CHAPTER 8

SUMMARY AND CONCLUSIONS

Nanoparticles have been highly exploited for controlled and site – specific delivery of drugs. The site specific delivery of drugs by them has shown promising results for the treatment of various diseases including cancer and human immunodeficiency virus infection. As compared with the bulk material, the formulated nanoparticles have a high surface /volume ratio and thereby result in a reduction in the dose as well as the frequency of administration of the drug with a consequent increase in the patient compliance.

Diseases of the Central Nervous System (CNS) such as schizophrenia, meningitis, migraine, Parkinson's disease and Alzheimer's disease require delivery of the drug to the brain for treatment. However such transport remains problematic, especially for hydrophilic drugs and large molecular weight drugs, due to the impervious nature of the endothelial membrane separating the systemic circulation and central interstitial fluid, the Blood–Brain Barrier (BBB).

Although a challenge, it is possible to transport of exogenous materials directly from nose-to-brain is a potential route for by-passing the BBB. This route, involves the olfactory or trigeminal nerve systems which initiate in the brain and terminate in the nasal cavity at the olfactory neuroepithelium or respiratory epithelium, respectively. They are the only externally exposed portions of the CNS and therefore represent the most direct method of non-invasive entry into the brain. The strategy of applying drugs that are encapsulated into particulate vectors such as nanoparticles to the olfactory has been deeply studied for improvement of the direct CNS delivery of drugs—including biologics.

Alzheimer's disease is characterized by memory dysfunction, loss of lexical access, spatial and temporal disorientation and impairment of judgment. The symptomatic course of disease is generally 5 or more years; although a 7-year preclinical period of stepwise decline in memory and attention span has been described. Synapse loss also occurs and has been shown to be the best correlate of cognitive decline. This multifactorial disorder combines both genetic and non-genetic components. The major non genetic risk factors are advanced age, diabetes, obesity, trauma, or cardiovascular

diseases. The genetic mutations share common biochemical pathways that include the altered production of the amyloid $-\beta$ with an overabundance of the $A\beta_{1\text{-}42}$ fragment, the principal constituent of senile plaques. The major genetic risk factor for the sporadic form of Alzheimer's is the inheritance of the $\varepsilon 4$ allele of apolipoprotein E, a gene located on chromosome 19q13. The apolipoprotein allele can affect the rate of progression of the disease, the extent of the neuronal cell loss, cholinergic activity, and accumulation of amyloid plaques in hippocampus and cortical areas and total $A\beta$ production and deposition in the brain of Alzheimer's subjects.

During the past two decades, one of the foremost challenges in health research was to understand better the cause(s) of Alzheimer's for the development of safe and effective pharmacological treatments. Irrespective of the form of therapy, the current approaches provide only temporary symptomatic relief, improve cognitive function, but do slow the long term progression of this disorder with several side effects. Moreover, these treatments have a modest effect on the progression of Alzheimer's from mild cognitive impairment to disabling dementia and death.

Currently available treatments for Alzheimer's disease are designed to address the symptoms. A new genre of agents for the disease, are also targeting the underlying pathology, particularly with the aim of reducing amyloid load in the brain, in the hope of modifying the disease process. One of the popular approaches using the above mentioned concept is the immunotherapy approach. This involves passive immunization using monoclonal antibodies, directed at known epitopes. This would ensure the likelihood of treated patients achieving an adequate level of antibody response.

Acetylcholine esterase inhibitors are the most successful class of therapeutic agents to decrease the progression of Alzheimer's disease. Out of that, **Galantamine hydrobromide** is one such drug. It is approved by the USA FDA and the European Medicines Agency for the symptomatic treatment of Alzheimer's disease due to its ability to moderate acetylcholinesterase inhibition in the CNS. Gal is commercially available as tablets and oral suspension. However, when administered via the oral route, it leads to severe nausea and vomiting because of its motor and evacuative

function on the intestinal tissue. Additionally, recent reports have shown that Galantamine also has anti amyloid activity. But to fully exploit its potential, it has to be delivered properly and that necessitates surpassing the Blood Brain Barrier (BBB).

Bapineuzumab is an IgG1, N – terminus anti amyloid humanized monoclonal antibody. It has been proven to reduce the amyloid plaques which are in excess in the brain. This occurs by binding to fibrillar and soluble Aβ and activates microglial phagocytosis and cytokine production.

