CHAPTER 2

LITERATURE REVIEW

2.1 Nanoparticles:

Nanotechnology is a rapidly expanding field, encompassing the development of manmade materials in the 5–200 nanometer size range. This dimension vastly exceeds that of standard organic molecules, but its lower range approaches that of many proteins and biological macromolecules. The first practical applications of nanotechnology can be traced to advances in communications, engineering, physics, chemistry, biology, robotics, and medicine. Nanotechnology has been utilized in medicine for therapeutic drug delivery and the development of treatments for a variety of diseases and disorders. The rise of nanomaterials correlates with further advances in these disciplines. Nanoparticles appeal to scientists across many disciplines due to the opportunity to engineer many properties that might otherwise be incompatible on a single device. Relevant attachments include biologically active molecules, targeting sequences, fluorescent or other imaging devices, biocompatible coatings, and others. Furthermore, the engineering of the particle backbone structure and the size and shape of the nanoparticle core provides yet another dimension of physical control that can be exerted toward the specific tailoring of function (Faraji & Wipf, 2009).

In recent years, medical therapies have become more tailored to specific diseases and patients in recent years. Most pharmaceutical agents have primary targets within cells and tissues; ideally, these agents may be preferentially delivered to these sites of action within the cell. Selective subcellular delivery is likely to have greater therapeutic benefits. Cytosolic delivery, for instance, is desirable for drugs that undergo extensive exportation from the cell via efflux transporters such as multi-drug resistance proteins and P-glycoproteins (Panyam J, 2004).

2.2 Polymeric Nanoparticles:

Polymeric nanoparticles have the advantage of being biodegradable and biocompatible and hence suitable for drug delivery. They have a good capability of entrapping a wide range of therapeutics having varying physicochemical properties. Pertinent nanoparticle formulations include those made from gelatin, chitosan, poly(lactic-co-glycolic acid) copolymer, polylactic acid, polyglycolic acid,

poly(methylmethacrylate), and poly(butyl)cyanoacrylate. Furthermore, polymer-based coatings may be functionalized and developed into other types of nanoparticles to change and improve their biodistribution properties. The biologically inert polymer, poly(ethylene glycol) (PEG) has been covalently linked onto the surface of nanoparticles (Alyautdin et al., 1997; Kreuter et al., 2003).

Additionally, polymeric nanoparticles have been formulated for gene therapy to target breast cancer cells, resulting in anti proliferative effects (Prabha & Labhasetwar, 2004b).In such cases, the polymer matrix prevents drug degradation and may also provide management of drug release from these nanoparticles. Varying the drug-to-polymer ratio and molecular weight and composition of the polymer can modify the extent and level of drug release (Prabha & Labhasetwar, 2004a). The surface properties of these polymeric nanoparticles are also a vital component of their targeting characteristics. Since nanoparticles come into direct contact with cellular membranes, their surface properties may determine the mechanism of internalization and intracellular localization (Murakami, Kobayashi, Takeuchi, & Kawashima, 1999).

The general biocompatibility and biodegradation profiles of polymeric nanoparticles are attractive; this is especially true with formulations that require more chronic dosing, perhaps in contrast to many inorganic nanoparticles. Practically, large-scale production and manufacturing remains an issue with polymeric nanoparticles. For instance, PLGA nanoparticles are mostly formulated using a double emulsion solvent evaporation system, utilizing water and oil with poly(vinyl alcohol) (PVA) as an emulsifier.

2.2.1 Methods of Preparation of Polymeric Nanoparticles

Nanoparticles can be prepared from a variety of materials such as proteins, polysaccharides and synthetic polymers. The selection of matrix materials is dependent on many factors including: (a) size of nanoparticles required; (b) inherent properties of the drug, e.g., aqueous solubility and stability; (c) surface characteristics

such as charge and permeability; (d) degree of biodegradability, biocompatibility and toxicity (Kreuter, 1994).

2.2.1.1 Nanoparticles Prepared by Polymerization Process of Monomers:

In this method, monomers are polymerized to form nanoparticles in an aqueous solution. Drug is incorporated either by being dissolved in the polymerization medium or by adsorption onto the nanoparticles after polymerization completed. The Nanoparticle suspension is then purified to remove various stabilizers and surfactants employed for polymerization by ultracentrifugation and re-suspending the particles in an isotonic surfactant-free medium. Nanocapsules formation and their particle size depend on the concentration of the surfactants and stabilizers used. Two types of polymerization processes have been adopted to prepare polymeric nanoparticles (Chen, 2006; R. A. Jain, 2000) .

a) Dispersion Polymerization: Dispersion polymerization starts with monomer, an initiator, solvent in which the formed polymer is insoluble, and a polymeric stabilizer. Polymer forms in the continuous phase and precipitates into a new particle phase which is stabilized by the polymeric stabilizer. Small particles are formed by aggregation of growing polymer chains precipitating from the continuous phase as these chains exceed a critical chain length. Coalescence of these precursor particles with themselves and with their aggregates results in the formation of stable colloidal particles, which occurs when sufficient stabilizer covers the particles.

b) Emulsion Polymerization: In this technique the monomer is emulsified in nonsolvent containing surfactant, which leads to the formation of monomer swollen micelles and stabilized monomer droplets. The polymerization is performed in the presence of initiator. Emulsion polymerization may be performed using either organic or aqueous media as continuous phase. Poly (methyl methacrylate), poly (alkyl cyanoacrylate), acrylic copolymer, polystyrene, poly (vinyl pyridine) and polyacrolen nanoparticles are prepared by emulsion polymerization technique.

2.2.1.2 Nanoparticles Prepared from Dispersion of Preformed Polymers:

Several techniques have been suggested to prepare the biodegradable polymeric nanoparticles from preformed polymers such as poly (D,L-lactide) (PLA), poly (D,L-glycolide) (PLG) and poly (D,L-lactide-co-glycolide) (PLGA) (Kompella UB, 2001)1(Kompella et al, 2001, Ravikumar et al; 2004). The basic methodologies of the commonly used preparation methods are as follows:

a) Emulsion/evaporation

This is one of the most frequently used methods. The preformed polymer and drug are first dissolved in a water-immiscible organic solvent, which is then emulsified in an aqueous solution containing stabilizer. The emulsification is brought about by subsequent exposure to a high-energy source such as an ultrasonic device, homogenizer, or colloid mill. The organic phase is evaporated under reduced pressure or vacuum, resulting in the formation of the aqueous dispersion of nanoparticles. The nanoparticles are collected by ultracentrifugation and washed with distilled water to remove stabilizer residues or any free drug and lyophilized for storage (Guarrero et al 1998; Song; 1997). Modification of this method, known as high-pressure emulsification solvent evaporation (HPESE), has been reported by (Jaiswal et al; 2004) This method involves preparation of a coarse emulsion, which is then subjected to homogenization under high-pressure followed by overnight stirring to remove organic solvent. This method has the advantage of obtaining small, monodispersed nanoparticles with high encapsulation efficiency and reproducibility. The emulsion evaporation method can be used for preparation of particles with sizes varying from a few nanometers to micrometers by controlling the stirring rates and conditions (Ubrich; 2004). A diagrammatic representation of this method is shown in Fig. 2.5

b) Double emulsion process

The emulsion evaporation method suffers from the limitation of poor entrapment of hydrophilic drugs because of their diffusion and partitioning from the dispersed oil phase into the aqueous continuous phase. Therefore, to encapsulate hydrophilic drugs and proteins, the double-emulsion technique is employed, which involves the addition of aqueous drug solution to organic polymer solution under vigorous stirring to from a w/o emulsion. This w/o emulsion is added into second aqueous phase containing more

stabilizers with stirring to form the w/o/w emulsion. The emulsion is then subjected to solvent removal by evaporation (Vandervoort et al; 2002). A number of hydrophilic drugs like the peptide leuprolide acetate, a lutenizing hormone-releasing agonist, vaccines, proteins/peptides and conventional molecules have been successfully encapsulated by this method. After evaporation of organic solvent under reduced pressure, the polymer precipitates and nanoparticles can be isolated by centrifugation at high speed. The formed nanoparticles must be thoroughly washed before lyophilization (Jain; 2000). A diagrammatic representation of this method is shown in Fig. 2.6.

c) Salting - out

This technique involves the addition of polymer and drug solution in a slightly water miscible solvent such as acetone to an aqueous solution containing the salting out agent and a colloidal stabilizer under vigorous mechanical stirring. When this o/w emulsion is diluted with a sufficient volume of water, it induces the formation of Nanoparticles by enhancing the diffusion of acetone into the aqueous phase. The remaining solvent and salting-out agent are eliminated by cross-flow filtration (Allemann et al; 1998). Several manufacturing parameters can be varied including stirring rate, internal/external phase ratio, concentration of polymer in the organic phase, type of electrolyte, concentration, and type of stabilizer in the aqueous phase. By considering the entrapment efficiency of nanoparticles, this method is most suitable for water insoluble drugs. Salt permeate biological systems and are crucial for life. However salts also affect the stability of proteins. It has been reported since many years that neutral salts perturb various protein structures in ways that go well beyond simple, non-specific charge effects (Doming et al;2002).

d) Emulsification - diffusion

This is another widely used method involving polymer solution in partially water miscible solvent (such as ethyl acetate, benzyl alcohol, propylene carbonate) presaturated with water, added to an aqueous solution containing stabilizer under vigorous stirring. The subsequent addition of water to the system destabilizes the equilibrium between the two phases and causes the solvent to diffuse into the external

phase, resulting in reduction of the interfacial tension and in nanoparticle formation, which gradually becomes poorer in solvent.

