CHAPTER II

LITERATURE REVIEW

1. Nasal Physiology and Anatomy (37, 39, 49)

The nose is one of the parts of upper airway including the mouth, nasopharynx, and larynx. The nasal cavity extends approximately 12-14 cm in length from nostril to the nasopharynx and is divided into two halves by the nasal septum. It has large surface area of approximately 150-160 cm^2 and volume of about 15 cm^3. The nasal cavity comprises various major differentiated regions.

1.1. *Nasal vestibule*, the anterior part of nasal cavity, opens through the nostril serving as a baffle system. There are nasal hairs playing roles in resistance to dehydration, noxious inhaled substances and filtering airborne particles.

1.2. *Atrium* is located between nasal vestibule and respiratory region and is the narrowest region. Anterior part of atrium presents stratified squamous cells, while its posterior part is pseudostratified cells with microvilli.

1.3. *Respiratory region* is one of the most important parts in nasal cavity. In human, it occupies about 80-90% of areas in nasal cavity because of 3 bony structures called superior, middle and inferior turbinates, while in rat it is about 50% (50). The surface area is covered with pseudostratified ciliated columnar cells with microvilli and richly blood supply playing roles in heating and humidicating inhaled air. It also helps to remove particles, microorganisms, and allergens which are beneficial for host body; however, such a function has an adverse impact on nasal drug absorption. Four different cell types constitute the epithelium as shown in **Figure II-1**, basal cells as precursor cells located on the basement membrane, goblet cells to produce surface mucus, ciliated and non-ciliated columnar cells for permeability of nasal epithelium. Between neighboring cells, apical tight junctions are formed. Importantly, the branches of trigeminal nerve spread through both respiratory and olfactory epithelium (50). Additionally, apical

surface of the epithelium is covered with sol-state and gel-state mucous layers secreted by seromucous glands and goblet cells. Mucus, composed of ~95% of water, ~2% of mucin, 1% of other proteins, ~1% of inorganic salts, and ~1% of lipid, is secreted 1.5-2.0 ml per day (49, 51). The movement of mucus for refreshing mucus and removing any substances on the nasal mucosa with the rate of 5 mm/min is MCC. Therefore, nasally administered substance will be cleared within 15-20 minutes at the normal stage (51). However, this system may be modified by several factors which will be discussed in the next topic.

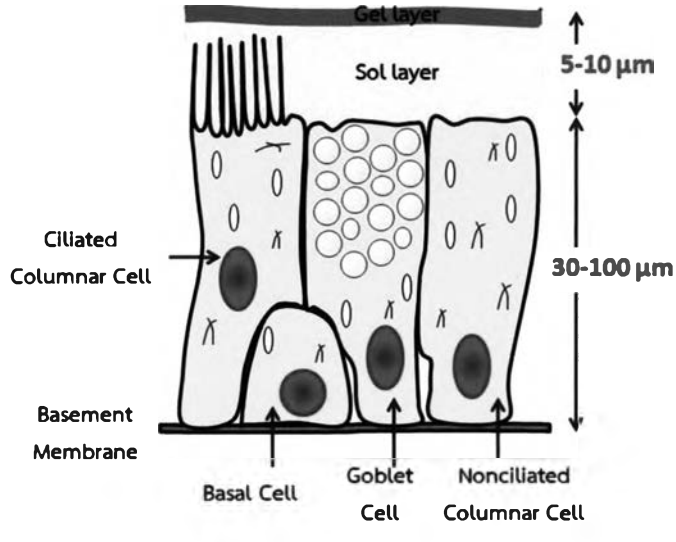

Figure II-1 Four major cell types in nasal epithelium; basal cell, goblet cell, non-ciliated columnar cell and ciliated columnar cell.

1.4. *Olfactory region* is normally known to serve as olfaction, because it has olfactory nerve cells. It also receives maxillary and ophthalmic nerve branches of trigeminal nerve. Olfactory region is composed of 10% of the nasal area in human which is situated on the roof of the nasal cavity. This region consists of various types of cells; olfactory sensory neuron (OSN), basal cell, supporting cell, Bowman's gland and ducts. OSN are bipolar neurons. Their dendritic branches are located in distal epithelium which ends as enlarged vesicles with several non-motile cilia that are directly opened to

the external circumstances. They regenerate every 3-4 weeks and as a result, protein characteristics, which are present in nasal mucosa, are not fully functional during the maturation of OSN (52). Their axons extend through the basal epithelium, lamina propria and encounter other axons to form nerve bundles called filia olfactoria. Filia olfactoria is surrounded by olfactory ensheathing cells (OEC) as well as fibroblasts, travels past the cribriform plate of the ethmoid bone, and then ends on the dendrite of a mitral cell in glomeruli of the olfactory bulb in the brain. All information is transmitted through a mitral cell to many regions in the brain including olfactory tubercle, anterior olfactory nucleus, piriform cortex, amygdala, and entorhinal cortex (50, 53). It is due to filia olfactoria or nerve bundle creating space of 10-15 nm (51). Therefore, transport by perineurons of molecule to olfactory bulb is limited by size of these spaces.

1.5. *Nasopharynx* is the last part in nasal cavity having a function as receiving nasal drainage. It comprises ciliated cells in the upper part and squamous epithelia in the lower part.

