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Prevention of diabetes-associated fibrosis : Strategies in FcRn-targeted nanosystems for oral drug delivery

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Prevention of diabetes-associated fibrosis: Strategies in FcRn-targeted nanosystems for oral drug delivery

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Abbreviations

ABD, albumin-binding domain; **AGEs**, advanced glycation end-products; **BSA**, bovine serum albumin; **β2m**, beta-2 microglobulin; **DM**, diabetes *mellitus*; **DPP-4**, dipeptidyl peptidase-4; **ECM**, extracellular matrix; **EDC/NHS**, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide/N-hydroxysuccinimide; **Fab**, fragment antigen-binding; **Fc**, fragment crystallizable; **Fc-NPs**, Fc-decorated nanoparticles; **FcRn**, neonatal Fc receptor; **FcRnBPs**, neonatal Fc receptor-binding peptides; **GIT**, gastrointestinal tract; **GLP-1**, glucagon-like peptide-1; **hFcRn**, human neonatal Fc receptor; **hIgG**, human immunoglobulin G; **HPMC**, hypromellose acetate succinate; **HSA**, human serum albumin; **HSA-NPs**, HSA-decorated nanoparticles; **IgG**, immunoglobulin G; **MAL**, maleimide; **mFcRn**, mouse neonatal Fc receptor; **MHC-I**, major histocompatibility complex class I; **mIgG**, mouse immunoglobulin G; **MSA**, mouse serum albumin; **NPs**, nanoparticles; **OI**, Oral insulin; **PEG**, polyethylene glycol; **PLA**, poly(lactic acid); **PLGA**, poly(lactic-co-glycolic acid); **RAAS**, renin-angiotensin-aldosterone system; **scFv**, single-chain variable fragment; **SiNPs**, silica nanoparticles; **T1DM**, type 1 diabetes *mellitus*; **T2DM**, type 2 diabetes *mellitus*; **TGF-β**, transforming growth factor beta; **WT**, wild-type.

Abstract

Diabetes *mellitus* is a chronic disease with an elevated risk of micro- and macrovascular complications, such as fibrosis. To prevent diabetes-associated fibrosis, the symptomatology of diabetes must be controlled, which is commonly done by subcutaneous injection of antidiabetic peptides. To minimize the pain and distress associated with such injections, there is an urgent need for non-invasive oral transmucosal drug delivery strategies. However, orally administered peptide-based drugs are exposed to harsh conditions in the gastrointestinal tract and poorly cross the selective intestinal epithelium. Thus, targeting of drugs to receptors expressed in epithelial cells, such as the neonatal Fc receptor (FcRn), may therefore enhance uptake and transport through mucosal barriers. This review compiles how in-depth studies of FcRn biology and engineering of receptor-binding molecules may pave the way for design of new classes of FcRn-targeted nanosystems. Tailored strategies may open new avenues for oral drug delivery and provide better treatment options for diabetes and, consequently, fibrosis prevention.

Keywords: Antidiabetic peptides, FcRn, IgG, albumin, intestinal epithelium, transcytosis, half-life, nanoparticles, active targeting, transgenic mouse model.

Teaser: This review provides a brief overview of FcRn biology, engineering of receptor-binding molecules, and recent advances in FcRn-targeted nanosystems with the aim of improving oral drug transport of antidiabetic peptides across selective intestinal epithelial barriers for the treatment of diabetes *mellitus* and, consequently, prevention of fibrosis.

Graphical abstract:

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1. Introduction

Diabetes *mellitus* (DM) is a chronic metabolic disease that develops when the pancreas fails to produce insulin or when the body does not fully respond to insulin, leading to impaired glucose tolerance. More than 460 million people worldwide are suffering from DM, and the number of cases is predicted to rise to 700 million by 2045 [1, 2]. Prolonged exposure to hyperglycemia may cause tissue injury and lead to fibrosis, which increases the risk of micro- and macrovascular complications in several organs, such as the eyes (diabetic retinopathy), kidneys (diabetic nephropathy), skin, heart, liver, lungs, nerves (neuropathy) and blood vessels (angiopathy) [3, 4]. Fibrosis is characterized by abnormal deposition of the extracellular matrix (ECM), and there is currently no specific treatment available [5]. As such, there is an urgent need for strategies that could prevent or delay the progression of diabetes-associated fibrosis.

Conventional treatment of DM requires subcutaneous injection of peptide-based antidiabetic drugs for glycemic control, which is considered a painful and invasive approach. The preferred route of administration is oral delivery via mucosal surfaces, due to its ease of administration and high patient compliance, without the need for syringes, needles, and trained health workers [6, 7]. Notwithstanding, orally administered antidiabetic peptides are susceptible to the harsh environment of the gastrointestinal tract (GIT) and the selective barrier of the intestinal epithelium [8, 9]. Hence, there is a demand for innovative strategies to overcome these limitations.

Biodegradable and biocompatible nanoparticles (NPs) have emerged as promising oral drug delivery systems that protect encapsulated drugs from acidic pH and enzymatic degradation in the GIT, while also providing controlled and sustained release and enhanced bioavailability of drugs to target sites. However, the transport of NPs across intestinal mucosa is restricted by polarized epithelial cell layers [8, 10]. Several recent studies support that targeting of the neonatal Fc receptor (FcRn), which is expressed by such epithelial cells and mediates bidirectional transport and recycling of immunoglobulin G (IgG) and albumin, may be used as a gateway for more efficient transcellular transport of NPs [11-15]. Furthermore, in light of successes in the development of engineered ligands and alternative scaffolds with improved FcRn binding and transport properties, researchers may now harvest from these advances in the design of new classes of NPs decorated with FcRn-targeted molecules, with the aim of enhancing transmucosal delivery upon oral administration. This review provides a brief overview of FcRn biology and discusses how receptor-binding molecules may be incorporated into nanosystems for improved FcRn-mediated oral delivery of antidiabetic peptides for the treatment of DM and the prevention of fibrosis.

2. Diabetes-associated fibrosis

Fibrosis is one of the major complications of DM, induced by chronic hyperglycemia, which leads to an excessive accumulation of ECM macromolecules and an imbalance in tissue homeostasis [3, 5]. Normally, ECM is responsible for the mechanical and structural support of cells and mediates intercellular communication. However, under cell and tissue injury, the formation of ECM is dysregulated. This is a complex and multifactorial process involving increased production of advanced glycation end-products (AGEs) and circulating growth factors, and activation of protein kinase C as well as the renin-angiotensin-aldosterone system (RAAS) and plasminogen and protease systems [3, 16]. A schematic overview of fibrosis development as a result of diabetes-induced hyperglycemia is shown in **Fig. 1**.

Fig. 1 | Fibrosis as a complication of diabetes. In DM, impaired pancreatic function or insulin resistance causes blood glucose levels to rise above normal. Prolonged hyperglycemic condition may induce severe tissue injury by the action of AGEs, RAAS, oxidative stress, plasminogen and protease systems, protein kinase C and circulating growth factors. This leads to dysregulation of the ECM, characterized by excessive accumulation of ECM components and alteration in ECM quality, which triggers the formation of fibrosis in several organs. The figure was created with BioRender.

AGEs are lipids or proteins that are glycated upon exposure to sugars, which are prevalent in patients with DM. Under hyperglycemic conditions, AGEs stimulate the excess production of ECM macromolecules, which affects the ECM integrity and the profibrotic process [3, 5]. RAAS, in which angiotensin II is considered the main effector molecule, promotes the accumulation of ECM components as well as the release of inflammatory cytokines and growth factors, such as transforming growth factor beta (TGF- β), vascular endothelial growth factor, platelet-derived growth factor, and connective tissue growth factor [3, 5]. In addition, oxidative stress can accelerate the progression of fibrosis [2]. In particular, the increase in TGF- β stimulates the epithelial-to-mesenchymal transition, which contributes to the accumulation of ECM components, culminating in tissue fibrosis [17, 18].

Interestingly, antidiabetic peptides have great potential in the treatment of fibrosis. For instance, dipeptidyl peptidase-4 (DDP-4) inhibitors, known as gliptins, and glucagon-like peptide 1 (GLP-1) agonists, have demonstrated mild antifibrotic properties in models of liver fibrosis by reducing inflammation and oxidative stress [19]. Owing to the antidiabetic activity of gliptins and GLP-1 analogs, they are widely used in the treatment of type 2 diabetes (T2DM) [20]. Furthermore, encapsulation of these peptide-based drugs into nanosystems targeting FcRn, by taking advantage of the protective effect of NPs and receptor-targeting ligands with improved FcRn binding and transport properties, may lead to enhanced transmucosal delivery upon oral administration. In addition, these antidiabetic peptides may be engineered for half-life extension using FcRn-binding molecules. Notably, FcRn is expressed by cells of the lungs, eyes and kidneys [21-23], where fibrosis typically occurs.

3. The biology and molecular mechanisms of FcRn

3.1. Structure and binding of ligands

FcRn is a heterodimeric glycoprotein that is structurally similar to major histocompatibility complex class I (MHC-I) molecules, consisting of a unique transmembrane heavy chain (40-46 kDa) that is non-covalently associated with the common beta-2-microglobulin (β 2m) light chain (12 kDa) [24, 25]. The heavy chain contains three extracellular domains (denoted α 1, α 2 and α 3) where the α 1 and α 2 domains assemble a platform of eight antiparallel β -strands topped by two α -helices followed by the α 3 domain, which is folded up on the soluble β 2m [26, 27].

