CHAPTER II

LITERATURE REVIEW

Anatomy and physiology of nasal cavity

Nasal cavity is divided by the nasal septum, with each half opening at the face (via the nostrils). There is also a connection to the oral cavity provided by the nasopharynx (Figure 1). The anterior and posterior vestibules, the respiratory region, and the olfactory region are the three main areas of the nasal cavity. The lateral walls comprise a folded structure. This folded structure further comprises the superior, median, and inferior turbinates, providing a total surface area of about 150 cm^2 in humans (Costantino et al.,2005). The epithelial tissue within the nasal cavity is relatively highly vascularized, and thus provides a potential conduit for drug delivery. The cellular makeup of the nasal epithelial tissue consists mainly of ciliated columnar cells, non-ciliated columnar cells, globet cells and basal cells, with the proportions varying the different regions of the nasal cavity (Figure2). Ciliated cells facilitate the transport of mucus towards the nasopharynx. Each ciliated cell contains approximately 100 cilia, and both ciliated and nonciliated cells possess approximately 300 microvilli each. Basal cells, which are differentiated act as stem cells to replace other epithelial cells. Collectively, the epithelium and lamina propia are called respiratory mucus membrane or respiratory mucosa. The respiratory mucosa is the region where drug absorption is optimal. A thin sheet of mucus produced from the seromucus glands and goblet cells cover the nasal turbinate and the atrium(Ugwoke et al.,2001). Mucus secretion is a complex mixture of many substances and consists of about 95%water, 2%mucin, 1%salts, 1% of other proteins and < 1% lipids(Ugwoke et al.,2005). Mucin is a high molecular mass (MW 2,000,000 – 4,000,000 Da) glycoprotein crosslinked with disulphide bridges, ionic bonds and physical entanglements. The carbohydrate side groups attached to the protein backbone include galactose, L-fucose, N-acetylglucosamine, N-acetylgalactoseamine and N-acetylneuraminic acid (sialic acid). The carbohydrate side chains terminate with a

sialic acid or L-fucose group, which make mucin an anionic polyelectrolyte at neutral pH. Due to the multiplicity of hydroxyl groups of the carbohydrate side chains, mucin easily forms hydrogen bonds with other suitable polymers(Ugwoke et al.,2001).

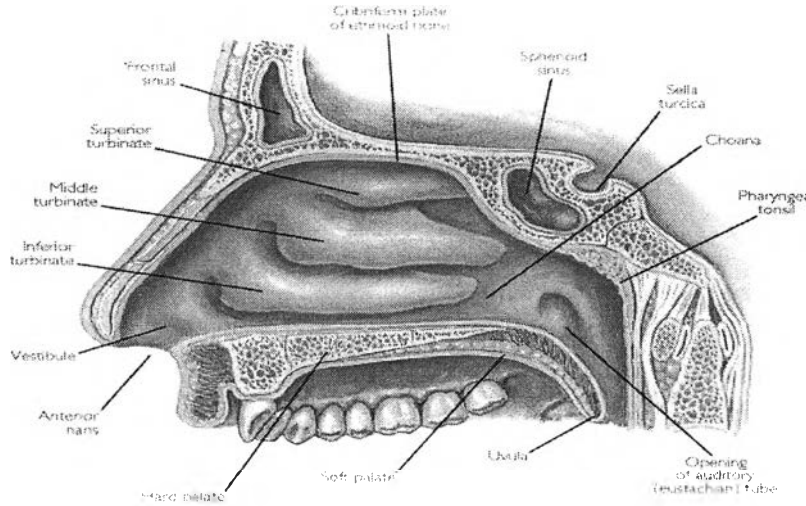

Figure 1 The anatomy of nasal cavity

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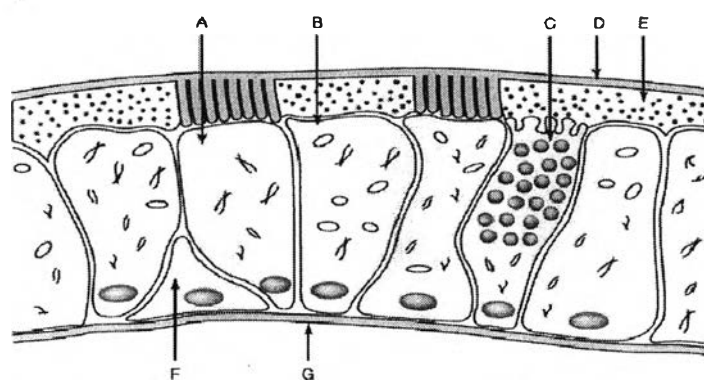

Figure 2 Cell types of the nasal epithelium showing ciliated cell (A), nonciliated cell (B), globet cell (C), gel mucus layer (D), sol layer (E), basal cell (F) and basement membrane (G).

About 1.5 – 2 ml of nasal mucus is produced daily. The mucus blanket, about 5 μm thick, consists of two layers, a lower sol layer and an upper gel layer. The lower layer, which bathes the cilia, is low viscosity, whereas the upper gel layer that rests on the cilia is a high viscosity fluid. Consequently, the viscosity of both layers would affect ciliary beating and the transport of the overlying mucus, the mucociliary clearance (MCC). The viscosity is very sensitive to even small changes in the mucin

content. A small increase in mucin causes a very large increase in mucus viscosity with a resultant prolongation of the mucociliary clearance time (Ugwoke et al.,2001). The mucus moves toward the posterior of the nose by beating of the cilia at a rate of 5 – 24 mm/min. Thus the nasal mucus is replaced with a fresh layer every 10 -15 min. Under normal conditions, inhaled substances or delivery systems are cleared from the nose within 15 – 20 min.(Chein et al.,1989; Dondeti et al.,1996). Pathological conditions with increased MCC rate reduce contact time of the drug with the absorptive nasal mucosa, whereas decreased MCC rate has the opposite effect. Nasal hypersecretion dilutes nasally-administered drug solutions leading to reduced concentration gradient, with a possible influence on absorption. A change in the pH of the mucus can affect the ionization of some drugs, and this can have a significant influence on nasal drug absorption(Morita et al.,2007). The site of deposition and the mucociliary clearance of the nasal formulation after administration are distinct but interrelated factors of importance for the nasal absorption of peptides and proteins.

Nasal drug delivery system

Nasal drug delivery offers many advantages that include the nose has a large surface area available for drug absorption due to the coverage of the epithelial surface by numerous microvilli, a thin, porous and very vascularized epithelium with high total blood flow per cm^3, a porous endothelial basement membrane which is the directly transport of absorbed substances into the systemic circulation (or even directly into the CNS), thereby avoiding the first-pass effect attendant with peroral drug administration, lower enzymatic activity compared with the GIT and liver, amendable to self-medication, which increase patient compliance, possibility of pulsatile delivery of some drugs such as human growth hormone and low risk of over-dosage(Dondeti et al.,1996; Ugwoke et al.,2001; Türker et al.,2004). However, the limitations of a nasal delivery include: potential local tissue irritation ; rapid mucocilliary clearance of the therapeutic agent from the site of deposition resulting in a short span of time available for absorption; low permeability of the nasal membrane for the larger macromolecules(Dondeti et al.,1996; Costantino et al.,2005).

