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Strategies to overcome hepatic clearance of endogenous proteins – molecular and formulation approaches

Kirstin Meiners,[a] Tobias Stepic,[a] Lorenz Meinel[ab] and Tessa Lühmann*[a]

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

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1. Liver and hepatic clearance

The liver is the body's most important metabolic organ, responsible for detoxification, and the 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, and about 16% is extracellular space.[2] Fenestration in the liver sinusoid endothelial cells (LSECs) of about 100 to 200 nm

[a] *Institute of Pharmacy and Food Chemistry, University of Würzburg, Am Hubland, Würzburg, 97074, Germany. E-mail: Tessa.luehmann@uni-wuerzburg.de*
[b] *Helmholtz Institute for RNA-Based Infection Research (HIRI), Josef-Schneider-Straße 2, 97080 Würzburg, Germany*

Kirstin Meiners

Dr Kirstin Meiners (Pharmacist) is a scientist in drug formulation and delivery. She completed her doctoral research in pharmaceutics and drug delivery at the University of Würzburg under the supervision of Prof. Tessa Lühmann. Her doctoral research focused on formulation strategies of tPA, particularly for the design of advanced therapeutic delivery systems.

Tobias Stepic

Tobias Stepic (Pharmacist) is a PhD-candidate in drug delivery at the University of Würzburg under the supervision of Prof. Tessa Lühmann. His doctoral research focuses on ASO based therapeutics, particularly on the design of bioresponsive delivery systems.

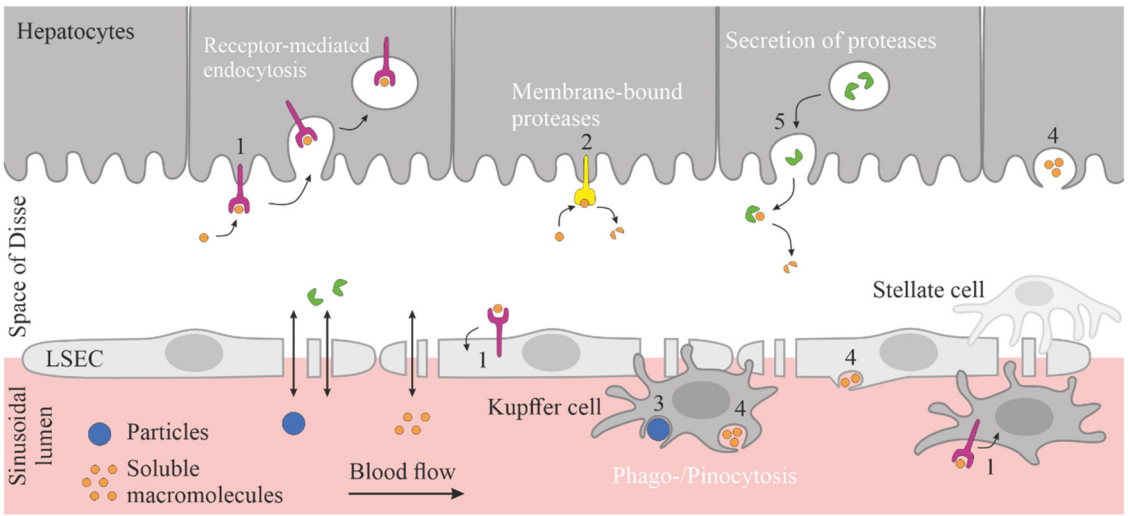

Fig. 1 Illustration of liver sinusoids with sinusoidal lumen and space of Disse, separated by fenestrated liver sinusoidal endothelial cells (LSEC) (pores 100–200 nm). Schematic representation of elimination pathways of particles (blue) and soluble macromolecules (orange) by (1) receptor-mediated endocytosis,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

allows for unhindered plasma flow between blood and the liver cells (Fig. 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 (Fig. 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

Lorenz Meinel

Lorenz Meinel is a Professor and Chair of Drug Formulation and Delivery at the University of Würzburg, where he has held a full professorship since 2010. Before joining the University of Würzburg, Prof. Meinel worked at ETH Zurich and the Massachusetts Institute of Technology and later held a leadership position in pharmaceutical development at Novartis Pharmaceuticals in Basel. Prof. Meinel's research focuses on drug delivery, biomaterials, pharmaceutics, and biopharmacy, with a particular emphasis on formulation technologies, controlled drug release, and translational pharmaceutical research.

