Microencapsulation of Cells

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Abstract

A method of microencapsulating Leydig cells for the long-term time release of male reproductive hormones in vivo *is desired. These cells are currently being studied for use in anti-aging therapy. The final design must avoid issues of biocompatibility, immune responses and hypoxia commonly associated with microcapsule implantation. Currently, the encapsulation of mouse Leydig cells with diacrylated polyethylene glycol (PEGDA) using a constructed microfluidic device is being pursued. Crosslinking is achieved using a photoinitiator and 365 nm ultraviolet light. The viability of encapsulated cells is being examined; future work entails microencapsulation optimization and diffusion studies to assess capsule function* in vitro.

Introduction

Hormone replacement

The hypothalamic-pituitary-gonadal (HPG) axis regulates the hormones needed for reproduction including testosterone, inhibin, activin and others. Normally, in the male, production of testosterone is stimulated by release of leutinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary (Figure 1). These hormones act on Leydig cells to release testosterone, and on Sertoli cells to release inhibin and activin. Negative feedback to the hypothalamus and anterior pituitary acts to keep LH and FSH levels within normal limits (Widmaier *et al.*, 2004). Natural aging results in the dysregulation of the HPG axis, and thus the loss of reproductive ability. It is hypothesized that with aging, a decrease in the ability to release adequate levels of testosterone and inhibin causes overproduction of LH and FSH by the HPG axis, and that such unregulated activity may be a causative agent for the wide-ranging diseases of aging in humans (Bowen and Atwood, 2004).

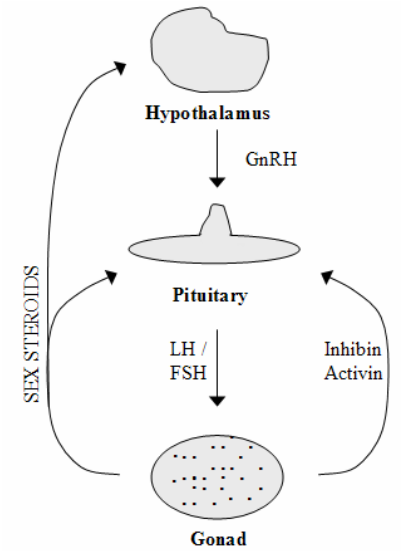

Figure 1. Hypothalamic-pituitary-gonadal axis, showing negative feedback loop from the gonads (Adapted from Morohashi, 1997).

Currently, androgen replacement therapy, which attempts to restore normal testosterone levels in the male, is achieved via oral administration, injections, or skin patches (Machluf *et al.*, 2003). Successful testosterone therapy maintains normal serum levels of testosterone and its derivatives, and has been shown to increase muscle strength, stabilize bone density, and restore secondary sexual characteristics. However, significant side effects, such as hypertension and bone density loss, can arise from long term use and unsteady dose administration (Machluf *et al.*,

2003). Another disadvantage of these therapies is the requirement of multiple treatments and consequently, multiple clinical visits.

Encapsulation of cells

An alternative method for restoring the HPG axis is transplanting cells. This study attempts to design a novel method of encapsulating Leydig cells, which will provide an *in vivo* mechanism for the release of testosterone while providing a physical barrier to the host's immune system. Such assisted hormone regulation, through the use of functional cells, should restore the normal function of the HPG axis by decreasing LH and FSH production in response to normal hormone release.

Cell encapsulation provides a physical barrier for transplanted cells against a host immune response, as the capsule prevents infiltration by immune cells and antibodies. For the purpose of treating hormonal disorders, the technology aims to allow therapeutic tissue transplantation without the use of immunosuppressant drugs, which can have serious side effects. Encapsulation of living cells was first described in the early 1950s, but only in the last decade have significant advances been made in the area of microencapsulation (Uludag *et al.*, 2000). In particular, microencapsulation is advantageous due to small diffusion distances for nutrients, gases, waste products, and hormones to travel to and from the encapsulated cells.

Hydrogels are crosslinked hydrophilic polymers that have been used extensively in the field of microencapsulation. While hydrogels provide a desirable immunoprotective barrier, their mesh structure also makes them highly permeable to oxygen, nutrients and other molecules—a necessary and attractive quality for maintaining viable encapsulated cells. Most importantly, efficient transport of hormone(s) in the HPG axis must be maintained for functionality of the encapsulation system.

Depending on the hydrogel polymer, crosslinking can be achieved via chemical, temperature, or light methods. Photocrosslinking involves converting the liquid polymer to a hydrogel by free radical addition using a photoinitiator, which generates radicals following absorption of ultraviolet (UV) or visible light. The reaction is fast, controlled, and can be carried out under ambient or physiological conditions (Nguyen and West, 2002). In the context of cell encapsulation, there are obvious concerns about the potential cytotoxicity of the photocrosslinking process. However, limitations can be overcome using mild conditions such as a low light intensity, short irradiation time, and low photoinitiator concentrations in the presence of cells (Nguyen and West, 2002). Prior to crosslinking, the polymer must often be modified to include two or more reactive groups.

Figure 2 outlines the basic principles of encapsulation using mouse Leydig cells and a photocrosslinkable polymer. Cells are first isolated from an animal donor, and may be expanded *in vitro* under standard culture conditions prior to encapsulation. The cell suspension is then combined with a photocrosslinkable polymer solution. When combined with a photoinitiator species, exposure to low intensity, long wavelength UV light results in the crosslinking of the polymer-cell suspension. Formation of a crosslinked meshwork results in the essential suspension of cells within the polymer. Due to the hydrogel's

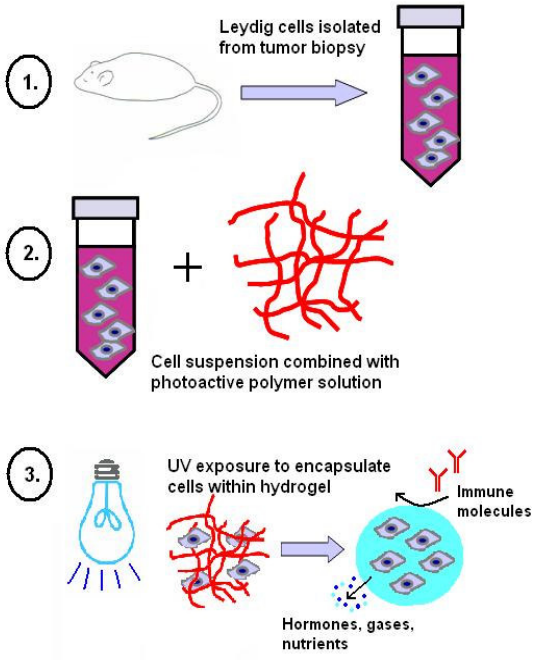

Figure 2. Basic steps in cell isolation and encapsulation within hydrogel process (Adapted from Masters, 2005).

inherent mesh network, nutrients, gases, wastes, and hormones are allowed to diffuse in and out of the capsule freely. However, the mesh size is small enough that larger, immune molecules and cells are prevented from entering the capsule.

