Chapter II

Literature Review

Novel drug delivery systems have revolutionized the methods of medication and provide therapeutic benefits. The goal in designing a new drug delivery system is to reduce the side effects and frequency of dosing and above all providing new therapeutic effect to the patient in addition to controlling the parent disease. Such a dosage form will be best suited for a multi-dose or long-term therapy of Diabetes mellitus and associated neurodegenerative disorder like Alzheimer's disease.

2.1 DIABETES

Diabetes mellitus (DM) is now one of the most common non-communicable diseases in the world. It is the fourth or fifth leading cause of death in most high-income countries and there is substantial evidence that it is epidemic in many economically developing and newly industrialized nations. The most common form of diabetes is type II diabetes and about 90 to 95% percent of total diabetic people suffer with this type of diabetes. Type II diabetes is often part of a metabolic syndrome that includes obesity, elevated blood pressure, and high levels of blood lipids. Complications from diabetes, such as coronary artery and peripheral vascular disease, stroke, diabetic neuropathy, amputations, renal failure and blindness are resulting in increased disability, reduced life expectancy and enormous health costs for virtually every society. Diabetes is undoubtedly one of the most challenging health problems in the 21st century. As per Diabetes Atlas released by WHO, the prevalence of DM is highest in middle-east countries. The number of studies describing the epidemiology of diabetes over the last 20 years has been extraordinary. It is now recognized that it is

the low and middle income countries that face the greatest burden of diabetes. However, many governments and public health planners still remain largely unaware of the current magnitude, or, more importantly, the future potential for increases in diabetes and its serious complications in their own countries. In addition to diabetes, the condition of impaired glucose tolerance also constitutes a major public health problem, both because of its association with diabetes incidence and its own association with an increased risk of cardiovascular disease (IDF Atlas, Ed. 3rd, 2006). When type II diabetes is diagnosed, the pancreas is usually producing enough insulin, but, for unknown reasons, the body cannot use the insulin effectively, a condition called insulin resistance. After several years, insulin production decreases. The result is the same as for type I diabetes i.e. glucose builds up in the blood and the body cannot make efficient use of its main source of fuel. This build up of glucose may lead to degradation of cell constituent proteins essential for vital functions (Li and Holscher, 2007).

2.1.1 EPIDEMIOLOGY OF DIABETES

The global prevalence of diabetes in the age group of 20-79 yrs in the year 2010 was projected to be 220 billion in the year 1999 but this number was reported to be 285 million i.e. much above the projections. Stated in percentage wise, 6.6% of world population was suffering with diabetes in 2010. This data is for adults and includes types 1 and 2 diabetes combined.

As per report, the deaths attributable to DM during the year 2010 in India in the age group of 20-79 were 1007642 i.e. 0.1% of total population of country gets eliminated with this disease every year. Unexpectedly, the majority of this population was women (57.73%) and the rest as men. However the number of deaths increases linearly with age in the whole women and men population but in case of males it is highest in the age group of 50-59 (IDF Atlas, Ed. 4th, 2009).

2.2 ALZHEIMER'S DISEASE

Alzheimer's disease (AD), the most common cause of dementia in elderly humans, occurs when neurons in the memory and cognition regions of the brain are accompanied by massive accumulation of abnormal fibrous amyloid β-protein (Aβ). Aβ is deposited as extracellular senile plaques of the neocortex, particularly in the temporal and parietal lobes. It is composed of 39–43 amino-acid long peptides derived from the amyloid precursor protein (APP) by cleavage with β- and γ-secretase. Mutations in AD genes cause an increase in the anabolism of Aβ-42, leading to Aβ deposition and accelerating AD pathology. **Thus, reducing Aβ production in the brains or the activation of mechanisms that accelerate its clearance from brains has become major targets for the development of drugs, since the metabolic imbalance between Aβ anabolic and catabolic activities might be responsible for AD** (Glabe, 2000).

Neurodegeneration affects the cognition (learning, abstraction, judgment, etc.) and the memory with behavioral consequences such as aggression, depression, hallucination, delusion, anger and agitation (Levy et at., 1996). Pathologically, ventricular enlargement and atrophy of the hippocampus (the limbic structure responsible for the memory) and cerebral cortex can be seen. At present, the definitive diagnoses of AD are made upon histological verification of the plaques (or the hyperphosphorylated tau protein) at autopsy.

2.2.1 GENOMICS AND PROTEOMICS OF ALZHEIMER'S DISEASE

The amyloid beta peptide is a normal metabolic byproduct of the amyloid precursor protein (APP). The gene encoding the amyloid precursor protein is located on chromosome 21, and it has been shown that trisomy 21 (Down's syndrome) leads to the neuropathology of AD (Hardy and Selkoe, 2002). APP is normally cleaved by proteases called α-, β-, and γ-secretases, however, mutations

along the gene encoding APP occur at these cleavage sites, eventually leading to the abnormal intra-membranous processing of APP, and the consequential extracellular deposition of Aβ. Likewise, these mutations influence the self-aggregation of Aβ into amyloid fibrils (Walsh and Selkoe, 2004). In addition, the presenilin proteins (PS1 and PS2, located on chromosomes 14 and 1, respectively) have been found to alter the APP metabolism by the direct effect of γ-secretase (Wolfe, 2001). Fagan et al. have shown that the E4 isoform of the apo-lipoprotein E (chromosome 19) facilitates the formation of Aβ fibrils in genetically engineered mice (Fagan et al., 2002). The collective effect of these processes is the increased production and accumulation of 1–42 fragment of amyloid beta peptide. The Aβ (1–42) oligomerizes and deposits in the synaptic space as diffuse plaques. The result is synaptic injury, preceded by the microglial activation, followed by oxidative stress, neuronal death and dementia.

2.2.2 DIABETES AS PROSPECTIVE ENHANCER OF INFLAMMATORY PROCESSES IN ALZHEIMER'S DISEASE

As an inevitable part of aging, advanced glycation end products (AGEs), also known as Maillard products, are sugar-derived oxidation products that covalently attach to long-lived proteins, including collagen, eye lens crystalline and any other deposited protein in the brain (Dukic-Stefanovic et al., 2003; Li et al., 1995). Once a protein has been modified by the addition of AGEs, it becomes cytotoxic, with the number or degree of modification determining the level of toxicity (Gasic-Milenkovic et al., 2001).

As one of the most common non-enzymatic modifications of proteins, the addition of sugar generally takes place on an arginine or lysine residue and results in the formation of a chemically reversible Schiff base or Amadori product. Although the formation of these initial products is reversible,

subsequent alterations to the sugars, including rearrangements, dehydrations and oxidations gives rise to the complex structures known as advanced glycation end products, which are chemically irreversible (Bierhaus et al., 1998; Valencia et al., 2004). AGEs have been demonstrated to have a broad range of effects, two of primary concern being the ability to cross-link proteins and to activate cellular pathways via cell surface receptors (Vlassara and Palace, 2002). The nature of these effects has linked AGEs not only to AD, but to several other diseases, including b2-microglobulin deposits in haemodialysis (Miyata et al., 1994; Miyazaki et al., 1995) and diabetes mellitus, where high glucose levels induce chronic production of AGEs that leads to vascular complications & inflammation (Schmidt et al., 1995; Schmidt and Stern, 2000; Vlassara and Palace, 2002).

2.3 BLOOD-BRAIN BARRIER (BBB)

The blood-brain barrier (BBB) is the specialized system of capillary endothelial cells that protects the brain from harmful substances in the blood stream, while supplying the brain with the required nutrients for its proper function. Unlike peripheral capillaries that allow quite free exchange of substances across/among cells, the BBB strictly limits transport into the brain through both physical (tight junctions) and metabolic (enzymes) barriers (Begley, 2004). Thus, BBB is often the rate-limiting factor in determining permeation of therapeutic's into the brain.

Innovation of drug delivery vehicles that provide temporal and spatial distribution of bioactives is a tough task. In the context of drug delivery to brain, the BBB plays a crucial role to regulate the access of drug molecules. It serves as a major constraint for the entry of hydrophilic drugs and the efflux pumps present on its surface restrain the intracellular accumulation of pharmacological moieties in the brain. Since last two decade, the transport of drugs across the brain has

been the most challenging and the field being most explored by research scientists. BBB is composed of capillary endothelial cells, interspersed with tight junctions, and is cased in astrocyte processes. BBB acts as a semi-permeable membrane that allows the access of only limited molecules. Moreover, the presence of efflux pumps actively takes the bioactives out of the CNS (Pardridge, 2003). Any attempt to alter the chemical structure of a bioactive may lead to the loss of its activity. Hence, the drug may be delivered to the site of action by using some delivery vehicle which may overcome the hindrance of crossing BBB.

2.3.1 STRUCTURE AND MOLECULAR ANATOMY OF BBB

The BBB is a unique, selective barrier formed by the endothelial cells that line cerebral capillaries, together with perivascular elements such as closely coupled astrocytic end-feet processes, perivascular neurons and pericytes (Cecchell et al., 2007; Newton, 2006). Astrocytic end-feet processes form around 99% of the CNS capillaries, yet they do not contribute to the physiological activity of BBB. They however secrete atropic factor necessary for the differentiation, function and health of the BBB endothelial cells. Vascular pericytes also release various soluble factors which help in differentiation and maintenance of BBB (Abbott et al., 2006; Newton, 2006). The endothelial cells of BBB are distinguished from those in the periphery by increased mitochondrial content, a lack of fenestrations, minimal pinocytotic activity (Hawkins and Davis, 2005) and the presence of complex tight junctions formed by the interaction of several transmembrane proteins (like occludin, claudins and junctional adhesion molecules-JAMs) that effectively seal the paracellular pathway (Cecchell et al., 2007; Newton, 2006) and divide the membranes of the endothelial cells into two distinct sides, luminal (blood side) and abluminal (brain side) (Hawkins et al., 2006; Pardridge, 2005). Attached at irregular intervals to the abluminal membrane of the endothelium are pericytes.

