DEVELOPMENT OF MICROPARTICLES WITH ENTRAPPED DUST MITE ALLERGEN EXTRACT

By
Tittaya Suksamran

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree
MASTER OF PHARMACY
Program of Pharmaceutical Technology
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การพัฒนาไมโครพาร์ทิเคิลกักเก็บสารก่อภูมิแพ้สกัดจากไรฝุ่น

โดย
นางสาวทิษฏยา สุขสำราญ

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The Graduate School, Silpakorn University has approved and accredited the thesis title "Development of Microparticles With Enrapped Dust Mite Allergen Extract" submitted by Miss Tittaya Suksamran as a partial fulfillment of the requirements for the degree of Master of Pharmacy in Pharmaceutical Technology.

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The entrapment of allergen in biodegradable microparticles offers the potential for novel forms of hyposensitization therapy since it can reduce the number of doses through controlled release of allergens, and may be used for oral administration. In this study, allergen (Al) from dust mite (*Dermatophagoides pteronyssinus*) was entrapped in 4 types of microparticles; calcium alginate microparticles (Ca-alginate), chitosan microparticles (CS-TPP), Ca-alginate coated CS-TPP microparticles and CS-CA-mPEG micelles. The effects of amount of polymer, crosslinking agents, and preparation methods on the physical characteristics of microparticles i.e. size and shape, the entrapment efficiency and the *in vitro* release were investigated. It was found that Ca-alginate microparticles prepared from 0.05% alginate low viscosity and 4% $CaCl_2$ had spherical shape and small size. The mean particle sizes of Bare-Ca-alginate and 5%Al-Ca-alginate microparticles were 3560 ± 131 nm and 598 ± 44 nm, respectively. The preparation of CS-TPP microparticles using 0.1% chitosan–0.2% TPP yielded spherical shape and small particles with the average size of 1740 ± 159 and 2343 ± 328 nm for Bare-CS-TPP and 2.5% Al-CS-TPP microparticles, respectively. Ca-alginate coated CS-TPP microparticles which were prepared from 0.25% alginate high viscosity -6% $CaCl_2$ and then coated with 2.5%Al-CS-TPP also showed spherical shape about 14.88 ± 1.84, 17.12 ± 2.04 µm. In Al entrapment study, initial Al of 0.25-10% w/w of polymer was incorporated into microparticles. The results revealed that Ca-alginate, CS-TPP, Ca-alginate coated CS-TPP microparticle and CS-CA-mPEG micelles showed the highest entrapment efficiency of 61.65$\pm$0.01%, 1.16$\pm$0.27%, 26$\pm$8.7%, 2.5$\pm$1.3% and loading content of 3.34$\pm$0.76, 1.16$\pm$0.2, 0.4$\pm$0.19, 0.141$\pm$0.05 mg/g, respectively. The *in vitro* release of Ca-alginate coated CS-TPP showed lower burst release and more sustain release than other types of microparticles, and therefore have a potential to be applied for mucosal vaccine delivery.

Program of Pharmaceutical Technology Graduate School, Silpakorn University Academic Year 2008
Student's signature .....
Thesis Advisors' signature 1. ……. 2. …….

50353203 : สาขาวิชาเทคโนโลยีเภสัชกรรม
คำสำคัญ : ไมโครพาร์ทิเคิล/อัลจิเนตไมโครพาร์ทิเคิล/ไคโตซานไมโครพาร์ทิเคิล

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การกักเก็บสารก่อภูมิแพ้ไว้ภายในไมโครพาร์ทิเคิลซึ่งเตรียมจากพอลิเมอร์ที่สลายได้เองในร่างกายนั้นมีศักยภาพเพียงพอที่จะเป็นแนวทางใหม่ในการรักษาโรคภูมิแพ้ในผู้ป่วย เนื่องจากสามารถควบคุมการปลดปล่อยของสารก่อภูมิแพ้ที่ใช้ได้จึงสามารถลดปริมาณของสารก่อภูมิแพ้ที่ใช้ในการรักษาลง และอาจนำมาใช้ในรูปแบบทางการรับประทาน การศึกษาครั้งนี้ใช้สารก่อภูมิแพ้สกัดจากไรฝุ่น (*Dermatophagoides pteronyssinus*) มาทำการกักเก็บอยู่ในไมโครพาร์ทิเคิล 4 ชนิดได้แก่ แคลเซียมอัลจิเนต ไคโตซานไตรพอลิฟอสเฟต ไคโตซานไตรพอลิฟอสเฟต เคลือบด้วยแคลเซียมอัลจิเนต และ ไคโตซานโคลิคพอลิเอทิลีนไกลคอลไมเซลล์ ศึกษาปัจจัยต่างๆ เช่น ปริมาณของพอลิเมอร์ สารเชื่อมพันธะ และวิธีการเตรียม ต่อลักษณะทางกายภาพของไมโครพาร์ทิเคิล เช่น ขนาดและรูปร่าง ความสามารถในการกักเก็บสารก่อภูมิแพ้ และการปลดปล่อยสารก่อภูมิแพ้ภายนอกร่างกาย ผลการทดลองพบว่าแคลเซียมอัลจิเนตไมโครพาร์ทิเคิลซึ่งเตรียมจาก 0.05% อัลจิเนตชนิดความหนืดต่ำและ 4% แคลเซียมคลอไรด์ เป็นสูตรที่ให้ขนาดเล็กและมีรูปร่างกลม อัลจิเนตไมโครพาร์ทิเคิลเปล่าและผสมกับสารก่อภูมิแพ้ความเข้มข้น 5% มีขนาดเท่ากับ 3560 $\pm$ 131 นาโนเมตร และ 598 $\pm$ 44 นาโนเมตรตามลำดับ ไคโตซานไตรพอลิฟอสเฟตเตรียมจาก 0.1 %ไคโตซาน และ 0.2 % ไตรพอลิฟอสเฟตเป็นสูตรที่ให้ขนาดเล็กและมีรูปร่างกลมโดยไคโตซานไตรพอลิฟอสเฟตเปล่าและที่ผสมสารก่อภูมิแพ้ที่ความเข้มข้น 2.5% มีขนาดเท่ากับ 1740 $\pm$ 159 และ 2343 $\pm$ 328 นาโนเมตรตามลำดับ สำหรับไคโตซานไตรพอลิฟอสเฟตเปล่าและไคโตซานไตรพอลิฟอสเฟตผสมสารก่อภูมิแพ้ที่ความเข้มข้น 2.5% เคลือบด้วยแคลเซียมอัลจิเนตโดยใช้ 0.25 % อัลจิเนตความหนืดสูง และ 6% แคลเซียมคลอไรด์ นั้นมีรูปร่างที่ค่อนข้างกลมมีขนาดอยู่ในช่วง 14.88 $\pm$ 1.84, 17.12 $\pm$ 2.04 ไมโครเมตร ผลการหาปริมาณการกักเก็บสารก่อภูมิแพ้นั้นใช้สารก่อภูมิแพ้ในช่วง 0.25 - 10% w/w ต่อน้ำหนักพอลิเมอร์ ผลการทดลองพบว่าปริมาณการกักเก็บสารก่อภูมิแพ้ในแคลเซียมอัลจิเนต ไคโตซานไตรพอลิฟอสเฟต ไคโตซานไตรพอลิฟอสเฟต เคลือบด้วยแคลเซียมอัลจิเนต และ ไคโตซานโคลิคพอลิเอทิลีนไกลคอลไมเซลล์ มีค่าสูงสุดของการกักเก็บในแต่ละชนิด เท่ากับ 61.65 $\pm$ 0.01%, 1.16 $\pm$ 0.27%, 26 $\pm$ 8.7%, 2.5 $\pm$ 1.3% และปริมาณสารก่อภูมิแพ้ต่อน้ำหนักไมโครพาร์ทิเคิลมีค่าเท่ากับ 3.34 $\pm$ 0.76, 1.16 $\pm$ 0.2, 0.4 $\pm$ 0.19 และ 0.141 $\pm$ 0.05 มิลลิกรัมต่อกรัมตามลำดับ ผลการทดสอบการปลดปล่อยภายนอกร่างกายพบว่า ไคโตซานไตรพอลิฟอสเฟตเคลือบด้วยแคลเซียมอัลจิเนต ให้ผลการปลดปล่อยยาช่วงแรกที่ช้ากว่าและเนิ่นนานกว่าไมโครพาร์ทิเคิลชนิดอื่น ดังนั้นจึงสามารถนำผลที่ได้ดังกล่าวมาศึกษาเพิ่มเติมเพื่อประยุกต์ใช้ในการนำส่งวัคซีนรักษาโรคภูมิแพ้ทางการรับประทานได้

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CHAPTER I
INTRODUCTION

1. Statement and significance of the research problem

Allergy is one of the most widespread diseases of the modern world. More than 25% of the population in developed countries suffer from allergies (Valenta 2002 : 446-453). Allergies, also known as hypersensitive reactions occur when the immune system overreacts to substances that do not affect most people. These substances also known as allergens, could be pollen, animal dander, chemicals, fungi, dust mites, or foods such as nuts, eggs, shellfish, fish, and milk**.** Different people show different symptoms of allergies ranging from mild (runny nose) to severe (anaphylaxis). The immune system protects our body against pathogens and other foreign substances by producing a kind of glycoprotein known as immunoglobulin (Ig) or antibodies from plasma cells or B-cells (a type of lymphocyte). Antibodies are mainly of five types, each one having a different function. The type involved in allergy is immunoglobulin E (IgE), which is over produced during an allergic response. On the very first exposure to an allergen, an allergic person becomes sensitized by producing allergen specific IgE that binds with IgE receptors on mast cells (in tissues) and basophils (in circulation) (Suri 2006 : 1-12). If the sensitized person has another exposure to this specific allergen, then this allergen will bind to the antigenic determinant site (Fab) of IgE attached to the mast cells and basophils and trigger the release of mediators, which are responsible for the symptoms (hay fever, asthma, urticaria, food allergy, anaphylactic shock). T-helper (Th) lymphocytes of the Th2-type as well as mast cells are predominantly responsible for interleukin (IL)-4 and IL-13 production and the isotype switch towards IgE. The resulting immune reactivity pattern in allergy is, therefore, called Th2 response. Nowadays, the only causative treatment is the immunotherapy, which consists of repetitive allergen applications and results in permissiveness of the allergen. Allergen immunotherapy (modulated Th2 response) is the process by which increasing doses of an allergen are injected subcutaneously (under the skin) over time as a treatment to prevent allergic

symptoms. These antibodies trap the allergen before triggering of effector cells can occur (blocking antibodies). In murine models, a shift towards Th1-type antibody IgG2a is indicative for successful therapy (Schöll et al. 2004 : 104). However, most of vaccinations to treat with immunotherapy are performed by parenteral administration. Parenteral vaccinations require trained personnel and sterilized materials, and infusion often causes noncompliance to patients (Hori et al. 2005 : 297).

Then mucosal administration of vaccines offers a number of advantages over the traditional approach to vaccine delivery, which normally involves systemic injection using a needle and syringe. Mucosal delivery of vaccines would avoid the pain and discomfort associated with injections, and would also eliminate the possibility of infections caused by inadequately sterilized needles, or needle re-use. Moreover, mucosal vaccines would be less expensive to produce, since they would not need to be manufactured under such stringent conditions as systemic vaccines. In addition, mucosal vaccines would be less expensive to administer, since trained personnel would not necessarily be required for immunization. Mucosal administration of vaccines might also result in improvement in vaccine efficacy, since mucosal delivery would stimulate mucosal immunity at the sites where most pathogens initially infect hosts. In contrast, systemic immunization does not normally result in the induction of mucosal immunity. The induction of mucosal immunity might prove to be particularly advantageous in the elderly, since unlike systemic immunity, mucosal immunity does not appear to be subjected to age-associated dysfunction. Mucosal immunization might also be an attractive approach in the very young, since mucosal immunity appears to develop earlier than systemic immunity. In addition to oral delivery, intranasal immunization is also attractive, since the nose is readily accessible and does not present the problems of low pH and abundant luminal enzymes which are inherent for the oral route. Alternative routes of mucosal immunization which are less attractive, but might be successfully exploited in certain circumstances, include pulmonary inhalation, rectal and ocular immunization.

The use of polymeric microparticles offers significant potential for the development of mucosal administered vaccines. Microparticles can be prepared from a range of different polymers which can be designed to protect entrapped vaccines against degradation in the gut, to delay the gastric transit of the vaccine or to target

vaccines for uptake into the mucosal associated lymphoid tissues (MALTs) of the Peyer's patches (PPs). In addition, similar microparticles can be applied intranasally for the delivery of antigens to the MALTs of the upper respiratory tract. For uptake into the MALT of the gut or the respiratory tract, microparticles need to be prepared with the appropriate dimensions (i.e. 10 nm). As an alternative approach to oral delivery, microparticles may also be designed simply to protect the vaccine against degradation in the gut and to release it in the vicinity of the PPs for subsequent uptake (Derek et al. 1994 : 34). A number of different polymers have been evaluated for the development of oral vaccines, including naturally occurring polymers (e.g. starch, alginates and gelatin) and synthetic polymers (e.g. polylactide-co-glycolides (PLG), polyanhydrides and phthalates). However primary concern are considerations of toxicity, irritancy and allergenicity, and the need for a biodegradable or a soluble coating (Singh et al. 1998 : 34). The advantages of using natural polymers include their low cost, biocompatibility and aqueous solubility. The most commonly studied polymers are alginate and chitosan. There has been increasing interest in the study in connection with various applications mainly for their non-toxic, biocompatible, biodegradable and mucoadhesive properties. They have shown their potential for use as scaffolds in tissue-engineered medical products (Pariente et al. 2001 : 33-39), as an encapsulating matrix for immobilization of living cells (Iyer et al. 2005 : 493-497) and as drug delivery systems (Tozaki et al. 1999 : 1107–1112, 2002 : 51-61; Giunchedi et al. 2002 : 233-239; Hejazi and Amiji 2003 : 151-165). Among the various drug delivery systems investigated to achieve efficient and site-specific delivery microparticles have received considerable attention (Mladenovska et al. 2007 : 345). These polymers have been approved for use in humans (e.g. as sutures, bone implants and screws as well as implants for sustained drug delivery) and have been extensively studied for use in the formulation of vaccine antigens (i.e. proteins, peptides, DNA, etc.). Microparticle encapsulation has been employed for a number of different vaccines, like meningococcal C conjugate (Baudner et al. 2002 : 4785–4790), diphtheria (Lubben et al. 2003 : 1400–1408) and tetanus toxoid (Jaganathan et al. 2005 : 4201-4211; Vila et al. 2004 : 123-131). However, few studies about the encapsulation of dust mite (*Dermatophagoides pteronyssimus*) allergen, the major

causes of allergies, have been reported. Therefore, the encapsulation aimed for this purpose seems to be an interesting research.

2. Objective of this research

The objective of this research was to formulate a high loading and high stability of allergen in Calcium alginate, Chitosan-tripolyphosphate and Calcium alginate coated chitosan-tripolyphosphate microparticles and Chitosan-cholic-polyethylene glycol micelle. The formulation factors such as the initial allergen added and the concentration of the polymers on the physicochemical property of the microparticles (i.e. particle size, size distribution, droplets surface charge), percent entrapment efficiency and percent *in vitro* allergen release were evaluated.

3. The research hypothesis

Different formulation factors involved in the preparation of microparticles significantly influence on the physicochemical properties, entrapment efficiency and *in vitro* release of allergen from the yielded microparticles.

CHAPTER II
LITERATURE REVIEW

1. Allergy

Allergy is one of the most widespread diseases of the modern world. More than 25% of the population in industrialized countries suffers from allergies (Valenta 2006 : 446-453). Allergy, or hypersensitivity type I, applies to an abnormal reaction against innocuous environmental compounds (allergens) and involves complex interactions between exogenous and genetically determined factors (Gómez et al. 2008 : 711-717). Different people show different symptoms of allergies, which can be mild (runny nose) to severe (anaphylaxis). Symptoms generally depend upon the part of body contacted by the allergen, e.g. pollens from the air enter the respiratory tract via the nose and cause respiratory symptoms such as cough, itchy and runny nose, nasal congestion, sneezing, and wheezing. Food allergy related symptoms include vomiting, nausea, abdominal pain, and diarrhea. Skin allergy symptoms are lesions, rashes, blisters, redness and itchiness, and so on. The immune system protects our body against pathogens and other foreign substances by producing a kind of glycoprotein known as immunoglobulin (Ig) or antibodies from plasma cells or B-cells (a type of lymphocyte). Antibodies are mainly of five types, each one having a different function; the type involved in allergy is immunoglobulin E (IgE). Immunoglobulin E (IgE) is overproduced during an allergic response. On the very first exposure to an allergen, an allergic person becomes sensitized by producing allergen specific IgE that binds with IgE receptors on mast cells (in tissues) and basophils (in circulation). If the sensitized person has another exposure to this specific allergen, then this allergen will bind to the antigenic determinant site (Fab) of IgE attached to the mast cells and basophils. Binding of two or more IgE molecules to mast cells (crosslinking) is required to activate the mast cells. These activated cells result in the release of certain chemicals, such as histamine, serotonin, proteoglycans, serine protease, leukotriene C4 and heparin that will further bind with their receptors

present in other cells (e.g. histamine receptors of blood vessels) and lead to inflammation, irritation, redness and other allergic symptoms.

The primary function of our immune system is to defend against infection; however, during an allergic reaction the immune system responds against a substance that is harmless to most people. There are two subpopulations of T helper cells, Th1 and Th2. Th1 cells are helpful in protecting against invading microbes and other particles by producing interferons and some cytokines. Th2 cells are responsible for triggering allergies by the overproduction of IgE, and are also involved in the struggle against parasitic worms. Th2 cells produce cytokines like interleukins (such as IL-5) that enhance the production of specific IgE antibodies by B cells and result in hypersensitivity, eosinophil activation, mucus production and IgE secretion (Drouin et al. 2001 : 4141-4145). The resulting immune reactivity pattern in allergy is, therefore, called Th2 response.

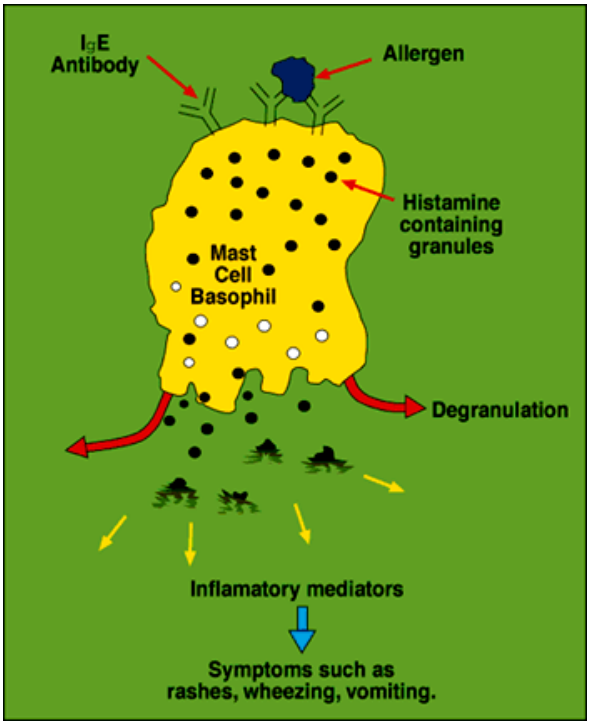


Figure 1 Mechanism of allergy

Source : A. Pipet et al. “Allergen-specific immunotherapy in allergic rhinitis and asthma; Mechanisms and proof of efficacy,” Respiratory Medicine, 2009. (Mimeographed)

2. Allergen

The most important sources of allergens are wind-dispersed pollen grains from trees, grasses, and weeds, followed by excretions of house dust mites and cockroaches, fungal spores, and animal dander and insect venoms. The route of exposure, dose, and function of the allergen are crucial to mount an allergic sensitization. Sensitization occurs at the site of allergen exposure, such as the airways and skin, but can also occur through the gastrointestinal tract. In general terms, exposure to low allergen concentrations induces IgE production and allergy, whereas exposure to high allergen doses induces tolerance through regulatory T cells, a modified Th2 cell response with the production of high levels of allergen-specific IgG4 antibodies, or both that can block the binding of IgE-allergen complexes to effector cells of the allergic immune response (Hoffmann et al. 2009 : 558-566). Allergens that elicit type I allergies are mostly proteins or glycoproteins and cluster in less than 2% of 9318 known protein families (Radauer and Breiteneder 2006 : 141-147; 2008 : 847-852). This would seem to imply that structural and biochemical similarities between allergenic proteins and the comparison of allergenic and nonallergenic members of the same protein family could explain what determines allergenicity. The primary structure (amino acid sequence) of a protein allows its physicochemical properties, such as molecular weight, isoelectric point (charge), hydrophobicity, and stability, to be predicted. Computational analysis of the major allergens has shown that most are relatively small (<70 kDa) negatively charged proteins with low hydrophobicity and high stability (Chapman 2007 : 414-420). In addition, posttranslational modifications, such as glycosylation or the presence of disulfide bonds, can increase the stability and bioavailabilty of allergens (Claudia et al. 2009 : 558-566).

Indoor allergen exposure to sources such as house-dust mites, pets, fungi, and insects plays a significant role in patients with allergic rhinitis and asthma. The examples of indoor allergen are described as follow.

2.1 House dust mites

The house dust mite is a cosmopolitan guest in human habitation. Dust mites feed on organic detritus such as flakes of shed human skin and flourish in the stable environment of dwellings. House dust mites are a common cause of asthma and

allergic symptoms worldwide. Some of the gut enzymes (notably proteases) produced by the house mite persist in their fecal matter, and can be strongly allergenic. The European house dust mite (*Dermatophagoides pteronyssinus*) and the American house dust mite (*Dermatophagoides farinae*) are two different species, but are not necessarily confined to Europe or North America; a third species *Euroglyphus maynei* also occurs widely. The body of a house dust mite is visible against a dark background in a normal light. A typical house dust mite measures 420 μm in length (almost 0.5 millimeters) and 250 to 320 μm in width. Both male and female adult house dust mites are creamy white and have a globular shape. The body of the house dust mite also contains a striated cuticle. Like all acari, house dust mites have eight legs (except 3 pairs in the first instar). Dust mites can be transported airborne by minor air currents generated from normal household activities. The average life cycle for a male house dust mite is 10 to 19 days. A mated female house dust mite can live for 70 days, laying 60 to 100 eggs in the last 5 weeks of her life. In a 10 week life span, a house dust mite will produce approximately 2000 fecal particles and an even larger number of partially digested enzyme-infested dust particles. A simple washing will remove most of the waste matter. Both being exposed to temperatures of over 60°C (140°F) for a period of one hour, freezing, or exposure to temperatures below 20°C, will typically prove fatal to house dust mites; a relative humidity less than 50 may also be fatal. Ten minutes in a household clothes dryer at lethal temperatures has been shown to be sufficient to kill all the dust mites in bedding (Miller and Miller 1996 : 423). House dust mites reproduce quickly enough that their effect on human health can be significant. The house dust mite survives in all climates, even at altitude. If trying to control house dust mites, humidity should be kept low. House dust mites thrive in the indoor environment provided by homes specifically in bedrooms and kitchens. Dust mites survive well in mattresses, carpets, furniture and bedding, with figures around 188 animals/g dust. Even in dry climates, house dust mites survive and reproduce easily in bedding (especially in pillows) because of the humidity generated by the human body during several hours of breathing and perspiring. House dust mites consume minute particles of organic matter. Like all acari, house dust mites have a simple gut; they have no stomach but rather diverticulae, which are sacs or pouches that divert out of hollow organs. Like many

decomposer animals, they select food that has been pre-decomposed by fungi. House dust mites eat the same particle several times, only partially digesting it each time; between feedings, house dust mites leave particles to decompose further. Only when the particles are fully digested do they enter the dust mite's fecal matter. A person sheds about 1.5 grams of skin cells and flakes every day (approximately 0.3–0.45 kg per year), which is enough to feed roughly a million house dust mites under ideal conditions. House dust mites in bedding derive moisture from human breathing, perspiration, and saliva. Allergens produced by house dust mites are among the most common triggers of asthma. This allergen is mainly associated with, and conveyed by, the faecal matter of the house dust mite *Dermatophagoides pteronyssinus,* which originally appears as spherical particles ranging between 10 and 40 μm in diameter. However, fragmentation of the faecal particles is likely to result in some house dust mite *Dermatophagoides pteronyssinus* carrying particles with dimensions in the range of tenths of a micron. Their wastes are found predominantly in bedding, woven material covered soft fumishings, soft toys, carpets, curtains and clothing house. Dust mite allergen-carrying particles can easily become airborne when physically disturbed and, consequently, may be inhaled. An immune response may then be initiated that, in allergic individuals, will induce their characteristic symptoms.

2.2 Pets

The most common household pets are dogs, cats, birds, hamsters, rabbits, mice, gerbils, rats and guinea pigs. Larger animals such as horses, goats, cows, chickens, ducks and geese, even though kept outdoors, can also cause problems as pets. Pets can cause problems to allergic patients in several ways. Their dander, or skin flakes, as well as their saliva and urine, can cause an allergic reaction. The animal hair is not considered to be a very significant allergen. However, the hair or fur can collect pollen, dust, mold and other allergens. Both feathers and the droppings from birds, another common pet, can increase the allergen exposure. The allergic patient should not use feather pillows or down comforters. If a feather pillow is used, it should be encased in plastic. An encasing with a zipper is recommended, so none of the feathers can escape. Bird droppings can be a source of bacteria, dust, fungi and mold. This also applies to the droppings of other caged pets, such as gerbils, hamsters and mice.

2.3 Insects

Insect allergens can trigger asthma symptoms in people who have the kind of asthma known as allergic asthma. The most common type of insect allergen that is a factor in triggering asthma symptoms is the cockroach. Most people do not like to think about cockroaches, but a protein in their droppings is one of the most common indoor asthma triggers for people who live in densely-populated, urban environments. Cockroaches have existed for hundreds of millions of years. They thrive in warm, moist environments, and they love moving into the homes and offices of humans. Cockroaches get into a house through wall cracks, windows, gaps in floors or wood trim, cellars, drains, and doors that leads outdoors. They need water in order to live and they will seek wet areas in the home such as leaky pipes and faucets. Cockroaches also tend to be found where there are open garbage containers or food left out in the open.

Other insect droppings may also trigger asthma symptoms in certain people. An emerging problem appears to be Asian Ladybugs. These insects were purposely introduced in the United States to fight aphids, but have since become a problem on the East Coast and in the South, especially during cold weather when they seek warm indoor spaces. There is some evidence that Asian Ladybugs may cause the same kinds of asthma problems as cockroaches. Another type of insect allergen is found outdoors, in the form of stinging insects (fire ants, honeybees, hornets, paper wasps, yellow jackets, etc.). Stinging insects that can cause allergies belong to the class of insects known as *Hymenoptera*. Stinging insects trigger allergies, not asthma. For most people who are stung by a stinging insect, there may be some short-term discomfort, but no serious side effects. Other people may have a mild allergic reaction to the venom of a stinging insect and notice some mild allergy symptoms such as sneezing, itching, nasal congestion, and watery red eyes. However, a small percentage (5%) of people are extremely allergic to stinging insects. These people may have more severe symptoms that combine into a condition known as anaphylaxis, or allergic shock.

3. Allergen-specific immunotherapy

Mainstay treatment for allergic diseases is allergen avoidance, where feasible, and the use of non-specific pharmacotherapy including antihistamines, beta-

2 agonists and corticosteroids for symptomatic relief. Severe, life-threatening anaphylactic reactions require treatment with adrenaline. Although effective at controlling symptoms, administration of anti-inflammatory treatments is not without side effects especially with prolonged use. In selected patients where there is a clear demonstration of symptoms on exposure to the offending allergen and documentation of allergen-specific IgE, allergen-specific immunotherapy (SIT) to target the underlying disease process may be instigated. This treatment is attractive as it selectively modulates the allergen specific immune response and is potentially curative (Rolland et al. 2009 : 273–28). Allergen immunotherapy is described to induce a so called modulated Th2 response, which is characterized by antibodies of the IgG4 subclass (Woodfolk et al. 2002 : 277– 285).

In retrospect, the rationale for using allergen to treat an allergen-induced disease as pioneered by Noon and Freeman in 1911 (Noon 1911 : 1572-1573) seems counter-intuitive. However, inspired by other vaccination successes of the day, these workers sought to vaccinate allergic individuals to induce ‘anti-toxins’ which would prevent disease. Indeed they found that appropriate doses of allergen could induce a state of clinical and immune tolerance to that allergen. Since this time, subcutaneous allergen-specific immunotherapy (SCIT) has been developed as a routine therapy and best practice protocols have been defined (Bousquet et al. 1998 : 439-446). Conventional SCIT involves injection of increasing quantities of allergen extract during an updosing phase followed by repeated injection of allergen at a maximum tolerated dose over three to five years in a maintenance phase (Rolland et al. 2000 : 515-527). Controlled trials have demonstrated efficacy in appropriately selected patients with venom hypersensitivity (Lerch et al. 1998 : 606-612), seasonal allergic rhinitis/conjunctivitis (Varney et al. 1991 : 265-269) and mild asthma. Importantly, SCIT can prevent the development of new sensitivities in monosensitized patients (Roches et al. 1997 : 450-453; Purello-D'Ambrosio et al. 2001 : 1295-1302) as well as the progression to asthma in patients with allergic rhinitis (Jacobsen et al. 2007 : 943-948; Moller et al. 2002 : 251-256). However, despite advances in standardization of allergen extracts and treatment protocols, SCIT is not recommended for patients with moderate to severe asthma, and immunotherapy with unfractionated extracts of potent allergens such as natural rubber latex, peanuts and shellfish is not currently available

due to the high risk of systemic adverse events (Rolland et al. 2000 : 515-527). Modification of SIT regimens, especially allergen preparations, is required to permit more widespread use of this therapy in clinical practice. Conventional SCIT is administered by subcutaneous injection of unfractionated extract in either alum-precipitated or aqueous form. Aqueous allergen preparations allow more rapid allergen absorption but are often highly potent. To minimize adverse reactions due to inappropriate dosage, allergen extracts are standardized as "Bioequivalent Allergy Units" or by quantitation of the dominant allergen. Although attractive for treatment of patients with multiple sensitivities, use of mixtures of unrelated allergens may be less efficacious and only proven mixtures containing known amounts of each component should be used (Bousquet et al. 1998 : 439-446). During the updosing phase, a small dose of allergen extract is given first followed by slowly increasing increments to minimize adverse allergic reactions, particularly anaphylaxis. The standard SCIT dosage schedule involves injections given once to twice weekly during the induction phase followed by maintenance injections at increasing intervals as tolerated up to monthly. Regimens are usually continued for three to five years (Durham et al. 1999 : 468-475; Lerch et al. 1998 : 606-612). More rapid dose escalations may be required for urgent treatment of anaphylaxis and venom allergy. For these regimens, the induction phase is completed over a period of about five days (rush) or even one day (ultra-rush) with typically injection intervals of one hour and treatment is usually performed in a hospital setting due to the higher incidence of systemic reactions (Cox 2008 : 432-434; Hejjaoui et al. 1990 : 473-479; Nelson 2007 : 769-779). Not withstanding the regimen, all immunotherapy injections require close medical supervision with adrenaline and adequate resuscitation facilities available. Immunotherapy is rarely started in children before the age of five years.

Sublingual immunotherapy (SLIT), an alternative route of administration for allergen-specific IT, is performed by the patients themselves and most commonly requires the placement of 1 to 5 drops of allergen under the tongue each day. The drops are kept in place for approximately 2 minutes, then swallowed. In contrast to SCIT that typically has an escalation phase of many months, maintenance dosing for SLIT is frequently reached in 4 to 5 weeks. Although severe adverse reactions during SLIT have been limited to a few recent case reports, SCIT has a systemic reaction rate

of 0.5% to 5.6% (Dunsky et al. 2006 : 1235; Passalacqua et al. 2006 : 43-51; Antico 2006 : 1236-1237).