Bapineuzumab entered a phase III trial and released preliminary analysis of the phase II results. The phase II trial was a randomised, double-blind, placebo controlled trial testing three doses of a humanized Aβ antibody in 240 participants. In each of the escalating doses of the antibody, about 32 patients received active agent and 28 received placebo. Although the study did not attain statistical significance on the primary efficacy endpoints in the whole study population over the 18-month trial period, in the subgroup of participants who did not have the *APOE* ε4 allele clinically significant benefits were recorded on several scales, including the mini-mental state examination and the Alzheimer's disease assessment scale battery. Furthermore, in the same subgroup, MRI showed less loss of brain volume in treated patients than in control patients. These findings suggest that this form of therapy might be effective. However, some patients in the treatment group, but not in the control group, had vasogenic oedema, a serious adverse event.

Such an adverse reaction can be avoided by targeting the agent at the proper site of action. This could be achieved by using a nanocarrier. The advantage associated with nanosystems is that the absorption, biodistribution and elimination of drug in the body are dependent on the inherent properties of nanosystems like size, surface properties and charge.

The objective of this work was to develop nanoparticulate drug delivery platform which would be employed to carry an acetylcholine esterase inhibitor (Galantamine hydrobromide) and a monoclonal antibody (Bapineuzumab) to be administered via the nasal route.

Such an endeavour is expected not only to reduce side effects of orally administered acetylcholinesterase but also would improve its targeting to the real site of action that is the brain. In addition, this investigation would open avenues towards possibility of immunization utilizing Bapineuzumab against Alzheimer's disease via the nasal route.

Analytical methods

For Galantamine hydrobromide loaded nanoparticles, the analytical method was standardized using UV – Visible Spectroscopy and High Performance Liquid Chromatography respectively. Calibration was done using standard concentrations for both the methods. The analytical methods were developed for estimating the entrapment efficiency and for *in vivo* studies of Galantamine hydrobromide loaded nanoparticles.

For Bapineuzumab loaded the analytical method was developed using Size Exclusion Chromatography and Affinity Column Chromatography. Size Exclusion Chromatography was used to separate the modified antibody from the unmodified one, where as Affinity Column Chromatography was used to calculate the entrapment efficiency in the antibody loaded nanoparticles.

Galantamine hydrobromide loaded Nanoparticles

The Galantamine Hydrobromide loaded Nanoparticles were prepared by desolvation method using Bovine Serum Albumin. The formulation was optimized step by step. Initially preliminary optimization was conducted on the basis of particle size. Herein, the choice of desolvating agent, the rate of addition of desolvating agent, bovine Serum albumin content in the solution, centrifugation speed for collecting nanoparticles were initially optimized. The risks were identified using the Ishikawa diagram and Failure Mode and Effect Analysis. Also Quality Target Product Profile and Critically Quality Attributes were identified. The major risks that could be seen through Failure Mode and Effect Analysis were drug amount, polymer amount, rate of addition of desolvating agent, pH of aqueous agent, incubation time between drug and polymer and stirring speed. Based on the outcomes of Failure Mode, Plackett Burman design was applied with particle size and entrapment efficiency as the responses.

After the risk assessment with Plackett-Burman Design, the pH of the aqueous phase and rate of addition of desolvating agent was kept constant for optimization, since they were not as significant as incubation time for entrapment efficiency. Further optimization of the formulation was done by selecting the Box − Behnken Design. Through this design, the optimization of the formulation could be achieved. To have a meticulous estimation of various factors of the responses, the significant variables affecting the particle size and entrapment efficiency were visually presented in contour and response surface plots. The design space was also established targeting particle size lower than 140 nm and encapsulation efficiency higher than 75%.

