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Study of the Aggregation of Human Insulin Langmuir Monolayer

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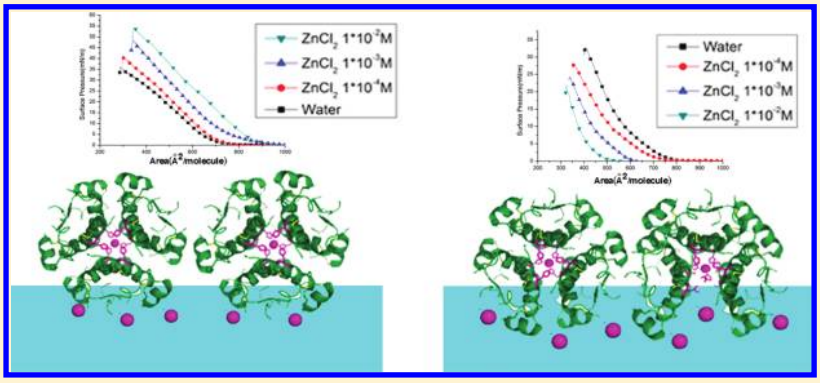

ABSTRACT: The human insulin (HI) Langmuir monolayer at the air−water interface was systematically investigated in the presence and absence of Zn(II) ions in the subphase. HI samples were dissolved in acidic (pH 2) and basic (pH 9) aqueous solutions and then spread at the air−water interface. Spectroscopic data of aqueous solutions of HI show a difference in HI conformation at different pH values. Moreover, the dynamics of the insulin protein showed a dependence on the concentration of Zn(II) ions. In the absence of Zn(II) ions in the subphase, the acidic and basic solutions showed similar behavior at the air−water interface. In the presence of Zn(II) ions in the subphase, the surface pressure−area and surface potential−area isotherms suggest that HI may aggregate at the air−water interface. It was observed that increasing the concentration of Zn(II) ions in the acidic (pH 2) aqueous solution of HI led to an increase of the area at a specific surface pressure. It was also seen that the conformation of HI in the basic (pH 9) medium had a reverse effect (decrease in the surface area) with the increase of the concentration of Zn(II) ions in solution. From the compression−decompression cycles we can conclude that the aggregated HI film at air−water interface is not stable and tends to restore a monolayer of monomers. These results were confirmed from UV−vis and fluorescence spectroscopy analysis. Infrared reflection−absorption and circular dichroism spectroscopy techniques were used to determine the secondary structure and orientation changes of HI by zinc ions. Generally, the aggregation process leads to a conformation change from α-helix to β-strand and β-turn, and at the air−water interface, the aggregation process was likewise seen to induce specific orientations for HI in the acidic and basic media. A proposed surface orientation model is presented here as an explanation to the experimental data, shedding light for further research on the behavior of insulin as a Langmuir monolayer.

1. INTRODUCTION

Insulin is a hormone with the crucial function of regulating glucose metabolism in the human body.[1] Human cells can be stimulated by insulin to absorb glucose from the bloodstream and store it as glycogen in liver and muscle.[2] Thus, insulin is an important protein in the control of blood glucose levels. Malfunction of this protein has the potential of causing diabetes, which had been a deadly disease in the past until insulin was discovered by Banting and Best in 1922.[3] Because insulin is so important for human metabolism, many properties of insulin have been well studied previously.[4,5] It has been confirmed that the active form of insulin is the monomer, which contains two peptide chains covalently connected by two disulfide bridges:[6,7] a hydrophilic chain named chain A built by 30 amino acid residues and the other hydrophobic one called chain B with 21 amino acid residues.[8] The secondary structure arrangement for insulin has also been confirmed before. The chain A forms two antiparallel α-helices, and the chain B forms α-helix, β-turn, and β-strand conformations.[9]

Although the insulin is effective as monomer, it is stored as a hexamer form with Zn^{2+} ions at the center of the assembly.[10] The hexameric structure of insulin contains 6 insulin

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monomers and 3 Zn^{2+} ions that are united by three equivalent dimers, each containing two insulin monomers and one Zn^{2+} ion.[9] In the bloodstream, the three structure levels of insulin, mainly consisting of monomers, dimers, and hexamers, are in equilibrium with each other.[11,12] Such equilibrium can be affected by different environmental conditions such as pH and temperature as well as by the concentration of insulin or metal ions.[13] In modern medical treatments for diabetes (especially type 1 and 2), most drugs that are used for helping patients with glycemic control are based on zinc-containing insulin in the hexameric state.[14] Therefore, research on the aggregation properties of insulin is of great importance for the development of medical treatment.

The Langmuir monolayer is a monomolecular film formed at the air−water interface by spreading a small amount of the compound of interest in an aqueous subphase solution.[15] It is an ideal in vitro model to study the target compound in biological membranes. In this two-dimensional system (2-D), the surface chemistry of the insulin Langmuir monolayer can be studied in terms of surface pressures and surface potential−area isotherms, compression−decompression isotherms, and stability measurements, while pertinent information regarding structure can be obtained through UV−vis, fluorescence, and FTIR spectroscopic methods. The common surface pressure environment of biomembrane is at about 30−35 mN/m,[16] which can be simulated by Langmuir monolayer approach. Insulin aggregation equilibrium has been studied through the Langmuir monolayer approach by using different experimental conditions such as the pH and the concentration of zinc ions in the subphase.

