Discussion
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Vaccines are considered as the most successful medical interventions against infectious disease (Hilleman, 2000). In spite of great success with vaccines against infectious diseases such as small pox, polio, tetanus, diphtheria, hepatitis B and several others (Weiner and Kennedy, 1999), the programme of vaccination is facing many obstacles because of logistical difficulty to attain immunization coverage with existing vaccines in certain part of the world, especially in developing countries, these disease are still leading killers. Moreover, many significant obstacles, such as improving sub optimal vaccines, developing new ones against diseases for which no vaccines yet exist and responding rapidly to new emerging pathogens remain unsolved (Kieny et al, 2004). In designing an effective vaccine against particular pathogen involves certain key factors to target and trigger the immune response (Hoeb et al, 2004). Thus inclusion of immunopotentiators i.e. adjuvants to trigger early innate immune responses to aid in the generation of robust and long lasting adaptive immune response is therapeutically very important. Therefore, traditionally, adjuvants have been used in the vaccine formulation to improve vaccine efficacy (Glenny et al, 1926; Lewis and Lommis, 1924).

During the last 70 years, many adjuvant formulations have been developed and a few of these have been evaluated in clinical trials. However, most of these were not accepted for routine vaccines, mainly due to their toxicity and side effects. Aluminum compounds are still the only adjuvants approved by US FDA for human use. Aluminum compounds used as vaccine adjuvants include aluminum hydroxide and aluminum phosphate. This may be due to higher adsorption capacity at neutral pH. Conventional alum type vaccines require multiple recall injection at approximate time intervals in order to achieve long lasting and optimal immune responses.
However, it is very difficult to maintain especially in developing countries. Therefore, development of more efficient and safe adjuvant requiring single administration to obtain and long lasting is of primary importance. In recent years great efforts have been made to improve the efficacy of vaccination by using novel adjuvants or antigen delivery systems.

Controlled release of antigens from polymer microparticles has been of particular interest to those interested in the development of vaccines, which would be effective in a single contact immunization (Aguado, 1993). In contrast to aluminum salts, polymeric controlled release systems may be designed to release entrapped antigens for long times i.e weeks to months after a single administration of vaccine and therefore the booster dose can be avoided. Moreover, polymeric microspheres are capable of protecting antigens from rapid destruction in vivo, allowing for presentation of antigens in its native form to the cells of immune system. Several recent studies have demonstrated the possible benefits of single dose vaccines in developed countries (Zell et al, 1992; Goldstein et al, 1993; Rapuoli, 1993 and NVAC, 1991). Continuous release of antigens from polymeric microspheres mimics the prime and boosting effect of immunization. Hepatitis B vaccine has been available for over 20 years. World Health Organization has recommended that hepatitis B vaccination should be included immunization for all children world wide. Accordingly, hepatitis B vaccine has been included in the national immunization programme of more than 130 countries (WHO, 2001 and Kane, 1998). As a result, various immunization strategies have been developed for routine infant vaccination, prevention of perinatal transmission and catch up vaccination for old age groups. Currently available hepatitis B vaccines are immunogenic, efficacious and safe. But the immune response varies with individual and declines with increasing age. Yet
there is justification for producing more immunogenic vaccines that could eventually be more efficacious in defined groups such as non responders to yeast derived vaccines or travelers and health care workers who need rapid protection even after a priming dose of vaccine. A more immunogenic vaccine may also enable a reduction in the number of injections in order to combat the social and cultural resistance to the use of syringes for long term protection against hepatitis B virus infection. The conversion of multiple dose vaccine in to single dose vaccine may represent an important advancement to overcome the dropout rates due to cultural ethics against the use of syringes and economical crisis of people especially in under developed countries. Taking this consideration in to account the ultimate goal of this research work is to develop a single contact hepatitis B vaccine that can be administered soon after birth will be the greatest development towards the betterment of human health care.

1 Preparation of microspheres

Prior to the loading of hepatitis B vaccine in to various polymeric microspheres, an extensive pre-formulation studies were carried out through which the effect of various parameters on the formulation of hepatitis B vaccine encapsulated polymeric microspheres were established. The parameters selected and optimized through pre formulation studies were used for the formulation of HBsAg encapsulated polymeric microspheres. In this research work the suitability of PLGA, PLA, Chitosan 50cps, 150 cps & 300cps, albumin and dextran polymers to encapsulate HBsAg either with glutaraldehyde or dextran as cross linking agent (stabilizing agent) was investigated. The PLGA and PLA microspheres were prepared by solvent evaporation technique. The effectiveness of the solvent evaporation method to produce microspheres depends on the successful entrapment of antigen
with in the particles, and thus, this process is most successful with drugs which are
either insoluble or poorly soluble in the aqueous medium, which comprises the
continuous phase (Bodmeier et al, 1987). Recently several studies showed the
successful entrapment of highly water soluble drugs (Aftabrouchand et al, 1993; O'
Donnel et al, 1993), reactive compounds such as amine based drugs (Maulding et al,
1986), proteins (Conway et al, 1996 and Jeffery et al, 1993) peptides (Hermann et al,
1995 and Mehta et al, 1996) and vaccines (Sturesson et al, 1996 and Koneberg et al,
1995) in to PLGA / PLA polymeric system. Polyvinyl alcohol (PVA) was used as
continuous phase in formulation of PLGA / PLA polymeric microspheres. PVA is a
hydrophilic, semi crystalline powder that swells in water but do not dissolve and can
stabilize the microspheres. However, to avoid, the burst effect the foremost problem
associated with microspheres, additional cross linkers either glutaraldehyde or dextran
was used to control the cross linked structure and reduce the number of
cntanglements. Bachtsi et al (1995) investigated the effect of glutaraldehyde as a
cross linking agent for PVA microspheres and the effect of a number of different
variables on the release of protease enzymes. Chitosan, a natural linear bio poly
amino saccharide is obtained by alkaline deacetylation of chitin (Muzzarelli, 1977 and
Roberts, 1992). Chitin is the principal component of protective cuticles of crustaceans
such as crabs, shrimps, prawns, lobsters and cell walls of some fungi such as
aspergillus and mucor. Chitin is a straight homo polymer composed of β - (1-4) –
linked N - acetyl glucosamine units while chitosan consists of glucosamine and N -
acetyl glucosamine (Kas, 1997; Singla et al, 2001 and Kato, 2003). Chitosan is weak
base and insoluble in water and organic solvents, however, it is soluble in dilute acetic
acid solution (pH < 6.5), which can convert the glucosamine units in to soluble form
R- NH₂⁺ ³³. Chitosan salts combined strongly to negatively charged materials such as
cell surfaces, mucus and chemicals. These properties may be due to strong hydrogen bonding groups like -OH, -COOH (Schipper et al, 1997), strong charges (Dodane et al, 1999), high molecular weight (Schipper et al, 1996 and Kotze et al, 1998), sufficient chain flexibility (He et al, 1998) and surface energy properties favouring spreading in to mucus (Lueben et al, 1994).

Albumin is a natural polymer made up of 585 amino acids mainly tryptophan as the major constituent. The exploitable feature of albumin includes biodegradable, lack of toxicity (Schafer et al, 1994 and Muller et al, 1996) and poor immunogenic. Albumin can become non immunogenic when it cross linked with glutaraldehyde. Dextran is a complex, branched polysaccharide made of many glucose molecules joined into chains of varying lengths. The straight chain consists of α1->6 glycosidic linkages between glucose molecules, while branches begin from α1->3 linkages and in some cases, α1->2 and α1->4 linkages. Biodegradable microspheres based on dextran have shown attractive systems for the controlled release of proteins (Hennink et al, 1996, 1997; Franssen et al, 1997 and Van Dijk Wolthuis et al, 1997).

1.1 Effect of continuous phase on the formation of microspheres

1.1.1 PLGA and PLA polymeric systems

Polyvinyl alcohol in the external phase is known to be a key factor that influences the size of microspheres (Yang, 2001). The concentration of PVA could influence the microsphere formation and its size (table 1 & 2). It is very obvious that in both PLGA / PLA polymeric system, the microspheres were not formed at low concentrations of PVA, possibly due to instability. However, stable microspheres were formulated by increasing the concentration from 1.5 to 3 % w/v of PVA. It can be observed from table 1 & 2 that significant decrease in microsphere size could be achieved by increasing the PVA concentration from 1.5 to 3 % w/v. The exact reason
may be by increasing PVA concentration from 1.5 to 3 % w/v, the emulsion droplets were stabilized to avoid coalescence, resulting in smaller microspheres. However, particle aggregation was observed when the PVA concentration was increased above 3 % w/v. Therefore, in this study 3 % w/v of polyvinyl alcohol was selected because at this concentration the size of microspheres was very less when compared to the rest. Moreover the microspheres were more stable at 3 % w/v of polyvinyl alcohol. This might be due to increased viscosity of PVA, which lead to particles aggregation. Similar type of results was observed previously by Maia et al (2004) during the formulation of PHB & PHB – HV microspheres. In this work significant decrease in size was observed by increasing concentration of PVA in the water phase from 0.5 to 2 % w/v. From the observation it is very obvious that the size of microspheres greatly depends on the stability of the emulsion droplets formed during agitation. Young – Hong Liao et al (2005) explained that the biological activities of the protein were preserved for a minimum of 12 weeks when PVA was mixed with a sugar. Importantly, PVA conferred excellent suspension stability and aerosolisation performance with out the addition of surfactants.

1.1.2 Chitosan / Albumin / Dextran polymeric systems

Emulsion cross linking technique was employed to prepare chitosan, albumin and dextran polymer microspheres. Totally four oil phases were screened for continuous phase to formulate W / O type of emulsion. The results tabulated in table 3 showed that the viscosity grade of various oil phases were found to be 57.96, 63.3, 138.9 and 138.91 centi poise for sunflower oil, olive oil, paraffin oil and linseed oil respectively. From the results observed, it is clear that the size of microspheres is directly proportional to the viscosity grade of oil except in the case of olive oil where the particles were not formed by chitosan 50 cps, albumin and dextran polymer.
Among all oil phases used sunflower oil was found to be the most suitable continuous phase due to the formation of lower particle size with smooth morphology of microspheres. The results tabulated in table 4, 5, & 6 and fig 3 and 4 explain the comparative size distribution analysis of chitosan microspheres of three different grades i.e 50, 150 and 300 cps by employing two different cross linkers. The chitosan 50 cps polymer grades failed to develop microspheres when olive oil was used as external phase (table 4). This might be due to the lesser viscosity of polymer grade and could not be able to interact with external phase to form microspheres. The results tabulated in table 5 apparently explain that the size of the microspheres was increased with the viscosity grade of external phase. However, in the case of linseed oil and paraffin oil the viscosity grade was found to be same but the size of microspheres is more when paraffin oil was used as external phase rather than linseed oil in both the cases. The same type of result was observed (table 6) when chitosan 300 cps was used as polymer with either glutaraldehyde or dextran as crosslinker. Fig 3 and 4 reveal the effect of various continuous phases on the formation of microspheres with three different grades of chitosan polymer. The observation made on table 7 and fig 5 explain the effect of various continuous phases on the size of albumin / dextran microspheres with glutaraldehyde as cross linker. In both the polymeric system, the microspheres were not formed when olive oil was used as continuous phase. This is mainly due to the lack of interaction between glutaraldehyde and either with albumin or dextran.

1.2. Effect of polymer and cross linking agent on microsphere formation

Investigation brought through the effect of polymer and cross linker concentration to formulate microspheres was confirmed the ideal concentration of polymer and cross linker in order to formulate stable microspheres. From the studies it
is understood that the physical characteristics of microspheres such as size and surface appearance, and even in vitro release profile were greatly influenced by the concentration of polymer as well as cross linker. In our study, in the case of polyester polymers we have investigated that 2% w/v of glutaraldehyde or 2% v/v of dextran was ideal concentration to fabricate stable microspheres (table 8 & 9). Korsmeyer et al (1981) investigated the effect of the degree of cross linking and relaxation properties of PVA on the diffusion of drugs. In their study glutaraldehyde was used as cross linker and theophylline was the model drugs. They discovered that at low concentration of glutaraldehyde the effect on drug release was very less, while at high concentration of glutaraldehyde the degree of cross linking had a much greater effect on the drug diffusion.

