

RSC Chemical Biology

Accepted Manuscript

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Strategies to Overcome Hepatic Clearance of Endogenous Proteins

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DOI: 10.1039/C6CB00041J

Molecular and Formulation Approaches

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Keywords: hepatic clearance; elimination; genetic engineering; bioconjugation; fusion proteins; Drug delivery systems

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1. Abstract

The medical use of biologics is often limited by rapid elimination from circulation. This review explores formulation strategies for enhancing the pharmacokinetic profiles of therapeutic proteins with hepatic clearance as major route of elimination. Pharmacokinetic and hepatic clearance mechanisms of selected endogenous proteins – tissue-type plasminogen activator, glucagon-like peptide 1, insulin and interferon- β – are detailed and discussed. Molecular optimization and formulation strategies for these four biologics illuminate the overcoming of pharmacokinetic limitations resulting in successful clinical approvals. The assessment of genetic engineering, bioconjugation, fusion proteins, and protein drug delivery systems for their potential to prolong serum half-life should facilitate prospective development



of pharmacological active macromolecules failing due to their rapid elimination in the liver.

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Overall, this review elucidates the relationships among formulation design, protein structure, and hepatic clearance, and their combined impact on therapeutic efficacy.

2. Liver and Hepatic clearance

The liver is the body's most important metabolic organ, responsible for detoxification, synthesis of plasma proteins, and biochemical substances that are necessary for digestion and growth. It receives oxygen-rich blood from the aorta via the hepatic artery, but also digested nutrients from the gastrointestinal tract, the spleen and pancreas via the portal vein. The blood must be freed from toxins, excess endogenous hormones or proteins and converted into more soluble and excretable metabolites.¹ For this purpose, these two large vessels are subdivided into small capillaries (liver sinusoids), which form functional units of the liver (hepatic lobules), each consisting of millions of hepatocytes – the basic metabolic cells. Hepatocytes make up 70 to 85% of the liver volume, while 2.8% consists of endothelial cells, 2.1% of Kupffer cells and 1.4% of hepatic stellate cells, about 16% are extracellular space.² Fenestration in the liver sinusoid endothelial cells (LSEC) of about 100 to 200 nm allows for unhindered plasma flow between blood and the liver cells (**Figure 1**).

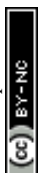
The liver plays a major role in drug metabolism of small molecules by providing metabolizing enzymes (e.g. cytochrome P450s), transferase enzymes, and transporter proteins.¹ The underlying metabolism pathways are different for Biologicals. Small peptides and proteins (< 60 kDa) are mainly eliminated renally by glomerular filtration.³ Similar to the liver endothelial cells, the glomerular barrier contains fenestrated endothelial pores (about 60 nm). However, in addition, a complex structure of charged proteoglycans in the basement membrane and podocytes with slit membranes (25-60 nm) makes the glomerular barrier in the kidneys a selective and fine filtration system.⁴ Small filtered proteins such as hormones (e.g. insulin) or



vitamin carrier proteins are reabsorbed endocytically in the proximal tubule by megalin and cubulin. Following endocytosis, they are either degraded lysosomally or, more rarely in the case of the latter, re-excreted into the blood by specific transport mechanisms.^{5, 6}

In contrast, the liver's LSECs facilitate the interaction of soluble macromolecules including proteins, peptides, metabolites or nanoparticle-based protein drug delivery systems with three predominant cell types involved in hepatic elimination: Hepatocytes, Kupffer cells and LSECs, which act together in hepatic metabolism (**Figure 1**). The most predominant pathway is receptor-mediated endocytosis (RME). The cargo molecule is internalized via clathrin-coated pits and finally degraded within lysosomal compartments. Interestingly, the expression profile of RME relevant receptors depend on cell type and location in the liver.⁷ In addition to specific receptors dedicated for hormones such as the insulin receptor, some receptors share affinity for a group of macromolecules, including the low-density lipoprotein (LDL) receptor for lipoproteins and the mannose/N-acetylglucosamine receptor for glycoproteins.⁸ Moreover, the internalization of soluble macromolecules can occur through non-specific fluid phases named pinocytosis. Kupffer cells, the largest tissue-resident macrophage population in the body, are the primary hepatic cells responsible for phagocytosis of larger particles and macromolecules.⁹⁻¹¹ Besides endocytosis pathways in hepatic elimination, the hepatocytes as the liver's parenchymal cell express a high density of membrane-bound proteases for example cleaving dipeptides from the N-terminus of polypeptides (dipeptidyl peptidase IV) or removing C-terminal amino acids (carboxypeptidases).^{12, 13} Protein-degrading enzymes are also secreted by the liver, including matrix metalloproteinases or cathepsins, both degrading extracellular matrix proteins.^{14, 15} The resulting peptide metabolites are often excreted renally.¹⁰

Due to the high filtration and metabolic capacity of the liver, the development of long-acting therapeutic proteins with hepatic clearance as major route of elimination needs to consider the mode of the elimination pathway in the liver.



3. Proteins with hepatic clearance as major route of elimination

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DOI: 10.1039/D6CB00041J

In the following, we detail four complementary endogenous proteins and peptides with major hepatic clearance from circulation, and how formulations strategies lead towards successful therapeutical use.

3.1. Tissue-type plasminogen activator

Tissue-type plasminogen activator (tPA) is a glycoprotein of about 65 kDa in either a single-chain form (sc-tPA; 527AA) or two-chain form (tc-tPA; 275AA and 252AA) connected via disulfide bridges.¹⁶ As a serine protease, it activates plasminogen to the fibrin-degrading enzyme plasmin. The high affinity of tPA for fibrin (dissociation constant of complex ~ 150 nM) and the stimulation of tPA by fibrin to the 1000-fold compared to absence of fibrin lead to specific activation of plasminogen at a blood clot and less activation of circulating plasminogen.¹⁷

The amount of circulating active tPA is regulated by rapid inhibition by its main inhibitor plasminogen activator inhibitor-1 (PAI-1). Depending on the tPA form, PAI-1 inhibits with a second-order rate constants of about 5.5×10^6 1/(M·s)(sc-tPA) and about 1.8×10^7 1/(M·s) (tc-tPA) (**Figure 2A**).¹⁸⁻²⁰

Transwell et al. showed a plasma half-life of 4.4 minutes for tPA in pharmacokinetic studies with 18 male subjects. Several studies confirmed this value with half-lives between 3 to 5 minutes.²¹⁻²³ The liver is the superior clearance organ compared to the kidney, which was as demonstrated by Brommer et al. by measuring tPA activity in renal and hepatic vein and arteries. Thereby, the high molecular mass of tPA is probably responsible for its inability to pass the glomerular barrier of the kidney.²³ Investigations of the elimination route in rats with ¹²⁵I marked tPA, revealed that 80% are rapidly cleared in the liver, whereby 54.5 % in parenchymal cells, 39.5 % in endothelial cells and 6 % in Kupffer cells.²⁴ The main clearance



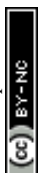
of tPA occurs there via two pathways. First, the mannose receptor on liver endothelial cells and Kupffer cells mainly binds free tPA. Second, the low-density lipoprotein (LRP) receptor interacts with free tPA and tPA complexed with its inhibitor PAI-1.²⁵⁻²⁷ Thereby, Chandler et al. confirmed that the tPA-PAI-1 complex is removed from the blood more slowly (48%) than active tPA (89%) when flowing through the liver, as the total tPA antigen rose even though tPA activity fell.²⁸

Despite its poor pharmacokinetic profile, recombinant tPA has been approved since 2000 in Europe for acute myocardial infarction, followed by approval for acute ischemic stroke and use in pulmonary embolism (Actilyse®).²⁹ To overcome rapid inhibition and hepatic clearance, high doses (0.9 mg/kg bodyweight) must be administered as a 10% initial intravenous bolus over 1-2 min, followed by a 60 min intravenous infusion of the remaining dose to achieve the required tPA concentration at the target site of the thrombus.³⁰ These concentrations cannot be compensated by PAI-1 in the rest of the body, leading to severe side effects such as intracranial hemorrhage. The mechanical properties of the thrombus evolve significantly over time, transitioning from a soft, compliant, and cell-rich structure into a stiffer, more fibrotic, and dense mass that is increasingly resistant to thrombolysis by tPA. Together these factors limit the treatment with tPA to a therapeutic window of up to 4.5 hours after symptoms onset.³¹⁻³³