Factors influencing nasal drug absorption

1. Molecular Weight

There are two major barriers in the mucosal absorption of peptide and protein- a penetration barrier and an enzymatic barrier, which are relevant to nasal delivery (Morita et al.,2007). The most important factor limiting the nasal absorption of polar drugs and especially large molecule weight polar drugs such as peptides and proteins is the low membrane permeability. Drugs can cross the epithelial cell membrane either by the transcellular route or by the paracellular route. In using the transcellular pathway, drugs can pass through the cell by a mechanism of concentration-dependent passive diffusion or endocytotic processes. Normally, lipophilic drugs can diffuse through the cells, whereas large hydrophilic molecules such as proteins have been seen to pass through via an endocytotic process (Illum,1999; Illum,2003). Using the paracellular pathway the drug passes between the cells through the intercellular tight junctions which is available only for hydrophilic drugs with molecular weight not more than 1000 daltons(Chein et al.,1989; Illum,1999; Costantino et al.,2005; Ugwoke et al.,2005). However, a typical datum, using radiolabeled dextrans in rats, shows clearly the inverse correlation between molecular weight and percentage absorbed of the nasal dose for a series of non-degradable dextran derivatives with molecular masses of 1260-45,500 Da (Fisher et al.,1992).

2. Environmental pH

Most drugs can be ionized and their partition coefficients are dependent upon environmental pH (unionized compounds will have a higher oil/water partition coefficient than ionized one) (Taylor,2007). The pH at the surface of the mucosal cells has been reported to be 7.39 and the mucus layer itself is slightly acidic, at the pH 5.5 – 6.5 (Illum,1999; Morita et al.,2007; Taylor,2007). This physiological pH of the nasal cavity may neutralize the pH of the formulation by its buffering capacity and can affect microenvironmental pH surrounding drug molecules during the absorption process. A greater absorption was achieved at pH lower than the drug's intrinsic pKa because under such conditions, the molecules exist as an unionized, hydrophobic form (Chein et al.,1989; Morita et al.,2007).

Strategies to improve nasal absorption

Most researchers have exploited the use of absorption enhancers in order to improve nasal absorption of peptides and proteins. A large number of absorption enhancers have been evaluated in animal model and in humans, including mucolytic agents such as *N*-acetyl-L-cysteine; transcellular and paracellular membrane modifiers such as surfactants, bile salts, bile salt analogues, EDTA, and cyclodextrins; and enzyme inhibitors such as aprotinin, bestatin, chymostatin, and amastatin. Generally, these enhancers are successful in improving bioavailability of the drug, but quit often the degree of absorption enhancement is closely related to the degree of damage encountered in the cell membrane.

Permeability of the mucous membrane

Transportation of substrate from the mucosal site into the bloodstream begins with drug diffusion through mucus or unstirred water layer, follow by two major routes, transcellular (intracellular) and paracelular (intercellular) pathways. After that, the substrate is transported into the lamina propria, which is richly endowed with capillaries, and where the substrate can readily be taken.

1. Transcellular Pathway

The transcellular pathway involves permeation across the apical cell membrane, the intracellular space and the basolateral membrane by passive transport (partition nad diffusion) or by active transport (carrier-mediated diffusion, endocytosis, and transcytosis). This pathway is important for the absorption of lipophilic molecules (passive transport) or molecules capable of specific recognition of a membrane site (active transport).

2. Paracellular Pathway

The paracellular pathway involve passage between the cells through the intercellular lipid material of the intercellular space. There are two components of paracellular pathway including the tight junction and underlying the extracellular space. They are variable by various physiological stimuli. For instance, lowering the extracellular Ca^{2+} concentration induces the opening tight junctions. This pathway is

important for the absorption of hydrophilic compounds, which cannot transport by an active transport process (Worakul et al.,2002).

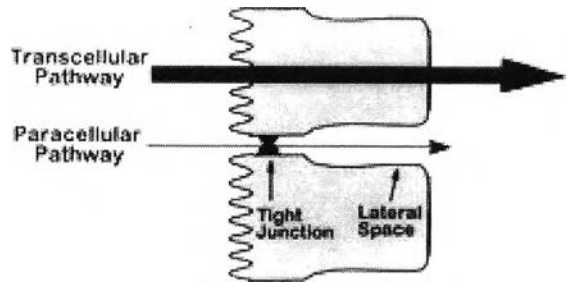

Figure 3. Transportaion of substrates pass through mucous membrane

Mucoadhesive drug delivery

An alternative to the use of absorption enhancers is to use mucoadhesive drug delivery systems that can prolonged contact between the drug and nasal cavity by delaying mucociliary clearance of the formulation. Systems that have been described in the literature include liquid bioadhesive systems, self gelling bioadhesive systems, bioadhesive powder systems, bioadhesive microsphere systems (Ugwoke et al.,1999; Callens et al.,2000; Ikechukwu Ugwoke et al.,2000; Hamman et al.,2002; Rajinikanth et al.,2003; Gavini et al.,2005; Benchabane et al.,2007; Gavini et al.,2008). These systems are achieved by the use of bioadhesive polymers. A bioadhesive polymer is defined as a compound that can interact with biological materials through interfacial forces and being on such material for prolonged periods of time. If the biological material is mucus membrane, the bioadhesive polymers is termed a mucoadhesive (Illum,1999). It involves interaction between mucin and a synthetic or natural polymer leading to a net attraction (Ugwoke et al.,2001). For mucoadhesion to occur, there are two basic steps. In step I, the *contact stage*, an intimate contact between the mucosdhesive agent and the mucus gel layer is formed. In step II, the *consolidation stage*, the joint between mucoadhesive agent and mucus gel layer is strengthened and consolidated, providing a prolonged adhesion(Bernkop-Schnurch,2002).On a molecular level, mucoadhesion can be explained on the basis of attractive molecular interactions involving forces.

Chemical principles of mucoadhesion

1. Noncovalent bonds

Formation of noncovalent bonds include: *hydrogen bonding*,which is based on hydrophilic functional groups such as hydroxylic groups, carboxylic groups, amino groups, and sulfate groups; *ionic interaction*, such as interaction of the cationic polymer with anionic sialic acid moieties of the mucus; and *van der Waals forces,* based on variousdipole-dipole interactions (Worakul et al.,2002).

2. Covalent bonds

Covalent bonds are not any more influenced by parameters such as ionic strength and pH value. Functional groups that are able to form covalent bonds with the mucuslayer are over all thiol groups. Thiolated polymers are able to mimic the naturally occurring mechanism whereby mucus glycoproteins are immobilized in the mucus.

