Pursuit of a perfect insulin

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Abstract | Insulin remains indispensable in the treatment of diabetes, but its use is hampered by its narrow therapeutic index. Although advances in peptide chemistry and recombinant DNA-based macromolecule synthesis have enabled the synthesis of structurally optimized insulin analogues, the growing epidemics of obesity and diabetes have emphasized the need for diabetes therapies that are more efficacious, safe and convenient. Accordingly, a broad set of drug candidates, targeting hyperglycaemia plus other disease abnormalities, is now progressing through the clinic. The development of an insulin therapy that is responsive to glucose concentration remains an ultimate goal, with initial prototypes now reaching the proof-of-concept stage. Simultaneously, the first alternatives to injectable delivery have progressed to registration.

Diabetes mellitus
A metabolic disease associated with elevated levels of glucose that results from pancreatic insufficiency in insulin production and/or reduced target-tissue insulin sensitivity. Type 1 or juvenile diabetes is caused by immunological destruction of insulin-producing pancreatic β-cells. Type 2 or adult-onset diabetes is a progressive condition characterized by insulin resistance and is often associated with obesity.

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For nearly a century, insulin has proven to be a life-saving medicine for individuals with diabetes mellitus. The pathobiology of diabetes that leads to insulin therapy has been characterized as either juvenile-onset, based upon immune-mediated destruction of pancreatic islets (type 1 diabetes (T1D)), or adult-onset pancreatic exhaustion, which is accelerated by the insulin resistance that is commonly associated with obesity (type 2 diabetes (T2D))[1]. The majority of insulin use is in T2D, given the much higher prevalence of this disease compared to T1D.

The first synthesis of human insulin by recombinant DNA (rDNA) technology occurred in the 1970s, and its commercialization shortly thereafter in the early 1980s represents a seminal milestone in the history of this hormone[2–4] (FIG. 1). Biosynthesis provided an alternative to animal-derived insulins and, more importantly, enabled production of virtually unlimited quantities of the human protein. Once established, rDNA technology offered a cost-effective means of producing non-native insulin analogues capable of delivering superior pharmacology[5].

Important advances in insulin therapy, such as the development of rapid- and sustained-action analogues, currently represent state-of-the-art therapy for millions of patients with insulin-dependent diabetes[6]. However, despite these improvements, insulin treatment still imposes a challenging regimen and provides sub-optimal outcomes for the majority of patients[7,8]. The pursuit of a therapy that can normalize blood glucose with enhanced safety and convenience continues, with reports of advances in pharmacokinetics and selectivity for target tissues and receptors, as well as polypharmacy to achieve superior glucose and body weight management[9–13]. In addition, concurrent developments in materials science and microfabrication are enabling the development of closed-loop systems for real-time glucose sensing and controlled insulin release, with the aim of achieving near-physiological precision in glucose control[14,15].

Consequently, we find ourselves in the midst of a period of renewed interest in insulin, which is delivering unprecedented insights into its mechanism of action and advancing the design of clinical candidates. The first century of insulin therapy focused largely on hormone supply, physical characterization, purity and improved pharmacokinetics. The focus for the future is increasingly on pharmacology, specifically on safer, more user-friendly therapies that address the heterogeneity of disease and, most notably, excess body weight. Chemical synthesis and semisynthesis of insulin and related peptides were first reported 50 years ago and proved to be instrumental to current progress[16]. The purpose of this Review is to present recent advances in the field, the status of the latest therapeutic analogues and priorities for further developments in insulin therapy.

Development of insulin therapy

Following the discovery of insulin in 1921, initial research focused on establishing a commercial supply of the hormone and therapeutic approaches to reduce the longer-term consequences of diabetes[17–19]. Animal-sourced insulin could be obtained in sufficient quantity to meet the clinical need. Furthermore, such insulin maintained biological function when applied to humans and, despite its low purity, immunogenicity leading to drug neutralization was an infrequent occurrence. As a higher purity and more reliable supply of commercial hormone was being secured, the need for longer-acting forms and non-injectable delivery first emerged[19–21].

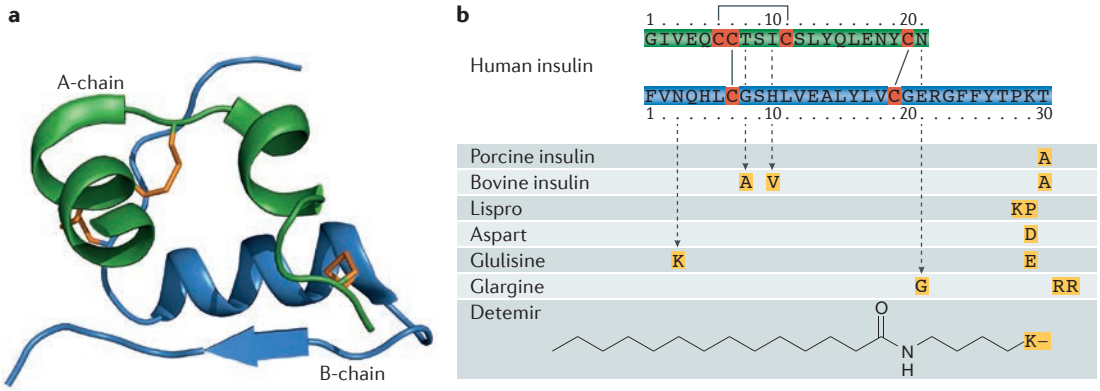

Figure 1 | **Structure and sequences of human insulin and analogues. a** | The structure of insulin (RCSB Protein Data Bank (PDB) entry 3INS; illustrated in PyMol). The A-chain (green) consists of two α-helices. The B-chain (blue) features an α-helix and a β-strand. **b** | Sequences of human insulin and insulin analogues that have been approved and used for treatment of diabetes. Sequence differences between human insulin and the insulin analogues are highlighted in yellow. Residues involved in disulfide bridges are highlighted in red.

The development of zinc-based suspensions to extend insulin action was a seminal accomplishment in patient care and for decades represented the primary form of insulin therapy[19,20,22]. Non-injectable forms of administration were far less successful, and it is only in the past decade that pulmonary and potentially oral delivery have shown promising results[23,24]. One of the most important developments in the treatment of insulin-dependent diabetes was the establishment of blood glucose monitoring[25]. This diagnostic test is essential to patient care, has inspired subsequent improvements in the therapeutic use of the hormone and has directed research leading to enhanced pharmacokinetics and safety. Following the introduction of blood glucose monitoring was the development of mechanical devices capable of providing a more precise and sustained subcutaneous delivery of insulin[26].

Insulin was a life-saving drug for three decades before its chemical structure was determined[27–29]. It could be argued that the era of medicinal protein chemistry was launched by insulin structure–function studies[16]. The first chemical syntheses of insulin occurred in the 1960s and, although it was far too inefficient to have commercial relevance, the technology (in concert with advances in semisynthesis) enabled the early structural interrogation of the molecule[30–32]. The initial commercial supply of human insulin was derived from the enzymatic conversion of porcine insulin[33], which was followed by biosynthetic production in bacteria and, later, in yeast[4,34]. Following this came the search for insulin analogues with improved glycaemic control, despite the fact that definitive proof of a relationship between diabetic complications and glucose control was not established until a decade later. Indeed, it was not until 1993 that the importance of insulin therapy capable of achieving more-precise glucose control was validated by the results of the Diabetes Control and Complications Trial (DCCT)[35]. This study showed that chronic microvascular disease complications such as retinopathy, nephropathy and neuropathy in patients with insulin-dependent diabetes, were sizably reduced with intensified insulin therapy.

