Production Considerations (I)

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Objectives of this lecture

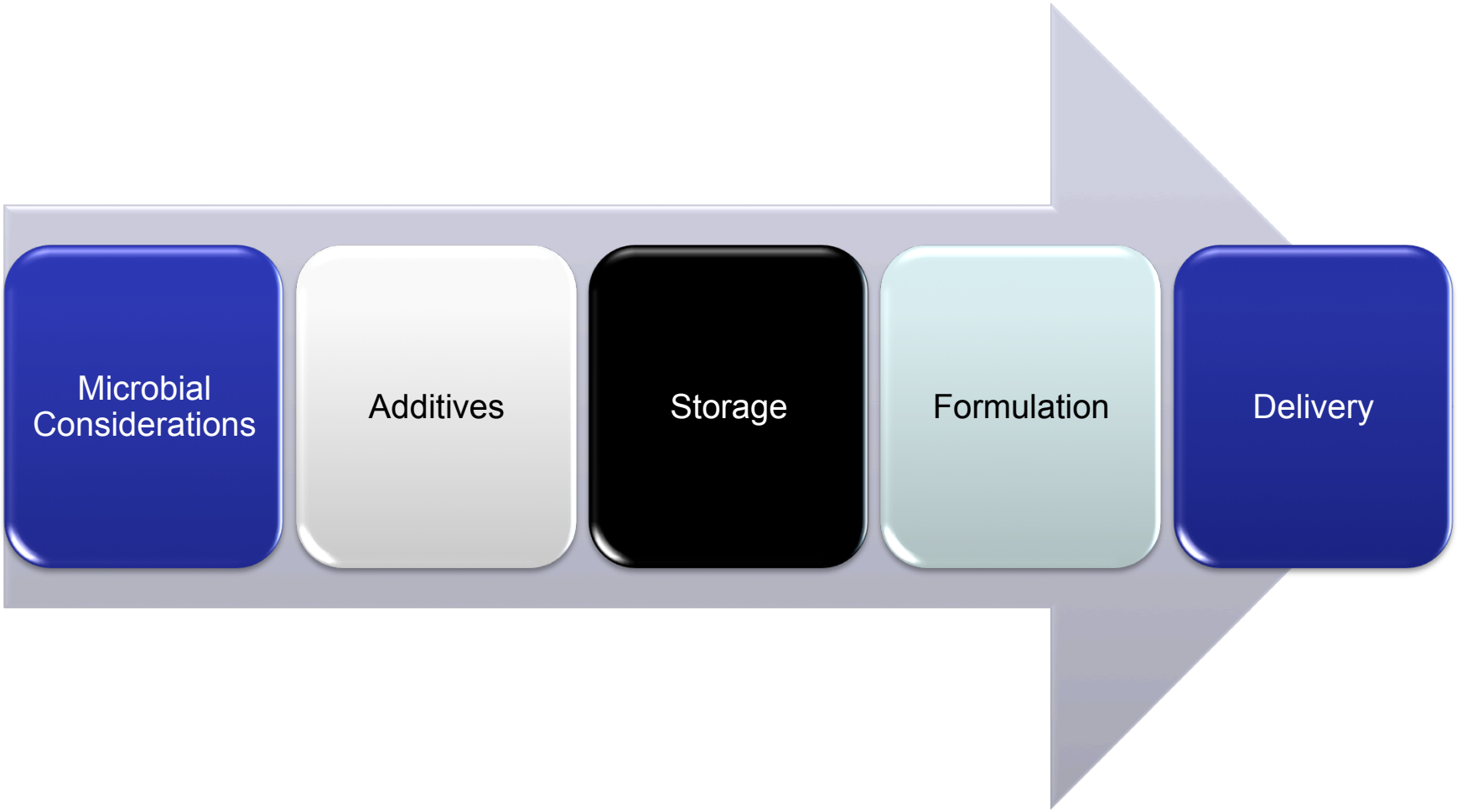
By the end of this lecture you will be able to:

1. Describe the problems associated with protein formulations
2. Numerate strategies to improve protein formulations
3. Understand the difficulty of scaling up pharmaceutical protein industry

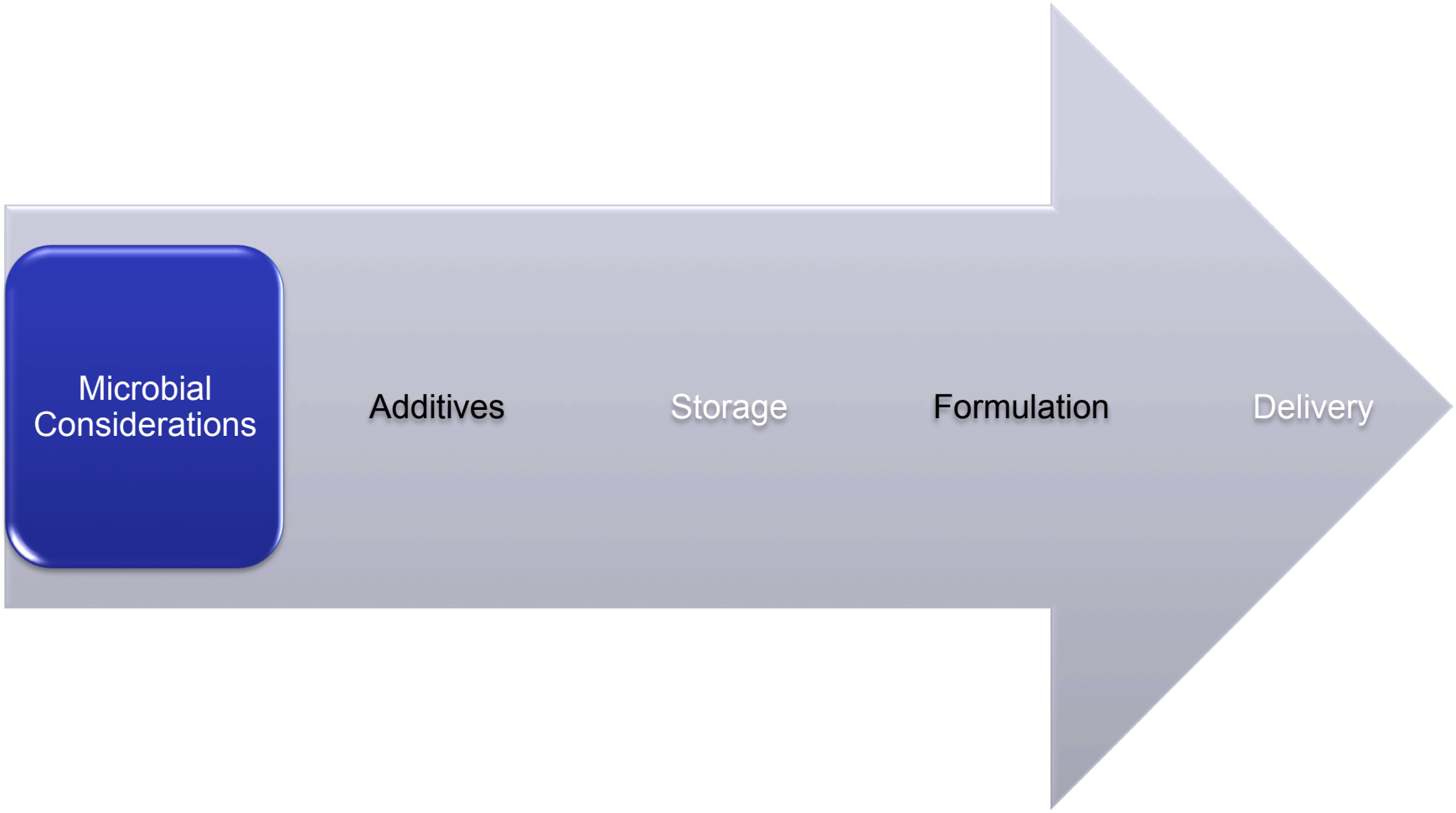
Problems with proteins

- **Large molecules**
  - Very hard to be synthesized chemically
- **Unstable:**
  - Held by weak forces
  - Easily destroyed *in vitro* and *in vivo*
- **Hard to obtain in large quantities by extraction**
  - Loss or denaturation of many proteins during the process
- **Easy to contaminate**
  - Most proteins are given parenterally
