PREPARATION AND EVALUATION OF ALGINATE-PECTIN-POLY-I-LYSINE PARTICULATES FOR DRUG DELIVERY AND EVALUATION OF MELITTIN AS A NOVEL ABSORPTION ENHANCER

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PREPARATION AND EVALUATION OF ALGINATE-PECTIN-POLY-*l*-LYSINE PARTICULATES FOR DRUG DELIVERY

AND

EVALUATION OF MELITTIN AS A NOVEL ABSORPTION ENHANCER

by

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Abstracts

I

Drug delivery particulates were prepared using alginate (ALG), poly-l-lysine (PLL) and pectin (PEC). Theophylline, chlorothiazide and indomethacin were used as the model drugs for *in vitro* dissolution, and mannitol was used as the model for assessing paracellular drug absorption across Caco-2 cell monolayers. ALG and PEC served as the core polymers; PLL helped to strengthen the particulate and PEC also helped to modulate the release profiles of the encapsulated model drugs. *In vitro* dissolution tests of ALG-PEC-PLL in acidic and alkaline media showed sustained drug release of the model drugs. *In vitro* bioadhesive tests indicated that the particulates had good bioadhesive properties. ALG and PEC also enhanced paracellular absorption of mannitol across Caco-2 monolayers by about three times. Use of ALG-PEC-PLL particulates is expected to combine the advantages of bioadhesion, absorption enhancement, and sustained release. This particulate system may have potential use as a carrier for poorly absorbed drugs by the oral route of administration.

II

In this study, the possibility of using melittin as a novel absorption enhancer was investigated. The membrane fusion peptide, melittin, is the major active

ingredient of honey bee venom. Its action as a membrane fusion peptide on membrane has a strong concentration dependency. By incorporating melittin, which is itself a peptide, along with the drug in a particulate system, such as ALG-PEC-PLL particulates, we anticipate that a sufficient quantity of the drug and the enhancer will be delivered at the absorption site. Caco-2 cell line was used as the model cell line and mannitol was used as the model drug for transport study. MTT assay was performed to evaluate the cytotoxicity of melittin. The results indicated that at a concentration below 2.42 μM melittin did not show any cytotoxic effects on Caco-2 cells. Transport study showed that 1.20 - 1.50 μM of melittin was able to increase the absorption of mannitol across Caco-2 cell monolayer by a factor of 3.5.

Linking part I and II of the project

The particulate system developed in part I, could be used to carry a drug and melittin (as an enhancer). It is envisaged that the pectin-alginate-poly lysine particulate will protect melittin (a peptide compound) in a biological environment that is 'hostile' to peptide molecules, thus enabling melittin to exercise its action as an absorption enhancer. This is an ongoing project and further work will be done in our laboratory.

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Glossary of Abbreviations and Symbols

λ_{max}	Maximum absorbance wavelength
ALG	Alginate
BDDS	Bioadhesive drug delivery system
$CaCl_2$	Calcium chloride
CDDS	Controlled-release drug delivery system
cm	Centimetre
CMC	Carboxymethyl cellulose
CO_2	Carbon dioxide
Da	Dalton
DMEM	Dulbecco's modified eagle's medium
dpm	Disintegrations per minute
FCS	Fetal calf serum
g	Gram(s)
G	Guluronate
GI	Gastrointestinal
h	Hour(s)
HCl	Hydrochloric acid
HM	High degree of methoxylation
HPC	Hydroxypropyl cellulose

KH_2PO_4	Potassium phosphate monobasic
L	Litre
LM	Low degree of methoxylation
LSC	Liquid scintillation counting
M	Mannuronate
μCi	Micro curie
μg	Microgram
μm	Micrometre
μM	Micromolar concentration
mCi	Millicurie
min	Minute(s)
mL	Millilitre
mm	Millimetre
MMC	Migrating myoelectrical complex
mmol	Millimolar
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
MW	Molecular weight
NaOH	Sodium hydroxide
NEAA	Non-essential amino acids
nm	Nanometre
P_{app}	Apparent permeability coefficient

PBS	Phosphate buffer saline
PEC	Pectin
PLL	Poly-*l*-lysine
psi	Pounds per square inch
rpm	Rotations per minute
SEM	Scanning electron microscopy
SD	Standard deviation
TEER	Transepithelial electrical resistance
UV	Ultraviolet (spectrometry)

Chapter I

1. INTRODUCTION

1.1 Development of bioadhesives in drug delivery systems

1.1.1. Controlled-release drug delivery systems (CDDS)

Over the past 30 years, the therapeutic advantages of CDDS have been recognized and greater attention has been paid to the development of CDDS. The goals of CDDS are to maintain effective blood concentrations of therapeutic agents throughout the dosage interval, to reduce the frequency of dosing or to increase effectiveness of the drug by localization at the site of action, to reduce the dose required and drug-induced side effects, or to provide uniform drug delivery. During the 1950's and early 1960's, the approach adopted to maintain effective plasma drug levels was through the use of dissolution/diffusion devices (Robinson, 1990). It is possible to only partially achieve these goals with the traditional CDDS such as controlled-release tablets and capsules, etc., especially when orally administered, because the dosage form leaves the absorption site before it releases the drug completely. It is now generally accepted that drug absorption is largely dependent on the transit of the dosage form through the absorption sites, and variability in absorption is expected for most of the drugs (Park, 1986). Traditional CDDS are not retained at the absorption site for a relatively long period of time, hence, they can not offer significant therapeutic advantages over conventional, prompt release dosage forms.

Oral route of drug administration is the most accepted and popular administration route. Besides acceptance by the patients for its convenience of administration, it is more popular in the pharmaceutical industry because it can be mass produced easily with less cumbersome facilities. However, the development of oral CDDS has been limited by the inability to restrain and localize the system in selected regions of the gastrointestinal (GI) tract. The transit time of most dosage forms in the GI tract is about 8-12 h, and it is difficult to increase the absorption phase of administration beyond this time frame. Only absorption from the colon may allow continued drug delivery for up to 24 h. If a drug is absorbed by active transport, or at a specific region of the intestine, conventional sustained-release preparations may be unsuitable for absorption. One method to provide sustained delivery for such drugs is with low density pellets, capsules or tablets that can float in gastric juice, thus the gastric emptying can be delayed (Thanoo, et al., 1993 and Xu, 1991). However, this method is not suitable for drugs that are predominantly absorbed in the small intestine (Ichikawa, et al., 1991). In this context, the use of polymeric drug delivery systems that adhere to GI mucus layer or epithelial cell surfaces holds promise and has attracted lots of attention. Such polymers are referred to as "bioadhesives". These bioadhesive polymeric CDDS find potential application at many absorption sites with mucous membrane such as, nasal, buccal, intestinal and vaginal. In this research project we have developed a polymeric particulate system with bioadhesive property. The novelty of our DDS is in combining the advantages of particulates and bioadhesive technologies by using

polymers of natural origin, alginate (ALG) and pectin (PEC).

1.2. Bioadhesives

A bioadhesive is defined as a synthetic or biological material which is capable of adhering to a biological substrate or tissue (Peppas, et al., 1985). The following characteristics are believed to be essential for exhibiting the bioadhesive properties: i) molecular flexibility; ii) hydrophilic functional groups; and iii) a specific molecular weight (MW), chain length and conformation (Chien, 1992). The bioadhesive polymer binding with mucus at the mucosal membrane is referred to as a "mucoadhesive" polymer (Park, 1989). Since most of the bioadhesives interact with mucus before they can reach the mucosa, most bioadhesives presently available are actually mucoadhesives. In this thesis, the term bioadhesive is used to refer to all types of bioadhesives.

A further classification of bioadhesives and bioadhesion is based on the presence or absence of non-biological (artificial) materials in the adhesion process (Park, et al., 1990). The types of bioadhesion which have been identified are divided into three types. Type I refers to the adhesion of two biological substrates (e. g. cell aggregation), type II refers to the adhesion of a biological substrate to an artificial material (e. g. barnacle adhesion to a rock surface) and type III refers to the adhesion of artificial substances to biological substrates (e. g. the adhesion of polymers to mucosal epithelial). Type III bioadhesion has been investigated most by many research groups, including Robinson's group and Peppas's group and

others (Robinson and Gauger, 1986; Robinson, 1990; Peppas, 1983; Peppas and Buri, 1985; Peppas and Robinson, 1995).

1.2.1. Rationale for using bioadhesives

Presently, extensive research has been done to investigate the mechanisms of bioadhesion, and to understand the phenomena at the molecular level (Kamath, et al., 1995). Mucus is the major factor in bioadhesion and has been studied thoroughly. Mucus usually refers to the layer covering the mucosa. The composition of mucus varies widely depending on animal species and anatomical locations. The mean thickness of the mucus layer varies from 50 to 450 μm in humans and about half of this thickness in the rat, depending on the site of location (Allen, et al., 1985). It is secreted by the goblet cells in the epithelium or by special exocrine glands. Mucus is a translucent and viscid secretion, which forms a thin, continuous gel and adheres to the epithelial surface to protect the mucosa from external environment. The major components of mucus are high MW glycoproteins attached to oligosaccharide units. These units contain an average of about 8-10 monosaccharide residues of five different types, and many of the terminal residues in the oligosaccharide side chains are sialic acid. Sialic acid has an axial carboxyl group which is an important source of negative charge for most mucus glycoproteins. Mucus is negatively charged at neutral pH and not charged at acidic pH. Numerous hydroxyl groups of carbohydrates on mucin molecules have the potential to interact with other polymers that can form hydrogen bonds (Johnson, et

al., 1972 and Kamath, et al. 1995). Mucus also contains other fractions such as fatty acids and lipids, which aid in its protective function (Murty, et al., 1984).

1.2.1.1. Mechanisms of bioadhesion

The characteristics of mucus, such as negative charge and potential to form hydrogen bonds, are of great importance in promoting bioadhesion. Theories have been proposed to explain the bioadhesion phenomena which include electron transfer (Derjaguin, 1965), wetting (Baier, et al., 1968), diffusion (Peppas, et al., 1983 and 1985), adsorption (Tabor, 1977), fracture and mechanical interlocking (Ponchel, et al., 1987 and Mikos, et al., 1988). Although these theories have provided some insights, no single theory can completely account for the bioadhesion phenomena and a combination of one or more factors are involved in the actual process.

The adhesion between mucus and bioadhesives also has been analyzed based on molecular interactions, which provide further insights into bioadhesion. The interaction between two molecules is composed of attraction and repulsion. Attractive interactions arise from Van der Waal forces, hydrogen bonding, electrostatic attraction and hydrophobic interaction. Repulsive interactions occur because of electrostatic and steric repulsion. The magnitude of these two phenomena would determine how the molecules would bind. For bioadhesion to occur, the attractive interaction should be larger than nonspecific repulsion (Kamath, et al., 1995).

1.2.1.2. Advantages of bioadhesion

Since most of the administration routes, such as oral, nasal, buccal, ocular, rectal, and vaginal, are coated with the mucus layer, bioadhesive drug delivery system (BDDS) would be useful when applied at any of these mucosal membranes. Bioadhesives may be able to delay the transit of pharmaceutical dosage forms and increase the intimacy through their interaction with either the mucus or mucosa of the absorption sites (Veillard, 1990). Therefore, bioadhesion could overcome several problems of the traditional CDDS. For instance, the extent of drug absorption from traditional CDDS is limited by the residence time at the absorption site. As for orally administered dosage form, absorption is limited by the GI transit time of the dosage form. In addition, many drugs are absorbed only from the upper part of the small intestine. Localizing oral drug delivery systems in the stomach or in the duodenum would significantly improve the extent of drug absorption. Bioadhesives may localize drugs in a particular region and prolong the residence time, thereby improve the bioavailability of those drugs with low bioavailability problems.

In addition, strong interaction between bioadhesives and mucus on the tissue provides intimate contact between a dosage form and the epithelium which may result in high drug concentration in the local area. This intimate contact may increase the local permeability of poorly absorbed drugs by loosening tight junction or altering apical membrane of the epithelium (Harris, et al., 1990). Also,

bioadhesives may inhibit the enzymatic metabolism in a localized area, and protect the drug locally (Robinson, 1990 and Luessen et al., 1997).

The above advantages put together seem very valuable for promoting the absorption of drugs that are poorly absorbed including macromolecules and peptides at mucosal sites.

1.2.2. Factors influencing bioadhesion

There are many factors that are important for the interaction between the bioadhesive and the mucus, which determine the strength and the extent of bioadhesion. These factors are described below.

Initial contact time and initial pressure

The initial contact time and the initial pressure between a bioadhesive and the mucus layer determine the extent of swelling and interpenetration of polymer chains. They can dramatically affect the performance of the bioadhesive. In general, the bioadhesive strength increases as the initial contact time increases. If a high pressure is applied for a sufficiently long period of time, polymers become bioadhesive even though they may not have attractive interactions with mucus (Harris, et al., 1990). It is easy to control the initial contact time and pressure when bioadhesives are applied to exposed areas such as nose and mouth. However, for the application of bioadhesives to the GI tract, the initial contact time cannot be controlled which is one of the difficulties in applying bioadhesives to the GI tract.

pH

pH is one of the important factors that affect bioadhesive strength, especially for polycationic bioadhesives, since mucus is negatively charged at neutral or alkine pH. Because of the strong interactions between oppositely charged electrolytes, polycations can be excellent bioadhesives at neutral pH. At low pH mucus is not charged and polycationic bioadhesives are far less effective. However, at low pH, polyanions are excellent bioadhesives due to their ability to form hydrogen bonds with numerous carbohydrate hydroxyl groups of mucin molecules.

Extent of hydration

The extent of hydration of the dosage form is related proportionally to the strength of bioadhesion. Hydration of the dosage form occurs by migration of water from the surrounding medium through the peripheral part of the dosage form. It is assumed that the greater the surface area of a dosage form, the greater the dosage form's bioadhesive strength. Therefore, it is better to have a lot of smaller microgranules or minitablets than a large size, thin tablet. On the other hand, over hydration will weaken the strength of the bioadhesion (Duchene, 1988). Thus, the extent of hydration should be monitored and controlled.

Mucus turnover

The natural turnover of mucus at the mucosal site is possibly the biggest

barrier to BDDS. The mucus turnover limits the residence time of the bioadhesives on the mucus layer. No matter how high the bioadhesive strength, bioadhesives are sloughed off from the surface while mucus turnover happens. The turnover rate may be different in the presence of bioadhesives, but no information is available on this aspect. Mucus turnover leaves substantial amount of soluble mucin molecules on the epithelial membrane, which interact with bioadhesives before they have a chance to interact with the epithelial membrane. Mucus layer is very viscous and most bioadhesives can not penetrate this protective layer to interact with the epithelial membrane. This is why almost all the bioadhesives used now are actually mucoadhesives. From a few assumptions and experimental measurements of mucus output using total hexose of the perfusion solution, Allen et al (1985) calculated a mucin turnover time of 47-270 min. The ciliated cells in the nasal cavity are known to transport the mucus to the throat at a rate of 5 mm/min (Gandhi, et al., 1988). The mucociliary clearance in the tracheal region has been found to be in the range of 4 to 10 mm/min (Murty, et al., 1984). Peppas et al (1995) reported that residence time of the drug dosage forms for all mucosal routes was typically less than an hour unless some form of adhesive was employed as part of the drug delivery system.

GI motility and transit

Oral administration is one of the most sought routes since it is the most convenient administration route but it also has the most complicated biological

environment. As for oral dosage forms, GI motility and transit are very important which control the residence time and the absorption of the drug. They also affect the bioadhesion. GI motility is under both nervous and hormonal control. Gastric emptying in the fasted state has a pattern termed the "migrating myoelectric complex" (MMC) which can be divided into four discrete phases of motility. The intensity of contractions (potential for gastric emptying) increases from almost zero in Phase I, to moderate in Phase II (mixing contractions), to very high in Phase III. The force of Phase III is strong enough to clear all indigestible materials from the stomach and the small intestine, but it lasts only for a short duration. Phase III is also called the housekeeper phase. Phase IV is the transitional period between Phases III and I. A complete cycle of these four phases has an average duration of 90-120 min. This cycle of motility repeats itself until food is ingested, after that the stomach returns to its fed-state pattern of motility. Once in stomach the food goes through fed state, and the time for gastric emptying is about 2-6 h (Chien, 1992; Helliwell, 1993).

The presence of the MMC has important implications for the oral delivery of BDDS. BDDS would need to be administered on an empty stomach to prevent adsorption to any ingested food. Ideally, one would hope to abtain attachment of the BDDS to the mucosa of the stomach during Phase I of its cycle, at least initially, so that intimate contact could be achieved with the resultant formation of bioadhesive bonds. The high motility pattern of the stomach during Phase III of the MMC might not allow strong enough bioadhesion to occur if this was in operation during drug

administration. The optimal bioadhesive should be capable of adhering to the mucosal membrane strongly enough to withstand the shear force produced in the Phase III. However, the currently used bioadhesives can not meet such requirements. It has been suggested that it may be prudent to search for polymers that are able to induce a fed-like pattern of motility to the stomach in order to delay gastric emptying and hence, increase dosage form transit time (Gruber, et al., 1988).

The MMC also exists in the small intestine (but not in the large intestine), and each cycle lasts for up to half-an-hour longer than that experienced in the stomach (Chien, 1992). Motility within the small intestine takes the form of segmentation of mixing contractions and propulsive or peristaltic contractions.

The transit of pharmaceutical dosage forms along the GI tract has been extensively studied with the aid of gamma scintigraphy (Davis, et al., 1989; Roca, et al., 1991; Blok, et al., 1991 and Coupe, et al., 1992). The gastric emptying of doasge forms has been reviewed by Meyer (1989). It is evident that the presence of food in the stomach delays the emptying of dosage forms; the extent to which it is achieved depends on the food type. If the stomach is in Phase III of the MMC, then the dosage form may be ejected after only a few minutes. Most research on this topic has attempted to correlate the size of the dosage form with gastric emptying, but, no firm conclusions have been drawn yet. The maximum threshold size for gastric retention has been shown to range from 3 to over 10 mm, and hence it can only be stated that size has some poorly characterized effect on gastric emptying.

Small intestinal transit of dosage forms has been reviewed by Gupta and Robinson (1991). Briefly, in all instances the transit time of the small intestine is 3-4 h. It also has been reported that during the MMC, solid dosage forms would reach the ileocaecal region from the pylorus in just under 2 h, compared with up to 6 h while in the fed state pattern of motility (Davis, 1989). Small intestinal transit is relatively constant and seems to be unaffected by pharmaceutical factors such as dosage form type, physiological factors such as age and exercise, and phathological conditions such as ulcerative colitis. In summary, the total transit time of foods and dosage forms in humans from stomach to the ileocecal junction is approximately 3-6 h in the fasted state and 6-10 h in the fed state. This sets an approximately 10 h limit for the delivery of drugs absorbed solely from the small intestine region (Davis, 1989 and Chien, 1992).

Transit through the colon is highly variable and depends to some extent on the rate of gastric emptying. Colonic transit has been reviewed by Hardy (1989). The conclusions are listed as following. (i) Transit time for tablets and capsules is 20-30 h, although much wider ranges have been reported. (ii) Transit time for solutions and microparticulates is about 30-40 h, which represents the greater spreading of such dosage forms in the ascending colon. (iii) Colonic transit is longer in females than in men, but it is generally unaffected by age in the range 20-70 years. It was concluded that the range of transit times experienced in the colon must be taken into account during the administration of the formulations intended for drug delivery to the colon.

1.3. Current research in drug delivery using BDDS

1.3.1. General information

The first generation of bioadhesives has been available for some time. At the very beginning, bioadhesion dealt mostly with the buccal administration of solid dosage forms and especially tablets (Nagai, et al., 1990). This was due to the easy accessibility of the mucosa, and the simple process of tablet manufacture. This concept rapidly led to the idea that bioadhesion could be used advantageously to improve drug absorption through other administration routes, such as the nasal, ocular, vaginal and colonic routes (Duchene, et al., 1988; Saettone, et al., 1989 and Hou, et al., 1985). A significant progress has occurred in this field and a better understanding of the mechanism of interaction between the bioadhesive and the mucosa has resulted in the targeting of polymers to specific receptors (Peppas, 1995).

Each administration route has its advantages and drawbacks. For instance, administration of drugs by buccal route helps in avoiding destruction of drugs by the GI fluids and bypassing hepatic first pass metabolism, but it is difficult to retain the dosage form in the mouth for prolonged period without swallowing it. Rectal administration of drugs, for systemic treatment, may have improved absorption and higher plasma concentration due to avoidance of hepatic first pass but retaining at the absorption site for a prolonged period is a major concern. For all these routes such as buccal, nasal, rectal, vaginal and oral, it is desirable that the dosage form

is not eliminated prematurely from the absorption site. Bioadhesives may provide the possibility of retaining the dosage forms at the absorption sites and prolonging the residence time.

In recent years, nasal administration of drugs has achieved great success as an alternative to oral route. Commercially available products that are given via the nasal route include LHRH, vasopressin and analogues, and calcitonin. However, mucus turnover in the nasal cavity is still a major limitation for sustained nasal drug delivery. Some bioadhesives such as microcrystalline cellulose, neutralized carboxymethylcellulose and chitosan, have been employed to improve the residence time of the drug on the absorption site. Use of bioadhesives in nasal drug delivery in prolonging the residence time showed great potential. Few polymeric bioadhesives such as chitosan also exhibited permeability enhancement (Nagai, et al., 1984 and Illum et al., 1994a). In vaginal administration, a non-hormanal drug-free bioadhesive vaginal moisturizer, Replens®, has been used to treat the vaginal dryness. Replens® has significantly improved therapeutic effect and patient compliance compared to estrogen vaginal cream (Nachtigall, 1994).

As eluded above, GI transit and degradation limit the usefulness of orally administered drugs. Such limitations have led to several approaches such as the incorporation of drugs into bioadhesive polymers for the preparation of oral BDDS. For water soluble drugs it is possible to use bioadhesive polymers to coat the surface of the dosage form, either totally or partially, which results in the prolonged retention on the mucosa, and the release rate of the drug is controlled by the

dissolution rate across the polymer used. For sparingly water soluble drugs, a gelling polymer can be used to trap the drug which would result in a slow release of drug because of reduced diffusion. Drugs also can be directly dispersed into a bioadhesive polymer to form a matrix type drug delivery system, or the matrix formulation can be further coated by a bioadhesive with a similar or different structure (Chien, 1992). Experiments in rats showed that bioadhesive polymers prolonged stomach emptying and increased the residence time of the drug in stomach. Some bioadhesives, such as polyacrylic polymers, are also speculated to inhibit the proteolytic activity of trypsin and carboxypeptidase A. Therefore, they are expected to improve oral absorption of drugs including peptides and proteins (Ch'ng, et al., 1985; Luessen, et al., 1993 and 1994).

1.3.2. Some commonly used bioadhesives

Earlier work examined binding of a broad spectrum of polymers to mucin-epithelial cell surfaces. The studies indicate that in general, anionic polymers are preferred over neutral or cationic polymers because they are relatively non-toxic and are suitable to form strong adhesion through hydrogen bonds. Water-insoluble polymers provide greater flexibility in dosage form design as compared with rapidly or slowly dissolving water-soluble polymers (Park, et al., 1984). Polycations are believed to interact with membrane-associated anionic sites to induce morphological alterations that could result in the loss of membrane integrity and cellular rigidity (Quinton, 1973).

It has been suggested that polyanions with a high charge density are highly active bioadhesives (August, et al., 1988a). Various polyanions have been evaluated, and the polymers containing carboxylic functional groups, such as polyacrylic polymers, show very high levels of bioadhesion (Scheuplein, 1966). Table 1 lists some commonly used pharmaceutical excipients in relation to their bioadhesive properties (Chien, 1992).

Hydrophilic polymers, such as carboxymethylcellulose (CMC) and hydroxypropylcellulose (HPC), are commonly used in pharmaceutical industry. Besides other polymeric properties, they have very good bioadhesive property. In the table presented above, they are on top rank of bioadhesive strength. They also have been used in combinations, such as a mixture of Carbopol® and HPC (Nagai, 1985), combinations of CMC, pectin and gelatin (Ranga Rao and Buri, 1989), etc.. Presently polyacrylic polymers (e.g. polycarbophil and Carbopol®) have been widely studied as model bioadhesives. *In vitro and in vivo* tests showed that polycarbophil and Carbopol® had very high bioadhesive strengths (Smart, et al., 1984 and Anlar, et al., 1993) and could inhibit the intestinal proteolytic enzyme trypsin (Luessen, et al., 1997). Harris et al (1989) investigated the feasibility of using bioadhesive polymers to extend the GI transit time in rats and humans. The results obtained in rats indicated that among the investigated polymers, polyacrylic polymers, such as polycarbophil and Carbopol®, had potential in delaying GI transit. However, the major delay was due to a decrease in the gastric emptying time. Hydrogels, such as Carbopol® 934p, may be useful for formulation of a sustained-release nasal

delivery system since they can achieve a long-term intimate contact with the nasal mucosa and thus provide a prolonged therapeutic effect without suppressing mucociliary functions (Zhou, 1996).

Gums, alginate, carrageenan, gum arabic and pectin usually form a mucilaginous solution in water and become sticky. They are another group of bioadhesives with good bioadhesive properties. Alginates have been studied extensively because they can form hydrogel.