Pericytes and endothelial cells are ensheathed by the basal lamina (a 30 to 40 nm thick membrane composed of collagen type IV, heparin sulfate proteoglycans, laminin, fibronectin, and other extracellular matrix proteins). The basal lamina is contiguous with the plasma membranes of astrocyte end-feet, which ensheathe cerebral capillaries (Hawkins and Davis, 2005). The interrelationship between the endothelium, the pericyte and the astrocyte foot processes is as intimate as any cell–cell interactions in biology. The space filled by the basement membrane and situated between the endothelium/pericyte and the astrocyte foot process forms the interface between blood and brain **(Fig. 2.1).**

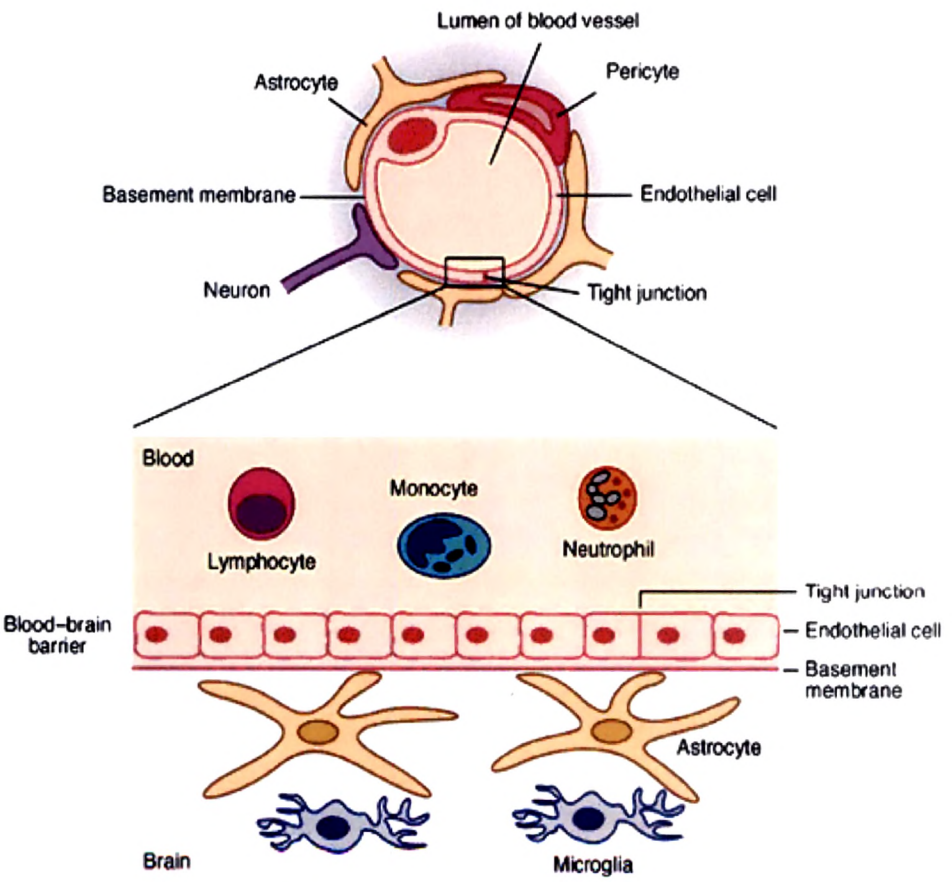


Figure 2.1 A representative cross-section of a cerebral capillary of the BBB

The transport of a ligand or a substrate either from brain to the blood, or from blood to the brain requires movement across the capillary endothelial plasma membrane. The solute transfer across the capillary endothelial barrier is a process of transport through two membranes in series. However, for a molecule to move from blood to brain interstitial space beyond the astrocyte foot process, the molecule must also escape the immediate perivascular space bordered by the plasma membrane of capillary endothelial cells, pericytes and astrocyte foot processes. Hence, the transport of nutrients or drugs across the BBB may be the result of a complex interplay between active efflux systems located on the endothelial plasma membrane, active transporters within the astrocytic foot process and ectoenzymes present on the pericyte plasma membrane (Pardridge, 2005).

Brain endothelial cells also contain numerous membrane transporters (e.g. P-glycoproteins; P-gp and multi drug resistance associated protein family; MRP) both on the luminal and abluminal membranes of the capillaries that regulate the transcellular traffic of essential molecules between brain and blood, as well as effluxing potentially harmful substances and waste products. BBB transporters exist for a variety of molecules, such as amino acids, glucose, micronutrients, electrolytes, hormones and peptides, and all of them do not operate equally well in both, the blood-to-brain and brain-to-blood direction. The structural difference between the brain capillaries and the nonbrain capillary endothelium is associated with the endothelial tight junctions. The nonbrain capillaries have fenestrations (openings) between the endothelial cells through which solutes can move readily via passive diffusion, whereas in brain capillaries, the endothelium epithelial is unfenestrated (tight junctions without openings). These unfenestrated capillaries are cemented together by intercellular tight junctions and the electrical resistance across intra-parenchymal endothelial cells may be as

high as 2000 Ω cm^2. The presence of tight junctions thus eliminates a paracellular pathway of solute movement across the BBB and there is a virtual absence of pinocytosis across brain capillary endothelium (Golden and Pardridge, 1999).

As a direct consequence of the existence of BBB, there is a clinical difficulty in delivering therapeutic compounds to the CNS. Polar drugs cannot diffuse freely into the brain (Kreuter, 2001), as the paracellular pathway is absent, thus, unlike capillaries in the persipheral circulation, the diffusion of aqueous solutes from the blood to the brain extracellular fluid or in the reverse direction cannot occur. The presence of few endocytic vesicles in the CNS capillaries, further removes a transcellular route for free diffusion of substances into the interstitium (Newton, 2006). Further a large number of more lipophilic drugs are also subject to the activity of efflux transporters. As a result of which, a significant number of CNS diseases have poorly met therapy largely due to the difficulty of delivering drugs across the BBB (Begley, 2003; Begley and Brightman, 2003). The ability of a particular substance to cross the BBB and enter the brain is dependent upon several factors:

1. *Drug related factors at the BBB:* Concentration at the BBB and the size, flexibility, conformation, ionization (nonionized form penetrates BBB) (Newton, 2006) and lipophilicity of the drug molecule, its cellular enzyme stability and cellular sequestration, affinity for efflux mechanisms (i.e. P-glycoprotein), hydrogen bonding potential (i.e. charge), affinity for carrier mechanisms, and effect on all of the above by the existing pathological conditions (Levin, 1980; Golden and Pardridge, 1999).

2. *The physicochemical characteristics:* e.g. log $P_{o/w}$ of the therapeutic agent is one of the most informative parameter. In this regard the rule of 2 is generally accepted i.e. the value of log $P_{o/w}$ nearing 2 is considered optimal (Gupta, 1989;

Levin, 1980). However, increasing the lipophilicity with intent to increase permeability would increase the volume of distribution (V_d) and also the rate of oxidative metabolism by cytochrome P450 (Levin, 1980; Lewis and Dickins, 2002; van deWaterbeemd et al., 2001). Peripheral factors including systemic enzymatic stability, plasma protein binding affinity, uptake of the drug into other tissues, clearance rate, and effects of existing pathological conditions are also important.

2.4 STRATEGIES FOR ENHANCED DRUG DELIVERY TO CNS

To circumvent the multitude of barriers inhibiting CNS penetration by potential therapeutic agents, numerous drug delivery strategies have been developed which can be categorized as follows:-

1. **Invasive procedures:** These include transient osmotic opening of the BBB, (Kroll and Neuwelt, 1998; Rapoport, 2000) shunts, (Alexander, 2000) and biodegradable implants (Benoit et al., 2000; Emerich et al., 1999). These procedures can be highly traumatic and often have low therapeutic efficiency with substantial side effects.

2. **Pharmacologically-based approaches:** These enhance the passage through the BBB by increasing specific biochemical attributes of a compound. This may be accomplished either by chemical modification of the therapeutic molecule itself, or by the attachment or encapsulation of the drug in a substance that increases permeability, stability, bioavailability, and/or receptor affinity. In addition, modification of drug structure and/or addition of constituents (eg, lipophilicity enhancers, polymers, antibodies) may enhance drug concentration within the CNS, with a reduced toxic profile. These include liposomes, nanoparticles, nanoconjugates and chemical drug delivery.

3. **Physiologically based strategies:** They exploit the various carrier mechanisms at the BBB, which have been characterized in recent years for nutrients, peptide and nonpeptide hormones, and transport proteins (ligand binding proteins).

These strategies can be combined, dependent of the nature of a given drug molecule, creating "hybrid" molecules, resulting in synergistic CNS delivery and end-effect.

4. **Drug modification:** Drug modification can be broadly divided into several categories such as lipidization, increasing affinity for nutrient transporters, prodrugs, vector-based, cationization, and polymer conjugation/ encapsulation.

(a) Lipidization

Lipid solubility is a key factor in determining the rate at which a drug passively crosses the BBB (Chikhale, 1994). There are many approaches which can be used to enhance lipid solubility of drugs, but lipidization does have several limitations. Since highly lipid-soluble drugs may be extensively bound to plasma proteins, there is a potential for reduction in the amount of free or exchangeable drug in the plasma, thereby compromising brain uptake. (Audus et al., 1992) The site of modification or attachment of substances/ molecules to increase lipophilicity must also be taken into account, as receptor binding affinity may be diminished if alterations are within the pharmacophore region, thus reducing biological activity. Thus enhancement of lipophilicity alone may not necessarily improve BBB transport. Factors such as molecule size, stability, intracellular sequestration, non-target organ uptake, efflux rates, and P-glycoprotein (P-gp) efflux affinity must also be considered.

