

Molecular Aspects of Insulin Aggregation and Various Therapeutic Interventions

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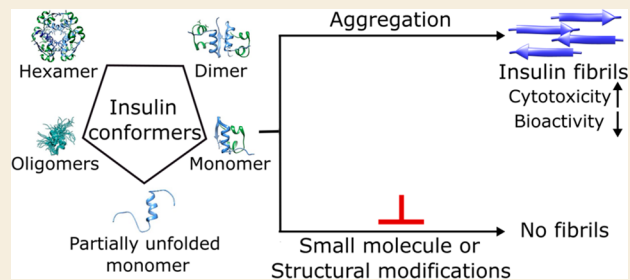
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ABSTRACT: Protein aggregation leading to the formation of amyloid fibrils has various adverse effects on human health ranging from fatigue and numbness to organ failure and death in extreme cases. Insulin, a peptide hormone commonly used to treat diabetes, undergoes aggregation at the site of repeated injections in diabetic patients as well as during its industrial production and transport. The reduced bioavailability of insulin due to aggregation hinders the proper control of glucose levels in diabetic patients. Thus, it is necessary to develop rational approaches for inhibiting insulin aggregation, which in turn requires a detailed understanding of the mechanism of fibrillation. Given the relative simplicity of insulin and ease of access, insulin has also served as a model system for studying amyloids. Approaches to inhibit insulin aggregation have included the use of natural molecules, synthetic peptides or small molecules, and bacterial chaperone machinery. This review focuses on insulin aggregation with an emphasis on its mechanism, the structural features of insulin fibrils, and the reported inhibitors that act at different stages in the aggregation pathway. We discuss molecules that can serve as leads for improved inhibitors for use in commercial insulin formulations. We also discuss the aggregation propensity of fast- and slow-acting insulin biosimilars, commonly administered to diabetic patients. The development of better insulin aggregation inhibitors and insights into their mechanism of action will not only aid diabetic therapies, but also enhance our knowledge of protein amyloidosis.

KEYWORDS: aggregation, insulin, amyloid, inhibitors, insulin analogs



1. INTRODUCTION

1.1. Protein Aggregation and Disease

Proteins are involved in a host of cellular functions like transport across membranes, catalyzing chemical reactions, organizing DNA, etc.¹ In order to be functional, proteins need to fold into their native structures. Disruption of protein folding due to cellular stress leads to the disruption of biological function. The loss of native protein structure may lead to misfolding or unfolding, and in several cases to the formation of fibrillar structures. The structures, termed amyloids, are proteinaceous deposits in which multiple copies of aberrantly folded protein molecules stick together to form elongated fibrils.² These long, straight, unbranched fibers have a characteristic cross- β structure, with β -strands arranged perpendicular to the fibrillar axis.^{3–5} The fibrillar structures are thermodynamically very stable and are resistant to proteolytic degradation. Interestingly, although the proteins that form fibrils in various diseases are completely unrelated in structure and function, the final fibril structure is remarkably similar. The exact mechanism by which the diverse proteins aggregate to give rise to a common structure is still not clearly understood and is an active area of research.²

The formation of amyloid fibrils has been linked to several neurodegenerative diseases like Alzheimer's disease, Parkinson's

Disease, Creutzfeldt–Jakob disease, and motor neuron diseases. They also include polyglutamine disorders like Huntington's disease and familial amyloid polyneuropathy (FAP).^{6–8} Among these, Alzheimer's and Parkinson's disease, which most commonly affect the aging population,⁴ are linked to the aggregation of $A\beta$ and α -synuclein, respectively. The aggregation of another protein, islet amyloid polypeptide (IAPP), has been linked to type-II diabetes. The hormone insulin, commonly used for the treatment of diabetes, forms amyloids at the site of repeated injections in diabetic patients.^{9,10} Insulin also aggregates during its industrial production, purification, and transportation. Notably, the aggregation of insulin has only been observed with externally administered forms of insulin, and not when it is normally produced in the human body. However, the serum samples from Parkinson's patients show an autoimmune response to insulin oligomers and fibrils, suggesting the

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possibility of their presence in Parkinson's disease.¹¹ These observations have prompted an interest in studying insulin fibrillation and to find ways to mitigate the effects. As insulin is a simple, highly studied, and readily accessible protein, it has also served as a model system to study amyloidosis. In this review, we will focus primarily on inhibition of insulin aggregation using organic molecules, with a brief reference to alternative strategies related to development of stable and/or orally bioavailable insulin analogs resistant to aggregation.

1.2. Insulin and Its Aggregation

Insulin, a peptide hormone, is secreted by the pancreatic β -cells of the islets of Langerhans.⁹ Insulin is a highly conserved protein composed of two chains A and B, of 21 and 30 amino acids respectively,¹² linked together by three disulfide bridges. Insulin can exist as a hexamer, tetramer, or dimer depending on the solution conditions.^{13,12} The functional form of insulin is a monomer, which via binding to its receptor regulates important cellular processes. For example, insulin helps in carbohydrate metabolism by facilitating glucose diffusion into fat and muscle cells by modulating translocation of the glucose transporter GLUT 4.^{14,15} It plays a key role in the synthesis and storage of fatty acid in adipose tissue, liver, and lactating mammary glands. Insulin is also involved in phospholipid metabolism¹² and the urea cycle.¹⁵ Further, it promotes cell proliferation¹⁶ in connective and musculoskeletal tissues by binding to the insulin growth factor receptor (IGF). Thus, insulin plays an invaluable role in various biological processes.

One of the key functions of insulin is the regulation of blood glucose level and prevention of hyperglycemia. However, in diabetic patients, the body either does not make enough insulin or cannot effectively use the insulin produced, which leads to impaired blood glucose levels. Such patients depend on insulin replacement therapy through subcutaneous insulin injections or insulin infusion pumps. However, numerous cases in diabetic patients highlight the formation of insulin fibrils at the site of repeated injections.^{10,17–20} In 1988, Dischhe et al. described amyloid deposits in a young man who was under porcine insulin administration twice daily.¹⁸ Repeated injections caused an increase in insulin dosage from 36 units to 176 units per day along with the development of a subcutaneous mass. The mass, sometimes called an “insulin ball”, consisted of amyloid deposits of insulin as determined from biophysical and antibody-based assessments. Similar observations were also reported for recombinant human insulin based on characteristic apple green birefringence from Congo Red,²¹ and studies with Thioflavin T (ThT), a well-known amyloid marker.²² Two recent reports also highlighted progressive insulin-derived amyloidosis in patients with Type-II diabetes.^{19,23}

Insulin-derived amyloidosis impairs blood glucose levels and results in increased insulin dose requirements.²⁴ Poor control of blood glucose might lead to several complications,²⁵ including diabetic retinopathy, hypoglycemic coma, cerebral infarction, and diabetic gangrene.²⁶ Subcutaneous amyloidosis is often treated by surgical removal of the affected area, which in turn may lead to postoperative complications like wound infections or delayed wound healing, especially when there is poor control of blood glucose.²⁷

The aggregation of insulin also poses a problem in commercial settings, where insulin and its analogs are produced in large quantities to cater to diabetics.¹⁴ Factors like acidic pH,²⁸ agitation,^{29,30} elevated temperature,³¹ contact with hydrophobic surfaces,³² or variation in ionic strength aggravate insulin

aggregation.³³ This reduces the bioavailability of insulin and results in inadequate functional activity. Thus, it is important to develop strategies to prevent insulin aggregation. Although insulin therapy was first introduced about 100 years ago, generating small molecules that can specifically and effectively inhibit insulin aggregation remains a challenge.

1.3. Interaction of Insulin with Metal Ions

The interaction of insulin with various components of the cellular milieu has as important effect on its activity. At the site of production, i.e., in the pancreas, insulin is coordinated with Zn^{2+} ions. *In vitro* studies have shown that Zn^{2+} and other metal ions like Co^{2+} and Cd^{2+} facilitate the formation of insulin crystals. Hudecek et al. found that Zn^{2+} , Co^{2+} , and Cd^{2+} cause a red shift in tyrosyl bands in insulin absorbance spectra while Ca^{2+} and Mg^{2+} were not found to exhibit any such effect.³⁴ This was attributed to conformational changes in the insulin hexamers due to the presence of the interacting metal ions near the tyrosine residues.

Coffman and Dunn synthesized two insulin hexamers, with or without Co^{3+} ions bound to HisB10.³⁵ They found that only one Co^{3+} ion at the HisB10 site was sufficient to stabilize the hexameric form of insulin. Apart from HisB10, metal ions like Zn^{2+} , Cd^{2+} , and Pb^{2+} also bind to another amino acid residue, GluB13.³⁵ While Zn^{2+} can bind to both B10 and B13 residues, coordination to HisB10 provides additional thermodynamic stabilization to insulin. Xu et al. found that excess zinc drives insulin to the hexameric form, while it is dimeric under zinc free conditions at low ionic strength and a pH close to its pI.³⁶ Other metal ions like Co^{2+} bind to HisB10, while Ca^{2+} binds preferentially to GluB13. Ca^{2+} acts similarly to Zn^{2+} and stabilizes the insulin hexamer. The knowledge of such interactions is valuable, since the industrial preparation(s) of insulin may be contaminated with metal ions during purification and packaging, and may adversely affect insulin aggregation.³⁷

1.4. Binding of Insulin to Its Receptor—Molecular Insights

Insulin exerts its biological activity via binding to its receptor on the cell surface. Mutagenesis and cross-linking studies have identified a group of residues which play an important role in this interaction.³⁸ Studies involving insulin–receptor interactions often use the insulin microreceptor (μ IR),³⁹ which comprises receptor domains L1 (the first leucine rich domain) and CR (a cysteine rich region) combined with a peptide spanning the C-terminal region of receptor α -chain (α CT), and contains the primary binding site of insulin. The interacting residues of insulin include GlyA1, IleA2, ValA3, GluA4, GluA17, TyrA19, and CysA20 on chain A and ValB12, GluB13, TyrB16, ArgB22, PheB24, PheB25, and TyrB26 on chain B of insulin. It is thought that these residues interact with the microreceptor μ IR, by inserting themselves between the α -CT subunit and the L1 β -sheet of the receptor through van der Waals interactions. The PheB25 residue of insulin inserts into a shallow pocket located in the α -CT subunit between Pro718 and Val715 of the insulin receptor and assists in binding. Insulin PheB24 projects itself into a hydrophobic pocket³⁹ where it interacts with Phe714 of the μ IR as well as with insulin ValB12, LeuB15, and TyrB26. Kristensel et al. determined that replacing the TyrA19 residue of insulin with Leu or Phe decreased binding by 1000-fold and 4–5-fold, respectively,⁴⁰ indicating the importance of this aromatic ring in receptor binding. Pullen et al. also reported that TyrB16, GlyB23, and GlnA5 of insulin play important roles in the insulin–receptor interaction, though the exact mode is yet to be deciphered.⁴¹

2. INSULIN AGGREGATION

2.1. Insulin Aggregation Pathway

Insulin generally exists as a hexamer, comprising three dimers held together by two zinc ions,^{42,43} which dissociates to the biologically active monomer for receptor binding and glucose uptake. Under conditions prone to fibrillation, e.g., change in pH, elevated temperature, and contact with hydrophobic surfaces, insulin may undergo partial unfolding (Figure 1). These partially unfolded intermediates stick together to form a nucleus, which elongates further to form the fibrillar amyloid structure of insulin.