Problem Statement

The goal of this work is to create microcapsules that will sustain viable Leydig cells for at least six months. This extended lifespan, relative to the aforementioned therapies, is preferable, as the clinical applications for such technology are expected to be expensive and inconvenient on a per treatment basis. However, it is widely recognized that current microcapsule designs fail prematurely for three primary reasons: insufficient biocompatibility, limited immunoprotective qualities, and hypoxia (de Groot *et al.*, 2004). A novel method for cell encapsulation must overcome these issues.

Biocompatibility

One of the most common causes of microencapsulation failure is an inflammatory response to the transplant site, resulting in overgrowth of the capsules by fibroblasts and macrophages. The incitement of the host inflammatory response can be reduced by using biocompatible materials.

Immunoprotection

Preventing the entry of leukocytes and antibodies is the primary purpose for cell encapsulation. As human antibodies are on the order of 150 kDa, and leukocytes much larger, a molecular weight cutoff (MWCO) of 75-100 kDa is capable of blocking the entry of antibodies and cells (Machluf *et al.*, 2003). This MWCO will also allow desired hormones from the HPG axis such as testosterone (300 Da), inhibin (32 kDa), activin (28kDa), FSH (36 kDa), and LH (1.2 kDa) to diffuse through the capsule. Oxygen radicals and nitric oxide (NO) will also diffuse

readily, and may exert a negative effect on cell viability. It is noted that activated macrophages often release nitric oxide and cytokines, and are partly responsible for encapsulated islet graft failure (de Groot *et al.*, 2003). In the Leydig cell system, co-encapsulation with Sertoli cells may aid in providing immunoprotection via their natural release of several immunosuppressive factors (de Groot *et al.*, 2003).

Encapsulated cell necrosis

A significant complication arising from encapsulated systems is the decreased efficiency by which oxygen, nutrients, and metabolic wastes diffuse in and out of the microcapsules. This causes cell necrosis, especially at the center of encapsulated cell masses. The poor diffusion of these molecules is not primarily a result of small mesh size in the hydrogel, but also arises due to the long diffusion distances across the capsule wall. By limiting this distance to 100 μm or less, hypoxia can often be avoided (Murphy, 2005, personal communication). Other solutions depend on the location of capsule injection. Often, capsules are injected into the peritoneal cavity where the oxygen supply is by diffusion only (de Groot *et al.*, 2004). Subcutaneous injection sites report longer viability of Leydig cells, possibly due to a better oxygen supply (Machluf *et al.*, 2003).

Design Specifications

The proposed design should allow for the controlled release of testosterone from a murine cancer Leydig cell line. In addition, LH and FSH must diffuse readily across the capsule surface in order to ensure adequate HPG axis regulation. The microcapsules must provide adequate immunoprotection *in vivo*. Based on these requirements, a MWCO of about 75 kDa is desired. Specifically, this means the design must demonstrate its ability to block the entry of macrophages and antibodies.

The proposed design (along with its manufacturing process) must be sufficiently cytocompatible. Ideally, the encapsulated cells would remain viable for several years and maintain adequate hormone release throughout that duration. At minimum, cells should remain viable in a non-degraded scaffold for 6 months. An extended lifespan is necessary to keep the costs of any clinical treatment sufficiently low.

In order to validate such technology, the proposed method of encapsulation must be repeatable. Experimental data must show consistent results in order to claim design success. As such, for a specific microcapsule production method, the mesh and capsule sizes must be predictable within certain ranges so as to ensure adequate performance of the capsules *in vivo*.

For a detailed outline of the design specifications, see Appendix.

Biomaterials for cell encapsulation strategies

Alginate/poly-L-lysine/alginate (APA)

The first encapsulation scheme considered incorporates a three-layered capsule consisting of alginate and poly-L-lysine (PLL). Comprising the core is a spherical bead of alginate, a natural polymer derived from brown algae. Alginate is composed of the polysaccharides β-D-mannuronic acid (M) and 1,4-linked α-L-guluronic acid (G) (Figure 3).

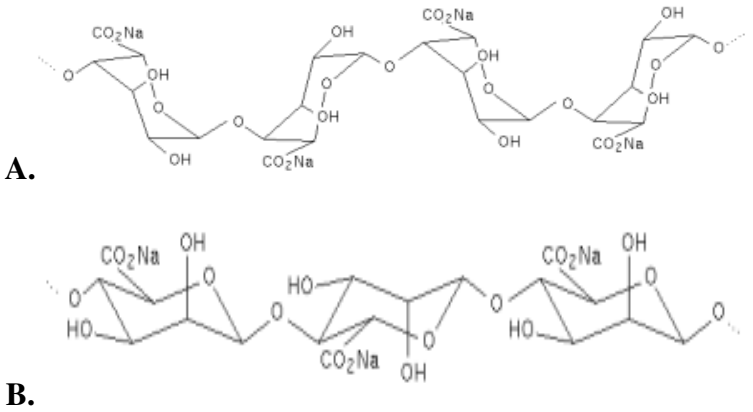

Figure 3. A. Two repeating units of guluronic acid; **B.** Two repeating units of mannuronic acid. Figure from http://www.cybercolloids.net/library/alginate/structure.php.

The ratio of these two acids (G:M) controls numerous microcapsule properties including mechanical strength and permeability (de Groot *et al*., 2003).

The encapsulation procedure involves suspending the cells of interest in a solution of alginate. In aqueous solution, alginate is polyanionic and can be crosslinked by a divalent cation, such as Ca^{2+}. Upon gelation of the alginate core, a layer of PLL is added. PLL is polycationic and readily binds to alginate (Figure 4).

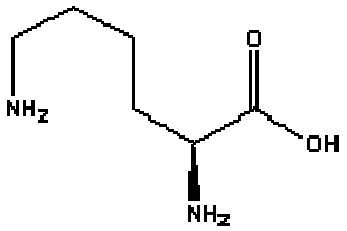

Figure 4. Lysine structure. Figure from chemfinder.com.

To a large extent, it is the PLL that determines microcapsule permeability; it can be modified to achieve varying degrees of pore size. Following the layer of PLL, the entire microcapsule is coated with alginate, which is essentially inert in physiological conditions when compared to PLL. This layer prevents a host response to the positively charged components of PLL (de Groot *et al*., 2003).

The APA system has a few advantages that were considered in the decision process. Being a natural polymer, alginate ranks high among materials in terms of biocompatibility when properly purified. That is, these microcapsules generally do not elicit an inflammatory response. Furthermore, this system has been used extensively, and copious literature has been published concerning the modifications made to improve efficacy. Such widespread study indicates promise with this system. That the materials are readily available and alginate crosslinking and subsequent PLL layering are very simple to carry out also makes the APA system attractive.

Unfortunately, these advantages cannot compete with this system's numerous shortcomings. Repeatable results are highly desired; however, alginate varies widely depending on its source. Inconsistent G:M ratios may lead to misinterpreted results. In addition, the aforementioned biocompatibility can only be achieved via thorough purification and sterilization, which greatly increase the cost. Furthermore, the method of encapsulation does not guarantee complete cell mass confinement. In this case, the host's immune system may respond, causing a buildup of macrophages and fibroblast overgrowth on the microcapsule. This leads to loss of graft function and ultimately encapsulated cell death. Finally, the degradation of APA microcapsules *in vivo* is essentially uncontrollable. For a long-term graft, control of this property is imperative.