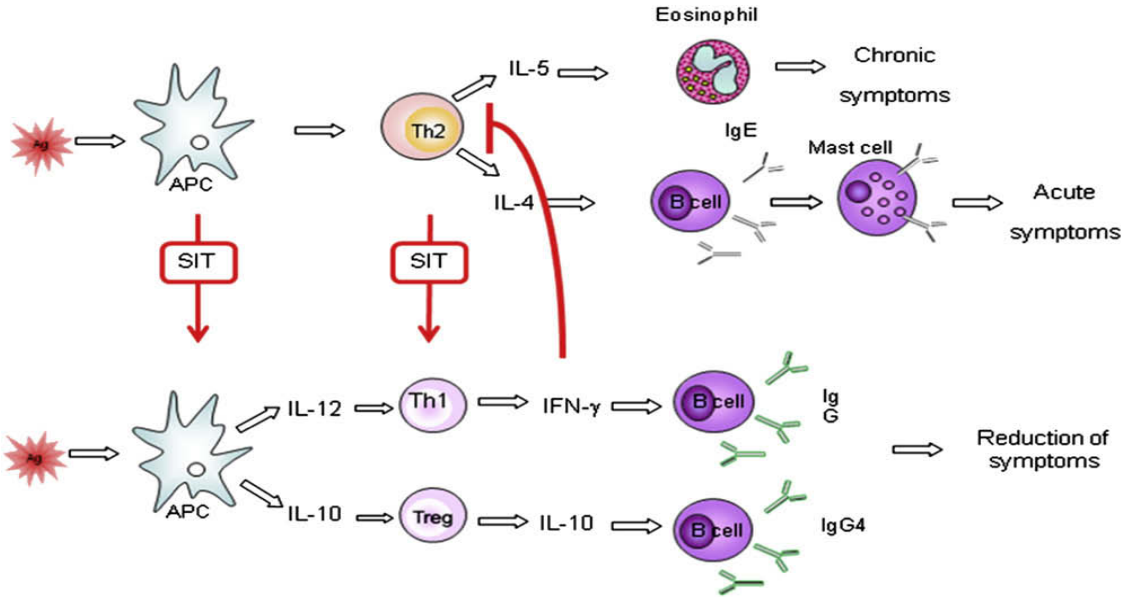


Figure 2 Diagram of allergen immunotherapy, antigen presentation results in a Th2 differentiation in allergy, leading to the eosinophil and mast cell mediated inflammatory reaction. Specific immunotherapy (SIT) acts both on antigen presenting cells and T cells to prevent symptoms. Ag: antigen, APC: antigen presenting cell.

Source : A. Pipet et al. "Allergen-specific immunotherapy in allergic rhinitis and asthma; Mechanisms and proof of efficacy," Respiratory Medicine, 2009. (Mimeographed)

Although SLIT is currently used in many European countries, questions about optimal dose, efficacy, and safety remain. In a multinational, randomized, double-blind, placebo-controlled study, 628 adults with grass pollen–induced rhino conjunctivitis were assigned to either 100 index of reactivity (IR), 300 IR, or 500 IR of a standardized 5-grass pollen extract or placebo administered sublingually using a once-daily tablet form. The 300 IR/mL dose was approximately equivalent to 25 mg/mL of the group 5 major allergens. Treatment was started 4 months before the estimated pollen season and continued throughout the season. Both the 300- and 500-IR doses significantly reduced mean rhinoconjunctivitis total symptom scores compared with placebo (37% and 35% improvement, respectively). The 100-IR group

was not significantly different from the placebo group. No serious side effects were reported, although the 500-IR tablet induced more adverse effects than the 300-IR dose. The authors concluded that the 300-IR 5-grass pollen tablet was the optimal dose for treatment. In a study of birch pollen SLIT, researchers examined the effects on apple allergy or oral allergy syndrome to apple. Nine of 20 SLIT-treated subjects experienced a decrease in nasal response to birch pollen extract, a decrease in skin test reactivity to birch, and an improvement of allergic symptoms during the birch pollen season. However, no subject showed a reduced sensitivity to oral challenge with apple or reported improvement in oral allergy symptoms with eating. SLIT significantly increased levels of Bet v 1 (from birch pollen)–specific IgE and IgG4 and decreased PBMC responses to Bet v 1 but failed to induce similar humoral and cellular responses to Mal d 1 (from apple). This contrasts with a previous report of SCIT with birch pollen reducing oral allergy syndrome to apple, indicating that SLIT might not be as efficacious as SCIT (Saltoun et al. 2007 : 481-487).

The major disadvantage of immunotherapy is its requirement of a prolonged course of weekly injections. The process is as follows; injections of diluted extracts of the allergen are given on a regular schedule, usually twice per week to weekly at first, then in increasing doses until a maintenance dose has been reached. At that time, intervals between shots can be two to four weeks, and the treatment is continued for up to three to five years. Patients can experience some relief within three to six months; if there is no benefit within 12 to 18 months, the shots should be discontinued. After stopping immunotherapy, about one third of allergy sufferers no longer have any symptoms, one third have improved symptoms and one third relapse completely. Injections for ragweed and, possibly, excessive doses of dust mites, have higher risks for side effects than other allergy shots. If complications or allergic reactions develop, they usually occur within 30 minutes although some can develop up to two hours after the shot is given. Side effects include general itching, swelling, red eyes, hives and/or soreness at the injection site. Rarely, low blood pressure, asthma exacerbation or difficulty breathing is observed. This is due to an extreme hypersensitivity response called anaphylaxis. It can also occur if excessive doses are given. In rare cases, particularly because of excessive doses or if a patient has a serious lung problem, severe reactions can occur, which can be life threatening. It

should be noted that in one 10-year study, the incidence of any adverse effect was less than two-tenths of one percent, and the great majority of events were mild. The risk for a fatal response is estimated to be one per 63 million injections. By comparison, the risk for a fatal reaction to penicillin is much higher, one per 7.5 million injections. In addition, in 1986, the British Committee for the Safety of Medicine documented several deaths caused by SCIT and raised concerns regarding the safety of SCIT. This prompted interest in additional routes of administration for IT such as intranasal, oral, bronchial, and sublingual.

4. Mucosal drug delivery

The mucosal route of delivery is the most attractive and acceptable, but is also the most challenging and difficult to exploit for proteins, peptides and other high-molecular-mass molecules. Mucosal administration of vaccines offers a number of advantages over the traditional approach to vaccine delivery, which normally involves systemic injection using a needle and syringe. Mucosal delivery of vaccines would avoid the pain and discomfort associated with injections, and would also eliminate the possibility of infections caused by inadequately sterilized needles, or needle re-use. Moreover, mucosal vaccines would be less expensive to produce, since they would not need to be manufactured under such stringent conditions as systemic vaccines. In addition, mucosal vaccines would be less expensive to administer, since trained personnel would not necessarily be required for immunization. Mucosal administration of vaccines might also result in improvements in vaccine efficacy, since mucosal delivery would stimulate mucosal immunity at the sites where most pathogens initially infect hosts. In contrast, systemic immunization does not normally result in the induction of mucosal immunity. The induction of mucosal immunity might prove to be particularly advantageous in the elderly, since unlike systemic immunity, mucosal immunity does not appear to be subjected to age-associated dysfunction. Mucosal immunization might also be an attractive approach in the very young, since mucosal immunity appears to develop earlier than systemic immunity. In addition to oral delivery, intranasal immunization is also attractive, since the nose is readily accessible and does not present the problems of low pH and abundant luminal enzymes which are inherent for the oral route. Alternative routes of mucosal

immunization which are less attractive, but might be success fully exploited in certain circumstances, include pulmonary inhalation, rectal and ocular immunization (Derek et al. 1998 : 305–320). Certainly, the manner in which antigen is perceived at mucosal surfaces is crucial in the initiation of effective immunological identification and the subsequent orchestration of effective immune responses. The formulation of antigenic material in particulate delivery systems is potentially highly advantageous for the generation of effective immune responses via mucosal, as well as parenteral, routes (Alpar et al. 2005 : 411–430).

5. Microparticles for mucosal vaccine or peptide drug delivery

An amazingly high number of particulate antigen delivery systems have come up in the last two decades. Major interest of many researches is the development of vaccines for the treatment of IgE-mediated hypersensitivity and the possible application of the various particles for the entrapment of allergens. There are various methods of delivering antigen, and biodegradable microparticles which have been extensively evaluated for mucosal immunization. Microparticles with diameter of 10 μm or less are taken up by M-cells in Peyer's patches of the small intestine of respiratory tract and induce strong mucosal immune response. The term "microparticles" is defined, as a spherical particle with the size varying in between 50 nm to 2 μm containing a core substance. Microparticles are in strict sense, spherically empty particles. However the terms microparticles are often used synonymously. In addition, some related terms are used as well. For example, "microbeads" and "beads" are used alternatively. Sphere and spherical particles are also employed for a large size and rigid morphology. The efficacy of microparticles following mucosal delivery is, at least in part, a consequence of their uptake into the intestinal Peyer's patches following oral administration or the nasal-associated lymphoid tissue (NALT) following i.n. administration (Almeida et al. 1996 : 455–467; Beier et al. 1998 : G130–G137). The intranasal route has been shown as a highly efficient mucosal route for the induction of antibody responses in the serum, as well as local and distal mucosal secretions (Almeida et al. 1996 : 455–467; Roussel et al. 2000 : 969–972; Liang et al. 2001 : 5416–5420). It is thought that, whilst nonciliated cuboidal cells

have a particle sampling capacity, particulate antigens are preferentially taken up by M-cells, which overlay NALT (Wu et al. 1997 : 187–201).

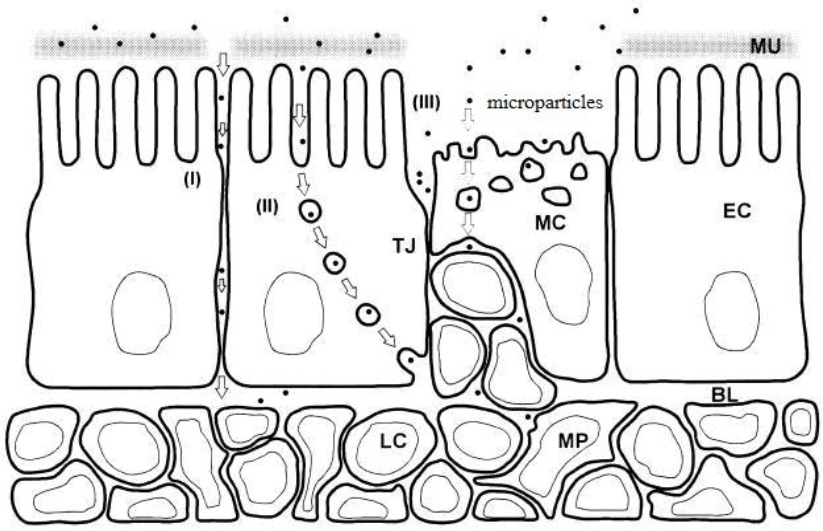


Figure 3 Schematic drawing of mucus (MU) covered absorptive enterocytes (EC) and M cells (MC) in the small intestine. Lymphocytes (LC) and macrophages (MP) from underlying lymphoid tissue can pass the basal lamina (BL) and reach the epithelial cell layer which is sealed by tight junctions (TJ). Possible translocation routes for microparticles are (I) paracellular uptake, (II) endocytotic uptake by enterocytes and (III) M cells.

Source : T. Jung et al. “Biodegradable nanoparticles for oral delivery of peptides: is there a role for polymers to affect mucosal uptake?,” European Journal of Pharmaceutics and Biopharmaceutics 50 (2000): 147-160.

The ability of polymeric microparticles to induce following systemic administration has been known for some times. It is also well established that particulate antigens are more effective for oral immunization than soluble antigens. The ability of particulate antigens to induce enhanced immune responses following oral immunization is mainly a consequence of their greater uptake into intestinal Peyer’s patches. However, the appropriate particle characteristics for optimal uptake at these sites and optimal stimulation of immune responses are comparatively poorly understood. Considerations such as particle size dependent uptake across mucosal epithelia remain as areas of some controversy. A number of researchers have

repeatedly demonstrated the uptake of microparticles across the gut or respiratory tract following mucosal administration (Hagan 1990 : 265–285; 1996 : 477–482). However, the reported sites of uptake and the mechanisms involved have differed. Overall, four alternative sites and mechanisms of uptake have been emphasized; the villus tips, intestinal macrophages, ordinary enterocytes and the epithelium of the Peyer's patches. It is possible that all of these mechanisms may be operating simultaneously to some extent. Le Fevre et al. (Fevre et al. 1980 : 691–704) challenged the earlier data of Volkheimer (Volkheimer 1977 : 163–187) and evaluated the effect of particle size on the uptake of polystyrene microparticles in mice. They showed that 5.7 μm particles were taken up into the Peyer's patches, but 15.8 μm particles were not. Meanwhile, Ebel (Ebel 1990 : 848–851) showed that in mice, 2.65 μm polystyrene particles were taken up to a greater extent than particles of 9.13 μm. Eldridge et al. (Eldridge et al. 1990 : 205–214) suggested that the upper limit for particle uptake into mouse Peyer's patches was about 10 μm. Eldridge et al. (Eldridge et al. 1990 : 205–214) further suggested that larger particles (5–10 μm) remained in Peyer's patches, while smaller particles (5 μm) were transported systemically in the lymph as shown in Table 1.

6. Biodegradable mucoadhesive particulates for mucosal antigen delivery

The use of polymeric microparticles offers significant potential for the development of orally administered vaccines. Microparticles can be prepared from a range of different polymers which can be designed to protect entrapped vaccines against degradation in the gut, to delay the gastric transit of the vaccine or to target vaccines for uptake into the mucosal associated lymphoid tissues (MALTs) of the Peyer's patches (PPs). In addition, similar microparticles can be applied intranasally for the delivery of antigens to the MALTs of the upper respiratory tract. For uptake into the MALT of the gut or the respiratory tract, microparticles need to be prepared with the appropriate dimensions (i.e. < 10 μm) (Derek et al. 1998 : 305-320). In the design of oral delivery of peptide or protein drugs, pH sensitive hydrogels have attracted increasing attention. Swelling of such hydrogels in the stomach should be minimal and thus the drug release will also be minimal. The extent of swelling increases as the hydrogels pass down the intestinal tract due to an increase in pH. A

variety of synthetic or natural polymers with acidic or basic pendant groups have been employed to fabricate pH sensitive hydrogels. Alginate and chitosan are naturally occurring biopolymers that are finding widespread applications in food and pharmaceutical industry. Alginate is used extensively in food industry as a thickener, emulsifier and as a stabilizer. Chitosan is a potentially useful pharmaceutical material owing to its good biocompatibility and low toxicity. This review describes the sources, physical and chemical properties of these polymers that enable them to become suitable for protein delivery, the mechanisms of hydrogel formation, modifications which increase their protein encapsulation efficiency and the recent trends in their application.

6.1 Alginate

6.1.1 Sources

Commercial alginates are extracted from three species of brown algae. These include *Laminaria hyperborean*, *Ascophyllum nodosum*, and *Macrocystis pyrifera*; in which alginate comprises up to 40% of the dry weight (Smidsrod 1990 : 71–78; Sutherland 1991 : 309–331). Alginate exists as a mixed salt of various cations found in the seawater such as Mg^{2+}, Sr^{2+}, Ba^{2+}, and Na^{+}. Bacterial alginates have also been isolated from *Azotobacter vinelandii* and several Pseudomonas species.

6.1.2 Chemical structure

Alginate is a water-soluble linear polysaccharide extracted from brown seaweed and is composed of alternating blocks of 1–4 linked α-L-guluronic and β-D-mannuronic acid residues. Fig. 4 shows the structures of mannuronic and guluronic acid residues and the binding between these residues in alginate. Because of the particular shapes of the monomers and their modes of linkage in the polymer, the geometries of the G-block regions, M-block regions, and alternating regions are substantially different. Specifically, the G-blocks are buckled while the M-blocks have a shape referred to as an extended ribbon. If two G-block regions are aligned side by side, a diamond shaped hole results. This hole has dimensions that are ideal for the cooperative binding of calcium ions. The homopolymeric regions of β-D-mannuronic acid blocks and α-L-guluronic acid blocks are interdispersed with regions of alternating structure (β-D-mannuronic acid–α-L-guluronic acid blocks). The

composition and extent of the sequences and the molecular weight determine the physical properties of the alginates.

Table 1 The mechanism of immune stimulation depends on the size and administration route of particles.

Particle size	**Route**	**Effect**	**References**
200 nm	i.v.	Phagocytosed by Kupffer cells (macrophages), reach the lysosomal compartment in liver tissues	Lima et al. 1999 : 171– 180; Eldridge et al. 1989 : 59-66, 191–202; Tabata et al.1988 : 837– 858.
1–10µm	s.c.	Phagocytosed by macrophages, which are recruited to the site of administration after s.c. injection intracellular delivery of the antigen for processing by *the MHC II exogenous antigen pathway ZTH-response, antibody production, or *MHC I endogenous pathway CTL-response, cytotoxic lymphocytes	Eldridge et al. 1989 : 59-66, 191–202.
>10 µm	s.c.	Act as a depot, release the antigen in a second step	Eldridge et al. 1989 : 59-66, 191–202.
<5 µm	orally	Effectively taken up by M-cells of PP and carried by macrophages to the mesenteric lymph nodes and spleen	Eldridge et al. 1989 : 59-66, 191–202.
5–10µm	orally	Remain in the PP during 35 days	Eldridge et al. 1989 : 59-66, 191–202.
>10 µm	orally	Not absorbed at any point in the gastrointestinal tract	Lima et al. 1999 : 171– 180.

Source : I. Schöll et al. “Review of novel particulate antigen delivery systems with special focus on treatment of type I allergy,” Journal of Controlled Release 104 (2005): 1–27.

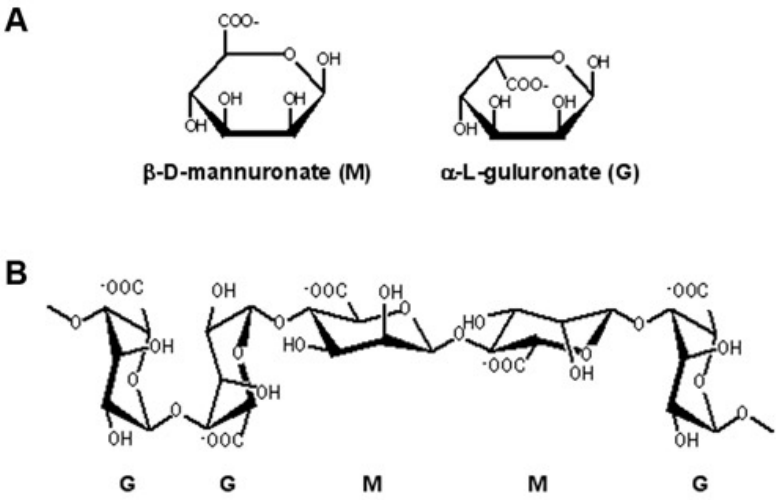


Figure 4 Chemical structure of alginate (a) the monomers in alginate; (b) the alginate chain.

Source : Pornsak Sriamornsak and Srisagun Sungthongjeen "Modification of theophylline release with alginate gel formed in hrad capsules," AAPS PharmSciTech 8 (2007): 1-8.

6.1.3 Gel formation

The gelation of alginate can be carried out under an extremely mild environment and uses non-toxic reactants. The most important property of alginates is their ability to form gels by reaction with divalent cations such as Ca^{2+}. Alginate beads can be prepared by extruding a solution of sodium alginate containing the desired protein, as droplets, in to a divalent cross-linking solution such as Ca^{2+}, Sr^{2+}, or Ba^{2+}. Monovalent cations and Mg^{2+} ions do not induce gelation.

6.1.4 Biocompatibility

Alginate is used extensively in food industry as a thickener, emulsifier and as a stabilizer. Alginates are included in a group of compounds that are generally regarded as safe (GRAS) by the FDA. The oral administration of alginate has not been shown to provoke much immunoresponses unlike the intravenously administered forms and it is reported that alginate is non-toxic and biodegradable when given orally (Espevik et al. 1993 : 255–261). Although alginate biocompatibility has been extensively investigated, there is a disagreement in the

literature. In case of intravenous administration, induction of foreign body reaction and fibrosis have been reported for most commercial alginates (Cole et al. 1992 : 231–237; Vos et al. 1996 : 893–899), while other reports show little or no immunoresponse around alginate implants (Zimmermann et al. 1992 : 269–274). Commercially available alginates when tested after purification by free-flow electrophoresis, did not provoke foreign body reactions at least 3 weeks after implantation in the peritoneal cavity of rodents (Mumper et al. 1994 : 241–251). The immunogenic response at intravenous injections would have been due to toxic contaminants in commercial alginates.

6.1.5 Bioadhesiveness

Mucoadhesive drug delivery systems work by increasing the drug residence time at the site of activity or resorption. The mucoadhesive feature of alginate may aid in its utility as a potential delivery vehicle for drugs to mucosal tissues such as the GI tract (Gombotz and Wee 1998 : 267–285). Studies have shown that polymers with charge density can serve as good mucoadhesive agents (Chickering and Mathiowitz 1995 : 251–261; Chang et al. 1985 : 399–405; Kwok et al. 1989 : 170–171; 1991 : 341–344). An increased charge density will give better adhesion. Alginate, being an anionic polymer with carboxyl end groups, is a good mucoadhesive agent. Studies have shown that alginate has the highest mucoadhesive strength as compared to polymers such as polystyrene, chitosan, carboxymethylcellulose and poly(lactic acid) (Chang et al. 1985 : 399–405; Kwok et al. 1989 : 170–171). Due to the adherence of alginate particles to the mucosal tissues, protein transit time is delayed and the drug is localized to the absorptive surfaces. It improves drug bioavailability and effectiveness.

6.1.6 pH sensitivity

Release of macromolecules from alginate beads in low pH solutions is also significantly reduced which could be advantageous in the development of an oral delivery system (Kim and Lee 1992 : 11–19; Sugawara et al. 1994 : 272–277). Theoretically, alginate shrinks at low pH (gastric environment) and the encapsulated drugs are not released (Chen et al. 2004 : 285–300). In gastric fluid, the hydrated sodium alginate is converted into a porous, insoluble so-called alginic acid skin. Once passed into the higher pH of the intestinal tract, the alginic acid skin is converted to a

soluble viscous layer. This pH dependent behaviour of alginate can be exploited to customize release profiles. However, the rapid dissolution of alginate matrices in the higher pH ranges may result in burst release of protein drugs and subsequently their denaturation of the protein drugs by proteolytic enzymes. Therefore many modifications in the physicochemical properties are needed for the prolonged controlled release of protein drugs.

6.1.7 Protein entrapment in alginate

Sodium alginate solution containing the desired protein, form droplets, when added into a divalent cross-linking solution such as Ca^{2+}, Sr^{2+}, or Ba^{2+}. The biological activity of drugs can be retained in the calcium-cross-linked alginate encapsulation process. Although the microenvironment in an alginate bead can be relatively inert to protein drugs and cells, a positively charged protein can potentially compete with calcium ions for available carboxylic acid sites on the alginate. This has been observed with small drugs and has been shown to result in protein inactivation in the case of the protein transforming growth factor-beta (TGA-β1). In such cases, it may become necessary to include additives which protect the active agent from the alginate polymer. The addition of the anionic polymer poly(acrylic acid) shields the TGF-β1 from interaction with the alginate and allows its activity to be retained (Espevik et al. 1993 : 255–261). Alginate as plain beads, coated beads and microcapsules has been used to entrap proteins like melatonin (Lee and Min 1996 : 37–46), heparin (Edelman et al. 2000 : 2279–2286), hemoglobin (Rasmussen et al. 2003 : 395–405), vaccines (Kim et al. 2002 : 191–202; Romalde et al. 2004 : 119–129), etc. Coated beads and microspheres are found to be better oral delivery vehicles.

6.1.8 Protein release from alginate

Proteins encapsulated in alginate matrices are released by two mechanisms. They are: (1) diffusion of the protein through the pores of the polymer network and (2) degradation of the polymeric network. For the controlled delivery of protein drugs, the degradation of the gel network may not be the suitable method, as it may result in the rapid release of the protein. Therefore the matrix being intact and the drug being diffused out through the pores will be the most suitable approach for protein delivery. The pore size of alginate gel microbeads has been shown to be between 5 and 200 nm. It is also suggested that the bead surface pores are more

constricted than that in the gel core (Martinsen et al. 1991 : 171-193). Unlike small chemical drugs, diffusion of larger proteins from the gels are dependent on their molecular weight. The diffusion of several proteins from alginate beads has been reported including IgG, fibrinogen and insulin, etc. The charge on a protein can also influence its rate of diffusion from the alginate matrix. If the entrapped protein is with a net positive charge, it can interact with negatively charged alginate, thus inhibiting its diffusion from the gel (Smidsrod et al. 1990 : 71–78; Espevik et al. 1993 : 255–261), whereas a protein with a net negative charge may be released more rapidly from the matrix. Therefore the charge of the entrapped protein drug is an important factor determining the efficiency of the controlled release of the drug from alginate matrix. The porosity, which is an important factor in determining the drug release from alginate, can be significantly reduced by partial drying of the beads. Complete dehydration may, however result in surface cracking which can facilitate the surface erosion of the beads upon rehydration (Kim and Lee 1992 : 11–19). This surface cracking and erosion will limit the efficiency of alginate matrix for protein delivery. Therefore selecting the most suited drying method and the extent of drying are important factors that influence drug release rates. A reduction in pore size of alginate occurs in low pH and therefore release of macromolecules from the gel is significantly reduced. This could be advantageous in the development of an oral delivery system (Espevik et al. 1993 : 255–261; Kim and Lee 1992 : 11–19; Sugawara et al. 1994 : 272–277). Also, the percentage of α-Lguluronic acid influences the protein release rate. Low α-L-guluronic acid content alginate and low molecular weight alginate are known to release encapsulated proteins at a much faster rate (Murata et al. 1993 : 21–26). Therefore, high guluronic acid content and relatively high molecular weight alginate is a preferred matrix for protein entrapment.

6.1.9 Alginate microparticles for mucosal administration

Sodium alginate has been used for preparing nanoparticles microspheres, microcapsules (Esquisabel et al. 2000 : 363–372) and beads (Kulkarni et al. 2001 : 127–133), for oral delivery. In particularly, the use of alginate microparticles as an antigen delivery system has been described in several publications and there are some indications that they are able to induce a mucosal and systemic immune response in a variety of animal species by both oral and intranasal

administration (Bowersock et al. 1999 : 1804–1811; Rebelatto et al. 2001 : 93–105). Encapsulation of antigen in alginate microspheres requires mild conditions and is compatible with the encapsulation of a variety of antigen. Alginate microspheres act as adjuvants, and vaccine containing alginate microspheres are effective for nasal vaccination in animal species (Rebelatto et al. 2001 : 93-105). Oral immunization with alginate microsphere-encapsulated live virised and proteins induced immune responses in mice (Offit et al. 1994 : 134-143; Periwal et al. 1997 : 2844-2850) and in cattle (Bowersock et al. 1998 : 37-43). Soheila et al. (Soheila et al. 2007 : 4595–4601) have compared immune responses in BALB/c mice immunized with BCG encapsulated in alginate microspheres by oral route with those of immunized orally and parenterally with free BCG. The result showed that proliferative and delayed-type hypersensitivity (DTH) responses and IFN-γ production were significantly higher in mice immunized orally with encapsulated BCG in comparison with results of mice immunized orally with free BCG. Following systemic infection with BCG, mice vaccinated with encapsulated BCG had lower mean bacterial count compared to those vaccinated orally with free BCG. The immune responses induced by oral administration of encapsulated BCG were equal to or better than the responses induced by standard BCG vaccination. For evoking mucosal immunity, alginate microparticles was shown to be effective in oral immunizations of BALB/c mice with entrapped cholera toxin B subunit plus a pneumococcal capsular polysaccharide PS19, or a capsular streptococcal polysaccharide (Cho et al. 1998 : 215–224; Seong et al. 1999 : 3587–3592). During these feedings, high levels of anti-PS19 mucosal IgA in small intestine wash samples and systemic IgM in sera were induced in animals treated with up to 25 Ag antigen, whereas higher amounts of antigen possibly induced tolerance. Interestingly, there was a lack of antigen-specific IgG in serum. Murine studies with microencapsulated plasmid DNA for bacterial h-galactosidase (LacZ) and/or bovine adenovirus type 3 (BAd3) showed that the route of administration is a critical factor for the type of immune response also with this particle material (Mittal et al. 2000 : 253–263). In general, IgG titers were higher after parenteral (i.m., s.c., i.p.) than after mucosal applications. On the other hand, the specific IgA titers in serum, lung lavages and feces were two times higher after i.n. administration compared to the parenteral treatment. The observed results indicate that the alginate

preparations can be used for mucosal as well as for parenteral treatment. This makes these particles interesting for application in diseases like viral infections, cancer or allergy.

6.2 Chitosan

6.2.1 Sources

Chitosan is a cationic polymer, which is the second most abundant polymer in nature after cellulose (Roberts 1992 : 274–315). Chitin is the primary structural component of the outer skeletons of crustaceans, and is also found in many other species such as molluscs, insects and fungi. The most commonly obtained form of chitosan is the α-chitosan from crustacean chitin obtained from crab- and shrimp shell wastes (Roberts 1992 : 274–315; Shepherd et al. 1997 : 535–542). This accounts for approximately 70% of the organic compounds in such shells. In preparing chitosan, ground shells are deproteinated and demineralized by sequential treatment with alkali and acid, after which the extracted chitin is deacetylated to chitosan by alkaline hydrolysis at high temperature. Production of chitosan from these sources is inexpensive and easy. It has also been suggested that other sources of chitin, e.g. β-chitin from squid pens, may be valuable in relation to the preparation of chitosan (Shepherd et al. 2000 : 337–344; Wuolijoki et al. 1999 : 357–361). Chitosan as such is rare in nature, except in certain fungi. In recent years, the production of chitosan from fungi, using fermentation methods is also gaining much interest (New et al. 2002 : 235–237).

6.2.2 Chemical structure

Chitosan is a linear co polymer polysaccharide consisting of β-(1–4)-linked 2-amino-2-deoxy-D-glucose (D-glucosamine) and 2-acetamido-2-deoxy-D-glucose (N-acetyl-D-glucosamine) units (Fig. 5). The structure of chitosan is very similar to that of cellulose (made up of β-(1–4)-linked D-glucose units), in which there are hydroxyl groups at C2 positions of the glucose rings. Chitosan is poly [β-(1–4)-2-amino-2-deoxy-D-glucopyranose]. The term chitosan is used to describe a series of polymers of different degrees of deacetylation (DD), defined in terms of the percentage of primary amino groups in the polymer backbone, and average molecular weights (Mw) (Roberts 1992 : 274–315). The DD of typical commercial chitosan is usually between 70% and 95%, and the Mw between 10 and 1000 kDa. The

properties, biodegradability and biological role of chitosan is frequently dependent on the relative proportions of N-acetyl-D-glucosamine and D-glucosamine residues.

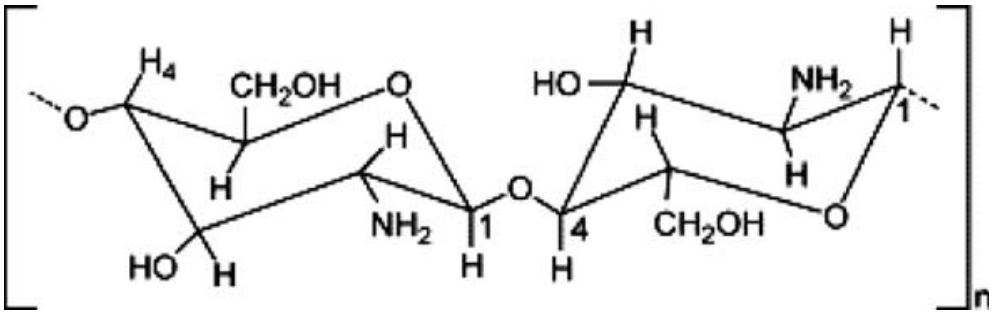


Figure 5 Chemical structure of chitosan.

Sources : M. George and T. E. Abraham "Polyionic hydrocolloids for the intestinal delivery of protein drugs: Alginate and chitosan — a review," Journal of Controlled Release 114 (2006): 1–14.

6.2.3 Biocompatibility and biodegradability

Chitosan has been widely used in food industry and is an approved food additive in Japan. Chitosan has also been marketed throughout the world as a component in non-medical products, as a fat binder in cholesterol-lowering and slimming formulations (Shahidi et al. 1999 : 37–51). It has been claimed the chitosan entraps lipids in the intestine, because of its cationic nature (Kanauchi et al. 1995 : 786–790; Wuolijoki et al. 1999 : 357–361). It has also been studied in biomedical field and has been found to be highly biocompatible. Also, chitosan is metabolized by certain human enzymes, especially lysozyme, and is considered biodegradable (Muzzarelli et al. 1990 : 71–78). Chitosan has also recently been approved by the authorities, and a monograph relating to chitosan hydrochloride was included in the fourth edition of the European Pharmacopoeia (2002).

