Hepatitis B virus (HBV) infection is a worldwide public health setback. It is estimated that there are 240 million HBV carriers in the globe, of whom approximately 600,000 die per annum from HBV-related liver disease. Hepatitis B is an infectious disease caused by the hepatitis B viral infection comprising clinically evident illness by the presence of the biological agent (Virus) in a host individual. In certain cases, the hepatitis B viral infection is asymptomatic (not shows any detectable symptoms in a host individual) for long periods of time or the entire course. Transmission of the pathogen from host individuals to another can be accomplished by several ways including using contaminated food items, physical contacts with infected individuals, coming in contact with the infected body fluid, and airborne inhalation. Infection towards various pathogenic diseases does not result in the death of the infected individual and the pathogen (Bacteria, viruses prions) will be cleared by the activation of the host immune system. Generation of immune responses against incoming pathogens requires processing and presentation of small peptides on the MHC molecules to provide signal to respective T cells for the generation of cytokines responsible for the B & T cells proliferation. Immunity mediated by B and T cells may be manifested to, direct consequence upon a pathogen, such as antibody-initiated to generate complement molecules these molecules are specially promotes bacteriolysis by means of opsonoization & phagocytosis. Secreted antibodies in the serum binds to the viral particles and block the viral adherence on the cell membrane so viral particles cannot inter in the cells. Activation of T lymphocytes which will kill the infected cells (cells having parasite inside the cells).

1.1. Overview:
Infectious diseases remain a significant public health problem world-wide, and are associated with increased morbidity and mortality. Although as in case of chronic hepatitis B infection the majority of people [approximately 350 million] with chronic HBV infection may remain in an inactive phase associated with low viral replication and histological remission, a significant proportion will develop chronic hepatitis B, which is characterized by the presence of high HBV replication and chronic necro-inflammation of the liver. It is estimated that about 15–20% of patients with chronic hepatitis B develop cirrhosis within 5 years, and only 55–85% of those with active HBV-related cirrhosis survive for more than 5 years. Moreover, although all patients with chronic HBV infection are at higher risk for hepatocellular carcinoma when
compared with the general population, the risk becomes much higher when cirrhosis develops. Thus, it is estimated that over 250,000 patients world-wide die annually from HBV-related liver disease.

1.1.1: Acute Hepatitis B:
More than 65% of Hepatitis B patients have reported for acute Hepatitis B virus infection and have a have a easy-going, asymptomatic and subclinical illness that usually goes unnoticed by the currently used diagnostic protocols (McMahon, Alward et al. 1985). While left over patients (roughly 35%) with acute hepatitis B viral infection develops clinically diagnosable alterations and signs of hepatitis infection, which range from mild constitutional symptoms of tiredness and queasiness, to more marked symptoms and jaundice, and rarely it grows to severe liver failures. The proven incubation periods of acute hepatitis B ranges from two to three months and it can be grow up to six months after initial exposure to the virus (HBV). The time duration for exposure to infection (incubation period) correlating to some amount with the level of HBV exposure and their root of exposure (infection route) (Barker and Murray 1972).

The viral incubation period is followed by a little preicteric or prodromal phase of reasonable symptoms such like fever, nausea, fatigue, anorexia, and body aches. During this phase (preicteric phase) plasma ALT concentration was boosted and high levels of HBsAg and viral (HBV) DNA was detected. The preicteric phase persists for a few days to as long as about 10 days and is further followed by commencement of jaundice or dark urine with increased levels of uric acid. The icteric phase of hepatitis B viral infection stands for a variable period of about 10–20 days, during this phase viral capsid antigen (HBsAg) and viral DNA concentration decrease in plasma. In convalescence, jaundice resolves but constitutional symptoms may last for weeks or even months. Throughout this phase, HBsAg is cleared followed by the disappearance of detectable amount of surface antigen (HBsAg) and viral genome (HBV) from serum. Acute liver failure is hardly reported in some patients of patients with acute hepatitis B and jaundice (approximately 1%) (Berk and Popper 1978). The commencement of fulminant hepatitis is typically marked by the sudden symptoms of abdominal pain, fever, vomiting, and jaundice, followed by disorientation, perplexity, and coma. Viral surface antigen (HBsAg) and HBV DNA levels usually drop quickly in the serum as liver failure develops. Some patients are showing HBsAg-negative serum profiles by the time of commencement of hepatic coma. Hepatitis B patients with acute liver
failure require careful supervision and monitoring and should be referred quickly to a tertiary medical centre (Hoofnagle, Carithers et al. 1995).

1.1.2. Chronic Hepatitis B:
Chronic hepatitis B has an unpredictable and pulsating course. During early infection, viral envelop proteins like (HBeAg, HBsAg), and HBV DNA are usually present in high concentration in serum, and reporting mild to moderate rise in serum amino-transferase concentrations. Disease activity can determined either with the immune tolerance phase ie persistence of high levels of HBeAg and HBV DNA or with inactive carrier state with loss of HBeAg and drop of HBV DNA to lowest serum concentrations or undetectable levels. Other patients persist to have chronic hepatitis B, although some misplace of HBeAg and develop anti-HBe antibodies (HBeAg-negative chronic hepatitis B) (McMahon 2009).

Generally prophecy of patients with chronic hepatitis B is directly connected to the severity of viral (HBV) infection and condition of infected individual. About 50% of severally infected patients of chronic hepatitis and cirrhosis, the 5-year survival rate was considered. (Weissberg, Andres et al. 1984; de Jongh, Janssen et al. 1992) Among patients with chronic hepatitis (improved ALT and inflammation and/or fibrosis on liver biopsy) many patients are reported with having nonspecific disease symptoms, such as exhaustion and discomfort at mild right upper quadrant. Patients with highly severe disease or cirrhosis could have significant legitimate symptoms like jaundice, and peripheral stigmata of end-stage liver disease including palmar erythema, spider angiomata, gynecomastia, splenomegalgy and fetor hepaticus. Symtomes like peripheral edema, encephalopathy, gastrointestinal bleeding and Ascites are seen in patients with more superior cirrhosis. ALT and AST are often prominent but could not correlate well with severity of liver disease. Prothrombin, Bilirubin, and albumin often turn into abnormal/ elevated concentrations with progression of disease while decrease in platelet count is often a poor prognostic sign.

Patients having chronic hepatitis B infection may develop acute exacerbations with markedly improved serum ALT levels. This situation is more often described in patients with HBeAg-negative chronic hepatitis B [6]. To differentiate between acute hepatitis B and chronic hepatitis B, anti-HBC IgM plays a significant role as marker peptide. While anti-HBc antibodies of the IgM class can be detected rarely in patients with chronic hepatitis B infection. Alpha-fetoprotein (AFP), used as a marker for
Chronic hepatitis C, is habitually elevated in equivalent with ALT at the time of acute exacerbation (Lok and Lai 1989). However, it is unlikely to exceed 400 ng/ml serum concentrations. In patients with elevated levels of AFP more than 400ng/ml serum, expansion of HCC might be suspected (Spangenberg, Thimme et al. 2006). Approximately one-third of persons with chronic Hepatitis B Virus infection will sooner or later develop long-term upshots of the disease, such as end-stage liver disease, HCC, or cirrhosis. The determinants of conclusion of chronic hepatitis B viral infection appear to be both viral (Viral genotype, viral DNA levels, some viral mutation patterns) and host-specific (immune status, genetic background, age and gender) serum distributions.