Although this method is a modification of the salting out procedure, it provides the advantage of avoiding the use of salts and thus eliminates the need for intensive purification steps. While this method also suffers from low entrapment efficiency of hydrophilic drugs in nanoparticles, incorporation of medium chain glyceride into aqueous solution has been found to improve the encapsulation efficiency of water soluble drugs into nanospheres offering the advantage of simplicity, narrow particle size distribution, and ready dispersibility of the resultant particles (Jain; 2000).

e) Nanoprecipitation

In nanoprecipitation, introduced by Fessi and co-workers (Fessi et al; 1995), the particle formation is based on precipitation and subsequent solidification of the polymer at the interface of a solvent and a non-solvent. Thus, the process is often called solvent displacement or interfacial deposition. This method is usually employed to incorporate lipophilic drugs into the carriers based on the interfacial deposition of a polymer following displacement of a semi-polar solvent miscible with water from a lipophilic solution (Molpeceres et al; 1996, Barichello et al; 1999)

The polymer is dissolved in a water miscible organic solvent (or solvent mixture) and added to an aqueous solution, in which the organic solvent diffuses (Fig. 2.7). Particle formation is spontaneous, because the polymer precipitates in the aqueous environment. According to the current opinion, the Marangoni effect is considered to explain the process: solvent flow, diffusion and surface tensions at the interface of the organic solvent and the aqueous phase cause turbulences, which form small droplets containing the polymer. Subsequently, as the solvent diffuses out from the droplets, the polymer precipitates. Finally, the organic solvent is typically evaporated with the help of a vacuum.

The injection rate of the organic phase into the aqueous phase affects the particle size. It was observed that a decrease occurs in both particle size and drug entrapment as the rate of mixing of the two phases increase. This method gave relatively narrow particle size distribution for different formulations evaluated. The drug loading efficiency was found to be lower for the hydrophilic drugs than lipophilic drugs because of their poor

interaction with the polymer leading to diffusion of the drug, from the polymer in the organic phase, to the external aqueous environment, although exceptions were found, as seen in case of proteins and peptides Govender et al (1999) showed improved incorporation of the water-soluble drug, procaine hydrochloride, into PLGA nanoparticles by increasing the aqueous phase pH and replacing procaine hydrochloride by procaine dehydrate base. The difficulty faced in this preparation technique is the choice of drug/polymer/solvent/nonsolvent system in which the nanoparticles would be formed and the drug efficiently entrapped.

f) Emulsion-diffusion-evaporation

Employing the emulsion-diffusion-evaporation method, Nanoparticles are prepared by dissolving PLGA in ethyl acetate at room temperature. The organic phase is then added to an aqueous stabilizer mixture containing PVA and chitosan in water under stirring.

The emulsion is stirred at room temperature for 3 hours before homogenizing for 10 minutes.

To this emulsion, water is added under stirring, resulting in Nanoprecipitation (Ravikumar et al; 2004). Stirring is continued in a water bath maintained at 40 °C to remove organic solvent. Stirring causes the dispersion of the solvent as irregularly sized globules in equilibrium with the continuous phase, and the stabilizer is then adsorbed on the larger interface created. Homogenization further results in smaller globules. Addition of the water and heating step destabilizes the equilibrium and causes the diffusion of organic solvents to the external surface.

2.2.2 Characterization of Nanoparticles

The unique qualities and performance of nanoparticulate systems as device for drug delivery arises directly from their physicochemical properties. Hence, determining such characteristics is essential in achieving the mechanistic understanding of their behaviour. A good understanding allows prediction of *in vivo* performance as well as allowing particle designing, formulation development and process troubleshooting to be carried out in a rational fashion. After preparation, nanoparticles are characterized at two levels. The physicochemical characterization consists of the evaluation of the

particle size, size distribution, and surface properties (composition, charge, hydrophobicity) of the nanoparticles. The biopharmaceutical characterization includes measurements of drug encapsulation, in *vitro* drug release rates, and *in vivo* studies revealing biodistribution, bioavailability, and efficacy of the drug. Nanoparticles are generally characterized for the following parameters:

Particle size

Surface charge (Zeta potential)

Crystalline state

Surface morphology

Drug release studies

Stability

2.2.2.1 Particle size

The most basic and important property of any nanoparticulate system is its size. The saturation solubility, dissolution velocity, physical stability and even biological performance of these systems depend on their particle size. Saturation solubility and dissolution velocity showed considerable variation with change in particle size of the drug(Müller & Peters, 1998) . The most frequently used techniques for particle size measurement of nanosized systems are dynamic light scattering techniques, static light scattering techniques and microscopy. Each method has its own advantages as well as disadvantages. The mean size and width of distribution (polydispersity index) is typically determined by photon correlation spectroscopy (PCS). This technique can be used for rapid and accurate determination of the mean particle diameter of nanoparticles (B. W. Muller & Muller, 1984). It records the variation in the intensity of scattered light on the microsecond time scale (Pecora, 2000) . The measuring range of PCS is limited to approximately 3 nm–3mm. Therefore, Laser Diffractometry (LD) is also used to detect any particles in the micrometer range or aggregates of drug nanoparticles. For nanoparticles intended for intravenous use, particle size determination by coulter counter is also essential as few particles with particle size more than 5 μm may cause problem of blockage of blood vessels. Depending on the type of equipment employed, the measuring size range is approximately 0.01–80 μm.

The instrument and the material to be analyzed are important parameters which will affect the accurate particle size measurement. The stability of the sample during analysis is the most important requisite for correct and reproducible results (Keck, 2010). Thus, all above things must be considered during selection of appropriate technique for particle size determination for a particular sample.

2.2.2.2 Surface charge (Zeta potential)

Particle charge is a stability determining parameter in nanoparticles. It is measured by electrophoresis and typically expressed as phoretic mobility [(mm/S) / (V/cm)] or zeta potential (mV). Zeta potential is used as surrogate for surface charge, and is often measured by observing the oscillations in signal that result from light scattered by particles located in an electric field. There are a number of instrumental configurations with different approaches implemented in different equipments, with mostly used Doppler shift. The zeta potential of a nanosuspension is governed by both the surfactant and the drug itself. For a physically stable nanoparticulate suspension solely stabilized by electrostatic repulsion, a zeta potential of ±30 mV is required as minimum. In case of a combined electrostatic and steric stabilization, ±20mV is sufficient as a rough guideline (R. H. Muller, Jacobs, & Kayser, 2001).

2.2.2.3 Crystalline state

Drug particles in amorphous form are likely to be generated when nanoparticles are prepared. Hence, it is essential to investigate the extent of amorphous drug particles generated during production of nanoparticles. The crystalline status of the nanosuspension can be assessed by differential scanning calorimetry (DSC) (Müller & Peters, 1998). This is particularly very important when the drug exhibits polymorphic forms. The changes in the physical state of the drug particles as well as extent of amorphous fraction can be determined by X-ray diffraction analysis(R. H. Muller, Grau, M. J., 1998) and can be supplemented by DSC studies. The assessment of the crystalline state and particle morphology together helps in understanding the polymorphic and morphological changes that a drug undergoes when subjected to nanosizing.

2.2.2.4 Surface morphology

Nanoparticles can be directly observed by Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) with the former method being better for morphological examinations (Molpeceres, Aberturas, & Guzman, 2000). TEM has a smaller size limit of detection and provides structural information via electron diffraction, but staining is usually required. Researchers must be cognizant of the statistically small sample size and the effect of applied vacuum on the particles during analysis. Very detailed images can be obtained from freeze fracture approach in which a cast is made of the original sample (Mosqueira et al., 2001). Sample corruption resulting from the extensive sample preparation is always a possibility, though lower vacuum instrumentation reduces this manipulation, albeit at the loss of some resolution(Nizri, Magdassi, Schmidt, Cohen, & Talmon, 2004) . Atomic force microscopy (AFM) microscopy can also be used to confirm the size and shape of nanosized particles. AFM is capable of scanning the surfaces in controlled environmental conditions and is a complementary to SEM imaging (Moribe, Limwikrant, Higashi, & Yamamoto, 2012).

