Development of novel artificial cell microcapsule for targeted delivery of thalidomide

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ABSTRACT

Crohn's disease is an inflammatory disease of the intestines that may affect any part of the gastrointestinal tract from mouth to anus. It's classified as one type of inflammatory bowel disease and causes a variety of symptoms including vomiting, diarrhea and abdominal pain. The aim of this thesis is to investigate the characteristics of a new delivery method for the oral administration of thalidomide in treating Crohn's disease. The suitability of Alginate-poly-L-lysine-alginate (APA) microcapsules for oral delivery of thalidomide is first evaluated in-vitro in a simulated gastrointestinal environment. The anti-inflammatory activities of microencapsulated thalidomide are then investigated on lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. Finally, the targeting delivery ability of APA microcapsules is tested in-vivo using a murine model of Crohn's disease. Results show that APA microcapsules enable a slow release of thalidomide in a pH-dependent manner. Moreover, this work demonstrates the efficiency of microencapsulated thalidomide in lowering the inflammation related to Crohn's disease, both in-vitro and in-vivo. However, further animal study is required to evaluate the full potential of this approach.

RESUME

La maladie de Crohn est une maladie inflammatoire chronique intestinale qui peut atteindre une ou plusieurs parties du tube digestif. Les symptômes de cette maladie inflammatoire se manifestent par des vomissements, des diarrhées chroniques et des douleurs abdominales. L'objectif de cette thèse est d'étudier les caractéristiques d'un nouveau mode de prestation pour l'administration orale de la thalidomide dans le traitement de l'inflammation reliée à la maladie de Crohn. Le potentiel des Alginate-poly-L-lysine-alginate (APA) microcapsules pour l'administration orale de la thalidomide est d'abord évaluée in-vitro en utilisant un modèle de simulation gastro-intestinal. Les activités anti-inflammatoires des microcapsules ont ensuite été examinés en utilisant des cellules RAW 264.7 stimulée avec du lipopolysaccharide (LPS). Enfin, la capacité de livraison de ciblage des microcapsules APA est testé in-vivo en utilisant un modèle animale de la maladie de Crohn. Les résultats montrent que les microcapsules APA permettent une libération lente de la thalidomide dépendente du pH de l'environnement extérieur. En outre, ce travail démontre le potentiel des microcapsules contenant de la thalidomide d'abaisser l'inflammation reliée à la maladie de Crohn à la fois in-vitro et in-vivo. Cependant, une étude plus approfondie des animaux est nécessaire pour pleinement évaluer le potentiel de cette approche.

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PREFACE

In accordance with the thesis preparation and submission guidelines, I have taken the option of writing the experimental section of this thesis as a compilation of original papers appropriate for publication.

This section is provided in the Thesis Preparation and Submission Guidelines, which reads as follows:

"As an alternative to the traditional thesis style, the research may be presented as a collection of papers of which the student is the author or co-author (i.e., the text of one or more manuscripts, submitted or to be submitted for publication, and/or published articles (not as reprints) but reformatted according to thesis requirements as described below). These papers must have a cohesive, unitary character making them a report of a single program of research."

The original papers presented in this thesis are divided into sections consisting of an Abstract, Introduction, Materials and Methods, Results and Discussion, and Conclusions. A common Abstract, Introduction, Literature Review, Summary of Results, Conclusions and References are included in this thesis in accordance to the guidelines.

LIST OF ABBREVIATIONS

DMSO	Dimethyl Sulfoxide
APA	Alginate-PLL-Alginate
PBS	Phosphate Buffer Solution
PLL	Poly-l-lysine
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H- tetrazolium),
LPS	Lipopolysaccharide
TNBS	2,5,6-trinitrobenzene sulfonic acid

Units

cm	Centimeter
g	Grams
pg	Picograms
Kg	Kilograms
mg	Milligrams
mL	Milliliter
nm	Nanometer
mM	Millimolar
h	Hours
S.D.	Standard deviation
U/g	Units per gram

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1.0. GENERAL INTRODUCTION

1.1. Overview

Crohn's disease is a type of inflammatory bowel disease (IBD) that affects any part of the gastrointestinal tract, form the mouth to the anus. Between 400 000 and 600 000 people in North America have this disease, males and females being equally affected [1]. Although the exact cause of Crohn's disease is not well known, genetic and environmental factors seem to play a crucial role in the predisposition of this disease [2, 3]. Crohn's disease is thought to be an autoimmune disease, with inflammation stimulated by an overactive T-helper 1 (Th1) response, leading to the production of pro-inflammatory cytokines. It has been shown that an enhanced secretion of the pro-inflammatory cytokines TNF-α, IL-6 and IL-1β is observed in patients affected by Crohn's disease [4].

Crohn's disease cannot be cured by surgery, and most anti-inflammatory drugs and immunomodulators currently available on the market show limited clinical efficiency in the remmision of this disease. One promising drug that has been successfully used in the remission of Crohn's disease is thalidomide. This drug has antiangiogenic activities and can inhibit inflammatory cytokines that are up-regulated in Crohn's disease [5]. However, it has been shown that thalidomide can cause drowsiness, hypertension, skin rash, oedema and neutropenia [6]. Therefore, an appropriate delivery platform must be introduced to limit the major side-effects associated with thalidomide.

In this thesis, artificial cell microcapsules have been designed and investigated for their clinical efficiency. Alginate-Poly-L-Lysine-Alginate (APA) microcapsules can be used for the delivery and targeting of thalidomide to desired locations of the Gastrointestinal Tract, thus optimizing clinical efficiency.

1.2. Thesis Hypothesis

Novel artificial cells loaded with sufficient amount of thalidomide will selectively target areas of the gastrointestinal tract affected by Crohn's disease, allowing effective thalidomide delivery and lowering intestinal inflammation with minimal associated side effects.

1.3. Thesis Research Objectives

The thesis research objectives are to:

i. Design and prepare artificial cells containing thalidomide and evaluate their stability in a simulated GI model,
ii. Analyze the anti-inflammatory effects of artificial microcapsules containing thalidomide in-vitro using cell lines, and
iii. Investigate the efficacy of APA microcapsules containing thalidomide in lowering intestinal inflammation in a murine model of Crohn's disease.

1.4. Thesis Outline

This thesis is divided into 6 chapters. Chapter 1 describes the background and research objectives of this thesis. Chapter 2 consists of an extensive literature review of the subject matter while Chapters 3-5 are the collections of original papers ready to be submitted. These research papers talk about the main studies performed to achieve the objectives discussed above. Chapter 6 is a general discussion of the present study. Finally, Chapter 7 summarizes the findings of the present study and provides future recommendations.

CHAPTER 2: BACKGROUND AND LITERATURE

2.1. Introduction and current research objectives

Inflammatory bowel disease (IBD) is defined as a chronic intestinal inflammation that results from host–microbial interactions in a genetically susceptible individual [7]. IBD is classified into two major types of diseases: Crohn's disease (CD) and ulcerative colitis (UC) [8]. In Crohn's disease, inflammation can affect any part of the Gastrointestinal Tract, while ulcerative colitis is characterized with an inflammation localized at the large intestine. The prevalence of Crohn's disease is relatively high in highly industrialized countries. Medical treatment of Crohn's disease and other inflammatory diseases is strongly dependent on the use of immunosuppressive drugs and anti-inflammatory compounds [9]. Although immunosuppressive therapies such as azathioprine, mercaptopurine, and methotrexate are currently available, remission of Crohn's disease in clinical patients remains a challenge [10]. This class of therapeutic is very efficient in reducing the extent of inflammation, but presents a wide range of side effects. For instance, administration of such pharmaceutical compounds can cause fluid retention, insomnia, weight gain, drowsiness, hypertension, constipation, and vomiting. Therefore, in the ongoing research for additional therapeutics, this work focuses on the development of an artificial cell formulation containing thalidomide to treat Crohn's disease. Thalidomide is an anti-inflammatory drug that significantly reduces the amount of pro-inflammatory cytokines, thus dampening the inflammation associated with Crohn's disease. The characteristics and effectiveness of this formulation is explored in details both in vitro, in a simulated dynamic gastrointestinal model, and in-vivo using experimental animals.

2.2. Inflammation and Crohn's disease

T helper cells are a sub-class of lymphocytes that play a key role in mediating an immune response. They are involved in activating other immune cells and in determining the specificity of antibodies secreted by B cells.

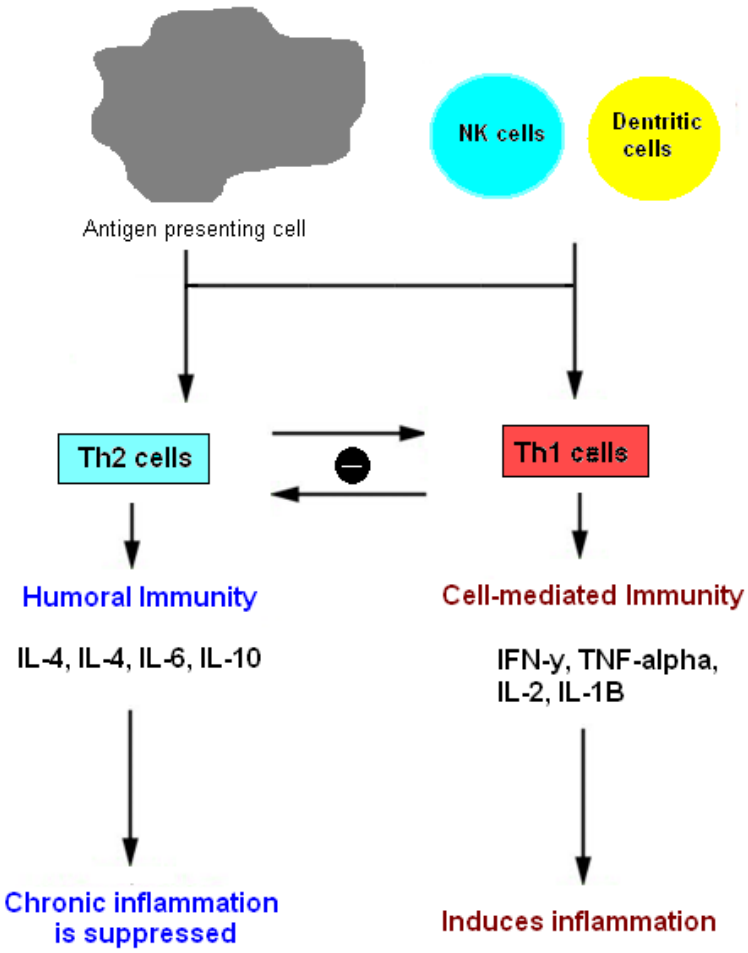

Figure 2.1: Factors determining T-lymphocytes differentiation in Crohn's disease.

Upon proliferation, a helper T cell differentiates into Th1 and Th2 cells. Th1 cells mediate the production of pro-inflammatory cytokines such as IFN-γ, TNF-α and IL-2, part of the cell mediated immunity. Th2 cells lead to the production of anti-inflammatory cytokines including IL-4, IL-5, IL-6, and IL-10 which constitutes the humoral immune

response. There is a mutual interaction between Th1 and Th2 lymphocytes: Th1 cytokines suppress the proliferation of Th2 cytokines and vice versa. In a normal situation, there is a balance between the amount of Th2 and Th1 cells [11]. The development of Crohn's disease is strongly related to a polarized type-1 immune response that causes chronic gut inflammation [12]. Administration of anti-inflammatory drugs will protect the individual from Crohn's disease by attenuating the Th1 response and enhancing the Th2-mediated activity. This immune modulation will cause the switch of the immune response from a Th1 to a Th2-mediated activity, thus significantly reducing the extent of bowel inflammation [13, 14].

2.3. Introduction to the Gastrointestinal Barrier

Being able to understand the properties and characteristics of the gastrointestinal tract can help clarify the bio-molecular mechanisms of inflammatory bowel diseases. The gastrointestinal tract consists of four different layers that reflect specialization in functional anatomy: Mucosa, submucosa, muscularis externa and adventitia. The mucosa is an inner layer of the gastrointestinal tract that plays a very important role in the digestion of food. It forms a barrier between the internal organs and the lumen of the gastrointestinal tract. It mainly consists of epithelial cells that are connected by tight junctions. The role of these tight junctions is to efficiently allow the transport of nutrients across the epithelium, while preventing the passage of molecules that might be harmful to the host [15]. These epithelial linings also contain goblet cells and endocrine cells [16]. Goblet cells sole function is to secrete mucin, which forms mucus when dissolved in water. Endocrine cells, which are commonly found in the epithelium wall, secrete a type of hormone that plays a crucial role in the regulation of digestive processes. External

microorganisms that are able to breach the epithelial wall will have unrestricted access to the circulatory system, which is one of the main causes of gastrointestinal diseases. Indeed, murine and human studies have demonstrated that Crohn's disease is characterized by a defect in the epithelial barrier and an alteration in mucus production, leading to an increase in intestinal permeability and in toxins adherence [17, 18].

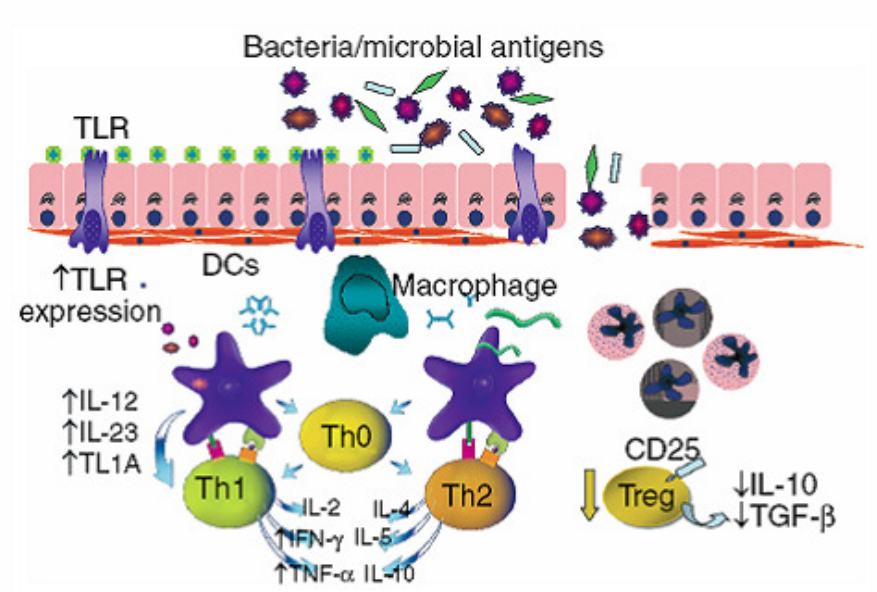

Figure 2.2: Triggering of immune response in Crohn's disease [19].

As illustrated in Figure 2.2, Crohn's disease is characterized by a defect in epithelial barrier integrity, resulting in the translocation of microbial antigens and other external agents [19]. Several studies have demonstrated that there is an increase in adherent bacteria in patients with Crohn's disease [17]. Exposure of the mucosal immune system to external luminal contents will significantly increase the production of pro-inflammatory cytokines and favor a differentiation of T cells to Th1-type effector cells.

2.4. The role of TNF-α in Crohn's disease

TNF-α is a pro-inflammatory cytokine that plays a major role in the inflammation caused by Crohn's disease [20]. Intestinal inflammation is characterized by a significant increase in TNF-α. Indeed, clinical studies have demonstrated that the level of TNF-α in serum was found to be elevated in patients affected by Crohn's disease [4]. It can be found in higher amount in both histologically normal mucosa and inflamed mucosa [21, 22]. An increase in TNF-α induces cell proliferation and differentiation [23], and leads to an up-regulation of adhesion molecules on endothelial cells [24]. TNF-α binds on its receptor, TNF-R1 and TNF-R2, which can be found in most cells of the immune system. TNF-α is also involved in the following pathways, that play a major role in intestinal inflammation.

a) TNF-α can activate the NFkB pathway

First of all, TNF-α can activate the classical NFkB pathway, leading to the production of more pro-inflammatory cytokines. The transcription of NF-Kappa B is crucial for several biological processes including inflammation, apoptosis, cell survival and differentiation [9]. IkBα is an inhibitory protein which normally binds to NF-KappaB and inhibits its translocation into the nucleus, thus preventing its transcription. However, in the presence of TNF-α, The IκB (IKK) complex is recruited. This kinase can phosphorylate the inhibitory proteins of NF-kappaB and mark them for polyubiquitination, leading to their subsequent degradation and translocation of NF-kappaB [25].

b) TNF-α plays a major role in the activation of the MAPK pathways

Indeed, the activation of the MAPK (Mitogen-activated protein kinases) pathway is strongly dependent on the presence of TNF-α. MAPK are serine/threonine-specific protein kinases that are sensitive to several external stimuli, such as heat shock, osmotic stress and pro-inflammatory cytokines [26]. The MAPK pathway eventually leads to the translocation of JNK to the nucleus, which plays a crucial role in various biological activities, including cell proliferation, apoptosis, mitosis and gene expression [27].

c) TNF-α is involved in death signaling

Various members of the TNF family have been shown to regulate apoptosis, a process of programmed cell death [28]. The binding of TNF-α to its receptor will lead to the recruitment and auto-proteolytic activation of caspase 8. This caspase protein amplifies the apoptotic cascade and promotes cell death by mediating the cleaving of effector caspases [29]. Current research tries to focus on designing an anti-TNF-α therapy in order to efficiently dampen the inflammation related to Crohn's disease in clinical patients.

2.5. Types and symptoms of Crohn's disease

There exist five different subtypes of Crohn's disease which are classified according to the area of the gastrointestinal tract where the inflammation takes place.

a) Gastroduodenal Crohn's disease mainly affects the stomach and the duodenum. This type of disease is found endoscopically in 20% [30] and up to 40% of cases histologically [31, 32]. The most frequent symptoms include dysphagia, odynophagia, nausea, anorexia, epigastric pain and dyspepsia [33].

b) Jejunoileitis Crohn's disease: This disease affects the jejunum, which is the longest portion of the small intestine located between the duodenum and the ileum. It's one of the most difficult locations in the gastrointestinal tract to treat, and patients may experience intestinal obstruction [34].

c) Ileitis: This disease affects the ileum, which is the lowest part of the small intestine, as illustrated in figure 2.3. The patient affected by this disease has a higher risk of developing anemia.

