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PROTEIN CHIMERIZATION: A NEW FRONTIER FOR ENGINEERING PROTEIN THERAPEUTICS WITH IMPROVED PHARMACOKINETICS

A. R. Satvik Iyengar, Shreya Gupta, Snehal Jawalekar, Abhay H. Pande

Department of Biotechnology, National Institute of Pharmaceutical Education and Research (NIPER), Sector 67, S.A.S. Nagar (Mohali)-160062, Punjab, India (A.R.S.I., S.G., S.J., A.H.P.)

Current address: School of Biomedical Sciences and Pharmacy, Faculty of Health and Medicine, The University of Newcastle, Callaghan, NSW 2308, Australia (Current address for A.R.S.I.)

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Running Title: Improving pharmacokinetics of therapeutic proteins by chimerization

*__To whom correspondence should be addressed__: Abhay H. Pande, Department of Biotechnology, National Institute of Pharmaceutical Education and Research (NIPER), Sector 67, S.A.S. Nagar, (Mohali) -160 062, Punjab, India. Tel.: +91 172 2214 682, Fax: +91 172 2214 692. E-mail: apande@niper.ac.in ; abbupande@yahoo.co.in

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Abbreviations: ABD, Albumin binding domain; CTP, Carboxy terminal peptide, dAB, domain antibody; ELP, Elastin-like peptide; FcRn, neonatal Fc receptor; GLK, gelatin-like protein; HAP, homo amino-acid polymer; HLEP, half-life extension partner; PEG, polyethylene glycol; scFv, single-chain variable fragment; Tf, Transferrin.

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ABSTRACT

With the advancement of medicine, the utility of proteins therapeutic is increasing exponentially. However, a significant number of protein therapeutics suffer from grave limitations, which includes their subpar pharmacokinetics. Here, we have reviewed the emerging field of protein chimerization for improving the short circulatory half-life of protein therapeutics. We have discussed various aspects of protein therapeutics aiming at their mechanism of clearance, and various approaches utilized to increase their short circulatory half-life with principle focus on the concept of chimerization. Furthermore, we have comprehensively reviewed various components of chimera, such as half-life extension partners and linkers; their shortcomings, and prospective work to be undertaken for developing effective chimeric protein therapeutics.

Introduction

With the advent of 21st century and the growth of recombinant DNA technology, there has been a significant progress in the field of biopharmaceuticals, most of which could be attributed to the development in protein therapeutics. In addition, the development of cloning methods and systems for recombinant protein production have certainly advanced the research and development of protein therapeutics (Gellissen, 2005). Recently, it has been reported that 129 distinct biopharmaceuticals have entered the market between Jan 2014 to July 2018 in the US and EU regions (Walsh, 2018). The approval of Humulin™ in 1982 has been a pioneering landmark in journey of recombinant protein therapeutics (De Meyts, 2017). Some other major approvals that followed the approval Humulin™, are Nutropin™ (somatropin) in 1985, Alteplase™ (tissue plasminogen activator) in 1987, followed by Epogen™ (erythropoietin) and Neupogen™ (granulocyte colony stimulating factor) in 1989 and 1991 respectively (De Meyts, 2017). Since the achievements of these milestones, protein therapeutics have come a long way and have proved to be extremely effective for treatment of various diseases (Leader et al., 2008; Lagassé et al., 2017; Walsh, 2014, 2018). It is estimated that at the global level, the share of biopharmaceuticals entities amongst clinically studied pharmaceutical products is around forty percent (Walsh, 2018).

In comparison to the small molecule drugs, the use of protein therapeutics offer substantial advantages, which include lower propensity of adverse events, better tolerance and the wide ranging application of protein therapeutics as a replacement therapy for a variety of disorders (Leader et al., 2008; Walsh, 2010, 2014, 2018). In addition, since protein therapeutics offer shorter timeline of approval from various drug regulatory agencies and better patent protection, this makes them commercially lucrative for the pharmaceutical enterprises (Leader et al., 2008).

However, the application of protein therapeutics still suffers from serious limitations that include poor pharmacokinetics and immunogenicity (De Groot and Scott, 2007; Strohl, 2015). Several mechanisms of protein metabolism can contribute to poor pharmacokinetics of protein therapeutics (Kontermann, 2011, 2016; Meibohm, 2012). In addition, immunogenicity of recombinant protein therapeutics is also one of the major challenges as it leads to development of neutralizing antibodies and undesirable immune responses in the patients, thus resulting in reduced efficacy, rapid excretion and multiple adverse effects (Baker et al., 2010; Chirmule et al., 2012; De Groot and Scott, 2007; Kimchi-Sarfaty et al., 2017; Purcell and Lockey, 2008). In this review, we have particularly focussed upon the use of chimerization approach to improve the pharmacokinetics of protein therapeutics.

Pharmacokinetics of Protein Therapeutics

Amongst various pharmacokinetic parameters, half-life, defined as the time required for the drug concentration to be reduced to one-half in the body, is of particular importance, as it is pivotal in determining the dosage frequency of the drug (Meibohm, 2012).

a) Issues with Non-conjugated Protein Therapeutics. As mentioned above, several protein therapeutics show suboptimal pharmacokinetic attributes. For instance, exenatide, which is an incretin mimetic, has a half-life of ~2.4 hrs. Similarly, glucagon like peptide (GLP-1) has a half-life of upto 2 mins, since it is cleaved readily by dipeptidyl peptidase 4 (DPP-4) (Bond et al., 2006; Diao et al., 2013; Strohl 2015). The short circulatory half-life of the protein therapeutic may result in issues like frequent dosing and patient incompliance, thereby making it important to address the metabolic mechanisms of protein therapeutics (Lagassé et al., 2017; Strohl, 2015).

b) Mechanisms of Protein Metabolism and Clearance. A protein, whether endogenous or externally administered, goes through the process of absorption, distribution, metabolism &

elimination in the body (Strohl, 2015). In terms of delivery, oral route is the most convenient, patient compliant and most preferred route for small molecule drugs (Meibohm, 2012). Although, a recent study has demonstrated successful oral delivery of insulin, but owing to gastrointestinal degradation and issues with bioavailability, oral route is generally not suitable for delivery of therapeutic proteins (Abramson et al., 2019; Meibohm, 2012; Tibbits et al., 2016). Interestingly, protein and peptide therapeutics have been delivered in the form of oral inhalations, as inhaled protein therapeutics with molecular weight of upto 40 kDa could achieve significantly high systemic bioavailability (de Kruijf and Ehrhardt, 2017). It is important to note that inhalable versions of insulin, marketed as Exubera® and Afrezza®, have been approved by the US-FDA (de Kruijf and Ehrhardt, 2017). In practice, the parentral route is considered to be most suitable for administration of protein therapeutics (Meibohm, 2012). Hence, intravenous (i.v.), subcutaneous (s.c.) and intramuscular routes (i.m.) are viable options for administration of protein therapeutics (Meibohm, 2012; Strohl, 2015).

