Polyglycerol for Half-Life Extension and Increased Stability of Biopharmaceuticals

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by

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Date of Defense:	25th August 2021

Statutory Declaration

Hereby I, Michael Tully, declare that I have independently authored the submitted thesis with the topic "*Polyglycerol for Half-Life Extension and Increased Stability of Biopharmaceuticals"*. I also confirm that this work as well as parts of this work have not been previously published or accepted for the award of any other degree or diploma in any university or other tertiary institution in my name.

Berlin,

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Michael Tully

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1 Introduction

The development of recombinant DNA technology in the late 1970s has been a milestone in medical research, as it enabled tailor-made synthesis and expression of therapeutic biomolecules, like antibodies, proteins, and peptides. The unique properties of these molecules resulted in one of the fastest growing markets in the pharmaceutical industry, with by today more than 200 protein- and peptide-based drugs approved by regulatory authorities, covering a wide range of human diseases.[1] The first generation of these drugs was designed to mimic (as replacement therapy) or inhibit (e.g., by monoclonal antibodies) the function of native proteins, but have also recently been structurally modified to create new moieties for better control of receptor binding and other attributes.[2]

Despite their high specificity, biopharmaceuticals bear some downsides, namely a poor physicochemical and pharmacokinetic profile. They often display low thermal stability, limited solubility and a propensity to aggregate, which create challenges regarding their manufacturing, formulation and shelf-life. In contrast to small molecule drugs, which can mostly be applied as oral dosage forms (tablets, granules, capsules), the oral bioavailability of biopharmaceuticals is typically very low and demands high-frequent injections *via* the subcutaneous or intravenous route, thereby impeding patient compliance and increasing the risk of side effects.[3] Biomolecules with molecular weights below the renal cutoff (50–70 kDa) undergo fast elimination from the bloodstream *via* the kidneys and therefore often display a circulation half-life being only in the range of minutes to hours.[1,4] Additionally, biopharmaceuticals are prone to proteolysis and, as often of non-human origin, bear immunogenic potential, which further impairs blood circulation time.[4]

To address these drawbacks and to create more stable molecules with an enhanced pharmacokinetic profile, several approaches have been pursued, including the design of new protein scaffolds[5] (DARPins, anticallins, affibodies, adnectins), encapsulation of proteins into nanogels[6] or micelles[7] or covalent modification with synthetic and natural polymers. Among the latter, the most prominent example is poly(ethylene glycol) (PEG), as it reached the market with by today ≥14 PEGylated protein drugs (Table 1) and several more in clinical trials. PEGylation is defined as the covalent attachment of one or multiple PEG-chains to an active pharmaceutical ingredient (API), in this case proteins, and was first described by Abuchowski and Davies in 1977, who successfully improved the circulation time and immunogenicity of bovine liver catalase and bovine serum albumin by the attachment of PEG.[8,9] Following these initial attempts led to the approval of the first PEGylated protein Adagen® in 1990.[10] Several other PEGylated proteins followed and gained market authorization thereafter.

Table 1. Regulatory approved PEGylated Biopharmaceuticals. (based on data from ref. 11, 12)

Trade name	API	Indication	Approval	Company
[a,c]Adagen®	PEG-adenosine deaminase, Pegademase	Severe combined immunodeficiency disease (SCID)	1990	Enzon
Oncaspar®	PEG-asparaginase, Pegaspargase	Acute lymphoblastic leukemia	1994	Enzon
PegIntron®	PEG-Interferon α-2b	Hepatitis B and C	2000	Schering-Plough/ Enzon
Pegasys®	PEG-Interferon α-2a	Hepatitis B and C	2001	Hoffmann-La Roche
Neulasta®	PEG-Granulocyte colony stimulating factor, Pegfilgrastim	Neutropenia	2002	Amgen
Somavert®	PEG-Human growth hormone receptor antagonist Pegvisomant	Acromegaly	2003	Pfizer
Macugen®	PEG-anti-VEGF aptamer, Pegabtanib	Age-related macula degeneration	2004	Pfizer
Mircera®	PEG-Erythropoietin beta	Anemia associated with chronic kidney disease	2007	Hoffman-La Roche
Cimzia®	PEG-Certolizumab	Rheumatoid arthritis, Crohn's disease, Axial spondyloarthritis and psoriatic arthritis	2008	Nektar/UCB Pharma
[b]Krystexxa®	PEG-Uricase, Pegloticase	Gout	2010	Savient
[b,c]Omontys®	PEG-Erythropoietin-mimetic peptide, Peginesatide	Anemia associated with chronic kidney disease	2012	Affymax/ Takeda
Plegridy®	PEG-Interferon beta-1a	Relapsing forms of multiple sclerosis	2014	Biogen
Adynovi®/Adynovate®	PEG-recombinant Factor VIII Antihemophilic Factor	Hemophilia A	2015	Baxalta
Refixia®/Rebinyn®	PEG-recombinant Factor IX Antihemophilic Factor	Hemophilia B	2017	Novo Nordisk
[a]Revcovi™	Elapegademase	Severe combined immunodeficiency disease (SCID)	2018	Leadient Biosciences
[a]Asparlas™	Calaspargase pegol	Acute lymphoblastic leukemia	2018	Servier
Palynzig™	PEG-Phenylalanine ammonia-lyase, Pegvaliase-pqpz	Phenyl-ketonuria	2018	Biomarin
Jivi®	PEG recombinant Factor VIII antihemophilic factor	Hemophilia A	2018	Bayer

[a]US-approved only. [b,c]Withdrawn from marketing in [b]EU or [c]US.

Generally, PEG is regarded as an inert and safe excipient and is approved by the Food and Drug Administration (FDA) and European Medicine Agency (EMA) for the use in foods, cosmetics and pharmaceuticals. Within recent years, however, PEG's image as non-immunogenic macromolecule has been challenged by numerous reports on anti-PEG antibodies in clinic, that diminished the initial benefits of PEGylation, in some cases even leading to a

neutralized therapeutic effect. Therefore, many alternative polymers for half-life extension of biopharmaceuticals are currently investigated.

In this work, polyglycerol (PG) is evaluated as alternative polymer to PEG on different therapeutic proteins and peptides, with the aim of disclosing differences in hydrodynamic size, stability, and activity between PGylated and PEGylated biopharmaceuticals, followed by an *in vivo* comparison on half-life extension and prolonged therapeutic activity.

2 Theoretical Background

2.1 Concept of Half-Life Extension through PEGylation

2.1.1 Half-Life Extension of Biopharmaceuticals

Half-life extension is one of the key technologies to generate sufficient blood levels of therapeutic proteins and peptides with a molecular mass below 50 kDa, including enzymes, coagulation factors, cytokines, growth factors and hormones.[1] Upon subcutaneous injection, those molecules usually move through the extracellular matrix (ECM) of the hypodermis by diffusion and convection entering systemic circulation *via* lymphatic or blood capillaries.

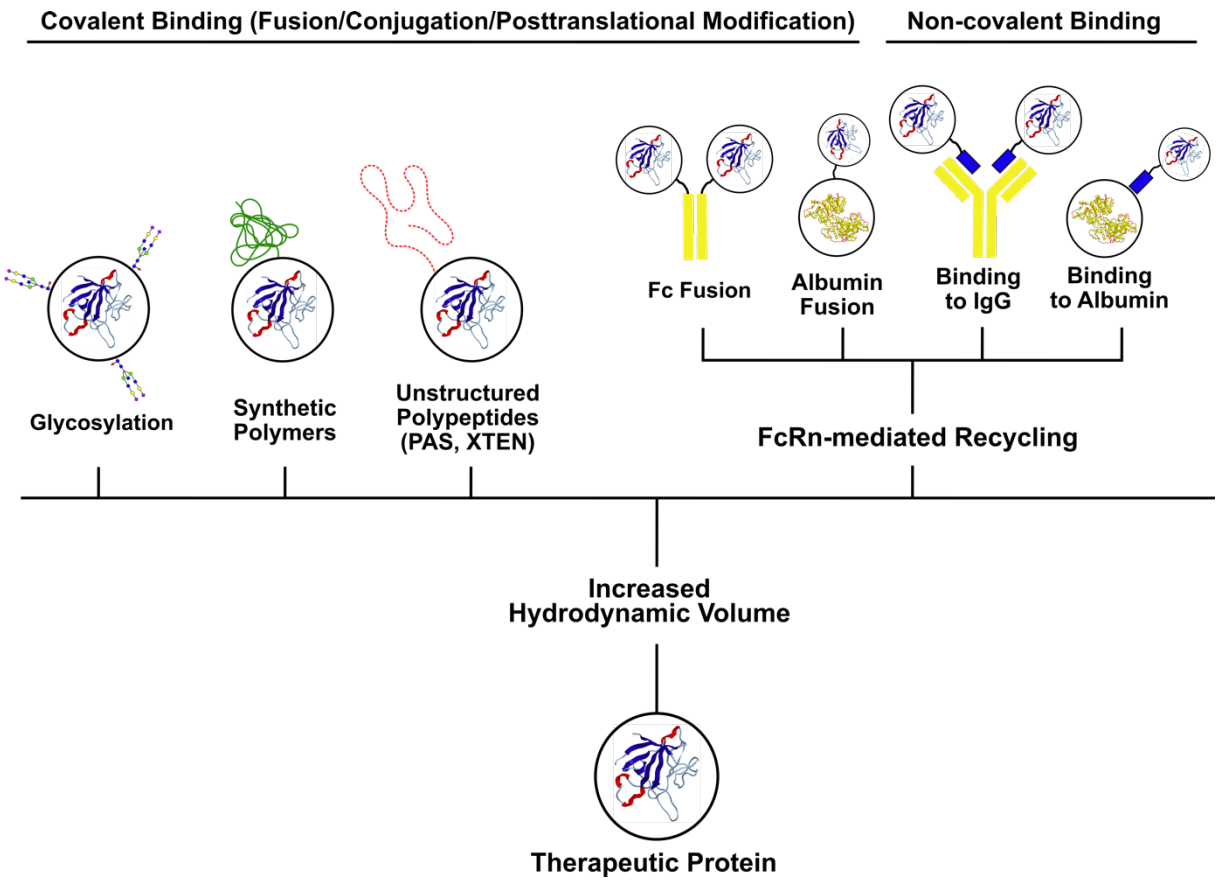

Figure 1. Overview of different half-life extension strategies for therapeutic proteins (adapted with permission from ref. [1]). Protein structures are from protein data base (PDB-entry: 1ilr, 1ao6).

Intravenous application circumvents this pathway, as the therapeutic proteins are directly exposed to the blood stream followed by distribution into the respective tissues. Elimination typically occurs by renal filtration and proteolytic degradation in plasma or, after pinocytosis-mediated cell-uptake, in lysosomes. Of these, kidney elimination displays the largest dependance on the mass of therapeutic proteins.[13]

The general approach to reach half-life extension of biopharmaceuticals is to focus on an increase of the hydrodynamic volume to reduce renal clearance (Figure 1). Therefore, the therapeutic biomolecule of interest is covalently attached to synthetic polymers or recombinantly expressed as a fusion protein resulting in longer blood circulation. The latter approach additionally leverages the neonatal Fc receptor (FcRn)-mediated recycling mechanism, where proteins fused to an albumin-molecule or the Fc-part of an IgG antibody are able to escape from endosomal degradation and can subsequently be released again into the blood stream.[14] Increase of negative charge (repulsion at negatively charged glomerular basement membrane) or replacement of degradation-prone amino acid residues can be pursued as well for half-life extension. Often several of these approaches are combined to exploit synergistic effects.[15]

2.1.2 Properties of PEG and its Impact on Biopharmaceuticals *in vivo*

Poly(ethylene glycol) (PEG), also called poly(ethylene oxide) (PEO), is a non-ionic macromolecule consisting of a polyether backbone and two functional end groups, which can be further modified (Figure 2). It is typically synthesized by anionic ring opening polymerization, initiated by the nucleophilic attack of a hydroxide ion on the monomer ethylene oxide (M_w = 44 g/mol), resulting in a bifunctional PEG-diol.[16] For protein modification, monofunctional methoxy-PEG (mPEG) is typically used to avoid cross-linking being synthesized by the same method but with a methoxide ion as initiator. The residual end group can be further modified and serves as functional moiety for covalent linkage to the respective biomolecule. PEGs employed for the modification of therapeutic proteins usually display a low dispersity (< 1.1)[16] and are used in linear or branched architectures,[17] with molecular weights between 2–40 kDa.[11]

Due to its high biocompatibility and low toxicity, PEG is generally regarded as safe and turned into one of the most frequent used excipients in drug delivery and pharmaceutical formulations. Also termed as macrogols, PEGs are listed in all main pharmacopoeias (United States, European and Japanese Pharmacopoeia) and are approved for the use in oral, topical and intravenous formulations. Depending on the molecular weight, PEGs can serve as stabilizers in liquid formulations, as binders and plasticizers in solid dosage forms or as base in creams and ointments.[18] Furthermore, PEG-based surfactants are applied for the stabilization of protein pharmaceuticals against aggregation and surface adsorption (Polysorbate: Tween®-20, Tween®-80) or as block-copolymer (Poloxamer 407, 188, Pluronic®) in the production of pharmaceuticals and cosmetics (Figure 2).[19]

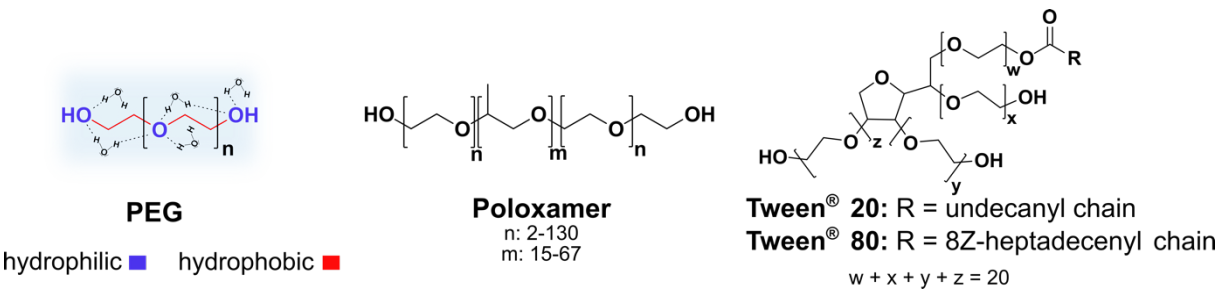

Figure 2. Structural overview of water-associated PEG and some of its employed forms in industry.

Covalent conjugation of PEG to liposomes or other nanocarriers leads to PEG's well-known stealth effect, which prevents interaction with plasma proteins and macrophages, thereby enhancing circulation time, biocompatibility and targeting of drugs.[18,20] Furthermore, PEG shows amphiphilic properties and is soluble in water and many organic solvents, therefore enhancing the solubility of many poorly soluble compounds.[21] Two structural components are responsible for this ambivalent behavior: ethylene groups represent the hydrophobic moiety, whereas the oxygen groups of PEG reflect hydrophilic properties (Figure 2). The presence of oxygen atoms is also responsible for the formation of a highly stable hydration layer around PEG, that usually consists of two to three water molecules per monomer unit, and results in a highly flexible polymer chain.[22] The oxygen spacing of the PEG backbone thereby nearly matches the hydrogen bonding of water and plays an important role in water solubility, as PEG's two "chemical neighbors", poly(methylene glycol) and poly(propylene glycol) are insoluble in water.[22]

Large PEGs up to a molecular weight of 50 kDa are pre-dominantly cleared without structural change *in vivo* by the urine, whereas at higher molecular weights, non-specific cell uptake, metabolism in the liver and clearance *via* feces also play a role.[23–25] The glomerular filtration barrier is considered to allow the passage of molecules smaller than 50–70 kDa in weight and 3–5 nm in size.[26] Although glomerular permeability is often related only to the molecular weight of a hydrophilic macromolecule, one should bear in mind that other parameters like charge, shape and size play a significant role as well.[4] For example, in the case of 30 kDa PEG with a diameter of about 8 nm, its high flexibility still enables filtration, despite its size being larger than the kidney pore size.[27]

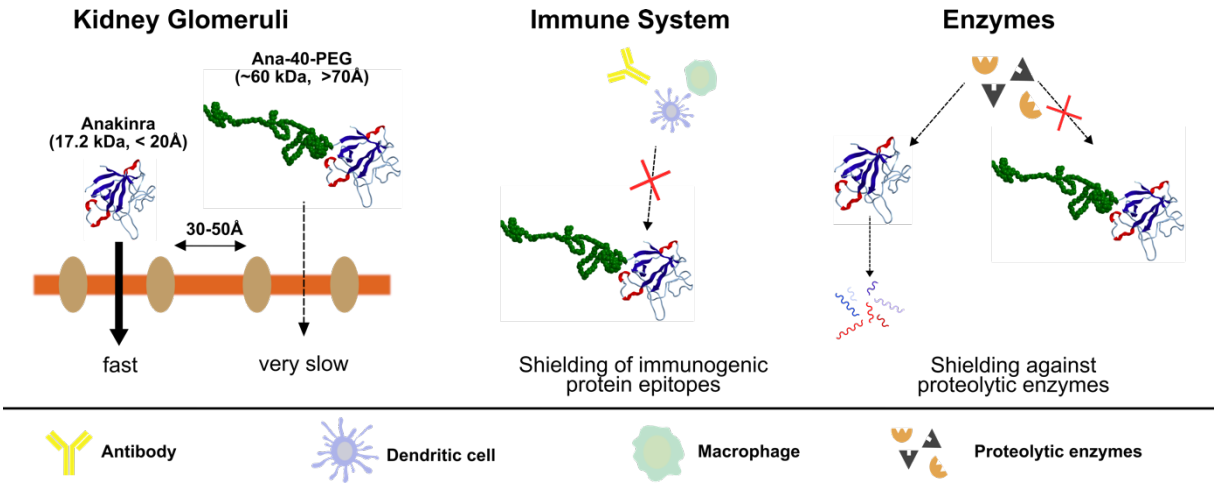

Figure 3. Impact of PEGylation on biopharmaceuticals.[28]

Upon conjugation to biopharmaceuticals, PEG drastically increases the hydrodynamic size and thereby reduces the glomerular filtration rate in the kidneys leading to an extended circulation time, a prolonged therapeutic effect, and a reduced dosing frequency (Figure 3).[28] One of the most prominent examples of that effect is probably the PEGylated form of Interferon-α2a (IFN-α2a, PEGASYS®). PEG-IFN displays only 7 % of initial bioactivity *in vitro* but led to a 50-fold extension of circulation half-life compared to the unmodified protein and therefore resulted in an extended therapeutic activity as well.[29] This phenomenon can be observed also for other PEGylated proteins like G-CSF[30] or TNF-α.[31]

Another major benefit introduced by PEGylation is its ability to alterate the immunogenic properties of proteins.[28] Due to their often non-human origin, many biopharmaceuticals are highly immunogenic and impede therapeutic usage. PEG is known to sterically shield immunogenic epitopes of proteins and to thereby prevent activation of macrophages, dendritic or other antigen-presenting cells of the immune system (Figure 3). The result is a protein-conjugate with better "acceptance" of the body, as immune response is diminished. This effect can be exemplarily observed in the case of PEGylated uricase. Humans and higher primates lack the enzyme uricase which is essential in reducing uric acid levels, especially in the disease state of gout. As uricase is of non-human origin it displays high immunogenicity, which was avoided and reduced upon PEGylation.[32] Additionally, PEGylation enhances proteolytic stability by its steric shielding effect on proteins towards metabolic enzymes thereby further extending overall half-life.[28]

The physical stability of proteins can be positively affected by PEGylation. Conformational and colloidal stability are the two main thermodynamic factors that impact physical stability of proteins in solution. Conformational stability is characterized by the protein's free energy of unfolding (ΔG_{unfold}) and correlates with its melting temperature T_m.[33,34] In most cases,

PEGylation increases the thermodynamic stability of proteins in solution.[35–38] Furthermore, PEGylation can improve colloidal stability through reduced protein-protein interactions, reduced aggregation and increased solubility[39,40] which prolongs the shelf-life and facilitates the storage of biopharmaceuticals.

2.1.3 PEGylation Chemistry

The covalent attachment of PEG to proteins can be achieved in a random- or site-selective manner using linear or branched PEGs, typically in the molecular weight range of 2–40 kDa. An overview of coupling chemistries of approved PEGylated biopharmaceuticals can be found in Table 2.

Table 2. Overview of coupling chemistries of approved PEGylated biopharmaceuticals.

PEGylated Drug [M_w in kDa]	PEG-M_w [No. of PEG/ Protein]	PEG-Architecture/ PEG-Linker	Modification Site [Resulting Linker Structure]
Pegademase (Adagen®) [96–126]	5 kDa [11–17]	linear/mPEG-SS	ε-amino groups of lysine residues [amide]
Pegaspargase (Oncaspar®) [483–548]	5 kDa [69–82]	linear/mPEG-SS	ε-amino groups of lysine residues [amide]
PEG-Interferon α-2b (PegIntron®) [31]	12 kDa [1]	linear/mPEG-SC	mixture of several isomers: $his^{7,34}$, several lys, cys^{1}, ser^{163}, tyr^{129} [amide]
PEG-Interferon α-2a (Pegasys®) [60]	40 kDa [1]	branched *via* lysine moiety/mPEG-NHS	ε-amino groups of lysine residues [amide]
Pegfilgrastim (Neulasta®) [39]	20 kDa [1]	linear/mPEG-propionaldehyde	*N*-terminal α-amino group [sec. amine]
Pegvisomant (Somavert®) [42–52]	5 kDa [4–6]	linear/mPEG-NHS	ε-amino groups of lysine residues [amide]
Pegabtanib (Macugen®) [50]	40 kDa [1]	branched *via* lysine moiety/mPEG-NHS	amine at the 5′end [amide]
PEG-EPO beta (Mircera®) [60]	30 kDa [1]	linear/mPEG-NHS	ε-amino groups of lysine residues [amide]
PEG-Certolizumab (Cimzia®) [91]	40 kDa [1]	branched *via* lysine/mPEG-mal	cysteine residue [thioether]
Pegloticase (Krystexxa®) [~545]	10 kDa [40.8 on avg.]	linear/mPEG-pNPC	ε-amino groups of lysine residues [amide]
Peginesatide (Omontys®) [45]	40 kDa [1]	branched *via* lysine moiety/mPEG-NHS	sec. amine on linker between the peptide-dimer [amide]
PEG-Interferon beta-1a (Plegridy®) [44]	20 kDa [1]	linear/mPEG-O2-propionaldehyde	*N*-terminal α-amino group [sec. amine]
PEG-Factor VIII (Adynovate®) [330]	20 kDa [2 on avg.]	branched *via* glycerol moiety/mPEG-NHS	ε-amino groups of lysine residues on B domain [amide]
PEG-Factor IX (Refixia®/Rebinyn®) [98]	40 kDa [1]	linear/PEG-conjugation by enzyme	*N*-linked glycans [-]

Elapegademase (Revcovi™) [115]	5.6 kDa [13]	linear/mPEG-SC	ε-amino groups of lysine residues [amide]
Calaspargase pegol (Asparlas™) [~313]	5 kDa [31–39]	linear/mPEG-SC	ε-amino groups of lysine residues [amide]
Pegvaliase-pqpz (Palynzig™) [~1,000]	20 kDa [32–36]	linear/mPEG-NHS	ε-amino groups of lysine residues [amide]
PEG Factor VIII (Jivi®) [234]	60 kDa [1]	branched /mPEG-maleimide	genetically engineered cysteine (K1804C) in A3 domain [thioether]

Data adapted from ref. 11, 12, 41, 42, 43 or public available sources (EU: European public assessment reports and Summary of Product Characteristics). For further references, see text. M_w: molecular weight; avg: average.

