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Aggregation of Insulin at the Interface

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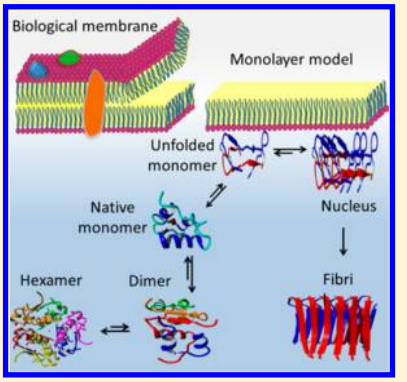
ABSTRACT: Insulin has so far been the most important pharmaceutical peptide for diabetes treatment, assisting to regulate carbohydrate and fat metabolism in patients. However, aggregation of insulin occurs readily in almost every biopharmaceutical process, ranging from production, purification, storage, transportation, delivery, to *in vivo* utilization at the terminal. As interfaces and surfaces are ubiquitous in each process and strongly influence physical/chemical properties of insulin, it is necessary and fundamentally important to investigate the aggregation of insulin at various interfaces, such as aqueous−solid interface, water−oil interface, and air−water interface. The objective of this article is to briefly summarize recent progress on insulin aggregation at different interfaces, with special focus on the air−water interface using the Langmuir monolayer technique.



1. INTRODUCTION

It has been known for several decades that failure to adopt or remain native conformations of some specific peptides or proteins can result in a wide range of human diseases.[1] The pathological conditions of these diseases are now known to be commonly associated with protein misfolding processes. More than 20 human peptides or proteins have been found to be able to misfold and develop aberrant self-assemblies *in vivo*, which are characterized by conformational conversion of soluble peptides or proteins into insoluble amyloid-like fibrils.[2] The misfolding peptides or proteins associated with serious human amyloidogenic diseases include amyloid-β peptide (Aβ) in Alzheimer's disease (AD), islet amyloid polypeptide (IAPP) in type 2 diabetes, α-synuclein in Parkinson's disease (PD), and prion protein in the spongiform encephalopathies.[3,4] Although these amyloidogenic peptides or proteins are not found to share sequence homology or related native structures with each other, their self-assembled fibrils possess strikingly similar characteristics (e.g., cross-β diffraction pattern, nucleation-dependent fibrillation, elongated morphology, Congo Red birefringence, and thioflavin T fluorescence), suggesting that the fibrillation process of these biomolecules may share a common molecular mechanism.[1,4,5] However, the current understanding of the mechanism and the structure evolution during aggregation or fibrillation is still very limited.

Insulin is a small polypeptide hormone composed of 51 amino acids with a largely α-helical structure in its native conformation. This hormone is produced by pancreatic β cells and stored predominantly as zinc-coordinated hexamers in the secretory granules within pancreatic islets. When released into bloodstream, insulin binds to its receptor in a monomeric form to regulate glucose metabolism in biological systems.[6] The insulin monomer has some hydrophobic amino acid residues exposed outside and tends to associate into dimer. The dimer-forming surface of the insulin monomer is almost flat and mainly composed by aromatic and aliphatic residues from the B chain: B8 Gly, B9 Ser, B12 Val, B13 Glu, B16 Tyr, B24 Phe, B25 Phe, B26 Tyr, B27 Thr, and B28 Pro.[7−11] This information is deduced from X-ray crystal structures or NMR structures of insulin or mutant insulin (PDB ID: 4INS, 2A3G, 2JV1, 1MHJ, 1MHI, 1MSO). The driving forces leading to dimerization are predominantly non-polar, reinforced and given direction by the antiparallel β sheet of hydrogen bonds.[11] Under certain conditions, such as higher insulin concentration, basic pH, and presence of zinc ions, three dimers associate to form a stable torus-shaped hexamer, in which both polar and non-polar residues are buried between the dimers.[11]

In vitro studies have shown that fibrillation of insulin can be induced under various conditions such as acidic pH, elevated temperature, hydrophobic interface, ionic strength, and mechanical agitation.[12,13] As its fibrils share common structures with other amyloidogenic proteins, insulin has been widely used as a suitable model system to fundamentally understand the pathologies of protein conformational misfolding diseases.[5,14,15] More than a model protein for fibril formation *in vitro*, insulin has been observed to develop fibrils *in vivo* near the sites of repeated insulin injection in the pathogenesis of some diabetic patients.[16] Insulin is also found in clinical practice to form aggregates immediately after injection in the bloodstream, exhibiting a reduced biological activity and an increased immunogenicity.[17] Therefore, it is necessary and crucial to fundamentally understand conformational changes that insulin undergoes in various conditions and the underlying mechanism involved in the reaction pathway.