Unlike MHC-I molecules that present antigenic peptides via their peptide binding groove to cytotoxic T cells, the corresponding binding groove of FcRn is closed [26-29]. Instead, FcRn has evolved to bind IgG and albumin, two structurally and functionally unrelated proteins. Both ligands bind the receptor in a pH-dependent manner, binding at acidic pH (6.5-5.0) and no binding or release at physiological pH (7.4) [30-35]. The strict pH-dependent mode of action is mediated by protonation of the imidazole side chains of conserved histidine residues found on both the receptor and the ligands. When the pH increases towards neutral, protonation of the histidine residues is gradually lost, and the ligands are released from the receptor [31, 33, 34, 36, 37]. Importantly, FcRn binds both ligands simultaneously in a non-cooperative manner as a ternary complex (**Fig. 2**) [32, 35, 37].

Fig. 2 | Crystallographic illustration of the ternary complex of hFcRn bound to hIgG and HSA. The three domains of the hFcRn heavy chain (denoted $\alpha 1$, $\alpha 2$, and $\alpha 3$) are shown in green, and the $\beta 2m$ subunit in gray. The hIgG1 Fc fragment is shown in brown. The subdomains DI, DII, DIIIa and DIIIb of HSA are colored in orange, wheat, light cyan and light blue, respectively. (A) Close-ups showing key amino acid residues involved in the interaction between hIgG1 (H310, I253, H435, N434) and hFcRn (E115, W131, D130). (B and C) Close-ups of the interactions between two loops in DI of HSA (loop I; R81, D89, and loop II; N109, N111) and hFcRn (T153, K63, S58). (D) Close-ups of the intramolecular interactions between H166 of the $\alpha 2$ domain and E54 and Y60 of the $\alpha 1$ domain in hFcRn, which form at acidic pH and in turn stabilize the loop containing W51, W53, W59 and W61. (E and F) Close-ups showing W59 and W53 of hFcRn inserted into hydrophobic pockets in DIIIa (T422, V426, L460, L463, H464 and T467) and DIIIb (F502, F507, F509, T527 and F551) of HSA, respectively. The figure was made using PyMol and the crystal structure data deposited of hFcRn bound to hIgG1 Fc and HSA (PDB 4N0U) [35].

IgG (150 kDa) is the most prevalent antibody in blood, with concentrations ranging from 2-14 mg/mL in mice, and 8-17 mg/mL in humans [38-42]. It is produced by B and plasma cells and protects the body from foreign substances, such as pathogens and toxins. This Y-shaped homodimeric protein contains four polypeptides that make up two fragment antigen-binding (Fab) arms, linked via a hinge to a constant N-glycosylated fragment crystallizable (Fc) part [38, 43, 44]. While the Fab arms recognize foreign antigens, the Fc is responsible for binding to effector molecules, including FcRn [38, 43]. The interaction between FcRn and IgG occurs primarily at the CH2-CH3 elbow region with a stoichiometry of two FcRn molecules per monomeric IgG, where the conserved residues I253, H310 and H435 are key players [27, 35, 45-

50]. Specifically, protonation of the histidine residue H310 at acidic pH enables interaction with E115 on the α 2-domain of the human FcRn (hFcRn) heavy chain [35, 50]. Moreover, I253 interacts with W131 of the receptor, whereas the side chain of H435 does not make any direct contacts but has been shown to regulate a loop within the Fc, including residues 429-436, prior to binding [27, 35, 50, 51]. Replacement of these residues with alanine abrogates receptor binding, and results in inefficient transport and short half-life [22, 46, 49, 50, 52-54]. Also, the soluble β 2m of rat FcRn has been shown to contribute to binding of rat IgG via residue Ile1 by making van der Waals contacts with residues V309 and P307 of the Fc [27, 55]. In addition, increasing evidence supports the possibility for Fab regions to modulate the interaction with FcRn [56-59]. The key interactions between hFcRn and human IgG1 (hIgG1) Fc are given in **Fig. 2**.

2.

The other ligand, albumin (66.5 kDa), is the most prevalent blood protein, with a concentration of 20-40 mg/mL and 35-50 mg/mL in mice and humans, respectively [32, 60, 61]. Its natural function is to transport a range of small insoluble cargos, including fatty acids, hormones, metal ions and waste products, for delivery to tissues and organs [60]. Due to its hydrophobic binding capacity and high aqueous solubility, albumin may be used as a transporter for poorly soluble drugs [62]. Also, it is an attractive fusion partner for protein-based drugs for half-life extension [63]. In contrast to the multi-domain structure of IgG, human serum albumin (HSA) contains a non-glycosylated single polypeptide made up of 585 amino acids, which forms a heart-shaped structure with three homologous domains (denoted DI, DII and DIII) [64]. These are connected via flexible loops, where each domain can be divided into two subdomains (A and B) [64]. While DIII contains the principle-binding site for hFcRn, DI modulates the interaction [34, 35, 37, 65]. Hydrophobic residues of the subdomains DIIIA and DIIIB make contacts with the hFcRn residues W59 and W53, located within a pH-sensitive α 1-domain loop [35, 65, 66]. At acidic pH, the histidine residue at position 166 within the FcRn α 2-domain (H166) is protonated and interacts with E54 and Y60 in the α 1-domain loop, which stabilizes the orientation of the loop required for efficient albumin binding [34, 35, 37, 65, 66]. Substitution of H166 with an alanine abolishes binding to albumin [33, 34, 66]. In addition, other DIII residues, including histidine residues (H464, H510 and H535) and K500, are required for strong binding at acidic pH [34, 35, 65]. Targeting of these residues by mutagenesis reduces receptor binding considerably, which results in short half-lives in hFcRn transgenic mice [34]. The contribution from DI is minor but important for optimal hFcRn binding. Notably, a recombinant form of HSA DIII binds 10-fold weaker to hFcRn than the full-length version [67, 68]. Structurally, the DI residues involved are N109, N111 and R81-D89 that make contacts with K63, S58, and T153 of hFcRn, respectively [35, 65, 67]. Close-ups of the interactions between hFcRn and HSA are given in **Fig. 2**.

3.2. Recycling and transcytosis of ligands

hIgG and HSA share a half-life of 3 weeks in humans on average [69-71]. This results from their molecular sizes above the renal clearance threshold and FcRn-mediated rescue from intracellular degradation [32, 72-75]. The astonishing role of FcRn in half-life regulation is evident in patients suffering from the rare human disease hypercatabolic hypoproteinemia, who have remarkably low blood amounts of both ligands due to deficient $\beta 2m$ expression and, consequently, low levels of functional FcRn [76, 77]. In line with this are data from clinical trials where blocking of the IgG binding site on FcRn using anti-FcRn antibodies rapidly reduced the levels of endogenous IgG by up to 80% [78-81]. In addition, mice lacking FcRn expression have about 4-fold and 2-fold lower levels of IgG and albumin than normal mice, respectively [32, 73, 74]. As such, FcRn is a key homeostatic regulator of IgG and albumin by transporting the ligands within and across cells via two cellular pathways, recycling and transcytosis. Both processes are regulated by strict pH-dependent binding-and-release interactions between FcRn and its ligands [68, 72, 75, 82-84].

Insights into how FcRn mediates cellular recycling have been extensively studied by *in vitro* cellular transport assays combined with advanced imaging technologies, mainly with a focus on IgG [72, 75, 82, 85-91]. However, recent studies have also revealed knowledge about how FcRn transports IgG and albumin simultaneously [72, 84, 92]. Together, the studies demonstrate that the ligands are taken up via passive fluid-phase pinocytosis at neutral pH, followed by entering of acidified endosomal compartments where FcRn is predominantly located. The mildly acidic pH (6.0-6.5) herein facilitates protonation of the histidine residues and engagement of the receptor. Bound ligands are then rescued from intracellular lysosomal degradation by sorting of the FcRn-ligand complexes to the cell surface, where exposure to neutral pH triggers ligand release from FcRn into the extracellular space. Proteins that do not bind the receptor are rapidly degraded in lysosomes [72, 82]. Studies in mice have shown that both non-hematopoietic and hematopoietic cells are equally important for FcRn-mediated half-life regulation [83, 93-99].

Furthermore, FcRn can transport the ligands in both directions across polarized cellular barriers, such as the epithelial cell layers of the lungs and the intestine [22, 52, 68, 83, 100-108]. Regarding the polarized intestinal epithelium of the GIT, IgG and albumin are taken up at the basolateral side via fluid-phase pinocytosis, where the pH is neutral, for subsequent delivery to acidified endosomes in which engagement of FcRn directs the transport across the cells. If the pH at the apical side is neutral, the ligands will be released. However, where the intestinal lumen milieu is mildly acidic, the ligands may remain bound to the receptor and, again, be taken up by

the cells, either in a monomeric form, or as IgG-containing immune complexes. This may result in either recycling at the apical side or transcytosis through the polarized epithelial cell layers for release at the basolateral side into lamina propria. The ligands may then be transported via the lymphatic system and enter the systemic circulation via the thoracic lymph nodes, or be processed by immune cells as IgG-containing immune complexes, as recently reviewed [99, 109]. FcRn-mediated transcytosis has been studied *in vitro* by the use of *Transwell*® assays with polarized epithelial cell lines that either endogenously express the receptor or are manipulated to overexpress the receptor [52, 68, 83, 102-104, 106, 108]. In addition, transcytosis across mucosal epithelial barriers has been studied *in vivo*, either by administering the ligands systemically followed by sampling at mucosal sides [101, 105, 107], or by giving them via mucosal sites in a needle-free manner followed by sampling of tissues or blood over time [22, 53, 68]. The principles of FcRn-mediated recycling and bidirectional transcytosis are shown in Fig. 3.

