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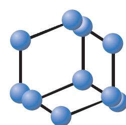

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Recent Advances in Half-life Extension Strategies for Therapeutic Peptides and Proteins

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Abstract: Peptides and proteins are two classes of molecules with attractive possibilities for therapeutic applications. However, the bottleneck for the therapeutic application of many peptides and proteins is their short half-lives *in vivo*, typically just a few minutes to hours. Half-life extension strategies have been extensively studied and many of them have been proven to be effective in the generation of long-acting therapeutics with improved pharmacokinetic and pharmacodynamic properties. In this review, we summarize the recent advances in half-life extension strategies, illustrate their potential applications and give some examples, highlighting the strategies that have been used in approved drugs and for drugs in clinical trials. Meanwhile, several novel strategies that are still in the process of discovery or at a preclinical stage are also introduced. In these strategies, the two most frequently used half-life extension methods are the reduction in the rate of renal clearance or the exploitation of the recycling mechanism of FcRn by binding to the albumin or IgG-Fc. Here, we discuss half-life extension strategies of recombinant therapeutic protein *via* genetic fusion, rather than chemical conjugation such as PEGylation. With the rapid development of genetic engineering and protein engineering, novel strategies for half-life extension have been emerged consistently. Some of these will be evaluated in clinical trials and may become viable alternatives to current strategies for making next-generation biodrugs.

Keywords: Peptides and proteins, half-life extension strategies, long-acting, renal clearance, FcRn, pharmacodynamic properties.

1. INTRODUCTION

Therapeutic peptides and proteins have received increasing attention because of their high binding affinity, specificity and solubility as well as their low toxicity. Since the first recombinant insulin was approved by the FDA in 1982, biopharmaceutical drugs have revolutionized the treatment of a broad spectrum of diseases, including cancer, metabolic disorders, and infectious diseases [1]. By April 2017, a total of 239 therapeutic peptides or proteins had been approved by the FDA, including clotting factors, hormones, growth factors, cytokines, enzymes, and antibodies [2, 3]. However, many therapeutic peptides and proteins exhibit short plasma half-lives, typically from a few minutes to a few hours, leading to the necessity for frequent or continuous injections.

There are several mechanisms responsible for the removal of therapeutic peptides and proteins from circulation. Enzymatic degradation by proteases and rapid clearance by renal filtration are the major reasons for short half-life. Numerous proteolytic enzymes, such as peptidases, are involved in peptide and protein degradation in the blood plasma and digestive system [4]. For instance, the half-life of glucagon-like peptide-1 (GLP-1) is only 1-2 min, because of inactivation by dipeptidyl peptidase-4 (DPP-4) [5]. Secondly, the threshold of renal filtration is about 40-50 kDa; biopharmaceuticals with a molecular weight below 50 kDa are rapidly eliminated by kidney filtration. The growth hormone, with a molecular weight of 22 kDa, has a half-life of 0.36 h and 3.4 h after intravenous and subcutaneous injection, respectively [6]. Additionally, the surface charge of biopharmaceuticals plays an important role in renal elimination [7]. Molecules with a negative surface charge are eliminated less rapidly than neutral and positive molecules due to repulsion by the negatively charged polysaccharides on membranes in the glomerular filter. The hepatic mechanism also contributes substantially to peptide and protein clearance through various processes of carrier- or receptor-mediated uptake of molecules into hepatocytes. A final route of clearance of proteins is lysosomal degradation *via* endocytosis by endothelial cells and other nucleated cells [2]. In short, the molecular size and physicochemical properties of a peptide or protein influence its longevity in circulation.

In order to extend the half-lives and improve the pharmacokinetic/pharmacodynamic properties of therapeutic peptides and proteins, much effort has been invested in the area of chemical and genetic engineering, resulting in the development of various effective strategies. In this review, strategies proven to be efficient are summarized and some newly emerging technologies, potentially capable of making great improvements to the pharmacokinetic/pharmacodynamic properties of peptides and proteins, are also discussed. We focus on recombinant therapeutic protein production by genetic fusion, not chemical conjugation such as PEGylation.

With the development of biotechnology, three key strategies have been proposed and employed to extend the half-lives of peptides and proteins (Fig. **1**). Firstly, chemical modification and amino acid substitution have been used to prevent enzymatic degradation of peptides. This includes N-/C- termini modification, Liable amino acid replacement, D-amino acid utilization, and cyclization [8]. Taking GLP-1 as an example, enzymatic stability is improved significantly *via* N-terminus modification (N-acetyl-GLP-1 and N-pyroglutamyl-GLP-1) [9], substitution of the second amino acid of the N-terminal (Ala) with Ser or Gly [10, 11], and introduction of two or three lactam bridges resulting in bicyclic and tricyclic analogues [12]. Octreotide, too, is designed with two unnatural amino acids, D-Phe and D-Trp, resulting in substantially longer half-life

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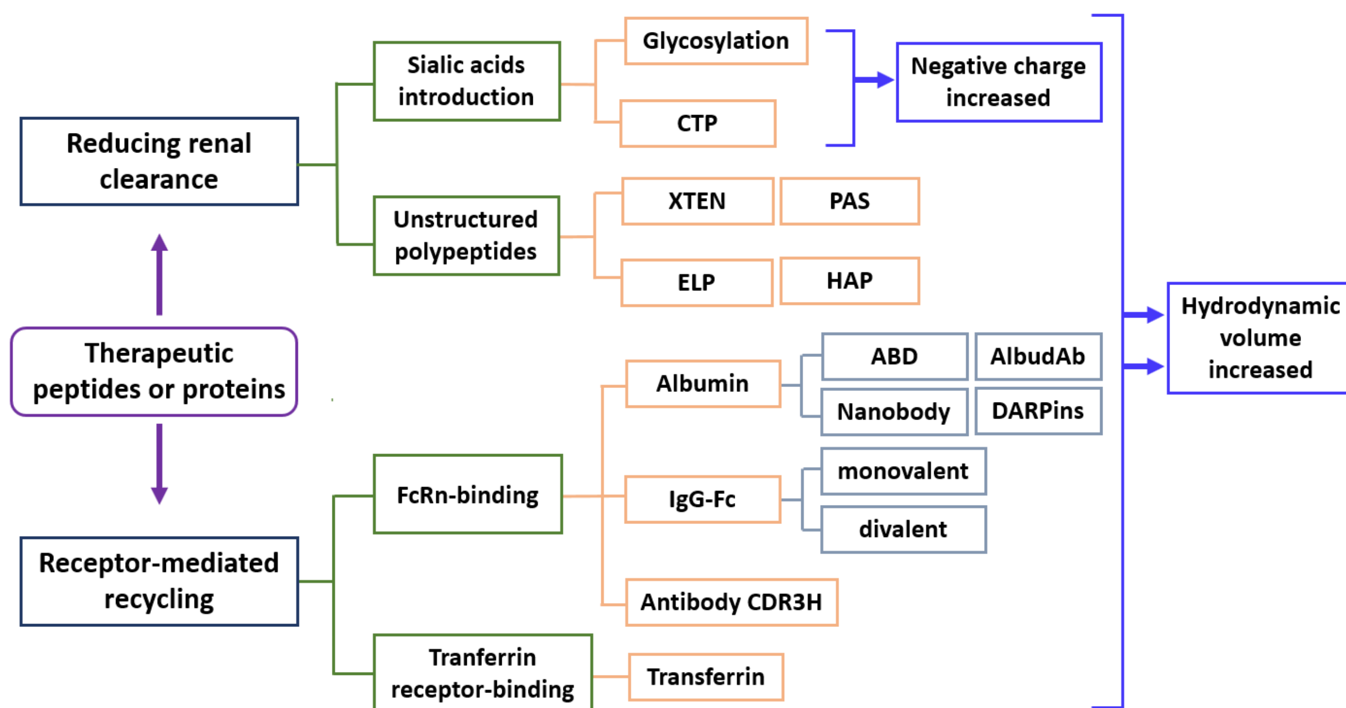

Fig. (1). Half-life extension strategies. Reducing renal clearance and exploiting the recycling mechanism of FcRn by binding to the albumin or IgG-Fc are the two most frequently used half-life extension strategies for therapeutic peptides and proteins.

(1.5-2 h) and enhanced pharmacological efficacy compared to the native somatostatin [13]. This part is not the main scope of the review.

Secondly, the neonatal Fc receptor (FcRn), which plays an important role in the regulation of levels of IgGs and albumin throughout the body, has been successfully developed as a target for drug delivery and therapy [14, 15]. A longer half-life can be achieved *via* combination of a biologic agent with the Fc region of IgG or albumin, responsible for FcRn-binding, in a process of FcRn-mediated recycling [16, 17] (Fig. **2A**). Thirdly, reducing the rate of renal clearance by increasing the molecular size (hydrodynamic volume) or increasing the negative surface charge of the peptide or protein has proven to be an effective way to prolong half-life [7] (Fig. **2B**). Linking the drug to a bulking moiety, such as PEG, or to a polypeptide, such as XTEN, is the most common strategy to increase the molecular size [18]. The introduction of glycosylation sites into a peptide or protein increases the negative charge, resulting in slower renal clearance [19]. Some strategies for half-life extension exploit more than one of these principles with synergistic effects. For instance, the half-life extension of a peptide or protein by fusion to albumin depends not only on the FcRn-mediated recycling system, but also on the resulting increased hydrodynamic volume. A protein engineered through post-translational glycosylation has an increased negative charge and enhanced molecular size, reducing renal clearance, modulating receptor-mediated endocytosis, and preventing proteolytic degradation [20] (Fig. **1**).

