Journal of Controlled Release 301 (2019) 176–189

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Review article

Current strategies in extending half-lives of therapeutic proteins

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ABSTRACT

Macromolecular protein and peptide therapeutics have been proven to be effective in treating critical human diseases precisely. Thanks to biotechnological advancement, a huge number of proteins and peptide therapeutics were made their way to pharmaceutical market in past few decades. However, one of the biggest challenges to be addressed for protein therapeutics during clinical application is their fast degradation in serum and quick elimination owing to enzymatic degradation, renal clearance, liver metabolism and immunogenicity, attributing to the short half-lives. Size and hydrophobicity of protein molecules make them prone to kidney filtration and liver metabolism. On the other hand, proteasomes responsible for protein destruction possess the capability of specifically recognizing almost all kinds of foreign proteins while avoiding any unwanted destruction of cellular components. At present almost all protein-based drug formulations available in market are administered intravenously (IV) or subcutaneously (SC) with high dosing at frequent interval, eventually creating dose-fluctuation-related complications and reducing patient compliance vastly. Therefore, artificially increasing the therapeutic half-life of a protein by attaching to it a molecule that increases the overall size (eg, PEG) or helps with receptor mediated recycling (eg, albumin), or manipulating amino acid chain in a way that makes it more prone towards aggregate formation, are some of the revolutionary approaches to avoid the fast degradation *in vivo*. Half-life extension technologies that are capable of dramatically enhancing half-lives of proteins in circulation (2–100 folds) and thus improving their overall pharmacokinetic (PK) parameters have been successfully applied on a wide range of protein therapeutics from hormones and enzymes, growth factor, clotting factor to interferon. The focus of the review is to assess the technological advancements made so far in enhancing circulatory half-lives and improving therapeutic potency of proteins.

1. Introduction

Introduction of peptides/proteins as bio-therapeutics is probably one of the biggest successes in the field of modern health science. Since insulin was used as the first therapeutic protein in clinical setting, the field of protein therapeutics has rapidly expanded with development of hormones, enzymes, clotting factors and antibodies [1]. At present a number of potential peptide drugs are under investigation for clinical uses. One of the greatest challenges to be addressed in peptide and protein therapeutic clinical administration is the fast degradation as a result of short half-lives of protein drugs. Due to rapid renal clearance and obvious enzymatic activity while in systemic circulation, protein molecules usually have short residence time in blood [2]. Thus, injection of a higher dose is usually required to maintain a therapeutic concentration for a desired period of time in serum environment. However, fluctuating concentration of a therapeutic protein with a high initial peak creates side effects. Besides, a number of proteins have very narrow therapeutic range. If the plasma half-life of a therapeutic protein could be extended, it would address both of these issues, thus, longer action can be maintained with lower doses [1].

Abbreviations: GRAS, Generally Regarded as Safe; GIT, Gastro-Intestinal track; IV, intravenous; SC, subcutaneous; IM, intramuscular; MW, molecular weight; PEG, polyethylene glycol; hours, hrs; Minute, min; G-CSF, granulocyte colony-stimulating factor; ADA, adenosine deaminase; FVIII, factor VIII; FIX, factor IX; Co_3AP, carbon apatite; CPA, carboxypeptidase A; PAS, porline, alanine and serine; ELP, elastin-like polypeptides; GLK, gelatin-like protein; GLP-1, glucagon-like peptide-1; ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity; Tf, transferrin fusion; T_{max}, the time it takes for a drug or other substance to reach the maximum concentration C_{max}; PSA, polysialic acid; CA, colomic acid; HES, hydroxyethyl starch; HEP, heparosan; HA, hyaluronic acid; PK, pharmacokinetics; PD, pharmacodynamics; PLGA, Poly-Lactic-Co-Glycolic Acid; kDa, kilo Dalton

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https://doi.org/10.1016/j.jconrel.2019.02.016
Received 12 January 2019; Received in revised form 12 February 2019; Accepted 13 February 2019
Available online 05 March 2019
0168-3659/

Over the past years, there have been tremendous efforts in development of protein half-life extension technologies. A number of technologies have been proposed and tested for long acting formulation of therapeutic proteins, like, amino acid alteration, genetic fusion of other proteins (immunoglobulin domains or serum proteins, like, albumin), conjugation with polymers (polysialylation, HESylation, PEGylation etc) [3]. These technologies can be divided in 4 major categories: amino acid manipulation, bio-conjugation, post-translational modification by attaching the peptide to polymers (natural/synthetic) and carrier mediated delivery. The first 3 technologies are based on half-life and circulation time modulation, whereas carrier mediated delivery concept is based on creating some kind of depot and slowly releasing the therapeutic in controlled and sustained manner in the circulation, thus enabling to form long-acting formulations.

2. Mechanism of protein regulation *in vivo* and recognition of "non-self" proteins to affect protein half-life

A main concern about protein therapeutic administration is the high elimination rate. Protein molecules are removed from the circulation if they are recognized as toxic to cells due to mis-folding or damage; also regulatory proteins are removed from circulation to maintain their level. Cell removes any foreign protein that it cannot recognize as "self". Proteins, both endogenous and foreign can be degraded due to adaptive immune response [4]. Recognizing a foreign protein is crucial yet very challenging job for cellular proteasomes. To select all the foreign proteins, cellular proteasome need to be tricky. Here, proteasome cannot be sequence specific so that any single proteasome unit can work on a diverse range of different proteins. On the other hand, it has to be selective and discriminatory to avoid any unwanted destruction of cellular component [4]. How proteasomes do that is not fully understood yet.

3. Factors determining protein half-life in circulation

Apart from monoclonal antibodies having week-long serum half-lives, mostly protein therapeutics get eliminated from body very fast [1]. Short half-life and quick elimination is the result of protein pharmacokinetic properties [5]. Rate of protein elimination is dependent on few factors like, size, molecular weight and surface charge [1,6]. Thus success of a half-life extension strategy is dependent on whether the technique can manipulate one or more of these factors. Protein elimination occurs by enzymatic degradation, renal clearance and hepatic metabolism.

Size of the protein and their hydrophobicity make therapeutics prone to kidney filtration and liver metabolism. An example, kidney filtration cut off size for a peptide is < 70 kDa; that is, a peptide smaller than that will easily get cleared by kidney filtration system [5]. Renal clearance rate can be reduced by increasing protein size (hydrodynamic volume) and molecular weight. One way of doing that is by attaching protein to a moiety, such as, polyethylene glycol (PEG). On the other hand, molecules with negative surface charge are able to avoid renal clearance by repelling the negative surface charge of basement membrane. This makes protein surface modification another option for half-life extension. During metabolism by liver, proteins are taken by hepatocytes. The uptake is in general receptor mediated. The protein molecules then get degraded in lysosome by enzymes and cleared out of circulation, like other proteins. However there are endogenous proteins present in circulation that can avoid liver metabolism by specific receptor mediated recycling. Most studies of this kind focused on FcRn receptor coupled recycling of albumin and IgG. And it was observed that binding of therapeutic protein to albumin or Fc portion of IgG helps target protein recycle back to the circulation, just like their moieties. One of the first half-life extended protein therapeutics that was designed using albumin conjugation is insulin detemir, a long acting insulin analog [6]. Fig. 1 is a diagrammatic representation for half-life extension strategies by avoiding renal clearance and receptor mediated recycling.

Enzymatic degradation which is probably the biggest factor in determining protein half-life, can be site specific, or non-site specific taking place in any part of the body independent of any specific organ. Also it can be intracellular or extracellular. Enzymatic degradation is dependent on size as well as secondary and tertiary structure of a protein. Also level of glycosylation affects the process. Extracellular degradation of therapeutic proteins is same as degradation of endogenous or dietary proteins, while intracellular degradation usually happens in lysosomes [6]. One of the few strategies for preventing extracellular degradation of a protein in GIT or other organs is masking the target protein with the help of a moiety (like, PEG) or incorporating inside a cover or matrix made of a complex drug delivery system that can protect enzymatic action. One example is modified carbon apatite-mediated delivery attempt of insulin [7,8]. Fig. 2 summarizes the mechanisms that limit protein residence time in circulation with few examples to address them.

4. Approaches to overcome *in vivo* instability of protein therapeutics

A number of strategies have been found effective for half-life extension of therapeutic proteins and peptides. The technologies fall in few categories:

- Amino acid manipulation- insertion/deletion or alteration of one or few amino acids in peptide chain has been proven to reduce immunogenicity and proteolytic instability *in vivo* [3].
- Bio-conjugation- Conjugation of peptide with serum proteins, such as albumin or immunoglobulin. Genetic engineering approach can also be applied to produce such fusion proteins [3,5].
- Post-translational modification- attaching the peptide to polymers (natural/synthetic). A very good example is PEGylation.
- Carrier mediated delivery- attaching a carrier to protect the peptide molecule *in vivo* environment. Some carriers are capable of slow and sustained release of therapeutics in serum. Carrier could be a microsphere, liposome, matrix and micro−/nanoparticle [3].