2. Transport and distribution from the nose-to-brain

The exact mechanism of nasal drug delivery to CNS is not completely understood, but much evidence indicates that mechanisms which involve nerves linking from the nasal epithelium to the brain and spinal cord are essential (52). Moreover, pathways relating to the blood vessels, cerebrospinal fluid, and lymphatic system are associated with the transport from the nose to the brain. However, two main pathways can be described. Firstly, olfactory nerve pathways are relevant to olfactory nerves which lie at olfactory region. Transport by these pathways, drug concentrations are mostly found in olfactory bulb. Secondly, trigeminal pathways are related to trigeminal nerves which lie both respiratory and olfactory epithelium. Drug concentrations enter to the brainstem at pons level. On the other hand, transports and distribution of macromolecules, such as peptides and proteins by intranasal administration can be divided into three steps: (1) transport across the respiratory and olfactory epithelia, (2) transport from the nasal epithelium to the sites of brain

entrance, and (3) transport from the initial brain entrance to the other sites within the brain.

2.1. Transport across the respiratory and olfactory epithelia

Delivery of macromolecules across the respiratory and olfactory epithelia, which serve as the barrier, may occur either by intracellular or by extracellular pathways as shown in **Figure II-2** and **Figure II-3** (50, 52). Intracellular pathways crossing epithelium probably include transcytosis over other cells to the lamina propria or endocytosis into OSN and then interneuronal delivery to the olfactory bulb or endocytosis through trigeminal nerve ending and later intracellular delivery to the brainstem. For example, large molecule like horseradish peroxidase (HRP) is internalized via fluid-phase endocytosis, and wheat germ agglutinin-horseradish peroxidase (WGA-HRP) is taken up via adsorptive endocytosis from nasal epithelium and then delivered by intracellular transport through the axon of OSN to the olfactory bulb by the anterograde direction (54). Similarly to the observation in olfactory region, WGA-HRP is taken up and delivered via intraneuronal transport through the trigeminal nerve branches to the brainstem (55).

Extracellular pathways consist of paracellular diffusion transport reaching to the laminar propria. Balin and colleagues (56) found that HRP, which was intranasally given in mice and squirrel monkey, was additionally internalized by OSN and then transported by using open intercellular clefts to reach olfactory bulb. It may be explained that tight junction had continuous rearrangement and loosening owing to regular turnover of nasal epithelium cells creating potential spaces for facilitating paracellular delivery of large MW drugs (50).

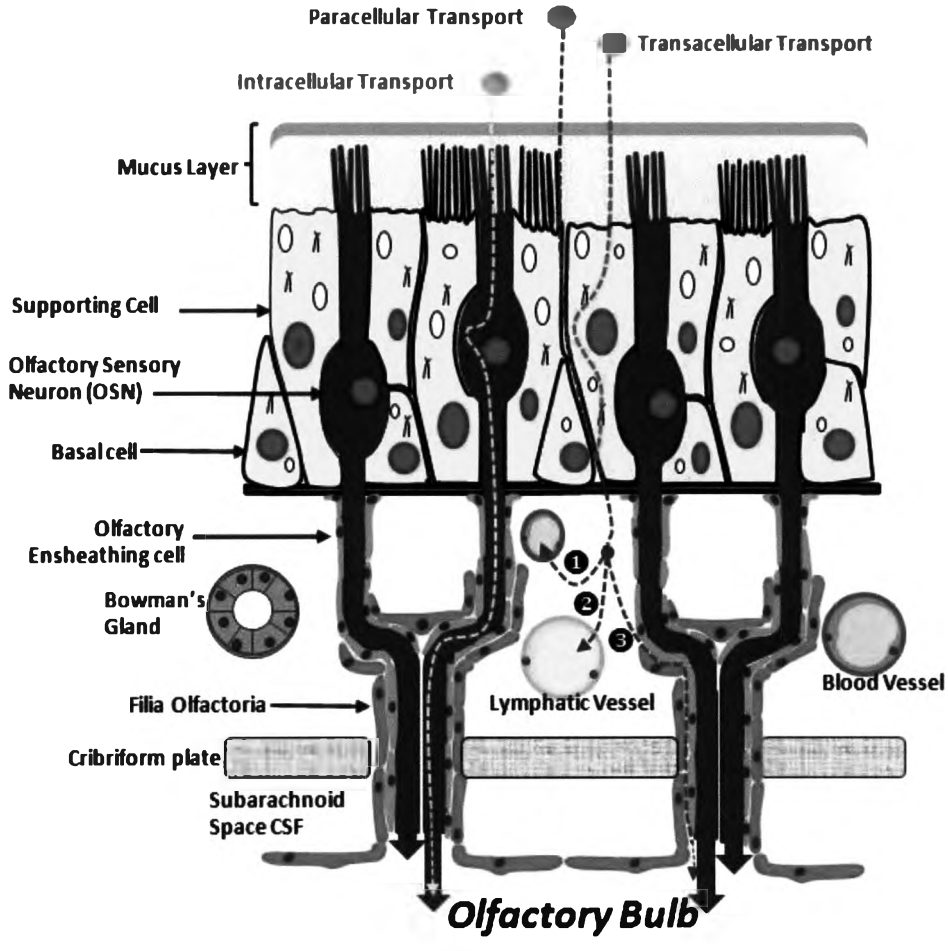

Figure II-2 Pathways of peptide and protein transport in the nasal cavity to CNS via the olfactory region. Some drugs may be delivered by an intracellular pathways from olfactory epithelium via olfactory sensory neurons by adsorptive or fluid-phase endocytosis to the olfactory bulb. Others may across the epithelium by paracellular or transcellular transport to reach the lamina propria, and subsequently possible extracellular pathways for distribution, as demonstrated: ❶absorption into blood vessels and pass into the systemic blood circulation; ❷absorption into lymphatic systems via lymphatic vessel draining to the lymph nodes at the neck; and ❸bulk flow or diffusion of convection through nerve bundles and subsequently pass into the cerebral compartment (50, 52).