Two major classes of insulin analogues — fast-acting and basal — have now emerged. The first of the so-called mealtime or fast-acting analogues to be developed was insulin lispro (Humalog, Eli Lilly), which was commercially launched in 1996. Insulin lispro is based on the principle of weakened self-association resulting from inversion of the ProB28LysB29 native sequence[36–38]. The second entry in this class — insulin aspart (Novolog, Novo Nordisk) — was first marketed in 2000 and utilizes an Asp at position B28 (REFS 39–41). The most recent rapid-acting analogue, insulin glulisine (Apidra, Sanofi), was introduced in 2006 and is based on replacements of Lys at B29 with Glu and of Asn at B3 with Lys[42]. Clinical results with insulin aspart and insulin glulisine have confirmed that these two insulin analogues provide similar pharmacokinetic and pharmacodynamic performance to that of first-in-class insulin lispro[43,44]. Basal insulin therapy is the second essential component of glucose control in insulin-dependent diabetes. A basal insulin analogue is intended to mimic the steady, unprovoked secretion profile of a healthy pancreas[45,46]. Historically, neutral protamine Hagedorn (NPH) and the lente and ultralente insulins served the purpose for sustained basal insulin action[20,47]. Preceding the advent of insulin biosynthesis, these classical insulin suspensions were used for decades, and their primary limitations were their insufficient duration of action and high degree of variability. The primary strategy used in the discovery of superior basal insulin therapy relies upon decreased solubility at the site of injection. Insulin glargine (Lantus, Sanofi), which was launched in 2001, uses a shift in isoelectric point to dramatically lower solubility at physiological pH, rendering the insulin far less soluble at the injection site[48,49]. This results in an extended time–action profile as the analogue slowly re-solubilizes. The increased isoelectric point is achieved through two additional Arg residues at positions B31 and B32. Gly is introduced at A21 to maintain chemical stability in the aqueous, acidic formulation. The isoelectric shift approach was also used in the development of insulin NovoSol (Novo Nordisk); however, this programme was terminated owing to issues

Insulin analogues
Compounds derived from insulin that has been altered in its structure for the primary purpose of enhanced pharmaceutics or pharmacology. The two main types of insulin analogues are basal or sustained-action analogues, which are used for daytime and night time glucose control, and bolus or rapid-acting analogues, which are used for mealtime glucose control and pump administration.

Subcutaneous delivery
The drug is delivered through injection to the subcutis, the layer of tissue located immediately beneath the skin layer. It remains the most common method of insulin administration.

Extended time–action profile
Characteristic of long-acting insulin analogues, which produce relatively stable insulin levels for 12–24 hours after injection. These analogues are sometimes referred to as 'peakless' insulins by virtue of their flat pharmacokinetic profiles.

Therapeutic index
The ratio between the dose of the drug that causes an adverse effect relative to the therapeutic dose. Insulin has an inherently low therapeutic index. This represents a persistent risk for overdosing that can result in life-threatening hypoglycaemia.

with inflammatory reactions at the injection site[50,51]. An alternative approach uses a long-chain fatty acid to slow adsorption and facilitate extended plasma circulation through non-covalent albumin binding. This strategy was reported by Eli Lilly with insulin lipidated at LysB29 with palmitic acid (W99-S-32) and by Novo Nordisk with LysB29-myristyl desB30-insulin[52,53]. The latter was eventually approved as the basal analogue insulin detemir (Levemir), which launched in 2006 (REFS 54,55). Recently, two novel basal insulin analogues — insulin degludec and insulin peglispro — completed Phase III clinical trials (BOX 1; TABLE 1).

After nearly a century of progress, the chemical character and biological performance of insulin have evolved, but it remains a drug of last resort. During the early stages of diabetes, preference is given to the use of non-insulin based medication, such as metformin and sulfonylurea therapy, although new evidence supporting early initiation of insulin therapy is now emerging[56,57]. The limitations of insulin are multiple and include a narrow therapeutic index, with an associated risk of life-threatening hypoglycaemia, as well as weight gain, which compromises its use in overweight individuals. In addition, insulin must typically be injected several times daily, a cumbersome

Box 1 | **Insulin degludec and insulin peglispro**

Insulin degludec (Tresiba, Novo Nordisk) is a des-B30 human insulin that is uniquely fatty-acylated at LysB29. This structural change (see the figure, top panel) introduces a novel time-extension mechanism, which is responsible for the pharmacodynamic profile of insulin degludec[10]. When formulated in the presence of phenol and zinc, insulin degludec maintains a stable di-hexameric structure. Following injection, phenol dissociates quickly and the ensuing conformational change mediates the formation of a soluble, multi-hexameric structure that slowly releases monomers of insulin degludec for absorption[175]. The oligomeric structure of insulin degludec is mainly responsible for its extended duration of action, whereas its affinity to plasma albumin is believed to provide a buffering effect and lessen variability once absorbed into the circulation. Clinical evaluation of insulin degludec therapy has indicated a glucose-lowering effect that persists for up to 42 hours and fewer episodes of hypoglycaemia, particularly nocturnal hypoglycaemia, when compared to insulin glargine therapy[176,177]. Importantly, these studies also confirmed that insulin degludec maintains glucose control, even when used in a flexible regimen permitting administration at any time of day[178]. Despite favourable clinical results and regulatory approval in Europe, the US Food and Drug Association (FDA) requested a cardiovascular outcome study before approval, in line with current guidance for new anti-diabetic agents[179]. Insulin degludec has recently been approved by the FDA in the United States[69].

Insulin peglispro (LY2605541, Eli Lilly) is derived by covalent attachment of a linear 20 kD polyethylene glycol (PEG) polymer to the LysB28 side-chain in insulin lispro (see the figure, bottom panel). Extended action is achieved through increased hydrodynamic size of the analogue owing to the PEG conjugate, which is more than four times the size of the unmodified insulin. This increase in size results in slower subcutaneous absorption as well as a substantially reduced glomerular filtration rate, both of which contribute to the appreciable prolongation of the half-life of the peptide[9]. Clinical studies with insulin peglispro confirmed the preclinical pharmacokinetics with a sustained glucose-lowering effect that persisted for up to 36 hours[180]. A head-to-head comparison of insulin peglispro to insulin glargine in patients with type 1 diabetes indicated that patients treated with insulin peglispro achieved better glycaemic control as measured by mean plasma glucose concentration (HbA_{1c}) (–0.6% versus –0.4%, respectively)[181]. A unique feature of this novel insulin analogue is its seemingly hepatoselective action, which was established through euglycaemic clamp studies performed in dogs and humans[98]. The subcutaneous administration of insulin peglispro exhibited a preferential hepatic effect as evidenced by a relative shift from net hepatic glucose output to peripheral glucose utilization. This suggests that this analogue may function in a more physiological manner than conventional insulins and that it may offer less hypoglycaemic risk and body weight gain. In fact, patients treated with insulin peglispro exhibited subtle loss of body weight, which is atypical for insulin treatment[181–183]. No apparent difference was observed in overall rates of hypoglycaemia in patients receiving insulin peglispro-treatment relative to those receiving insulin glargine, although the former exhibited a lower frequency of nocturnal hypoglycaemia. A concern was raised regarding elevated levels of liver enzymes (alanine aminotransferase and aspartate aminotransferase), of which the clinical significance remains unclear and should be addressed in longer-term studies[70,71]. Eli Lilly first delayed the submission of insulin peglispro to further evaluate safety of this candidate in continuing Phase III trials and very recently discontinued its development[16,22,184–187].

