A SWINE MODEL FOR THE QUANTIFICATION OF PELVIC ADHESIONS AND
THE ENCAPSULATION OF KETOROLAC TROMETHAMINE FOR THE
PREVENTION OF ADHESION FORMATION

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A SWINE MODEL FOR THE QUANTIFICATION OF PELVIC ADHESIONS AND
THE ENCAPSULATION OF KETOROLAC TROMETHAMINE FOR THE
PREVENTION OF ADHESION FORMATION

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I would like to dedicate this thesis to Dr. Stephanie Lopina. She was an amazing woman whose inspiration abilities were boundless. Without her guidance and inspirational personality, I may not have ever attended The University of Akron. It was because of her that I started this research and was able to make the connections and have the experiences that led me to attend medical school. She was professionally a top-notch researcher and advisor, and personally, an extremely good friend. She was full of life and passion. She was my advisor, mentor, and friend. Without her this thesis would not be possible.
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CHAPTER 1—INTRODUCTION

Following pelvic and abdominal surgeries, the development of postoperative adhesions are unavoidable, resulting in a significant, costly morbidity associated with pain and infertility [1, 2, 3]. Adhesion formation occurs in 90% of major abdominal surgery patients and in 55%-100% of women following pelvic surgery [1, 3]. A reliable method to decrease pelvic adhesions would be a useful addition to current surgical practice; however, a reliable, objective, replicable, and quantitative method for adhesion assessment must first be developed, as has been done in other fields [4, 5]. Currently, only a graded qualitative scale, with a variety of grading and injury methods, has been used in existing studies [6]. Without an objectively determined quantitative method for assessing pelvic adhesions, comparisons of adhesion barriers’ efficacy cannot be made.

Creation of a quantitative model suitable for testing adhesions depends on several principles. First, a technique must be devised that reliably creates significant adhesions that are neither too strong to be prevented, nor too weak to be insignificant. Significant adhesions are usually defined qualitatively as dense, thick, and vascular [3]. Secondly, the adhesions created must be physically appropriate for a quantitative analysis. Finally, the experimental adhesions should have clear validity for the surgical methods under consideration; in this case, replicating pelvic and abdominal organ adhesions following open or closed surgery.
The first part of the work described in this thesis is the development of a model created to meet the above criteria. Namely, the creation of reproducible significant, but not obliterative, experimental adhesions of the pelvic viscera to the sidewall following a laparotomy with manipulation of the pelvic organs, followed by excision and quantification of the experimental adhesions.

Once the model was created, work to develop a reliable method to decrease pelvic adhesions continued. This would be a useful addition to current surgical practice; preventing the need for subsequent surgeries to break the adhesions and preventing some postoperative complications created by adhesions such as, small bowel obstructions, pain, and infertility. The ideal adhesion prevention adjuvant would effectively prevent adhesions from forming for the first 14 days post-surgically. Adhesion formation generally occurs within the first 5 days and this would allow for complete healing of peritoneal injuries while the adjuvant is present and active.

There are two clinically approved industry standard adhesion barriers. However, their efficacy and utility in laparoscopic surgery is limited because both are difficult to work with. Our long term goal is to create a microsphere encapsulated drug that can be easily applied in both open and laparoscopic surgeries to prevent adhesion development.

We propose to utilize the analgesic and anti-inflammatory properties of ketorolac tromethamine (KT), an NSAID cox-2 inhibitor, to attempt to decrease the occurrence and severity of adhesions. KT has been explored for use in adhesion prevention previously [7, 8] and it is our belief that it can be encapsulated and utilized as a local adhesion prevention agent. Other potential candidates for encapsulation and successful adhesion
prevention include statins, ibuprofen, and other non-steroidal anti-inflammatory drugs (NSAIDs).

The pharmaceutical agent will be encapsulated into PLGA microspheres. PLGA is a well characterized biodegradable and bioabsorbable material which is FDA approved and has been previous utilized in microsphere formulations [9, 10]. Low molecular weight PLGA composed of a 50/50 mixture of lactate to glycolic acid was selected as it has been shown to possess the desired degradation time frame [11, 12, 13, 14]. The work described in this thesis represents the initial development of microsphere encapsulated KT and subsequent research that will be performed in the pursuit of an anti-adhesive agent.

1.1—Objectives of the Study

The objectives of the research performed were to create a quantitative model of adhesion strength assessment, to successfully encapsulate ketorolac tromethamine (KT) into poly(lactic-co-glycolic acid) microspheres, and to characterize of the microspheres for use in preventing adhesion formation.

To achieve the quantitative model, studies were preformed to:

1. Create a consistent method of injury
2. Successful harvest the complex
3. Quantify the adhesion strength through analytical methods
4. Compare the quantification to previously acceptable methods of adhesion assessment.
To achieve the encapsulation and characterization of the KT microspheres, studies were performed to:

1. Determine the optimal loading method, utilizing two different solvents: PVA and PVP. This was accomplished by varying the amount and the solvent and comparing the efficiencies of loading of the drug encapsulated microspheres.
2. Determine drug loading efficiencies and surface morphologies of the drug encapsulated microspheres. In these studies, the primary objective is to examine the effects of the drug loading on the surface of the microparticles and to determine the efficiency of the KT encapsulation on PLGA microspheres.
3. Determine the duration of drug release microspheres under in vitro conditions.

1.2—Hypotheses of Quantitative Model

1.2.1—Research Hypothesis of Quantitative Model

A method of consistent injury creation and specimen harvest can be established to quantitatively evaluate the strength of a pelvic adhesion.

1.2.2—Null Hypothesis 1

There is no significant difference between the quantitative method of adhesion evaluation and the previously accepted methods of visual and histological grading.
1.3—Hypotheses of Ketorolac Tromethamine Encapsulated Microspheres

1.3.1—Research Hypothesis

The pharmaceutical agent ketorolac tromethamine USP can be encapsulated into PLGA microspheres for sustained local drug delivery applications.

1.3.2—Null Hypothesis 1

There is no significant difference between the loading efficiencies of the solvents PVA and PVP.

1.3.3—Null Hypothesis 2

There is no significant difference between the release of KT, KT microspheres, and unloaded microspheres under in vitro conditions.
Peritoneal adhesions can be categorized into two classes; congenital or acquired [15]. Congenital adhesions are those which are present from birth. They occur as embryological anomalies during the development of the peritoneal cavity [3]. They can for example manifest as vitellointestinal bands. Generally, congenital adhesions are harmless [3]. Acquired adhesions are often the result of either inflammation or surgery.

![Adhesion Forming on Peritoneum](image)

Figure 1. Representative illustration of adhesion formation between two peritoneal surfaces. Used with permission from Dr. DiZerega [19].

Unlike skin, which consists of epidermis, dermis, and subcutaneous layers, the peritoneum consists only of a single layer of mesothelial cells. This allows damage to the surface lining very easily. Injury to the peritoneum results in an initial formation of loose
fibrous bridges between serosal surfaces which will subsequently become infiltrated by fibroblasts. These in turn produce the permanent fibrous adhesions [16].

An inflammation induced adhesion can arise following any intra-abdominal inflammatory process. Some examples are: appendicitis, acute cholecystitis, acute diverticulitis, pelvic inflammatory disease, and use of intrauterine contraceptive devices [1, 3, 15].

The clinical definition of post-surgical adhesions is a consequence resulting when injured tissue surfaces, following incision, cauterization, suturing, or other means of trauma, fuse together to form scar tissue [3]. Post-surgical adhesion formation can occur following any procedure, including cholecystectomy, gastrectomy, appendectomy, hysterectomy, colectomy, abdominoperineal resection, and abdominal vascular operations [3, 19,17, 18]. There are several factors which have been shown to contribute to adhesion formation. These include level of trauma, thermal injury, subsequent infection, ischemia (often due to suturing), and foreign bodies such as powders talc, lint, and fibers [1, 3].

The incidence of adhesions formation following gynecological operations has been reported to be as high as 90% [17], and 97% following laparotomy procedures [18]. The most common complications in gynecology associated with adhesion formation include: chronic pelvic pain (20-50% incidence), small bowel obstruction (49-74% incidence), intestinal obstruction in ovarian cancer patients (22% incidence), and infertility due to complications in the fallopian tube, ovary, and uterus (15–20% incidence) [17]. Adhesions are a costly, painful, and life threatening complication. They are however preventable.
2.1—Adhesion Formation

As early as 1919, it was recognized that peritoneum heals differently than skin heals [19]. Skin healing takes place through the proliferation of epithelial cells from the periphery toward the center of the wound [3, 20] (see Figure 2). In contrast, peritoneum healing occurs by simultaneous mesothelialization (see Figure 3).

![Figure 2. Schematic of dermal wound repair progression. Adapted by L.T. Mellert from Vaalamo [20].](image)

The peritoneum healing occurs as a two-stage process. The first stage involves a wave of phagocytic cells. These are responsible for destroying and clearing of the traumatic debris in and around the wound. The next stage involves subperitoneal perivascular connective tissue cells. These are responsible for the healing of the defect and the re-mesothelialization [1]. Regardless of the wound size, new mesothelium will develop from many different sites, which later proliferate into sheets of cells [3, 19] (see
Figure 3). Thus, peritoneal healing occurs at essentially the same rate no matter the size of the wound.

Figure 3. (A) Illustrative image of the peritoneum. The peritoneal surface is comprised of mesothelial cells, supported by a scaffold of connective tissue. This connective tissue is represented by the white strands. The red represents the microcirculation supplying the peritoneum. (B) Illustrative image of the peritoneum with a denuded area following an injury. (C) Illustrative image of peritoneal healing. Healing occurs via re-epithelialization. The new mesothelial cells migrate to the site of injury via chemotactic messengers. (D) Illustrative image of a newly re-epithelialized injury site. Typically occurs within 5-7 days followed by remodeling for a few months. Image used with permission of Dr. DiZerega; caption adapted from Dr. DiZerega [19].

When undisturbed, peritoneal defects with acquire mesothelial integrity within 48-72 hours, a process known as re-peritonization or re-epithelialization [1]. For parietal peritoneum, complete wound healing occurs within 5-6 days of injury; for visceral mesothelium of the terminal ileum and the parietal peritoneum, healing occurs within 5-8 days [3, 19]. Adhesion formation is a result of a disruption from this normal physiological process. For a simplified breakdown of the adhesion formation see Figure 4.
Figure 4. Summarized breakdown of adhesion formation. Adapted from DiZerega [19].

When normal peritoneal healing occurs, an injury is followed by an initial release of histamine and vasoactive kinins. This causes increased capillary permeability. Within three hours, serosanguineous fluid exits the capillaries. This fluid is extremely protein
filled and causes coagulation and the production of fibrinous bands between and within injured areas [1, 21, 22, 23]. The fibrinous bands attract a variety of inflammatory cells, including monocytes, plasma cells, polymorphonuclear cells, and histocytes. Next the fibrinolytic system is triggered. This causes the lysis of the fibrinous bands. This lysis and inflammatory response usually occurs within 72 hours of injury [1, 23]. The denuded area then undergoes re-epithelialization and the wound is healed.

An important aspect to adhesion formation is the organization of the fibrin gel matrix [24]. This matrix forms following the outpouring of serosanguineous fluid from the capillaries. It starts from fibrinogen which becomes fibrin monomer, then soluble fibrin polymer and finally forms insoluble fibrin polymer which interacts with proteins such as fibronectin to form the fibrin gel matrix [3]. As mentioned above, under normal healing conditions, the fibrinolytic activity occurs and re-mesothelialization results. If a disturbance occurs to disrupt the equilibrium between fibrin deposits and fibrinolytic activity, the fibrous bands will persist. This allows for infiltration by fibroblasts, which proliferate. New angiogenesis will occur and under ischemic conditions the bands will become organized into adhesions [1, 15, 19, 22]. If two damaged peritoneal surfaces come into opposition while covered with a fibrin gel matrix, they will form an adhesion [3, 24]. Once adhesions have formed, they will develop vascularity and even become innervated [1, 3, 15-19].

In the comparison between peritoneal fibroblasts and the fibroblasts present in adhesions, interesting differences were found on the molecular level between adhesion and normal peritoneal fibroblasts. It was observed that the adhesion fibroblasts have a significant higher level of basal messenger ribonucleic acid for collagen I, fibronectin,
matrix metalloproteinase-1 (MMP-1), tissue inhibitor of metalloproteinase-1 (TIMP-1), transforming growth factor-beta-1 (TGF-β1), transforming growth factor-beta 2 (TGF-β2), and interleukin-10 (IL-10). Additionally, adhesion fibroblasts have a decreased apoptosis rate compared with normal peritoneal fibroblasts [1, 25].

Ischemia is believed to be a major contributing factor in adhesion formation [1-3, 19-29]. This can occur to tissues when they are exposed to excessive or rough handling, ligated, sutured, crushed, cauterized, or when the peritoneum is stripped [1]. While other causes have been suggested, such as the presence of intraperitoneal blood, it was found that many of these do not cause adhesion formation when occurring without the ischemia present [1, 26]. Another interesting observation is that adhesion development is significantly less likely in the presence of a foreign body without coincidental injury to the peritoneum [1, 26]. This would suggest that the inflammatory pathway for adhesion formation alone does not cause the majority of post-surgical adhesions.

2.2—Clinical Significance of Adhesions

Adhesions, post-surgical and non-surgically related adhesions, severely affect the quality of life for millions of individuals worldwide. As previously mentions, they can cause complications such as small-bowel obstruction [15, 27, 28], dyspareunia, difficult re-operative surgery [3], chronic pain [29], and infertility [1-3, 28-39].
Figure 5. Laparoscopic views of pelvic adhesions in female patients. (A) Note the circle region; here tension is being placed on both of the connected structures. This is thought to be one possible cause of pelvic pain. (B) Note the circle region; here vertical bands of collagen are visible. This is a major component of adhesions. (C) Note the circled region; here the vascularity of adhesions is visible. Adhesions will develop their own blood supply or vascular aspects, this can cause bleeding in subsequent surgeries.

One example of adhesion occurrence that can occur without previous surgery is endometriosis. Endometriosis is a debilitating condition where endometrial-like cells present in areas outside of the uterine cavity [30]. One of the main indications of endometriosis is pain. It is believed that this pain is a result of adhesion presence and innervation [3, 31]. It was found in a study of the nerve fibers and histopathology of post-surgical, post-infectious, and endometriosis-related adhesions that this innervation is present in all adhesion types [32]. As with other pathologies related to adhesions, one of
the primary treatments for endometriosis adhesions is adhesiolysis which comes with the normal associated risk of reoccurrence [1, 3, 31].

Re-operation through previous wounds and operation due to adhesion presence can be difficult due to adhesion formation. Adhesiolysis adds to the difficulty of a procedure, extending operating time, anesthesia exposure, and recovery time. It can also have the added risks of blood loss, visceral damage such as injury to the bladder, enterocutaneous fistulas, and bowel resection [3, 15].

