# Protein and Peptides Drug delivery, PEGylations and Biotech based products, Immunomodulated molecules

# Protein and Peptides Drug Delivery

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## INTRODUCTION:-

In recent years, the number of protein-based pharmaceuticals reaching the marketplace has increased exponentially. Innovations in biotechnology have led to a significant increase in the number of protein and peptide therapeutics and other macromolecular drugs. Approximately 84 macromolecules are currently approved for marketing in the United States, and almost 350 more are in clinical development. Further, recent advances in genomic and proteomic technologies are expected to continue to increase the pipeline of macromolecular therapeutic candidates. Working with macromolecules typically poses a number of challenges, however, that drug developers must overcome in order to successfully develop these compounds into safe and effective therapeutics.

## STRUCTURE OF PROTEINS AND PEPTIDES:-

Pharmaceutical proteins are built up of amino acids chains (their primary structure). If the polypeptide chain contains less than 40 amino acids than it known as ‘peptides’ and if contains more than 40 amino acids than known as ‘proteins’.

Parts of the proteins will fold in locally identifiable, discrete structure such as α-helices and β-sheets (their secondary structures). The overall (tertiary) structure of protein is established by proper positioning of different subunits relative to each other. In some cases individual proteins molecules form a quaternary structure, in which the individual protein molecules interact and build a larger, well defined structure. The biological responses of Proteins & Peptides depend upon their three-dimensional structures.

Many of them can bear a number of conformations and only a few of these that meet the conformational requirement of the respective receptors are of biological significance. This vital conformational flexibility assumes an iceberg importance in developing formulations with Proteins & Peptides as A.P.I.

Based on the conformation we can divide the proteins into two major categories: one consisting of fibrous proteins, which are composed of polypeptide chains that are arranged in parallel position along a single axis and results into long fibrous or sheets. Example includes keratin, collagen. Second consist of globular proteins, where the polypeptide chains are tightly folded into compact globular or spherical shapes. Example includes albumin

## CLASSES OF PHARMACEUTICAL PROTEINS:-

The pharmaceutical proteins are classified in four classes as shown below:

- Vaccines (Peptides, parts of proteins, Killed bacteria)
- Peptides (Oxytocin, Pitocin)
- Blood products (Factor X, Factor VIII, Gamma globulin, Serum albumin)
- Recombinant therapeutic proteins (Herceptin, Humulin, Alferon, etc.)

## PROBLEMS WITH PROTEINS AND PEPTIDES:-

The pharmaceutical proteins offers special challenges to the formulation scientists because of there are certain major issues are present in their formulation and developments.

As they are delicate, large molecules with many functional groups with a relatively weak physical bond make the vulnerable to readily and irreversible change. Same time it is very difficult to get them in large quantity but now a day because of advance in biotechnology they are obtained in large quantity by certain bio-techniques.

In vivo the protein pharmaceutical are recognized as foreign substance and so eliminated by B and T cells. They are susceptible for Proteolysis by endo/exo peptidases. Small proteins are easily and quickly filtered out by kidney so this type of protein required frequent dosing. Unwanted allergic reaction and even toxicity may develop after administration of protein pharmaceutics.

## IN VITRO - IN THE BOTTLE PROBLEM WITH PROTEINS AND PEPTIDES:

Proteins & Peptides are most susceptible molecules for almost all kind of stability problems as depicted through Physical instability (non covalent), Chemical instability (covalent), and Biological instability.

## Physical instability (Non covalent):-

### ❖ Denaturation:

If denaturation is reversible than the subjected molecule can be refold to its original form.In case when it is irreversible then unfold proteins fails to regain their original structures.

**Solvent**: Occurs due to solvent changes or use of blend of solvents

**pH alteration :**Alters the charge of molecules by inducing ionization of carboxylic acid and amino groups of amino acids and leading to unfolding of molecules

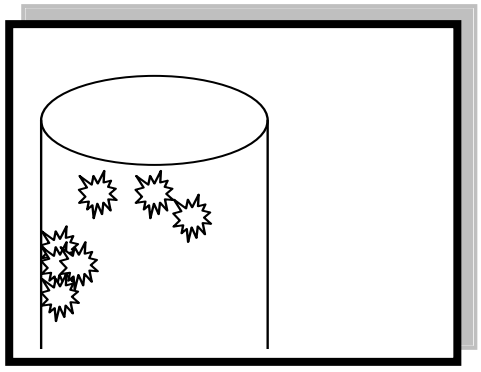
**Ionic strength**: Change in ionic strength of surrounding environment leads to reduction in resultant charge by grouping of oppositely charged around the molecule

**Temperature fluctuations:**

Induces rearrangement of structure leading to vanishing of inherent activity plus generation of totally new range of activity

### ❖ Adsorption:

Surface-adsorption of Proteins & peptides reduces the concentration of drug available to elicitate its function.



Both proteins & peptides are amphiphilic molecules and thus gets adsorb at interfaces like air-water, air-solid

**Chances of its occurrence**: during

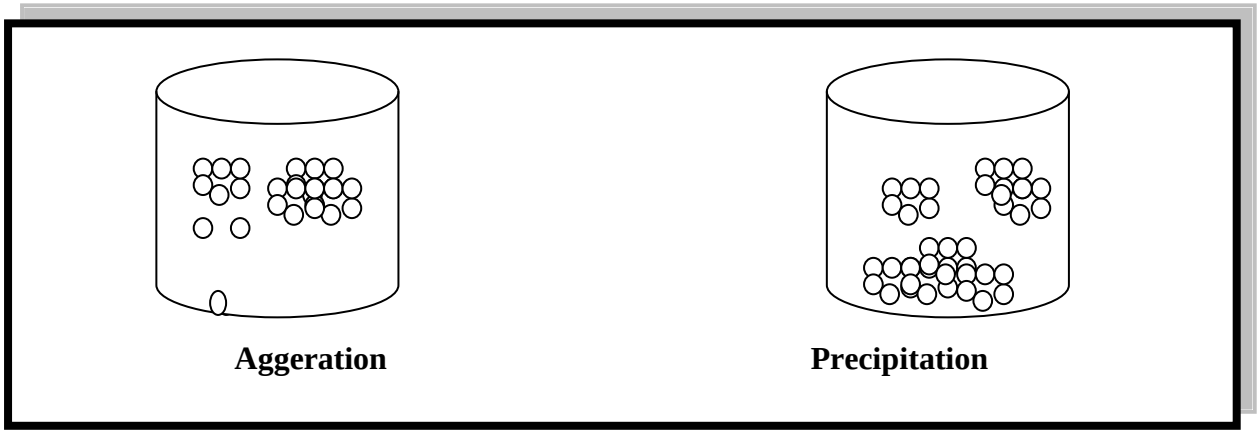
purification, formulation, storage and/or delivery.

Adsorbed proteins at surface when subjected to desorption, they desorbs leaving their hydrophobic residues exposed leading to opening of windows for unexpected aggregates & precipitation.

## ❖ Aggreation & Precipitation:

The extent of aggregation and precipitation of protein molecules depends upon the relative hydrophilicity of surfaces to which the polypeptide/protein solution are in contact with. Presence of large air-water interface accelerates these processes.

E.g. agitation of polypeptide and protein solutions introduces air bubbles, thereby increases the hydrophobic air interface leads to aggregation and precipitation of them**.**




## Chemical instability (Covalent):-

### ❖ Deamidation:

There is hydrolysis of the side chain amide linkage of an amino acid residue leading to the formation of a free carboxylic acid. The produced carboxylic acid leads to conversion of a neutral residue to a negatively charged residue and primary sequence isomerization.

### ❖ Oxidation & Reduction:

Oxidation is likely to occur during isolation, synthesis and storage of proteins. Oxidation occurs at side chains of histidine, lysine, tryptophan, thyronine residues in proteins. Even the atmospheric oxygen induces auto-

oxidation. Oxidation of amino acid residues is followed by loss of biological activity.

❖ **Proteolysis:**

Proteolysis occurs on exposing the proteins to harsh conditions, such as prolonged exposure to extremes of pH or high temperature or proteolytic enzymes. Bacterial contamination is the most common cause of proteases. Proteolysis is avoided by storing the proteins in cold under sterile conditions.

❖ **Disulphide exchange:**

Breaking and reformation of disulphide bonds results into an alteration in three dimensional structure followed by reduced activity. Disulphide exchange is more in case of molecules having large no. of disulphide bonds. This reaction occurs in neutral or alkaline medium and accelerates by thiols which are generated by disulphide exchange.

❖ **Racemization:**

With an exception of glycine all amino acids are chiral; their racemization will lead to increase in susceptibility of peptide bonds toward proteolytic enzymes. Presence of thiol scavengers like copper ions, N-thylmaleimide prevents disulphide exchange as well as racemization. Racemization is catalyzed in neutral and alkaline media by thiols generated through disulphide exchange.

## Biological instability problems:-

❖ **Enzymatic barrier:**

Proteolytic enzymes are located at various sites of our body making the proteolysis at site of administration, blood, vascular endothelia, kidney, liver etc. Chemical alteration of protein like phosphorylation by kinases, oxidation by xanthine oxidase or glucose oxidase. All the structural features of peptides and proteins are vulnerable to the protease activity. Hence each and every peptide bond in a molecule had to be protected from protease activity.

❖ **Intestinal epithelial barriers:**

Stereoisomerism, side-chain length, and N- and C-terminal substitution are reported to affect absorption of dipeptide.

# ISSUES TO BE CONSIDERED IN EFFECTIVE AND SAFE DELIVERY OF PROTEIN PHARMACEUTICAL:-

The way of processing peptides and proteins and their formulations such that the active maintains its optimal stability and activity. Their stabilization, Processing and formulation receives, attention for effective and safe protein Pharmaceutical

| Problems | Solutions |
|---|---|
| Deamidation, cyclic imide formation | pH optimization |
| Aggregation, precipitation | pH optimization; addition of sugars, salts, amino acids, and/or surfactants |
| Truncation | pH optimization, protease removal |
| Oxidation | Excipient purity analysis, addition of free-radical scavengers (mannitol, sorbitol), use of a competitive inhibitor (methionine) |
| Surface denaturation, adsorption | Addition of surfactants or excipient proteins |
| Dehydration | Addition of sugars or amino acids |

## Storage of proteins & peptides:-

### Refrigeration:

Storage of proteins and peptides at a low temperature leads to

- Reduction in microbial growth and metabolism
- Reduces thermal or spontaneous denaturation
- Reduces adsorption
- Freezing is best for long-term storage
- Freeze/Thaw can denature proteins & peptides so in such a case the storage condition will depends on the nature and properties of that particular proteins and peptides.

## Packaging:

- Packaging materials also plays importance role in proteins and peptides stabilization.
- The packaging component should be that it not adversely affects the stability of proteins and peptides.
- Like smooth glass walls best to reduce adsorption or precipitation while polystyrene or containers with silanyl or plasticizer coatings may leads to aggregation and precipitation. Air-tight containers or argon atmosphere reduces air oxidation.

## Additive:

Various types of additives are used in proteins and peptides formulations depending upon delivery route and delivery system to stabilize them on storage. Various additives used are solubility enhancing substance, stabilizing ions/salts, surfactants etc...

# Formulation of proteins and peptides:-

Various formulation aspects which should be considered in formulation include mainly selection of additives and manufacturing techniques. In most of formulation the need of absorption enhancers and enzyme inhibiter arrives because of basic nature of macromolecules.

**(A) Use of Buffers:**

Because of the extremely diverse structure and related properties of proteins, it may not be possible to predict a priori the “best” buffer for any given protein molecule. However, some correlative generalizations can be attempted—recognizing that these may not necessarily be causative in nature. Buffers that may best protect a given protein from a variety of denaturing stresses should possess the following attributes:

- Should be zwitter ionic i.e. Ability to incorporate the electron-donating and electron-accepting sites on one molecule.
- Preferentially be excluded from the protein domain (i.e., increase the surface tension of water) and **incorporate kosmotropic ions** (these are anions which

are polar water structure making in nature & stabilizes the protein molecules. E.g. Citrate, Sulphate, Biphosphate etc.)

- Scavenge free radicals
- Possess a low heat of ionization
- Decrease the mobility of water molecules
- Cause negligible change in the denaturation Gibbs energy, for that protein
- Not undergoes or catalyze complexation with the carbohydrate part of the glycosylated protein
- Inhibit the nucleophilic attack of the thiolate anion on disulfide links, thus preventing thiol–disulfide interchange.
- Unless intended, not act as a substrate for the enzyme, not catalyze metal mediated redox reactions or alter surfactant binding characteristics to the protein
- Not render the protein more susceptible to mechanical stress
- Not cause an increase in the proton amide exchange rate for the protein residues with the buffer vis-à-vis an "inert" buffer medium.

## (B) Freeze drying of Proteins & Peptides:

FT IR spectroscopy examination during freeze drying of the proteins showed that **lyoprotectants (sucrose) are needed to prevent the protein inactivation during drying**. The results showed the decrease in α-helix and random structure and an increase in β-sheet structure. It is well established that structural changes during freeze-drying are responsible for low activity of freeze-dried powders in nearly anhydrous medium. The outcome of the studies revels that freeze-drying is not an innocuous process for proteins and peptides.

## (C) Use of super critical fluids for drying:

Quality of proteins dried with SCF depends upon the operating conditions, formulation and the nature of proteins. Regarding protein stability, resistance to SCF drying processing is clearly protein dependent, but process conditions like temperature, pressure, and composition of the SCF–cosolvent–solvent mixture are important as well.

## PROTEIN FORMULATION:-

- Protein sequence modification (site directed mutagenisis)
- PEGylation
- Proteinylation
- Microsphere/Nanosphere encapsulation
- Formulating with permeabilizers

## Protein sequence modification (site directed mutagenisis):

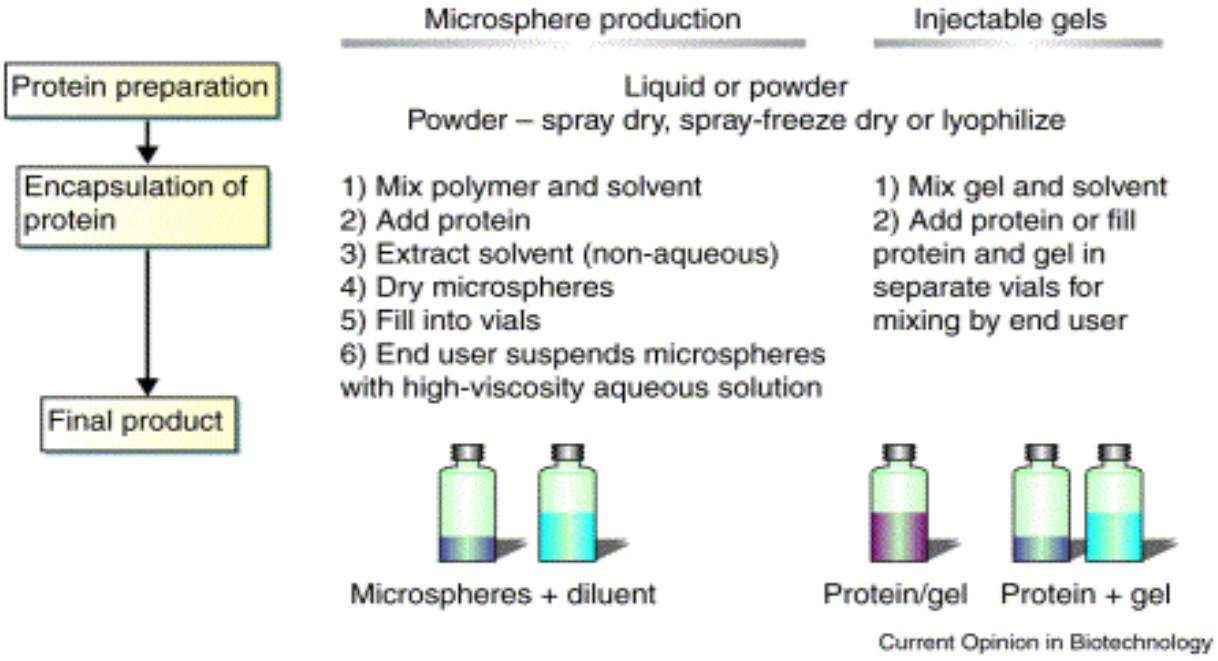
- Allows amino acid substitutions at specific sites in a protein
- i.e. substituting a Met to a Leu will reduce likelihood of oxidation
- Strategic placement of cysteines to produce disulfides to increase Tm
- Protein engineering (size, shape, etc.) plays important role.

