# Methodology to encapsulate progesterone-producing prostate cancer PC3 cells in polyethylene glycol diacrylate thin films

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**Abstract**
One approach to solve the problem of hypogonadism is to restore the steriodogenic function of Leydig cells through cell transplantation. In order to dodge the immune response, the implanted cells need to be micro-encapsulated in PEGdA hydrogel. The size of the microcapsules affects the diffusion of oxygen and nutrients, which in turn have an impact on cell viability. We determined the three-dimensional expansion ratio of 10% (w/v) PEGdA (MW12000) hydrogel to be 3.80 and the one-dimensional expansion ratio to be 1.54. We have also described a method to create PEGdA hydrogel thin films of preset thickness, with deviation of 10%. Additionally, we showed that the hydrophobic sex steroid progesterone can diffuse out of the PEGdA hydrogel network into aqueous environment in as little time as 0.5 hours. The presented information may aid in the development of a micro-encapsulation system that could be used for the treatment of hormone related diseases.

Keywords: *Encapsulation, polyethylene glycol, progesterone, prostate cancer cell, cell viability, ultrasound, photo-polymerization.*

## Introduction

Hormone regulation within the human body is governed by the HPG axis comprised of the hypothalamus, the anterior pituitary gland, and the gonads. The HPG axis, through various feedback loops, regulates the production and release of several important hormones including the sex steroids progesterone and testosterone. The upkeep of this regulatory axis is vital to human health, because it controls biological phenomena such as the differentiation of gender, human physiology, and emotions [1].

The HPG axis can be partially or completely disabled when the feedback loops are disrupted and/or the hormone-producing organ fails. Hypogonadism, which is the reduction or loss of function of the gonads, is one example of hormone-producing organ failure [1]. Hypogonadism is frequently observed in older individuals and it has been suggested to be one of the causes of aging [1].

One approach to combat the problem of hypogonadism is to restore the steroidogenic function of the cells found in the gonads through cell transplantation. Unfortunately, cells inserted into the patient through traditional cell transplantation die due to the immune response of the host. To counter the host's immune response, researchers have been deploying the technique of micro-encapsulation [10]. One type of micro-encapsulation involves entrapment of target cells in a porous, bio-inert material such that the host's immune system finds difficulty in detecting and labeling the implanted cells as foreign. The advantages of micro-encapsulation to treat hormone related diseases include immuno-isolation for prolonged cell viability and sustained hormone release by the encapsulated cells to restore hormonal levels. Researchers have had some success in encapsulating pancreatic islet cells for the treatment of diabetes. Similarly, the presented project plan is to encapsulate gonadal cells for the treatment of hypogonadism and the restoration of the HPG axis.

In order to employ the technique of micro-encapsulation, four parameters need to be addressed, namely biocompatibility, material biodegradation rate, material mesh/pore size, and microcapsule size [3]. Biocompatibility is a measure of the toxicity of the material to the host; the material used for encapsulating the targeted cells should be tolerable to the cells and the host. Biodegradation rate is the time scale of the material degradation within the host due to biological processes; the material should be non-biodegradable such that the encapsulated cells will not be exposed to the immune system [7]. Material mesh size allows for the selective diffusion of substances into and out of the microcapsules [8]; appropriate material mesh size allows for the diffusion of oxygen, nutrients and waste while blocking out large antibodies. Microcapsule size affects the diffusion rate into and out of the microcapsule; effective microcapsule size allows for sufficient diffusion of oxygen and nutrients to reach all of the encapsulated cells.

To address the parameters of biocompatibility, biodegradation rate, and mesh size, polyethylene glycol diacrylate hydrogel was chosen to be the encapsulation material. Polyethylene glycol (PEG) is a ubiquitous synthetic material commonly found in wound dressing and food addictives. PEG is biocompatible and assumed to be non-degradable within the human body [4]. The molecular weight (MW) of PEG determines the mesh size and MW12000 was calculated to be appropriate to block out antibodies (calculations not shown). The diacrylation (dA) of PEG allows the PEG hydrogel to be photo-polymerized and cross-linked using UV radiation.

The biological requirements of the target cell type determine the effective microcapsule size. The diffusion of oxygen and nutrients is inversely related to the microcapsule radius; therefore, the more oxygen and nutrients a targeted cell type requires, the smaller the microcapsule radius needs to be to sustain cell viability. The target cell type in this project is the testosterone producing Leydig cells with the following biological requirements: oxygen consumption rate ($18 \times 10^{-17}$ mol/cell/sec) and cell density ($10^6$ cells/mL). Using the equation developed by Muschler et. al., which not only takes into account the above biological requirements, but also the diffusivity of oxygen ($2 \times 10^{-5}$ $cm^2$/sec @ 37 $^oC$) and oxygen level at implantation site (0.07 mM on average), the effective microcapsule radius was calculated to range from 25 μm to 250 μm [9]. Above 250 μm, oxygen diffusion (assumed to be the most important parameter) drops below 50%, at which the cell viability is thought to be un-sustainable.