In this research work, 3 different viscosity grades of polymer were screened because chitosan is a natural polymer having specialized characters such as biodegradability, low toxicity and good biocompatibility, which make it suitable for the biomedical and pharmaceutical application. However, from the present study it could be seen that the viscosity grade of chitosan polymer had influences on the physical characteristics of chitosan microspheres. Chitosan 50 cps produced smaller particles when compared to chitosan 150 cps and 300 cps (table 10, 11 & 12). The chitosan 50 and 300 cps failed to fabricate ideal microspheres to encapsulate HBsAg because in these grades the encapsulation efficiency was found to be very less and moreover, the microspheres were not stable. However, in all the three grades the formation of microspheres was greatly influenced by polymer and cross linker concentration. Berthold et al (1996) stated that the higher concentrations of chitosan polymer were led to the formation of agglomerates. In this study, chitosan 150 cps was found to be ideal to encapsulate hepatitis B vaccine. Microspheres made up of 1
% w/v of chitosan 150 cps with 2% v/v of glutaraldehyde and 2% w/v of chitosan 150 cps with 1 % w/v of dextran were ideal because of more encapsulation efficiency and stability. In this study, the albumin and dextran polymer failed to encapsulate HBsAg and this might be due to the protein – protein interaction in the case of albumin microspheres or this might be due to the difference in the molecular weight of polymers. Some of earlier workers reported that the molecular weight of polymer had a great impact on the formulation of micro encapsulation (Maria Alanso et al, 1994 and 1999; and Inez van der Luben et al, 2003).

2 Characterization of microspheres

2.1 Physical Characterization

2.1.1 PLGA polymeric system

Generally, the size of microspheres dictates the immune response. The larger microspheres show the slower release of vaccine and the longer immunogenicity (Gupta et al, 1997). In addition to the depot effect, smaller PLGA microparticles were demonstrated to have adjuvant activity via their uptake by macrophages and dendritic cells and their localization in lymph nodes (Singh et al, 1992) and to induce CTL response (Maloy et al, 1994; Moore et al, 1995; Nixon et al, 1996; Partidos et al, 1997 and Men et al, 1997). In this study, the size of glutaraldehyde or dextran cross linked microspheres was quite less in vaccine unloaded microspheres when compared with vaccine loaded microspheres. SEM micrographs of glutaraldehyde cross linked hepatitis B vaccine encapsulated PLGA microspheres showed that they were uniformly distributed and minute pores were present on the microspheres (Plate 1). Moreover, the size was still reduced and morphology became very smooth and discrete after sonification. Similar type of observation was noted by Xingguo Mei et
al (2006), while encapsulating Leutinizing Hormone – Releasing Hormone in PLGA microspheres. They have investigated that the microspheres prepared with a W/O/W technique, morphological investigations by SEM showed the spherical shape as well as the internal sporadic porosity due to solvent evaporation technique. The microspheres prepared using the W/O/W technique were prepared from a clear homogenous solution of polymer and drug, and the drug would like to be molecularly distributed in the PLGA matrix. However, the appearance of microspheres prepared by an O/W technique was relatively different. The surface was smooth, compact and exempt of pores. They have stated that lack of pores was due to lack of the internal aqueous phase during the process and increasing the internal aqueous phase and hence a more porous matrix was observed. The size of vaccine encapsulated or unencapsulated PLGA microspheres was 150.36 ± 0.3 and 45.02 ± 0.4 µm respectively, when glutaraldehyde was used as cross linker before sonification. However, the sonification process drastically altered the size of the microspheres and found as 35.02 ± 0.9, 27.8 ± 0.2 µm for vaccine encapsulated and unencapsulated microspheres respectively. Panyam et al (2003) stated that various factors such as surface morphology, particle size, polymer composition, viscosity, molecular weight of the polymer etc have shown to affect the release of antigen from microspheres.

The dextran cross linked hepatitis B vaccine encapsulated PLGA microspheres were aggregated to some extend. From this experimental study it is proven that smooth, discrete microspheres could not be produced with out sonification process and this might be due to the lack of interaction between polymer and cross linker while encapsulating the HBsAg. The particles aggregation of dextran cross linked HBsAg encapsulated microspheres were rectified by sonification process. However, it is note worthy that vaccine unencapsulated dextran cross linked PLGA microspheres
did not produce particle aggregation before sonification. The size of vaccine unencapsulated and encapsulated PLGA microspheres were 55.02 ± 0.9 and 138.18 ± 3.5 µm respectively before sonification. After sonification the size of vaccine encapsulated and unencapsulated PLGA microspheres was 24.8 ± 0.2 and 34.06 ± 0.9 µm respectively when dextran was used as cross linker. It is generally considered that the mechanism of degradation of aliphatic polymer microspheres is a hydrolytic mechanism (Anderson, 1995; Okada et al, 1995; Li et al, 1995). Vert and coworkers have carried out extensive studies on the size dependence of the hydrolytic degradation of devices based on lactic acid and glycolic acid polymers (Grizzi and Vert, 1995). They have stated that in large sized PLA / GA polymer devices, microspheres less than 300 microns in diameter undergo a homogenous degradation with the rate of degradation of the core being equivalent to that at the surface.

2.1.2 PLA polymeric system

Studies to date indicate that PLA microspheres containing bioactive agents are biocompatible, when used in therapeutic applications in vivo do not exhibit untoward reactions either locally or systemically. The biodegradation of PLA microspheres occurs through homogenous hydrolytic chain cleavage mechanism where the rate of degradation is predominately size dependent (Anderson et al, 1997). From our studies, it could be seen that PLA polymer either vaccine encapsulated or unencapsulated microspheres by using glutaraldehyde or dextran as cross linker produced smooth particles and spherical in shape (Plate 5, 6, 7 & 8). The size of vaccine encapsulated or unencapsulated PLA microspheres was 94.09 ± 1.2 and 27.5 ± 0.3 µm respectively when glutaraldehyde was used as cross linker before sonification. However, the sonification process considerably altered the size of the microspheres, 38.09 ± 2 and 26.1 ± 0.3 µm for vaccine encapsulated and
unencapsulated microspheres respectively. Pores could not be able to detect on the glutaraldehyde cross linked PLA microspheres. Therefore, the surface of the glutaraldehyde cross linked PLA microspheres was very smooth. Lacasse et al (1998) reported that the pores were unable to observe from the micrograph and stated that very smooth microspheres were produced and concluded the usage of PEG - distearate decreased the roughness of surface. They also stated that the mean particle size was greatly influenced by the polymer concentration. In the present study the dextran cross linked PLA microspheres either vaccine encapsulated or unencapsulated produced particle clump before sonification process. On the other hand this technical problem was over come by sonification process as previously discussed for PLGA polymeric system. It might be due to the improper interaction between polymer and cross linker, when poly vinyl alcohol was used as external phase. However, this problem was overcome by sonification process.

Nikolaus Kofler et al (1996) stated that the method of preparation also influenced the size of PLA microspheres. They have prepared the PLA microspheres by solvent evaporation and solvent extraction method and both were exhibiting heterogenous particle size. Solvent evaporation microspheres were porous particles with a rough surface and more heterogenous particle size, whereas solvent extraction particles showed an intact smooth surface with small pores and greater particle size. In contrast, in this study, only solvent evaporation method was employed and we observed smooth and smaller particles without pores.

2.1.3 Chitosan polymeric system

The utilization of chitosan as sustained drug carrier system is very interesting. A large number of studies in the last few years indicate the high efficiency of chitosan on production of various sustained release drug carrier system such as microparticles
(Lin and Lin, 1992; Inez van der Lubben et al, 2003; Seyed Alireza Mortazavi et al, 2004). From the present study it could be seen that chitosan polymer 50 cps either vaccine encapsulated or unencapsulated microspheres using glutaraldehyde or dextran as cross linker produced rough particles and less spherical in shape (Plate 9, 10, 11 &12). However, smooth particles were not formed even after sonification in the case of chitosan 50 cps polymer (Plate 10). This might be due to the less viscosity of polymer that could not able to interact with external phase even after sonification. Oliveria et al, (2005) investigated the effect of viscosity of polymer on the size as well as microsphere formation. They have stated that larger microspheres with greater size dispersion were formed from more concentrated and more viscous solution. They concluded that the effect of the solution viscosity on the size of droplets formed during the automization step. The similar trend was observed in our experimental work that microspheres were not formed with 1 % w/v of chitosan polymer 50cps with 0.5 % v/v of glutaraldehyde as cross linker. However, the microspheres were not formed at higher concentration of chitosan polymer i.e 2 % w/v of chitosan with 3 %w/v of dextran and 3% w/v of chitosan with more than 1 % v/v of dextran as a cross linker failed to form microspheres. Berthold et al (1996) investigated that higher concentration of chitosan did not form microspheres because the viscosity was too high and lead to agglomerates. They also stated that an addition of polysorbate 80 was needed to get the microspheres. But in this study, Tween 80 was employed as surfactant and addition of more Tween 80 also favourable to form microspheres before and after sonification. The sizes of chitosan 50 cps vaccine encapsulated and unencapsulated microspheres were 85.09 ± 0.9 and 37.03 ±0.9 μm respectively when glutaraldehyde was used as cross linker before sonification. However, after sonification the size was reduced to 73.6 ± 1.2 and 27.3± 0.5 μm for vaccine
encapsulated and unencapsulated chitosan 50 cps microspheres using glutaraldehyde as cross linker (table 14). The dextran cross linked chitosan 50 cps microspheres was rough and more or less spherical in shape before and after sonification (Plate 11 and 12). The sizes of chitosan 50 cps vaccine encapsulated and unencapsulated microspheres were 125.1 ± 5.1 and 41.6 ± 0.7 µm respectively when dextran was used as cross linker before sonification. However, after sonification the size was 78.6 ± 2.1 and 35.7 ± 0.5 µm for vaccine encapsulated and unencapsulated chitosan 50 cps microspheres using dextran as cross linker after sonification.

The observation made on chitosan polymer 150 cps either vaccine encapsulated or unencapsulated microspheres by using glutaraldehyde as cross linker produced rough particles and less spherical in shape before sonification (Plate 13). The size of vaccine encapsulated and unencapsulated chitosan 150 cps was 145.2 ± 1.8 and 77.9 ± 6.2 µm respectively before sonification, when glutaraldehyde was used as cross linker. The sonification process produced spherical, smooth, porous, discrete and non homogenous microspheres with glutaraldehyde as cross linker (Plate 14). The microspheres were spherical, smooth, porous, discrete and non homogenous. The size of vaccine encapsulated and unencapsulated chitosan 150 cps microspheres was 87.9 ± 3.1 and 47.27 ± 3.3 µm respectively after sonification process when glutaraldehyde was used as cross linker. The dextran cross linked hepatitis B vaccine encapsulated chitosan 150 cps microspheres showed that the microspheres were less spherical in shape, porous and non homogenous before sonification (Plate 15). The size of the vaccine encapsulated and unencapsulated microspheres were 121.3 ± 4.9 and 80.7 ± 5.2 µm respectively before sonification process. However, after sonification the particles become very smooth, more or less spherical in shape and discrete particles (Plate 16). The observed size of the vaccine encapsulated and unencapsulated
microspheres were $81.4 \pm 4.7$ and $66.9 \pm 4.4 \mu m$ respectively after sonification process. In the present study, 3 % w/v of chitosan 150 cps solution with the concentration of glutaraldehyde more than 1.5 % v/v did not form any microspheres and also 3 % chitosan with dextran 1.5 % w/v did not produce any microspheres before sonification but the microspheres were obtained after sonification process. Moreover, the microspheres did not form above 1.5 % w/v concentration of dextran. This might be due to excess viscosity of polymer as well as cross linker that could not be able to interact with each other and failed to produce microspheres. Thus optimizing polymer and cross linker concentration with respect to vaccine loading is an important parameter to fabricate microspheres.

From the experimental data, it is understood that 1 % w/v of chitosan 150 cps with 2 % v/v of glutaraldehyde and 2 % w/v of chitosan with 2 % w/v of dextran were found to be ideal because the microspheres were stable and the encapsulation efficacy was high (discussed separately under loading of vaccines). Seyed Alireza Mortazavi et al (2004), investigated that 1% w/v of chitosan solution with different molecular weights was found to be most appropriate concentration for preparing chitosan microspheres to encapsulate diphtheria toxoid. Akbuga et al (1994), stated that the important factors related are the viscosity grade of the chitosan - acetic acid solution. In their studies microspheres did not form at low concentration of chitosan - acetic acid. However, higher concentration of solution also did not form any microspheres and therefore they found that 4% w/v of chitosan was found to be ideal to encapsulate furosemide. From the data obtained in this experimental work, the chitosan 300 cps also followed the similar trend as chitosan 150 cps. But the particles were very big, collapsed and clumped. However, few microspheres were freely present and their size was 179.5 ± 5.2 µm for vaccine encapsulated and 166.7 ± 2.4 µm for vaccine
unencapsulated microspheres before sonification (Plate 17). Besides, more or less spherical particles could be obtained after sonification process (Plate 18) and the size of vaccine encapsulated chitosan 300 cps was $124.5 \pm 4.1$ and $152.4 \pm 5.5$ μm before and after sonification process respectively. The dextran cross linked chitosan 300 cps microspheres produces spherical, non porous and most particles were clumped and the size was $321.3 \pm 1.7$ μm for vaccine encapsulated and $321.3 \pm 1.7$ μm for vaccine unencapsulated microspheres before sonification process. Moreover, the size of vaccine encapsulated and unencapsulated microspheres were $176.6 \pm 3$ μm in both the cases after sonification (table 14). Thus in this study we have investigated that chitosan 50 cps and 300 cps were not preferred as an ideal carrier system since it produced unstable, collapsed, bigger particles and vaccine loading was also not significant.

