Pharmaceutical Biotechnology

Excipients used in parenteral formulation of biotech products

Introduction

- Molecular Biologist can produce
  - More recombinant protein drugs
  - Produce Pure Products

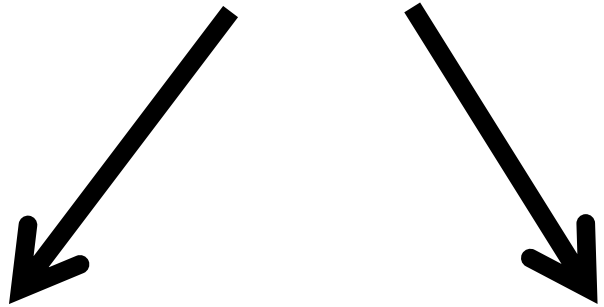
- Purification process of proteins strips away carbohydrates, salts, lipids and other proteins which are in natural environment.

- This purification process could make the protein drug less stable.

- Protein is sensitive to process such as
  - Shear
  - Agitation
  - Enzymatic and Chemical Degradation
  - Aggregation

Biotech products

(composition)



Active substance

Excipients

Goal of protein formulation

- The formulation should stabilize protein from chemical and physical degradations.

- Goals of Formulation
  - Easily administered
  - Efficacious
  - Adequate stability for shelf life for marketing.
  - Simple
    - excipient has potential interaction with protein drug which could block activity.

- To stabilize the protein using
  - Chemical Additives (Excipients)
  - Physical Methods (Lyophilization or Spray Drying)

- For parenteral formulation, several factors should be considered such as sterility, isotonicity, pH, and preservatives.

- Container and delivery devices are important
  - protein can be adsorbed to surfaces.
  - protein can be destabilized by extractable compounds from the packaging material (extractables and leachables).

Components found in parenteral formulations of biotech products .

- Active ingredient
- Solubility enhancers
- Anti-adsorption and anti-aggregation agents
- Buffer components
- Preservatives and antioxidants
- Lyoprotectants/cake formers
- Osmotic agents
- Carrier system

Excipients

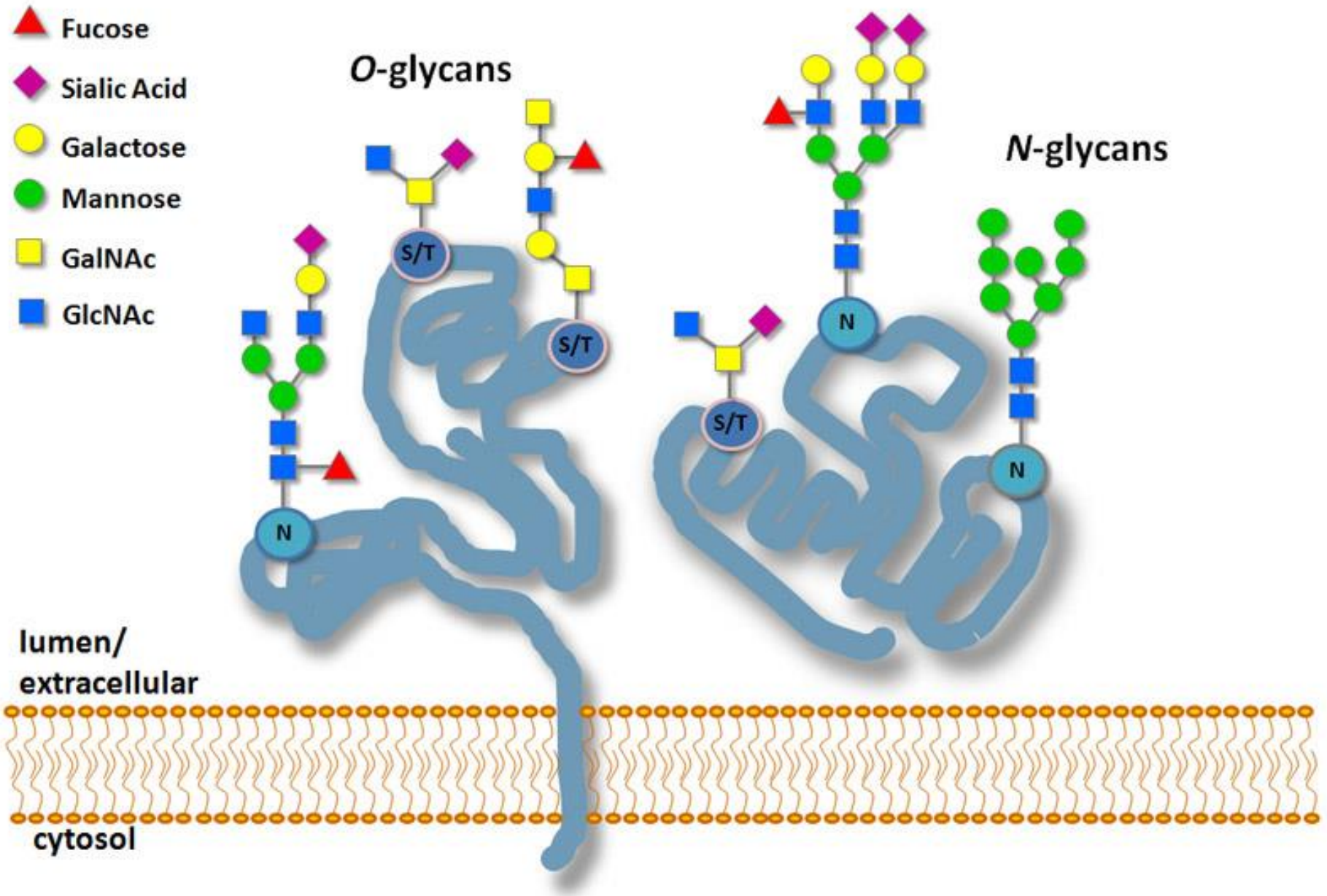
** Not necessarily all of the above are present in one particular protein formulation