6.2.4 Mucoadhesiveness

One area in which interest is growing is use of chitosan as a bioadhesive material. The mucoadhesive properties of chitosan have been illustrated by its ability to adhere to porcine gastric mucosa *in vitro* (Gåserød et al. 1998 : 237–246), and hence it could be useful for in site specific drug delivery. Many

commercially available chitosans exhibit fairly good mucoadhesive properties *in vitro* (Lehr et al. 1992 : 43–48). It has been suggested that residence time of formulations at sites of drug action or absorption could be prolonged through the use of chitosan. It has also been suggested that chitosan might be valuable for delivery of drugs to specific regions of the gastrointestinal tract like the stomach (Gåserød et al. 1998 : 237–246; López et al. 2000 : 69–76), small intestine (Lehr et al. 1992 : 43–48; He et al 1998 : 75–88; Shimoda et al. 2001 : 567–576), and buccal mucosa (Miyazaki et al. 1995 : 257–263; López et al. 1998 : 143–152). The adhesive properties of chitosan in a swollen state have been shown to persist well during repeated contacts of chitosan and the substrate (Lehr et al. 1992 : 43–48) which implies that, in addition to the adhesion by hydration, many other mechanisms, such as hydrogen bonding and ionic interactions might also have been involved. Important mechanism of action was suggested to be ionic interactions between positively charged amino groups in chitosan and the negatively charged mucus gel layer. In the interactions between chitosan and mucus (Fiebrig et al. 1995 : 239–244; He et al. 1998 : 75–88), the primary mechanism of action at the molecular level was found to be electrostatic (Deacon et al. 2000 : 557–563). The interactions are strong at acidic and slightly acidic pH levels, at which the charge density of chitosan is high (He et al. 1998 : 75–88). Increase in molecular weight of chitosan results in stronger adhesion (Lehr et al. 1992 : 43–48). He et al. (He et al 1998 : 75–88) showed that amounts of chitosan microspheres adhering to the intestine were greatest when the density of cross-linking of chitosan was least, i.e. when the number of free amino groups in chitosan was greatest. This finding also suggests that the adhesive properties of chitosan should become more marked as degree of deacetylation increases, and cross-linking reduces the mucoadhesive effects of chitosan (Schnürch et al. 1998 : 217–225; Genta et al. 1999 : 305–313).

6.2.5 Permeation enhancing effect

In recent years, chitosan has attracted a lot of attention as a potential absorption enhancer across mucosal epithelia especially for peptide drugs (Kotze et al. 1997 : 243–253; Lueßen et al. 1996 : 1668–1672; Illum et al. 1994 : 1186–1189). It has been reported that chitosan acts as a permeation enhancer by opening epithelial tight junctions (Junginger and Verhoef 1998 : 370–376; Kotze et al. 1999 : 341–385).

It was in 1994 that Illum et al. showed the permeation enhancing capabilities of chitosan for the first time (Illum et al. 1994 : 1186–1189). Chitosan is able to enhance the paracellular route of absorption, which is important for the transport of hydrophilic compounds such as therapeutic peptides and antisense oligonucleotides across the membrane. The mechanism underlying this permeation enhancing effect seems to be based on the positive charges of the polymer, which interact with the cell membrane resulting in a structural reorganization of tight junction associated proteins (Schipper et al. 1997 : 923–929). Chitosan display certain advantages over low molecular mass enhancers. For example, the additional mucoadhesive properties, which allow them to remain concentrated at the area of drug absorption (Lehr 1996 : 139–148). The ability of chitosan to work as an absorption enhancer was proven on Caco-2 cells, which serve as a model of intestinal epithelium (Artursson et al. 1994 : 1358–1361; Borchard et al. 1996 : 131–138; Dodane et al. 1999 : 21–32; Smith et al. 2004 : 43–49), as well as in *in vitro* experiments on nasal, buccal, vaginal and urinary bladder mucosa of different animals. Chitosan also increased the bioavailabilty of a peptide drug buserelin, which was intraduodenally applied in the *in vivo* experiments in rats (Natsume et al. 1999 : 1–12; Senel and Hincal 2001 : 133–144; Grabnar et al. 2003 : 167–173; Sinswat and Tengamnuay 2003 : 15–22; Sandri et al. 2004 : 351–359).

6.2.6 pH sensitiveness

Chitosan exhibits a pH-sensitive behavior as a weak polybase due to the large quantities of amino groups on its chain. Chitosan dissolves easily at low pH while it is insoluble at higher pH ranges. The mechanism of pH sensitive swelling involves the protonation of amine groups of chitosan under low pH conditions. This protonation leads to chain repulsion, diffusion of proton and counter ions together with water inside the gel and dissociation of secondary interactions (Yao et al. 1994 : 1213–1223). This property has helped it to be used in the delivery of chemical drugs to the stomach and has been widely investigated as a delivery matrix. But for the delivery of protein drugs to the intestine, this property pause a limitation. Because, as the matrix gets dissolved in the stomach, the released protein drugs will get denatured. Moreover, the pH sensitivity of the native chitosan is not suitable for protein delivery. To overcome this, many modifications can be done to improve the stability of

chitosan in the stomach and the subsequent controlled delivery of protein drugs in the intestine.

6.2.7 Mild gelation conditions

The hydrogel preparation and drug entrapment can be done under relatively mild gelation conditions. Chitosan solution containing the desired protein can be formed into a gel upon contact with a cross-linking agent. One of the commonly used cross-linking agents for the ionic gelation of chitosan is tripolyphosphate (TPP). It is a non-toxic polyanion which can interact with chitosan via electrostatic forces to form ionic cross-linked networks. It can be used for the preparation of chitosan beads and microspheres because of its quick gelling ability (Mi et al. 1999 : 1551–1564). Covalently cross-linked chitosan hydrogels can be prepared by treating chitosan with various chemical reagents. The cross-linking procedure helps to reinforce the chemical and mechanical properties of chitosan, making it a more stable network. Thus it can perform controlled protein release at higher pH of intestine instead of rapidly releasing the protein drugs by rapid dissolution in the stomach. The most common cross-linkers used to cross-link chitosan are dialdehydes such as glyoxal (Khalid et al. 1999 : 359–364; Patel and Amiji 1996 : 588–593) and glutaraldehyde (Aly 1998 : 13–18; Yamada et al. 2000 : 252–258; Denkbas et al.2000 : 33–38). The aldehyde groups form covalent imine bonds with the amino groups of chitosan, due to the resonance established with adjacent double ethylinic bonds (Monteiro and Airoldi 1999 : 119–128; Yao et al. 1995 : 77–82) via a Schiff reaction. Dialdehydes allow the cross-linking to happen by direct reaction in aqueous media and under mild conditions and it does not require the addition of auxiliary molecules such as reducers (Khalid et al.1999 : 359–364). It also adds to retaining the biocompatibility of the polymer. Diethyl squarate (Angelis et al. 1998 : 1595–1601) and oxalic acid (Hirano et al. 1990 : 145–149) have also been found to act as direct cross-linkers for chitosan. Natural crosslinker like genipin (Mi et al. 2000 : 2804–2814) is gaining wide acceptance for crosslinking chitosan.

6.2.8 Chitosan micro/nanoparticles for mucosal administration

Chitosan suspensions or microparticles have also been shown to possess immune stimulating capacity, such as the accumulation (via chemotaxis) and specific activation (NO production) of macrophages (Peluso et al. 1994 : 1215– 1220)

suppress tumor growth (Qin et al. 2002 : 111–117) induce non-specific resistance to infections, induce cytokines like IL-2, IL-10, TNF-a, IL-12,IFN-g (Lee et al. 2002 : 645–648; Porporatto et al. 2004 : 433–441), enhance antibody responses and delayed type hypersensitivity (Shibata et al. 2001 : 6123–6130) and activate cytotoxic T-cells as well as natural killer cells. In an *in vitro* Caco-2 cell monolayer system, chitosan suspensions were able to penetrate the tight junctions and, therefore, allowed the enhanced paracellular transport of co-administered drugs across the epithelium (Schipper et al. 1999 : 335–343; 1997 : 923–929; 1996 : 1686–1692).

Depending on the chitosan preparation, the model drugs atenolol or 14C-mannitol was transported in 10–40 times higher amounts across the Caco-2 cell layers compared to baseline permeability. In contrast to the soluble formulation, chitosan particles were endocytosed by the M-cells of PP and biodegraded by macrophages (Lubben et al. 2001 : 687–694; 2002 : 449–456). By derivatization with different coupling reagents and thiol groups, respectively (thiolated chitosans, thiomers), the mucoadhesiveness, the paracellular transport, the in situ gelling features, the cohesion and the stability could be increased, guaranteeing a prolonged release of embedded substances (Bernkop-Schnurch et al. 2004 : 177–186; Bernkop-Schnurch et al. 2003 : 95–103; 2004 : 9–17). In an approach to cure OVA sensitized mice, animals were treated intranasally (i.n.) with plasmid DNA coding for IFN-g in chitosan nanoparticles (CIN) (Kumar et al. 2003 : 3). Indeed, airway hyperresponsiveness and lung eosinophilia could be reduced two times and six times, respectively, in CIN-treated mice compared to the untreated or chitosan-treated control group. Th2- cytokines IL-4 and IL-5 were reduced fourfold in a therapeutic and also in a prophylactic approach. In addition, induction of IgE-antibodies was prevented in CIN-treated mice (10 ng/ml vs 61 ng/ml in PBS treated animals). Roy et al. showed the potency of chitosan in targeting, transfection and immunologic protection against peanut allergy in oral gene delivery studies (Roy et al. 1999 : 387–391). Mice were fed with a gene coding for the dominant peanut allergen Ara h 2 entrapped in chitosan nanospheres with a diameter of 150–300 nm. These animals were protected from a consecutive allergic reaction upon peanut challenge. This could be due to a three times higher IgG2a concentration in serum compared to the untreated group and four times higher mucosal IgA levels in feces compared to the

group treated with naked Ara h 2-plasmid. The increase in Ara h 2-specific IgE was suppressed to 25%.In another study porous chitosan microspheres were prepared for the controlled delivery of the antigen of new castle disease, which was immobilized into the pores of microspheres. High porosity of microspheres with an open structure could be obtained by changing the pH value of the coagulation medium, i.e. aqueous tripolyphosphate solution to 8.9. Higher adsorption efficiency and slower release rate of the antigen were obtained by chemical modification of the microspheres with 3-chloro-2-hydoxypropyltrimethyl ammonium chloride (Mi et al. 1999 : 1603–1612).

7. Preparation of alginate coated chitosan microparticles for mucosal drug delivery

Recently, chitosan microparticles have been used for the delivery of antigens to Peyer's patches (Lubben et al. 2001 : 687-694; 2002 : 449-456). Chitosan itself exhibits mucobioadhesive properties to the mucosal membrane probably because of its cationic and viscous properties, and is considered suitable for the delivery to specific sites of the intestine. However, as chitosan is easily dissolved in the acidic stomach, simple oral administration results in the dissolution or collapse. Then coating chitosan microparticles with polyanionic polymers, such as alginate, has been commonly used to protect chitosan microparticles from dissolution or collapse under gastric conditions and extend the drug release. Over the last years, sodium alginate has also been used as a coating material for cells with some advantages. Alginate coated–chitosan microparticles erodes slowly in phosphate buffer at pH values higher than 6.5 suppressing the initial drug release in the upper segments of the intestine occurring for uncoated microparticles and controlling the release in the colon whereas pH value is in the range of 6.5–7.0. Borges et al. (Borges et al 2005 : 155–166) have described the development and the characterization of a new nanosized delivery system, consisting of chitosan nanoparticles with ovalbumin adsorbed at the surface and coated with sodium alginate. The coating of the chitosan nanoparticles with sodium alginate has inverted the surface charge of the particles from positive to negative values. These particles were designed for crossing mucosal barriers and releasing the antigen into lymphoid tissue, in particular in the ileal Peyer's patches. In the present manuscript, successive studies related to this new delivery system are

presented. Moreover the further study they have assess the model vaccine release profiles from coated nanoparticles in order to examine the nature of the interactions between the chitosan core and the alginate coating and to evaluate the potential of these coated particles for being taken up by Peyer's patches and the cytotoxicity of the coated and uncoated nanoparticles in comparison to the polymers used to prepare these particles. The result of their study showed that the adsorption of therapeutic proteins and model antigens onto chitosan nanoparticles has proven to be a very mild process resulting in a very high loading efficacy. In this study, it was shown that coating of this delivery system with sodium alginate yielded coated particles, in the nanosize range, with a much better stability and controlled release properties for vaccine delivery, than the chitosan loaded cores themselves. It was also demonstrated that the preparation process of the nanoparticles did not introduce any toxic compound on the particles. On the contrary, a slight stimulation of the splenocytes co-cultured with the nanoparticles was observed. Finally it was shown that these hydrophilic coated nanoparticles even with a negative surface charge were taken up by rat Peyer's patches, which made them promising carriers for mucosal vaccination. *In vivo* studies with a real vaccine are underway to investigate the efficacy of this new mucosal delivery system (Borges et al. 2006 : 348–358).

8. Methods of preparation microparticles

Encapsulation techniques usually involve preparation of polymeric microparticles of a desired size and shape containing entrapped biologically active material. The main methods of microencapsulation have been extensively reviewed. In general, the most important requirement in the development of microencapsulation techniques, especially for protein delivery is avoiding conditions that can cause adverse effect on the biological material, such as excessive heat, organic solvents, strongly acidic or basic solutions. Other critical considerations include particle size, distribution control, and microencapsulation efficiency (Table 2). Microparticles can be prepared using any of the following techniques.

Table 2 Comparison of the various processes used for the preparation of microparticles

Process used	Particle size (μm)	Polymers	Comments
Solvent evaporation	1–100	Relatively stable polymers, e.g. polyesters, polystyrene	Labile polymers may degrade during the fabrication process due to the presence of water
Solvent removal	1–300	High melting point polymers especially polyanhydrides	Avoids use of water, only organic solvents are used
Spray drying	1–10	-	Primarily for microspheres used for intestinal imaging
Ionic gelation and size extrusion	1–300	Chitosan, CMC, alginate	Used for encapsulation of live cells or allergen
Phase inversion	0.5–5.0	Polyanhydrides	Involves low polymer loss and low drug loss during fabrication process

Sources : J. K. Vasir, K. Tambwekar and S. Garg "Bioadhesive microspheres as a controlled drug delivery system," International Journal of Pharmaceutics 255 (2003): 13–32.

8.1 Emulsion cross-linking or solvent evaporization

This method involves the formation of an emulsion between polymer solution and an immiscible continuous phase whether aqueous (o/w) or non-aqueous (w/o). This method is the most extensively used of microencapsulation, first described (Ogawa et al. 1988 : 1095–1103). A buffered or plain aqueous solution of the drug (may contain a viscosity building or stabilising agent) is added to an organic phase consisting of the polymer solution in solvents like dichloromethane (or ethyl acetate or chloroform) with vigorous stirring to form the primary water in oil emulsion. This emulsion is then added to a large volume of water containing an emulsifier like PVA or PVP to form the multiple emulsion (w/o/w). The double emulsion, so formed, is

then subjected to stirring until most of the organic solvent evaporates, leaving solid microspheres. The microspheres can then be washed, centrifuged and lyophilised to obtain the free flowing and dried microspheres.

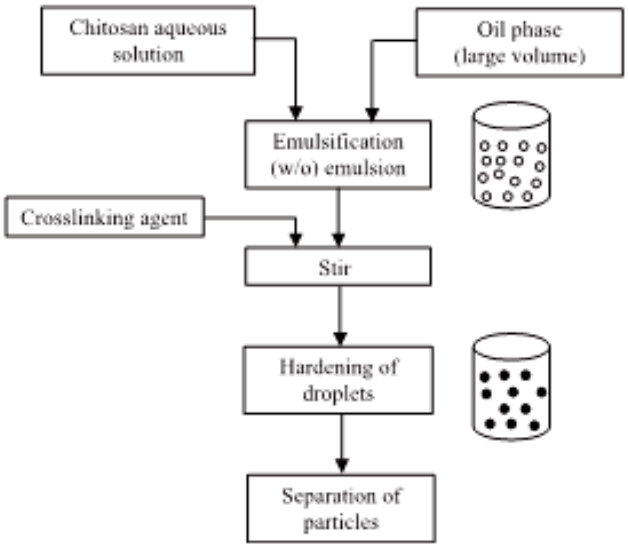


Figure 6 Schematic diagram of preparation of chitosan microparticles with emulsion cross-linking method.

Sources : L. Chenguang et al. “Preparations, Characterizations and Applications of Chitosan-based Nanoparticles,”. Journal of Ocean University of China 6 (2007): 237-243.

8.2 Ionic gelation

This method is based on the conjugation of oppositely charged macromolecules for preparing microparticle. Microparticles made of gel-type polymers, such as alginate, are produced by dissolving the polymer in an aqueous solution, suspending the active ingredient in the mixture and extruding through a precision device, producing microdroplets which fall into a hardening bath, that is slowly stirred. The hardening bath usually contains calcium chloride solution, whereby the divalent calcium ions crosslink the polymer forming gelled microspheres. The method involves an “all-aqueous” system and avoids residual solvents in microspheres. Lim and Moss (Lim and Moss 1981 : 351–354) developed this method for encapsulation of live cells, as it does not involve harsh conditions, which could kill the cells. The surface of these microspheres can be further modified

by coating them with polycationic polymers, like polylysine after fabrication. The particle size of microspheres can be controlled by using various size extruders or by varying the polymer solution flow rates. Dambies et al. (Dambies et al. 2001 : 1198–1205) prepared chitosan gel beads using molybdate as the gelling agent. It was observed that this new gelation technique led to a structure different from one produced during alkaline coagulation of a chitosan solution. Instead of a morphology characterized by large open pores, gel beads produced in a molybdate solution, under optimum conditions (pH 6; molybdate concentration, 7 g/l), were found to have a double layer structure corresponding to a very compact 100 μm thick external layer and an internal structure of small pores.

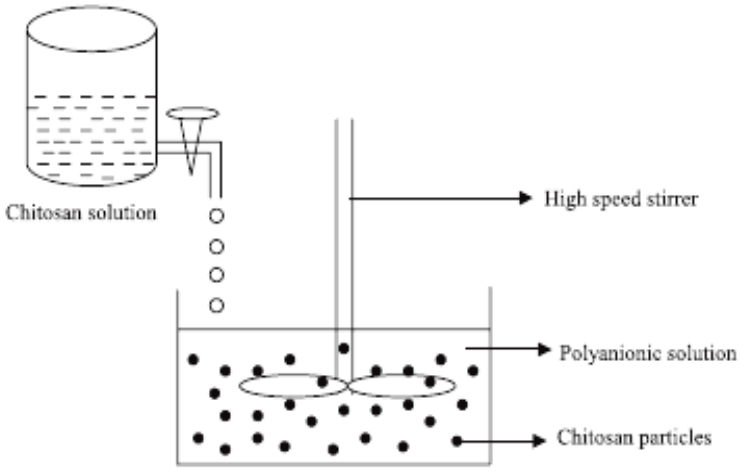


Figure 7 Schematic diagram of preparation of chitosan microparticles with ionic gelation method.

Sources : L. Chenguang et al. “Preparations, Characterizations and Applications of Chitosan-based Nanoparticles,”. Journal of Ocean University of China 6 (2007): 237-243.

8.3 Spray drying

In this process, the drug may be dissolved or dispersed in the polymer solution and spray dried. The quality of spray-dried microspheres can be improved by the addition of plasticizers, e.g. citric acid, which promote polymer coalescence on the drug particles and hence promote the formation of spherical and smooth surfaced microspheres. The size of microspheres can be controlled by the rate of spraying, the

feed rate of polymer drug solution, nozzle size, and the drying temperature. This method of microencapsulation is particularly less dependent on the solubility characteristics of the drug and polymer and is simple, reproducible, and easy to scale up. He et al. (He et al. 1999 : 343–355) further reported a novel method in which cimetidine and famotidine were entrapped in microspheres prepared by spray drying of multiple emulsion (o/w/o or w/o/w). They found that the release of the drugs from microspheres by this novel method was significantly sustained as compared to those prepared by conventional spray drying or o/w emulsion method.

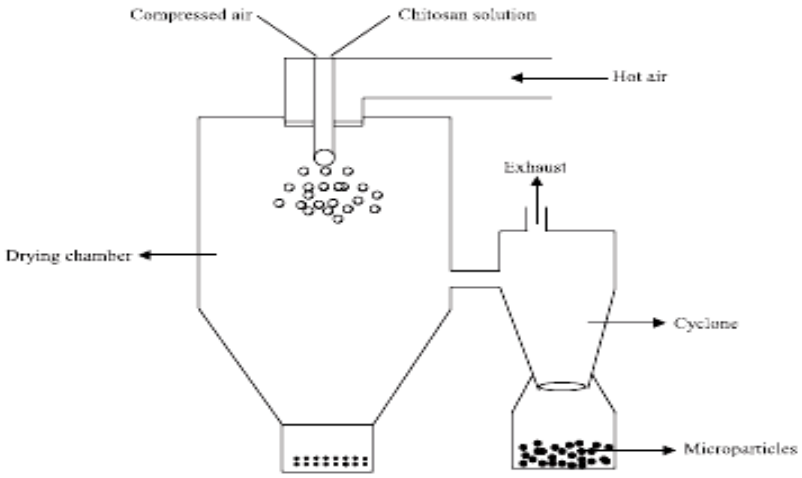


Figure 8 Schematic diagram of microparticles preparation with spray dry method.
Sources : L. Chenguang et al. “Preparations, Characterizations and Applications of Chitosan-based Nanoparticles,”. Journal of Ocean University of China 6 (2007): 237-243.

8.4 Reverse micellar method

Polymeric micelles are core–shell structures formed through the self-assembly of amphiphilic polymers in a solvent that is considered hostile towards either moiety. In water, these micelles are characterized by a hydrophobic core shielded from the external medium by a hydrophilic shell. This particular type of micelles has been extensively studied with a particular attention to their ability to improve the aqueous solubility of hydrophobic therapeutic agents (Liggins and Burt 2002 : 191–202; Gaucher and Dufresne 2005 : 169–188). Recently, ultrafine polymeric nanoparticles with narrow size range can be prepared with this method.

The surfactant was dissolved in an organic solvent to prepare reverse micelles. Aqueous solution of chitosan was added with constant agitation to avoid any turbulence. The aqueous phase was regulated in such a way as keeping the entire mixture in an optically transparent microemulsion phase. More water should be added in case nanoparticles of larger size should be prepared (Sunil et al. 2004 : 5-28).

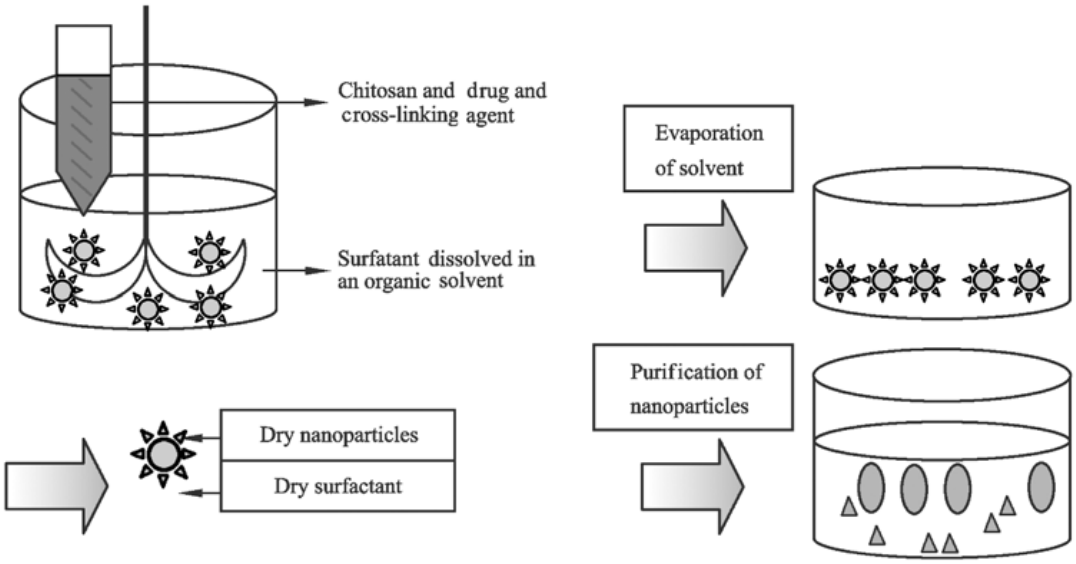


Figure 9 Schematic diagram of preparation of chitosan nanoparticles with reverse micellar method.

Sources : L. Chenguang et al. “Preparations, Characterizations and Applications of Chitosan-based Nanoparticles,”. Journal of Ocean University of China 6 (2007): 237-243.

9. Factors affecting of microparticles

9.1 Factors affecting the entrapment efficiency of drugs in the microparticles

Many factors affect the entrapment efficiency of the drugs in the microparticles, e.g. nature of the drug, chitosan concentration, drug polymer ratio, stirring speed, etc. Generally a low concentration of chitosan shows low encapsulation efficiency (Orienti et al. 1996 : 463–472). However, at higher concentrations, chitosan forms highly viscous solutions, which are difficult to process. A number of reports have shown that entrapment efficiency increases with an increase in chitosan concentration. This may be explained on the basis that an increase in viscosity of the

chitosan solution with increase in concentration prevents drug crystals from leaving the droplet (Sinha et al. 2004 : 1–33). A study carried out by Nishioka et al. (1990) also revealed that the cisplatin content increased with increasing chitosan concentration. Further Nishioka et al. (Nishioka et al. 1990 : 2871-2873) also proved that the incorporation of chitin in the carrier matrix produced a more pronounced increase in drug content.

9.2 Factor affecting of particlesizes

BirÓ et al. (BirÓ et al. 2009 : 771–779) was investigated the influence of process parameters on the mean particle size of chitosan microspheres produced by water-in-oil (w/o) emulsion crosslinking method. Chitosan particles for enzyme immobilization were prepared by w/o emulsion crosslinking method. For this, aqueous solution of chitosan in 2% (w/w) acetic acid was dispersed in an oil phase composed of 40% (v/v) sunflower oil and 60% (v/v) *n*-hexadecane in the presence of Tween 80 surfactant. The resulting droplets were chemically crosslinked by glutaraldehyde to obtain solid chitosan microspheres. Investigation was carried out to elucidate the influence of process parameters on the mean particle size of chitosan microspheres high chitosan, high surfactant and low glutaraldehyde concentration should be used with low stirring rate to avoid the coagulation of particles. Increasing the relative quantity of glutaraldehyde together with high stirring speed, the hardness of particles can be increased while maintaining the relatively low mean particle size (between about 230 and 240 μm).

9.3 Parameters affecting the release characteristics of drugs from microparticles

9.3.1 Effect of molecular weight of polymer

Drug release studies from chitosan microspheres have generally shown that the release of the drug decreases with an increase in molecular weight of polymer. In another study, Polk et al. (Polk et al. 1994 : 178–185) reported that chitosan molecular weight was a key variable in the release of albumin from chitosan microspheres. The molecular weight of chitosan was varied from 1.25×106 to 0.25×106 through a nitrite oxidation reaction with sodium nitrite. Decreasing the molecular weight increased the release of albumin (from 37% release at 4 h with high molecular weight chitosan to 77% release with low molecular weight chitosan).

Capsules produced with high molecular weight chitosan and a combination of high and low molecular weight chitosan gave the best results for reducing elution of albumin in the first 4 h and increasing elution in the following 20 h. However, Genta et al. (Genta et al. 1998 : 779–784) reported that the fastest ketoprofen dissolution profile from chitosan microspheres was obtained from medium molecular weight chitosan. This may be attributed to swelling behavior of chitosan microspheres. An increase in molecular weight of chitosan leads to increase in viscosity of the gel layer, which influences the diffusion of the drug as well as erosion of the microspheres.

9.3.2 Effect of concentration of polymer

Nishioka et al. (Nishioka et al. 1990 : 2871–2873) reported that the rate of cisplatin release reduced with the increasing concentration of chitosan. Aiedeh et al. (Aiedeh et al. 1997 : 567–576) observed that the method of chitosan interfacial crosslinkage by ascorbyl palmitate in water/oil dispersion was suitable to produce biodegradable system for insulin. The microcapsules obtained had release kinetics approaching zero order and a release rate, which could be increased by decreasing the chitosan content in the preparative solution.

CHAPTER III
MATERIALS AND METHODS

1. Materials

1.1. Alginic acid sodium salt from brown algae, high viscosity 40000 cps. (Sigma-Chemical Company St. Louis, MO, USA)

1.2. Alginic acid sodium salt from brown algae, low viscosity 250 cps. (Sigma-Chemical Company St. Louis, MO, USA)

1.3. Allergenic extract STANDARD MITE (*Dermatophagoides pteronyssinus*) : Dust mite allergen (Lot : 7F00642, ALK ABELLó , USA)

1.4. Calcium chloride dihydrate (Merck, Germany)

1.5. Chitosan hydrochloride with MW of 20, 45, 200, and 460 kDa and 85% degree of deacetylation

1.6. Chitosan-cholic-polyethylene glycol (CS-CA-mPEG)

1.7. Coomassie Brilliant Blue G-250 (Sigma-Chemical Company St. Louis, MO, USA)

1.8. Dimethyl sulfoxide (Fisher Scientific; analytical reagent grade)

1.9. Ethanol absolute (Scharlau® ET0016, Scharlau® Chemie SPAIN analytical reagent grade)

1.10. Ethylenediaminetetraacetic acid solution : EDTA (Sigma-Chemical Company St. Louis, MO, USA)

1.11. Hydrochloric acid (Scharlau Chemie S.A. (Analytical grade, ACS)

1.12. Hydroxy naphthol Blue (Sigma-Chemical Company St. Louis, MO, USA)

1.13. Penta-sodium triphosphate (Sigma-Chemical Company St. Louis, MO, USA)

1.14. Sodium chloride (UNIVAR®Ajax Finechem; analytical reagent grade)

1.15. Sodium citrate (Ajax Finechem Australia, New Zealand)

1.16. Sodium hydroxide pellet BP (Ajax chemicals, New South Wales, Australia)

2. Equipments

2.1 15 ml, 50 ml Centrifuge tubes-Sterile (BIOLOGIX RESEARCH COMPANY)

2.2 96-well Cell Culture Cluster (Costar®; Corning Incorporated)

2.3 Analytical balance (Sartorius CP224S, Sartorius CP3202S ; SCIENTIFIC PROMOTION CO.,LTD)

2.4 Blunt needle 24, 26-gauge (TERUMO® SYRING with Needle)

2.5 Cellulose Acetate Filter 0.2 μm (Sartorius AG . 37070 Goettingen, Germany)

2.6 Centrifuge (HERMLE Z300K ; Labnet® ; LAB FOCUS CO.,LTD.)

2.7 Dialysis bag (CelluSep® MWCO 6,000-8,000 Membrane Filtration Product, Inc., USA)

2.8 Eppendrof® tubes (Corning Incorporated)

2.9 Freeze-dryer (LABCONCO, Freezone 2.5,USA)

2.10 Fusion Universal Microplate Analyzer (Model No : AOPUS01 and A153601 ; A Packard BioScience Company)

2.11 High Performance Liquid Chromatography (HPLC) instrument consisted with the following

- Liquid chromatography pumps (P-1500, Thermo Separation Products, USA
- UV-VIS detector (UV-1000, Thermo Separation Products, USA)
- Autosampler (AS-3000, Thermo Separation Products, USA)
- Degasser (Thermo Separation Products, USA)
- Software ChromQuest (Thermo Separation Products, USA)
- Column HPLC (VetiSep GES C18 5 μm,4.6x250 mm, Waters, USA)

2.12 Inverted Microscope (ECLIPSE TE 2000-U ; Model : T-DH Nikon® Japan)

2.13 Magnetic stirrer and magnetic bar (Becthai Bangkok Equipment & Chemical Co.,Ltd.)

2.14 Micropipette 0.1-2 μl, 2-20 μl, 20-100 μl, 100-1000 μl, 1-5 ml (masterpette ; Bio-Active Co.,Ltd.)

2.15 Micropipette tip (Bio-Active Co.,Ltd.)

2.16 Sartorius® filter set (Sartorius BORO 3. 3 Goettingen, Germany)

2.17 Shaking Incubator (GFL 3031)

2.18 Sonicator (Transsonic T460/H, Elma Hans Schmidbauer, Germany)

2.19 Syringe filter (25 mm diameter, 0.45 μm pore size, Chrom Tech Inc., USA)

2.20 Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK)

3. Methods

3.1 Preparation of allergen-loaded microparticles

3.1.1 Preparation of allergen-loaded alginate microparticles (Al-Ca-alginate)

Ca-alginate microparticles were prepared by ionotropic gelation. The gelation and crosslinking of the polymers are mainly achieved by the exchange of sodium ions from the guluronic acids with the divalent cations, and the stacking of these guluronic groups to form the characteristic egg-box structure (George and Abraham 2006 : 1–14). The simplest method of producing Ca-alginate microparticles is based on dropwise sodium alginate in $CaCl_2$, using a syringe with a needle or a pipette (Kumar 2000 : 234-258). Briefly, the powders (alginate and $CaCl_2$) were weighed separately and dissolved in water under magnetic stirring to prepare stock solutions of 0.001, 0.01, 0.05, 0.1% w/v alginate (high viscosity; AHV and low viscosity; ALV) and 3 and 4% w/v $CaCl_2$. Forty ml alginate solutions were dropped through a needle placed into a 10 ml glass syringe capped with a 24-gauge blunt needle (an internal diameter of needle is 0.9 mm) into a 50 ml of $CaCl_2$ solution while the solution was stirred at ambient temperature. Stirring continued for another 30 min, and then the resultant particles were collected for morphology and particle size determination. The spherical and smallest particle sizes were selected for allergen-loaded alginate microparticles.