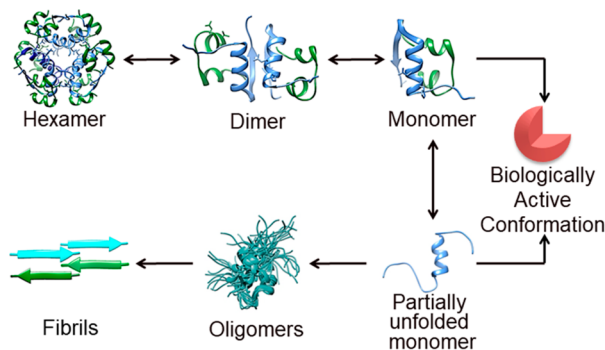


Figure 1. Proposed pathway of insulin aggregation. (Adapted with permission from ref 127. Copyright 1969, Elsevier).

The residues of the B-chain including GlyB8, SerB9, GluB13, ValB12, TyrB16, GlyB23, PheB24, PheB25, TyrB26, and ThrB27 play an important role in insulin self-assembly.⁴⁴ The resulting dimers interact to propagate nucleus formation. The most important residues for driving the formation of insulin nucleus include PheB1, ValB2, GluB13, GlnB4, AlaB14, LeuB17, ValB18, CysB19, and GlyB20 from the insulin B-chain and LeuA13, TyrA14, and GluA17 from the insulin A-chain. Hydrogen bonding and hydrophobic interactions are the major driving forces for nucleus formation and insulin

aggregation. It is thought that three to four insulin monomers comprise the building blocks of a nucleus.⁴⁵ However, it is unclear whether the growth of the mature fibril proceeds through the addition of monomeric or oligomeric species to the growing fibril.⁴⁶ It is important to note that several of the residues, e.g., ValB12, LeuB15, PheB24, and TyrB26, are also important for interaction of insulin with its receptor. Thus, designing a small molecule that can disrupt insulin self-assembly without affecting receptor binding remains a difficult task.

2.2. Structure of Insulin Oligomers and Fibrils

Various microscopy, X-ray, and NMR techniques have been employed to investigate the structural features of insulin and other amyloids.^{47,48} The cryo-electron microscopy structure of insulin amyloid protofilaments by Jimenez et al. revealed that the protofilaments arranged themselves into pairs or wound around one another with a long-range twist around the constituent β -strand (Figure 2A).³ The twists around the protofilaments were proposed to be left-handed, and non-interacting regions on the protofilament surface were free to interact with other monomeric proteins without disrupting the packing. Moreover, the inter- and intrachain disulfide bonds played an important role in fibril packing and were retained in the amyloid form. The interchain disulfide bonds made the strands run in a parallel direction, while the intrachain disulfide bond caused a bulge in one of the sheets.⁴⁹ Other proteins like lysozyme and A β (1–42) also form similar fibrils where 2–6 protofilaments twist around one another to form a mature fibril.²

Vestergaard et al. determined the structure of the oligomeric species of insulin through small angle X-ray scattering (SAXS) analysis (Figure 2B).⁵⁰ The oligomers were found to be helical in nature, and two helical oligomers interwound with each other to form a protofibril having an average diameter of 100 Å. They proposed that the helical oligomer is preorganized for interaction with other oligomers to help elongate the protofibrils to the mature fibril.

Ivanova et al. reported that the B-chain residues LVEALYLV form the core of insulin fibrils.⁵¹ Strands of LVEALYL pack together to form parallel β -sheets, which run the entire length of

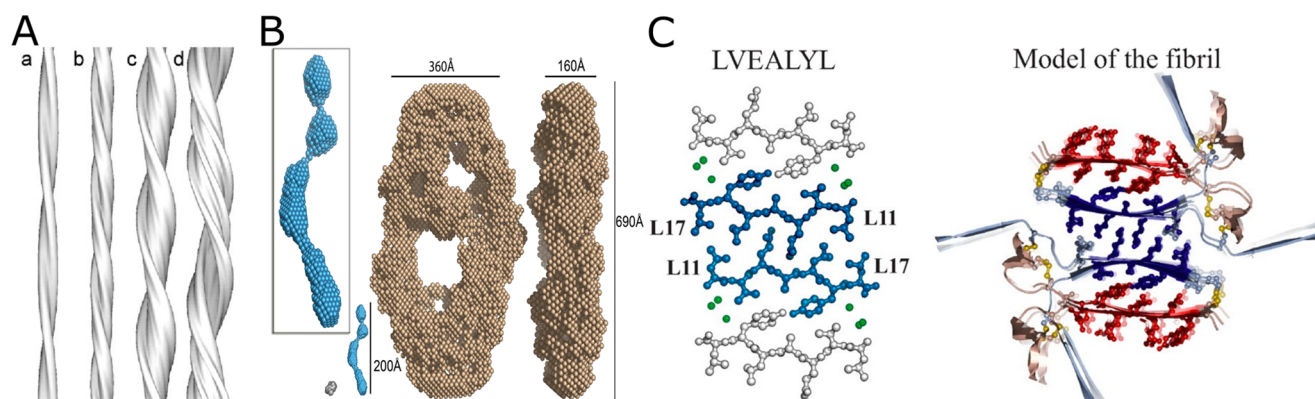


Figure 2. (A) Cryo-EM structure of the insulin fibril with (a) two, (b) four, and (c) six-protofilaments twisting around each other, and (d) a ribbon twisted to form protofibril (Reprinted with permission from ref 3, Copyright 2002 National Academy of Sciences). (B) The structure of the insulin monomer from SAXS analysis is shown in the lower left corner (gray), the *ab initio* helical oligomer is in blue, and the repeating helical unit of the fibril (front and side view) is shown in brown. The boxed upper left corner represents the insulin oligomer (Reprinted with permission from Vestergaard et al.⁵⁰ Copyright 2007). (C) (Left) X-ray crystal structure of four β -sheets formed from B chain residues LVEALYL. The two sheets forming the steric zipper interface are in blue. The green spheres indicate water molecules. (Right) Fibril model of insulin A and B chains depicted in pale red and pale blue, respectively. The LVEALYL fragment, that forms the spine of the fibril, is shown in dark blue. The SLYQLENY region, from the A chain, which forms auxiliary sheets to the spine of the fibril, is colored dark red. The disulfide bonds are shown in yellow (Reprinted with permission from ref 51. Copyright 2009 National Academy of Sciences).

the fiber. Alternating side chain residues L, E, L, and L from one sheet interact with the identical residues from another sheet to form a hydrophobic, highly complementary interface called the “steric zipper”. Two identical steric zippers are present alongside a pair of internal dry sheets, surrounded by six water molecules. The α -helix spanning region B11–B17 LVEALYL undergoes a transformation from the α -helix in native insulin to the β -sheet in the fibril structure. The dry steric zipper surface of LVEALYLV is packed together by the stacking of SLYQLENY from insulin A chain (Figure 2C). The Tyr residue interacts with the Tyr of LVEALYL thus maintaining the “kissing tyrosine” interaction along the wet surface of the fibril. Moreover, X-ray diffraction studies of insulin fibrils suggested that there are two insulin molecules per 4.7 Å layer of fibril, in agreement with Jimenez et al. Surin et al. determined the polypeptide regions that form the spine of insulin fibrils using bioinformatics, limited proteolysis, and mass analysis.^{52,53} They observed that for human insulin as well as its analogs, the regions consisting of A8–A15, B12–B19, and B23–B25 formed the spine of the fibrils.

Kourouski et al. used tip-enhanced Raman scattering (TERS) to characterize the surface of the insulin fibril, which exhibited a chiral polymorph.⁵⁴ The secondary structure on the fibril surface was very heterogeneous and consisted of both twisted and flat topologies. The TERS data further suggested that Tyr, Pro, and His residues usually dominated the twisted fibrils, whereas Cys preferentially occupied the flat surfaces. The difference in the distribution of the amino acid residues on the surface was proposed to be a result of the difference in the aggregation mechanism of fibril formation. On the other hand, the surface of the protofibrils was found to contain very few Tyr and Phe residues in comparison to the mature fibril.⁵⁵ Phe and Tyr, being buried, may be involved in the propagation of protofilaments to form mature fibrils. However, the frequency of Cys was found to be twice as high in protofibrils compared to twisted fibrils and almost similar to that found on flat surfaces. This indicates that Cys might be involved in the propagation of twisted fibrils but does not get involved in the propagation of protofilaments with a flat morphology.

Atomic force microscopy-infrared spectroscopy (AFM-IR) of the insulin fibrils revealed that insulin fibrillation led to formation of two different polymorphs.⁵⁶ The first polymorph was primarily rich in β -sheet structure, while the surface of the other was composed of both unordered protein and β -sheets. The β -sheet on the first polymorph surface promoted attachment of further β -sheets for fibril elongation. This was consistent with the report by Jimenez et al., who found that fibril elongation took place laterally, by attachment of a layer of β -sheet on the fibril surface. In the other polymorph, the unordered portion interacted with similar hydrophobic counterparts for fibril elongation.