2.1.4 Albumin Polymeric system

Albumin microspheres have been extensively investigated as controlled release systems for the delivery of vehicles (Karmer, 1974). In the present research work, only glutaraldehyde was used as cross linker because albumin is a protein which can easily bind with any aldehyde group. Eventhough albumin and glutaraldehyde strongly combined to form good spherical, smaller microspheres with smooth surfaces (Plate 21 and 22), the antigen was unable to encapsulate in to the albumin polymeric microsphere system. This might be due to the protein - protein interaction between albumin and HBsAg that failed the encapsulation process. This was physically proved by observing the size of the microspheres and the antigen loading was also tested and discussed in forth coming chapter. The size of albumin microspheres for vaccine encapsulated and unencapsulated was $33.1 \pm 1.6$ and $35.1 \pm 2.4$ μm respectively before sonification process when glutaraldehyde was used as cross
linker. The size of glutaraldehyde cross linked vaccine encapsulated albumin microspheres was 17.8 ± 1.8 μm for vaccine encapsulated and 19.4 ± 1.2μm for vaccine unencapsulated microspheres after sonification process (table 14).

2.1.5 **Dextran Polymeric system**

Biodegradable hydrogels based on cross-linked dextrans have been shown to be attractive systems for controlled release of proteins (Hennink *et al*, 1996, 1997; Franssen *et al*, 1997 and Van Dijk Wolthuis *et al*, 1997). In this research work, the dextran microspheres were developed newly by emulsion cross linking technique. Glutaraldehyde is homo-bi-functional cross linker can bind with dextran to form microspheres. However, microspheres were formed but failed to encapsulate the HBsAg and moreover, the microspheres were not stable. This was physically proved by observing the size of the microspheres and the antigen loading was also tested and discussed in forth coming chapter. This might be due to lesser interaction of glutaraldehyde and dextran. The microspheres were not formed properly; some were formed and most of the particles were clumped together (Plate 21), but sonification process produced more or less spherical microspheres, smaller in size with slighter smooth surface (Plate 22). The size of dextran microspheres for vaccine encapsulated and unencapsulated was 73.58 ± 1.2 and 85.01 ± 0.91 μm respectively before sonification process when glutaraldehyde was used as cross linker. The size of glutaraldehyde cross linked vaccine encapsulated dextran microspheres was 60.7 ± 0.8 μm for vaccine encapsulated and 73.12 ± 1.2 μm for vaccine unencapsulated microspheres after sonification process. From this study, it is understood that either synthetic or natural polymeric systems the formation of microspheres was greatly influenced by the type of polymer, cross linker and their concentrations utilized for
the preparation. However, the size of microspheres reduced significantly after sonification process.

2.2 Determination of Vaccine entrapment

The encapsulation efficiency of hepatitis B vaccine in PLGA / PLA / chitosan polymeric system was determined by four methods termed as centrifugation (Inez van der Lubben et al, 2003), digestion, filtration and extraction methods (Maria Alonso et al, 1999). The encapsulation efficiency was determined either by quantification of total protein or antigenically active protein. Total protein could be detected successfully by all the four methods. However, antigenically active protein is more important and could be determined successfully by centrifugation process followed by filtration method. But digestion and extraction methods failed to determine antigenically active protein. In contrast the extraction and digestion method resulted in an under estimation of the entrapped hepatitis B vaccine as previously reported by Maria Alonso et al (1999) for tetanus toxoid. Li Feng et al (2006) reported that the total amount of HBsAg encapsulated in to microspheres could not be determined accurately by extraction method. However, they have investigated that the digestion method is relatively accurate technique for the determination of total protein entrapped in PLGA. In the present research work, the data revealed that the most accurate method of determining antigenically active protein is the centrifugation method (table 15).

2.2.1 PLGA and PLA polymeric systems

The data presented in table 16 and 17 show the actual amount of hepatitis B vaccine entrapped in the 50 mg of PLGA and PLA microspheres. The proportion of vaccine loading in both PLGA and PLA microspheres were 40 – 43 % and 35- 43 respectively and rest of the vaccine was lost during microencapsulation process.
During the cross linking and hardening process the vaccine could be lost either due to stirring process or chemical interaction. Alex et al, (1990) previously reported that drug loss during a microencapsulation process using a double emulsion technique occurred because of the migration of inner water droplets to the external aqueous phase. It can be seen from fig 25 and 26 that the maximum loading of vaccine in to the PLGA polymeric system was attained, when 2 % w/v of PLGA cross linked with 2 % v/v of glutaraldehyde was used. However, in the case of dextran cross linked PLGA microspheres the maximum vaccine entrapment was achieved at 2 % w/v of PLGA cross linked with 2 % w/v of dextran. In the case of PLA polymeric system, the maximum vaccine entrapment could be detected when 2 % w/v of PLA polymer cross linked with 2 % v/v of glutaraldehyde or 2 % w/v of dextran (Fig 27 & 28). The nature of vaccine loading is similar in both the polymeric systems since both are aliphatic polyester and PLGA is the co polymer of PLA. Among these two polymers PLGA was showed a better vaccine entrapment. Maria Alonso et al, 1994 investigated that the both ILA and PLGA polymeric system displayed a high protein loading efficiency. But the release pattern varies among PLA and PLGA; the release pattern was lower in PLA when compared to the PLGA. In order to increase the loading of vaccine large amount of hepatitis B vaccine was incorporated during microencapsulating process, which increased the loading capacity but it resulted in particle aggregation. Moreover, it was very difficult to resuspend and syringeable after washing process. Xingguo Mei et al (2006) investigated that the encapsulation efficiency was highly dependent on the LA / GA ratio of PLGA. The encapsulation efficiency reduced significantly from 74.7 % with LA / GA (75:25) ratio to 55.8 % with LA / GA (40:60). They concluded that increase of GA ratio, the hydrophilicity of the polymer increased that led to increased loading capacity.
Shaobing Zhou et al (2004) investigated the microspheres prepared from PELA – 10 copolymer achieved the highest encapsulation efficiency among all polymer microspheres. They had stated that, it might be a result of the existence of certain amount of hydrophilic PEG segments in the polymer chains, which improved the affinity of the polymer in the polymer chains, resulting in the improved affinity to the protein molecule. In particular, PELA – 10 appears to have the most suitable ratio of hydrophilic / hydrophobic domains in the copolymer, making it a better candidate polymer for the development of controlled delivery systems of water soluble drugs, peptides and vaccines. In the present study, it was observed that the loading efficiency of PLGA and PLA polymer varied with polymer concentration with respect to cross linking agent concentration. Therefore, a significant (P<0.05, n= 6) hepatitis B vaccine loading could be observed, when microspheres prepared by using 2% w/v of PLGA with 2 % v/v of glutaraldehyde as well as 2 % w/v of PLGA with 2 % w/v of dextran. However, glutaraldehyde cross linked PLA microspheres prepared at the concentration of 2% w/v of PLA with 2 % v/v of glutaraldehyde showed extremely (P < 0.001, n= 6) significant vaccine loading of 41 ± 1.4% but dextran cross linked PLA microspheres prepared at the concentration of 2 % w/v of PLA with 2 % w/v of dextran revealed significant vaccine loading (P<0.05, n 6) of 35.8 ± 2 % (fig 28).

2.2.2 Chitosan Polymeric system

The data presented in table 18 and 19 showed the actual amount of hepatitis B vaccine entrapped in the 50mg of chitosan 50, 150 and 300 cps microspheres. From the studies it could be seen that chitosan 150 cps viscosity grade was successful to encapsulate hepatitis B vaccine, when compared to the 50 and 300 cps viscosity grade. In this research work, the proportion of vaccine loading in chitosan 150 cps microspheres was 68 – 70 % in both glutaraldehyde and dextran cross linked
microspheres (table 18; fig 29 and 30) and 30 % vaccine lost was observed during microencapsulation process. Similar type of results was observed, when acetic anhydride was encapsulated in chitosan sponges by Kwunchit Oungbho and Muller et al (1997). They observed 68 – 79 % of the drug was incorporated in the final sponge matrices. They concluded that the loss of drug during loading was mainly due to the process of soaking the chitosan gels with many times of large volume of water. On the other hand the drug content in cross linked sponges was easily adjusted by dispersion of the desired amount of drug in the solutions cross linked chitosan. Jameela and Jayakrishnan (1995) encapsulated mitoxantrone in chitosan microspheres. They observed 50 % drug loss during microencapsulation process and stated that during the cross linking and hardening process water was exuded from the microspheres along with dissolved drug and this appears to be responsible for the low incorporation efficiency. In order to increase the loading of vaccine more amount of hepatitis B vaccine was incorporated during microencapsulating process, which increased the loading capacity but it resulted in particle aggregation with stick appearance and failed to resuspend for making injection. The similar type of result was observed previously for chitosan microparticles for mucosal vaccination against diphtheria: oral and nasal efficacy studies in mice (Inez van der Lubben et al, 2003).

Concentration of a polymer is a key factor to prepare microspheres. In this research work, 3 concentrations such as 1, 2 and 3 % w/v of 3 different viscosity grades of chitosan 50, 150 and 300 cps were screened to encapsulate hepatitis B vaccine. It is very obvious that the maximum vaccine entrapment was achieved, when 1 % w/v of chitosan 150 cps polymer cross linked with 2% v/v of glutaraldehyde and 2 % w/v of chitosan 150 cps polymer with 2 % w/v of dextran (fig 30). In the case of chitosan 50 and 300 cps viscosity grades polymeric system, there were no significant
vaccine loading could be observed (table 19 and fig 31-34). However, the percentage of vaccine loading was 6 - 40 % in 50 mg of microspheres. Therefore, it is very obvious that the viscosity grade of polymer and concentrations of either polymer or cross linker played a key role in the encapsulation of hepatitis B vaccine. Akubga and Durmaz (1994) encapsulated furosemide in 4 % w/v of chitosan with glutaraldehyde as cross linker. In contrast, Berthold et al (1996) investigated that higher concentrations of chitosan were not successful to entrap prednisolone sodium phosphate because the viscosity became too high. As a consequence, a homogenous distribution of the added prednisolone sodium sulphate was not possible, which led to the formation of agglomerates. Therefore they utilized 0.25 % w/v of chitosan to encapsulate prednisolone sodium phosphate. In contrast of these studies hepatitis B vaccine could be encapsulated in all the three different concentrations (1 - 3% w/v) of three different viscosity grades (50, 150 and 300 cps) of chitosan polymer. However, it was estimated that extremely significant (p< 0.001, n = 6) vaccine loading could be observed when 1% w/v of chitosan 150 cps with 2 % v/v of glutaraldehyde and 2 % w/v of chitosan 150 cps with 2 % v/v of dextran revealed 69.6 ± 1.6 and 68.4 ± 2.2 % loading respectively.

2.3 Compatibility studies using FT Infra Red spectrum

The final appearance of infra red spectrum is very important in the interpretation process. The FT IR spectrum is an efficient tool to monitor the presence of chemical groups. In this study, the IR spectrum of hepatitis B vaccine has displayed (fig 36) broad band at 3436 cm⁻¹, which indicated the stretching band of heterocyclic amine and a sharp band at 2919 cm⁻¹ indicates the asymmetrical stretching vibrations of methylene group. Bands at 1657 and 1021 cm⁻¹ proved the presence of alkenyl and primary amine groups with stretching vibrations. Moreover, few very short bands
were also observed at 2850 and 1261 cm\(^{-1}\), which proved the presence of methylene group stretching vibrations and primary or secondary OH group in bending vibration. The FTIR spectrum of PLGA polymer (fig 37) showed the characteristic peak at 3401 cm\(^{-1}\) indicated the presence of aromatic amine (N-H) stretching vibration. The sharp band at 2919 cm\(^{-1}\) is the characteristic peak of methylene group (C-H) asymmetric stretching and the sharp band at 2850 cm\(^{-1}\) is the characteristic peak of methylene group (C-H) symmetrical stretching. The peaks at 1655 and 1463 cm\(^{-1}\) proved the presence of open chain immuno group (C=N) stretching and alkane (C-H) bending vibration.