Physical principles of mucoadhesion

One theory explaining the phenomenon of mucoadhesion is based on a macromolecular interpenetration effect. The interpenetration of the chains oc polymer and mucus to a sufficient enough depth to create a semi-permanent adhesive bond (Peppas et al.,1996). Basically, during interpenetration the molecules of the mucoadhesive agent and the glycoproteinic network are brought in intimate contact, and due to the concentration gradient, the mucoadhesive chains penetrate at rates which are dependent on the diffusion coefficient of a macromolecule through a crosslinked network. This interpenetration phenomenon can be observed with liquid and uncrosslinked solid (or swollen) mucoadhesive controlled release systems(Peppas et al.,1996; Bernkop-Schnurch,2002). And another theory, dehydration of a mucus gel layer increase its cohesive nature. Dehydration essentially alters the physicochemical properties of a mucus gel layer, making it locally more cohesive and promoting the retention of a delivery system. The mucoadhesive as well as cohesive properties of polymers can also be explained by physical entanglements of polymer chains.

Factors that influence the mucoadhesiveness of a polymer include the type of functional groups, polymer molecular mass, contact time with mucus, polymer concentration, environmental pH and physiological variables (Ugwoke et al.,2001).

The chemical class of a polymer has a strong influence on its mucoadhesive properties. Polymer molecular mass influences its bioadhesion characteristics. There is a critical polymer molecular mass below or above which there is reduced mucoadhesive power, and this is dependenton the type of polymer. There is a report about the *in vivo* nasal mucoadhesive capability of two polymers, CMC and Carbopol, and found that CMC was cleared faster than Carbopol even up to 6 hours after administration in rabbits (Ugwoke et al.,2000a).

There is an optimum polymer concentration required at the polymer-mucus interface for bioadhesion, beyond which few polymer chains are available for polymer-mucus interpenetration. This requirement may be relevant only for bioadhesion in the gel form.

Hydration condition and swelling are related to the polymer and the environment. An excessive amount of hydration fluid lead to inordinate swelling of the polymer, which reduces its adhesive strength. The swelling-hydration rate should not be too rapid in order to prolong adhesion time.

The environmental pH affects mucoadhesion due to its influence on the charge characteristics of the mucus and polymer depending on its type. The maximum adhesion of ionizable polymers such as polycarbophil occurred at pH 3 and below when the polymeris unionized.

It has been reported that increased contact time and applied pressure increase the adhesiveness of a polymer.

MCC, mucus turnover and disease states are physiological factors which influence nasal mucoadhesion. Mucoadhesion can slow down MCC, but with time, mucus production reduces the mucoadhesion bond strength, allowing a recovery of MCC to normal clearance rates(Kamath et al.,1994).

Mucoadhesive polymers

A system for mucoadhesive polymerscan based on their surface charge can be divided into anionic, cationic, nonionic, and ambiphilic polmers.

1. Anionic polymers

For anionic polymers mainly –COOH groups are responsible for their adhesion to the mucus gel layer. Carbonic acid moieties are supposed to form hydrogen bonds with hydroxyl groups of the oligosaccharide side chains on mucus

proteins. There are several anionic polymes such as alginate, Carbomer, Chitosan-EDTA, Hyaluronic acid, NaCMC, Pectins, and Polycarbophil. Their swelling behavior depends on the pH value. At lower pH values is the swelling behavior, leading to a quite inadequate adhesion in many cases. A further disadvantage of anionic mucoadhesive polymers is their incompatibility with multivalent cations like Ca2+, Mg2+, and Fe3+ and leading to a strong reduction in their adhesive properties (Bernkop-Schnurch,2002).

2. Cationic polymers

For cationic polymers such as chitosan and polylysine, the strong mucoadhesion can be explained by ionic interactions between these polymers and anionic substructures such as sialic acid moieties of the mucus gel layer. Their swelling behavior is strongly pH dependent as well.

3. Nonionic polymers

Nonionic polymers are mostly independent from pH value. Whereas the formation of secondary chemical bonds due to ionic interaction can be completely excluded for this group of polymers, some of them such as poly(ethylene oxide) are able to form hydrogen bonds. Apart from these interactions, their adhesion to mucosa seems to be rather based on interpenetration followed by polymer chain entanglement. They are adhesive only if applied in dry form. Hence, nonionic polymers are in most cases less adhesive than anionic and cationic mucoadhesive polymers. Well- known representatives of this group mucoadhesive consist of Hydroxypropyl cellulose, Hydroxypropyl methylcellulose, poly(ethylene oxide), poly(vinyl alcohol), poly(viyl pyrrolidone).

4. Ambiphilic polymers

Ambiphilic polymers display cationic as well as anionic substructures on their polymer chains. The combination of positive and negative charges on the same polymer, however, seems to compensate both effects, leading to strongly reduced adhesive properties of ambiphilic polymers (Bernkop-Schnurch,2002).

Type of nasal dosage form and delivery system

The final dosage form used for nasal drug delivery of chosen after consideration of a wide range of issues, covering patient convenience, efficiency of drug delivery and formulation reasons. The specifics of the dosage form or delivery system plays a major role in the absorption of the drug by influencing its deposition. For example, drugs deposited in the anterior part of the nasal cavity will be better absorbed than those applied further back (Taylor,2007).

Deposition mechanism

There are three main ways of depositing inhaled particles on the nasal lining.

Impaction Impaction occurs when there is a change in direction of the airflow – as happens when inspired air passes through the nasal valve – and the inertia of large or fast-moving particles carriers them in their original direction. This is usually the main way of depositing particles in the turbulence caused by fast slow rates, or with particles larger than 0.5 - 1μm. Varying the flow rate from the device or the aerodynamic particle size are the mean by which the formulator can influence this type of deposition.

Sedimentation Sedimentation happens when the air is moving slowly and the particles settle slowly under the force of gravity. This mode of deposition us described by Strokes' equation. The only control

Diffusion Diffusion occurs by Brownian motion and is thus limited to very small particles (< 0.5 μm). Normally diffusion is not important, as the inspired particles are too large (Taylor,2007).

Nasal dosage forms

Liquid formulation

Liquid formulation are usually aqueous solutions of the drug and thus have the general benefit and drawbacks of pharmaceutical solutions. They are relatively simple

to develop and manufacture compare to solid dosage forms, but often have a lower microbiological and chemical stability, requiring the use of various preservatives.

The liquid form can be soothing to the nasal lining but this may be countered by excipients, such as the antimicrobial preservatives, which can cause irritation or ciliotoxicity. The simplest way to give a liquid is by nose drops, however, the exact volume of dosing is difficult to determine, and rapid drainage from the nose is another problem with drops. Usually, droplet administered by sprays reach a more posterior region of the nose than do drops. As posterior region is rich in ciliated cells, it consists of a large surface area and contributes to the absorption of drugs (Morita et al.,2007). For example, the bioavailability of nasally administered desmopressin has been significantly increased by sprays, compare with drops (Harris et al.,1988). The size of the sprayed droplets is fairly affected by the performance of administration devices. Generally, droplet less than 10 μm in diameter will reach the upper respiratory tract. As various types of devices for nasal administration, such as unit dose containers, metered dose sprays, compressed air nebulizers, and so on, are becoming available(Taylor,2007).