(b) Nutrient Transporters

Drug or drug delivery vehicles may also incorporate specific molecular characteristics that enable the drug to be transported by one or more of the inwardly directed nutrient carriers. The BBB expresses several transport systems for nutrients and endogenous compounds (Begley, 1996; Tsuji and Tamai, 1999). Utilization of these transport systems is a potential strategy for controlling the delivery of drugs into the brain. These drugs/carriers must have a molecular structure mimicking the endogenous nutrient. The prototypical example is levodopa, a lipid-insoluble precursor of dopamine that has been used for the treatment of Parkinson's disease because it contains the carboxyl and α-amino groups that allow it to compete for transport across the BBB by the large neutral amino acid carrier (Wade and Katzman, 1975). Chemical groups could be designed with the ability to attach to specific drugs rendering them substrates for carriers, or drugs specifically designed for a carrier mechanism. The hexose and large neutral amino acid carriers have the highest capacity and presently are the best candidates for delivery of substrates to the brain. Lower capacity carriers may also be utilized for highly potent drugs. Drugs, which generally require low concentrations to induce effect, are most appropriate for this type of targeting design. Yet, the complexity in tailoring a bioactive molecule to target specific nutrient transporters requires a great deal of knowledge of both carrier and transporter. Nevertheless, these transport mechanisms may also be advantageous targets for prodrug and vector-mediated approaches to enhance peptide delivery to the brain.

(c) Prodrugs

Prodrugs contain a pharmacologically active moiety that is either conjugated to a molecule with a known transporter or to a lipophilicity enhancer, which is

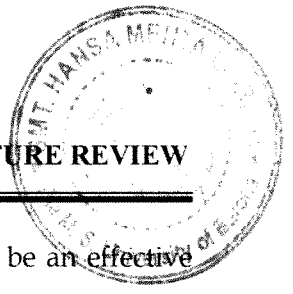
cleaved at or near the site of action, allowing drug to induce its effect. The rationale for prodrug design is that the structural requirements necessary to elicit a desired pharmacological action and those necessary to provide optimal delivery to the target receptor site may not be the same. The ideal prodrug is enzymatically stable in the blood, but rapidly degraded to the active parent compound once it is within the target tissue. Esters have shown particular promise in the area of prodrug design for brain delivery, owing to the abundance of endogenous esterases in the CNS. Esterification or amidation of amino, hydroxyl, or carboxylic acid-containing drugs may greatly enhance lipid solubility, and thus brain entry (Ghosh and Mitra, 1992; Lambert et al., 1995). Once in the CNS, hydrolysis of the modifying group releases the active compound. Both aromatic benzoyl esters (Ghosh and Mitra, 1992) and branched chain tertiary butyl esters (Greig et al., 1990) have shown stability in plasma, while still adequately cleaved within the CNS. Another prodrug design focuses on the redox system, (Bodor et al., 1975; Prokai et al., 2000) in which a lipophilic attachment (eg, 1,4-dihydro) is converted to the hydrophilic quaternary form, effectively "locking" the drug in the tissue (Bodor and Buchwald, 1999) When a drug is conjugated to a methyldihydropyridine carrier and subsequently oxidized by NADH-linked dehydrogenases in the brain, it results in a quaternary ammonium salt, which does not cross back through the BBB endothelium (Brewster et al., 1988). Similar design has been explored with a wide variety of drugs, such as steroids, antivirals, neurotransmitters, anticonvulsants, and peptides (leucine enkephalin analog and TRH analog) (Bodor and Buchwald, 1999; Prokai-Tatrai et al., 1996). The primary difficulties with the redox design are that any tissue may take up the lipophilic moiety, and rapid elimination of the charged salt form occurs. The difficulty with prodrugs lies primarily within

the pharmacokinetics. Precise placement and choice of cleavable moiety must be optimized to obtain the most efficacious pharmacokinetic profile.

(d) Polymer conjugation/encapsulation

Structural modifications can be obtained by the covalent conjugation of bioactives to polymers. Polymer conjugations are used to increase stability, reduce elimination, and reduce immunogenicity (Francis et al., 1998; Mumtaz and Bachhawat, 1991; Reddy, 2000; So et al., 1999; Tsutsumi et al., 2000). Chemical modification with macromolecular polymers, such as poly(ethylene glycol) (PEG), (So et al., 1999; Tsutsumi et al., 2000) and poly(styrene maleic acid), (Duncan, 1992; Mu et al., 1999) shows significant promise.

Pegylation is a procedure of growing interest for enhancing the therapeutic and biotechnological potential of drugs and proteins. When PEG is correctly linked to a drug/peptide it will modify many of the pharmacokinetic features, while theoretically maintaining the primary biological activity (i.e., enzymatic activity or receptor recognition). PEG chains may contain linear and branched structures, which can be conjugated directly to the drug or linked in a prodrug manner. PEG conjugation masks the drug's surface and increases the molecular size, thereby reducing immune response, enzymatic degradation, toxicity and renal ultrafiltration (Reddy, 2000). PEGs may also produce improved physical and thermal stability, as well as increased solubility (Veronese, 2001). The principal disadvantage of pegylation is the potential loss of activity with improper choice of PEG (i.e., length, branching, chemical design) or unfavorable choice of attachment site. Another considerable disadvantage to pegylation, in regard to CNS focused drug delivery, is the enhanced hydrophilicity and molecular size that can bring about significant reductions in passive diffusion across the BBB.



Encapsulation, via nanoparticles and liposomes, may also be an effective manner by which increased delivery to the brain can be achieved. Nanoparticles are polymeric particles ranging in size between 10 and 1000 nm, which may have a polysorbate overcoating. Drugs can be bound in the form of a solid solution or dispersion, or they can be adsorbed on to the surface of the particles or chemically attached. Polysorbate-80 coated nanoparticles have been shown to enhance delivery of the leu-enkephalin analog dalargin (Kreuter et al., 1995). The particles are thought to mimic low density lipoproteins (LDL) and could therefore be taken up into the endothelium of the BBB via the LDL receptors. Enhanced transport of these nanoparticles may also involve tight junction modulation (Kreuter, 2001) or P-gp inhibition (Woodcock et al., 1992). Liposomes are composed of a phospholipids bilayer that may act as a carrier for both hydrophilic and hydrophobic drugs. The beneficial attributes of liposomes are enhanced plasma half-life, decreased clearance, and decreased toxicity of associated drug (Reddy, 2000). One such liposome formulation is the pluronic copolymer P85, a self-forming micelle preparation that encapsulates a drug. The P85 formulation has been shown to enhance the delivery of digoxin into the brain, (Batrakova et al., 2001) and to enhance the analgesic profile of biphalin, DPDPE, and morphine, (Witt et al., 2002) with the mechanism of action theorized to be the inhibition of P-gp. One of the disadvantages of the P85 micellar formulation is the observation that P85 may function via depletion of ATP, which would reduce P-gp activity, yet may also result in undesired responses within the endothelium (Batrakova et al., 2004).

(e) Vector-based approach

Physiologically vector-based strategy involves the use of existing BBB transport properties to enhance brain entry of a specific drug, much the same as prodrugs.

The principle of the vector-mediated or chimeric delivery strategy lies in the coupling of a moderately impermeable drug to a substance that increases the affinity to and transport across a biological membrane via receptor-mediated or absorptive mediated endocytosis. After entry into the brain, these chimeric drugs are released via enzymatic cleavage, allowing the drug to initiate a pharmacological action in the brain. This technology may be adapted for use with drug pharmaceuticals, peptides, nucleic acid therapeutics and small molecules. Important design considerations include vector specificity for the brain, pharmacokinetics of the vector, and placement and cleavability of the linker between the drug and vector. Multiple concepts for such systems exist, with present focus on antibody attachment and chemical linker strategies (Bickel et al., 2001; Pardridge et al., 1995; Pardridge, 1999; Zlokovic, 1995).

Receptor-mediated vectors for brain delivery must be specific. There are several potential receptors at the BBB that may serve this purpose. The plasma protein transferrin is able to bind and undergo endothelial endocytosis in brain capillaries and has proven to be a suitable vector. A murine monoclonal antibody (OX26) to the transferrin receptor has been successfully used to increase brain uptake of proteins and peptides. An analog of the opioid peptide dermorphin ([Lys7] dermorphin) was conjugated to the OX26 vector, demonstrating analgesia, which was reversed by naloxone (Bickel et al., 1995). Cationized albumin has also been used as a vector to enhance peptide uptake, via adsorptive-mediated endocytosis. When cationized albumin was conjugated to β-endorphin, it yielded increased uptake into isolated brain endothelial cells, as compared with β-endorphin alone (Pardridge et al., 1990).

Several additional limitations should be considered with the use of a receptor-mediated vector design. There exists the potential competition between

the vector-drug and the endogenous ligand for the receptor, as such endocytotic mechanism tends to have low capacity in the brain. This phenomenon would result in decreased vector transport and/or decreased concentration of a required nutrient to the brain, resulting in a subsequent pathology. In addition, the quantity of drug delivered to the brain is directly limited by transporter concentration. Transporter capacity may become saturated or downregulated over time, decreasing the ability to deliver an adequate and consistent dosage of drug to elicit a pharmacological effect. This possibility is important if the use of a drug requires consistent and repeated doses. Therefore, this approach would likely require extremely potent therapeutic peptides, which may be most effective for acute disease states.

(f) Cationization

Cationization of drugs is a manner of increasing membrane entry via absorptive-mediated endocytosis (Deguchi et al., 2003; Deguchi et al., 2004; Hardebo and Kahrstrom, 1985; Pardridge et al., 1995; Vehaskari et al., 1984). Presence of anionic sites on BBB endothelium brings about attraction of cationized substances to the membrane surface (Chakrabarti and Sima, 1990). The dynorphin-like analgesic peptide E-2078, and the adrenocorticotropic hormone (ACTH) analog, ebiratide, are polycationic peptides at physiologic pH which have shown to internalize into brain capillaries by absorptive-mediated endocytosis (Shimura et al., 1992; Terasaki et al., 1989; Terasaki et al., 1992; Yu et al., 1997). Cationized albumin attached to beta-endorphin, in a vector based manner, showed significant increase in membrane uptake by AME (Kumagai et al., 1987). Cationized albumin displayed longer serum half-life and a general selectivity to the brain (Bickel et al., 2001). In addition, avidin-cationized albumin conjugates have been proposed to affect the delivery of biotinylated,

phosphodiesterase, antisense oligodeoxynucleotides to the brain. These conjugates retain function (i.e. inactivation of target mRNAs) and impart a degree of stability to serum and cellular enzymes (Boado and Pardridge, 1994). Unfortunately the potential toxicity of this strategy limits its clinical use for therapeutics. Cationized proteins have been shown to induce immune complex formation with membranous nephropathy, (Adler et al., 1983; Huang et al., 1984) and increased cerebral and peripheral vascular permeability (Hardebo and Kahrstrom, 1985; Nagy et al., 1983; Vehaskari et al., 1984). Cationized albumin has been shown to be significantly cleared by the kidney and liver, posing a potential toxicological threat as well (Bergmann et al., 1984; Bickel et al., 2001; Pardridge et al., 1989). This approach is also non-specific as to tissue uptake, unless additionally coupled to a selective vector.