Therapeutic peptides and proteins modified through these strategies approved by the FDA or EMA are listed in Table **1**. The number of approved peptides and proteins suggests that these strategies are relatively safe and effective. Meanwhile, some strategies are still in the process of verifying safety and effectiveness in clinical trials or in the preclinical stage (Table **2**). All data used in this review were collected from various publicly available sources, including published literature, reports of scientific meetings, the FDA website, the ClinicalTrials.gov website, pharmaceutical company websites, and the prescription information released by the pharmaceutical manufacturers.

2. FCRN-MEDIATED RECYCLING MECHANISM

Human IgG isotypes (except IgG3) and albumin bind FcRn simultaneously at different sites, with neither competition nor cooperation [21], in a strictly pH-dependent manner at acidic pH (~6.0), but not at a physiological pH (7.4) [15, 22, 23]. However, whether a single FcRn molecule can simultaneously transport both IgG and albumin has not yet been determined [14]. The mechanisms of interaction between IgG-FcRn and albumin-FcRn are substantially different. One IgG molecule can simultaneously bind two FcRn molecules due to the homodimeric nature of IgG, while the ratio for the albumin-FcRn interaction is 1:1 [14]. The IgG-FcRn interaction is predominately driven by electrostatics between histidine residues in the Fc CH2-CH3 domain and acidic residues on the α2-domain of FcRn [14], while the albumin-FcRn interaction is predominately driven by the hydrophobic force between the HH-loop of albumin and WW-loop of FcRn [14, 21]. The IgGs and albumin are rescued from lysosomal degradation by a pH-dependent FcRn-mediated recycling and transcytosis process, resulting in an extraordinarily long half-life (Fig. **2A**): 19 days for albumin and 21 days for IgGs [24]. This mechanism has been successfully used to prolong the half-lives of therapeutic peptides and proteins by chemical conjugation or genetic fusion to the albumin or IgG-Fc [25]. Two other half-life extension approaches that rely on FcRn are the use of affibody molecules with FcRn affinity and of antibody fusion proteins.

2.1. Fc Fusion

Fc fusion represents one of the most clinically successful half-life extension strategies to date, and has been used in the development of a major portion of the fusion proteins approved by the FDA. The concept of applying IgG-Fc as a scaffold to increase the half-lives of therapeutic peptides and proteins primarily utilizes bivalency of the molecule to improve bioactivity and extend the

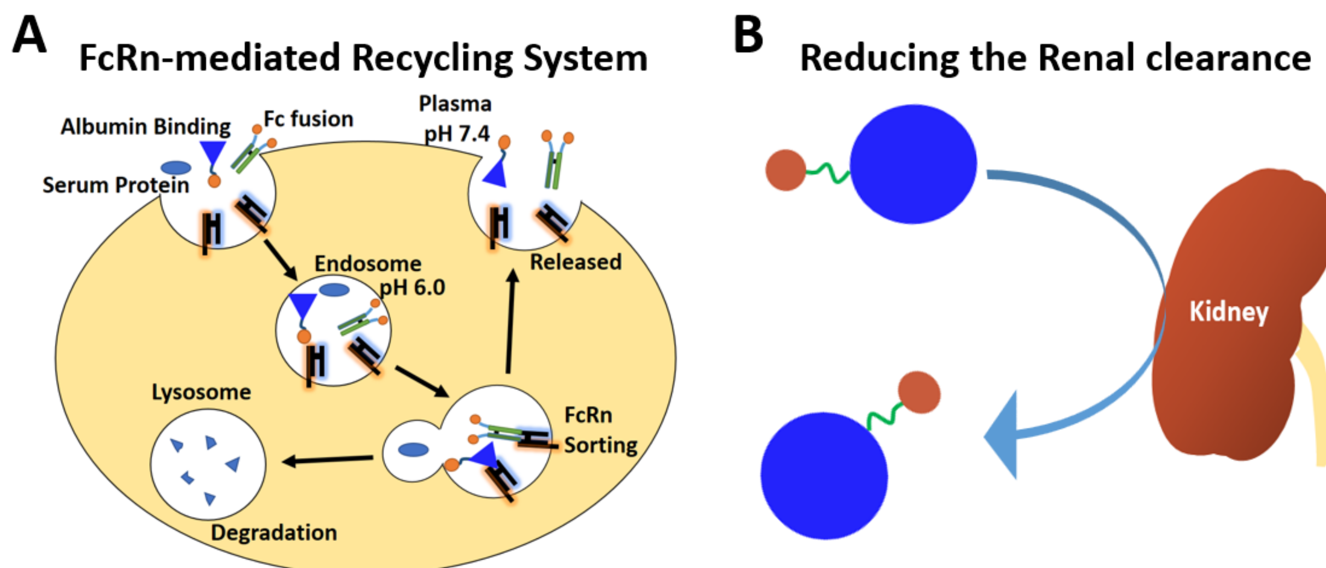

Fig. (2). The two most-used methods for half-life extension. A longer half-life can be achieved through a process of pH-dependent FcRn-mediated recycling by genetically fusing the peptide or protein to IgG-Fc or binding it to albumin (**A**). Additionally, reducing renal clearance of a peptide or protein by increasing its molecular size has been proven to be an effective way to prolong the half-life (**B**).

molecule's half-life [26] (Fig. **3A**). A wide range of molecules, from small peptides to larger proteins are suitable for fusion to the Fc region; suitable molecules include hormones, growth factors, blood proteins, and protein or peptide mimetics.

Etanercept (Enbrel), the first Fc fusion protein, was approved by the FDA in 1998 for the treatment of rheumatoid arthritis. With a half-life of 102 h, etanercept consists of the extracellular region of TNFR2 genetically fused to the Fc region of IgG1 [27] (Table **1**). In the past twenty years, ten Fc fusion proteins have been approved by the FDA, and many more are in clinical trials [7].

Pharmacokinetic profiles of peptides can be improved by genetic fusion to the two arms of an Fc domain, resulting in bivalent fusion proteins (Fig. **3A**). Dulaglutide (Trulicity), a GLP-1 receptor agonist administered once weekly, was approved in 2014 by the FDA for the treatment of type 2 diabetes (T2DM). Dulaglutide consists of two identical GLP-1 molecules with several mutations (A8G/G22E/R36G), fused to the IgG4-Fc region (F234A/L235A) through a small peptide linker. The half-life of dulaglutide is prolonged to about 5 days; much longer than the half-lives of exenatide, liraglutide, and lixisenatide, which have to be administered once or twice daily [28]. Romiplostim (Nplate), approved for the treatment of thrombocytopenia in patients with chronic immune thrombocytopenic purpura, is constructed by genetic fusion of two thrombopoietin (TPO)-mimetic-peptides to the Fc region of IgG1, thereby achieving a half-life of 3.5 days. The TPO-mimetic-peptide consists of two repeats of 14 amino acids, capable of binding to the extracellular domain of the TPO receptor and stimulating megakaryocyte growth and platelet production [29, 30].

Most of the functional moieties in approved Fc fusion proteins are portions of the receptors, such as TNF receptor (etanercept), IL-1 receptor (rilonacept) [31], VEGF receptor (aflibercept and ziv-aflibercept), and CTLA-4 (abatacept and belatacept). The half-lives of these fusion proteins are in the range of 3-13 days *in vivo*. Rilonacept (Arycalyst) is an IL-1 inhibitor, consisting of portions of IL-1R extracellular domains and the IL-1R accessory protein linked in-line to the Fc region of IgG1, possessing a half-life of 8.6 days. Rilonacept was approved to treat cryopyrin-associated periodic syndromes (CAPS) [31]. Aflibercept (Elyea) and ziv-aflibercept (Zaltrap) have the same ingredient, consisting of VEGF-binding portions from extracellular domains of human VEGF receptor 1 and 2 fused to the Fc region of IgG1. Aflibercept was approved by the FDA for the treatment of neovascular (wet) age-related macular degeneration (AMD), developed for intravitreal injection. Ziv-aflibercept was approved for use in metastatic colorectal cancer, with a different osmolarity from aflibercept. The half-lives of aflibercept and ziv-aflibercept are both approximately 6 days by intravenous administration [32, 33]. Abatacept (Orencia) is constructed by fusion of the extracellular domain of CTLA-4 to the modified Fc region of IgG1, it binds to CD80/CD86 to prevent co-stimulation and inhibit T-cell activation. The FDA approved abatacept for the treatment of rheumatoid arthritis. However, abatacept does not completely block CD80/CD86 co-stimulation signal, resulting in lower effectiveness in non-human primate transplant models [34, 35]. Finally, belatacept (Nulojix) was designed with two amino acid substitutions (L104E/A29Y) in the ligand-binding region of CTLA-4, resulting in an enhanced affinity for CD80/CD86 and greater immunosuppressive activity than abatacept in non-human primates [36]. Belatacept, with a half-life of 8 days, was approved by the FDA for the prophylaxis of organ rejection in adult patients receiving a kidney transplant [34].

Ligands have also been used as binding partners of the Fc domain for the purposes of half-life extension. Examples of ligand binding partners are the extracellular domain of lymphocyte function-associated antigen 3 (LFA-3, alefacept), and GLP-1, TPO-mimetic-peptide, which are mentioned above. Alefacept (Amevive) is composed of the first extracellular CD2 receptor-binding domain of LFA-3 fused to the Fc portion of IgG1. Alefacept has a half-life approximately 12 days [37].