Powder dosage forms

Nasal drug delivery is not limited to liquid drug formulations or suspensions. Powder dispensing systems can be more advantageous in dispensing many drug substances. In particularly, the biotechnological drugs, mostly represented by proteins and peptides (Bommer,2007). One of the advantages of the powder formulation is a higher chemical stability than solution, which lead to the possible administration of large amounts of the drug and the excipients (Morita et al.,2007). In addition, The powder systems have been suggested to slow down the mucocilliary clearance and, in some cases, to affect the paracellular pathway by removal of water from the cell membrane. Another possibility noted is that powder formulation can reduce the dilution of drugs by nasal endogeneous secretion, which lead to an important stratrgy of keeping a high drug concentration at the absorption site (Morita et al.,2007).

Nasal dry powder delivery systems can be divided into passive and active systems. Passive systems are powder devices where the act of sniffing delivers the powder. An active system is based mostly on an air pressure driven mechanism. The powder is dispensed through a rapid airflow, which passes through the container and carries the drug into the nasal cavity(Bommer,2007). A nasal dry powder device take advantage of the fact that one of the functions of the nose is the filtering of pollutants. A recently developed passive dry powder bi dose system (Figure 4) consists of two prefilled blister, which assures optimal protection against vapor transmission, oxygen and light. The aerodynamic feature, combined with easy actuation mode, facilitated accurate dosing in terms of expelled amount and inhalation forces. An optimal coverage of the mucosa is facilitated through a very low air flow resistance of the device. It is very convenient for the patient to sniff the powder out of the blister. As soon as the velocity of the inhalation airflow reaches 8 L/min or more, the airflow in the nasal cavity becomes turbulent (Bommer,2007). The turbulent in the nasal cavity allows the powder to become evenly dispersed, which contribute to an optimal therapeutic effect.

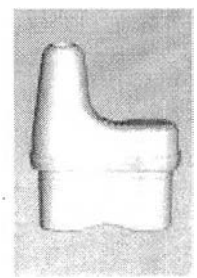

Figure 4. Powder bi-dose system

The particle size of nasal powders is not regarded as critical as for inhalation powders. Particles with size between 5–7 μm will be retained in the nasal cavity and subsequently permeated (Arora et al.,2002). For inhalation, the particle size should be in the range of 1-5 μm. To allow deposition in the nasal cavity, this fine particular fraction must be avoided. The possibility of inhaling particles larger in diameter than 10 μm, or less than 5 μm, is unlikely. Active systems are fitted with a mechanism that allows pressure to build up and eject the powder into the nostril. These devices are

especially suitable for children, where it may be difficult to carry out the required inhalation process.

Delivery of pharmaceutical proteins

Protein structures

Primary structure The primary level of structure refers to linear sequence of amino acids along a protein chain and to the location of covalent bonds, namely disulfide bonds, between chains or within a chain. The primary structure identifies a protein unambigueously, determines its chemical and biological characteristics and specifies the higher levels of protein structure (Cozzone,2002).

Secondary structure In principle, a polypeptide chain could assume great flexibility owing to the free rotation of the atoms around different bonds along the chain. If it did so, it would behave like a random coil and could theoretically adopt a myriad of conformations of similar energies. In fact, in biological conditions, each protein adopts essentially only conformation because the side-chains of its amino acid residues associate locally with one another and with the solvent to yield a global structure of maximum stability. Several folding patterns occur repeatedly in parts of protein molecules. They are known collectively as secondary structure, which constitutes the next level of protein structure after the primary structure (Cozzone,2002).

The α helix structure is a right-handed rodlike structure. Its inner part is formed by the coiled polypeptide main chain and the surface by the side-chains projecting outwards in a helical arrangement.

The other major type of periodic secondary structure is coiled the β sheet. In a b sheet, strands of protein lie adjacent to one another, interacting laterally via H bonds between backbone carbonyl oxygen and amino H atoms. The strands may be parallel (N-termini of both strands at the same end) or antiparallel. Because of the tetrahedral nature of carbon bonds, a b-sheet is puckered, leading to the designation pleated sheet.

Tertiary Structure The tertiary level of structure refers to the spatial arrangement of a polypeptide chain through folding and coiling to produce a compact globular shape. The tertiary structure is essentially determined by the packing of the secondary structures, α helices and β sheets, which combine to form one or several units called 'domains'. These combinations are limited in number, and some of them are especially frequent in proteins. They represent the fundamental elements of globular polypeptide chains in terms of three-dimensional structure as well as in terms of function.

Quaternary Structure Many proteins are made up from two or more polypeptide chains, called subunits or monomers, which may have identical or different amino acid sequences, Such polypeptide aggregation, which represents the quaternary structure, is generally of critical importance to the proper functioning of these oligomeric proteins. Quaternary structure tends to be stabilized mainly by weak interactions between residues exposed on surfaces polypeptides within a complex (Cozzone,2002).

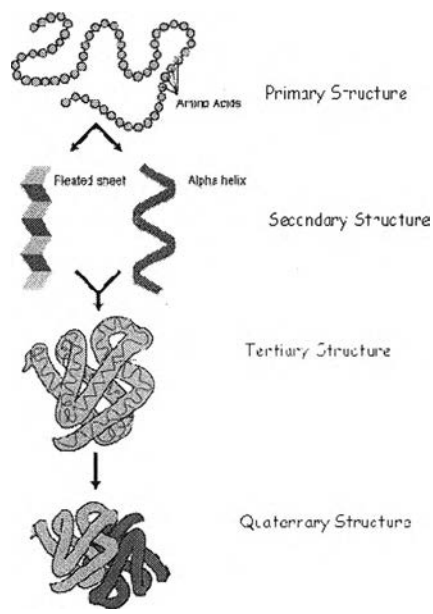

Figure 5. Protein structure

Formulation of Pharmaceutical proteins

For effective product development the formulator must have a good understanding of the mechanism of degradation of the macromolecule of interest, and its potential impact on such areas as its biological activity, metabolic half-life and immunogenicity. These mechanisms can, however, be complicated; encompassing many interrelated chemical and physical processes. As degradation may occur during the production, isolation, purification, formulation, storage and delivery of the macromolecule, it is necessary for the formulator to work closely with those developing the bioprocessing and analytical methodologies.

Chemical instability

Deamidation is a rather common degradation reaction in water. Deamidation reaction kinetics depend on pH and neighbouring amino acids. Oxidation is catalysed by traces of transition metal ions. Incorrect choice of excipients may also cause degradation reactions. For example, sugars are often used as excipients but reducing sugars can react with free primary amino groups of the protein molecule via the so-called Maillard reaction and form brownish reaction products (Parkins et al.,2000; Crommelin et al.,2007).

Physical instability

Degradation rates depend on environmental conditions. For example, elevated temperatures can cause denaturation of proteins in aqueous solution. Interestingly, low temperatures may also induce destabilization. Besides, protein aggregation is often initiated by adsorption of the protein monomer on the walls of the container. Proteins may also aggregated by shaking or by exposure to shear forces. This can cause hydrophobic parts of the molecule to be exposed to interfaces (air/water). In turn this can result in unfolding of the protein and aggregation occurs (Parkins et al.,2000; Crommelin et al.,2007).