Over the years, a number of technologies surfaced based on these strategies. Fig. 3 summarizes the protein half-life extension strategies reported till now.

5. Attaching the therapeutic protein/peptide to a polymer

5.1. PEGylation

PEGylation, a very popular technology for modification of proteins, was introduced in early 70s [9–11]. Peptide chain is covalently attached to one or more high molecular weight chains of PEG [1], a molecule built of repetitive units of ethylene oxide (CH_2-CH_2-O) [12]. This elongation works by hiding antigenic determinant of the peptide so that antibodies cannot recognize it as a foreign body, and increasing protein size so that it won't be cleared out by kidneys very easily [1,11]. PEG itself is an uncharged hydrophilic polymer which is non-biodegradable, mostly non-toxic and non-immunogenic. Highly flexible PEG molecules can be easily attached to a therapeutic molecule [5]. PEGylation also helps absorption enhancement by making the proteins more water soluble, and masks the molecules from proteolytic degradation [11].

First generation PEGylation used linear chain of PEG attached to peptide while 2nd generation came up with the idea of branched chain PEG and PEG derivative attachment [12]. Over the years far better PEGylation strategies were developed to reduce non-specific activity of peptides as well as increase their stability. There are > 10 peptide therapeutics already in the market, with a number of potential drugs

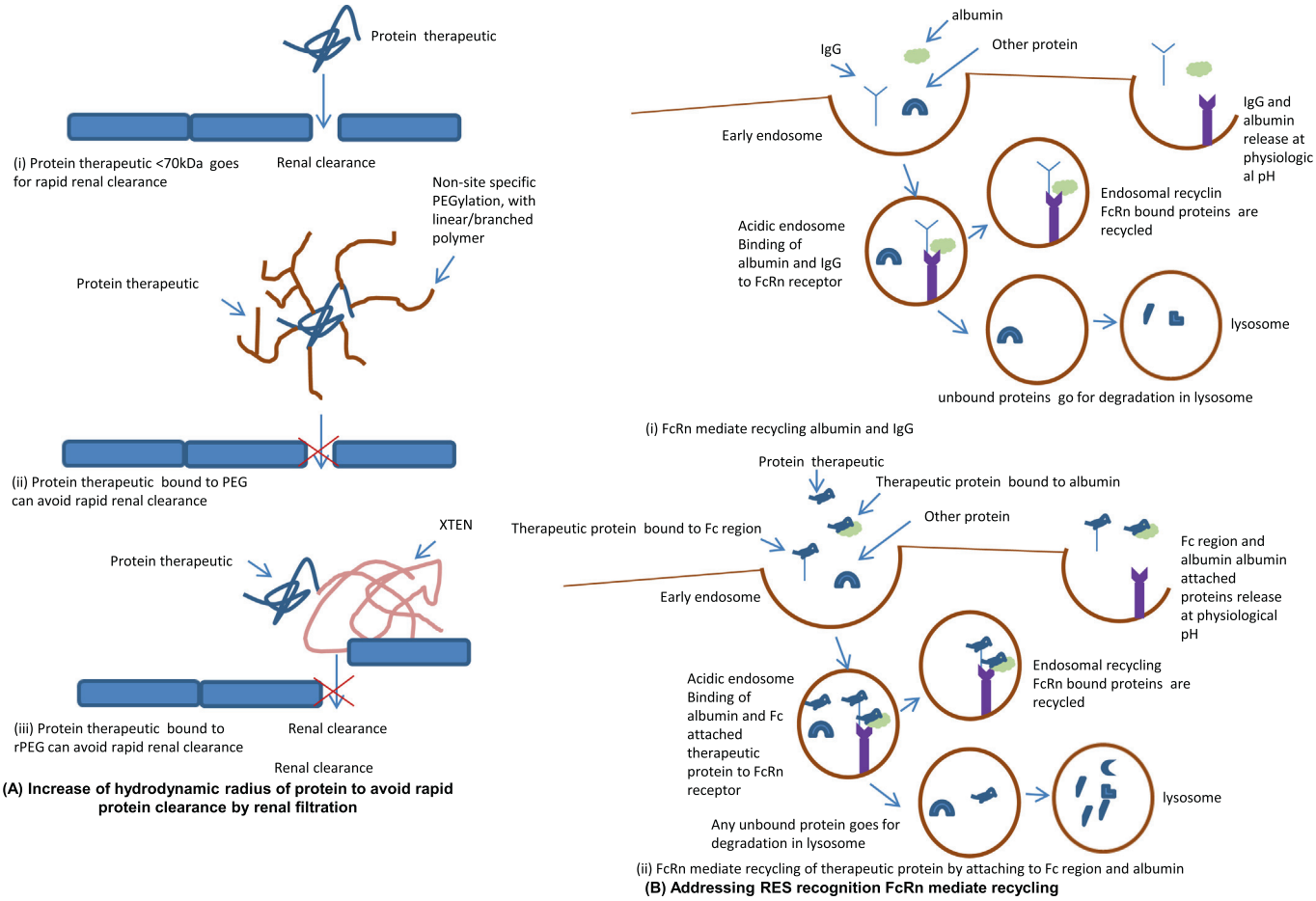

Fig. 1. Different protein half-life extension strategies: avoidance of rapid renal clearance and receptor mediated recycling. (A) Shows few strategies to avoid renal clearance. Protein molecule < 70 kDa undergoes renal clearance very fast (A.i). A good strategy to avoid that is to increase hydrodynamic radius of a protein by attaching it to another molecule. A very good example is PEGylation (A.ii). A polymer, PEG (liner/branched) can be attached to a peptide chain. The attachment could be site specific (not shown in the picture) or non-site specific. The attachment helps protein avoid elimination by kidney. XTEN, also sometimes called as rPEG, is another example (A.iii). This peptide polymer also attaches itself to protein therapeutic and increases its size. Fig. B shows a classic example of receptor mediated recycling of protein. (B.i) Proteins get eliminated from circulation by recognition and uptake by body immune cells thus gets degraded in lysosome, with exception of two unrelated proteins IgG and albumin that share a similar mechanism of FcRn receptor bound recycling. (B.ii) Therapeutic proteins bound to albumin and Fc portion of IgG take advantage of FcRn receptor recognition to recycle back to circulation.

designed based on this technology currently under clinical trial [13]. The very first works of Frank Davis and coworkers in '70s generated first commercial PEGylated form of protein drug, modified adenosine deaminase (ADA) [11,14,15]. Enzyme adenosine deaminase (ADA) is used to treat severe combined immunodeficiency disease (SCID) mainly in young adults [15,16]. Like most of the enzymes, ADA has a very short plasma half-life of few minutes. Conjugating PEG to bovine ADA prolonged its half-life to 24 h in mice. In subsequent studies, PEG-ADA was tested on two children by intramuscular (IM) injection. The enzyme was found to be absorbed rapidly from injection site to plasma, showing an elongated half-life of 48–72 h. Once a week administration of 15 U/kg body weight of the enzyme successfully maintained a plasma ADA activity which is 2–3folds higher than erythocyte ADA activity in normal subjects [11,15].

Filgrastim is a recombinant methionyl human granulocyte colony-stimulating factor (G-CSF) (rmetHuG-CSF) required to stimulate the supply of neutrophil during chemotherapy to prevent febrile neutropenia (FN). Previously, it was administrated as daily injections. A PEGylated form of filgrastim is Pegfilgrastim which requires only once-per-cycle administration [17,18]. During animal study, Pegfilgrastim boosted and sustained neutrophil count in both normal and neutropenic mice. A single dose of Pegfilgrastim in normal mice elevated neutrophil count to 15.5×10^9/L. This result is equivalent to regular doses of filgrastim twice daily for 4 days. The elevated level of neutrophil was maintained for 5 days. Similarly, increased level of neutrophil was observed in neutropenic mice and myelo-suppressed rhesus monkeys [19]. Pegfilgrastim has increased size because of PEG moiety and does not get cleared by kidney easily. Extended serum half-life of pegfilgrastim is 42 h compared to 3.5–3.8 h of half-life of Filgrastim [17].