- **Difficult to formulate for large scale purposes**
  - Reproducibility is a challenge

Solving the problems



Solving the problems



Microbial consedirations

- **Sterilization:**
  - It is impossible to sterilize the end product
  - All equipments must be sterilized
  - Assembled in aseptic conditions **“BSL and Cleanroom”**

- **Quality control:**
  - Viral testing
  - Bacterial testing
  - Pyrogen testing

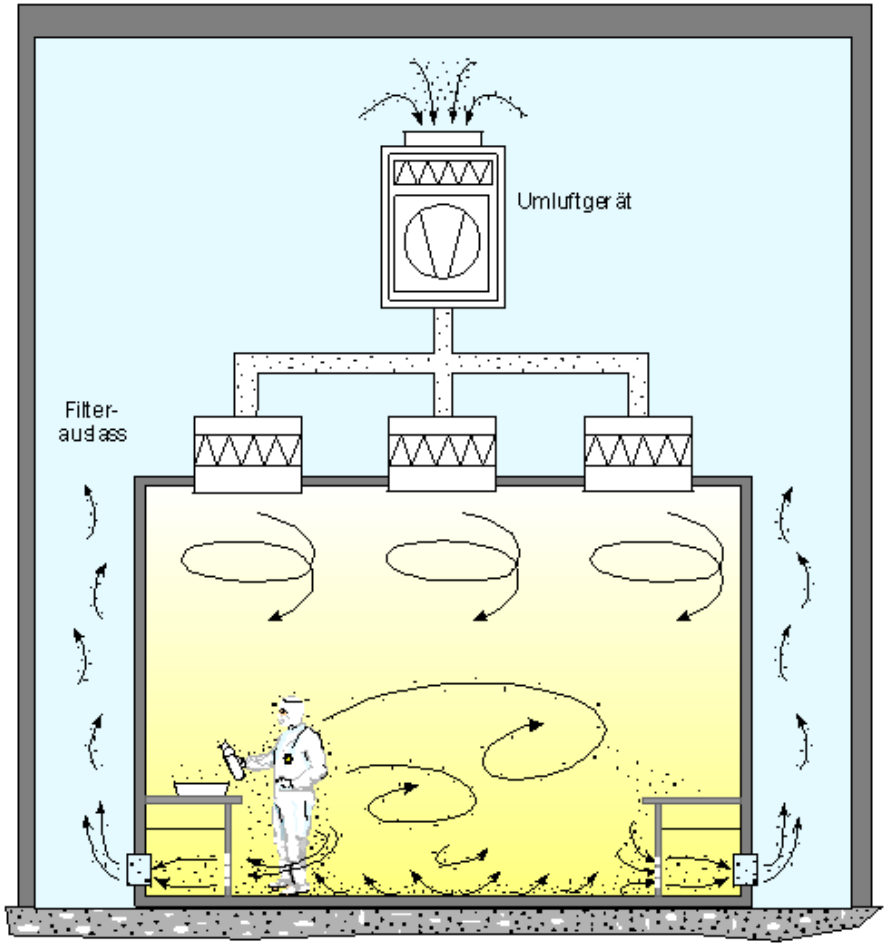
Cleanroom

- **An environment of controlled level of contamination that is specified by:**
  1. The number of particles/volume
  2. Particles size