2.1.3.1 First Generation PEGylation

First generation PEGs are mainly used for random PEGylation by targeting amine groups of lysine residues utilizing linear PEGs with a molecular weight < 12 kDa.[16] Lysine residues are one of the most abundant amino acids found in proteins therefore enabling an easy approach with high yields. Drawbacks include a drastic decrease of bioactivity, unstable linkages and challenges in purification and analysis of these conjugates which impede batch-to-batch reproducibility or subsequent approval of the respective PEGylated protein.[44]

The activated PEG typically carries an electrophilic group that can be attacked by nucleophilic amine groups. The most widely used first generation PEGs belong to the group of carbonates (Figure 4: **3a-3d** and **4**),[45] which form a carbamate/urethane linkage upon reaction with a lysine. Out of these, PEG-succinimidyl carbonate (PEG-SC, **3a**)[46] and PEG-*p*-nitrophenyl carbonate (PEG-pNPC, **3c**) were employed in the synthesis of several approved PEGylated proteins. Furthermore, PEG-succinimidyl succinate (PEG-SS, **5a**)[47] can be used which is, however, highly prone to hydrolysis at neutral pH due to its ester bond.[48] Unstable linkers can be a problem, as they can serve as a new hapten on the protein surface thereby enhancing immunogenicity, which has been demonstrated already for PEG-asparaginase.[49] PEG-dichlorotriazine (**1**) or PEG-tresylate (**2**) result in a stable, secondary amine linkage but are rarely used anymore due to toxicity or unspecific conjugation leading to a mixture of products.[28]

2.1.3.2 Second Generation PEGylation

Second generation PEGs consist of higher molecular weight (> 12 kDa) and are methoxy-capped on one end (mPEG). They display a higher linker stability, higher coupling selectivity and are employed in linear and branched architectures. Branched, y-shaped PEG reagents can

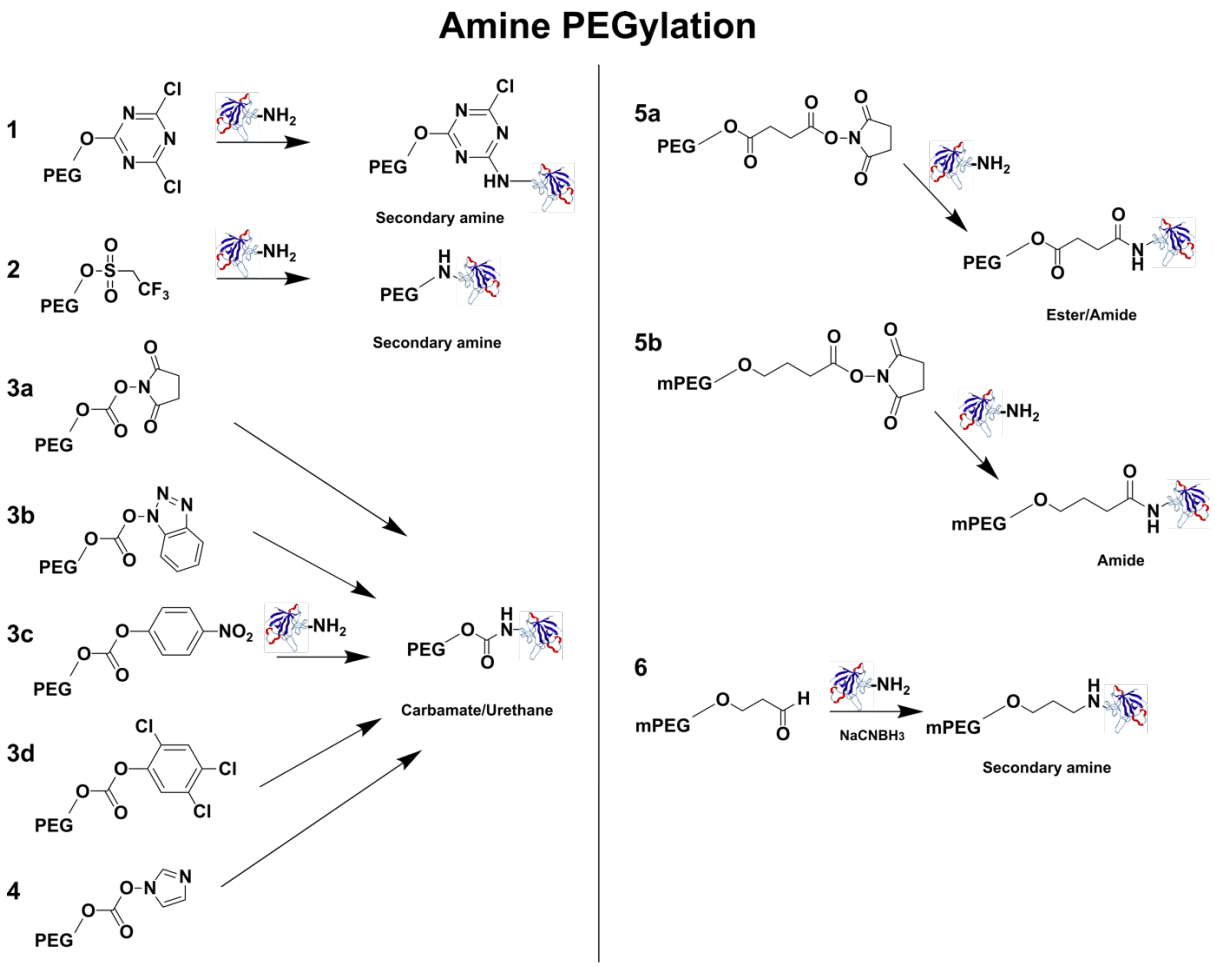

Figure 4. Overview of chemistries for amine PEGylation. Protein structure: PDB-entry 1ilr.

be generated, e.g., based on a lysine core and are more effective in reducing antigenicity, immunogenicity, and proteolysis than their linear analogs.[50] The following section provides an overview of second-generation PEG-chemistry.

Second Generation PEGs for Amine Modification

A more selective targeting of amine groups can be achieved using mPEG-*N*-hydroxy succinimide (Figure 4, mPEG-NHS, **5b**) or mPEG-propionaldehyde (Figure 4, mPEG-CHO, **6**). Methoxy-PEG-NHS is an improved variant to the initially used carbonate and succinate PEGs, leading to a more stable amide bond.[51] Control of reactivity and modification site can be achieved through adjustment of the spacer between the PEG backbone and the NHS-moiety,[52] where propionic- and butanoic-spacers are typically used. Half of all approved PEGylated products contain mPEG-NHS-based coupling chemistry (Table 2).

Another more selective approach is the employment of mPEG-propionaldehyde for conjugation to the *N*-terminal α-amino group. In this so-called reductive amination approach the electrophilic aldehyde group of mPEG is attacked by an amine residue to form an imine

bond, which is *in situ* reduced by a mild reducing agent like $NaCNBH_3$ (Figure 5). The result is a stable secondary amine linkage. In contrast to amide formation, this coupling strategy retains the charge properties of the native protein.[53] The control of attachment site can be achieved through pH adjustment, as the pk_a values between the *N*-terminal α-amino group (≈ 6–9) and the lysine ε-amino groups (≈ 10.5) differ therefore resulting in lower reactivity of the latter at acidic pH.[54,55] This technique has been employed for the approved drugs PEGfilgrastim (Neulasta®)[56] and PEG-IFN beta-1a (Plegridy®).[57]

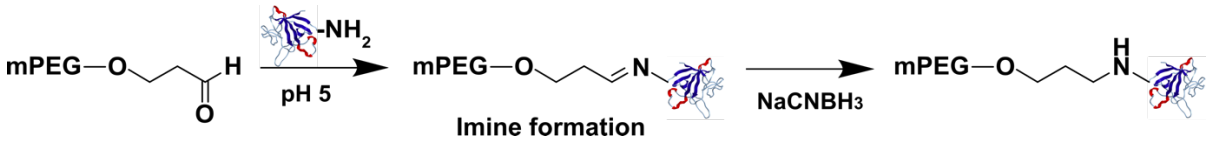

Figure 5. *N*-terminal ligation of a protein *via* reductive amination. The relative selectivity is achieved at acidic pH through differences in pk_a-values between ε-amino side chain groups (lysine) and the *N*-terminal α-amino group. Protein structure: PDB-entry 1ilr.

Second Generation PEGs for Thiol-Modification

Thiol groups at cysteines are one of the most attractive target residues for site-specific PEGylation, as cysteines are rarely present in proteins. PEGs used for this purpose include mPEG-maleimide (Figure 6, mPEG-mal, **7a**), mPEG-vinylsulfone (mPEG-VS, **7b**), mPEG-

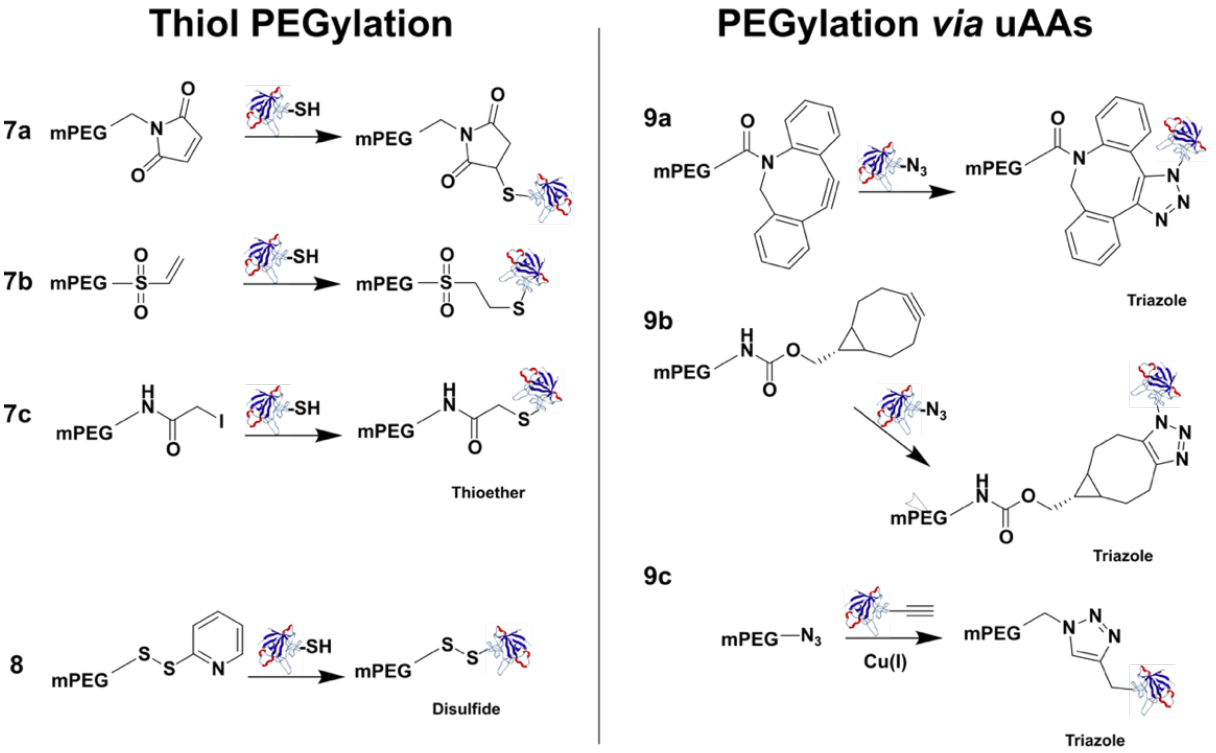

Figure 6. Overview of chemistries for thiol PEGylation (left) and site-specific PEGylation (right). uAA: unnatural amino acid.

iodoacetamide (mPEG-IA, **7c**) and PEG-*ortho*pyridyldisulfide (mPEG-OPSS, **8**). mPEG-mal (**7a**) is the most used reagent for thiol-modification and results in formation of a stable thioether linkage at slightly acidic pH. mPEG-IA and mPEG-VS are less reactive and specific and display coupling to lysine-residues as well (mPEG-VS). MPEG-OPSS can be employed to yield a stable disulfide linker, which is cleavable in a reductive environment.[16] Unfortunately, due to its hydrophobicity, many cysteines are buried inside the protein structure and are therefore not accessible for polymer conjugation. Cysteines can also be introduced to the protein by genetical engineering. However, care must be taken in its downstream processing and handling to prevent dimer formation *via* disulfide bridges. Several proteins and peptides have already been successfully PEGylated by thiol-maleimide coupling[58–60] with certolizumab Pegol (Cimzia®) and PEG-Factor VIII (Jivi®) being approved.[61]

Site-specific PEGylation by Azide-Alkine Cycloaddition

To enable absolute site-specificity and control of the polymer attachment site, unnatural amino acids (uAA) can be incorporated into a protein sequence, which carry functional groups that are only able to react with the corresponding "pair" on the polymer side. A major advantage of this strategy is that the conjugation site can be exceeded without drastically impairing the protein's bioactivity. A way to achieve this is by employing Cu(I)-catalyzed (CuAAC, Figure 6, **9c**) or strain-promoted azide-alkyne cycloaddition (SpAAC, **9a, 9b**). PEGylation of azide-modified human growth hormone (hGH) has already been demonstrated *via* SpAAC,[62] whereas Tamshen *et al.* followed a *vice versa* approach (Figure 7), where hGH was expressed as a propargyl-tyrosine variant and linked to PEG-azide under Cu-catalysis.[63] Other biomolecules have been PEGylated with a similar strategy[64,65] demonstrating the great versatility of this approach. However, site-specific coupling is not always feasible or leads to low yields, which makes this technology so far limited to the lab-scale.[44]

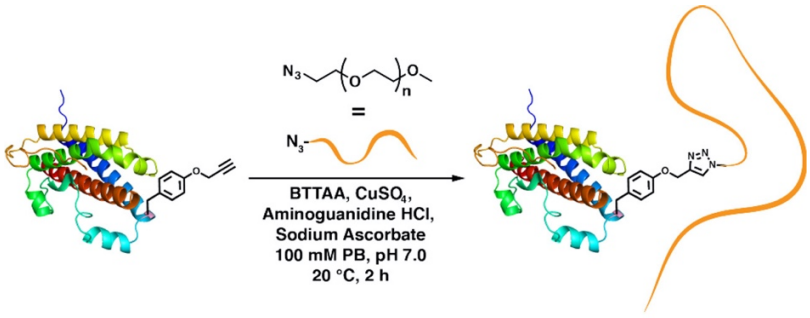

Figure 7. Site-specific PEGylation of human growth hormone *via* CuAAC. Reprinted with permission from ref. [63]. Copyright 2020, American Chemical Society.

Site-specific enzymatic PEGylation

For enzymatic PEGylation, the protein molecule is modified with a specific genetically encoded tag, that is recognized from the respective enzyme. The latter then catalyzes ligation of the PEG-substrate that carries a certain biorthogonal functional group.[66] The enzyme sortase uses an amino acid sequence (LPXTG) on the protein as recognition motif subsequently reacting with a polyglycine-terminated molecule[67] and has already been successfully applied for PEGylation of IFN-α2a and G-CSF.[68] Another enzyme for PEGylation is transglutaminase (TGase), that catalyzes an acyl transfer reaction between the γ-carboxamide group of glutamine residues and the primary amine groups of proteins or synthetic polymers.[43] Different variants of TGase exist, which impact reaction selectivity towards different glutamine residues[69] therefore demanding the appropriate enzyme form for site-specific PEGylation. Other enzymes include formylglycine generating enzyme, farnesyltransferase and sialyltransferase for glycoPEGylation.[43] The first approved PEG-protein generated by enzymatic PEGylation is Rebinyn®/Refixia®, a PEGylated recombinant blood coagulation factor IX.

2.1.4 Limitations and Drawbacks of PEG

2.1.4.1 Quality of PEGs, Impurities and Stability Issues

Despite its broad use, PEG also bears some disadvantages that may be problematic for its use in medical applications. PEGs are characterized by a certain polydispersity (M_w/M_n) which ranges from 1.01 (PEGs < 5 kDa) to 1.1 (PEGs > 50 kDa).[16,70] Despite approaches towards monodispersed PEG, dispersity must still be seen as a critical quality attribute for process control of manufacturing PEG-protein conjugates, as batch-to-batch variability may occur.

Another important aspect regards impurities and potentially toxic moieties involved during the synthesis of PEG. The gaseous monomer ethylene oxide employed for PEG-polymerization is toxic. Furthermore, a prominent impurity formed during synthesis is 1,4-dioxane, the cyclic dimer of ethylene oxide, which is classified as “possibly carcinogenic in humans” by the International Agency for Research on Cancer (IARC).[20] Additionally, mPEG, that is mainly used in the approved PEGylated biopharmaceuticals, contains up to 15 % of diol PEG which results from trace amounts of water during synthesis.[16] This could lead to cross linking of the respective conjugates and therefore demands purification of the PEG-reagent prior to protein conjugation. PEG also needs to be activated for protein functionalization which may create impurities that impact conjugate synthesis and stability within the final product.[71] Using a suitable initiator and/or termination reagent during polymerization circumvents this, however, demanding the validation of a new synthetic process.

Moreover, by-products formed during storage need to be considered, as they can alter properties of the protein drug. Due to its ether linkages, PEG is susceptible to form peroxides upon high-temperature treatment or light exposure leading to chain scission and increased polydispersity.[72] This is not only limited to PEG, but has also been reported for ethylene glycol-based surfactants like polysorbate, which is often used in commercial protein formulations.[73] PEGs are therefore recommended to be stored light-protected, under an inert atmosphere (argon, nitrogen) and at lower temperatures (below –15 °C).[72] Impurities related to PEG need to be avoided and continuously monitored by a variety of analytical techniques to ensure a reproducible PEGylation process, high product quality and safety for the patient.[74]

In *in vivo* surroundings, PEG shows relatively high stability which is not always desired especially when it comes to metabolism and elimination. Once the PEG portion is cleaved from the protein drug, it follows fast elimination *via* the kidney/urine (< 30 kDa) or non-specific uptake into cells *via* pinocytosis for larger PEGs.[25] Due to the non-biodegradability and the lack of mammalian etherase, PEG might persist in cell vacuoles for up to 2 months, which was assumed for PEGylated TNF.[75] Furthermore PEG-induced vacuolation has been reported for several organs of different animal species with no clinical manifestations or relevant toxicities so far being observed, even in the case of larger vacuoles.[76,77] However, these reports demand an even more critical evaluation regarding the safety of a PEGylated protein drug.

2.1.4.2 Anti-PEG Antibodies and Immunogenicity

Generally, PEG is seen as a non-toxic, non-immunogenic and biocompatible polymer. However, the recent two decades have unraveled some findings about PEG that were thought to be negligible. Already in the 1980s, Richter, *et al.* reported the prevalence of antibodies (Abs) against PEG in healthy individuals (0.2 %, 1984),[78] which continuously increased within the last decades to 27 % (Armstrong, *et al.*, 2003)[79] and 72 % (Yang, *et al.*, 2016)[80] (Figure 8). It is speculated that the continuous exposure to PEG and PEG-based surfactants in daily

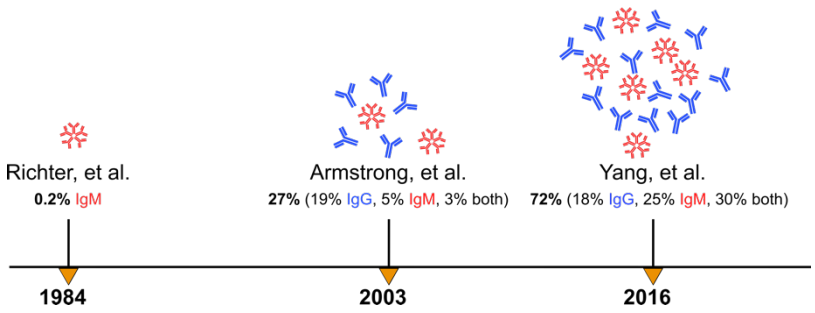

Figure 8. Evolution of anti-PEG antibodies among the healthy population.[78–80]

household and cosmetic products could lead to the formation of Abs, but the mechanism behind is not yet fully understood.[81] Those antibodies are not necessarily linked to a neutralizing effect in PEGylated therapies,[49] but could play a role in some cases of hypersensitivity reactions (HSR) upon treatment with PEGylated drugs.[82] IgE-mediated[83] and non-IgE-mediated (pseudoallergic)[84] HSRs against PEG-containing products have been observed in a few number of cases. Rare anaphylactic reactions reported for the new SARS-CoV-2 mRNA vaccine by BioNTech-Pfizer might be attributed to the 2 kDa PEG-moieties located on the surface of their lipid nanoparticle formulation, which led to the general recommendation to exclude patients with a history of immediate-type hypersensitivity against PEG or PEG-similar excipients (e.g., Polysorbate 80) from vaccination, until further information is available.[85]

Anti-PEG Antibodies (Abs) have also been reported for PEGylated proteins in clinic and were associated with a loss of therapeutic efficacy,[86] for example in the case of Pegloticase (PEG-Uricase).[87,88] Rapid drug clearance of PEG-asparaginase was correlated with the formation of anti-PEG Abs[49,89] where the PEG-protein linker might affect the immune response. For example, bulky aromatic or heterocyclic groups can promote antibody formation against PEG.[90] Furthermore, different linker structures can result in a different antibody response, as demonstrated for PEG-asparaginase.[49,91] An accelerated blood clearance (ABC) phenomenon induced by anti-PEG Abs has further been widely reported for repeated injections of PEGylated nanoparticles (for detailed reviews see ref. 92 and 93) and was also described for PEGylated ovalbumin, where the IgM-subtype was a major contributor for ABC.[94] Interestingly, anti-PEG Abs induced by PEGylated liposomes did not lead to ABC of PEGylated ovalbumin, but *vice versa*, suggesting a high specificity for IgM anti-PEG Abs.

Typically, 3–6 oxyethylene groups of PEG are needed to bind to its anti-PEG antibody.[95,96] Studies on antibody specificity in rabbits revealed an impact of end-group hydrophobicity on the immunogenicity of PEG, following the order of tert-butoxy-PEG (t-PEG) > mPEG > OH-PEG. Larger end group hydrophobicity led to the formation of an additional antibody population which displayed relatively higher selectivity to the PEG end group than to its backbone. In the case of OH-PEG, only anti-PEG Abs against PEG-backbone were observed.[97,98]

The mechanism behind anti-PEG Abs formation is still not fully understood. In general, one must distinguish between PEGylated nanoparticles, that usually carry a higher PEG density on their surface, and PEGylated proteins, where ~2/3 of the approved drugs bear only one or two PEG chains per protein. Ishida *et al.* suggested a T-cell independent (TI) mechanism for anti-PEG IgM antibody formation of PEGylated liposomes. Thereby, the PEG-coated

liposomes act as a T-cell independent type 2 (TI-2) antigen, that usually correlates with the presence of repetitive structures like polysaccharides,[99] and is recognized by marginal zone B-cells in the spleen that lead to formation of anti-PEG IgM antibodies.[100] A similar mechanism has been supported for PEGylated proteins by Kloos *et al.*, who suggest that the ethylene oxide repeating units of PEG could serve as TI-2 antigen for IgM-induction.[49] Other findings by Mima *et al.* propose a distinct mechanism of antibody formation between PEGylated proteins (in their case PEGylated ovalbumin) and PEGylated liposomes by employing the hapten-carrier system. They argue that PEG serves as a hapten that only elicitates immune response upon conjugation to a carrier in a T-cell dependent (ovalbumin as carrier) or T-cell independent (liposome as carrier) manner.[94]

Further studies need to be done to elucidate the mechanisms behind anti-PEG antibody formation and its clinical relevance. To avoid vanishing of the initial benefits introduced by PEGylation, routine screening on pre-existing anti-PEG Abs could be employed to survey clearance and therapeutic efficacy and might also be a helpful tool in deciding for non-PEGylated alternative therapies.[89] Other studies suggest the pre-treatment with free PEG to prevent neutralization by anti-PEG antibodies.[101]

2.1.4.3 Reduced Affinity and Activity of Biomolecules

Upon PEG-conjugation, many proteins and peptides display a reduced receptor affinity and bioactivity *in vitro*. The decrease in activity upon PEGylation correlates with 1) an increase in PEG molecular weight, 2) a higher number of attached PEGs, 3) randomly attached PEGs, 4) linear PEG architecture (compared to branched). Steric hindrance plays the main role in this mechanism, as PEG behaves like a highly flexible coil which shields active protein sites from interaction with their receptor.[70] However, a reduction in receptor affinity can be balanced by a prolonged circulation time, which leads to an overall extension of therapeutic activity. For example, PEGylated IFN-α2a displays only an *in vitro* activity of 5–10 % but a significantly extended antitumor effect *in vivo*, compared to the unmodified protein.[29]

Better control of activity can be gained by a site-specific PEGylation approach supported by computational studies to unravel protein sites located further away from the active binding pocket. Tamshen and co-workers found superior activity of site-specific mono-PEGylates of human growth hormone (hGH) compared to the commercial random- and multi-PEGylated PEGvisomant.[63] Similar results were obtained from Basu *et al.* for mono-PEGylated IFNβ-1b.[39] Furthermore, PEG architecture plays a role in bioactivity, where branched PEGs seem to

perform better than their linear analogs.[50] The type and number of PEG-molecules used for modification should therefore be considered when designing a PEGylated biopharmaceutical.

2.1.4.4 Effect on Protein Stability and Viscosity

PEGylation of proteins can lead to a decrease in thermodynamic stability. For example, Plesner *et al.* have found an ~2 °C lower T_m for PEGylated bovine serum albumin (BSA), compared to the unmodified protein. Interestingly, the length of PEG had no significant effect on T_m-values of PEGylated BSA.[102] Similarly, a decrease in melting stability has been found for PEGylated lysozyme[103] and myoglobin.[104] Additionally, PEG bears a high solution viscosity due to chain entanglement at high concentrations, which increases in a molecular weight-dependent manner.[105] This can impede certain manufacturing processes as well as limit its applicability for the injection of high-concentrated protein-formulations.

2.2 Analytical Techniques for the Characterization of PEG-Protein Conjugates

2.2.1 Sodium-dodecylsulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE is a relatively easy technique to monitor the reaction process and the purity of PEG-protein conjugates. The principle relies on the mass-based separation of denatured protein molecules in an electric field, with the negatively charged surfactant SDS surrounding the protein moieties enabling their migration towards the anode. A typical SDS-gel consists of a stacking gel (loading and up-concentration of the samples) and a subsequent separation gel. The latter separates the samples solely depending on their molecular weight and is stained thereafter by Coomassie or silver staining to visualize the protein bands. In the case of PEGylated proteins, the PEG-portion can also be stained by a protocol involving barium iodide.[106]

Care must be taken in the interpretation of the molecular weight when employing a protein standard molecular weight ladder. The mobility of PEG-conjugates in the gel is always slightly diminished, which is possibly due to interference between SDS and the PEG-part of the conjugate thereby slowing down the migration in the gel.[106,107] Furthermore, the dispersity of polymers sometimes creates smeared bands, which might impede distinguishing different species on the gel.