Table 1. Relative mucoadhesive performance of some potential and commonly used bioadhesive pharmaceutical polymers

Polymers	Relative mucoadhesive force[a]	Qualitative bioadhesive property[b]
Carboxymethylcellulose	193	Excellent
Carbopol®	185	Excellent
Polycarbophil	—	Excellent
Tragacanth	154	Excellent
Na Alginate	126	Excellent
HPMC	125	Excellent
Gelatin	116	Fair
Pectin	100	Poor
Acacia	98	Poor
Providone	98	Poor

[a] Percentage of a standard, tested *in vitro* (Smart, et al., 1984)

[b] Assessed *in vivo* (Satoh, et al., 1989)

1.3.3. Some novel bioadhesive polymers

Although hydrogels and hydrophilic polymers have been traditionally thought of as good bioadhesive systems, new results suggest that novel materials with greater bioadhesive properties may exist (Lehr, et al., 1993). The results obtained by Chickering (1995) group using a novel electrobalance-based method showed that one group of bioerodible polyanhydrides (co-polymers of fumaric acid and sebacic acid) produced strong bioadhesive interactions in *in vitro* experiment, compared to other thermoplastic and hydrogel materials. These findings strongly supported the existence of bioadhesion between soft tissue and hard, bioerodible, thermoplastic polymers. Correlation between the *in vitro* findings and their *in vivo* performance is still under investigation.

1.3.4. Development of bioadhesive formulations in the market

At present, several drugs in the market have bioadhesive formulations. They are listed in Table 2.

Table 2. Some bioadhesive formulations in the market (Kamath, 1995)

Name and Form	Drug	Mucoadhesive	Application Site
Aftach tablet	Triamcinolone Acetonide	Hydroxypropyl cellulose, Carbopol 934	Oral Cavity
Susadrin tablet	Nitroglycerin	Synchron (modified HPMC)	Buccal
Buccastem tablet	Prochlorperazine maleate	Ceratonia, Xanthan gum	Buccal
Salcoat powder spray	Beclomethasone dipropionate	Hydroxypropyl cellulose	Oral cavity
Solcoseryl paste	-		Gingival
Orabase gel	-	NaCMC, pectin, and gelatin in polyethylene-mineral oil base	Oral cavity
Orahesive bandage	-	NaCMC, pectin, and gelatin in poly-isobutylene spread onto polyethylene film	Oral cavity
Rhinocort powder spray	Beclomethasone dipropionate	Hydroxypropyl cellulose	Nasal
Replens gel	-	Polyacrylic acid	Vaginal
Sucralfate	Aluminum hydroxide	Sucorse octasulfate	GI tract ulcers

1.3.5. Oral drug delivery using novel bioadhesives

Most of the currently used bioadhesive polymers can not achieve the desirable prolonged residence time by oral route. Hence, research groups are exploring new bioadhesive candidates, which have site-specific adhesion property, to meet this challenge. In the last few years, lectins have attracted the interest of pharmaceutical scientists because of their ability to accomplish specific binding to membrane bound sugar moieties located at the surface of epithelial cells. Another advantage of lectins is their generally good resistance to digestion within the GI tract (Haltner, et al., 1997). Several lectins have already been investigated in the light of possible pharmaceutical applications (Florence, et al., 1995, Lazarova, et al., 1993 and Irache, et al., 1994). Tomato lectin, a non-toxic dietary glycoprotein of molecular mass 71 kDa, has been studied thoroughly by Naisbett et al (1994a, 1994b and 1995) in both *in vitro* and *in vivo* experiments. The *in vitro* results showed very exciting promise that tomato lectin bind with the enterocyte cell surface due to the specific lectin-sugar interaction. However, the *in vivo* results did not correlate with the *in vitro* results, and this might be due to interactions of tomato lectin with intestinal mucus limiting its use as an intestinal bioadhesive. Other *in vivo* experiments are under progress.

The concepts of specific endo- and transcytosis of bioadhesive ligands have been introduced into this field. These include receptor-mediated endo- and transcytosis, adsorptive endo- and transcytosis mediated by electrostatic (charge-mediated) and sugar-specific (lectin-mediated) interaction. As a major difference to

the previous attempts to nonspecifically increase the permeability of an epithelial barrier in general, such specific, receptor-mediated absorption enhancement are expected to affect only the substrate concerned, whereas the barrier function of the epithelium against all other solutes remains intact. The uptake of Vitamin B12 is receptor-mediated transcytosis through epithelial cells. Russel-Jones and de Aizpurua (1988) prepared conjugates of Vitamin B12 and an analogue of luteinizing hormone-releasing hormone (LHRH). The oral administration of this conjugate to mice stimulated ovulation in developing follicles whereas the free hormone did not. This result suggested that the Vitamin B12 pathway provided a means to deliver macromolecules at physiologically relevant quantities. However, whether the total capacity of this transport mechanism will be sufficient for systemic drug delivery or merely for the delivery of trace amounts of macromolecular antigens still remains to be proved (Lehr, 1994).

1.3.6. Colonic drug delivery by oral route

The delivery of drugs to the colon for systemic action or a local effect is valuable in a variety of circumstances. Many studies indicate that due to the lower proteolytic activity in the colon compared to the small intestine, colon-specific drug carriers may potentially be used for the delivery of peptide and protein drugs (Rubinstein, et al., 1997).

Several bioadhesives, such as polycarbophil, Carbopol® 934 and pectin, have been investigated for drug delivery to the colon. Besides having bioadhesive

properties, the loosely crosslinked acrylic polymers Carbopol® 934 and polycarbophil have been shown to inhibit enzymatic degradation (Luessen. et al., 1993 and 1994). However, a major drawback of polycarbophil and Carbopol® 934 is their extremely high swelling properties, which cause them to disperse in aqueous solutions within several minutes (Luessen. et al., 1994). A possible outcome of these studies is that a core tablet made of polycarbophil or Carbopol® 934, coated with a colon-specific biodegradable polymer, could be an efficient delivery system for peptide and protein drugs to the colon.

The natural polysaccharide pectin or its low water soluble product, calcium pectinate, is non-toxic, almost totally degraded by colonic bacteria and is not digested by gastric or intestinal enzymes (Ashford, et al., 1993a, 1994 and Rubinstein, et al., 1995). It has been demonstrated that compression coats of pectin in human (Ashford, et al., 1993b) and calcium pectinate in dogs (Rubinstein, et al., 1995) were able to retard the release of model drugs long enough for the drugs to arrive to the large intestine. In this research project we have explored the possibility of using alginate and pectin together to benefit from both their properties.

1.4. Bioadhesive particulate system

Much advancement in drug delivery could come from improvement of the existing systems. The concept of combining the advantages of particulates and bioadhesive technologies in one delivery system is attractive because of its potential capability of delivering peptide and protein drugs (Ponchel, et al., 1997). While the

bioadhesive part of the formulation could enhance the peptide delivery by prolonging its retention, localizing the drug at the site of absorption and intensifying the concentration gradient (Lehr, et al., 1994), the particulate part of the formulation could act as a carrier for the drug and protect it from the harsh physiological environment.

Attempts to use bioadhesive particulates to deliver drugs including peptides and proteins have been made by some groups such as Illum group and Mathiowitz group (Chickering et al., 1997), and the results showed some promise for the drug delivery. For instance, for the development of bioadhesive particulate systems, albumin, starch and diethylaminoethyl (DEAE)-dextran were used as the formulation base and administered by nasal route. This study demonstrated that it is possible to control the release of drugs from the bioadhesive particulate system. The results showed that the particulate prepared from starch and DEAE-Sephadex® were effective in delaying nasal muco ciliary clearance (Illum, et al., 1987). Illum group (1994) also reported that hyaluronic acid ester microspheres significantly enhanced the intranasal absorption of insulin in sheep, and bioadhesive starch microspheres, especially in combination with an absorption enhancer, lysolecithin, had a profound effect on the absorption of desmopressin in sheep, with bioavailabilities reaching nearly 10% compared with 1.2% for a simple nasal solution of desmopressin.

Likewise, a microcapsule system (100 - 500 μm) with a bioadhesive coating that consists of polyhydroxy methcrylate (p-HEMA) and a bioadhesive coating, Carbopol® 943/Eudragit® RL-100 (9:1) blend has been developed. This system was

examined and found to substantially increase the mean residence time in the GI tract by two-fold when compared to uncoated microcapsules. The adhesive interaction was also studied and the results showed that the bioadhesive coated microcapsules clearly adhered to the mucosal tissue, whereas the non-coated microcapsules circulated freely in the solution (Chien, 1992).

Investigation of bioadhesive polymer polycarbophil-coated albumin beads indicated that the gastric retention time was substantially prolonged by the bioadhesive coating. Therefore, the systemic bioavailability of the incorporated drug was significantly improved with an increase in the drug plasma concentration (Longer, et al., 1985).

Besides, some other bioadhesive particulate systems have been developed such as poly (fumaric-co-sabacic anhydride) microspheres, etc. However, these methods either use organic solvent during preparation or have complicated preparation procedures, which are incompatible with many labile drugs including the peptide and protein drugs. The methods involving organic solvent also have the possibility of residual toxicity due to trace amount of organic solvent left in the preparation.

Anionic polysaccharide ALG and cationic polysaccharide chitosan, used for various purposes in pharmaceutical drug formulations due to their good biocompatibility, biodegradability and low toxicological properties, have good bioadhesive properties (Table 1 and Smart, et al., 1984). There is a relatively simple procedure for the preparation of both ALG and chitosan particulates since they can

form the hydrogel by ionotropic interactions. The procedure is performed in a mild aqueous environment which would be more suitable for carrying labile drugs including peptide and protein drugs (Bodmeier, et al., 1989, 1990 and 1994). However, ALG particulates are fragile in the presence of monovalent cations (e.g., sodium ions), and chitosan particulates are fragile in the presence of monovalent anions (e.g., chloride ions).

Alginate-poly-*l*-lysine-alginate (ALG-PLL-ALG) microcapsules containing bioactive cells have been reported and patented to be used for transplantation (Lim, et al., 1980 and Uludag, et al., 1993). Calcium ions cross-link the ALG species, initially in solution as the sodium salt, and form the calcium-ALG hydrogel. The addition of an oppositely charged polyelectrolyte (e.g. PLL) allows a layer of insoluble polyelectrolyte complex to form at the interface of the hydrogel. Finally, the calcium ions remaining in the interior of the microcapsule are exchanged with an excess of sodium ions in diluted sodium ALG solution, rendering the interior fluid again. The microcapsules are formed under mild conditions (e. g., no extremes of pH, no chemical reactions). Their ability to entrap the cells also makes them an attractive candidate for controlled-release dosage forms.

Pectin has been used to coat the tablets due to its characteristics of forming insoluble pectate in the presence of calcium ions, and this formulation has been used to deliver the drug to the colon. The experimental results showed that drug could reach the colon after administered this dosage formulation (Ashford, et al., 1993).

As eluded earlier all of these particulates individually have limited utility due to either lack of robustness or inability to control drug release. Hence, we have attempted to combine the properties of alginate, PLL and pectin in preparing a novel particulates that is robust, able to sustain drug release and has bioadhesive property.

1.5. Test methods used to study bioadhesion

The main purpose of bioadhesives is to secure the dosage form to a certain site in the body, thereby increase the drug absorption. Bioavailability can be used as an ultimate parameter to test the performance of bioadhesives. However, *in vivo* bioavailability test is very expensive and time-consuming, and therefore cannot be used as a routine method to prescreen the bioadhesive properties of various polymers. For this reason, many investigators proposed and used simple test methods, such as measurement of residence time, measurement of *in vitro* adhesive strength, etc., to study the bioadhesive properties. These tests are very useful and necessary because they can not only screen a large number of bioadhesive candidates, but also help in elucidating the mechanisms of bioadhesion without affecting the complicated physiological factors. Some of the commonly used methods for BDDS study are briefly described below.

1.5.1. *In vitro* test methods

In vitro tests are preformed in controlled environments and some of them

might bear no relationship to the ultimate performance of the BDDS. Nevertheless, *in vitro* tests provide useful and preliminary information of the dosage form and can be correlated to *in vivo* situation to some extent.

1.5.1.1. Adhesive strength tests

Adhesive strength tests include tensile, shear and peel strength. Of these, and tensile strength test is the most commonly employed *in vitro* test. It measures the weight per area or the force required to detach a bioadhesive substance from a biological substrate, such as a mucosal epithelium (Smart, et al., 1982 and 1984; Peppas, et al., 1985; Park, et al., 1985; Ponchel, et al., 1987; Leung, et al., 1988; Robert, 1988; Lehr, et al., 1990; Bottenberg, et al., 1991 and Smart, 1991). Briefly, the bioadhesive under examination is attached to the under-surface of a solid support, which is connected to a torsion balance via a pulley. The test bioadhesive is lowered onto the biological substrate and left for a predetermined period to allow interaction between the material and the biological tissue. After this period, the solid support is raised at a constant rate until total detachment occurs and the maximum weight required indicates the adhesive strength of the test sample. Numerous modifications of the standard have been reported to be used for this test. Since, there is no criterion for this test, different equipments give different values of the adhesive strength.

1.5.1.2. Perfusion tests

Falling liquid-film test (Teng, et al., 1987; Rao, et al., 1989 and Pimienta, et al., 1990) is one of the perfusion tests measuring the adhesion of particulate formulations. It involves placing the test material onto a section of excised stomach or intestine, mounted on an inclined platform and washing it with simulated stomach or intestinal solutions. The eluted particles are collected and quantified. The percentage of the particles adhering to the tissue is determined and used as an index of bioadhesive strength.

Another perfusion technique is similar to the falling liquid-film test except that an entire segment of intestine is used rather than a part of the intestine. The radio-labeled bioadhesive formulation is allowed to interact with the tissue for a period of time and then the perfusion is commenced. The radioactivity of the eluted samples is determined, which is used as an index of bioadhesion (King, et al., 1991).

1.5.1.3. Rheological tests

Rheological evaluation of mucus and polymer mixtures gives information on the extent and magnitude of interaction between the two, since the increase in viscosity resulting from mixing the two has been claimed to correlate with bioadhesion (Allen, et al., 1986; Hassan, et al., 1990). As with other experimental techniques, here also the *in vitro - in vivo* correlation is questionable. Besides the methods mentioned above there are other methods which have been investigated. Some methods such as the fluorescent probe technique (Park, et al., 1984) and the

colloidal gold technique (Park, 1989) do not bear much resemblance to bioadhesion within the GI tract and will not be discussed here.

1.5.1.4. Cell culture study

Conducting studies to evaluate the *in vivo* oral absorption of test substances is expensive, time consuming and labor intensive. Use of various epithelial cell lines as model membranes to study drug absorption is well accepted in the scientific world for preliminary screening of drugs and excipients since the results obtained are close to *in vivo* tests (Brayden, 1997). One could choose human cell lines to mimic human conditions to study aspects covering toxicity, absorption, mechanism of transport of the compounds, etc..

1.5.1.4.1. Cytotoxicity study

Cell lines can be used to screen a preliminary cytotoxicity of the test compounds. Assays for cell viability (e.g. the MTT-transformation, the release of Lactate Dehydrogenase, Propidium Iodide Staining and Trypan blue test, etc.) and transepithelial electroresistance (TEER) can be used to assess the cytotoxicity of the compounds (Anderberg, et al., 1992 and Werner, et al., 1996). These results can be used as predictions for further studies to be done in animal models.

1.5.1.4.2. Drug transport study

Cell lines such as Caco-2 and HT29 have been suggested for screening drug

absorption at the early stage of drug development. Several advantages of applying them to pre-clinical studies have been suggested by many investigators including, the fact that they are human original, provide a high degree of reproducibility and a means of rapid assessment of the permeability of a drug (Hilgers, et al., 1990; Wilson, et al., 1990; Hillgren, et al., 1995 and Sattler et al., 1997). Drug transport study is carried out using either Transwell® insert plates or modified Ussing diffusion chambers. Drug candidates are usually added to the donor compartment in the form of a radio-labeled or fluorescent molecule and detection is made by sampling both donor and receiver compartments to determine the amount of the drug transported across the monolayers.

1.5.1.4.3. Mechanisms of transport study

Many researchers have used the different cell lines to decipher the permeation pathway of a specific candidate compound across the epithelium. The transport of paracellular tight junctional markers such as ^{14}C-mannitol or the TEER are usually used to monitor the tight junction of the epithelium. Apparent permeability coefficient (P_{app}) for ^{14}C-mannitol of about 0.8-2.5 X 10^{-7} cm/sec, and TEER of about 250-600 Ω cm^2, indicate intact epithelial tight junctions. If the flux of a drug candidate is found to be unsaturable, equal in both the donor and receiver sides, pH independent, and cause no effect on the flux of ^{14}C-mannitol or on the TEER, it indicates that the absorption mechanism of the drug is passive and transcellular. Agents causing a reversible increase in the flux of ^{14}C-mannitol with

an associated drop in TEER suggest that the tight junctional paracellular passive pathway may play a role in its permeation. If the flux of the agent follows an active, carrier-mediated pathway, then the concentration in the donor side is found saturable, pH dependent, and there is an unequal net flux in both directions (Brayden, 1997).

1.5.1.4.4. Caco-2 cell line

Caco-2 cell line is the most popular commercially available human intestinal cell line for drug absorption studies. The cells were derived from a human colonic adenocarcinoma which differentiate after 15 to 20 days on polycarbonate filters to express many carriers of the small intestinal villi such as those for dipeptides, bile acids and Vitamin B12. It has been widely used to study drug transport mechanisms (Artursson, 1990b and 1990c; Thwaites, et al., 1996; Rubas, et al., 1996 and Yamashita, et al., 1997) as well as absorption enhancement (Anderberg, 1992, 1993; Schipper, et al., 1996 and Werner, et al., 1996). Caco-2 monolayer has also received considerable attention from the pharmaceutical industry because the correlation between drug permeability on Caco-2 monolayer and *in vivo* drug absorption is very high (Artursson, et al., 1991). Yamashita et al (1997) provided a theoretical rationale illustrated in Figure 1 for using the cultured monolayer system to predict *in vivo* drug absorption. The jejunal membrane has a villous structure that increases the effective surface area of absorption. Drugs can enter the blood circulation *in vivo*, just after crossing the epithelial absorptive cell layer from

capillaries in the lamina propria of the villi (Trier, 1981). In contrast, *in vitro* permeation needs the diffusion of drugs from the mucosal to the serosal side of the membrane through the villi. This simple concept shown in Figure 1 is applicable not only to Caco-2 monolayer, but also to other monolayer systems and is very important when considering differences between the *in vitro* permeation and the *in vivo* absorption of drugs.

In our study, Caco-2 cell monolayer was used to carry out transport study to investigate the effect of the alginate and pectin on enhancing the permeability of the epithelial membrane.

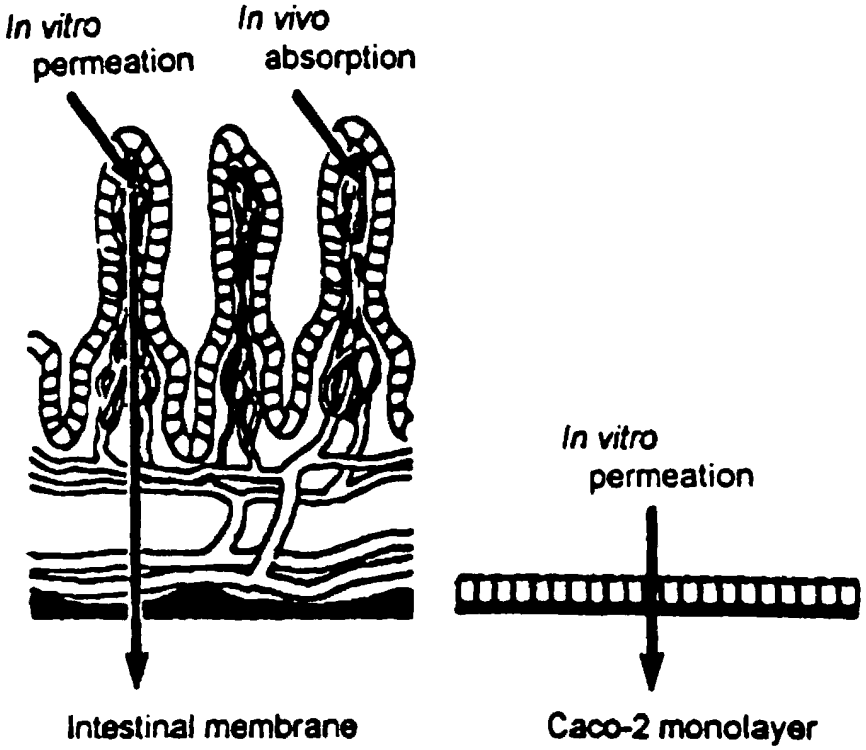


Figure 1. Schematic representation of *in vitro* permeation and *in vivo* absorption of drugs

1.5.2. *In vivo* test methods

1.5.2.1. Bioavailability studies

Some research groups have investigated the bioavailability of bioadhesive dosage forms using animal models or human volunteers (Longer, et al., 1985; Chickering III, et al., 1997 and Santus, et al., 1997). The results are controversial because some were opposite to the *in vitro* results and some did not show the same extent of improvement as that shown in the *in vitro* test due to complications in the *in vivo* situations. Especially for oral administration route, biological variables such as GI motility, mucus turnover, presence of endogenous materials (e.g. enzymes, electrolytes, bile salts) and exogenous materials (e.g. food, drink, drugs) are very difficult to mimic in an *in vitro* model.

1.5.2.2. Measurement of residence time

The three main techniques which have been used to measure the residence time of bioadhesives *in vivo* include gamma scintigraphy (Khoshla, et al., 1987; Santus, et al., 1997 and Jacques, et al., 1997), perfused intestinal loops (Lehr, et al., 1990 and 1991) and transit studies with radio-labeled dosage forms (Ch'ng, et al., 1985; Ponchel, et al., 1997 and Chickering III, et al., 1997). Although the *in vivo* techniques listed above may be able to indicate the prolonged retention of a BDDS at a particular site, they do not necessarily correlate with clinical efficacy of the incorporated drug. Hence, the extent of drug absorption can not be predicted from transit studies alone. Pharmacokinetic data can be obtained in conjunction with

each of these techniques which will provide more complete information about the BDDS.

1.6. Bioadhesive candidates

In our research project, initially we screened a number of polymers to make the final selection of the few polymers most suitable for our work. The following section describes the polymers selected for pre-screening.

1.6.1. Alginate (ALG)

ALGs are natural, non-toxic, polysaccharides found in the cell walls and intercellular spaces of brown seaweed. ALGs are necessary for plant growth in the sea to provide the plant with both flexibility and strength. They are widely used in food and pharmaceutical industries, such as disintegrant, tablet binder, thickening/stabilizing agents in mixtures and as gelling agents in confectionary. Recently they have been employed as a matrix for entrapment of drugs (Bodmeier, et al., 1989 and Rubio, et al., 1994), macromolecules (Mumper, et al., 1994 and Kim, et al., 1992) and biological cells (Lanza, et al., 1995). ALG acid is a linear copolymer of β-D-mannuronic acid and α-L-guluronic acid linked by (1→4)-glycosidic bonds. When producing ALGs, uronic acid is converted into the salt forms mannuronate (M) and guluronate (G) (see Figure 2a). It has been shown that the G and M units are joined together in blocks. Three types of blocks may be found, homopolymeric blocks MM and GG, heteropolymeric sequentially alternating blocks MG

(Yotsuyanagi, et al., 1991). The different block types are shown in Figure 2b. Regions of G monomers in one ALG molecule can be linked to a similar region in another ALG molecule by means of calcium or other multivalent cations such as aluminum and zinc (Figure 3a). The calcium cations fit into the G structures like eggs in an 'egg-box' (see Figure 3b). This binds the ALG polymers together by forming junction zones, thus leading to gelling of the solution. ALGs contain various proportions of M and G monomers, and gel formation depends on this distribution. To form a gel by reaction with calcium, ALG needs to contain a sufficient level of G monomer and a certain proportion of G monomers must be available in a block. The ALG containing the highest GG-fractions possesses the highest ability to form gels. The viscosity of an ALG solution depends on the length of the ALG molecules, such as the number of monomer units in the chains. The longer the chains the higher the viscosity at similar concentrations. We used sodium ALG with medium viscosity in our study.