Fig. 3 | Schematic illustration of FcRn-mediated recycling and bidirectional transepithelial transport. (A) Recycling: (1) IgG and albumin are internalized into cells expressing FcRn, such as vascular endothelial cells, via fluid-phase pinocytosis, and subsequently (2) bind FcRn located within acidic endosomes. (3) The receptor-ligand complex is then recycled back to the cell surface, (4) where the ligands are released into the systemic circulation upon exposure to the neutral pH following exocytosis. (5) Proteins that do not

bind FcRn are catabolized in lysosomal compartments. **(B) Transcytosis:** **(1)** In polarized epithelial cells, such as enterocytes, IgG and albumin are taken up via fluid-phase pinocytosis at the basolateral side where the pH is neutral. **(2)** Ligands are subsequently sorted into acidified endosomes where they bind to FcRn. **(3)** The receptor-ligand complex is then directed to the apical surface of the cells, where it promotes the release of ligands upon exposure to acidic pH of the intestinal mucosa. In addition, the ligands may remain bound to the receptor and be taken up by the cells again. The figure was created with BioRender.

4. IgG- and albumin-based drug delivery of antidiabetic peptides

About 90% of patients diagnosed with DM have T2DM, while the remaining have type 1 DM (T1DM) [1]. The primary aim of T2DM treatment is to maintain glycemic control by antidiabetic medication. This can be achieved by the use of GLP-1 peptide analogs that stimulate insulin secretion by pancreatic beta cells, or insulin analogs that promote efficient glucose disposal. However, such peptides have a very short half-life of about a few minutes, due to their small size and rapid clearance from the circulatory system via kidney filtration. Hence, efficient treatment would require regular monitoring and frequent dosing schedules [1]. To improve the efficacy and patient compliance of DM therapy, the natural long half-life of hIgG4 Fc and HSA may be used in drug design, which have already been explored for a range of antidiabetic peptides (**Table 1**).

The first GLP-1 analog to reach the market was liraglutide (Victoza®), which is linked to a fatty acid (palmitic acid) that binds reversibly to hydrophobic pockets of HSA [110]. As such, it associates non-covalently with endogenous HSA in the subcutaneous tissues and bloodstream. This strategy extends the half-life to 11-15 hours and allows for once-daily dosing [110-112]. Another example is the zinc-based taspeglutide, where aminoisobutyric acid is covalently linked to two positions on GLP-1. This was shown to extend the half-life up to 2-3 days in humans [113, 114]. However, clinical testing was halted due to severe gastrointestinal side effects and hypersensitivity reactions [115]. Furthermore, for the treatment of not only T2DM but also T1DM, other fatty acid-based strategies have been developed for insulin. Examples are insulin detemir (Levemir®) [116, 117] and insulin degludec (Tresiba®) [118, 119]. Both are recombinant human insulin analogs with a fatty acid linked to a lysine residue, while insulin degludec also lacks a threonine residue. Insulin detemir and insulin degludec have distinct half-lives of 18-26 hours and 42 hours, respectively [63, 116-120]. An insulin analog with an activity profile close to that of insulin detemir is insulin glargine (Lantus®) with a half-life of about 24 hours [121]. Instead of modification of the fatty acid side chains, insulin glargine includes two additional arginine residues in the beta chain and an amino acid replacement of asparagine at position 21 with glycine. Nevertheless, these products have gained market approval for once-daily dosing.

To further extend half-lives of insulin analogs, insulin icodec has been developed as a once-weekly insulin that is currently in phase I and phase III clinical trials in patients with T1DM and T2DM, respectively [122, 123]. As a result of three amino acid substitutions and the addition of an icosane fatty diacid to this insulin analog, it shows an extended half-life of 8 days in humans and improved stability and solubility.

Despite the fact that these strategies provide prolonged blood persistence compared with native peptides, treatment relies on subcutaneous injections. To allow oral administration, semaglutide has been developed [124]. It is a modified GLP-1 analog where alanine and lysine residues at positions 8 and 34 have been replaced with 2-aminoisobutyric acid and arginine, respectively, to prevent degradation by dipeptidyl peptidase-4 (DPP-4). In addition, lysine at position 26 is engineered to contain a spacer and a fatty acid that can bind endogenous HSA upon injection. This formulation provides a half-life of about 7 days in humans [125, 126]. When semaglutide is co-formulated with an absorption enhancer, namely sodium N-(8-[2-hydroxybenzoyl]amino) caprylate, which neutralizes the pH of the gastric fluid and hinders enzymatic activity, it is protected from degradation in the GIT following oral administration [124]. The injected version of semaglutide (Ozempic®) was clinically approved in the US and Europe in 2017-2018, while the oral formulation (Rybelsus®) was approved last year [124, 127-129]. The latter is the first GLP-1 receptor agonist to be approved for oral administration. Notably, there may be room for further optimization as its oral bioavailability is limited ($\leq 1.0\%$) [130, 131].

Currently, there are no oral insulin (OI)-based drugs available for DM treatment. However, very recently, the first clinically tested once-weekly OI analog, named OI338, demonstrated efficacy near subcutaneously injected insulin glargine [132], which has led to the development of long-acting OI formulations [133]. Commonly, a tyrosine at position 14 and a phenylalanine at position 25 are replaced by glycine and histidine, respectively, in addition to a deletion of threonine at position 30 [133]. These amino acid modifications make OI less susceptible to degradation in the GIT upon oral administration [132, 133], in combination with the use of an absorption enhancer, such as Gastro Intestinal Permeation Enhancer Tablet I (GIPET®1) based on sodium caprate [134]. To gain improved HSA binding, the lysine residue at position 29 of the OI is coupled to a strong albumin binding molecule (C18 fatty diacid) via a hydrophilic linker. Moreover, OI molecules can be designed for prolonged half-life by engineering for decreased insulin receptor affinity. This design resulted in OI338 with a half-life of 70 hours in T2DM patients [132].

In the context of FcRn, it has been demonstrated that binding of fatty acids to the hydrophobic pockets of HSA fact negatively affects receptor binding, in which the effect depends on the

length of the fatty acid [65]. As such, half-life extension upon conjugation to fatty acids will have limitations. Regarding oral delivery, this means that fatty acid-based strategies may not benefit from albumin binding to FcRn expressed at epithelial cell barriers in the GIT. This is a highly interesting topic that should be addressed in future studies.

An alternative strategy to non-covalent association with HSA is site-specific conjugation. One example is GLP-1 receptor agonist exendin-4 [135-137]. Notably, exendin-4 increases insulin secretion in a glucose-dependent manner at the same time as it stimulates β -cell proliferation and restores sensitivity to glucose [138]. When injected in the absence of HSA, it has a half-life below 1 hour [138, 139]. However, when linked to HSA (CJC-1134-PC), it has been shown to gain a half-life of about 8 days in a phase II clinical trial [111, 136, 140]. This allows for once-a-week dosing. Specifically, exendin-4 is linked to HSA via the PC-DAC™ technology that utilizes maleimide (MAL) chemistry for stable conjugation to the thiol group of a free cysteine residue (C34) in HSA DI [112, 139]. The same technology has been used for conjugation of insulin (PC-DAC™:Insulin), which is under preclinical development and has demonstrated 30 times longer half-life compared with native insulin in rats [141].

Importantly, C34 is distant from the hFcRn interaction site in HSA DI [65, 142]. However, it has been demonstrated that conjugation to C34 may compromise pH-dependent FcRn binding [143-145]. How pronounced this effect could be will depend on the biophysical properties of the conjugated drug and, as such, needs to be addressed in a case-to-case scenario. Notably, the loss of receptor binding may be restored by taking advantage of engineered HSA variants containing amino acid substitutions in the C-terminal DIII that improve binding to FcRn, as discussed in **Section 5**.

Another alternative strategy is to genetically fuse the peptide of interest to the N- or C-terminal end of HSA. In the context of DM, a di-peptide of GLP-1 has been fused to wild-type (WT) HSA (albiglutide) [146-148], a product known as Tanzeum® in the US and Eperzan® in Europe, which was recently withdrawn from the market due to economic reasons. Nevertheless, the design gave rise to a half-life of 5 days in humans, allowing for weekly subcutaneous injection [146-148]. This fusion was based on the addition of the di-peptide to the N-terminal of HSA, which leaves the C-terminal end free. Interestingly, the last leucine residue of the C-terminal end of HSA may be enzymatically cleaved in blood, which has been demonstrated to reduce hFcRn binding and lead to a short half-life in mice and humans [149]. Hence, this biological phenomenon should be considered when designing HSA fusions. A way to compensate for loss of receptor binding is to take advantage of engineered HSA variants with improved receptor affinity, as recently demonstrated [149].