## Proteinylation:

- Attachment of additional or secondary (nonimmunogenic) proteins for in vivo protection.
- Increases in vivo half-life (10X)
- Cross-linking with Serum Albumin
- Cross-linking or connecting by protein engineering with antibody fragments

## Microsphere/Nanosphere encapsulation:

- Process involves encapsulating protein or peptide drugs in small porous particles for protection from “insults” and for sustained release




## Formulating with permeabilizers:

Various permeabilizers are used to increase the absorption of proteins and peptides. Selections of them are based on the delivery route and regulatory aspects. i.e. anything that is known to “punch holes” into the intestine or lumen.

# PEGylation



## 1. INTRODUCTION

### 1.1 ATTEMPTS TO IMPROVE PROTEIN DELIVERY

Scientists have searched for years to overcome the problems associated with protein delivery. Initial approaches tried were:

- Alteration of amino-acid sequences to reduce degradation by enzymes and antigenic side-effects
- Fusing them to immuno-globulins or albumin to improve half-life
- Polymer-entrapment in insoluble matrices
- Glycosylation
- Incorporating them into the drug delivery vehicles such as Liposomes to prolong action
- Immobilization onto polymer resins

**Although occasionally successful, these methods have limitations.**

- For example: Liposomes besides delivering drugs to diseases tissues, also rapidly enters liver, spleen, kidneys and reticulo-endothelial systems and leak drugs while in circulation. Liposomes also activate complement systems, which causes pseudo allergic reactions that damage heart and liver cells.

- So, an alternative solution required for efficient protein delivery

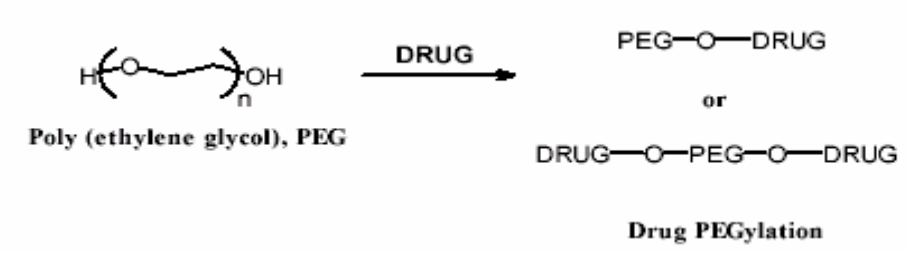
### 1.2 POLYMER THERAPEUTICS – NOVEL APPROACH FOR PROTEIN DELIVERY

- ‘Polymer therapeutics’ is an ***umbrella term*** used to describe Macromolecular drugs, Polymer-drug and Polymer-protein conjugates, Polymeric micelles containing covalently bound drug, and Polyplexes DNA delivery.
- All the subclasses use specific water soluble polymers, either as the bioactive itself or as an inert functional part of multifaceted construct for improved drug, protein and gene delivery.
- Among these, most popular approach for Protein delivery is Polymer-protein conjugation, and the polymer most commonly used for conjugation is PEG.
- Hence the conjugated protein is termed as PEGylated protein and the process as PEGylation

## 2. PEGylation – IN GENERAL

### 2.1 PEGylation - THE CONCEPT

- It involves the attachment of PEG (Poly-ethylene Glycol) to therapeutic proteins or small molecules for the purpose of enhancing the therapeutic value




Poly (ethylene glycol), PEG

Drug PEGylation

### 2.2 PEGylation – MERITS

- A longer in vivo (circulating) half-life especially of smaller peptides and proteins, which normally have a rapid glomerular filtration rate and are cleared on the basis of size. This results in a much less frequent dosing regimen
- Reduced immunogenecity and toxicity and prevents *neutralizing-antibody* formation to certain proteins and therapeutic enzymes.

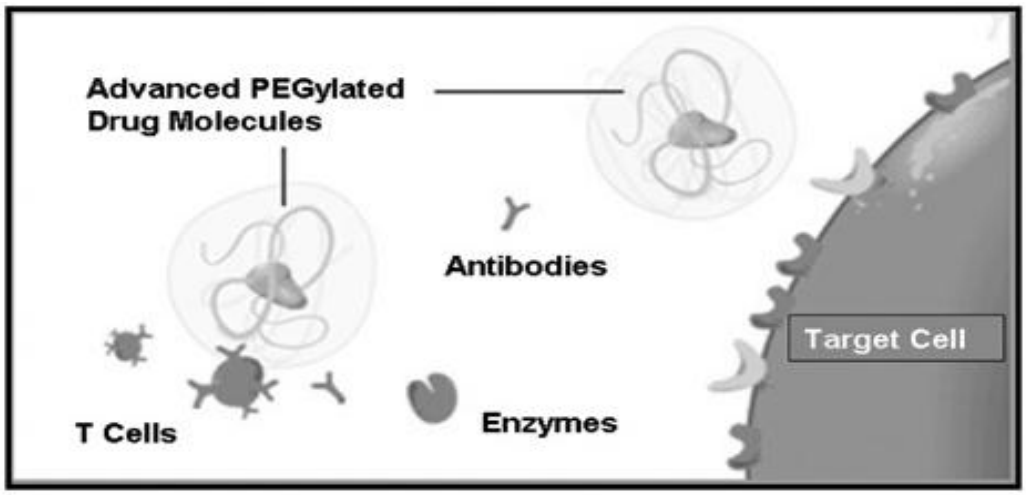
- Protection against proteolysis by forming shell around the protein
- Avoidance of reticulo-endothelial (RES) clearance
- Reduce clearance rate through the kidneys leading to improved bioavailability.
- Improved bioavailability via reduced losses at subcutaneous injection sites
- A low volume of distribution and sustained absorption from the injection site useful for slow release depot administration strategies i.e. Optimized pharmacokinetics resulting in sustained duration
- Improved safety profile resulting with lower toxicity, immunogenecity and antigenecity.
- Due to the amphiphilic nature of PEG, PEGylation improves formulation properties of the protein i.e. improve the solubility and physical-chemical stability of proteins

### 2.3 PEGylation – SPECIFICATIONS

- FDA has approved PEG for use as a vehicle or base in foods , cosmetics and pharmaceuticals
- It shows little toxicity and is eliminated by kidneys (for PEGs < 20kDa) or through faeces (for PEGs > 20kDa)
- It can produce a decrease in the in-vitro activity of proteins, but generally this negative effect is offset in the biological systems by increased half-life
- It lacks immunogenecity, but antibodies to PEG are generated in rabbits; only if PEG is combined with highly immunogenic proteins
- No one has ever reported the generation of antibodies to PEG under routine clinical administration

## 3. PEGylation – MECHANISM OF ACTION

- After administration, when PEG comes in contact of aqueous environment, ethylene glycol sub-unit gets tightly attached to the water molecule. This binding to water renders them high mobility and hydration.
- Hydration and rapid motion causes PEGylated protein to function, as it causes PEG to sweep out a large volume which acts like a shield to protect the attached drug from enzymatic degradation and interaction with cell surface proteins.
- This increased size also helps to prevent rapid renal filtration and clearance sustaining the drug bioavailability




## 4. PEG - CHEMICAL PROPERTIES

- General formula: $HO\text{-}(CH_2CH_2O)_n\text{-}CH_2CH_2OH$
- Inert, non-toxic and non-immunogenic
- Highly soluble, amphiphilic polyether diol
- Does not harm active protein or cells
- Molecular weight: Few hundred to approximately 20,000
- High mobility in solutions
- It contain two OH-groups that can be activated
- Currently, there are two main types of commercially available PEG: Linear and Branched
- PEG is a viscous liquid at molecular weights lower than 1000 and solid at higher molecular weights.
- Poly-dispersity i.e. Molecular weight distribution is narrow (1.01 – 1.1)

## 5. FACTORS AFFECTING PERFORMANCE OF PEGylated PEPTIDES

**Molecular Weight and Structure**

- Molecular weight < 1000 Da: broken down into sub-units, and have some toxicity.
- Molecular weight > 1000 Da: does not demonstrate any toxicity in vivo.
- Molecular Weight upto 40,000 – 50,000 Da: used in clinical and approved pharmaceutical application.
- The molecular weight of PEG has a direct impact on the activity; Higher molecular weight PEGs tends to have lower in-vitro activity but have higher in-vivo activity due to the improved pharmacokinetic profile

**Number of PEG chains**

- Two or more lower molecular weight chains can be added to increase total molecular weight of PEG complex

**Specific location of PEG site of attachment to the peptide**

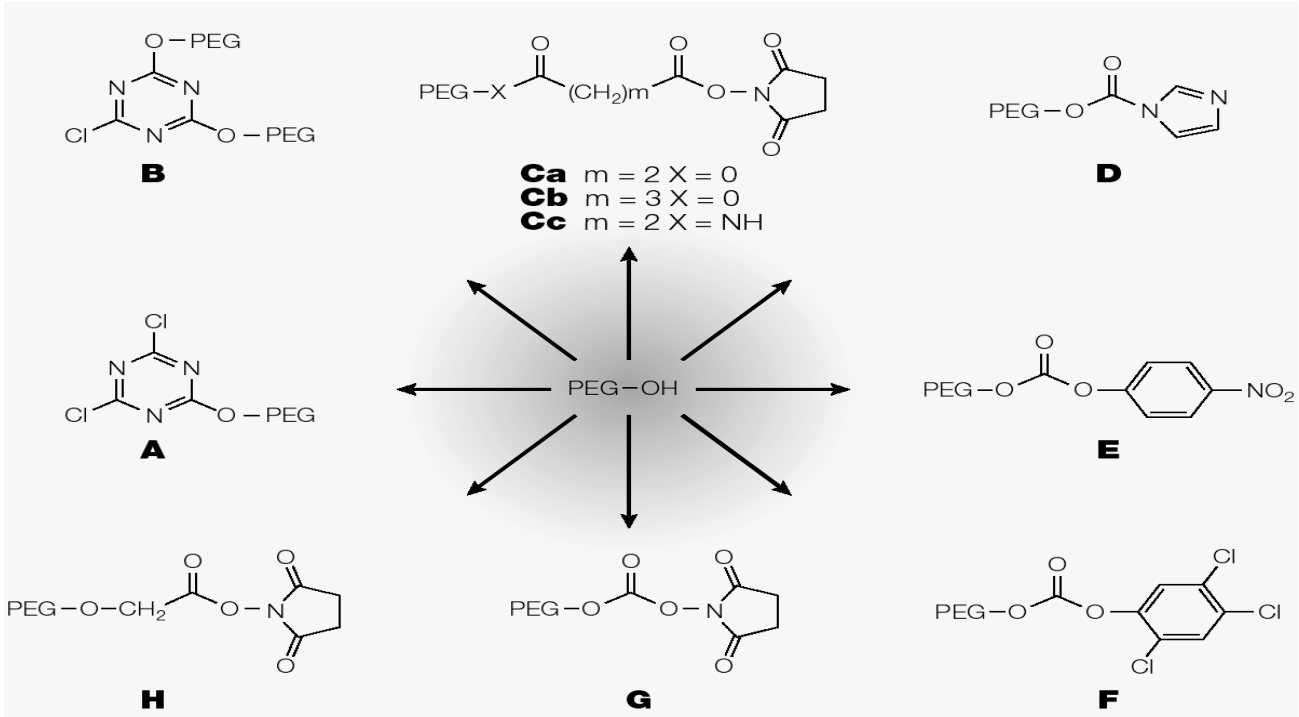
- It should be carefully engineered to retain the highest possible binding efficiency and activity of the peptide ligand

**Chemistry used to attach PEG to peptide, purity of raw materials, intermediate and final products**

- It is very important factor. Peptide and linker must be very pure and very stable to yield pure conjugate with high efficiency.
- Speed of conjugation reaction can be hours or days to completion and critical parameters must be optimized and monitored to maximize yield and purity

## 6. PEGylation CHEMISTRY

- To maximize the pharmacological benefits of PEGylation, a stable bond is formed between the PEG polymer and polypeptide drug of choice.
- In general, a PEG polymer is first chemically activated in order to react with a polypeptide drug.
- A variety of chemical modifications are used to prepare an active PEG derivative with a functional group – such as active carbonate, active ester, aldehyde, or tresylate as illustrated in figure.
- The activated PEG derivative is then covalently linked to a reactive group on the polypeptide drug.
- The most common reactive sites on polypeptides for attaching PEG polymers are the α or ε groups of lysine or the N-terminal amino acids group of other amino acids.




| **Method for the activation of PEG molecules. A** | Cyanuric chloride method. **B** | A variation on the cyanuric chloride method. **Ca** | Polyethylene glycol (PEG)–succinimidyl succinate method. **Cb** | Substitution of the succinate residue by glutarate. **Cc** | Substitution of the aliphatic ester in **Ca** by an amide bond. **D** | Imidazoyl formate method. **E** and **F** | Variations using phenylcarbonates of PEG. **G** | Succinimidyl carbonates of PEG. **H** | Succinimidyl active ester of PEG.

- The careful selection of PEGylation chemistries and reaction conditions yield PEGylated polypeptides with different therapeutic properties. For example: PEGylation of granulocyte colony stimulating factor (G-CSF) through an amine linkage increases the liquid phase stability of G-CSF five times compared to the PEGylation of the amide bond.

## 7. PEGylation PROCESSES

It is classified into two types:

- Early PEGylation technology (First generation PEGylation)
- Advanced PEGylation technology (Second generation PEGylation)

### 7.1FIRST GENERATION PEGylation

- First generation PEGylation methods were fraught with difficulties.
- With first generation PEGylation, the PEG polymer was generally attached to ε amino group of lysine, and gave mixtures of PEG isomers with different molecular masses. The existence of these isomers makes it difficult to reproduce drug batches, and can contribute to the antigenecity of the drug and poor clinical outcomes.

