General Discussion and Conclusion
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Hepatitis B is an acute systemic infection, which turn into a major public health problem all over the world. Hepatitis B virus infection can lead to fulminant hepatic failure (FHF), which threatens the life of affected patients. Hepatitis B vaccine has now been used extensively throughout the world and is currently being incorporated into the Expanded Programme on Immunization of the World Health Organization. New information, vaccines, and technology will have implications for this effort, and adjustments and changes are expected to occur over the years.

The development of new vaccines, both more efficacious and easier to deliver, has become an area of research that can certainly benefit from recent controlled release technology. In particular, the conversion of multiple dose vaccines in to single dose vaccines may represent an important advancement towards the betterment of human health care and welfare. The development of vaccine delivery systems prepared from biodegradable polymers has received considerable attention for past two decades. Vaccine research is often focused on the identification and application of novel antigens. The immune response to these antigens is routinely optimized by assessing the dose and number of injections. Due to the advances in biotechnology, many future vaccines will be peptides or proteins made by recombinant DNA technology. Subunit vaccines are poorly immunogenic and therefore required several boosters with standard adjuvant. Currently, alum is the only adjuvant that is approved for clinical use. The use of alum type adjuvant for immunization, however, has several disadvantages because it induces inflammation and stimulates the local production of granuloma. In addition alum is not a universal adjuvant as it is not suitable for small peptides, recombinant proteins and alum cannot be frozen or lyophilized. Conventional alum type vaccines require multiple recall injections at
appropriately timed intervals in order to achieve long lasting and optimal immune response. However, it is very difficult, especially in developing countries, to maintain a high reimmunization rate in the case of multiple administration immunization programs. Therefore, development of more efficient and safe adjuvant / vaccine delivery systems requiring single administration to obtain high and long lasting immune responses is of primary importance. This research work is mainly focused towards the formulation of biodegradable microspheres as sustained release carrier system for hepatitis B vaccine. Totally 5 different polymers were screened in order to establish the exact carrier system for hepatitis B vaccine. PLGA and PLA microspheres were successfully formulated using solvent evaporation technique; chitosan, albumin and dextran microspheres were successfully formulated by emulsion cross linking technique. However, in these work two types of crosslinker, viz glutaraldehyde and dextran; 3 grades of chitosan polymer of 50, 150 and 300 cps were screened. 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 this study, in the case of polyester polymers, it is found that 2% w/v of glutaraldehyde or 2% v/v of dextran was ideal concentration to fabricate stable microspheres. The microencapsulation of hepatitis B vaccine was achieved only in PLGA, PLA and chitosan polymer. Besides, albumin and dextran failed to encapsulate HBsAg. The amount of vaccine loading in to the microspheres with respect to total amount of vaccine incorporated to prepare the microspheres was greatly influenced by the
polymer and cross linking agent concentrations. The encapsulation efficiency was determined either by quantification of total protein or antigenically active protein. Among 4 methods employed, centrifugation method was very ideal when compared to the rest. It was concluded that the total amount of antigen encapsulated in to microspheres could not be detected efficiently by the methods of extraction, filtration and digestion. In the present research work, the data revealed that the most accurate method of determining antigenically active protein was the centrifugation method. The FT IR spectrum of vaccine loaded and unloaded microspheres were shown marked differences and proved the presence of specific groups. The water uptake of microparticles after 24hrs incubation leads to burst effect. The weight and size of the microspheres were increased even after drying the microspheres. However, it is very obvious that water absorption is less when glutaraldehyde was used as cross linking agent rather than dextran as cross linking agent.

In PLGA polymeric system, the average 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 average 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 mean weight of glutaraldehyde cross linked chitosan microspheres was increased to 22 mg with an increased in size of 4 μm and the mean weight of dextran cross linked chitosan microspheres was increased to 25 mg with an increased in size of about 6 μm. 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.

The stability study was ascertained by observing the size and vaccine loading of polymeric microspheres. 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. 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. In the case of glutaraldehyde or dextran cross linked PLGA microspheres drastic loss of vaccine could be observed
at 50°C. 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. Chitosan microspheres either with glutaraldehyde or with dextran as cross linker showed better carrier system for vaccine due to its 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. 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 extend 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. Moreover, among all polymeric system chitosan microspheres were more stable when compared to the rest and dextran was considered as a better cross linker when compared to glutaraldehyde. In the case of in vitro release profile of hepatitis B vaccine encapsulated PLGA (glutaraldehyde cross linked) & PLGA (dextran cross linked) microspheres, it is very obvious that the cumulative percentage release of hepatitis B vaccine was found to be much sustained with an initial burst effect. The peak antigen release was observed on 70th day almost 50 % of vaccine was released. It is understood that after attaining peak antigen release, the release was prolonged and sustained for about 105 days (15 weeks). Observation made on the release pattern of PLGA microspheres, the release was more in the case of dextran as cross linker and the peak antigen release was on 77th day almost 56 % of vaccine was released. The hepatitis B vaccine release was more irregular and sustained for a period of 45 days. The peak antigen release was observed on 63rd day in both PLA (glutaraldehyde cross linked) and PLA (dextran cross linked) microspheres and almost 50% vaccine was released. In the case of PLA polymeric system the release patterns in both the cases were more or less same. The release pattern was sustained over a period of 105 days. In chitosan microparticulate system the peak antigen release observed on 84th day (12 weeks) in both the cases and the level was mainained for about 105 days by chitosan (glutaraldehyde cross linked) & chitosan (dextran cross linked) microspheres. It is understood that the antigen was released with initial burst effect almost 12 % percent vaccine was released during first
week of *in vitro* release study. However, the initial burst effect is quite less when chitosan microspheres were formulated with dextran as cross linker. Hepatitis B vaccine was released in sustained manner from either glutaraldehyde cross linked microspheres or dextran cross linked chitosan microspheres. 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 was the most successful polymeric system either glutaraldehyde or dextran as cross linker to encapsulate HBsAg. 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. Moreover, in all polymeric microparticulate system, the release pattern varied with the type of cross linker used, concentrations of cross linker and polymer.

Among two cross linker screened, dextran was found to be a better cross linker when compared to glutaraldehyde because dextran cross linked microspheres were smaller in size and more stable. It released antigen in much sustained manner for longer duration and universally it was less toxic when compared to glutaraldehyde. Therefore, dextran cross linked microspheres was preferred for immunogenicity study. The immune response was determined by measuring specific antibodies and immunoglobulin levels. Totally 15 groups were segregated containing 6 animals per
each group, immunized with different combinations of vaccine preparations and the maximum duration was predetermined for 120 days. 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 anti body 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 was found to be 10 IU/1 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 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 induce a robust immune response for longer duration. It is noteworthy 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. The development of single contact hepatitis B vaccine based on chitosan polymer is very important advancement towards the betterment of human health care.