The last example is the use of hIgG Fc fusion technology, where a GLP-1 receptor agonist, again engineered to be resistant to enzymatic degradation, is fused to a hIgG4 Fc fragment via a short linker (dulaglutide, Trulicity®) [150, 151]. IgG4 is likely to be chosen instead of IgG1 due to safety concerns, as IgG4 Fc is less potent in induction of effector functions [43], without altering the ability to engage FcRn. Dulaglutide has a half-life of nearly 5 days in humans, and the product is approved for once-weekly subcutaneous administration [150, 151].

Hitherto, only one of these strategies has been approved for oral delivery. However, in principle, all the antidiabetic peptide strategies may be combined with nanosystems tailored for oral administration to achieve more efficient delivery across the selective mucosal barriers. Although, this will require a sophisticated design that allows the nanosystems to be taken up and delivered to the circulatory system more efficiently than what has been demonstrated to date. This may be achieved by harvesting from recent knowledge about FcRn biology and advances in the engineering of receptor-targeting molecules with favorable binding and transport properties. Such molecules may be directly fused to the peptides, but they may also be used to decorate nanosystems, as discussed in **Section 7**.

Table 1 | hIgG Fc and HSA as platform for half-life extension of antidiabetic peptides in clinic.

Analog	Name	Ligand	Drug type	Clinical status	$t_{1/2}$	ROA	Company	Reference
GLP-1 (T2DM)	Victoza®	HSA	Liraglutide	Marketed	11-15 h	SC	Novo Nordisk	[110, 111]
	Rybelsus®	HSA	Semaglutide	Marketed	6-7 d	Oral	Novo Nordisk	[124, 128, 129]
	Ozempic®	HSA	Semaglutide	Marketed	7 d	SC	Novo Nordisk	[127]
	Trulicity®	hIgG4 Fc	Dulaglutide	Marketed	4.7-5 d	SC	Eli Lilly and Company	[150, 151]
	CJC-1134	HSA	Exendin-4	Phase II	8 d	SC	ConjuChem	[135, 136, 139]
	R1538	HSA	Taspoglutide	Halted phase III	2-3 d	SC	Roche/Ipsen	[113-115]
	Tanzeum®/ Eperzan®	HSA	Albiglutide	Withdrawn	5-8 d	SC	GlaxoSmithKline	[146-148]
Insulin (T1DM, T2DM)	Lantus®	HSA	Insulin glargine	Marketed	24 h	SC	Sanofi	[121]
	Levemir®	HSA	Insulin detemir	Marketed	18-26 h	SC	Novo Nordisk	[116, 117]
	Tresiba®	HSA	Insulin degludec	Marketed	42 h	SC	Novo Nordisk	[118, 119]
	LAI-287	HSA	Insulin icodec	Phase I/III	> 8 d	SC	Novo Nordisk	[122, 123]
	OI-338	HSA	Insulin-338	Phase II	70 h	Oral	Novo Nordisk	[132, 133]
	PC-DAC™	HSA	Insulin	Preclinical	5-15 h	SC	ConjuChem	[141]

$t_{1/2}$: half-life; ROA: route of administration; SC: subcutaneous administration.

5. Ligand engineering for improved FcRn binding

The therapeutic efficacy of many protein-based drug candidates is hampered by their short half-life, and those that have reached the market require frequent administration, which is a burden for the patients. As hIgG and HSA have a naturally long half-life, they are extensively used in therapy, either as full-length hIgG antibodies, or as Fc or HSA fused to therapeutic peptides or proteins of interest [63, 152, 153].

The in-depth knowledge gained from dissecting of the relationship between FcRn and its ligands at molecular and cellular level has been used in the design of hIgG and HSA variants with improved hFcRn binding and transport properties [38, 63, 68, 153-155]. One motivation for this is the high clinical approval rate of monoclonal hIgG antibodies and HSA fusions used to treat a range of different diseases. In fact, hIgG antibodies are the fastest growing class of protein-based drugs, with more than 100 products on the market and impressively over 800 antibodies under clinical testing [156, 157]. One example is orelizumab (TRX4), a hIgG1 antibody targeting CD3 to suppress pathogenic T cells, which is used to treat T1DM caused by antigen-specific T cells that infiltrate pancreatic beta cells and lead impaired insulin secretion [158]. While many of the early developed antibodies are based on WT hIgG1, next-generation of monoclonal antibodies will be engineered for either lack or improved effector functions, including optimal hFcRn-mediated transport properties [155]. In addition, recent research has also resulted in engineered HSA variants with improved half-life [65, 68, 159]. Moreover, several alternative scaffolds with FcRn-binding properties have been reported [160-164], but a major challenge for such engineering is fine-tuning of strict pH-dependent receptor binding in order to gain favorable recycling and transcytosis capacity. Improvements in FcRn engagement and transport may result in more favorable pharmacokinetic profiles, and consequently less frequent administration and higher patient compliance.

5.1. IgG Fc-engineering

Scrutinizing the FcRn-IgG interaction has led to the development of Fc-engineered variants (**Fig. 4** and **Table 2**) where amino acid substitutions within the CH2 and CH3 domains have been modified to gain improved pH-dependent binding to hFcRn. Importantly, a prerequisite for successful engineering is to augment receptor binding at acidic pH while, at the same time, avoiding increase in receptor affinity at neutral pH [38, 154, 165, 166]. The first proof-of-concept was given in 1997 by isolation of mouse IgG1 (mIgG1) Fc fragments by phage display that gave 3.5-fold improved binding towards mouse FcRn (mFcRn), which resulted in about 1.6-fold extended half-life in conventional mice [167]. This was followed by a generation of hIgG Fc-

engineered variants with increased affinity for hFcRn, which provided a several fold extended half-life in mice, non-human primates and humans.

One example is the YTE triple mutant variant, with three mutations in the CH2 domains (M252Y/S254T/T256E) (**Fig. 4**). The variant has 10-fold improved binding at pH 6.0, which resulted in about 3.5-fold extended half-life in non-human primates and humans [154, 168, 169]. Another well-studied hIgG1 variant harbors two substitutions of amino acid residues in the CH3 domains (M428L/N434S: LS variant) (**Fig. 4**). The LS variant has 11-fold increased binding to hFcRn at acidic pH and negligible binding at neutral pH, which gave more than 3-fold extended half-life in hFcRn transgenic mice and non-human primates [170]. Importantly, such engineering has shown to improve therapeutic efficacy in mouse tumor models when combined with antibodies targeting tumor antigens [170]. In addition, extended half-life improves antibody-based prophylaxis, as shown in non-human primates upon viral challenge [171, 172]. Clinical data on the use of the LS substitutions have revealed that the antibody can reach a half-life of over 100 days compared to 20 days for the WT counterpart [173, 174].

A range of additional hIgG Fc-engineered variants have been reported with increased persistence over the WT antibody when tested in hFcRn transgenic mice or non-human primates [165, 166, 175-182], but these do not reach the same half-life values as that of YTE and LS. However, a few recent examples are of interest. In one report, three double CH2 mutant variants (M252Y/T256D (YD), T256D/T307Q (DQ) and T256D/T307W (DW)) with enhanced pH-dependent binding to hFcRn resulted in similar extended half-lives in hFcRn expressing mice and non-human primates as that of LS used for benchmarking [183]. Another recent Fc-engineered variant, combining two mutations in the CH2 domains with one mutation in the CH3 domains (L309D/Q311H/N434S (DHS)) (**Fig. 4**) showed a half-life of 2 to 3-fold beyond that of the clinical stage YTE and LS variants in hFcRn expressing mice [89-91, 95]. Intriguingly, it has only 5-fold improved affinity to hFcRn at acidic pH and no detectable binding at pH 7.4 [184]. The authors speculate that this may be explained by complete lack of binding at neutral pH, which warrants further studies on how binding throughout the pH gradient regulates FcRn-mediated transport within and across cells.

These Fc-engineered variants may be used as fusion partners for therapeutic proteins, such as antidiabetic peptides, to achieve extended half-life and improved bioavailability. Preclinical examples are the combination of Fc-containing YTE or LS variants fused to cytotoxic T lymphocyte-associated protein 4, where both Fc fusion products yielded more than 2-fold extended half-life in non-human primates [185, 186]. In addition, *in vitro* studies on transcytosis have demonstrated that Fc-engineering can enhance delivery across polarized epithelial cell monolayers, as shown for hIgG1 antibodies containing LS or YTE substitutions that were

transported almost twice as efficiently across human epithelial cells as the WT counterpart [43]. Interestingly, the capacity to be transported in a hFcRn-dependent manner could be further enhanced when the Fc-engineered antibodies were administered as multivalent immune complexes [52]. As such, FcRn-directed transepithelial transport may be enhanced by Fc-engineering for improved pH-dependent receptor binding combined with increased avidity, factors that should inspire the design of nanosystems aiming for enhanced mucosal delivery.

Fig. 4 | Crystallographic illustrations of hIgG and HSA and targeted residues to modify FcRn binding. (A) The CH2 and CH3 domains of the hIgG1 Fc are shown in sand and brown, respectively. Residues in the CH2-CH3 elbow region that have been targeted by mutagenesis to increase FcRn binding are indicated. (B) The subdomains DI, DII, DIIIa and DIIIb of HSA are colored in orange, wheat, light cyan and light blue, respectively. Residues in the DIIIb subdomain that have been targeted by mutagenesis to increase FcRn binding are indicated. The free cysteine residue, C34, in DI is indicated. The figure was made using PyMol and the crystal structure data deposited of hIgG1 Fc (PDB 1H3W) [187] and HSA (PDB 1BM0) [188].