2.5 NOVEL DRUG DELIVERY SYSTEM APPROACH

Two main reasons for the failure of drug delivery to the brain are:

- Poor penetration of the drug molecule across the BBB.
- Back transport (efflux) of drugs from the brain to the blood.

Various colloidal delivery systems have been tried upon by different researchers to overcome, especially the first aspect. These systems include liposomes, microspheres, lipid microspheres, niosomes, polymeric nanoparticles, and solid lipid nanoparticles (SLNs) (Alyautdin et al., 1997; Azmin et al., 1985; Chen and Lee, 1993; Fresta et al., 1994; Minagawa et al., 1996; Muller, et al., 1996; Siekmann and Westesan, 1992). For a delivery system to be effective, a high drug loading, physical and chemical stability and a low incidence of toxicity of the carrier used are a few important factors. Further the *in vivo* fate of the carrier, the chances of scaling up the process of producing the drug delivery system and the

overall cost are other considerations to be kept in mind before deciding on the suitability of the system (Gasco, 1993; Gualbert et al., 2003; Siekmann and Westesan, 1992; Schwarz and Mehnert, 1999). The cerebral distribution parameters of drugs are further governed by a number of factors like plasma protein binding, cerebral blood flow rate, influx and efflux rates at the BBB, blood-CSF barrier and the brain parenchyma, rate of drug metabolism in the brain, drug-tissue interactions/binding in the brain and flow rate. Thus it becomes important to consider and optimize these parameters while selecting a suitable delivery system for brain delivery.

Various newer techniques like genomics and proteomics could be used to direct the novel drug delivery system to their target sites by unearthing pathways involved in disease pathogenesis and thus identifying candidate brain specific transport systems that could be exploited to ferry the drug cargo from the blood to the brain as a mode of noninvasive delivery (Shusta, 2005).

2.5.1 DRUG-NANOCARRIERS

The treatment of brain related disorders is limited by the inadequacy in delivering therapeutic agents in such a way that drug molecules reach the desired targets. In order to achieve efficient treatments of CNS, it is necessary to transport therapeutic agents across the specialized vascular system of the brain, the BBB, which can present formidable challenges. These include the definition of the properties of cerebral vascular system during cancer progression and the development of biotechnology to prepare biomarker-targeted delivery of multiple therapeutic agents, coupled to the possibility of avoiding various resistance mechanisms. A great deal of effort, therefore, is presently focused on improving CNS bioavailability of therapeutic drugs that can be specifically targeted to diseased tissue, improving therapeutic opportunities, efficiency, and

patient survival, while decreasing side-effects to normal cells. Devices, such as functionalized drug colloidal carriers, can take advantage of these features and act as vehicles to deliver, selectively and specifically, anti-cancer drugs to tissues, either using passive mechanisms relying on increased vascular permeability in a defined location, or using active targeting of chemically modified drugs or nanoparticles. Alternatively, direct modification and/or functionalization of drugs, involving the chemical conjugation of drugs to disease-targeting biological markers, can be used to achieve direct active targeting of drugs.

Drug-carrier nanoparticles (Beduneau et al., 2007; Juillerat-Jeanneret, 2006; Koo et al., 2006) are defined as submicroscopic colloidal systems that may act as drug vehicles, either as nanospheres (matrix system in which the drug is dispersed) or nanocapsules (reservoirs in which the drug is confined in a hydrophobic or hydrophilic core surrounded by a single polymeric membrane). Clinical status of polymeric nanoparticles for drug delivery under development (Alexis et al., 2008) is listed in **Table 2.1**. Micelles are self-assembling amphipathic colloidal aggregates of hydrophilic and hydrophobic block copolymers in which hydrophobic or hydrophilic drugs are physically trapped or covalently bound. Following systemic administration, drug delivery devices must be transported across the vascular wall into the surrounding tissues and the interstitial space. The structure of the polymer and the method of trapping drugs in the nanoparticles will define the drug release kinetics and characteristics (Juillerat-Jeanneret, 2006).

To achieve this goal, however, it is necessary to develop tools to entrap drugs into vectors capable of releasing the drugs at the right place and time from their vector. In addition, the delivery systems and the therapeutic agents must resist hydrostatic, hydrophilic/hydrophobic and biophysical/biochemical

barriers, resistance to treatment developed in brain delivery and biotransformation, degradation, and clearance mechanisms. The use of biodegradable polymeric matrices solves the issue of removal of the device after delivery of the drug. Surface modification of nanospheres made of polymer (synthetic or natural) aggregates or of nanoliposomes in which the drug is either dissolved, entrapped, encapsulated, or covalently attached, is possible. Drugs may also be attached to their vector carriers via linkers. The design of the chemical bonds linking the drugs to their carriers is also of potential interest for the selective release of the therapeutic agents (Reents et al., 2002; Schoenmakers et al., 2004). However drug-loaded targeting and transport-enhancing nanoparticles must match the mechanical properties and degradation rates that are needed for the application (Grazia et al., 2002; Missirlis et al., 2005). The polymers need to have features such as controllable mechanical properties, degradation rates, minimal toxicity, and immune response (Liu et al., 2004). Conjugation of ligands targeting the BBB on the surface of colloidal carriers, either by covalent or non-covalent linkage, increases selectivity for brain disorders and the future of development for transporting therapeutic agents across the BBB for treatment of brain disorders probably relies in the development of targeting transport-enhancing nanocarriers. Active targeting requires that reactive groups exist at the surface of nanoparticles for chemical coupling and that selective ligands for defined cell markers are presented in adequate configuration and concentration at the surface of nanoparticles. Drug release by the carrier must follow extravasation and transvascular transport and therapeutic concentration of drug must be attained.

Composition (Trade Name)	Clinical Status	Particle Size (nm), Drug, Circulation T½	Efficacy
methoxy-PEG-poly (D,L-lactide) Taxol (Genexol-PM)	Phase II	30-60, paclitaxel, 12 h (human)	75% of metastatic breast cancer patient showed 2 years overall survival
HPMA-DACH palatinate (ProLindac, previously AP5346)	Phase II	6-15, DACH-platinum, 70 h (human)	NA
PEG-arginine deaminase (Hepacid, previously ADI-SS PEG 20000 MW)	Phase I/II	NA , arginine deaminase, 7days (human)	double median survival time of patients with metastatic melanoma; 47% response rate in HCC patients (n=19)
PEG-camptothecin (Prothecan)	Phase I/II	NA, camptothecin, 40 h (human)	NA
Pluronic-doxorubicin (SP1049C)	Phase II	~25, doxorubicin,3 h (human)	3 patients over 21 showed response
polycyclodextrin camptothecin (IT-101)	Phase I	~40, camptothecin, 38 h (mice)	preliminary data reported stable disease rate in patients with solid tumors
Polyglutamate-camptothecin (CT-2106)	Phase I/II	NA, camptothecin, 44-63 h (human)	Less toxicity than free drug
polyglutamate paclitaxel (Xyotax)	Phase III	NA, paclitaxel, 100 h (human)	response rate of 10% for 99 patients & a median time to disease progression of 2m
dextran-doxorubicin (AD-70)	Phase I	NA, doxorubicin, 3-12 h (human)	discontinued due to severe hepatotoxicity limiting the dose at 20 mg/m^2
dextran-camptothecin (DE-310)	Phase I/II	NA, camptothecin, 300-400 h (human)	no new major toxicity compared to drug beside reversible hepatotoxicity
HPMA-paclitaxel (PNU166945)	Phase I	NA, paclitaxel, 3-12 h (human)	discontinued due to severe neurotoxicity
HPMA-doxorubicin (PK1)	Phase II	NA, doxorubicin, 93 h (human)	hepatic toxicity at doses >120 mg/m^2; two partial & two minor responses in over 36 patients
PEG-aspartic acid-doxorubicin (NK911)	Phase I	30-50, doxorubicin, 1.6-4.7 h (human)	no severe toxicity; Phase II clinical trial for pancreatic cancer
HPMA-doxorubicin with galactosamine (PK2)	Phase I	~8.4, biphasic clearance with half-lives of 2.9 and 26.7 h when 120 mg/m^2 administered by 24 h infusion (human)	Of 18 patients, three responded to treatment, with two in partial remission for >26 and >47 months

Table 2.1: Clinical Status of Polymeric Nanoparticles for Drug Delivery under Development (Alexis et al., 2008)

Nanoparticles are generally internalized into cells via fluid phase endo-cytosis, receptor-mediated endocytosis, or phagocytosis. Nanoparticle surface manipulations may be performed to increase cell uptake and the potential delivery of the nanoparticles in different cell compartments (Cengelli et al., 2006; Dubowchik and Walker, 1999; Panyam and Labhasetwar, 2004; Savic et al., 2003).

2.5.2 MECHANISM OF NANOPARTICLE - MEDIATED DRUG TRANSPORT TO THE BRAIN

Fabricating nanoparticles with certain targeting ligands or coating them with some agent can lead to the development of carrier systems of diversified nature and ultimately target specific. The nanoparticles can gain access in the brain via, employment of a number of possible mechanisms. These mechanisms either alone or in combination can elucidate the movement of therapeutic bioactives across the BBB. A number of possibilities exist that could explain the mechanism of delivery of above mentioned substances across the blood-brain barrier (Agarwal et al., 2009):

1. An increased retention of nanoparticles in the brain blood capillaries combined with an adsorption to the capillary walls. This could create a higher concentration gradient that would enhance the transport across the endothelial cell layer and as a result the delivery to the brain.
2. A general surfactant effect characterized by a solubilisation of the lipids of the endothelial cell membrane that would lead to membrane fluidisation and enhanced drug permeability through the blood-brain barrier.
3. The nanoparticles could lead to an opening of the tight junctions between the endothelial cells. The drug could then permeate through the tight junctions in the free form or together with the nanoparticles in bound form.

