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Terahertz Spectroscopy Analysis of Amyloid Fibrils Derived from Human Serum Albumin

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ABSTRACT

Amyloid fibrils, also referred to as protein fibrils, represent irregular assemblies of proteins exhibiting a cross-β configuration. Terahertz radiation, a segment of the electromagnetic spectrum spanning 0.3 to 10 terahertz (THz), is known to interact with these fibrils due to their cylindrical shape. This study's objective was to explore the potential of terahertz spectroscopy as a non-invasive means to detect the progressive growth of protein fibrils. In this investigation, human serum albumin (HSA) was synthetically induced to form protein fibrils. Confirmation of fibril formation was done by fluorescence spectroscopy, Fourier transform infrared (FTIR) spectroscopy, UV-Vis spectroscopy, and Terahertz time-domain spectroscopy (THz-TDS). To initiate the process, HSA was dissolved in distilled water at a concentration of 160 µM, followed by the addition of 60% ethanol to both samples for dilution. The HSA to Cu (II) ratio was maintained at 1:1 (160 µM) for stock solutions containing Cu (II). Subsequently, each set underwent a 6-hour heating phase at 65 °C to cultivate fibrils, which were then stored at room temperature for 30, 60, and 90 days before evaluation. Incubation of human serum albumin yielded amyloid fibrils as evidenced by ThT fluorescence, FT-IR spectroscopy revealed an emergence of fibrils within the amide I bands, extending from 1630 cm^{-1} to 1650 cm^{-1} and UV-Vis spectroscopy disclosed the augmentation of protein fibril absorbance over the incubation period. THz spectroscopy absorbance progressively heightened with prolonged heating of the protein fibrils due to the hydration shell surrounding them. Through these spectroscopic methods, it was elucidated that the fibrillation process led to the gradual development of β-sheet and unordered helix structures as well as the potential of THz spectroscopy as non-invasive tool for detection and monitoring of amyloidal diseases, promising insights into early diagnosis and treatment strategies.

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1. Introduction

Amyloid disease is a rare disease that occur when abnormal protein builds up and deposited in the organs and disturbed the normal function of human system. These diseases occur when proteins or peptides fail to fold correctly or preserve their native structure. Alzheimer, Parkinson, Mad Cow Disease and Diabetes Type 2 disease are some examples of the amyloid diseases. Proteins with a cross-β shaped structure known as amyloid fibril proteins are deposited as insoluble fibrils, mostly in the subendothelial space of tissues and organs as a result of the series of modifications in protein folding that lead to amyloidosis [1]. The genetic analysis, shape, morphology, degree of expression, or function of these disease-associated proteins are not particularly notable. All these proteins, however, misfold from their normal forms resulting in intermolecular β-sheet-rich structures that range in size from tiny oligomers to massive fibrillar complex. Staining with certain dyes, such as Congo red, thioflavin, and their variants, is the most common method used to identify amyloids [2].

A multidomain, globular protein with 585 amino acids is called human serum albumin, which comprises 35 cystine residues and joined by 17 internal disulphide linkages. Human serum albumin (HSA) carries neutral and acidic ligands throughout the body. Depending on the pH of the environment, it can take one among five isomeric forms that can change its morphology. Human serum albumin (HSA), a crucial transporter protein that binds different metal ions like copper, iron, calcium, and magnesium, also transports and stores fatty acids, bilirubin, hormones, and amino acids. Given that HSA normally have a stable α-helical structure, specific destabilising conditions that are common in partially folded species are required for HSA to fibrillate [3]. Consequently, under conditions that promote partially destabilised monomers and dimers, such as low pH, elevated temperature, and the existence of chemical denaturation of protein, human serum albumin aggregation is enhanced.