Many macromolecules have a relatively short shelf life in aqueous medium, which can lead to both poor bioavailability and poor stability in the final product. To improve bioavailability the macromolecule may be formulated as an oily injection, sustained release product, particulate delivery system or implant; these types of products often have the advantage of improved stability over an aqueous formulation. Where the formulation does not have adequate stability, then stability might be improved through the exclusion of water from the product or the addition of stabilizing excipients (Parkins et al.,2000).

By removing water protein stability usually greatly increases. This is related to the reduced mobility of the protein and to the absence of certain degradation pathways facilitated by water. Upon drying the proteins are incorporated into a matrix, where the type of matrix depends on the glass transition temperature (T_g) of the compounds in the formulation. A low T_g yields a crystalline solid, which often results in phase separation and may destabilize the protein, whereas a higher T_g results in an amorphous solid. Under the dry conditions the amorphous matrix is maintained as long as the temperature is kept below the glass transition temperature, enhancing the long-term stability of the protein. In addition, excipients stabilise the protein by water replacement and provide protection against structural perturbations upon drying as well as aggregation upon rehydration (Maltesen et al.,2008).

Drying techniques for protein pharmaceuticals

Numerous techniques can be used to dry proteins and obtain protein powders with desirable characteristics. The most widely used technique is freeze-drying followed by spray drying, with supercritical fluid (SCF) drying emerging as a viable alternative.

Freeze-drying

Freeze drying or lyophilisation is a three-step batch drying method. The first step is a controlled freezing step, where the protein solution is cooled down under controlled conditions vitrifying the protein formulation. The second step, known as

the primary drying, is conducted at low temperature under vacuum, and involves removal of the frozen water by sublimation. The final step, known as secondary drying, involves removal of unfrozen water by sublimation. In the latter step the temperature is usually increased to raise the drying rate (Maltesen et al.,2008).

The main source for protein stress during freeze-drying, apart from the drying itself, is associated with the freezing process. The destabilizing effects of freezing are not trivial, but known to be highly protein dependent. Cold denaturation of proteins is caused by a decrease in hydrophobic effects and the hydration of nonpolar residues, and may explain the denaturation of some proteins eventhough cold denaturation kinetics might be too slow to unfold the protein during the freeze-drying process (Bhatnagar et al.,2007). The ice formation and concentration of solutes and proteins also affect the protein stability during the freezing process. In particular the increase in ionic strength may pose a problem for protein stability (Bhatnagar et al.,2007). Furthermore, the cooling rate affects the crystal formation of the water molecules: a slow cooling rate yields larger crystals, whereas a rapid cooling rate enhances the formation of small crystals with a higher specific surface area (SSA). A larger SSA could result in larger protein adsorption to the interface, thereby increased denaturation and aggregation. In contrast, a slow cooling rate tends to enhance phase separation between proteins and excipients, which can destabilise the protein. Finally, some component of the buffer system may crystallize resulting in a change in pH in the concentrated sample; a well-known example is sodium phosphate buffer, where the pH may drop almost four units upon freezing.

The amorphous matrix often has a relatively high SSA and is readily reconstituted. Freeze-drying is thus a good manufacturing method for producing protein powders for parenteral administration, but lacks the opportunity to make particles instead of cakes. This problem has been solved by spray-freeze-drying, where the protein feed solution is sprayed directly into a cryogenic medium followed by the typical primary and secondary drying in a normal freeze-drying process. Spray-freeze-drying causes and extremely rapid vitrification because of the large SSA and direct contact with the freezing medium, which makes it difficult to control the

freezing process. However, the ability to make particles instead of the cake, as well as the possibility to control the particle characteristics to some extent, makes it an attractive alternative for applications where particles are required (Maltesen et al.,2008).

Spray drying

Although Freeze-drying has been the method of choice to process therapeutic proteins and peptides into dry powder formulations, spray drying is a suitable process for production of protein dry powders which particle size is must controlled.

Spray drying has also become a mainstream process to stabilise proteins by rendering them into the dry stage in the presence of stabilizer as an alternative to freez-drying. Other pharmaceutical applications of spray drying include the production of active pharmaceutical ingredient (API) when control of particle properties such as crystallinity, particle size, residual moisture content, bulk density, and morphology is desired. Additional spray drying applications in drug delivery include the production of rapidly dissolving tablets, microspheres, nanoparticles, and liposomes.

The spray drying process is conceptually simple; a solution is pumped through an atomizer, a plume of liquid droplets containing solid component is created and subsequently exposed to a suitable gas stream to promote rapid evaporative mass transfer of the liquid carrier into the gas. When sufficient liquid mass has been transformed to vapour, the remaining solid material in the droplet forms an individual dried particle which is then separated from the gas stream. The particle formation process involves the conversion of the atomized spray droplet into solid particles and includes multiple steps as the droplets are exposed to the drying gas medium. First, the droplet must adjust to the temperature of the environment near the nozzle. During this period, the type of atomizer will play the role in the local droplet environment and hence impact the early droplet temperature. For twin-fluid nozzles utilizing air to blast the liquid into droplets, the expansion of the high pressure atomization gas will decrease the local temperature. The droplet temperature subsequently rises as the

atomized droplets and drying gas mix. This mass transfer process can occur very quickly, in a less than one millisecond, for droplet diameter of 10 μm or less. The second stage occurs when the liquid droplet has established equilibrium evaporation of the carrier solvent into the surrounding gas stream. During this period, the evaporation rate will be driven by the heat transfer to the droplet which must balance the liquid vaporizing energy and the liquid droplet temperature is below the local drying gas temperature. The third stage of particle formation occurs after a portion of the solvent has been evaporated and the solid content within the droplet influences the evaporation rate into the gas medium. At this time, while the solute concentration reaches its solubility, the droplet surface has started to solidify forming a shell. This creates an internal droplets diffusion controlled mass transfer process slowing the rate of solvent escape from the inner core to the surface prior to evaporation into the gas medium. For solution based feed stocks the particle morphology is set in this third drying stage. The particle formation control the morphology of the spray dried particles by the evaporation rate with the rising droplet viscosity. The particles produced from the higher drying rate displayed a dimpled or first-order shell collapse mode while the lower drying rate particles tend to be more spherical, with smaller surface wrinkles. As mentioned above, shell formation will occur when one of the formulation component reaches its solubility and precipitates leading to the formation of a solid shell that may be either amorphous or crystalline. For formulation with different physicochemical properties of the components, low aqueous solubility components tend to precipitate early in the drying process and tend to form corrugated particles (Snyder,2008). On the contrary, highly water soluble components continuously reduced as the liquid droplet dries, resulting a smooth, spherical particle.

A clear advantage of spray drying processes is that they are readily scalable for clinical and commercial manufacturing. Dry particles produced from spray drying are single particles composed of drug alone or drug and excipients with low levels of residual solvent. Particle properties such as particle size, morphology, and composition can be readily controlled to obtain highly dispersible particles, avoiding the use of a secondary processing, such as blending with a larger particle size carrier. It has been demonstrated that spray drying is suitable for the production of dry

powder containing protein and peptide since they can be processed from a stable solution and biological activity is kept intact upon reconstitution (Snyder,2008).