1.2. Hepatitis B virus (HBV):
The hepatitis B virus is a small DNA virus with the characteristics similar to retroviruses (Liang 2009) and belongs to the *Hepadnaviridae* family. Viruses of the *Hepadnaviridae* family are mostly found in ground squirrels, woodchucks, herons, Peking ducks and tree squirrels. On the basis of genetic sequence (DNA sequencing) comparison Hepatitis B virus (HBV) can be classified into eight genotypes, namely A, B, C, D, E, F, G and H. individual genotype has its distinct geographic distribution. On the basis of shape and size three types of viral particles were visualized in the serum of infected individual using electron microscopy. HBV particles are of spherical shape with a diameter of about 20 nm with filaments of inconsistent lengths with a width of 22 nm. HBV spheres (capsid) and filaments are composed of Glyco-protein majorly hepatitis B surface antigen (HBsAg) and host-derived lipids as bilayer structure (forming capsid). Without viral nucleic acids (HBV-DNA) these capsids are not infectious(Gavilanes, Gonzalez-Ros et al. 1982; Gavilanes, Gomez-Gutierrez et al. 1990). HBV virion (infectious) has a globular, double-walled arrangement and sized up to 42 nm in diameter (Figure 1.1).

Major constituents of the inner virion are HBsAg embedded in the lipid bilayer forming an envelope that surrounds an inner HBV-DNA and peptide complex (nucleo-capsid) composed of hepatitis B core antigen (HBcAg) complexed with the viral DNA genome and virally encoded polymerase. HBV has partially double-stranded genome formed by a rounded DNA of about 3.2 kilobase pairs (kb). The viral polymerase is covalently attached to the 5’ end of the negative strand of HBV-DNA(Gerlich and Robinson 1980)
1.2.1. HBV Transmission:

The hepatitis B virus can survive for about 7 days outside the host body. During this time, virus (HBV) can infect an individual if it comes in contact with the individual which is non vaccinated for hepatitis B. The incubation period of the HBV comprises for about 75 days, but it can vary from 30 days to 180 days in different host individuals. The HBV presence in the serum can be detected within 30 to 60 days after viral infection and can extend into chronic hepatitis B.

![Morphology of HBsAg Virus by TEM analysis of infected serum 42nm viral particles as well as 22nm capsid (A), diagrammatic representation of HBsAg virus and their structural features (B). As represented by Liang et al. (Liang 2009)](image)

In highly endemic areas, HBV is usually spread from mother to child at birth or through horizontal transmission by exposure to contaminated blood. The progression of chronic stage of Hepatitis B infection is very common in infants infected from their mothers (perinatal transmission) or the children got infection at the age bellow the 5 years. The foremost Hepatitis B infection route covered by percutaneous or mucosal contact to infected body fluid (vaginal, menstrual, seminal fluids and saliva) and blood contact. Mostly Hepatitis B infection in middle age leads to chronic stage in less than 5% of cases. HBV can spread through the reuse of needles and syringes. Moreover HBV infection can occur at the time of dental and surgical procedures, or by using
contaminated razors and other similar objects that are unhygienic and contaminated with HBV infected blood.

1.2.2. Diagnosis
A laboratory verification of the diagnosis is essential to differentiate hepatitis B from hepatitis caused by different viral agents. A number of blood tests based on the ELISA are available to diagnose and monitor people with hepatitis strain as well as to distinguish between acute and chronic HBV infections.

Majorly laboratory diagnosis of hepatitis B infection performed by the ELISA focuses on the detection of the presence of hepatitis B surface antigen HBsAg in Blood/serum samples.

- Acute Hepatitis B infection is characterized by the presence of HBsAg and HBcAg specific immunoglobulin M antibody (IgM). At the initial stage of infection, patients are also seropositive for HBeAg. Generally HBeAg is an indicator of elevated levels of replication of the HBV genome. Presence of HBeAg in the serum indicates that the blood and body fluids of the individual are greatly contagious.

- Chronic Hepatitis B infection is characterized by the persistence of HBsAg for at least 6 consecutive months. This phenomenon is the major indicator of risk for budding chronic liver disease and can develop into liver cancer at latter stage in life.

1.2.3. Available treatment for HBV infection
There is no precise treatment available for acute hepatitis B infection. Therefore, concerns are expected towards maintaining comfort and enough nutritional balance in regular diet. Chronic hepatitis B infection can be treated with antiviral agents. Treatment can reduces the development of cirrhosis, reduce the chances for liver cancer and improve survival time.

WHO recommends use of oral treatments using antiviral agents ie. Tenofovir or Entecavir. However, in most cases, the treatment does not cure hepatitis B infection, but only suppresses the replication of the virus. Therefore, most people who start hepatitis B treatment must continue it for life.
Treatment using cytokines treatments like interferon injections may be considered in some people, but its use is not feasible in poor people due to its high cost and significant adverse effects requiring careful monitoring.

Limited access to diagnosis and treatment of hepatitis B in many part of population was observed, and many people are diagnosed only when they already have advanced liver disease. Liver cancer progresses very rapidly, and since treatment options are limited, the outcome is not satisfactory. In low-income population, most people with liver cancer die within months of diagnosis while in high-income countries, surgical procedure and chemotherapy can extend life for up to a few years.

1.2.4. Prevention

The hepatitis B vaccination is the only present prevention for hepatitis B viral infections. WHO recommends HBsAg vaccination for all infants as soon as possible after the birth by means of IM injection of recombinant HBsAg vaccine. The birth dose should be followed by 2 or 3 booster doses to complete the primary series of vaccination.

The complete vaccination sequence induces protective antibody titers in more than 95% of young adult, children and newborn Childs. Protection from the HBV lasts minimum up to 20 years and is probably observed up to lifelong periods in some cases. The vaccine has a sparkling eyewitness of safety and effectiveness towards protection from HBV infection. Since 1982, more than one billion doses of hepatitis B vaccine formulation have been administered to men globally. In many countries where 10–15% of children used to get infection of HBV and prone to become chronically infected, vaccination program has reduced the rate of HBV infection the as well as chronic infection to less than 1% among immunized children.

1.3. HBV genome

The genome of HBV encodes for the 4 overlapping open reading frames (ORFs) viz. S, C, P and X. S-ORF sequence of the viral genome encodes for the viral surface (envelop) peptides and C- ORF encodes for core peptides. The P-ORF (precore) codes for a signal peptide molecule that guide the translation product to the endoplasmic reticulum for further processing (Glycosylation) and formed the complete HBeAg molecule ready to be secreted. The functions of HBeAg peptide remains largely undefined, even though it has been concerned as an immune tolerogen, which functions
as to promote persistent infection (Milich and Liang 2003). The last segment of HBV genome X-ORF encodes for 16.5 kd protein molecule (HBxAg) that shows numerous functions in assembling the newly synthesized virus particles, DNA repair, signal transduction, activation and inhibition of protein degradation (Cross, Wen et al. 1993; Hu, Zhang et al. 2006). Other functionally active component of the Hepatitis B viral genome is the DRs (direct repeats) found at both the ends of genome and are responsible for the sequence specific replication of DNA. Two enhancer sequences (En₁ and En₂) confers liver specific signaling and expression of HBV gene products.

Viral infection in the host cells begins with the receptor mediated viral particle adherence (receptors for the surface envelop of HBV) at the cell surface. In this process many receptors were involved but only carboxypeptidase D have been shown to play an essential role in viral entry for the duck HBV (Liang 2009). The mechanism of the viral particle entry inside the nucleus of the host cell was not properly understood and it may involve structural changes in the nucleocapsid core protein to facilitate its transport towards nucleus permeabilizing the nuclear membrane (17). After entry of the viral genome into the nucleus of the host cell it get in the form of covalently closed circular DNA (cccDNA) this form of the HBV DNA serve as the template strand for the further transcription of all genes sequences and DNA replication purposes. Replication of the virus particles begins with the packaging of the nucleic acid content in the nuclear envelop proteins in the cytosol and these packed nucleic acid in the core proteins was further packed by the surface envelop proteins those come together after the nessesory modifications (Glycosylation) at the endoplasmic reticulum.