Tessa Lühmann

Tessa Lühmann is a Professor of Drug Delivery and Food Technology at the University of Würzburg. Before joining the University of Würzburg, Prof. Lühmann conducted research at several international academic institutions, including ETH Zurich, focusing on biomaterials and nanomedicine. Prof. Lühmann's research interests are centered on biomaterials, drug delivery systems, and protein engineering, with a particular focus on bioprinted materials, controlled drug release, and bioresponsive drug delivery systems for regenerative medicine and pharmaceutical applications.

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 a major route of elimination needs to consider the mode of the elimination pathway in the liver.

2. Proteins with hepatic clearance as a major route of elimination

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

2.1. 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 second-order rate constants of about 5.5×10^6 1 M^{-1} s^{-1} (sc-tPA) and about 1.8×10^7 1 M^{-1} s^{-1} (tc-tPA) (Fig. 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 and 5 minutes.[21–23] The liver is the superior clearance organ when compared to the kidney, which was 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 ^{125}I marked tPA, revealed that 80% are rapidly cleared in the liver, of which 54.5% occurs 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 use in 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^{-1} bodyweight) must be administered as a 10% initial intravenous bolus over 1–2 min, followed by a 60 min

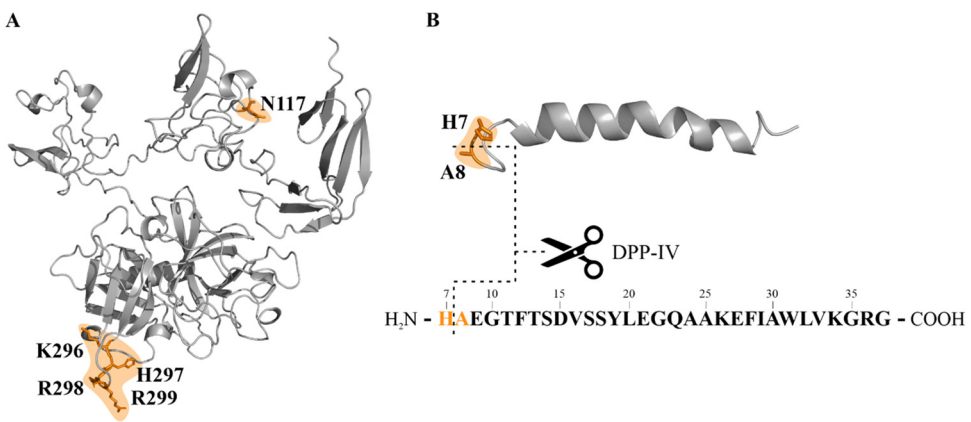

Fig. 2 (A) Human tPA modelled by alpha-fold (UniProt: P00750) with orange highlighting the PAI-1 binding site (K296–K299) and mannose-containing glycosylation site (N117) leading to hepatic elimination *via* mannose receptors. (B) The structure and primary sequence of Human GLP-1-(7–37) (UniProt: P01275)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

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]

2.2. 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 residue (3.3 kDa), amidated form (7–36) or as a 31 residue, glycine-extended peptide (7–37), whereby the former is predominant in humans with a prevalence of $>80\%$.[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.[39–44]