Another application of Fc as a means for half-life extension is monomeric Fc utilization, in which the therapeutic protein is fused to only one arm of a dimeric Fc (Fig. **3B**). Although the resultant therapeutic protein is monovalent, improved pharmacokinetics and pharmacodynamics are still achieved [38]. Both of the monovalent Fc-fusion proteins approved by the FDA are for the treatment of hemophilia. Eftrenonacog-α (Alprolix), approved for the treatment of hemophilia B, consists of a single factor IX, a coagulation factor, fused to an Fc dimer from IgG1. The half-life of eftrenonacog-α is 53-82 h, 3-5 times longer than that of the recombinant factor IX (19 h) [39, 40]. Efraloctocog-α (Eloctate) comprises a single B domain of coagulation factor VIII fused to the Fc dimer, and is approved for the treatment of hemophilia A. The half-life of efraloctocog-α (19

h) was extended 1.5-fold compared with recombinant factor VIII [41].

Besides the approved Fc fusion proteins, many biodrugs have been in clinical trials, such as CNTO 528 [42], FP-1039[43] and sotatercept (Table **2**). Sotatercept, which is in phase 2 clinical trials for improving bone remodeling in multiple myeloma patients with osteolytic lesions, consists of the extracellular domain of activin receptor type IIA (ActRIIA) linked to the IgG1 Fc domain. Sotatercept possesses an extraordinarily long half-life of 22-26 d, allowing for a monthly injection regime [44].

Fc fusion proteins exhibit shorter half-life when compared with the whole IgG (which has a half-life of 3 weeks). The factors influencing this are complex, and include a generally lower binding affinity to FcRn, lower stability, the clearance pathway of effector molecules and a lack of the Fab domain [14, 45]. Several studies have shown that an improved half-life of IgG-Fc can be achieved by increasing Fc-FcRn affinity. Substitutions of IgG (M252Y/S254T/T256E) resulted in an approximately 10-fold pH-dependent affinity increase for IgG-FcRn and a 2- to 4-fold increase in half-life in human [46]. Yeung *et al.* have found that the Fc variant (N434A), which displays increased binding affinity to FcRn at pH 6.0, but negligible binding to FcRn at pH 7.4, exhibits a 2-fold decrease in clearance in cynomolgus monkey [47].

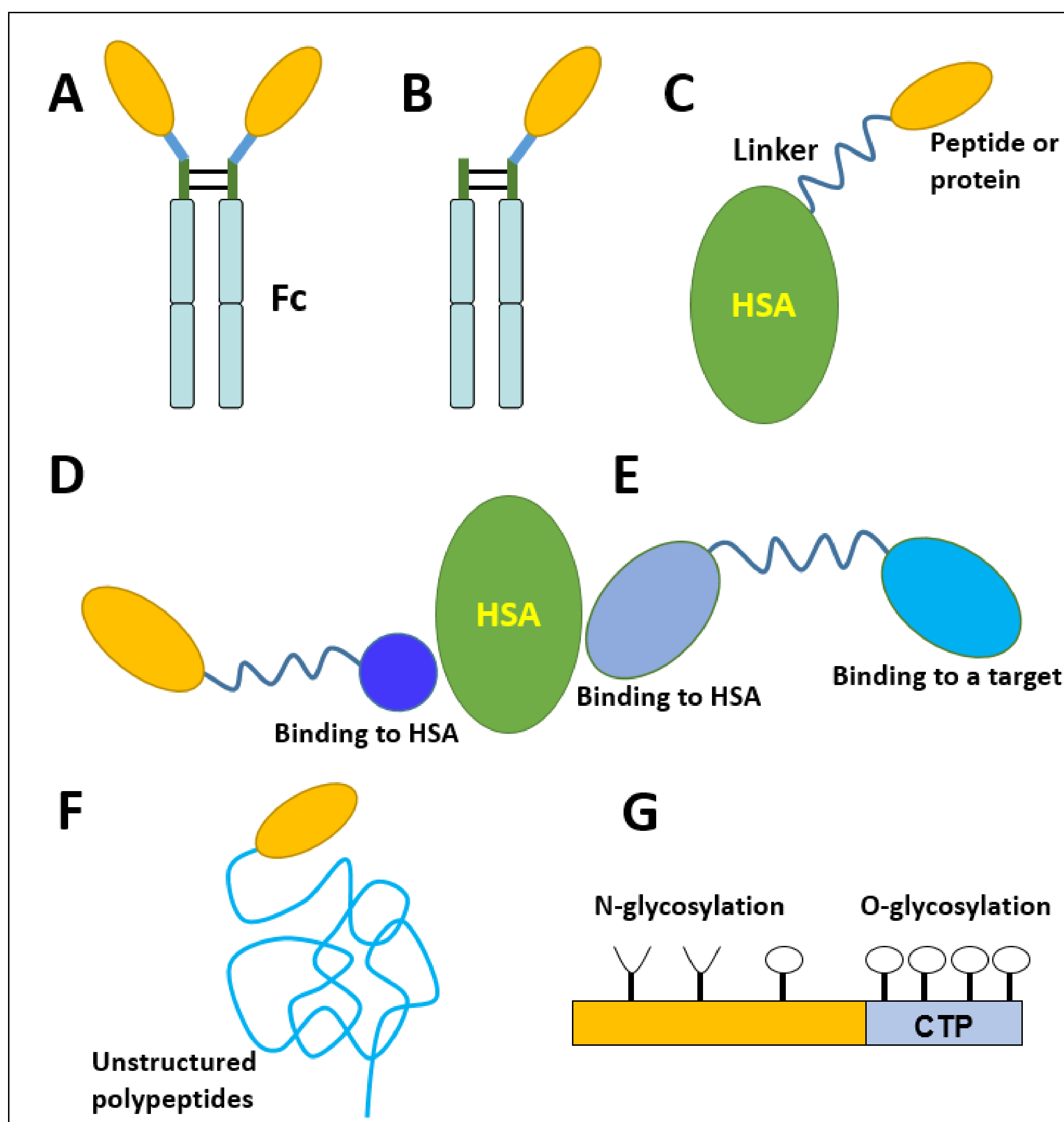

Fig. (3). Schematic representations of the different strategies for half-life extension of therapeutic peptides and proteins. The half-lives of therapeutic peptides and proteins can be prolonged by genetic fusion to two arms of Fc region (**A**), one arm of Fc region (**B**) or HSA (**C**) through the FcRn-mediated recycling system. Domains with high affinity for HSA have been developed for half-life extension by indirect targeting to FcRn. ABD and AlbudAb with HSA binding properties have been developed for half-life extension of therapeutic peptides and proteins by genetic fusion (**D**). The half-lives of nanobodies and DARPins with therapeutic effects can be extended by genetic fusion to others that bind to HSA (**E**). Unstructured polypeptides, such as XTEN, ELP and PAS, substantially increase the hydrodynamic volume of therapeutic peptides or proteins, resulting in an extended half-life (**F**). Increasing the negative charge of therapeutic peptides and proteins by introduction of glycosylation sites or fusion to CTP can also prolong half-life (**G**).

Table 1. Approved therapeutics with extended half-lives by a carrier.

Drug	Peptide/protein	Approval Date[a]	Half-life Modified	Carrier	Indication
Semaglutide (Ozempic)	GLP-1	2017	6-7 d	Albumin	Type 2 diabetes mellitus
Albutrepenonacog alfa (Idelvion)	Coagulation factor IX	2016	4 d	Albumin	Hemophilia B
Insulin Degludec (Tresiba)	Insulin	2015	25 h	Albumin	Diabetes mellitus
Liraglutide (Saxenda)	GLP-1	2014	13 h	Albumin	Obesity
Dulaglutide (Trulicity)	GLP-1	2014	5 d	IgG-Fc	Type 2 diabetes mellitus
Efraloctocog-α (Eloctate)	B-domain-deleted Factor VIII	2014	19 h	IgG-Fc	Hemophilia A
Albiglutide (Tanzeum)	GLP-1	2014	5 d	Albumin	Type 2 diabetes mellitus
Eftrenonacog-α (Alprolix)	Factor IX	2014	53-82 h	IgG-Fc	Hemophilia B
Ziv-aflibercept (Zaltrap)	Portions of VEGFR extracellular domains	2012	6 d	IgG-Fc	Metastatic colorectal cancer
Aflibercept (Elyea)	Portions of VEGFR extracellular domains	2011	4.6 d	IgG-Fc	Macular degeneration
Belatacept (Nulojix)	Extracellular domain of CTLA-4	2011	8 d	IgG-Fc	Kidney transplant rejection prophylaxis
Lirglutide (Victoza)	GLP-1	2010	13 h	Albumin	Type 2 diabetes mellitus
Corifollitropin-alfa (Elonva)	FSH	2010 (EMA)	95 h	CTP	Infertility in women
Romiplostim (Nplate)	TPO mimetic peptide	2008	3.5 d	IgG-Fc	Chronic immune thrombocytopenic purpura
Rilonacept (Arcalyst)	Portions of IL-1R extracellular domains and IL-1R accessory protein linked in-line	2008	8.6 d	IgG-Fc	Cryopyrin-associated periodic syndromes
Abatacept (Orencia)	Extracellular domain of CTLA-4	2005	13.1 d	IgGFc	Rheumatoid arthritis
Insulin detemir (Levemir)	Insulin	2005	5-7 h	Albumin	Diabetes mellitus
Alefacept (Amevive)[b]	First extracellular CD2 receptor binding domain of LFA-3	2003	12 d	IgG-Fc	Chronic plaque psoriasis
Darbepoetin-α (Aranesp)	Mutated EPO	2001	3 d	Glycosylation	Anemia
Etanercept (Enbrel)	Extracellular domain of TNFR2	1998	3 d	IgG-Fc	Rheumatoid arthritis

Note: [a]The date listed is the time of approval by the FDA, with the exception of corifollitropin-alfa, which has not been approved by the FDA.
[b]In 2011, Amevive was withdrawn from the market due to business considerations, not as a result of safety or efficacy concerns.