Allergen-loaded alginate microparticles containing dust mite allergen were prepared. Briefly, sodium alginate (AHV and ALV) was dissolved in distilled water with constant stirring to prepare stock solutions of 0.1% w/v. Dust mite allergen (0, 1, 2.5, 5% w/w) was premixed with 0.05% w/v alginate solution to make 10 ml of final alginate solution and microparticles were prepared by the same procedures as of bare alginate microparticles.

3.1.2 Preparation of allergen-loaded chitosan microparticles (Al-CS-TPP)

Chitosan microparticles were prepared by ionotropic gelation. Shortly, chitosan hydrochloride (CS) was dissolved in distilled water to a concentration of 0.1% w/v. Penta-sodium triphosphate (TPP) was dissolved in distilled water to a concentration of 0.2% w/v. Allergen-loaded chitosan microparticles were prepared by drop-wise addition of 10 ml TPP solution containing dust mite allergen (0.1, 0.5, 2.5 % w/v) to a 40 ml CS solution while the solution was stirred at 700 rpm for 20 min. The resultant particles were collected and washed with distilled water by centrifugation at 3000 rpm for 15 min. The allergen-loaded microparticles were lyophilized and stored at 4°C. Bare microspheres were also prepared in the same manner without added allergen.

3.1.3 Preparation of allergen-loaded CS-TPP microparticles coated with calcium alginate

Firstly, 2.5% allergen-loaded chitosan-TPP microparticles were prepared according to 3.1.2 and then washed with distilled water by centrifugation at 3000 rpm for 15 min. Secondly, allergen-loaded chitosan microparticles were coated with calcium alginate. Shortly, alginate (AHV) was dissolved in water under magnetic stirring to prepare stock solutions of 0.025, 0.05, 0.1, 0.25, 0.5% w/v. Fifty microliter of sodium alginate solution was mixed with allergen-loaded chitosan-TPP microparticles and stirred until a uniform mixture was obtained. The mixture were dropped through a needle placed into a 10 ml glass syringe capped with a 26-gauge blunt needle (an internal diameter of needle is 0.5 mm) into 100 ml of $CaCl_2$ solution (4 and 6% w/v) while the solution was stirred at ambient temperature. Stirring continued for another 30 min, and then the resultant particles were collected and washed with distilled water by centrifugation at 3000 rpm for 15 min. The allergen-

loaded CS-TPP microparticles coated with calcium alginate were lyophilized and stored at 4°C. Bare microspheres were also prepared in the same manner without added allergen. The morphology and particle size were determined.

3.1.4 Preparation of allergen-loaded chitosan cholic polyethylene glycol micelles (Al-CS-CA-mPEG)

The incorporation of allergen into polymeric micelles was carried out by dialysis method. Briefly, 5 mg of CS-CA-mPEG polymer were dissolved in 2 ml of DMSO and stirred at room temperature until completely dissolved. Dust mite allergen (0.25-0.75 % of polymer) was added to polymer solution. The mixture was then placed in a dialysis bag (membrane: Spectra/Por® 1,000 MWCO, Spectrum Laboratories, USA) and dialyzed against distilled water or 0.9% sodium chloride overnight. In the case of reverse micelles, CS-CA-mPEG polymer was dissolved in 2 ml of distilled water and then dialyzed against DMSO. After dialyzed CS-CA-mPEG solution with DMSO, these reversed micelles were placed in a dialysis bag and dialyzed against distilled water to remove DMSO and to change the reversed micelles to become micelles.

3.2 Characterization of allergen-loaded microparticles

3.2.1 Particle size and zeta potential

The mean particle diameter and zeta potential of the allegen-loaded microparticles were determined in triplicate at 25°C by photon correlation spectroscopy (PCS) using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). The microparticle samples were diluted with distilled water that had been filtered through a 0.22 μm membrane filter.

3.2.2 Morphology

Morphological characterization of the microparticles was performed by inverted microscopy (Inverted Research Microscope; ECLIPSE TE 2000-U, Japan) using the magnification of 20X and by transmission electron micrograph (TEM). Briefly, solution of formvar (3%w/v) was prepared in spectroscopic-grade chloroform. Then, one drop of allegen-loaded microparticles sample solution was put on a formvar-coated carbon ultra-thin grid and air-dried. The dried grid was then examined under a transmission electron microscope (JEOL JEM1230, Tokyo, Japan).

3.3 Allergen entrapment efficiency

3.3.1 Ca-alginate microparticles

3.3.1.1 Allergen assay

The total content of allergen in the allergen-loaded alginate microparticles was determined. Accurately weighed amounts (about 0.1 g) of the dried allergen-loaded alginate microparticles were placed in 15 ml centrifuge tubes containing 2 ml of 2 % tri-sodium citrate buffer followed by continuous shaking in a shaker incubator (Orbital Shaking Incubator Model : SI4) at 200 rpm until the allergen-loaded alginate microparticles were totally dissolved. The supernatant was measured using Bradford assays to quantify the protein concentration in solution. The absorbance of each sample was measured on absorbance at 550 nm using a microplate reader (Fusion Universal Microplate Analyzer Model: A153601).

3.3.1.2 Calcium assay

Reagents

a) Preparation of 0.05 M EDTA solution

9.3 g of EDTA was dissolved in 200 ml of distilled water and then adjusted to 500 ml in a volumetric flask.

b) Preparation of hydroxy naphthol blue indicator (0.1 % stock solution)

0.125 g of hydroxy naphthol blue indicator was dissolved in 40 ml of distilled water and then adjusted volume to 50 ml in a volumetric flask.

c) Preparation of standard 0.002 M calcium solution

Accurately weighed 0.2 g of $CaCO_3$ was dissolved in concentrated hydrochloric acid until a clear solution was obtained (no CO_2 evolution, usually 4-5 drops of conc. HCl) and then adjusted to 100 ml with distilled water in a volumetric flask.

The total content of calcium in the allergen-loaded alginate microparticles was determined by EDTA tritration as following procedure:

Determination of molarity of standard EDTA

One hundred milliliter of calcium standard was added into 500 ml Erlenmeyer flask. After 0.25 ml of hydroxy naphthol blue indicator was added to

calcium standard solution, titration was performed using EDTA solution as a titrant until wine red color turned into blue (Fig. 10). The volume of EDTA was used to calculate the molarity of EDTA by equation 1. The titration was done in duplicate.

$$\text{Molarity of EDTA} = \text{mg } CaCO_3/(\text{MW } CaCO_3 \text{ x ml EDTA}) \quad (1)$$

Analysis of calcium in samples

Ten milliliter of $CaCl_2$ solution before and after Ca-alginate microparticle preparation was added into 500 ml Erlenmeyer flask. To neutralize the samples, Five ml of 1 M sodium hydroxide solution was added and then a few drops of hydroxy naphthol blue indicator solution was added and mixed with the samples. The solution should be in wine red color. Sample solution was titrated with standard EDTA solution with continuous swirling until the wine red color turned into blue. The volume of EDTA was used to calculate the amount of $CaCl_2$ by equation 2. The titration was done in duplicate.

$$\text{Amount of } CaCl_2 \text{ (g)}/\text{MW } CaCl_2 = \frac{(\text{molarity EDTA x ml EDTA})}{1000} \quad (2)$$

a) b) c)

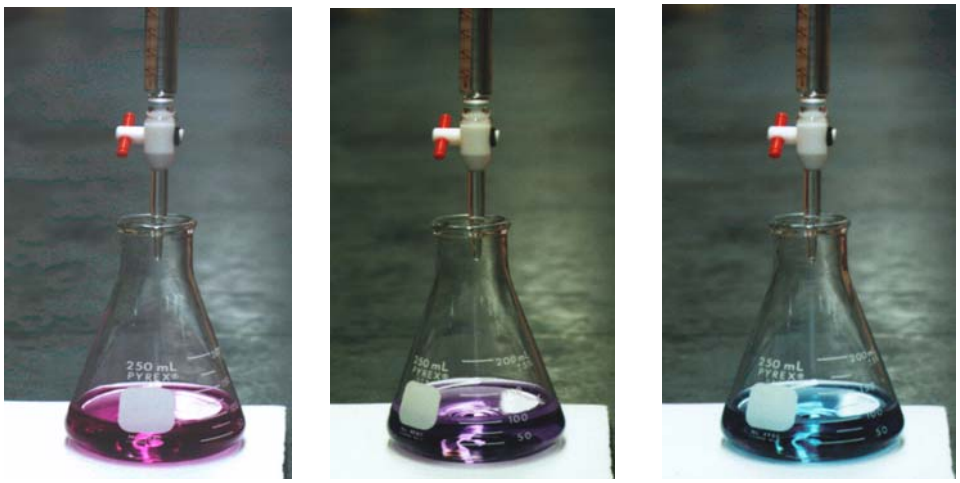


Figure 10 Analysis of calcium by titration (a) before titration (wine red color) (b) during titration with standard EDTA solution (c) at the end point

3.3.1.3 The percentage yield, percentage entrapment efficiency and loading capacity

The percentage yield, percentage entrapment efficiency and loading capacity were calculated by using equation 3-5, respectively (Min et al. 2007 : 5-9).

$$\% \text{ yield} = Wm/Wt \quad (3)$$

where Wm is the weight of microparticle, and Wt is the theoretical weight of microparticle.

$$\% \text{ allegen entrapment efficiency} = (Pt/ Lt) \text{ X } 100 \quad (4)$$

where Pt is the amount of allergen embedded in microparticles. Lt is the theoretical amount of allergen (obtained from feeding condition) incorporated into microparticles.

$$\text{allegen loading capacity} = Pt \text{ (mg)} / Mt \text{ (g)} \quad (5)$$

where Pt is the amount of allergen embedded in microparticles, and Mt is the total amount of microparticle harvested.

3.3.2 CS-TPP microparticles

One hundred milligrams of allergen-loaded CS-TPP microparticles were dissolved in 2 ml of 5 N HCl (pH 1.2). The mixture was stirred at room temperature until completely dissolved. The amount of allergen incorporated into microparticles was determined by using a reverse-phase HPLC. The % yield, % allegen entrapment efficiency and allegen loading capacity of microparticles were calculated by equation 3-5 as described above.

3.3.3 CS-TPP microparticles coated with calcium alginate

One hundred milligrams of allergen-loaded CS-TPP microparticles coated with calcium alginate were dissolved with 2 ml of PBS pH 7.4 and followed by continuous shaking in a shaker incubator (Orbital Shaking Incubator Model : SI4) at 200 rpm until the microparticles were totally dissolved. The amount of allergen

incorporated into microparticles was determined by using reverse-phase HPLC. The % yield, % allegen entrapment efficiency and allegen loading capacity of microparticles were calculated as by equation 3-5 as described above.

3.3.4 Chitosan cholic polyethylene glycol micelles (CS-CA-mPEG)

0.2 ml of allergen-loaded CS-CA-mPEG micelles was dissolved in distilled water 1.8 ml (1:9), and followed by continuous shaking in a shaker incubator (Orbital Shaking Incubator Model : SI4) at 200 rpm until the micelles were totally dissolved. The amount of allergen incorporated into microparticles was determined by using a reverse-phase HPLC. The percentage of allergen entrapment efficiency and allergen loading capacity of CS-CA-mPEG micelles were calculated as presented in equation 4-5 as described above.

3.4 *In vitro* allergen release

In vitro release studies of allergen-loaded microparticles were performed by suspending 10 mg of allergen-loaded microparticles tubes with 1 ml of PBS pH 7.4 or simulated gastric fluid without pepsin (pH 1.2) in 1.5 ml microcentrifuge. All tubes were then incubated at 37°C under shaking at 200 rpm to maintain the particles in suspension (sink conditions). To determine the amount of allergen released after a given time (1, 2, 4, 8, 12, 24, 48, 72 and 96 h), the sample was centrifuged for 15 min at 3000 rpm. The supernatant was measured in triplicate by a reverse-phase HPLC using fluorescence detector.

For allergen-loaded CS-CA-mPEG micelles, 1 ml of micelles was placed in a dialysis bag (membrane: Spectra/Por® 12,000–14,000 MWCO, Spectrum Laboratories, USA) and immersed in 15 ml of medium (PBS pH 7.4 or in simulated gastric fluid without pepsin (pH 1.2)) at 37 ± 0.1 °C under shaking at 200 rpm. At certain time intervals (1, 2, 4, 8, 12, 24, 48, 72 and 96 h), 1 ml aliquots of the medium was withdrawn and the same volume of fresh medium was added. The sample solution was analyzed by a reverse-phase HPLC using fluorescence detector. All experiments were performed in triplicate.

3.5 Analysis of allergen

3.5.1 Bradford assays

The Bradford assay was used to determine the concentration of proteins in solution. The procedure is based on the formation of a complex between the dye, Brilliant Blue G, and proteins in solution. The protein dye complex causes a shift in the absorption maximum of the dye from 465 to 595 nm. The amount of absorption is proportional to the protein present.

- **Bradford reagent**

50 mg of Coomassie Brilliant Blue G-250 is dissolved in 2.5 ml of 95% ethanol in 50 ml volumetric flask until completely dissolved. The solution is added to 5 ml of 85% (w/v) phosphoric acid, and diluted to 50 ml with water. The Bradford reagent should be light brown in color. It is stable for weeks in a dark bottle at 4°C.

- **Protein Standards**

A protein standard curve was made using bovine serum albumin (BSA) with concentrations of 0, 12.5, 25, 50, 100 and 200 µg/ml for the standard assay of dust mite allergen.

- **Procedures**

The sample was a blank, a protein standard, or an unknown sample. The blank consisted of buffer (or deionized water) with no protein. The protein standard consisted of a known concentration of protein, and the unknown sample was the solution to be assayed. 20 µl of standard BSA and dust mite allergen samples were added into 96 well-plate and then 200 µl of Bradford reagent was added and gently mixed. The mixture was assayed on absorbance at 550 nm using a microplate reader (Fusion Universal Microplate Analyzer Model: A153601).

3.5.2 Reverse-phase HPLC

Concentrations of allergen were determined using a reverse phase-HPLC system (Agilent 1100 series, USA) at a flow rate of 1.0 ml/min. For separation, a Waters C_{18} reverse-phase column (Water, USA), 5 µm, 150 mm x 0.5 mm was used. The mobile phase was composed of 20:80 v/v 0.085% trifluoroacetic acid (TFA) in

acetonitrile and 0.1% TFA in water. The injection volume was 20 µl. The detection was performed using a fluorescence detector with an excitation wavelength of 280 nm and emission wavelength of 304 nm.

3.6 Statistical analysis

All experimental measurements were performed in triplicate. Result values were expressed as mean value ± standard deviation (S.D.). Statistical significance of differences were examined using one-way analysis of variance (ANOVA) followed by LSD post hoc test. The significance level was set at $p < 0.05$.

CHAPTER IV
RESULTS AND DISCUSSION

1. Preformulation

1.1 Calcium alginate (Ca-alginate) microparticles

Sequential crosslinking and formation of polymeric networks result in hydrogel structured drug delivery carriers such as micro- and nanoparticles upon the addition of counter-ions to alginate. Any possible cationic species can initiate the reaction sequence, but calcium chloride ($CaCl_2$) is favorably utilized by most researchers (Hamidi et al. 2008 : 1638–1649). The advantages of ionic crosslinking method are that it is an "all-aqueous" system and avoids residual solvents in microspheres. Moreover, this method is suitable for protein drug and allergen because it does not involve harsh conditions, which can destroy protein drug or allergen (Vasir et al. 2003 : 13–32). Therefore, in this study Ca-alginate microparticles were prepared by ionic gelation method. In this process, alginate solution was extruded through a precise device, producing microdroplets which fell into a hardening bath, that was slowly stirred. Hardening bath contains calcium chloride solution, whereby the divalent calcium ions crosslinked with alginate forming gelled microspheres. The aim of this part was to control the gelation phenomenon, which leaded to desired size ranges depending on various factors including alginate concentration, viscosity and counter-ion concentration. The mean particle size of Ca-alginate microparticles in different formulation are shown in Table 3. The mean particle size was about 1-6 μm depending on the type, amount of alginate and $CaCl_2$. The result showed that 0.05 mg/ml alginate, medium viscosity with 4 mg/ml $CaCl_2$ (A4) and 0.05 mg/ml alginate, low viscosity with 4 mg/ml $CaCl_2$ (A8) yielded the smallest particles of 2683.3 ± 700 nm and 2250 ± 992 nm, respectively. Increasing the concentration of alginate resulted in the increase in mean particle size. However, the increase of concentration of $CaCl_2$ resulted in the decrease in mean particle size. The results were in agreement with previous report which showed that the concentration of polymer and $CaCl_2$ had the most significant effect on the mean particle size of microparticles. Biró et al. (Biró et

al. 2009 : 771–779) elucidated the influence of process parameters on the mean particle size of chitosan microspheres produced by water-in-oil (w/o) emulsion crosslinking method. The result showed the mean particle size increased with the increasing chitosan concentration. SMRDEL et al. (SMRDEL et al. 2008 : 76-89) evaluated the influence of various processing parameters such as hardening time, temperature and concentration of $CaCl_2$ solution and drying conditions on size and morphology of alginate beads prepared by ionotropic gelation method. It was found that increasing the calcium chloride concentration yielded, smaller beads. The beads hardened in 0.1 and 0.27 M calcium chloride solution had significantly lower mean values of frontal diameters than beads prepared using 0.5, 0.68 and 1.0 M calcium chloride solution. Østberg et al. (Østberg et al. 1993 : 183-193) prepared small calcium alginate matrices by ionotropic gelation of droplets of an alginate solution containing dispersed theophylline, followed by air-drying of the gel beads. The effect of various production factors on the size, composition and drug release properties was investigated in two separate studies. The result of their study showed the alginate concentration and the calcium concentration used for gelation appeared to have a significant influence. These two factors, together with the amount of drug dispersed, determined the drug content in matrix. Moreover, the mean diameter increased as concentration of alginate was increased. The reason might be that alginates are not random copolymers but consist of blocks of similar and regularly alternating residues of mannuronic acid (M) and guluronic acid (G) (i.e., MMMM, GGGG and GMGM), and the relative abundance of the different blocks depends on the algae source. When alginate solution was dropped into $CaCl_2$, strong interaction between Ca^{2+} and carboxylic groug in poly-G chains of sodium alginate in egg-box structure was obtained (Schoubben et al. 2009 : 226–234), then viscosity of alginate the block copolymer was increased, and resulted in stronger interaction of Ca^{2+} and poly-G chains. Therefore, the particle sizes of higher concentration of polymer were larger than lower concentration. In this study, two formulae (A4 and A8) were selected to prepare allergen-loaded alginate microparticles in the next experiment.

Table 3 Mean particle size of Ca-alginate microparticles prepared by different formula

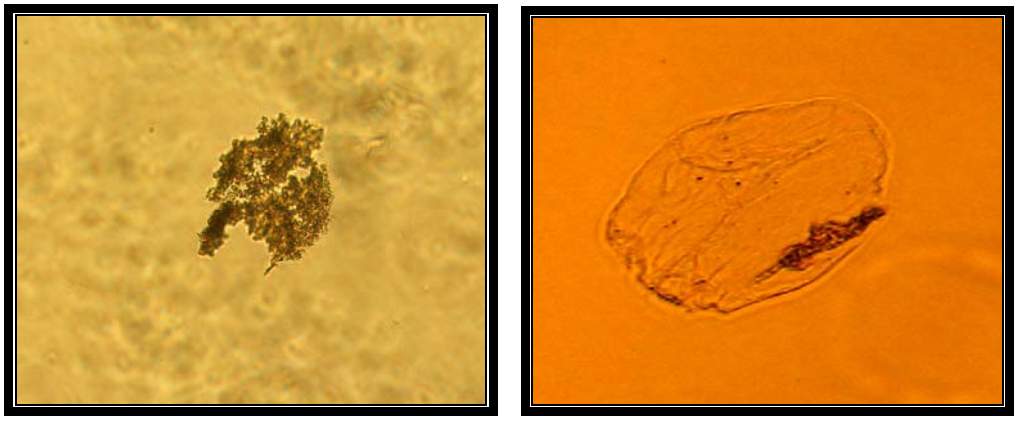
Code	**Alginate viscosity**	**Na alginate (mg/ml)**	**$CaCl_2$ (mg/ml)**	**Alginate:$CaCl_2$ (w/w)**	**Diameter ± SD (nm)**	**PDI**
A1	HV	0.05	3	1 : 150	3890 ± 2792	0.55
A2	HV	0.1	3	1 : 75	3117 ± 848.5	0.92
A3	HV	0.5	3	1 : 15	3670 ± 490	0.75
A4	HV	0.05	4	1 : 200	2683 ± 700	0.71
A5	HV	0.1	4	1 : 100	4593 ± 2809	0.75
A6	LV	0.05	3	1 : 150	5580 ± 2237	0.84
A7	LV	0.1	3	1 : 75	4527 ± 626	0.47
A8	LV	0.05	4	1 : 150	2250 ± 992	1
A9	LV	0.1	4	1 : 75	5200 ± 985	1

1.2 Ca-alginate coated CS-TPP microparticles

Ca-alginate coated CS-TPP microparticles were prepared by coating CS-TPP microparticles with sodium alginate solution (AHV) of different concentrations (0.025, 0.05, 0.1, 0.25, 0.5% w/v) and crosslinking microparticles with 4 or 6 % w/v $CaCl_2$. The preparation method followed the method of Yu et al. (Yu et al. 2009 : 245–249), which described a suitable and relatively simple technique to obtain Ca-alginate coated CS-TPP microparticles. The aim of this part was to find suitable concentration of alginate and $CaCl_2$ that produced microparticles in spherical shape. Table 4 shows morphology of CS-alginate coated CS-TPP microparticles obtain from various formulations observed under inverted microscope using the magnification of 20X.

Table 4 Morphology of Ca-alginate coated CS-TPP prepared from different formulations observed by inverted microscopy using the magnification of 20X.

Alginate conc. (mg/ml)	**$CaCl_2$ solution (mg/ml)**	**Alginate:$CaCl_2$ (w/w)**	**Morphology of microparticles**
0.025	4	1 : 400	small amorphous form
0.025	6	1 : 600	small amorphous form
0.05	4	1 :2000	small amorphous form
0.05	6	1 : 3000	small amorphous form
0.1	4	1 : 100	small tail particles
0.1	6	1 : 150	small tail particles
0.25	4	1 : 40	small semi-circles particles
0.25	6	1 : 60	small semi-circles particles
0.5	4	1 : 20	large semi-circles particles
0.5	6	1 : 30	large semi-circles particles



(a) (b)

Figure 11 CS-TPP microparticle (a), Ca-alginate coated CS-TPP microparticle (b) by inverted microscopy using the magnification of 20X.

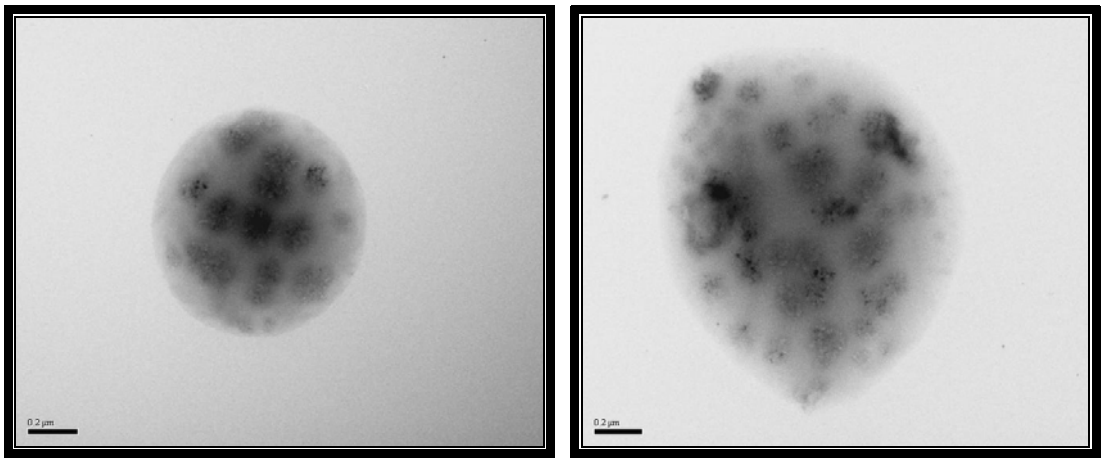
It is shown that Ca-alginate prepared from 0.25% w/w sodium alginate and 6% w/v $CaCl_2$ was able to coat CS-TPP and the shape of particles was more spherical than other formulations. Therefore, 0.25 mg/ml sodium alginate and 6 mg/ml $CaCl_2$ solution was chosen to coat allergen loaded CS-TPP at the concentration of 2.5% w/w for *in vitro* release study.

2. Physicochemical properties of allergen loaded microparticles

2.1 Ca-alginate microparticles

Microparticles were successfully prepared using alginate and chitosan by ionotropic gelation method. In case of Ca-alginate microparticles, they were produced by ionic gelation and crosslinked with $CaCl_2$. Fig. 12 shows the shape of Ca-alginate microparticles from formulae A8 before and after the incorporation with allergen. The shape of microparticles was generally spherical, and the surface before and after allergen loading was smooth. The mean particle sizes of Bare-Ca-alginate and Al-Ca-alginate microparticles were 3560 ± 130 nm and 598 ± 43 nm, respectively. The charge of Bare-Ca-alginate and Al-Ca-alginate microparticles were -7.7 ± 2.9 and -22.7 ± 2.6, respectively. Before allergen loading, Ca-alginate microparticles showed a slightly negative zeta potential. However, after the incorporation of allergen, more negative zeta potential was obtained (Table 5). This probably is due to the negative charge of allergen. This result was corresponded with previous report of Beatriz et al.

(Beatriz et al. 2008 : 98–108). They study about CpG oligonucleotide Th1-biased adjuvant activity can be improved when closely associated with a variety of antigens in PLGA microparticles. From this results found that the zeta potential was moved towards slightly negative when CpG motifs were encapsulated 3.1 ± 0.8 to -5.1 ± 0.3 mV.



(a) (b)

Figure 12 TEM micrographs of Ca-alginate microparticles before (a) and after (b) incorporation with allergen (Magnification 5,000 and 10,000x).

According to the characteristic FT-IR spectra of the microparticles, an attempt was made to elucidate the eventual existence and type of interactions between the polymers and the drug. Sodium alginate as a carboxylate salt showed a strong asymmetric stretch at 1605 cm^{-1}. The frequency of carbonyl absorption is lower compared to the value found for the parent carboxylic acid due to a resonance phenomenon. The carboxyl and carboxylate groups are present at wave number of about 1000–1400 cm^{-1} (Borges et al. 2005 : 155–166). The characteristic alginate spectra have been previously described (Wang and He 2002 : 117–126, Soares et al. 2004 : 57–64) and they correspond to CH (2950 cm^{-1}), COO (1613 cm^{-1}), CH (1415 cm^{-1}) and C-O-C (1033 cm^{-1}). The FT-IR spectra of Ca-alginate microparticles in Fig. 13 showed that the absorption band around 2950, 1620, 1438 and 1040 cm^{-1} corresponds to the stretching of -CH, COO-/, -/CH and C-/O- C, respectively, while

the band at 3440 cm^{-1} belongs to the stretching vibrations of the hydroxyl groups bonded via hydrogen bonds.

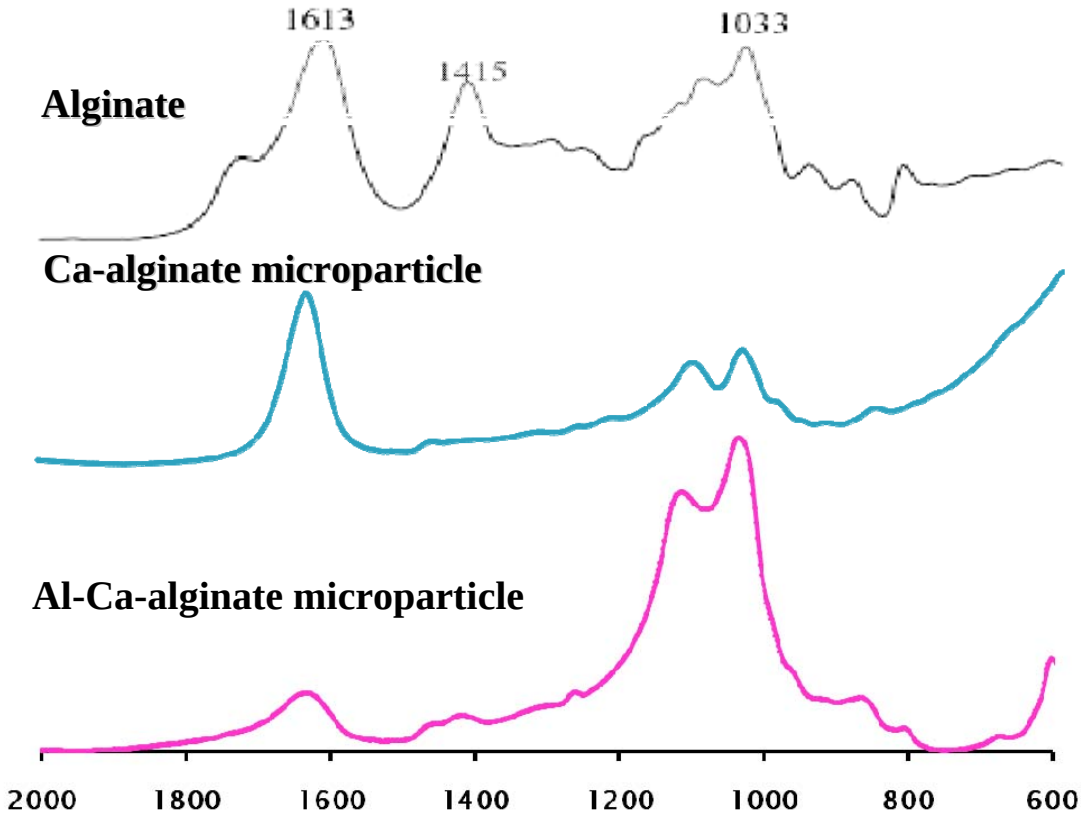
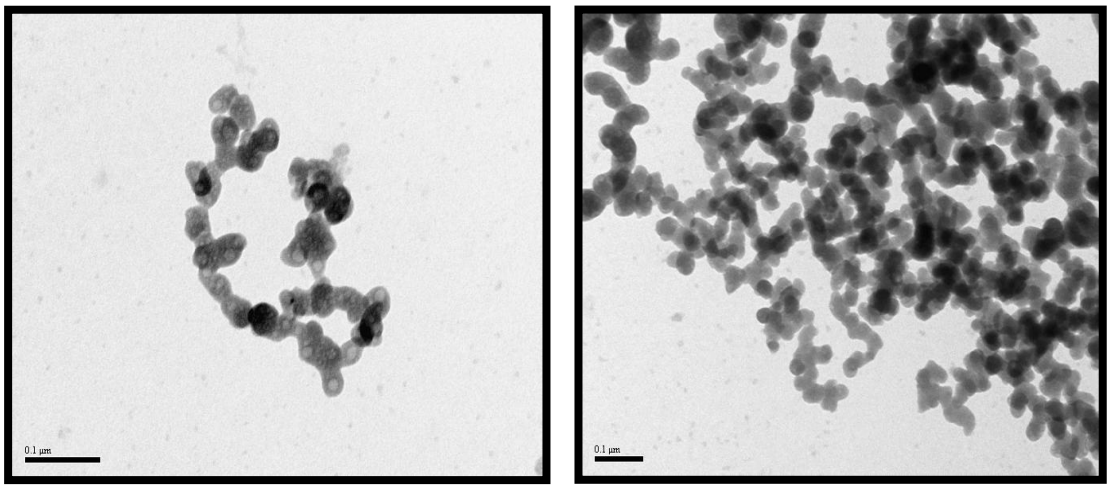


Figure 13 The FT-IR (KBr) spectra of the alginate,the Bare-Ca-alginate microparticles and Al-Ca-alginate microparticles at the range of 900 – 3500 cm^{-1}

2.2 CS-TPP microparticles

The characteristics of CS-TPP microparticles depended on the ratio of chitosan and TPP. In the present study, microparticles were also successfully prepared by drop-wise addition of 10 ml TPP solution (0.2% w/v) containing dust mite allergen to a 40 ml CS-HCl solution (0.1% w/v) while the solution was stirred. In Table 5, the mean particle size of Bare-CS-TPP and Al-CS-TPP microparticles was 1740 ± 158 and 2343 ± 328 nm, respectively. The charge of Bare-CS-TPP and Al-CS-TPP microparticles was 11.3 ± 2.9 and -20.1 ± 3.0 mV, respectively. The polydispersity

index (PDI) is a measure of the distribution of microparticle population which it is zero for monodisperse particles. The PDI of allergen loaded microparticles showed in the range of 0.7 to 0.8. These results represented that the narrow size distribution was obtained in all prepared formulations. The allergen loading only slightly decreased zeta potential of the CS-TPP microparticles. This is as expected since allergen entrapment in and adsorption on the particle would have reduced the positive surface charge of cationic chitosan molecules. By imaging with TEM, an acceptable spherical morphology was observed. The particles tended to agglomerate (Fig. 14a), probably due to the specific localization of the polymers and the existence of attractive electrostatic forces (Mladenovska et al. 2007 : 59-69). All bare microparticles were characterized by TEM similar to that of the allergen loaded microparticles (Fig. 14b). In addition, the optical microscopy was used to observe the morphological parameters of the particles after suspending in distilled water. Disaggregation of the particles was observed, and spherical shape of the particles was obtained as shown in Fig. 11a.