3.2. Glucagon-like Peptide 1

Glucagon-like Peptide 1 (GLP-1) is a gastrointestinal hormone released as proglucagon from enteroglucagon cells in the small intestine.³⁴⁻³⁶ GLP-1 is post-translationally obtained from proglucagon in a 30 residues (3.3 kDa), amidated form (7-36) or 31 residues, glycine-extended peptide (7-37) whereby the former is predominantly in human with > 80 %.^{37,38}

Binding of GLP-1 to GLP-1R (glucagon like peptide 1 receptor) activates intracellular signaling pathways leading to insulin secretion and β -cell proliferation in the pancreas, inhibition of gastric emptying, acid secretion and motility in the stomach, and protective and



regulatory effects in various regions of the body, such as the brain, heart, and adipose tissue.³⁹

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GLP-1 is rapidly inactivated by ubiquitous dipeptidyl peptidase IV (DPP-IV). DPP-IV has a high specificity for N-terminal α -amino group of His7 and Ala8 cleavage resulting in GLP-1-(9-36)amide, followed by no further proteolytic cleavage (**Figure 2B**).⁴⁵⁻⁴⁷ Kieffer et al. showed that 50% of ¹²⁵I labeled GLP-1-(7-36)NH₂ was metabolized within 2 minutes to GLP-1-(9-36)NH₂ after infusion in rats. In DPP-IV-deficient rats, the cleavage products were not present.⁴⁸ The plasma half-life of the GLP-1 metabolites are about 3 minutes, cleared by the kidney.⁴⁹ While the kidney is the major extraction route for endogenous and exogenous GLP-1, the liver provides a high concentration of DPP-IV on hepatocytes, and is therefore one of the main sites of inactivation in addition to the soluble form of DPP-IV in plasma.⁵⁰

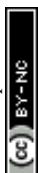
Due to the rapid clearance from plasma, GLP-1 administration was by 24 h infusion in type 2 diabetes mellitus patients. Despite the positive effects on the glycemic control, a continuous therapy with GLP-1 is impossible and demanded the development of long-acting derivatives.

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3.3. Insulin

Insulin consists of two polypeptide chains (A and B) linked by two disulfide bridges (MW 6 kDa).^{53, 54} Besides insulin's role in fat and protein metabolism, one of its main physiological functions is the stimulation of glucose uptake in muscle and fat cells via glucose transporter type 4 (GLUT4)-mediated exocytosis activated by binding to insulin receptor (IR).^{55, 56} Together with the hormone glucagon, insulin is therefore responsible for maintaining plasma glucose levels within a narrow range.⁵⁷

The clearance of insulin is predominantly within the liver.^{58, 59} Meier et al. confirmed by direct hepatic vein sampling in healthy humans that 80% of insulin secreted in pancreatic islet β -cells is extracted during the first liver passage.⁶⁰ Eaton et al. confirmed the hepatic removal



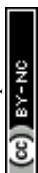
of insulin decreases from 67% to 42% by increasing insulin exposure from 2.6 to 13.2 U during dose-response glucose ingestion.⁶¹ This results in a short plasma half-life of 3 to 6 minutes.⁶²⁻⁶⁴ Although the kidney is also responsible for insulin excretion via glomerular filtration, the high density of insulin receptors (IR) and insulin-degrading enzymes (IDE) in the liver, which activate the insulin degradation after binding, makes it the main site of excretion.⁶⁵ Binding of insulin to IR initiates a two-step internalization process. First, the redistribution of the receptor from the microvilli to the non-villous membrane of the cell and second, the anchoring of the insulin-IR complex in clathrin-coated pits, which is mainly mediated by a transmembrane glycoprotein, CEACAM1.⁶⁶ Depending on the insulin plasma concentration, this degradation can be precisely controlled via CEACAM1 expression.⁵⁸

Although insulin offers unparalleled pharmacology, its pharmacokinetics make it difficult to use in chronic therapy. Nevertheless, the first basal insulin formulation with zinc and protamine crystallization (neutral protein Hagedorn, NPH), has been used for diabetes mellitus treatment since 1950, requiring multiple subcutaneous administrations per day. The first insulin preparations were associated with the risk of hypo- or hyperglycemia and unstable insulin plasma levels. Various formulation strategies were pursued to develop long-acting basal insulin analogs that maintain flatter and more stable basal insulin plasma levels.⁶⁷

3.4. Interferon- β

Interferon- β (IFN- β) is a cytokine (20 kDa) forming 5 α -helices in the secondary structure and containing a single-glycosylation site (Asn 80).⁶⁸ IFN- β is secreted in fibroblasts and as with other type-1 interferons, IFN- β activates JAK/STAT-pathway by binding to interferon receptor 1 and 2 (IFNAR1/2), leading to the expression of antiviral, antiproliferative and antitumor products.⁶⁹

While other interferons such as IFN- α are mainly renally excreted, IFN- β is predominantly cleared by the liver. Tokazewski et al. investigated that nephrectomy had no effect on the



circulating plasma level of IFN- β , while IFN- α was impaired. They hypothesized that this behavior may be explained by the increased average hydrophobicity of IFN- β .⁷⁰ Bocci et al. made similar observations by showing the decreased biological activity of IFN- β after 1-hour perfusion of rabbit liver compared to IFN- α , which was not affected.⁷¹ Intravenous, subcutaneous and intramuscular administration of IFN- β to healthy volunteers showed that the elimination half-lives were 4.0 h, 10.0 h to 8.6 h depending on the application route.⁷²

IFN- β is approved for the treatment of relapsing multiple sclerosis since 1997 as a glycosylated recombinant protein (IFN- β 1a, Avonex®).⁶⁹ The beneficial therapeutic outcome is probably explainable by the multiple effects of IFN- β on the immune system.⁷³ Avonex® is intramuscularly administered once weekly (30 μ g), although the plasma half-life is 10.0 h. The approval of another IFN- β 1a prepartate (Rebif®) with repeated subcutaneous injection three times per week (44 μ g) could extend the half-life to 50-60 hours, nevertheless, requires a high expenditure for patients and physicians.^{74, 75}

4. Formulation strategies

In the following paragraph, formulation strategies of the protein drugs are presented, which resulted in the successful approval by modulating their physiological elimination pathways.

4.1. Genetic engineering

Substitution of single or multiple amino acids in the protein is one promising strategy to alter pharmacokinetic properties. A crucial requirement for successful genetic engineering of a therapeutic protein essential consists of a deep understanding of its mode of action, its secondary and tertiary structure, potential binding sites to inhibitors, enzymes, and/or receptors. Systemic L-alanine scans of the entire protein sequence or selected protein sites are routinely performed to identify residues or domains important for function, stability or structure.⁷⁶ Apart from the exchange of inhibitor and enzymatic binding sites, substitutions may change the



physicochemical properties of the protein by introducing charged amino acids. However, the precise selection of appropriate sites for half-life extension without losing protein activity has been challenging.⁷⁷

Tenecteplase

The predominant clearance pathway of free tPA involves binding to the mannose receptor in the liver.²⁵ To reduce affinity to the mannose receptor, recombinant tissue-type plasminogen activator (tPA, Alteplase) was altered by several amino acid substitutions. Hotchkiss et al. confirmed that removal of the highly mannose-containing glycosylation site at Asn117 by exchanging the asparagin by glutamin resulted in a 2-fold decrease of the clearance in rabbits.^{78, 79} In addition, Bennett et al. identified Lys296-His297-Arg298-Arg299 as the major PAI-1 recognition site in tPA by replacing these residues with a quadruple alanine sequence.⁸⁰ Madison et al. showed that inhibition of the tPA mutant could be reduced 465- and 2800- fold by deleting this sequence or substituting it with negatively charged glutamic residues compared to wild-type tPA (pseudo-first-order rate constant 1.4×10^6 1/(M·s) vs 3.0×10^3 1/(M·s) and 5.0×10^2 1/(M·s)).⁸¹