Hyaluronic acid (HA)

The synthesis of bioactive hydrogels from natural polymers such as glycosaminoglycans (GAGs) has recently become a topic of intense investigation, as their physiological activity has been shown to promote cell and tissue propagation (Segura, *et al.,* 2005). In particular, hyaluronic acid (HA) has been extensively studied, due to its numerous natural functions, physical properties and biocompatibility (Hahn, *et al.*, 2004). HA is a natural, linear, and negatively charged polysaccharide, composed of disaccharide repeat units of D-glucuronic acid and N-acetyl-D-glucosamine (Figure 5).

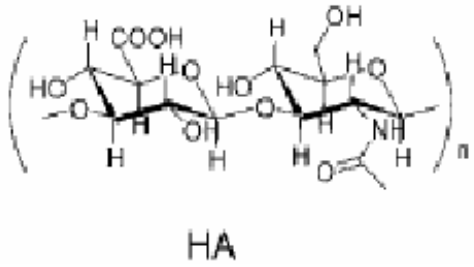

Figure 5. Hyaluronic acid consists of disaccharide repeat units of D-glucuronic acid and N-acetyl-D-glucosamine, with a (1→4) linkage. Figure from Hahn, *et al.*, 2004.

In a natural setting, intracellular HA serves several important functions, including the provision of cell migration, growth and differentiation, and assistance in wound repair. When uncrosslinked, soluble HA has limited mechanical properties, and degrades rapidly. However, chemical modification or photocrosslinking forms HA hydrogels and improves these characteristics. Chemical change usually involves the carboxyl or hydroxyl groups of the HA backbone; the carboxylic acid groups can be modified with methacrylamide, while the alcohol groups have been modified with divinyl sulfone and diglycidyl ethers (Segura, *et al.,* 2005).

Hyaluronic acid is considered advantageous because it is naturally derived, non-immunogenic, and bioactive. Disadvantages arise in its limited mechanical strength and its somewhat rapid enzymatic degradation by hyaluronidase. However, it has recently been shown that HA-composite hydrogels can be created that are hydrated, pliable, bioactive, and allow for the controlled release of a model protein (Leach, *et al.* 2005). By restricting macromolecule diffusion, protein delivery is possible for extended time periods—on the scale of weeks. (Note: limited diffusion may also adversely affect the diffusion of soluble factors needed by the encapsulated cells.) A hybridization of materials is also possible (e.g. PEG and HA), and may increase crosslinking density, thus improving mechanical strength and lengthening capsule lifespan.

Polyethylene glycol (PEG)

Polyethylene glycol (PEG) is a synthetic polymer composed of repeating C-C-O sequences (Figure 6) of specified length.

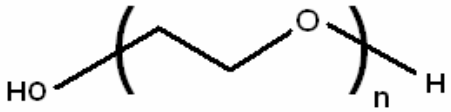

Figure 6. Repeating unit of polyethylene glycol.

Common examples include PEG 3400 and PEG 8000, where the number indicates the molecular weight of the chain. The terminal groups are initially hydroxyls, though these can be replaced with photoactive acrylate groups prior to crosslinking. Encapsulating cells using PEG involves exposing a solution of the acrylated polymer, a photoinitiator, and the cells to light of selected wavelengths, thereby photocrosslinking the PEG capsule and suspending the cells in the hydrogel.

One advantage of using PEG is its intrinsic ability to minimize protein adsorption which consequently reduces fibroblast overgrowth. This property has been found to be a function of chain length, as longer chains have better repellent properties (Sawhney, 1999). Another advantage of PEG over other materials is its synthetic nature, which allows for reproducibility in results. Synthetic polymers such as PEG avoid the issue of source variability that accompanies natural polymers such as alginate. Perhaps most important is PEG's ability to be controlled within the laboratory. Chemical modification allows for the precise tailoring of PEG to a specific application. Various polymer variables can be manipulated by changing parameters such as concentration, molecular weight, and degree of functionalization.

While minimal protein adsorption is advantageous in warding off an immune response, it can also be considered an obstacle to cell adhesion, which follows protein adhesion. To incur any kind of cell response, PEG must modified, which adds greater complexity to the system. Procedures such as adding adhesion peptide sequences allow for precise control over ligand presentation, but are more time consuming and costly.

Past Work: Droplet Generation

Previously, a piezoelectric Bioprinter approach was developed as a method of microcapsule production due to its low cost, high repeatability, and spatial control. By applying a

voltage to two piezoelectric devices placed near a nozzle outlet, droplets of solution can be squeezed out at precise rates and sizes. An Epson® R200 inkjet printer was reconfigured to release controlled droplet sizes on standard microscope slides based on a graphical software control interface (Figure 7, left). Spatial and volume control were achieved, but mass production scalability was limited (Figure 7, right). Several thousands capsules must be produced to release measurable hormone concentrations *in vitro*. Tens of thousands will be required for *in vivo* studies.

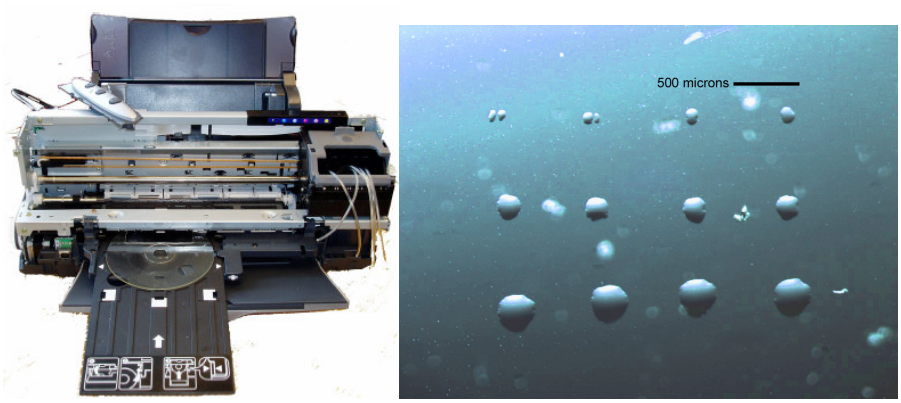

Figure 7. (**Left**) Epson® R200 printer after 'bioprinter' conversion. Printer casing was removed, ink cartridges replaced with polymer dispensers, and electronic ink chips reset using a universal Epson® chip resetter. The CD tray was modified to hold a standard microscope slide. Printing control is achieved using GIMP software on a Linux operating system and a bioprinter driver (Victorey, 2005). (**Right**) PEG (8 kDa, 10% w/v) printed on a dry glass microscope slide, viewed at 40x using a stereroscope.

