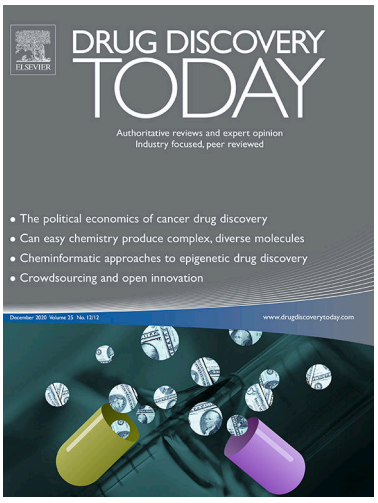
Journal Pre-proofs



Keynote (green)

Building better biobetters: from fundamentals to industrial application

Karin M. Torres-Obreque, Giovanna P. Meneguetti, Jorge J. Muso-Cachumba, Valker A. Feitosa, João H. P. M. Santos, Sónia P. M. Ventura, Carlota O. Rangel-Yagui

PII: S1359-6446(21)00369-X
DOI: https://doi.org/10.1016/j.drudis.2021.08.009
Reference: DRUDIS 3089

To appear in: *Drug Discovery Today*

Received Date: 2 March 2021
Revised Date: 28 May 2021
Accepted Date: 23 August 2021

Please cite this article as: K.M. Torres-Obreque, G.P. Meneguetti, J.J. Muso-Cachumba, V.A. Feitosa, J. H. P. M. Santos, S. P. M. Ventura, C.O. Rangel-Yagui, Building better biobetters: from fundamentals to industrial application, *Drug Discovery Today* (2021), doi: https://doi.org/10.1016/j.drudis.2021.08.009

Building better biobetters: from fundamentals to industrial application

Karin M. Torres-Obreque,[1] Giovanna P. Meneguetti,[1,2] Jorge J. Muso-Cachumba,[1] Valker A. Feitosa,[1,2] João H. P. M. Santos,[1,3] Sónia P. M. Ventura[3] and Carlota O. Rangel-Yagui[1*]

[1]Department of Biochemical and Pharmaceutical Technology, School of Pharmaceutical Sciences, University of São Paulo, São Paulo, Brazil
[2]Bionanomanufacturing Center, Institute for Technological Research (IPT), São Paulo, Brazil
[3]CICECO–Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, Aveiro, Portugal

Corresponding author: Rangel-Yagui, C.O. (corangel@usp.br)

Keywords: biobetter; protein engineering; PEGylation; glycoengineering; biodegradable polymer; fusion protein.

Highlights:

- Biobetters are improved biological drugs, usually with enhanced pharmacokinetics.
- Biobetters can be considered to be intermediates between biosimilars and innovative biological drugs.
- It takes about 10 years and an investment of about US$500 million to develop a biobetter.
- Glycosylation and PEGylation are the most common strategies for the development of biobetters.
- Innovative trends in genetic engineering show promise in the development of biobetters.

Author biographies:

Karin Torres-Obreque has a BSc in Biotechnology Engineering (2015) from the University of La Frontera (Chile) and a MSc in Biochemical-Pharmaceutical Technology (2017) from the University of São Paulo (USP, Brazil). From 2018 to 2019, she worked as an R&D analyst at Prozyn®, Brazil, developing enzyme and bioingredient formulations. At present, she is a PhD candidate in the Biochemical-Pharmaceutical Technology Graduate Program at USP, where she works in the Nanobio group with Prof. Rangel-Yagui as her advisor. Her experience focuses on the nanobiotechnological development of protein drugs by bioprocess including the use of recombinant bacteria, protein PEGylation and nanoencapsulation.

João Santos has a MSc in Biotechnology (2014) from the University of Aveiro, Portugal, and a PhD in Chemical Engineering (Bioengineering) awarded jointly by the University of Aveiro and the University of São Paulo (USP, Brazil) (2019). At present, he is a post-doctoral fellow in the

Nanobio group, School of Pharmaceutical Sciences (USP) and a visiting researcher at the Institute of Technological Research (IPT) in São Paulo. His research is focused on the development and implementation of cost-effective biotechnological and nanotechnological solutions in the (bio)pharmaceutical industry. In addition, he seeks sustainable processes using greener solvents, such as ionic liquids and deep eutectic solvents.

Prof. Rangel-Yagui has a BSc in Pharmacy-Biochemistry (1997) and a PhD in Biochemical-Pharmaceutical Technology (2003) from the University of São Paulo (USP, Brazil), having spent 18 months studying in the Chemical Engineering Department of the Massachusetts Institute of Technology (MIT, USA). Since 2006 she has been a professor at the School of Pharmaceutical Sciences, USP, where her work has focused on the development of biological drugs using strategies such as PEGylation and nanoencapsulation. She is currently a full professor and head of the Nanobio group at USP, and also an Associate Editor of the Brazilian Journal of Pharmaceutical Sciences.

Off-patent biological drugs or biopharmaceuticals have the promise to fill a potentially large market in follow-on biologics: biosimilars and biobetters. Biobetters are new drugs that are designed from existing ones but with improved properties, such as increased selectivity, stability and half-life and/or lower toxicity and immunogenicity. Glycosylation is one of the most used strategies to improve biological drugs. Bioconjugation is an alternative strategy involving the covalent attachment of polymers to biological drugs. Extensive research on novel polymers for the delivery of biologics is underway, but at present, PEGylation is still the best alternative with the longest clinical track record. Innovative trends based on genetic engineering techniques such as fusion proteins and PASylation are also promising. In this review, all these alternatives are explored, as are current market trends, legislation and future perspectives.

Biological drugs: new opportunities in the pharmaceutical industry

The activities of the pharmaceutical industry worldwide are still mainly focused on the production and development of synthetic drugs used in traditional therapies. In recent years, however, interest in biological drugs (also known as biologicals or biopharmaceuticals) has increased considerably as they might represent solutions for diseases, including cancer, Alzheimer's, hemophilia, diabetes, arthritis and some immune system diseases, that have not been treated effectively by synthetic drugs.[1]

The term 'biopharmaceutical' emerged in the 1980s to describe products obtained by modern techniques of biotechnology and molecular biology, distinguishing them from traditional biological products such as vaccines, blood factors and immunoglobulins.[2] In contrast to chemically synthesized drugs, biological drugs are complex products manufactured by living organisms (microorganisms, animals or plants) and usually composed

of peptides, proteins or genetic materials. One of the most used definitions for this type of drug was coined by Walsh,[3] who defined a biological drug as a 'protein or nucleic acid-based pharmaceutical substance used for therapeutic or *in vivo* diagnostic purposes, which is produced by means other than direct extraction from a native (non-engineered) biological source'. Other authors define biologics as drugs that originate from biotechnological processes in which the active pharmaceutical ingredient (API) is obtained from microorganisms or genetically modified cells.[1] The definition varies among regulatory agencies, with different terms referring to different subsets of therapeutics within the general biological products category. Most regulatory agencies, including the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA), consider biologicals to include a wide range of products including vaccines, blood and blood components, allergens, somatic cells, gene therapies, tissues and therapeutic proteins.

The biopharmaceutical market has been growing exponentially, and the attrition rate of biologics is generally considered to be lower than that for conventional small molecule drugs as their development is a highly regulated industry with a highly skilled workforce.[4] Nonetheless, the number of biological drugs available in the market is limited when compared to the number of synthetic drugs, and biological drugs usually cost much more, making them less accessible, especially to low-income patients and health systems.[4]

Another important aspect to consider is the 'Patent Cliff', the well-described marketing phenomenon of a sharp drop in the sales of a successful product as it approaches the end of its patent period.[5] Once the patent expires, it becomes possible to develop and commercialize biosimilars, drugs that are similar to the reference biopharmaceutical in terms of structure, efficacy, safety, target, formulation, dosage and administration.[4] More specifically, the EMA defines a biosimilar as 'a medicine highly similar to another biological medicine already marketed in the EU (so-called 'reference medicine')' and the FDA as 'a biological product that is highly similar to and has no clinically meaningful differences from an existing FDA-approved reference product'.[6,7] Similarity must be based on pre-clinical and clinical studies demonstrating purity, safety and efficacy in all of the conditions for which the reference product is licensed.

At present, a number of different terminologies are used for biosimilars, such as subsequent entry biologics, follow-on biologics, follow-on proteins, biocomparables, similar biotherapeutic products and intended copies. Nonetheless, biologics must be clearly distinguished on the basis of the evidence available to demonstrate their similarity. Hence, they can be classified into one of the following categories: 'approved biosimilars' (from a regulatory standpoint), 'proposed biosimilars' (which are currently under development, but not yet approved by regulatory authorities), and 'intended copies' (which have not undergone rigorous comparative evaluations according to the relevant World Health Organization (WHO) recommendations, but are commercialized in some countries).[8] The concept of generics, that is identical copies, of biological drugs is still contentious because the characteristics of these biomolecules depend on the manufacturing process. To date, no legislation about biological generics has been brought forward in any country worldwide.

Despite some economic success, biological drugs present drawbacks such as immunogenicity and short plasma half-life. Their immunogenicity is mainly due to the production of anti-drug antibodies, resulting in reduced clinical efficacy, hypersensitivity and anaphylactic reactions.[9] The short plasma half-life usually necessitates recurrent administrations to achieve the desired clinical effect. With the advent of better manufacturing processes, new administration procedures and continuous advances in molecular biology, biological drugs are logically expected to improve leading to the generation of biobetters, biologicals developed through the chemical or molecular modification of an originator product.[10]

The term 'biobetter' was first introduced in 2007 by G.V. Prasad, executive director of Dr Reddy's Laboratories in India.[4] It refers to a biological drug that is better than or superior to the reference molecule in one or more parameters but that preserves the therapeutic objective (**Figure 1**). Superiority may result from a difference in amino acid sequence or protein folding, from a chemical modification, from a difference in the humanization process, or from differences in the production process, such as a more efficient purification protocol. These differences can influence the pharmacokinetics of biological drugs, for example increasing half-life.[4]

Research related to biosimilars and biobetters has been increasing. Nonetheless, a PubMed database search found the publication of only 94 articles mentioning biobetters in the past 10 years, highlighting the need to better discuss the research and development of this branch of biological drugs. Biobetters form an appealing part of the biopharmaceutical market; they present reduced inherent drug development risks and decreased production costs as some of the R&D tests have already been performed for the reference molecule and the mechanism of action is already known. Consequently, the overall probability of success is higher. The costs associated with launching a biobetter on the market are two to four times higher than those for a biosimilar, but the economic return and market share gain based on patent exclusivity compensate for these additional costs.[10,11]

The first biobetter to be approved was an improved version of infliximab (Remicade®, Janssen), a murine monoclonal antibody (mAb) that recognizes anti-tumor necrosis factor (TNFα) and is associated with high immunogenicity rates in patients. The improved version adalimumab (Humira®, AbbVie/MedImmune) corresponds to a fully human monoclonal antibody.[12] As we mentioned above, biobetters can be understood as drugs presenting improvements based on changes in amino acid sequence, chemical modification, protein folding and humanization process, like adalimumab. We understand the definition is still controversial and some authors might not consider adalimumab to be a biobetter. Nonetheless, with advances in chemical and molecular biology techniques, as well as a better understanding of the mechanisms of action and the characteristics of biological drugs, several other biobetters have been introduced to the market.

One of the strategies used most commonly to develop biobetters is protein mutation, which can be achieved using traditional molecular biology tools and bioinformatics. Nevertheless, strategies that preserve the original amino acid sequence of a protein drug are

also interesting, especially because of their potential for fast regulatory approval. In this review, we explore the main strategies used to develop biobetters while preserving the original amino acid sequence of a protein drug **{AuQ: Edit OK?}**. More specifically, we provide an updated overview of the difficulties in and future possibilities for polymer conjugation, glycoengineering and selected protein engineering strategies.

Conventional polymer conjugation: PEGylation

PEGylation is the covalent attachment of polyethylene glycol (PEG) chains to a functional molecule.[13] The PEGylation of biological drugs, and particularly protein drugs, is very effective in improving pharmacokinetics and in reducing toxicity and immunogenicity.[14] It has become the main approach to overcoming the limitations of biological drugs, and this is reflected by the increasing number of PEGylated products on the market.[15]

PEGylated proteins are characterized by: (i) increased size and hydrodynamic volume, which reduces renal clearance and, consequently, prolongs circulating half-life; (ii) reduced immunogenicity because the protein immunogenic epitopes are shielded; (iii) protection against *in vivo* proteolysis and endocytosis, (iv) greater solubility in water, which decreases protein aggregation due to steric repulsion among the PEGylated surfaces; and (v) increased thermal and mechanical stability.[13,16,17] Often, these characteristics are achieved with no change in the secondary and tertiary structures of the protein.[17] PEG-biological drugs are mainly eliminated by renal clearance, and toxicity studies in rabbits (measuring vacuolization in the epithelial cells of the proximal renal tubule) indicated that PEG is toxic only at doses much higher than those usually found in biological drug formulations.[17]

Interest in the PEGylation of therapeutic proteins is well-documented and has been increasing since Abuchowski *et al.*[18] discovered this method. PEG is a biocompatible, non-biodegradable and hydrophilic polymer, and several successful examples of PEGylated biological drugs are available.

