Optimizing therapeutic proteins through PEGylation: key parameters and impacts

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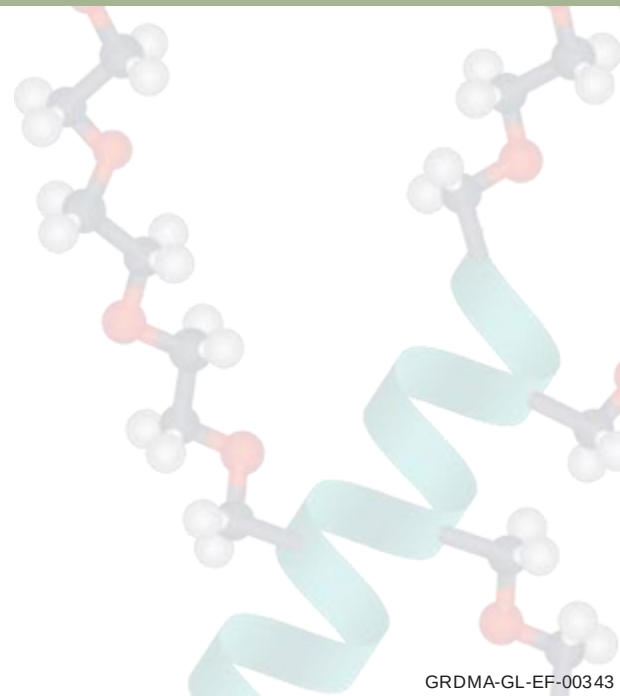

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Disclaimer

This educational webinar is based on the presenters' manuscript titled "Optimizing Pharmacological and Immunological Properties of Therapeutic Proteins Through PEGylation: Investigating Key Parameters and Their Impact", published in *Drug Design, Development and Therapy*.

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Disclosures

João Gonçalves

- Astra-Zeneca, Celltrion, Chiesi, Lundbeck, LxBio, Pfizer, Sanofi, TechnoPhage

Paolo Caliceti

- Contracts with: BrYet, Bio-Ker, Chiesi, Keryos, Laboratori Farmaceutici KRYMI, ITN – European Union, Lunex University / Cognos International, Nuova Ompi, PharmEste, Schering Plough, Unifarco

Q&A session

You can ask questions at any time during this presentation

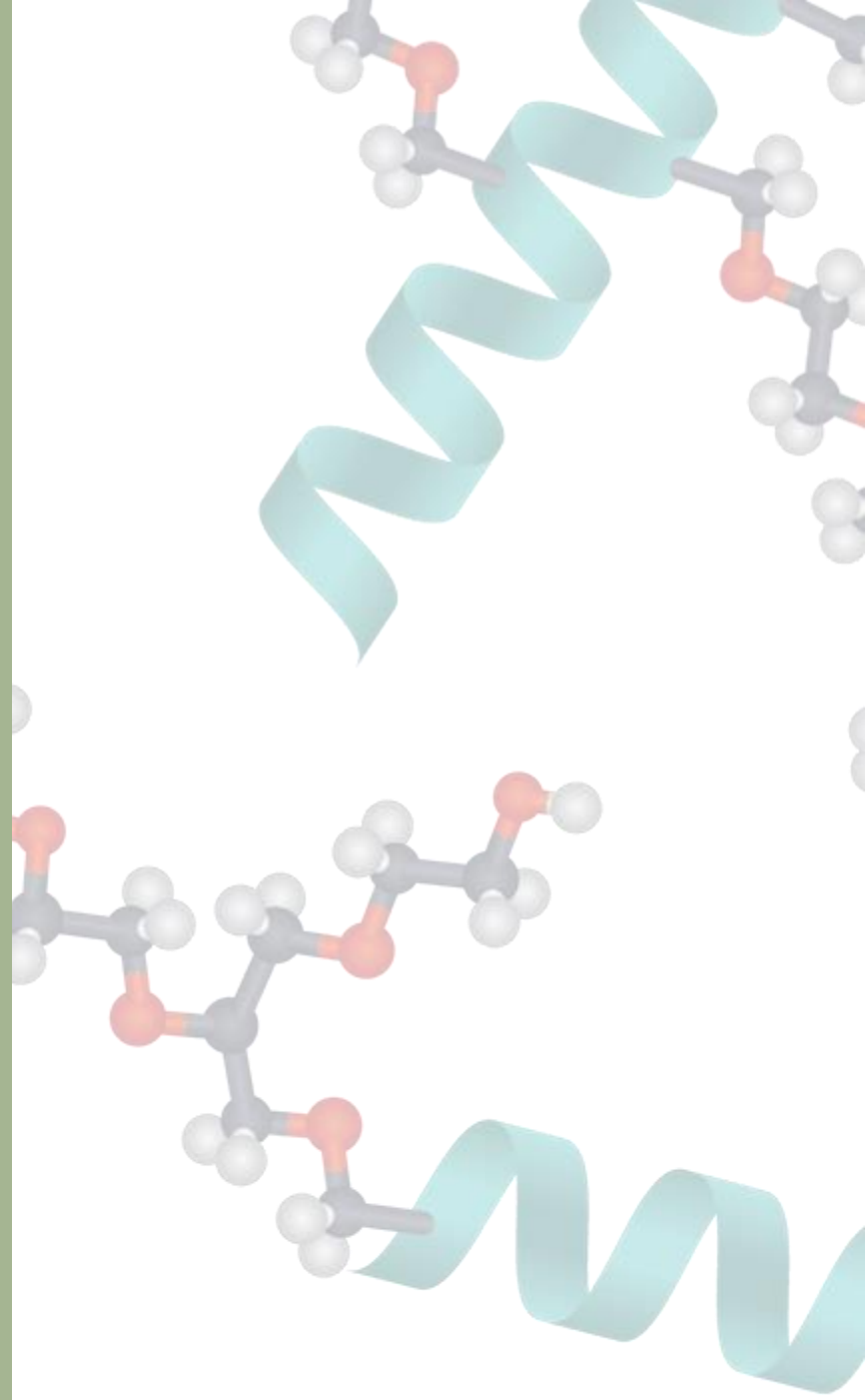
To ask a question, please click on the button below

Audience questions will be answered during a separate follow-up video

What is PEGylation and why is it important?

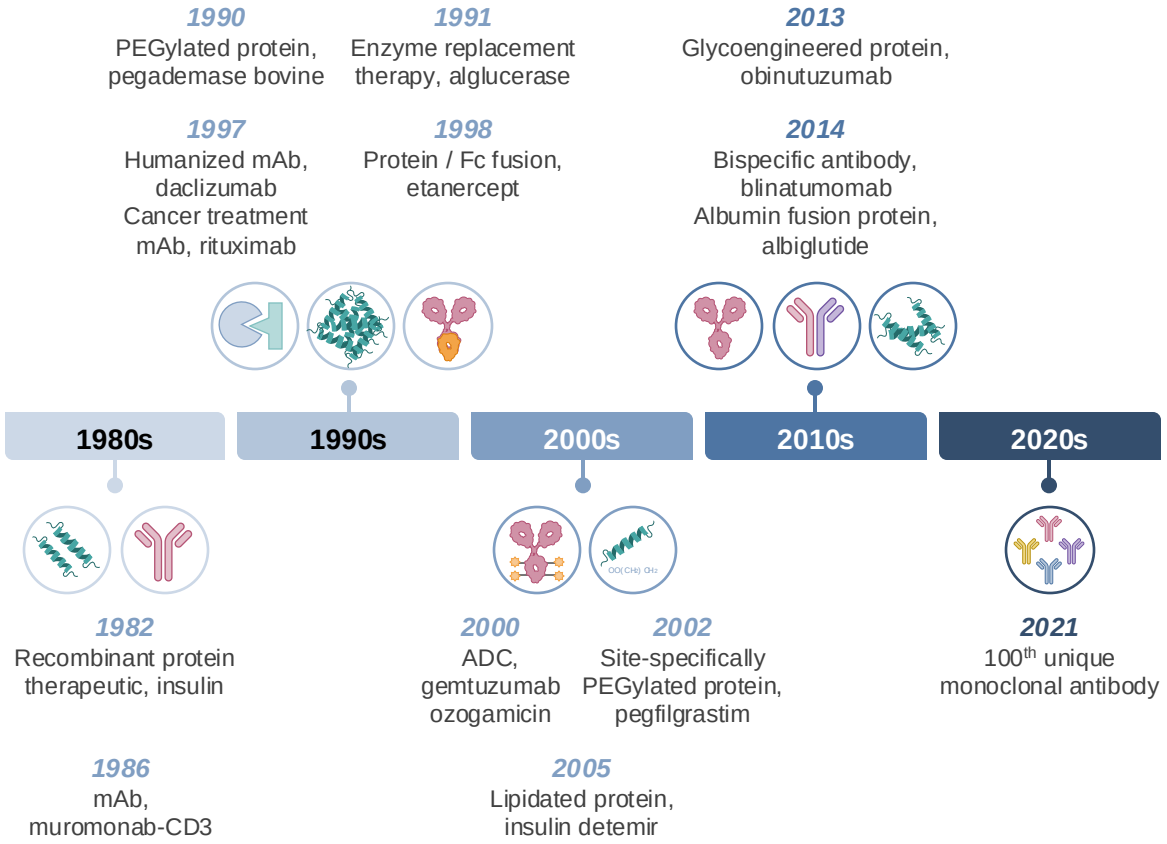
João Gonçalves

To ask a question, please click on the button below



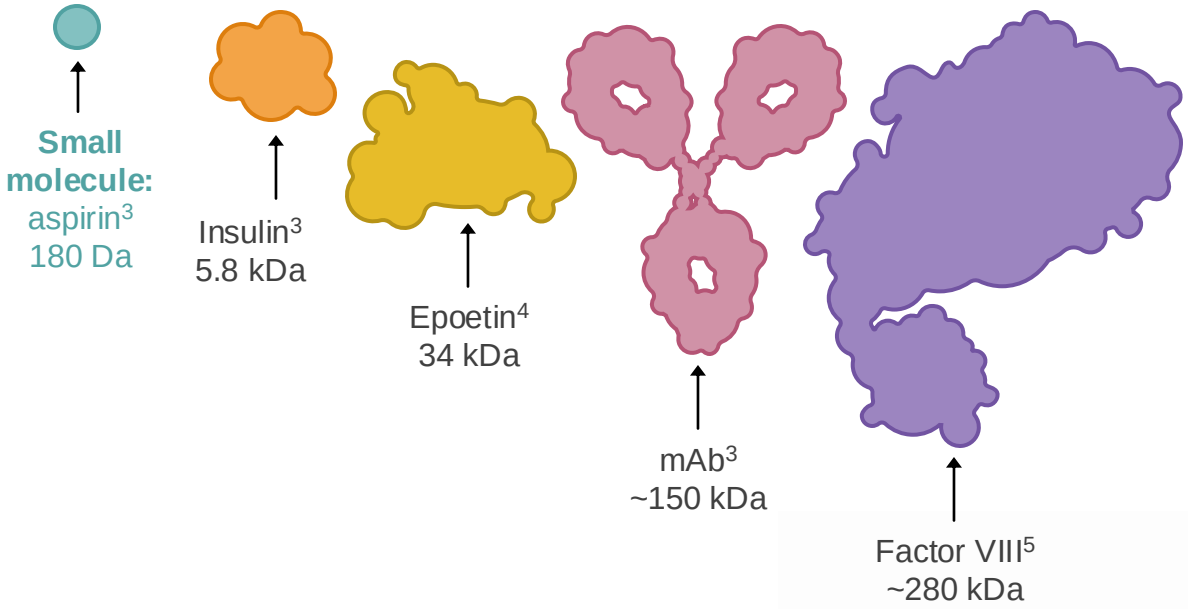
Therapeutic proteins are widely used in medicine

First FDA approvals of therapeutic proteins[1]



Represented with changes from Ebrahimi S, Samanta D. Nat Commun 2023;14:2411, licensed under CC-BY 4.0 (https://creativecommons.org/licenses/by/4.0/)

Therapeutic proteins vary considerably in source (e.g. plants, animals, fungi, bacteria), structure, and function[2]



Structures shown are not to scale

Molecular weight and structural complexity

ADC, antibody-drug conjugate; CD3, cluster of differentiation 3; Da, dalton; Fc, fragment crystallizable; FDA, US Food and Drug Administration; kDa, kilodalton; mAb, monoclonal antibody; PEG, polyethylene glycol