Current design

PEG biomaterial

The current design uses PEG 8000 (Fisher Scientific) that has been functionalized via the derivatization of acrylate end groups (Figure 8). This reaction was performed as

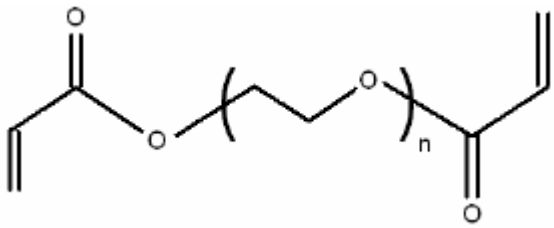

Figure 8. PEG diacrylate, which is photocrosslinkable.

described (Cruise *et al*., 1999). Briefly, 8.0 g of PEG 8000 was dissolved in benzene and azeotropically distilled to remove water. Four times molar excess (per end group) of triethylamine was added to deprotonate the hydroxyl group. Four times molar excess (per end group) of acryloyl chloride was then added dropwise, and the reaction was stirred overnight. The solution was filtered to remove triethylamine salts and precipitated by addition of ethyl ether. Upon complete evaporation of ethyl ether, the product was dissolved in deionized water, frozen, and lyophilized. Figure 9 depicts the complete reaction mechanism.

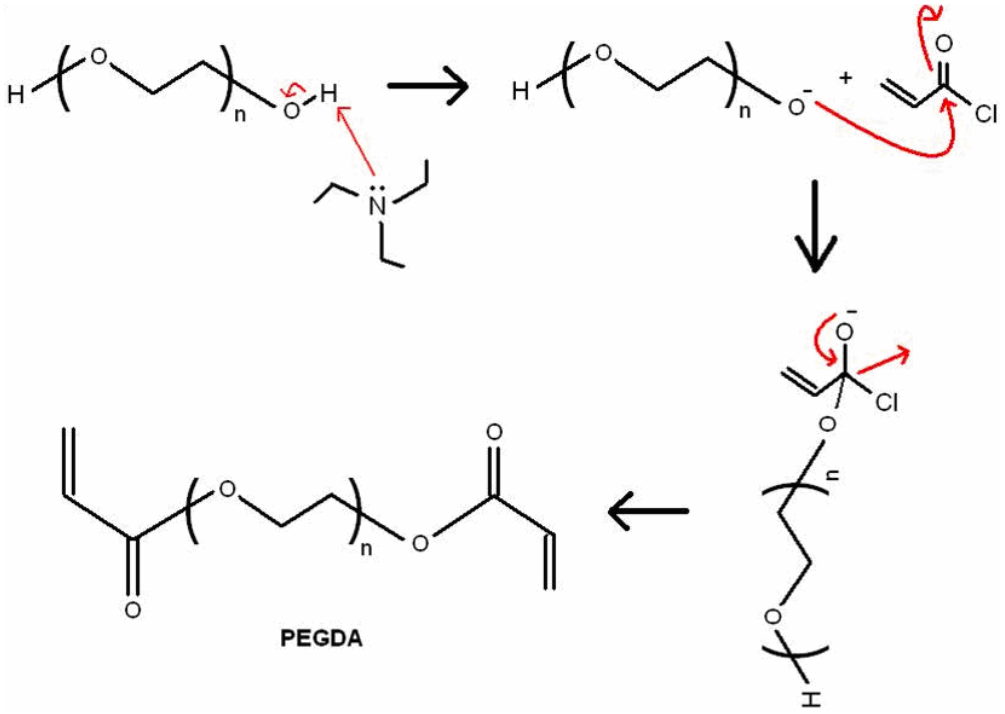

Figure 9. Reaction mechanism of the derivatization of the acrylate groups on PEG.

Photocrosslinking scheme

Because PEG is highly hydrophilic, it seems feasible to combine the polymer and cell suspension with an organic phase (such as mineral oil or a more cell-compatible triglyceride solution) to form small droplets of encapsulated cells prior to UV light exposure. The ensuing radical reaction then crosslinks the hydrogel, and the organic phase can be washed away.

Previous studies on the cytocompatibility of various photoinitiating systems have shown that Irgacure 2959® (2-hydroxy-l-[4-(hydroxyethoxy)phenyl]-2-methyl-1-propanone) (Ciba Specialties®) provides the best cell survival rates in the presence of low intensity UV light (Bryant *et al.,* 2000). However, Irgacure 651® (Figure 10) is a faster acting photoinitiator, allowing for shorter crosslinking times. Furthermore, microcapsule production using a microfluidic device may dictate shorter UV exposure time due to limited tubing lengths. This necessitates the use of a faster photoinitiator. In both cases, 3.9 mW/cm^2 intensity and 365 nm wavelength are used.

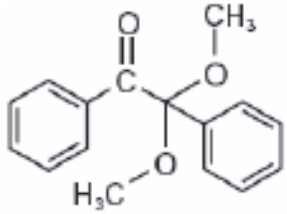

Figure 10. Irgacure 651® is the fastest acting photoinitator from Ciba Specialties®.

Mouse MA-10 Leydig tumor cell line

A murine cancer Leydig cell serves as a model cell type for microencapsulation studies (Figure 11).

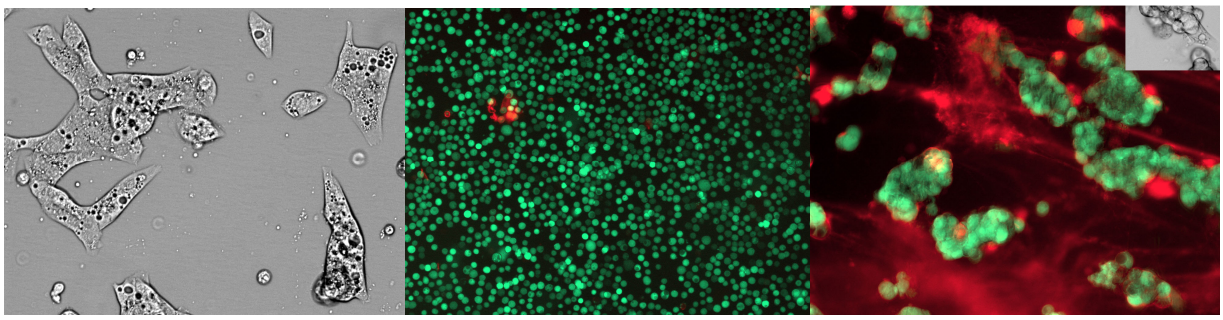

Figure 11. Mouse MA-10 cell histology. (**Left**) Cells at 3rd passage adhered to culture flask surface at 200x. A LIVE/DEAD assay was conducted in a 96-well plate at 4th passage. (**Center**) Fluorescent image 10 min after plating at 100x and (**Right**) 1 d after plating at 200x. Inset shows cells under visible light at 200x.

Cells were obtained from Dr. Craig Atwood's laboratory at the VA Hospital (Madison, WI). MA-10 cells were cultured in Waymouth's complete medium, 15% heat-inactivated donor herd horse serum, and 1% penicillin/streptomycin and maintained at 37°C under 5% CO_2. Cells were

passaged using 10x trypsin/EDTA near 70-80% confluence. In previous independent study work, testosterone secretion in response to LH and FSH stimulation was shown for this cell type (Figure 12).