The optimization of the drug loaded nanoprticles were characterized Differential Scanning Calorimetry, X − Ray Diffraction, zeta potential, Transmission Electron Microscopy and Atomic Force Microscopy. Pure Galantamine hydrobromide showed a sharp exothermic peak at 280 °C, representing its melting point as well as confirming its crystalline nature. The physical mixture of BSA and Galantamine hydrobromide showed characteristic peaks of both BSA and Galantamine hydrobromide. The DSC of Galantamine hydrobromide did not show any peak at the melting point of Galantamine hydrobromide demonstrating its encapsulation into polymer matrix and also the possibility of it being in amorphous state. The diffraction pattern of Galantamine hydrobromide nanoparticles showed diminished peaks of the drug. A negative zeta potential was observed for Galantamine hydrobromide nanoparticles, ranging from -13 mV± 0.25 to -11 mV±0.32. Transmission electron microscopy showed that the Galantamine hydrobromide nanoparticles were spherical, uniform in size and had size less than 200 nm.

In vitro drug release studies from plain drug solution and Galantamine hydrobromide loaded nanoparticles is shown in. The plain drug solution released completely from the dialysis membrane in 1h. On the other hand, 99.67 ± 4.78% Galantamine hydrobromide was released in 8 h .The drug release from nanoparticles followed the biphasic model, with an initial burst attributed to the drug-associated near-particle

surface which may have got desorbed upon contact with the dissolution medium The drug release from nanoparticles was slow and sustained.

For *ex vivo* studies the drug release from plain drug solution was faster. Complete drug release was achieved in 2 hour. The nasal mucosa was a thicker barrier through which the drug molecules had to pass, and hence it became a rate limiting step. Therefore, the drug release from nanoparticles did not show a burst release in *ex vivo* diffusion. For the nanoparticles, 98.12 ± 3.18 % drug was released in 8 h.

Histopathology of nasal tissue of rat was done after keeping it in contact with Galantamine hydrobromide nanoparticles. The rat nasal tissue after its contact with Galantamine hydrobromide nanoparticles did not show any damage to cilia and epithelial cells of the nasal mucosa. There was no evidence of cellular necrosis. The epithelial cells were also intact and not uprooted from the mucosal base. Thus the formulation was found to be safe and non – toxic for the nasal mucosa.

Nanoparticles have high therapeutic benefits but also the potentially unpredictable and adverse consequences of human exposure thereto. For nanoparticles, the administered dose, route of administration and extent of tissue distribution are important parameters in nano-cytotoxicity. The MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is one of the most commonly used techniques to evaluate cytotoxicity of nanoparticles. In the MTT assay, a significant ($p<0.05$) reduction in the enzymatic activity of succinate dehydrogenase was observed upto 67 and 65% (relative to 100% of control) at 250 and 300 μg/ml of Galantamine hydrobromide nanoparticles exposure after 48 hr in IMR 32 cells. However, after 24 hr exposure, no significant cytotoxicity was observed. The nanoparticles demonstrated time- dependent and concentration – dependent uptake.

In comparison to control, which had no nanoparticles in contact with the cell line, the nanoparticles demonstrated time- dependent and concentration – dependent uptake.

There was a two – fold increase in uptake at 24 h for a concentration of 300 μg/mL Galantamine hydrobromide loaded nanoparticles. This observation can be extrapolated to its in vivo performance after being administered through the nasal route. The

nanoparticles after reaching the target area, for example the olfactory lobe; would be taken up by the neuronal cells and quantity of nanoparticles would increase by two fold at 24 h.

The *in – vivo* studies were conducted on male Sprague - Dawley rats. After nasal administration, C_{max} for plain drug solution in Cerebrospinal fluid was only 0.82 µg/ml, due to the inability to effectively pass the blood brain barrier. C_{max} for the nanoparticles in Cerebrospinal fluid was found to be 1.58 µg/ml, which was significantly higher than the plain drug solution ($p < 0.01$). The AUC $_{0-\infty}$ for plain drug solution was 3.06 µg/mL*h whereas for nanoparticles it was 6.28 µg/mL*h. Hence there was a twofold improvement in CNS bioavailability of Galantamine after nasal delivery ($p < 0.01$). This could be attributed to various transport mechanisms that are widely reported for direct nose to brain delivery. Also, the Drug Targeting Index (DTI) was found to be 6.91 whereas the Direct Brain Transport (DBT) percentage was found to be 84.87%. It is assumed that if drug concentration in the brain is significantly higher after intranasal administration , or DTI >1, a direct pathway from the nasal olfactory region to the brain exists. The higher the DTI is, the better the drug targeting to brain can be expected after intranasal administration.