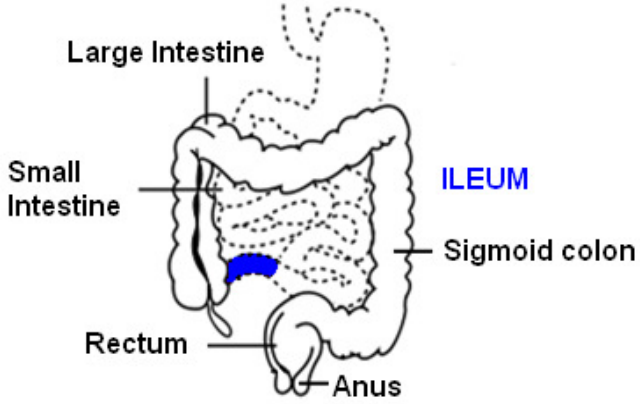

Figure 2.3: Areas of the gastrointestinal tract affected by Ileitis Crohn's disease.

This disease accounts for 20% of the cases. Symptoms include diarrhea, cramping or pain in the abdominal region.

d) Ileocolic Crohn's disease: It's the most common type of disease which accounts for fifty percent of the cases. As represented in figure 2.4, it affects both the ileum, which is the lowest part of the small intestine, and the colon.

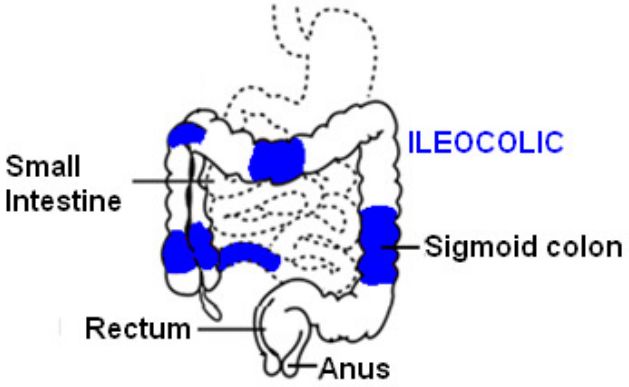

Figure 2.4: Areas of the gastrointestinal tract affected by Ileocolic Crohn's disease.

e) Granulomatous Colitis

Granulomatous Colitis is a disease which affects the colon. As illustrated in Figure 2.5, the disease is not spread entirely over the colon. There are often areas of healthy tissues between areas of diseased tissues.

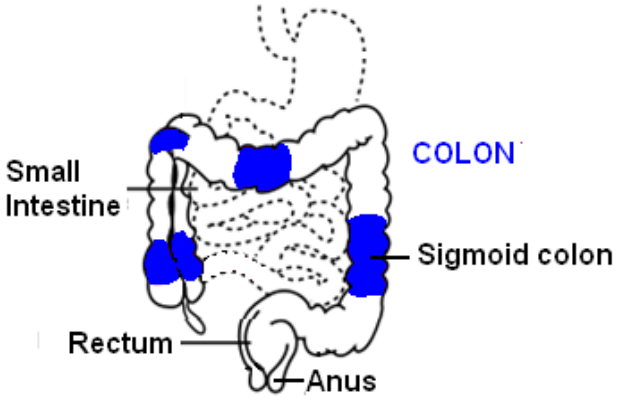

Figure 2.5: Areas of the gastrointestinal tract affected by Crohn's Colitis.

The main gross alterations found in granulomatous colitis are inflammation, edema and thickening of the tissues underlying the affected bowel.

2.6. Diagnosis of Crohn's disease

Several techniques and medical tests are currently available to help the physician provide an effective diagnosis of Crohn's disease. The use of capsule endoscopy is one of the best tests for making an effective diagnosis of Crohn's disease. The capsule, which is of the size of a pill, can take pictures of the inside of the gastrointestinal tract when swallowed by a patient. These endoscopic images can localize small erosion and ulcer along the gastrointestinal tract and help physicians identify the type of Crohn's disease [35]. An alternative to endoscopy is the use of radiology tests that can aid in the diagnosis of the disease. A barium follow-through procedure is a useful medical imaging technique used when only the small intestine is involved in Crohn's disease [36]. In this technique, the patient drinks a solution containing barium-sulfate that appears white on X-rays, displaying the internal lining of the bowel. X-ray computed tomography (CT) and Magnetic resonance imaging (MRI) scans are also commonly used for looking at intra-abdominal complications of Crohn's disease such as small bowel obstruction, abscesses or fistulae [37]. Diagnosis of Crohn's disease can also be done by testing blood samples of patients. Laboratory blood tests may show elevated sedimentation rates and white cell counts, both of which are associated with intestinal inflammation. Complete blood count from patients affected with Crohn's disease may reveal anemia, caused by vitamin B12 deficiency and autoimmune hemolysis [38]. Moreover, increasing amounts and levels of serological antibodies including ASCA, anti-laminaribioside, anti-chitobioside, anti-mannobioside, anti-Laminarin and anti-Chitin may be useful in the prognosis of Crohn's disease [39, 40].

2.7. Causes of Crohn's disease

a) Genetic factors

The exact cause of Crohn's disease is not well known, but its appearance seems to be strongly dependent on genetic factors. Several factors such as bacterial contamination, change in the immune system and genetic variations, appear to be strongly correlated to Crohn's disease. For instance, a mutation in NOD2 gene is associated with an increase susceptibility to Crohn's disease [41]. Indeed, wild-type NOD2 can activate the nuclear factor NF-kB, leading to the production of pro-inflammatory cytokines. Furthermore, the XBP1 gene has recently been linked to the unfolded protein response pathway of the endoplasmic reticulum, which is characteristic of inflammatory bowel diseases [42, 43].

b) Environmental factors

While genetic predisposition plays a key role in immune-mediated diseases, the major influence appears to be due to environmental factors. When individual moves from a low prevalence to a high prevalence area, their children have the same risk of developing autoimmune diseases as people born in extremely hygienic countries. While the environment present in highly industrialized countries produces a risk for immune-mediated diseases such as Crohn's disease, environmental factors in less-developed regions protects the host system from such diseases. Indeed, current research suggests that autoimmune diseases, such as Crohn's disease, are most prevalent in highly industrialized nations but very rare in less developed countries [2]. Diet seems to be strongly dependent to its higher prevalence in industrialized regions. Studies have shown that increased consumption of milk protein, animal protein and polyunsaturated fatty acids can increase the risk of getting inflammatory bowel diseases [44]. Furthermore, it has

been suggested that smoking is also involved in the development of Crohn's disease. This strong association could be due to tobacco's influence on mucus permeability [19].

2.8. Crohn's disease, available treatments and their limitations

a) Background information on available treatments

There is no known pharmaceutical or surgical cure for the treatment of Crohn's disease. Treatment of this disease involves the use of anti-inflammatory drugs that significantly reduce the symptoms of the disease and help maintain its remission. Medications used to treat the symptoms of Crohn's disease include anti-inflammatory drugs such as 5-aminosalicylic acid (5-ASA) [45], and immunomodulators such as azathioprine, mercaptopurine, methotrexate, infliximab, adalimumab, certolizumab and natalizumab. These pharmaceutical compounds will regulate the immune system by efficiently triggering a Th2-mediated response that dampens Th1-mediated inflammation. This will result in the production of anti-inflammatory cytokines such as IL-4, IL-5, IL-6 and IL-10 that inhibits the production of pro-inflammatory cytokines. Oral delivery of such as compounds has shown to be successful against intestinal inflammation [46], but presents several limitations when administered in high dose. These limitations are mainly due to the fact that most of the administered drug is delivered to non-specific cells of the body.

b) Lifestyle changes

A change in lifestyle can help reduce the symptoms of Crohn's disease and significantly improve the quality of life of patients. This includes regular exercise and

enough sleep to avoid fatigue. Moreover, a proper adjustment in the diet can help the patient manage the symptoms of Crohn's disease. For instance, eating small, frequent meals, instead of a few big ones can help control the symptoms. Finally, avoiding alcohol and smoking can make the symptoms of Crohn's disease go away, since tobacco is directly involved in mucus production and epithelial barrier integrity [47].

c) Use of monoclonal antibodies

Anti-TNF-α antibodies are frequently used for the treatment of Crohn's disease because they are able to efficiently reduce the amount of TNF-α in the body [48]. Recent studies suggest that infliximab is a potential antibody to treat Crohn's disease since it neutralizes TNF-α by preventing it from interacting with its receptor [49, 50, 51]. Infliximab is a monoclonal antibody against TNF-α, and was approved by the FDA for the treatment of Crohn's disease in August 1998. The complementarities between TNF-α molecule and the variable region of infliximab make this antibody a promising treatment for Crohn's disease and other autoimmune diseases. Other antibodies, such as adalimumab and certolizumab, have shown significant positive results in the treatment of inflammatory bowel diseases [52]. The major limitation associated with infliximab is that patients can develop problems with tuberculosis as a result of TNF-α neutralization [53]. Delayed hypersensitivity reactions are also frequently observed following administration of infliximab [54]. Moreover, infliximab cannot be administered orally, since the digestive system would destroy it. Administration of infliximab must be done by intravenous infusion at 6-8 week intervals. Therefore, it's more suitable to eliminate this method and treat Crohn's disease using oral drug delivery.

2.9. Introduction to thalidomide and its potential in Crohn's disease

Thalidomide was first manufactured by a German pharmaceutical company Grünenthal and found to be an effective sedative and tranquilizer for insomnia, colds and headaches. It was introduced in the market in 1957 in several countries as an immunomodulatory agent to treat diseases such as multiple myeloma. However, it was withdrawn from the market in 1961 after being found to be associated with severe birth defects when taken during pregnancy [55]. The use of thalidomide for the treatment of the lesions associated with erythema nodosum leprosum (ENL) was approved in July 1988 by the FDA. The drug can now be administered to patients in the United States under strict guidelines in accordance to the STEPS program (System for Thalidomide Education and Prescribing Safety). The systematic name of thalidomide is (*RS*)-2-(2,6-dioxopiperidin-3-yl)-1*H*-isoindole-1,3(2*H*)-dione. As illustrated in figure 2.6, thalidomide is a tricyclic molecule. Its half-life in blood plasma ranges from 5 to 7 hours following administration of a single dose. Thalidomide is often solubilized in dimethyl sulfoxide (DMSO) since its solubility is high in this organic solvent. Because thalidomide has an absorbance at 220 nanometers, spectrophotometry can be used to detect the concentration of this drug in solution.

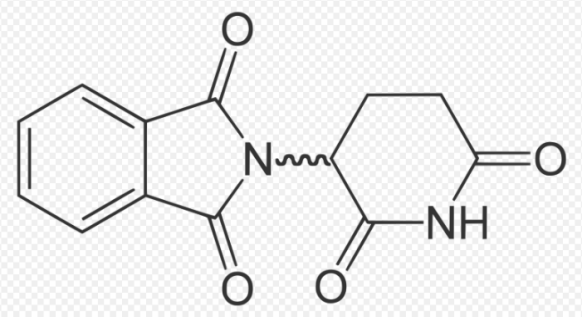

Figure 2.6: Structure of thalidomide.

The mechanisms of action of thalidomide are not fully understood. This drug has antiangiogenic effects and can inhibit inflammatory cytokines [5]. It also stimulates the production of cytotoxic T-cells, resulting in an increase in the number of T-lymphocytes [56]. Thalidomide is involved in the pathophysiology of Crohn's disease since it suppresses all major cytokines that participates in intestinal inflammation. The production of pro-inflammatory cytokines as seen in Crohn's disease mainly depends on the activity of NF-kB. As previously discussed, the activation of this transcription factor is tightly regulated [57, 58]. Several external stimuli, including some tumor necrosis factor receptors (TNFR), can activate the NF-kB pathway [57]. When translocated inside the nucleus, NFKB is responsible for secretion of several pro-inflammatory cytokines such as IL-2, TNF-α and IFN-γ. This translocation inside the nucleus depends on the action of the IKB kinase. It was shown that thalidomide is able to suppress the activity of IKB kinase, leading to the inhibition of NFkB transcription [59]. Moreover, thalidomide destabilizes TNF-α mRNA, leading to a significant decrease in the amount of the TNF-α protein, a pro-inflammatory cytokine responsible for Crohn's disease inflammation [60, 61]. Although thalidomide is beneficial in dampening the production of pro-inflammatory cytokines, it presents several limitations. Such limitations include drowsiness, hypertension, skin rash, oedema and neutropenia [6]. In order to minimize the side effects of thalidomide, an appropriate delivery platform must be used to successfully deliver this drug to affected parts of the gastrointestinal tract. This research focuses on the development of a new delivery method using artificial microcapsules.

2.10. Introduction of microencapsulation and its potential in targeted delivery of thalidomide in Crohn's disease

Microencapsulation is a promising tool in scientific research that allows the delivery of pharmaceutical compounds at specific tissues in the body, in a time dependent fashion [62]. An artificial cell involves the preparation of an artificial structure of cellular dimensions using different types of polymers and proteins. It can contain a variety of molecular structures including DNA, drugs, enzymes, antibodies, bacteria cells, mammalian cells and other microorganisms [63]. Alginates are certainly the most frequently employed polymer for microencapsulation due to their abundance, easy gelling characteristics and apparent biocompatibility. Although the suitability of other biomaterials is under investigation, none has reached the same level of performance as alginates [64]. APA microcapsules are formed by the ionic interactions between negatively charged alginate molecules and positively charged calcium ions. Furthermore, the membrane coating in an APA microcapsule is held by electrostatic and chemical interactions between alginate and Poly-L-Lysine layers.

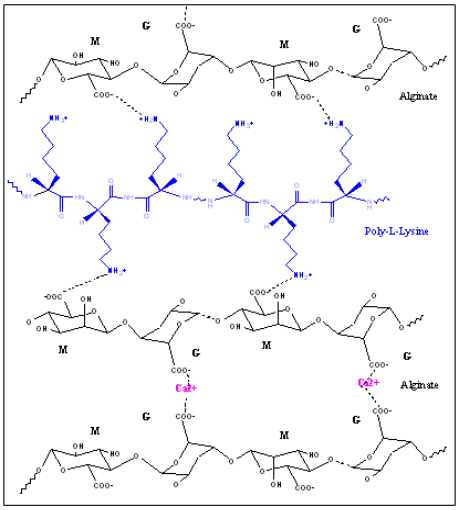

Figure 2.7: Molecular structure of the Alginate-Poly-L-Lysine-alginate formulation.

Poly-L-Lysine is a polymer of lysine units, an essential amino acid. It's used as a control of membrane porosity and protection from external environment. Several publications suggest the use of APA microcapsules as an efficient platform for the delivery of pharmaceutical compounds [62]. Preliminary data suggest that APA capsules remain intact in low pH environment normally encountered in the stomach [65]. In the small intestine, the capsules degrade at a slow rate and release its content in a time-dependant manner. By enabling successful delivery of thalidomide to specific sites of the Gastrointestinal Tract, APA microcapsules will limit the major side effects associated with thalidomide.

2.11. In-vitro and in-vivo models for studying Crohn's disease

a) *In-vitro* model of Crohn's disease

Crohn's disease can be modeled by the use of appropriate mammalian cells that expresses the inflammatory markers when grown in culture. The appearance of this disease in individuals is largely due to environmental and genetic factors that elicit an immune response leading to increased pro-inflammatory cytokine production. The lipopolysacharide (LPS) layer of bacteria have shown to efficiently create a strong immune response that result in an increased production of pro-inflammatory cytokines by cells in the intestinal epithelial, submucosal and endothelial layers [66]. Upon stimulation of monocytes and macrophages by LPS, an increased production of pro-inflammatory cytokines including TNF-α and IL-1 ensues [15]. In this study, RAW 264.7 mouse macrophage cells derived from Balb/c mice are used to mimic intestinal inflammation. LPS-activated RAW 264.7 macrophages cells have shown to produce pro-inflammatory

cytokines and can thus be used to closely mimic the inflammation related to Crohn's disease [62].

b) *In-vivo* model of Crohn's disease

Crohn's disease is an inflammatory disease that affects any part of the gastrointestinal tract, from the mouth to the anus. Several animal models, including chemically-induced and genetically modified, have been developed in order to study the different characteristics of the disease pathogenesis [67]. Murine model are ideal for studying Crohn's disease and mimicking gut inflammation with regard to disease location and clinical response to therapy. The use of genetically modified mice requires the understanding and proper use of transgenic and knockout methodologies. Transgenic technology is a technique in which foreign genes are introduced into the germ line of a mouse while gene knockout refers to the "silencing" of a gene using appropriate genetic techniques [68]. Examples of genetic models include the IL-2, T-cell receptor (TCR) a/b, IL-10 and knockout models [67]. These genetic models can greatly contribute in understanding the key biochemical and immunological pathways involved in the inflammation associated with Crohn's disease.

The chemically-induced group of animals requires the administration of an external chemical agent for induction of intestinal inflammation, including trinitrobenzene sulfonic acid (TNBS), dextran sodium sulfate (DSS), and oxazolone [67]. The advantage of using these models is their relatively low cost compared to genetically modified animals while sharing similar biochemical and immunological pathologies as seen in Crohn's disease. This research focuses on the use on chemically-induced model for the study and treatment of Crohn's disease. In order to induce intestinal inflammation, TNBS will be

administered to Balb/c mice by anal injection. Acute inflammation in response to TNBS administration seems to be associated with increased levels of TNF-α and other pro-inflammatory cytokines such as IFN-γ in this strain of mice [69].

PREFACE FOR CHAPTERS 3-5:

In order to evaluate the suitability of microencapsulated thalidomide for the treatment of Crohn's disease, artificial cells were designed using an Alginate-Poly-L-Lysine-Alginate (APA) membrane. Chapter 3 describes the design and preparation of APA microcapsules containing thalidomide. Moreover, Chapter 3 investigates the characteristics of APA microcapsules containing thalidomide. In-vitro experiments are conducted in the proposed paper to better understand the drug release profile using a simulated gastrointestinal model.

The objective of Chapter 4 is to further understand the anti-inflammatory properties of artificial cells containing thalidomide. In this study, RAW 264.7 mouse macrophage cells derived are used to mimic intestinal inflammation. Activation of RAW 264.7 cells with Lipopolysacharides (LPS) will stimulate the production of pro-inflammatory cytokines, thus closely mimicking intestinal inflammation as seen in Crohn's disease.

Chapter 5 investigates the potential of this approach in an experimental murine model of Crohn's disease. In this paper, 2,4,6-trinitrobenzene sulfonic acid (TNBS) is used to induce Crohn's disease in Balb/c mice. After treating the mice with APA microcapsules containing thalidomide, several parameters are measured to assess the extent inflammation.

Chapter 3

Artificial cell microcapsule for oral delivery of thalidomide: design, preparation, and in-vitro characterization for Crohn's disease application

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PREFACE FOR CHAPTER 3

Preliminary data have shown that alginate-poly-L-lysine-alginate (APA) microcapsules could successfully enable the delivery of thalidomide in a pH-dependent fashion [30]. In this work APA microcapsules containing thalidomide were prepared and characterized in-vitro. Moreover, simulated intestinal fluid (SIF) and simulated gastric fluid (SGF) were prepared to mimic the gastrointestinal tract and to evaluate the drug delivery potential of APA microcapsules.