Small bowel obstructions due to pelvic adhesions are the cause for 5.5% of readmissions [16], and adhesions are responsible for approximately 60-70% of small bowel obstructions [3]. Intestinal obstruction by adhesion is usually the result of kinking, angulation, or compression [3]. It is rare for congenital or inflammatory adhesions to result in intestinal blockages and thus post-surgical adhesions are the primary cause [33]. About 20% of patients presenting with adhesive obstructions occur within 1 month of surgery, 40% within 1 year [15].

The risk associated with post-surgical adhesion formation increases with age, the number of laparotomies, and the complexity of surgical procedures [1, 3, 34]. A strong indicator for adhesion obstruction is prior incidence of adhesion presence. Often, it is possible to treat a stable patient with a first time adhesive obstruction with non-operative management; as reoccurrence is experienced, patients with operative strategies appear more useful [34].

Gynecologic and obstetric events are a major source of intraperitoneal adhesion formation and thus the gynecological consequences of post-surgical adhesions are extremely prevalent. Endometriosis has already been discussed above with moderate to
severe consequences ranging from occasional discomfort to infertility and radical hysterectomies [1, 3, 31-34]. Among the most common procedure resulting in intestinal obstruction is abdominal hysterectomies [3]. Another gynecology procedures resulting in adhesions is a myomectomy. This is associated with increased adnexal adhesions, especially when the incision is performed on the posterior uterine wall [35]. Ovarian cancer debulking surgery, and other surgical treatments of gynecologic malignancies have also been associated with intestinal obstruction due to tumor growth or post-operative adhesions [3, 29, 33, 35, 36, 37].

The financial repercussions of adhesion formation are significant. In 1988, a study by Ray et. al. was conducted which attributed 281,982 hospitalizations and approximately $1.2 billions dollars in hospital and surgeon expenditures to lower abdominal adhesiolysis [38]. In 1994, Fox Ray et. al. performed a study which documented 303,836 hospitalizations for adhesiolysis-related procedures in the United States alone [17]. Of these 81% were performed following an unrelated procedure. An estimated 846,415 inpatient days were associated with adhesiolysis and the total cost during these hospitalizations was estimated to be $1.33 billion [17]. The cost of adhesions is immense and an effective method of prevention would significantly affect the quality of life and financial strain due to adhesions.

2.4—Current Models of Adhesion Formation

There is no currently described method for the creation, excision, and quantitative testing of experimentally created pelvic or abdominal adhesions. Even so, a number of methods have been developed to create and qualitatively assess postoperative adhesions.
Experimental adhesions in the rat model have shown variations with breed, sex, injury methods, and grading methods [39, 40, 41, 42]. Injury methods include scratching of the cecum with a scalpel, scratching of the peritoneum, burning the uterine horns by electrocautery, crushing of the uterine horn, scraping of the uterine horn, closing of hemostat along cecum, and injection with a bacterial charge [39-42]. Similar methodological variability occurs in studies of rabbits [43, 44], dogs [45, 46], and swine [7, 47, 48, 49, 50, 51, 52], each devoid of quantitative adhesion assessment. While most studies employ a visual macroscopic or histological grading method [6-52], these grading scales are very subjective and controlling for observer bias is difficult. Other grading methods employed include immunohistological [40], area of adhesion [42,48-50,52], and behavior performance status [42], none of which provide a mechanical assessment of the adhesion strength. Some studies took advantage of the bifid uterus of swine, leaving one side untreated, acting as an internal control [47, 49].

2.3—Current Clinical Adhesion Prevention Methods

Currently, adhesion prevention at the time of surgery can be accomplished using several methods beyond appropriate tissue handling techniques. Interceed, a commercially produced mesh of oxidized cellulose, has been used in several clinical trials and is currently indicated for the prevention of postoperative adhesions [53, 43]. Other adhesion barriers are also available, including a hyaluronic acid derivative named Seprafilm [54, 55]. Given the importance of adhesion prevention during surgery, more products are anticipated to become available.
Both Interceed® and Seprafilm™ are adjuvants that work by mechanical separation of the peritoneal surfaces. Interceed® is an oxidized regenerated carboxymethylcellulose (ORC) sheet which is placed between the injury surfaces. Seprafilm™ is a hyaluronic acid (HA) and carboxymethylcellulose (CMC) film. HA is a glycosaminoglycan. When exposed to an aqueous environment, it forms a viscous solution [56]. CMC is high-molecular weight polysaccharide. The film is a strong, flexible sheet prior to exposure to aqueous solutions. When it is exposed to any small amount of water, the areas which contact it become sticky making it difficult to place in laproscopic surgeries.

The two industry standards mentioned above have several negative qualities. Most important of these is that both Interceed® and Seprafilm™ are difficult to work with in open surgeries, and nearly impossible to apply during laparoscopic surgeries. With this in mind, the research performed was based on the idea of a local drug delivery system which could be used in both open and closed procedures with ease.

2.5—Current Proposed Adhesion Prevention Methods

The ideal treatment for adhesion prevention would be one which was safe, effective, non-inflammatory, non-immunogenic, lasts through re-epithelialization, is stationary without the need for staples or suture, remains active in the presence of blood, is biodegradable, does not interfere with healing, does not increase the potential for infections, and does not induce adhesion formation.

There are currently several classes of adhesion-reducing adjutants under consideration which attempt to meet the above criteria. The main one currently in use is
the mechanical separation method. Additional methods include 1) fibrinolytic agents, 2) anticoagulants, 3) anti-inflammatory agents, 4) antibiotics, and 5) the before mentioned mechanical separation either by intra-abdominal instillates or barriers [1]. See Table 1 for a summary of these classes and their proposed mechanism of action; Table 1 was adapted from El-Mowafi and Diamond [1].

As is obvious from Table 1, the two main approaches which exist for adjunctive prevention are pharmaceutical administration or physical separation. There are several principles that are common to all pharmacological adjuvant therapies. First, adhesion are more prevalent at ischemic sites. At these sites there is little to no systemic blood supply, therefore, a systemic drug delivery would be ineffective [3]. Secondly, the rapid absorption ability of the peritoneal membrane limits the half-life and efficacy of intraperitoneal agents [3]. And lastly, adhesion formation is very similar to the nature wound healing mechanism; therefore it is imperative that the agent be specific to adhesions and does not interfere with wound healing [3].
<table>
<thead>
<tr>
<th>Class of Adjuvant</th>
<th>Proposed Mechanism of Action</th>
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<tr>
<td>I. Fibrinolytic agents</td>
<td>- Fibrinolysis</td>
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<tr>
<td>Fibrinolysin</td>
<td>- Stimulation of plasminogen activators</td>
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<td>Urokinase</td>
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<td>Hyaluronidase</td>
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<td>Chymotrypsin</td>
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<td>Trypsin</td>
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<td>Pepsin</td>
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<td>Plasminogen activators</td>
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<td>II. Anticoagulants</td>
<td>- Prevention of clot and fibrin formation</td>
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<td>Citrates</td>
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<td>Oxalates</td>
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<td>III. Anti-inflammatory Agents</td>
<td>- Reduced vascular permeability</td>
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<td>Corticosterios</td>
<td>- Reduce histamine release</td>
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<td>Nonsteroidal anti-inflammatory agents</td>
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<td>Anti-histamines</td>
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<td>Progesterone and progestogens</td>
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<td>Calcium channel blockers</td>
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<td>Colchicine</td>
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<td>IV. Anti-biotics</td>
<td>- Prevent infection</td>
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<td>Tetracyclines</td>
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<td>v. Mechanical Separation</td>
<td>- Surface separation</td>
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<td>a. Intra-abdominal Instillates</td>
<td>- Hydroflotation</td>
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<td>Dextran</td>
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<td>Mineral oil</td>
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<td>Silicone</td>
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<td>Vaseline</td>
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<td>Crystalloid solutions</td>
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<td>Carboxymethylcellulose</td>
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<td>Hyaluronic acid</td>
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<td>Chelated hyaluronic acid</td>
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<td>Poloxamer</td>
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<td>b. Barriers</td>
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<td>Omental grafts</td>
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<td>Bladder strips</td>
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<td>Fetal membranes</td>
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<td>Fibrin glue</td>
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<td>Polytetrafluoroethylene</td>
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<td>Oxidized cellulose</td>
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<td>Oxidized regenerated cellulose</td>
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<td>Gelatin</td>
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<td>Seprafilm</td>
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<td>Spraygel</td>
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Table 1. Summary of adjuvant class and its proposed mechanism of action. Adapted from El-Mowafi and Diamond [1].
2.5.1—Class I: Fibrinolytic Agents

Class I, fibrinolytic agents, is considered on the basis that they can induce or stimulate the production of plasminogen activator inhibitor-1 (PAI-1). To date there have been many animal studies but none of the agents have been investigated in humans for adhesion reducing ability [1]. The peritoneal cells possess the ability to prevent the deposition of intra-abdominal fibrin through plasminogen-activating activity. This is mainly through PAI-1 and PAI-2 [1]. PAI-1 is produced in both the mesothelial and endothelial cells of the sub-mesothelial blood vessels, while PAI-2 is produced by the mesothelial cells and the macrophages within peritoneal tissue. This inhibitors are produced when there are high concentrations of tumor necrosis factor (TNF), interleukin-1 (IL-1), and interleukin-6 (IL-6) which are inflammatory cytokines [1, 57]. Each of these cytokines individually and synergistically can result in an increased production of PAI-1 [1, 57]. It is believed that enhancing the peritoneal fibrinolytic activity through stimulation of these PAIs or the reduction of inflammation could reduce adhesion prevalence. While there have been many different studies, the findings have often been contradictory [1].

In a study by Vipond et al., recombinant tissue plasminogen activator (rtPA) was delivered locally in rats. They reported that this successfully reduced the formation of adhesions [1, 58]. A study completed by the same group showed that this method was superior to carboxymethyl-cellulose (CMC) gel. LeGrand and colleagues investigated the utility of imidazole, a thromboxane synthetase inhibitor, in a rabbit model. They found that adhesion formation was significantly reduced. They also reported that Ridogrel, an inhibitor of thromboxane synthetase, also showed significant ability in
reducing peritoneal adhesion severity [1, 8]. The results of these studies were in direct contradiction to two other studies.

One, performed by Gehlback et. al. on rabbits, showed that the combination of IP, rtPA, and CMC was not successful in significantly reducing post-surgical adhesion reformation and induced hemorrhagic complications [1, 59]. Another studies on rats reported that rtPA and oxidized regenerated cellulose (ORC), a barrier, did not decrease adhesion formation. The purpose of rtPA in the study was to mitigate the adhesion-promoting effects of ORC, which it did; but when Evans et. al. studied the effects of the required levels of rtPA, they found a significant impairment of wound healing [1,60, 61].

A more recent study performed in rats, showed that the use of nimesulide, a selective cyclooxygenase-2 inhibitor, both preoperative IM and postoperative IP administration at the site of injury, was able to decrease adhesion formation [1, 62]. While this class shows some promise in adhesion reduction, corroboration and clinical trials necessary prior to use as an adhesion barrier.

2.5.2—Class II: Anticoagulants

The only class II adjuvant that has been studied extensively is heparin. Heparin is believed to work through one of three mechanisms [1, 63]. First, it may work by combining with anti-thrombin III [64]. Second, it may directly increase the activity of plasminogen activators, thus promoting fibrin clot breakdown [65]. Third, by biding to fibroblast growth factor (FGF); this has been shown to significantly improve healing of cutaneous wounds [66].
Heparin has been added to peritoneal irrigants [67], a method that has been effective in animal models [68, 69]. It has also been added in low-molecular-weight form to Na-CMC solution [70] with positive effects. Heparin has been combined with amniotic membrane, also with successful results [71]. But similar to other anti-adhesive studies, contradictory studies were performed. Diamone et. al. reported that heparin delivery via IP lavage, IV injection, intra-abdominal formation, or in conjunction with IP instillates of CMC or 32% Dextran 70® were not significantly effective [43, 72, 73]. In these studies however, they did find that Interceed® with heparin did inhibit adhesion reformation significantly. Again the results are contradicted when Reid et. al performed a clinical trial. In this, the addition of heparin did not enhance the adhesion-reducing ability of Interceed TC7® [74]. Once again although there were some successful trials, there is a lack of consistency and corroboration between the studies.

2.5.3—Class III: Anti-inflammatory Agents

Class III, anti-inflammatory agents, is used to do exactly what they sound like, reduce the inflammatory response. This decrease of the initial response to the tissue injury is believed to be the mechanism for decreasing adhesion formation [1]. The agents investigated are those which fall under the generic categories of corticosteroids, nonsteroidal anti-inflammatory agents (NSAIDs), progesterone and progestogens, anti-histamines, and calcium channel blockers. They are capable of reducing vascular permeability, inhibition of synthesis and release of histamines, lysosome stabilization or some combination there of [1].
2.5.3.1—Corticosteroids

While some success was shown in adhesion prevention in small animals \([75,76]\), a large dose is required which increase the risk of immuno-suppression, infection, and wound disruption complications \([77]\). Most other animal studies failed to prove any significant reduction of adhesion formation with corticosteroid therapy. diZerega and Hodgen showed perioperative dexamethason and promethazine were not effective after trauma to fallopian tubes \([78]\); Seitz et al. showed that the use of normal saline, low-molecular-weight dextran, and dexamethason/promethazine, were all similar in prevention the reformation of adhesions in monkeys \([79]\), and the additional corticosteroid clinic trials all failed to prove efficacy for adhesion reduction \([45, 80]\).

2.5.3.2—Nonsteroidal anti-inflammatory drugs (NSAIDs)

The potential pathway for NSAID adhesion prevention is numerous. Of these, the most likely is anti-prostaglandins effect (blocking the adhesiogenic action of prostaglandins instilled IP) \([81]\), inhibition of platelet aggregation, leucocyte migration, phagocytosis, and lysosome release \([28]\). In most animal studies, the efficacy of NSAIDs was significant for the prevention of adhesion formation and reformation \([82, 83, 84]\). Tolmetin \([8, 85]\), IP indomethacin \([77]\), oxyphenbutazone perioperatively \([86]\), ibuprofen \([8]\), aspirin \([8]\), indomethacin \([8]\), and ketoprofen \([7, 8]\), have all been proven to be successful in animal models. This would indicate that all NSAIDs share some common pathway for adhesion prevention. Similar to other adjuvants discussed, there has not been extensive clinical trials published although several are known to have been conducted.
2.5.3.3—Anti-histamines

The only use of anti-histamines for the prevention of adhesions has been in conjunction with corticosteroids. As discussed previously, these studies have no proved efficacy. The method by which anti-histamines are thought to work is via inhibition of the inflammatory response, stabilization of lysosomal membranes, and the inhibition of fibroblast proliferation [1, 87].