After administration, the absorption of therapeutic proteins occurs mostly through the blood vessels and lymphatics (Strohl, 2015; Meibohm, 2012; Meibohm and Braeckman, 2007; Supersaxo et al., 1990). The distribution of proteins is mostly dependent upon their molecular weight, binding to other proteins in the plasma, their overall charge and their extent of lipophilic nature, however, owing to their large size, their distribution is largely confined to the extracellular compartment (Diao and Meibohm, 2013; Meibohm, 2012; Strohl, 2015). The metabolism and elimination of proteins occurs significantly by proteolytic mechanisms (Meibohm, 2012; Strohl, 2015). The renal route of elimination also contributes considerably to the metabolism of proteins and is mostly selective towards molecular size and charge (Meibohm, 2012; Strohl 2015). Proteins with molecular weight less than 60-70 kDa are eliminated relatively swiftly through glomerular filtration and the passage of negatively charged molecules is impeded in comparison to neutral or cationic molecules (Deen et al.,

2001; Meibohm, 2012; Strohl 2015; Tibbits et al., 2016). There is also a substantial contribution of liver to the metabolism of protein and depending on their size, proteins therapeutics may be taken up into the hepatocytes by passive diffusion or via uptake facilitated by carriers or receptors (Meibohm, 2012). The protein therapeutics also undergo receptor-mediated cellular uptake via the target receptor of the protein therapeutics expressed by any cell, by a process known as "target-mediated drug disposition", which can be regarded as stepping-stone in the elimination of protein therapeutic (Mager, 2006; Meibohm, 2012; Tang et al., 2004). In addition, the neonatal Fc receptor (FcRn) plays a pivotal role in the disposition of IgGs, and protein therapeutics fused with a fraction of Ig (e.g., Fc), or albumin, by a process called FcRn-mediated recycling (**Fig. 1**) (Kim et al., 2007; Kontermann, 2011; Meibohm, 2012; Roopenian and Akilesh, 2007; Schmidt, 2013b; Sockolosky and Szoka, 2015; Wang et al., 2008). It has been observed that, FcRn-mediated recycling, owing to differential affinity of Fc region towards FcRn, is responsible for varying circulatory half-lives of IgG subtypes, with IgG1, IgG2 and IgG4 having substantially long half-lives (~18 to 21 days) in comparison to IgG3 (~ 7 days) (Dirks and Meibohm, 2010; Kim et al., 2007; Meibohm, 2012). Thus, various mechanisms of clearance can be targeted for improving the pharmacokinetics of therapeutic proteins.

Strategies for Improving Pharmacokinetics of Protein Therapeutics

To improve the pharmacokinetic attributes of therapeutic proteins several approaches have been devised (**Fig. 2**) (Kontermann, 2011, 2016; Strohl, 2015; Zaman et al., 2019).

a) Mechanisms for Improvement in Pharmacokinetics of Protein Therapeutics. One of the most common approach is to increase the hydrodynamic radius of protein therapeutics, which would lead to reduced renal clearance and increased residence time in the circulation (Kontermann, 2011, 2016; Strohl, 2015). Second approach is to utilize formulations that

entrap the therapeutic proteins thereby resulting in decreased proteolysis and recognition by phagocytic cells (Hartung and Bendas, 2012). A third approach is to impart negative charge onto the proteins, which would selectively impede their renal clearance (Kontermann, 2011, 2016; Meibohm, 2012; Strohl, 2015). Lastly, attachment or genetic fusion with another protein/domain or recombinant polymeric peptide repeats that have inherently long half-life can be used as an approach to increase the hydrodynamic radius and facilitate FcRn-mediated recycling (Kontermann 2016; Meibohm, 2012; Strohl, 2015). These approaches of improving pharmacokinetics of therapeutic proteins are discussed in this section.

b) Conjugation, Attachment and Modification. Researchers have devised several methods of chemical conjugation; with one of the classical method being PEGylation (Kontermann, 2012; Zaman et al, 2019). PEGylation is described as covalent bonding of polyethylene glycol (PEG) moieties with therapeutic proteins (Kontermann, 2012). It has been observed that, binding of a few molecules of water to the ethylene glycol subunit results in up to 10-fold increase in size of the PEGylated molecule in comparison to a protein of similar mass (Swierczewska et al., 2015). Therefore, binding to PEG leads to considerable increase in the size and mass of PEGylated molecule, thereby increasing the hydrodynamic radius and impeding the clearance of PEGylated molecule, consequently resulting in increased half-life (Kontermann, 2012). Furthermore, the PEG conjugation also protects the therapeutic protein from proteolysis and immunological response (Jevševar and Kunstelj, 2012; Swierczewska et al., 2015). In addition, conjugation with other carbohydrates (e.g., glycosylation, HESylation, polysialylation) and synthetic polypeptides (e.g., PEPylation) have also gained attention as an alternative to PEGylation (Fares, 2012; Hou et al., 2019; Li and d'Anjou, 2009; Kontermann 2011, 2012, 2016; Sinclair and Elliott, 2005; Solá and Griebenow, 2010; Vugmeyester et al., 2012).

c) Encapsulation and Surface Binding. Several pharmaceutical delivery systems, such as liposomes and other polymeric formulations, have also been utilized to improve the circulatory half-life of therapeutic proteins (Colletier et al., 2002; Hartung and Bendas, 2012; Landfester et al., 2012). Liposomes are bilayered phospholipid vesicles with hydrophilic interior; moreover, PEG modification of liposomes helps surpass clearance by reticuloendothelial system or by phagocytic cells, thereby leading to increased half-life (Hartung and Bendas, 2012). Encapsulation in liposomes have been shown to increase the half-life of tumor necrosis factor (TNF-α) and interleukin 2 (IL-2) (Hartung and Bendas, 2012). Another approach to improve pharmacokinetics using liposomes, is non-covalent interaction of the protein (for e.g., recombinant factor VIII (rVIII)) on the surface of PEGylated liposomes (Hartung and Bendas, 2012). In addition, certain polymeric nanoparticles, nanocapsules and nanoghosts are also used for pharmacokinetically efficient delivery of peptides and proteins (Kontermann, 2012; Krishnamurthy et al., 2019; Landfester et al., 2012; Pisal et al., 2010; Swed et al., 2014).

d) Focus on Chimerization of Protein Therapeutics.