1. Ebrahimi S, Samanta D. Nat Commun 2023;14:2411; 2. Singh DB, Tripathi T. Protein-Based Therapeutics. Singapore: Springer Nature, 2023; 3. Bawa, R. Current Immune Aspects of Biologics and Nanodrugs: An Overview. In: Immune Aspects of Biopharmaceuticals and Nanomedicines. Jenny Stanford Publishing; 2019:1–82; 4. García JM, et al. Clin Transl Oncol 2007;9:715–722; 5. Graf L. Transfus Med Hemother 2018;45:86–91

Types of therapeutic proteins and their clinical uses

Type	Function	Examples of clinical use*
Enzymes and regulatory proteins *Enzymes, hormones, interferons, interleukins, cytokines*	• **Replace** deficient or abnormal protein • **Augment** existing pathway • Provide **novel function**	Diabetes, growth failure, Fabry disease
Targeted proteins *Monoclonal antibodies, antibody-drug conjugates*	• **Interfere** with a molecule or organism • **Deliver** cytotoxic payload to cancer cells	Cancer, autoimmune disorders
Protein vaccines	• **Protect** against foreign agents or cancer cells	Vaccines against hepatitis B and HPV, and cancer†
Protein diagnostics	• **Detect** disease	Tuberculosis detection test, pancreatic dysfunction test

*The list is not exhaustive; †Vaccines against cancer are currently in clinical development
AIDS, acquired immunodeficiency syndrome; HPV, human papillomavirus; IVF, in-vitro fertilization
Singh DB, Tripathi T. Protein-Based Therapeutics. Singapore: Springer Nature, 2023

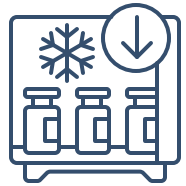
Potential limitations of therapeutic proteins

Poor in-vivo stability[1,2]

Prone to proteolysis

Poor in-vitro stability[3]

Denaturation and degradation during handling, formulation, and storage

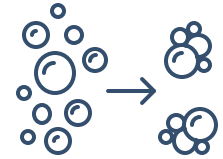


Low bioavailability[1,2]

Low cellular uptake and activity

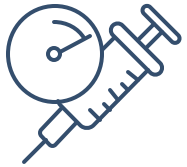
Aggregation during storage[1,2]

Aggregates can cause immune response



Short half-life[1,2]

Need for more frequent administrations, which can increase immunogenicity



Immunogenicity[1,2]

Immune responses to therapeutic proteins may affect their efficacy and safety

First-generation therapeutic proteins had many inherent limitations[2]

Strategies are still needed to overcome the potential limitations of recent therapeutic proteins[1]

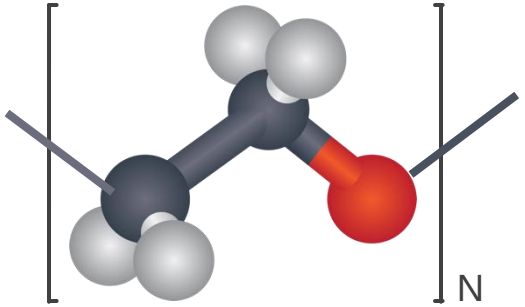
1. Singh DB, Tripathi T. Protein-Based Therapeutics. Singapore: Springer Nature, 2023; 2. Gonçalves J, Caliceti P. Drug Des Devel Ther 2024;18:5041–5062; 3. Lee P, et al. Macromol Biosci 2015;15:1332–1337

What is PEGylation?

PEGylation is a key strategy, increasingly used to address the challenges faced by native proteins[1]

PEGylation is the **conjugation of polyethylene glycol (PEG)** to functional amino acid groups on the protein surface[1,2]

Polyethylene glycol[3]



$HO\text{-}(CH_2\text{-}CH_2\text{-}O)_n\text{-}H$

- Linear polymer
- MW = 0.4–150 kDa
- Low cytotoxicity
- Biocompatible
- Soluble
- Clearable[4]

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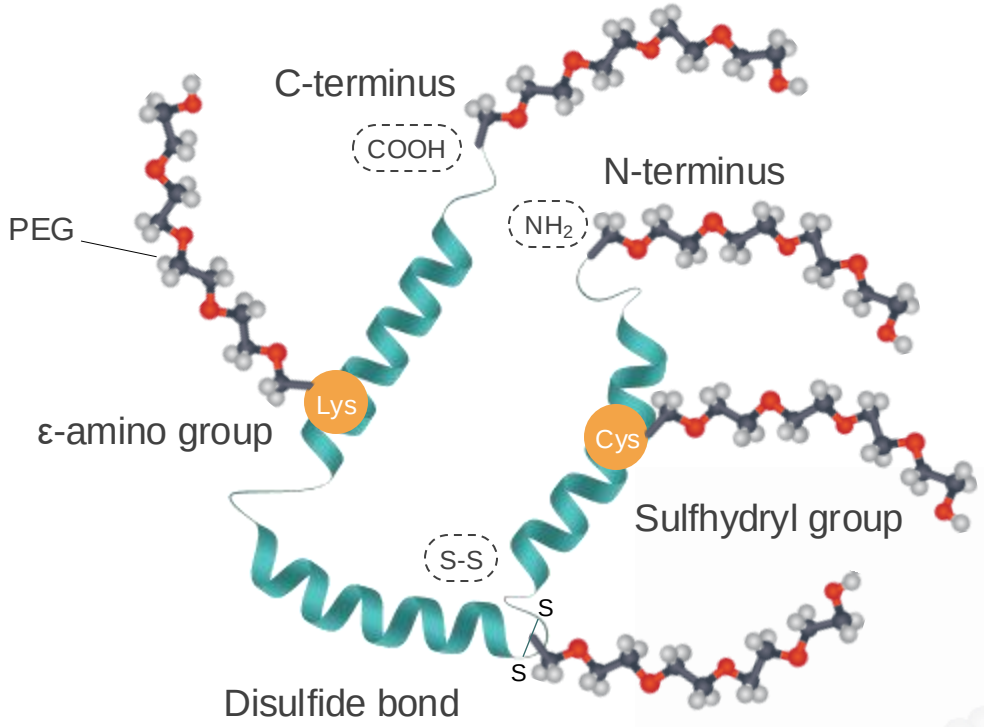
PEGylation **modifies the characteristics of a protein**, such as the pharmacokinetics and immunogenicity[5]

PEGylation sites include:[1]



Cys, cysteine; kDa, kilodalton; Lys, lysine; MW, molecular weight; PEG, polyethylene glycol. 1. Gonçalves J, Caliceti P. Drug Des Devel Ther 2024;18:5041–5062; 2. Abuchowski A, et al. J Biol Chem 1977;252:3578–3581; 3. Li C, et al. Front Pharmacol 2024;15:1353626; 4. Webster R, et al. Drug Metab Dispos 2007;35:9–16; 5. Singh DB, Tripathi T. Protein-Based Therapeutics. Singapore: Springer Nature, 2023

PEGylation is a significant technological advancement

Poor in-vivo stability

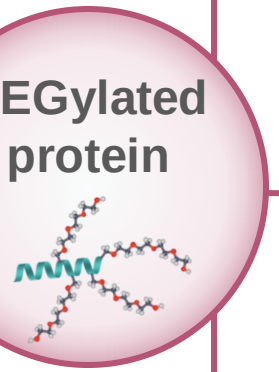
Improved in-vivo stability[1]

Low bioavailability

Increased bioavailability[1]

Short half-life

Potential for longer half-life[1]



Poor in-vitro stability

Improved in-vitro stability[2]

Aggregation during storage

Less aggregation in storage[1]

Immunogenicity

Potential for lower immunogenicity[1]

PEGylation may enhance the properties of some native therapeutic proteins[1]

PEG, polyethylene glycol

1. Salmaso S, Caliceti P. Peptide and protein bioconjugation: a useful tool to improve the biological performance of biotech drugs. In: Van Der Walle C, ed. Peptide and Protein Delivery. Academic Press; 2011:247–290;
2. Lee P, et al. Macromol Biosci 2015;15:1332–1337

Clinical use of PEGylated proteins

Widely used*[1]

Cancer
- Melanoma | **peginterferon alfa-2b** (2011)

Blood disorders
- Anemia | **methoxy peg-epoetin beta** (2007)

Immune system-related
- ADA-SCID | **pegademase bovine** (1990)
- Hepatitis | **peginterferon alfa-2a** (2002)
- Rheumatoid arthritis | **certolizumab pegol** (2008)

The earliest approved PEGylated proteins **decreased the immunogenicity** of the native xenogeneic proteins[2]

Recently approved by FDA*[1]

Ocular disease
- Geographic atrophy | **avacincaptad pegol** (2023)

Rare diseases
- Paroxysmal nocturnal hemoglobinuria | **pegcetacoplan** (2021)
- Fabry disease | **pegunigalsidase alfa** (2023)

Blood disorders
- Neutropenia | **pegfilgrastim** (2022)

The production of high-quality PEGylated proteins is **expanding**, with many new drugs in clinical development[1]

*Year of FDA approval in brackets. The list is not exhaustive
ADA-SCID, adenosine deaminase-deficient severe combined immunodeficiency; FDA, US Food and Drug Administration; PEG, polyethylene glycol
1. Biopharma PEG. FDA approved pegylated drugs by 2024. Available from: https://www.biochempeg.com/article/58.html. Accessed October 23, 2024; 2. Rondon A, et al. Adv Funct Mater 2021;31:2101633

Biopharmaceutical and immunological properties of PEGylated proteins

Paolo Caliceti

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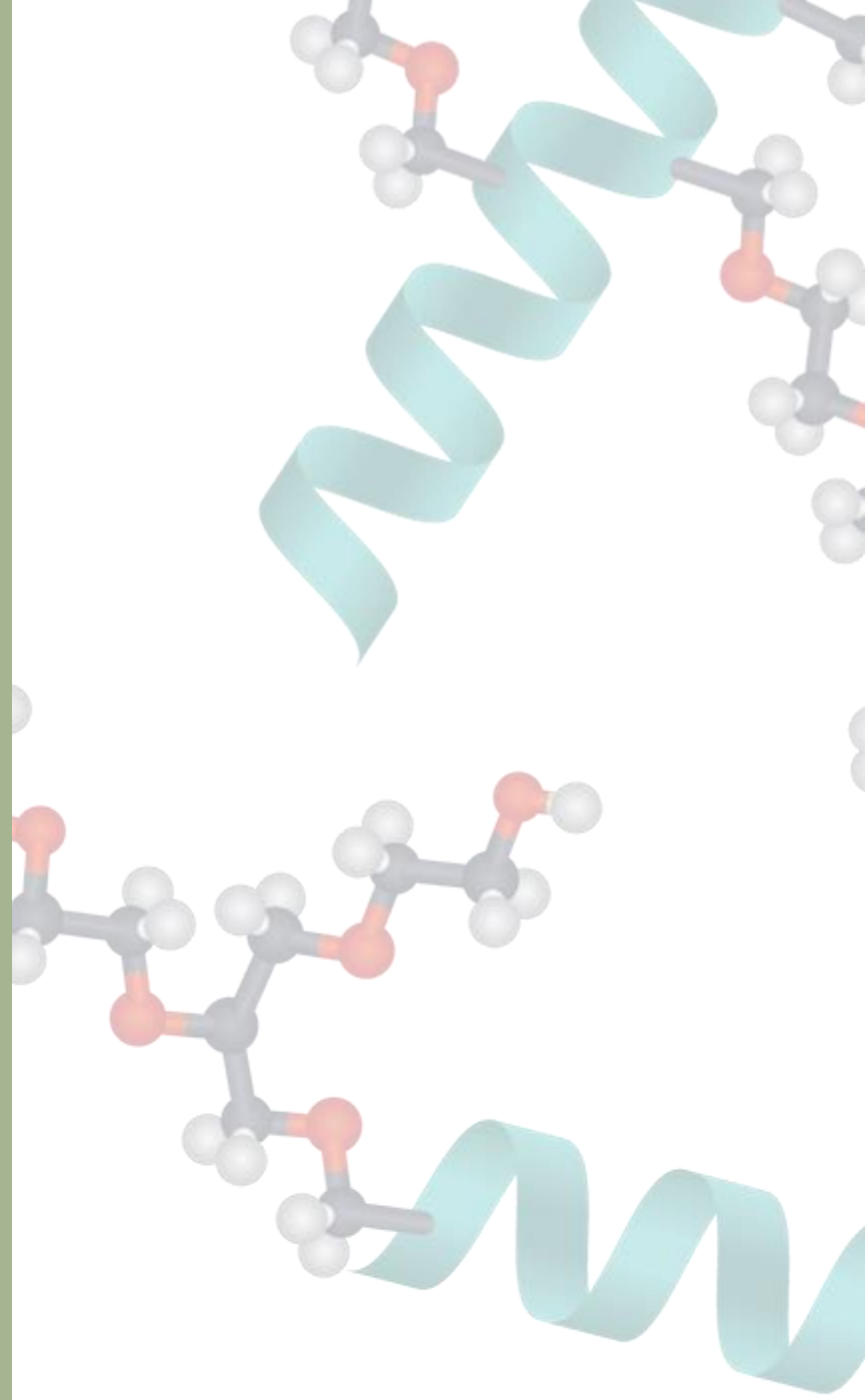
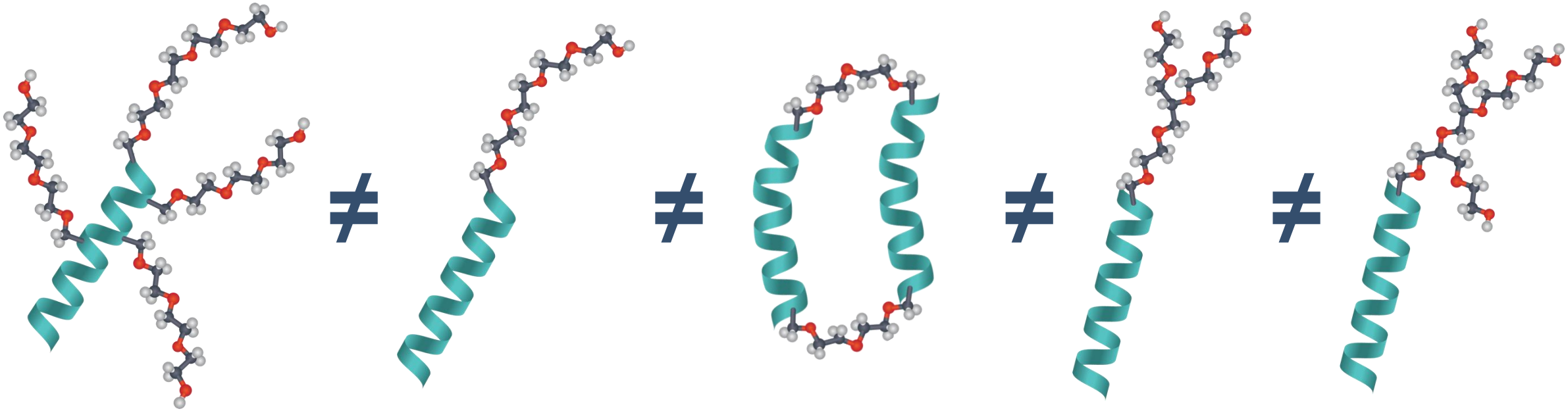