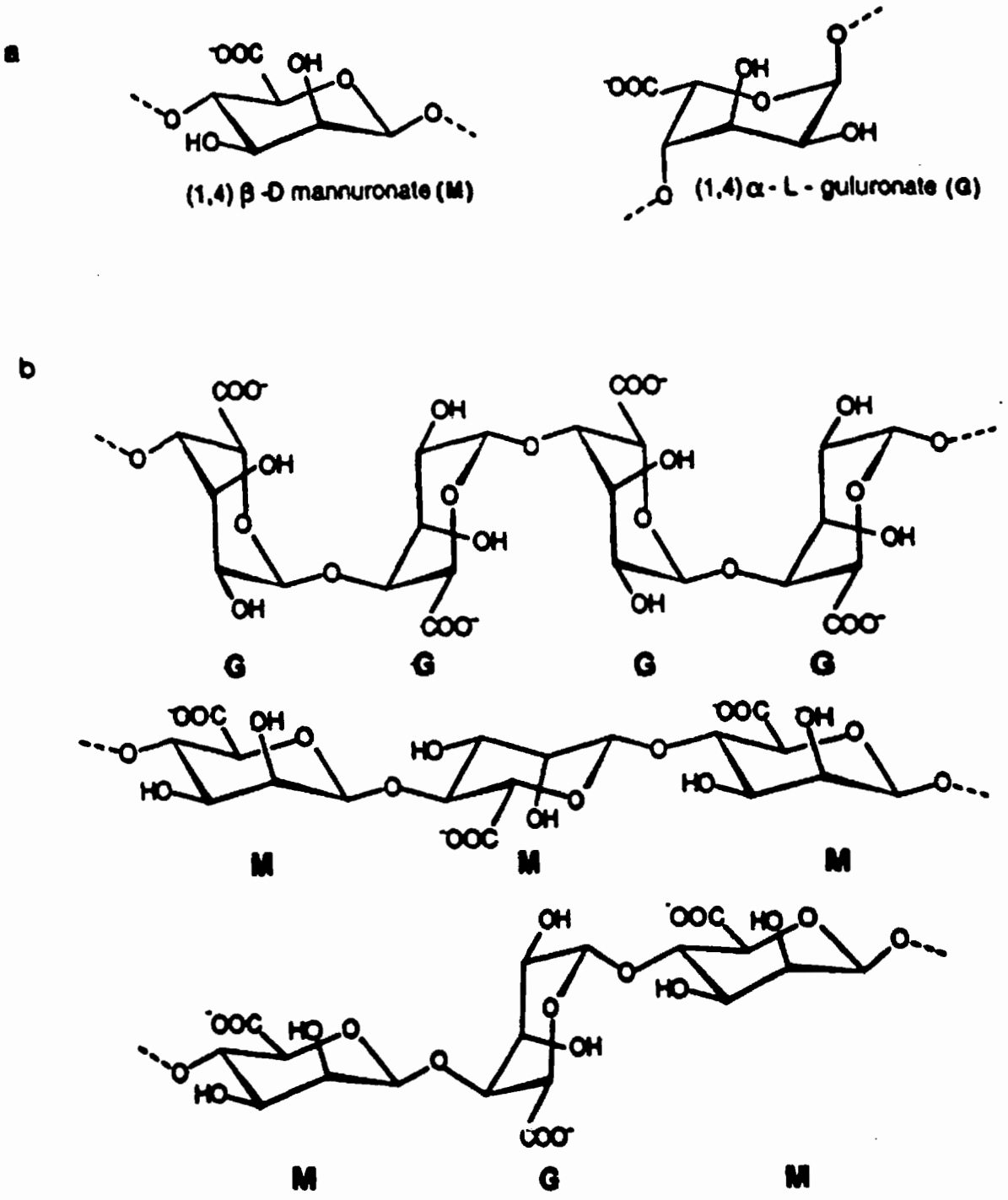


Figure 2. a) ALG block types; b) the monomers of ALG

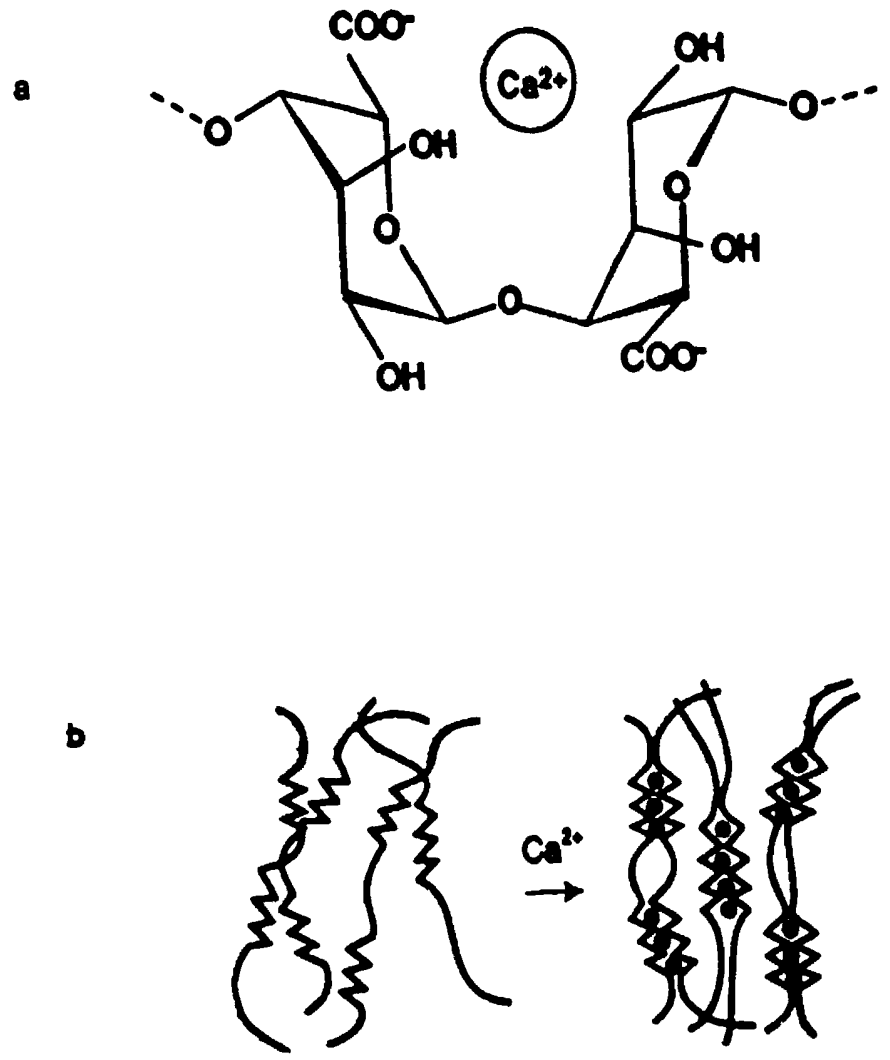


Figure 3. a)Calcium-bonding site in G-blocks; b)"Egg-box" model for calcium-ALG gel formation

1.6.2. Chitosan

Chitosans are biodegradable, high MW cationic polysaccharides. Industrially they are produced from chitin, the world's second most abundant biopolymer, by a deacetylation process involving alkaline hydrolysis. Chitosan has been used for a range of applications as diverse as for water purification, as a food ingredient and as a pharmaceutical excipient in oral drug formulations for the improvement of the dissolution of poorly soluble drugs or to obtain controlled drug release (Li, et al., 1992 and Hou, et al., 1985). It has been previously shown that chitosan has a great potential as a nasal delivery system, facilitating the passage of large hydrophilic molecules such as salmon calcitonin and insulin, through the nasal mucosa into the systemic circulation (Illum, et al., 1994a). The tests of *in vitro* tensile strength show that chitosan has a very strong adhesion strength (Smart, 1984). Cell culture study indicates that chitosan can improve the absorption of the drug significantly as an absorption enhancer (Schipper, et al., 1996).

1.6.3. Poly-*l*-lysine (PLL)

PLL, a lysine polypeptide or homopolymer, is cationic polysarccharide with adsorption characteristics on membranes. The chain length of the polymer depends on the method of preparation. PLL has been known to have ionic interaction with polyanions such as ALG. It has been used in the preparation of ALG-PLL-ALG microcapsules, and its primary function is to form a strong membrane complex to stabilize and strengthen the ionic hydrogel.

Due to its polycationic nature, PLL has been reported to stimulate endocytosis of different cell types (Duncan, et al., 1979 and Ginsburg, et al., 1984). Literature data shows that PLL is immunogenic. Therefore, ALG-PLL-ALG microcapsules have the outer coating of ALG (PLL-ALG) to neutralize the non-reacted PLL and generate a negatively charged surface to avoid attachment of cells to the capsule membrane. This is expected to mask the unwanted immune responses of PLL.

Shimi et al (1991) has examined the permeability, robustness and ultrastructure of ALG-PLL-ALG microcapsule made by PLL of different MW. The results indicated that PLL of low MW produced a relatively permeable and robust membrane whereas the high MW produced capsules with the reverse characteristics. A MW of 22,000 appears to be optimal in forming robust capsules which are relatively impermeable to high MW species such as immunoglobulins. Based on these reports, we selected the PLL with low MW (22,000) for our work.

1.6.4. Pectin (PEC)

PEC is a heterogeneous polysaccharide composed mainly of galacturonic acid and its methyl ester linked by α (1 -4) glycosidic linkage. It is nontoxic and used in many foods and confectionaries. It is resistant to gastric and intestinal enzymes (Sandberg, et al., 1981), but it is almost completely degraded by the colonic bacterial enzymes to produce a series of soluble oligogalacturonates (Cummings, et al., 1979 and Englyst, et al., 1987). It is isolated from plant cell walls by a number of processes to produce PECs with varying degrees of methoxylation. PECs with high

degrees of methoxylation (HM) are poorly water-soluble. However, PECs with low degrees of methoxylation (LM) are more water-soluble and can be cross-linked by divalent cations, most commonly calcium, to produce an insoluble pectate gel. We used PEC with LM for our study. PEC has been recently used as a matrix for oral sustained release tablet and as a carrier for colonic drug delivery due to its prolonged retention at colon (Ashford, et al., 1993b).

In our study we have used ALG as the core material for preparing drug-loaded particulates. The hydrogel structure of calcium-ALG is fairly labile in the presence of monovalent cations, and the gel structure could be strengthened by using different compositions of ALG, calcium ion concentrations, gelation time etc. (Bodmeier, et al., 1993; Kikuchi, et al., 1997). Attempts to strengthen ALG particulates by complexing with cationic chitosan (Murata, et al., 1996) or PLL (Thu, et al., 1996) have also been reported in the literature with varying degree of success. However, all these attempts only improved the release profiles of the drug from ALG particulates marginally. The difficulty in sustaining drug release is more for aqueous soluble drugs and those with smaller molecular weights, such as theophylline, acetaminophen and chloramphenicol (Østberg, et al., 1994). We aimed to develop a novel bioadhesive particulate system, using ALG with the help of PLL and PEC strengthening the partiuclates, to allow controlled release of small molecular weight drugs from the particulates.

1.7. Model drugs

Conventional drug with molecular weight less than 500 were chosen for this project. The three model compounds chosen (Figure 4) had a wide range of solubility to represent a wide range of compounds available in the market.

1.7.1. Theophylline

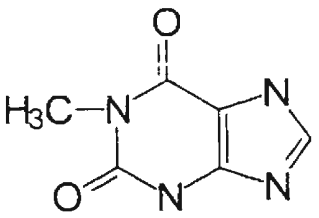
Theophylline is widely used in the treatment of asthma and other respiratory diseases. It has a narrow therapeutic window, and it is rapidly absorbed and eliminated. These attributes make theophylline a good candidates for sustained release. It has a solubility of 8.8 mg/mL (Robinson, et al., 1986).

1.7.2. Chlorothiazide

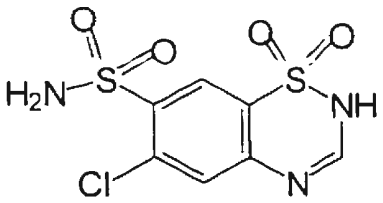
Chlorothiazide, a diuretic and antihypertensive drug, is slightly soluble in water; 400 mg/L at pH 4 and 650 mg/L at pH 7. Some research data suggests that the absorption of chlorothiazide from the GI tract in humans may be saturable or site-specific (Longer, et al., 1985).

1.7.3. Indomethacin

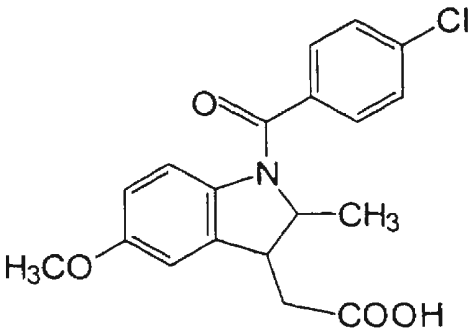
Indomethacin, an anti-inflammatory drug, is frequently used in studies on hydrophilic matrix systems due to its poor water solubility. It is practically insoluble in water (the Merk Index) and represents the extreme in the solubility scale.



Theophylline



Chlorothiazide



Indomethacin

Figure 4. Structures of model drugs

2. RESEARCH OBJECTIVES

The primary objective of the research is to prepare polymeric particulates that combine the advantages of particulates and bioadhesive drug delivery systems. To achieve the above objective the following secondary objectives were laid out:

i) Develop an efficient method to prepare polymeric particulates using ionotropic gelation technique.

ii) Explore the use of polymers which have both particulate forming property (by gelation technique) as well as bioadhesive property.

iii) Study the *in vitro* release profiles of model drugs in the finally chosen polymeric particulates.

iv) Study the *in vitro* bioadhesive property of the polymeric particulates.

v) Evaluate the absorption enhancing effect of the polymers using cell culture experiments.

3. RESEARCH HYPOTHESES

To achieve these research objectives, the following hypotheses are proposed:

1. The merits of bioadhesive and particulate technologies could be combined by selecting suitable polymeric materials and excipients to prepare particulates with bioadhesive properties.
2. These particulates would also be suitable as sustained release dosage forms.

3. Bioadhesive polymers would be able to adhere to mucus layer and increase the permeability of the epithelium.
4. Due to their large molecular weight, polymers themselves are not likely to be absorbed and to produce undesired systemic effects.

4. MATERIALS AND METHODS

4.1. Materials

4.1.1. Chemicals and equipments

Sodium alginate (suppliers' specification: viscosity of 2% solution at 25^{0}C, 3500 cps), chitosan (Supplier's specification: minimum 85% deacetylated), poly-*l*-lysine hydrochloride (MW: ~22,000/~48,000 Da), pectin as a potassium salt (degree of esterification: 28%), calcium chloride ($CaCl_2$), tripolyphosphate, Tween 20, glass beads (700~1,200 μm), mannitol, theophylline, chlorothiazide, indomethacin, hydroflumethiazide, Dulbecco's Modified Eagle's Medium (DMEM, with 4.5g/L glucose), Non-essential amino acids (NEAA) and Trypsin (0.25%)-EDTA (1 mM) were all purchased from Sigma Chemicals, MO, USA. Fetal calf serum (FCS), penicillin-streptomycin (5,000 I. U./mL and 5,000 μg/mL) and sodium bicarbonate solution (7.5%, w/v) were obtained from Gibco BRL, Life Technologies, NY, USA. Concentrated hydrochloride acid (HCl), ammonium chloride, sodium phosphate monobasic, potassium phosphate monobasic and potassium phosphate dibasic were purchased from Fisher Scientific Co., NJ, USA. Scintillation cocktail (Formula 989®) was purchased from Packard Instrument Company Inc. CT, USA. Methanol and acetonitrile were of HPLC grade and purchased from Fisher Scientific Co., NJ, USA. Deionized water was obtained using the Barnstead Nanopure II system (MA, USA).

A homogenizer (Brinkmann Instruments Company, Switzerland) was used to mix the dispersions, Corning stirrer plate and magnetic stirrer (Fisher Scientific Co., NJ, USA) were used for agitation and Accumet® pH Meter 10 (Fisher Scientific Co., NJ, USA) was used to precisely measure the pH value of the buffer solution. Freeze dryer (Labconco Corp., MO, USA) was used during the freeze drying procedure. A custom-designed artist airbrush (*Paasche*, CA, USA) was used to create a spray of polymer solution for the preparation of microspheres. A custom made tensiometer was used to measure the *in vitro* tensile strength. A humidity chamber was applied to offer an environment with high humidity.

Water-jacketed incubator (Isotemp® Incubator Model 546, Fisher Scientific Co., NJ, USA) was employed to provide suitable environment for cells growth. Hund Wetzlar Wilovert® microscope (Wetzlar/Nauborn, Germany) and Hemacytometer (Hausser Scientific, PA, USA) were used for cells counting. Tissue culture flasks and pipettes were purchased from Fisher Scientific Co., NJ, USA. Costar Transwell® 6-well plate inserts (pore size 0.4μm, Costar, MA, USA) were used for transport study.

4.1.2. Instruments

A liquid scintillation counter (Beckman LS 5000 TD, CA, USA) was used to measure radioactivity of ^{14}C samples. A USP type I (basket) dissolution apparatus (Vanderkamp 600, Vankel Industries Inc. NJ, USA) attached to a fraction collector and a programmable dissolution sequencer (Vanderkamp model 10, Vankel

Industries Inc. NJ, USA) was used for *in vitro* release studies. A Beckman DU® - 70 spectrophotometer was used to measure the absorbance of the model drugs. Computerized image analysis (BIOQUANT® system IV, USA) was performed to analyze the particulate size and scanning electron microscope (Hitachi S570 SEM, Japan) was operated to obtain the micro-structure of cross-section of the particulates.

4.1.3. Radioisotope

^{14}C-mannitol (specific activity: 50 mCi/mmol, 0.25μCi/mL) in ethanol solution was purchased from Sigma Chemicals, MO, USA.

4.1.4. Cell line

The Caco-2 cell line was obtained from American Type Culture Collection (MD, USA) at passage 17 and used for transport study.

4.1.5. Animals

Male Sprague Dawley rats weighing between 250g - 350g (Animal Care Center, Memorial University of Newfoundland, NF, Canada) were used for obtaining parts of the GI tract for *in vitro* bioadhesive property tests. Plasma from the healthy rats was used for the preparation of the calibration curve of chlorothiazide in HPLC analysis and validation of the analytical procedure.

4.2. Methods

4.2.1. Preparation of particulates

4.2.1.1 Preparation of ALG particulates

The method to prepare ALG particulates was adopted from Bodmeier's work (1989) and changed to suit our need. A solution of sodium ALG (1.5% w/v) was prepared in deionized water. The model drug was dispersed in the sodium ALG solution using a homogenizer (~1000 rpm). Then 20 mL of the dispersion, containing about 0.4 g model drug, was added drop-wise using a disposable syringe (# 20 gauge) into 50 mL gently agitated aqueous $CaCl_2$ solution (2% w/v). After allowing 10 min for gelation, the particulates were harvested and rinsed with deionized water to remove loosely adhered drug molecules. The ALG particulates were then dried using a freeze dryer without any pre-cooling step. Remaining traces of moisture were removed by desiccating under vacuum for at least 48 h. A few test samples were also air dried for comparison with the freeze dried products.

4.2.1.2. Preparation of chitosan particulates

The method to prepare chitosan particulates was also adopted from Bodmeier's work (1990). A chitosan solution (1% w/v) was prepared in diluted acetic acid (1%, v/v). The model drug was dispersed in the chitosan solution using a homogenizer. Twenty mL of the dispersion was added drop-wise using a disposable syringe (# 20 gauge) into 50 mL gently agitated tripolyphosphate solution (2%, w/v).

After 30 min gelation, the particulates were harvested and rinsed with deionized water to remove loosely adhered drug molecules. The particulates were then freeze dried without any pre-cooling step. Remaining traces of moisture were removed by desiccating under vacuum for at least 48 h.

4.2.1.3. Preparation of ALG and chitosan microspheres

A laboratory method to produce microspheres was developed using an artist airbrush. This method allowed to produce microspheres of any size by properly adjusting the air pressure and nozzle sizes. Mostly, we used nozzle size 3 (diameter 1 mm) at the air pressure of 4-5 psi. The dispersions of sodium ALG or chitosan containing model drug described above were sprayed into either $CaCl_2$ (2% w/v) or tripolyphosphate (2% w/v) solution. The gelation time ranged from 5 to 15 min. The microspheres were filtered out and freeze-dried for 12 h. During the preparation of chitosan microspheres, 0.5% Tween 20 was added to the tripolyphosphate solution to prevent agglomeration of microspheres.

4.2.1.4. Preparation of ALG-PLL particulates

We attempted to prepare ALG-PLL particulates by two different methods which were described below.

4.2.1.4.1. Method of preparation of ALG-PLL I particulates

The procedure was essentially similar to that of ALG particulates described

above (3.2.1.1.) with an additional step where the ALG particulates were coated with a PLL solution (0.1% w/v). Essentially 20 mL sodium ALG solution (1.5% w/v) containing the dispersed model drug were added drop-wise to 50 mL $CaCl_2$ solution (2%, w/v) and allowed to form temporary gel for 10 min. The particulates were harvested and resuspended in PLL solution (0.1%, w/v) for 6 min to allow cross-linking to occur between ALG (anionic) and PLL (cationic) and form a permanent layer. Thereafter, the particulates were filtered out, washed with deionized water and then suspended in 20 mL sodium ALG solution (0.05% w/v) with gentle agitation for 4 min which reacted with any excess PLL at the outface of the particulates. The ALG-PLL I particulates were washed twice with deionized water and freeze-dried for 24 h as described above. A few test samples were also air-dried for performing a comparative evaluation.

In this study, we used PLL with a mean MW of ~ 22,000 in all cases except one where MW ~ 48,000 was used. Particulates prepared with the two MW PLL were compared with respect to their *in vitro* release profiles.

4.2.1.4.2. Method of preparation of ALG-PLL II particulates

At first, the $CaCl_2$ solution (2% w/v) was mixed with PLL solution (0.1% w/v), then sodium ALG dispersion (1.5% w/v) was added drop-wise into this mixed solution and gelated for 10 min to form the temporary gel particulates. The following steps are similar to those of ALG-PLL I particulates. Briefly, ALG particulates were suspended in PLL solution (0.1%, w/v) for 6 min, then filtered out, washed with

deionized water and resuspended in sodium ALG solution (0.05%, w/v) for 4 min to neutralize any excess PLL at the surface. Finally these particulates were freeze dried.

4.2.1.5. Preparation of ALG-PEC-PLL particulates

The preparation was similar to that of ALG-PLL I particulates with a slight modification. In this procedure, firstly, 1.5% (w/v) of sodium ALG and 3.7% (w/v) of PEC were dissolved in deionized water, then the model drug was dispersed in the polymer solution using a homogenizer. The rest of the steps followed the preparation of ALG-PLL I particulates. Briefly, twenty mL of the dispersion was added drop-wise into 50 mL $CaCl_2$ solution (5%, w/v) to form the gel particulates for 20 min, then the particulates were suspend in 15 mL PLL solution (0.1%, w/v) for 6 min. After washing with deionized water, they were resuspended in 20 mL sodium ALG solution (0.05%, w/v) for 4 min. Finally the particulates were freeze dried.

Besides, during the preparation of ALG-PEC-PLL particulates, for some batches of theophylline loaded particulates the gelation time was 10 min, which were prepared to compare the *in vitro* release profiles with those prepared with 20 min gelation time.

4.2.2. Particle size analysis

The mean diameter and the size distribution of the dried and resuspended microspheres were determined by computerized image analysis. The freeze-dried

ALG and chitosan microspheres were suspended in phosphate buffer (pH 6.0) and dilute HCl (pH 1.0), respectively, soaked for 10 min and subjected to image analysis. Parameters such as periphery and the longest diameter were determined.

4.2.3. Scanning Electron Microscopy (SEM)

SEM was performed to characterize the cross-sectional view of the particulates in order to study the effects of the polymers and drying techniques. Cross-sections were obtained by cutting the particulates with a razor blade. The samples were then coated with gold-palladium for 70 seconds under an argon atmosphere and observed with a scanning electron microscope. In this study, we measured five different types of the particulates: 1. ALG particulates (air-dried); 2. ALG particulates (freeze-dried); 3. ALG-PLL I particulates (freeze-dried); 4. ALG-PLL II particulates (freeze-dried); 5. ALG-PEC-PLL particulates (freeze-dried).

4.2.4. *In vitro* release studies

4.2.4.1. Preparation of dissolution test solutions

Phosphate buffer (0.05M, pH 7.5 ± 0.1) were prepared by mixing 250 mL of KH_2PO_4 solution (6.8 g in 250 mL) and 190 mL 0.2 M NaOH. The pH was measured and if required, adjusted with 0.2 M NaOH. The final volume was made up to 1000 mL with deionized water.

The 0.1 M HCl (pH 1.0 ± 0.1) was prepared by diluting 6.25 mL of concentrated HCl acid (37%, v/v) to 1000 mL with deionized water. The final pH

was determined using a pH meter.

4.2.4.2. Dissolution tests

The release studies were performed using a USP Type I (basket) dissolution apparatus attached to a fraction collector and a programmable dissolution sequencer. The stirrer speed was set to 120 rpm and the thermostat controlled water bath was set to 37 °C. In this study, we used two different dissolution fluids, 0.1 M HCl (pH 1.0 ± 0.1) and phosphate buffer (0.05M, pH 7.5 ± 0.1). The total volume of the dissolution fluid was 900 mL. Drug-loaded particulates weighing between 40 and 100 mg (depending on the type of model drug) were placed in the basket. Dissolution test was performed over 8 h. Five mL of dissolution fluid was sampled at predetermined time intervals, viz., 15 min, 30 min, 45 min, 1 h, 1.5 h, 2 h, 3 h, 4 h, 6 h, 8 h. The sample volume was replenished with fresh dissolution fluid after sampling. In order to determine the total content of the drug in the particulates, we also took the samples at 24 h and 72 h when the dissolution medium was phosphate buffer (pH 7.5 ± 0.1). All experiments were performed in triplicate and the mean cumulative % of drug released ± SD are reported.

4.2.4.3 . Analysis of the model drugs

Standard solutions of the test compound were prepared in phosphate buffer. After scanning the solutions, the λ_{max} were selected for analysis. These values were 272 nm for theophylline, 294 nm for chlorothiazide and 266 nm for indomethacin.

Standard curves were prepared by plotting UV absorbance vs. concentrations of the standard solutions.

The test samples were measured either directly or after diluted with suitable dissolution medium such as 0.1 M HCl (pH 1.0) and phosphate buffer (pH 7.5). The amount of drug released was quantified spectrophotometrically from the standard curves.

4.2.4.4. Drug content

The cumulative amount of drug dissolved after 72 h of dissolution in phosphate buffer (pH 7.5) was taken as the total amount of drug in the particulates. All the three particulates, ALG, ALG-PLL and ALG-PEC-PLL, were observed to completely disintegrate after 48 h of dissolution, and hence, 72 h was considered as the adequate time period to release all the encapsulated drugs.