Another noted success using PEGylation technology was long-lasting hemophilia drugs. Hemophilia is a rare blood disorder due to clotting factor deficiency. Therefore, it is treated with recombinant factor VIII products. Products available in market had half-lives of 12 h and needed at least 3 injections per week [20]. FDA approved PEGylated anti-hemophilia agent, Adynovate (PEG-FVIII) has extended the half-life to 13.4–14.7 h [20]. Similarly, a phase III clinical product with PEGylation technology, like BAY94-9027 from Bayer's has half-life of 19 h. Likewise is Baxalta's Bax855. A list of PEG-conjugated therapeutics available in the market and under clinical trials are provided in Table 1.

PEGylation is considered as the most popular protein half-life extension technology. FDA recognizes PEG as GRAS (generally recognized as safe). However, PEG is not 100% safe, since it is non-biodegradable, immunogenic and shown to create vacuoles in renal cortical tubular epithelium cells [5].

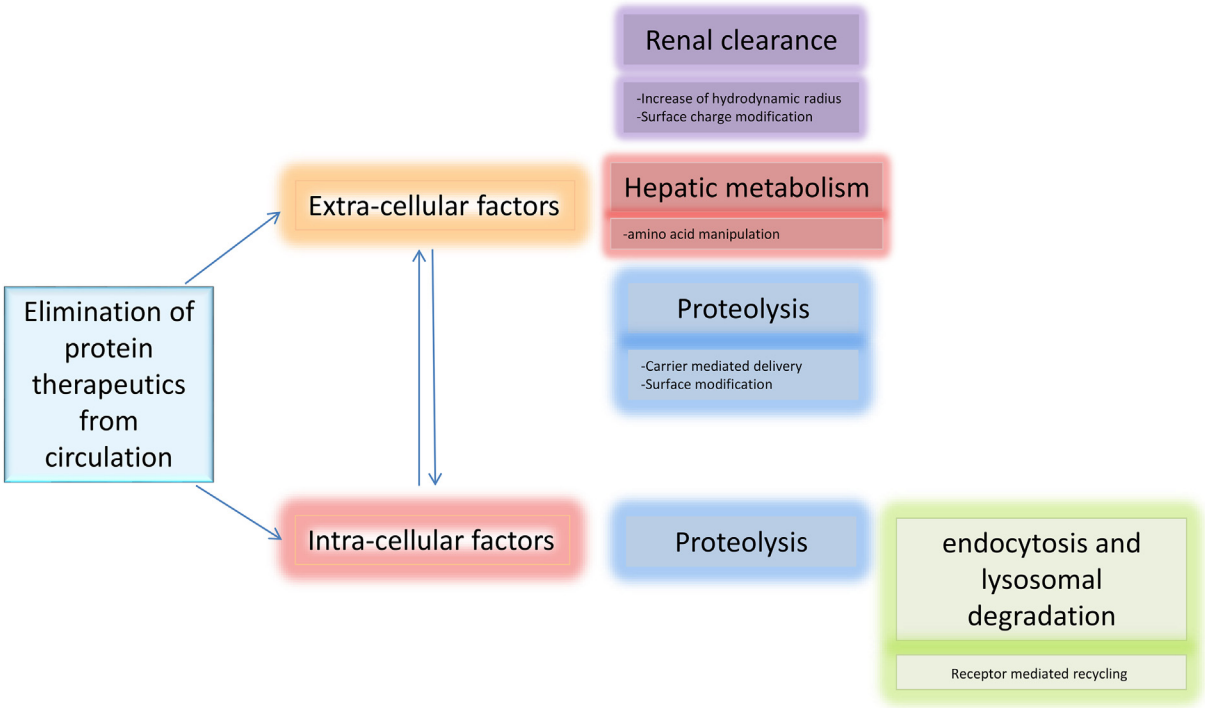

Fig. 2. Factors that limit residence time of protein in circulation and a few ways to address them.

5.2. Modified PEGylation

Over the decades there have been efforts to further develop PEG-conjugated therapeutics by modifying and conjugating PEG to other moieties. Conjugating PEG to another molecule is relatively new approach. So far no product was commercialized, but there have been several reports revealing their potential.

5.2.1. Glyco-PEGylation

Conventionally PEG is attached to a protein covalently by attaching to the reactive groups on amino acids. The new approach of "Glyco-PEGylation" approach attaches PEG to O-glycans. Thus it is "site directed PEGylation" by glycosyltransferases. The method claims to minimize isoform formations as well as enhances PEG performance on half-life [36]. Different therapeutic peptides were conjugated and shown to increase the circulatory half-life [36]. Table 2 summarizes efficiency of Glyco-PEGylated formulations compared to their native and PEGylated counterparts.

5.2.2. Biotinylated PEG

A modified version of PEG was developed by conjugating PEG to streptavidin very recently [39]. Biotinylated PEG was applied for surface modification of carbon apatite nanoparticle (Co_3AP NP) carrying Gemcitabine. Biotinylated PEG was found to enhance residence time of Co_3AP-Gemcitabine complex in blood circulation in mice model [39]. Earlier, in another study S. Ke, John C. Wright and G S. Kwon (2007)

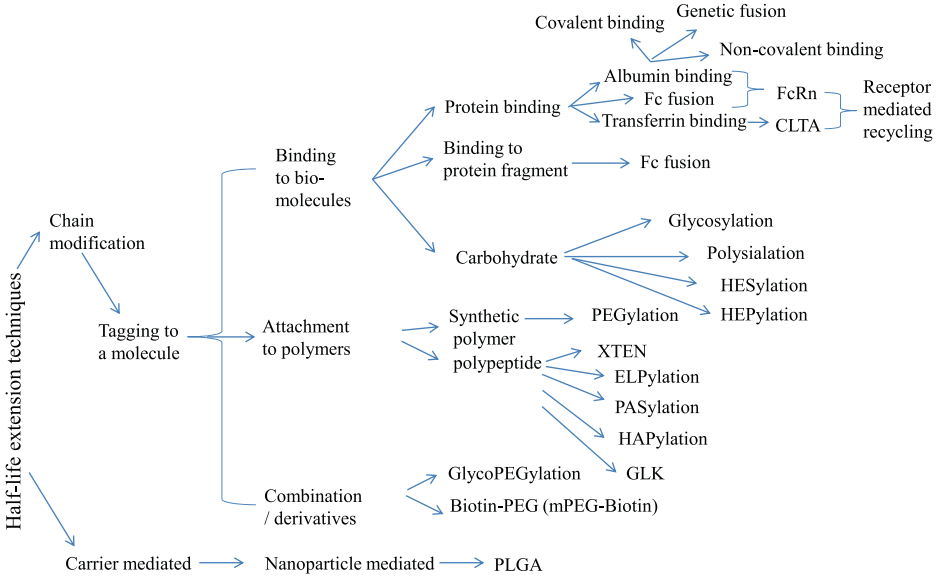

Fig. 3. Protein half-life extension techniques at a glance.

Table 1
Efficiency of PEGylated therapeutics available in market and under clinical and pre-clinical trials compared to the native peptide formulations.

Protein therapeutics	Plasma half-life	PEGylated formulations (patent name)	Plasma half-life	animal model/pre-/clinical trial	Ref.
Filgrastim recombinant human granulocyte colony-stimulating factor (G-CSF) (rmetHuG-CSF)	3.5–3.8 h	Pegfilgrastim (PEG-rmetHuG-CSF, Neulasta®)	42 h	In market	[5,17,19]
Recombinant factor VIII	12 h	Adynovate (PEG-FVIII)	13.4–14.7 h	In market	[13,20]
Recombinant factor VIII	12 h	BAY94–9027	19 h	PhaseIII	[13,20]
Enzyme adenosine deaminase (ADA)	few min	Adagen®Pegademase bovine	48-72 h; 3-6 dys	In market	[15,21,22]
L-asparaginase	34 ± 8 h	PEGylated L-asparaginase (Oncaspar®; Enzon)	357 ± 243 h	In market	[21,23,24]
Erythropoietin	> 24 h	Mycera®	142 h	In market	[5,25,26]
alfa-2a	5 h	PEGASYS (Roche)	160 h	In market	[16,21,24,27]
alfa-2b	2.3 h	PegIntron	4.6 h	In market	[24,28]
Erythropoietin mimicking novel protein	……	Peginastide (brand name: Hematide/Omontys)	21.5 h 59.7 h	In market	[16,24]
Synthetic integrin-binding peptide	28 min	HM3	162 min	rat	[16]
Recombinant growth hormone (GH)	4–5 min	NNC126–0083 (Novo Nordisk)	47.6 h	Phase I	[7,29–31]
GH	4–5 min	Somavert/ pegvisomant	6 days	In market	[24,30–32]
GLP-1	2 min	PEG- biotinylated GLP-1 (DBP-GLP-1)	Elongated half-life. Activity for 3 h	preclinical	[7,33]
Rasburicase (recombinant) Urate oxidase	8 h	Krystexxa	10–12 dys; application just once every 2 to 4 wks	In market	[24,34]
Tumour necrosis factor receptor type I	…..	r-Hu-sTNF-RI	3–29 h	Preclinical: Chimpanzee	[35]

Table 2
Efficiency of potential Glyco-PEGylated therapeutics under pre-clinical and clinical trials compared to the native and PEGylated peptide formulations.