Interfaces and surfaces are ubiquitous environments and play important roles in a multitude of physical and chemical

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processes, including biomedical engineering, catalysis, chemical sensors, and drug delivery.[18,19] As a large number of important physicochemical processes occur at the place of interfaces in biological systems, much attention has been drawn to the properties of biomolecules at interfaces, such as liquid−solid interface,[20,21] liquid−liquid interface,[22−26] and gas−liquid interface.[27,28] Adsorption of proteins at the interface often results in conformational changes, reducing the biological activity and physical/chemical stability of proteins.[29,30] This process has been shown to be very complex and can be influenced by many factors, such as electrostatic interactions, surface roughness and curvature, and hydrophilicity/hydrophobicity, to name a few.[30−35] In the case of insulin, the great interest for studies at various interfaces started from the early observation that insulin was vulnerable to change conformation and develop aggregates in storage vials, infusion pumps, controlled release devices, etc.[36−39] Since then, studies of adsorption, aggregation, and fibril formation of insulin have been performed at various interfaces, such as the aqueous−solid interface,[31,40−45] water−oil interface,[22,23,25,26] and air−water interface.[46−52] Although much progress has been achieved in the past few years, a molecule-level understanding of the detailed structure and property of insulin at the interfaces remains a great challenge.

The objective of this article is to provide readers an overview on the current state of our understanding of insulin aggregation at interfaces. We will first briefly summarize the recent understanding of the insulin fibrillation mechanism at a molecular level. Then, we will switch to the aggregation of insulin at the interfaces, with particular attention to the Langmuir monolayer technique at the air−water interface. Factors that may contribute to the aggregation or fibrillation of insulin at the air−water interface will be discussed, such as pH of subphase solution, zinc concentration, and presence of lipid. Finally, a summary of aggregation of insulin at the interfaces and outlook on the challenges and future studies will be given.

It is worth noticing that we summarize in this article the recent progress of insulin at the interfaces without distinction of the source of insulin used in experiments (human, bovine, or porcine). Due to the fact that the amino acid sequence of insulin is highly conserved within mammals, properties and functions of insulin from diverse species and even insulin mutants are very similar.[53,54] All of these insulin molecules undergo a similar chemical mechanism during the process of fibril formation in spite of the difference in speed of fibrillation.[54]

2. MECHANISM OF INSULIN AGGREGATION OR FIBRILLATION

A number of efforts have been devoted to elucidate the molecular mechanism of insulin aggregation or fibrillation by kinetic and structural studies.[5,13,41,45,54−60] Various kinetic studies have demonstrated that insulin fibril binds with dyes such as thioflavin T and Congo Red with characteristic enhancement of fluorescence emission.[13,41,54,58] Spectroscopic methods, such as Fourier transform infrared (FTIR) and circular dichroism (CD), have been widely used to monitor the protein conformation changes during fibrillation in aqueous solutions.[54,58,61] However, FTIR and CD provide limited information in structural studies, as the results from these spectroscopies only indicate the secondary structure changes, i.e., the components of α-helix, β-sheet, and random coil. Electron microscopy (EM) and atomic force microscopy (AFM) have been applied to directly visualize the morphologies in the pathway of insulin fibrillation.[60,62,63] Although the microscopic images are able to clearly reveal the morphology of insulin fibril architectures as unbranched, long, straight structures with periodic twists, neither EM nor AFM approaches atomic level resolution. More sophisticated and precise techniques such as small-angle X-ray scattering, X-ray microcrystallography, and solid-state nuclear magnetic resonance (NMR) spectroscopy have recently been emerging as new attempts to elucidate the atomic level structures of proteins in three dimensions.[55,59,63] However, the insoluble uncrystallizable nature of insulin fibrils is still severely challenging the applications of these structure determination methods.