2.5.3.4—Progesterone and Progestogens

Progesterone possesses the ability to inhibit leukocyte migration, T-cell activation, and reduce humoral antibody production [1]. It was observed that following ovarian wedge resection, when the ovary contained active corpus luteum at the time of surgery, post-operative adhesions were reduced [88]. This coupled with its anti-inflammatory and immunosuppressive effects [89,90,91], led to its investigation for anti-adhesive purposes. Progesterone was investigated by Maurer and Bonaventura via IP and IM routes; they found that it successfully decreased adhesion formation [91]. There were however contradictory studies [92, 93]. In these studies, there was actually an increased adhesion development following IP administration of progesterone. In a study by Freeman et al., they found no reduction in adhesions following progesterone treatment [94]. Thus the ability of progesterone to prevent post-operative adhesions is non-conclusive at this point.

2.5.3.5—Calcium Channel Blockers

The mechanism by which calcium channel blockers could prevent adhesions is via an inhibition of the release of vasoactive inflammatory mediators such as histamine
and prostaglandings E and F [95]; they may also reduce tissue ischemia [96], inhibit platelet aggregation [97], and inhibit fibroblast migration [98]. The research performed to test the preventative effects of calcium channel blockers is limited and is further hindered by an inability to reproduce the effects in other models [99]. While these may be an effective method of adhesion prevention, more research is necessary.

### 2.5.3.6—Colchicine

Colchicine is known to inhibit histamine secretion, mitotic activity, and collagen synthesis [1]. It has also been shown to have inhibitory effects on adhesion formation in two rat studies [100, 101]. Although these were promising, there have been no additional studies to date.

#### 2.5.4—Class IV: Antibiotics

Antibiotic use has been suggested as a mean of prophylaxis in surgery. Its rational is merely that by preemptively guarding against infection, the inflammatory response will be subdued and thus adhesion development prevented or retarded [1]. There has not be extensive research into the adhesion prevention ability of antibiotics although it has been suggested as a potential adjuvant.

#### 2.5.5—Class V: Mechanical Separation

The idea of mechanical separation is focused on peritoneal surfaces and the surfaces of the pelvic organs. During the first day of healing, contact between surfaces and the fibrin matrix can result in adhesion formation. A practical way to prevent this adhesion formation is to mechanically separate the surfaces [1].
There are many methods of mechanical separation and the adjuvants include both intra-abdominal instillates and physical barriers [1]. Crystalloid solutions are one such adjuvant which has been explored for adhesion prevention. The instillate, placed into the abdominal cavity after the completion of the surgical procedure prior to closing, was thought to separate peritoneal surfaces, dilute fibrin and fibrinous exudates [102]. It was found however that the absorption rate of such a solution is on the order of 35mL/hr. This means that for a 6 days postoperative presence, 5L of solution would need to be placed [1, 103]. This is obviously far too large of a volume to be realistic and thus the idea has not been extensively pursued.

Another adjuvant pursued is carboxymethylcellulose or CMC. This is a high molecular weight polysaccharide which is suggested as a coating for the intraperitoneal surfaces and is thought to create hydroflotation of the intra-abdominal surfaces [1]. CMC has been explored as a tissue coating [104], a sponge [105], and a sheet [85].

2.6—Microsphere Drug Delivery System

Microsphere drug delivery systems have been considered since 1964 when Chang first published the idea in Science magazine [106]. Prior to this article, the idea of polymers for controlled release had been explored by Folkman and Long, and Desai et al. [107]; however, it was not until the 1970s that the idea of utilizing biodegradable polymers was explored. This idea was incorporated with microspheres by Mason et al. in 1976 [107]. The exploration and characterization of microspheres is continuously being expanded.
The novelty of utilizing biodegradable polymeric microspheres is the ability to control the drug release properties of the delivery system. Drug release from microspheres are affected by many different factors including polymer molecular weight, microsphere size, drug distribution, microsphere morphology, and composition [107]. By controlling and changing these aspects, a drug delivery platform can be created to specifically meet the desired release profile and even target conditions or organs [107].

Additionally, a biodegradable, local delivery system, such as poly(lactic-co-glycolic acid) (PLGA) microspheres, has the potential to deliver multiple pharmaceutical agents with guaranteed patient compliance. In nearly every area of medicine, patient compliance is a concern and often a serious issue [108]. In the realm of surgery, a local delivery system, applied during operation, which releases the necessary amount of therapeutic agents within a particular time frame would be very advantageous and the potentials for delivery of additional agents including analgesics and antibiotics would be useful.

2.6.1—Preparation techniques

There are several methods for preparing polymer microspheres. In this section, the main methods encountered in literature will be briefly reviewed.

2.6.1.1—Microspheres via polymerization

One method of microsphere preparation involved the polymerization of monomers to polymers via an emulsion technique. In this method, monomers are dispersed in a medium of opposite solubilities. This dispersion is referred to as oil-in-water (O/W) when the oil-soluble monomer is dispersed into an aqueous media and
water-in-oil (W/O) when the opposing circumstance arises [109]. Usually, for an O/W emulsion, a water soluble initiator is utilized and a surfactant is present to ensure uniform sphere formation. The polymerization itself takes place within the micelles as this is where the initiator is located. The resulting spheres typically have a size on the nanometer level [107, 109].

Another method of microsphere formation with polymerization is a dispersion technique. This technique results in particles which are larger than those formed via the emulsion technique, with a particle size range of 0.5-10 µm [107, 109, 110]. In this method, the monomer, initiator, and a stabilizer are all dissolved into an organic medium. The polymerization occurs inside monomer droplets and as the polymer beads are formed they precipitate out of solution with the stabilizer ensuring flocculation does not occur [107, 111, 112].

The final method of polymerization microsphere formation found commonly in literature is suspension formation. In suspension polymerization, micron-sized particles are usually obtained (50-500µm). For this method, monomer is dispersed into an aqueous phase with stabilizer and initiator. The initiator is soluble within the monomer phase and thus the polymerization occurs within these droplets [107, 110].

In each of these methods, a polymerization occurs with subsequent of concurrent microsphere formation. There are however methods of preparing microspheres from polymers.
2.6.1.2—Microspheres from polymers

The most commonly used polymers are linear polymers. As the polymer of interested (PLGA) is a linear polymer, only linear polymer microsphere formation methods will be explored. Several reviews exist which treat microspheres formation and degradation of linear polymers [113, 114, 115]. The information below is a brief summary of the two most commonly encountered microsphere preparation methods; namely solvent evaporation technique (a.k.a emulsion technique) and the spray drying technique.

For solvent evaporation, the microspheres are formed from the evaporation of the organic solvent from an aqueous medium. As the solvent evaporates, the polymer shell hardens forming microspheres [116]. If the drug is soluble in the organic medium, a single emulsion can be utilized, but if the drug is water soluble, a double emulsion technique is required. In the double emulsion technique, the drug is first dissolved into an aqueous phase. It is then dispersed into an organic solvent containing the degradable polymer. This constitutes the first W/O emulsion. Next, the W/O mixture is added to an aqueous medium usually containing a stabilizer such as poly(vinyl alcohol). This constitutes the final O/W emulsion. As the organic solvent evaporates, the polymer hardens and the drug becomes encapsulated within the microspheres [107, 113-116, 117, 118].

The alternative method of spray drying is a rapid, convenient method which requires only mild conditions. One of its major advantages is that it is less dependent on the solubility parameters of the drug and the polymer [114, 119]. It also offers application and scale up advantages due to its relatively continuous process capabilities.
Spray drying does however have its disadvantages. One issue encountered is the formation of crystals on the microsphere surface when a hydrophilic drug was encapsulated [114]. Another potential problem is the significant loss of product which can occur during the spray drying process due to the adhesion of the microparticles to the inside of the spray-drier apparatus [121]. However, many groups have used this particular technique successfully for the microencapsulation of low-molecular weight substances [114, 122].

2.7—Ketorolac Tromethamine (KT)

As was discussed earlier, it has been suggested that a Cox-inhibitor, such as ketorolac tromethamine, could interrupt the inflammatory response and thus the fibrin gel matrix formation and prevent adhesion formation [7]. This section will explore the specifics of ketorolac tromethamine and its potential utilization as an anti-adhesive adjuvant.

KT is a pyrrolizine carboxylic acid derivative structurally related to indomethacinis [123]. Since its approval by the U.S. FDA in 1997 [124], KT has been used as an alternative to opioids following a variety of procedures [25, 7, 125, 126]. Unlike opioids, KT is not habit forming, does not depress respiration, has low incidence of nausea and vomiting, does not cause urinary retention and does not have a sedative effect [124, 127].

KT is classified as a non-steroidal, anti-inflammatory drug, otherwise known as an NSAID; it is available in intravenous, intramuscular, and oral formulations. It is indicated as a short-term analgesic for acute pain [25, 7, 123-132]. It is said to be 800
times more potent than aspirin when administered in oral formulation [128] and that 30mg of KT has the efficacy equivalent of 12mg morphine sulphate when administered via intramuscular injection (IM) [129]. It also exhibits pronounced analgesic effect, a moderate anti-inflammatory activity [128], and has been shown to have anti-pyretic tendencies [130]. In the body, KT has a half life of approximately 5-6 hours and the major excretory system is through the kidney following hepatic metabolism [124, 131].

![Figure 6. Chemical structure of Ketorolac Tromethamine (KT).](image)

KT acts by inhibiting the cyclooxygenase pathway and therefore will also inhibit platelet aggregation [124]. One study examined the effect of KT and baicalin on the levels of TNF-α, IL-6, and IL-1β; this study reported that KT with baicalin did inhibit the expression of these inflammatory molecules [126]. As mentioned above, adhesions are formed when the inflammatory response is triggered after injury; this starts a cascade which leads to the formation of a fibrin matrix which becomes the adhesions [25]. It is believed that by interrupting the inflammatory response, the adhesion formation can be decreased if not completely prevented. KT has been used in both a porcine and a rabbit study for its potential use as an anti-adhesion treatment [7, 8]. These studies did show positive results and a reduction of adhesion formation in the presence of KT treatment. However, they utilized qualitative grading methods and did not use a localized delivery
system. They also did not completely eliminate adhesion formation. This suggests that KT has promise as an adhesion prevention adjuvant.

KT is an inexpensive and commonly used drug. It is often used as a substitute for opioids; it is safe and generally well tolerated by patients [132]. Although KT is considered safe, it does show more side effects when administered in the oral dosage form [132]. The major side effects of KT are gastrointestinal complications; these are believed to be dose related. A localized delivery of KT could alleviate these side effects. Because of its potency in systemic delivery, a local drug delivery system for KT would be advantageous independent of the prevention of adhesions and thus would be beneficial to society regardless of the outcome of this study.

2.8—Poly(lactide-co-glycolide) (PLGA)

The use of poly(lactide-co-glycolide) (PLGA) is an FDA approved polymer for a variety of uses [133]. It is a well characterized and widely used polymer [133]. Like many polymers, PLGA can be manipulated to the desired degradation timeline and release profile by changing its composition and encapsulation parameters [134].

![Figure 7. Structure of PLGA co-polymer. X represents the number of lactic acid units, Y the number of glycolic acid units.](image-url)

32
2.8.1—Biodegradation

The main mechanism of aliphatic polyesters, such as PLGA, degradation is via hydrolytic action [135, 136]. There are several methods of adjusting and modulating the hydrolysis of lactide/glycolide polymers, both homo and copolymers.

The adjustable factors include, water permeability and solubility, chemical composition, additive contributions (such as acidic, basic, monomers, solvents, and drug contributions), morphology, porosity, \( T_g \), molecular weight and distribution, sterilization, implantation location, device dimensions, hydrophilicity/hydrophobicity, and physicochemical factors such as pH and ionic strength [135,].

One example of degradation rate adjustment was shown in a study on rats. In this study, the chemical composition of the PLGA microspheres was adjusted and it was found that by increasing the glycolide mole ratio of the co-polymer, the rate of degradation was increased [135, 137, 138].

An interesting phenomenon occurs in the biodegradation of microspheres less than 300 microns in diameter. In the case of PLGA, PLA, and PGA devices within this size range, homogeneous degradation occurs; i.e. the surface degradation rate is equal to the core degradation rate [135, 139].

In a study by Tabata and Ikada, the degradation rate of microspheres in mouse peritoneal macrophage cell culture system was studied [140, 141]. This study found that by changing molecular weight and monomer composition, they were able to control the degradation rate. A ratio of 50/50 PLGA was found to be the fastest degrading in this system and was completely degraded within the macrophages within seven days [140, 141].
While changing of certain factors can affect the degradation rate of PLGA microspheres, it is difficult to predict the interaction and thus the optimal conditions [135]. Information in the literature is useful for determining an educated starting point, but in vivo studies are necessary to validate the release and biodegradation of any pharmaceutical microsphere. Following in vivo studies, parameters can be adjusted to fine tune the device to the desired degradation and release rates.

2.8.2—Biocompatibility and tissue/material interaction

Following any injury, a cascade of reactions follows. In the case of microsphere placement, the accepted order of events is as follows [135]. First, acute inflammation occurs with the presence of polymorphonuclear leukocytes. This is followed by chronic inflammation characterized by the presence of monocytes and lymphocytes. Then comes tissue granulation with fibroblast presence and the creation of new blood capillaries. This is followed by foreign body reaction with macrophages and foreign-body giant cells (FBGCs) at the material-tissue interface.

For microspheres of greater than 10 micron diameter, the foreign body response is seen within two to three weeks following placement. This response continues until the diameter is reduced below 10 microns at which point, the microspheres are phagocytized by macrophages and FBGCs [142, 143]. Finally, fibrosis occurs and the foreign material is encapsulated into a fibrous shell [135].

The fibrosis is similar to all such fibrosis in vascularized connective tissue. This means that it is metabolically active and collagen is produced and metabolized and in
time the fibrosis present following complete degradation will be reduced with only a small amount of residual fibrotic tissue or scar formation [135].

This cascade is known as the tissue response continuum. While microsphere presence does elicit the phases of tissue response continuum, it is worth noting that the initial acute and chronic inflammatory responses are minimal and often it is difficult to determine if the response is elicited by the presence of the biocompatible microspheres or the injury which occurred during the implantation or the therapeutic agent within the microsphere [135].
CHAPTER 3—QUANTITATIVE MODEL

The objective of this model is to create consistent adhesion formation over a 2-week survival, ending with adhesion quantification using a Material Testing System (MTS™ System Corp, Eden Prairie, MN) machine platform. The development of this technique required several revisions and 19 swine were utilized before a consistent acceptable technique was established; 11 swine were then employed to establish the accuracy and reliability of the final technique. The process for quantitative assessment of pelvic adhesions in a swine model can be divided into several steps: the initial laparotomy with fixation of the uterine cornu to the pelvic sidewall; creation of the adhesion by inducing injury; the harvesting of the adherent uterine horn and adjacent sidewall complex following a two week survival; and finally the mounting and quantitative testing of the uterus/sidewall adhesion complex.

