Discovery Research - Preclinical Research

Models in biological research.

- Experimental design (Some ideas for planning?)

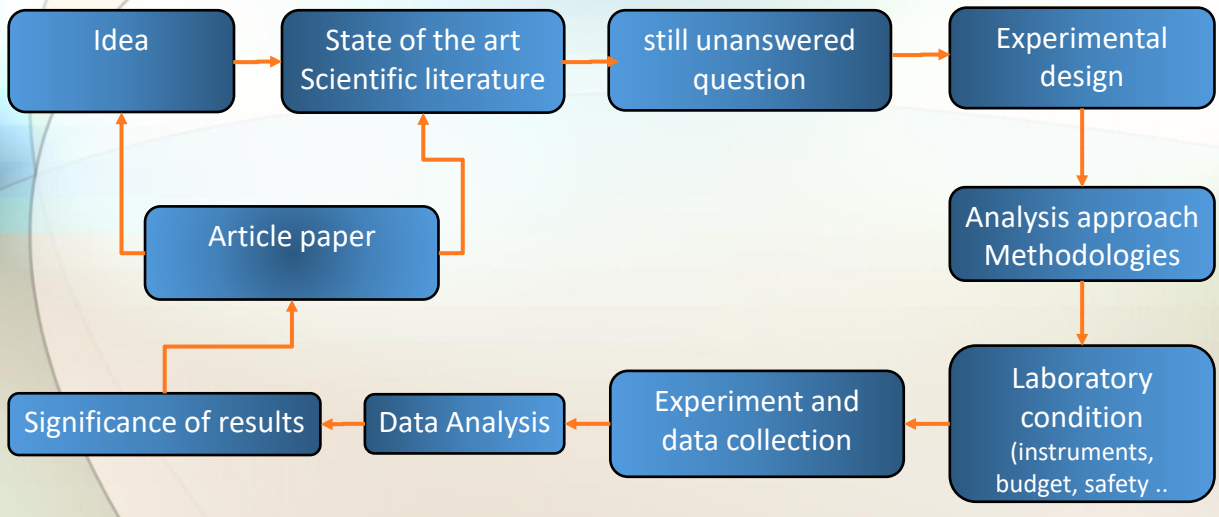
→Evaluation of Scientific Literature

→Scientific Databases

→Specific Techniques

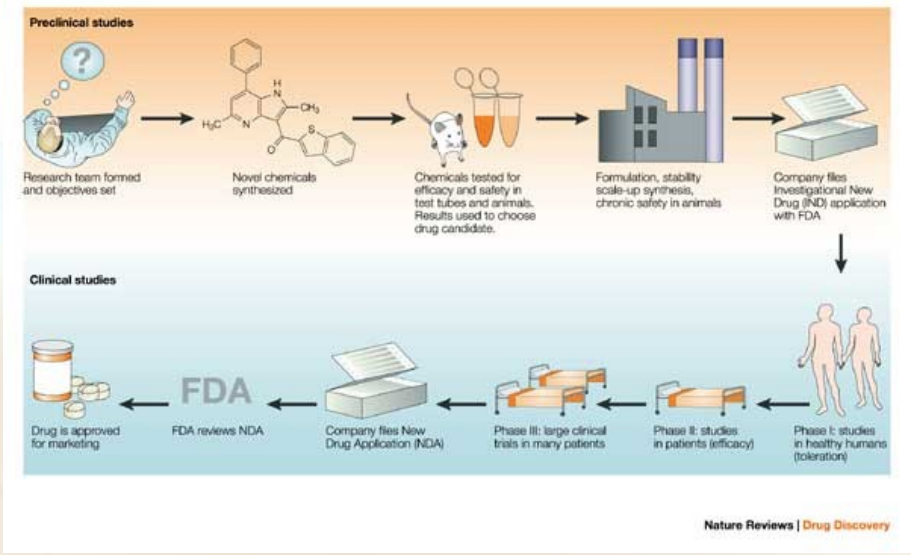
1

Work-flow



2

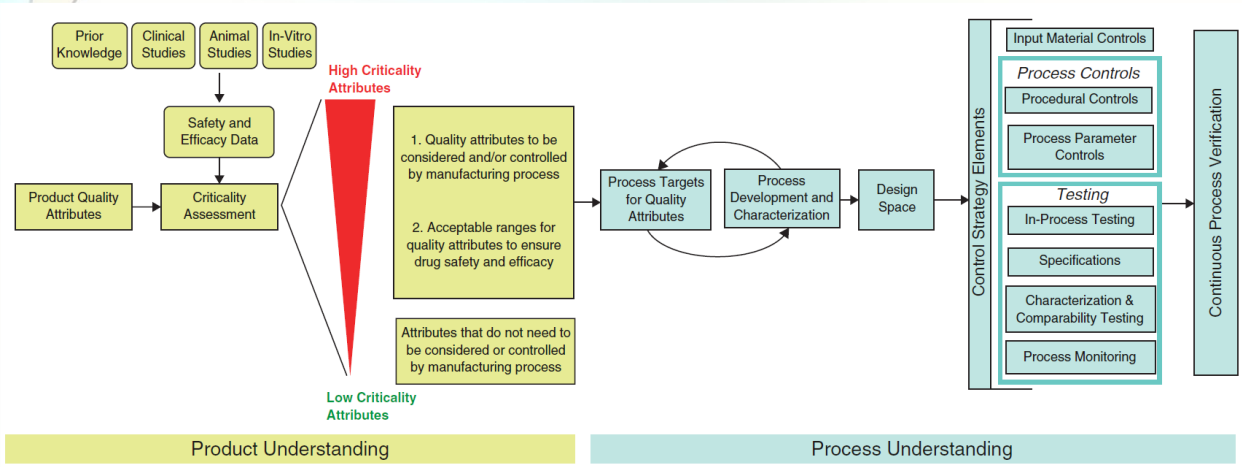
Pharmaceutical Biotechnologies



3

Sequence of activities

(starts with the design of the molecule → development process → → final process and control strategy used for commercial-scale manufacturing)



