Incorporating Filastatin into medical plastics to minimize nosocomial fungal infections

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Abstract

Candida albicans commonly infect medically implanted devices. To combat C. albicans' increasing resistance to current treatment methods, a preventative measure was developed by incorporating Filastatin into silicone catheters. Filastatin was shown to inhibit the adhesion of cells by constraining the development of hyphae that allow the cells to adhere to one another and to surfaces. Different drug incorporation methods were developed and tested to determine the best way to utilize Filastatin in order to inhibit C. albicans from adhering to silicone urinary catheters.

Background

Candida albicans is the fourth most common source of hospital-acquired infections in the United States (Wisplinghoff *et al*., 2004). It primarily affects immunocompromised individuals with a thirty to fifty percent mortality rate (Pappas *et al.*, 2003). *C. albicans* can cause two types of infections: mucosal infections (oral and vaginal thrush) and systemic infections (candidemia) (Fidel and Sobel, 1996). These infections usually reside from medical devices including catheters, prosthetics, grafts, and cardiac devices (Kojic & Darouiche, 2004). The estimated annual cost of treating a systemic *C. albicans* infection exceeds $200 million per year due to its increasing resistance to antifungal drugs (Millar *et. al*, 2001). *C. albicans* is becoming resistant to first-line and second-line antifungal medications, namely, Pyrimidine, Azoles, Polyenes and Echinocandins (Morgan, 2005). For example, approximately seven percent of all *Candida* bloodstream isolates tested at the Center for Disease Control (CDC) are resistant to fluconazole (a type of azole) (Cleveland *et al.*, 2012). Overall, antifungal resistance is becoming more and more of an issue. It will continue to worsen unless more is done to prevent further resistance and the spread of these infections.

C. a*lbicans* cells are not harmful at 30°C, 5.4 pH and remain as single round budding yeast cells (blastospore). It is only once they are induced by several environmental conditions including pH, temperature, or a carbon lacking environment that they pose a threat to humans. (Fazly *et. al.,* 2013) Once the C. a*lbicans* are induced, they start filamentation in which the cells form small projections called "germ tubes" and continue to divide at the apical tip of the tubes to form extended filaments or hyphae (Odds, 1988; Lo *et al.*, 1997; Brown, 2002; Saville *et al*, 2003). Hyphae allow the cells to adhere to implanted medical devices and human epithelial cells by exerting force from the growth of the apical tip of the tubes on the surfaces (Kumamoto and Vinces, 2005). This same force allows the cells to adhere to one another and form biofilm over the surface with which they come into contact. Biofilm is a polysaccharide extracellular matrix produced by the cells in order to shield them from external threats

like antibodies and antibiotics (Chandra *et al.*, 2001). Biofilm formation is harmful because once it forms on a medical device, the device is difficult to remove and can cause diseases as described previously. These medical devices often have to be surgically removed (Chauhan, 2012).

Urinary catheters are the most commonly used devices in the U.S. that acquire *C. albicans* infections (Kojic & Darouiche, 2004). They have the greatest overall infection rate between 10-30%. A urinary catheter is a tube placed in the body to collect and drain urine from the bladder. Catheters come in various sizes, plastics and types (Lawrence and Turner, 2005). The most common catheter plastics are latex, thermoplastic polyurethane and silicone. There are two main types of catheters: indwelling and intermittent.

Indwelling urinary catheters can be left in the bladder for either a short (<14 days) or long period of time (>14 days) (Pickard *et al.,* 2012). An indwelling catheter collects urine by attaching to a drainage bag. Recently developed catheters have valves that can be opened to allow urine to flow out. An indwelling catheter may be inserted into the bladder in two ways. Most often, the catheter is inserted through the urethra. This is the tube that carries urine from the bladder to the outside of the body. Sometimes, the provider will insert a catheter into your bladder through a small hole in your abdomen. Intermittent urinary catheters are used when the individual only needs the catheter for a short period (a matter of hours) or they do not want to wear a bag (Wilde *et al.*, 2011). The individual will insert the catheter to drain the bladder and then remove it him/herself. The highest incidence of healthcare-associated infection is associated with long-term indwelling catheterization (National Institute for Clinical Excellence (NICE), 2012).

To combat the increasing resistance of *C. albicans* to typical treatment methods, scientists including Dr. Paul Kaufman at the University of Massachusetts conducted a high-throughput phenotypic screening of small molecules to identify compounds that inhibit adhesion of *C. albicans* to polystyrene

plates (Fazly, 2013). Adhesion is the first step of the pathogenesis of *C. albicans*, and without it, the fungus cannot form pathogenic biofilm. From the screening of small molecules, they found a candidate that prevented *C. albicans'* adhesion called Filastatin (Fazly, 2013). Screening was conducted by co-incubating molecules with cells grown in Spider media (a carbon-lacking media that induces hyphal formation) and quantifying how many cells adhered to the surface. What they found was that Filastatin significantly decreased the amount of cells adhering to the various surfaces from 50-75%. Assays comparing the effect of co-incubating cells with Filastatin, after 8 hours of cells incubating alone, showed that Filastatin also has an effect on cells already bound to a surface. These tests showed that Filastatin affects hyphal formation, which in turn, affected biofilm formation (Fazly, 2013).

Filastatin diminishes yeast-to-hyphal transformation and therefore reduces fungal pathogenesis. The exact mechanism for the interaction between Filastatin and *C. albicans* is unknown. It is hypothesized that it is caused by disrupting multiple signaling pathways. The first pathway is the cAMP-PKA pathway which causes hyphal induction by Spider media. Cells constantly overexpressing the G protein-coupled receptor Gpr1 became hyperfilamentous in Spider media by PKA stimulation, and Filastatin inhibits this. PKA pathway stimulation also drives transcription factor Efg1 phosphorylation, activating Efg1 to increase expression of genes required for hyphal morphogenesis. This was confirmed with an experiment that involved hyperactive Ras1 signaling protein mutant, another upstream signal that governs hyphal development. When Filastatin was introduced to the Spider media with the modified cells, the inhibitory effect was comparable to that of WT cells.

Other experiments suggested that Filastatin affected more than one signaling pathway. For example, the modified sugar GlcNac stimulates hyphal morphogenesis independently of the cAMP-PKA pathway; instead, it activates the transcription factor Cph1 (Midkiff and White, 2013). On testing morphogenesis driven by GlcNac-containing media or constitutive overexpression of Cph1, Fazly's lab found that Filastatin still inhibited hyphal formation.

Filastatin's effects on *C. albicans'* morphogenesis have been clearly documented but not understood to the extent that it can be used to treat patients. *C. albicans* infections have been identified as a public health issue (Pfaller 2007), but it could take years for Filastatin to be used therapeutically. There are various drugs as well as antifungal treatments used to prevent infection, but these are becoming ineffective quickly (Pfaller 2007).

Materials and compounds capable of inhibiting *C. albicans* adhesion onto medical materials have been identified as the best preventative measure (Palza, 2015) (Zhou, *et al.*, 2010) (Onaizi, 2011). These material or compounds can have several incorporation strategies to transfer their antifungal properties to the medical material. Four of the most common drug incorporation methods are polymerization, simple coating, absorption, and controlled release via the layer of polymers.