(a) (b)

Figure 14 TEM micrographs of (a) Bare-CS-TPP microparticles, and (b) Al-CS-TPP microparticles (Magnification 100,000 x).

In the chitosan spectra, the strong and broad peaks in the 3400–3200 cm^{-1} ranges correspond to combined peaks of OH stretching and intermolecular hydrogen bonding. The NH stretching from primary amines are overlapped in the same region.

The FT-IR spectrum of CS-HCl spectra has been also recorded (Mi et al. 2002 : 61–67) and they are characterized by a broad absorption around 1660 cm^{-1} (amide I, C-O stretching mode conjugated with N-H deformation mode) and 1600 cm^{-1} (δNH_2 bend of non-acetylated NH_2 groups). Characteristic for its saccharide structure are absorption bands at 1154 cm^{-1} (asymmetric stretching vibration of the C-O-C bridge), 1083 cm^{-1} and 1038 cm^{-1} (skeletal vibration involving C-O stretching). Fig. 15 shows FT-IR spectra between pure CS solution and CS-TPP microparticles. The result showed that CS-TPP had characteristic peak at 3442 (OH), 2949 and 2910 (C-H stretching), 1662 (amide I), 1554 (amide II), 1084 (C-O-C) and 866 cm^{-1} (pyranose ring). These results were similar with CS-HCl. Moreover, broad band around 1824 was found, and more peaks in range between 862 and 1167 cm^{-1} were observed. These peaks are specifically of phosphate group, therefore it can be concluded that complexation between chitosan and TPP has occurred.

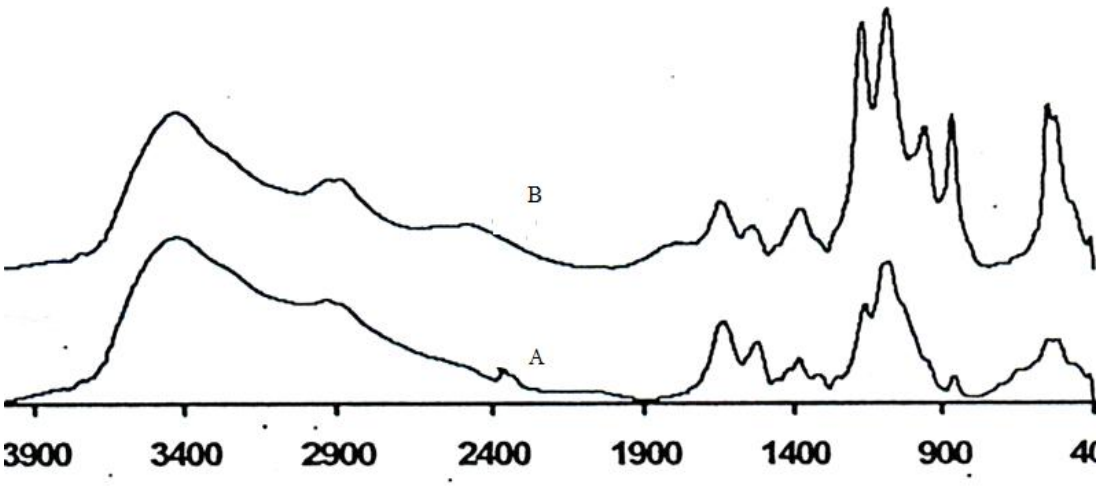


Figure 15 The FT-IR spectra of the CS-HCl (A), CS-TPP microparticles (B) at the range of 400 – 3500 cm^{-1}

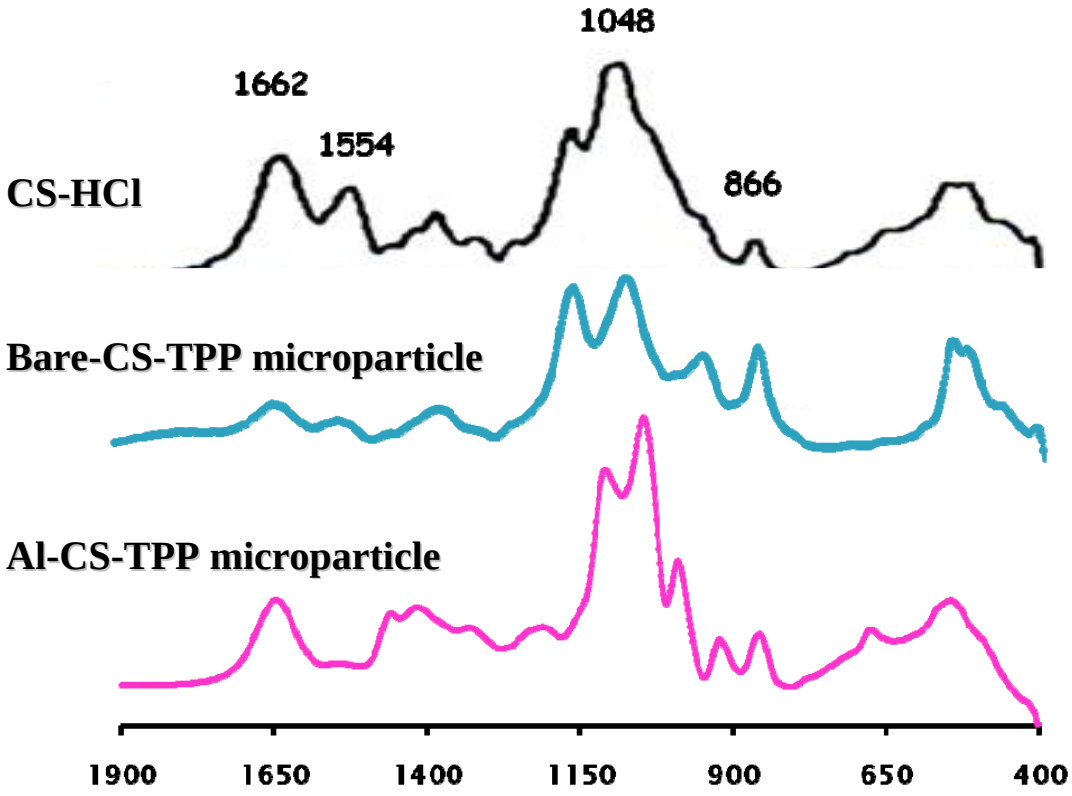


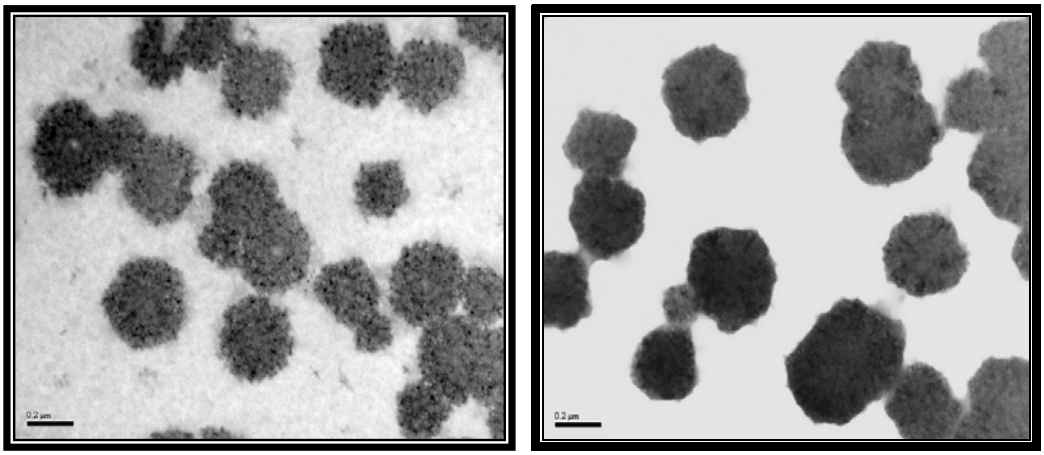
Figure 16 The FT-IR spectra of CS-HCl, Bare-CS-TPP microparticles, Al-CS-TPP microparticles at the range of 900 – 3500 cm^{-1}

2.3 Ca-alginate coated CS-TPP microparticles

From Table 5 the mean size of Bare-Ca-alginate coated CS-TPP and Al-Ca-alginate coated CS-TPP microparticles were about 14.88 ± 1.84 and 17.12 ± 2.04 µm, respectively. The charge of Bare-Ca-alginate coated CS-TPP and Al-Ca-alginate coated CS-TPP microparticles were -3.8 ± 2.2 and -7.2 ± 1.1 mV, respectively. Coated microparticles were negatively charged due to the contribution of the alginate. By imaging with TEM, an acceptable spherical morphology was observed (Fig. 17). The microscopic observation is in good agreement with the data obtained by the particle size analyzer.

From the FT-IR spectra of the coated particles, three peaks that are different from those of the chitosan uncoated particles could be found. The results clearly show the existence of alginate coating layer around the chitosan particles. Figure 18 showed

Ca-alginate coated CS-TPP microparticles. Some peaks disappeared or became weak due to interaction or superposition between groups of Ca-alginate and CS-TPP. However FT-IR spectra of Ca-alginate coated CS-TPP microparticles represented unreacted –COOH groups of alginate. The characteristic peak observed at 1613 cm^{-1} (salt of carboxyl group) in the FT-IR spectrum of microparticles was attributed to the ionic interaction between these two reactive groups.



(a) (b)

Figure 17 TEM micrographs of Ca-alginate coated CS-TPP (a), Al-Ca-alginate coated CS-TPP (b) microparticles (Magnification 100,000x).

2.4 CS-CA-mPEG micelles

The CS-CA-mPEG polymers were synthesized, and the chemical structure was depicted. Initially, suitable solvents for dissolving CS-CA-mPEG were investigated. Since CS-CA-mPEG was completely dissolved in dimethylsulfoxide (DMSO) and water, in this study they were chosen as a solvent to dissolve CS-CA-mPEG. Micro-size micelles were formed when it was dialyzed against water. The tendency of these polymers to form self-assembling micelles might be based on the hydrophobic cholic groups on the chitosan chain and hydrophilic mPEG chain. The mean particles size of Bare-CS-CA-mPEG and Al-CS-CA-mPEG micelles was 918 ± 418 and 1099 ± 240 nm, respectively (Table 5). The charges of CS-CA-mPEG and Al-CS-CA-mPEG micelles were -2.0 ± 0.4 and -5.7 ± 0.2 mV, respectively. The size

distributions were also narrow in the range of 0.7 to 0.8. These results indicated that the addition of allergen in formulation had the effect both on size and charge. The particles was enlarged and the charge was more negative. Fig. 19a showed TEM image of Bare-CS-CA-mPEG micelles. The micelles was spherical and the size was in micron range. In contrast, the TEM image of Al-CS-CA-mPEG micelles (Fig. 19b) showed agglomeration of particles. Typically, the formation of macromolecular micelles depended on the balance of the attractive and the repulsive forces, including the stretching of the core-forming segments, the intercoronal interactions, and the free energy of the interface between the solvent and the micelles (Peng et al. 2009 : 21-25). Due to higher negatively charged of Al-CS-CA-mPEG micelles, it might cause higher attractive forces which the particles agglomerated.

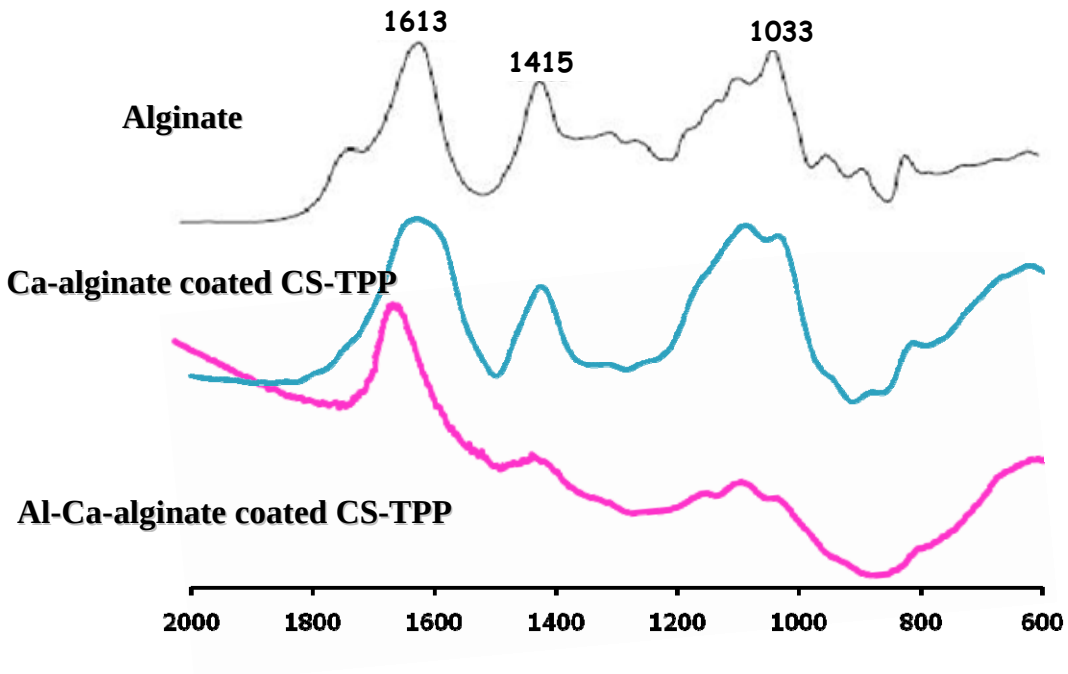
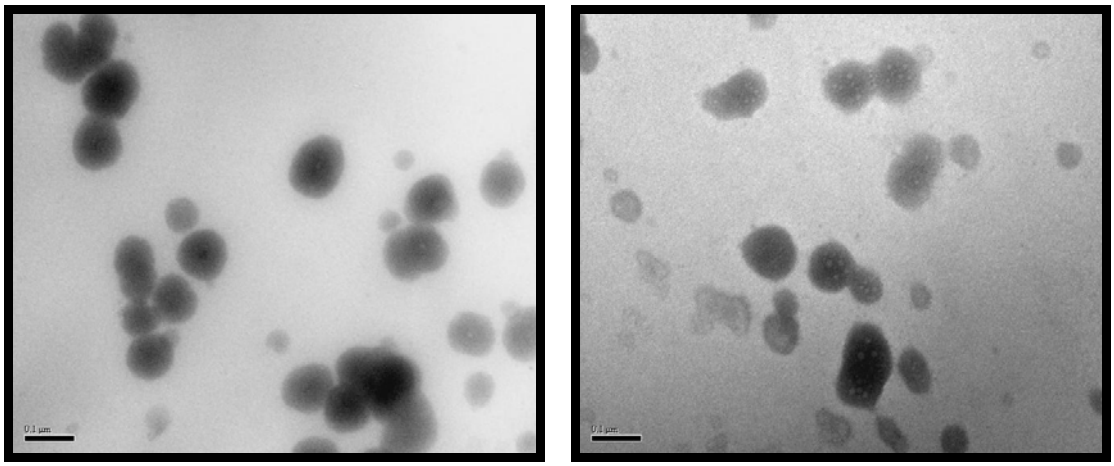


Figure 18 The FT-IR spectra of the Bare-Ca-alginate coated CS-TPP microparticles, Al-Ca-alginate coated CS-TPP microparticles in the range of 500 – 3500 cm^{-1}



(a) (b)

Figure 19 TEM micrographs of (a) Bare-CS-CA-mPEG micelles, and (b) Al-CS-CA-mPEG micelles (Magnification 100,000 x)

The FT-IR spectra of CS-CA-mPEG showed in Fig 20. CS-CA-mPEG showed the specific spectra of chitosan in the range between 895 and 1153 cm^{-1} (pyranose ring). However, the intensity or peak height at 1644 cm^{-1} (amide) and 2881 cm^{-1} (CH_3) was increased and new peak at 1734 (-COOR-) was observed. It can be concluded that chitosan graft cholic with polyethylene glycol methyl ether was successfully synthesized.

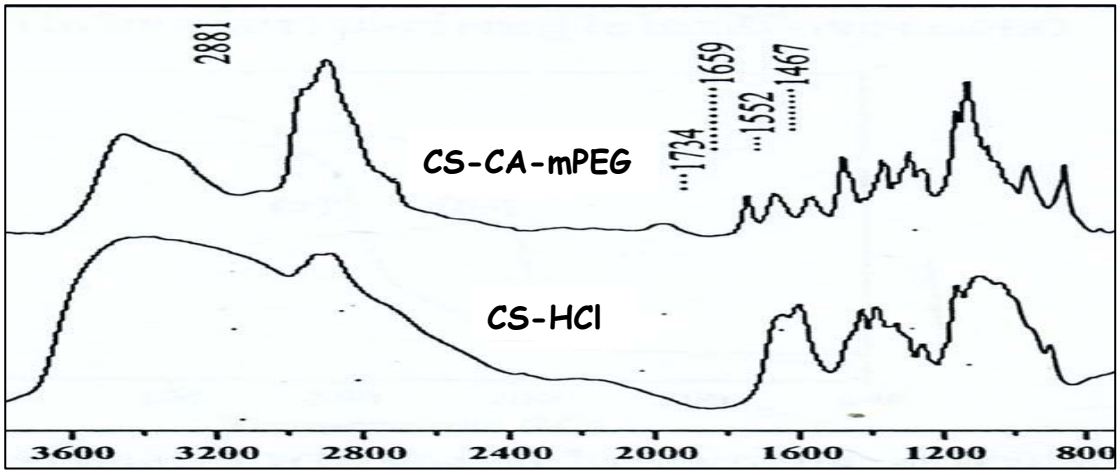


Figure 20 The FT-IR spectra of CS-CA-mPEG (A), CS-HCl (B) at the range of 800 – 3600 cm^{-1}

Table 5 Particle size and zeta potential of Ca-alginate, CS-TPP microparticles and CS-CA-mPEG micelles with and without allergen (Al)

Samples	Size (nm) Ave ± SD	Zeta potential (mV)	Polydispersity index
CS-TPP microparticles	1740 ± 158	11.3 ± 2.9	0.75
Al-CS-TPP microparticles	2343 ± 328	-20.1 ± 3.0	0.83
Ca-alginate microparticles	3560 ± 130	-7.7 ± 2.9	0.71
Al-Ca-alginate microparticles	598 ± 43	-22.7 ± 2.6	0.89
Ca-alginate coated CS-TPP microparticles	14880 ± 1840	-3.8 ± 2.2	-
Al-Ca-alginate coated CS-TPP microparticles	17120 ± 2040	-7.2 ± 1.1	-
CS-CA-mPEG micelles	918 ± 418	-2.0 ± 0.4	0.72
Al-CS-CA-mPEG micelles	1099 ± 240	-5.7 ± 0.2	0.83

3. Entrapment efficiency

3.1 Analysis of allergen content

A protein standard curve was made using bovine serum albumin (BSA) with concentrations of 0, 12.5, 25, 50, 100 and 200 µg/ml for the standard assay of commercial dust mite allergen which was used throughout the experiments by Bradford assay. The result showed that concentration of allergen was 260 µg/ml.

3.2 Ca-alginate microparticles

Allergen loaded Ca-alginate microparticles can be prepared by extruding a solution of sodium alginate containing the allergen in different concentration, as droplets, into a divalent cross-linking solution. $CaCl_2$ was used as a divalent cross-

linking agent. Various amounts of initial allergen (1, 5, 10% w/w to polymer) were incorporated into the Ca-alginate microparticles (A4 and A8). Table 6 shows % yield, %entrapment efficiency and allergen content of Ca-alginate microparticles. The result showed that the %yield of Ca-alginate microparticle was in the range 23-90% depending on the process of microparticles preparation (Fig. 21). The %entrapment efficiency and allergen content increased when viscosity of sodium alginate was increased (Fig. 22). Sodium alginate is a family of polysaccharides composed of α-L-guluronic acid and β-D-mannuronic acid residues, arranged in homopolymeric blocks of each type and in heteropolymeric blocks (Demiröz et al. 2007 : 491–497). Therefore, when calcium ions are added to a sodium alginate solution, such an alignment of the G blocks occurs, and the calcium ions are bound between the 2 chains like eggs in an egg box, then allergen can be retained in the calcium-cross-linked alginate encapsulation process. Therefore, when molecular weight of alginate increased, viscosity of alginate was increased, and swelling property was increased. Moreover, an increasing allergen concentration can be expected to lead to an increasing replacement of Ca^{2+} ions during drug loading, and resulting in less dense polymer networks, enabling to take up more allergen solution in aqueous medium (Moebus et al. 2009 : 42–53). Therefore higher entrapment efficiency and allergen content in Ca-alginate (HV) microparticles were obtained. A similar observation was reported by Segi et al. (Segi et al. 1989 : 3092–3095) for propranol soaked into alginate beads. The drug loading increased in a sigmoidal manner with increasing bulk drug concentration when loaded at a pH > 2.5 accompanied by an abrupt contraction of the beads. However, the swelling properties of alginate polymer have limited when Ca-alginate microparticles was entrapped allergen at the maximum concentration. When more allergen was added in microparticles, the entrapment efficiency was decreased.

Therefore, formula A4 was chosen for *in vitro* release in the next study because allergen content and entrapment efficiency were higher than A8.

Table 6 Percent yield, percent entrapment efficiency, and allergen content of A4 and A8

Formulation	%Yield	Allergen content (mg/g)	%Entrapment efficiency
1%Al+A4	90.81	0.71 ± 0.38	43.85 ± 5.44
5%Al+A4	23.33	3.34 ± 0.76	61.65 ± 13.95
10%Al+A4	37.05	3.30 ± 1.50	30.54 ± 13.94
1%Al+A8	19.80	0.68 ± 0.35	59.38 ± 17.50
5%Al+A8	50.45	1.89 ± 0.73	44.39 ± 6.73
10%Al+A8	96.04	3.97 ± 0.72	36.79 ± 6.65

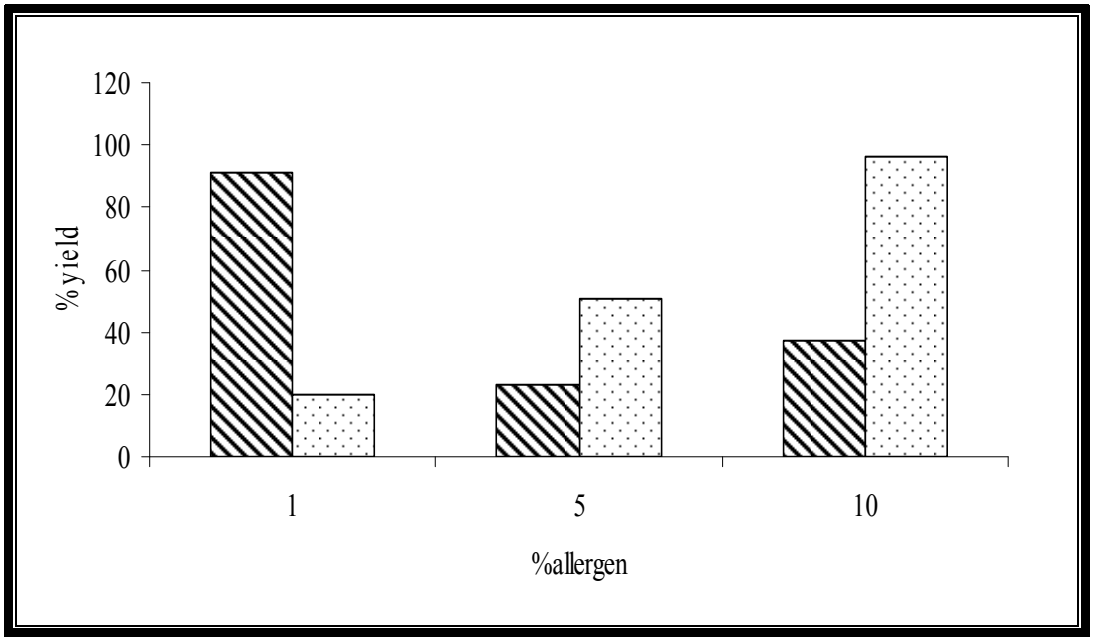
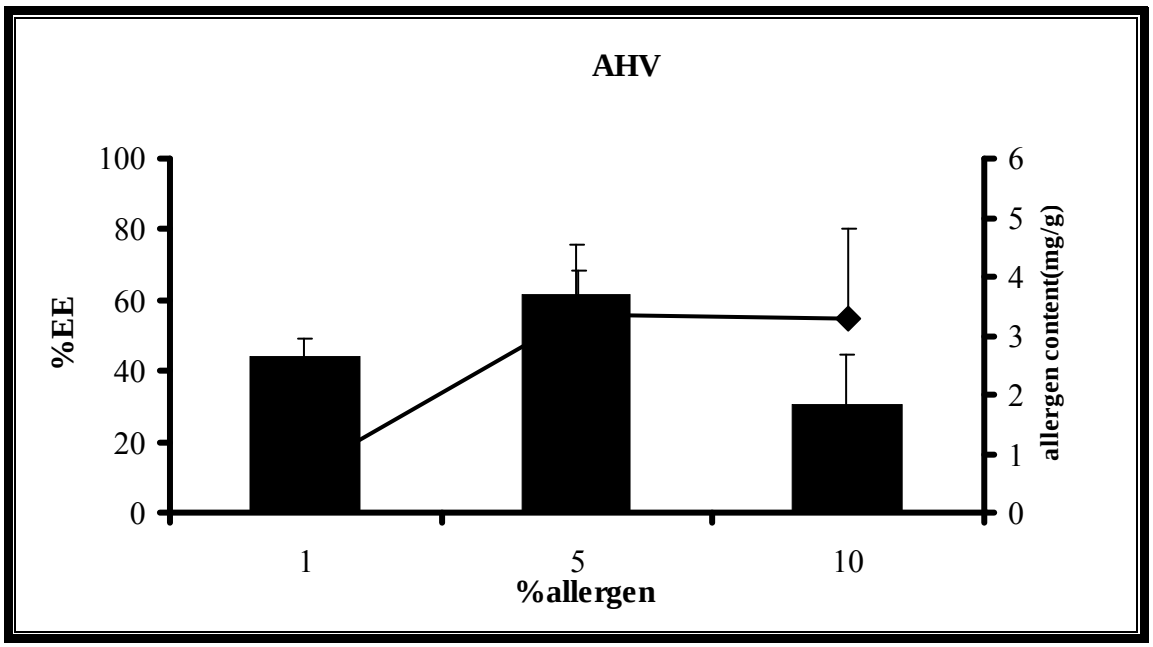


Figure 21 Percent yield of Ca-alginate microparticles prepared from (▨) alginate high viscosity (AHV), (⬚) alginate low viscosity (ALV).

(A)



(B)

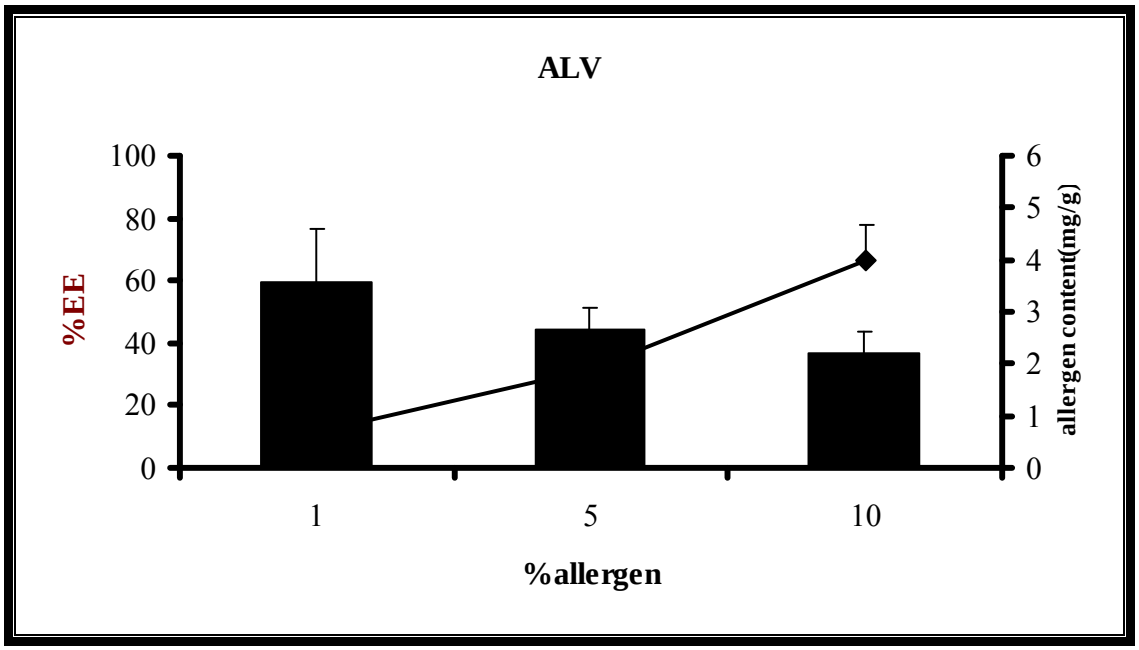


Figure 22 Percent entrapment efficiency and allergen content of Al-Ca-alginate microparticles prepared from (A) alginate high viscosity (AHV), (B) alginate low viscosity (ALV).

