milliliters (mL) of Dulbecco’s modified eagles medium (DMEM), 10 mL of penicillin/streptomycin, 10 mL of l-glutamine, and 100 mL of fetal calf serum (FCS). Two other important materials used in this experiment are trypsin and phosphate buffer saline (PBS), which are used for detaching the cells from the walls of the flask.

Cell culturing is a continuous process that entails growing cells under aseptic conditions. Once all safety and sterile measures have been taken, the first step is removing the flask of cells from the incubator. The incubator maintains the proper conditions for cells by controlling temperature and humidity, thereby creating an environment that is conducive to cell growth. In this case, cells are incubated at 37°C and 5% CO_2. After the cells are under a sterile fume hood and the spent media has been disposed of, 3T3 fibroblast cells need to be removed from the surface of the flask. Ten mL of PBS are added to the flask and then disposed of to wash off any loose or dead cells. One mL of this enzyme is pipetted into the flask, followed by placing the flask into the incubator for another six to eight minutes (longer amount of time if more cells present). Trypsin is the enzyme used in facilitating the detachment of cells at a faster pace. Re-suspending the enzyme and cells with an additional nine mL of media is essential before centrifuging the cells. The Model TJ-6 centrifuge (Beckman) is used at 800g for five minutes. Using the centrifuge, cells are spun down into pellet form and then after removal of trypsin and the old media, they are re-suspended in 10 mL of fresh media. If the goal of the process is to simply sub-culture to provide new area and media for growth, then one mL of the re-suspended cells is pipetted into a T10 tissue culture flask along with nine mL of fresh media. If additional experiments are planned for these cells then 2.5 mL are placed into each of three different T25 flasks along with 22.5 mL of media. The process of culturing cells should be carried out every three to four days to eliminate the competition of resources and promote cell growth.

2.2. autoclaving:

Sterilization is the chief component that allows for the killing of bacteria, germs, fungi or any other harmful diseases that would contaminate the fibroblast 3T3 cells. Specifically, autoclaving is the methodological process by which any traces of these contaminants would be destroyed. Before they were used for experimentation, scaffolds were sterilized in an autoclave at 121°C and a pressure of 110 kPa.

2.2.1. materials:

In past experiments Burton determined the material that is best suited for designing a scaffold[9]. Choosing a material that is both compatible with the rapid prototyping machines and also autoclaveable was a requirement when designing the multilayered scaffold. During this research a test was completed in order to ensure both of those requirements were meet. In the Rapid Prototyping Center (RPC) at the Milwaukee School of Engineering (MSOE) there were eight materials that could be examined for experimentation. The materials included: DuraForm GF (glass filled), DuraForm Flex, DuraForm PA (polyamide), Accura25, Polycarbonate, Acrylonitrile-Butadiene Styrene(ABS), Polyphenyl Sulfone (PPSF), and ZP-131.

2.3. poly(*N*-isopropylacrylamide):

Poly(*N*-isopropylacrylamide) (PIPAAm) can be grafted onto a surface using many different methods. The most common practice among researchers is the electron beam grafting method described by Okano's group[10]. In this method, *N*-isopropylacrylamide (IPAAm) monomer is dissolved in isopropyl alcohol, added to a polystyrene tissue culture dish and then irradiated with an electron beam. With this procedure IPAAm was both polymerized and grafted onto the surface of the dishes using the electron beam. However, since a high-powered electron beam was unavailable for this research, other methods were examined.

A different method of applying PIPAAm to substrata in a tissue culture dish was researched by Callewaert's group[11]. Briefly, this method dissolved PIPAAm in Milli-Q water and then heated the solution to both 25°C and 50°C. The solution was then applied to glass substrata and analyzed.

