A survey of literature on the research topic makes the researcher familiar and more knowledgeable with the existing studies and provides further information, which helps to focus on a particular problem and lay the foundation for newer and greater knowledge. It creates an accurate picture on the information presently available on the subject.

A review of literature for the present study is to gather knowledge about:

a) a major silent killer disease such as hepatitis B.

b) a mucosal immune system, which plays a major role in oral immunization.

c) a suitable and effective delivery system with different biodegradable polymers and adjuvants for oral administration of vaccines.

HEPATITIS B – THE DISEASE

Hepatitis B is a serious liver disease, caused by hepatitis B virus. The disease is transmitted through human body fluids such as blood and serum. It is an alarming public health problem worldwide. Its methods of transmission include through mother to baby (perinatal), sexual contact and the use of improper injection techniques. More than two billion amongst the population alive today, would have been infected at some time or other in their lives by the hepatitis B virus (HBV) and approximately 350 million of them are the carriers of the chronically infected disease (Kane, 1995). Out of these 25-30% would die as a consequence of the infection (WHO, 2003). These carriers are at high risk of this serious illness and death from cirrhosis of liver and/or primary liver cancer would kill more than one million of them, per year. They also constitute a reservoir of infected individuals, who perpetuate the infection from generation to generation.
The Hepatitis B - The Virus

Hepatitis B is a DNA Virus of the hepadnaviridae family of viruses. It replicates within infected liver cells (hepatocytes). The hepatitis B virus particle (virion), also known as the Dane Particle, consists of an inner core and an outer surface coat.

Virions are 42nm in diameter and possess an isometric nucleo-capsid or ‘core’ of 27 nm in diameter, surrounded by an outer coat approximately 4nm thick. The
The protein of the virion coat is termed ‘surface antigen’ or HBsAg. It is sometimes extended as a tubular tail on one side of the virus particle. The surface antigen is generally produced in vast excess, and is found in the blood of infected individuals in the form of filamentous and spherical particles. Filamentous particles are identical to the virion ‘tails’ – they vary in length and have a mean diameter of about 22nm. The outer surface coat surrounds an inner protein shell, composed of HBc protein (hepatitis-B core protein) or HbcAg (hepatitis B core antigen). This inner shell is referred to as the core particle or capsid. Finally, the core particle surrounds the viral DNA and the enzyme, DNA Polymerase.

![Dane particle and sphere](image)

**Figure 3**: Detailed structure of hepatitis B virion

**Epidemiology**

The researchers have divided the world into areas of high, intermediate, and low HBV endemicity. This division is based on the prevalence of HBV markers and the primary modes of HBV transmission.
Areas of high endemicity include those, where most of the population becomes infected with the virus. In areas of high endemicity, the most common route of transmission is perinatal or the infection acquired during the pre-school years. Africa, Asia, East of the Indian sub-continent, the Pacific Basin, the Amazon Basin, the Arctic Rim, and the portions of the Middle East, Asia Minor, and the Caribbean are the areas of high endemicity.

![Figure 4: Geographical distribution of hepatitis B infection](image)

Areas of ‘intermediate endemicity’ generally have an HBV carrier prevalence of 2 to 5%, and 30 to 50% of the population has serological evidence of prior HBV infection. In these areas both child to child and adult transmission occur. Acute viral hepatitis with jaundice is a major cause of morbidity.

In the areas of low endemicity, perinatal and child to child transmission is relatively uncommon, and most infections occur in adults through sexual activity, needle sharing during drug abuse, or during occupational exposure to blood.
Epidemiology in India

India is in the intermediate zone of endemicity with a prevalence of 4.7% and contributing to 10-15% of the total infected population, worldwide. In India, 2,50,000 infants get infected every year and 90% of them, develop chronic infection. There are wide variations in social, economic, and health factors in different regions of India, which may explain the difference in HBV carrier rates, reported by investigators in different parts of the country. Professional blood donors constitute the major high-risk group for HBV infections in India, with a hepatitis B surface antigen positivity rate of 14%.

Signs and Symptoms

The incubation period averages six weeks, but may be as long as six months. About 30% of persons have no sign or symptoms. Signs and symptoms are less common in children than adults. However a large proportion of children may become chronic carriers, compared to adults. Any common symptoms, that might show up, are usually flu-like symptoms, including fever, fatigue, muscle or joint pain.

Severe symptoms include yellow eyes and skin called jaundice and a bloated or swollen stomach. The symptoms may last several weeks or months. A laboratory blood test is required for confirmation. Most acute infection in adults is followed by a complete recovery. However, many children become chronic carriers. People, who recover from acute hepatitis and not becoming chronic carriers, are protected from being infected again, throughout their lives.

However, to a limited number of the population, the acute infection could be severe and lead to death. The most serious complications including chronic hepatitis, cirrhosis, liver failure, and liver cancer, occur in people with chronic infection.
Mode of Transmission

The hepatitis B virus is carried in the blood and other body fluids. The virus is present in the blood, saliva, semen, vaginal secretions, menstrual blood, and to a lesser extent, perspiration, breast milk, tears and urine of infected individuals. The highly resilient virus, is easily transmitted through contact with infected body fluids. It is usually spread by contact with blood in the following ways.

1) Perinatal (mother to child) transmission is one of the most common and serious mode of HBV transmission. Perinatal transmission occurs from mothers, who are positive for both the hepatitis B surface antigen (HBsAg). More than 90% of these women are chronic HBV carriers, although those acutely infected with the virus, during pregnancy, may also transmit to their children. Infected newborns rarely develop acute hepatitis, although reports of fatal fulminant hepatitis have been reported. These carriers form a pool of infectious individuals, who will infect others in the community and eventually, their own offspring.

2) Child to child transmission, also called horizontal transmission, is responsible for majority of HBV infections and their carriers. Transmission between children, occurs during social contact through cuts, scrapes, bites and scratches. The skin lesions, such as, impetigo, scabies, abrasions and infected insect-bites, play an important role. These lesions provide a route for the virus to leave the body of the infected children, as well as, one to enter into the body of susceptible children.

3) Transmission through an unsafe injection, needle-prick or reuse of unsterile needles, and use of contaminated needles (Kane et al., 1999; Simonsen et al., 1999; Jodar et al., 2001) and other medical and dental equipments. Survey in developed countries have revealed, that upto 30% of injections used for immunization, are not found sterile. Disposable
syringes are reused and reusable syringes are improperly sterilized, resulting in a significant risk of transmission of blood-borne pathogens. Auto-destruct syringes and single use pre-filled devices, can reduce the transmission by averting inappropriate use. In some western countries, needle-sharing by drug abusers is also causative. If sterile needles are not used, it is possible to transmit hepatitis B, through body-piercing, tattooing, drug injection and acupuncture. The hepatitis B virus can also be transmitted by sharing razors, tooth brushes, nail-clippers and earrings.

4) Transmission during sexual intercourse through contact with blood or other body fluids.

Hepatitis B is the major infective occupational hazard to the following:

- Healthcare workers.
- Emergency personnel.
- Staff of jails, prisons and group homes.

Hepatitis-B is not transmitted casually. It cannot be spread through sneezing, coughing, hugging or eating food prepared by someone, who is infected.

**Diagnosis**

A simple blood test could easily diagnose hepatitis B infection. The test looks for antigens and antibodies in blood. If recently infected, it will take 4 to 6 weeks, before the virus could be found in the blood.

A blood test will show whether the patient:

- Has been infected previously.
- Has an acute hepatitis B infection, presently.
- Has recovered from a past infection and is now immune.
- Has a chronic hepatitis B infection and the virus is present in the blood.
- Is immune to hepatitis, due to vaccination.

**Serology**

The first readily identifiable marker of acute HBV infection is the presence of HBsAg. It appears before transaminase levels are elevated and remains detectable by conventional means for a few weeks to months. Persistence of HBsAg for more than six months suggests the development into the chronic carrier state.

The risk of viral persistence and development of chronic carrier state is related to age at the time of initial infection. While in adults this risk has been estimated to be 10%, it may exceed to 60% in infants. The reasons for non-clearance of HBsAg and the development of chronic carrier state are poorly understood. Abnormalities of cytokine function and aberrations of immune mechanisms are believed to play a prominent role.

**Table 1: Risk of developing into the carrier state in relation to age**

<table>
<thead>
<tr>
<th>Age of infection</th>
<th>Carrier risk (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birth</td>
<td>90</td>
</tr>
<tr>
<td>1-6 months</td>
<td>80</td>
</tr>
<tr>
<td>7-12 months</td>
<td>60</td>
</tr>
<tr>
<td>1-4 years</td>
<td>35</td>
</tr>
<tr>
<td>Adults</td>
<td>10</td>
</tr>
</tbody>
</table>

**Treatment**

Antiviral therapy is not warranted for acute hepatitis B, because the infection will resolve on its own, in most symptomatic individuals. Acute liver failure, which, as mentioned above, may develop in less than 0.5% of adults with acute hepatitis B. Potential candidates for anti-viral treatment are those with active
viral reproduction, elevated liver tests and no signs of advanced liver disease. The immediate goals of anti-viral therapy in chronic hepatitis B viral infection are to suppress viral reproduction and improve liver tests. The ultimate goals are to prevent further liver injury and scarring, stop the progression towards cirrhosis, and thereby prevent the complications of cirrhosis, including liver cancer.

There are four medications currently approved by the Food and Drug Administration (FDA) for treatment of chronic hepatitis B infection.