- In addition, first generation methods mainly used linear PEG polymers of 12 kDa or less.
- Unstable bonds between the drug and PEG were also sometimes used, which leads to degradation of PEG-drug conjugate during manufacturing and injection
- Early PEGylation was performed with methoxy-PEG (m-PEG), which was contaminated with PEG diol and which resulted in the cross-linking of proteins to form inactive aggregates. Diol contamination can reach upto 10-15 %.

| | |
|---|---|
| Linear PEG-OH | $H-(OCH_2CH_2)_n-OH$ |
| Linear m–PEG-OH | $CH_3-(OCH_2CH_2)_n-OH$ |

- Despite these limitations, several first generation PEGylated drugs receive regulatory approval.

**Examples:**

- Still in use today are Pegademase (Adagen), a PEGylated form of the enzyme adenosine de-aminase for the treatment of Severe Combined Immuno-Deficiency (SCID) and Pegaspargase (Oncaspar), a PEGylated form of enzyme asparginase for the treatment of Leukemia.

### 7.2 SECOND GENERATION PEGylation

- Second generation PEGylation strives to avoid pitfalls associated with the first generation PEGylation.
- Overall goal of this technology is to create larger PEG polymers to improve the Pharmacokinetics and Pharmacodynamic effects seen with lower molecular mass PEGs

***It includes several techniques as follows:***

**1) Array of chemistries to improve PEG derivatives and their linkages to drug**

- For example: Generating Carboxylic acid intermediates of PEG permits up to 97 % of diol impurities to be removed by ion-exchange chromatography before PEG attachment to polypeptide drugs

**2) PEGylation at the –SH (thiol) groups of Cysteine of polypeptides**

- PEGylating site specifically can minimize the loss of biological activity and reduce immunogenecity.
- -SH groups are ideal site for PEGylation compared to lysine as it causes reduction in immunogenecity, but the problem is that there are far fewer cysteine residues than lysine groups on polypeptides.
- For this, cysteines can be added to polypeptides precisely where they are desired by genetic engineering

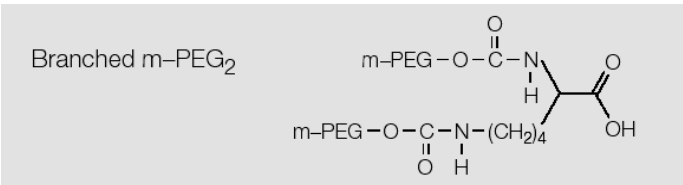
**3) Incorporation of the degradable linkages to release drug at the targeted sites**

**4) Hetero-bifunctional PEGs**

- Hetero-bifunctional PEGs are the one bearing dissimilar terminal groups
- Such hetero-bifunctional PEGs bearing appropriate functional groups may be used to link two entities where a hydrophilic, flexible, and biocompatible spacer is needed.
- Hetero-bifunctional PEGs can be used in a variety of ways that includes linking macromolecules to surfaces (for immunoassays, biosensors or various probe applications), targeting of drugs, liposomes and viruses to specific tissues, liquid phase peptide synthesis and many others.
- Preferred end groups for hetero-bifunctional PEGs are NHS esters, maleimide, vinyl sulfone, pyridyl disulfide, amine, and carboxylic acids.

**5) Use of Branched structures**

- Second generation PEGylation uses branched structures of PEG, in contrast to the solely linear structures found in first generation PEGs.
- Branched PEGs of greatly increased molecular masses – upto 60 kDa or more, as compared with the 12 kDa or less found in the first generation PEGs – have been prepared.
- A branched PEG 'acts' as if it were much larger than a corresponding linear PEG of the same molecular mass.




- Branched PEGs are also better at cloaking the attached polypeptide drug

from the immune system and proteolytic enzymes, thereby reducing its antigenecity and likelihood of destruction.

**Example:**

- A competing treatment for chronic hepatitis C utilizes IFN (Interferon) – α2a coupled to PEG.
- The first formulation in 1999 used a first generation linear PEG of 5 kDa. In the first clinical trials, this PEGylated drug was administered to patients with chronic hepatitis C once weekly and compared with its un-PEGylated counterpart administered three times a week. The PEGylated IFN – α2a produced no clinical advantages
- A second generation, branched PEG of 40 kDa, was then coupled to IFN – α2a. This version (Pegasys) under clinical investigation, showed nearly constant blood concentration of IFN – α2a and renal clearance is reduce to 100-fold related to un-PEGylated IFN – α2a. PEGylated also increased the half-life of IFN – α2a from 9 to 77 hours.

## 8. PEG – BASED HYDROGELS

- PEG can be chemically cross-linked to form polymer networks that swell and form gels.
- These swollen jelly-like materials are called hydrogels, and are well suited for a range of medical applications. The bio-compatibility of hydrogels makes them ideal for wound – healing applications.

### 8.1 FocalSeal

- In 2000, FDA approved surgical sealant **FocalSeal** to prevent air leaks in lungs following the removal of lung tumours and other chest surgeries.
- FocalSeal uses a PEG that is applied as a liquid, and then transformed into a waterproof hydrogel seal by irradiation. The sealant protects the wound sites from leaking during tissue healing, and then naturally degrades and dissolves.
- Clinical trial show that 93-100 % of surgery patients treated with FocalSeal remain free of air leaks, compared with 20-30 % of patients who do not receive the treatment.

### 8.2 SprayGel

- **SprayGel**, another biodegradable hydrogel, prevents post-operative adhesion formation.
- Following surgery, internal wounds often develop an adhesion – a type of scar tissue – that causes severe pain, and which are a leading cause of small bowel obstructions and infertility in women.
- SprayGel is sprayed onto the wound site and acts as a protective barrier during healing. This material also dissolves and degrades at a programmed rate.
- Other PEG-based hydrogels under development deliver encapsulated drugs as implants. Degradable linkages between hydrogels and incorporated drugs allow drugs to be slowly and specifically released in the body.

## 9. PEGylation – HURDLES

### 9.1 PEGylation – DEMERITS

- Proteins as well as the PEG agents are expensive
- Losses during the PEGylation process of 25% in late stage/commercial production to 50-75% in early clinical production are not uncommon
- Maximizing the benefits require Yield improvement, but usually not performed due to time constraints

### 9.2 PEGylation – MANUFACTURING CHALLENGES

- When optimizing a process for the manufacturing of a protein drug, there are three main considerations: high product quality (purity, stability, and activity), process robustness, and low cost.
- Purifying and characterizing a particular positional isomer are recognized challenges associated with manufacturing of a PEGylated bio-molecule.
- To lower analytical and downstream processing hurdles, it is preferred that the selectivity of the PEGylation reaction to be optimized.
- For a large-scale production of a PEGylated protein drug, a higher yield of the target product coupled with a lower yield of secondary PEGylation products is extremely advantageous and to have it is a challenge.
- However, because the progression of the PEGylation reaction depends on several variables, the stoichiometry and the attachment site are hard to control. Ideally, the reaction parameters can be fine-tuned to achieve the

desired stoichiometry of PEG conjugates, to produce predominantly mono-, di, or other target PEG conjugates.

- The scaling-up of size exclusion chromatography (Purification technique), however, is a significant challenge in the biopharmaceutical industry because of its low throughput and high cost.
- These challenges in developing PEGylated protein drug still not have been resolved.

## 10. PEGylated PRODUCT – PURIFICATION AND ANALYSIS

### 10.1PURIFICATION OF PEGYLATED PRODUCT:

- Size-exclusion chromatography (SEC) can separate un-PEGylated from PEGylated proteins but not the PEG dimer from the PEG monomer.
- Hydrophobic interaction chromatography generally works poorly for this application (depending on the hydro-phobicity of the protein relative to the PEG)
- Ion-exchange chromatography seems to be the method of choice for purification of PEGylated product. Here, the separation of the PEG dimer from the other components in the reaction mixture appeared to be based on the PEG-to-protein ratio.

### 10.2 ANALYSIS OF THE PEGYLATED PROTEIN PRODUCT:

- Characterization of PEGylated proteins is difficult due to the fact that the PEG molecule is more polydisperse than the protein and imparts size heterogeneity to the conjugated protein.
- Size-exclusion chromatography (SEC) has often been used to characterize these conjugates. This tool is limited by the extra peak-broadening due to the polydispersity of the PEG conjugate. SEC also will not detect "PEGylation site isomers" in which the protein is PEGylated at different residues.
- Cation-exchange HPLC is a powerful tool for resolving these PEGylation site isomers.

## 11. PEGylation – PROCESS OPTIMIZATION

- Rational design and High throughput experimentation are very effective and efficient ways to optimize the PEGylation process

- There are several process parameters to be considered in developing a PEGylation procedure. Optimization of the Process Parameters should be done by using Statistical Design of Experiments
- Parameters to be considered include:
  Protein concentration, PEG-to-protein ratio (on a molar basis), Temperature, pH, Reaction time, Protein characteristics (molecular weight, surface area, polarity, local amino acid conditions at the PEGylation site, such as lysine pKa, and site accessibility).
- Objectives should be set before optimization of the process parameters such that the process should: Reduce cycle time, Minimize impurities, Maximize yield (> 85 %)

## 12. PEGylation – NOVEL APPLICATIONS

- There are just a few of the biomedical applications of PEGylation either approved by the FDA or undergoing clinical investigation.
- Although proteins and peptides have been the main targets for PEGylation, other molecules, including small molecule drugs, co-factors, oligonucleotides, lipids, saccharides and biomaterials can be PEGylated as well.
- The benefits of PEGylated catalase, uricase, honey bee venom, hemoglobin, Pyrrolidone and dextran are also under investigation.
- Other researchers are designing PEGylated nano-particles to cross the blood-brain barrier or using PEGylated DNA – containing liposomes with tethered antibodies to provide targeted gene therapy

**CONCLUSION**

- PEGylation has taken 20 years to emerge as a viable pharmaceutical tool.
- During this time, there have been important advances in the chemistry of PEGylation, in the generation of bio-molecule therapeutics and in understanding PEG-biomolecule conjugates.
- PEGylation has now been established as a method of choice for improving pharmacokinetics and Pharmacodynamics of protein pharmaceuticals,
- Application of PEG – based hydrogels and PEG – modified liposomes (stealth liposomes) have become increasingly important.

- New frontiers for the technology are now emerging, for example, in small molecule modification, and it is certain that PEGylation will play an increasingly important role in pharmaceutical science and technology.
- Despite these advancements in PEGylation techniques, issues regarding purification and characterization of PEG still needs to be solved

## 13. APPROVED AND LAST STAGE PEGylated PRODUCTS

| Brand Name | Product | Company | Indication |
|---|---|---|---|
| PEGasys | PEG-IFN α-2a (interferon) | Hoffmann – La Roche | Hepatitis |
| PEG-Intron | PEG-IFN α-2b (interferon) | Enzon | Hepatitis |
| Neulasta | PEG-filgrastim (granulocyte colony-stimulating factor) | Amgen | Neutropenia |
| Adagen | PEG-adenosine deaminase | Enzon | Immuno-deficiency |
| Oncaspar | PEG-aspargase (asparaginase) | Enzon | Cancer |
| Somavert | PEG-visomant (growth hormone) | Pfizer | Acromegaly |
| PEG-Hirudin | PEG-recombinant hirudin | Abbot | Thrombosis (phase III) |
| PEG-monoclonal antibody | PEG-CDP 870 | Pfizer | Rheumatoid Arthritis (phase III) |
| PEG-Axokine | PEG-ciliary neurotrophic Factor | Regeneron | Obesity (phase III) |
| Pre-clinical testing | PEG-erythropoietin | Prolong Phamaceuticals | Anemia |

## DELIVERY TECHNIQUES FOR PROTEINS & PEPTIDES:-

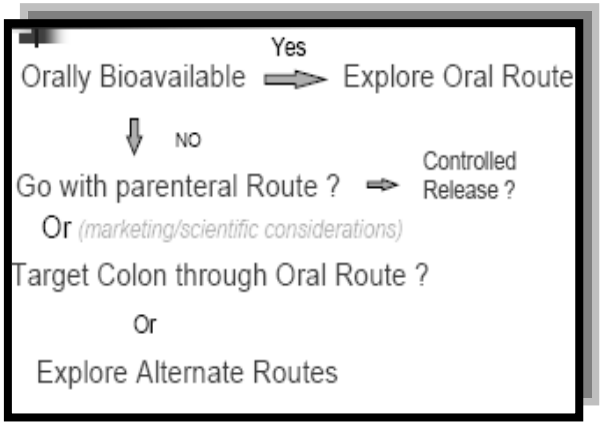
The most common method for protein and peptide-based drug delivery is by injection. The obvious downside of this delivery method is patient acceptance and compliance, limiting most macromolecule development to indications in which the need to use invasive administration routes are not outweighed by associated expenses or inconvenience. Hence various newer routes for effective delivery of Proteins and Peptides are implemented.

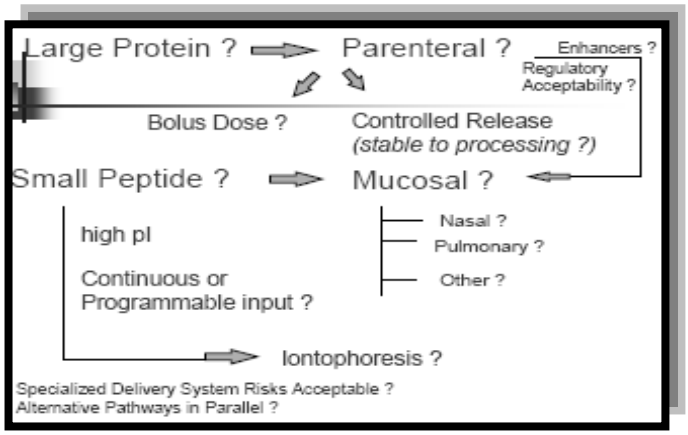
The choice of a delivery route is driven by patient acceptability, the properties of the drug (such as its solubility), access to a disease location, or effectiveness in dealing with the specific disease.

**Selection of Delivery technology for Proteins & Peptides:**

Based on the following listed parameters the delivery technology for Proteins & Peptides should be selected.

- Optimum delivery of proteins & peptides to specific cells / diseased sites (cancer cells).
- Reduction in potential side effects (specially immune response)
- Improvement upon the benefit / risk ratio.
- Delivery at a controlled fashion to the pharmacological receptor.
- Protection of intactness of protein & peptide from the body and vice versa until they reaches to their site of action**.**




## NASAL ROUTE:-

The nasal route for drug delivery is attracting increased attention and the application of this route is feasible for both local and systemic drug delivery. The nasal route has one of the most permeable and highly vascularized mucosa, which would exponentiate rapid absorption of drugs and quick onset of its therapeutic effect.

**The advantages of the nasal route:**

Nasal delivery provides

- Rapid onset of therapeutic effect,
- Comparable to injection, but without the discomfort and inconvenience of an injection.
- Ease of administration,
- Comparable to oral delivery, but without the delay of onset of effect for oral delivery, due to first pass metabolism.

**The disadvantages of nasal route**:

- Long-term use may lead to mucosal and cilial toxicity.
- Alteration in nasal environment may take place in some disease states.