Similar to other amyloidogenic proteins, fibrillation of insulin is proposed to occur under various conditions via the aggregation of partially folded intermediates through a nucleation mechanism.[14,56,57,64] The kinetic process of fibril formation in experiments is commonly characterized as an apparent lag period followed by an exponential growth regime, and a final plateau regime, as shown in Figure 1. The apparent

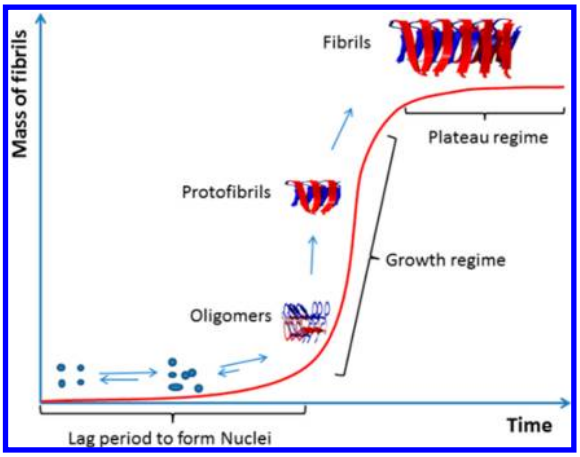

Figure 1. A typical kinetic graph of the insulin aggregation model.

lag period is mainly due to a reversible nucleation process, in which the amount and size of insulin aggregates (oligomers) are not significant enough to be detectable by current methods. Previous studies suggest that nucleation of insulin results from the simultaneous assembly of a few misfolded insulin monomers into oligomers via their hydrophobic surfaces.[13,57] Once the concentration of nucleation reaches its critical state, the exponential growth regime occurs, in which the subsequent addition of insulin monomers or oligomers to the nuclei elongates into long unbranched fibrils.[13] This eventually leads to the irreversible formation of large fibrils. When the concentration of insulin in solution falls below the threshold, a final stable plateau regime is achieved, aborting further fibril extension.

The driving forces transforming native monomeric insulins to oligomers and eventual mature fibrils in the process of insulin fibrillation are assumed to (1) minimize the exposure of the hydrophobic residues to aqueous environment, (2) saturate hydrogen bonding, and (3) reach an alternative non-native global free energy minimum.[60,64] It has been suggested that the initial step is probably to form a partially folded intermediate by a misfolding monomer (Figure 2), in which the hydrophobic residues, normally buried in the dimer and hexamer, become

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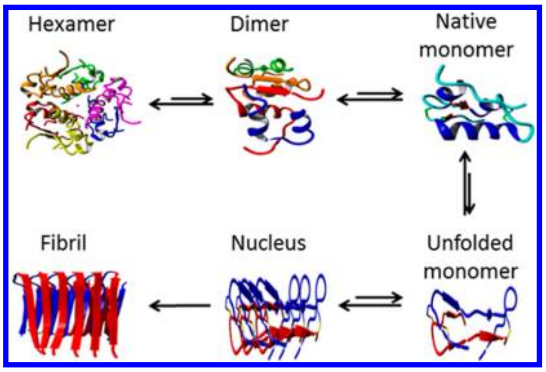

Figure 2. Schematic mechanism of the proposed pathway of insulin fibrillation (please notice that these are not X-ray crystal structures except hexamer).

exposed to solvent.[13,55] When two conformationally changed monomers assemble together, a shared antiparallel β-sheet structure is formed with the structure different from that of the normal insulin dimer. This shared structure in the assembly makes the monomer−monomer binding even stronger than the interaction involved in the dimer and hexamer.[65] The assembly evolves into an oligomer and forms a nucleus (Figure 2). The subsequent step of assembling insulin molecules to the nuclei may result from an effort to minimize the exposure of hydrophobic residues. This process eventually promotes fibril formation with rich cross-β structures, which are energetically favored through forming intermolecular and intramolecular hydrogen bonding.[60]

3. INSULIN AGGREGATION AT THE AQUEOUS−SOLID AND WATER−OIL INTERFACE

Like many other protein drugs, insulin is inevitably exposed to a diversity of interfaces, such as the aqueous−solid interface, water−oil interface, and air−water interface during production, purification, storage, delivery, and *in vivo* utilization. Particular interest of insulin at these interfaces is mostly based on observations that its adsorption and aggregation have long been recognized at the surfaces of syringes, infusion pumps, storage vials, and drug delivery materials, significantly decreasing the biological activity and stability of insulin.[36−39] Understanding the dynamics, properties, and structures of insulin exposed at the interfaces will be of great benefit to future development of insulin product. In the following part, we will briefly summarize the progress of insulin fibrillation studies at the aqueous−solid and water−oil interfaces. After that, we will focus on the air−water interface with the Langmuir monolayer approach.