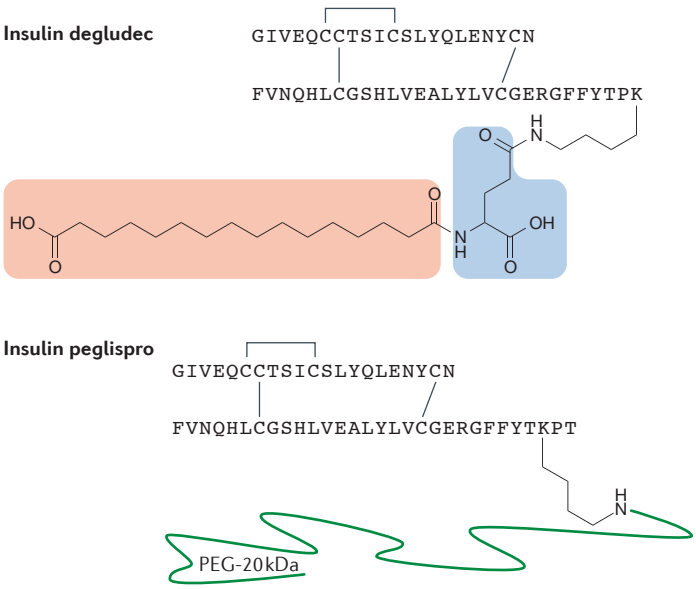

Table 1 | **Selected insulin analogues that are recently approved or in development**

Name	Company	Description	Mechanism	Development phase	Refs
Insulin degludec (Tresiba)	Novo Nordisk	Fatty-acylated insulin analogue	Basal	• Approved in the EU in 2013 • Approved in the US in 2015	10, 175–178
Insulin peglispro (LY2605541)	Eli Lilly	Insulin lispro with 20 kDa PEG	Basal	Programme terminated	9,71, 180–182, 188
Afrezza	Sanofi	Insulin powder in thumb-size inhalation devices	Inhalation	Approved in 2014	112–114
Dance-501	Dance Biopharm	Solution-based formulation in pocket-size device	Inhalation	Phase II	189
ORMD-0801	Oramed Pharmaceuticals	Formulation using POD technology	Oral	Phase II	116,173
NN1953	Novo Nordisk	Formulation using GIPET technology	Oral	Phase II	69
NN1957	Novo Nordisk	Formulation using GIPET technology	Oral	Phase I	69
U-strip	Transdermal Specialties	Insulin patch with ultrasonic trigger	Transdermal	Not disclosed	190,191
AB101	AntriaBio	Insulin encapsulated in PLGA microspheres	Ultralong	Preclinical	61
TransCon insulin	Ascendis	Chemically-immobilized insulin on PEG hydrogel	Ultralong	Phase II	62,63
HM12470 (^{LAPS}Insulin115)	Hanmi	Fc–insulin conjugate	Ultralong	Phase I	64,65
PE0139 (Insumera)	Phase Bio	Elastin-like polypeptide fusion	Ultralong	Phase II	67,68
NN1436 (LAI287)	Novo Nordisk	Not disclosed	Ultralong	Phase I	69
NN1438 (LAI338)	Novo Nordisk	Not disclosed	Ultralong	Phase I	69
IDegLira (Xultophy)	Novo Nordisk	Liraglutide and insulin degludec combination	Co-therapy with GLP1	• Approved in the EU in 2014 • FDA submission	13,69, 192
BioChaperone lispro	Adocia	Lispro formulation	Rapid acting	Phase III	78
BIOD-123	Biodel	Formulation using Linjeta technology	Rapid acting	Phase II	77
LixiLan	Sanofi	Lixisenatide and insulin glargine combination	Co-therapy with GLP1	FDA submission	193, 194
Ryzodeg	Novo Nordisk	Insulin degludec and insulin aspart	Basal and fast acting	• Approved in the EU in 2013 • Approved in the US in 2015	69, 195–197
Insulin-327	Novo Nordisk	Hepatoselective insulin	Experimental	NA	11
INS-A and INS-B	Novo Nordisk	Receptor-selective analogues	Experimental	NA	12
PBA-insulin	MIT	Glucose-responsive insulin analogue	Experimental	NA	123

Fc, crystallisable fragment; FDA, US Food and Drug Administration; GIPET, gastro-intestinal permeation enhancement technology; GLP1, glucagon-like peptide 1; MIT, Massachusetts Institute of Technology; NA, not applicable; PEG, polyethylene glycol; PLGA, poly(lactic-co-glycolic acid); POD, protein oral delivery.

task for the patient, particularly when coordinated with a separate glucose measurement. These three areas represent the forefront of current research seeking a breakthrough in the treatment of insulin-dependent diabetes.

Emerging directions in insulin therapy

Advances in insulin chemistry and formulation sciences, coupled to the developments in glucose monitoring, have provided sophisticated, highly cost-effective options for the management of insulin-dependent diabetes. Nonetheless, insulin remains a drug of narrow therapeutic index that only infrequently normalizes blood glucose in chronic use[8,58]. Current insulin therapies suffer from serious deficiencies owing to inconsistency of therapeutic action from dose to dose and from patient to patient[8,59]. Synthetic chemistry in its broadest scope provides the means to refine insulin for its use as a drug (see FIG. 2 and below). Optimization of insulin will be directed beyond pharmacokinetics to alteration of the pharmacodynamic properties of insulin, and the identification of insulin therapies that are more selective and less variable in their effect is a future priority. Insulin action that is responsive to changes in blood glucose

Incretin hormone
A gut-derived peptide hormone that stimulates insulin secretion after food consumption. Additional functions of incretin hormones include inhibition of glucagon secretion, restriction of gastric motility and appetite suppression. The two most prominent physiological hormones within this class are glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP1).

concentration remains a primary goal, which ideally will be coupled with additional pharmacology that addresses underlying molecular pathology inherent to the heterogeneity of human diabetes.

Refined basal insulin therapy. Existing basal insulin treatment is limited by a relatively short time–action profile and variability in glucose lowering, which ultimately result in suboptimal glucose management[45,59]. Current long-acting insulin analogues provide control of basal glucose over a period of 12–24 hours in most patients. Consequently, basal therapy is typically administered as a once- or twice-daily injection, which is a source of poor patient compliance. Therefore, an insulin analogue with a longer time–action profile that requires less-frequent injections could lead to improved treatment outcome[60]. Extension of therapeutic action is accomplished by adjusting the physicochemical properties of insulin to promote formation of subcutaneous depots and to diminish the rate of clearance. However, although these approaches produce the desired duration of action, they suffer from substantial inter- and intra-patient variability[59]. Developing a near-peakless basal insulin replacement therapy will probably require novel conceptual approaches.

Several strategies are currently being explored to achieve ultra-long insulin action. AntriaBio Inc. is advancing a formulation that utilizes polyethylene glycol (PEG)ylated insulin encapsulated in a poly-lactic, poly-glycolic microsphere (AB101)[61]. The modified insulin is enclosed within an injectable microgel to provide controlled release from a subcutaneous depot through hydrolysis of the supporting polymeric matrix. A sustained therapeutic concentration of insulin for periods lasting more than 1 week has been reported[61]. Meanwhile, Ascendis Pharma has developed a different depot formulation that utilizes a prodrug technology[62]. Insulin is immobilized within an injectable PEG hydrogel using a proprietary chemical linker (TransCon), which degrades in a controlled fashion under physiological conditions to provide insulin release for a sustained period. The feasibility of this approach has been successfully demonstrated with growth hormone, which Ascendis Pharma recently advanced to Phase II study, and the application of this technique to insulin was conducted in partnership with Sanofi[63]. Hanmi Pharmaceutical has reported early clinical assessment of ^{LAPS}Insulin115 (HM12470), which comprises insulin linked to a biosynthetically derived, aglycosylated crystallizable antibody fragment (Fc)-carrier protein. Initial results indicate a favourable pharmacokinetic profile that is consistent with once-a-week dosing and substantiate the reported demonstration of glucose control in db/db mice when administered weekly over a 5-week period[64,65]. In addition, ^{LAPS}Insulin115 is being developed as part of combination therapy with an analogue of glucagon-like peptide 1 (GLP1) — an incretin hormone that stimulates glucose-dependent insulin secretion — utilizing the same technology[64,65]. PhaseBio seems to have the most clinically advanced fusion protein directed at insulin delivery[66]; Phase IIa trials with PE0139 (Insumera), a biosynthetic fusion of insulin with an elastin-like polypeptide, are ongoing[67,68]. The preliminary results support extended circulation of PE0139 in individuals with T2D for a period of 7 days, during which it provided a reduction in levels of fasting glucose. Last, a long-acting lipidated insulin, LAI287 (also known as NN1436, Novo Nordisk), which promises prolonged action, entered Phase I study in early 2013, but at this time there is no additional information regarding its composition or therapeutic profile[69].