2.2. Albumin Fusion

Human serum albumin (HSA) is the most abundant protein in plasma, with a molecular weight of about 67 kDa. The functions of HSA in plasma include stabilization of plasma pH and maintenance of blood pressure; it also acts as a carrier of many metabolites and fatty acids. Albumin is also known as a transporter of drugs [48]. In the past decades, HSA has become a versatile carrier for therapeutic peptides and proteins and diagnostic agents. A peptide or protein with a short half-life can have its half-life significantly prolonged by genetic fusion to the N- or C-terminal of HSA (Fig. **3C**), or by chemical conjugation to a free cysteine residue (Cys-34), employing the FcRn-mediated recycling system [49].

The application of the HSA has been extensively studied; however, there are only two fusion proteins on the market (Table **1**). Albiglutide (Tanzeum) was the first therapeutic protein to be directly fused to HSA; it was approved by the FDA in 2014 for the treatment of T2DM [50]. Albiglutide is a GLP-1 receptor agonist composed of two copies of modified GLP-1 molecules with a Gly substitution for Ala at position two. Albiglutide has a half-life of about 5 days, allowing for once-weekly administration [51]. Albutrepenonacog alfa (Idelvion) is a recombinant albumin-human coagulation factor IX (FIX) fusion protein, consisting of FIX genetically fused to albumin *via* a novel cleavable linker peptide derived from the endogenous activation peptide in native FIX. Albutrepenonacog alfa was approved in 2016 by the FDA for the treatment and prevention of bleeding in patients with hemophilia B. The half-life of albutrepenonacog alfa is 90-104 h in patients, 5-fold longer than the half-life of native FIX (18-34 h). This allows routine prophylaxis to be administered once every 7-14 days [52].

In addition to the approved protein drugs, several biopharmaceuticals based on HSA half-life extension strategy have been in clinical trials (Table **2**). Extensive studies have been conducted to apply HSA to the production of long-acting coagulation factors, including factor IX, VIII and VIIa, for the prevention or treatment of bleeding disorders. CSL689 is a long-acting FVIIa-HSA fusion protein being developed by CSL Behring for hemophilia patients

with inhibitors to factor VIII or factor IX. The half-life of CSL689 is extended *via* the fusion of human albumin to the C-terminus of FVIIa by a flexible glycine-serine linker. In rats, CSL689 has a 6-fold longer half-life compared to the native FVIIa [53]. CSL689 completed phase 2 clinical trials, but phase 3 clinical trials were terminated as the result of a business decision unrelated to the drug's safety (Table **2**).

HSA is also used to extend the half-lives of hormones. Albutropin constructed by the fusion of growth hormone (GH) to the N-terminal of HSA, is used to treat GH deficiency. The half-life of albutropin was 13-15 h in cynomolgus monkey; much longer than GH (2-3 h). A single s.c. administration of albutropin increased plasma insulin-like growth factor 1 (IGF-1) levels for up to 7 days in rats [54]. However, Teva has terminated a phase 3 trial in the USA and terminated development of albutropin (Table **2**). CJC-1134-PC is an exendin-4 analog containing a C-terminal maleimide modification at Lys40 which allows for conjugation to the Cys34 in HSA [55]. The half-life of CJC-1134-PC is approximately 8 days in human, and entered phase 2 clinical trials for the treatment of T2DM [56]. CJC-1134-PC showed promise as a drug with a once-weekly injection in phase 1/2 clinical trials. It faced competition, however, from marketed drugs, and clinical trials were thus terminated [57] (Table **2**).

There are also some albumin fusion proteins which have failed or been discontinued after clinical trials due to safety problems. These include G-CSF-HSA (Egranli and Albugranin), IFN-α2b-HSA (Albuferon), Her3-HSA-Her2 (MM-111) and GLP-1-HSA (CJC-1131) [7].

HSA also binds FcRn in a strictly pH-dependent manner. Increasing the affinity of HSA to FcRn may increase the half-life of HSA even further. Andersen *et al*. have designed a series of HSA variants with a mutation at position 573 within the C-terminal helix of domain III (DIII). They found that the K573P mutation in HSA had a more than 12-fold improved affinity to FcRn, resulting in an approximately 1.5-fold extension of serum half-life in WT mice and cynomolgus monkeys [49]. It is expected that application of the K573P mutant of HSA will lead to the development of biopharmaceuticals with longer half-lives than those that use native HSA.

2.3. Non-covalent Binding to HSA

Peptides and proteins with the property of HSA binding *via* non-covalent association have also been used to prolong the half-lives of the peptides and proteins by indirect targeting of FcRn through processes such as lipidation, or by using albumin-binding domains (ABD), affibodies, anti-albumin antibodies, nanobodies, or DARPins.

2.3.1. Lipidation

Peptides and proteins conjugate with HSA in a non-covalent manner by fatty acid binding. This is called lipidation [58]. HSA contains nine different fatty acid binding sites, which may bind free fatty acids as well as fatty acids conjugated to larger molecules [58, 59]. Biopharmaceuticals containing a fatty acid are released gradually into circulation, resulting in a protracted half-life [58]. Lipidation strategy has been well established in approved peptide pharmaceuticals from Novo Nordisk [59]. So far, four lipidated biopharmaceuticals, based on insulin and GLP-1, have been approved by the FDA, and are all approved for the treatment of diabetes. Insulin detemir (Levemir) is the first lipidated polypeptide approved for the treatment of type 1 diabetes (T1DM). It is a long-acting insulin analog with Thr30 deletion and a C14 fatty acid attached to the Lys29 in chain B, allowing reversible binding to HSA [60]. Insulin detemir has a half-life of 5-7 h following s.c. administration due to albumin binding and self-association at the injection site, allowing for once-daily injection [58]. Insulin degludec (Tresiba) is a new generation of basal insulin for the treatment of T1DM with a C16 fatty acid conjugation *via* a γ-L-glutamic acid spacer at the ε-amino group of Lys29 in chain B. The half-life of insulin degludec can be longer than 25 h, depending on the albumin and oligomerization [58, 61].

Lipidation has also successfully improved the pharmacokinetic properties of GLP-1. Liraglutide, a lipidated once-daily GLP-1 analog, has been marketed for the treatment of T2DM and obesity. The backbone of liraglutide is a modified GLP-1 with a K34R substitution and a palmitic acid (C16) conjugated to Lys26 *via* a γ-L-glutamic acid spacer. Albumin binding through the fatty acid prolongs the half-life of liraglutide to 13 h after s.c. administration [62]. Semaglutide (Ozempic), approved by the FDA in 2017, is the next generation of liraglutide, with two differences in structure: the Ala8 in semaglutide is replaced by Aib, an unnatural amino acid, and the albumin binding moiety is changed into C18 fatty acids conjugated to Lys26 through a γGlu-2xOEG linker. These alterations increase the albumin affinity and resistance to DPP-4, resulting in a half-life of up to 165 h, allowing once-weekly injection [63, 64].

2.3.2. ABD

The naturally occurring albumin-binding domain (ABD) with an HSA-binding property is a small, three-helical protein domain found in various surface proteins expressed by gram-positive bacteria [65]. The binding site on HSA for FcRn is located in domain III, and does not interfere with ABD binding [66]. In addition, ABD can be expressed in *E.coli* at a high level, due to the small molecular weight. ABD has been utilized to prolong the half-lives of peptides or proteins by indirect targeting of FcRn (Fig. **3D**). For instance, the half-life of a bispecific single-chain diabody (ScDb) was extended to 27.6 h in mice, substantially longer than the natural ScDb (5.6 h), *via* genetic fusion to ABD3, which derives from streptococcal protein G and has been most extensively studied [67, 68].

Inspired by the promising feature of ABD3 as a half-life extension fusion partner for peptides and proteins, variants with improved affinity for HSA were designed and obtained. The consensus sequence design method was applied by Jacobs *et al*. to construct an ABD3 variant, named ABDCon, based on the consensus sequence of the 20 closest homologs to ABD3. ABDCon is highly stable, with a melting temperature of 81.5°C. A high affinity of 75 pM between ABDCon and HSA was also obtained, resulting in a long half-life for the fusion partner: 182 h in cynomolgus monkeys. The study showed that the half-life of the fusion molecules can be tuned using ABDCon variants with introduced mutations at the albumin binding interface. It demonstrated that the higher binding affinity of ABDCon variants for HSA, ranging from 3 nM to 1600 nM, resultes in a longer half-life for the fusion partner in mice [69]. This is, to some extent, not in agreement with the conclusion from Hopp *et al*., who indicated that the half-life extending property of ABD in mice is only weakly influenced by affinity for HSA [70]. The discrepancy may be explained by the fact that the weakest affinity in the latter study was 600 nM, while in the former, the weakest was 1600 nM [69].

Phage display technology has also been used to select an ABD with a higher affinity for HSA. One variant, denoted ABD035, has shown an extremely high affinity of 500 fM, corresponding to a more than 2400-fold improvement over the affinity of the original ABD3 [71]. By genetic fusion to ABD035, Guo *et al*. have successfully prolonged the half-life of HER2-specific immunotoxin Z_{HER2}-PE38 from 13.5 min to 330.8 min [72]. ABD035 endowed the human tumor necrosis factor-related apoptosis-inducing ligand (hTRAIL) with HSA binding affinity, and significantly extended the half-life of hTRAIL from 0.32 h to 14.1 h [73].