4. The nanoparticles may be endocytosed by the endothelial cells followed by the release of drug within these cells and delivery to the brain.

5. The nanoparticles with bound drugs could be transcytosed through the endothelial cell layer.

6. The polysorbate 80 used as the coating agent could inhibit the efflux system, especially P-glycoprotein (Pgp).

2.6 TECHNIQUES FOR PREPARATION OF NANOPARTICLES

Nanoparticles can be obtained by (a) polymerization of monomers entrapping the drug molecules or (b) from preformed polymers (Couvreur et al., 1995).

A. Nanoparticles prepared by polymerization process:

Two types of polymerization processes are used to prepare polymeric nanoparticles.

I. **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 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.

II. **Emulsion polymerization:** Emulsion polymerization is one of the fastest methods for the preparation of nanoparticles and is readily scalable (Kreuter, 1990). Emulsion polymerization may be performed

using either organic or aqueous media as continuous phase. In this technique, the monomer is emulsified in non-solvent containing surfactant, which leads to the formation of monomer swollen micelles and stabilized monomer droplets. The continuous organic phase methodology involves the dispersion of monomer into an emulsion or inverse microemulsion, or into a material in which the monomer is not soluble (nonsolvent) (Ekman and Sjfholm , 1978; Lowe and Temple, 1994).

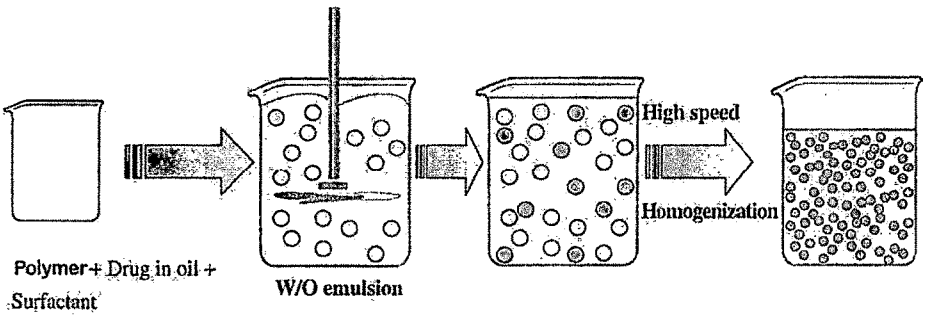


Figure 2.2 Schematic representation of Emulsion Polymerization technique.

The monomer is dissolved in a continuous phase that is usually an aqueous solution, and the surfactants or emulsifiers are not needed. The polymerization process can be initiated by different mechanisms. Initiation occurs when a monomer molecule dissolved in the continuous phase collides with an initiator molecule that might be an ion or a free radical. Alternatively, the monomer molecule can be transformed into an initiating radical by high-energy radiation, including γ-radiation, or ultraviolet or strong visible light. Chain growth starts when initiated monomer ions or monomer radicals collide with other monomer molecules according to an anionic

polymerization mechanism (Vauthier et al., 2003). Phase separation and formation of solid particles can take place before or after termination of the polymerization reaction (Kreuter, 1982).

B. Nanoparticles prepared from 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). The basic methodologies of the commonly used preparation methods are as follows:

I. **Emulsion-evaporation:** Emulsification-solvent evaporation involves two steps. The first step requires emulsification of the polymer solution into an aqueous phase. During the second step polymer solvent is evaporated, inducing polymer precipitation as nanospheres.

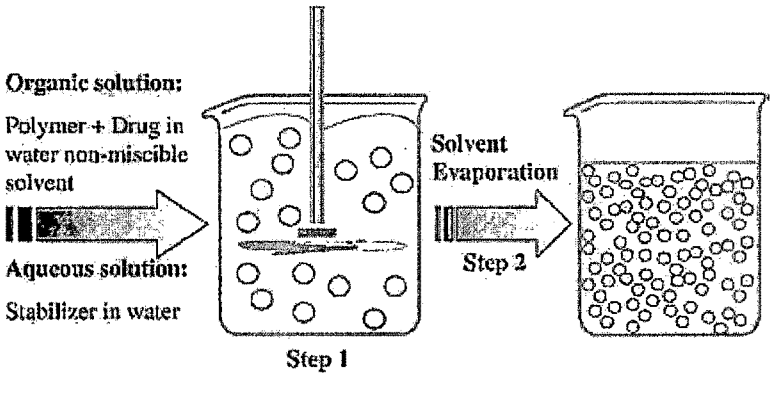


Figure 2.3 Schematic representation of the Emulsion-Evaporation Technique

A polymeric solution in organic solvent containing the dissolved drug is dispersed into nanodroplets, using a dispersing agent and high-energy homogenization, in a nonsolvent or suspension medium (Tice and Gilley, 1985). The polymer precipitates in the form of

nanoparticles in which the drug is finely dispersed in the polymer matrix network. The solvent is subsequently evaporated by increasing the temperature under pressure or by continuous stirring (Soppimath et al., 2001).

II. **Double-emulsion evaporation:** This procedure is used to prepare nanoparticles encapsulating hydrophilic drugs and proteins.

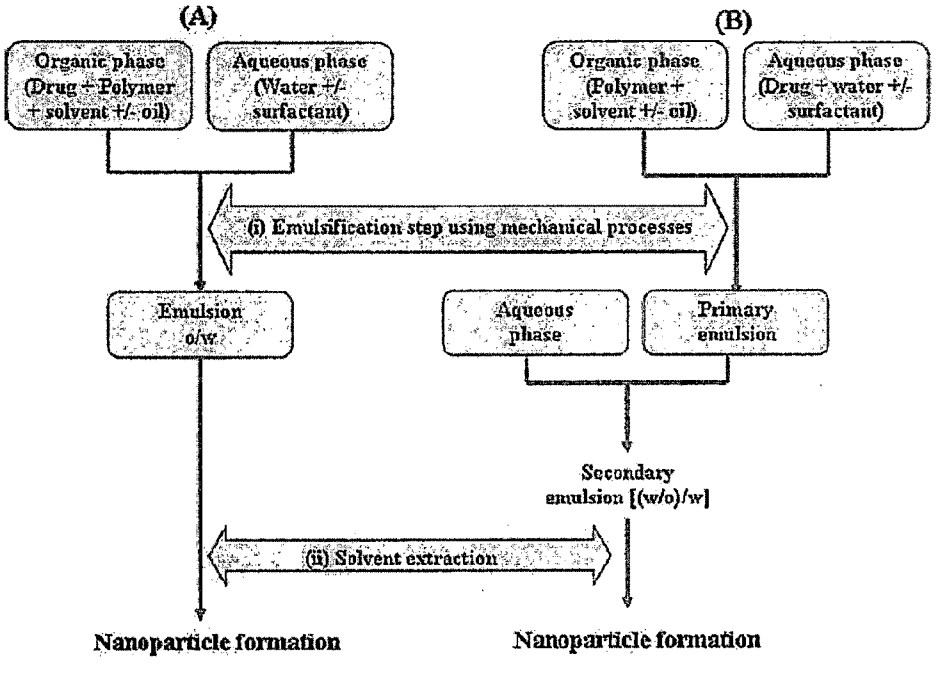


Figure 2.4 Schematic Representation of (A) Single and (B) Double Emulsion Techniques for preparation of Nanoparticles (Vauthier and Bouchemal, 2009)

III. **Salting-out:** Salting-out is based on the separation of a water miscible solvent from aqueous solution via a salting-out effect. Polymer and drug are initially dissolved in a solvent such as acetone, which is subsequently emulsified into an aqueous gel containing the salting-out agent (electrolytes, such as magnesium chloride, calcium chloride, and

magnesium acetate, or non-electrolytes such as sucrose) and a colloidal stabilizer such as polyvinylpyrrolidone or hydroxyethylcellulose. This oil/water emulsion is diluted with a sufficient volume of water or aqueous solution to enhance the diffusion of acetone into the aqueous phase, thus inducing the formation of nanospheres (Quintanar-Guerrero et al., 1998).

IV. **Emulsion-diffusion:** This method is derived from the salting-out procedure. It involves adding of a polymer solution, in partially water miscible solvent (such as ethyl acetate, benzyl alcohol, propylene carbonate) presaturated with water, 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 formation of nanoparticles (Quintanar-Guerrero et al., 1998).

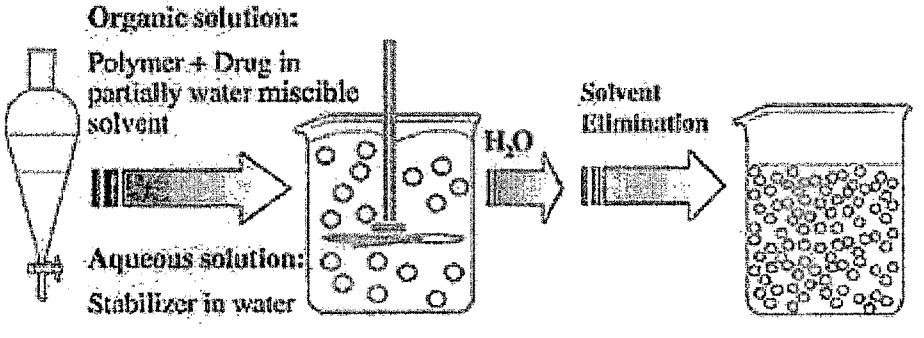


Figure 2.5 Schematic description of the proposed formation mechanism of nanocapsules by emulsification/solvent diffusion.