PEGylation reactions evolved from random conjugation, which resulted in mixtures with limited reproducibility and compounds with variable properties, to site-directed reactions that are intended to provide more homogeneous and potentially more effective products.[13,17] Although the products available on the market were obtained mainly by non-specific PEGylation, industrial and regulatory requirements point to the development of therapeutic proteins with improved structural control. Regulatory agencies used to require only information on the degree of PEGylation, but the distribution of positional isomers might also become a requirement.[19]

When forming a product by PEGylation, one should consider the molecular weight and chemical structure (linear, branched) of the polymer, the steric hindrance of the polymer **{AuQ: Edit OK?}**, the reactivity of both the biomolecule and the polymer and the final application.[13,17] The number of PEG molecules and conjugation sites on the protein should also be considered and will influence the pharmacokinetics and pharmacodynamics of the product.[19] The activated PEG derivatives that are **{AuQ: Edit OK?}** used for the PEGylation reaction have two ends with unique characteristics. One end acts as a selective ligand that

attaches to specific residues at the protein surface, while the other is usually methylated to reduce reactivity (methoxy-PEG).[15,17] The PEGylation of therapeutic proteins should be carried out under mild conditions that avoid lowering the activity or stability of the protein and that prevent hydrolysis of the activated PEG.[19] The thiol group of cysteine residues and the amine groups of both lysine residues and the N-terminus exhibit significantly higher chemical reactivity than any other natural amino acid residue, and are thus preferred for PEGylation. Free cysteines (not forming disulfide bridges) are relatively rare, so most PEGylation agents react with amine groups.[13] Santos *et al.*[16] have reviewed the most common reactive groups for protein PEGylation.

PEGylation has some drawbacks and potential safety risks that cannot be neglected. Among them are: (i) a decrease in protein activity resulting from steric hindrance and/or conformational restriction, (ii) heterogeneous reaction products, (iii) cytoplasmic vacuolization; and (iv) unexpected immune responses to PEG itself.[14] The first two can be tackled by site-directed PEGylation that results in products with a higher degree of homogeneity. Cytoplasmic vacuolization seems to present no toxicological significance but, nonetheless, it can be avoided by modulating the PEG chain size.[20] It is important to mention that the hydrodynamic volume of a free PEG molecule is larger than that of a globular protein of the same molecular weight[21] because of the higher frequency of hydrogen bonds between the oxygen atoms and water molecules.[19]

PEGylated biological drugs are generally less immunogenic than the naked protein, but many clinically approved PEGylated proteins still induce immune responses in a significant fraction of patients.[22] PEG itself also has the potential to induce an immune response, generating anti-PEG antibodies that, unfortunately, have been found to be cross-reactive among PEGylated products.[23] In this regard, the FDA recently updated its guidance for industry on *Immunogenicity Assessment for Therapeutic Protein Products* (2014) and on the *Assay Development and Validation for Immunogenicity Testing of Therapeutic Protein Products* (2016). These two documents provide recommendations for the industrial development and validation of immunoassays of therapeutic proteins during clinical trials. For PEGylated proteins, the anti-drug antibody assays should be able to detect both anti-protein antibodies and anti-PEG antibodies. Despite several studies showing that anti-PEG antibodies were responsible for an attenuated response, few studies have investigated the specificity of these antibodies and whether they significantly influence the pharmacokinetics of PEGylated proteins.[22]

PEGylation usually reduces the drug's affinity for the target and, consequently, its efficacy.[24] To overcome this problem, releasable PEGylation can be used. In this case, the PEGylated molecule will be stable under storage conditions and will dissociate under physiological conditions.[16] The use of releasable PEGylation enhances the shelf-life stability of the biological drug but might not improve its pharmacokinetics and immunogenic profile. Another approach is noncovalent PEGylation, based on hydrophobic interactions, ionic interactions, and chelation between the biological drug and PEG. The first attempt to achieve

noncovalent PEGylation described in the literature exploited hydrophobic interactions between a modified PEG moiety (dansyl-PEG) and salmon calcitonin (sCT) protein.[25]

Recently, an increase in the use of branched PEGs has been observed. These polymers are formed by two or more PEG molecules attached to a central core, from which extends a tethered reactive moiety that binds to the drug molecule. Branched PEGylation decreases immunogenicity and increases half-life more than linear PEGylation, but might lower activity.[26] This form of PEGylation can be found in some of the biobetters that are currently on the market, namely PEG-interferon-α-2a, PEGaptanib, and certolizumab pegol, while several other molecules involve branched PEGs are in advanced stages of clinical investigation.[27] This strategy is also of interest for small molecules drugs. The antineoplastic irinotecan conjugated to a four-arm PEG by a cleavable ester linkage has recently entered phase III trials. Onzeald™ (etirinotecan pegol) is the first long-acting topoisomerase I-inhibitor (Topo I) designed to concentrate in tumor tissue, enabling it to provide sustained tumor suppression throughout the entire chemotherapy cycle.[28]

Another form of branched PEG named PolyPEG® (Polytherics Ltd) comprises a poly(methacrylate) backbone with short pendent PEG teeth attached, stretching out in parallel like teeth on a comb. This form of branched PEG is still under investigation for protein PEGylation and does not accumulate in vacuoles in the liver and kidneys upon repeated administration.[14] Interferon-alpha (IFN) was conjugated via its N-terminal amino group by reductive amination to α-aldehyde functional comb-shaped PolyPEG polymers (50 and 70 kDa) and linear PEG (30 kDa).[29] Both PolyPEG-IFN conjugates (50 and 70 kDa) **{AuQ: Edit OK?}** retained a level of potency similar to that of the (linear PEGylated) reference drug, but with longer half-lives. In addition, linear PEG-IFN was twice as viscous as the PolyPEG-IFN forms. Innovative PEGylation strategies are awaited and will certainly contribute to the development of biobetters.

Alternative polymers for conjugation

Many synthetic biomaterials, including PEG and essentially all polymers derived from radical polymerization, are non-biodegradable.[30] Extensive research is underway to develop polymers as alternatives to the current gold-standard polymer for conjugation, PEG, but most of them are still in early stages of development. The *in vivo* distribution, mechanism of degradation, route of elimination and immunogenicity of these alternative polymers have not been investigated to the same extent as they have for PEG.[31] **Figure 2** outlines a representative sample of polymers for bioconjugation that have been developed in the past decade.

Small and water-soluble polymers, such as poly(2-methyl-2-oxazoline) (MeOx) or poly(2-hydroxypropylacrylamide) (PHMPA) present a hydrodynamic radius or diameter that is below the renal filtration threshold (approx. 3.8 nm) and, therefore, are rapidly cleared by the kidneys. Other polymers based on (meth)acrylates bearing oligoethylene glycol side chains (OEGMA), such as diethylene glycol methacrylate (DEGMA) or polyethylene glycol methacrylate (PEGMA), are of increasing interest because they have properties such as high

solubility in water, low immunogenicity and toxicity, lower critical solution temperature (LCST) and enhanced blood circulation times.[32]

Poly[*N*-(2-hydroxypropyl)methacrylamide] (HPMA) is an FDA-approved polymer that is considered to be an alternative to PEG, especially in nanomedicine applications, as it forms nanosized (5–20 nm) and water-soluble conjugates with proteins.[33] HPMA is a semisynthetic hydrophilic, non-immunogenic, non-toxic and biocompatible solid polymer.[34] In addition, HPMA–protein conjugates showed improved stability when subjected to heat and autolysis.[35] Conjugation with HPMA demonstrated potential to deliver antineoplastic and anti-angiogenic drugs selectively to solid tumors that resulted from the enhanced permeability and retention (EPR) effect, which decreased the dose-limiting toxicity.[36] Tao *et al.*[34] investigated lysozyme–pHPMA bioconjugates and observed that the number of polymer chains that were attached to the protein significantly influenced the enzyme activity (based on the *Micrococcus lysodeikticus* method) and, therefore, the antimicrobial potency of the conjugate. The number of polymer chains attached to lysozime could be controlled by both the reaction pH and the molecular weight of the polymer. The same group created a branched mid-chain-functional polyHPMA containing a thio-reactive group that was conjugated to bovine serum albumin (BSA), thereby improving the protein stability and enhancing circulation time of the conjugate.[34] More recently, an interleukin-2-poly(HPMA) conjugate was synthesized, containing 2–3 polymer chains per IL-2 molecule. IL-2–pHPMA had longer *in vivo* half-life than IL-2, which counteracts its reduced ability to interact with the cytokine's receptor. Thus, the *in vivo* activity of IL-2–pHPMA was significantly higher than that of IL-2.[37]

Poly(vinylpyrrolidone) (PVP) is a nonionic water-soluble polymer, with molecular mass ranging from 40 to 360 kDa, that is FDA approved. It has universal solubility in hydrophilic and hydrophobic solvents. PVP conjugation to proteins results in the longest mean residence time (MRT) after i.v. injection of all nonionic polymers of the same molecular weight.[38] Nonetheless, the main disadvantage of PVP is the immunological response, which involves anti-PVP antibody production.[35]

Another promising alternative to PEG is poly(*N*-acryloylmorpholine) (pNAcM), which has stealth behavior and easy renal clearance similar to those of PEG. pNAcM is also easily modified and thermoresponsive, but may elicit stronger immunological responses than PEG.[35] Caliceti *et al.*[39] compared different polymer–uricase conjugates (PVP, pNAcM, linear PEG and branched PEG, all with the same molecular weight) and showed that pNAcM–uricase had the longest blood half-life. On the other hand, it also had the highest liver accumulation rates (up to 25.5% of the dose) and considerable accumulation in other organs. Recently, Morgenstern *et al.*[40] investigated conjugates of lysozyme with PNAcM, which had improved protein solubility and preserved or even increased enzyme activity.

Poly(2-oxazoline)s (Pox) is another type of polymer, synthesized in the 1950s and considered promising for biomedical applications since the 1990s, particularly for the fabrication of artificial membranes from PMOXA–lipid conjugates.[41] POx is chemically described as a poly(ethyleneimine) backbone with amide-bond side groups in the repetition unit. Pox polymers are low-dispersity peptidomimetics, that are structural isomers of

polypeptides **{AuQ: Edit OK?}**. Pox-based polymers might be less prone to degradation than PEG, and therefore POx conjugation (sometimes termed POxylation, POXAylation or POzylation) has been investigated for decades as an alternative to PEG–protein conjugation.[41–44] At present, four **{AuQ: Edit OK?}** heterocyclic monomers are commercially available: MeOx (2-methyl-2-oxazoline), EtOx (2-ethyl-2-oxazoline), iPrOx (2-isopropenyl-2-oxazoline) and PheOx (2-phenyl-2-oxazoline).[45] Other POx polymers have been synthesized, such as poly(2-dialkylamino-2-oxazoline)s (PAmOx), a new class of thermoresponsive polymers.[46] POx polymers do not suffer from macrophage metabolism and phagocytic activity after incubation.[45] Pidhatika *et al.*[47] demonstrated that MeOx–protein conjugates were significantly more stable than the PEG-based equivalent **{AuQ: Edit OK?}** under physiological conditions, maintaining their non-fouling properties while PEG suffered degradation over time. Farkaš *et al.*[48] prepared POx–BSA conjugates and successively POx–BSA-antigen conjugates for vaccination against *Vibrio cholera*.