Solubility enhancers

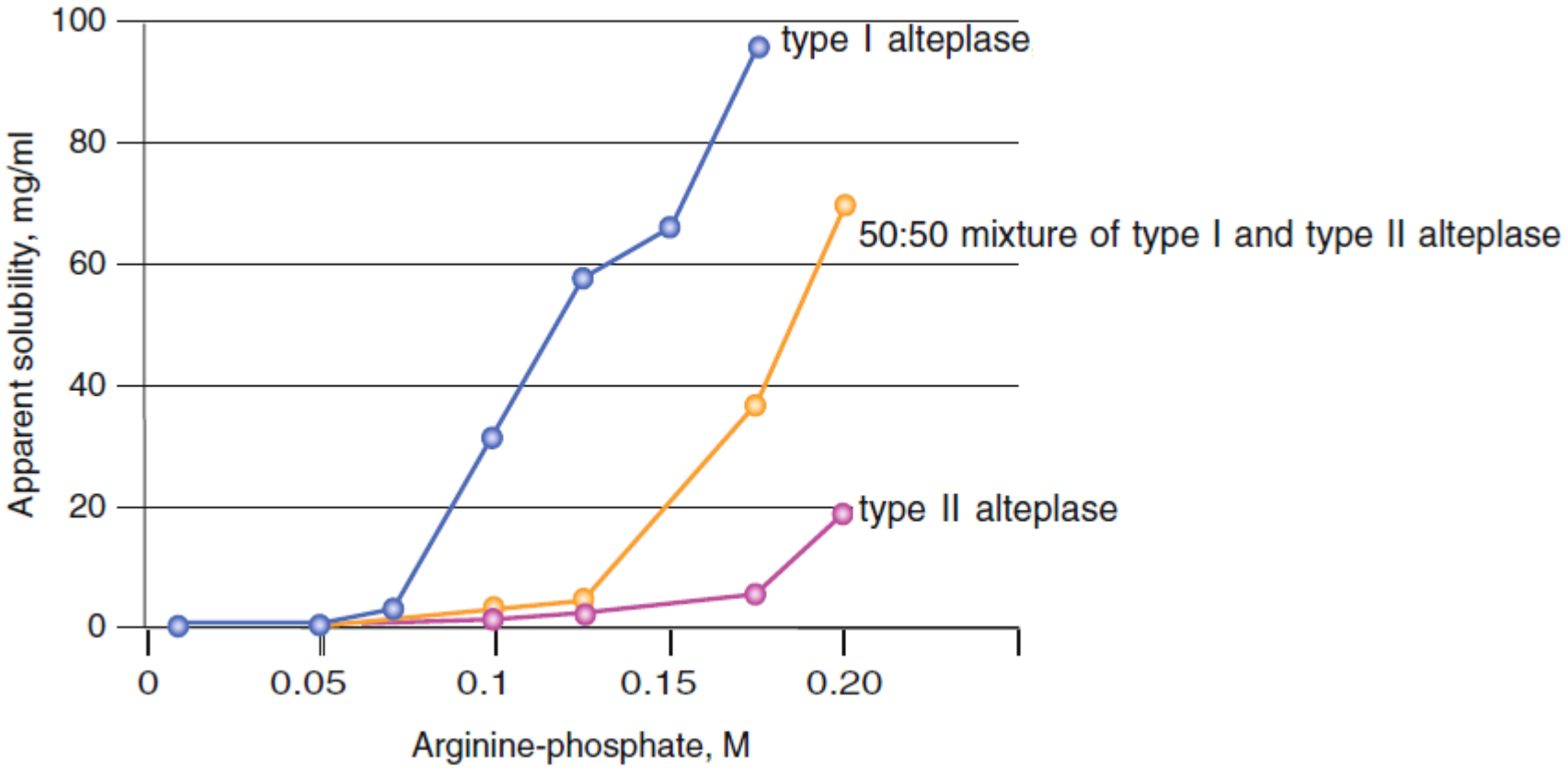
- Proteins, in particular those that are non-glycosylated, may have a tendency to aggregate and precipitate! **(H.W. types of protein glycosylation)**
- Therefore, several approaches can be used to improve protein solubility and reduce aggregation such as:

  - **Proper pH and ionic strength** conditions.
  - Addition of **amino acids** such as lysine or arginine (used to solubilize tissue plasminogen activator, t-PA) or
  - **Surfactants** such as sodium dodecyl sulfate (SDS) to solubilize non-glycosylated IL-2

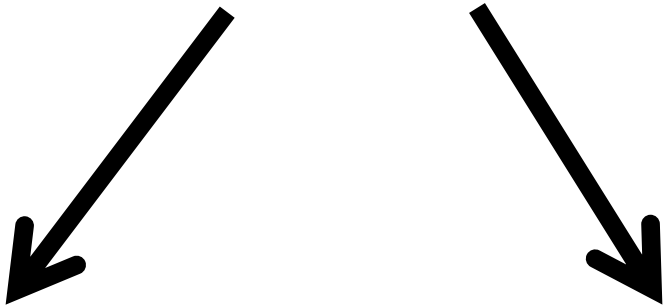
**Mechanisms are poorly understood and usually depends on type of protein and type of solubility enhancer!


Fucose
Sialic Acid
Galactose
Mannose
GalNAc
GlcNAc
O-glycans
N-glycans
S/T
S/T
S/T
N
N
N
lumen/
extracellular
cytosol

Effect of arginine on type I and type II alteplase at pH 7.2 and 25 °C



Aggregation



Physical

based on hydrophobic and/or electrostatic interactions between molecules

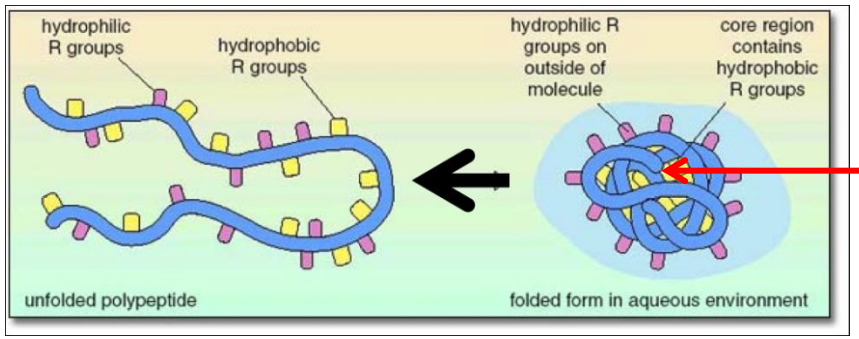
Chemical

based on the formation of **covalent bridges** between molecules through disulfide bonds and ester or amide linkages. In those cases, proper conditions should be found to avoid these chemical reactions.

Feature	Physical Aggregation	Chemical Aggregation
Driving force	Non-covalent interactions (hydrogen bonding, hydrophobic interactions, van der Waals forces, electrostatic interactions)	Covalent bond formation (disulfide bonds, cross-linking)
Influencing factors	Temperature, pH, ionic strength, crowding agents, interfaces	Oxidative stress, reactive chemicals, enzymes
Reversibility	Can be reversible, depending on conditions	Irreversible
Examples	Heat-induced aggregation, protein precipitation	Disulfide bond formation in the lens of the eye, protein cross-linking by glycation

Anti-adsorption and Anti-aggregation Agents

- Some proteins tend to **expose hydrophobic sites**, normally present in the core of the native protein structure when an **interface** is present.