1.4. Hepatitis B surface antigen (HBsAg)

HBV particles (42 nm) consisting of a pretentious core that surrounds the viral genome this core proteins were further surrounded by envelop made of phospholipids and protein mainly responsible for major antigenic determinants Hepatitis B surface antigen protein (HBsAg). HBsAg may be found in both glycosylated or non-glycosylate forms (Laub, Rall et al. 1983) with the molecular weight of 27 and 25kd respectively as measured by SDS PAGE analysis. HBsAg isolated from the HBV infected patients consists of a major protein molecule (P24) and its glycosylated form known as (GP27). HBsAg purified by gel filtration and affinity chromatography shows about 20-nm large particles. Further twofold-glycosylated form, GP36 was also found in infected serum (Julithe, Abou-Jaoudé et al. 2014). The amino-terminal protein sequence of HBsAg has
been analyzed and aligned with the peptide sequence of HBsAg. P24 gene carries a nucleotide sequence of 226 triplets codons ending with a stop codon was identified that begins at the fourth possible start codon of a larger open reading frame (ORF). By calculating the mass of the 20 nm HBsAg particle it was concluded that the 20 nm particles contain about 114 monomer units of HBsAg.

1.5. Vaccination strategies for HBV

Once the immune system is taught to resist a disease, it is said to be immune for particular disease. Previous to vaccination programs, the only way to become immune to a particular disease was to actually get it (viral infection) and with chance, survive from the disease. It is the process termed as naturally acquired immunity. With naturally acquired immunity, individual suffers the symptoms of the disease and also risk the complications of the infection, these can be quite serious or even deadly for some time. Additionally, during certain stages of the disease, these may be contagious and may pass to other family members, friends, or the individuals come in contact with diseased individual. Vaccines, which provide artificially acquired immunity, are an easier with least risky way to become immune. Vaccines are capable of preventing a disease from occurring in the first place, rather than attempt to cure it after the infection. It was also observed that the vaccination is also much cheaper to prevent a disease than to treat it.

Considering that most HBV infections occur via parental administration, complete infant vaccination or mass vaccination is the key to eradication and subsequent elimination of HBV. First HBV vaccine, made from human carrier plasma, was approved for human use in the USA in 1981. In 1991, the WHO recommended it for all countries to implement a policy of universal HBV vaccination up to 1997.

1.5.1. Drawback of conventional mode of vaccine delivery

- Requires primary course of injections followed by boosters
- Requires adjuvant
- Fails to elicit cell mediated Immunity (CMI)

The major disadvantage of the conventionally used vaccine delivery systems is that the intramuscular injection provides IgG and IgM responses against used antigen but totally fail to elicit mucosal IgA antibody production against antigens.
1.5.2. Currently used adjuvant and their adverse effects

Adjuvants play an important role in development of effective vaccine formulations but unfortunately some few adjuvants have succeeded in regulatory approval protocols for human administration. Some of the important characteristics of adjuvants selection are the type of antigen, route of administration, species to be vaccinated, and probability of side-effects generated by the its administration in host body. Ideal adjuvants should have the properties like -

- Non immunogenic for host individual.
- Promote an appropriate immune response (i.e. cellular or antibody mediated immunity depending on requirements for protection from pathogen).
- Stable with long shelf life, biodegradable and cheap, producible at industrial scale.

There are marked differences in the efficacy of adjuvants depending on the rout of administration (e.g. between mucosal and parenteral routes). Hence new vectors, antigen delivery systems or adjuvant compounds need to take into account the characteristics of the proposed administration route.

The most common adjuvant used for human vaccination is alum, however, it is not suitable for eliciting cell mediated immunity and therefore not appropriate adjuvant for vaccines. Furthermore using alum is not suitable for mucosal vaccine vaccination and in general vaccines formulated with alum do not yield mucosal antibodies following oral/nasal administration. A new potent, safe and non-toxic adjuvant for human use was needed with the capacity to elicit both cellular as well as humoral immunity. Combinations of lipidic adjuvants (bacterial or synthetic) with peptide antigen were tested to produce self adjuvanting lipo-peptide vaccine formulations were prepared. These lipid peptide combinations were tested in human clinical trial and demonstrated improved degree of safety with reduced side effects and these formulations were also demonstrated to be effective after mucosal administration.

Moreover, whole conventional licensed hepatitis B vaccines in human use contain alum as adjuvant. Even though it is a potent B cell stimulator, alum is less effective in inducing a TH\(^1\) response while given via intramuscular route. Other most potent and
widely used adjuvants for vaccination are LT (closely related E. coli heat-labile enterotoxin) and CT (cholera toxin). Both were demonstrated as unsafe for human use in their native form.

Vitamin B12 till now used as targeted delivery of bio-actives from GIT enhancing GIT absorption via receptor mediated endocytosis. Scalabrino G et al (2002) has reported role of vitamin B12 in up regulation of IL 6 in rat cerebrospinal fluid. Up regulation of IL 6 plays an important role in mucosal immunology.

1.5.3. Oral Immunization for Hepatitis B

Oral route of the vaccination having major advantage in case of mucosal transmitting infectious diseases like hepatitis B by means of providing additional protection at the mucosal surfaces by synthesizing pathogen specific secretary IgA antibodies. These secreted antibodies on the mucosal surfaces capture antigens coming in contact with Abs and present the processed antigen to the T cells located in the nearby lymph node (MALT) to provoke immune response against the pathogen. Subsequently pathogen deactivation at the initial phase of inoculation by means of activated cellular and humoral immunity at the local site (mucosal surfaces). Peptides and proteins cannot be delivered by the oral route of administration because of their sensitivity towards proteolytic enzymes and alterations in the pH at gastric and intestinal conditions. To overcome these barriers a delivery system (nanoparticle / microparticle) is necessary to encapsulate the peptide for its delivery as functional peptide/Antigen.

1.5.4. Nanotechnology based delivery to mucosal immune cells

The field of nanotechnology is rapidly growing in medicine and has generated considerable attention in mucosal delivery of therapeutic molecules (immunogen, drugs, RNA) over the past few years. It has led to important research output in the field of vaccinology (Kasturi, Skountzou et al. 2011) and molecular imaging. (Hikage, Gonda et al. 2010) In immunology, nanotechnology-based research is mainly focused on the development of optimally designed therapeutic agents for mucosal surfaces.(de la Fuente, Csaba et al. 2008) Nanomaterials are a small nanoscale particles with size ranging about 10–100 nm, but this fact is much debated regarding the suitability of this simple criterion for the delivery of large molecules like peptides and nucleic acids.(Maynard 2011) An advantageous condition for mucosal drug/Ag delivery would be to have efficacious and self determined drug-release agents that integrate with the
physiological conditions of gastro intestinal tract (GIT) without causing any harmful side effect. The challenge towards efficient antigen delivery towards specific sites or mucosal target location (peyer’s patches and dendritic cells) while minimizing systemic out flow of delivery system. The use of nanomaterials creates endless possibilities for their modifications in respect to modulate therapeutic and immunogenicity efficacy. Thus, nanotechnology-based particles may hold great potential as novel drug/Ag delivery systems for targeted and controlled release in mucosal delivery.

