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‘Smart’ insulin-delivery technologies and intrinsic glucose-responsive insulin analogues

Mark A. Jarosinski[1], **Balamurugan Dhayalan**[1], **Nischay Rege**[2], **Deepak Chatterjee**[1], **Michael A. Weiss**[1,3,4]

[1]Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, IN, USA

[2]Department of Medicine, Yale University School of Medicine, New Haven, CT, USA

[3]Department of Chemistry, Indiana University, Bloomington, IN, USA

[4]Weldon School of Biomedical Engineering, Purdue University, West Lafayette, IN, USA

Abstract

Insulin replacement therapy for diabetes mellitus seeks to minimise excursions in blood glucose concentration above or below the therapeutic range (hyper- or hypoglycaemia). To mitigate acute and chronic risks of such excursions, glucose-responsive insulin-delivery technologies have long been sought for clinical application in type 1 and long-standing type 2 diabetes mellitus. Such ‘smart’ systems or insulin analogues seek to provide hormonal activity proportional to blood glucose levels without external monitoring. This review highlights three broad strategies to co-optimise mean glycaemic control and time in range: (1) coupling of continuous glucose monitoring (CGM) to delivery devices (algorithm-based ‘closed-loop’ systems); (2) glucose-responsive polymer encapsulation of insulin; and (3) mechanism-based hormone modifications. Innovations span control algorithms for CGM-based insulin-delivery systems, glucose-responsive polymer matrices, bio-inspired design based on insulin’s conformational switch mechanism upon insulin receptor engagement, and glucose-responsive modifications of new insulin analogues. In each case, innovations in insulin chemistry and formulation may enhance clinical outcomes. Prospects are discussed for intrinsic glucose-responsive insulin analogues containing a reversible switch (regulating bioavailability or conformation) that can be activated by glucose at high concentrations.

Keywords

Artificial pancreas; Glucose-responsive insulin; Glucose-responsive polymers; Glucose sensor; Hormone-receptor recognition; Review

Corresponding author: Michael A. Weiss, weissma@iu.edu.

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Introduction

Insulin replacement therapy (IRT) is essential for the treatment of type 1 diabetes mellitus and often required by patients with late-stage type 2 diabetes. Advances in diabetes technologies have been broadly motivated by the aim to link optimisation of IRT (including individualised glycaemic goals in type 2 diabetes [1, 2]) and healthcare outcomes [3–8]. During a typical 24 h period, patients on insulin therapy often exhibit episodes of hyperglycaemia or hypoglycaemia [9], despite individualised dosing regimens [10, 11] and the broad use of engineered basal and rapid-acting insulin analogues [12–14]. Indeed, glycaemic excursions outside the narrow blood glucose range classified as normoglycaemia (4.4–6.7 mmol/l) [15] are frequent despite strict adherence to dietary and lifestyle recommendations [16, 17]. These challenges have motivated innovation in ancillary technologies: formulation chemistry, protein engineering, glucose-sensing technologies and delivery devices [18, 19]. Integrating IRT with engineered mechanisms of feedback (whether at the level of protein, cell or device) represents a critical current frontier of innovation, with the overarching goal of fewer hyperglycaemic excursions and reduced time in hypoglycaemia (i.e. increased time in range [TIR]). TIR is a major issue, ever-present in the daily life of an individual with type 1 diabetes [20, 21] and in a subset of those with type 2 diabetes [22].

In this review we outline a range of glucose-responsive (‘smart’) approaches to control TIR and discuss the prospects for mechanism-based molecular design of intrinsic glucose-responsive insulin (GRI) analogues [23]. Such delivery systems or insulin analogues seek to optimise TIR by the combined approaches of molecular design of new chemical entities and increased engineering controls to deliver insulin therapeutics. Like endogenous beta cells, such systems (broadly designated GRIs) would provide insulin activity proportionate to the metabolic state. Accordingly, the GRI concept has attracted the attention of the research community [24–27] and funders [28, 29] alike.

GRIs may be broadly classified as: (1) algorithm-based mechanical GRI systems (closed-loop delivery systems as an ‘artificial pancreas’) based on continuous glucose monitoring (CGM)-coupled insulin pumps [30]; (2) polymer-based systems, wherein insulin is encapsulated within a glucose-responsive polymeric matrix-based vesicle or hydrogel [31]; or (3) molecular GRI analogue systems, which involve the introduction of a glucose-sensitive motif to the insulin molecule or its formulation that, in either case, confers glucose-responsive changes to bioavailability or hormonal activity [32] (Fig. 1). We highlight potential synergies among these technologies as molecular GRIs may, in principle, be delivered via a closed-loop system (for further review, please see a related article in this special series by Boughton and Hovorka [33]). Cell-based therapies, which provide biological feedback regulation [34, 35], are beyond the scope of this review.

Mechanical systems

Closed-loop systems

Mechanical GRI systems [36] integrate: (1) CGM to provide real-time measurement of interstitial glucose concentration; (2) an insulin pump receiving CGM input; and (3) a

control algorithm specifying the appropriate dose of insulin for minute-by-minute s.c. injection [11, 37, 38]. Although these systems promise to improve 24 h glycaemic control in diverse patient populations (including adults, adolescents and children), CGM-based measurements of interstitial glucose concentrations lag by ~20 min behind changes in blood glucose levels, complicating prediction of future glycaemic trends. Reliable prediction is important because a ‘rapid-acting’ insulin analogue (once injected) may require 20–40 min for absorption and, once in the blood stream, may exert biological effects lasting 3–4 h [39]. The robustness of current predictive algorithms is likely to be enhanced by development of ‘ultra-fast’ pump insulin analogue formulations [40], such as Fiasp (Novo Nordisk) [41, 42], ultra-rapid lispro (URLi; known as Lyumjev in Europe; Eli Lilly) [43, 44], and respective reformulations of prandial analogues insulin, including insulin aspart (Pro^{B28}→Asp) and insulin lispro (Pro^{B28}→Lys and Lys^{B29}→Pro) [45–47]. The progress of CGM along with automated real-time insulin titration and bolus calculators, has enabled initial regulatory approval of mechanical GRI systems [11].

Dual-hormone pumps

To prevent or treat hypoglycaemia more effectively, bihormonal pumps have been developed to provide either insulin or its counter-regulatory hormone glucagon [48–53]. Dual-hormone algorithms trigger s.c. injections of a stabilised glucagon formulation based on trends in CGM readings predicting an impending hypoglycaemic excursion, thereby maintaining blood-glucose levels within range more efficiently than conventional CGM-coupled insulin pumps [54, 55]; the extent of advantage and its clinical impact have been the subject of debate. Fibrillation-resistant formulations of glucagon or glucagon analogues would be required for practical bihormonal systems [56].

Insulin stability in closed-loop systems

Ultra-miniaturisation of sensors and improved accuracy of pumps [36, 57–58] have encouraged reconsideration of i.p. delivery of insulin via i.p. infusion devices [59, 60]. Implanted refillable pumps [61] have promised ultra-rapid pharmacokinetic dosing profiles of insulin with first-pass hepatic signalling in the portal circulation. These advantageous features have stimulated interest in novel i.p-compatible insulin analogue formulations; desiderata include insulins with even more rapid onset of activity, shorter duration and greater formulation stability compared with the insulin therapeutics currently on the market.