	Half-life			Glyco-PEGylated formulations (patent name)	Animal model/clinical trial	Ref
	Native peptide	PEGylated peptide	Glyco-PEGylated peptide			
G-CSF	1.71 h	7.74 h	8.78 h	…….	Rats	[36]
IFN-α2b	2.3 h	–	± 18 h	…….	Rats	[36]
FVIII	12 h	13.4–14.7 h; 19 h	19 h; 1.6 fold	N8-GP	PhaseIII	[13,20,37]
FIX	18–20 h	–	93 h	N9-GP	PhaseIII	[13,20,38]

used bovine carboxypeptidase A (CPA) as target enzyme and successfully prepared few different formulations of avidin-biotin-PEG-CPA complex using different molecular weight PEG combining with avidin-biotin. *In vitro* results showed half-life of 3–4 days depending on PEG chain length, indicating a good improvement in circulation residence time [40]. Therefore, it has enormous potential as pharmacokinetics enhancer for protein and peptide therapeutics.

6. Attaching therapeutic protein/peptide to recombinant polypeptide

In recent years, new technologies have surfaced for protein half-life extension, which comprises unstructured polypeptide chain. Just like PEG, attachment of the polypeptide polymers to protein therapeutics increases their hydrodynamic radius, making them less prone to kidney filtration. But unlike PEG, the peptide polymeric alternatives are biodegradable, non-toxic, non-immunogenic, hydrophilic and neutral polymers which are also much cost-effective because of their simple synthesis techniques [5,41].

6.1. XTEN

XTEN is protein polymer developed by Amunix, Versatis as an alternative to non-biodegradable polymers for half-life extension. XTEN is a non-structured polypeptide consists of a limited number of monomers. Unlike chemical polymers XTENs do not have a defined structure (coils randomly). Like PEG, XTEN also increases protein half-life by increasing size and hydrodynamic radius of molecule. In other words, XTEN is a good alternative to PEG (also called rPEG). XTEN comprises a library of amino acid sequences with A, E, G, P, S, and T which were expressed using *E.coli*. XTEN sequences have been tested for several protein therapeutics with enhanced half-lives and reduced immunogenicity [5,42]. According to Amunix pipeline product list, there are at least 4 XTEN conjugated protein therapeutics that are in different phases of clinical trials [42].

6.2. PASylation

PAS is another peptide polymer developed by Technical University of Munich, which also works like PEG and possesses all the properties of a biodegradable peptide molecule. “PAS” which stands for Porline, Alanine and Serine is an unstructured peptide polymer of 100–200 repeated sequence created by random arrangement of these three amino acids as building blocks [5,43]. Initially 3 proteins were tested as model therapeutics: a recombinant Fab fragment, IFN α2b and human growth factor (somatropin). Different PAS sequences attached to Fab fragment showed 2–25 fold increase in half-life. PASylated form was to shown to have half-life of 2.71–37 h, whereas rFab itself had only 1.34 h. Similarly PASylated IFN showed 5–30 folds and hGH showed 94 fold increase in plasma half-life. There are some 6 PASylated protein therapeutics in the pipeline of XL-Protein GmbH, most of which are in preclinical trial [43].

6.3. ELPylation

Another polypeptide fusion technique available is ELPylation. Like XTEN and PAS, ELP (Elastin-like polypeptides) are also random sequences containing repeats of V-P-G-x-G, mostly found in elastin, where x refers to any protein other than proline. Sequence has high similarity to elastin that makes it prone to human elastases, conferring its

biodegradable feature. ELPs can attach to any peptide therapeutic at genetic level and create a thermally responsive reversible phase transition. Thus, above a certain threshold temperature, they precipitate out of solution by forming aggregate. However, ELP acts as a half-life extension agent, just like PEG and other polypeptide formulations, by increasing hydrodynamic radius [5,44].

6.4. HAPylation

Repeated sequence of a glycine rich $(Gly_4Ser)_n$ polypeptide is the active molecule of HAPylation, which consists of a repeated residue of 100–200. HAPylation showed to increase Fab's half-life in animal model [5]. 200 residues HAP conjugated Fab showed a prolonged 6 h of half-life *in vivo*. A major setback was encountered with $(Gly_4Ser)_n$ solubility. Solubility for HAP depends on chain length, with an increase in length vastly decreasing solubility. Comparatively shorter chains are not very efficient enough to increase half-life [41].

6.5. Gelatin-like protein (GLK)

Gelatin-like protein (GLK) is a group of biologically active recombinant polypeptide with extended *in vivo* half-life and has a $(Gly\text{-}XY)_n$ structure, where X and Y refer to any natural amino acid except Cys and n stands between 60 and 1500 amino acid residues (preferably 200–1000) with mass of 6-150 kDa (preferably a 20–80 kDa) [45]. Different GLK formulations prepared by genetic fusion increased G-CSF half-life from 1.76 h to 10 h in rats. On the other hand, chemical conjugation of succinylated gelatins to soyabin trypsin inhibitor and Cu/Zn superoxide dismutase showed 6–7 fold increase in half-life [5,46] (Table 3).

7. Attaching therapeutic protein/peptide to bioactive natural protein or protein domain (protein fragment)

There are few natural protein molecules that have exceptionally long circulation half-life. Therefore, proteins like serum albumin and transferrin were first considered as half-life extension moieties of other therapeutic proteins. The long half-life of transferrin is due to clathrin-dependent transferrin receptor binding, whereas albumin's long half-life is the result of neonatal Fc receptor (FcRn) dependent recycling. Same mechanism of pH dependent FcRn receptor mediated recycling works for human immunoglobulin (Ig)G antibody as well. Human IgG_1, IgG_2, and IgG_4 have *in vivo* half-lives of 14–21 days. A fragmented portion of immunoglobulin molecule (Fc region) was also proven to be successful as half-life extension agent [5]. Structurally and also biologically, IgG and albumin do not have any similarity. However they share few exclusive characteristics. Both proteins have distinctively longer half-lives, which are inversely proportional to serum concentration. These characteristics are due to binding to FcRn receptor and subsequent transcytosis and recycling mechanism [54].

7.1. Albumin binding

Albumin has some unique features like a plasma half-life of ~19 d and multivalent binding sites with negatively charged surface area, making the molecule a perfect candidate as drug delivery vehicle and half-life extension agent for peptide drugs. Peptides can easily get attached to albumin by covalent and non-covalent bonds [52,53]. Albumin binding to peptide can be facilitated in more than one ways and with the help of different classes of helper molecules like, antibody fragments (Fab, scFv, domain antibodies (dAbs), nanobodies), scaffold proteins, bacterial albumin-binding domains, fatty acids and other peptides [6,52,53]. Figs. 4 and 5 are simplified diagrammatic representation of different modes of albumin binding to therapeutic proteins and their mechanisms of action *in vivo*.

7.1.1. Non-covalent binding of albumin

Indirect binding can be achieved by conjugating albumin to peptide therapeutic with the help of a helper molecule, for example, attaching fatty-chain by non-covalent binding. Albumin, a well-studied transport protein, is efficient enough to reversibly bind to a range of endogenous ligands like fatty acids, bilirubin and few exogenous ligands, like, penicillin, warfarin and diazepam [6,52,53]. Use of fatty acid as ligand is also known as “lipidation” [55]. Albumin binding to these molecules enhances their bioavailability. Two very famous peptide therapeutic formulations developed exploiting this technology are Levemir® (Insulin detemir) and Victoza® (Liraglutide). Insulin detemir was developed by attaching insulin molecule to a fatty acid (myristic acid). Glucagon-like peptide-1 agonist (GLP-1), Victoza® (Liraglutide) was also created by attaching a fatty acid (myristic acid) to an N-terminal lysine. As soon as the formulations get into the blood stream, the fatty acid forms a non-covalent binding to albumin present in circulation and then dissociates slowly, therefore, increasing the residence time of therapeutic in body. Both of the formulations are prescribed to use once daily instead of multiple SC administrations [6,52,53].