The hepatitis B encapsulated glutaraldehyde cross linked PLGA microspheres showed a characteristic peaks at various frequencies (fig 40). The wide band at 3444 cm\(^{-1}\) indicates the presence of heterocyclic amine group with stretching vibration. But the frequencies obtained in hepatitis B vaccine before microencapsulation was 3436 cm\(^{-1}\). This indicates that there is some interaction between either with polymer or with glutaraldehyde, which led to a shift from 3436 to 3444 cm\(^{-1}\). The band at 2924 cm\(^{-1}\) proved the presence of methylene group with asymmetrical stretching. However, asymmetrical stretching with methylene group could be found at 2919 cm\(^{-1}\) before encapsulation of hepatitis B vaccine. The short band at 1081 cm\(^{-1}\) is a characteristic peak of primary amine with stretching vibrations. However, similar type of bands could be observed at 1021 cm\(^{-1}\) before encapsulation of hepatitis B vaccine. The comparative study with bands of glutaraldehyde cross linked PLGA microspheres without encapsulation of hepatitis B vaccine did not produce any characteristic peaks (fig 38) as like HBsAg encapsulated PLGA microspheres. But some peaks were short displayed at 2946 and 2995 cm\(^{-1}\) proved the presence of methylene group with asymmetrical stretching. Similar type of band could
be observed at 2919 and 2850 cm\(^{-1}\) of PLGA polymer (fig 37). From the comparative results, it is understood that significant absorption shift was detected after the formation of microspheres. This might be due to the interaction between PLGA polymer and glutaraldehyde. Therefore, these bands at various wave cycles at cm\(^{-1}\) proved the encapsulation of hepatitis B vaccine in PLGA microspheres using glutaraldehyde as cross linker. Sunil Agnihotri et al (2006) investigated novel interpenetrating network chitosan – poly (ethylene oxide - δ- acrylamide) hydrogel microspheres for the controlled release of capecitabine. In their study the grafting, interpenetrating network formation and chemical stability of the capecitabine after encapsulation into microspheres was confirmed by FTIR analysis. The spectral data confirmed the presence of characteristic band at 2888 and 1464 cm\(^{-1}\) indicate the presence of CH\(_2\) group with stretching and bending vibrations respectively. The bands at 1412 cm\(^{-1}\) indicate the presence of CH\(_2\) group with stretching and bending vibrations respectively. The bands at 1412 cm\(^{-1}\) indicate the C-H bending vibrations, while the bands at 1097 cm\(^{-1}\) confirm the presence of ethereal C-O stretching. The hepatitis B vaccine encapsulated, dextran cross linked PLGA microspheres displayed characteristic peaks at 3436, 2924 and 2853 cm\(^{-1}\) and indicated the presence of heterocyclic amine stretching vibration, asymmetrical and symmetrical stretching of methylene group (fig 41). The presence of peak at 3436 cm\(^{-1}\), a shift from 2919 to 2924 and 2850 to 2853 cm\(^{-1}\) indicated the presence of hepatitis B vaccine in dextran cross linked PLGA microspheres. Peaks at 1751, 1631 and 1091 cm\(^{-1}\) indicated the presence of alkyl carbonate (C=O) stretching, (N-H) primary amine bending and (C-N) primary amine. The comparative study with bands of dextran cross linked PLGA microspheres without encapsulation of hepatitis B vaccine did not produce any characteristic peaks (fig 39) as like HBsAg encapsulated PLGA microspheres.
Therefore, these bands at various wave cycles at cm\(^{-1}\) proved the encapsulation of 
hepatitis B vaccine in PLGA microspheres using glutaraldehyde as cross linker.

The FT IR spectrum of PLA polymer displayed characteristic peak at 3503 
\(\text{cm}^{-1}\) specified the presence of aromatic amine (NH) stretching, 2998 & 2947 cm\(^{-1}\) 
aliphatic stretching (C-H), 1756 cm\(^{-1}\) alkyl carbonate (C=O) stretching, 1457 cm\(^{-1}\) (C-H) 
methyl group bending, 1384 cm\(^{-1}\) di methyl (iso) group, 1187 cm\(^{-1}\) (C-N) 
secondary amines stretching, 1088 cm\(^{-1}\) (C-N) primary amine stretching. The hepatitis 
B vaccine encapsulated PLA polymeric system with glutaraldehyde as cross linker 
revealed characteristic peak (fig 45) at 3436 cm\(^{-1}\) specified the presence of 
heterocyclic amine stretching. Similar type of band could be observed for hepatitis B 
vaccine before microencapsulation process. The band at 1748 cm\(^{-1}\) showed the 
presence of alkyl carbonate group stretching vibration. Similar type of band could be 
observed in PLA polymer at 1756 cm\(^{-1}\) and 1755 cm\(^{-1}\) in the case of PLA 
microspheres with out vaccine. Moreover, the bands at 1653, 1459, 1187 and 1083 
\(\text{cm}^{-1}\) indicated the presence of alkenyl stretching (C=C), methyl asymmetrical 
stretching, secondary amine (C-N) stretching and primary amine stretching 
respectively. The glutaraldehyde cross linked PLA microspheres without vaccine 
showed a wide band at 3445 cm\(^{-1}\) (fig 43), which indicated the presence of 
heterocyclic amine stretching. But after encapsulation of hepatitis B vaccine similar 
type of band could be observed at 3436 cm\(^{-1}\), which was similar as in FT IR spectrum 
of hepatitis B vaccine. This proved the encapsulation of hepatitis B vaccine in the 
glutaraldehyde cross linked PLA microspheres. Moreover, all the other bands were 
the indicative of chemical shifts due to microencapsulation. In the case of dextran 
cross linked, hepatitis B vaccine encapsulated PLA microspheres depicted various 
characteristic peaks (fig 46) at 3468 cm\(^{-1}\) and indicated the presence OH group
stretching vibration, which was an indicative of chemical interaction either with polymer or with dextran during microencapsulation. All other groups were similar with slight chemical shift occurred at 1751, 1457, 1387 and 1086 cm\(^{-1}\), when compared with dextran cross linked PLA microspheres with out vaccine.

The FT-IR spectrum of chitosan polymer (Fig 47) described characteristic peaks at 3435 cm\(^{-1}\) (N-H) heterocyclic amine stretching, a sharp band at 2924 cm\(^{-1}\) and indicated the presence of (C-H) methylene asymmetrical stretching, 2854 cm\(^{-1}\) methylene (C-H) symmetrical stretching, 1646 cm\(^{-1}\) alkenyl stretch (C=C), 1463 cm\(^{-1}\) methyl (C-H) asymmetrical bending, 1377 cm\(^{-1}\) methyl (C-H) symmetrical bending, 1066 cm\(^{-1}\) primary amine (C-N) stretching. The FT-IR spectra of glutaraldehyde cross linked chitosan microspheres with out vaccine (fig 48) revealed a broad band at 3400 cm\(^{-1}\), indicating polymeric OH stretching vibration. However, the peak was compared with the polymer spectra in that a wide peak was obtained at 3435 cm\(^{-1}\). But the hepatitis B vaccine encapsulated, glutaraldehyde cross linked chitosan microspheres displayed a wide peak at 3400 cm\(^{-1}\). FT-IR spectrum of hepatitis B vaccine has displayed broad band at 3436 cm\(^{-1}\), which indicated the stretching band of heterocyclic amine. Moreover, the similar type of peak could be obtained in the case of hepatitis B vaccine encapsulated chitosan microspheres at 3400 and very short band at 3775 cm\(^{-1}\) (fig 50). From these data, it is obvious that chemical interaction could be takes place while encapsulating the hepatitis B vaccine in chitosan microspheres with glutaraldehyde as cross linker. The peaks at 2919 and 2854 cm\(^{-1}\) in vaccine encapsulated microspheres proved the presence of the asymmetrical and symmetrical stretching vibrations of methylene group. Similar type of asymmetrical stretching band could be observed for hepatitis B vaccine at 2919 cm\(^{-1}\). But asymmetrical stretching of methylene group took place at 2921 cm\(^{-1}\) in the case of
glutaraldehyde cross linked chitosan microspheres with out vaccine. All other groups displayed in the hepatitis B vaccine encapsulated chitosan microspheres were similar with modification occurred due to chemical shift at 1742, 1656, 1456 and 1015 cm⁻¹ when compared with glutaraldehyde cross linked chitosan microspheres with out vaccine. In the case of dextran cross linked, hepatitis B vaccine encapsulated chitosan microspheres revealed various characteristic peaks (fig 46) at 3400 cm⁻¹, indicating the presence polymeric OH group stretching vibration, but the dextran cross linked chitosan microspheres with out vaccine revealed same type peak at 3435 cm⁻¹ which was an indicative of presence of (N-H) heterocyclic amine group with stretching vibration. This might be due to the chemical interaction either with polymer or with dextran during microencapsulation. Devika Bhunkar and varsha Pokharkar, 2006 studied the effect of pH on cross linking of chitosan with sodium triphosphate. They observed a characteristic band at 3449 cm⁻¹ was attributed to —NH₂ and —OH groups with stretching vibrations and the band for amide I at 1655 cm⁻¹ was seen in the IR spectrum of chitosan polymer. Where as in the FTIR spectra cross linked chitosan the peak at 1655 cm⁻¹ disappeared and 2 new peaks at 1645 cm⁻¹ and 1554 cm⁻¹ appeared. They have stated that the disappearance of the band could be attributed to the linkage between the phosphoric and ammonium ions. The cross linked chitosan also shoed a peak for P=O at 1155 cm⁻¹. Xu et al, 2003; Knaul et al, 1999 and Wang et al, 2001 observed similar results in their study of formation of chitosan nanoparticles and chitosan film treated with phosphate.

Hepatitis B vaccine encapsulated and unencapsulated (fig 49 & 51), dextran cross linked chitosan microspheres also revealed characteristic peaks at 2925 and 2854 cm⁻¹ indicated the presence of (C-H) methylene group asymmetrical stretching and (C-H) methylene group symmetrical stretching respectively. Chemical shift
occurred at 1639, 1457, 1251 and 1075 cm\(^{-1}\) when compared with dextran cross linked chitosan microspheres with vaccine. These data can be well compared with peaks of chitosan polymer and it is obvious that chemical interaction could be takes place while encapsulating the hepatitis B vaccine in chitosan microspheres with dextran as cross linker. Lian Yan Wang \textit{et al}, (2006) investigated the improvement of release behavior of chitosan microspheres containin insulin. They found that in glutaraldehyde cross linking method, the peak of 1635 cm\(^{-1}\) stands for stretching vibration of C = N in Schiff base. In TPP combination with glutaraldehyde cross linking method, there were two peaks in 1653 cm\(^{-1}\) and 1534 cm \(-1\) to sand for chitosan salt formation, indicating ionic cross linking method. The albumin showed characteristic peaks in FT IR spectrum (Fig 52) at 3400 cm\(^{-1}\) aliphatic primary amine stretching, 919 cm\(^{-1}\)(C-H) methylene asymmetrical stretching, 2851 cm\(^{-1}\) (C-H) methylene symmetrical stretching, 1657 cm\(^{-1}\) (C=C) alkeynl stretching & 801 cm\(^{-1}\) (C-H) phenyl ring substitution.

The FT IR spectrum (fig 53 & 54) of hepatitis B vaccine encapsulated unencapsulated albumin microspheres showed at marked differences at 3400 cm\(^{-1}\) with respect to polymer. The vaccine encapsulated albumin microspheres showed characteristic peak at 3401 cm\(^{-1}\) and showed hydroxy group (OH) stretching vibration. But vaccine unencapsulated albumin microspheres showed a vshort peak at 3011 cm\(^{-1}\) (CH) stretching cis or trans isomer. The bands at 2926 and 2855 cm\(^{-1}\) is an indicative (C-H) methylene asymmetrical stretching and methylene symmetrical stretching of vaccine unencapsulated albumin microspheres. But the bands were shifted to 2929 and 2859 cm\(^{-1}\) respectively after encapsulation of vaccine. Certain isolated peak could be observed at 2348 cm\(^{-1}\) in albumin microspheres without vaccine encapsulation, which was not seen after encapsulation as well as in polymer. Even though certain
interaction between albumin polymer, cross linker and hepatitis B vaccine could be observed in the FT IR spectra but failed to determine the vaccine entrapment. This might be due to denaturation of vaccine during microencapsulation process.

