Review

MOLECULAR METABOLISM

Review: Glucose-sensitive insulin

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ABSTRACT

Background: Hypoglycemia, the condition of low blood sugar, is a common occurance in people with diabetes using insulin therapy. Protecting against hypoglycaemia by engineering an insulin preparation that can auto-adjust its biological activity to fluctuating blood glucose levels has been pursued since the 1970s, but despite numerous publications, no system that works well enough for practical use has reached clinical practise.

Scope of review: This review will summarise and scrutinise known approaches for producing glucose-sensitive insulin therapies. Notably, systems described in patent applications will be extensively covered, which has not been the case for earlier reviews of this area.

Major conclusions: The vast majority of published systems are not suitable for product development, but a few glucose-sensitive insulin concepts have recently reached clinical trials, and there is hope that glucose-sensitive insulin will become available to people with diabetes in the near future.

Keywords Hypoglycaemia; Glucose binders; Insulin conjugates; Polymers; Time lag; Toxicity; Stability

1. INTRODUCTION

Glucose is the main fuel for the human body, and blood glucose values are tightly regulated in healthy individuals. Between meals, blood glucose is near 5 mmol/L (mM), and when blood glucose concentrations rise after a meal, the value is quickly adjusted back toward 5 mM by the action of insulin. The insulin hormone is glucose-responsively secreted from pancreatic beta cells, and when insulin binds to insulin receptors on cells all over the body (for example muscle and fat), the cells are stimulated to absorb glucose by translocation of glucose transporters from storage vesicles to the cell surface (GLUT4) [1].

People with diabetes either lose their ability to produce insulin due to autoimmunity against the beta cells (type 1) or have low sensitivity to insulin in combination with impaired insulin secretion (type 2). Those with type 1 diabetes rely on multiple daily insulin injections, both for basal coverage, typically once a day, and with meals (bolus) to adequately control their glucose levels [2]. Handy pen systems with virtually pain-free needles are convenient for subcutaneous injections, but the timing and dosing of insulin administration is a delicate balance that requires several daily decisions, in which the user must try to match each insulin dose to the size and character of their meals. In addition, insulin doses must be adjusted to consider a number of other factors that influence glucose levels and insulin sensitivity, such as physical exercise [3], time of day (circadian rhythms) [4], and infections. Overall, glucose values can fluctuate unpredictably, so perfect insulin dosing day after day is impossible. Injection site location can also play a role in the insulin absorption rate; an injection near a blood vessel or into muscle can result in faster absorption than injecting into the intended subcutaneous compartment. Modern handy glucose monitors are helpful for judging optimal insulin doses, but blood glucose measurements require painful finger pricks or the wearing of invasive electrodes. For people with type 1 diabetes, it is not uncommon that they experience wide swings in glucose levels, sometimes with blood glucose values above 20–30 mM, as well as frightening hypoglycaemic events with values below 2–3 mM [5,6]. Hypoglycaemia can manifest in sweating, shaking, seizures, coma, or death. In particular, nocturnal hypoglycaemia can be dangerous because the person may not be aware of the problem and is thus unable to take remedial action. Good glucose control, that is, maintaining fasted glucose values in a target range, approximately 5–10 mM, is known to decrease the risk of long-term complications from diabetes, such as damage to the eyes, heart, kidneys, and circulation. However, fear of experiencing hypoglycaemic episodes often results in people underdosing their insulin such that they on average live with too high blood glucose.

Type 2 diabetes can be treated with lifestyle changes or a range of medications, including metformin, DPP-IV, and SGTL2 inhibitors, which do not induce hypoglycaemia. GLP-1 analogues can provide good glucose control while at the same time lowering body weight and improving cardiovascular outcome [7,8] with little or no hypoglycaemia since GLP-1's effect on insulin secretion is based on physiological glucose sensing [9]. However, the efficacy of medications for type 2 diabetes tends to diminish over time, and most people with type 2 diabetes will sooner or later require insulin to control their blood glucose, and with this comes the risk of experiencing hypoglycaemic episodes. Despite many technological advances in diabetes treatment, we are currently seeing a worsening of long-term glucose control and outcomes from diabetes [10]. Thus, there is certainly an unmet medical need for glucose-responsive insulin, or as it has been termed, "smart insulin" or "the holy grail of insulin."

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The discovery of insulin nearly 100 years ago was a lifesaving miracle for people with diabetes [11], but native insulin itself is not ideal for use as an injectable drug. The pharmacokinetics of injected native insulin provide neither a good basal nor adequate bolus profile when absorbed from subcutaneous fat tissue. Chemical changes to insulin have provided analogues with better basal and bolus profiles [12–14], but they still occasionally result in hypoglycaemia. The latest developments are once-weekly insulin [15] and attempts at oral basal insulin [16,17], but these projects are striving towards dosing convenience and thus patient compliance more than hypoglycaemia safety. The first reports on glucose-sensitive insulin preparations (GSI) appeared in the 1970s, and they have since been followed by thousands of publications and patent applications. The vast majority of published systems use insulin encapsulations in polymers intended for subcutaneous depots and include some sort of glucose-binding or glucose-reactive motif, thus enabling insulin release in response to glucose spikes. However, these systems are hardly suitable for use as therapeutic products, mainly because they respond too slowly, as discussed to follow. None of them have reached clinical trials. A few described systems can provide glucose-responsiveness of insulin in the circulation, which seems to be a better principle, but is more difficult to engineer.

The current review summarises the most important developments in the GSI area. Notably, many studies in this field have been published in patent applications, but patent work has largely been overlooked by prior reviews of the field [18–25]. There is a race to bring the first GSI product to market (a 40-year race at this time), and some teams have been tempted to oversell their results and/or publish sketchy ideas in attempts to attract financing. Many of the published patent applications are based on good ideas, but unfortunately do not include examples demonstrating that the systems work. The related field of non-invasive glucose monitoring devices has been similarly crowded with good science, bad science, and illusions, as wisely and entertainingly described in John Smith's (J&J) e-book with the subtitle from Mark Twain's novel "Hunting the Deceitful Turkey." [26] The current review is a personal reflection with a critique of the many attempts and postulates in the GSI field, but also with the sincere hope that people with diabetes will soon have access to glucose-sensitive insulin treatments.

2. CHALLENGES

A series of challenges must be overcome to develop a glucose-sensitive insulin product for therapeutic use:

The hypoglycaemic to normoglycemic to hyperglycaemic glucose range is narrow (approximately 2–5–20 mM); hence, a system is necessary in which the insulin level or activity is altered significantly over a narrow glucose concentration range. Many reports show shallow glucose responses or tests *in vitro* at glucose concentrations well above physiological values (50–100 mM).