Despite the above theoretical advantages and the convenience of long-term i.p. reservoirs of insulin (up to 3 months), catheter occlusion [62] remains a concern that has limited the feasibility of such systems in otherwise encouraging clinical trials [63]. Occlusions are often mediated by immunogenic and proinflammatory insulin-derived amyloid fibrils [64, 65]. Insulin instability and fibrillation can also occur within the pump reservoir to inactivate the hormone and provide seeds for further cycles of fibrillation.

Risk of insulin degradation in i.p. systems might be mitigated through design and development of ultra-stable, fibrillation-resistant analogues. Examples are provided by single-chain insulins (SCIs) containing a foreshortened C domain [66] and by two-chain insulin analogues containing an engineered non-canonical disulfide bridge between A- and

B-chains [67–69] (Fig. 2). The altered topology (connectivity) of such analogues appears incompatible with cross-β assembly, the canonical core structure of an amyloid [70–72]. Their topologies may, in principle, alter the signalling properties of the hormone. Insertion of non-canonical cystine B4–A10 (Fig. 2b), for example, is associated with anomalously prolonged duration of activity of insulin upon intravenous bolus injection in rat studies [67], an unfavourable property of a pump insulin, the safety of which relies on fast-on/fast-off pharmacodynamics. Clinical data are not available.

Polymer-based GRI systems

Polymer-based technologies exploit sequestration of native or derivatised insulin within a matrix suitable for s.c. injection by directly integrating glucose-responsive components into delivery systems [73, 74]. The matrix, in principle, senses the glucose concentration and releases a proportional amount of insulin. Three classes of glucose-sensitive motifs have enabled such feedback: (1) glucose-binding proteins, a class that includes lectins, like concanavalin A (ConA); (2) glucose oxidase, an enzyme that catalyses oxidation of glucose to gluconic acid with release of a proton (hence lowering the pH); and (3) boronate-based chemistries, exemplified by phenylboronic acid (PBA; see below), which form reversible ester linkages with diol-containing molecules [75], including glucose [76]. In addition to these categories, an innovative recent technology envisions endogenous biological systems (e.g., the mannose receptor and even components of the erythrocyte) as a ‘smart’ matrix-based insulin deliver system [77, 78].

Insulin–lectin complexes

More than 40 years ago, Brownlee and Cerami pioneered a model GRI system: glycosylated insulin complexed with ConA [24, 79]. This complex was designed to sequester insulin in the s.c. space during normoglycaemia and release the hormone during hyperglycaemia via competition with ambient glucose molecules (Fig. 3a). Although the strategy was successful in vitro, its competitive set point was above the range of typical hyperglycaemic concentrations [79]. ConA’s immunogenicity and mitogenicity might limit clinical translation [80, 81].

Glucose-responsive polymers

Advances in materials science have enabled design of diverse candidate polymer-based GRI systems. These were based on initial observations of Lorand and Edwards [75] that boronic acids react reversibly with vicinal (1,2-) diols (like those that occur naturally in carbohydrates and catechol); their reaction yields boronate esters. Norrild and Eggert first provided NMR evidence that binding of PBA to D-glucose is primarily mediated by the α-D -glucofuranose conformer (Fig. 4) [76]. Subsequently, Wang’s group [82, 83] described a mechanism/mechanisms of binding between diols and boronic acids (reviewed by Joop A. Peters in relation to monosaccharides [84]). Such systems envisaged encapsulation of insulin within polymeric matrices to form a ‘smart’ s.c. depot. The set points of chemical equilibrium-based glucose-recognition technologies (relative to enzyme- or protein-based schemes) may be more amenable to optimisation than ConA systems were found to be.

Polymer-based GRIs can, in principle, employ a variety of encapsulation chemistries, including polyethylene glycol (PEF), poly(*N*-vinyl-pyrrolidone) and succinyl-amidophenyl-glucopyranoside [31, 74, 85]. Whereas the matrices are impermeable to insulin during normoglycaemic or hypoglycaemic conditions, their permeability may increase as a result of physical changes that cause swelling or increased water solubility of the polymer in response to an increase in interstitial glucose concentration. Examples include matrices co-derivatised with PBA, other boronic acids [86–88] and dot-immobilised glucose ester-based crosslinks [74, 85, 89].

Immobilised glucose-binding proteins and enzymes may also be integrated with glucose-modified polymers [77, 90]. Of particular interest, glucose oxidase has been encapsulated in matrices that are chemically sensitive to H_2O_2, hypoxia or decreases in local pH. Unlike PBA and non-enzymatic glucose-binding-protein-based technologies, polymeric matrices in glucose oxidase-based systems exploit its catalytic activity to increase the polymer's water permeability and, so, regulate hormone release [91, 92] (for review, see Wang et al [73]).

Polymer-based GRI technologies are challenged by limited particle stability [93, 94], lag times and suboptimal insulin-response rates leading to hyperglycaemic or hypoglycaemic excursions. Addressing these limitations has encountered a catch-22: sensitising matrices to hyperglycaemia, for example, can limit their ability to attenuate insulin release at low glucose concentrations. The latter problem can be exacerbated by matrix degradation, in principle raising the risk of severe hypoglycaemic episodes in patients due to bolus over-delivery [95, 96].

Intrinsic GRI systems

Intrinsic (or unimolecular) GRIs define a novel class of analogues wherein the modified hormone itself confers glucose-dependent activity or bioavailability. Initial candidate technologies relied on sequestration of active insulin hormone within the s.c. space or within the bloodstream (as inactive complexes) with enhanced release or activation only during hyperglycaemia. Recent bio-inspired advances exploit specific endogenous features of the s.c. space, potential hormone-carrier proteins or cellular clearance systems. Although these elegant strategies remain in the early stages of development, their simplicity, convenience and potential cost-effectiveness make them an attractive target of ongoing research. Because standard insulin products are becoming a commodity in the pharmaceutical industry, clinical introduction of chemically modified insulins that are glucose responsive will likely require detailed analysis of cost effectiveness despite their elegance.

Insulin fusion proteins

A pioneering approach employed an insulin–glucose oxidase fusion molecule [97]. A cysteine-based linkage was broken as the enzyme oxidised glucose. Although proof of principle was obtained in vitro, the low K_m of the enzyme for glucose led to liberation of the hormone under hypoglycaemic, as well as hyperglycaemic conditions [97]. In addition, release of H_2O_2 by glucose oxidase (as a byproduct of glucose oxidation) and generation of reactive oxygen species (ROS) could cause tissue injury. In a complementary approach, glucose oxidase-dependent changes in s.c. pH were exploited to modulate the solubility

(and, hence, rate of absorption) of insulin glargine, the isoelectric point-shifted active component of Lantus (Sanofi) [98]. Because this basal analogue is insoluble at neutral pH but soluble under acidic conditions, glucose oxidase-mediated acidification (via production of gluconic acid), in principle, enhances bioavailability [99] (Fig. 3b). Although in vitro and pilot animal studies appear promising, clinical data has not yet been obtained [100].