By combining these two aspects, TNK-tPA (Tenecteplase) was developed, Alteplase with two point mutations (T103N, N117Q) and one tetra-alanine substitution (KHRR296-299AAAA) (**Figure 3A**). This mutant showed an altered *in vivo* clearance of 1.9 mL per minute per kg in rabbits compared to wild type tPA (16.1 mL/min/kg). Furthermore, Tenecteplase demonstrated 80 % higher resistance to PAI-1 and 14-fold enhanced relative fibrin selectivity, while demonstrating 8- and 13-fold higher efficacy against whole blood and platelet-enriched blood clots, respectively.⁸² Comparison of Tenecteplase with Alteplase showed a significantly longer half-life of about 20 minutes compared to 4 minutes (**Table 1**). Due to its improved pharmacokinetic, Tenecteplase, which was approved as Metalyse® since 2001, allows the use of a single-bolus application in the thrombolytic treatment of acute



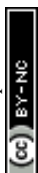
myocardial infarction.⁸³⁻⁸⁵ Additionally Tenecteplase has received regulatory approval for the treatment of acute ischemic stroke in adults, including approval by the European Medicines Agency (based on a positive CHMP assessment report in 2023) and by the U.S. Food and Drug Administration in February 2025.^{86, 87} In contrast, Alteplase still has to be administered as 90 minute-infusion to maintain the required plasma concentration.

GLP-1 (DPP-IV resistant)

The cleavage site for DPP-IV (His7-Ala8) in GLP-1 has been clearly identified, with cleavage leading to a rapid loss of biological activity.⁸⁸ L-alanine scans revealed that residue His7 at the N-terminus is directly involved in the interaction with GLP-1R and thus would lead to a loss of activity by substitution.⁸⁹ Deacon et al. synthesized four analogues with N-terminally substituted Ala8 against threonine, glycine, serine and α -aminoisobutyric acid (Aib). *In vivo* all analogs had longer N-terminal half-lives after infusion than GLP-1(7-36)amide (3.3-3.9 min vs. 0.9 min) but only the glycine- and α -aminoisobutyric acid-substituted analogs showed similar binding affinities to their receptor GLP-1R (IC_{50} ; Thr⁸ = 49 nmol/L; Gly⁸ = 2.8 nmol/L; Ser⁸ = 9 nmol/L; Aib⁸ = 0.45 nmol/L; GLP-1(7-36)amide = 0.78 nmol/L).⁹⁰ This improvement in pharmacokinetics alone was not sufficient to enable treatment with GLP-1, although this strategy could be combined with other formulation approaches as described above.

Insulin glargine

In contrast to amino acids substitutions described above to reduce affinity of binding sites of degrading enzymes and inhibitors, changes in the primary sequence of proteins or peptides can also be used to alter their physicochemical properties. Insulin glargine (Lantus®) was developed by extending the C-terminus of the human insulin B-chain by two positive arginine residues, changing the isoelectric point from 5.4 to 6.7 (**Figure 3B**). In the acidic formulation of pH 4, insulin glargine is soluble but crystallizes immediately at physiological pH of



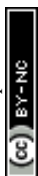
subcutaneous tissue.⁹¹ As a consequence of the acidic formulation, the acid-sensitive asparagine residue Asn21 in the A-chain, which can lead to deamidation or dimerization, was charge-neutrally replaced by a glycine resulting in good stability of the human insulin analogue.⁹² The 25% disappearance study of a subcutaneous bolus injection of radiolabeled insulin glargine compared to NPH (neutral protamine Hagedorn) insulin showed that insulin glargine was adsorbed 3 to 3.5 times slower, depending on the zinc content (15 to 80 $\mu\text{g/mL}$).⁹³ Although changes in the primary sequence of insulin to insulin glargine do not alter the hepatic elimination pathway, its prolonged pharmacokinetic profile is driven by delayed absorption, consistent with absorption rate limited (flip-flop) kinetics, resulting in once-daily application with more stable plasma curves and a lower risk of hypoglycemia compared to NPH.^{94, 95}

4.2. Conjugation strategies

Conjugation strategies offer the possibility of posttranslational modification of proteins and peptides to insert target structures, shielding effects and changes in physicochemical properties. The 20 standard amino acids provide several functionalities as attachment points for reactive functional groups such as activated ester or aldehydes. However, unselective conjugation can lead to heterogeneity of the bioconjugate, disrupting the active site of enzymes or binding sites with receptors, which in turn results in a loss of biological activity.

To overcome this challenge, site-specific conjugation strategies are required. Several reviews outlined how the N- or C-terminus, single cysteines, or other amino acids can be used as selective reaction sites.⁹⁶ Using mutagenesis, amino acid residues can be inserted or replaced to create a single attachment points or entire amino acid sequences for enzymatic reactions. Even unnatural amino acids can be incorporated by genetic code expansion to pave the way for new chemical approaches.⁹⁷

In our recent work, we demonstrated that selective conjugation to a single-free cysteine via maleimide reaction maintained full bioactivity of PEGylated tPA, whereas PEGylation at the



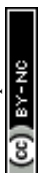
N-terminus via reductive alkylation at low pH led to reduction in activity to 68 %. Since the structure of tPA remained intact, the decreased activity may result from shielding of the catalytic triad from substrate access, depending on the PEGylation site.⁹⁸ This highlights that, in addition to site-specific conjugation strategies, detailed knowledge of protein structure is crucial.

4.2.1. Fatty acid acylation

Human serum albumin (HSA) is an important carrier protein for delivering endogenous substances, metabolites and xenobiotics among various organs, providing a high capacity due to a plasma concentration of about 42 g/L.⁹⁹ For fatty acids, it's the major carrier, providing hydrophobic pockets in which large insoluble anions can be absorbed. Longer fatty acids with 14 to 18 carbons have dissociation binding affinity constants to the fatty acid pocket of HSA below 50 nM, while shorter fatty acids (8 to 12 carbons) have a 10-fold lower affinity between 0.5 and 60 μ M.¹⁰⁰ Therapeutics with problematic pharmacokinetics can benefit from these properties by acylation of fatty acids, increasing the interaction and carrier time with HSA and thus the plasma half-life.

Liraglutide

The half-life of GLP-1 could be extended from 1.5 hours to 11-13 hours after subcutaneous administration by covalently binding a fatty acid (16 carbons) to Lys26 via a γ -glutamoyl (γ -Glu) linker.^{101, 102} Knudsen et al. discovered in the investigation of various GLP-1 derivatives with different fatty acid lengths that all derivatives with more than 12 carbon atoms made once-daily administration possible. Fatty acids conjugations with up to 16 carbon atoms, almost anywhere in the C-terminal portion, did not result in any appreciable loss of GLP-1 efficacy, whereas two fatty acid substitutes and longer fatty acids led to a severe loss of potency.¹⁰³ To ensure site-specific conjugation of the fatty acid to the Lys26 residue, the single other lysine

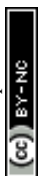


Lys34 of GLP-1 was replaced by Arg34 (**Figure 4A**). This GLP-1 analogue Liraglutide was approved 2009 (Victoza®, 2015: Saxenda®).¹⁰²

However, the once-daily use of Liraglutide required high compliance and time commitment from both patient and physician, so further research and efforts were made to enable the once-weekly use of GLP-1 analogs. Approaches include the addition of acidic functional groups to increase binding affinity to basic residues in albumin, bulkier or longer linkage structures to gain distance from the shielding HSA protein, and combination with sequence modifications to protect DPP-IV degradation.¹⁰⁴

Semaglutide

By systematically screening different fatty acid lengths (C12 to C22, monoacids and diacids) and linker structures Lau et al. discovered C18 diacid bound to Lys26 via γ -Glu-2xOEG (aminodiethoxyacetyl) to be the derivative with the highest albumin affinity and GLP-1 receptor affinity.¹⁰⁴ This optimized GLP-1 derivative was further optimized by two amino acid substitutions. First, as described for Liraglutide, the replacement of Lys34 with Arg34 and second, the substitution of Ala8 by Aib as described above. Combining both strategies, the amino acid substitution and the increasing affinity, resulted in a GLP-1 analog (Semaglutide, **Figure 4B**) allowing once-weekly subcutaneous administration with a plasma half-life of 7 days (**Table 1**).^{105, 106} In addition to the benefit of once-weekly administration of Semaglutide, a meta-analysis showed a significantly greater HbA1c reduction and a dose-dependent significant weight reduction compared to once-daily application of Liraglutide.¹⁰⁷ Furthermore, Semaglutide was approved as an oral tablet formulation (Rybelsus®) in 2020. The oral route of administration results in low absolute bioavailability and variable absorption, necessitating daily application at higher doses than the subcutaneous formulations Wegovy® and Ozempic® (3-14 mg vs. 0.25-2.4 mg). However, peroral administration is a major benefit for patients in terms of avoiding complications at the injection site and compliance.