3.3 CS-TPP microparticles

The aim of this study was to investigate the influence of molecular weight (MW) of CS-HCl on %entrapment efficiency and allergen content. In this study CS-HCl MW of 20, 200 and 460 kDa was used to incorporate with allergen in different concentrations. Table 7 shows %entrapment efficiency and allergen content of Al-CS-TPP microparticles. The result showed that as MW of CS-HCl increased, % entrapment efficiency and allergen content increased. Allergen was entrapped in CS-TPP microparticles with electrostatic interaction of the positively charged chitosan (amino group) and the negatively charged allergen. A number of reports have shown that entrapment efficiency increases with an increase of MW of CS. This might be explained on the basis that an increase in viscosity of the CS solution with increase in MW prevents allergen leaving the droplet. In this study, as MW of CS was increased from 20 to 460 kDa, the viscosity of CS solution increased and the entrapment efficiency also increased (Table 7). These results correlated with the study of Bazzo et al. (Bazzo et al. 2009) who prepared Poly (3-hydroxybutyrate)/chitosan/piroxicam or ketoprofen composite microparticles by the solid-in-water-in-oil emulsion-solvent evaporation technique with the aim of reducing the burst effect and controlling the drug release. Their results showed that encapsulation efficiency decreased from 74.9 to 34.6 % and from 33.5 to 23.3 %, respectively, when the chitosan concentration decreased from 3.0 to 1.0 % w/v. The increase in the solution viscosity when higher chitosan concentration was used could be a major factor to the increase of %entrapment efficiency. It has been reported that the enhancement of the polymeric solution viscosity enhanced the % entrapment efficiency because it was more difficult for the drug to diffuse into the outer phase (Hasan et al. 2007 : 53-61). In fact, it has previously been demonstrated that the spread length of chitosan chain in solution may vary in correlation with the MW, which may affect protein interaction and encapsulation. It was found that BSA encapsulation efficiency tended to increase from 61.1 to 69.9 and 78.2% when molecular weight of chitosan was changed from low to medium and high, repectively (Gan and Wang 2007 : 24-34). Therefore in this study, the best formulation was CS 9 (2.5%Al+CS-HCl 460 kDa), and it can be concluded that MW of CS-HCl was increased, %entrapment efficiency and allergen content

increased (Fig 23). Therefore, chitosan HCl MW 460 kDa was chosen to incorporate with allergen in different concentrations for the next study.

Table 7 Percent yield, percent entrapment efficiency and allergen content of CS-TPP microparticles in different molecular weight

Code	**Formulation**	**%Yield**	**%EE± SD**	**Allergen content (mg/g) ± SD**
CS 1	0.1%Al + CS-HCl 20 kDa	59.75	1.86±0.17	0.0078±0.01
CS 2	0.5%Al + CS-HCl 20 kDa	63.00	0.49±0.03	0.0147±0.01
CS 3	2.5%Al + CS-HCl 20 kDa	34.88	0.33±0.11	0.0551±0.02
CS 4	0.1%Al + CS-HCl 200 kDa	76.04	1.48±0.11	0.0159±0.01
CS 5	0.5%Al + CS-HCl 200 kDa	63.39	0.52±0.13	0.0387±0.01
CS 6	2.5%Al + CS-HCl 200 kDa	95.3	0.77±0.47	0.1926±0.12
CS 7	0.1%Al + CS-HCl 460 kDa	71.74	2.62±0.82	0.0393±0.01
CS 8	0.5%Al + CS-HCl 460 kDa	66.51	0.83±0.01	0.0619±0.01
CS 9	2.5%Al + CS-HCl 460 kDa	52.48	1.16±0.27	0.2899±0.07

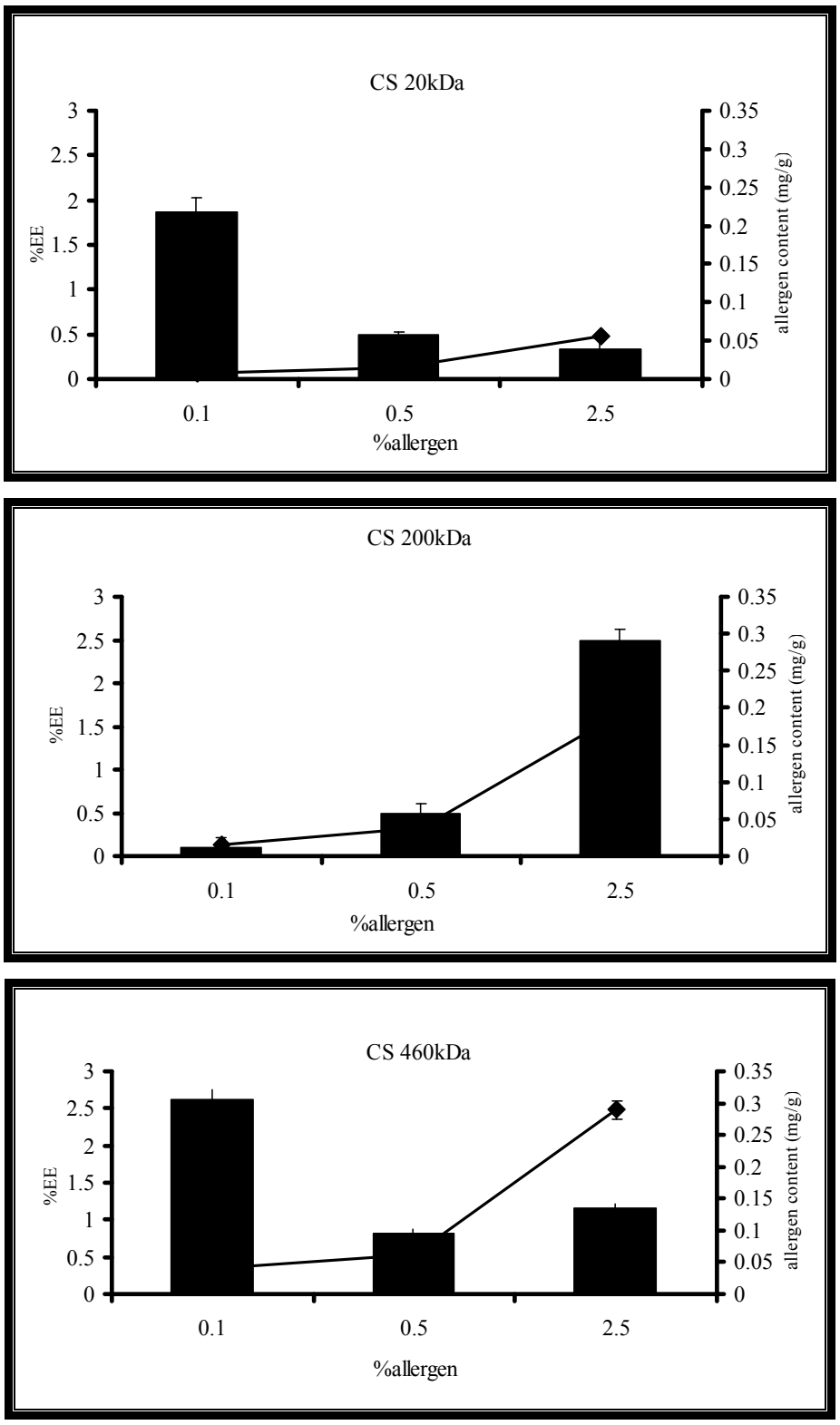


Figure 23 Percent entrapment efficiency and allergen content of CS-TPP microparticles in different molecular weight of chitosan.

3.4 Ca-alginate coated CS-TPP microparticles

In this part, the composite microparticle drug delivery systems were prepared using chitosan amd alginate. Since allergen is electrically negative and chitosan is positive in particular pH range, polyelectrolyte complexes could be formed between allergen and chitosan via electrostatic interactions during the microparticle preparation. To achieve a high encapsulation efficiency, allergen was first mixed with chitosan and then coated by alginate, and finally ionically gelated in $CaCl_2$ solution to form calcium alginate coated CS-TPP microparticles. It should be noted that the microparticles could not be prepared by directly mixing chitosan and alginate solutions together because immediate gelation occured and un-uniform bulk precipitated due to the relatively high viscosity of the solutions, leading to very low encapsulation efficiency of the drug. In this study, in order to ensure that the chitosan chains and alginate chains can contact each other well to form a uniformly cross-linked network, and to achieve a high encapsulation efficiency, the allergen encapsulated TPP cross-linked chitosan microparticles were prepared first and then the Al-CS-TPP microparticles were dispersed in alginate solutions to form a uniform suspension and to achieve the maximized electrostatic interaction (Yu et al. 2009 : 245–249). Allergen content and entrapment efficiency obtained by dissolving microparticle samples in phosphate buffer solution pH 7.4 could be considered as the total allergen loaded into the microparticles. Entrapment efficiency of Al-Ca-alginate coated CS-TPP microparticles was found about 19% and allergen content was 0.3 mg/g.

3.5 CS-CA-mPEG micelles

Allergen was incorporated into CS-CA-mPEG micelles using dialysis method. The entrapment efficiency of Al-CS-CA-mPEG with different medium was evaluated (Fig. 24). Table 8 shows the loading capacity of allergen in loaded CS-CA-mPEG micelles prepared by dialysis method against water, sodium chloride solution and DMSO. The results of allergen loading content revealed that CS-CA-mPEG micelles with the initial allergen of 0.75% w/w and dialyzed against DMSO showed the highest allergen loading content of 0.154 mg/g of particles, followed with the initial allergen of 0.5% w/w and dialyzed against NaCl solution as allergen capacity of 0.141 mg/g of particles. Al-CS-CA-mPEG micelles dialyzed against water showed

the lowest allergen loading capacity. This phenomenon might be due to the structure of CS-CA-mPEG. When micelles were formed by dialyzed against water, the tendency of these polymers to form self-assembling micelles might be based on the hydrophobic CA groups on the chitosan chain and hydrophilic mPEG chain (Liu et al. 2009 : 21-25). Allergen is hydrophilic, therefore, when CS-CA-mPEG formed micelles, partial amount of allergen was entrapped into hydrophobic inner core of micelles. In case of CS-CA-mPEG micelle dialyzed against NaCl solution, the more allergen loading content were observed. This is in agreement with the previous study which found that the micelles exhibited responsive behavior to ionic strength where a contraction of the micelles was observed as the carboxylate charges were balanced by sodium ions (Stubenrauch et al. 2009 : 1178-1191). Therefore, the addition of NaCl in the dialysis medium might induce the entrapment of allergen with a higher extent than micelles formed by dialyzing against water. CS-CA-mPEG micelles dialyzed against DMSO showed the highest allergen loading content. This reversed micelles entrapped allergen method was done by two steps, the first step was dialyzed against DMSO to form reversed micelles, and the second step was the conversion of the reversed micelles to normal micelles by dialyzing against water. In this step, allergen could be more entrapped into inner core than the simple micelle dialysis against water.

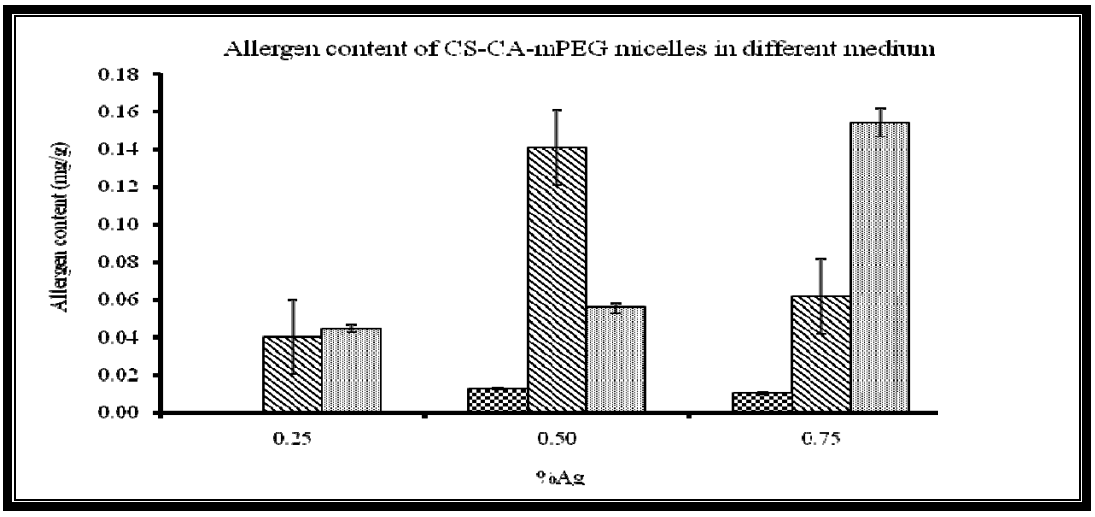


Figure 24 Allergen content (mg/g) of CS-CA-mPEG micelles in dialysis against (▩) H_2O, (■) 0.9% NaCl solution and (▨) DMSO

Table 8 Allergen loading capacity of Al-CS-CA-mPEG micelles prepared by dialysis method against water, sodium chloride solution and DMSO

Code	**Sample name**	**%EE**	**Content (mg/g)**
CA 1	0.25%Al-CS-CA-mPEG -H_2O	*N/D*	*N/D*
CA 2	0.5%Al-CS-CA-mPEG -H_2O	0.003	0.012
CA 3	0.75%Al-CS-CA-mPEG -H_2O	0.002	0.010
CA 4	0.25%Al-CS-CA-mPEG -NaCl	1.310	0.040
CA 5	0.5%Al-CS-CA-mPEG -NaCl	2.501	0.141
CA 6	0.75%Al-CS-CA-mPEG -NaCl	0.607	0.062
CA 7	0.25%Al-CS-CA-mPEG -DMSO	2.777	0.044
CA 8	0.5%Al-CS-CA-mPEG -DMSO	1.791	0.056
CA 9	0.75%Al-CS-CA-mPEG -DMSO	3.332	0.154

***** *ND = cannot be detected*

4. The *in vitro* release study

4.1 Ca-alginate microparticles

Allergen at different concentrations were incorporated into Ca-alginate microparticles. The results revealed that formula A4 showed the highest allergen entrapment, and was therefore, chosen for *in vitro* release study in different medium. Ca-alginate microparticles are known to release incorporated proteins by two mechanisms; diffusion of the protein through the pores of the polymer network and degradation of the polymer network (Gombotz and Wee 1998 : 267–285). Figure 25(A) shows the release profiles of allergen from Ca-alginate microparticles in PBS pH 7.4. The result showed that all formulations exhibited an initial burst of approximately 50% within 2 h, followed by the slow release. After 4 h, the release of allergen from microparticles was constant to 24 h. These phenomenon might be the result of the deformation of crosslink once alginate gel is suspended in a chelating agent such as lactate, citrate and phosphate (Sutherland 1991: 309–331). As Ca^{2+} ions are removed, the crosslinking in the gel decreases and the gels are destabilized. This can lead to the leakage of entrapped material and solubilization of the high molecular weight alginate polymers. Alginate gels degrade and precipitate in a 0.1 M phosphate buffer solution and completely dissolve in 0.1 M sodium citrate at pH 7.8. If Ca^{2+} is used in the crosslinking solution and phosphate is used as the dissolution medium, the dissolution medium turns turbid due to the dissociation of Ca^{2+} from the polymer network and the formation of insoluble calcium phosphate. This phenomenon is obviously seen when a high guluronic content alginate is used (Murata et al. 1993 : 21–26). Figure 25(B) shows the release profiles of allergen from Ca-alginate microparticles in simulated gastric fluid without pepsin (pH 1.2). The allergen release was about 50% within 4 h, then increased to 80%, 70% and 60% after 48 h, for Ca-alginate microspheres loading with 1, 2.5 and 5% allergen, respectively. The burst effect within initial 4 h might be due to the release through macroporous structure of Ca-alginate microparticles. In the previous study where calcium alginate gels microbeads has been analysed by electron microscopy, it was found that the pore size ranged from 5 nm to 200 nm in diameter and exhibited the highest diffusion rates for small molecules such as glucose, proteins or allergen (Gombotz and Wee 1998 : 267–285). Therefore the porosity of Ca-alginate microparticles might be a major factor for

allergen release from microparticles. The slow release of allergen at the later stage might be explained that theoretically, alginate shrinks at low pH (gastric environment) and the encapsulated drugs are not released (Chen et al. 2004 : 285–300). In the gastric fluid, the hydrated sodium alginate is converted into a porous, insoluble so-called alginic acid skin (Yotsuyanagi et al. 1987 : 1555–1563). The sustained release profile of allergen from Ca-alginate microparticles was therefore obtained after 4 h until 48 h.

4.2 CS-TPP microparticles

Figure 26 shows the *in vitro* release of allergen from CS-TPP microparticles in PBS pH 7.4 and simulated gastric fluid without pepsin (pH 1.2) medium. The results showed that the amount of allergen released were approximately 80% in simulated gastric fluid without pepsin (pH 1.2) medium over a period of 8 h and 60% in the PBS 7.4 over a period of 24 h. These results indicated that in simulated gastric fluid without pepsin (pH 1.2) solution, the release rate of allergen from Al-CS-TPP microparticles was faster than in PBS pH 7.4. This phenomenon might be due to a pH-sensitive behavior of chitosan. Chitosan dissolves easily at low pH while it is insoluble at higher pH ranges. The mechanism of pH sensitive swelling involves the protonation of amine groups of chitosan under low pH conditions. This protonation leads to chain repulsion, diffusion of proton and counter ions together with water inside the gel and dissociation of secondary interactions. Therefore this property produced the matrix which can dissolve in the stomach. (George et al. 2006 : 1-14). In our study burst release of allergen in pH 7.4 receptor medium was about 30-40 % in 1 h, suggesting the burst was likely a consequential effect of rapid surface desorption of large amount of protein molecules from a large specific surface area provided by large numbers of particles at micro-scale, and a larger proportion of protein molecules may not truly embedded in the inner structure of microparticles (Gan and Wang 2007 : 24–34). After 1 h, the release was sustainded up to 24 h. This phenomena might be cause by the swelling process during 6 to 12 h, which reduced the density, and increased the particle size. Moreover hydrolytic reaction may take place on matrix and allergen was released from microparticles.

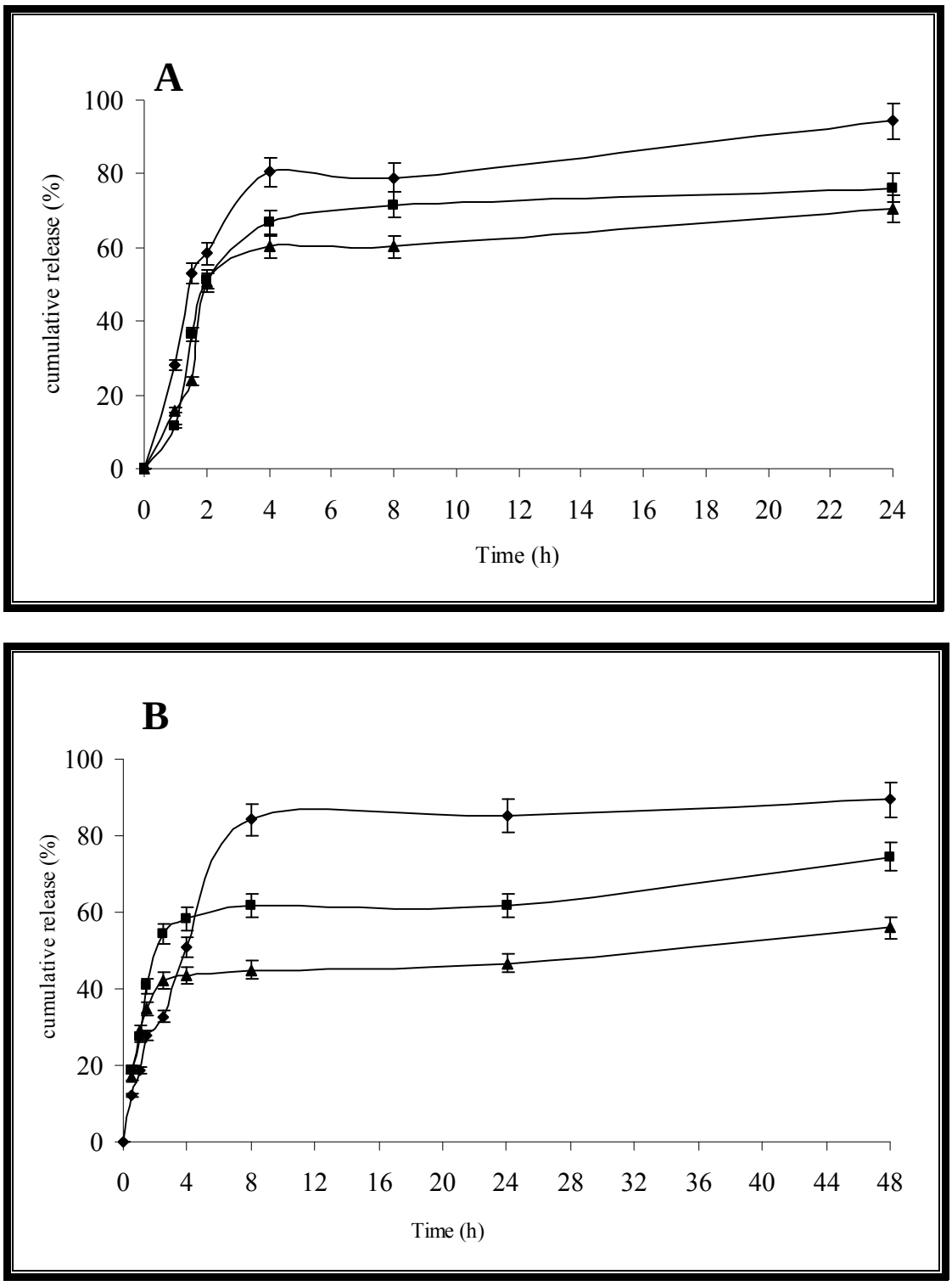


Figure 25 *In vitro* release of allergen from Ca-alginate microparticles in PBS pH 7.4 (A), simulated gastric fluid without pepsin (pH 1.2) (B); (♦) 1% Al, (■) 2.5% Al and (▲) 5% Al

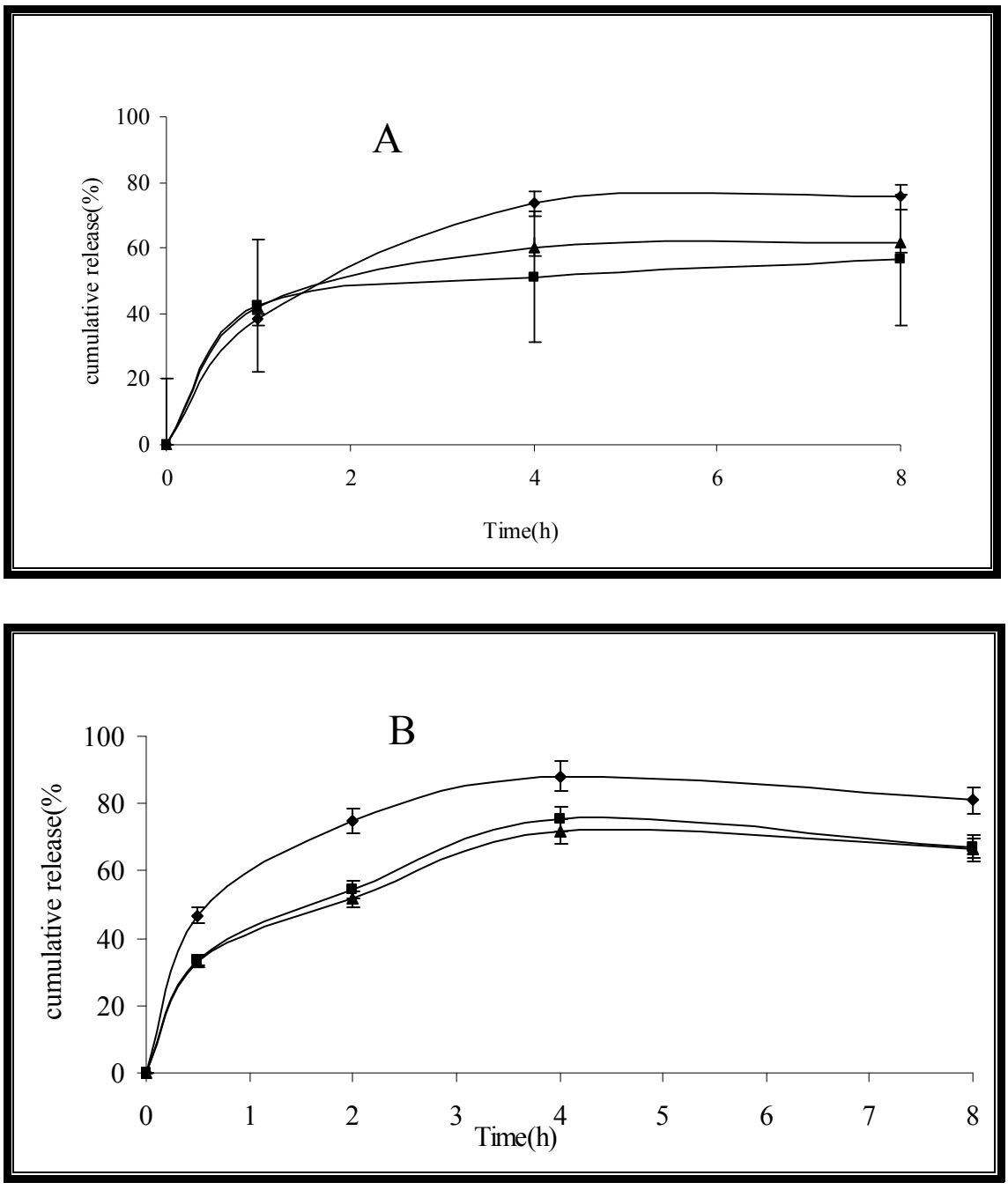


Figure 26 *In vitro* release of allergen from CS-TPP microparticles in PBS pH 7.4 (A), simulated gastric fluid without pepsin (pH 1.2) (B); (♦) 0.5% Al, (■) 1% Al and (▲) 2.5% Al.

4.3 Ca-alginate coated CS-TPP

In the section 4.2, the release rate of allergen from CS-TPP microparticles in simulated gastric fluid without pepsin (pH 1.2) receptor medium was faster than in PBS pH 7.4 (Fig 27). In this section, 2.5% Al-CS-TPP microparticles was chosen to coat with Ca-alginate. Alginate with carboxylate groups shrinks at low pH and dissolves at high pH. Therefore the drug release was retarded by Ca-alginate coating. The chitosan with amine groups dissolves at low pH and is insoluble at high pH. Upon mixing, the carboxylate groups of alginate and the ammonium groups of chitosan ionically interact to form the polyelectrolyte complex. Complexation of alginate with chitosan reduces the porosity of the matrix and decreases the diffusion of encapsulated drugs (Yu et al. 2008 : 15–21). However, the burst effect of coated microparticles occurred, but the time for prolong release of allergen from these microparticles was more obvious than that in uncoated CS-TPP microparticles.

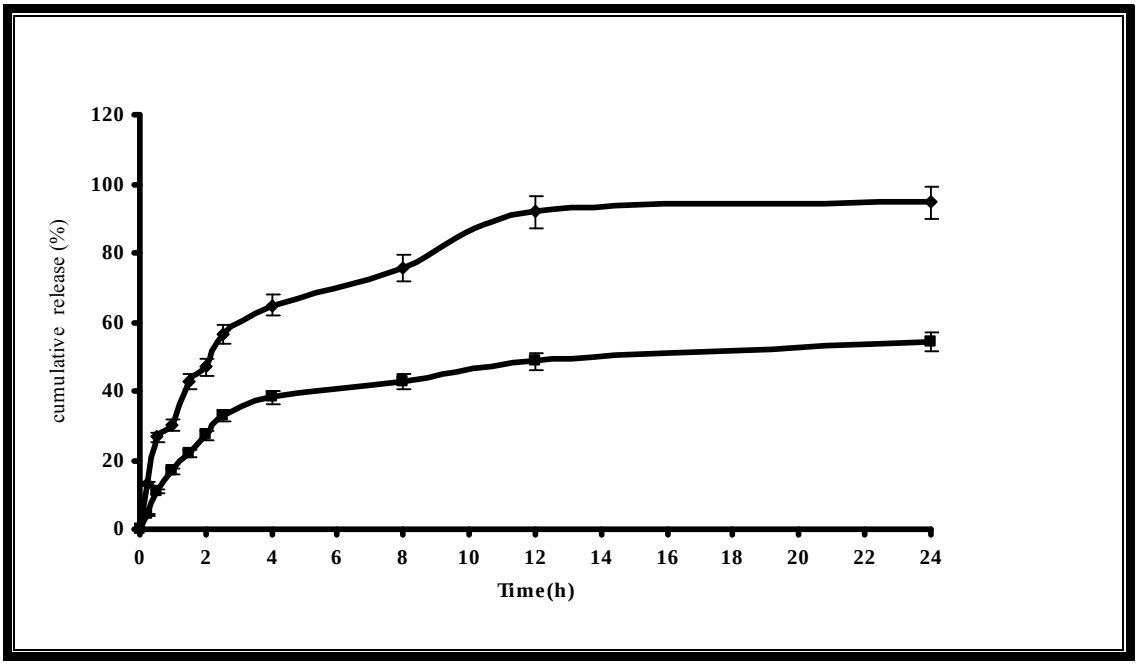


Figure 27 *In vitro* release of allergen from Ca-alginate coated CS-TPP microparticles in PBS pH 7.4 (♦), simulated gastric fluid without pepsin (pH 1.2) (■)

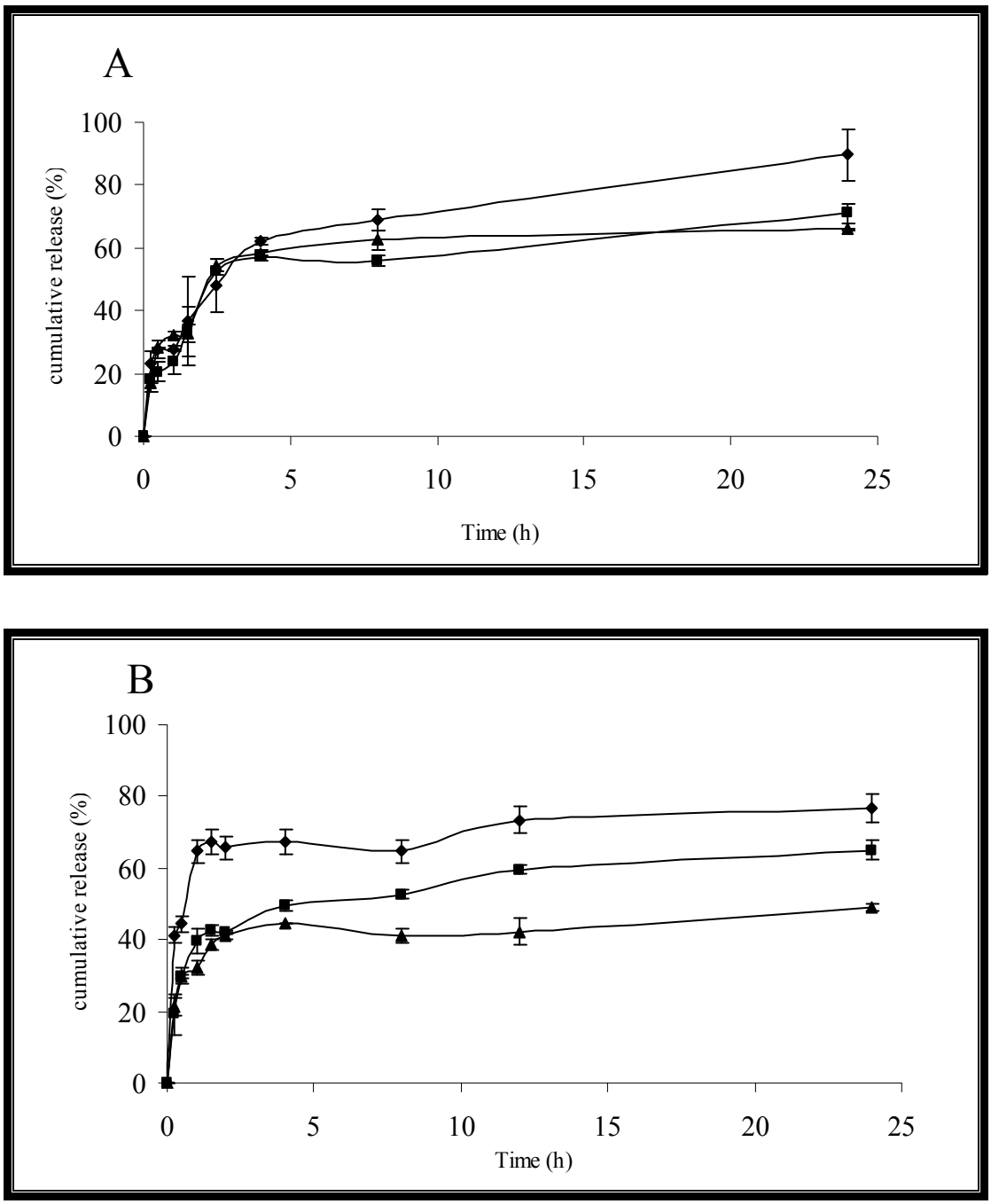


Figure 28 *In vitro* release of CS-CA-mPEG micelles against NaCl in pH 7.4 (A) and pH 1.2 (B); (♦) 0.5% Al, (■) 1% Al and (▲) 2.5% Al.