Biology-inspired GRI systems

An attractive frontier of GRI engineering takes advantage of an endogenous biological system, 'hijacked' in its native form to provide an active component of a glucose-dependent regulatory scheme. Such an approach was developed by Merck, based on competitive clearance of a saccharide-modified insulin by the endogenous mannose receptor [101, 102]. The saccharide adduct presented terminal mannose moieties and so provided a substrate for clearance by the ubiquitous mannose receptor system. The essential idea envisioned rapid clearance of the modified insulin under conditions of hypoglycaemia but slow clearance under conditions of hyperglycaemia due to low-affinity binding of glucose to (and, hence, competition at) the mannose receptor. The glucose-dependent differences in rates of clearance, although not large, were sufficient to provide partial protection from hypoglycaemia relative to the same dose of an unmodified insulin. Although animal-based and pilot clinical studies were pursued, this approach is no longer under development. These challenges to create a modified insulin with an improved therapeutic index that enables tighter glycaemic control with a reduced risk for hypoglycaemia, as well as the challenges of translating in vivo animal models and data for application in humans, highlight the importance of developing better in silico GRI modelling in the developmental pipeline [23, 103–105].

PBA-modified insulin derivatives

PBA is a diol-binding element that is able to sense carbohydrates [106–108]. A PBA-modified insulin derivative was first described as a potential intrinsic GRI by Hoeg-Jensen et al [32]. This work established that PBA could be coupled to the insulin B29 position without affecting its biological activity, in turn enabling the analogue to bind diol-containing sequestering agents (Fig.3). The investigators further demonstrated that a modified insulin carrying both a PBA and a polyol group attached to the Lys^{B29} sidechain could form high molecular weight multimeric complexes that dissociate under control by D-sorbitol or D-glucose. (Fig. 3d) [109, 110], A sidechain glutamic acid linker was required for in vitro activity, as is the case for insulin degludec, a second-generation basal acylated insulin [111]. To our knowledge, no in vivo results have been described.

Novel use of a PBA-based GRI was described by Chou and colleagues [112] who employed an acylated insulin analogue (insulin detemir) in which myristic acid was coupled onto lysine (Lys^{B29}) to mediate binding to serum albumin and, so, provide a long-lived circulating depot of insulin. The hydrocarbon acyl tag was further derivatised with PBA so that its affinity for albumin might be glucose-responsive. The essential idea thus envisioned albumin as a glucose-dependent carrier, similar, in spirit, to the reported use of erythrocyte membranes as glucose-dependent carriers [77, 113]. Although this goal was not achieved in vitro with this analogue (i.e. the albumin-binding properties of the analogues were not

glucose-dependent), several such candidate GRI analogues exhibited glucose-responsive biological activity in a peritoneal glucose-infusion assay in mice [73].

In a similar manner to the above, Jensen and colleagues [114] took advantage of albumin binding as a plasma depot to demonstrate novel GRI activity acceleration, which was mediated by aldehyde-responsive capture of released insulin. The strategy biased for glucose reactivity over fructose by virtue of aldehyde capture based on the increased reactivity of acyclic glucosyl aldehyde over fructosyl ketone and the fact that fasting glucose concentrations are about >1000-fold greater than those of fructose (Fig. 4) [114]. In the same study [114], in glucose clamp models, some insulin analogues demonstrated GRI activity, but their in vitro hydrolysis rates were >6 h, suggesting that lag time might be an issue.

Glucose-regulated conformational switches

A reversible conformational cycle between active and inactive states of insulin may, in principle, be regulated by a ligand, such as glucose (Fig. 1b). A new avenue for molecular GRI design was inspired by crystallographic and cryogenic electron microscopy (cryo-EM) studies of insulin bound to fragments of the insulin receptor [115, 116], including the intact ectodomain [117, 118]. Such studies revealed a major change in the conformation of insulin on receptor binding in which the hormone ‘opens’ with detachment of the C-terminal B-chain segment; this enables intimate contact between the N-terminal A-chain α-helix and the receptor complex [117, 118].

It may be possible to exploit the mechanism of insulin–insulin receptor binding and signalling to design a glucose-dependent conformational switch, such that binding of the modified insulin to the insulin receptor is impaired under hypoglycaemic conditions. A glucose-displaceable bridge between a glucose-binding element attached to one position in the insulin molecule and an internal ligand (such as a diol or saccharide) may, in principle, be placed at any pair of sites, such that the ‘closed state’ is inactive and the ‘open state’ is active. Proof of principle was recently provided in studies of a fructose-responsive insulin (FRI), in which *meta*-fluoro-PBA was attached to the A-chain N-terminus, and an aromatic diol group was attached to the ε-amino group of Lys^{B28} to provide an internal tether between the A- and B-chains (see ‘open, active’ form in Fig. 1b) [119]. Whilst the baseline activity of this analogue was low, near-native activity (as assessed in studies of the liver-derived cell line HepG2) was restored by 50 mmol/l fructose, but not by 50 mmol/l glucose [119].

The binding preference of PBA and its derivatives for fructose (relative to glucose) reflects their respective conformational equilibria and, in particular, the subpopulation of conformers displaying *cis*-1,2-diols that have hydroxyl groups that are oriented *syn*-periplanar (same side) for joint presentation to the boronic acid (Fig. 4c) [108]. Such alignment depends on the conformational equilibrium of a monosaccharide, as is observed in the β-D-fructofuranose form (Fig. 4c). Because the analogous conformation of glucose (α-D-glucofuranose; Fig. 4c) is >100-fold less populated compared with β-D-fructofuranose at equilibrium in solution [84, 120], binding of PBAs to fructose is significantly favoured. Improved glucose-binding elements will be required to extend the FRI proof-of-principle

results to obtain bona fide GRIs. A chemical diversity of candidate boronate-based glucose sensors has been described [73, 82, 121–124], as well as non-boronate-based chemistries [125–127]. In addition, the above FRI's bridge between the A-chain N-terminus and B-chain C-terminus follows naturally from the hormone's mechanism of insulin receptor binding [116], but a wide variety of bridges might exhibit analogous glucose-responsive properties. Such design options promise an opportunity to co-optimise other GRI molecular features, including stability and immunogenicity.

To define potential non-canonical pairs of conformational switch sites suitable for GRI design, DiMarchi and colleagues [128] undertook a systematic survey of fourth disulfide bridges in an insulin analogue (*des*-[B29,B30]-Lys^{B28}-insulin; 'DesDi' [129]). Whereas previous efforts to engineer additional disulfide bridges into insulin were motivated by stability [67–69], this study's emphasis was on differences in insulin receptor binding on closure of the fourth bridge. Six such pairs of putative switch sites were identified based on this functional criterion (Fig. 5). These pairs are distant in the native structure of insulin; formation of the engineered disulfide bridge (forced by selective chemical tactics) presumably distorts the conformation of the hormone, including its insulin receptor-binding surface. Predicted distances between the unpaired cysteines in the framework of native insulin (T state) are given in the Fig. 5 and reported as interatomic distances.

To visualise these novel analogues on closure of the non-native disulfide bridge, we undertook molecular modelling of the six constrained insulin analogues based on distance-geometry and restrained molecular dynamics. A baseline set of restraints was provided by prior NMR analysis of engineered insulin monomers [130, 131]. Of such fourth disulfide bridges, only cystine A0–B26 (Fig. 6c) could be accommodated within a native-like protein conformation (Fig. 7). A glucose-displaceable tether between amino acid residues A0 and B26 would, thus, be analogous to the fructose-regulated switch described above (residues A0–B26) [119].