Models and methods to create various thicknesses of PEGdA (MW12000) thin hydrogel films encapsulating progesterone producing prostate cancer cells were developed in this project to simulate the microcapsule radius encapsulating testosterone producing Leydig cells. The thicknesses of the thin films correspond to the capsule radii of the microcapsules, while the progesterone used in this project models after the testosterone. Progesterone and testosterone are both sex steroids derived from cholesterol and the prostate cancer cells and the Leydig cells are both found in the gonads. Due to the similarity, prostate cancer cells are a favorable and readily available candidate to be used as a model for Leydig cells for this project.

## Materials and methods

*PEG diacrylate (PEGdA) synthesis*

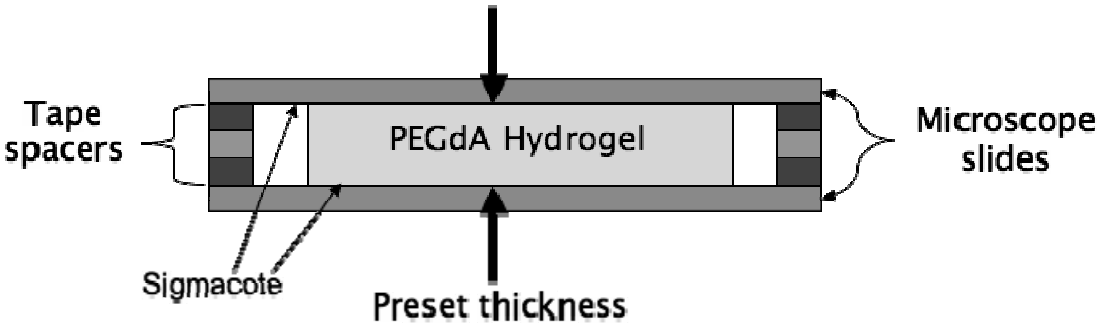
A 250 mL round-bottom flask was flamed dried and purged with nitrogen for ten minutes prior to the diacrylation reaction. 10.0g of dry PEG (MW12000; Sigma Aldrich, St. Louis, MO) was dissolved in dichloromethane. Triethylamine was added in 2.5 molar excess (roughly 0.29mL) of PEG followed by ten-second-interval dropwise addition of acryloyl chloride (roughly 0.31mL) [5]. The reaction flask was purged again with nitrogen for fifteen minutes and stirred overnight. PEGdA was precipitated in excess cold diethyl ether, filtered with Whatman® filter, and dried by vacuum. The product was then dissolved in deionized (DI) water, dialyzed against DI water with 3500MW cutoff dialysis tubing, frozen, and lyophilized.

*Determination of PEGdA hydrogel swelling*

1 mL of hydrogel pre-cursor solution was prepared to 10% (w/v) PEGdA and 0.05% (w/v) Irgacure 2959 photoinitiator (CIBA, Tarrytown, NY) with deionized water as the solvent. The PEGdA solution was dispensed via pipette at 0.1 mL per sample into 6-well microplates. This dispensation occurred at one minute intervals from 4~15 minutes and 13.5~15 minutes with the first sample dispensed corresponding to 15 minutes of UV exposure. The UV radiation had a wavelength of 365 nm and output power of 3.9 $mW/cm^2$ at a distance of 3 inches. When the appropriate UV exposure time had elapsed, the just formed hydrogels were weighed and submerged in 6 mL of deionized water. The microplates were wrapped in aluminum foil and allowed to sit for 48 hours at room temperature. Prior to post-swell weighing, the hydrogel samples were removed from the wells and extraneous moisture was removed using a Kimwipes®. The mass of each hydrogel sample was measured using an analytical balance. The swelling ratio was computed by taking a ratio of the post-swelled mass to the pre-swelled mass.

*Thin PEGdA film production for swelling experiments*

The thicknesses of the thin PEGdA films were controlled by stacking layers of Kapton® Polyimide film, single-sided tape, and/or double-sided tape (25.4, 63.5, 101.6 μm respectively; CS Hyde company, Lake Villa, IL) on the short edges of the microscope slides (Schematic 1). Droplets of 100 μm PEGdA solution were placed on the Sigmacote™ treated microscope slides and a separate microscope slide was placed on top of it. Sigmacote™ (Sigma Aldrich) is a silicon-based solution that provides non-stick coatings and for our application aids in the removal of the hydrogel films from the microscope slides with minimal deformation of the thin gel surface. Metal clamps were affixed on the side of the microscope slides over the tape spacers to create compression and ensure the preset thickness over the tape. This microscope slide assembly with PEGdA solution in between was exposed to UV radiation for 14.5 minutes.




