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Insulin amyloid fibrillation studied by terahertz spectroscopy and other biophysical methods

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ABSTRACT

Assembly and fibrillation of amyloid proteins are believed to play a key role in the etiology of various human diseases, including Alzheimer's, Parkinson's, Huntington's and type II diabetes. Insights into conformational changes and formation processes during amyloid fibrillation are essential for the clinical diagnosis and drug discovery. To study the changes in secondary, tertiary, quaternary structures, and the alteration in the collective vibrational mode density of states during the amyloid fibrillation, bovine insulin in 20% acetic acid was incubated at 60 °C, and its multi-level structures were followed by various biophysical techniques, including circular dichroism (CD), thioflavin T fluorescence (ThT), dynamic light scattering (DLS), electron microscopy, and terahertz (THz) absorption spectroscopy. The experimental data demonstrated a transformation of α-helix into β-sheet starting at 26 h. This was followed by the aggregation of insulin, as shown by ThT binding, with a transition midpoint at 41 h, and by the bulk formation of mature aggregates after about 71 h. THz is a quick and non-invasive technique, which has the advantage of allowing the study of the conformational state of biomolecules and tissues. We first applied THz spectroscopy to study the amyloid fibrillation. At the terahertz frequency range of 0.2–2.0 THz, there was an apparent increase in both the absorbance and refractive index in THz spectra. Thus, THz is expected to provide a new way of looking into amyloid fibrillation.

Introduction

Protein misfolding and fibrillation is implicated in a number of human degenerative diseases, such as Alzheimer's, Parkinson's, Huntington's diseases and type II diabetes [1–3]. The process of fibrillation is accompanied by a conformational transition of amyloid proteins from their natural fold into a predominantly β-sheet secondary structure. This eventually leads to the formation of amyloid fibrils. Insight into the conformational changes occurring during amyloid fibrillation is critical for the development of drugs as well as for improvements in the stability of the proteins. The changes in the structure and the morphology of amyloid proteins during fibrillation have been investigated by a number of spectral methods and by electron microscopy. However, a THz spectral change in the collective vibrational mode density has never been utilized to study conformational changes during the amyloid fibrillation at the lowest frequency regime.

Insulin is a desirable model system for the study of fibrillation kinetics and conformation changes, because it is an important hormone and a drug for the regulation of blood glucose levels and is implicated in diseases such as type I and type II diabetes. Insulin is a 51-residue hormone and consists of two chains, that are predominantly α-helix structures linked together by two disulfide bonds [4,5]. Insulin exists as an equilibrium mixture of monomers, dimers, tetramers, hexamers, and possibly higher associated states in solution, depending on the environmental conditions. For example, it is predominantly monomeric in 20% acetic acid [6], dimeric in HCl solution [7], and hexameric in phosphate buffer [8]. It is well recognized that insulin can form amyloid fibrils under certain conditions, after undergoing three steps: nucleation, elongation and precipitation. The fibrillation kinetics and conformational changes will be influenced by temperature, insulin concentration, pH, and ionic strength [9,10]. At the clinical level, the ability of insulin to undergo fibrillation poses problems in its production, storage, and delivery.

Terahertz (THz) absorption spectroscopy, with its unique time-resolved feature and high signal-to-noise ratio, provides a new method for study of structural changes and conformational flexibility of biomolecules by using collective vibrational modes in the terahertz frequency range of 0.1–3 THz. Protein motion is

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thought to represent a superposition of many normal modes of vibration that link protein structure and function. The normal mode vibrations interact with electromagnetic radiation, since proteins are composed of polar and polarizable molecular units. THz absorbance agrees well with the density of low frequency vibrational modes predicted by normal mode analysis [11–14]. Therefore, THz absorption spectroscopy can be used to probe the normal modes, and hence, protein structure.