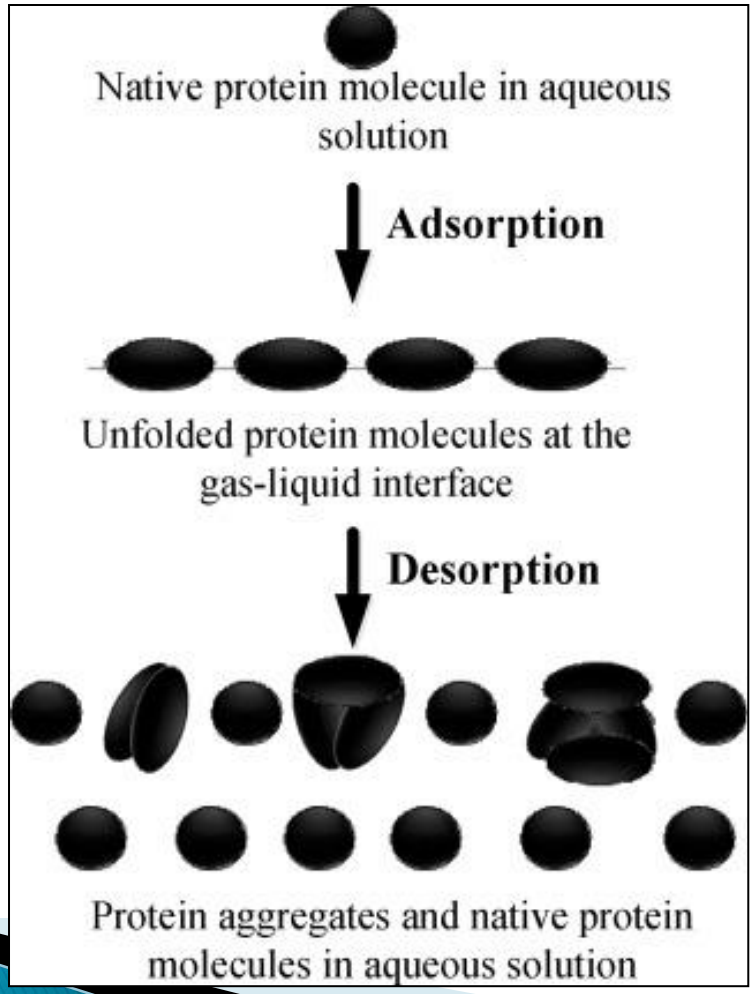


Hydrophobic sites usually reside inside the core of the folded protein!

- These interfaces (which are usually hydrophobic) can be:
  - water–air interface,
  - water–container wall interface,
  - or interfaces formed between the aqueous phase and utensils used to administer the drug (e.g., catheter, needle)
- Therefore, Anti-adsorption agents are added to reduce adsorption of the active protein to these interfaces.

What can happen upon adsorption?

- The adsorbed, partially unfolded protein molecules form aggregates, leave the surface, return to the aqueous phase, form larger aggregates, and precipitate.



Insulin case study

Insulin description

- Insulin molecule is composed of two polypeptide chains that are connected by **two inter-chain** disulfide bonds.
- The A-chain is composed of 21 amino acids
- The B-chain is composed of 30 amino acids.
- The inter-chain disulfide linkages occur between A^{7} -B^{7} and A^{20} -B^{19}.
- A **third intra-chain** disulfide bond is located in the A-chain, between residues A^{6} and A^{11}

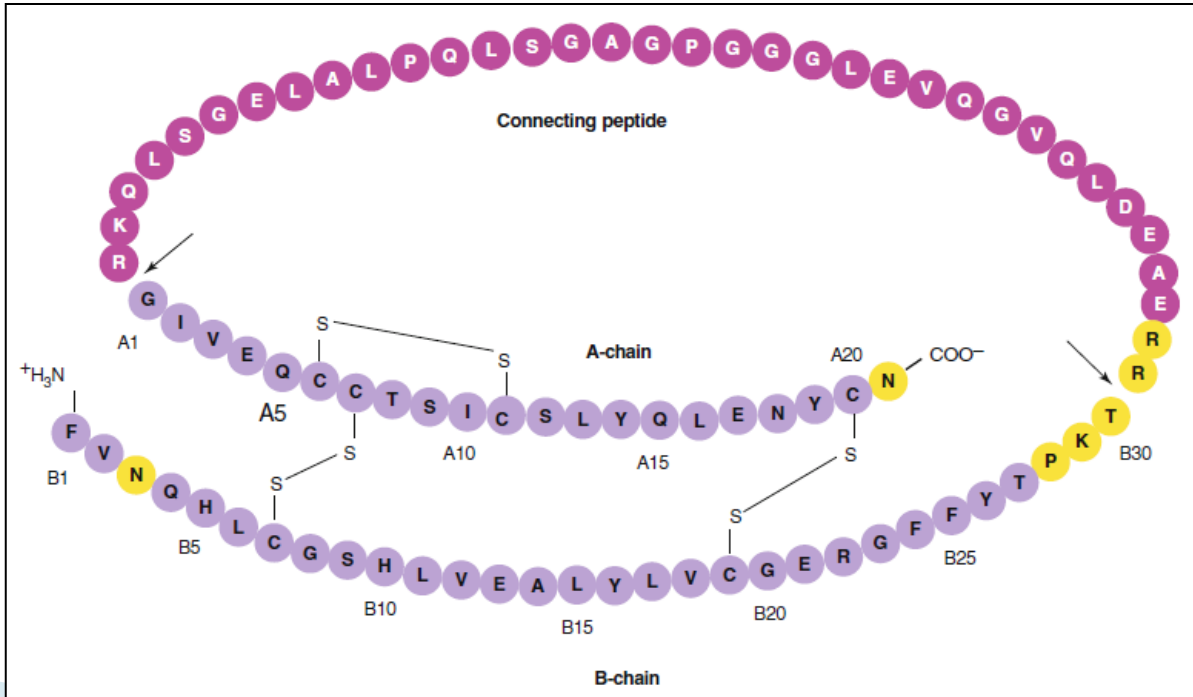


Figure 12.1 ■ Primary sequence of insulin. The shaded amino acids represent sites of sequence alterations denoted in Table 12.1.

- Native insulin in solution is in an equilibrium state between monomeric, dimeric, tetrameric, and hexameric forms.