A glucose-binding/reactive molecular motif is necessary that will selectively respond to glucose in the relevant concentration range. The glucose-binding constant (displacement constant, Kd) should be balanced toward physiological glucose values so the system can respond to relevant glucose concentrations at the steepest part of the binding curve. Glucose is a small polar molecule, and it is generally difficult to bind glucose in aqueous solvents with sufficient affinity and selectively over related substances [27–29]. Established options are limited to a few native proteins/enzymes or designed boronic acids/boronates or macrocycles, each with their challenges, as will be discussed to follow.

A fast responding system is necessary. Human blood glucose fluctuations can occur within a few minutes, and GSI systems that rely on polymers in subcutaneous depots are slow. There is time lag in glucose diffusion from blood to subcutaneous tissue (10–15 min) [30], as well as into the polymers, and a time lag in insulin transport from subcutaneous depots to the circulation (30–60 min). Furthermore, insulin release from subcutaneous and particulate depots is irreversible; thus, if too much insulin is released initially, there is no remedy. Reversible glucose responsiveness occurring in the circulation seems to be needed.

The GSI drug must be designed for either basal or bolus use (or both). People with type 1 diabetes require both slow-acting basal as well as fact-acting bolus insulin types, and many hypoglycaemic events occur in connection with mealtime dosing since it is difficult to always get the timing and dosing right when trying to match the diverse character of meals and snacks. Thus, it can be argued that bolus GSI would be most helpful to people with type 1 diabetes. However, hypoglycaemic events in connection with bolus dosing happen during the daytime when the person and their surroundings can often react to developing hypoglycaemia with extra sugar/juice or if necessary, an emergency glucagon injection. Night-time hypoglycaemia can be more dangerous, and these events are mostly attributed to long-acting basal insulin. In addition, people with type 2 diabetes primarily use basal insulin only. Taken together, since 90% of those with diabetes are type 2, there is also the view that basal GSI is very important. Different GSI mechanisms may lend themselves more or less obviously to either bolus or basal profiles. Some people have the hope that a basal GSI can also cover meals ("inject once a day and forget it"). This performance of a GSI system is yet to be shown, and it seems to be a steep requirement for a basal GSI to be able to cover all kinds of meals and snacks. Less than optimal GSI performance could still give a significant advantage over insulin therapies that are not glucose-sensitive, even if a basal GSI needs bolus-dosing supplements for some or all meals.

Which *in vitro* and *in vivo* systems should be used for compound evaluations? [31,32] How glucose-responsive must your lead molecule be at high glucose vs normal glucose vs low glucose? In healthy individuals, the circulated insulin concentration increases approximately 5- to 10-fold if blood glucose levels double from 5 mM to approximately 10 mM after a meal, that is, 5- to 10-fold gearing. Such a steep response seems difficult to achieve with a GSI system, but a shallower glucose response can still benefit the patient. Which animal models can best assess glucose sensitivity? The ultimate read-out would be fewer hypo events in humans over longer periods of time. However, glucose sensitivity should preferably be assessed before embarking on phase 3 clinical trials. Few GSI publications include *in vivo* data, and if they do, the majority only assess glucose profiles in comparison to some reference insulin, which can be misleading, as discussed to follow. Measuring glucose is straightforward, whereas measuring insulin profiles (pharmacokinetic profiles and clearance rates) is best done with specific assays that can measure the GSI construct without interference from native insulin.

Materials that are safe for life-long use must be utilised, for example, those that are not allergenic or toxic. This factor is rarely discussed in any reports. Many GSI systems use non-biodegradable polymers of unknown toxicity or even glucose binders that are known to be toxic, such as ConA.

Materials that are stable over long term must be used; current insulin products are aqueous solutions (or suspensions) with a shelf-life of at least 2 years. Most reports do not address this issue, and polymers,

proteins, or boronic acids from most GSI publications would likely be too unstable over the long term in aqueous formulations [33]. Proteins are rarely stable in aqueous solutions for prolonged periods of time unless extensive formulation optimisations are done. Trace level deamidations and formation of cross-linked species are common (high molecular weight products). These impurities may be tolerable, but they must be assessed and controlled for drug products.

The challenges outlined are discussed in connection with a variety of GSI concepts.

3. POLYMER-BASED CONCEPTS

In 1979, Brownlee et al. reported conjugations of insulin to carbohydrates and showed that the conjugates could bind to polymers incorporating Concanavalin A (ConA) and be released by glucose [34,35]. The applied polymers were hardly suited for pharmaceutical use (non-biodegradable Sepharose). Furthermore, ConA is notoriously toxic (mitogenic) [36,37], so ConA is not relevant for drug use. Although PEGylation of ConA was later pursued, this approach will not mitigate the issue of toxicity [38]. Despite these obvious problems, Brownlee's work illustrated the principle of glucose-sensitive insulin release from polymer systems, and the report has since been followed by hundreds of publications, in which the polymer, glucose binder, or insulin type have been varied and combined in numerous ways [39]. Most of the systems use polymers that are hardly biodegradable or at least will require significant efforts to illuminate the polymer residue remaining in the body. Polylactic-co-glycolic acid (PLGA) is well established for use in normal sustained-release formulations [40], but it does not overcome the lag issues with subcutaneous and particulate depots. Furthermore, PLGA degradation releases lactic acid that can change local pH, which may skew the function of the glucose binder and the long-term stability of the components.

Other proteins than ConA have been used for polymer systems, mainly glucose oxidase (GOx) [41]. GOx is an enzyme that oxidises glucose to gluconic acid + hydrogen peroxide. This reaction can result in glucose-responsive pH changes that can change the permeability of the given polymer and/or promote glucose-responsive oxidations of the polymer components via the hydrogen peroxide released [42,43]. The main source of GOx is fungus (*Aspergillus niger*), so using GOx in drugs will carry a risk of immunogenicity, and the long-term stability of GOx in aqueous solution is questionable [33]. GOx works well in glucose-monitoring strips and electrodes, but these are not aqueous formulations, and the electrodes are easily pulled from the body upon use, whereas a subcutaneous GSI depot remains in the body. Some projects have used enzyme catalase to remove released peroxide, but catalase has not been qualified for drug use, and it will be a challenge to keep catalase stable over the long term in aqueous formulations. Galactose/glucose-binding protein (GGBP) has been tried for GSI systems, but such a large bacterial protein comes with a high risk of immunogenicity if used in a human drug [44]. Glucokinase (GK) is the human glucose binder/sensor found in beta cells, but GK has 8 unpaired cysteines [45]; hence, GK is only stable at reducing conditions when insulin (with 3 disulphides) is not stable.

Microneedle patches for dermal delivery have been promoted as improvements over subcutaneous depots both for normal insulin delivery and GSI systems (Gu/Buse/Zenomics Inc.) [42,46–49]. Patch-based dosing to the dermis can result in faster insulin absorption than subcutaneous dosing, but there is still a lag of glucose and insulin transport from and to the blood, and insulin release is irreversible. Dosing of regular insulin using patches has been pursued by several groups [48], but none have made it to market. Mechanical robustness of the patches is a problem as well as loading enough insulin into the microneedles.