Several authors have used THz to investigate single amino acids, crystalline peptides, proteins, and nucleic acids. For example, THz has been used to study the conformational changes in native and thermally denatured bovine serum albumin [15], the role of flexibility in the function of cytochrome *c* [16], and the comparison of the dynamics of photoactive yellow protein (PYP) between the rigid and partially unfolded state [17]. THz is a quick and non-invasive method that provides information about protein structure without disruption, as the low energy of THz radiation does not induce any photochemical processes [18]. THz technology, therefore, provides a new way to look into insulin fibrillation and is expected to have a wider application in biomedicine, especially in human diseases that involve protein misfolding and fibrillation.

In the present work, we have probed the fibrillation of monomeric insulin, using a combination of ThT fluorescence, CD, DLS, and electron microscopy, to obtain detailed information of insulin kinetics, conformational changes, and morphology. The absorbance and refractive index of insulin monomers and fibrils were determined and analyzed by THz, and these results were compared with those derived by other techniques. THz showed promise as a new way of detecting the fibrillation of amyloid proteins and pathological tissues.

Materials and methods

Materials. Bovine insulin, a small monomeric protein with a molecular weight of 5800 Da, and thioflavin T (ThT) were obtained from Sigma. All other common reagents and solvents were of analytical grade from commercial sources. A 2 mg/mL bovine insulin solution was freshly prepared in 20% acetic acid. A 20 μM ThT solution was prepared in 20 mM Tris–HCl buffer, pH 7.2 and stored at 4 °C, protected from light to prevent quenching until used.

Fibrillation of insulin upon heat treatment. The 2 mg/mL insulin solution was incubated at 60 °C in a water bath without agitation. Aliquots from this solution were taken at desired time intervals for further analysis.

Far-UV circular dichroism (CD). Far-UV CD spectra were collected at room temperature using a Jasco 810 spectrophotometer (Jasco Inc., Tokyo, Japan) from 250 to 200 nm. Aliquots of insulin solution (200 μL) were injected into a 0.2-mm path length quartz cuvette. A background CD spectrum of 20% acetic acid blank solution was subtracted from the sample spectra for baseline correction. Spectra were recorded using a resolution of 0.5 nm and a scanning speed of 50 nm/min, with a response time of 1 s and a bandwidth of 1.5 nm. Spectra presented were an average of three consecutive measurements. The derivative analyses were performed on Microcal Origin™ 7.5 software.

ThT fluorescence. For determining the kinetics of fibrillation, ThT fluorescence measurements were performed on a Cary Eclipse fluorescence spectrophotometer (Varian Inc., Palo Alto, USA) at room temperature. Aliquots of insulin solution (10 μL) were added to 3 mL ThT solution, and fully shaken for a few minutes in a 1-cm path length quartz cuvettes. A background fluorescence spectrum was obtained by running free ThT solution. Each sample was excited at 450 nm, and the emission signal was obtained as the ThT intensity at 480 nm. The ThT readings were an average of 10 replicates.

Dynamic light scattering (DLS). Dynamic light scattering (DLS) measurements were performed on a BI-200SM DLS system (Brookhaven Instruments Corporation, New York, USA) equipped with a MGL-III model 100 mV He–Ne laser (λ = 532 nm), a computer-controlled BI-200SM goniometer, a BI-9000AT digital correlator, and a signal processor. Light scattering was monitored at a 90° angle and the temperature of the sample holder was controlled at 60 °C via a recirculating water bath. Insulin solutions were carefully filtered through a 0.45 μm membrane directly into a borosilicate glass tube and measured within 5 min. The particle size distribution (1–1000 nm) of species in the insulin solutions as a function of heating time was obtained using the CONTIN model.

Transmission electron microscopy (TEM). The morphology of insulin fibrils under different heating times were examined with an electron microscope (JEOL Inc., Tokyo, Japan) with an acceleration voltage of 100 kV. Samples of insulin fibrils were placed onto carbon-coated Formvar membranes supported by 300 mesh copper grids and negatively stained with phosphotungstic acid solution for 2 min. Excess stain was removed with the edge of a filter paper, and the grids were left to air dry at ambient temperature. TEM images were obtained with scale bars of 5 and 10 μm.