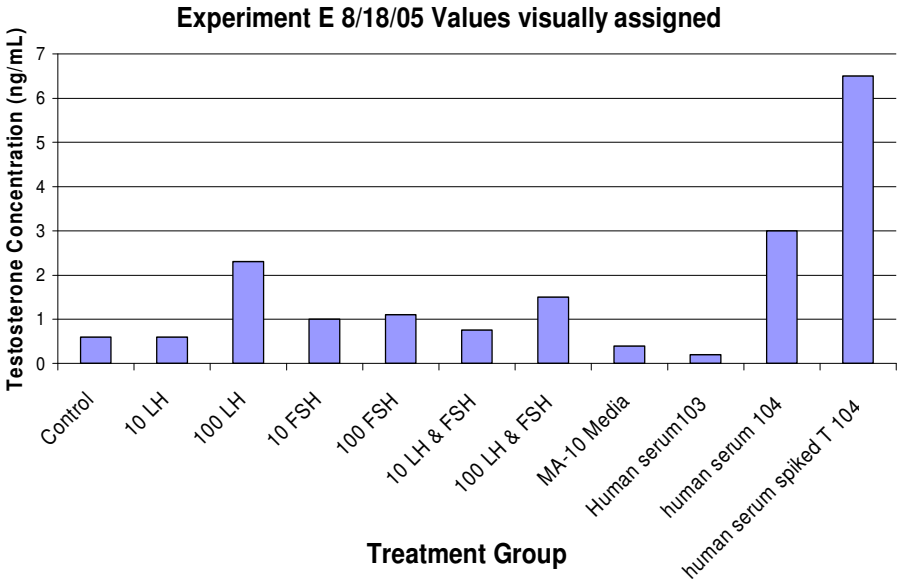

Figure 12. Testosterone release by mouse MA-10 cells in response to LH and FSH stimulation, using a competitive ELISA kit (ALPCO Diagnostics).

Stimulating Leydig cells with LH or FSH is an integral part of the *in vivo* HPG axis as described earlier; physiological concentrations of LH and FSH are on the order of 10 mIU/mL in rats and humans (Machluf *et al*., 2003; Atwood, 2005, personal communication). A competitive testosterone ELISA kit (ALPCO Diagnostics) was used to measure hormone release.

Cell seeding densities for microencapsulation experiments will be on the order of $1x10^6$ cells/mL (Machluf *et al*., 2003). A sufficient quantity of cells must be transplanted so as to sustain an animal model or patient near normal serum testosterone levels, namely 1.7 ng/mL for rats and 2.0 ng/mL for humans, for at least 6 months. This includes contributions from transplanted cells as well as the individual's innate capability to produce some testosterone.

Microfluidic capsule generation

To form microcapsules we used a microfluidics device based on the design that Jeong *et al*. (2005) successfully used for the formation of 75-275 µm capsules composed of PEG with proteins included for drug delivery applications.

Production of Device

Specifically molded polydimethylsiloxane (PDMS) devices were formed by attaching plastic anchors to a petri dish and attaching 14 and 19 gauge needle tips in the desired positions to the plastic anchors (Figure 13, left). PDMS prepolymer and curing agent (Dow Corning) were mixed in a 10:1 PDMS:curing agent ratio according to instructions and poured over the petri dish setup. The PDMS was cured at 80°C for 1.5 h. Following PDMS solidification, the needles were removed to form the channels for fluid flows. The channels were joined by removal of the cured PDMS remaining at the tip of the 14 gauge needle site, allowing for interconnected fluid flow throughout the device.

Next, 1 mm outer diameter (OD)/0.5 mm inner diameter (ID) borosilicate glass pipettes (Sutter Instruments) were pulled to ~20 µm inner tip diameter for inlet aqueous sample flow. For outlet flow, 1 mm OD/0.5 mm ID pipettes were used. These were inserted into the 19 gauge PDMS channel from opposite sides and the pulled pipette was inserted into the outlet pipette a distance of ~1 mm to allow smooth sheath flow and droplet release (Figures 13-14, right). Next, 3 mm OD/1 mm ID flexible tubing was inserted into the 14 gauge channel to allow for inlet organic sheath flow. The pipettes and tubing were affixed to the PDMS with a tight seal using silicone-based adhesive sealant (General Electric) and allowed to cure overnight.

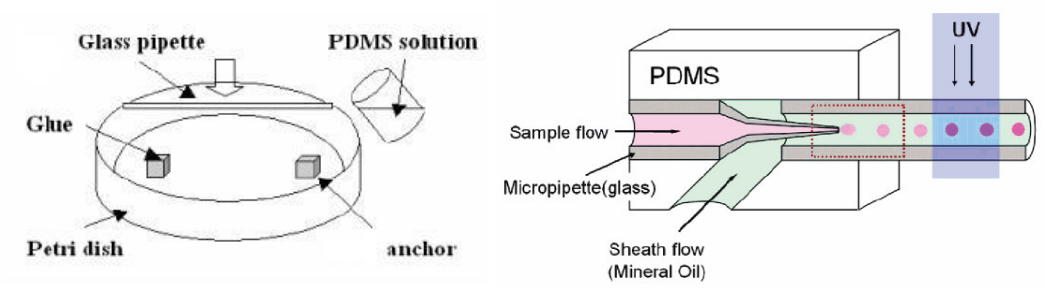

Figure 13. Microfluidic device construction (**left**) showing layout using anchored needles and PDMS. (**right**) Schematic of microfluidic operation. Figures from Jeong *et al*. (2005).

Production of Capsules

To connect syringes, 3 mm OD/1 mm ID tubing sections were attached to the inlet and outlet pipettes. Syringes for sample and sheath flow were connected to the tubing using 19 gauge needles, and controlled using Genie® syringe pumps (New Era Pump Systems) (Figure 14, left). The tubing from the outlet pipette was placed in a microcentrifuge tube to collect all outlet flow.

Jeong *et al*. (2005) showed that the size of the capsules can be precisely controlled by varying the inlet flows of the sample and sheath solutions (Figure 14, right). They report using a sample flow of 1 µL/min and sheath flow of 225 µL/min to produce 90 µm diameter droplets. The entire PDMS device was set under a long-wave UV light (wavelength 365 nm) at a power of 3.9 mW/cm^2. The syringes and pumps were placed outside of the range of the UV light. The device was covered up to the outlet pipette with aluminum foil in order to prevent the premature crosslinking of the PEG solution prior to the formation of droplets. The droplets traveled through the outlet tubing under the UV light for approximately 15 seconds before being deposited into the microcentrifuge tube. Droplets were then pipetted to a 96-well plate for imaging.