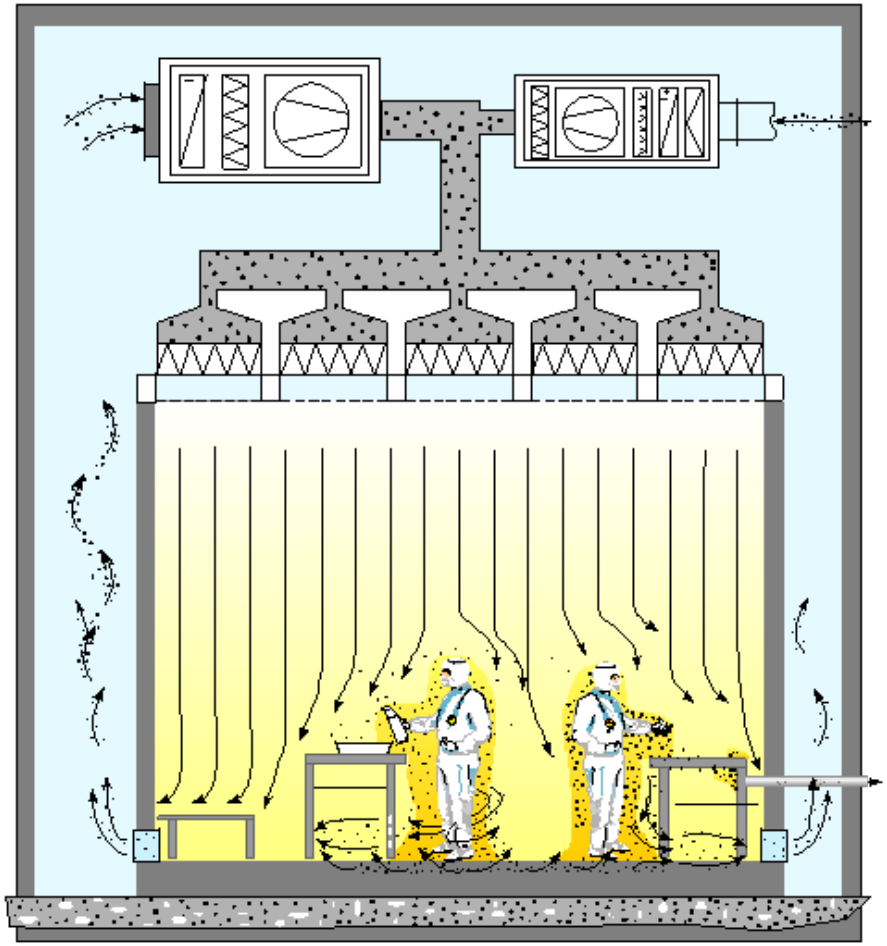
- **Air entering is HEPA filtered to exclude dust, airborne microbes, and aerosol particles**

- **Working staff must wear personal protective equipments (PPE)**

Cleanroom



Turbulent Cleanroom



Laminar Flow Cleanroom

Cleanroom

US FED STD 209E cleanroom standards

Class	Maximum Particles/ft³					ISO equivalent
	≥0.1 μm	≥0.2 μm	≥0.3 μm	≥0.5 μm	≥5 μm	
1	35	7.5	3	1	0.007	ISO 3
10	350	75	30	10	0.07	ISO 4
100	**3,500**	**750**	**300**	**100**	**0.7**	**ISO 5**
1,000	35,000	7,500	3000	1,000	7	ISO 6
10,000	350,000	75,000	30,000	10,000	70	ISO 7
100,000	3.5×10^6	750,000	300,000	100,000	700	ISO 8