GLP-1 is rapidly inactivated by ubiquitous dipeptidyl peptidase IV (DPP-IV). DPP-IV has a high specificity for the N-terminal α-amino group of His7 and Ala8 cleavage resulting in GLP-1-(9–36)amide, followed by no further proteolytic cleavage (Fig. 2B).[45–47] Kieffer *et al.* showed that 50% of ^{125}I labeled GLP-1-(7–36)NH_2 was metabolized within 2 minutes to GLP-1-(9–36)NH_2 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 is 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 demands the development of long-acting derivatives.[51,52]

2.3. 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 receptors (IRs).[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 (IRs) and insulin-degrading enzymes (IDEs) in the liver, which activate the insulin degradation after binding, makes it the main site of excretion.[65] Binding of insulin to IRs 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]

2.4. 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, INF-β activates the 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 was approved for the treatment of relapsing multiple sclerosis in 1997 as a glycosylated recombinant protein (IFN-β1a, Avonex®).[69] The beneficial therapeutic outcome is probably explainable by the multiple effects of IFN-β on the immune system.[73] Avenox® is intramuscularly administered once weekly (30 μg), although the plasma half-life is 10.0 h. The

approval of another IFN-β1a preparate (Rebif®) with repeated subcutaneous injection three times per week (44 μg) could extend the half-life to 50–60 hours, nevertheless, it requires a high expenditure for patients and physicians.[74,75]

3. Formulation strategies

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

3.1. Genetic engineering

Substitution of single or multiple amino acids in the protein is one promising strategy used to alter pharmacokinetic properties. A crucial requirement for successful genetic engineering of an essential therapeutic protein 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]

3.1.1. 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 asparagine by glutamine 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^{-1} s^{-1} *vs.* 3.0×10^3 1 M^{-1} s^{-1} and 5.0×10^2 1 M^{-1} s^{-1}).[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) (Fig. 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^{-1} kg^{-1}). 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 pharmacokinetics, Tenecteplase, which was approved as Metalyse® in 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 a 90 minute-infusion to maintain the required plasma concentration.

3.1.2. GLP-1 (DPP-IV resistant). The cleavage site for DPP-IV (His7-Ala8) in GLP-1 has been clearly identified, with

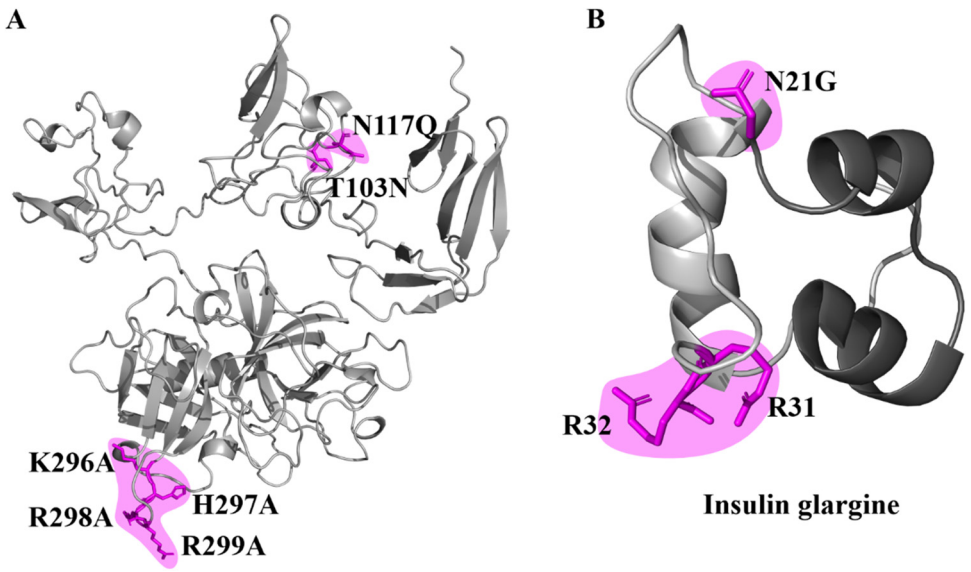

Fig. 3 Genetic engineering as formulation strategy for tPA and insulin. (A) Human tPA modelled by alpha-fold (UniProt: P00750) with purple marking point mutations T103N, N117Q and K296A–R299A for the development of the tPA analog Tenecteplase. (B) Insulin glargine with the A-chain (dark grey) and B-chain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