3.1—Material and Methods

All surgical procedures are conducted in accordance with the regulations and approval of the Summa Health System Institutional Animal Care and Use Committee and in compliance with standards issued by the United States Department of Agriculture, Public Health Service and the American Association for Accreditation of Laboratory Animal Care.
3.1.1—Materials

Female domestic Yorkshire cross virgin swine, weighing 80-100 lbs, were purchased from Pineview Farms. Tiletamine-zolazepam was acquired from Fort Dodge Animal Health (Fort Dodge, IA). Atropine was bought from the American Regent Inc. (Shirley, NY). Isoflurane was purchased from Baxter Healthcare Corp. (Deerfield, IL). The analgesic ketoprofen was received from Teva Pharmaceuticals USA (Sellersville, PA). Formalin (10%, neutral buffered) was acquired from Fischer Scientific Co. L.L.C. (Kalamazoo, MI), Spill-X-FP was bought from Ansul Fire Protection (Marinette, WI). Bupivicaine (0.5%) was purchased from Hospira, Inc. (Lake Forest, IL).

3.1.2—Laparotomy and Adhesion Injury

Female domestic Yorkshire cross virgin swine were received and conditioned a minimum of 10 days prior to surgery. Anesthesia was induced with tiletamine-zolazepam (6.6 mg/kg, IM) and atropine (0.05 mg/kg, SQ.); subsequent delivery of isoflurane (0.5-2.5%) was used to maintain anesthesia throughout surgery. The analgesic ketoprofen (1-3mg/kg, PO) was administered before anesthesia was delivered and after surgery as needed.

The midline infraumbilical area was clipped, shaved, and prepared for aseptic surgery with a chlorhexidine solution followed by application of an iodine impregnated adhesive surgical barrier. Following a midline infraumbilical laparotomy, the bowel was packed and retracted, allowing adequate exposure of the uterine horns and the adjacent pelvic sidewall.
3.1.2.1—Preparation of the uterine horn

A salpingostomy was made approximately 1 cm caudal to the junction of the uterus and fallopian tube using electrocautery. Through this incision was placed a 7 cm segment of an 8 fr. latex urinary catheter, which is reinforced with a coaxial internal semi-rigid 5 fr. polypropylene catheter. The catheters were inserted from cranial toward the caudal aspect of the uterus to lie entirely within the midportion of the uterine cornu. Once the uterus is cannulated, a 10 cm segment of 6.35mm inside diameter latex rubber drain tubing is secured longitudinally to the dorsal aspect of the broad ligament just medial to the horn of the uterus using two 2-0 delayed absorbable monofilament sutures. This tubing was placed to prevent adhesion of the sidewall to the broad ligament without interfering with the adhesion of the uterus to the sidewall.

3.1.2.2—Attachment of the uterine horn to the sidewall

The cannulated uterus and underlying latex rubber drain were then attached to the sidewall of the pelvis. This was done by first placing a suture of 2-0 silk through the peritoneum on the pelvic sidewall in a position to secure the end of the uterus without putting excessive strain on the broad ligament. Determination of the exact location of the suture placement must be done by first laying the uterus along the sidewall, and visually inspecting the tension on the broad ligament compared to an optimal position on the lateral aspect of the pelvic sidewall. Once the silk suture was placed, four square knots were tied, leaving the needle on the suture and the tied ends long enough for a second knot. The needle of the silk suture was then passed through the uterus, into the open end of the 8 fr. catheter, and back out through the wall of the catheter and uterine wall. Thus,
approximately 2-3 mm of uterus and underlying catheter are attached by a length of four knots of silk suture to the sidewall. This procedure was repeated for the opposite end of the uterus so that it lies affixed by both ends to the pelvic sidewall, hanging loosely by the four knots in each silk suture. See Figure 8 for a depiction of the final placement of the created adhesion complex.

Figure 8. The cannulated uterus is suspended from the pelvic sidewall by sutures at either end. The latex rubber drain under the broad ligament is visible, extending beyond the uterus on the left and right. The coagulated sidewall is visible behind the uterine horn. Final placement of adhesion complex in Swine S0763 on 19 October 2007.

3.1.2.3—Creation of surgical injury

The peritoneum of the pelvic sidewall just lateral to the attached uterus was coagulated at a setting of 6/10 using a Valleylab Surgistat B®, which gives an output of 17 Watts, along the full length of the cannulated uterus using a shielded electrocautery tip. This was accomplished by gently pulling the attached uterine horn off of the sidewall.
The area cauterized should correspond to the lie of the cannulated uterine horn, and should be limited to the peritoneum only, avoiding deep injury to the underlying muscle and attempting to avoid fenestrating the peritoneum. The overall area cauterized was approximately 8 cm long by 1 cm wide. Once the sidewall was injured, electrocautery at the same setting was used to injure the sidewall side of the cannulated uterus. This was done to desiccate the superficial layer of the uterus in a single, longitudinal line.

Coagulation does not involve the entire circumference of the uterus or the ventral aspect. This process was repeated on the contralateral side.

3.1.2.4—Closure

Upon completion of the bilateral uterine horn and pelvic wall injury, an antibiotic rinse of 1g cephazolin in 500cc sterile saline was added to the abdomen; excess fluid is removed via suction. The packing and retractors were removed and the abdomen was closed using a running mass closure of 0 polyglycolic acid, including peritoneum and fascia. The subcutaneous tissue was closed with a 2-0 polyglycolic acid suture, and the skin is reapproximated using a 2-0 polyglycolic acid subcuticular closure. The incision site was infiltrated with 10 cc of 0.5% bupivicaine (2.5mg/mL). Anesthesia was discontinued and, once the animal was extubated and breathing normally, it was returned to the cage. The animals were observed twice daily to check general health and ensure dressings are intact.

3.1.3—Surgical Removal and Testing

Following a 2-week survival, animals were anesthetized as previously described. Once full plane anesthesia was reached, a midline laparotomy was performed through the
previous incision, and visual measurement of the pelvic uterine horn adhesions is assessed by the surgeon according to the visual grading scale adapted from several sources [39,43,44]. An intravenous bolus of 60 mL saturated potassium chloride solution was delivered to achieve euthanasia. The entire complex of cannulated uterine horn, attaching sutures, and adherent underlying pelvic sidewall with muscle was removed en bloc.

3.1.3.1—Visual Grading

Prior to excision, the uterine horn adhesion was assessed by the surgeon. This assessment was based on a visual grading scale that was adapted from several sources [39,43,44]. See Table 2 for the visual grading scale.

<table>
<thead>
<tr>
<th>Visual Grading Scale</th>
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<tbody>
<tr>
<td>0</td>
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<tr>
<td>1</td>
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<td>2</td>
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<td>3</td>
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</table>

Table 2. Visual Grading Scale. Adapted from sources.

- No adhesions present
- thin, filmy adhesions
- translucent but not transparent adhesions
- minimal vascularity
- allows motions of horn away from wall
- Dense, opaque
- Vascular elements
- Limited motion of horn
- Dense, opaque
- Vascular elements
- involves other organs

3.1.3.2—Histological Testing

Histological testing was performed after the complex has been quantitatively tested. For this, two representative slices are taken adjacent to the test location. See
Figure 9 for a detailed schematic. These two slices are then evaluated by a pathologist using the scoring system found in Table 3.

### Table 3. Histological scoring evaluation of post-surgical adhesions.

<table>
<thead>
<tr>
<th>Adhesion Thickness</th>
<th>Adhesion Density</th>
<th>Adhesion Organization</th>
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<tbody>
<tr>
<td>0</td>
<td>No collagen</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>Fine collagen (individual Strands)</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Thicker collagen</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>High density (scar)</td>
<td>3</td>
</tr>
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</table>

Score range = 0 - 8

3.1.3.3—Quantitative Testing

The sample was marked cranially and caudally with a clip or suture to distinguish these for future reference. Once excised, the muscle, broad ligament and latex rubber drain were dissected away leaving only the uterine horn and adherent pelvic sidewall. The muscle was cautiously removed using dissecting scissors. Once the excess muscle was removed, the latex rubber drain was opened along its length, removing the attachment of the broad ligament to the uterine horn. The latex rubber drain and attaching sutures were removed. The medial attachment of the broad ligament was severed, resulting in a specimen consisting of only peritoneal sidewall with adhesion attaching the uterine horn to the wall.

With the sample oriented, a ruler was placed at the silk cranial suture to mark cut points at 1.5 cm and 4.5 cm distally. A scalpel blade was used to cut through at the marked points, extending vertically through the peritoneal sidewall so that a 3 cm testing section of sidewall and adherent horn remain. The excised cranial and caudal portions
were saved for histological assessment. Figure 9 demonstrates a schematic of the final complex of uterus and sidewall with markings for preparation and histology specimens.

![Left Uterine Horn Diagram](image)

Figure 9. A schematic of the final complex of uterus and sidewall with markings for preparation and histology specimens.

The cannulating catheters were removed and a 0.64 cm outside diameter (OD) by 8 cm length stainless steel rod was inserted through the lumen of the uterine horn. The length of uterus being tested was measured and recorded. The uterus was centered onto the rod and held in place using small rubber O-rings (0.95 cm OD) placed against each end of the specimen to prevent any lateral movement. Larger O-rings (1.28 cm OD) were placed at each end of the stainless steel rod to stabilize the specimen during MTS testing. Finally the peritoneal sidewalls are tightly secured within a jig clamp. The complex is loaded onto the MTS machine platform, which consists of a hydraulically controlled ram and previously calibrated dynamic load cell. For our purposes, the ram moves a total of 40 mm at a rate of 1.6 mm/sec. Figure 10 demonstrates the complex ready for quantitative testing. The clamp containing the peritoneum draped sidewall was secured to the stationary head of the platform, while the stainless steel rod side was affixed to the hydraulically controlled ram. Under displacement control, the adhesion complex was
pulled apart. Force and displacement measurements were recorded during the pull. This quantitative data collected was then be analyzed to assess adhesion strength.

![Figure 10](image-url)

Figure 10. The complex of the uterine horn, adhesion, and attached pelvic sidewall is mounted onto the MTS testing device. The rod runs through the uterus while the sidewall is grasped within the clamp. The adhesion is being slowly pulled apart by the MTS. (a) complex loaded at beginning of test (b) complex being pulled apart mid-test (c) complex nearing the end of the test.

3.1.3.4—Statistical Method

To establish the statistical significance of this study SigmaStat® (Systat Software, Inc., Ashburn, VA) was employed. Pearson and Spearman analyses were performed to identify correlations among variables, and an analysis of variance (ANOVA), blocking on the animal was performed using a post hoc Tukey standardized range test to evaluate the main effects. Also, each grading method was tested with paired t-test to establish statistical differences while blocking on the animal. Significance was accept at p<0.05. For detailed information regarding the statistical analysis see Appendix I.
3.2—Results and Discussion

3.2.1—Rationale for New Procedure

This procedure was developed in an attempt to develop a reproducible model for the creation and quantitative evaluation of pelvic and abdominal adhesions. Small animal models, mainly rats and rabbits, have been explored; but these models lack uniformity of injury, grading or results [6-52]. The lack of a uniform injury and grading system makes it difficult to determine the best method for creating adhesions. Another disadvantage of the small animal models is their discrepancy with human anatomy and physiology. While the mechanism for peritoneal healing in rats and rabbits has not be extensively investigated, it can be argued that because the wound healing of these small animals varies from human wound healing, similarly peritoneal repair will also vary [144].

Larger animal models, mostly dog and swine, have also been studied. While larger animal models have the benefit of similar organ size and weights to humans, especially the swine [144], these animal studies showed similar grading bias and inconsistencies as the small animal studies [6, 45-52]. In swine models, it is also observed that the mechanism for wound healing, namely re-epithelialization, is the same as human wound healing. These wound healing characteristics differ from small mammals, which heal primarily through wound contraction [144]. In an assessment of 25 wound therapies, various models were compared to human studies to test for concordant results. Results showed that 78% of the time swine and human results coincided, versus 53% in small-animals [144]. An additional advantage of a swine study is the potential to test both laparoscopic and open procedures.
3.2.2—Advantages of new procedure

The significant advantage of this method is that the adhesions are created in such a manner as to be appropriate for quantitative assessment using the MTS system. A representative graph of one tested adhesion complex is demonstrated in Figure 11. The numerical information from the quantitative testing takes many forms, including the maximum strength of the adhesion (maximum peak on the graph), total work (the total area under the curve), and the displacement required for adhesion rupture. The corresponding histological images can be found in Figure 12.

This method for adhesion creation was specifically designed to permit MTS testing. As such, the method has undergone extensive analysis and revision to optimize the testing results while minimizing artifacts. In optimizing this technique, 19 swine were utilized. The extent of electrocautery injury, the placement of a reinforced uterine cannula, and the placement of the latex rubber drain on the dorsal side of the broad ligament are all refinements of the original concept. Due to the size requirements of the MTS testing platform, swine of an adequate weight are required, which is advantageous due to their physiological similarities to humans [144].
Figure 11. The quantitative data generated by MTS testing of experimental pelvic adhesions. The displacement (mm) of the MTS clamp and rod is plotted against the force (Nts) required to create the displacement. Note how the sidewall completely separates from the uterus at the end of the graph.
Figure 12. Histological findings for P0751L. The upper left slide was taken at 10X magnification; A,B,C are at 400X magnification.

3.2.3—Statistical Findings

Following the optimization of the described technique, 11 swine were employed to establish the accuracy and reliability of the final technique. Of these 11 swine, 2 were excluded from statistical analysis leaving 9 swine total. One of the swine was excluded because it experienced postoperative malignant hyperthermia, and upon harvest bowel and bladder adhesions were observed making it difficult to excise and the left uterine horn was torn during this attempt at excision. The other swine also developed small bowel adhesions; in attempting to excise the left uterine horn, the adhesions were torn.

From statistical analysis, it was found that the animal side, left or right, from which the uterine-peritoneal complex was harvested, had no effect on any tested parameter. The left and right values for the MTS parameters, histology gradings, and
visual assessments were not statistically different. This establishes the internal consistency of utilized the bifid anatomy of porcine. Furthermore, the cranial and caudal histology scores were shown to have a positive correlation and were not significantly different from one another, establishing a uniform testing complex.

The visual scoring method, adapted from accepted models of adhesion formation, showed no correlative relationship with either histological score, average, cranial or caudal, or with any MTS force parameters. This is a noteworthy discovery as many previous studies utilize the visual scoring method as the primary method for determining adhesion strength and comparing potential adhesion prevention adjuvants.