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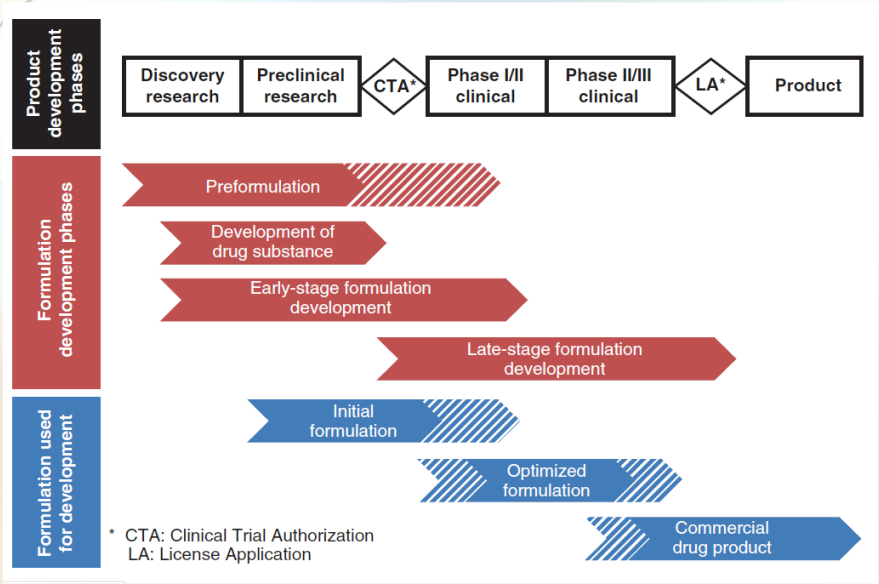
4

Pharmaceutical Biotechnologies



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5

POINTS TO CONSIDER IN THE PROCESS OF FORMULATING A THERAPEUTIC PROTEIN

- Formulating a protein is not a one-step or fixed strategy
  - starts with pre-formulation activities
  - ends up with optimized product composition dosage form.
- An early and deep understanding of the structural properties of the protein --> will speed up the formulation process