1) **Interferon-alpha-2b**: Interferon-alpha 2b was first shown to be effective in chronic hepatitis-B virus patients in 1988. In addition to its direct anti-viral effects, interferon works against hepatitis B virus by stimulating the body’s immune system to clear the virus. For the treatment of chronic hepatitis B, a four to six month course of interferon-alpha is given.

2) **Lamivudine**: In the last five years, the focus of treatment for chronic hepatitis B virus has turned to nucleoside drugs. A number of nucleoside drugs that are used to treat HIV by slowing down the reproduction of that virus, therefore, have been tried for the treatment of hepatitis B virus. Nucleoside drugs are man-made molecules that closely resemble the biochemical units that make up genetic material (DNA and RNA). The nucleosides, therefore, work as imposters to trick hepatitis B virus genetic material and thereby slow down reproduction. Unlike interferon, the nucleoside class of compounds has no known direct effect on the immune system.

3) **Adefovir dipivoxil (Hepsera)**: inhibits DNA polymerase activity and reverse transcriptase. This drug is administered orally on a daily basis and is typically well tolerated.
4) **Baraclude (Entecavir):** is the latest drug approved by the FDA for treatment of chronic hepatitis B. It works by inhibiting the function of Hepatitis B virus polymerase. Side effects include headache, fatigue, dizziness, nausea, and transient elevation in liver enzymes. This drug is taken orally, once daily and the optimal duration of therapy is not yet established.

In patients with severe liver dysfunction, a liver transplant may be required.

**Prevention**

The best way to prevent HBV infection is through vaccination. Hepatitis vaccines are alum adjuvanted highly purified preparations of hepatitis B surface antigen (HBsAg), the glycoprotein that forms the outer coat of the hepatitis B virus.

**Vaccines for hepatitis B**

The discovery of the etiologic agents for hepatitis B and the development of safe and effective vaccines for this virus are among the remarkable scientific achievements of the 20th century. The first vaccines to be introduced were made from the blood of infected individuals, which was treated to destroy any live virus. As of date, two kinds of vaccines for hepatitis B are available:

1. Plasma – derived vaccines.
2. Recombinant DNA vaccines.

1. **Plasma derived vaccines:** In natural HBV infections, liver cells produce much more HBsAg than is needed to coat viral particles, and the excess HBsAg forms 22nm spherical and long tubular particles. Plasma derived HB vaccines are prepared by purifying HBsAg particles from the plasma of HBsAg positive donors. These vaccines are inactivated to ensure that no infectious viral or other micro-organisms are present, and then are alum adjuvanted. Plasma derived vaccines have been available since 1981 and have been used in more than 70 million individuals with an outstanding record of safety and efficacy.
2. **Recombinant hepatitis B vaccines** are produced from HBsAg derived from yeast or mammalian cells that have replicating plasmids containing the viral HBsAg gene inserted into the cells. The HBsAg forms spherical particles similar to the natural 22nm spherical particle in both chemical composition and immunogenicity. Recombinant HBsAg for vaccines may be produced in almost unlimited amounts in brewery-like fermentation vats, so there need be no concern that availability of antigen will compromise future vaccine supply.

**Hepatitis B immunoglobulin**

If hepatitis B immune globulin (HBIG) is given to newborns of HBeAg positive mothers in addition to HB vaccine, the efficacy in preventing the carrier state may be slightly increased. Use of HBIG adds considerably to the cost of treatment, since it is expensive, and since it requires serological testing of mothers to determine their HBsAg status. Such testing is itself expensive, and requires laboratories and prenatal testing programmes that are generally unavailable in developing countries. For these reasons it is generally accepted that it is more cost-effective to devote resources to routine infant immunization, and that most developing countries will elect to forego the use of HBIG.

**IMPORTANCE OF IMMUNIZATION**

Immunization is the most important preventive action for protection against disease, disability and death resulting from an infection. It is the act of artificially inducing an active or passive immune response, desired to ward off or even eradicate an infectious pathogen in the system. Passive or short term immunity is conferred on an individual by exogenously formed antibodies that prevent or ameliorate the advent of an infection. Two situations in which passive immunization occurs are the transplacental transmission of antibodies to the foetus and the injection of immunoglobulins for a specific preventive purpose. The protection bestowed by passive immunity usually lasts for a short term and
therefore, may require repeated immunization each time. Active immunization involves the induction of antibodies to develop defensive capability against the infection and is accomplished by exposing an individual to the immunogens. This concept serves as a corner-stone of immunoprophylactic or active vaccination (Spector, 1986). The three major approaches to active immunization employ the use of live-attenuated, inactivated, killed, or detoxified infectious agents, the extracts of infectious agents and the use of live-vectors capable of producing specific antigens to stimulate the immune response against the infectious agents. Live-attenuated vaccine produce an immunological response most like that occurring from a natural infection and generally confer a life-long protection with a single dose (Spector, 1986). By contrast, the other forms of immunogens do not induce a permanent immunity with one dose, making repeated vaccination and booster doses necessary to develop and maintain sufficient levels of antibody.

THE IMMUNE SYSTEM
The immune system is a remarkably adaptive defence system that has evolved in vertebrates to protect them from invading pathogens or cancerous cells. Immune system is capable of generating an enormous variety of cells and molecules capable of specifically recognizing and eliminating an apparently limitless variety of foreign invaders. Immune response against any agent can be divided into two activities, i.e., recognition and response. Immune recognition is remarkable in its specificity. The immune system can recognize subtle chemical differences that distinguish one foreign invading organism from another. Simultaneously the system can discriminate between foreign molecules and body’s own cells and proteins. Recognition of foreign organism is followed by initiation of an appropriate response with the participation of a variety of cells and molecules to eliminate or neutralize the organism.
THE COMMON MUCOSAL IMMUNE SYSTEM

Traditionally vaccine development has been concerned mainly with the induction of systemic immunity by parenteral immunization. Because most infectious agents were detected parenterally following the course of infection, it appears to be the most direct mode of warding off the infection. This is also due to the fact that the antigens present in the vaccines are particles or large peptides which are poorly delivered to the site of specific immunity. The poor delivery is commonly due to spontaneous or enzymatic breakdown and/or poor absorption. Consequently, a substantial number of vaccines developed employ the parenteral route of administration. Until 1960s, it was perceived that only systemic mechanisms existed for the production of immunity, following an antigenic stimulus, and the detection of antibodies in external secretions was the result of an overflow of the conferred immunity (McGhee and Mestecky, 1990). However, the success of polio vaccine by an oral administration and the poor induction of mucosal immunity by parenteral administration of cholera vaccine suggested the presence of an immunological system characteristic of certain external secretions. Considerable evidence has been gathered that indicates the existence of an independent mucosal immune system (Tomasi et al., 1965), that can be anatomically and functionally divided into at least two distinct interconnected compartments. The immunoglobulin-A (IgA) derived from mucosal effector sites represents more than 75% of all antibody isotypes produced in humans (McGhee et al., 1992). These mucosal inductive sites include the gut associated and the nasal associated lymphoreticular tissue, strategically placed in the gastrointestinal tract and the nasopharyngeal tonsil area, respectively.

A number of studies in several experimental models including mice have provided compelling evidence that the stimulation of IgA precursor cells in the GALT with orally administered antigens leads to the dissemination of B and T cells to mucosal effector tissues such as lamina propria regions of the intestinal,
respiratory and genitourinary tracts and various secretary glands for subsequent antigen-specific S-IgA responses. Further several oral vaccines have been shown to induce appropriate S-IgA responses in remote secretions including saliva, tears and fluids obtained from nasal and gastrointestinal washes (Mestecky J, 1987). Thus it is feasible to consider that most vaccines, which are currently under development, could be considered for oral administration. Oral immunization is both practical and safe in terms of lack of side effect and can induce protective mucosal S-IgA responses.

**POTENTIAL OF MUCOSAL IMMUNIZATION THERAPY**

Because most infectious agents enter the body through mucosal surfaces, immunization of these surfaces will represent a potent mechanism of warding off the pathogens at the site of entry. However, most of vaccinations are performed by parenteral administration, in which systemic immune responses are induced but mucosal immune responses are not. Oral vaccination can induce both systemic and mucosal immune responses (O’Hagan et al., 1989; Tabata et al., 1996; Trolle et al., 1998; Wikingsson and Sjoholm, 2002; Minato et al., 2003). The mucosal route covers aero-digestive and urinogenital tracts, as well as, the eye conjunctiva and the inner ear and the ducts of endocrine glands endowed with powerful mechanical and chemical cleansing mechanisms that degrade and repel most foreign matter. It is comprised of anatomically defined lymphoid micro-compartments, such as peyer’s patches, the mesenteric lymph nodes, the appendix and solitary follicles in the intestine, and the tonsils and adenoids, at the entrance of the aero digestive tract, which serve as the principal mucosal inductive sites where immune responses are initiated. In a healthy human adult, mucosal immune system contributes almost 80% of all immunocytes.