**Factors Influencing Nasal Drug Absorption:**

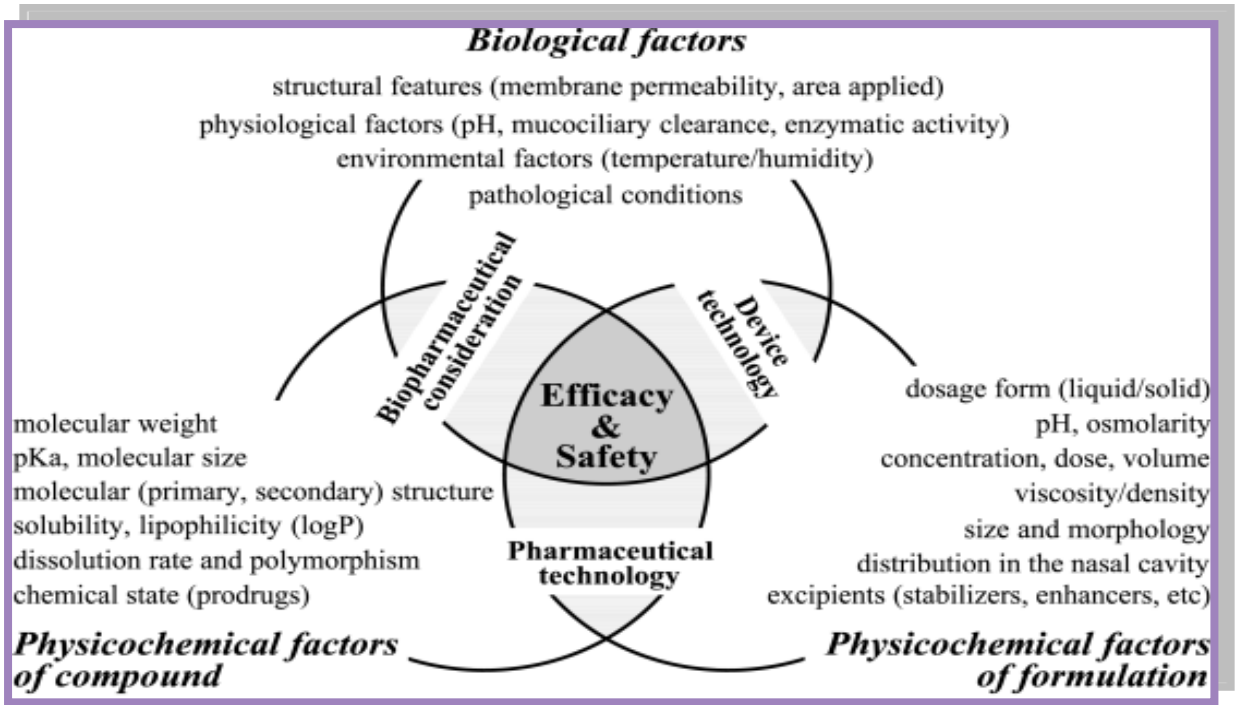
Figure given below summarizes various factors that should be taken into account when studying and developing nasal delivery systems of general drug compounds. Although conveniently classified into three categories, these are intimately related. It is desirable that almost all factors be considered together when designing a new nasal formulation, whereas problems originating in the said barriers must be overcome for attaining practical application.

- **Molecular Weight:**

Using radiolabelled dextrans in rats, it was found clearly the inverse correlation between molecular weight and percentage absorbed of the nasal dose for a series of nondegradable dextran derivatives with molecular masses of 1260–45,500 Daltons.

- **Enzymatic Degradation:**

The nasal epithelium possess oxidative enzymes (e.g., cytochrome P450, carboxy esterase, aldehyde dehydrogenase, and carbonic anhydrase), conjugative enzymes (e.g., glucuronyl transferase and glutathione transferase), and exopeptidases and endopeptidases (e.g., aminopeptidase, carboxypeptidase, trypsin like activities, and cathepsins) this wide variety of enzymes is creating a “pseudo-first-pass effect,” obstructing the absorption of peptide and protein drugs.




- **Environmental pH :**

The pH of the nasal mucus layer varies between 5.5 and 6.5 in adults and between 5.0 and 7.0 in infants. 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. In addition, the environmental pH may be an important key factor when facing the enzymatic barrier because some endogenous enzymes have specific optimal pH for exerting their activities.

## Strategies to Improve Nasal Absorption of Peptides and Proteins:

Following enlisted strategies are investigated for nasal delivery of Peptides and Proteins:

- **Absorption Enhancers:**

Most of all chemical enhancers are not approved for mucosal usage by any regulatory authority; for which a special concern is required to prove the safety parameters of them.

Example of such agents includes Surfactants, Bile salts and their derivatives, Chelators, Fatty acid salts, Phospholipids, Glycyrrhetinic acid derivatives, Cyclodextrins, Glycols etc.

- **Enzyme Inhibitors:**

The nasal tissue has various enzymes, as mentioned above, including peptidases and proteases, in the mucus, on the membrane surface, and in the intercellular space. The prominent enzyme appears to be **aminopeptidase**.
Enzyme inhibitors such as bacitracin, bestatin, and amastatin have been found to improve nasal absorption of peptide drugs such as LHRH and calcitonin through their peptide like structure which exhibits the competitive mechanism with peptides and proteins.

- **Synthesis of Peptide Analogues:**

Success of such an analog depends upon the affinity of the generated analogues to the targeted receptor which in case of proteins may not always be holds true. Successful examples are found in LHRH analogues such as buserelin, leuprolide, and nafarelin. Although the nasal bioavailability of these analogs appeared to be 2-3%, they appeared to be about 50-200 times more potent than the parent compound LHRH.

## Formulation Approach:

For the purpose of improving absorption efficiency by prolonging nasal retention, recent progresses based on the formulation approach are:

**Delivery mode: Sprays / Drops:**

The limitation over here is that the entire dose must be given in a volume of 25–200 µL, depending on the formulation. Nasal drops are the simplest and most convenient form. However, the exact volume of dosing is difficult to determine, which may be device-related, and rapid drainage from the nose is another problem with drops.
For example, the bioavailability of nasally administered desmopressin has been significantly increased by sprays, compared with drops.

Various types of devices for nasal administration, such as unit dose containers, metered dose sprays, compressed air nebulizers, and so on, are becoming available; formulation should be developed as per the designed dosage form. Nasal sprays containing viscous polymer like methyl cellulose retards the nasal clearance of the spray, showed delayed absorption of peptides and proteins.

**Solutions/ Powders:**

One of the advantages of the powder formulation is a higher chemical stability than the solution, which leads to the possible administration of large amounts of the drug. When designing powder formulation for nasal peptide delivery, substantial care must be taken when controlling the particle size.

**Bioadhesives:**

Chitosan has been found to be useful as a potent absorption enhancer for nasal peptide delivery. Other bioadhesive polymer systems, such as polyacrylic acid, cellulose derivatives, and hyaluronate, have also been used in nasal peptide delivery.

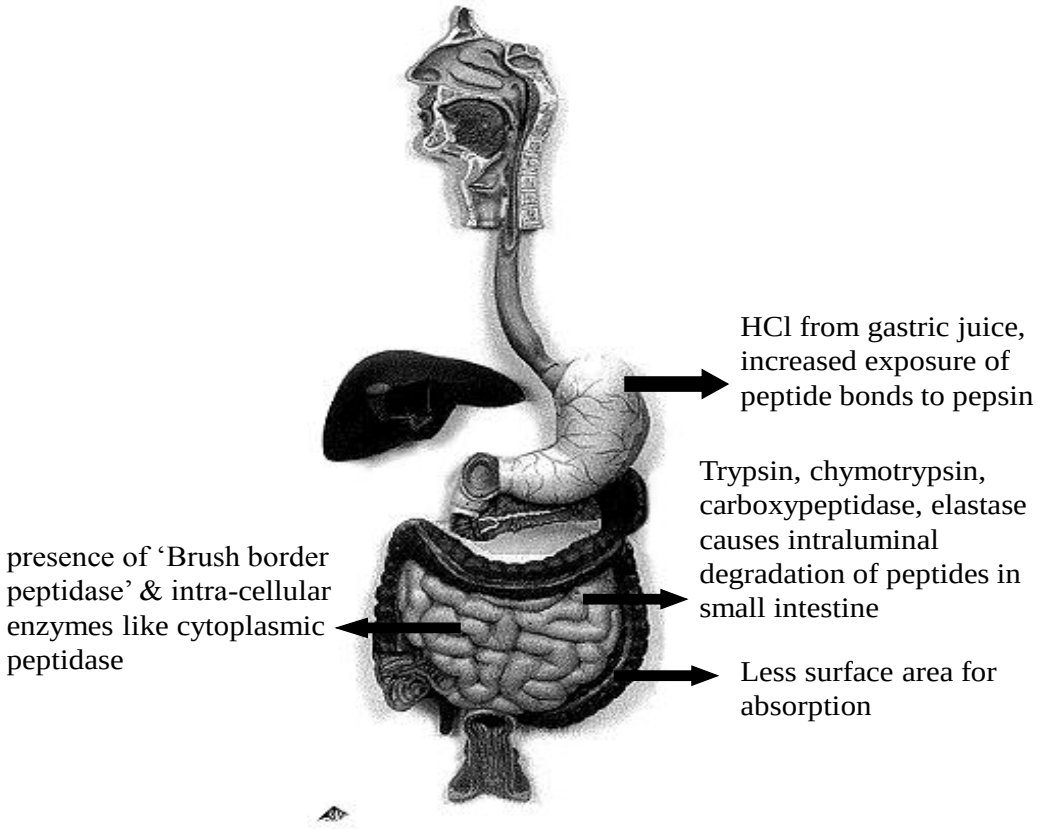
**Other specialized systems:**

Liposomes, lipid emulsion, microspheres, nanoparticles, niosomes, ointment, and so on, have also been tried in recent decades, although they are not confined to nasal peptide and protein delivery. Some of these offer a better chance of permeation for drugs as they can provide an intimate and prolonged contact with the nasal membrane.

**Marketed products:-**

| **Peptide/protein** | **M wt.** | **Formulation** | **Company/ stage** |
|---|---|---|---|
| Desmopressin | 1,183 | Solution | Aventis, Ferring |
| Salmon calcitonin | 3,432 | Solution | Novartis, Aventis |
| Nafarelin | 1,322 | Solution | Pfizer |
| Oxytocin | 1,007 | Solution | Novartis |
| Buserelin | 1,300 | Solution | Aventis |

## ORAL ROUTE:-




**Characteristic features associated with different regions of GIT with respect to oral delivery of proteins and peptides.**

**Absorption of amino acids and small peptides:**

Amino acids are absorbed through brush border plasma membrane into the epithelial cells through specific amino acid transporters. The intestinal absorption of amino acids is stereochemically specific. The rate of absorption of the L-isomer is greater than that of the corresponding D-isomer when racemic mixtures of the amino acid are introduced in the small intestine. Di- and tripeptides are absorbed through the active transport utilizing the specific carrier systems across the epithelial cell membrane.

**Absorption of polypeptides and proteins:**

Enterocytes of the intestinal membrane do not have transporters to carry polypeptides and proteins across the intestinal membrane, and they certainly cannot permeate through tight junctions of cells because of their size. Also, polypeptides and proteins are substrates for luminal brush border, and cytolytic enzymes.

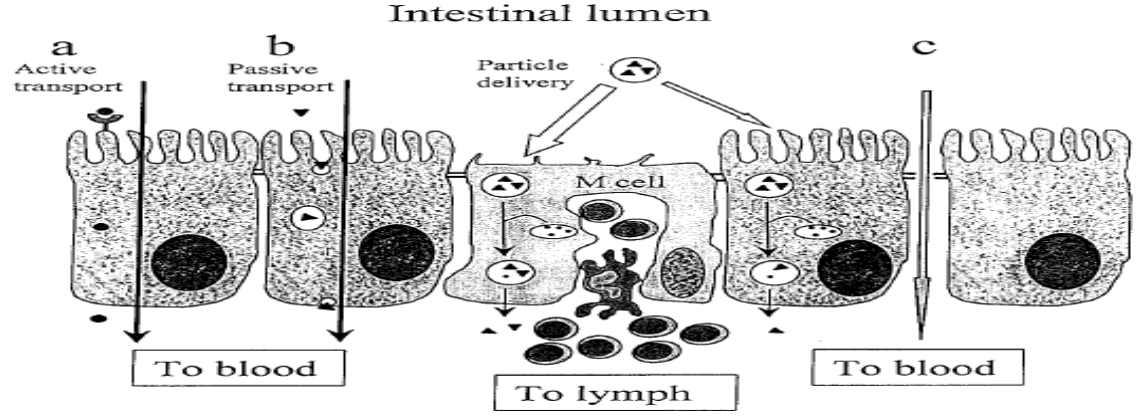
There are **two possible mechanisms associated** with the absorption of intact proteins: endocytosis and transport through the epithelium of Peyer's patches.

- **Endocytosis :**

Macromolecules such as proteins may be absorbed from the intestinal lumen by cellular vesicular processes, through fluid-phase endocytosis (pinocytosis), or by receptor-mediated endocytosis.

- **Transport through the epithelium of Peyer's patches :**

Payer's patches are made up of 30-40 lymphoid nodules on the outer wall of intestine. They have specialized epithelial cells called M (membranous or microfold) cells which possess apical luminal surface with many microfolds, truncated microvilli and low levels of membrane-bound enzymes. Through all




these characters, M cells can be exploited for the endocytosis of macromolecules or particles.

**Strategies for oral delivery:**

The dosage form must initially stabilize the drug, while making it to take orally. Once delivered to the stomach or intestine tract, the protein has to be protected from enzymatic degradation since digestive processes are to brake down proteins and peptides without any discrimination in favour of therapeutically active compounds.

One strategy for overcoming the body's natural process is to alter the environment for maximum solubility and enzyme stability of proteins and peptides by using formulation excipients such as buffer, surfactant and protease inhibitors or by carrier system. If the protein can be defeated or delayed, the protein can be presented for absorption.

## Carrier systems:

### Proteinoid Microspheres

Proteinoids are thermally condensed amino acids and spontaneously form microspheres when exposed to an acidic medium. The microsphere size is approximately 1–5 µm in diameter. They are very stable at lower pH conditions of 1 to 3 but unstable at the pH range of 6 to 7. Therefore, the proteinoid microspheres are able to protect the peptide and protein drugs from the gastric acid and enzymes while in the stomach and release the encapsulated drug in the small intestine to be available for absorption. Oral administration of proteinoid encapsulated insulin in diabetic rats has shown a significant hypoglycemic effect and provided a longer duration of action than when administered subcutaneously.

### Liposomes

Liposomes have been employed as a delivery system for the molecule especially the insulin and the oral administration of liposome-entrapped insulin into diabetic rats has produced a significant fall in blood glucose levels. However, in general liposomes have not been successful for oral delivery. Key of the reasons may be related to: their stability in the GI tract and their susceptibility to changes in pH, bile salts, and lipases. In addition, it has been shown that liposome systems are immunogenic.

### Lectins

Lectins are known to have specific binding properties to the epithelial cell surface and have been tested as a possible oral delivery system for peptide and protein drugs. The lectin-coated nanoparticles that contain peptide or protein drugs can protect against degradation in the lumen of the small intestine and facilitate the uptake of peptide or protein drugs across M cells by acting as a specific targeting ligand.