Selivanova et al. proposed that the formation and the lengthening of fibrils mainly involved the disappearance of oligomeric structures.⁵⁷ Using a high magnification electron microscope, they observed that ring-like oligomers were involved in the formation of mature fibrils with an outer diameter of about 6–7 nm, an inner diameter of 2 nm, and a height of 3–4 nm. These oligomers were either positioned in a ring-to-ring fashion touching the lateral sides or slightly overlapping with each other. The authors proposed that the ring-like oligomers, which consisted of hexamers formed by the interaction of misfolded monomers, were the building block of insulin fibrils. The irregular association of the oligomers

accounted for the polymorphism of insulin fibrils due to breaking, branching, and surface roughness of the oligomers.

Cannon et al. compared the structure of insulin spherulites, large structures formed by the self-assembly of amyloid fibrils, with that formed by $A\beta$ (1–40) and β -lactoglobulin (β -LG).⁵⁸ Insulin and $A\beta$ spherulites were dominated by β -sheets at the edges, with predominantly random coil structure at the center. In contrast, the β -LG spherulites were dominated by β -sheet structures at the center. Moreover, although insulin and $A\beta$ formed similar fibrils, the amyloid fibrils of β -LG had a different orientation. Overall, the structural insights could be valuable tools for rational design of small molecule inhibitors of amyloid aggregation.

2.3. Insulin Aggregation in the Presence of Ions

While studying insulin aggregation in artificial delivery systems, Loughheed et al. found that Zn^{2+} , Cu^{2+} , and Fe^{2+} cause insulin to form large aggregates at neutral pH with molecular weight ranging from 10^5 to 2×10^5 Da.³⁷ When insulin was subjected to slow rocking at physiological pH, they observed visible precipitation in vials containing Cu^{2+} within 4 h, while in controls without copper, visible aggregation appeared only after 3–4 days. The effect of zinc on insulin aggregation, on the other hand, is more complex due to its physiological role in promoting insulin hexamer formation. In the absence of zinc, the insulin dimer is considered as the starting species for aggregation, either because it is the predominant species or because it aggregates more rapidly.⁵⁹ Consistent with a nucleation dependent pathway, insulin has a higher propensity to aggregate at higher monomer concentration. In solutions of Zn-insulin at physiological pH, the opposite trend is observed and a decrease in insulin concentration leads to more fibrillation. This is because the coordination of Zn^{2+} with HisB10 favors the formation of insulin hexamers at concentrations above 0.01 mM Zn-insulin.⁶⁰ Consistent with this, the aggregation of insulin requires the dissociation of hexameric Zn-insulin to the dimeric form.⁶¹ At low pH, insulin exists in its monomeric state owing to the protonation of HisB10 which prevents zinc coordination, resulting in a higher aggregation propensity of insulin. In a neutral environment in the presence of zinc, the increased tendency of insulin to form hexamers at concentrations above 0.01 mM leads to less fibrillation.

The effect of anions on insulin aggregation has also been reported. Owczarz and Arosio reported that sulfate anions inhibited the conversion of insulin monomer to aggregates by modifying the insulin aggregation pathway.⁶² At low sulfate concentrations (0–5 mM) and acidic pH, insulin followed a nucleation dependent polymerization pathway and formed thin fibrils as normally observed. Beyond 5 mM, sulfate ions induced salting out of ~18–20% insulin monomers into reversible amorphous aggregates rich in α -helix character, leading to slower fibrillation. Over time, the amorphous aggregates structurally rearranged themselves into β -sheet-rich fibrils, which elongated into mature fibrils by recruiting monomer molecules. In contrast to sulfate, chloride ions did not show an inhibitory effect on insulin aggregation at any concentration, but rather induced an increase in the aggregation rate due to shielding of charge on the insulin surface. The aggregation of insulin in the presence of sulfate ions, on the other hand, depended on sulfate ion concentration. In the presence of a large excess of sulfate, aggregation was characterized by a higher activation energy with respect to that of chloride. This may be related to either structural rearrangements of insulin due to

sulfate or the elongation reaction between monomeric proteins and seed-component aggregates.

2.4. Interaction of Insulin with Model Membranes

The chronic administration of insulin leads to insulin aggregation at the site of injection. The molecular interactions between insulin and the membrane surface, which is different from the native membrane surface in pancreatic β -cells, play a very important role in insulin aggregation. Insulin injected subcutaneously encounters cholesterol, which is typically absent in pancreatic β -cells. To investigate the molecular interactions between insulin and human model membranes, Ratha et al. used two model membrane surfaces: 7:3 DOPC:DOPG LUVs, mimicking the pancreatic β -cell membrane, and 6:4 POPC:Cholesterol LUVs,⁶³ which mimic the eukaryotic cell membrane.⁶⁴ The presence of cholesterol accelerated insulin aggregation, in contrast to the β -cell membrane model which did not have a significant effect. The enhanced aggregation propensity in the presence of cholesterol has also been reported for other amyloidogenic proteins like $A\beta$ ^{65,66} and IAPP.⁶⁷

Grudzielanek et al. studied the effect of model phospholipid membranes on the aggregation of insulin.⁶⁸ They found that the length of the saturated lipid chain and the hydrophilic head groups had differential effects on insulin aggregation kinetics, depending on the pH and salt conditions. The rate of insulin aggregation dominated the overall aggregation kinetics at pH 1.9 and 0.1 M NaCl. When insulin was exposed to lipid bilayers under these conditions, the overall aggregation rate was decreased likely due to insertion of insulin in the lipid bilayer, thereby reducing the exposed hydrophobic patches. Interestingly, in the presence of the salt, insulin aggregation was faster for all lipid additives, likely due to greater accumulation at the lipid surface. At physiological pH, insulin aggregation was also inhibited mainly due to the partial insertion of the insulin into the lipid bilayers, except for DOPS. Thus, the presence and composition of specific lipids is an important factor to be considered for insulin aggregation while administering it to diabetic patients.

3. PREVENTION OF INSULIN AGGREGATION

As described before (Figure 1), the insulin aggregation pathway consists of insulin hexamers, dimers, monomers, partially unfolded insulin intermediates, oligomers, and fibrils. The association of insulin monomers to form a nucleus is the rate-determining step of aggregation. Once formed, the nuclei attract other monomers through hydrophobic and H-bonding interactions for fibril elongation. The kinetics of insulin fibrillation is characterized by a lag phase, during which the insulin hexamer converts to partially unfolded monomers, which associate to form a nucleus. This is followed by a fibril growth phase, during which the oligomers and/or monomers associate to form protofibrils and ultimately the mature fibrils.

To inhibit insulin aggregation, several of the above species could be targeted by small molecules. For example, stabilization of the hexameric, dimeric, or monomeric forms of insulin using small molecules will prevent their progression to the fibril state while maintaining the biological activity of the protein (Figure 3). Stabilizing off-pathway intermediates, on the other hand, might help prevent formation of toxic oligomers. Since the mature amyloid fibrils are thought to be nontoxic, it has also been suggested that small molecules that bind to fibrils and stabilize them might help alleviate toxicity due to oligomers.⁶⁹

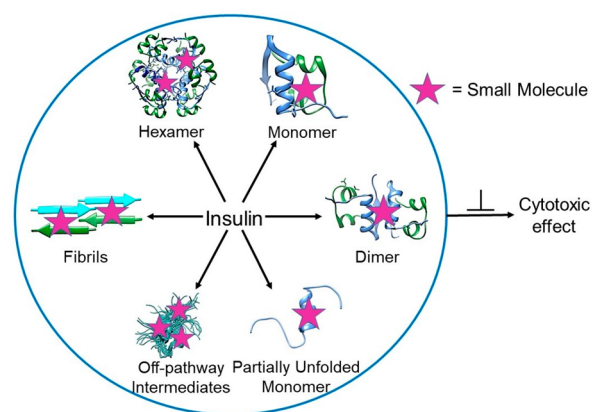


Figure 3. Possible modes of intervention by a small molecule during insulin fibrillation.

Scaffolds ranging from peptides and small molecules, both naturally occurring and synthetic, have been used to inhibit insulin aggregation. The reported molecules target either the nucleation or the elongation step, thereby preventing insulin aggregation. A few molecules have been reported to disintegrate mature insulin fibrils, while the modes of action of a few are still unknown. Here, we discuss some representative examples of insulin aggregation inhibitors categorized based on their mode of action.

3.1. Targeting the Hexameric State

The earliest strategy to inhibit the aggregation of insulin was to facilitate the self-association of insulin by addition of Zn^{2+} or Ca^{2+} .^{70,71} These ions stabilized the hexameric state of the protein, which had a lower aggregation propensity than the monomer. Currently, phenolic excipients are used as preservatives in insulin solutions. These compounds serve two purposes: maintaining the sterility of the solution and preventing insulin aggregation by stabilizing the hexameric state. Commercial insulin formulations contain phenol, *m*-cresol, or both as preservatives in considerable amounts: 29–32 mM or 2.7–3.2 mg/mL.⁷² Promotion of the hexameric association of insulin, however, slows down the biological activity of the protein and is undesirable.

3.2. Targeting the Nucleation Step

Small molecules like betaine and trehalose inhibited insulin aggregation when used in large excess over insulin (~ 1700 -fold).⁷³ However, investigations into the mode of action of trehalose indicated that it increased the lag time for insulin aggregation considerably by inhibiting nucleation, but did not inhibit the overall aggregation.³¹ Similarly, paclitaxel, an FDA approved drug for antimitotic and antitumor activity, inhibited insulin aggregation at a sub-stoichiometric ratio.⁷⁴ However, it could not inhibit insulin aggregation in the presence of preformed seeds which act as templates for fiber elongation, indicating that paclitaxel mainly inhibited the primary nucleation stage of aggregation.