Schematic 1: Device to create thin PEGdA hydrogel films. The present thickness is determined by the layering of film and/or tape spacers.

*Ultrasound determination of hydrogel thickness*

The pre and post-swell thicknesses of a supposed 200 μm sample were measured via ultrasound to check the accuracy of our method to create thin films of PEGdA hydrogel with preset thickness. A 60 MHz ultrasound transducer (NIH Resource Center for Medial Ultrasonic Transducer Technology, fractional bandwidth = 80%, focal length = 5 mm, aperture diameter = 2.5 mm) was used to measure thickness every millimeter along the lateral position of a microscope slide (50 mm total) with the thin PEGdA hydrogel film on top [11]. The surrounding media was deionized water and the resolution of the transducer was approximately 10 μm. The pre-swell thicknesses were measured right after gelation by UV exposure for 14.5 minutes and the post-swell thicknesses were measured after the hydrogel thin film had been immersed in deionized water for at least 48 hours. The distances from the transducer, corresponding to the top and bottom of the hydrogel film, were tabulated by measuring the echo time and the equation: $distance = \left( {echo\ time}/{2} \right) *$ (speed of sound in water at room temperature) [11].

*Progesterone diffusion*

Three samples of 10% (w/v) PEGdA hydrogel containing 0.0031M of progesterone were created to test the diffusion of the sex steroid out of the hydrogel network [2]. To photo-crosslink the hydrogel, three aliquots of 0.3 mL PEGdA solution were exposed to UV radiation for 15 minutes. Upon gelation, each of the three hydrogel samples containing progesterone was immersed in 10 mL of deionized water. The diffusion experiments were carried out in three separate wells in a 6-well microplate. To determine the diffusion progression, 150 μL samples of the surrounding liquid were drawn at various time points from 0.5 hours to 75.5 hours. The progesterone content within the media samples was analyzed using an ELISA detection kit.

*Competitive Enzyme-Linked ImmunoSorbent Assay (ELISA)*

The full protocol for the ELISA used for measuring progesterone levels can be found at http://www.alpco.com/pdfs/11/11-PROGH-305.pdf.

*Prostate cancer cell (PC3) isolation and culture*

Human prostate cancer cells (PC3 cell line) were cultured in F12 cell culture media supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin/streptomycin in a 37 $^{o}$C incubator.

*PC3 cell encapsulation and PEGdA hydrogel fabrication*

Cells were cleaved from culture flasks with 0.5% trypsin/EDTA and resuspended in serum free F12 cell culture media. This cell suspension was incorporated into a pre-cursor solution containing PEGdA (10% w/v), Irgacure 2959 photoinitiator (0.05% w/v), and phosphate buffered saline (PBS) at pH 7.4.

Kapton® Polyimide spacer tapes and films (CS Hyde Company), microscope slides, and metal clips were sterilized via germicidal UV irradiation for 30 minutes. The microscope slide assemblies were then partially assembled with the lower slides and spacers. For hydrogel fabrication, 10 µL of pre-cursor solution was added in triplicate onto microscope slides. Another microscope slide was then added on top of the liquid and metal clips affixed to the assembly to form thin circular films of liquid. The microscope slide assembly was placed under 365 nm UV light for 14.5 minutes at a distance of 3 inches (3.9 mW/cm$^2$). The newly formed thin gels were then removed using sterile tweezers and transferred into a 24-well microplate. 400 µL of serum free cell culture media was added to each well containing gels and to empty wells for negative controls. Samples were then incubated at 37 $^{o}$C for subsequent experimentation.

*Cell Titer-Blue$^{TM}$ viability analysis*

Cell viability was assessed via a Cell Titer-Blue$^{TM}$ Cell Viability Assay (Promega Corporation, Madison, WI). In following with assay instructions, the Cell Titer-Blue reagent was added to all sample unknowns and controls, which were then incubated for 2 hours at 37 $^{o}$C. After incubation, 100 µL of surrounding solution from sample unknowns and controls was transferred to a 96-well microplate and fluorescence was read at 560/590 using a plate reader. Blanks containing only the assay reagent were prepared and were set up to serve as a "low cell" reference to which all other measurements were scaled. Negative control (serum free media and assay reagent) fluorescence values were subtracted from encapsulated cell fluorescence values to obtain the fluorescence from only the released fluorescent compound. Cell viability was assessed over the course of three days. Basic statistics were computed using Microsoft Excel and a 2-sample t-test was used to determine whether there were statistically significant changes over time (day-by-day basis), using Minitab statistical software.