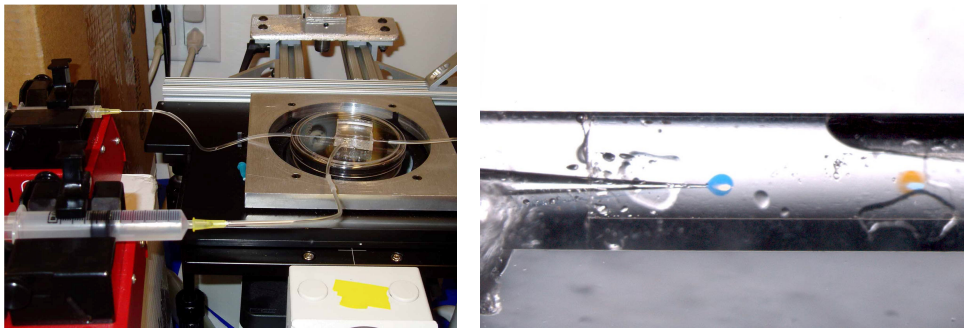

Figure 14. Microfluidic device (**left**) set up on microscope stage showing flow controllers at left and exit tube at right. (**right**) Magnified view using stereoscope reveals the pulled pipette nozzle with approximate 20 µm diameter.

Current Experiments

Crosslinking Studies

Hydrogels swell when immersed in aqueous solutions; the amount of swelling can be quantified as a change in mass from freeze-dried and wet masses to infer the degree of crosslinking for a particular hydrogel. We investigated the degree of crosslinking in 1 µL PEGDA droplets produced manually with pipettes under various UV exposure times, but the results were inconclusive (data not shown). Specifically, the total mass and number of droplets created were too small to measure with significant resolution on a standard laboratory scale.

Viability assays

Viability studies were conducted in cell-seeded 1 µL PEGDA droplets and in small cell-seeded PEGDA cylinders to investigate the effects of the proposed PEGDA crosslinking scheme on MA-10 cells. The proposed crosslinking scheme uses low photoinitiator concentrations (0.05 wt.%) and minimal UV exposure time (less than one minute), in accordance with widely accepted crosslinking standards (Bryant *et al*., 2000).

Using manually produced 1 µL PEGDA droplets, the Live/Dead® assay (Invitrogen) was conducted to qualitatively measure cell viability up to 5 d in *in vitro* culture (Figure 15, top row).

Studies in small cylinders (1 mm diameter x 4 mm length) were conducted up to 1 d after crosslinking (Figure 15, bottom row). Measuring viability of MA-10 cells encapsulated in small microspheres (~100 µm) using a microfluidic device remains a work in progress.

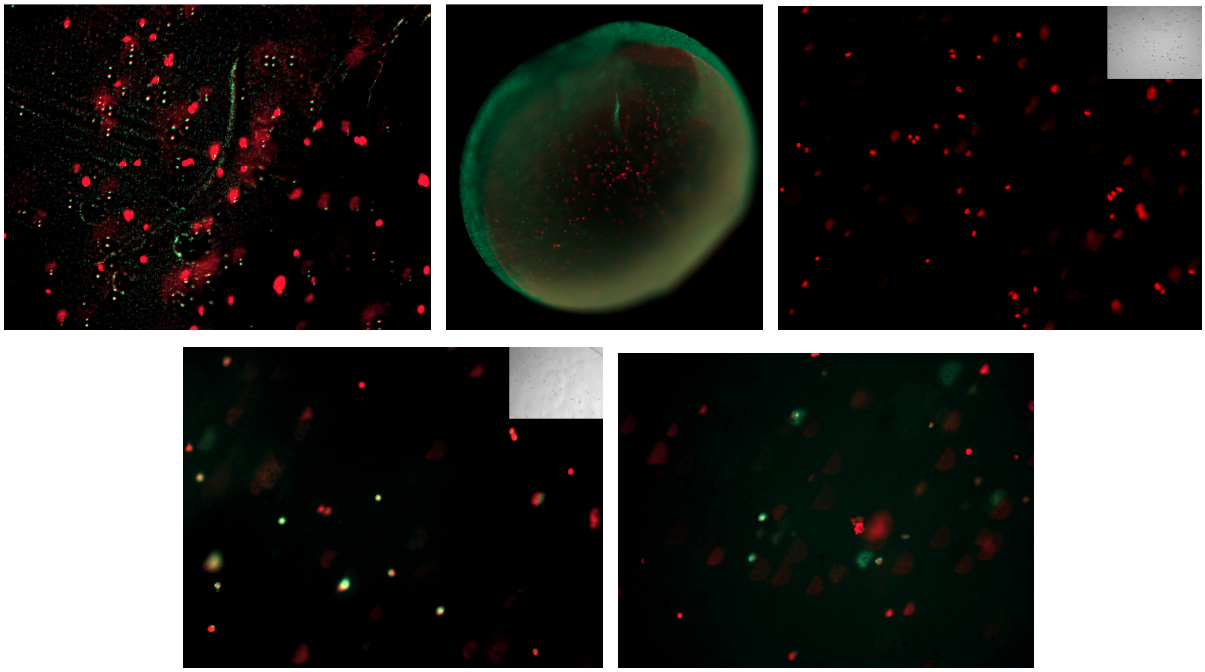

Figure 15. Top row is Live/Dead® on 1 µL capsules crosslinked for 30 s. (**left**) Capsule placed immediately into Live/Dead® solution and imaged after 15 min at 100x. (**middle**) Whole capsule after 2 d at 40x. Note PEG may autofluoresce. (**right**) Hydrogel after 5 d at 100x. Bottom row is Live/Dead® on PEG crosslinked within entrance tube of microfluidic device. Hydrogel was sectioned into small cylinders (1 mm diameter x 4 mm length), kept in media, and imaged (**left**) 1 h and (**right**) 1 d after UV exposure. Insets are bright field images of cells at 100x. All cells at passage 4.

Microfluidic operation and cell encapsulation

Before using the microfluidic device with cell suspensions, its ability to produce droplets was tested using water and mineral oil as the sample and sheath flows, respectfully. Before use, the syringes were manually advanced to remove air bubbles and 'prime' the device. Because the device was constructed using similar dimensions and materials as that described by Jeong *et al.* (2005), the reported flow rates for sample (1 µL/min) and sheath (225 µL/min) were initially used. Significant variation existed between multiple constructed microfluidic devices in that

different sample flow rates (1-20 μL/min) were needed to start producing droplets. Often a threshold value was required whereby a lesser flow rate would not produce any droplets. Additionally, though both syringe pumps were held at constant flow rates, the rate of droplet production fluctuated significantly, at times ceasing entirely. This may be due to pump inconsistencies and air in the tubing, which would alter flow rates. Friction in the syringe barrels may also be a cause and was observed when manually operating the syringes. During experiments, it was frequently necessary to manually advance the sample syringe every few minutes to resume droplet production. As a result, droplets of varying size and frequency were produced.

A second step was running PEGDA solutions without cells through the microfluidic device surrounded by a mineral oil sheath flow. Using the same flow rates as for water, many droplets were produced but in varying size and frequency. The rate of droplet formation was unstable, as with water. A particular challenge was preventing crosslinked PEGDA from clogging the device, both in the entrance sample tube and at the pipette tip, after approximately 1 h of device use. Ambient light or reflected UV light caused unwanted crosslinking of PEGDA in the device, even though aluminum foil was used to shield the entrance tube and PDMS portion of the device. Appropriate UV exposure occurred in the exit tubing portion of the device, namely a section of tubing approximately 15 cm in length placed under a UV lamp (365 nm, 3.9 mW/cm^2). Steps were also taken to rinse the device with 70% ethanol and deionized water prior to and after use. Unwanted PEGDA crosslinking within the device is still a significant problem, especially if single devices are needed for multiple day-to-day tests.