V. **Solvent displacement:** In this strategy, the encapsulating polymer is dissolved in a partially water miscible solvent and saturated with

water to ensure the initial thermodynamic equilibrium of both liquids. In fact, to produce the precipitation of the polymer and the consequent formation of nanoparticles, it is necessary to promote the diffusion of the solvent of the dispersed phase by dilution with an excess of water when the organic solvent is partly miscible with water or with another organic solvent in the opposite case. Subsequently, the polymer-water saturated solvent phase is emulsified in an aqueous solution containing stabilizer, leading to solvent diffusion to the external phase and the formation of nanospheres or nanocapsules, according to the oil-to-polymer ratio. Finally, the solvent is eliminated by evaporation or filtration, according to its boiling point (Quintanar-Guerrero et., 1998).

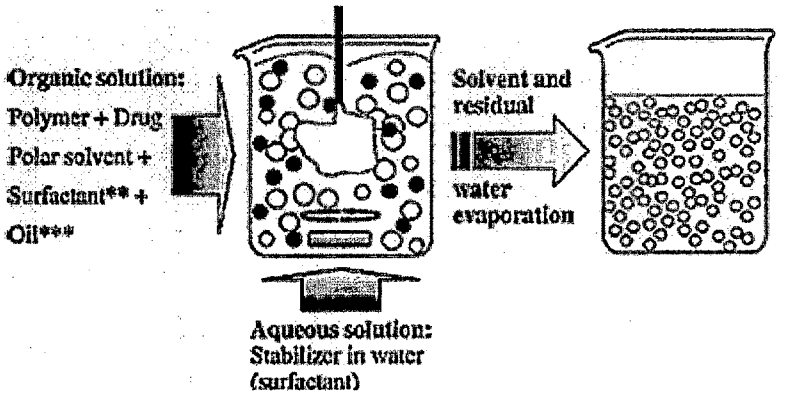


Figure 2.6 Schematic representation of formation of nanoparticles by Solvent Displacement Technique

2.7 STABILITY OF NANOPARTICLES

Lyophillization is one of the well established strategies for the preservation of unstable molecules for prolonged time period (Corveleyn and Remon, 1996; Diminsky et al., 1999; Li et al., 2000). Freeze-dried nanoparticles possess certain desirable characteristics, including: i) the preservation of the primary physical

and chemical characteristics of the product (elegant cake appearance, short reconstitution time, an acceptable suspension and low or unmodified particle size distribution of nanoparticle suspensions, unchanged activity of encapsulated drug), ii) an acceptable relative humidity, and iii) long-term stability. A number of studies have shown a good preservation of the physio-chemical properties of the particles when the cryoprotectant was employed in a sufficient concentration.

2.8 NANOPARTICLES CONJUGATED TO LIGANDS IN RECEPTOR MEDIATED TRANSPORT SYSTEMS

Nanoparticles are well-defined, solid, colloidal particles, ranging in size approximately from 1 to 1000 nm (usually 200-300 nm), having a core-shell structure (nanocapsules) or a continuous matrix structure (nanospheres). Nanoparticles can serve as a potential module for ferrying large doses of drugs across the BBB. They may be instrumental to deliver high therapeutic payload of drug in the brain, helping to obtain optimal therapeutic response with the commencement of minimal side effects (Kreuter, 1994; Ringe et al., 2004).

In order to achieve specific targeting, it is required that there should be some specific carrier molecule to carry the drug. The target ligand is responsible for targeting and providing site specificity to the drug delivery system, at the same time the modifier acts as a lipophilizer and prevents unwanted metabolism of the system.

Receptor-mediated transport (RMT) may serve as a vital strategy responsible for the trafficking of larger molecules such as transferrin. In this approach, a molecule binds to the specific receptor expressed on the endothelial cell. The complex so formed is endocytosed into the cell and transported via

vesicles across the basolateral membrane, thereby gaining access to the CNS. The approach is promising, particularly for delivery of large agents such as antibodies and genes. However this technique is limited by transport systems that have low affinity and capacity, such that basal levels of the endogenous substrate may interfere with the binding of engineered ligands. Examples of receptors involved in RMT are the insulin receptor, the transferrin receptor, and the transporters for low-density lipoprotein, leptin and insulin-like growth factors. In general, RMT occurs in 3 steps: (i) receptor-mediated endocytosis of the compound at the luminal (blood) side, (ii) movement through the endothelial cytoplasm, and (iii) exocytosis at the abluminal (brain) side of the brain capillary endothelium.

2.8.1 TARGETED DELIVERY MEDIATED THROUGH TRANSFERRIN RECEPTORS

2.8.1.1 Transferrin

Transferrin (Tf) molecule consists of a single polypeptide chain of molecular weight about 80,000 organized into two domains of approximately equal size. Each domain contains one Fe-binding site (Harris and Aisen, 1989). Tf is a member of a closely related family of Fe-binding glycoproteins which also include lactoferrin, melanotransferrin, and ovotransferrin (Morgan, 1996). The primary function of plasma Tf is to act as Fe carrier in the plasma and interstitial fluids of the body. Iron (Fe) is an essential component of virtually all types of cells and organisms (Baker and Morgan, 1994). Iron-containing transferrin has a high affinity for the transferrin receptor, which is present on all cells with a requirement for Fe. Plasma Tf is synthesized in the liver (Morgan, 1983) but similar proteins are also synthesized in the brain (Bloch *et al.*, 1985, 1987; Dickson

et al., 1985; Dziegielewska *et al.*, 1980), testes (Skinner and Griswold, 1980), and mammary glands (Jordan and Morgan, 1969).

The degree of expression of transferrin receptors on most types of cells is determined by the level of Fe supply and their rate of proliferation. In plasma and other extracellular fluids, Tf is present as a mixture of iron free (apo-Tf), monoferric Tf, and diferric Tf (holo-Tf). The relative abundance of each form depends on the concentrations of iron and Tf. The brain, like other organs, needs Fe for metabolic processes. Transferrin is present in blood plasma and brain extracellular fluids, and the transferrin receptor is present on brain capillary endothelial cells, choroid plexus epithelial cells, neurons, and probably glial cells also. Many investigators across the world have worked on delivering transferrin conjugated nanocarriers for brain targeting. Mishra et al. have successfully transported azidothymidine to brain, after incorporation in albumin nanoparticles and conjugating with transferrin (Mishra et al., 2006). Soni et al. have shown the improved brain uptake of 5-fluorouracil in the form of Tf conjugated liposomes (Soni et al., 2008). Other groups have shown the enhanced *in vitro* intracellular uptake of Tf conjugated nanocarriers (Gan et al., 2010; Li, et al., 2009; Sahoo and Labhasetwar, 2005).

2.8.1.2 Transferrin Receptor

The human transferrin receptor (TfR) is a transmembrane glycoprotein consisting of two 90-kD subunits linked by disulfide bonds. Each subunit can bind one Tf molecule. In the brain, TfR is expressed on choroid plexus epithelial cells and neurons. The function of TfR is to mediate the cellular uptake of Fe bound to Tf. In most types of cells this is achieved by receptor-mediated endocytosis. The endocytosis occurs via coated pits. This is followed by uncoating of the pits with the formation of endosomes, and acidification to a pH of 5.5–6.5 due to the

function of an endosomal H1-ATPase. The Fe is then released from Tf and transported across the endosomal membrane into the cytosol. The apo-Tf remains bound to the TfR and is recycled to the cell membrane by exocytosis. At the extracellular pH, the apo-Tf has a poor affinity for the TfR and is released into the extracellular medium, to be replaced by iron-containing Tf, and the cycle is repeated. Tf is not degraded in the process.

The TfR mediates cellular uptake of iron bound to Tf. The expression level of TfR depends on the level of iron supply and rate of cell proliferation. For example, in malignant cells an elevated level of TfR expression is found. This is caused by the high iron requirements for malignant growth (Huebers and Finch, 1987; Ponka, 1999). The iron concentration determines TfR synthesis and expression via an iron-responsive element (IRE) in the mRNA of the TfR (Casey et al., 1989; Kuhn, 1991). This IRE is also found in the mRNA of ferritin, a protein that can store iron. In cases of low iron concentrations, a so called IRE binding protein stabilizes the mRNA of the TfR, which can therefore be translated. The mRNA of ferritin is in low-iron situations less stable and is therefore translated to a lesser extent.

Recently, a second TfR (TfR-2) has been identified (Trinder and Baker, 2003), which does not contain IRE in its mRNA. TfR-2 is differentially distributed from TfR and has a 25-fold lower affinity for Tf. Finally, a soluble or serum TfR is present in the circulation (Kohgo, 1986). During the process of recycling of the TfR, some receptors are shed, in which case they appear in truncated form in the blood circulation (Shin et al., 1990). It has been shown that serum TfR to ferritin ratios have significant predictive value for differentiating iron deficiency anemia from non-deficiency anaemia (Kohgo, 2002).

2.9 NASAL ANATOMY AND PHYSIOLOGY

The human nasal cavity has a total volume of about 16-19 ml, and a total surface area of about 150-180 cm^2 (measured using a computed tomography scan). The nasal septum divides the nasal cavity along the center into two halves, one opening to the facial side and the other to the rhinopharynx, through the anterior and via the posterior nasal apertures, respectively. The nasal septum is not very accessible for the penetration of drugs into the human system because it consists mostly of cartilage and skin. The volume of each cavity is approximately 7.5 ml, having a surface area around 75 cm^2. The most efficient area for drug administration is the lateral walls of the nasal cavity, which consist of highly vascularized mucosa (Mygind and Anggard, 1984).