2.2.2 Size-exclusion Chromatography (SEC)

Size-exclusion chromatography is employed to determine purity and molecular weight of macromolecular species in solution. Separation in SEC is based on the hydrodynamic size of a macromolecule and its distinct permeation/diffusion through the pores of a stationary phase (dextran- or polystyrene-based) being monitored by refractive index- or UV-detection.[108] Large molecules are excluded more from the pores therefore eluting earlier than smaller moieties, which allows the determination of the distribution coefficient K_{av} (= $(V_R–V_0)/ (V_c–V_0)$), where V_R, V_0, and V_c represent elution volume of the analyte, void volume, and the total column bed volume, respectively. The K_{av} of reference standards can be plotted against their molecular weights to create a calibration curve, where the molecular weight of the unknown analyte is determined.[109] Upon PEG-conjugation, a drastic increase in hydrodynamic size is usually observed shifting the elution in SEC to earlier time points. However, the molecular weight cannot be directly derived from a pure protein or PEG calibration curve, as PEGylated proteins behave as "hybrid" forms.[109] In this case, SEC needs to be coupled to other detectors, (multi-angle light scattering (MALS), MS-detector) to determine the absolute molecular weight. Furthermore, SEC can be employed to determine and predict the hydrodynamic radii of PEGylated proteins, where a column-specific, universal calibration curve is used.[109,110]

2.2.3 Dynamic Light Scattering (DLS)

Dynamic light scattering is a possible technique to determine the hydrodynamic size of PEGylated proteins. It measures the intensity fluctuations of scattered monochromatic light, which are caused by the size-based diffusion of particles in solution (Brownian motion) over a certain time.[111] The diffusion depends on solution temperature (T), solution viscosity (η) and the size of the macromolecular particles (R_h), with larger molecules diffusing more slowly therefore displaying less fluctuations. Digital correlation of the intensity fluctuations in regard to time (ns-µs) allows the determination of the translational diffusion coefficient D_τ, which can be further used to calculate the hydrodynamic radius R_h following the Stokes-Einstein equation:

$$D_\tau = \frac{k_B T}{6\pi\eta R_h}$$

2.2.4 Enzyme-linked Immunosorbent Assay (ELISA)

ELISA is a method to quantify and detect a specific substance, often an antigen, in a complex sample. The antigen of interest is typically coated either directly or by employing a

specific capture antibody on a microplate at basic pH.[112] An enzyme-labeled, primary detection antibody is added and forms an antigen-antibody complex which leads to enzyme-mediated colour formation upon addition of a substrate and can be detected in a microplate reader (direct ELISA, Figure 9A). The enzyme can also be attached to a secondary detection antibody, that

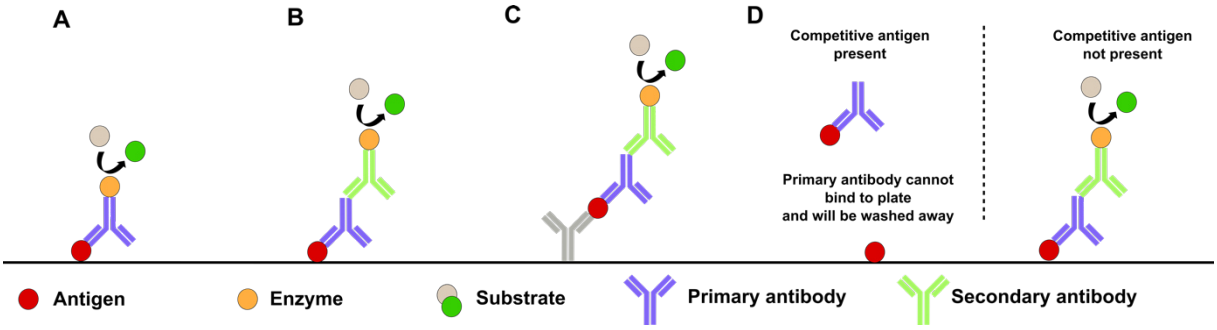

Figure 9. Overview of different ELISA methods.

binds to the Fc-part of the primary antibody to increase specificity (indirect ELISA, Figure 9B). More complex samples are analyzed by a "sandwich ELISA", where the antigen is captured by an immobilized antibody and subsequently detected by addition of the primary antibody and the enzyme-labeled secondary antibody (Figure 9C).[112] Another approach employs competitive binding of an immobilized antigen and the same antigen in an analytical sample, where colour formation is suppressed when the free antigen is present (competitive ELISA, Figure 9D). A modified version of the latter was used to detect the cross-reactivity of anti-PEG-antibodies towards other synthetic macromolecules.[113]

ELISA is an important tool to detect anti-PEG antibodies and to determine the immunogenicity of PEGylated proteins, even though the lack of reference sera and standardized assay procedures still impede data assessment.[114] Furthermore, the *in vivo* half-life of a PEGylated protein is typically determined by ELISA through quantification of the remaining analyte in blood samples from different time points.

2.3 Alternative Macromolecules for Half-Life Extension

The following chapter provides an overview of PEG-alternative macromolecules for the half-life extension of biopharmaceuticals.

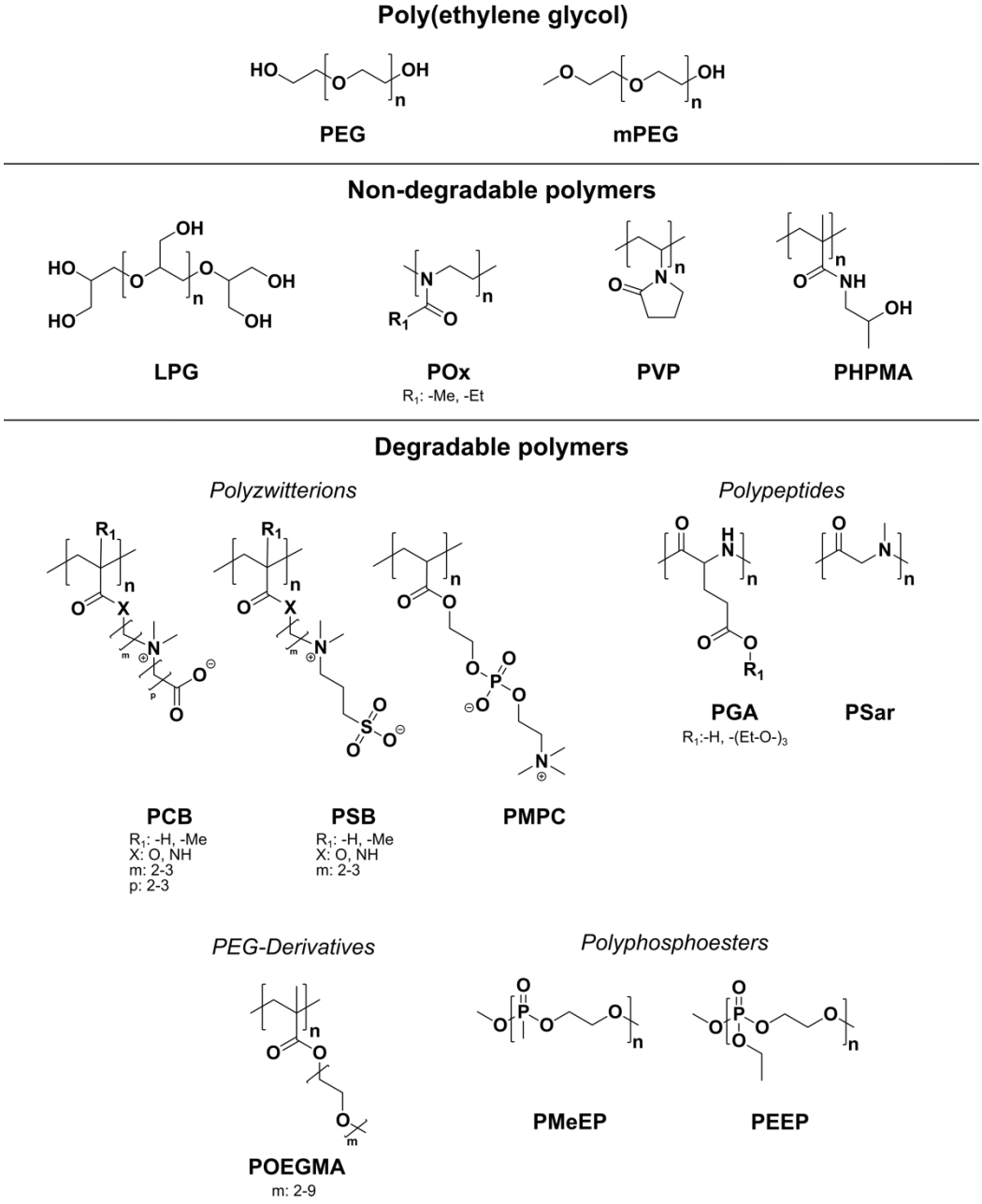

Figure 10. Overview of synthetic PEG-alternative macromolecules for half-life extension discussed in this thesis.

2.3.1 Non-degradable synthetic Polymers

2.3.1.1 Polyglycerol (PG)

Synthesis of end-functional linear Polyglycerol (LPG)

Linear Polyglycerol (also named polyglycidol) is a water-soluble and highly hydrophilic polymer, consisting of a polyether backbone with methyl hydroxy groups on the side chain. It

can be generated in various architectures, such as linear, hyperbranched (HPG) or dendronized brush-type (denPG), to name just a few (Figure 11B).[115,116] LPG is typically synthesized in a three-step process, that involves (1) protection of the glycidol monomer with ethyl vinyl ether to yield ethoxyethyl glycidyl ether (EEGE), (2) anionic ring opening polymerization (AROP) and (3) acidic deprotection (Figure 11A). It is important to protect the monomer hydroxyl groups prior to polymerization to avoid proton transition and gain better control over polymerization.[115] Various glycidol derivatives have already been exploited as monomers for polymerization that allow the introduction of different backbone-functionalities or -architectures.[116,117] However, due to the facile acidic deprotection under mild conditions, EEGE is mostly used.

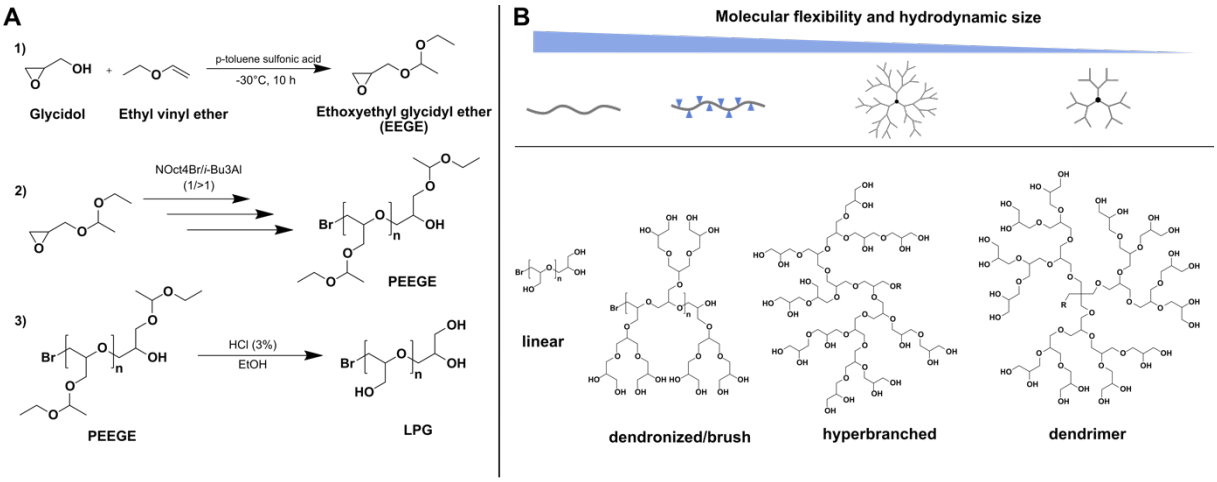

Figure 11. A) Synthetic scheme for linear polyglycerol (LPG). B) Different architectures of PG for potential conjugation to biopharmaceuticals. The size and molecular flexibility decrease with more rigid, branched polymer structures.

A major advantage is that glycidyl ethers are liquid and therefore easier to handle than the gaseous, toxic ethylene oxide (EO), which is employed as monomer in the synthesis of PEG.[117] Alkali metal alkoxides or ammonium salts typically serve as initiators and are often combined with activators like triisobutyl aluminum (*i*-Bu_3Al). The latter increases the reactivity of the monomer towards nucleophiles and simultaneously reduces the basicity of the growing chain end thereby preventing transfer reactions of protons adjacent to the oxirane ring, which usually limit the final molecular weight to around 30 kDa.[117]

To enable a more controlled polymer conjugation toward therapeutic proteins, end-functional polymers are desired that enable further modification with linkers or direct coupling to the respective biomolecule. Gervais *et al.* employed tetraoctylammonium bromide as initiator to synthesize end-functionalized LPG-Br with molecular weights (M_n) up to 85 kDa and dispersities (M_w/M_n) below 1.3. Higher molecular weights were thereby achieved by increasing

the [*i*-Bu_3Al]/[initiator] ratio up to 5.[118,119] The same authors also reported tetrabutylammonium azide as initiator to directly enable mono-functional LPG-N_3,[119,120] which can be additionally modified or directly used for bioconjugation. Further reduction of LPG-N_3 yields LPG-NH_2 to allow the introduction of functional moieties (e.g., aldehyde-linker) for site-directed protein modification. Alternative mono-LPGs have been described in literature including LPG-NH_2,[121,122] -SH,[122,123] -propargyl[124] or -vinylsulfonate.[125]

Biocompatibility, Conformation and Applications on Protein Delivery

Polyglycerol can be generally considered as a biocompatible and non-toxic polymer. It is approved as non-ionic surfactant (oligoglycerol-monoesters of fatty acids) by regulatory authorities to be used as pharma and food additive since several decades.[126] Early studies by the Brooks group examined the blood compatibility of polyglycerol (M_w ~6 kDa) with linear or hyperbranched architectures. They found no significant effects on blood coagulation and complement activation up to concentrations of 44 mg/mL and 20 mg/mL, respectively. Furthermore, no significant increase in blood viscosity or aggregation of red blood cells was observed,[127] which was similarly confirmed for hyperbranched polyglycerols (HPG) of very high molecular weights (M_n: 540 kDa, 871 kDa).[128] Another interesting study of the Kizhakkedathu group compared LPG, HPG and PEG of similar molecular weights (M_w: 100–120 kDa) regarding their biocompatibility *in vitro* and circulation time *in vivo*.[129] No significant impact on complement activation, blood coagulation parameters or red blood cell (RBC) aggregation was found for LPG and HPG (max. tested concentration: 10 mg/mL)

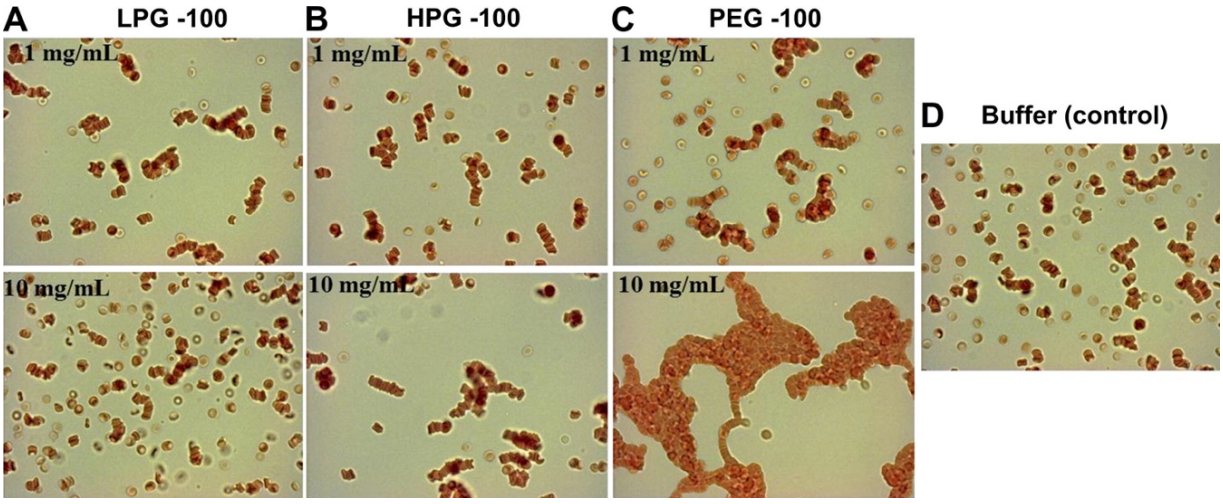

Figure 12. Optical micrograph analysis of red blood cells after incubation with polymers for 1 h at 37 °C, compared to control. Final polymer concentrations: 1 or 10 mg/mL. A) LPG-100, B) HPG-100. C) PEG-100. D) Buffer control. Reprinted from ref. [129]. Copyright 2012, with permission from Elsevier.

(Figure 12A/B). In contrast, PEG led to platelet and complement activation, cell death and severe aggregation of RBCs at 10 mg/ml (Figure 12C). Furthermore, it displayed a much higher intrinsic viscosity than LPG or HPG, which is of disadvantage in applying highly concentrated drug formulations.[129] Despite its small hydrodynamic size, the blood circulation time of LPG in mice was considerably longer than that of other linear polymers, including PEG, suggesting a promising potential for LPG as tool for half-life extension of therapeutic proteins. The authors attributed this to the more compact and less flexible conformation of LPG exhibiting a very small frictional ratio (a parameter that defines shape of polymers), which resulted in lower glomerular permeability but longer vascular residence time.[129]

A major problem reported for PEG conjugated to proteins or nanocarriers is the formation of antibodies, that vanish the initial benefits of PEGylation through a phenomenon termed as accelerated blood clearance (ABC). PEGylated liposomes exhibit ABC, that, however, strongly depends on dosing regimen, PEG-density and other factors.[93] PG bears protein-resistant properties with a similar stealth effect to PEG.[126,130,131] Studies by Abu Lila *et al.* showed the prevention of ABC on liposomes[132] and lipoplexes[133] when replacing the PEG-moiety with LPG which could be beneficial also to prevent the formation of anti-polymer antibodies for PEGylated proteins.

LPG has been conjugated so far to model proteins like bovine serume albumin (BSA) and lysozyme[121]. Additionally, a grafting-from approach employed BSA to synthesize linear PG-conjugates with controlled branching on its backbone.[134] Furthermore, PG-based nanogels (NG) were successfully applied in the field of protein delivery.[135–137] Steinhilber *et al.* synthesized dendritic polyglycerol (dPG)-based nanogels for highly efficient encapsulation of asparaginase, that still showed full activity and structural integrity upon release.[135] Another study by Witting *et al.* described the synthesis of thermoresponsive PNIPAM-dPG nanogels for intraepidermal delivery of asparaginase, which was first encapsulated and successfully delivered in barrier deficient skin upon release at ≥ 35 °C.[137] This further demonstrates the great versatility of PG-based systems as a tool for delivery of biopharmaceuticals.

2.3.1.2 Poly(2-oxazoline) (POx)

Polyoxazolines (POx) have been shown to be a promising polymer class for the use in medical applications and drug delivery systems.[138–140] They are synthesized by cationic ring opening polymerization (CROP) employing an electrophilic initiator, like alkyl-tosylate or -triflate, which is added to the respective 2-oxazoline monomers in a dry organic solvent and under inert atmosphere (Figure 13).[141,142] The polymerization is terminated by adding a

nucleophile, such as OH^-, $-NH^-$, $-S^-$ or $-COO^-$.[143] To shorten the reaction time a microwave-assisted synthesis of POx has been developed recently.[144] POx allows the introduction of side-chain functionalities to control solubility, thermal properties or targeting. Due to their similar

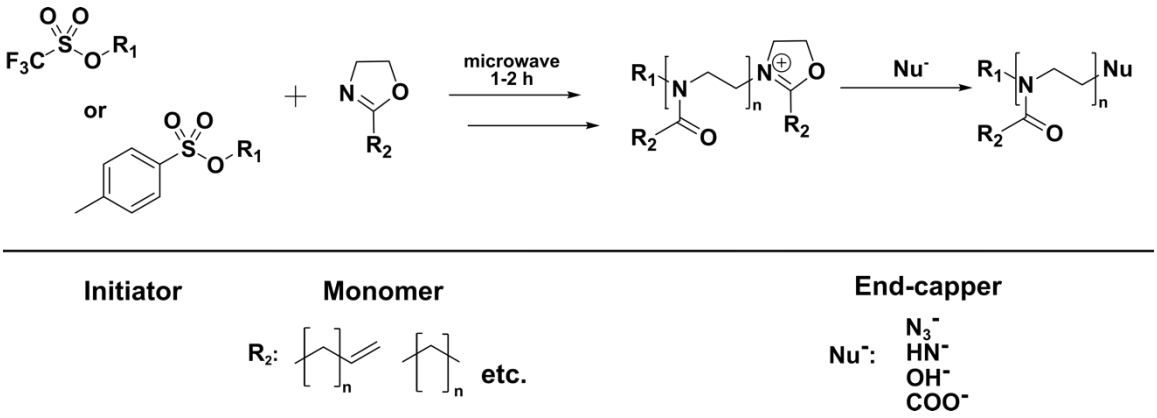

Figure 13. Synthetic scheme for cationic ring opening polymerization of poly(2-oxazolines).

chemical composition and the presence of a peptide bond per repeating unit, POx can also be regarded as pseudo-polypeptides.[145] For protein conjugation, 2-methyl-(PMeOx) and 2-ethyl-2-oxazolines (PEtOx) are mainly employed and described here in more detail.

PMeOx and PEtOx show a good hemocompatibility and cytotoxicity profile *in vitro*,[146–148] with no significant accumulation in organs up to 30 kDa.[149,150] POx did not induce antibody-formation in rabbits[151] and prolonged the blood circulation of liposomes upon grafting, thereby suggesting a similar stealth behavior than PEG.[152,153] With increasing chain length, differences in amphiphilicity can be observed where PMeOx displays a hydrophilicity similar to PEG, whereas PEtOx shows more hydrophobic character.[143] The hydrodynamic sizes of PMeOx and PEtOx in aqueous solution are similar, but slightly smaller compared to PEG, therefore suggesting a smaller extent of solvation for POx.[143,154] Nevertheless, Gubarev *et al.* found a flexible, PEG-like conformation and rigidity for PEtOx in buffer.[155]

Polyoxazolines have already been successfully conjugated to proteins like G-CSF,[156] EPO[157] or others[158] by employing random or site-selective (*N*-terminal, enzymatic) conjugation chemistry. Lühmann *et al.* employed bioorthogonal CuAAC to synthesize site-specific conjugates of interleukin-4 with PMeOx of 4 kDa.[65] Conjugation yields were higher than with the respective PEG of same molecular weight. The thermal stability upon conjugation was improved while the bioactivity of IL-4 *in vitro* was retained.

During the synthesis of POx, various side reactions can occur that result in high molecular weight impurities of up to 10 % with dispersities (Đ) of >1.3, that are sometimes difficult to remove and can impede scale-up and reproducibility.[143] Under oxidative stress conditions, POx

is less stable than other linear polymers like PEG or PVP.[159,160] Additionally, the amphiphilicity and comparably low cloud point in salt solutions could lead to higher aggregation, especially in the case of PEtOx, that could hamper further protein conjugation and decrease coupling yields.[161] PEtOx displays slightly worse stealth characteristics compared to PEG, as demonstrated for the immunocamouflage of polymer-grafted blood cells.[162] Additionally, accelerated blood clearance was found for POxylated liposomes upon repeated administration, which was correlated with the formation of anti-POx IgM antibodies.[163] Furthermore, Luxenhofer *et al.* found complement activation for PMeOx and and PEtOx block copolymers that were, even though moderate, still significant above negative control.[164]

2.3.1.3 Poly(*N*-vinylpyrrolidone) (PVP)

Poly(*N*-vinylpyrrolidone) (PVP) is a regulatory approved polymer, that is widely used as pharmaceutical excipient, e.g., as solubility enhancer, stabilizer, binder, disintegrant or coating in tablet formulation.[165] Its applicability for biomedical applications was strongly limited as its synthesis followed the conventional free radical polymerization mechanism which hampered the production of end-functional polymers with low polydispersity.[166] Recently PVP has been successfully synthesized from its monomer *N*-vinylpyrrolidone by reversible addition-fragmentation chain transfer (RAFT) polymerization to enable lower dispersity and functional thiol- or aldehyde-endgroups, that were successfully conjugated to model proteins.[167,168] Earlier studies reported the conjugation of PVP (5 and 6 kDa) to amine groups of the therapeutic protein TNF-α by employing NHS-based chemistry. The resulting conjugates displayed longer half-life[169] than PEG-analogs of same molecular weight and had an increased antitumor potency in mice.[170] However, the molecular weights used in these studies were quite low and therefore still leave room for optimization of PVP in the field of half-life extension of biomolecules.

2.3.1.4 Poly(*N*-(2-hydroxypropyl) methacrylamide) (PHPMA)

Poly(*N*-(2-hydroxypropyl) methacrylamide (PHPMA) is a neutral, hydrophilic and biocompatible polymer. It has been investigated intensively as drug delivery vehicle for anticancer drugs, site specific drug delivery in tumor and gastrointestinal compartments and as scaffold for hydrogels. Similar to PVP, free-radical polymerization as synthesis pathway limited the applicability of PHPMA for protein conjugation until a RAFT-based polymerization method was developed by the McCormick group.[171] Following that approach, Davies and co-workers synthesized PHPMA (3.5 and 6.6 kDa) with a terminal thiazolidine-2-thione group which was used for conjugation of lysozyme.[172] The same group also developed a thiol-reactive

branched PHPMA (~20 and 30 kDa), which was successfully conjugated to the free cysteine of BSA.[173] Recently, the conjugation of NHS-activated PHPMA (5.5 kDa) to asparaginase was reported. PHPMA led to a strong disruption of asparaginase's secondary structure, however, increased its stability against freeze-thaw stress for up to six cycles.[174]

2.3.2 Degradable synthetic Polymers

Degradable synthetic polymers for conjugation to therapeutic proteins include the classes of polyzwitterions (PZIs/polybetaines), polypeptides (PGA, PSar), PEG-derived methacrylates (POEGMA) and polyphosphoesters (PPEs). They are all biodegradable to a certain extent and therefore avoid accumulation in organs, a problem often observed for PEG. Remaining PEG-linker fragments on the protein were shown to serve as haptens acting as a trigger for an immunologic reaction against the protein part. Therefore, the haptenic character of such hydrolyzed polymer-components should always be considered.