One example of polymerizing a drug into a medical material is the incorporation of ester and cyclic hydrocarbon moieties into silicone. Silicone polymerized with these molecules reduced adhesion of three pathogenic bacteria by 96% (Hook *et al*., 2012). These results were demonstrateed *in vitro* with lesser results in a mouse model. While these methods were effective with bacteria strains, similar results have yet to been seen with *C. albicans.*

Parylene is an example of an antimicrobial simple coating method. It is used to coat many different surfaces including metal, glass, paper, resin, plastic, and ceramic by chemical vapor deposition and polymerization of pare-xylene (Demirel, 2008). Parylene is thought to cut the bond between silanol groups (Si-OH), like those between the silicone on the surface and hydrogen atoms of proteins on the *C. albicans'* surface (Zhou, *et al.*, 2010). *C. albicans* adhesion to silicone elastomer surfaces coated with Parylene is less than uncoated samples, on the magnitude of 4.5x fewer cells (2.18×10^7 uncoated vs. 0.48×10^7 coated) (Zhou, *et al.*, 2010).

Although effective in limiting *C. albicans'* adhesion, there are some issues associated with this coating technique. One includes the moisture barrier performance. Parylene moisture barriers are susceptible to failure after prolonged exposure at higher temperatures. Under such conditions, resistance to corrosion can decline due to contaminants or particles trapped inside the coating-film (Li, *et al.*, 2008). Parylene's biggest flaw is adhesion to the surface being coated. Coating can only be done on devices that will fit in the deposition system's coating chamber (Li, *et al.*, 2008). Furthermore, while Parylene coatings are typically thin, they also deposit relatively slowly; it would take over an hour to deposit enough parylene onto the surface of silicone (Tooker 2007).

Absorption of drugs by plastics was effective in preventing *C. albicans* infections. One study used fluconazole loaded polyurethane with pore-forming agents (Donelli, 2009). The hope was to release the antifungal drug over time, inhibiting fungal biofilm formation on medical devices. By releasing the drug over time, the local drug concentration at the surface remains high enough to inhibit pathogenesis for a longer time. Fluconazole was adsorbed in higher amounts by the most hydrophilic polymers and its release was influenced by the degree of polymer swelling in water. Drug release was noticeable through UV spectroscopy at 215 nanometers up to nine days after incubation with *C. albicans*. This method inhibited *C. albicans* growth and biofilm formation (undetectable cell count) on polymeric surfaces for up to eight days.

There are a few issue associated with this method. One is that the polymer uses fluconazole as the antifungal agent. Fluconazole is a type of azole which, among other antifungal agents, has increased resistance by *C. albicans*. In addition, this treatment method elutes fluconazole for a nine day period with no detectable biofilm formation for only eight days. Indwelling long-term catheters can be implanted for up to month (Saint and Lipsky, 1999), so this coating method would not work for these types of catheters.

The final method is controlled release. A study involving the controlled-release method released silver ions through a polydopamine film. Metals can be extremely toxic to bacteria and yeast at exceptionally low concentrations (3-5% of the total composition of the polymer) (Palza, 2015). Polydopamine is a self-polymerizing molecule that forms a thin adherent film on virtually any surface under mild alkaline aqueous conditions and in the presence of oxygen. Moreover, exposed reactive groups of the polydopamine coatings enable further functionalization of the coatings through covalent grafting of polymers and allow reduction of metal ions to be released (Lee, 2007). Sustained silver release was observed for a little over 7 days from silver-coated substrates, and the release kinetics could be modulated via additional polydopamine overlayers. *In vitro* functional assays employing both gram negative and positive strains demonstrated dual fouling resistance and antibacterial properties of the coatings due to the antibacterial effect of silver. Silver ions *in vivo* have not produced effective results, as expressed by what is currently on the market.

Materials and Methods

Incorporating Filastatin

Filastatin incorporation was measured using absorbance. In a 24-well plate, plastic squares (0.8 x 0.8 cm.) were placed in 1.5 ml of 25uM Filastatin in Tris buffer. The plate was then placed on a rotator at roughly 80 RPM at room temperature and at various time points from 0 to 2880 min (2 days); 1 ml of solution was taken out and measured using a spectrophotometer at 400 nanometers. The 1 ml of solution was put back into the plate each time and allowed to continue rotating. Absorbance readings were recorded in an Excel document and plotted against time. To make sure that the concentration of Filastatin correlated well with the absorbance at 400 nm, a standard curve was created. Filastatin was serially diluted from 25uM to 1.5uM using 10uM Tris pH 8.5 to make sure that the small amount of molecules that were eluted over time could be measured. The absorbance reading correlated linearly with concentration (R^2 = 0.9897). Based off of this correlation, approximate concentration in solution can be calculated.

Yeast Culture and Maintenance

The cells used for this experiment were the SC5314 strain of *C. albicans*. This wild-type strain is more filamentous and more invasive than other wild-type strains of *C. albicans* (like VE175) and representative of strains that would be most likely seen in a hospital (Hua *et al.,* 2009). The cells were grown on a yeast peptone dextrose (YPD) agar plate. Individual colonies were grown in supplemented YNB broth (Yeast Nitrogen Broth + 2 % glucose + 0.1 mg/ml uridine), in a 30^{o}C shaker at 140RPM for up to 24 hours (Daniels *et al.*, 2013). The OD of the culture was measured at 12 hours and adjusted so that the cells remained in log phase. In order to induce filamentation during incubation with samples, spider media (1% Difco nutrient broth, 1% mannitol, 0.2% dibasic potassium phosphate, pH 7.2) was used and the cells were incubated at 37^{o}C in a shaker at 80RPM (Daniels *et al.*, 2013).

Preparing Samples

Plastic samples were cut from either a silicone sheet or a silicone catheter. Samples were cut to size (0.8 cm squares or 2 x 11 mm rings) using a razor blade and then sterilized by immersion in ethanol. Samples were weighed to assure that they were equal by mass. The samples were soaked in ethanol after being cut to keep them clean. On the day that they were to be used for the cell adhesion assay they underwent four doses of UV radiation at a radiant exposure of 3 mJ/cm2 (Dai *et al.*, 2011).

Measuring Cell Adhesion

Cell adhesion was measured by testing *C. albicans'* ability to stick to the catheter plastics. Cells were incubated with the samples at 37°C rotating at 80RPM. One Optical Density (OD) of *C. albicans* at 600 nm wavelength is approximately 3 x 10^7 cells. The cell density for the initial assays was 0.3 OD/ml (total 0.45 OD), based off a cell seeding experiment on silicone elastomers (Chandra, Jyotsna, et al., 2001). The final assays used a starting cell count of 1000 cells per milliliter to more closely mirror the minimum infectious does of *C. albicans.*

Cell Staining

Crystal violet, a triarylmethane dye that stains DNA within cells and has an absorbance around 590 nm, was used for cell quantification after incubation (Adams and Ludwig, 1914). Samples were removed from the 24-well plate in which they were incubated and washed using PBS. Samples were submerged in 1.5 mL PBS for five minutes on a rotator at roughly 80RPM. The samples were moved into a fresh 24-well plate and 800 uL of crystal violet was added directly to each sample. After incubating cells with crystal violet for 15 minutes, the samples were washed again using PBS. Three consecutive washes were done, aspirating the PBS each time. Washes were done to assure that any crystal violet

remaining had effectively stained only cells that had adhered to the samples. Samples were next submerged in 750 uL of acetic acid in 1.5 mL Eppendorf tubes to remove the crystal violet from the sample. The tubes were vortexed until the samples were no longer stained. Absorbance readings were measured in duplicate by reading 100 uL of acetic acid in a 96-well plate. Absorbance readings were measured in duplicate to measure the approximate number of cells in the sample.

Analysis

Outliers from the absorbance readings were identified using Turkey's boxplot (Rousseeuw, 2011). The interquartile distance from the upper and lower quartiles of data were multiplied by 1.5 (1.5*IQR). The interval or "fence" is defined as the upper quartile plus 1.5*IQR and the lower limit minus 1.5*IQR. Any values outside this range were labeled as outliers and rejected. The computations for this were carried out using excel. Next, background readings were subtracted from the readings. Background was a control sample that measured either the fluorescence or absorbance of the dye itself when no cells were present.