Each PEGylated protein is unique



PEGylated proteins vary substantially in PEG architecture, MW, and degree of conjugation[1]

Each PEGylated protein has a unique structure and composition that impacts its properties[2]

Properties of one PEGylated protein cannot be extrapolated to another[2]

MW, molecular weight; PEG, polyethylene glycol

Gonçalves J, Caliceti P. Drug Des Devel Ther 2024;18:5041–5062

The characteristics of PEGylated proteins affect their clinical properties

Permanence in circulation[1]

Hydrophilicity and high hydrodynamic volume of PEG

↓

Hydrated cloud **increasing protein's functional size**

↓

Slower renal filtration rate

↓

Prolonged systemic half-life

Important for small proteins under the kidney ultrafiltration cutoff

PEGylated alfa-GAL-A*: half-life ~80 hours vs ≤3 hours for non-PEGylated ERTs[2–5]

PEG biodistribution and elimination

Rate depends on PEG size and MW[6]

Kidneys: main route of elimination for PEGs ≤190 kDa[6]

Liver: minor route of elimination for some PEGs >20 kDa[6]

Cellular vacuolation has been reported with PEGs ≥30 kDa, with **no functional impact[7]**

86% and 96% of PEG1000 and 6000 were **excreted in the urine** 12 h after IV administration[6]

Protein stability to proteases[8]

Hydrated cloud masks protein surface

↓

Reduced protease degradation by mechanical repulsion and derivatization of amino acids

Protection from proteases: observed *in vitro* with PEGylated proteins, e.g. amylin[9]

Anti-drug immunogenicity[8,10]

Both xenogeneic and human proteins can elicit reaction

↓

PEGylation can mask surface proteins from immune recognition

↓

Potential for reduced immunogenicity

PK benefits may reduce administration frequency

↓

Potential for reduced immunogenicity

PEGylated L-asparaginase has masked antigenic epitopes and reduced immunogenicity[11]

***Clinical benefit has not been demonstrated for the longer half-life of pegunigalsidase alfa. Infusions are every two weeks.**

alfa-GAL-A, alpha-galactosidase A; EPO, erythropoietin; GH, growth hormone; kDa, kilodalton; MW, molecular weight; PEG, polyethylene glycol; PK, pharmacokinetics. 1. Rondon A, et al. Adv Funct Mater 2021;31:2101633; 2. Schiffmann R, J Inherit Metab Dis 2019;42:534–544; 3. Ries M, et al. J Clin Pharmacol 2007;47:1222–1230; 4. European Medicines Agency. Replagal: summary of product characteristics. 2008. Accessed December 16, 2024; 5. US Food and Drug Administration. Fabrazyme: prescribing information. 2010. Accessed October 24, 2024; 6. Webster R, et al. Drug Metab Dispos 2007;35:9–16; 7. Ivens IA, et al. Toxicol Pathol 2015;43:959–983; 8. Gonçalves J, Caliceti P. Drug Des Devel Ther 2024;18:5041–5062; 9. Böttger R, et al. J Control Release 2018;292:58–66; 10. Jevševar S, et al. Biotechnol J 2010;5:113–128; 11. Li C, et al. Front Pharmacol 2024;15:1353626

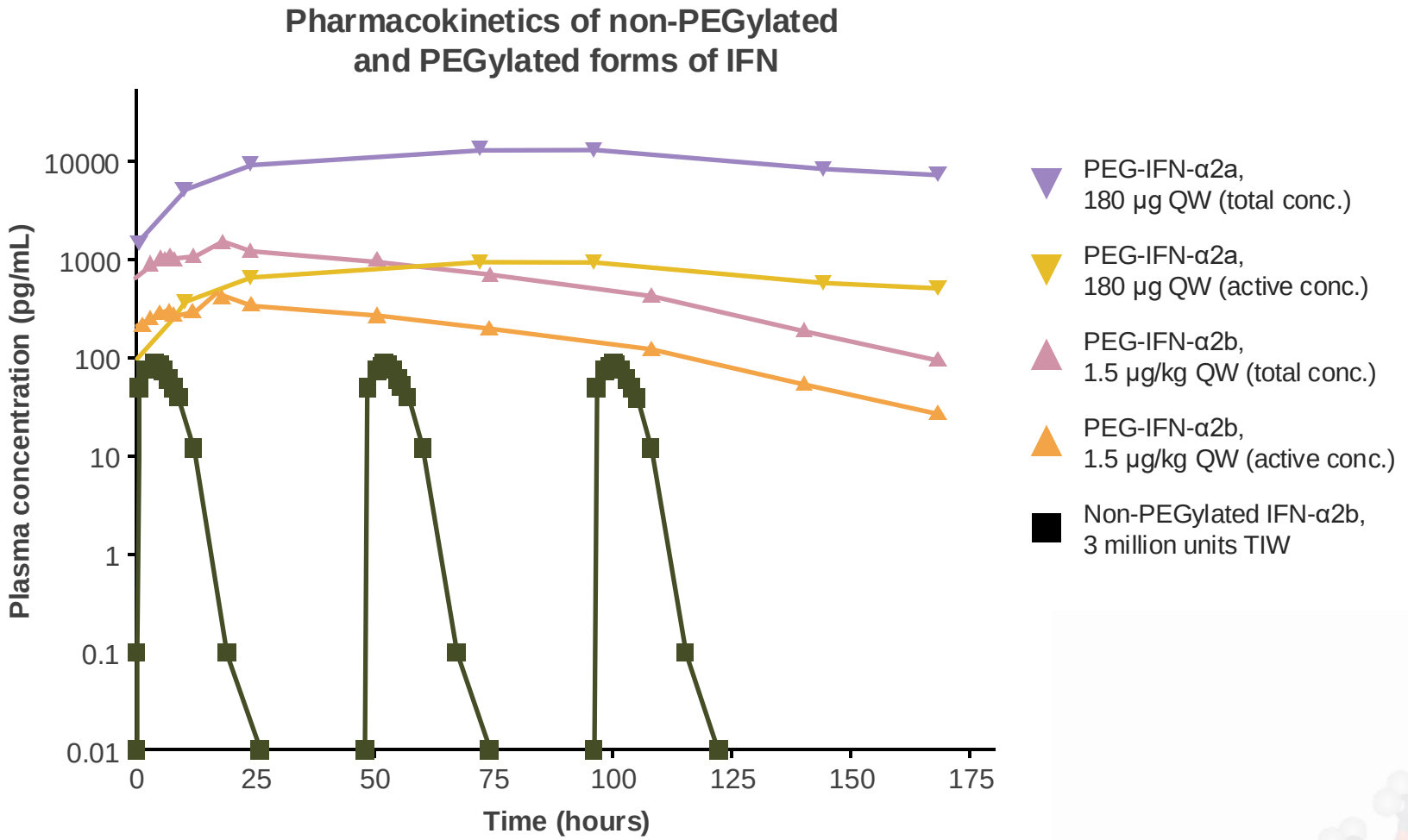
Benefits of PEGylation: interferon

IFNs are the most effective drugs against hepatitis C

First-generation recombinant IFNs:
- Short half-life (4–8 hours)
- Administered three times a week

IFN-α:
- Administration every 2 days led to viral rebound between injections
- Daily injections were poorly tolerated

PEGylated IFNs:
- Almost absent immunogenicity
- Longer half-life and sustained blood levels
- Enhances effectiveness and reduced AEs



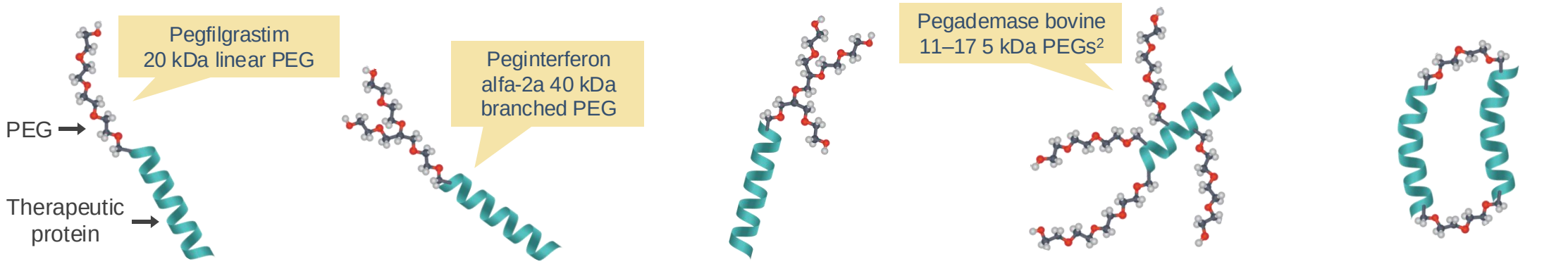
Adapted with permission from Milla P, et al. Current Drug Metab 2012;13:105–119

AE, adverse event; conc., concentration; IFN, interferon; PEG, polyethylene glycol; QW, once weekly; TIW, three times weekly

1. Milla P, et al. Current Drug Metab 2012;13:105–119

PEG structure affects the properties of PEGylated proteins

PEG structure includes **MW, shape, and number of PEG chains** attached to the protein[1]



PEGylated protein	PEG structure	Impact on function
Peginterferon alfa-2a[1]	One branched 40-kDa PEG	**Decreased** in-vitro bioactivity by 93%
Pegunigalsidase alfa*[3,4]	Crosslinking by a bifunctional 2-kDa PEG	**Improved** in vitro stability and **prolonged** half-life
Pegloticase[1]	One linear 10-kDa PEG	**Extended** half-life
Pegfilgrastim (G-CSF)[1]	One linear 20-kDa PEG	**Prolonged** in-vivo stabilization without compromising bioactivity
Certolizumab pegol; peginterferon alfa-2a[1]	One branched 40-kDa PEG	

***Clinical benefit has not been demonstrated for the longer half-life of pegunigalsidase alfa. Infusions are every two weeks.**