7.1.2. Covalent attachment to albumin

Covalent attachment of therapeutic protein to albumin molecule is another way of increasing protein half-life. It could be done by either conjugation of protein to albumin chemically [6,52]. Direct fusion of protein therapeutic to albumin gives the flexibility to further increase half-life of protein by conjugating it to a genetically modified albumin

Table 3
Efficiency of recombinant polypeptide sequence attached protein therapeutics in pre-clinical/clinical trials compared to the native protein formulations.

Protein therapeutics	Plasma half-life	patent name	Plasma half-life	Animal model/ clinical trial	Technology used	Ref.
GH (rhGH)	4–5 min	VRS-317	131 h	Phase3	**XTENylation**	[30,41,42,47]
FVIII	12 h	BIVV001	72 h	Phase1	**XTENylation**	[13,20,41,48]
GLP1	2 min	NB1001	30 days	Phase1	**XTENylation**	[6,41,42,49–51]
GLP2	2 min	NB1002	Mice = 34 h, rat = 38 h, monkey = 120 h; Estimated half-life in human = 240 h	Pre-clinical	**XTENylation**	[7,33,41]
FVIII	12 h	BIVV001	37 h	Phase2	**XTENylation**	[13,20,38,41,48]
FIX	18–20 h	BIVV002	Half-life in human: unknown; once in every 2 weeks formulation	Pre-clinical	**XTENylation**	[13,20,38,41,48]
rFab	1.34 h		2.71-37 h	Pre-clinical	**PASylation**	[5,43]
IFN α2b	.54 h		2.64–15.85 h	Pre-clinical	**PASylation**	[5,43]
hGH	0.047 h		4.42 h	Pre-clinical	**PASylation**	[5,43]
GLP-1	2 min	Glymera™ (PB1023)	Once weekly formulation	phase 2	**ELPylation**	[5,7,33]
Insulin	4–5 min	PE0139	Once weekly formulation	phase 1	**ELPylation**	[5,6,52,53]
Vasoactive intestinal peptide (VIP; VPAC2 receptor agonist)	……	Vasomera™ (PB1046)	Once weekly formulation	……..	**ELPylation**	[5]

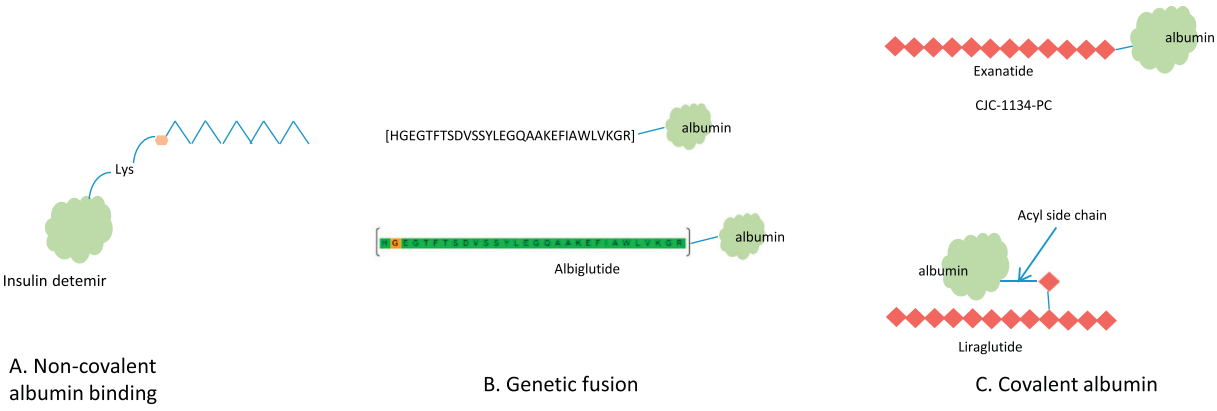

Fig. 4. Different mechanisms applied for attaching albumin to protein. (A) Insulin detemir was created by attaching a fatty acid (myristic acid) to amino acid lysine at B29 position. While in circulation, the fatty acid forms non-covalent bond to endogenous albumin. (B) GLP-1 was attached to albumin in genetic level and then expressed into a host to get a recombinant protein. (C) Albumin is chemically attached to a therapeutic protein by covalent bond. Exanatide and Liraglutide are the two examples.

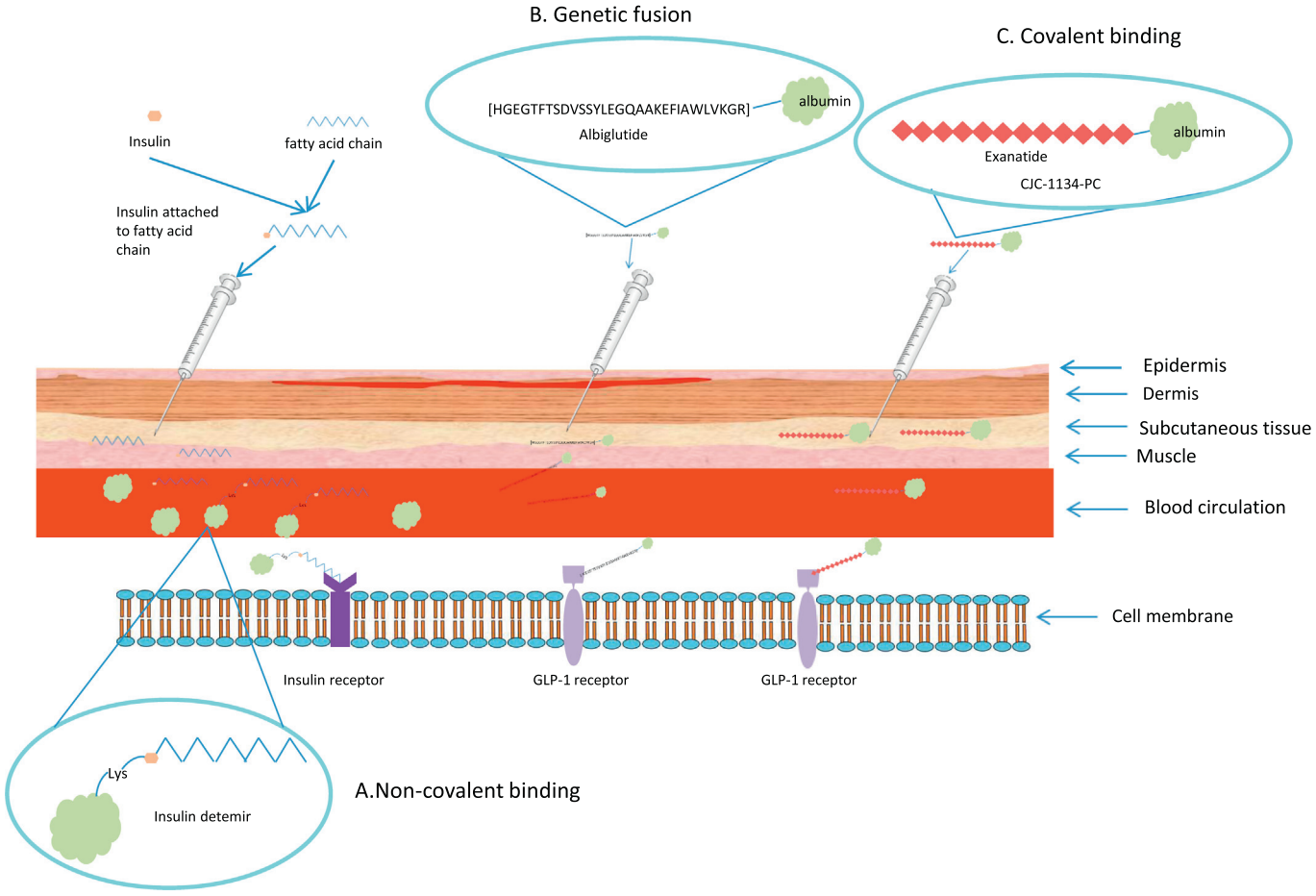

Fig. 5. Popular mechanisms of peptide half-life extension by attachment of therapeutic protein to albumin. (A) Insulin detemir was created by attaching a fatty acid (myristic acid) to amino acid lysine at B29 position. While in circulation, the fatty acid forms non-covalent bond to endogenous albumin. (B) GLP-1 was attached to albumin in genetic level and then expressed into a host to get a recombinant form of long acting GLP-1 protein. (C) Albumin is chemically attached to a therapeutic protein by covalent bond. Exanatide is a good example.

or albumin derivative, instead of endogenous albumin. Albumin derivatives are recombinant proteins with extended serum half-life [6].