Polycyclic aromatic compounds are known to inhibit protein aggregation owing to the presence of several $-OH$ groups. The $-OH$ groups interact with insulin through electrostatic and hydrogen bonding interactions, thereby stabilizing the protein and preventing aggregation. Curcumin and epigallocatechin gallate (EGCG) are well-known polycyclic compounds that act as protein aggregation inhibitors. Curcumin, a natural molecule found in turmeric can inhibit insulin aggregation at sub-

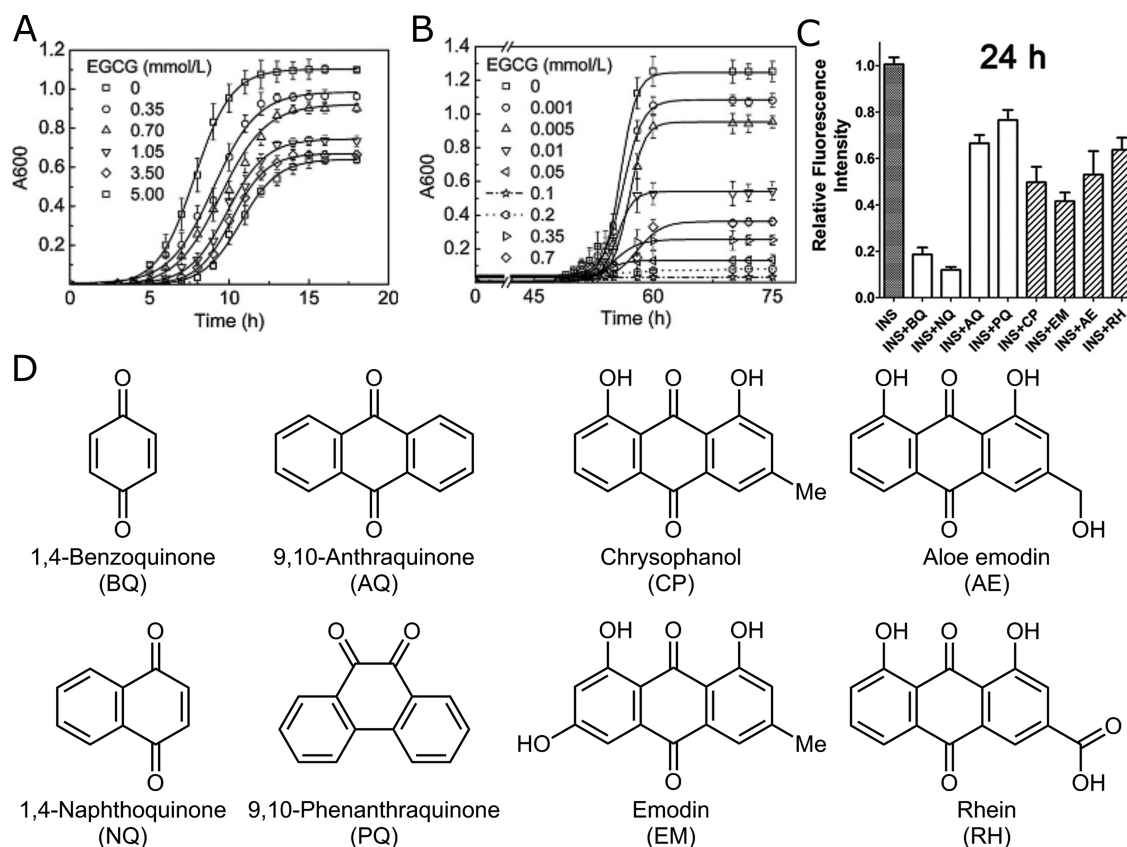


Figure 4. Molecules that target insulin nucleus formation. Aggregation kinetics of insulin in the presence of EGCG under two different pH and temperature conditions: (A) pH 2.0, 60 °C; (B) pH 7.4, 37 °C (Reprinted from ref 76. Copyright 2012, with permission from Elsevier). (C) Effect of various quinones on insulin aggregation, as measured using ThT assay (Reprinted with permission from ref 79. Copyright 2014 Springer Nature). (D) Structures of the corresponding quinones used.

micromolar concentration, though it suffers from the disadvantage that it is relatively insoluble in aqueous solution at room temperature.⁷⁵ Molecular docking studies have revealed the B-chain of insulin as the possible interaction site of curcumin. Wang et al. have shown that EGCG, found mainly in green tea,⁷⁶ can be used to inhibit insulin aggregation under two different pH conditions, and at 14–15-fold molar excess over insulin. Due to the protonation of –OH groups at low pH, EGCG partially inhibits the aggregation of insulin at pH 2.0 and 60 °C, conditions under which insulin exists predominantly as a monomer (Figure 4A). At pH 7.4 and 37 °C, EGCG interacts with insulin through H-bonds and electrostatic interactions thereby acting as a bridge for its association to the tetramer/hexamer form, thus slowing down the denaturation and dissociation of insulin⁷⁷ (Figure 4B). However, EGCG suffers from disadvantages like poor stability and low bioavailability. Moreover, both curcumin and EGCG fall into a class of compounds known as “PAINS” (pan-assay interference compounds).⁷⁸ PAINS are small molecules that can give false positive results owing to promiscuous binding to certain metal ions, covalent modification of the target, aggregation, or interference in fluorescence-based assays. These properties make PAINS unsuitable for aggregation inhibition.

Huang group used quinones to modulate insulin aggregation,⁷⁹ as several quinones are known to effectively inhibit the aggregation of tau and A β (Figure 4C,D). It was observed that some quinones increased the lag phase of insulin aggregation and inhibited nucleus formation likely by binding to insulin monomer/dimer. Others affected secondary nucleation, thus

decreasing the aggregation to ~40%, as measured from ThT fluorescence. The authors proposed that quinones likely destabilized the intermolecular cross- β strand H-bonds via electrostatic interactions with the protein backbone. The quinone moiety may hydrogen bond to the carbonyl oxygens and amide hydrogens of the peptide backbone and allow intercalation of the quinone molecule into protein oligomers. The aromatic ring of quinones may competitively interact with protein aromatic residues, thereby resulting in quino-protein oligomers preventing further insulin aggregation. The inhibitory effect may also be influenced by substituent groups. For example, substituents containing benzene ring(s) may interact better with hydrophobic residues, and thus inhibit aggregation more efficiently. Several such naturally occurring molecules have been used to inhibit insulin aggregation,⁸⁰ though their major disadvantage is that they are relatively nonspecific in their action, and affect the activity of other proteins.

Bhunia group reported two synthetic peptides, KR7 (KPW-WPRR-NH₂) and NK9 (NIVNVSLVK) for the inhibition of insulin aggregation.^{81,82} NK9, derived from the SARS coronavirus-E protein sequence, had β -sheet breaker amino acids like Ile, Val, and Leu. It was found to inhibit the nucleation of insulin resulting in reduced fibril formation and decrease in amyloid-mediated cytotoxicity in HT1080 cells. The maximum inhibitory effect was observed at 1:1 molar ratio of insulin:NK9. To probe whether NK9 inhibited the nucleation phase, NK9 was added at different time points during insulin aggregation. When NK9 was added at later stages of insulin aggregation, its effect on insulin aggregation decreased significantly. This indicates that

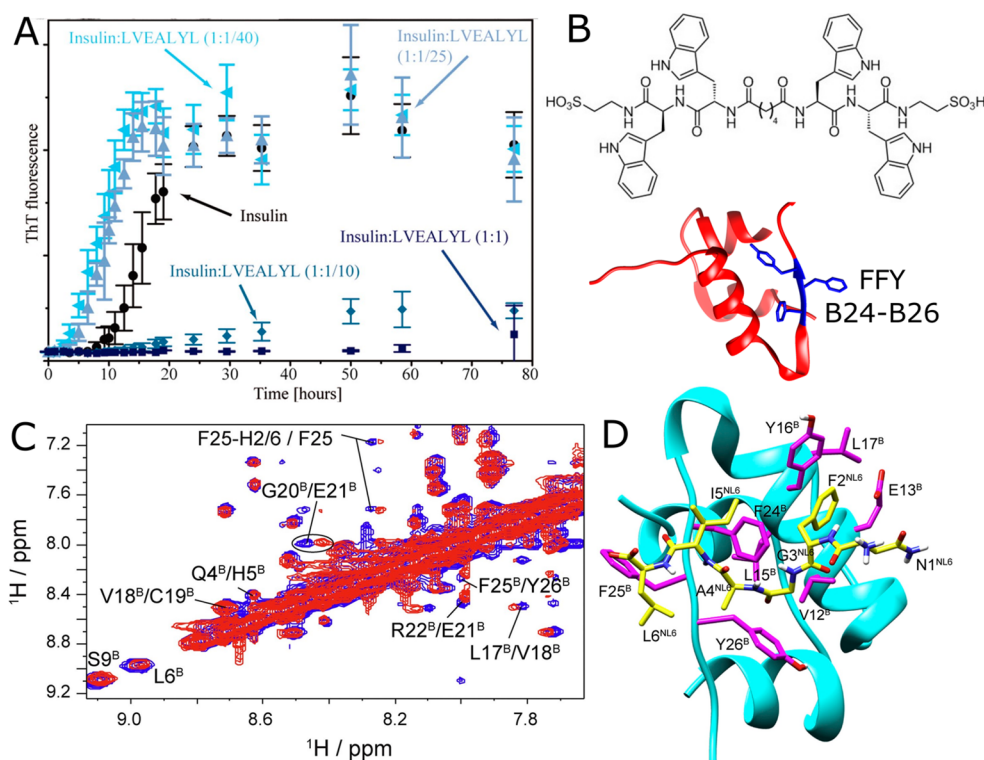


Figure 5. Synthetic peptides as modulators of nucleation during insulin fibrillation. (A) The effect of LVEALYL peptide on insulin aggregation as measured by ThT assay (Reprinted with permission from ref 51. Copyright 2009 National Academy of Sciences). (B) Peptide used by Verma and co-workers to inhibit insulin aggregation. The proposed binding site, B24–B26 (FFY) on the insulin monomer (red), is shown and the interacting residues are in blue (Reprinted from ref 83. Copyright 2013, American Chemical Society). (C) 2D ^1H – ^1H NOESY NMR showing chemical shift perturbations of insulin in the presence of NL6 peptide at a 1:1 molar ratio. (D) Residues of insulin that interact with NL6 as determined by docking. Insulin is shown in cyan, the interacting residues are in violet, and NL6 is in yellow (panels C,D are reprinted from Ratha et al.⁸⁴ Copyright (2019) with permission from Elsevier).