2.2.2.5 Drug release studies (*In – vitro* studies) (D'Souza & DeLuca, 2006)

In vitro release studies are generally performed to accomplish one or more of the following aims:

1. As an indirect measurement of drug availability, especially in preliminary stages of product development.
2. Quality control to support batch release and to comply with specifications of batches

proven to be clinically and biologically effective.

3. Assess formulation factors and manufacturing methods that are likely to influence bioavailability
4. Substantiation of label claim of the product
5. As a compendial requirement

Currently, research is focused on shortening the time span of *in vitro* release experiments with the aim of providing a quick and reliable method for assessing and

predicting drug release. For commercial dosage forms that release drug for 30 to 90 days or even longer, accelerated or short-term release provides the potential for conducting an *in vitro* release test in a matter of days rather than months. Release testing of these dosage forms at 37ºC would require the addition of preservatives and impose certain limitations on the *in vitro* method, such as stability and compatibility of the components of the release device, like tubings and membranes. Therefore, a short term release test might even be more reliable for quality-control purposes. In addition, short term studies can provide a rapid assessment of formulation and processing variables that affect drug release from the delivery system, especially in the developmental stage. These short-term studies can be performed by accelerating one or more conditions employed in a real-time *in vitro* release study. Such accelerating conditions include elevated temperature, altering pH, and use of surfactants. As with the real-time *in vitro* release study, the method should be simple, reproducible under the conditions of study, inexpensive, and applicable to biodegradable nanoparticulate formulations that have varying duration of action *in vivo*. Generally aqueous media such as simulated gastric fluid without enzymes, simulated intestinal fluid without enzymes, water and buffers have been employed to study release of water soluble drugs. For water insoluble drugs, surfactants, bile acids, bile salts and lecithins have been shown t increase the rate of drug release. The level of interest in the *in vitro* dissolution of poorly water soluble drugs has increased in recent years due to the need of finding a suitable dissolution media for pharmaceutical formulations that may reflect their *in vivo* performance.

In vivo poorly water-soluble drugs are solubilised through complex endogenous surfactants such as bile acids, bile salts and lecithin. However, *in vitro* dissolution models in less complex micelle systems have been used. The use of surfactants in the dissolution system for poorly water-soluble drugs may be physiologically more meaningful due to the presence of natural surfactants in the gastrointestinal tract.

Additionally, the following should be considered prior to studying drug release:

1. Sink conditions: Although sink conditions may not exist at the *in vivo* site of action, it is wise to employ sink conditions during *in vitro* testing. In the event that a small volume of media can be used (based on the method employed and assay sensitivity),

total media replacement may be used to ensure drug solubility, maintain sink conditions, and prevent accumulation of polymer degradation products.

2. Burst release: The release method employed should be able to identify a high initial release or burst from the formulation. Additionally, the method should provide information about the onset and duration of burst to assess its influence on the *in vivo* efficacy and safety window of the drug being studied.

3. Robustness of technique: The *in vitro* release method employed should be able to assess the influence of changes in the manufacturing procedure on the formulation. This would be useful from a quality-control standpoint and could also aid in the design and development of drug delivery systems. Ideally, an *in vitro* test method should mimic *in vivo* conditions and release mechanism as much as possible. Methods to study the *in vitro* release are: (i) side-by-side diffusion cells with artificial or bio logical membranes; (ii) dialysis bag diffusion technique; (iii) reverse dialysis sac technique; (Pili, Bourgaux, Meneau, Couvreur, & Ollivon, 2009) ultra centrifugation; (v) Ultra filtration; or (vi) centrifugal ultra filtration technique. Despite the continuous efforts in this direction, there are still some technical difficulties to study *in vitro* drug release from NPs. These are attributed to the separation of NPs from the release media. In order to separate NPs and to avoid the tedious and time-consuming separation dialysis has been used; here, the suspension of NPs is added to the dialysis bags/ tubes of different molecular mass cut-off. These bags are then incubated in the dissolution medium. Another technique involves the use of a diffusion cell consisting of donor and acceptor compartments; this technique was used to separate through the artificial / biological membranes. In this method kinetic study was not performed under the perfect sink conditions, because the NPs were not diluted in the release media, but were separated from the release media through the membrane. Thus, the amount of drug in the release media did not reflect the amount of drug released. In order to avoid the enclosure of NPs in the dialysis bag, Leavy and Benita (Couvereur P, 1996) used a reverse dialysis technique for o/w emulsion. In this method, NPs were added directly into the dissolution medium. The same technique was adopted by Calvo et al. for the release from the NPs, nanocapsules and nanoemulsions (Soppimath, Aminabhavi, Kulkarni, & Rudzinski, 2001).

The release rates of NPs depend upon (i) desorption of the surface-bound /adsorbed drug; (ii) diffusion through the NP matrix; (iii) diffusion (in case of nanocapsules) through the polymer wall; (Pili et al., 2009) NP matrix erosion; and (v) a combined erosion / and diffusion process. Thus, diffusion and biodegradation govern the process of drug release. Release profiles of the drugs from NPs depend upon the nature of the delivery system. In the case of a matrix device, drug is uniformly distributed / dissolved in the matrix and the release occurs by diffusion or erosion of the matrix. If the diffusion of the drug is faster than matrix degradation, then the mechanism of drug release occurs mainly by diffusion, otherwise it depends upon degradation. Rapid initial release is attributed to the fraction of the drug which is adsorbed or weakly bound to large surface area of the NPs, than to the drug incorporated in NPs.

2.2.2.6 Stability (Wu, Zhang, & Watanabe, 2011)

Physical stability is crucial in formulation of drug nanosuspension. As nanoparticles have mean particle diameter in nanometer range, they are prone to aggregation of the particles. The aggregation may be due to Ostwald ripening which occurs due to different saturation solubilities in the vicinity of very small and larger particles. Stabilizers like surfactants or polymeric macromolecules are required to stabilize the nanoparticles against inter-particulate forces and prevent them from aggregation. Surfactants are used to minimize the free energy and stabilize the system. The stabilization provided by the stabilizers is by steric, electrostatic or combination of these two processes. Steric stabilization is achieved by adsorbing surfactants/polymers onto the particle surface while electrostatic stabilization is obtained by adsorbing charged molecules, which can be ionic surfactants or charged polymers, onto the particle surface. Generally, steric stabilization alone is sufficient to provide stability to the nanosized particles but electrostatic stabilization is often combined with it as an additional measure.

Formation of impurities due to process and formulation parameters must be studied. The impurities could be identified by various techniques such as infrared spectroscopy (IR), high performance liquid chromatography (HPLC) and mass spectroscopy (MS). Beside characterization of above properties, additional characterization of the

nanoparticles is required if surface modification is done for particles. The parameters for which surface modified nanoparticles are evaluated include adhesion properties, surface hydrophilicity/hydrophobicity and interaction with body proteins. The adhesiveness of the drug nanoparticles is considered to be a major factor contributing towards increasing the bioavailability and reducing variability of absorption. Surface hydrophobicity determines the interaction with the cells prior to phygocytosis and is relevant parameter for adsorption of plasma proteins. It is considered as important parameter affecting *in vivo* organ distribution after i.v. injection.

2.3 Nose to Brain delivery using Polymeric Nanoparticles:

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) (Pardridge, 1999) .

Macromolecular drugs such as peptides and proteins, termed 'biologics,' are too large and too hydrophilic to penetrate the BBB from the systemic circulation and would be rapidly degraded by gastrointestinal enzymes or the liver cytochromes, if taken orally. A non-invasive therapy would be desirable for patients particularly for diseases that require chronic dosing such as those related to dementia.

It has been shown in the literature from animal and human investigations, that transport of exogenous materials directly from nose-to-brain is a potential route for by-passing the BBB (Hinchcliffe & Illum, 1999). 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 represents the most direct method of non-invasive entry into the brain. However, the quantities of drug administered nasally that have been shown to be transported directly fromnose-to-brain are very low, normally less than 0.1%, and hence the system is not currently

used therapeutically and no product is licensed specifically via this route (Illum, 2004).

The strategy of applying drugs that are encapsulated into particulate vectors (such as synthetic nanoparticles) to the olfactory has been deeply studied for improvement of the direct CNS delivery of drugs—including biologics.

2.3.1 Anatomy and Physiology of the Nasal Passage

In order to understand the aspects of nose-to-brain drug delivery it is necessary to have a reasonable understanding of the nasal anatomy and physiology relating to the field.