1) The Concept and Molecular Biology of Chimerization. The word ‘chimaera’ also spelled as chimera, hold its origins in the Greek mythology, where chimaera is defined as a monstrous creature of Lycia, which is believed to be a hybrid (Peck, 1898). Chimaera is portrayed as having combination of physical attributes of a lion, a goat and a snake (Peck, 1898). The concept of chimerism is significant in terms of genetics because it exists in both animal and plant kingdoms, furthermore, experimentally generated chimeras have been serving as useful tools for developmental biologists (Dunsford, 1953; Eckardt et al., 2011; Fontaine-Pérus, 1999; Norris et al., 1983; Santelices, 2004). Moreover, certain chimeras, in their incipient stages, could also act as source of organs for transplantation in the future (Blakemore, 2017).

In terms of protein therapeutics, chimerization is a process where a hybrid is generated with genetic fusion of multiple distinct entities (Baldo, 2015). Hence, in protein chimerization, the idea behind the mythological creature 'chimera' is utilized to produce a molecule with superior properties. A typical protein chimera is produced by connecting genes of the protein molecule of therapeutic interest ('effector') to another protein/domain (referred to as 'helper') with the help of a 'linker', where the effector molecule possesses a myriad of pharmacological activities (Czajkowsky et al., 2012; Baldo, 2015; Schmidt, 2013a). The protein chimera or chimerized protein is also referred to as a 'fusion protein' or 'chimeric protein', and these terms are used interchangeably (Baldo, 2015; Kontermann, 2012, 2016). The 'helper' imparts stability to the molecule and helps in targeting the effector (Baldo, 2015). Several of these helpers utilized as half-life extension partners (HLEP) are either full length proteins or truncated domains of proteins, and result in a considerable increase in the half-lives of the effector molecules (Kontermann, 2011, 2016; Strohl, 2015). The purpose of the 'linker' is to connect the 'effector' and 'helper' in a way that allows optimum functionality so that the whole chimerized molecule can execute its operation (Baldo, 2015, Schmidt, 2013a).

The protein chimerization for half-life extension involves, fusing the therapeutic protein with a HLEP, which has inherently longer half-life for e.g., albumin fusion (**Fig. 3**). (Meibohm, 2012; Strohl, 2015; Sun and Micheals, 2018). Fusing multiple repeats of amino acid sequences (recombinant polymeric peptide repeats) with the effector protein, also lead to increased hydrodynamic radius, for e.g., elastin like polypeptides (ELPs) (Kontermann 2016; Strohl, 2015) (**Fig. 3**). Using a negatively charged protein fragment that decelerates renal elimination has also been utilized as HLEP, for e.g., C-terminal peptide (CTP) of human chorionic gonadotropic hormone (Kontermann 2016; Meibohm, 2012; Strohl, 2015; Sun and Micheals, 2018) (**Fig. 3**).

2) Various HLE Partners Utilized to Enhance Half-Life of Protein Therapeutics. As discussed above, a typical chimeric protein therapeutic is composed of three components; the effector protein is fused to half-life extension partner (HLEP) via a linker peptide. Various partners/helpers utilized for improving the pharmacokinetics of therapeutic effector proteins are elaborated in this section (**Fig. 3**).

i) Crystallisable Fragment of Immunoglobulin (Fc). Fusion with the Fc region of IgG is one of the most popular approach utilized to prolong the half-lives of protein therapeutics (Kontermann, 2016; Richter et al., 2019; Strohl, 2015; Wu and Sun, 2014; Zaman et al., 2019). As discussed above, FcRn-mediated recycling plays an important role in recirculation of proteins, particularly in case of fusions containing the Fc region (**Fig. 1**) (Meibohm, 2012; Rath et al., 2015; Sockolosky and Szoka, 2015; Ward and Ober, 2018). Since the immunoglobulins, contain the Fc-region, they have substantially long half-lives for e.g., IgG1, IgG2 and IgG4 (Huang, 2009; Kontermann, 2016; Meibohm, 2012; Sockolosky and Szoka, 2015). In case of therapeutic antibodies, half-life extension of as much as four weeks have been observed (Keizer et al., 2010; Kontermann, 2016). The function of Fc-fusion is to bestow properties such as FcRn-mediated recycling to decrease the metabolism of the therapeutic proteins (Kontermann, 2016; Rath et al., 2015; Strohl 2015). However, Fc fusions do not possess half-lives as long as the immunoglobulins, which could be in part due to the involvement of fragment antigen binding (Fab) of Ig towards FcRn binding (Schoch et al., 2015; Souders et al., 2015; Suzuki et al., 2010; Unverdorben et al., 2016). Several undesirable effects are also associated with Fc fusion, such as, it may facilitate antibody dependent cellular cytotoxicity (ADCC) and phagocytosis and complement fixation (Kontermann, 2016). By introducing mutations at specific positions in the Fc region and using particular isotypes of IgG that do not bear such effects, such as IgG4, these undesirable effects can be subsided (Kontermann, 2016). Fc fusion also provides the flexibility of fusing

effector molecule(s) at either or both *N*- and *C*-terminus (Kontermann, 2016). Several Fc fusions are developed and approved by various regulatory authorities, including etanercept (TNF receptor fusion), aflibercept (VEGF receptor fusion) and rilonacept (IL-1 receptor fusion) (Huang, 2009; Jazayeri and Carroll, 2008; Kontermann, 2016; Strohl, 2015). Mutations in the Fc regions have been performed for optimizing the properties of the resultant fusions and to overcome undesirable effects. For example, dulaglutide, a fusion of GLP-1 with Fc region of IgG4, is a GLP-1 receptor agonist with mutations F234A and L235A (Kontermann, 2016). These mutations decease Fcγ receptors interaction and ADCC induction (Kontermann, 2016). The mutation S228P in dulaglutide prevents the formation of half-antibodies (Glaesner et al., 2010; Kontermann, 2016). When compared to other GLP-1 agonists, such as exenatide and liraglutide, which require daily administration, dulaglutide has to be applied only once in a week (Kontermaan, 2016). Mutations which cause an upsurge in FcRn binding, thereby increasing the half-life, can also be incorporated in the Fc region (Kontermann, 2016; Kuo and Aveson, 2011; Presta, 2008; Wang et al., 2014a). Several mutations that have led to considerable half-life improvements of IgGs are reported in the literature (Bas et al., 2019; Kontermann, 2016; Kuo and Aveson, 2011; Presta, 2008; Wang et al., 2014a). For example, motavizumab which is a monoclonal antibody for respiratory syncytial virus incorporates three mutations in the Fc region namely M252Y, S254T and T256E (Kontermann, 2016; Robbie et al., 2013). These mutations increase the FcRn binding upto 10-fold and the half-life in serum from 2- to 4-fold (Kontermann 2016; Liu et al., 2018; Robbie et al., 2013). Moreover, it has been reported that hypersialylation of aspargine residue at 297 position, achieved through deletion of glutamate residue at 294, could prolong the residence of Fc in the serum (Bas et al., 2019). Also, fusion with certain FcRn binding peptides (FcRnBPs), has also led to enhancement in the half-life of Fab domain (Datta-Mannan et al., 2019). Apart from direct Fc chimerization, fusion with IgG-binding