One final method found in literature is explained by Morra's group and uses UV irradiation with benzophenone (BP) to both polymerize and graft IPAAm in a Petri dish[12]. This method dissolves IPAAm and BP in isopropyl alcohol and then irradiates the solution in the wells for approximately 30 minutes at a maximum of 365 nanometers (nm). Because Morra's group concluded that an IPAAm concentration between 30% and 40% wt. allowed for the best yield of cells recovered, a percent weight of 35% was used in these experiments. Following the irradiation was an extensive wash of the wells with isopropyl alcohol (to remove any unreacted IPAAm) and cold Milli-Q water (to remove any ungrafted PIPAAm).

2.4. fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FT-IR) is a type of measuring technique for collecting infrared spectra. It is commonly used in research to analyze compounds and specimens on the molecular level. FT-IR is especially useful to determine if a polymer layer has attached itself to plastic. In this case, the Nicolet 6700 FT-IR (Thermo Electron Corporation) model was used for the FT-IR scan.

2.5. cell seeding:

Cell seeding has a profound effect on how the cell is able to proliferate within the scaffold. In literature, methods have been reported with the purpose of figuring out how to efficiently seed the cells onto a scaffold, ranging from simple techniques such as static seeding all the way to more advanced seeding methods similar to pulsatile perfusion seeding**.** Depending on the function of the scaffold, this will determine which method would be applicable for cell seeding. In this experiment, centrifugal force was the method that was employed. Centrifugal force seeding allows high efficiency seeding at low cell concentrations using fewer disposal items. Also being able to yield a fairly even distribution of cells throughout the entire construct made this an appropriate technique to replicate. This procedure is a step that determines how well the fibroblast cells are able to attach and proliferate throughout the scaffold. For the case of this research the device used to seed the cells was a Red Rotor shaker Model PR-70 (Hoefer Scientific Instruments, San Francisco) set at a very low setting for 10 minutes. The six-well plate with each well having approximately 100,000 cells was put on the shaker for this time period before the three to four days of incubation.

2.6. cell counting:

The method utilized to calculate cell population involved using a Neubauer cell counting chamber as part of a hemocytometer. Once the cells have been counted, an equation can be used to determine the total number of cells in each container. Equation 1 (shown below) is used as follows:

$$\frac{\text{Total cells counted}}{\text{Number of squares}} \times \text{dilution factor} \times 10{,}000 = \text{cells/mL} \qquad (1)$$

2.7. rapid prototyping:

The goal of the scaffold is to create a design in such a way that the cells would be in close proximity but at the same time allow the growth of cells to be separated in layers mimicking *in vivo*. Rapid prototyping is a unique process that allows a scaffold to mimic the function of a natural extra cellular matrix in the body. The potential to increase the bioactivity in the scaffold makes this technology an advancement in designing a scaffold because of the increased cell proliferation. In general, rapid prototyping takes virtual designs from computer aided design (CAD) software and then builds the structure up by cross-sections in physical space. This process is replicated layer after layer until the model comes to completion. There are several machines by which this process can be completed. In the case of this research, the 3D CAD design software known as SolidWorks™ was used to design the scaffold by first designing a tetrahedral base unit and combining base units to build the structure. After design, the selective laser sintering (SLS) method was applicable to the scaffold design because the SLS machine is constructed in such a way that uses a high power laser to selectively fuse small particles of powder that are later represented as a three-dimensional object. SLS process creates solid 3-D objects, layer-by-layer, from plastic, metal, or ceramic powders that are "sintered" or fused using CO_2 laser energy[13]. Various plastic-based powders are used to produce functional models directly in the SLS process. This preferred fabrication process is ideal for producing an intricate structure that requires precision and accuracy.