Although the mechanism of insulin aggregation equilibrium is not completely understood, several studies have shown that specific conditions can have an effect on the structural equilibrium.[17,18] These studies have been mainly performed in aqueous and crystalline phases of insulin; however, research has seldomly reported insulin evaluations at the air−water interface. Pérez-López et al.[19] have recently shown that the presence of Zn^{2+} ions in the subphase has a profound effect on the surface chemistry of the insulin Langmuir monolayer. Therefore, we have extended the study of the insulin aggregation process by comparing surface chemistry and spectroscopy of the insulin Langmuir monolayer in the absence and presence of Zn^{2+} ions in the subphase. In order to analyze the aggregation of insulin at the air−water interface, surface pressure and surface potential−area isotherms were compared at different concentrations of zinc ions in the subphase. Absorbance and fluorescence spectra were measured in 2-D using the same experimental conditions in order to analyze the structural changes due to insulin aggregation. A sample of insulin was taken as a reference to the 2-D approach, where insulin was dissolved in the aqueous phase at the same pH and Zn^{2+} ion concentration as in the subphase and then spread in 2-D. IRRAS spectroscopy was used to study human insulin Langmuir monolayer in order to gather more information on the secondary structure and surface orientation of the insulin aggregates. Aqueous solutions of HI under different environment parameters were also investigated by circular dichroism (CD) spectroscopy to observe the changes in the secondary structure of HI. Through our research we were able to confirm that insulin aggregation and its change on secondary structure and orientation at the air−water interface were induced by the presence of zinc ions in the subphase.

2. EXPERIMENTAL SECTION

2.1. Materials. Pure recombinant human insulin (HI), expressed in *S. Cerevisae*, is now commercially available from MP Biochemicals, LLC (Santa Ana, CA), with less than 1% zinc and a molecular weight of 5807. The isoelectric point of the HI is at pH 5.3. The zinc chloride was purchased from Alfa Aesar (Ward Hill, MA) with purity of 99%. All the pure water used in these experiments was obtained from a Modulab 2020 water purification system (Continental Water System Corp., San Antonio, TX) with a resistivity of 18 MΩ·cm and a surface tension of 72.6 mN/m at 20.0 ± 0.5 °C.

2.2. Methods. Surface pressure− and surface potential−area isotherms were determined using a Kibron μTrough S (Helsinki, Finland). Surface area was regulated through two symmetrical barriers controlled by computer software. Surface pressure was measured by the Wilhelmy method with a 0.51 mm diameter alloy wire probe with a sensitivity of ±0.01 mN/m. Surface potential was measured with a Kelvin probe consisting of a capacitor-like system. The vibrating plate was set at about 1 mm above the surface of the Langmuir monolayer, and a gold-plated trough acted as a counter electrode. The size of the Langmuir microtrough was 23 cm × 5.9 cm.

The UV−vis spectra for Langmuir monolayers were measured by a Hewlett-Packard (HP) 8452A diode array spectrophotometer. The in situ fluorescence spectra for Langmuir monolayers were measured by an optical fiber detector connected to a Fluorolog-3 spectrofluorimeter (Horiba Scientific, Edison, NJ). The slit widths for excitation and emission were each set at 5 nm. Both spectra were collected on the top of a KSV mini-trough (size 225 cm^2) with a quartz window located in the center of the trough. UV−vis and fluorescence spectra for the aqueous solutions of HI were measured by a Perkin-Elmer Lambda 900 UV/vis/NIR spectrophotometer (Norwalk, CT) and a Fluorolog-3 spectrofluorimeter (Horiba Scientific, Edison, NJ), respectively.

The IRRAS spectra were obtained using p-polarized light from a XA-511 accessory attached to a Bruker Optics (Billerica, MA) Equinox55 FTIR instrument with a mercury cadmium telluride liquid nitrogen-cooled detector. All spectra scans and baseline corrections were maintained in Bruker Optics' OPUS 3.0 software with parameters of 8 cm^{-1} resolution and 1200 scans for each spectrum.

The CD spectra were obtained using a JASCO J-815 spectropolarimeter (Easton, MD). The sample was examined in a quartz cell with 1 mm optical path length, and the scan range was in the far-UV region covering the region between 190 and 260 nm. The parameter of the scan was a response time of 8 s and a scan speed of 20 nm/min. The concentration of sample was 10^{-4} M. The Softsec software was used to estimate the second structure composition of the sample.

All experiments were performed in a clean room (class 1000) with constant temperature of 20.0 ± 0.5 °C and humidity of 50 ± 1%.

3. RESULTS AND DISCUSSION

3.1. Surface Pressure− and Potential−Area Isotherms: Effect of Zinc Concentration in the Subphase. Previous experiments have observed a change in the conformation of insulin aqueous phase with the variation of pH.[20] Taking such observations into account, we have conducted our experiments by using two different spreading aqueous solutions or samples, one at pH 2 and another at pH 9, into which ground human insulin (HI) was dissolved. All the experiments were performed with these HI samples, and the data were reproduced in the absence and presence of Zn(II) ions in the aqueous subphase (pH 5.6). The HI samples were prepared and spread without Zn(II) ions, and the $ZnCl_2$ solutions were only used as subphase so that the effect of Zn(II) ions at the air−water interface could be well addressed. Aqueous solution at pH 2 with HCl is typical for maintaining a stable acidic conformation of insulin. Pure water subphase was used due to the fact that $ZnCl_2$ precipitates at pH 9 and because of the adequacy of working at the same experimental conditions of the subphase. Pure water at pH 5.6 was thus used to prepare all the subphases

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differing in Zn(II) ion concentration. Figures 1A and 2A present the surface pressure (π)− and surface potential (ΔV)−