Terahertz absorption spectroscopy (THz). The THz measurements were performed at room temperature in a photoconductive switch-based Terahertz Time-Domain system, which consists of four parabolic mirrors in an 8-F confocal geometry, with a useful bandwidth of 0.2–2.0 THz and an amplitude signal-to-noise ratio greater than 1.5×10^4:1. The 8-F confocal system compressed the terahertz beam to a frequency-independent diameter of 3.5 mm and the THz radiation was excited by optical pulses from a mode-locked Ti-sapphire laser (λ = 800 nm, T_{FWHM} = 25 fs). The insulin powder was naturally placed, without applying additional pressure, in a silicon cell composed of two parallel, 0.64-mm-spaced, and 0.64-mm-thick windows. An identical empty cell was used as a reference during the data acquisition.

Results and discussion

Secondary structural analysis of insulin fibrillation

Far-UV CD was used to investigate the secondary structure and folding properties of insulin in 20% acetic acid as a function of heating time. Far-UV CD spectra of insulin obtained at various times of incubation are shown in Fig. 1A. The native insulin, without incubation, exhibited a typical spectrum of an α-helical protein, with the characteristic minima at 208 and 222 nm. Significant increases in the overall intensity of the CD signal were observed after 8 h heating, which might reflect the dissolution of insulin. As the heating time was further increased, transitions from α-helix into β-sheet occurred between 8 and 26 h. The heat-induced changes in the secondary structure during the fibrillation of insulin were monitored both at 208 nm (Fig. 1B) and 222 nm (Fig. 1C). For up to 72 h heating, no significant difference could be found in comparison with the 26 h spectra. However, after 72 h heating, the spectral shape shifted the minima from 208 to 212 nm, indicating an increase in β-sheet structure. After 120 h incubation, the β-sheet became the predominant structure, showing that α-helix characteristic peaks were changing to β-sheet characteristic peaks (218 nm).

The analysis of spectroscopic data by the phase diagram method is an extremely sensitive way to detect intermediate states. The procedure of the phase diagram method is to plot $I(\lambda_1)$ *versus* $I(\lambda_2)$ in a diagram, where $I(\lambda_1)$ and $I(\lambda_2)$ are the spectral intensity values measured at λ_1 and λ_2, respectively. The non-linearity of the $I(\lambda_1) = f(I(\lambda_2))$ then reflects the existence of structural transitions [19–21].