domain of *streptococcal* G protein, which possesses binding capability towards Fc, has also been utilized for half-life extension (Unverdorben et al., 2015; Zong et al., 2019). Eftrenonacog-α, marketed as Alprolix®, which is a Fc-fused-factor IX for treatment of haemophilia, is an example of recently approved Fc fusion therapeutic **(Table 1)** (Graf, 2018; Shapiro et al., 2012; Shapiro et al., 2019; Strohl, 2015). In addition, Eloctate® (Fc fused factor VIII) has also been studied clinically for the treatment of haemophilia (Mahlangu et al., 2018). Studies with Fc-fused cocaine hydrolase for the treatment of cocaine abuse and fusion of growth hormone with single chain Fc-dimer have also been performed (Chen et al., 2018; Zhou et al., 2017).

ii) Albumin. It has been observed that the half-life of albumin, a predominantly abundant protein in the serum, is upto 19 days (Kontermann, 2016; Sleep, 2015). FcRn-mediated recycling also plays a pivotal role in preventing albumin catabolism, but since albumin binds to FcRn at a site different from IgGs, there is no interference with the recycling of IgGs (Larsen et al., 2018; Sand et al., 2015). The first and the third domain of albumin are found to interact with FcRn in a pH-dependent manner (Sand et al., 2014). Since albumin acts as a transport protein, it can be used for half-life improvement of protein therapeutics (Kontermann, 2016; Sleep, 2015). Half-life extension approaches using albumin involve either binding to albumin, via various interactions and conjugations, fusion of target proteins to albumin-binding moieties or by generating chimeras with direct albumin genetic fusion (Fuchs and Igney, 2017; Kontermann, 2016; Larsen et al., 2016; Lee and Youn, 2016; Ramírez-Andersen et al., 2018; Taraghdari et al., 2019; Zaman et al., 2019). Albumin-binding moieties include certain fragments of antibodies (such as Fab), single chain variable fragment (scFv), domain antibodies (dAbs), nanobodies, albumin-binding domains (ABDs) and DARPin® domains and albumin binding peptides (Goodall et al., 2015; Ikeda et al., 2019; Jacobs et al., 2015; Jank et al., 2019; Khodabakhsh et al., 2018; Kontermann, 2016; Li et al.,

2016; Rycroft and Holt, 2012; Schmidt et al., 2013; Seijsing et al., 2018; Sleep, 2015; Sleep et al., 2013; Steiner et al., 2017; Tijink et al., 2008; Van Roy et al., 2015). When albumin binding moieties, attached to therapeutic proteins by genetic fusion, are administered, they bind to serum albumin thereby increasing the half-life of fused therapeutic (Kontermann, 2016). However, direct fusion with albumin is one of the significant approaches for half-life extension and has been utilized for a wide variety of molecules with therapeutic properties (Hoogenboezem and Duvall 2018; Kontermann, 2016, Müller et al., 2007, Strohl, 2015). One notable example is the fusion of human serum albumin with GLP-1, referred to as albiglutide, has a half-life ranging from 4 to 7 days, and has been approved for diabetes treatment **(Table 1)** (Bush et al., 2009; Trujillo et al., 2014; Strohl, 2015). Another example is the fusion of albumin to factor-IX, referred to as albutrepenonacog alfa and marketed as Idelvion® for haemophilia treatment (Chia et al., 2018; Graf, 2018; Santagostino et al., 2016). Moreover, other clotting factors such as factor VIIa, VIII and X have also been fused with albumin (Ferrarese et al., 2019; Schulte, 2009; Tiede et al., 2015). Recently, fusion of albumin with glucarpidase and Kunitz protease inhibitor domain of protease nexin 2 have also been investigated (AlQahtani et al., 2019; Sheffield et al., 2018). Derivatives of albumin with enhanced properties can be produced by introducing mutations at certain positions. Such as a mutation K573P results in enhanced FcRn affinity by upto 11-fold, thereby leading to increased half-life (Andersen et al., 2014; Kontermann, 2016). In addition, fusion to albumin can be performed at both *N*- or *C*-terminus or individually at either *N*- or *C*- terminus (Andersen et al., 2014; Kontermann, 2016; McDonagh et al., 2012; Müller et al., 2007; Rogers et al., 2015; Strohl, 2015).

iii) Transferrin. Transferrin (Tf), which is a highly abundant monomeric glycoprotein of 80 kDa and takes part in uptake of iron in the cells by exocytosis and receptor-mediated endocytosis (through Tf receptor), is also used as HLEP (Chen et al, 2013a; Kontermann,

2016; Li and Qian, 2002; Strohl, 2015). It is found to have a half-life of upto 17 days, however the glycosylated counterpart of Tf may have a reduced half-life of upto 10 days, and hence the non-glycosylated Tf is preferred as a fusion partner (Kim et al., 2010; Kontermann, 2016; Strohl, 2015). The prominent example of Tf fusion is the fusion of GLP-1 agonist and exendin-4 with non-glycosylated Tf (Kim et al., 2010). Fusions of GLP-1 with Tf have shown a half-life of 44 hrs in cynomolgus monkey model and resulted in decrease in blood glucose and increase in insulin secretion (Kim et al., 2010; Kontermann, 2016). In another study, the Tf-proinsulin fusion displayed a substantial increment in the elimination half-life of 15-folds in comparison to the half-life of unfused proinsulin **(Table 1)** (Wang et al., 2014b). Moreover, a recent study with Tf-proinsulin fusion demonstrates that the fusion possessed a lowering effect on blood glucose levels for upto 40 hrs (Shao et al., 2016). Other examples include development of Tf fusions with growth hormone and granulocyte colony stimulating factor (Chen et al, 2011; Kontermann, 2016).