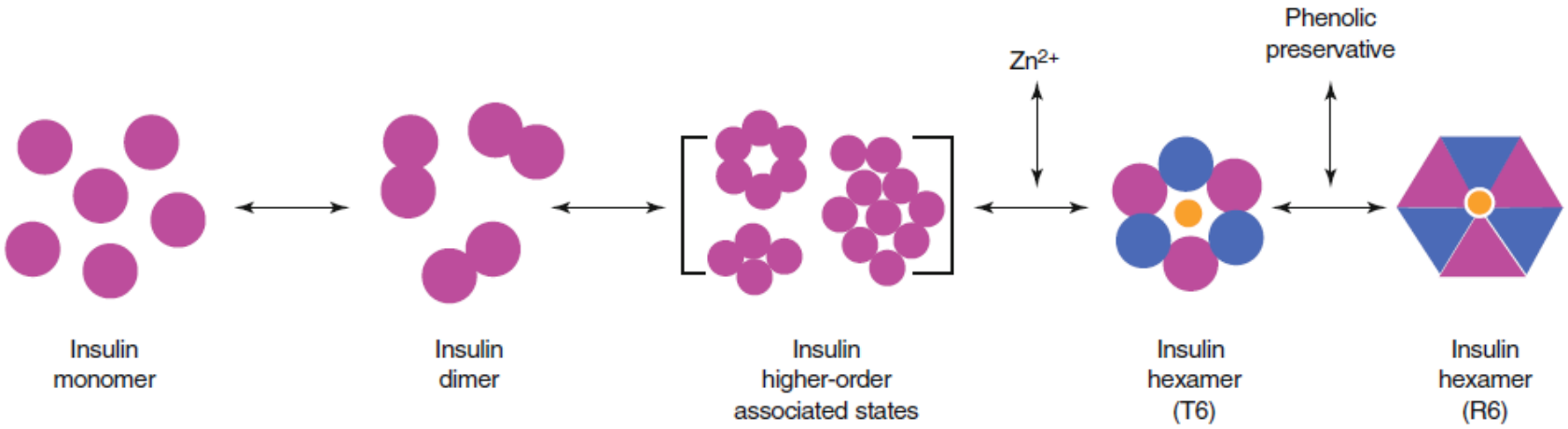


Figure 12.2 ■ Schematic representation of insulin association in the presence and absence of zinc and phenolic, antimicrobial preservatives.

- An intrinsic property of insulin is its ability to **associate into dimers** and higher-order associated states.
- The driving force for dimerization appears to be the formation of **favorable hydrophobic interactions** at the C-terminus of the B-chain
- The relative abundance of the different aggregation states **depends on** the pH, insulin concentration, ionic strength, and specific excipients (e.g., Zn^{2+} and phenol).

Importance of zinc

- Insulin can associate into **discrete hexameric complexes** in the presence of various divalent metal ions, such as **zinc** at 0.33 g-atom/monomer
- Each zinc ion (a total of two) is coordinated by a His B^{10} residue from three adjacent monomers
- Physiologically, insulin is stored as a zinc containing hexamer in the β-cells of the pancreas
- the ability to form discrete hexamers in the presence of zinc has been used to develop therapeutically useful formulations of insulin

Phenolic excipients in insulin formulation

- Commercial insulin preparations contain phenolic excipients (e.g., phenol, m-cresol, or methylparaben) as **antimicrobial agents**
- These phenolic species also bind to specific sites on insulin hexamers, causing a conformational change that **increases the chemical stability** of insulin in commercial preparations (additional advantage!)
- The phenolic ligands are stabilized in a binding pocket between monomers of adjacent dimers by **hydrogen bonds** as well as numerous **van der Waal contacts**.

Shown from X-ray crystallographic studies

Insulin aggregation and anti-adhesion agents

- The proposed mechanism for aggregation of insulin in aqueous media through contact with a hydrophobic surface (or water-air interface) is presented in this figure:

Water-air interface (hydrophobic)