NK9 mainly acted to prevent primary nucleation, and ultimately the formation of soluble toxic oligomers of insulin.

Ivanova et al. tested two peptides LYQLEN (residues A13–A18) and LVEALYL (residues B11–B17) based on insulin A and B chains, respectively, for insulin aggregation inhibition.⁵¹ They found that the latter peptide was able to attenuate insulin aggregation significantly at 0.1 molar ratio compared to insulin, while the maximal effect was observed at equimolar concentration. Paradoxically, the peptide was found to promote insulin aggregation at a concentration 40-fold less than insulin (Figure 5A). The authors concluded that LVEALYL mainly contributed to the spine of insulin fibrils, and when used at a low concentration could mimic the insulin B chain to accelerate aggregation.

The Verma group reported the inhibition of both human and bovine insulin aggregation by a Trp-Trp-Tau tripeptide at submicromolar concentration (Figure 5B).⁸³ CD spectroscopy revealed that the peptide was able to maintain the secondary structure of insulin. A time-dependent kinetic assay suggested that the peptide could prevent insulin aggregation even when added 6 h after the start of aggregation, though the effect was less pronounced than addition at early stages of aggregation. This suggested that the molecule bound to insulin during early nucleation. Mechanistic studies using NMR spectroscopy revealed that the peptide bound to the FFY residues of insulin B chain (B24–B26), which play a crucial role in β -sheet formation.

Bhunia and co-workers used the fibrillating core from human IAPP (N²²FGAIL²⁷, called NL6) and its derivative NFGAXL

(NL6X, X = 2-aminobenzoic acid) for the inhibition of insulin amyloidogenesis.⁸⁴ The NL6 peptide was found to be more potent than NL6X. Several NOE cross peaks for E13, A14, L15, L17, E21, G23, and F25 residues of the insulin B-chain showed significant perturbations in their chemical shift upon interaction with the NL6 peptide (Figure 5C). This was supported by molecular modeling and MD simulations which showed that NL6 formed a stable contact with the B-chain of insulin (Figure 6D). CD and Raman spectroscopy further confirmed that NL6 was successful in keeping insulin in its native form. Although peptides can potentially be excellent inhibitors of insulin aggregation, the major disadvantage is that they are prone to proteolytic degradation, and thus have limited therapeutic utility.

Supramolecular moieties like cucurbituril (Figure 6A,D,E),⁸⁵ calixarenes,⁸⁶ and β -cyclodextrins⁸⁷ have been shown to inhibit insulin fibrillation. Kim group employed cucurbit[7]uril (CB[7]), which has a strong tendency to bind Phe residues in proteins, as a supramolecular agent to inhibit insulin aggregation. CB[7] binds to PheB1 to impede insulin oligomerization (Figure 6B). It was also found to interact with PheB24, which is exposed during partial unfolding of insulin and contributes to the monomer–monomer interaction. Thus, CB[7] prevented intermolecular contact after partial unfolding of the protein and subsequently inhibited insulin aggregation (Figure 6C). Webber et al. reported that cucurbit[7]uril-PEG conjugates were able to prevent insulin aggregation more effectively even under harsh conditions for more than 100 days.⁸⁸ The effect was more pronounced as the molecular weight

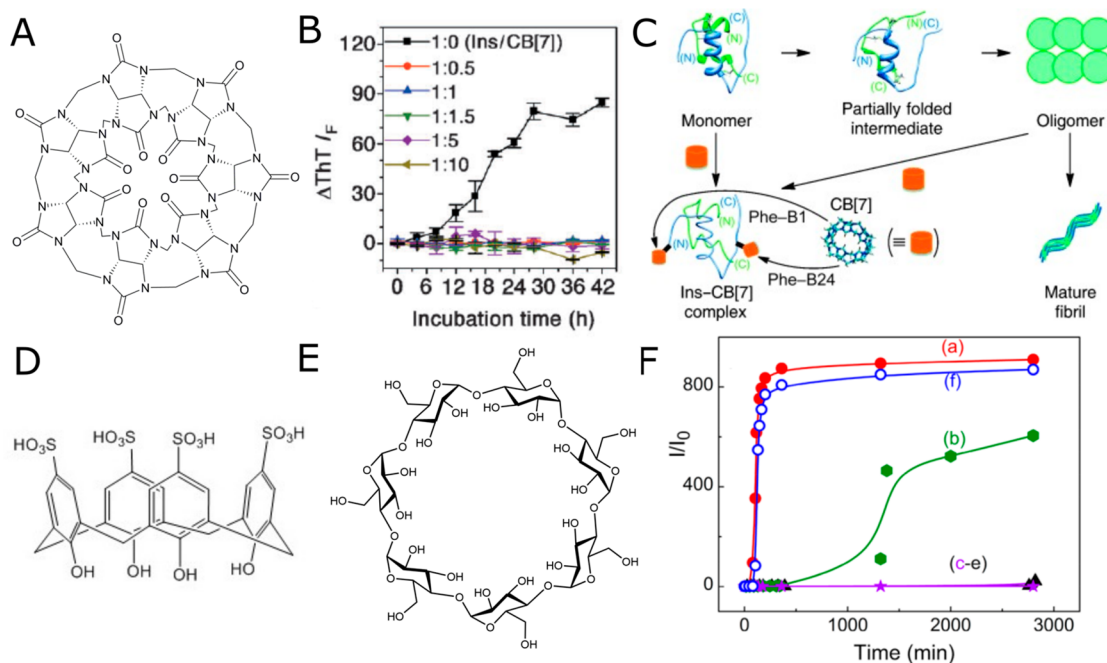


Figure 6. Supramolecular agents and their effect on insulin aggregation inhibition. (A) Structure of cucurbit[7]uril. (B) Concentration dependent effect of cucurbit[7]uril on insulin aggregation as measured by ThT assay. (C) Proposed mechanism of the inhibitory effect of CB[7] on insulin aggregation. CB [7] binds to Phe residues of insulin to stabilize the protein and arrest fibrillation. (panels B, C were reproduced from Kim et al.⁸⁵ with permission from John Wiley and Sons). (D) *p*-Sulfonatocalix[4]arene. (E) β -cyclodextrin. (F) Dose dependent effect of sulfobutylether- β -cyclodextrin (SBE7- β -CD) on insulin aggregation (Ins: 250 μM ; Ins: SBE7- β -CD ratios were as follows: (a) no host (1:0), (b) 1:0.2, (c) 1:1, (d) 1:4, (e) 1:8, and (f) in the presence of β -CD (1:8) (Reprinted from ref 89. Copyright 2017 American Chemical Society).

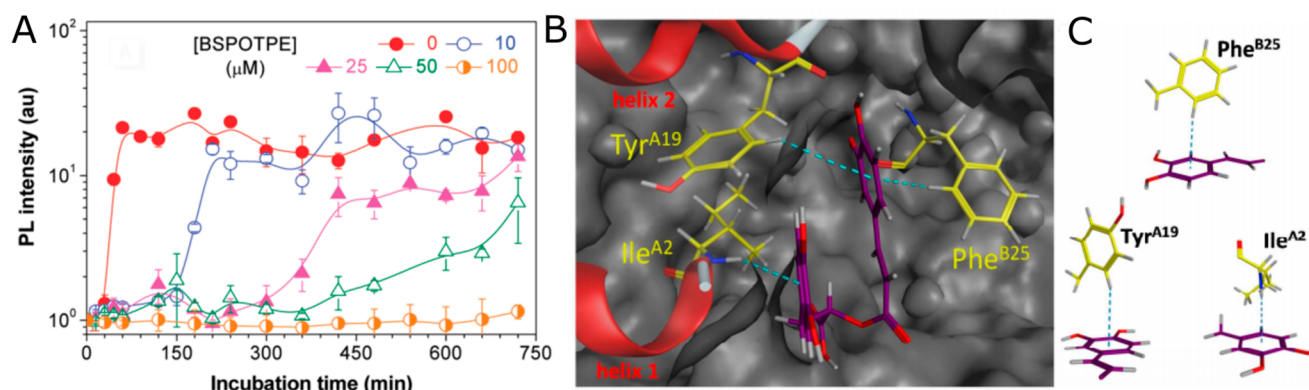


Figure 7. (A) ThT assay showing concentration dependent inhibition of insulin aggregation by BSPOTPE (Reproduced from ref 49. Copyright 2012, American Chemical Society). (B) Molecular docking study depicting the interaction of rosmarinic acid with human insulin. (C) Rosmarinic acid showed strong interaction with aromatic residues like B25Phe and A19Tyr (panels B,C were reproduced from ref 95. Copyright 2018, American Chemical Society).

of the PEG chain increased from 5 to 30 kDa. This study highlighted the importance of supramolecular PEGylation in stabilizing insulin.

Mohanty and co-workers used *p*-sulfonatocalix[4/6]arene (SCX4/6) (Figure 6D) to inhibit insulin aggregation at very low inhibitor ratio (Ins:SCX4 = 1:0.2).⁸⁶ Sulfonatocalixarene exhibited a stronger binding to insulin monomer compared to cucurbituril owing to the presence of the macrocycle and sulfonate groups. The authors proposed that the inhibition of fibril formation by SCX4/6 was either due to a decrease in the availability of free insulin because of encapsulation of the insulin monomer inside the supramolecular cavity, or due to the sequestration of specific amino acid residues which contribute to fibril formation of insulin. For example, amino acid residues such

as Arg, Lys, Glu, Ala, and Phe show strong binding with SCX4/6, thus inhibiting their interaction with the other hydrophobic residues. The same group reported the inhibition of insulin aggregation using a sulfobutylether- β -cyclodextrin (SBE7- β -CD) (Figure 6F).⁸⁹ It is a water-soluble macrocycle that is also capable of breaking mature fibrils to smaller nontoxic species. The mechanism of action is similar to the other two macrocycles, i.e., via encapsulation of certain protein side chains, preferably Tyr to prevent the transformation of insulin to toxic fibrils.