## Results

*Determination of PEGdA hydrogel swelling*

The effects of UV exposure time on the degree of PEGdA hydrogel swelling were investigated. Assuming water is incompressible, the increase in mass of the hydrogel sample is directly related to the expansion in volume (swelling). After a 48 hour waiting period, the

hydrogel samples with 4 minutes of UV exposure time swelled the most, about 3.83 times the original size (Figure 1). As UV exposure time increased, the degree of hydrogel swelling dropped and started to plateau at 13 minutes. At 15 minutes, the hydrogel was assumed to be completely cross-linked and the sample expanded 3.80 times its original volume.

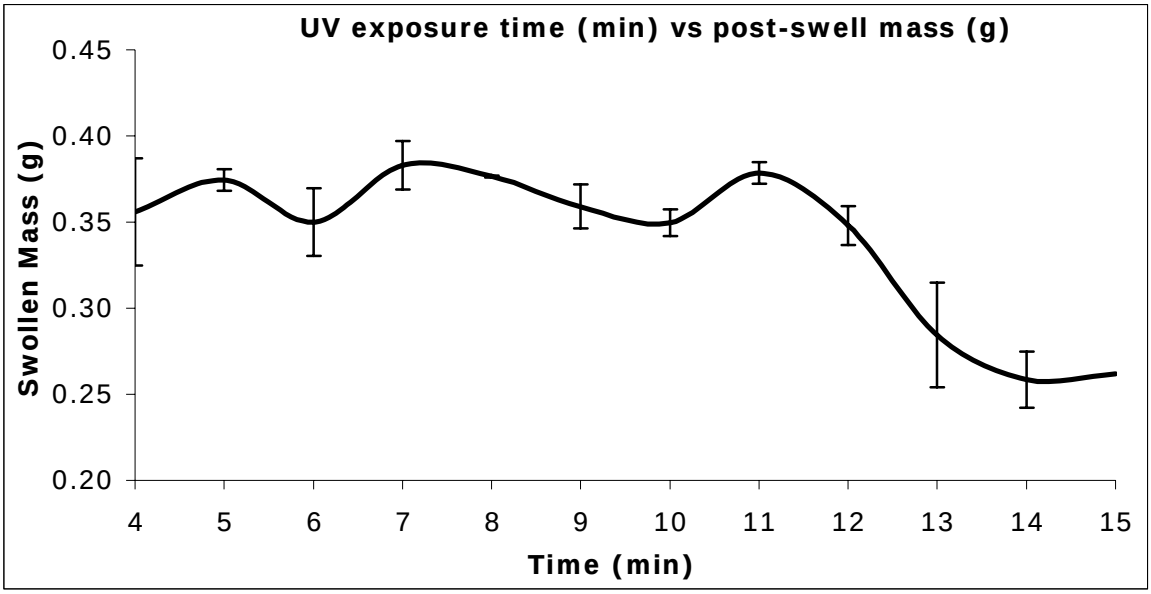



Figure 1: Effect of UV exposure time (4~15 min) on degree of hydrogel swelling. As UV exposure time increased, the degree of hydrogel swelling decreased. The swelling started to plateau after 13 minutes.