The mucosal associated lymphoid tissues represent a highly compartmentalized immunological system. They are capable of trapping antigen and provide sites
for mature lymphocytes, to interact with antigen. The mucosal sites, which
comprise the common mucosal immune system include Gut Associated
Lymphoid Tissues (GALT), Bronchus Associated Lymphoid Tissues (BALT),
genital tract, salivary glands, ocular tissues, and mammary glands. These sites
are in intimate and constant contact with the external environment, contain
mucocilliary epithelium, possess secretory component S-IgA in the epithelium
and lamina propria, as well as, organized lymphoid follicles in subepithelial
regions. These tissues participate in circulation of antigen reactive IgA B
lymphocytes and specifically sensitized T cells to other distant sites, after in vivo
stimulation in the GALT or BALT (Tomasi et al., 1965; McGhee et al., 1992).
IgA is the principal immunoglobulin produced on mucosal surfaces after mucosal
immunization and SIgA antibodies generated in the gastrointestinal, respiratory
or genito-urinary mucosal tissues, which can confer protection against infections
affecting or originating in these sites.

There is an enormous challenge for development of vaccine delivery systems
targeted to induce immunity that can prevent the infectious agent from attaching
and colonizing at the mucosal epithelium or from penetrating and replicating in
the mucosa.

**ROUTES OF MUCOSAL IMMUNIZATION**

It is of relevance to select an appropriate immunization route to develop mucosal
vaccines because almost all viral and bacterial pathogens invade mucosal tissues
and because majority of the current vaccines are administered parenterally, a
route that does not induce significant mucosal immune responses (Ogra et al.,
2001; Yuki et al., 2003).

The theoretical basis of oral immunization is described to the existence of the
compartmentalized common mucosal immune system. Following this concept,
antigenic stimulus in the gut associated lymphoid tissue or bronchus-associated
lymphoid tissue will stimulate IgA precursor cells that migrate to distant surfaces, where they express secretory IgA specific for that antigen. Oral immunization is both practical and relatively safe and is the preferred route compared with parenteral administration. Oral immunization thus should effectively stimulate antibody production in all associated secretory sites (Waldman et al., 1986; Cuff et al., 1992; McGhee and Kiyono, 1993). The feasibility of such oral immunization has been examined in a limited number of studies. Moderate immunogenic response achieved by this route has prompted the concurrent administration of adjuvants, such as cholera toxin, to amplify the immune response.

GUT ASSOCIATED LYMPHOID TISSUE
The gastrointestinal tract is constantly confronted by potentially harmful antigens, which are usually destroyed by the mucosal barrier - a combination of immunological barriers, such as gastric acidity, proteolytic enzymes, peristalsis, commensal microflora and mucus (Walker and Owen, 1990; Takagi et al., 2003). The immune response is stimulated, when antigens gain access to lymphoid tissue within gastrointestinal tract.

The gut associated lymphoid tissue (GALT) is distributed in four anatomical regions (O’Hagan et al., 1987).

A. The lamina propria which contains large number of plasma cells, as well as, macrophages, neutrophils, eosinophils and mast cells.
B. The intraepithelial lymphocytes, which are dispersed between the epithelial cells of the mucosal membrane.
C. Isolated lymphoid follicles, present throughout the intestine and colon.
D. The peyer’s patches (PP) which is a cluster of lymphoid follicles found along the wall of the small intestine.
Lymphoid tissue of the lamina propria and intraepithelial lymphocytes are collectively known as the diffuse lymphoid tissue. An immune response is elicited through lymphoid tissue of the PP and isolated lymphoid follicles.

**Peyer’s Patches**

Peyer’s patches are located along the entire length of the gastrointestinal tract with more numbers in the colon and rectum regions. The peyer’s patches contain a dome region enriched with lymphocytes, macrophages and some plasma cells. This dome area is covered by a unique epithelium, enriched with specialized antigen-sampling cells, termed follicle-associated epithelial cells (FAE). The FAE is enriched in specialized antigen sampling cells, known as microfold or M cells. Antigen uptake by M cells does not result in degradation, but instead, deliver intact antigen into the underlying lymphoid tissue (Wolf and Bye, 1984).

![Figure 5: Schematic diagram of the dome region of the Peyer’s Patches](image-url)

The importance of PP lymphocytes in dissemination to other exocrine tissues of antigen sensitized precursors of IgA plasma cells was demonstrated by the use of ligated ileal loops (Robertson and Cebra, 1976). The two isolated ileal loops, one with and one without PP, were constructed without the interruption of the flow of blood and lymph. After the introduction of antigens into the loop that contained the PP, a S-IgA response was detected in the loops, while these antigens...
introduced into a loop devoid of PP, did not induce an immune response (Robertson and Cebra, 1976). These observations directly implicated the PP as the source of precursor cells that are capable of undergoing direct antigen-driven stimulation to migrate, divide and differentiate, and repopulate the intestinal lamina propria with cells that secrete specific IgA antibodies.

Thus, all necessary immunocompetent cells are present in IgA inductive sites. These cells are engaged in regulation of induction of antigen-specific effector cells, which will ultimately mediate both humoral and cellular immune responses. Thus, by the use of appropriate oral antigen delivery, one can now immunize from various life threatening diseases.

ANTIGEN UPTAKE AT MUCOSAL SURFACES
The mucosal sites continuously sample foreign material via specific cells, such as M cells and dendritic cells. Many new ways of antigen uptake have been described recently, especially by M cells and dendritic cells in the intestine. Depending on various factors, antigen presentation in the mucosal, can lead to tolerance or initiation of an immune response. Mucosal vaccine strategies will certainly require the elicitation of specific antigen uptake, because this initial step has a crucial role in controlling the outcome of immune responses (Florence and Kweon., 2005).

The M cell is a specialized cell type, initially described as being restricted to the epithelium associated with lymphoid tissues of the digestive and respiratory tracts. They can be distinguished from the neighbouring epithelial cells (Kraehenbuhi and Neutra, 2000). A hallmark of M cells is their capacity to internalize material from the lumen and to transfer it efficiently to the underlying lymphoid cells. Initially described only in the epithelium associated with lymphoid tissues, recently M cells have been observed in intestinal villi of mice,
where they internalize bacteria, suggesting that they have the same role of antigen sampling in villi as in a follicle-associated epithelium.

**Figure 6: Induction of Mucosal S-IgA response**

Dendritic cells are professional antigen presenting cells that are found in various sites of the body, where they survey and process micro-organisms. At mucosal interfaces, DCs are found at various locations - the lamina propria, the sub-epithelium, T-cell zones of lymphoid tissue associated with the mucosa, and draining lymph nodes. The DCs situated inside or below the epithelium are ideally placed to sample antigens that have crossed the epithelial layer via the specialized M cells (Niedergang et al., 2000).

**THE ORAL IMMUNE RESPONSE**

The oral immune response is stimulated when antigens gain access, often through M cells to lymphoid tissue, within the gut. The main determinant of humoral mucosal immunity is secretory immunoglobulin A(S-IgA) (Mestecky and Mc Ghee, 1987). Secretory immunoglobulin A(S-IgA) response is induced, when
antigen is taken up by M cells in the GALT. Th cells and B cells committed to specific IgA synthesis are rapidly generated in peyer’s patches and migrate to mesenteric lymphnodes, thoracic duct and systemic circulation. Partly matured lymphocytes return to the mucosal membranes and to the lamina propria of GALT, to become IgA plasma cells or effector T cells. Within plasma cells two IgA monomers complex with the J chain to form polymeric IgA. Polymeric, J chain-containing IgA is released by subepithelial plasma cells and interact with a secretory component (SC) on the surfaces of the epithelial cells. This binding results in endocytosis and subsequent movement of the membrane SC-polymer IgA complex through the epithelial cell. The complex is then extruded on the luminal surface by reverse endocytosis. The secretory IgA (SIgA) is protected from proteolysis by the secretory component.

From the above overview of the oral immune system, it would seem that oral route of administration could be utilized not only to provide protection against enteric pathogens, but also to immunize against a wide range of pathogens.

MUCOSAL IMMUNIZATION DELIVERY SYSTEMS
The current strategy for an induction of a mucosal immune response involves the use of particulate delivery systems. Formulation of antigens into particulate carrier systems offers the potential of optimizing delivery to immuno-responsive sites and also protection of the antigen against proteolytic degradation in the gastrointestinal tract.

Replicating antigen delivery systems
The replicating antigen delivery systems proliferate in the host-tissues, following immunization, resulting in a more prevalent and effective presentation of the antigen and therefore, are more likely to be effective at stimulating an immune response following oral delivery. Replicating systems may also be able to induce
a cytotoxic T-lymphocyte response that can result in longer lasting immunity. Typically a replicating delivery system involves the genetic alteration of a live virus to function as a vector. Multiple foreign genes can replace the non-essential regions of the viral genome, resulting in an immune response against multiple pathogens. This approach enables the recombinant virus to function as a vaccine for two or more infectious agents. The consequences of the host recombinant virus, however, need to be better defined before replicating systems can gain broad acceptability as a preferred antigen delivery system.

**Non replicating antigen delivery systems**

Recent advances in DNA-recombinant technology has led to some novel approaches that are focussed on the production of particulate antigen presentation systems. The Ty-VLP are produced by transposing the fusion proteins of the yeast on Ty-encoded particle, forming protein and the viral protein of interest. Each particle is approximately 50 nm in diameter and contains several hundred copies of coupled antigens, expressed in a polyvalent form on the particulate surface. For HBsAg, the antigenic protein self-assemble to form 22 nm particles that serve as the immunogen. Another interesting approach of antigen presentation involves the construction of solid matrix antibody-antigen complexes by attachment of monoclonal antibodies to a suitable solid matrix. The desired antigens are complexed on to the monoclonal antibodies to produce particulates that are capable of presenting multiple antigens to the immune system and thereby evoking an enhanced multivalent immune response. The enhancement of the immune response is believed to be due to the particulate character of the system and the presence of antibody antigen complex, which is a potent stimulant of the humoral and cell-mediated immunity.