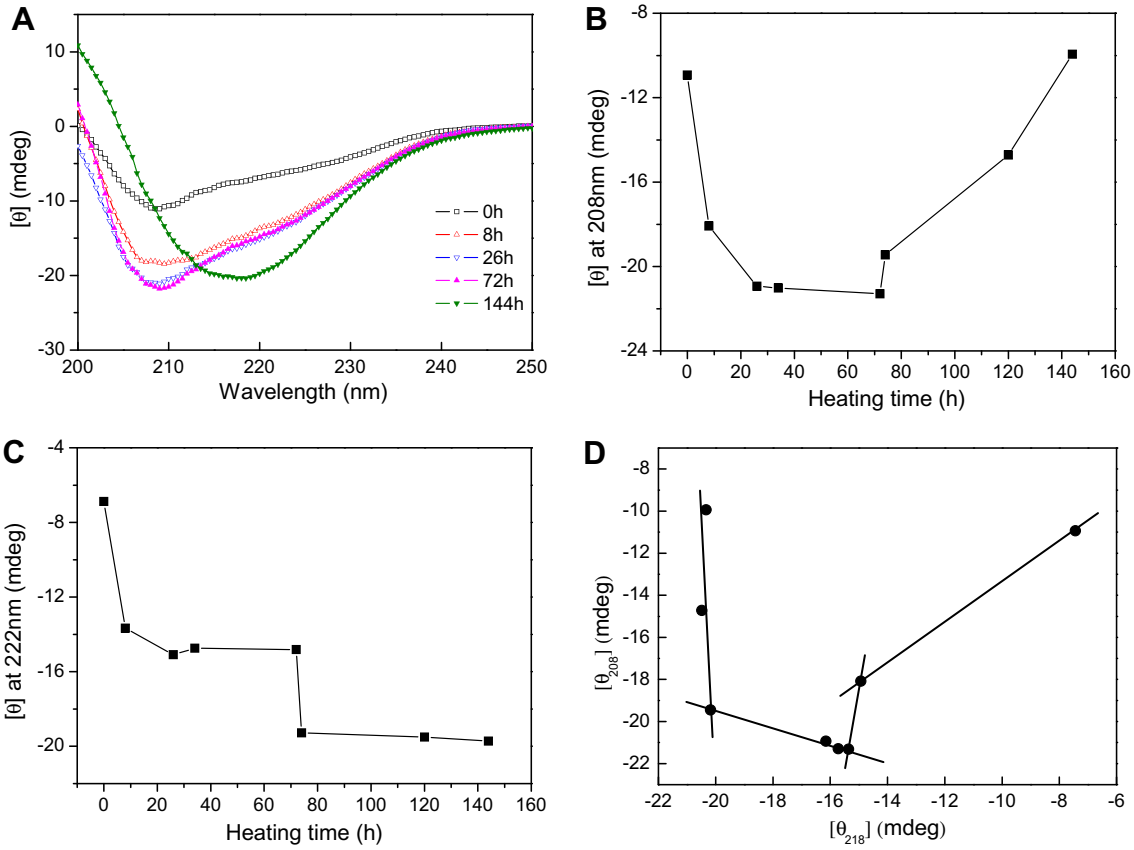

Fig. 1. Secondary structural changes of insulin fibrillation. (A) Far-UV CD spectra of insulin fibrillation. (B) and (C) Structural changes of insulin fibrillation monitored by at 208 and 222 nm. (D) CD phase diagram of insulin fibrillation.

Fig. 1D displays the phase diagram obtained by plotting ellipticity at 208 nm against 218 nm. Four linear segments, corresponding to: 0–8, 8–26, 26–72, and 72–144 h, were noted, indicating that there were at least four different structural transitions occurring during the process of insulin fibrillation. The initial region of 0–8 h represented the dissolution of insulin and an increase in α-helical conformation. The second transition (8–26 h) could be attributed to the changes within monomeric insulin in the nucleation phase, along with a continued increase of α-helical conformation. The third transition (26–72 h) showed the reduction of α-helix into β-sheet conformation. After 72 h, the conformation of the insulin solution was predominantly β-sheet. The analysis of CD data in the form of phase diagrams indicated that these changes in the secondary structure represented a stepwise process during insulin fibrillation.

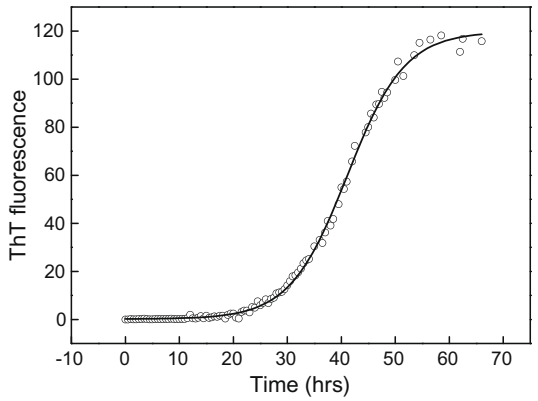

Fig. 2. Fibrillogenesis of insulin monitored by ThT fluorescence.