The use of boronic acids in GSI polymer systems was pioneered in the early 1990s [50,51]. Boronic acids bind to glucose by covalent boronate formation, but the reaction is fast and reversible; therefore, it behaves similar to non-covalent binding [52]. The main driving force behind boronate/glucose recognition is the formation of a tetrahedral boronate geometry fitting to the 1,2-*cis*-diol of glucose. The pKa values of simple boronic acids are >9, so pKa adjustments of aryl boronic acids must be done for glucose binding at a neutral pH. Most GSI systems have used monoboronic acid, but monoborons have glucose-binding constants in the range of 10–50 mM (Kd), which does not optimally match with physiological glucose fluctuations. Monoboronic acids also bind stronger to fructose than glucose. Diboronic acids can give stronger glucose affinity (Kd 1 mM or lower) as well as better selectivity over other carbohydrates [53], and there are many publications describing the use of diboronic acids in pursuit of optical glucose sensors [54]. Most work in the optical sensor area focuses on glucose/fructose selectivity of diboronates, but this detail is somewhat irrelevant for pharmaceutical applications, since blood fructose concentrations never reach mM values [55,56]. However, the blood lactate concentration is approximately 2 mM at rest and can reach 10–20 mM during exercise; hence, lactate seems more of an issue than fructose. Glucose/lactate selectivity has been established for certain types of boronates [57]. Most publications on optical glucose sensors employ fluorescent probes, which are not desirable in drug products, as they tend to be coloured, carry a risk of being unstable due to photobleaching, and incorporate polyaromatic hydrocarbons with unknown toxicity. Notably, boronates are also known to be fragile in aqueous solution long term [58–61]. Hydrolysis and oxidations can occur, ArB(OH)2 → ArH and ArB(OH)2 → ArOH, but none of the many papers on boronate-based GSI polymers addressed long-term stability. In addition to stability challenges, there are also reports that certain boronic acids can be genotoxic in bacteria, although at high concentrations [62–65]. Some types of boronic acids are described as safe with regard to genotoxicity [65], but for drug use, a thorough preclinical toxicological investigation of engineered diboronate is probably necessary. There are 3 approved drugs on the market containing boronic acids: bortezomib/Velcade [66], which is a proteasome inhibitor for cancer treatment, tavaborole/Kerydin [67], which is used for treatment of nail fungi, and crisaborole/Eucrisa [68], which is used sub-chronically to treat eczema/psoriasis. Eversense is a diboronate optical glucose sensor on the market for use as a continuous glucose monitor [69].

To address the challenges implicated with non-biodegradable polymers in GSI systems, researchers at Novo Nordisk have produced insulins that self-assemble in a carbohydrate-sensitive manner. Inspired by the discovery of multihexamer-forming insulins such as NN344 [70] and degludec [13], which provide good basal profiles, insulin was equipped with pairs of boronates and polyols, which led to sugar-responsive high molecular weight self-assemblies (multihexamers, Figure 1) potentially useful for making subcutaneous GSI depots [71–73]. However, it was not possible to engineer glucose sensitivity strong enough for drug use, and polymer-free GSI subcutaneous depots still pose challenges with respect to time lags as discussed in Section 2.

4. MANNOSYL CONCEPTS

Around 2010, Zion et al./SmartCells Inc. pursued a concept similar to Brownlee's polymer system using ConA combined with

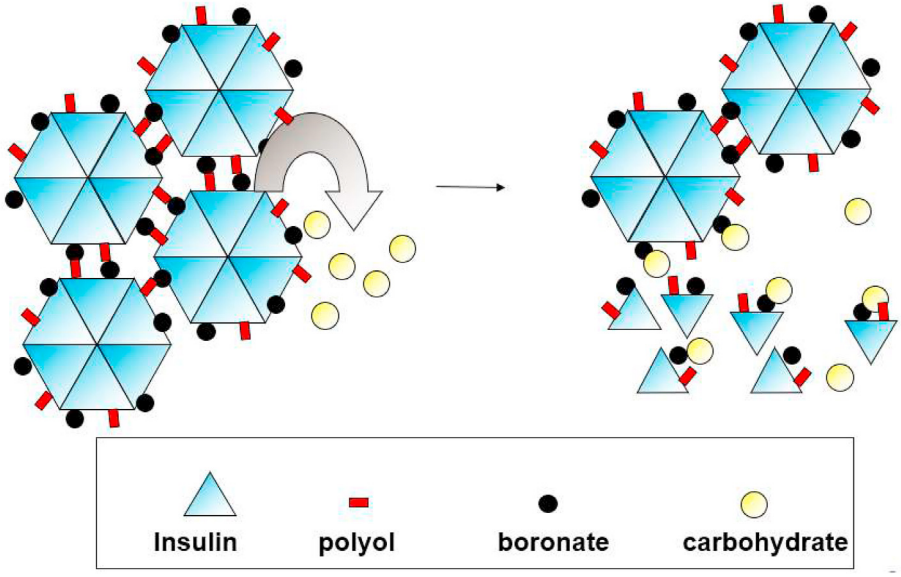

Figure 1: Carbohydrate-sensitive insulin multihexamers. Reprinted with permission from J. Am. Chem. Soc. 2005, 127, 6158–6159. Copyright 2005 American Chemical Society.