7.1.3. Recombinant albumin

Synthetic plasma albumin derivatives are great options to further modify and design albumin bound extended half-life protein therapeutics. Recombinant albumins are also great source to examine albumin-peptide binding in details. Recombinant human albumin from yeast is identical to serum albumin and therefore, found to be the safest [51].

7.1.4. Genetic fusion to albumin

Like covalent attachment, genetic fusion technique also creates fusion-protein attached to albumin. By genetic fusion target protein

Table 4
Efficiency of albumin attached protein therapeutics available in market and under pre-clinical/clinical trials compared to the native peptide formulations.

Binding towards albumin	Protein therapeutics	Plasma half-life	Albumin bound formulations (patent name)	Half-life extension technology	Plasma Half-life	Animal model/ clinical trial	Ref.
Non-covalent binding	Insulin	4-5 min	Levemir® (Insulin detemir)	Conjugation of fatty acid chain: myristic acid modified insulin analog	Half-life: 5–7 h; long distribution phase of approx. 8 h. Once-daily administration sc	In market	[6,52,53]
Non-covalent binding	GLP-1	2 min	GLP-1 agonist Victoza® (Liraglutide)	Conjugation of a fatty acid chain: palmitic acid is attached	13 h; once-daily administration sc	In market	[6,52,53]
Genetic fusion	GLP-1	2 min	GLP-1 receptor agonist; GSK2374697	Domain antibodies to serum albumin (AlbudAb)	6–10 days	Phase 1	[6]
Genetic fusion	pleiotropic cytokine interleukin-6 (IL-6)	4.3 h	IL-6 receptor agonist; ALX-0061	albumin-binding nano-bodies	6.6 days; iv	monkey	[6,56]
Genetic fusion	GLP-1	2 min	Albiglutide; Eperzan/Tanzeum	GLP-1-human serum albumin (HSA) fusion protein;	6–8 days	In market	[6,49–51]
Genetic fusion	FIX	18–34 h	rIX-FP; Idelvion®, CSL-Behring	Albumin fusion	102 h; 5 fold increase,	Phase 3	[6,38,51,57,58]
Genetic fusion	FVIII	10–14 h	CSL627	Single-chain factor VIII fused to albumin	18 h (1.5-fold)	Phase 1	[6,51,58,59]
Genetic fusion	FVIIa	11.8 h	FVIIa-HSA fusion protein	Glycine-serine linker was used to connect factor VIIa to albumin.	3–4 fold increase	Phase 1	[6,51,52,60]
Genetic fusion	hGH	4-5 min	hGH-HSA fusion protein (TV-1106),	Albumin fusion	3–35 hrs; up to 7 days after dosing	Phase 1	[6,30,51,61]
Genetic fusion	G-CSF	3.5–3.8 h	Balugrastim	Albumin fusion	1–2 (18–40 h)	MAA submitted (Europe)	[5,17,19,51,62]
Covalent attachment	Exendin-4	< 1 h	CJC-1134-PC	Albumin fusion	8 days; sc	Phase 2	[52,63]
Genetic fusion	Granulocyte colony stimulating factor protein(G-CSF)	2.5 h	Balugrastim/Albugranin™/ Neugranin™	Albumin fusion	18–40 h	Phase3	[51,52,64,65]

molecule gene is fused to albumin gene and the synthetic fusion gene is then transferred and expressed in a suitable host and that host starts producing a synthetic fusion peptide (protein + albumin) [6,52]. This provides a simpler process since the manufacturing takes place inside the organism or cell line and fully folded functional protein can be collected directly. Therapeutic peptide can be attached to albumin in one end (N- or C-terminus of albumin) or both end (bi-specific albumin fusion) [51]. This technique has proven to be very popular and successful in terms of albumin conjugated drug development; over the last two decades a number of recombinant protein therapeutics found their way to pharmaceutical market. These include peptides (GLP-1), hormones (insulin), growth factors (G-CSF), and coagulation factors. Table 4 has a list of protein therapeutics that is in clinical trial or in market.

7.2. Fc fusion

A recent review on Fc fusion proteins estimated that there are some 350 1gG based protein therapeutics which are either in pharmaceutical pipeline or already approved and available in market [66]. This number is the biggest compare to any other available protein extension technology we have in our hand. A very important milestone for IgG coupled protein technology was invention of hybridoma technology [66,67], which allowed speedy production of chimeric and humanized antibodies with much decreased immunologic concern and improved PK and PD properties [66]. IgG based extended half-life proteins are mostly based on Fc domain fusion coupling. Structurally, constant region of immunoglobulin G (IgG), Fc region is divalent, which provides flexibility for drug binding. Conceptually divalent binding is an added benefit for avidity effects. Protein molecules can be added to Fc region fusion sites (either N-terminus or C-terminus) or inserted into loop formation [68,69]. Apart from half-life extension, Fc fusion sometimes shows to improve physiological properties of a protein, like, increase of solubility and stability [68].

There are few things which are of concern for Fc mediated half-life extension formulations. They are ADCC (Antibody-dependent cellular cytotoxicity) and CDC (Complement-dependent cytotoxicity). This has been addressed by CH3−CH3 heterodimerization vastly [68,69].

Hypothetically, albumin or Fc-fused proteins follow their conjugates extended half-life. However, it was a surprise when few Fc fusion proteins were found to have a half-life slightly shorter compared to their half-life of Fc counterpart. Etanercept, abatacept, and alefacept are good examples. It seems like protein binding somehow agitates Fc region such a way that changes its affinity towards FcRn receptor [66,68–70] (Tables 5 and 6).

7.3. Transferrin fusion

Serum transferrin has a well-known function of reversibly binding and transferring iron molecules to tissues. With long serum half-life of 7–10 days (unglycosylated Tf is 14–17 days), transferrin is a good candidate for protein half-life extension conjugate [5,85]. GLP-1 or exendin-4 (EX-4) conjugated to non-glycosylated transferrin (BRX-0585) showed some 1000 fold increase of half-life (apprx. 2 days). Unfortunately the approved formulation was taken out of clinical trial due to significantly high side-effects (increased pulse and blood pressure) [85]. Another study attempted a new proinsulin-transferrin (ProINS-Tf) fusion protein. ProINS-Tf fusion protein showed a slow sustained hypoglycemic affect with longer half-life in mice. After SC administration slow absorption t_{max} was 5.5 h compared to free ProINS, which showed a t_{max} of 0.3 h. ProINS-Tf serum half-life was 7.29 h compared to ProINS half-life of only 0.5 h [5,86].

8. Attaching the therapeutic protein/peptide to carbohydrate

In biological system, protein stabilization and protection from

Table 5
Efficiency of Fc fragment attached protein therapeutics available in market and under pre-clinical/clinical trials compared to the native peptide formulations.

Protein therapeutics	Plasma Half-life	Fc fusion formulations (patent name)	Plasma Half-life	Animal model/ clinical trial	Ref.
FIX	18-20 h	lprolix®, Biogen Idec/Sobi	82 h	In market	[13,20,38]
rFIX	18-20 h	–	56.7 (42.4–74.5) h	Clinical trial	[66,71]
rFVIII	12 h	Elocta®/Eloctate®, Biogen Idec/Sobi	19 h	In market	[13,20,38]
Human granulocyte colony-stimulating factor (G-CSF) (rmetHuG-CSF)	3.5–3.8 h	G-CSF/IgG1-FcL protein	5- to 8-fold; > 48 h	Rats	[5,17,19,72–74]
Receptor tumour necrosis factor (TNFR)	.....	Etanercept	102 ± 30 h	In market	[75]
IL-2 fused to diphtheriotoxin; enzyme activity and membrane translocating domain	.....	Denileukin	70–80 min	In market	[5,76]
GLP-1	2 min	Trulicity®(dulaglutide)	5 days	In market	[5,7,33,77]
B-domain-deleted FVIII	12 h	ELOCTATE	19 h	In market	[5,13,20,37,78]
FIX (Recombinant)	18–20 h;	ALPROLIX®	97 h	In market	[5,13,20,38,70]
VEGFR	70 min	Aflibercept; Zaltrap®	6 days (range, 4 to 7 days)	In market	[5,66,79]
CTLA	.....	NULOJIX; Belatacept	9.8 days	In market	[66,80]
2 components of ACVR2A	.....	Sotatercept	23.7 to 31.8 days	Clinical trial	[66,81]
Angiopoietin-2	.....	AMG 386	3.1 to 5.1 days	Clinical trial	[66,82]
CD95 receptor	.....	APG101	3 (1) to 14 (0.5) days	Clinical trial	[66]
Extracellular CD2- binding portion of human LFA-3	.....	Alefacept	11 days	In market	[66]
Thrombopoietin mimetic peptides	45 min	Romiplostim; Nplate	1 to 34 days (median: 3.5 days).	In market	[66,83,84]

enzymatic degradation takes place by glycosylation which is the covalent modification of a protein with carbohydrates [87]. Protein intrinsic properties are altered by sugar molecules attached to its chain, which eventually changes its quaternary structure stability and resistance from proteases [76]. This natural protection system can be manipulated to design new half-life extended bio-therapeutics [87]. Besides, the abilities of oligosachharide moieties to increase size and decrease renal clearance by adding negative charge to the protein have made glycosylation a popular approach to increase therapeutic half-life. O-linked oligosachharides also protects the chimeric proteins from protease degradation by blocking its access to the core of the protein [88].