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<td>0749</td>
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<tr>
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<td>0.44</td>
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The ultimate utility of the technique described here lies not with the testing of adhesions themselves, but in testing and comparing of adhesion prevention techniques. As such, it is necessary to demonstrate that treated and untreated adhesions can be quantified and compared with this model. This will be the focus of up-coming studies.
CHAPTER 4—KETOROLAC TROMETHAMINE ENCAPSULATED MICROSPHERES

4.1—Optimization of External Aqueous Phase Stabilizer

Stabilizer optimization involves the comparison of two different stabilizers at three different concentrations. These are studied to determine the optimal external aqueous phase for encapsulation of KT. The stabilizers of interest are poly(vinyl-alcohol) (PVA) and poly(vinyl pyrrolidone) (PVP). In a study by Capan et al., the effect of PVA versus PVP at varying compositions on microsphere size, release, and surface morphology was studied [145]. They found that PVA produced significantly smaller microspheres than PVP, and that with increasing concentrations of PVA, the initial release rate increased. This study was performed on the encapsulation of pDNA. A similar study has not been performed on encapsulation of KT, nor has it been replicated in other areas. As this could significantly affect the release rate, it should be investigated to determine any effects. The different solutions consist of 1\%PVA, 2\%PVA, 5\%PVA, 1\%PVP, 2\%PVP, and 5\%PVP. The loading efficiency was determined by UV/VIS Spectrometry and the optimal stabilizer determined.
4.1.1—Materials

Ketorolac Tromethamine, USP grade, was purchased from Spectrum Chemicals (Gardena, CA, USA). Poly(lactic-co-glycolic acid) (PLGA) 50/50 (inherent viscosity: 0.15-0.25dl/g) was purchased from Sigma (St. Louis, MO, USA). Dichloromethane (DCM) was bought from Aldrich (Milwaukee, WI, USA) and used as received, without further purification. Poly(vinyl alcohol) (PVA) and Poly(vinyl pyrolidone) (PVP) were purchased from Sigma Aldrich. Phosphate buffered saline (PBS) was bought from Aldrich (Milwaukee, WI, USA), dissolved in 1 L of distilled-deionized water as directed to yield a concentration of 0.01 M PBS.

4.1.2—Microsphere Preparation and Drug Loading

Microsphere preparation will involve a standard w/o/w (water-in-oil-in-water) emulsion method that has been established [114, 146, 147, 148]. The double emulsion process is utilized because KT is highly water soluble. See Figure 13 for a breakdown of the W/O/W emulation technique.

Briefly, 2g of PLGA was dissolved in 20mL of DCM to constitute the organic phase. A corresponding amount of drug (KT) was dissolved in 40mL de-ionized water (dH₂O). The amount of KT was determined according to the theoretical loading of either 0.1 or 0.2; the loading value is defined in Equation 1 below. This constitutes the first aqueous phase.

\[
\text{Loading \text{Theoretical (mg)} = \frac{\text{Weight of drug (mg)}}{\text{Weight of polymer (mg)}}}
\]

Equation 1. Theoretical Loading Value.
Figure 13. Flow diagram of double emulsion technique for the production of KT encapsulated in PLGA microspheres.

The drug containing aqueous phase is added to the organic phase. This solution is emulsified with vigorous stirring at room temperature using a stirring motor with an impeller (LR400D, Yamato Scientific Co. Ltd.) at a constant speed of 350rpm for 10 minutes, this constitutes the w/o emulsion. It is then added gently with stirring to 50mL external aqueous solution (either PVP or PVA at 5%, 2% or 1%), stirring continues for 10 minutes after addition. The organic solvent is permitted to evaporate under atmospheric conditions on a lab stirring plate (Fisher Scientific) for 5 hours at a speed of 300rpm.
As the solvent evaporates, the drug contained in the internal aqueous phase becomes trapped within the polymer microparticle. The evaporation leads to the hardening of the initially soft and viscous microparticles droplets. The microspheres are then collected by centrifugation at 2500rpm for 10 minutes (Sorvall), washed with distilled-de-ionized water and re-centrifuged; the wash is repeated 3 times. The microparticles are then shell frozen and lyophilized (Labconco) for complete removal of water from the system and the final product is obtained. The final product is a free flowing powder of PLGA microspheres loaded with KT. Blank PLGA microspheres, with no drug present, were prepared in a similar manner with 40mL of dH$_2$O without drug constituting the internal aqueous phase for control purposes.

4.1.3—Drug Loading Studies

The efficiency and actual loading of all samples was determined by the following process. A known amount of loaded microsphere, approximately 0.03g, was placed in 2mL of DCM and allowed to stand for 30 minutes. This allows the polymer to dissolve. Then 10mL of PBS (pH = 7.4) was added; the mixture was vigorously shaken for 2 minutes. It was then allowed to settle for one hour. Care was taken not to disturb the aqueous/organic layer separation. Once the layers had separated, the aqueous layer was collected. The drug presence was then measured by UV/VIS Spectrophotometer. The wavelength of interest is 322nm and the concentration of the drug was determined by a standard calibration curve for KT in PBS. Triplicate runs were completed for each drug loading. The encapsulation efficiency, expressed as a percentage, was calculated by Equation 1, Equation 2, and Equation 3, with the later two defined below.
Encapsulation Efficiency = \frac{\text{Actual Drug Loading}}{\text{Theoretical Drug Loading}}

Equation 2. Encapsulation Efficiency.

\text{Actual Drug Loading} = \frac{\text{Weight of Drug (analytical)}}{\text{Weight of Microspheres - Weight of Drug (analytical)}}

Equation 3. Actual Drug Loading.

4.1.4—Statistical Methods

To establish statistical significance in this section, SigmaStat® (Systat Software, Inc., Ashburn, VA) was employed. Whenever possible, Student’s t-tests were used; when normality or variance did not allow for this method, Mann-Whitney Rank Sum Tests were utilized. Analysis of variance (ANOVA) was employed when more than two variables were compared. Significance was accepted at \( p < 0.05 \). For detailed information regarding the statistical analysis, see Appendix II.

4.2—Microsphere Encapsulation Characterization and Analysis

All six solutions were used to produce microspheres at both theoretical loadings. Additional characterization and analysis were performed. These studies include the before mentioned drug loading study and yield studies, as well as microsphere surface morphology determination, particle size analysis, release studies, and differential scanning calorimetry studies. Microsphere synthesis and drug loading efficiencies were determined as described in 4.1.
4.2.1—Microsphere Surface Morphology

The microsphere surface morphology was analyzed with the assistance of an optical microscope (Olympus IX 71) and a scanning electron microscopes (Hitachi & JEOL-JSM35C).

An optical microscope, also known as a light microscope, consists of a series of lenses with a variety of magnifications. It utilizes visible light to view magnified images of small samples. Samples are generally prepared on a glass slide and can be viewed in either liquid suspension or solid state.

A scanning electron microscope images the surfaces of an item with great resolution and depth of field, allowing for direct imaging of microscale structures. Typically, a 5-100keV electron beam is focused on the specimen in a raster pattern. The primary electron emerges from the high-energy beam and interacts with the sample; this causes the emission of secondary electrons of low-energy. The intensity of the secondary emission is a result of the geometry of the features under observation and the atomic composition of the sample. These electrons are collected by a detector which counts the response at each raster point; they are then spatially reconstructed on a phosphor screen. Thus a digital image of the surface is displayed. The SEM only allows for shallow penetration thus only the secondary electrons generated near the surface are detected; therefore, it is primarily used for surface analysis [149, 150].

To determine the surface characteristics of both loaded and unloaded microspheres, a known amount of microspheres was suspended in distilled-deionized water utilizing a sonicator; 200μL of the suspension was transferred slowly over aluminum foil placed on aluminum stub using double-sided copper tape to form a drop
covering the entire area of the stub. The stubs were dried overnight and then sputter coated with silver and viewed at an accelerated voltage of 15 and 20kV.

4.2.2—Release Study

The release profile of the microspheres was determined utilizing dialysis bags and a PBS bath on a heated stir plate. Thirty milligrams of the pure drug, drug-loaded microspheres, or unloaded microspheres were placed in a dialysis bag. 4mL of PBS was added to each bag. These were then suspended in 50mL of PBS in a beaker, and the beaker was maintained at 37ºC under continuous agitation at 300rpm stirring. Samples (1mL in volume) were withdrawn at specific time intervals. These time intervals were 1, 2, 4, 6, 8, 13, 16, 20, 24, 48, 120, 168, 240, 360, 720, and 1080 hrs. The withdrawn volume was replaced with fresh PBS to maintain sink conditions. The release was determined by UV/VIS Spectrophotometry at a wavelength of 322nm. Each experiment was performed in triplicate.

4.2.3—Size Analysis

In order to determine the particle size distribution of the microspheres, a Malvern Zetasizer Nanoseries Nano-25 (Malvern, Worcesthershire, UK) dynamic light scatterer was utilized. A minimum of 5 milligrams of sample was placed in an aqueous solution. It was then sonicated to suspend the microspheres and then dynamic light scattering was performed. All samples were run in triplicate.
4.2.4—Differential Scanning Calorimetry

A common method of verifying encapsulation is via DSC. In DSC, the reference and the sample are heated at the same constant rate. The energy which is absorbed or released during the endo or exothermic event require subsequent compensation in order to maintain the constant heating rate. This energy is electronically monitored and analyzed [151]. In this method, the $T_g$ of the polymer and microspheres are compared. If an interaction exists between the KT and PLGA the $T_g$ will be affected [151, 152]. If no interaction exists, the PLGA $T_g$ will appear identical to the microsphere samples and if desired, the KT $T_m$ can also be measured and compared.

Approximately 2mg samples will be measured and sealed in aluminum pans. These are then heated under nitrogen purging at 50mL/min with an empty pan reference. The equipment was calibrated with indium samples. All samples were run at 3ºC/min from 0 to 60ºC. DSC scans were performed using a DSC Q100 TA (TA Instruments, USA). All determinations were performed in duplicate.

4.2.5—Statistical Methods

To establish statistical significance in this section, SigmaStat® (Systat Software, Inc., Ashburn, VA) was employed. Whenever possible, Student’s t-tests were used; when normality or variance did not allow for this method, Mann-Whitney Rank Sum Tests were utilized. Analysis of variance (ANOVA) was employed when more than two variables were compared. Significance was accepted at $p<0.05$. For detailed information regarding the statistical analysis, see Appendix II.
4.3—Results and Discussion

4.3.1—Microsphere Yield

The microsphere yield was to be utilized in determining an optimum stabilizer. When all external aqueous phases were utilized in triplicate to synthesize 0.2 KT loaded microspheres, the respective yields were compared. Figure 13 depicts the yields of KT microspheres generated in the six solutions (i.e. 1 wt.%, 2 wt.% and 5 wt.% of PVA or PVP in water). The results show yields between 60 – 80%. Slightly higher yields (75-80%) were noticed for the 1wt.% PVA and 2 wt.% PVA. When the statistical analysis was performed, it was found that no statistical difference existed between the 6 groups when they were treated independently. However, the desired power was not satisfied. It was noted that there appeared to be a trend in the mean values, therefore the groups were redefined and the PVA solutions were compared to the PVP solutions.

![KT Microsphere Yield]

Figure 14. Graphical representation of microsphere yield with a KT loading of 0.2 when various solvents were utilized.
In order to achieve this comparison, a Mann-Whiney Rank Sum Test was required. This method showed a significant difference between PVA and PVP with a p=0.037. PVA proved to be the more effective stabilizer. It was decided that an internal comparison between the different PVA formulations should be performed. This did not show any significant difference between the groups.

While it was established that PVA was statistically superior to PVP when utilized at three different concentrations, the overall analysis for an optimal stabilizer did not result in a specific answer, it was decided to utilize all six solvents at a KT loading of 0.1 in an attempt to rectify the situation.

4.3.2—Drug Loading Efficiency

Drug loading was determined by the complete degradation and aqueous recovery of the pharmaceutical agent. Although some trends appear present, no statistical significant was noted between the external aqueous phases. The external phase of 1% PVA solution for a theoretical loading of 0.2 did have the smallest standard deviation and a relatively high loading efficiency of 14.8% ± 0.5%, however, both PVP 5% and 2% have ranges which overlap with that of PVA 1% making it difficult to draw any conclusions. See Figure 15 for a visual representation of the drug loading efficiency at a theoretical KT loading of 0.2; the error bars represent the standard deviation with the bar height at the average value.
Figure 15. Drug loading efficiency summary for a theoretical loading of 0.2.

Figure 16. Drug loading efficiency summary for a theoretical loading of 0.1.
4.3.3—Microsphere Surface Morphology

To analyze the microsphere surface morphology, both optical and SEM microscopes were utilized. The figures below are representative images of the findings from both the SEM and optical microscopic views. It is obvious from these images that in most samples, there are three distinct microsphere sizes present.

Figure 17. Optical microscope view of 0.2 loading microspheres made with PVP 5% at 40X. Representative of optical views. Show many small spherical objects with some small amount of aggregation visible (marked A).
Figure 18. Polarized optical microscope view of 0.2 loaded microsphere made with PVP 5%. Representative of findings. Above is a single spherical particle which is view at 40X with polarized filter. It shows a distinctly larger size microsphere particle than those seen in Figure 17. This is consistent with the size analysis findings and SEM views.

Figure 19. Optical view of 0.2 loaded microsphere synthesized with PVP 5% at 40X with polarized filter. Shows two distinct microsphere sizes. One similar to that seen in Figure 18 the other distinctly larger.
Although the microspheres are visible under optical observation, the surface morphology is not evident. Therefore, SEM imaging was performed. Under this observation the smooth surface and spherical formation is visible. It is also evident that, although the majority of the microspheres fall within the smaller size ranges, there are three distinct microsphere sizes present in most samples. This is an observation which was confirmed by the particle sizing analysis (discussed later in section 4.3.5). See Figure 20, Figure 21, Figure 22 for dry microsphere images.
Figure 21. SEM image of cluster of microspheres at high magnification. Microspheres were 0.2 loaded with utilization of PVA 2%. Note the spherical formation and smooth surface morphology.
Figure 22. SEM image of a single microsphere with 0.2 loading and PVP 2%. Representative of the smooth surface morphology and spherical formation visible in the dry microsphere samples. Note the 'pitted' region on the left hemisphere of the sphere; this was caused by the increased intensity of the electron beam and was not present prior to magnification and imaging. It is not a natural defect.

Figure 23 and Figure 24 also show the microspheres under SEM examination, however these samples were exposed to an aqueous solution for 26 hours prior to imaging. These figures show the agglomeration which occurs as the PLGA is hydrolyzed. The hydrolysis begins earlier in this instance than previous studies have shown [13, 14]. It is possible that this is due to interaction between the PLGA and the KT.

KT has a carbonyl functional group with an electronegative oxygen atom attached to it. It is a pyrolizine carboxylic acid derivative. The oxygen atom in close proximity to
the carbonyl group leads to a change in reactivity. This could lead to an interaction between the functional group of KT and the ester linkage present in PLGA. In the presence of water, hydrolysis degrades PLGA at these ester linkages, if KT were to also interact with the ester linkages, it could increase this degradation causing an increased initial rate of degradation and accounting for the agglomeration visible in the SEM images.