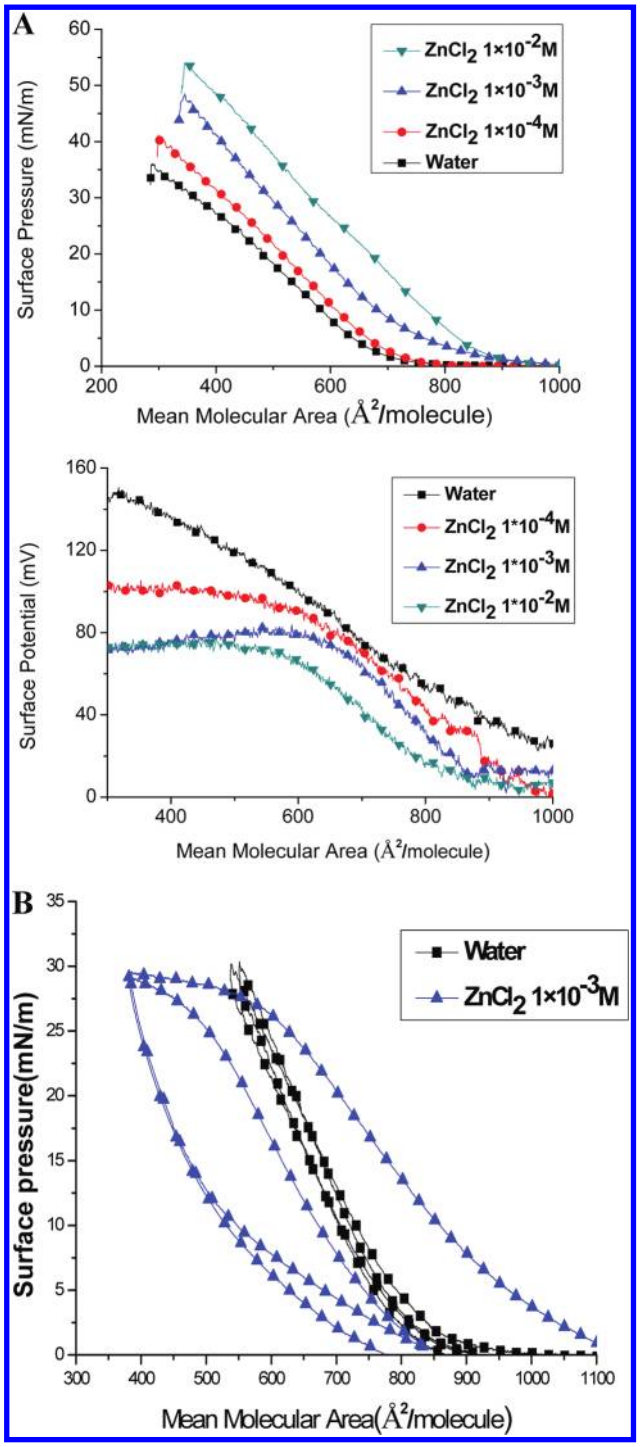

Figure 1. Surface pressure− and surface potential−area isotherms of recombinant human insulin aqueous solution (pH 2) spread at the air−water interface in absence and presence of $ZnCl_2$ in the subphase (A); compression−decompression isotherm cycles in absence and presence of $ZnCl_2$ in the subphase (B).

area (A) isotherms of the human insulin aqueous solutions prepared at pH 2 and pH 9, respectively, in the absence and presence of Zn(II) ions in the subphase.

Figure 1A shows that (i) the lift-off area per molecule increases with the increase in the concentration of Zn(II) ions;

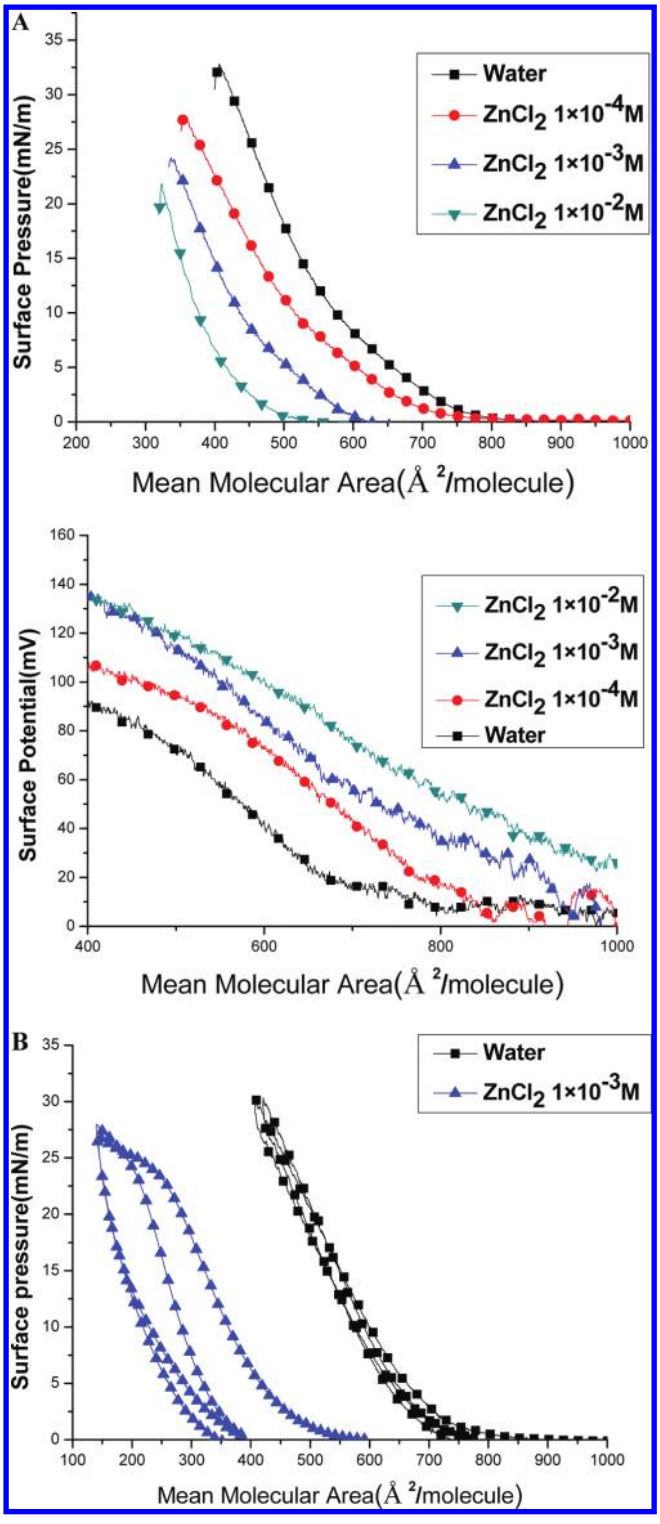

Figure 2. Surface pressure− and surface potential−area isotherms of recombinant human insulin aqueous solution (pH 9) spread at the air−water interface in absence and presence of $ZnCl_2$ in the subphase (A); compression−decompression isotherm cycles in absence and presence of $ZnCl_2$ in the subphase (B).