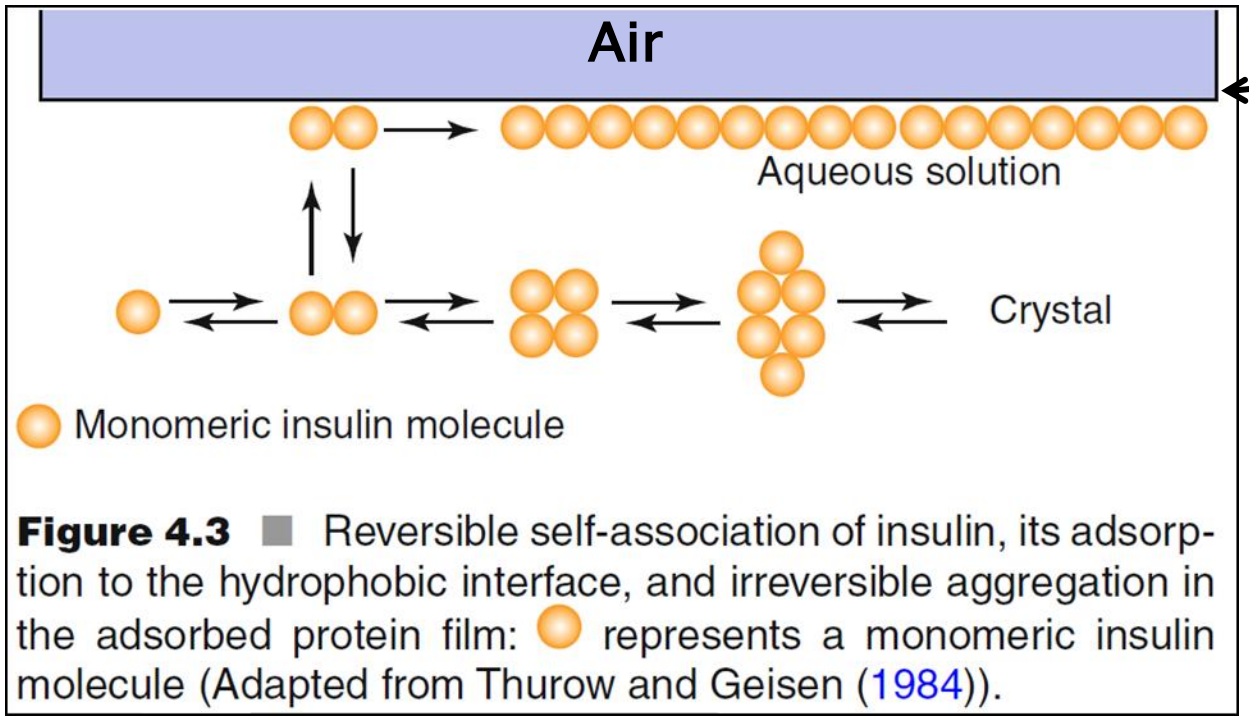


Figure 4.3 ■ Reversible self-association of insulin, its adsorption to the hydrophobic interface, and irreversible aggregation in the adsorbed protein film: ● represents a monomeric insulin molecule (Adapted from Thurow and Geisen (1984)).

Blue is usually for water!!!

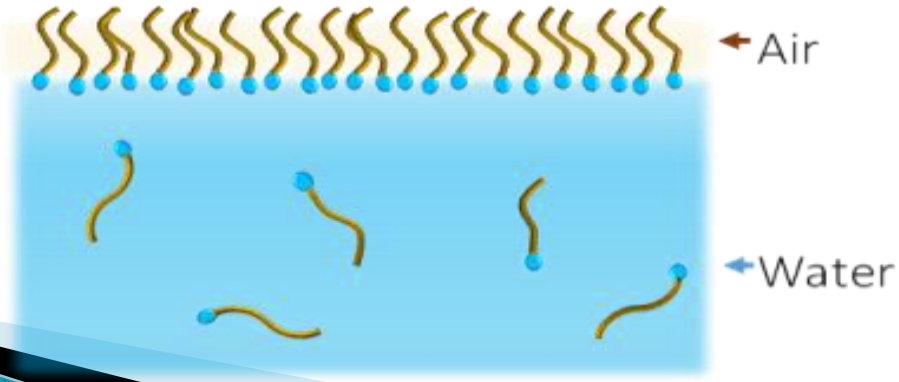
- It has been suggested that the dimeric form of insulin adsorbs to hydrophobic interfaces and subsequently forms larger aggregates at the interface. **This explains why anti- adhesion agents can also act as anti-aggregation agents.**

Notes

- Insulin can form **fibrillar precipitates** (long rod-shaped structures with diameters in the 0.1 μm range).
- Low concentrations of phospholipids and surfactants have been shown to exert a fibrillation-inhibitory effect.

Surfactants as anti-adhesion agents

- surfactants can also prevent adhesion to interfaces and precipitation.
- Mechanism: These molecules readily adsorb to hydrophobic interfaces with their own hydrophobic groups and render this interface hydrophilic by exposing their hydrophilic groups to the aqueous phase.
- Examples include polysorbate 20 and 80 (tween 20 and tween 80)



Albumin can act as anti-adhesion agent

- Albumin has a strong tendency to adsorb to surfaces and is therefor added in relatively high concentrations (e.g. 1 %) to protein formulations as an anti- adhesion agent.
- Albumin competes with the therapeutic protein for binding sites and supposedly prevents adhesion of the therapeutically active agent by a combination of its binding tendency and abundant presence.

Summary:

Low concentration of PL and surfactants and relatively high conc. of albumin can work as anti-adhasion agents and prevent protein aggregation. Also selecting the proper pH can also help to prevent this unwanted phenomenon.

Buffer components

Buffer selection is an important part of the formulation process, **because the protein solubility , physical and chemical stability** **are dependent on pH.**

Buffer systems regularly encountered in biotech formulations includes:

1. **phosphate**
2. **citrate**
3. **acetate**

Importance of protein isoelectric point (pI)

pI is the pH of a solution at which the net primary charge of a protein becomes zero.