## Co-administration of Enzyme Inhibitors :

The known inhibitors employed are aprotinin, bacitracin, Bowman–Birk inhibitor, camostat mesilate, soybean trypsin inhibitor, sodium glycocholate, and

chymotrypsin inhibitor (FK-448). Some of these inhibitors have absorption-enhancing activity in addition to enzymatic inhibition activity. As an example, insulin has often been used as a model protein drug in studies on enzymatic inhibition activity. Trypsin inhibitor and aprotinin have shown a marginal effect on increasing insulin absorption in rats. However, a significant hypoglycemic effect has been observed after administration of insulin with sodium glycocholate, camostat mesilate, and bacitracin, which may be related to that these compounds act as enzymatic inhibitors and absorption enhancers.

## Co-administration of Absorption Enhancers :

Most polypeptide and protein drugs show low permeability across the intestinal membrane because of their polarity and size. Therefore, one approach to increase the permeability of these drugs is to co-administer with absorption enhancers. The known enhancers tested for the oral delivery of peptide and protein drugs are bile salts, nonionic surfactants, anionic surfactants, lysolecithin, amines, medium chain glycerides, and salicylates.

- The proposed mechanisms for their action in relation to insulin are as bellow:
- Bile salts, act as an absorption enhancer as well as an enzyme inhibitor
- Enhancers act as a dispersing agent to prevent aggregation of peptide and protein molecules in solution, resulting in increased solubility of the drugs
- Enhancers reduce the viscosity of the membrane mucous layer and increase the membrane fluidity, resulting in increased absorption by opening up the aqueous channel on the cell membrane; and
- Positively charged enhancers may interact with the negatively charged epithelial cell membrane and neutralize the membrane surface, resulting in increased absorption of the protein.

Long term usage is associated with the toxicity produced by most of the enhancers hence it has to be characterized and established the safety data of their usage.

### ✘ Chemical Modifications:

This approach is deal by medicinal chemists, who mainly applied three main types of chemical modifications for improvement of protein and peptide absorption which includes analogs, irreversible derivatives, and prodrugs.

The ***irreversible derivative*** and the ***analog*** approaches are usually applied when compounds shows poor absorption because of in vivo metabolism. Peptide drug examples include enkephalins, TRH, and vasopressin, and insulin can be a model for protein drugs.

The ***prodrug approach*** is applied in cases particularly to deal with the improvement in lipophilicity and degradation minimization without affecting the intrinsic bioavaibility of the parent molecule. A good example is an ACE inhibitor, enalapril, which was found to be orally well absorbed and metabolized to the active form, enanaprilat, in the liver. In contrast, the parent drug, enanaprilat, is very poorly absorbed via the oral route.

## COLON DELIVERY:

Colon has intrinsic advantages of having low almost nil peptidase activities but is having the following disadvantages:

- ♦ Less surface area for absorption than small intestine.
- ♦ High concentration of anaerobic bacteria in the colon leads to faster degradation of majority of drugs.

Degradation by colonic microflora had been explored by the scientists to reach site-specific delivery of peptide and proteins. For the approach, the mechanisms like coating of molecules with azoaromatic groups or conjugation of molecule with azoaromatic groups has been implemented in which the entire molecule becomes impermeable to the upper git and reaches the colon where through the action of microbial flora, the molecule is released. This polymeric system was demonstrated to protect and deliver orally administered insulin and vasopressin in rats.

## RECTUM DELIVERY:

Recent study of peptides like des-enkephalin-gamma-endorphin and desglycinamide arginine vasopressin, and proteins e.g., albumin, insulin, and a

somastatin analog has shown the promising results with absorption enhancers in the rectum delivery.

The enhancers used for the rectal absorption of insulin are surface active agents; bile acids; EDTA; and phospholipids such as lecithin, saponins, sodium salicylate, organic alcohols, acids, amines, and fats.

## PULMONARY ROUTE:-

The ability to deliver proteins and peptides to the systemic circulation by inhalation has contributed to a rise in the number of inhalation therapies under investigation. For most of these therapies, aerosols are designed to comprise small spherical droplets or particles of mass density near 1 $g/cm^3$ and mean geometric diameter between ~1 and 3 μm, suitable for particle penetration into the airways or lung periphery.

If other noninvasive routes have not been successful, **why should pulmonary delivery be any different?**

Most basically, the biology of the lung makes it a favorable environment for noninvasive drug delivery. Studies have shown that most large-molecule agents are absorbed naturally by the lungs, and once absorbed in the deep lung, they pass readily into the bloodstream without the need for enhancers used by other noninvasive routes.

Logically, there is no reason to **expect safety problems** related to the inhalation of a substance any different from those associated with the injection of the same amount of the substance. A growing quantity of safety data indicates that inhaling proteins can be safe for patients with healthy or diseased lungs. The safety of therapeutic inhalation is further supported by the existence of more than 20 small-molecule and one large-protein drug inhalation products approved by the U.S. Food and Drug Administration (FDA); this group of therapeutic inhalants contains 13 different excipients.

## Mechanisms of drug deposition in respiratory track:

Particle deposition in the human respiratory tract takes place primarily by three mechanisms.

**Inertial impaction** causes filtering of particles according to their aerodynamic size and velocity. This mechanism of deposition is important in the upper and central airways. In particular, oropharyngeal deposition increases approximately in proportion to the particle velocity and the square of its aerodynamic size. Therefore, particles with large aerodynamic diameters, especially if they are inhaled rapidly, will not be delivered into the lung for absorption.

**Gravitational sedimentation and diffusion** are important for deposition of particles in airways and the alveolated regions. The extent of deposition by both of these mechanisms increases with breath-holding. The aim with protein and peptides is to produce a slow, moving aerosol cloud with drug containing particles in the size range 1–3 μm to minimize oropharyngeal deposition since peptides or protein are poorly absorbed from this region.

# The challenges of aerosol formulations:

Although a natural and safe route, pulmonary delivery has its challenges. **The key challenge is getting the drug to reach the deep lung.** Historically, aerosol formulations have not been able to move the medication into the deep lung efficiently, and until recently, companies developed pulmonary drug delivery systems to dispense drugs to the airways only for local applications. Metered dose inhalers (MDIs), breath-activated dry powder inhalers (DPIs), liquid jet, and ultrasonic nebulizers have proved useful in the management of asthma, but such devices are not designed to deliver drugs into the deep lung. They are not practical for the delivery of most macromolecules because of their low system efficiency, low drug mass per puff, poor formulation stability for macromolecules, and poor dosing reproducibility.

For optimal deep lung delivery of costly proteins and peptides**, it is important to use the correct aerosol particle size.** Studies have established that these particles should range from 1 to 3 μm in diameter for optimal deposition efficiency. As the amount of drug deposited from the device is highly dependent

on the patient's inhalation technique, any truly effective delivery device for proteins and peptides needs to optimize a patient's ability to inhale correctly.

For inhalation therapies to accomplish their medical goals, macromolecule delivery to the lungs must be precise and consistent at every inspiration. Although the natural bioavailability of the deep lung epithelia appears difficult to change, the efficiency of drug deposition offers some opportunity for adjustments. Deposition efficiency from traditional devices has been low, however, with less than 10% of the total inhaled dose reaching the deep lung.

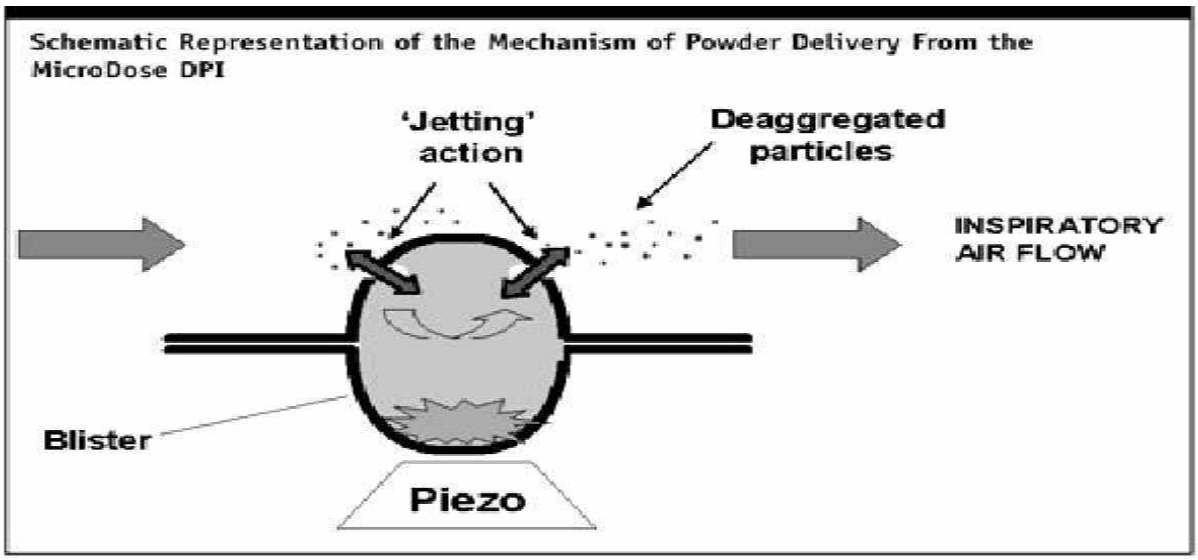
| **Physiological parameter** | **Breathing maneuver for optimum peptide and protein absorption** |
|---|---|
| Lung volume prior to inhalation | Full exhalation |
| Volume inhaled at the time of actuation of aerosol delivery | Minimum volume inhaled prior to actuation of drug delivery |
| Inspiratory flow rate | The minimum flow rate consistent with acceptable performance of the aerosol delivery device |
| Total volume inhaled during delivery | Close to vital capacity (shown to be important for insulin) |
| Respiratory pause between inspiration and exhalation | Several seconds (minimum time to be determined by experimentation) |

## Stability issue:

A key issue for effective protein and peptide absorption is the preservation of biochemical and structural integrity during the preparation, storage, and aerosolization of the drug molecule. The process of aerosolization can cause damage to the active molecule. The generation of small droplets provides a vast increase in the air-liquid interfacial area, which may cause unfolding of proteins followed by **aggregation**. This is particularly likely to happen for hydrophobic proteins that undergoes multiple recirculations in jet nebulizers or during spray-drying and may be prevented by addition of suitable surfactants. **Thermal denaturation** can occur also during the high temperature spray-drying or with some ultrasonic nebulizers that warm the solution for nebulization during their operation. Freeze-dried parenteral protein preparations are typically unsuitable

for delivery by inhalation for at least two reasons: they often contain excipients such as citrate that could cause irritation if inhaled in sufficient quantities. The freeze-dried materials form **cohesive powders** that do not lend themselves to be dispersed into respirable particles. Spray-drying has been employed to make respirable protein powders.

## The AERx® system:-




The device uses a piezo vibrator to deaggregate the drug powder packaged in aluminum blisters. Actuation took place by the threshold level of inspiratory airflow by the patient (sensed by directional inhalation sensor). The piezo vibrator converts the electrical energy to mechanical motion, which is transferred through the blister into the powder.

Aradigm (Hayward, California) is behind the development of AERx, a hand-held liquid aerosol inhaler that calibrates airflow and dosage. It is licensed to

The future of drug administration by inhalation will expand vastly. The development of new macromolecule drugs that can treat diseases that previously were either not treatable or only partially treatable has led to renewed interest in noninvasive drug delivery technology. Many new agents are now under investigation for pulmonary delivery: interleukin-1 receptor (asthma therapy), heparin (blood clotting), human insulin (diabetes), α-1 antitrypsin (emphysema and cystic fibrosis), interferons (multiple sclerosis and hepatitis B and C), and calcitonin and other peptides (osteoporosis). Inhalation delivery methods may

apply gene therapy via tissue targeting and organ targeting. Inhale's novel dry powder formulation, processing, and filling, combined with aerosol device technology, will provide many patients who previously received injections with the ability to independently and painlessly inhale medicine into the deep lung, where it will be absorbed into the bloodstream naturally and efficiently.

## TRANSDERMAL ROUTE:

Various approaches for transdermal delivery of peptidal drugs are tried to deliver proteins and peptides. Approaches include Iontophoresis, Electroporation, Sonophoresis, Use of penetration enhancers, Prodrug approaches, and some recent innovations.

### Recent innovations:-

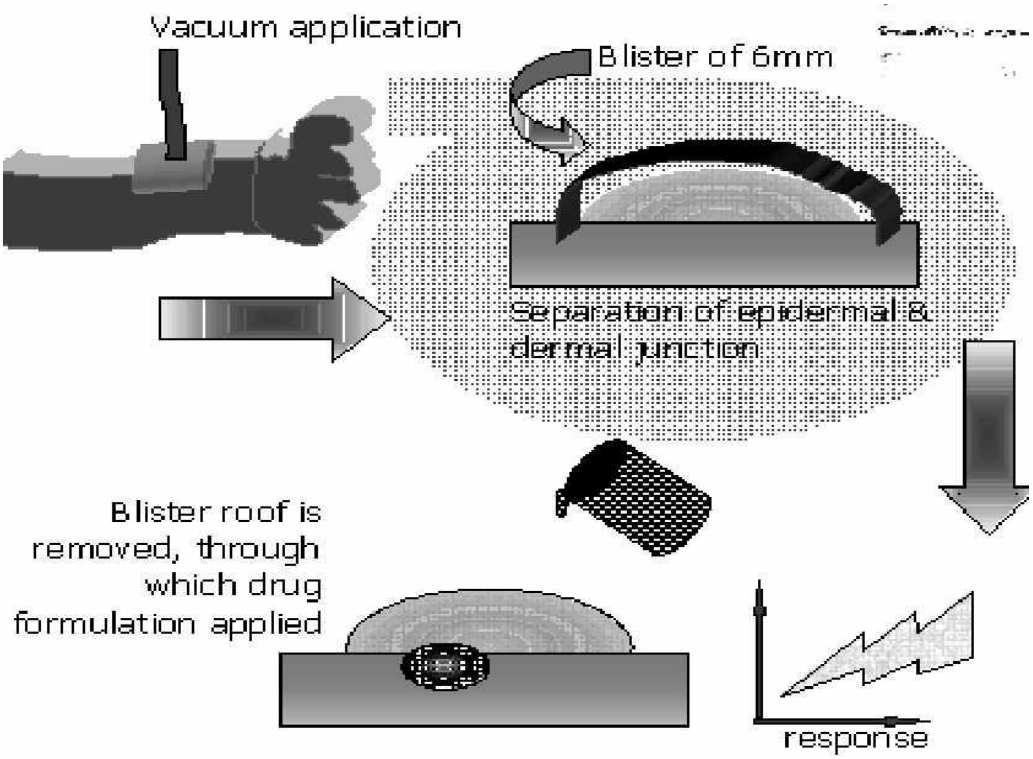
#### *Use of electroporation:-*

Electroporation (EP), the application of pulsed electric fields to target tissue, has been found to be an effective technique to overcome the membrane barrier in clinical applications of genes and DNA. Scientists at Genetronics have developed the drug delivery applicators. These applicators composed of six needles arranged in a regular hexagon array.

In clinical study, the anticancer drug is injected into a sold tumor and needle array is inserted into the tumor and a surrounding margin of healthy tissues. Needle pairs are energized in a rotating fashion as EP occurs at the apexes of a cell in the direction of the field. The needle pairs are energized in polarities as membrane pore formation is polarity dependent. Needle length and angle may be chosen by physician to allow optimal effect.