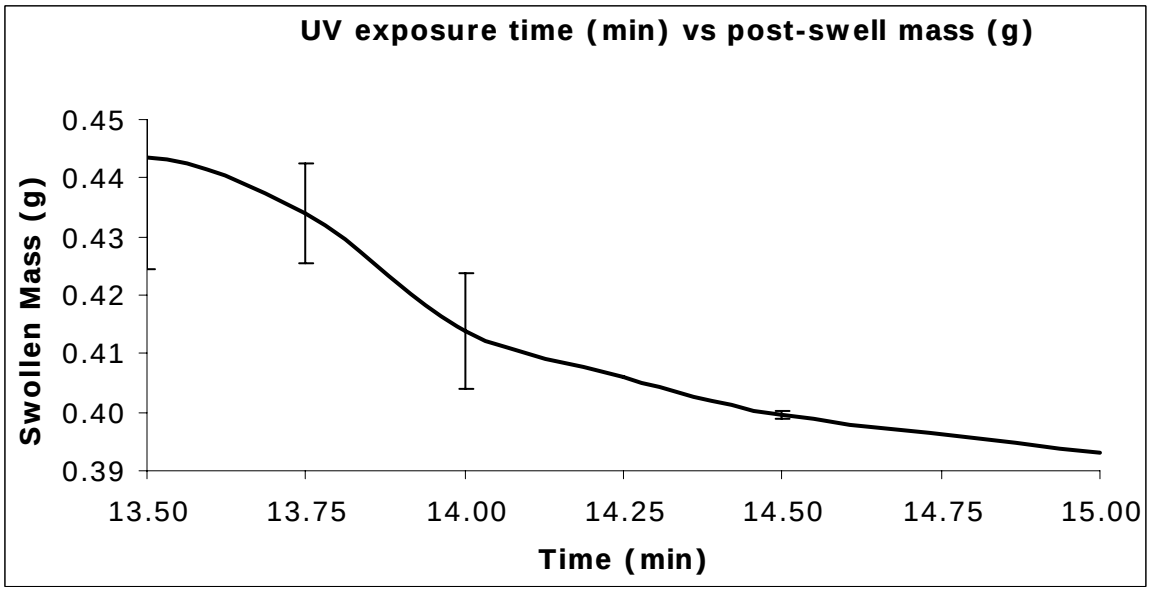



Figure 2: Effect of UV exposure time (13.5~15 min) on degree of hydrogel swelling. 14.5 minutes of UV exposure time was decided to be sufficient for photo crosslinking of PEGdA solution.

A second experiment was run to determine the appropriate time to expose the PEGdA solution with PC3 cells to UV for gelation zooming in on the 13.5 to 15 minute interval. It was decided that at 14.5 minutes, the hydrogel had been sufficient crossed linked (Figure 2).

*Ultrasound determination of hydrogel thickness*

Ultrasound was used to confirm the accuracy of our method to create thin PEGdA films with preset thickness. Both the pre-swell curve and the post-swell curve have lower values in the center of the microscope slide. For a supposed thickness of 200 μm, the average pre-swell hydrogel film measured 220 μm (Figure 3), a 10% difference from preset. The average post-swell thickness measured 340 μm. The linear expansion coefficient calculated by dividing the post-swell by the pre-swell thickness was 1.54.

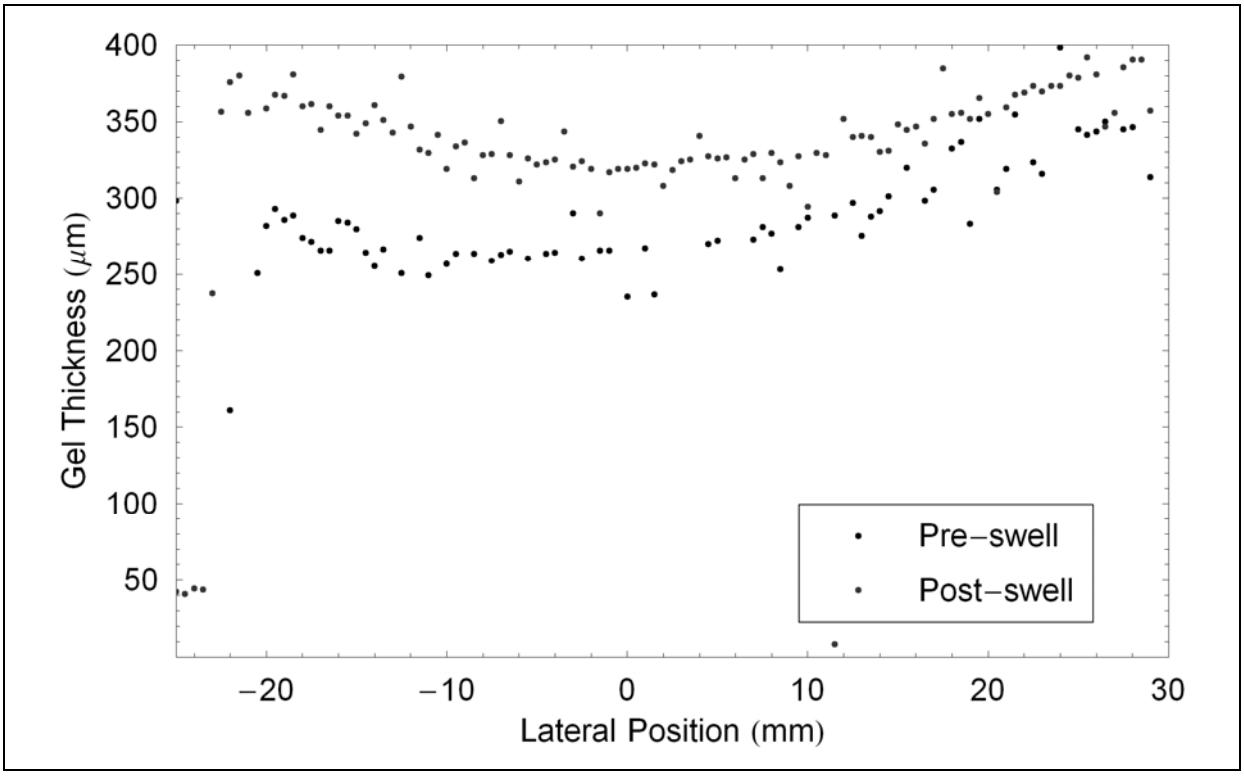



Figure 3: Thickness of pre-swelled (lower curve) and post-swelled (upper curve) hydrogel films as measured by ultrasound. Both curves are concave downward with the lowest value in the middle of the microscope slide.