2.3.2.1 Polyzwitterions (PZIs/Polybetaines)

Polyzwitterions (PZIs), also named polybetaines, are a special class of polyampholytes that contain both a positively and a negatively charged moiety on the same monomer leading to an overall neutrally charged macromolecule. The backbone usually consists of poly(meth)acrylic acid linked to the charged linker chain *via* an amide or ester bond. Quarternized ammonium groups represent the cationic element whereas the anionic moiety stems from sulfonate-, carboxy- or phosphate-groups leading to various subclasses, that are polysulfobetaines (pSB), polycarboxybetaines (pCB) or polyphosphobetaines (pPB), among others.[175] Polybetaines are mostly generated by controlled radical polymerization (ATRP or RAFT) on either zwitterionic monomers or uncharged monomers followed by zwitterionic functionalization of the resulting polymer.[175] PZIs are considered as highly hydrophilic, biocompatible materials and display strong anti-fouling properties outperforming PEG in regard to non-specific protein adsorption from blood plasma and serum.[176,177] This effect is mostly attributed to a strong hydration layer formed by the polymer. However, in contrast to other hydrophilic macromolecules, that achieve hydration through hydrogen bonding,[176] PZIs display even stronger hydration and water binding *via* electrostatic interactions.[178–180] Their conformation in aqueous solution is highly dependent on pH, ionic strength and salt type and can be described by the so-called "antipolyelectrolyte effect" which leads to a chain expansion and an increase in viscosity upon addition of low molecular weight electrolytes.[175,181] This effect, and the strong intramolecular

interactions of PZIs, both result in a high solution density, compact structure and smaller hydrodynamic size than PEG of same molecular weight.[182]

Besides pPB, which has been successfully conjugated as methacryloyl-derivative (PMPC) to IFN-α[183,184] and exenatide,[185] one of the most widely used PZI-subclasses for protein conjugation is polycarboxybetaine (pCB). pCB consists of a quarternary amine group and a negatively charged carboxylate with varying distance possible between the charged groups. (Figure 13D). NHS-based coupling chemistry is mainly employed and has already been exploited for several proteins.[182–189] Interesting features of pCB have been unraveled, regarding the often-observed diminished bioactivity of PEGylated proteins. Keefe and Jiang synthesized 5 kDa PEG conjugates of α-chymotrypsin and compared them with pCB analogs of similar molecular weight or size.[182] They found an unaltered or even increased enzyme-substrate affinity for pCB-chymotrypsin of PEG-similar weight or size, respectively. To explain

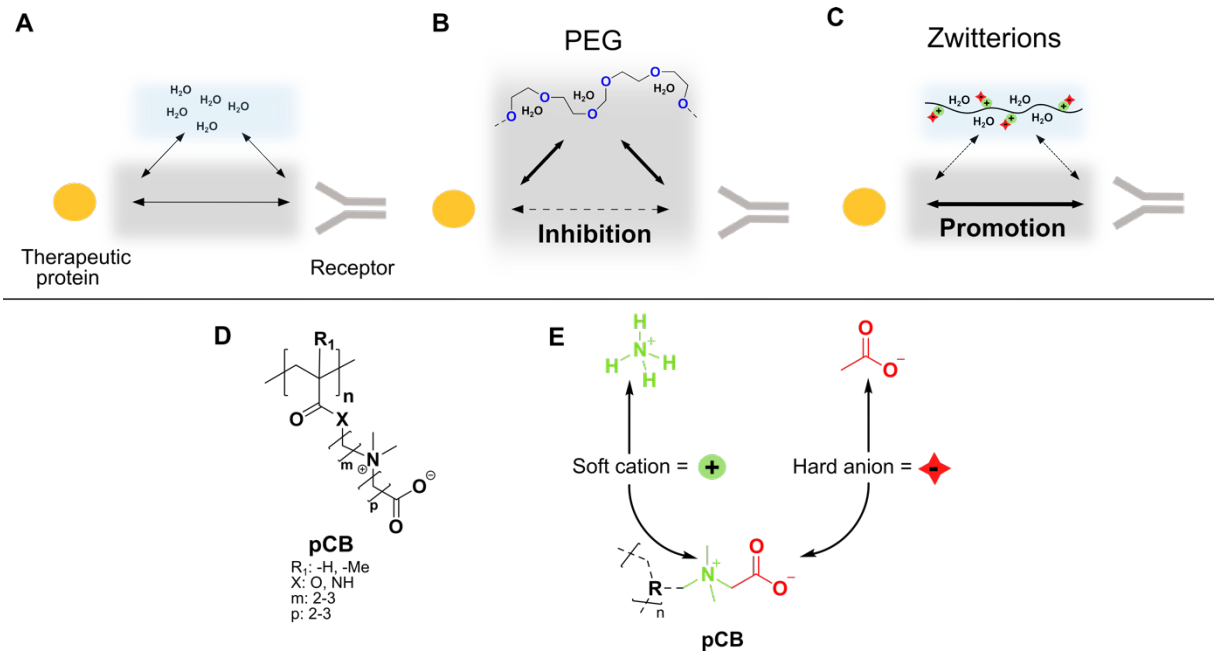

Figure 14. Proposed model for the involvement of PEG or zwitterionic polymers in the receptor-interaction of therapeutic proteins. Adapted by permission from Springer Nature, Nature Chemistry, ref. [182], Copyright 2011.

this phenomenon, the authors proposed a model where PEG with its amphiphilic character competitively reduces hydrophobic interactions between the substrate and its enzyme binding-site, while simultaneously imposing steric hindrance, leading to an overall diminished activity (Figure 14B). pCB on the other hand displays super-hydrophilic character and a strong hydration drawing water molecules away from the hydrophobic protein binding-sites thereby allowing unhindered interaction between the enzyme and its substrate (Figure 14C).

Furthermore, the authors correlate this feature to pCB's monomer structure, a derivative of glycine betaine, reflecting properties of the two most protein stabilizing ions ammonium (soft cation) and acetate (hard anion) in the Hofmeister series (Figure 14E). Further evidence for PEG interacting with hydrophobic protein surfaces can be found in a molecular dynamics simulation study, where solutions of oligoethylene glycol (OEG) reduced the solvent accessible surface area of hydrophobic protein domains by 1/3, whereas carboxybetaine-solutions only showed a decrease of 1/10.[190]

Another study about pCB- and PEG-IFN-α2a conjugates confirmed the compact structure of pCB also after protein conjugation, as double the molecular weight of pCB was needed to reach the same molecular size of a PEG-conjugate.[189] pCB_{20k}-IFN displayed 4.4-fold higher antiproliferative bioactivity compared to its PEG-conjugate of same molecular weight (62.1 % of IFN-activity vs. 14.2 %), which was similarly found for the 10 kDa conjugates. Again, the authors speculate that steric hindrance and non-specific hydrophobic interactions between PEG and the IFN-binding domain, respectively, IFN-receptor, decrease bioactivity, which was mitigated in the case of pCB-conjugates. Notably, the circulation time of pCB_{20k}-IFN in rats was comparable to PEG_{40k}-IFN, despite half of the molecular weight. Furthermore, PEG-IFN conjugates showed accelerated blood clearance due to IFN- and polymer-specific antibodies, whereas PCB-conjugates displayed negligible antibody formation, confirming earlier results for pCB-modified nanoparticles[191] and conjugates of uricase.[187]

Similar results on immunogenicity between PEG and pCB of 5, 10 and 20 kDa were found when conjugated to a series of proteins with varying immunogenicity by thiol-maleimide coupling (Figure 15A).[192] SPR and ELISA-techniques were employed to analyze the formation of anti-polymer antibodies after repeated injection in mice. The authors found an increasing amount of PEG-specific IgM and IgG antibodies which could be linearly correlated with the

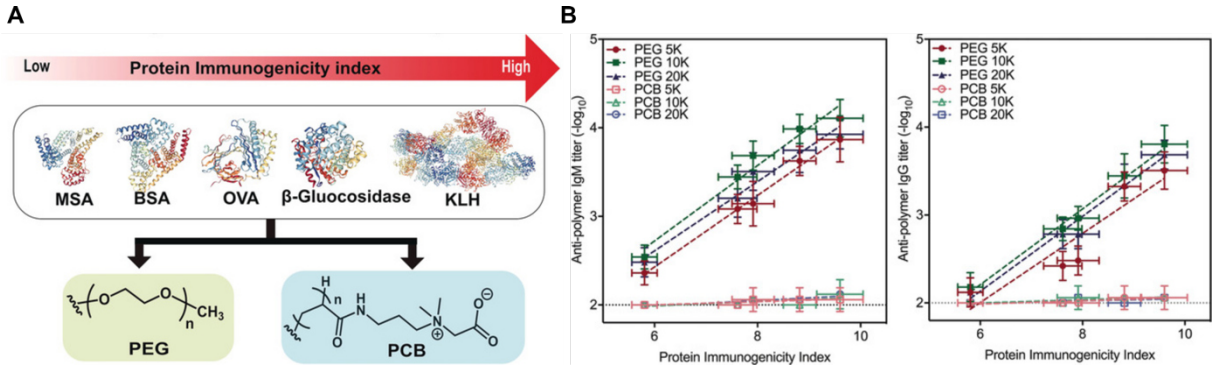

Figure 15. A) Immunogenicity index of various proteins used for conjugation to PEG or pCB. B) Correlation of protein immunogenicity index and anti-polymer antibody formation after four weekly subcutaneous injections of PEG- or pCB-conjugates in C57BL6 mice. Reprinted from ref. [192]. Copyright 2018, with permission from Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

immunogenicity of the protein moiety thereby confirming the haptenic character of PEG (Figure 15B). In contrast, minimal immunogenicity was found in the case of pCB-conjugates.

2.3.2.2 Polypeptides (PPs)

Polypeptides (PPs) are synthetic macromolecules comprising of natural amino acids as non-toxic building blocks. They are generated by polymerization of highly reactive *N*-carboxy-anhydrides as monomers and are considered as biodegradable.[145] Poly-γ-glutamic acid (γ-PGA) is one of the most widely used PPs being approved by the FDA for cosmetic applications and has also been exploited in various architectures for the conjugation to biomolecules.[193–195] An interesting study by Lu and co-workers focused on the conformation of PGA within a conjugate of the therapeutic protein IFN.[196] 20 kDa brush-type PGA bearing three ethylene glycol (EG) units on its backbone was fused to the *N*-terminus of IFN and compared with a PEGylated IFN-analog of same molecular weight. The authors used PGA with either unstructured (*DL*-PGA) or helical (*L*-PGA) conformation to examine the impact of polymer conformation on the properties of the resulting conjugate (Figure 16). The rigid, helical *L*-PGA-IFN conjugate displayed a higher binding affinity and antiproliferative activity *in vitro* than IFN attached to the two unstructured polymers PEG or *DL*-PGA. Furthermore, *L*-PGA-IFN showed significantly slower tumor growth *in vivo*, no accelerated blood clearance upon repeated administration and almost no detectable anti-polymer antibodies in rats, which was in contrast to PEG-IFN and *DL*-PGA-IFN. Similar results were found for polymer conjugates of human growth hormone in the same study indicating the relevance of polymer conformation in conjugates of biotherapeutics.[196]

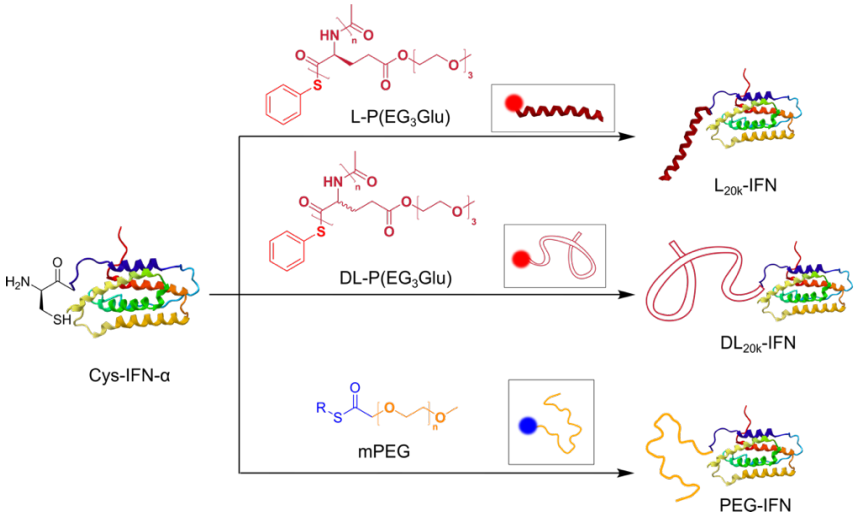

Figure 16. Synthesis of IFN-conjugates with either helical or unstructured PGA-polypeptides. Adapted with permission from ref. [196]. Further permissions related to the adapted material must be directed to the ACS.

Another polymer within this class is the non-ionic and hydrophilic macromolecule polysarcosine (PSar) (also called poly(*N*-methylglycine)), a polypeptoid based on the

endogenous but non-proteinogenic amino acid sarcosine. Due to its large hydrodynamic volume, PSar displays anti-fouling properties and is considered as a stealth polymer[197,198] with a slightly lower flexibility than PEG.[199,200] In a recent study by the company BioNTech, PSar has been proposed as PEG-alternative surface modification on lipid nanoparticles for mRNA-delivery, a technology platform that is already in use for the application of the new Sars-Cov2 vaccine by the same company.[201] In contrast to PEG, PSar-modified liposomes were shown to evade accelerated blood clearance and exhibited lower antibody-formation upon repeated administration with, however, a higher tendency to accumulate in the liver.[202] Lu and co-workers were the first ones to report the successful conjugation of PSar to a therapeutic protein and found higher *in vitro* activity and slower tumor growth *in vivo* for a site-specific *N*-terminal PSar-IFN, with the terminal half-life being comparable to PEG-IFN.[197] Furthermore, PSar-IFN displayed significantly lower anti-IFN antibody formation than its PEGylated analog upon repeated administration in mice and a higher tumor accumulation with less exposure to the liver.

2.3.2.3 Poly[oligo(ethylene glycol) methyl methacrylate] (POEGMA)

Several approaches focused on the generation of PEG-derivatives with labile functional groups (e.g., disulfide[203] or ester[204] groups) that allow hydrolysis of PEG into lower molecular weight fragments therefore making it, in principle, biodegradable. Among these, one of the most prominent examples is the PEG-based, comb-like polymer poly[oligo(ethylene glycol) methyl methacrylate] (POEGMA), which gained more and more attraction since the report of its controlled synthesis in the 1990s.[205] POEGMA consists of a methacrylate backbone decorated with side-chain ethylene glycol (EG) monomers of up to nine units. The sidechain EGs are connected by an ester linkage that allows hydrolysis and enzymatic breakdown. Studies on ATRP-mediated grafting of POEGMA from a large variety of biomolecules have been mainly conducted by the Chilkoti group.[206–213] Conjugates of salmon calcitonin[214] and lysozyme[215] were produced by direct conjugation of the pre-synthesized polymer.

POEGMA bears a hydrophobic backbone and amphiphilic ethylene glycol side-chains, whose length can be tuned to alter hydrophilicity and hydration state of the polymer.[205] This so-called “bottlebrush” structure shows excellent protein- and cell-resistant properties, because of the high density of oligoethylene glycol (OEG) moieties.[216] POEGMA displays a compact and rather rigid conformation in solution, with a smaller hydrodynamic size than a PEG molecule of same molecular weight.[211] All these offer interesting features in regard to bioactivity and shielding of immunogenic epitopes on proteins, two key factors in extending therapeutic activity of biomolecules. For example, Hu *et al.* reported higher *in vitro* bioactivity,

similar half-life and a longer anti-tumor effect *in vivo* for a site-specific C-terminal IFN-POEGMA$_{66k}$ conjugate, compared to PEGASYS (random PEGylation with a branched mono-PEG$_{40k}$). POEGMA was thereby grafted from IFN, which was previously functionalized with the initiator for polymerization by employing Sortase A (Figure 17). Despite of IFN-POEGMA$_{66k}$ bearing a larger molecular weight, it showed higher bioactivity than PEGASYS, which could most likely be attributed to the difference in conjugation site but also suggests an impact of polymer conformation.[213]

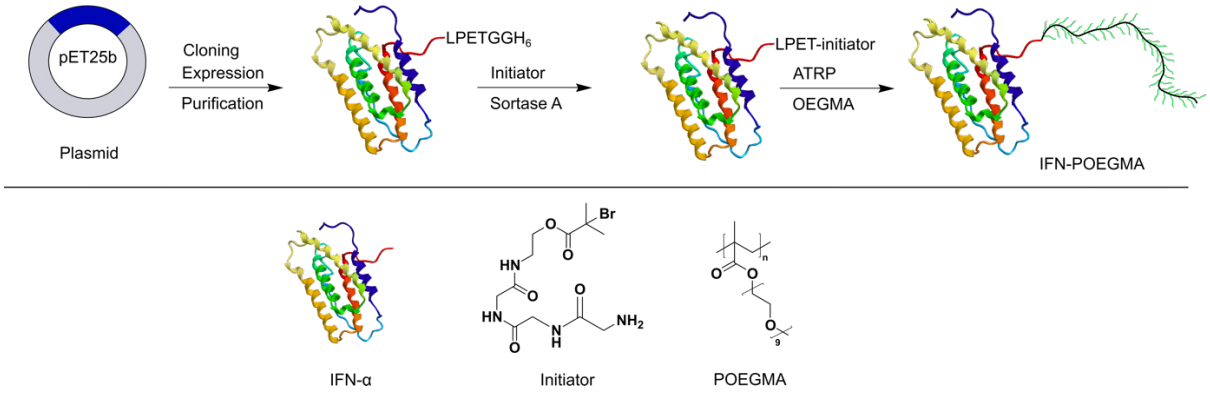

Figure 17. Grafting-from approach of POEGMA on initiator-modified IFN *via* ATRP. Adapted from ref. [213]. Copyright 2016, with permission from Elsevier.

This structural effect is even more pronounced for the shielding of immunogenic epitopes on biomolecules. Qi *et al.* synthesized exendine-POEGMA conjugates with varying polymer side chain length bearing either three or nine EG units.[212] Analysis of anti-PEG-positive patient plasma samples revealed a significant reduction of anti-PEG antigenicity for EG9 exendine-POEGMA, compared to the two approved PEGylated proteins Krystexxa® and Adagen®. When further reducing the side chain length to three EG units, the anti-PEG antigenicity was virtually eliminated without compromising the extended half-life of exenatide. Studies on POEGMA grafted on planar surfaces revealed an optimum of two EG units for minimizing anti-PEG antigenicity and non-specific adsorption.[216] Following these results, the side chain hydrophilicity of the polymer seems to play a major role in anti-PEG antigenicity of POEGMA-conjugates.

2.3.2.4 Polyphosphoesters (PPEs)

Polyphosphoesters (PPEs) are a relatively young polymer class applied to the field of bioconjugation. They carry a biodegradable phosphoester group in their backbone,[217,218] display low toxicity and high biocompatibility, with analog structural features to the naturally occurring teichoic acids and nucleic acids.[219–221] PPEs can be generated by organo-catalyzed anionic ring

opening polymerization of cyclic phosphate monomers and are typically employed as poly(ethyl ethylene phosphate) (PEEP) or poly(methyl ethylene phosphonate) (PMeEP) for conjugation to proteins.[218] PPEs exhibit a stealth effect, that is similar to PEG.[222] Several fundamental studies on solvation and relaxation dynamics of PPE conjugated to different model proteins have been reported over the last few years expanding the knowledge about the solution properties of those materials.[223–227] Bioconjugates of PEEPs (2–33 kDa) were so far reported for BSA and catalase, where the *in vitro* bioactivity of the latter decreased upon polymer modification but was in similar range to its PEGylated analogs.[228] Similar results were obtained for PMeEP-uricase-conjugates which, however, displayed a smaller hydrodynamic volume in SEC than PEG-uricase. Following the authors' explanation, this originates from the difference in chain length of PEG and PMeEP, as the molecular weight of their repeating units differ approximately by a factor of three therefore leading to smaller hydrodynamic sizes.[229] Other studies examined the thermal stability of PPE-myoglobin(Mb)-conjugates in regard to polymer length, grafting density[230] and polymer hydrophilicity.[104] PPE-variants with larger hydrophilicity displayed better thermal stabilization and protease resistance than their hydrophobic analogs highlighting the importance of polymer hydrophilicity for the thermal stabilization of proteins.

2.3.3 Degradable natural Polymers: Polysaccharides

2.3.3.1 Hydroxyethylstarch (HES)

Hydroxyethylstarch (HES) is a highly biocompatible, modified natural polymer structurally derived from amylopectin. It is predominantly produced from maize starch, whose high molecular weight amylopectin molecules are transformed into smaller fragments by acid hydrolysis and mechanical stress followed by modification with hydroxyethyl-groups, which allow tuning of the pharmacokinetic properties and water-binding capacity. The final step includes ultrafiltration to control the molecular weight and dispersity of HES.[231]

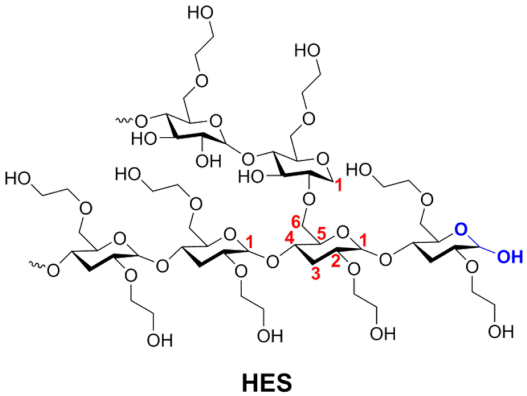

Figure 18. Structure of HES.[232] The oxidation site at the reducing end is marked in blue.

HES is available in molecular weights from 70–670 kDa and is widely used as a plasma volume substitute with high water-solubility and low viscosity.[232,233] Cleavage and degradation in blood takes place by the enzyme α-amylase but can be successfully reduced

through a higher content of hydroxyethyl groups. To enable mono-functional HES, the reducing end (Figure 18, marked in blue) can be oxidized resulting in an aldonic acid (oxHES), which can further be transformed into its lactone by extensive drying. Subsequent activation by NHS-esters enabled conjugates of serum albumin or oligonucleotides.[234,235] Other strategies involved bifunctional linkers to introduce maleimide-, aldehyde- and amine-groups.[236] Next generation-HES employs modification of its non-oxidized reducing end with 1-amino-3,3-diethoxypropane by reductive amination. Acidic deprotection under selective conditions yields HES-propionaldehyde for site-selective modification of *N*-terminal α-amino groups on proteins.[231,237]

Erythropoietin (EPO) and IFNα-2b have already been successfully HESylated on their *N*-terminii, where HES-IFNα-2b displayed higher *in vitro* bioactivity than PEGasys, possibly attributed to the different polymer attachment sites of the two conjugates.[231] Pharmacokinetic studies following a single subcutaneous injection in rabbits unraveled faster absorption and longer half-life in the case of HESylated IFNα-2b, compared to PEGasys.[231] Furthermore, freeze-drying of an *N*-terminally HESylated IFNα-2b resulted in better storage stability at elevated temperatures than its PEGylated analog.[238] Liebner *et al.* reported lower viscosity, higher storage stability and better receptor-binding *in vitro* for *N*-terminally HESylated anakinra compared to its PEGylated analog.[239] Furthermore, the terminal half-life of HES-anakinra was extended about 6-fold compared to the unmodified protein.[240]

Despite HES being evaluated on several biopharmaceuticals already showing potential for half-life extension, its naturally large dispersity might still be a problem from a regulatory perspective. Additionally, safety concerns need to be addressed, where some types of HES are possibly connected to impaired renal function, tissue storage, anaphylactic reactions and mortality.[241–243]

2.3.3.2 Polysialic Acid (PSA)

Polysialic acid (PSA) is a naturally occurring, linear and polydisperse macromolecule consisting of *N*-acetylneuraminic acid (sialic acid) moieties connected by α-glycosidic linkages. It naturally occurs in capsules of several gram-negative bacteria like *Neisseria meningitidis* serogroup B and C and *Escherichia coli* strains, where it mainly serves as a shield to evade the host immune response.[244] The diverse isotypes found in bacteria strains

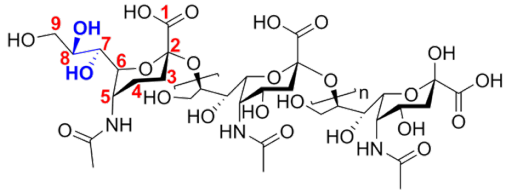

Figure 19. Structure of PSA[246] with the vicinal diol marked in blue.

display distinct differences in their *in vivo* circulation time, which depends, for example, on the degree of phospholipid acylation or the type of glycosidic linkage.[245]

PSA is described as nonimmunogenic and safe. Furthermore, it is negatively charged (and therefore highly hydrophilic), stable at physiological pH, but biodegradable due to digestion through cellular neuraminidases.[246] It displays similar hydration-properties to PEG leading to a 5–10-fold increase in hydrodynamic volume of a protein and can additionally diminish renal clearance through electrostatic repulsion at the negatively charged glomerular membrane, leading to an extended circulation half-life.[247] Furthermore, IgM antibody formation and subsequent accelerated blood clearance has not been observed in the case of PSAylated liposomes, further highlighting its stealth properties.[248] Tissue-permeability can be improved by PSAylation, where, e.g., a PSAylated antibody fragment displayed an up to 30-fold improved tumor uptake, compared to its unmodified analog.[249,250] PSA seemed to have a drastic influence on the hydrodynamic size of the ab-fragment, as the conjugates eluted in the void volume of SEC, which, however, might also be attributed to the high negative charge of PSA.[252]

PSA derived from *E. coli* K1 strain (also called colominic acid) consists of α-(2->8) linked sialic acid groups and is mainly used for the conjugation to protein therapeutics, as it bears a single vicinal diol to be mildly oxidized by $NaIO_4$ at its non-reducing end (Figure 19, C7 and C8 bearing the vicinal diol are marked in blue).[251] The resulting aldehyde can be further modified with linkers or directly used for protein coupling by reductive amination. The latter has already been exploited to several proteins,[252–255] with erythropoietin and deoxyribonuclease I[256] being also evaluated in clinical trials. The technology of PSAylation (PolyXen™) has been commercialized by the company Lipoxen PLC (now Xenetic Biosciences).

