Artificial cell microcapsules for oral delivery of thalidomide for use in Crohn's Disease: design, preparation, and in-vitro analysis.

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Abstract

A delivery method for the oral administration of thalidomide for lowering tumor necrosis factor alpha (TNF-α), and thus Crohn's disease-related inflammation in the proximal small intestine, using two separate engineered polymer microcapsules is explored in this thesis. Membrane formation of both alginate-polylysine-alginate (APA) and alginate-chitosan (AC) capsules are discussed and thalidomide encapsulation procedures have been described. *In-vitro* tests have been used to monitor capsule degradation and drug release in a simulated gastrointestinal environment. Also, culture and use of RAW 264.7 mouse macrophage cells for the simulation of the human intestine is described. Results show two separate methods of drug delivery by the APA and AC capsules. APA capsules release thalidomide in a timed-release fashion whereas AC capsules release the drug in a burst manner. These results implicate the benefits of the use of both capsules to target separate sites along the small intestine. Also the cultured macrophage studies conclude that the proposed encapsulation therapy does indeed lower TNF- α levels and could therefore be of benefit for the lowering of inflammation associated with Crohn's disease. However, further animal study is needed before full potential of this approach can be realized.

Resume

Une methode pour la libération de la thalidomide par la voie orale en utilisation deux familles de microcapsules polymeriques est explorée dans cette thèse. Ceci avait pour but de diminuer le niveau de la sécrétion du facteur nécrose des tumeurs alpha (TNF-α) ainsi que l'inflammation associée à la maladie de Crohn's dans l'intestin proximal. La préparation des membranes à base d'alginate-polylysine-alginate (APA) et d'alginate-chitosan (AC) est discutée. Nous avons également présenté la procédure détaillée pour l'encapsulation de la thalidomide. Les essais *in-vitro* ont été utilisés pour étudier la dégradation des capsules et le relargage de la drogue dans un milieux gastrointestinal simulé. De plus, le protocole de la culture des macrophages RAW 264.7 pour la simulation de l'intestin humain est décrit. Les résultats ont démontré deux méthodes distinctes de la libération de la drogue pour les deux familles de microcapsules. Les capsules d'APA libèrent la thalidomide graduellement tandis que les microcapsules à base d'AC libèrent la drogue très rapidement. Ces résultats indiquent les avantages de chaque famille pour contrôler la cinétique de la libération aux plusieurs sites au long de l'intestin proximal selon la thérapie requise. Les études de la culture des macrophages indiquent que la therapie en utilisant la méthode d'encapsulation de la drogue diminue le niveaux du TNF-α, indiquant ainsi une baisse de taux d'inflammation associée avec la maladie de Crohn's. Cependant, les essais *in vivo* s'avèrent nécessaires afin de conclure sur les effets bénéfiques de microencapsulation pour le traitement de la maladie de Crohn's.

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Cellular Biochemistry, I would like to acknowledge intermittent laboratory assistance and advice given by my co-authors.

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Preface

In accordance with the thesis preparation and submission guidelines, I have taken the option of writing the experimental portion of this thesis in the form of original papers suitable for publication. This option is provided by **Section I-C** in the **Thesis Preparation and Submission Guidelines**, which reads as follows:

> As an alternative to the traditional thesis format, the dissertation can consist of a collection of papers of which the student is an author or co-author. These papers must have a cohesive, unitary character making them a report of a single program of research.

In this thesis, manuscripts of original papers are presented in **Chapters 3-5**. Each experiment based paper has its own Abstract, Introduction, Materials and Methods, Results, Discussion, and References section. A common Abstract, Introduction, Literature Review, a final overall Conclusion, Summary, Claims to Original Contributions to Knowledge, and Recommendations are also included.

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Figures 5.5b: Graph of stimulated mouse macrophage TNF secretion with and without APA-containing thalidomide. TNF-α secretion from RAW 264.7 cells in comparison to RAW 264.7 cells incubated with APA encapsulated thalidomide at time intervals from 0.5, 1, 3, 12, 24, and 48 hours. Black and blue lines in the graph represent cells stimulated with 100 μg/ml of LPS versus the green and red lines which represent cells stimulated with 10 μg/ml of LPS. Experiments were done in triplicate and standard deviations were calculated using Microsoft's Excel calculator.

Figure 5.6: Comparison photomicrographs of RAW 264.7 macrophage cells before (A) and after (B and C) exposure to encapsulated thalidomide.

Chapter 1

General Introduction

1.1 Crohn's disease, treatment methods, limitations, and thesis research objectives

Crohn's Disease (CD) is one of the most common inflammatory bowel diseases (IBD) and is characterized by such symptoms as abdominal pain, diarrhea, and cramping. Once thought to be rare among the pediatrics population, about 20-30 % of all causes of inflammatory bowel disease (IBD) are now regularly diagnosed before the age of 20 [2]. The specific etiology and pathogenetic mechanisms, however, remain unknown [3]. IBD is now considered one of the most common chronic diseases affecting the population. Hundreds of thousands of Canadians are affected by CD. The primary goal in treating CD is to control gastrointestinal tract inflammation. The choice of treatment for CD depends on the severity of the disease [4]. Several procedures such as dietary adjustments, surgery, and medications have been suggested, but a perfect treatment procedure for CD is yet to be found [5,6]. The drug infliximab has shown notable results but shows limited clinical efficacy [7-10]. Another drug, thalidomide, has been tested in patients as a replacement of infliximab. Though thalidomide has been effective in lowering inflammation as well as maintaining remission, it is not used because of its side effects and high dose requirement to be effective in CD [11-13]. Effective dosages of between 50 and 300 mg/day have been used in patients and given in the form of a solid pill. The drug is absorbed in the stomach and circulated throughout the entire system. As a result of this systemic circulation, effective therapy is achieved to a greater extent at higher doses. Although, at these doses, side effects ranging from sedation and rash to neuropathy can result [14,15]. In order to negate these effects, therapy methods protecting drug absorption until a target area is necessitated. This research concentrated on the design and development of a novel artificial cell formulation containing thalidomide to design a therapy and delivery system to decrease inflammation and

induce and maintain remission in the gastrointestinal (GI) tract in CD. We explored in detail the properties of these formulations vis-à-vis their design, process optimization and effectiveness in the oral delivery of the above formulation. We anticipate that the results of this research will further our understanding of artificial cell microcapsule design and will lead to the development of microcapsule formulations suitable for CD applications and delivery of small molecules into the GI tract.

Chapter 2

Literature Review

2.1 Crohn's disease, presently available treatment modalities, and associated limitations:

Once thought to be rare, CD is now regularly diagnosed [16]. CD causes severe inflammation of the gastrointestinal (GI) tract. It commonly affects the small intestine, but can strike at any area of the GI tract, from the mouth to the anus and causes complications such as the occurrence of fistulae. In addition to the pain caused by inflammation, it can cause diarrhea, stomach ulcers, malnutrition and other complications [17-20]. Mucosal inflammation such as that associated with CD was found to be enhanced by molecules such as TNF-α and other cytokines through experimental mouse models of IBD with silenced cytokine genes[21,22]. Specifically, molecular studies have shown that the molecule TNF-α is highly responsible for the inflammation in CD. The molecule is increased in CD and remains localized in the mucosa and intestinal lumen[1]. Because of this, targeted oral therapy, rather than systemic, is an area of interest when developing CD therapies. TNF-α plays a role in the immune regulation pathway and is secreted by cells such as macrophages, monocytes, and T cells in a mature form as a 51kD homotrimer. The molecule itself can be down regulated by corticosteroids, has a low mRNA half-life due to it's multiple RNAse attack sites, and is regulated at multiple points along the transcription and translation pathways[1]. The existence of multiple regulatory mechanisms imply the importance and sensitivity of TNF-α in the inflammatory pathway and also give insight into possible target areas for the regulation of this molecule's secretion. TNF-α induces inflammation through its two receptors TNFRI and TNFRII which activate nuclear factor kappa B (NFκB) to induce transcription of several proinflammatory genes. Increased serum concentrations of both the receptors I and II can be found in active CD patients[23]. In addition, TNF-α has multiple biologic activities involved in apoptosis, metabolism, and activation of

granulocytes, lymphocytes, eosinophils, fibroblasts, chondrocytes, and endothelial cells. The exact etiology and mechanism for CD, however, is still unknown [24,25]. Though, it is known that Crohn's disease is marked by the histopathological appearance of granulomas, or an accumulation of multiple immune cells recruited in part by TNF-α[26,27]. The pathway of granuloma formation is shown below in figure 2.1 emphasizing the integral role TNF plays in the granuloma formation process.

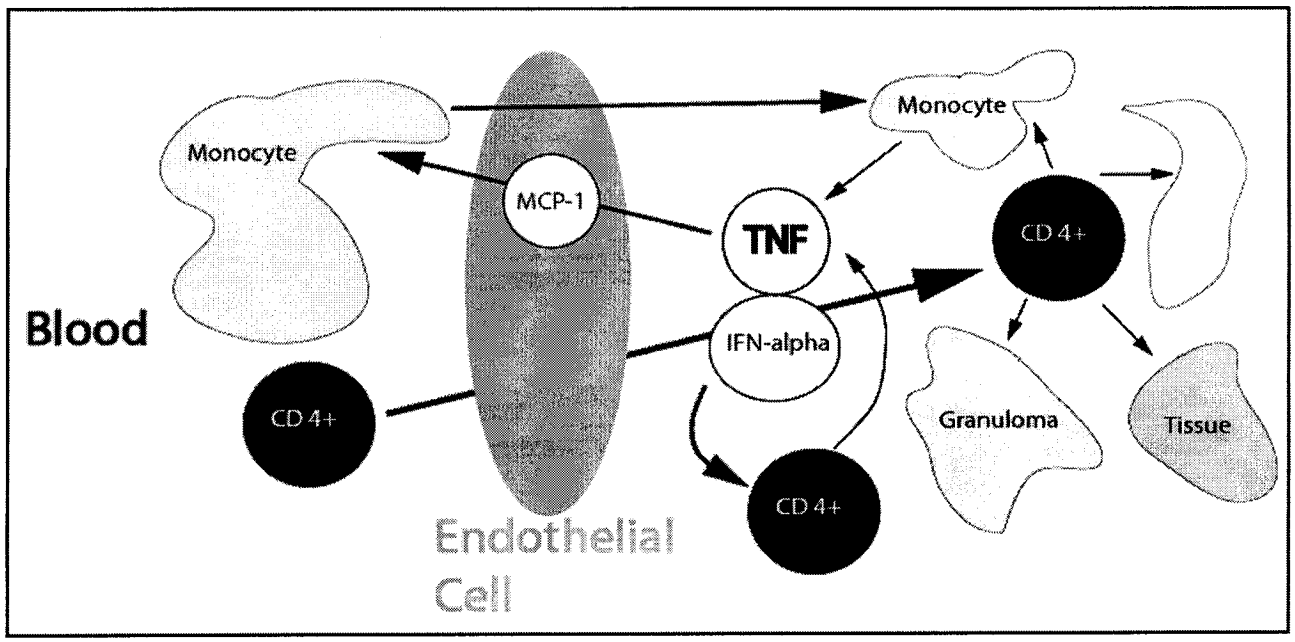


Figure 2.1: The role of Tumour Necrosis Factor (TNF) in granuloma formation. (adapted from van Deventer[1])

A cure for CD is still not available [28,29]. Presently available therapy procedures include combinations of surgery, anti-inflammatory drugs, steroids, immunosuppressants, antibiotics, anti-Tumor Necrosis Factor (anti-TNF) drugs, antidiarrheals and other symptom-suppressing drugs. Lifestyle and dietary changes have also been found to be helpful. Crohn's treatment varies

widely, as does the location and severity of the disease, and response to therapy differs from individual to individual [20,30-33].

Therapies such as antibiotics, immunomodulating agents such as azathioprine and 6-mercaptopurine, methotrexate, aminosalicylates, and corticosteroids have been tested and used on CD patients. Standard treatments include corticosteroids and immunosuppressive drugs such as those mentioned above. However, in clinical testing a substantial amount of patients either did not retain remission or remained in the refractory state with these standard therapies. It was found that roughly 30% of patients failed to have induced remission[34]. Other drugs or target molecules such as peroxisome proliferator activated receptor γ (PPARγ), TNF-α converting enzyme (TACE) inhibitors, phospodiesterase inhibitors, signal transduction inhibitors, and eicosanoids have been researched, but have had low efficacy due to problems such as the lack of maintenance of remission mentioned above, intolerable side effects, toxicity, and other problems. PPARγ is a receptor recognized for its role in inflammation and in specific for governing the inhibition of NFκB and thus the inhibition of TNF-α. However, so far no further research has been done to target this receptor molecule for lowering GI inflammation. TACE systems are responsible for processing TNF molecules before secretion at the cell membrane but inhibitors of this enzyme are not specific enough to warrant trial experiments. Phosphodiesterase inhibitors, which block the regulatory effects of phosphodiesterase on cyclic AMP (CAMP) and thus block the expression of multiple proinflammatory cytokines, have proven to be ineffective in patients[35,36]. Certain signal transduction inhibitors can block pathways such as that of the mitogen activated protein kinases (MAP), which can regulate the transcription and translation of TNF-α, but do not block at early enough segments along these specific pathways and thus do not block cytokine secretion. Also, these therapies can lead to uncontrolled cellular proliferation.

Finally, eicosanoids were found to be present in mucosal inflammation but targeting these specific molecules has not been shown to be clinically relevant[34].

Newer treatments have concentrated on the use of biological agents such as monoclonal antibodies, antisense oligonucleotides, and therapeutic peptides but non-oral delivery methods create problems such as cost and difficulty of administration[34]. Only when drug therapy is not successful or in certain complications, surgery is indicated. Fulminate life-threatening Crohn's disease is rare, but due to therapeutic failure and severe and disabling side effects of corticosteroids and immunosuppressant drugs alternative therapies are needed. Therefore, anti-TNF therapy specifically designed to lower TNF-α secretion is presently used as a main form of CD treatment. Of these therapies, the monoclonal antibody infliximab has been used most frequently but due to complications, still other anti-TNF agents are desired. Specifically, thalidomide is a promising treatment but side effects associated with the drug must be decreased in order to use the therapy on a regular basis in the clinic.

2.2 Drugs used for treating Crohn's disease

2.2.1 Infliximab for use in Crohn's disease

One therapy in particular, infliximab, has shown significant positive results in patients with CD [37-40]. Infliximab is a chimeric anti-TNF antibody shown *in vitro* to bind to TNF-α, neutralizing its biological activity and inhibiting binding to its receptors. In a recent study treatment with infliximab increased the closure of fistulas in 46 percent of patients as opposed to 13 percent of the placebo group [41]. Histological tests indicate reductions in inflammation due to

lowered mononuclear cells and disappearance of neutrophils, as well as near-complete epithelial damage disappearance after four weeks of infliximab treatment within patients [42]. Lowering of activity indices such as the Crohn's Disease Activity Index (CDAI) and the serum concentration of the C-reactive protein (CRP) occurs after just two weeks of treatment and continues on for eight weeks before re-treatment has been suggested. In another study it has been used for patients who had not responded to other conventional drugs such as 6-mercaptopurine, azathioprine, cyclosporine, methotrexate and aminosalicylates [43]. However, later studies showed its limited clinical efficacy [44-47]. Problems such as the development of tuberculosis as a result of the neutralization of TNF-α occur in approximately 1:2000 patients[48]. Infliximab was also found to produce delayed hypersensitivity reactions because of its administration procedures [49,50]. All hypersensitivity affected patients had, in their initial treatment, received a liquid formulation of infliximab. It was noticed that the liquid formulation had a higher turbidity (possibly caused by infliximab self-association) than lyophilized infliximab used in other studies where no delayed hypersensitivity reactions were observed. Characterization of the molecular integrity of the reconstituted lyophilized product showed that the monomer content of the product was only 98%, which is still a major concern in infliximab applications. Other problems related to immunotherapy such as infliximab is that patients can develop human antichimeric antibodies (HACAs). These circulating molecules then lower the efficacy of the antibody treatment[51].

Limitations

Therefore, problems with tuberculosis, hypersensitivity, HACAs, and the lack of long term disease remission lead to the necessity of designing a formulation utilizing a more clinically efficient and targetable drug . Also, the nature of intravenous (IV) administration of systemic

therapies like infliximab necessitates the hospitalization of patients for several days per week to receive treatment. It is desirable to eliminate this method of delivery by creating an oral therapy.

2.2.2 Thalidomide for use in Crohn's disease

Thalidomide was first introduced as a sedative for use during pregnancy in the 1960s. The drug was withdrawn from the market after researchers found an association with severe teratogenicity, or birth defects. Until recently, it has received limited investigation for other potential applications. There are now various reports on the results of several new studies using thalidomide for therapy. And, thalidomide's effectiveness in treating Hanson's disease (leprosy) has led to studies of the drug in other inflammatory conditions such as CD. In July 1998, the FDA approved the use of thalidomide for the treatment of the debilitating and disfiguring lesions associated with erythema nodosum leprosum, a complication of leprosy. The drug can now be dispensed to patients in the United States under stringent guidelines administered through the STEPS (System for Thalidomide Education and Prescribing Safety) program.

Thalidomide's structure is shown below, in figure 2.2, as a tricyclic molecule with the chemical formula of $C_{13}H_{10}N_2O_4$. It has a molecular mass of 258.23 and an ultraviolet absorbance at 220 nanometers. Also its solubility is low in water but is at 55.2mg/ml in dimethyl sulfoxide (DMSO), an organic solvent. For these reasons, thalidomide can be solubilized in a small amount of DMSO and detected by spectrophotometry.

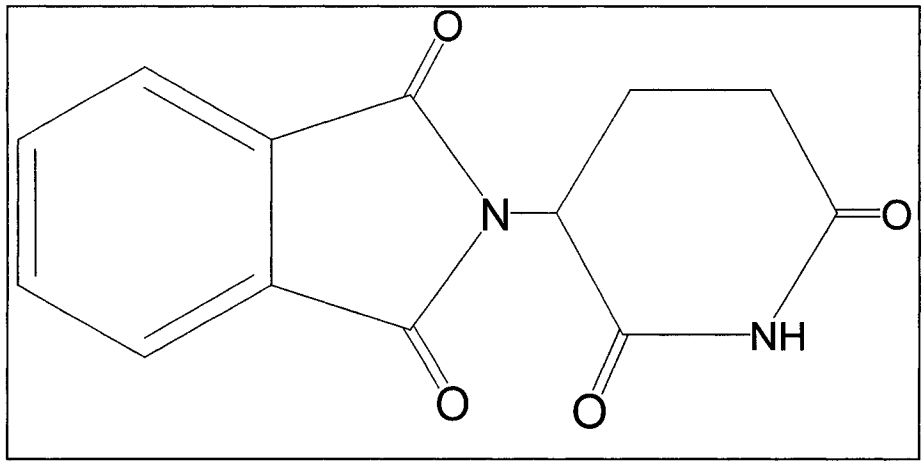


Figure 2.2: The tricyclic structure of thalidomide.