*Progesterone diffusion*

The amount of progesterone diffusion was determined by measuring the level of progesterone in the surrounding liquid at various time points with an ELISA kit. Progesterone was detected as early as 0.5 hours of incubation (122.4ng/ml) and it reached the high point at 5.5 hours (130.1ng/ml) (Figure 4). After 5.5 hours, the progesterone levels were too high to be measured correctly by the ELISA kit.

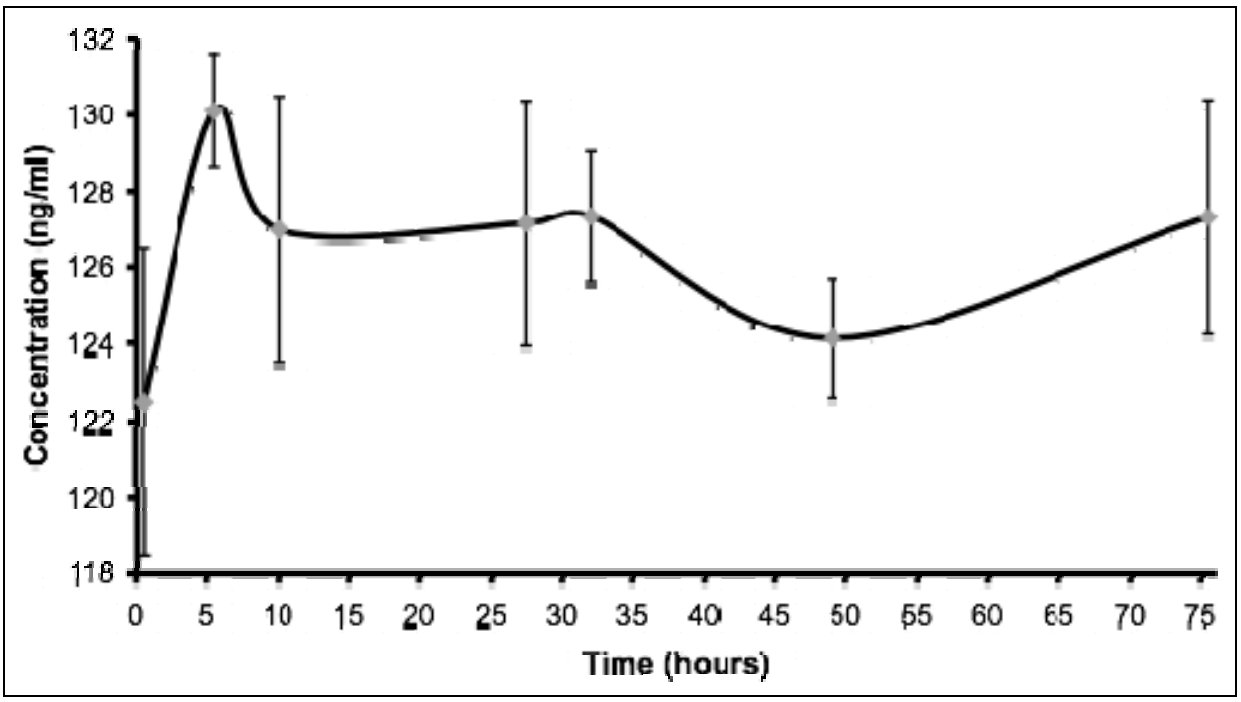



Figure 4: Progesterone levels in the surrounding liquid at various time points. Progesterone was detected as early as 0.5 hours.

*Encapsulated PC3 cell viability*

The viability of PC3 cells encapsulated in various thicknesses of PEGdA hydrogel was measured using the Cell Titer-Blue™ Cell Viability Assay. For any thickness, the viability at day 2 was higher than at day 3, and similarly for day 3 to day 4. At day 4, all cells in any thickness appeared to be non-viable. The validity of the data is questionable because the fluorescence of the sample unknowns for any day was very close to that of the negative control without cells. The fluorescence values shown in Figure 5 are those corrected for the presence of the serum free cell culture media.