The growth of cells during the cell adhesion assays was slightly different from experiment to experiment. In order to be able to compare the results of each experiment to one another, the data was later normalized to the uncoated control samples. Normalization allowed for comparative decreases to be compared between conditions and experiments. The data was plotted with a standard error bar to explore variability within a condition between experiments. Lastly, a T-test and an ANOVA were conducted to prove statistical significance or difference between different conditions, most specifically comparing samples containing Filastatin to the controls.

Long Term Simulated *in vivo* Testing

The OD/mL used in the previously described experiments, 0.3, is higher than any cell count that would cause infections in humans. The 2.5 hour incubation period is significantly shorter than the time that catheters are left in patients. To address these problems, a long term assay was conducted. The goal with this assay was to more closely simulate the conditions a catheter would be exposed to while operating within the constraints of our lab. The same cell adhesion assay using crystal violet was used.

The initial cell count used was 1000 cells per mL, or 0.000001 OD/mL. The experiment was set up such that each condition occupied a 24 well plate. This cell count was chosen to more closely simulate the number of cells found *in vivo.* At 24 and 48 hours, six samples were removed. There were duplicate samples for the 48 hour reading; one to show the effects of washing and one to determine the effects of prolonged incubation. Washing the final set of samples consisted of removing one mL from each well and replacing it with fresh spider media. These extracts were measured to track the change in OD over two days. Washing slowed the growth of the cells as well as simulate movement of urine through the urethra.

Results

Designing a method of incorporating Filastatin into plastics already used for urinary catheters would decrease *C. albicans* treatment costs around the world. Studies done at the University of Massachusetts by Fazly et al have demonstrated that solubilized Filastatin at a concentration of 25uM inhibits hyphal morphogenesis of *C. albicans*, effectively preventing cell adhesion and therefore infection. These results were found with regards to adhesion onto polystyrene plates. As promising as the results are, they do not indicate whether or not Filastatin can retain a therapeutic effect over a longer period of time or if Filastatin could be incorporated into medical grade plastics. This study aims to analyze these possibilities by developing an incorporation method for Filastatin into medical grade plastics that will significantly decrease cell adhesion for up to 48 hours. In order to test whether or not incorporating Filastatin was possible, experiments to reaffirming effectiveness and test the versatility of the incorporation method were done. After effectiveness was demonstrated, Filastatin's elution rate was calculated, on the market catheters were tested, and forecasts were done to approximate the cost per catheter for each incorporation method.

Cell adhesion was tested by incubating samples in Spider Media with *C. albicans* cells. Crystal violet was used to stain cells that had adhered to the samples during incubation. After releasing the crystal violet from the samples using acetic acid, absorbance readings were measured at 590 nm; the absorbance values correlated to the amount of cells adhered to the sample. Four experimental conditions were used along with a positive and negative control. The positive control was uncoated samples and the negative control was samples incubated in water without cells. Given that solubilized Filastatin effectively inhibited cell adhesion as demonstrated by Fazly et al, this was used as a control on Filastatin's effect on the *C. albicans* cells.

Once solubilized Filastatin proved to inhibit cell adhesion experiments were done to incorporate Filastatin into the samples by absorption. The samples were soaked in 25uM Filastatin in Tris pH 8.5 for 24 hours. The samples were incubated with cells for 2.5 hours and then stained with crystal violet and analyzed. Samples with Filastatin absorbed into them decreased cell adhesion by roughly 50% during preliminary testing (Figure 1). This incorporation method demonstrated the ability to significantly inhibit cell adhesion. Augmenting the release of Filastatin was the next goal of experimentation.

Polydpamine was added to samples that had absorbed Filastatin as the next experimental condition. Polydopamine is a self-assembling polymer that was allowed to cover the samples after they had absorbed Filastatin for 24 hours. Samples were left in a solution of polydopamine (2mg per mL) for 24 hours, washed, and incubated with cells for 2.5 hours. Samples coated only in polydopamine were included to determine whether or not the polymer alone would affect cell adhesion. Comparison of these conditions would determine if the two molecules would have no effect, and additive effect, or act synergistically. Absorbance readings from these samples are presented in Figure 1. Preliminary testing of samples coated with both Filastain and polydopamine resulted in approximately 65% fewer cells adhered to the samples. The polydopamine coating alone decreased cell adhesion by 57%.

Each condition was replicated 18 times, adding to the inherent variability seen in the large error bars of some conditions. The absorbance of the crystal violet that stained the adhered cells was measured at 590 nm; this is the y-axis. Each condition is compared on the x-axis. The values were normalized to the uncoated sample, allowing for comparison of Filastatin's effect on cell adhesion and causing the uncoated condition to have an absorbance reading of one. The absorbance readings of each condition (excluding the controls) were not statistically different from one according to a student T test (95% confidence). The separate treatments had similar effects on cell adhesion after 2.5 hours of

incubation. This proved that the incorporation of Filastatin was effective but did not clearly show why Filastatin was working because polydopamine treatments had very little impact on efficacy. The significant effect on cell adhesion led to the decision to test the incorporation method on similar plastics used to make urinary catheters.

Preliminary testing proved that the incorporation methods decreased *C. albicans'* ability to adhere to silicone. The subsequent experiments tested two other catheter plastics, thermoplastic polyurethane (TPU) and pellethane, using the same incorporation methods that were effective with silicone. The versatility tests were done because an incorporation method that is applicable to more than one type of catheter plastic would make a much more significant impact on the frequency of *C. albicans* infections. Both of these plastics were acquired from Bentec Medical in the form of long sheets. They were comparable to silicone in rigidity, texture and opacity. The only notable difference between the materials during the cell adhesion assays was absorptivity; pellethane swelled more than thermoplastic polyurethane or silicone, especially when soaked in ethanol.

The same experimental conditions from the cell adhesion assay using crystal violet to stain the cells adhered to silicone were used for both plastics. There were no notable qualitative differences between the assays done on silicone and those done on TPU and pellethane. The differences between the assays was in the absorbance readings. Figure 2 shows that there was no noticeable change in cell adhesion throughout the separate conditions for pellethane; each experimental condition absorbed a similar amount of crystal violet. It also shows the results for the assay done with thermoplastic polyurethane. The decrease in adhesion on the sample coated with both Filastatin and polydopamine was 28.0%, not as drastic as it was on silicone (64.9%). In addition, Filastatin co-incubation had a lower decrease in cell adhesion on thermoplastic polyurethane than it did on pellethane (40.1% v. 66.5%). Neither the Filastatin incorporation nor the polydopamine treatments inhibited cell adhesion on these

plastics. The incorporation method used for silicone was not transferable to either of the two other medical grade plastics that were tested. Without the option of testing other medical grade materials, the focus for the next series of experiments shifted to testing the effectiveness of the incorporation method.

Qualitatively testing the incorporation of Filastatin into silicone was simple; the molecule is bright yellow. After allowing the samples to soak in silicone for 24 hours, they absorbed enough Filastatin to turn yellow. The next series of experiments were chosen in order to determine the rate at which the molecules were incorporated into the samples and in turn eluted. First, a standard curve for the concentration of Filastatin was created. Filastatin was serially diluted from a concentration of 25uM to 1.5uM. Absorbance values were measured at 400 nm to test the correlation between concentration and absorbance. Figure 3 shows that the relationship is linear with an R^2 value of 0.9897. The equation for the trend line was used to calculate Fialastatin's concentration in solution as it was absorbed and then eluted from the samples. After determining the correlation, absorption and elution tests were done to measure the incorporation of Filastatin into silicone samples.