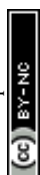
Insulin detemir

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A similar strategy was used to develop long-acting insulin analogs mimicking the basal level of insulin. Kurtzhals et al. prepared insulins acylated at the ϵ -amino group of Lys29 at the B-chain with saturated fatty acids (C10-C16 carbon atoms). The association constants of the conjugates to human serum albumin were lower than those of the free fatty acids (10^8 1/M), but still about 10^4 to 10^5 1/M.¹⁰⁸ Further studies demonstrated that myristic acid (C14) acylated at Lys29 and deletion of Thr30 of the B-chain, is the most protracted insulin conjugate with a 50% disappearance time after subcutaneous injection of 14.3 hours in pigs compared to NPH insulin with 10 hours (**Figure 4C**). Thereby, the 50% disappearance time correlated with the affinity of the conjugate for bind to albumin.¹⁰⁹ Nevertheless, the terminal half-life of 7 hours in humans is similar to that of NPH insulin. However, acylation of a fatty acid resulted in lower intra-patient variations in absorption, such that the maximum plasma concentration of insulin detemir was reached between 6 and 8 hours, compared with NPH between 2-18 hours.^{110, 111} Several studies comparing this insulin detemir with NPH in type I diabetes mellitus patients confirmed that insulin detemir resulted in more predictable fasting blood glucose with lower risk of hypoglycemia (22%) and nocturnal hypoglycemia (34%).^{112, 113}

Insulin degludec

As with the GLP-1 analogs, efforts have also been made to further improve the pharmacokinetic behavior of insulin. Insulin degludec (Tresiba®) was developed by acylating a fatty diacid (C16) side chain to Lys29 in the B-chain via an acidic γ -L-glutamic acid linker, similar to Semaglutide (**Figure 4D**). On the one hand, this new conjugation partner led to the formation of di-hexamers in a phenol-zinc formulation, which then formed self-associated stable depots of multi-hexamer chains at the injection site, resulting in a slow and continuous release of insulin degludec in the circulation. On the other hand, the acidity of the side chain is responsible for strong binding to HSA of more than 99% after absorption.^{114, 115} Once daily



administration of insulin degludec resulted in longer plasma half-lives at steady state (25 hours) and more stable 24 hour glucose infusion rate profiles compared to other long-acting insulin analogs.¹¹⁶

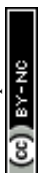
4.3. PEGylation

Another approach to altering the pharmacokinetic properties of proteins is the conjugation of polymers, with polyethylene glycol (PEG) being the most widely used conjugation agent. This can influence various physicochemical properties, such as the conformation, steric hindrance, electrostatic binding properties and the hydrophobicity of the parent protein. In particular, the size and molecular weight changes due to the bulky hydrophilic shield generated by two or three water molecules per ethylene glycol subunit. This behavior is primarily known to reduce the glomerular filtration in the kidney by increasing the size of the parent protein beyond the renal cut off.³

PEGylation reduces plasma clearance rates of protein therapeutics in the liver. PEGylation can mask binding sites responsible for specific enzymatic deactivation, receptor-mediated uptake or inhibitor recognition.¹¹⁷ In addition, PEG shielding reduces non-specific proteolytic digestion, immunogenic recognition and cellular protein clearance by diminished elimination by the reticuloendothelial system.¹¹⁸

Pegylated IFN- β

Baker et al. modified recombinant IFN- β -1a with 20 kDa mPEG-O-2-methylpropionaldehyde at the N-terminal alpha-amino group (**Figure 5**). The obtained bioconjugate retained only 50% of its activity but showed significantly improved pharmacokinetic properties after intravenous application in rats. Especially the elimination half-life of the PEGylated protein was about 13-fold longer than the parent protein.¹¹⁹ Although the bioactivity of PEGylated IFN- β -1a was reduced, it could be shown in an animal model that the inhibition of blood vessels entering the periphery of human SK-MEL-1 melanoma tumors



in mice was higher (58%) for PEGylated IFN- β -1a than for unmodified IFN- β -1a (29%) using the same dose.¹²⁰

PEGylated IFN- β -1a (Plegridy®) has been approved for multiple sclerosis (MS) since 2014. Plegridy® can be administered every 2 or 4 weeks, leading to a 30% reduction in relapses in patients with relapsing-remitting MS compared to placebo and comparable to non-pegylated interferon- β (**Table 1**).^{121, 122} This represents a high benefit compared to non-pegylated IFN- β -1a (Rebif®), which had to be administered three times per week.⁷⁵

4.4. Fusion proteins

Fusion proteins extend the plasma half-lives of the parent proteins by utilizing the long half-lives of the fused proteins. In general, there are three promising candidates already used in approved therapeutics: human serum albumin (HSA), human IgGs1, 2, 4 and transferrin.¹²³ This section will focus on the first two proteins as they have led to approvals of the endogenous proteins discussed here.

Both HSA and IgG have a long plasma half-lives of 19 and 14 to 21 days, respectively.⁹⁹ The neonatal Fc receptor (FcRn) is responsible for the homeostasis of both. After pinocytosis of serum proteins by cells of the reticuloendothelial system, they are usually lysosomal degraded in acidic vesicles. In contrast, HSA and IgGs bind to FcRn in a pH-dependent manner, followed by translocation back to the cell surface. There, at neutral pH, the affinity for FcRn decreases, allowing the release and recycling of both proteins into the circulation.^{124, 125}

4.4.1. Albumin fusion protein

As for acylation with fatty acids, albumin fusion proteins also take advantage of the long plasma half-life of human serum albumin (HSA). In addition to the recycling of HSA by FcRn, the negative electrical potential and size of HSA also inhibits the glomerular filtration.¹²⁶ However, increasing size and molecular weight can lead to steric hindrance and loss of biological activity of the therapeutic protein.¹²⁶ Yang et al. confirmed that this issue can be



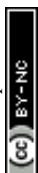
addressed by using longer linker lengths, which in his case resulted in higher cytotoxic effect on tumor cells *in vitro* using recombinant protein Onconase fused with HSA than shorter linker lengths.¹²⁷ Further adjustments to achieve high bioactivity of the fused protein are the consideration of the N- or C-terminus as junction between the two proteins and the use of tandem copies of the fused protein.¹²⁸

Albiglutide

Albiglutide is the first albumin fusion protein approved 2014 (Eperzan®).¹²⁹ It was engineered by fusing the C-terminus of GLP-1 to the N-terminus of HSA, as the N-terminus of GLP-1 is decisive for receptor binding (**Figure 6A**).⁸⁹ Additionally, the strategy of amino acid substitution was applied by substituting Ala8 by a glycine residue to increase stability towards DPP-IV. Two tandem copies of GLP-1 were fused due to the loss of affinity to the receptor by the steric hindrance of HSA, but also by amino acid exchange.^{130, 131} Nevertheless, higher doses of albiglutide (30-50 mg; ~ 410-685 nmol) compared with semaglutide (0.25-2 mg; ~ 60-485 nmol) are required to overcome the reduced potency.^{106, 129} In pharmacokinetic and pharmacodynamic studies, albiglutide demonstrated serum half-lives of 6 to 7 days after subcutaneous injection with concomitant improvement in fasting plasma glucose, HbA1c levels and postprandial blood glucose levels, with no hypoglycemic episodes compared to placebo in type 2 diabetes patients.¹³²⁻¹³⁴ Despite the positive effects, the approval of Eperzan® was withdrawn worldwide in 2018 due to its low commercial success, mainly explained by the lower reduction of body weight compared to other GLP-1 analogs.¹³⁵