(ii) the slope of each isotherm in the region of 400−700 Å^2 molecule^{-1} is approximately the same, but the limiting molecular area, which indicates the closest packing of the human insulin molecule, increases with the concentration of Zn(II) ions, i.e., from 660 (pure water) to 700, 780, and 850 Å^2

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molecule^{-1}; (iii) the increase of Zn(II) concentration increases the surface collapse pressure, from 35 (pure water) to 40, 48, and 55 mN/m, respectively. From previous studies we can deduce that our data shows formation of aggregates at the air−water interface, i.e., dimers and hexamers.[21,22]

Figure 2A shows a behavior opposite to the one seen in Figure 1A: with the increase of the Zn(II) concentration, the lift-off (from 800 to 500 Å^2 molecule^{-1}), limiting molecular area (from 630 to 450 Å^2 molecule^{-1}) and surface pressure collapse area (from 425 to 250 Å^2 molecule^{-1}) are reduced. From the data we can see that the basic (pH 9) human insulin spreading aqueous solution (BI) had an insulin conformation different than the one in the acidic (pH 2) medium (AI), as shown in Figure 5 and Tables 1 and 2. The presence of Zn(II) ions has a great influence on the different conformations since both the basic (BI) and acidic human insulin (AI) aqueous spreading solvent on pure water surface give a similar surface pressure−area isotherm.

Table 1. Band Assignment of the FTIR and IRRAS Spectra Related to the Secondary Structure of Human Insulin in Aqueous Solution and as Langmuir Monolayer

band position, cm^{-1} (surface pressure, incident angle, pH)	band assignment
1630 (10 mN/m, 64°, pH 2)	β-sheet (amide I)
1655 (aqueous solution and 10 mN/m, 64°, pH 2)	α-helix (amide I)
1650 (5 and 25 mN/m, 60°, pH 2 and 9)	
1620 (aqueous solution, pH 2)	β-strands
1544 (aqueous solution, pH 2)	α-helix (amide II)
1550 (10 mN/m, 64°, pH 2)	
1525 (5 and 25 mN/m, 60°, pH 2)	
1560 (5 and 25 mN/m, 60°, pH 2)	
1570 (5 and 25 mN/m, 60°, pH 9)	β-sheet (amide II)
1720 (5 and 25 mN/m, 60°, pH 9)	β-turn

The surface potential−area isotherms of AI and BI are shown in Figures 1A and 2A. The trend observed in the π−A curves is followed by the ΔV−A isotherms when one increases the concentration of Zn(II) ions.

Figures 1B and 2B show the compression−decompression isotherms of the AI and BI in the absence and presence of Zn(II) ions in the subphase, respectively. For both AI and BI the compression−decompression isotherms (two cycles) in absence of Zn(II) ions were the same within experimental error (4.4%). However, a large difference in the hysteresis was observed in presence of Zn(II) ions, particularly for the AI cycles. For AI, there was certainly a high effect of Zn(II) ions on the conformation and orientation of the insulin in 2-D.

3.2. UV−vis and Fluorescence Spectroscopy of Human Insulin in Aqueous Phase and as Langmuir Monolayer.

Figures 3A−D show the photophysical properties of human insulin in two different phases: in aqueous solution (Figure 3A,B) and in Langmuir monolayer (Figure 3C,D). The controlled parameters were (i) pH, (ii) absence and presence of Zn(II) ions and (iii) surface pressure of the Langmuir monolayer.

UV−vis and fluorescence spectra of aqueous solution at pH 2 and 9 in the absence of Zn(II) ions showed tyrosine-corresponding peaks at 276 and 304 nm, respectively (Figure 3A,B), while the same peak maxima were seen in the presence of Zn(II) ions (10^{-3} M). A peak at 222 nm was observed in all insulin Langmuir monolayer UV−vis spectra. However, this peak is too strong to be observed in aqueous solution even when the concentration is very low. As reference, the pure tyrosine aqueous solution has been analyzed by UV−vis spectroscopy and shows both absorption peaks at 222 and 276 nm. Therefore, those two peak maxima are particular to the tyrosine absorption. This observation has been previously published (ref 23) for the tyrosine aqueous solution at pH 6.0. It is known that the band maxima corresponding to the tyrosine groups refer to the characteristic bands that are observed for phenol, which is a component of the tyrosine molecule. Regardless of the experimental conditions selected for the aqueous phase, a change in the band position was not observed. However, the shape of the spectra for the aqueous solution at pH 2 in the absence of Zn(II) ions and for the aqueous solution at pH 5.6 in the presence of Zn(II) ions differ from the one at pH 9 in the absence of Zn(II) ions. Such a difference in shape can be explained by a different conformation of the human insulin at these two pH values (see section 3.4).

Figures 3C and 3D present the UV−vis properties of the human insulin Langmuir monolayer at two surface pressures, 5 and 25 mN/m, respectively. From these figures, similar peak positions are identified in the UV−vis spectra when the Langmuir monolayer was compressed to 5 mN/m (Figure 3C) and 25 mN/m (Figure 3D) in absence of Zn(II) ions. However, in presence of Zn(II) ions a shift is observed from 224 to 234 nm, while the peak at 276 nm remains the same at both surface pressures (Figure 3C,D). The fluorescence spectra for the Langmuir monolayer were also examined (not shown), but it is difficult to clearly observe insulin fluorescence peaks due to the small amount and low quantum yield of tyrosine. From our results we have suggested a mechanism for the observed shift in the 224−234 nm range of the absorption spectra. In this mechanism, the Zn(II) ions would induce an aggregated state of the human insulin in 2-D, regardless of the pH and of the surface pressure imposed on the system. Hereon we have demonstrated the aggregation of human insulin induced by the Zn(II) ions in the subphase. To support the interpretation of our data, we have examined the effect that the