3. Experimentation:

The set up for this experimentation involved using sterile tissue cultured treated polystyrene (TCPS) six-well plates. Initial testing for grafting of PIPAAm was carried out by directly coating the surface of the wells. However, once the grafting method was determined, three different scaffolds with varying lattice structure porosities or "volume ratios" were placed inside the six wells plates. Figure 1 shows the three different scaffolds used in experimentation. On both the top and the bottom of the well plate there were three different lattice structures with varying volume ratios, together holding six scaffolds in the first well plate. This is shown in Figure 2 below. The well plate had two of each scaffold volume ratio. The top half was used for PIPAAm coating, while the other half had no PIPAAm coating. A second well plate was utilized to act as a control without using any scaffolds. Each of the scaffolds was seeded with 100,000 cells in approximately 6.5mL of media so that each well would be starting with the same amount of cells.

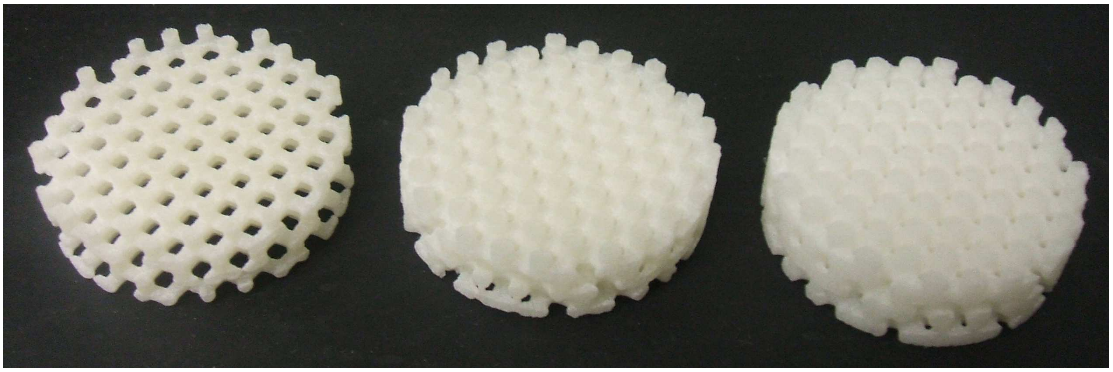

Figure 1. Scaffolds with three different volume ratios. (from left to right: 28%, 44%, 61%).

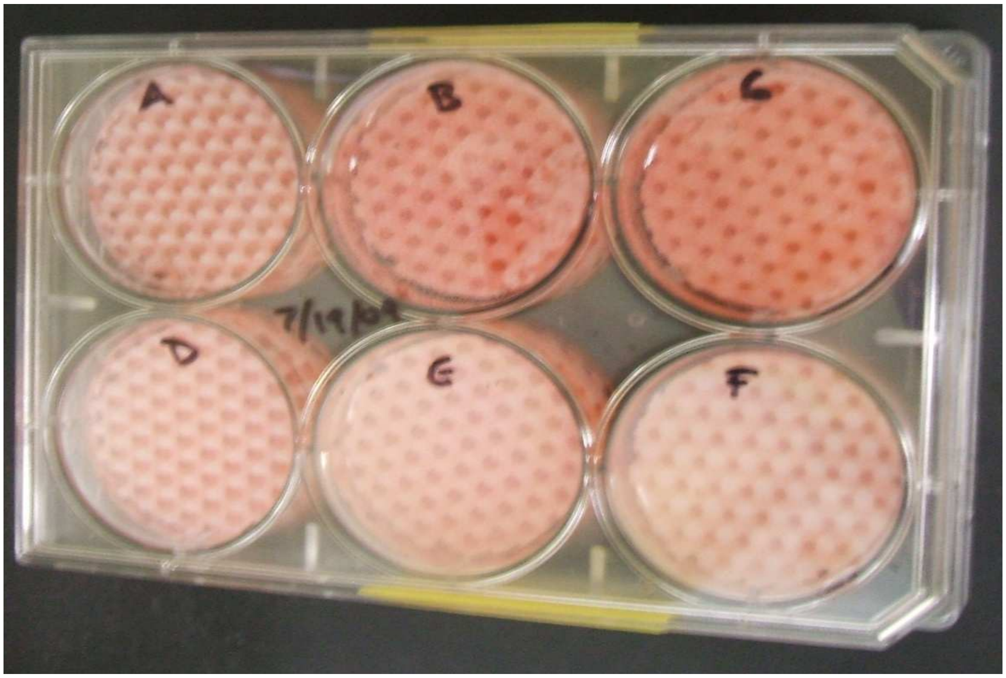

Figure 2. Set-up of scaffolds in six-well plate.