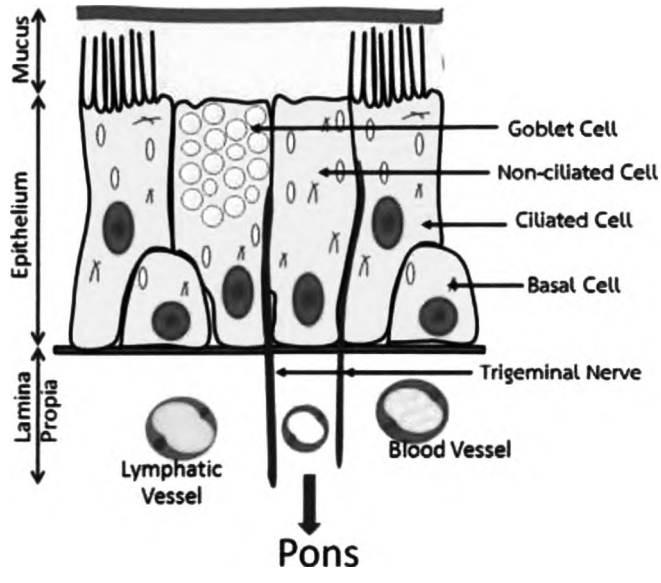

Figure II-3 Pathways of peptide and protein transport in the nasal cavity to CNS via the respiratory region. Trigeminal nerve fibers extending in both the respiratory and olfactory epithelia send chemosensory, touch, nociceptive and temperature information to CNS at pons level (50, 52).

2.2. Transport from the nasal epithelium to sites of the brain entrance (50)

In theory, delivery from the nasal epithelium reaching into the olfactory bulbs or brainstem at pons level may happen via both intracellular (endocytosis and intraneuronal transport) and extracellular (diffusion or bulk flow transport) pathways. Intracellular pathways have been aforementioned. The possible extracellular pathways include (1) absorption into blood vessels and enter into the systemic blood circulation, (2) absorption into lymphatic systems via lymphatic channels and drain to deep cervical lymph nodes, and (3) diffusion or convection (bulk flow) using perineural or perivascular spaces via nerve bundles, and subsequently enter into the cerebral compartment.

When a drug reaches into lamina propria, drug absorption may occur through nasal blood vessels which are rich at respiratory region, enter to the general circulation, and cross the BBB to reach the CNS. Although an observation showed

that the olfactory bulb and cortex of rats were permeable to serum albumin while the respiratory and olfactory regions were not when injected Evan blue as indicator, interpretation of Evan blue was complicated due to non-negligible fractions of free drug, binding between drug and non-albumin proteins in the serum also happened (57). Moreover, radiolabeled [^{125}I]-Insulin like growth factor-1 (IGF-1) concentration in olfactory bulb and frontal cortex after intranasal administration in adult male Sprague–Dawley rats did not correspond with that in blood (26); therefore, supported that the CNS transport mechanism of IGF-1 might not be associated with distribution from the blood circulation.

In the lamina propria, intranasal administration of protein drugs may be absorbed via lymphatic channels draining to the cervical lymph nodes of the neck, so they may be found at least in part at such lymph nodes. A study (26) suggested that nasal epithelium and deep cervical lymph nodes communicated with each other. The concentrations of IGF-1 measured by gamma counting were magnitude higher in deep cervical lymph node and other parts in CNS of male rats following intranasal than intravenous administration (IV). As cervical lymph nodes are an augmentation of the brain's immune, studying of the lymphatic drainage system is prone to acquire knowledge with promising for monitoring and curing multiple sclerosis (MS), an immune-mediated inflammatory disease, damaging the myelin and the axon in CNS with variable degrees (58). Nose-to-brain peptide delivery technology is currently entering phase II clinical trials in patients suffering from MS, and has shown safety, tolerability and efficacy in a phase I/IIa clinical trial (59).

Apart from absorption from epithelium into the systemic circulation and lymphatic system at the neck, a remaining drug may enter the CNS by perineural pathway. Fluorescently labeled 3 kDa dextran was found in the perineural spaces of the sheath around olfactory nerve bundle, lamina propria, and the outer layer of olfactory bulb. This result suggested some direct pathways from nasal epithelium to the CNS (60). Moreover, the perineural spaces of olfactory and trigeminal nerves could communicate with the cerebrospinal fluid (CSF) of the subarachnoid space, providing a promising pathway to enter the CNS (50, 61). However, not all proteins

delivered by intranasal administration are able to detect in the CSF, for example, IGF-1 (26).

The vast majority of published studies of drug delivery from the nose-to-brain demonstrated rapid delivery of peptides and proteins with high CNS concentration. The most likely mechanism of transport and distribution for these proteins in adult rat may be summarized that convection or bulk flow transport through olfactory and trigeminal nerves to the brain is only plausible transport process (26).

2.3. Transport from the initial brain entrance to the other sites within the brain

When drugs are transported to the initial brain entry, the final step is distribution to other areas. The distribution may be related to either by intracellular transport such as transferring and uptaking to higher order neurons which synapse with peripheral OSN or trigeminal ganglion cell, or by extracellular transport such as widespread distribution through cerebral perivascular spaces using convective transport and diffusion into the parenchyma. Rapid transportation results from extracellular flow occurring along the nerve fibers in the nasal mucosa to the CNS targets. The most promising convective transport that could account for the further distributing of macromolecules from the brain entry sites to other far areas in the brain is bulk flow mechanism through the perivascular spaces. Moreover, the rostral migratory stream (RMS) was suggested to play a possible role in transport macromolecules between periventricular regions and the olfactory bulb by using neuronal progenitors. Levels of [^{125}I]-calcitonin and [^{125}I]-erythropoietin (EPO) in the brain after intranasal administration decreased when RMS was dissected (62).