Current therapeutic research towards targeting mucosal immune cells aims to engineer nanoparticles using biocompatible and biodegradable materials that carry peptide (Ag) or drug of interest or other molecules to target formulated nanoparticle to desired site at specific mucosal tissues, such as peyer’s patches in GIT (Laroui, Wilson et al. 2011). Nanoparticle formulations for the oral delivery may be designed to demonstrate improved release the Ag at a specific pH conditions, mucoadhesion or mucopenetration properties, improving the stability of encapsulated antigen towards luminal enzymes, or shows bacterial dependency on the cleavage for site-specific commencement. In a study, the Laroui et al (Wilson, Dalmasso et al. 2010) successfully demonstrated the use of thioketal nanoparticles as an oral delivery system for siRNA of TNF α to block inflammation in Dextran sodium sulfate (DSS) induced colitis. Thioketal obtained from a polymer that showed its selective degradation at specific sites in GIT (areas of high reactive oxygen), that induces the localized delivery / release of siRNA specifically towards inflamed intestinal tissues.

Nanoparticulate system is a complex delivery system with a wide variety of characteristics. Distinct nanoparticles can exploit diverse means to gain entry into a cell.(Hess and Tseng 2007) Rather than passing directly in the course of a cell wall, nanoparticles may be actively endocytosed or phagocytosed by the targeted cells. Several physicochemical and biological characteristics of nanoparticles (not only size, shape, and charge) could transform the individual cell’s responses (Dwivedi, Tripathi et al. 2011). Wide range of immune-modulatory profiles response studies are lacking on of biodegradable and biocompatible organic nanoparticles in mucosal immune cells. Presently any foreign substance at mucosal surfaces should be considered with concern to various immunological alterations at the site. Variety of metal or inorganic material nanoparticles considered as biologically inert can be recognized as “non-self” by the host innate immune system.
Recent advancements towards the delivery of peptides and proteins have highlighted the great promise of nanoparticle-based drug delivery for the immunization via mucosal surfaces. It is also obvious that nanotechnology-based drug delivery technologies at mucosal surfaces are not likely to reach to the clinical practices in the recent very near future because of its stability and their variability of results in different human subjects. The emerging field of “nano-immunology” is still in its early life, opening a mass of exciting research prospects for mucosal immunologists. Findings may help to understand targeted nanoparticulate delivery system of a drug/Ag up to a different extent of cellular interactions with mucosal cell populations.

1.5.5. Factors affecting nanoparticle uptake by intestinal M cells and dendritic cells

Size and surface morphology

Size, surface charge and morphology of the nanoparticles plays a key role in their cell interaction with lipid bilayer of cell promoting its cell uptake. In the case of nanoparticle delivered in vivo penetrate deeply into the lung parenchyma and mucus membrane. Different sized nanoparticles show specific bio-distribution patterns given in vivo. Many studies have examined the in vivo distribution of nanomaterials and found particles diameter higher than 6 nm cannot be excreted by the kidneys and retained in the blood circulation. Further these particles were accumulate in specific organs, such as the liver and spleen, and finally cleared by the macrophages. (Albanese, Tang et al. 2012)

Surface properties of the nanoparticulate delivery system play an important role in their interactions towards cell membrane. Biological interactions of the nano-material were driven by nanoparticles surface characteristics because it comes in direct contact with the cells membrane. In case of nanoscale surface roughness on the nanoparticles made somehow increased contact area between the nanoparticle and lipid bilayer membrane comparing to smooth nanoparticles (Figure 1.2). Adhesion towards the cell membrane was monitored by non-covalent interactions between cell surface and surfaces of nanoparticulate delivery system. In case of oral delivery of nanoparticles surface characteristics plays major role not only with the lipid membrane but also with the mucus layer present throughout the GIT.

Mucus layer in the GIT plays as a significant and limiting role for the various lipid based delivery system due to their less density and poor mucus penetration properties.
Orally delivered vaccine cannot prove its best therapeutic efficacy until it has a good mucus penetration/diffusion capability (Khan, Iliboshi et al. 1999). Moreover, the relatively thin mucus layer over M cells supports penetration of very small size nanoparticles (about 50 nm) (Frey, Giannasca et al. 1996). Polymeric coating and induces surface roughness over liposomes or nanoparticles significantly improves mucus diffusion that supports effective M cell targeting by means of liposomes.

Figure 1.2: Adhesion of rough and smooth nanoparticles on the lipid bilayer membrane.

1.6. Solid Lipid nanoparticles for oral vaccine delivery system

Immunogenic potential of peptide (Ag) as well as their clinical application is significantly hampered by number of obstacles to their successful oral delivery. Protein stability is the major concern towards the destabilizing and stabilizing forces. The folding of peptide to their secondary, tertiary and quaternary structure is based on the week non-covalent interactions (i.e., Electrostatic, hydrogen bonding, hydrophobic interaction and Vander wall forces) forces between the peptide chains. Any disturbance in this non-covalent interaction leads to the destabilization the protein structure and whole peptide may loss its activity by shift in their folding patterns. There for activity of the peptide will be compromised by the external factors like pH, ionic strength, temperature, non-aqueous solvents. Most of these factors were present in the common formulation methods including sterilization and lyophilisation resulted in the loss of activity and precipitation. Diffusion transport of macromolecules (proteins) through epithelial barriers is generally slow ensuing in poor absorption, unless specific transport receptors are available. Physicochemical properties proteins make them unsuitable for absorption by the main routes and mechanisms. In the GI track the condition is more challenging due to presence of proteolytic enzymes and variation in the physiological pH (Wang 1999; Frokjaer and Otzen 2005).
Since their first depiction by Müller et al., Solid lipid nanoparticles have attracted increased consideration as an efficient and non-toxic opportunity for lipophilic colloidal carrier system prepared with lipids used as common pharmaceutical excipients. Two main production techniques were often applied for the preparation of SLNPs: the high-pressure homogenisation (HPH)/ Microfluidization described by Müller and Lucks (Muller, Rdite et al. 2002; Wissing, Kayser et al. 2004) and the micro/nano-emulsion based technique by Gasco (Gasco, Priano et al. 2009). Unlike polymeric nanoparticles SLNPs do not need any additional chemical needing for their crosslinking which may have impact on the activity of encapsulated peptide. These lipid nanoparticles were formed due to their hydrophobic interactions between the aqueous phase and the lipid molecules that force them to form small size particles in the presence of suitable stabilizer. Under optimal condition these lipid nanoparticles were found suitable for the encapsulation of lipophilic drug molecules as well as hydrophilic peptide macro-molecules and seem to fulfill the requirements for an optimum nanoparticulate carrier system (Wissing, Kayser et al. 2004; Jain, Jain et al. 2014).

Lipidic delivery systems are the preferred approach towards the oral delivery of peptides /drugs due to their hydrophobic nature. Due to hydrophobic inner core possibility towards the permeation of GIT fluid inside the nanoparticle core was limited that reflect in the improved gastric and intestinal stability of encapsulated peptide. Their colloidal dimensions and the controlled release behaviour of encapsulated peptides enable protection and administration by mucosal and non-mucosal routes thus emphasizing the adaptability of this nanoparticulate carrier. Publications have described the use of solid lipid nanoparticles by oral routes including bio-distribution and pharmacokinetic studies of encapsulated peptides/drugs.

Characterization of the mechanisms of particulate uptake and translocation at mucosal surfaces now widely established and opened a massive field for the investigations on the therapeutic applications of solid lipid nanoparticles via mucosal routes.