It is still too early to predict whether an insulin therapy with a sustained time–action profile will emerge from the current field of clinical candidates or, more importantly, whether improved therapeutic outcomes can be achieved with less-frequent dosing or daily microdosing. The different approaches aiming to achieve extended pharmacology have several elements in common. They all rely to a variable degree upon delayed absorption from a subcutaneous administration site, either through polymeric encapsulation, protein fusions or directed precipitation. Variability in drug pharmacokinetics attributed to pharmaceutical dynamics at the injection site is a major contributing factor to inconsistencies in therapeutic action[59]. How well each approach minimizes this traditional obstacle remains to be determined. Polymer-encapsulated analogues are claimed to have vastly improved control in drug release through the precision in controlled chemical hydrolysis[61–63]. Fusion proteins use enhanced biophysical properties to maintain solubility from the point of injection to appearance in the blood, thus eliminating the inherent variability in physical phase transfer[64–68]. The extended action of fusion proteins once in circulation is dependent on various mechanisms that protect them from clearance, as in the case of antibody fusion proteins that bind to neonatal Fc-receptors[64,65]. An additional inherent challenge to less-frequent administration is the need for an increased dose per injection (and the associated risk of premature action). The design in which insulin is most securely sequestered from inopportune action by chemical, physical or biological methods would constitute the preferred approach to extending the time–action profile. Finally, the supplemental ingredients used to achieve the targeted effect must be taken into consideration. TransCon and AntriaBio rely upon polymer-based formulations for their analogues and, although there is no report of polymer-mediated toxicity, the demonstration of safety following chronic use has not yet been confirmed. In this regard, the reported elevation in liver enzymes for certain patients using insulin peglispro is a worrisome observation[70,71]. Clinical studies of appreciable size and heterogeneity are required to discover and assess the potential risks in these related-but-differing long-action candidates.

Ultra-rapid insulin analogues. The improved performance of the first-generation rapid-acting insulin analogues relative to native insulin has encouraged further studies to achieve even faster action and greater precision. The primary clinical objective is an increase in the speed to onset of action with more-rapid termination

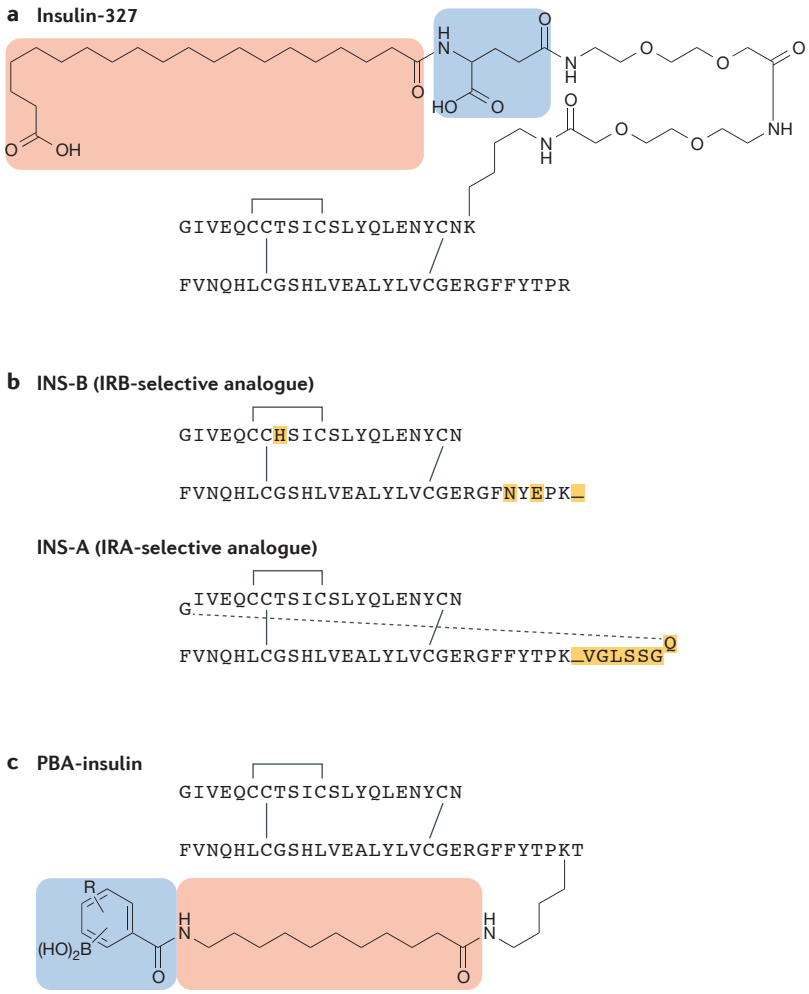

Figure 2 | **Experimental insulin analogues. a** | Structure of insulin-327, which was demonstrated to have preferential hepatic versus peripheral activity in dogs. This preference is attributed to the modification of insulin with a fatty diacid (highlighted in red), which slows adsorption and extends plasma circulation through non-covalent albumin binding[11]. The fatty acid part is separated from the core of the insulin molecule by two short polyethylene glycol (PEG) spacers and a glutamic acid spacer (highlighted in blue). **b** | Two analogues of insulin, INS-B and INS-A, with 2–4 fold higher affinity for their respective isoform of the insulin receptor (the B and A isoforms, IRB and IRA, respectively) compared to human insulin[12]. Differences in amino acid sequence as compared to human insulin are highlighted in yellow. **c** | A general structure of insulin analogues derivatized with phenyl boronic acid (PBA; blue) to display glucose-sensing behaviour *in vivo* and an aliphatic linker (red) to prolong the half-life of the insulin derivative in the circulation[123].

of action. This might enable injection simultaneous to a meal[72] as well as having applications in pump-infused delivery guided by online glucose monitoring[73]. Several novel monomeric analogues have been reported, notably AlaB16 insulin, AlaB22 insulin and (4-ClPhe)B24 insulin, which have demonstrated preclinical differences relative to first-generation rapid-acting insulin comparators[74–76]. When studied in pigs, the pharmacodynamic profile of these analogues indicated a twofold increase in the rate of glucose lowering for AlaB16 and AlaB22 insulin and reduced time to onset and termination of action for the chlorinated analogue compared to those of human insulin and insulin lispro. Simultaneously, a number of innovative formulations of conventional insulins are presently in clinical evaluation, including BIOD-123 (Linjeta, Biodel) and Adocia's biochaperone formulation of insulin lispro[77,78]. The primary measure of clinical performance remains the quality of mealtime glucose control.

Single chain insulin analogues. With the exception of proinsulin, all of the insulin analogues that have advanced to clinical practice so far have been two-chain peptides, which are typically equipotent to native insulin. However, this trend may change with the recent disclosure of single-chain insulin (SCI) analogues, which retain potency and can be prepared by rDNA or chemical methods[79–84]. Unlike conventional two-chain insulins, the production of SCI analogues eliminates the steps required to excise a connecting peptide, thereby decreasing cost and simplifying subsequent modifications. Furthermore, active insulin analogues enhanced with additional functional entities can be produced in a single step. Examples of functional entities include carrier proteins such as albumin, transferrin or Fc for extended *in vivo* circulation[80,85]. The use of tissue-targeting probes, inteins, for controlled release of insulin activity and glucose-sensing elements can also be envisioned, but these modifications have yet to be achieved using the SCI approach. Finally, SCI analogues have also been reported to possess superior thermal and chemical stability, which might constitute a sizable advantage for use in geographies that lack access to refrigeration[84,86].