Besides classical chemical ligation strategies, polysialic acid has also been exploited for recombinant[257] or enzymatic[258] protein modification. Another recent study describes the polysialylation on glycan chains of human coagulation factor VIII (FVIII) which was conducted by the company Baxalta.[246] In this sophisticated approach, a 20 kDa oxPSA was linked to several oxidized carbo-hydrate moieties on *N*-glycans of FVIII resulting in stable oxime-linkages, with 6–11 mol PSA per mol of FVIII conjugated (Figure 20, BAX 826). Extensive

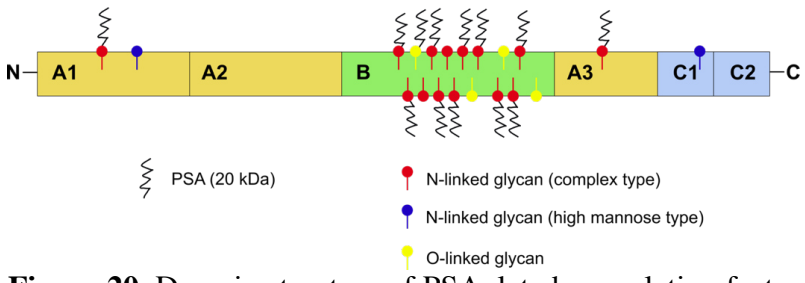

Figure 20. Domain structure of PSAylated coagulation factor VIII. Adapted from ref. [246], Copyright 2020, with permission from Elsevier.

characterization was followed by pharmacokinetic *in vivo* evaluation where BAX 826 was found to exhibit prolonged circulation time in hemophilia A mice, wild-type rats and cynomolgus monkeys, compared to the unmodified protein.[259] A phase 1 clinical study in humans further demonstra-ted the capability of BAX 826 to extend the mean residence time by factor 1.5, compared to FVIII alone, while maintaining high tolerability and safety.[260]

2.3.4 Recombinant Half-Life Extension Strategies

Recombinant strategies for half-life extension include the technologies of albumin-/Fc-fusion proteins, XTENylation and PASylation. These approaches allow expression of the full conjugate via biotechnological engineering resulting in a monodisperse construct with favorable properties. All mentioned technologies are already present on the market (albumin-/Fc-fusion, Albufuse®) and/or involved in several clinical trials (XTENylation, PASylation).

2.3.4.1 Albumin- and Fc-fusion Proteins

Albumin and IgG antibodies are ubiquitously present molecules in humans and exhibit long circulation times, both with a half-life of around 3 weeks.[261–263] The longevity of those two molecules can be explained by their high affinity to the neonatal Fc receptor (FcRn), which is widely expressed in several cell types throughout the body and prevents ligands from intracellular degradation through a pH-dependent recycling process (Figure 21). Following

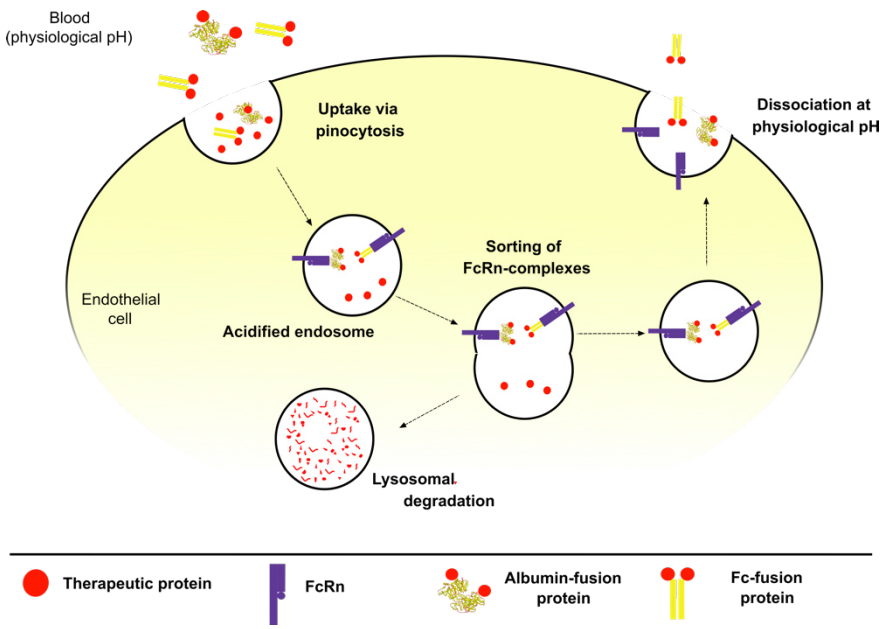

Figure 21. FcRn-mediated recycling mechanism of albumin- and Fc-fusion proteins.

uptake into cells by fluid-phase pinocytosis, albumin and IgG form complexes with endosomal FcRn at acidic pH and get released again into the blood stream upon exposure to the neutral pH of the extracellular space.[264–268] This process can be exploited for half-life extension by genetically fusing either human serum albumin or the *N*-terminus of the Fc-part of an IgG antibody to a therapeutic protein or peptide of interest (mostly on its C-terminus).[269] Several fusion products have already employed this concept and are under clinical development, with by today thirteen approved Fc-fusion proteins[269] and one albumin-fusion construct[268,270] on the market. Other strategies focus on the non-covalent association of proteins and peptides equipped with an albumin-binding domain (e.g., C-14 or C-16 fatty acids) to target endogenous albumin for half-life extension. This strategy has also proven to be successful, with marketed products including insulin and GLP-1.[268]

Despite their extremely long half-life, the production of Fc-fusion proteins is cost-intense and demands extensive characterization of the respective glycosylation pattern,[269] that, in turn, is important in regard to circulation half-life.[271] Their complex structure requires a large battery of analytical methods to ensure safety and stability while excluding aggregation, charge variation or immunogenicity of the antibody-fusion protein.[269] Furthermore, albumin- and Fc-fusion proteins do not allow the fine-tuning of half-life, which might be problematic with drugs bearing a small therapeutic window. Nevertheless, they have been proven to be a quite useful tool for half-life extension as demonstrated by several products on the market.

2.3.4.2 XTENylation

XTEN describes a class of negatively charged, unstructured polypeptides consisting of random sequences of the six hydrophilic amino acids alanine, glutamic acid, glycine, proline, serine and threonine. In contrast to IgG- or albumin-fusion proteins, XTEN polypeptides can be expressed in bacterial cell lines (typically *E.coli*) and are mostly encoded together with the target gene of the protein of interest yielding a homogenous, monodisperse product after expression and purification. This methodology allows high yields and absolute site-specificity of XTEN-attachment without the need of cost-intense purification, as compared to PEGylation, where typically the removal of multi-PEGylated species and free PEG is necessary.[272] The parental XTEN-molecule, first described in 2009, consisted of 864 residues (XTEN864, 79 kDa) and was followed by the development of shorter XTEN polypeptides in recent years. XTENs are highly flexible, unstructured macromolecules that display an extended conformation with a very large hydrodynamic volume.[273] As demonstrated for several XTEN-fusion constructs,[272–275] this leads to extremely long circulation half-lifes which can further be

fine-tuned by varying the length of the XTEN sequence. The technology of XTEN has been commercialized by the company Amunix Pharmaceuticals Inc. (USA), with several therapeutic biomolecules in clinical trials.[276,277]

Fusion of XTEN is predominantly exploited to the *N*- and C-termini of therapeutic biomolecules but can in principle be implemented at any position of the sequence which allows even multiple XTENylation of complex molecules like blood factor VIII.[272] XTEN is thermally stable, biodegradable and considered as non-immunogenic, as it lacks any hydrophobic residues, that could trigger aggregation or immune response. Elimination of a radiolabeled XTEN864 was primarily observed *via* the kidneys and no accumulation in organs was found in mice and rats.[272] Despite of all those unique advantages, the recombinant fusion of XTEN protein polymers excludes post-translational modification or other payloads like nucleic acids. Therefore, amine- or thiol-moieties can be encoded to the XTEN-sequence which allow further chemical ligation with linkers or peptides.[272,278,279]

2.3.4.3 PASylation

PAS is an unstructured, hydrophilic, neutral polypeptide consisting of random sequences of the amino acids proline (P), alanine (A) and serine (S), that can be genetically fused to the

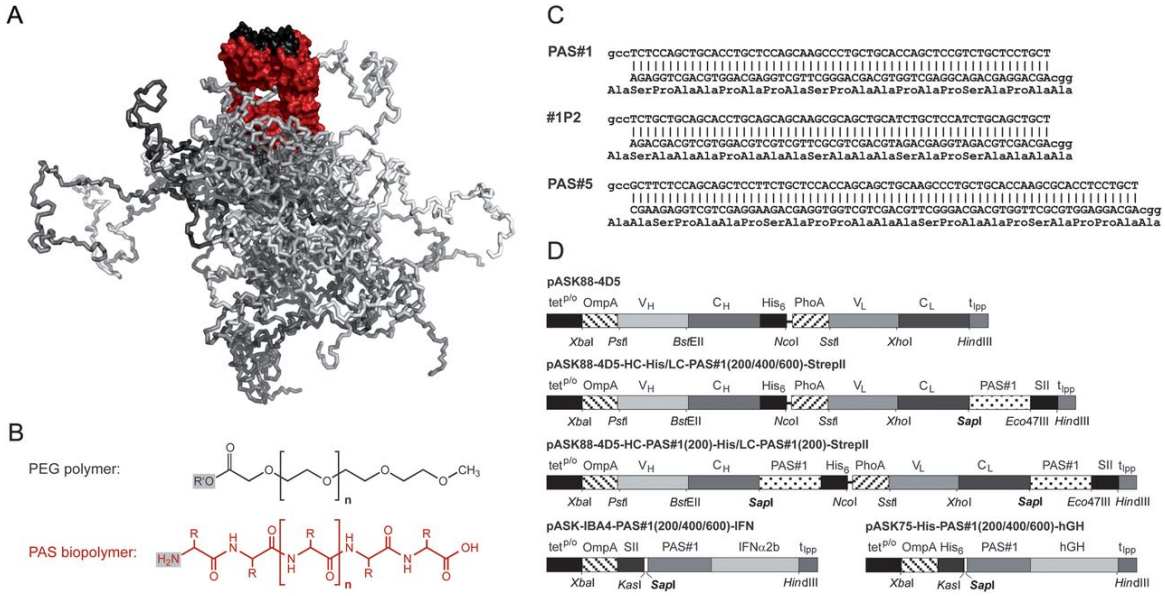

Figure 22. Concept of PASylation. A) Model structure of a C-terminally PASylated Fab fragment. The Fab-fragment is coloured in red, with the antigen-binding site in black. B) Comparison of chemical structures of PEG and PAS (side chains abbreviated as R). C) Nucleotide and encoded amino acid sequences of the building blocks for different PAS gene cassettes. D) Schematic representation of expression cassettes on the plasmids used for PAS-generation. The PAS can be directly expressed together with the respective protein, as shown for the two lowest expression cassettes bearing IFN (lower left) or hGH (lower right). Reprinted from ref. [280].

protein drug of interest followed by direct expression in bacterial (or mammalian) cell lines (Figure 22). The resulting homogenous, monodisperse product displays a large hydrodynamic volume, long circulation time and extended therapeutic activity.[280] Depending on the PAS-length attached (100–1000 residues possible) the half-life can typically be extended 10–100 fold.[293] PASylation technology is commercialized by the company XL-protein GmbH (Freising, Germany) and has already been applied to more than 10 biologicals of therapeutic interest, including antibody fragments, proteins, peptides and nanocarriers.[280–295] PAS can also be equipped with functional groups for chemical conjugation to target proteins, typically established by genetical engineering of the PAS-sequence (e.g., cysteine, *N*-terminal serine, etc.), which also allows branched architectures.[293]

PAS polypeptides are biodegradable but stable in plasma and are described as non-immunogenic, as they lack any hydrophobic epitopes (like XTEN) typically involved in aggregation or a potential immune response. The proline-, alanine- and serine-moieties of PAS show no intramolecular interactions besides solvent molecules resulting in a fully solvated peptide backbone with high solubility and a native disorder.[293] This random-chain behavior is similar to PEG, however, resulting in a slightly larger hydrodynamic volume for PAS, which displays a more elongated shape in solution. Furthermore, PAS shows higher hydrophilicity and lower viscosity compared to PEG of similar molecular weight. Interestingly, serine plays only a minor role for the hydrophilicity and biophysical properties of PAS, as serine-free sequences displayed the same biophysical behavior.[288] In contrast, a decrease in proline content resulted in a more compact and less random structure of PAS. Increasing the proline portion in the polypeptide promotes an elongation and therefore larger hydrodynamic volume of the polymer chain.[280,288]

Interestingly, the receptor binding affinity of biologicals decreased only slightly upon PASylation and was almost unaffected by PAS-length, suggesting a minor role of hydrodynamic volume and steric repulsion in the receptor binding of PASylated molecules.[280] This is in contrast to XTEN, which bears negative charge due to its glutamate residues therefore promoting repulsion between a protein drug and its receptor. Simultaneously, receptor-mediated clearance can be hampered, as demonstrated for XTEN-hGH.[280,294] The negative charge has also been proposed to affect tissue distribution of XTENylated and PASylated biopharmaceuticals, with, however, no significant differences observed so far.[291]

3 Scientific goals

The employment of PEGylation to increase the circulation half-life of biopharmaceuticals has clearly been a revolution in the field of protein drug delivery, with more than ten PEGylated products on the market. The technology not only improved patient compliance and safety but also expanded treatment options to highly immunogenic, non-human proteins like uricase. However, the loss of bioactivity upon conjugation and the formation of antibodies are two major shortcomings of PEG, which led to a large body of macromolecules explored as alternatives for the purpose of half-life extension of biopharmaceuticals. This work aimed to establish linear polyglycerol, a highly hydrophilic and biocompatible polymer, as an alternative polymer platform to PEG for the conjugation to therapeutic biomolecules. Several model proteins were selected for this approach with the goal to reveal the impact of polymer length and type (LPG or PEG) on the performance of the respective protein-conjugates in different *in vitro* and *in vivo* settings.

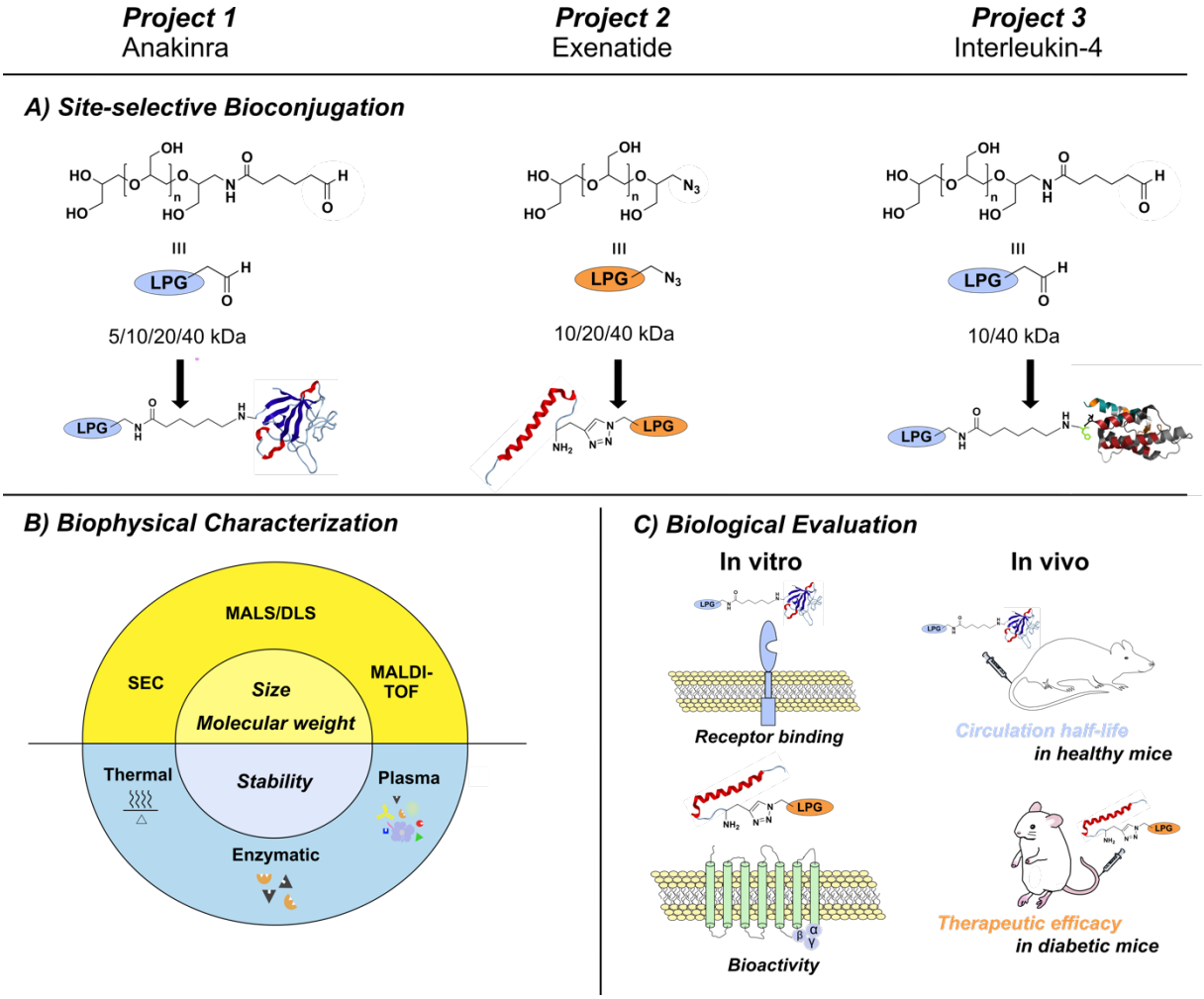

Figure 23. Schematic representation of the three projects covered in this thesis (protein structures from PDB-entries 1ilr, 1jrj, 2b8u).

The conjugation of polymers to a biopharmaceutical demands a highly selective and well-controlled ligation-process to adjust number and location of the attached polymer chains and to avoid a loss in bioactivity. Therefore, we aimed to synthesize site-selectively modified protein-conjugates with a 1:1 protein-polymer ratio by employing mono-functional LPGs of various molecular weights. Instead of model proteins like lysozyme or BSA, we selected the therapeutically active biomolecules anakinra, interleukin-4 and exenatide to address alterations of their biological activity after polymer modification. PEG-conjugates of the respective proteins were synthesized as reference in a similar manner (Figure 23A).

To ensure a valid comparison between LPG and the benchmark PEG in further characterization studies, the molecular weights of the conjugates needed to be similar. Besides molecular weight, the hydrodynamic size of a therapeutic protein-PEG conjugate is one of the key parameters for its renal elimination and circulation half-life. LPG alone was found to have a smaller, more compact, and less flexible structure in solution, compared to the highly expanded PEG.[129] Additionally, the stability of biopharmaceuticals often suffers from aggregation at elevated temperatures and proteolytic degradation, a process which can be prevented by the conjugation of PEG. Therefore, a second goal of this work was to conduct an extensive biophysical characterization of LPG-and PEG-conjugates to reveal differences in their molecular weight, size and stability (Figure 23B).

The final goal of this thesis was to determine whether LPG is capable to extend the half-life and therapeutic efficacy of biopharmaceuticals to a similar extent than PEG. Therefore LPG-conjugates of various biomolecules were biologically evaluated in different *in vitro* and *in vivo* settings to gain insights on their bioactivity and pharmacokinetic behavior (Figure 23C).

4 Publications and Manuscripts

4.1 Polyglycerol for Half-Life Extension of Proteins–Alternative to PEGylation?

Michael Tully,* Mathias Dimde, Christoph Weise, Paria Pouyan, Kai Licha, Michael Schirner, Rainer Haag* *Biomacromolecules*, **2021**, *22*, 1406–1416.
https://doi.org/10.1021/acs.biomac.0c01627

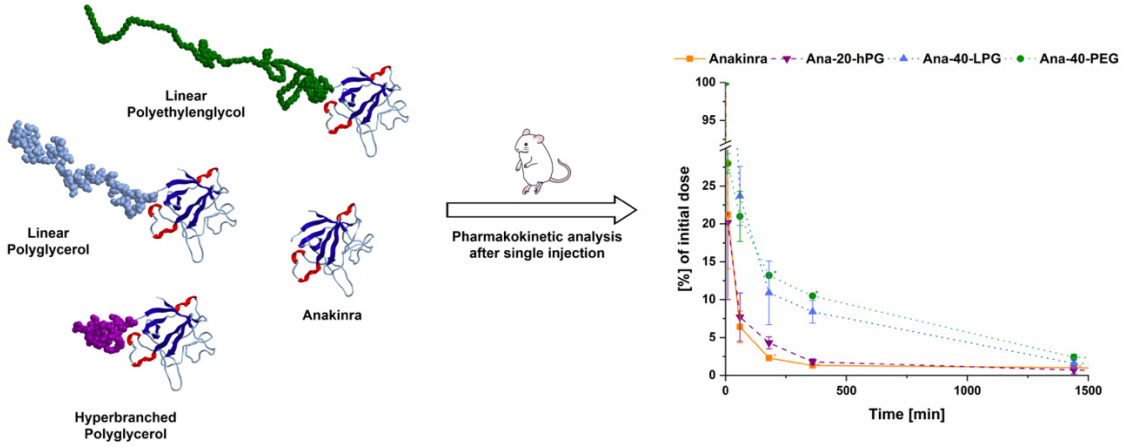

Figure 24. Reproduced with permission from ref. [296]. Copyright 2021, American Chemical Society.

I functionalized linear polyglycerol-amine of various molecular weights with the respective aldehyde-linker. Furthermore, I synthesized and purified all protein conjugates and conducted their characterization *via* SDS-PAGE, dynamic light scattering (DLS), size-exclusion multi angle light scattering (SEC-MALS), circular dichroism (CD), enzyme-stability assay and surface-plasmon-resonance (SPR). Moreover, I wrote the manuscript and planned the *in vivo* experiments including evaluation of the final data. Mathias Dimde synthesized and purified hyperbranched polyglycerol-aldehyde and was a great support in all kinds of chemical questions. Christoph Weise conducted the MALDI-TOF experiments. Paria Pouyan synthesized linear polyglycerol-amine of different molecular weights. Michael Schirner was involved in planning and consulting of the *in* vivo experiments. Kai Licha and Rainer Haag contributed to questions regarding conjugate synthesis, aquired funding and supervised this project.

4.2 Prolonged Activity of Exenatide: Detailed Comparison of Site-specific linear Polyglycerol- and Poly(ethylene glycol)-Conjugates

Michael Tully, Stefanie Wedepohl, Daniel Kutifa, Christoph Weise, Kai Licha, Michael Schirner, Rainer Haag* *European Journal of Pharmaceutics and Biopharmaceutics*, **2021**, *164*, 105–113.

https://doi.org/10.1016/j.ejpb.2021.04.019

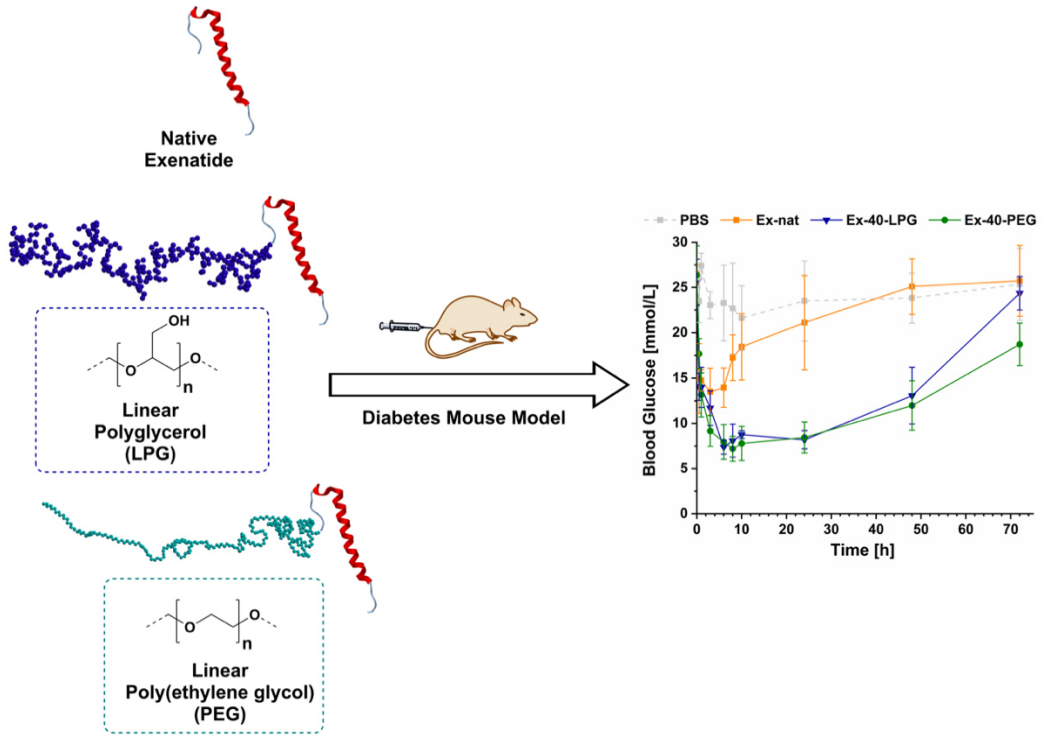

Figure 25. Reproduced with permission from ref. [297]. Copyright 2021, Elsevier.

I synthesized and purified all LPG- and PEG-exenatide conjugates, conducted their characterization *via* SDS-PAGE, dynamic light scattering (DLS), size-exclusion chromatography (SEC) and circular dichroism (CD) and wrote the manuscript. Moreover, I initiated and guided the conduction of *in vivo* experiments at Pharmacelsus GmbH, including evaluation of the final data. Stefanie Wedepohl performed the cAMP-bioactivity assay and greatly supported in all kinds of biological questions. Daniel Kutifa was synthesizing linear polyglycerol-azide of different molecular weights. Christoph Weise conducted the MALDI-TOF experiments. Michael Schirner consulted in the *in vivo* experiments. Kai Licha and Rainer Haag contributed to questions regarding chemistry, aquired funding and supervised this project.

4.3 Linear Polyglycerol for N-terminal-selective Modification of Interleukin-4

Michael Tully,‡ Niklas Hauptstein,‡ Kai Licha, Lorenz Meinel, Tessa Lühmann, Rainer Haag,* **2021**, *submitted, Journal of Pharmaceutical Sciences*

(‡ These authors contributed equally.)