Thalidomide has anti-inflammatory effects, although the mechanisms are not fully understood [52,53]. The current interest of IBD research in this drug stems from the observation that the levels of an inflammatory inducing factor, tumor necrosis factor-alpha (TNF-α), are increased in CD. Thalidomide appears to inhibit TNF-α. It also decreases the ration of helper T cells, which play a role in inciting inflammation. Although unlike the above mentioned infliximab which can induce remission in active CD,[54,55] thalidomide is found to maintain remission [56]. Part of thalidomide's action is on the suppression of the inhibitory κB (IκB) kinase resulting in the inhibition of NFκB transcription [57]. Thus, it could be implicated that by inhibiting transcription, a more complete remission of the disease is achieved. It was originally found that thalidomide enhanced TNF-α mRNA degradation specifically[58,59]. This is a benefit due to the maintenance of immunity within the body during thalidomide treatment; contrasting the immunological effects of infliximab mentioned above. Also, it has been found that patients with no response to infliximab therapy have responded to thalidomide treatments [14]. Further success has been reported with the use of thalidomide as a treatment for CD in children where major clinical response was reported in just 3-5 weeks and in two cases, patients were able to discontinue steroid treatment entirely[60]. Other

significant outcomes were published in two studies for the use of thalidomide to treat both orofacial granulomatosis and vulvar ulcerations related to CD[61,62].

Limitations

However, although shown effective with patients in clinical trials, thalidomide shows side effects such as drowsiness and to a much lesser extent skin rash, hypertension, constipation, oedema, and neutropenia.[14,63]. As well there are concerns of possible neuropathy resulting from the treatment [64-66]. Therefore, though promising, its use has been limited [67,68]. A suitable formulation to improve its clinical efficacy and safe use is urgently needed.

2.3 Artificial cell microencapsulation

Given the limitations of the presently available therapy procedures for CD, development of suitable therapy procedures, therefore, is necessary and urgent [69,70]. The aim of the thesis is to engineer a novel artificial cell formulation containing thalidomide to design a therapy and delivery system to decrease inflammation and induce and maintain remission in the gastrointestinal (GI) tract in CD.

Artificial cell microencapsulation is a technique to encapsulate biologically active and other materials in a specialized ultrathin semi-permeable polymer membrane originally established by Thomas Chang [71-73]. Artificial cells can protect the encapsulated materials from harsh external environments while at the same time allowing for metabolism of selected solutes capable of passing into and out of the microcapsules. In this manner, the enclosed material can be retained and separated from the external environment. Also, artificial cells can be engineered to degrade upon exposure to certain environments such as a change in pH level. This allows for

the selected release of encapsulated materials. Microcapsules are known to protect live cells, enzymes, DNA etc. from immune rejection and other extreme environments and control delivery of molecules and have a number of other biomedical and clinical applications [74-81]. Shown below in figure 2.3, artificial cells can maintain molecules inside while preventing external substances from entering or internal encapsulated substances from exiting. Also the membrane provides a protective layer from harsh GI environments.

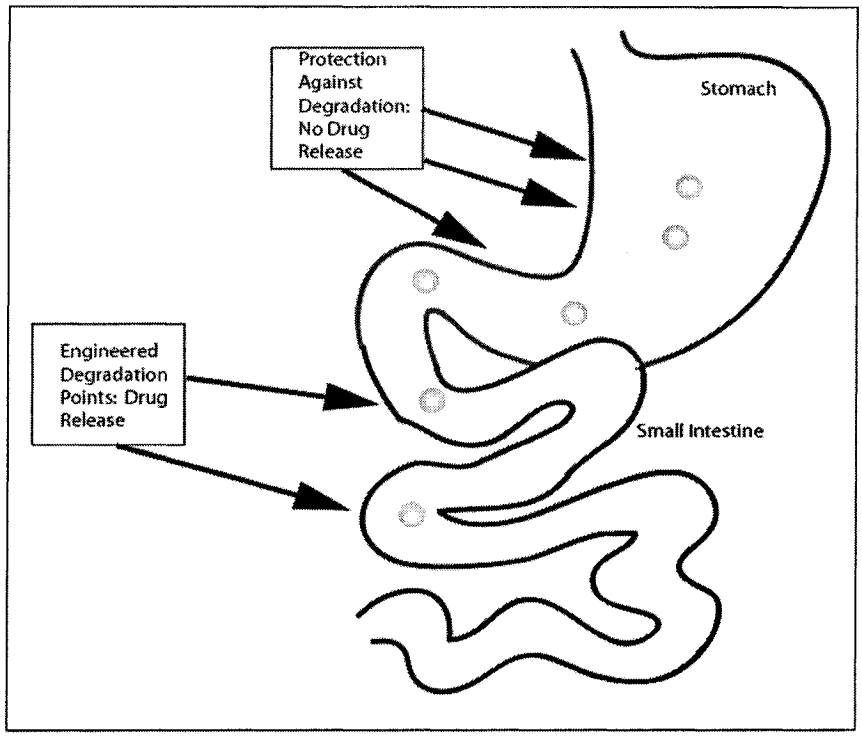


Figure 2.3: Protective properties of artificial cells in the harsh environment of the stomach. Also, several points in the small intestine at which artificial cells can be engineered to degrade are indicated.

It was first reported in 1980 that live islet cells for transplantation into the liver could be encapsulated and implanted into the body while maintaining normal function of the cells[82]. Further research has concentrated on using multilayer polymer membranes to protect

encapsulated materials from degradation, exposure, or delivery in undesired environments. Microcapsules can thus be designed for various purposes by varying membrane component concentrations and thus changing the artificial cell's degradation profile.

2.3.2 Background and rational for using AC membrane

Crohn's Disease related inflammation can occur at any point along the gastrointestinal tract. Thus, it is desirable to be able to tailor a drug delivery method, such as microencapsulation, to design several membranes which degrade at different areas along this tract. This would enable one to choose from a variety of capsule membranes and then select the capsule that would degrade closest to a specific patient's site of inflammation. In this thesis, development of two separate membranes, alginate-chitosan and alginate-polylysine-alginate, was carried out for investigation of their respective degradation targeting abilities. Alginate-chitosan is a more recent development in the field of polymer engineering. Originally, developments of calcium alginate-chitosan beads had lead to their use for drug delivery of nitrofurantoin in the intestine[85]. Further evaluation of alginate-chitosan capsules as an oral therapy for protein delivery was also done[86]. The study found that alginate-chitosan capsules retain protein through low pH values of 1.5, such as those encountered in the stomach, and then release the protein in a higher pH of 7.5, similar to the environment of the small intestine.

Chitosan, a polymer isolated from chitin in the hard exoskeleton of shellfish[87], adds structural rigidity to the standard alginate bead. For this reason it is layered over the initial alginate bead. Alginate beads are formed by the laminar liquid jet frequency superimposition method and then dipped into a 0.5% chitosan solution in acetic acid. Previous characterization

studies of alginate and chitosan where done in 1998 by Gaserod et al.[88] to assess both of the polymers' binding and interaction properties. It was found that chitosan with a higher molecular weight contained a larger number of acetyl chains causing a lower binding of chitosan to alginate. This was postulated to be due to the acetyl blockage of chitosan from diffusing further into alginate pores and therefore more significantly binding[89]. It is due to these findings that in this thesis, we have used a lower molecular weight chitosan. Also, it was found that chitosan binds to alginate in much greater quantities if the alginate beads are formed and first put in a calcium chloride solution, and then incubated in a chitosan solution as opposed to just dropping alginate beads directly in chitosan. As described by Gaserod et al., this is most likely due to the increased porosity in the alginate when calcium, from calcium chloride, is present.

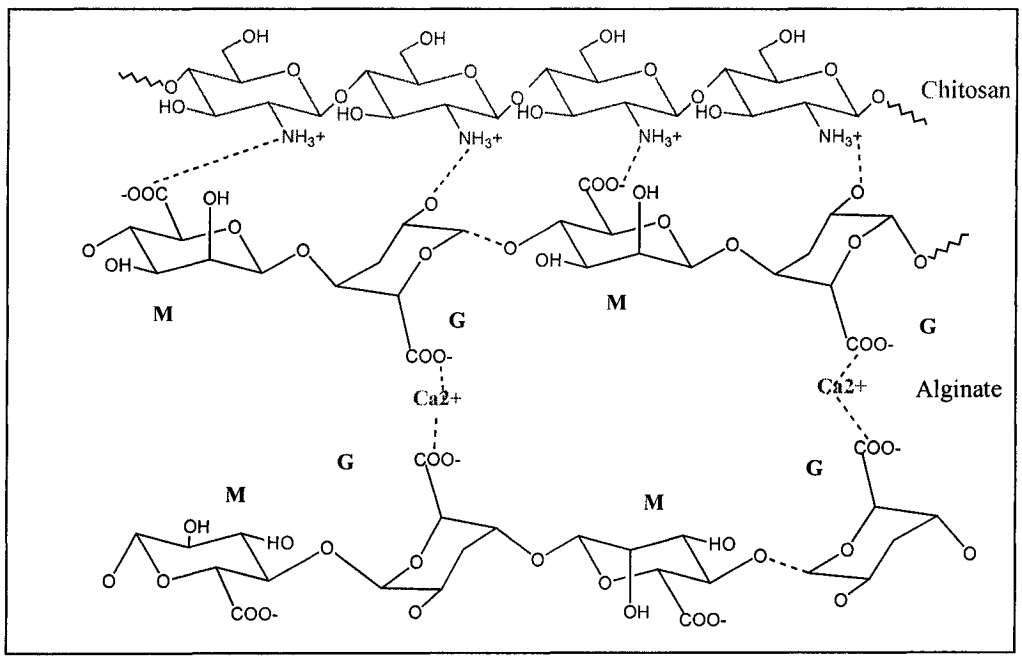


Figure 2.4: Electrostatic interactions of both the G and M confirmations of alginate with chitosan as well as the cross-linking ability of the divalent calcium cation in the alginate-chitosan (AC) membrane.

Further studies by Gaserod et al.[90] concluded these findings and strengthened the hypothesis that a two-step method of alginate-chitosan bead formation is most effective for creating stable capsules. This method of first exposing alginate beads to a calcium chloride solution is what was used in the experiments carried out and reported upon in this thesis. Figure 2.4 below shows the chemical binding of alginate and chitosan.

2.3.3 Background and rational for using APA membrane

The cross-linking of sodium alginate with poly-l-lysine, and their binary mixture with calcium and other ions through ionotropic gelation is well established[91,92]. The combination of both alginate and polylysine to form a multilayer polymer membrane was first used in the 1980 Lim and Sun study to encapsulate live islet cells[82]. The alginate beads containing the islet cells were originally formed through a droplet technique and then submerged in a polylysine solution to form the second layer of the capsule. These microcapsules were then washed and stored in a 1% calcium chloride solution to provide a calcium ion for binding to the membrane layers. This allows for a more stable membrane and also protects newly-formed cells from agglomeration. Initially, the AP membrane worked well for cell encapsulation but it was not until the further addition of another alginate layer, creating an alginate-polylysine-alginate (APA) membrane, was done and varying membrane thickness studies in 1994 were completed that the further potential of this formulation was realized. Ma and Sun[93] found that thicker membranes provided a longer lifetime before the microcapsules leaked or degraded.

Membrane thickness of the capsules was increased by increasing either or all concentrations of the alginate, polylysine, and calcium solutions. It thus became apparent that thickness could be altered and optimal membrane thicknesses could be achieved. Figure 2.5 below shows a diagram of the chemical interactions between each of the three layers: alginate, polylysine, and alginate.

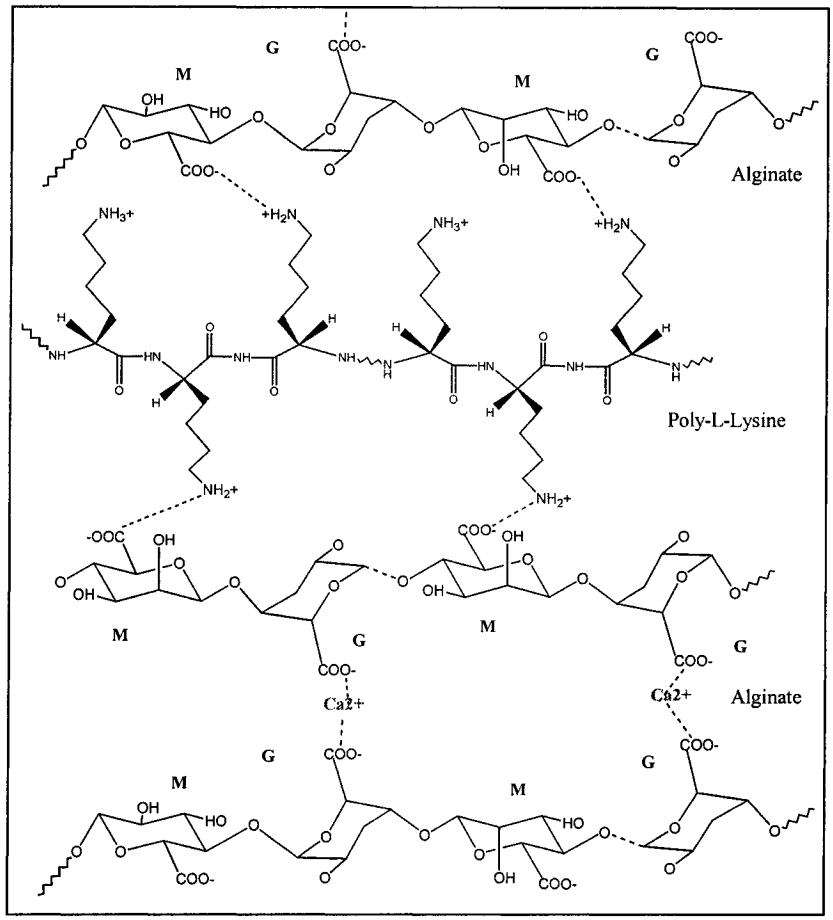


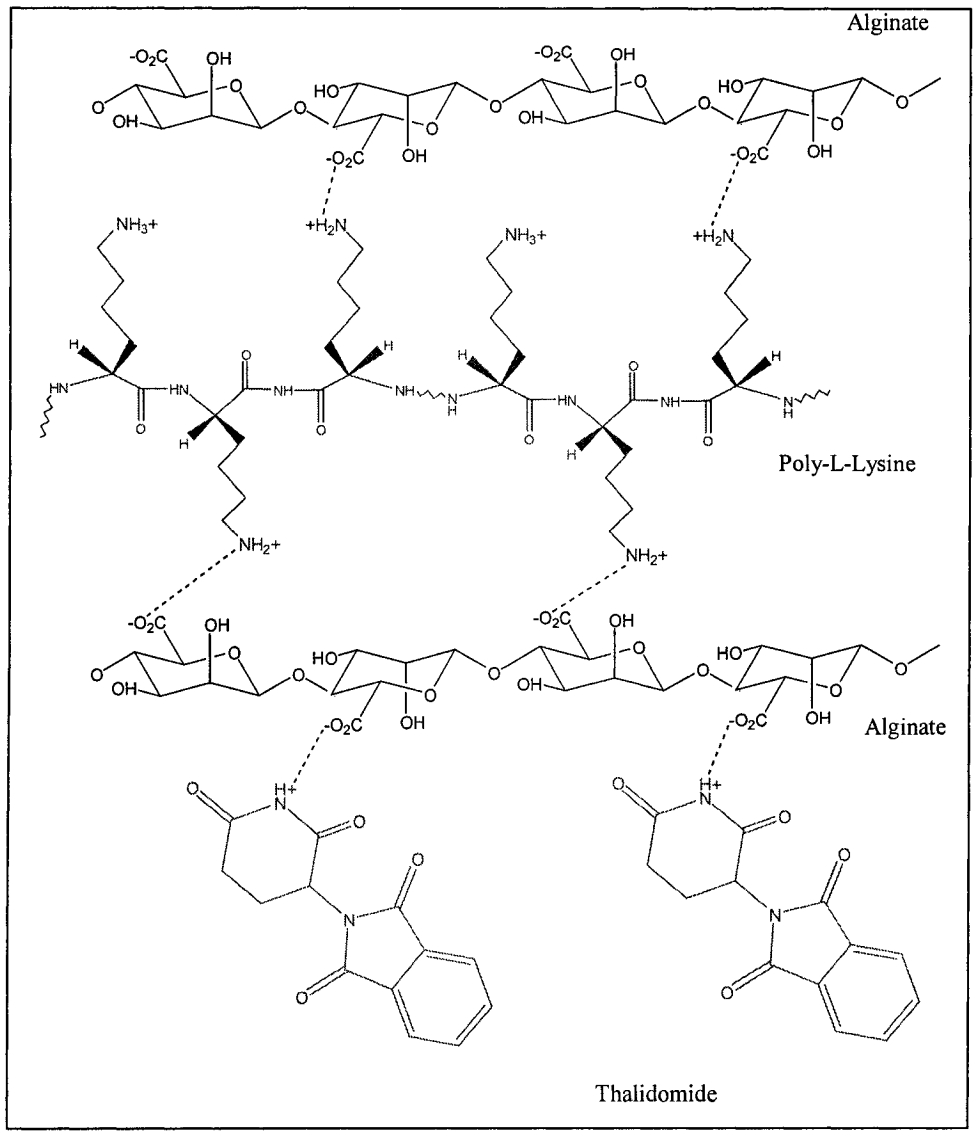
Figure 2.5: Electrostatic interactions between the inner layer of alginate and the cross-linking of calcium, the middle layer of polylysine, and an outer layer of alginate in alginate-polylysine-alginate (APA) membranes.

By changing membrane properties, one can alter the behaviour and degradation characteristics of

the capsules. Additionally, the outer alginate layer was added to the capsules to increase stability by lowering the susceptibility of enzyme action upon the polylysine.