5.2. HSA-engineering

IgG antibodies and Fc fusions have the ability to engage effector molecules, such as the complement factor C1q and the classical Fcγ receptors, and as such induce a range of effector functions, such as antibody-mediated cellular cytotoxicity or complement dependent cytotoxicity [43, 157]. Although these immune responses are therapeutically attractive, they may also cause undesirable negative side effects [157, 189]. To avoid the latter, a solution may be to use the effector-negative HSA as a fusion partner. As the C-terminal DIII of HSA contains the principal binding sites for hFcRn [34, 35, 37, 65, 190], engineering for improved binding has thus far focused on this domain (**Fig. 4** and **Table 2**). Among the first examples to be published

was a single amino acid substitution introduced in the last α -helix (K573P (KP)) (**Fig. 4**), which gave a 12-fold stronger binding to hFcRn at acidic pH with negligible binding at neutral pH [159]. This resulted in about 1.5-fold extended half-life in hFcRn transgenic mice and non-human primates compared with WT HSA [159]. Moreover, this albumin variant has been shown to be transported up to 2.5 times more efficient across polarized epithelial cell lines [15, 68]. Similarly, another single amino acid substitution (V547A (VA)) (**Fig. 4**) showed 13-fold stronger binding to hFcRn at acidic pH, and 1.5-fold longer half-life in hFcRn transgenic mice [65]. However, in an unrelated study, VA gave comparable improved binding to hFcRn to that of KP, but was less well transcytosed across polarized human epithelial cells [68].

Very recently, a triple mutant variant (E505Q/T527M/K573P (QMP)) (**Fig. 4**) was reported with impressively more than 180-fold increased hFcRn binding affinity without affecting binding at neutral pH [68]. QMP translated into 4-fold enhanced transcellular transport *in vitro*, and upon intranasal delivery in hFcRn transgenic mice, 2-fold more of the engineered variant reached the blood compared with WT HSA. Importantly, following intravenous injection, QMP gained 2-fold longer half-life *in vivo* in the presence of strong competition with endogenous albumin [68]. The QMP variant has been benchmarked toward a panel of engineered HSA variants and shown to display more favorable binding and transport properties [68]. As such, QMP should be an attractive fusion partner for antidiabetic peptides to achieve prolonged half-life upon subcutaneous or mucosal delivery. The encouraging data on enhanced delivery across polarized lung epithelial barriers should inspire research exploring whether QMP can be used for oral drug delivery to facilitate more efficient uptake and delivery across the selective intestinal epithelium in the GIT.

Table 2 | Examples of engineered hIgG and HSA variants with improved pH-dependent hFcRn binding.
Fold compared with WT hIgG or WT HSA.

Ligand	Variant	Mutation	Increase in hFcRn binding at pH 6	Increase in transcytosis	Increase in half-life	Reference
IgG	YTE	M252Y/S254T/T256E	10-fold	1.6-fold	3.5-fold (humans), 3.7-fold (NHP)	[154, 168, 169]
	LS	M428L/N434S	11-fold	1.9-fold	3.2-fold (NHP)	[170]
	QL	T250Q/M428L	29-fold	-	2.5-fold (NHP)	[177]
	YD	M252Y/T256D	6.8-fold	-	1.5-fold (hFcRn Tg mice)	[183]
	DQ	T256D/T307Q	7-fold	-	2.1-fold (hFcRn Tg mice)	[183]
	DW	T256D/T307W	9.8-fold	-	1.7-fold (hFcRn Tg mice)	[183]
	DHS	L309D/Q311H/N434S	5-fold	-	5.9-fold (hFcRn Tg mice)	[184]
HSA	VA	V547A	13.2-fold	-	1.5-fold (hFcRn Tg mice)	[65]
	KP	K573P	12-fold	1.5-fold	1.4-fold (hFcRn Tg mice), 1.6-fold (NHP)	[68, 159]

QMP	E505Q/T527M/K573P	180-fold	4-fold	2-fold (hFcRn Tg mice)	[68]
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NHP: non-human primates; Tg mice: transgenic mice.

5.3. Alternative engineered FcRn-binding scaffolds

5.3.1. FcRn-binding peptides

Besides engineering of hIgG Fc and HSA, alternative scaffolds with FcRn-binding properties have been developed. One example is the selection of FcRn-binding peptides (FcRnBPs) by phage display technology that bind in the micromolar to nanomolar affinity range at both acidic and neutral pH, and with only minor differences in binding under both conditions (**Table 3**) [160, 161]. As such, these peptides are not directly attractive for half-life extension. However, a 26-amino acid peptide (SYN1436), which blocks the IgG-binding site of FcRn, has been shown to lower the systemic levels of endogenous IgG in mice and non-human primates by up to 80%, but this required the peptide to be dimeric [160, 161]. Conjugation of polyethylene glycol (PEG) chains to the dimeric peptide affected the binding of FcRn to different degrees [191]. Still, the most optimal PEG-peptide conjugate could accelerate the catabolism of hIgG when administered to hFcRn transgenic mice [191]. Notably, a range of on-going clinical trials are evaluating FcRn-targeting molecules for blockage of the IgG site, as a strategy to lower the levels of pathogenic IgG in autoimmune diseases, as reviewed [192-194].

Inspired by the work on SYN1436, Sockolosky and colleagues created an engineered version by introducing a single point substitution (Y12H) to make it bind more pH-dependently to the receptor [195]. When fused to the N- or C-terminal of a model fusion partner, or both, it bound hFcRn without affecting the biological function of the fusion partner. Notably, the fusion proteins harboring an FcRnBP on each end, bound hFcRn with an apparent K_d comparable to that of hIgG1 at pH 6.0, but unlike for hIgG1, binding was also detected at pH 7.4. Furthermore, one of the fusion proteins was shown to be transcytosed 14-fold more efficiently than the non-fused counterpart across hFcRn-expressing polarized epithelial cell monolayers, but not to the same extent as that to hIgG1 [195, 196]. The ability of the fused FcRnBPs to extend the half-life or deliver the fusion partner across mucosal barriers *in vivo* was not addressed in this study. However, in another study, Y12H-containing FcRnBPs were used to build a panel of antibody Fab fusions with linear and cyclic versions of the peptides that were fused to the light chain or heavy chain of the Fab at either the N- or C-terminal end, or both, via a glycine-serine-linker [196]. The fusion proteins were shown to have varying degrees of receptor binding activity and ability to be transported across polarized human epithelial cells *in vitro*. In addition, determination of the half-life in non-human primates revealed that a fusion with four linear FcRnBPs fused to the N-

and C-terminal ends of both light and heavy chains gave the greatest systemic exposure [196]. While the half-life of a Fab fragment was measured to be only about 3-5 hours due to its molecular weight below the renal clearance threshold, the fused FcRnBPs could extend the half-life to 67 hours, which is far from that of IgG and albumin with a half-life of about 5 days in non-human primates [159, 168]. In spite of this, the study pinpoints the importance of avidity for efficient receptor engagement and transport.

5.3.2. Engineered protein domains

Protein domains (16kDa) with FcRn-binding properties have been generated by combining the CH2 domain of an IgG1 Fc (m01s) with the grafting of a stretch of five amino acids derived from the CH3 domain, containing the key FcRn interaction residues H433 and H435 (**Table 3**) [163]. Although this fusion bound hFcRn, further engineering was done by the use of phage display to select mutant versions with increased and strict pH-dependent binding to the receptor. One such variant (2C10) showed the capacity to be transcytosed across polarized epithelial cells in a *Transwell*[®] system but did not perform as well as the hIgG1 Fc fragment. When tested in non-human primates, the half-life was measured to be 40-44 hours, which was more than 3-fold longer than that of m01s [163]. While this example shows that protein domains with a size below the renal clearance threshold combined with FcRn-binding properties can be rescued from degradation *in vivo*, the gain in half-life was modest compared with full-length hIgG.

In another phage display approach, single chain variable fragments (scFv) were selected towards hFcRn, which yielded candidates (FnAb-8 and FnAb-12) with nanomolar affinity at acidic pH (**Table 3**) [164]. These scFv bound 10 to 100-fold more strongly to hFcRn than hIgG, and with no or slightly weak binding observed at neutral pH. While FnAb-8 showed overlapping binding with that of IgG, FnAb-12 strengthened IgG binding to the receptor. Further, fusion of FnAb-8 and FnAb-12 to GLP-1 resulted in maintained pH-dependent hFcRn binding as well as binding to the GLP-1 receptor. When FnAb-8 was studied in non-human primates, it revealed a half-life of 100-200 hours, which was about 2-fold longer than that measured for 2C10. Curiously, while the GLP-1 analog exenatide reduced blood glucose concentrations for 2 hours in WT mice, engineered scFvs fused to GLP-1 prolonged this effect for up to 8 hours, despite their inability to engage mFcRn [164].