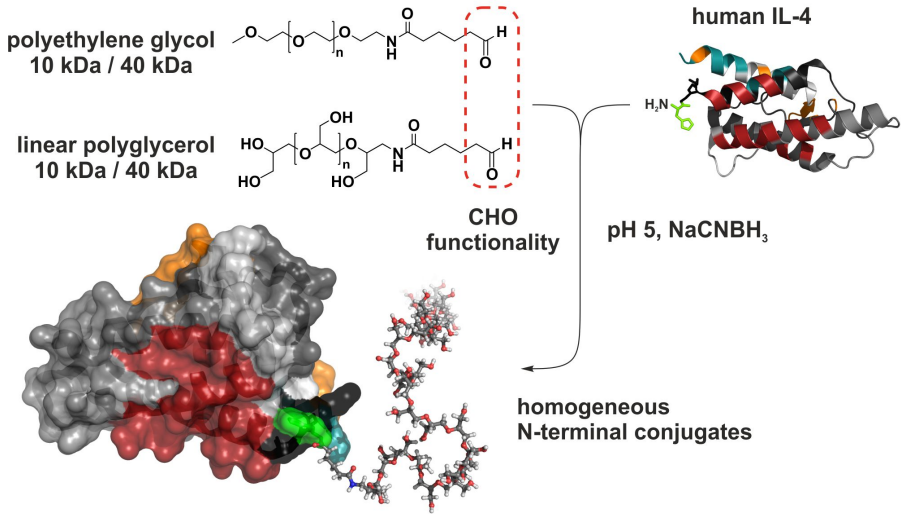

Figure 26. Overview of *N*-terminal coupling strategy for Interleukin-4 conjugates.

I synthesized and purified all LPG- and PEG-Interleukin-4 conjugates and conducted their characterization *via* SDS-PAGE, dynamic light scattering (DLS), size-exclusion chromatography-multi angle light scattering (SEC-MALS), circular dichroism (CD) and microscale thermophoresis (MST). Niklas Hauptstein expressed and purified wt-IL-4, conducted SDS-PAGE analysis, the bioactivity assay of the conjugates, HPLC-analysis and a plasma stability assay. Furthermore, he initiated and prepared samples for the MALDI-TOF experiments. I wrote the manuscript together with Niklas Hauptstein. Kai Licha was giving chemical support on the conjugation process. Lorenz Meinel, Tessa Lühmann and Rainer Haag developed the project idea, aquired funding and supervised this project.

Linear Polyglycerol for N-terminal-selective Modification of Interleukin-4

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KEYWORDS: protein delivery, polymeric drug delivery systems, Pegylation, polyethylene glycol (PEG), conjugates, polymers, biopharmaceutical characterization,

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ABSTRACT

Polymer conjugation to biologics is of key interest to the pharmaceutical industry for the development of potent and long acting biotherapeutics, with poly(ethylene glycol) (PEG) being the gold standard. Within the last years, unwanted PEG-related side effects (immunological reactions, antibody formation) arose, therefore creating several attempts to establish alternative polymers with similar potential to PEG. In this article, we synthesized *N*-terminal bioconjugates of the potential therapeutic human wild-type interleukin-4 (hIL-4 WT) with linear polyglycerol (LPG) of 10 and 40 kDa and compared it with its PEG analogs of same nominal weights. Polyglycerol is a highly hydrophilic polymer with good biocompatibility and therefore represents an alternative polymer to PEG.

Both polymer types resulted in similar conjugation yields, comparable hydrodynamic sizes and an unaltered secondary structure of the protein after modification. LPG- and PEG-bioconjugates remained stable in human plasma, whereas binding to human serum albumin (HSA) decreased after polymer modification. Furthermore, only minor differences in bioactivity were observed between LPG- and PEG-bioconjugates of same nominal weights. The presented findings are promising for future pharmacokinetic evaluation of hIL-4-polymer bioconjugates.

INTRODUCTION

The clinical use of small, but potent biologics with molecular masses up to 50 kDa is still vastly expandable. Due to their small size, most of these drugs display a limited blood circulation time, therefore leading to fast elimination, which makes high frequent dosing necessary.[1,2] To eliminate this problem, several techniques are available ranging from encapsulation of proteins into micelles[3], or nanogels[4] to covalent modification, with poly(ethylene glycol) (PEG) representing the most prominent polymer in that field.[5] PEG is an amphiphilic polymer that equips biologics with desirable properties including a diminished renal excretion through size expansion as well as a stealth behavior, that leads to reduced opsonization and clearance through the immune system.[6] Drawbacks of PEG include a reduced bioactivity after

conjugation[7] and its tendency to accumulate in the body, due to its non-degradability.[6] Within recent years the formation of anti-PEG antibodies was reported[8] which led to an accelerated blood clearance (ABC) in some cases thereby impairing PEG's initial benefits.[9,10] Rare anaphylactic reactions observed for the new SARS-CoV-2 mRNA vaccine by BioNTech-Pfizer might be attributed to the PEG-moiety located on the lipid nanoparticle for vaccine delivery.[11] However, it is not clear yet how serious these PEG-related issues really are, especially in the clinical field. PEG is still regarded as a safe excipient and is the preferred macromolecule for half-life extension and drug delivery, with other polymers being studied as well.[12,13]

Polyglycerol (PG) is a highly hydrophilic polymer showing excellent biocompatibility[14–1718] and further displays stealth properties, that are similar to PEG.[19–21] Its half-life is longer than PEG of similar molecular weight,[14] which led us to the idea of employing linear polyglycerol (LPG) as a polymer for bioconjugation to extend the blood circulation time of biopharmaceuticals. We chose Interleukin-4 (IL-4) as a model protein, a potent cytokine bearing highly anti-inflammatory attributes through polarization of macrophages into the beneficial M2 type.[22,23] IL-4 has several potential applications to target chronic inflammations in wounds, arthritic joints and similar affected tissues.[23,24] For site-selective conjugation, we decorated IL-4's *N*-terminus with LPG-variants of 10 or 40 kDa *via* a reductive alkylation approach, which has already been used previously for other proteins.[25] The LPG-conjugates were then systematically compared to PEG-analogs of same nominal weights and characterized mainly regarding their hydrodynamic size and structural changes followed by evaluation of their bioactivity and behavior towards human plasma components *in vitro*.

EXPERIMENTAL SECTION

Materials

10 kDa methoxy-PEG-hexylaldehyde (mPEG-aldehyde) was from Rapp Polymers (Tübingen, Germany). 40 kDa mPEG-amine-HCl was from JenKem Technology (Texas, USA). Highly purified water from a MilliQ®-system was used for all biological experiments. Buffers were degassed and filtered through 0.22 μm regenerated cellulose (RC) filter (Sartorius, Göttingen, Germany) before use. All other chemicals and

solvents were obtained from Sigma Aldrich (Steinheim, Germany) and used without further purification, unless otherwise noted.

Synthesis of LPG-10-, LPG-40- and mPEG-40-aldehyde

A detailed description of the synthesis part can be found in the Supporting Information.

Expression, purification and characterization of wild-type Interleukin-4

Human Interleukin-4 (hIL-4 WT) was expressed as described before.[24,26] In brief hIL-4 WT was cloned into the pET21a-vector between the NdeI and BamHI restriction site, bearing an ampicillin resistance. Expression took place in *E. coli* BL21(DE3) Star bacteria (Thermo Fisher scientific) in Terrific Broth Medium at 37 °C. The bacteria were induced at an OD_{600} of 0.6 with 1 mM IPTG and incubated for 5–6 h. Afterwards, bacteria were harvested by centrifugation at 4 °C and 5000 x g for 20 min, resuspended in lysis buffer (50 mM TrisHCl pH 8.0, 50 mM NaCl, 1 mM EDTA) and sonicated. The pellet was then washed twice with lysis buffer containing 1 % Triton X-100, followed by a lysis buffer wash, centrifuged and subsequently unfolded in lysis buffer containing 5 M guanidinium hydrochloride, 2 mM reduced glutathione and 0.2 mM oxidized glutathione. Refolding was performed as described in literature.[27] The refolded protein was dialyzed against PBS overnight. On the next day its pH was adjusted to 5 with AcOH, followed by purification on an ÄKTA pure 25 FPLC system (GE Healthcare, Freiburg, Germany) employing ion exchange chromatography (IEX) using a HiTrap Q XL 5 mL column (Cytiva Europe GmbH, Freiburg, Germany) (Buffer A: 25 mM NaOAc, pH 5.0, Buffer B: 25 mM NaOAc pH 5.0 + 2 M NaCl) with a linear gradient from 0–40 % B in 12 CV, with hIL-4 WT eluting at 30 % B. hIL-4 WT containing fractions were pooled, dialyzed against buffer A and applied to an additional IEX purification step (same buffers as above) using a YMC Biopro IEX smart sep S20 1 mL column (YMC Europe GmbH, Dinslaken, Germany) with a gradient from 12–60 % B. Collected hIL-4 WT fractions were evaluated on purity by SDS-PAGE and subsequently pooled followed by determination of hIL-4 WT concentration by standard BCA assay (Pierce BCA Assay Kit, Thermo Fisher scientific) following the manufacturer's instructions.

Synthesis and purification of PG- and PEG-Interleukin-4 bioconjugates

Linear polyglycerol or polyethylene glycol, all mono-functionalized with a single aldehyde group, were conjugated to hIL-4's *N*-terminus *via* reductive amination at pH 5, as described previously.[28] In short, 600 μg of protein were diluted into 1 mL pre-chilled 0.1 M NaOAc pH 5 in a 2 mL glass vial followed by addition of three-fold molar excess of activated polymer in the same buffer. After gentle mixing, the reaction was initiated by adding freshly prepared $NaCNBH_3$ solution (0.5 M) as reducing agent to a final concentration of 20 mM. The reaction mixture was shaken on a bioshaker (Quantifoil instruments, Jena, Germany) for 16 h at 4 °C. The batch was then diluted 10-fold in 25 mM NaOAc pH 5 and subsequently loaded on an ÄKTA pure 25 FPLC system equipped with two HiTrap SP FF 1 mL columns (both GE Healthcare, Freiburg, Germany) connected in series (flow rate 1 ml/min), where 25 mM NaOAc pH 5 served as eluent A. To isolate the monoconjugates from free polymer, unreacted protein and multi-PEGylated/PGylated species, first a washing step with 5 % eluent B (B = eluent A + 2 M NaCl) for five column volumes (CV) was conducted followed by a linear gradient from 10–50 % B for 16 CV to elute multi- and mono-PEGylated/PGylated proteins. The fractions with mono-conjugated product were collected, pooled and rebuffered against phosphate-buffered saline (PBS) pH 7.4 (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) in Amicon Ultra 2 centrifugal filter units (MWCO 3 kDa, Sigma Aldrich, Steinheim, Germany). Concentration was determined by Nanodrop 2000c (Thermo Fisher, Dreieich, Germany) at 280 nm with an extinction coefficient of 8860 M^{-1} cm^{-1}.[24] Samples were then aliquoted, snap frozen in liquid N_2 and stored at -80 °C until further use.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE analysis was performed under standard denaturing Laemmli-conditions with acrylamide concentrations of 5-12 % in the separation gel. Purified fractions were analyzed on gradient gels with a concentration of 5–12 %. Gels were run at 200 V and analyzed *via* ImageLab software 5.2.1. Standard coomassie staining was used to visualize the protein bands.[29]

Size-exclusion multi-angle light scattering (SEC-MALS)

For molecular weight determination, size-exclusion chromatography coupled to multi-angle light-scattering (SEC-MALS) was performed on a Hitachi L-2130 HPLC system that was equipped with a UV-Vis absorption detector (Hitachi L-2400), a DAWN 8+ MALS detector, and an Optilab refractive index detector (both Wyatt Technology, Dernbach, Germany). For size exclusion, a Superdex 200 Increase 10/300 GL column (GE Healthcare, Uppsala, Sweden) was used with the mobile phase consisting of PBS pH 7.4 operated at a flow rate of 0.5 mL/min. 50 μg of bioconjugate (based on protein weight) were injected in each run. Data analysis was followed by the software Astra 6.0 (Wyatt Technology, Dernbach, Germany). Protein conjugate analysis was performed by the Wyatt protein-conjugate application, which was embedded in the Astra software. UV extinction coefficient for hIL-4 WT was 8860 M^{-1} cm^{-1}, for *dn/dc* (differential refractive index) of hIL-4 WT, a typical value of 0.185 mL/g was used. *dn/dc* for PEG and LPG were measured at 25 °C on a SEC-3010 RI detector (WGE Dr. Bures GmbH, Dallgow, Germany) which was calibrated against potassium chloride and determined as 0.143 mL/g (PEG) and 0.142 mL/g (LPG).

Reversed Phase (RP)-HPLC analysis

RP-HPLC analysis was performed on an Agilent 1260 Infinity II system, equipped with a VWD detector. The wavelength was set to 214 nm and column oven temperature to 30 °C. 5 μg of each sample were applied to a ZORBAX 300SB-CN column (4.6 x 150 mm, particle size = 5 μm) (Agilent, Santa Clara, CA, USA) with an autoinjector. For elution of hIL-4 WT or its bioconjugates, a linear gradient of 5–60 % was used over 30 min at a flow rate of 1 mL/min (eluent A: Water + 0.1 % TFA, eluent B: Acetonitrile + 0.1 % TFA).

Matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS)

The samples were desalted using ZipTipC18-tips (Pierce C18 Tips, Thermo Fisher Scientific) following the manufacturer's instructions. Matrix-assisted laser desorption ionization (MALDI-MS) spectra were acquired in the linear positive mode using an ultrafleXtreme mass spectrometer (Bruker Daltonics, Bremen), equipped with a 355 nm smartbeam-II™ laser. Mass spectra were calibrated with bovine serum albumin. Sinapinic acid was used as a matrix.

Dynamic Light Scattering (DLS)

For determination of the hydrodynamic size of hIL-4 WT and its bioconjugates, dynamic light scattering was performed on a Malvern Zetasizer ZS (Malvern Panalytical, Herrenberg, Germany). All measurements were done at 25 °C in PBS pH 7.4 (c = 0.4 mg/mL) and samples were centrifuged at 10,000 g for 5 min prior to measurements. The hydrodynamic radius is expressed as volume value, as displayed in the Zetasizer software version 7.13.

Far-UV Circular Dichroism (CD)

CD spectra were recorded on a Jasco J810 (Bruker Instruments, Massachusetts, USA) from 190–250 nm at 20 °C to monitor changes in the far-UV region of hIL-4 WT after polymer modification. Measurements were done in 0.1 M phosphate buffer pH 7.2 at a concentration of 0.10–0.11 mg/mL using a bandwidth of 2 nm and a 1 cm-path-length cuvette. Each spectrum was baseline corrected using a blank spectrum of buffer.

HEK Blue IL-4/IL-13 Cells *in vitro* secreting alkaline phosphatase (SEAP) assay

The cell culture assay was performed according to the manufacturer's instructions. The used cells (HEK-Blue™ IL-4/IL-13 Cells), colorimetric reagents and antibiotics were bought from Invivogen (Toulouse, France).

Plasma stability in human serum

For plasma stability, human AB+ plasma from healthy men was used (Sigma Aldrich, Germany, P9523). Plasma was thawed on ice only once for aliquotation and thawed once again on ice when used. The plasma was incubated in an Eppendorf thermoshaker at 37 °C, 600 rpm. hIL-4 WT or its bioconjugates were spiked into the plasma at a final concentration of 1 ng/mL, small aliquots were taken at predetermined time points and frozen in liquid nitrogen until further characterization. For quantification of hIL-4 WT or its bioconjugates an IL-4 Human ProQuantum Immunoassay Kit (Thermo fisher scientific, A35587) was used according to

the manufacturer's instructions with an ABI Prism 7900 HT Real time PCR machine using the standard temperature ramp protocol and micro Amp^{tm} 96-well qPCR plates (Applied Biosystems, Germany, N8010560).

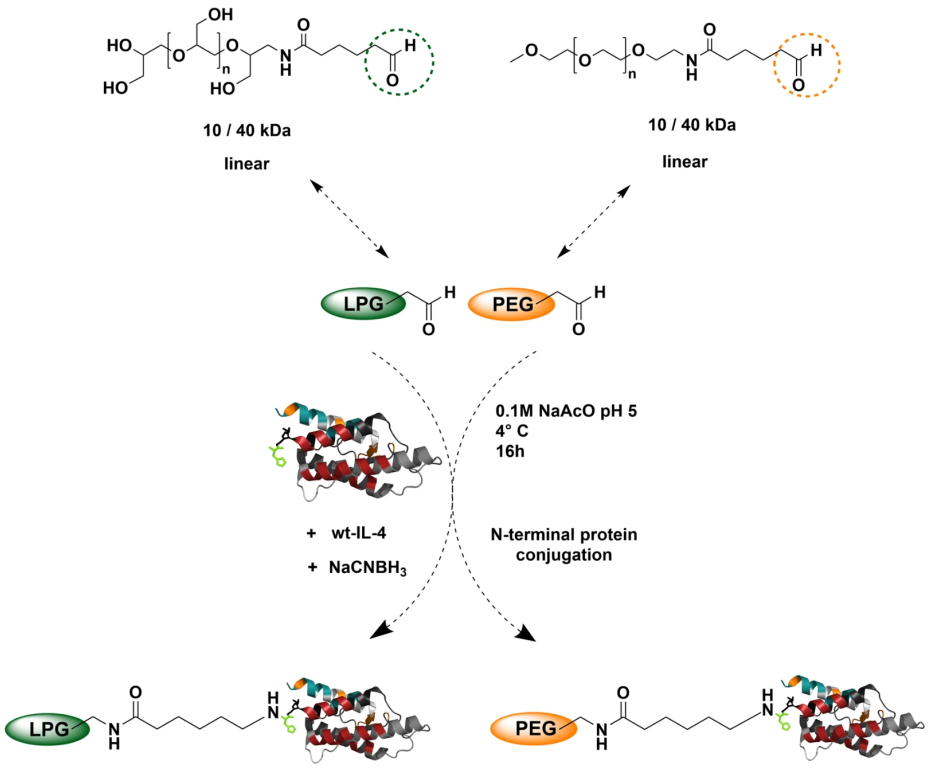
Microscale-thermophoresis (MST) for determination of HSA-binding

Microscale thermophoresis experiments were carried out on a Monolith NT.115 instrument (NanoTemper technologies GmbH, München, Germany) using red laser with LED settings of 40 % and low MST power. HSA was fluorescently labeled with Alexa Fluor™ 647 NHS dye (Thermo Fisher Scientific, Massachusetts, USA) prior to measurements using the standard protocol provided by the manufacturer. To minimize alterations in binding due to a high degree of labeling, we used a neutral pH for labeling to favor modification at the protein's *N*-terminus rather than its lysine-residues.[30] After purification, the degree of HSA-labeling was determined as 0.33 according to the manufacturer's instructions. Without the use of surfactant, HSA was sticking to the capillaries during measurement causing fluctuations in fluorescence, that was also present when using premium type capillaries (NanoTemper technologies GmbH). Therefore, we selected PBS pH 7.4 including 0.05 % Tween®20 as buffer system for our measurements. Concentration of HSA was set to 50 nM whereas the hIL-4 WT samples were added in a concentration range between 0.4–10,250 nM. Samples were centrifuged at 13,000 g for 5 min directly before measurements and subsequently loaded into normal Monolith NT.115 capillaries. All samples were prepared and measured in triplicates, with 2–6 runs per measurement.

RESULTS AND DISCUSSION

Expression of hIL-4 WT and synthesis of its mono-PG and -PEG bioconjugates

LPG-mono-aldehyde as well as mPEG-40-aldehyde were synthesized as described previously (for polymer characteristics see table S1).[31] To exclude a variety in linker structure, the same moiety consisting of a hexyl-spacer between polymer backbone and reactive group was used (Scheme 1). hIL-4 WT was expressed in *E. coli* using a simple expression protocol yielding sufficient amounts, as demonstrated in Figure 1 (Lane 2: appearance of protein band at 15 kDa). The *N*-terminus of hIL-4 WT is not engaged into



Scheme 1. Reaction scheme for the synthesis of *N*-terminal hIL-4 WT bioconjugates

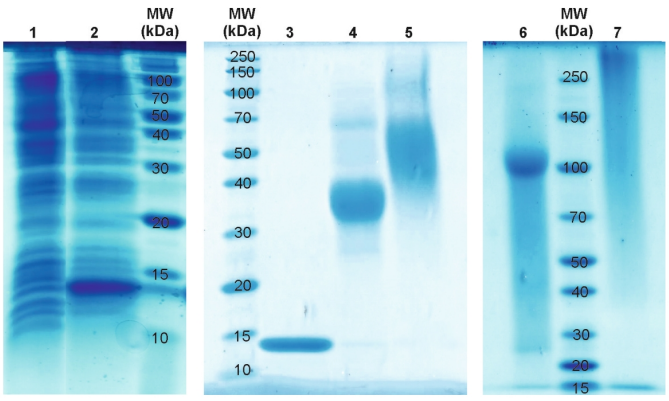


Figure 1. SDS-PAGE of IL-4 WT and its purified bioconjugates. **(1)** IL-4 WT uninduced **(2)** IL-4 WT induced for 6 h **(3)** IL-4 WT, **(4)** IL-4-NH-10-PEG, **(5)** IL-4-NH-10-LPG, **(6)** IL-4-NH-40-PEG **(7)** IL-4-NH-40-LPG.

any molecular interactions regarding hIL-4 WT tertiary structure or during receptor binding with IL-4 receptor α (IL-4Rα) in its Type I and II receptor binding complex (Figure 2) therefore representing a suitable attachment site for polymer modification. Targeting the *N*-terminus for selective bioconjugation bears some advantages, as it is usually solvent exposed thereby enabling direct use of the wild-type protein. Its *N*-terminal α-amino group shows a pk_a-value ≈ 6–9 and therefore displays higher reactivity at acidic pH than lysine ε-amino groups (pk_a ≈ 10.5).[30,32]

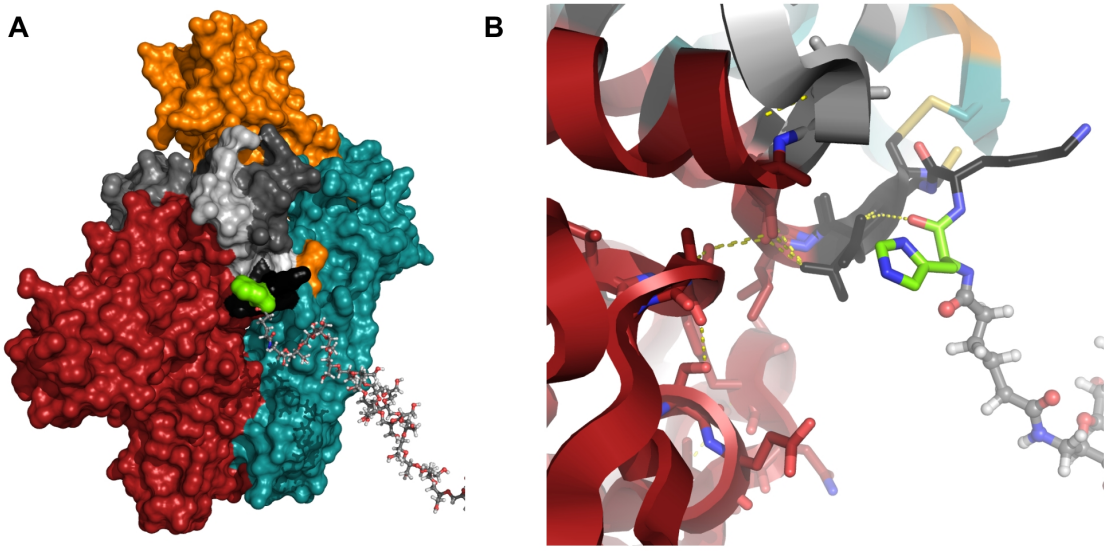


Figure 2. (A) hIL-4 WT engaged in its receptor binding complex(es) Type I and II (PDB: 2b8u, 3bpl and 3bpn are superimposed). hIL-4 WT is displayed in black to white (N to C terminus) with its *N*-terminal residue highlighted in green. Side chain residues engaged into receptor binding are displayed in the color of the receptor. IL-4Rα is displayed in red. IL-4Rγc is displayed in petrol (Type I). IL-13Rα1 is displayed in orange (Type II). LPG is displayed as a stick model and was attached artificially to the *N*-terminal histidine for demonstration purposes of its positioning. **(B)** Polar contacts in the *N*-terminal environment during Type I and II receptor binding.

On the SDS-gel, a shift to higher molecular weight can be observed after polymer conjugation, with the mono-conjugated product being the preferred one under acidic conditions (Figure 1). The diminished gel-

migration of the bioconjugates (compared to M_w-marker) is possibly due to polymer specific interactions with SDS and has already been described for PEG.[33,34] hIL-4 WT LPG bioconjugates showed even shorter migration confirming earlier findings for N-terminally modified anakinra,[31] and suggests a reduced interaction with SDS for LPG-bioconjugates. Due to reactions with hIL-4's side chain lysine-NH_2 groups, multiple bands occurred on the gel reflecting di- or multi-PEGylated/PGylated species (Figure S1), with the mono-product being the preferred one under acidic conditions. The mono-conjugated hIL-4 WT was isolated by ion exchange chromatography, where unmodified hIL-4 WT as well as multi-conjugated hIL-4 WT varied in their elution profile due to a different extent of charge shielding of the protein surfaces. (Figure S2). Overall yields of mono-hIL-4 WT after purification were around 32 % (LPG) and 42 % (PEG) in the case of the 10 kDa polymers and expectedly decreased for the larger 40 kDa chains (12 % and 32 %, respectively).

Characterization of hIL-4 WT bioconjugates by MALDI-TOF MS, SEC-MALS and RP-HPLC

MALDI MS spectra of hIL-4 WT and its 10 kDa bioconjugates showed molecular weights close to their theoretical values (≈ 25 kDa, Figure 3B/C) and further confirmed good comparability between LPG- and PEG-variants of the protein.