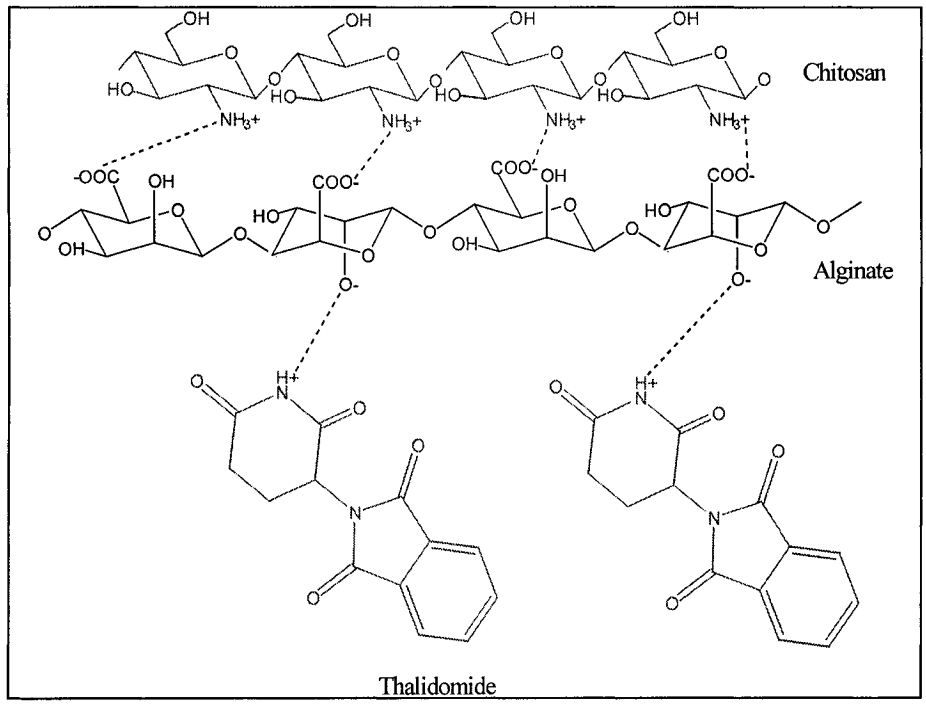
2.3.4 Proposed artificial cell design strategy for thalidomide

It is known that in its cationic state, thalidomide can gain a hydrogen atom causing its lone 6-membered ring to become positively charged. As well, alginate can exist in an anionic form causing a weak electrostatic bond to form within both the AC and APA capsules with thalidomide and the inner alginate layer of each respective capsule. It is hypothesized in proposed research that this bond may be strong enough to hold thalidomide inside the capsules and will break, and thus releases thalidomide, upon capsular degradation. This would result in the transition of exposed alginate back to its neutral form. Figure 2.6a and b shows a diagram of thalidomide design and thalidomide binding strategy in AC and APA microcapsules.



A

Figure 2.6 A: Binding of cationic thalidomide to anionic alginate layers in APA capsules.



B

Figure 2.6 B: B inding of cationic thalidomide to anionic alginate layers in AC capsules.

2.4 The present research objectives are:

1) To design artificial cell microcapsules containing thalidomide and optimize the encapsulation process and molecular membrane degradation,
2) To evaluate, *in-vitro*, the efficacy of the artificial microcapsule in delivering its content to the desired location of the gastrointestinal tract in a pH controlled gastrointestinal simulation, and
3) To evaluate preclinical efficacy tests for diminished or lowered inflammation *in vitro* in an experimental mouse macrophage intestinal model.

Preface for Chapters 3 to 5

To evaluate the feasibility of the novel approach of encapsulating thalidomide within an alginate-chitosan membrane to lower the inflammation associated with Crohn's Disease, alginate encapsulated beads were formed using the laminar liquid jet frequency superimposition method. Beads were then coated with a specifically designated concentration of chitosan to form an outer layer and these capsules were tested *in vitro* for degradation and thalidomide delivery as described in **Chapter 3**. Also included in this chapter are descriptions of the *in vitro* tests I designed and developed to simulate the human gastrointestinal tract by using varying pH physiological buffer solutions.

To further investigate polymer microcapsules for targeted thalidomide delivery in Crohn's disease, I have proposed and designed further experiments to test and compare alginate-chitosan beads with another engineered membrane design, alginate-polylysine-alginate. The methods of developing and testing the APA microcapsules as well as the comparison methods between both capsules' drug delivery are described in **Chapter 4**.

Chapter 5 describes the details of both APA and AC-encapsulated thalidomide's effects upon 264.7 mouse macrophage cells. These cells are similar to those responsible for stimulating inflammatory reactions in the small intestine exhibited in Crohn's disease and thus provide a good model for research. Both the efficacy of drug delivery and the resulting therapeutic effects are described.

The results obtained and reported upon in my research have been presented and/or published in the following papers:

Research Articles:

1) **Terrence Metz**, Devendra Amre, Mitchell L. Jones, Wei Ouyang, Hongmei Chen, Tasima Haque, Christopher Martoni, and Satya Prakash. New Technology for Crohn's disease: Polymeric artificial cell microcapsules for targeted delivery of thalidomide; preparation and in-vitro characterization. (Submitted). Drug Delivery.

2) **Terrence Metz**, Hongmei Chen, Wei Ouyang, Tasima Haque, Christopher Martoni, Devendra Amre, Satya Prakash. Polymeric microcapsules for thalidomide delivery for the treatment of Crohn's disease. (Submitted). Journal of Biochemistry and Biophysics

3) **Terrence Metz**, Tasima Haque, Hongmei Chen, Devendra Amre , Satya Prakash. Preparation and In Vitro Analysis of Artificial Microcapsules Containing Thalidomide for Targeted Suppression of TNF-α. (Submitted). Molecular and Cellular Biochemistry.

All above listed articles will follow as chapters of this thesis. In accordance with McGill University regulations, the three research articles are reported in their original form as individual chapters (Chapters 3 to 5). During my master's of engineering studies I was also able to publish and contribute to the following proceedings, abstracts, and papers which are not included in this thesis.

Proceedings, Abstracts, and Papers:

1) **Terrence Metz**, Devendra Amre, Mitchell L. Jones, Wei Ouyang, Hongmei Chen, Tasima Haque Christopher Martoni, and Satya Prakash. Artificial cell microcapsules

containing Thalidomide as an alternative oral therapy method for Crohn's Disease (CD). Journal of Pediatric Gastroenterology and Nutrition. July 2004.

2) **Terrence Metz**, Devendra Amre, Hongmei Chen, Wei Ouyang, Tasima Haque, Christopher Martoni, Satya Prakash.(2004) Artificial Cells Containing Thalidomide as an Alternative Oral Therapy Method for Crohn's Disease. Gastroenterology (Supplement). April 2004.

3) **Terrence Metz**, Mitchell Jones, Philip Magown, Wei Ouyang, Hongmei Chen, Satya Prakash. (2003) Polymer Microcapsules for Targeted Oral Drug Delivery in Crohn's Disease. Annals of Canadian Biomaterials Society 23rd Annual Meeting; 89. May 2003

4) Mitchell Jones, Hongmei Chen, Wei Ouyang, **Terrence Metz**, and Satya Prakash. (2003). Method for Bile Acid Determination by High Performance Liquid Chromatography. Journal of Medical Sciences; 23(5):277-280.

5) Mitchell Jones, Hongmei Chen, Wei Ouyang, **Terrence Metz**, and Satya Prakash. (2003). Microencapsulated genetically engineered Lactobacillus plantarum 80 (pCBH1) for bile acid deconjugation and its implication in lowering cholesterol. Journal of Biomedicine and Biotechnology. (In press).

6) Wei Ouyang, Hongmei Chen, Mitchell Jones, **Terrence Metz**, Christopher Martoni, Tasima Haque, Satya Prakash. Artificial cell microcapsule for oral delivery of live cells for therapy: design, preparation and in-vitro characterization. (Submitted) Journal of Pharmacy & Pharmaceutical sciences.

7) Wei Ouyang, Hongmei Chen, Mitchell Jones, **Terrence Metz**, and Satya Prakash. (2003) Novel multilayer microcapsule and its GI stability. 23rd CBS Symposium. Montreal.

8) Wei Ouyang, Hongmei Chen, Mitchell Jones, **Terrence Metz**, and Satya Prakash. Novel artificial cell formulation: design, preparation and morphology studies. (2002) Polymer in Medicine and Biology, American Chemical Society Symposia, California.

9) Tasima Haque, Hongmei Chen, Wei Ouyang, **Terrence Metz**, Christopher Martoni, and Satya Prakash. Investigation of a novel microcapsule membrane integrating polyethylene glycol to alginate, poly-l-lysine and chitosan microcapsules for the application of liver cell transplantation. XX International Congress of the Transplantation Society, 5-10 September 2004, Vienna. Published in: Transplantation (Supplement).

10) Tasima Haque, Hongmei Chen, Wei Ouyang, **Terrence Metz**, Bisi Lawuyi, and Satya Prakash. Effect of Integrating Polyethylene Glycol to Alginate-poly-L-lysine and Alginate Chitosan Microcapsules for Oral Delivery of Live Cells and Cell Transplant for Therapy. (Submitted) 28th Canadian Medical and Biological Engineering Society (CMBES) conference, Quebec City.

11) Tasima Haque, Hongmei Chen, Wei Ouyang, **Terrence Metz**, Christopher Martoni, and Satya Prakash. Design of a New Microcapsule Membrane Combining Alginate, Chitosan, Polyethylene Glycol, and Poly-L-lysine for Cell Transplantation. (Submitted) European Society for Artificial Organs (ESAO) conference, 8-11 September 2004, Warsaw, Poland.

12) Hongmei Chen, Mitchell Jones, Wei Ouyang, **Terrence Metz**, Christopher Martoni, Tasima Haque, Rebecca Cohen, Bisi Lawuyi, and Satya Prakash. (2004) Design, preparation and in-vitro characterization of genipin cross linked alginate-chitosan microcapsules for live cell encapsulation for cell therapy. (Submitted) Cell Biochemistry and Biophysics.

13) Hongmei Chen, Wei Ouyang, Mitchell Jones, **Terrence Metz**, and Satya Prakash. (2003) Alginate-chitosan microcapsules cross-linked by naturally occurring genipin. 23rd CBS Symposium. Montreal.

14) Hongmei Chen, Wei Ouyang, Mitchell Jones, **Terrence Metz**, and Satya Prakash. (2002) Preparation alginate/chitosan based multilayer microcapsules for biomedical application. Polymer in medicine and Biology, American Chemical Society Symposia, California.

15) Hongmei Chen, Wei Ouyang, Mitchell Jones, **Terrence Metz**, and Satya Prakash. (2002) New artificial cells: preparation and in-vitro characterization. 5th CBGRC, Montreal. September 2004.

Chapter 3

Original Paper: New Technology for Crohn's disease: Polymeric artificial cell microcapsules for targeted delivery of thalidomide; preparation and in-vitro characterization.

Terrence Metz, Devendra Amre, Mitchell L. Jones, Wei Ouyang, Hongmei Chen, Tasima Haque, Christopher Martoni, and Satya Prakash *

Manuscript submitted to Drug Delivery

*** Corresponding Author**

3.1 Abstract

Oral administration of thalidomide has been used for the treatment of inflammatory bowel diseases such as Crohn's disease (CD). In spite of its potential therapeutic benefits, known adverse effects associated with the drug limit its widespread utilization. In this study artificial cell alginate-chitosan (AC) membrane microcapsules containing thalidomide have been designed and their drug release characteristics and effectiveness for targeted thalidomide gastrointestinal (GI) delivery has been evaluated. Results show that AC capsule thalidomide release was immediate. In the absence of degradation, no release of the drug takes place from the microcapsules. Thus, microencapsulation of thalidomide for targeted delivery in Crohn's disease is possible and will be advantageous. However, further study is required.

3.2 Introduction

Inflammatory bowel diseases (IBD) such as Crohn's disease are some of the most common chronic diseases especially in the western world [105,106]. Although substantial progress has been made in determining therapeutic strategies, presently no single treatment is entirely effective. Heterogeneity in disease, variability in disease course and potential steroid resistance has prompted research into other treatment modalities. Recently the therapeutic potential of thalidomide has been explored [14,15,107-110]. Preliminary results from clinical trials indicate that thalidomide can reduce the inflammation associated with CD especially in more severe phenotypes. Notably in the past five years, two clinical trials have proven the effectiveness of thalidomide in the treatment of adult cases of Crohn's disease[14,110]. Both Ehrenpreis and Vasiliauskas reported clinical response and remission of inflammation in patients after the

administration of thalidomide. In 2001 Facchini et al. tested the use of thalidomide in children who had no response to conventional immunosuppressive treatments. Remission was achieved in four out of five patients after at least 20 months. Also several single case studies exist. Odeka and Miller (1997) [111] used thalidomide to depress previously and unsuccessfully treated ulcerations of an 8-year-old patient with IBD. Pain was instantaneously relieved and after two weeks, complete disappearance of ulcers was apparent. Wettstein and Meagher (1997) treated a 55-year-old patient who experienced continual ileac and colic bleeding and inflammation[112]. Previous steroid treatment had resulted in no change in symptoms. Upon treatment with thalidomide, bleeding had completely stopped after 3 weeks [113]. The anti-inflammatory effects of the drug are thought to be due to its stimulatory effects on TNF-α mRNA degradation through inhibiting certain transcription factor activities. Given the potential for systemic toxicity and adverse effects such as teratogenecity and neuropathy, it is unlikely that thalidomide could be used for prolonged periods of time. Thus, though certain trials using thalidomide therapy have been successful, its widespread use has not occurred as higher thalidomide dosages were potentially needed to overcome drug dilution within the GI tract causing these side effects. Therefore, there is an immediate need to develop suitable methods for optimal delivery of thalidomide for CD therapy. In the present article, we hypothesized that this can be avoided by using artificial cell polymeric microencapsulation procedures, giving opportunity to use a much lower amount of thalidomide to achieve the same therapeutic effects while avoiding high dosage side effects. Using artificial cell microencapsulation oral delivery of thalidomide, it will then be possible to directly target sites of inflammation where drug action is needed. Membranes such as AC have been used previously in numerous cases for encapsulation purposes based on their stability, biocompatibility and reproducibility [114-116]. Objectives of this study include determining

AC membrane degradation in a dynamic pH environment, testing the feasibility of thalidomide encapsulation within the AC membrane microcapsules, and in-vitro analysis of thalidomide release upon capsule exposure to simulated gastrointestinal pH conditions for possible IBD therapy applications.

3.3 Materials and Methods

Chemicals and Laboratory Equipment:

The chemicals thalidomide, alginic acid and dimethyl sulfoxide were supplied by Sigma-Aldrich Canada. Chitosan was supplied by Wako Chemicals USA. The Research IER-20 cell encapsulator was supplied by Inotech Biosystems International. The Varian Cary 100 Bio Spectrophotometer was supplied by Varian and the Lab-Line Environ Shaker 3527 was supplied by Lab-Line Designers and Manufacturers.

Preparation of AC microcapsule containing thalidomide

To prepare AC microcapsules containing thalidomide, alginic acid was purchased and added to deionized water to make a 1.5% alginate solution. Thalidomide ((±)-2-(2,6-Dioxo-3-piperidinyl)-1H-isoindole-1,3(2H)-dione) was dissolved in dimethyl sulfoxide and diluted with deionized water. 1ml of solution containing 0.7mg of thalidomide was added to the alginate solution. Alginate was additionally added to maintain a 1.5% concentration after the thalidomide and water solution was included. AC beads were then formed by running the above solution through an Inotech (Inotech Inc. Rockville, Maryland) encapsulator pump using a 300μm nozzle. Frequency was set to 528 Hz, flow rate to 20.8 ml/min and voltage to 0.348 kV. Formed beads

were collected directly in a prepared 0.1M calcium chloride solution to avoid cell aggregation. The beads were then washed with deionized water two times and soaked in a 0.5% chitosan in 1% acetic acid bath for 25 minutes. Soaking time in the chitosan solution was increased to 25 minutes to insure adequate membrane coating. Final washing was done with water and beads were transferred into 0.1 M calcium chloride for storage. The capsules were visually evaluated for uniformity and integrity through a Lomo light microscope with 250X magnification.

Testing AC microcapsule degradation in a varying pH environment

AC capsules were prepared and counted. The microcapsules were then washed using deionized water and were exposed to a pH 1.5 buffer solution. At five minute increments, small amounts of pH 7.5 buffer solution were added and pH levels were monitored. At each interval capsules were counted. AC degradation was microscopically observed until all AC microcapsules were destroyed.

Measuring the efficacy of thalidomide encapsulation

Thalidomide encapsulation was evaluated initially through AC membrane degradation. Samples weighing 35 mg of AC beads containing thalidomide were soaked in a prepared solution of 3% sodium citrate for 12 hours in order to dissolve the cell membranes. Samples from these preparations were analyzed in a Varian Cary 100 UV-visible spectrophotometer for thalidomide detection and compared with the supernatant of the beads prior to the sodium citrate soaking.

Testing the microcapsules in simulated gastrointestinal fluid and evaluating thalidomide release

Samples of alginate-chitosan (1.3g dry weight) beads containing thalidomide were washed, filtered and added to a simulated GI fluid buffer solution (pH 1.5) for 10 minutes to simulate acidic conditions normally encountered in the stomach. The solutions were shaken at 125 rpm in an Environ shaker at room temperature. The microcapsules were then transferred to another simulated GI fluid buffer solution (pH 7.5) and shaken to simulate proximal small intestine conditions for 60 minutes. For the duration of both tests, supernatant samples were analyzed at every 10 minutes using a Varian spectrophotometer.

3.4 Results and Discussion

Results

Experiments were designed to prepare artificial cell AC microcapsules containing thalidomide by encapsulation. Magnification of formed AC beads containing thalidomide revealed regular and uniformly shaped cells (Fig.3.1). Bead diameter was measured to be 300 ± 50 μm. No deformities, cell breakage or membrane damage was observed because of thalidomide loading procedures in the AC microcapsule. AC exposure to a varying pH environment experiment (Fig. 3.2), from pH 1.2 to 7.5 in selected time intervals, revealed microcapsule stability from pH 1.2 through pH 3.18. Between pH values from 1.2 to 3.18, 100% of microcapsules were intact. Between pH 3.18 and 4.77, less than 7% of the AC capsules had burst. Bursting of the AC membrane occurred most rapidly between pH 4.77 and 6.15 resulting in full degradation of 80% of the microcapsules. At pH 7.5, all capsules had been destroyed.

Figure 3.3 is a photograph through a light microscope of AC microcapsules during the degradation process.