As mentioned above, peptides and proteins are far more stable in the solid state compared to the liquid state. Delicate proteins often significantly degrade within hours when held at room temperature in the liquid state. In spray dried powders, residual moisture can also have an effect on powder dispersion and powder flow characteristics. The presence of moisture may influence the cohesive nature of the powder and lead to the formation of aggregates. Even though low moisture content is desired for improved stability, too low a moisture content may lead to increased oxidation of amorphous formulations and may encourage the desired electrostatic charging during powder handling.

The three main sources of protein stress during the spray drying process are heat stress, the mechanical stress and the adsorption to the air liquid interface during atomization. Although the drying air temperature may exceed 100°C in normal spray drying conditions, thermal denaturation of proteins is rarely observed because the temperature of the droplet containing a dilute protein solution hardly exceeds the wet bulb temperature of water (≈40°C) and the drying process last only a fraction to a few of seconds. The protein denaturation temperature is a function of water content (or protein concentration in the dilute solution) and increase sharply with the decreasing water content (Maltesen et al.,2008; Snyder,2008). Besides the stress caused by the thermal dehydration, the protein is subjected to shear stress and exposed to an increased air-water interface during the atomization event. It has been shown that proteins can resist shear rates of spray drying except when shear stress is combined with the rapid formation of an air-liquid interface. Owing to the rapid and large expansion of the air-liquid interface during atomization denaturation and aggregation at the air-water interface is often the dominant source for protein denaturatin in the spray-drying process. Most protein are amphiphilic, and therefore surface active, and thus prone to adsorption at the air-liquid interface. Upon adsorption the protein may change conformation exposing the ghydrophobic residues towards the air-liquid interface to avoid contact with water. Interaction between hydrophobic regions may

then lead to aggregation and ultimately precipitation. To circumvent denaturation at the air-liquid interface surfactants are often used to prevent protein adsorption at the interface (Maltesen et al.,2008; Snyder,2008).

Supercritical fluid drying

Supercritical fluid (SCF) drying is s relatively new drying methodint the pharmaceutical field. The method is based on the anti-solvent properties of a SCF for the protein, which results in protein precipitation and water extract from the formulation. SCFs exist at a temperature and pressure above their critical points in a single phase with characteristics if both liquid and gas. The density of a SCF increases with pressure and can exceed that of the liquid , whereas diffusivity and viscosity are gas-like and ensure a high mass transfer. The density of the SCF is directly correlated with the salvation power and thus a small change in pressure the salvation power dramatically. The critical temperature and pressure varies for different SCFs and the exact values are important for the applicability of a given SCF. Supercritical carbon dioxide is often used, because of its low critical temperature (31.5 °C) compared with water (374.4°C), and it is generally regard as safe by FDA.

The method of SCF in the drying of protein has been applied in two different experimental setups. The first procedure utilizes spray drying of the protein formulation into SCF. The setup is in principle similar to the regular spray drying, with the SCF being used as the drying gas instead of hot air or nitrogen. The drying is driven by the precipitation of the solute in the droplet because the SCF is dissolved in the solvent, which subsequently loses solvent power. The supersaturated protein solution in the droplet precipitates as the water is extracted to the SCF and the protein is vitrified in an amorphous matrix. In the second setup the SCF and the protein solution is mixed with the SCF before atomization and sprayed to atmospheric conditions. In this step the SCF is primarily used to enhance the atomization of the protein solution because the drying takes place under a nitrogen flow.

The main challenges of SCF drying are much less understood compared with freeze-drying and spray drying, and only a limited number of studies have addressed

the effect of SCF drying on protein structure and stability. Whilst the stresses from dehydration are also presented for SCF drying, other stresses common with the alternative processes are the adsorption and denaturation at the air-water interface of the droplets and possible thermal stress. The high pressure necessary for the SCF might have an effect on the protein stability and the tendency to unfold should be taken into consideration for each protein. The dissolution of carbon dioxide in the protein solution may result in a significant decrease in pH if the solution is not buffered correctly which may also affect the protein stability. Furthermore, the use of organic solvents in the protein solution may result in aggregation (Winters et al.,1996).

For the concept of particle engineering of SCF dried proteins, the primary parameters influencing the particle morphology are the operating temperature and pressure, and the atomization conditions of the nozzle, similar to spray drying and spray-freeze-drying. The pressure of the SCF affects the viscosity, which in turn affects the atomization of the solution into droplets. The temperature affects the solubility of the protein and thus the precipitation rate. Generally, a fast precipitation rate is desired to avoid crystallization. When excipients are used they are often present in much higher concentrations than the protein; it is important to use excipients with a high T_g to produce amorphous matrix and to avoid crystal formation (Maltesen et al.,2008). SCF drying is likely to remain a marginal method in the pharmaceutical industry for the drying of protein pharmaceuticals, but it may become a useful technique for the preparation of advanced drug delivery system, also those containing proteins.

Biopolymers

Natural polymers such as collagen, alginate, and chitosan have featured frequently as drug delivery devices in the literature. Natural polymers such as these carry a number of advantages over the synthetic polymers such as the reduced need for harsh processing conditions (e.g., high temperatures).

1. Collagen

Collagen is the major structural protein found in animal tissues. Because of its unique structural properties it has found a variety of biomedical applications such as sutures, wound dressings, facial reconstructive surgery, and as drug delivery vehicles. It is biocompatible and nontoxic to most tissues and has been used as film ocular delivery of several ophthalmic drugs. Collagen has also been used for protein and peptide delivery. For example, water-soluble protein fractions isolated from bone matrix have been incorporated into collagen matrix and shown to induce bone and cartilage formation *in vivo* (Nan et al.,2005).

2. Cellulose derivative

Cellulose is the most abundantly available organic material; half of all organic carbon in nature is in cellulose form. Cellulose is a polymer of glucose with the glucose units connected by β-(1,4) linkage. Cellulose polymer chains are stabilized by hydrogen bonds between adjacent hexose units.

Cellulose is a hygroscopic material, insoluble but able to swell in water, dilute acid, and most solvents. Solubility can be achieved in concentrated acids but at the expense of considerable degradation through acetal (glycosidic) hydrolysis. Alkali solutions lead to considerable swelling and dissolution of hemicellulose present.

Ethylcellulose is a cellulose ether derivative which is one of the most widely used water-insoluble polymers for coating of solid dosage forms. Besides the predominant use as controlled release barriers, they have also been used as moisture barriers to improve stability of hydrolytically labile drugs or for taste masking purposes. Usually low molecular weight grades are used for coating while high molecular weight grades are used for microencapsulation. Water-soluble cellulose ether derivatives such as hydroxylpropyl methylcellulose (HPMC) are often used to modify drug release from delivery systems. Ethyl cellulose and HPMC have been extensively used in a variety of drug delivery models such as matrix preparations and microspheres (Nan et al.,2005).