3.1. Insulin Aggregation at the Aqueous−Solid Interface. It has long been suggested that aggregation of dissolved proteins can occur at hydrophobic interfaces in a general process.[41,44,66] This process is initiated by diffusion and adsorption of protein molecules to the interface. It is widely accepted that the adsorbed protein changes its conformation upon adsorption, and thus, some hydrophobic regions are exposed to contact with the hydrophobic interface.[44] Subsequently, the protein molecules in the boundary layer associate over these exposed hydrophobic regions, forming aggregates. The aggregates eventually desorb from the surface to aqueous solution and may serve as nuclei for further fibrillation.

In the case of insulin at the aqueous−solid interface, Sluzky et al. verified the above model that insulin aggregation initiates with conformational changes of the monomer at hydrophobic interfaces by UV−vis absorption spectroscopy, quasi-elastic light scattering, and mathematical calculations.[45,57] The driving force is mainly hydrophobic interaction between the monomer and the hydrophobic interface. Recent investigations with new techniques provide more evidence. For example, using Fourier transform infrared spectroscopy, attenuated total reflection spectroscopy, thioflavin T fluorescence measurement, dynamic light scattering, and atomic force microscopy, Smith et al. found that the nucleation and fibril growth of insulin at the hydrophobic polystyrene surfaces follow the model pattern.[41] More than insulin itself, fluorescein isothiocyanate labeled insulin,[67] mutated insulin,[67] and acylated insulin[68] also show high affinity with hydrophobic surfaces.

Compared to hydrophobic surfaces, a much longer lag time of aggregation of insulin has been observed in the presence of a highly hydrophilic surface. This phenomenon may be attributed to the less conformational change of insulin induced by the hydrophilic surface.[69]

Besides the hydrophobicity of a surface, other parameters also contribute to the aggregation rate of insulin at the aqueous−solid interfaces, such as surface roughness[31,40] and additive in the aqueous solution.[45,57,70] Pandey et al. recently demonstrated that a larger initial surface roughness results in a faster rate of insulin adsorption and aggregation.[31] Stabilizing additives such as zinc and non-ionic surfactants are able to inhibit conformational changes of insulin. Therefore, they can prevent insulin fibril formation at the hydrophobic interfaces.[45,57]

3.2. Insulin Aggregation at the Water−Oil Interface. Similar to the aggregation process of insulin at the aqueous−solid interface discussed above, one will expect that the aggregation can also happen at the boundary between water and non-miscible liquid, i.e., a water−oil interface. As both solvent molecules are able to move freely in their own phase and penetrate from one phase to the other to a certain degree, the water−oil interface is considered to be much more continuous than the aqueous−solid interface.[23] Therefore, insulin molecules dissolved in aqueous solution can easily reach the hydrophobic interface, promoting unfolding and eventually fibril formation. Indeed, the rate of insulin aggregation induced by the water−methylene chloride interface was found by Kwon et al. to be an order of magnitude higher than that at the aqueous−solid interface.[23] Some factors contributing to the rate of aggregation at the water−oil interface are determined as associated states of insulin (such as monomer, dimer, and hexamer), charge of surfactant (non-ionic and anionic), agitation, and presence of another polymer component in the organic phase.[23]

4. INSULIN AGGREGATION AT THE AIR−WATER INTERFACE BY THE LANGMUIR MONOLAYER APPROACH

The air−water interface possesses hydrophilicity from the aqueous solution and hydrophobicity from the air phase, sharing some features of both the aqueous−hydrophobic solid interface and the water−oil interface. Thanks to the Langmuir monolayer technique established and developed by Irving Langmuir, characterization of physical/chemical properties of various molecules at the air−water interface was made possible. This technique is a typical two-dimensional (2-D) surface chemistry approach, widely applied to investigate structures and properties of amphiphilic molecules at the interface, such as