Absortion of Filastatin by silicone samples was measured by soaking the samples in 1.5 ml of 25 uM Filastatin in Tris pH 8.5. for two days. Figure 4 shows how much Filastatin was absorbed over time through this experiment. The concentration of Filastatin in Tris pH 8.5 plateaued around 3uM, showing that Filastatin had been incorporated into the silicone. The amount of Filastatin incorporated into the samples was observed by periodically measuring the absorbance of Filastin at 400 nm over the course of two days. During the first two hours, the six samples varied greatly. The samples' absorption began to converge after 24 hours, and by 48 hours they were almost exactly the same. The final Filastatin concentration was roughly 22uM less than where it began; the samples had each taken up 33.71 nanomoles of Filastatin. After measuring absorption, the rate of elution was tested.

Once the samples had reached equilibrium with Filastatin in solution, they were moved to a fresh plate with 1.5 mL 10mM Tris pH8.5. The absorbance of each sample was read periodically at 400 nm over two days. Some samples that had absorbed Filastatin were then coated in polydopamine. The rate of elution for the two separate conditions were compared. Figure 5 shows the release of Filastatin over time from each condition measured by absorbance of the solution at 400 nm. Time points up to 720 minutes created a roughly linear elution rate of 0.833 nanomoles per hour for the silicone squares and 1.08 nanomoles per hour for squares coated in polydopamine after Filastatin. The elution rate that was measured was generated from samples prepared in the same manner as the cell adheison assay, making it safe to assume that 0.8 nanomoles per hour would be enough for a therapeutic effect. These elution values were used to calculate the theoretical cost of a Filastatin incorporated catheter.

Determining if the incorporation method was sustainably manufacturable started with a cost analysis of catheter that was incorporated with Filastatin. Cost analysis was performed to figure out how much money it would cost to produce one full catheter with the absorption of Filastatin and Filastatin with a polydopamine layer. Using the moles of Filastatin used to treat each silicone sample was used as the basis of the analysis. The small samples were used as the base unit for the catheter. Taking into consideration only the Filastatin and silicone, the total cost of treating a silicone catheter with Filastatin would be $9.97; the addition of polydopamine increased cost to $10.68. The breakeven point was calculated to be $24.03 per catheter based on what *C. albicans* infections cost. The cost of this catheter is comparable to on the market antimicrobial catheters and less than the cost of treating one systemic infection. After showing production was feasible, cell adhesion assays were repeated with catheter segments.

The silicone used in the previous assays was a sheet, the next assays tested catheter segments. The silicone catheter from Seeking Health™ had an outer diameter (OD) of 11 mm and inner diameter

(ID) of 8 mm. When testing this catheter, two options for cutting were tested: rings and curved squares. Rings were cut to a depth of 2mm and curved squares were cut 8 x 8 mm. Rings were chosen over curved square segments due to difficulty cutting the squares. Uneven square curvature lead to an increased error between replicates, even within the same assay. The decision to use rings was made after considering the results of the cell adhesion assay done as previously described. Figure 6 shows the same experimental conditions as the assay done using silicone sheets. In comparing the decrease in adhesion, the assay done using rings was 65% more effective. This is the ultimate reason that rings were chosen over squares.

Cutting the rings to 2mm deep rings proved to be variable. To account for the rings not being a consistent shape (some were lopsided), they were weighed to assure that size was equal by mass. Once preliminary testing determined a proper cutting method, a cell adhesion assay was performed in triplicate to determine the decrease in cell adhesion for each incorporation method. The results are presented in Figure 7. Comparing the normalized absorbance values to the positive control revealed that the polydopamine coating alone had the largest decrease in cell adhesion (71.7%), followed by Filastatin then Polydopamine (70.5%) and Filastatin (67.6%). All of these values were statistically similar to one another as well as Filastatin co-incubated. These results are roughly congruent with those from the previous assay done using silicone sheets. Again, the incorporation of Filastatin proved to be successful but not different from nor affected by polydopamine treatments. Since both incorporation and decreasing cell adhesion were effective with catheter segments, they were used for the completion of the project. In order to distinguish between treatment conditions, a long term assay was chosen as the next stage of experimentation.

Previous cell adhesion assays were done with a 2.5 hour incubation time and a starting cell count of 0.3OD per milliliter. This assay was adjusted to more closely replicate *in vivo* conditions. The

starting cell count was changed to 1000 cells per milliliter, much closer to the minimum infectious dose of *C. albicans.* Two sets of samples were incubated concurrently. Treatment with crystal violet and subsequent absorbance readings were done at 22 hours and 45 hours. The hope of a longer incubation time was to distinguish between treatment methods; results are presented in Figure 8. The 22 hour reading demonstrated that Filastatin, both absorbed and in soluble form, had an expected effect on cell adhesion. Filastatin in solution decreased cell adhesion by a third and Filastatin absorbed into the samples halved cell adhesion. The polydopamine treatments had an adverse effect, the absorbance of crystal violet was higher than the positive control. The samples that were read at 45 hours produced different results. Polydopamine treatments did not significantly decrease cell adhesion and the absorbance readings were widely variable. The solubilized Filastatin condition revealed a decrease in cell adhesion of 60% and samples with Filastatin absorbed into them decreased adhesion by 80%. The absorbance of the Filastatin absorbed condition was not significantly different from the negative control. That is to say that the decrease in cell adhesion was comparable to no cells adhering to the silicone ring.

Discussion

This project refined incorporation methods of Filastatin into silicone to decrease *C. albicans'* ability to adhere to silicone. The final incorporation method controlled pH and incubation time to maximize the final product's antifungal properties. Tris at pH 8.5 proved to be the optimal solvent for the interaction between Filastatin and the silicone samples. Repeated experimentation demonstrated that Filastatin absorbed into silicone decreased cell adhesion by 50-80%. This result was slightly amplified by the addition of a polydopamine layer on top of the Filastatin at short time points; the average decrease rising to almost 70%. While the polydopamine layer covering Filastatin initially increased its effectiveness, longer incubation proved to show the opposite effect. Filastatin absorbed into silicone samples was the most effective method.

Incorporation of Filastatin into the silicone samples was demonstrated by color change (the clear plastic turned yellow) and was reaffirmed by testing the samples' elution rates by absorbance readings when left in Tris pH 8.5. Starting with a 25 uM concentration of Filastatin, the silicone squares absorbed Filastatin until the concentration of the solution dropped to 3uM. When samples eluted Filastatin, they did so until the concentration in solution was 3uM. These results suggest that Filastatin's movement into and out of silicone is controlled by a strong concentration gradient. This may not be the saturation point of Filastatin in silicone, but rather appears to be the point of equilibrium between the solution and silicone samples. It is possible that more Filastatin could move into or out of the samples if the starting concentration was higher. Nonetheless, it was an effective concentration of Filastatin to maintain a therapeutic effect.

Although absorption of Filastatin into silicone was consistently effective, the incorporation methods were not replicable using materials other than silicone. Tests on thermoplastic polyurethane and pellethane showed insignificant decreases in cell adhesion. These results may be in part due to the

cell quantification assays used; crystal violet stained the negative controls more for these plastics than it did when using silicone. It is possible that another cell adhesion assay would produce different results.