Huang and Tang reported a small molecule called BSPOTPE, consisting of a tetraphenylene moiety functionalized with two sulfonate groups, which inhibited insulin aggregation completely at sub-stoichiometric concentrations, and could report on ex situ

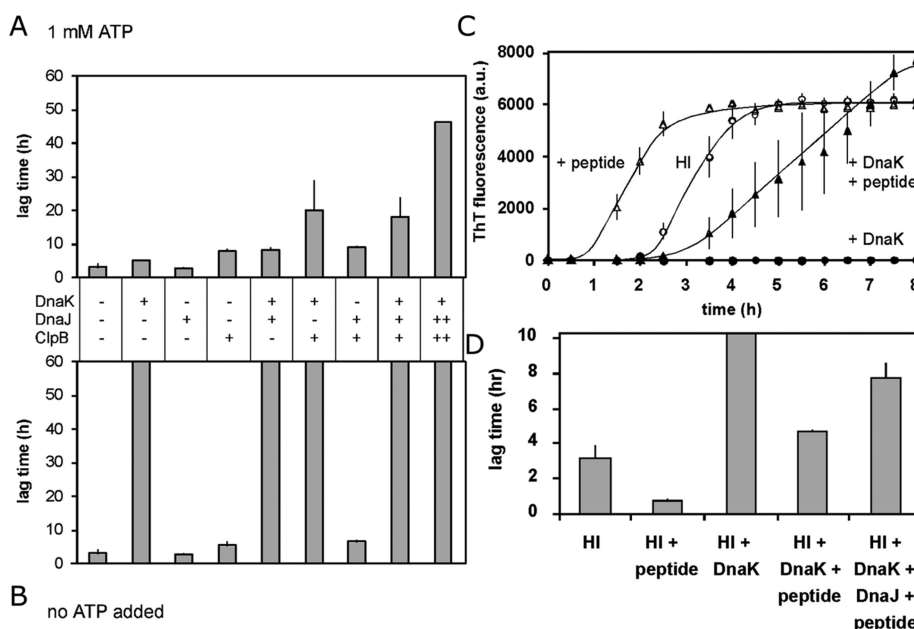


Figure 8. Effect of bacterial chaperones on insulin aggregation (A) in the presence and (B) absence of ATP. (C) Effect of DnaK on insulin aggregation in the presence and absence of the peptide LVEALYL. (D) Effect of chaperones (DnaK and DnaJ) on the lag time of fibril formation in the presence and absence of the LVEALYL peptide (reproduced from ref 101. Copyright 2012, American Chemical Society).

insulin aggregation (Figure 7A).⁹⁰ BSPOTPE was shown to bind the partially folded conformers of insulin thus inhibiting nucleation. This was supported by molecular docking studies, which revealed that the molecule binds to the unfolded hydrophobic patch B11-19, thereby stabilizing the partially folded structure of insulin. BSPOTPE could also be used as an amyloid sensor as it shows enhanced fluorescence on fibril binding due to restricted rotation of its phenyl rings. Later, this molecule was also shown to inhibit the aggregation of IAPP,⁹¹ suggesting that it is likely a general amyloid inhibitor. Zeng group reported the use of equimolar quercetin to inhibit insulin aggregation, but the molecule was insoluble in water at room temperature.⁹²

Hosur and co-workers used small osmolytes, namely, betaine, citrulline, proline, and sorbitol, to inhibit insulin amyloidogenesis.⁹³ These molecules, like trehalose, were used at a relatively high concentration of about 250 mM (~500-fold molar excess over insulin). The authors proposed that these molecules either increased hydration around insulin to enhance intramolecular hydrophobic association instead of intermolecular ones or directly interacted with the insulin surface to inhibit fibrillation. Another naturally occurring small molecule, morin hydrate, dose-dependently inhibited insulin aggregation with 80% inhibition at equimolar concentration, while preserving the α -helical native structure of insulin.⁹⁴ Based on computational analyses, the authors proposed that the molecule bound to the “LVEALYLV” region of the insulin B-chain and prevented structural transformation leading to fibril formation.

Zheng et al. showed that a polyphenol called rosmarinic acid completely inhibited the aggregation of insulin at pH 2.⁹⁵ ¹H NMR studies, along with NOE and molecular docking (Figure 7B), revealed that rosmarinic acid bound to a pocket on the surface of insulin. This resulted in the formation of a tetramolecular aromatic network on the surface of the dimer with increased resistance to thermal unfolding (Figure 7C).

The Gazova group highlighted the use of glycoacridine derivatives as small molecule inhibitors of insulin aggregation.⁹⁶

The IC₅₀ values for such molecules were in the high μ M range. Their *in silico* analysis revealed that the various side chain conformations of glycoacridine, along with the acridine core, played an important role in binding to insulin and preventing its aggregation. Kinetic studies found that the molecules significantly prolonged the lag phase of insulin aggregation, thereby slowing down nucleus formation and subsequent formation of oligomers. However, none of the molecules were able to inhibit the aggregation of insulin completely.

Jayamani and Shanmugam reported the use of gelatin, a common gelling agent, in the prevention of insulin aggregation.⁹⁷ Based on spectroscopic studies and ThT assays, they proposed that gelatin stabilized the native structure of insulin. Between concentrations of 0.3% and 0.9 wt %, gelatin increased the lag phase of insulin aggregation in a dose dependent manner. The increased lag phase along with lowered fibrillation suggested slower rate of nuclei formation, presumably by protecting the native structure of insulin. IR data revealed the preservation of α -helix character (1656 cm⁻¹) on treating insulin with gelatin. In contrast, insulin alone showed a peak at 1627 cm⁻¹ indicating β -sheet formation. As gelatin is commonly used in drug formulations, its anti-amyloidogenic property can be beneficial for insulin preparations. The same group also reported that gallic acid inhibits insulin fibrillogenesis at a 50-fold molar excess,⁹⁸ likely by delaying the lag phase of nucleus formation. Based on the structure of gallic acid, they proposed that its aromatic moiety may interfere with the aromatic interactions involved in fiber formation, and polyhydroxy groups might form H-bonds with amino acid residues on the insulin surface protecting the native structure.

Chatterjee and co-workers used the dye methylene blue (MB) to disrupt insulin aggregation at an insulin:MB ratio > 1:50,⁹⁹ which inhibited nucleus formation. When HepG2 cells were treated with insulin aggregates in the absence and presence of MB, only MB treated insulin was able to induce phosphorylation of Akt. This suggests that after MB treatment, insulin was active and able to exert its normal biological function. The same group

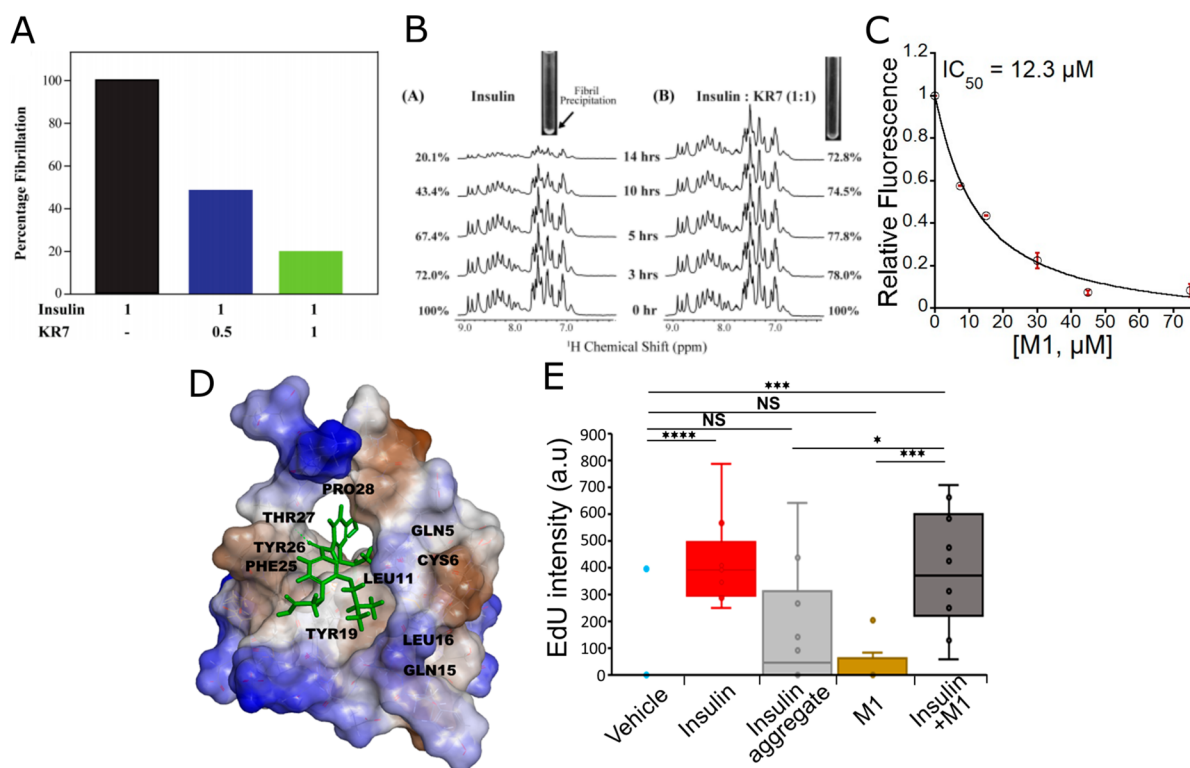


Figure 9. Inhibitors that prevent insulin fibril elongation. (A) Bar diagram representing the concentration dependent inhibitory effect of KR7 peptide on insulin aggregation. (B) NMR data showing inhibition of insulin aggregation by KR7, as depicted by the retention of aromatic peaks in KR7 treated insulin over time (panels A, B were reproduced with permission from ref 82. Copyright 1969, Elsevier). (C) Concentration dependent effect of M1 on insulin aggregation. (D) Molecular docking showing the probable binding mode of M1 to partially unfolded insulin (PDB ID: 1SF1). The key interacting residues are labeled. (E) Relative EdU (*S*-ethynyl-2'-deoxyuridine) intensity depicting actively dividing cells, which reports on the biological activity of insulin in *Drosophila melanogaster* wing epithelial cells (panels C–E were reproduced from ref 102. Copyright 2020, American Chemical Society).

recently reported a prion derived tetrapeptide, VYYR (named IS1), to effectively inhibit the heat and storage-induced fibrillation of insulin, and maintain the bioactive form of insulin.¹⁰⁰ IS1 treated insulin showed adequate glucose control in mouse models of diabetes. The authors proposed that IS1 trapped insulin in the dimeric form, thereby preventing its aggregation and preserving the normal bioactivity of insulin. IS1 was also successful in preventing the aggregation of various insulin analogs that may be useful for therapeutically relevant insulin formulations.