Polyglycerols (PG) are flexible hydrophilic polymers that are similar to PEG in terms of biocompatibility (nontoxicity). Linear or (hyper)branched PG can be produced by different synthetic methods and have low polydispersity.[49] *In vivo* circulation half-life is higher for branched PG than for linear PG, owing to the relatively rigid structure of PG that slows glomerular filtration rate.[50] Moreover, hyperbranched PG are thermally and oxidatively more stable than PEG. Unfortunately, like PEG, PG are non-degradable and present chronic toxicity.[51] If protein–polymer conjugates are used as replacement therapies, non-biodegradable polymers may accumulate in the body. In this sense, the molecular mass of the conjugate should not exceed the renal clearance threshold in order to allow complete excretion. The ideal molecular mass for non-degradable polymers is 20–60 kDa, which corresponds to a hydrodynamic radius of approximately 3.5 nm, the limit of albumin excretion.[52]

Linear PG are attractive because of their ease of synthesis and functionalization; their main disadvantage is their rapid clearance from circulation when compared to branched PG. Ul-Haq *et al.*[50] reported the unusually compact nature of high-molecular-weight linear polyglycerols (LPG). The properties of LPG have been compared to those of hyperbranched polyglycerols (HPG) and linear PEG of similar molecular weight, but LPG showed better biocompatibility and longer *in vivo* circulation time when compared to PEG. These polymers have also been used to prepare a diverse library of BSA and lysozyme–PG conjugates, and significantly higher activities were observed for conjugates prepared from synthetic branched copolymers (PEG–co-PG) than for conjugates prepared from linear polymers (PEG or PG) of similar molecular weight.[49]

Biodegradable polymers are an interesting alternative to PEG. Polyglutamic acid (PGA) is a polymer that is biodegradable by lysosomal cathepsin B and well tolerated in high doses as a therapeutic. Among the advantages of PGA over other biodegradable polymers, its multivalency allows post-polymerization modifications that can produce a large variety of polyglutamates.[53] The conjugation occurs at the carboxyl groups of glutamic acid.[54] There have been several studies on PGA conjugation to small molecule drugs such as doxorubicin,[54]

paclitaxel,[55] camptothecin[56] and retinoids.[57,58] Nonetheless, no reports on biological drug–PGA conjugation are available in the literature. The main characteristics, advantages and disadvantages of alternative polymers for protein conjugation are presented in **Table 1**.

Carbohydrates for conjugation

The strategy that is most frequently used for the conjugation of carbohydrates to proteins is glycosylation, the covalent addition of glycans to the amide group of an asparagine residue (*N*-glycans) or to the hydroxyl group of a serine or threonine residue (*O*-glycans). It occurs through the action of a series of enzymes that are localized in the endoplasmic reticulum and Golgi apparatus.[59] Glycosylation is the most prevalent and complex posttranslational modification to proteins that occurs naturally[60]; it improves protein solubility and stability. The binding of glycans to some proteins also assists in the correct folding and protects the protein from cleavage by proteases by masking cleavage sites.[60] The number and composition of the glycans also play important roles in protein folding, solubility and intracellular trafficking.[61] The biological activity and clearance rates of a protein are also highly influenced by its glycosylation, with the possibility of increasing its half-life, a key attribute for patients' adherence to treatment.[60]

Glycoengineering is considered to be a valuable tool for producing biobetters by engineering glycoprotein expression in several systems, including yeast, plant, and mammalian cells, with the possibility of reaching humanized glycosylation patterns. Glycoengineering falls into two main approaches: genetic and metabolic.[62] These approaches can be used to achieve the optimization and remodeling of naturally expressed glycans in a protein, the elimination of glycans or the incorporation of new glycans. Glycoengineering by *N*- and/or *O*-hyperglycosylation introduces potential sites for the addition of *N*- and/or *O*-glycosyl groups to proteins.[63]

The production of several protein drugs relies on mammalian cell expression systems, owing to their natural ability to express human-compatible glycosylation patterns. Substantial efforts have been made in recent years to overcome glycan heterogeneity and to produce homogeneous therapeutic glycoproteins.[61] The most common strategies are: (i) knock-out mutagenesis to delete specific genes that encode enzymes involved in the glycosylation process, thereby eliminating immunogenic sequences and/or unwanted sugar residues; (ii) inhibition of specific enzymes of the glycan biosynthetic pathway to generate simpler and uniform glycoforms and to prevent the introduction of unwanted sugar residues; and (iii) overexpression of the enzymes involved in the glycoprocessing of proteins **{AuQ: Edit OK?}** (belonging to the expression system or not) to alter the glycosylation profile and increase the production of the desired glycoform.[64]

The glycosylation of protein drugs can also be achieved in microbial systems (fungi or yeast). These expression systems are attractive due to their low cost, high productivity and fast implementation. Platforms based on fungi and yeast are capable of performing *N*-glycosylation, sharing the first reaction step with mammalian cells but with different final processing and thus producing a potentially immunogenic structure. For proteins with defined

human-like *N*-glycans, glycoengineering strategies such as the inhibition or deletion of genes (knock-out) that are involved in glycoprocessing and the introduction of mammalian enzymes (GlicoSwitch® technology) have been successfully applied in microorganisms such as *Saccharomyces cerevisiae* and *Pichia pastoris*. Sinclair and Elliott[65] established that *N*-glycosylation relies on the recognition of a consensus sequence, and therefore occurs at desired positions on the protein. Therefore, *N*-glycosylation sites have been successfully engineered in recombinant proteins. Song *et al.*[66] introduced an *N*-glycosylation site in the light chain of an anti-CD4 monoclonal antibody that is capable of neutralizing HIV-1, thereby increasing the plasma half-life of this antibody. Progress has also been made in the production of proteins that have human *O*-glycan patterns in eukaryote hosts by removing the *O*-mannosylation feature of the host and genetically introducing human cellular machinery.[64] In recent innovative work, glycoengineering was used to developed a specific glycosylation pattern in erythropoietin (EPO) to block the undesired erythropoietic activity and at the same time preserve the neurotrophic and cytoprotective properties.[67]

Plant cells share the same initial steps of glycosylation with mammalian cells.[68] Efforts to produce humanized glycoproteins in plants have included the elimination or shutdown of the expression of specific endogenous plant enzymes that produce immunogenic structures. This may be coupled to the transfer of the *N*-branching machinery present in human glycans. Plants do not possess enzymes that are able to sialylate proteins, but introduction of the complete sialylation biosynthetic pathway into plant cells can enable these cells to produce proteins with sialylation levels higher than those in proteins produced in mammalian cells.[64]

Metabolic glycoengineering (MGE) refers to the regulation of natural flux through a biosynthetic pathway and is the second major strategy used to control glycosylation. In MGE, living cells and entire organisms are supplemented with monosaccharide precursors. MGE is simple to perform in the laboratory because the precursors can be directly added to culture medium without any need to manipulate the host cell genetically. The monosaccharides required for MGE need to be synthesized, however, making the technique expensive at the industrial scale.[62]

Chemical glycosylation, also called neo-glycoconjugation, can change the structure, function and thermodynamic stabilization of proteins.[69] Human brain natriuretic peptide (hBNP) heterologously expressed in *Escherichia coli*, for example, was chemically glycosylated and formulated as a citrate salt; it was approved by the FDA in 2001 as Natrecor®. Another chemically glycosylated biobetter introduced to the market in 2007 is the recombinant glycosylated EPO-α (Aranesp®), which has five amino acid changes (N30, T32, V87, N88, T90) that result in two new sites for the addition of *N*-linked oligosaccharide chains (recombinant human EPO has three chains). This modification resulted in increased half-life, which was approximately three-fold longer than that of wild EPO-α when administered intravenously.[70] Another study describes a long-acting hyperglycosylated EPO analog prepared by the addition of *N*-linked oligosaccharides to the backbone of the protein.[71] This analog was bioconjugated with a carboxyl-terminal peptide (CTP) sequence and it significantly increased the *in vivo* potency and half-life of the EPO.

Other biodegradable carbohydrates such as hydroxyethyl starch (HES) and PG have also been shown to improve the therapeutic properties of protein drugs.[72] Recombinant human epidermal growth factor conjugated to dextrin (rhEGF–dextrin) demonstrated better wound healing *in vivo* than the protein in isolation.[73] Dextrins are composed of D-glucopyranosyl units of shorter chain lengths than dextrans; the first studies on protein conjugation to this polymer were on trypsin.[74] This conjugation reduced the enzyme activity of trypsin by 34–69% depending on the molecular weight and succinylation level of the dextrin. However, incubation with α-amylase resulted in activity recovery of 92–115%, showing that unmasking could be a strategy for therapeutic application.

The concept of masked-unmasked polymer protein therapy (PUMPT) refers to the use of protein–drug conjugates with biodegradable carriers that mask the protein activity during transport, with an unmasking effect triggered specifically at the site of action. A multifunctional and biodegradable polymer (such as dextrin, hyaluronic acid (HA) or polyglutamic acid) is used to envelop the protein (for example, trypsin, melanocyte-stimulating hormone (MSH),[74] phospholipase A2 (PLA2)[75] or rhEGF),[73] thereby masking the bioactivity, minimizing toxicity in transport, and protecting against premature proteolytic inactivation.[74] Degradation of locally triggered polymer, for example using α-amylase for dextrin and hyaluronidase for HA, allows the time-dependent 'unmasking' of the protein and controlled local bioactivity. This strategy has potential application in cancer therapy, where it could present a therapeutic effect in the tumor microenvironment or intracellularly, and in enzyme replacement therapy in lysosomal storage diseases, where it could unmask the protein in lysosomes through disulfide cleavage and enzymatic degradation.[53]

HA conjugation, also known as HAylation, has also been shown to preserve enzyme activity and thermal stability. A HA–insulin conjugate, for example, was effective in lowering blood-glucose levels for up to 6 h, whereas free insulin was effective for only 1 h.[76] The main limitations of HAylation are the formation of soluble aggregates, undesired cross-linking, and immune reactions.[76] As for PEGylation, *N*-terminal site-specific HAylation reactions are possible. HA conjugation has also demonstrated potential in PUMPT because it is enzymatically degraded by hyaluronidases (HAase) to smaller oligosaccharides.[77,78]

HESylation is coupling with the biodegradable polymer HES.[79] The pharmaceutical company Fresenius Kabi pioneered the study of HESylation to improve the pharmacokinetic and pharmacodynamic properties of therapeutic proteins. The company showed comparable *in vivo* and *in vitro* bioavailability of HESylated and PEGylated EPO, with a three-fold increase in half-life when compared to the native form.[35] HESylation can provide formulation advantages when compared to PEGylation, especially for highly concentrated protein solutions. Liebner *et al.*[79] compared HESylation with PEGylation for the drug anakinra (an interleukin 1 receptor antagonist) and found that the coupling of HES or PEG had practically no effect on the secondary structure of the protein, but the viscosity of HESylated anakinra at protein concentrations of up to 75 mg.mL^{-1} was approximately 40% lower than that of PEG–anakinra.

Although HESylated products have good pharmacokinetic properties and are widely used as plasma volume expanders, they have been shown to accumulate in the liver, kidney and bone marrow,[80] increasing the risks of kidney damage and death in critically ill patients. Therefore, further studies should be carried out on the prolonged use of HESylated biological drugs and the associated side effects.

Polysialic acid (PSA) is also under development for clinical use, and polysialylated versions of insulin and EPO have shown improved tolerance and pharmacokinetics.[53] PSA–insulin was found to have increased half-life and therefore reduced blood glucose levels in rats.[81] PSA–uricase conjugates have also shown increased half-life and doubled catalytic activity when compared to native uricase in the treatment of hyperuricemia.[82] PSA is metabolized by sialidases, resulting in the release of **{AuQ: Edit OK?}** natural sugar molecules. Nonetheless, PSA conjugation is technically complex and expensive, with random attachment patterns and undesirable heterogeneity. Owing to these limitations, studies have been carried out in recent years to optimize the bioprocesses for PSA production, with bacteria and metabolic engineering being used as an important tool. In addition, research has been carried out on the production of PSA-conjugated proteins in *E. coli* through glycoengineering and without the need for *in vitro* chemical modification, envisioning a direct, rapid, and cost-effective process.[83,84]

Another important glycoengineering strategy that is used to produce biobetters is defucosylation, which has been used to develop therapeutic antibodies that have superior properties.[85] Defucosylation refers to the production of monoclonal antibodies that are engineered so that they do not present a fucose sugar unit in the oligosaccharides of the Fc region. Core fucosylation plays a critical role in modulating the effector functions of therapeutic antibodies such as antibody-dependent cellular cytotoxicity (ADCC), by adversely affecting the affinity of the antibodies for Fcγ receptors.[86] Defucosylated antibodies have improved binding to FcgγIIIa, allowing them to evade the inhibitory effect on ADCC of plasma IgG (which is fucosylated and binds to FcgγIIIa with lower strength). Thus, this strategy is important both for functional studies and for an enhanced therapeutic efficacy of monoclonal antibodies.[87]

The main advantages and disadvantages of carbohydrate conjugation are presented in **Table 1**.

Novel trends

Fusion proteins

Fusion proteins are created by combining genes that originally encoded separate proteins. The novel single polypeptide that is produced displays the functional properties of both originator biomolecules. These proteins can be categorized according to their incorporated domains. Commonly, one fusion partner has a molecular recognition function whereas the other partner transfers a certain functionality, such as decreased cytotoxicity, improved half-life and stability, novel targeting or a new delivery route.[88] Therapeutic fusion proteins are normally from one of three different families: fragment crystallizable Fc-fusions, albumin

fusions and transferrin fusions. The advantages and disadvantages of each family are presented in **Table 1**. Fusion proteins have been widely investigated for several pathologies, with notable recent successes coming to market.