The last example is the selection of affibody molecules by phage display selection. This scaffold is a three-helix bundle based on a domain (6 kDa) derived from IgG-binding Protein A from *Staphylococcus aureus* [197]. The identified FcRn binders (Z_{FcRn_2} , Z_{FcRn_4} and $Z_{\text{FcRn}_{16}}$) (**Table 3**) were shown to bind to the receptor in a pH-dependent manner, not only to hFcRn but also

mFcRn, where binding was mediated by the presence of two histidine residues that gave 20 to 43-fold stronger binding than that of hIgG [162]. Fusion of the affibody molecules to an albumin-binding domain (ABD), which will hijack albumin *in vivo*, resulted in a half-life of 91-73 hours, which was 1.6 to 2.7-fold longer than that of a control protein when injected in mice expressing mFcRn [162]. Notably, ABD interacts with albumin across species without affecting FcRn binding [198]. Lastly, when ABD-fused Z_{FcRn_2}, which bound less pH-dependent, was tested for its ability to out-compete IgG binding to the receptor, a reduction of up to 40% in IgG levels was measured in mice [199].

Table 3 | Engineered FcRn binding peptides and domains.

Ligand	Name	Sequence ^a	K _d at pH 6.0 (nM)	K _d at pH 7.4 (nM)	Reference
FcRnBP	SYN722	QRFGTGHFGGLYPCNGP	5,700*	45,000*	[160, 161]
	SYN723	GGGCVTGHFGGIYCNYQ	5,200*	35,000*	
	SYN724	KIICSPGHFGGMYCQ GK	22,000*	78,000*	
	SYN725	PSYCIEGHIDGIYCFNA	8,800*	76,000*	
	SYN726	NSFCRGRPGHFGGCYLF	9,400*	93,000*	
	SYN746	Ac-NH-[QRFGTGHFGGLYPCNGP]-CONH ₂	5,100*	30,000*	
	SYN1327	Ac-NH-[RF-Pen-TGHFG-Sar-NMeLeu-YPC]-CONH ₂	31 ± 4*	170 ± 20*	
	SYN1436	Dimer of -[RF-Pen-TGHFG-Sar-NMeLeu-YPC]-CONH ₂	< 0.5*	< 0.8*	
Protein domain	m01s	-	Low*	Low*	[163]
	a2b	(m01s)-RCPRWMRTPHNHYT	2,200*	Negligible*	
	2C10	(m01s)-FGNCAYLRPHNHYT	1,100*	Low*	
scFv	FnAb-8	-	4.32*	Negligible*	[164]
	FnAb-12	-	43.2*	Low*	
Affibody	Z _{FcRn_2}	VDAKYAKEQDAAAHEIRWLPNLTFDQRFVAFIHKLADDPS QSSELLSEAKKLND SQAPK	12 [#]	> 500 [#]	[162]
	Z _{FcRn_4}	VDAKYAKEFESAAHEIRWLPNLTYDQRFVAFIHKLSDDPSQ SSELLSEAKKLND SQAPK	50 [#]	> 1,000 [#]	
	Z _{FcRn_16}	VDAKYAKEWMRAAHEIRWLPNLTFDQRFVAFIHKLEDDPS QSSELLSEAKKLND SQAPK	14 [#]	> 500 [#]	

(a) Single-letter amino acid codes are used except for Pen, L-penicillamine; NMeLeu, L-N-methylleucine; Sar, sarcosine. Amino acids colored in orange are connected by a disulfide bridge. Binding affinity (K_d) was determined by (*) surface plasmon resonance and (#) biosensor analysis.

6. The importance of considering cross-species FcRn differences for preclinical evaluation

Non-human primates are considered the most suitable animal model for preclinical testing of hIgG- and HSA-based drugs, but economic and ethical reasons exclude them from routine screening of drug candidates and exploration of proof-of-concepts [200]. On the other hand,

mouse models are more suitable for initial preclinical assessment due to their convenience, cost-effectiveness and genetic background. However, the examples given in this review on engineered hIgG Fc and HSA variants with improved FcRn binding properties, which will be presented in **Section 5**, were all evaluated in mice transgenic for hFcRn prior to testing in non-human primates. The reason for this is that there are large cross-species ligand binding differences between the mouse and human forms of FcRn that must be taken into consideration to obtain reliable preclinical *in vivo* data.

6.1. Biology of FcRn and its ligand in mice and humans

FcRn is expressed in almost all cell types and tissues of the body, with a few exceptions, as described [201, 202]. Regarding its role in transcytosis of IgG, FcRn has been shown to mediate the delivery of endogenous IgG from the mother to the fetus via transplacental transport during the third trimester of pregnancy [203, 204]. This secures passive immunity of the newborn at a critical time of life. In mice, such transport occurs via the placenta, but primarily via intestinal uptake of neonatal mice during the suckling period, when large amounts of IgG are taken up from the mother's milk via pH-dependent FcRn-directed transcytosis [205]. Interestingly, while the intestinal tissue expression of FcRn in humans is maintained throughout life, it is developmentally downregulated in conventional mice [93, 206]. For this reason, caution should be taken when addressing FcRn-targeting approaches and it has been suggested that such studies should be performed no later than 6-16 weeks after weaning in mice expressing mFcRn [207]. Notably, mFcRn is expressed at high levels in the duodenum and jejunum during neonatal life [206, 208, 209]. In humans, hFcRn is expressed in different sections of the human intestine, including the colon and ileum, but somewhat less in the jejunum and duodenum [108, 201, 207, 210]. Although the FcRn expression of intestinal tissues is down-regulated in mice, it persists in other tissues throughout life and, as such, regulates half-life and biodistribution of IgG and albumin.

Despite the fact that the mouse and human forms of the FcRn heavy chain share nearly 70% similarity in the amino acid composition, and that the key interacting residues responsible for pH-dependent binding are conserved [26, 211], there are distinct variations that result in large differences in cross-species binding. Specifically, this results in no or very poor binding of mIgG to the human receptor, while hIgG binds more strongly to the mouse receptor than to the human form [212, 213]. As such, hIgG Fc-engineering for improved binding to hFcRn does not translate into extended half-life in conventional mice, but rather shorter half-life than the WT counterpart, as engineering for more favorable pH-dependent binding is not preserved [72,

214]. This is the case for all hIgG Fc-engineered variants reported to date, which means that they cannot be used in mouse models expressing mFcRn.

In contrast to hIgG, HSA binds very poorly to mFcRn, with more than 20-fold weaker binding than mouse serum albumin (MSA), whereas MSA binds slightly stronger than HSA to hFcRn [34, 200, 212, 215, 216]. Thus, HSA injected into conventional mice is out-competed for binding to mFcRn in the presence of high amounts of natural endogenous MSA, produced continuously by the liver. This means that HSA is not rescued from intracellular degradation in mice expressing mFcRn, as illustrated by the fact that WT HSA has a nearly identical half-life as HSA engineered for considerably reduced binding to hFcRn [159]. Therefore, studies conducted under such conditions do not take into consideration the contribution of FcRn and, as such, this preclinical setting is non-physiological. The molecular explanations for the large cross-species differences have been described in depth elsewhere [75, 159, 200, 215, 217, 218].

6.2. Human FcRn transgenic mouse models

To solve this preclinical challenge, several genetically engineered mouse strains have been developed where the mFcRn heavy chain has been replaced with the human counterpart, either under the control of mouse or human promoter elements [32, 74, 181, 216, 219-223]. These strains are the current gold standards for the evaluation of hFcRn-targeting strategies addressing pharmacokinetics, pharmacodynamics and delivery across selective mucosal barriers prior to studies in non-human primates.

The most commonly used hFcRn-expressing strains are generated from conventional C57BL/6J mice, named Tg32 and Tg276 (**Fig. 5A**), which are extensively used to assess the half-life of hIgG and HSA [32, 74, 181, 220, 222, 223]. Both strains have substituted the mouse heavy chain with that of the human form, which can pair non-covalently with the endogenous mouse β_2m [32, 74, 181, 222, 223]. A distinct difference between these strains is that the Tg32 mice express the heavy chain under the control of its human regulatory elements [74, 181, 220, 222, 223], while the expression in Tg276 mice is regulated by a constitutive CAG promoter, a human cytomegalovirus enhancer fused to chicken β -actin/rabbit β -globin hybrid [32, 181]. This results in a much more widespread expression profile of hFcRn in Tg276, whereas the receptor expression profile in Tg32 mice has been mapped to be similar to that in humans [201, 224]. There is also a modified version of Tg32 that expresses human β_2m , but no difference in half-life of the ligands has been reported that can be related to whether the mice carry a mouse or human form of β_2m [222]. In general, the half-life of hIgG is shorter in Tg276 homozygous mice relative to the Tg32 strain, and longer in the Tg32 hemizygous than the homozygous strain [222]. Since the expression profile of the Tg32 strains resembles more closely to that of humans,

studies of FcRn in mucosal immunity and receptor targeting for delivery across the epithelial barriers have been mainly studied in these mice, as recently reviewed [99]. Therefore, the Tg32 strains should be used for studies addressing the mucosal delivery of FcRn-targeted molecules or nanosystems.