4.2.5. Determination of bioadhesive strength

4.2.5.1. Preparation of isolated rat stomach and jejunum

Unfasted male Sprague Dawly rats (250-350 g) were sacrificed using an overdose of urethane. As for tensile strength test, the jejunum was cut and kept in phosphate buffer solution (pH 6.0) and used within 4 h of sacrifice. Before the test, the jejunum (2.5-3 cm) was cut longitudinally, gently washed with phosphate buffer and mounted on a platform to expose 2 cm^2 of tissue. As for falling liquid film test,

the stomach and jejunum were cut and stored in 0.1 M HCl and phosphate buffer (pH 6.0), respectively, and used within 2 h of sacrifice. Before the test, the stomach and jejunum (5-7 cm) were cut open longitudinally, emptied of food and washed with 0.1 M HCl (20 mL/min) and phosphate buffer (pH 6.0, 20 mL/min), respectively, until they were clean. Pieces (5-7 cm) of jejunum or stomach were placed on one half of a longitudinally cut rubber tube (2 cm diameter) with the help of pins. They were used as the bio-surface for the *in situ* test.

4.2.5.2. *In vitro* tensile strength test

A custom made tensiometer (Figure 5) was used to measure the tensile strength of polymer candidates when brought in contact with rat intestinal mucosa. Freshly excised rat jejunum was used as the bio-surface for this test. Glass slides (22X22X0.15 mm) were coated with the polymer solution and air dried to form a thin film. The weight of the glass slide was counterbalanced with a plastic container having requisite amount of water on the other side. Then the polymer film was kept in close contact with the tissue mounted on the platform by placing a 2 g weight on the glass side of the glass slide for 5 min. Just before the measurement of tensile strength, the weight was removed. Water was added drop-wise (4.5 mL/min) to the container with the help of a peristaltic pump that ensured a smooth flow of water and avoided any jerky movement. The pump was switched off as soon as the polymer film got detached from the tissue. The experiment was repeated five times to determine the average tensile strength, and each time a fresh piece of mucosal

tissue and a new polymer covered glass slide were used. (Lehr, et al., 1990; Smart 1991 and Parodi, et al., 1996)

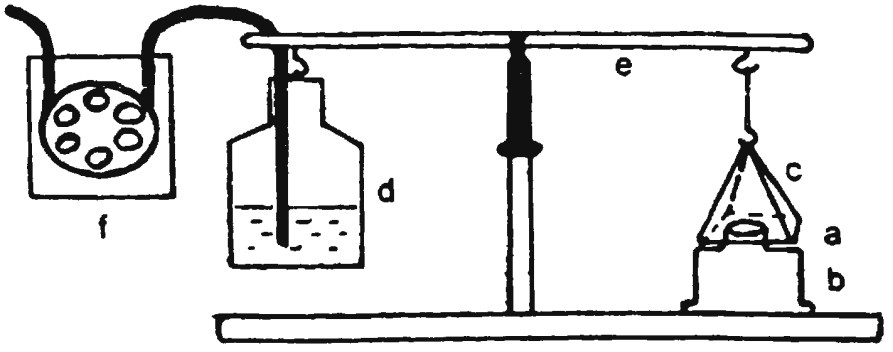


Figure 5. Schematic diagram of the modified tensiometer used to test the tensile strength of the bioadhesive polymers: a) disk; b) platform; c) stopper; d) plastic container; e) balance; f) pump.

4.2.5.3. *In situ* falling liquid film test

This procedure was adopted from Rango Rao and Buri's method (1989). Isolated rat jejunum and stomach pieces were used as the bio-surface to test the bioadhesive strength of the test particulates. Silica coated glass beads (700~1,100 μm) were used as the control samples. A humidity chamber (80% RH) was prepared by saturating it with a saturated solution of ammonium chloride at room temperature (25 ± 1 ^{0}C). About 50 mg of test samples were placed on the piece of tissue (stomach or jejunum) and incubated for 20 minutes in the humidity chamber. This procedure allowed the particulates to hydrate and interact with the mucosal surface of the gut. Then the tissue-particulate assembly was placed on the plastic support, fixed at an angle of 45^{0} (Figure 6). A rubber tube connected to a peristaltic pump was placed about 1 cm above the tissue sample to obtain an even flow of liquid. The particulates were washed by pumping either dilute HCl (for stomach tissue) or phosphate buffer solution (for jejunum tissue) at 30 mL/min. The percentage of the particulates retained on the test tissue was used as an index of the bioadhesive property of the particulates. It was determined by collecting the washed particulates and counting them.

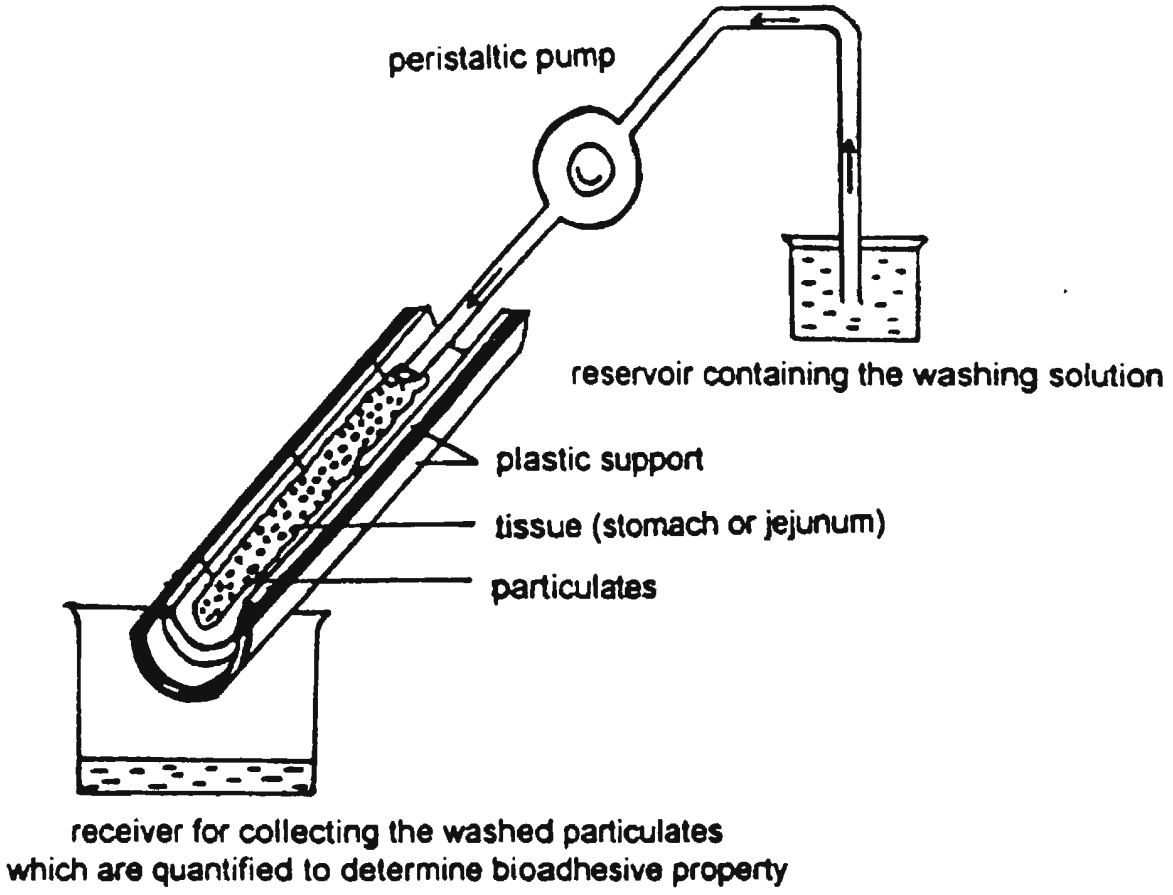


Figure 6. Schematic diagram of the assembly used to test the bioadhesion of the particulates (Ranga Rao and Buri, 1989)

4.2.6. Transport studies using Caco-2 monolayers

4.2.6.1. Cell cultures

Caco-2 cells were cultivated with completed DMEM (supplemented with 10% FCS, 1% NEAA, 2% sodium bicarbonate and 1% penicillin-streptomycin) and incubated in tissue culture flasks in an atmosphere of 5% CO_2 and 95% air at 37 °C. The medium was changed every 48 h. The confluent cells were passed by using trypsin-EDTA to detach the cells from the flasks. Cells with passage number 26-29 were used for the experiments.

4.2.6.2. Preparation of Caco-2 monolayer

Initially, confluent Caco-2 cells (passages 26-29) were harvested with trypsin-EDTA and cultivated on tissue culture treated polycarbonate filters in Costar Transwell® 6-well plate inserts at a seeding density of 3 X 10^5 cells/well. Completed DMEM was used as culture medium. The medium was added to both the donor (1.5 mL) and the receiver compartment (2.5 mL), and it was replaced every 48 hours. The cell were incubated in an atmosphere of 5% CO_2 and 95% air at 37 °C. The cells were used for the transport study between 21-23 days after seeding.

4.2.6.3. Preparation of polymer solutions

For the transport experiment, two different test samples were prepared. One contained 1% ALG, 1% PEC and 0.1% mannitol in DMEM, and the other one contained 1% ALG, 2% PEC and 0.1% mannitol in DMEM. Just before the addition

of the polymers, twenty μL of radio-labeled ^{14}C-mannitol alcohol solution (Specific activity: 0.25 μCi/mL) was added into 40 mL of the above solution as a tracer to facilitate the analysis by liquid sintillation counting (LSC).

4.2.6.4. Transport of ^{14}C-mannitol across Caco-2 monolayers

Caco-2 monolayers were used to perform transport studies in this experiment. During 21-23 days after seeding, the Caco-2 cells became confluent and totally differentiated with villi and carriers. The morphological development in the cells were noted primarily based on the observation of the monolayers under microsocpe. The old medium was removed from both the donor and the receiver compartments of the Transwell® inserts. Two mL fresh completed DMEM was added to the receiver compartment and 1.5 mL polymer solution sample was add to the donor compartment of each well. The control solution only consisted of 0.1% (w/v) mannitol and the tracer in DMEM without the polymers. Transport studies were performed over 180 min in the incubator. Samples (100μL) were taken from the receiver compartment at 0 min, 30 min, 60 min, 90 min, 120 min, 150 min, 180 min. The volume removed from the receiver compartment was replenished with fresh completed DMEM. Samples (100μL) were also taken from the stock polymer solutions to determine the total radioactivity. To each of the test samples, 5 mL of scintillation cocktail was added before determining the disintegrations per minute (dpm) using the liquid scintillation counter. The background radioactivity was

measured using 100 μL of completed DMEM. All of the samples were counted at a window setting for standard ^{14}C with a preset time of 15 min. The background radioactivity was subtracted from each sample reading.

4.2.6.5. Calculation of apparent permeability coefficients (P_{app})

The P_{app} (cm/sec) were determined using the following equation (Artursson, 1990):

$$P_{app} = \frac{\Delta Q}{\Delta t \times 60 \times A \times C_0}$$

Where $\Delta Q/\Delta t$ is the permeability rate (dpm/minute),

C_0 is the initial concentration of mannitol in the donor compartment (dpm/mL)

A is the surface area of the membrane (4.71 cm^2).

In order to observe the effects of the test samples on permeability as a function of time, the P_{app} values were calculated for each time point separately. All experiments were carried out in triplicate and the mean P_{app} ± SD are reported.

4.2.7 Statistical evaluation

In this study, Sigmaplot® 4.0 and SPSS 7.5 for Windows program were used to analyze the data. All data are expressed as the mean ± SD. Statistical differences were determined using one-way ANOVA and Bonferroni t-tests for multiple comparisons. Differences between group means were judged with $p = 0.05$.

5. RESULTS AND DISCUSSION

5.1. Bioadhesive particulates

The important features of a good drug delivery system include versatility to carry drugs with different physicochemical properties, simplicity of the method of preparation and feasibility for mass production. We have attempted to bear these factors in mind while formulating the ALG-based particulates. With the use of organic solvents for preparing particulates such as polylactide glycolide microspheres, there are two main problems: toxicity due to the residual organic solvents, and instability of certain drugs, especially those belonging to the class of peptides and proteins (Cleland, 1996). Our method to prepare drug-loaded particulates involves only aqueous solvents. Although at this initial stage of development we have used conventional drug molecules as model compounds, the technique is also expected to be applicable to peptide and protein drugs. Although the samples for our experiments were prepared using a syringe technique, micro-drops of the polymer solution can be sprayed using an air-brush spray device (Kwok et al. 1991). We have attempted this technique in our laboratory to successfully prepare microspheres which are 100-200 μm in size. Although the feasibility has not been checked, the technique seems to have potential for larger scale production.

5.1.1. ALG particulates

The mechanism of ALG gelation with calcium ions is quite well understood. It is believed that junctions are formed by an interchain chelation of calcium ions between homopolymeric G-blocks. Above a certain threshold ALG concentration the gel strength increases in proportion to the square of the ALG concentration, and there is an indication of bimolecular mechanism for junction formation (Martinsen, et al., 1989). Therefore, the higher the concentration, the stronger the particulates. Different concentrations of $CaCl_2$ and gelation times have also been studied to optimize the strength of ALG particulates. Martinsen's research group also investigated the effects of changing the concentration of $CaCl_2$ and of storing the gel particulates. Their results indicated that above a certain concentration of calcium ions (depending upon the type of ALG) and above certain storage times, the gel beads are quite stable with respect to changes in volume and strength. Other groups have reported the use of 1-1.5% $CaCl_2$ and 5 min gelation time for the preparation of ALG particulates due to the quick interaction between ALG and calcium ions (Bodmeier, et al., 1991; Shiraishi, et al., 1993). Here, we used 2% $CaCl_2$ solution and 10 min gelation time in most of our experiments.

We tried different concentrations of sodium ALG solution to prepare ALG particulates by syringe technique. Two percent (w/v) sodium ALG solution was very viscous, thereby it was very difficult to push the solution through the narrow syringe needle. In addition, it was difficult to disperse the drug powder homogeneously in the solution. A concentration of 1.5% (w/v) sodium ALG was found suitable for the

particulate preparation by syringe technique.

The spherical particulates were formed spontaneously as soon as the sodium ALG drop touched the $CaCl_2$ solution. Freshly prepared particulates were spherical and flexible. It has been shown earlier that the elasticity of ALG gels was energetic rather than entropic because the stiff ALG chains were densely cross-linked with calcium ions (Smidsrød, 1990). Multivalent ions such as calcium, exchange with sodium ions of sodium ALG solution to form calcium ALG gel. On placing the gel in a solution containing monovalent cations the reversible process takes place, resulting in the gel to sol transformation. Thus, the dried ALG particulates swell in aqueous solution and disintegrate in the presence of monovalent cations. As a result of this erosion, the encapsulated compound get released from the particulates. The release profiles will depend on the strength of the gel and the rate of particulate erosion (Shenouda, et al., 1990).

5.1.2. Chitosan particulates

Chitosan is a cationic polymer and can interact with anionic compound, tripolyphosphate, to form a hydrogel. The hydrogel is stable in aqueous solution containing monovalent cations, but disintegrates in acidic medium such as in the stomach.

The shape of the chitosan particulates was not as spherical as that of ALG particulates. Some particulates even had small spikes. This was probably because the interaction between the cationic polymer and the anions was not spontaneous

enough, or maybe the droplets from the syringe were not totally spherical due to their high viscosity. Another drawback was that the particulates were very soft after gelation, and the longer gelation time did not make the particulates hard. We tried to increase the gelation time to 30 min and 1 h, and found no change in the hardness. The particulates could keep the spherical shape when they were in the solution, but became somewhat flat when filtered out and stuck to each other. Use of Tween 20 (0.5%) prevented interparticle sticking in the solution helped only marginally. Freeze drying seemed to alleviate the problems and the dried particles were spherical.

Many studies have been done to investigate the potential use of chitosan as a particulate system (Bodmier, et al., 1990 and 1993). Although chitosan has a few desirable properties such as high *in vitro* bioadhesive strength and good permeability enhancement, we did not find it to be a suitable candidate in our study since it did not allow us to integrate polymers to take advantage from their individual properties.

5.1.3. ALG microspheres and chitosan microspheres

It has been reported that smaller sized particles could be prepared by using compressed air to spray the dispersion through a narrow nozzle, and the size of the microspheres could be controlled by using different nozzle sizes and air pressure (Kwok, et al., 1991). In our study, for both ALG and chitosan particulates, a spray technique by using an artist airbrush was developed to explore the possibility of obtaining smaller particulate size. We employed the size 3 nozzle to prepared the

ALG and chitosan microspheres at the air pressure of 4-5 psi, and the particle size ranged from 300 μm to 700 μm. However, the preparation procedure itself was fraught with numerous problems. It was very labor intensive and time consuming. Attempt to produce microspheres using the spray technique was cumbersome due to constant blocking of the outlet pore. Because of these difficulties, we did not pursue with ALG and chitosan microspheres further. Therefore, we prepared the particulates using syringe technique for the *in vitro* studies although the particle size was relatively large (diameter ~ 2.5 mm).

5.1.4. ALG-PLL I and II particulates

In order to prepare particulate carriers which are resistant to degradation and able to improve drug release profiles, attempts were made to combine two or more polymers. Gel particulates composed of different negatively and positively charged polymers represent a type of drug delivery system that can be prepared without a tedious process. It should be possible to control the release of various drugs by a proper selection of these polymers, by modification of the gel matrix and by improvement of the gel preparation. Coating ALG particulates with PLL is reported in the literature (Uludag, et al., 1993). In this work Uludag et al. have coated the ALG particulates externally to prolong the life of ALG particulates. PLL, which is positively charged, showed a very strong affinity to the negatively charged ALG.

The difference between the ALG-PLL I particulates and ALG-PLL II particulates could not be observed by the size or the shape. We assumed that the

preparation procedure of particulates II would allow the inside cross-linking between ALG and PLL to occur when sodium ALG form the hydrogel, and this might improve the micro-structure of the particulates to make them stronger. This assumption has been proved by SEM photographs (please see section 4.3.3.). As predicted, although the ALG-PLL I particulates did not disintegrate for more than 24 h, there was no significant improvement in prolonging the release of the encapsulated drugs with low MW. While ALG-PLL II particulates had a more stable matrix due to the inside cross-linkings and improved drug release profiles (please see section 4.4.3.). It has been reported that PLL is immunogenic and high concentration of PLL can cause immunological toxicity in animal models (Thu, et al., 1996). Although the ALG-PLL II particulates held promise with regard to improving the release profiles of the encapsulated drugs (please see Section 4.5), we did not pursue with it further due to the high content of PLL which has potential of causing toxicity.

5.1.5. ALG-PEC-PLL particulates

In our attempt to improve ALG particulates, we now incorporated PEC in the particulates. PEC also forms hydrogel with divalent cations such as calcium. Ca-PEC hydrogel is stable in the stomach and small intestine, but breaks down in the colon region in the presence of microflora. This property of Ca-PEC gel has been employed to coat tablets for colonic drug delivery (Puttipipatkhachorn, et al., 1997; Ashford, et al., 1993, 1994). To our knowledge both ALG and PEC together have not been tried in the preparation of particulates for obtaining sustained release of

drugs. An outer mask of PLL would serve the same purpose as in ALG-PLL particulates.

Although both sodium-ALG and PEC are known to form hydrogels upon contact with calcium ions (Bodmeier, et al., 1991 and Ashford, et al., 1994), we observed that the ionotropic gelation was spontaneous for ALG while that for PEC took some time and the particulates did not assume a spherical shape. Therefore, we increased the gelation time to 20 min for the preparation of ALG-PEC-PLL particulates. We anticipated that the longer gelation time would ensure complete cross-linking between PEC and calcium ions.

Formation of ALG-PEC-PLL particulates was as spontaneous similar to the formation of ALG and ALG-PLL particulates and the shape remained spherical. Hence, it seems that the presence of ALG is very much essential for spontaneity of ionotropic gelation and for the maintenance of a spherical shape of the particle. Besides, being polyanionic ALG also served as a template to bind to the polycation, PLL, which helped in prolonging the life of ALG particulates in aqueous solutions. Although the concentration of PLL in the particulates was low, a final coating of sodium-ALG was done to neutralize any unreacted PLL. This was done to minimize any unwanted immune responses to PLL, and also to provide a negative surface charge to the particulates that would allow them to bind to mucous membranes (Thu, et al., 1996). PEC was used as the co-core material and its ability to withstand degradation in gastric pH and the gut made the particles more robust. As a result the particulate's ability to sustain drug release was significantly improved. After

performing the initial *in vitro* tests for the various particulates, ALG with PLL and PEC were found most promising. Hence, from hereon, the results and discussion will mostly pertain to them.

5.2. Gelation time

Gelation time played an important role in the performance of the particulates. It affected the strength of the particulates, the drug loading and release profiles of the drugs. The effect of gelation time on release profiles of the drug is discussed in section 5.5.3.

5.2.1. Strength of ALG/ALG-PEC-PLL particulates and gelation time

As mentioned before, Martinson et al. (1989) has studied the relationship between the strength of the ALG particulates and the gelation time. The strength of the ALG particulates increased as the gelation time was increased up to certain point. Further increase in the gelation time did not make any significant difference in the particulate strength. Based on our study we determined that 10 min of gelation time was optimum for ALG and ALG-PLL particulates and 20 min of gelation time was optimum for ALG-PLL-PEC particulates.

5.2.2. Drug loading efficiency and gelation time

Drug loading depends on both the solubility of the drug and the gelation time allowed. Longer gelation time would lead to leaching of soluble drugs from the

particulates to the surrounding aqueous medium, thereby reducing drug loading. Hence, we kept the gelation time as short as possible.

In our experiments, during the preparation of ALG and ALG-PLL particulates, the gelation time of calcium-ALG spheres was kept 10 min, while for those containing PEC it was kept 20 min. Twenty min gelation time was anticipated to be sufficient to allow PEC and calcium ions to cross-link completely. For soluble drugs gelation time was a major concern that would affect drug loading. Due to the relatively high water solubility, theophylline loaded ALG-PEC spheres were allowed only 10 min gelation time. The time allowed for the interaction between ALG and PLL were kept uniform in all the particulates in order to avoid interparticulate differences.

The drug loading and encapsulation efficiency of the model drugs in the freeze-dried particulates are given in Table 3. Drug loading represents the percentage of the particulate that is occupied by the drug. For example, 22.4% of theophylline in ALG particulates indicates that 22.4% of the particle is the drug and the remaining 77.6% is the polymer. The values, given within the brackets, are the encapsulation efficiency of the preparation procedure which indicate the percentage of the original drug that has been incorporated in the particulates. The gelation time for ALG and ALG-PLL particulates were 10 min and that for ALG-PEC-PLL was 20 min.

Although use of aqueous medium has its advantages (described in section 5.1), one of the problems is the relatively lower drug-loading efficiency for the water

soluble drugs. This problem may be minimized by reducing the total contact time of the drug in the calcium chloride solution. The unloaded drug present in the left-over calcium chloride solution may be salvaged by extracting with an organic solvent, such as chloroform or hexane.

Table 3. Drug loading and encapsulation efficiency of the model drugs in the different freeze-dried particulates

	% Actual drug loading (encapsulation efficiency)			
	ALG	ALG-PLL(low MW)[a]	ALG-PLL(high MW)[b]	ALG-PEC-PLL
Theophylline	22.6 (41.8)	26.2 (48.1)		12.5 (36.6)
Chlorothiazide	38.0 (77.7)	45.5 (92.9)		23.4 (93.2)
Indomethacin	34.3 (68.0)	46.7 (94.1)	47.0 (94.7)	21.8 (94.1)

1. PLL MW 22,000 Da;
2. PLL MW 48,000 Da;

The values outsides the brackets are actual drug loading and those within the brackets are encapsulation efficiencies of the model drugs; Gelation time allowed for ALG, ALG-PLL was 10 min, and for ALG-PEC-PLL was 20 min.

From the results it is evident that for the most soluble drug theophylline, encapsulation efficiencies were the lowest. For the less soluble drugs chlorothiazide and indomethacin, they had similar drug loading and encapsulation efficiencies and their encapsulation efficiencies were higher than that for theophylline. Another obvious observation was that for theophylline increasing the gelation time from 10 min (in the ALG and ALG-PLL particulates) to 20 minutes (in the ALG-PEC-PLL particulates) resulted in a significant decrease in encapsulation efficiency. However, for the less soluble drugs, chlorothiazide and indomethacin, the encapsulation efficiency remained unchanged.

In summary, encapsulation efficiency is an important parameter that could be used to compare different preparation procedures. Drugs that are soluble in the medium of preparation (within this case is water) are more susceptible to the changes made in the process.

5.3. Factors influencing the characteristics of the particulates

5.3.1. Effect of drying techniques on physical characteristics of particulates

The drying technique used in this study influenced the final shape and size of the particulates. Freshly prepared particulates were spherical and flexible. After freeze drying, the final particulates were white and spherical and could be flattened out rather than brittle. The dried particulates kept almost the same shape and there was no significant shrinkage in the size. The particulates prepared using a syringe (20 gauge) were about 2-3 mm in diameter. Those prepared by air-spray technique

were about 300 - 700 μm in diameter.