oligosaccharide-insulin conjugates [38,74,75]. Incidentally, Zion et al. discovered that the bioactivity of oligosaccharide insulins was glucose-sensitive in the circulation, that is, without polymer/ConA subcutaneous depots [76,77]. It was thus discovered that the oligomannosyl-insulin conjugates could bind to native mannose receptor (MR or mannose-binding lectin, MBL) in a glucose-sensitive manner. MR is part of the innate immune system and can clear oligomannosyl-rich entities such as bacteria [78]. The SmartCells system thus works via a glucose-sensitive balance between oligomannosyl binding and clearance at mannose receptors at lower glucose values vs insulin binding and activation/clearance at insulin receptors at higher glucose values. This system is highly advantageous in that glucose-sensitive balancing occurs in the circulation. Merck acquired the system and took it to phase 1 clinical trials, the lead being an A1,B29-oligofucosyl-insulin (MK-2640, Figure 2) [79–87]. However, this insulin conjugate is hardly useful as a basal insulin (with no protracting element) and is likely not an ideal bolus insulin either; the insulin receptor affinity of MK-2640 is approximately 5% compared to human insulin, and such low affinity can give relatively slow action in comparison to current insulin products that are optimised for meal use (insulin aspart, lispro, and glulisine). Note that human insulin receptor (HIR) affinities are generally reported in percentages compared to human insulin, mainly because this helps comparisons between different bioactivity assays (HIR binding, HIR phosphorylation, AKT signalling, and glucose uptake). It is conceivable that SmartCells/Merck had plans to initially test the concept in humans and planned to later address the pharmacokinetic issues, but the project was terminated after phase 1. The main problem was too low insulin potency, likely because of high background clearance via mannose receptor. MK-2640 showed approximately 25-fold lower glucodynamic potency than regular insulin [88], which is not surprising since similar lower potency had been observed in animal models. Elegant clinical clamp studies showed a 44% change in the glucose infusion rate when comparing MK-2640 vs human insulin, but there was no change in the insulin clearance rate at high blood glucose vs normoglycemia, which contrasts with a 30% difference observed for MK-2640 in preclinical studies [85]. Saturation of human MR clearance using high doses of MK-2640 seems a likely explanation. Low insulin potency can be compensated for by increasing doses, but injection volumes cannot readily be increased because of limitations to the size of insulin pens and vials. Very large injection volumes would be painful; current insulin products are injected in low microlitre quantities. Alternatively, the insulin concentration in the pens can be increased, as has been done with insulin detemir [89], but likely not beyond a 5- to 10-fold concentration increase because of limited insulin solubility. Most commercial insulin products use c = 600 μM = 100 units/mL, and it is difficult to produce stable insulin solutions with concentrations >10 times above 600 μM. Apart from the solubility issue, there is also a manufacturing/cost issue associated with the production of larger amounts of insulin.

Research related to oligomannosyl insulins led to the development of interesting *in vitro* and *in vivo* models. Animal studies could be conducted by administering oligosaccharide insulins + beta-methyl-mannopyranoside as a probe [83,84]. Mannoside can displace the oligosaccharide insulins from MR, that is, mannoside could be used as a mimic for high glucose levels, with the advantage that mannoside does not induce native insulin release. As previously mentioned, clinical trials were conducted using glucose clamps to evaluate glucose profiles, glucose infusion rates, and insulin profiles, but the low glucodynamic potency of oligomannosyl/fucosyl insulin hampered the project's further progression.

Dimarchi et al. pursued the oligosaccharide-insulins route using a single-chain insulin with C-peptide carrying a known O-mannosyl-rich domain that was expressed in yeast and shown to work in the mannoside model as previously described [90]. However, the O-mannosyl insulins were heterogeneous both in terms of glycosylation sites and oligosaccharide lengths, whereas all current insulin products are highly homogeneous.

Weiss et al. patent applied a synthetic insulin with O-mannosyl, albeit with little biodata [91]. The construct required full chemical synthesis of insulin, which is hardly realistic economically; all current insulin products are produced fully recombinantly or semi-recombinantly (that is, insulin conjugates). Notably, Merck produced a homogeneous recombinant glycosylated insulin using N-oligomannosylations via the GlycoFi platform [92], but Merck's chosen lead was the semi-synthetic

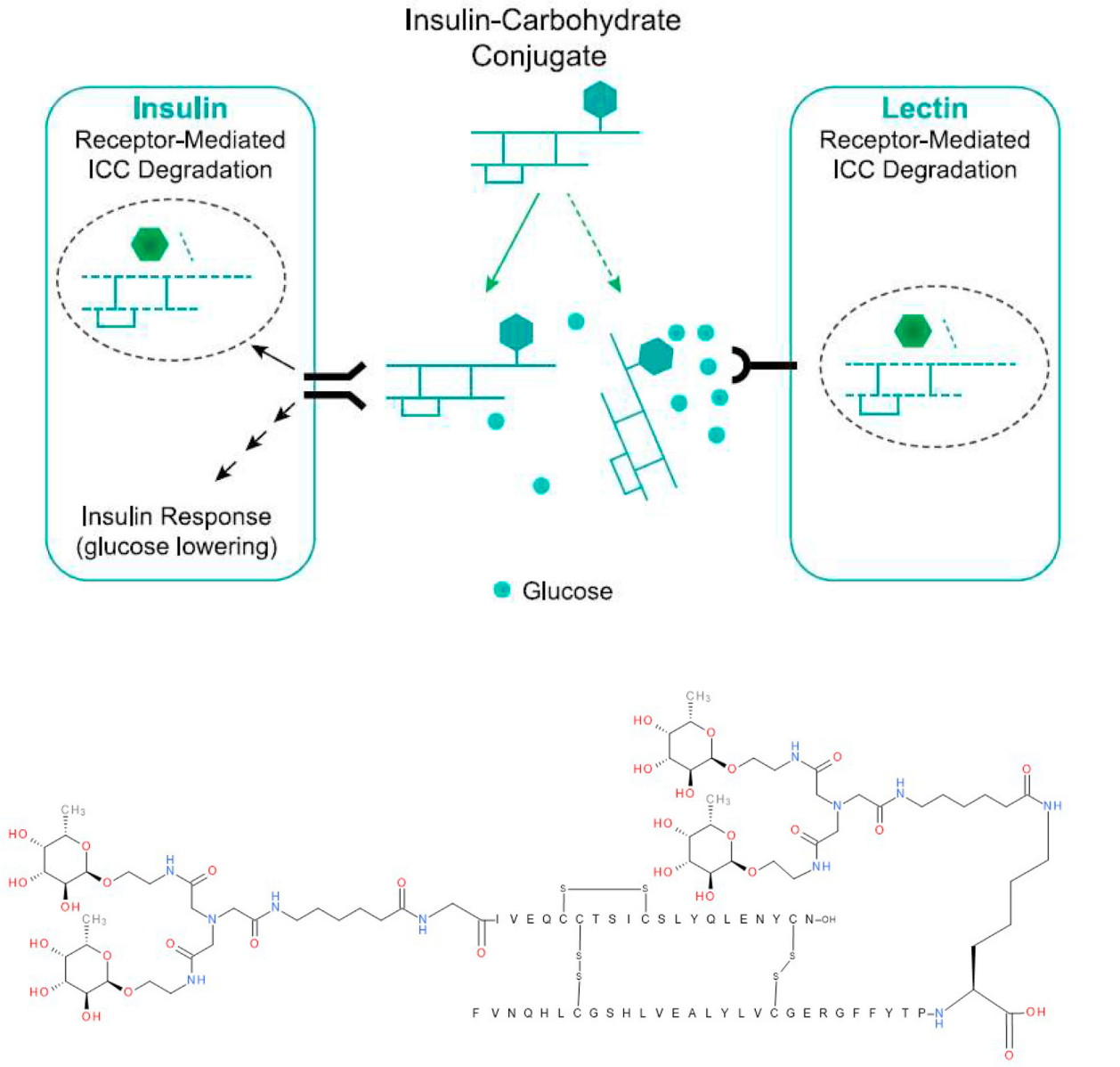

Figure 2: MK-2640 and its balanced clearance between MR and HIR. Reprinted with permission from Diabetes 2018, 678, 299—30. Copyright 2018 American Diabetes Association.