The main functions of the nose are olfaction, regulation of humidity and temperature of inhaled air, and removal of large particulates including microorganisms from the inhaled air. In humans, the total surface area and volume of the two sides of the nasal cavity has been measured using computed tomography (CT) scans as 150.4cm^2 (made possible by three protrusions or 'turbinates' within the cavity) and 13.0 ml, respectively (Ménache et al., 1997) . The nasal septum divides the nasal cavity along the centre into two halves open to the facial side and to the rhinopharynx, through the anterior and via the posterior nasal apertures, respectively. Each nasal cavity can be divided into three regions; the nasal vestibule, the olfactory region and the respiratory region. The olfactory epithelium is located high in the nasal cavity in man. It partly overlies the cribriform plate, a bony structure that contains many pores that allow the passage of neuronal bundles from the olfactory epithelium to pass into the CNS. Olfactory epithelium may also lie partly on the nasal septum and on the superior turbinate. It is above the normal path of the airflow which means that odorant molecules normally reach the sensitive receptors by diffusion. The act of sniffing enhances the diffusional process by increasing the airflow rate and changing it from continuous to pulsatile in nature. This behaviour increases the turbulence within the nasal cavity and therefore allows greater interaction of the inspired air with the olfactory region at the roof of the nasal cavity. The respiratory region is dominated by

the large inferior turbinate, the middle turbinate and further back in the nasal cavity, the superior turbinate.

The respiratory epithelium is composed of four types of cells, namely, non-ciliated and ciliated columnar cells, basal cells and goblet cells. These cells facilitate active transport processes such as the exchange of water and ions between cells and motility of cilia (where applicable). They may also serve to prevent drying of the mucosa by trapping moisture. About 15–20% of the respiratory cells

are covered with a layer of long cilia, which move in a coordinated way to propel mucus towards the pharynx. Mucus (or nasal secretion) is a complex mixture of materials consisting of approximately 95% water, 2% mucin, 1% salts, 1% of other proteins such as albumin, immunoglobulins, lysozymes and lactoferrin, and <1% lipids (Kaliner, Marom, Patow, & Shelhamer, 1984).

Mucus is present in two layers on the epithelium in order to facilitate mucociliary clearance. A viscous gel layer, the 'mucus blanket' (Figure 2.1c; 'gel layer', 2–4_m thickness) floats on the serous fluid layer (Figure 2.1e 'sol layer', 3–5_m thickness). The viscous gel layer is moved along by the hook shaped cilia termini during the energy dependent 'effective stroke' phase of the ciliary motion (Figure 2.1a). Cilia are up to 7µm in length when fully extended but can fold to half this length during the recovery stroke where the hook terminus detaches from the gel layer and moves immersed in the sol layer in the opposite direction to the gel layer movement (Figure 2.1b). The cilia beat with a frequency of 1000 strokes per min. Hence the mucus moves only in one direction from the anterior to the posterior part of the nasal cavity to the nasopharynx. Therefore, particles applied to the nasal respiratory mucosa will be transported on the mucus to the back of the throat with a speed of 5mm per min (Lenaaerts V, 1990).

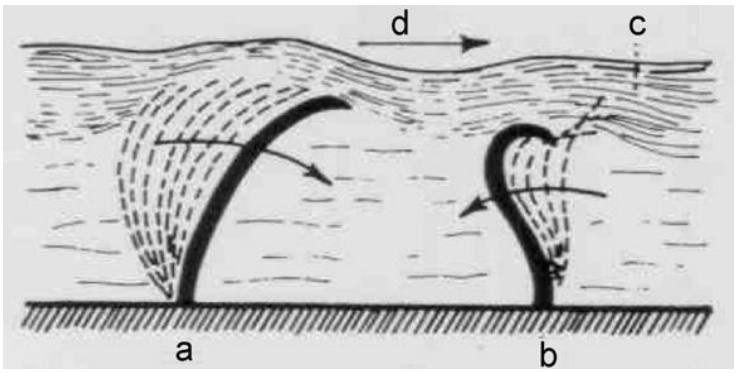

Figure 2.1: The relationship between ciliary motion and mucus layer composition that allows mucociliary clearance. (a) effective stroke, (b) recovery stroke, (c) gel layer, (d) direction of gel layer movement, and (e) sol layer. Adapted from (Alpesh Mistry, Snjezana Stolnik, & Lisbeth Illum, 2009)

The olfactory epithelial layer predominantly contains three cell types: the olfactory neural cells, the sustentacular (also known as supporting) cells and the basal cells. Basal cells are progenitor cells (of supporting cells) that also provide mechanical support via anchorage to other cells. The olfactory neural cells or the axons are un-myelinated and interspaced between the supporting cells (Figure 2.2). They originate at the olfactory bulb in the CNS and terminate at the apical surface of the olfactory epithelium.

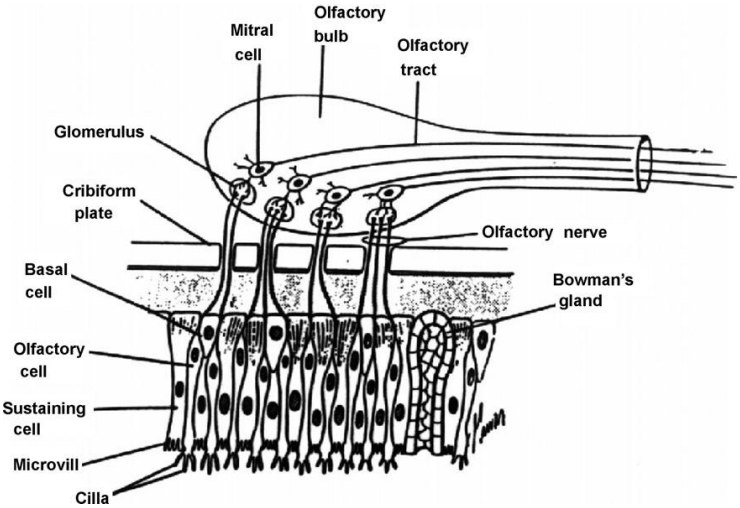

Figure 2.2: Diagram of the olfactory area showing the olfactory epithelium, bulb and tract. Adapted from(Alpesh Mistry et al., 2009)

The olfactory knob (or vesicle) protrudes out from and above the apical surface of the olfactory epithelium. Approximately 10–23 cilia project from the basal bodies of the knob, each of length up to 200 μm. The cilia contain chemical detectors that, once activated by odorants, initiate depolarisation of the olfactory axon by either direct ion-gated channels or cAMP operated ion-channels(Gartner LP, 2000). The cilia entangle with the thick brush border of microvilli of the supporting cells at the air/mucus/tissue interface. The cilia are non-motile in the olfactory region (in contrast to respiratory tissue) since they lack the dynein arms which contain the Mg^{2+}-ATPase that generates the force for ciliary motility (Moran, Rowley, Jafek, & Lovell, 1982).

The *lamina propria* of the olfactory epithelium, which is located beneath the epithelial layer(s), contains the blood supply, mucus secreting acinar glands (Bowman's glands), nasal lymphatics, and
a neuronal supply that consists of olfactory axon bundles, autonomic nerve fibres and the maxillary branch of the trigeminal nerve (Alpesh Mistry et al., 2009). Bowman's glands are under the control of the parasympathetic nervous system. These acinar type glands produce nasal secretions in the *lamina propria* and secrete them through a narrow tube-like opening into the luminal space. The Schwann-sheathed axonal bundles then pass through the *lamina propria* and into the porous structure of the cribriform plate.

Around 1500 sensory neurones synapse on one mitral cell in the glomeruli of the olfactory bulb. The mitral and tufted cells are branched; they project one dendrite to each glomerulus. These neurones
then pass through to the olfactory tubercle. From there, the third-order neuronal projections pass to the amygdala, prepyriform cortex, the anterior olfactory nucleus and the entorhinal cortex as well as the hippocampus, hypothalamus and thalamus(Pierre - Marie Lledo & Vincent, 2005). It is envisaged based on findings for large proteins (Shipley, 1985) and studies on nanoparticles (Oberdorster, Oberdorster, & Oberdorster, 2005) that nanoparticles of sufficiently small size could potentially be

transported via axons through the olfactory bulb into the olfactory cortex and from there to the caudal pole of the cerebral hemisphere and into the cerebrum and the cerebellum. Hence, these are all potential delivery sites for the nose-to-brain drug transport route via olfactory epithelium.