The FT IR spectral pattern (Fig 55) of dextran has displayed characteristic absorption peaks in 3436 cm\(^{-1}\) (N-H) heterocyclic amine stretching, 2998 & 2946 cm\(^{-1}\) (C-H) aliphatic stretching, 1752 cm\(^{-1}\) (C=O) stretching, 1630 cm\(^{-1}\) (C=C) alkenyl stretching, 1457 cm\(^{-1}\) (C-H) methylene bending, 1278 cm\(^{-1}\) (O-H) bending, 1086 cm\(^{-1}\) (C-N) primary amine stretching and 865 cm\(^{-1}\) (C-H) phenyl ring substitution. The FT IR spectrum (fig 56) of hepatitis B vaccine in unencapsulated dextran microspheres showed the characteristic peak at 3436 cm\(^{-1}\), the same peak was also observed in FT IR spectroscopy of dextran polymer and hepatitis B vaccine. However, after vaccine encapsulation the spectrum (fig 57) was shifted to 3401 cm\(^{-1}\). This proved the degeneration of hepatitis B vaccine either might be due to glutaraldehyde or due to incompatibility during microencapsulation process. Band shift could be observed at peak 2926 and 2853 cm\(^{-1}\) in the case of vaccine unencapsulated dextran microspheres and 2921 and 2854 cm\(^{-1}\) in the case of vaccine encapsulated dextran microspheres. But the bands were characteristic of methylene asymmetrical stretching group and methylene symmetrical stretching group. The band observed at 1632 cm\(^{-1}\) of dextran microspheres without vaccine encapsulation is an indicative of (C=C) alkenyl group with stretching vibration. But after vaccine encapsulation the band was shifted to 1654 cm\(^{-1}\) and similar type of peak was observed at 1657 cm\(^{-1}\) in the case of hepatitis B vaccine is an indicative of alkenyl (CH) group with stretching vibration. Even though certain interaction between dextran polymer and hepatitis B vaccine could be observed in the FT IR spectra but failed to determine the vaccine entrapment. This might be due to denaturation of vaccine during microencapsulation process.
2.4 Water uptake of microspheres

The water uptake of microspheres is an important parameter to find out the burst effect. In PLGA polymeric system, the weight of glutaraldehyde cross linked PLGA microspheres increased around 23 mg with an increase in size of 2 µm and the weight of dextran cross linked PLGA microspheres was increased around 24 mg with an increased in size of 3 µm. In the case of PLA polymeric system, the weight of glutaraldehyde cross linked PLA microspheres increased to 20 mg with an increased in size of about 1 µm. and the weight of dextran cross linked PLA microspheres increased to 22 mg with an increase in size of 2 µm. On the other hand, the weight of glutaraldehyde cross linked chitosan microspheres was increased to 22 mg with an increased in size of 4 µm and the weight of dextran cross linked chitosan microspheres was increased to 25 mg with an increased in size of about 6 µm (table 22). Generally, it is very clear that the water absorption is less when glutaraldehyde was used as cross linking agent rather than dextran as cross linking agent. Conventionally poly vinyl alcohol has been used as stabilizing agent for preparing poly ester polymers PLGA and PLA microspheres. However, in this research work an additional cross linker either glutaraldehyde or dextran was employed inorder to stabilize more and to avoid burst effect even though, only 15 – 18 % of water absorption could be observed. Maria Alonso et al, (1999) investigated high water absorption of PLGA microspheres with various stabilizers like dextran, heparin and trehalose. On the other hand, 18 – 22 % of water absorption of chitosan microspheres could be observed either with glutaraldehyde or dextran as cross linker in this study. Kwunchit Oungbho and Muller (1997) found that the uptake of water in both N-acetylchitosan and cross linked chitosan sponges was more than 20 times of their weight. Anthony Amachei Attama et al, (2007) observed 2 to 5 times of water
absorption in mucuna gum microspheres with glutaraldehyde as cross linker. Therefore, it is presumed that water penetrates into the microparticles, dissolves the cross linking agent and causes osmotic pressure. Accordingly, the pore size of microparticles increased and thus the vaccine was released to induce burst effect. The burst effect showed impact on *in vitro* release profile. Matsumoto *et al.*, (2005) reported that the PLGA microspheres with the inner drug holding layer of gelatin showed rapid water uptake, which led to destruction of the outer layer occurred at the early period and caused initial burst as observed in the study. They investigated that the PLGA showed a higher water uptake than PLA.

2.5 Stability studies

The stability studies were demonstrated by the size of microspheres and percentage vaccine loading in polymeric microspheres were carried out in hepatitis B vaccine loaded and unloaded microspheres at various temperatures over a period of 8 weeks. In PLGA polymeric system, the glutaraldehyde cross linked PLGA microspheres with either vaccine loaded or vaccine unloaded were stable at 4°C. The size and percentage vaccine loading were not changed at 4°C. However, the size was reduced after 8 weeks of storage. The size and percentage loading of vaccine did not alter even after 8 weeks of storage. Therefore, the glutaraldehyde cross linked vaccine loaded and unloaded microspheres were stable at 4°C for about 8 weeks of storage. However, the size of vaccine loaded and unloaded microspheres were reduced at room temperature and no shape could be observed at 50°C. Therefore, the microspheres were totally disintegrated and the size of microspheres was unable to measure. On the other hand, reduced percentage loading could be observed after 8 weeks of storage at room temperature and 50°C. The percentage of vaccine loading was extremely significant at 4°C, significant at room temperature and non significant
at 50°C (table 28). In the case of dextran as cross linker, the vaccine loaded and unloaded microspheres were stable at 4°C and room temperature. The percentage of loading in PLGA microspheres was also not affected even after storage at room temperature for 8 weeks. Moreover, disintegration of microspheres at 50°C led to non shape of PLGA microspheres and the percentage of vaccine loading was also drastically decreased. The significant percentage vaccine loading could be observed at 4°C and non significant percentage of vaccine loading could be observed at room temperature and 50°C after 8 weeks of storage (table 23). In the case of glutaraldehyde or dextran cross linked PLGA microspheres drastic loss of vaccine could be observed at 50°C. Therefore, at 50°C the microspheres were disintegrated and the nature of vaccine also destroyed. This might be due to the glass transition temperature (Tg) of PLGA polymer. The Tg of PLGA polymer is 40 – 60°C, which was not modified either with glutaraldehyde or with dextran as cross linker while preparing microspheres. Similar type of result was observed earlier by Xinagguo Mei et al, (2006) PLGA microspheres as sustained release carrier for leutinizing hormone. They investigated that PLGA 75 / 25 polymer had a Tg value of 38°C, whereas drug loaded microspheres exhibited considerably higher Tg values of 42°C. In the present work there could be increased in Tg value after encapsulation of vaccine in PLGA microspheres but it also led to particle degradation.

In the case of PLA polymeric system, the glutaraldehyde cross linked PLA microspheres either with vaccine loaded or vaccine unloaded microspheres were stable at 4°C. The size of glutaraldehyde PLA microspheres did not change at 4°C and at room temperature after 8 weeks of storage. However, the size of vaccine loaded microspheres was not changed at 4°C but the size was reduced at room temperature. This reflects in vaccine loading also, which was not changed at 4°C and decreased at
room temperature after 8 weeks of storage. In the case of dextran cross linked PLA microspheres the size of PLA microspheres was not changed at 4°C and at room temperature even after 8 weeks of storage. With respect to particle size the vaccine loading also not changed at 4°C and room temperature after 8 weeks of storage. In the case of glutaraldehyde or dextran cross linked PLA microspheres drastic loss of vaccine could be observed at 50°C, followed by the disintegration of microspheres, resulting in the destruction of the nature of vaccine. This might be due to the glass transition temperature (Tg) of PLA polymer. The Tg of PLA polymer was indicated as 50 – 80°C, which was not modified either with glutaraldehyde or with dextran as cross linker while preparing microspheres.

Chitosan microspheres either with glutaraldehyde or with dextran as cross linker showed better carrier system for vaccine due to it’s stability. The size of both vaccine loaded and unloaded, glutaraldehyde cross linked microspheres did not change at 4°C, but the size was increased at room temperature and 50°C after 8 weeks of storage. The percentage of vaccine loading was not affected with respect to the size of microspheres at 4°C, but the loading was reduced at room temperature and at 50°C. This might be due to the interaction of glutaraldehyde with hepatitis B vaccine in microsphere system during storage for a period of 8 weeks. Inez van der Lubben et al, (2003) investigated that diphtherial toxoid encapsulated chitosan microspheres did not show any morphological change, when stored at 4°C and room temperature for at least 3 months. In addition, no changes in size or vaccine release were observed over this period. The results indicated that vaccine loaded chitosan microparticles need not be prepared or resuspended freshly before vaccination. On the other hand, the dextran cross linked chitosan microspheres was stable at 4°C and room temperature. At this temperature the size and vaccine loading were not changed after 8 weeks of storage.
However, the size was slightly decreased with decreased in vaccine loading to some extent after storing at 50°C for a period of 8 weeks. In PLGA, PLA and chitosan polymeric system, among two cross linker were tested for formulating PLGA, PLA and chitosan microspheres, dextran was found to be ideal eventhough the percentage loading was less when compared to glutaraldehyde cross linked microspheres because the dextran cross linked microspheres were stable even at room temperature after 8 weeks of storage.

3 In vitro release profile

The antigen was dispersed with in polymer matrix in a dry state, thus providing an extended self life compared with traditional vaccine formulation. This would be considerably advantages in developing countries to reduce the overall immunization cost. For investigating in vitro release profile, the best formulation method was selected through vaccine loading and size distribution analysis. Many factors such as concentrations of cross linker, type of cross linker, polymer type and polymer concentration influence the antigen release from polymeric microspheres. In this study, the release pattern was characterized by a bi phasic system with typical initial release, followed by sustained release for a particular period. However, the initial antigen release also depended on the total surface of the microspheres and was influenced by the method of preparation and polymer used. Erosion and formation of pores was time dependent and largely influenced by the half life of polymer and the surface area. Gradual and continuous release of antigen occured from the microspheres at different rates. O'Donnel et al (1997) reviewed that antigen release was greatly influenced by the preparation techniques and ratio of co polymer. In present research work, the gradual release of HBsAg from polymeric microspheres might be caused by degradation of the polymer latex leading to a swollen inner
structure followed by contact of the microspheres matrix with an aqueous release medium and antigen diffusion through the swollen phase. It is very clear from table 24 and fig 59 that the pattern of antigen release from PLGA microspheres was greatly influenced by the concentration of glutaraldehyde. From the vaccine entrapment studies, it is concluded that the HBsAg was encapsulated in to PLGA microspheres when 1.5 to 3% v/v of glutaraldehyde was used as cross linker. It was found that microspheres prepared with 2% v/v of glutaraldehyde as cross linker were ideal to encapsulate HBsAg and to fabricate PLGA microspheres (table 16). At this concentration the release of antigen was sustained with less burst effect. Almost 3 - 4% of antigen release was observed during 7 days in both the cases. The release of antigen was much sustained and very slow up to 56 days. However, the antigen release was faster and increased release could be observed between 56th to 63rd and 63rd to 70th day. Therefore, sudden release that is almost 22% rise in antigen release was observed (fig 59 & 69). The release of antigen was maintained up to 84 days. Xingguo Mei et al (2006) stated the burst effect of the microspheres attracted much attention because it might result in the severe adverse action or economically waste. In most cases, the burst release was attributed to the release of drug dispersed on the surface of the microspheres. So the burst release was connected closely with those factors that influenced the drug dispersion in microspheres and the porosity caused by organic solvent evaporation. Nikolaus Kofler et al (1996) investigated that initial burst release depended on the total surface of the microspheres and also influenced by the method of preparation and the polymer used, as observed in the present study. Decreasing the concentration of glutaraldehyde to 1.5% v/v the initial release was very less up to 14 days but sudden burst effect after 14 days could be detected and almost 18% increase in the release of antigen was observed during next seven days that is 14
to 21st day. The PLGA microsphere system prepared with 1.5% v/v of glutaraldehyde as cross linker released antigen, with a peak level on 35th day and maintained up to 49th day. Almost 31 to 33% of antigen was released and the system was unable to release the HBsAg more due to instability of microspheres (fig 59). Increasing the concentration of glutaraldehyde to 2.5% v/v released antigen in much sustained manner, reached peak antigen release on 56th day with 20 – 22% of release during the period. However, the microspheres were ruptured after 56 days and antigenically active HBsAg could not be detected further. PLGA microspheres prepared with 3% v/v of glutaraldehyde released HBsAg up to 63 days with 25 to 28% of antigen release could be observed (table 16). Rest of the vaccine was inactivated due to higher glutaraldehyde concentration. Shaobing Zhou et al, (2004) detected with an initial burst effect of 20.5 % of HBsAg from PELA microspheres during the first day of incubation.

Gideon Kersten et al (1996) investigated that TT encapsulated PLGA microspheres preparation released their TT content quickly that is with in 3 or 4 days for certain PLGA micropsheres and certain other preparations the release was maintained up to 3 weeks to 5 months. This might be due to the instabilization of microspheres, which eroded quickly. In contrast to their findings, in this research work only 3 - 4% antigen release could be observed in first seven days because of the addition of either glutaraldehyde as stabilizing agent. Thus the microparticles stabilized more and initial burst effect was reduced. However, the grades and half life of PLGA polymer also showed the impact on in vitro release pattern of HBsAg. Li feng et al (2006) investigated that the half life of PLGA 50: 50, 75: 25 grades of polymer was 41.5 and 116.8 days respectively. Therefore, they observed incomplete erosion and break down of polymers on 40th day. In this research work 50: 50 PLGA
polymer grade was utilized to encapsulate HBsAg. In contrast with findings of Li Feng
et al (2006), the microspheres were not broken down even up to 80 days when
 glutaraldehyde was used as cross linker.