Table 2. Secondary Structure Estimation of the Aqueous Human Insulin CD Spectra Using Softsec Software

conditions		helix (%)	β-sheet (%)	others (%)	scale factor
HCl, pH 2	fraction	75	20	5	0.999
	standard error	1.8×10^{-3}	3.9×10^{-3}	3.2×10^{-3}	
NaOH, pH 9	fraction	61	33	6	1.000
	standard error	1.2×10^{-3}	2.9×10^{-3}	2.3×10^{-3}	
ZnCl, 2×10^{-4} M	fraction	69	23	8	0.999
	standard error	1.9×10^{-3}	2.2×10^{-3}	3.2×10^{-3}	
$ZnCl_2$, 10^{-2} M	fraction	59	22	19	0.999
	standard error	2.0×10^{-3}	3.3×10^{-3}	3.0×10^{-3}	

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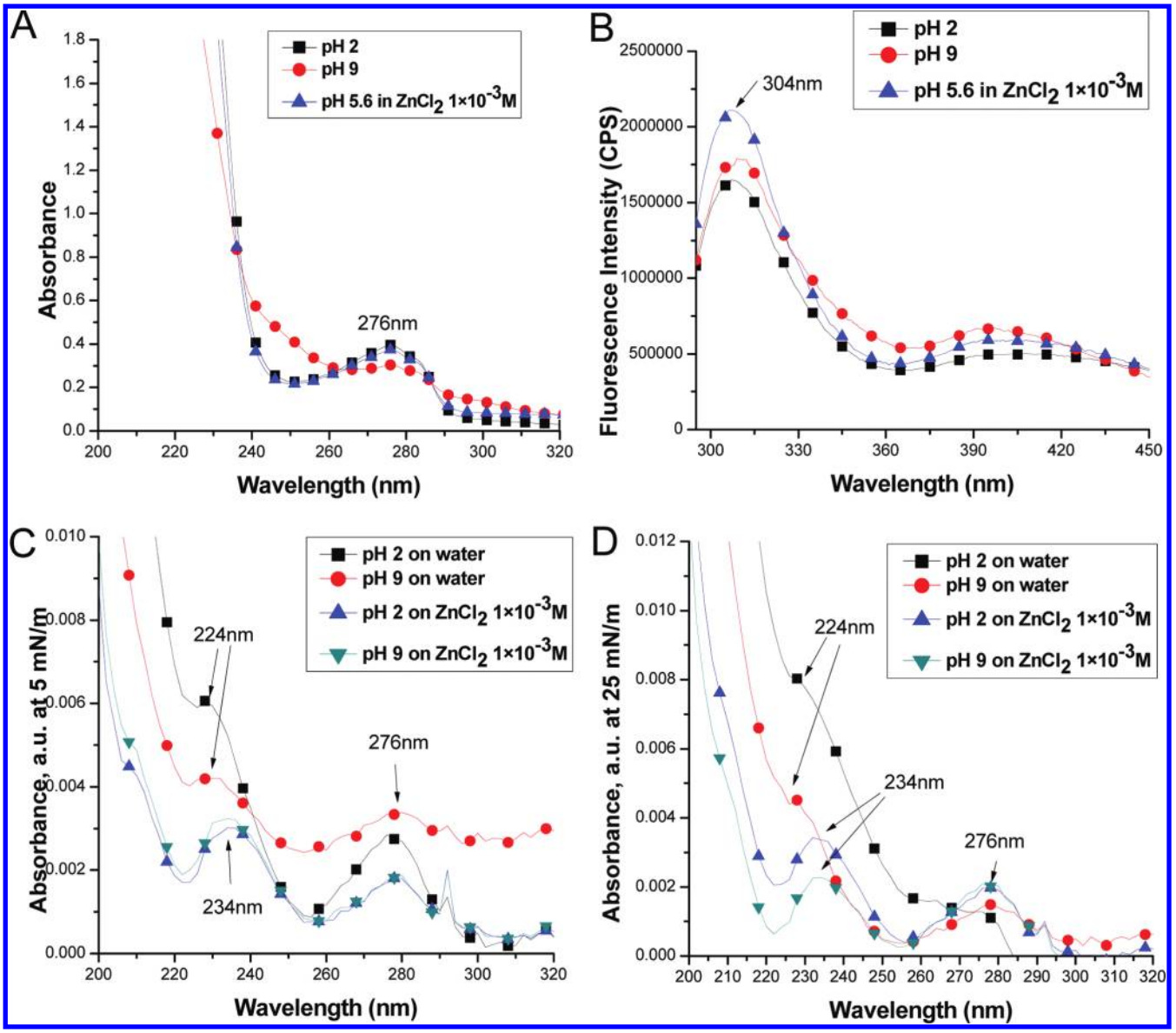

Figure 3. UV−vis and fluorescence (excitation at 270 nm, Raman at 297 nm) spectra of 10^{-4} M human insulin aqueous solutions (A, B) and UV−vis spectra of human insulin Langmuir monolayer at pH 2 and pH 9 in absence and presence of $ZnCl_2$ (10^{-3} M) in the subphase at 5 mN/m (C) and at 25 mN/m (D).

Zn(II) ion presence may have on the human insulin in 2-D and aqueous phase by employing Fourier transform infrared (FTIR) and circular dichroism (CD) spectroscopy, respectively.

3.3. FTIR and IRRAS of Human Insulin in Aqueous Phase and Langmuir Monolayer. Information on the secondary structure of protein in IR spectroscopy was first elucidated by Elliot and Ambrose in 1950,[24] who observed a correlation between the frequency of the amide I and amide II IR absorptions and the secondary structure of the protein. Through the use of both IR and X-ray diffraction techniques, such study had shown that the position of amide I and amide II IR absorption might have been influenced by the hydrogen bonding in the protein, which is closely connected to the protein's secondary structure in space.[25] For most proteins in aqueous solution, an α-helix-predominated structure will exhibit amide I and II absorptions in the spectral range from 1650 to 1660 cm^{-1} and 1540 to 1550 cm^{-1}, respectively, and a structure predominant on β-sheets exhibits similar absorptions at 1620−1635 and 1520−1535 cm^{-1}.[26] Figure 4 shows the FTIR (Figure 4A) and IRRAS (Figure 4B−D) spectra of human insulin aqueous solutions differing in pH in the absence and presence of Zn(II) ions (Figure 4A). Likewise, we have reported its Langmuir monolayer at a specific surface pressure, varying in the angle of incidence (Figure 4B) and at a specific angle, with p-polarization varying the surface pressure (Figure 4C,D).