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surfactants, proteins, lipids, and other materials.[47,48,50,71−73] A Langmuir monolayer is formed at the air−water interface when a one-molecule-thick layer of amphiphilic organic or inorganic matter is spread onto an aqueous subphase. Advantages of the Langmuir monolayer technique lie in the possibility of controlling both the intermolecular structure and ordering of the amphiphilic molecules via controllable variables, such as the surface pressure, surface potential, monolayer and subphase component, temperature, pH, and packing status.[74] Characterizations can be directly applied with ease at the air−water interface due to the accessibility from the air phase, such as UV−vis absorption, fluorescence, epi-fluorescence, infrared reflection−absorption spectroscopy (IRRAS), vibrational sum frequency generation spectroscopy (SFG), Brewster angle microscopy (BAM), and grazing incidence X-ray diffraction.[75] However, direct characterization at the aqueous−solid interface and especially water−oil interface is more difficult and challenging, and even impossible in some cases due to the shielding from the solid or organic phase. Furthermore, Langmuir monolayer of lipid is a well-accepted *in vitro* model to mimic biological membranes,[76] which can be considered as two weakly assembled Langmuir monolayers, as illustrated in Figure 3. In the following, we will focus on the aggregation of

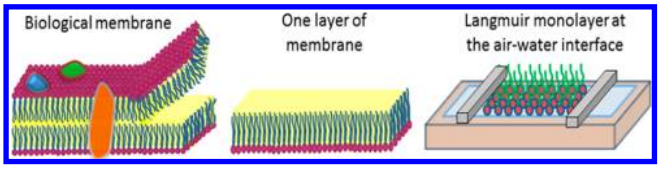

Figure 3. Cartoons of a biological membrane (left), one layer of membrane (middle), and Langmuir monolayer at the air−water interface to mimic the biological membrane (right).

insulin using the Langmuir monolayer technique at the air−water interface in the absence and presence of lipid monolayer, respectively.

4.1. Aggregation of Insulin at the Air−Water Interface in the Absence of Lipid Monolayer. Several studies of insulin aggregation have been investigated at the air−water interface under various conditions using the Langmuir monolayer technique combined with spectroscopy and microscopy.[49,50,52] When deposited at the air−water interface, molecules of insulin can stay at the air−water interface and form a uniform Langmuir monolayer due to the amphiphilicity of insulin. Before the monolayer is compressed, the distance between insulin molecules is so large that insulin molecules are extremely loosely packed. When compressed gradually, insulin molecules in the monolayer become closer and interact with each other. Therefore, the Langmuir monolayer is a good method to study intermolecular interactions induced by surface pressure.

Johnson et al. found that insulin spread from acidic insulin solution (pH 2) forms a homogeneous Langmuir monolayer at 20 °C at the air−water interface.[50] The conformational changes of the insulin Langmuir monolayer are monitored by infrared reflection−absorption spectroscopy (IRRAS), which is a good and sensitive technique to check secondary structure changes of protein molecules at the air−water interface. Results of IRRAS show that insulin adopts mainly α-helix and slightly β-sheet structures under different surface pressures at the air−water interface, but the spectra of amide I (1700−1600 cm^{-1}) and amide II (1600−1500 cm^{-1}) bands at the air−water interface are quite different from those obtained in aqueous solution. The spectra differences indicate that the air−water interface does change the conformation of insulin. Hydrophobic residues from insulin monomer probably misfold and are exposed to the hydrophobic air phase, while the hydrophilic residues are mainly submerged in the subphase of water. Furthermore, the air−water interface can anchor the misfolded insulin monomers from moving freely under a certain surface pressure (i.e., less freedom degree at the air−water interface than in aqueous solution).[75] As a result, it is probably more difficult or takes more time for insulin monomers to aggregate or form fibrils at the air−water interface. Actually, no insulin aggregates are examined in experiments during compression by fluorescein isothiocyanate labeled insulin epi-fluorescence or Brewster angle microscopy (BAM).[50] A similar homogeneous Langmuir monolayer without fibril formation is also observed by Pérez-López et al. using BAM at the air−water interface.[48]