Although the silicone samples did not produce data as variable as the other plastics, initial adhesion assays produced somewhat inconsistent data. A large variable may have been the overnight culture. Originally, a colony of *C. albicans* was taken from a YPD (yeast extract peptone dextrose) plate and incubated in 0.2% glucose YNB (yeast nitrogen base) media. The next day the O.D was adjusted to 0.3 to complete the experiment. Repeating the cell adhesion assay with different overnight cultures revealed that a reading of 10 OD/ml or greater before adjusting for the assay did not work well. These cells were presumed to be in death phase. When the initial OD of the overnight culture was 5-7 OD/ml, the cells grew as expected through the assay and were more consistent among replicates. These cells were presumably in log phase. Controlling cell growth resulted in more consistent readings, between replicates as well as experimental conditions on separate occasions.

The long term assay gave the most conclusive data; it demonstrated that Filastatin that is absorbed into silicone retains antifungal properties for 45 hours. The polydopamine samples were wildly variable and resulted in adhesion values close to the positive control while the Filastatin absorbed samples were increasingly effective over time. One explanation for Filastatin's increased effect was that a longer incubation time allowed for an increased effect in transcriptional inhibition. As cells began to adhere to the samples, Filastatin may have permanently blocked transcriptional factors, blocking hyphal morphogenesis. An increased amount of time may have allowed Filastatin to interact with more of the cells that tried to adhere. The adverse effect of polydopamine that was seen at later time points may be explained by its self-assembling properties; the polydopamine layer may have been able to remain well organized at short time points but began to disassemble over time. Cells that did adhere may have been polymerized over, trapping them and allowing them to adhere and replicate.

The future of this project begins with continued experimentation on incorporation. Absorbing Filastatin into medical materials can be affected by things the team did not have time to look into: swelling the material, using other solvents, Filastatin concentration. Swelling was identified as a possibility but the only experimentation demonstrated that Tris buffer was more effective than water. The team tested buffers ranging from pH 5-10, but only two buffers were used and no other solvents were tested. Incorporation of Filastatin was completed at 25uM concentration, but it is possible that the material would retain a therapeutic effect at lower concentrations. Determining the minimum concentration would lead to designing a more cost effective manufacturing process.

The final suggestion for future work is to determine the activity of Filastatin with regards to *C. albicans.* Filastatin's exact mechanism remains unknown. Future work could include using GFP tags to observe if Filastatin is taken into the cell and if it is degraded at any point. Another option is to allow cells to soak in Filastatin and then filter the cells out. If less Filastatin is left in solution it may show that Filastatin is either being taken up by the cells or at least adhering to them.

Improving the incorporation method would have further effects that just preventing *C. albicans* infections in patients requiring urinary catheters. The incorporation methods could have considerable impact on fungal and bacterial infections that begin with cell adhesion. Identifying the interaction between Filastatin and *C. albicans* would allow a target to be identified. Many pathogens cause infection by means of adhesion and biofilm formation; inhibiting this pathway could stop many pathogens from becoming infectious. The preventative measures could be applied to implanted devices other than urinary catheters as well. Catheters are used intravenously, and many other medically implanted devices are made of plastics similar to silicone. Using absorption as an incorporation method for plastics is simple and easily scalable. Developing this incorporation method, as well as the molecule itself, has broad reaching implications across medical devices and individual pathogens.

Figures

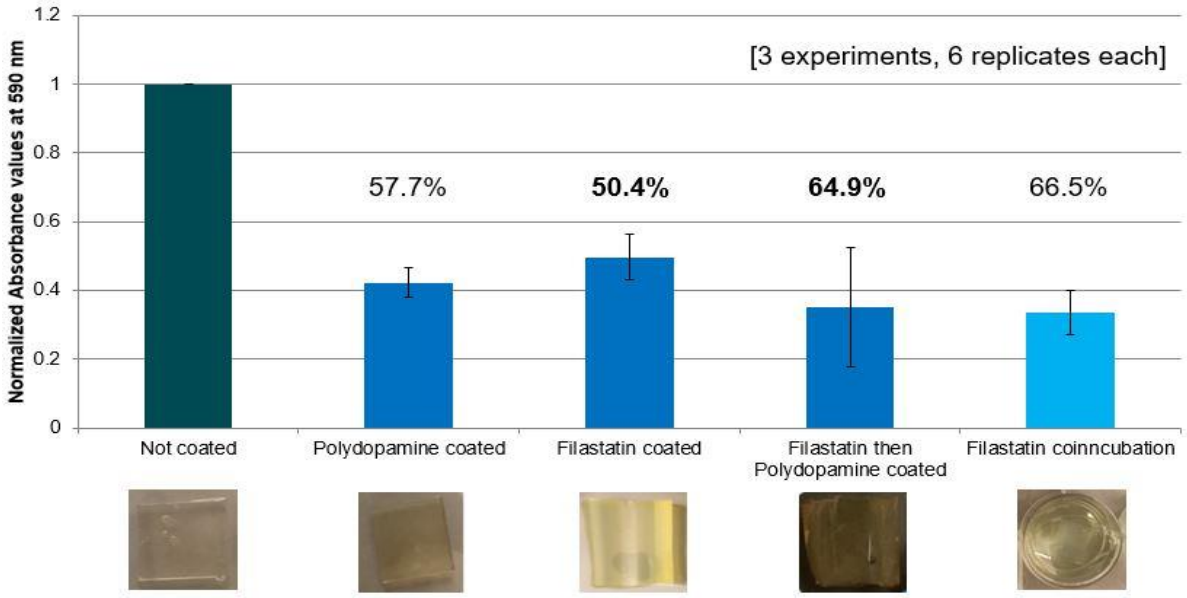


Figure 1: Absorbance readings at 590 nm of cell adhesion assay done on silicone squares

A cell adhesion assay using crystal violet was performed using silicone squares. Absorbance readings of the six biological replicates of each condition over three separate experiments was averaged and normalized to the positive control. Comparison shows the percent decrease of cell adhesion.