At a solution pH that is above the pI; the surface of the protein is predominantly negatively charged and like-charged molecules will exhibit repulsive forces.

At a solution pH that is below the pI; the surface of the protein is predominantly positively charged and repulsion between proteins occurs.

At the pI the negative and positive charges cancel out; repulsive electrostatic forces are reduced and the attraction forces predominate. ***The attraction forces will cause aggregation and precipitation.***

The pI of most proteins is in the pH range of 4-6.

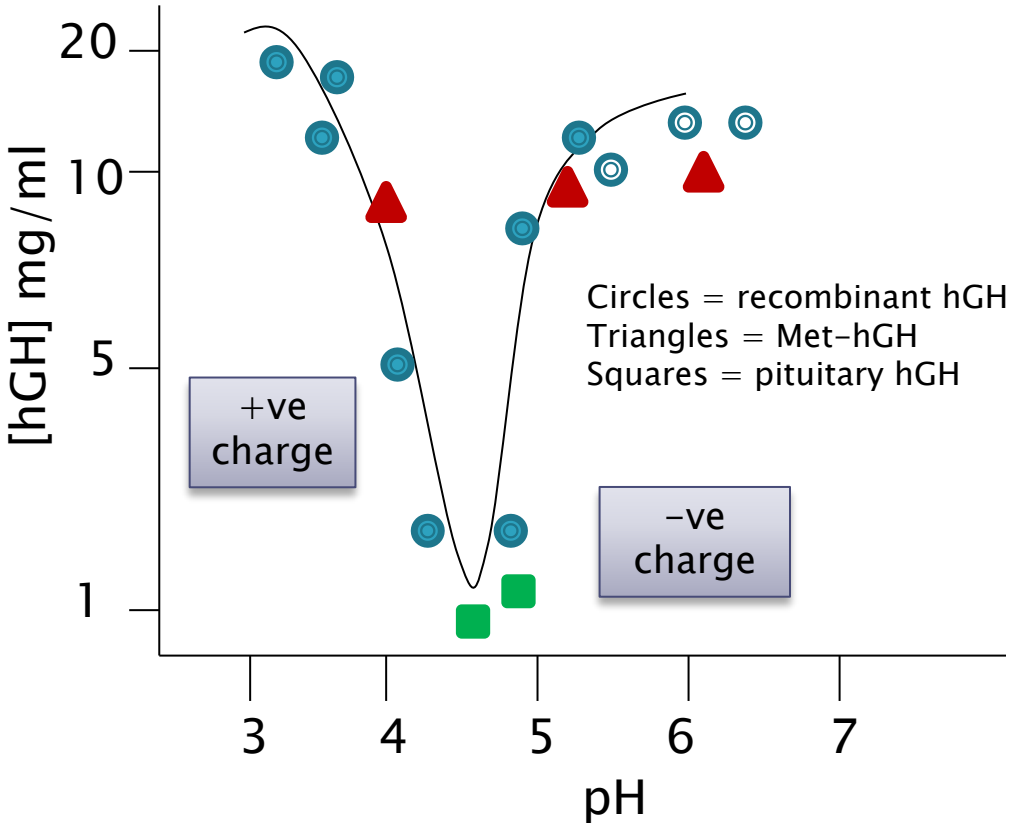


Figure 1. A plot of the solubility of various forms of hGH as a function of pH. The closed symbols mean that precipitate was present in the dialysis tube after equilibration, whereas open symbols mean that no solid material was present, and thus the solubility is at least this amount.

A good example of importance of the **isoelectric point** (its negative logarithm [pH] is equal to pI) is the **solubility profile of human growth hormone** (hGH, pI around 5) as presented in Figure 1:

pI: is the pH at a particular molecule carries no net electrical charges (overall charge).
Thus molecule is affected by pH of its surrounding environment and can become more positively or negatively charged due to the gain or loss, respectively, of (H^+).
Such molecules have minimum solubility in water or salt solutions at the pH that corresponds to their **pI** and often ppt. out of solution.

Even short, temporary pH changes can cause aggregation. Explain why?

- These conditions can occur, for example, during the freeze-drying process, when one of the buffer components is crystallizing and the other is not.
- In a phosphate buffer, Na_2HPO_4 crystallizes faster than NaH_2PO_4.

drop in pH during the freezing step.

- While other buffer components do not crystallize, but form amorphous systems and then pH changes are minimized.

Preservatives and antioxidants

Antioxidants

- **Methionine, cysteine, tryptophan, tyrosine and histidine** are amino acids that are readily oxidized.
- Proteins rich in these amino acids are susceptible to oxidative degradation.

The solution….

1. Replacement of oxygen by inert gases in the vials helps to reduce oxidative stress.
2. Addition of anti-oxidant such as ascorbic acid or sodium formaldehyde sulfoxylate can be considered.