G-CSF, granulocyte colony-stimulating factor; kDa, kilodalton; MW, molecular weight; PEG, polyethylene glycol

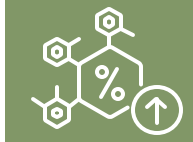
1. Gonçalves J, Caliceti P. Drug Des Devel Ther 2024;18:5041–5062; 2. Rondon A, et al. Adv Funct Mater 2021;31:2101633; 3. Schiffmann R, J Inherit Metab Dis 2019;42:534–544;
4. Kizhner T, et al. Mol Genet Metab 2015;114:259–267

Clinical advantages of PEGylation

↑ Half-life

↓ Administration frequency

↓ Protein aggregation



↑ Stability, bioavailability, and shelf life

Potential for reduced immunogenicity

PEGylation can increase half-life of the protein by up to 20-fold in humans, enabling less frequent administration[1]

- **Pegunigalsidase alfa*** (Fabry disease) | 4 PEGs; ~116 kDa | half-life of **53–121 h** vs a mean of **≤3 h** for non-PEGylated agalsidase alfa (~51 kDa) and beta (~100 kDa)[2–4]

Protection from proteolysis by PEG has been observed in mice[5]

- **PEGylated taspoglutide, amylin, and pramlintide** (diabetes) | Native proteins prone to proteases | PEGylated versions protected within the prodrug, especially the N-terminal sequences near the PEG | degraded PEG-taspoglutide was **<2%** at 24 hours

The PEG shell also increases thermal stability and slows down diffusion[1]

PEGylation decreases immune reactivity, enabling the use of non-human and highly reactive human proteins[1]

- **Pegaspargase** (leukemia) | Asparaginase from *E. coli* was withdrawn by the FDA due to hypersensitivity in up to one-third of patients | pegaspargase induces allergic reactions in **~10%** of treatment-naïve patients

***Clinical benefit has not been demonstrated for the longer half-life of pegunigalsidase alfa. Infusions are every two weeks.**

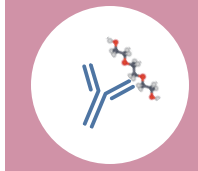
E. coli, *Escherichia coli*; FDA, US Food and Drug Administration; h, hours; kDa, kilodalton; PEG, polyethylene glycol. 1. Rondon A, et al. Adv Funct Mater 2021;31:2101633; 2. Schiffmann R, et al. J Inherit Metab Dis 2019;42:534–544; 3. Ries M, et al. J Clin Pharmacol 2007;47:1222–1230; 4. US Food and Drug Administration. Fabrazyme: prescribing information. 2010. Accessed October 24, 2024; 5. Böttger R, et al. J Control Release 2018;292:58–66

Potential drawbacks of PEGylation

↓ Bioactivity

PEG, especially of high MW, can reduce the ability of proteins to interact with large substrates or receptors on the cell surface[1]

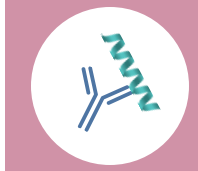
- **Peginterferon alfa-2a** (hepatitis C) | one branched 40-kDa PEG | overall bioactivity decrease of **93%**[2]



Anti-PEG antibodies

Anti-PEG response, especially with high MW or non-human* proteins, may lead to increased drug clearance and allergic reactions[1,3]

- **Pegloticase** (gout) | **40%** of 169 patients developed antibodies against PEG, which was associated with infusion reactions



Immune reaction due to altered protein conformation

Random PEGylation can alter the structure of proteins, exposing new epitopes to the immune system[1]

Factors that affect the potential risks associated with PEGylated proteins:[1]

- **PEG**-related (e.g. size, structure)
- **Protein**-related (e.g. immunogenicity)
- **Drug**-related (e.g. administration frequency)
- **Patient**-related (e.g. genetics)

*Other scenarios include a human protein that is not recognized as self, and a human protein that is deficient in the patient[3]

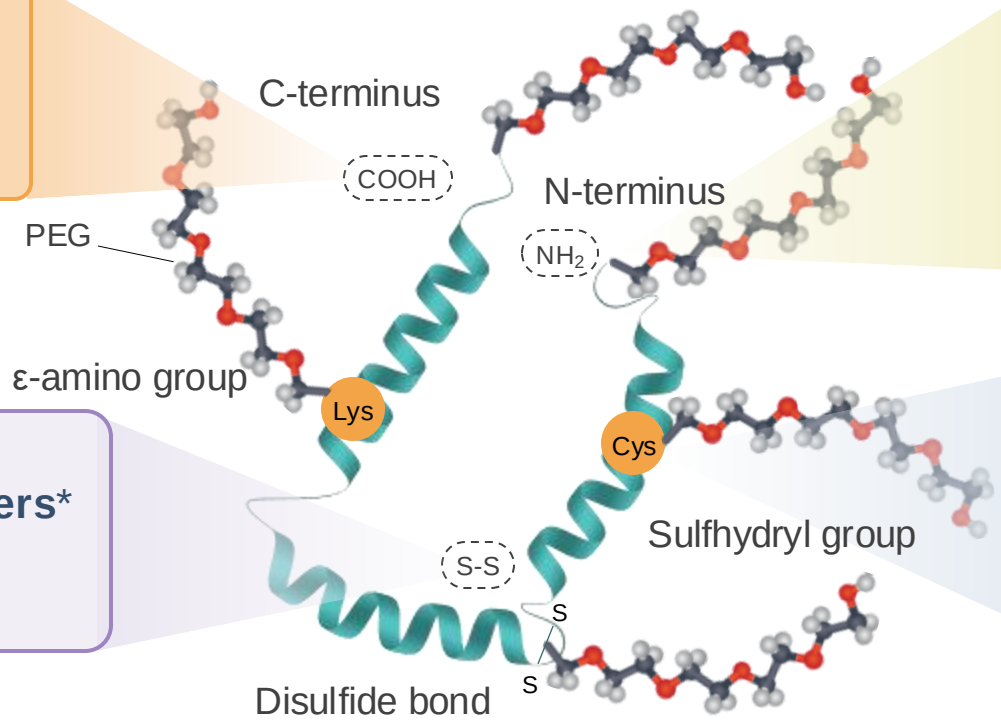
MW, molecular weight; PEG, polyethylene glycol

1. Gonçalves J, Caliceti P. Drug Des Devel Ther 2024;18:5041–5062; 2. Bailon P, et al. Bioconjug Chem 2001;12:195–202; 3. Chen BM, et al. ACS Nano 2021;15:14022–14048

PEG conjugation techniques vary substantially

PEGylation chemistry has progressed from random to **amino acid–specific** conjugation with **minimal-to-no impact** on inherent protein function[1]

PEGylation types by conjugation site[1,2]



Carboxyl modifiers*

- PEG-amine
- PEG-hydrazide

Amino modifiers*

- PEG-propionaldehyde
- mPEG-succinimidyl carbonate

G-CSF, GH, interferon beta-1a

Disulfide bond modifiers*

- PEG-bis-sulfone

Sulfhydryl modifiers*

- PEG-vinylsulfone
- PEG-maleimide
- PEG-iodoacetamide
- PEG-dithyopyridine

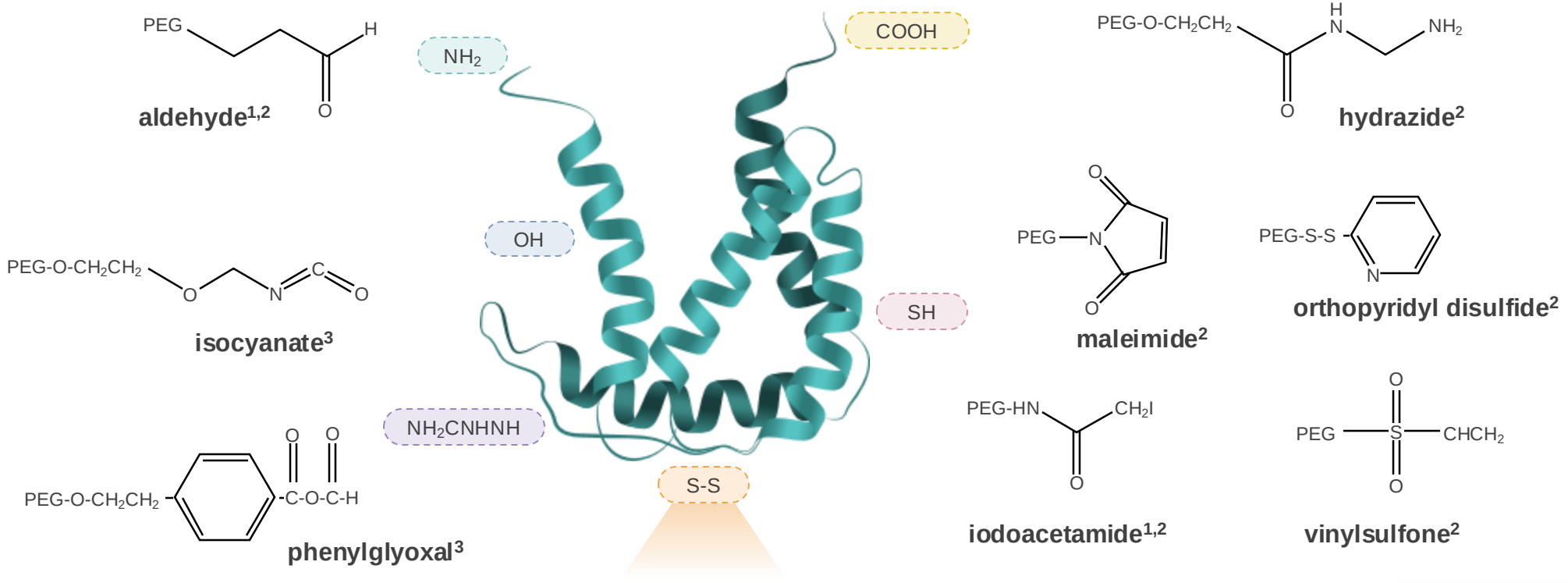
Certolizumab pegol

*The list is not exhaustive

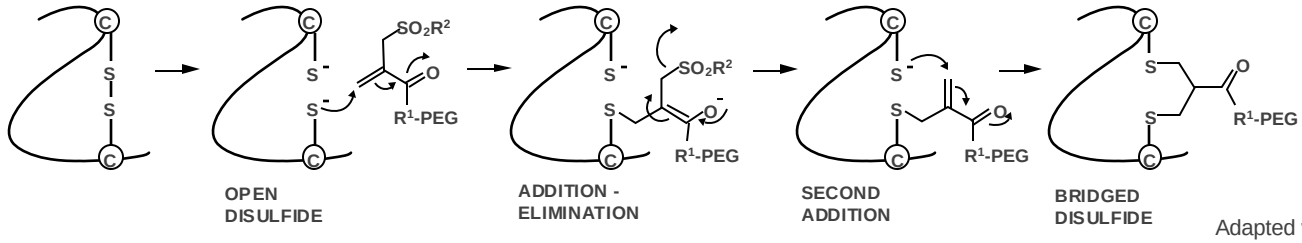
Cys, cysteine; G-CSF, granulocyte colony stimulating factor; GH, growth hormone; Lys, lysine; mPEG, monomethoxy polyethylene glycol; PEG, polyethylene glycol

1. Gonçalves J, Caliceti P. Drug Des Devel Ther 2024;18:5041–5062; 2. Li C, et al. Front Pharmacol 2024;15:1353626

Chemical conjugation of PEG



Conjugation to the disulfide bond:[1]



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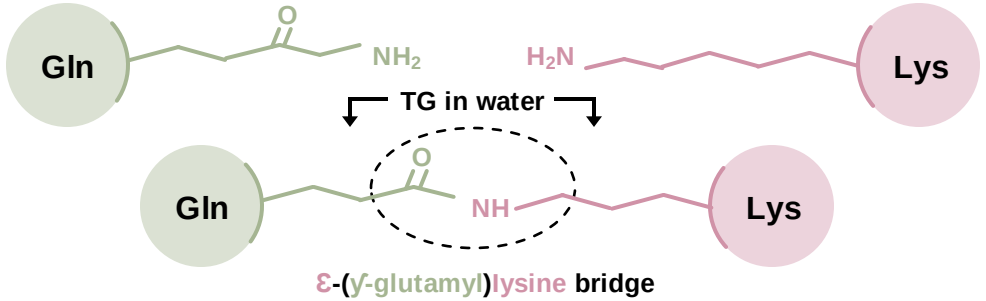
PEG, polyethylene glycol