### The MicroPors™

The MicroPors™ technology consists of directing tightly focused thermal energy into the SC to create micropores. The skin is contacted by a wire mesh through which a current is passed that causes local heating sufficient to burn small holes in the barrier. Delivery of insulin by this approach has been suggest




A more macroscopic method to create erosion in the SC is via “suction de-epithelialization”.

High-velocity particle delivery across the skin is the technology of a major drug delivery operation based in the UK and USA (PowderJect, Inc.). Once a drug has been formulated as an appropriate and well-characterized powder, it is then introduced into a compact hand-held device in which a supersonic flow of gas accelerates the particles to a speed high enough that they can collide with the skin having enough energy to penetrate the outer layers and affect drug delivery. The depth and extent of delivery depends on the speed, diameter, and density of the drug particles. Again, insulin has been a peptide of choice for study by this method.

## OCULAR ROUTE:-

Concept was developed to achieve systemic delivery of protein and peptide though ocular route. The basis behind the concept was to explore the lachrymal system that exports the drug to nasal cavity from where the systemic absorption took place.

However the localized bioavaibility of the drugs remains poor through this route due to physiological hurdles like tear distribution, lachrymal drainage and protein binding. Various protein and peptides tried via ocular route include:

| Peptide / protein | Therapeutic indications |
|---|---|
| Enkephalins, substance P | Anti-inflammatory |
| Cyclosporine, interferon | Generate immune response |
| Insulin like growth factor | Healing of wounds |

## MISCELLANEOUS APPROACHES:-

Various sophisticated systems and techniques have been developed to control and target the delivery of proteins and peptides. These techniques include:

- ☞ On-demand systems
- ☞ Self-regulated systems
- ☞ Temperature sensitive systems
- ☞ Pumps

### On demand systems:-

Here the device consists of an ethylene-vinyl acetate matrix with magnetic beads or cylinders. The magnetic beads alternatively compress and expand the matrix in the presence of a magnetic field. On exposure to external oscillating magnetic field the drug (insulin) release was increased upto 30 times. On removal of the magnetic field, the drug release rates returned to normal. The position, orientation and strength of the embedded magnets, the amplitude and frequency of the applied magnetic field and the mechanical properties of the polymer matrix influence the release rate.

### Self-regulated systems:

**(A) Polymer based systems:**

It utilizes a cationic hydrogel polymeric membrane with immobilized glucose oxidase. Glucose diffuses into the polymer, the immobilized glucose oxidase catalyses its biochemical conversion into gluconic acid. The microenvironmental pH within the membrane is lowered and the amine groups in the membrane get protonated. Hence, the membrane swells and its permeability to the insulin held

in the adjacent reservoir increases. This approach is applicable to only small peptide molecules.

**(B) Affinity based systems:**

It is based on the competitive binding between the glucose and glycosylated insulin (G-insulin) to Concanavalin A (lectin). Here affinity ligand (glucose) occupies the binding site while low affinity ligand (insulin) is released into the environment where it acts as a therapeutic entity. With an increase in the glucose level, the influx of glucose pouch increases and thereby G-insulin is displaced form the Concanavalin A substrate. The increase in displaced G-insulin in the pouch results in efflux of G-insulin from the system into the circulation for pharmacodynamic activity.

## Temperature sensitive systems:

Here temperature sensitive swelling polymers like polyacrylamide derivatives are used for the pulsatile delivery of peptides and proteins.

Example: insulin permeation through poly-N-isopropylacrylamide Dibutyl-methactylate copolymer membrane varies with the composition of membrane affects the release rate. The thermosensitive permeation determines reversibility without any noticeable lag times.

## Pumps:

In pumps the primary driving force for the delivery is the pressure difference and not the concentration difference of the drug between the formulation and the surroundings. Pump can either be implantable or externally portable.

**Advantages of pumps:**

- Better flexibility and freedom for the patient.
- The desired physiological levels of the drug can be easily attained.

**Disadvantages of pumps:**

- Possibility of mechanical or electrical failure.
- Inconvenient and complicated for the patient for use.
- Prohibitive cost of the device.
- Chances of dermatological complications.

- ❖ Aggregation of peptide/protein drug may be undesirable and adverse since most of the pumps rely on a linear relationship between volumetric flow rate and actual drug delivery rate.

## Mechanical pumps

Major classes of these portable pumps, for insulin delivery are syringe driven, either lead screw or direct drive or roller peristaltic. The other class of mechanical pump is Infusaid. Examples of direct drive pumps: Autosyringe, microjet, mililll infuser penpump, pharmajet and examples of roller peristaltic pumps: Medtronic, Siemens Promedos, Zyklomat.

### Infusaid:

It comprises of a hollow titanium disk divided into two chambers. Outer chamber carries fluorocarbon liquid, which exerts vapor pressure well abouve the atmospheric pressure at 37°C. This pressure propels the drug solution in the chamber through the capillary flow resistant into a suitably placed catheter. Use of infusaid (infusion pump) is restricted to subcutaneous insulin delivery.

### Osmotic pump

The pump consists of a central drug and osmogen core with semipermeable polymeric rigid lamella. Drug release took place based on osmosis phenomenon. These pumps can be implanted subcutaneously. Important aspects for these pumps include stability of drug at 37°C within the pump for the entire infusion period and compatibility of drug with interiors of pump. These pumps had been tried with Insulin, calcitonin, growth hormone and vasopressin.

Chief development in osmotic pump is based on principle of Electroosmosis.

The Teorell-Meyer™ dosage forms depend on bioelectricity for their function. They obey the equations generated by Drs. Torsten Teorell and Karl Meyer in their work on membranes. This work has recently been applied in a new way, by using the hydrophilic properties of ionic molecules. Ions follow the course of the anatomy and biologically closed electric circuits (BCEC). Buffering a recipient compartment at a chosen pH with respect to the target site allows us to determine the length of the delivery vector according to the anatomy. By

controlling the electric vector and direction of current in the BCEC, drug molecules may be driven to the delivery site in ionic form, along with suitable companion molecules. By complexing with a suitable molecule, they globally become neutral at the end of the vector, and penetrate the membrane at the delivery site in the usual way. BCEC between the mouth and nose has been reported in the literature and is awaiting independent confirmation.

## Controlled release micropumps:

The pumps were developed for intraperitoneal delivery of insulin. Concentration difference between drug reservoir and drug delivery site causes the diffusion of drug to the delivery site. No external power source is required. Current is applied through solenoid coil, which causes the piston to compress the foam disk repeatedly and thus release the drug.

## SITE SPECIFIC DELIVERY:-

1. Macromolecular drug carriers
2. Soluble polymer conjugates
3. Particulate carriers

**Macromolecular drug carriers and soluble polymer conjugates**

| **Macromolecular drug carriers & Soluble polymer conjugates** | |
|---|---|
| **Polymer employed** | **Suggested use** |
| Polylysine | General carrier for targeting to cancer cells |
| Polypeptide-mustard conjugates | Lung targeting, tumor targeting |
| Polyglutamic acids | General carrier for tumor targeting |
| Pyran copolymers | General carrier of cytotoxics |
| Polyethylene glycol | General carrier and protective agent against immune recognition |
| Poly-L-aspartic acid | Hydrolysable targeting carrier for cancer |

## Particulate carriers:

They may be monolithic or capsular. The carrier systems include liposomes, emulsions, stabilized micellar system, proteinoid microspheres, retroviral vectors, native or modified endogenous particles.

They all possess major disadvantages of being subjectable to mononuclear phagocytic system of host.

**COMPENDIAL LIST OF PROTEINS, PEPTIDES & AMINO ACIDS:-**

| | | |
|---|---|---|
| Albumin human | Antirabies serum | Arginine |
| Aspartic acid | Bleomycin | Botulism antitoxin |
| Cholera vaccine | Cyclosporine | Cysteine |
| Enalapril | Glycine | Gonadotropin |
| Heparin | HepatitisB vaccine | Histidine |
| Insulin | Levothyroxine | Lysine |
| Measles virus vaccine | Meningococcal polysaccharide vaccine | Mumps skin test antigen |
| Pancreatin | Plague vaccine | Plasma protein fraction |
| Protein hydrolysate injection | Rabies immunoglobulin | Rabies vaccine |
| Rubella & Mumps virus vaccine live | Serine | Smallpox vaccine |
| Trypsin | Typhoid vaccine | Tyrosine |
| Vaccinia immune globulin | Valine | Varioella-Zoster immune globulin |
| Yellow fever vaccine | | |

**The Future:** Greater use of Nanotechnology in biopharmaceutics (nanopharm) Using cells as "Protein Factories" or as targetable "Nanosensors & Nanorobots". Artificial or Synthetic Cells as drug delivery agents will be utilized in near future.

## Delivery of proteins and peptides to brain via nasal route:-

The blood brain barrier (BBB) represents one of the strictest barriers of *in vivo* therapeutic drug delivery. The barrier is defined by restricted exchange of hydrophilic compounds, small proteins and charged molecules between the plasma and central nervous system (CNS).

An attempt to overcome the barrier *in vivo* has focused on bypassing the BBB by using a novel, practical, simple and non-invasive approach *i.e*. **intranasal delivery.** This method works because of the unique connection which the olfactory and trigeminal nerves (involved in sensing odors and chemicals) provide between the brain and external environments.

Intranasal delivery does not require any modification of the therapeutic agents and does not require that drugs be coupled with any carrier like in case of drug delivery across the BBB. A wide variety of therapeutic agents, including both small molecules and macromolecules can be successfully delivered, including to the CNS, using this intranasal method.

***Advantages:***

The advantages of intranasal delivery are considerable. This method is:

1. Non-invasive, rapid and comfortable
2. Bypasses the BBB and targets the CNS, reducing systemic exposure and thus systemic side effects
3. Does not require any modification of the therapeutic agent being delivered
4. Works for a wide range of drugs. It facilitates the treatment of many neurologic and psychiatric disorders
5. Rich vasculature and highly permeable structure of the nasal mucosa greatly enhance drug absorption
6. Problem of degradation of peptide drugs is minimized up to a certain extent
7. Easy accessibility to blood capillaries
8. Avoids destruction in the gastrointestinal tract, hepatic “first pass” elimination and gut wall metabolism, allowing increased, reliable bioavailability.

***Limitations***

1. Concentration achievable in different regions of the brain and spinal cord, varies with each agent
2. Delivery is expected to decrease with increasing molecular weight of drug
3. Some therapeutic agents may be susceptible to partial degradation in the nasal mucosa or may cause irritation to the mucosa
4. Nasal congestion due to cold or allergies may interfere with this method of delivery
5. Frequent use of this route results in mucosal damage (e.g. infection, anosmia).

# Biotech based products & Immunomodulated molecules

## INDEX



## INTRODUCTION TO BIOTECHNOLOGY

The emergence of biotechnology in the 1970s created new opportunities for the world. Almost forty years later, biotechnology is still in its infancy; yet, the industry is quickly. Establishing itself, not as another area of scientific inquiry, but as a core technology upon which many industries can base their future innovations.

The classic example of biotechnological drugs was proteins obtained from recombinant DNA technology. Biotechnology now encompasses the use of tissue culture, living cells or cell enzymes to make a defined product.

### APPLICATION IN VARIOUS FIELD LIKE

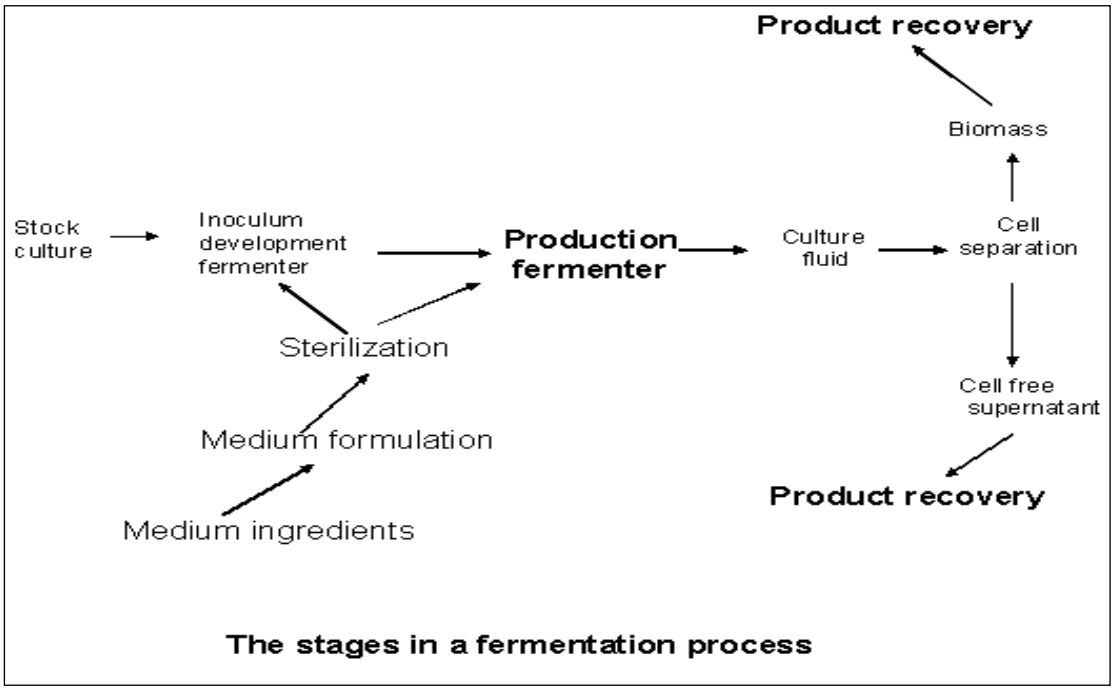
- Pharmaceutical – Protein, Gene, Vaccine
- Agriculture – Plant Tissue Culture
- Industry – Lactic/Citric acid, ethanol
- Beverages – Whisky, Beer
- Dairy products – Bread, Cheese
- Amino acid production.

## TECHNIQUES USED TO PRODUCE BIOTECHNOLOGICAL PRODUCTS

A. Fermentation process
B. Genetic engineering
    1. Recombinant DNA (rDNA) technology
    2. Monoclonal antibodies
    3. Cell therapy products
    4. Gene therapy products
    5. Polymerase chain Reaction PCR
    6. Peptide technology.
C. Enzyme technology

## FERMENTATION PROCESS

Fermentation shares with biocatalysts the distinction of being the oldest form of biotechnology. Traditionally, fermentation has meant the production of potable alcohol from carbohydrates. However, fermentation-that is the application of microbial metabolism to transform simple raw material into a valuable product like citric acid, antibiotics, biopolymers and single cell proteins. What is needed is the knowledge of these micro-organism and growth and the ability to handle them on the large scale.