Figure 23. SEM view of 0.1 loaded, PVA 1%, microspheres after 26 hours in aqueous solution without agitation. This is a representative image of the microspheres left in solution for 26 hours prior to SEM imaging. It shows the agglomeration which is evident within these samples.
4.3.4—Release Study

Release studies were performed utilizing a simple dialysis experiment with in vitro conditions of 37ºC and continuous stirring. See Figure 25 for a representative image and a depiction of the concept behind dialysis bag experiments. The KT released was determined utilizing UV/VIS Spectroscopy at 322nm.
As KT was released from the microspheres, measurements were taken to
determine the concentration of KT and thus determine the amount of KT that had been
released from the microspheres. It was noted that although the amount of KT released
was variable, the percentage of KT released was not (see Figure 26 and Figure 27 for
visual depictions). It was also observed that there appears to be three distinct regions of
release: an initial release, a transition period, and an extended release. This prompted an
in-depth analysis of the release rates. The initial release was defined for the purposes of
analysis as $t = [0, 24]$ hours, the transition region as $t = [24, 240]$ hours, and the extended
release as $t = [240, 1080]$ hours.

The release rates were examined for each external aqueous phase individually. It
was observed that there did exist a distinct difference in the release rate of KT in $\mu$g/h for
the 6 groups. It was interesting to observe that the differences were mirrored in the initial
burst and transition regions but varied slightly in the extended release phase. See Figure
28, Figure 29, and Figure 30.
Figure 26. Graphical representation of release data for a theoretical loading of 0.1 KT. The values represent the % of KT release versus time. The % release was determined using the drug loading data obtained during the drug loading efficiency studies.

Figure 27. Graphical representation of release data for a theoretical loading of 0.2 KT. The values represent the % of KT release versus time. The % release was determined using the drug loading data obtained during the drug loading efficiency studies.
Figure 28. Graphical representation of initial burst release rates for each external aqueous phase at 0.1 and 0.2 theoretical loadings. The initial burst period was defined as the time from 0 to 24 hours. The error bars represent the standard deviation.

Figure 29. Graphical representation of transition period release rates for each external aqueous phase of 0.1 and 0.2 theoretical loadings. The transition period was defined as the time from 24 hours to 240 hours. The error bars represent the standard deviation.
There are several possible explanations for the variable release rates. After reviewing the size analysis data it would seem that the most plausible is that the initial burst and transition regions are directly affected by the particle sizes. Table 5 gives a brief summary of the particle size distributions and most importantly the volume percentages of each representative size.

For example, in the size analysis it was found that PVA 5% 0.1 loading contained a larger volume of 4.9μm diameter particles (68%) and a smaller volume of 101.6μm diameter particles. As the smaller particles have more surface area per unit mass, this would result in a larger total presence of KT on or near the surface of the particles. It is conceivable that the increased release is due to this large presence of smaller diameter particles. This would account for the increased release rates in the initial and transition
regions. When the extended release period is reached, polymer degradation becomes a significant factor and the effect of particle surface area is diminished.

4.3.5—Size Analysis

A common way of reporting the size analysis data collected via dynamic light scattering (DLS) is through a $Z_{\text{avg}}$ and a correspondent PDI or polydispersity index. For DLS, $Z_{\text{avg}}$ is a measure of the average particle size and the PDI is a measure of the relative variance assuming that only one particle population is present [153]. As the SEM and optical images indicated this would be an incorrect assumption; therefore a multimodal setting was utilized for the size analysis of the samples. In this mode, the assumption of one particle size is not utilized and thus multiple populations can be discovered.

The particle size analysis revealed a striking correlation to the release data collected and visual observations made during surface morphology analysis. It was discovered that there were indeed three distinct particle diameter ranges. While the visual observations would have led to the belief that three distinct sizes would be observed in all samples, the small sample size associated with the particle size analysis could cause those samples with a lower ratio of one size to go undetected. Also, while the test was run on multimodal settings, the wide width of several of the curves and the raw correlation data would indicate that there is the possibility of two size populations being treated as one during the testing. If the two sizes are indistinguishable within the parameters of the testing equipment it would difficult to differentiate and thus they would be recorded and treated as one intermediate population with an increased volume %.
Table 5. Particle size analysis summary. Note the size distributions are consistent with those observed in visual observations and seem to have a correlation to release data.

<table>
<thead>
<tr>
<th></th>
<th>Diameter</th>
<th>Diameter</th>
<th>Diameter</th>
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<tbody>
<tr>
<td></td>
<td>Peak 1 (μm) vol %</td>
<td>Peak 2 (μm) vol %</td>
<td>Peak 3 (μm) vol %</td>
</tr>
<tr>
<td>0.2 loading</td>
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<td></td>
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<tr>
<td>PVA 5%</td>
<td>19.665</td>
<td>60.58</td>
<td>89.66</td>
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<tr>
<td>PVA 2%</td>
<td>12.936</td>
<td>95.4</td>
<td>72%</td>
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<td>PVA 1%</td>
<td>15.578</td>
<td>47.94</td>
<td>111.2</td>
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<tr>
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<td>12.47</td>
<td>54.94</td>
<td>94.02</td>
</tr>
<tr>
<td>PVP 2%</td>
<td>17.884</td>
<td>46.433</td>
<td>101.1</td>
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<tr>
<td>PVP 1%</td>
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<tr>
<td>0.1 loading</td>
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<td>44.06</td>
<td>44.06</td>
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It is interesting to note that the control microspheres did not appear to contain any particles <30μm. This is also consistent with SEM observations. As discussed previously, KT does have a functional carbonyl group with a reactive oxygen atom adjacent. This could cause a surface interaction with PLGA which results in the lower microsphere size production. The variable size of the microspheres has been found in literature for PLGA microspheres produced via emulsion techniques [154, 155]. It is moderately confounding that there is no apparent correlation between the external aqueous phase utilized and the particle sizes observed during the two different theoretical loadings. While the loading efficiency of the 0.2 theoretical loading was lower, the actual mass of KT loaded was higher which would lead to a higher volume of smaller particles, however the size analysis does not reflect this. Statistically, the data between stabilizers is not significant and conclusions about the individual external aqueous phases remain ambiguous.
Figure 31. Size distribution by volume of unloaded microspheres produced with PVA 5% external aqueous solution.

Figure 32. Graphical representations of size analysis data observed during analysis. A) Size distribution by volume of microspheres with a theoretical loading of 0.2 and an external aqueous phase of PVA 5%. B) Size distribution by intensity of the same sample.
4.3.6—Differential Scanning Calorimetry

The differential scanning calorimetry was utilized with the purpose of examining the effect of microsphere synthesis and KT encapsulation on the glass transition temperature. The machine was first calibrated and then pure samples of PLGA and KT were analyzed. The PLGA revealed a \( T_g \) of 39.3 ± 1.8°C; this is consistent with literature values [156, 157]. KT showed a \( T_m \) at 168°C and representative peaks at 84.5°C and 103°C.
The microsphere samples were then analyzed. The table below shows a summary of the results reported by external aqueous phase. The T\textsubscript{g}’s of the microspheres varied from 24.7\textdegree C to 34.2\textdegree C or about 10\textdegree C. All microsphere samples revealed a T\textsubscript{g} less than that of the pure PLGA sample. Most of the samples also revealed a peak between 97.7 and 103.6\textdegree C. This is often referred to in literature as ‘complex remodeling’ [156, 157]. From a comparison with the loading efficiencies, it would appear that this peak is only observed when the loading efficiency exceeded 10\%. This peak is useful as it verifies the encapsulation of KT into the microspheres and maybe used in the future for rough estimation of loading efficiency.

Table 6. Summary of DSC results reported by external aqueous phase.

<table>
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<tr>
<th></th>
<th>T\textsubscript{g} (\textdegree C)</th>
<th>StDev</th>
<th>CR (\textdegree C)</th>
<th>StDev</th>
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<td>KT *Tm</td>
<td>168.07</td>
<td>84.54</td>
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<tr>
<td>PLGA</td>
<td>39.31</td>
<td>1.82</td>
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<tr>
<td>0.1 TL</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>PVA 1%</td>
<td>29.77</td>
<td>0.54</td>
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<td>PVA 2%</td>
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<td>PVP 2%</td>
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<tr>
<td>PVP 5%</td>
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<tr>
<td>PVP 5%</td>
<td>29.94</td>
<td>0.53</td>
<td>103.18</td>
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</table>

The above DSC data was examined in relation to size distribution. When the data is taken as a whole compared to the average diameter (a weighted average based on volume percentages), no distinct overall relation can be found. To get a better idea of any relationships, the data was grouped by theoretical loading and external aqueous phase. It was found that at theoretical loading of 0.1 and external aqueous phase of PVA, as the T\textsubscript{g}
and average diameter both increased. This was also observed at a theoretical loading of 0.2 and external aqueous phase of PVP. A confounding observation was that at a theoretical loading of 0.1 and external aqueous phase of PVP, as $T_g$ increased the average diameter decreased. In an attempt to get a better understanding of the phenomena, the DSC data was compared to the dominant diameter size. When taken as a whole this again did not reveal any overall trends. The data was again divided by external aqueous phase and similarly nothing was distinguishable. The data was then divided based on theoretical loading. When this was done, both PVA groups revealed a positive relation between $T_g$ and diameter. No similar relation was observed in the PVP samples. The analysis above, while interesting, does not reveal any way to predict average size based on $T_g$ or vice versa.

This analysis was also compared to the release rates. It was found that at a theoretical loading of 0.2, as the PVA concentration increased the $T_g$ and the initial burst rates decreased. While this is an interesting observation, a similar effect was not seen in any other combinations. It is also interesting and perhaps mildly frustrating to observed that the lowest $T_g$ of 24.7°C was associated with PVA 5% and theoretical loading of 0.2; this particular sample showed the lowest initial burst rate. The next lowest $T_g$ of 26.8°C was associated with PVA 5% and theoretical loading of 0.1; this sample showed the highest initial burst rate. There were no other trends or relationships that could be discovered.

The utility of the DSC to verify encapsulation and examine its effect on $T_g$ was useful. When a relationship between size or release rates was attempted to be determined, the data proved confounding. There have been several contradictory studies
related to glass transition temperature and size relationships and the observations of this study do not aid in determining a correct correlation [158].
CHAPTER 5—Conclusions and Future Works

The results presented and discussed in the above chapter represent the initial steps toward the creation of a pharmaceutical adjuvant for the prevention of pelvic adhesions. The analysis shows that KT can be successfully encapsulated into PLGA. While the release time seen above was longer than the originally sought release of 14 days, the desired initial burst effect was large and this could be potentially advantageous.

The burst effect, which lasted for about 24 hours following aqueous exposure, involved the release of about 60-70% of the loaded KT. As Figure 4 explained, inflammatory responses to injury begin within the first three hours and the fibrin matrix formation and inflammatory exudates are prominent up to the first 72 hours. KT is thought to prevent adhesion formation via an interruption in this inflammatory cascade, therefore its increased presence during the first 24 hours may be enough to cause this interruption.

An additional factor which could increase this burst effect or extend its duration is macrophage presence. When a microsphere is reduced to <20 micron diameter, studies have shown that the degradation rate and thus the release is significantly accelerated due to the phagocytosis and subsequent degradation due to macrophage presence [141]. As the size and SEM analyses would indicate that there exists microspheres which fit this description, the in vitro degradation rates may be significantly accelerated and thus fall...
within the desired time frame. It is necessary to complete in vitro studies prior to any changes in formulation or size distributions.

If additional increase in release and degradation are desired, other possibilities include adjusting the encapsulation parameters or the lactide to glycolide ratio.

5.1—Future works

The next important step to take in developing KT microspheres for pharmaceutical drug delivery is to determine the effect of sterilization on the microsphere structure, size, and duration of drug release under in vitro conditions. Once microsphere sterilization has been characterized and if there are no significant issues, in vitro and in vivo testing can be pursued.

Sterility is an important aspect of any pharmaceutical agent. Post production sterilization can affect polymers and thus must be evaluated. It is suggested that sterilization be accomplished via gamma sterilization [146]. Once sterilization is completed, SEM, thermal analysis (DSC) and release studies of the sterilized and un-sterilized samples should be evaluated for differences.

As this study showed, the manufacturing of these microspheres currently yields several different sized particles. In order to determine the optimum single size or combination of sizes, it is necessary to determine an optimum KT concentration for adhesion growth prevention. It is also necessary to get a better estimate of in-vivo microsphere degradation rates. This can be done via in-vitro cell culture studies.

For these purposes, I propose a co-culture of mouse fibroblast cells and macrophage cells. The fibroblasts are the most important precursor of adhesion
formation and therefore should be used. The macrophage cells are an important immunological response cell. In the case of microspheres, they are particularly pivotal as they are responsible for the increased degradation rate of microspheres in vivo. As mentioned previously, macrophage cells will phagocytize microsphere particles with <20μm diameters; this increases the rate of adjuvant release [141]. Because this co-culture would be necessary for the testing of the microspheres, it should be used in both experiments. From this in-vitro study, an optimum, minimum, and toxicity level for KT can be determined. Provided this information, the efficacy, size, and release rate of the produced microspheres can be reevaluated and a single or combination of sizes can be isolated via filtration. A brief description of the procedure is provided below.

The in-vitro degradation of microspheres is pivotal to the advancement of any potential pharmaceutical agent. In this instance, it is particularly important as previous studies have reported the at a particle size of <20μm in diameter, the microspheres can become phagocytized and the release rate of the adjuvant increased [141]. For the purposes of this study, mouse fibroblast and macrophage cells co-culture will be utilized for cell culture studies. The cells will be allowed to proliferate at 37°C in a humidified, 5% CO₂ atmosphere in an automatic CO₂ incubator. The cells will be passed every week and passages 3 and 6 used for the drug and device efficacy studies.

For efficacy studies, cells will be plated sparsely in 12 well flat-bottomed microplate at a density of 2000cells/cm² in cell media. After 24 hrs of subconfluency, a solution of either (1) no treatment, (2) various concentrations of ketorolac free drug solution, (3) known amount of unloaded microspheres, (4)/(5) known amount of loaded microspheres of each loading value will be added. The media will be routinely changed;
to maintain drug concentration solution, each of the above will be added each time the media is changed. On days 4 and 7, the cells will be trypsinized, according to a standard protocol and counted using a hemacytometer with trypan blue staining for assessment of cell viability. Upon completion of in-vitro studies, additional steps can be taken.

Without in-vitro studies, it is impossible to move forward. From the studies, the synthesis technique can be refined to produce microspheres with the most desirable properties following sterilization. Once this is completed, animal studies and eventual implementation as a pharmaceutical adjuvant for the prevention of pelvic adhesions can commence.
Appendix I—Quantitative Model Detailed Statistics

ANOVA Results (Blocking on subject)

Friedman Repeated Measures Analysis of Variance on Ranks  Tuesday, March 30, 2010, 12:58:11 AM

Data source: Data 6 in Model data

Normality Test:  Failed (P < 0.050)

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Chi-square= 25.784 with 5 degrees of freedom.  (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference  (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Tukey Test):

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<td>------</td>
</tr>
<tr>
<td>R Histo vs L Histo</td>
<td>1.000</td>
<td>0.178</td>
<td></td>
</tr>
<tr>
<td>L Histo vs R Visual</td>
<td>13.500</td>
<td>2.405</td>
<td></td>
</tr>
<tr>
<td>L Histo vs R Visual</td>
<td>13.500</td>
<td>2.405</td>
<td></td>
</tr>
<tr>
<td>R Visual vs R Visual</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
</tr>
</tbody>
</table>

Note: The multiple comparisons on ranks do not include an adjustment for ties.