Figure 4A shows two peaks at approximately 1544 and 1655 cm^{-1} which are attributed to the α-helix of the protein. Through the comparison of the aforementioned spectra, it was observed that: (i) The absorption peak positions for amide I and II in the aqueous solution at pH 9.0 were slightly lower in magnitude than the ones present at pH 5.6 and pH 2.0. This difference in magnitude can be related to the difference in protein conformation between basic and acidic media. (ii) Because the peak positions assigned to the α-helix structure are identified in acidic and neutral aqueous phase, we can deduce

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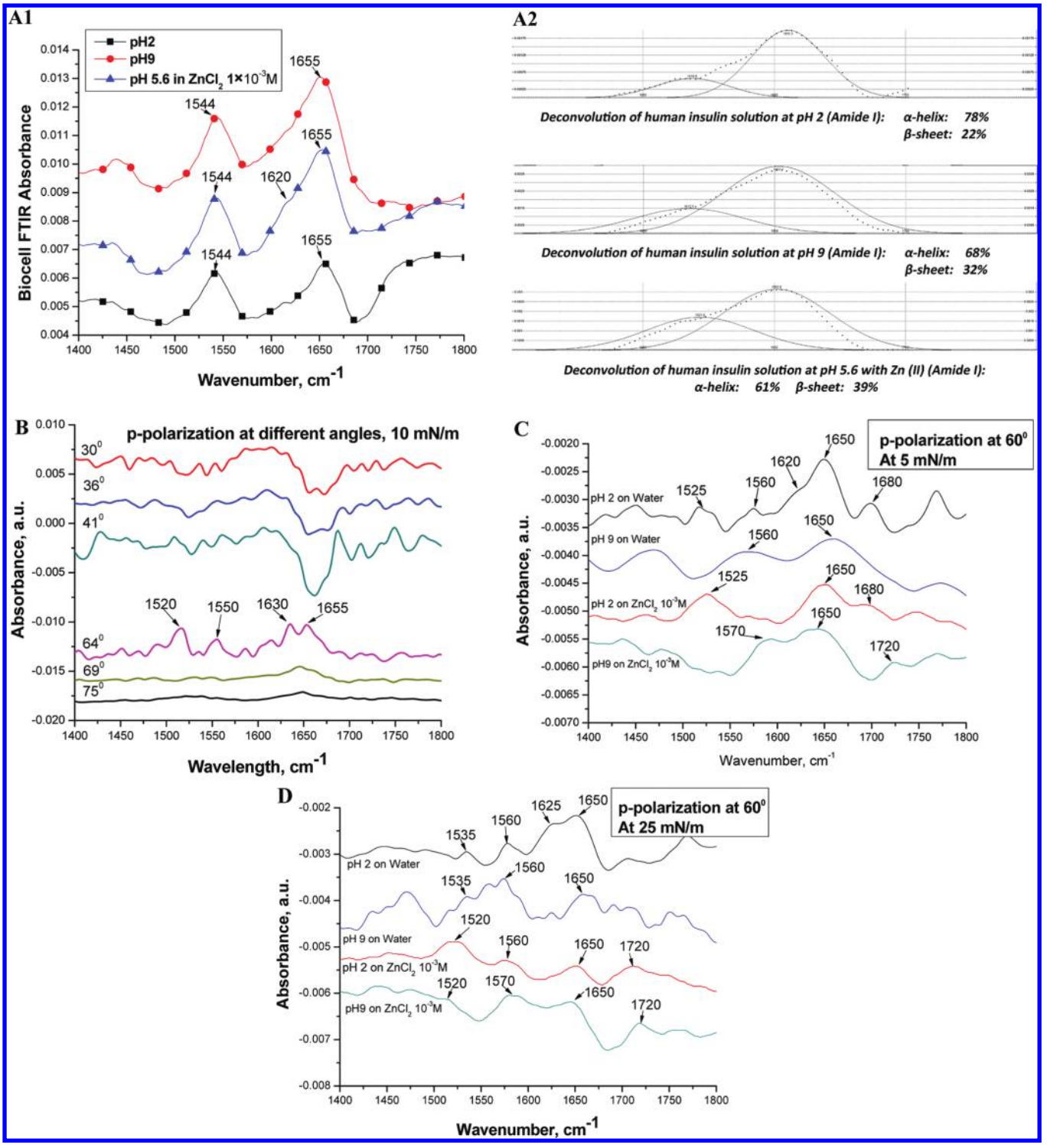

Figure 4. FTIR spectra of human insulin aqueous solution and IRRAS spectra of insulin Langmuir monolayer: Bio-ATR cell spectra of insulin (1.5 mg/mL) in absence and presence of $ZnCl_2$ at pH 2 and pH 9 (A1); Bio-ATR spectra deconvolution estimation of insulin aqueous solution in different conditions (A2); p-polarization IRRAS spectra of insulin Langmuir monolayer at a surface pressure of 10 mN/m and various angles of incidence at pH 2 (B); p-polarization at 60° IRRAS spectra of insulin Langmuir monolayer at pH 2 and pH 9 in absence and presence of $ZnCl_2$ in the subphase at surface pressure 5 (C) and 25 mN/m (D).

that the α-helix conformation is mostly maintained in the aggregates. (iii) The shoulder at 1620 cm^{-1} that is seen from the plot corresponding to human insulin at pH 5.6 in the presence of Zn(II) ions (Figure 4A) elucidates the formation of a hexamer, i.e., from the increase in the β-strand structure as aggregation occurs.[27]

Infrared reflection−absorption spectroscopy (IRRAS) has been used to analyze the insulin protein at the air−water interface. Through the analysis we have determined the orientation and the secondary structure of the protein in 2-D. Figure 4B shows the spectra taken at 10 mN/m at different angles of incidence (pH at 2). This was done in order to select

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the appropriate angle of incidence of the light with highest signal-to-noise ratio. At an angle of 64°, clear peaks of α-helix at 1550 and 1655 cm^{-1} and β-sheet at 1520 and 1655 cm^{-1} were obtained with highest resolution. Because of our observation, we selected an angle of 60° to measure the spectra at two surface pressures varying in pH and in the presence and absence of Zn(II) ions as shown in Figure 4C,D.