1.6.1. Protein encapsulation in SLNPs

Formulation of SLNPs is based on the controlled lipid aggregation in the aqueous phase in the presence of suitable stabilizer that can stabilize the formulated nanoparticle by steric stabilization by providing repulsion between nanoparticles. Therefore due to their hydrophilic nature most of the peptides/ proteins were expected to be poorly encapsulated in the SLNPs core. However, lipids are versatile in nature that may form
different structured solid matrices, such as lipid peptide conjugated (by non-covalent interactions) and nano-structured lipid carriers (NLCs) towards improve the peptide encapsulation in SLNPs core (Wissing, Kayser et al. 2004; Fan, Chen et al. 2014). Since the mid 1990’s authors have regularly publishing the results of the peptide loaded SLNPs by using the Insulin, bovine serum albumin and Lysozymes.

1.6.2. Methods of SLNPs preparation

*High pressure homogenisation (HPH)/ Microfluidization*

![Figure 1.3: Microfluidizer™ M-110P (Microfluidics corp UK)](image)

The HPH/Microfluidization technique has been extensively explored for the encapsulation of the peptides and drug molecules with providing reproducible batch results and encapsulation efficiencies above 90% (68–73). Various Lysozyme formulations were prepared with varying the concentrations of lipid and lipid ratios results in a pharmaceutically active encapsulated enzyme. After processing at 1000bars for 3 cycles at 50 °C encapsulated enzyme was found pharmaceutically active. Loading of the enzyme was found 0.03% (wt/wt) of SLNPs with only 60 % of the encapsulation efficiency with total enzyme taken but Lysozymes did not suffer any detectable instability by performing PAGE analysis. This is not surprising like Lysozymes have higher protein stability due to stronger internal coherence; human insulin as well as
CyA were also remain stable throughout the formulation using high pressure homogenizer. X-ray investigation of formulated delivery system shows that the hydrophobic CyA molecules are dissolved in the lipid matrix (inner core) and were not found on the surface of nanoparticles. An oral formulation of SLN (157 nm) containing 20% of CyA was compared for *in-vivo* activity with the commercially available CyA formulation (Sandimmun Neoral/Orptoral®) young pigs. The blood profiles observed after oral administration revealed a fast absorption and comparable mean plasma profiles among the SLNPs and the commercially available formulation.

### 1.6.3. Surface modified SLNPs for improved M cells targeting

Various modifications on the surface of nanoparticle were done to improve M cells targeting present in peyer’s patches in GIT. Some glycopeptides like lectin WGA has roles in the receptor mediated targeting towards intestinal M cells. Mannose receptor is also found over expressed on the surface of antigen presenting cells (APCs) of the intestinal lumen. Targeting vaccine formulation towards intestinal APCs can evoke the mucosal immune response. However combination strategies for the mucosal vaccine delivery system were not yet reported as the oral vaccine formulation.

### 1.7. Liposomes for mucosal vaccine delivery

Liposomes are the unique delivery system promising suitability towards both for the hydrophobic and hydrophilic cargo. Core of the liposome is made of the aqueous phase and surrounded by the hydrophobic lipid bilayer.

Liposomes have been extensively used as potential delivery systems for a variety of compounds primarily due to their high degree of biocompatibility. Presence of both hydrophilic and hydrophobic compartments confers liposomes suitably for the long range of molecules like peptides nucleic acids and drugs. Liposomes are classified according to their size range, about 50–5000 nm in diameter. This resulted into two categories of liposomes namely multilamellar vesicles (consist of two or more lipid bilayers250-5000nm) and unilamellar vesicles (single bilayer 50-200nm). (Bharali, Khalil et al. 2009)
1.7.1. Method of preparation

Liposomes were made by the lipid hydration method a thin film of the lipid molecules was formed in round bottom flask by evaporating the organic solvent (chloroform). Formed lipid film was hydrated with appropriate volume of aqueous phase. Hydrated lipids form large size multilamellar vesicles (MLVs) further to get the desired size, formulated MLVs were subjected to sequential filtration using extruder. Sequential filtration was performed at 10-15 PSI pressure maintained by N2 gas supply and was filtered sequentially from 800, 400, 200 and 100 nm pore size polycarbonate filter for 5 cycles at each filter.

Figure 1.4: Liposome extruder.

1.7.2. Polymer coating on liposomes for mucosal delivery

Oral peptide delivery systems have not yet reached their full potential. The main hurdles need to be overcome towards making effective oral protein/peptide delivery are first to protect enzymatic degradation of peptides before reaching the target site like intestinal M cells and APCs, and second the low permeability of such large molecules as well as nanoparticulate delivery system across the intestinal mucosa (Shaji and Patole 2008; Park, Kwon et al. 2011). Polymeric coatings on the liposomes significantly improve the hydrophilicity of the surface of liposomes that results in the improved mucus diffusion and permeation. Liposomal delivery system were suffered from having a soft structure that makes it unfit for their application in oral deliver. At the GIT condition liposomal ruptures were observed that makes encapsulated peptide freely available to proteolytic enzymes. Surface of liposomes was coated with the additional polymer will improve the structural and physical stability of the liposomes at the GIT (Sehgal and Rogers 1995; Zhou, Liu et al. 2014). These changes on the
liposome surface improves protection of encapsulated peptide from the proteolytic enzymes of the GIT (Gradauer, Barthelmes et al. 2013).

1.8. Formulation excipients and their role in immune modulation
Nanoparticles made by the biodegradable polymeric materials have an advantage of having some of immune modulatory function of polymers.

1.8.1. Vitamin B12 and its derivatives
As the recent reports show, the Vitamin B metabolites are capable of evoking MAIT activated T cell mediated immune response. Because bacteria synthesize vitamin B, our immune system uses this as a point of difference to recognize infection. Mucosal associated invariant T cells (MAIT cells), recognize products of vitamin B synthesis from bacteria and yeast in an early step to activating the immune system. The research revealed how by-products of bacterial vitamin synthesis, including some derived from Folic acid or vitamin B9 and Riboflavin or vitamin B2, could be captured by the immune receptor MR1 thus tuning the of MAIT cell activity in Peyer’s patches. (Patel, Kjer-Nielsen et al. 2013)

1.8.2. Chitosan
Biodegradation, immunological activity and high viscosity make chitosan an excellent candidate as an adjuvant system for parenteral vaccination. Further some researchers have explored immune properties by inducing both humoral and cell mediated immunity. David A et al (Zaharoff, Rogers et al. 2007) found peptide delivered by chitosan depot enhanced antigen-specific antibody titers and antigen-specific splenic CD4+ proliferation over 5 and 6 fold respectively in mice model. Mechanistic studies of chitosan revealed that it exhibited at least two major characteristics that might allow it to function as an immune adjuvant.

- The chitosan solution creates an antigen depot at the site of injection that makes slow release of used Ag.
- Chitosan induced a transient 67% cellular expansion in draining lymph nodes that makes Ag more available to the immune cells.
1.8.3. Sodium alginate
Sodium Alginate is a widely used polymer biomaterial extracted from the algae it stimulates NF-kB translocation into the nucleus of macrophage cells (RAW 264.7). (Yang and Jones 2009) Initial translocation began within 2 h of exposure and extended up to 24 h, depending on amount of sodium alginate administered. Tight regulation of NF-kB activation is necessary for the inflammatory signalling while after treatment with sodium alginate it was observed that there has been feedback inhibition of NF-kB after 48 hrs. It was confirmed that the alginate stimulated significantly greater NF-kB translocation. Further it stimulates release of inflammatory cytokines and activation of a transcription factor common to many inflammatory pathways.