In the other five cases, formation of the fourth disulfide seems to require partial unfolding of the protein, perturbing one or more α-helical elements (Fig. 6a,b,d–f); these distortions are incompatible with the structure of the primary insulin-binding site in the insulin receptor. For example, imposing cystines A8–B10 or A14–B10 in our modelling (Fig. 6d,e) distorted the central B-chain α-helix and N-terminal A-chain α-helix, key insulin receptor-binding elements [116]. Displacement of such aberrant bridges by glucose would presumably relieve the distortion and, so, restore activity. A rational path towards switchable intrinsic GRIs, based on strained disulfide engineering, was envisaged by DiMarchi and colleagues [128]. It would be of future interest to probe the structures and stabilities of these strained analogues, whether they contain cystine or reversible glucose-displaceable tethers. We anticipate that the set point for glucose displacement would be modulated by the degree of conformational strain.

Clinical significance and conclusions

The centennial of the discovery of insulin marks a time of continuing innovation in insulin technologies. Even as the events leading to insulin discovery in Toronto in 1921 are

celebrated as a landmark in molecular medicine, a comprehensive history recognises not only the contributions of Frederick Banting, Charles Best, James B. Collip and John J. R. Macleod, but also the key insights and advances made by others in the preceding five decades, beginning with Oskar Minkowski and Joseph von Mering (Germany) and continuing with Étienne Lancereaux (France), Nicolae C. Paulescu (Romania) and Israel Kleiner (USA) [132].

The present review highlights a vibrant frontier of insulin technologies. Whereas closed-loop systems have recently become a clinical reality [133–135], polymer-based and intrinsic GRIs promise to enhance the safety and efficacy of IRT. In addition to the elegance of the associated chemistries and macromolecular structures, ongoing research has a compelling clinical motivation: to enhance the health and quality of life of patients with type 1 diabetes and of patients with type 2 diabetes refractory to oral therapy—and with less burden on patients [136]. To bridge the valley between basic science and clinical applications, in silico simulations of animal physiology and human patients are likely to provide key guidance [23, 103–105].

All three classes of GRI technologies considered here, namely closed-loop delivery (as an ‘artificial pancreas’), polymer-based systems and molecular GRI analogue systems, seek to achieve optimal TIR. Standard IRT faces a trade-off: on the one hand, strict glycaemic control has been shown to retard or prevent microvascular complications in type 1 diabetes [6] and is likely to be beneficial in early stages of type 2 diabetes [137–139] but, on the other hand, aggressive glycaemic targets increase the acute and long-term risks of hypoglycaemia [10, 140–143]. Whereas CGM pump-based GRIs presently employ the most mature component technologies, recent innovations in matrix-based and unimolecular GRIs suggest promising routes towards safe and effective approximation of pancreatic beta cell function. We anticipate continuing progress in the coming years to reduce the burden of diabetes. Given the balance of price and access to new therapeutics (especially derivatised insulin analogues [144]) and the high human and economic costs of long-term diabetes complications, such innovative technologies are likely to be cost-effective when considering the integrated impact on society.

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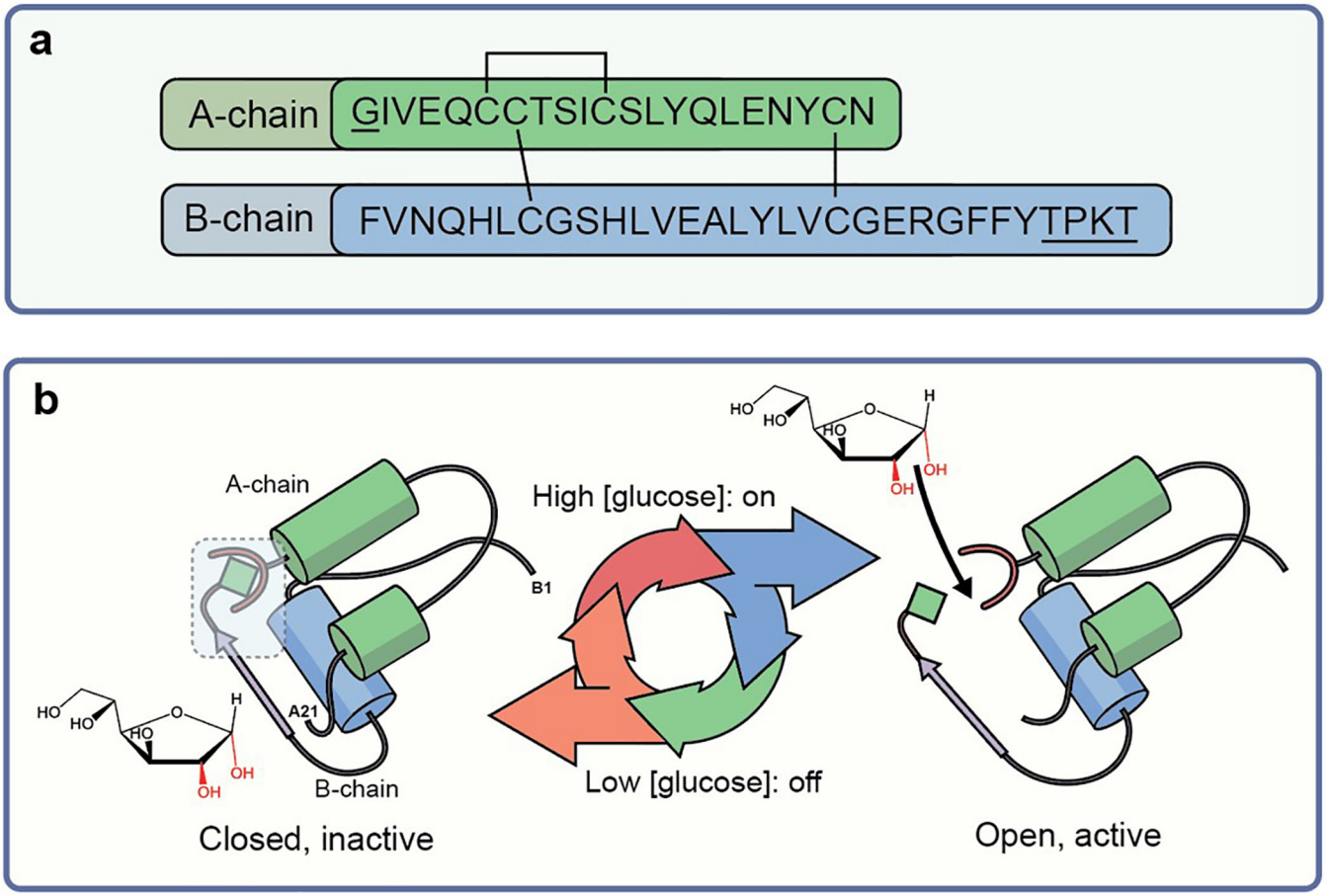

Fig. 1.