The air-dried particulates could not retain the spherical shape of the freshly prepared particulates, and shrank more than 50% in size. It has been reported that the higher the values of the L-guluronic acid content and the average length of the G-blocks, the lower the shrinkage of the ALG particulates (Martinsen, et al., 1989). The air dried particulates were irregular in shape, hard and more off-white than the freeze-dried particulates. However, they could swell up to about the same size as the freshly prepared ones (approximately 2-3 mm) when placed in an aqueous solution, especially in the presence of monovalent cations.

The freeze-drying technique seemed superior with regard to retaining a spherical shape, size and color of the particulates. It would also be more suitable for drugs that are thermolabile and susceptible to degradation on prolonged contact with moisture since the drying occurs at sub-zero temperature and time taken is far less than air-drying.

5.3.2. Effect of drying techniques on the micro-structures of the particulates

The internal micro-structure of the particulates were observed using scanning electron micrographs of the particulates. The cross sections of freeze-dried particulates viewed under a scanning electron microscope showed numerous, honeycomb-like open cavities (Fig 8, 9, 10 and 11), as opposed to a less open and denser structure for the air-dried ones (Fig 7). This suggested that drying techniques played an important role in the micro-structure of the particulates. Water

inside the particulates helped in giving the particulates a spherical shape. In the freeze-drying procedure, the particulates were frozen very quickly at a freezing temperature (~ -50 °C) before the water inside was drawn out of the particulates so that the spherical shape of the particulates was retained. The water droplets inside became icelets and were removed by sublimation, leaving behind numerous cavities inside. However, when the particulates were air-dried, the water inside evaporated very slowly and the particulates shrunk with it, giving it a denser and irregular final shape.

Figure 7. Scanning electron micrograph of the cross-sections of ALG particulates prepared by air-drying technique (Magnification X100)

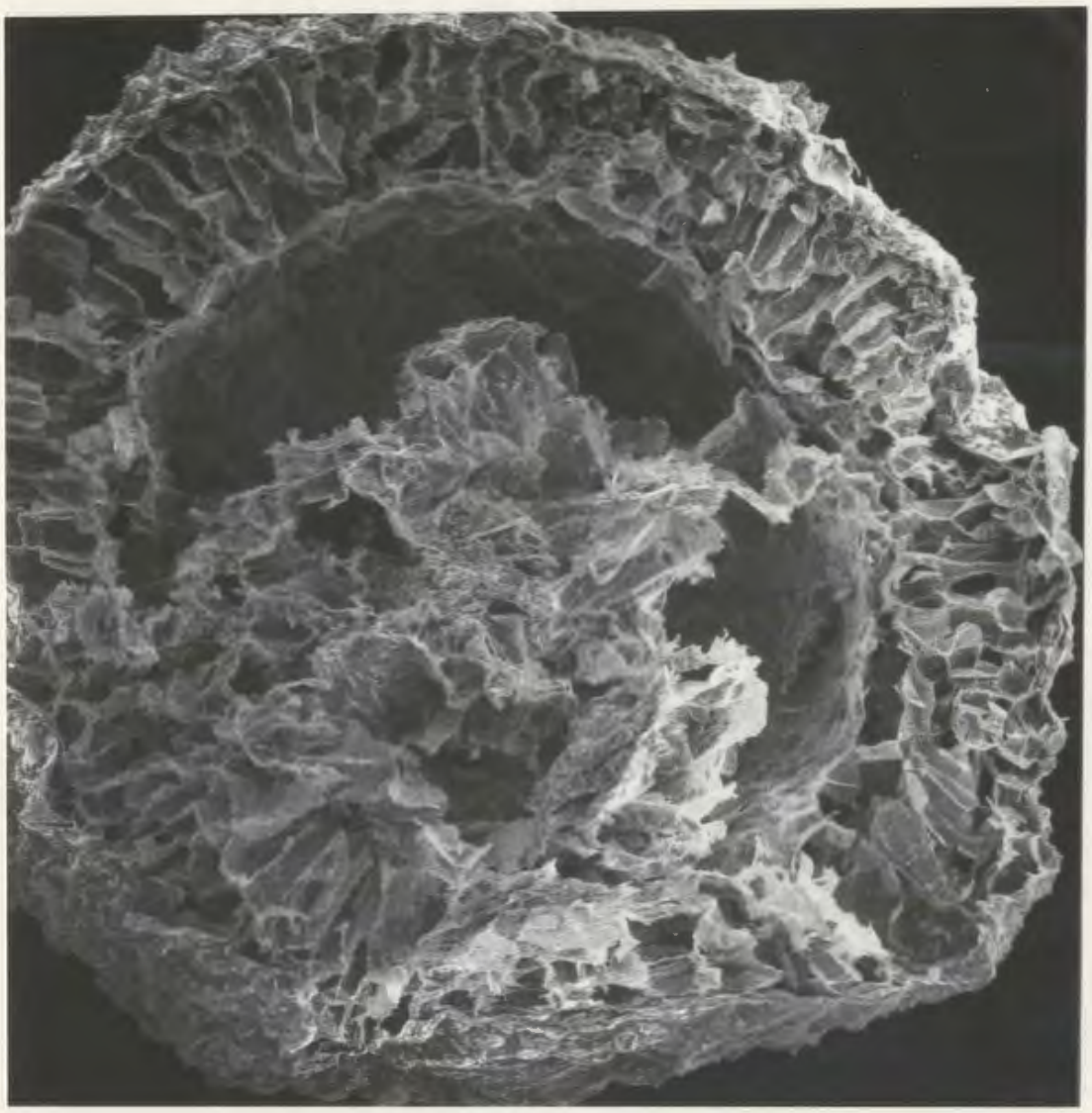


Figure 8. Scanning electron micrograph of the cross-sections of ALG particulates prepared by freeze-drying technique (Magnification X35).

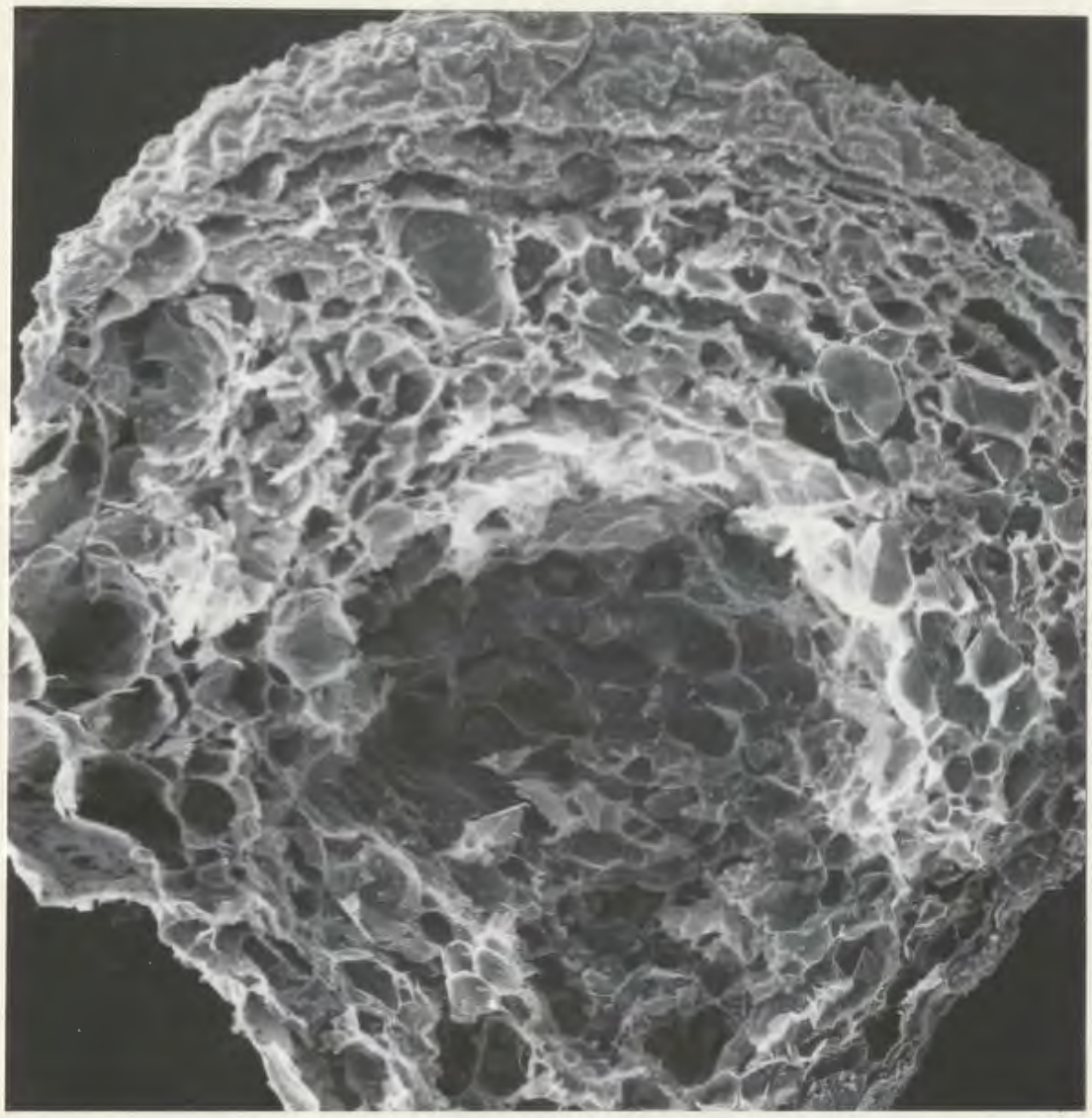


Figure 9. Scanning electron micrograph of the cross-section of ALG-PLL I particulates prepared by freeze-drying technique (Magnification X35).

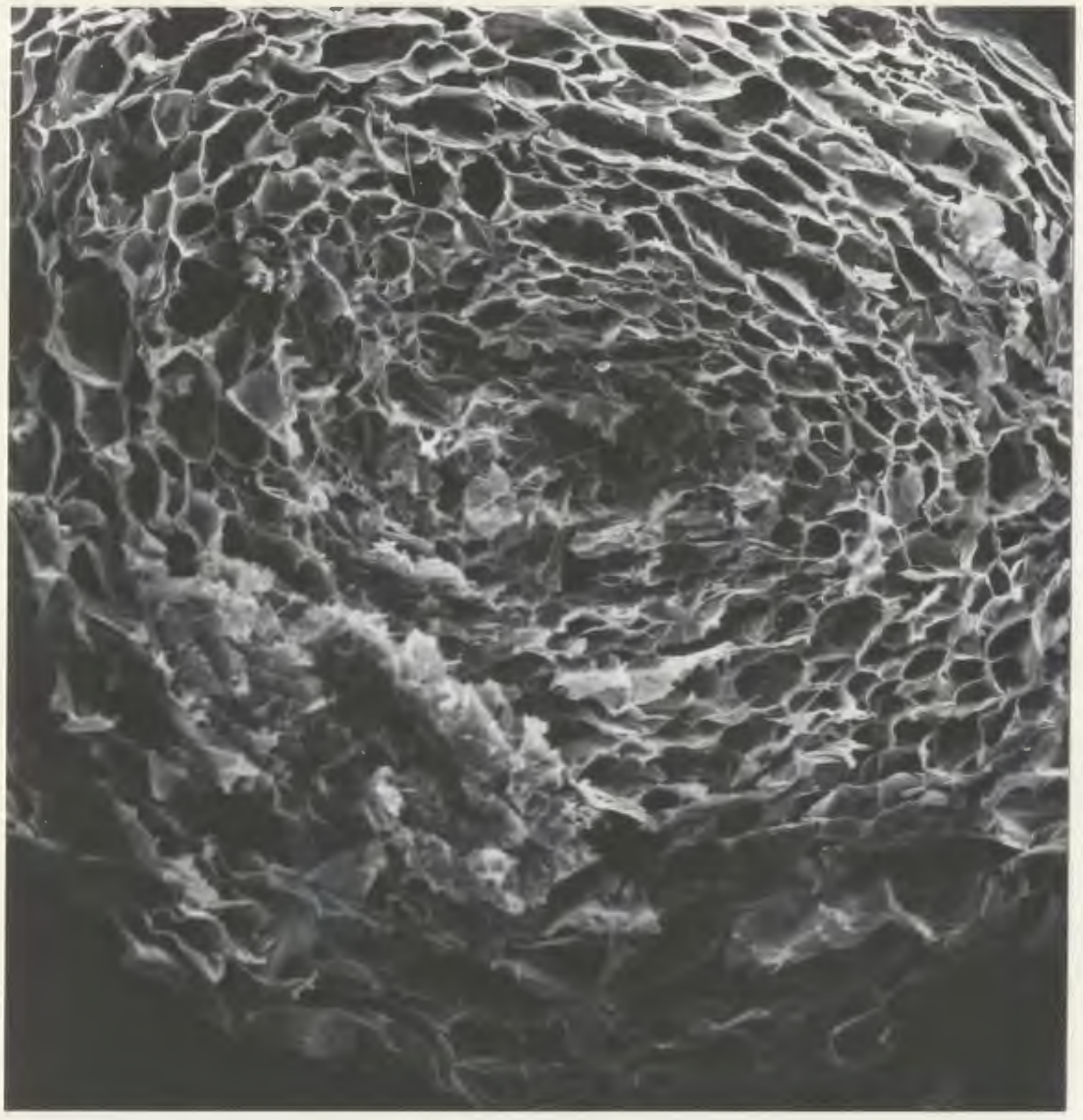


Figure 10. Scanning electron micrograph of the cross-sections of ALG-PLL II particulates prepared by freeze-drying technique (Magnification X35).

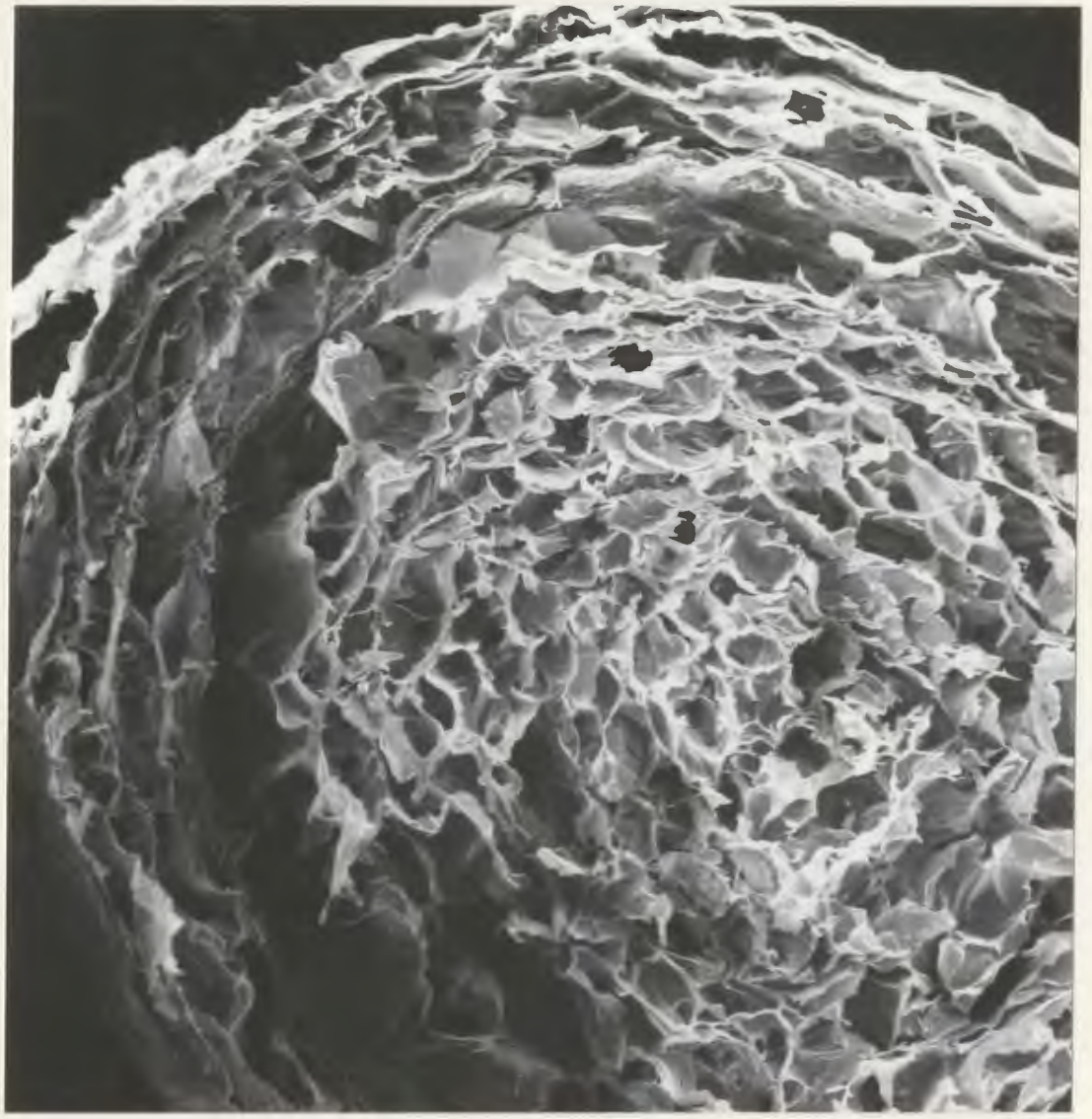


Figure 11. Scanning electron micrograph of the cross-sections of ALG-PEC-PLL particulates prepared by freeze-drying technique (Magnification X35).

5.3.3. Effect of polymers on the micro-structures of the particulates

Figures 8-11 show the SEM graphs of the freeze-dried particulates. The SEM graphs of the cross-sections of the particles were taken to observe the internal structures. There was a big cavity in the center of the ALG (Fig. 8) particulate. ALG-PLL I (Fig. 9) particulates had relatively larger honeycomb-like cavities. The SEM graphs of the cross-section of ALG-PLL II (Fig. 10) and ALG-PEC-PLL (Fig. 11) particulates were similar and looked denser. This could result in increasing the diffusional barrier for the encapsulated drugs in these two particulates.

5.4. *In vitro* release profiles of drugs from particulates

The three model drugs chosen, represented the wide range of physicochemical properties of conventional drugs presently. Theophylline is relatively soluble (8.33 g/L), chlorothiazide is slightly soluble (0.4 g/L at pH 4 and 0.65 g/L at pH 7) and indomethacin is practically insoluble in water. The drug release profiles were studied at two pH conditions, in 0.1 M HCl medium (pH 1.0 ± 0.1) simulating the gastric condition, and phosphate buffer medium (pH 7.5 ± 0.1) simulating the intestinal condition. After initially screening different particulates, three particulates, viz., ALG, ALG-PLL and ALG-PEC-PLL, were chosen for the tests. The results of other particulates which were not of interest are not discussed further. ALG particulates were used as a relative control in the dissolution tests. Model drug loaded particulates were prepared for ALG, ALG-PLL and ALG-PEC-

PLL and their dissolution profiles were studied in details. The release profiles of the three model drugs from the particulates at two pH conditions were plotted as cumulative % of drug released over a time period of 8 h and are shown in Figure 12-21. The underlying fact in all the figures is the influence of drug solubility on the release profiles, the higher the solubility the faster the release (Theophylline > Chlorothiazide > Indomethacin). The dissolution time for releasing 50% and 90% drug, viz., $t_{50\%}$ and $t_{90\%}$, are given in Table 4.

Table 4. Dissolution half life, $t_{50\%}$ and $t_{90\%}$ of the particulates in acidic and alkaline media

In 0.1 M HCl pH 1.0

	$t_{50\%}$ (h) ± SD			$t_{90\%}$ (h) ± SD		
	ALG	ALG-PLL	ALG-PEC-PLL	ALG	ALG-PLL	ALG-PEC-PLL
Theophylline	0.49±0.008	1.05±0.004	1.70±0.008	3.91±0.028	4.60±0.017	6.05±0.015
Chlorothiazide	1.38±0.021	———	———	———	———	———
Indomethacin	———	———	———	———	———	———

"-" indicates that less than 50% of drug was released in 8 hours.

In phosphate buffer pH 7.5

	$t_{50\%}$ (h) ± SD			$t_{90\%}$ (h) ± SD		
	ALG	ALG-PLL	ALG-PEC-PLL	ALG	ALG-PLL	ALG-PEC-PLL
Theophylline	0.21±0.007	0.24±0.008	0.90±0.007	0.96±0.033	1.32±0.028	3.50±0.015
Chlorothiazide	0.80±0.009	0.82±0.012	1.50±0.015	3.00±0.048	2.20±0.008	6.02±0.04
Indomethacin	1.00±0.004	1.92±0.011	2.70±0.016	7.02±0.023	8.10±0.014	9.21±0.013

5.4.1. Standard curves for the model drugs

The calibration curves for the model drugs were established. The relationship between the absorbance and the concentration were as follows:

Theophylline: Abs = Conc. X 0.0563 + 0.00814, r^2 = 0.9999;

Chlorothiazide: Abs = Conc. X 0.0342 - 0.00044, r^2 =0.9998;

Indomethacin: Abs = Conc. X 0.0506 + 0.0198, r^2 = 0.9997.

5.4.2. Drug release in acidic solution

In the acidic solution there was minimal release of chlorothiazide and indomethacin. Only the very soluble drug, theophylline, was released from all the three particulates, ALG, ALG-PLL and ALG-PEC-PLL (Figure 12). The $t_{50\%}$ of theophylline from the three particulates were 0.49h, 1.05h and 1.70h, respectively. The $t_{90\%}$ also showed a similar trend (Table 4) viz., fastest from ALG (3.91 h), intermediate from ALG-PLL (4.6 h) and slowest from ALG-PEC-PLL (6.05 h).

Chlorothiazide being only slightly soluble in acidic medium (<0.4 g/L at pH 1), was released only to a small extent from the most permeable ALG particulates. Its $t_{50\%}$ was 1.38 h (Table 4, Figure 13). However, in the 8 h study less than 50% of the drug was released from ALG-PLL and ALG-PEC-PLL particulates. For the poorly soluble drug indomethacin, the amount of drug released from all the three particulates were far less than their $t_{50\%}$ values (Figure 14). No more than 3% of drug were released from both ALG-PLL and ALG-PEC-PLL particulates within the 8 h duration.

We speculate that drug release occurs by both diffusion and erosion in the ALG particulates and primarily by diffusion in ALG-PLL and ALG-PEC-PLL particulates. ALG particulates are more fragile to break and release the encapsulated drugs. Hence, dissolution is fastest from ALG particulates, ALG-PEC-PLL is more resistant to degradation in the acidic medium. This is primarily due to the presence of the pectin gel which is stable in acidic pH and disintegrates only in the presence of microflora (Sandberg, et al., 1981; Englyst, et al., 1987). As a result, drug release from ALG-PEC-PLL is primarily occuring by diffusional process. ALG-PLL is in the intermediate category. PLL affords some robustness to ALG particulates but can not prevent the degradation of calcium ALG inside. Since the ALG-PEC-PLL particulate retained its shape for a prolonged period and, released only a small quantity of the encapsulated drug in the acidic medium, it may be reasonable to speculate that the particulate will protect drugs which are susceptible to degradation in the acidic environment. But this has to be confirmed by conducting further experiments with acid labile drugs.