1.8.3. Calcium Phosphate
Biodegradable calcium phosphate nano/micro-particles were investigated as an alternative towards aluminum adjuvants for parenteral delivery of vaccine formulations. Clinical studies conducted in France described the potential of a calcium phosphate as an adjuvant system used in booster immunizations against tetanus and diphtheria. Further Qing He et al (2002) (He, Mitchell et al. 2002) reported that calcium phosphate nanoparticles (CAP) represents a superior alternative towards alum adjuvants in mice immunized with viral peptides.

1.9. M cell structure and function
The mucosa-associated lymphoid tissues (MALT) are the only sites for the antigen-sampling and stimulation of mucosal immune responses from mucosal surfaces of oral (GIT) and nasal vaccination. At MALT, phagocytosed antigens were transported across the mucosal epithelial barrier to lymph nodes for subsequent immunological reactions (Didierlaurent, Sirard et al. 2002). For this specially dedicated cell population was present for the antigen sampling from MALT and were termed as membranous epithelial (M) cells are accountable for this transepithelial antigen transport (Gebert 1997; Gebert and Pabst 1999). The M cells are present at the epithelial cell lining of follicle-associated epithelium (FAE) overlying on the aggregated lymphoid follicles (clusters of B and T cells) present at the small and large intestines. Proportion of M cells occupying total FAE varies between species, with M cells comprising about 50% of the total cell population of FAE in rabbit Peyer’s patches. While M cell population in the man and mouse FAE reduced to only 10% of the total FAE total cell population.
Scattered M-like cells have also irregularly been reported in the villous epithelium of rabbit intestine (Borghesi, Taussig et al. 1999; Iwatsuki, Ogawa et al. 2002). M cells are characterised by their asymmetrical cell surface microvilli (helps in antigen sampling by improved contact surface area) and existence of a basolateral cytoplasmic invagination that creates a pocket like structure containing immune cells (lymphocytes and occasional macrophages) (Ermak and Giannasca 1998; Kraehenbuhl and Neutra 2000).

Intestinal M-cells provide an efficient site for the antigen sampling from the gut and delivering towards underlying sub-mucosal tissues. Although the sampling of the antigen is carried out by the non-selective manner as a consequence of bulk sampling of particulate material comes in contact via intestinal lumen. In respective of these M cells expresses variety of surface immunological receptors that enable them to sample a large verity of microbial pathogens from intestinal lumen (Mabbott, Donaldson et al. 2013).

Figure 1.5: M cells located in mucosal epithelium endocytose Ag loaded nanoparticle in the lumen and transport it for processing to sub-mucosal pockets of immune cells. [Sansonetti at al. (2009)].
1.9.1. M cell targeting strategies

**Lectin receptors:**

Wheat germ agglutinin (WGA) is isolated from the wheat (Triticum vulgaris) with a two subunit macro molecule having molecular weight of 36 KD. Transcytosis and cellular uptake of many dietary lectins occurs by means of receptor-mediated endocytosis (Gupta and Vyas 2011). During this process lectins were poorly digested in the intestine can bind through their sugar reactive sites to glycoproteins and glycolipids via \( \alpha-1, 2 \) fucosylation, N-Acetyl glucosamine (GlcNAc) and N-acetyleneuraminic acid (sialic acid) residues of glycoproteins generally present on the surface of M cells at FAE. The subsequent fate of these bounded lectin molecules depends upon the temperament of the ligand to which they bind. In certain cases the lectin is subsequently transported across the intestinal epithelial cell (enterocyte) towards the blood circulation, where it can stimulate surface immune response to the lectin while combating with circulating B and T cells. (Chionh and Sutton 2010)

**Claudin 4 receptors**

Claudin 4 receptors are generally present on the tight junctions of the endothelial cells. Its role in the M cell endocytosis was observed and may also be redistributed to the cytoplasm of M cells. An antigenic peptide of the C-terminal domain of the Clostridium perfringens enterotoxin binds to the external domain of claudin 4 situated on the surface of the M cells and mediate bacterial cellular uptake. (Rajapaksa, Stover-Hamer et al. 2010) Moreover, this claudin 4- binding peptide when coupled to Ag is effective in enhancing mucosal Ag-specific secretory IgA responses after (Kim, Seo et al. 2010).

**C5aR ligands**

C5aR expressed commonly on a wide variety of cells but particularly on the surface of immune cells like macrophages, neutrophils and T cells. C5aR is a typical G protein-coupled receptor that signals through \( G_{a16} \) and \( G_{ai} \) membrane bound proteins (Kim and Jang 2014). C5aR Present on the apical surface of the M cells shows analogy with the with OmpH of *Y. enterocolitica*, and each of these peptides appeared to bind to a C5aR on the apical surface of M cells. (Kim, Jung et al. 2011)
Reovirus hemagglutinin protein σ1
As reovirus was found to bind with M cells in vivo M-cell in MALT. Nanoparticle targeting can be attempted to improve the effectiveness of the DNA vaccine delivery through mucosal lymphoid tissues.

Secretory immunoglobulin A (IgA) receptors
Secretory IgA antibodies provides first line of defense in the intestinal micro flora, these antibodies binds to the pathogen (bacteria & viruses) and inhibit their attachments towards intestinal epithelial cells. IgA-pathogen complex also showed reduced mucus penetration capability to bind the epithelial cells. (Michetti, Mahan et al. 1992; Boullier, Tanguy et al. 2009) Targeting strategies towards IgA mediated complex of antigen by M cells was facilitated by the receptor mediated endocytosis by means of MNP peptides on the M cells. This mechanism improves antigen sampling by the M cells using IgA bound antigens. (Rey, Garin et al. 2004; Kadaoui and Corthesy 2007)

Other M-cell specific proteins
A number of M-cell-specific proteins have been identified those are selectively binds to the surface antigens of potentially pathogenic microorganisms. However, experiments are required to determine their in vivo functions in intestinal M cell functioning. ANXA5 receptor is expressed on the surface by immature and mature M cells (Verbrugghe, Waelput et al. 2006) and capable to bind via the lipid A domain of lipopolysaccharide (LPS) of Gram-negative bacteria. (Rand, Wu et al. 2012)

1.10. Antigen sampling by the gut dendritic cells
Over the past some years one of the most significant concepts that emerged in the area of gut immunology resulted from the observation by Rescigno et al. (2001) (Rescigno, Urbano et al. 2001).
Functioning of intestinal dendritic cells (DCs) mediate by expanding their cellular extensions between epithelial cells of the villi, to internalize bacteria from intestinal lumen. Intestinal DCs plays an important role in antigen sampling and further migrated towards lamina propria for needed immunological activities. The migration of phagocytes from intestinal epithelial cells towards gut lumen was described in the past
repots and it was interpreted as a mechanism of cellular control of the gut associated pathogens. (Regoli, Borghesi et al. 1994)

![Image](image.jpg)

Figure1.6: TEM image showing a dendritic cell embedded between epithelial cells moving into the intestinal lumen following inoculated with non-invasive salmonella (ΔSPI1) (MC-migrating cell, E-epithelial cell and CE-extra cellular extension). Nicoletti et al (2009). (Nicoletti, Regoli et al.)