The vestibular region is present at the opening of nasal passages lined with keratinized stratified squamous transitional epithelium with no or few cilia. These cells also contain vibrissae, or nasal hairs, for filtering out airborne particles. Drug deposition in this region will remain stationary, be sucked along the floor to the pharynx by sniffing, drip out, or wiped off. The vestibule includes an important region called the nasal valve, which is the narrowest segment in the respiratory tract, accounting for 80% of nasal resistance and 50% of total airway resistance. The olfactory region is of about 10 cm^2 in surface area, and it plays a vital role in transportation of drugs to the brain and the CSF (Mathison et al., 1998; Schipper et al., 1991). The three distinct anatomical regions present in the nasal cavity and its cross sectional sketch is shown in **Fig. 2.7.**

Olfactory epithelial deposition may also involve drug delivery via the trigeminal nerve, although there is no evidence at present to support this mechanism. The trigeminal nerve is the largest of the cranial nerves and, among other functions, enables sensory perception in the nasal cavity. The trigeminal

nerves innervate the entire nasal mucosa. They have three major branches, the ophthalmic nerve, maxillary nerve, and mandibular nerve. The ophthalmic and maxillary branches of the trigeminal nerve are important for nose-to-brain drug delivery because neurons from these branches pass directly through the nasal mucosa. Hence, in contrast to the nostral entry of a drug via the olfactory pathway, trigeminal nerve was shown to enhance nose-to-brain delivery to caudal brain areas (Dhanda et al., 2005). Portions of trigeminal nerve enter the brain both through the pons and separately through the cribriform plate under the olfactory bulbs, and deliver the drug to the upper spinal cord and the brain.

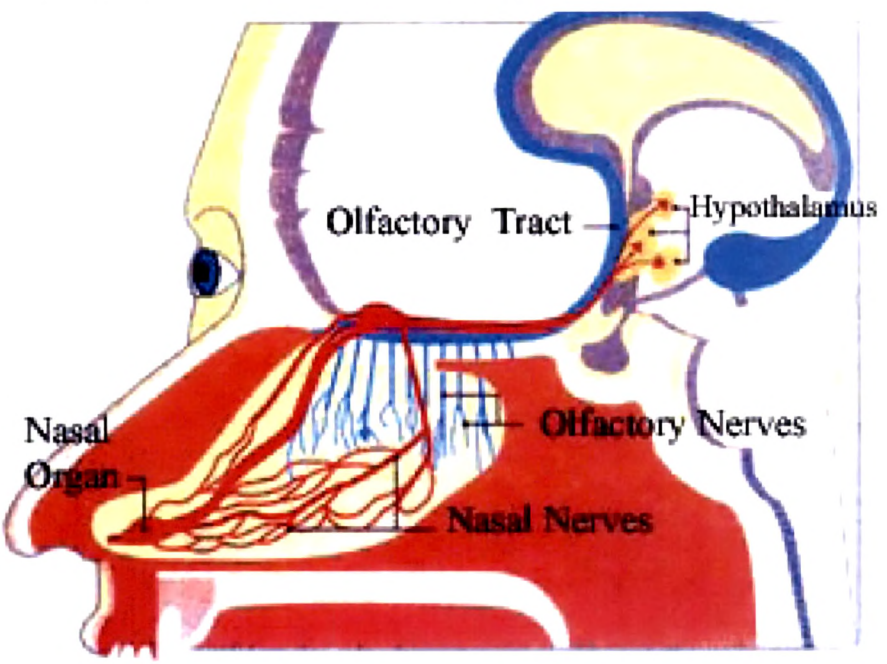


Figure 2.7: Pathophysiologic connection between Nose and Brain.

The epithelium of the nasal passage is covered by a mucus layer and the pH of mucosal secretions ranges from 5.5 to 6.5 in adults and 5.0 and 6.7 in children, which entraps particles and is cleared from the nasal cavity by cilia. The mucus moves through the nose at an approximate rate of 5 to 6 mm/min

resulting in particle clearance within the nose every 15 to 20 minutes. Numerous enzymes for example, cytochrome P450, enzyme isoforms (CYP1A, CYP2A and CYP2E), carboxylesterases and glutathione S-transferases are found in nasal cavity (Chein et al., 1987; Chein et al., 1989; Reed et al., 1993).

2.9.1 ADVANTAGES OF INTRANASAL DELIVERY

Intranasal delivery is a noninvasive method of drug delivery, associated with little pain and preferable to other methods such as injections. Directly targeting drugs to the brain and reducing systemic exposure would likely result in a decrease in unwanted side effects. Several nasal spray devices are in the market for intranasal delivery that can target drugs to the upper portion of the nasal cavity (Dhanda et al., 2005). Systemic dilution effect and first pass metabolism are also avoided. Direct transport could result rapid and/or higher uptake in brain, which provides an alternative option of self-medication in management of emergencies.

2.9.2 DEMERITS OF INTRANASAL DELIVERY

The difficulties that have to be overcome in nasal delivery of drugs include active degradation or alteration by enzyme, low pH of nasal epithelium, the possibility of mucosal irritation or possibility of large variability caused by nasal pathology, such as common cold. The limitations of intranasal delivery are low dose/volume especially when compounds have less aqueous solubility and are difficult to formulate. High lipophilicity and preferably low molecular weight of drug are the prerequisites as it could influence the uptake across nasal mucosa. Drug compounds devoid of offensive/pungent odor/aroma and non-irritant nature are highly desirable for designing intranasal drug delivery systems (Vyas et al., 2005).

2.9.3 INTRANASAL DELIVERY – A STRATEGY TO TARGET CNS, LYMPHATICS & PERIVASCULAR SPACES OF CEREBROVASCULATURE

Many drugs are not being effectively and efficiently delivered using conventional drug delivery approach to brain or central nervous system (CNS) due to its complexity. The brain and CNS both have limited accessibility to blood compartment due to a number of barriers. Many advanced and effective approaches to brain delivery of drugs have emerged in recent years. Intranasal drug delivery is one of the focused delivery options for brain targeting, as the brain and nose compartments are connected to each other via the olfactory route and via peripheral circulation. Realization of nose-to-brain transport and the therapeutic viability of this route can be traced from the ancient times and has been investigated for rapid and effective transport in the last two decades. Various models have been designed and studied by scientists to establish the quantitative and qualitative transport through nasal mucosa to brain. In 1989, Frey first developed a noninvasive, intranasal method of bypassing the BBB to deliver therapeutic agents to CNS (Frey 1991). This method facilitated drugs that do not cross BBB and required to be delivered to CNS. It also directly targets the drugs that do cross the BBB to the CNS, eliminating the need for systemic delivery and thereby reducing unwanted systemic side effects. Delivery from nose to the CNS occurs within minutes along both the olfactory and trigeminal neural pathways. Delivery occurs by an extracellular route and did not require the drugs to bind to any receptor or undergo axonal transport. In addition to targeting the CNS, the intranasal delivery method also targets the nasal associated lymphatic tissues, the deep cervical lymph nodes, the perivascular spaces and blood vessel walls associated with cerebrovasculature (Hanson et al., 2007). In an animal model of

stroke, intranasal IGF-I given up to 4h after stroke markedly reduced infarct volume and improved neurological function (Liu et al. 2004). Intranasal erythropoietin, a 30,400-Da glycoprotein, also protected against focal cerebral ischemia (Yu et al., 2005). Intranasal NGF, a 26,500-Da protein, bypasses the BBB and targets the CNS (Chen et al., 1998; Frey et al., 1997). Intranasal NGF, administered once every 2 days according to a procedure modified from Frey et al. was found to successfully protect against and even reverse neurodegeneration in a transgenic mouse model of Alzheimer's disease (Capsoni et al., 2002; Frey et al., 1997). De et al. further reported that intranasal NGF rescued recognition memory deficits in this same Alzheimer's disease mouse model, the AD11 mouse (De et al., 2005). Intranasal neurotrophins FGF-2 or heparin binding EGF has also been shown to stimulate neurogenesis in adult mice (Jin et al., 2003).

Direct delivery of a wide variety of therapeutic agents to the CNS following intranasal administration, as well as the therapeutic benefit of intranasal drug treatments, has previously been demonstrated in mice, rats, primates, and humans by many groups including Born et al., 2002; Dhanda et al., 2005; Frey 2002; Leah at al., 2007; Thorne et al., 2001. From drug distribution studies, it appeared that drug traveling along the olfactory neural pathway distributes into rostral brain structures including the olfactory bulb, anterior olfactory nucleus, frontal cortex, and hippocampus. In addition, drug traveling along the trigeminal nerve distributes into caudal brain structures including upper cervical spinal cord, midbrain, pons, and hypothalamus. This general distribution can be altered by the presence of CNS receptors that may bind to the therapeutic agent and it may vary between species. Intranasal delivery of insulin improved the memory and mood in healthy adults (Benedict et al., 2004) and improved memory in patients with Alzheimer's disease without altering blood

levels of insulin or glucose (Reger et al., 2006). On a side note, intranasal delivery of insulin has also been shown to reduce body fat in normal, but not obese men (Hallschmid et al., 2004, 2006). Researchers have demonstrated that intranasal exendin is directly delivered to the brain and improved memory, cognition, and neuronal survival (Banks et al., 2004; During et al., 2003). Intranasal delivery has also been used to target NAP and ADNF to the brain to treat anxiety and neurodegeneration (Alcalay et al., 2004; Gozes et al., 2000).

While intranasal delivery can reduce systemic exposure, this effect is dependent on the size and charge of the molecule being delivered. For example, charged molecules such as insulin reach the brain from the nasal cavity without altering blood levels of insulin or glucose (Born et al., 2002). However, formulation of such agents with permeation enhancers can increase delivery to the systemic circulation if this is desired. On the other hand, small, lipophilic molecules can readily enter the blood stream from the nasal mucosa and may subsequently reach the CNS by crossing the BBB.

The investigations till date have attracted researchers to place the intranasal drug delivery option under the microscope. Nevertheless, it is imperative to understand the uptake of drug across the nasal mucosa. From a kinetic point of view, nose is a complex organ since three different processes, such as disposition, clearance and absorption of drugs, simultaneously occur inside nasal cavity. For effective absorption of drugs across nasal mucosa, it is essential to comprehend the nasal anatomy and related physiological features of the nose. Insulin-like growth factor-I [IGF-I, a 7,600-Da protein], has been intranasally delivered to the brain and spinal cord. In addition, very high delivery of IGF-I was observed to the lymphatics (i.e., NALT and deep cervical lymph nodes) and walls of the cerebrovasculature (Thorne et al., 2004).

The development of nasal drug products for brain targeting is still faced with enormous challenges. A better understanding in terms of properties of the drug candidate, nose-to-brain transport mechanism, and transport to and within the brain is of utmost importance in the development of intranasal delivery products.