The trigeminal nerve is the largest of the cranial nerves and, amongst other functions, enables sensory perception in the nasal cavity. It has three major branches namely: the ophthalmic nerve, maxillary nerve and mandibular nerve. The ophthalmic and maxillary nerves only have a sensory function whereas the mandibular nerve has both sensory and motor functions. The three branches
meet at the trigeminal ganglion which contains the cell bodies of these sensory nerve fibres. Afferent neurones synapse at the trigeminal ganglion to form a single incoming nerve that enters the brainstem at the level of the pons.
The ophthalmic and maxillary branches of the trigeminal nerve are also important for nose-to-brain drug delivery since neurones from the branches pass directly through the nasal mucosa.
This has been extensively studied and reported.
Thorne et al (Thorne, Pronk, Padmanabhan, & Frey, 2004) investigated the CNS delivery of insulin-like growth factor-I (IGF-I), a 7.65 kDa protein neurotrophic factor, following intranasal administration and the possible pathways and mechanisms underlying transport from the nasal passages to the CNS. Anesthetized adult male Sprague-Dawley rats were given [^{125}I]-IGF-I intranasally or intravenously and then killed by perfusion-fixation within 30 min. Other animals were killed following cisternal puncture and withdrawal of cerebrospinal fluid or intranasal administration of unlabeled IGF-I or vehicle. Both gamma counting of micro dissected tissue and high resolution phosphor imaging of tissue sections showed that the tissue concentrations and distribution following intranasal administration were consistent with two routes of rapid entry into the CNS: one associated with the peripheral olfactory system connecting the nasal passages with the olfactory bulbs and rostral brain regions (e.g. anterior olfactory nucleus and frontal cortex) and the other associated with the peripheral trigeminal system connecting the nasal passages with

brainstem and spinal cord regions. Intranasal administration of [125I]-IGF-I also targeted the deep cervical lymph nodes, consistent with their possible role in lymphatic drainage of both the nasal passages and the CNS. Cisternal CSF did not contain [125I]-IGF-I following intranasal administration. Intravenous [125I]-IGF-I resulted in blood and peripheral tissue exposure similar to that seen following intranasal administration but CNS concentrations were significantly lower. Finally, delivery of IGF-I into the CNS activated IGF-I signaling pathways, confirming some portion of the IGF-I that reached CNS target sites was functionally intact. The results suggest intranasally delivered IGF-I can bypass the blood-brain barrier via olfactory- and trigeminal-associated extracellular pathways to rapidly elicit biological effects at multiple sites within the brain and spinal cord. Similarly, brain targeting has been reported by Chemuturi et al for Dopamine via the trigeminal pathway across the nasal pseudoepithelium wherein large trigeminal pathways are present.

Additionally, Robert Scantron et al (Scranton, Fletcher, Sprague, Jimenez, & Digicaylioglu, 2011) demonstrated the role of Rostral Migratory System in the CNS delivery of intranasally administered drug. The Rostral Migratory Stream (RMS) connects the olfactory bulb to the periventricular regions. The scientists have extensively studied this pathway through surgical and radiolabelling methods. Thus, the identification of the RMS as the major access path for intranasally administered drugs into the CNS may contribute to the development of treatments that are tailored for efficient transport within this structure. RMS needs to continue to elucidate its limitations, capabilities, mechanisms of transport and potential hazards before we are able to advance this technique into human research. Kandimalla et al utilized molecules like hydroxyzine and tripolidine to understand its brain uptake by nasal route. Their work demonstrated that lipophilic drugs with low molecular weight can be transported to the brain passively despite the presence of efflux transporters and metabolizing enzymes. The study also involved effect of pH on the transport of drug to the brain.

2.3.2 Scientific reports on Nanoparticles for nose to brain delivery

There are numerous reports on attempts for transporting various types of drugs directly to the brain via the nasal route. Some of the recent work would be described in the below paragraphs. The focus would be on the use of nanoparticles for the nose to brain delivery.

Sharma D et al (D. Sharma et al., 2015) formulated diazepam-loaded poly(lactic-co-glycolic acid) nanoparticles to achieve delivery in the brain through intranasal administration. Diazepam nanoparticles were formulated by nanoprecipitation and optimized using Box-Behnken design Developed nanoparticles showed z-average 148-337 d.nm, polydispersity index 0.04-0.45, drug entrapment 69-92%, and zeta potential in the range of -15 to -29.24 mV. Brain/blood uptake ratios, drug targeting efficiency, and direct nose-to-brain transport were found to be 1.23-1.45, 258, and 61% for ^{99m}Tc-DNP (i.n) compared to ^{99m}Tc-DS (i.n) (0.38-1.06, 125, and 1%). Scintigraphy images showed uptake of Diazepam from nose-to-brain, and this observation was in agreement with the biodistribution results.

Jafareih O et al (Jafarieh et al., 2014) investigated the possibility of targeting an anti-Parkinson's drug Ropinirole to the brain using polymeric nanoparticles. Ropinirole hydrochloride - loaded chitosan nanoparticles were prepared by an ionic gelation method. The nanoparticles showed sustained release profiles for up to 18 h. The Ropinirole concentrations (% Radioactivity/g) in the brain following intranasal administration (i.n.) of nanoparticles were found to be significantly higher at all the time points compared with plain solution. From gamma scintigraphy imaging in rats, the brain/blood ratios obtained were 0.251 ± 0.09 and 0.386 ± 0.57 respectively for Ropinirole solution (i.n.) and nanoparticles (i.n.), at 0.5 h indicative of direct nose to brain transport, bypassing the blood-brain barrier (BBB).

Sharma D et al (D. Sharma, Maheshwari, & Philip, 2014) optimized Lorazepam loaded PLGA nanoparticles (Lzp-PLGA-NPs) by nanoprecipitation method using PLGA as polymer, poloxamer as surfactant and acetone as organic phase. In vitro drug release behavior followed Korsmeyer-Peppas model and showed initial burst release of 21.7 ± 1.3% with prolonged drug release of 69.5 ± 0.8% from optimized NPs up to

24 h. In vitro drug release data was found in agreement with ex vivo permeation data through sheep nasal mucosa. In vitro cell viability study on Vero cell line confirmed the safety of optimized NPs. Optimized Lzp-PLGA-NPs were radiolabelled with Technitium-99m for scintigraphy imaging and biodistribution studies in Sprague-Dawley rats to establish nose-to-brain pathway.

Mittal D et al (Mittal, Md, et al., 2014) prepared and evaluated Rasagiline-loaded chitosan glutamate nanoparticles (RAS-CG-NPs) by ionic gelation. The loaded nanoparticles after complete characterization were studied for biodistribution in the brain and blood of mice following intranasal (i.n.) and intravenous (i.v.) administration. The drug concentrations in brain following the i.n. of nanoparticles were found to be significantly higher at all the time points compared to both drug (i.n.) and drug CG-NPs (i.v.). The C_{max} (999.25 ng/ml) and AUC (2086.60 ng h/ml) of formulated nanoparticles (i.n) were found to be significantly higher than nanoparticles (i.v.) and Rasagiline solution (i.n.). The direct transport percentage (DTP%) values of nanoparticles (i.n.) as compared to drug solution (i.n.) increased from 66.27 ± 1.8 to $69.27 \pm 2.1\%$. The results showed significant enhancement of bioavailability in brain, after administration of the Rasagiline loaded nanoparticles which could be a substantial achievement of direct nose to brain targeting in Parkinson's disease therapy.

Bhatt R et al (Bhatt, Singh, Prakash, & Mishra, 2015) developed solid lipid nanoparticles as a drug delivery system to enhance the brain-targeting efficiency of Rosmarinic acid following intranasal (i.n.) administration. The Rosamarine - loaded solid lipid anoparticles was prepared by the hot homogenization technique and were completely characterized. Nasal delivery of the developed formulation followed by the study of behavioral (locomotor, narrow beam, body weight) and biochemical parameters (glutathione, lipid peroxidation, catalase and nitrite) in wistar rat was carried out. The treatment significantly improved behavioral abnormalities and attenuated the oxidative stress in 3NP-treated rats. The nasal delivery of drug loaded solid lipid nanoparticles produced significant therapeutic action as compared to

intravenous application. In the organ distribution study, brain drug concentration was found to be 5.69 µg, in pharmacokinetic study C_{max}, t_{max}, $t_{1/2}$, AUC values were found to be 0.284 µg/ml, 1.5 h, 3.17 h, and 1.505 µg/ml/h, respectively. The encouraging results confirmed the developed optimized Rosamarine-loaded solid lipid nanoparticles formulation following the non-invasive nose-to-brain drug delivery as a promising therapeutic approach for the effective management in Huntington disease.

Lipid nanoparticles with solid matrix encapsulating basic fibroblast growth factor (bFGF) were developed by Zhao ZY et al (Zhao et al., 2014) to target the brain via nasal administration. Treatment effects were assessed by quantifying rotational behavior, monoamine neurotransmitter levels and tyrosine hydroxylase expression in 6-hydroxydopamine induced hemiparkinsonian rats. The gelatin nanostructured lipid carriers prepared by a water-in-water emulsion method possessed better profile than gelatin nanoparticles. The intranasal nanoparticles efficiently enriched exogenous bFGF in olfactory bulb and striatum without adverse impact on the integrity of nasal mucosa and showed obvious therapeutic effects on hemiparkinsonian rats. Thus, they were found to be effective carriers for nose-to-brain drug delivery, especially for unstable macromolecular drugs such as bFGF.