iv) Fusion with Other Half-Life Extension Partners. As a substitute to PEGylation, certain approaches have been developed which utilize recombinant fusion of target protein with recombinant polymeric peptide repeats that are also termed as PEG mimetics (Kontermann, 2012, 2016; Strohl, 2015; Sun and Micheals, 2018). Fusion to these recombinant polypeptide chains leads to increase in the hydrodynamic radius of the chimeric proteins thereby, causing impeded renal elimination (Kontermann 2016; Strohl, 2015). The most prominent examples to this approach include XTENylation, ELPylation and PASylation (Kontermann 2016; Strohl, 2015; Sun and Micheals, 2018). XTENylation is a half-life extension approach developed by Amunix, which utilizes fusion of polymeric amino acid sequences (alanine, glutamic acid, glycine, proline, serine and threonine), to the target protein (Schellenberger et al., 2009; Strohl, 2015). XTEN polymer of 864 amino acids in length has been shown to extend half-lives of several therapeutically important proteins both in the form of fusion and

chemical conjugation (Alters et al., 2012; Podust et al., 2013, 2016; Schellenberger et al., 2009; Strohl, 2015). One of the examples for XTEN fusions is VRS-317 (somavaratan), a fusion of XTEN and human growth hormone (hGH) **(Table 1)** (Cleland et al., 2012; Moore et al., 2016; Yuen et al., 2013). Another example of polypeptide repeats used for half-life extension is ELPylation, which utilizes peptide polymer repeats that are generally found in hydrophobic domain of Elastin, hence the term, Elastin-like peptides (ELPs) (Hassouneh et al., 2012; MacEwan and Chilkoti, 2014; Strohl, 2015). ELPs are formed with combination of five peptides valine-proline-glycine-x-glycine, where x could be any guest amino acid apart from proline (Floss et al., 2010, 2013; Hassouneh et al., 2012; MacEwan and Chilkoti, 2014; Strohl, 2015). ELPs are biodegradable owing to their metabolism by the elastases in the body (Strohl, 2015). ELPylation impedes kidney clearance by increasing the hydrodynamic radius, thereby enhancing the half-life of protein therapeutics (Conrad et al., 2011). Some examples of ELPylated fusions include Glymera ^{TM} (PB1023) which is a GLP-1 fusion, and Vasomera^{TM}, a fusion with vasoactive intenstinal peptide **(Table 1)** (Strohl, 2015). Recently, a study has shown that ELPylation of interferon alpha (IFN-α) led to the formation of sustained release depot that significantly prolonged the action of IFN-α for anti-tumor activity (Wang et al., 2019). PASylation is another approach towards half-life extension of therapeutic proteins, where recombinant polymeric repeats are formed by using proline, alanine and serine amino acids (therefore termed as PASylation) (Binder and Skerra, 2017; Breibeck and Skerra, 2017; Gebauer and Skerra, 2018; Schlapschy et al., 2013). Several studies for half-life extension using PASylation are reported in the literature, one of the notable examples being the PASylation of exenatide where a PAS repeat of 600 amino acids led upto 100-folds increment in the half-life (Harari et al., 2014; Gebauer and Skerra, 2018; Strohl, 2015; Schlapschy et al., 2013). A recent example for PASylation, is the fusion of Adnectin C with PAS repeat of 200 amino acids, which led to increase in half-life by a factor

of 4.5 **(Table 1)** (Aghaabdollahian et al., 2019). In addition to the above-mentioned approaches for half-life extension, some others include HAPylation, which utilizes homo-amino-acid polymers (HAPs) that consist of glycine rich repeat sequences, and Gelatin-like protein (GLK) (Kontermann 2009; Huang et al., 2010; Schlapschy et al., 2007; Strohl, 2015). Apart from using polypeptide repeats, fusions of therapeutic proteins with carboxy terminal peptide (CTP) of the β-subunit of human chorionic gonadotrophic hormone have also been generated (Calo et al, 2015; Fares et al, 1992; Fares and Azzam, 2019). This imparts or increases the negative charge on the chimeric protein thereby impeding renal elimination (Calo et al., 2015; Fares et al., 1992). Some recent examples of CTP fusion include MOD-4023, which is a fusion with human growth hormone and MOD-5014 that is CTP fusion with factor VIIa **(Table 1)** (Bar-Ilan et al., 2018; Hershkovitz et al., 2016; Strasburger et al., 2017).

v) Linkers: the Bond Between the 'Effector' and 'Helper'. The selection or rational design of a linker to join the effector and HLEP protein is a critical area in chimeric protein technology (Chen et al., 2013a, 2013b; Kontermann, 2011, 2016). The linker peptide helps to connect the protein components, and could also have a pivotal role in inter-domain/ inter-protein interactions and in preserving the respective biological activity (Gokhale et al., 2000). Moreover, linkers can also have positive effects on the stability, activity and pharmacokinetics of chimeric proteins (Chen et al., 2013b). Direct fusion of proteins to generate a chimera without any linker may lead to unwanted effects, such as low yield and reduced activity (Amet et al., 2009; Bai et al., 2005; Bai and Shen., 2006; Chen et al., 2013b; Zhao et al., 2008). Thus, choice and design of linkers is very important. Based on their attributes, linkers can be classified as flexible, rigid and cleavable **(Table 2)** (Chen et al., 2013a, 2013b). Flexible linkers are used when the protein partners in the chimera require movement, interaction and maintenance of a certain distance between them. They are

composed of small, non-polar or polar amino acids. The polar amino acids facilitate hydrogen bonding and the small size of amino acids helps to achieve flexibility (Argos et al., 1990; Chen et al., 2013a, 2013b). The most commonly used flexible linkers is the GS linker with sequences consisting of glycine and serine residues and the sequence formula (Gly-Gly-Gly-Gly-Ser)n or $(G_4S)_n$ is the most popular among flexible linkers; where n is the number of repeats (Chen et al., 2013a, 2013b) **(Table 2)**. Other examples of flexible linkers include $(Gly)_8$ or $(G)_8$ linker, and the linkers used to create single-chain variable fragment (scFv) such as KESGSVS and EGKSSGSGSESKST (Bird et al., 1988; Chen et al., 2013a, 2013b; Sabourin et al., 2007). The flexible linkers however, owing to their high flexibility, might not allow the separation of the operational domains/protein partners (Chen et al., 2013b). Moreover, it has been reported that the use of flexible linker may result in failed expression of certain chimeric constructs (Amet et al., 2009; Bai and Shen, 2006; Chen et al., 2013b).

Therefore, where a spatial separation of domains/protein partners is required, rigid linkers are utilized since they act as inflexible spacer peptides that separate domains/protein partners **(Table 2)**. Due to this inflexible distance, the domains/protein partners are relatively free to perform their respective functions (Chen et al., 2013a, 2013b). Two types of rigid linkers are predominantly mentioned in the literature. The first is the α-helical linker with the formula $(EAAAK)_n$, where n is the number of repeats, the second is the rigid $(XP)_n$ linker, where n is number of repeats and P is proline; here X can be any amino acid, however, generally alanine, lysine and glutamine are utilized **(Table 2)** (Chen et al., 2013a, 2013b). Due to the inability of proline to form hydrogen bonds, the domain-linker/protein-linker interaction is avoided, and this increases the rigidity leading to efficient separation of protein partners in the chimera (Chen et al., 2013a, 2013b). The first two categories of linkers contain non-cleavable and stable sequence of peptides that can impart several advantages including conformational flexibility, improved stability and activity (Chen et al., 2013a, 2013b).