Preservatives

- Certain **proteins** are formulated in the container **designed for multiple injection** schemes.
- After administering the first dose, contamination with microorganism may occur and the preservatives are needed to minimize growth.
- Usually, these **preservatives** are present in concentrations that are **bacteriostatic rather than bactericide** in nature.
- Antimicrobial agents mentioned in the USP XXIV are the mercury-containing **pheylmercuric nitrate, thimerosal, p-hydroxybenzoic acids, phenol, benzyl alcohol and chlorobutanol.**

Osmotic agents

- For proteins, adjusting the tonicity-of parenteral products by using (Saline and mono- or disaccharide solutions).

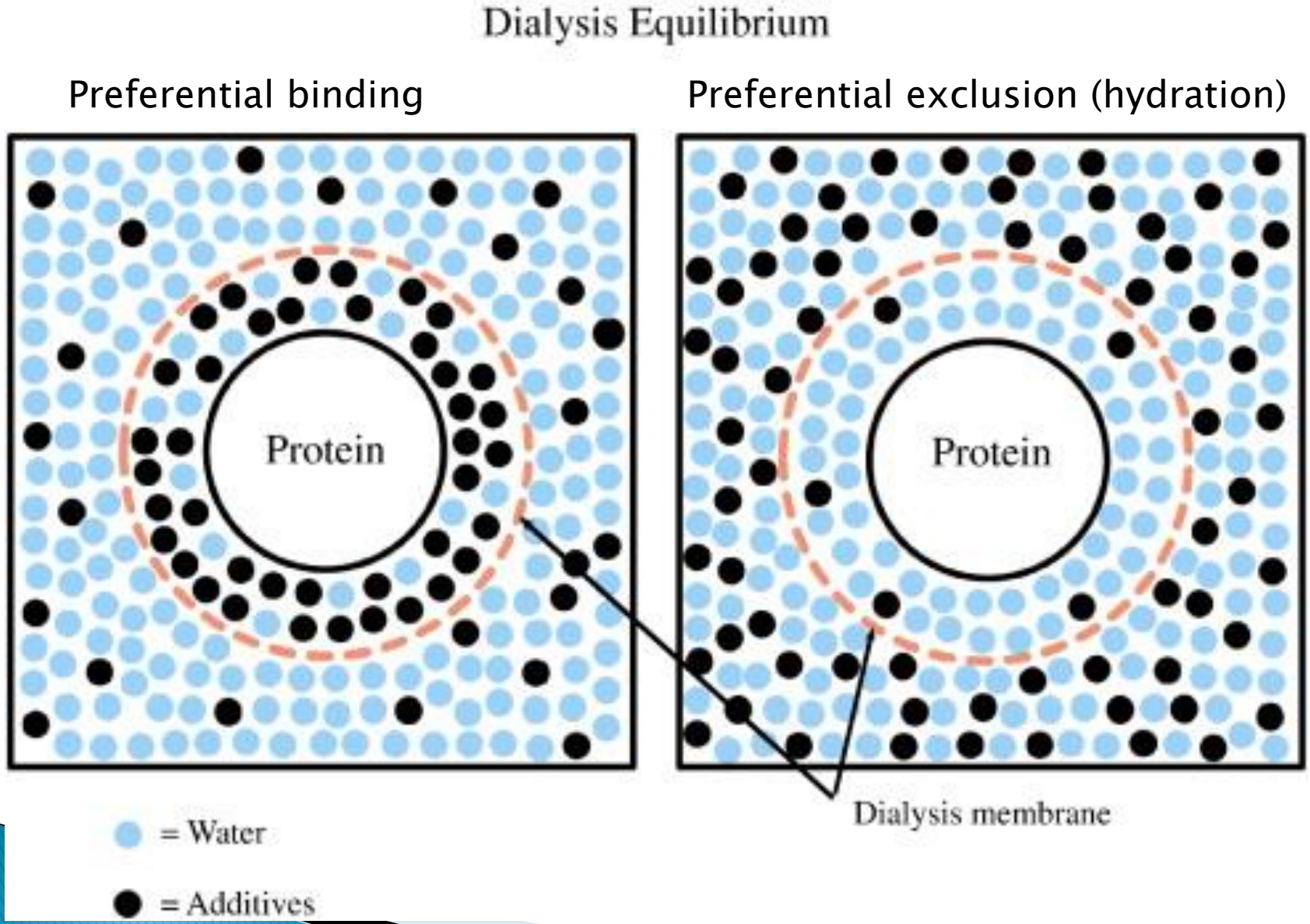
But

These excipients may not be inert; they may influence protein structural stability.

Example: sugars and polyhydric alcohol can stabilize the protein structure through the principle of preferential exclusion.

Enhance the interaction of the solvent (water structure promoters) with the protein and are themselves excluded from the protein surface layer; the protein is preferentially hydrated.

- **This phenomenon can be monitored through an increased thermal stability of the protein.**


Dialysis Equilibrium
Preferential binding
Preferential exclusion (hydration)
Protein
Protein
Dialysis membrane
= Water
= Additives