Therefore, a brain targeted nasal nanoparticulate formulation was developed for Galantamine hydrobromide. This novel formulation would aid in surpassing the adverse effects, improve patient acceptance and target the real site by circumventing the Blood Brain Barrier.

Bapineuzumab loaded nanoparticles

The antibody was linked to blank Bovine Serum Albumin by bioconjugation. The antibody was firstly modified by introduction of thiol groups using 2 – imminothiolane. To optimize the amount of 2 – iminothiolane and the reaction time for modification of antibody, studies were carried out with 50 – fold molar excess of 2 - iminothiolane and 100 – fold of 2 - iminothiolane molar excess for 2 and 5 hour respectively for each. A 50-fold molar excess of 2 – iminothiolane with an incubation period of 2 hours was set for modification of antibody. The modified antibody was

separated from the unmodified antibody by size exclusion chromatography using gravity column. This was followed by calculation of thiol groups attached to the modified antibody using Ellman's reagent. Thiol quantification showed thiolated antibodies had 12 – 15 times more thiol group in comparison to unmodified antibody. Therefore these additional thiol groups can get involved in bond formation with the surface modified Bovine Serum Albumin through the cross linker. For linking the modified antibody to nanoparticles, blank Bovine Serum Albumin nanoparticles were prepared. Its surface was modified using maliemide-ω-ester. To this modified antibody was added and was allowed to incubate. After incubation, the nanoparticles were separated by centrifugation. The antibody linked nanoparticles were characterized with respect to particle size, zeta potential, entrapment efficiency, Transmission Electron Microscopy, Atomic Force Microscopy, Sodium DodecylSulphate PolyAcrylamide Gel Electrophoresis (SDA – PAGE) and Indirect Enzyme Linked Immunosorbent Assay (ELISA).

69.89% ± 2.45 % of thiolated antibody got bound to the surface modified bovine serum albumin nanoparticles.

The particle size of actively loaded nanoparticles was found to be 110.45 ± 6.21 nm with a polydispersity index of 0.11 ± 0.02.

For Atomic Force Microscopy, the surface topography of blank nanoparticles was smooth with a particle size range of 98.12nm to 105.47 nm and a particle height of 4.6nm to 5.8 nm. Bapineuzumab loaded nanoparticles showed a rough surface, with undulation throughout indicating the presence of antibody on the surface of nanoparticles. The particle size was found to be in the range of 118.12 nm to 131.01 nm with a particle height of 12.76 nm to 15.42 nm.

SDS – PAGE gel was viewed utilizing Gel Doc XR+ Imager (Bio Rad, India) using the Image Lab Software. Gel showed the presence of antibody in the nanoparticles with a typical band at 25 kD and approximately 50kD respectively. Results of Indirect ELISA proved the efficacy of antibody loaded nanoparticles. The antibody attached to the surface of nanoparticles showed binding to the coated antigen. This was calculated against a standard curve obtained through varying concentration of the antibody.

Therefore the integrity of the antibody was maintained with its N – terminal being free to bind on the epitope of antigen (amyloid peptide).

Bapineuzumab loaded nanoparticles when administered intranasally to reduced the amyloid load significantly (p < 0.01) in comparison to intravenous administration. The data recorded for intranasal administration was significantly higher (p < 0.01) than what was achieved for intravenous administration. The amyloid reduction after intra nasal administration relative to sham control group was 139 % on the first day which progressively increased to 219 % on the 7th day and, 309.85% on the 14th day. This indicated towards Bapineuzumab's ability to deplete amyloid from the affected site in the brain. The antibody being an anti amyloid antibody, its repeated administration increased the antibody generation in the animals leading to decreased amyloid load. This mechanism forms the basis of passive immunization. Additonally, the active immunization techniques and its effect on amyloid deposition can be mimicked by passive administration of anti – amyloid antibodies.

Therefore, nasal administration of Bapineuzumab loaded nanopaticles open up possibilities of passive immunization, by promising delivery of the antibody to the actual target that is brain.

To sum up, the work would contribute towards improving the entire line of treatment for Alzheimer's disease. With accurate targeting, the drug or the antibody would reach the site of action without adverse effects. The total cost of patient care would reduce, which is the significant part in Alzheimer's disease management. However, proper and elaborate toxicological studies, preclinical studies followed by clinical studies on human subjects would be required to make this project into a commercially viable venture.