The first attempt at grafting PIPAAm onto the wells of the 6-well plates was done using the adsorption method employed by Callewaert's group[14]. However after two attempts at grafting the polymer in this manner, no cells could be detached from the surface of the wells. Fourier transform infrared (FT-IR) spectroscopy (described further below) confirmed that PIPAAm was coated onto the surface. When the FT-IR outcome showed that PIPAAm was indeed attaching to the surface of the wells, other steps were added to better assist in the detachment of the cells. The two main steps added onto the process used by Callewaert's group were the use of ethylenediaminetetraacetic acid (EDTA) to inhibit the calcium of the cells and a rotor shaker to further enhance the detachment of the cells from the surface. EDTA is often used in tissue cultures to bind to calcium and prevent calcium caused cell adhesion between cells, which often prevents detaching of adherent cells, or clumping of cells grown in liquid suspension. Because EDTA can be toxic in large amounts, this research made sure to use it sparingly and at low concentrations.

A second method of grafting the polymer onto the surface, employed by Morra's group[15], was also tested. In this case, the monomer *N*-isopropylacrylamide (IPAAm) was directly polymerized on the surface. This process used benzophenone, IPAAm, and isopropyl alcohol as previously discussed to both graft and polymerize IPAAm. After the surfaces were coated with PIPAAm, cells were seeded and incubated in the same manner as they were for the adsorption method. To release the cells from the surface, EDTA and a rotor shaker were again both used in conjunction with the decrease in temperature.

4. Results:

4.1. rapid prototyping materials:

According to Burton, out of the eight RP materials available at MSOE, five could not withstand the harsh conditions required for autoclaving[16]. Those materials included: DuraForm Flex, Accura25, Acrylonitrile Butadiene Styrene(ABS), Polyphenyl Sulfone (PPSF), and ZP-131. Warping, bending, cracks in the structure, and a change of the color and texture of the materials were all indications that these materials could not be used to design a multilayered structure. Of the three remaining materials that could be autoclaved only DuraForm PA was used to carry out Burton's experiment. Since Duraform PA was previously chosen as the best material available, it was used again for this research.

4.2. Fourier transform infrared spectroscopy of PIPAAm

When two PIPAAm-coated surfaces (using the adsorption method) were tested with FT-IR to verify that PIPAAm was indeed coating the surface properly, the results were consistent with the literature[17]. Both the literature and this research found major peaks at around 3000 cm^{-1} and 1500 cm^{-1} with many smaller peaks between 500 cm^{-1} and 1700 cm^{-1}. More specifically, the peak at approximately 1530 cm^{-1} is known to represent the presence of the acrylamide group. Figure 3 below depicts one of these findings.