A newer approach to the presentation of peptide antigens is by linking multiple radially branching peptide epitopes, nine to fifteen residues in length, to a core
matrix of a tri-functional amino acids, such as lysine. These multiple antigens peptide systems may also be sufficiently flexible to permit the inclusion of the multiple antigenic peptides such as the T and B cell epitopes to produce an enhanced antibody response to the proteins, from which, these peptides were derived.

The use of biodegradable polymeric particulate carrier systems that has been pursued fairly vigorously during the past decade. The choice of the polymeric materials has been primarily serum albumin, chitosan, polylactide and polyglycolides and polyacrylic acid systems. Liposomal systems have also been investigated for the delivery of antigenic load.

**ORAL IMMUNIZATION**

There are two possible ways for oral immunization, firstly by the use of live attenuated organisms and secondly, by using peptides which have the capacity to bind and be absorbed at the intestinal levels and generate both a local response and a systemic immune response.

Most of the oral vaccines available so far, are based on live attenuated organisms, as these have the capacity to colonize the intestine and elicit an immune response in a manner analogous to the natural infection. The other approach to oral vaccination is by the use of synthetic peptide vaccines to stimulate a mucosal immune response. Synthetic peptides with the same structure as protective antigens isolated from the whole microorganism, for example, the hepatitis B surface antigen (HBsAg). Advantages of synthetic peptide vaccines include (Liew, 1988) (1) chemically defined, generally indefinitely stable products; (2) absence of infectious agents; (3) usually non-requirement of large production culture plant; and (4) relatively easy yet difficult, to formulate into oral dosage form, without damage to protective antigen. The major disadvantage is that synthetic peptides are not usually as immunogenic as the proteins, from
which they were derived, and this require the presence of adjuvants to elicit an appropriate immune response.

Through studies on vaccination by oral route, several points have now emerged: higher doses and more frequent administration, when compared to systemic immunization, are required for orally administered antigens, due to enzymatic degradation, acid sensitivity or poor bio-availability (Hemings, 1978). Particulate antigens, rather than soluble antigens, provide a more effective stimulus for the induction of a local, generalized secretory and systemic immune response (Ebersole and Molinari, 1978; Cox and Muench, 1984; Cox and Taubman, 1984). DNA recombinant vaccines are usually not transmissible to contacts and are suitable for mass application, without the need for highly trained staff.

Oral vaccination may fail due to several factors: failure to swallow the vaccine; inactivation by gastric acid and intestinal enzymes; poor bioavailability and too rapid transit of the vaccine through the intestine, limiting its binding to mucosal cell receptors, and hence, non-stimulation of an adequate immune response.

In an attempt to overcome the need for higher and more frequent dosing, required by oral administration, and to minimize vaccine failure, several strategies have now been followed.

**PARTICULATE DRUG DELIVERY SYSTEMS**

For the treatment of infectious diseases, the direct induction of an appropriate immune response is the ultimate goal. This can be achieved by novel vaccination strategies using effective antigen delivery systems. Hence, recent research has included development of particulate system for the oral delivery of antigens (Tazio et al., 2005). Particle formulations are made of a variety of materials, including lipids, proteins or amino acids, polysaccharides, polyacrylic substances or organic acids. Microparticles serve as vehicles and provide a depot for the
entrapped or coupled antigen. The release occurs in a pulsatile or continuous manner, a feature, which is well controllable for many particulate systems.

The particulate characteristics facilitate uptake by APCs and transport to secondary lymph organs, resulting in the induction of immune responses (O’Hagan and Valiante, 2003). Importantly, particles also protect the entrapped substance. This is especially necessary after oral application to avoid gastric or trypic breakdown. They are recruited to the injection site or, upon oral application, adhere to the peyer's patches (PP), which could facilitate the antigen presentation. Particulate antigens are most likely delivered from the gut lumen to the underlying lymphoid tissue by M cells in peyer’s patches. An amazingly high number of particulate antigen delivery systems has come up in the last two decades e.g., emulsions, microspheres, immune stimulating complexes (ISCOMS) and liposomes, noisomes etc (Isabella et al., 2005).

Polymethacrylic microspheres with ovalbumin (OVA) were used for oral immunization of rats. They were capable of sustaining a memory secretory IgA immune response, which was significantly higher than in the control group fed with soluble antigen alone (O’Hagan, 1989).

Distribution studies by Arora and Gangal in mice revealed that liposome-entrapped radiolabelled Artemisia pollen allergens were retained for a longer time period in the tissues after i.p. injection compared to free allergen. Regarding immune responses of these animals, the IgG/IgE ratio was four to eight times higher in the groups treated with liposome entrapped pollen allergens than after immunization with free allergen (Arora and Gangal, 1990).

Nakoda et al incorporated the model antigen human gammaglobulin (HGG) into gelatin microspheres and the immune response examined after s.c. injection in mice. The antigen-specific IgG levels induced by this preparation were 10 times
higher compared to soluble antigen and even 3–4 times compared to antigen in Freund's incomplete adjuvant (FIA). These findings were paralleled by enhanced delayed type hypersensitivity, as the footpad thickness was about 10 times higher after HGG in gelatin microspheres than after free HGG (Nakoda et al., 1995).

The effects of PLGA-entrapped antigen on the immune response have been shown to be superior to application of soluble, free antigen (Igartua et al., 1998). After oral administration in mice, OVA-filled niosomes increased the specific IgG and IgA titers, which were 10 times higher than after feedings with soluble OVA (Rentel et al., 1999; Chattaraj and Das, 2003).

In order to promote oral absorption of antigens from gastro intestinal tract, many components of the enzymatic barrier must be controlled. This can be achieved by

1. Restricting the release of antigens in a region of the G.I.tract that favors its absorption.
2. Co- administration of protease inhibitors.
3. Co- administration of intestinal permeation enhancer.
4. Antigen may be housed within a delivery system that is designed not only to protect the drug from contact with the luminal proteases, but also to release the drug only upon reaching an area favourable to its absorption.

The vaccines may be prepared by entrapping antigens in various carrier systems prepared from biodegradable polymers, which can be administered orally. The carrier systems can be designed to release entrapped antigen at the appropriate site of the gut. The inhibition of protease activity with suitable inhibitor can protect the vaccine from degradation. The improvement of oral bioavailability can be achieved with suitable permeation enhancers. The primary candidates for the development of polymeric release vaccines are microparticles and microspheres. Since the microparticles are taken up from the intestine into lymphoid follicles (peyer’s patches) following oral administration, they have considerable potential as oral vaccines.
Microspheres and nanoparticles

Over the past several decades, there has been an enormous increase in the development for novel vaccines and improvement of existing vaccines. Now it has become a major goal of the WHO global Programme for vaccines and immunizations to promote and support the research and development of oral vaccines. However, the simple oral administration of antigens elicit little immune responses because gastric acid, various hydrolytic enzymes, thick mucus etc degrade or remove the ingested antigens (Walker and Taylor, 1978; Kunisawa et al., 2001; Takagi et al., 2003). Therefore, various delivery systems have been investigated in order to make oral immunization more effective.

Microspheres and nanoparticles which can be adapted for delivery of antigens were studied with model protein such as albumin (O’Hagan et al., 1993) and can be adapted to other antigens. In particular, microspheres with a diameter less than 10μm are reported to show good adjuvant effects (Eldridge et al., 1990; Tabata et al., 1996; Nakamura et al., 1998). Such particles can protect the antigens from degradation in the intestine, and deliver them into gut associated lymphoid tissue (GALT) located in the lower portion of the small intestine. When such microspheres reach the peyer’s patches in the GALT, they can be taken up by M- cells located between the epithelial cells. The internalization by M cells appears to be an important step for oral vaccination (Tabata et al., 1996).

The ability of microparticulate delivery systems to induce a specific antibody response has been extensively studied in laboratory animals using different antigens and delivery routes. Using intraperitoneal and oral immunization routes for toxoided staphylococcal enterotoxin β, it was shown that the antigen entrapped in biodegradable polymer is far more superior to the free form for immunization. These results provided a clear indication that biodegradable microspheres can act as an adjuvant system with potential ability to stabilize
proteins and with possible wide spread application for induction of both circulating and mucosal immunity (Eldridge et al., 1991).

Various studies have shown that microencapsulation of proteins can lead to increased stability of protein antigens. There have been many reports of successful immune responses generated to encapsulated antigens, in particular to encapsulated tetanus toxoid vaccines (Almeida et al., 1993; Gupta et al., 1993).

Jaganathan et al developed single dose tetanus toxoid formulation based on polymeric microspheres and the results indicated that a single injection of PLGA and chitosan microspheres containing tetanus toxoid could maintain the antibody response at a level comparable to booster injections of conventional alum adsorbed vaccines (Jaganathan et al., 2005). Several studies have also demonstrated the effective protective immunity elicited by various biodegradable polymer encapsulated diphtheria toxoid and Bordetella pertussis antigens (Cahil et al., 1995; Jones et al., 1995) in different animal models and routes of immunization.

The preparation of microspheres should satisfy certain criteria such as,

1. The ability to incorporate reasonably high concentration of the drug
2. Stability of preparation after formulation with a clinically acceptable shelf life
3. Controllable particle size and dispersability in aqueous vehicles
4. Release of active agents with a good control over wide time scale.
5. Biocompatibility with a controllable biodegradability.