3. Strategies to improve nasal absorption

Although there is possibility to utilize nasal administration of peptides and proteins, their nasal bioavailability is limited which may be one of the reasons of difficulties in development. Two ways that have been used to enhance bioavailability of intranasal peptides and proteins, which are naturally very poorly absorbed, are either chemical approach or formulation approach.

3.1. Chemical approach

Because of biochemical environment of epithelial membrane being an obstacle to transport peptides and proteins, several strategies have been challenged to struggle the barrier. There are two strategies used; absorption enhancers and enzyme inhibitors.

3.1.1. *Absorption enhancers* (63-65): The ideal properties of absorption enhancer should be practical in small amount, non-irritating, non-toxic, temporary as well as reversible effect, and compatible with others. The functions of absorption enhancers for improving nasal bioavailability include increasing the membrane fluidity, decreasing mucus viscosity, prohibiting proteolytic enzyme, disrupting the tight junction, enhancing the paracellular and/or transcellular transport, improving blood circulation, dissociating aggregation of protein, initiating pore formation, or combinations. To date, there are about 11 types of nasal absorption enhancing agents as shown in **Table II-1.**

3.1.2. *Enzyme inhibitors* (37, 63, 64, 66-70): the nasal mucosa has several enzymes such as trypsin-like activities, glutathione transferase, carboxylesterases, aldehyde dehydrogenase, cytochrome P450, carbonic anhydrase, cathepsins, carboxypeptidase, glucuronyl transferase, aminopeptidase, and others. Therefore, to protect peptides and proteins from enzymatic degradation, co-administer of peptidase/protease inhibitors is established. Due to similar structure of enzyme inhibitors, their mechanism is competitive mechanism. Examples of enzyme inhibitors using in research include apotinin, bacitracin, bestatin, amastatin, boroleucin, puromycin, camostat mesilate, nafamostat mesilate, etc. They are effective for improving nasal absorption of several therapeutic peptides such as salmon calcitonin, vasopressin, luteinizing hormone releasing hormone, and desmopressin.

Table II-1 Nasal absorption enhancing agents (71-73)

Absorption Enhancers	Examples
Surfactants	polyethylene-9-lauryl ester, Brij 35, polysorbate 80, quillaja saponin, sterol glucoside, soybean-derived sterol, lysophosphatidylcholine (LPC), dodecylmaltoside, sucrose cocoate etc.
Bile salt and derivatives	sodium glycocholate, sodium taurodihydrofusidate (STDHF), taurocholic acid, sodium glycodeoxycholate (GDC), sodium taurocholate, alkyl glycosides and sodium deoxycholate
Chelators	EDTA
Fatty acid salts	oleic acid, sodium caprate, sodium caprylate, and sodium laurate
Phopholipids	lysophospholipid, didecanoylphophatidyl-choline (DDPC)
Glycerrhetinic acid derivatives	carbenoxolone, glycyrrhizinate
Cyclodextrins (CD)	α-CD, dimethyl-β-CD, hydroxypropyl-β-CD, methylated β-CD and γ-CD
Glycerols	N-glycofurols, N-ethylene glycols
Cationic polymer	chitosan and its derivatives, aminated gelatine, poly-L-arginines,
Anionic polymer	sodium hyaluronate, N-acetyl-L-cysteine
Others	nitric oxide donor, borneol/menthol eutectic mixture, polyacrylic acid

3.2. Formulation approach (63)

The rapid clearance of the dosage form owing to MCC may be a disadvantage in obtaining reproducible absorption kinetics. Therefore, in order to enhance the absorption efficiency of therapeutic peptides, prolonging nasal retention time based on the formulation approach should be noted.

3.2.1. Dosage form: The dosage form of any therapeutic drugs is selected from the expected indication, drug being used, other drugs in the

market, and patient population. There are four kinds of dosage forms, each of which has advantages and disadvantages as in **Table II-2**.

3.2.2. Bioadhesives: A bioadhesive formulation may be one of the promising strategies to improve contact time with nasal mucosa. In fact, bioadhesive microsphere containing polymer such as starch, albumin, and Sephadex® with a particle size range of 40-60 μm had been observed to be cleared from nasal cavity quite slower than solutions (63). Bioadhesive polymers used in nasal peptide and protein drugs include carbopol, chitosan and their derivatives, polyacrylic acid, cellulose derivatives and sodium hyaluronate (39, 63, 74).

Table II-2 Summary of intranasal dosage forms and their characteristics.

	Dosage Form	Characteristics
Liquid Dosage form	**Nasal Drops** (63, 66, 75)	• The most simple and comfortable nasal delivery systems. • More widely distribute throughout the nasal cavity. • Reaching the posterior part of nasal cavity. • Lacking of dose precision and rapid drainage from the nose.
	Nasal Sprays (66, 75, 76)	• More accuracy by using metered-dose pumps and actuators. • A dose of spray per naris from 25 to 200 μl. • Slow clearance because of anterior part of nasal cavity deposition.
	Nasal Emulsions, Microemulsions, & Nanoemulsions (66, 77, 78)	• Better viscosity & physical stability. • Having a mucoadhesive property. • Microemulsion providing longer residence time. • Enhanced uptaking drug across nasal mucosa.

Table II-2 Summary of intranasal dosage forms and their characteristics (cont.)