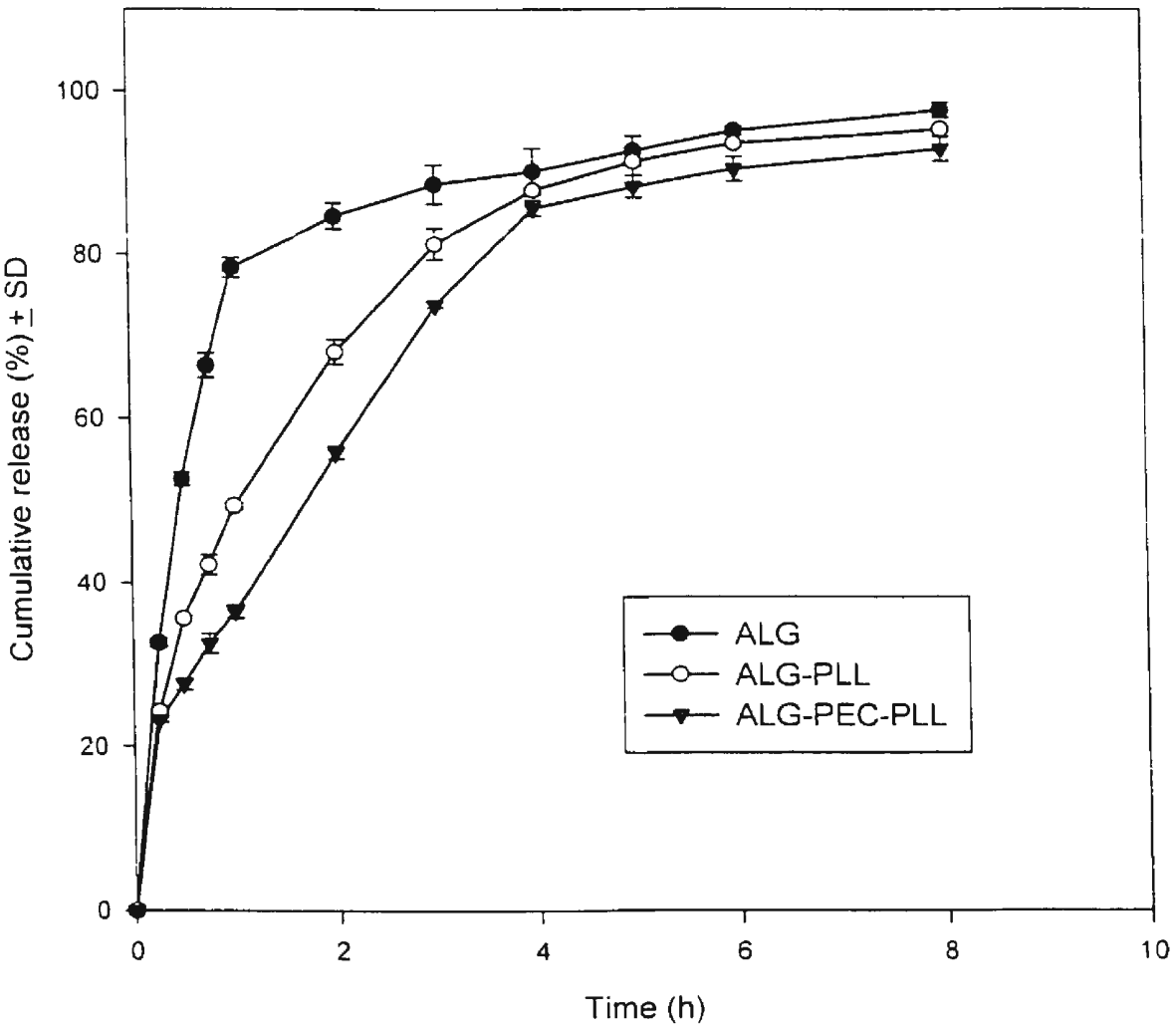


Figure 12. Cumulative release profiles of theophylline from different particulates in 0.1 M HCl (n = 3)

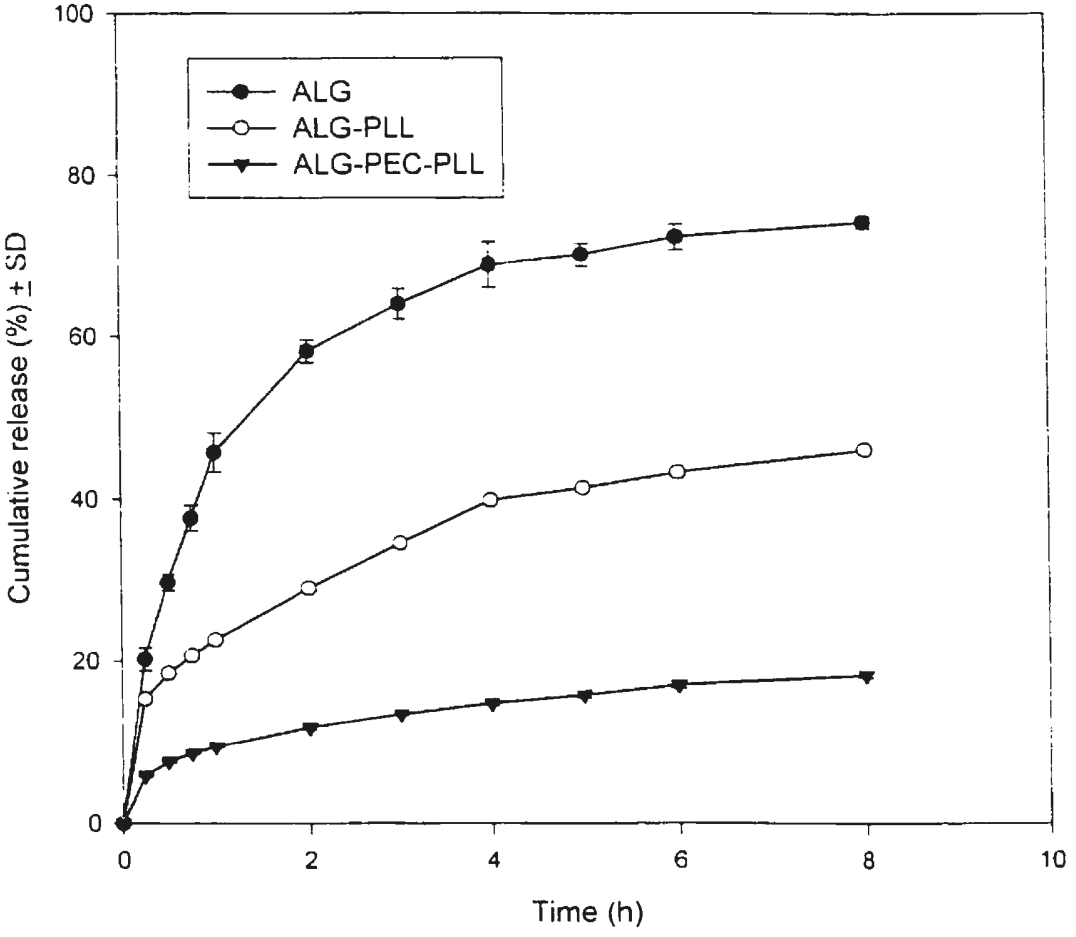


Figure 13. Cumulative release profiles of chlorothiazide from different particulates in 0.1 M HCl (n = 3)

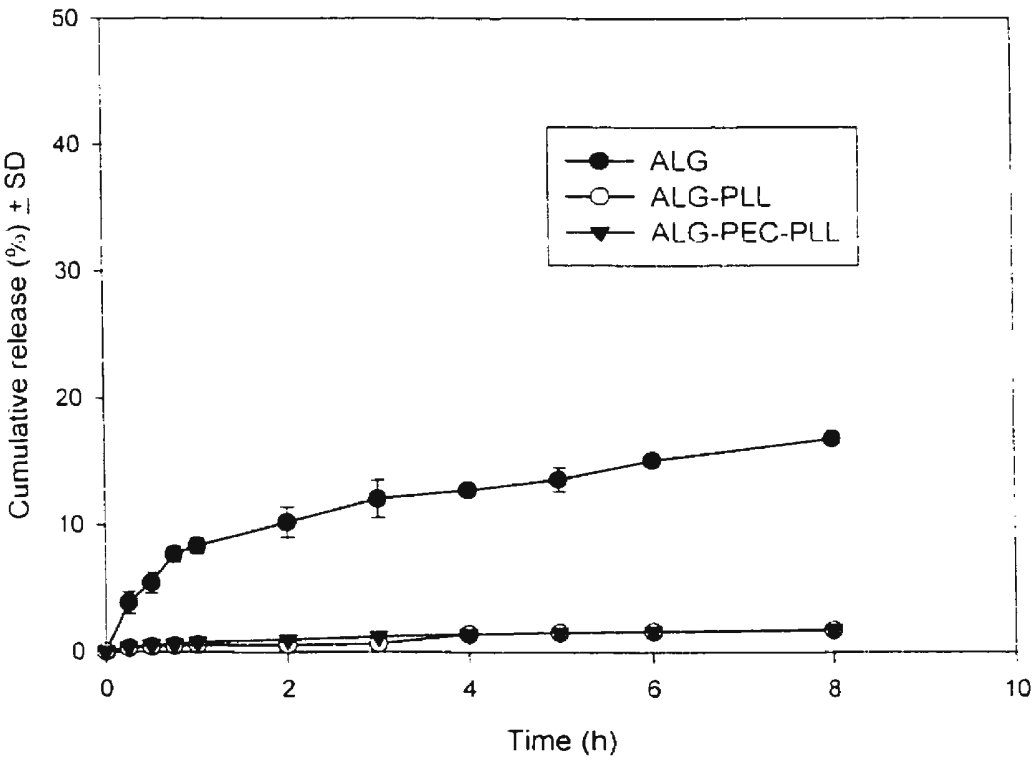


Figure 14. Cumulative release profiles of indomethacin from different particulates in 0.1 M HCl (n = 3).

5.4.3. Drug release in alkaline solution

For theophylline $t_{50\%}$ from ALG, ALG-PLL and ALG-PEC-PLL particulates were 0.21, 0.24 and 0.9 h, respectively and the $t_{90\%}$ were 0.96, 1.32 and 3.50 h, respectively (Table 4). As shown in Figure 15, plain ALG particulates, although somewhat stable in acidic pH conditions, were unable to control the release of the very soluble drug, theophylline beyond one hour. The release profiles of theophylline also showed a high burst effect; about 70% theophylline was released in15 minutes (Figure 15). Coating with PLL helped only marginally in prolonging the release of theophylline because the out layer formed by cross-linking ALG and PLL had very large pore size. The most significant difference was obtained by ALG-PEC-PLL particulates where the $t_{90\%}$ was 3.5 hours and the burst effect was significantly reduced. This could be attributed to the presence of PEC gel which is stronger and stabler than ALG gel in both acidic and alkaline solutions.

Chlorothiazide and indomethacin had the similar trends (Fig 16 and 17). The $t_{50\%}$ of chlorothiazide from ALG and ALG-PEC-PLL particulates were 0.8 h and 1.5 h, respectively, and their $t_{90\%}$ were 3.0 h and 6.02 h, respectively. The $t_{50\%}$ of indomethacin from ALG and ALG-PEC-PLL particulates were 1.0 h and 2.7 h, respectively, and their $t_{90\%}$ were 7.02 h and 9.21 h, respectively. The $t_{50\%}$ of indomethacin from ALG-PEC-PLL was about 2.7 times longer than that from ALG, while its $t_{90\%}$ was longer by 1.3 times.

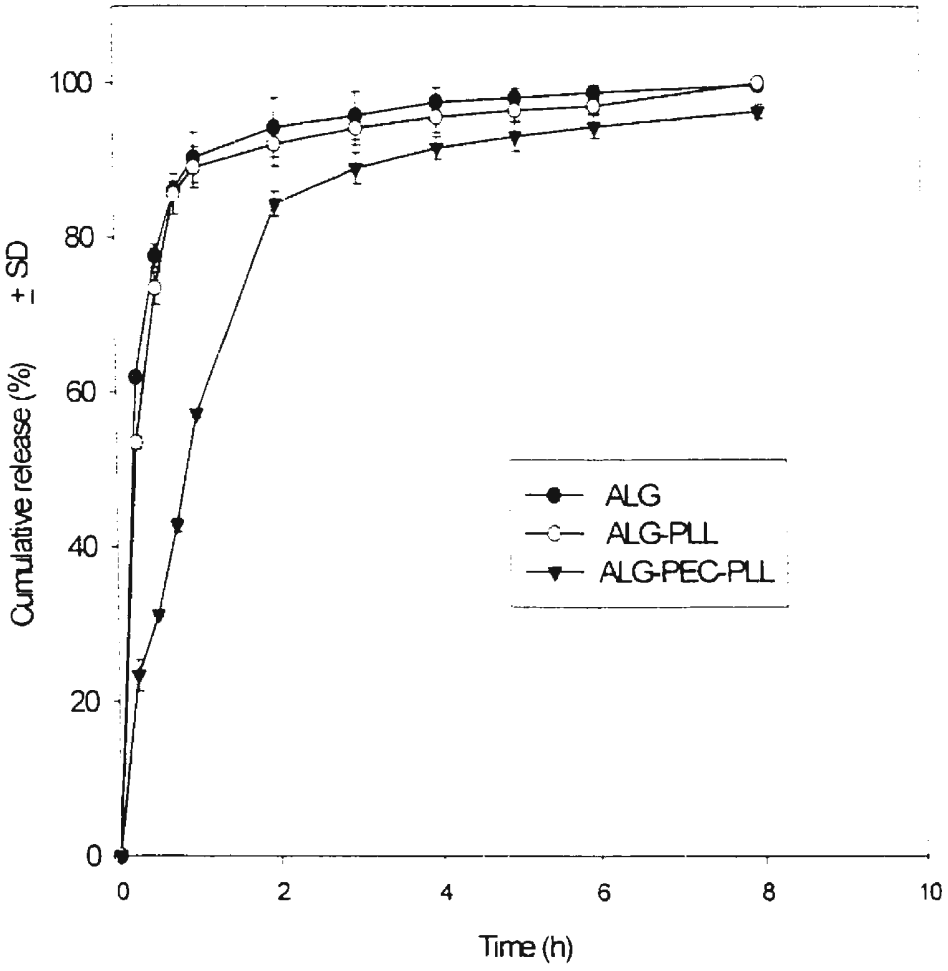


Figure 15. Cumulative release profiles of theophylline from different particulates in phosphate buffer pH 7.5 ± 0.1 (n = 3)

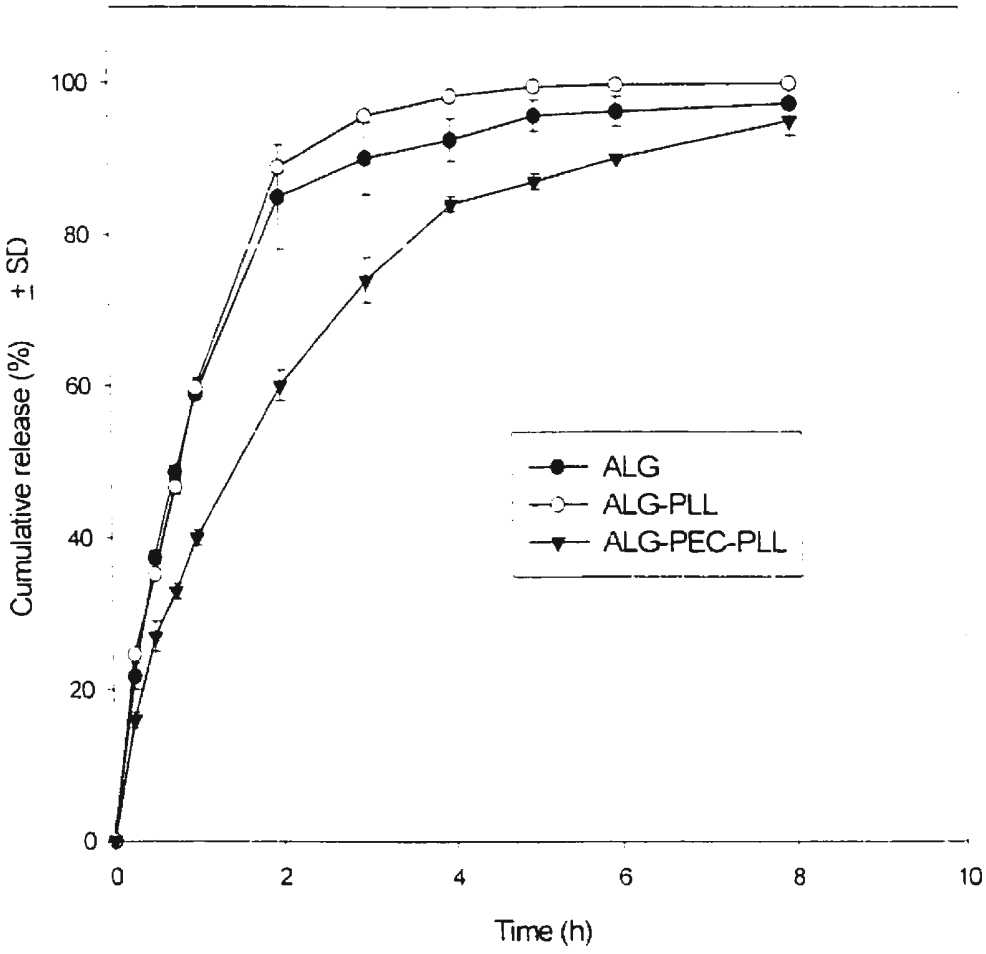


Figure 16. Cumulative release profiles of chlorothiazide from different particulates in phosphate buffer pH 7.5 ± 0.1 (n = 3)

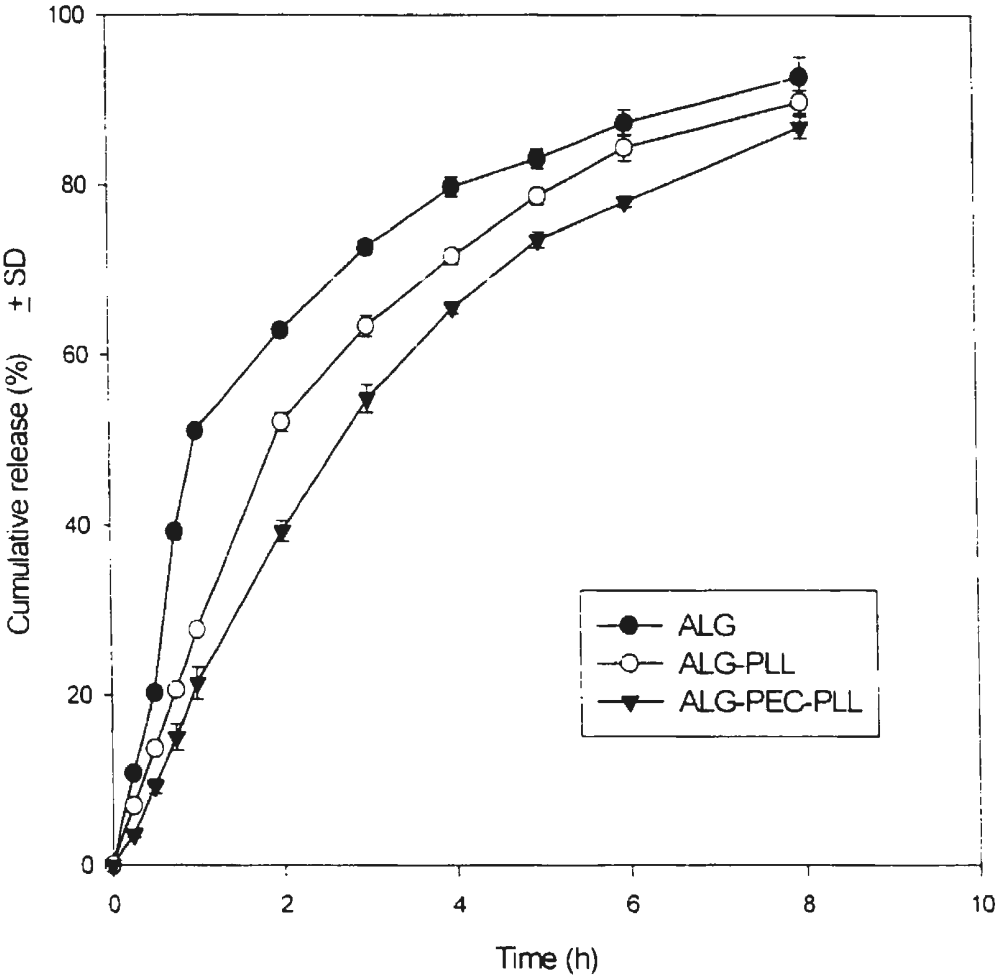


Figure 17. Cumulative release profiles of indomethacin from different particulates in phosphate buffer pH 7.5 ± 0.1 (n = 3)

From above observations, we can see that the drug release was fastest from the ALG particulates, intermediate from ALG-PLL particulates and slowest from the ALG-PEC-PLL particulates. It also showed that release of all the three drugs from ALG-PEC-PLL particulates were slower and more towards sustained release in the alkaline medium.

Contradictory results are reported in the literature with regard to the dissolution rate of ALG particulates in the acidic and alkaline media. While Bodmeier and coworkers (Bodmeier, et al., 1989) have reported a slower release of drug from ALG particulates in acidic solution than in alkaline solution, Østberg and coworkers (1994) have reported a faster release in acidic solution. Our observation was similar to the results reported by Bodmeier et al (1989). This could be attributed to the differences in the chemical composition of the sodium ALG used by the different groups. Commercially available sodium ALG comes in various grades that differ in the G/M ratios and the level of free acid functions (Timmins, et al., 1992). Since the ALG used by Bodmeier's group (1989) and us were similar and obtained from the same supplier (Sigma Chemicals, St. Louis, USA, 2% solution with a viscosity of 3,500 cps), it is reasonable to assume a similar chemical composition and hence, similar physical properties. Østberg and coworkers (1994) used sodium ALG from a different source (Pronova Biopolymer, Drammen, Norway, MW 200,000-270,000 Da) which had a different composition from that of ours.

5.4.4. Release profiles and gelation time

Some research groups have tried to increase the gelation time of ALG particulates. For example, Murata et al (1996) increased the gelation time of ALG particulates to 48 h and incubated the particulates in $CaCl_2$ at 37 °C in order to modify the release pattern. The particulates were supposed to be more robust. However, their results showed that ALG particulates disintegrated gradually in the dissolution test solution (JP XII 2nd fluid, pH 6.8), and about 90% of the drug was released after 45 min (Murata, et al., 1996). This release profile was similar to our ALG particulates, prepared by 10 min gelation time, in phosphate buffer (pH 7.5). It is evident coating ALG with PLL alone can not improve drug release profile in alkaline medium beyond a certain extent. For any significant improvement in the release profile a different approach has to be adopted. We achieved this by incorporating PEC in the particulates.

The effect of gelation time on the performance of ALG-PEC-PLL were studied. This was done by comparing the release profiles of theophylline from ALG-PEC-PLL particulates, prepared by 10 and 20 min gelation time, in 0.1M HCl solution and phosphate buffer (Figure 18). In 0.1M HCl solution the $t_{90\%}$ of ALG-PEC-PLL particulates with 10 and 20 min gelation time were 4 and 6 h, respectively, while in phosphate buffer their $t_{90\%}$ were 2 and 3.5 h, respectively. The particulates prepared by 20 min gelation time demonstrated a slower release in both dissolution fluids. Since the interaction between calcium and PEC was relatively slow, the relatively

longer gelation time would allow the interaction to complete. The results indicated that gelation time played an important role in the intensity of the ALG-PEC-PLL particulates which affected the release pattern. Therefore, we choose 20 min as the optimal gelation time for the preparation of ALG-PEC-PLL particulates.

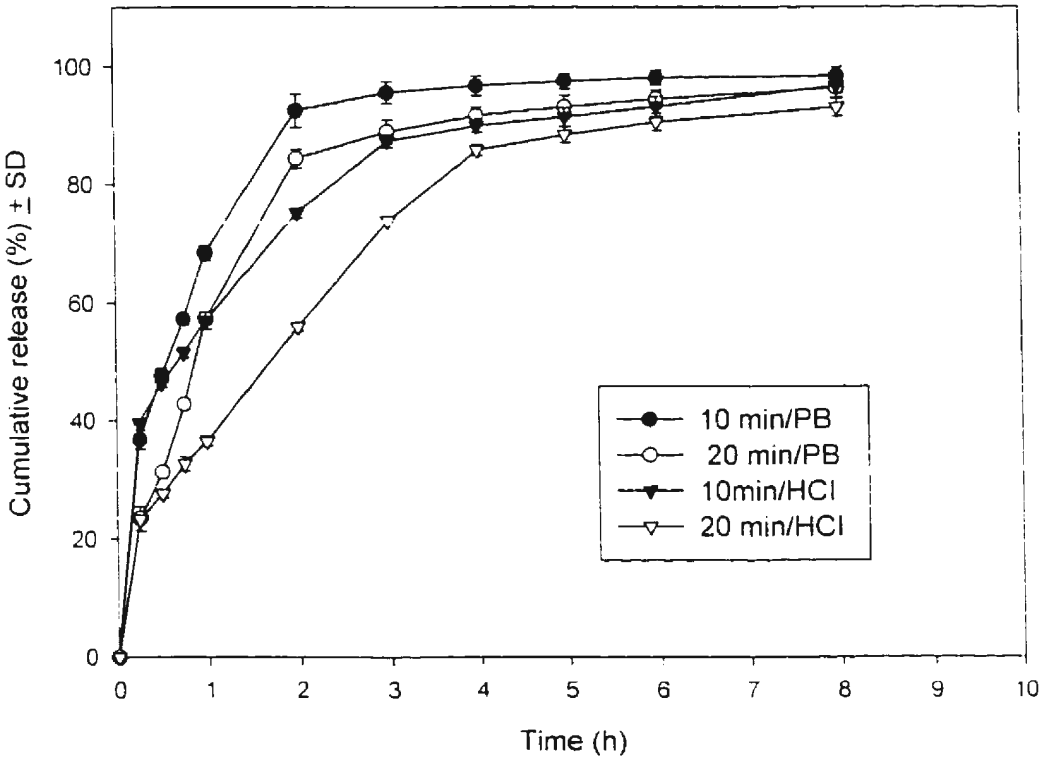


Figure 18. Cumulative release profiles of theophylline loaded ALG-PEC-PLL particulates with different gelation time (10 and 20 min) in 0.1 M HCl and phosphate buffer pH 7.5 ± 0.1 (n = 3)

5.4.5. Release profiles of ALG-PLL I and II particulates

The release profiles of ALG-PLL I (PLL coated on ALG particulates) and ALG-PLL II (PLL incorporated in the matrix of the ALG particulates and also as a coat outside the particulates) were similar. Since the reaction between polyanions and polycations itself was expected to be very rapid, the rate limiting step was probably the diffusion of PLL into the ALG gel core, where the free diffusion of PLL was restricted by the gel porosity and the relative MW of PLL. During the preparation of ALG-PLL II particulates, we assumed that at the beginning of the formation of calcium-ALG gel, the gel porosity was large, and PLL could diffused into the calcium-ALG gel core to interact with ALG inside.

We used indomethacin as the model drug in this comparative study, and the release profiles showed the slower release of the drug from ALG-PLL II particulates in phosphate buffer (Figure 19). $t_{50\%}$ of ALG-PLL I particulates was 1.92 h and $t_{50\%}$ of ALG-PLL II particulates increased to 2.8 h. Besides the micrographs of the structure of the particulates, this result also proved our assumption that ALG-PLL II particulates had cross-linkings between ALG and PLL inside the particulates. However, PLL is known for its immunogenic activity, thereby increased usage of PLL is not suitable for the delivery system. Hence, ALG-PLL II particulate still was not the optimal particulate formulation. In this study, we also used PEC as an ingredient to strengthen the central structure of the particulates. ALG-PEC-PLL particulates had a sustained drug release pattern similar to ALG-PLL II particulates.