In order to ascertain the linearity of the release of antigen linear regression
between the duration in days and cumulative percentage release of hepatitis B vaccine
was calculated. Table 25 describes the linear regression of *in vitro* release pattern of
hepatitis B vaccine from PLGA microspheres prepared by varying the concentrations
of glutaraldehyde cross linked with 2% w/v of PLGA polymer. Fig 38, 39, 40 and 41
explain that the linear regression of *in vitro* release pattern of HBsAg from PLGA
microspheres prepared by varying the concentration of glutaraldehyde from 1.5 to 3%
 v/v of glutaraldehyde cross linked 2% w/v of PLGA microspheres. From the Run’s
test, it is understood that the linear trend of antigen release was followed when the
concentration of 1.5, 2.5 & 3% v/v of glutaraldehyde were used. However,
microspheres prepared with 2% v/v of glutaraldehyde did not release antigen in linear
fashion because even amount of vaccine did not release at specific time interval. Even
though the release pattern was so sustained, uneven antigen release could be observed
but antigenically active HBsAg was released more when compared to the rest.
Moreover, PLGA microspheres prepared with 2% v/v of glutaraldehyde was more stable
and capable of release antigen for about 84 days.

Maria Alonso et al (1994) investigated the immunopotentiating activity of
PLGA / PLA microspheres of various sizes and release profiles with the final aim of
designing a formulation which induces a prolonged immune response against tetanus.
They found that each of microencapsulated preparation induced higher levels of IgG
antibody and tetanus antitoxin activity than fluid tetanus toxoid. The experiment
clearly demonstrated that microencapsulation of tetanus toxoid was associated with a
significant adjuvant effect. However, none of the preparations induced higher levels of more prolonged tetanus toxoid antibody responses than the control with aluminium phosphate. They have concluded that the protein released by microspheres after the initial period of release was denatured and thus no longer antigenically active.

The results tabulated in table 26 and graphical represented in fig 64 explain the effect of various concentrations of dextran on in vitro release pattern of HBsAg encapsulated, dextran cross linked PLGA microspheres. From the fig 64, it is understood that PLGA microspheres prepared with 1.5% w/v concentration of dextran released antigen in sustained manner up to 49th day. A sudden increase in antigen release could be observed after 49th day with a peak antigen release on 77th day and the increased level of release was maintained up to 91 days. However, the percentage of release was quite less and only 25 – 27 % of antigen release was observed. This might be due to the microspheres was hardened more while preparation. Increasing the concentration to 2% w/v of dextran showed a sustained release of antigen with a peak level on 77th day and maintained up to 91 days. In this system, almost 56 to 57% of antigen release was observed. Increasing the concentration of dextran to 2.5 % w/v showed sustained release of antigen with a peak antigen release on 63rd day and maintained up to 91 days. In this system almost 41% of antigen release could be observed.

It is very interesting to note that the PLGA microspheres prepared with 1.5, 2 & 2.5% w/v of dextran was used as cross linker released and maintained the antigen release up to 91 days. Further release of antigen could not be observed because of degradation of microspheres and unable to detect antigenically active HBsAg. Increase in the concentration of dextran to 3% w/v showed the increased release of antigen level for first seven days and almost 12 – 13% of antigen release could be
observed. Following the first phase the release antigen was more in the next seven
days and reached peak level on 70th day. Furthermore, no significant release could be
observed after 70th day. Therefore, 2% w/v of dextran was more ideal to release
antigenically active HBsAg. Table 27 showed the linear regression of in vitro release
pattern of hepatitis B vaccine from PLGA microspheres prepared by varying the
concentrations of dextran cross linked with 2% w/v of PLGA. Fig 43, 44, 45 and 46
explains that the linear regression of in vitro release pattern of HBsAg from PLGA
microspheres prepared by varying the concentration of dextran from 1.5 to 3% v/v
and cross linked with 2% w/v of PLGA microspheres. From the Run's test, it is
understood that the linear trend of antigen release was followed when 1.5% w/v of
dextran was used to prepare microspheres. However, microspheres prepared with 2,
2.5 and 3% w/v of dextran did not release antigen in linear fashion because even
amount of vaccine was not released at specific time interval. From the loading studies
and in vitro release profile confirmed that 2% v/v or 2% w/v of either glutaraldehyde
or dextran was found to be ideal to encapsulate HBsAg. At this concentration the
antigenically active HBsAg was retained more when compared to the rest. Moreover,
the release pattern of HBsAg was much sustained. However, it is necessary to find out
the effect of polymer concentration on in vitro release profile. Therefore, by keeping
2% v/v of glutaraldehyde or 2% w/v of dextran as constant concentration of cross
linker and the concentration of PLGA polymer was screened (table 28 & 29).

The table 28 and graphical representation in fig 69 explains the effect of
various concentrations of PLGA polymer with 2 % v/v of glutaraldehyde as a cross
linking agent on in vitro release pattern of hepatitis B vaccine encapsulated PLGA
microspheres. From the study it is understood that 2% w/v of PLGA polymer with 2%
v/v of glutaraldehyde was ideal to encapsulate HBsAg because the antigenically
active HBsAg was able to be released up to 84 days. In this system the antigen release was much sustained and 41 - 42 % of antigen was released. Decreasing the concentration of PLGA polymer to 1% w/v showed an initial burst effect of about 12 - 15 % of antigen release during first seven days and reached the peak on 35th day by releasing 38- 41 % of antigen but failed to maintain antigen level after 35th day (fig 69). When the concentration of PLGA polymer was increased to 3% w/v, the antigen release was found to be sustained and reached peak antigen level on 70th day by releasing 44 - 46 % of antigen. Eventhough the percentage of antigen release was more, when compared to rest but unable to maintain the level of release because of the degradation of the polymer when the concentration was increased to 3% w/v of PLGA polymer, an initial burst effect of about 18 – 20% release with in 7 days was resulted and the release was sustained with peak antigen release observed on 77th day. But antigenically active antigen release could not be observed after 77th day. Table 29 and fig 47 explain the linear regression of in vitro release pattern of hepatitis B vaccine from PLGA microspheres prepared by varying the concentrations of PLGA polymer cross linked with 2% v/v of glutaraldehyde. Fig 48, 49 and 50 explain the linear regression of in vitro release pattern of HBsAg from PLGA microspheres prepared by varying the concentration of PLGA polymer to 1, 2 and 3% cross linked with 2% v/v of glutaraldehyde. However, linear trend was followed when 1 and 3% of PLGA polymer was used. Microspheres prepared with 2% w/v of PLGA polymer did not release antigen in linear fashion but it released antigen in sustained manner and the percentage of antigenically active HBsAg was also more when compared to rest. From the Run’s test it is understood that microspheres prepared with 3% w/v of PLGA polymer displayed the linear trend of antigen release in vitro. Besides 1and 2% did not follow the linear trends but percentage of antigenically active HBsAg was
more. More over the initial antigen release was very high that is 20% of antigen was released with first seven days when 3% w/v of PLGA polymer. In all the models of in vitro release study was found to be biphasic process either with less antigen release or with more antigen release. This is mainly due to erosion and water penetration due to PLGA degradation resulting in the release of antigen. The percentage of antigen release was greatly depending on water penetration in to the microspheres. In this study, 18 – 21% of water absorption (table 22) was observed for either glutaraldehyce cross linked or dextran cross linked microspheres. As discussed earlier only few models showed significant initial burst effect. This is mainly due to the penetration of water in to microspheres and saturated the HBsAg – stabilizing excipient depots, generating an osmotic pressure which caused them to release HBsAg content in burst manner. A similar behaviour was previously observed for gelatin core coated microspheres prepared by anhydrous procedure (Tobio et al, 2000). Lin Du et al (2006) investigated the effect of LA / GA ratio of PLGA polymer to release luteinizing hormone - releasing hormone from PLGA microspheres. The hormone release from PLGA microspheres was governed by an asymptotic profile in which up to 87% release from 40:60 PLGA polymeric microspheres, 64% release from 50: 50 PLGA polymeric microspheres and 60% release from 60:40 PLGA polymeric microspheres were noticed. In addition, the size of the microspheres is also an important parameter that influences the rate at which HBsAg was released. As the particle decreased in size, the relatively high surface area per unit volume of the microspheres facilitates contact with the buffer or penetration in to the microspheres and at the same time allows a faster diffusion of monomers and oligomers formed as a result of the degradation of the polymer. Then, the acidic (lactic acid and glycolic acid) monomers and oligomers further catalyze the degradation of PLGA
microspheres (Bittner et al., 1999; Sah et al., 1995; Ghaderi et al., 1996 and Cleland, 1995).

Alonso et al. (1999) reported that PLGA microspheres with a particle size of 20 – 35\(\mu\)m released antigen about 50 – 90\% of tetanus toxoid with in first day. In contrast to the findings of Alonso et al. (1999) in the present work the size of glutaraldehyde cross linked or dextran cross linked PLGA microspheres was 35.02±0.9 and 34.06±0.9 respectively. But the release of antigen was found about 2-4\% in both the cases during first seven days. This proves that the microspheres were stabilized more either with glutaraldehyde or with dextran to release the HBsAg in a sustained manner. It is obvious from fig 77 that the dextran cross linked PLGA microspheres was more capable to release antigenically active HBsAg when compared to glutaraldehyde cross linked PLGA microspheres. Moreover, the release is much sustained in both the cases significantly deviated from the linearity.

It is very obvious from the fig 78 that the pattern of antigen release was much sustained, when 2% v/v of glutaraldehyde was used as cross linker. The microspheres released antigen for about 84 days with an initial release about 16 – 19\% in first seven days. Decreasing the concentration of dextran to 1.5\% w/v resulted in the release of antigen for about 49 days and almost 39 to 42\% of antigen release was observed. In this system sudden release of antigen that is around 18 to 20\% of increase in antigen release could be observed during 14 to 21th day. Increased concentration of dextran to 2.5\% w/v led to increase antigen release level up to 70th day with an initial release of about 11 to 13\% during first seven days. As like previous system the sudden release of antigen can be quantified between 7th to 14th day and 21st to 28th day. But the percentage of burst antigen release was quite less. Totally 50 to 52 \% of antigenically active HBsAg was released in 70 days. Increased concentration of dextran to 3\% w/v
led to increased antigen release of about 16 to 18% with in first seven days and the release of antigen was much sustained up to 70th day and almost 47 to 48% of antigenically active HBsAg was released. Nikolaus Kofler et al, (1996) investigated that the release profile of antigen from PLA microspheres was characterized by an initial burst with in the first three days and followed by sustained release of antigen. They have stated that the initial antigen release depends on the total surface area of the microspheres and was influenced by the method of preparation. It is generally accepted that the adjuvant mechanism is based on effective delivery of antigen in to antigen presenting cells (Eldridge et al, 1991 and Challocombe et al, 1992). From the linear regression study (fig 79, 80, 81 & 82) it was observed that the antigen release from PLA polymeric system was not deviated from linearity. Therefore, PLA microspheres prepared with 1.5 to 3% v/v of glutaraldehyde were capable of releasing antigen in linear fashion. From the study it is understood that microspheres prepared with 1.5 and 2% w/v of dextran were capable of releasing antigen for about 84 days (fig 83). However, the percentage of antigen release and initial amount of antigen release was more when microspheres prepared with 2% w/v of dextran. In both the cases the antigen release was much sustained over a period of 84 days. However, in the case of microspheres prepared with 1.5% w/v of dextran the antigen release was much sustained after 14th day. But in the case of microspheres prepared with 2% w/v of dextran almost 40 to 42% of antigen was released in 28 days and further release was much sustained for a period of 84 days. Totally 54 to 56% and 45 to 47% of antigenically active HBsAg was released from microspheres prepared with 1.5 and 1% w/v of dextran respectively. The size of the microspheres is also an important parameter that influences the rate at which HBsAg was released. As the particle decreased in size, the relatively high surface area per unit volume of the microspheres
facilitates contact with the buffer or penetration into the microspheres. Matsumoto et al. (2005) concluded that the drug release rate from microsphere system was governed by the erosion of the polymer in drug holding layer. Increasing the concentration of dextran to 2.5% w/v increased the initial release of antigen in first seven days. After seven days the release of antigen was much sustained for 70 days and 44 to 47% of antigen was released during that period. However, further increase in dextran concentration that is microspheres prepared with 3% w/v of dextran released lesser percentage of antigen initially when compared to microspheres prepared with 2 and 2.5% w/v of dextran. The percentage of antigenically active HBsAg was released from microspheres prepared with 3% w/v of dextran was quite less when compared to the rest. From the Run's test, the microspheres prepared with 1.5 and 2.5% w/v of dextran showed the linear trends in antigen release. However, the microspheres prepared with 2 and 3% w/v of dextran showed the significant deviation from the linearity. Besides, the antigenically active HBsAg was released more when microspheres were prepared with 2% w/v of dextran. The effect of polymer concentration also influenced the release pattern of HBsAg from PLA microspheres. It is also noticed that the percentage of antigenically active HBsAg release was more, when 2% w/v of PLA was used. Decreasing the concentration of PLA to 1% w/v resulted in 19 to 20% of antigen release in first seven days (fig 88). Almost 19% of antigen release was observed during 14th to 21st day. In addition increasing the concentration of PLA to 3% w/v released antigen in sustained manner with an initial release of antigen was about 18 to 20%. Among 3 various concentrations of PLA 2% w/v was found to be ideal when compared to the rest. Maria Alonso et al. (1994) found that polymer molecular weight also influenced the \textit{in vitro} release profile. The higher release rate from lower molecular weight PLA microspheres was attributed.
The linear regression analysis showed that the percentage of antigenically active HBsAg release was more when 2% w/v of PLA was used. The system was able to release antigen for 84 days with an initial release of 16 to 19% during first seven days. After seven days the antigen release was much sustained and approximately 57% of antigen release was observed (table 38). Decreasing the concentration of PLA led to more release in antigen initially and about 20 – 23% of antigen was released during first seven days. Approximately 48 – 50% of antigen was released in 77 days. Further increase in the concentration of PLA to 3% w/v led to 18 to 20% of antigen release during first seven days. After seven days the antigen release was much sustained and 43 – 45% of antigen was released for 77 days. Nikolaus Kofler et al, (1996) observed that the release of antigen is characterized by a typical initial protein burst with in the first week, due to antigen release from the surface and slow release of antigen thereafter.