Ballet et al. observed that the bacterial chaperones DnaK, DnaJ, and ClpB were able to protect insulin from aggregation. In the presence of 1 mM ATP, the combined effect of the three proteins delayed recombinant human insulin aggregation *in vitro* (Figure 8A).¹⁰¹ Surprisingly, DnaK, which typically uses ATP, was able to prolong insulin aggregation significantly even in the absence of ATP (Figure 8B). The authors reasoned that in the absence of ATP, DnaK bound directly to the aggregation prone “LVEALYL” site (B11–B17) of insulin and prevented self-assembly. This was supported by competition experiments with the LVEALYL peptide (Figure 8C). DnaJ synergistically interacted with DnaK to stabilize the DnaK–insulin complex and prolonged the lag time (Figure 8D). Overall, the bacterial chaperones worked together to inhibit insulin aggregation by delaying the nucleus formation. Although it is unlikely for chaperones to be used for insulin aggregation inhibition directly, such studies can inform small molecule inhibitor design.

3.3. Targeting Insulin Fibril Elongation

A few molecules have been reported to inhibit the elongation of insulin fibrils. KR7, derived from the antimicrobial peptide indolicidin, showed good insulin aggregation inhibition at equimolar concentration (Figure 9A).⁸² Biophysical and NMR studies suggested that KR7 primarily targeted the fiber elongation step with little effect on the early oligomerization events. In the absence of KR7, the intensity of the aromatic and amide peaks in ¹H NMR was reduced by almost 5-fold after 14 h due to the formation of aggregates. In contrast, in the presence of an equimolar concentration of KR7, >70% of the signal could be detected after 14 h (Figure 9B). The NMR results agree with ThT data, which suggests that KR7 suppresses the formation of large aggregates. NMR analysis also showed that KR7 interacted with B11–B18 (LVEALYLV) residues of insulin through favorable binding via “PWWP” residues of KR7 as indicated by broadening of the two tryptophan peaks of KR7 in the NOESY spectrum.

Our group has reported the dose-dependent inhibitory effect of an oligopyridylamide-based small molecule, M1 on insulin aggregation with 80–90% inhibition ($IC_{50} = 12 \mu M$) (Figure 9C).¹⁰² M1 did not inhibit the aggregation of another amyloidogenic protein, α -synuclein, suggesting that M1 is not a generic inhibitor of amyloidogenic proteins. Detailed biophysical analyses suggested that M1 primarily disrupted fibril elongation by binding to a partially unfolded form of insulin. Docking suggested that “FFY” (B24–B26) residues of insulin were the primary interacting site of M1 (Figure 9D). Moreover, M1-treated insulin was less cytotoxic and was

biologically active in promoting cell proliferation in *Drosophila* wing epithelial tissue (Figure 9E). Subsequent SAR studies identified BPAD2 which inhibited insulin aggregation with an IC₅₀ value of ~1 μM. Mechanistic investigations led to the conclusion that BPAD2 mainly inhibited the elongation step of insulin fibrillation, thereby slowing aggregation.¹⁰⁵

3.4. Disintegration of Mature Insulin Fibrils

The development of small molecules for the disintegration of amyloid fibrils is a challenging task as mature fibrils are thermodynamically very stable. Further, partial fibril disintegration might result in the formation of prefibrillar intermediates, that may be more toxic than mature fibrils,^{104,105} limiting the use of such molecules. The greater toxicity of shorter fibrils or oligomers generated by the disintegration of mature fibrils is thought to arise from the availability of larger hydrophobic surfaces for interaction with the cellular surface, ultimately leading to its disruption.^{106,107} It is not surprising then that only a few molecules have been reported to successfully disintegrate mature insulin fibrils.

The Khan group reported structured short peptide fragments VITYF (P4) and VVVVV (P5) for the inhibition of aggregation.¹⁰⁸ P4 was more effective than P5 in its inhibitory property likely due to the presence of aromatic residues in the peptide sequence, underlining the importance of π–π interactions between the peptide and insulin. The peptides were also capable of disaggregating preformed fibrils providing valuable insights into peptide-based inhibition chemistry. The same group reported that ascorbic acid dose-dependently inhibited insulin aggregation.¹⁰⁹ Their study also reported that ascorbic acid was successful in disaggregation of insulin fibrils, though the exact mechanism of action was unclear. Later, Yang and Yang reported that the degradation products of ascorbic acid were more potent than ascorbic acid in inhibiting insulin aggregation.¹¹⁰ Moreover, the degradation species, dehydroascorbic acid and diketogulonic acid, showed a much stronger disruptive effect on mature amyloid fibrils and exhibited less cytotoxicity. Wang et al. reported the use of two naturally occurring anthraquinones, alizarin and purpurin, in the disintegration of mature insulin fibrils to shorter ones.¹¹¹ TEM imaging and DLS analysis of the samples confirmed the shorter size of fibrils after small molecule treatment compared to untreated insulin aggregates, although the authors did not comment on the cytotoxicity of the degraded products.

4. INSULIN ANALOGS AND THEIR FIBRILLATION

Currently, several insulin variants are available for the treatment of diabetes depending on the severity of the disease. These variants, also called biosimilars, are mutants of human insulin that are functionally identical with insulin and are categorized as fast- and slow-acting insulin analogs. The fast-acting analogs are absorbed quickly after injection and act faster than normal insulin. They act to supply a bolus of insulin at mealtime, which acts quickly to maintain the glucose level after a meal. The slow-acting analogs are released over a period of 8–24 h, which helps in maintaining the basal insulin level throughout the day and particularly at night. The most common fast-acting insulin analogs comprise Lispro, Aspart, and Glulisine, while Glargine is a slow-acting analog.

In Lispro, the amino acid residues at B28 (Pro) and B29 (Lys) of human insulin are swapped. Aspart consists of aspartic acid instead of proline at B28.^{112,113} Notably, both Lispro and Aspart have a substitution at ProB28, which is involved at β-sheet

interfaces formed between insulin monomers, important for dimerization of the protein. This amino acid substitution usually prevents dimerization, thereby increasing the monomeric form of the protein. When phenolic excipients are present, these analogs can form hexamers owing to certain allosteric interactions, which are rapidly dissociated upon injections due to insulin dilution, zinc dissociation, and the diffusion of phenolic excipients. In Glulisine, the mutations are at B3 (NB3K) and B29 (KB29E) residues. Glulisine cannot form hexamers due to steric and electrostatic repulsions from the B3 lysine substitution. The glutamic acid at B29 also reduces the tendency of dimer formation significantly. Glulisine contains a salt bridge between the N-terminal and B29E, thereby increasing monomer stabilization.

Woods et al. studied the aggregation of fast-acting insulin analogs.¹¹⁴ Kinetic analysis showed that at pH 7.4, the three fast-acting analogs, namely, Lispro, Aspart, and Glulisine, had slower aggregating propensity than human insulin under similar experimental conditions. The slower fibrillating property and increased lag time can be attributed to greater stability of the monomer, slower nucleation, and slower elongation of the fibrils. For both Lispro and Aspart, this may be due to the substitution at B28 proline, which inhibits the formation of β-sheet-rich fibrils. In Glulisine, the intramolecular salt bridge enhances the stability of the monomer, which slows down its partial unfolding and subsequent dimerization. However, at pH 3 (insulin manufacturing conditions), seeded aggregation kinetic studies showed that Lispro aggregated at a faster rate than wild-type human insulin, likely due to a reduced propensity to reversibly self-associate.

The slow-acting insulin biosimilar, Glargine, contains an AspA21 to glycine mutation along with addition of two arginine residues at the C-terminus of the B chain. These modifications shift the pI of insulin from 5.4 to 6.7, causing Glargine to precipitate at the site of injection and slowing absorption, resulting in a slow mode of action. Coppolino et al. studied the aggregation of Glargine at physiological pH using light scattering, and found that it is also prone to aggregation and forms compact structures with radii of hundreds of nanometers.¹¹³ Bhunia group compared the aggregation of Glargine with human and bovine insulin and found that Glargine aggregated at a slower rate than both human and bovine insulin.⁸⁴ They proposed that the two arginine residues at the C-terminal end strengthened the salt bridge between ArgB31, LysB29, and GluA4, thereby reducing the extensive thermal distortion and tertiary collapse of the protein.

Thus, insulin biosimilars are also prone to varying degrees of aggregation, and strategies to keep them in bioactive form are desirable. Appel and co-workers reported the use of amphiphilic polymers as excipients for stabilization of monomeric Lispro against aggregation.¹¹⁵ Their ultrafast absorbing insulin lispro (UAFI) formulation was stable under stress aging conditions for 25 ± 1 h compared to 5 ± 2 h for Humalog, a commercial fast-acting Lispro analog. This stable ultrafast human insulin formulation is an important lead to improve diabetes management in patients.