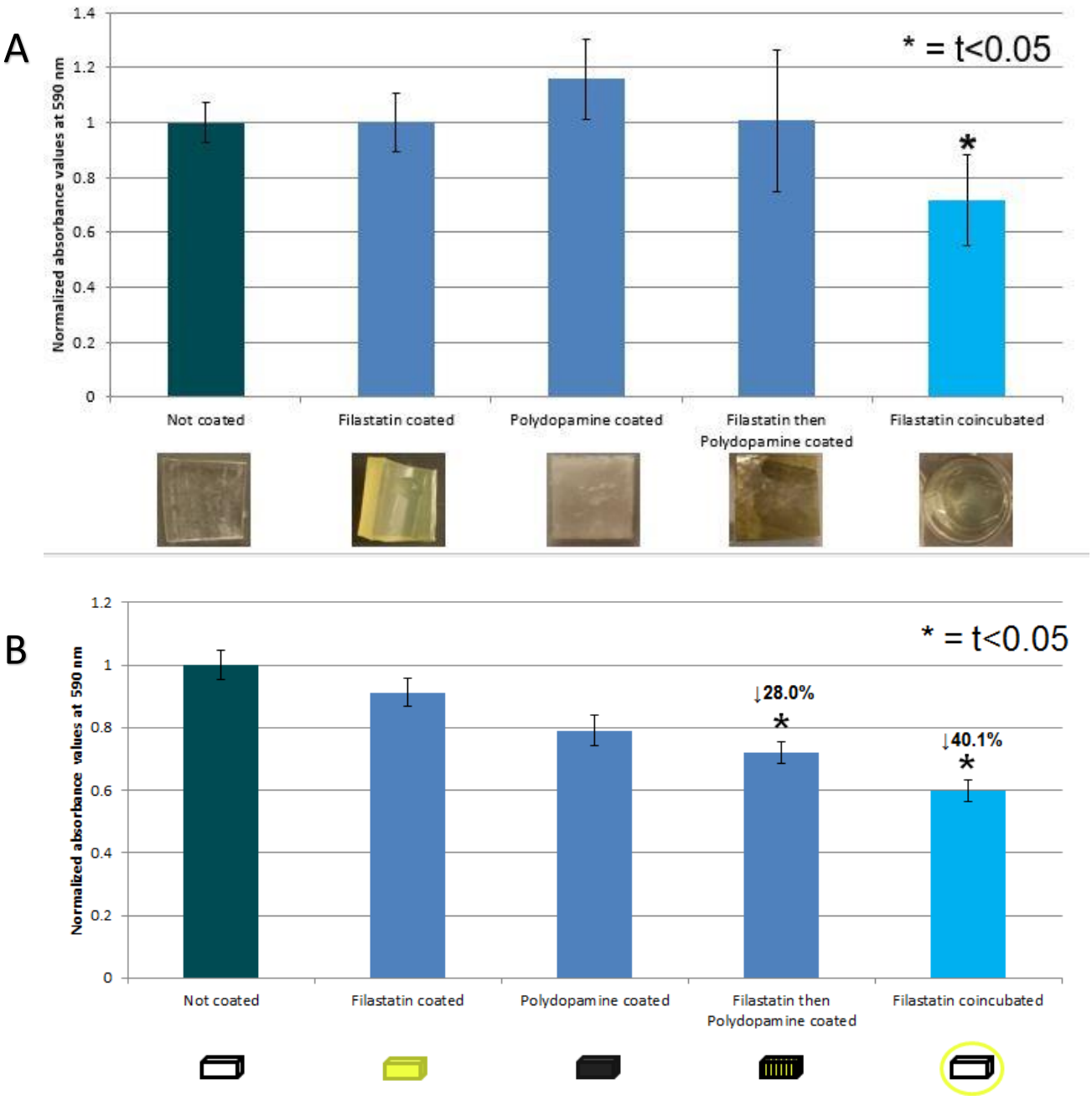


Figure 2: Absorbance of cell adhesion assay on A) Pellethane and B) thermoplastic polyurethane

The same short term cell adhesion assay was repeated using two other plastics. Absorbance values of six biological replicates for each experiment were averaged and normalized against the positive control. A) Pellethane produced inconsistent readings close to the control, suggesting incorporation is not possible. B) Thermoplastic polyurethane resulted in statistically significant decrease for the Filastatin absorption treatment, but even this was only a 28% decrease.

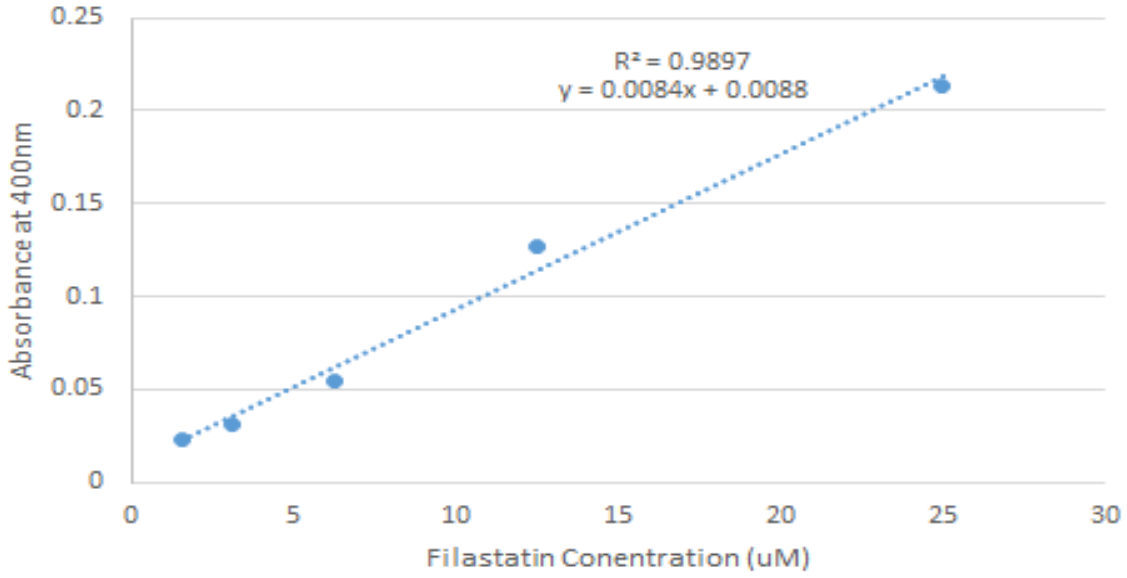


Figure 3: Absorbance versus Filastatin concentration correlation of Filastatin serial dilution

Stock solution of Filastatin at 25uM was serially diluted six times. The absorbance of each solution was read at 400nm. The correlation is almost perfectly linear, with an R^2 value of 0.9897. The best fit line for the absorbance readings was used to calculate the concentration of Filastatin at absorbance readings taken during absorption and elution testing.

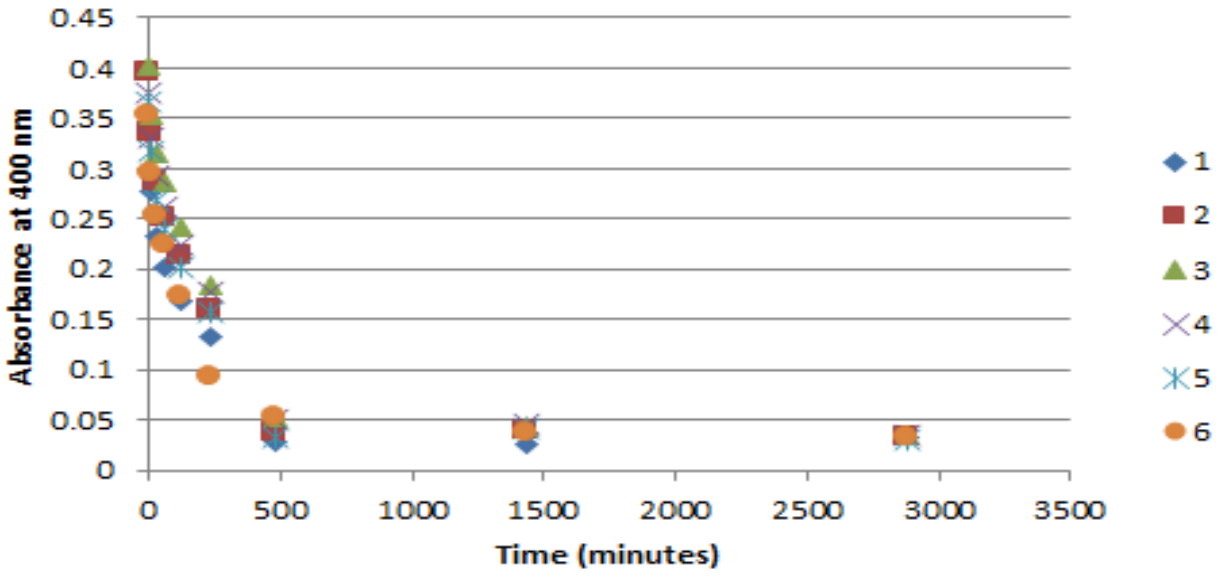


Fig.4: Concentration of Filastatin as it is absorbed into silicone over two days

Six silicone square samples were left in 1.5mL of 25uM Filastatin for two days. Absorbance readings were periodically taken at 400 nm and showed a consistent decrease in the concentration of Filastatin left in solution. This proved that Filastatin was being taken up by the samples and incorporated into the silicone.