MK-2640. Recent Merck patent applications revealed attempts to produce basal versions of oligofucosyl insulins by conjugating albumin-binding motifs to MK-2640 and other constructs [93], but these compounds are likely not pursued in development. With the termination of the SmartCells/Merck project, there seems no way around the high background MR clearance of the constructs.

5. SWITCH-BASED CONCEPTS

A switch is a molecular motif that can control the function of the system to which it is attached [94]. Commonly described are photo switches based on *cis*/*trans*-isomerisation of diazobenzenes [95], which can work as molecular transistors controlled by light pulses. Switches that respond to small molecules are less common [96], but systems have been described that respond to for example the concentration of metal ions, that is, bipyridines that bind Fe^{2+} and change their system's topology [97]. In some sense, insulin itself is a switch system, with Zn^{2+} controlling the equilibrium between the insulin monomer/dimer and hexamer. An intramolecular switch in insulin could be envisioned to control insulin folding, which might toggle insulin between biologically inactive and active states. For a switch to respond to glucose, a proper glucose binder is needed, in addition to a partner/acceptor that can bind to the glucose binder at low glucose values (closed state) and be displaced/opened at higher glucose values (see Figure 3). The synthetic methodology for attaching the switch components to insulin in positions that enable inactivation in closed form + activation in open form including finding orthogonal chemistry for conjugating two different motifs (if using an asymmetric switch) poses significant challenges. The Novo Nordisk boronate/polyol insulin system described in Section 3 to produce carbohydrate-sensitive insulin multihexamers exemplifies an intermolecular symmetric switch [71].

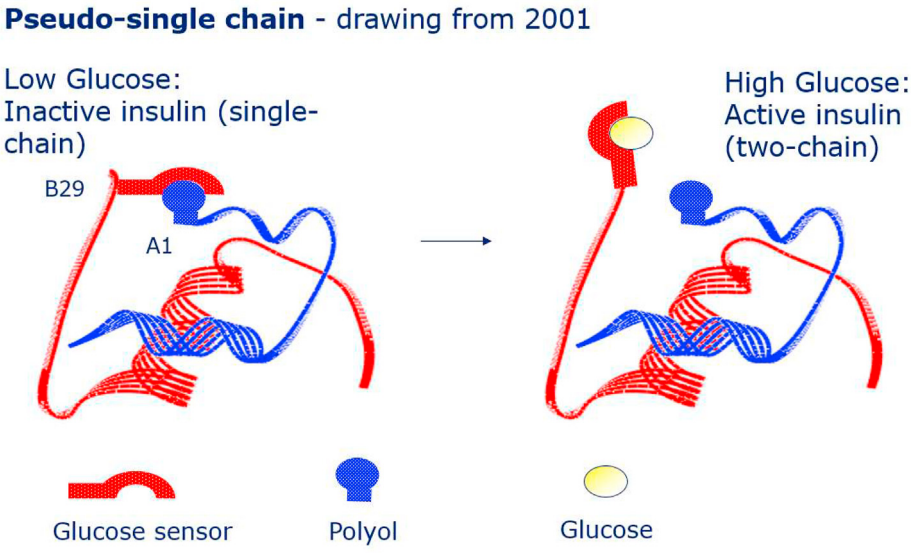

Figure 3: Early diagram of how a glucose-sensitive switch might work.

Insulin consists of two chains (A and B chains) and is expressed in the pancreas as a single-chain form with low bioactivity (proinsulin). Proinsulin has a connecting peptide (C-peptide) between B30 and A1 and becomes active only when the C-peptide is split off to produce two-chain native insulin. One idea for equipping insulin with a glucose-sensitive switch could be to conjugate the two partners of a switch near B30 and A1, thus providing a reversibly glucose-responsive opening/closing C-peptide. Novo Nordisk pursued this idea (referred to as pseudo-single-chain insulin; see Figure 3) around 2000—2005, but did not find compounds that worked well, so did not publish them. SmartCells suggested a similar idea in WO2010107520 [98], but did not reveal any functional compounds. In fact, WO2010107520 did not even reveal a working glucose-binding motif. WO2010107520 was issued with claims of using DNA aptamers as the glucose-binding motif, but WO2010107520 has no data showing that the aptamers in fact bind to glucose (no Kd values), much less that insulin equipped with a switch could control bioactivity. Shultz et al. suggested a similar idea in WO2015057852 and WO2015095406 with the desire to use an antibody as the glucose binder. However, a glucose-binding antibody was never reported, much less a glucose-sensitive insulin [99,100]. A similar idea was suggested by Protomer Inc. in WO2016179568, but no glucose binder or any supporting data were disclosed [101]. Weiss/Thermalin suggested a similar idea in WO2016149222 and WO2017070617 [102,103] and showed data for an insulin conjugate with monoboronate in A1 and a polyol in B29, which allegedly rendered a hexamer formulation of this insulin conjugate sensitive to 25 mM glucose. Notably, insulin hexamer equilibria has no impact on the biological activity of insulin in the circulation, and hexamers only exist in the vial/pen and at the injection site (at uM insulin concentrations), not in the circulation, where insulin is diluted to pM concentrations of the monomeric form. Thus, in the best case, the given compound might work *in vivo* from a subcutaneous depot, which would render the system subjective to time lags. Chou pursued a similar switch-based idea using polyols and monoboronates in A1 and B29, respectively, and found compounds responding to mM concentrations of fructose, but unresponsive to glucose [104,105].

Glycostasis has pursued a project of glucose-sensitive binding to a carrier protein, probably an antibody, and Eli Lilly has invested in the project, but no details about the work have been published [106].

Dimarchi et al. designed insulins with extra disulphide bridges, seeking pairs of positions that would inactivate insulin with the disulphide in closed form vs providing active insulin when the same disulphide was in open form using Acm-blocked Cys. Disulphide cross-links A1-B22 and A8-B10 worked best [107]. However, for the design to provide GSI leads, the chosen disulphide would need to be replaced with a glucose binder and an acceptor (for a switch) in the same positions as the disulphide. Any glucose binder and acceptor will inherently be much larger than the disulphide link. Glucose itself contains 12 heavy atoms (not counting protons), whereas the Cys-Cys sidechain contains 4 heavy atoms (CH2SSCH2). Any glucose binder will be larger than glucose itself, so it is hard to imagine how Cys-Cys links could be replaced with a glucose-sensitive switch.