Chapter 3: Artificial cell microcapsule for oral delivery of thalidomide: design, preparation, and in-vitro characterization for Crohn's disease application

3.1. Abstract

Crohn's disease is a chronic inflammatory disorder of the gut and is classified as a type of inflammatory bowel disease. The anti-inflammatory drug thalidomide has shown to be very effective against Crohn's disease, but presents several limitations such as drowsiness, skin rash and hypertension. Therefore, development of novel delivery system is urgent and necessary. The aim of this paper is to present the formulation of Alginate-Poly-L-Lysine-Alginate (APA) microcapsules for the delivery of thalidomide to desired locations of the Gastrointestinal Tract. APA microcapsules were designed, prepared and characterized in-vitro for thalidomide release. Mechanical stability of capsules and the drug release profile were monitored in a simulated gastrointestinal model. Data suggest that APA microcapsules enable a slow release of thalidomide in a pH and time-dependent manner. Indeed, the characteristics of APA microcapsules make it a suitable carrier for the targeted delivery of thalidomide to specific areas of the gastrointestinal tract.

Keywords: Alginate-Poly-L-Lysine-Alginate (APA); Simulated gastric fluid (SGF); Simulated Intestine fluid (SIF)

3.2. Introduction

Crohn's disease is a chronic inflammatory disorder of the gut and is classified as one type of inflammatory bowel disease. More than 400 000 people are affected by Crohn's disease in North America [1]. Epidemiological studies suggest that this disease occurs in

genetically susceptible individuals as a result of defects in mucosal barrier function and dysregulated Th1-type mucosal inflammation [70, 71]. In Crohn's disease, inflammation can affect any part of the Gastrointestinal Tract, from the mouth to the anus. Most common symptoms include abdominal pain, diarrhea, vomiting, and weight loss [72, 73].

There is no cure for Crohn's disease and surgery cannot be used to treat this disease [74]. Medications used to treat Crohn's disease include anti-inflammatory drugs such as 5-aminosalicylic acid (5-ASA) and immunomodulators such as azathioprine, mercaptopurine, methotrexate, infliximab, adalimumab, certolizumab and natalizumab [75, 76]. Although these pharmaceutical compounds have shown to be effective against Crohn's disease, they show limited clinical efficiency and several side effects. The anti-inflammatory drug thalidomide has shown to be very successful in inhibiting the inflammation associated with Crohn's disease but presents side effects due to his high dose requirements [62].

Appropriate delivery systems must be developed in order to overcome the limitations and issues of the currently available treatment for Crohn's disease. Artificial cell microencapsulation is a promising tool in scientific research that allows for the delivery of pharmaceutical compounds to specific tissues in the body, in a time dependent fashion [77, 78]. Creating an artificial cell involves the preparation of artificial structure of cellular dimension using different types of polymers and proteins. Although several encapsulation techniques are being used, the most promising formulation is the encapsulation of calcium alginate beads with poly-L-lysine (PLL), forming alginate-poly-L-lysine-alginate (APA) microcapsules [64]. APA microcapsules are formed by the ionic interaction between negatively charged alginate molecules and positively charged calcium ions. This allows the entrapped material to be protected from the external environment. This paper

proposes the use of APA microcapsules for the specific delivery of thalidomide to treat Crohn's disease. The goal of this study is to evaluate the drug release characteristics in different intestinal segments and to determine the effect of thalidomide on a simulated intestinal inflammation by using RAW 264.7 macrophage cells.

3.3. Material and methods

Chemicals and laboratory equipment

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

Preparation of APA microcapsules containing thalidomide

Alginic acid was 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 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 were included. APA beads were then formed by running the above solution through an Inotech encapsulator pump using a 300µm nozzle. Frequency was set to 528 Hz, flow rate to 20.8 ml/min and voltage to 1.48 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 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.

Characterization of thalidomide

A standard curve for thalidomide in deionized water was prepared. Thalidomide was dissolved in deionized water at a concentration of 0.035 mg/ml by stirring and heating for 24 hours. The absorbance of different dilution factors was measured at 220 nm and plotted against the concentration of thalidomide in mM (Figure 3.1).

Physical integrity of APA microcapsules

In-vitro experiments were conducted to study the physical integrity of APA microcapsules containing thalidomide. In order to simulate *in vivo* shear stress, microcapsules were incubated for 48 h in saline solution and shaken at 150 rpm [79]. The percentage of damaged and undamaged microcapsules were visually determined using a light microscope from supernatant samples taken at 1h, 9h, 12h, 24h and 48h after incubation. Pictures were taken before and after incubation to analyze the morphology of the microcapsule. Moreover, the release of thalidomide was measured spectrophotometrically over time as a marker of membrane permeability.

Evaluating thalidomide release in simulated gastric and intestine fluid

Simulated gastric fluid (SGF) and simulated intestine fluid (SIF) were prepared in order to mimic the external environment encountered in the stomach and small intestine.

SGF was prepared by dissolving Sodium Chloride (NaCl) and pepsin in deionized water. Hydrochloric acid was added to acidify the solution. The final pH of the solution was 1.5. Then, 250 mg of dried APA microcapsules containing thalidomide was added to 1 ml of SGF and shaken at 125 rpm for 30 min. The optical density at 220 nm (OD 220) was measured from the supernatant every 10 min and the results are shown in figure 4. SIF was prepared by dissolving potassium phosphate monobasic, sodium Hydroxide and pancreatin in deionized water. The pH of the solution was 6.5. In this experiment, 305 mg of APA microcapsules containing thalidomide were added to 1 ml of SIF and shaken at 125 rpm from 60 min. The absorbance was measured from the supernatant every 10 min and plotted on Figure 3.5.

In-vitro analysis of APA microcapsules in full simulated gastrointestinal model

The aim of this experiment was to evaluate the resistance and the survival of APA microcapsules containing thalidomide in the gastrointestinal environment. Different solutions were prepared in order to mimic the stomach, the small intestine, the colon ascendans, the colon transversum and the colon descendans with respect to their pH. Table 3.1 describes the retention time and pH of each solution. 15.65 g of dry capsules was initially added to the first solution and shaken at 250 rpm for 46h. HCl (0.102 M) and NaOH (0.2 M) are added throughout the experiment in order to reach the desired pH. OD 220 of supernatant was measured at several time points and the concentration of thalidomide was calculated in g/ml as shown in figure 3.6. Furthermore, the pH of the solution was monitored throughout the experiment to determine the effect of thalidomide on the pH of the surrounding solution.

Statistical analysis

Values are expressed as mean ± SD. Study was considered a randomized balanced design. Statistical comparisons between various biomarkers were carried out by repeated measures analysis of variance (ANOVA). Statistical comparisons between various treatment groups were carried out by using the general linear model (GLM). Statistical significance was set at $p < 0.05$.

3.4. Results and Discussion:

Thalidomide standard curve

The optical density at 220 nm was measured from the supernatant and plotted in Figure 3.1. Data suggest a direct proportionality between the optical density and thalidomide concentration. The mathematical equation y=45.464x, where y represents the absorbance at 220nm and x the concentration in mM, will be used for calculating the amount thalidomide in samples.

Physical integrity of APA microcapsules

In order to mimic in vivo shear stress, the APA microcapsules were incubated in saline solution for 48h and shaken at 150 rpm. The percentage of intact APA microcapsule was 96% ± 1 after incubation in saline for 48h. APA microcapsules displayed a consistent spherical shape with a diameter of 350–400 μm (Figure 3.2). The majority of APA membrane is found intact after 48h hours of incubation in saline solution. The intact ratio of APA microcapsules is illustrated in table 3.2. Although the capsules were shaken at 150 rpm for 48h in saline solution, thalidomide was largely maintained inside the APA

microcapsules. Data illustrated in Figure 3.3 suggest a minimal release of thalidomide after 48h (10.26 mM ± 1.27 x10^-6 mM).

Evaluating thalidomide release in simulated gastric and intestine fluid

The release of thalidomide was kept relatively constant following incubation of APA microcapsules in simulated gastric solution for 30 min. Results show that after 30 min of incubation and shaking in simulated gastric fluid, thalidomide release was minimal (9.43%). However, after incubation in simulated intestine fluid, the percentage release of thalidomide significantly increased (Figure 3.5). After 10 min incubation in SIF, the percentage release of thalidomide was 72.89%, and reached a value of 82.79% following 30 min incubation. Maximal release of thalidomide was achieved after 60 min incubation in SIF.

In-vitro analysis of APA microcapsules in full simulated gastrointestinal model

Figure 3.6 illustrates the release profile of thalidomide in solutions simulating the acidic and basic conditions normally encountered in the stomach, the small intestine, the colon ascendans, the colon transversum and the colon descendans. Data suggest that thalidomide release from APA microcapsules is pH-dependent. Thalidomide release is minimal at pH 2.32 (6.25x10^-7 g/ml) but dramatically increases when the pH is above 5.31 to finally reach a peak of (18.43x10^-7 g/ml).

3.5. Conclusion:

This experiment was aimed at determining the mechanical strength of APA microcapsules in vitro and analyse the drug release profile with respect to change in pH

and external environment. Despite the large size of formed beads, this study showed that thalidomide was mainly maintained within the microcapsules after 48h of incubation in saline solution at 150 rpm. This was largely due to the presence of electrostatic forces between the positively charged drug and the negatively charged alginate molecule.

Moreover, the results suggest that the mean intact ratio of APA microcapsules after vigorous shaking for 48h is always above 95%. This characteristic of APA microcapsules makes it an appropriate carrier for drug delivery by oral administration.

In the present study, we showed that the release of thalidomide was minimal when the microcapsules were incubated in simulated gastric fluid, but dramatically increased upon incubation of beads in simulated intestine fluid. These results demonstrated the fact that APA microcapsules are specifically designed to protect the entrapped material from the harsh acidic conditions normally found in the stomach, while allowing the encapsulated material to be slowly released in the intestines.

Transferring the APA microcapsules from a low pH (2.32) to a high pH (5.31) significantly increased the release of thalidomide. This burst release of thalidomide strongly suggests that the pH of the external solution plays a crucial role on the mechanical strength and stability of the APA membrane. Thus, APA microcapsules can be efficiently used for the targeted delivery of thalidomide in the regions that are the most affected by the inflammation related to Crohn's disease.

3.6. Acknowledgments

This study was supported by research operating grant MOP 64308 from the Canadian Institute of Health Research (CIHR). Marc Fakhoury also acknowledges the help and advice and all co-authors and his supervisor Dr. Satya Prakash.

SOLUTION	INTESTINAL SEGMENT	Retention Time(h)	pH
1	stomack	2	2.32
2	small intestine	6	5.31
3	colon ascendans	9	5.85
4	colon transversum	18	6.20
5	colon descendans	11	6.74

Table 3.1: Retention time and pH of gastrointestinal solutions. Solutions 1 and 2 represent the stomach and small intestine respectively, while vessels 3, 4 and 5 represent the ascending colon, the transverse colon and the descending colon respectively. Fixed amount of APA microcapsules were added into these gastrointestinal solution and the concentration of thalidomide released was measured over 46h incubation.

Incubation time	1h	9h	12h	24h	48h
% intact APA capsules	100	99.7 ± 0.58	98 ± 1.0	96.7 ± 0.58	96 ± 1.0

Table 3.2: Percentage of intact APA microcapsules after incubation in saline solution for 48h and vigorous shaking at 150 rpm. Values are expressed as mean + S.D. of three independent experiments.

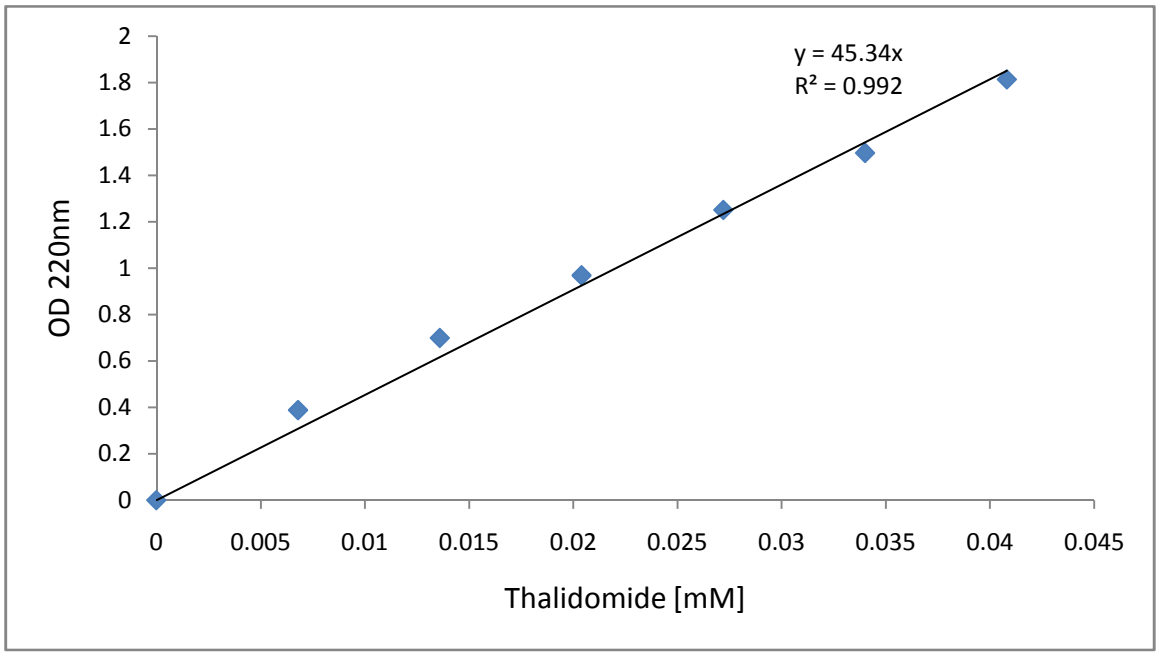

Figure 3.1: Standard curve for calculating thalidomide concentration. All data are done in triplicate and the equation displayed on the chart was used for calculating the concentration of thalidomide in supernatant samples.

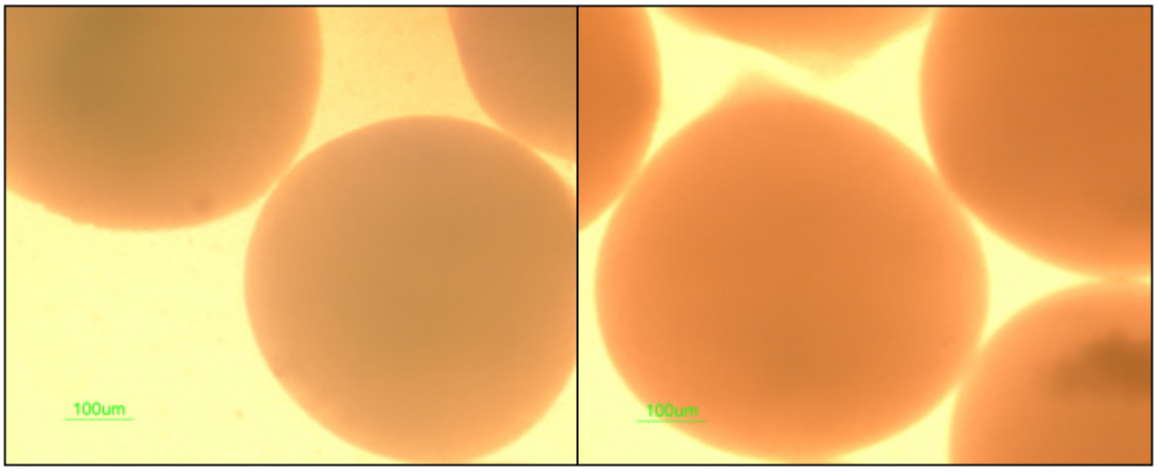

Figure 3.2: Photomicrographs of APA microcapsules containing thalidomide before (left) and after (right) incubation in saline solution at 150 rpm under 250X magnification. Size ranges from 300-350 µm.

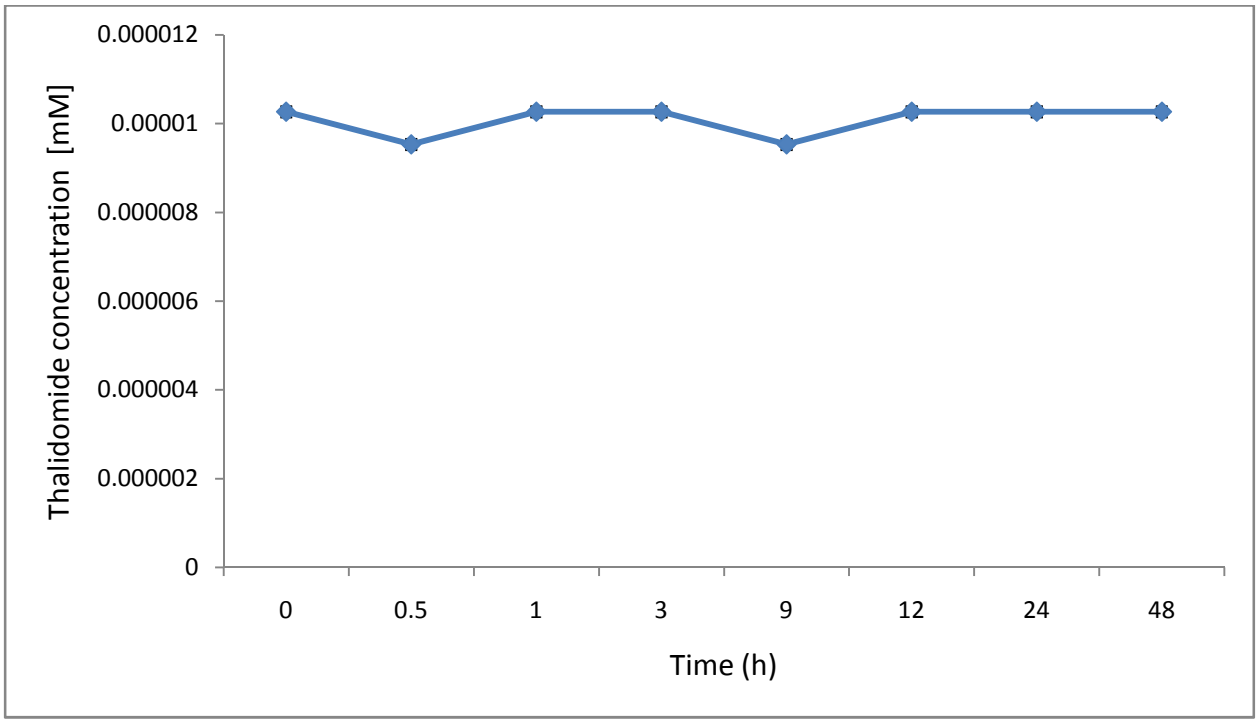

Figure 3.3: Thalidomide retention efficacy profile of APA microcapsules in saline. After 48h of incubation, the concentration of thalidomide measured from the supernatant was 12.29x10^-6 mM.