Experiments were also designed to determine the release of drug in simulated gastrointestinal pH conditions. For this we prepared simulated GI fluid as described above, added the drug loaded microcapsule, and analyzed the release of the drug. The gastrointestinal environment contains physiological fluid, enzymatic systems, and varying pH levels. The pH levels were simulated here with the buffer solution, a method capable of maintaining a constant pH near that of each GI compartment. Results show that after 10 minutes of incubation shaking in pH 1.5 buffer solutions, thalidomide release from the AC capsules was minimal. Almost immediately after transferring alginate-chitosan (AC) microcapsules to the pH 7.5 environment the microcapsules burst, releasing most of the encapsulated thalidomide. Further release was negligible after 20 minutes of shaking. Observation of alginate-chitosan microcapsules after pH 7.5 exposure for 20 minutes revealed degradation and destruction of the microcapsule membrane. And after 60 minutes the capsules were completely dissolved, having released the entire loaded drug (Fig. 3.4).

Discussion

Several clinical trials have suggested the importance and efficacy of thalidomide treatment for symptoms associated with Crohn's disease [117-119]. However, toxicity, sedative effects, and other side effects and concerns were noted in several patients[14,120] To relieve such effects, use of lower doses of the drug is warranted [121-123]. Thus, it is of interest to deliver thalidomide specifically to areas of inflammation in order to decrease the total amount of the drug needed for proper treatment. In the present study we observed that microencapsulation of

thalidomide by AC membranes provides an efficient means for delivering thalidomide to targeted regions in the small intestine. AC capsules release thalidomide almost immediately following pH changes and in a burst-type manner due to their immediate degradation upon exposure to changing pH environments such as those experienced in the present study. Though the molecular weight permeability of the AC membrane is higher than thalidomide's molecular weight, its encapsulation within the microcapsule is hypothesized to be due to the attraction between the positively charged thalidomide and the negatively charged alginate. This interaction formed is possibly suitable for retaining the drug inside the AC microcapsule. However this bond is weak and breaks when AC capsules burst and alginate dissociates from its membrane as a result of a change in pH. Thus, this membrane has features that allow the drug to be delivered at proximal sections of the intestine. In Crohn's disease, alginate-chitosan encapsulation of thalidomide, thus, could provide a vehicle for concentrated drug delivery.

This study has shown that microcapsules containing thalidomide can be designed and that they have several advantages. Polymeric membrane microencapsulation renders the potentially useful method of thalidomide delivery, while at the same time avoiding the problems associated with oral administration of thalidomide for Crohn's disease therapy. However, further research is required to substantiate these results; in particular *in-vivo* affirmation of the drug delivery efficacy and therapeutics of these microcapsules is required before complete potential of this research can be comprehended.

3.5 Acknowledgements

This work was supported by the Faculty of Medicine, McGill University, the Natural Science & Engineering Research Council of Canada (NSERC), and the Canadian Institute of Health Research (CIHR).

Figures:

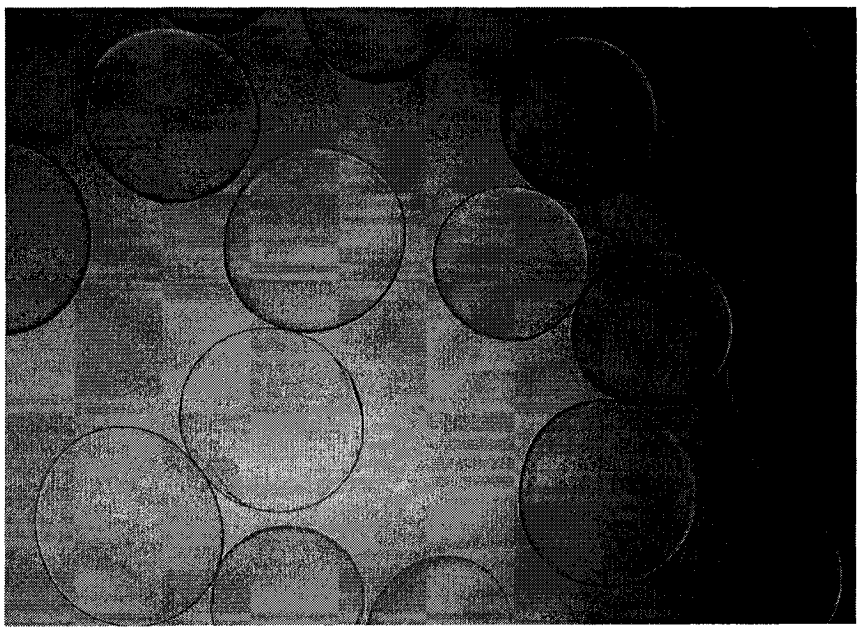


Figure 3.1: Experiment to determine bead uniformity. Photomicrograph of the freshly prepared alginate-chitosan microcapsules containing thalidomide (under 250 X magnification).

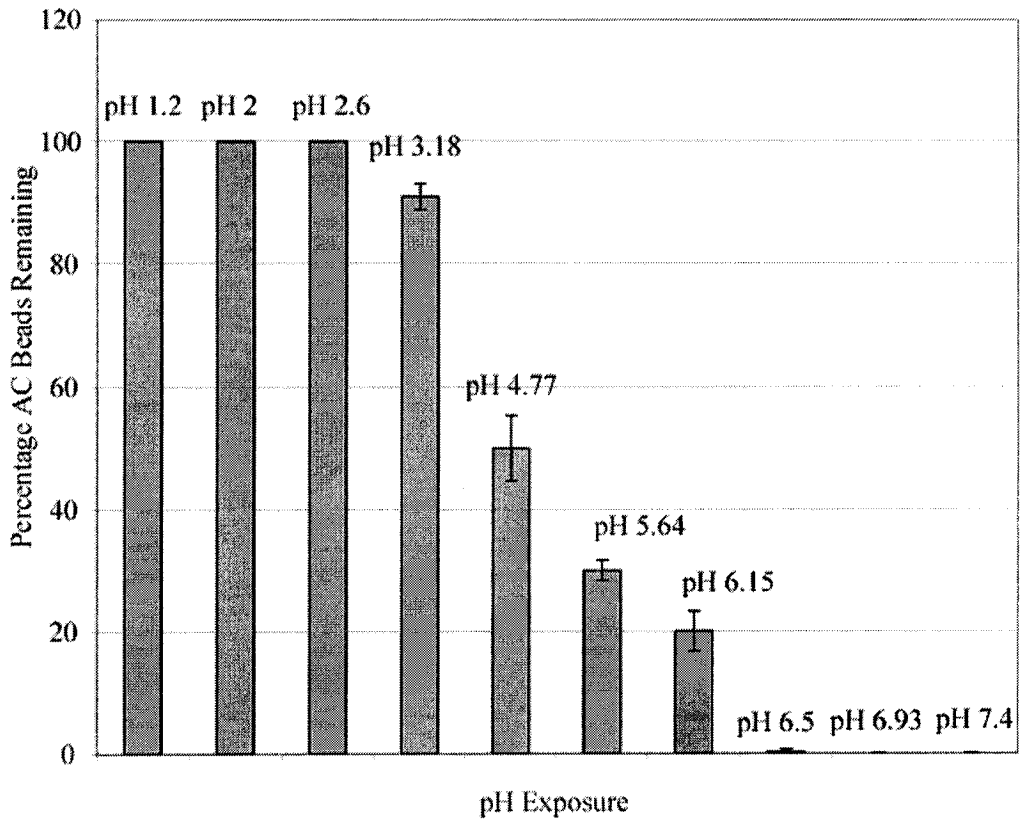


Figure 3.2: Experiments to analyze alginate chitosan bead degradation.

Alginate-Chitosan (AC) bead degradation in a pH varied environment. Experiments were done in triplicate and error bars were calculated as one standard deviation using Microsoft's Excel calculator.

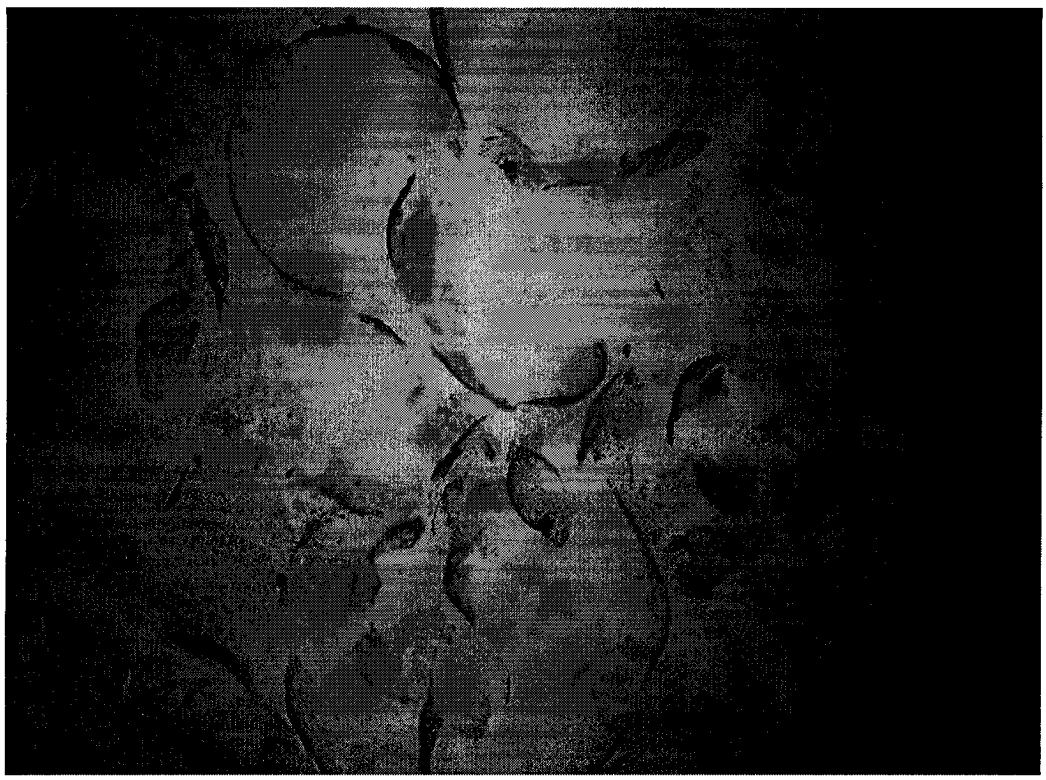


Figure 3.3: Experiment to visualize alginate chitosan degradation. Alginate-chitosan membranes in a dynamic state as degradation occurs (250X magnification).

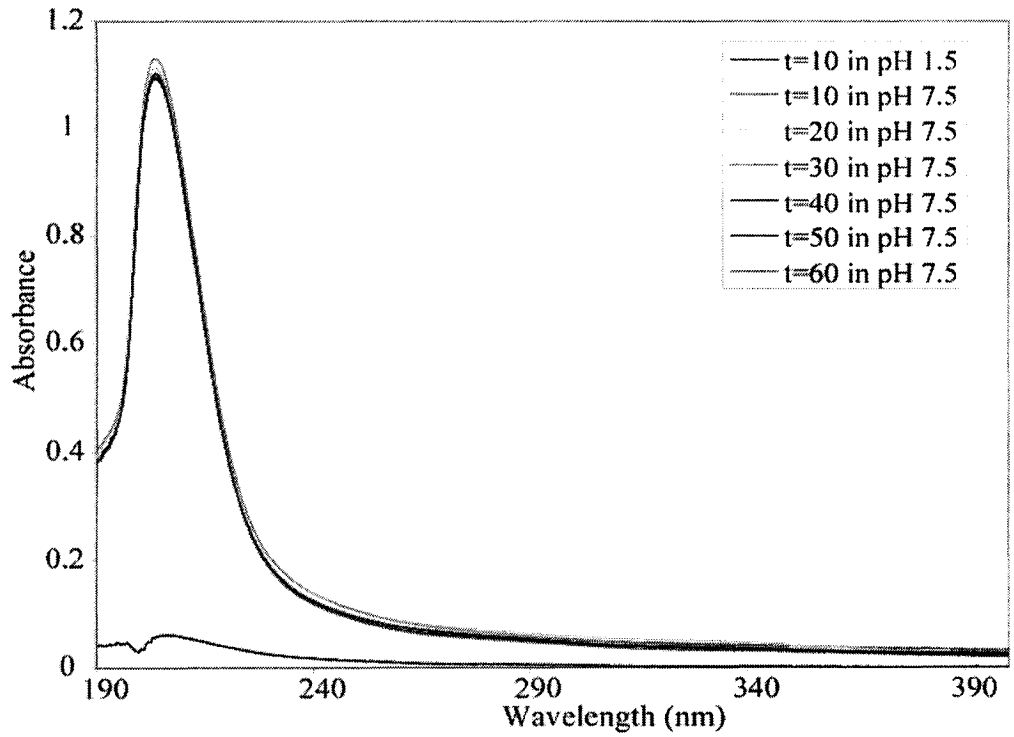


Figure 3.4: Experiment to determine drug release from alginate chitosan beads. Thalidomide release from alginate-chitosan microcapsules in simulated gastrointestinal pH conditions. Graph was taken directly from one of three experiments, all replicating exact conditions and showing similar results.

Chapter 4

Original Paper: Polymeric microcapsules for thalidomide delivery for the treatment of Crohn's disease

Terrence Metz, Hongmei Chen, Wei Ouyang, Tasima Haque, Christopher Martoni, Devendra Amre, Satya Prakash *

Manuscript submitted to the Journal of Biochemistry and Biophysics.

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4.1 Abstract

Recent evidence suggests that thalidomide could potentially be used to treat inflammation associated with Crohn's disease. However, systemic side effects associated with large doses of this drug have limited its widespread use. Treatment with thalidomide would prove more efficacious, if the drug could be delivered directly to target areas in the gut and systemic circulation is reduced. Microcapsule encapsulation of the drug could enable direct delivery of the drug. In order to assess the latter, we designed and tested drug targeting release characteristics in simulated gastrointestinal environments of Alginate-polylysine-alginate (APA) microcapsules compared to alginate chitosan (AC) capsules. Results show that AC capsules degrade rapidly in the simulated proximal small intestinal fluid. APA capsules enabled delivery of thalidomide further along the small intestine. Results show that both APA and AC capsules allow for successful delivery of thalidomide in the gut and could prove beneficial in the treatment of Crohn's disease. However, further research is required.

4.2 Introduction

Crohn's disease, an Inflammatory Bowel Disease, is characterized by inflammation in specific areas of the gastrointestinal tract. Adequate treatment of the disease has been hampered by the inability to determine specific factors perpetuating and maintaining the inflammation. Nevertheless based on evidence implicating the tumor necrosis factor alpha (TNF-α) secretion pathway in Crohn's related inflammation [124-128], current treatment modalities focused on anti-TNF- α therapy have achieved some measure of success in inducing and maintaining remission of inflammatory processes [14,43,129]. Among the latter, thalidomide has shown promise. Evidence

from open-label clinical trials and other case-series suggest that systemic administration of the drug could prove effective in providing pain relief, reducing ulcer formation, and stopping intestinal bleeding [14,15,107-112]. However, well known teratogenecitic and neuropathic effects of the drug could limit usage of the drug for long periods, a pre-requisite for maintaining remission and preventing relapse.

The initial promise shown by thalidomide could be enhanced if methods to limit systemic administration and simultaneous targeting to inflamed areas in the gut could be designed. The latter could substantially augment the clinical efficacy of the drug and reduce associated side effects. We have proposed that a therapy designed to orally deliver thalidomide within an artificial cell membrane engineered to degrade at a certain site along the gastrointestinal path would overcome the limitations previously associated with systemic delivery of thalidomide. Encapsulation therapy would maintain the drug within the microcapsule through the stomach protecting it from being absorbed into the systemic circulation. Microencapsulation methods for various applications, such as live cell implantation and drug delivery, have used both APA and AC polymers due to their biocompatibility and stability [114,130,131]. Earlier in-vitro studies show that alginate chitosan (AC) membrane formulations have a burst-type thalidomide release from the capsules after transfer from low to high pH [132]. This encourages us to develop a new formulation that can possibly have the capacity to target other therapy areas of the intestine. The goal of this study is to design an alginate-poly-l-lysine-alginate (APA) membrane thalidomide formulation and evaluate its thalidomide release characteristics for targeted thalidomide delivery.

4.3 Materials and Methods

Chemicals and Laboratory Equipment:

The chemicals thalidomide, alginic acid, poly –L-lysine (hydrobromide) and dimethyl sulfoxide were supplied by Sigma-Aldrich Canada. Sodium citrate was supplied by Fisher Scientific Canada. Chitosan was supplied by Wako Chemicals USA. The Research IER-20 cell encapsulator was supplied by Inotech Biosystems International. The Varian Cary 100 Bio Spectrophotometer was supplied by Varian and the Lab-Line Environ Shaker 3527 was supplied by Lab-Line Designers and Manufacturers.

AC encapsulation of thalidomide

To prepare AC microcapsules containing thalidomide, alginic acid was purchased and added to deionized water to make a 1.5% alginate solution. Thalidomide ((±)-2-(2,6-Dioxo-3-piperidinyl)-1H-isoindole-1,3(2H)-dione) was dissolved in dimethyl sulfoxide and diluted with deionized water. 1ml of solution containing 0.7mg of thalidomide was added to the alginate solution. Alginate was additionally added to maintain a 1.5% concentration after the thalidomide and water solution was included. AC beads were then formed by running the above solution through an Inotech (Inotech Inc. Rockville, Maryland) encapsulator pump using a 300μm nozzle. Frequency was set to 528 Hz, flow rate to 20.8 ml/min and voltage to 0.348 kV. Formed beads were collected directly in a prepared 0.1M calcium chloride solution to avoid cell aggregation. The beads were then washed with deionized water two times and soaked in a 0.5% chitosan in 1% acetic acid bath for 25 minutes. Soaking time in the chitosan solution was increased to 25 minutes to insure adequate membrane coating. Final washing was done with water and beads were transferred into 0.1 M calcium chloride for storage. The capsules were visually evaluated for uniformity and integrity through a Lomo light microscope with 250X magnification.