3. Polysaccharides

3.1 Dextran

Dextran is composed entirely of glucose residues with primarily α-(1,6), and occasionally α-(1,2), α-(1,3), and α-(1,4) linkages for branching. This gives it an open helix conformation. Dextran has been used as a blood plasma substitute and drug carriers (Schacht et al.,1985; Nan et al.,2005; Ramirez et al.,2007).

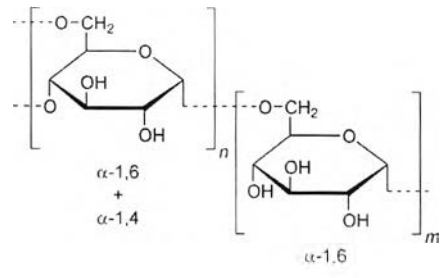

Figure 6. Chemical structure of dextran

Dextrans are readily soluble in water and electrolytes, and very concentrated aqueous solution of dextran fractions may be prepared. Dextran is also soluble in dimethylsulfoxide, formamide, ethylene glycol, and glycerol but is insoluble in monohydric alcohols. It should be noted that dextran fractions of molecular weight <20,000 may tend to adopt a certain degree of crystallization and may require strong heating to bring them into solution (Belder,1996). Dextran behaves in solution as a flexible coil, and its dimensions are dependent on, inter alia, molecular weight, and solvent.

3.2 Alginate

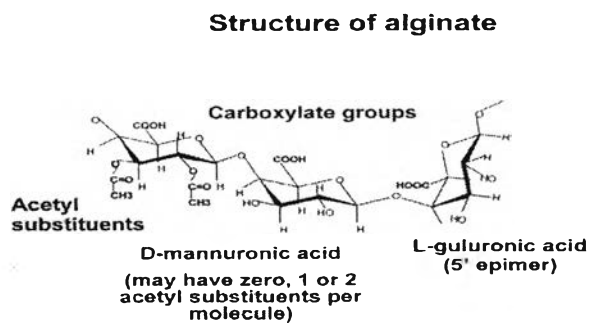

Figure 7 Chemical structure of alginate

Alginate is a family of unbranched binary copolymers of (1→4)-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues of widely varying composition and sequential structure (Draget et al.,2006). It is well known established that except for some bacterial alginate, alginate contains L-guluronic acid in various proportions depending on the organism and tissue from which it is isolated. It is an anionic polysaccharide derived from brown algae. When complexed with divalent cation (Ca^{2+}), alginate forms a gel, almost independent of temperature, unlike many other biopolymers that require ~37°C (Ginty et al.,2006). The gelation of alginates is based on their affinity towards specific ions, with the G units responsible for specific ionic binding. This high level of binding gives the G units more rigidity and mechanical strength. Therefore, the ratio of G units to M units can be altered to change the properties of the gel, a useful tool when designing drug delivery devices. Despite the fact that alginate has been shown to induce inflammatory response, it is well tolerated and has been used to produce drug delivery matrices with some success. Alginate microspheres have been used to encapsulate protein (Wells et al.,2007) and controlled release of drugs (González-Rodríguez et al.,2002{Nokhodchi, 2004 #96; Rajinikanth et al.,2003).

3.3 Chitin and chitosan

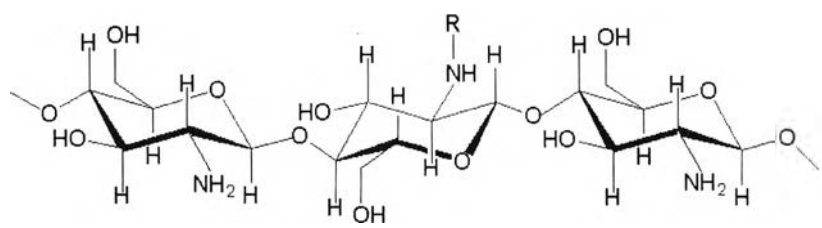

Figure 8 Chemical structure of chitosan

Chitin is the second most abundant organic compound and is similar to cellulose, except it is composed of *N*-acetylglucosamine in a β-(1,4) linkage. Chitin is readily available and occurs naturally in many insects and marine organisms. It is popular component of cosmetic and health care products. A close derivative is chitosan, a linear glycosaminoglycan made up of β-(1,4) linked 2-amino-2-

deoxyglucopyranose. Chitosan can be dissolved in inorganic or organic acids to obtain protonization of the amino group resulting in positively charged chitosan salts. Chitosan is available in a broad range of molecular weights and salt forms such as glutamate, lactate, and chloride. The properties of chitosan such as pKa and solubility, can be modified by changing the degree of deacetylation and the environment of the formulation, such as pH and ionic strength.

Chitosan has been used as a pharmaceutical excipient in oral drug formulation to improve the dissolution of poorly soluble drugs (Portero et al.,1998; Maestrelli et al.,2004) and for sustained release of drug (Nunthanid 2004). It has been shown to be mucoadhesive in nature, due to an interaction of the positive charges on the molecule with the negatively charged sialic acid groups of the mucin (Illum,1999). Moreover, chitosan has shown to enhance the nasal absorption of drugs such as polar, low-molecular-weight molecules as well as proteins, due to prolonging the resident time of chitosan formulation in the nasal cavity.

3.4 Pectin

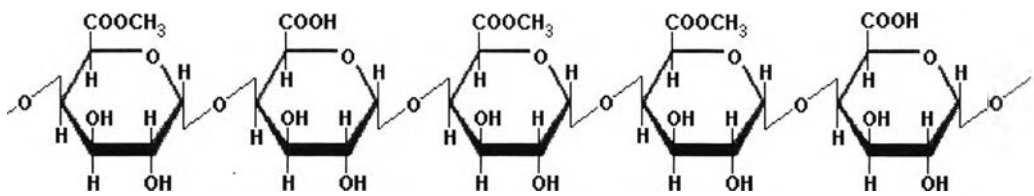

Figure 9 The chemical structure of pectin

Pectin is a cell wall structural carbohydrate present in all higher plants. Commercially available pectin is obtained from edible plants. Like many naturally occurring polysaccharides, pectin is heterogenous with respect to chemical structure (Figure 6) and molecular weight. Primarily, pectin contains large amounts of poly(D-galacturonic acid) bond via α– 1, 4-glycosidic linkage (Rolin et al.,1998). Pectin also cantains neutral sugars such as L-rhamnose, which are either inserted in or attached to the main chains. In pectin from all souces the carboxyl groups are partially in the methyl ester form. The degree of esterification (DE) varies depending on the source of the pectin and enzymatic activity in the process of ripening and maturation, and the

conditions under which the isolation is conducted. Some of carboxyl groups may be converted to carboxamide groups, when ammonia is used in the process of de-esterification, producing amidated pectin. The DE and the degree of amidation (DA) determine the content of carboxylic acid in pectin chains. The most attractive property of pectin for industrial applications is its gelling activity. Studies on the congelative property of pectin were initiated by Vauquelin in 1790. These led to applications of pectin in the food industry as gelling or thickening agentin the beginning, and then as an excipient for pharmaceutical purposes. Factor which determine whether gellation can occur and which influence gel characteristics are the types and concentration of pectin, DE, DA, the modifications of hydroxyl group, solution pH, temperature and the presence of cations. All these parameters are interdependent. For high DE pectins, the formation of hydrophobic areas parallel to the helix axes can expand to such an extent as to dramatically reduce the solubility of pectin. High DE pectins also gel in the presence of large concentrations of sugar. Aqueous solutions of univalent salts of pectins exhibit low viscosity at physiological pH (Paoletti et al.,1986). Univalent salts of low DE pectins are highly water soluble and gel only at extremely low solution pH or in the presence of divalent cation (Rolin et al.,1998; Liu et al.,2003). The introduction of amide groups in low DE pectin reduces the hydrophilic property with an increasing tendency to form gels. Those pectins of low DE, while having high DA, have received attention in the development of colon-specific drug delivery systems (Liu et al.,2003).