Fc-based fusion proteins are obtained by the replacement of an antibody-combining region (Fab) by an effector molecule. The remaining constant (Fc) and hinge regions exhibit the potential to provide immune functions and to extend the half-life of the fusion protein. These Fc-fusion proteins also enable interaction with the Fc-receptors (FcRs) found on immune cells, an important feature in oncological therapies, vaccines and other conditions. The first report of Fc-fusion proteins, published in 1989, showed that the protein inhibited entry of the human immunodeficiency virus into T cells. Since then, promising outcomes have resulted from the development of novel approaches to improve efficacy and safety, while also broadening clinical applications to other uses. In this context, Fc-fusion proteins of great therapeutic potential have been developed as possible treatments, such as eflapegrastim (Rolontis®) for neutropenia and efpeglenatide for type-2 diabetes, both in phase III clinical trials. In the case of eflapegrastim, Spectrum Pharmaceuticals, Inc. developed a fusion protein formed by a recombinant human G-CSF analog (Ser-G-CSF, without additional N-terminal Met) and the human immunoglobulin IgG4 Fc fragment, linked through a 3.4 kDa PEG to produce a longer-acting G-CSF.[89] Efpeglenatide (an Exendin-4 analog) was developed by Hanmi Pharmaceutical Co. by conjugation of the long-acting glucagon-like peptide-1 receptor agonist (GLP-1RA) (a modified version of Exendin-4) and a non-glycosylated human IgG4 Fc fragment, which offered increased plasma half-life and decreased immunogenicity relative to that of the Exendin-4 peptide **{AuQ: Edit OK?}**. The fusion occurs through a 3.4-kDa PEG linker that is present in the Lys^{27} residue of the modified Exendin-4 analog, which has a replacement of the N-terminal histidine with a 4-imidazoacetyl group.[90] Fc-fusion proteins with significantly improved half-life binding to the neonatal Fc-receptor (FcRn) are especially interesting as treatments for autoimmune disorders that are caused by the reaction of IgG to self-antigens. FcRn extends IgG half-life, and as the binding of Fc-fusion proteins to FcRn can disrupt the IgG–FcRn interaction, the inflammation that occurs in response to self-antigens is controlled.[91] Enbrel® (etanercept) was the first successful IgG Fc-linked soluble receptor therapeutic, and it works by binding and neutralizing the pro-inflammatory cytokine TNF-α. Rilonacept (Arcalyst®), romiplostim (Nplate®), abatacept (Orencia®) and belatacept (Nulojix®) are also biologicals that are based on the binding of Fc-fusion proteins to FcRn.[92]

Another protein that is employed in fusion proteins is albumin, the most abundant human plasma protein (35–50 $g.L^{-1}$ of human serum) with an impressive average half-life of 19 days. The long-term stability of albumin provides the promising ability of albumin fusion to prolong the serum half-life of biological drugs. The Albumin Fusion Technology developed by Human Genome Sciences Inc. is an example of the industrial relevance of this technology in extending the half-life and stability of therapeutic proteins while reducing their immunogenicity. Albumin can be fused to either the N- or the C- terminus of the effector protein to avoid changes in biological activity. Albuferon-α, for example, is a biobetter resulting from albumin fusion that is currently in phase III clinical studies for hepatitis C. This

biobetter is superior even to the various PEGylated variants of the original drug **{AuQ: Edit OK?}** as regards pharmacodynamics properties and half-life. Further albumin fusion proteins have been developed for cytokines, peptides, hormones and growth factors.

The third potential fusion molecule that can be applied to improve the stability and half-life of protein drugs is transferrin, although few studies applying this technology are available. Biorexis Pharmaceutical Corp. (acquired by Pfizer Inc. in 2007) conducted a preclinical study based on transferrin fused with glucagon-like peptide and interferon-β.[93] As far as can be determined, no BioRexis-derived fusion proteins are currently in the clinic.

EKylation

An alternative strategy to stabilize proteins refers to the genetic fusion of repeated amino acid sequences. On protein surfaces, a balanced ratio of cationic lysine (K) and anionic glutamic acid (E) residues exists to promote stabilization. Repeated EK sequences (alternated or mixed) have been shown to confer non-fouling zwitterionic characteristics upon surfaces and nanoparticles.[94] This poly(EK), a natural analog to zwitterionic poly(carboxybetaine) (pCB), is ideal for medical applications due to its biological chemistry, high biocompatibility and enzymatic degradability.[95] Liu *et al.*[95] demonstrated that a strategy in which poly(EK) tails of well-defined lengths are appended to the C-terminus of β-lactamase via *E. coli* expression is effective for protein stabilization. This bioinspired 'EKylation' method not only confers the stabilizing benefits of poly(zwitterions) but also allows for rapid biosynthesis of target constructs and stability when exposed to environmental stressors such as high temperature and highly salty solutions. This one-step strategy provides broadly applicable alternatives to synthetic polymer conjugates that are biocompatible and biodegradable.

XTEN technology

Schellenberger *et al*.[96] developed the XTEN technology, also called XTENylation. XTENs are genetic fusions of an unstructured recombinant polypeptide of 864 amino acids, composed entirely of alanine, glutamate, glycine, proline, serine, and threonine residues, therefore highly hydrophilic and anionic.[97] The XTEN sequence has been demonstrated to increase the serum half-life of peptides and proteins controllably, and simultaneously to increase the water solubility and stability of proteins, allowing their expression in solution and facilitating manufacturing.[98] XTEN is typically attached to either the N- or the C-terminus of the protein and either bacterial or mammalian cells can be use as expression systems for the sequence.

As the XTEN sequence is composed of only natural amino acids, it is efficiently biodegraded. XTEN lacks hydrophobic amino acid residues, so it contains few, if any, T-cell epitopes responsible for immunogenicity. Consequently, studies have demonstrated low rates of immunogenicity in animals, even in the presence of adjuvants.[96] Moreover, BLASTP (basic local alignment search tool for protein) analysis suggests that the XTEN sequence does not possess any known homology to natural human proteins, so cross-reactivity autoimmunity is not observed.[96]

The recombinant nature of XTEN provides several advantages over traditional PEGylation. Genetic fusion of a defined amino acid sequence results in homogeneous end-products, which contrast with the more heterogeneous PEGylated proteins. Further, XTEN products have lower costs and higher yields than PEGylated products, which need chemical coupling and purification from poly-PEGylated species, non-modified species and free PEG.[96,99]

Amunix Pharmaceuticals has developed a highly flexible method based on XTEN technology that has enabled the formulation of multiple products in a broad range of therapeutic areas. Geething *et al.*[98] showed that glucagon–XTEN is effective in preventing hypoglycemia overnight (12 h) without the associated hyperglycemia observed for unmodified glucagon. They demonstrated that the solubility and stability of glucagon were also significantly improved by fusion to XTEN. Multivalent antiviral T-20, which is effective in inhibiting HIV, was also conjugated to an XTEN sequence (T-20-XTEN), resulting in a half-life that was 20 times longer than that reported for free T-20.[97]

PASylation

Like EKylation, PASylation is based on polypeptide genetic fusion. In PASylation, an amino acid sequence of proline, alanine, and serine is designed to achieve an unstructured and uncharged polypeptide with high water solubility.[100,101] Different polypeptide lengths (200, 400 and 600 amino acids) were fused with therapeutic proteins, that is, IFN, hGH and Fab fragments, resulting in an increase in *in vivo* half-life in mice.[101] Accordingly, fused proteins were stable in blood circulation, but their biodegradability prevented their accumulation in organs. Moreover, the toxicity of the fused proteins was negligible and their unstructured nature presumably explained the absence of immunogenicity in mice. Together with other genetic fusion approaches, PASylation may not be the most appropriate method for highly immunogenic proteins because the fused polypeptide is attached only at the N- and/or C-terminus and, consequently, does not fully shield the protein surface in the same way as polymer conjugation does. Nevertheless, PASylation presents great potential for proteins of low water solubility.[102] **Figure 3** summarizes the novel trends discussed in this section.

Market analysis and regulatory approval

The pharmaceutical industry is one of the most lucrative and has the fastest growth rate of any industry worldwide, with sales reaching a total of USD 844 billion in 2019. These sales are expected to grow at an annual rate of 6.3%, reaching approximately USD 1200 billion in 2022.[103] To reach this level, however, high capital investment and time for research and development (R&D) of new drugs are required. In 2015, for example, the pharmaceutical industry invested about USD 150 billion in R&D and this value is likely to increase to USD 182 billion by 2022.[104]

Biological drugs have proven to be a revolutionary innovation in the pharmaceutical industry. According to figures for global revenues in 2018, 10 biotechnological-related products were listed among the top-20 best-selling drugs. Among the top-100 pharmaceutical

products in terms of worldwide sales, biotechnology products increased from 34% to 53% between 2010 and 2018 (**Figure 4**).[103] More than 300 mAbs, 250 vaccines and 100 other biologicals, including cell and gene therapies, are currently under clinical development. By 2030, the biosimilar market is expected to be greater than USD 240 billion, as patents on major biologics continue to expire.[1] Today, biological drugs, used mainly in the treatment of autoimmune or inflammatory diseases (such as rheumatoid arthritis) and cancer, form a multibillion-dollar industry.[105]

Many of the patents and regulatory protection periods for the cloning and production of original-generation (branded) biological drugs have expired, or their expiry is looming within the next few years (**Table 2**). Biological drugs with sales of approximately USD 110 billion are expected to be off patent by the year 2020. These patent expirations, combined with rising healthcare costs and worldwide aging of the population, are paving the way for the development of biosimilars and biobetters, opening new commercial opportunities.[106] In fact, several biosimilars are currently under development,[4] and follow-on biologics will inevitably play an increasing role in healthcare in the coming years.[107]

The main barriers to market access for biosimilars are: (1) expensive and complex manufacturing processes, (2) regulatory processes, (3) intellectual property rights, (4) lack of incentive, (5) the impossibility of interchangeability, and (6) difficulties in reaching the status of the reference drug that has already gained the confidence of the prescribers and patients.[108] As biobetters offer advantages over both the reference molecule and biosimilars, they can demand a premium price. **Table 3** lists biobetters that are currently available in the market.

Regulatory agencies around the world have similar definitions for biosimilars and emphasize quality, safety and efficacy aspects. Biobetters, on the other hand, have no legal or regulatory recognition and a definitive regulatory pathway is still not available for these products. Biobetters are considered as investigational new drugs (IND) and are subject to the same regulatory guidance. From the developers' perspective, biobetters can be considered to be in between biosimilars and reference biological drugs,[1] with no need to wait for patent expiration, but it is sometimes difficult to have biobetters patented because their therapeutic focus is similar to that of the reference drug.[4]

Biosimilars and biobetters differ in terms of development time and probability of success (**Table 4**),[109–112] with biosimilars having a better chance of success from the earliest stages of development. By contrast, biobetters have the same rate of success as a new drug in preclinical development. Nonetheless, once past preclinical development, biobetters have a significantly better chance of success in Phase I clinical trials than new drugs, close to the rate observed for biosimilars. The same is observed for Phase III clinical trials. As already mentioned, the overall probability of success of follow-on biologics is higher than that for new drugs as their mechanism of action has already been clinically validated. A new drug takes about 15–20 years to develop before entering the market, with investments of approximately USD 1.2 billion. A biosimilar usually takes 5–8 years to develop and investments of

approximately $100–200 million, whereas a biobetter takes about 10 years and USD 500 million.[112,113]

Conclusions

In spite of all the promising alternatives discussed in this review, glycosylation and PEGylation still represent the main strategies to improve protein drugs, that is for the development of biobetters, a situation that might persist for the next five to ten years. The main challenge facing the development of biological drugs is drug-related immunogenicity, and glycosylation and PEGylation have already proved to be efficient and safe in reducing the generation of anti-drug antibodies. Even when the recent debate on PEG immunogenicity is considered, none of the other polymers investigated to date for chemical conjugation has proved to be superior. Nonetheless, we believe genetic engineering strategies to attach specific amino acid sequences to a protein drug, such as EKylation, PASylation and XTEN technology, are promising strategies that will result in an increased number of biobetters in the future.

The diversity of available techniques makes it clear that there is no single pathway that must be followed by companies who choose to development biobetters. Significant knowledge on the structure of the starting molecule, the disease involved, and the route of administration is necessary, and this information will drive the choice of the most suitable techniques for chemical and/or molecular modification. As the scientific knowledge advances in this area, driven by the economic, regulatory and therapeutic characteristics of this new generation of biological drugs, novel possibilities may arise soon.