APA encapsulation of thalidomide

Alginic acid was purchased from Sigma (Oakville, Ontario, Canada) and added to deionized water to make a 1.5% alginate solution. (±)-Thalidomide ((±)-2-(2,6-Dioxo-3-piperidinyl)-1H-isoindole-1,3(2H)-dione) (Sigma) was dissolved in deionized water at a concentration of 0.035 mg/ml by stirring and heating for 24 hours and added to the alginate solution. Alginic acid was additionally added to maintain a 1.5% concentration after the thalidomide and water solution was included. APA beads were then formed by running the above solution through an Inotech (Inotech Inc. Rockville, Maryland) encapsulator pump using a 300μm nozzle. Frequency was set to 528 Hz, flow rate to 20.8 ml/min and voltage to 0.348 kV. Formed beads were collected in a prepared 0.1M calcium chloride solution to avoid cell aggregation. The beads were then washed with deionized water and soaked in a 0.1% poly-l-lysine (Sigma) bath for 10 minutes. Beads were washed again and soaked in 0.15% alginate solution for 15 minutes. Final washing was done with water and beads were transferred into calcium chloride for storage. The capsules were visually evaluated for uniformity and integrity through a Lomo light microscope with 250X magnification.

Similar procedures were used to create alginate-chitosan capsules. 1.5% alginate solution was used to immobilize thalidomide. Resulting beads were soaked in a solution of 0.5% chitosan (Sigma) in 1% acetic acid. Soaking time in the chitosan solution was increased to 25 minutes to insure adequate membrane coating.

Measuring the efficacy of thalidomide encapsulation

Thalidomide encapsulation was evaluated initially through APA and AC membrane degradation. Samples of beads weighing roughly 35.1 mg were soaked in a prepared solution of

3% sodium citrate for 12 hours in order to dissolve the cell membrane. Samples from these preparations were analyzed in a Varian Cary 100 UV-visible spectrophotometer (Varian Canada, St. Laurent, Quebec) for thalidomide detection and compared with the supernatant of the beads prior to the sodium citrate soaking.

Testing capsules in simulated gastrointestinal fluid and evaluating thalidomide release

Samples of APA (1.22g dry weight) and alginate-chitosan (1.30g dry weight) beads containing thalidomide were washed, filtered and added to a prepared pH 1.5 buffer solution for 10 minutes to simulate acidic conditions normally encountered in the stomach. The solutions were shaken at 125 rpm in an Environ shaker. The microcapsules were then transferred to a pH 7.5 buffer solution and shaken to simulate proximal small intestine conditions for 60 minutes. For the duration of both tests, supernatant samples were spectrophotometrically analyzed every 10 minutes.

Testing capsules in variable pH environments

Similar quantities of both AC and APA capsules were counted and put in 5cm Petri dishes with 10mL of pH 1.5 physiological buffer solution. Measured quantities of pH 7.4 physiological buffer solution were added every five minutes in order to gradually increase the pH of each individual dish to values of 1.2, 2, 2.6, 3.18, 4.77, 5.64, 6.15, 6.5, 6.93, and 7.4 respectively. After each addition of pH 7.4 buffer, capsules in each dish were counted and observed for degradation.

4.4 Results and Discussion

Results

We studied the feasibility of delivering thalidomide to the proximal and middle small intestine where Crohn's-related inflammation most commonly occurs. Experiments were designed to formulate APA capsules containing thalidomide. Figure 4.1 shows the APA capsules containing thalidomide. APA microcapsules were compared with AC for their alternate delivery mechanisms for thalidomide in areas of the small intestine. Both of these membranes deliver the drug to a separate area of the gut due to differing degradation patterns. Our aim was to successfully encapsulate thalidomide within both APA and AC membranes as well as analyze the drug's release in simulated gastrointestinal pH conditions. We observed that APA beads containing thalidomide were regular in formation and the bead diameters measured 300 ± 50 μm (Fig. 4.1). After dissolving beads in sodium citrate for 12 hours, spectrophotometric analysis of the remaining solution indicated the high concentration of thalidomide present after membrane degradation signifying successful encapsulation. Analysis of membrane supernatant prior to sodium citrate exposure indicated the absence of thalidomide.

After 10 minutes of shaking in pH 1.5 buffer, thalidomide release from both the APA and alginate-chitosan capsules was minimal. Almost immediately after transferring alginate-chitosan beads to the pH 7.5 environment the cells burst, releasing most of the encapsulated thalidomide. Further release was negligible after 20 minutes of shaking. However, release of thalidomide from the APA capsules acts as a timed mechanism slowly allowing the drug to escape as the membrane degrades. APA membranes indicated capsule stability from pH values from 1.2 to higher pH values of 5.8. Membrane thinning was not detected to a great extent until pH values reached the range of 6.15-6.5. It should be noted that for APA tests, observed membranes did

not degrade as in the case of AC, which allowed thalidomide retention in the membrane for a longer time period (Fig. 4.2). Full release of thalidomide was achieved after 60 minutes of shaking. Thalidomide peaks remained constant at an absorbance of 1.6 after 60 minutes indicated by the highest peak detected (Fig. 4.3). APA degradation was observed as a membrane thinning effect seen in figure 4. Rather than completely degrading, the membrane's alginate layers degraded leaving a polylysine membrane intact. Although this occurred, thalidomide was still released. Observation of alginate-chitosan capsules after pH 7.5 exposure for 20 minutes revealed degradation and destruction of the membrane itself. After 60 minutes the capsules had completely dissolved. Degradation differences between AC and APA capsules after a varying pH study can be seen (Fig. 4.5). AC capsule degradation, as reported above, occurred in a burst-like fashion. Specifically, 80-100% of capsules were intact throughout the test until pH levels rose above 3.18. Between pH 3.18 and 5.64, nearly 70% of AC capsules had burst. Conversely, the APA capsules thinned out as the alginate coatings degraded to produce a ghosting effect (Fig. 4.4). Nevertheless, this was sufficient to deliver thalidomide as suggested by the spectrophotometric results described above, suggesting that AC capsules were capable of delivering thalidomide to the most proximal sections of the small intestine such as the duodenum or the upper jejunum. Alternatively, APA capsules would retain thalidomide and release the drug further along the small intestine.

Discussion

Previous studies have evaluated treatment with thalidomide for oral, vulvar, distal intestinal (jejunum and ileum), and colonic localized Crohn's disease[14,15,60-62]. However, the full potential of thalidomide is not realized because of its associated side effects. It has been

suggested that targeted delivery could be ideal for this. We have shown that encapsulation could be an alternative method for thalidomide delivery. In this comparative study we observed the different mechanisms of drug delivery from both APA and AC membranes (Fig. 4.6). Maintenance of thalidomide within the microcapsules is thought to be due to ionic interactions between the positively charged thalidomide and the negatively charged alginate. Upon membrane degradation, these interactions are broken and thus drug is released. APA capsules slowly release thalidomide after exposure to a pH change from 1.5 to 7.5. AC capsules release thalidomide almost immediately following pH changes. Specifically, APA capsules degrade at higher pH values and are thus more resilient than AC capsules.

A method that could ensure delivery of thalidomide specifically to areas of inflammation would enhance the treatment efficacy of the drug substantially, reducing the amount of drug required for therapy and considerably reducing systemic side effects. Our observations strongly suggest that microencapsulation would be an ideal delivery system. APA capsules, as shown, can be used for a timed-release delivery mechanism of thalidomide and enable shuttling the drug to more distal sections of the intestine. The ability of AC capsules to release thalidomide in a burst-type manner (due to their immediate degradation upon exposure to changing pH environments such as those experienced in the gastrointestinal tract) would make them suitable for drug delivery to the proximal sections of the intestine. Although pore sizes can reach up to 60KD in size which is much larger than the thalidomide molecule, we hypothesized that the drug was maintained within the capsules due to binding forces between the negatively charged alginate and the positively charged thalidomide.

In conclusion, our preliminary studies indicate that microencapsulation of thalidomide would enhance its potential therapeutic benefits for Crohn's disease. Further studies will evaluate

the effects on the microcapsules of other elements within the gastrointestinal tract (viz. enzymes) and the effects of varying concentrations of poly-l-lysine and chitosan on capsule stability and thalidomide release.

4.5 Acknowledgements

This work was supported by the Faculty of Medicine, McGill University, the Natural Science & Engineering Research Council of Canada (NSERC), and the Canadian Institute of Health Research (CIHR).

Figures:

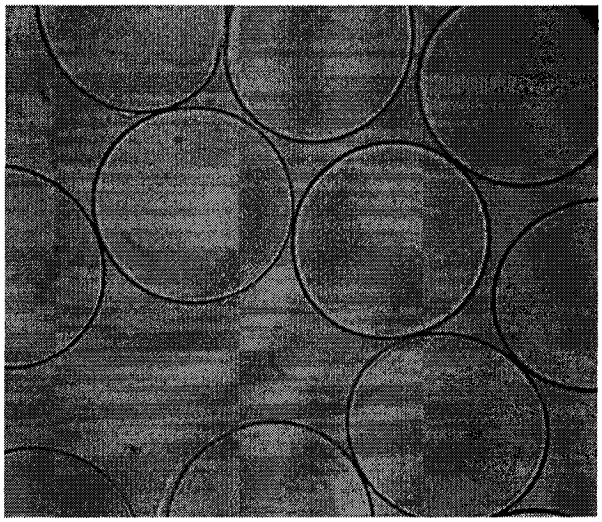

Figure 4.1: Experiment to visually analyze alginate-polylysine-alginate bead's uniformity. Photomicrograph of APA microcapsule thalidomide formulation under 250X magnification.

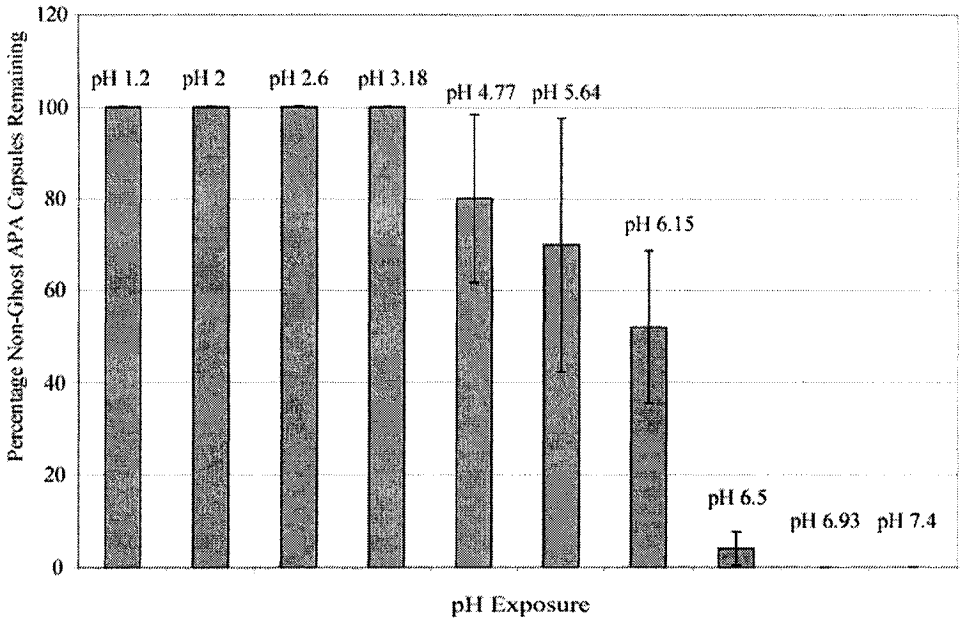

Figure 4.2: Experiments to observe alginate-polylysine-alginate degradation. APA capsule degradation in a pH varying environment. Experiments were done in triplicate and error bars were calculated as one standard deviation using Microsoft's Excel calculator.

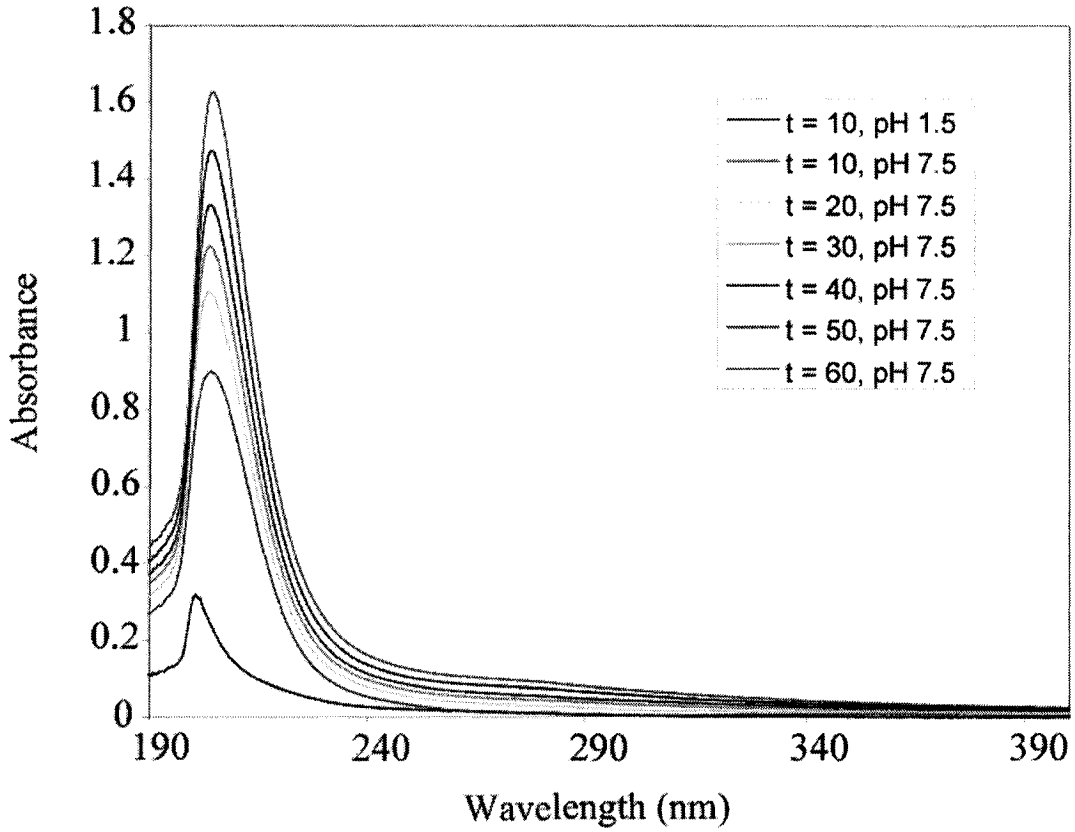

Figure 4.3: Experiment to determine drug release from alginate-polylysine-alginate beads. Simulated gastrointestinal pH test of thalidomide release from APA microcapsules. Graph was taken directly from one of three experiments, all replicating exact conditions and showing similar results.

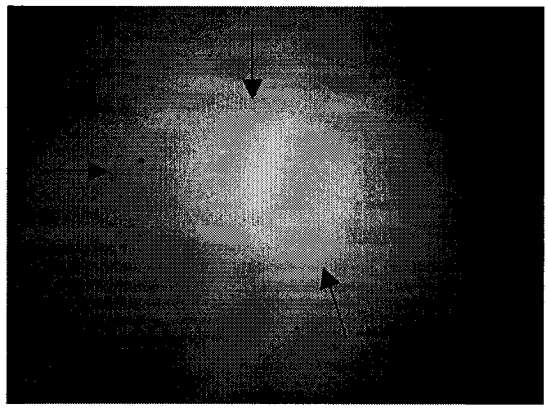

Figure 4.4: Experiment to visually analyze alginate-polylysine-alginate degradation. APA membranes after exposure to pH 7.5 conditions for 20 minutes (250X magnification).

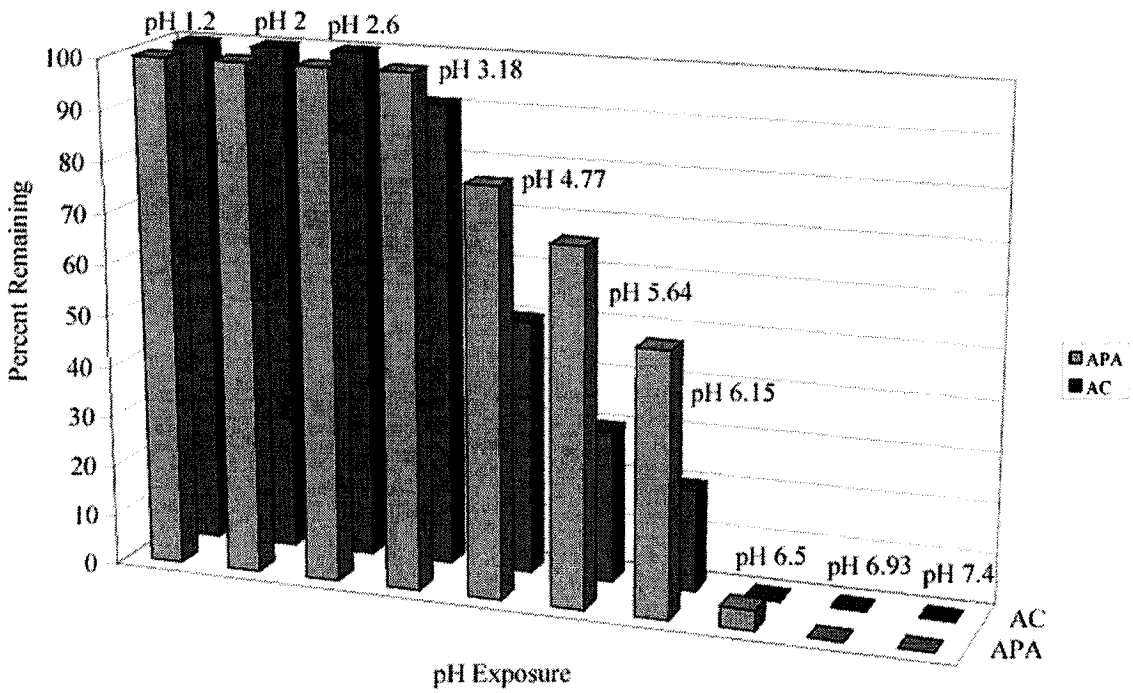

Figure 4.5: Graphical plot of both APA and AC degradation tests done previously in triplicate in a varying pH environment.