These studies have all confirmed that it is possible to induce specific protective immune response by delivering antigens in microspheres.
BIODEGRADABLE POLYMERS

For more than two decades use of polymeric materials to deliver bioactive agents has attracted the attention of investigators throughout the scientific community.

When attempting to design a microparticulate antigen delivery system for targeting mucosal sites, it is important to choose a polymer that is biodegradable, bio-compatible and safe for use in humans. Biodegradable polymers are natural or synthetic polymers, which degrade to natural products, such as, small acids formed in metabolic pathways (Wise et al., 1987). Biodegradable polymers have properties of degrading in biological fluids with progressive release of dissolved or dispersed drug. A biodegradable polymer is ideal for immunization purposes because it can release antigen at the desired rate and does not necessitate an additional surgical step for retrieval of the depleted system. Release of entrapped molecules occurs primarily by bulk erosion of the polymer, although a combination of surface erosion, diffusion of the active agent through the polymer itself or its release through the pores may also play a part. In addition, their breakdown products are natural, biocompatible which overcomes the problem of toxicity. The timescale over which the control release is envisaged can be weeks or frequently in months. Many natural and synthetic biodegradable polymers have been investigated as implants, microcapsules, microparticles, microspheres, and nanocapsules in order to achieve prolonged release and targeting of a variety of drugs (Deasy 1984, 1993; Venkatesan et al., 1995).

Natural biodegradable polymers

Natural polymers, particularly proteins, have been extensively investigated as drug-carrier systems to achieve prolonged and site-specific targeted drug delivery. The use of natural polymers to deliver drugs continues to be an area of active research despite the advent of synthetic biodegradable polymers. Natural polymers remain attractive primarily because they are natural products of living
organisms, readily available, relatively inexpensive, and capable of a multitude of chemical modifications. A majority of investigations of natural polymers as matrices in drug delivery systems have centered on proteins (collagen, gelatin, albumin, haemoglobin, casein, etc.) and polysaccaride (chitosan, dextran, starch hyaluronic acid etc.).

**Chitosan**

Chitosan is a natural polymer obtained by deacetylation of chitin. Chitin is the second most abundant polysaccharides in nature after cellulose. Chitin found in nature is a renewable bioresource. The main commercial sources of chitin are the shell wastes of shrimp, crab, lobster and squid. This can also be isolated from fungi such as Aspergillus niger (Felt et al., 1998). Among several sources, the exoskeleton of crustaceans consist of 15% to 20% chitin of dry weight. It is a biologically safe, biocompatible and biodegradable polysaccharide. Because of its superior characteristics together with a very save toxicity profile, chitosan is widely used as a pharmaceutical excipient.

![Figure 7: Preparation of Chitosan from Chitin](image)

Chitosan is made by alkaline N-deacetylation of chitin. The term chitosan does not refer to a uniquely defined compound; it merely refers to a family of
copolymers with various fractions of acetylated units. It consists of two types of monomers; chitin-monomers and chitosan-monomers. Chitn is a linear polysaccharide consisting of (1-4)-linked 2-acetamido-2-deoxy-b-D-glucopyranose. Chitosan is a linear polysaccharide consisting of (1-4)-linked 2-amino-2-deoxy-b-D-glucopyranose.

Chitosan can be described in general by the following parameters:

- degree of deacetylation in %,
- dry matter in %,
- ash in %,
- protein in %,
- viscosity in Centipoise,
- intrinsic viscosity in ml/g,
- molecular weight in g/mol, and
- turbidity in NTU units.

All of these parameters can be adjusted to the application for which chitosan is being used. The deacetylation is very important to get a soluble product. Almost all functional properties of chitosan depend on the chain length, charge density and charge distribution. Numerous studies have demonstrated that the salt form (Lehr et al., 1992), molecular weight, degree of deacetylation (Sabnis et al., 1997) as well as pH at which chitosan is used (Artursson et al., 1994) influences the properties of this polymer in drug delivery systems. Therefore these factors must be considered carefully during formulation for optimization dosage forms. In addition, regulatory requirements concerning the use of chitosan in humans will be far more demanding. It has been reported that the purity of chitosan influences its toxicological profile. Therefore, it would stand to reason that only the highest purity of chitosan would satisfy the standards set by the regulatory agencies.
The intriguing properties of chitosan is known for years and the polymer has been used in the field of agriculture, industry and medicine. In agriculture, chitosan has been described as a plant antiviral, an additive in liquid multicomponent fertilizers (Struszczyk et al., 1989). Chitosan has been noted for its application as a film forming agent in cosmetics (Lang and Clausen, 1989), a dye binder for textiles, a strengthening additive in paper and a hypolipidaemic material in diets (Fukunda et al., 1991). It has also been used extensively as a biomaterial, owing to its immunostimulatory activities, anticoagulant properties, antibacterial and antifungal action and for its action as a promoter of wound healing in the field of surgery Dutkiewicz and Kucharska, 1992). Roy et al. showed the potency of chitosan in targeting, transfection and immunologic protection against peanut allergy in oral gene delivery studies (Roy et al., 1999).

In addition, Chitosan has a variety of promising pharmaceutical uses (Dodnea and Vilivalam, 1998) and is presently considered as a novel carrier material in drug delivery systems, as indicated by the large number of studies published over the last few years.

**Ophthalmic delivery**

Various studies showed the potential of chitosan-based systems for improving the retention and biodistribution of drugs applied topically onto the eye. In addition to its low toxicity and good ocular tolerance, chitosan exhibits favorable biological behavior, such as bioadhesion, permeability-enhancing properties, and interesting physico-chemical characteristics. The microparticulate drug-carrier (microspheres) seems a promising means of topical administration of acyclovir to the eye (Genta et al., 1997).

**Nasal delivery**

Chitosan drug delivery systems, such as microspheres, liposomes, and gels, have been demonstrated to have good bioadhesive characteristics and swell easily.
when in contact with the nasal mucosa. Sankar et al has reported that bioadhesive chitosan microspheres of pentazocine for intranasal systemic delivery significantly improved bioavailability with sustained and controlled blood level profiles compared to intravenous, oral administration (Sankar et al., 2001). Chitosan may be a good option in nasal delivery as it binds to the nasal mucosal membrane with an increased retention time and it is a good absorption enhancer (Illum, 2003; Ravi Kumar et al., 2004). Furthermore chitosan is an excipient able to enhance the dissolution rate of low watersoluble drugs (Giunchedi et al., 2002; Maestrelli et al., 2004).

**Buccal delivery**

Chitosan is an excellent polymer to be used for buccal delivery because it has muco/bioadhesive properties and can act as an absorption enhancer. Miyazaki et al reported that directly compressible bioadhesive tablets of ketoprofen containing chitosan and sodium alginate in the weight ratio 1:4 showed sustained release 3 hours after intraoral (sublingual site of rabbits) drug administration (Miyazaki et al., 1994). Studies by Park et al on bioadhesive tablets of nicotine containing 0% to 50% w/w glycol chitosan showed the good adhesion (Park et al., 2004).

**Oral drug delivery**

Because of the mucoadhesive properties of chitosan and most of its derivatives, a presystemic metabolism of peptides on the way between the dosage form and the absorption membrane can be strongly reduced. Based on these unique features, the coadministration of chitosan and its derivatives leads to a strongly improved bioavailability of many perorally given peptide drugs, such as insulin, calcitonin, and buserelin (Bernkop-Schnurch, 2000). They have reported that a protective effect for polymer-embedded peptides toward degradation by intestinal peptidases can be achieved by the immobilization of enzyme inhibitors on the polymer. Serine proteases are inhibited by the covalent attachment of
competitive inhibitors, such as the Bowman-Birk inhibitor; metallo-peptidases are inhibited by chitosan derivatives displaying complexing properties, such as chitosan-EDTA conjugates.

Nifedipine embedded in a chitosan matrix in the form of beads showed prolonged-release of drug compared to granules. Studies by Hari et al on Chitosan/calcium alginate microcapsules containing nitrofurantoin (NF) showed sustained release of drug. They have also reported that drug release into the gastric medium is found to be relatively slow compared to that into the intestinal medium (Hari et al., 1996). Chitosan-coated nanosphere reduced significantly the blood calcium level compared with uncoated nanospheres, and the reduced calcium level was sustained for a period of 48 hours (Kawashima et al., 2000).

**Transdermal delivery**

Chitosan has good film-forming properties. The drug release from the devices are affected by the membrane thickness and cross-linking of the film. Chitosan gel beads are a promising biocompatible and biodegradable vehicle for treatment of local inflammation. Chitosan gel beads containing the anti-inflammatory drug prednisolone showed sustained release of drug with reduced inflammation indexes that resulted in improved therapeutic efficacy (Kofuji.K et al., 2004).

**Periodontal delivery**

Local delivery of drugs and other bioactive agents directly into the periodontal pocket has received a lot of attention lately. For example, for moderate to severe periodontal diseases, antimicrobial agents are used to eradicate and/or suppress the plaque bacteria. However, systemic administration of these drugs has certain disadvantages, such as the necessity for frequent dosing to maintain the drug concentrations at the therapeutic level in the plasma, poor patient compliance, super infections caused by resistant organisms, and gastrointestinal and systemic side-effects.
Chitosan gel and chitosan film containing chlorhexidine gluconate for local delivery were developed. Chitosan itself showed antifungal activity. Also, a prolonged release was observed with chitosan films. A monolayer and multilayered film of chitosan/PLGA containing ipriflavone were showed to prolong drug release for 20 days in vitro (Perugini et al., 2003).