8.1. Glycosylation

Glycosylation is a way of natural co- or post translational modification, which is carried out in endoplasmic reticulum and golgi where carbohydrate moieties are covalently attached to asparagine residue (N-glycosylation) and threonine/serine residues (O-glycosylation). The type and number of ingredient sugars, length, branching and other properties depend on the expression systems in which the recombinant proteins are produced in and thus play roles on the protein's three-dimensional structure, pharmacokinetics, efficiency, immunogenicity and half-life.

Genetically modified, methylotrophic yeast, *Pichia pastoris* can produce few proteins, such as EPO, with their human like glycosylation [89]. But recombinant proteins are produced in mammalian system, *i.e.* CHO cells, followed by human cell lines recently, because of their similarity in glycosylation of the protein to their natural human derived counterparts [90].

The first generations of recombinant proteins prepared with this technology, usually bear glycans similar to their native forms. For example, fusion of a 28-amino acid long sequence from the carboxy terminal region (C terminal peptide or CTP) of human chorionic gonadotropinβ subunit has increased the half-lives of some hormones. The coding sequence for CTP was ligated to the N-terminal coding sequence of hormones- EPO, FSHβ and TSHβ and to both C- and N-terminal of GH. TSH-CTP expressed in COS-7 cells (African green monkey) showed prolonged half-life compared to the TSH-wt. EPO-CTP showed 2–3 times increase in half-life compared to its native peptide (5 h). FSH-CTP was approved in 2010 (brand name ELONVA). Chimeric GH (CTP-GH-CTP-CTP) showed 4–5 fold longer half-life compared to its unglycosylated native form (12 min) [88]. Fig. 6 is simplified diagrammating representation of site-specific mutagenesis.

Site directed mutagenesis of protein coding DNA without interrupting the receptor binding and catalytic sites is a popular strategy to increase half-life *via* glycosylation. Insertion of (N-X-S/T) codes, where X is any amino acid except proline introduces the site for N-glycosylation. Commercially available EPO analog, Darbapoietin alfa bears 2 extra N-glycans which confer it a half-life of 26.3 h, 3 times higher than the native one [88,91]. Adding 3 extra N glycosylation sites (N-X-S/T) in human growth hormone prolonged its half-life by 13–24 folds compared to the native form. However, relationship between efficiency and number of N-glycan insertion is not always linear and directly proportional. While the heavily sialylated form of the recombinant protein improved half-life to 5.6 h in comparison to 0.23 h for native protein, the moderately sialylated three extra N-linked oligosachharide form showed best IGF-1 response with a half-life of 3.10 h [92].

Mpl ligand, a hematopoietic growth factor, belonging to glycolytic hormone family, contains only O-linked glycosylation. On the other hand, the satiety hormone contains no glycans at all. S. Elliott *et al* added 4 and 5 N-glycans *via* site directed mutagenesis to these hormones respectively, which resulted in better efficiency and prolonged circulation time for both in mouse model [93].

Glycosylation is very important for proteins function and efficiency. Any aberrant glycosytion of important proteins may lead to pathological conditions [94], which must be under consideration while preparing any new therapeutic formulation using this technique.

8.2. Polysialylation

Naturally occurring PSA (or colomic acid; CA) polymers are carbohydrate chains which are biodegradable, highly hydrophilic in nature and have no identified receptors in the body. Thus, the posttranslational modification of therapeutic peptides and proteins with terminal sialic acid improves drug pharmacokinetics and reduces immunogenicity, thereby, improving their overall efficiency. As reported, certain bacteria use their polysialic acid coat in order to escape recognition by the immune system of the host [95], correlating with the low immunogenicity of the polymer. Fernandes et al. demonstrated that shorter PSA (molecular weight of 22 kDa or less) conjugated with anticancer agent, aspariginase for the treatment of leukemia increased enzyme stability and serum half-life, as well as significantly enhanced the area under the curve of asparaginase [96]. Another research

Table 6
Efficiency of polysaccharides attached protein therapeutics available in market and under pre-clinical/clinical trials compared to the native peptide formulations.

Binding agent	Protein therapeutics	Plasma Half-life	polysaccharides bound formulations (patent name)	Half-life extension technology	Plasma Half-life	Animal model/ clinical trial	Ref.
N-X-S/T codes, where X is any amino acid except proline	EPO	8 h	Darbapoietin alfa	Glycosylation	26.3 h	In market	[88,91]
……	FIX	18-20 h	…….	Glycosylation	22 h	……	[113]
……	Human interferon alpha (IFNα)	.54 h	…….	Glycosylation	> 48 h	……	[114]
90 kDa heavily glycosylated	rThrombopoietin/Mpl ligand	45 min	Tebiao	Glycosylation	40 h	Clinical trial	[84,115]
70 kDa dextran moiety	Somatostatin	< 2 h	……	Dextran conjugate	27 h	Phase I	[55]
5 kDa dextran with a hexyleneamine carboxymethyl dextran (CMD)	Catalase	0.8 h	……	Dextran conjugate	16 h	Rats	[55]
	insulin	12.4 min (Zn-insulin)		Dextran conjugate	114.1 min	Sprague-Dawley rats	[55,103]
1:6 ration HA: INFa	INFa	24 h	…..	HAylation	50–110 h	rats	[55]
100 kDa HA variant containing a vinyl sulfone functionality at the COOH position (VS-HA)	Exendin-4 (Ex4)	< 1 h	….	HAylation	> 96 h; up to 3 days	Human serum; db/ db mice	[52,55,63]
Covalent binding	Insulin	4–5 min	Insulin polysialic - Xenetic Biosciences	Polysialylation	6 h	In market	[95]
Covalent binding	Asparginase	6 h	–	Polysialylation	38 h	*In vivo*	[96]

published by Jain et al. showed that polysialylated insulin offered significant enhancement of therapeutic value for the treatment of diabetes. They found that insulin polysialylated with the 22-kDa PSA exerted a more prolonged reduction of blood glucose levels compared to intact insulin treatment *in vivo* [95]. In addition, inclusion of a long PSA (*e.g.* average molecular weight of 60 kDa) greatly prolonged the circulation time of a small model drug, fluorescein which possesses a very short half-life [97]. Similar results have been attained with several other protein therapeutics, like aprotinin and IgG immunoglobulin [98]. To date, PSA, as a promising "stealth polymer" could be conjugated with protein drugs *in vitro* by chemical or chemo-enzymatic strategies. However, advancement in research has developed a biosynthetic pathway for site-specific polysialylation of recombinant proteins in the cytoplasm of *Escherichia coli.* as well [99].

8.3. Other polysaccharide based approaches

Apart from popular technologies like, glycosylation and polysialylation, there have been few other approaches that came into light in last 2 decades. Most of them have limited success, but a few of the formulations were promising enough to go for clinical trials. However, all of them show promising potential as half-life extension agents.

8.3.1. Dextran conjugation

Dextran polymers are D-glucose polymers with α (1–3) linkage. Dextran and dextran derivatives are chemically conjugated to protein by several different methods including periodate oxidation and also by enzymatic approaches. Like PEG, dextran also increases protein hydrodynamic radius and decreases kidney filtration. As a compound it is recognized as GRAS by the FDA. Dextran conjugated somatostatin was reported to be in clinical trial [55]. Several other reports mention about dextran conjugated proteins like uricase [100], superoxide dismutase [101], hemoglobin [102], insulin [103], hemoglobin [104,105], asparaginase [106], carboxypeptidase G2 [107,108]. Unfortunately, there is not much progress of this approach after 2006.

8.3.2. HESylation

A modified form of plant polysaccharide amylopectin is Hydroxyethyl starch (HES) which is basically a natural polymer. HES is produced by hydroxyethylation of amylopectin. Hydroxyethylation makes the polysaccharide more water soluble and prevents degradation due to a-amylase [55]. HESylation technology showed promising success in half-life extension of several protein therapeutics including erythropoietin and FVIII [55]. However recent studies revealed connection of HES with increased risk of kidney failure and death in critically ill patients [109]. Also few of the earlier clinical trial reports were retracted due to research misconduct [110]. Overall safety issue made HESylation approach questionable.