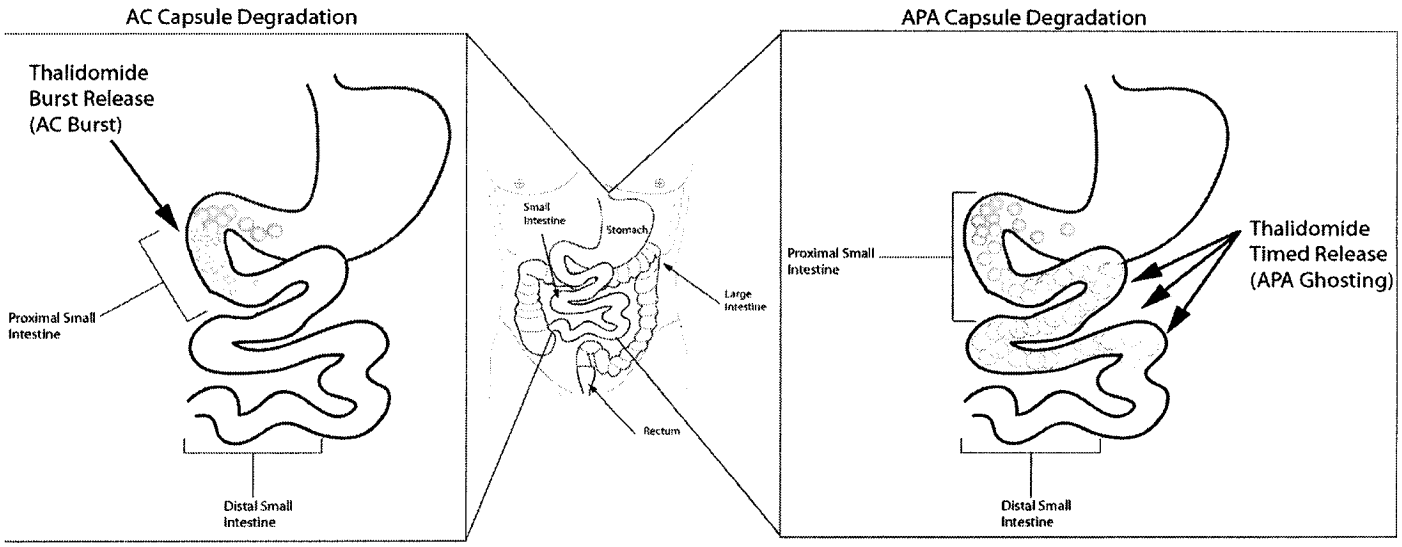

Figure 4.6: Schematic drawing showing thalidomide targeted delivery from both AC and APA capsules in different areas of the small intestine and by different degradation methods.

Chapter 5

Original Paper: Preparation and In Vitro Analysis of Artificial Microcapsules Containing Thalidomide for Targeted Suppression of TNF-α

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5.1 Abstract

Recent studies have implicated the cytokine Tumor Necrosis Factor-alpha (TNF-α) in the inflammation associated with Crohn's disease. Also, thalidomide has been shown to decrease this inflammation due to it's suppression of TNF-α secretion. Direct delivery of the drug to the site of inflammation by specifically engineered alginate-chitosan (AC) and alginate-polylysine-alginate (APA) polymer microcapsules would prevent side effects previously associated with thalidomide and would provide an efficient way to target therapy. In order to test this hypothesis, both AC- and APA-encapsulated thalidomide were transferred through simulated gastrointestinal (GI) fluids and incubated with lipopolysaccharide-stimulated murine RAW 264.7 macrophage cells. Thalidomide released from AC capsules lowered TNF-α secretion from activated cells but did not completely arrest TNF-α production. However, drug release from APA capsules almost entirely prevented TNF-α secretion from activated RAW 264.7 cells. Degradation differences between AC and APA capsules result in different thalidomide release mechanisms and thus would treat Crohn's inflammation differently.

5.2 Introduction

Inflammatory bowel diseases (IBD) such as Crohn's disease are some of the most common chronic diseases especially in the western world [133,134]. Although substantial progress has been made in determining therapeutic strategies, presently no single treatment is entirely effective. Heterogeneity in disease, variability in disease course and potential steroid resistance has prompted research into other treatment modalities. TNF-α, a proinflammatory cytokine secreted from cells such as macrophages in the immune system, has been implicated in Crohn's disease-related inflammation[1]. Recently the anti-TNF therapeutic potential of thalidomide has

been explored [14,15,59,107-110]. Preliminary results from clinical trials indicate that thalidomide can reduce the inflammation associated with CD especially in more severe phenotypes. Notably in the past five years, several clinical trials have proven the effectiveness of thalidomide in the treatment of Crohn's disease[14,110-112]. The anti-inflammatory effects of the drug are thought to be due to its stimulatory effects on TNF-α mRNA degradation through inhibiting certain transcription factor activities[58]. Given the potential for systemic toxicity and adverse effects such as teratogenecity and neuropathy, it is unlikely that thalidomide could be used for prolonged periods of time. Thus, though certain trials using thalidomide therapy have been successful, its widespread use has not occurred as higher thalidomide dosages were potentially needed to overcome drug dilution within the GI tract causing these side effects. Therefore, there is an immediate need to develop suitable methods for optimal delivery of thalidomide to avoid systemic circulation for CD therapy. In the present article, we hypothesized that this can be avoided by using artificial cell polymeric microencapsulation procedures, giving opportunity to use a much lower amount of thalidomide to achieve the same therapeutic effects while avoiding high dosage side effects. Using artificial cell microencapsulation oral delivery of thalidomide, it will then be possible to directly target sites of inflammation where drug action is needed. Membranes such as AC and APA have been used previously in numerous cases for encapsulation purposes based on their stability, biocompatibility and reproducibility [93,114,135-137]. Our present tests were done using lipopolysaccharide(LPS)-activated RAW 264.7 murine macrophage cells to simulate TNF-secreting cells encountered in the GI tract. LPS has been shown previously to activate TNF-α secretion[100-104]. Objectives of this study include using previously designed AC and APA membranes[138] to encapsulate thalidomide, deliver thalidomide from the capsules after they are exposed to simulated GI conditions, and monitor the effects of

the capsular drug delivery system on lowering TNF-α secretion from cultured RAW 264.7 mouse macrophage cells.

5.3 Materials and Methods

Chemicals and Lab Equipment:

The chemicals thalidomide, alginic acid, poly –L-lysine (hydrobromide) and dimethyl sulfoxide were supplied by Sigma-Aldrich Canada. Sodium citrate was supplied by Fisher Scientific Canada. Chitosan was supplied by Wako Chemicals USA. The Research IER-20 cell encapsulator was supplied by Inotech Biosystems International. The Varian Cary 100 Bio Spectrophotometer was supplied by Varian and the Lab-Line Environ Shaker 3527 was supplied by Lab-Line Designers and Manufacturers.

Macrophage Cell Line and Growth Conditions

The RAW 264.7 cells from the American Type Culture Collection (ATCC) were stimulated by Lipopolysaccharide from Escherichia Coli 055:B5 supplied by Sigma-Aldrich and grown in Dulbecco's Modified Eagle's Medium and Fetal Bovine Serum from ATCC and supplemented with Penicillin Streptomycin supplied by Sigma-Aldrich Canada. Polyoxyethylene (Tween 20) Sorbitan Monolaurate was purchased from VWR International and used as an ELISA agent. A Sanyo MCO-18M Oxygen/Carbon Dioxide Incubator and a Sanyo MDF-U50V -86 degrees Celsius Freezer were used for cell incubation and storage and were supplied by SANYO Canada. A Lomo Biological Inverted Microscope BIOLAM P was supplied by LOMO America and used for microscopic cellular observation. ELISA testing was done with an EBioscience Mouse TNF-alpha ELISA Ready-SET-Go! Kit supplied by EBioscience. A

NAPCO 2028R Centrifuge was supplied by Precisionand was used for cell centrifugation. A Bio-Tek μQuant Universal Microplate Spectrophotometer from Fisher Scientific was used for ELISA plate analysis.

Formation of Microcapsules Containing Thalidomide

Alginic acid was purchased from Sigma (Oakville, Ontario, Canada) and added to deionized water to make a 1.5% alginate solution. (±)-Thalidomide ((±)-2-(2,6-Dioxo-3-piperidinyl)-1H-isoindole-1,3(2H)-dione) (Sigma) was dissolved in dimethyl sulfoxide and diluted with deionized water. 1ml of solution containing 0.7mg of thalidomide was added to the alginate solution. Alginate was additionally added to maintain a 1.5% concentration after the thalidomide and water solution was included. APA beads were then formed by running the above solution through an Inotech (Inotech Inc. Rockville, Maryland) encapsulator pump using a 300μm nozzle. Frequency was set to 528 Hz, flow rate to 20.8 ml/min, and voltage to 0.348 kV. Formed beads were collected in a prepared 0.1M calcium chloride solution to avoid cell aggregation. The beads were then washed with deionized water and soaked in a 0.1% poly-l-lysine (Sigma) bath for 10 minutes. Beads were washed again and soaked in 0.15% alginate solution for 15 minutes. Final washing was done with water and beads were transferred into calcium chloride for storage.

Similar procedures were used to create alginate-chitosan capsules. Again, 1.5% alginate solution was used to immobilize thalidomide. Resulting beads were soaked in a solution of 0.5% chitosan (Sigma) in 1% acetic acid. Soaking time in the chitosan solution was increased to 25 minutes to insure adequate membrane coating.

APA and AC beads were prepared as previously described using 12.7μg/ml of thalidomide per milliliter of alginate solution.

Macrophage Cell Culturing

Mouse RAW 264.7 macrophage cells were purchased from the American Type Culture Collection (ATCC) ATCC, P.O.Box 1549, Manassas, VA 20108 USA and cultured according to standard culture procedures using Dulbecco's Modified Eagle's Medium, purchased from ATCC, and supplemented with 10% Fetal Bovine Serum and 0.1% penicillin streptomycin antibiotic[139-143]. The cells were incubated in a 37° Celsius and 5% CO_2 environment in a Sanyo MCO-18M Oxygen/Carbon Dioxide incubator.

Stimulating TNF-α Secretion

LPS dilutions of 0.1, 1, 10, and 100 μg/mL in autoclaved PBS solution were prepared for use in stimulating 264.7 macrophage cells. Standard TNF-α secretion experiments were performed by adding 0.23mL media solution containing 300,000 264.7 macrophage cells to wells within a Falcon Brand 24-well Flat Bottom Tissue Culture Plate. 0.23mL of each of the four LPS dilutions were added and allowed to incubate in the individual wells containing the media and cells. Six samples of each of the four dilutions were prepared for sampling at six separate time points: 0.5 hours, 1 hour, 3 hours, 12 hours, 24 hours, and 48 hours. Control samples containing just cells in media were also prepared. All samples were incubated in a standard 5% CO_2 environment. After supernatant from the appropriate wells were withdrawn, these solutions were frozen for storage in a Sanyo MDF-U50V -86° Celsius freezer prior to ELISA analysis.

APA and AC Capsule Testing in the Presence of 264.7 Macrophage Cells

APA and AC capsules were initially submerged in 1.5 pH buffer solutions to mimic stomach acidic conditions. These capsules were then added to cultured 264.7 macrophage cells in media of pH of approximately 7.4. The cells were stimulated using LPS and quantities of cells and LPS solution were the same as previously described. Similar amounts of APA and AC capsules, approximately 0.116g, were added to the culture plate wells by weighing the samples prior to their addition. Samples of LPS, cells in culture media, and APA or AC beads were incubated in a 5% CO_2 environment and withdrawn at time points of 0.5 hours, 1 hour, 3 hours, 12 hours, 24 hours, and 48 hours. After supernatant from the appropriate wells were withdrawn, the solutions were frozen for storage prior to ELISA analysis in a Sanyo MDF-U50V -86° Celsius freezer.

TNF-a ELISA Analysis

In order to detect TNF-alpha secretion levels from mouse 264.7 macrophage cells an ELISA test was done using an EBioscience Mouse TNF-alpha ELISA Ready-SET-Go! kit. Nunc Maxisorb 96 well plates were coated with capture antibody and incubated at 4 degrees Celsius overnight. Wells were then manually washed three times with phosphate buffer solution (PBS) containing 0.05% Tween-20 and aspirated. Assay diluent was then added to each well and allowed to incubate for 1 hour at room temperature. Manually washing was repeated three times, plates were aspirated, and then 8 concentrations of two-fold serial dilutions of the included top standard of 1000 pg/ml were added to the appropriate wells. Samples of supernatant were spun down in a NAPCO 2028R centrifuge for 5 minutes at 1000 rpm to separate remaining cells

out. These samples were then added to the appropriate wells. The plates were sealed and incubated at room temperature for 2 hours. Manual washing was performed 5 times before aspiration and detection antibody was then added to the plates. The plates were again sealed and incubated for 1 hour at room temperature. Wells were washed 5 times again, aspirated, and Avidin-HRP enzyme was added. The plates were sealed and incubated at room temperature for 30 minutes. Wells were washed for 7 times and wash buffer was allowed to soak in each well for 1-2 minutes before plate aspiration. Substrate solution was added to the wells and allowed to incubate at room temperature for 15 minutes. 2N sulfuric acid was added to wells as a stop solution for color development and the optical density of the plates was read at 450nm and 570nm on a Bio-Tek μQuant Universal Microplate Spectrophotometer.

5.4 Results and Discussion

Results

Capsule formation and TNF-α standard analysis

AC and APA capsules were formed containing thalidomide to a diameter of 300μm ± 50μm and analyzed by light microscopy for membrane integrity, stability, and uniformity. Photomicrographs of both of these capsular formulations reveal spherical and stable capsules (Fig. 5.1). Differences in membrane thickness between both AC and APA capsules can be seen upon visual observation. AC membranes appear thinner due to a double layer of alginate and chitosan. APA membranes appear thicker as a result of a triple layering technique with alginate, polylysine, and alginate. Additionally, pictures were taken of the cultured RAW 264.7 cells to monitor growth in media (Fig. 5.2). TNF-α standards were analyzed using ELISA analysis with

an included top standard in the purchased EBioscience TNF-α ELISA kit. The analysis of tests revealed a linear graph with an R squared value of 0.9916 (Fig. 5.3).

AC thalidomide delivery induced and maintained lower TNF-α secretion levels following incubation with LPS-stimulated cells

ELISA analysis for cytokine content revealed control 10μg/ml LPS-stimulated RAW 264.7 cell's secretion of up to 1.23 ng/ml of TNF-α after a period of 24 hours. After the addition of 0.116 g of AC capsules containing 1.5 μg of thalidomide, TNF-α secretion initially rose to similar concentrations as controls. Though, after 12 hours, TNF levels stayed at concentrations of 0.79 ng/ml-0.86 ng/ml and remained between 68% and 72% of control concentrations (Fig. 5.3a). Similar analysis from 0.5 to 48 hours was done with the addition of the same quantities of AC capsules to 100μg/ml LPS-stimulated RAW 264.7 cells. Control cells secreted a maximum of 1.35 ng/ml of TNF-α after a period of 12 hours. Results indicate an initial rise in TNF-α levels in samples but a leveling off occurred after 12 hours at concentrations of between 0.88 ng/ml and 0.90 ng/ml and remained between 65% and 70% of control concentrations (Fig. 5.3b). Comparisons between both the 10 and 100μg/ml LPS-stimulated RAW 264.7 cells are graphed (Fig. 5.5a).

APA thalidomide delivery suppressed TNF-α secretion after incubation with LPS-stimulated cells

Supernatant from the incubation of 10 μg/ml LPS-stimulated RAW 264.7 cells with 0.116g of APA capsules containing 1.5 μg of thalidomide was compared to control samples and analyzed for TNF-α content by ELISA. Initially TNF-α levels remained quite low at

concentrations of 0.086 ng/ml or 7% of control levels up to 12 hours. Between 12 and 24 hours, TNF levels rose to roughly 80% of control concentrations. After 24 hours, TNF secretion was suppressed significantly to concentrations of 0.056 ng/ml or 5% of control concentrations (Fig. 5.4a). Experiments with APA capsules containing thalidomide were repeated in the presence of RAW 264.7 cells stimulated with 100 μg/ml of LPS. TNF-α secretion was delayed in the presence of the capsule formulation. Cytokine concentrations of 0.23 ng/ml, or 24% of control concentrations, were detected after 3 hours of incubation. TNF levels rose in a lag-response fashion to 96% of control concentrations. After 24 hours, concentrations of TNF-α decreased to 87% of controls and by 48 hours, secretion was maintained at 0.051 ng/ml or 4% of the control concentrations of 1.17 ng/ml (Fig. 5.4b). Comparisons between both the 10 and 100μg/ml LPS-stimulated RAW 264.7 cells are graphed (Fig. 5.5b).

RAW 264.7 cell viability

The effects of encapsulated thalidomide were tested by microscopic evaluation of RAW 264.7 cells. Cells were observed for growth and morphological differences between standard cultured cells and post-incubated cells with encapsulated thalidomide. Morphologically, after exposure, the macrophage cells were identical in shape and had ovular projections like the cells prior to thalidomide exposure (Fig. 5.6). Cells still maintained the ability to grow in culture in Dulbecco's modified Eagle's medium. Thus, encapsulated thalidomide lowered the secretion of TNF- α but did not affect the macrophage's overall growth. This is an essential property of the proposed therapy for the maintenance of a normal-functioning intestinal tract.

Discussion

Cytokines act as signal transmitters between cells in the immune system creating a response pathway to various external stimuli. Of these TNF-α has been implicated in processes such as inflammation, apoptosis or cell death, and thrombosis among others. Specifically, this molecule has been implicated in mediating the inflammatory mechanisms associated with inflammatory bowel diseases (IBD), such as Crohn's disease, by initiating an increase in inflammatory cells to areas of the gut[1]. Therapies for treatment of Crohn's disease have thus concentrated on anti-TNF-α mechanisms. Treatments with monoclonal antibodies, eicosanoids, PPARγ, phosphodiesterase inhibitors, signal transduction inhibitors, TNF-α converting enzyme inhibitors, and thalidomide have been tried[34]. Of these, thalidomide has shown notable results in clinical trials but has been associated with side effects forcing many patients to drop out[14,34]. Also, these effects limit the ability to fully explore thalidomide's potential benefits. We have tested a potential solution to eliminating systemic side effects with local targeted therapy by encapsulating the drug in artificial cell membranes of AC and APA. Delivery of the drug was successful and provided a means of decreasing TNF secretion in stimulated RAW 264.7 macrophage cells at two different LPS concentrations: 10μg/ml and 100μg/ml. APA membranes degrade slowly after transition from low to high pH as encountered from the stomach to the small intestine and seen in the simulated GI experiment. This profile enables drug release in a timed manner and thus increases total delivery time. These findings from previous studies [138] correlate to results shown here in which TNF secretion is lowered, beginning from macrophage stimulation onset, increases to levels near control values, and then is almost completely suppressed after 48 hours of incubation. Thalidomide effects are delayed for several hours due to the intranuclear site of action[58] of the drug. As it is steadily released, longer delivery time

could provide a more complete suppression of TNF-α. Conversely, degradation characteristics of AC membranes reveal a rapid loss of structure when submerged in a dynamic pH environment from low to high levels and thus deliver thalidomide shortly after capsules arrive in the small intestine[138]. Results observed with the AC capsules in this paper conclude a lowered TNF secretion level from RAW 264.7 cells in the presence of AC capsules. These levels are stabilized at values of 70% of control TNF secretion. This observation could be due to the sudden release of thalidomide after transition into the higher 7.4 pH solution of the cells. Because thalidomide was released as an entire bolus, long-term TNF secretion was not fully suppressed as it was with the timed delivery through the APA capsules. Also, encapsulated thalidomide did not have any effects upon the growth patterns of RAW 264.7 cells and thus would allow the normal proliferation of these cells in an *in vivo* model.