In humans, injected hIgG and HSA will compete for binding to FcRn in the presence of high concentrations of IgG (10 mg/mL) and albumin (40 mg/mL), produced by the plasma cells and liver, respectively. This is in stark contrast to what has been found in mice kept under pathogen-free housing, where the concentrations of endogenous mIgG are low (roughly 1.0-0.2 mg/mL) compared to those found in nature, while the concentrations of MSA are high (20-50 mg/mL) due to constitutive production by the liver [32, 225]. These conditions must be taken into consideration in the preclinical evaluation of FcRn-targeted strategies, since the outcome can be greatly affected by a lack of competition for receptor binding, as in the case of the IgG binding site, which is in contrast to strong competition for the albumin-binding site. To address how competition for the IgG binding site of FcRn affects targeting approaches, mice may be pre-loaded with excess amounts of hIgG prior to administration, which has been shown to modulate the half-life of injected IgG variants [181, 219, 226]. Interestingly, a modified version of the Tg32 strain has been recently reported to produce hIgG1 Fc combined with mIgG Fab arms, which is rescued by hFcRn (**Fig. 5B**) [219]. The levels of endogenous chimeric mouse-human IgG1 were shown to be greatly increased upon immunization, which modulated the half-life of injected hIgG. Thus, this Tg32-hFc mouse model may be attractive for studies addressing competition for the IgG binding sites. A similar mouse model that also expresses the human classical Fc γ receptors has been reported [227].

Regarding studies of HSA-based strategies, a version of Tg32 that lacks expression of MSA has been developed [221]. This model allows for studies in the absence of competition, but also in the presence of HSA following preloading (**Fig. 5C**). An alternative to this is a mouse strain, named AlbumusTM, that has been genetically modified to express both hFcRn and HSA (**Fig. 5D**) [216]. However, the expression of the heavy chain of hFcRn is not under the control of the human promoter. Despite this, studies of engineered HSA variants in Tg32 with competition and in the double transgenic mouse model have revealed similar half-lives [159, 216, 223]. Due to differences in the construction of these strains, they may differ in their expression of the receptor in different mucosal tissues, which has not yet been studied in detail. Importantly, it has recently been shown that competition for the albumin-binding site strongly affects the half-life of injected HSA [68, 221]. While the half-life in Tg32 mice lacking expression of albumin was measured to be around 3 weeks for injected WT HSA, it was only a few days in the presence of competition [68, 221]. This demonstrates that the competitive pressure is high for the albumin-

binding site of FcRn. As such, only minor alterations in the binding kinetics of WT HSA fusions may affect their half-life. To compensate for this, HSA engineered for improved hFcRn binding may be used. When an engineered albumin, called QMP HSA, was compared with WT HSA in Tg32 mice lacking albumin, it only showed a 1.2-fold longer half-life, whereas a 2-fold difference was measured in the presence of competition [68]. The same effect of competition was observed when the HSA variants were administered intranasally, followed by homing to the lungs and uptake into the bloodstream [68]. Moreover, when QMP was fused to an antibody fragment, more than 4-fold the amount of WT fusion was detected in the blood for 24 hours following intranasal administration, which corresponded to around 10% of the dose given [68]. Importantly, uptake and transport across the mucosal lung barriers were much more efficient for HSA than for hIgG [68]. As such, QMP should be an attractive fusion partner for more favorable pharmacokinetics and transepithelial delivery, as previously discussed.

Fig. 5 | hFcRn transgenic mouse models for evaluation of hIgG and HSA-based therapeutics. Conventional C57BL/6J mice have been used to generate humanized mouse strains that are transgenic for hFcRn. While (A) the Tg32 and Tg276 strains express mIgG, (B) the Tg32-hFc strain expresses chimeric hIgG1 (chIgG1). In comparison to these mouse strains, (C) Tg32 albumin knockout mice do not express MSA, while (D) the double transgenic mouse model (Albumus™) has both hFcRn and HSA. The figure was created with BioRender.

7. FcRn-targeted nanosystems for oral delivery of antidiabetic peptides

An attractive strategy for enhancing oral delivery of antidiabetic peptides is to encapsulate drugs into NPs [7, 228-230]. Such nanosystems can provide protection against harsh conditions in the GIT, which may enhance uptake and transport across the selective cellular barriers [8, 9]. While several reports have shown promising results, oral delivery of NPs is still limited by low bioavailability. Thus, there is a need to make a quantum leap in the design of drug-encapsulated nanosystems to make oral delivery far more efficient. One way to achieve this may be to combine the biotechnological advances of NP design with strategies to target FcRn at mucosal surfaces [11-15]. This may allow active transport across the polarized intestinal epithelium for delivery to the systemic circulation [11, 12, 15]. The NP design must then be done in a way that favors FcRn engagement and transcellular transport, parameters that are not yet fully defined. However, while NPs can be decorated with FcRn-targeting molecules, the encapsulated peptides may also be engineered for efficient FcRn-mediated recycling, as discussed in **Section 3**, which can improve half-life and bioavailability upon release from the NPs. Herein, a brief overview of parameters that are relevant to be considered in the context of NP design and FcRn-targeting and examples of FcRn-targeted nanosystems encapsulating antidiabetic peptides will be discussed.

7.1. Design of targeting nanosystems

The stability and characteristics of nanosystems, including their size, charge, shape, material and coating are parameters that dictate their internalization and transport properties (**Fig. 6**), as extensively reviewed [7, 8, 231-234]. However, for the NPs to be targeted to FcRn, they must be engineered to display receptor-targeting molecules on their surface in such a manner that will allow for efficient binding-and-release and stability throughout the endosomal pH gradient. Relevant parameters to be considered are how the pH-dependent binding kinetics of the FcRn-binding molecules affect the ability of the NPs to be transported, and in this context, how the number and density of the targeting molecules and, as such, avidity and flexibility are affecting the outcome [235].

Decoration of NPs with FcRn-targeting molecules could be achieved in a non-covalent manner by adsorption, but preferably covalently to secure stability [236]. To ensure the latter, conjugation should be irreversible and site-specific to fix the orientation of the ligands in such a way that guarantees availability and functional display. One strategy is to use MAL-thiol or carbodiimide chemistry for site-specific conjugation. Regarding the MAL-thiol reaction, it requires coupling of sulfhydryl reactive MAL on the surface of NPs to thiol groups present on

hIgG or HSA, which will result in irreversible thioether bonds [237]. As hIgG or its Fc fragment does not contain any free cysteine residues, functional groups using primary amine reactive reagents or crosslinkers must be used [11, 12]. However, when it comes to HSA, C34 in DI is an attractive site for conjugation [15]. On the other hand, carbodiimide crosslinking can be achieved using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide/N-hydroxysuccinimide (EDC/NHS) reaction to form an amide bond between the carboxylic groups of hIgG Fc or HSA and the functionalized aminated NPs [13, 14, 238, 239].

Fig. 6 | Design of FcRn-targeted nanosystems for oral delivery of antidiabetic peptides. The physicochemical characteristics of engineered NPs can be tailored by altering **(A)** the particle size, shape and composition as well as **(B)** their surface properties. To ensure efficient transport of the NPs across selective polarized epithelial barriers that express FcRn, the surface of NPs may be decorated with **(C)** receptor-binding molecules engineered for improved binding and transport properties, such as IgG Fc and albumin. Such nanosystems may be used in the treatment of DM and prevention of fibrosis by **(D)** encapsulating antidiabetic peptides or fusion proteins, in the internal core of the NPs, allowing for drug protection and controlled release upon oral administration. The figure was created with BioRender.

7.2. hIgG Fc-decorated nanosystems

The first FcRn-targeted nanosystems were developed using the Fc fragment of hIgG antibodies as a targeting moiety [11-13]. Several of these studies focused on the use of polymeric NPs as a

scaffold for drug encapsulation [11, 12, 240]. A major advantage of such polymeric NPs is their biocompatibility and biodegradability, which are desirable features to avoid potential toxicity of a range of inorganic classes of NPs when considering translation of developed nanosystems into a clinical setting [233, 234]. An example of this is polymer-based nanosystems of poly(lactic acid) (PLA), in which thiolated hIgG Fc was covalently conjugated to the MAL-functionalized NPs, with an average hydrodynamic diameter of about 55 nm, via MAL-thiol reaction [11]. The conjugation of Fc resulted in a slight increase in the particle size and showed a maintained monodispersity of the NPs before and after coupling. Since IgG antibodies generally carry a weakly negative charge under physiological conditions [241], the surface charge of the Fc-decorated NPs (Fc-NPs) became slightly more negatively charged when coupled to the Fc fragments. Interestingly, Fc-NPs gave a 2-fold rise in transcytosis *in vitro* compared with non-targeted NPs across a polarized epithelial cell line that endogenously expressed hFcRn, while transport was reduced in the presence of free hIgG Fc competing for receptor binding, suggesting that the increase in uptake and transcytosis was FcRn-dependent [11]. In conventional WT BALB/c mice, which express mFcRn throughout the small intestine and colon, Fc-targeting of FcRn enhanced *in vivo* biodistribution of the NPs in several organs, as well as increasing the absorption efficiency by 11.5-fold following oral administration. Importantly, insulin-loaded Fc-NPs showed prolonged hypoglycemic responses in fasted mice when administered orally at a clinically relevant insulin dose compared with non-targeted NPs and to injected insulin, while free OI solution did not generate a glucose response. This effect was not observed in mice lacking FcRn expression, which may indicate FcRn-induced transcytosis of Fc-NPs in the GIT and, as such, the release of insulin from the NP matrix into blood circulation. While these studies were done in WT mice, further studies on ligands engineered for improved hFcRn binding and transport properties should be performed in mice transgenic for the human receptor [220, 223].