There are many opportunities for the use of intranasal delivery to target therapeutics to the brain, lymphatics, perivascular spaces of the cerebrovasculature, and CNS for the treatment and prevention of AD.

2.10 INTRANASAL DELIVERY OF ANTI-DIABETICS FOR CNS TARGETING

The poor penetration of anti-diabetic agents into the CNS may potentially be overcome by intranasal delivery which will directly target the brain and reduce and/or eliminate advanced glycosylation end products, thereby preventing AD from ever developing. Intranasal delivery may be used to target any type of anti-diabetics to the CNS including biguanides, thiazolidinediones, DPP-IV inhibitors such as Vildagliptin, Sitagliptin, Denagliptin, and Saxagliptin, GLP-1 mimetics such as Liraglutide which have been proved beneficial in the simultaneous management of T2DM and AD.

A similar approach using intranasal delivery to target chemotherapeutics to the CNS has shown great promise for the treatment of brain tumors in preclinical studies (da Fonseca et al. 2006; Hashizume et al., 2006; Shingaki et al., 1999; Wang et al., 2004, 2005).

2.10.1 FORMULATION OF INTRANASAL THERAPEUTIC AGENTS

The intranasal method does not require any modification of the therapeutic agent. The method can deliver a wide variety of therapeutic agents to the CNS,

including both small molecules and macromolecules. However, this delivery method is not a panacea. It works best with potent therapeutic agents that are active in the nanomolar range (Dhanda et al., 2005). Because delivery occurs along the olfactory and trigeminal neural pathways, high concentrations of intranasal therapeutic agents are routinely observed in the olfactory bulb and trigeminal nerves; thus drug candidate should be screened for its potential side effects in these sensory neurons. Further more, P-gp has been reported to operate in the nasal epithelium (Graff and Pollack 2003, 2005; Kandimalla and Donovan 2005 a, b), reasonably good intranasal delivery to the brain has been reported for P-gp substrates (Graff and Pollack, 2003). However, it is not yet known whether nasal congestion due to allergies or colds may interfere with intranasal delivery. Finally, small lipophilic molecules that rapidly enter the blood from the nasal mucosa may require special formulation to enhance delivery to the CNS while reducing systemic exposure.

Many previously abandoned potent CNS drug candidates promise to become successful CNS therapeutic drugs via intranasal delivery. Recently, several nasal formulations, such as ergotamine (Novartis), sumatriptan (GlaxoSmith-Kline), and zolmitriptan (AstraZeneca) have been marketed to treat migraine (Jogani et al., 2008). Scientists have also focused their research towards intranasal administration for drug delivery to the brain, especially for the treatment of diseases, such as epilepsy, migraine, emesis, depression and erectile dysfunction.

Absorption of drug across the olfactory region of the nose provides a unique feature and superior option to target drugs to brain (Dominique, et al., 1993). When administered nasally to the rat, some drugs resulted in CSF and olfactory bulb drug levels considerably higher than those following intravenous

administration (Hussain et al., 1989; Chow et al., 1999; Sakane et al., 1994). Evidence of nose-to-brain transport has been reported by many scientists (Alcalay et al., 2004; Banks et al., 2004; Frey, 1991; Wang et al., 2004).

2.10.2 DOSAGE FORMS FOR INTRANASAL DELIVERY

Nasal Drops: Nasal drops are one of the most simple and convenient nasal delivery systems. It has been reported that nasal drops deposit human serum albumin in the nostrils more efficiently than nasal sprays. Drops are more widely spread through the nasal cavity and reach the posterior of the nasal cavity, where mucociliary clearance is more active, creating more rapid clearance from the cavity. The main disadvantage of this system is the lack of dose precision; therefore, nasal drops may not be suitable for prescription products.

Nasal Sprays: Both solution and suspension formulations can be formulated into nasal sprays. Due to the availability of metered-dose pumps and actuators, a nasal spray delivers a dose from 25 to 200 µl. In case of suspensions, the particle size and morphology of the drug and viscosity of formulation determine the choice of pump and actuator assembly. Sprays are cleared more slowly because deposition is local in the anterior part of the nasal cavity.

Nasal Emulsions, Microemulsions, and Nanoemulsions: Viscosity of nasal emulsions is generally an advantage for local application. The physical stability of emulsion formulations and specific delivery are the main issues. A mucoadhesive microemulsion will provide longer residence time in the nasal mucosa, indicating rapid and complete absorption of drugs. Vyas et al. and Jogani et al. have reported intranasal administration of mucoadhesive microemulsions for the effective management of various brain-associated disorders (Jogani et al., 2008; Vyas et al., 2005).

2.11 DRUG(S) RATIONALE

Thiazolidinediones such as pioglitazone are commonly used to treat type 2 diabetes (T2DM) because they exert a number of effects on glucose and lipid metabolism, resulting in reduced insulin resistance and increased glucose uptake and its utilization in non-cerebral tissues (Hammarstedt, et al., 2005; Kubota, et al., 2006). The finding that thiazolidinediones are potent inhibitors of inflammation (Heneka, et al., 2000; Cunard, et al., 2004; Schutz, et al., 2005) has prompted several clinical trials for testing the effects of thiazolidinediones in neurological diseases such as Alzheimer's disease and multiple sclerosis, in which inflammation contributes to pathogenesis (Feinstein, et al., 2004). The anti-inflammatory properties of pioglitazone in the brain have also been shown in murine models of acute (Breidert, et al., 2002) and chronic inflammation (Lacombe, et al., 2004), when it was administered to the animals orally. Both the metabolic and anti-inflammatory actions of thiazolidinediones have been attributed to changes in gene expression mediated by peroxisome proliferator-activated nuclear receptor (PPAR)-γ, of which thiazolidinediones are ligands (Heneka, et al., 2000). However, several recent studies point to the existence of PPAR-independent effects of thiazolidinediones, such as the regulation of mitochondrial respiration (Dello-Russo, et al., 2003; Brunmair, et al., 2004; Feinstein, et al., 2005; Konrad, et al., 2005). Impaired mitochondrial activity has been related to insulin resistance (Petersen, et al., 2005) and correlated with the clinical state in Alzheimer's disease (Bubber, et al., 2005). Considering the strict dependence that the brain has on glucose as a fuel (Sokoloff, 1981), a stimulatory action of thiazolidinediones on glucose metabolism would provide an additional therapeutic benefit in pathologies such as Alzheimer's disease. Regional decreases in the activity of glycolytic enzymes and in glucose utilization have

been detected in sporadic Alzheimer's disease (Sorbi et al., 1983; de Leon et al., 2001), and the administration of glucose (Craft et al., 1992) or insulin (Craft et al., 2003; Watson and Craft, 2004) can facilitate memory in patients. This evidence has led to the notion of metabolic insufficiency or gluco-regulatory impairment in Alzheimer's disease (Gibson et al., 1998; Convit et al., 2003; Hoyer, 2004) and has provided a strong rationale for the therapeutic use of drugs, such as thiazolidinediones, that increase insulin sensitivity and glucose consumption (Galea et al., 2006) Preliminary results of clinical studies consistently suggested that restoring adequate levels of insulin and glucose by using a thiazolidinedione, facilitates memory in patients with Alzheimer's disease (Watson et al., 2005). A very recent study in mice modeling Alzheimer's disease suggests that rosiglitazone attenuates learning and memory deficits by lowering glucocorticoid actions (Pederson et al., 2006). However, unlike pioglitazone, rosiglitazone does not cross the blood-brain barrier.

Watson et al. reported the outcome of a small clinical trial examining the effects of 6 months treatment with rosiglitazone on cognition and memory in AD patients (Watson et al., 2005). This small study of 30 patients with mild AD or amnestic mild cognitive impairment found that rosiglitazone therapy resulted in improved memory and selective attention. A large trial of rosiglitazone in AD patients has also been reported, in which patients (n=500), with mild to moderate AD were treated for 6 months with rosiglitazone. Drug treatment was associated with a significant improvement in cognition in those patients that did not possess an ApoE4 allele. Patients with ApoE4 did not respond to the drug and experienced no improvement in cognition or function. These authors favor the hypothesis that rosiglitazone acts on mitochondria, increasing their metabolic efficiency and number (Risner et al., 2006). The actions of TZDs on mitochondria

are largely PPARγ-independent (Feinstein et al. 2005). Importantly, this hypothesis is reliant upon penetrance of the drug into the brain and this poses a problem since rosiglitazone is reported not to pass the BBB (Pedersen and Flynn, 2004; Risner et al., 2006). Two studies investigating the effect of PPARγ agonist pioglitazone in animal models of AD have been reported. Pioglitazone passes the BBB, albeit poorly (Maeshiba et al., 1997). Yan and colleagues, found that 6-month treatment of Tg2576 mice with pioglitazone resulted in a modest reduction in soluble Aβ levels with no effect on plaque burden or inflammation (Yan, et al., 2003). The absence of a substantial effect was postulated to be due to poor drug penetration into the brain. Heneka et al. reported that treatment with a higher dose of pioglitazone in another animal model of AD resulted in the reduction of numbers of activated microglia associated with a lower plaque burden (Heneka et al., 2005). The observation that PPARγ agonist treatment is associated with reduction in amyloid pathology in animal models of the disease may be the result of the ability of PPARγ to affect Aβ homeostasis. Sastre and colleagues have reported that PPARγ agonists inhibit Aβ production stimulated by inflammatory cytokines. This effect is a consequence of the suppression of BACE1 expression through a PPARγ-dependent transcriptional silencing of the BACE gene promoter (Sastre et al., 2003, 2006). It has recently been reported that PPARγ activation can affect cellular APP levels and subsequent Aβ production by stimulating the ubiquitin-mediated degradation of APP (d'Abramo et al., 2005). PPARγ activation has also been associated with stimulation of Aβ clearance (Gary, 2006). Camecho and colleagues, reported that PPARγ activation in both glia and neurons resulted in the very rapid and efficient uptake and clearance of Aβ from the medium (Camecho, et al., 2004).

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