	Dosage Form	Characteristics
Serni-Solid Dosage	Nasal gels (66, 79)	• Decreased clearance rate of MCC. • Reducing postnasal drip due to high viscosity. • Reducing irritation due to incorporating. soothing/emollient excipients. • Enhanced absorption.
Solid Dosage Forms	Nasal Powders (63, 66, 80, 81)	• Simpler to formulate (fewer excipients needed) • Absence of preservatives. • Superior chemical and microbiological stability. • Higher bioavailability and patient acceptance. • Some challenges in dose accuracy and irritation to nasal mucosa.
Novel Formulation Approaches	Microspheres (63, 65, 66, 82-84)	• Reducing clearance rate and prolonging residence time with the nasal mucosa by using bioadhesive material such as starch, albumin, cross-linked dextran (Sephadex®), aminated gelatin etc. • Transient opening tight junction. • Toxicity/irritancy to the nasal epithelium must be evaluated
	Nanoparticles (13, 51, 65, 66, 85-87)	• Achieving for nose-to-brain drug delivery by surface modification of the nanoparticles. • Protection the encapsulated drug from decomposition and efflux transport by P-glycoprotein. • Simpler to prepare (compare to liposomes). • Simpler to scale up. • Highly stable both storage and *in vivo*.

Table II-2 Summary of intranasal dosage forms and their characteristics (cont.)

Dosage Form	Characteristics
Liposomes (37, 66, 79, 88)	• Providing bioadhesive effect especially positive charged liposomes. • Improved nasal permeability

4. The basic and mechanisms of mucoadhesion (89-91)

Mucoadhesion can be defined as the state in which two materials are held together for prolonged period of time by interfacial force (91). In the field of pharmaceutical sciences, one material is adhered to mucus or a mucus membrane. The adhesive materials must bind across the interface of mucus for adhesion to occur. These chemical bonds are ionic, covalent, hydrogen, Van-der-Waal, or hydrophobic bonds.

4.1. Theories of mucoadhesion

There are 6 theories that have been adapted from studies explaining the phenomenon as the following **Table II-3.**

Table II-3 Theories accounting for mucoadhesion (89-91).

The electronic theory	• Based on the opposing electrical charges of materials. Therefore, they form an electrical double layer at the interface when both materials occur upon contact of adhering surfaces.
The wetting theory	• Applying to liquid systems. • Based on the capability of a liquid to spread over a surface which is a prerequisite for the development of adhesion.
The adsorption theory	• Based on hydrogen bonding and Van-der-Waals' forces. Though they are independently weak, a numerous number of bondings lead to an intense adhesion.
The diffusion theory	• Based on interdiffusion of both polymers and mucin chains across an adhesive interface in order to create a semi-permanent adhesive bond. The interdiffusion rate is subject to the diffusion coefficient, chain flexibility, mobility, and the time of contact.
The mechanical theory	• Based on an interlocking of mucoadhesive liquid into the irregularities on a rough surface.
The fracture theory	• Associated with the strength of adhesion to the forces required for separating two surfaces after adhesion is established. This theory is suitable for use in the materials in which chains of polymer do not diffuse into the mucus layer.

4.2. Mechanisms of mucoadhesion

In the study of adhesion, the mucoadhesive process is frequently categorized into two stages, the contact stage and the consolidation stage (**Figure II-4**).

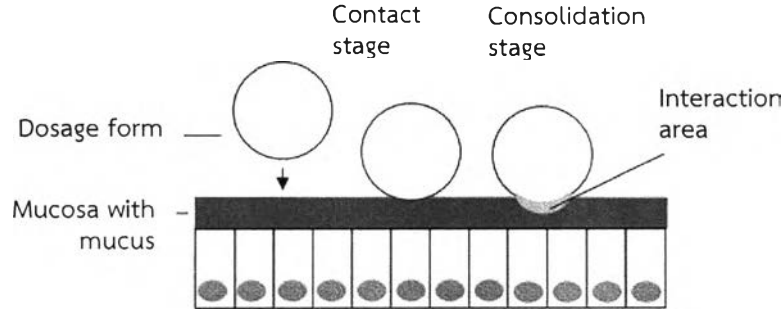

Figure II-4 The two stages in mucoadhesive process (modified from (91)).

The first step is the intimate contact between the mucoadhesive and mucosa. The formulation will spread and swell in order to initiate its deep contact with the mucus layer. Moreover, the attractive forces (Van-der-Waals' force, surface energy effects, and electrostatic interactions) of both formulation and mucus layer must be greater than their repulsive forces (osmotic pressure, steric forces, and electrostatic interactions).

The second step is to consolidate and strengthen the adhesive joint, resulting to extend adhesion. Moisture plays an important role by plasticizing the system that allows mucoadhesives to get free, conforming to the roughness of the surface, and predominant bonding by weak Van-der-Waals and hydrogen forces.

5. Peptides and proteins delivery

Currently, therapeutic proteins are more interesting among medical scientists because there are many peptides and proteins used for treatment diseases such as diabetics, growth hormone deficiency, gastrointestinal disorders, hemophilia, anemia, fungal infections, stroke, and cancer (16, 92-94). However, pharmaceutical formulation development of peptides and proteins has been challenged by their physical and chemical instability. Such challenges result in difficulties of clinical use such as inadequate blood concentration, poor oral bioavailability, poor BBB penetration, short shelf stability, rapid hepatic metabolism, and inefficient passing through the target cells (10, 16). Therefore, parenteral administrations are primarily main route for peptide and protein therapeutics.

5.1. Structures of peptides and proteins (16, 95)

Peptide and protein molecules can be divided into four levels of structure; primary, secondary, tertiary, and quaternary structures.

Primary structure refers to linear arrangement of simple building units called amino acids and the location of disulfide linkages in the component polypeptide chains.

Secondary structure describes spatial conformation of a polypeptide chain through hydrogen bonding or disulfide bridges of neighboring amino acids, and the common forms are the alpha-helix and beta-sheets.

Tertiary structure can be described as the 3-dimentional structure of polypeptide chains resulting from attractive hydrogen bond, ionic, or hydrophobic interactions of amino acids on secondary structure polypeptide chains in order to stabilize their structure.