As mentioned before, normal physiological conditions do not cause protein aggregation, but some trigger variables can cause it to happen. One of the catalysts that most actively encourages protein aggregation is metal ion concentration. Protein misfolding and aggregation are encouraged by dyshomeostasis and the metal ions' formation of reactive oxygen species such as copper. Copper is an essential mineral for human life, and metal breakdown is what causes the elevated serum level that speeds up protein aggregation [4]. Aside from its role in protein aggregation diseases, Cu (II) is required for human body homeostasis. Numerous processes including the synthesis of energy, the stimulation of neuromodulators, the metabolism of iron, and the synthesis of neurotransmitters all include the utilisation of copper [5]. Adults need copper doses of 50–80 mg, and quantities higher than this are harmful. Depending on the protein and the physicochemical conditions, the fibril proteins, and peptides that Cu (II) binds have been found to have both enhancing and restraining effects on the fibrillation process [6].

In our study, we used FTIR, fluorescence, and UV-VIS spectroscopy as potent analytical methods to evaluate the novel material's molecular structure, emission properties, and absorbance characteristics under diverse experimental settings. The Fourier transform infrared (FTIR) method is used to determine the infrared spectrum of solid, liquid, and gas sample absorption, emission, and photoconductivity [7].

Besides fluorescence spectroscopy, Fourier transform infrared and UV-visible spectroscopy, terahertz time domain spectroscopy is one of the tools used to monitor and analysed amyloid fibrils. Terahertz spectroscopy is a technology to obtain spectrum data in the far infrared (approximately 3 – 100 wavenumbers). Terahertz radiation is made up of electromagnetic waves with frequencies in the range of 0.3 and 10 terahertz, also known as terahertz waves, terahertz light, T-waves, or THz in physics (THz) [8]. Figure 1 below shows a schematic diagram of the THz spectroscopy system. To limit

water vapour absorption by the THz radiation, measurements are normally done in an atmosphere that has been nitrogen-purged [9].

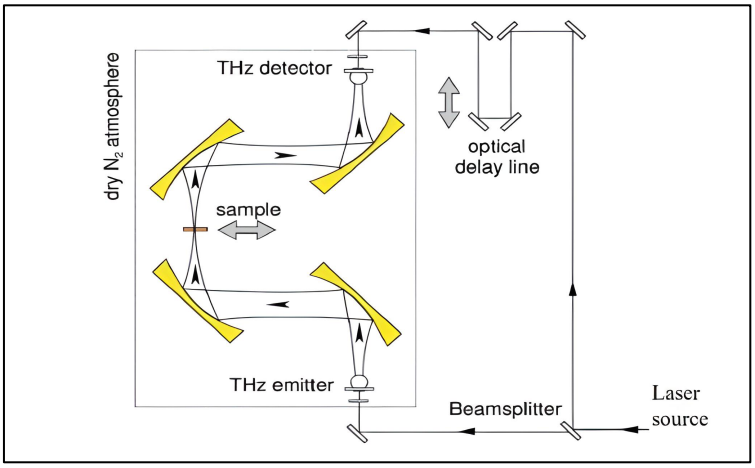

Fig. 1. Diagram of THz spectroscopy system adopted from Fischer [9]

Investigating the potential of THz spectroscopy to identify neurodegenerative illnesses in human brains is an intriguing topic due to the inherent capacity of THz rays to non-invasively permeate practically all materials [10]. Since amyloid fibrils are cylindrical and are known to scatter light in the THz spectral range, our findings suggest a non-invasive method to identify amyloid disorders in humans [11]. So, the objective of this project is to use the advent of THz spectroscopy, which is a relatively new generation of spectroscopic method to study the fibrillation stages of amyloid fibrils derived from human serum albumin with various copper concentration as catalyst for protein aggregation.

2. Methodology

2.1 Materials

Sigma Chemical Co. (St. Louis, MO) provided the Human Serum Albumin (HSA), Thioflavin T (ThT) and Copper II sulfate pentahydrate (CuSO4 ·5H2O) which were used exactly as received. Distilled water and 60% of ethanol also used throughout the experiment.

2.2 Protein Fibrillation

Distilled water was used to dilute HSA and 60% of ethanol was added to a concentration of 160 µM. Then, the stock solutions were divided into 2; one set contains no copper and the other stock solutions, 160 µM Cu (II) was added. The samples were diluted in phosphate buffer with a pH of 7.4 for each analysis. Each set was heated for 6 hours at 65 °C to produce fibrils, which were then stored for 30 days, 60 days and 90 days at room temperature before analysed [6].