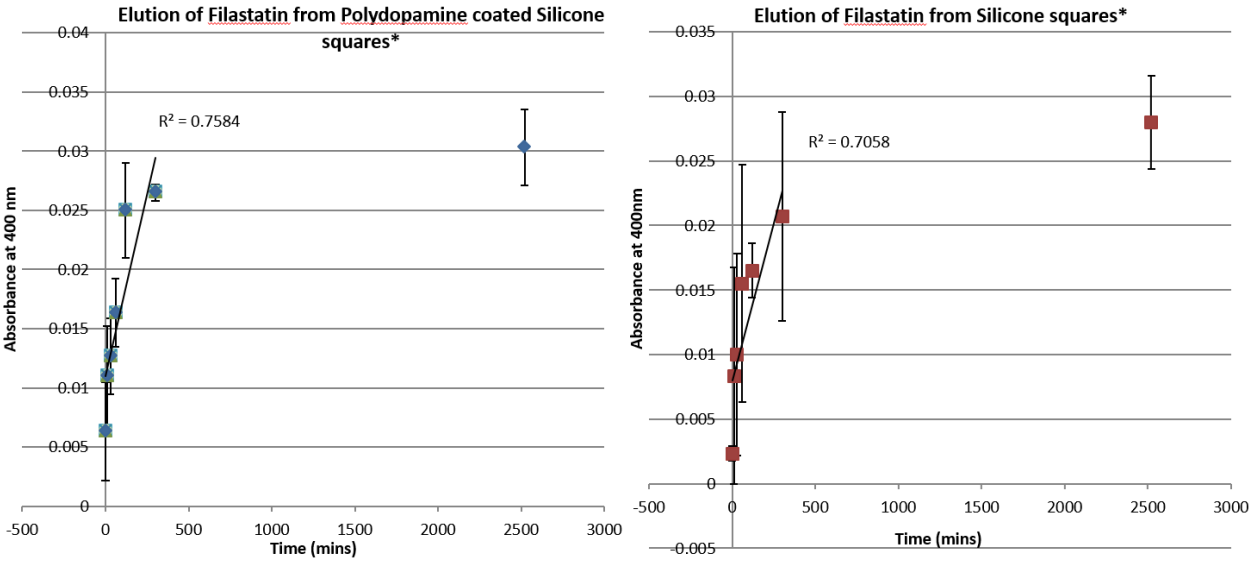
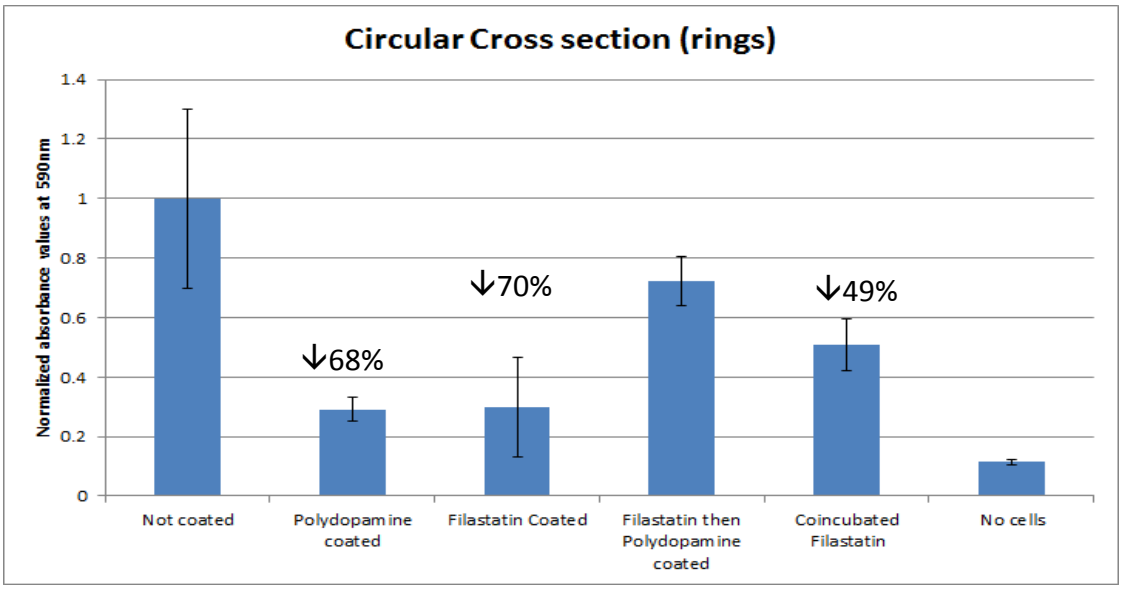


Figure 5: Absorbance of Filastatin eluted from silicone with and without polydopamine layer

Six of each silicone treated sample were left in 1.5 mL 10uM Tris pH 8.5. Absorbance readings were measured at 400 nm, the increase in absorbance showing that Filastatin was eluted slowly over time. The graph on the right shows the elution rate of samples treated with both Filastatin and polydopamine while the graph on the right shows the elution rate of samples that had absorbed Filastatin.

A



B

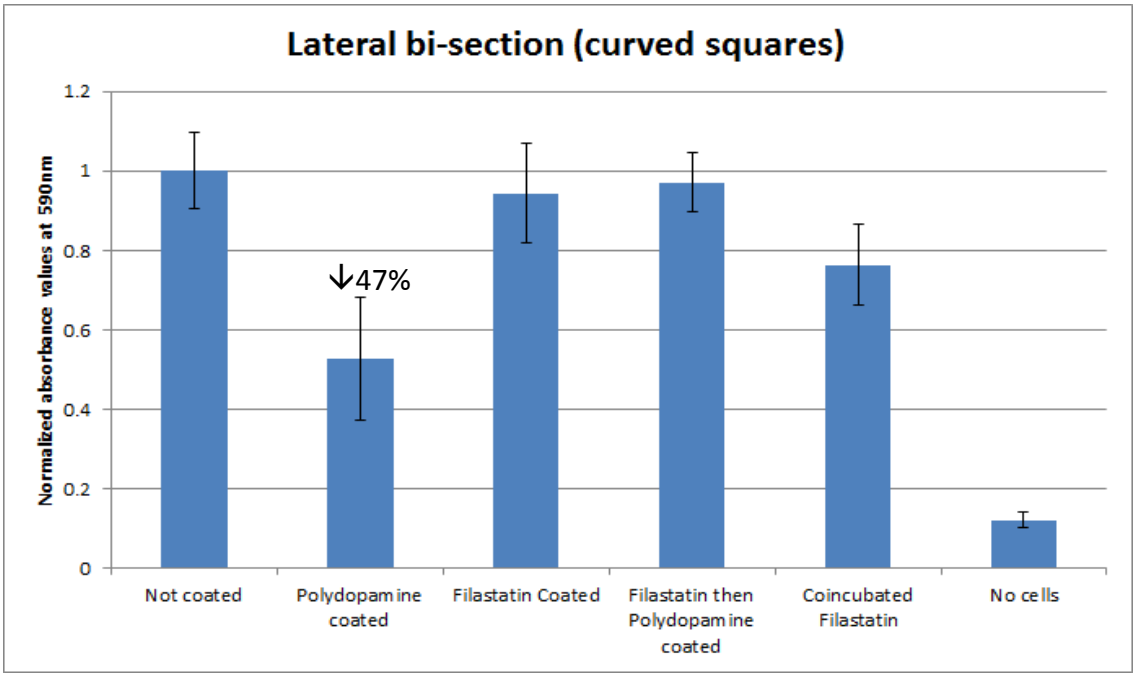


Figure 6: Absorbance readings from cell adhesion assay done with rings and squares side by side

A cell adhesion assay was performed using both squares and rings cut from a silicone catheter. A) Rings cut from the catheter significantly decreased cell adhesion over each condition, similarly to the previous assays. B) Squares cut from the catheter showed lesser decrease in cell adhesion and were more difficult to cut consistently.