2.3.3. AlbudAb

GlaxoSmithKline (GSK) has developed the AlbudAb (albumin-binding domain antibody) platform on the basis of a human domain

antibody (dAb) that binds to HSA with a high affinity (Fig. **3D**). The dAb (11-13 kDa) is the smallest functional binding unit of human immunoglobulins, comprising only a single heavy or light chain variable domain [74]. The AlbudAb platform has been applied to significantly extend the half-lives of therapeutic peptides and proteins. GSK2374697, a genetically engineered fusion protein joining an AlbudAb to exendin-4, had a half-life of 6-10 days in human, and retained full GLP-1R agonist activity. A single dose of GSK2374697 reduced postprandial plasma glucose and insulin, and delayed gastric emptying [75]. GSK2374697 has completed phase 1 clinical trials (Table **2**). An IFN-α2b genetically fused to an AlbudAb resulted in longer half-life after s.c. injection (28.3 h *versus* 19.7 h) and higher potency compared with the HSA-IFN-α2b [74]. Extended half-lives of IL-1 receptor antagonist (IL-1ra) [76] and GLP-1 [77] were also obtained using the AlbudAb platform. The AlbudAb platform can therefore potentially be applied to generate a range of long-acting biopharmaceuticals with improved dosing regimens or clinical effects.

2.3.4. Nanobodies

Nanobodies are therapeutic proteins developed by Ablynx that are based on the smallest functional fragment of heavy chain-only (V_{HH}) antibodies, naturally occurring in *camelidae* (*e.g.* camels and llamas). Albumin-binding nanobodies have been developed for half-life extension [78] (Fig. **3E**). Currently, several therapeutic proteins with extended half-lives based on albumin-binding nanobodies are being tested in clinical trials (Table **2**). ALX-0061 is a half-life-extended bispecific therapeutic molecule comprising two nanobody domains; one is directed against IL-6R with high affinity and the other interacts with HSA. The half-life of ALX-0061 was 6.6 days, much longer than the non-half-life-extended domain (4.3 h) in cynomolgus monkeys. ALX-0061 also demonstrated dose-dependent and complete inhibition of IL-6-induced inflammatory parameters. ALX-0061 is currently in phase 2 clinical trials [79]. ATN-103 (Ozoralizumab) is composed of one nanobody domain directed against human TNF-alpha for rheumatoid arthritis treatment fused to the other domain targeting HSA [80]. ATN-103 has completed phase 2 clinical trials. ALX-0761/M1095 (MSB0010841), being developed for the treatment of auto-immune disorders, is a trivalent, bispecific biological agent that blocks both IL-17A and IL-17F with a high affinity for HSA, designed to prolong the *in vivo* serum half-life [81]. ALX-0761/M1095 has completed phase 1 clinical trials. ALX-0141 is also a trivalent, bispecific therapeutic molecule, with a half-life of 8.9 to 20.6 days depending on the injection dose in human. Two nanobody domains of ALX-0141 are designed to target receptor activator of nuclear factor kappa-B ligand (RANKL), and one nanobody domain targets HSA for half-life extension [82, 83]. ALX-0141 is being developed for the treatment of bone-loss related disorders, including osteoporosis and bone metastasis, and has completed phase 1 clinical trials. The modular assembly property of nanobody platforms enables us to tailor the valency, specificity and half-lives of peptides and proteins, making it a promising technology for the improvement of pharmacokinetics and pharmacodynamics.

2.3.5. DARPins

Designed ankyrin repeat proteins (DARPins) are small, single-domain proteins derived from natural ankyrin repeat proteins, which are the most abundant binding proteins found in the human genome [84]. DARPins usually consist of an N-capping repeat, several internal repeats, and a C-capping repeat, with typically 33 amino acids in each repeat. The consensus design approach was applied to build DARPin libraries as alternatives to antibody-based scaffolds. A DARPin library comprises fixed positions, important for maintaining the fold, and variable positions, which engage in interaction with targets [85]. Thus, DARPins can be evolved to bind a given target protein with high affinity and specificity through mutations of variable positions.

Ribosome display technology has been used frequently to select specific DARPins with high affinity to many different types of proteins, such as proteases, kinases, cytokines and membrane proteins [84]. Likewise, the serum albumin-binding DARPins exhibiting high thermal stability and high expression levels in *E.coli*, useful for pharmacokinetic engineering of protein therapeutics, were selected by ribosome display technology (Fig. **3E**). The DARPin platform is very suitable for the generation of multi-functional constructs. Genetically fusing a serum albumin-binding DARPin domain to domains with other target specificities resulted in a prolonged half-life ranging from 27 h to 80 h in mouse, and 2.6 days to 20 days in cynomolgus monkey [86]. MP0250 is a tri-specific DARPin construct consisting of N- and C-terminal DARPin domains binding serum albumin, one DARPin domain binding vascular endothelial growth factor-A (VEGF-A), and one DARPin domain binding hepatocyte growth factor (HGF). The serum albumin-DARPin in MP0250 resulted in improved pharmacokinetic properties, with a half-life of 30 h in mouse and 5 days in cynomolgus monkey. MP0250 was highly potent in inhibiting the two key growth factors simultaneously, and was highly stable, exhibiting a 2.4% loss of monomer peak over a 6-month period at 25°C. The pharmacokinetic profiles and pharmacologic activity suggest that MP0250 is a promising candidate for cancer therapy. MP0250 is currently in phase 2 clinical trials [87, 88]. MP0274 is a tri-specific HER-2-targeted DARPin drug candidate, binding to two distinct, non-overlapping HER-2 epitopes and to serum albumin for half-life extension. The pharmacokinetics study in cynomolgus monkeys showed a half-life of ≥5 days at doses of 5 and 10 mg/kg. MP0274 is currently in phase 1 clinical trials [89, 90]. These examples indicate that serum albumin-binding DARPins are valuable tools for producing next-generation protein drugs with extended half-lives.

2.4. Affibody Molecules

Besides the utilization of Fc or albumin as a carrier for FcRn binding, affibody molecules that bind directly to FcRn have been screened for half-life extension. Affibody molecules, which can be efficiently produced in bacteria, are a class of small affinity proteins with high thermostability and anti-enzymatic properties that can be used as tools for molecular recognition in diagnostic and therapeutic applications [91, 92]. Phage display technology was used to select affibody molecules that bound directly to FcRn in a pH-dependent fashion. The resultant $Z_{FcRn-16}$ bound to human FcRn with a high affinity at pH 6.0, but a low affinity at pH 7.4. Significantly, the half-life of construct containing $Z_{FcRn-16}$ was approximately 3-fold longer than that of the control protein [93]. This suggests that FcRn-binding affibody molecules could serve as fusion tags for extending the half-lives of biopharmaceuticals.

2.5. Antibody CDR3H Fusion

In the structure of bovine antibody BLV1H12, Zhang *et al*. found a disulfide cross-linked “knob domain” in a novel ultralong heavy-chain complementary-determining region 3 (CDR3H) structure. The bovine granulocyte colony-stimulating factor (bGCSF) was first substituted for the knob region, forming the fusion protein Ab-bGCSF. With this fusion protein, half-life of bGCSF (3 h) was prolonged to approximately 10 days in mice, with enhanced bioactivity. Meanwhile, other studies revealed that the half-lives of erythropoietin (EPO) and C-X-C chemokine receptor type 4 (CXCR4) peptide agonists were both prolonged by fusion to the CDR3H of bovine antibodies [94, 95]. To avoid occurrence of immunogenicity, Zhang *et al*. demonstrated that bovine antibody could be replaced by human or humanized antibody. Thus, a novel, versatile half-life extension approach was developed by exploiting the CDR3H of human or humanized antibodies. In this approach, hGH was genetically fused to the CDR3H of Herceptin, an FDA-approved, fully humanized anti-HER2 receptor monoclonal antibody with minimal immunogenicity, *via* a rigid, stable, antiparallel coiled-coil stalk motif (hGH-CDR3H-coil-Herceptin). The

Table 2. Examples of therapeutics in clinical trials with extended half-lives.