Kumar M et al (Kumar et al., 2013) worked on nose to brain delivery of Leucine-enkephalin (Leu-Enk), which is a neurotransmitter or neuromodulator in pain transmission. Leu-Enk loaded N-trimethyl chitosan (TMC) nanoparticles were prepared by ionic gelation method and evaluated as a brain delivery vehicle via nasal route. N – trimethyl chitosan biopolymer was synthesized and analyzed by $^{(1)}$H NMR spectroscopy. Mean peptide encapsulation efficiency and loading capacity were 78.28±3.8% and 14±1.3%, respectively. Permeability of Leu-Enk released from nanoparticles was 35 fold improved from the nasal mucosa as compared to Leu-Enk solution. Fluorescent microscopy of brain sections of mice showed higher accumulation of fluorescent marker NBD-F labelled Leu-Enk, when administered nasally by TMC nanoparticles, while low brain uptake of marker solution was observed. Furthermore, enhancement in brain uptake resulted into significant

improvement in the observed antinociceptive effect of Leu-Enk as evidenced by hot plate and acetic acid induced writhing assay.

2.4 ALZHEIMER'S DISEASE: PATHOPHYSIOLOGY

Alzheimer's disease (AD) is a chronic and progressive neurodegenerative disorder set to become the developed world's largest socioeconomic healthcare burden over the coming decades. In AD, multiple regions of brain gray matter have a profound neuronal loss, including basal forebrain, hippocampus, entorhinal, and temporal cortices. The neurodegenerative process begins with neuronal loss in the glutamatergic pathways of the entorhinal cortex before extending to the hippocampus and amygdala and then more widely to neocortical and subcortical areas(Jacobson, 2005) .

Alzheimer's disease affects 24.3 million people worldwide and hence becomes one of the most severe socio – economical and medical burden all over the world (Ferri et al., 2005).The disease burden of Alzheimer's goes beyond the cost of medicines / therapy. The most common outcome of Alzheimer's is dementia. Around the world, the global market for Alzheimer's disease is slated to increase to $ 13.3 billion by 2023. Much of the costs incurred in Alzheimer's disease have been due to informal care or direct societal care. In India, the total societal cost of dementia was estimated to be Rs. 206.11 billion. The leading market areas for Alzheimer's are US, Europe and Japan. However, the future leading markets are anticipated to be located in China, India and the South Asian and Western Pacific countries. This is because the fastest growth of elderly population in these areas. In 2015, the Alzheimer's market in India is close to Rs. 300 crore (U. Sharma, 2011)

AD is characterized by the gradual development of forgetfulness, progressing to disturbances in language, dyscalculia/ acalculia, visuospatial disorientation, ideational and ideomotor apraxia, akinesia, and mutism. Synapse loss also occurs and has been shown to be the best correlate of cognitive decline (R.D. Terry et al., 1991) . 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. Genetic factor affecting <5 % of cases include mutation on three genes. These include the genes coding for the amyloid precursor protein on chromosome 21,

presenilin 1 on chromosome 14 and presenilin 2 on chromosome 1. The mutations on these genes share common biochemical pathways that include the altered production of the amyloid –β with an overabundance of the $A\beta_{1-42}$ fragment, the principal constituent of senile plaques. The major genetic risk factor for the sporadic form of Alzheimer's is the inheritance of the ε4 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 Aβ production and deposition in the brain of Alzheimer's subjects (Sahni, S., Ali, Baboota, & Dao, 2011) .

There are various theories to explain the pathophysiology of Alzheimer's disease. One of the most prominent amongst all is the "Amyloid Cascade Hypothesis". According to it, amyloid-β-peptide plays a main role in the pathogenesis of AD. On the basis of this theory,amyloid is produced by secretase-mediated cleavages of the amyloid precursor protein (APP) (Haass et al., 1992; Seubert et al., 1992; Shoji et al., 1992). The encoding gene for APP is located on the longharm of chromosome 21. β-Secretase, an aspartyl protease with a strong homology with the pepsin family, cleaves APP at the extracellular N-terminus, generating an extracellular soluble fragment called sAPPb and leaving an intramembrane fragment known as C99. Sequentially, γ-secretase, an aspartyl protease formed by four proteins such as nicastrin, presenilin, alphaprotein 1A and presenilin enhancer 2, cleaves the C99 C-terminal end, originating an intracellular fragment (amyloid intracellular domain, AICD) and releasing Amyloid – β - peptide. The product of these cleavages is either the 40- or 42-amino. acid fragment of Amyloid – β - peptide . Once produced, it forms the core of senile plaques (SP), which are undoubtedly implicated in AD pathogenesis, and the toxicity of this peptide is thought to be heavily dependent on self association. Amyloid monomers are considered to be less toxic, and in one study protective (Giuffrida et al., 2009) than low molecular weightoligomers of Amyloid – β - peptide which has been shown to cause significant adverse cellular events (Querfurth & LaFerla, 2010; Shankar et al., 2008). Preclinical evidence demonstrated that Amyloid – β – peptide protein oligomers, isolated from the cerebral cortex of AD patients and administered

to mice, severely inhibited long-term potentiation, reduced hippocampal dendritic spine density, and disrupted memory of a learned behavior (Shankar et al., 2008).

Additionally, Amyloid fibrils were shown to contribute to AD by stimulating the hyperphosphorylation of tau thus increasing the formation of neurofibrillary tangles (NFT) (Gotz, Chen, van Dorpe, & Nitsch, 2001). The link between Ab and tau was also strengthened by the evidence that two kinases, such as GSK3b and DYRK1A, which are activated by Amyloid – β - peptide and APP cleavage products, significantly increased tau phosphorylation (Ballard et al., 2011).

Another novel hypothesis was developed which focused on a direct toxic role played by APP and presenilins and support the idea that Amyloid –β - peptide is not causative but can be considered as an 'innocent bystander' in AD. Presenilins were shown to be involved in the regulation of many pro-survival intracellular pathways including the PI3K/Akt system, the mytogen-activated protein kinases MEK and ERK, the kinase GSK3b and calcium homeostasis. Additionally, presenilin-1 was demonstrated to promote the degradation of the transcription factor b-catenin and control the release of N-cadherin, the latter playing a pivotal role in hippocampal long-term potentiation.

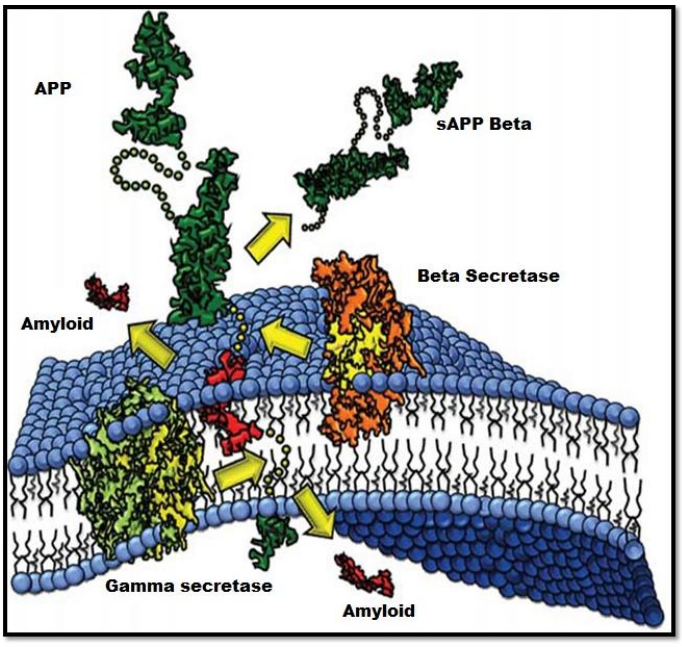

Figure 2.3: Metabolic pathway leading to amyloid –β-peptide formation in AD patients.(Mancuso, Siciliano, Barone, Butterfield, & Preziosi, 2011)

Other than the hypothesis based on Amyloid Protein Precursor. Hensley et al. were among the first to observe the free radical-generating properties of Amyloid – β-protein in brains from AD patients, providing a potential mechanism for pathology-induced neurodegeneration (Hensley et al., 1994). The oxidative stress hypothesis in AD is further supported by data showing little/no oxidative damage in neuronal areas that are not affected in AD, such as cerebellum. resulting in decreased enzymatic activity; these proteins are involved in glucose metabolism, mitochondrial electron transport, cytoskeletal maintenance and proteasomal function, all of which are altered in AD relative to brains from healthy controls(Butterfield, Drake, Pocernich, & Castegna, 2001; Reed et al., 2008) .The involvement of lipid oxidation and increased concentration of oxidized mitochondrial and nuclear bases were also found to be leading to AD.

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While the involvement of neuronal death undoubtedly contributes to AD progression, the underlying cause of this phenomenon remains elusive. Studies on postmortem brains of AD patients indicate the presence of neuronal apoptosis(Colurso, Nilson, & Vervoort, 2003). Sultana et al.(Sultana & Butterfield, 2013) reported on increased cytosolic levels of proapoptotic Bcl-2 and caspase-3 in amnestic MCI hippocampus, implicating apoptosis as an early event in AD pathogenesis. Therefore, pathways to programmed cell death may provide therapeutic targets for preventing neurodegeneration.