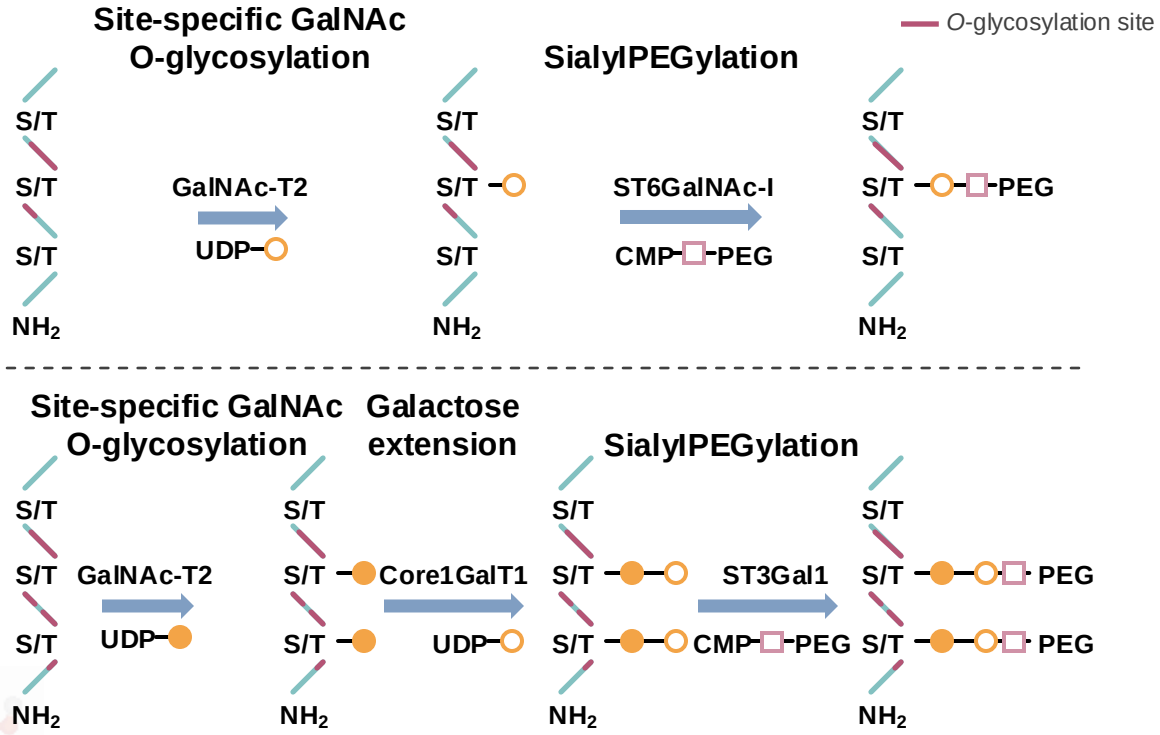
1. Salmaso S, Caliceti P. Peptide and protein bioconjugation: a useful tool to improve the biological performance of biotech drugs. In: Van Der Walle C, ed. Peptide and Protein Delivery. Academic Press; 2011:247–290;
2. Gonçalves J, Caliceti P. Drug Des Devel Ther 2024;18:5041–5062; 3. Koniev O, Wagner A. Chem Soc Rev 2015;44:5495–5551

Enzymatic conjugation of PEG

Site-specific PEGylation using TG[1]

- Alkylamine PEG derivatives are incorporated into intact or chimeric proteins without decreasing their bioactivities
- Incorporation site is limited to the substrate Gln residues for TG





GlycoPEGylation[2]

- Polypeptides (e.g. G-CSF; IFN-2b) may contain a single, natural non-utilized *O*-glycosylation site in which a serine (S) or threonine (T) is an acceptor for selective addition of GalNAc by a polypeptide GalNAc transferase (e.g. GalNAc-T2)
- GalNAc is an acceptor for PEGylated sialic acid transferred by ST6GalNAc-I
- GM-CSF may contain multiple closely-positioned natural non-utilized *O*-glycosylation sites

GalNAc, N-acetylgalactosamine; G-CSF, granulocyte colony-stimulating factor; Gln, glutamine; GM-CSF, granulocyte macrophage colony-stimulating factor; IFN, interferon; Lys, lysine; PEG, polyethylene glycol; TG, transglutaminase; UDP, uridine diphosphate. 1. Sato H. Adv Drug Deliv Rev 2002;54:487–504; 2. DeFrees S, et al. Glycobiology 2006;16:833–843

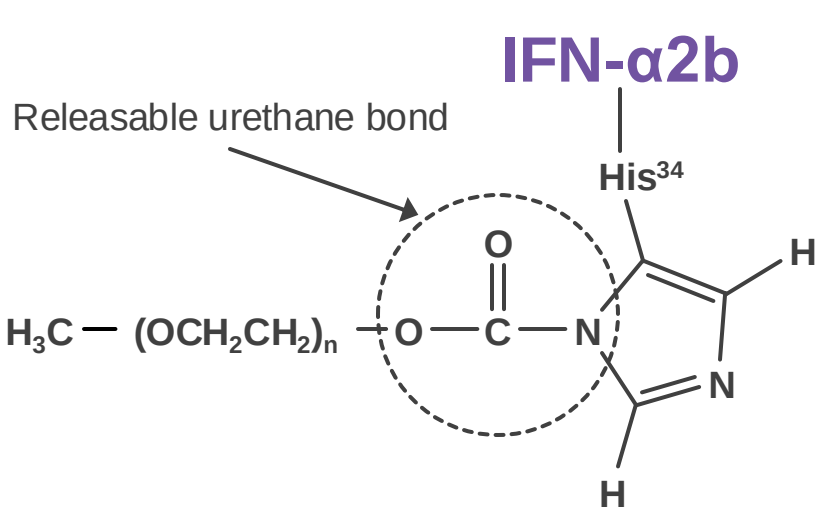
Differences in PEGylation methods: interferon

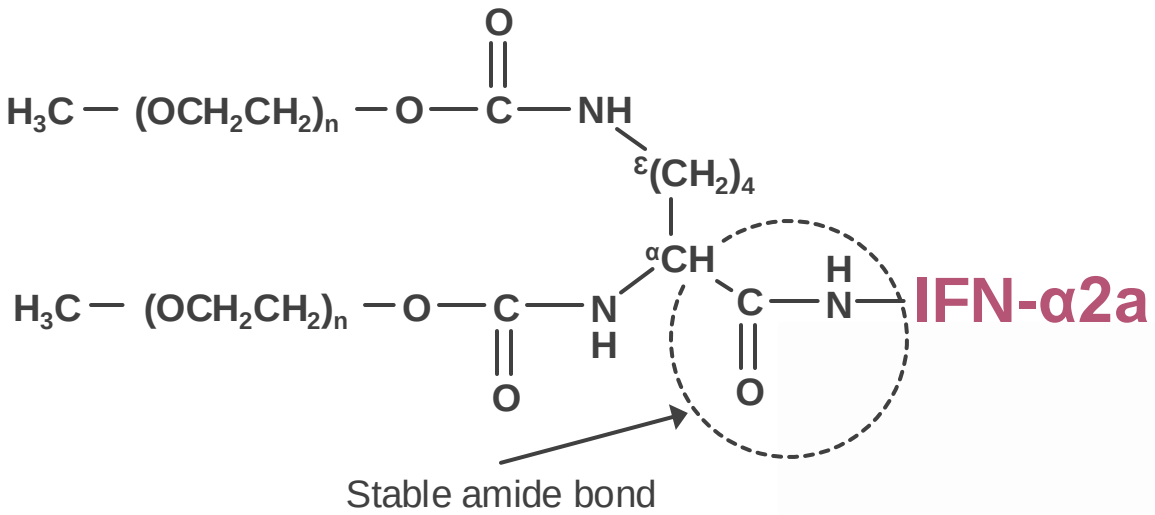
IFN-α2b

- Covalent linking of a 12-kDa linear mPEG via an **instable** urethane bond that slowly releases the free protein
- Linking at: His^{34} (~50%), lysine (35%), and other residues (~15%)

IFN-α2a

- Covalent linking of a 40-kDa branched mPEG via a **stable** amide bond
- Linking at: lysine residues 31, 121, 131, or 134 (94% overall)





Adapted with permission from Milla P, et al. Current Drug Metab 2012;13:105–119

IFN, interferon; kDa, kilodalton; mPEG, monomethoxy polyethylene glycol
Milla P, et al. Current Drug Metab 2012;13:105–119

The conjugation method affects protein properties

The choice of conjugation method can directly impact **drug efficacy and safety**, and offer more **predictable** patient outcomes

		Effect on protein property	Example
PEG activation method	Activation of the OH-PEG of monofunctional PEG (e.g. mPEG)	Conjugation under mild conditions; no crosslinking and heterogeneity; potential for immunogenicity due to the hydrophobic methoxy group	**Pegloticase**
	Activation of the HO-PEG-OH with bifunctional PEGs	A mix of crosslinked and non-crosslinked PEGs; improved stability; extended half-life; potential for reduced immunogenicity due to the lack of methoxy group	**Pegunigalsidase alfa***
Conjugation method	Random; PEG-succinimidyl succinate / PEG-p-nitrophenyl carbonate	Multiple reactions resulting in heterogeneity, poor stability, and toxicity	**Pegloticase**
	Partially selective; PEG-succinimide under controlled conditions	Cluster of isomers	**Peginterferon alpha-2a**
	Selective; pH-controlled; end-derivatization with PEG-methylpropionaldehyde	Reduced immunogenicity; extended half-life; improved specificity	**Peginterferon beta-1a**
	Highly selective; controlled; free Cys or Cys–Cys PEGylation		**Certolizumab pegol**

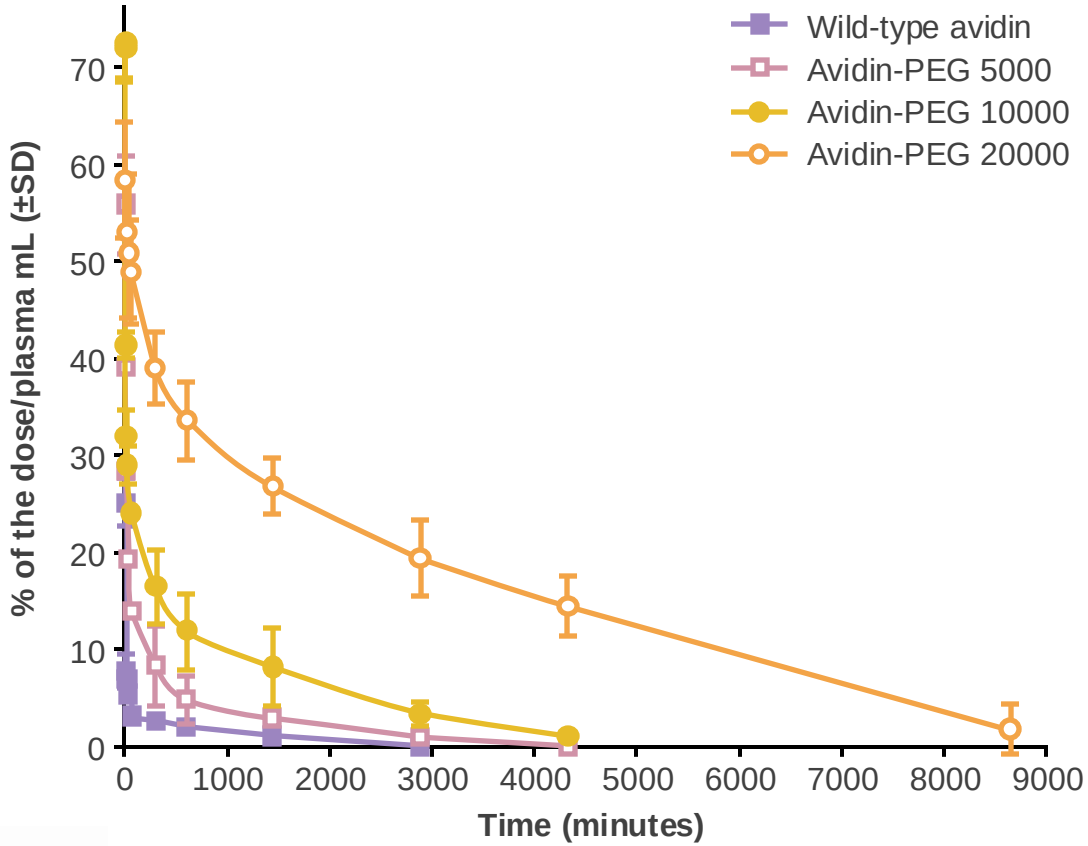
***Clinical benefit has not been demonstrated for the longer half-life of pegunigalsidase alfa. Infusions are every two weeks.**