Quaternary structure refers to the highest degree of protein organization. It is the association of more than one polypeptide chain combining to form dimers,

trimers, and oligomers, establishing the quaternary structure. In addition, almost all of the proteins having MW larger than 100 kDa have a quaternary structure.

5.2. Instability of peptides/proteins (15, 16)

One of the challenging issues for pharmaceutical protein development is to both physical and chemical instability of therapeutic peptides and proteins. Moreover, not only primary structure but also higher level structures of protein have an essential impact on their biological activity, unlike small organic molecules.

5.2.1. Physical instability

Peptide and protein instability by a change in higher level structure and no association with covalent modification can result in denaturation leading to aggregation, precipitation, and/or adsorption to surface.

Denaturation: Disruption of higher level structure of peptides and proteins causes from cooling, freezing, heating, extremes of pH, and contact with organic substances leading to losing of native state of protein called protein denaturation. Denaturation of protein is usually related to increased hydrophobic surface of protein. In solution, several protein molecules might self-associate and exclude the solution. This phenomenon is called aggregation. If aggregates are visually evident and separate from solution, the phenomenon is termed protein precipitation.

Aggregation and precipitation: Protein aggregation describes non-reversible interaction and clustering protein molecules which may be soluble or insoluble. There are many factors leading to protein aggregation/precipitation, such as shear forces, temperature, ionic strength, pH, and moisture.

Surface absorption: The exposure between hydrophobic interior of protein surface and non-polar surface of container or filter leads to adsorption to the surface. The pH and ionic strength of media as well as initial concentration of protein in solution may enhance or reduce protein to adsorption. Poly (oxyethylene oxide)

or Teflon® quite effectively reduces and prevents protein denaturation by adsorption.

5.2.2. Chemical instability

Chemical instability of peptides and proteins is associated with modification of peptides and proteins via bond formation or bond cleavage which yields a new chemical entity.

Hydrolysis: Proteolysis is the hydrolysis of peptide linkage, and rapid hydrolysis occurs at an extreme pH and temperature. Luteinizing hormone releasing hormone, human growth hormone, macrophage colony stimulating factor, and vasoactive intestinal peptide are known to hydrolyze by this reaction.

Deamination: Deamination is an acid and base-hydrolysis reaction of side chain linkage in glutamine or asparagine residues to form a carboxylic acid. So, optimized pH and lyophilization are often used to reduce deamination in peptides and proteins.

Oxidation: Oxidation is one of the main chemical degradations resulting from atmospheric oxygen, source of peroxide in formulation, Fe^{2+}/Fe^{3+} and Cu^{+}/Cu^{2+} contamination, light, acid/base, and free radicles. Therefore, there are several strategies preventing oxidation of peptides and proteins including low temperature storage, light protection, antioxidant usage, pH optimization, minimization of oxygen exposure, and lyophilization.

Racemization: Racemization is to process involved with the conversion of enantiomer of amino acid residues. The rate of racemization depends on temperature, pH, ionic strength, and metal ion chelation.

Disulfide exchange: Disulfide exchange is catalization process by thiol compounds which results in tertiary structure alteration. However, addition of thiol scavengers can prevent this reaction.

Maillard reaction: Maillard or browning reaction may occur in the protein formulation presenting reducing sugars, which is associated with non-enzymatic glycosylation of basic protein residues. Storage at low temperature, removing reducing agents, reducing water content can minimize the reaction.

6. Powder formulation for intranasal drug delivery

There are numerous intranasal preparations inventing and available in markets as well as research publications as previously mentioned. Each dosage form has its own unique and advantageous characteristics. However, solid dosage form, especially powder formulations, is useful over liquid formulations such as extending absorption time, enabling high drug concentration at the absorption site, avoiding preservative used, providing higher drug loading per delivery dose, improving stability of products, and minimizing temperature damages products during distribution or storage (38, 45-48).

Dry powder dosage forms can be formulated into two types; native drug powder and drug-polymer powder. Some native drug powder formulations could permeate through nasal mucosa without any absorption enhancer because of their properties and shows better drug concentration level in the brain than solution formulations (20), while adding polymers in formulations may enhance drug absorption properties and stability of formulations (42, 46, 81, 96). For example, apomorphine loaded polyacrylic acid powder formulation was less oxidized to green coloration at atmospheric environment depending on amount of polymer content (46).

6.1. Influencing parameters for nasal powder development

In addition to advantages of powder intranasal drug delivery over liquid ones, particle size and shape of powder formulations for intranasal products should be optimized because of influencing on potential to nasal irritation and deposition (97).

Needle-shaped particles are potentially more irritated the mucosa tissue than round-shaped ones. Sacchetti et al. (98) demonstrated that spray-dried caffeine

microparticles using mannitol as a filler and hydroxypropylmethyl cellulose (HPMC) as a shaper could modify the needle morphology of spray-dried pure caffeine to a more spherical morphology which was beneficial to nasal use.

Site of particle deposition in nasal cavity relies on target of action of therapeutic drugs. In case of rhinitis treatment, deposition of drugs over all entire nasal mucosa is required, while particles should deposit at the roof of nose around olfactory region for brain targeting therapeutics (46). There are several factors influencing on distribution of particles in nasal cavity such as shape, density, porosity, and so on (46, 99, 100). However, size of particles, inhalation flow rate, and a device produced cloud are the most three essential to nasal deposition (97, 101, 102).

According to US FDA draft guidance for industry sinusitis about designing clinical development programs of non-antimicrobial drugs for treatment (103), aerodynamic-based sizing of particles or droplets should be larger than 5 micron in order to prevent inhaling into the lung. Although the suitable size of particle for nasal delivery is limited, particles below than 5 micron are required in order to reach the olfactory region (45). Moreover, too large particles also do not reach the target site in nasal cavity but remain in the anterior part of nose which limit clinical efficacy (102). Studies were conducted on sizes of particles generated from devices to nasal and lung deposition as shown in **Table II-4**.