AP Davis has dedicated considerable efforts to the challenging task of designing and synthesising macrocycles that bind carbohydrates [29]. The main goal was to develop small molecule optical sensors for glucose, complementing currently available glucose monitors based on enzymatic systems. Enzymes may have limited stability in aqueous solutions, but this is not a problem for glucose monitoring that relies on strips and electrodes. The main limitation of current continuous glucose monitors is that they are not in direct contact with the blood, and a macrocycle-based sensor will hardly address this issue. Nevertheless, in 2012, the work of Davis et al. was spun out into a biotech company, Ziylo Ltd, to commercialise the invented macrocycles into optical glucose monitors. To provide optical sensing, Davis' macrocycles were typically equipped with a fluorescent motif as exemplified with structure **1** carrying anthracene (see Figure 4). At the time (2012), **1** was the only viable small molecule alternative to boronates [108]. However, for GSI drug development, a polyaromatic fluorescent motif is not desirable. In addition, the reported affinity of macrocycle **1** to glucose was rather weak, with a Kd of 20 mM, thus likely not adequate to respond well to glucose in the hypoglycaemic concentration range. Remarkably, by

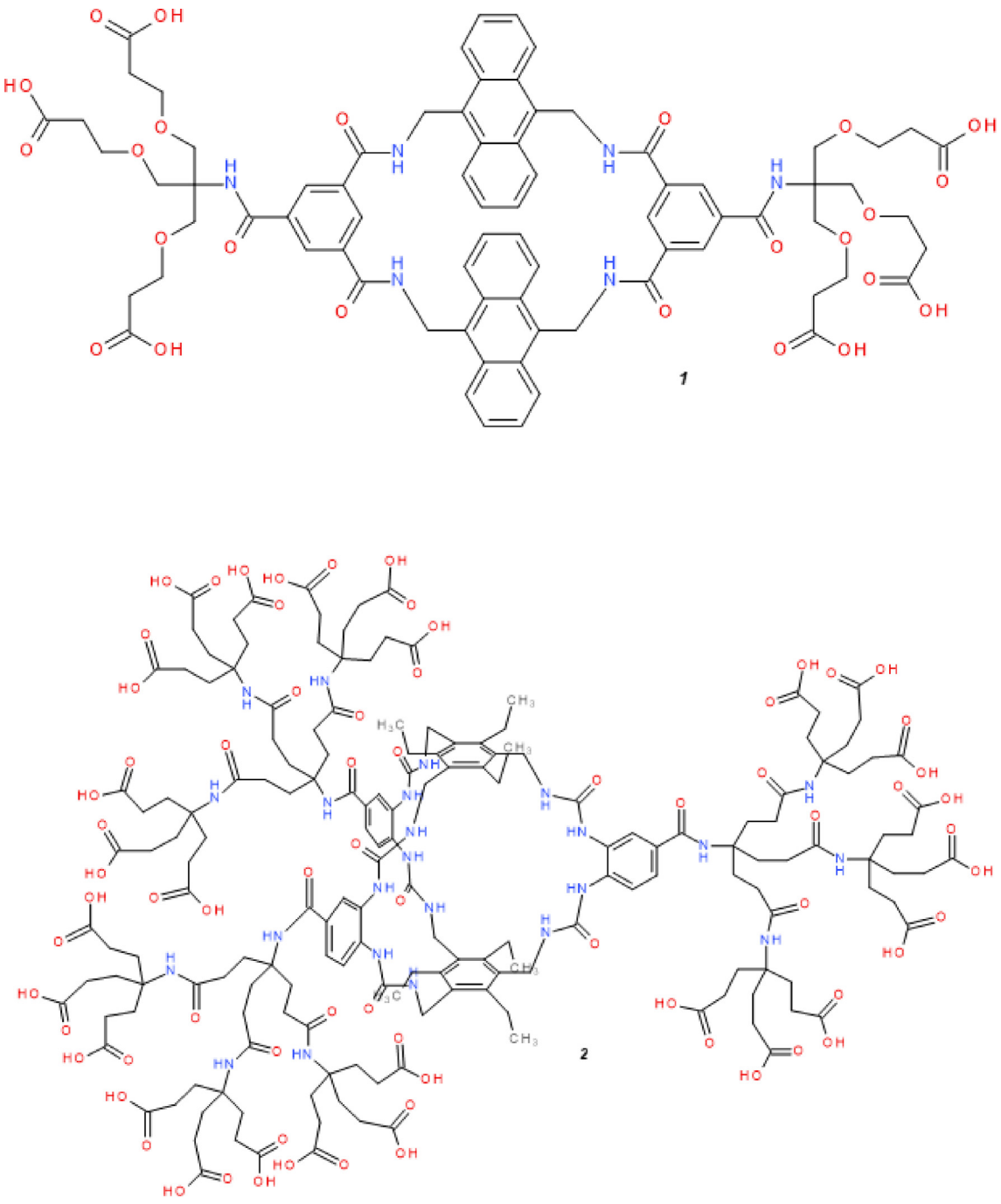

Figure 4: Davis' glucose-binding macrocycles **1** and **2**.

carefully redesign to macrocycle **2**, Davis et al. developed a glucose binder with a much stronger affinity, a Kd of 60 uM with no fluorescent moieties, and a high selectivity for glucose over other carbohydrates and biologically relevant compounds [109,110]. In 2018, Ziylo was acquired by Novo Nordisk and reorganised as Carbometrics Ltd. The companies are currently collaborating in attempts to employ macrocycles such as **2** in designs of glucose-sensitive insulins. WO2020058322 describes insulin conjugates incorporating macrocycle in B29 + glycoside in A1 or B1, which *in vitro* shows up to a 7-fold change in insulin receptor affinity when comparing HIR binding assays with and without 20 mM glucose [111].

6. SINGLE-CHAIN INSULIN

Amidebio's patent application WO2018187147 suggested incorporating a glucose-binding peptide into the C-peptide of a single-chain insulin [112]. However, it is not clear from Amidebio's presentations how glucose binding of C-peptide should reflect glucose-controlled