Along with the aforementioned oxidative stress, mitochondrial dysfunction and loss of phospholipids asymmetry (Takuma, Yan, Stern, & Yamada, 2005) which are related to oxidative stress, are major pathways leading to the induction of apoptosis. Mitochondria play a key role in cellular vitality since they are responsible for the generation of adenosine triphosphate (ATP) through oxidative phosphorylation, as well as regulation of intracellular Ca^{2+}. Cytochrome C oxidase deficiency in AD has been proposed as a possible cause of increased apoptosis (Ojaimi & Byrne, 2001). Another possible mechanism of ATP depletion is oxidation of enzymes involved in glycolysis, the Krebs cycle, or oxidative phosphorylation (Butterfield et al., 2006), possibly by Amyloid – β - peptide. Oxidative inactivation of key enzymes involved in

ATP production would undoubtedly impair mitochondrial respiration, cause loss of membrane potentials and ultimately leading to cell death.

Calcium is considered as an important second messenger in neurons, since it regulates membrane excitability, triggers neurotransmitter release at the synapse, mediates gene expression and modulates neuronal growth (Bezprozvanny & Mattson, 2008). Therefore, disruption of Ca^{2+} homeostasis within the neuron can have multiple adverse consequences leading to cell death. Ca^{2+} levels in the cytosol are regulated by a cross-talk between voltage-gated

Ca^{2+} channels, N-methyl-D-aspartate receptors (NMDA), uptake by mitochondria and endoplasmic reticulum stores(Berridge, Bootman, & Lipp, 1998) . Excessive amounts of cytosolic Ca^{2+} are sequestered by mitochondria to a point, at which Ca^{2+} can trigger mitochondrial mechanisms leading to the opening of the mitochondrial permeability transition pore (MPTP) and the release of cytochrome c from the mitochondrial matrix to the cytosol, a key event in the intrinsic apoptotic pathway. In AD, Aβ membrane interactions result in the formation of ion-conducting pores, leading to increased cytosolic Ca^{2+} and elevated vulnerability of neurons to excitotoxicity . Furthermore, oligomeric Amyloid – β - peptide can cause Ca^{2+}-related toxicity in cultured neurons. Lipid peroxidation, a well-established event in AD and MCI and oxidative modification of NMDA receptors, Ca^{2+} membrane channels, glutamate and glucose transporters would also contribute to elevated cytosolic Ca^{2+} in AD, leading to apoptosis (Sahni, S., et al., 2011).

2.5 CURRENT THERAPIES FOR ALZHEIMER'S DISEASE (R. S. Shah et al., 2008)

The important current therapeutic line of action for Alzheimer's is tabulated in Table 2.1

Table 2.1: Current pharmacotherapy for Alzheimer's Disease

Pathogenic mechanism	**Treatment**
Cholinergic deficiency	Cholinesterase inhibitors: 1st Generation: Tacrine 2nd Generation: Donepezil Galantamine Rivastigmine Butyrylcholinesterases NGF gene delivery
Oxidative stress	Selegeline Alpha - tocopherol
Amyloid cascade	Statins
Inflammation	NSAIDS
Excitotoxicity	Memantine
Other	Mediterranean diet

2.5.1 Acetylcholine choline esterase inhibitors:

The nucleus basalis of Meynert, a distinct population of basal forebrain neurons, is a major source of cholinergic innervations to the cerebral cortex. In 1982, Whitehouse and colleagues found that these neurons are selectively (>70%) degenerated in patients with AD (Whitehouse, 2006; Williams et al., 1986). Furthermore, the loss of cholinergic function has been found to be closely related to cognitive dysfunction (Farlow & Evans, 1998). Over the last two decades, a number of therapeutics targeting cholinesterase inhibition, choline precursors, postsynaptic cholinergic stimulation with muscarinic agonists, and presynaptic cholinergic stimulation with nicotinic agonists

has been investigated (Herbert et al., 1993). These drugs stabilize cognitive decline for up to 3 to 6 months (Giacobini, 2000), although no modification of disease duration or general disease progression has been accomplished.

Acetylcholinesterase inhibitors (ACIs) are the best developed therapy and are used for mild to moderate disease. The mechanism by which ACIs slow progression of disease is thought to be by decreasing levels of amyloid-b protein precursor (AbPP) and production of Ab and amyloidogenic compounds (Mori, Lai, Fusi, & Giacobini, 1995). Tacrine was the first widely used ACI (Summers, 2006). A 30-week randomized control clinical trial showed a significant dose related improvement in cognitive function (Whitehouse, 2006). However, subsequent studies were less impressive and a short half-life, hepatotoxicity, and cholinergic side effects have limited the use of this drug.

Second generation cholinergics, including Donepezil (trade name Aricept®, Eisai Company and Pfizer Inc.), Galantamine (Hoechst Marion Roussel Inc., Shire Pharmaceutical Group, and Janssen Pharmaceutical, trade names Reminyl® and Nivalin, U.S. trade name Razadyne®) and Rivastigmine (trade name Exelon®, Novartis Pharmaceuticals) have since been developed (Table 1). These drugs have fewer side effects, longer half-lives, and greater efficacy. In a meta-analysis of 29 randomized placebo-controlled trials, patients on cholinesterase inhibitors improved 0.1 standard deviations (SDs) on activity of daily living (ADL) scales and 0.09 SDs on instrumental ADL scales compared with placebo. This effect is similar to preventing 2 months per year decline in a patient with AD (Trinh, Hoblyn, Mohanty, & Yaffe, 2003). Typically, ACIs are started at low doses to minimize side effects such as facial flushing, dyspepsia, nausea, vomiting, and diarrhea. The dose is then titrated up to the maximum tolerated dose (Mulugeta et al., 2003)

2.5.2 Immunization for Alzheimer's disease:

The 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. Removal of excess Ab from the brain occurs by a reactive T cell response. In 1999, active immunization of transgenic mice producing human Ab was shown to produce a significant reduction of Ab plaques (Schenk et al., 1999). The PDAPP transgenic mouse was the model used and immunization with Ab was administered prior to the onset of AD-type neuropathologies or once these changes were already established. Immunization of the young animals prevented the development of Ab plaque formation, neuritic dystrophy and astrogliosis. Treatment of the older animals reduced the extent and progression of these neuropathologies (Schenk et al., 1999). There is evidence for a number of mechanisms of plaque removal: (1) solubilization by binding of antibody to Ab; (2) phagocytosis of opsonized Ab by microglial cells; and (3) the ‘‘sink’’ hypothesis in which Ab antibodies remain in the plasma and extract Ab from the brain by altering equilibrium across the blood brain barrier(Nicoll et al., 2006) . Subsequent studies indicated that these animals may be able to regain functional effects following immunization (Janus et al., 2000).

Passive transfer of exogenous monoclonal Aβ antibodies seems the easiest way to provide antibodies without eliciting Th1 – mediated autoimmunity. Transgenic mice treated this way had significant decreases in Aβ concentration and cognitive benefit. . This would ensure the likelihood of treated patients achieving an adequate level of antibody response (Janus et al., 2000; D. Morgan et al., 2000). However, major challenges of this approach are high costs, blood – brain barrier penetration, microhaemoorrhage, off – target cross reactivity, and loss of the antibody to a peripheral sink.

Passive immunotherapy of AD requires repeated administration of anti-AbP antibodies. For that reason, human anti-AbP antibodies should be used to prevent an immune response to the currently available murine monoclonal immunoglobulins. Several methods are known to obtain human anti-AbP antibodies. One method is humanization of murine anti-ABP antibodies by replacing framework portions of the murine anti-AbP antibodies with human framework sequences using recombinant DNA technology. Alternative methods are the generation of human monoclonal anti-AbP antibodies in vitro by phage library display techniques, or in vivo by

immunization of animals whose immunoglobulin loci have been replaced by human genes (Solomon, 2009).

Compared to active immunization, passive immunization would be heavier for patients, because of requiring frequent anti-AbP antigen administration to maintain steady-state levels of the antibody. But, on the contrary, it might offer more control over both safety and efficacy.

The passively administered antibodies were able to enter the central nervous system (CNS), either to decorate plaques and induce clearance of pre-existing amyloid in old mice or to prevent plaque formation in young mice. Such antibodies can provide a therapeutic benefit by acting either within the CNS or in the periphery as a peripheral sink . IgGs have limited access to the brain. Only 0.1% of an intravenous dose was shown to pass via extracellular pathways through the blood–brain barrier (BBB) into the brain(Banks et al., 2002). Antibody uptake to and clearance from the brain did not differ in mice overexpressing AbP compared with wild-type mice. Indeed, upon entering the brain and binding to AbP or amyloid plaques, the local half-lives of antibodies were not observed to be prolonged in the CNS. The potential benefit of anti-AbP antibodies may be derived from the difference between plasma levels of anti-AbP antibodies in AD-affected people and the levels in healthy individuals. Humans produce natural antibodies against AbP and healthy individuals have higher levels of plasma anti-AbP antibodies compared to Alzheimer's patients.