Table II-4 Comparison of intranasal delivery systems (modified from (104)).

Intranasal DDS	Particle size (μm)	Intranasal deposition	Intranasal drug retention	Pulmonary deposition
Small particle nebulizer	3-5	Antrum	Low	Yes
NasoNeb nasal nebulizer	23.3	Broad	High	No
Spray bottles	37-157	Antrum	High	No
Irrigation bottles	Fluid	Broad	Low	No

Inhalation flow rate is one of the factors having an impact on nasal deposition. Zwartz and Guilmette (105) conducting the effect of air flow rate to particle deposition in a replica of human nose demonstrated that more particle deposition occurred in the upper part of the nasal section than in the lower part, when air flow rate increased from 20 to 40 liter per minute. Such a phenomenon was explained by inertial impaction causing more particles to drop out of the air flow and rather deposit on the ceiling than the floor of the airway surface at high air flow rates.

Intranasal delivery devices for powder formulation have been developed for serving the formulations and improving clinical perspectives. Moreover, they may enhance reducing the risk of pulmonary deposition. Nasal powder devices can be classified into three main types based on different principles; breath actuated inhalers, mechanical powder sprayers, and insufflators, as shown in **Table II-5**. Among these devices, only insufflators, such Bi-Directional™ and DirectHaler™ technology, are less potential to the risk and problem related to lung. For instance, a comparative study in 16 healthy volunteers using radio-labeled particles with a mean particle size of 3.5 micron demonstrated that Bi-Directional™ insufflator could prevent lung deposition, whereas 12-39% fraction deposited in the lung in subjects using conventional inhaler (106).

Table II-5 Nasal powder delivery devices (107).

Devices types	Principles	Examples
Breath actuated inhalers	patient using his own breath to inhale the powder from a blister or capsule	Rhinocort Turbuhaler®, BiDose™/Prohaler™, and Twin-lizer™
Mechanical powder sprayers	Powder sprayed by utilizing a compressible compartment to provide a pressure	Fit-lizer™, UnidoseDP™, SoluVent™, and Monopowder™
Insufflators	subject exhales into a mouthpiece connected with a nosepiece to close the velum, and the airflow brings the powder particles into the nose via the device nosepiece	Bi-Directional™, DirectHaler™

6.2. Deposition mechanisms of powder for intranasal delivery (108)

Inhaled particles especially for nasal administration are deposited in nasal cavity by three essential mechanisms; inertial impaction, gravitational sedimentation, and Brownian diffusion (**Figure II-5**).

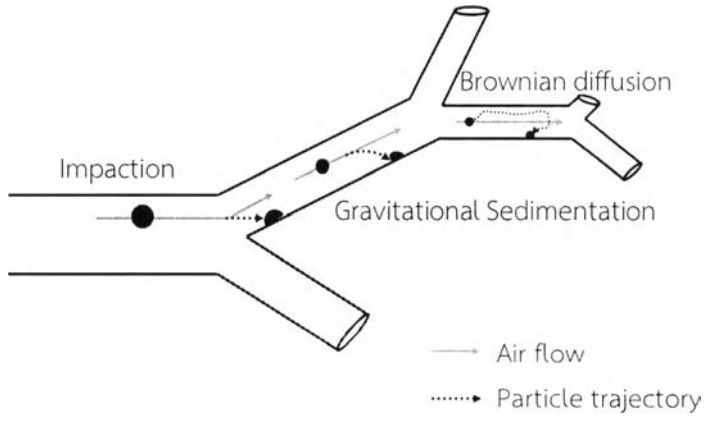

Figure II-5 Deposition mechanisms in respiratory airway (modified from (109, 110)).

Inertial impaction: When air stream carrying heavy, large, or fast-moving particles changes its direction, the particle flies off and impacts the airway wall, called inertial impaction. Furthermore, deposition by this mechanism can be influenced by the particle size larger than 0.5-1 micron and inhalation air flow rate. Therefore, inertial impaction may be essential and dominant mechanism for nasal powder deposition.

Gravitational sedimentation: This deposition mechanism dominantly occurs in the small conductivity airways and can be influenced by size of particle. The smaller particles will more slowly sediment than the larger ones.

Brownian diffusion: By collision with gas molecule, the particles whose diameter is less than 0.5 micron can be impacted to a surface wall and deposition depends on diffusion coefficient and inhalation flow. However, this mechanism can be ignored because suitable particle size for nasal delivery has to be larger than 5 micron.

7. Milling

Milling of materials is a common process in pharmaceutical industry because size reduction plays a significant role in several aspects affecting performance of pharmaceutical products. In general, milling provides pharmaceutical materials with increasing solubility rate having a positive impact on drug bioavailability (111, 112). Moreover, fine particles ranging from 1-5 micron are required for pulmonary drug delivery, especially dry powder inhaler (DPI) (113).

Three types of milling machines are focused.

7.1. Jet mill (JM)

Micronization by JM is used for dry comminution of powder for inhalation productions. JM utilizes high pressure and high velocity jets of gas in order to cause impaction between particle and particle and thus particle attrition (48). In short, the material with a size of approximately 1 to 2 mm is passed into the grinding chamber via a vibrating feeder or gas stream, as shown in **Figure II-6**. Prior to entering the grinding chamber, particles are accelerated through a pusher nozzle by air jet generated from compressed air. The particles, which undergo extreme turbulence flow and circular nature of the grinding chamber, are milled and crushed by impaction with each other or the chamber wall. As a result, the micronized particles are collected from grinding chamber to the collecting chamber by gas stream, while the larger particles still stay in the chamber by centrifugal force.