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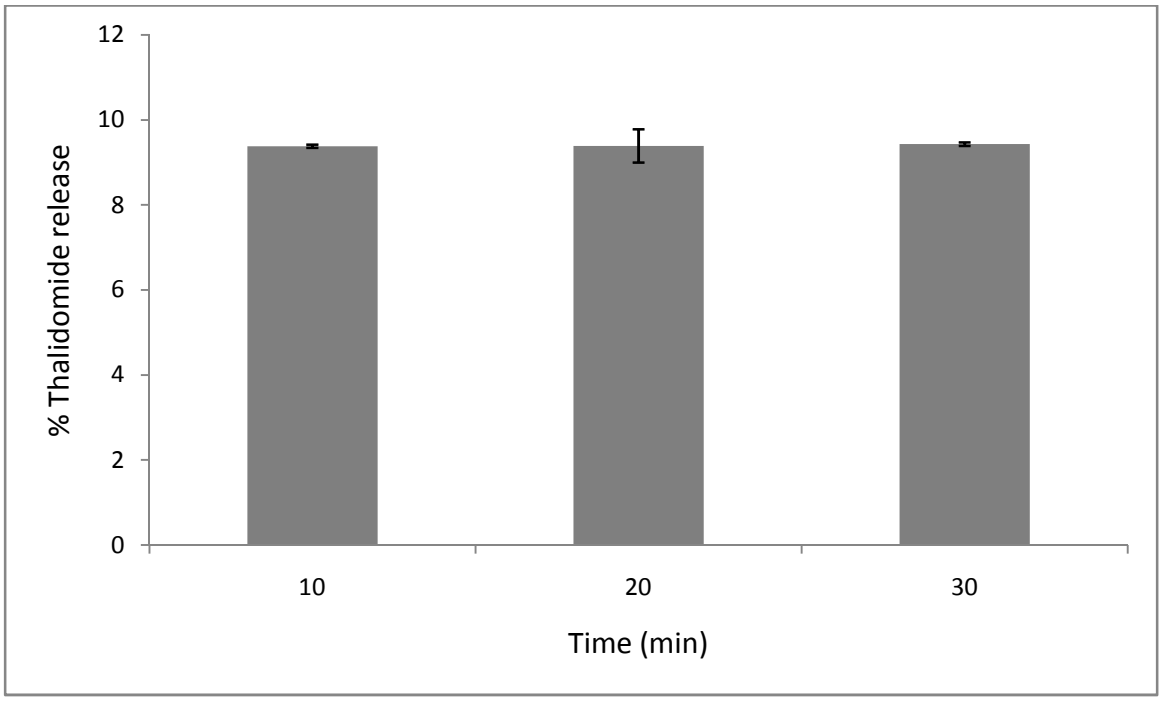

Figure 3.4: Thalidomide release from APA microcapsules in simulated gastric fluid. The percentage release of thalidomide is 9.38 ± 0.04 %, 9.39 ± 0.39% and 9.43 ± 0.04 % at time 10, 20 and 30 min respectively. Values are expressed as mean + S.D. of three independent experiments.

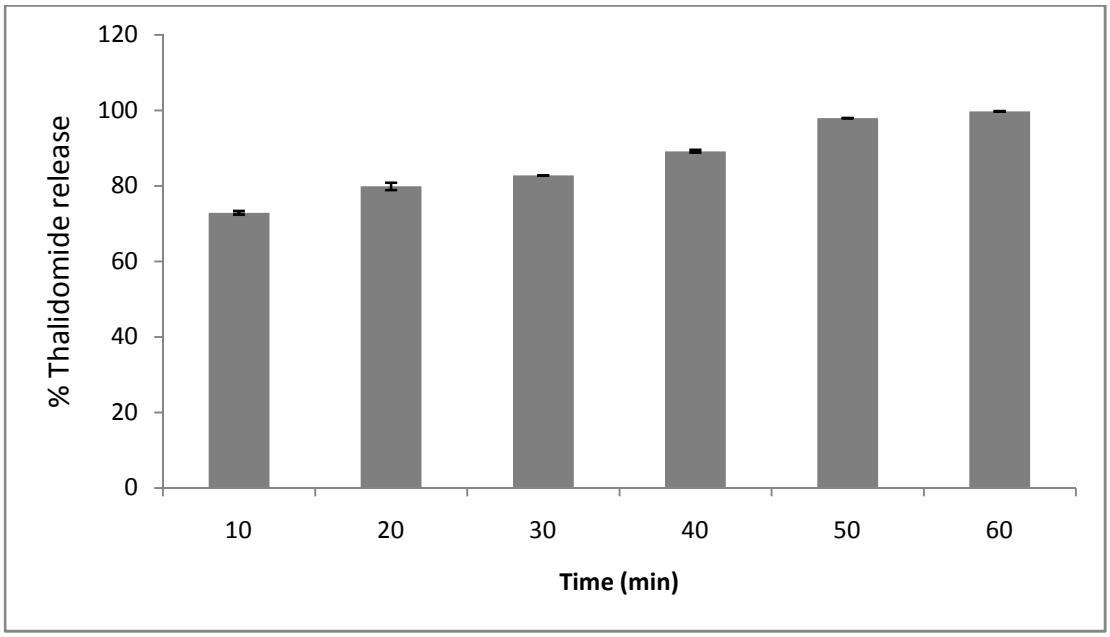

Figure 3.5: Thalidomide delivery profile of APA microcapsules in simulated intestine fluid. The average percentage release of thalidomide is 99.72 ± 0.06 % after 60 min of incubation. Values are expressed as mean + S.D. of three independent experiments.

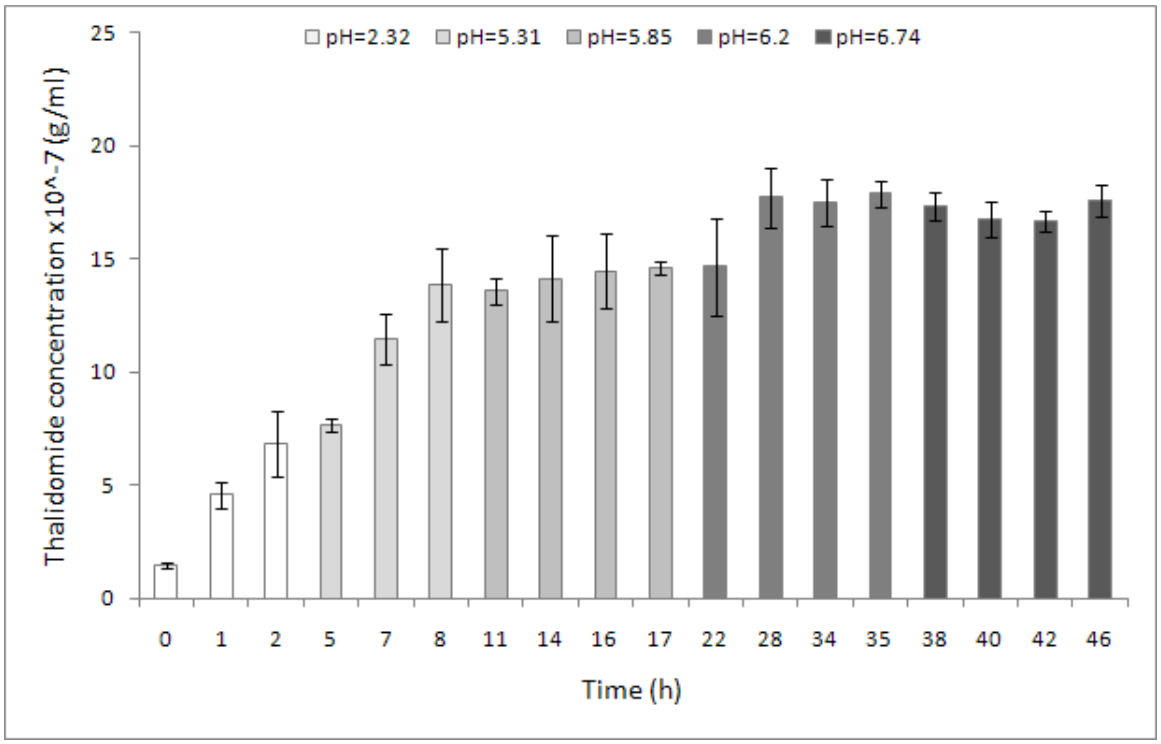

Figure 3.6: Thalidomide delivery by APA microcapsules in simulated gastrointestinal fluids. Results suggest a burst release of thalidomide when the APA microcapsules are transferred from low pH (2.32) to a high pH (5.31) environment. Maximal drug release is observed when the microcapsules are transferred in a pH=6.2 and pH=6.74 environments.

Chapter 4:

Anti-inflammatory potential of artificial microcapsules containing thalidomide for use in treating Crohn's disease

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PREFACE FOR CHAPTER 4

In the previous chapter, experiments were designed in order to evaluate the in-vitro characteristics of APA microcapsules and their potential of releasing thalidomide to specific areas of the gastrointestinal tract. The objective of Chapter 4 is to investigate the anti-inflammatory properties of artificial cells containing thalidomide using cell lines. In this study, RAW 264.7 mouse macrophage cells are activated with Lipopolysacharides (LPS) to create an in-vitro environment that closely mimics the inflammation associated to Crohn's disease.

Chapter 4: Anti-inflammatory potential of artificial microcapsules containing thalidomide for use in treating Crohn's disease

4.1. Abstract

Crohn's disease is a chronic inflammatory bowel disease associated with an abnormal immune response in the gastrointestinal tract. Several studies demonstrate that thalidomide could be effective in the treatment of refractory Crohn's disease. However, its widespread use has been limited because of potential side effects. In the present study, we investigated the inhibitory activity of alginate-poly-L-lysine-alginate (APA) microcapsules containing thalidomide on Lipopolysaccharide (LPS)-induced inflammation in RAW 264.7 macrophage cells and on 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced Crohn's disease. Results showed that APA microcapsules containing thalidomide inhibited the release of pro-inflammatory cytokines in cell supernatant following LPS activation. Moreover, treatment with microencapsulated thalidomide decreased the level of TNF-α, IL-6 and IL-1β by 49.3%, 62.3% and 54.6% respectively in TNBS-treated mice. The present project validates the efficiency of APA microcapsules in providing a targeted delivery of thalidomide for treating chronic conditions such as Crohn's disease.

Keywords: Crohns disease (CD) ,Lipopolysaccharide (LPS), Nitric Oxide (NO), Tumor necrosis factor-α (TNF- α); Interleukin-6 (IL-6); Interleukin-1B (IL-1B)

4.2. Introduction

Crohn's disease is an auto-immune disease characterized by an exaggerated Th1-mediated immune response that affects any part of the gastrointestinal tract from the mouth to the anus. Symptoms include diarrhea, abdominal pain, and weight loss. Although thalidomide exhibits anti-inflammatory properties useful for the treatment of Crohn's disease, it presents several harmful side effects such as fatigue, drowsiness and constipation [6]. Hence, appropriate delivery systems should be used in order to limit the side effects associated with thalidomide. In this study, we examine the potential of APA microcapsules containing thalidomide in reducing intestinal inflammation both in-vitro and in-vivo. LPS-activated RAW 264.7 mouse macrophage cells are commonly used to mimic intestinal inflammation [80, 81]. LPS-induced inflammation is characterized by an increase in inflammatory cytokines, and other crucial mediators of inflammation such as nitric oxide (NO) [82, 83]. The inflammatory cytokines investigated in the present study are TNF-α, IL-6 and IL-1β. TNF-α is a pro-inflammatory cytokine that plays a major role in the inflammation related to Crohn's disease [20]. In addition, acute inflammation is characterized by an increased level of IL-1β leading to cell or tissue damage [84]. Finally, IL-6 is a critical pro-inflammatory cytokine that is a primordial mediator of chemokine production and leukocyte apoptosis [85]. An animal model of intestinal inflammation is also used to mimic Crohn's disease. The model is based on the intrarectal administration of TNBS to Balb/c mice. This chemically-induced model has shown to trigger an inflammatory response similar to that in human Crohn's disease [86].

4.3. Material and methods

Chemicals and laboratory equipment

The Research IER-20 cell encapsulator was purchased from Inotech Biosystems International. The chemicals thalidomide, alginic acid, poly–L-lysine (hydrobromide) and dimethyl sulfoxide were purchased by Sigma-Aldrich Canada. The MTS Reagent Powder was purchased from Promega. Cells were incubated in a Sanyo MCO-18M Oxygen/Carbon Dioxide Incubator and stored in a Sanyo MDF-U50V -86 degrees Celsius Freezer, supplied by SANYO Canada. A Lomo Biological Inverted Microscope BIOLAMP, supplied by LOMO America, was used for microscopic cellular observations. ELISA testing was done on Mouse ELISA Ready-SET-Go! supplied by eBioscience. A Nitric Oxide Colorimetric Assay Kit from Biovision was used for calculating the amount of nitric oxide in cell supernatants. A Bio-Tek uQuant Universal Microplate Spectrophotometer from Fisher Scientific was used for ELISA plate analysis. Finally, cell centrifugation was performed on a NAPCO 2028R Centrifuge, supplied by Precision.

Preparation of APA microcapsules containing thalidomide

Alginic acid was 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 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. APA beads were then formed by running the above solution through an Inotech encapsulator pump using a 300 µm nozzle. Frequency was set to 528 Hz, flow rate to 20.8 ml/min and voltage to 1.48 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 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 200X magnification.

Macrophage cell culturing

Mouse RAW 264.7 macrophage cells were purchased from the American Type Culture Collection (ATCC) and cultured according to standard procedures using Dulbecco's Modified Eagles's Medium (DMEM). The cells were incubated in a 37˚ Celsius and 5% CO_2 environment in a Sanyo MCO-18M Oxygen/Carbon Dioxide incubator.

Cell viability assay

The aim of this study is to evaluate the effect of microencapsulated thalidomide on cell viability. Mitochondrial reduction of MTS into aqueous soluble formazan was used as an indicator of cell viability [87, 88]. The cells were cultured in a 96-well plate at a concentration of 5×10^4 cells/well. The plate was incubated for 24 hours at 37°C in a humidified, 5% CO_2 atmosphere. After aspirating the culture media, the wells were separately treated with empty APA microcapsules, APA microcapsules containing thalidomide (0.1g), thalidomide (0.7 mg/ml) and LPS (10 µg/ml). A control group consisting of no treatment was also included in the study. After incubating the plate for 24 hours at 37°C in a humidified, 5% CO_2 atmosphere, the cells were resuspended in 100

µL medium and cell viability was analyzed by MTS assay. Procedures were performed as described by the manufacturer's protocol. Briefly, 20 µL of MTS/PMS solution was added to 100 µL of culture medium. After incubating the plate for 1 hour at 37 degrees in a humidified, 5% CO_2 atmosphere, the absorbance was read at 490 nm using an ELISA plate reader. The amount of formazan product measured spectrophotometrically is directly proportional to the number of living cells in culture. The percentage cell viability is illustrated in Figure 4.1.

Induction of inflammatory cytokines by LPS

Lipopolysacharide (LPS) was used to stimulate RAW 264.7 macrophage cells. It was shown that a concentration of 10 µg/ml of LPS can significantly induce the macrophage cells to produce more pro-inflammatory cytokines such as TNF-α [62, 89]. 0.25 ml of media solution containing 380 000 RAW 264.7 cells were added to 0.25 ml of LPS (10 µg/ml) within a Falcon Brand 24-well Flat Bottom Tissue Culture Plate. The experiment was divided into four different groups. The first group consisted of cells treated with LPS alone. The second group consisted of cells treated with LPS and 0.15 g of APA microcapsules containing thalidomide solution (0.7 mg/ml). In the third group, 0.15 g of empty microcapsules was added to LPS-induced cells. The last group consisted of cells treated with LPS and free thalidomide in solution (0.7 mg/ml). The amount of inflammatory cytokines and nitric oxide was then measured from cell supernatant collected at five separate time points: 0h, 1h, 3h, 9h, 16h, 24h and 48h, after incubation in a standard 5% CO_2 environment, at 37 degree.

Cytokine production in cell supernatant

The amount of inflammatory cytokines produced in cell supernatant was measured using commercially available ELISA Kit for TNF-α, IL-6 and IL-1β. Briefly, replicate serial dilutions of the antigen standard and experimental samples were prepared. 50 µl of Assay Buffer was then added into each well of the 8-well ELISA strips. After transferring 50 µl of samples and standards to the appropriate wells, the plate was gently shaken for 10 seconds and allowed to incubate for 2 hours at room temperature. The ELISA plate was then manually washed with 1x washing buffer. This process was repeated twice. Then, 100 µl of Detection Antibody solution was added to each well. After incubating the plate for 1 hour at room temperature, the ELISA wells were washed again as described above. 100 µl of Avidin-HRP solution was then added to all wells, and the plate was incubated for 30 minutes at room temperature. After 4 washing steps, 100 µl of Development Solution was added to each well and the plate was incubated for 15 min at room temperature, in the dark. Finally, 100 µl of Stop solution was added and the absorbance was read at 450 nm and 570 nm.

Measurement of nitric oxide from cell supernatant

NO production was assayed by measuring nitrite (a stable degradation product of NO) in supernatant of cultured RAW 264.7 cells [90]. Nitric oxide plays a crucial role in inflammation and can be measured from cell supernatant [91, 92]. Nitric Oxide Colorimetric Assay was performed as described by the manufacter's protocol. 5 µl of the Nitrate Reductase mixture and enzyme cofactor was added to each well containing cell supernatant diluted in Assay buffer. The plate was then covered and incubated at room temperature for 1h in order to convert nitrate to nitrite. The enhancer (5 µL) was then

added to each well and was let incubated for 10 min. The last step uses Griess reagents to convert nitrite to a deep purple azo-compound. The absorbance was read at 540 nm using an ELISA plate reader.

Induction of inflammation using TNBS

Male Balb/c mice, 6 weeks old and weighing 23-26 g, were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in the Lyman Duff animal center of Mcgill University. TNBS (120 mg/kg/bodyweight) dissolved in 30% ethanol [86, 93] was used to induce intestinal inflammation in this strain of mice. This is a well-characterized model that has been shown to resemble human Crohn's disease [86]. The technique involves the use of a rubber catheter inserted 3-4 cm via the anus [69]. Prior to TNBS injection, the mice were slightly anesthetized with isoflurane gas. The mice were then kept in vertical position for 30s to prevent leakage of TNBS. After TNBS injection, the mice were monitored daily for survival and body mass. All animals were cared for in accord with the Canadian Council on Animal Care (CCAC) guidelines.