**Vaccine delivery**

Various chitosan-antigen nasal vaccines have been prepared. These include cholera toxin, microspheres, nanoparticles, liposomes, attenuated virus and cells, and outer membrane proteins (proteosomes). They induced significant serum IgG responses similar to and secretory IgA levels superior to what was induced by a parenteral administration of the vaccine (Illum et al., 2001).

Chitosan microparticles are very promising mucosal vaccine delivery systems. Van der Lubben et al reported that significant systemic humoral immune responses were found after nasal vaccination with diphtheria toxoid associated to chitosan microparticles. Diphtheria toxoid associated to chitosan microparticles results in protective systemic and local immune response against diphtheria toxoid after oral vaccination, and in significant enhancement of IgG production after nasal administration (van der Lubben et al., 2003). Chitosan microspheres cross-linked with glutaraldehyde were loaded by bovine serum albumin (BSA) and diphtheria toxoid and showed tissue compatibility with a long-lasting drug delivery system in wistar rats for several days (Jameela et al., 1994). Chitosan has also been successfully used as a nasal delivery system for subunit influenza vaccine (Read et al., 2005), tetanus toxoid (Vila et al., 2004) and diphtheria (McNeela et al., 2000).

Chitosan has unique properties of bioadhesion, absorption enhancement by increasing the residence time of dosage forms at mucosal sites, inhibiting proteolytic enzymes, and increasing the permeability of various drugs across mucosal membranes. Chitosan is degraded by the microbial flora that are present...
in the colon; as a result, chitosan is a good candidate for site-specific drug delivery. Low toxicity coupled with wide applicability makes it a promising candidate not only for the purpose of drug delivery for a host of drug moieties, but also as a biologically active agent.

Considering all the above properties, it has been decided to use chitosan as chitosan microspheres for the delivery of antigen in our present study. There are number of studies using chitosan microspheres as a novel carrier for drug delivery.


Hassan et al (1992) used emulsion/polymer cross-linking/solvent evaporation methods to prepare magnetic chitosan microspheres containing piroxantrone (oxantrazole) and effects of formulation factors on various response variables were examined using a central composite experimental design (Hassan et al., 1992).

Kumar et al (2002) prepared the microspheres of curcumin with biodegradable polymers like albumin and chitosan. Evaluation of antinflammatory activity using Freund’s adjuvant induced arthritic model in wistar rats revealed significance difference between both formulations as well as against control (Kumar and Misra, 2002).

Thakkar et al (2004) prepared celecoxib-loaded chitosan microspheres for intra-articular administration and compared the retention of the celecoxib solution and chitosan microspheres in the joint cavity. The study indicated that following intra-articular administration the distribution of the drug to the organs, like liver.
and spleen, is very rapid compared with that of the microspheres. Compared with the drug solution, a 10-fold increase in the concentration of the drug in the joint was observed 24 h post intra-articular injection (P < 0.005) when drug was encapsulated in microspheres (Thakkar et al., 2004).

Chitosan microspheres also found application in the nasal delivery of Insulin, reported by Varshosaz et al (2004). Microspheres containing 400mg of chitosan and 70mg ascorbyl palmitate caused a 67% reduction of blood glucose compared to i.v. route and absolute bioavailability of insulin was 44%. The results showed that chitosan microspheres of insulin are absorbable from nasal route (Varshosaz et al., 2004).

Studies by Gavini et al (2005) showed that alginate/chitosan spray-dried microspheres have promising properties for use as mucoadhesive nasal carriers of an antiemetic drug metaclopramide (Gavini et al., 2005).

Novel mucoadhesive chitosan microspheres were developed by Jain et al to explore the possibilities of non invasive delivery of insulin. The mucoadhesive chitosan microspheres were prepared by emulsification method. Glutaraldehyde cross-linked microspheres showed better reduction of blood glucose level than citric acid cross-linked microspheres. The in vivo performance of mucoadhesive microspheres showed prolonged and controlled release of drug as compared with the conventional dosage form (Jain et al., 2007). The chitosan microspheres were prepared by a membrane emulsification technique in this study. The microspheres with different size were prepared by using the membranes with different pore size, and there was a linear relationship between the diameter of microspheres and pore size of the membranes when the microspheres were in the range of micron size. Bovine serum albumin (BSA) as a model drug was loaded in the microspheres and released in vitro. The effects of pH value, diameter and
cross-linking degree of microspheres, and BSA concentration on loading efficiency and release behavior were discussed.

Encapsulation of α lipoic acid (LA) was carried out using chitosan as an encapsulant matrix was studied by Rangika et al. Placebo and LA-loaded chitosan microspheres were prepared by a spray-drying process. The antioxidant activity of encapsulated lipoic acid was studied using the free-radical scavenging assay. This study demonstrated significant retention of antioxidant activity of lipoic acid (75%) after encapsulation in the chitosan matrix (Rangika et al., 2008).

**Albumin**

Albumin is also being used as a carrier for microparticles and nanoparticles for sustained-release injectable drugs. A nanoparticulate formulation of paclitaxel containing albumin as the carrier was recently approved by the FDA. A number of researchers have also used albumin for sustained release of small molecules and proteins. Albumin’s capacity to adsorb hydrophobic molecules makes it, a unique carrier for controlled release because the drug gets released via desorption without significant burst effects. Albumin’s adsorption capacity has also been exploited in development of magnetic microparticles. Such particles were used for targeted delivery of chemotherapeutic agents, such as doxorubicin. The particles consisted of albumin for binding of drug and iron for magnetic behavior to facilitate targeting. Albumin microspheres have also been used in diagnostic applications to detect intravascular susceptibility.

The use of biodegradable polymeric microparticulate systems is an interesting application in the control of drug release and targeting. The yield, drug content, and particle size distribution depend on different factors such as the nature of the polymer, and the formulative and preparative methods.
Albumin microspheres are biodegradable particles that can be produced in a size range of 1 to 200 μm in diameter, by either physical or chemical solidification of an albumin emulsion in an organic phase. Bovine serum albumin (BSA) is widely used for microsphere preparation because it is nonantigenic, biodegradable, free from toxicity, able to control the physicochemical characteristics of the microspheres produced, and readily available. Albumin-based drug delivery systems are popular for the treatment of inflammation and arthritis because albumin has a tendency to deposit at the inflamed joints. Albumin microspheres are metabolized in the body, and the size of particles, degree of stabilization, and site of metabolism are the main factors influencing the extent of metabolism. Drug release from the microspheres can be controlled by the extent and nature of cross-linking, size, and drug incorporation level in the microspheres.

Nifedipine-loaded albumin microspheres were prepared by a chemical cross-linking method to develop a sustained release form by Thau et al. The effects of cross-linking agent (glutaraldehyde) on the percentage of drug loading, biodegradability of albumin microspheres and drug release kinetics were investigated in this study. Albumin microspheres prepared with different amounts of glutaraldehyde indicated different release kinetics. Besides, albumin microspheres gave an adequate fit to either zero order or spherical matrix model, depending on the extent of cross-linking reaction (Thau et al., 1996).

The particulate form of albumin has been regarded as a potential carrier of drugs for either site-specific localization or their local application into autonomically discrete sites. Studies conducted by Sankar et al on various formulations of metronidazole-loaded albumin microspheres prepared by heat stabilization process and chemical stabilization process. In vitro release profile for formulations containing metronidazole-loaded albumin microspheres with cross linking agent showed slow sustained release up to 24h. The studies conducted in
rabbits confirm sustained release. They have reported that albumin microspheres prepared by the heat stabilization process and chemical stabilization process could be used for the treatment of hepatic amoebiasis where the sustained action is needed (Sankar et al., 2001). Albumin microspheres containing methotrexate enhanced the drug localization in lung compared with free drug has been described by Dhanraj et al (Dhanraj et al., 2001).

Albumin microspheres loaded with terbutaline sulphate, a drug with short biological half life which is widely used as bronchodilator were studied by Sahin et al for characterization and in vivo distribution (Sahin et al., 2002). Biodistribution studies indicated that the degree of uptake by the lungs was higher than that of the other organs. The results demonstrated that terbutaline sulfate loaded microspheres can be used for passive lung targeting which is desirable to improve patient compliance.

Celecoxib-loaded albumin microspheres were prepared by Thakkar et al. The blood kinetic studies revealed that celecoxib-loaded albumin microspheres exhibited prolonged circulation than the celecoxib solution (Thakkar et al., 2005).

Microsphere formulations of vancomycin by the spray-drying method using bovine serum albumin (BSA) were reported by Netty et al (Netty et al., 2006). This study showed that encapsulation of vancomycin did not alter the bioactivity of the drug and it was more effective in killing Staphylococcus aureus than the solution form.

Mathew et al prepared albumin microspheres by emulsion cross-linking method. Selected formulations were characterized for their entrapment efficiency, particle size, surface morphology, and release behavior. From the experimental data obtained with respect to particle size and extent of drug release, it could be
concluded that the prepared microspheres are useful for once-a-day intramuscular administration of ketorolac tromethamine (Mathew et al., 2007).