Hepatoselective analogues. When insulin is secreted physiologically from the pancreas it is delivered through the portal vein directly to the liver. It is estimated that ~50% of this insulin is consumed hepatically, resulting in diminished insulin exposure of peripheral tissues and organs[87–89]. The enhanced insulin concentration at the liver ensures suppression of hepatic glucose production, which represents a major contributing factor to diabetic hyperglycaemia[11,90–92]. A perceived limitation of conventional insulin therapy originates in its subcutaneous administration, which results in high peripheral tissue concentration relative to hepatic exposure[93]. The interest in hepatoselective insulin analogues is driven by the belief that a more physiological balance between the hepatic and peripheral actions of insulin might enhance the safety of insulin treatment[94]. Preferential insulin tissue activity was initially demonstrated by Tomkins and co-workers, who noted that GlyA1,LysB29-diacetyl insulin acted predominantly by stimulating peripheral glucose uptake, whereas a related analogue cross-linked at the same residues exerted its hypoglycaemic effect largely through inhibition of hepatic glucose production[95]. Various degrees of hepatoselectivity have also been demonstrated in the case of proinsulin, thyroxyl–insulin conjugates, insulin detemir and insulin peglispro[85,96–99]. The tissue selectivity is believed to be a result of the increased molecular size of these analogues either through their design (proinsulin and insulin peglispro) or through binding to endogenous proteins (thyroxyl conjugates and insulin detemir). Hepatic endothelial

Single-chain insulin
(SCI). An insulin analogue or its precursor in which the two individual peptide chains (A and B) are covalently connected. The two chains can be linked by a connecting sequence (such as the proinsulin C-peptide), synthetic linker or fused directly through an amide bond.

blood flow is facilitated by larger vascular pore size, so particles of enhanced hydrodynamic radius can pass more freely, whereas in peripheral tissues their transport is impaired. A thyroxyl–insulin conjugate was the first targeted attempt at achieving hepatoselectivity, which was supported by euglycaemic clamp data from initial clinical studies[99]. More recently, a proinsulin–transferrin fusion protein was investigated for its preferential activity in diabetic mice, displaying a lack of peripheral activity in an immunoprecipitation assay[85]. Last, in dog portal infusion studies a novel analogue, insulin-327, was recently shown to invert the gradient typically associated with peripheral insulin administration[11] (FIG. 2a).

Receptor-isoform-selective analogues. The insulin receptor exists in two distinct but closely related isoforms (insulin receptor isoform A (IRA) and IRB), differentiated by an extracellular twelve-amino-acid carboxy-terminal sequence[100,101]. The isoforms differ in their relative expression in various tissues, and this differential expression is associated with differential biological functions[102–104]. IRB is purported to be responsible for mediating the metabolic activity of insulin and is abundant in organs involved in glucose metabolism, such as the liver, whereas IRA mediates the mitogenic activity of insulin and is predominantly expressed in the brain, spleen and transformed cells found in cancerous tissues[102–104]. The development of isoform-selective analogues could therefore serve to elucidate the importance of each isoform and potentially provide better glucose management. Novo Nordisk has recently disclosed insulin analogues with an enhanced affinity for IRB that have modifications at residues B25 and B27 and that exhibit 2–4-fold enhanced binding at IRB relative to IRA[12] (FIG. 2b). This IRB analogue, and a separate IRA-enhanced single-chain analogue, demonstrated *in vitro* and preliminary *in vivo* biological activity consistent with preferential receptor-isoform activity[105]. Another aspect of insulin receptor action that merits further investigation pertains to the biological role of hybrid receptors formed between IRA, IRB and insulin-like growth factor 1 receptor (IGF1R)[101,106]. Hybrid receptors result from heterodimeric associations that form owing to the close sequence similarity within the family of insulin receptors. Experimental evidence suggests that hybrid receptors could represent 60% of the total receptor expression level[101]. Characterization of the biological function of these hybrid receptors could be facilitated by the development of insulin analogues exhibiting differential ability to signal through or away from them. The enhanced structural diversity accessible through chemical synthesis may provide the structural variation needed to achieve higher receptor isoform selectivity.

Oral and pulmonary delivery. Macromolecular medicines typically require parenteral delivery, most commonly by subcutaneous and intravenous routes. The prospect of administering insulin by a non-injectable route has been the subject of considerable research since the discovery of this hormone. However, despite a range of approaches, including dermal, rectal, nasal, buccal, oral and pulmonary routes of administration, success has been limited[107,108]. The withdrawal of the inhalable insulin product Exubera (Pfizer) owing to poor sales was a notable event for the industry; however, alternatives to injection, particularly oral and pulmonary administration, continue to attract commercial interest[23,24,109–111].

The most advanced non-injectable approach is the recently approved Afrezza (Sanofi) — an inhalable prandial insulin based on Technosphere particle technology[112]. The Technosphere particles are composed of insulin formulated with fumaryl diketopiperazine powder and are administered through a thumb-sized inhaler[113]. A 52-week multicentre Phase III trial of poorly controlled T2D compared mealtime administration of Technosphere insulin plus insulin glargine at bedtime to insulin aspart administered twice daily[114]. The results demonstrated similar mean plasma glucose concentration (HbA_{1c}) values, but the patients treated with Technosphere gained significantly less weight and reported fewer hypoglycaemic episodes. More-frequent coughing and some change in pulmonary function were noted in the inhaled insulin cohort[87]. The commercial registration of Afrezza was associated with the requirement for longer-term clinical assessment to determine the risk of lung cancer and changes in pulmonary function. Sanofi has recently terminated its partnership with MannKind as a result of the poor commercial reception of Afrezza[115].

In addition to convenience and improved compliance, the appeal of oral delivery is based on the belief that this route of administration will mimic physiological secretion to the portal vein and more effectively suppress hepatic glucose production, minimizing hypoglycaemic risk. Two early stage oral insulin delivery development projects have gained visibility, but insufficient information exists to assess the likelihood of their ultimate success. Oramed Pharmaceuticals reported a pilot study involving eight patients with T1D who self-administered an oral insulin capsule ORMD-0801 (containing 8 mg of human insulin) three times daily for a 10-day period, in addition to their regular insulin treatment[116]. Continuous glucose monitoring indicated an average reduction of 16% relative to the pretreatment period[116]. Separately, Novo Nordisk has recently entered into Phase II trials[117] of an oral, long-acting insulin, NN1953, which is purportedly based upon proprietary gastro-intestinal permeation enhancement technology (GIPET) licensed from Merrion Pharmaceuticals[118].

Glucose-responsive systems. The development of an insulin therapy capable of mimicking pancreatic function under varying glycaemic conditions to maintain optimal glucose control has proved to be a nearly impossible task. However, recent progress in this area is summarized in several reviews[15,119,120]. Most approaches involve a blood glucose detection system coupled to a release mechanism, which liberates insulin when triggered by increased glucose levels. The majority of these systems can be categorized by their method of glucose detection or insulin release (FIG. 3). Presently, glucose sensing