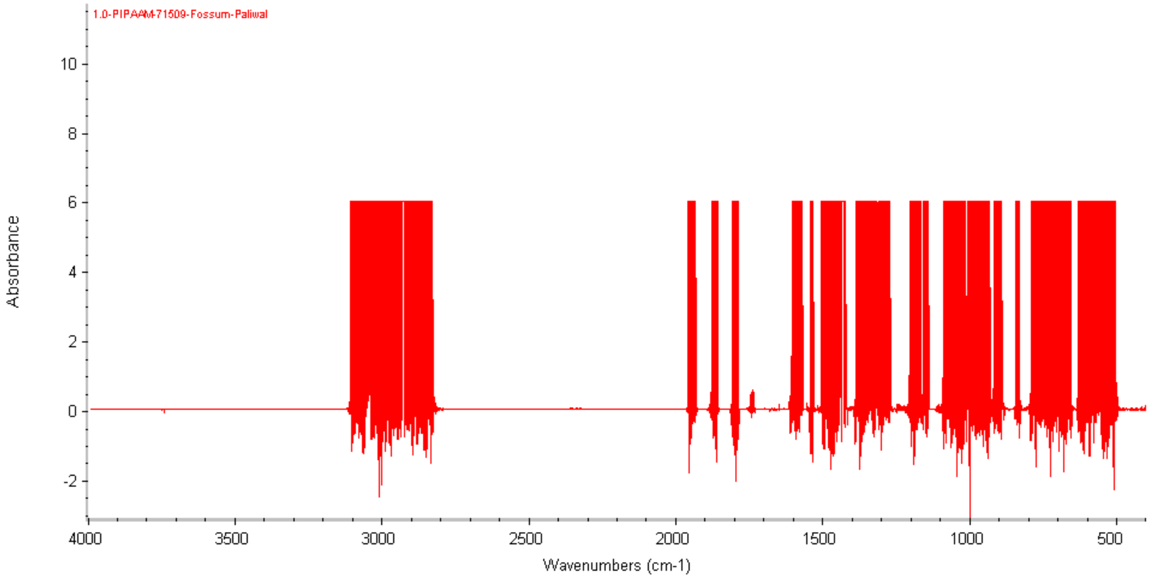

Figure 3. FT-IR spectra of 1.0 g/L PIPAAm.

4.3. cell growth:

Figures 4 and 5 below depict the results from the two methods of PIPAAm grafting tested in this research. Figure 4 shows the outcome from the adsorption grafting method, while Figure 5 shows the outcome of the UV monomer grafting method. It is important to note that both graphs use logarithmic scales for the y-axis to better portray the findings.

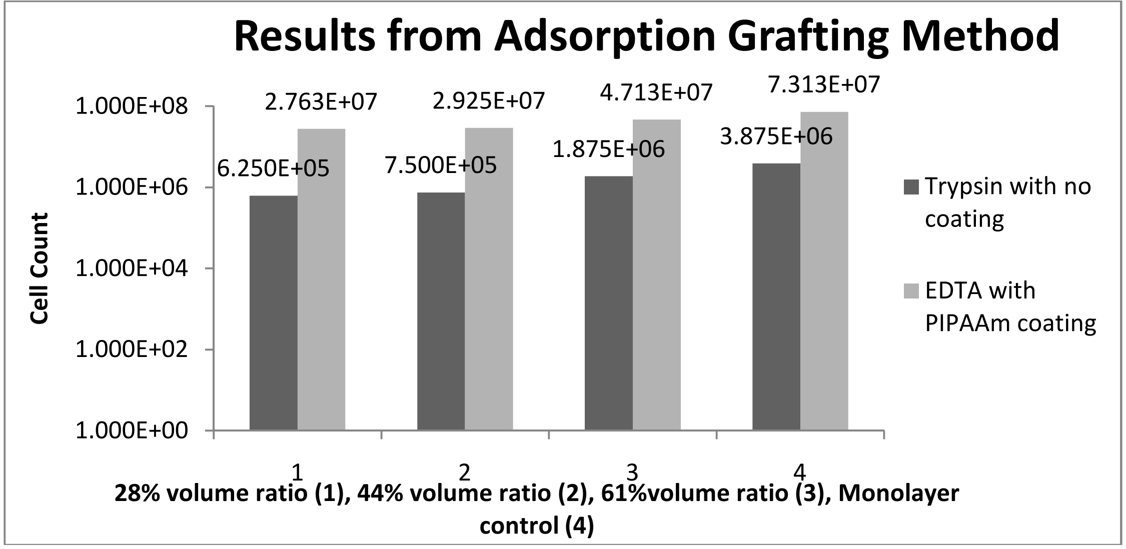

Figure 4. Cell population for control scaffold using trypsin for cell removal vs. for the PIPAAm coated scaffold.