4.4 CS-CA-mPEG micelles

Figure 28 shows *in vitro* release profiles of allergen from the CS-CA-mPEG micelles in simulated gastric fluid without pepsin (pH 1.2) and PBS pH 7.4. The result showed that in simulated gastric fluid without pepsin (pH 1.2) receptor medium, the release rate of allergen from Al-CS-CA-mPEG micelles was slower than in PBS pH 7.4. The burst release of allergen appeared at 2 h, attributed to the allergen adopted mostly on the micelle surfaces that was easily released. The release of the drug from micelles was probably caused by the concentration gradient between the core of the micelles and the surrounding aqueous solution. After the burst release, the allergen slowly released from the micelles. This might be due to the strong interaction between allergen and CS-CA-mPEG. This result suggests that the allergen encapsulated in the micelles can be slowly released in the basic environment such as in large intestine, colon and rectal mucosa.

CHAPTER V
CONCLUSIONS

In this study microparticles from calcium alginate, chitosan tripolyphosphate (CS-TPP), calcium alginate coated chitosan tripolyphosphate were successfully prepared by ionotropic gelation method and CS-CA-mPEG micelles was prepared by dialysis method. The morphology of microparticles entrapping dust mite allergen was spherical and their particles were in micron size.

1. Ca-alginate microparticles

1.1 Preformulation

The formulation that produced smallest and spherical Ca-alginate microparticles was 0.05% alginate +4% $CaCl_2$ (Formula code A4, A8). This formulation was chosen to incorporate with allergen.

1.2 Entrapment efficiency

Alginate high viscosity (AHV) showed higher entrapment efficiency and allergen content than alginate low viscosity (ALV). The highest entrapment efficiency and allergen content was found in 5% initial allergen of formula A4.

1.3 The *in vitro* release

The allergen release was about 50% within 4 h, then increased to 80%, 70% and 60% after 48 h, for Ca-alginate microspheres loading with 1, 2.5 and 5% allergen, respectively. The release rate and amount of allergen in simulated gastric fluid without pepsin (pH 1.2) receptor medium was slower than those in PBS pH 7.4 receptor medium.

2. CS-TPP microparticles

2.1 Entrapment efficiency

As molecular weight of chitosan and allergen initial added were increased, the entrapment efficiency and allergen content increase. The highest entrapment efficiency and allergen content were found in CS MW 460 kDa-TPP microparticles.

2.2 The *in vitro* release

The release rate and amount of allergen in simulated gastric fluid without pepsin (pH 1.2) receptor medium was higher than those in PBS pH 7.4 receptor medium.

3. Ca-alginate coated CS-TPP microparticles

3.1 Preformulation

The formulation that produced spherical particle was 0.25% alginate (AHV) + 6% $CaCl_2$ to coated 2.5% Al-CS-TPP microparticles.

3.2 Entrapment efficiency

The entrapment efficiency of allergen loaded Ca-alginate coated CS-TPP microparticles was 19 ± 0.08% and allergen content was 0.3 ± 0.19 mg/g respectively.

3.3 The *in vitro* release

The release rate and amount of allergen in simulated gastric fluid without pepsin (pH 1.2) receptor medium was higher than those in PBS pH 7.4 receptor medium. Moreover, retardation was found in Ca-alginate coated CS-TPP microparticles.

4. CS-CA-mPEG micelles

4.1 Entrapment efficiency

The method that produced the highest allergen entrapment was prepared by dialysis against NaCl solution. The highest entrapment efficiency and allergen content was found in 0.25% Al + CS-CA-mPEG dialysis against NaCl.

4.2 *In vitro* release

The release rate and amount of allergen in 0.1 N HCl solution pH 1.2 receptor medium was faster than those in PBS pH 7.4 receptor medium. The burst release of allergen appeared at 2 h. This might be attributed to the adoptation of allergen on the micelle surfaces, which is easily released.

It can be concluded that all formulation showed spherical shape and yielded micron size microparticles. Ca-alginate microparticles show the highest entrapment efficiency, whereas Ca-alginate coated CS-TPP microparticles show the highest

allergen entrapment. In case of *in vitro* release, Ca-alginate coated CS-TPP microparticles showed more sustained release pattern than other formulae and had a few burst effect at the initial time.

BIBLIOGRAPHY

Almeida, A. J., and H. O. Alpar. "Nasal delivery of vaccines." J. Drug Target 3 (1996) : 455–467.

Aly, A. S. "Self-dissolving chitosan: I. Preparation, characterization an evaluation for drug delivery system." Angew. Makromol. Chem 259 (1998) : 13–18.

Anaïs, P. et al. "Allergen-specific immunotherapy in allergic rhinitis and asthma. Mechanisms and proof of efficacy." Respiratory Medicine 2009. (Mimeographed)

Antico, A. et al. "Anaphylaxis by latex sublingual immunotherapy." Allergy 61 (2006) : 1236–1237.

Aiedeh, K. et al. "Chitosan microcapsules as controlled release systems for insulin." J. Microencapsul 14 1997 : 567–576.

Artursson, P. et al. "Effect of chitosan on the permeability of monolayers of intestinal epithelial cells (Caco-2)." Pharm. Res. 11 (1994) : 1358–1361.

Aurélie, S. et al. "Novel composite microparticles for protein stabilization and delivery." Eur. J. Pharm. Sci. 36 (2009) : 226–234.

Bazzo, G. C. et al. "Poly(3-hydroxybutyrate)/chitosan/ketoprofen or piroxicam composite microparticles: preparation and controlled drug release evaluation." Carbohydr. Polym. 2009. (Mimeographed)

Beier, R., and A. Gebert, "Kinetics of particle uptake in the domes of Peyer's patches." Am. J. Physiol 275 (1998) : 130–137.

Bernkop, S. A. "Permeation enhancing polymers in oral delivery of hydrophilic macromolecules: thiomer/GSH systems." J. Control. Release 93 (2003) : 95–103.

Bernkop, S. A. et al. "Thiolated chitosans: development and in vitro evaluation of a mucoadhesive, permeation enhancing oral drug delivery system." J. Control. Release 94 (2004) : 177–186.

Bernkop, S. A. et al. "Thiolated chitosans." Eur. J. Pharm. Biopharm. 57 (2004) : 9-17.

Biróa, E. et al. "5-ASA loaded chitosan–Ca–alginate microparticles:Preparation and physicochemical characterization." Int. J. Pharm. 345 (2007) : 59–69.

Borchard, G. et al. “The potential of mucoadhesive polymers in enhancing intestinal peptide drug absorption: III. Effects of chitosan glutamate and carbomer on epithelial tight junctions *in vitro*.” J. Control. Release 39 (1996) : 131–138.

Bowersock, T. L. et al. “Induced of pulmonary immunity in cattle by oral administration of ovalbumin in alginate microspheres.” Immunol. Lett. 60 (1998) : 37-43.

Bowersock, T. L. et al. “Oral vaccination of animals with antigens encapsulated in alginate microspheres.” Vaccine 17 (1999) :1804–1811.

Borges, O. et al. “Preparation of coated nanoparticles for a new mucosal vaccine delivery system.” Int. J. Pharm. 299 (2005) : 155–166.

Borges, O. et al. “Uptake studies in rat Peyer's patches, cytotoxicity and release studies of alginate coated chitosan nanoparticles for mucosal vaccination.” J. Control. Release 114 (2006) : 348–358.

Bruno, S. et al. “Characterization of insulin-loaded alginate nanoparticles produced by ionotropic pre-gelation through DSC and FTIR studies.” Carbohydr. Polym. 66 (2006) : 1–7.

Carol, S., and C. A. Pedro, “Advances in upper airway diseases and allergen immunotherapy.” J. Allergy Clin. Immunol. 122 (2007) : 481-487.

Claudia, T. H. et al. “Determinants of allergenicity.” J. Allergy Clin. Immunol. 123 (2009) : 558-66.

Chapman et al. “Nomenclature and structural biology of allergens.” J. Allergy Clin Immunol 199 (2007) : 414-20.

Chen, S.C. et al. “A novel pH sensitive hydrogel composed of N,O-carboxymethyl chitosan and alginate cross-linked by genipin for protein drug delivery.” J. Control. Release 96 (2004) : 285–300.

Chenguang, L. et al. “Preparations, Characterizations and Applications of Chitosan-based Nanoparticles.” J. Ocean University of China. 6 (2007) : 237–243.

Chickering, D. E., and E. Mathiowitz, “Bioadhesive microspheres: I. A novel electrobalance-based method to study adhesive interactions between individual microspheres and intestinal mucosa.” J. Control. Release 34 (1995) : 251–261.

Cho, N. H. et al. “Novel mucosal immunization with polysaccharide–protein conjugates entrapped in alginate microspheres.” J. Control. Release 53 (1998) : 215–224.

Cole, D. R. et al. “Microencapsulated islet grafts in the BB/E rat: a possible role for cytokines in graft failure.” Diabetologia 35 (1992) : 231–237.

Cox, L. “Advantages and disadvantages of accelerated immunotherapy Schedules.” J. Allergy Clin. Immunol. 122 (2008) : 432-434.

Dambies, L. et al. “Preparation of chitosan gel beads by ionotropic molybdate gelation.” Biomacromolecules 2 (2001) : 1198–1205.

Davis, P. et al. “Sustained release chitosan microspheres prepared by novel spray drying methods.” J. Microencapsul. 16 (1999) : 343–355.

Deacon, M. P. et al. “Harding, Atomic force microscopy of gastric mucin and chitosan mucoadhesive systems.” Biochem. J. 348 (2000) : 557–563.

De Angelis, A. A. et al. “Synthesis and 13C CP-MAS NMR characterisation of a new chitosan-based polymeric network.” Macromolecules 31 (1998) : 1595–1601.

De Vos, P. et al. “Association between capsule diameter, adequacy of encapsulation, and survival of microencapsulated rat islet allografts.” Transplantation 62 (1996) : 893–899.

Denkbas, E. B. et al. “Implantable 5-fluorouracil loaded chitosan scaffolds prepared by wet spinning.” J. Membr. Sci. 172 (2000) : 33–38.

Des Roches, A. et al. “Immunotherapy with a standardized *Dermatophagoides pteronyssinus* extract. VI. Specific immunotherapy prevents the onset of new sensitizations in children.” J. Allergy Clin. Immunol. 99 **(**1997) : 450-453.

Derek, T. “Microparticles and polymers for the mucosal delivery of vaccines.” Adv. Drug Deliv. Rev. 34 (1998) : 305–320.

Dodane, V. et al. “Effect of chitosan on epithelial permeability and structure.” Int. J. Pharm. 182 (1999) : 21–32.

Drew, A. C. et al. “Hypoallergenic variants of the major latex allergen Hev b 6.01 retaining human T lymphocyte reactivity.” J. Immuno. 173 (2004) : 5872-5879.

Drouin ,S. M. et al. “Cutting Edge: The absence of C3 demonstrates a Role for Complement in Th2 Effector Functions in a Murine Model of Pulmonary Allergy.” J. Immuno. 167 (2001) : 4141-4145.

Durham, S. R. et al. “Long-term clinical efficacy of grass-pollen immunotherapy.” N. Engl. J. Med 341 (1999) : 468–475.

Dunsky, E. et al. “Anaphylaxis to sublingual immunotherapy.” Allergy 61 (2006) : 1235.

Ebel, J. P. “A method for quantifying particle absorption from the small intestine.” Pharm. Res. 7 (1990) : 848–851.

Edelman, E. R. et al. “Perivascular graft heparin delivery using biodegradable polymer wraps.” Biomaterials 21 (2000) : 2279–2286.

Eldridge, J. H. “Controlled release in the gutassociated lymphoid tissues. I. Orally administered biodegradable microspheres target the Peyer’s patches.” J. Control. Release 11 (1990) : 205–214.

Espevik, T. “The involvement of CD14 in stimulation of cytokine production by uronic acid polymers.” Eur. J. Immunol 23 (1993) : 255–261.

Esquisabel, A. “Effect of lecithins on BCG-alginate-PLL microcapsule particle size and stability upon storage.” J. Microencapsul 17 (2000) : 363–372.

Fatmanur, T. D. et al. “Evaluation of alginate based mesalazine tablets for intestinal drug delivery.” Eur. J. Pharm. Biopharm. 67 (2007) : 491–497.

Fiebrig, I. “Transmission electron microscopy on pig gastric mucin and its interactions with chitosan.” Carbohydr. Polym. 28 (1995) : 239–244.

Gåserød, O. “The enhancement of the bioadhesive properties of calcium alginate gel beads by coating with chitosan.” Int. J. Pharm. 175 (1998) : 237–246.

Gan, Q., and T. Wang. “Chitosan nanoparticle as protein delivery carrier – Systematic examination of fabrication conditions for efficient loading and release.” Colloids and Surfaces B 59 (2007) : 24-34.

Gaucher, G. “Block copolymer micelles: preparation, characterization and application in drug delivery.” J. Control. Release 109 (2005) : 169–188.

Genta, I. et al. “Different molecular weight chitosan microspheres: influence on drug loading and drug release.” Drug Dev. Ind. Pharm 24 (1998) : 779–784.

Gombotz, R. and F. Wee. “Protein release from alginate matrices.” Adv. Drug Deliv. Rev. 31 (1998) : 267–285.

Grabnar, I. et al. “Influence of chitosan and polycarbophil on permeation of a model hydrophilic drug into the urinary bladder wall.” Int. J. Pharm 256 (2003) : 167–173.

Hasan, A. et al. “Effect of the microencapsulation of nanoparticles on the reduction of burst release.” Int. J. Pharm. 344 (2007) : 53-61.

He, P. et al. “*In vitro* evaluation of the mucoadhesive properties of chitosan microspheres.” Int. J. Pharm. 166 (1998) : 75–88.

Hejjaoui, A. et al. “Immunotherapy with a standardized *Dermatophagoides pteronyssinus* extract IV. Systemic reactions according to the immunotherapy schedule.” J. Allergy Clin. Immunol. 85 (1990) : 473- 479.

Hirano, S. et al. “A chitosan, oxalate gel: its conversion to an N-acetylchitosan gel via a chitosan gel.” Carbohydr 201 (1990) : 145–149.

Hori, H. O., and M**.** Yoshiharu. **“**Evaluation of Eudragit-coated chitosan microparticles as an oral immune delivery system.” Int. J. Pharm. 297 (2005) : 223–234.

Illum, L. et al. “Chitosan as a novel nasal delivery system for peptide drugs.” Pharm. Res. 11 (1994) : 1186–1189.

Jacobsen, L. et al. “Specific immunotherapy has long-term preventive effect of seasonal and perennial asthma: 10-year follow-up on the PAT study.” Allergy 62 (2007) : 943-948.

Jaspreet, K. et al. “Bioadhesive microspheres as a controlled drug delivery system.” Int. J. Pharm. 255 (2003) : 13–32.

Jennifer M. R. et al. “Allergen-related approaches to immunotherapy.” Pharmaco. & Therapeu. 121 (2009) : 273–284.

Jönsson, B. et al. Surfactants and polymers in aqueous solutions. New York : Wiley and Sons, 1998.

Junginger, H.E., and J. C. Verhoef. “Macromolecules as safe penetration enhancers for hydrophilic drugs—a fiction?” PSTT 1 (1998) : 370–376.

Kanauchi, O. et al. “Mechanism for the inhibition of fat digestion by chitosan and for the synergistic effect of ascorbate.” Biosci. Biotechnol. Biochem. 59 (1995) : 786–790.

Katrin, M. et al. “Alginate–poloxamer microparticles for controlled drug delivery to mucosal tissue.” Eur. J. Pharm. Biopharm. 72 (2009) : 42–53.

Khalid, M. N. et al. “Swelling properties and mechanical characterization of a semiinterpenetrating chitosan/polyethylene oxide network: Comparison with a chitosan reference gel.” STP Pharm. Sci. 9 (1999) 359–364.

Kim, C. K., and E. J. Lee. “The controlled release of blue dextran from alginate beads.” Int. J. Pharm. 79 (1992) : 11–19.

Kim, B. et al. “Mucosal immune responses following oral immunization with rotavirus antigens encapsulated in alginate microspheres.” J. Control. Release 85 (2002) : 191–202.

Kotze, A. F. et al. “Chitosans for enhanced delivery of therapeutic peptides across intestinal epithelia: in vitro evaluation of Caco-2 cell monolayers.” Int. J. Pharm. 159 (1997) : 243–253.

Kotze, A. F. et al. “Chitosan and chitosan derivatives as absorption enhancers for peptide drugs across mucosal epithelia.” Bioadhe. Drug Deliv. Syst. (1999) : 341–385.

Kumar, M. et al. “Chitosan IFN-gamma-pDNA Nanoparticle (CIN) Therapy for Allergic Asthma.” Genet. Vaccines Ther 1 (2003) : 3.

Kulkarni, A. R. et al. “In-vitro release kinetics of cefadroxil-loaded sodium alginate interpenetrating network beads.” Eur. J. Pharm. Biopharm. 51 (2001) : 127–133.

Kurt, S. et al. “pH and ionic strength responsive polyelectrolyte block copolymer micelles prepared by ring opening metathesis polymerization.” J. Polym. Sci. Part A: Polym. Chem. 47 (2009) : 1178-1191.

Kwok, K. K. et al. “Production of 5–15 μm diameter alginate–polylysine microcapsules by an air atomization technique.” Pharm. Res. 8 (1991) : 341–344.

Lee, B. J., and G. H. Min. “Oral controlled release of melatonin using polymer reinforced and coated alginate bead.” Int. J. Pharm. 144 (1996) : 37–46.

Lee, D. Y. et al. “Chitosan and D-glucosamine induce expression of Th1 cytokine genes in porcine spleen cells.” J. Vet. Med. Sci 64 (2002) : 645–648.

Lehr, C. M. et al. “In vitro evaluation of mucoadhesive properties of chitosan and some other natural polymers.” Int. J. Pharm. 78 (1992) : 43–48.

Lehr, C. M. “From sticky stuff to sweet receptors—achievements, limits and novel approaches to bioadhesion.” Eur. J. Drug Metab. Pharmacokinet. 21 (1996) : 139–148.

Lerch, E., and U. R. Muller. “Long-term protection after stopping venom imnotherapy: results of re-stings in 200 patients.” J. Allergy Clin. Immunol. 101 (1998) : 606-612.

Li, L. “Synthesis and self-assembly of chitosan-based copolymer with a pair of hydrophobic/hydrophilic grafts of polycaprolactone and poly(ethylene glycol).” Colloids and Surfaces A: Physicochem. Eng. Aspects. 337 (2009) : 21–25.

Liang, B. et al. “Nasal-associated lymphoid tissue is a site of long-term virus-specific antibody production following respiratory virus infection of mice.” J. Virol. 75 (2001) : 5416–5420.

Lima, K. M. “Poly-dl-lactide-co-glycolide microspheres as a controlled release antigen delivery system.” Braz. J. Med. Biol. Res. 32 (1999) : 171– 180.

Liggins, R. T., and H. M. Burt. “Polyether-polyester diblock copolymers for the preparation of paclitaxel loaded polymeric micelle formulations.” Adv. Drug Deliv. Rev 54 (2002) : 191–202.

López, C. R. et al. “Design and evaluation of chitosan/ethylcellulose mucoadhesive bilayered devices for buccal drug delivery.” J. Control. Release 55 (1998) : 143–152.

López, C. R. et al. “Chitosan microspheres for the specific delivery of amoxycillin to the gastric cavity.” STP Pharma. Sci. 10 (2000) : 69–76.

Lueßen, H. L. et al. “Mucoadhesive polymers in peroral peptide drug delivery: VI. Carbomer and chitosan improve the intestinal absorption of the peptide drug buserelin in vivo.” Pharm. Res. 13 (1996) : 1668–1672.

Majeti, N. V., and R. Kumar. “Nano and Microparticles as Controlled Drug Delivery Devices.” J. Phatm. Pharmaceut. Sci. (2000) : 234-258.

Manmohan, S., and T. Derek. “The preparation and characterization of polymeric antigen delivery systems for oral administration.” Adv. Drug Deliv. Rev. 34 (1998) : 285–304.

Martinsen, A. et al. “Comparison of different methods for determination of molecular weight and molecular weight distribution of alginates.” Carbohydr. Polym. 15 (1991) : 171–193.

Meera, G., and T. E. Abraham. “Polyionic hydrocolloids for the intestinal delivery of protein drugs: Alginate and chitosan — a review.” J. Control. Release 114 (2006) : 1–14.

Mehrdad, H. et al. “Hydrogel nanoparticles in drug delivery.” Adv. Drug Deliv. Rev. 60 (2008) : 1638–1649.

Mladenovska, K. et al. “5-ASA loaded chitosan–Ca–alginate microparticles: Preparation and physicochemical characterization.” Int. J. Pharm. 345 (2007) : 59–69.

Mi, F. L. et al. “Kinetic study of chitosan–tripolyphosphate complex reaction and acid-resistive properties of the chitosan–tripolyphosphate gel beads prepared by in-liquid curing method.” J. Polym. Sci., B, Polym. Phys 37 (1999) : 1551–1564.

Mi, F. L. et al. “Porous chitosan microsphere for controlling the antigen release of Newcastle disease vaccine: preparation of antigen-adsorbed microsphere and *in vitro* release.” Biomaterials 20, (1999) : 1603–1612.

Mi, F. L. et al. “Synthesis and characterization of a novel chitosan-based network prepared using naturally occurring crosslinker.” J. Polym. Sci., A, Polym. Chem 38 (2000) : 2804–2814.

Mi, F. L. et al. “Drug release from chitosan-alginate complex beads reinforced by a naturally occurring cross-linking agent.” Carbohydr. Polym. 48 (2002) : 61–72.

Miller, J. D., and A. Miller. “Ten minutes in a clothes dryer kills all mites in blankets (abstract).” J. Allergy Clin. Immunol. 97 (1996) : 423.

Min, J. K. et al. “Rapid communication Long acting porous microparticle for pulmonary protein delivery.” Int. J. Pharm. 333 (2007) : 5–9.

Mittal, S. K. et al. “Immunization with DNA, adenovirus or both in biodegradable alginate microspheres: effect of route of inoculation on immune response.” Vaccine 19 (2000) : 253– 263.

Miyazaki, S. et al. “Drug release from oral mucosal adhesive tablets of chitosan and sodium alginate.” Int. J. Pharm. 118 (1995) : 257–263.

Moller, C. et al. “Pollen immunotherapy reduces the development of asthma in children with seasonal rhinoconjunctivitis (the PAT-study).” J. Allergy Clin. Immunol. 109 (2002) : 251–256.

Monteiro, O. A. C., and C. Airoldi. “Some studies of crosslinking chitosan glutaraldehyde interaction in a homogeneous system.” Int. J. Biol. Macromol 26 (1999) : 119–128.

Muzzarelli, R. A. A. “Human enzymatic activities related to the therapeutic administration of chitin derivatives.” Cell. Mol. Life Sci. 53 (1997) : 131–140.

Mumper, R. J. et al. “Calcium-alginate beads for the oral delivery of transforming growth factor-β1 (TGF-β1): stabilization of TGF-β1 by the addition of polyacrylic acid within acid treated beads.” J. Control. Release 30 (1994) : 241–251.

Murata, Y. et al. “Influence of erosion of calcium-induced alginate gel matrix on the release of Brilliant Blue.” J. Control. Release 23 (1993) : 21–26.

Natsume, H. “Screening of cationic compounds as an absorption enhancer for nasal drug delivery.” Int. J. Pharm. 185 (1999) : 1–12.

Nelson, H. S. “Allergen immunotherapy: where is it now?.” J. Allergy Clin. Immunol. 119 (2007) : 769-779.

Nishioka, Y. et al. “Release characteristics of cisplatin chitosan microspheres and effect of containing chitin.” Chem. Pharm. Bull 38 (1990) : 2871–2873.

Nwe, N. “Production of fungal chitosan by solid state and submerged fermentation.” Carbohydr. Polym 49 (2002) : 235–237.

Offit, P. A. “Enhancement of rotavirus immunogenicity by microencapsulation.” Virology 203 (1994) : 134-143.

Olga, B. “Preparation of coated nanoparticles for a new mucosal vaccine delivery system.” Int. J. Pharm. 299 (2005) : 155–166.

Olga, B. “Evaluation of the immune response following a short oral vaccination schedule with hepatitis B antigen encapsulated into alginate-coated chitosan nanoparticles.” Eur. J. Pharm. Sci. 32 (2007) : 278–290.

O’Hagan, D. T. “Intestinal translocation of particulates implications for drug and antigen delivery.” Adv. Drug Deliv. Rev. 5 (1990) : 265–285.

O’Hagan, D. T. “The intestinal uptake of particles and the implications for drug and antigen delivery.” J. Anat 189 (1996) : 477–482.

Orienti, I. “Indomethacin loaded chitosan microspheres. Correlation between the erosion process and release kinetics.” J. Microencapsul. 13 (1996) : 463–472.

Oyam, A. H. “Biodegradable mucoadhesive particulates for nasal and pulmonary antigen and DNA delivery.” Adv. Drug Deliv. Rev. 57 (2005) : 411–430.

Passalacqua, G. “Non-injection routes for allergen immunotherapy: focus on sublingual immunotherapy.” Inflamma. Allergy Drug Target 5 (2006) : 43-51.

Patel, V. R., and M. M. Amiji. “Preparation and characterization of freeze dried chitosan–poly(ethylene oxide) hydrogels for site-specific antibiotic delivery in the stomach.” Pharm. Res. 13 (1996) : 588–593.

Peluso, G. et al. “Chitosan-mediated stimulation of macrophage function.” Biomaterials 15 (1994) : 1215–1220.

Periwal, S. B. “Orally administered microencapsulated reovirus can bypass suckled, meutralizing maternal antibody that inhibits active immunization of neonates.” J. Virol 71 (1997) : 2844-2850.

Polona, S. et al. “The Influence of Selected Parameters on the Size and Shape of Alginate Beads Prepared by Ionotropic Gelation.” Sci. Pharm. 76 (2008) : 77–89.

Polk, A. et al. “Controlled release of albumin from chitosan–alginate microcapsules.” J. Pharm. Sci. 83 (1994) : 178–185.

Porporatto, C. “Early events associated to the oral co-administration of type II collagen and chitosan: induction of anti-inflammatory cytokines.” Int. Immunol. 16 (2004) : 433–441.

Purello-D'Ambrosio, F. et al. “Prevention of new sensitizations in monosensitized subjects submitted to specific immunotherapy or not, A retrospective study.” Clin.Exp. Allergy 31 (2001) : 1295-1302.

Qin, C. “Enzymic preparation of water-soluble chitosan and their antitumor activity.” Int. J. Biol. Macromol. 31 (2002) : 111 – 117.

Radauer, C., and H. Breiteneder. “Pollen allergens are restricted to few protein families and show distinct patterns of species distribution.” J Allergy Clin. Immunol. 117 (2006) : 141-147.

Radauer , C. et al. “Allergens are distributed into few protein families and possess a restricted number of biochemical functions.” J. Allergy Clin. Immunol. 121 (2008) : 847-52.

Rasmussen, M. R. et al. “Numerical modelling of insulin and amyloglucosidase release from swelling Ca-alginate beads.” J. Control. Release 91 (2003) : 395–405.

Rebelatto, M. C. et al. “Induction of systemic and mucosal immune response in cattle by intranasal administration of pig serum albumin in alginate microparticles.” Vet. Immunol. Immunopathol. 83 (2001) : 93–105.

Rhazi, M. et al. “Investigation of different natural sources of chitin: influence of the source and deacetylation process on the physicochemical characteristics of chitosan.” Polym. Int. 49 (2000) : 337–344.

Rolland, J. “Current and new therapeutic strategies. Expert Opin Investig Drugs.” Allergen Immunother. 9 (2000) : 515-527.

Robert, K. et al. “Advances in environmental and occupational respiratory disease in 2007.” J. Allergy Clin. Immunol. (2008) : 1359-1362.

Romalde, J. L. “Oral immunization using alginate microparticles as a useful strategy for booster vaccination against fish lactoccocosis.” Aquaculture 236 (2004) : 119–129.

Roy, K. et al. “Oral gene delivery with chitosan–DNA nanoparticles generates immunologic protection in a murine model of peanut allergy.” Nat. Med 5 (1999) : 387–391.

Roberts, G. A. F. Chitin Chemistry. Vol. 1–110. London : The Mac Millan Press, 1992.

Sandri, G. et al. “Asessment of chitosan derivatives as buccal and vaginal penetration enhancers.” Eur. J. Pharm. Sci. 21 (2004) : 351–359.

Sara, G. et al. “Allergen immunotherapy with nanoparticles containing lipopolysaccharide from Brucella ovis.” Eur. J. Pharm. Biopharm. 70 (2008) : 711–717.

Sarah, K. et al. “Quality of life outcomes with sublingual immunotherapy.” Ameri. J. Otolaryng. – Head and Neck Medicine and Surgery (2009) : in press.

Schipper, N. G. et al. “Chitosans as absorption enhancers for poorly absorbable drugs: 1. Influence of molecular weight and degree of acetylation on drug transport across human intestinal epithelial (Caco-2) cells.” Pharm. Res. 13 (1996) : 1686– 1692.

Schipper, N. G. et al. “Chitosans as absorption enhancers for poorly absorbable drugs: 2. Mechanism of absorption enhancement.” Pharm. Res. 14 (1997) : 923–929.

Schipper, N. G. et al. “Chitosans as absorption enhancers of poorly absorbable drugs: 3. Influence of mucus on absorption enhancement.” Eur J. Pharm. Sci. 8 (1999) : 335– 343.

Schnürch, A. B. “Basic studies on bioadhesive delivery systems for peptide and protein drugs.” Int. J. Pharm. 165 (1998) : 217–225.

Schipper, N. G. M. et al “Chitosans as absorption enhancers for poorly absorbable drugs: 2. Mechanism of absorption enhancement.” Pharm. Res. 14 (1997) : 923–929.

Schöll, I. et al. “Review of novel particulate antigen delivery systems with special focus on treatment of type I allergy.” J. Control. Release 104 (2005) : 1 – 27.

Senel, S., and A. A. Hincal. “Drug permeation enhancement via buccal route: possibilities and limitations.” J. Control. Release 72 (2001) : 133–144.

Seong, S. Y. et al. “Protective immunity of microsphere-based mucosal vaccines against lethal intranasal challenge with Streptococcus pneumoniae.” Infect. Immun 67 (1999) : 3587–3592.

Shepherd, R. et al. “Chitosan functional properties.” Glycoconj. 14 (1997) : 535–542.

Shahidi, F. et al. “Food applications of chitin and chitosans.” Trends Food Sci. Technol. 10 (1999) : 37–51.

Shibata, Y et al. “Th1 adjuvant N-acetyl-d-glucosamine polymer up-regulates Th1 immunity but down regulates Th2 immunity against a mycobacterial protein (MPB-59) in interleukin-10-knockout and wild-type mice.” Infect. Immun. 69 (2001) : 6123–6130.

Shimoda, J. et al. “Bioadhesive characteristics of chitosan microspheres to the mucosa of rat small intestine.” Drug Dev. Ind. Pharm. 27 (2001) : 567–576.

Sinha, A. K. et al. “Chitosan microspheres as a potential carrier for drugs.” Int. J. Pharm. 274 (2004) : 1–33.

Sinswat, P., and P. Tengamnuay. “Enhancing effect of chitosan on nasal absorption of salmon calcitonin in rats: comparison with hydroxypropyl and dimethyl-β-cyclodextrins.” Int. J. Pharm. 257 (2003) : 15–22.

Smidsrod, O., and G. Skjak-Braek. “Alginate as immobilization matrix for cells.” TIBTECH 8 (1990) : 71–78.

Smith, J. “Effect of chitosan on epithelial cell tight junctions.” Pharm. Res. 21 (2004) : 43–49.