Drug	Peptide/Protein	Phase	Half-life Un-modified	Half-life Modified	Carrier	Indication	Company
CSL689	Factor VIIa	Phase 3 terminated (NCT02484638)	0.8 h (Rat)	5.1 h (Rat)	Albumin	Hemophilia	CSL Behring
Albutropin	Growth Hormone	Phase 3 terminated (NCT02410343)	3 h (Monkey)	15 h (Monkey)	Albumin	GH deficiency	Teva
CJC-1134-PC	Exendin-4	Phase 2 terminated (NCT01514149)	2.4 h (Human)	8 d (Human)	Albumin	Type 2 diabetes mellitus	ConjuChem
GSK2374697	Exendin-4	Phase 1 completed (NCT01545570)	2.4 h (Human)	6-10 d (Human)	AlbudAb	Type 2 diabetes mellitus	GSK
ALX-0061	Nanobody againsts IL-6R	Phase 2 (NCT02518620)	4.3 h (Monkey)	6.6 d (Monkey)	Nanobody	Rheumatoid arthritis	Ablynx
ATN-103	Nanobody againsts TNF-alpha	Phase 2 completed (NCT0103803)	Not determined (ND)	9.5-13.5 d (Human)	Nanobody	Rheumatoid arthritis	Ablynx
ALX-0761/M1095	Nanobody againsts IL-17A and IL-17F	Phase 1 completed (NCT02156466)	ND	ND	Nanobody	Auto-immune disorders	Merk
ALX-0141	Nanobody againsts RANKL	Phase 1 completed, as reported by company.	ND	8.9-20.6 d (Human)	Nanobody	Bone-loss related disorders	Ablynx
MP0250	DARPins binding VEGF-A and HGF	Phase 2 (NCT03418532)	ND	5 d (Monkey)	DARPins	Solid tumor	Molecular Partners AG
MP0274	DARPins binding HER-2 epitopes	Phase 1 (NCT03084926)	ND	5 d (Monkey)	DARPins	Breast cancer	Molecular Partners AG
MOD-4023	Growth Hormone	Phase 3 (NCT02968004)	3.4 h (Human)	37 h (Human)	CTP	GH deficiency	Opko Biologics
MOD-5014	Factor VIIa	Phase 1/2a (NCT02418793)	1.3 h (Rat)	6.5 h (Rat)	CTP	Hemophilia	Opko/Prolor
VRS-859	Exendin-4	Phase 1 completed, as reported by company.	2.4 h (Human)	128 h (Human)	XTEN	Type 2 diabetes mellitus	Diartis
VRS-317	Growth Hormone	Phase 3 terminated (NCT02413138)	3.4 h (Human)	131 h (Human)	XTEN	GH deficiency	Versartis
BIVV001	FVIIIFc-VWF	Phase 1 (NCT03205163)	13 h (Monkey)	25 h (Monkey)	XTEN	Hemophilia A	Bioverativ Therapeutics
PB1023	GLP-1	Phase 2b completed (NCT01658501)	1-2 min (Human)	36 h (Human)	ELP	Type 2 diabetes mellitus	PhaseBio
PB1046	Vasoactive intestinal peptide	Phase 2 (NCT03556020)	2 min (Human)	60 h (Human)	ELP	Pulmonary arterial hypertension	PhaseBio
PE0139 (Insumera)	Insulin	Phase 2a completed (NCT02581657)	ND	ND	ELP	Diabetes mellitus	PhaseBio
CNTO 528	EPO mimetic peptide	Phase 3 (NCT03158285)	ND	5.9 d (Human)	IgG-Fc	Anemia	Centocor
FP-1039	Extracellular domain of human fibroblast growth factor receptor 1c	Phase 1 completed (NCT00687505)	ND	3-5 d (Human)	IgG-Fc	Cancer	Five prime GSK
ACE-011 (Sotatercept)	Extracellular domain of human activin receptor type IIA (ActRIIA)	Phase 2 (NCT03496207)	ND	22-26 d (Human)	IgG-Fc	Myeloma	Acceleron Celgene

Note: NCT numbers refer to clinical trial identifiers on http://www.clinicaltrials.gov.

estimated half-life of hGH-CDR3H-coil-Herceptin after i.v. injection in SD rats was 47 h, approximately 100 times longer than the half-life of recombinant hGH (Genotropin, 0.5 h). Meanwhile, an increased and long-term *in vivo* efficacy was observed in rats. The hGH was then replaced by hLeptin, a construct denoted hLeptin-CDR3H-coil-Herceptin, resulting in a 24-fold longer half-life compared to the unmodified hLeptin in mice after i.v. injection [96]. Likewise, the half-life of exenatide was prolonged to four days in mice *via* the antibody fusion strategy, and the modified exenatide showed sustained control of blood glucose for more than a week in mice [95]. This indicates that long-acting therapeutic proteins can be developed using human or humanized antibodies by fusion to CDR3H region [96].

3. TRANSFERRIN FUSION

Transferrin (Tf) is a transport protein for transporting iron to control the levels of free iron in body fluids (679 amino acids; 80 kDa) [7, 97]. The half-life of Tf in circulation is 14-17 days for non-glycosylated form, and 7-10 days for glycosylated form [98]. The long half-life of Tf relies on a clathrin-dependent Tf receptor-mediated recycling mechanism which recycles Tf back into the circulation as well as on large molecular size [97]. Tf has been developed as a carrier for drug delivery, and it has been proven that Tf fusion proteins have improved pharmacodynamic and pharmacokinetic properties in animals when administered either orally or subcutaneously [98, 99].

The insulinotropic peptides GLP-1 and exendin-4 were genetically fused to non-glycosylated Tf, forming GLP-1-Tf and Ex-4-Tf fusion proteins. Both fusion proteins retained the pharmacological effects of native peptides while extending their length of action. GLP-1-Tf had a half-life of approximately 2 days in cynomolgus monkeys [98]. Proinsulin-transferrin (proINS-Tf), designed by fusing proinsulin to Tf, was converted to active INS-Tf through Tf receptor-mediated endocytosis in hepatic cells by specific cleavage of proinsulin. ProINS-Tf had a delayed but sustained *in vivo* hypoglycemic effect after s.c. injection, and had a 15-fold increase of half-life in diabetic mice. Interestingly, proINS-Tf only showed activation in the liver, not in muscle, decreasing the risk of hypoglycemia [100, 101]. Significantly, the half-lives of bifunctional Tf fusion proteins are determined not only by the affinity between the effector and the receptor, but also by the affinity between Tf and Tf receptor, as exemplified by GH-Tf and G-CSF-Tf fusion proteins. The results demonstrated that insertion of different linkers between Tf and GH or G-CSF altered the binding affinities of fusion proteins to both domain receptors, and that the half-lives of fusion proteins were greatly affected [102]. These applications demonstrate that Tf-based technology is a promising approach for extending the half-lives of peptides and proteins.

Recently, the oral bioactivity of Tf-fusion proteins was confirmed, suggesting that they may be promising candidates for orally delivered therapy. The oral bioactivity of proINS-Tf, expressed from the transgenic rice expression system, was determined in diabetic mice. The results showed that the blood glucose curve of orally administered proINS-Tf at a dose of 800 nmol/kg was similar to an s.c. injection at 22.5 nmol/kg [103]. The exendin-4-Tf fusion protein, expressed in a transgenic tobacco plant, was proven to significantly improve glucose tolerance when administered orally in mice [104]. G-CSF-Tf fusion protein significantly increased the absolute neutrophil counts after oral administration to mice, while G-CSF had no effect [99]. Although no Tf fusion proteins are currently in clinical trials, these observations provide an insight into the potential future development of orally administered fusion proteins based on Tf.

4. REDUCING RENAL CLEARANCE

Employing the receptor-mediated recycling system is one of the most successful strategies for half-life extension of peptides and proteins. However, the long half-lives of Fc or albumin fusion proteins depend not only on the receptor-mediated recycling system, but also on reduced renal clearance due to large molecular size. Reducing the rate of renal clearance, either through increasing the molecular size or increasing the negative surface charge, is another important tactic for half-life extension and has been applied widely. PEGylation is one of the most frequently used strategies for renal clearance reduction. More than ten PEG-modified drugs have been approved for the market. However, PEGylation has several drawbacks. PEGylation proteins have been observed to cause renal tubular vacuolation due to accumulation of the non-degradable PEG chain in the kidney. Additionally, immunogenicity of PEGylated therapeutics has been reported [105-107]. Therefore, to meet the need for long-acting drugs, alternative strategies have been extensively developed. A particular focus has been on strategies employing recombinant DNA, such as unstructured polypeptide XTEN, ELP (elastin-like polypeptide) and PAS (proline-alanine-serine), which shield the fusion partner by increasing its hydrodynamic volume. In addition, renal filtration can be slowed by increasing the negative surface charge of a peptide or protein through the introduction of glycosylation sites [7].

4.1. Recombinant Polypeptide

Because of the limitations of PEGylation, several new strategies have been developed as an alternative to chemical conjugation to PEG. The recombinant XTEN, ELP and PAS polypeptides are rationally designed homo-amino acids polymers that adopt extended conformations similar to PEG. The half-life of a biologically active molecule can be significantly extended by increasing its hydrodynamic volume through genetic fusion to an unstructured polypeptide (Fig. **3F**). Genetic fusion to polypeptides yields several advantages over the PEGylation: a more homogeneous end-product can be obtained; the resultant construct can be expressed in *E.coli* with high efficiency and low cost; the process of fusion protein purification is relative simple; and molecular half-life can be tuned by precisely adjusted polypeptide length [7, 108]. Because they possess these advantages, recombinant polypeptides fusion strategies have been developed extensively, and they have proven effective at extending the half-lives of peptides and proteins in clinical trials or in animal models.

4.1.1. XTEN

XTEN polymers, a class of unstructured biodegradable polypeptides developed by Amunix, consist of six hydrophilic, chemically stable amino acids A, E, G, P, S and T [18]. The XTEN polypeptide adopts an extended and unstructured conformation, resulting in abnormally large hydrodynamic volume [108]. For example, the 53 kDa XTEN576, containing 576 amino acids, eluted at essentially the same elution time as the 670 kDa thyroglobulin, and the 79 kDa XTEN864 corresponded to a 1.5 MDa globular protein when analyzed by size-exclusion chromatography. This indicates that the longer XTEN polypeptides contributes to larger hydrodynamic volumes, with the expectation of longer half-lives due to reduced rates of kidney clearance. In animals, the half-life of XTEN prolongs as the amount of amino acids (from 288 to 864) increases. The XTEN864 protein polymer exhibited about 10-fold longer half-life than XTEN288: 25 h *versus* 2.5 h in mice. Therefore, the half-life of a payload can be tuned by adjusting the length of the XTEN polymer. Furthermore, the XTEN polymer can completely degrade in cell homogenate of kidney, implying no accumulation in tissue. The XTEN polymer was found to have low immunogenicity and to be well-tolerated [18]. Consequently, the properties of XTEN polypeptides make it a promising half-life extension strategy for a variety of peptide- and protein-based therapeutics.