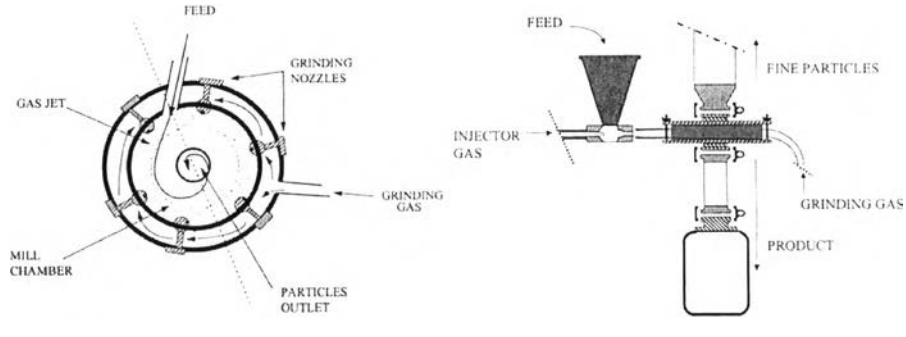

Figure II-6 Schematic figure of spiral JM machine (reproduced from (114)).

There are many parameters affecting on final particle size and size distribution, such as jet nozzle design and size, nozzle angle, nozzle pressure, feeding rate, particle size of the feed, as well as grinding chamber design and size, but only nozzle pressures, number of milling cycles and feeding rate can be adjusted to desirable product size (114-116).

In pharmaceutical industry, jet micronizer offers several advantages, such as producing high fineness particle less than 10 micron with narrow size distribution, enabling to control operating temperature in a chamber, producing one product stream, being simple to use and clean, no insusceptible to dust explosions, and no product contamination from autogenic grinding (48, 115, 117, 118). In view of product potency, LiCalsi and colleagues (48) conducted research on powder aerosol formulation of measles vaccine by using JM. The finding indicated that no major physical changes of obtained fine particle of measles vaccine were observed, and viral potency was retained. In addition, many macromolecules were investigated by micronized to particle size less than 5 micron such as BSA, human growth hormone, Interferon-β, granulocyte-colony stimulating factor, horseradish peroxidase, insulin, and salmon calcitonin (116, 119, 120).

7.2. Planetary ball mill (PBM)

PBM has the potential to comminute wide range of materials from soft, fibrous, hard to brittle materials. They are also appropriate for both dry and wet milling and suitable for several fields such as agriculture, biology, ceramics and glass, chemicals and plastics, construction materials, mineralogy and metallurgy, environmental research, and pharmaceuticals (121, 122). The mechanism of grinding is an interaction between frictional and impact forces. Briefly, a rotating pot made of zirconia placed on a revolving disk, as the grinding zirconia balls were mono-size spheres, as shown in **Figure II-7**. The pots are spun in the opposite direction against the revolving disk. The grinding balls in the grinding pot are based on superimposed rotational movements, and the difference in speeds between the balls and the pot produces such an interaction generating the high and very effective degree of size reduction (122, 123).

There are several factors influencing on milling resultants such as the diameter of pot, depth of pot, diameter of balls used, ball-filling ratio, revolving disk radius and the rotational speed (123, 124).

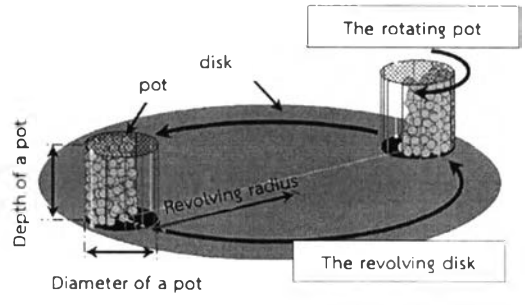

Figure II-7 Schematic figure of PBM (modified from (123)).

In addition to be a versatile mill, it accomplishes a very fineness down to the submicron range, uncomplicated handling, easy cleaning, and moderate budgets (122, 125). However, there are three problematic issues could occur. First, milled product may contaminate to the surrounding due to facing excessive temperatures over a long time interval resulting to loose integrity between the pot and the lid (126). Second, the product may decompose, evaporate or ignite during grinding due to excessive temperatures (126, 127). Third, it is the problem about technical difficulties in production scale (125).

7.3. Cryomill (CM)

CM is a cryogenic grinding by chilling the materials using cryogenic liquid such as liquid nitrogen in order to low temperature at which they are generally more brittle (128). Cryogenic mills are suitable for thermolabile, low-melting point, very elastic and volatile materials (129-131). In addition to prevent thermal damage to materials, they are also used to prepare the amorphous states of materials better than conventional grinding at room temperature, preserve crystallinity of drugs, prohibit the manifestation of unsatisfactory chemical reactions, and minimize particles aggregation (128, 132-135). Impaction and friction are grinding mechanisms of cryogenic mill (130). Briefly, CM consists of mill, grinding chamber, and grinding

rod. Sample is filled in the chamber with the rod and put in the mill. Before starting an operation, the mill is cooled down with large amount of liquid nitrogen to the desired temperature. Sample will be pulverized by mechanical stress from moving rod during the operation. On the other hand, liquid nitrogen may be present with milling sample (128). According to this process, CM could be used as a new and effective technique for producing submicron-sized powder (136).

One factor effecting to grinding results is vibrational frequency (130). The more frequency of the milling use, the finer grinding sizes are. Moreover, grinding time, are also influencing factors to the fineness of materials (134).

Although many studies indicated as previously mentioned that cryogenic milling could be suitable for heat-sensitive drugs, chemical decomposition of the drugs ground under cryogenic condition may be introduced (135). The other disadvantage of CM is the extensive cost associated with the used amount of liquid nitrogen during grinding period (128).