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

Therapeutic agent		Formulation strategy	Plasma half-life	Approval EMA	Therapeutical use	Ref.
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 and 176
GLP-1 receptor agonists	GLP-1	—	2 min (i.v.) 1.5 h (s.c.)	—	—	49 and 102
	Liraglutide	Fatty acid acylation	13 h (s.c.)	2009, Victoza® 2015, Saxenda®	Adjunct for DM type 2 Obesity	177 and 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 and 180
	Albiglutide	HSA fusion protein, point mutation, tandem copies	5 d (s.c.)	2014, Eperzan® Withdrawn: 2018	Adjunct for DM type 2	129 and 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
Long-acting insulin analogs	Human insulin	—	4–6 min (i.v.)	1997, Insuman Rapid®	DM	62 and 182
	NPH	Suspension formulation with protamine and zinc	Term $t_{1/2}$ 5–10 h	2002, Protaphane®	DM	110
	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 analog	IFN-β-1a	—	10 h (i.m.), 50–60 h (r.s.c.)	1997, Avonex® 1998, Rebif®	Relapsing MS	74 and 75
	PEG-IFN-β-1a	CHO-mPEG 20 kDa	78 ± 15 h (s.c.)	2014, Plegridy®	Relapsing remitting MS	185

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}; Thr^8 = 49 nmol L^{-1}; Gly^8 = 2.8 nmol L^{-1}; Ser^8 = 9 nmol L^{-1}; Aib^8 = 0.45 nmol L^{-1}; GLP-1(7–36)amide = 0.78 nmol L^{-1}).[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.

3.1.3. Insulin glargine. In contrast to the amino acid substitutions described above to reduce the 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 (Fig. 3B). In the acidic formulation of pH 4, insulin glargine is soluble but crystallizes immediately at the 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 μg mL^{-1}).[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]

3.2. Conjugation strategies

Conjugation strategies offer the possibility of posttranslational modification of proteins and peptides to provide 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 esters 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 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 study, 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 a 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.

3.2.1. 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^{-1}.[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 μM.[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.

3.2.2. 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 derivates with more than 12 carbon atoms made once-daily administration possible. Fatty acid conjugations with up to 16 carbon atoms, almost anywhere in the C-terminal region, 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 (Fig. 4A). This GLP-1 analogue Liraglutide was approved in 2009 (Victoza®, 2015: Saxenda®).[102]

However, the once-daily use of Liraglutide required high compliance and time commitments 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 the binding affinity to basic residues in albumin, bulkier or longer linkage structures to gain distance from the shielding HSA protein, and combinations with sequence modifications to protect DPP-IV degradation.[104]

3.2.3. 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, Fig. 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 the 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.

3.2.4. Insulin detemir. A similar strategy was used to develop long-acting insulin analogs mimicking the basal level of insulin. Kurtzhals *et al.* prepared insulin 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^{-1}), but still about 10^4 to 10^5 1 M^{-1}.[108] Further studies demonstrated that myristic acid (C14) acylated at Lys29 and deletion of Thr30 of the B-chain yield the most protracted insulin conjugate, with a 50% disappearance time of 14.3 hours after subcutaneous injection in pigs, compared to 10 hours for NPH insulin(Fig. 4C). Thereby, the 50% disappearance time correlated with the affinity of the conjugate for binding 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 and 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]