Finally, the capability for the microfluidic device to encapsulate MA-10 cells in PEGDA droplets was investigated, again using similar flow rates to those used for water and PEGDA

alone. Using a seeding density on the order of $1x10^6$ cells/mL, numerous cells were possibly encapsulated within PEGDA hydrogels (Figure 16, left). It is presently unclear whether the cells were fully encapsulated, as conclusions cannot be drawn from the microscope image. Several droplets were obtained with appropriate diameter near 100-200 µm (Figure 16, right). However, the size of droplets and number of cells per droplet each varied substantially, in large part due to the unstable response of the microfluidic device to constant flow rates, as described earlier.

Altering flow rates to obtain appropriately-sized droplets and maintaining sufficient UV exposure time remain ongoing difficulties with the microfluidic approach. Throughout testing, many PEGDA droplets aggregate upon entering the exit tubing, and further aggregate in the microcentrifuge collecting tube, limiting the quantity of microcapsules obtained from the device. Furthermore, microfluidic devices tend to clog after approximately 1 h of use, even when shielded with aluminum foil. Shielding and rinsing techniques could be improved for future experiments.

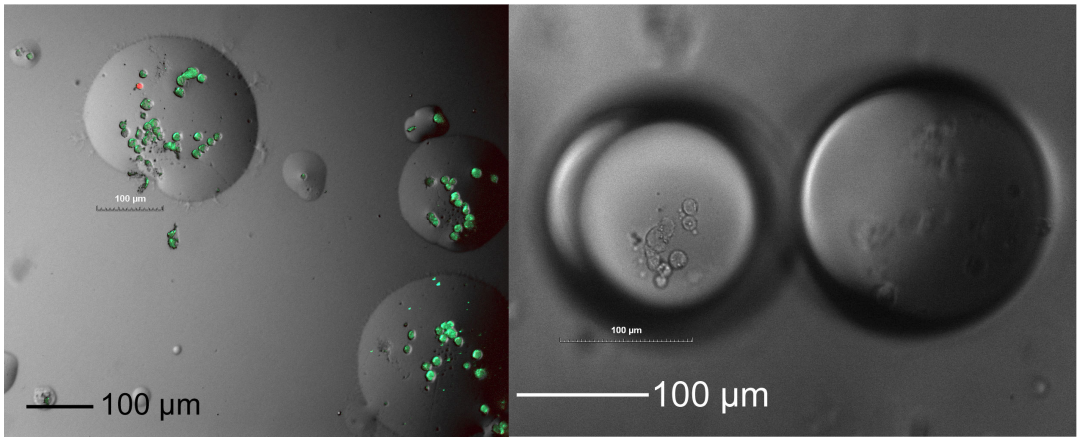

Figure 16. MA-10 cells in microcapsules produced using microfluidic device. (**left**) A composite image obtained by merging Live/Dead® and visible light images, viewed at 100x. The PEGDA appears to have fully crosslinked after coming into contact with the polystyrene 96-well surface, due to the flattened morphology of the droplets. Note that Live/Dead® solution was added before encapsulation in order to easily locate cells, and thus cannot be used to confirm cell viability after encapsulation. (**right**) Polymerized droplets of PEDGA, possibly including cells, viewed at 200x, after 5 d incubation in PBS.

Cost Analysis

Table 1 shows an estimated cost analysis for materials used this semester. The total spent is around $300.00. Important to note is that many, more expensive items were graciously provided by the team's advisor and client. Had the team been required to purchase lab equipment, cell culture, and biochemical assay supplies, the total would expectedly be much greater.

Table 1. Estimated costs of supplies used this semester.

Product		Cost
PEG diacrylate reagents	PEG 8000	$35.72
	Acryloyl chloride	$13.90
	Triethylamine (TEA)	$13.90
	Benzene, ethyl ether	$70.00
Microfluidic Device	Polydimethylsiloxane (PDMS)	$30.00
	Pipettes	$30.00
UV light		provided by Prof. Masters
Cell culture supplies		$100.00 / provided by client
Live/Dead® Cytotoxicity/Viability assay kit		provided by client
Total		**$293.52**

Future Work

As mentioned, the design team has struggled with accurately measuring the degree of crosslinking of the PEG microcapsules. The small scale of these hydrogels makes this parameter difficult to determine using conventional laboratory techniques such as lyophilization and simple swelling and weighing. A chemical assessment to determine how many acrylated ends have been crosslinked may be possible (Murphy, 2005, personal communication). Crosslinking is a parameter that can be tailored by modifying the polymer's molecular weight, concentration in solution, and degree of functionalization. Related to this is the minimal (and therefore most cytocompatible) UV exposure time required for complete crosslinking of the microcapsules, which still must be determined. Once a crosslinking protocol is established, various exposure

times can then be tested. The team has also noted the differences between several photoinitiators. The use of a less cytotoxic initiator (such as Irgacure 2959®) as opposed to the current, faster system (Irgacure 651®) is obviously desirable.

Several glitches in the microfluidic system must be worked out. Most notably, the aggregation of the PEGDA-cell suspension within the tubing must be eliminated, in order to precisely control capsule size. This may mean eliminating the glass-tubing junction in the device. In addition, the PEG-in-oil emulsion has caused some problems when trying to collect the actual crosslinked capsules at the end of the device. A more efficient method of capsule collection has yet to be determined.

Since PEG is bioinert, cell adhesion will not occur without incorporation of adhesive peptide sequences. Future work includes adding such peptide sequences to the PEGDA acrylate groups to improve cell adhesion. Selecting appropriate peptide sequences may involve studying MA-10 cell response to certain peptides; adhesive sequences common for many cell types include RGD and PHSRN.

Hormone release has yet to be measured in encapsulated MA-10 cells. Release of inhibin and testosterone can be quantitatively measured using ELISA assays kits. Once the diffusion of hormones is confirmed, further studies can be performed to look into the diffusion rates of various molecules through the PEG microcapsules. This is important, since cells require nutrients to survive, and slow diffusion rates may cause cell necrosis. A simple method of measuring release by the PEG capsules would involve tagging encapsulated molecules (e.g., proteins) with a dye (e.g., Evans blue) and using fluorometric methods to quantify diffusion.

Following further *in vitro* testing, a future application of this encapsulated cell system is *in vivo* implantation and monitoring. Only then will the true biocompatibility of this system be able to be accurately tested.

Conclusion

Microencapsulation of Leydig cells, by increasing testosterone secretion *in vivo*, may restore negative feedback to the hypothalamus and pituitary gland and subsequently decrease the release of FSH and LH. By balancing the HPG axis, microencapsulation treatments hope to counteract the degenerative effects of HPG dysregulation commonly associated with aging (Bowen and Atwood, 2004). This semester, the team has come much closer to the end goal of successfully encapsulating Leydig cells within a photopolymerizable polymer through the use of microfluidics.