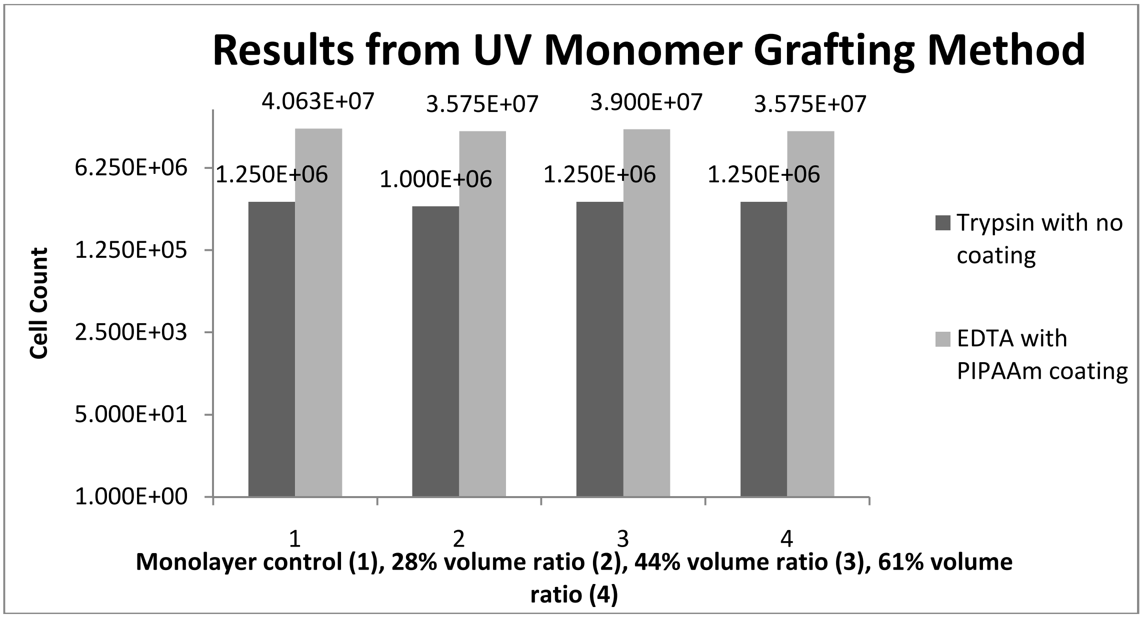

Figure 5. Cell population for control scaffold using trypsin for cell removal vs. for the PIPAAm coated scaffold.

5. Discussion:

Despite the fact that Callewaert's methods were followed very closely, this research originally failed to yield the desired results. It was concluded that by simply dissolving PIPAAm in Milli-Q water and then heating it to 50°C before applying it to a surface cannot be considered a viable option if the end goal is to use this surface to grow cells on and then have those cells detach when the temperature is dropped below the lower critical solution temperature (LCST) of 32°C. However, when additional steps were used to enhance the releasing of the cells, the results were much more desirable. In fact, when EDTA and the rotor shaker steps became part of the procedure the findings matched the hypotheses almost entirely. This was especially true for the results of the adsorption method of grafting.

As the graph suggests, when PIPAAm and EDTA were used instead of trypsin to release the cells, between 18 and 40 times as many cells were released. Furthermore, the highest volume ratio of 61% also had the most amount of cells released from the scaffold reaching 4.7125*10^7. The one peculiar result from both the adsorption method and the UV monomer grafting method is that the well that released the highest amount of cells was the control well without a scaffold. This is different than Burton's research that proved that using a scaffold for cell growth allows for more 3T3 fibroblasts to be harvested than when using a monolayer surface[18]. This result was most likely due to that not all of the cells were able to be released from the intricate nanostructure of the Duraform PA material as they were for the TCPS six-well plates.