Treatment protocol

The animal use protocol was approved by the Animal Care Committee of McGill University. In order to investigate the therapeutic effects of APA microcapsules containing thalidomide, mice were divided randomly into five groups. Control group (n=5, receiving 30% ethanol only and no treatment) and TNBS group (n=5, receiving TNBS and no treatment) were included in this study. The treated mice were divided into three distinct groups (n=10) that consist of daily gavaging the animals with empty APA

microcapsules, APA microcapsules containing thalidomide (100 mg/kg/bodyweight) and thalidomide (100 mg/kg/bodyweight) for two weeks five days following TNBS injection.

Assessment of inflammatory markers

In order to investigate the therapeutic effects of microencapsulated thalidomide, the level of TNF-α, IL-6, IL-1β and NO was measured from blood samples. These cytokines participate in the inflammation associated with Crohn's disease [94, 95]. Blood sample was collected from mice at weekly interval via the tail vein. The serum was then separated from the blood sample by centrifugation at 36,000 rpm for 8 min. The concentration of pro-inflammatory cytokines was measured in serum using ELISA analysis (eBioscience) and the level of NO was quantified using Nitric Oxide Colorimetric Assay (Biovision).

Statistical analysis

Values are expressed as mean ± SD. Study was considered a randomized balanced design. Statistical comparisons between various biomarkers were carried out by repeated measures analysis of variance (ANOVA). Statistical comparisons between various treatment groups were carried out by using the general linear model (GLM). Statistical significance was set at $p < 0.05$.

4.4. Results and Discussion

Cell viability assay

Experiments were designed to determine the non-cytotoxicity effect of microencapsulated thalidomide. MTS assay clearly suggests that the viability of RAW 264.7 macrophage cells remains intact after treating with APA microcapsules containing

thalidomide at a concentration of 0.7 mg/ml. As illustrated in Figure 4.1, the percentage viability of macrophage cells compared to the control group was 98.43 ± 0.79 %, 99.11 ± 0.36%, 98.99 ± 0.5 % and 98.55 ± 0.67 % after treating with empty APA microcapsules, microencapsulated thalidomide, free thalidomide and LPS respectively. Cells were then observed in the microscope under x200 magnification and no cell damage was observed. The RAW 264.7 macrophage cells retained their ability to proliferate in Dulbecco's modified Eagle's medium while maintaining identical shape.

Cytokine production in cell supernatant

The amount of pro-inflammatory cytokines TNF-α, IL-6 and IL-1β was measured from the supernatant after incubation of cells with 10 µg/ml of LPS. In the LPS control group, the initial concentration of TNF-α, IL-1β and IL-6 was 238.3 ± 5.8 pg/ml, 4.3 ± 0.1 pg/ml and 235 ± 10 pg/ml respectively. Activating RAW 264.7 cells with LPS significantly increased the production of pro-inflammatory cytokines. After 48h of incubation in LPS, the levels of TNF-α, IL-1β and IL-6 in cell supernatant reached a concentration of 911.7 ± 35.1 pg/ml, 10.2 ± 0.1 pg/ml and 451.7 ± 30.6 pg/ml respectively. Treating the cells with empty APA microcapsules did not significantly alter the expression profile of these inflammatory cytokines. However, free thalidomide was able to suppress the production of TNF-α, IL-1β and IL-6 immediately 3h following LPS induction. A delayed inhibition of cytokine production was observed when treating the cells with APA microcapsules containing thalidomide. The concentration of TNF-α, IL-1β and IL-6 measured after 9h of incubation with microencapsulated thalidomide was 625 ± 26.5 pg/ml, 8.13 ± 0.21 pg/ml and 345 ± 10 pg/ml respectively. However, after 48

hours of incubation, the concentration of TNF-α, IL-1β and IL-6 decreased to 415 ± 65.6, 5.4 ± 0.3 and 228.3 ± 25.2 (Figure 4.2, 4.3 and 4.4).

Nitric oxide production in cell supernatant

The concentration of nitric oxide in cell supernatant was measured to assess the extent of inflammation. Figure 4.5 illustrates the level of nitric oxide released after stimulation of RAW 264.7 macrophage cells with Lipopolysaccharides (LPS). It was shown that treating the cells with LPS alone (control group) for 48h increased the concentration of NO from 1.5 ± 0.2 μM to 42.6 ± 0.6 μM. Treating the LPS-activated cells with empty APA microcapsules did not significantly alter the production of NO compared to the control group. However, microencapsulated thalidomide and free thalidomide (0.7 mg/ml) lowered the concentration of NO to 25.9 ± 0.6 μM to 22.6 ± 0.7 μM respectively after 48h of incubation.

TNF-α, IL-6 and IL-1β level in serum

The concentration of TNF-α, IL-6 and IL-1β is measured in blood serum from Balb/c mice following TNBS administration (Figure 4.7). ELISA analysis of cytokine levels confirmed that experimental mice treated with TNBS have a higher level of TNF-α, IL-6 and IL-1β compared to the control group. It was shown that APA microcapsules containing thalidomide (100 mg/kg/bodyweight) caused a marked decreased in the level of pro-inflammatory cytokines. Treating the mice with empty microcapsules did not alter the concentration of cytokines following TNBS administration. Moreover, using APA microcapsules as a delivery carrier for thalidomide have proven to be much more successful in lowering TNF-α, IL-6 and IL-1β compared to free thalidomide. Treating the

mice with thalidomide (100 mg/kg/bodyweight) for a period of two weeks decreased the level of TNF-α, IL-6 and IL-1β from 2557.1 ± 22.5 pg/ml, 7561.8 ± 103.4 pg/ml, 2388.4 ± 64.2 pg/ml to 1295.8 ± 34.1 pg/ml, 2852.5 ± 90.6 pg/ml and 1084.2 ± 38.6 pg/ml respectively. However, treating the mice with microencapsulated thalidomide (100 mg/kg/bodyweight) for two weeks decreased the level of TNF-α, IL-6 and IL-1β to 803.25 ± 44.9 pg/ml, 1883 ± 124.9 pg/ml and 1165.5 ± 31.3 pg/ml respectively.

4.5. Conclusion

Results demonstrate that APA microcapsules containing thalidomide exert inhibitory effects on the secretion of NO, TNF-α, IL-6 and IL-1β from RAW 264.7 cell supernatant. The latter inflammatory markers are key mediators of host defense and inflammatory response associated with Crohn's disease [96, 97]. Moreover, it was shown that treating the cells with microencapsulated thalidomide resulted in a delayed inhibition of inflammatory markers of several hours compared to the cells treated with thalidomide alone.

This suggests that APA membrane enables a slow release of thalidomide, thus increasing total delivery time. This important characteristic of APA microcapsule could be useful in the treatment of Crohn's disease where local delivery of the encapsulated drug to affected sites of the gastrointestinal tract is needed [62].

Furthermore, results demonstrate that treating the cells with empty APA microcapsules, APA microcapsules containing thalidomide, thalidomide solution (0.7 mg/ml) and LPS (10 µg/ml) did not affect cell viability. Cell still maintain their ability to grow and proliferate in culture and the percentage of viable cells observed after 24h of incubation with the treatment was close to 100%. However, it was shown that treating

RAW 264.7 cells with LPS at a concentration of 10 µg/ml caused a change in cell morphology and size. More specifically, the majority of the macrophage cells lost their circular shape and became more elongated after stimulation with LPS.

Overall the results showed that artificial cell containing thalidomide could significantly suppress the formation of NO, TNF-α, IL-6 and IL-1β from RAW 264.7 cell supernatant after LPS stimulation. MTS assay confirmed the fact that treating the cells with APA microcapsules containing thalidomide does not affect their viability and ability to grow in cultured medium. Indeed, this characteristic of APA microcapsules makes it an ideal carrier for thalidomide delivery since the proposed therapy can significantly lower the production of pro-inflammatory cytokines while preserving the intestinal tract integrity. Our results also provide the in-vivo evidence that microencapsulated thalidomide exert anti-inflammatory properties using a murine model of Crohn's disease. Following induction of intestinal inflammation by TNBS injection, the level of pro-inflammatory cytokines TNF-α, IL-1β and IL-6 in blood serum significantly increased. Treating the mice with APA microcapsules containing thalidomide caused a marked decrease in the level of TNF-α, IL-1β and IL-6. Understanding the inhibitory effect of artificial cells containing thalidomide in the production of inflammatory markers from macrophage cells will help contribute to the development of novel therapies for Crohn's disease and other immune-mediated disorders.

4.6. Acknowledgment

This study was supported by research operating grant MOP 64308 from the Canadian Institute of Health Research (CIHR). Marc Fakhoury also acknowledges the help and advice and all co-authors and his supervisor Dr. Satya Prakash.

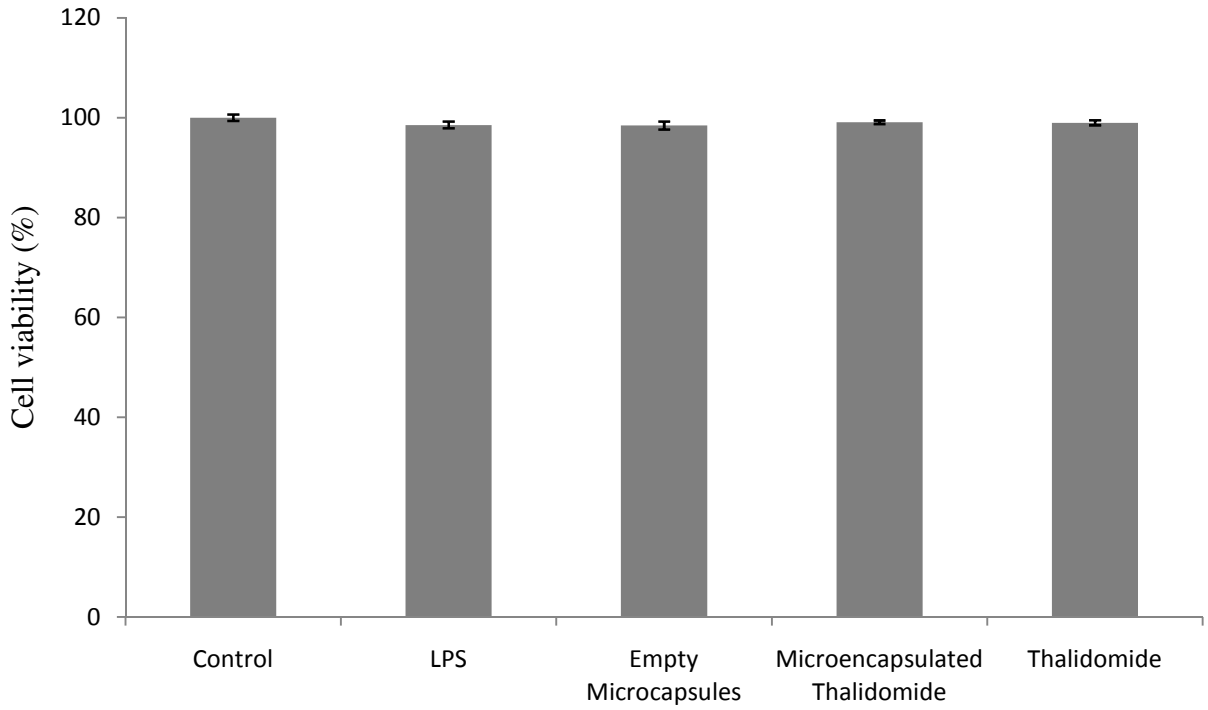

Figure 4.1: Effect of LPS (10 µg/ml), empty microcapsules, microencapsulated thalidomide (0.7 mg/ml) and thalidomide (0.7 mg/ml) on RAW 264.7 cell viability. Each column represents the percentage cell viability compared to the control group. Values are expressed as the mean + S.D.

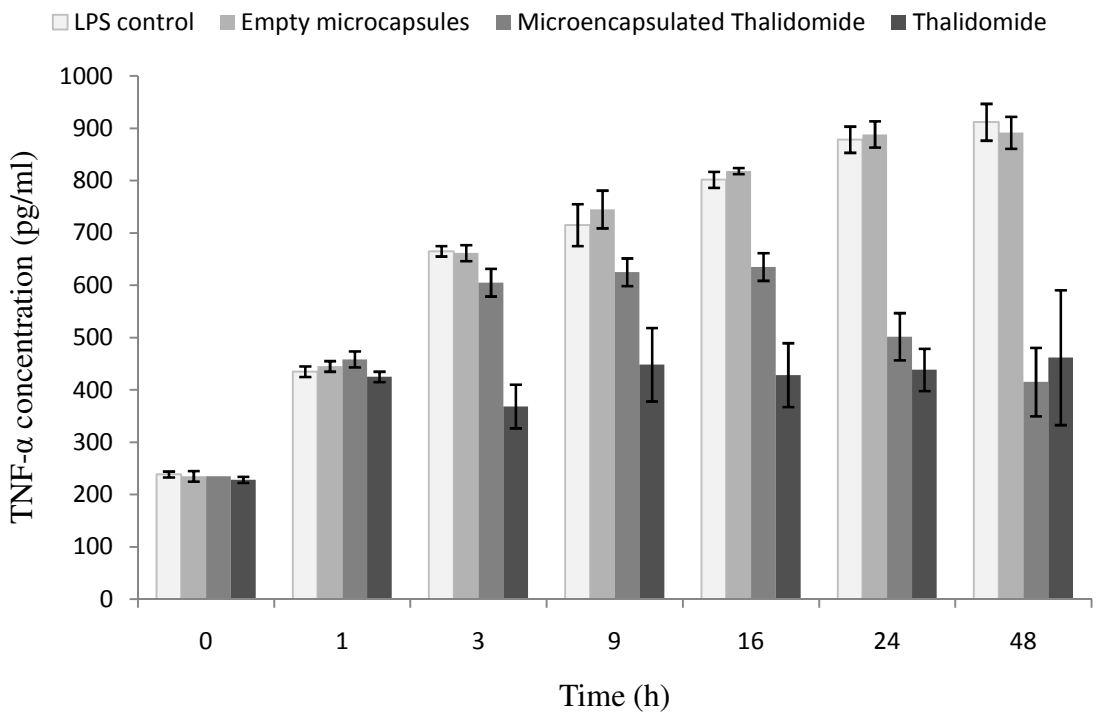

Figure 4.2: Effect of empty microcapsules, microencapsulated thalidomide (0.7 mg/ml) and thalidomide (0.7 mg/ml) on TNF-α concentration in cell supernatant from RAW 264.7 macrophage cells stimulated with 10 µg/ml of LPS. Comparisons were made after incubation times of 0, 1, 3, 9, 16, 24 and 48 hours. Values are expressed as the mean + S.D. of three independent experiments.

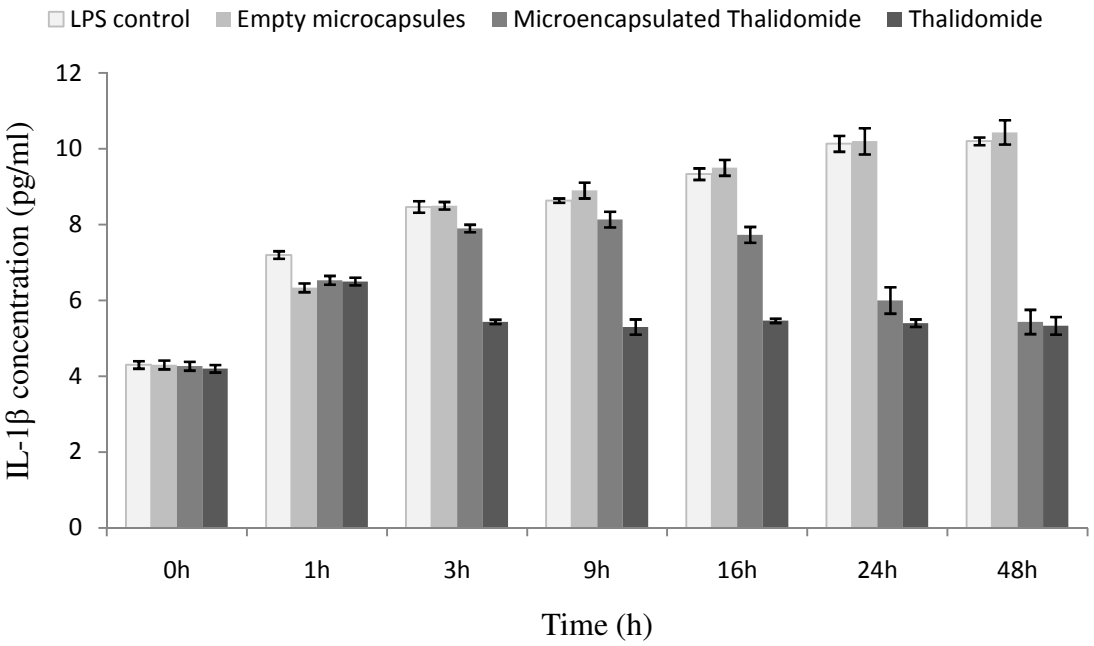

Figure 4.3: Effect of empty microcapsules, microencapsulated thalidomide (0.7 mg/ml) and thalidomide (0.7 mg/ml) on IL-1β concentration in cell supernatant from RAW 264.7 macrophage cells stimulated with 10 µg/ml of LPS. Comparisons were made after incubation times of 0, 1, 3, 9, 16, 24 and 48 hours. Values are expressed as the mean + S.D. of three independent experiments.