Thus, artificial cell encapsulation of thalidomide could be a possible solution to avoid side effects related to systemic drug delivery by locally delivering their contents within the GI tract. Differences in membrane construction between APA and AC capsules result in different degradation characteristics and thus different drug release mechanisms. As a result, TNF-α secretions are lowered with both of these capsule therapies but to different levels. Further development of polymer membranes for drug encapsulation is possible for the local delivery of small molecules to treat inflammation associated with Crohn's disease.

5.5 Acknowledgements

The authors would like to acknowledge the role of Dr. Devendre Amre in his support for this project. This project was funded in part by NSERC and CIHR.

Figures:

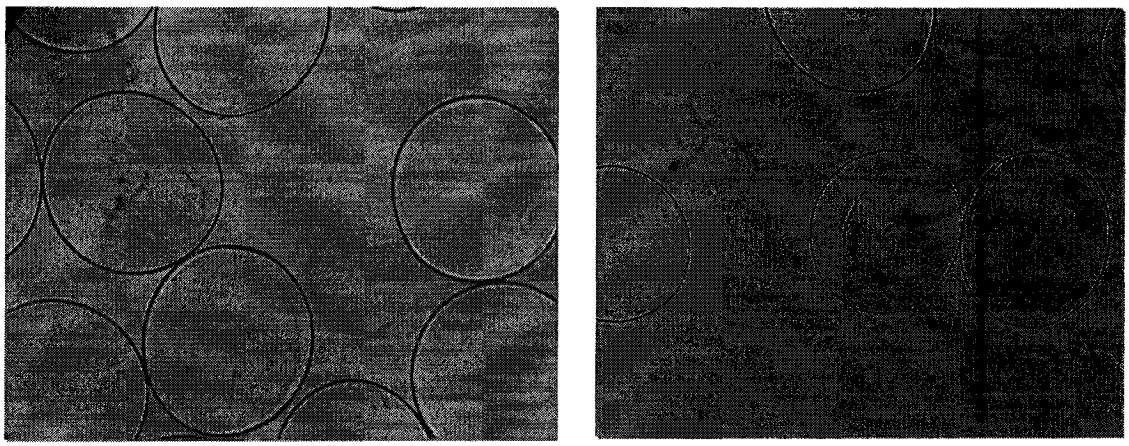


Figure 5.1: Experiment to visually analyze both APA and AC bead uniformity. Photomicrographs (250X) of APA (left) and AC (right) encapsulated thalidomide revealed stable and uniform spherical capsules.

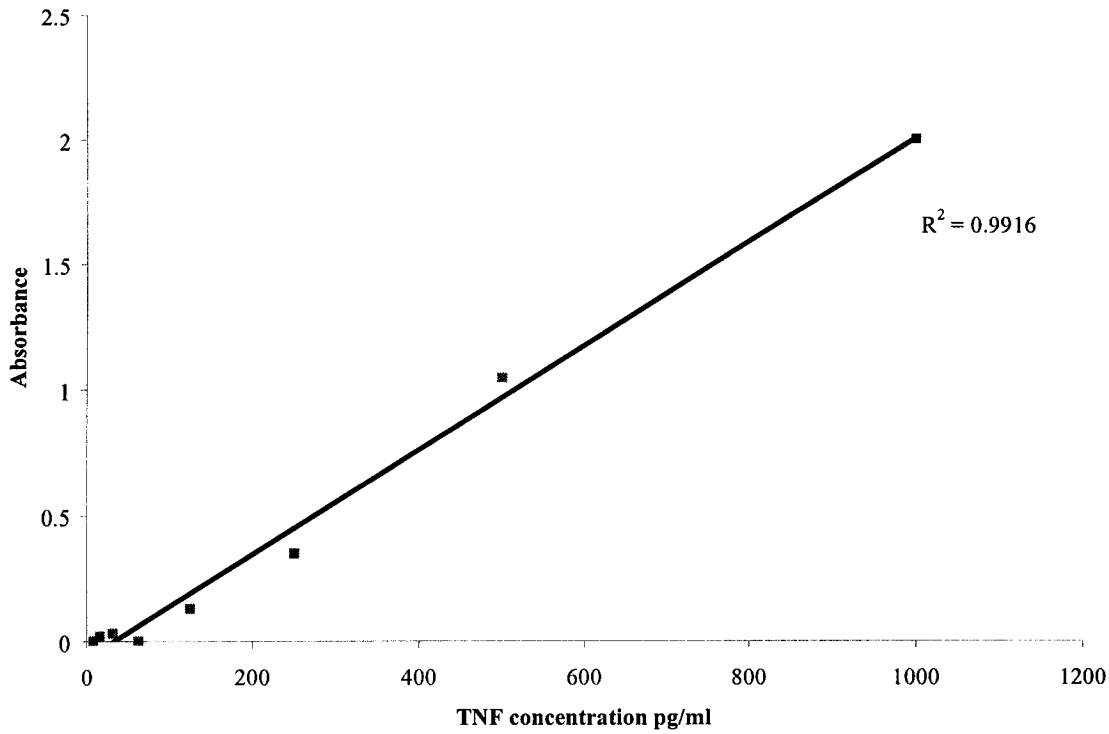
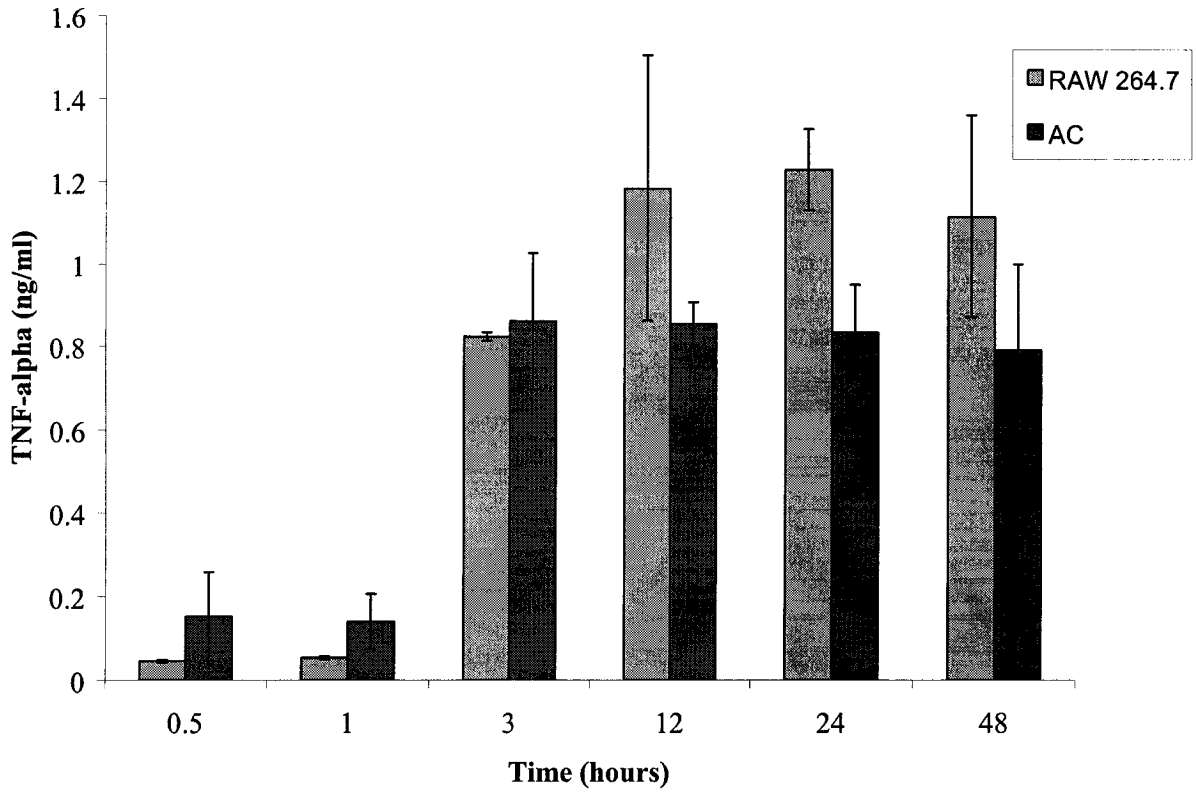
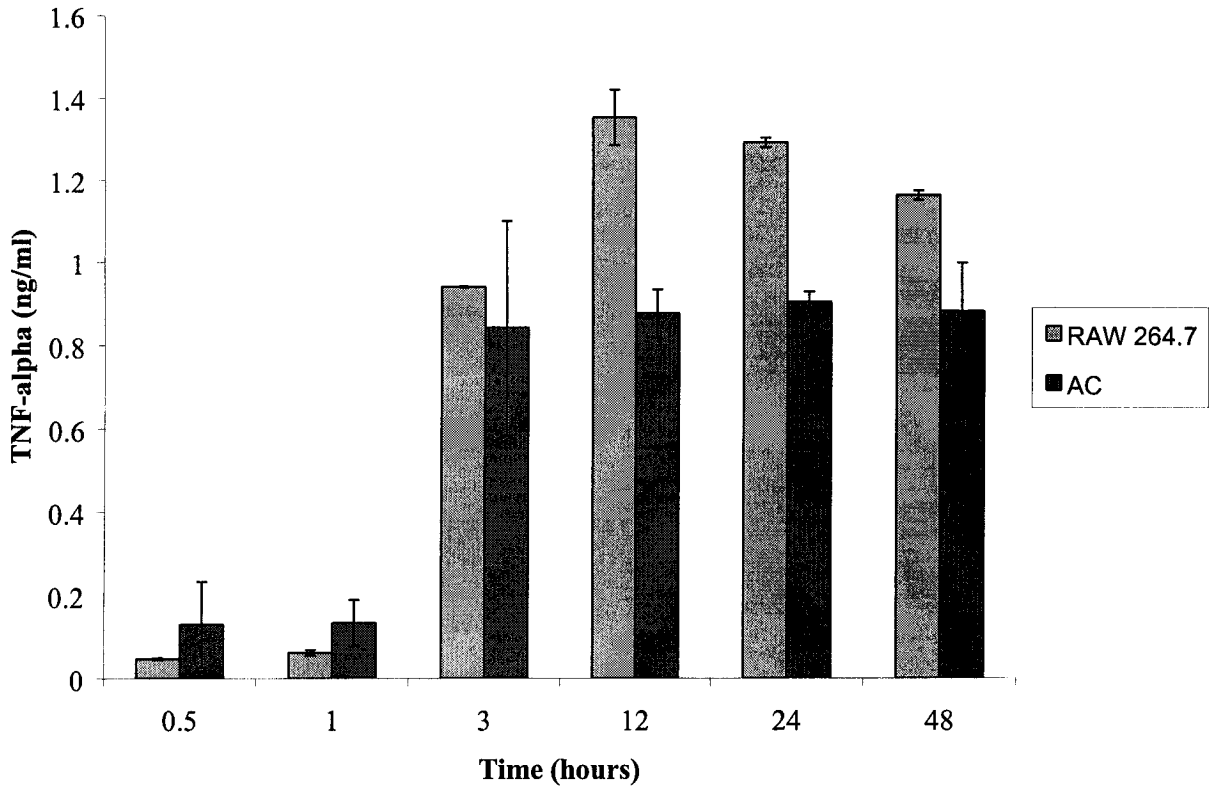


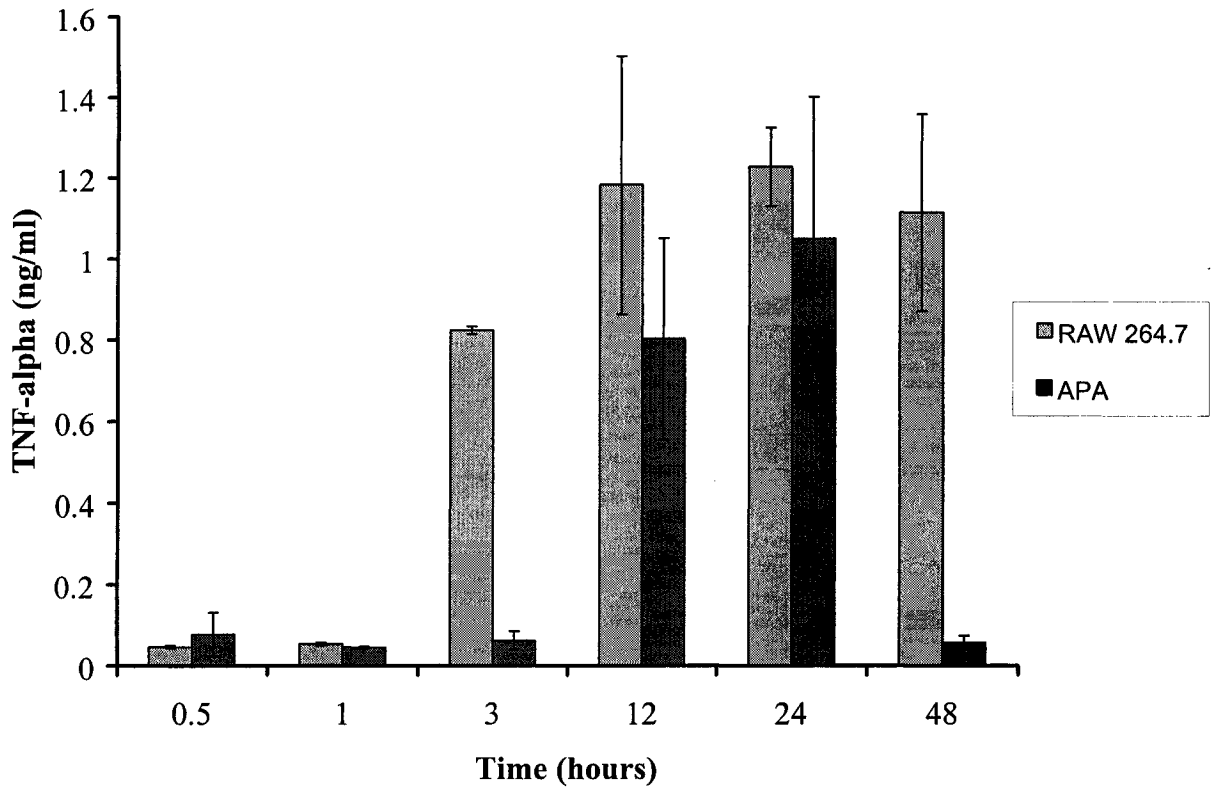
Figure 5.2: ELISA test standard. Absorbance versus TNF-α concentration graph for calculating a standard curve of TNF- α secretion from RAW 264.7 mouse macrophage cells.



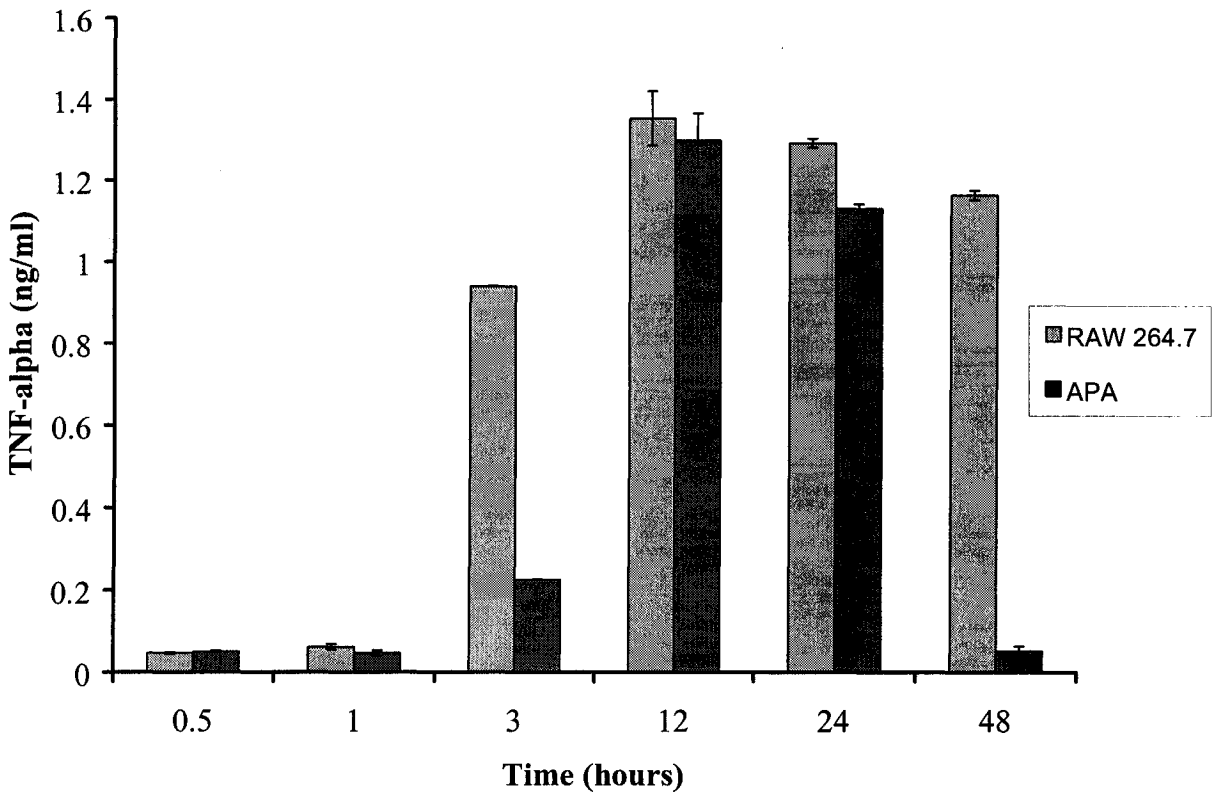
Figures 5.3a: Experiments to determine the effect of AC-delivered thalidomide on stimulated mouse macrophage TNF secretion. The concentration of TNF-α secretion from RAW 264.7 cells stimulated with 10 μg/ml of LPS was plotted in light blue. These values are compared to TNF-α secretion from stimulated 264.7 cells (with 10μg/ml) in the presence of AC encapsulated thalidomide, plotted in red. Comparisons were made after incubation times of 0.5, 1, 3, 12, 24, and 48 hours. Experiments were done in triplicate and error bars were calculated as one standard deviation using Microsoft's Excel calculator.