However, with the use of these stable peptide linkers several detrimental effects such as decrements in activity, steric hindrances in domains/protein partners have been observed (Chen et al., 2013a, 2013b). To overcome this, a third category, of the cleavable linkers, is used with the intention of releasing free functional domains/protein partners *in vivo* (Chen et al., 2010, 2013a, 2013b) **(Table 2)**. These linkers are cleaved under some specific conditions such as presence of reducing reagents or proteases. The first type in this category is the *in vivo* cleavable disulfide linker. One of the examples of this is the dithiocyclopeptide linker, which is cleaved in presence of a reducing environment (Chen et al., 2013a, 2013b). **(Table 2)**. The second type are the *in vivo* protease sensitive linkers where the chimera is designed with a linker containing a protease sensitive sequence (Chen et al., 2013a, 2013b). An example of this is a fusion between recombinant factor IX (rFIX) and albumin, where a sequence VSQTSKLTRAETVFPDV, from N-terminal region of FIX, which is susceptible to proteolytic cleavage, is incorporated **(Table 2)** (Chen et al., 2013a, 2013b; Schulte et al., 2009). This causes several fold increments in the clotting activity of the chimeric protein as compared to the chimeric protein with non-cleavable linkers (Chen et al., 2013b; Schulte et al., 2009).

Protein Chimerization for Miscellaneous Specialized Purposes

The choice of 'helper' protein is primarily based on the desired functions of the chimeric protein, since the helper partners (apart from half-life extension) can also be utilized to generate chimeras with other properties such as cancer targeting and permeation through blood brain barrier (BBB) (Hoogenboezem and Duvall 2018; Jank et al., 2019; Pardridge, 2015; Tijink et al., 2008). For instance, human paraoxanase-1 (PON-1) fused with the *C*-terminus of heavy chain of monoclonal antibody against human insulin receptor (HIRMAb) has the ability to cross BBB (Boado et al., 2008). In addition, NHS-IL12, a chimera of IL-2 and NHS76 (an IgG1 targeting tumor necrosis) not only displayed half-life extension, but

also showed selectivity in targeting tumors in mice (Fallon et al., 2014). In addition, an antibody developed with fusion of scFv with Fc, showed significant *in vitro* growth inhibition of *Staphylococcus aureus* (Wang et al., 2019**)**.

Chimerization with HLEPs could also have synergistic effect on the pharmacological activity of the effector, for eg., Fc fusions with Osteopontin and Follistatin-288 have shown to enhance the osteogenic effect and promote localized growth of skeletal muscles, respectively (Castonguay et al., 2019; Rattanpasit et al., 2019). In addition, fusion of canine interferon gamma with canine serum albumin not only lead to improved pharmacokinetics but also improved antitumor efficacy (Li et al., 2019). Similarly, fusion of recombinant immunotoxins with ABDs led to increased half-life and significant increment in the antitumor effects of these immunotoxins (Wei et al., 2018). Thus, effector proteins with different functions can be chimerized together to produce a multifunctional protein therapeutic (Jochems et al., 2019).

Comparison and Outlook on Shortcomings of Protein Chimerization versus Other Approaches Utilized for Improvement of Pharmacokinetics

Various approaches of half-life extension have their respective shortcomings. PEGylation for instance has many disadvantages that include reduced activity of the conjugated protein, increased aggregation, unusual behaviour of the therapeutic such as increase in activity in some cases (Kontermann, 2016; Pisal et al., 2010, Wang et al, 2007). Since PEG is non-degradable in circulation, it may lead to renal, hepatic and splenic vacuolization (Qi and Chilkoti, 2015; Pelegri-O'Day et al., 2014; Zhang et al., 2014). Immunological response subsequent to administration of PEGylated molecules is another prevailing issue (Swierczewska et al., 2015; Kontermann, 2016). Development of antibodies against PEGylated therapeutic products such as Krystexxa® (PEGylated Uricase) and

Oncospar® (PEGylated Asparginase) has been reported and may lead to accelerated clearance of the therapeutic (Armstrong, et al., 2007; Garay et al., 2012; Sundy et al., 2001; Swierczewska et al., 2015). In case of glycosylation, the performance of the therapeutic protein may be affected by aberrant glycosylation patterns that can further lead to rapid clearance through mannose- and asialoglycoprotien-receptors and in addition certain carbohydrate structures may lead to development of an immune response (Vugmeyester et al., 2012; Jenkins et al., 1994, 1996). In case of pharmaceutical formulation such as liposomes, their stability in blood is questionable due to low critical micelle concentration (CMC) (Landfester et al., 2012).

One of the important issues faced by protein therapeutics is the formulational stability of the finished product (Strohl, 2015). The critical aspect of formulation stability lies in poor solubility and tendency of chimeric proteins to form aggregates and micelles, however, the introduction of glycosylation sites in the chimera might help in overcoming aggregation (Huang and Swanson, 2013; Strohl, 2015). In addition, immunogenicity is the bottleneck for the application of therapeutic proteins (Baldo, 2015; De Groot and Scott, 2007; Jawa et al., 2013). It is crucial that the chimeric protein must elicit negligible to very low immune response, subsequent to administration, this is particularly important for chimeras developed for long-term therapy (Swierczewska et al., 2015; Strohl, 2015). Proteins are recognized by the immune system owing to the presence of T and B cell epitopes, in case of chimerized proteins, immunogenic response against the protein of interest could potentially worsen the disease condition in patients (Purcell and Lockey, 2008; Strohl, 2015). In addition, chimerization of proteins may lead to the formation of new epitopes that may elicit further immunogenic response (Strohl, 2015). Furthermore, several aspects relating to molecular structure and formulation affect the immunogenicity of therapeutic proteins (Purcell and Lockey, 2008; Schmidt, 2013a; Strohl, 2015; Strohl and Strohl, 2012). However, there have

been lesser incidences of immunogenic response and anti-drug antibody formation subsequent to administration of chimeric proteins (Strohl, 2015). One of the methods utilized to reduce immunogenicity, includes the prediction and elimination of T cell epitopes in a chimeric protein (Strohl, 2015). In addition, stability of finished product (such as optimal solubility and lack of aggregation) also help in eliminating the development of immunogenic response (Baldo, 2015; Jawa et al., 2013; Strohl, 2015; Strohl and Strohl 2012).