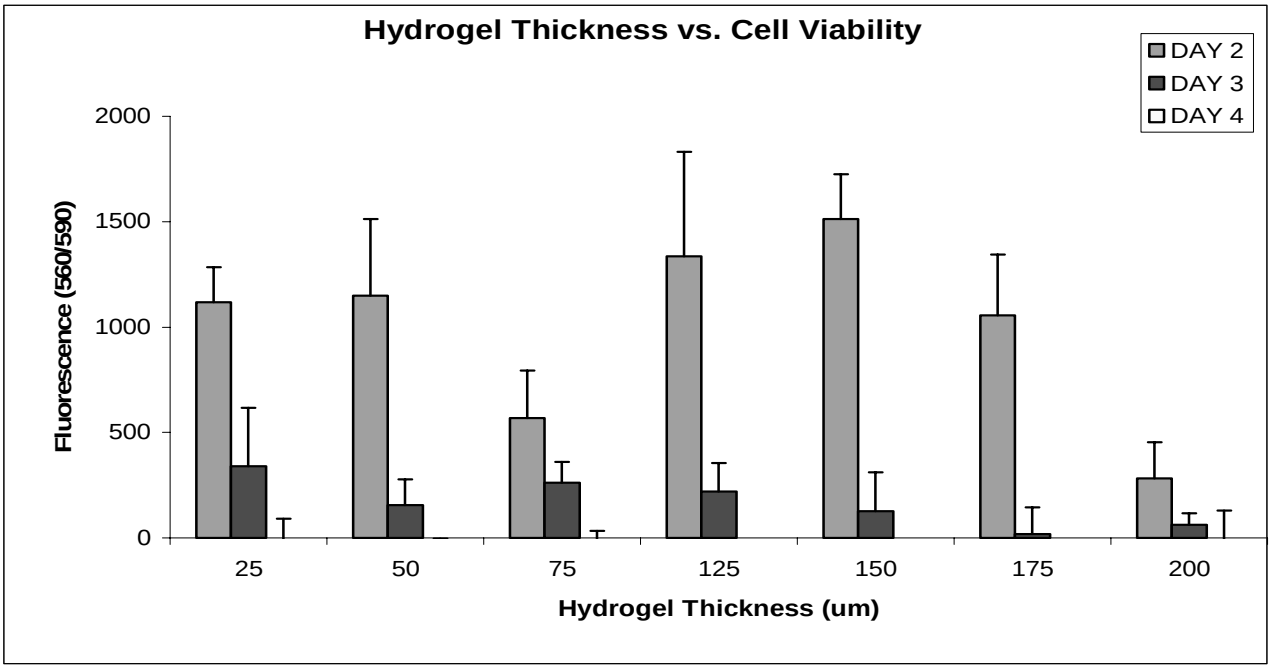



Figure 5: Cell viability of PC3 cells encapsulated in various thicknesses of PEGdA hydrogel. The fluorescence levels were very close to the negative control such that the apparent result is doubtful.

## Discussion

*Determination of PEGdA hydrogel swelling*

The degree of swelling decreased as UV exposure time (min) increased. This phenomenon could be explained by the degree of cross-linking within the PEGdA hydrogel. As UV exposure time increased, the degree of cross-linking within the PEGdA hyrogel also increased, making the PEGdA hydrogel much more rigid and stiff [6]. The increase in rigidity makes the water uptake much more difficult and thus less volume expansion. Additionally, a greater degree of cross-linking results in a smaller mesh or pore size and thus less space for water molecules to fill.

The expansion of the PEGdA hydrogel in deionized water at full polymerization (14.5 minutes UV exposure) was 3.80 times of the original size. In other words, a 1 $cm^3$ piece of our PEGdA hydrogel will expand to 3.8 $cm^3$ after long term implementation. Considering this expansion/swelling, in order to obtain post-swell capsules with radii ranging from 25~250 μm, one needs to make capsules with initial radii of 17.5~175 μm. It is important to note that all the samples within the experiment were conducted using de-ionized water with no cells, whereas the actual encapsulation system will incorporate cells and the hydrogel will be submerged in serum. The presence of cells and the usage of serum will cause the hydrogel to expand less than in the case of no cells and deionized water. The reduced expansion of the actual system leads to smaller size capsules than the ones observed in the experiment, and this is an advantage in terms of diffusion of oxygen and nutrients.

It was previously determined that the threshold of UV exposure time for PC3 cells was 15 minutes (data not shown). In order to obtain an appropriate mesh size, the PEGdA hydrogel needs to be completely cross-linked. The difficulty lies with finding the optimal UV exposure time such that the PEGdA hydrogel is completely polymerized but the PC3 cells remain viable. In the second swelling experiment where the UV exposure time ranged from 13.5~15 minutes, it was decided that at 14.5 minutes, the PEGdA hydrogel was sufficiently polymerized. At this time point, the UV exposure time had not passed the threshold of viability for the PC3 cells.