References

Bowen, R. and Atwood, C. 2004. Living and dying for sex: a theory of aging based on the modulation of cell cycle signaling by reproductive hormones. *Gerontology* 50: 265-290.

Bryant, S., Nuttelman, C., and Anseth, K. 2000. Cytocompatibility of UV and visible light photoinitiating systems on cultured NIH/3T3 fibroblasts *in vitro*. *Journal of Biomaterials Science. Polymer Edition* 11: 439-457.

Cruise, G., Hegre, O., Lamberti, F., Hager, S., Hill, R., Scharp, D., and Hubbell, J. 1999. *In vitro* and *in vivo* performance of porcine islets encapsulated in interfacially photopolymerized poly(ethylene glycol) diacrylate membranes. *Cell Transplant* 8:293-306.

Cruise, G., Scharp, D., and Hubbell, J. 1998. Characterization of permeability and network structure of interfacially photopolymerized poly(ethylene glycol) diacrylate hydrogels. *Biomaterials* 19: 1287 – 1294.

de Groot, M., Schuurs, T., and van Schilfgaarde, R. 2004. Causes of limited survival of microencapsulated pancreatic islet grafts. *Journal of Surgical Research* 121: 141-150.

Machluf, M., Orsola, A., Boorjian, S., Kershen, R., and Atala, A. 2003. Microencapsulation of Leydig cells: a system for testosterone supplementation. Endocrinology 144: 4975-4979.

Masters, K. 2005. BME 601: Tissue Engineering, Fall 2005. UW-Madison.

Morohashi, K. 1997. The ontogenesis of the steroidogenic tissues. *Genes to Cells* 2: 95-106.

Murphy, W. 2005. Personal Communication. February 3, 2005.

Nguyen, K., and West, J. 2002. Photopolymerizable hydrogels for tissue engineering applications. *Biomaterials* 23: 4307-4314.

Sawhney, A. 1999. Poly (ethylene glycol). In: Kuhtreiber, Lanza and Chick, editors. Cell encapsulation technology and therapeutics. Boston: Birkhauser. p 108-116.

Ulmke, H., Wriedt, T., Lohner, H., and Bauckhage, K. 1999. The piezoelectric droplet generator – a versatile tool for dispensing applications and calibration of particle sizing instruments. *Pat McKeown et al. (editor): Precision Engineering – Nanotechnology, Proceedings of the 1st International euspen Conference, Vol. 2, Shaker Verlag, Aachen,* 290-293.

Uludag, H., De Vos, P., and Tresco, P. 2000. Technology of mammalian cell encapsulation. *Advanced Drug Delivery Reviews* 42: 29-64.

Victorey, P. 2005. Epson® R200 Bioprinter driver. University of Wisconsin – Madison, Madison,Wisconsin.

Widmaier, E., Raff, H., and Strang, K. 2004. Human physiology: the mechanisms of body function, 9th ed. McGraw-Hill, New York.

Appendix

Product Design Specifications

Team:

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Joseph Zechinski
April Zehm

Problem Statement:

A method of treatment for various endocrine diseases incorporates the encapsulation of cells and tissues and the time-released delivery of chemical mediators. Presently, this method encounters a slew of problems, including a lack of biocompatibility, limited immunoprotective properties, and hypoxia. The client desires the development of microcapsules that would permit the successful release of hormones (namely, testosterone and inhibin) by encapsulated cells into animals, while avoiding the aforementioned problems.

Function:

Microcapsules should allow transplantation of hormone-releasing tissue without the use of immunosuppressant drugs by providing a mechanical barrier to immune system attack.

Client requirements:

- Microencapsulation of reproductive hormone-producing cells
- Must release molecules of interest (hormones) over time, while keeping harmful molecules (antibodies, etc.) out
- Biocompatible, immunoprotective, minimize mechanical, and chemical degradation

Design requirements:

Physical and operational characteristics

a. *Viability*: The encapsulation process may have adverse effects on initial cell viability in microcapsules. The proposed design should retain at least 75% cell viability measured 1 d post-encapsulation using a Live/Dead® assay.
b. *Hormone release*: Normal values of testosterone in human males is 2-12 ng/mL. Microencapsulated cell therapy should maintain serum testosterone concentrations of at least 2 ng/mL in patients, with contributions from both transplanted cells and patients' baseline testosterone secretion.
c. *Degradation*: The biomaterial should remain intact long enough to sustain a critical cell mass, such to provide adequate hormone release for at least 6 months.
d. *Capsule size*: Diameter of microcapsules should be such that 100 ± 20 um describes 90% of the capsule distribution. Sufficient diffusion of gases and nutrients is limited beyond 100 um (Murphy, 2005, personal communication).

e. *Molecular weight cutoff (MWCO)*: MWCO should be 75 kDa in order to allow diffusion of relevant molecules, specifically testosterone (300 Da), inhibin (32 kDa), activin, LH (30 kDa), FSH (36 kDa), but block antibody (150 kDa) diffusion.
f. *Immune response*: Microcapsules must not allow the diffusion of antibodies (molecular weight ~150 kDa) or immune system cells by providing a sufficiently small mesh size.
g. *Biocompatibility*: Biomaterial and its degradation products must be nontoxic and not cause inflammation within the body. Injected microcapsules must resist protein adsorption and fibroblast overgrowth. Previous designs suffer from fibroblast overgrowth resulting in cell graft death due to hypoxia (de Groot *et al*., 2004). In addition, the microcapsule production process including droplet formation and biomaterial crosslinking (if required) should be sufficiently cytocompatible.
h. *Life in service*: Therapy should sustain patient at minimum serum testosterone concentration (at least 2 ng/mL) for at least 6 months.
i. *Production timeframe*: Microencapsulated cell therapy, at present, will be prepared from start to finish as needed by patient demand. Microencapsulated cells will be implanted soon after production. The microencapsulated cells should be sustainable *in vitro* for at least several days.
j. *Operating environment*: *In vivo*, site of implantation to be determined. Subcutaneous and intraperitoneal injections are both possible (Machluf *et al*., 2003).

Product characteristics:

a. *Quantity*: Thousands to hundreds of thousands of microcapsules will be required per injection. The cell mass required to provide adequate hormone release is currently unknown, but likely on the order of 10^6 cells. Previous studies in rats used $5x10^6$ cells in transplant therapies (Machluf *et al*., 2003).
b. *Sterility*: Final product must be sterile prior to implantation.
c. *Target product cost*: Not specified.
d. *Research costs*: A budget of approximately $10,000 to $20,000 has been reserved for research and development of working microcapsule designs.

Miscellaneous:

a. *Standards and specifications*: FDA approval is required. University approval required prior to animal or human subject testing.
b. *Competition*: Several patents regarding cell encapsulation exist and define specific production protocols. These are US Patent 5,762,959, US Patent 5,100,673, and US Patent 5,164,126. This study is not expected to infringe upon past technology as a novel capsule design is desired.