Zinc is found to have a concentration as high as 11 mM in secretory granules in human pancreatic β-cells.[77] It has been known for many years that there exists a physical chemical relationship between insulin and zinc. Insulin is found to be stored predominantly as zinc-coordinated hexamers in the secretory granules of pancreas.[78] Therefore, it is necessary to study effects of zinc in the subphase on the aggregation of insulin at the air−water interface.[49,52,79] It has been previously known that zinc can induce the formation of dimers and hexamers in solutions.[45,80,81] Nieto-Suárez et al. observed using surface pressure−area isotherms that the presence of zinc ions has a profound effect on the lifting up molecular area and transition state at the air−water interface.[52] Liu et al. recently studied systematically the effects of zinc ions of the subphase on the aggregation of insulin at the air−water interface.[49] In this study, the insulin sample was first dissolved in either pH 2 HCl or pH 9 NaOH solution and then was spread at the air−water interface with different concentrations of zinc ions in the subphase. In both cases (pH 2 and 9), insulin molecules in the Langmuir monolayer are found to form aggregates (oligomers) at the air−water interface in the presence of zinc in the subphase. The aggregation process is confirmed by the secondary structure changes during compression, with a decreased component of α-helical conformation and increased component of β-sheet and β-strand structures.[49] When the insulin Langmuir monolayer is compressed to reach collapse in the presence of zinc in the subphase, long insulin fibers are observed at the air−water interface using BAM.[48]

Besides zinc concentration, other factors such as pH, ionic strength, and proteins in the subphase also contribute to the aggregation of insulin at the air−water interface.[52,75] Compared to the surface pressure−area isotherm of the insulin Langmuir monolayer at pH 5.7, lowering the pH to 1 results in a more expanded conformation of insulin with larger limiting molecular area, indicating more monomeric insulin present. Increasing the pH to 10 leads to the association of insulin molecules to form a more compact and rigid monolayer, shrinking the mean molecular area to a smaller number.[52] The larger ionic strength increases the molecular area occupied at a given surface pressure. This is probably due to the salting out effect: the presence of ions weakens the hydration of the polar residues of proteins, increasing intermolecular repulsion.[52,82] The presence of islet amyloid polypeptide (hIAPP) also contributes to the aggregation process of insulin due to the interaction and copolymerization between insulin and hIAPP.[75]

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4.2. Aggregation of Insulin at the Air−Water Interface in the Presence of Lipid Monolayer. Amyloid fibers isolated from patients are determined to have some lipid content.[12,83] Therefore, it is necessary to study how the formation of amyloid fibril is affected by the presence of lipid interface and surface. As the Langmuir monolayer of lipid is analogous to half of a biomembrane bilayer, it has been verified to be an excellent model to mimic *in vivo* conditions. Compared with a living cell, constituents in the Langmuir monolayer and subphase are known and can be controlled at ease, facilitating our understanding of the chemical/physical process at the interface of the lipid.

The property and aggregation of insulin have been investigated at the air−water interface in the presence of the lipid Langmuir monolayer, such as phospholipid and sphingomyelin.[46−48] Phospholipids containing phosphatidylcholine terminations are the main content of cellular membranes. Although sphingomyelin has a much lower content in mammals ranging from 2 to 15% in most tissues, it contributes significantly to the structural and functional roles in cellular membranes.[84] Pérez-López et al. investigated the behavior of the binary mixed Langmuir monolayer of phosphatidylcholine and bovine insulin spread at the air−water interface under various conditions.[48] They suggested that domain separation of phosphatidylcholine and insulin will probably promote insulin aggregation at the air−water interface. This assumption is supported by the observation that insulin aggregates are found on the monolayer edges and fractures using BAM.[48] In contrast with phosphatidylcholine, when sphingomyelin is present at the air−water interface, insulin does not form fibrils with various pH's and zinc concentrations in the subphase.[46] It is possible due to the miscibility and the strong intermolecular interaction between sphingomyelin and insulin associated states (such as dimer and hexamer), stabilizing insulin molecules from further fibrillation.