Soares, J. P. et al. “Thermal behaviour of alginic acid and its sodium salt.” Eclet. Quim. 29 (2004) : 57–64.

Sugawara, S. “The controlled release of prednisolone using alginate gel.” Pharm. Res. 11 (1994) : 272–277.

Stoscheck, C. “Quantification of Protein.” Methods in Enzymology 182 (1990) **:** 50-68.

Sunil, A. et al. “Recent advances on chitosan-based micro- and nanoparticles in drug delivery.” J. Control. Release 100 (2004): 5-28.

Sujata S. ABC’s Of Allergies[online]. Assessed August 2006. Available from http://www.csa.com/discoveryguides/discoveryguides-main.php

Sutherland, I. W. “Novel Materials from Biological Sources.” Biomaterials (1991) : 309–331.

Tone, Ø. et al. “Calcium alginate matrices for oral multiple unit administration: II. Effect of process and formulation factors on matrix properties.” Int. J. Pharm. 97 (1993) : 183-193.

Valenta, R. "The future of antigen specific immunotherapy of allergy." Nat. Rev. Immunol. 2 : 446-453.

Varney, V. A. et al. "Usefulness of immunotherapy in patients with severe summer hay fever uncontrolled by anti allergic drugs." Bmj 302 (1991) : 265-269.

Velge-Roussel, F. et al. "Intranasal immunization with toxoplasma gondiisag 1 induces protective cells into both NALT and GALT compartments." Infect. Immun. 68 (2000) : 969–972.

Van der Lubben, I. M. "Chitosan microparticles for oral vaccination: preparation, characterization and preliminary in vivo uptake studies in murine Peyer's patches." Biomaterials 22 (2001) : 687– 694.

Van der Lubben, I. M. "Transport of chitosan microparticles for mucosal vaccine delivery in a human intestinal M-cell model." J. Drug Target 10 (2002) : 449– 456.

Wang, K. "Alginate-konjac glucomannan-chitosan beads as controlled release matrix." Int. J. Pharm. 244 (2002) : 117–126.

Woodfolk, J. A., and T. A. Platts-Mills. "The immune response to intrinsic and extrinsic allergens: determinants of allergic disease." Int. Arch. Allergy Immunol. 129 (2002) : 277– 285.

Wu, H. Y., and M. W. Russell. "Nasal lymphoid tissue, intranasal immunization, and compartmentalization of the common mucosal immune system." Immunol. Res. 16 (1997) : 187– 201.

Wuolijoki, E. et al. "Decrease in serum LDL cholesterol with microcrystalline chitosan, Methods Find." Exp. Clin. Pharmacol. 21 (1999) : 357–361.

Xianghong, P. et al. "Self-assembled micelles of *N*-phthaloyl-carboxymethy chitosan for drug delivery." Colloids and Surfaces A: Physicochem. 337 (2009) : 21–25

Yamada, K. et al. "Chitosan based water-resistant adhesive. Analogy to mussel glue." Biomacromol. 1 (2000) : 252–258.

Yao, K. D. et al. "Swelling kinetics and release characteristic of crosslinked chitosan–polyether polymer network (semi-IPN) hydrogels." J. Polym. Sci., A, Polym. Chem. 32 (1994) : 1213–1223.

Yao, K. D. et al. “Investigation of pH sensitive drug delivery system of chitosan/gelatin hybrid polymer network.” Polym. Int 38 (1995) : 77–82.

Yu, C. Y. et al. “Sustained release of antineoplastic drugs from chitosan-reinforced alginate microparticle drug delivery systems.” Int. J. Pharm. 357 (2008) : 15–21.

Zimmermann, U. et al. “Production of mitogen contamination free alginates with variable rations of mannuronic to guluronic acid by free flow electrophoresis.” Electrophoresis 13 (1992) : 269–274.

APPENDIX

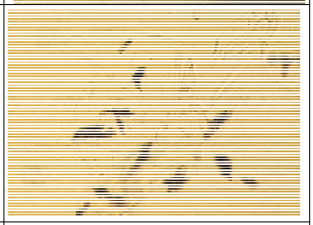
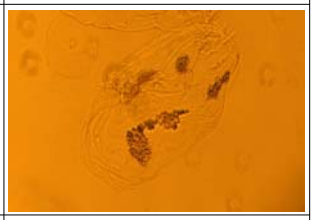
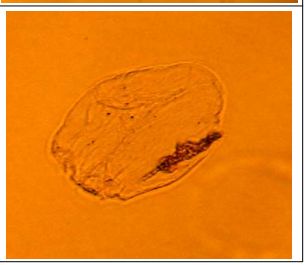
APPENDIX I

Preformulation of Ca-alginate microparticles

Table 9 Particle size of the Ca-alginate microparticles

Sample Name	Z-Average d.nm	AVE.	S.D.	PdI	AVE.	S.D.
0.001%AHV+3%Ca	7280 19500 42800	23193.3	18045.7	1.00 1.00 0.75	0.918	0.142
0.1%AHV+3%Ca	2790 1590 17100	7160.0	8629.2	1.00 1.00 0.60	0.867	0.231
0.5%AHV+3%Ca	3120 4060 3830	3670.0	490.0	0.27 1.00 0.99	0.752	0.422
0.05%AHV+3%Ca	52000 6730 1050	19926.7	27921.1	0.04 1.00 0.64	0.560	0.485
0.1%AHV+4%Ca	3160 7830 2790	4593.3	2809.1	1.00 0.28 1.00	0.760	0.416
0.01%AHV+4%Ca	15100 3870 1310	6760.0	7335.2	0.71 1.00 0.92	0.876	0.150
0.05%AHV+4%Ca	3250 2900 1900	2683.3	700.6	0.54 0.96 0.62	0.705	0.221
0.001%ALV+4%Ca	4850 3010 2620	3493.3	1191.0	1.00 1.00 1.00	1.000	0.000
0.05%ALV+3%Ca	5690 7760 3290	5580.0	2237.0	1.00 1.00 0.51	0.837	0.282
0.1%ALV+3%Ca	5220 4360 4000	4526.7	626.8	0.40 0.01 1.00	0.470	0.499
0.1%ALV+4%Ca	6190 5190 4220	5200.0	985.0	1.00 1.00 1.00	1.000	0.000

Table 10 Shape of the Ca-alginate coated CS-TPP microparticles in different alginate concentration by inverted microscope.

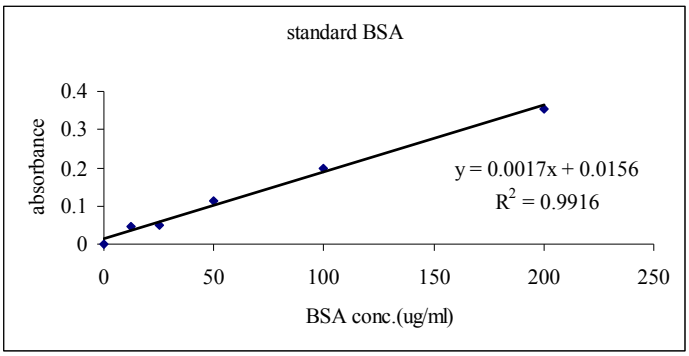
alginate conc. (mg/ml)	$CaCl_2$ solution (mg/ml)	Alginate : Cation (w/w)	Morphology (20x)
0.05	**4**	**0.0125:1**	
0.05	**6**	**0.0083:1**	
0.1	**4**	**0.025:1**	
0.1	**6**	**0.017:1**	
0.25	**4**	**0.0625:1**	
0.25	**6**	**0.041:1**	

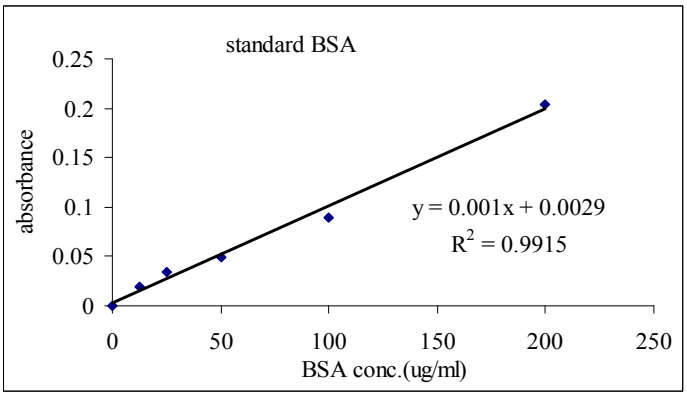
APPENDIX II

Entrapment efficiency and allergen content of microparticlexs

1. Determination the amount of dust mite allergen

Standard : BSA
Concentration : 12.5, 25, 50, 100, 200 µg/ml
Method : Bradford Assay
Absorbance : 550





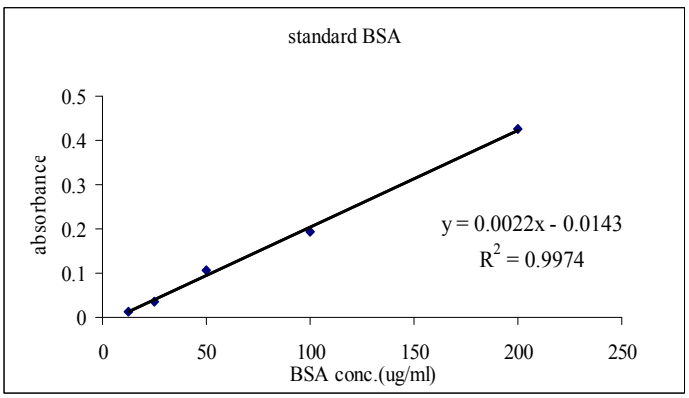


Figure 29 Standard curve of BSA

Table 11 Showed percent yield, entrapment efficiency, allergen content of Ca-alginate microparticles in different viscosity

Formulation	Allergen content(mg/g)		AVE.	SD	%Yield	%Entrapment efficiency		SD	AVE.
	n1	n2				n1	n2		
1%Al+A4	0.44	0.98	0.71	0.39	90.81	40.01	47.70	5.44	43.85
5%Al+A4	3.88	2.81	3.34	0.76	23.33	71.51	51.78	13.95	61.65
10%Al+A4	2.23	4.36	3.30	1.50	37.05	20.68	40.40	13.94	30.54
1%Al+A8	0.43	0.93	0.68	0.35	19.80	47.00	71.76	17.51	59.38
5%Al+A8	1.38	2.41	1.89	0.73	50.45	34.87	44.39	6.73	44.39
10%Al+A8	4.48	3.46	3.97	0.72	96.04	41.50	32.09	6.66	36.79

Analysis allergen by HPLC

Flow rate	:	1.0 ml/min.
Colum	:	Water C_{18} reverse-phase column (Water, USA), 5 µm, 150 mm x 0.5 mm was used.
Mobile phase	:	0.085% TFA in ACN and 0.1% TFA in water.
Injection volume	:	20 µl.
Detection	:	Fluorescence detector excitation wavelength of 280 nm and emission wavelength of 304 nm

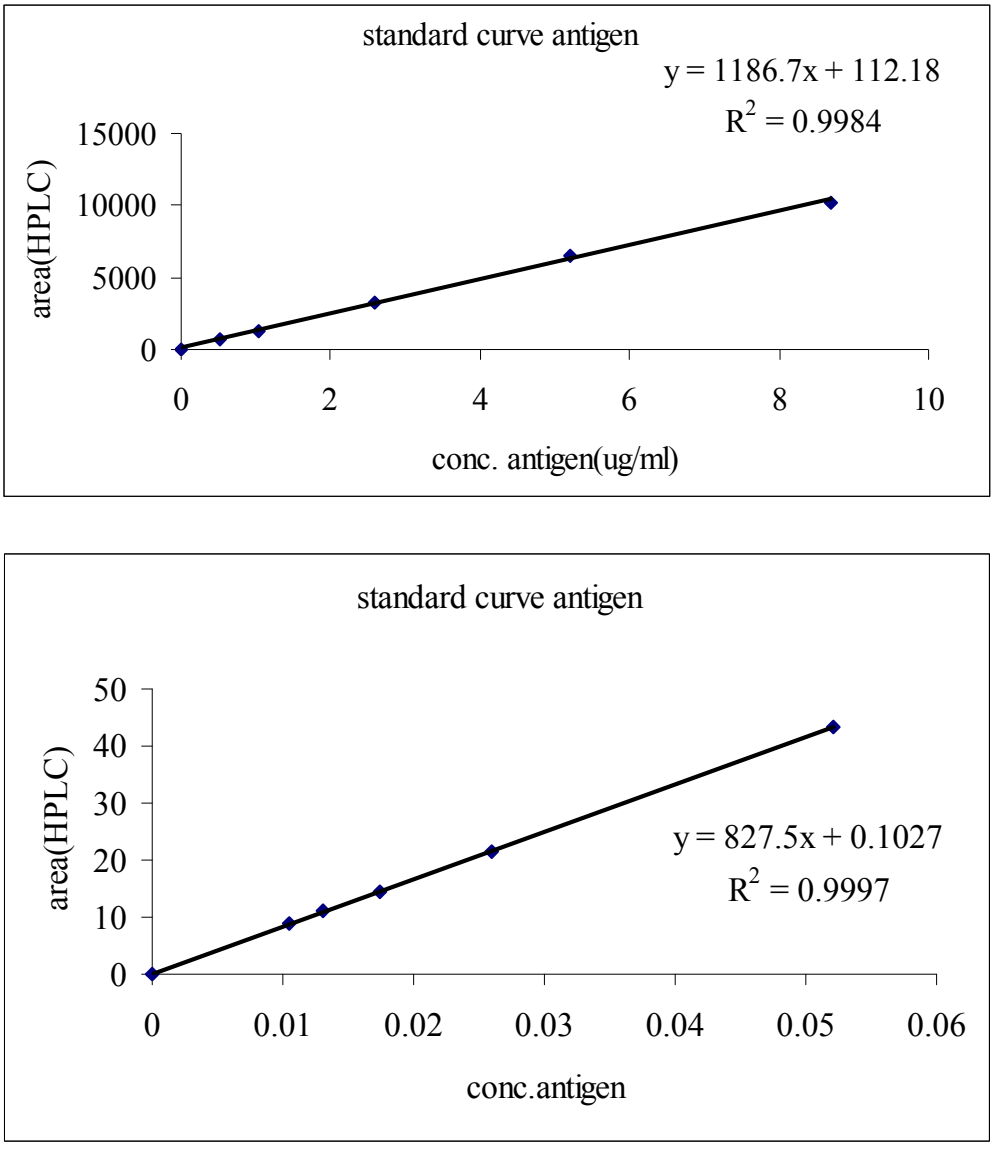


Figure 30 Standard curve of allergen detected by HPLC

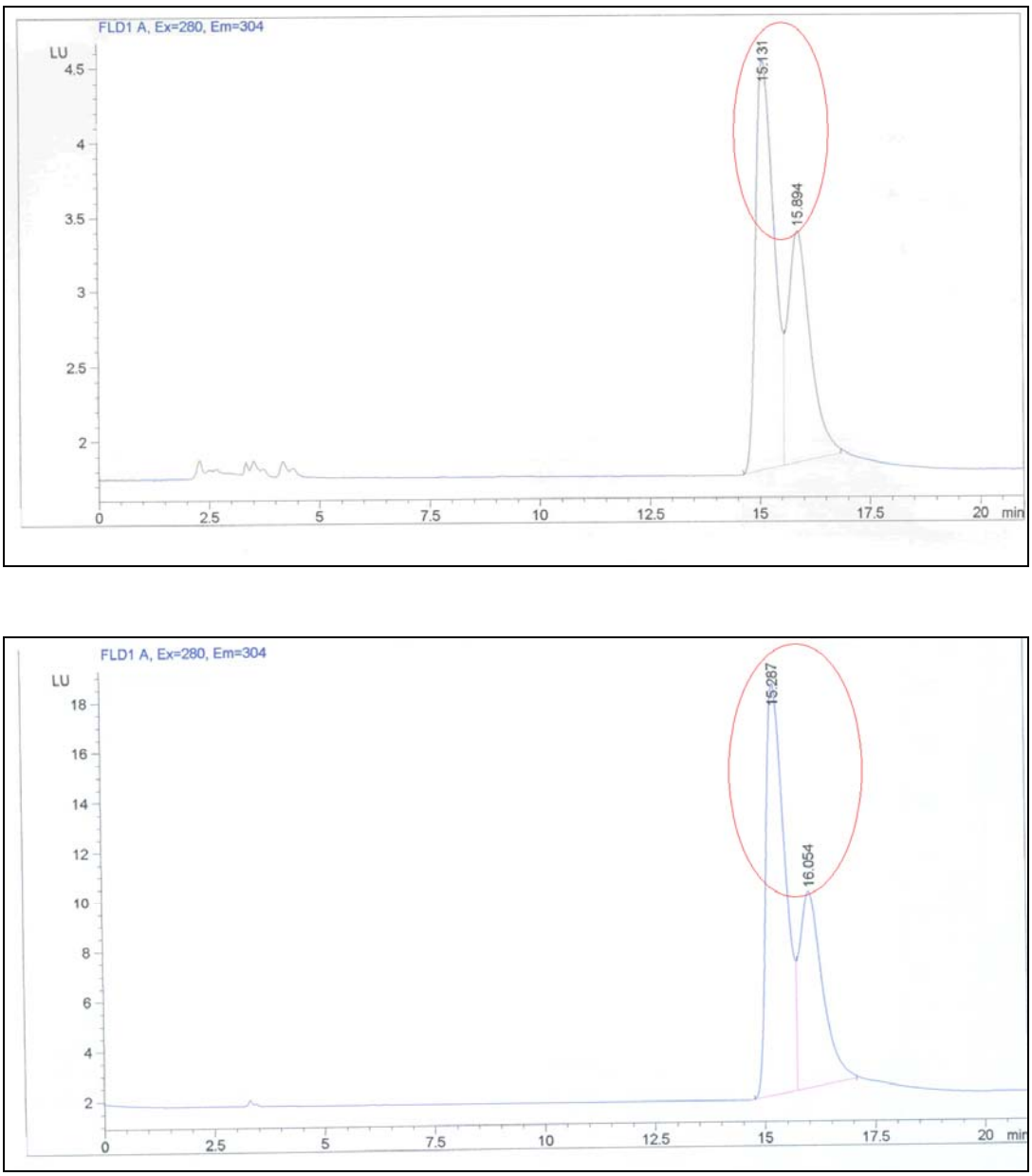


Figure 31 Showed peak of allergen detect by HPLC Ex = 280, Em = 304

Table 12 Showed percent yield, entrapment efficiency, allergen content of CS-TPP microparticles in different molecular weight

Formulation	%Encapsulation		AVE.	SD	Allergen content(mg/g)		AVE.	SD	%Yield
	n1	n2			n1	n2			
0.1%Al + CS-HCl 20K	2.45	2.36	1.86	0.55	0.02	0.01	0.01	0.01	59.75
0.5%Al + CS-HCl 20K	0.38	0.37	0.49	0.11	0.01	0.01	0.01	0.00	63.00
2.5%Al + CS-HCl 20K	0.25	0.41	0.33	0.11	0.04	0.07	0.06	0.02	34.88
0.1%Al + CS-HCl 200K	2.51	2.26	2.38	0.18	0.02	0.02	0.02	0.00	50.69
0.5%Al + CS-HCl 200K	0.96	1.36	1.16	0.28	0.03	0.05	0.04	0.01	42.26
2.5%Al + CS-HCl 200K	1.66	0.65	1.16	0.71	0.28	0.11	0.19	0.12	63.53
0.1%Al + CS-HCl 460K	7.21	4.59	5.90	1.85	0.05	0.03	0.04	0.01	47.83
0.5%Al + CS-HCl 460K	1.88	1.83	1.86	0.03	0.06	0.06	0.06	0.00	44.34
2.5%Al + CS-HCl 460K	1.45	2.03	1.74	0.41	0.24	0.34	0.29	0.07	34.98

APPENDIX III

The *in vitro* release of microparticles

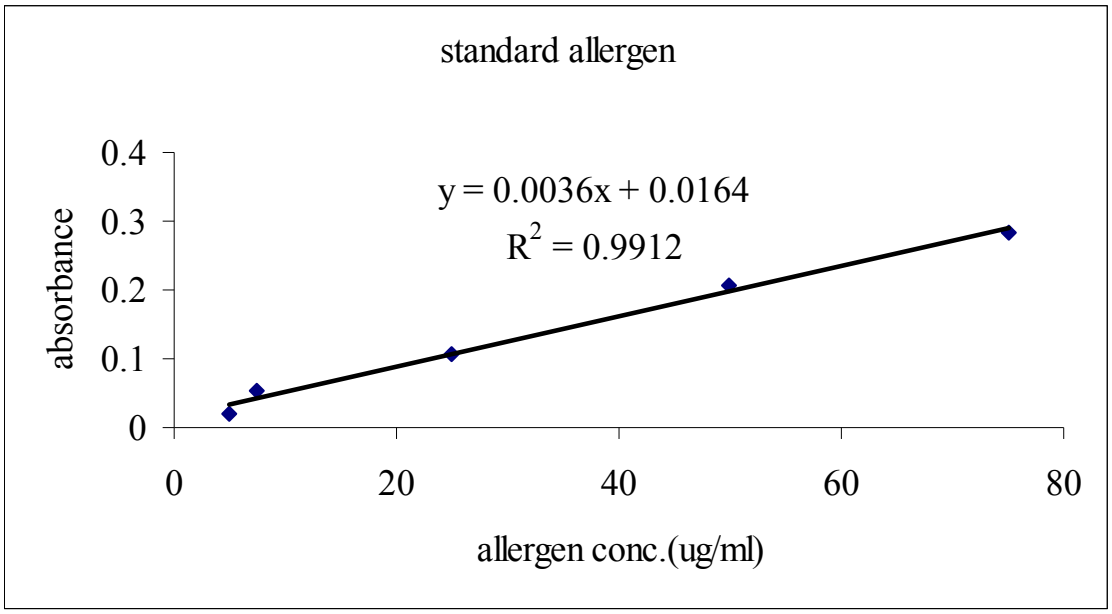


Figure 32 Standard curve of allergen for *in vitro* release

Table 13 Showed *in vitro* release profile of Ca-alginate microparticles in simulated gastric fluid without pepsin (pH 1.2)

Time (h.)	0.1%Al + A4				0.5%Al + A4				2.5%Al + A4			
	Cumulative (μg)		%Cumulative		Cumulative (μg)		%Cumulative		Cumulative (μg)		%Cumulative	
	AVE.	S.D.	AVE.	S.D.	AVE.	S.D.	AVE.	S.D.	AVE.	S.D.	AVE.	S.D.
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.5	14.59	0.51	12.16	0.42	15.09	9.75	18.86	12.18	16.93	2.42	16.93	2.42
1	22.33	2.12	18.60	1.77	21.93	12.52	27.41	15.65	28.97	1.93	28.97	1.93
1.5	33.32	2.44	27.77	2.04	32.57	12.42	40.71	15.53	34.76	10.52	34.76	10.52
2.5	39.34	0.00	32.78	0.00	43.55	11.32	54.43	14.15	42.07	3.66	42.07	3.66
4	61.00	14.49	50.83	12.08	46.55	12.80	58.19	16.01	43.56	1.94	43.56	1.94
8	101.07	13.15	84.22	10.96	49.46	14.52	61.82	18.16	44.98	0.70	44.98	0.70
24	102.42	0.16	85.35	0.13	49.25	14.14	61.57	17.68	46.60	0.51	46.60	0.51
48	107.35	8.57	89.46	7.14	59.62	13.14	74.53	16.42	56.05	4.62	56.05	4.62

Table 14 Showed *in vitro* release profile of Ca-alginate microparticles in PBS pH 7.4

Time (h.)	0.1%Al + A4				0.5%Al + A4				2.5%Al + A4			
	Cumulative (μg)		%Cumulative		Cumulative (μg)		%Cumulative		Cumulative (μg)		%Cumulative	
	AVE.	S.D.	AVE.	S.D.	AVE.	S.D.	AVE.	S.D.	AVE.	S.D.	AVE.	S.D.
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1	7.02	2.39	28.09	2.68	5.81	1.53	11.61	3.06	7.77	1.05	15.87	2.39
1.5	13.24	5.78	52.95	13.58	18.31	2.10	36.61	4.20	12.39	6.45	23.92	6.13
2.5	14.59	5.49	58.35	9.12	25.67	21.50	51.34	43.00	23.67	4.35	50.60	23.01
4	20.13	3.15	80.50	16.93	33.44	13.00	66.87	25.99	29.15	1.48	60.28	14.09
8	19.72	3.34	78.87	14.89	35.80	10.22	71.60	20.45	29.28	2.25	60.34	12.57
24	23.57	3.87	94.27	18.64	38.09	12.66	76.19	18.34	33.94	0.72	70.50	18.51
48	38.63	12.42	147.49	9.97	54.31	12.66	98.74	0.00	38.09	12.23	75.76	3.03

Table 15 Showed *in vitro* release profile of CS-TPP microparticles in simulated gastric fluid without pepsin (pH 1.2)

Time (h.)	0.5%Al + CS9				1%Al + CS9				2.5%Al + CS9			
	Cumulative (μg)		%Cumulative		Cumulative (μg)		%Cumulative		Cumulative (μg)		%Cumulative	
	AVE.	S.D.	AVE.	S.D.	AVE.	S.D.	AVE.	S.D.	AVE.	S.D.	AVE.	S.D.
0	0.00	0.0	0.00	0.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.5	0.13	0.0	46.73	5.1	0.10	0.02	33.36	6.11	0.22	0.01	33.14	0.80
1.5	0.12	0.0	48.85	0.4	0.09	0.03	36.02	8.36	0.24	0.01	36.40	1.49
2	0.18	0.0	74.74	6.2	0.19	0.02	54.33	5.69	0.34	0.01	51.58	1.35
4	0.23	0.0	88.21	8.0	0.23	0.01	75.52	2.70	0.47	0.02	71.68	3.26
8	0.20	0.0	80.92	3.0	0.20	0.01	67.26	2.07	0.43	0.01	66.26	2.27
24	0.13	0.0	52.52	2.3	0.18	0.01	61.31	1.82	0.40	0.02	61.10	2.82

Table 16 Showed *in vitro* release profile of CS-TPP microparticles in PBS pH 7.4

Time (h.)	0.5%Al + CS9				1%Al + CS9				2.5%Al + CS9			
	Cumulative (μg)		%Cumulative		Cumulative (μg)		%Cumulative		Cumulative (μg)		%Cumulative	
	AVE.	S.D.	AVE.	S.D.	AVE.	S.D.	AVE.	S.D.	AVE.	S.D.	AVE.	S.D.
0	0.00	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.00	0.00	0.00	0.00
1	0.14	0.0	38.24	11.8	0.17	0.0	42.39	0.56	0.25	0.00	41.99	0.56
2	0.15	0.0	49.52	15.8	0.13	0.0	32.21	0.97	0.26	0.01	32.98	1.75
4	0.17	0.1	73.57	23.4	0.20	0.0	51.15	2.71	0.48	0.02	60.34	2.61
8	0.23	0.0	75.68	14.3	0.23	0.0	56.42	2.50	0.49	0.05	61.79	6.39
24	0.29	0.0	97.57	12.0	0.29	0.0	72.84	3.3	0.53	0.06	65.93	7.64

Table 17 Showed *in vitro* release profile of Ca-alginate coated CS-TPP microparticles in different medium

Time (h.)	pH 7.4		pH 1.2	
	AVE	**S.D.**	**AVE**	**S.D.**
0	0.00	0.00	0.00	0.00
0.25	13.13	0.35	4.12	0.05
0.5	26.69	1.13	10.95	0.15
1	30.13	0.45	16.93	0.41
1.5	42.93	0.16	21.97	0.76
2	46.95	0.06	27.27	0.85
2.5	56.28	3.05	32.88	1.16
4	64.90	0.06	38.27	2.36
8	75.68	0.07	42.65	2.79
12	91.82	2.36	48.56	1.26
24	94.53	3.04	54.08	0.65

Table 18 Showed *in vitro* release profile of CS-CA-mPEG in simulated gastric fluid without pepsin (pH 1.2)

Time (h.)	0.25%Al + CS-CA-mPEG				0.5%Al + CS-CA-mPEG				0.75%Al + CS-CA-mPEG			
	Cumulative (μg)		%Cumulative		Cumulative (μg)		%Cumulative		Cumulative (μg)		%Cumulative	
	AVE.	S.D.	AVE.	S.D.	AVE.	S.D.	AVE.	S.D.	AVE.	S.D.	AVE.	S.D.
0	0.00	0.0	0.00	0.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.25	2.75	0.4	41.29	6.1	1.41	0.40	19.07	5.56	2.13	0.26	21.26	2.64
0.5	2.58	0.3	44.44	4.1	2.10	0.14	29.92	2.32	2.82	0.04	29.65	0.53
1	3.94	1.2	64.68	18.2	2.65	0.21	39.41	3.42	2.88	0.18	32.14	1.99
1.5	3.85	0.2	67.34	2.1	2.69	0.16	42.38	1.45	3.32	0.11	38.56	1.46
2	3.30	0.2	65.77	4.1	2.50	0.00	42.21	0.55	3.36	0.06	41.19	0.97
4	3.45	0.8	67.23	14.8	2.90	0.00	49.50	1.27	3.55	0.07	44.59	0.06
8	3.04	0.2	64.82	1.7	3.00	0.00	52.70	1.32	3.00	0.00	41.01	2.14
12	3.35	0.2	73.46	5.5	3.21	0.13	59.59	1.30	2.80	0.42	42.11	3.74
24	3.35	0.9	76.75	10.5	3.39	0.16	64.99	2.67	3.30	0.06	49.04	0.84

Table 19 Showed *in vitro* release profile of CS-CA-mPEG in PBS pH 7.4

Time (h.)	0.25%Al + CS-CA-mPEG				0.5%Al + CS-CA-mPEG				1%Al + CS-CA-mPEG			
	Cumulative (μg)		%Cumulative		Cumulative (μg)		%Cumulative		Cumulative (μg)		%Cumulative	
	AVE.	S.D.	AVE.	S.D.	AVE.	S.D.	AVE.	S.D.	AVE.	S.D.	AVE.	S.D.
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.25	2.25	0.35	23.30	3.70	2.10	0.14	18.01	1.23	2.25	0.35	16.89	2.68
0.5	2.70	0.28	27.64	2.71	2.25	0.35	20.51	3.15	3.59	0.02	28.14	0.02
1	2.35	0.07	27.77	0.79	2.50	0.42	23.96	3.96	3.90	0.14	32.33	1.24
1.5	3.06	1.35	36.76	14.22	3.40	0.85	33.19	7.88	3.70	0.42	32.78	2.98
2.5	4.50	0.71	48.03	8.43	5.40	0.14	52.47	0.20	6.25	0.35	53.97	2.71
4	5.05	0.07	62.00	1.05	5.65	0.35	57.17	1.31	6.50	0.00	58.38	0.68
8	5.45	0.07	68.82	3.30	5.25	0.35	55.98	1.60	6.75	0.07	62.54	3.06
24	6.70	0.64	89.68	8.11	6.24	0.47	70.92	3.24	6.34	0.00	66.00	0.21

APPENDIX IV

LIST OF ABBREVIATIONS

°C	Degree celsius
µg	Microgram
µm	Micrometer
%	Percent
%v/v	Percent volume by volume
%w/w	Percent weight by weight
%EE	Percent entrapment efficiency
AHV	Alginate high viscosity
Al	Allergen
ALV	Alginate low viscosity
Ca-alginate	Calcium alginate
$CaCl_2$	Calcium chloride
CS-HCl	Chitosan hydrochloride
Conc.	Concentration
CS-CA-mPEG	Chitosan cholic polyethylene glycol
CS-TPP	Chitosan tripolyphosphate
DMS	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid solution
et al.	and others
FTIR	Fourier transform infra-red
g	Gram(s)
h	Hour
HCl	Hydrochloric acid
HPLC	High Performance Liquid Chromatography
kDa	Kilo-Daltons
NaCl	Sodium chloride
NaOH	Sodium hydroxide
nm	Nanometer
mg	Milligram

LIST OF ABBREVIATIONS

ml	Milliliter
MWCO	Molecular weight cut-off
MW	Molecular weight
PBS	Phosphate-buffered saline
PDI	Polydispersity index
pH	Potentia hydrogenii (lat.)
R^2	Coefficient of determination
SD	Standard deviation
UV	Ultraviolet
TPP	Penta-sodium triphosphate
ZP	Zeta potential

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