Kinetics of insulin fibrillation and stability of the tertiary structure

ThT fluorescence has been widely used to investigate the kinetics of the fibrillation process. Fig. 2 shows the changes in ThT emission (at 480 nm) of insulin in 20% acetic acid at 60 °C, with a transition midpoint at 41 h. The fibrillation kinetics displayed a sharp time-dependence, with a pronounced lag phase and a subsequent explosive growth of insulin aggregates, followed by the bulk formation of mature fibrils, corresponding to the nucleation, elongation and precipitation phases. No apparent changes appeared in fluorescence during the nucleation phase (with a lag time of ~30 h), after which the fluorescence began to rise rapidly, indicating the formation of amyloid-like fibrils. The bulk formation of mature fibrils formed after ~62 h, as the signal intensity of this measurement came to a maximum, suggesting that the binding site of insulin reached saturation and that no significant amount of nonspecific binding of ThT occurred [22].

The stability of the tertiary structure could be partially described by the transition midpoint of the ThT kinetics curve. The transition midpoint had been investigated under different conditions, such as insulin concentration, agitation, pH, ionic strength, and anions [6,10,23]. These results are summarized in Table 1. Exposure of insulin to acidic pH, high temperature, and agitation would more easily result in aggregation from native unfolded proteins into β-sheet rich ordered fibrils. Thus, the control of agitation, pH, and temperature were important for the stability of insulin.

Table 1
Summary of the transition midpoint of insulin fibrillation under different conditions.

Conditions	Transition midpoint (h)	References
2 mg/ml, 60 °C, 20% acetic acid, pH 2, without agitation	~41	This work
4 mg/ml, 37 °C, 20% acetic acid, pH 2, agitation	~10	[6]
2 mg/ml, 60 °C, 0.025 M HCl, pH 1.6, 0.1 M NaCl	~4.5	[10]
2 mg/ml, 37 °C, 0.025 M HCl, pH 1.6, 0.1 M NaCl, agitation	~10.5	[23]
2 mg/ml, 37 °C, 20% acetic acid, pH 2, agitation	~14	[6]
2 mg/ml, 37 °C, 0.05 M phosphate buffer, pH 7.5, agitation	~40	[23]