6

Points for consideration in the formulation process of pharmaceutical proteins

Factor	Description/attributes/examples
API (active pharmaceutical Ingredient) or DS (drug substance)	Type of protein, **physico-chemical properties**, e.g., molecular weight, pI, hydrophobicity, solubility, post- translational modifications, pegylation, **physical and chemical stability and concentration, available amount**, purity
Clinical factors	Patient **population** (e.g., age and concomitant medication), self- **administration** versus administration by professional, compatibility with an infusion solution, indication (e.g., one-time application or chronical application)
Route of administration	Subcutaneous, intravenous injection or infusion, intramuscular, intravitreal, intra-articular, intradermal, pulmonal
Dosage form	**Single- or multi-dose**, prefilled syringe, dual chamber cartridge, pen cartridge; liquid, lyophilized, frozen liquid, API concentration, injection volume, injection rate, controlled delivery/release
Primary packaging material	Glass, polymers, rubber, silicone oil, metals, leachables (anti-oxidants, plasticizers, etc.)
Excipients	**Pharmaceutical quality**, safety record (for intended administration route and dose), manufacturer, tested for critical impurities, stability
Analytical methods	Characterization of API, stability-indicating assays, **quality control assays**

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7

Chemical and physical reactions protein stability

Proteins are unstable

Chemical instability	Physical instability
- Deamidation	- Conformational: Unfolding / Misfolding
- Oxidation	- Colloidal: Aggregation / Precipitation
- Proteolysis (hydrolysis)	- Adsorption
- Disulfide shuffling	
- Racemization	
- Beta elimination	
- ...	

Each degradation reaction can induce another one
Multiple degradation processes occur at different rates, yielding numerous degradation products

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8

POINTS TO CONSIDER IN THE PROCESS OF FORMULATING A THERAPEUTIC PROTEIN

In an early stage, data on:

- The physical stability (e.g. colloidal and conformational stability)
- The chemical stability of the API and formulations are collected

9

POINTS TO CONSIDER IN THE PROCESS OF FORMULATING A THERAPEUTIC PROTEIN

- This basic information helps to design a product that is stable under real-life conditions
  - Transportation
  - Compounding (e.g., dilution in an intravenous infusion bag)
  - Administration
- A product can encounter various stress factors

10

Stress factors a therapeutic protein may encounter

Stress factor	When encountered/examples
Elevated temperature, temperature excursions	Production (upstream and downstream processing); improper shipment; storage or handling deviations
Freezing, freeze-thawing	Storage of frozen (bulk) material; accidental freezing during storage or shipment; lyophilization
Mechanical stress	Production (e.g., pumping, stirring, filtration)
Light	Production; shipment; storage; handling
Oxidative stress	Production (exposure to oxygen); exposure to peroxide or metal ion impurities in excipients; shipment (cavitation)
pH changes	Production (downstream processing); freezing; formulation; dilution in infusion liquids; administration
Interfaces	Air-water interface; filters; primary packaging material; infusion bags and administration lines; particulate impurities
X-ray	Air freight transportation

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11

Analytical Toolbox

- An “analytical toolbox’ is necessary

→ to characterize a protein in various stages of formulation development in as much detail as possible

- Orthogonal analytical techniques

and

- Complementary analytical techniques

12

Orthogonal and Complementary analytical techniques

Orthogonal analytical techniques

- Techniques that monitor the same (or similar) properties of a protein (in its formulation) with a different measurement principle

Complementary analytical techniques

- Techniques that measure different properties of a protein (in its formulation) with a different measurement principle

13

Analytical techniques

Monitoring and controlling aggregate formation is particularly important because **protein aggregates** are readily formed under a variety of conditions → **associated with enhanced risk of immunogenicity**

Type of degradation product	Examples of analytical techniques
Soluble aggregates (dimers, trimers, oligomers) and fragments	Size-exclusion HPLC/UPLC, AF4, analytical ultracentrifugation, SDS-PAGE, CE-SDS
Nanometer-sized aggregates	Dynamic light scattering; nanoparticle tracking analysis; AF4; Taylor dispersion analysis; turbidimetry/nephelometry; static light scattering
Micrometer-sized aggregates	Light obscuration; light microscopy; flow imaging microscopy; coulter counter; fluorescence microscopy; turbidimetry/nephelometry; Raman microscopy
Visible particles	Visual inspection; (semi-)automated visual inspection
Conformational changes	Conformational changes Circular dichroism, infrared, intrinsic fluorescence, extrinsic fluorescence spectroscopy andsecondary- derivative UV spectroscopy
Chemical changes	Reversed-phase HPLC/UPLC; (HPLC-)mass spectrometry; ion-exchange chromatography;(capillary) isoelectric focusing

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14

Examples of accelerated stability and forced degradation studies

Aggregation of proteins can happen at:
- concentrations much below their solubility
- temperatures far below their denaturation temperature.