Cys, cysteine; mPEG, monomethoxy PEG; PEG, polyethylene glycol

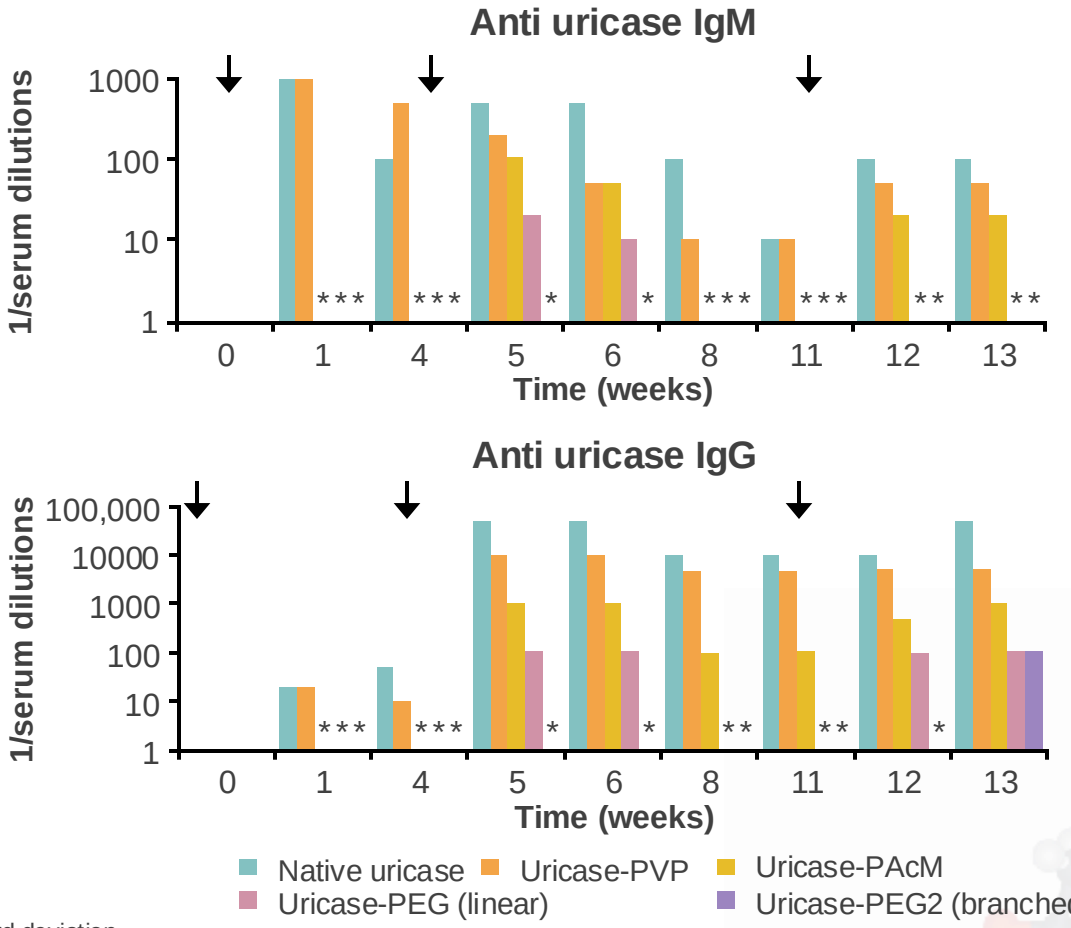
Gonçalves J, Caliceti P. Drug Des Devel Ther 2024;18:5041–5062

The conjugation method affects protein properties

Concentration-time profiles of avidin with different PEG conjugates[1]



Immunogenicity against different polymers conjugated to uricase[2]



Adapted with permission from Caliceti P, et al. J Controlled Release 2002:83;97–108

*Undetectable values; ↓, immunization time
Ig, immunoglobulin; PAcM, poly(N-acryloylmorpholine); PEG, polyethylene glycol; PVP, polyvinylpyrrolidone; SD, standard deviation

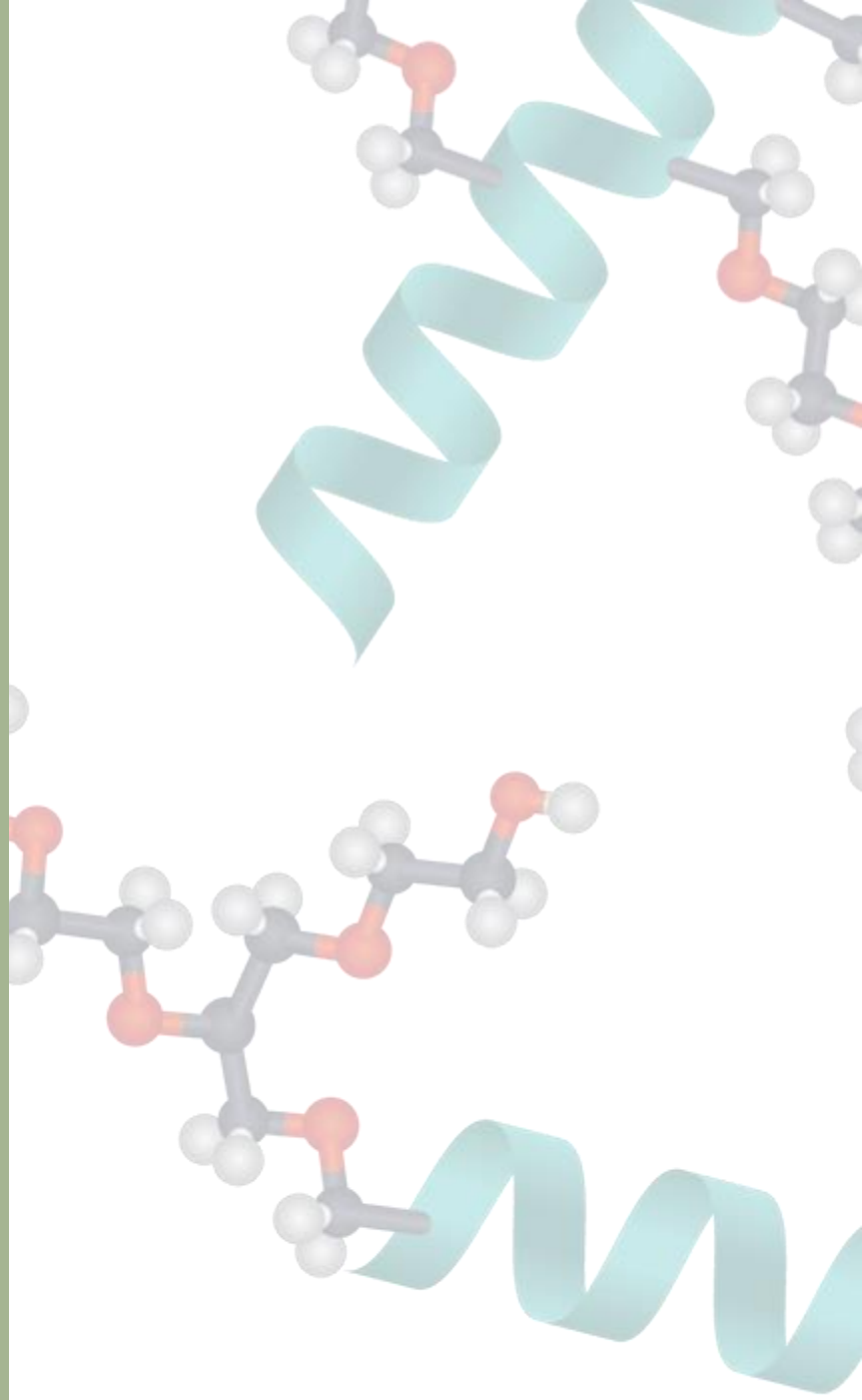
1. Caliceti P, et al. J Controlled Release 2002:83;97–108; 2. Caliceti P, et al. Bioconjugate Chem 2001;12:515–522

Adapted with permission from Caliceti P, et al. Bioconjugate Chem 2001;12:515–522

Immunogenicity

João Gonçalves

To ask a question, please click on the button below



Immunological aspects of therapeutic proteins

Immunogenicity: undesirable **host immune response** to administration of a therapeutic agent[1]

Cell-mediated[1]

Innate effector cells (non-T-cell mediated response)

Adaptive effector cells (T-cell mediated response)

Humoral[1]

Pre-existing and **treatment-emergent** anti-drug antibodies (ADAs)

Anti-drug immune responses can have a potential negative impact on the clinical benefit vs risk ratio of a drug by[1,2]

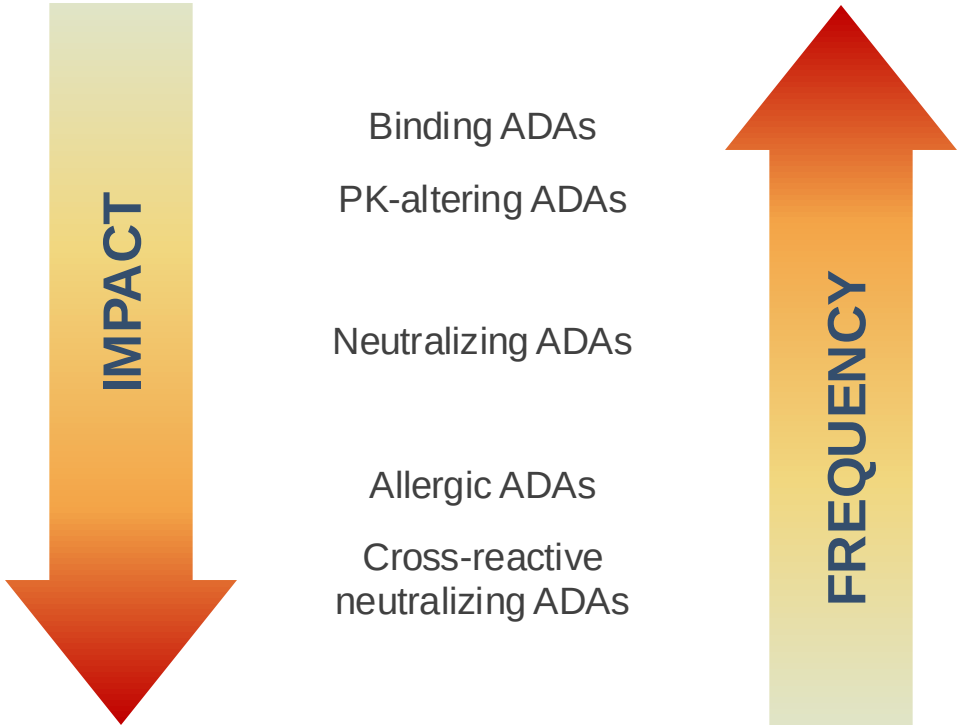
- Neutralizing the biologic activity and **inhibiting the efficacy** of the drug
- Accelerating the drug clearance, thus **reducing its efficacy**
- Impacting safety, sometimes causing **local or systemic AEs**
- Cross-reacting with, and neutralizing, **endogenous counterparts** of the drug

ADA, anti-drug antibody; AE, adverse event

1. Kuriakose A, et al. J Immunol Res 2016:1298473; 2. Immunogenicity assessment for therapeutic protein products. August 2014.
Available from: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/immunogenicity-assessment-therapeutic-protein-products. Assessed October 26, 2024

Clinical consequences of immunogenicity and strategies to overcome it

Clinical sequelae[1]



Examples of immunogenicity issues with therapeutic proteins

Anemia[2]
- 13 patients treated with recombinant EPO developed pure red-cell aplasia caused by neutralizing anti-EPO antibodies

IBD[3]
- Loss of response to murine–human IFX is common and associated with anti-IFX antibodies
- Pre-existing antibodies against the murine Fab of IFX predict loss of response

Mitigation strategies

Modulate patient's immune response (not always appropriate)[4]
- Immune suppression or immune tolerance induction