4.4.2 Fc fusion proteins

The binding site for the recycling mechanism of IgGs via FcRn is located at the Fc region of IgG antibodies between hinge CH2 and CH3. Therefore, this Fc region is decisive and sufficient to benefit from the pharmacokinetic of IgGs. The choice of IgG antibody subclass influences the immunological properties of the resulting fused Fc region and the desired

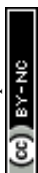


protein. IgG₁ bind to all IgG receptors (FcγR), especially the FcγRIIIA, which is localized on natural killer (NK) cells, inducing antibody-dependent cell-mediated cytotoxicity (ADCC). This property may be advantageous in the treatment of cancer cells. In contrast, this activation of complement and FcγRs must be avoided in other diseases to prevent unwanted immunogenicity. IgG₂ and IgG₄ have a lower affinity to FcγRs.^{136, 137} Changes in the amino acid sequence of the fused Fc region can also lead to changes in immunochemical properties or affinity to FcRn and therefore need to be carefully evaluated in a similar way to the genetic engineering considerations mentioned above.¹³⁸

Dulaglutide

Glaesner et al. developed a GLP-1 fc fusion protein (Dulaglutide) with fourfold higher GLP-1 receptor activation with a simultaneous extension of the half-life to 1.5 days in rats and over 2 days in monkeys. Dulaglutide is a DPP-4 protected GLP-1 peptide fused via its C-terminus to the N-terminus of the human IgG₄ hinge region (**Figure 6B**). Without an additional linker between these proteins, the *in vitro* activity of this fusion protein was drastically reduced by about 95%; with the optimal linker, it was four times higher than that of the free DPP-IV protected GLP-1. In addition, the IgG₁ Fc region first used was replaced by an IgG₄ isotype modified at two amino acid residues (F234A and L235A) to further reduce interaction with high affinity FcγRs, resulting in lower dose-dependent cytotoxicity. Further mutations were made in the Fc region (S228P) to eliminate half-antibody formation and GLP-1 (R36G) to de-immunize the fusion protein. As well as the removal of the C-terminal lysine of the IgG-Fc region.¹³⁹

Dulaglutide has been approved since 2014 (Trulicity®) with a half-life of 5 days after subcutaneous administration in humans (**Table 1**). Due to its high residual potency, treatment can be carried out with relatively low doses between 0.75 and 4.5 mg, in contrast to albiglutide.¹⁴⁰ Even though Semaglutide and Dulaglutide have similar pharmacokinetic



behavior, Semaglutide shows superior glycemic control and bodyweight reduction while having a similar safety profile.¹⁴¹

4.5. Depots to circumvent hepatic elimination

In addition to direct modifications of the protein drug, pharmacokinetics may be modulated via encapsulation in drug delivery systems. There are already several reviews on tPA¹⁴²⁻¹⁴⁴, GLP-1^{145, 146}, insulin^{147, 148} and IFN- β ¹⁴⁹ formulated in liposomes, nano- or microencapsulation and polymeric micelles. For the sake of completeness and to demonstrate their potential, the extension of their plasma half-life by these strategies is outlined for several examples of the proteins mentioned, even if they are not approved.

In all these examples, it must be considered that the specific and non-specific proteolytic elimination pathways are interrupted by the protection of the drug delivery systems, but the particulate systems also undergo clearance mechanisms in the body. In particular, the liver as a biological filter system is responsible for the recognition and excretion of particles larger than 6 nm.¹⁵⁰ Two types of cells are mainly involved in this process. Firstly, Kupffer cells, which are tissue-resident macrophages phagocytizing recognizing pathogens and foreign bodies. Uptake is highly dependent on the surface charge, ligand chemistry and size of the particulate system.¹⁵¹ The internalization occurs through multiple scavenger receptors leading to micropinocytosis, clathrin-mediated, caveolin-mediated endocytosis and other endocytotic pathways. Secondly, sinusoidal endothelial cells in the liver are involved in filtering blood from particulate systems via several receptor-ligand interactions.¹⁵²

4.5.1. Liposomes

Encapsulation of tPA in liposomes consisting of egg phosphatidylcholine (EPC), cholesterol, sodium cholesterol-3-sulfate (CS) and distearoylphosphatidyl ethanolamine-N-poly(ethylene glycol) 2000 (DSPE-PEG2000) could prolong the half-life 21-fold compared to free tPA without altering the fibrinolytic activity of intact tPA.¹⁵³ Without the use of DSPE-PEG2000,



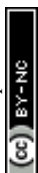
the plasma half-life could only be increased 16-fold. This can be explained because the rapid uptake of liposomes by cells of the mononuclear phagocytic system (MPS) in the liver and spleen can be shielded by PEG-modification.¹⁵⁴ Heeremans et al. confirmed that liposomally encapsulated tPA showed significantly better thrombolysis efficiency in a rabbit jugular vein thrombosis model.¹⁵⁵ By using different types of lipids, including PEGylation or providing modification options, targeted delivery is possible. For instance, Asahi et al. demonstrated in an established rat model of embolic focal emboli that antiactin-targeted immunoliposomes with tPA showed a reduction in the volume of tPA-induced intracerebral hemorrhage.¹⁵⁶

4.5.2. Cationic liposomes

Cationic or ionizable cationic liposomes have been widely studied as carriers for biomolecules. Insulin-loaded liposomes containing the cationic lipid DOTAP (dioleoyl-3-trimethylammonium-propane), cholesterol, DOPE (dioleoyl-sn-glycero-3-phosphoethanolamine) and DSPE-PEG showed increased encapsulation efficiency and prolonged *in vitro* release.¹⁵⁷ Shalaby et al. confirmed insulin encapsulation in chitosan-coated cationic liposomes of over 85%. In diabetic mice, oral administration of these cationic liposomes resulted in a reduction to normal glucose levels after 4 hours that lasted up to 8 hours, compared to the hypoglycemic effect of subcutaneously administered insulin after 3 hours.¹⁵⁸ However, their general translational potential is constrained by toxicity concerns, including charge-dependent cytotoxicity, elevated ROS production, and *in vivo* hepatotoxicity and inflammatory responses.¹⁵⁹

4.5.3. Nanoparticles

As already shown for cationic liposomes, nanoparticles can also be used to enable other administration routes. P(MAA-g-EG) hydrogel carriers (poly(methacrylic acid-g-ethylene glycol) loaded with IFN- β showed enhanced plasma IFN- β concentrations after administration in closed ileal segments of rats.¹⁶⁰ Kondiah et al. used pH-sensitive microparticles for the oral



delivery of IFN- β . They were able to show in vitro that only 3% was released from the oral tablet formulation at gastric pH (1.2), compared to 74% at intestinal pH (6.8). Studies in New Zealand White rabbits found higher IFN- β plasma concentrations for the oral formulation compared to a subcutaneous formulation.¹⁶¹ Intranasal formulations with chitosan/sulfobutylether- β -cyclodextrin nanoparticles led to an improvement in clinical symptoms in an experimental autoimmune encephalomyelitis (EAE) mouse model used to simulate clinical symptoms of MS, while free IFN showed no effect.¹⁶²

4.5.4. Microspheres

Bydureon®, a GLP-1 analogue (exenatide), is formulated in PLGA (polylactic-co-glycolic acid) microspheres. Exenatide is a naturally occurring peptide derived from Heloderma lizard venom with 53% homology to human GLP-1 (**Figure 6C**).¹⁰² The relatively short plasma half-life of exenatide after s.c. administration (2.4 hours) was prolonged by loading into PLGA microspheres, allowing for weekly administration.¹⁶³ As common drawback of PLGA microspheres as drug delivery system, Bydureon® exhibits a “lag phase”: an initial burst in the first two days, followed by delayed release in the next two weeks and a complete release of exenatide after 7 weeks.¹⁶⁴ Nevertheless, Bydureon® show a glucoregulatory effect lasting 7 days and a superior reduction of HbA1c levels and fewer side effects than the classical exenatide dosing formulation (Byetta®).¹⁶⁵

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5. Comparison of formulation strategies