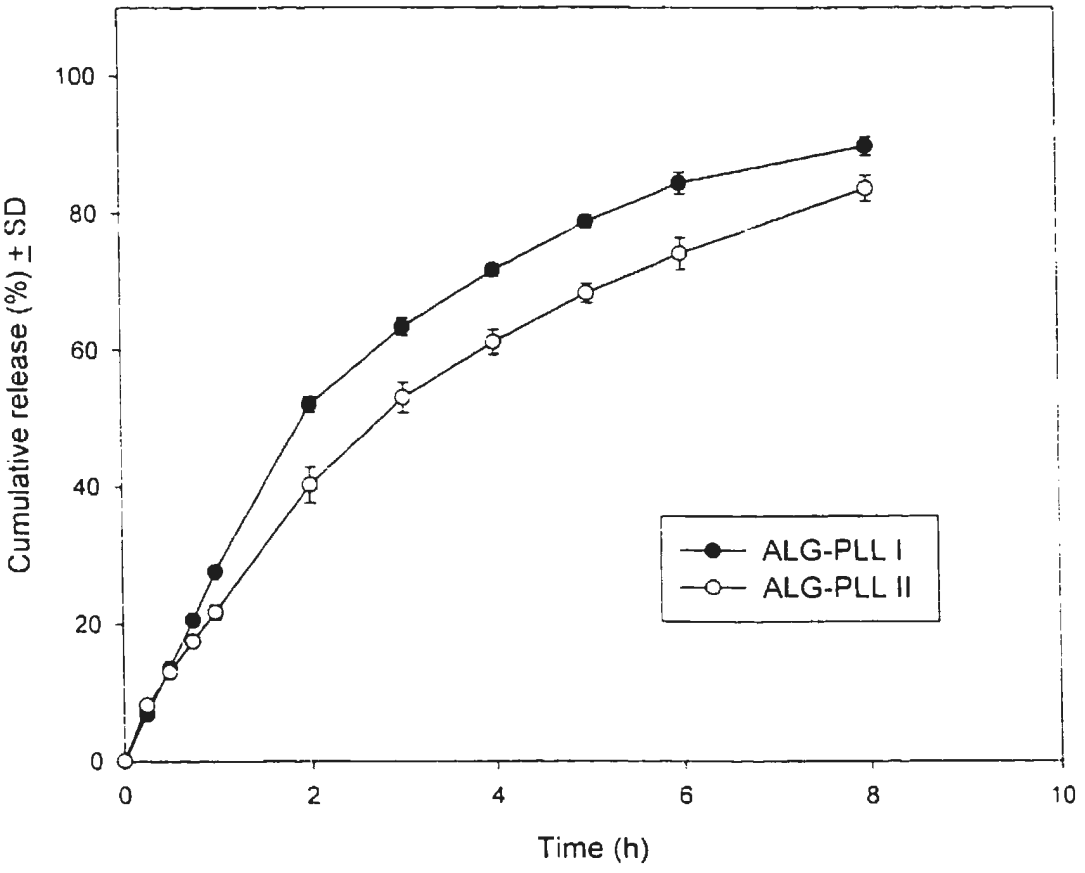


Figure 19. Cumulative release profiles of indomethacin loaded ALG-PLL I and ALG-PLL II particulates in phosphate buffer pH 7.5 ± 0.1 (n = 3)

5.4.6. Effect of MW of PLL on drug release

It has been reported that PLL of low MW produced a relatively permeable and robust membrane whereas a high MW produced capsules with the reverse characteristics (Shimi, 1991). Since the strong strength of the particulates was desirable for this drug delivery system as well as the low permeability of the particulates, we selected PLL with low MW.

ALG-PLL I particulates containing indomethacin were prepared using two different low MW of PLL, 22,000 Da and 48,000 Da. The release of indomethacin from the two particulates in alkaline buffer (pH 7.5) are given in Figure 20. There was a slight difference in their release. Particulates prepared with the high MW PLL had a faster and higher release than that with the lower MW PLL. The particulates with PLL MW 48,000 were less robust than that with PLL MW 22,000 resulting in faster release. These results were similar to the former results reported by Shimi (1991). They suggested that PLL of low MW (~22,000) was optimal in forming robust capsules which were relatively impermeable to the high MW species. For most of the particulates prepared in this project, PLL with 22,000 Da was chosen.

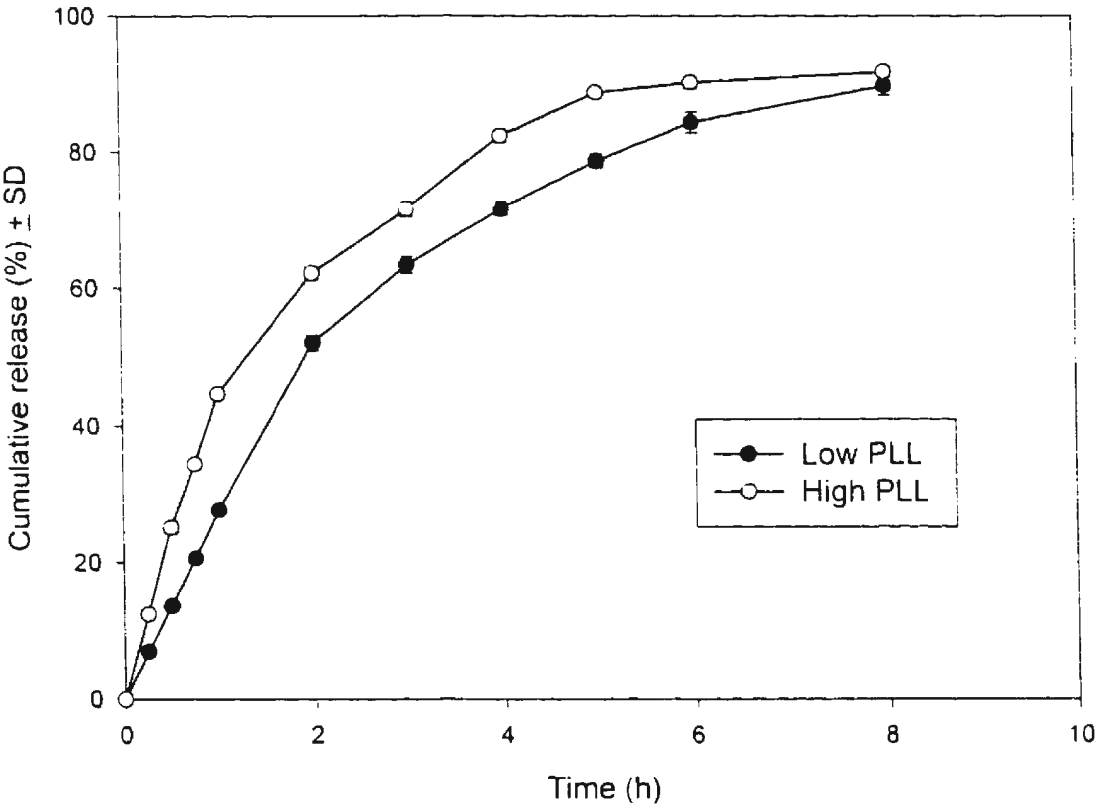


Figure 20. Cumulative release profiles of indomethacin loaded ALG-PLL I particulates using PLL with different MW in phosphate buffer pH 7.5 ± 0.1 (n = 3)

5.4.7. Effect of drying techniques on drug release

Drying technique had a profound effect on the drug release profile. Using indomethacin as the model compound, ALG and ALG-PLL I particulates were prepared by both air-drying and freeze-drying techniques. The air-dried particulates gave a higher and faster release of the encapsulated drug and the freeze-dried particulates gave a more sustained release (Figure 21). The $t_{90\%}$ of indomethacin for the air-dried ALG and ALG-PLL I particulates in phosphate buffer were 1.95 and 1.3 h, respectively, and their $t_{90\%}$ for freeze-dried ALG and ALG-PLL I particulates were 7.02 and 8.1 h, respectively. Freeze-drying technique resulted in forming honey-comb like structure inside the particulates. It is speculated that this would increase the tortuosity factor, which in turn would reduce the diffusion rate of the drugs. Freeze-dried particulates provided a slower and more sustained release which was one of the aims of this project. Therefore, for all our particulates, we used freeze-drying technique.

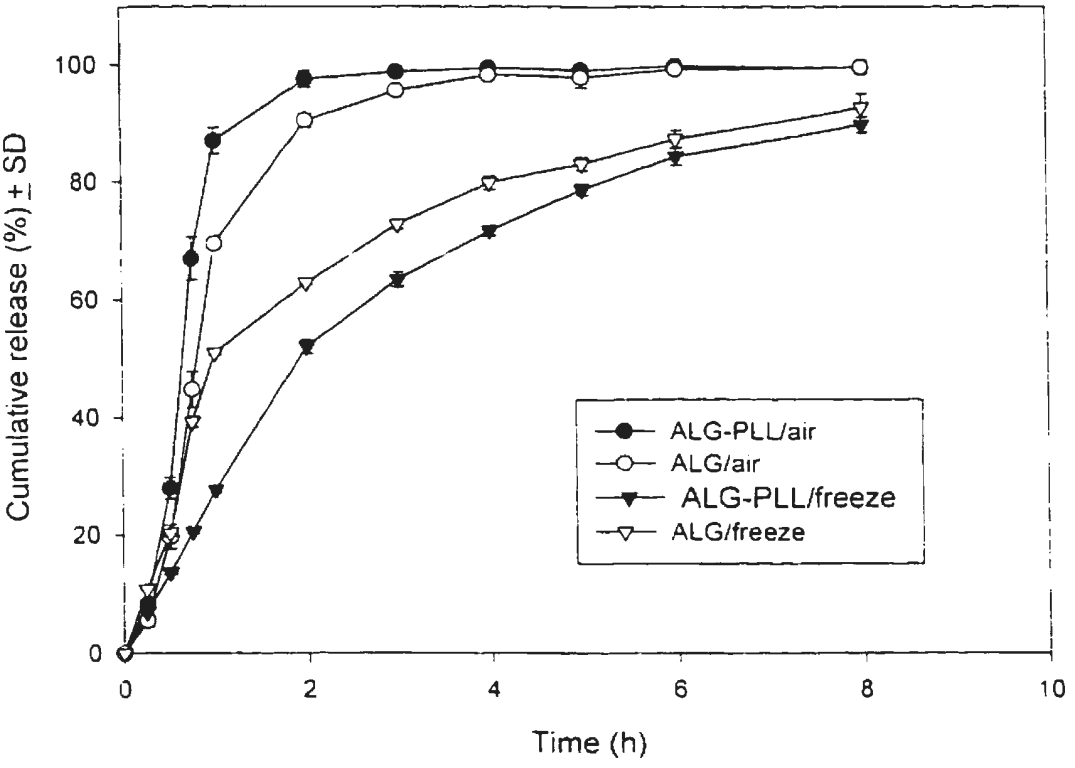


Figure 21. Cumulative release profiles of indomethacin loaded ALG and ALG-PLL particulates using air drying and freeze drying techniques, dissolution medium: phosphate buffer pH 7.5 ± 0.1 (n = 3)

5.4.8. Mechanism of drug release and release kinetics

The mechanism of release in all the particulates would be a combination of diffusion and erosion (Timmins, et al., 1992). Drug release from intact particulates would be predominantly by diffusion. ALG particulates being more labile are expected to be prone to erosion. The cross-linking in calcium-ALG gel gets destroyed as the calcium ions are exchanged in the presence of monovalent cations. Although morphologically there may not be a significant change in the swelling characteristics of the matrices, the gel structure that retards drug release gets destroyed by the loss of calcium ions (Østberg, et al., 1994). The ALG-PEC-PLL particulates with a rigid PEC gel inside would be expected to resist erosion and prolong drug release. We chose a PEC with a low degree of methoxylation (28%) that was expected to be more soluble in water and able to form a gel with calcium ions relatively easily (Ashford, et al., 1994). It should be possible to choose ALG and PEC with different chemical compositions to custom design the release profile desired for a particular drug.

To elucidate the kinetics of drug release from the particulates the percentage of drug remaining to be released (Q) were plotted as a function of time (t) in the following equations:

Q	$= k\,t$	zero order equation
$\ln Q$	$= k\,t$	first order equation
Q	$= k\,t^{1/2}$	square root time equation

Linear regression analyses were performed for the three equations of straight lines and their correlation coefficients (R^2) were determined. These values are given in Table 5. The release rate could be described as following first order or square-root time process depending on the drug load. For theophylline particulates (drug load 23-26%) the release rates were closer to first order process both in acid and alkaline solutions. For chlorothiazide (drug load 45%) and indomethacin (drug load 47%) particulates the amount of drug released in the acidic medium were proportional to the square root of time. Their release in the alkaline medium could be described either as first order or square root time process. This is in general agreement with the release mechanisms described for reservoir systems without a rate controlling membrane, in which there is an initial burst followed by a slower release that is proportional to the square root of the time (Baker, 1987).

Table 5. Correlation coefficients (R^2) calculated using different kinetic orders of drug release from particulates

In 0.1 M HCl

R^2	Zero-order			First-order			$t^{1/2}$		
	ALG	ALG-PLL	ALG-PEC-PLL	ALG	ALG-PLL	ALG-PEC-PLL	ALG	ALG-PLL	ALG-PEC-PLL
Theophylline	0.55	0.77	0.84	0.92	0.97	0.96	0.79	0.95	0.98
Chlorothiazide	0.71	0.82	0.81	0.84	0.88	0.83	0.91	0.97	0.96
Indomethacin	0.84	0.91	0.89	0.86	0.91	0.89	0.97	0.91	0.99

In phosphate buffer pH 7.5

R^2	Zero-order			First-order			$t^{1/2}$		
	ALG	ALG-PLL	ALG-PEC-PLL	ALG	ALG-PLL	ALG-PEC-PLL	ALG	ALG-PLL	ALG-PEC-PLL
Theophylline	0.34	0.38	0.69	0.91	0.86	0.91	0.58	0.62	0.88
Chlorothiazide	0.68	0.67	0.84	0.92	0.99	0.99	0.88	0.88	0.97
Indomethacin	0.79	0.89	0.93	0.97	0.99	1.00	0.94	0.97	0.96

5.5. *In vitro* bioadhesive strength studies

5.5.1. Tensile strength test

The tensile strengths of polymer films were measured using rat's intestinal mucosa as the binding surface. The modified tensiometer served the purpose quite well and helped in distinguishing between the polymer films studied. Between chitosan and sodium alginate, the former had more than twice as large tensile strength (Table 6). This could be attributed to the electrostatic force of attraction between the positively charged chitosan and the negatively charged mucosa. The bioadhesion of the negatively charged sodium alginate was attributed to the less strong hydrogen bonds. Both alginate 1.5% and 2% were tested and their tensile strengths were not statistically different ($p > 0.05$). Although the tensile strength did give us an idea of the type of binding that could be anticipated, it was less relevant to our project since the final use of the polymer was in the form of particulates and not films.

Table 6. Tensile strength measurement of polymer films when applied to rat's intestinal mucosa

Materials	n	Mean force (N/cm^{-2})	SD
Sodium ALG (2%)	8	380.15	56.32
Sodium ALG (1.5%)	8	327.92	63.14
Chitosan (1%)	8	746.48	102.79

5.5.2. Falling liquid-film test

To test the bioadhesive property of microspheres, Rao and Buri's (1989) method has been widely accepted (Pimienta, et al., 1990; Kamath, et al., 1995). We adapted this method to test our particulates and the percentage of particulates adhering to stomach/intestine was considered as a primary index of the bioadhesive property of the particulates (Table 7). The results indicated that this method was more qualitative rather than quantitative in nature. Silicon coated glass beads (0.7 - 1.1 mm in diameter), used as the control, had no bioadhesive property at all. These beads had comparable surface area to the test polymeric particulates and hence, were considered adequate as a control. In contrast, the test particulates, ALG, ALG-PLL and ALG-PEC-PLL, were 100% adhered to both the stomach and intestinal mucosae. Thus, from these results it is reasonable to conclude that ALG, ALG-PLL and ALG-PEC-PLL particulates could be expected to have good bioadhesive property.

Table 7. Percentage of bioadhesive particulates adhering to stomach and intestine segment after washing with 0.1 N HCl and phosphate buffer

Materials	% particulates adhering (n = 5)	
	Stomach	Intestine
Siliconized glass beads (control)	0	0
ALG particulates	100	100
ALG-PLL particulates	100	100
ALG-PEC-PLL particulates	100	100

5.6. Transport study

Use of Caco-2 cell monolayers for an *in vitro* estimation of the extent of drug absorption by oral route is well documented (Artursson 1990, Schipper et al 1996, Brayden 1997). Mannitol is typically used as the paracellular tight junctional marker (Brayden 1997). One of the limitations of the study is that only solutions can be test samples and drug loaded microspheres can not be used as test samples.

In this experiment, mannitol was used as the model drug for transport study. As shown in Table 8, P_{app} of mannitol ranged from 0.65 X 10^{-7} to 1.1 X 10^{-7} cm/sec in the control situation, and the P_{app} gradually increased and reached a plateau value of 1.1 x 10^{-7} cm/sec in 90 minutes. This result obtained was consistent with those reported by other research groups (Jorgensen, 1993 and Schipper, et al., 1996). The P_{app} value indicated that the Caco-2 monolayer on the filter was totally confluent and integrate.

The P_{app} of mannitol was affected in the presence of the test polymer solutions. Both test solutions contained 1% ALG and differed in their PEC content, viz., one had 1% and the other had 2% (w/v) PEC. Addition of PEC beyond 2% resulted in a very viscous solution that was difficult to handle. The time courses for the effects of test samples on the permeability of mannitol in Caco-2 monolayer were obtained by plotting mean P_{app} as a function of time. From Figure 22 , we can see for both the test samples there were significant enhancements in the apparent permeability of mannitol. In 30 minutes, the test sample with 1% PEC reached 87%

peak P_{app} value (3.0×10^{-7} cm/sec) whereas, the test sample with 2% PEC reached its peak P_{app} (3.1×10^{-7} cm/sec). There was no statistical difference ($p>0.05$) in their plateau P_{app} (range $2.9 - 3.0 \times 10^{-7}$ cm/sec) which was attained in about 60 minutes. The three-fold increase compared to the control, in P_{app} of mannitol across Caco-2 monolayers in the presence of ALG and PEC could be of significant importance in improving oral drug delivery of poorly absorbed drugs. Since mannitol is well recognized as a paracellular tight junctional marker (Brayden 1997), it may be speculated that alginate and pectin alter the cellular tight junctions, resulting in a net increase in mannitol transport across the Caco-2 cell monolayers.

Table 8. Effect of ALG and PEC on the mean permeability of mannitol across Caco-2 cell monolayers

Time (min)	Mean P_{app} value of mannitol X 10^{-7} (cm/sec) ± SD (n = 3)		
	Control	1% ALG and 1% PEC	1% ALG and 2% PEC
30	0.621 ± 0.037	2.554 ± 0.22	3.102 ± 0.328
60	0.896 ± 0.075	2.841 ± 0.079	2.517 ± 0.341
90	1.103 ± 0.027	3.03 ± 0.062	2.861 ± 0.372
120	1.087 ± 0.09	3.02 ± 0.081	2.825 ± 0.225
150	1.143 ± 0.034	2.91 ± 0.098	2.87 ± 0.226
180	1.116 ± 0.157	2.884 ± 0.046	2.849 ± 0.229

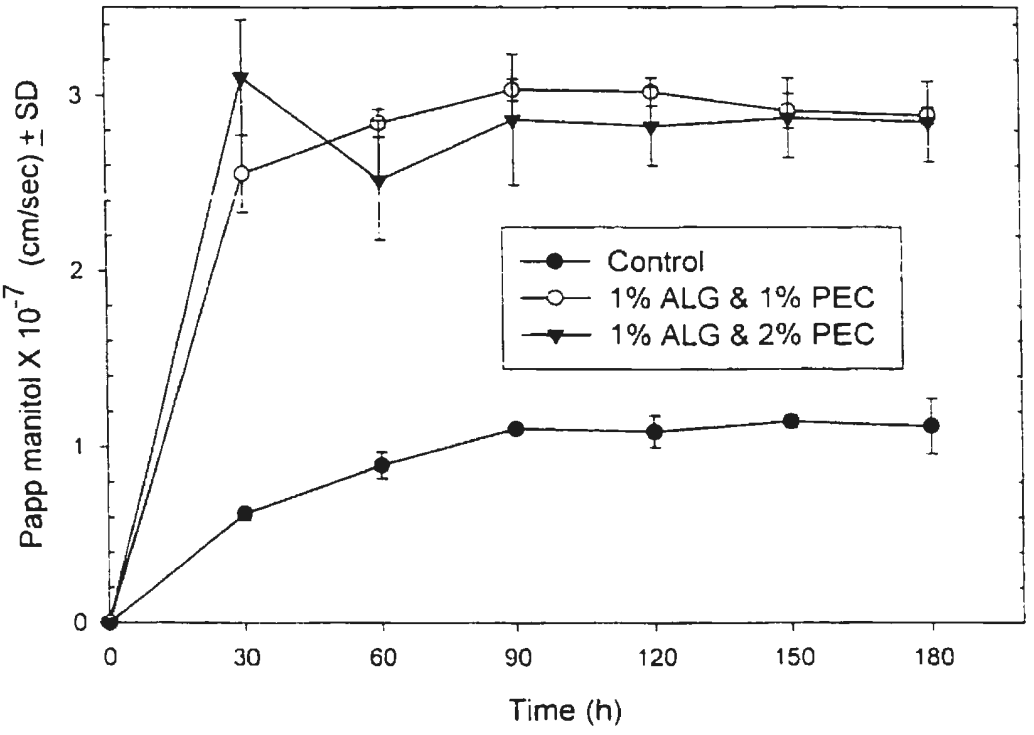


Figure 22. Transport of mannitol from ALG and PEC solutions across Caco-2 cell monolayers (n = 3)

It is generally believed that achieving an absorption enhancing effect quickly, with the simultaneous presence of high drug concentration at the site, is difficult to achieve (De Boer, 1994). In our case, the actual dosage form, viz., drug-loaded ALG-PEC-PLL particulates, would also have bioadhesive property. This would mean that these particulates could be expected to adhere to the mucosal sites in the gut and exhibit the permeability enhancing effect of ALG and PEC within 30 minutes of contact with Caco-2 monolayers. It would be reasonable to expect that our drug-loaded particulates would have the combined advantages of enhanced permeability and increased drug concentration at an absorption site.

It is reported that PLL exercises a permeability enhancing property on endothelial and epithelial tissues (McEwan, et al., 1993). The changes in permeability were more dependent on the amount of positive charge in the polycations than the type of cationic polypeptide used, and were related to alterations in membrane properties (Tzan, et al., 1993) as well as tight junctional integrity (McEwan, et al., 1993; Bentzel, et al., 1987). In the formulation of ALG-PEC-PLL particulates, PLL was presented in very small concentration and was neutralized with negatively charged ALG. Hence, we do not anticipate PLL by itself to exercise any of its permeability enhancing property.

The *in vitro* results from release, bioadhesive strength and transport studies showed that this novel particulate system, ALG-PEC-PLL had a good potential to be used as a carrier for poorly absorbed and/or erratically absorbed drugs by mucosal

route. Further work to test the drug loaded particulates in an *in vivo* animal model will be done in future in the laboratory.

6. CONCLUSIONS

In summary, the *in vitro* studies performed demonstrated that the three particulates, ALG, ALG-PLL, ALG-PEC-PLL, had good bioadhesive properties to both stomach and intestinal mucosal sections of rat's gut. ALG and PEC were used as core materials. PEC strengthened the particulates by modifying the core structure of the particulates, and PLL strengthened the particulates by forming permanent outer layer with ALG. The drug release was fastest from the ALG particulates, intermediate from ALG-PLL particulates and slowest from the ALG-PEC-PLL particulates in both acidic and alkaline dissolution medium. Among the three particulates, ALG-PEC-PLL was the most suitable particulate in sustaining drug release, and its stability in acidic medium made it a potential carrier for acid-labile drugs. Use of ALG and PEC resulted in increased absorption of mannitol across Caco-2 monolayer by about three times. Therefore, use of ALG-PEC-PLL particulates is expected to combine the advantages of sustained release, bioadhesion and absorption enhancement. This particulate system may have potential use as a carrier for poorly absorbed drugs by oral administration.

Chapter II

1. INTRODUCTION

Hydrophilic drugs including peptides and proteins in simple solution formulations usually have very low bioavailability when administered by mucosal route, such as nasal and oral route. The low mucosal absorption can be the result of poor membrane permeability, enzymatic degradation or due to the physicochemical properties of the drug. To overcome this problem, many research groups have investigated to use absorption enhancers to facilitate the transport of drugs and improve their bioavailability (Wheatley, et al., 1988; Aungst, et al., 1988a and O'Hagan, et al., 1990). Additionally, the rapid removal of the drug from the absorption sites by mucus turnover also results in the low bioavailability for this class of drugs. It is conceivable that the drug must have sufficient time to achieve maximal contact with the membrane before efficient transport can occur. It has been known that BDDS can be retained in contact with the mucosa for a prolonged period and the process of absorption can be improved because of the interaction of bioadhesive systems with the mucus. The development of bioadhesive particulates is discussed in Chapter I. In this chapter, we will describe another approach, using absorption enhancers to increase the low permeability of the epithelial membrane. Eventually the two approaches would be combined so that a suitable enhancer could be carried by the particulate carriers. These particulate carriers would be novel and

would be expected to have particulates' protective and bioadhesive properties and enhancer's permeation enhancing property. These unique particulates would then be very suitable to deliver the delicate peptide and protein drugs through mucosal routes that would be more patient compliant.