A result of "Do Not Test" occurs for a comparison when no significant difference is found between the two rank sums that enclose that comparison. For example, if you had four rank sums sorted in order, and found no significant difference between rank sums 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed rank sums is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the rank sums, even though one may appear to exist.

MTS Paired T-Test

Paired t-test: Tuesday, March 30, 2010, 1:15:06 AM

Data source: Data 6 in Model data

Normality Test: Passed (P = 0.148)

<table>
<thead>
<tr>
<th>Treatment Name</th>
<th>N</th>
<th>Missing</th>
<th>Mean</th>
<th>Std Dev</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>R MTS</td>
<td>9</td>
<td>0</td>
<td>8.663</td>
<td>4.788</td>
<td>1.596</td>
</tr>
<tr>
<td>L MTS</td>
<td>9</td>
<td>0</td>
<td>10.286</td>
<td>6.738</td>
<td>2.246</td>
</tr>
<tr>
<td>Difference</td>
<td>9</td>
<td>0</td>
<td>-1.623</td>
<td>9.881</td>
<td>3.294</td>
</tr>
</tbody>
</table>

t = -0.493 with 8 degrees of freedom. (P = 0.635)

95 percent confidence interval for difference of means: -9.218 to 5.972

The change that occurred with the treatment is not great enough to exclude the possibility that the difference is due to chance (P = 0.635)

Power of performed test with alpha = 0.050: 0.050

The power of the performed test (0.050) is below the desired power of 0.800. Less than desired power indicates you are more likely to not detect a difference when one actually exists. Be cautious in over-interpreting the lack of difference found here.

Visual Grading Paired T-Test

Paired t-test: Tuesday, March 30, 2010, 1:15:23 AM

Data source: Data 6 in Model data

Normality Test: Failed (P < 0.050)
Test execution ended by user request, Signed Rank Test begun

**Wilcoxon Signed Rank Test** Tuesday, March 30, 2010, 1:15:23 AM

**Data source:** Data 6 in Model data

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Missing</th>
<th>Median</th>
<th>25%</th>
<th>75%</th>
</tr>
</thead>
<tbody>
<tr>
<td>R Visual</td>
<td>9</td>
<td>0</td>
<td>3.000</td>
<td>2.750</td>
<td>3.250</td>
</tr>
<tr>
<td>L Visual</td>
<td>9</td>
<td>0</td>
<td>3.000</td>
<td>3.000</td>
<td>3.250</td>
</tr>
</tbody>
</table>

W= 5.000  T+ = 7.500  T- = -2.500  P(est.)= 0.424  P(exact)= 0.375

The change that occurred with the treatment is not great enough to exclude the possibility that it is due to chance  (P = 0.375).

Histology Grading Paired T-Test

**Paired t-test:** Tuesday, March 30, 2010, 12:14:00 AM

**Data source:** Paired T-Test in Notebook 3

**Normality Test:** Passed (P = 0.251)

<table>
<thead>
<tr>
<th>Treatment Name</th>
<th>N</th>
<th>Missing</th>
<th>Mean</th>
<th>Std Dev</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histo R</td>
<td>15</td>
<td>6</td>
<td>4.676</td>
<td>1.206</td>
<td>0.402</td>
</tr>
<tr>
<td>Histo L</td>
<td>15</td>
<td>6</td>
<td>4.739</td>
<td>1.275</td>
<td>0.425</td>
</tr>
<tr>
<td>Difference</td>
<td>15</td>
<td>6</td>
<td>-0.0627</td>
<td>1.244</td>
<td>0.415</td>
</tr>
</tbody>
</table>

* t = -0.151  with 8 degrees of freedom. (P = 0.884)

95 percent confidence interval for difference of means: -1.019 to 0.893

The change that occurred with the treatment is not great enough to exclude the possibility that the difference is due to chance  (P = 0.884)

**Power of performed test with alpha = 0.050:** 0.050

The power of the performed test (0.050) is below the desired power of 0.800. Less than desired power indicates you are more likely to not detect a difference when one actually exists. Be cautious in over-interpreting the lack of difference found here.
Histology Cranial versus Caudal

**Spearman Correlation**

**Spearman Rank Order Correlation**

*Data source:* Data 7 in Model data

Cell Contents:
Correlation Coefficient
P Value
Number of Samples

<table>
<thead>
<tr>
<th></th>
<th>Caudal</th>
<th>Ave R/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cranial</td>
<td>0.925</td>
<td>0.986</td>
</tr>
<tr>
<td></td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>Caudal</td>
<td></td>
<td>0.963</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18</td>
</tr>
</tbody>
</table>

**Ave R/L**

The pair(s) of variables with positive correlation coefficients and P values below 0.050 tend to increase together. For the pairs with negative correlation coefficients and P values below 0.050, one variable tends to decrease while the other increases. For pairs with P values greater than 0.050, there is no significant relationship between the two variables.

**Pearson Correlation**

**Pearson Product Moment Correlation**

*Data source:* Data 7 in Model data

Cell Contents:
Correlation Coefficient
P Value
Number of Samples

<table>
<thead>
<tr>
<th></th>
<th>Caudal</th>
<th>Ave R/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cranial</td>
<td>0.936</td>
<td>0.983</td>
</tr>
<tr>
<td></td>
<td>0.000000010</td>
<td>2.874E-013</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>18</td>
</tr>
</tbody>
</table>
The pair(s) of variables with positive correlation coefficients and P values below 0.050 tend to increase together. For the pairs with negative correlation coefficients and P values below 0.050, one variable tends to decrease while the other increases. For pairs with P values greater than 0.050, there is no significant relationship between the two variables.

Pearson Correlation

**Pearson Product Moment Correlation**

**Data source:** P and S again in Model data

Cell Contents:
Correlation Coefficient
P Value
Number of Samples

<table>
<thead>
<tr>
<th></th>
<th>Visual</th>
<th>Histo av</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTS</td>
<td>0.399</td>
<td>0.0847</td>
</tr>
<tr>
<td></td>
<td>0.101</td>
<td>0.738</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>Visual</td>
<td></td>
<td>0.107</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.674</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18</td>
</tr>
</tbody>
</table>

Histo av

There are no significant relationships between any pair of variables in the correlation table (P > 0.050).

Spearman Correlation

**Spearman Rank Order Correlation**

**Data source:** P and S again in Model data
There are no significant relationships between any pair of variables in the correlation table (P > 0.050).

Appendix II—Ketorolac Tromethamine Encapsulated Microspheres Detailed Statistics

Solvent Analysis Raw SigmaStat Output

Analysis of different solvents and concentrations

**One Way Analysis of Variance**

**Data source:** Data 1 in Solvent data analysis

**Normality Test:** Passed (P = 0.379)

**Equal Variance Test:** Passed (P = 0.368)
The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.287).

Power of performed test with alpha = 0.050: 0.119

The power of the performed test (0.119) is below the desired power of 0.800. Less than desired power indicates you are more likely to not detect a difference when one actually exists. Be cautious in over-interpreting the lack of difference found here.

Analysis of differences between solvents PVP and PVA

t-test

**Data source:** Data 2 in Solvent data analysis

**Normality Test:** Passed (P = 0.822)

**Equal Variance Test:** Failed (P < 0.050)

Test execution ended by user request, Rank Sum Test begun

**Mann-Whitney Rank Sum Test**

**Data source:** Data 2 in Solvent data analysis

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Missing</th>
<th>Median</th>
<th>25%</th>
<th>75%</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVA</td>
<td>10</td>
<td>0</td>
<td>76.177</td>
<td>62.878</td>
<td>93.917</td>
</tr>
<tr>
<td>PVP</td>
<td>9</td>
<td>0</td>
<td>62.253</td>
<td>57.621</td>
<td>69.360</td>
</tr>
</tbody>
</table>

T = 64.000  n(small)= 9  n(big)= 10  (P = 0.037)

The difference in the median values between the two groups is greater than would be expected by chance; there is a statistically significant difference (P = 0.037)

Analysis of PVA concentrations

**One Way Analysis of Variance**

**Data source:** Data 3 in Solvent data analysis
Normality Test: Passed (P = 0.700)

Equal Variance Test: Passed (P = 0.368)

<table>
<thead>
<tr>
<th>Group Name</th>
<th>N</th>
<th>Missing</th>
<th>Mean</th>
<th>Std Dev</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVA 1 %</td>
<td>3</td>
<td>0</td>
<td>74.541</td>
<td>4.501</td>
<td>2.599</td>
</tr>
<tr>
<td>PVA 2 %</td>
<td>4</td>
<td>0</td>
<td>81.805</td>
<td>17.587</td>
<td>8.794</td>
</tr>
<tr>
<td>PVA 5 %</td>
<td>3</td>
<td>0</td>
<td>71.393</td>
<td>23.754</td>
<td>13.715</td>
</tr>
</tbody>
</table>

Source of Variation | DF | SS   | MS  | F    | P    |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Groups</td>
<td>2</td>
<td>202.328</td>
<td>101.164</td>
<td>0.338</td>
<td>0.724</td>
</tr>
<tr>
<td>Residual</td>
<td>7</td>
<td>2096.993</td>
<td>299.570</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>9</td>
<td>2299.321</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.724).

Power of performed test with alpha = 0.050: 0.050

The power of the performed test (0.050) is below the desired power of 0.800. Less than desired power indicates you are more likely to not detect a difference when one actually exists. Be cautious in over-interpreting the lack of difference found here.

**Loading efficiency Statistics SigmaStat output**

Solvent independent 0.2 vs. 0.1 loading

Using a student’s t-test failed the normality therefore Mann-Whitney Rank Sum utilized

**t-test**  Monday, March 29, 2010, 10:38:58 PM

**Data source:** 0.1 vs 0.2 solvent independent in Drug Loading efficiency

**Normality Test:** Failed  (P < 0.050)

Test execution ended by user request, Rank Sum Test begun

**Mann-Whitney Rank Sum Test**  Monday, March 29, 2010, 10:38:58 PM

**Data source:** 0.1 vs 0.2 solvent independent in Drug Loading efficiency

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Missing</th>
<th>Median</th>
<th>25%</th>
<th>75%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 loading</td>
<td>52</td>
<td>0</td>
<td>0.0777</td>
<td>0.0470</td>
<td>0.142</td>
</tr>
<tr>
<td>0.1 loading</td>
<td>51</td>
<td>0</td>
<td>0.127</td>
<td>0.0909</td>
<td>0.182</td>
</tr>
</tbody>
</table>

T = 3139.000  n(small)= 51  n(big)= 52  (P = 0.001)
The difference in the median values between the two groups is greater than would be expected by chance; there is a statistically significant difference (P = 0.001)

Solvent dependent 0.1 vs. 0.2 loading

PVA 0.1 vs. 0.2 Loading

Utilizing Student’s t-test

\[ t = \frac{\text{difference}}{\text{standard error}} \]

Data source: PVA 0.1 vs 0.2 in Drug Loading efficiency

Normality Test: Passed (P = 0.091)

Equal Variance Test: Passed (P = 0.178)

<table>
<thead>
<tr>
<th>Group Name</th>
<th>N</th>
<th>Missing</th>
<th>Mean</th>
<th>Std Dev</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 loading</td>
<td>25</td>
<td>0</td>
<td>0.0941</td>
<td>0.0709</td>
<td>0.0142</td>
</tr>
<tr>
<td>0.1 loading</td>
<td>24</td>
<td>0</td>
<td>0.150</td>
<td>0.0500</td>
<td>0.0102</td>
</tr>
</tbody>
</table>

Difference -0.0563

\[ t = -3.198 \text{ with } 47 \text{ degrees of freedom. (P = 0.002)} \]

95 percent confidence interval for difference of means: -0.0916 to -0.0209

The difference in the mean values of the two groups is greater than would be expected by chance; there is a statistically significant difference between the input groups (P = 0.002).

Power of performed test with alpha = 0.050: 0.862

PVA 1% vs.2% vs. 5% at 0.1 loading

One Way Analysis of Variance

Data source: PVA 1 vs 2 vs 5% at 0.1 loading in Drug Loading efficiency

Normality Test: Passed (P = 0.263)

Equal Variance Test: Passed (P = 0.269)

<table>
<thead>
<tr>
<th>Group Name</th>
<th>N</th>
<th>Missing</th>
<th>Mean</th>
<th>Std Dev</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVA 5%</td>
<td>6</td>
<td>0</td>
<td>0.172</td>
<td>0.0261</td>
<td>0.0107</td>
</tr>
<tr>
<td>PVA 2%</td>
<td>9</td>
<td>0</td>
<td>0.144</td>
<td>0.0469</td>
<td>0.0156</td>
</tr>
<tr>
<td>PVA 1%</td>
<td>9</td>
<td>0</td>
<td>0.142</td>
<td>0.0640</td>
<td>0.0213</td>
</tr>
</tbody>
</table>
The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.492).

Power of performed test with alpha = 0.050: 0.049

The power of the performed test (0.049) is below the desired power of 0.800. Less than desired power indicates you are more likely to not detect a difference when one actually exists. Be cautious in over-interpreting the lack of difference found here.

PVA 1% vs. 2% vs. 5% at 0.2 Loading

**One Way Analysis of Variance**  Monday, March 29, 2010, 10:58:16 PM

**Data source:** PVA 1vs3vs5% at 0.2 loading in Drug Loading efficiency

**Normality Test:**  Passed (P = 0.514)

**Equal Variance Test:**  Passed (P = 0.424)

<table>
<thead>
<tr>
<th>Group Name</th>
<th>N</th>
<th>Missing</th>
<th>Mean</th>
<th>Std Dev</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVA 5%</td>
<td>6</td>
<td>0</td>
<td>0.105</td>
<td>0.102</td>
<td>0.0417</td>
</tr>
<tr>
<td>PVA 2%</td>
<td>9</td>
<td>0</td>
<td>0.0919</td>
<td>0.0421</td>
<td>0.0140</td>
</tr>
<tr>
<td>PVA 1%</td>
<td>10</td>
<td>0</td>
<td>0.0896</td>
<td>0.0770</td>
<td>0.0243</td>
</tr>
</tbody>
</table>

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.918).