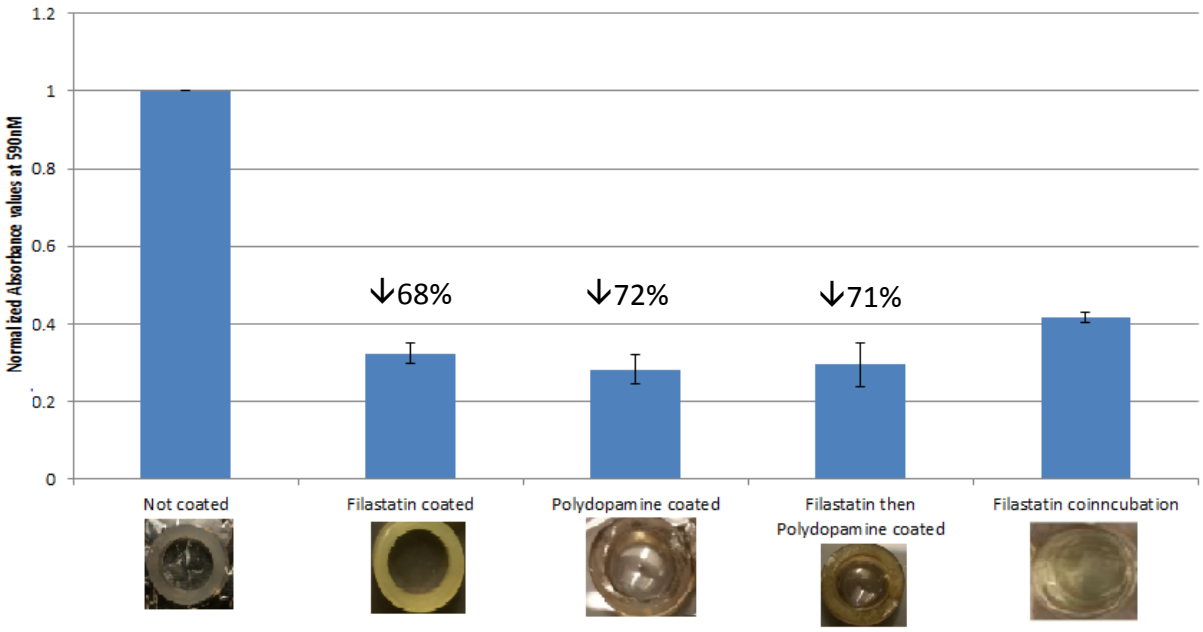


Figure 7: Absorbance readings from cell adhesion assay done on silicone catheter rings

A silicone catheter was cut into rings 2mm deep. A cell adhesion assay was completed and absorbance readings for the crystal violet were done at 590 nm. These results were congruent with results using the silicon sheet; all conditions were similar and significantly different from the uncoated samples.

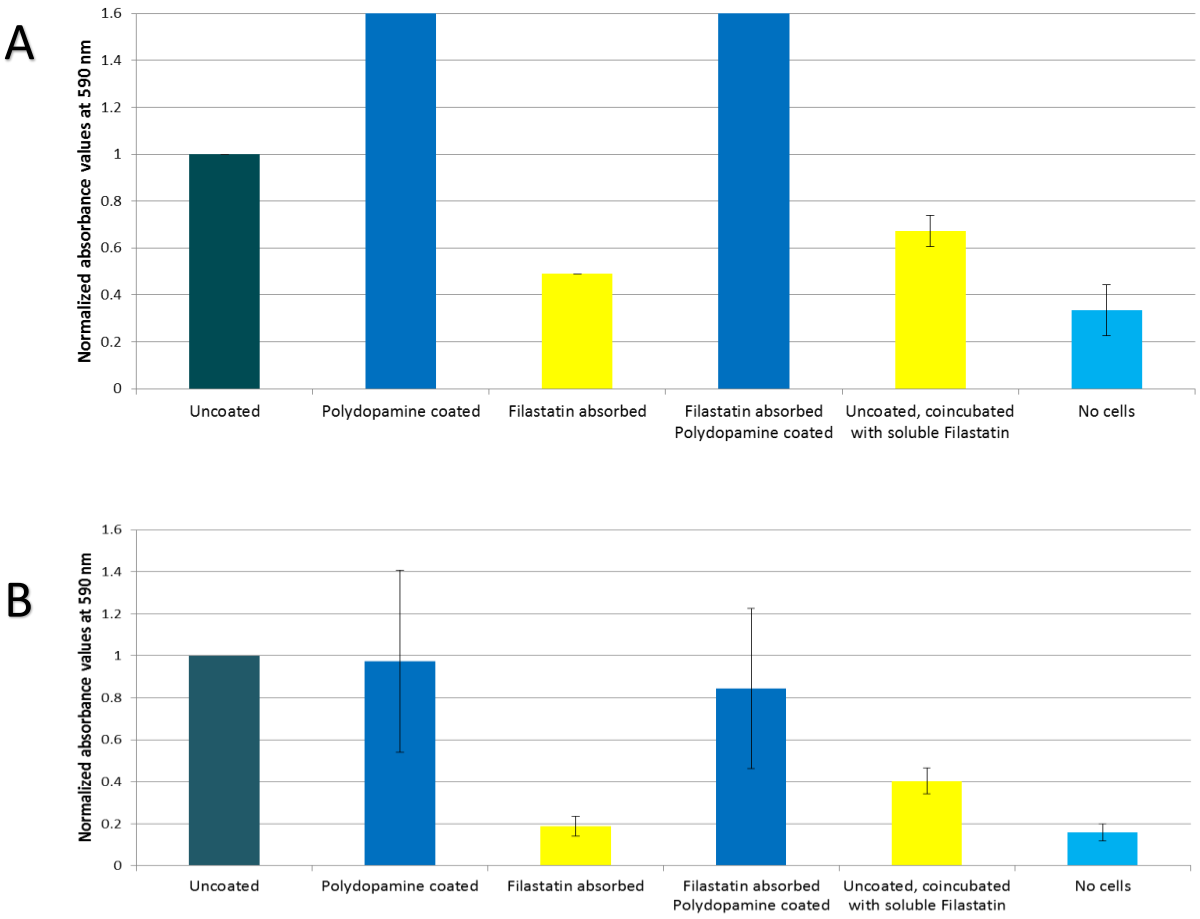


Figure 8: Absorbance readings of cell adhesion assay on silicone catheter rings over two days

The cell adhesion assay as previously described was adapted to more closely mirror *in vivo* conditions. The starting cell count was decreased from 1.5 x 10^7 to 1500 cells. Incubation time was increased from2.5 hours to 22 hours (A) and 45 hours (B). Samples were incubated with cells at 37° C and 80 RPM. After treatment with crystal violet, the absorbance was taken at 590nm. These readings were normalized to the positive control of an uncoated sample. A) Absorbance readings of crystal violet that was released from cells that had adhered to silicone rings after 22 hours of incubation. The samples that were co-incubated with Filastatin decreased cell adhesion by 30% and the sample that with Filastatin absorbed into them had a 50% decrease. Polydopamine treatments had an adverse effect. B) Absorbance readings at 45 hours showed that polydopamine treatments had no significant effect on cell adhesion. The soluble Filastatin samples had a decrease of 60% and the Filastatin absorbed samples had a decrease of 80%. The Filastatin absorbed sample was not significantly different from the negative control.

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