(**a**) Design strategy for intrinsic molecular GRIs. Sequence of insulin showing A-chain and B-chain with typical sites of chemical modification underlined (GlyA1 N-terminal and B-chain residues B27–B30) that affect pharmacokinetics and monosaccharide responsivity. Amino acid residues are labelled using their standard single letter codes. (**b**) Design scheme of monosaccharide-responsive insulin. The ribbon model of closed inactive insulin (T-state monomer) is shown (with a free glucose molecule adjacent to it); the blue box highlights sites of modification (red horseshoe shapes indicate glucose-binding element; green diamonds indicate internal diol). The envisioned glucose-regulated conformational cycle in which a monosaccharide acts as a competitive ligand to regulate a conformational switch between the closed state (inactive in absence of ligand) and the open state (active in presence of ligand) is illustrated.

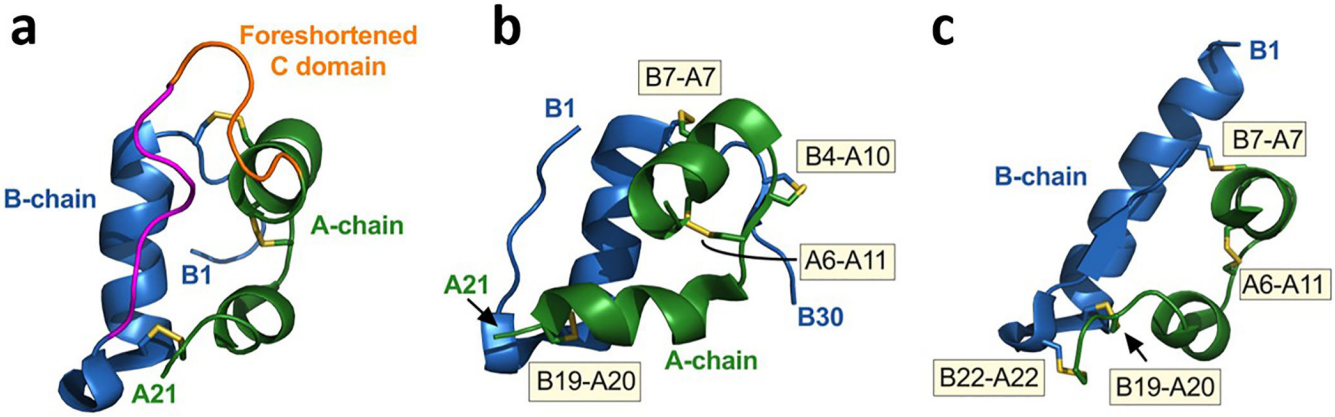

Fig. 2.

Ultra-stable insulin analogues. (**a**) Single-chain insulin (SCI) analogues exhibit native-like A and B domains (green and blue, respectively) with three native disulfide bridges (yellow). A foreshortened C-domain (5–8 residues; orange) connects the C-terminal B-chain β-strand (magenta) to the A-chain N-terminus. A favourable C-domain sequence can dampen conformational flexibility, augment thermodynamic stability and protect from fibrillation [66]. Protein Data Bank (PDB) ID: 2LWZ (www.rcsb.org/; accessed: 1 February 2021). Figure adapted from [145] with permission from Wolters Kluwer Health, Inc. (**b**, **c**) Ultra-stable two-chain insulin analogues with engineered fourth disulfide bridges. Crystal structure of the four-disulfide (4SS)-insulin analogues containing a disulfide bond in (**b**) position A10-B4 (PDB ID: 4EFX) [67] and (**c**) position A22-B22 (PDB ID: 6TYH) [69]. The disulfide bonds (shown in yellow) and labelled (yellow boxes). Images were created with PyMOL (https://pymol.org/2/; accessed: 1 February 2021).

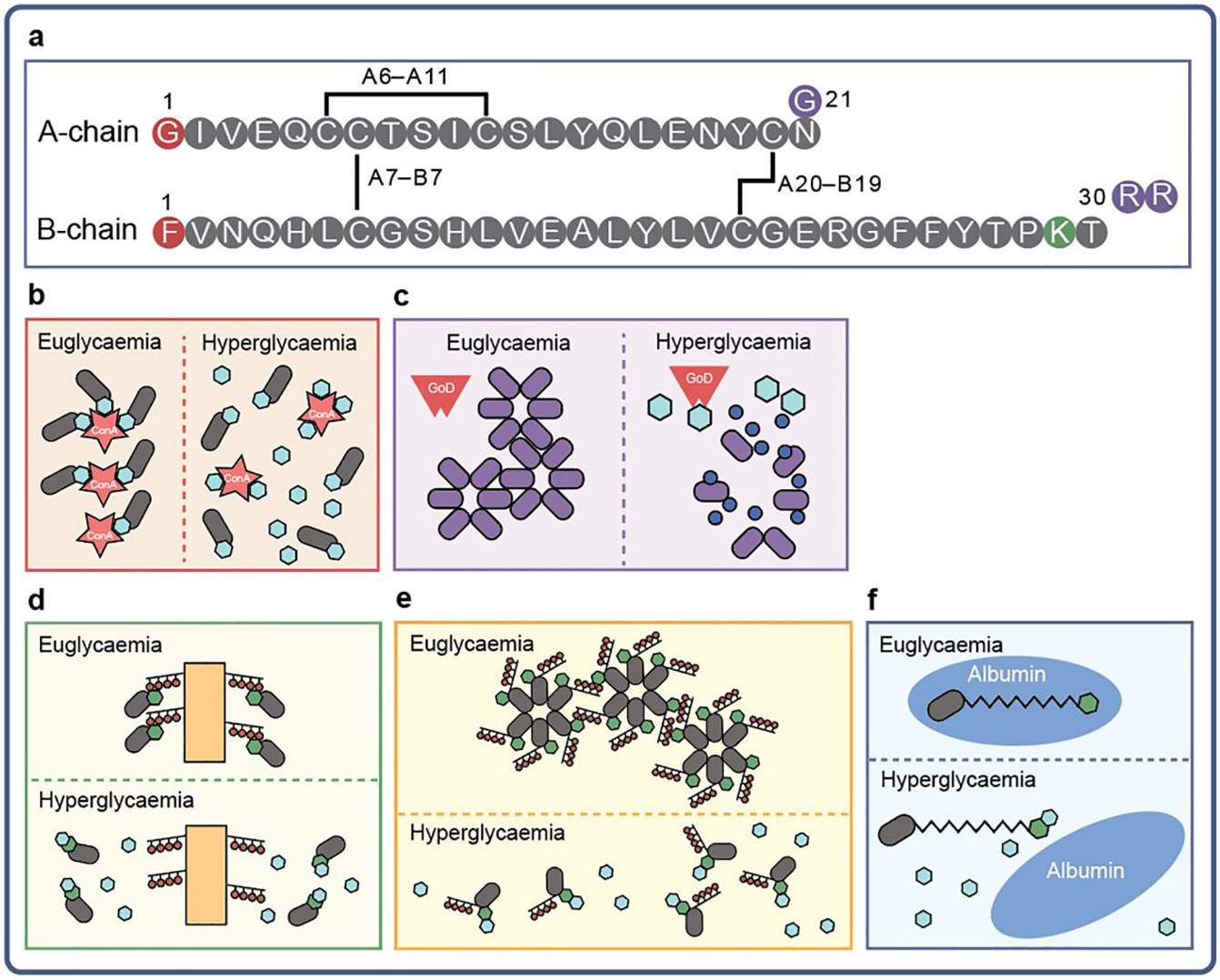

Fig. 3.