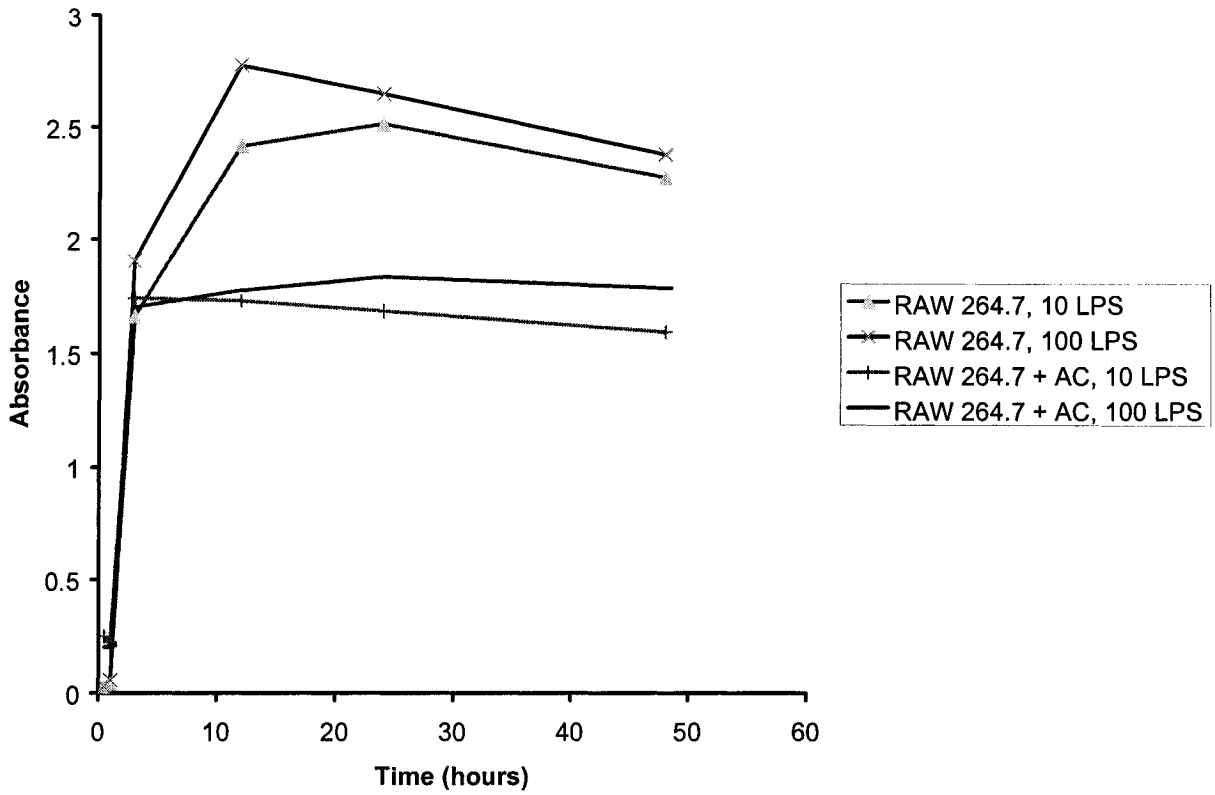
Figures 5.3b: Experiments to determine the effect of AC-delivered thalidomide on stimulated mouse macrophage TNF secretion. The concentration of TNF-α secretion from RAW 264.7 cells stimulated with 100 μg/ml of LPS was plotted in light blue. These values are compared to TNF-α secretion from stimulated 264.7 cells (with 100 μg/ml) in the presence of AC encapsulated thalidomide, plotted in red. Comparisons were made after incubation times of 0.5, 1, 3, 12, 24, and 48 hours. Experiments were done in triplicate and error bars were calculated as one standard deviation using Microsoft's Excel calculator.



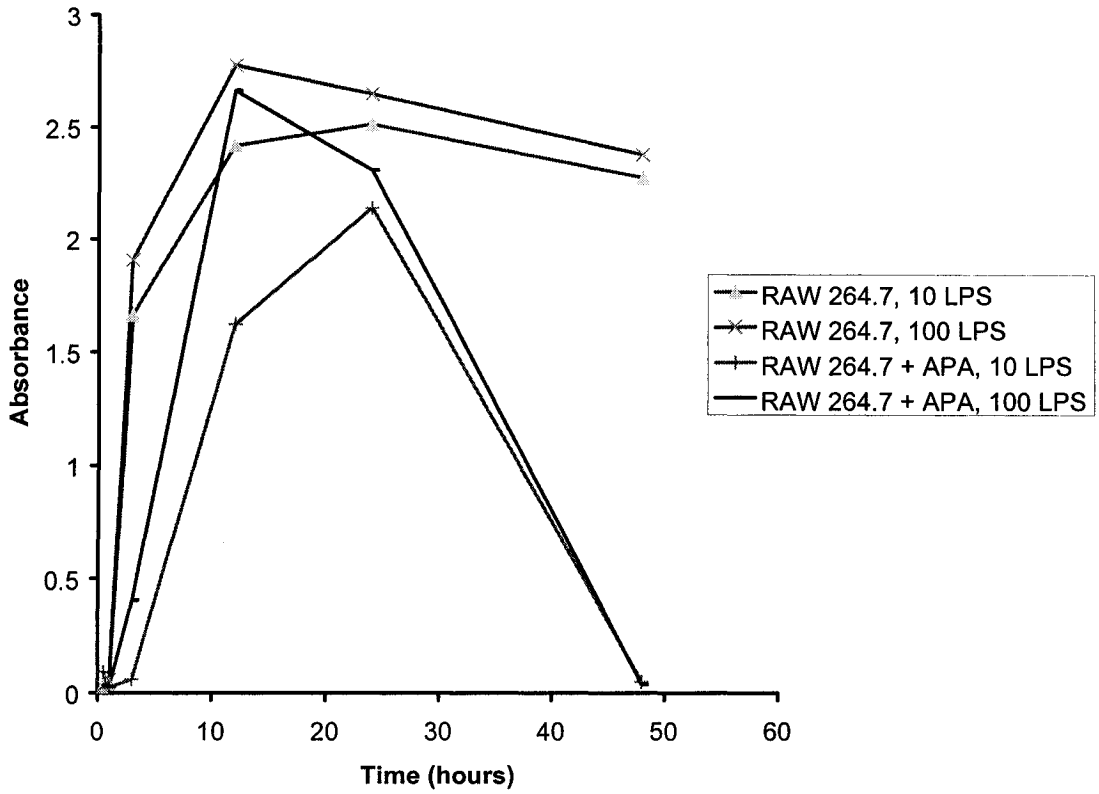
Figures 5.4a: Experiments to determine the effect of APA-delivered thalidomide on stimulated mouse macrophage TNF secretion. The concentration of TNF-α secretion from RAW 264.7 cells stimulated with 10 μg/ml of LPS was plotted in light blue. These values are compared to TNF-α secretion from stimulated 264.7 cells (with 10 μg/ml) in the presence of APA encapsulated thalidomide, plotted in red. Comparisons were made after incubation times of 0.5, 1, 3, 12, 24, and 48 hours. Experiments were done in triplicate and error bars were calculated as one standard deviation using Microsoft's Excel calculator.



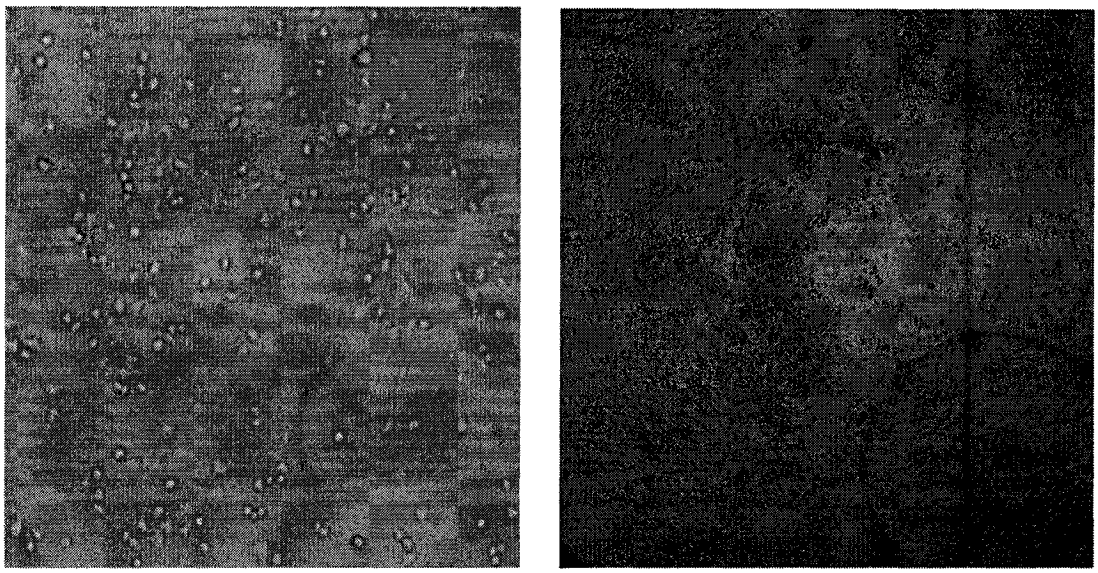
Figures 5.4b: Experiments to determine the effect of APA-delivered thalidomide on stimulated mouse macrophage TNF secretion. The concentration of TNF-α secretion from RAW 264.7 cells stimulated with 100 μg/ml of LPS was plotted in light blue. These values are compared to TNF-α secretion from stimulated 264.7 cells (with 100 μg/ml) in the presence of APA encapsulated thalidomide, plotted in red. Comparisons were made after incubation times of 0.5, 1, 3, 12, 24, and 48 hours. Experiments were done in triplicate and error bars were calculated as one standard deviation using Microsoft's Excel calculator.



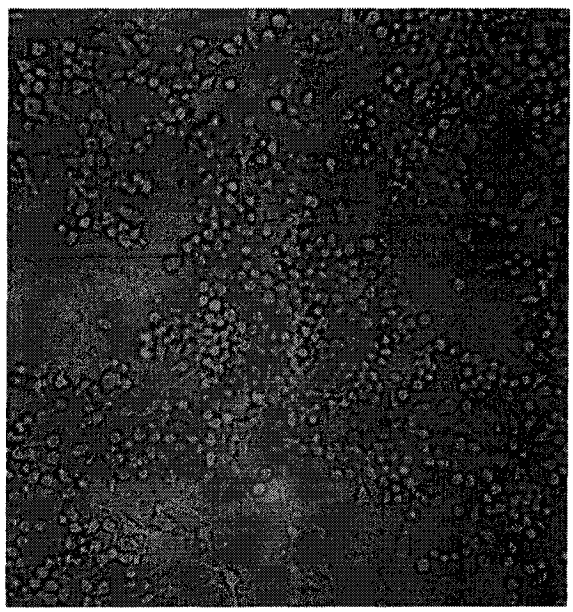
Figures 5.5a: Graph of stimulated mouse macrophage TNF secretion with and without AC-containing thalidomide. TNF-α secretion from RAW 264.7 cells in comparison to RAW 264.7 cells incubated with AC encapsulated thalidomide at time intervals from 0.5, 1, 3, 12, 24, and 48 hours. Black and blue lines in the graph represent cells stimulated with 100 μg/ml of LPS versus the green and red lines which represent cells stimulated with 10 μg/ml of LPS. Experiments were done in triplicate and standard deviations were calculated using Microsoft's Excel calculator.



Figures 5.5b: Graph of stimulated mouse macrophage TNF secretion with and without APA-containing thalidomide. TNF-α secretion from RAW 264.7 cells in comparison to RAW 264.7 cells incubated with APA encapsulated thalidomide at time intervals from 0.5, 1, 3, 12, 24, and 48 hours. Black and blue lines in the graph represent cells stimulated with 100 μg/ml of LPS versus the green and red lines which represent cells stimulated with 10 μg/ml of LPS. Experiments were done in triplicate and standard deviations were calculated using Microsoft's Excel calculator.



A B



C

Figure 5.6: Comparison photomicrographs of RAW 264.7 macrophage cells before (A) and after (B and C) exposure to encapsulated thalidomide.

Chapter 6

Conclusions, Summary, Claims to the Contribution of Knowledge, and Recommendations

6.1 Summary of Observations and Recommendations

1. For the targeted delivery of a drug for IBD therapy, microcapsules containing thalidomide were prepared. Alginate Chitosan (AC) and Alginate-Polylysine-Alginate (APA) membranes were designed and used for the above delivery applications.

2. Artificial microencapsulation of thalidomide within an AC membrane was successful and capsules were found to be stable in a static pH environment (Fig. 3.1). Despite the large molecular membrane permeability, thalidomide was maintained within the microcapsules. This is, as hypothesized, possibly due to the electrostatic force of the negatively charged alginate and the positively charged thalidomide.

2. The results show that AC capsules degrade in a burst-like manner when simulated gastrointestinal pH levels rise to levels between 4.77 and 6.18 (Fig. 3.3) but that 93% of AC microcapsules remained intact from increasing pH levels from 1.5 to 4.77. As much as 80% of capsules degrade by pH 6.15 and 100% of capsules have fully degraded by pH 7.5. Because of capsule bursting, 100% thalidomide drug release is detected almost immediately after pH transition by spectrophotometry as a strong absorbance peak of 1.15 at 220 nanometers.

3. Artificial microencapsulation of thalidomide within an APA membrane was successful and capsules were found to be stable in a static pH environment (Fig. 4.1). Despite the large molecular membrane permeability (60-76 kd), thalidomide was maintained within

the APA microcapsule due in part to the electrostatic force of the negatively charged alginate and the positively charged thalidomide.

4. The results show that APA capsules remain intact to larger pH values of up to 5.8. The capsules degrade at a much slower rate and maintain their core structure in the designed dynamic gastrointestinal model. Instead of fully degrading, the APA capsules were observed to thin or "ghost" and thus slowly release thalidomide in a time-dependant manner (fig. 4.4). At pH 6, roughly 50% or capsules had begun to thin and thus release thalidomide as seen in figure 4.2 as a plot of % non-ghost APA capsules remaining. Thalidomide release occurred in steps as it slowly leaked through the thinning membranes (fig. 4.3).

5. Results comparing AC and APA degradation revealed opposing mechanisms of drug release (fig. 4.6). With AC, thalidomide is released immediately. Thus, results suggest that oral alginate-chitosan capsule therapy could be of use when targeting Crohn's inflammation in the most proximal sections of the small intestine. Because nearly 100% of drug release was observed, lower drug concentration would be needed to have similar effects due to the direct delivery of microencapsulation.

6. Results comparing AC and APA degradation revealed opposing mechanisms of drug release (fig. 4.6). With APA, thalidomide release was released in timed-release mechanism. Thus, results suggest that the oral alginate-polylysine-alginate capsule therapy could be used to target inflammation in the medial aspect of the small intestine or

where disease has progressed further along the gastrointestinal tract. Because of this release, slightly more drug would need to be included in capsules.

7. Results from incubation of AC capsules containing thalidomide with 10 and 100μg/ml LPS-stimulated RAW 264.7 mouse macrophage cells showed that the designed therapy could lower TNF-α secretion levels and maintain these at values between 65 and 72% of control levels (fig. 5.3a and b). It is postulated that TNF-α levels did not decrease further due to the single dose bolus the stimulated cells received after the AC capsules burst.

8. Results from incubation of APA capsules containing thalidomide with 10 and 100μg/ml LPS-stimulated RAW 264.7 mouse macrophage cells showed that the designed therapy could lower and almost completely suppress TNF-α secretion levels and maintain these at values between 4 and 5% of control levels (fig. 5.4a and b). The action of thalidomide-loaded APA capsules is delayed but has more significant and longer lasting effects on lowering TNF-α secretion from macrophage cells presumably because of a longer lasting timed-release mechanism.

6.2 Conclusions

In the present project, we tested two novel treatment modalities, in several gastrointestinal environment simulations for the targeted drug delivery in the small intestine for the treatment of inflammation related to Crohn's disease. The therapies are based on the encapsulation of the drug thalidomide within two separated polymer artificial cell microcapsules:

alginate-chitosan and alginate-polylysine-alginate. Our hypothesis was that by encapsulating the drug, one can reduced the drug dosage and ensure its protection from absorbance in the stomach and thus avoid side effects associated with thalidomide's current delivery mechanism through the systemic circulation. This thesis project was designed and carried out to test and prove the above hypothesis. Specifically, our research objectives were to design artificial cell microcapsules containing thalidomide and optimize the encapsulation process and membrane molecular degradation, evaluate, the efficacy of the artificial microcapsule in delivering its content to the desired location of the gastrointestinal tract in a pH controlled gastrointestinal simulation *in-vitro* and to evaluate preclinical efficacy tests for diminished or lowered inflammation *in-vitro* in an experimental mouse macrophage intestinal model.

After reviewing the results obtained through our experiments, the following conclusions can be made:

1. Microencapsulation of thalidomide in alginate-chitosan and alginate-polylysine-alginate artificial cell microcapsules for the treatment of Crohn's disease was proposed and realized.

2. Both AC and APA microcapsules provide two separate oral delivery methods. The two capsules deliver thalidomide to lower Crohn's-related inflammation in the small intestine by targeting TNF-α secretion from intestinal macrophage cells. Each formulation for this therapy has the advantage of degrading at a specific site in the intestine and thus delivers thalidomide in a different manner.

3. TNF-α secretion from RAW 264.7 cells, a pathway known to initiate IBD/CD related inflammation, is suppressed by both alginate-chitosan and alginate-polylysine-alginate capsules containing thalidomide in-vitro in a gastrointestinal environment simulation indicating their IBD/CD therapeutic potentials.

4. This study shows the *in-vitro* feasibility of the approach. However, further *in-vitro* and *in-vivo* studies are required to establish this proposed therapy as an efficient way to treat inflammation associated with IBD and Crohn's diseases.

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