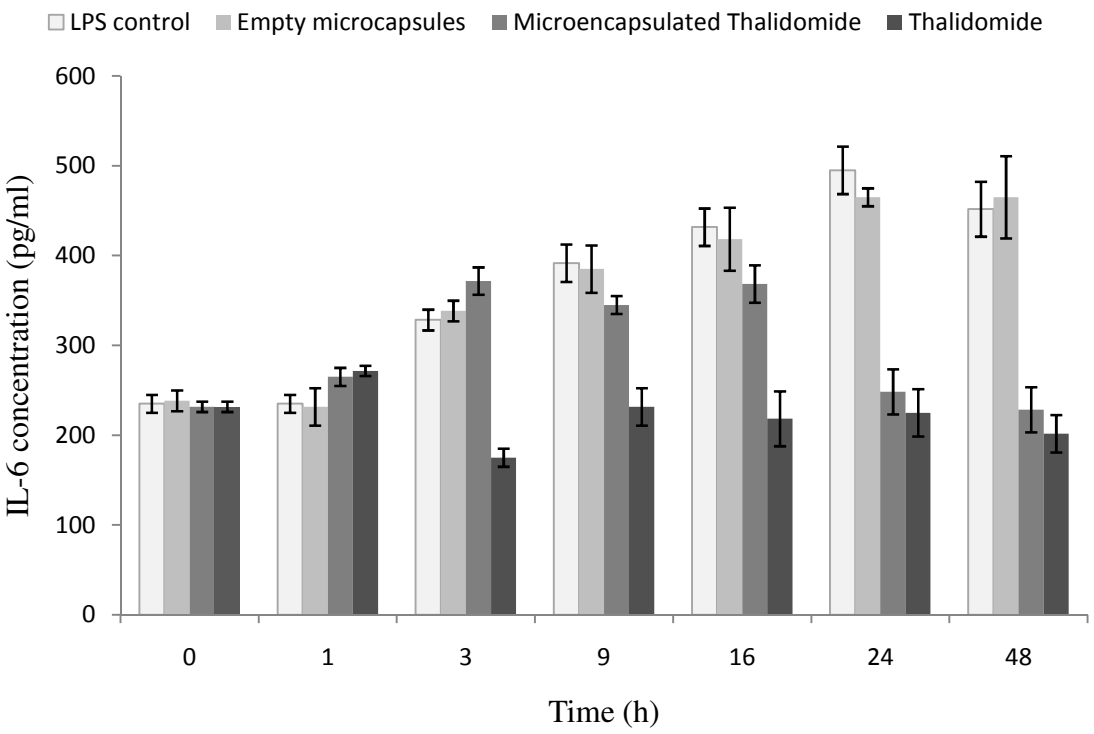

Figure 4.4: Effect of empty microcapsules, microencapsulated thalidomide (0.7 mg/ml) and thalidomide (0.7 mg/ml) on IL-6 concentration in cell supernatant from RAW 264.7 macrophage cells stimulated with 10 µg/ml of LPS. Comparisons were made after incubation times of 0, 1, 3, 9, 16, 24 and 48 hours. Values are expressed as the mean + S.D. of three independent experiments.

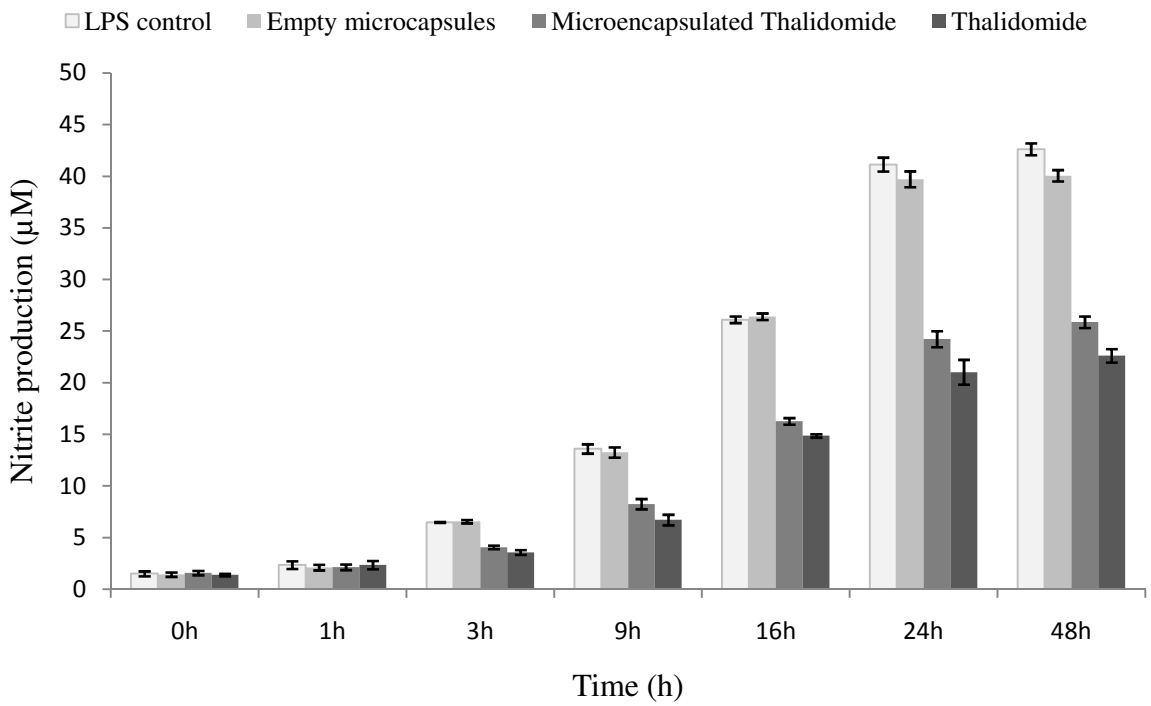

Figure 4.5: Effect of empty microcapsules, microencapsulated thalidomide (0.7 mg/ml) and thalidomide (0.7 mg/ml) on nitrite production from RAW 264.7 macrophage cells stimulated with 10 µg/ml of LPS. Comparisons were made after incubation times of 0, 1, 3, 9, 16, 24 and 48 hours. Values are expressed as the mean + S.D. of three independent experiments.

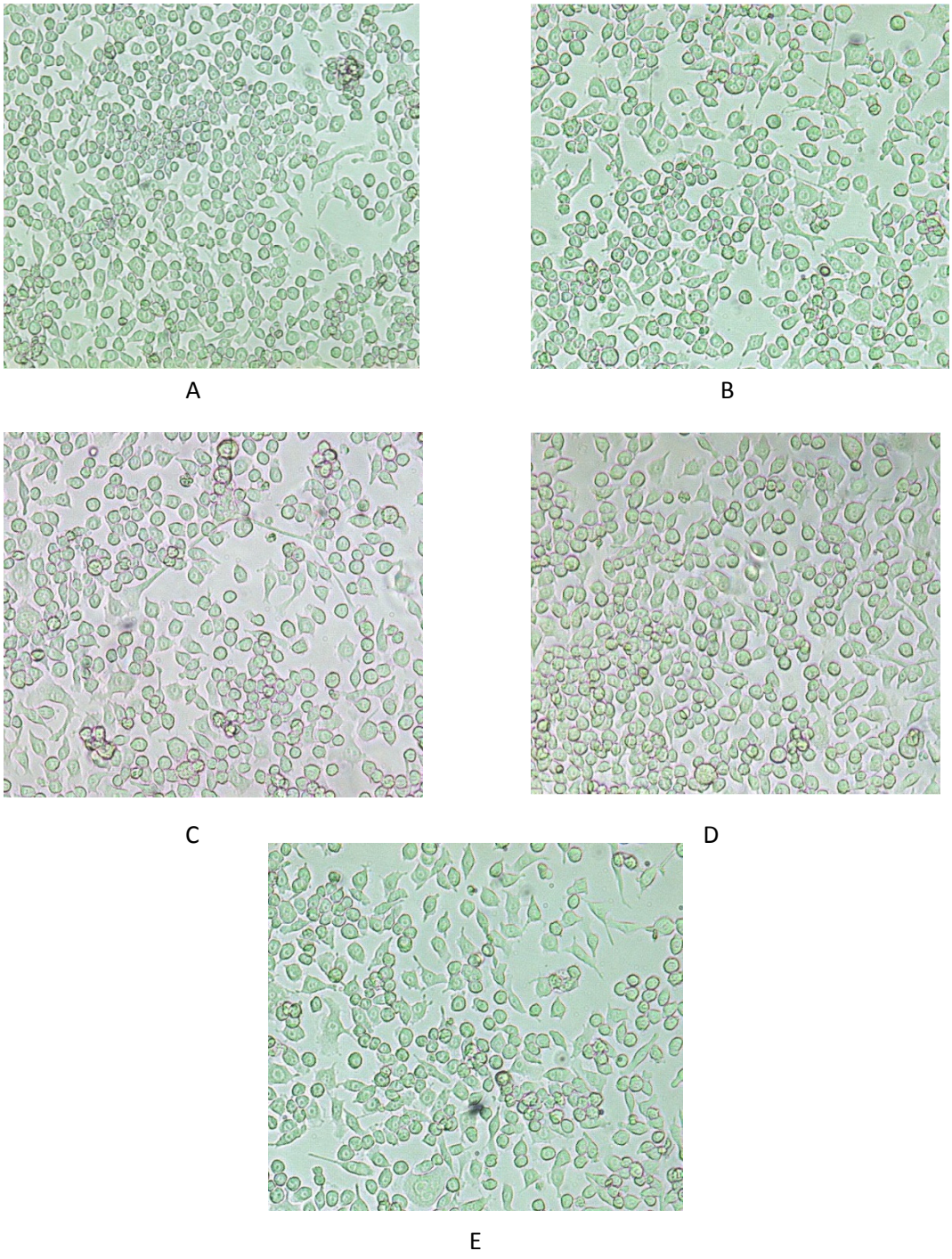

Figure 4.6: Comparison photomicrographs of RAW 264.7 macrophage cells treated for 24 hours at 37°C with: (A) Control group, (B) Empty microcapsules, (C) Microencapsulated thalidomide (0.7 mg/ml), (D) Thalidomide solution (0.7 mg/ml) and (E) LPS (10 µg/ml).

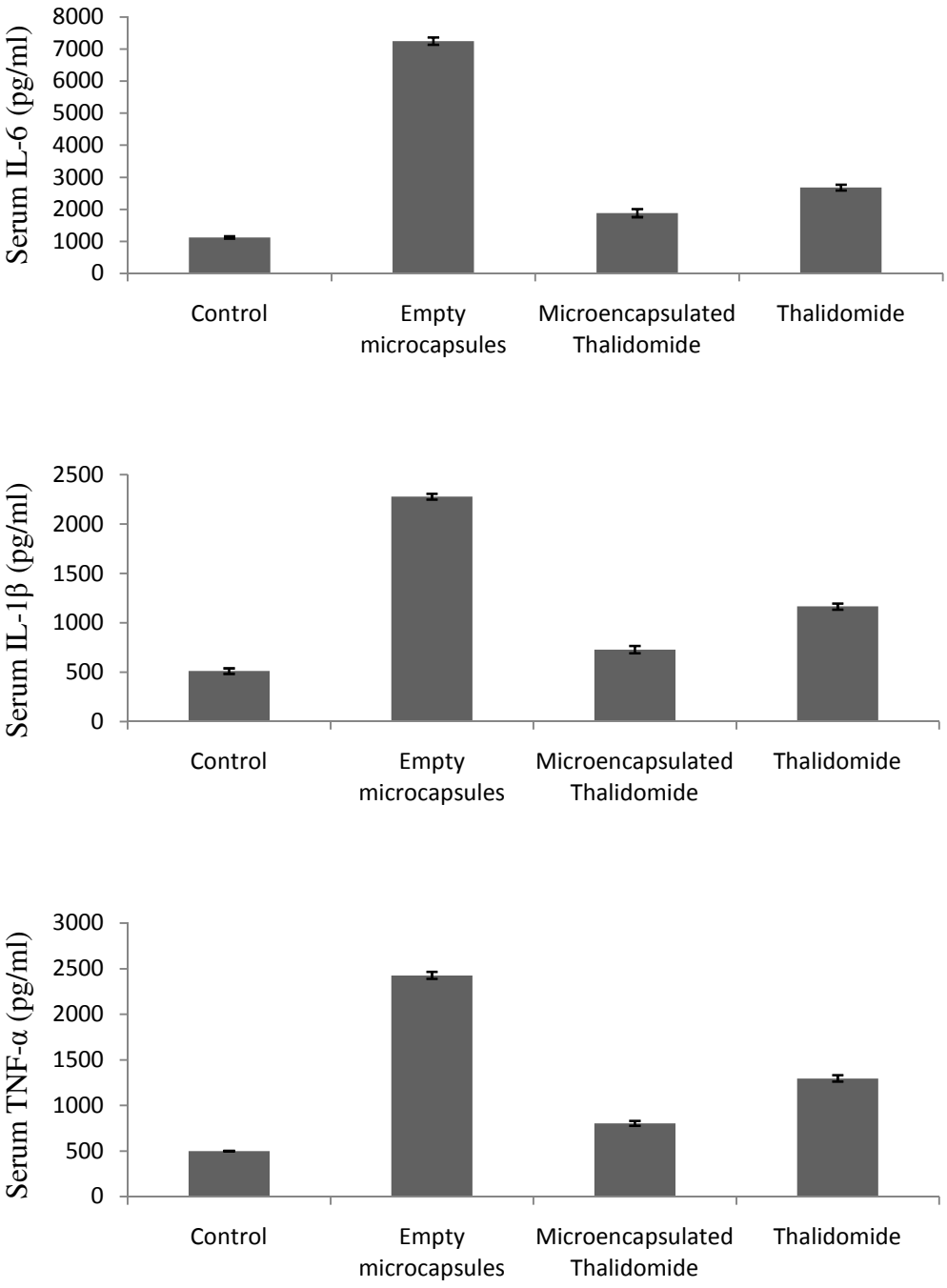

Figure 4.7: Effect of empty microcapsules, microencapsulated thalidomide (100 mg/kg/bodyweight) and thalidomide (100 mg/kg/bodyweight) on serum IL-6, IL-1β and TNF-α level following TNBS injection in Balb/c mice. The level of cytokines in the serum was measured after two-weeks treatment. Values are shown as mean + S.D. of mice for each group.

Chapter 5:

Use of artificial cell microcapsule containing thalidomide for treating TNBS-induced Crohn's disease in mice.

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PREFACE FOR CHAPTER 5

In the previous chapter, the anti-inflammatory properties of APA microcapsules containing thalidomide were evaluated using RAW 264.7 macrophage cells. Chapter 5 investigates the in-vivo characteristics of microencapsulated thalidomide using a murine model of Crohn's disease. 2,4,6-trinitrobenzene sulfonic acid (TNBS) is used to induce Crohn's disease in Balb/c mice and several biochemical parameters are evaluated to evaluate the clinical efficiency of different concentrations of thalidomide.

Chapter 5: Effect of different doses of artificial microcapsules containing thalidomide in TNBS-induced Crohn's disease in mice

5.1. Abstract

In this study, we examined the in-vivo characteristics of a novel microencapsulated thalidomide formulation in a murine model of experimental Crohn's disease. Crohn's disease was induced with a single intra-colonic injection of 120 mg/kg/bodyweight of 2,5,6-trinitrobenzene sulfonic acid (TNBS) dissolved in 30% ethanol in Balb/c mice. Level of tumor necrosis factor alpha (TNF-α), interleukin one beta (IL-1β), interleukin 6 (IL-6) and Nitric oxide (NO) were measured in tissue homogenate. Moreover, myeloperoxidase (MPO) activity was determined to assess the extent of neutrophil infiltration. Dose response study showed that treating the mice with microencapsulated thalidomide (100 mg/kg/bodyweight) for two weeks significantly decreased the degree of intestinal inflammation related to Crohn's disease. Higher and lower doses (0, 25, 50 and 200 mg/kg/bodyweight) did not exhibit comparable effects. The present study validates the success of APA microcapsules containing thalidomide in reducing colonic inflammation, and proposes a potential remedy for Crohn's disease.

Keywords: myeloperoxidase (MPO), tumor necrosis factor alpha (TNF-α), interleukin one beta (IL-1B), Nitric oxide (NO), 2,5,6-trinitrobenzene sulfonic acid (TNBS)

5.2. Introduction

Crohn's disease is one type of inflammatory bowel disease characterized by inflammation of the gastrointestinal mucosa [94, 98]. This chronic inflammatory disease can affect any part of the gastrointestinal tract, from the mouth to the anus [99]. Between 400,000 and 600,000 people in North America have active Crohn's disease [1].

Common drugs used for treatment of inflammatory bowel disease are corticosteroid, sulfasalazine, infliximab and different antibiotics [100]. Although these pharmaceutical coumpounds have demonstrated notable results, they show limited clinical efficiency [7, 101, 102]. Thalidomide is emerging as an alternative treatment for patients affected by Crohn's disease [103]. It was first introduced in 1956 as a hypnotic drug exhibiting anti-inflammatory and immunomodulatory characteristics [104]. Several studies have demonstrated that thalidomide could be used as an efficient treatment against Crohn's disease [103, 105]. Thalidomide displays immunomodulatory properties that can help in the remission of Crohn's disease. It causes the stimulation of cytotoxic T-cells and participates in the reduction of lipopolysacharide-induced cytokines like IL-6, IL-1β and TNF-α [104]. However, the use of thalidomide as an anti-inflammatory treatment is limited since this drug presents several limitations, such as drowsiness, hypertension, skin rash, and neutropenia [6]. Appropriate delivery systems should be used in order to deliver thalidomide to desired areas of the gastrointestinal tract.

Microencapsulation in specialized ultra-thin semi-permeable polymer membranes has been successfully shown to protect the encapsulated material and allow its release in a time-dependent fashion [65]. Oral administration of microcapsules containing pharmaceutical compounds has potential as an alternative treatment for several diseases [106]. Recent research has shown that APA microencapsulation technology could enable

successful delivery of thalidomide in-vitro, using simulated gastrointestinal environments [65]. The goal of the present study is to design an alginate-poly-L-lysine-alginate (APA) membrane thalidomide formulation and evaluate its potential performance in the remission of Crohn's disease, using a well-established murine model of 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced Crohn's disease. This chemically-induced model shares biochemical and immunological pathways with Crohn's disease and is very useful in studying epithelial response to injury and neutrophil infiltration [67].

5.3. Material and methods

Chemicals and laboratory Equipment:

The chemicals thalidomide, 2,4,6-Trinitrobenzenesulfonic acid solution (TNBS), alginic acid, poly-L-lysine and Dimethyl sulfoxide (DMSO) were supplied from Sigma-Aldrich Canada. The research IER-20 cell encapsulator was purchased from Inotech Biosystems International. A Mouse ELISA Ready-SET-Go! Supplied by eBioscience was used for ELISA analysis of cytokines in serum and tissue homogenates. ELISA plate analysis was performed using a Bio-Tek uQuant Universal Microplate Spectrophotometer from Fisher Scientific. A Nitric Oxide Colorimetric Assay Kit from Biovision was used for calculating the amount of nitric oxide in tissue homogenate. Finally, centrifugation of blood samples was performed on a NAPCO 2028R Centrifuge, supplied by Precision.

Preparation of APA microcapsules containing thalidomide

Alginic acid was 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 (DMSO) and diluted with deionised water. Alginic acid was additionally added to maintain a 1.5% concentration. APA beads were then formed by running the above solution through an Inotech encapsulator pump using a 300 µm nozzle. Frequency was set to 528 Hz, flow rate to 20.8 ml/min and voltage above 1.5 kV. Formed beads were collected in a prepared 0.1 M calcium chloride solution to avoid cell aggregation. The beads were then washed with deionized water and soaked in a 0.1% poly-l-lysine 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.