Indeed, human B-lymphocytes have the capacity to produce anti-AbP antibodies. A recent study showed that plasma anti-AbP antibodies that bind to aggregated AbP were significantly lower in people with AD than in healthy controls, while there was no difference in anti-AbP antibodies binding to AbP monomers(Du et al., 2001). Therefore, natural antibodies to aggregated AbP may have great importance against AD pathology (Xu & Gaskin, 1997).

2.6 Drug Profile : Galantamine Hydrobromide (Farlow, 2001; Novikova & Tulaganov, 2002)

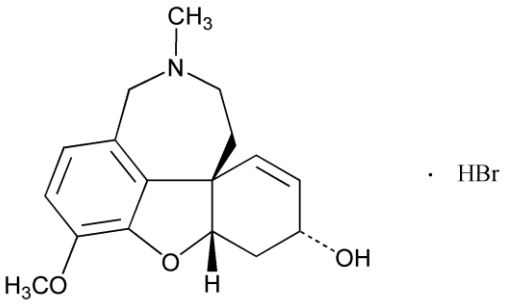
Category: Parasympathomimetics, Cholinesterase Inhibitors, Nootropic agents.

CAS No: 1953-04-4

Molecular Weight: 368.27

Molecular Formula: $C_{17}H_{21}NO_3$.HBr

Molecular Structure:



IUPAC:

(4a*S*,6*R*,8a*S*)-4a,5,9,10,11,12-hexahydro-3-methoxy-11-methyl-6*H*-benzofuro[3a,3,2ef][2]benzazepin-6-ol hydrobromide

Appearance: White to practically white crystalline powder

Physicochemical parameters:

Solubility: Sparingly soluble in water, insoluble in ethanol, chloroform and ether.

pKa: 8.2

Melting Point: 258 -264° C

Mechanism of action:

Galantamine is an anti alzheimer's drug, which competitively and reversibly inhibits cholinesterases, the enzymes responsible for inactivating acetylcholine**.** Acetylcholinesterase is the enzyme that is localized around cholinergic neuroeffector junctions in the periphery, brain and spinal cord and it specifically hydrolyzes the cholinergic neurotransmitter, acetylcholine. Butyrylcholinesterase, an enzyme synthesized in the liver, is primarily found in plasma and has the ability to cleave a variety of choline esters. By reducing the hydrolysis (and inactivation) of acetylcholine released from central cholinergic neurons, it is believed that galantamine

increases the concentration of functional acetylcholine. Although there is no evidence that galantamine alters the underlying dementia, it is postulated that the effect of galantamine may lessen advancement of the disease process by supporting functionally-active cholinergic neurons.

Adverse Effects:

Reported common adverse effects due to galantamine administration include nausea, vomiting, anoxia, diarrhea, abdominal pain, dizziness, headache, insomnia and muscle tremors. Less common adverse effects may include atrial arrhythmias, apathy, incontinence, epistaxis and upper and lower gastrointestinal bleeding.

Pharmacokinetics:

Galantamine (via immediate-release tablets) is well absorbed and almost completely absorbed following oral administration with time to peak plasma concentration (T_{max}) . occurring at about 1 hour; the oral bioavailability of the drug is approximately 90%. Food reduced the maximum plasma concentration (C_{max}) by about 25% and delayed the T_{max} by 1.5 hours. With the extended-release capsule preparation, the C_{max} and T_{max} were lower (about 25%) and occurred later (about 4.5 to 5.0 hours), respectively, compared to the immediate-release preparation.

This compound is not highly bound to plasma proteins (~ 18%) at therapeutic concentrations. Its mean volume of distribution (Vd) is high (175 L). In whole blood, about 53% is distributed to blood cells; the blood to plasma concentration ratio of galantamine is 1.2.

The major metabolic pathways of galantamine metabolism are glucuronidation, O-demethylation, N-demethylation, N-oxidation, and epimerization. *In vitro* studies showed that the CYP2D6 isozyme mediated O-demethylation of the compound to form O-demethyl-galantamine and the CYP3A4 isozyme mediated the formation of galantamine-N-oxide. Since the activity of the CYP2D6 isozyme is subject to genetic variability, about 7% of the population is characterized as “poor” metabolizers while the other 93% are considered as extensive metabolizers. After single oral doses of galantamine, CYP2D6 poor metabolizers showed a similar C_{max} but an increase in

area-under-the-curve (AUC) of about 35% of unchanged galantamine as compared to data from extensive metabolizers. There is no information that any of these metabolites have significant pharmacologic activity.

Elimination of the drug is mainly through the urine (about 95%) with the remainder being excreted into the feces (about 5%). After oral administration, about 20% of a dose was excreted unchanged in the urine in 24 hours. Using radioactivity studies with 3H-galantamine, approximately 32% of a dose was found in urine as the parent compound while approximately 5% was present as the galantamine glucuronide over 7 days after dosing.

The elimination half-life of galantamine ranges from about 6 to 16 hours.

Analytical Methods:

Galantamine Hydrobromide can be analysed by various chromatographic and spectroscopic methods. Out of that UV – Visible spectroscopic method and HPLC method are described below.

UV – Visible spectroscopic method:(Patel Hitesh, Patel amit, patel vishal, Dave Jayant, & Chhaganbhai, 2010)

Patel Hitesh et al reported a simple method for analyzing Galantamine hydrobromide from immediate release tablets, wherein standard calibration curve were made by dissolving the drug in distilled water and analyzing in UV – visible spectrophotometer at a λ_{max} of 289 nm.

HPLC method:

There are numerous reports on the use of HPLC for determination Galantamine Hydrobromide from animal or human plasma.

RPLC conditions:(Tencheva, Yamboliev, & Zhivkova, 1987)

Column: C_8

The methanol-water (40:60) mobile phase modified with $5X10^{-3}$ M dibutylamine at pH 7 (adjusted with 85% phosphoric acid) and a flow-rate of 1.2 ml/min were chosen. The presence of dibutylamine significantly improved the chromatographic behaviour of the substances investigated. The mobile phase was prepared by mixing 400 ml of methanol, 0.85 ml of dibutylamine and 0.2 ml of 85% phosphoric acid and making to

1000 ml with distilled water. The mixture was degassed before use. All chromatographic investigations were performed at room temperature.

Marketed Formulations:

Galantamine Hydrobromide tablets, capsules and oral solutions are available. *Razadyne*® is marketed as tablets containing galantamine hydrobromide in 4, 8, or 12 mg (as the base drug) and as an oral solution containing 4 mg/mL. It is recommended that these immediate-release formulations be given twice daily, preferably with the morning and evening meals. *Razadyne ER*® consists of extended-release capsule preparations containing galantamine hydrobromide in 8, 16, or 24 mg (as the base); once daily administration with one of these capsules with food is the recommended dosing schedule. The extended-release capsules deliver 25% of the galantamine dosage as an immediate-release dose and the remaining 75% of the dosage as a sustained-release dose.

Research work done on Galantamine Hydrobromide:

Hanafy et al (Hanafy, Farid, & ElGamal, 2015)developed chitosan nanoparticles to entrap Galantamine Hydrobromide. Here the challenge was to link cationic nanoparticles with the cationic drug. The nanoparticles were optimized; characterized and in vivo studies were done to understand its distribution in brain after intranasal administration.

Ligand functionalized nanoliposomes were also formulated by Mufamadi *et al* (Mufamadi et al., 2013) for Galantamine hydrobromide. The cellular uptake for the same evaluated using PC 12 neuronal cell line.

PLGA nanoparticles were formulated by (Fornaguera, Feiner-Gracia, Caldero, Garcia-Celma, & Solans, 2015) for intravenous administration of Galantamine hydrobromide. The enzymatic activity of the drug was maintained in 80% after its encapsulation in nanoparticles that were non-cytotoxic at the required therapeutic concentration.

2.7 Drug Profile of Bapineuzumab

Category: anti beta amyloid antibody

Solubility: Soluble in water, insoluble in ethanol, chloroform and ether.

Mechanism of action:

Bapineuzumab is an IgG1, N – terminus anti amyloid humanized monoclonal antibody. It has been proven to be reducing 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. (http://www.alzforum.org/therapeutics/bapineuzumab)

Adverse Effects:

Vascular emboli have been reported in few cases after intravenous administration of Bapineuzumab.

Pharmacokinetics:

Discrete details of pharmacokinetics of this antibody are not yet available.

Marketed Formulations:

Marketed formulations of Bapineuzumab are not available as it's currently under clinical study.

Research work done on Bapineuzumab:

Reports for Bapineuzumab were mainly associated with its clinical studies (Wyeth, 2008). Reports on formulation based work on Bapineuzumab are not yet available.

2.8 References

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