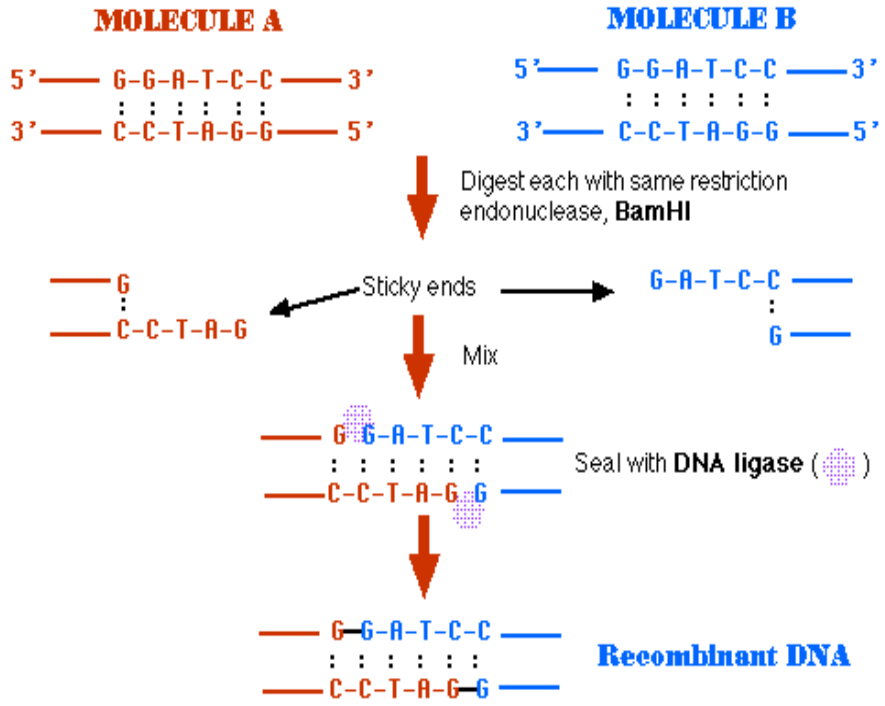
The stages in a fermentation process

The central part of fermentation process is the growth of the industrial organism in an environment which stimulates the synthesis of the desired commercial product. This is carried out in a fomenter which is, essentially, a large

vessel in which the organism may be maintained at the required temperature, pH, dissolved oxygen concentration and substrate concentration.

The medium on which the organism wish to grown has to be formulated for its raw material and sterilized. The fermenter has to be sterilized and inoculated with viable, metabolic active culture which is capable of producing the required product; after growth, The culture food has to be harvested, the cells separated from the supernatant and the product has to be accepted from the relevant fraction and purified. One most also visualize the research and development programme superimposed upon this process. First the successful fermentation is based on the skill of microbiologist, biochemist, geneticists, chemical engineers, and chemist and control engineers.

## RECOMBINANT DNA (rDNA) TECHNOLOGY



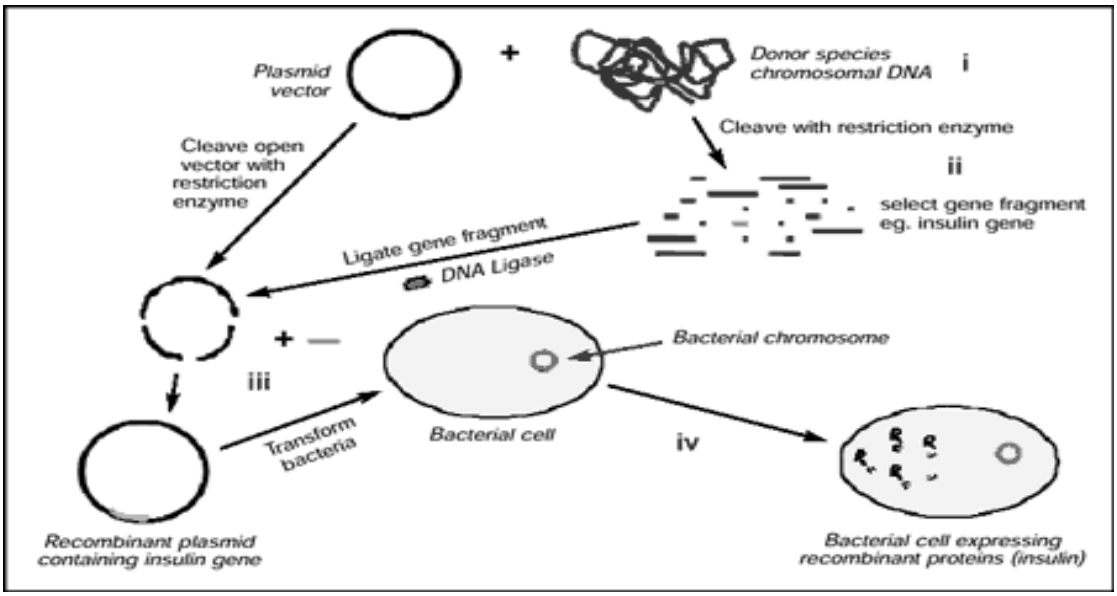

**a.** It facilitates the production of selective DNA fragments from larger and complex DNA molecule, in larger quantities

**b.** DNA from two or more sources is incorporated into a single recombinant molecule.

**c. Critical steps** in application of rDNA technology for production of desired protein….

1) Identification of protein that is to be produced.
2) DNA sequence coding for the desired protein is done.
3) Fully characterized gene is isolated using restriction enzymes
4) This gene is inserted into a suitable vector like plasmid (circular extra chromosomal segment of DNA found in certain bacteria) with DNA ligase.

5) The plasmid is then inserted into the host cell (eukaryotic of prokaryotic cells) (transformation process)
6) Clones of the transformed host cells are isolated and those producing protein of interest in desired quantity are preserved under suitable conditions as a cell bank.
7) As the manufacturing need arise, the cloned cells can be scaled up in a fermentation or cell culture process to produce the protein product.




## Prokaryotic (Bacterial) production

- *E. coli* is used as a bacterial strain for production of protein.
- **Advantages**

- Biology of bacteria is well understood.
- Safe and effective use of *E. coli* as a host organism is well documented.
- The expression of new protein is easier to accomplish than in other more theoretically suitable system.

- **Disadvantages**

- It produces proteins in a chemically reduced form.
- *E. coli* protein begins their sequence with N-formyle methionine residue and thus yields methionine derivative of desired natural protein.
- Potential for product degradation because of trace protease impurity.
- Requires endotoxin removal during purification.
- Expressed protein product may cause cellular toxicity or it is extremely difficult to purify as it is sequestered into bacterial inclusion body as large aggregates.

- **Recent advancement**

  Exploration of *E. coli* molecular biology have lead to the ability to express protein in periplasmic space, allowing the removal of unwanted terminal N-methionine group leading to more rapidly purified proteins.

## Eukaryotic (mammalian cell and yeast) production

- The use of yeast strain *Sachharomyces cerevisiae* for production has been explored.

**Advantages**

- Can produce large proteins or glycoproteins
- Secrete proteins that are properly folded and identical in their primary, secondary and tertiary structure to natural human protein.

**Limitation**

- Economy of the production is high to hinder development.

- **Recent advancement**

- Large scale culture using Chinese Hamster Ovary (CHO) cells and formulation of highly defined growth media have improve the economic feasibility of eukaryotic cell substrate.

**Application**

- Techniques used in research directed towards developing and generating new drugs
- Study and develop treatments for some genetic diseases.
- To produce molecules naturally present in human body in large quantities previously difficult to obtain from human sources. (hormones like insulin and growth hormone)

- **DNA probe technology for diagnosis of disease.** In this process…

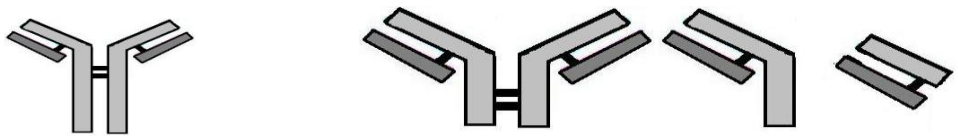
- Specific strand of DNA is synthesized with sequence of nucleotide matches with the gene under investigation.
- Now tag the synthetic gene with dye or radioactive isotope.
- When introduced into a specimen, the synthetic strand of DNA acts as a probe searching for complementary strand.
- When one is found, two are hybridized and dye/radio isotope reveals the location of the location of synthetic strand.

## FDA Approved rDNA Products

| Category | Generic Name of Drugs |
|---|---|
| Anti coagulants | Lepirudin |
| Clotting Factors | Systemic Antihemophilic factors<br>Recombinant factor VIII |
| Colony Stimulating Factors | Granulocyte CSF<br>G-CSF + Monomethoxy PEG<br>Granulocyte Macrophase CSF |
| Erythropoietins | Epoetin Alfa<br>Darbepoetin Alfa<br>Drotrecogin Alfa |
| Growth Factor | Becaplermin |
| Human Growth Factor (hGH) | System Growth Hormon |
| Interferon | Interferon beta 1-b<br>Interferon beta 1-a |
| Interleukins | Aldesleukin<br>Anakinra<br>Oprelvekin |
| Tissue Plasminogen Activator | Recombinant Alteplase<br>Recombinant Reteplase<br>Recombinant Tenecteplase |
| Tyrosine Kinase Inhibitor | Imatinib Mesylated |

## MONOCLONAL ANTIBODIES

- Antibodies are proteins produced by differentiated B lymphocytes.
- Antibodies produced in immunized animals are formed from different clones of B lymphocytes (polyclonal). Polyclonal means they all are not specific to only that antigen, and specific are less in number.
- Antibodies that are produced by immortalized cell lines (hybridoma) derived from single B cells are monoclonal antibodies. MAb – Monoclonal Antibody are Specific to only one Antigen.
- Lower part of antibody is called a constant region, identical in all immunoglobulin of specific class (e.g., IgG, IgM)
- The variable domain is highly hetrogenous and gives antibody its binding specificity and affinity.



**IgG molecule** **F(ab')$_2$** **Fab'** **sFv**

- Smaller fragments containing intact variable region like F(ab')$_2$, Fab' and sFv have following advantages:
- ✓ Do not contain the lower binding domain (constant region). Smaller molecule leads to less immunogenic effect and have a greater penetration capacity than larger molecule.
- ✓ In case of diagnostic imaging application, smaller fragments have greater renal, biliary or colonic uptake
- ✓ All three smaller antibody forms have had success in detecting smaller (<2cm) lesions not seen on Computed tomography.
- Monoclonal antibodies can be produced in two major ways: **murine (mouse) origin** and **human origin**

## Mouse origin

- Chemical induced fusion of mouse spleen cell with mouse myeloma cell.
- The resultant mouse-mouse hybridoma cell inherits the replication ability from myeloma cell and ability to produce the desired monoclonal antibody from spleen cell.
- **Limitation**: production of human Antimouse antibody responses against the MAbs – allergic reaction.

## Human origin

- Human B lymphocytes can be clonally selected for hepten binding specificity of their product antibodies.
- These selected cells are then immortalized by infection with virus.

**Cell banks of hybridoma cell** (fused or transformed cell) lines can be used to produce a continuous supply of monoclonal antibody by two ways:
In-vivo: by injection into mice and subsequent collection of the ascetic fluid.
In-vitro: by conventional cell culture techniques.

## Application

- Diagnostic as well as therapeutic.
- MAb can be coupled with other agents e.g., oncolytic agent, radio nuclide, toxins, etc. with the resultant antibody conjugate being final product of interest.

# Immunomodulated molecules

### Introduction

The modulation of immune system activity is a required pharmacological intervention for many medical conditions. Immunosuppression is integral to the treatment of autoimmune diseases and allergies, and the inhibition and prevention of immune responses also constitutes a prominent treatment aim for transplant recipients. Conversely, immune system stimulation is adopted for a number of infections and cancers.

The market for immunomodulators was valued at $43bn in 2006, and is expected to grow at a CAGR (**Compound Annual Growth Rate)** of 13% to reach $80bn by 2011.

Immune system-mediated diseases are significant medical problems. Immunological diseases are growing at epidemic proportions that require aggressive and innovative approaches to develop new treatments. These diseases include a broad spectrum of autoimmune diseases such as rheumatoid arthritis, type I diabetes mellitus, systemic lupus erythematosus, and multiple sclerosis; solid tumors and hematologic malignancies; infectious diseases; asthma; and various allergic conditions. Furthermore, one of the great therapeutic opportunities for the treatment of many disorders is organ transplantation.

Immunomodulator modulate the immune response in three ways: immunosuppression, tolerance, and immunostimulation. The "holy grail" of immunomodulation is the induction and maintenance of immune tolerance, the active state of antigen-specific nonresponsiveness. With this greater understanding of pathogenesis coupled with advances in biotechnology, many immunomodulatory agents have been introduced into the clinic for the treatment of various conditions

Immunomodulators mainly consist of biopharmaceuticals such as **Recombinant proteins, Vaccines and monoclonal antibodies**, but also include several small molecules.

In inflammatory and autoimmune disorders, immunomodulators in clinical development are predominantly aimed at **G protein-coupled receptors** (**GPCRs), leukocyte surface antigens, cytokines and kinases.**

**IMMUNOTHERAPY WITH UNCONJUGATED MONOCLONAL ANTIBODIES**

- Rituximab (Rituxan). This MoAb binds to the CD20 antigen of B-lymphocytes, which is expressed on >90% of non- Hodgkin's lymphoma B-cells. Upon binding, the Fc region of the MoAb recruits immune effector functions to mediate B-cell lysis, possibly by both CDC and ADCC mechanisms.

- Tratsuzumab (Herceptin) (phase III trial) binds to the extracellular domain of a transmembrane protein, human epidermal growth factor receptor 2 (HER2), which is overexpressed in 20–30% of primary breast cancer cells. It is thought to act primarily by ADCC.

## Radioimmunoconjugates

- Are MoAbs to which radionuclides have been conjugated, to provide cytotoxic radiation after the MoAb binds to its target antigen.
- The isotopes most commonly used are Iodine-131 and Yttrium-90, both of which are *β* emitters. The former is covalently bound to tyrosine residues of the MoAb by standard chemical techniques, whereas the latter is chelated to a ligand that has been conjugated to the MoAb.
- Radionuclide emissions from both 131I and 90Y can extend to 1–5mm of their final location, corresponding to several cell diameters. Thus, their chief advantage resides in their ability to kill tumor cells that are poorly accessible and/ or antigen-negative. Unlike conventional radiation therapy, radioimmunoconjugates provide continuous radiation from the decay of the radionuclide, which allows less opportunity for the tumor cells to repair sublethal damage.
- Depending on the type of MoAb, the antibody itself may trigger CDC and ADCC mechanisms that supplement the effect of the radionuclide.

- Although no radioimmunoconjugates have progressed to the market, a number have been examined in clinical trials.
- Bexxar (131I-tositumomab) is an anti-CD20 MoAb examined in Phase III trials for non-Hodgkin's lymphoma

## Immunoliposomes

- Generally, the antigens expressed by tumor cells are not specific but are merely present in higher ratio than on the normal cells. Hence, systems such as immunoliposomes have been developed to exploit these opportunities, as they are expected to bind to a greater extent to high antigen density tumor cells than to low antigen density normal cells.
- The increase in the valency of liposomes (i.e., number of antibody molecules per liposome) increased their binding with low as well as high antigen density cells, and thus the low valency immunoliposomes were found to allow better discrimination between target and normal cells.
- An additional advantage of immunoliposomes is that a relatively high drug loading can potentially be accommodated, with the result that a small number of antibody molecules conjugated to the surface of an immunoliposome can deliver many more drug molecules to the target than is otherwise possible.
- Once the drug is released into the target cell, no further transformation is needed, because the entrapment process does not involve any chemical modification of the drug.
- Heath et al. have proposed the use of immunoliposomes for the intracellular delivery of compounds that intrinsically do not enter diseased cells. These compounds are cytotoxic if they are transported intracellularly. Methotrexate-*g*-aspartate, a good example of this type of compound
- Extensive work is being pursued to assess the potential of immunoliposomes for the targeted drug delivery to CD4 positive cells in patients with HIV infection.
- The HIV infected cells possess CD4, which can be targeted by conjugating anti-Leu3A (CD4) MoAbs onto the surface of drug-loaded liposomes.
- Heat-sensitive immunoliposomes have also been evaluated for the feasibility of drug delivery. These liposomes release the entrapped drug at temperatures above the phase transition temperature of the lipid(s).