6. Conclusion:

To sum up, the data from this research supports the hypotheses made before beginning the experimentation. The highest volume ratio of 61% allowed for the maximum amount of 3T3 fibroblasts to be harvested. Moreover, using a PIPAAm coating in concurrence with EDTA greatly increased the number of live cells that were harvested when compared with the number of cells that were harvested using trypsin. This is another important step in showing that trypsin is not needed for the cell culturing process as it is known to cause damage to or alter the functionalities of the cells on which it is being used. Future work in this area could look further into using PIPAAm together with other materials (i.e. collagen) to increase cell growth and number of cells released. Other topics that could be further researched include using a biodegradable material for making the scaffold from rapid prototyping, altering the structure of the scaffold from the tetrahedral structure to some other structure that may increase the number of cells able to be released from the scaffold, or using an electron beam to polymerize and graft IPAAm onto the surface since it is the most common method used in today's research.

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8. References:

1. Whitney Burton, "Designing a Scaffold for High Density Mammalian Cell Growth," Milwaukee School of Engineering Rapid Prototyping Center, Research Experience for Undergraduates, 2008.
2. M. Callewaert, Ch. Grandfils, L. Boulange-Petermann, P. G. Rouxhet, "Adsorption of Poly(N-isopropylacrylamide) on Glass Substrata," *Journal of Colloid and Interface Science,* Volume 276, Issue 2, (August 2004): 299-305.
3. Marco Morra and Clara Cassinelli, "Thermal Recovery of Cells Cultured on Poly(*N*-isopropylacrylamide) Surface-grafted Polystyrene Dishes," *Surface Modification of Polymeric Biomaterials* (1996): 175-181.
4. Yoshito Ikada, "Challenges in Tissue Engineering," *Journal of the Royal Society Interface* 3 (2006): 589–601, http://rsif.royalsocietypublishing.org/content/3/10/589.full.html#ref-list-1.
5. "Understanding: Statistics/Facts," The International Association for Organ Donation, 2009, http://www.iaod.org/newsroom-fact-sheets.htm.
6. Hiroshi Takamatsu, Satoru Uchida, Takehisa Matsuda. "In Situ Harvesting of Adhered Target Cells Using Thermoresponsive Substrate under a Microscope: Principle and Instrumentation," *Journal of Biotechnology* 134 (2008): 297-304.
7. T. Okano, N. Yamada, M. Okuhara, H. Sakai, Y. Sakurai, "Mechanism of Cell Detachment from Temperature-modulated, Hydrophilic-hydrophobic Polymer Surfaces," *Biomaterials* **16** (4), (1995): 297-303.
8. Martin T. Moran, William M. Carroll, Alexander Gorelov, Yuri Rochev, "Intact Endothelial Cell Sheet Harvesting from Thermoresponsive Surfaces Coated with Cell Adhesion Promoters," *Journal of the Royal Society Interface* 4 (2007): 1151-1157.
9. Burton, "Designing a Scaffold," 2008.
10. Okano, "Mechanism of Cell Detachment," 297-303.
11. Callewaert, "Adsorption of Poly(N-isopropylacrylamide)," 299-305.
12. Morra, "Thermal Recovery of Cells," 175-181.
13. Solid Freeform Fabrication. 2009. Milwaukee School of Engineering http://www.msoe.edu/academics/research_centers/reu/solid_freeform_fabrication.shtml.
14. Callewaert, "Adsorption of Poly(N-isopropylacrylamide)," 299-305.
15. Morra, "Thermal Recovery of Cells," 175-181.
16. Burton, "Designing a Scaffold," 2008.
17. Diannan Lu, Zhixia Liu, Minlian Zhang, Xiaogong Wang, Zheng Liu. "Dextran-grafted-PNIPAAm as an Artificial Chaperone for Protein Refolding" *Biochemical Engineering Journal* 27 (2006): 336-343.
18. Burton, "Designing a Scaffold," 2008.