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Formulation strategies of protein drugs to circumvent hepatic clearance must be evaluated regarding (i) protein structure and pharmacological performances and (ii) manufacturing from the initial process at research (low scale) towards GMP grade at production scale. Therapeutic proteins with alterations from genetic engineering or expressed as fusion protein are based on established biotechnological tools, although post-translational modification needs to be considered in each case. The fusion protein of HSA and GLP-1 is produced in efficient yeast expression systems as it has no endogenous glycosylation sites. In contrast, production of Fc fusion proteins is routinely performed in eucaryotic cell lines to enable glycosylation within the Fc region.¹⁶⁶ Fusion proteins either prolonged on the N- or C-terminus are structurally complex and possess multiple cysteines that can interact with free cysteines in the parent protein, resulting in incorrect folding and function. The design of the linker structure between the parent and the fused protein can influence stability, folding, expression, biological activity, and targeting, depending on flexibility (e.g. Gly-Ser structures) or rigidity (e.g. alpha helix, multiple Pro-residues) and cleavability (disulfide bonds or protease-sensitive linker).¹⁶⁷ Preserving its functionality, the development of an optimized fusion protein may be time-consuming and challenging.⁷⁷

Conjugation approaches such as acylation and PEGylation require an additional production step after expression, considering site-specificity of the modification, additional purification and characterization steps.⁷⁷

Unmodified peptides and proteins are used for formulation in particulate or liposomal drug delivery systems. Their manufacturing is demanding, requiring a high level of development effort in terms of reproducibility, and transfer from low- to high scale production. Moreover, stability of the peptide within the formulation is crucial, which has been only achieved for a limited number of approved peptides.¹⁶⁸

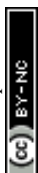


Genetic engineering is a facile way to inhibit the selective elimination pathway of proteins, as described for tPA and GLP-1 *vide supra*. Nevertheless, in these two examples, the success of prolonging their half-life was rather limited, not allowing once weekly or even daily administration. Both examples demonstrate the complex interplay of the hepatic and the remaining non-selective proteolytic degradation mechanism in the body. In summary, genetic engineering is a versatile approach for optimizing physicochemical properties (Insulin glargine), reducing immunogenic potential (Dulaglutide), and facilitating conjugation selectivity (Liraglutide, Semaglutide).

Formulation strategies considering steric hindrance to receptor sites and entry to the liver have been so far most successful for hepatic half-life extension. Interestingly, strategies to enhance size of GLP via direct fusion to HSA or attachment of a fatty acid to enable HSA binding in circulation (Semaglutide) resulted in similar pharmacokinetic outcome, indicating the important role of reducing diffusion kinetics of free GLP into the liver/kidney from circulation (**Table 1**).

In general, the herein described fusion proteins and the conjugation of PEG or fatty acids with an optimized linker structure resulted in at least weekly dosing, except for insulin analogues. This longer half-life, together with higher doses, compensates for the often-observed loss of bioactivity, which is due to the steric hindrance caused by the bulky structure, but also to the change in protein surface and charge.¹⁶⁹

Beyond directly reducing hepatic elimination, a more innovative strategy involves targeting the delivery of proteins to specific tissues within body. This approach minimizes systemic circulation and consequently reduces elimination via liver and kidneys. For precise and targeted delivery, small peptide sequences are commonly employed. For instance, Huang et al. modified the surface of tPA-loaded liposomes with cRGD (Arg-Gly-Asp) motif, which binds with high affinity and specificity to $\alpha_{IIb}\beta_3$ integrins on activated platelets. This, combined with activated

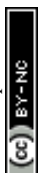


platelet-induced liposomal membrane destabilization, allowed them to selectively target blood clots and reduce clot lysis time compared to non-cRGD-coated liposomes.¹⁷⁰

We recently demonstrated a magnetically guided micro robotic drug delivery system for tPA delivery that enables precise, targeted therapy while reducing off-target effects of tPA.¹⁷¹ The platform approach was effective in accurate navigation and controlled drug delivery in both *in vitro* vascular models and *in vivo* large animal studies.

Covalent anchoring of proteins therapeutics to extracellular matrix cues may be another strategy to form local depots and evade elimination. Hamm et al. deployed the D-domain of insulin-like growth factor-I (IGF-I) to modify anti-myostatin peptides, taking advantage of its high-affinity substrate properties for factor XIIIa, which forms covalent bonds via transamidation.^{172, 173} This strategy may be expanded to store tPA within circulation, as factor XIIIa activity is known to increase at thrombolytic sites, where it is also responsible for cross-linking fibrin within the thrombus.¹⁷⁴ Using a similar strategy, Mergenthaler et al. demonstrated pH-responsive binding of N-terminal boronic acid-modified peptides to diol-rich glycans in extracellular matrices, enabling dynamic drug storage and targeted delivery of anti-myostatin peptides.¹⁷⁵

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6. Conclusion

Specific formulation strategies to reduce liver uptake of protein drugs are largely underexplored considering the predominant role of renal uptake on protein clearance. Considering mechanism of hepatic clearance in formulation design were successfully applied for few protein drugs, such as GLP-1, insulin, tPA and interferon β -1-a. These include genetic engineering to circumvent hepatic protease degradation and precise steric hindrance to avoid liver uptake, and endocytosis. These may be expanded in the future by targeting covalent formation of depots of protein drugs on extracellular matrix in diseased tissues to avoid liver uptake, and elimination.

Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Acknowledgements

We thank the European Union's Horizon 2020 research and innovation program under grant agreement No 952152, project ANGIE (Magnetically steerable wireless Nanodevices for the targeted delivery of therapeutic agents in any vascular region of the body) for kindly supporting out research. The Interdisciplinary Center for Clinical Research at the University of Würzburg (IZKF), project No S542 (Bioinspired nucleic acid therapeutic depots for the therapy of liver fibrosis) is also acknowledged for financial support.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.



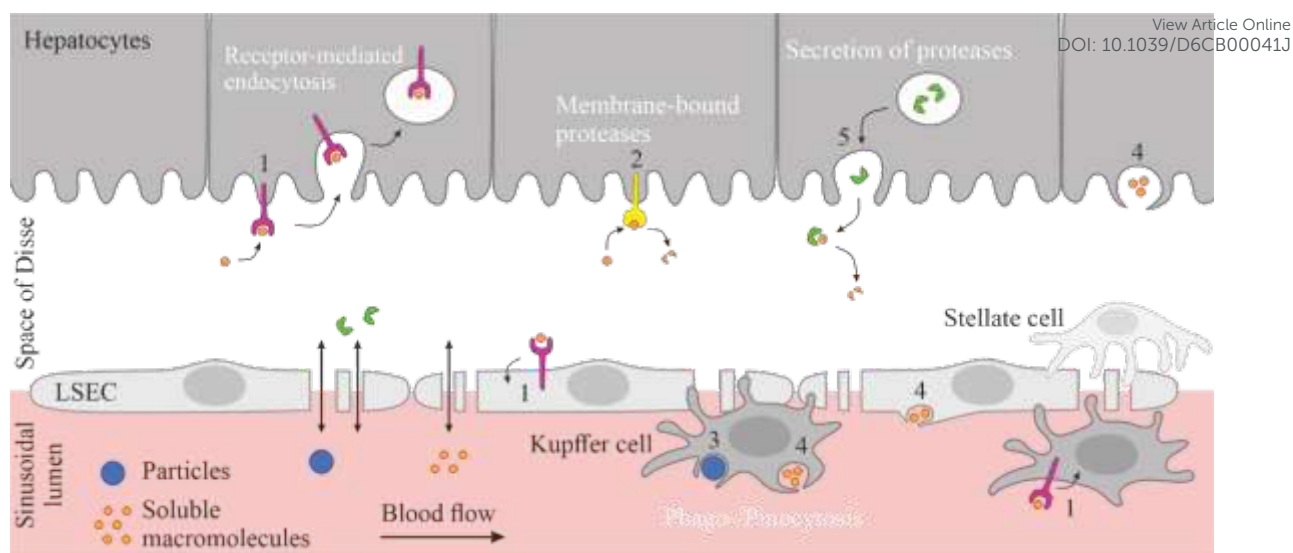


Figure 1. Illustration of liver sinusoids with sinusoidal lumen and space of Disse, separated by fenestrated liver sinusoidal endothelial cells (LSEC) (pores 100-200nm). Schematic representation of elimination pathways of particles (blue) and soluble macromolecules (orange) by (1) receptor-mediated endocytosis, (2) membrane-bound proteases, (3) phagocytosis, (4) pinocytosis or (5) secreted proteases in the respective cells: Hepatocytes, LSEC or Kupffer cells.