In November 2001, US FED STD 209E was cancelled

Cleanroom

ISO 14644-1 cleanroom standards

Class	Maximum Particles/m³						FED STD 209E equivalent
	≥0.1 µm	≥0.2 µm	≥0.3 µm	≥0.5 µm	≥1 µm	≥5 µm	
ISO 1	10	2.37	1.02	0.35	0.083	0.0029	
ISO 2	100	23.7	10.2	3.5	0.83	0.029	
ISO 3	1,000	237	102	35	8.3	0.29	Class 1
ISO 4	10,000	2,370	1,020	352	83	2.9	Class 10
ISO 5	**100,000**	**23,700**	**10,200**	**3,520**	**832**	**29**	**Class 100**
ISO 6	1.0×10^{6}	237,000	102,000	35,200	8,320	293	Class 1,000
ISO 7	1.0×10^{7}	2.37×10^{6}	1,020,000	352,000	83,200	2,930	Class 10,000
ISO 8	1.0×10^{8}	2.37×10^{7}	1.02×10^{7}	3,520,000	832,000	29,300	Class 100,000
ISO 9	1.0×10^{9}	2.37×10^{8}	1.02×10^{8}	35,200,000	8,320,000	293,000	Room air

Viral Decontamination

- **There is no well-determined mean to detect viruses in the cell culture**

- Each lab has a level of biocontaminents AKA Biosafety Level (BSL):
  1. **BSL1:**Well-characterized agents not know to cause disease to a healthy adult human being
  2. **BSL2:** BSL1+ agents of moderate potential hazard to personnel and environment (e.g. HBV and Salmonella)
  3. **BSL3:** Agents which may cause serious or potentially lethal disease after inhalation but to which treatment is available (e.g. TB, Anthrax, and SARS)
  4. **BSL4:** High individual risk of aerosol-transmitted lab infection that cause severe or fatal diseases to which no treatment or vaccine is available (e.g. Ebola and Marburg)

Viral Decontamination

- **Viral contamination can be from the host cell line or nutrients present in the growth media (e.g. FCS)**

Category	Type	Example
Inactivation	Heat	Pasteurization
	Radiation	UV-light
	Dehydration	Lyophilization
	Cross linking	Formaldehyde
	Neutralization	Antibodies
Removal	Chromatography	Affinity chromatography
	Filtration	Ultrafiltration
	Precipitation	Cryoprecipitation

but these processes may be harmful to the product

Bacterial Decontamination

- **Filtration sterilization of the final product by bacterial filter "0.22 μm membrane filter"**
- **Antibiotics must be added to the cell culture to inhibit bacterial contamination**
  - *What if the expression system is bacterial?*
- **Complete removal of antibiotic residues from the final product is very difficult**