The extent of antigen burst with in the first three days was 36% in PLG solvent evaporation microspheres where as in PLG solvent extraction microspheres the initial antigen release was about 25% and in PLA solvent evaporation microspheres about 19% of initial antigen release was observed. Thus initial antigen release depends on the total surface area of the microspheres and is influenced by the method of preparation and the polymer used. Following the initial protein burst, a steady release of antigen over the next 30 days was observed, where as PLA solvent evaporation microspheres showed significant antigen release. After 33 days, 68% of antigen was released from PLG solvent evaporation microspheres, 37% from PLG solvent extraction microspheres and 23% from PLA solvent evaporation microspheres. Since the erosion and formation of pores is time dependent and largely influenced by the half life of the polymer and surface area, gradual and continuous
release of antigen from the microspheres occurs at different rates as observed in present study. It is very obvious from the Run’s test that PLA microspheres prepared with 2% and 3% w/v of PLA polymer showed linear trends in releasing antigen. However, microspheres prepared with 1% w/v of PLA released antigen in non linear fashion. From the observation in fig 96, it is understood that the release of antigenically active HBsAg was more when dextran was used as cross linker. Moreover, dextran cross linked PLA microspheres released antigen about 23 – 25% during first seven days and almost 40 – 42% of antigen was released during 21 days. But the release of antigen was so sustained during the next 21 days that is 42 days. After 42 days the release of antigen was slightly increased and maintained up to 84 days. However, the glutaraldehyde cross linked PLA microspheres released antigen in biphasic manner that is initial release of about 16 to 19% during first seven days. After seventh day the antigen release was maintained over a period of 84 days (table 41). From the fig 75, it is understood that microspheres prepared with 2 and 2.5% v/v of glutaraldehyde released antigenically active HBsAg for 105 days with an initial release about 14 – 15% and 16% respectively. However, both the concentrations were able to release peak antigen level on 84th day and maintained up to 105 days. Almost 64 to 66% and 61 to 64% antigen release was observed for microspheres prepared with 2 and 2.5% v/v of glutaraldehyde as cross linker. Decreasing or increasing the concentration of glutaraldehyde did not release the antigen significantly. However, the initial release of antigen was directly proportional to the increased concentration of glutaraldehyde. From the Run’s test, it is understood that the linear trends of vaccine release was followed when microspheres prepared with 0.5, 1.5 and 3% v/v of glutaraldehyde as cross linker (table 43). However, microspheres prepared with 1, 2 and 2.5% v/v of glutaraldehyde released antigen in non linear fashion.
The percentage of antigenically active HBsAg released more when microspheres prepared with 2 and 2.5% v/v of glutaraldehyde. Kwunchit Oungbho and Muller et al (1997) investigated that the drug release from chitosan sponges was depend on the pH of the dissolution media. They have stated that the drug release from the N- acetyl - chitosan and the cross linked chitosan sponges was however sustained at both pH. Kanke et al (1989) and Akbuga and Durmaz (1994) suggested that this is due to the decrease in solubility and permeability of the chitosan matrices by either the acetylation or the cross linking of the chitosan. Jameela and Jayakrishnan (1995) have demonstrated the in vitro release of mitoxantrone in to phosphate buffer from chitosan microspheres having different cross linking densities. They investigated that increasing the concentration of cross linker did not release more drug. They have stated that the effect of cross linking density on drug release from chitosan matrix appears to be more remarkable from a protein matrix. This might be due to the poor affinity of chitosan towards water. It was found that only 25 – 60 % of drug was released from microspheres having high cross linking densities even after 1 month in the dissolution medium, in the view of good solubility of the drug. Jelena Filipovic et al (2003) investigated that the in vitro release profile of hydrocortisone from chitosan microspheres was influenced by the drug / polymer ratio in a manner that an increase in the release rate was observed when the drug loading was reduced.

From the Run's test it is quite interesting to note that the linear trends of vaccine release was followed when microspheres prepared with all the concentration of dextran that is from 0.5 to 3% w/v (table 45). From the results, it is understood that the dextran is highly compatible as cross linker with chitosan and chitosan is highly compatible polymer with the dextran cross linker when compared to the rest of the formulation screened in this research work. Berthold et al (1996) investigated a linear
correlation between the logarithm of the molecular weight and the amount of sodium sulfate necessary for the reduction of transmission to 50% was observed, \( r = 0.9998 \) and \( y = 5.791x + 3.8283 \). They have concluded that the result was probably due to the fact that the solubility of chitosan depends on the number of positive charges on its surface. A specific amount of positive charge is necessary for the dissolution of one chitosan polymer molecule. In the present study, it is noticed that 1% w/v of chitosan with 2% v/v of glutaraldehyde was ideal to encapsulate HBsAg because the antigen release was maintained up to 105 days (table 46). During this period almost 64 – 66% of antigen release was observed. Moreover, microspheres prepared with 2 and 3% w/v of chitosan did not release antigen not more than 70 days, about 66 – 68% and 70 – 74% of antigen was released respectively during 70 days. Microspheres prepared with 1 & 2% w/v of chitosan released antigen in non linear fashion. But microspheres prepared with 3% w/v of chitosan released antigen in linear fashion (Table 47). In the case of dextran cross linked microspheres, microspheres prepared with 1% w/v of chitosan with 2% w/v of dextran released antigen only up to 63rd day (table 48). However, microspheres prepared with 2% w/v of chitosan with 2% w/v of dextran released antigen over a period of 105 days. Besides, microspheres prepared with 3% w/v of dextran released antigen not more than 70 days. Linear regression analysis and Run's test showed that the release of antigen was linear in all the cases.

Hepatitis B vaccine was released in sustained manner from either glutaraldehyde cross linked microspheres or dextran cross linked microspheres (table 50). However, the initial antigen release was same in both the cases during first seven days and the release of antigen was much sustained when dextran was used as cross linker. Moreover, the percentage of antigenically active HBsAg from dextran cross linked microspheres was more when compared to glutaraldehyde cross linked
chitosan microspheres. Therefore, chitosan and dextran were highly compatible to each other to encapsulate HBsAg. From this study, it is understood that chitosan polymer is the most successful polymeric system either glutaraldehyde or dextran as cross linker to encapsulate HBsAg (table 51). However, PLGA and PLA either with glutaraldehyde or dextran proved as a good carrier system for HBsAg. When the release pattern is compared, PLGA microspheres showed contained antigen release during first seven days in both the system. The PLA polymeric system either with glutaraldehyde or dextran as cross linker released antigen more during first seven days. The size of microspheres is an important parameter that influences the rate of antigen release. As the particle size decreases, the relatively high surface area per unit volume of the microspheres facilitates contact with the buffer or penetration in to microspheres and allows faster diffusion. This was reflected in all the microparticle system. Maria Alonso et al (1999) reported that PLGA microspheres with a particle size of 20 – 35 µm released about 50 – 90% of tetanus toxoid with in a first day itself. Brittner et al (1999) reported that particles of 18 µm with 9% loading released 60% of bovine serum albumin in an initial burst with in first day. In contrast to the present study, the size of PLGA and PLA microspheres was in between 20 - 40 µm and they released antigen sustained in a manner for about 84 and 91 days. Moreover, the size of chitosan microspheres was 80 – 90 µm released antigen in sustained manner for 105 days. This proved that the microspheres were well stabilized by the addition of either glutaraldehyde or dextran as cross linker. Among two cross linker screened, dextran was found to be a better cross linker when compared to glutaraldehyde because dextran cross linked microspheres are smaller in size and more stable. It released antigen in much sustained manner for longer duration and universally it is
less toxic when compared to glutaraldehyde. Therefore, dextran cross linked microspheres was preferred for immunogenicity study.

4 **In vivo immune response**

Immune response is a complex and intricately regulated sequence of events involving several types. Generally the immune response is triggered when an antigen enter the body. The immune system has evolved two main functions: to react quickly (with in minutes) to molecular pattern found in microbes and to develop slowly, precisely targeted specific adaptive immune responses. The faster acting innate immune responses provide a necessary first line of defence because of relatively slow nature of adaptive immunity (Medzhitov and Janeway, 1997). In contrast, adaptive immunity uses selection and clonal expansion of immune cells harboring made to order somaticaly rearranged receptor genes (T and B cell receptor) recognizing antigens from the pathogen, thereby providing specificity and long lasting immunological memory (Gourley et al, 2004). Innate immune responses, among their many effects, lead to a rapid burst of inflammatory cytokines and activation of antigen presenting cells (APCs) such as macrophages and dendritic cells. These nonclonal responses also lead to a conditioning of the immune system for subsequent development of specific adaptive immune responses. Adjuvants are functionally defined as components added to vaccine formulations that enhance the immunogenicity of antigens remain a focus in vaccine development. Potent adjuvants can improve the effectiveness of vaccines by accelerating the generation of robust immune responses, sustaining immune responses for a longer duration, inducing local immune responses, generating antibodies with increased avidity and neutralization capacity, eliciting cytotoxic T lymphocytes (CTLs), enhancing immune responses in individuals with weakened immune systems, increasing the response rate in lower
responder individuals and reducing the amount of antigen needed, thus reducing the cost of vaccination programme. Therefore, the development of a delivery system for the hepatitis B virus, which could induce the desired antibody response from a single injection, would be of enormous benefit.

In the present study a comparative study on anti HBs and immunoglobulin titre of Wistar rats subjected to various types of treatment groups was carried out. In order to differentiate the status of antibody level after a single step immunization certain groups of animals received booster dose with different combinations. From the results it is very obvious that the anti HBs response on 45th, 90th and 120th day after primary immunization that is without booster was found to be satisfactory with all the preparations. The required amount of anti HBs level for protection is 10 IU / l after vaccination. In this study, all the vaccine combinations with conventional hepatitis B vaccine as booster dose elicited robust immune response. However, HBsAg encapsulated in chitosan microspheres as a single dose or the same vaccine preparation as a booster dose elicited good immune response and the antibody level was increased sustainedly. In contrast to the study carried out by Sivakumar (1999) the tetanus toxoid encapsulated in chitosan microspheres either with the same preparation as booster dose or conventional alum adsorbed vaccine as booster dose produces higher antibody titre. In this research work HBsAg encapsulated PLGA, PLA and chitosan microspheres induced antibody level not less than 10 IU / l even up to 120th day after a single administration. However, in the case of PLGA and PLA microspheres, the induction of specific antibody level was increased up to 90th day and the level was slightly decreased on 120th day but not less than 10 IU / l.