Figure 4 C shows the spectra of human insulin Langmuir monolayer at 5 mN/m at various pH values in the absence and presence of Zn(II) ions. A peak at 1650 cm^{-1} that corresponds to α-helix was observed in all spectra. Through further analysis of this spectra we have seen that: (i) The spectra of HI with basic spreading solvent (lines 2 and 4 from top) showed different peak maxima and shape from the spectra with acidic spreading solvent (lines 1 and 3 from top), indicating a conformational and orientational difference of the human insulin Langmuir monolayer that exists at different pH levels.[13] (ii) An increase in the quantity of β-strands on the solution in the presence of Zn(II) ions to the solution in absence of Zn(II) ions has been observed as a wider peak at around 1650 cm^{-1} as observed in the plot representative of the HI solution with Zn(II) ions. The new absorption peak at around 1720 cm^{-1} in the presence of zinc ions was assigned to β-turn structures. From the data (Figure 4C) we thus concluded that the hexameric state of insulin was formed at the air−water interface. (iii) The insulin Langmuir monolayer with acidic and basic spreading solvent had unique absorption peaks at around 1525 and 1570 cm^{-1}, respectively. This observation shows a significant difference between samples under acidic and basic conditions, implying that the human insulin Langmuir monolayers under different pH levels have different orientations at the air−water interface.

The spectra of human insulin Langmuir monolayer at 25 mN/m at various pH values in absence and presence of Zn(II) ions are shown in Figure 4D. In contrast to the spectra obtained at 5 mN/m, the spectra seen at 25 mN/m (Figure 4C) showed similar peaks; even though the intensity of each peak increased when the surface pressure increased, the position and shape remained close to each other. From such observation we can conclude that there were few, if any, conformational and orientational changes when the surface pressure increased. We can justify the small shift of the peaks in the spectra, e.g., from 1560 to 1570 cm^{-1} and 1525 to 1520 cm^{-1}, by the mechanism of intermolecular hydrogen bonding, which is induced by the increase of the surface pressure, making the peptide chains from different molecules close enough to interact with each other.[28]

Since the p-polarized IRRAS is more sensitive to the vibration perpendicular to the air−water interface,[29,30] pertinent information can be obtained on the orientation of the human insulin Langmuir monolayer in 2-D. Therefore, the IRRAS data were used to propose an orientation model of the human insulin Langmuir monolayer subject to different pH values of spreading solvent in the presence of Zn(II) ions in the subphase (see section 3.5)

3.4. CD Spectroscopy of Human Insulin Aqueous Solution. CD spectroscopy is an appropriate technique to measure the secondary structure of the protein molecule in the far-UV region (190−260 nm).[31] The CD spectra of human insulin aqueous solution are shown in Figure 5 at various pH and in the absence and presence of Zn(II) ions. Because of the precipitation of $ZnCl_2$ at basic conditions, one can only obtain the CD spectrum of human insulin with Zn(II) ions at pH 2

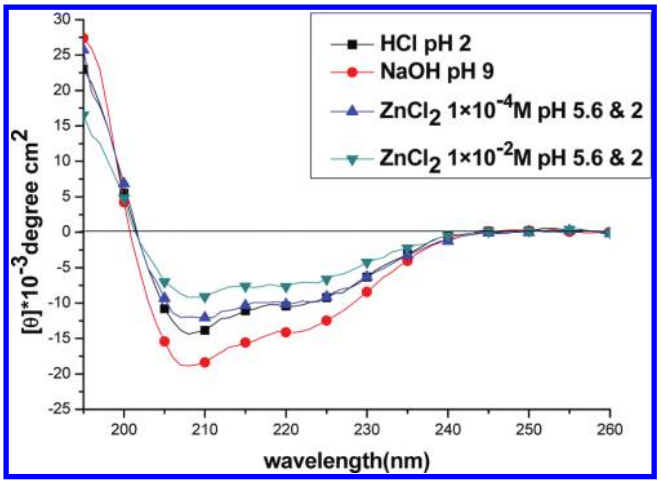

Figure 5. CD spectra of human insulin aqueous solutions at pH 2 and pH 9 in the absence of $ZnCl_2$ and at pH 5.6 in the presence of $ZnCl_2$ (concentration of solution 10^{-4} M; optical path length: 1 mm).

and pH 5.6, where they showed identical spectra in the figure. The CD spectra showed maxima at about 208 and 220 nm with a crossing wavelength at 0° of ellipticity at about 203 nm. The positions of the maxima confirm the importance of the α-helix of the protein in aqueous solution. Table 2 shows Softsec software estimation of the secondary structure of the human insulin protein in the different aqueous environments previously chosen. The data showed that the β-sheet conformation is more important at pH 9 than at pH 2. At low concentration of Zn(II) ions (10^{-4} M), one does not observe a significant change in the α-helix and β-sheet content, but at a higher concentration (10^{-2} M), the α-helix content is reduced in favor of other conformations, including β-strand and β-turn. This result corresponded to the deconvolution estimation in Bio-ATR spectra and can be justified by the fact that an induced aggregation on insulin reduces the α-helix and increases β-strand as well as β-turn content. This deduction is congruent to the works already reported[32,33] on the hexameric state of insulin, which showed more β-strands and β-turn conformations.