Experimental animals

Male Balb/c mice, 6 weeks old and weighing 23-26 g, were obtained from The Jackson Laboratory (Bar Harbor, ME). This mouse is commonly used for the study of immunological and inflammatory diseases. Trinitrobenzene sulfonic acid (TNBS) was used to induce intestinal inflammation in this strain of mice. Chemically induced Crohn's disease models using TNBS are commonly used in research because of the immediate inflammation, the high reproducibility, and the simplicity of the induction process [107]. All animals were housed in the Lyman Duff Medical Building. The animal use protocol was approved by the Animal Care Committee of McGill University. All procedures were performed according to Standard Operating procedures (SOP), and the animals were cared for in accord with the Canadian Council on Animal Care (CCAC) guidelines.

Induction of Crohn's disease using TNBS

TNBS (120 mg/kg/bodyweight) dissolved in 30% ethanol [86, 93] was used to induce Crohn's disease in experimental mice by anal injection. The technique involves the use of a rubber catheter inserted 3-4 cm via the anus [69]. Prior to TNBS injection, the mice were slightly anesthetised with gas isoflurane. The mice were then kept in vertical position for 30s to prevent leakage of TNBS. After TNBS injection, the mice were monitored daily for survival and body mass. The mice was discarded from the experiment if one or more of the following clinical endpoints were observed: TNBS backflow, diarrhea, anal prolapse, lack of grooming, lethargy and decrease body weight by more than 20%.

Animal test groups

The treated mice were divided into five distinct groups (n=10). The treatment consisted of daily gavaging a solution of APA microcapsules containing thalidomide using different concentrations of drug (0, 25, 50, 100 and 200 mg/kg/bodyweight) five days following TNBS injection. The model was established for two weeks. A control group (n=5) that consists of no TNBS injection and no treatment, was also included in this study. All animals were closely monitored for body weight and body mass following TNBS injection. Moreover, in order to prevent dehydration of the mice, 0.85% saline solution was administered to the animals by subcutaneous injection.

Histological assessment

The distal part of the colon was flushed with ice-cold saline solution, dissected and fixed with formaldehyde (10% solution). Tissues were embedded in paraffin and sections (4 µm) were stained with haematoxylin and eosin (H & E) for histological assessment [108,

[109]. Stained sections were observed under the light microscope (x200) for mucosal damage and leukocyte infiltration.

Morphometric study

The morphology of the organs was studied in order to evaluate the therapeutic effect of APA microcapsules containing thalidomide. It has been shown that colon and spleen enlargement is a clear indicator of the inflammation associated with Crohn's disease [86]. After rapid removal of the organs from all groups, the length and weight of the colon and spleen were recorded.

Myeloperoxidase assay

Myeloperoxidase enzyme has been extensively used in biomedical research to assess the extent of neutrophil infiltration into gastrointrestinal tissues [110]. A 4-cm segment of the distal part of the colon was removed and gently rinsed with ice-cold saline, and blotted dry [111]. The segment was then homogenized in ice-cold potassium phosphate buffer (20 mM, pH 7.4) and the homogenate was centrifuged at 4000 xg for 20 min at 4C. The pellet was then ice-homogenized in a tube containing 1 ml/100 mg of tissue of 50 mM PBS, pH 6.0 and 0.5% hexadecyltrimethylammonium bromide (HTAB) [112, 113]. HTAB is used as a detergent that releases MPO from the primary granules of neutrophils [114]. The homogenate was then freeze/thawed three times and centrifuged at 12,000 x g for 10 min at 4 C [112, 115]. The supernatant was diluted with assay buffer, and an aliquot (50 µL) was mixed with 25 µL of o-dianisidine (0.167 mg/mL) and 25 µL of 0.0005% H_2O_2.

The change in absorbance at 490 nm was measured with a microplate reader for a period of 10 min. MPO activity is expressed as Units/g tissue sample [69, 86].

Assesment of Nitric oxide production

Nitric oxide (NO) plays a crucial role in inflammation and can be measured from tissue homogenate sample. Intestinal inflammation is associated with increased level of NO [116]. Distal part of the colon was homogenized in ice-cold physiological saline and the production of nitric oxide was measured as described by the manufacture's protocol. Briefly, 5 µl of the Nitrate Reductase mixture and enzyme cofactor was added to each well containing cell supernatant diluted in Assay buffer. The plate was then covered and incubated at room temperature for 1h in order to convert nitrate to nitrite. The enhancer (5 µL) was then added to each well and was let incubated for 10 min. The last step uses Griess reagents to convert nitrite to a deep purple azo-compound. The absorbance was read at 540 nm using an ELISA plate reader.

ELISA analysis on colon homogenate

Colon homogenates were prepared as previously described [117]. A 1 cm segment was taken from the distal part of the colon. Colon tissue samples were homogenized in PBS and centrifuged at 12,000 x*g* for 10 min. The supernatants were stored at −80°C until used for further analysis. The amount of TNF-α, IL-6 and IL-1β were measured from colon homogenate by ELISA analysis as described by the manufacturer (eBioscience). Briefly, replicate serial dilutions of the antigen Standard and experimental samples were prepared. 50 µl of Assay Buffer was then pipetted into each well of the 8-well ELISA strips. After transferring 50 µl of samples and standards to the appropriate wells, the plate

was gently shaken for 10 seconds and allowed to incubate for 2 hours at room temperature. The ELISA plate was then manually washed with 1x washing buffer. This process was repeated twice. Then, 100 µl of Detection Antibody solution was added to each well. After incubating the plate for 1 hour at room temperature, the ELISA wells were washed again as described above. 100 µl of Avidin-HRP solution was then added to all wells, and the plate was incubated for 30 minutes at room temperature. After 4 washing steps, 100 µl of development solution was added to each well and the plate was incubated for 15 min at room temperature, in the dark. Finally, 100 µl of stop solution was added and the absorbance was read at 450 nm and 570 nm.

Statistical analysis

Values are expressed as mean ± SD. Study was considered a randomized balanced design. Statistical comparisons between various biomarkers were carried out by repeated measures analysis of variance (ANOVA). Statistical comparisons between various treatment groups were carried out by using the general linear model (GLM). Statistical significance was set at $p < 0.05$.

5.4. Results and discussion:

TNBS induces Crohn's disease in Balb/c mice

Anal injection of TNBS at a concentration of 120 mg/kg/bodyweight has proven to be successful in inducing Crohn's disease while maintaining the mortality rate very low (Table 5.1). Following TNBS administration, the majority of the mice assumed hunch-backed postures suggesting excessive ongoing pain. Other symptoms observed in TNBS-treated mice were severe diarrhea and poor clinical state.

Effect of TNBS on body, colon and spleen weight

A significant decrease in bodyweight and colon length, and increase in colon and spleen weight was observed in all mice treated with TNBS compared to the control group (Figure 5.2). Treating the mice with microencapsulated thalidomide has shown to significantly attenuate the body weight loss of the mice in a dose dependent fashion. Moreover, this treatment was successful in restoring initial colon and spleen morphology, demonstrating the effectiveness of thalidomide in treating TNBS-induced Crohn's disease. The best results were obtained when using a dose of 100 mg/kg/bodyweight.

Histological assessment

Sections of the distal colon were stained with H&E and observed under the microscope for sign of colon damage, mucosal damage and leukocyte infiltration. As illustrated in Figure 5.3, stained sections showed severe areas of mucosal and colon damage after treating with microencapsulated thalidomide (0 mg/kg/bodyweight). Moreover, the mucosa showed marked lymphocyte infiltration compared to the control group. Treating the mice with microencapsulated thalidomide (100 mg/kg/bodyweight) significantly reduced the extent of neutrophil infiltration and mucosal damage.

NO production in colonic tissues

The level of NO in colon sample significantly increased following TNBS injection. The concentration of NO measured in tissue homogenate was 7.29 mmol/mg ± 0.04 in microencapsulated thalidomide (0 mg/kg/bodyweight) treated group compared to 2.55 mmol/mg ± 0.036 in the control group. APA microcapsules containing thalidomide was

shown to significantly inhibit the level of NO in colonic tissues in a dose dependent fashion. A dose of 100 mg/kg/bodyweight yielded the best efficacy.

Myeloperoxidase activity in colon

The myeloperoxidase (MPO) activity was measured in colon homogenate sample from Balb/c mice. The level of MPO in tissue samples is a clear indicator of neutrophil infiltration that frequently takes place in Crohn's disease. The level of MPO was found to be relatively low in the control group (1.43 U/g ± 0.14) but significantly increased following TNBS administration. Treating the mice with microencapsulated thalidomide reduced the level of MPO in colon samples in a dose dependent manner. Maximal inhibition was observed when using a concentration of 100 mg/kg/bodyweight.

TNF- α, IL-6 and IL-1β level in colon homogenate

In the homogenate of full thickness strips isolated from colonic segments of mice, the level of TNF-α, IL-1β and IL-6 was relatively low in the control group (2.73 ± 0.13, 7.52 ± 0.33 and 20.13 ± 0.72 pg/mg of tissue respectively). Treatment with microencapsulated thalidomide was shown to reduce the production of pro-inflammatory cytokines in a dose dependent manner following TNBS injection. The best results were obtained when using a drug concentration of 100 mg/kg/bodyweight. Indeed, following treatment with microencapsulated thalidomide (100 mg/kg/bodyweight) after TNBS injection, the concentration of TNF-α, IL-1β and IL-6 was 3.03 ± 0.32, 9.86 ± 0.311 and 30.76 ± 0.36 pg/mg respectively.

5.5. Conclusion:

Anti-inflammatory drugs such as thalidomide are very effective in dampening intestinal inflammation, but presents major limitations such as skin rash and drowsiness [48]. Appropriate delivery methods should be used to target thalidomide to specific sites of the gastrointestinal tract, thus reducing its side effects and improving its clinical efficiency. The present study has demonstrated that artificial cells can be successfully used for the targeting and delivery of thalidomide in order to treat Crohn's disease. The results showed that TNBS induces colonic inflammation in Balb/c mice. The degree of inflammation was assessed by the amount of pro-inflammatory cytokines in blood and colon samples, the extent of neutrophil infiltration, and the level of nitric oxide in colon homogenate. Moreover, TNBS administration caused a marked decrease in the body weight of the mice and affected the morphology of the spleen and colon.

The spleen is an organ part of the lymph system which plays a crucial role in the defense mechanism of the immune system. It contains in its reserve most of the body's monocytes, that turn into dendritic cells and macrophages upon migration to inflamed tissues [118]. An enlarged spleen was observed in mice following TNBS injection, suggesting that this organ was overactive in destroying blood cells. Moreover, following TNBS injection, the colon of Balb/c mice increased in weight and decreased in length. Indeed, colon enlargement can frequently take place following TNBS-induced Crohn's disease. It's mainly caused by constipation which results in the accumulation of feces in the colon, and its dilation. This condition is referred to as toxic megacolon, which is frequently observed in patients affected by Crohn's disease and other immune-mediated disorders [119].

Treating the mice with APA microcapsules containing thalidomide reduced colonic inflammation in a dose dependence manner. A dose of 100 mg/kg/bodyweight of thalidomide yielded the best efficacy. The level of TNF-α, IL-6 and IL-1β level in colon homogenate significantly reduced following treatment with artificial cells containing thalidomide. Furthermore, treating the mice with thalidomide caused a significant decrease in the level of NO and MPO in colon homogenate. Histological analysis showed considerable reduction in neutrophil infiltration and mucosal damage following treatment. In conclusion, the anti-inflammatory effect of microencapsulated thalidomide presented in this study shed new light on our understanding of the efficiency of APA microcapsules as a delivery system for the treatment of Crohn's disease.

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Thalidomide concentration (mg/kg/bodyweight)	0	25	50	100	200
% Mortality Rate	30	10	10	0	0

Table 5.1: Animal mortality rate of the mice after treating with APA microcapsules containing thalidomide (0, 25, 50, 100 and 200 mg/kg/bodyweight)

Thalidomide concentration (mg/kg/bodyweight)	0	25	50	100	200
% Survival Rate	70	90	90	100	100

Table 5.2: Animal survival rate of the mice after treating with APA microcapsules containing thalidomide (0, 25, 50, 100 and 200 mg/kg/bodyweight)

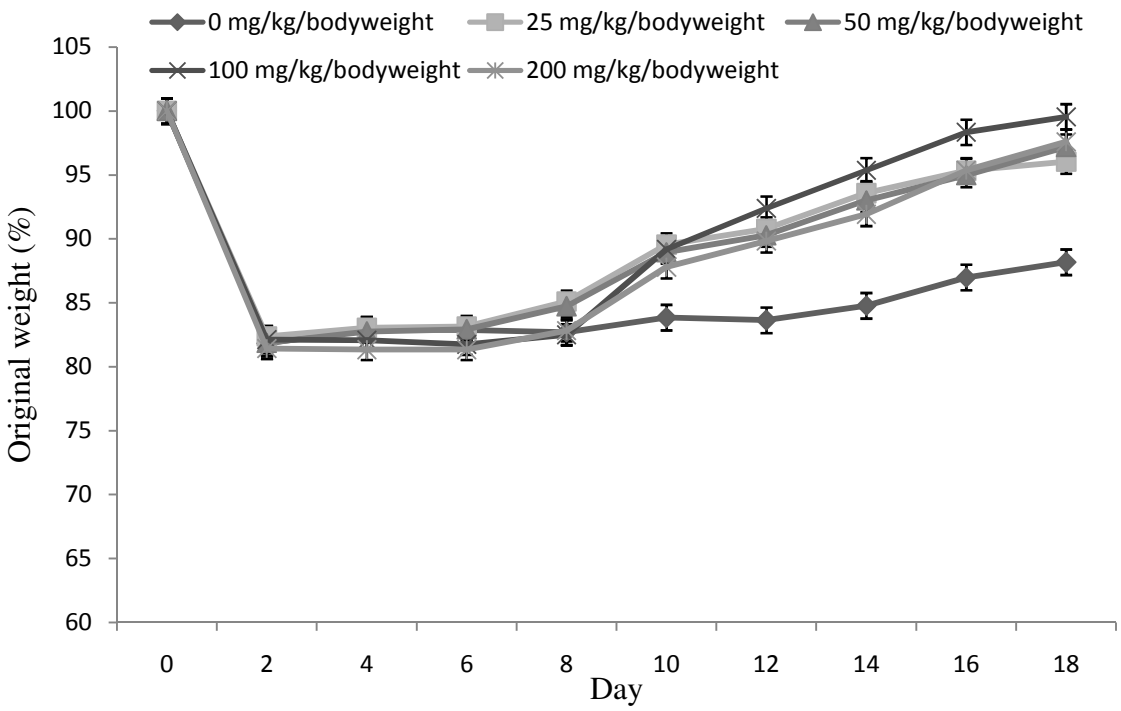
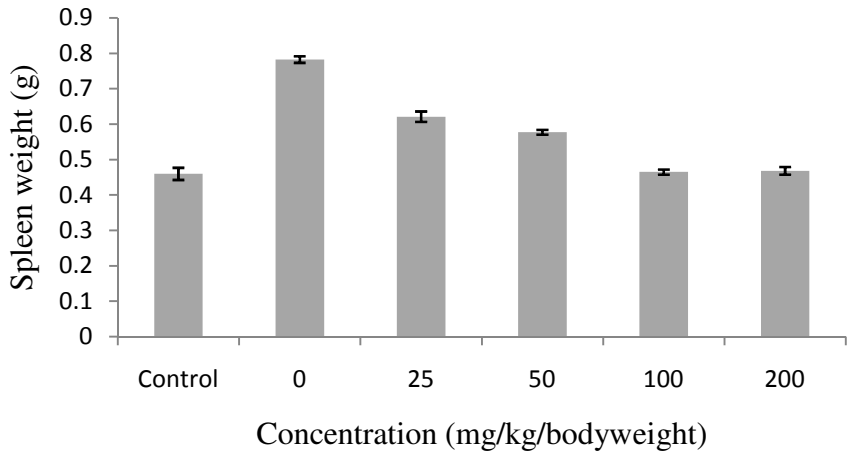
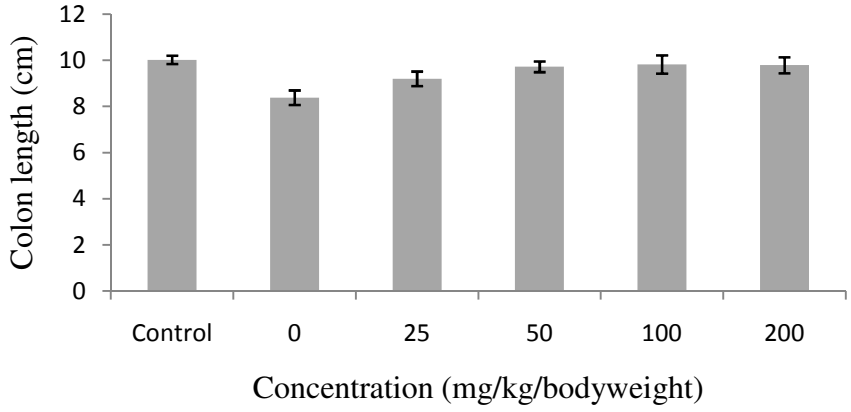


Figure 5.1: Effect of APA microcapsules containing thalidomide (0, 25, 50, 100 and 200 mg/kg/bodyweight) on bodyweight of mice following TNBS injection at day 0. Mice were treated 5 days following TNBS injection for a total period of two weeks. Values are shown as mean + S.D. of mice for each group.