- Similarly, selection of appropriate lipids can also allow synthesis of pHsensitive liposomes. Inclusion of target-cell specific immunogenic moieties in these colloidal particles leads to preparation of pH-sensitive immunoliposomes.
- A great deal of attention has been paid in recent years to long-circulating (also called sterically stabilized or ‘‘stealth’’) liposomes, in which polyethylene glycol (PEG) molecules have been grafted to the surface of the liposomes by covalent attachment of PEG to liposomal phospholipids (specifically phosphatidylethanolamine).
- These long-circulating liposomes have been shown to avoid the rapid uptake by the reticuloendothelial system (RES), which normally plagues conventional liposomes without PEG; the circulating half-life can be increased by an order of magnitude.In fact, the presence of a MoAb on the surface of a conventional immunoliposome may actually increase the uptake by the RES system, suggesting that modification to insure long-circulation may be especially important for immunoliposomes.
- When designing PEG-modified immunoliposomes, the composition must be optimized for both antigen binding and extended circulating lifetimes. Antigen recognition by the liposomal antibody can be sterically hindered by the presence of the PEG. This can be overcome by either reducing the polymer size to 2000, or by moving the antibody out to the terminus of the PEG rather than the liposome surface.
- Despite the extensive in vitro and in vivo research on immunoliposomes, these MoAb targeted systems have apparently not yet reached clinical trials.
- This may be because of a variety of factors, including the difficulty of clearly demonstrating efficacy in suitable animal models, and the obstacles associated with scaleup and manufacture of system as complex as a sterically stabilized MoAb-targeted liposome.
- Other potential problems of immunoliposomes are that they may not adequately penetrate the vasculature of solid tumors; they may not adequately release the loaded drug into the target cells; and they may demonstrate immunogenicity. Use of humanized antibodies may alleviate the latter effect to some extent

## MoAb targeted emulsions

A related approach for lipophilic drugs is MoAb targeted emulsions. For example, a lung-targeted MoAb 34A was conjugated to the surface of a long-circulating emulsion composed of castor oil, phosphatidylcholine, and pegylated phosphatidylethanolamine. Upon intravenous injection into mice, 30% of the injected emulsion dose became preferentially associated with lung tissue within 30 min.

## Immunomicrospheres

- In view of the availability of a wide variety of biocompatible and biodegradable polymers, and the ease of preparation of stable microparticles with predictable physicochemical characteristics, antibodies have been conjugated to polymeric microparticles for controlling their in vivo deposition.
- Although a few in vitro studies have demonstrated promising results with immunomicrospheres, limited information has been published on the in vivo efficacy of immunomicrospheres for drug delivery.

## **Antibody-directed prodrug therapy** (ADEPT)

- Recent approach combines MoAb targeting with enzymatic prodrug activation. In this therapeutic method, called antibody-directed prodrug therapy (ADEPT), an enzyme–antibody conjugate is administered and allowed to accumulate in the target site (e.g., tumor). A latent, non-toxic prodrug is then injected, which on contact with the enzyme is converted into the active parent drug and subsequently kills the tumor cells
- The ADEPT technique has been tested clinically in colorectal cancer patients using para-N-(mono-2- chloroethyl monomesyl)-aminobenzoyl glutamic acid as the prodrug and an antibody conjugate of glutamate hydrolase as the activating enzyme, with temporary regression of disease in two out of five patients.

## FDA approved immunomodulated molecules

**Cytokine inhibitors**

1. **Infliximab**, a chimeric anti–TNF-a mAb initially approved in 1998;

2. **Etanercept**, a recombinant soluble p75 TNF receptor (CD120b)–IgG Fc fusion protein initially approved in 1998;
3. **Adalimumab**, a human anti–TNF-a mAb initially approved in 2002;
4. **Etanercept** and **adalimumab** have been approved for the treatment of juvenile idiopathic arthritis.
5. **Certolizumab pegol**, a pegylated Fab9 fragment of a human anti–TNF-a antibody initially approved in 2008;
6. **Golimumab**, a human anti–TNF-a mAb initially approved in 2009
   They are used for the treatment of RA, PsA, AS, Crohn disease, juvenile idiopathic arthritis, and psoriasis ulcerative colitis.
7. **Anakinra**, approved in 2001 for the treatment of RA, is a recombinant IL-1ra that differs from the endogenous IL-1Ra by a single amino acid addition at the amino terminus
8. **Rilonacept** (previously known as IL-1–Trap), which was approved in 2008 for the treatment of cryopyrin-associated periodic syndromes, is a fusion protein comprised of the extracellular domain of the IL-1 accessory protein and IL-1 receptor type 1 attached to the Fc portion of IgG1
9. **Tocilizumab** is a humanized anti–IL-6 receptor mAb

## T-cell modulators

1. Humanized antibody **daclizumab** is in phase II trials for the treatment of MS
2. **Basiliximab**
   They are indicated for the prevention of organ transplant rejection, particularly kidney grafts, and have been suggested for the management of autoimmune disorders.
3. **Abatacept**, approved in 2005 for the treatment of RA, is a soluble protein consisting of the extracellular domain of CTLA-4 linked to the Fc portion of IgG1
4. **Alefacept**, approved in 2003 for the treatment of chronic plaque psoriasis, is a fusion protein of a soluble form of the extracellular domain of lymphocyte function–associated antigen (LFA) 3 attached to the Fc portion of an IgG1 molecule.

## β-cell modulators

1. **Rituximab** is a chimeric IgG1 mAb directed against the Blymphocyte surface antigen CD20. It was initially approved in 1997 for the treatment of CD201 B-cell non-Hodgkin lymphoma and later for the treatment of RA in 2006.
2. Anti-IgE antibody: **Omalizumab**
   This antibody was developed to aid in the management of severe asthma with an allergic component.

## Adhesion cell modulators

1. **Natalizumab**, approved in 2004 for the treatment of MS, is a recombinant humanized IgG4 mAb However, shortly after FDA approval; natalizumab was temporarily withdrawn from the market after 3 cases of PML (Progressive multifocal leukoencephalopathy) were reported. Similar to rituximab, the exact role of natalizumab in the development of PML remains unknown.
2. **Efalizumab**, approved in 2003 for the treatment of psoriasis, is a humanized IgG1 mAb However, the development of PML among several patients treated with efalizumab led to its withdrawal in 2009.

## Cytokines

Recombinant IL-2 has been approved by the US Food and Drug Administration (FDA) for the treatment of metastatic renal cancer and malignant melanoma

**Others**

Gemtuzumab, Ozogamicin: Acute Myeloid Leukemia
Ibritumomab,Tiuxetan: Radiolabeled for cancer
Murononab CD3: Block T-cell activity – Immunosuppresive
Omalizumab: Asthma
Trastuzumab: Breast cancer

## Recent innovation:

- Development of transfectomas, E. coli and bactriophage based production scheme which may offer advantages for future production of monoclonal antibodies.
- Super Antigen + MAb technology: (staphylococcal enterotoxin A) – toxin is attached to MAb. Thus, Super Antigen binds to macrophages and activates them. e.g., if super antigen is linked to antibody having specificity for tumor associated antigen, it targets activated macrophages to the tumor cell. This is very Novel approach, and it is under Phase I trials.

### CELL THERAPY PRODUCTS

- Recent advances in biotechnology have resulted in two new categories of product: Cell therapy product and gene therapy product.
- Cell therapy products contain living mammalian cells as one of their active ingredient while gene therapy products contain piece of nucleic acid, usually DNA as their active ingredients.
- Some of the products combine both categories, resulting in therapy that uses cells that express new gene product.
- These are the products with live cells that replace, augment or modify the function of patient's cells that are diseased or dysfunctional or missing.
- e.g., transplantation of bone marrow to replace marrow that has been destroyed by chemotherapy and radiation is an example of cell- therapy product.
- These therapy products are referred to as somatic cell therapy products as non-germ cells are used in the product.

### ➢ Sources of donor for cell therapy products

1. The patient's own cell (autologous cell product)
2. The cells from another human being ( allogeneic cell product)
3. Cells derived from animals such as pigs, primates or cows (xenogenic cell products)

- Autologous cells are not rejected by patient but they are not available for many treatments as they are missing, dysfunctional or diseased. In such situations, allogenic or xenogenic cells are used.
- The advantage of allogenic cells is that, they do not trigger a rejection reaction as strong as xenogenic cells.
- Xenogenic cells are used when human cells with desired characteristics are not available or supply of human donor is too limited.
- Cell therapy products are sometimes encapsulated in a device that prevents patient's cells and antibodies from killing xenogenic cells.
- Much research is focused on identifying and propagating stem cells regardless of the source as stem cells can be manipulated to differentiate either during manufacturing or after administration.
- Stem cells are unspecialized cells, can divide and renew themselves for long periods of time and become specific specialized cell types of the body.

## Unique properties of stem cell

| Property | Description |
|---|---|
| Unspecialized | Not have any tissue-specific structures & Functions |
| Dividing & renewing | They divide many times over it is called proliferation. |
| Rise to specialized cell | Differentiation:-<br>1.Internal signals (Genes)<br>2.External signals(chemicals, physical contact, microenvironment) |

## Manufacturing challenges

- ✓ They cannot be terminnally sterilized or filtered. So removal or inactivation of micro-organisms or virus without killing the cells in a problem.
- ✓ Every raw material in manufacturing has potential of remaining associated with the cells. So quantification of these raw materials is critical to produce a safe and effective product.
- ✓ Storage of these products is a challenge as freezing is the main mode for long term storage while some of the cell therapy products cannot be frozen without

changing the basic characteristics. So, these products have to be administered within hours or days at most after manufacturing process.

- ✓ Some products consist of a batch size as small as one dose.

## Cell therapy products

| Indication | Product |
|---|---|
| Bone marrow transplantation | Devices and reagents to propagate stem and progenitor cells or remove diseased cell |
| Cancer | T cells or macrophages exposed to cancer specific peptides to elicit immune response |
| Pain | Cells secreting endorphins or chatecholamines |
| Diabetes | Encapsulated β-islet cells secreting insulin in response to glucose level |
| Tissue repair | Autologus or allogenic chondrocytes in a biocompatible matrix |
| Neurodegenerative diseases | Allogenic or xenogenic neuronal cells |
| Liver assist | Allogenic or xenogenic hepatocytes |
| Infectious disease | Activated T cell |

## GENE THERAPY PRODUCTS

- ➢ These are the products in which nucleic acids are used to modify the genetic material of cells.
- ➢ E.g., a retroviral vector used to deliver gene for factor IX to cells of patients with hemophilia B
- ➢ These products can be classified broadly on the bases of their delivery system.

1. Viral vector: viruses with genes of interest but usually without the mechanism of self replication in vivo.
2. Nucleic acid in a simple formulation (nacked DNA)
3. Nucleic acids formulated with agents (such as liposomes to enhance penetration)
4. Antisense oligonucleotide (complementory to naturally occurring RNA and block its expression.

- Most of the clinical work is done using viral vector. The most common viruses used till date includes murine retrovirus, human adeno virus and human adeno associated virus.

## Manufacturing challenges

- Analytical methodologies for viruses are still being developed.
- Manufacturing of large batches of viral vectors with no or minimal amount of replication component viruses (RCV) is challenging.
- Detecting of small number of RCV particles in the presence of large amount of replication-defective vector is difficult.
- Sourcing of raw material is difficult.
- Defining purity is an issue for enveloped viral vector such as retro viruses or herpes viruses as they incorporate cellular proteins in their envelop when they bud from the cells.

## Safety concerns related to therapy

- Integration of gene therapy products into somatic cell DNA carries a theoretical risk of mutation which could lead to modified gene expression and deregulation of cell.
- Patients need to be monitored in case of viral gene therapy for presence of RCV.
- To address risk associated with specific products, preclinical studies, QC and patient monitoring strategies need to be developed in accordance with applicable regulations and guidance documents.

## Gene therapy product

| Categories | Indication | Product |
|---|---|---|
| Gene replacement<br>Sort term<br>Long term | CVS disease<br>Cystic fibrosis | Growth factor vector<br>Transmembrane conductance regulatory vector |
| Immuno therapy | Cancer<br>Arthritis | Autologous tumor cells<br>Autologous lymphocytes |
| Conditionally lethal genes | Cancer solid tumor | Thymidine kinase (TK) or Cytocine Deaminase (CD) vector |

| | | |
|---|---|---|
| Antisense | Cancer<br>Cytomegalovirys retinitis | Anti- oncogene vector<br>Antiviral vector |
| Ribozymes | HIV | Antiviral ribozyme vector into autologous lymphocytes |

**EQUIPMENTS USED FOR MANUFACTURING**

- Fermenter
- Bioreactor
- Sterilizer
- Centrifugation
- Filtration
- Ultra Filtration
- Extraction
- Ion Exchanger
- Gel Chromatography
- Affinity Chromatography
- HPLC, RP-HPLC
- Distillation
- SCF extractor
- Cell Immobilization
- Enzyme Immobilization

**ANALYTICAL TESTING**

***Major techniques are as follows…***

- Protein Content Analysis
- Amino Acid Analysis
- Protein sequence
- Peptide Mapping
- Immunoassay
- Electrophoresis
- Chromatography
- DNA determination

**MAJOR IMPURITIES MAY BE PRESENT IN FINAL PRODUCT**

- Endotoxins
- Host cell Protein
- From Media
- DNA

- Protein mutants
- Formyl Methionine
- Oxidised Methionine
- Protelytic Clevage residues
- Aggregated Protein
- MAb
- Amino Acids
- Bacteria, yeast, fungi, virus

**REGULATION**

- The first time need for the regulation in biotechnology was arise in 1976, the first guideline - RAC Guidelines – given by Recombinant DNA Advisory Committee by the US National Institute of Health (NIH).
- To ensure compliance with RAC Guidelines, Institutional Biosafety Committees (IBCs) were set.
- Various countries may have different guidelines for approval, production, sale of biotechnology base pharmaceutical products.
- **At present in USA:-**
  - Food and Drug Administration (FDA)
  - **CBER – Center for Biological Evaluation and Research**
  - NCTR – National Center for Toxicological Research
  - United States Department of Agriculture (USDA)
  - Environmental Protection Agency (EPA)
- The regulatory body of Australia - TGA – Therapeutic Goods Administration has given an Annexure 2 related to manufacture of biological medical products for human use.
- The regulatory body of European Union – EMEA- European Medicine Evaluation Agency has made various committees for regulation of biotech products like BWP – Biological Working Party, BMWP – Biosimilar Medicinal products Working Party, VEG – Vaccine Expert Group.
- In Uk's MHRA also, there is one sub committee has been dedicated to biological under the advisory body.
- The globally accepted WHO guidelines also cover the biological manufacturing related guidelines. The WHO has also constituted the ECBS – Expert Committee on Biological Standardization under the Executive board of WHO.

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