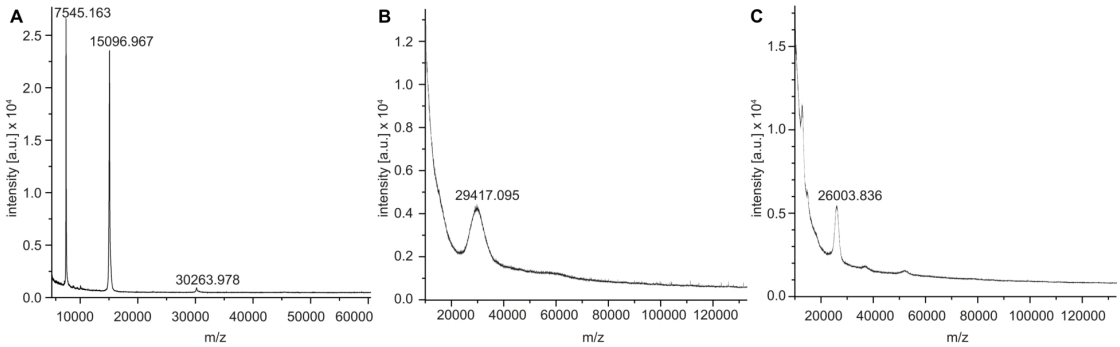


Figure 3. MALDI-TOF analysis of **(A)** hIL-4 WT (expected Mass +1H⁺: 15095.40) **(B)** hIL-4-NH-10-LPG **(C)** hIL-4-NH-10-PEG.

Enzymatic in-gel digestion of hIL-4 WT and hIL-4-NH-10-PEG was employed to proof the *N*-terminal conjugation of the polymers. The resulting peptide digests contained different fragments including one *N*-

terminal peptide bearing the 10 kDa PEG-moiety that was only found in the bioconjugate. We therefore assumed the *N*-terminus being the main polymer attachment site on the protein (Figure S4).

Further analysis by SEC-MALS confirmed the good comparability between masses of hIL-4-NH-10-LPG and -PEG, respectively. For the 40 kDa-bioconjugates, a mass close to its theoretical value (≈ 55 kDa) was obtained for hIL-4-NH-40-PEG, whereas a slightly larger molecular weight was determined for the 40 kDa LPG-analog, most likely due to its larger dispersity being also observed in SDS-PAGE (Table 1, Figure 1).

Table 1. Molecular weight (M_w) of hIL-4 WT and its bioconjugates determined by SEC-MALS and MALDI-TOF-MS

	SEC-MALS [kDa]	Đ	MALDI-TOF-MS [kDa]
hIL-4 WT	15.6	1.00	15.097
hIL-4-NH-10-LPG	29.8	1.06	29.417
hIL-4-NH-10-PEG	30.9	1.01	26.003
hIL-4-NH-40-LPG	72.9	1.08	n.d.
hIL-4-NH-40-PEG	59.9	1.01	n.d.

n.d.: not determined, Đ: dispersity

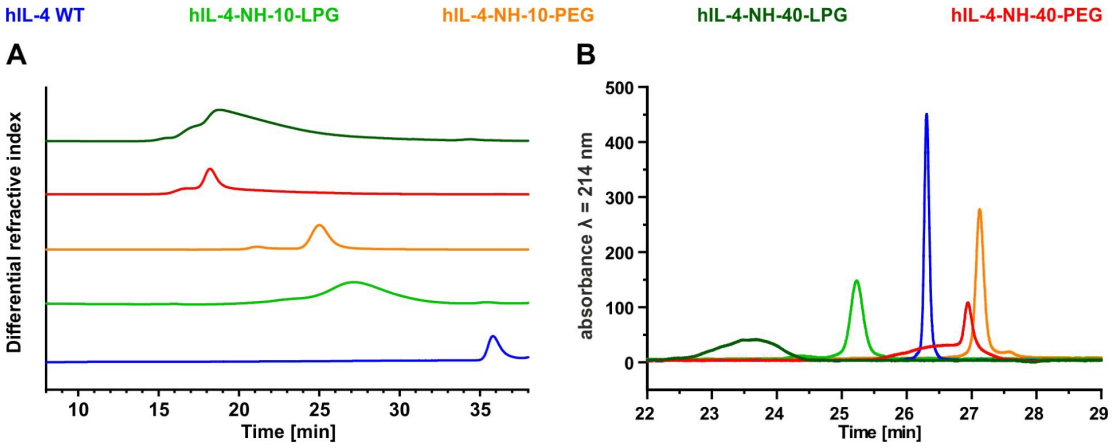


Figure 4. (A) SEC-traces and **(B)** RP-HPLC analysis of hIL-4 WT and its bioconjugates.

In SEC, hIL-4 WT was retained longest on the column (retention time (r.t.) 35.80 min), as it displayed the smallest hydrodynamic size of all analyzed compounds (Figure 4A). Upon polymer conjugation, elution was shifted to earlier time points, where the LPG-variants of hIL-4 WT eluted a bit later than their PEG-analogs being substantiated by LPG's slightly more compact structure[14] (r.t. 27.11 min (10-LPG) vs. 25.00 min (10-PEG) and 18.79 min (40-LPG) vs. 18.18 min (40-PEG)). We observed this phenomenon previously as well for another *N*-terminally modified protein, displaying a similar SEC-profile of LPG- and PEG-conjugates.[31] The hydrophobic elution behavior of IL-4 WT and its bioconjugates was examined by RP-HPLC analysis using a CN column, which shows stronger separation according to protein hydrophobicity than common C18 columns. As demonstrated from the elution profile in reference to hIL-4 WT (r.t. 26.31 min), LPG and PEG significantly impact the protein's overall hydrophilicity (Figure 4B). Conjugation of PEG to hIL-4 WT decreased its hydrophilicity and led to stronger retention on the column, which was quite similar for 10 kDa (r.t. 27.13 min) and 40 kDa PEG (r.t. 26.95 min). The slightly earlier elution of the 40 kDa PEG bioconjugate might be attributed to its larger sterical shielding against interaction with the column matrix. Due to the combination of its hydrophilic oxygen atoms and hydrophobic ethylene units, PEG displays amphiphilic properties. The stronger retention on reversed-phase columns has been shown previously for other PEGylated biomolecules which confirms our findings from HPLC analysis.[35–37] In contrast, 10 kDa LPG (r.t. 25.23 min) distinctly increased the hydrophilicity, therefore displaying earlier elution. This effect was even more pronounced in the case of 40 kDa LPG (r.t. 23.66 min), confirming a chain-length dependent effect for LPG.

Determination of secondary structure and hydrodynamic size

To exclude perturbance of hIL-4's secondary structure after polymer modification, circular dichroism was employed for structural characterization. hIL-4 WT shows a strong alpha helical motif, displaying three extrema at 193, 208 and 222 nm.[24] After polymer conjugation, no significant changes in the overall alpha-helical structure of hIL-4 WT were observed. (Figure 5A). There was no evidence of random-coil or beta-sheet formation present therefore supporting structural retainment of the bioconjugates, independent of polymer type or length.

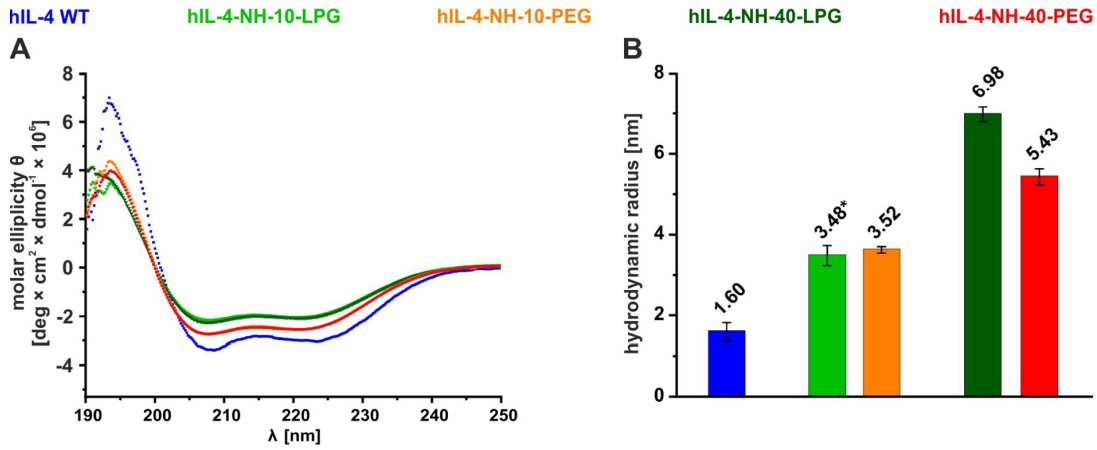


Figure 5. (A) CD-spectra of hIL-4 WT and its bioconjugates at 20 °C (n = 3). Data from hIL-4 WT were published already.[24] **(B)** Hydrodynamic radius of hIL-4 WT and its bioconjugates in PBS pH 7.4 (n = 3, *n = 2).

Hydrodynamic size of hIL-4 bioconjugates

Dynamic light scattering was employed to determine the hydrodynamic size of the bioconjugates. hIL-4 WT showed a radius of 1.6 nm at pH 7.4, which was close to earlier reported values obtained from similar molecular weight proteins (Figure 5B).[38] Conjugation of a single polymer led to a distinct increase in size, that was even more pronounced in the case of the 40 kDa bioconjugates thereby confirming a chain-length dependent volume expansion. PEG's known ability to form stable hydration layers consisting of around three water molecules per monomer unit[39] impacts the overall hydrodynamic volume of protein conjugates, possibly attributed similar for LPG. Furthermore, the highly flexible PEG chain of a mono-bioconjugate usually shows random-coil structure adjacent to the protein (dumbbell-model),[40] leading to a larger overall size.

Despite its higher abundance of OH-groups in polymer backbone, differences between 10 kDa LPG- and PEG-conjugates were not substantial, with the LPG-10-variant being slightly more compact, which confirms our findings from SEC. The hIL-4-NH-40-LPG bioconjugate was slightly larger than its PEG-analog, which be attributed to a salt effect in DLS, whereas SEC confirmed comparable hydrodynamic sizes between the LPG- and PEG-40-bioconjugate. Previous studies by various methods showed, that high molecular weight

LPG alone (100 kDa) displayed a distinct smaller size than its PEG-analog of same nominal weight (7.4–7.6 nm difference in radius).[14] However, in our case we did not observe such a clear difference between the LPG- and PEG-bioconjugates within the molecular weight range used in this study. We speculate this distinct compactness might only be pronounced for the free polymer bearing larger chain lengths and therefore higher molecular weights.

In vitro activity of hIL-4 bioconjugates

To assess the impact of polymer conjugation on hIL-4's *in vitro* activity, we tested our bioconjugates in a HEK 293 cell line expressing IL-4-Rα and IL-13Rα1. Binding of hIL-4 WT to IL-4-Rα is recognized by IL-13Rα1 resulting in a dimerization of the receptor, which triggers a tyrosine kinase (Tyk2, JAK1)-mediated translocation of STAT6 into the nucleus.[41] The latter promotes expression of secreted embryonic alkaline phosphatase (SEAP), which can be finally determined in a colorimetric assay.

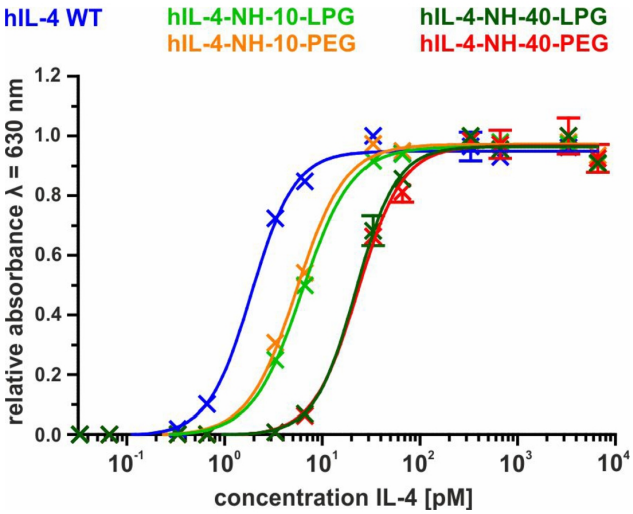


Figure 6. SEAP assay of HEK Blue IL-4/IL-13 cells after 20 h of stimulation with hIL-4-WT and its bioconjugates. Data points represent mean with SD (n = 3).

hIL-4 WT binds to its IL-4Rα-subunit in the pM range,[42] which was confirmed by an EC50-range of 1.7–2.1 pM in our assay (Figure 6, Table 2). Upon polymer conjugation, the biological activity of hIL-4 WT diminished, with hIL-4-NH-10-LPG and hIL-4-NH-10-PEG displaying an approximately three-fold reduced

bioactivity. Further extension of the polymer chain resulted in an almost twelve-fold decrease of receptor activation. (EC50: 20.0–24.2 pM and 20.8–25.9 pM for hIL-4-NH-40-LPG and -40-PEG, respectively). The loss in bioactivity thereby scaled proportionally to the increase in polymer molecular weight, as each additional 10 kDa polymer-unit resulted in an approximately three-fold reduction of *in vitro* activity of hIL-4 WT (Table 2). Despite their different hydrophilicity profile, LPG- and PEG-bioconjugates of same nominal weights displayed comparable biological activities, which is in line with other LPG- and PEG-bioconjugates reported.[31,43] Therefore, we assume the rationale behind diminished biological potency originates from steric hindrance caused by larger hydrodynamic sizes rather than from variations in the bioconjugates hydrophilicity.

In another study by Lühmann *et al.*, 4 kDa poly(2-methyloxazoline) (PMeOx) was employed for the site-specific modification of hIL-4 followed by characterization of its *in vitro* activity with a similar SEAP-assay as applied here.[22] The SEAP-expression level of PMeOx-IL-4 was close to unmodified IL-4 but diminished about factor 1.2–1.4, which is in line with the results obtained for our LPG- and PEG-hIL-4 WT bioconjugates. PMeOx displays comparable hydrophilicity to PEG, but a slightly smaller hydrodynamic size, which might be beneficial in maintaining the biological activity of proteins.[44,45] Coupling of PMeOx occurred at an alkyne-functionalized lysine at position #K42, which is in close proximity to IL-4's *N*-terminus therefore allowing a certain comparability with the bioconjugates investigated here. However, the molecular weight of the polymers is different (4 kDa vs. 10 and 40 kDa, respectively) which impedes a direct contrasting of the *in vitro* activity of the respective bioconjugates.

Plasma stability and HSA-binding of hIL-4 WT and its bioconjugates

To reveal potential benefits through polymer conjugation impacting the plasma stability of hIL-4 WT, we incubated the free protein or its bioconjugates for 24 h in human plasma and collected samples at predetermined timepoints. No significant decrease in hIL-4 WT content in plasma was observed for up to 24 h therefore demonstrating good stability *in vitro* (Figure 7A). Conjugation of LPG or PEG of different molecular weights to hIL-4 WT had no significant impact on its plasma stability, as all bioconjugates remained stable for up to 24 h, which confirms previous data for PEGylated murine IL-4.[24]

Table 2. Important biological parameters of hIL-4 WT and its bioconjugates. EC50 and K_D values are given as 95% confidence intervals.

	EC50 [pM]	K_D to HSA [nM]
hIL-4 WT	1.7-2.1	19.5-65.3
hIL-4-NH-10-LPG	5.8-6.7	281.1-347.2
hIL-4-NH-10-PEG	5.1-5.8	93.7-255.1
hIL-4-NH-40-LPG	20.0-24.2	126.1-286.6
hIL-4-NH-40-PEG	20.8-25.9	298.9-332.0

Besides stability, we further investigated binding of hIL-4 WT and its bioconjugates to the most abundant plasma protein human serum albumin (HSA). In pharmaceutical development, plasma protein binding is of high interest, as its extent usually impacts bioavailability and -distribution of drugs. HSA is often used as a target to increase blood circulation time by incorporating albumin-binding motifs on the target protein,[46,47] or direct fusion to an albumin molecule.[48] Figure 7B shows the binding curves for hIL-4 WT and its LPG- and PEG-bioconjugates, where free hIL-4 WT displayed relatively strong binding in the nM-range (k_D = 42.4 nM). Upon polymer conjugation, the affinity to HSA decreased with ascending polymer length in the case of hIL-4 WT PEG-conjugates (k_D = 159.4 nM and 315.5 nM for hIL-4-NH-10-PEG and -40-PEG, respectively), which we mostly attribute to steric hindrance and the stealth effect of PEG.[49] Furthermore, PEG might prevent hydrophobic interactions between hIL-4 WT and HSA due to its amphiphilic nature.[50–52] The LPG-variants of hIL-4 WT on the other hand showed an inverse behavior regarding HSA-affinity, with the larger hIL-4-NH-40-LPG displaying superior affinity to HSA (k_D = 206.3 nM) compared to its 10-kDa analog (k_D = 314.1 nM). We assume that the distinct higher hydrophilicity of the hIL-4-NH-40-LPG bioconjugate might promote HSA-binding to hIL-4 WT, even though differences are only modest. Despite their overall HSA binding diminished in comparison to hIL-4 WT, this minor effect will be likely negligible for the circulation half-life of hIL-4 WT LPG bioconjugates. hIL-4 WT alone shows a serum half-life of only 19 min in humans,[53] which can already be extended six-fold in mice through the addition of a 10 kDa PEG moiety[24] and resulted in similar HSA binding properties as hIL-4-NH-40-LPG (Figure 7B). Therefore, an

extended circulation time of LPG-hIL-4 WT bioconjugates will be mostly accounted to an increase in hydrodynamic size rather than to their binding to HSA.

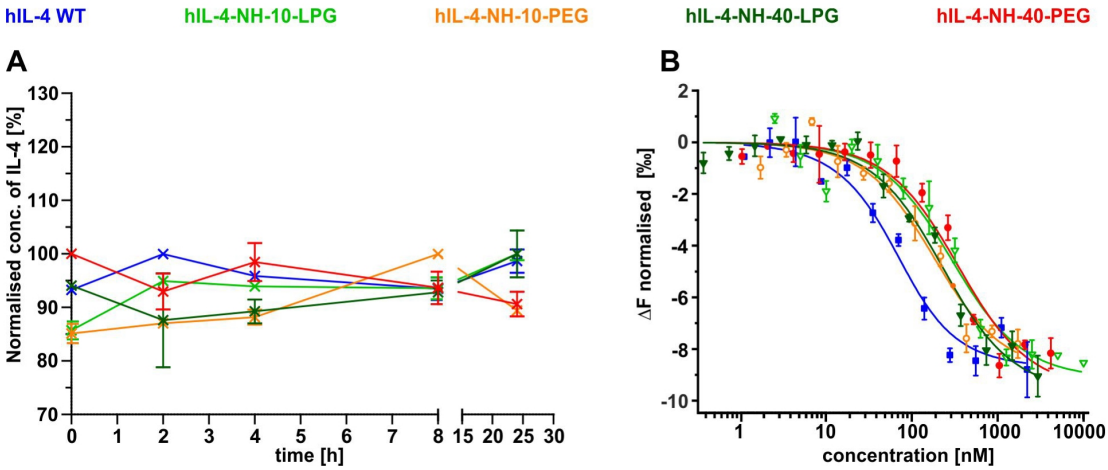


Figure 7. (A) ELISA quantification of hIL-4 WT and its bioconjugates after incubation in human plasma for 24 h. **(B)** Binding analysis of hIL-4 WT and its bioconjugates with fluorescently labeled HSA upon thermophoresis. Each data point represents mean with SD (n = 3).

CONCLUSION

In this article, we demonstrate site-selective *N*-terminal ligation of human interleukin-4 WT with the two distinct polymer types, linear polyglycerol and poly(ethylene glycol). *N*-terminal conjugation was achieved through a reductive alkylation approach at acidic pH yielding the respective mono-conjugates of hIL-4 WT, which were subsequently contrasted in terms of molecular size, *in vitro* stability, bioactivity and HSA-binding properties. LPG- and PEG-hIL-4 WT of same nominal weights showed comparable molecular masses and hydrodynamic sizes. The alpha-helical structure of hIL-4 WT was retained after polymer modification, whereas the bioactivity decreased in a molecular weight dependent manner, where LPG- and PEG-hIL-4 WT of same nominal weights behaved similar. All bioconjugates as well as the free protein showed no degradation for up to 24 h in human plasma therefore indicating good *in vitro* stability. Finally, affinity to the plasma protein HSA was determined, where binding of hIL-4 WT generally diminished after polymer conjugation.

To our knowledge, this is the first study describing the *N*-terminal polymer modification of hIL-4 WT. We believe the data presented here could serve as a fundamental to unravel differences in the pharmacokinetic and pharmacodynamic *in vivo* profile of PEGylated and PGylated forms of hIL-4 WT. From an *in vitro* perspective, *N*-terminal LPG-hIL-4 WT bioconjugates showed equivalent biological performance to their PEG-analogs therefore highlighting the good potential of LPG as an alternative polymer platform to improve the pharmacokinetics of small biologics.

ASSOCIATED CONTENT

Supporting Information Available

The following files are available free of charge: Polymer synthesis and characterization, SDS-gels, chromatograms, mass spectra.

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Notes

M. Tully and N. Hauptstein have contributed equally to this work. The authors declare no competing financial interest.

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Supporting Information (SI)

Linear Polyglycerol for N-terminal-selective Modification of Interleukin-4

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EXPERIMENTAL

Dialysis tubes, molecular weight cut-off (MWCO) 1 kDa, were from Carl Roth (Karlsruhe, Germany). 40 kDa LPG-N_3 was purified additionally by Tangential Flow Filtration (RC-membrane, MWCO 10 kDa). Gel-permeation chromatography (GPC) of the LPG-N_3 polymers was performed on an Agilent HPLC system containing an IsoPump (G1310A), a refractive index detector (G1362A) and a manual injection unit (G1328B) (Agilent 1100 Series, PSS, Mainz, Germany). 0.1 M $NaNO_3$ was used as mobile phase on three Suprema size exclusion columns (calibrated against pullulan (342–708,000 Da)) connected in series to determine molecular weight distribution of the LPGs (**Table S1**).

Synthesis of LPG-10, LPG-40 and PEG-40-aldehyde

The synthesis of LPG- and PEG-aldehyde follows a previous protocol[1] and is described here in short.

LPG-N_3 was synthesized starting from the monomer ethoxy ethyl glycidyl ether (EEGE)[2] through anionic ring-opening polymerization according to Richter, *et al.*[3] In short, tetraoctylammonium bromide as initiator was added to a dried Schlenk flask under inert atmosphere, melted under vacuum and dissolved in dry toluene after cooling to room temperature. Subsequently, EEGE was added while cooling to 4 °C with an ice bath and the polymerization was initiated by fast addition of the catalyst triisobutylaluminium *via* syringe. After 16 h at room temperature, the reaction was quenched with ethanol and further purified by several cycles consisting of freezing and subsequent centrifugation, followed by final dialysis against acetone. The obtained product LPEEGE-Br was then deprotected over night in 3 % HCl (conc.), azidated and subsequently reduced with Tris(2-carboxyethyl)phosphine-hydrochloride (TCEP-HCl) to yield the respective LPG mono amine. In a final step, LPG-amine was modified with the short linker 6,6-dimethoxyhexanoic acid (synthesized from 2-Hydroxycyclohexanone dimer, as described previously[4,5]) in DMF in the presence of N,N,N′,N′-Tetramethyl-O-(N-succinimidyl)uronium hexafluorophosphate (HSTU) and N,N-Diisopropylamine. After 72 h, the reaction mixture was dialyzed against water (three days)

followed by acidic deprotection (3 % HCl conc.) and lyophilization to yield the aldehyde-bearing polymer as slightly yellow solid.

Synthesis of mPEG-40-aldehyde followed the same protocol as described above, starting from the commercially available mPEG-40-NH_2-HCl.

Endoproteinase GluC in-gel digest

In gel digest of IL-4-PEG 10 kDa was performed as described before with GluC (Promega) in 100 mM ammonium bicarbonate buffer instead of Elastase.[6] After 5% TFA extraction following overnight digest MALDI-MS was performed as described in the experimental section. GluC was chosen over Trypsin to yield a longer amino acid chain rest, as Il-4 contains a lysine at position 2, leaving only a 2 AA residue.

Table S1. Molecular weight characteristics and level of aldehyde content of the different polymers used in this study. (see also ref [1])

polymer sample	M_n [g/mol]	M_w [g/mol]	Đ	end group conversion to protected aldehyde [%]
10-LPG-N_3	12222	14620	1.20	66
40-LPG-N_3	49377	64421	1.30	61
10-mPEG-CHO	-	11153[a]	1.05	>95[c]
40-mPEG-NH_2-HCl	-	42266[b]	1.03	13

The number in the polymer sample describes the nominal molecular weight in kDa. The properties of PEG were used as supplied by the manufacturer's data sheet. The calculation of the number of protected aldehyde-functionalized polymer chains is described previously.[1] Đ = dispersity. [a]M_p-value. [b]MALDI-TOF. [c]Value for unprotected aldehyde, as supplied by manufacturer.

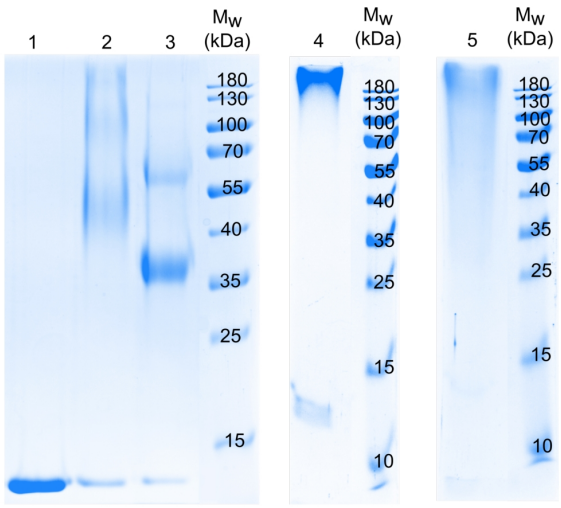

Figure S1. SDS-PAGE of coupling reactions of IL-4 WT with its different polymers. **(1)** IL-4 WT, **(2)** IL-4-NH-10-LPG, (**3**) IL-4-NH-10-PEG, **(4)** IL-4-NH-40-LPG, **(5)** IL-4-NH-40-PEG.