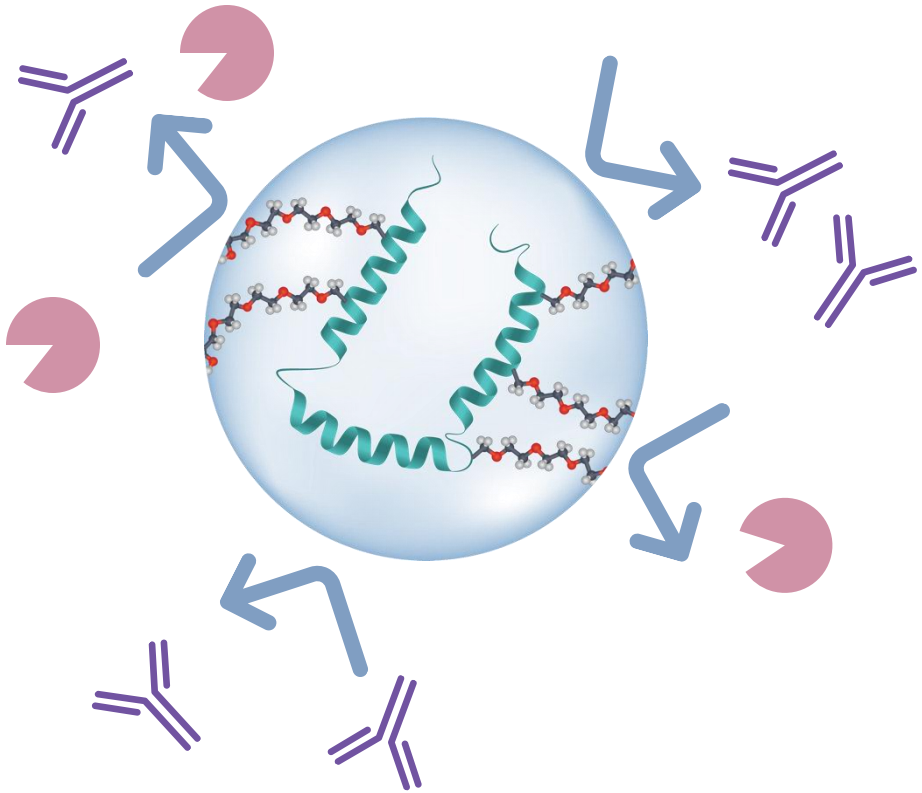
Modify protein characteristics
- Reduce administration frequency[5]
- Remove T/B-cell epitopes in inherently immunogenic proteins[4]
- Alter propensity to aggregate, deamidate, and oxidize[5]
- **Shield epitopes and extend half-life, e.g. using PEGylation**[5]

ADA, anti-drug antibody; EPO, erythropoietin; Fab, fragment antigen-binding region; IBD, inflammatory bowel disease; IFX, infliximab; PEG, polyethylene glycol; PK, pharmacokinetics

1. Strand V, et al. Nat Rev Rheumatol 2021;17:81–97; 2. Casadevall N, et al. N Engl J Med 2002;346:469–475; 3. Steenholdt C, et al. Aliment Pharmacol Ther 2013;37:1172–1183;
4. Mazor R, et al. Am J Pathol 2018;188:1736–1743; 5. US Food and Drug Administration. Immunogenicity assessment for therapeutic protein products. August 2014. Available from: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/immunogenicity-assessment-therapeutic-protein-products. Assessed October 26, 2024

PEGylation may mask epitopes and reduce anti-drug immunogenicity and antigenicity

PEGylation provides a **hydration shell** on the protein surface, which **may mask from, and repel, immune system components**[1,2]



Additional immunogenicity-related benefits of PEGylation:

↑ Size of PEGylated proteins; masking from proteases

↓

Longer half-life

↓

Potential for reduced drug administration frequency

↓

Potential for reduced immune reactions perpetuated by frequent administration of highly immunogenic protein

PEG, polyethylene glycol

1. Rondon A, et al. Adv Funct Mater 2021;31:2101633; 2. Gonçalves J, Caliceti P. Drug Des Devel Ther 2024;18:5041–5062

PEGylation can sometimes trigger an immune response against the PEG or PEGylated protein

Despite the potential of PEGylation to reduce immunogenicity, the **PEG moiety may induce an immune response**[1]

Additionally, a PEGylated protein **may induce an immune reaction** that is more severe than that against the native protein[1]

Anti-PEG response can be weak and may not cause adverse reactions[1]

- PEGylated COVID-19 vaccines have been safely administered to most patients[1,2]

Anti-PEG antibody response may be increased when the therapeutic protein is:[3]

- A human protein that is deficient in the recipient
- A highly immunogenic non-human protein
  - E.g. pegloticase, pegaspargase, and pegvaliase

Neutralizing ADAs against a PEGylated protein can occasionally cause serious conditions[4]

- E.g. **PEG-MGDF** (PEGylated thrombopoietin)[4]

Immunogenicity against PEG may:[5]

- Decrease efficacy due to neutralization
- Decrease bioavailability due to accelerated clearance
- Cause hypersensitivity / allergic reactions

ADA, anti-drug antibody; COVID-19, coronavirus disease; MGDF, megakaryocyte growth and development factor; PEG, polyethylene glycol; TPO, thrombopoietin

1. Gonçalves J, Caliceti P. Drug Des Devel Ther 2024;18:5041–5062; 2. Xu W, et al. Vaccines (Basel) 2023;11:1118; 3. Chen BM, et al. ACS Nano 2021;15:14022–14048; 4. Li J. et al. Blood 2001;98:3241–3248; 5. Rondon A, et al. Adv Funct Mater 2021;31:2101633

Impact of pre-existing anti-PEG or anti-drug antibodies

Pre-treatment exposure to PEG

Anti-PEG antibodies were found in 72% of the general US population in 2016[1]

- Exposure to PEG happens through **food, cosmetics, and medicines**[1]
- Patients treated with PEGylated drugs can have **both** pre-existing and induced anti-PEG antibodies[2]

Although immune responses to PEG seem to be longstanding[1], **most individuals with anti-PEG response have low antibody titers**[3]

The presence of anti-PEG antibodies **may not always be clinically relevant**[4]

Exposure to pre-existing ADAs

Patients previously treated with non-PEGylated therapeutic proteins may have **pre-existing antibodies** that **cross-react with the new PEGylated protein**, especially if the two drugs are structurally similar[5]

ADA, anti-drug antibody; PEG, polyethylene glycol

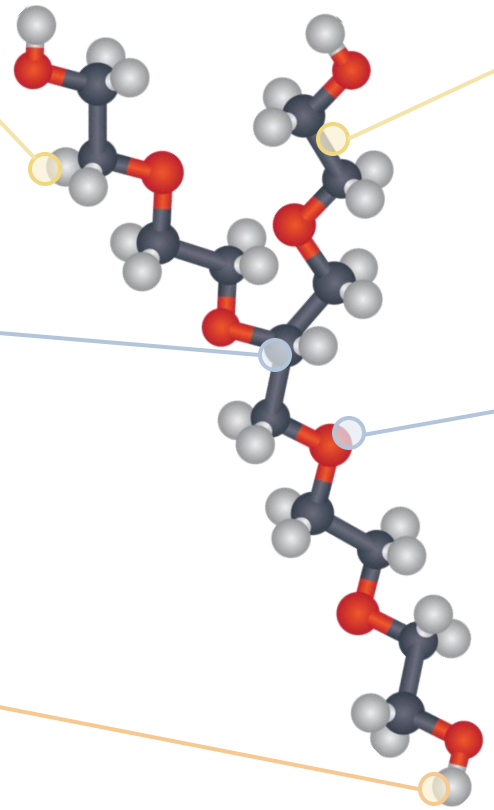
1. Yang Q, et al. Anal Chem 2016;88:11804–11812; 2. Hershfield MS, et al. Arthritis Res Ther 2014;16:R63; 3. Chen BM, et al. ACS Nano 2021;15:14022–14048;
4. Carreño JM, et al. Vaccine 2022;40:6114–6124; 5. Gonçalves J, Caliceti P. Drug Des Devel Ther 2024;18:5041–5062

The risk of an immune response is influenced by the unique properties of the PEGylated protein

PEGs of high MW and size are associated with higher reactivity[1–3]

Branched vs linear PEGs are associated with enhanced immune shielding[4,5]

Site of attachment and linker properties: Cys residues or terminal NH_2 groups cause less immunogenicity[2,6,7]



Site-specific PEGylation minimizes exposure of new epitopes and ADA formation[6–8]

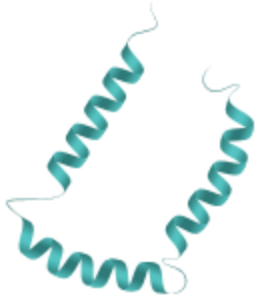
Alternatives to **hydrophobic end groups** (e.g. mPEG) could reduce immunogenicity[8,9]

PEG by itself is weakly immunogenic, but this can increase when conjugated to a macromolecule[10]

ADA, anti-drug antibody; Cys, cysteine; mPEG, methoxy-PEG; MW, molecular weight; PEG, polyethylene glycol

1. Chen BM, et al. ACS Nano 2021;15:14022–14048; 2. Huckaby JT, et al. Commun Chem 2020;3:124; 3. Wenande W, Garvey L. Clin Exp Allergy 2016;46:907–922; 4. Lu X, Zhang K. Nano Res 2018;11:5519–5534; 5. Shi D, et al. Adv Drug Deliv Rev 2022;180:114079; 6. Zuma LK, et al. Biomed Res Int 2022;2022:8929715; 7. Li C, et al. Front Pharmacol 2024;15:1353626; 8. Zhang P, et al. J Control Release 2016;244:184–193; 9. Sherman MR, et al. Bioconjug Chem 2012;23:485–499; 10. Shiraishi K, Yokoyama M. Sci Technol Adv Mater 2019;20:324–336

Protein- and patient-related factors also impact the risk of immunogenicity

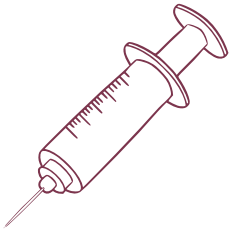


Therapeutic protein-related factors[1–3]

- Native immunogenicity (e.g. T-cell epitopes)
- Origin (human vs xenogeneic)
- Post-translational modifications
- Formulation / impurities / aggregates
- Stability

Patient-related factors[1,4–6]

- Age and sex
- Genetics, e.g. haplotype
- Previous exposure to PEG/PEGylated drugs; pre-existing ADAs
- Disease; previous and concomitant treatments
- Immunologic status (e.g. autoimmunity)
- Protein endogenous amount / deficiency / tolerance



Drug administration and target factors[7]

- Dosing
- Frequency and duration of treatment
- Route
- Target nature (e.g. cellular / soluble)

ADA, anti-drug antibody; PEG, polyethylene glycol

1. Chen BM, et al. ACS Nano 2021;15:14022–14048; 2. Zuma LK, et al. Biomed Res Int 2022;2022:8929715; 3. Jevševar S, et al. Biotechnol J 2010;5:113–128; 4. Matzaraki V, et al. Genome Biol 2017;18:76; 5. Gubbels et al. Front Immunol 2018;9:1269; 6. Yang Q, et al. Anal Chem 2016;88:11804–11812; 7. U.S. Food and Drug Administration. Immunogenicity assessment for therapeutic protein products. August 2014. Available from: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/immunogenicity-assessment-therapeutic-protein-products. Accessed October 23, 2024

Monitoring for immunogenicity

Immunogenicity and adverse events associated with PEGylated proteins can **vary widely** and must be assessed by preclinical screening, clinical trial monitoring, and postmarketing surveillance[1]

Multiple assays are available to measure ADAs,[2] and some protocols exist to measure anti-PEG antibodies[3]

Patient groups that will benefit most from monitoring[1]

Atopic patients[1,4] • Heightened immune response to allergens	• Monitor for signs of immunogenicity, e.g. reduced efficacy or allergic reactions • Assess ADA levels regularly
Patients with certain HLA types[1,5] • Some HLA types are associated with higher immunogenicity to biologics	• Genetic screening could identify patients at higher risk, allowing for personalized treatment
Patients pre-treated with non-PEGylated proteins[1,6] • Pre-existing ADAs could cross-react with PEGylated versions	• Consideration of the patient's treatment history is essential
Patients with compromised immune systems[1] • May be less likely to develop ADAs but, if they do, response could be more serious	• Monitor immunosuppressed and elderly patients
Patients deficient in the native protein[1] • More likely to develop ADAs toward the protein itself and to PEG	• Assess ADA levels regularly

ADA, anti-drug antibody; HLA, human leukocyte antigen; PEG, polyethylene glycol

1. Gonçalves J, Caliceti P. Drug Des Devel Ther 2024;18:5041–5062; 2. Wadhwa M, et al. Biologicals 2015;43:298–306; 3. Yang Q, et al. Anal Chem 2016;88:11804–11812;
4. Freire Haddad H, et al. Regen Eng Transl Med 2022;8:32-42; 5. Harris CT, et al. BioDrugs 2024;38:205–226; 6. Singh DB, Tripathi T. Protein-Based Therapeutics. Singapore: Springer Nature, 2023

Measurement of ADAs

Screening assay[1,2]

Detection of ADAs; methods include direct and indirect ELISA, antigen binding tests, RIAs

Confirmation assay[1]

Determination of specificity

Characterization assay[1]

Titration and neutralization assays

ADA assays lack comparability due to:

- Variable tolerance for therapeutic drug in the sample[3]
- Susceptibility to interference by serum components[3]
- Variable specificity for ADAs[3]
- Ability to detect multiple isotypes of an ADA[1]

Consistency in ADA testing would allow:[4]

- Comparability when switching to other protein therapies – faster and better choice of drug
- Better pharmacovigilance assessment

ADA, anti-drug antibody; ELISA, enzyme-linked immunosorbent assay; RIA, radioimmunoassay.