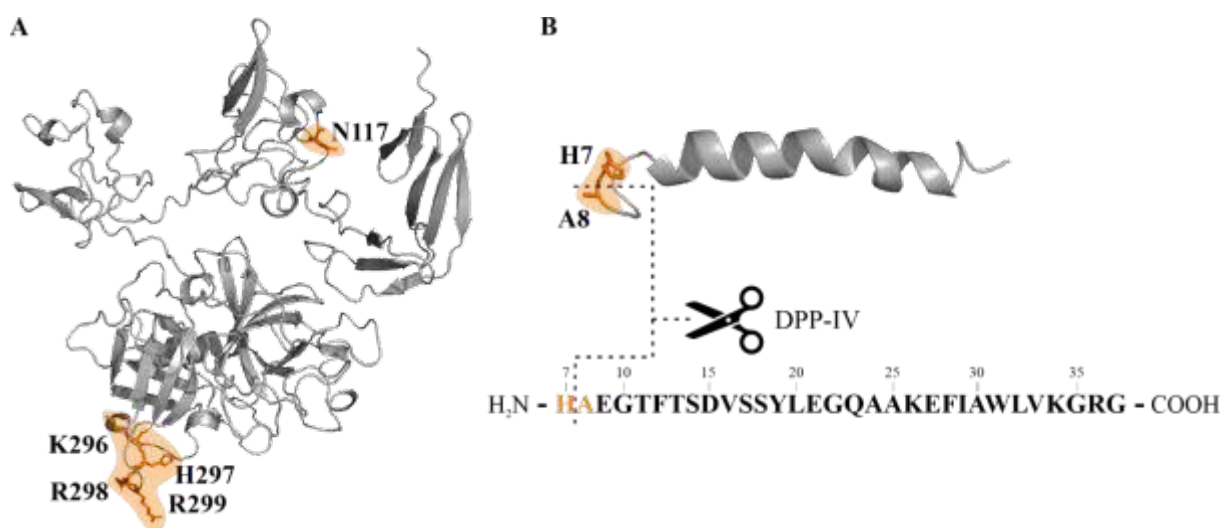


Figure 2. (A) Human tPA modelled by alpha-fold (UniProt: P00750) with orange highlighted PAI-1 binding site (K296-K299) and mannose-containing glycosylation site (N117) leading to hepatic elimination via mannose receptor. (B) Human GLP-1-(7-37) (UniProt: P01275) structure and primary sequence with N-terminal DPP-IV cleavage site between H7 and A8 (orange).



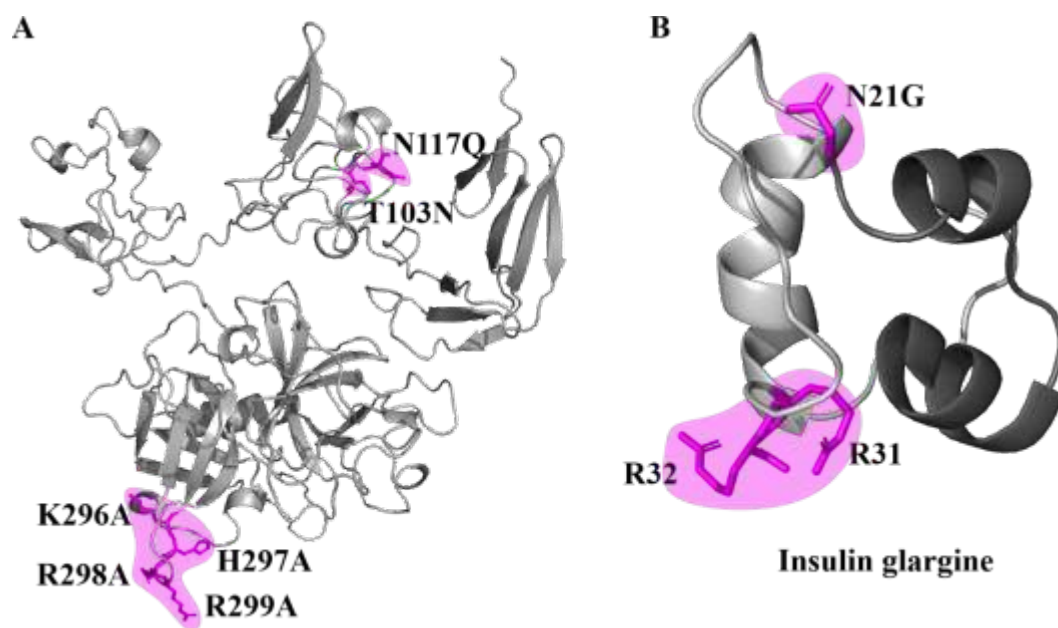


Figure 3. Genetic engineering as formulation strategy for tPA and insulin. (A) Human tPA modelled by alpha-fold (UniProt: P00750) with purple marked point mutations T103N, N117Q and K296A-R299A to the development of the tPA analog Tenecteplase. (B) Insulin glargine with A-chain (dark grey), B-chain (light grey) and purple marked point mutation N21G in A-chain and addition of R31 and R32 in B-chain.