Acknowledgements

This work was funded by the Coordination of Superior Level Staff Improvement (CAPES/Brazil, finance code 001), the State of São Paulo Research Foundation (FAPESP/Brazil, process 2016/22065-5) and the National Council for Scientific and Technological Development (CNPq/Brazil, Fellowship #301832/2017-0 to C.O.R.Y.). This work was developed within the scope of the CICECO-Aveiro Institute of Materials project, UIDB/50011/2020 & UIDP/50011/2020, financed by national funds through the Portuguese Foundation for Science and Technology/MCTES. J.H.P.M. Santos acknowledges FAPESP for awarding the post-doctoral grant FAPESP #2018/25994-2.

References

1. Kesik-Brodacka M. Progress in biopharmaceutical development. *Biotechnol Appl Biochem* 2018; 65: 306–22. doi:10.1002/bab.1617.
2. Walsh G. Biopharmaceutical benchmarks 2018. *Nat Biotechnol* 2018; 36: 1136–45. doi:10.1038/nbt.4305.
3. Walsh G. Biopharmaceuticals and biotechnology medicines: an issue of nomenclature. *Eur J Pharm Sci* 2002; 15: 135–8. doi:10.1016/S0928-0987(01)00222-6.

4. Sharma A, Kumar N, Kuppermann BD, Francesco B, Loewenstein A. Biologics, biosimilars, and biobetters: different terms or different drugs? *Eye* 2019; 33: 1032–4. doi:10.1038/s41433-019-0391-5.
5. Calo-Fernández B, Martínez-Hurtado JL. Biosimilars: company strategies to capture value from the biologics market. *Pharmaceuticals* 2012; 5: 1393–408. doi:10.3390/ph5121393.
6. Biosimilar and Interchangeable Products 2017. Food and Drug Administration. https://www.fda.gov/drugs/biosimilars/biosimilar-and-interchangeable-products. Published October 23, 2017. Accessed November 10, 2019.
7. Biosimilars in the EU: information guide for healthcare professionals. European Medicines Agency. https://www.ema.europa.eu/en/documents/leaflet/biosimilars-eu-information-guide-healthcare-professionals_en.pdf. Published October 02, 2019. Accessed February 21, 2020.
8. Jacobs I, Ewesuedo R, Lula S, Zacharchuk C. Biosimilars for the treatment of cancer: a systematic review of published evidence. *BioDrugs* 2017; 31: 1–36. doi:10.1007/s40259-016-0207-0.
9. Barbosa MDFS, Kumar S, Loughrey H, Singh SK. Biosimilars and biobetters as tools for understanding and mitigating the immunogenicity of biotherapeutics. *Drug Discov Today* 2012; 17: 1282–8. doi:10.1016/j.drudis.2012.07.003.
10. Satterwhite C. Assessing development needs for biobetters and biosimilars. *Biopharm Int* 2013; 26: 1–4. https://www.biopharminternational.com/view/assessing-development-needs-biobetters-and-biosimilars. Published February 1, 2013. Accessed November 10, 2019.
11. Dinwoodie N. Biobetters and the future biologics market. *BioPharm Int* 2011; 24: 31–5. https://www.biopharminternational.com/view/biobetters-and-future-biologics-market. Published November 1, 2011. Accessed November 10, 2019.
12. Burchiel SW, Aspbury R, Munday J. The search for biosimilars and biobetters. *Drug Discov Today* 2019; 24: 1087–91. doi:10.1016/j.drudis.2019.03.016.
13. Ginn C, Khalili H, Lever R, Brocchini S. PEGylation and its impact on the design of new protein-based medicines. *Future Med Chem* 2014; 6: 1829–46. doi:10.4155/fmc.14.125.
14. Zhang F, Liu M-R, Wan H-T. Discussion about several potential drawbacks of PEGylated therapeutic proteins. *Biol Pharm Bull* 2014; 37: 335–9. doi:10.1248/bpb.b13-00661.
15. Zhang C, Yang XL, Yuan YH, Pu J, Liao F. Site-specific PEGylation of therapeutic proteins via optimization of both accessible reactive amino acid residues and PEG derivatives. *BioDrugs* 2012; 26: 209–15. doi:10.1007/BF03261880.
16. Santos JHPM, Torres-Obreque KM, Meneguetti GP, Amaro BP, Rangel-Yagui CO. Protein PEGylation for the design of biobetters: from reaction to purification processes. *Brazilian J Pharm Sci* 2018; 54: e01009. doi:10.1590/s2175-97902018000001009.

17. Pasut G, Veronese FM. State of the art in PEGylation: the great versatility achieved after forty years of research. *J Control Release* 2012; 161: 461–72. doi:10.1016/j.jconrel.2011.10.037.
18. Abuchowski A, van Es T, Palczuk NC, Davis FF. Alteration of immunological properties of bovine serum albumin by covalent attachment of polyethylene glycol. *J Biol Chem* 1977; 252: 3578–81.
19. Pfister D, Morbidelli M. Process for protein PEGylation. *J Control Release* 2014; 180: 134–49. doi:10.1016/j.jconrel.2014.02.002.
20. Rudmann DG, Alston JT, Hanson JC, Heidel S. High molecular weight polyethylene glycol cellular distribution and PEG-associated cytoplasmic vacuolation is molecular weight dependent and does not require conjugation to proteins. *Toxicol Pathol* 2013; 41: 970–83. doi:10.1177/0192623312474726.
21. Kolate A, Baradia D, Patil S, Vhora I, Kore G, Misra A. PEG—a versatile conjugating ligand for drugs and drug delivery systems. *J Control Release* 2014; 192: 67–81. doi:10.1016/j.jconrel.2014.06.046.
22. Verhoef JJF, Carpenter JF, Anchordoquy TJ, Schellekens H. Potential induction of anti-PEG antibodies and complement activation toward PEGylated therapeutics. *Drug Discov Today* 2014; 19: 1945–52. doi:10.1016/j.drudis.2014.08.015.
23. Schellekens H, Hennink WE, Brinks V. The immunogenicity of polyethylene glycol: facts and fiction. *Pharm Res* 2013; 30: 1729–34. doi:10.1007/s11095-013-1067-7.
24. Turecek PL, Bossard MJ, Schoetens F, Ivens IA. PEGylation of biopharmaceuticals: a review of chemistry and nonclinical safety information of approved drugs. *J Pharm Sci* 2016; 105: 460–75. doi:10.1016/j.xphs.2015.11.015.
25. Mueller C, Capelle MAH, Arvinte T, Seyrek E, Borchard G. Noncovalent PEGylation by dansyl-poly(ethylene glycol)s as a new means against aggregation of salmon calcitonin. *J Pharm Sci* 2011; 100: 1648–62. doi:10.1002/jps.22401.
26. Fishman A, Farrah ME, Zhong JH, Paramanantham S, Carrera C, Lee-Ruff E. Synthesis and investigation of novel branched PEG-based soluble polymer supports. *J Org Chem* 2003; 68: 9843–6. doi:10.1021/jo034868u.
27. Rajender Reddy K, Modi MW, Pedder S. Use of peginterferon alfa-2a (40 KD) (Pegasys) for the treatment of hepatitis C. *Adv Drug Deliv Rev* 2002; 54: 571–86. doi:10.1016/S0169-409X(02)00028-5.
28. Hoch U, Staschen CM, Johnson RK, Eldon MA. Nonclinical pharmacokinetics and activity of etirinotecan pegol (NKTR-102), a long-acting topoisomerase 1 inhibitor, in multiple cancer models. *Cancer Chemother Pharmacol* 2014; 74: 1125–37. doi:10.1007/s00280-014-2577-7.
29. Podobnik B, Helk B, Smilović V, Škrajnar, Fidler K, Jevševar S, *et al*. Conjugation of PolyPEG to interferon alpha extends serum half-life while maintaining low viscosity of the conjugate. *Bioconjug Chem* 2015; 26: 452–9. doi:10.1021/bc500523t.

30. Ulbricht J, Jordan R, Luxenhofer R. On the biodegradability of polyethylene glycol, polypeptoids and poly(2-oxazoline)s. *Biomaterials* 2014; 35: 4848–61. doi:10.1016/j.biomaterials.2014.02.029.
31. Qi Y, Chilkoti A. Protein-polymer conjugation-moving beyond PEGylation. *Curr Opin Chem Biol* 2015; 28: 181–93. doi:10.1016/j.cbpa.2015.08.009.
32. Ekladious I, Colson YL, Grinstaff MW. Polymer–drug conjugate therapeutics: advances, insights and prospects. *Nat Rev Drug Discov* 2019; 18: 273–94. doi:10.1038/s41573-018-0005-0.
33. Kopeček J, Kopečková P. HPMA copolymers: origins, early developments, present, and future. *Adv Drug Deliv Rev* 2010; 62: 122–49. doi:10.1016/j.addr.2009.10.004.
34. Tao L, Liu J, Xu J, Davis TP. Synthesis and bioactivity of poly(HPMA)-lysozyme conjugates: the use of novel thiazolidine-2-thione coupling chemistry. *Org Biomol Chem* 2009; 7: 3481–5. doi:10.1039/b907061c.
35. Pelegri-O'Day EM, Lin E, Maynard HD. Therapeutic protein—polymer conjugates: advancing beyond PEGylation. *J Am Chem Soc* 2014; 136: 14323–32. doi:10.1021/ja504390x.
36. Shamay Y, Raviv L, Golan M, Voronov E, Apte RN, David A. Inhibition of primary and metastatic tumors in mice by E-selectin-targeted polymer–drug conjugates. *J Control Release* 2015; 217: 102–12. doi:10.1016/j.jconrel.2015.08.029.
37. Votavova P, Tomala J, Subr V, Strohalm J, Ulbrich K, Rihova B, *et al*. Novel IL-2-poly(HPMA)nanoconjugate based immunotherapy. *J Biomed Nanotechnol* 2015; 11: 1662–73. doi:10.1166/jbn.2015.2114.
38. Kaneda Y, Tsutsumi Y, Yoshioka Y, Kamada H, Yamamoto Y, Kodaira H, *et al*. The use of PVP as a polymeric carrier to improve the plasma half-life of drugs. *Biomaterials* 2004; 25: 3259–66. doi:10.1016/j.biomaterials.2003.10.003.
39. Caliceti P, Schiavon O, Veronese FM. Biopharmaceutical properties of uricase conjugated to neutral and amphiphilic polymers. *Bioconjug Chem* 1999; 10: 638–46. doi:10.1021/bc980155k.
40. Morgenstern J, Gil Alvaradejo G, Bluthardt N, Beloqui A, Delaittre G, Hubbuch J. Impact of polymer bioconjugation on protein stability and activity investigated with discrete conjugates: alternatives to PEGylation. *Biomacromolecules* 2018; 19: 4250–62. doi:10.1021/acs.biomac.8b01020.
41. Barz M, Luxenhofer R, Zentel R, Vicent MJ. Overcoming the PEG-addiction: well-defined alternatives to PEG, from structure–property relationships to better defined therapeutics. *Polym Chem* 2011; 2: 1900–18. doi:10.1039/c0py00406e.
42. Konradi R, Pidhatika B, Mühlebach A, Textor M. Poly-2-methyl-2-oxazoline: a peptide-like polymer for protein-repellent surfaces. *Langmuir* 2008; 24: 613–6. doi:10.1021/la702917z.
43. Glassner M, Vergaelen M, Hoogenboom R. Poly(2-oxazoline)s: a comprehensive overview of polymer structures and their physical properties. *Polym Int* 2018; 67: 32–45. doi:10.1002/pi.5457.