*Ultrasound determination of hydrogel thickness*

The ultrasound experiment determined the accuracy of our method to create PEGdA hydrogel thin films with preset thickness. The data showed that there is a 10% deviation from the preset thickness of 200 μm. In order to obtain more precise deviations and to determine the accuracy of our method in creating hydrogel thin films of other thicknesses, more samples at various thickness needs to be evaluated via ultrasound.

Unlike the swelling experiment, which measures three-dimensional expansion, the ultrasound measures one-dimensional expansion in thickness. The linear expansion ratio was calculated to be 1.54. This linear expansion ratio is close to what one would expect for the theoretical ratio tabulated from the three-dimensional expansion value. The cube root of the three-dimensional ratio is 1.56, only 1.3% different from the ratio that was measured by ultrasound.

With the particular device setup and preset thickness, one would expect the PEGdA hydrogel thin film created would have a uniform surface; however, the ultrasound data shows that the thin films had lower thickness toward the mid section of the microscope slides than at the edges. Both the pre and post-swell thickness curves had the same shape of concaving downward. This phenomenon could have resulted from the disruption of the hydrogel surface when the microscope slides were peeled apart prior to measurement or it could have resulted from the transducer being closer to the surface of the hydrogel in the mid section due to heavy machine weight. In the ultrasound measurement, the transducer is moved by a precision motor attached to a horizontal supporting beam in the machine set up. In the mid section, the weight from the heavy precision motor could have presented a classic three-point bending situation in which the middle of the supporting beam is actually lower than the edges [11]. One would need to measure a piece of solid material of know constant thickness across the lateral direction to determine whether or not the apparent downward concave curve of the data is due to disruption of hydrogel uniformity or due to lowering of transducers because of heavy weight.

*Progesterone diffusion*

The progesterone diffusion experiment was carried out to determine whether or not the hydrophobic sex steroid could diffuse out of the PEGdA hydrogel into an aqueous environment. The diffusion of sex steroids out of the PEGdA hydrogel microcapsule is important because it dictates whether or not cell transplantation with micro-encapsulation is a feasible way to treat hormone related diseases. In the progesterone diffusion data, one could observe progesterone in the surrounding liquid as early as 0.5 hours. After 5.5 hours, all samples from the surrounding media had high levels of progesterone. In fact, the progesterone was at such high levels that they

were at least one order of magnitude above the calibration curve. One could predict the progesterone level within the surround liquid would eventually reach an equilibrium level of 0.2659 mg/mL (0.117 mL of 25 mg/mL progesterone solution added originally in 11 mL of deionized water)—a value too high for the ELISA kit to accurately detect. Regardless, the data points show that progesterone does diffuse out of the PEGdA hydrogel and micro-encapsulation is still a viable way to treat hormone related diseases.

*Encapsulated PC3 cell viability*

Cell-Titer-Blue® assays were carried out to determine the cell viability of the PC3 cells encapsulated in various thicknesses of the PEGdA hydrogel. The validity of the data was doubtful because the fluorescence values from the released fluorescent compound was close to that of the negative control (cell culture media and assay reagent). In order to improve the precision of the data, one would need to increase the fluorescence response from the encapsulated cells by increasing the density of cells per well or lengthening the incubation period from 2 hours to 4 hours to allow more time for the cells to convert the non-fluorescent dye. Additionally, more controls should be pursued in the future, namely a positive control (non-encapsulated PC3 cells cultured in tissue culture polystyrene microplates) and another negative control involving the hydrogel thin films without PC3 cells.

## Conclusion

With the results that we have gathered in our experiments, we determined that the three-dimensional expansion ratio is 3.80 and the one-dimensional expansion ratio is 1.54 for the 10% (w/v) PEGdA (MW 12000) hydrogel. We also present a method to create PEGdA hydrogel thin films of preset thickness, with 10% deviation, by using Sigmacote® treated microscope slides and film/tape spacers of known thickness. The thin hydrogel films can be used to simulate capsule diameter for the encapsulation of gonadal cells. Although currently the system is targeted towards testosterone producing Leydig cells, it is not hard to imagine that one could extend our method to test for appropriate microcapsule size of other cell types.

## Acknowledgements

Special thanks to Amy Chung, Dr. Kristyn Masters, Dr. William Murphy, and Dr. Brenda Ogle from the University of Wisconsin-Madison, Dr. Craig Atwood from the Veteran's Administration hospital in Madison, WI, and Lydia Hwang from Promega Corporation for their continued support of this project.

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