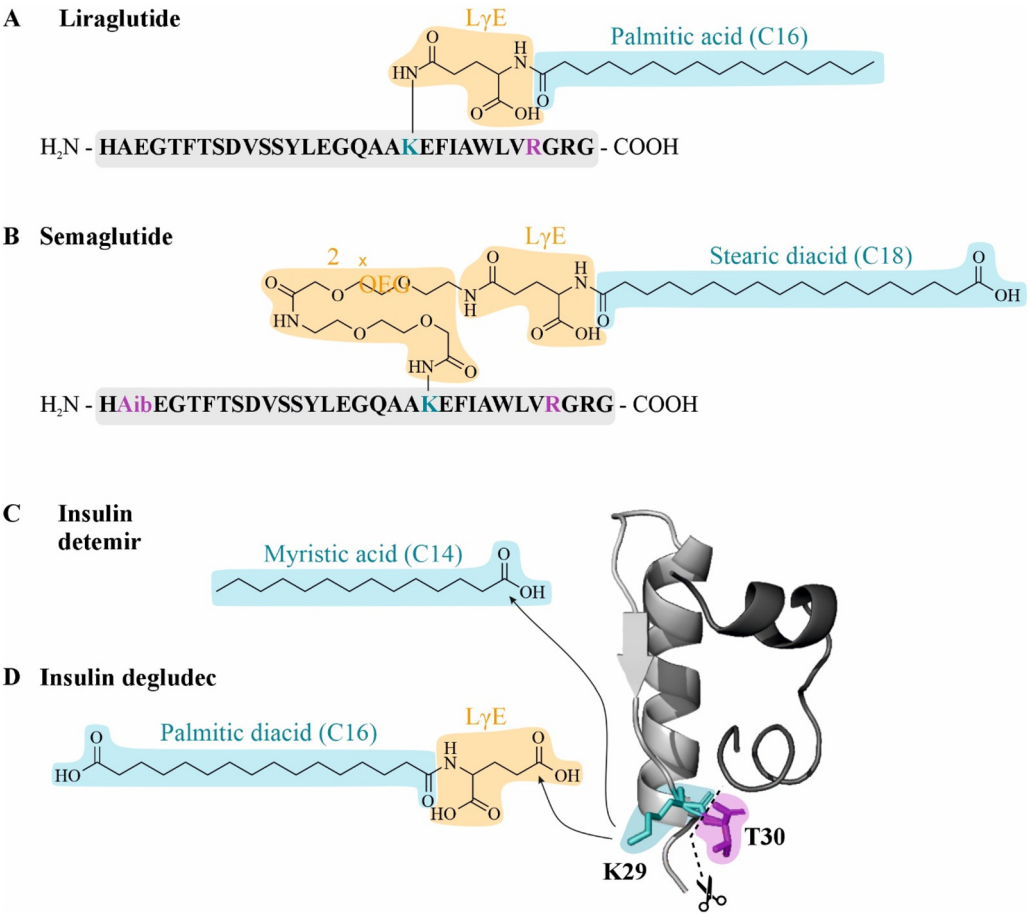

Fig. 4 Fatty acid acylation as a formulation strategy for GLP-1 and insulin. Purple labeled residues indicate point mutations compared to human GLP-1 or insulin, and blue labeled residues indicate conjugation sites. Orange labels indicate linkage structures, and blue labels indicate conjugated fatty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

3.2.5. 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 (Fig. 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 a steady state (25 hours) and more stable 24 hour glucose infusion rate profiles compared to other long-acting insulin analogs.[116]

3.2.6. 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 change 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]

3.2.7. 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 (Fig. 5). The obtained bioconjugate retained only 50% of its activity but showed significantly improved pharmacokinetic properties after intravenous application in rats. In particular, the elimination half-life of the PEGylated protein was about 13-fold longer than that of the parent protein.[119] Although the bioactivity of PEGylated IFN-β-1a was reduced, it could be shown in an animal model that the