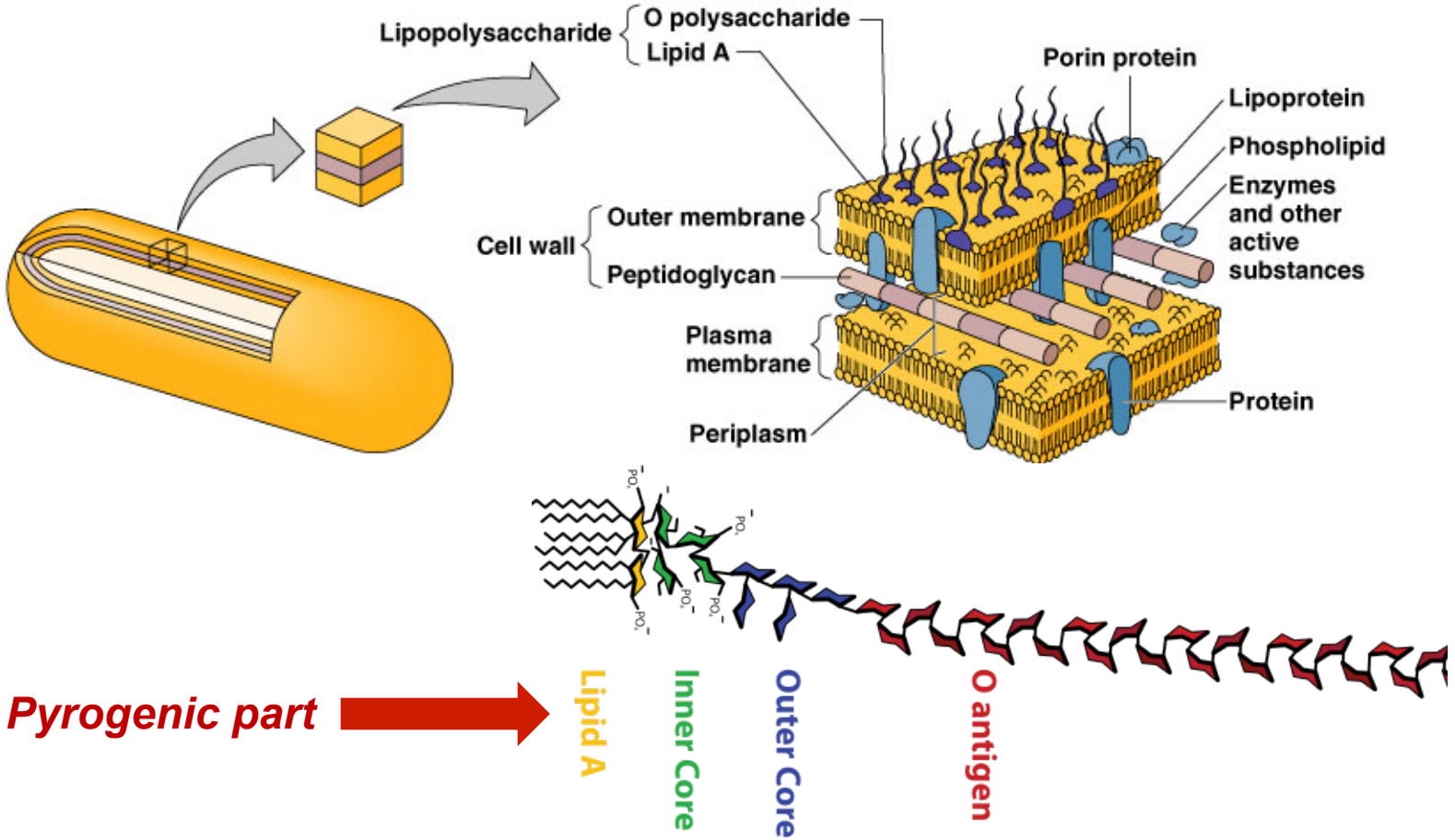
Pyrogens

- **The process of pyrogen removal AKA depyrogenation refers to the removal of pyrogens such a *"endotoxins"* from solutions.**

Property	Exotoxin	Endotoxin
Chemistry	Secreted proteins	Shed lipopolysaccharide
Source	Gram (+ve) or Gram (-ve) bacteria	Gram (-ve) bacteria
Symptoms	Specific action on target tissue	Fever, diarrhea, vomiting, shock
Toxicity	High / Fatal	Weak / Rarely fatal
Immunogenicity	Causes neutralizing Ab production	Insufficient Ab production
Toxoid potential	After formaldehyde treatment	None
Fever potential	Rarely	Pyrogenic

Pyrogens

- **Lipopolysaccharide is a component of Gr (-ve) bacteria cell wall**



Pyrogen Testing

- **Rabbit Test:**
  - Rabbits have similar endotoxin tolerance to humans
  - Costly method and time consuming
  - Inability to quantify the endotoxin level

- **LAL Test:**
  - Limulus Amebocyte Lysate (LAL) test
  - FDA-approved for *in vitro* pyrogen testing
  - High sensitivity 0.005 EU/mL
  - Only detects LPS
  - Gives false positives with Glucans