A similar strategy has been explored to improve oral efficacy of exenatide, a GLP-1 receptor agonist for T2DM treatment, in which the peptides were loaded into poly(lactic-co-glycolic acid) (PLGA) NPs using the same coupling chemistry as PLA-based nanosystems [12]. The particle size of native PLGA NPs was about 109 nm, which was larger than that of the PLA NPs [11]. The difference in hydrodynamic diameters observed can be explained by the methods used to synthesize NPs and the chemical composition of NP formulations. Similar increase in particle size and surface charge of hIgG Fc-decorated PLGA NPs as that of PLA NPs was observed. In this study, relative to non-targeted nanosystems, Fc-NPs showed to be more efficiently taken up and transcytosed across polarized epithelial cells expressing endogenous hFcRn [12]. Notably, it would be of importance to compare to what degree the particle size may affect the internalization of NPs [242, 243]. A mouse model of human T2DM (db/db) was used to study the

hypoglycemic response of the peptide-loaded NPs. Upon oral administration, the Fc-NPs showed a higher reduction in blood glucose levels and particle retention in the GIT compared with non-decorated NPs [12]. Furthermore, FcRn-targeting of Fc-decorated nanosystems exhibited a prolonged hypoglycemic response relative to injected exenatide, although the reduction effect of the injection solution was shown to be more efficient in the first few hours following subcutaneous injection.

In addition to polymer-based NPs, nanosystems may be designed with inorganic components having unique electrical, magnetic and optical properties [233, 234]. Such inorganic NPs allow for fine-tuning of the size, structure and geometry for enhanced performance that may benefit oral delivery across epithelial barriers of the tailored-made nanosystems. Nevertheless, due to the toxicity and solubility limitations of such NPs, the use of inorganic materials in humans must be carefully examined [244, 245]. An example of inorganic nanosystems explored for oral delivery of antidiabetic peptides is undecylenic acid-coated silicon NPs (SiNPs) that were loaded with GLP-1 [13]. In contrast to the examples of polymeric NP formulations given [11, 12], SiNPs were functionalized with amine-modified Fc via EDC/NHS reaction. The NPs were then treated with mucoadhesive chitosan and eventually entrapped into a pH-responsive polymeric matrix of hypromellose acetate succinate (HPMC) to improve drug adsorption at mucosal sites and prevent premature release of encapsulated drugs under strong acidic conditions in the GIT [13]. The Fc-NPs showed enhanced transcytosis *in vitro* in polarized epithelial cell lines expressing hFcRn compared with non-targeted NPs, which resulted in higher amounts of GLP-1 released from the NP matrix at the basolateral side of *Transwell*[®] system [13].

Overall, these findings have demonstrated that decoration with hIgG Fc fragments may enable the oral delivery of peptide-loaded NPs across epithelial barriers for delivery to the systemic circulation [11-13]. However, in addition to the lack of use of hFcRn transgenic mouse models, pH-dependent binding affinity and functional avidity for the receptor of the Fc ligands decorating the surface of NPs have yet to be examined to ensure optimal receptor-mediated binding and transepithelial transport of nanosystems. Furthermore, the mechanisms of action of the FcRn-targeted NPs should be studied in detail.

7.3. HSA-decorated nanosystems

Although hIgG Fc have been explored as FcRn-targeting moieties, their use is impeded due to the need for chemical modification for conjugation. As such, HSA may be used for decoration of NPs owing to low immunogenicity and ease of production [246, 247]. Furthermore, it was very recently shown that HSA was transported more efficiently than hIgG across mucosal lung barriers *in vivo* in hFcRn transgenic mice [68]. On this basis, HSA may be an attractive targeting

molecule for favorable FcRn-mediated transepithelial delivery of nanosystems for oral administration of antidiabetic peptides [14, 15, 240].

An example of nanosystems that has been combined with albumin is polymeric alginate-dextran sulfate-based (ADS) NPs with an average hydrodynamic diameter of 233 nm, that were coated with chitosan [240]. In contrast to synthetic PLA and PLGA, the polymers alginate, dextran and chitosan used to synthesize NPs are natural product-derived compounds that provide nanosystems with mucoadhesive characteristics [247]. In this study, bovine serum albumin (BSA) was non-specifically adsorbed onto the surface of ADS NPs [240], in contrary to site-directed chemical coupling of Fc-decorated NPs [11-13]. In an *in vitro* model of polarized epithelial cells expressing hFcRn combined with mucus-producing goblet cells, BSA-decorated NPs loaded with insulin showed an improved permeability profile of the peptides compared to free insulin [240]. However, cautious should be taken when interpreting the result since the binding kinetics of BSA for hFcRn is not identical to that of HSA [32]. Aside from that, strategies for surface modification of NPs and pH-dependent functionality of ligands are two other important parameters to address.

An attempt was made to decorate the surface of HPMC-treated undecylenic acid-coated SiNPs with HSA via EDC/NHS reaction [14]. The chemical surface modification of NPs with HSA showed an increase in particle size after conjugation, while maintaining high monodispersity. Interestingly, HSA-conjugated NPs (HSA-NPs) exhibited improved cytocompatibility in human epithelial cell lines relative to non-conjugated NPs, which suggests that decorating nanosystems with biocompatible compounds, such as HSA, may reduce the toxicity of inorganic nanomaterials. Furthermore, the presence of HSA on the NP surface demonstrated enhanced cellular uptake and internalization of nanosystems *in vitro* with the aid of hFcRn expressed on the cell surface [14]. Done in this fashion, HSA-NPs encapsulating insulin showed enhanced transport of loaded peptides across polarized epithelial cell monolayers in *in vitro* transcytosis assays. The data also suggested an improvement in the transepithelial delivery of encapsulated antidiabetic peptides when comparing HSA-NPs with IgG Fc-NPs [13], which encourages research on the use of engineered HSA variants tailored for improved hFcRn binding and transport properties as targeting ligands, as discussed in **Section 5** [68].

Recently, the KP variant of HSA, which shows a 12-fold improved hFcRn binding compared to WT HSA [159], has been explored in an NP formulation of polymeric PLGA with an average hydrodynamic diameter of about 150 nm [15]. In this study, the surface of PLGA NPs was functionalized with HSA through MAL-thiol conjugation chemistry, in which no or slight increase in particle size was observed as a result of protein coupling. Most importantly, the pH-dependent binding characteristics for hFcRn of HSA-NPs were in line with reported studies for

native HSA [68, 159]. Compared to WT-decorated NPs, KP-decorated NPs showed enhanced binding to hFcRn at mildly acidic pH (5.5), while receptor binding was not observed for NPs decorated with an engineered HSA variant that does not bind hFcRn [15]. Similar results were demonstrated in *in vitro* transcytosis assays, where KP-decorated NPs were transported 2-fold more efficiently than WT-decorated NPs across polarized epithelial cells expressing hFcRn [15]. Intriguingly, when studying the effect of HSA functionalization on the hypoglycemic response of insulin-loaded PLGA NPs in T1DM-induced mice transgenic for hFcRn, KP-decorated NPs showed the most efficient glycemic reduction following oral administration compared with other HSA-NPs and to OI solution.

Altogether, engineered HSA shows promise in the next-generation design of FcRn-targeted nanosystems for oral delivery of antidiabetic peptides. The ultimate aim now will be to tailor-design such strategies for further improvement in receptor engagement and transport properties to favor efficient transepithelial transport of NPs across the GIT upon oral administration. The QMP HSA variant should be an attractive FcRn targeting partner for decoration of NPs to achieve not only enhanced mucosal delivery but also prolonged half-life of fused peptides [68].

8. Conclusions

Nanosystems as drug carriers have the potential to make a significant impact on the treatment of many diseases, including diabetes and fibrosis. While a number of NP-based strategies have entered clinical trials or have been approved for use, they can only be administered by parenteral delivery methods. An alternative and preferable approach would be oral administration due to patient convenience and compliance, but this remains a challenge because uptake and delivery across selective intestinal epithelial barriers are limited. Furthermore, frequent administration is often required. As such, design of nanosystems tailored for oral drug delivery is extensively explored to increase the bioavailability of encapsulated drugs. This review highlights how in-depth insights into the biology of FcRn may be used to engineer NPs decorated with receptor-binding molecules with the aim to overcome the highly inefficient uptake at the intestinal epithelial barriers. If this major challenge is solved, it will allow for more cost-effective self-administered methods. In addition, the encapsulated drugs, such as antidiabetic peptides, may also be engineered for extended half-life and increased bioavailability by taking advantage of pH-dependent FcRn engagement. For preclinical testing of such strategies, considerable cross-species FcRn binding differences must be taken into account to secure reliable results from mouse model systems.

Conflict of interest

The authors declare no conflict of interest.

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Highlights

- The symptomatology of diabetes must be controlled to prevent fibrosis development.
- FcRn is responsible for the efficient transcytosis and half-life extension of drugs.
- Engineered FcRn-targeted ligands are a strategy to enhance receptor-binding affinity.
- FcRn-targeting nanosystems can improve antidiabetic peptides' oral bioavailability.