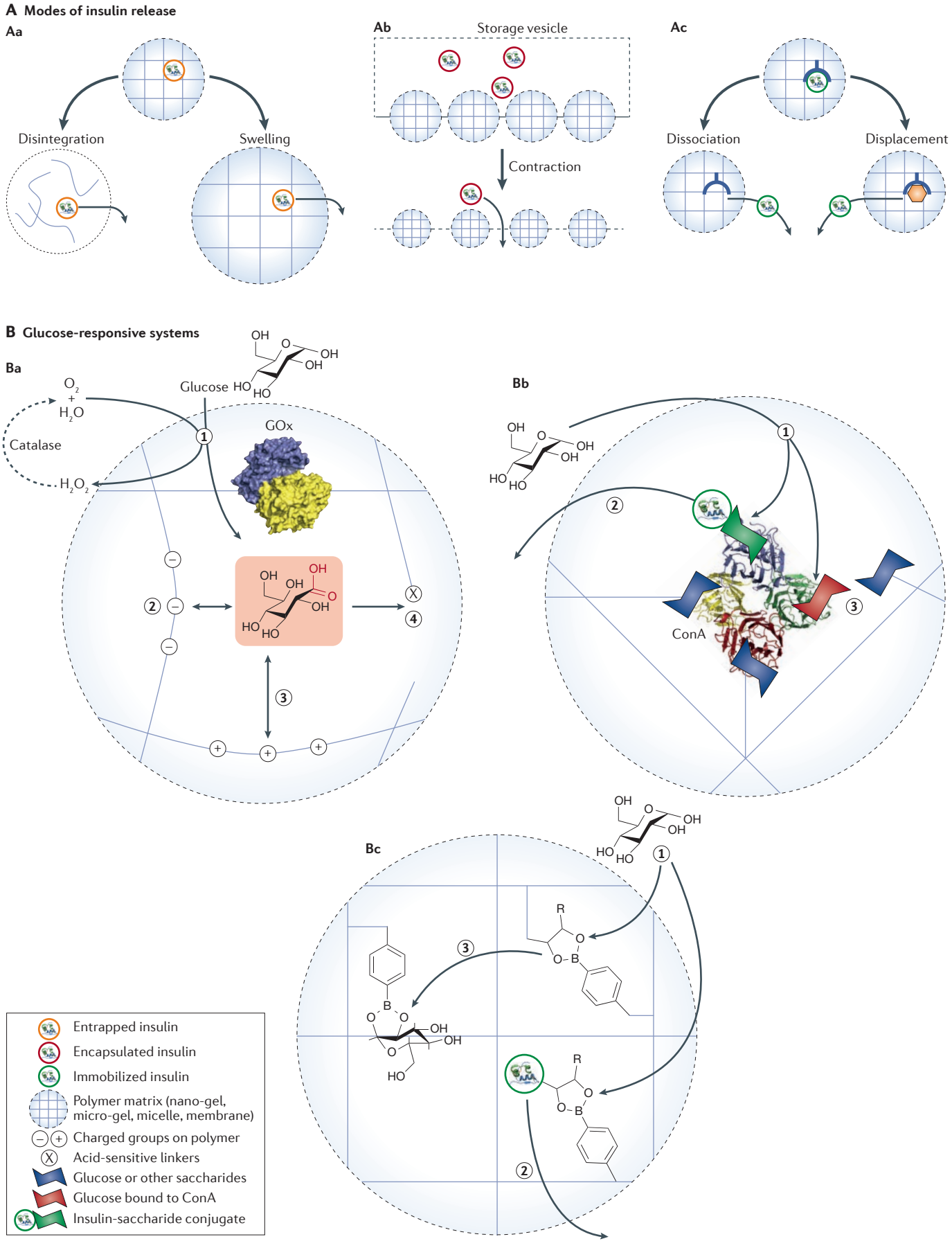

Figure 3 | **Design elements of glucose responsive systems. A** | Physical and chemical transitions that were used to enable release of insulin. **Aa** | Insulin is entrapped within a polymer matrix and liberated upon polymer disintegration or swelling. **Ab** | Insulin is contained inside a storage vesicle and its release is regulated by a membrane, the pore size of which is modulated through swelling and contraction. **Ac** | Insulin is chemically or biochemically immobilized on a solid support. Equilibrium dissociation, decomposition of a linker or competitive displacement by another ligand can serve as mechanisms for insulin release. **B** | Mechanisms of glucose detection and insulin release. **Ba** | This system is based on enzymatic conversion of glucose to gluconic acid (highlighted in red) by glucose oxidase (GOx) (step **1**). GOx is often coupled with a secondary enzyme, catalase, which recycles the by-product peroxide back into oxygen (required for GOx activity). The drop in pH caused by gluconic acid acts as a trigger for a number of possible steps: contraction of a polymer with acidic functional groups (step **2**), swelling of a basic polymer (step **3**) or acid-mediated degradation of a polymer support (step **4**). **Bb** | Glucose detection is derived from its affinity to concanavalin A (ConA) (step **1**). Sugar-modified insulin is immobilized on ConA and released upon displacement by glucose (step **2**). The tetrameric structure of ConA, with four sugar-binding sites, serves as a crosslinker to a polymer functionalized by sugar molecules (blue). Competition of free glucose for ConA will cause degradation of the polymer (step **3**). **Bc** | A mechanism based on the interaction between glucose and phenyl boronic acid (PBA) (step **1**). As with ConA, PBA can be used to anchor insulin labelled with a sugar (or diol) molecule, which will dissociate in the presence of glucose (step **2**), or can provide structural integrity to a polymer matrix (step **3**).

is accomplished by one of three methods: a system based on the enzymatic conversion of glucose to gluconic acid by glucose oxidase (GOx), a system based on glucose detection derived from its affinity to concanavalin A (ConA), or a system based on the interaction between glucose and phenylboronic acid (PBA)[119]. Insulin storage and its glucose-dependent release typically rely on some form of entrapment within polymeric matrices or micelles capable of chemical or conformational change upon interaction with elevated glucose levels[120]. The chemical nature of the polymeric support, the size of the individual particles and their formulated composition are the predominant variations between reported methods. Of particular note is the so-called SmartCells technology, which represents the first commercial attempt at using a glucose-sensitive approach to insulin therapy and is currently in clinical assessment at Merck Research Laboratories[121,122]. Surprisingly, there are only a few examples of single-molecule systems that are based on the direct modification of insulin with a glucose-sensitive functionality[123,124]. From a synthetic standpoint, the smaller molecular size of PBA when compared to macromolecular proteins (GOx and ConA) enhances the prospect for integrating glucose-sensing and insulin bioactivity within a single peptide. One such example of a boronate- and carbohydrate-derivatized insulin is reported to form supramolecular aggregates that are susceptible to dissociation in the presence of glucose[124]. The most recent report demonstrated that PBA-modified insulin is capable of blood glucose control in a streptozotocin (STZ)-induced mouse model of diabetes[123] (FIG. 2c). The structure of this PBA-modified insulin analogue is closely related to those previously developed by Novo Nordisk[125,126]. This single-molecule design possesses considerable promise, but further advancement requires independent validation of the approach.

Co-therapy with incretin hormones. There is accumulating evidence of benefits associated with the simultaneous treatment of insulin and incretin-based hormones such as GLP1 (REFS 127–130). GLP1 stimulates glucose-dependent insulin secretion to supplement basal insulin therapy and minimize hyperglycaemic surges[131]. Suppression of glucagon release by GLP1 further enhances glucose lowering, and its ability to suppress appetite lowers body weight[131]. Clinical studies demonstrate statistically significant improvement in glucose management in patients on basal insulin upon addition of GLP1 treatment[128,129,132,133]. In the Phase IIIa DUAL-I extension trial, treatment with IDegLira (a combination of insulin degludec and the GLP1 analogue liraglutide) enabled the use of a reduced insulin dose, leading to less weight gain and a reduced frequency of hypoglycaemia[13,134] (TABLE 2). The most common adverse effects of GLP1 therapy are gastrointestinal, including flatulence, nausea and in the most extreme cases vomiting. Nonetheless, these late-phase clinical results are of utmost importance in setting future directions in the management of diabetes associated with excess body weight. These results demonstrate the ability to achieve enhanced metabolic outcomes with reduced body weight and hypoglycaemia through decreasing the use of insulin.