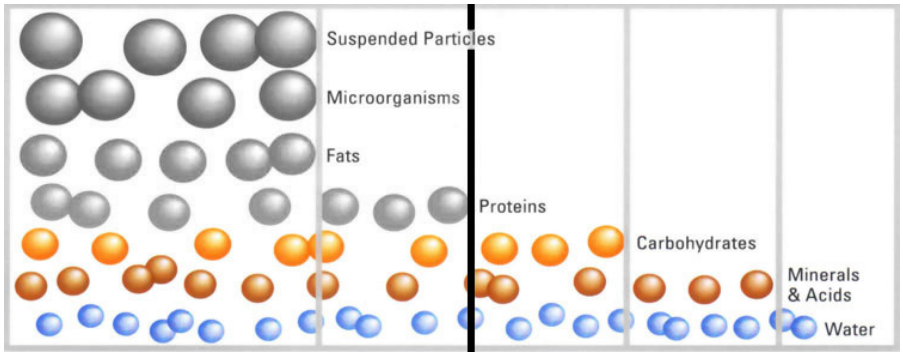
Pyrogen Removal

- **Simple filtration sterilization and standard autoclaving conditions do not remove pyrogens**
- **Dry heat for 30 min at 250 ^{o}C would breakdown the endotoxin**
- **All equipments used in the production process must be endotoxin free**
- **The FDA's maximum permissible endotoxin limit is *5 EU/kg/hr***
- **Intrathecal endotoxin limit is *0.2 EU/kg/hr***
- **Sterile water for injection is allowed to contain *0.25-0.5 EU/mL***

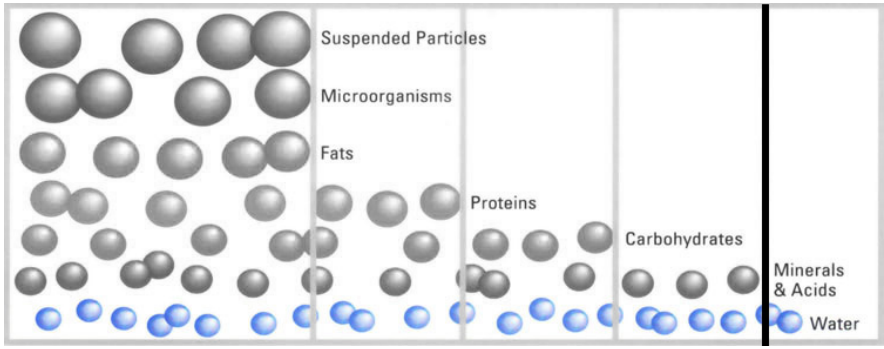
Pyrogen Removal

- **Ion Exchange Chromatography:**
  - LPS is highly negative
  - Anion exchanger

- **Ultrafiltration and Reverse Osmosis:**
  - LPS has high molecular weight >10kDa



Ultrafiltration



Reverse Osmosis

Pyrogen Inactivation

- **Hydrolysis** in order to cleave Lipid A from the polysaccharide component, **Oxidation** using hydrogen peroxide, and **Heating** at 250 ^{o}C for 30 minutes are commonly used methods inactivate endotoxin on solid surfaces. ***However, these methods would harm the therapeutic protein***. Therefore, it is important to work with sterile endotoxin-free equipments under aseptic condition.

Cellular DNA

- Mammalian expression systems are immortalized cell lines by stable oncogene transfection
- Recombinant products may get contaminated with oncogen-bearing DNA fragments in the final product
- Purification process **MUST** remove cellular DNA and RNA
- DNA concentration in the final product should not exceed **10 pg/dose**

Protein Contaminants

- **Source:**
  1. Growth media (FBS)
  2. Host cells
  3. Ligands from affinity chromatography columns

- Host version of the protein can be co-purified with the protein of interest

- Large-scale production prefers the use of **serum-free media** (e.g. in mAbs production) but this causes insufficient growth and lower yield of production

- Foreign protein contaminants can be hazardous and immunogenic. If not purified, they lead to miss-interpretation of the produced protein's immunogenicity profile

You are now able to:

- ✓ Describe the problems associated with protein formulations
- ✓ Numerate strategies to improve protein formulations
- ✓ Understand the difficulty of scaling up pharmaceutical protein industry