1.1 Absorption enhancers

Absorption enhancers are compounds that can increase the mucosal membrane permeability and hence the absorption of poorly permeable drugs that are coadministered with the enhancer. In general, enhancers improve the absorption of the drug by one or several of the following mechanisms: i) increase the membrane fluidity and reduce the viscosity of the mucus layer, thereby increase membrane permeability (Yamamoto, et al., 1990 and Hussain, et al., 1990); ii) inhibit proteolytic enzymes at the absorption site (Yamamoto, et al., 1990 and Gros, et al., 1990); iii) transiently loosen the tight junctions between certain epithelial cells (Lasdun, et al., 1989); iv) increase paracellular or transcellular transport (Lasdun, et al., 1990); v) dissociate protein aggregation (hanson, et al., 1989); vi) initiate membrane pore formation (Lejczak, et al., 1989). These mechanisms were summarized in Table 9. Various types of absorption enhancers have been evaluated. Examples include surfactants, steroidal detergents (bile salts), salicylate and analogues, EDTA and other chelators and Enamines/N-acyl amino acids (Aungst, 1994). The results have shown that they do affect membrane permeability, and the proposed mechanisms are enumerated in Table 10. However, most absorption enhancers are fraught with the problem of toxicity such as, cell erosion,

excessive mucus discharge, membrane protein removal, etc. (Gizurarson et al., 1990; Hermens et al., 1990; Jorgensen. et al., 1993). The practical use of these absorption enhancers required toxicological assessment and in particular, their potential to cause irritation and their effect on the integrity of the epithelium. For instance, steroidal surfactants have been by far the most frequently evaluated for facilitating the nasal absorption of peptide and protein drugs, and some steroidal surfactants even have been examined in clinical trials. However, in most cases, there were adverse reactions such as stinging or burning sensation, discomfort, or a certain degree of pain, indicative of irritation potential. Thus, although many compounds have been studied to determine an acceptable balance between absorption enhancing and toxicological effects, only a few have been found acceptable (Ennis et al., 1990; Chandler, et al., 1991). As result the quest for a good absorption enhancer is still continuing. Researchers in the field of bioadhesion and absorption enhancers are now focusing on natural compounds (Lehr 1994), in search of novel bioadhesives and enhancers. Although melittin is generally considered as toxic substance, its action as a membrane fusion substance has a strong concentration dependency (Ohki et al. 1994). Ours is a curiosity-driven research and we envisage that the concentration of melittin could be optimized, and used as an effective absorption enhancer, without any undesirable effects on membranes.

Table 9. Possible mechanisms of enhancing transmucosal absorption.

A.	Barrier disruption (permeability enhancement)
	1. Mucus layer
	2. Transcellular
	3. Paracellular (tight junctions)
	4. Epithelial cell loss
B.	Altered physicochemical properties of drug in vehicle
C.	Inhibition of metabolism

Table 10. Proposed mechanisms of mucosal membrane permeability enhancement for various enhancers (Aungst, 1994)

Surfactants
Extraction of membrane proteins or lipids
Membrane fluidization
Steroidal detergents
Extraction of membrane proteins or lipids
Membrane fluidization
Reverse micellization in membrane, creating aqueous channels
EDTA and other chelators
Chelates calcium at tight junctions, increasing paracellular transport
Salicylate and analogues
Decreases nonprotein sulfhydryls in membrane
Interacts with membrane proteins
Calcium chelation
Enamines/N-acyl amino acids
Surface-active: membrane fluidization or extraction of membrane proteins or lipids
Calcium chelation

1.2. Melittin

In this study to improve the membrane permeability of poorly absorbed drugs, we have investigated the possibility of using melittin as a novel absorption enhancer. Melittin, the major active ingredient of honey bee venom, *Apis mellifera*, consists of 26 amino acids (sequence see Figure 23). Similar to other membrane-binding peptides and membrane proteins, melittin is predominantly hydrophobic. The peptide has a net charge of +6; four of these charges are in the highly basic C-terminal tetrapeptide sequence Lys-Arg-Lys-Arg. There are no acidic groups in melittin. Although it has high proportion of hydrophobic sidechains, melittin is very soluble in water (> 250 mg/mL). Melittin reaches membranes through the aqueous phase. In aqueous solution melittin adopts different conformations and aggregation states which result from two opposing forces: the hydrophobic effect promotes self-association, and the high positive-charge density of melittin resists self-association. Its conformation depends on several factors including peptide concentration, pH, ionic strength and the nature of the negative countreion (Lauterwein, et al., 1980; Quay, 1983; Dawson, et al., 1978 and Knoppel, et al., 1979).

Melittin has been used as a model compound for studying the general features of membrane proteins and their interactions with membrane lipids (DeGrado et al, 1982; Tosteson et al 1987; Stankowski et al., 1991 and Benachir et al., 1995). There is no consensus on the mechanism of interaction of melittin with membrane lipids because several actions of melittin on membranes have been suggested, such as hemolytic activity, voltage-gated channel formation and melittin-induced bilayer

micellization and fusion, which are not necessarily related (Dempsey, 1990). It is used in homeopathy for treating allergies, inflammation and burns (Picard et al., 1995). Its action as a membrane fusion peptide has been reported to be very efficient and have a strong concentration dependency (Thomas, et al., 1994). Based on these results, we envisage that the concentration of melittin could be optimized, and it could be used as an effective absorption enhancer without any undesirable effects on membranes.

Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-Gln-NH

Figure 23. Amino acid sequence of melittin

It is known that both metabolism and low membrane permeation can contribute to the poor bioavailability of many drugs including peptides and proteins administered by mucosal routes. To address the problem of metabolism, many delivery systems and enzymatic inhibitors have been investigated. Particulate delivery system is one of these applications which has been extensively studied for the delivery of peptides and proteins (Wood, 1982; Wang, et al., 1991; Sah, et al., 1994; Yeh, et al., 1995 and 1996). One of the advantages of the particulate system is that it could protect the peptides and proteins from metabolism occurring at the surface of the epithelial membrane to some extent. By incorporating melittin, which is itself a peptide, along with the drug in a particulate delivery system, we anticipate that a sufficient quantity of the drug and the enhancer will be delivered at the absorption site.

1.3. Caco-2 cell line for the study of absorption enhancers

The efficacy of absorption enhancers on the permeability of epithelial membrane has been investigated in numerous *in vivo* and *in vitro* models. Various epithelial cell lines have been reported to be used as *in vitro* model membranes for the study of drug transport and metabolism at biological barriers. The *in vitro* cell culture model has many advantages over conventional methods for the study of absorption enhancers, which include rapid assessment of the permeability of a drug, elucidation of mechanisms of the various pathways in transepithelial transport, easy construction of dose-response curves with high degree of reproducibility.

Among these, the Caco-2 cell line, originating from a human colonic adenocarcinoma, is the most commonly used cell line for absorption enhancer studies because it resembles the human intestinal epithelial membrane in that it differentiates to express structures similar to that of small intestinal villi. It has been widely used to study drug transport mechanisms (Artursson, 1990a, 1990b; Thwaites et al., 1996; Rubas et al., 1996; Yamashita et al., 1997), cytotoxicity (Jørgensen, et al., 1993 and Schipper, et al., 1996;), and absorption enhancement (Andernberg et al. 1992, 1993; Schipper et al., 1996 and Werner et al., 1996), which have been described in section 1.5.2.4., Chapter I.

In this study, we investigated the *in vitro* cytotoxicity of melittin on the Caco-2 cell membrane using MTT assay to establish its safe concentration range. We also demonstrated the effective and safe use of melittin as an absorption enhancer to transport mannitol across Caco-2 cell monolayers.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals and equipments

Melittin (79% purity), mannitol, Dulbecco's Modified Eagle's Medium (DMEM, with 4.5g/L glucose), Non-essential amino acids (NEAA), and Trypsin (0.25%)-EDTA (1 mM) were all purchased from Sigma Chemicals, MO, USA. Fetal calf serum (FCS), penicillin-streptomycin (5,000 I. U./mL and 5,000 μg/mL) and sodium bicarbonate solution (7.5%, w/v) were obtained from Gibco BRL, Life Technologies, NY, USA. Potassium phosphate monobasic, dibasic were purchased form Fisher Scientific Co., NJ, USA. Scintillation cocktail (Formula 989®) was purchased from Packard Instrument Company Inc. Meriden, CT, USA. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) was purchased from Bio-Rad Laboratories, Inc. CA, USA.

Water-jacketed incubator (Isotemp® Incubator Model 546, Fisher Scientific Co., NJ, USA) was employed to provide a suitable environment for cells growth. Hund Wetzlar Wilovert® microscope (Wetzlar/Nauborn, Germany) and Hemacytometer (Hausser Scientific, PA, USA) were used for cells counting. Tissue culture flasks and pipettes were purchased from Fisher Scientific Co., NJ, USA. Ninety six-well tissue culture plates (Costar, MA, USA) were used for MTT assay. Transwell® 6-well plate inserts (pore size 0.4 μm, Costar, MA, USA) were used for

the transport study.

2.1.2. Instruments

A Microplate-Reader® (Model 550, Bio-Rad Laboratories, Inc. CA, USA) was used to determine the absorbance in MTT assay, and a liquid scintillation counter (Beckman LS 5000 TD, CA, USA) was used to measure the radioactivity of ^{14}C samples.

2.1.3. Radioisotope

^{14}C-mannitol (specific activity: 50 mCi/mmol, 0.25mCi/mL) in ethanol solution was purchased from Sigma Chemicals, MO, USA.

2.1.4. Cell line

The Caco-2 cell line was obtained from American Type Culture Collection (MD, USA) at passage 17 and used for MTT assay and transport study.

2.2. Methods

2.2.1. Preparation of Caco-2 monolayers

For MTT assays, the cells (passages 26-29) were harvested with trypsin-EDTA and seeded in 96-well tissue culture plates at a density of 10^4 cells/well, and cultivated with completed DMEM. About 100 μL DMEM was added into each well and was changed every 48 hr. The cells were incubated at 37 °C, in an atmosphere

of 5% CO_2 and 95% air for 21 days before starting MTT assays.

For the transport studies, preparation of Caco-2 cell monolayers was the same as that discussed in section 4.2.6.2. Chapter 1. Briefly, Caco-2 cells (passages 26-29) were cultivated on polycarbonate filters of Transwell® inserts at a seeding density of 3 $x10^5$ cells/well. After 21-23 days of seeding, the cells were suitable for conducting the transport study.

2.2.2. MTT assay

The effect of melittin on intracellular dehydrogenase activity, which has been used to indicate the viability of the cells, was determined by the MTT method. MTT is a tetrazolium salt that can be cleaved by mitochondrial dehydrogenases in living cells to give a dark-blue formazan product (Lappalainen, et al., 1994). Damaged or dead cells show reduced or no dehydrogenase activity. Thus the amount of formazan produced during the MTT assay could be used as an index of melittin's effect as a cellular toxin. For the MTT assay, the cells were first incubated with various concentrations of melittin ranging from 0.61 µM to 5.45 µM in 100 µL DMEM for a period of 2 hours at 37 °C. Then they were washed with 100 µL phosphate buffer saline (PBS), and incubated for another 4 hours at 37 °C with 100 µL of MTT in PBS. Thereafter, the solution was removed and formazan crystals in the cells were dissolved by adding 200 µL dimetheyl sulfoxide (DMSO). The resulting color was measured at 570 nm using a microplate reader. The control consisted of 100 µL DMEM without melittin.

2.2.3. Transport study of ^{14}C- mannitol

The procedure was similar to that used for the transport study of the polymer solutions discussed in Section 4.2.6.4-6. Chapter I. Briefly, in all the transport experiments, DMEM was used as the solvent and medium. It was added to the receiver compartment of the Transwell® containing the cultured Caco-2 cell monolayers. Mannitol (0.1% w/v) was dissolved in DMEM and was spiked with ^{14}C-mannitol (0.25 μCi/ml) for liquid scintillation assays. To this solution different amounts of melittin were added to obtain four test solutions, 0.34 µM, 0.68 µM, 1.2 µM and 1.5 µM. The control solution consisted of only mannitol in DMEM. The test solution was added to donor compartment, and the transport studies were conducted for a duration of 3 hours. Aliquot samples (100 μL) were taken from the receiver side every 30 minutes, which was replenished with fresh DMEM. The donor side solutions were also analyzed to determine the total radioactivity of the test solution applied to the cells. To 100 μL of the aliquot samples, 5 mL scintillation cocktail were added before quantifying in the liquid scintillation counter. The background radioactivity was determined using DMEM. The P_{app} was calculated using the equation described in Section 4.2.6.6. Chapter I, and the mean P_{app} ± SD were determined.

2.2.4. Statistical Evaluations

In this study, Sigmaplot® 4.0 and SPSS 7.5 for Windows program were used to analyze the data. Statistical differences were investigated using one-way ANOVA

followed by Bonferroni t-tests for multiple comparisons. Differences between group means were judged significant at $p < 0.05$. Results are expressed as mean ± SD (n= 6 for MTT assay and 3 for mannitol transport study).

3. RESULTS AND DISCUSSION

3.1. MTT assay

Melittin produced a concentration dependent decrease in the dehydrogenase activity on Caco-2 cell monolayers (Figure 24). For making comparative analysis, the dehydrogenase activity of the control sample was considered 100%. Compared to control there was no significant ($p>0.05$) decrease in the enzyme activity for melittin concentrations below 2.42 µM. Melittin concentrations above 2.42 µM caused a significant ($P < 0.05$) decrease in cellular dehydrogenase activity and the 4.5 µM solution produced 50% inhibition (IC_{50}) of the dehydrogenase activity. From these results, we could infer that a melittin concentration below 2.42 µM would produce negligible cytotoxicity and could be considered safe. However, to ensure further safety we limited the highest melittin concentration to 1.5 µM for the drug enhancement study.

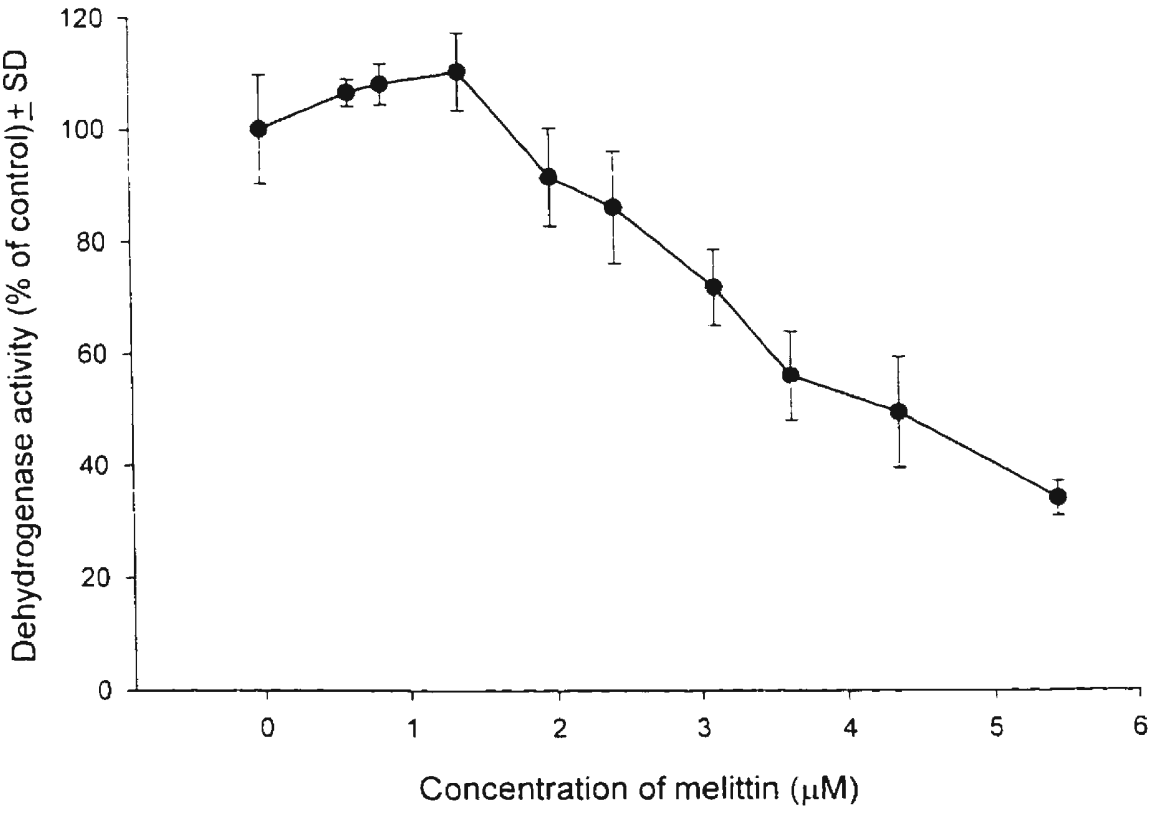


Figure 24. Effect of melittin on intracellular dehydrogenase activities (n = 3)

3.2. Transport study of ^{14}C- mannitol across Caco-2 monolayers

Mannitol is typically used as a paracellular tight junctional marker (Brayden, 1997). The cumulative percentage of mannitol transported across the Caco-2 cell monolayers are indicated in Figure 25. The concentrations of melittin chosen for transport study were well within the safety limit (2.42 μM) deduced from the MTT assay. With 1.20 μM and 1.50 μM of melittin, the percentage of mannitol transported across Caco-2 cell monolayers in three hours increased by 3.5 fold compared to the control. With the lower concentrations of melittin viz., 0.34 and 0.68 μM, there were no significant ($P>0.05$) differences from the control. The P_{app} values of mannitol were calculated at different time intervals (Figure 26). The P_{app} for mannitol from the control sample ranged between 0.8-1.0 x 10^{-7} cm/sec which is in agreement with the published report of Schipper et al. (1996). This is also an evidence for the confluence and cellular integrity of the Caco-2 cell monolayers. The P_{app} values obtained with the control solution, and with 0.34 μM and 0.68 μM of melittin solutions were not statistically ($P>0.05$) different. However, the P_{app} for 1.20 and 1.50 μM of melittin solutions were similar and statistically different ($P<0.05$) from the control, 0.34 μM and 0.68 μM solutions. From the transport study it is apparent that it is necessary to reach a critical threshold concentration of melittin in order to evoke its absorption enhancing effect. Permeabilization of membranes at low concentrations of melittin has been attributed to alteration of the organization properties of membrane lipids (Dempsey, 1990). Other investigators have shown that melittin induces a lamellar (bilayer) to micellar phase transition in model phospholipid

membranes when the melittin concentration in the bilayers is between 3 and 5 mol% (Dempsey, et al., 1987a and 1987b). We speculate that a similar mechanism is responsible for the increased permeability of Caco-2 cells. It is clear from our toxicity determinations that these transitions are not extensive enough to cause cell lysis in this cell line.

Since this critical concentration of melittin (1.20-1.50 μM) for observing an absorption enhancement effect is within the safe concentration limit (2.42 μM), it will be worthwhile to pursue further the use of melittin as a viable absorption enhancer.

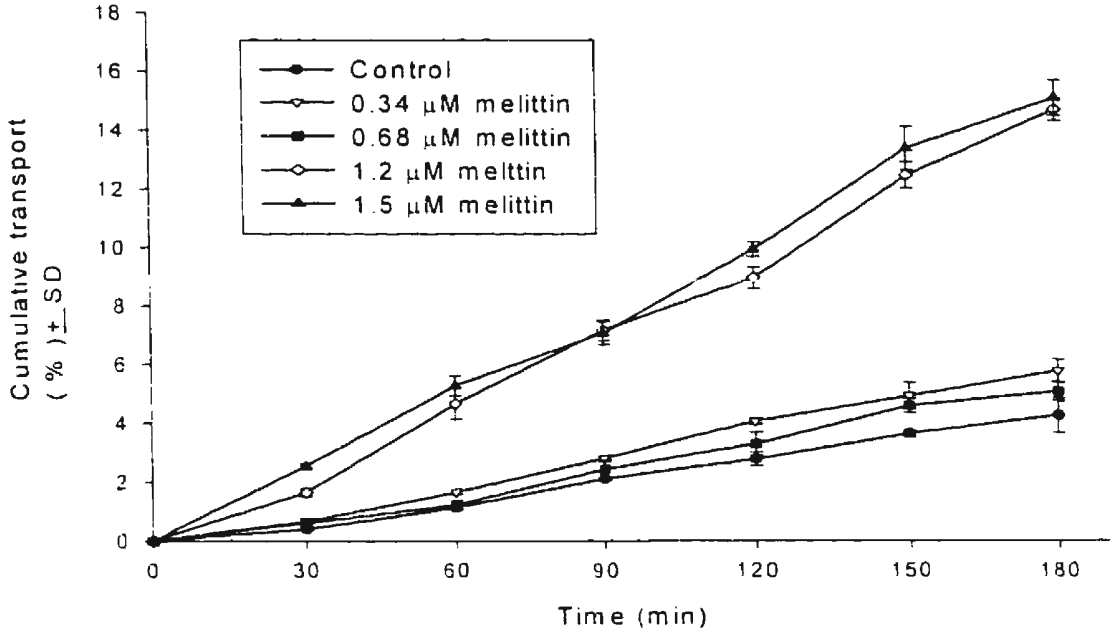


Figure 25. Cumulative transport of 14C-mannitol through Caco-2 monolayers during incubation with melittin and control solutions (n = 6)

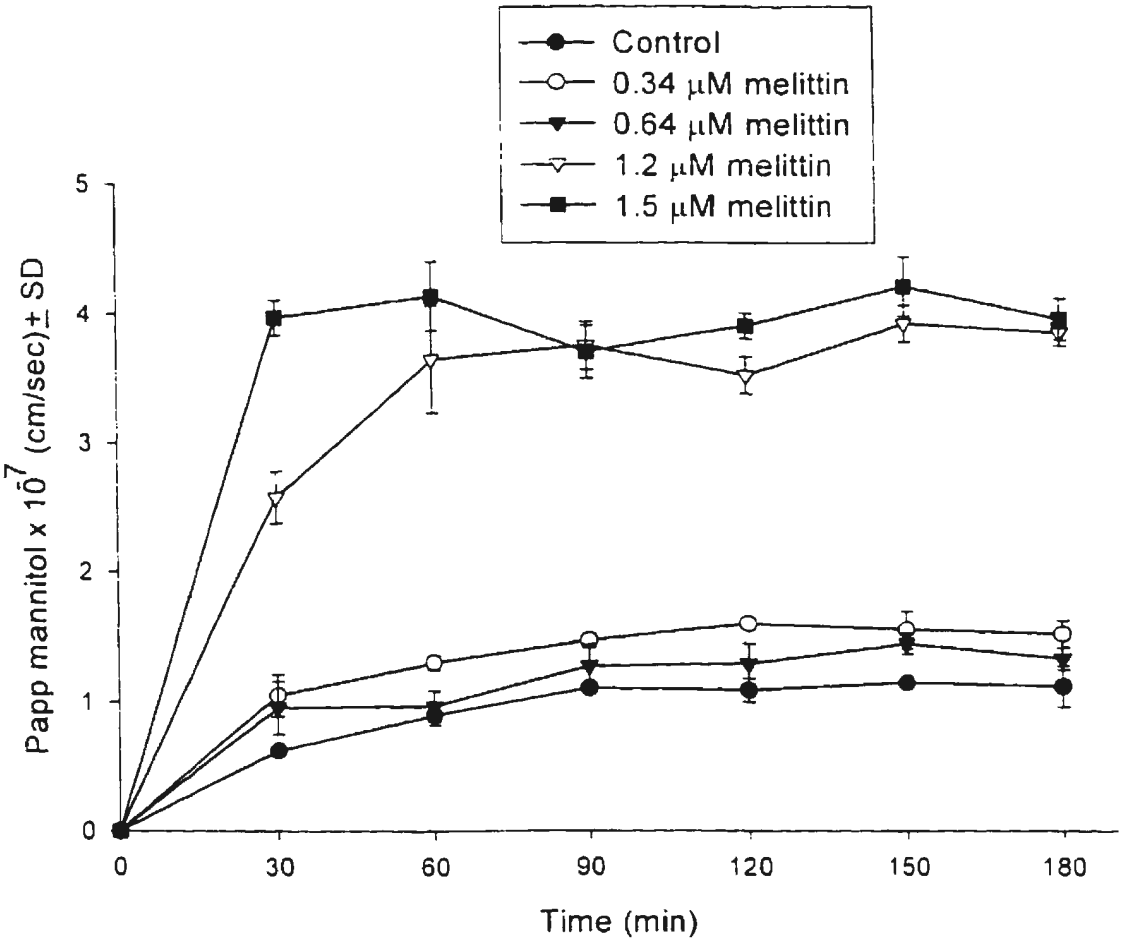


Figure 26. Time courses for the effects of melittin on the permeability of mannitol in Caco-2 monolayers (n = 3)

4. CONCLUSION

This study indicated the possibility of using melittin as an absorption enhancer. At a concentration below 2.42 μM it did not show any cytotoxic effects on Caco-2 cells. 1.20-1.50 μM of melittin solution was able to increase the transport of mannitol across Caco-2 monolayer by a factor of 3.5. Further studies to determine the long-term safety of melittin are required to be done to prove its utility as a safe absorption enhancer.

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