Rathod and Deshpande prepared pilocarpine nitrate loaded egg albumin microspheres were by thermal denaturation process in the size range of 1-12 µm. Biological response of microspheric suspension was measured by reduction in intraocular pressure in albino rabbit eyes and compared with marketed eye drops. Various pharmacokinetic parameters viz. onset of action, duration of action, Tmax and AUC were studied. A measurable difference was found in the mean miotic response, duration and AUC of pilocarpine nitrate microspheric suspension (Rathod and Deshpande, 2008).

Considering the wide application of albumin microspheres, it has been decided to use albumin as a polymer for oral delivery of antigens.

MICROENCAPSULATION

Several techniques are available for microencapsulation and the choice of a method depends on the physical and chemical properties of the polymer and the antigen to be encapsulated, and the function and desired size of microspheres. A high ratio of antigen to polymer is preferred to minimize the amount of mass that needs to be administered, without compromising the release kinetics. In addition, the microencapsulation technique must afford a pharmaceutically acceptable product, relative to residual solvents and processing aids, batch to batch reproducibility, ease of scale up and high encapsulation efficiency and yields. For commercialization, cost effectiveness is also an important requirement, especially for product isolation and drying and for solvent disposal.

The most commonly used methods of antigen microencapsulation encompass solvent extraction or evaporation from a W/O/W-dispersion, coacervation and spray-drying (Jain et al., 1998). Each of these methods employs a similar first
step, where an aqueous antigen solution is emulsified in an organic polymer solution to form a water-in-oil dispersion. If appropriate, the antigen may also be dispersed as solid powder in the organic polymer solution, or co-dissolved in a common solvent with the polymer. The solution or dispersion is then processed according to one of the mentioned microencapsulation methods.

**Solvent extraction and solvent evaporation**

The extraction-evaporation microencapsulation methods have been widely used because that can be easily set up in a laboratory and do not require any specialized equipment. A good review of procedures and modifications of solvent extraction and evaporation methods used for microsphere manufacture has been presented (Arshady, 1991). In both these processes, the polymer is first dissolved in a suitable volatile solvent, usually methylene chloride for solvent evaporation or acetonitrile for solvent extraction. Either the active agent can be incorporated into the polymer solution as an aqueous solution to form a primary emulsion or as a solid matrix, which forms a dispersion. In such a system droplet formation is a dynamic process in which droplets constantly form, collide, coalesce or re-divide. In the solvent extraction process, the solvent for polymer is dissolved away, when the emulsion is added to a suspension medium that is a non-solvent for the polymer. This leads to the formation of solid microspheres in a short period, the microspheres can be recovered either by filtration or centrifugation. Solvent extraction has been used for encapsulation of various peptides and proteins (Reid et al., 1993).

On the other hand, in the solvent evaporation process, droplet solidification occurs by evaporation of the volatile solvent at the continuous phase-air phase interphase. Most commonly the primary water-in-oil (W/O) emulsion is formed by the aqueous solution of antigen in the polymer solution, which is later emulsified into a large volume of aqueous phase to form an water-in-oil-in-water
(W/O/W) emulsion. In general, longer processing time is required to obtain solid microspheres by solvent evaporation. The mixing rate and evaporation time needs to be carefully controlled for re-producibility. Solvent evaporation has been used to successfully encapsulate proteins, including bovine-serum albumin, ovalbumin, tetanus toxoid, HBsAg, staphylococcal enterotoxin B toxoid and peptides, such as leuprolide acetate (Eldrige et al., 1991; Cohen et al., 1991; Alonso et al., 1994). Subjecting the microsphere to vacuum drying can significantly reduce entrapped volatile solvent in the microspheres; however, trace amounts of organic solvents are often difficult to remove. To minimize the safety and the regulatory concerns when dealing with organics solvents, liquid carbon dioxide under super-critical conditions has recently been used as a non-solvent for the polymer. In general, porous, spherical particles, with a broad-sized distribution, that provide rapid release of incorporated active agents that are obtained by the solvent extraction technique, whereas, less-porous microspheres are obtained by solvent evaporation.

**ORAL ADJUVANTS**

The design of vaccines primarily requires the identification of immunological correlates of protection, i.e., the immune effector mechanism(s) responsible for protection against disease, and the subsequent selection of an antigen that is capable to elicit the desired adaptive response. Once this appropriate antigen has been identified, it is essential to deliver it effectively to the host's immune system (Degen et al., 2003; Sachijns, 2003).

Traditionally, vaccines come in several forms: live-attenuated, replicating pathogens and non-replicating, inactivated pathogens or their subunits. A drawback of live vaccines or inactivated virus or bacteria is potential adverse effects, as reported for whole pertussis vaccine (Donnelly et al., 2001) and measles vaccine (Piyasirisilp and Hemachudha, 2002). During the last 25–30
years, research and development of alternative particulate adjuvants and vaccines has increased. The latter, non-viable category is the safest one, but because of poor or no immunogenicity, it often requires adjuvants to elicit an adequate immune response. In the absence of adjuvant, a lack of responsiveness may occur, and naïve antigen-specific T cells may recognize the antigen but are not sufficiently activated or they even become tolerated.

Adjuvants are defined as a group of structurally heterogeneous compounds, used to evoke or increase an immune response to an antigen (Gupta et al., 1993). Classically recognized examples include oil emulsions, saponins, aluminium or calcium salts, non-ionic block polymer surfactants, derivatives of lipopolysaccharide (LPS), mycobacteria and many others (Marciani, 2003). Theoretically, each molecule or substance that is capable to favour or amplify a particular step in the cascade of immunological events, ultimately leading to a better immunological response can be defined as an adjuvant. Obviously, the first step is very important. However, the *in vivo* molecular and cellular mechanisms required for the generation of an effective immune response, which depends critically on co-injection of adjuvant, are poorly understood. The structural requirements of adjuvants are similarly poorly understood. Adjuvants have therefore been surrounded by obscurity and called “the immunologist's dirty little secret”.

The only adjuvants suitable for human use are aluminium salts and gels. Aluminium adjuvanated vaccines have a number of limitations, for example, cell-mediated immunity is difficult to achieve and adverse stimulation of local Ig-E responses can occur. Other major disadvantages of aluminium are unpredictable extent of adsorption of certain proteins and low stability of some adsorbates (Matheis et al., 2001). Nevertheless, aluminium salts are the benchmark to which other adjuvants must compare (Mahon et al., 1998).
For these reasons a number of alternative technologies have been investigated, whereby the immunogenicity of such vaccines can be increased, while at the same time reducing the number of doses required. To promote retention in the gastrointestinal tract to aid absorption, mucoadhesive microspheres can be used. Mucoadhesive microspheres, when used as antigen-carrier for oral delivery, may achieve increased residence time within the gastrointestinal tract. Chitosan has been shown to have mucoadhesive properties.

**Absorption enhancers**

Oral route is without question the most popular, but yet the most complex route of drug delivery. The epithelial surface of the small and large intestine is different from that of skin, buccal, and nasal epithelial surface. In order to promote oral absorption of antigens, co-administration of intestinal permeation enhancer is needed.

Intestinal epithelial cells can be divided into several types; the globlet cells are mucus-secreting cells; the enterocytes, the most numerous of the epithelium, are absorptive cells; and there are also some endocrine cells. Epithelial cells originate from crypts of lieberkuhm and differentiate into various types of cells during migration to the top of the microvilli.

The primary function of the intestine is absorption of nutrients and its permeability properties are quite different from those of other mucosal epithelium. Because of the mucus covering of the epithelial surface of the intestine, the layer of water, adjacent to the mucosal surface is essentially unstirred. This layer is often called the unstirred water layer, with a thickness upto 400 μm. Studies have shown that if the absorption of substances by this cell is fast, diffusion across this layer becomes a rate-limiting step. Most small lipid soluble unionized drugs can be absorbed by diffusion across the epithelial
membrane; small polar drugs are absorbed by a transporter-mediated absorption or via the paracellular route.

Absorption enhances, commonly called penetration enhancers, facilitate transport of co-administered substances across biological epithelial barriers. Human epithelial membranes exclude many substances from entry into the human body and limit others with low absorption rate, based on their physical and chemical properties. As a result, many drug candidates with low permeability across human epithelial membranes have to be administered intravenously or intramuscularly. This greatly limits dosing frequency, can cause low patient compliance and in many cases, renders otherwise effective drugs useless. With a rapid development of bio-technology, more and more protein, peptide, and nucleotide drugs are becoming available, most of which have low membrane absorption characteristics due to

1. A large size with high molecular weight.
2. Domains of different hydrophobicity.
4. Delicate structures easily inactivated.

These drugs are unable to cross membrane barriers in therapeutic amounts and thus research into penetration enhancers becomes given more important.

**Designing a Good Penetration Enhancer**

Many penetration enhancers are both drug and site specific. A good penetration enhancer for a certain drug at a certain site may not be a good enhancer for another drug at another site. Different drugs have different physicochemical characteristics and compatibility with various penetration enhancers. Similarly, each administration site has its own distinct characteristics in terms of membrane thickness, membrane composition, lipid organization and enzyme activity. All these differences should be considered in the selection and design of an effective
penetration enhancer. It is necessary to thoroughly understand the physical and chemical characteristics of the drug and application site before selecting or designing an effective penetration enhancer.

The penetration enhancer selected for the present study is bile salts especially sodium taurocholate. Various relevant studies reported that bile salts can be used to enhance protein and peptide absorption by various routes of administration. There are three major hypothesis to explain the mechanism of mucosal enhancement of protein absorption by bile salt.