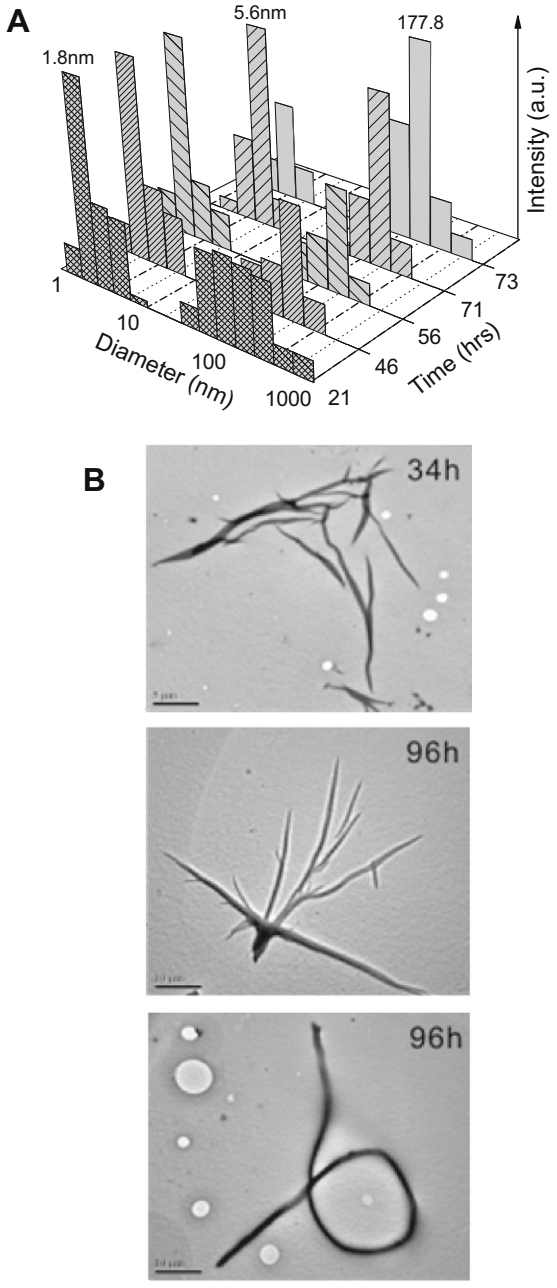

Fig. 3. Quaternary structural changes and morphology of insulin fibrillation. (A) Particle size distribution of insulin fibrillation detected by DLS system. (B) TEM images of insulin fibrils incubated at 60 °C for 34 and 96 h. (Scale bars represent 5 μm (34 h) and 10 μm (96 h), respectively.)

Analysis of quaternary structure and morphology of insulin fibrillation

Alterations in quaternary structure during insulin fibrillation were followed by DLS. Fig. 3A shows the particle size distribution of insulin plotted as a function of the heating time. After 21 h incubation at 60 °C, two discrete sizes of particles were identified by DLS as monomers and oligomers, with hydrodynamic radii (d_H) of 1.8 nm/5.6 nm and 177.8 nm, respectively. No significant changes in the concentration of monomers, oligomers, or nuclei were found between 21 and 56 h, whereas a slight increase in mature fibrils (>1000 nm) was later detected by TEM, corresponding to the telophase of nucleation and the elongation phase. The oligomers, with a d_H of 5.6 nm, were significantly populated at 71 h and decreased rapidly at 73 h. The dramatic transition taking place between 71 and 73 h was consistent with the results of CD spectra and corresponded to the bulk formation of mature insulin fibrils.

Our DLS data showed that the predominant insulin monomers, around 1.8 nm in diameter, coexisted with the oligomers (with d_H of 5.6 nm) and protofibrils (with d_H of 177.8 nm) for up to 21 h incubation. Stefan et al. found that the process of insulin fibrillation in water/HCl (pH 2) at 60 °C had a lag time of ~30 h. Additionally, their DLS data suggested the initial diameter of most of insulin molecules was around 3 nm, which agreed well with the diameter of the predominantly dimeric insulin, and particles around 250 nm in diameter were substantially produced after ~30 h [24]. Therefore, the size distributions obtained by DLS were different, due to the different solution conditions, which would alter the initial and the oligomeric states. In other words, insulin fibrillation was highly sensitive to solution conditions and to the primary structure of the protein.

To further evaluate the heat-induced changes, the morphology of the insulin fibrils was investigated using TEM. We observed mature fibrils after 34 h incubation, as shown in Fig. 3B. This was in good accordance with the DLS analytical results, which had showed a decrease in both monomers/oligomers and nuclei between 21 and 56 h, accompanied with the production of fibrils. Compared with fibrils after 34 h incubation, the mature fibrils after 96 h incubation were larger in size, and had a ribbon-like structure with a slight twist. From TEM analysis, it might be concluded that the size of fibrils increased with time in the absence of additional ionic salts, agitation and nuclei. It should be noted that the white points in the images are the holes on the TEM support membrane due to acidic corrosion by the 20% acetic acid.

THz spectra analysis of monomeric insulin and insulin fibrils

The THz absorption and refractive index spectra of insulin samples in the monomeric and aggregative states are shown in Fig. 4. The frequency range was set as 0.2–3.0 THz, since the THz signal was too highly attenuated to gain information above 3 THz. As shown in Fig. 4, it was easy to distinguish between insulin monomers and fibrils. Although there were no apparent absorption peaks observed in the THz spectra, a significant increase in absorbance after the formation of insulin fibrils was seen in the spectral range. The refractive indexes of insulin monomers and fibrils were stabilized at 1.20 and 1.44, respectively. However, the refractive index of insulin fibrils decreased slightly when the frequency was above 1.64 THz. Previous research has indicated that several proteins share similar spectral characteristics, lacking prominent