GRI-inspired derivatisation of insulin. Insulin contains two peptide chains (designated ‘A’ and ‘B’) connected by two disulfide linkages (cystines A7–B7 and A20–B19). (**a**) Brownlee and Cerami [24, 79] created a molecular GRI by coupling saccharides to the N-termini of one or both polypeptide chains of insulin (shown as red circles in the A- and B-chain schematic). The analogues (grey ovals) were bound to ConA (red star) before administration in rats. ConA was expected to sequester the modified insulin in the s.c. space under euglycaemic conditions, but allow its liberation following competitive binding of ambient glucose (blue hexagons) during hyperglycaemia [24, 79]. (**b**) A GRI system employing insulin glargine (purple ovals; its three amino acid substitutions denoted as purple circles in the A- and B-chain schematic) was co-injected with glucose oxidase (GoD; red polygon), which was expected to lower local pH by oxidising glucose to gluconic acid (dark blue circles) at a rate proportional to the glycaemic state, thereby increasing the solubility and, hence, pH-dependent bioavailability of the analogue. (**c**–**e**) A number of groups have modified the ε-amino group of Lys^{B29} of the insulin molecule with PBA derivatives (green

hexagons) to create candidate GRI systems. (**c**) Hoeg-Jensen et al [32] directly coupled Lys^{B29} with PBA derivatives, enabling binding to diol-containing polymer carriers (orange rectangles with red circles) in a glucose-dependent fashion [32]. (**d**) The same group also derivatised residue B29 with a molecule containing a PBA and a polyol group (black lines with red circles), leading to multi-hexameric complexes in vitro that could dissociate in a glucose-dependent fashion [110]. (**e**) Chou and colleagues [112] created a GRI that contained a PBA derivative coupled via a fatty-acyl linker (black jagged line) to Lys^{B29}. They hypothesised that this analogue would bind to albumin (blue oval) below a threshold level of blood glucose, being liberated during hyperglycaemia as glucose-modified PBA–insulin, which was envisioned to have decreased affinity for albumin; however, the GRI-responsive mechanism of lower affinity of the glucose-modified PBA–insulin for albumin was not confirmed [112]. Figure adapted from [145] with permission from Wolters Kluwer Health, Inc.

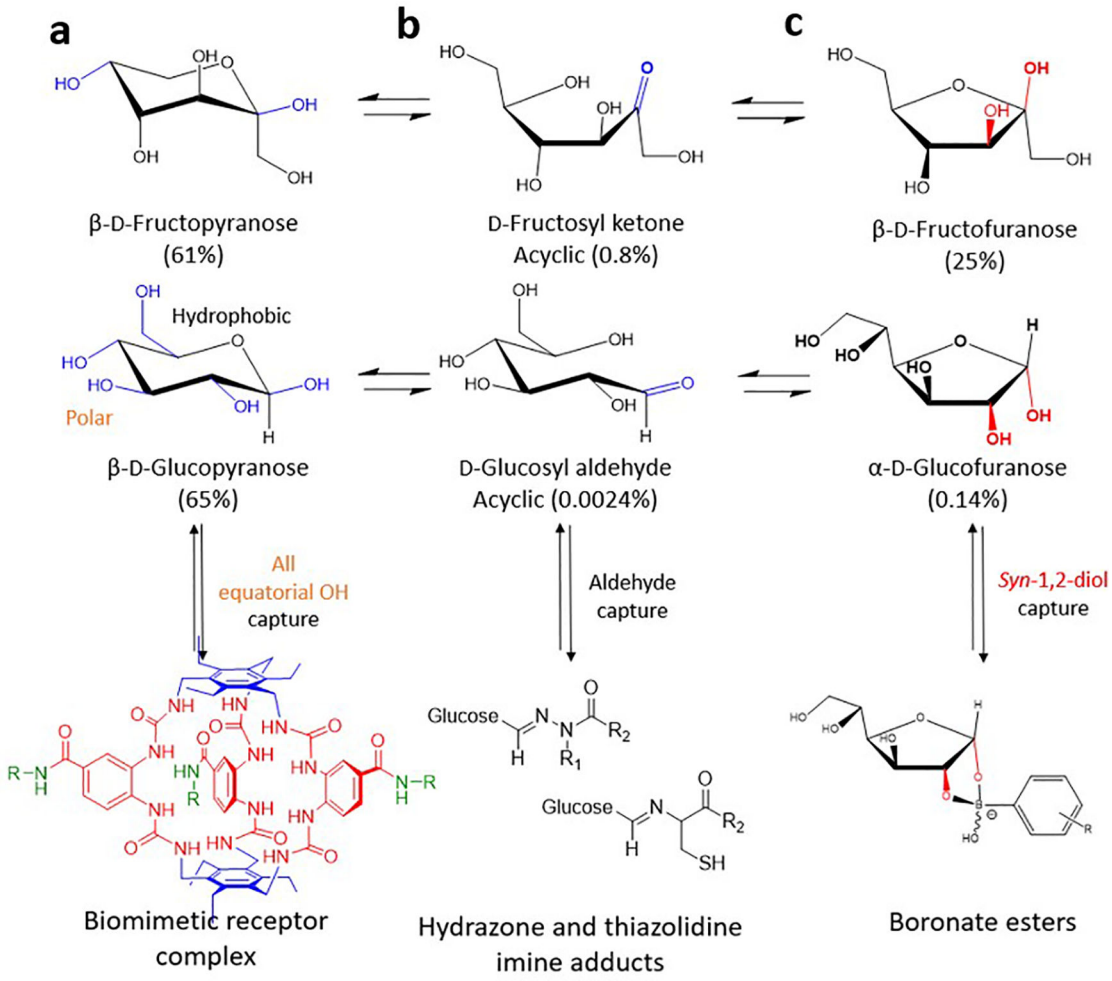

Fig. 4.

Biomimetic and chemical strategies to bind glucose vs fructose. Selective but reversible capture strategies exploit different molecular features and reactivity in conformational equilibria in water at pH 7.4 between saccharides, such as D-fructose and D-glucose conformations. D-Fructose populates β-D-fructopyranose, D-fructosyl ketone and β-D-fructofuranose (per cent population: 61%, 0.8% and 25%, respectively) and D-glucose populates β-D-glucopyranose, D-glucosyl aldehyde and α-D-glucofuranose (per cent population: 65%, 0.0024% and 0.14%, respectively) (data from [75]). (**a**) The biomimetic approach has achieved selective binding of β-D-glucopyranose over β-D-fructopyranose (dissociation constant [K_a] ~18,000 M^{-1} and K_a ~51 M^{-1}, respectively). This is owing to the all equatorial polar hydroxy groups in β-D-glucopyranose (shown in blue) that form specific hydrogen bonds with the preorganised urea-based cage elements and the dual phenyl groups in the biomimetic receptor complex (shown in red and blue, respectively), which make apolar hydrophobic contacts with the glucopyranose ring with solubilising groups (shown in green) [146]. Neither D-fructose nor other saccharides accommodate these requirements for interaction with the biomimetic receptor complex [125]. (**b**) Jensen and colleagues [114] demonstrated a novel chemical approach to D-glucose selectivity by exploiting its open chain, acyclic D-glucosyl aldehyde. A series of masked cleavable hydrazones or thiazolidines