1) The reduction of protein degradation by the enzymes adsorbed within the mucosal layer.
2) The interaction of bile salts with cell membranes to form a reverse micelle, which act as a channel to increase membrane permeation.
3) The dissociation of molecular aggregates through micellar solubilization (Yamamoto et al., 1994)

Protease Inhibitors
The oral administration of vaccines is a major challenge to pharmaceutical science. In order to provide a sufficient bioavailability of these therapeutic agents after oral dosing, several barriers encountered with the gastrointestinal (GI) tract have to be overcome by a suitable method. One of these barriers is caused by proteolytic enzymes, leading to a severe presystemic degradation in the GI tract.

The use of inhibitory agents has gained considerable scientific interest, as various in vivo studies could demonstrate a significantly improved bioavailability of therapeutic peptides and proteins, due to the co-administration of such excipients (Bernkop-Schnurch, 2001). He has described the various techniques to evaluate the potential of inhibitory agents. In vitro techniques to evaluate the actual potential of inhibitory agents is incubation with pure proteases, freshly collected gastric or intestinal fluids, mucosal homogenates, brush border vesicles and
freshly excised mucosa. In situ techniques are based on single-pass perfusion studies cannulating different intestinal segments and determining the amount of un-degraded model drug in perfusion solutions or blood. For in vivo studies, insulin is mostly used as a model drug, offering the advantage of a well-established method to evaluate the biological response after oral dosing by determining the decrease in blood glucose level.

Generally, inhibitory agents can be divided into:

1) Inhibitors which are not based on amino acids such as p-aminobenzamidine, FK-448 and camostat mesilate;
2) Amino acids and modified amino acids such acid derivatives;
3) Peptides and modified peptides; bacitracin, antipain, chymostatin and amastatin;
4) Polypeptide protease inhibitors e.g. aprotinin, Bowman-Birk inhibitor and soybean trypsin inhibitor.

Tozaki et al studied the effects of protease inhibitors on the absorption of calcitonin from different regions of the intestine in rats. Of the co-administered protease inhibitors, bacitracin strongly promoted calcitonin absorption from the jejunum, ileum and colon. A significant hypocalcaemic effect was also obtained after intestinal administration of calcitonin with soybean trypsin inhibitor, camostat mesylate or aprotinin. The results suggested that endo- and exopeptidases might be responsible for the hydrolysis of calcitonin and that protease inhibitors might be useful to improve absorption of calcitonin to the systemic circulation from the large intestine (Tozaki et al., 1998).

The feasibility of microcapsules containing a protein and a proteinase inhibitor in order to allow the oral administration of proteic or peptidic drug has been described by Larionova et al. The protective effect of microcapsules with aprotinin for bovine serum albumin was revealed in vitro. The presence of the
native bovine serum albumin was demonstrated after incubation of the microcapsules with aprotinin in a mixture of alpha-amylase and trypsin for 3 h at 37 degrees C, whereas the protein was completely degraded in the release medium of the microcapsules without aprotinin (Larionova et al., 1999).

**Bacitracin**

Bacitracin is a mixture of related cyclic polypeptides produced by organisms of the licheniformis group of Bacillus subtilis, isolation of which was first reported in 1945. The drug's unique name derives from the fact that it was isolated from a girl named Tracy. Bacitracin is used in human medicine as a polypeptide antibiotic and is "approved by the FDA for use in chickens and turkeys. It is also commonly used as an aftercare antibiotic on tattoos. It is preferred over Neosporin because of its fewer ingredients, which lowers chances of an allergic reaction. In infants, it is sometimes administered intramuscularly for the treatment of pneumonias. Bacitracin is also used as protease inhibitor in different studies.

Gotoh et al studied the absorption enhancement effects of three types of protease inhibitors, aprotinin, bacitracin and soybean trypsin inhibitor, in the rat intestine. Of these protease inhibitors, bacitracin enhanced the absorption of FD-4 and phenol red from the rat small and large intestine without mucosal toxicity. Thus, it was suggested that bacitracin has not only a protease-inhibitory but also an absorption-enhancing capability (Gotoh et al., 1995).

The absorption enhancement effects of three types of protease inhibitors, aprotinin, bacitracin, and soybean trypsin inhibitor, on the small and large intestinal absorption of phenol red (PR) and fluorescein isothiocyanate dextrans (FDs) were examined in rats by Shinji et al. Of these protease inhibitors, only bacitracin enhanced the absorption of PR and FDs from the rat small and large intestine. Thus, they suggest that bacitracin has not only a protease-inhibitory but
also an absorption-enhancing capability. Therefore, bacitracin may be a good model adjuvant for improving the intestinal absorption of poorly absorbable drugs because it did not cause serious intestinal mucosal damage, as seen in the case of BL-9 (Shinji et al., 2000).

Liu et al. studied the effects of protease inhibitors on the absorption of insulin in-situ from closed small and large intestinal loops in rats and to investigate the mechanism of various protease inhibitors in different intestinal loops. The intestinal absorption of insulin was evaluated by its hypoglycaemic effect and serum insulin level in the presence or absence of luminal contents. No marked hypoglycaemic effect was observed after administration of insulin alone in either region in the presence or absence of luminal contents. A significant hypoglycaemic effect of insulin was obtained in the large intestinal loop in the presence or absence of luminal contents when insulin was co-administered with bacitracin (20, 30 mM), sodium glycocholate (20, 40 mM), bestatin (29 mM), leupeptin (21 mM) and cystatin (0.8 mM) (Liu et al., 2003).

Kumar and Misra conducted a study to enhance the bioactivity of insulin by the pulmonary route using a combination of absorption promoters. Aliquots (100 µL) containing 1.0 IU/kg to 7.0 IU/kg doses of porcine insulin solutions with different classes of absorption promoters and combinations of these at 3 concentration levels were instilled intratracheally to the anesthetized rats. Blood concentrations of glucose were measured at specific time points. Out of 3 concentration levels of each of the absorption promoters used, the formulations having the least concentration with the maximum percentage of blood glucose reduction were selected for combining absorption promoters, and their pharmacodynamic parameters related to insulin absorption were determined. Absorption promoters in combination have significant potential for increasing the pulmonary bioactivity of insulin. These studies support the argument that
pulmonary administration of insulin is a viable alternative to subcutaneous administration for diabetic patients (Kumar and Misra, 2003).

**Aprotinin**

Aprotinin, also known as bovine pancreatic trypsin inhibitor, BPTI. It is a monomeric (single-chain) globular polypeptide derived from bovine lung tissue; it has a molecular weight of 6512 and consists of 16 different amino acids arranged in a chain of 58 amino acid residues. Aprotinin is a member of protein family of serine protease inhibitors. Aprotinin inhibits several serine proteases, trypsiin, chymotrypsin and plasmin at a concentration of about 125,000 IU/ml, and kallikrein at 300,000 IU/ml. This inhibition is provided by inactivation of the active serine of the protease by the lysine residue at position 15 of the aprotinin molecule.

Aprotinin has been used over decades as an intensive care drug and is recommended for shock of traumatic or septic origin, fibrinolytic hemorrhage. Due to its low toxicity and strong inhibition of luminally secreted and brush border membrane bound proteases, aprotinin has been used to overcome the enzymatic barrier of perorally administered therapeutic peptides and proteins.

The activity of aprotinin is expressed in various ways, kallikrein inhibitory units (KIU) and trypsin inhibitor units (TIU) have been commonly used. It has its inhibitory effect on target serine protease by forming reversible stoichometric enzyme inhibitor complexes.

In pure chemical systems (those without other plasma proteins) the concentration of aprotinin required to inhibit serine proteases that occur in nature, such as, trypsin, plasmin or tissue and plasma kallikrein, varies for each of the enzymes with concentrations of approximately 50 KIU per ml to inhibit plasmin and approximately 200 KIU per ml to inhibit plasma kallikrein.
Thus, in order to increase the oral bioavailability of antigen, we have designed a delivery system containing antigen (hepatitis B surface antigen), aprotinin and bacitracin (a protease inhibitors) and sodium taurocholate (an absorption enhancer). The drug delivery system chosen for this study is microspheres to intestinal peyer’s patches by encapsulating in enteric-coated gelatin capsule cell. This enteric-coating may protect the microspheres in contact with gastric medium and allows the microspheres to release in the intestine.

**DElIVERY SYSTEM FOR MICROSPHERES**

For commercial use, microspheres should be placed in a pharmaceutically acceptable oral delivery system. Potential oral delivery systems include tablets, capsules and dry powder for reconstitution into a suspension. The former two are the most widely used oral delivery systems. Tablets are prepared by compression forces may deform and fuse the microspheres, rendering them into large aggregates on disintegration in aqueous media. Also compression forces and heat generated during compression may affect the stability of encapsulated antigen. A capsule formulation, although providing more favourable manufacturing conditions could lead to chemical instability. Commercial capsules are made of gelatin or starch and may have upto 12% moisture to maintain flexibility. The moisture maintains a high relative humidity inside the capsule, promoting chances hydrolytic cleavage of the bio-degradable polymer. Hydrolysis of the polymer may increase antigen release rate and make the polymeric matrix acidic, resulting in potential stability problem for the antigen. Placing the microspheres in a moisture resistant container for reconstitution with suitable vehicle before administration presents a suitable alternative. This approach is commercially used with hydrolytic unstable antibiotics and has been extended to clinical investigations of oral microsphere delivery (Tacket et al, 1994).

In the present study, microspheres are filled into hard gelatin capsules and then enteric coated to protect the drug.