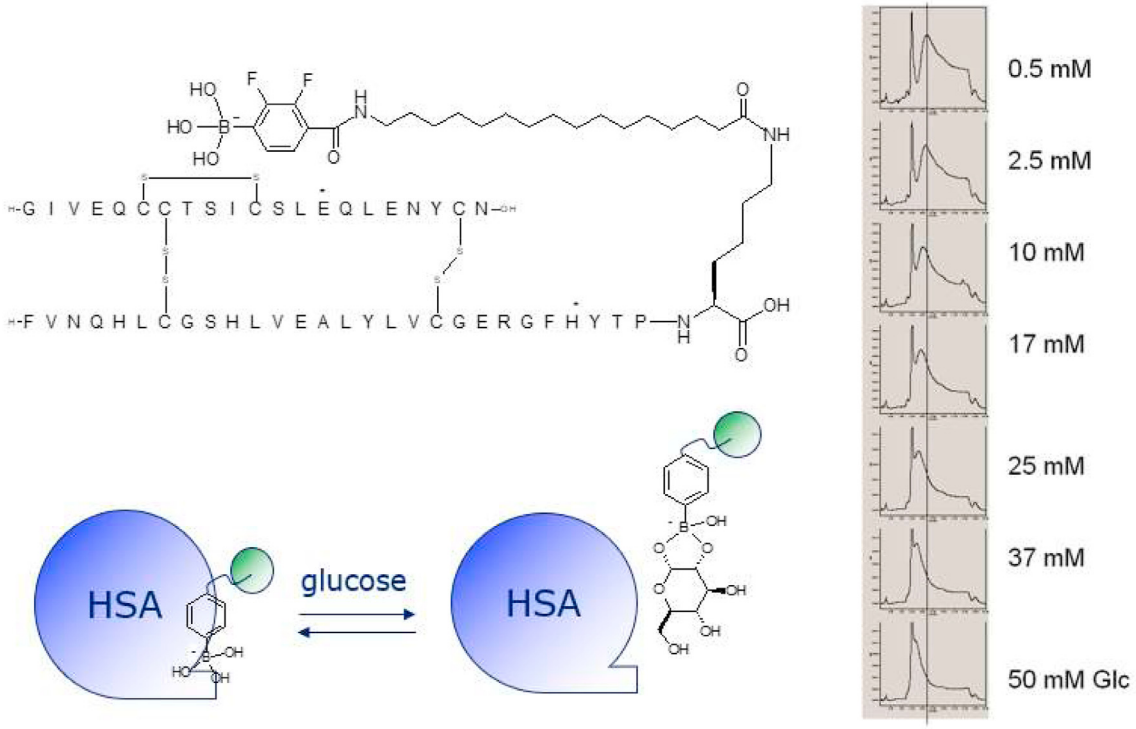

Figure 5: Boronate fatty acid insulin for glucose-sensitive albumin binding.

insulin bioactivity. No glucose-binding peptides that work in aqueous solutions are currently available, and identifying such a peptide (that furthermore is fit as C-peptide) poses a formidable challenge. Attempts to engineer and screen carbohydrate-binding short peptides have been reported, but affinities obtained remained very weak (Kd $>$ 100 mM) [113,114].

Carbohydrate-binding peptides were reported to be found by monitoring for the yellow colour of erythrose bound to peptide-containing bead libraries [115]. Erythrose is obviously colourless; it has no chromophore; thus, yellow colour in erythrose solutions must be due to impurities. The paper postulated binding tetrapeptides to erythrose with low uM Kd. Considering all of the other data about peptide/carbohydrates interactions, these results seem unlikely to be reproducible.

Odorranalectin is a peptide from frog skin that has been reported to bind to fucose [116], but other researchers could not reproduce the results [117].

7. ALBUMIN CONCEPTS

Novo Nordisk has pioneered the use of albumin-binding motifs to prolong the pharmacokinetic profiles of peptide- and protein-based drugs. Human serum albumin/HSA is the most abundant protein in human blood, with a concentration of approximately 500 uM, and HSA is known to bind a range of hormones, fatty acids, and small molecule drugs. The myristoyl conjugate insulin detemir [118,119] was the first albumin-binding peptide conjugate to reach the market, followed by liraglutide (GLP-1) [120], insulin degludec [13], and semaglutide (GLP-1) [7]. In most cases, the conjugations of fatty acids or fatty diacids not only provide albumin binding, but can also modulate the self-assembly character of the peptides, which can increase the *in vivo* half-life, as mainly exploited with insulin degludec multihexamers [13] that provide basal insulin coverage well beyond 24 h.

Further equipping fatty acid insulin conjugates with glucose-binding boronates was envisioned to provide glucose-sensitive albumin binding as first described by Novo Nordisk in WO2011000823 (2011) [121]. This concept would enable glucose responsiveness in the circulation from an albumin-bound depot. Boronates were coupled to omega-amino fatty acids that were conjugated to insulin, and the glucose sensitivity of the constructs was evaluated via elution through commercial HPLC columns with immobilised HSA using buffer +/− 0.5−50 mM glucose (see Figure 5). The most glucose-responsive compound was also tested *in vivo* in normal rats compared to diabetic rats, that is, normal vs high blood glucose, to assess the differences in insulin clearance. The glucose sensitivity appeared rather weak, and the compounds were not pursued in further development. In PNAS 2015 [122,123], Chou/Langer et al. published nearly identical compounds without referencing WO2011000823. The insulin conjugates (called F-PBA-Ins) were found to be equipotent with native insulin *in vitro* using an insulin receptor phosphorylation assay (Figure S3 in the given reference), but the compounds were not tested *in vitro* at varying glucose concentrations. The conjugates were tested in mice in comparison to myristoyl insulin, but only glucose profiles were measured, not insulin profiles. The authors claimed that the longer effect on glucose from F-PBA-Ins compared to myristoyl insulin should document glucose sensitivity, but the given insulins surely have very different pharmacokinetics; thus, the longer effect on glucose could simply be due to longer PK of F-PBA-Ins, not glucose sensitivity.

Hanmi pursued long-acting GLP-1-PEG-Fc and insulin-PEG-Fc conjugates [124]. Sanofi was involved, but stopped their development

project. Hanmi published WO2019066603 in which the insulin-PEG-Fc was further equipped with fatty acid boronates as previously described [125] but WO2019066603 included no data showing that the compounds were in fact glucose-sensitive.
Novo Nordisk published WO2019092125 disclosing glucose-sensitive HSA-binding diboronates for producing insulin conjugates. 19-Fluorine NMR studies showed that the diboronates bind to albumin in a glucose-dependent manner [126].

8. GLUT1 CONCEPTS

Similar to SmartCells' oligomannosyl concept (Section 4), Gu's GLUT1 concept employs conjugating insulin to ligands of the native glucose binder GLUT1, the glucose transporter found on among other red blood cells [127]. Contrary to GLUT4, GLUT1 is not controlled by insulin or internalised like GLUT4. GLUT1 thus always transports glucose into cells irrespective of whether the insulin levels are high or low. Various small molecule GLUT1 binders are known, mainly GLUT1 inhibitors, and by conjugating these to insulin, Gu/Buse envisioned establishing a glucose-sensitive insulin depot functioning in the circulation [128,129]. While this concept is appealing, reduction to practise raises questions. A 2-fold increase in insulin levels was observed when glucose increased 4-fold from 5.6 to 22 mM blood glucose using an *in vitro* erythrocyte assay (100—400 mg/dL glucose, Figure 2E in the given reference) [130], which seems to be a shallow response. The *in vivo* results (mice studies) did not include insulin pharmacokinetic/clearance data, and glucose clamp conditions were not used. Sanofi pursued the concept as described in WO2017207754 and WO2019106122 with hundreds of small molecule GLUT1 binders [131,132], but only a few insulin conjugates were revealed, notably with no associated biodata.