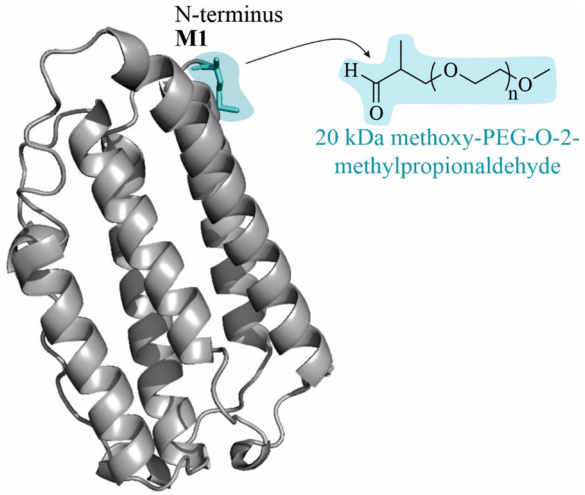

Fig. 5 Site-specific PEGylation at the N-terminus M1 (blue) of Interferon-β-1a (UniProt: P01574) with 20 kDa methoxy-PEG-*O*-2-methylpropionaldehyde (blue).

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 a placebo and comparable to non-pegylated interferon-β (Table 1).[121,122] This is highly beneficial compared to non-pegylated IFN-β-1a (Rebif®), which had to be administered three times per week.[75]

3.3. 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 been approved for 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 degraded in lysosomes 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]

3.3.1. 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 the 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 effects on tumor cells *in vitro* using recombinant protein Onconase fused with HSA, compared with using shorter linker lengths.[127] Further adjustments to achieve high bioactivity of the fused protein require consideration of the N- or C-terminus as the junction between the two proteins and the use of tandem copies of the fused protein.[128]

3.3.2. Albiglutide. Albiglutide was the first albumin fusion protein approved in 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 a determining factor for receptor binding (Fig. 6A).[89] Additionally, the strategy of amino acid substitution was applied by substituting Ala8 with 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

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

postprandial blood glucose levels, with no hypoglycemic episodes compared to the 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 due to the lower reduction in body weight reported compared to using other GLP-1 analogs.[135]

3.3.3. Fc fusion proteins. The binding site for the recycling mechanism of IgG *via* FcRn is located in the Fc region of IgG antibodies between the hinge and the CH2 and CH3 domains. Therefore, this Fc region is sufficient to enable FcRn-mediated recycling, resulting in the prolonged half-life of IgGs. The choice of IgG antibody subclass influences the immunological properties of the resulting fused Fc region and the desired protein. IgG_1 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. IgG_2 and IgG_4 have a lower affinity toward 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]

3.3.4. 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 IgG_4 hinge region (Fig. 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 IgG_1 Fc region first used was replaced by an IgG_4 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 for 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]

3.5. 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 tPA,[142–144] 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]

3.5.1. Liposomes. Encapsulation of tPA in liposomes consisting of egg phosphatidylcholine (EPC), cholesterol, sodium cholesterol-3-sulfate (CS) and distearolyphosphatidyl 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]

3.5.2. 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]

3.5.3. 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]

3.5.3. 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 (Fig. 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] A common drawback of PLGA microspheres as a 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]

4. Comparison of formulation strategies

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 productions 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 exhibit multiple cysteines that can interact with free cysteines in the patent 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, the stability of the peptide within the formulation is crucial, which has only been 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 the 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 outcomes, 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 the body. This approach minimizes systemic circulation and consequently reduces elimination *via* the 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 the cRGD (Arg–GlyvAsp) 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 protein 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]

5. Conclusion

Specific formulation strategies to reduce liver uptake of protein drugs are largely underexplored considering the predominant role of renal uptake in protein clearance. Considering the mechanism of hepatic clearance in formulation design was successfully applied for a 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 the covalent formation of depots of protein drugs on the extracellular matrix in diseased tissues to avoid liver uptake, and elimination.

Author contributions

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

Conflicts of interest

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.

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

Data availability

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

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