44. Lorson T, Lübtow MM, Wegener E, Haider MS, Borova S, Nahm D, *et al*. Poly(2-oxazoline)s based biomaterials: a comprehensive and critical update. *Biomaterials* 2018; 178: 204–80. doi:10.1016/j.biomaterials.2018.05.022.
45. Luxenhofer R, Han Y, Schulz A, Tong J, He Z, Kabanov AV, Jordan R. Poly(2-oxazoline)s as polymer therapeutics. *Macromol Rapid Commun* 2012; 33: 1613–31. doi:10.1002/marc.201200354.
46. Sedlacek O, Bera D, Hoogenboom R. Poly(2-amino-2-oxazoline)s: a new class of thermoresponsive polymers. *Polym Chem* 2019; 10: 4683–9. doi:10.1039/c9py00943d.
47. Pidhatika B, Rodenstein M, Chen Y, Rakhmatullina E, Mühlebach A, Acikgöz C, *et al*. Comparative stability studies of poly(2-methyl-2-oxazoline) and poly(ethylene glycol) brush coatings. *Biointerphases* 2012; 7: 1. doi:10.1007/s13758-011-0001-y.
48. Farkaš P, Korcová J, Kronek J, Bystrický S. Preparation of synthetic polyoxazoline based carrier and *Vibrio cholerae* O-specific polysaccharide conjugate vaccine. *Eur J Med Chem* 2010; 45: 795–9. doi:10.1016/j.ejmech.2009.11.002.
49. Wurm F, Dingels C, Frey H, Klok HA. Squaric acid mediated synthesis and biological activity of a library of linear and hyperbranched poly(glycerol)–protein conjugates. *Biomacromolecules* 2012; 13: 1161–71. doi:10.1021/bm300103u.
50. Ul-Haq IM, Lai BFL, Chapanian R, Kizhakkedathu JN. Influence of architecture of high molecular weight linear and branched polyglycerols on their biocompatibility and biodistribution. *Biomaterials* 2012; 33: 9135–47. doi:10.1016/j.biomaterials.2012.09.007.
51. Kainthan RK, Gnanamani M, Ganguli M, Ghosh T, Brooks DE, Maiti S, *et al*. Blood compatibility of novel water soluble hyperbranched polyglycerol-based multivalent cationic polymers and their interaction with DNA. *Biomaterials* 2006; 27: 5377–90. doi:10.1016/j.biomaterials.2006.06.021.
52. Knop K, Hoogenboom R, Fischer D, Schubert US. Poly(ethylene glycol) in drug delivery: pros and cons as well as potential alternatives. *Angew Chem Int Ed Engl* 2010; 49: 6288–308. doi:10.1002/anie.200902672.
53. Talelli M, Vicent MJ. Reduction sensitive poly(l-glutamic acid) (PGA)–protein conjugates designed for polymer masked-unmasked protein therapy. *Biomacromolecules* 2014; 15: 4168–77. doi:10.1021/bm5011883.
54. Guan H, McGuire MJ, Li S, Brown KC. Peptide-targeted polyglutamic acid doxorubicin conjugates for the treatment of αvβ6-positive cancers. *Bioconjug Chem* 2008; 19: 1813–21. doi:10.1021/bc800154f.
55. Auzenne E, Donato NJ, Li C, Leroux E, Price RE, Farquhar D, *et al*. Superior therapeutic profile of poly-L-glutamic acid–paclitaxel copolymer compared with taxol in xenogeneic compartmental models of human ovarian carcinoma. *Clin Cancer Res* 2002; 8: 573–81.

56. Singer JW, De Vries P, Bhatt R, Tulinsky J, Klein P, Chun LI, *et al*. Conjugation of camptothecins to poly-(L-glutamic acid). *Ann N Y Acad Sci* 2000; 922: 136–50. doi:10.1111/j.1749-6632.2000.tb07032.x.
57. Zou C, Brewer M, Cao X, Zang R, Lin J, Deng Y, *et al*. Antitumor activity of 4-(N-hydroxyphenyl)retinamide conjugated with poly(l-glutamic acid) against ovarian cancer xenografts. *Gynecol Oncol* 2007; 107: 441–9. doi:10.1016/j.ygyno.2007.07.077.
58. Zhu G, Cao X, Chang JY, Milas L, Wallace S, Li C. Polymeric retinoid prodrug PG-4HPR enhances the radiation response of lung cancer. *Oncol Rep* 2007; 18: 645–51. doi:10.3892/or.18.3.645.
59. Spahn PN, Lewis NE. Systems glycobiology for glycoengineering. *Curr Opin Biotechnol* 2014; 30: 218–24. doi:10.1016/j.copbio.2014.08.004.
60. Costa AR, Rodrigues ME, Henriques M, Oliveira R, Azeredo J. Glycosylation: impact, control and improvement during therapeutic protein production. *Crit Rev Biotechnol* 2014; 34: 281–99. doi:10.3109/07388551.2013.793649.
61. Dicker M, Strasser R. Using glyco-engineering to produce therapeutic proteins. *Expert Opin Biol Ther* 2015; 15: 1501–16. doi:10.1517/14712598.2015.1069271.
62. Buettner MJ, Shah SR, Saeui CT, Ariss R, Yarema KJ. Improving immunotherapy through glycodesign. *Front Immunol* 2018; 9: 2485. doi:10.3389/fimmu.2018.02485.
63. Gugliotta A, Ceaglio N, Etcheverrigaray M, Kratje R, Oggero M. Strategies to develop therapeutic N- and O-hyperglycosylated proteins. *Methods Mol Biol* 2018; 1674: 163–81. doi:10.1007/978-1-4939-7312-5_13.
64. Wang H, Zhang W, Zhao J, Zhang L, Liu M, Yan G, *et al*. N-glycosylation pattern of recombinant human CD82 (KAI1), a tumor-associated membrane protein. *J Proteomics* 2012; 75: 1375–85. doi:10.1016/j.jprot.2011.11.013.
65. Song R, Oren DA, Franco D, Seaman MS, Ho DD. Strategic addition of an N-linked glycan to a monoclonal antibody improves its HIV-1-neutralizing activity. *Nat Biotechnol* 2013; 31: 1047–52. doi:10.1038/nbt.2677.
66. Sinclair AM, Elliott S. Glycoengineering: the effect of glycosylation on the properties of therapeutic proteins. *J Pharm Sci* 2005; 94: 1626–35. doi:10.1002/jps.20319.
67. Bürgi M, Aparicio GI, Dorella A, Kratje R, Scorticati C, Oggero M. Novel erythropoietin-based therapeutic candidates with extra N-glycan sites that block hematopoiesis but preserve neuroplasticity. *Biotechnol J* 2021; 16: e2000455. doi:10.1002/biot.202000455.
68. Picanco-Castro V, Biaggio R, Cova D, Swiech K. Production of recombinant therapeutic proteins in human cells: current achievements and future perspectives. *Protein Pept Lett* 2013; 20: 1373–81. doi:10.2174/09298665201213111213032 2.
69. Behren S, Westerlind U. Glycopeptides and -mimetics to detect, monitor and inhibit bacterial and viral infections: recent advances and perspectives. *Molecules* 2019; 24: 1004. doi:10.3390/molecules24061004.

70. Elliott S, Lorenzini T, Asher S, Aoki K, Brankow D, Buck L, *et al*. Enhancement of therapeutic protein in vivo activities through glycoengineering. *Nat Biotechnol* 2003; 21: 414–21. doi:10.1038/nbt799.
71. Fares F, Havron A, Fima E. Designing a long acting erythropoietin by fusing three carboxyl-terminal peptides of human chorionic gonadotropin subunit to the N-terminal and C-terminal coding sequence. *Int J Cell Biol* 2011; 2011: 275063. doi:10.1155/2011/275063.
72. Calderon J, Zavrel M, Ragni E, Fonzi WA, Rupp S, Popolo L. PHR1, a pH-regulated gene of *Candida albicans* encoding a glucan-remodelling enzyme, is required for adhesion and invasion. *Microbiology* 2010; 156: 2484–94. doi:10.1099/mic.0.038000-0.
73. Hardwicke J, Ferguson EL, Moseley R, Stephens P, Thomas DW, Duncan R. Dextrin–rhEGF conjugates as bioresponsive nanomedicines for wound repair. *J Control Release* 2008; 130: 275–83. doi:10.1016/j.jconrel.2008.07.023.
74. Duncan R, Gilbert HRP, Carbajo RJ, Vicent MJ. Polymer masked-unmasked protein therapy. 1. Bioresponsive dextrin-trypsin and -melanocyte stimulating hormone conjugates designed for α-amylase activation. *Biomacromolecules* 2008; 9: 1146–54. doi:10.1021/bm701073n.
75. Ferguson EL, Duncan R. Dextrin-phospholipase A2: synthesis and evaluation as a bioresponsive anticancer conjugate. *Biomacromolecules* 2009; 10: 1358–64. doi:10.1021/bm8013022.
76. Mero A, Pasqualin M, Campisi M, Renier D, Pasut G. Conjugation of hyaluronan to proteins. *Carbohydr Polym* 2013; 92: 2163–70. doi:10.1016/j.carbpol.2012.11.090.
77. Kogan G, Šoltés L, Stern R, Gemeiner P. Hyaluronic acid: a natural biopolymer with a broad range of biomedical and industrial applications. *Biotechnol Lett* 2007; 29: 17–25. doi:10.1007/s10529-006-9219-z.
78. Ferguson EL, Alshame AMJ, Thomas DW. Evaluation of hyaluronic acid–protein conjugates for polymer masked-unmasked protein therapy. *Int J Pharm* 2010; 402: 95–102. doi:10.1016/j.ijpharm.2010.09.029.
79. Liebner R, Meyer M, Hey T, Winter G, Besheer A. Head to head comparison of the formulation and stability of concentrated solutions of HESylated versus PEGylated anakinra. *J Pharm Sci* 2015; 104: 515–26. doi:10.1002/jps.24253.
80. Lameire N, Hoste E. What's new in the controversy on the renal/tissue toxicity of starch solutions? *Intensive Care Med* 2014; 40: 427–30. doi:10.1007/s00134-013-3191-6.
81. Jain S, Hreczuk-Hirst DH, McCormack B, Mital M, Epenetos A, Laing P, *et al*. Polysialylated insulin: synthesis, characterization and biological activity in vivo. *Biochim Biophys Acta* 2003; 1622: 42–9. doi:10.1016/S0304-4165(03)00116-8.
82. Punnappuzha A, Ponnanettiyappan J, Nishith RS, Hadigal S, Pai PG. Synthesis and characterization of polysialic acid–uricase conjugates for the treatment of hyperuricemia. *Int J Pept Res Ther* 2014; 20: 465–72. doi:10.1007/s10989-014-9411-2.

83. Keys TG, Wetter M, Hang I, Rutschmann C, Russo S, Mally M, *et al*. A biosynthetic route for polysialylating proteins in *Escherichia coli*. *Metab Eng* 2017; 44: 293–301. doi:10.1016/j.ymben.2017.10.012.
84. Lin BX, Qiao Y, Shi B, Tao Y. Polysialic acid biosynthesis and production in *Escherichia coli*: current state and perspectives. *Appl Microbiol Biotechnol* 2016; 100: 1–8. doi:10.1007/s00253-015-7019-x.
85. Shields RL, Lai J, Keck R, O'Connell LY, Hong K, Meng G, *et al*. Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human FcÎ3RIII and antibody-dependent cellular toxicity. *J Biol Chem* 2002; 277: 26733–40. doi:10.1074/jbc.M202069200.
86. Li C, Li T, Wang LX. Chemoenzymatic defucosylation of therapeutic antibodies for enhanced effector functions using bacterial α-fucosidases. *Methods Mol Biol* 2018; 1827: 367–80. doi:10.1007/978-1-4939-8648-4_19.
87. Morgan C, Fernandes D. Designing biobetter monoclonal antibody therapeutics by glycoengineering. *Int Pharm Ind* 2009; 38–44. https://www.ludger.com/articles/ludger-m-00308-intpharmind-2009-biobetter-mAbs.pdf Accessed May 18, 2021.
88. Berger S, Lowe P, Tesar M. Fusion protein technologies for biopharmaceuticals: applications and challenges. *MAbs* 2015; 7: 456–60. doi:10.1080/19420862.2015.1019788.
89. Vacirca JL, Chan A, Mezei K, Adoo CS, Pápai Z, McGregor K, *et al*. An open-label, dose-ranging study of Rolontis, a novel long-acting myeloid growth factor, in breast cancer. *Cancer Med* 2018; 7: 1660–9. doi:10.1002/cam4.1388.
90. Pratley RE, Kang J, Trautmann ME, Hompesch M, Han OP, Stewart J, *et al*. Body weight management and safety with efpeglenatide in adults without diabetes: a phase II randomized study. *Diabetes Obes Metab* 2019; 21: 2429–39. doi:10.1111/dom.13824.
91. Rath T, Baker K, Dumont JA, Peters RT, Jiang H, Qiao SW, *et al*. Fc-fusion proteins and FcRn: structural insights for longer-lasting and more effective therapeutics. *Crit Rev Biotechnol* 2015; 35: 235–54. doi:10.3109/07388551.2013.834293.
92. Huang C. Receptor-Fc fusion therapeutics, traps, and MIMETIBODY technology. *Curr Opin Biotechnol* 2009; 20: 692–9. doi:10.1016/j.copbio.2009.10.010.
93. Strohl WR. Fusion proteins for half-life extension of biologics as a strategy to make biobetters. *BioDrugs* 2015; 29: 215–39. doi:10.1007/s40259-015-0133-6.
94. Nowinski AK, Sun F, White AD, Keefe AJ, Jiang S. Sequence, structure, and function of peptide self-assembled monolayers. *J Am Chem Soc* 2012; 134: 6000–5. doi:10.1021/ja3006868.
95. Liu EJ, Sinclair A, Keefe AJ, Nannenga BL, Coyle BL, Baneyx F, *et al*. EKylation: addition of an alternating-charge peptide stabilizes proteins. *Biomacromolecules* 2015; 16: 3357–61. doi:10.1021/acs.biomac.5b01031.