were tuned for aldehyde reactivity (carbonyl groups shown in blue). These GRI designs relied on hydrolysis of a hydrazone or thiazolidine linker that was covalently attached to insulin at one end and C18 fatty acid at the other. On hydrolysis, D-glucosyl aldehyde reacts with the free unmasked linker-moiety, resulting in a shift of equilibrium towards a free active insulin analogue. Increasing concentrations of glucose (from normoglycaemia to hyperglycaemia) are proposed to drive this shift. In contrast, D-fructose's acyclic form (D-fructosyl ketone [shown in blue]) is reactive to the unmasked linkers but not generally found in the body [114]. (**c**) PBAs bind most strongly to aligned 1,2-diol elements, such as those found in β-D-fructofuranose; respective conformational equilibria, thus, favour selective binding to fructose [84]. The acidity of PBAs is also known to affect diol reactivity, with vicinal diols known to produce boronate esters [75, 83], as illustrated by the α-D-glucofuranose/boronate ester in the figure. Key *cis*-hydroxyl groups are coloured red. D-Fructopyranose, β-D-fructofuranose, β-D-glucopyranose and α-D-glucofuranose images adapted from [108], published by The Royal Society of Chemistry under the terms of the Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/). Biomimetic receptor complex image adapted from [146] by permission from Springer Nature, ©2018.

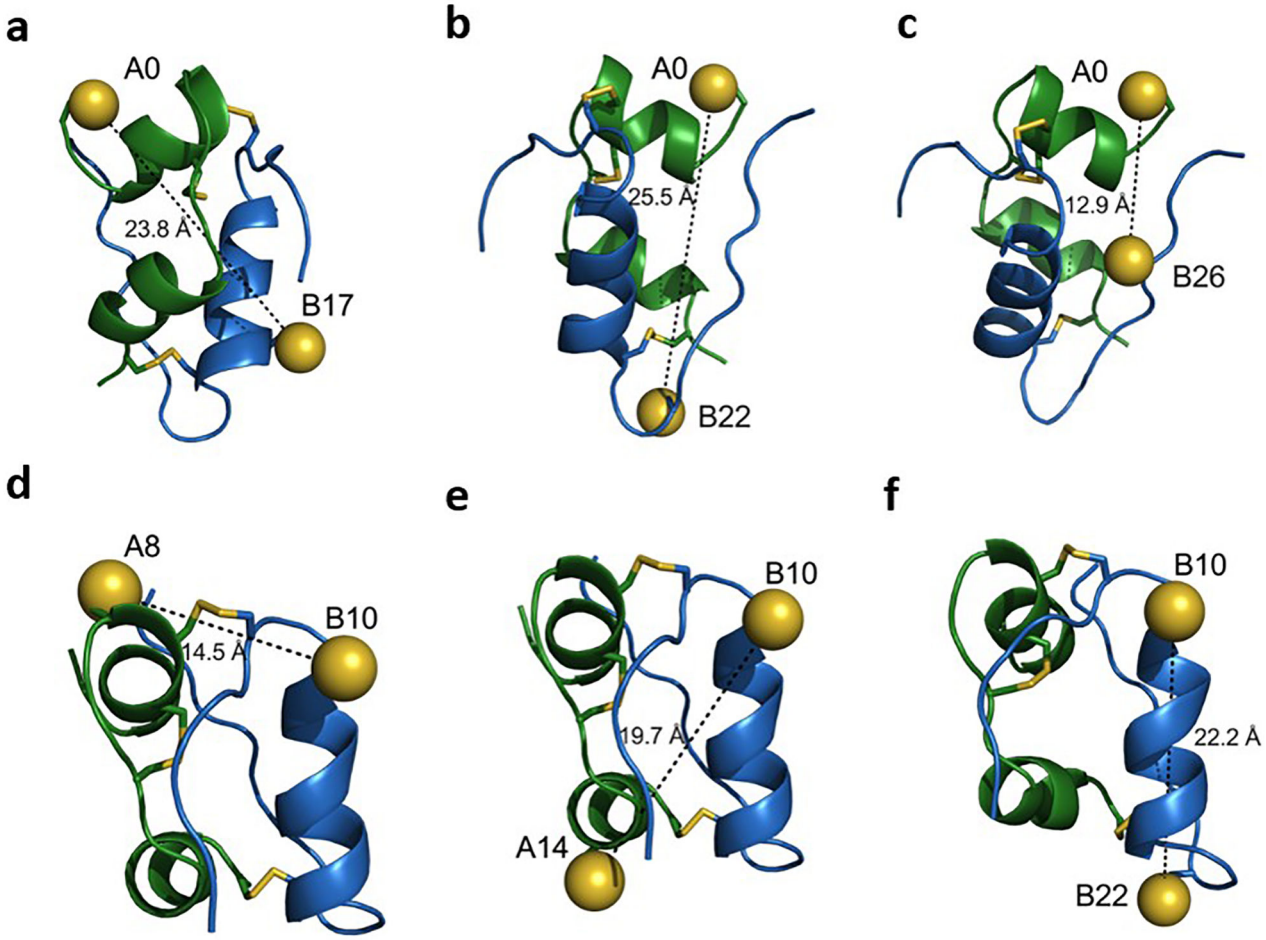

Fig. 5.

Search for non-canonical disulfide-based conformational switch sites as designed by Brunel et al [128]. Location of fourth disulfide bonds and their predicted interatomic distances (indicated by dashed lines between sulphur atoms [yellow spheres]; predicted interatomic distances shown in angstroms [Å]) in the insulin monomer (crystallographic T state; Protein Data Bank [PDB] ID: 4INS [www.rcsb.org/; accessed: 1 February 2021]). The disulfide pairs in (**a**–**f**) were chosen based on their reported insulin receptor binding activity, the open:closed ratio of which was >100; they were associated with >100-fold decrease in activity on fourth disulfide-bond formation. The pairs are: (**a**) A0–B17; (**b**) A0–B22; (**c**) A0–B26; (**d**) A8–B10; (**e**) A14–B10; and (**f**) B10–B22. Sulphur atoms are shown in the substituted cysteines. Native disulfide bonds are shown as yellow lines; A-chain shown in green and B-chain in blue. Data were taken from [128]; images were created with PyMOL (https://pymol.org/2/; accessed: 1 February 2021).