In case of Fc fusions, the issue of inconsistent glycosylation either in linker or in chimera and the functionality of Fc domain needs attention (Strohl, 2015). Orientation of the effector molecule may also play a key role, as the binding of effector towards *N*- or *C*-terminus of the HLE partner may significantly affect its activity (Schmidt, 2013a). Hence, an attempt for fusion of target proteins should be made at both *N*- and *C*-terminus, to design chimera with maximum activity. In addition, since linkers also affect the activity and utility of chimeric proteins, the choice of linkers (rigid, flexible and cleavable) should be made as per the desired therapeutic effect of the target protein (Chen et al, 2013b; Schmidt, 2013a). Moreover, research should also be focussed towards exploring the different avenues of linker design, to provide much wider variety and combinations.

Studies done on recombinant polymeric peptide repeats have been limited in contrast to Fc, HSA and transferrin fusion (Strohl, 2015). Therefore, much is unknown about these approaches and elaborate studies are required to establish them as candidates for HLEP, and since these platforms are unnatural repeats of amino acids, use of these approaches may also raise several multifaceted issues (Strohl, 2015). Approaches such as HAPylation and GLK fusion offer small half-life improvements, and mostly are in their incipient stages, and much study is required to establish a clinical basis for the use of these approaches (Strohl, 2015). In case of CTP fusion, owing to the strong negative charge, the biological activity of the chimera may be affected (Strohl, 2015). However, considering the half-life extension

afforded by the fusion with recombinant polymeric peptide repeats and CTP, their use seems to be more beneficial than approaches involving conjugation and encapsulation (Bar-Ilan et al., 2018; Gebauer and Skerra, 2018; Hershkovitz et al., 2016; Strasburger et al., 2017; Strohl, 2015).

Therefore, considering all the currently known facts, it seems that the protein chimerization, is more advantageous in comparison to other approaches for half-life extension (e.g., PEGylation, glycosylation, liposome formulation) for delivery of protein therapeutics.

Conclusion and Future Prospects

Biopharmaceuticals are clearly leading the way for pharmaceutical therapy. The number of approvals from Jan 2015 to July 2018 (~ 3.5 years) were almost double in comparison to approvals in each five-year span from 1995-2014 (Walsh, 2018). Interestingly, amongst the products approved between Jan 2014 to July 2018, >90% were protein therapeutics that include mAbs, clotting factors, enzymes and vaccines (Walsh, 2018). This suggests that protein therapeutics have become the cornerstone of biological therapy. Furthermore, since their inception, half-life extension technologies have come very far and with the development of newer approaches like Fc fusion, albumin fusion, Tf fusion etc., we now have wide variety of approaches to choose from (Strohl, 2015; Kontermann, 2012, 2016). However, even with such remarkable advancements there are considerable challenges that need to be addressed.

With the increasing discovery of novel pathophysiological mechanisms of various diseases, the significance of protein therapeutics in the current scenario, for disease interventions is more than ever before. However, the application of an emerging protein therapeutic may be hindered due to its poor pharmacokinetic attributes. This clearly

emphasizes that mere discovery may not be enough for most protein therapeutics, engineering them for optimum pharmacokinetic and pharmacodynamics is equally important. Furthermore, creation of computational methods, programs or software applications for the design and engineering of the chimera, with integrated systems for the prediction of immunogenicity, can ease out and accelerate the development of chimeric proteins with improved of pharmacokinetic and pharmacodynamic properties (Paladino et al., 2017; Wang et al., 2018). It is important to note that programs for designing linkers for chimeric proteins are already available, e.g., LINKER and SynLinker (Crasto and Feng, 2000; Liu et al., 2015). Such advancements in computational approaches may also lead to the development of 'tailored protein therapeutics' with customizable half-life, which may even have possible applications in personalized medicine. Finally, the development of multifunctional half-life extended chimeric proteins (with multiple effector proteins) for complex disease interventions must be explored in much detail (Chen et al., 2011; Jochems et al., 2019).

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Figure legends:

Fig. 1. Schematic depiction of FcRn-mediated recycling of chimeric proteins: Proteins chimerized with Fc portion of Ig or albumin is taken up by non-specific mechanisms into the cell. Once taken up into the cell, the chimeric proteins bind to FcRn at pH 6.0 in the endosome and are recycled back into the circulation where the chimeric proteins dissociate from the FcRn, as a result of low affinity, due to shift in pH to 7.4**.** Unfused proteins that do not bind to FcRn are degraded in the lysosome at pH 4.0-5.0 **(**Sockolosky and Szoka, 2015).

Fig. 2. Various approaches available to increase circulatory half-life of protein therapeutics.

Fig. 3. Various HLEPs available for generation of chimeric protein therapeutics (Strohl, 2015).

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Table 1: Examples of protein partners used in the generation of chimeric protein therapeutics with improved half-life.

HLEP	Effector protein (drug name)	Half-life with and without chimerization		Disease indication	Development Status	References
		Without	With			
Fc fusion	Factor IX (eftrenonacog-α)	~18 hrs	~57 hrs	Haemophilia	Approved by US-FDA in 2014	Shapiro et al., 2012; Strohl, 2015
Albumin fusion	GLP-1 (albiglutide)	1-2 mins	~4-7 days	Diabetes Mellitus	Approved by US-FDA in 2014	Kontermann, 2016; Strohl, 2015
Tf fusion	Proinsulin	0.5 hrs	>7 hrs	Diabetes Mellitus	Preclinical (BALB/c mice)	Wang et al., 2014b
XTENylation	Growth hormone (GH) (VRS-317 /somavaratan)	1.7 hrs	131 hrs (in adult)	GH deficiency	Phase 2 for adults (NCT02526420) Phase 3 for children (NCT0233	Fares et al., 2010; Moore et al., 2016; Strohl, 2015; Yuen et al., 2013

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PASylation	Adenectin	49 mins	226 mins	Cancer	Preclinical (BALB/c mice)	Aghaabdollahian et al., 2019
ELPylation	GLP-1 (PB1023)	1-2 mins	~ 36 hrs	Diabetes Mellitus	Phase 2 (NCT01658501)	Strohl, 2015
CTP fusion	Growth hormone (GH) (MOD-4023)	1.7 hrs	~33-37 hrs	GH deficiency	Phase 3 (NCT02968004)	Fares et al., 2010; Hershkovitz et al., 2016; Strasburger et al., 2017

The NCT number represents the clinicaltrials.gov identifier for the clinical study of the respective drug molecule. Web addresses for information taken from clinicaltrials.gov (Accessed 02-04-2019):

https://clinicaltrials.gov/ct2/show/NCT02526420;

https://clinicaltrials.gov/ct2/show/NCT02339090;

https://clinicaltrials.gov/ct2/show/NCT01658501;

https://clinicaltrials.gov/ct2/show/NCT02968004.