96. Schellenberger V, Wang C, Geething NC, Spink BJ, Campbell A, To W, *et al*. A recombinant polypeptide extends the in vivo half-life of peptides and proteins in a tunable manner. *Nat Biotechnol* 2009; 27: 1186–90. doi:10.1038/nbt.1588.
97. Ding S, Song M, Sim BC, Gu C, Podust VN, Wang CW, *et al*. Multivalent antiviral XTEN–peptide conjugates with long in vivo half-life and enhanced solubility. *Bioconjug Chem* 2014; 25: 1351–9. doi:10.1021/bc500215m.
98. Geething NC, To W, Spink BJ, Scholle MD, Wang CW, Yin Y, *et al*. Gcg-XTEN: an improved glucagon capable of preventing hypoglycemia without increasing baseline blood glucose. *PLoS One* 2010; 5: e10175. doi:10.1371/journal.pone.0010175.
99. Haeckel A, Appler F, Figge L, Kratz H, Lukas M, Michel R, *et al*. XTEN-annexin A5: XTEN allows complete expression of long-circulating protein-based imaging probes as recombinant alternative to PEGylation. *J Nucl Med* 2014; 55: 508–14. doi:10.2967/jnumed.113.128108.
100. Ahmadpour S, Hosseinimehr SJ. PASylation as a powerful technology for improving the pharmacokinetic properties of biopharmaceuticals. *Curr Drug Deliv* 2017; 15: 331–41. doi:10.2174/1567201814666171120122352.
101. Schlapschy M, Binder U, Börger C, Theobald I, Wachinger K, Kisling S, *et al*. PASylation: a biological alternative to PEGylation for extending the plasma half-life of pharmaceutically active proteins. *Protein Eng Des Sel* 2013; 26: 489–501. doi:10.1093/protein/gzt023.
102. Gebauer M, Skerra A. Prospects of PASylation® for the design of protein and peptide therapeutics with extended half-life and enhanced action. *Bioorganic Med Chem* 2018; 26: 2882–7. doi:10.1016/j.bmc.2017.09.016.
103. EvaluatePharma. World Preview 2019, Outlook to 2024. https://www.evaluate.com/thought-leadership/pharma/evaluatepharma-world-preview-2019-outlook-2024 Published June 4, 2019. Accessed November 10, 2019.
104. am Ende DJ, am Ende MT. Chemical engineering in the pharmaceutical industry. In: Chemical engineering in the pharmaceutical industry: active pharmaceutical ingredients, Hoboken, NJ: John Wiley & Sons, Inc.; 2019: 1–17. doi:10.1002/9781119600800.ch51.
105. Blankenship K. The top 20 drugs by 2018 U.S. sales. https://www.fiercepharma.com/special-report/top-20-drugs-by-2018-u-s-sales. Published June 17, 2019. Accessed October 9, 2020.
106. Kresse GB. Biosimilars—science, status, and strategic perspective. *Eur J Pharm Biopharm* 2009; 72: 479–86. doi:10.1016/j.ejpb.2009.02.014.
107. Polimeni G, Trifirò G, Ingrasciotta Y, Caputi AP. The advent of biosimilars for the treatment of diabetes: current status and future directions. *Acta Diabetol* 2015; 52: 423–31. doi:10.1007/s00592-015-0771-7.
108. Moorkens E, Jonker-Exler C, Huys I, Declerck P, Simoens S, Vulto AG. Overcoming barriers to the market access of biosimilars in the European union: the case of

biosimilar monoclonal antibodies. *Front Pharmacol* 2016; 7: 193. doi:10.3389/fphar.2016.00193.

109. Paul SM, Mytelka DS, Dunwiddie CT, Persinger CC, Munos BH, Lindborg SR, *et al*. How to improve RD productivity: the pharmaceutical industry's grand challenge. *Nat Rev Drug Discov* 2010; 9: 203–14. doi:10.1038/nrd3078.
110. DiMasi JA, Feldman L, Seckler A, Wilson A. Trends in risks associated with new drug development: success rates for investigational drugs. *Clin Pharmacol Ther* 2010; 87: 272–7. doi:10.1038/clpt.2009.295.
111. Reichert J. Monoclonal antibodies as innovative therapeutics. *Curr Pharm Biotechnol* 2008; 9: 423–30. doi:10.2174/138920108786786358.
112. Kumar S, Chawla S, Dutta S. Biobetters: betting on the future. *J Ration Pharmacother Res* 2018; 4: 13–21.
113. Brumano LP, da Silva FVS, Costa-Silva TA, Apolinário AC, Santos JHPM, Kleingesinds EK, *et al*. Development of L-asparaginase biobetters: current research status and review of the desirable quality profiles. *Front Bioeng Biotechnol* 2019; 6: 212. doi:10.3389/fbioe.2018.00212.

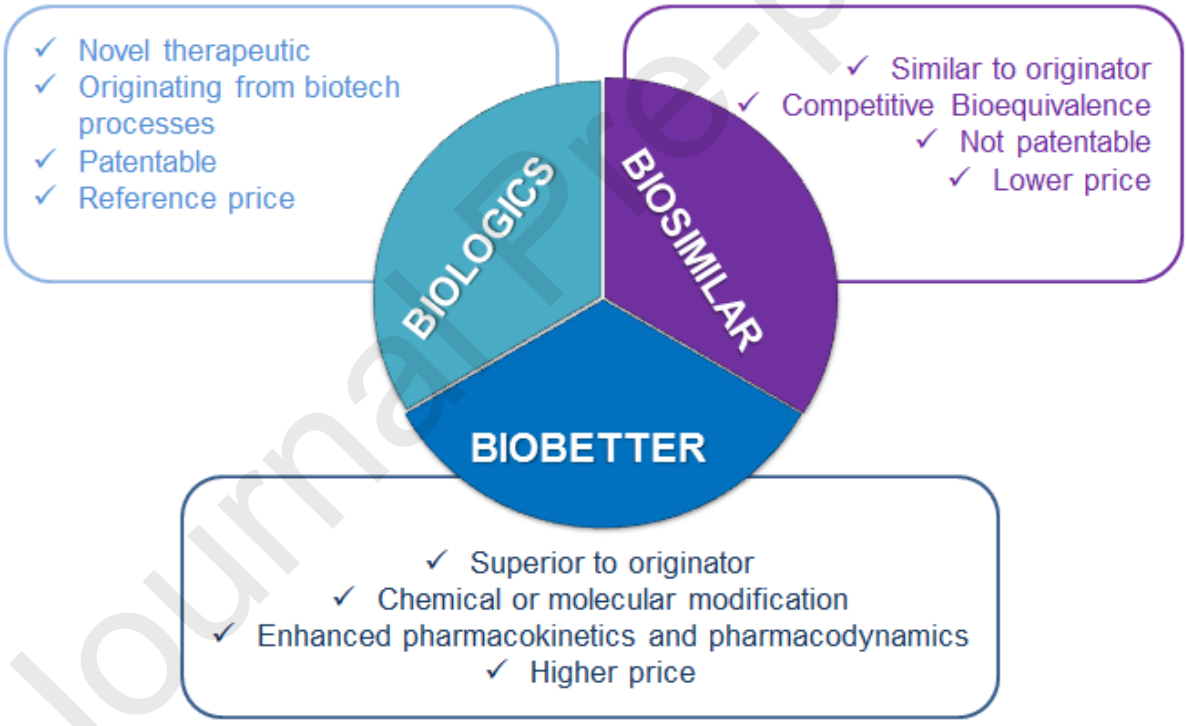


Figure 1. Comparison of the main characteristics of biological reference drugs, biosimilars and biobetters.

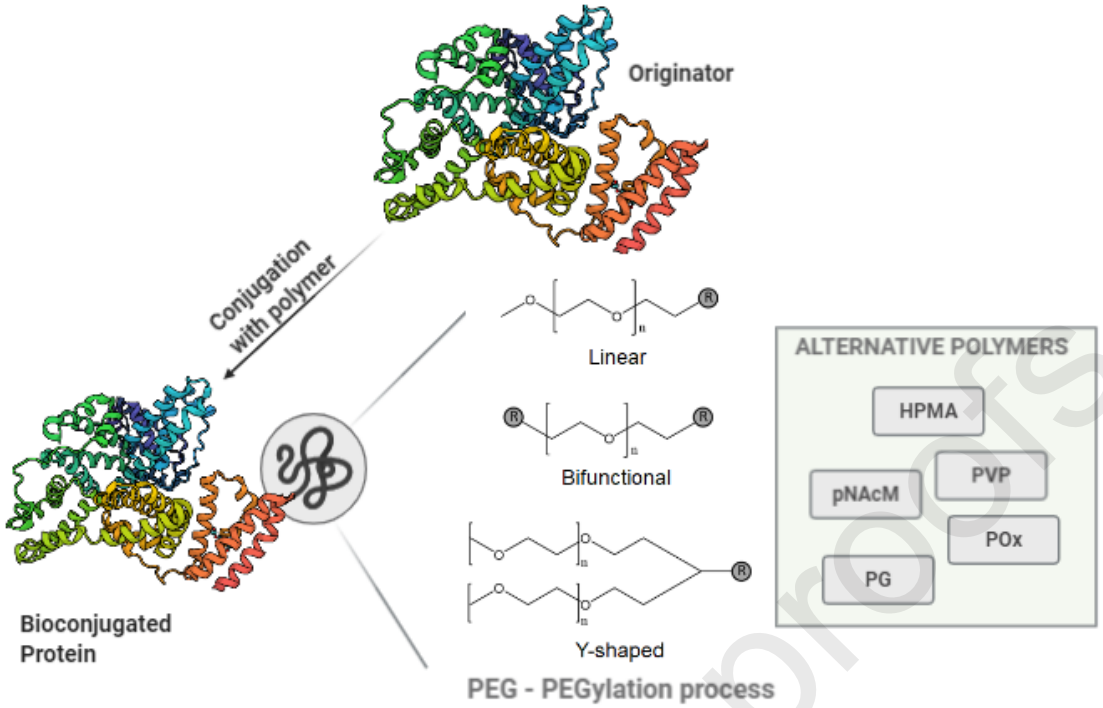


Figure 2. Schematic representation of PEGylation and alternative polymers for bioconjugation. HPMA, poly(*N*-(2-hydroxypropyl) methacrylamide); PG, polyglycerols; pNAcM, poly(*N*-acryloylmorpholine); Pox, poly(2-oxazoline)s; PVP, poly(vinylpyrrolidone).

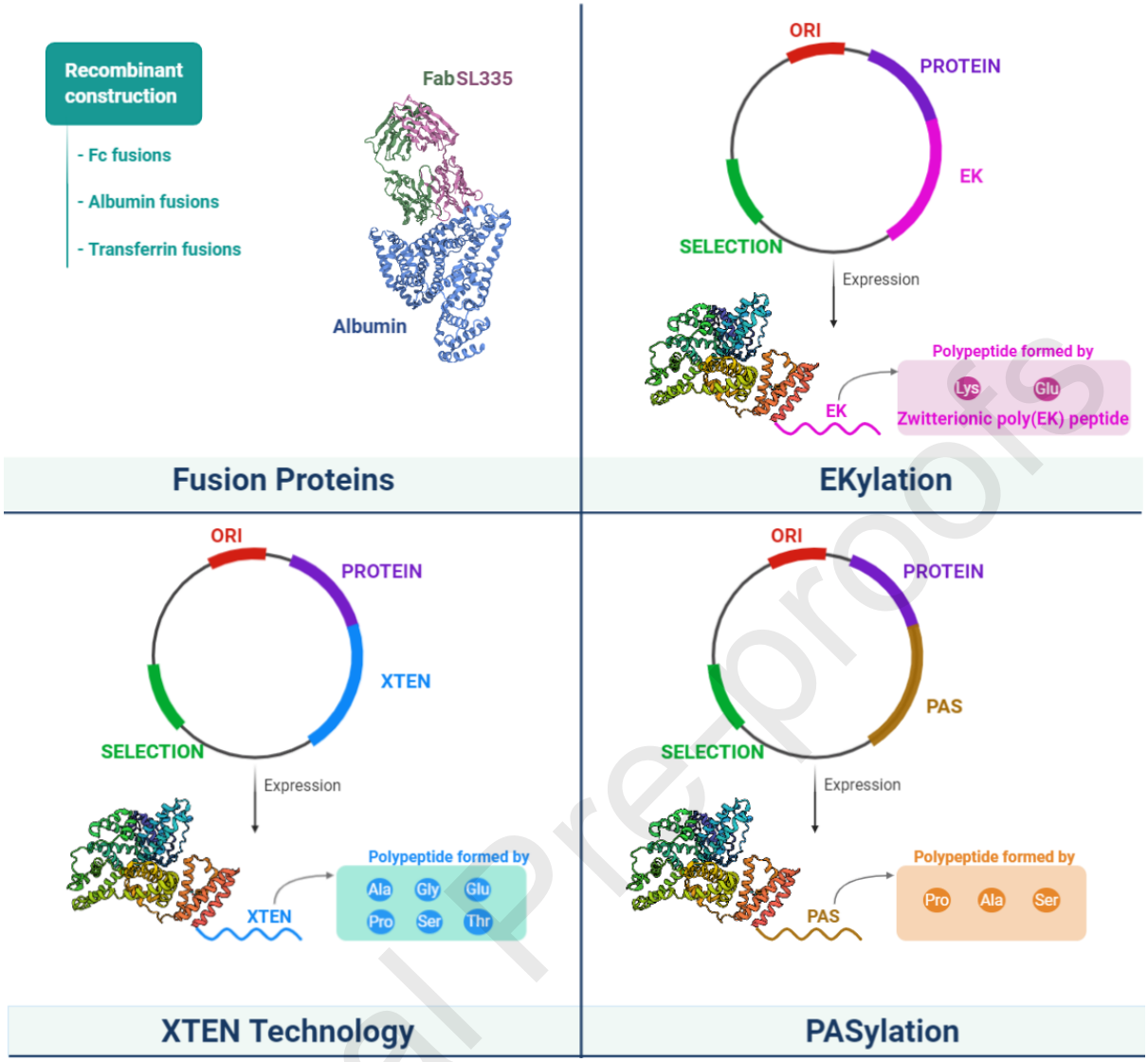


Figure 3. Schematic representation of novel trends in the development of biobetters.