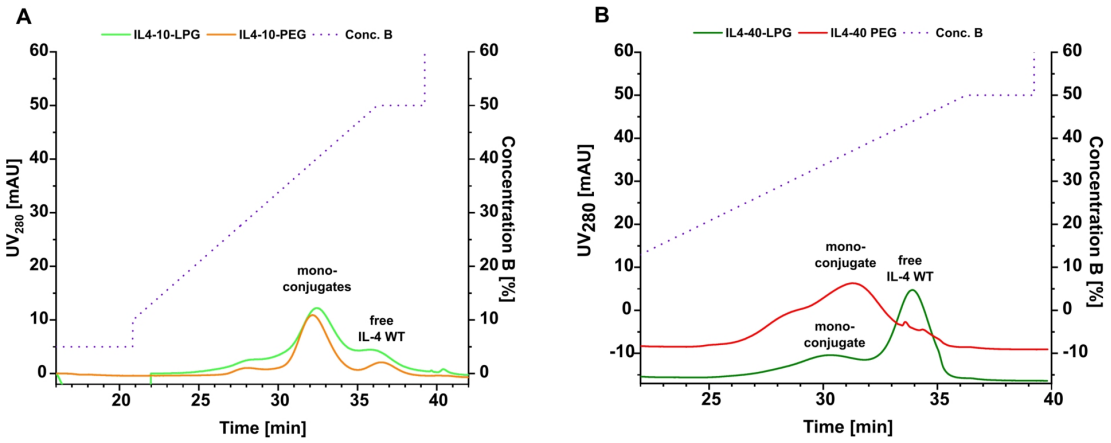

Figure S2. FPLC cation exchange chromatogram of purification of **(A)** IL-4-NH-10 kDa and **(B)** IL-4-NH-40 kDa conjugates.

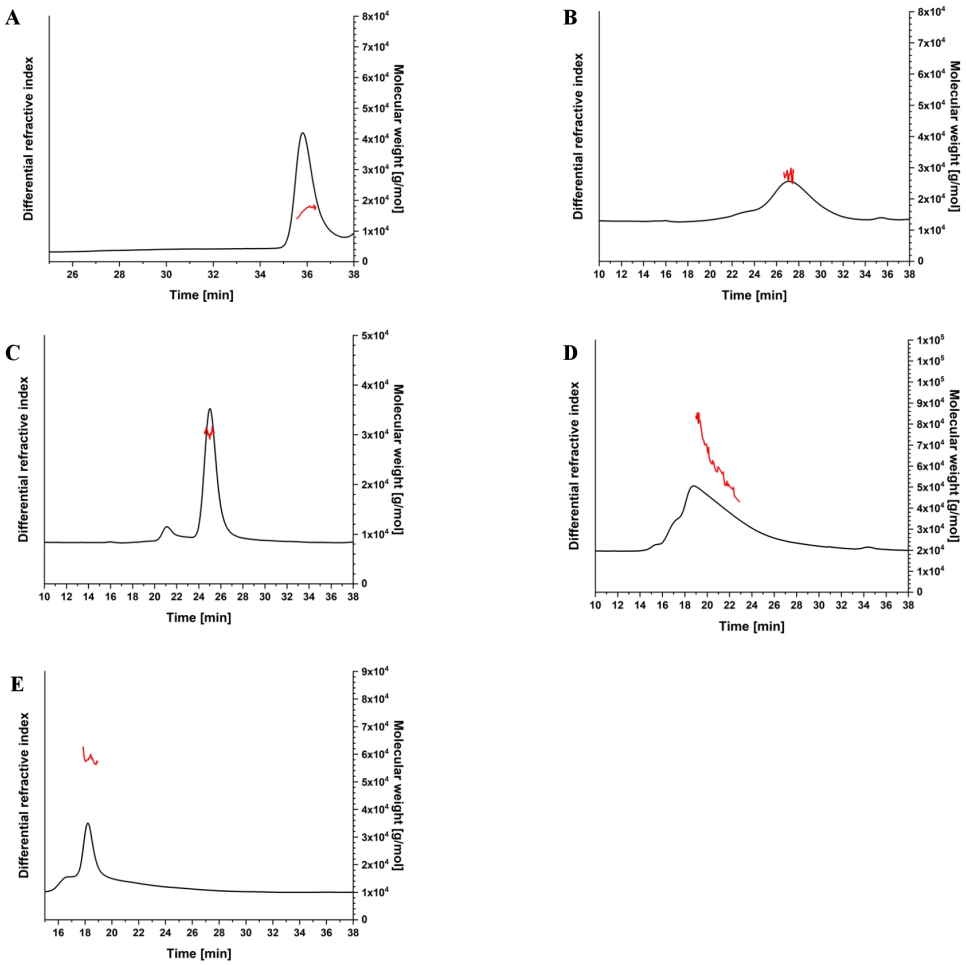

Figure S3. SEC-MALS data of IL-4 WT and its LPG- and PEG-conjugates. **(A)** IL-4 WT **(B)** IL4-NH-10-LPG, **(C)** IL-4-NH-10-PEG, **(D)** IL-4-NH-40-LPG, **(E)** IL-4-NH-40-PEG.

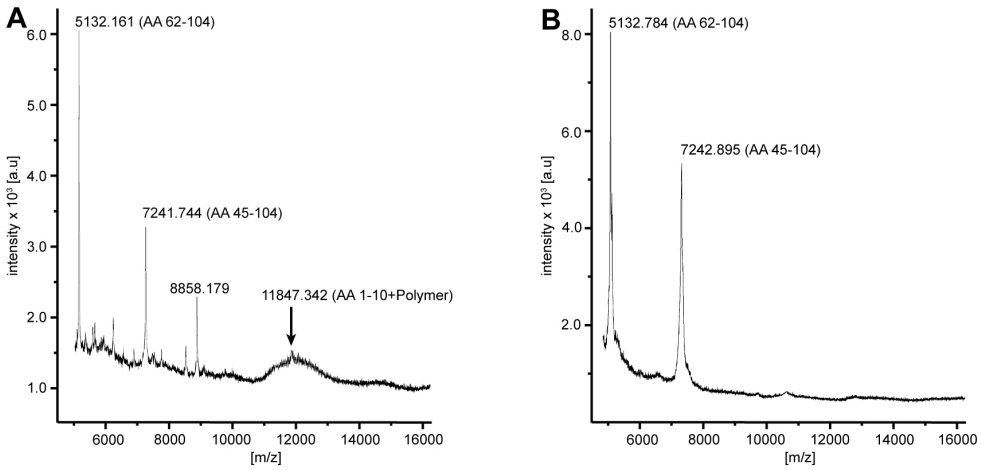

Figure S4. Mass spectra of peptide mixtures after GluC in-gel digestion. (**A**) GluC in-gel digest of IL-4-NH-PEG 10 kDa. The arrow indicates the N-terminal fragment bearing the 10 kDa PEG, which was only found in the conjugate, with a peak mass of 11,847 Da (1-10: 1275.48 Da + 10572 Da PEG). (**B**) GluC in-gel digest of IL-4 WT.

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5 Conclusion and Outlook

In this thesis, the overall goal was to establish linear polyglycerol as PEG-alternative polymer platform for the half-life extension of therapeutic proteins and peptides. The aim was to design polymer-protein conjugates with a 1:1 ratio of polymer to biomolecule by employing mono-functional LPG and site-selective coupling chemistries. Furthermore, this work aimed to reveal differences between LPG- and PEG-bioconjugates in regard to their hydrodynamic size, bioactivity, and stability *in vitro* and their extended half-life and therapeutic efficacy *in vivo*.

In the first project, a site-selective *N*-terminal conjugation strategy was developed which enabled modification of the therapeutic protein anakinra by employing LPG-aldehyde of 5, 10, 20 and 40 kDa. PEG-conjugates of the same protein were synthesized as reference. The molecular weights of the isolated mono-conjugates were systematically analyzed by multi-angle light scattering and mass spectrometry and confirmed close values for LPG- and PEG-conjugates of same nominal weight, with dispersities below 1.1.

The hydrodynamic size of the bioconjugates increased with ascending polymer length, where the LPG-conjugates displayed slightly smaller values than their PEG-analogs. This finding confirms the general picture of LPG as a more compact polymer than PEG, even though differences were not as large as found for the free polymers.[129] Studies on enzymatic and thermal degradation revealed a somewhat better performance for the more hydrophilic LPG-conjugates, which aids in designing more stable protein-conjugates in the future. Additionally, binding affinity to IL-1 receptor 1 decreased about factor 3–7 upon polymer conjugation, with no significant differences between LPG- and PEG-conjugates observed. Finally, a pharmacokinetic study of a selected Anakinra-40-LPG conjugate revealed a similar performance and terminal half-life than its PEGylated analog of same molecular weight.

The second project focused on the site-specific C-terminal conjugation of the propargyl-modified diabetes therapeutic exenatide. LPG-N_3 of 10, 20 and 40 kDa was employed to synthesize conjugates by Cu-catalyzed click-chemistry. PEG-conjugates of similar molecular weights again served as reference. The secondary structure of exenatide was not altered by polymer conjugation, whereas its hydrodynamic size increased with ascending polymer length. The values were higher than expected, as exenatide is known to exhibit oligomerization in solution, which was not prevented by conjugation of LPG or PEG. Exenatide-LPG-conjugates displayed smaller sizes and a more compact structure than their PEG-analogs of same molecular weight. Furthermore, thermal denaturation studies showed a slightly larger retainment of exenatide's α-helical structure upon PEG-conjugation, which was independent of the length of the polymer. Determination of the *in vitro* bioactivity of the conjugates by GLP-1 receptor-

mediated cAMP-response revealed no significant differences between LPG- and PEG-exenatide of same molecular weight. Despite the bioactivity diminished after conjugation, maximum receptor response was still enabled at slightly higher concentrations. The main goal of this project was the evaluation of LPG-conjugates in a therapeutic disease model. A single injection of a selected exenatide-40-LPG conjugate in diabetic mice led to a significant blood glucose reduction for up to 72 h. This extended therapeutic activity was comparable to its exenatide-40-PEG analog, but 9-fold longer than native exenatide (8 h).

The third project aimed to focus on the plasma characterization of LPG-and PEG-conjugates. The same coupling strategy as in project 1 was employed to generate *N*-terminal bioconjugates of interleukin-4. Comparable masses and hydrodynamic sizes between LPG- and PEG-IL-4 of same nominal weights were confirmed by SEC-MALS, MALDI-TOF and DLS, respectively. HPLC analysis revealed the large impact of LPG on the overall hydrophilicity of the conjugate, which increased with ascending polymer length. In contrast, PEG-conjugation resulted in a more hydrophobic character of IL-4. Polymer modification diminished the bioactivity of IL-4 in a proportional manner, where each 10 kDa polymer unit attached resulted approximately in a three-fold reduction of potency compared to the unmodified protein. The hydrophilicity had no substantial impact on the bioactivity *in vitro*, as LPG- and PEG-modified IL-4 displayed similar activation of the respective receptor. All conjugates remained stable in human plasma for up to 24 h and displayed diminished binding to human serum albumin (HSA), with no significant impact of polymer type or length observed.

To conclude, this thesis has demonstrated comparable *in vitro* and *in vivo* performances of LPG- and PEG-conjugates of various therapeutic proteins and thereby verified polyglycerol's great potential as an alternative to PEG for half-life extension. The impact of LPG on the bioactivity, terminal half-life and *in vivo* efficacy of selected biopharmaceuticals was similar to PEG. Differences were observed in thermal and proteolytic stability, overall hydrophilicity, and hydrodynamic size of the conjugates.

In the future, questions regarding the immunogenicity and antigenicity of LPG need to be answered. Several setups employing ELISA or SPR already exist that enable the detection of anti-polymer specific antibodies in serum from healthy animals that received weekly doses of a specific PEGylated protein.[11,298] Other studies evaluated polymers of different chemical structures on their cross-reactivity towards anti-PEG antibodies.[113] This could be useful to determine the antigenicity of LPG outruling concerns about its reactivity towards pre-existing

anti-PEG antibodies in the healthy population, which might impede the therapeutic efficacy of PGylated proteins.

Another aspect covers the lyophilization of biopharmaceuticals. Due to its high content of hydroxy groups, LPG (free or conjugated) could serve as a stabilization tool during freeze-drying of therapeutic proteins. So far, high concentrations of sugars (trehalose, sucrose) are used to replace the water hydration shell upon drying.[299] Conjugation of LPG might serve as a similar tool and could potentially maintain protein structure, stability and bioactivity after drying without the need of complex formulation studies.

6 Zusammenfassung

Ziel dieser Arbeit war es, Polyglycerol als PEG-alternative Polymer-Plattform zur Halbwertszeitverlängerung von therapeutischen Proteinen und Peptiden zu etablieren. Der Fokus lag dabei auf Polymer-Protein Konjugaten mit definiertem Verhältnis von einer Polymerkette pro Protein, was durch die Anwendung von monofunktionalem LPG und ortsspezifischer Konjugationschemie erreicht werden sollte. Weiterhin sollten Unterschiede zwischen LPG- und PEG-Biokonjugaten bezüglich deren hydrodynamischer Größe, Bioaktivität und Stabilität *in vitro* sowie ihrer verlängerten Halbwertszeit und therapeutischen Effektivität *in vivo* untersucht werden.

Im ersten Projekt dieser Arbeit wurde eine ortsspezifische, *N*-terminale Konjugationsstrategie entwickelt, bei der LPG-Aldehyd mit Molekulargewichten von 5, 10, 20 und 40 kDa an das therapeutische Protein Anakinra konjugiert wurde. PEG-Konjugate des gleichen Proteins wurden als Vergleichsmaterial hergestellt. Die Molekulargewichte der gereinigten Mono-Konjugate wurden systematisch mittels multi-angle light scattering und Massenspektrometrie untersucht, wobei ähnliche Werte für LPG- und PEG-Konjugate gleichen, nominellen Molekulargewichts erhalten wurden. Die Dispersität lag hierbei bei unter 1,1.

Die hydrodynamische Größe der Biokonjugate nahm mit steigender Polymerlänge zu, wobei die LPG-Konjugate ein etwas kleineres Volumen als ihre jeweiligen PEG-analoga zeigten. Die Ergebnisse unterstützen hierbei das Bild von LPG als etwas kompakterem Polymer, verglichen mit PEG, obwohl die Unterschiede bei den Konjugaten nicht so deutlich waren wie bei den freien Polymeren in Lösung.[129] Studien zum enzymatischen und thermischen Abbau zeigten eine höhere Stabilität der hydrophilen LPG-Konjugate, was hilfreich beim zukünftigen Design von stabileren Proteinkonjugaten sein könnte. Zusätzlich wurde die Affinität der Konjugate an den IL-1 Rezeptor 1 untersucht, wobei die Bindung um Faktor 3–7 abnahm und die LPG- und PEG-Konjugate ähnliches Verhalten zeigten. Schließlich bestätigte eine pharmakokinetische Studie eines ausgewählten Anakinra-40-LPG Konjugats eine vergleichbare Zirkulationsdauer zum jeweiligen PEG-Analogon.

Im zweiten Projekt lag der Fokus auf der ortsspezifschen, C-terminalen Konjugation des Diabetestherapeutikums Exenatid. LPG-Azid (10, 20 und 40 kDa) wurde mittels Kupfer-katalysierter „Klick“-Chemie an das Propargyl-modifizierte Peptid angebracht, wobei PEG-Konjugate gleichen Molekulargewichts erneut als Vergleichsmaterial dienten. Die Sekundärstruktur von Exenatid wurde durch die Polymerkonjugation nicht beeinflusst, wohingegen die hydrodynamische Größe mit aufsteigender Polymerkettenlänge zunahm. Die

erhaltenen Werte waren hierbei größer als erwartet, da Exenatid zur Bildung von Oligomeren in Lösung neigt, was durch Konjugation von LPG oder PEG nicht unterbunden werden konnte. Die hydrodynamischen Durchmesser der LPG-Exenatid-Konjugate waren kleiner als die der PEG-Konjugate gleichen Molekulargewichts. Die α-helikale Sekundärstruktur von Exenatid konnte bei Erhitzen besser durch PEG stabilisiert werden als durch LPG, wobei die Länge der Polymerkette keine große Rolle spielte. Eine Aktivierung des GLP-1 Rezeptors wurde für LPG-Exenatid in ähnlichem Maße erreicht wie für PEG-Exenatid gleichen Molekulargewichts. Hierbei wurden jedoch höhere Konzentrationen der größeren Konjugate benötigt, um die gleiche, maximale Rezeptorantwort von unmodifiziertem Exenatid zu generieren. Schließlich konnte gezeigt werden, dass ein ausgewähltes LPG-Exenatid-Konjugat die Blutglukose in diabetischen Mäusen ähnlich stark und lange reduzieren kann wie ein PEG-Konjugat gleichen Molekulargewichts (bis zu 72 Stunden).

Im dritten Projekt wurde der Fokus auf die Charakterisierung von LPG- und PEG-Konjugaten gegenüber Plasmabestandteilen gelegt. Hierfür wurde die etablierte Kupplungsstrategie aus dem ersten Projekt angewandt, um *N*-terminale Konjugate des Proteins Interleukin-4 (IL-4) zu erhalten. LPG- und PEG-IL-4 gleichen, nominellen Molekulargewichts zeigten vergleichbare Massen und hydrodynamische Größen. HPLC-Analysen bestätigten den großen Einfluss von LPG auf die generelle Hydrophilie des Konjugats, die mit steigender LPG-Kettenlänge zunahm. Im Gegensatz dazu führte die Konjugation von PEG zu einem eher hydrophoben IL-4-Konstrukt. Die Bioaktivität wurde durch Polymerkonjugation reduziert, wobei das Anbringen einer 10 kDa Polymereinheit zu einer ungefähr 3-fachen Abnahme der *in vitro* Wirksamkeit führte. Große Unterschiede zwischen LPG- und PEG-IL-4 konnten hierbei nicht festgestellt werden. Alle Konjugate waren stabil in Humanplasma (bis 24 Stunden) und zeigten verminderte Affinität zum Plasmaprotein Human Serum Albumin (HSA).

Zusammenfassend konnte in dieser Arbeit dargestellt werden, dass LPG- und PEG-Konjugate verschiedener therapeutischer Proteine ein ähnliches *in vitro* und *in vivo* Verhalten zeigen, was das große Potential von Polyglycerol als PEG-Alternative zur Halbwertszeitverlängerung unterstreicht. Der Einfluss von LPG auf Bioaktivität, terminale Halbwertszeit und *in vivo* Wirksamkeit von ausgewählten Biopharmazeutika war ähnlich wie der von PEG. Unterschiede konnten in den Bereichen thermischer und proteolytischer Stabilität, genereller Hydrophilie und hydrodynamischer Größe festgestellt werden.

Um die Anwendbarkeit von Polyglycerol als PEG-Alternative weiter zu verfestigen, sollten in Zukunft Fragen zu dessen Immunogenität und Antigenizität beantwortet werden. Mehrere

Möglichkeiten zur Detektion von anti-Polymer-spezifischen Antikörpern im Serum mittels ELISA oder SPR wurden bereits beschrieben.[11,298] Zeitlich begrenzte, wöchentliche Injektionen eines bestimmten PG-Konjugats im gesunden Versuchstier würden hierbei eingesetzt, um die entsprechenden Polymer-Antikörper zu erzeugen. Andere Studien befassen sich mit der Kreuz-Reaktivität verschiedener Polymertypen gegenüber anti-PEG Antikörpern.[113] Dies könnte hilfreich sein, um Bedenken gegenüber der Reaktivität von LPG auf bereits bestehende anti-PEG Antikörper in der gesunden Bevölkerung auszuräumen, die eine Anwendbarkeit von PGylierten Proteinen limitieren würde.

Ein anderer Aspekt betrifft die Gefriertrocknung (Lyophilisation) von Biopharmazeutika. Durch den hohen Gehalt an Hydroxygruppen könnte LPG (frei oder konjugiert) die Struktur von therapeutischen Proteinen während des Gefriertrocknungsprozesses stabilisieren. Bisher werden hierfür hohe Konzentrationen von Zuckern (Trehalose, Sucrose) eingesetzt, die die Hydrathülle des Wassers nach Trocknung ersetzen.[299] Konjugation von LPG könnte einen ähnlichen Effekt bewirken und somit die Durchführung komplexer Formulierungsstudien zur Proteinstabilität bei Lyophilisation vereinfachen.

7 References

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List of Abbreviations

ABC	Accelerated Blood Clearance
ABS	Antibodies
API	Active pharmaceutical ingredient
AROP	Anionic ring opening polymerization
ATRP	Atom transfer radical polymerization
BSA	Bovine serum albumin
BTC	Benzotriazole carbonate
CD	Circular Dichroism
CDI	Carbonylimidazole
CHO	Chinese Hamster Ovary
CROP	Cationic ring opening polymerization
CuAAC	Copper(I)-catalyzed azide-alkyne cycloaddition
DARPin	Designed Ankyrin Repeat Proteins
DCC	Dicyclohexylcarbodiimid
DLS	Dynamic Light Scattering
dPG	Dendritic Polyglycerol
E.coli	Escherichia coli
ECM	Extracellular matrix
EG	Ethylene glycol
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
EO	Ethylene oxide
EPO	Erythropoietin
FcRn	Neonatal Fc receptor
FDA	Food and Drug Administration
GCS-F	Granulocyte colony-stimulation factor
GFP	Green fluorescent protein
GLP-2	Glucagon-like peptide 2
HES	Hydroxyethylstarch
HGH	Human growth hormone
hPG	Hyperbranched Polyglycerol
HSR	Hypersensitivity reactions
IFN	Interferon
IgG	Immunoglobulin G
LPG	Linear Polyglycerol
LPXTG	Leucine-Proline-X-Threonine-Glycine
MALDI	Matrix-assisted laser desorption ionization
M_n	Number-average molecular weight
M_w	Weight-average molecular weight
mPEG	Methoxy-poly(ethylene glycol)
NG	Nanogel
NHS	N-Hydroxysuccinimide
OEG	Oligoethylene glycol
pCB	Polycarboxybetaine
PDB	Protein data bank
PEEP	Poly(ethyl ethylene phosphate)
PEO	Poly(ethylene oxide)
PEG	Poly(ethylene glycol)
PG	Polyglycerol

PGA	Polyglutamic acid
PHPMA	Poly(N-(2-hydroxypropyl) methacrylamide)
PMeEP	Poly(methyl ethylene phosphonate)
PMPC	Poly(2-methacryloyloxyethyl phosphorylcholine)
PNIPAM	Poly-N-isopropylacrylamid
pNPC	*para*-nitrophenyl carbonate
POEGMA	Poly[oligo(ethylene glycol) methyl methacrylate]
POx	Poly(2-oxazoline)
PP	Polypeptides
PPB	Polyphosphobetaine
PPE	Polyphosphoester
PSA	Polysialic acid
PSar	Polysarcosine
PSB	Polysulfobetaine
PVP	Poly(N-vinylpyrrolidone)
RAFT	Reversible-addition-fragmentation chain-transfer polymerization
RBC	Red blood cell
SC	Succinimidyl carbonate
SCID	Severe combined immunodeficiency disease
SDS	Sodium dodecylsulfate
SDS-PAGE	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
SEC	Size-exclusion chromatography
SEC-MALS	Size-exclusion chromatography-multi angle light scattering
SpAAC	Strain-promoted azide-alkyne cycloaddition
SPR	Surface plasmon resonance
SS	Succinimidyl succinate
TCP	Trichlorophenyl carbonate
TNF-α	Tumor necrose factor-α
TOF	Time of flight
UAA	Unnatural amino acid
VEGF	Vascular endothelial growth factor

List of Publications and Conference Contributions

PUBLICATIONS

[1] **Tully, M.**;* Dimde, M.; Weise, C.; Pouyan, P.; Licha, K.; Schirner, M.; Haag, R.* Polyglycerol for Half-Life Extension of Proteins–Alternative to PEGylation? *Biomacromolecules*, **2021**, *22*, 1406–1416.

[2] **Tully, M.**; Wedepohl, S.; Kutifa, D.; Weise, C.; Licha, K.; Schirner, M.; Haag, R.* Prolonged Activity of Exenatide: Detailed Comparison of Site-specific linear Polyglycerol- and Poly(ethylene glycol)-Conjugates. *European Journal of Pharmaceutics and Biopharmaceutics*, **2021**, *164*, 105–113.

[3] **Tully, M.**;‡ Hauptstein, N.;‡ Licha, K.; Meinel, L.; Lühmann, T.; Haag, R.* Linear Polyglycerol for N-terminal-selective Modification of Interleukin-4. **2021**, *submitted.*
(‡ These authors contributed equally.)

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POSTER PRESENTATIONS

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[2] **Tully, M.;** Hauptstein, N.; Licha, K.; Lühmann, T.; Meinel, L.; Haag, R. Half-life extension of Interleukin-4: Polyglycerol as an alternative to PEGylation. *Annual Meeting of the German Local Chapter of the Controlled Release Society* **2020**, Munich, Germany.

[3] **Tully, M.;** Dimde, M.; Licha, K.; Haag, R. Polyglycerol as an alternative Polymer-Platform for PEGylation of biopharmaceutics. *Workshop on Protein Stability* **2020**, Munich, Germany.

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[7] Kapourani, E.; **Tully, M.**; Pouyan, P.; Haag, R. Dendritic polyglycerol-based microgels as potential cell encapsulation therapeutics. *12th International Symposium on Polymer Therapeutics* **2018**, Valencia, Spain.

Curriculum vitae

For reasons of data protection, the curriculum vitae is not included in the online version.