Currently, multiple clinical trials are in progress to test the safety and efficacy of the XTEN-modified therapeutics. VRS-859, constructed by genetic fusion of exenatide to the 864 amino acid

XTEN sequence (XTEN864), exhibited a slow absorption phase and a relatively flat peak before reaching a linear elimination phase after s.c. injection into monkeys. VRS-859 had a half-life of 128 h in human, allowing for once-monthly injection [57, 108]. VRS-859 has completed the phase 1 clinical trials for the treatment of T2DM (Table **2**) [18]. VRS-317 was being developed for use as a long-term replacement therapy in adults and children with growth hormone deficiency, designed by genetically fusing XTEN1 (83.6 kDa) to the N-terminal and XTEN2 (13.3 kDa) to the C-terminal of GH [109]. However, the phase 3 clinical trials of VRS-317 were terminated, because the primary endpoint of non-inferiority to daily therapy in the pediatric was not achieved (Table **2**). The half-life of VRS-317 (131 h in human) was much longer than that of other recombinant GH products, such as CTP-GH and MOD-4023 [110]. BIVV001 is a complex protein, uniquely constructed by fusing coagulation factor VIII to the Fc region, Von Willebrand factor (VWF) and XTEN polypetide (FVIIIFc-VWF-XTEN). BIVV001 showed 2-fold improvement in pharmacokinetic properties compared to rFVIII-Fc in cynomolgus monkeys, resulting in a dosing regimen of once weekly or less [111]. The BIVV001 is in phase 1 clinical trials. Additionally, the XTEN polymer is widely applied as a carrier and has successfully extended the half-lives of glucagon [112], GLP-2 [113], coagulation factors VIIa, VIII and IX, as well as others [18].

4.1.2. ELP

Elastin-like polypeptides (ELPs) are a class of recombinant polymers composed of short, repeating peptide motifs containing VPGXP, where X is a guest residue that can be any amino acid except proline [114]. ELP polymers are fully soluble in aqueous solution below the inverse transition temperature (T_t), and aggregate above the T_t. The T_t of ELP polymers can be adjusted by varying the guest residue and the length of the ELP [115]. This unique property of ELP polymers leads to the formation of a subcutaneous depot for the sustained release of biopharmaceuticals constructed by fusing the peptides or proteins to ELP polymers. Over time, the ELP fusion protein can be released from the depot, allowing the prolonged release of a high molecular weight drug into circulation [115]. Taking advantage of this property, ELP polymers have been used to control the half-lives and bioavailability of therapeutic peptides or proteins. ([G8E22]GLP)$_{X6}$-ELP$_{Low}$240 is constructed by fusing six repeats of GLP-1 molecules with two mutations (A8G and G22E) to an ELP polymer containing 240-VPGXG repeats with a T_t that is below body temperature. The ([G8E22]GLP)$_{X6}$-ELP$_{Low}$240 formed a stable depot after s.c. injection that was able to reduce blood glucose levels in mice for up to 5 days, 120 times longer than native GLP-1 [116, 117]. Recently, Luginbuhl *et al.* designed a GLP1-ELP$_{opt}$ fusion protein by optimizing the ELP molecular weight and T_t. GLP1-ELP$_{opt}$ controlled glucose for up to 10 days, the longest duration reported, in three different and challenging diabetic mouse models. GLP1-ELP$_{opt}$ also showed 17 days of sustained release in monkeys, superior to Tanzeum and Trulicity [118].

The above designs demonstrate the utility and flexibility of ELP polymers as a scaffold for drug delivery. Several ELP fusion proteins have been taken into clinical trials for efficacy verification. PB1023 is a GLP-1 analogue genetically fused to an ELP polymer, containing 636 amino acids expressed in *E.coli*, used as a once-weekly treatment for T2DM. In phase 1/2a clinical trials, PB1023 exhibited a slow, flat absorption profile with a sustained duration of exposure to support once weekly dosing. PB1023 has completed phase 2b clinical trials (Table **2**). PB1046, designed by the fusion of vasoactive intestinal peptide (VIP) to an ELP polymer, was highly resistant to enzymatic degradation and maintains drug levels over the course of one week, with a half-life of 60 h in human [119]. PB1046 is now in phase 2 clinical trials. PE0139 is a native insulin molecule genetically fused to an ELP polymer, resulting in a novel, super-long-acting basal insulin for treatment of hyperglycemia associated with diabetes, dosed as a once-weekly injection [120]. PE0139 has completed phase 2 clinical trials [7].

4.1.3. PAS

PAS polymers are a class of polypeptides composed of the small amino acids P, A and S. PAS polymers are hydrophilic, highly soluble, and are easily and efficiently biosynthesized. Like PEG, PAS polypeptides exhibit a disordered random coil conformation in aqueous solution, resulting in an expanded hydrodynamic volume. By size exclusion chromatography, a PAS polymer comprising 600 residues with a total mass of about 50 kDa corresponded to an effective size of more than 600 kDa. Furthermore, PAS polymers are resistant to serum proteases, while still being degradable by kidney proteases. The PAS polypeptide is non-toxic, lacks T-cell epitopes, and shows no signs of immunogenicity in animal experiments [121, 122].

PAS polypeptides have been applied to a wide range of therapeutic peptides and proteins, including hormones, cytokines, antibody fragments and enzymes [123]. Previous studies have indicated that the half-lives of biopharmaceuticals can also be easily adjusted to a specific application by varying the length of the PAS polymers. PAS polymers of 30-200 repeats in length were demonstrated to prolong the half-lives of proteins by 2- to 90-fold over the wild type proteins. For instance, the half-life of a Fab fragment bound to a PAS polypeptide was prolonged from 2.71 to 28.19 h when the length of PAS polypeptide was increased from 100 to 600 residues. All of the Fab-PAS combinations showed half-lives that were much longer than unmodified Fab with its half-life of 1.34 h in mice [121]. A 600-residue PAS polymer prolonged the half-life of YNSα8, an interferon superagonist, from 3 h to 30 h, with no detectable loss in biological potency in mice [124]. PAS600-leptin, designed by genetically fusing a 600-residue PAS polypeptide to leptin, had a 40-fold longer half-life and more potent bioactivity than unmodified leptin in mice [125]. Likewise, a PAS600-modified hGH showed a 94-fold longer half-life of 4.42 h with no bioactivity loss in mice, compared to unfused hGH (0.047 h) [121]. To date, more than 20 peptides or proteins have been modified with PAS polymers of different lengths by the company XL-protein. However, no PAS-modified biopharmaceuticals are in clinical trials [7, 121, 123].

4.1.4. Other Polypeptides

A few other structurally disordered polypeptides with expanded hydrodynamic volumes have been proposed for half-life extension, such as glycine-rich homo-amino-acid polypeptides (HAPs) and gelatin-like-protein polymers (GLKs). Schlapschy *et al.* designed HAPs based on the repetitive sequence (GGGGS)$_n$ with large hydrodynamic volumes. The anti-HER2 Fab fragment 4D5 was fused to a 200-residue HAP polypeptide, leading to a 3-fold longer half-life than that of the unfused Fab fragment [126]. An artificial 116-residue GLK polymer with an enlarged hydrodynamic radius was applied to prolong the half-life of G-CSF, resulting in an approximately 6-fold extension of half-life and improved neutrophilic hematopoiesis effects in rats [127].

4.2. Glycosylation and CTP

A vast number of studies have demonstrated that glycosylation, a common form of post-translational modification, can lead to improved the solubility, stability, and pharmacodynamic properties of proteins [128, 129]. The circulatory time of glycosylation proteins can be also prolonged, mainly through increases in molecular size, prevention of proteolytic degradation and increased sialic acid content [130]. Sialic acids are negative charged monosaccharides found on the terminus of glycans. Therapeutic peptides or proteins with negative surface charge are repulsed by negatively charged polysaccharides on membranes in the glomerular filter, resulting in slowed renal clearance and less rapid elimination [7, 19]. Therefore,

the negatively charged sialic acids, attached to the terminus of glycan chains, have been proven to play an important role in half-life extension for many glycoproteins with N- or O-glycosylation [2, 131, 132].

Half-life extension of peptides or proteins can be achieved by the introduction of additional N-glycosylation *via* site-directed mutagenesis. Aranesp (darbepoetin-α) is a hyperglycosylated, engineered EPO containing 5 amino acid changes (A30N, H32T, P87V, W88N, P90T), resulting in two extra N-glycosylation sites (5 in total) with increased sialic acid content. Darbepoetin-α had a 3.5-fold longer half-life and is 13- to 14-fold more potent than recombinant EPO in dogs [133]. The half-life of darbepoetin-α was determined to be 70 hours in patients with chronic kidney disease [134]. Darbepoetin-α was approved by the FDA for the treatment of anemia in patients with chronic renal failure. It could be administered once every two weeks, but even monthly administration was effective [135]. Flintegaard *et al.* introduced three N-glycosylation sites into the GH sequence (3N-GH). The terminal half-life of 3N-GH with the most pronounced sialylation was 24-fold prolonged compared with the wild-type GH, 13-fold prolonged for that of less sialylated in rats. And the less sialylated 3N-GH exhibited a profound pharmacodynamic effect in GH-deficient rats. Thus, the sialic acids play a pivotal role for the properties of glycosylated GH [19].