3.5. A Proposed Model of the Human Insulin Langmuir Monolayer. A proposed model of the human insulin (HI) Langmuir monolayer is presented in Figure 6 as an

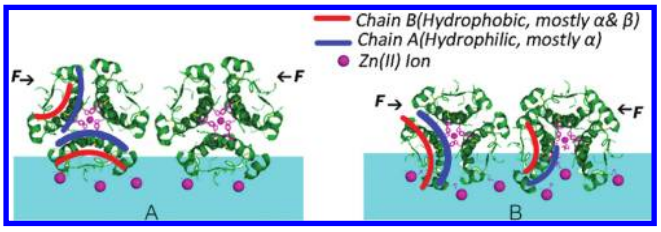

Figure 6. The proposed insulin hexamer orientation at the air−water interface with acidic (A) and basic human insulin samples (B).

explanation to the properties of insulin at different pH and Zn(II) ions concentrations. The following interpretation of data support our hypothesis: (i) From the surface pressure−area isotherms at pH 2 (Figure 1), one noticed that the limiting molecular area of HI Langmuir monolayer is significantly larger when Zn(II) was present in the subphase, which is a direct evidence that monomer of HI did aggregate into dimer or hexamer. This conclusion can also be confirmed by observing

peak shifting in UV−vis spectroscopy (Figure 3C,D). Furthermore, the pressure−area isotherm of pH 9 samples (Figure 2) shows that the limiting molecular area of HI Langmuir monolayer shrinks when Zn(II) was present in the subphase. On the basis of the fact that both acidic and basic HI monolayers were aggregated with Zn(II) in subphase but showing different surface properties, the only explanation for different limiting molecular area of HI Langmuir monolayer in acidic and basic conditions is the differences in surface orientation. We, therefore, assume that the major aggregated form of HI Langmuir monolayer is hexamer because different orientations of HI dimer have little effect on surface physical property. (ii) The proposed model of HI hexamer Langmuir monolayer at different pH is shown in Figure 6. When HI monolayer is formed in acidic condition, the orientation of hexamer is more likely to have hydrophobic chain B (Figure 6A, red curve) in contact with subphase. In this orientation, the hydrophilic chain A is not accessible to zinc ion from subphase so it is relatively more stable than the basic case. The limiting molecular area is expected to increase in this orientation, which corresponds to the surface pressure−area and surface potential−area isotherms in acidic condition (Figure 1). On the other hand, the basic HI hexamer Langmuir monolayer has a different orientation (Figure 3B). As shown in the model, chain A is partially submerged into the subphase in this orientation so that the hydrophilic chain A is open to the attack of Zn(II) ions. The basic condition make this attack easier to take place because the insulin in basic condition has a relatively loose structure due to the titration of the A1 α-amino group.[21] When chain A of HI was attacked by Zn(II) ions, the hexamer is easy to disaggregate and the chain A immerges more into subphase. This destruction process is irreversible and leads to lower limiting molecular area, which well interprets the surface pressure−area and surface potential−area isotherms in basic condition (Figure 2). (iii) This model is also confirmed by IRRAS spectroscopic data. As presented in Figure 4 and interpretation in section 3.3, it shows that the aggregated HI Langmuir monolayer at pH 2 has mostly α-helix and the aggregated HI Langmuir monolayer at pH 9 has more β-sheet and β-strands. In the CD analysis in section 3.4 we have discussed the aggregation effect that the formation of hexamer increases the percentage of β-sheet and β-strands in HI. However, this effect is not significant according to the secondary structure estimation of CD spectra (Table 2) and previous research.[32,33] Therefore, the significant differences between spectra of HI hexamer Langmuir monolayer in acidic and basic conditions can be only explained by the difference in orientation. At p-polarization, the IRRAS spectra were selectively sensitive to the vibration bands parallel to the air−water interface. As shown in Figure 6, the chain A (mostly α-helix) has a vertical direction in acidic orientation, where α-helix vibrations of amide I and amide II are most likely at parallel direction (A), while the chain B (mostly β-sheet and β-strands) sits in a vertical direction with chain A immerge into subphase and removed from surface in basic orientation, where β-sheet and β-strands vibrations of amide I and amide II are most likely at parallel direction (B). This model of HI Langmuir monolayer well explains the spectroscopic data and confirms our assumption that major aggregated conformation of HI is hexamer because the IRRAS spectra cannot be explained by HI dimer monolayer. Furthermore, when human insulin with basic spreading solvent forms a Langmuir monolayer, the submerged part of the chains in insulin are more prone to attack due to the abundance of Zn(II) ions from the subphase (Figure 6B). This can lead to a partial destruction of the insulin hexamer. This concept of the partial destruction of the insulin hexamer in basic condition can also be used to explain the data on the surface pressure− and surface potential−area isotherms. Overall, this model well explained all experimental data so far.

4. CONCLUSION

Several studies on insulin aggregation have been done in the aqueous and crystalline phases. However, such research done at the air−water interface has been seldom. We have reported the dynamics of insulin aggregation at the Langmuir monolayer including the effect that the aggregation has from Zn(II) in the subphase, while the secondary structural change in the aggregation process has also been reported. The aggregation of human insulin was confirmed by measurements of surface pressure−area and surface potential−area isotherms and spectroscopic data. In general, the aggregation dynamics of insulin as a monolayer has been observed to be particularly similar in the aqueous and crystalline phases.

Nonetheless, through the peculiar environment of the air−water interface we have observed certain contrasting properties on the aggregation process as the environmental conditions were changed. The infrared absorption and CD spectroscopy approach of our study has confirmed that the secondary structure of the insulin protein possessed a higher content of β-sheet and β-strands as aggregation occurred under basic conditions. On the basis of the experimental data, we have proposed a human insulin (HI) monolayer model of the insulin conformation and dynamics under acidic and basic conditions. Overall, we have shown the contrasting aggregation dynamics that human insulin has on pH at the air−water interface, shedding light for promising research on the effect that pH has on protein aggregation at cell membranes.

AUTHOR INFORMATION

Notes

The authors declare no competing financial interest.

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