8.3.3. HEPylation

Heparosan, an active polymer of HEPylation is a natural polysaccharide and a biosynthetic precursor of heparin. Because of its relation to heparin, it is recognized as "self" inside our body environment and doesn't have any immunogenicity issue. Therefore, it is considered as a good option for a biodegradable protein half-life extension moiety. The simplified formula is $[-4\text{-}N\text{-acetylglucosamine-a1, 4-glucuronic acid-b 1-}]_n$ [111]. Heparosan and its derivatives are of interest for their potential as pharmacological agents for diversified field [111]. Heparosans show 15 h to 8 day long half-life in bloodstream depending on MW and route of administration [112].

8.3.4. HAylation

Hyaluronic acid (HA) is a natural polysaccharide with a linear chain and negative charges (repeating disaccharide: $[b(1–4)\text{-GlcA-b}(1–3)\text{-GlcNAc}]_n$). It is found in different tissues of human body. Like other half-life extension agents, conjugation of HA to any peptide can

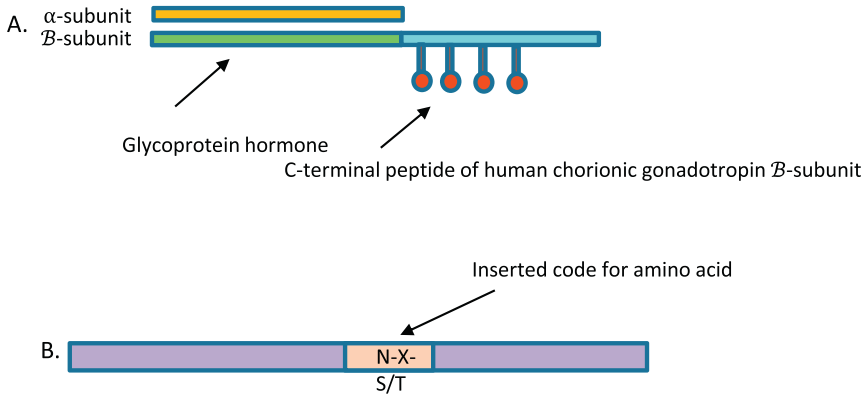

Fig. 6. Strategies to increase half-life *via* glycosylation. A. Chimeric protein by fusing CTP of human chorionic gonadotropin β-subunit. B. Site directed mutagenesis.

increase size of the protein decreasing renal clearance. Also HA retains water. This hydrodynamic property works like a wall or insulation between protein molecules [55].

9. Half-life extension by genetic manipulation

9.1. Protease-resistant point mutations

Mutation in one or more genes has successfully generated few long-acting counter parts of native short-acting peptide therapeutics. Alteration of one or more amino acids in a peptide chain can enhance the PK and PD properties of the protein, keeping its activity intact. Long acting insulin formulations are good example for that (mentioned earlier in albumin binding section).

Lately, point mutation has surfaced as a new technology for protein pharmacokinetic enhancement. A single amino-acid substitution/alteration can make a molecule highly protease resistance *in vivo*. Nautilus Biotech came up with two protein formulations interferon beta (IFNß) & Somatropin [1]. Patent filing for IFNß was on 2006 and until now no report with progress details has been publicized [116] and somatropin formulation was discontinued from phase1 trial after 2017 [117]. Even with slow progress and delay to commercialization, the technology itself remains promising and could be explored for betterment.

10. Nanoparticle (NP) mediated approaches

NP mediated controlled drug delivery vehicles are designed with various purposes, like, targeted delivery of a drug to specific organ, or to protect a therapeutic from degradation in an environment that is harsh for it. Use of NP mediated drug delivery to modulate PK and PD of a therapeutic is another approach to create long acting therapeutic formulations [7]. Drug delivery systems do not modify PK and PD of a therapeutic molecule in a conventional sense. Rather the concept relies on incorporating a native or recombinant peptide molecule in a matrix or inside another molecule that works like a protective covering for it. This system works like a “depot” and instead of introducing the therapeutic amount all at a time, this releases the drug to systemic circulation slowly creating a long acting formulation [7].

A popular NP system is PLGA (Poly-Lactic-*Co*-Glycolic Acid). Few very promising PLGA and modified PLGA (for example, PEGylated PLGA) formulations of therapeutic proteins are either in pre-clinical/clinical trial stage. One of the examples is biodegradable soluble thermo sensitive tri-block copolymer of PLGA-PEG-PLGA, trade name ReGel™. ReGel™ is water soluble in room temperature, but inside body temperature it forms highly viscos gel which maintains its structure for a month. A number of therapeutic proteins have been tested for ReGel™. ReGel™ incorporated insulin [7,118,119] and GLP-1 [7,120] showed very good sustained release profiles in pre-clinical study.

Another good example for drug delivery vehicle is colloidal vesicular liposomes. Liposomes are lipid formulations mimicking bi-layer amphipathic cell membrane structure. There are several formulations designed, which are different from each other in lipid composition and particle size, thus having different PD and PK properties. Liposome itself has very short half-life *in vivo* (< 2 h) but liposome coupled with a polymer moiety (like, PEG) has extended half-life (45 h in human) and can be used for long-acting protein formulation preparation. Liposome incorporated coagulation factor showed success in preclinical testing [69].

Though conceptually being very promising, major drawbacks of known drug delivery systems lie with challenges of manufacturing and administration. Also burst release of protein and poor loading efficiency are common problems [69]. Nutropin is a long acting growth hormone formulation which was approved in 1999. It is a month-long formulation of human growth hormone incorporated inside PLGA. The product was discontinued on 2004 due to side-effects associated with burst-release (over 50% of the therapeutic are released in first few days only) [7,29,121]. The future of this technology lies on successful addressing of associated drawbacks [69].

11. Other approaches for half-life extension (biodegradable and non-biodegradable polymers and chemical modifications)

There have been several polymers proposed and reported as protein half-life extension moiety and alternative to PEG, which show promising profiles mostly *in vitro* and in animal models. Although the technologies look promising, they still need at least few years of research to evaluate whether they have any real potential in pharmaceutical market as half-life extension agents. Table 7 reports some of these “not so popular yet” half-life extension agents with their preclinical findings.

12. Conclusion

Half-life extended “bio-better” versions of the protein therapeutics have been proven to be very effective in every way. Over the last few decades research and development for new formulation of protein expended a lot. Biotechnological advancement has contributed a lot in booming the field. However, still there are challenges need to be addressed. Clinical safety and immunogenicity issue has been always there. Instability of protein *in vivo* environment is a contributing factor for immunogenicity. Even half-life extended formulation can contribute to immunogenicity. If protein stability is not maintained during sustained release, generated particulate can induce immune response, which might even be another contributing factor for stability of protein [125]. In a word, extensive PK and PD study of any half-life extended protein formulation is a must to avoid all the drawbacks and also for eliminating any unwanted side-effect.

Table 7
lists some other half-life extension agents.

Type of modification	Half-life extension agent	Successful conjugation of protein	Ref.
Chemical chain modification	Bipyridyl	2,2′-bipyridyl (bipy) conjugated to insulin; *in vitro*	[55]
	Perfluoroalkylation	Perfluoroalkyle conjugated insulin; *in vitro*	[55]
	Bile acid derivatives	Cholic acid and derivatives conjugated to insulin; animal model (pigs)	[55]
	Ion conjugation	Zn^{2+} conjugated to insulin; animal model	[7,55]
Non-degradable polymers	Poly(N-vinylpyrrolidone) (PVP)	TNF which showed a longer half-life than PEG-TNF; animal model (mice)	[6,87]
	Polyglycerol (PG)	BSA as model drug; *in vitro*	[6,122]
	Poly(N-(2-hydroxypropyl) methacrylamide) (PHPMA)	BSA as model drug; *in vitro*	[6]
	Polyoxazolines (POZs)	G-CSF conjugated with a 20 kDa POZ; Insulin conjugated to 10 kDa MW PEOZ; animal model (rats)	[6,87,123,124]
Degradable Synthetic polymers	poly[oligo(ethylene glycol) methyl methacrylate] (POEGMA)	Exendin conjugated POEGMA; *in vitro*	[6]

Acknowledgements

The project was funded by a FRGS grant from Ministry of Education Malaysia (FRGS/2/2014/SG05/MUSM/03/1). Authors declare no conflict of interest.

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