Besides site-directed mutagenesis, sialic acid content can be increased to extend the half-lives of proteins by introduction of modules containing glycosylation sites. Previous studies found that the half-life of human chorionic gonadotropin (hCG) is significantly longer than that of other glycoprotein hormones, such as follicle-stimulating hormone (FSH), luteinizing hormone (LH) and thyroid stimulating hormone (TSH). It was confirmed that a carboxyl-terminal peptide (CTP) with four O-glycosylation sites, ended with sialic acid, extended the half-life of hCG because its negative charge decreased glomerular filtration [132]. Therefore, the CTP can be used as a module fused to the terminals of proteins for half-life extension (Fig. **3G**). Some studies have demonstrated that fusing CTP to FSH, TSH, EPO, and GH did not affect receptor binding affinity or *in vitro* bioactivity, but substantially increased half-life [136].

Corifollitropin-α (FSH-CTP) was the first CTP fusion protein approved by the EMA in 2010 for the treatment of infertility in women (Table **1**). FSH-CTP was constructed by fusion of CTP to the β-subunit of FSH to increase four O-glycosylation sites, resulting in a 2- to 3-fold prolongation of the *in vivo* half-life compared with recombinant FSH [137, 138]. MOD-4023 is a novel, long-acting version of GH, generated by fusing three CTP sequences to the N- and C-terminals of GH (CTP-GH-CTP-CTP) [139]. The half-life of MOD-4023 in human was about 12-fold longer than recombinant GH (3.4 h). MOD-4023 is currently in phase 3 clinical trials for the treatment of GH deficiency with a once-weekly dosing regimen (Table **2**) [140]. MOD-5014, a coagulation factor VIIa-CTP fusion protein, is in phase 2a clinical trials for the treatment of bleeding episodes with hemophilia A or B [7].

Additionally, EPO has been further modified by fusing CTP to its C-terminal (EPO-CTP). The half-life of EPO-CTP was increased 2- to 3-fold compared to wild type EPO, and the *in vivo* potency was significantly enhanced [141]. A new analog, CTP-EPO-CTP-CTP, obtained by fusing three CTPs to the C- and N-terminals of EPO, was also designed. The estimated half-lives of wild type EPO, Aranesp, and CTP-EPO-CTP-CTP were 4.4 h, 10.8 h and 13.1 h in mice, respectively. The addition of CTP sequences significantly increased *in vivo* potency [142]. Furthermore, the pharmacokinetic and pharmacodynamic properties of coagulation factor IX, INF-β and oxyntomodulin were indicated to be improved by CTP modification [7].

5. POTENTIAL ISSUES

PEGylation has been applied widely for half-life extension of therapeutic peptides and proteins since 1990, when the first PEG-modified adenosine deaminase (Adagen) was approved by the FDA. However, the problems of nondegradation and immunogenicity have received growing attention. There is, therefore, an urgent need for the development of alternative, safer strategies for the prolongation of the half-lives of therapeutic peptides and proteins. Genetic fusion technology based on recombinant DNA, such as Fc- or albumin-fusion, makes these strategies safer, more effective and diverse, and promotes the development of biopharmaceuticals.

The strategy of genetic fusion has many advantages. In general, the genetic fusion facilitates manufacturing, as it yields homogeneous products. The process of recombinant protein purification is also relatively simple, avoiding the additional steps of conjugation and subsequent downstream purification. In addition, recombinant production solves source availability problems, and is considered to be a bio-safe and green process [143]. Furthermore, the half-life of a peptide or protein can be tuned by modification of carrier sequence. As mentioned above, the increased affinity of Fc or albumin to FcRn by point mutation contributed to longer half-lives of Fc- and albumin-fusion proteins. The half-life can also be controlled by precisely adjusting the length of a bound polypeptide, such as XTEN, ELP and PAS.

Although fusion proteins have many advantages, some limitations still exist. Therapeutic proteins based on the Fc-, albumin- or CTP-fusion strategies must be expressed in mammalian cells or yeast cells (albumin-fusion) with high associated costs and long production periods. Other carriers, expressed in *E.coli*, can not be applied to modify some complicated or glycosylated proteins. Moreover, fusion proteins modified through the reviewed strategies are usually of large molecular size, making them unable to penetrate into the blood-brain barrier (BBB), and thereby restricting the application of the drugs targeting to central nervous system [144]. Additionally, the most common administration route of fusion proteins is currently intravenous injection (i.v.) or subcutaneous injection (s.c.), which is expensive, painful and inconvenient. Therefore, a great efforts have been made to design and develop alternative administration routes, such as transdermal delivery, pulmonary delivery and oral delivery [145]. Semaglutide is the first oral GLP-1 analog, taken once daily as a tablet. Semaglutide was formulated with the absorption enhancer sodium N-[8-(2-hydroxybenzoyl) amino] caprylate (SNAC), developed by Emisphere Technologies, Inc. Clinical trials demonstrated that the oral semaglutide resulted in better glycemic control than placebo over 26 weeks [146]. Finally, the genetic fusion process may result in low production, low solubility or lack of stability. Thus, rational design and optimization is essential.

Aside from these limitations, immunogenicity and bioactivity of the fusion proteins are two important issues that must be considered. The carriers of peptides or proteins responsible for half-life extension mainly come from naturally occurring proteins in human, such as albumin, IgG-Fc and transferrin. However, with the trend of rapid developments of advanced genetic engineering and protein engineering technologies over the past decades, it is clear that therapeutic proteins will be more extensively modified or engineered in the future by protein engineering and computer design. Artificially designed polymers, such as XTEN, ELP and PAS, are emerging and developing rapidly. Domains from other species have also been successfully used for half-life extension; such are the cases with ABD, DARPins, and nanobodies. In light of these technologies, one or multiple domains of interest from different proteins or different species may be fine-tuned and rationally designed to construct a new protein that targets one or many receptors and has an enhanced half-life and bioactivity. These novel proteins are never found in nature. Thus, their immunogenicity, a critical safety concern, needs to be determined. The amino acid sequence, solubil-

ity, stability, formulation, aggregation tendency, and route of administration are key factors that must be carefully considered for the minimization of immunogenicity.

Bioactivity of a long-acting protein can be tuned. Direct fusion of functional modules to a carrier without a linker may result in loss of bioactivity, due to steric hindrance. In fusion proteins, the length and the flexibility of the linker affect the spatial distance between the functional modules and the carrier, which in turn influences the steric hindrance. The linker thus plays a crucial role in the production of therapeutic peptides and proteins, affecting half-life length and retention of bioactivity, and needs to be evaluated and selected carefully.

A deep understanding, then, of the relationship between protein structure and function, between receptor and ligand, and of the molecular mechanism of disease, will be greatly beneficial for the rational design of next-generation fusion proteins or biobetters.

Besides the genetic fusion, other strategies for half-life extension are under constant development. The half-life of a peptide or protein can be prolonged through lipidation, for instance, conjugating with HSA by a fatty acid chain in a non-covalent manner [58]. Chemical conjugation to a macromolecular carrier is another promising strategy for improving the pharmacokinetics of a peptide or protein, using hydroxyethyl starch (HES), poly(N-vinylpyrrolidone) (PVP) or polyglycerol (PG) [147]. In addition, the controlled release of a biopharmaceutical can be achieved by encapsulation into nanoparticles, such as PEGylated nanoparticles, poly(lactic-co-glycolic acid) (PLGA) nanoparticles or poly(lactic acid) (PLA) nanoparticles [148, 149].

CONCLUSION

With the exception of PEGylation, we have reviewed current half-life extension strategies, including those that are already approved, as well as those still in a clinical or preclinical stage. The FcRn is the most successful target for half-life extension based on the FcRn-mediated recycling mechanism. There are eighteen long-acting biopharmaceuticals exploiting the IgG-Fc or HSA-binding strategy that have been approved for treatment. Another two approved, long-acting biodrugs (Elonva and Aranesp) were constructed by the introduction of glycosylation sites *via* site-directed mutagenesis or CTP fusion. Other fusion strategies, utilizing transferrin, AbudAb, nanobodies, DARPins, XTEN, ELP, and PAS, still require further testing in clinical trials to establish their efficacy and safety.

In the long term, although many novel half-life extension strategies are still at a preclinical stage, it is to be expected that an increasing number of these technologies will overcome the obstacles of development and be evaluated in clinical trials in the future. It can also be anticipated that even more advanced strategies to extend the half-lives and improve the pharmacokinetic and pharmacodynamic properties of therapeutic peptides and proteins will emerge in the future. These, too, will benefit patients by reducing the required frequency of injections.

LIST OF ABBREVIATION

GLP-1	=	Glucagon-like Peptide-1
DPP-4	=	Dipeptidyl Peptidase-4
FcRn	=	Neonatal Fc Receptor
TPO	=	Thrombopoietin
EPO	=	Erythropoietin
TNF	=	Tumor Necrosis Factor
IL-1	=	Interleukin-1
VEGF	=	Vascular Endothelial Growth Factor
CTLA-4	=	Cytotoxic T-lymphocyte-associated Protein-4
LFA-3	=	Lymphocyte function-associated antigen 3
HSA	=	Human Serum Albumin
GH	=	Growth Hormone
IGF-1	=	Insulin-like Growth Factor 1
T2DM	=	Type 2 Diabetes Millitus
ABD	=	Albumin-binding Domains
AlbudAb	=	Albumin-binding Domain Antibody
DARPins	=	Designed Ankyrin Repeat Proteins
CDR3H	=	Complementary-determining Region 3
GCSF	=	Granulocyte Colony-stimulating Factor
Tf	=	Transferrin
ELPs	=	Elastin-like Polypeptides
PAS	=	Proline-alanine-serine
HAPs	=	Homo-amino-acid Polypeptides
GLKs	=	Gelatin-like-protein Polymers
FSH	=	Follicle-stimulating Hormone
CTP	=	Carboxyl-terminal Peptide

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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