9. ALDEHYDE/OXIME/HYDRAZONE CONCEPT

In aqueous solutions, glucose exists mainly as a hemiacetal in equilibrium with its aldehyde form. Aldehydes are generally reactive towards hydrazines and oxyamines, thus forming hydrazones and oximes [133]. KJ Jensen and researchers at Gubra thus envisioned how insulin with hydrazone or oxime linkers could be hydrolysed/cleaved and react with the aldehyde form of glucose, thus providing glucose-sensitive insulin conjugates [134,135]. For example, an oxime-based linker between insulin and an albumin-binding motif should be subject to hydrolysis and react with glucose aldehyde, which should provide glucose sensitivity. However, the aldehyde form of glucose in water constitutes only approximately 1%, whereas 99% is in the hemiacetal form (see Figure 6). In addition, transformations of hydrazones or oximes with aldehydes are generally very slow and may take days to reach equilibrium. Even when using acetaldehyde as a probe (100% aldehyde, no acetal form), the system takes days for a significant response. These systems are thus likely too slow to use in diabetes.

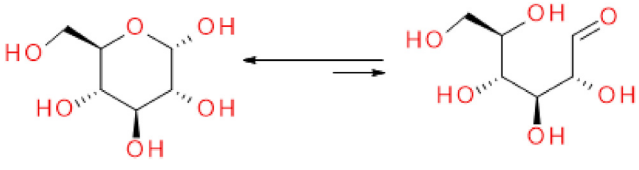

Figure 6: Glucose exists in water as 99% hemiacetal and 1% aldehyde.

10. GOX OR BORONATE GLARGINE

Insulin glargine is a basal insulin with an altered isoelectric point (pI), thereby rendering glargine poorly soluble at a neutral pH but more soluble at an acidic pH. Human insulin has a pI value of approximately 5, and by adding two arginines B31 and B32, Hoechst/Sanofi engineered glargine with a pI value of approximately 7 [136,137]. Glargine is thus supplied as an acidic formulation, which upon injection is neutralised, producing glargine precipitate in the subcutaneous space, from where it slowly redissolves and provides basal insulin profiles with near 24 h coverage. NN had a similar project, NovoSol, but it was stopped due to injection site irritations [138]. Prior to glargine, the most common basal insulin formulation was based on protamine-insulin precipitates (neutral protamine Hagedorn, NPH), which the patient needed to resuspend and inject as particles, typically twice daily. NPH tends to give variations in day-to-day doses and thus hypoglycaemia risk. Biodel co-formulated glargine with GOx with the aim that the solubility of glargine in this system should follow the glucose concentration, since GOx oxidises glucose to gluconic acid and thus alters pH [139]. There has been no sign of development with this idea since the patent application was published in 2010. Chou equipped a glargine-like insulin with monoboronates with the expectation that glucose binding to the boronates should increase the solubility of the resulting insulin conjugates, thus equipping a glargine-like insulin with glucose-sensitive solubility [140,141]. The *in vivo* data showed glucose profiles, not insulin profiles, rendering the concept hard to evaluate. The protraction of both systems would occur in subcutaneous tissues, and the glucose response would accordingly be subject to lag and irreversible release, analogous to that of the polymer systems discussed in Section 2.

11. CONCLUSIONS

When a proper glucose-sensitive insulin finally reaches the market, it will be a long sought-after relief for people with diabetes and their families. Type 1 diabetes often starts in childhood with immense implications for the affected child. Parents are frequently reminded of the health risks associated with hypoglycaemic episodes, which may result in loss of consciousness and hospitalisation. The fear of hypoglycaemia can limit the ambition to strive for optimal insulin dosing, which would otherwise lower the risk of long-term complications. Glucose-sensitive insulin therapies aim to lower the risk of hypoglycaemia and improve treatment outcomes to benefit both patients and society.
Of note, I doubt that glucose-sensitive insulin will completely eliminate hypoglycaemia; glucose responsiveness is based on chemical equilibria controlled by a narrow physiological glucose range; thus, it may still be possible to observe skewed insulin dosing at times due to the many factors influencing blood glucose. But glucose-sensitive insulin should hopefully help considerably. Modern insulin degludec already provides lower risks of hypoglycaemia compared to older basal insulins, not because insulin degludec is glucose-sensitive, but because insulin degludec provides a flatter and longer insulin profile than older basal insulins [142]. However, the average patient still experiences hypoglycaemia many times during their lifetime and therefore tends to dose insulin conservatively. This situation could be changed with a glucose-sensitive insulin. Of note, it is generally not possible to demonstrate improved glucose control with a new insulin in clinical development, since regulatory agencies demand that the studies are conducted as treat-to-target studies, that is, the patient and caretaker

titrate insulin to find the dose that reaches a certain fasted glucose goal in the individual patient. The number of hypoglycaemic events is then typically compared to the standard of care, that is, an insulin already on the market. To document both improved glucose control and a lower risk of hypoglycaemia will demand new and innovative clinical studies in the future.

Some people have high hopes for insulin pumps controlled by continuous glucose monitors (closed-loop systems) or even pumps also equipped with glucagon, which stimulates glucose release from the liver, thereby assisting recovery from hypoglycaemic events [143]. For closed-loop systems, there are challenges associated with glucose electrodes and insulin tubes placed in subcutaneous tissue, and as discussed in Section 2, there are issues with time lags of transport of glucose and insulin between the blood and subcutaneous tissues. A glucose electrode may be positioned in direct contact with the blood, as has been demonstrated clinically (arterial insertion) [143–145], but this is hardly viable for home use. Furthermore, sterile tubes and injection ports for use with pumps are expensive, and the individual must tolerate wearing a pump + invasive sensor, including frequent changing of tubes and electrodes. Not least, electronics have dropouts. All computer systems encounter software crashes or short circuits from humidity or battery failures. Conversely, chemistry as such is reproducible; the affinity of a given glucose binder for glucose will always be the same if conditions are maintained constant. Chemical equilibria can fluctuate when conditions change, but chemistry will never crash similar to a computer system. Closed-loop systems will surely find their use, but probably never be the solution for the broadest base of diabetes patients.

Attempts to engineer beta cells from stem cells could potentially offer a "cure" for diabetes, and this monumental work will likely continue for decades [146]. Glucose-responsive insulin-secreting cells need to be engineered, and the immune-driven destruction of native beta cells in type 1 diabetes must be dodged, along with the issue of low insulin sensitivity in type 2 diabetes. Production, transport, and dosing of living cells to millions of patients will surely be challenging. Beta cell engineering and replacement may well succeed one day, but it will hardly be a solution for the broadest base of diabetes patients. Glucose-sensitive insulin will.

CONFLICT OF INTEREST

THJ is an employee and stockholder of Novo Nordisk A/S.

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