The second of the two physiological incretin hormones is glucose-dependent insulinotropic polypeptide (GIP, also known as gastric inhibitory polypeptide), which is also capable of glucose-dependent insulin release but with no apparent effect on gastrointestinal motility or appetite[131]. The development of GIP for therapeutic purposes has trailed that of GLP1 largely for two reasons: its purportedly diminished efficacy in patients with diabetes and the initial preclinical findings suggestive of weight gain[135,136]. More-recent reports have challenged these results, with the most recent pharmacological studies reporting modest body weight reduction in obese mice treated with GIP, which is enhanced when GIP is administered with GLP1 (REFS 137–140). Therefore, theoretically, GIP could supplement insulin and GLP1 treatment to achieve a more balanced action and to reduce GLP1-associated side effects. A unique difference in GLP1 and GIP pharmacology is the proven ability of GIP to enhance glucagon secretion in clinical hypoglycaemia, which could be of great value in combination with insulin[131].

It seems that incretin supplementation is destined to be an important addition to conventional insulin therapy. Whether the pharmacological functions of GIP and GLP1 are comparable to their integrated roles in physiology (providing enhanced efficacy and safety) remains uncertain but worthy of study. Dual GIP and GLP1 co-agonists have delivered improved metabolic effects in lowering blood glucose and body weight relative to comparable GLP1-selective agonists, when studied in appropriate preclinical models of diabetes and obesity[140]. The identification of additional pharmacology that might reduce the need for insulin could lead to further reductions in body weight and hypoglycaemia relative to therapy with insulin and GLP1 combinations[134].

Table 2 | **Recent data from the Phase IIIa DUAL I extension trials of IDegLira***

Treatment	Number of patients	Daily dose	Final HbA_{1C} (difference)	Mean change in body weight (lbs)	Hypoglycaemic events (per patient, per year)
Insulin degludec	414	62 units	6.9% (–1.4%)	+ 5.1	2.6
Liraglutide	415	1.8 mg	7.1% (–1.2%)	–6.6	0.2
IDegLira	834	39 units	6.4% (–1.8%)	–0.9	1.8

*A group of 1,663 patients with insulin-naive type 2 diabetes (aged 55 ± 10 years; HbA_{1C} 8.3 ± 0.9; BMI 31.2 ± 4.8) were administered IDegLira (1 unit of insulin degludec and 0.036 mg of glucagon-like peptide 1 (GLP1) analogue liraglutide) over 52 weeks[13,134]. HbA_{1C}, mean plasma glucose concentration.

Novel chemically synthesized analogues. For chemical synthesis to be successfully used in the drug discovery process, it must be able to outperform alternative methods, specifically rDNA protein expression[141]. One clear benefit of chemical synthesis is the ability to access chemical diversity that is impossible to obtain by biosynthesis, such as analogues incorporating non-native amino acids and pharmacophores[142]. Another underappreciated aspect inherent to chemical preparation is the substantially increased speed of production of the target peptides, as the intermediate biosynthetic step of gene synthesis is eliminated. Novel synthetic insulin analogues can be obtained and biochemically characterized in less than a week in amounts that can immediately support *in vivo* studies[143–145]. Although rDNA-based biosynthesis remains a more cost-effective method for large-scale manufacture, it only pertains to the drug substance, which is a fraction of the cost in a formulated commercial drug product. Any increased cost in a chemically prepared insulin analogue would need to be justified by improved pharmacology. Semisynthesis utilizing rDNA-derived intermediates that are subsequently modified by chemical optimization has been validated in the commercial synthesis of the lipidated, basal insulin analogues and establishes the precedent for mixed-mode industrial synthesis[146].

Interest in the synthesis of insulin and insulin analogues by chemical methods has recently increased owing to improvements in reagents, resins and methodology. Two methodologies, using either the single-chain insulin (SCI) approach or the two-chain combination scheme, were recently validated as effective methods of insulin synthesis, with overall yields of the final peptide approaching 25%[144,145,147–149] (FIG. 4). Such a dramatic increase in production quality coupled with the speed of chemical synthesis makes these methods valuable tools for investigation of the structure–activity relationships of insulin and the identification of new therapeutic analogues.

Challenges and future directions

Insulin has been a cornerstone of diabetic care for nearly a century. The therapeutic modality has evolved but not fundamentally changed since its inception, with therapy continuing to be injectable and focused on controlling hyperglycaemia. As long as insulin therapy remains to be a preferred method of glucose management, the risk of life-threatening hypoglycaemia driven by its narrow therapeutic index will remain. Incretin-based therapies, such as GLP1 mimetics, have the potential of lessening the intensity of insulin treatment, thus improving its safety, with additional beneficial effects on body weight[13]. Alternatives to insulin — such as antibodies, non-insulin peptides, aptamers and small-molecule insulin receptor agonists — have also appeared in the literature, although these approaches are in the early stages of development and will require additional validation[150–156]. The chronic concerns of diabetes continue to be the minimization of microcardiovascular disease (which is closely linked to hyperglycaemia) and macrocardiovascular disease (largely accelerated by excessive body weight, blood pressure and lipid abnormalities)[1].

In the past four decades, insulin therapy has advanced to a point of unparalleled supply of high-purity insulins with refined pharmacokinetics that are administered using state-of-the-art injection devices. Although there remains room for further improvement in these areas, it is expected that continued investments are likely to be driven by consumer appeal. The forefront for medicinal advances resides in enhanced therapeutic efficacy and safety as assessed by intermediate measures in glucose, lipid and body weight management and ultimately by measures of disease mortality. In this respect, a current priority is glucose control in a timely, closed-loop fashion, closely simulating pancreatic function[132]. Improvements in glucose sensing and miniaturization of biomechanical devices are steps towards highly convenient and potentially implantable insulin pumps, striving for closed-loop performance[15,157–159]. A biological route to this same goal relies on stem cell and tissue-engineering technologies[160–162]. Although still in early development, the restoration of β-cells is already being pursued by at least two companies — ViaCyte and Semma Therapeutics. Such a cell-based therapy would be an unprecedented achievement and would move us closer to the ultimate solution to insulin-dependent T1D, which would also require complementary suppression of β-cell immune-based destruction. A glucose-responsive therapy can also conceivably be achieved through an insulin analogue that is engineered to include a glucose-sensitive element within its structure or, alternatively, engineered as part of a multicomponent formulation[119,120,123] (FIG. 3).

In many ways, maturity-onset diabetes represents a more complex disease than insulin-dependent, juvenile-onset diabetes. Maturity-onset diabetes occurs over a broader age range than that of juvenile-onset diabetes, and its presentation is associated with multiple

β-cells
Functional endocrine cells that are located in pancreatic islets and are responsible for biosynthesis, storage and secretion of insulin under glucose control.