Power of performed test with alpha = 0.050: 0.049

The power of the performed test (0.049) is below the desired power of 0.800. Less than desired power indicates you are more likely to not detect a difference when one actually exists. Be cautious in over-interpreting the lack of difference found here.
PVP 0.1 vs. 0.2 Loading

Utilizing a Student’s T-Test—Normality failed therefore Mann-Whitney Rank Sum Test was used

_t-test_ Monday, March 29, 2010, 10:42:52 PM

Data source: PVP 0.1 vs 0.2 in Drug Loading efficiency

Normality Test: Failed  (P < 0.050)

Test execution ended by user request, Rank Sum Test begun

Mann-Whitney Rank Sum Test  Monday, March 29, 2010, 10:42:52 PM

Data source: PVP 0.1 vs 0.2 in Drug Loading efficiency

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Missing</th>
<th>Median</th>
<th>25%</th>
<th>75%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 loading</td>
<td>27</td>
<td>0</td>
<td>0.0768</td>
<td>0.0522</td>
<td>0.132</td>
</tr>
<tr>
<td>0.1 loading</td>
<td>27</td>
<td>0</td>
<td>0.104</td>
<td>0.0841</td>
<td>0.175</td>
</tr>
</tbody>
</table>

T = 654.000  n(small)= 27  n(big)= 27  (P = 0.128)

The difference in the median values between the two groups is not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference  (P = 0.128)

PVP 1%—0.1 vs. 0.2 loading

t-test  Monday, March 29, 2010, 11:35:31 PM

Data source: PVP 1%--0.1 vs 0.2 loading in Drug Loading efficiency

Normality Test: Passed (P = 0.756)

Equal Variance Test: Passed (P = 0.718)

<table>
<thead>
<tr>
<th>Group Name</th>
<th>N</th>
<th>Missing</th>
<th>Mean</th>
<th>Std Dev</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 loading</td>
<td>9</td>
<td>0</td>
<td>0.0714</td>
<td>0.0505</td>
<td>0.0168</td>
</tr>
<tr>
<td>0.1 loading</td>
<td>9</td>
<td>0</td>
<td>0.103</td>
<td>0.0494</td>
<td>0.0165</td>
</tr>
</tbody>
</table>

Difference  -0.0312
t = -1.325 with 16 degrees of freedom. (P = 0.204)

95 percent confidence interval for difference of means: -0.0811 to 0.0187

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0.204).

Power of performed test with alpha = 0.050: 0.122

The power of the performed test (0.122) is below the desired power of 0.800. Less than desired power indicates you are more likely to not detect a difference when one actually exists. Be cautious in over-interpreting the lack of difference found here.

PVP 2%—0.1 vs. 0.2 loading

t-test  Monday, March 29, 2010, 11:36:28 PM

Data source: PVP 2%—0.1 vs. 0.2 loading in Drug Loading efficiency

Normality Test: Failed (P < 0.050)

Test execution ended by user request, Rank Sum Test begun

Mann-Whitney Rank Sum Test  Monday, March 29, 2010, 11:36:28 PM

Data source: PVP 2%—0.1 vs. 0.2 loading in Drug Loading efficiency

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Missing</th>
<th>Median</th>
<th>25%</th>
<th>75%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 loading</td>
<td>9</td>
<td>0</td>
<td>0.104</td>
<td>0.0798</td>
<td>0.178</td>
</tr>
<tr>
<td>0.2 loading</td>
<td>9</td>
<td>0</td>
<td>0.0735</td>
<td>0.0675</td>
<td>0.259</td>
</tr>
</tbody>
</table>

T = 89.000  n(small)= 9  n(big)= 9  (P = 0.791)

The difference in the median values between the two groups is not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.791)

PVP 5%—0.1 vs. 0.2 loading

t-test  Monday, March 29, 2010, 11:37:05 PM
Data source: PVP 5%--0.1 vs 0.2 loading in Drug Loading efficiency

Normality Test: Passed (P = 0.169)

Equal Variance Test: Passed (P = 0.225)

<table>
<thead>
<tr>
<th>Group Name</th>
<th>N</th>
<th>Missing</th>
<th>Mean</th>
<th>Std Dev</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 loading</td>
<td>9</td>
<td>0</td>
<td>0.134</td>
<td>0.0999</td>
<td>0.0333</td>
</tr>
<tr>
<td>0.1 loading</td>
<td>9</td>
<td>0</td>
<td>0.149</td>
<td>0.0606</td>
<td>0.0202</td>
</tr>
</tbody>
</table>

Difference -0.0155

t = -0.399 with 16 degrees of freedom. (P = 0.695)

95 percent confidence interval for difference of means: -0.0981 to 0.0670

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0.695).

Power of performed test with alpha = 0.050: 0.050

The power of the performed test (0.050) is below the desired power of 0.800. Less than desired power indicates you are more likely to not detect a difference when one actually exists. Be cautious in over-interpreting the lack of difference found here.

PVP vs. PVA at 0.2 loading

Data source: PVA vs. PVP at 0.2 in Drug Loading efficiency

Normality Test: Failed (P < 0.050)

Test execution ended by user request, Rank Sum Test begun

Mann-Whitney Rank Sum Test Monday, March 29, 2010, 10:50:04 PM

Data source: PVA vs. PVP at 0.2 in Drug Loading efficiency

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Missing</th>
<th>Median</th>
<th>25%</th>
<th>75%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
PVA  25  0  0.0785  0.0389  0.145
PVP  27  0  0.0768  0.0522  0.132

T = 629.000  n(small)= 25  n(big)= 27  (P = 0.546)

The difference in the median values between the two groups is not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference  (P = 0.546)

PVP vs. PVA at 0.1 loading

t-test  Monday, March 29, 2010, 10:48:40 PM

Data source: PVA vs. PVP at 0.1 in Drug Loading efficiency

Normality Test:  Failed (P < 0.050)

Test execution ended by user request, Rank Sum Test begun

Mann-Whitney Rank Sum Test  Monday, March 29, 2010, 10:48:40 PM

Data source: PVA vs. PVP at 0.1 in Drug Loading efficiency

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Missing</th>
<th>Median 25%</th>
<th>Median 75%</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVA</td>
<td>24</td>
<td>0</td>
<td>0.144</td>
<td>0.111 0.184</td>
</tr>
<tr>
<td>PVP</td>
<td>27</td>
<td>0</td>
<td>0.104</td>
<td>0.0841 0.175</td>
</tr>
</tbody>
</table>

T = 721.000  n(small)= 24  n(big)= 27  (P = 0.069)

The difference in the median values between the two groups is not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference  (P = 0.069)
Appendix III—Data and Regression Analysis for KT Calibration

Prior to beginning the analysis of drug loading and release studies, it was necessary to find a method of testing for KT presence and concentration. This was accomplished by UV/VIS spectroscopy. See Figure 34 for a visual representation of the calibration for KT in PBS at 323nm and Figure 35 for KT in PBS at 250nm.

Raw data and calibration of KT at wavelength of 323 nm.

<table>
<thead>
<tr>
<th>μL 0.01</th>
<th>μL PBS</th>
<th>molarity KT</th>
<th>Absorbance</th>
<th>376.4 MW KT</th>
</tr>
</thead>
<tbody>
<tr>
<td>900</td>
<td>2600</td>
<td>1.41E-04</td>
<td>2.418</td>
<td>0.0103 g KT</td>
</tr>
<tr>
<td>700</td>
<td>2800</td>
<td>1.09E-04</td>
<td>1.898</td>
<td>50 mL PBS</td>
</tr>
<tr>
<td>500</td>
<td>3000</td>
<td>7.82E-05</td>
<td>1.549</td>
<td>0.05 L</td>
</tr>
<tr>
<td>250</td>
<td>3250</td>
<td>3.91E-05</td>
<td>0.774</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>3400</td>
<td>1.56E-05</td>
<td>0.314</td>
<td>0.206 g/L</td>
</tr>
<tr>
<td>50</td>
<td>3450</td>
<td>7.82E-06</td>
<td>0.162</td>
<td>0.00054729 mol/L</td>
</tr>
</tbody>
</table>

transposed (*10^4)

1.41
1.09
0.78
0.39
0.16
0.08
**Figure 34.** Graph of KT molarity versus absorbance for 323 nm. Calibration data for KT in PBS.

![Graph of KT molarity versus absorbance for 323 nm. Calibration data for KT in PBS.](image)

**SUMMARY OUTPUT: constant 0**

<table>
<thead>
<tr>
<th>Regression Statistics</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiple R</td>
<td>0.998302757</td>
</tr>
<tr>
<td>R Square</td>
<td>0.996608394</td>
</tr>
<tr>
<td>Adjusted R Square</td>
<td>0.796608394</td>
</tr>
<tr>
<td>Standard Error</td>
<td>0.092356019</td>
</tr>
<tr>
<td>Observations</td>
<td>6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ANOVA</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>df</td>
<td>SS</td>
</tr>
<tr>
<td>-------</td>
<td>--------</td>
</tr>
<tr>
<td>Regression</td>
<td>1</td>
</tr>
<tr>
<td>Residual</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Coefficients</th>
<th>Standard Error</th>
<th>t Stat</th>
<th>P-value</th>
<th>Lower 95%</th>
<th>Upper 95%</th>
<th>Lower 95.0%</th>
<th>Upper 95.0%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>#N/A</td>
<td>#N/A</td>
<td>#N/A</td>
<td>#N/A</td>
<td>#N/A</td>
<td>#N/A</td>
<td>#N/A</td>
</tr>
<tr>
<td>transposed (*10^7)</td>
<td>1.775961458</td>
<td>0.046332844</td>
<td>38.33090794</td>
<td>2.3E-07</td>
<td>1.65685909</td>
<td>1.895063826</td>
<td>1.65685909</td>
</tr>
</tbody>
</table>

This indicates that the model should not include an intercept as the p-value is 0.2385 with a t-stat of 1.38, therefore the model with constant of 0 is accepted.

**SUMMARY OUTPUT: constant not 0**

<table>
<thead>
<tr>
<th>Regression Statistics</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiple R</td>
<td>0.996590919</td>
</tr>
<tr>
<td>R Square</td>
<td>0.99030225</td>
</tr>
<tr>
<td>Adjusted R Square</td>
<td>0.991287781</td>
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<tr>
<td>Standard Error</td>
<td>0.084904702</td>
</tr>
<tr>
<td>Observations</td>
<td>6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ANOVA</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>df</td>
<td>SS</td>
</tr>
<tr>
<td>-------</td>
<td>--------</td>
</tr>
<tr>
<td>Regression</td>
<td>1</td>
</tr>
<tr>
<td>Residual</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Coefficients</th>
<th>Standard Error</th>
<th>t Stat</th>
<th>P-value</th>
<th>Lower 95%</th>
<th>Upper 95%</th>
<th>Lower 95.0%</th>
<th>Upper 95.0%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>0.080082143</td>
<td>0.057852803</td>
<td>1.384239638</td>
<td>0.2385</td>
<td>-0.080542988</td>
<td>0.240702724</td>
<td>-0.080542988</td>
</tr>
<tr>
<td>transposed (*10^7)</td>
<td>1.697171155</td>
<td>0.046332844</td>
<td>38.33090794</td>
<td>2.3E-07</td>
<td>1.65685909</td>
<td>1.895063826</td>
<td>1.65685909</td>
</tr>
</tbody>
</table>

This indicates that the model should not include an intercept as the p-value is 0.2385 with a t-stat of 1.38, therefore the model with constant of 0 is accepted.
Raw data and graph for 250nm:

**Calibration for KT in PBS at 250nm**

![Graph of KT molarity versus absorbance for 250nm. Calibration curve for KT in PBS at 250nm.](image)

\[ y = 5944.2x \]
\[ R^2 = 0.989 \]

A similar regression was performed for the absorbance of KT at ~250nm.

---

<table>
<thead>
<tr>
<th>376.4 MW KT</th>
<th>0.0103 g KT</th>
<th>0.206 g/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mL PBS</td>
<td>0.000547 mol/L</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>μL 0.01</th>
<th>μL PBS</th>
<th>molarity KT</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>900</td>
<td>2600</td>
<td>0.000140732</td>
<td>0.8055</td>
</tr>
<tr>
<td>700</td>
<td>2800</td>
<td>0.000109458</td>
<td>0.6393</td>
</tr>
<tr>
<td>500</td>
<td>3000</td>
<td>7.81843E-05</td>
<td>0.5197</td>
</tr>
<tr>
<td>250</td>
<td>3250</td>
<td>3.90922E-05</td>
<td>0.2599</td>
</tr>
<tr>
<td>100</td>
<td>3400</td>
<td>1.56369E-05</td>
<td>0.1033</td>
</tr>
<tr>
<td>50</td>
<td>3450</td>
<td>7.81843E-06</td>
<td>0.0558</td>
</tr>
</tbody>
</table>

transposed (*10^7)

1407.317443
1094.580234
781.8430241
390.9215121
156.3686048
78.18430241

---

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Appendix IV. DSC Graphs

The following graphs are the graphical representation of the DSC data. As there were many samples and each was run multiple times, not all graphs have been included. They are summarized in a table below and included on the data disc provided.

<table>
<thead>
<tr>
<th></th>
<th>Tg (°C)</th>
<th>stddev</th>
<th>CR (°C)</th>
<th>stddev</th>
</tr>
</thead>
<tbody>
<tr>
<td>KT *Tm</td>
<td>168.07</td>
<td>84.54</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>PLGA</td>
<td>38.02</td>
<td>--</td>
<td>103.19</td>
<td>0.25</td>
</tr>
<tr>
<td>0.1 TL</td>
<td>29.77</td>
<td>0.54</td>
<td>103.19</td>
<td>0.25</td>
</tr>
<tr>
<td>PVA 1%</td>
<td>30.97</td>
<td>0.94</td>
<td>97.70</td>
<td>8.10</td>
</tr>
<tr>
<td>PVA 2%</td>
<td>26.80</td>
<td>0.94</td>
<td>103.14</td>
<td>0.88</td>
</tr>
<tr>
<td>PVA 5%</td>
<td>31.19</td>
<td>1.18</td>
<td>103.56</td>
<td>0.57</td>
</tr>
<tr>
<td>PVP 1%</td>
<td>30.71</td>
<td>0.98</td>
<td>103.29</td>
<td>--</td>
</tr>
<tr>
<td>PVP 2%</td>
<td>33.92</td>
<td>1.51</td>
<td>102.80</td>
<td>0.78</td>
</tr>
<tr>
<td>PVP 5%</td>
<td>34.21</td>
<td>2.31</td>
<td>103.27</td>
<td>--</td>
</tr>
<tr>
<td>0.2 TL</td>
<td>33.56</td>
<td>1.51</td>
<td>103.45</td>
<td>--</td>
</tr>
<tr>
<td>PVA 1%</td>
<td>30.22</td>
<td>1.58</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>PVA 2%</td>
<td>24.68</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>PVA 5%</td>
<td>31.23</td>
<td>1.86</td>
<td>103.45</td>
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</tr>
<tr>
<td>PVP 1%</td>
<td>34.21</td>
<td>2.31</td>
<td>103.27</td>
<td>--</td>
</tr>
<tr>
<td>PVP 5%</td>
<td>29.94</td>
<td>0.53</td>
<td>103.18</td>
<td>0.26</td>
</tr>
</tbody>
</table>
References