Proteins in solution have an increased tendency to aggregate
→upon mechanical stress and interaction with interfaces.

However, a shelf life of at least 18–24 months for the drug product in its final primary packaging container (e.g., vial, syringe, cartridge pen, autoinjector) **is desired.**

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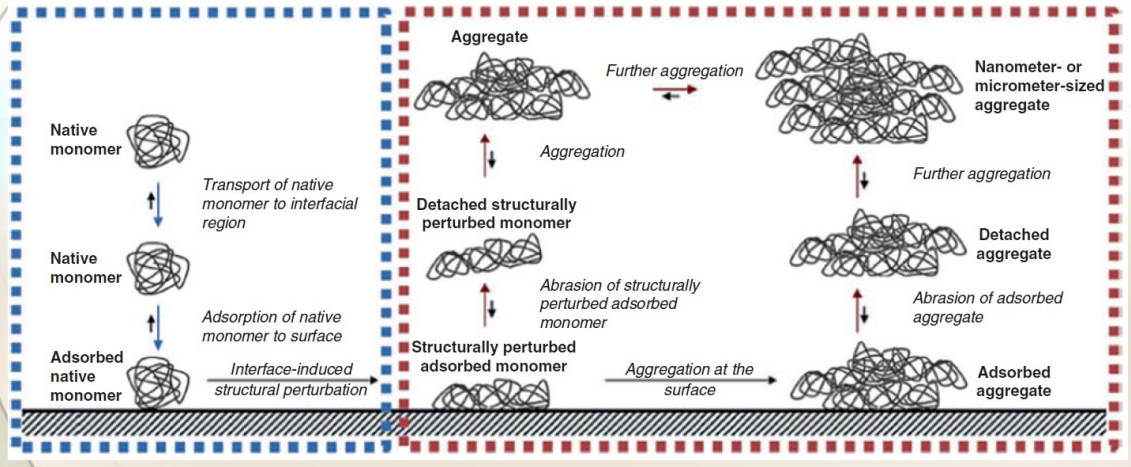
15

Suggested mechanism of stirring-induced protein aggregation.



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16

Lists of a typical set of stress test conditions that are used in practice.

Type of stress	Examples of stress conditions	Anticipated instability types
Temperature	Real time (2–8 °C; up to several years) Accelerated (e.g., 25 °C, 40 °C, up to several months)	Aggregation, conformational changes, chemical changes
Mechanical	Shaking (50–500 rpm, hours-days) Stirring (50–500 rpm, hours-days) Freeze-thawing, (1–5 cycles, from 25 °C to −20 or −80 °C)	Aggregation, adsorption, conformational changes
Oxidation	H_2O_2 (1–5%, 1–2 days)	Chemical changes, aggregation, conformational changes
Humidity (lyophilized products)	0–100% relative humidity	Aggregation, conformational changes, chemical changes

NB: Aggregation of proteins can happen!!

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17

Excipients (selected to serve different purposes)

Excipient	class Function	Examples
Buffers	pH control, tonicity	Histidine, phosphate, acetate, citrate, succinate
Salts	Tonicity, stabilization, viscosity reduction	Sodium chloride
Sugars[a], polyols	Tonicity, stabilization, cryoprotection, lyoprotection[b], bulking agent[b], reconstitution improvement[b]	Sucrose, trehalose, mannitol, sorbitol
Surfactants	**Adsorption prevention**, solubilization, stabilization, reconstitution improvement[b]	Polysorbate 20, polysorbate 80, poloxamer 188
Amino acids	Stabilization, viscosity reduction, tonicity, pH control, bulking agent[b]	Arginine, glycine, histidine, lysine, proline
Anti-oxidants	Oxidation prevention	Methionine, sodium edetate
Preservatives[c]	Bacterial growth prevention	m-cresol, benzyl alcohol, phenol

a: Only non-reducing sugars
b: For freeze-dried products
c: Multi-dose containers

E.G.: different selection of excipients
- an intravenously administered product (**hospital setting**) versus a subcutaneously administered product (**self-administration**).
- the choice of the excipient and its concentration are important.