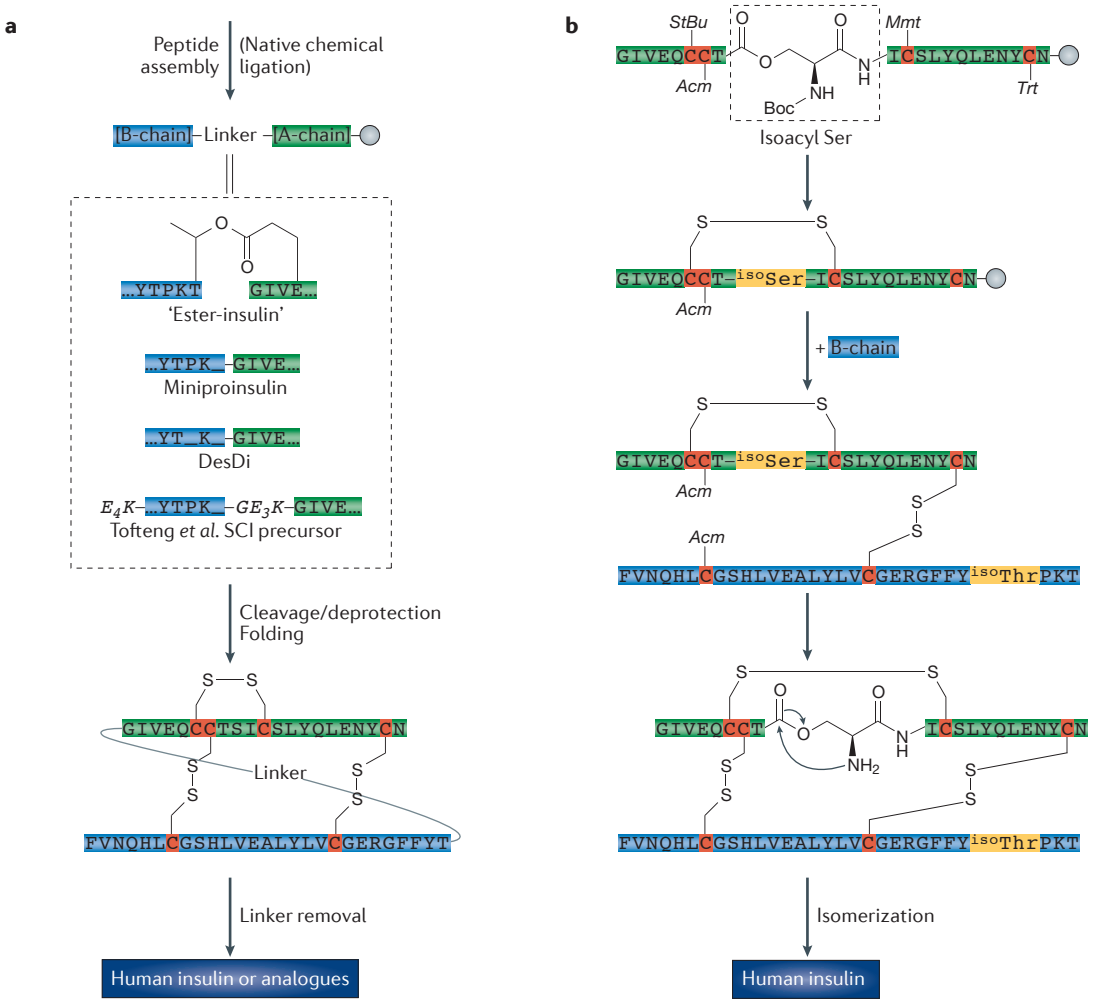

Figure 4 | **Chemical synthesis of insulin and related analogues.** The newer chemical synthesis methods draw on two validated precedents: the single-chain insulin (SCI) approach (panel **a**), first utilized in biosynthesis, and the historical two-chain combination scheme augmented by directed disulfide bond formation (panel **b**). **a** | SCI precursors are first prepared as linear peptides using solid-phase peptide synthesis (SPPS). This is accomplished either through a direct full-length peptide assembly or through intermediate fragments conjoined by native chemical ligation. The linear precursor is then folded under oxidative conditions to produce the three intramolecular disulfide bonds. Last, the folded peptide is processed to a two-chain form by enzymatic or chemical means. The provided examples allow synthesis of insulin or its analogues in a 6–25% yield calculated from the loading of resin used for peptide assembly (miniproinsulin 7–12%[145], DesDi 15–20%[145], ester-insulin 15%[147,148] and GE_3K-linked precursor 6%[149]). **b** | Chemical synthesis of insulin using a chain-combination method was not suitable for drug discovery until rather recently, which is mainly owing to very low synthetic yields (below 2%) and an inability to tolerate most mutations. This new method has allowed synthesis of human insulin in a 24% yield, which is attributable to improvements in the quality of peptide assembly and commercial availability of isoacyl-dipeptide fragments (which enhance both SPPS yields and solubility of individual fragments)[144]. The three disulfide bonds were formed sequentially using orthogonal protecting groups at Cys residues (highlighted in red).

Gastric bypass surgery
A bariatric procedure that surgically reduces the size of the stomach. This restricts the quantity of absorbed nutrients and alters local hormone production and action to collectively achieve body weight reduction.

other abnormalities, most notably obesity. Obesity is by far the predominant contributor to deterioration of the diabetic condition, and development of an effective weight loss therapy constitutes a priority for diabetes care. Bariatric surgeries (such as gastric bypass surgery) have demonstrated unparalleled weight loss and restoration of insulin sensitivity that results in full or partial reversal of diabetes[163–165]. These surgeries set a benchmark for future therapeutics as surgical procedures are expensive, often irreversible and fraught with complications. Advances in pharmacology have provided a set of first-generation anti-obesity drugs of modest efficacy[166]. The integration of incretin and insulin biology has encouraged the search for other agents that might adjunctively function to lessen the need for insulin through further reductions in body weight. In this regard, leptin, fibroblast growth factor 21 (FGF21), melanocortin receptor 4 (MC4R) agonists and peptide YY (PYY) are four agents among many that are receiving considerable attention[167–170].

An additional priority in insulin therapy applies to approaches that enhance patient compliance, leading to improved therapeutic outcomes. Subcutaneous injections are invasive, but insulin administration has been improved owing to progress in insulin pen and needle design[171]. In addition, the potential of inhalable insulin is currently being assessed through a commercial product, and subsequent alternatives are advancing to registration[112]. Oral administration represents a preferable route of insulin delivery, but this system has not yet proven to be successful[172]. The current development of oral insulin is focused on optimizing formulations, which protects them from degradation and facilitates their absorption[69,116,172,173]. The relatively low bioavailability of insulin and its high variability in absorption constitute the two fundamental challenges to achieving glycaemic control with a drug of such low therapeutic index. Effective oral administration may be limited to conditions of fasting, when food is not compromising performance, which could restrict this approach to longer-acting insulin therapy. If so, conventional approaches that rely on a subcutaneous depot to extend duration of action are not likely to be viable. A potential perceived virtue of oral delivery is the expectation of enhanced first-pass metabolism that leads to increased hepato-selectivity, but this may not be fully compatible with approaches to extend duration of action. However, given the magnitude of disease and the heterogeneity in insulin use, it is likely that no single delivery method will meet all patient needs.

Conclusions

Insulin has played a central part in our understanding of metabolic disease and in the advancement of biochemistry, peptide chemistry and structural biology. The global epidemic of adult-onset diabetes has increased the demand for insulin to unprecedented levels. Despite the maturation of insulin therapy, management of glucose and body weight is far from ideal, and use of insulin is associated with the persistent risk of developing life-threatening hypoglycaemia. Advances in synthetic chemistry and rDNA biosynthesis have enabled optimization of the insulin molecule for enhanced efficacy and safety[6,16]. Biosynthesis has provided insulin in a virtually unlimited quantity and high purity for global distribution and has also provided the core technology for the development of the first-generation insulin analogues, such as insulin lispro[4,36]. Subsequent insulin analogues have delivered enhanced basal glucose control and secured a sustained interest in further optimization of the hormone and associated therapies[45].

The objective for diabetes remains the elimination of insulin therapy, and for this to happen, further advances in diagnostics and preventive therapy are needed. Glucose-sensitive cellular delivery of insulin is highly desired but still in its infancy regarding production and implementation. In the meantime, the development of insulin that is responsive to glucose, or at least that is much less variable in its action within and across broad patient subgroups, is crucial. Supplementation of insulin therapy with incretin-based drugs has already demonstrated its value, and further expansion of this pharmacological toolset will probably lead to new and improved treatments. Of utmost importance is cardiovascular health, as it remains the primary cause of premature death in diabetes[174]. Therefore, novel therapeutic supplements and alternatives ideally should minimize the heightened risk inherent to the disease. In this regard, tissue- and receptor-selective insulin analogues are of great interest given their potential to restore metabolic homeostasis without excessive insulin action at undesired sites, which might promote vascular disease or even, conceivably, oncogenesis. Last, the therapy must be cost accessible, as the majority of individuals with diabetes reside in countries where the expense of the therapy constitutes a barrier to improved health.

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Competing interests statement
The authors declare competing interests: see Web version for details.

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