Table 2: Various linkers used for chimerization of proteins (Chen et al., 2013a; Chen et al., 2013b).

Type of Linker	Properties	Example
Flexible Linkers	➢ Composed of small polar/non polar amino acids. ➢ Used when domains require movement. ➢ Do not allow separation of operational domains/protein partners.	$(G_4S)_n$, $(G)_8$
Rigid Linkers	➢ Used when spatial distance between domains/protein partners is required. ➢ Freedom for domains to perform respective functions.	$(EAAAK)_n$, $(XP)_n$
Cleavable linkers	➢ Free functional protein partners released subsequent to cleavage *in vivo*. ➢ Cleaved in the presence of proteases or reducing agents.	VSQTSKLTRAETVFPDV, dithiocyclopeptide linker

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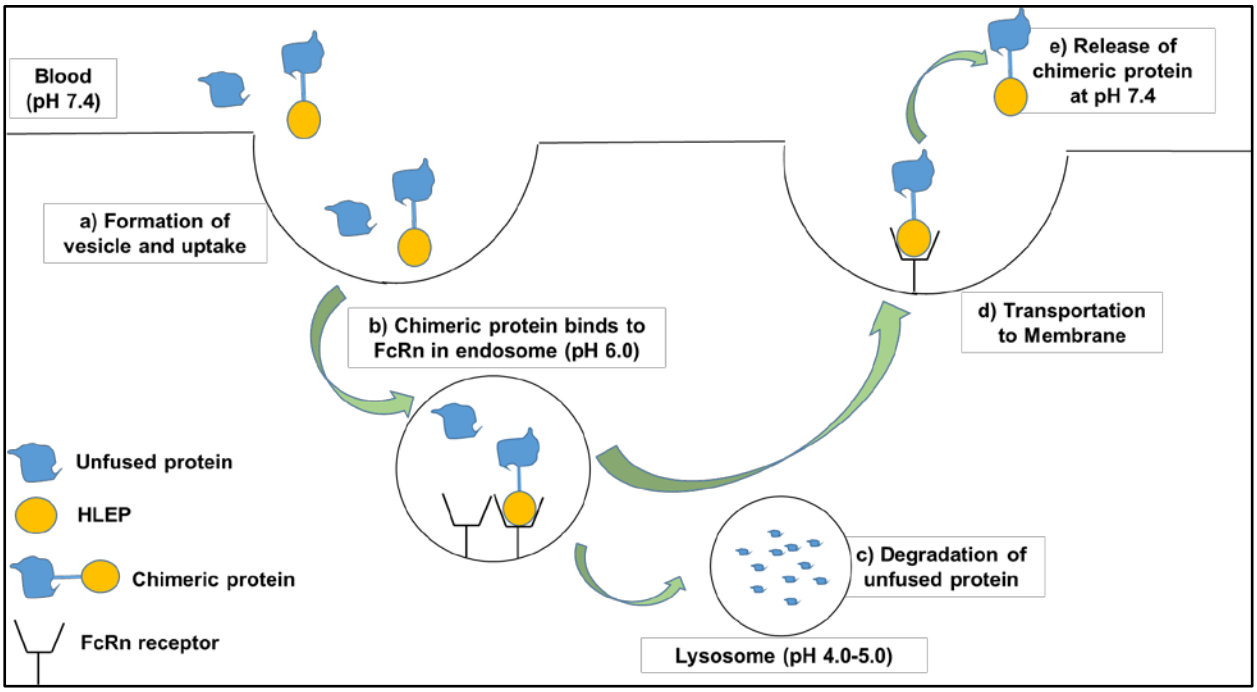
Figures:

Figure 1



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This article has not been copyedited and formatted. The final version may differ from this version.

Figure 2

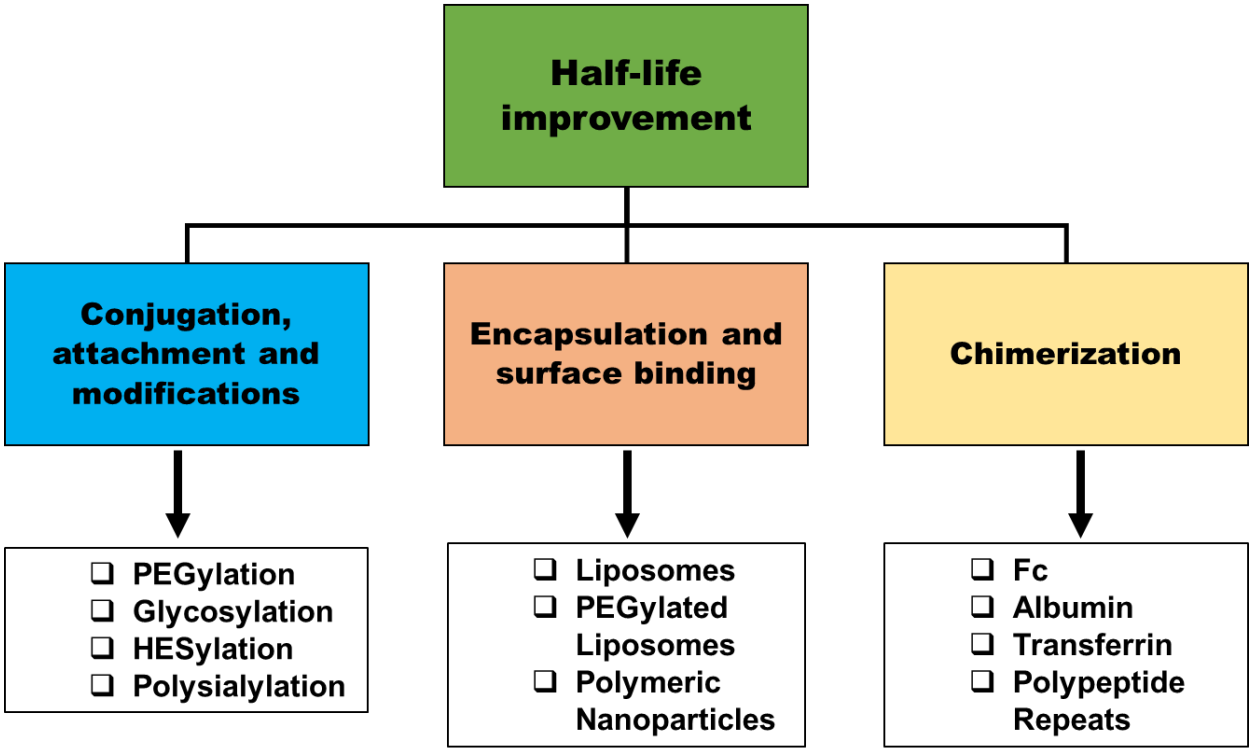

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Figure 3