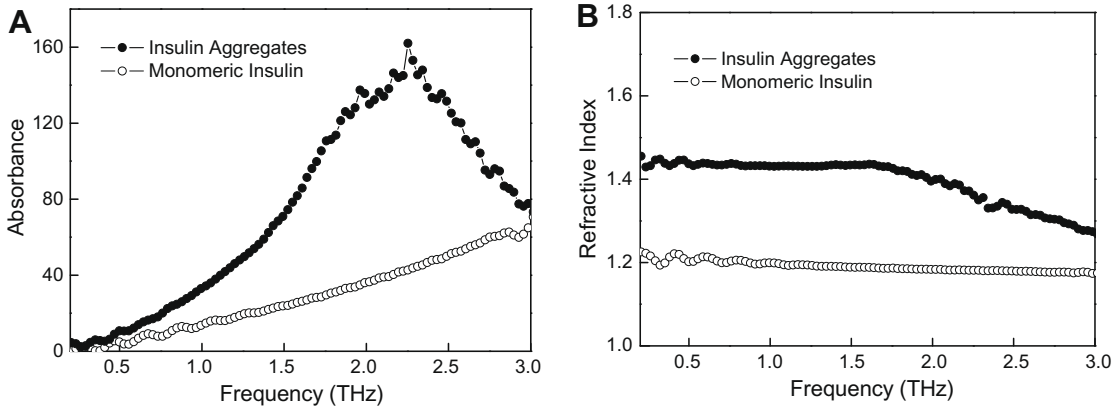

Fig. 4. The absorption coefficient (A) and refractive index (B) of insulin in the frequency range 0.2–3.0 THz at 293 K.

absorption peaks in THz range, such as thermally denatured BSA [15,25] and cytochrome *c* [16].

THz was expected to indicate the changes in intermolecular and intramolecular vibrational modes of the samples in powder form in this frequency range. The differences in THz spectra of insulin monomers and fibrils implied that the low frequency collective vibrational modes of insulin were changed greatly by incubation at 60 °C in 20% acetic acid. Low frequency vibrational modes are known to be related to hydrogen bonding and other weak interactions; hence, the THz technique would be sensitive to hydration [11], protein–ligand binding [26], photoactive processes [17], oxidation [16], denaturation [27,28], and fibrillation, which would provide unique information that is relevant to the secondary and tertiary structure of specific molecules.

Following the heating time, the formation of fibrils involved a significant increase in β-sheet structure, as revealed by CD. The mature fibrils observed in TEM indicated that the initial native monomers generated twisted fibrils. The intermediates were distinguished by DLS, while the kinetics of insulin fibrillation were monitored by ThT, which reflected significantly different underlying structures and identified the formation of amyloid fibrils. Either of these tools was required to prove that amyloid fibrillation had occurred. Even so, the molecular mechanism of amyloid fibrillation remained unclear and the detection of amyloid fibrils was only possible by in vitro examination.

The THz absorption spectra revealed that the dynamic transition was independent of either tertiary or secondary structure, in agreement with the results of He et al. [29]. Importantly, the details of dynamics transition, conformational flexibility, structural changes, and alteration in density of states derived from the original THz absorption spectra could be further developed by comparison with the results from other techniques. Qu et al. [28] determined that THz absorption spectroscopy was more sensitive to thermal changes of membrane proteins than FT-IR spectroscopy and it was not always necessary to correlate changes of the low frequency collective vibrational modes to the secondary structure changes. CD data showed that the conformation of an insulin solution was predominantly characterized by β-sheets after 72 h. The increases in low frequency collective vibrational modes and in the refractive index of insulin fibrils, therefore, implied that the tertiary structure of the fibrils had been strengthened during heat-induced fibrillation by hydrogen bonding or hydrophobic interactions.

In conclusion, the predominant structural transitions of insulin have been monitored by several techniques. This study has demonstrated that it is possible to distinguish between monomeric insulin and fibrils in the terahertz range by using either the absorption coefficient or refractive index spectra. We expect that the THz method will play an important role in future research into insulin fibrillation and will be further applied to the detection of amyloid aggregation.

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