### 1.10.1. Targeting strategies towards gut dendritic cells

DCs of GIT were also reported to over express some of the common DCs specific ligands. In past DCs targeting was extensively explored for the targeting of sub coetaneous and intra muscular immunization (Keler, Ramakrishna et al. 2004) as well as for the therapeutic purposes for the DC related protozoan dieses like Leishmania. (Singodia, Verma et al. 2012)

**Mannose receptors**

Mannose receptor (MR) is a carbohydrate-binding surface receptor expressed by various populations of macrophages and dendritic cells (DCs) and nonvascular endothelium.
Figure 1.7: A typical structure of mannose receptor expressed on dendritic cells. (CR)-Cystine rich domain binds to sulfated carbohydrates. (FNII)- Fibronectine type II domain binds to collagen and (CTLD)-C-type lectin like domain mannose ended chains of carbohydrates.

MR mediates pathogen recognition and their internalization by means of receptor mediated endocytosis in macrophages and dendritic cells (DC) further it helps in the modulation of cellular activation and trafficking of T cell population at nearby lymph nodes. MR shows its affinity towards both sulfated and mannosylated sugars due to the presence of two independent carbohydrate-binding domains at the apical site of the ligand. MR is the only one member of the MR family receptors that contains a functional CR peptide domain that facilitates its binding with the sulfated carbohydrates, mainly galactose or GalNAc sulfated at Position 3 or 4. (Leteux, Chai et al. 2000). The CTLD domain of MR binds glycoconjugates terminated in mannose, fucose, or GlcNAc using a calcium-dependent mechanism. (Taylor, Bezouska et al. 1992; Taylor, Gordon et al. 2005)

MR is a highly efficient endocytic receptor that recycles constantly between the plasma membrane and the early endosomal compartment (Gazi and Martinez-Pomares 2009) due to its high rate of encounter with antigen. MR-mediated endocytosis is clathrin-independent and it requires the engulfment of motif (FENTLY) (Gazi and Martinez-
Endosomal acidification is thought to be a mechanism by which it induces ligand release, resulted with the empty receptor fixing back to the cell surface. MR was initially recognized as a major system for antigen internalization in cultured human DCs that carry out antigen processing via endosome and presented towards TH\textsubscript{2} cells through the surface bound MHC II molecule. In fact, MR targeting has been afterward considered as a approach to increase antigen immunogenicity. Involvement of MR in antigen presentation \textit{in vivo} requires an unambiguous understanding of MR expression in professional APCs. MR having DCs are present in selected Lymph Nodes (LNs) (Linehan, Martinez-Pomares et al. 1999) with numbers rising after innate immune stimulation (McKenzie, Taylor et al. 2007). Furthermore, comparable to the tolerogenic macrophages present at the lamina propria of the small intestine, the nearby CD11b\textsuperscript{+}CD11c\textsuperscript{−} cells express mannose receptor (MR) and class-2 retinaldehyde dehydrogenase. (Mascarell, Lombardi et al. 2008; Song, Kim et al. 2009) On the other hand, studies on both mice models and human subjects suggest that the sublingual mucosa is an attractive immunological site to induce antigenic tolerance for improved immunological responses.

1.11. Dual ligand targeting approach towards mucosal immune cells

Multifunctional targeting approach extensively explored in the case of cancer targeting using more than one targeting ligands on the surface of same nanoparticle. This includes both the ligands target towards single cell while in other approach two different ligands have their different target cells. Previous publications have proved the potential of multiple ligand conjugation theory with single nanoparticles for selective targeting and improved cell uptake in the tumor cells. Nanoparticles with the multiple ligands on the surface encounter to the cell surface receptors and as the nanoparticles comes in contact with more surface ligands (than a single ligand conjugated nanoparticle) increased cell interaction with the nanoparticle resulted in the stable anchorage of the dual ligated nanoparticle. This mechanism somewhat prevents the possibility of detachment of nanoparticles from target cells. Intestinal immune cells comprise both the M cells and dendritic cells at the mucosal surfaces. Targeting towards both the immune cells could improve the vaccine efficacy by improving Ag presentation by both the cells.
**1.12. Function of mucosal SIgA towards mucosal immunity**

Mucosal route of the pathogen encounter is most susceptible because of its structural arrangements and direct exposure towards ingested food materials. Mucosal antibody (sIgA) secreted from the mucosal plasma & B cells plays an important role in the pathogen neutralization at the mucosal surfaces.

*In vivo* relevance of IgA can be best demonstrated in the patients with severe IgA deficiency and common variable immunodeficiency. In these primary immunodeficiencies, reduced SIgA production shows persistent symptoms of autoimmunity, allergy/asthma, as well as respiratory and gastrointestinal infections (Cunningham-Rundles and Ponda 2005). Development of intestinal inflammations and small intestinal nodular lesions is commonly seen in IgA-deficient patients, an incident attributed to unusual expansion of commensal bacteria.

Due to their diametric nature SIgA Abs are ideally intended to cross-link target microbial antigens in the mucosal atmosphere. They do so by impeding their circulation through the glycocalyx at the surface of the mucosal epithelial cells, by blocking pathogen adhesion or by satirically hindering their contact with the surface of mucosal epithelial cells and by inhibiting pathogens motility or facilitating their adherence in mucus layer. The ability of secretory Abs to identify intact bacteria, viruses or parasites at mucosal surfaces is a must be needed for the protection of mucosal surfaces.
However, due to the several layers of protection linked with the mucosal immune system, the specific demonstration of the role of sIgA in the process of immune defence at mucosal surfaces is not well established till now. Intestinal Peyer’s patches and their associated M cells correspond to the primary site for uptake followed by presentation of ingested antigens towards immune cells (Niedergang and Kraehenbuhl 2000). Remarkably, sIgA has ability to selectively adhere on mucosal M cells but not IgG or IgM can. This adherence phenomenon was attributed by some receptors expressed on the M cells which can bind to α2 domain in the heavy chain of IgA (Mantis, Cheung et al. 2002; Rescigno 2010). Additionally in an experiment using caco2 cells were infected with the bacterial strain added with the sIgA within the apical compartment of caco2 cells culture, delayed bacterial infection was observed due to formation of Ab–bacteria aggregates, an observation that did not happens in the presence of bivalent IgG of the same specificity the delay in cell damages (caco2 cells) observed resulted into preserved cells structure and decreases in the secretion of pro-inflammatory messengers.

Range of actions for sIgA would find its superior prospective in mucosa-associated pathogenesis and involving the presence of microorganisms with the mucosal tissues. Passively administered sIgA has proven thriving results in numerous viral and bacterial infections through intestinal route; however the course from laboratory experiments to patient appliance and clinical trial data remains untrustworthy. Necrotizing enterocolitis in pre-term, undersized weight infants is accompanied by the recognition of a broad range of bacterial and viral antigens were detected within samples of the peritoneal fluids like damaged tissues and stools. Breast-feeding IgA/IgG-enriched preparations established an encouraging effect on the growth and harshness of the symptoms within low birth weight newborns of a randomized controlled clinical trial held in 1988. Combinations of anti-viral/anti-bacterial and anti-inflammatory activities of the sIgA antibodies might result in decreasing the release of inflammatory cytokines such as interleukin 6 (IL-6), tumour necrosis factor-α (TNF-α) was supposed to responsible for the obstruction of necrotizing enterocolitis in treated patients.

sIgA has also been understood to be capable of blocking the pathogens from attaching to sub mucosal intestinal epithelial cells by direct recognition and binding to the specified binding domains on the pathogen (Ag), as established in the case of reovirus type 1. sIgA is necessary at the mucosal sites for full defence against intestinal reovirus infection, as revealed by performing experiments on the IgA deficient (knockout)
mice. (Silvey, Hutchings et al. 2001) To investigate the molecular machinery fundamentals of sIgA-mediated immunity to reoviruses, a group of reovirus-specific IgA antibodies was screened for those that had protected mice from type 1 Lang challenge given via oral route. Protection of mice was conferred by IgA antibodies directed against antigenic peptide (adhesion fibber protein) on the capsid of virus known for improving viral adherence towards sub mucosal epithelial cells on mucosal surfaces. (Helander, Silvey et al. 2003)

Administration of immune-regulatory sIgA with a broad range of specificity may well be regarded as a precious contributor to the weapon store for future therapeutic challenges. The well-known manifestation that sIgA stably found along the GIT, adheres itself deep in the mucus layer surrounding the epithelium layer in GIT. SIgA Abs showed great stability and remains stable in the harsh environment of the GIT and further recognizes passing or adhering antigenic structures via glycol-conjugates, and promotes the secretion of non-inflammatory cytokines by the immune cells at intestinal mucosa.