1. Immunogenicity Testing of Therapeutic Protein Products – Developing and Validating Assays for Anti-Drug Antibody Detection. January 2019. Available from: https://www.fda.gov/media/119788/download. Assessed December 5, 2024; 2. Suh K, et al. J Sep Sci 2022;45:2077–2092; 3. Wadhwa M et al. Biologicals 2015;43:298-306; 4. Jordan G, Staack R. Bioanalysis 2020;12:1021–1031

Further research to address remaining challenges and enhance the therapeutic potential of PEGylated proteins

More research is essential to **further enhance** the therapeutic potential of PEGylated proteins

Understanding immunogenicity

Improve the strategies to mitigate immune responses

Understand if low levels of pre-existing ADA are clinically meaningful

Optimizing PEGylation

Develop novel PEGylation techniques that maximize therapeutic efficacy while minimizing adverse immune reactions

Factors influencing long-term safety

Conduct long-term studies

Understand the impact of pre-existing ADAs

Personalized medicine

Tailor PEGylation design to individual patients

ADA, anti-drug antibody; PEG, polyethylene glycol

Gonçalves J, Caliceti P. Drug Des Devel Ther 2024;18:5041–5062

Discussion and conclusions

João Gonçalves
Paolo Caliceti

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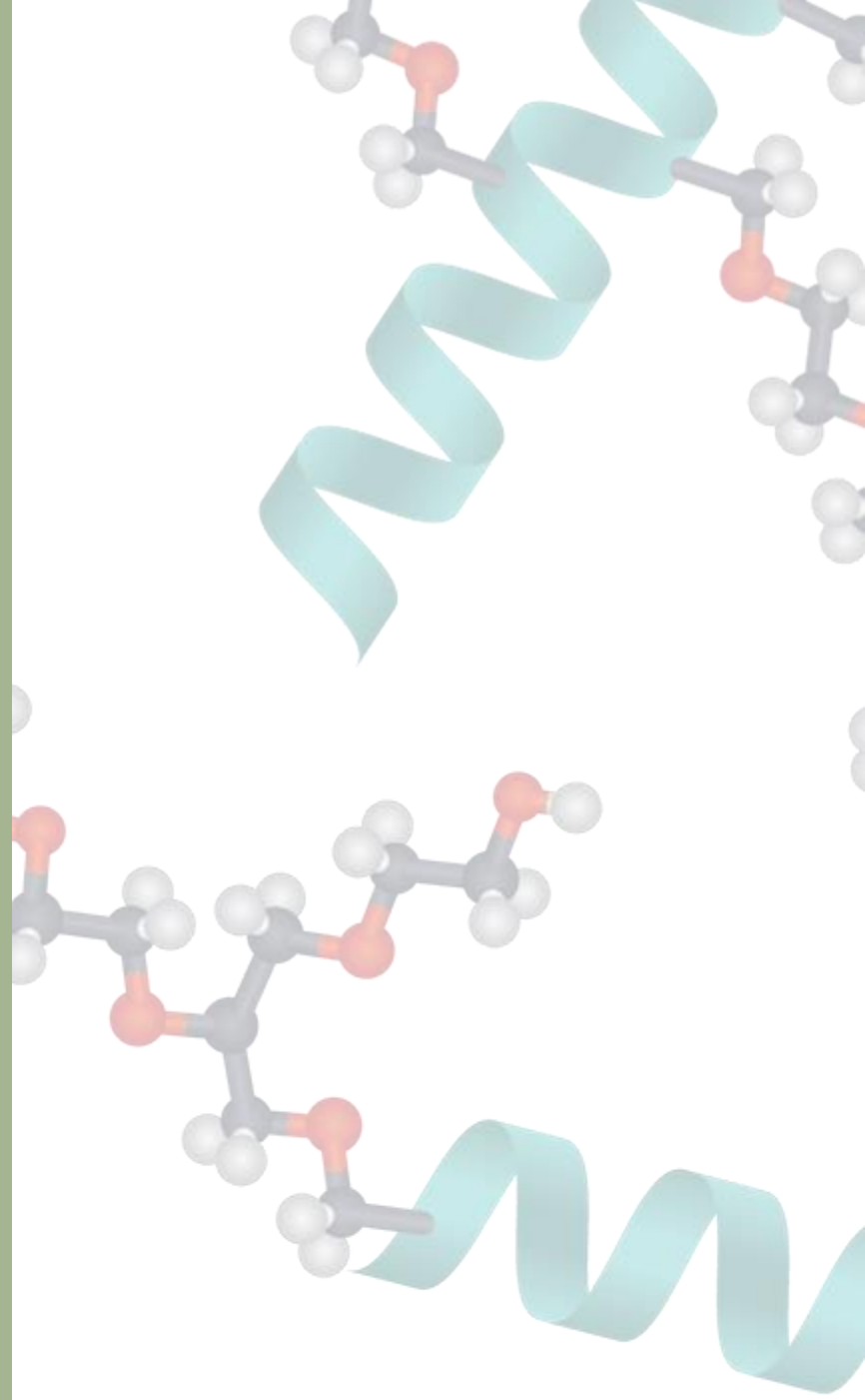
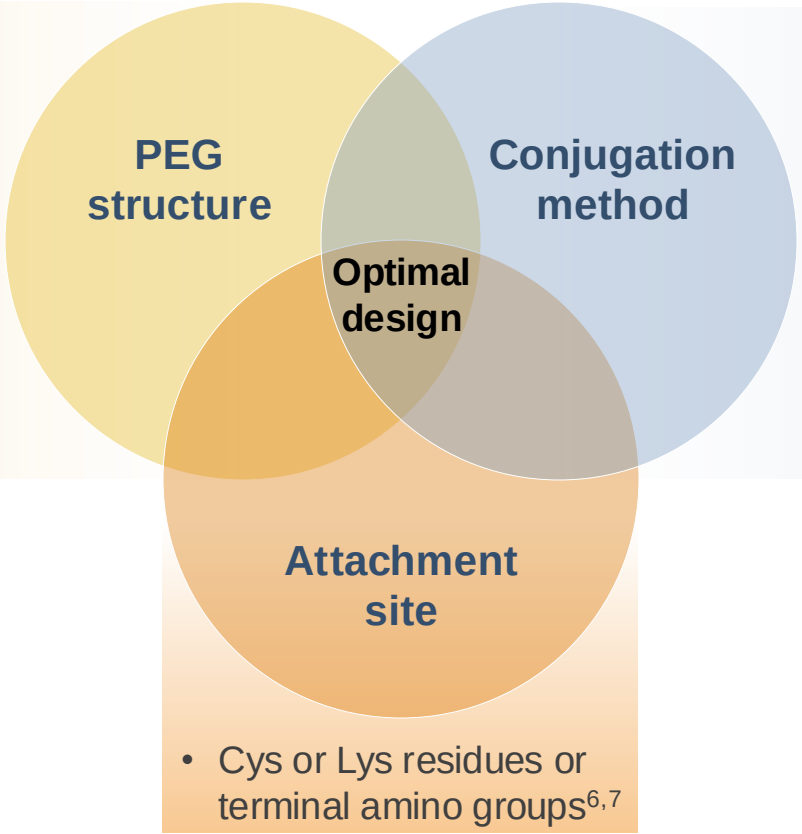


Strategies for optimal design of PEGylated proteins

PEGylation design should consider several factors,
to **enhance clinical performance** and **overcome immunogenicity**[1]

- Branched vs linear mPEG[2,3]
- Alternatives to hydrophobic methoxy-PEG, such as hydroxy-PEG[4]
- PEG oligomers administered before PEGylated drug[5]



- Site-specific vs random conjugation[6,7]
- Controlled vs less controlled conditions[6,7]

- Cys or Lys residues or terminal amino groups[6,7]

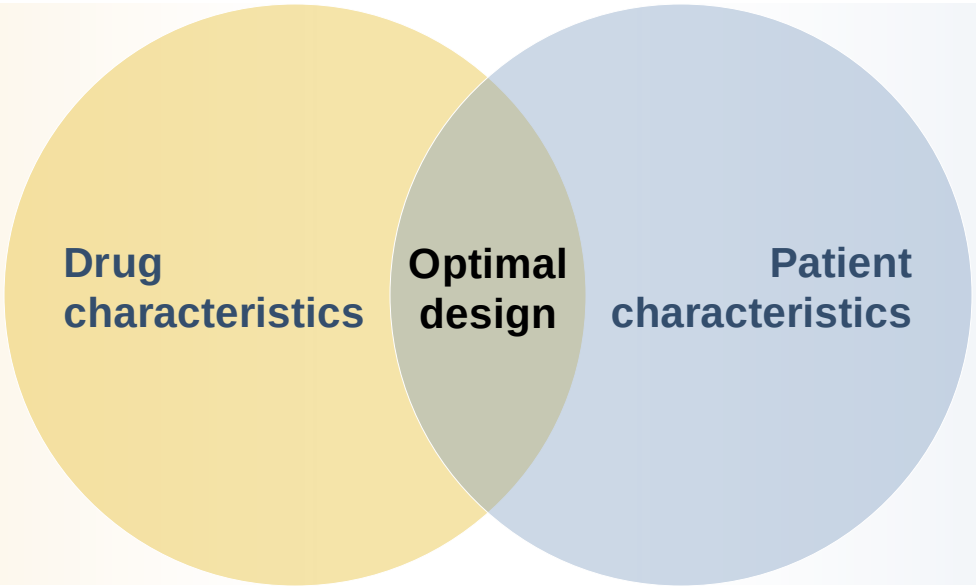
Cys, cysteine; Lys, lysine; mPEG, monomethoxy PEG; PEG, polyethylene glycol

1. Gonçalves J, Caliceti P. Drug Des Devel Ther 2024;18:5041–5062; 2. Caliceti P, et al. Bioconjug Chem 2001;12:515–522; 3. Shi D, et al. Adv Drug Deliv Rev 2022;180:114079; 4. Sherman MR, et al. Bioconjug Chem 2012;23:485–499; 5. Zhang F, et al. Biol Pharm Bull 2014;37:335–339; 6. Zuma LK, et al. Biomed Res Int 2022;2022:8929715; 7. Li C, et al. Front Pharmacol 2024;15:1353626

PEGylation design strategy should consider both drug and patient characteristics

PEGylation design should consider **both target patient population and therapeutic protein**, as unknown factors (e.g. individual genetic characteristics) can affect the immunogenic profile of the drug

- PEG size, structure, MW, and conjugation site and method
- Immunogenicity, structure, and stability of the protein
- Frequency and route of administration



- Age and sex
- Genetics
- Previous exposure to PEG
- Health conditions and treatments
- Pre-existing ADAs
- Therapeutic protein deficiency

ADA, anti-drug antibody; MW, molecular weight; PEG, polyethylene glycol

Gonçalves J, Caliceti P. Drug Des Devel Ther 2024;18:5041–5062

Conclusions

PEGylation offers clinical benefits to protein-based drugs, in potentially lowering immunogenicity and improving stability, bioavailability, and half-life*

PEGylation is most beneficial for therapeutic proteins that have higher immunogenicity and suboptimal pharmacokinetic features, e.g. short half-life and poor stability

Certain properties of PEGylated proteins affect immunogenicity in different ways, and can either reduce or increase immune reactions

Further research is needed to optimize PEGylation design strategies to overcome the risk of triggering an immune response

***PEGylation effect is specific to each protein and not all effects are observed in every PEGylated product**

PEG, polyethylene glycol

Gonçalves J, Caliceti P. Drug Des Devel Ther 2024;18:5041–5062

Q&A session

To ask a question, please click on the button below

Audience questions will be answered during a separate follow-up video

THANK YOU!