5. STABLE INSULIN ANALOGS WITH REDUCED AGGREGATION PROPENSITY

Apart from small molecule inhibitors, stable insulin analogs less prone to aggregation have been developed by introducing an additional disulfide bond in the insulin structure. Disulfide bonds have long been known to increase protein stability owing

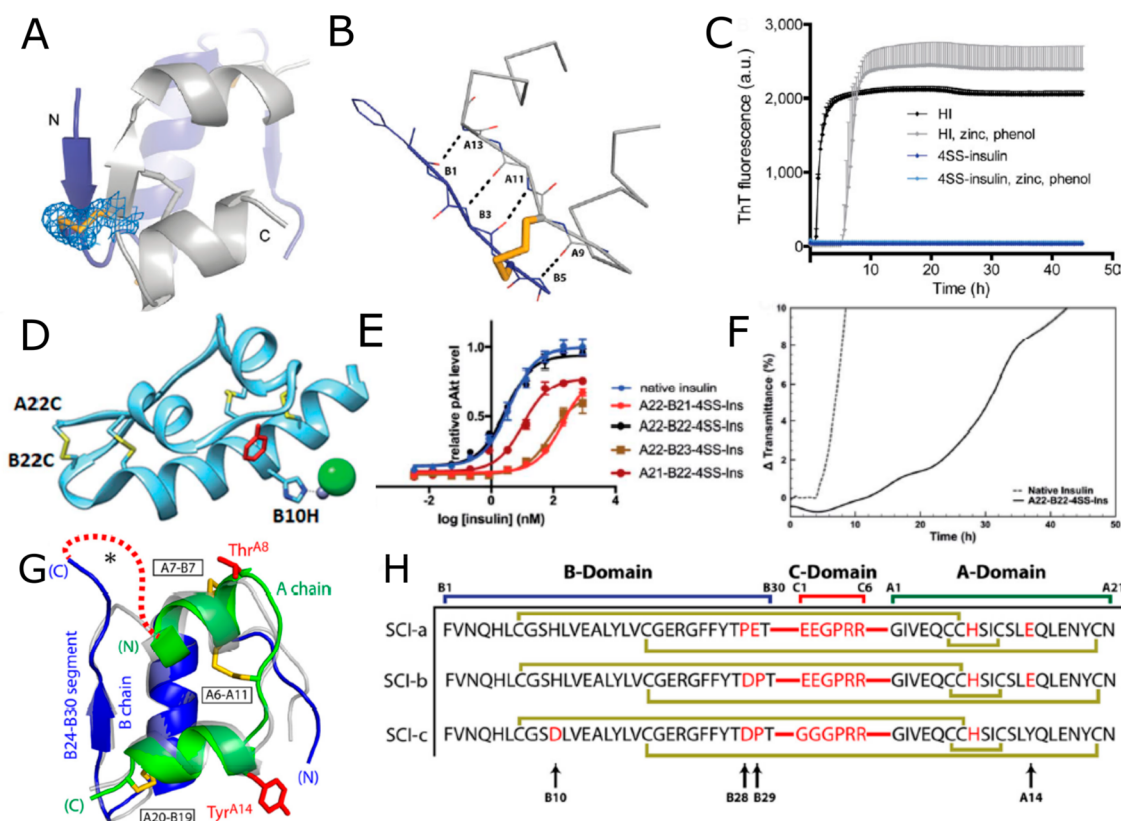


Figure 10. Stable insulin analogs with reduced aggregation propensity. (A) X-ray crystal structure of the 4SS-insulin analog (PDB code 4EFX, A-chain (gray) and B-chain (blue)). The additional disulfide bond between B4C and A10C is represented in yellow. The B-chain N- and C-termini are marked. (B) Introduction of an extra disulfide bond allows formation of additional hydrogen bonds between the A (gray) and B (blue) chains. (C) ThT assay for HI (black), HI with 3 Zn ions per hexamer and 30 mM phenol (gray), the 4SS-insulin analog (dark blue), and the 4SS-insulin analog with 3 Zn ions per hexamer and 30 mM phenol (light blue) (panels A–C were reproduced from Kjeldsen et al.¹¹⁷ with permission from John Wiley and Sons). (D) The crystal structure of one of the A22-B22-4SS-Ins subunits from the corresponding hexamer. The phenol ligand is in red, Zn in gray, and Cl⁻ ions in green. The coordinating histidine residues and disulfide bonds (yellow) are also shown. (E) Bioactivity profile of the insulin analogs compared to native insulin. (F) Change in transmittance to monitor insulin aggregation kinetics, at 1 mg mL⁻¹ under continuous agitation at physiological conditions (37 °C, pH 7.4 PBS) (panels D–F were reproduced with permission from Xiong et al.¹¹⁸ Copyright 2020 The Royal Society of Chemistry) (G) T state protomer of B29–A1 single-chain insulin (gray; PDB code 6INS) overlaid with porcine insulin (PDB code 4INS). A and B chains are marked in green and blue, respectively, and three native disulfide bridges are in yellow. ThrA8 and TyrA14 side chain residues of the WT insulin are shown as red sticks. The C domains of proinsulin and SCIs are shown as a red dashed line and an asterisk. (H) Protein sequences of SCI-a, SCI-b, and progenitor SCI-c. The red lines represent the peptide bonds connecting the A and B domains to the C domain. The red letters mark the substitutions relative to WT insulin. The three native disulfide bonds are shown in gold; black arrows at the bottom mark the key sites of substitution: B10, B28, B29, and A14. (Panels G–H were reproduced with permission from ref 122. Copyright 1969, Elsevier.)

to both entropy and enthalpy effects.¹¹⁶ Vinther et al. attempted to increase insulin dimer stabilization by covalently linking B25 via a disulfide bond, but found the resulting protein to be biologically inactive.¹¹⁷ Later, they synthesized a new insulin variant containing an additional S–S bond between A10 and B4 (Figure 10A), as the two C_α atoms were in close proximity (5.4 Å) in the crystal structure (Figure 10B).¹¹⁷ From HPLC analysis, it was found that the novel insulin analog showed a remarkable change in hydrophobicity, owing to the substitution of Ile with Cys for the fourth disulfide bond, and due to shielding of hydrophobic residues. The addition of a disulfide bond resulted in a large increase in thermal stability (34.6 °C), likely due to the reduction in entropy owing to stabilization of the N-terminus of the protein, and inhibited fibril formation under high stress conditions for a prolonged period (Figure 10C). The 4SS insulin analog also had an increased insulin receptor affinity of 156% relative to human insulin owing to decreased flexibility of the N-terminus of the B chain upon introduction of the disulfide bond.

Xiong et al. synthesized a novel insulin analog with an extra interchain disulfide bond.¹¹⁸ Four insulin analogs were prepared: A22-B21, A22-B22, A22-B23, and A21-B22. Among these, the A22-B22-4SS-Ins (Figure 10D) displayed high stability and activity, as determined by a phospho-Akt assay (Figure 10E). The other linkages resulted in much reduced bioactivity (50- and 20-fold for A22-B21 and A22-B23-4SS-Ins, respectively, 4-fold for A21-B22) compared to native insulin. X-ray crystal structure revealed that A22-B22-4SS-Ins maintained the tertiary structure analogous to native insulin, and an insulin tolerance test in mice revealed similar *in vivo* activity of the new analog. Moreover, the A22-B22-4SS-Ins was found to resist aggregation for a prolonged period (42 h), allowing potential use as a more stable therapeutic insulin in formulations (Figure 10F).

Brunel et al. reported a general strategy to introduce a fourth disulfide bond in insulin in the presence of the other three disulfide bonds. Insulin B10 residue was found to be a favorable anchor site to obtain bioactive analogs.¹¹⁹ Other stable insulin

analogs include a chemically synthesized glycosylated insulin (disialo-glycoinsulin) with reduced aggregation propensity and improved serum stability, while exhibiting insulin receptor binding affinity comparable to native insulin.¹²⁰ Similarly, a methylene thioacetal human insulin analog (SCS-Ins), with enhanced serum stability and reduced aggregation propensity, was reported to be bioactive.¹²¹

Glidden et al. synthesized three 57-residue single chain insulin (SCI) analogs with several amino acid substitutions (Figure 10G,H).¹²² They linked the A- and B-chains using a C-chain (EEGPRR) which maintained the net negative charge on the protein at physiological conditions. SCI-a was the best insulin analog with similar in vivo mode of action as human insulin. It was a Zn free analog with the native insulin fold, as determined by X-ray crystal structure. The analog was found to be more stable ($\Delta G_U = 5(\pm 0.1)$ kcal/mol) in comparison to native insulin ($\Delta G_U = 3.3(\pm 0.1)$ kcal/mol). It was also bioactive for >140 days at 45 °C and >48 h at 75 °C, indicating strong resistance to aggregation. While human insulin was found to aggregate at 0.6 mM, SCI-a did not fibrillate even in the concentration range of 1–7 mM and remained primarily in the dimeric form. Surprisingly, SCI-b showed monomer retention property at all concentrations tested. Thus, these authors show ultrastable SCI as a therapeutic protein.

More recent developments have included the generation of mini-Ins, an insulin analog which acted with potency similar to human insulin.³⁹ Mini-Ins lacks the B-chain C-terminal octapeptide and is monomeric. Mini-Ins showed excellent in vivo properties and exhibited efficacy similarly to Lispro in controlling glucose levels. As it is monomeric, mini-Ins will be important for the development of prandial insulins. Its aggregation behavior, however, remains to be studied.

6. CONCLUSIONS AND FUTURE OUTLOOK

Diabetes, a common disease affecting millions, has drastically increased the need for insulin injections. This necessitates the safe and proper delivery of insulin to diabetic patients, which is a challenge, since insulin undergoes aggregation during its production and transportation, and also at the site of repeated injection in diabetic patients.³⁷ The inhibition of aggregation, although challenging, has been actively pursued using small molecules, peptide-based inhibitors, and supramolecular entities. However, no molecule has advanced to the clinic so far.

Currently, *m*-cresol and phenol are used as preservatives in insulin formulations to maintain sterility as well as to stabilize the hexameric state of insulin, thereby slowing aggregation. However, prolonged exposure to phenol in insulin injections may result in phenol-based toxicity like dysrhythmia, seizures, and coma, mainly affecting the central nervous system.¹²³ Moreover, injections containing cresols can cause gastrointestinal damage, skin problems at the site of injections, and even cell death.^{124,125} Weber et al. reported the cytotoxic effects of insulin formulations in vitro, which may be caused by excipients phenol and *m*-cresol.¹²⁵ These studies highlight the need to replace currently used excipients with molecules that have a better biocompatibility profile.

An alternative to small molecule inhibitors is to generate new insulin analogs with enhanced stability and resistance to aggregation. A radically different approach is to make insulin orally bioavailable, which is fraught with many challenges, although some progress in this direction has been made.¹²⁶ Nonetheless, it will be important to ensure that the alternate forms of insulin do not aggregate on storage. In this review, we

have discussed key features of insulin aggregation with emphasis on molecular details, which we hope will stimulate interest in the design of novel insulin aggregation inhibitors. These studies will be important not only for the inhibition of insulin aggregation but also to inform the development of aggregation inhibitors for other amyloid diseases.

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Notes

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DEDICATION

Dedicated to Prof. T. N. Guru Row on the occasion of his 70th birthday.

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