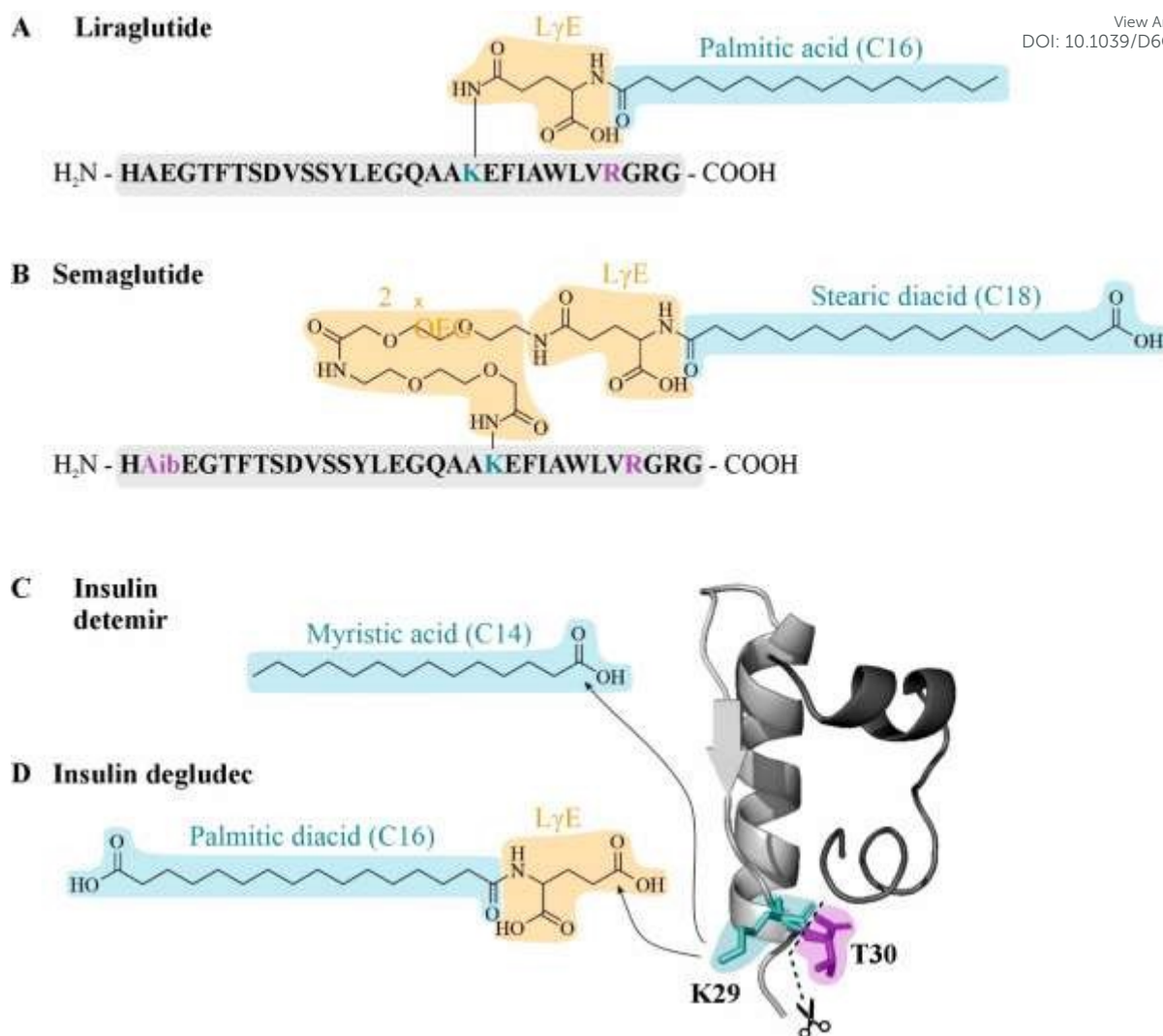


Figure 4. Fatty acid acylation as formulation strategy for GLP-1 and insulin. Purple labeled residues show point mutations compared to human GLP-1 or insulin, blue labeled residues show conjugation sites. Orange labels show linkage structures, blue labels show conjugated fatty acids. Primary sequence of the GLP-1 receptor agonists (A) Liraglutide and (B) Semaglutide; long-acting human insulin (UniProt: P01308) analogs (C) Insulin detemir and (D) Insulin degludec.



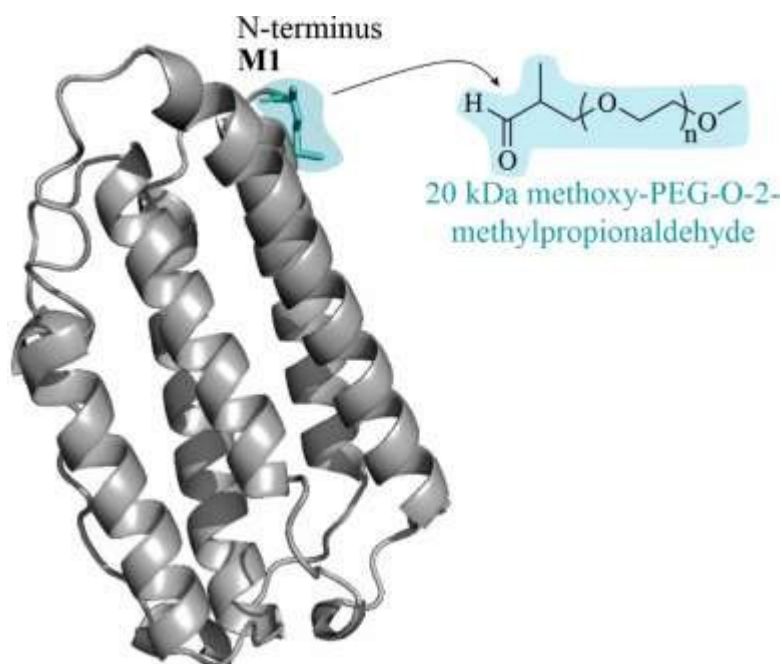
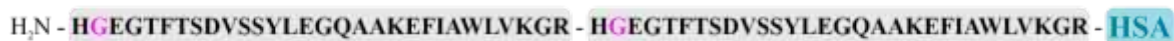
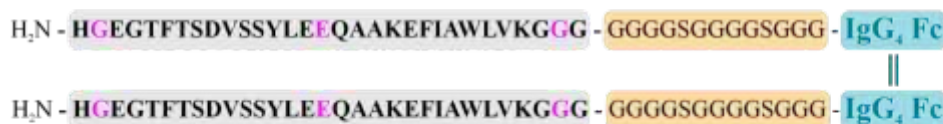


Figure 5. Site-specific PEGylation at the N-terminus M1 (blue) of Interferon- β -1a (UniProt: P01574) with 20kDa methoxy-PEG-O-2-methylpropionaldehyde (blue).

A Albiglutide



B Dulaglutide



C Exenatide



Figure 6. Primary sequences of the GLP-1 receptor agonists (A) Albiglutide, (B) Dulaglutide and (C) Exenatide. Purple labeled residues show point mutations compared to human GLP-1, green labeled residues show difference to GLP-1 found in Heloderma lizard venom, blue labeled residues show conjugation sites. Orange labels show linkage structures, blue labels show fused proteins.



Table 1. Selection of approved tPA, GLP-1, insulin and IFN- β analogs, their formulation strategy and corresponding plasma half-lives.

Therapeutic agent	Formulation strategy	Plasma half-life	Approval EMA	Therapeutical use	Reference	
tPA analogs	recombinant tPA, Alteplase	-	4-5 min (i.v.)	2000, Actilyse®	Thrombolytic treatment of AMI, APE, AIS	29
	Tenecteplase	Multiple mutations	24 min (i.v.)	2001, Metalyse®	Thrombolytic treatment of MI, AIS	86,176
GLP-1 receptor agonists	GLP-1	-	2 min (i.v.) 1.5 h (s.c.)	-	-	49, 102
	Liraglutide	Fatty acid acylation	13 h (s.c.)	2009, Victoza® 2015, Saxenda®	Adjunct for DM type 2 Obesity	177, 178
	Semaglutide	Fatty acid acylation, point mutation	7 d (s.c., p.o.)	2018, Ozempic® (s.c.) 2020, Rybelsus® (p.o.) 2022, Wegovy® (s.c.)	Adjunct for DM type 2 Obesity,	106, 179, 180
	Albiglutide	HSA fusion protein, point mutation, tandem copies	5 d (s.c.)	2014, Eperzan® Withdrawn: 2018	Adjunct for DM type 2	129, 135
	Dulaglutide	IgG ₄ -Fc fusion protein, multiple point mutation	5 d (s.c.)	2014, Trulicity®	Adjunct for DM type 2	140
	Exenatide	Origin: Heloderma lizard venom	term $t_{1/2}$ 2.4 h (s.c.)	2006, Byetta®	Adjunct for DM type 2	181
	Exenatide	PLGA microsphere	term $t_{1/2}$ 2.4 h (s.c.)	2011, Bydureon®	Adjunct for DM type 2	163



	Human insulin	-	4-6 min (i.v.)	1997, Insuman Rapid®	DM	62, 182
	NPH	Suspension formulation with protamine and zinc	term $t_{1/2}$ 5-10 h	2002, Protaphane®	DM	110
Long-acting Insulin analogs	Insulin glargine	Multiple point mutation	18-19 h (s.c.)	2000, Lantus®	DM	183
	Insulin detemir	Fatty acid acylation	term $t_{1/2}$ 5-7 h (s.c.)	2004, Levemir®	DM	111
	Insulin degludec	Fatty acid acylation, point mutation	term $t_{1/2}$ 25 h (s.c.)	2013, Tresiba®	DM	184
	IFN- β -1a	-	10 h (i.m.), 50-60 h (r.s.c.)	1997, Avonex® 1998, Rebif®	Relapsing MS	74, 75
IFN- β -1a analog	PEG-IFN- β -1a	CHO-mPEG 20kDa	78 \pm 15 h (s.c.)	2014, Plegridy®	Relapsing remitting MS	185

Abbreviations:

EMA: European Medicines Agency, i.v.: intravenous, s.c.: subcutaneous, p.o.: per oral, i.m.: intramuscular, r.s.c.: repeated subcutaneous, term $t_{1/2}$: terminal plasma half-life, AMI: acute myocardial infarction, APE: acute pulmonary embolism, AIS: acute ischemic stroke, DM: diabetes mellitus, MS: multiple sclerosis



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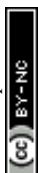
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View Article Online
DOI: 10.1039/D6CB00041J

Data Availability Statement:

No primary research results, software or code have been included, and no new data were generated or analysed as part of this review.