Li feng et al (2006) investigated that the HBsAg encapsulated PLGA 50 / 50 – COOH microspheres produced anti-HBs antibody rapidly which continued to fall
from 6 weeks onwards when compared to the HBsAg encapsulated PLGA 50 / 50 and 75 / 25 microspheres. HBsAg – PLGA 50/ 50 – COOH microspheres might have released antibody sufficiently during 6 weeks and lacked of a persistent antigen stimulation to maintain the antigen concentration in the germinal center of lymphoid tissues in the later stage. Their study suggested that HBsAg – PLGA 50 / 50 COOH microspheres released antibody sufficiently during the 6 weeks and lacked of an effective booster in the later time. The fact that HBsAg – PLGA 75 / 25 microspheres released antigen slightly slower than HBsAg – PLGA 50 / 50 microspheres might be due to the higher molecular weight and higher ratio of L / G of the copolymer. The mixture of these three microspheres showed intermediate immune response induced by the rapid release of HBsAg PLGA 50 / 50 – COOH microspheres. However, the slow release of HBsAg – PLGA 50 /50 and HBsAg – 75 / 25 microspheres. Until 3 months post immunization, the antibody responses for the PLGA 50 / 50 microspheres. Charrie Chong et al, (2005) stated that currently there is a need of therapeutic vaccines, that are effective in inducing robust T helper type 1 (Th1) immune responses capable of mediating viral clearance in chronic hepatitis B infection. Therefore, they prepared hepatitis B therapeutic vaccine by encapsulating the hepatitis B core antigen (HBc Ag) in to poly (D, L lactic acid – co – glycolic acid) (PLGA) with or with out monophospho lipid A (MPLA), a Th1 favouring immunomodulator. They observed, a single immunization with a vaccine formulation containing (MPLA + HBcAg) co formulated in PLGA nano particles induced a stronger Th1 cellular immune response with a predominant interferon γ (IFN - γ) profile than those induced by HBcAg alone, free (HBc Ag + MPLA) simple mixture or HBcAg loaded nanoparticles in a mixture model. More importantly, the level of HBcAg specific IFN - γ production could be increased further significantly by a
booster immunization with the (HBc Ag + MPLA) loaded nano particles. These results demonstrated that the co delivery of HBcAg and MPLA in PLGA nano particles promoted HBCag specific Th1 immune responses with IFN - γ production and they suggested that appropriate design of the vaccine formulations and careful planning of the immunization schedule are important in the development of effective HBV therapeutic vaccines.

It is quite interesting to note that HBsAg encapsulated chitosan microspheres after a single shot elicited antibody cumulatively even up to 120th day. However, after booster dose with the same preparation elicited antibody cumulatively up to 90th day. After 90th day the antibody level was decreased. This might be due to lack of antigen release from microspheres or the negative phase was due to antigen antibody interaction after 90th day. Inez van der Lubben et al (2003) investigated that strong immune response was developed after oral vaccination of DT loaded chitosan microparticles. They found that mice vaccinated with 10 Lf DT associated to chitosan microparticles were found to have a significantly lower systemic immune responses than the groups vaccinated with 20 or 40 Lf DT associated to chitosan microparticles. They observed that the systemic immune response was strongly enhanced after incorporating the DT vaccine in to chitosan microparticles. In addition, significantly higher levels of specific anti DT IgA in the gastrointestinal tract were detected. From the observation, it is understood that HBsAg encapsulated PLGA, PLA and chitosan microspheres were capable of boosting the immune response significantly after a single step immunization. The conventional alum adsorbed hepatitis B vaccine produced satisfactory level up to 120 days but the anti HBs level was quite less when compared to the HBsAg encapsulated polymeric microspheres. Jennifer Moynihan et al (2001) investigated that single step immunization of HBsAg encapsulated PLGA
microspheres was produced high anti HBs level. They have stated that the response was long lasting and was superior to that obtained using the same peptide adjuvant with Freund’s complete adjuvant. The immunoglobulin levels were determined in order to know about the mode of immune response. Dunnet multiple comparison test was performed to compare the immunoglobulin levels of test samples with control values. From the test it is understood that all the values were extremely significant (P< 0.01) when compared with control groups except dummy batches. In contrast to the study carried out by Tobio et al (2000) the HBsAg encapsulated in polymeric microspheres either with single immunization or with booster dose elicited robust immunoglobulin titre. Gutierrez et al (2002) investigated the ability of BSA encapsulated PLGA microspheres to stimulate a specific T_h cell subset. They determined the IgG2a / IgG1 ratio at weeks 3 and 5 for the highest oral and intranasal doses. They concluded that robust immunoglobulin production was observed in after an injection of BSA encapsulated PLGA microspheres. In this study the immunoglobulins level was measured but sub typing were not performed. Maria Alonso et al (1994) investigated microencapsulated tetanus toxoid induced higher levels of IgG antibody and teanus antitoxin activity than fluid tetanus toxoid. They have stated that the levels are considerably higher than the estimated minimum protective levels in humans. This clearly demonstrated that microencapsulation of tetanus toxoid produced significant adjuvant effect.

The relative independence of the circulatory IgA is also evident for immune response induced by systemic (Russell et al 1992). Parenteral immunization with certain antigens induces strong IgA response in the circulatory compartment. The origin of serum IgA antibodies specific for parenterally introduced antigens remains unclear, but regional lymphnodes and spleen are probably involved. In this research
work it has been determined significant IgA antibody was produced and suggested that the release of IgA antibody was due to the local action after forming depot at the site of injection. The route of immunization and types of antigens that might induce an effective immune response in human bone marrow has not been clarified. However, specific IgA antibody that is initially polymeric appears in serum of parentally immunized or mucosally infected individuals, irrespective of the type of antigen (Russell et al, 1992). The effectiveness of systemic immunization on the induction of IgA antibodies appears to depend on the route of previous exposure to the antigen. Svennerholm et al (1980) observed IgA antibodies to *Vibrio cholerae* lipopolysaccharide in milk and saliva of lactating Pakistani women but not Swedish women after systemic immunization.

The IgA response to protein antigens such as tetanus toxoid, influenza virus haemagglutinin are of IgA1 sub class whereas responses to carbohydrate antigens are predominately of the IgA2 sub class (Brown et al, 1985; Lue et al, 1988; Tarkowski et al, 1990 and Lue et al, 1988). In this study significant IgM antibody was produced by polymeric microspheres either with a single dose or with booster dose (fig 124). This might be due to the direct trigger of B lymphocytes and no immunological memory is effected. In this research work the IgE level was measured in order to ascertain the hypersensitivity of HBsAg encapsulated polymeric microspheres. However, the microparticulate vaccine was inducing lesser amount of IgE but dummy microspheres did not elicit significant IgE level. Moreover, the total system did not produce any skin rash at the site of the injection. From this, it is understood that all polymeric microspheres were highly biocompatible in the biological system. Antigen release profiles from parenteral vaccine delivery systems are matter of dispute. Although some immunologists insist on a truly pulsatile release profile, characterized
by defined, short lasting intervals of antigen release, followed by absolutely no release at all, to avoid immunological tolerance to the antigen, others claim that a continuous exposure to a small amount of antigen may be beneficial in terms of maximizing the immune response (Kissel and Koncberg, 1996). Singh et al (1991) demonstrated that continuous release of DT from PLA microspheres led to antibody titres in mice comparable with those achieved by the conventional injection therapy, using three subcutaneous injections, on 0, 30 and 60, of a DT adsorbate vaccine. The antibody titres increased after one injection of DT microspheres up to 70 days and then steadily declined, up to day 240 (Singh et al, 1992). In contrast to the above study in this research work all polymeric microspheres elicited good immune response even better than conventional HBsAg vaccine for about 120 days in the present study. Raghuvanshi et al (1993) encapsulated TT in microspheres using PLGA (65: 35; Mw 75, 000) and investigated both the in vitro release and in vivo antibody response in rats. Under in vitro conditions, an initial burst of TT of about 12% was followed by a continuous release of ELISA reactive antigen for 60 days. Therefore, the release of antigen was bi phasic in vitro but in vivo no such biphasic release could be seen. Similar type of release pattern was observed in HBsAg encapsulated PLGA, PLA and chitosan microspheres both in vitro and in vivo. In both the studies of Singh et al, (1991&1992) and Raghuvanshi et al, (1993) the antigen was released in continuous fashion from PLA and PLGA microspheres under in vitro conditions, leading to the induction of IgG antibodies against TT and DT.

Both the activation of T-cells and B-cells are very important for effective immunogenic reactions because in a complex way they interact with one another, either directly or through interleukins. The helper T (T_h) cells are the principal orchestrators of the immune response because they are needed for the activation of the
two other lymphoid effector cell types: cytotoxic T (T\(_C\)) cells and antibody secreting plasma cells. The effector T cells are differentiated into two called Th\(_1\) and Th\(_2\). The Th\(_2\) cells initiate the humoral immune response by activating native antigen specific B cells to produce IgM antibodies. The Th\(_2\) cells can subsequently stimulate the production of different isotypes, including IgA, IgE, as well as neutralizing and weakly opsonizing sub types of IgG. Th\(_1\) cell activation occurs early in an immune response and requires at least two signals. One signal is provided by binding of the T cell antigen receptor to the antigenic peptide – MHC complex on the APC surface and is transmitted through the CD3 protein complex. The second, costimulatory signal also require close contact between APC and Th\(_1\) cell surface and is usually delivered by the Th\(_1\) cell protein called CD28 when it binds to either one of a pair of B7 proteins on the APC surface. Microspheres are capable of forming antigen depots from which the antigen is slowly released at the injection site. It is now known that microspheres enhance the immune response to antigen by several mechanisms in addition to the depot effect. For example, microspheres are capable of providing enhanced antigen processing through their ability to target phagocytosis by professional antigen presenting cells (APCs). Microspheres less than 10 microns in diameter are readily phagocytosed by macrophages the primary APCs in the body, leading to direct intracellular delivery of antigen for processing by the major histocompatibility complex (MHC) class II pathway (exogenous antigen). Recently, it has also been shown that the encapsulation of antigen within particulates (Ying Men et al., 1995, Anne Moore et al., 1995 and Douglas Nixon et al., 1996) or on their surface (Kovacs-Sovics-Bankowski, 1993; Falo, 1995 and Rock, 1996) can lead to antigen presentation by the MHC class I pathway (endogenous antigen) as well. Presentation of antigen by MHC class II molecules generally leads to enhanced antibody
production (i.e., the induction of a humoral immune response), whereas antigen presentation by MHC class I molecules primes for a cytotoxic T lymphomencyte (CTL)-mediated immune response. A humoral immune response is generally effective for protection from blood-borne pathogens and toxins, while a cellular immune response is thought necessary for the eradication of infected or altered cells of the body, as is the case with cancerous or virus-infected cells. However, in the case of vaccines the effective immunogenic response can be obtained by the activation of T cells and B cells. The Th cells are attracted and activated by two signals:

1. Binding of the T cell antigen receptor to MHC complex
2. Production of interleukins -1 by the APC

The activated Th cells generate a complex cascade and releases lymphokines, which stimulates B lymphocytes to proliferate and produce specific antibodies. Moreover, it also activates cytotoxic T lymphocytes, granulocytes, macrophages and natural killer cells. Moreover, some long-lived Th cells seem to provide a memory function in the T cell compartment of the immune system. The release of antigen from microspheres is controlled by a variety of factors such as morphology and size of microspheres. Shi et al., (2002) reported that HBsAg loaded PLGA microspheres 25 – 50μm in size did not induce a significant immune response after a single administration with a dose of 12 μg of HBsAg but a single injection of 3μg of HBsAg aluminum vaccine plus 9μg of HBsAg PLGA microspheres did induce a response. They suggested that the priming the mice with HBsAg aluminum vaccine might lead to a greater initial response than immunization with the microspheres alone. Gupta et al., (1997) and Eldridge et al., (1991) investigated that the smaller particles less than 10 μm can easily phagocytozed and transported by phagocytic APCs into the draining lymph nodes for rapid antigen release, inducing rapid antibody response, while larger...
particles more than 30 μm are too large for phagocytosis so they remain at the injection site (depot) and have the effect of continuously stimulating the immune system. In this research work, the size of all the microspheres was not less than 30μm except PLA with dextran as cross linker. Eventhough the size of dextran crosslinked PLA microspheres was 23 to 24μm, which induces a robust immune response for longer duration. It is note worthy from this research work that all polymeric microspheres elicited satisfactory antibody level than the required amount for protection as a single dose for about 120 days. Among the various polymeric microspheres screened in this work, the chitosan microspheres were found to be better adjuvant for hepatitis B vaccine since it induced robust immune response after a single step immunization when compared to alum adsorbed hepatitis B vaccine. Moreover, the chitosan is non toxic, highly biocompatible, biodegradable and inexpensive when compared to PLGA and PLA polymer. Therefore, it is possible to modify the immunization schedule of hepatitis B vaccine by encapsulating in to chitosan microspheres to save the people from hepatitis B after single step immunization.