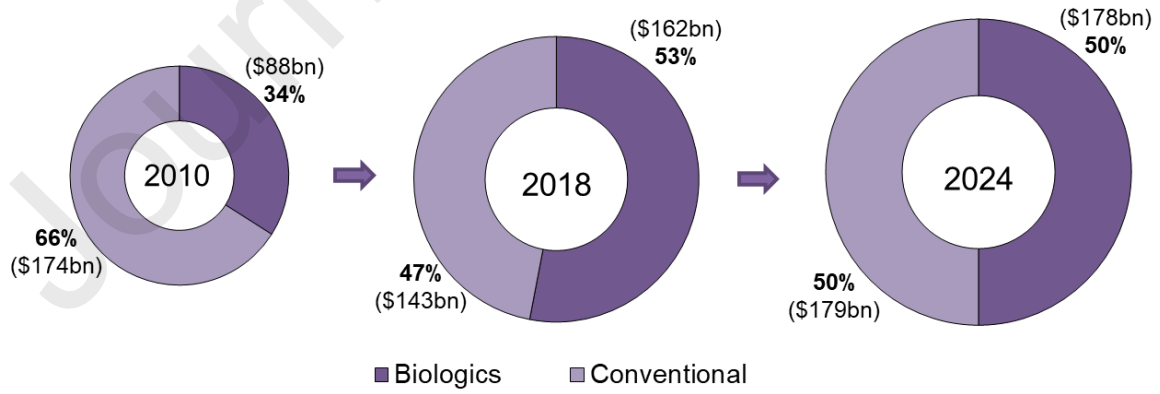
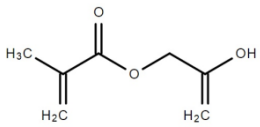
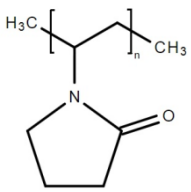
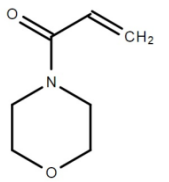
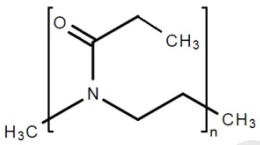
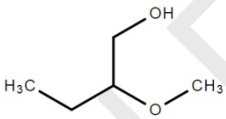


Figure 4. Global pharmaceutical market: biotechnological vs conventional drugs in the top 100 pharmaceutical products by sale figures (billions).

Table 1. Advantages and disadvantages of different techniques for the development of biobetters.

Polymers (alternatives to polyethylene glycol (PEG))			
Polymer	**Structure**	**Advantages**	**Disadvantages**
HPMA Poly[*N*-(2-hydroxypropyl) methacrylamide]		Good circulation in blood plasma; FDA approved; stability against heat and autolysis	Chronic toxicity associated with non-biodegradable polymers
PVP Poly(vinylpyrrolidone)		Water-soluble polymers; FDA approved; long residence time; show the minimum volume of tissue distribution	Antibody production
pNAcM Poly(N-acryloylmorpholine)		Easily modifiable; thermoresponsive	Antibody production
Pox Poly(2-oxazoline)		High quality (low dispersity); not influenced by metabolic macrophages and phagocytic activity	Chronic toxicity associated with non-biodegradable polymers
PG Polyglycerols **{AuQ: Edit OK?}**		Water soluble; low polydispersity; linear or (hyper)branched; easy synthesis; longer *in vivo* circulation time than PEG	Linear PG have rapid clearance

Carbohydrates		
Strategy	**Advantages**	**Disadvantages**
Glycosylation	Most prevalent post-translational modification naturally occurring in proteins; improves solubility and stability; approved by the FDA	Glycosylation patterns depend on the host cell, and the glycosylation sites at the protein must be determined
Neo-glycoconjugation	May change the structure, function and thermodynamic stabilization of proteins; approved by the FDA; promotes increased half-life	Expensive, usually involves an enzyme-catalyzed step
Hydroxyethyl starch (HES)	Good pharmacokinetic properties	Accumulates in the liver, kidney and bone marrow
HAylation	Preserves enzyme activities and thermal stabilities	Formation of soluble aggregates; undesired cross-linking; immune reactions

Polysialic acid (PSA)	PSA–insulin conjugation reduces blood glucose; metabolized as a natural sugar molecule by sialidases; prolongs half-life	Expensive; random attachment patterns; undesirable heterogeneity

Fusion proteins		
Fusion protein	**Advantages**	**Disadvantages**
Fc	Increased half-life; increased solubility; increased avidity for multivalent ligands; secondary cytotoxic functions can be tuned by the selection of the Fc isotype; secretion in the culture medium (simplifies the downstream processing)	High production costs (eukaryotic expression systems are required); decreased diffusion rate due to the increased size of the Fc fusion protein
Albumin	Increased half-life, resulting in decreased dose; simplified and low-cost production (can be expressed in yeast); large-scale production; blocking of the active N- and C- termini of the fusion partners can be avoided	Albumin only acts as a stabilizing agent and does not offer any additional function such as cytotoxicity
Transferrin	Increased half-life; low production costs (can be expressed in yeast); flexibility of fusion to either the N- or the C-terminus; can cross the blood-brain barrier by receptor-mediated transcytosis	This technology is not mature as other fusion proteins

Table 2. Expiration of patents for biological drugs.

Reference biologic	**Manufacturer**	**Type**	**Target**	**Clinical use**	**Patent expiration**
Tysabri® (natalizumab)	Biogen Idec	Humanized monoclonal antibody (mAb)	Cell adhesion molecule α4-integrin	Multiple sclerosis and Crohn's disease	2020
Lucentis® (ranibizumab)	Genentech/Novartis	Humanized Fab	VEGF-A	Age-related macular degeneration	2020
Soliris® (eculizumab)	Alexion Pharmaceuticals	Humanized mAb	Complement protein C5	Paroxysmal nocturnal hemoglobinuria, atypical hemolytic uremic syndrome, and neuromyelitis optica	2021

Stelara® (ustekinumab)	Janssen Biotech	Human mAb	IL-12 and IL-23	Psoriasis, Crohn's disease and ulcerative colitis	2023
Prolia®/Xgeva® (denosumab)	Amgen	Human mAb	RANK ligand	Osteoporosis, bone loss and bone tumors	2023
Cimzia® (certolizumab pegol)	Union Chimique Belge	Humanized Fab	TNF-α	Crohn's disease, rheumatoid arthritis, psoriatic arthritis and ankylosing spondylitis	2024
Simponi® (golimumab)	Janssen Biotech, Schering-Plough, Mitsubishi Tanabe Pharma	Human mAb	TNF-α	Rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis and ulcerative colitis	2024
Yervoy® (ipilimumab)	Bristol-Myers Squibb	Human mAb	CTLA-4	Melanoma	2025
Avonex (interferon β-1a)	Biogen	Cytokine	–	Multiple sclerosis	2026
Enbrel (etanercept)	Amgen/Wyeth Pharmaceuticals	Fusion protein (TNF receptor 2–Fc)	TNF-α	Rheumatoid arthritis, juvenile idiopathic arthritis and psoriatic arthritis, plaque psoriasis and ankylosing spondylitis	2028

Sources: EvaluatePharma, Cortells.

Table 3. Biobetters on the market.

Biobetter	**Reference biologic**	**Manufacturer**	**Approved in (by)**	**Specifications**	**Improved characteristics compared to the original**
ARANESP® (alfadarbepoetina)	Erythropoietin alpha	Amgen	2001 (FDA/EMA)	Recombinant human erythropoietin containing 5 N-glycosylation	Reduced dosage frequency to once every fortnight

NATRECOR® (citrato de BNPh)	Nesiritida	Janssen-Cilag	2001 (FDA)	Recombinant form of the 32 amino acid human B-type natriuretic peptide	Plasma levels increase from baseline endogenous levels by approximately 3-fold to 6-fold
ELONVA® (corifollitropin-alpha)	Follicle-stimulating hormone (FSH)	Merck	2010 (EMA)	Recombinant fusion of FSH and the C-terminal peptide of human chorionic gonadotropin (hCG)	Single subcutaneous injection instead of seven daily injections of a FSH preparation
KADCYLA® (trastuzumab emtansine or T-DM1)	Trastuzumab	Genentech	2013 (FDA)	Antibody drug conjugate, combining the HER2 inhibition of trastuzumab and the microtubule inhibition of DM1	Combination with efficacy greater than that of the current standard of care
GAZYVARO® (obinutuzumab)	Rituximab	Roche	2013 (FDA)	Glyco-engineering of humanized anti-CD20 monoclonal antibody	Improved pharmacokinetics
ELOCTATE™ (alfaefmoroctocog ue)	Recombinant antihemophilic factor	Biogen Idec	2014 (FDA)	B-domain-deleted recombinant Factor VIII, Fc fusion protein (BDDrFVIIIFc)	Reduced dosage frequency
TANZEUM® (albiglutide)	Glucagon-like peptide-2	GlaxoSmithKline	2014 (FDA), disc. 2017	GLP-1 receptor agonist–albumin fusion	Extended half-life allowing once-weekly dosage
STRENSIQ® (alfa-asfotase)		Alexion Pharmaceuticals	2015 (FDA/EMA)	Alkaline phosphatase enzyme/Fc fusion/deca-aspartate (D10) peptide	Lower production of anti-alfa-asfotase antibody
IDELVION® (albutrepenonacog alfa)	Factor IX	CSL Behring Recombinant Facility AG	2016 (FDA/EMA)	Recombinant factor IX albumin fusion	Prolongs the elimination half-life in the circulation allowing administration once every 7–14 days (in children and adults)

ROLONTIS® (eflapegrastim)	Neulasta	Spectrum Pharmaceuticals	October 2020 (FDA)	Recombinant human granulocyte-colony stimulating factor (rhG-CSF) conjugated to a human IgG4 Fc fragment via a short PEG linker	Enhanced efficacy, increased therapeutic potential (pharmacodynamic)

Data source: Statements of the listed companies.

Table 4. Probability of technical and regulatory success for biological drugs.[100–103]

Phase	**Probability of success**		
	New drug	**Biosimilar**	**Biobetter**
Preclinical development	86%	95% Abbreviated tests—focus on comparability	86%
Phase I clinical trials	53%	90% Large trial—focus on comparability	84% Large trial—focus on comparability
Phase II clinical trials	74%	–	74%
Phase III clinical trials	53%	80%[a] One pivotal trial—might be extrapolated to other approved indications of the originator product	80%[a] Trials for each indication, non-inferiority trials
Registration	96%	96%	96%
Total PTRS[b]	**27%**	**65%**	**41%**

[a]Higher because the target has been clinically validated

[b]PTRS, probability of technical and regulatory success

Highlights

- Biobetters are improved biological drugs, usually with better pharmacokinetics.
- Biobetters can be considered in between biosimilars and innovative biological drugs.
- Biobetters take about 10 years of development with investments of $500 millions.
- Glycosylation and PEGylation are the most common strategies to develop biobetters.
- Innovative trends of genetic engineering are promising for Biobetters development.