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18

Solubility Enhancement

- Selection of the proper pH
- Addition of amino acids or surfactants

The mechanism of action of **these solubility enhancers depends** on the type of enhancer and the protein involved and **is not always fully understood**.

19

Protection Against Adsorption, Interfacial Stress and Aggregation in the Bulk Solution

Many protein **formulations include a surfactant** to reduce protein **adsorption**.

- **Surfactants readily adsorb to hydrophobic interfaces** with their own hydrophobic groups
  → exposing their hydrophilic groups to the aqueous phase.
- **Protein accumulation** at the interface **is suppressed** and **thereby aggregate** formation.

E.G. the 2-hydroxypropyl-beta- cyclodextrin can prevent adsorption and is accepted as an excipient for parenteral use as well.

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20

Protection Against Adsorption, Interfacial Stress and Aggregation in the Bulk Solution

Aggregates may be **formed in the bulk**
→ because of **colloidal and/or conformational instability**

- **Sugars and polyhydric alcohols:**

→ enhance the interaction of the solvent with the protein

→ are themselves excluded from the protein surface layer;

→ the protein is preferentially hydrated.

→ increasing conformational stability of the protein

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21

Protection Against Adsorption, Interfacial Stress and Aggregation in the Bulk Solution

Aggregates may be **formed in the bulk** of a solution
→ because of **colloidal and/or conformational instability**

NOTE:
Sugars, selection of a proper pH value and buffer components **may mitigate the tendency** to this bulk aggregation.

→ ***Sucrose*** *should* ***not be used below pH 6*** *because of hydrolysis, leading to the* ***formation of fructose and glucose****, both being* ***reducing sugars****.*

→ ***Primary amino groups*** *of the proteins* ***react*** *with the* ***reducing sugar****, resulting in brownish/yellow solutions. (Maillard reaction)*

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22

Buffer Selection

The pH and the buffer species can **have profound effects** on the **physical (aggregation)** and **chemical stability of proteins**

- **Buffer systems** in protein formulations are often:
  phosphate, citrate, histidine, succinate, glutamate and acetate

→ *Even short, temporary pH changes can cause protein aggregation.*

For example, during the ***elution of a monoclonal antibody from a protein A column at low pH.***

→ ***In the presence of high concentrations of sugar*** *(typically added as lyo- and cryoprotectant during lyophilization),* ***the effect of pH changes is less pronounced***.

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23

Protection Against Oxidation

Oxidative degradation is a regular threat to the stability of proteins.

→ ***Replacement of oxygen by inert gases*** in the vials or minimizing the headspace, such as in pre-filled syringes, helps reducing oxidative stress.

→ ***addition of antioxidants***, such as methionine, which competes with methionine residues for oxidation

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24

Preservation – Protection Against Freezing and Drying

Formulations in multi-dose containers must contain a **preservative** as **antimicrobial agents**.

→ *NOTE: These preservative molecules can interact with the protein, which may compromise both the activity of the protein*

Cryoprotectants are excipients that protect a protein during freezing or in the frozen state → enhancing the interaction of the solvent (water) with the protein and are themselves excluded from the protein surface layer.

Lyoprotectants protect the protein in the lyophilized state
(1) the '**water replacement theory**': replacement of water by forming hydrogen bonds with the protein
(2) the '**vitrification theory**': formation of a glassy amorphous matrix keeping protein molecules separated from each other.

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25

Remarks

To **formulate a protein API successfully** and turn it into a medicinal product

- **In-depth understanding of the chemical and physical characteristics** of the molecule in question, including its stability under the preferred storage conditions.
- A set of stability-indicating, **complementary and orthogonal analytical techniques should be available** to help in successfully selecting the route of administration, the proper excipients, and the packaging material for a stable product (freeze-dried or not).
- To date the **parenteral route is the only one that allows us to administer protein-based medicines for systemic delivery** to the patient.

26