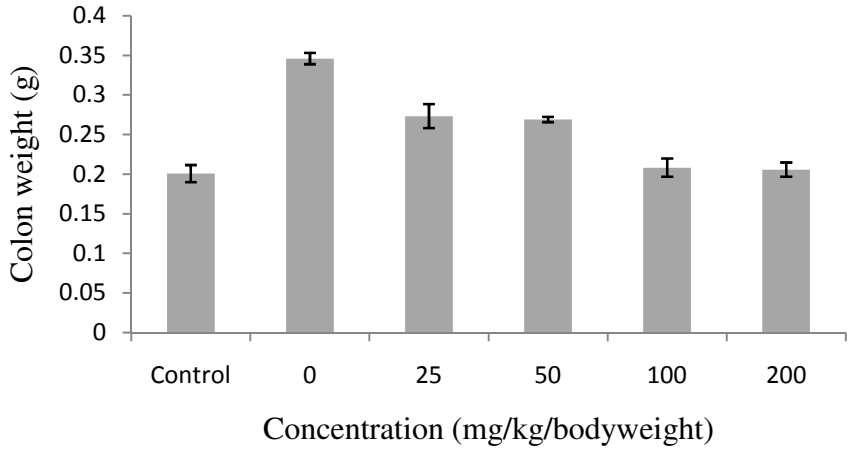
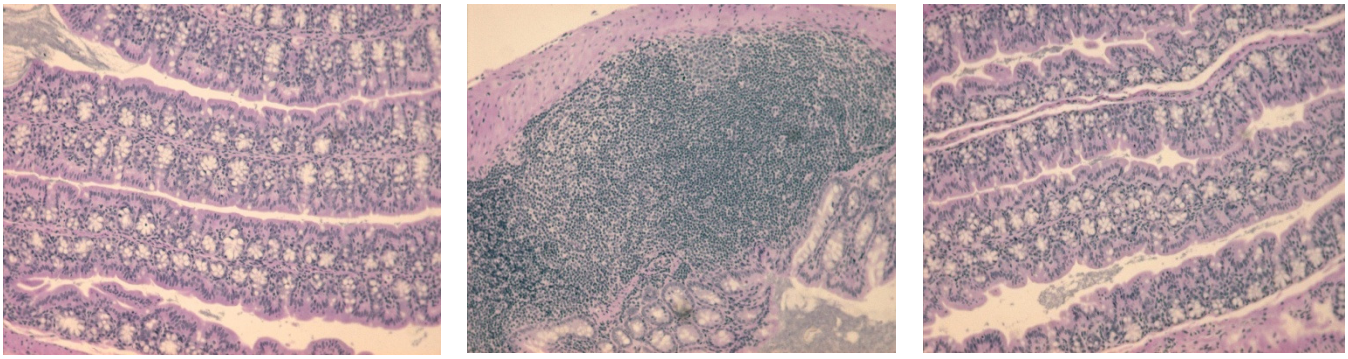


Figure 5.2: Effect of APA microcapsules containing thalidomide (0, 25, 50, 100 and 200 mg/kg/bodyweight) on spleen weight, colon weight and colon length of mice following TNBS injection. Values are shown as mean + S.D. of mice for each group.



(A) (B) (C)

Figure 5.3: Photomicrographs of H&E stained full thickness sections of distal colon taken from Balb/c mice in the control group (a), microencapsulated thalidomide (0 mg/kg/bodyweight) treated group (b) and microencapsulated thalidomide (100 mg/kg/bodyweight) treated group (c) (Magnification x200).

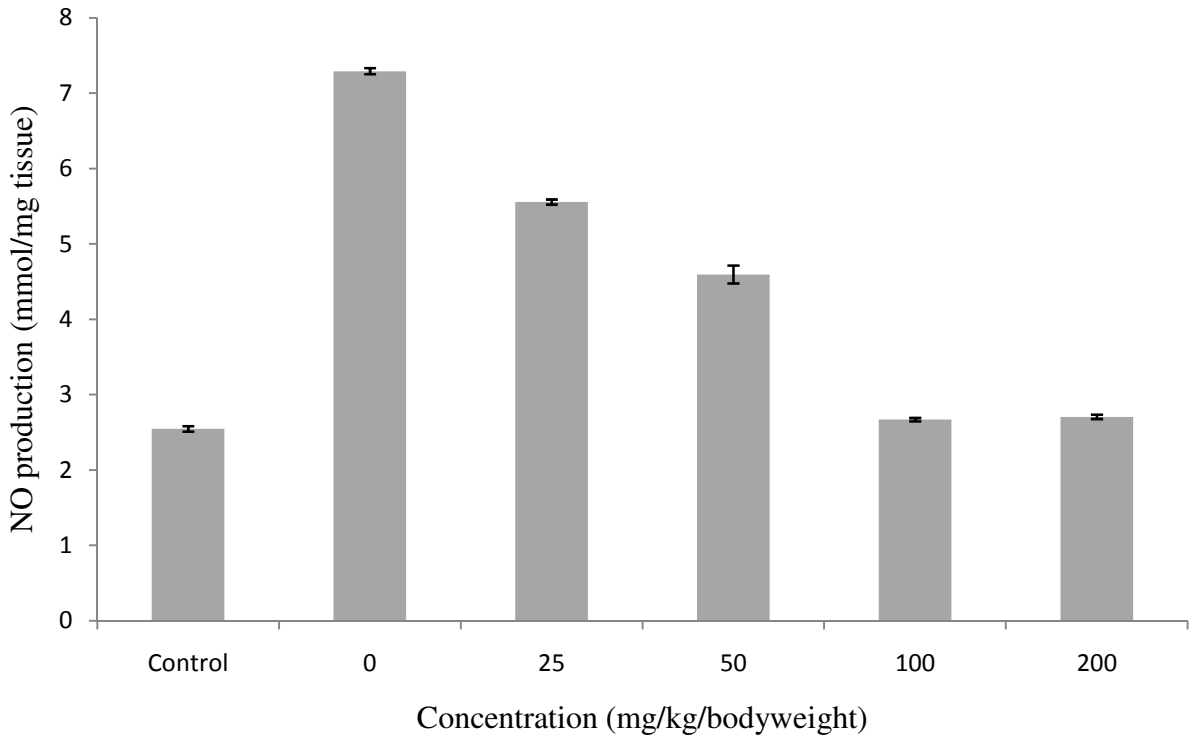


Figure 5.4: Effect of APA microcapsules containing thalidomide (0, 25, 50, 100 and 200 mg/kg/bodyweight) on NO production in colonic tissues following TNBS injection. Values are shown as mean + S.D. of mice for each group.

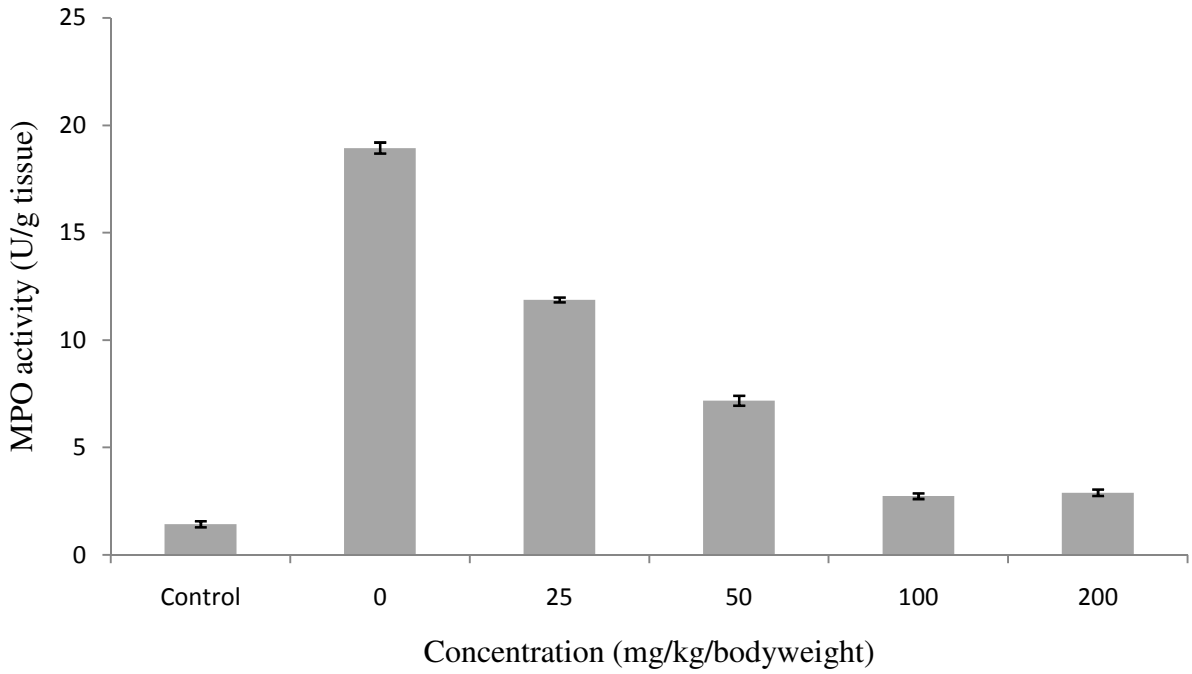


Figure 5.5: Effect of APA microcapsules containing thalidomide (0, 25, 50, 100 and 200 mg/kg/bodyweight) on MPO production in colonic tissues following TNBS injection. Values are shown as mean + S.D. of mice for each group.

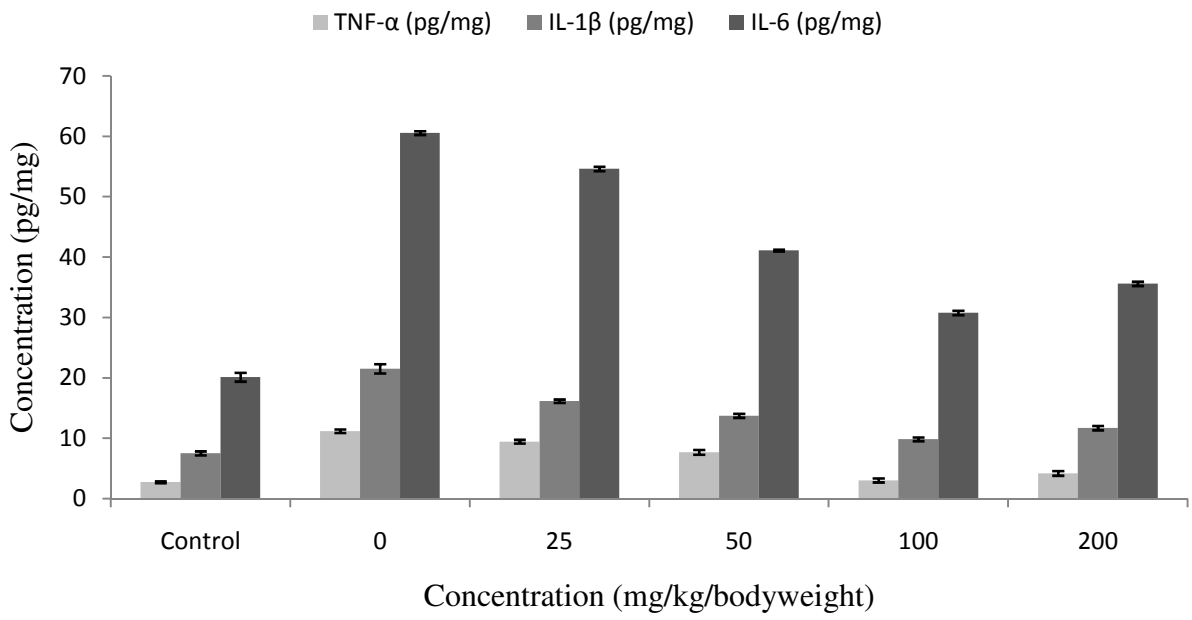


Figure 5.6: Effect of APA microcapsules containing thalidomide (0, 25, 50, 100 and 200 mg/kg/bodyweight) on cytokine production in colonic tissues following TNBS injection. Values are shown as mean + S.D. of mice for each group.

Chapter 6

General Discussion

In the present study, we investigated the anti-inflammatory properties of APA microcapsules containing thalidomide both in-vitro and in-vivo for their ability to dampen the inflammation associated in Crohn's disease.

Experiments were designed in order to test the drug release profile with respect to change in the external pH. APA microcapsules containing thalidomide were successfully prepared and designed. It was shown that incubating the capsules in saline solution for 48h did not disrupt the membrane, even after vigorous shaking at 150 rpm. This is in accordance with the literature, showing that the membrane coating remained intact following incubation in saline solution [79]. Furthermore, simulated gastric and intestine fluids were prepared in order to mimic the pH and chemical conditions normally encountered in the gastrointestinal tract. Results showed that after incubating the beads in simulated gastric fluid, the release of thalidomide was minimal, but dramatically increased following incubation in simulated intestine fluid. Moreover, a set of solutions of different pH were prepared in order to mimic the pH condition encountered in the stomach, the small intestine, the ascending colon, the transverse colon and the descending colon. Results showed a burst released of thalidomide from a low pH (2.32) to a high pH (5.31) suggesting that APA microcapsules can be efficiently used for the targeted delivery of thalidomide to regions of the gastrointestinal tracts that are the most affected by Crohn's disease.

The anti-inflammatory properties of microencapsulated thalidomide were also tested using an in-vitro model of intestinal inflammation. Lipopolysacharide (LPS) was used to

trigger inflammation in RAW 264.7 macrophage cells. Increased secretion of NO, TNF-α, IL-6 and IL-1β from RAW 264.7 cells has been demonstrated following LPS activation [62, 89]. Treating the cells with microencapsulated thalidomide significantly inhibited the production of inflammatory markers suggesting its ability to dampen the inflammation related to Crohn's disease. Moreover, a delayed inhibition of inflammatory markers of several hours was observed when treating the cells with microencapsulated thalidomide compared to the cells treated with thalidomide alone. Indeed, APA microcapsules can be used as an efficient delivery system since it allows the entrapped drug to be "slowly" released into the external environment [62, 65]. This formulation has also proven to be non-toxic since it did not affect the macrophage cell viability following 24h of incubation. Moreover, a murine model of Crohn's disease was studied by intra-rectal injection of TNBS to Balb/c mice. This is a well-established model of intestinal inflammation that closely resembles human Crohn's disease [69, 86]. Results showed that treating the mice with microencapsulated thalidomide caused a significant reduction in the concentration of pro-inflammatory cytokines in serum of experimental animals. In conclusion, the results obtained are comparable to other studies, validating the fact that thalidomide presents anti-inflammatory activities useful for the treatment of Crohn's disease [103,104], and that optimal inhibition is observed when using APA microcapsules as a delivery system.

Finally, several concentrations of microencapsulated thalidomide were tested in the TNBS-induced model of Crohn's disease. The concentration of pro-inflammatory cytokines TNF-α, IL-6 and IL-1β in serum of experimental animals significantly increased following TNBS injection. Moreover, TNBS was shown to cause a marked decreased in the bodyweight of the mice and an overall increase in the spleen weight.

Furthermore, following TNBS injection, the colon of Balb/c mice increased in weight and decreased in length. Treating the mice with microencapsulated thalidomide for a period of two weeks following TNBS administration significantly inhibited the inflammation related to Crohn's disease. The inhibitory properties of microencapsulated thalidomide were assessed by its ability to lower the concentration of inflammatory markers in serum of experimental animals and to restore the morphology of affected organs. The best results were obtained when treating the mice with microencapsulated thalidomide at a concentration of 100 mg/kg/bodyweight. Higher and lower doses (0, 25, 50 and 200 mg/kg/bodyweight) did not exhibit comparable effects. In conclusion, the present study indicated the efficacy of thalidomide in treating TNBS-induced Crohn's disease, as shown in previously published papers [10, 56], and demonstrated the fact that APA microcapsules can be used as an efficient tool for targeted drug delivery.

Chapter 7

Summary of results, Conclusions, and Future directions

7.1. Summary of results

In this thesis, a novel microcapsule formulation containing thalidomide was prepared, optimized and characterized. The feasibility of using this delivery system in targeting and delivery of thalidomide for treating Crohn's disease was investigated. Both in-vitro and in-vivo analysis were conducted to study the potential of APA microcapsules containing thalidomide in inhibiting intestinal inflammation.

Following are a summary of the results:

1. APA microcapsules containing thalidomide were successfully prepared and they displayed a consistent spherical shape with a diameter of 350–400 μm (Figure 3.2).
2. Because of the presence of electrostatic forces between the positively charged drug and the negatively charged alginate molecule, thalidomide was largely maintained within the microcapsules after 48h incubation in saline.
3. Results show that thalidomide release was minimal when the microcapsules were incubated in SGF, but significantly increased upon incubation in SIF.
4. Results also showed that thalidomide release from APA microcapsules is dependent on the pH of the external environment. Transferring the APA microcapsules from a low pH (2.32) to a high pH (5.31) significantly caused a burst release of thalidomide.
5. Results from LPS-induced RAW 264.7 macrophage cells study demonstrated that APA microcapsules containing thalidomide inhibited the secretion of NO, TNF-α, IL-6 and IL-1β.

6. It was also shown that treating the cells with microencapsulated thalidomide resulted in a delayed inhibition of inflammatory markers of several hours compared to the cells treated with thalidomide alone.
7. Results also showed that treating RAW 264.7 cells with empty APA microcapsules, APA microcapsules containing thalidomide, thalidomide solution (0.7 mg/ml) and LPS (10 µg/ml) did not affect cell viability.
8. After treatment with LPS (10 µg/ml), the macrophage lost their circular shape and became more elongated.
9. In-vivo analysis showed that thalidomide is much more effective in reducing the concentration of pro-inflammatory cytokines in serum of TNBS-induced animals when administered inside APA microcapsules.
10. In-vivo studies showed that anal injection of TNBS (120 mg/kg/bodyweight) was successfully able to induce Crohn's disease in Balb/c mice while maintaining the mortality rate very low.
11. Microencapsulated thalidomide was effective in reducing the amount of NO, MPO, TNF-α, IL-6 and IL-1β in serum of experimental animals, in a dose dependence manner.
12. Administration of TNBS caused a marked decreased in the body weight of the mice and affected the morphology of the spleen and colon. Moreover, following TNBS injection, the colon of Balb/c mice increased in weight and decreased in length. Treating with microencapsulated thalidomide was shown to restore the bodyweight of the mice and the morphology of affected organs.

13. A dose of 100 mg/kg/bodyweight of thalidomide yielded the optimal results. Thalidomide was shown to be more effective in reducing intestinal inflammation when administered using APA microcapsules.

7.2. Conclusions and future directions

In the present study, a novel microencapsulation formulation of thalidomide was tested for its ability in treating Crohn's disease. In-vitro study using several gastrointestinal solutions was performed to characterize the drug profile release. LPS-induced RAW 264.7 macrophage cells were also used to mimic inflammation associated with Crohn's disease and test the anti-inflammatory properties of artificial cells containing thalidomide. Moreover, we examined the in-vivo characteristics of a novel microencapsulated thalidomide formulation in a murine model of experimental Crohn's disease. Crohn's disease was induced with a single intra-colonic injection of 120 mg/kg/bodyweight of 2,5,6-trinitrobenzene sulfonic acid (TNBS) dissolved in 30% ethanol in Balb/c mice. The objective was to evaluate the in-vivo ability of APA microcapsules in delivering it content to desired locations of the gastrointestinal tract that are the most affected by Crohn's disease. The results obtained from the present study can lead to the following conclusions:

1. This study proposes the use of microencapsulated of thalidomide using an alginate-polylysine-alginate membrane for the treatment of Crohn's disease.
2. This formulation was effective in providing a "slow" release of thalidomide in a pH dependent manner.
3. APA microcapsules containing thalidomide was effective in reducing the amount of inflammatory markers in LPS-activated RAW 264.7 macrophage cells.
4. Moreover, this study was able to demonstrate the efficiency of APA microcapsules containing thalidomide in treating inflammation associated with Crohn's disease.

This study has established the feasibility of this novel formulation in reducing the inflammation associated with Crohn's disease using in-vitro and in-vivo models. However, more research needs to be performed to further investigate the biocompatibility of APA microcapsules containing thalidomide and its suitability in treating human Crohn's disease.

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