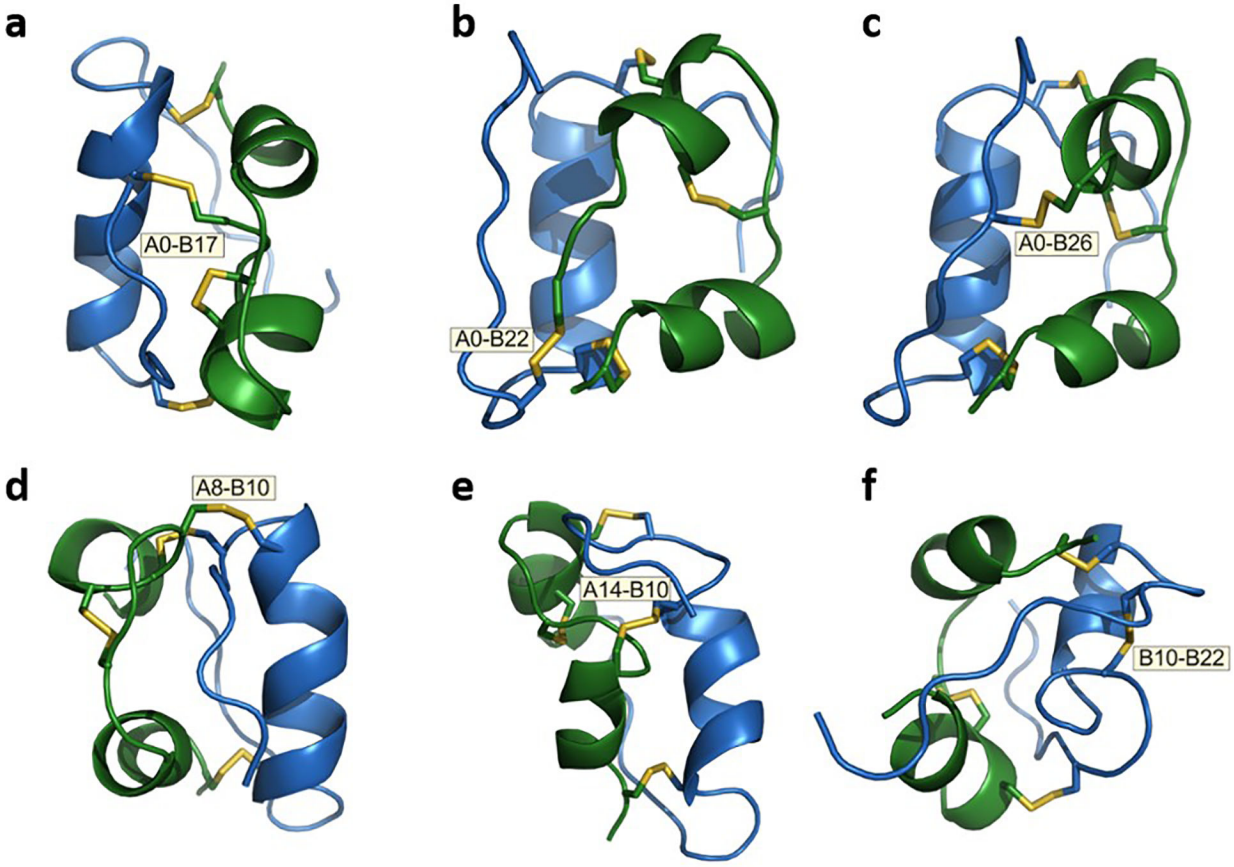

Fig. 6.

Structural distortions among putative disulfide switch analogues [128] predicted on closure of a fourth disulfide bridge involve (**a**) cystine A0–B17, (**b**) cystine A0–B22, (**c**) cystine A0–B26, (**d**) cystine A8–B10, (**e**) cystine A14–B10 and (**f**) cystine B10–B22. A0 represents N-terminal extension of the A-chain by cystine. Only the model in (**c**) contains native α-helical segments and a native-like tertiary structure; the other five models are remarkable for segmental unfolding of helical segments and distortion of helix–helix orientations. Models were calculated using Xplor-NIH (https://nmr.cit.nih.gov/xplor-nih/; accessed: 1 February 2021), based on distant restraints (~750 per model) derived from NMR analysis of an engineered insulin monomer (Protein Data Bank [PDB] ID: 2JMN [www.rcsb.org/; accessed: 1 February 2021]; [147]). Specific subsets of native distance restraints were removed to enable the designated additional disulfide bridge to be formed; distance restraints were omitted involving residues in: B9–B22 (**a**,**b**); B25–B30 and A0–A3 (**c**); B9–B16 and A2–A9 (**d**); A11–A16 and B9–B16 (**e**); and B9–B22 (**f**). Representative models were visualised and analysed using PyMOL (https://pymol.org/2/; accessed: 1 February 2021). A-chain shown in green and B-chain in blue.

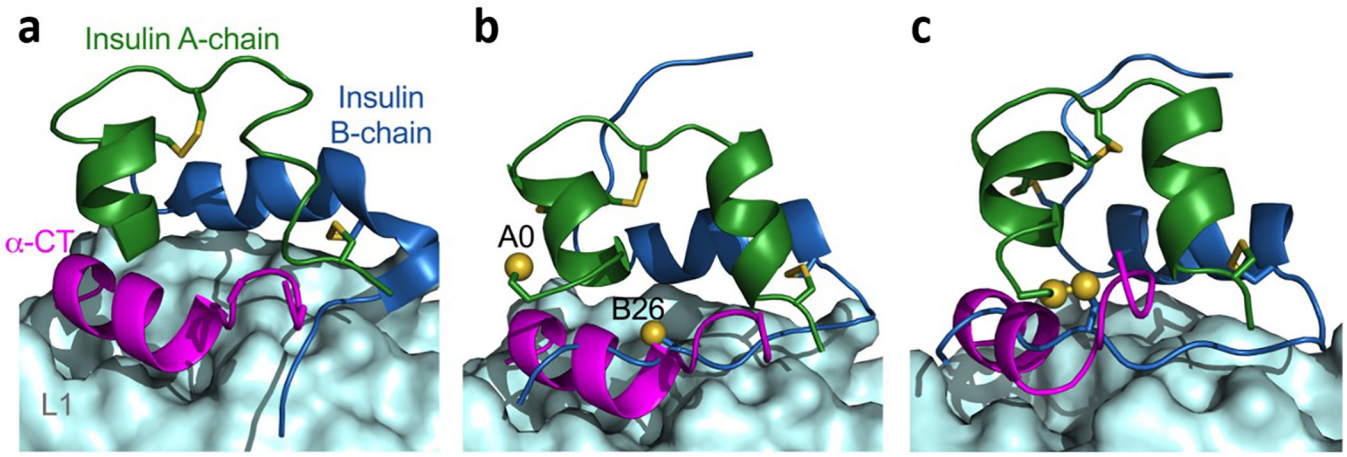

Fig. 7.

Mechanism of insulin–insulin receptor binding excludes certain engineered disulfide bridges. (**a**) Co-crystal structure of wild-type insulin bound to the micro-receptor (μIR) (Protein Data Bank [PDB] ID: 4OGA [www.rcsb.org/; accessed: 1 February 2021]). Upon insulin binding to its receptor, the B-chain C-terminus moves away from A-chain, giving way to the α-CT domain. (**b**) A0–B26 three-disulfide (3SS) insulin monomer bound to the μIR. The location of sulphur atoms in the fourth cysteine pairs is shown. (**c**) Modelled structure of four-disulfide (4SS) insulin (A0–B26; see Fig. 6c) bound to the μIR. If the normal mode of binding takes place, the fourth A0–B26 disulfide bridge hinders the opening of the B-chain and causes a steric clash with the α-CT domain. Images were created with PyMOL (https://pymol.org/2/; accessed: 1 February 2021). L1 domain shown as a pale cyan surface; α-chain of carboxyl terminal (α-CT) domain shown in magenta; insulin A-chain shown in green and B-chain in blue; sulphur atoms shown as yellow sphere.