However, until now, there has been no investigation on the secondary structure and orientation changes of insulin when it interacts with lipid at the air−water interface, which will benefit our understanding of insulin aggregation in a biological environment. Methods such as polarization modulation infrared reflection−absorption spectroscopy (PM-IRRAS) and vibrational sum frequency generation (SFG) spectroscopy have recently been successfully applied to study the secondary structure changes of proteins at the air−water interface.[27,85,86] PM-IRRAS is a highly surface specific Fourier transform infrared method that is able to detect chemical compositions and orientations down to one molecule thick films at the interface. Compared with regular IRRAS, the polarization modulation at high frequency of PM-IRRAS can almost completely eliminate the background interferences from environmental factors such as atmospheric water vapor, carbon dioxide, and instrumental noise.[27,87] The elimination of water background absorption of IR is extremely important, as it overlaps with the IR absorption of proteins in amide I and amide II regions. SFG has recently emerged as a novel second-order non-linear optical technique, which allows us to obtain vibrational spectra at surfaces and interfaces with high surface selectivity and resolution.[88,89] SFG is generated by one visible beam at a fixed frequency ω_{vis} and another beam at a scanning frequency ω_{ir} in the infrared region with an observation of a sum frequency $\omega_{SFG} = \omega_{vis} + \omega_{ir}$. When ω_{ir} is equal to a vibrational level of the molecule, the SFG signal is resonantly enhanced. Due to properties of non-centrosymmetry at the air−water interface, SFG is sensitive only to molecules at the interface but not in bulk solutions. It is therefore capable to provide direct information about the structure and orientation of proteins at the interface.[85,86] Most recently, Yan's group at Yale University established vibrational SFG spectroscopy for the characterization of protein secondary structures. By probing the vibrational chirality of protein backbones at the interfaces, they found that the SFG signals of proteins are unique and sensitive to the secondary structures in amide I and N−H stretch spectra.[85,90] It is expected that PM-IRRAS and SFG will soon be used to characterize the structure and orientation changes of insulin aggregation in the presence of lipid at the air−water interface.

5. CONCLUSION AND OUTLOOK

In this article, the recent progress of insulin aggregation is briefly summarized at the aqueous−solid, water−oil, and air−water interfaces. The aggregation of insulin initiates with diffusion and adsorption of insulin at the hydrophobic solid or oil interfaces, resulting in conformational changes of the monomeric insulin to expose the hydrophobic residues.[41,45,57] The driving force is mainly hydrophobic interaction between the unfolded monomer and the hydrophobic interface.[44] Subsequently, the dissolved insulin molecules associate over the exposed hydrophobic regions, eventually forming aggregates. Compared to hydrophobic solid or oil surfaces, the hydrophilic surface induces less conformational change of insulin.[69] As a result, a much longer lag time of aggregation of insulin has been observed at the hydrophilic interfaces. To mimic a cellular environment, the Langmuir monolayer technique has been utilized to study the aggregation of insulin through intermolecular interaction in the absence and presence of lipid monolayer at the air−water interface. In the absence of lipid, insulin forms a homogeneous Langmuir monolayer on the pure water subphase without fibril formation at the air−water interface.[49,50,52] In the presence of a lipid Langmuir monolayer at the air−water interface, the component of lipid has a profound effect on the insulin aggregation: phosphatidylcholine promotes insulin aggregation, while sphingomyelin stabilizes insulin at the air−water interface.[46−48] Other factors in the subphase, such as pH, zinc concentration, ionic strength, and presence of other proteins, also contribute to the aggregation of insulin at the air−water interface.[49,52] Higher pH and concentration of zinc promote the aggregation of insulin.

Although some progress has been recently made on the aggregation of insulin at the interfaces, a large number of open questions still exist about the structural changes and pathways during the process of insulin aggregation. These questions include the following: (1) a molecular level understanding of the aggregation pathway from native insulin to insulin oligomers and mature fibers is still lacking; (2) once monomeric insulin molecules are adsorbed at various interfaces, it is still unclear to what extent these molecules can unfold, and what will be the conformation of the molecules after desorption from the interface; (3) the structure and misfolding pathway of transiently populated oligomeric insulin species remain to be detected and elucidated; (4) particularly at the air−water interface, the secondary structure and orientation changes of insulin are still lacking when insulin interacts with lipids and other proteins under various conditions of subphase. Although the first three questions are still challenging for the current techniques, the last one is expected to be answered in the near future by spectroscopic studies, such as polarization modulation

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infrared reflection−absorption spectroscopy (PM-IRRAS) and vibrational sum frequency generation spectroscopy (SFG).

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Roger M. Leblanc is a professor of chemistry at the University of Miami. He received his B.S. in chemistry in 1964 from Université Laval, Canada, and Ph.D. in physical chemistry in 1968 from the same university. From 1968 to 1970, he was a postdoctoral fellow in the laboratory of Prof. George Porter, FRS, in Davy Faraday Research Lab, the Royal Institution of Great Britain. His current research interest is to apply two-dimensional (2-D) surface chemistry combined with spectroscopy and microscopy to investigate the properties of biomolecules at the air−water interface. He is also interested in synthesis and properties of nanomaterials, such as quantum dots, gold nanoparticles, and graphene oxide.

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