1.13. Fate of orally delivered vaccine

1.13.1. Uptake and processing of Ag at intestinal mucosal surfaces

Antigen uptake from the intestinal surfaces is primarily expected by two pathways. The first pathway is still moderately poorly understood, involves uptake of foreign particles by means of the normal epithelium overlying spread over the mucosal associated lymphoid tissues (MALT). For example retrovirus adheres selectively on epithelial cells and expected to be endocytosed beside the basal surface of absorptive cells in isolated monolayer of intestinal epithelial cells. These mucosal epithelial cells are proficient of antigen presentation and initiating T cell proliferation at least in in-vitro conditions. Following the cellular uptake, intracellular digestion of the antigens takes place in the lysosomes while small amount of antigen escape this degradation process to be exocytosed into the interstitial spaces of cells. The initial phase of this type of transport is initiated by the binding of antigens to the specific receptors on the epithelial cell linings.

Second pathway has been extensively studied as the microfold cell (M cell) mediated uptake of intestinal pathogens. These M cells overlie the lymphoid follicles of the gastro-intestinal tract beneath a very thin layer of mucus. The M cell typically has a central pocket or extracellular space which carries macrophages, plasma cells,
lymphocytes, and occasionally polymorphonuclear leukocytes. The M cells have reduced numbers of lysosomes so that particulate antigens entering in the M cells have a improved chances of escaping liposomal degradation and passes into the central hollow cavity further to presentation to the immune cells residing in the M cell pocket. Processed antigens subsequently taken up by the lymphocytes and migrate to nearby lymph nodes through pores in the basal lamina. Effective immune responses towards administered antigens require the intervention of T lymphocytes. For these to be stimulated, the antigen must be presented by antigen presenting cells (APCs) to their respective T cells. APCs digest and link antigen fragments to a surface glycoprotein (MHC) which interacts with a T-cell receptor. These M cells can function not only as sampling but can modify itself as APCs when M cells take up antigen complexes with secretory IgA and expresses MHC Class II HLA-DR antigen in a similar mechanism like dendritic cells and macrophages. T cells that over express the CD8 molecule are also observed showing close similarity with to M cells and may therefore also receive the processed antigens by means of MHC II presentation pathway. A wide variety of macromolecules, peptides, nanoparticles, and microparticles have now been shown to be effectively taken up by M cells.

It is well established that particulate matter absorbed by Peyer’s patches may go through lymphatic transport system to the mesenteric lymph nodes and, rarely in some cases, diffused via the lymphatic vessels to the systemic circulation and reached to the major organs like kidney, liver spleen and lungs. The biological fate of particulate matter internalised by the Peyer’s patches is majorly influenced by the size of particulate materials. Larger particles (>5-µm) are naturally engaged in the Peyer’s patches, while smaller particles (50-200nm) run off this site quickly to distribute via the lymphatic system to more distant sites. Although only a very small fraction of the orally given formulation is absorbed from the peyer’s patches, such least amount of absorbed amount may still be appropriate to evoke a strong immune response. This phenomenon is supported by the mechanism of antigen presentation of the follicle associated endothelial cells which delivered directly to the inductive membrane associated lymphoid tissues.
1.14 Research envisaged

Advancement in the biotechnological research provides new possibilities for prevention and cure of various diseases in the form of genetically engineered proteins, nucleic acids, peptide hormones and interleukins. Despite of all these advancements stability of peptide and nucleic acid is a major challenge towards the delivery of these bioactive agents due to presence of proteolytic lytic enzymes as well as nucleases \textit{in-vivo}. However oral administration of the peptide drugs is of a great challenge due to the presence of gastric and intestinal secretions which contains variety of proteases and nuclease to digest dietary meal further mucus layer on the surface of endothelial cells caps whole intestinal lumen and protects our body by capturing pathogens in to mucus layer and excreted out without making their contact with endothelial cell surface this phenomenon makes a big challenge towards oral delivery of peptides and drugs and making them unavailable for epithelial cell until they have mucoadhesion and mucus penetration properties.

An intra-muscular or intra peritoneal injection of peptides ensures that whole drug was directly injected in the body but its diffusion and accumulation at the site of injection makes several complications and provokes immune inflammatory responses at the site of drug administration. In this regard oral drug delivery makes several advantages due to its study state absorption from the intestinal mucosa and transported to the blood stream (by means of passive diffusion from tight junction and uptake by endothelial cells) or lymph vessels (by means of paysers patches targeting facilitated by M cell uptake). Oral delivery strategy towards mass vaccination is help full because it does not require any trained personals, prevents speeding communicable diseases by means of injection or blood contact and provides pain less vaccine administration.

For the pathogens speeding by mans of mucosal communication oral delivery route for these type of pathogen related vaccine is desired due to its unique ability to provide mucosal defense in the form of secretory IgA antibodies (sIgA) that plays a major role in pathogen neutralization at the mucosal sites and improves immunological responses against the pathogen by means of actively M cell engulfment of the sIgA bound pathogen.

The study aims to develop nanoparticulate vaccine delivery system that enables effective protection of encapsulated Ag (HBsAg) from the outer physiological conditions and proteolytic enzymes at GIT as well as sustain the release of HBsAg and provide effective targeting towards M ells in the peyer’s patches. Two different lipid
based nanoparticle formulations were developed and surface was decorated in response to provide stability of encapsulated HBsAg and delivery system, flexibility of nanoparticulate system to target and receptor mediated targeting towards M cells. Designed delivery system also proves its multi-functionality by targeting M cells as well as intestinal dendritic cells. That phenomenon was not studied till now and proves the effectiveness by means of improved mucosal immune responses.

1.14.1. Objectives of the study

- Design and develop nano-particulate delivery system which would serve to protect encapsulated antigen from proteolytic enzymes in intestinal conditions.
- Develop nanoparticle formulations with the improved colloidal stability (in stooge conditions and in the biological fluids).
- Improved structural stability of developed nanoparticle formulation at increased pressure and intestinal conditions.
- Developed nanoparticle system will contain increased antigen encapsulation
- Provides improved mucus penetration to get more cellular interaction with M cells in payer’s patches and dendritic cells.
- Investigate the potential of surface roughness on the nanoparticle mucus penetration and uptake by intestinal epithelial cells.
- Improve the lipid membrane interactions with the nanoparticles to get phagocytosed/ endocytosed quickly while contacting with the cells.
- Design and study the malty ligand approach to target dendritic cells and M cells by dual ligated nanoparticle.
- Delivery system should enhance the systemic as well as mucosal immune responses against encapsulated antigen.
- Improved B cell proliferation in response to the nanoparticle formulations.
- Improved Cytotoxic T cell (CTL) responses against the immunogen.
- Long lasting circulating serum antibodies against delivered immunogen.
- Developed delivery system will be safe and does not contain any side effects due to presence of surfactants and adjuvants.