Pectins can gel in various ways depending on the type and structure of the pectin molecule. When solution pH is lowered, the ionization of carboxylate groups on pectins is repressed. Pectin molecule no longer repel each other over their entire chains, and thus can associate over a portion of their chains to form acid-pectin gels. For acid-induced pectin gels, the hydration of pectin is reduced and there is less water incorporated into interchain entanglements. When solution pH is raised, the polycarboxylate groups are ionized, and able to react with calcium ions to form calcium-pectinate gels. The interaction of calcium ions and the carboxylate groups in pectin involves intermolecular chelate binding of the cation leading to the formation of macromolecular aggregates.

Pectin-based drug delivery vehicles have been developed from pectin, either plain or chemically modified, in combination with other polymers, either naturally or synthetic. The composites take the advantages of their parent polymers and/or create useful new properties. For example, the composite of pectinand ethylcellulose combines the enzymatic susceptibility of pectin and the protective properties of ethylcellulose. Composites of pectin and chitosan or pectin and the polymethylmethacrylate derivatives carrying various amounts of $-NH_4^+$ groups (Eudragit® RL, RS) are enzymaticaclly degradable and more water resistant, although the three polymers in origin are water soluble. These composite matrices are often used for tablet coating in the forms of aqueous film-coating dispersion or blended powder, or for drug loading in the form of coacervate. In the later case, a drug can be loaded into the composite matrix either by the diffusion method or mixed with one of the components prior to coacervation. And, sometimes the composites are used as excipients in tablet formation(Liu et al.,2003). Pectins are natural polymers in all land plants, which have been discovered in the 18th century and coined as such in the 19th century after being crudely characterized as the active fruit component responsible for gel formation. As mentioned above, pectic substances are structural polysaccharides of the plant cell walls, which play an important role as cementing material in the middle lamellae and form one of the two independent but interactive polysaccharide networks of the primary cell walls in flowering plants.

Durian (*Durio zibethinus* Murr), a tropical fruit native to Southeast Asia, has been reported that its rinds could be a profitable agricultural waste as a potential source of the pectin. Polysaccharides have been extracted from durian rinds by hot water extraction followed by ethanol precipitation (Figure 7) (Hokputsa et al.,2004).

Durian rinds extract is composed of pectin as the principal component and starch as a contaminant (Hokputsa et al.,2004), which is often found in commercial pectins and referred as a ballast compound. This polysaccharide demonstrated a high viscosity and may therefore be used as an alternative viscosity enhancer instead of commercial pectins. Therefore it has been found to be useful in preparation of jellies and jams (Pongsamart et al.,2001), and as excipient in pharmaceutical preparations

such as tablet, suspension, and emulsion (Umprayn et al.,1990). Toxic effects of a high oral dose of polysaccharide gel were also investigated in mice and rats, with the results indicating that polysaccharide gel did not induce acute toxicity at dose of 0.125, 0.25 and 0.5 g/kg/day for 10 days when compared with water and pectin as a control and a standard polysaccharide, respectively (Pongsamart et al.,1989b). In addition, the research of long-term consumption in mice has been indicated that no toxic effect was induced in treated mice with dose of o.25 g/kg/day or 0.5g/kg/day for 60 and 100 days (Pongsamart et al.,2002).

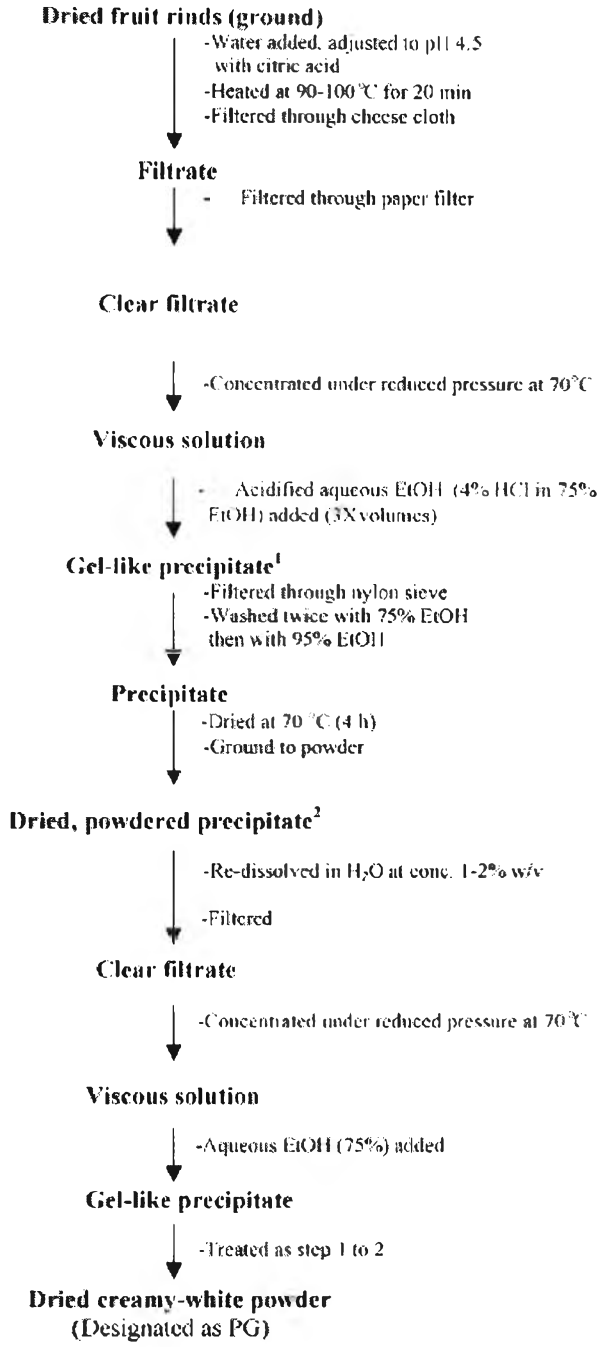

Figure 10 Schematic presentation of isolation of water soluble polysaccharides from *Durio zibethinus* Murr Rinds (Hokputsa et al.,2004).