2.3 Spectroscopic Analysis of Protein Fibrils

2.3.1 ThioflavinT (ThT) fluorescence assay

Thioflavin T is a dye that can be used to study the fibrillation kinetics of protein. In this method, ThT was added to the samples after incubation period at room temperature, and they were incubated for 5 minutes before being measured with a Cary Eclipse fluorescence spectrophotometer. The ThT

content in the working solutions was held at 2 µM while the samples of protein solutions with and without Cu (II) were extracted and diluted to a final concentration of 1 µM with phosphate buffer. Prior to taking measurements, samples were incubated for 10 minutes. The integration time was set at 60 s, the slit width remained at 5 nm, and the excitation and emission wavelengths were adjusted to 450 and 482 nm. The spectra were all corrected in relation to the corresponding blank before taking the reading [6].

2.3.2 Fourier transform infrared spectroscopy (FTIR)

The FTIR spectra of HSA were acquired using an FTIR spectrometer with a horizontal ZnS ATR accessory (Shimadzu A217051). A signal-to-noise ratio was attained by accumulating 200 scans at a resolution of 2 cm^{-1} for the spectra. Each sample solution was run three times with the sample concentration held constant at 160 µM before being averaged to create a single spectrum.

2.3.3 UV–visible spectroscopy

Shimadzu-TCC 240A UV-Vis spectrophotometer was used to collect optical absorption spectra in the wavelength range 350-700 nm at room temperature. The concentration of HSA was fixed at 5 µM. For all measurements, disposable polystyrene cuvettes with a 1 cm path length were used. As a control, cuvettes containing only distilled water were used. 80% of the cuvette was filled with the samples before loaded into the spectroscopy. Then, the reading was obtained using UV probe software.

2.3.4 THz time domain spectroscopy

THz spectroscopy measurements were executed at the THz lab of University Technology Malaysia (UTM) in Skudai, Johor using Batop TDS 1008. To lessen the amount of water vapour that THz radiation absorbs during the measurements, the atmosphere of spectroscopy is nitrogen-purged. The sample is placed between the THz emitter and the sensor along the course of the THz wave. The sample was exposed to a THz wave from the THz emitter, and the THz detector picked up the transmitted THz pulse. The signal from the THz detector will then be Fourier converted to produce the complex valued spectra of the THz waves using T3DS V5.0 software.

The frequency band of terahertz spectroscopy used in this study was 0.05-4.0 THz in which THz-TDS offers an unparalleled window into the unique spectral properties of materials. The spectral resolution was up to < 2 GHz, enabling us to discern subtle molecular signatures and intricate structural details with exceptional clarity. Moreover, THz-TDS achieves an impressive peak dynamic range of ≥ 85 dB, allowing for the detection of weak signals in the presence of strong interference. Lastly, the size of the spot generated by THz-TDS is a versatile 10 mm x 10 mm, facilitating both micro-scale investigations and larger-scale analyses.

3. Results

3.1 Fluorescence Assay

Human serum albumin fibrils were formed during heat treatment at 65 °C for 6 hours, followed by incubation time for 30 days, 60 days and 90 days. The formation of fibrils in heat-treated HSA was confirmed by a Thioflavin T fibrillar assay due to a stronger binding of Thioflavin T to the intermolecular β-pleated sheets that present in the fibrils.

The most distinctive and thoroughly researched feature of ThT is fluorescence intensification upon binding to fibrils. It is thought that the selective immobilisation of a portion of ThT conformers is what causes the substantial rise in ThT fluorescence. ThT's benzylamine and benzathiole can freely spin and share their carbon-carbon bond due to the relatively low energy gap in a solution. From this, it may be concluded that amyloid fibrils have ThT-binding sites that substituent "lock" the attached dye, enhancing ThT fluorescence [12].

We used ThT, an amyloid-specific dye, to measure the degree of fibrillation in HSA. When ThT binds to β-sheet-rich structures like amyloid fibrils, it exhibits intensified fluorescence and a pronounced blue shift in the emission spectra, going from approximately 510 nm to 482 nm when bound to amyloid fibrils [13]. HSA solutions incubated with and without copper II were investigated for its degree of fibrillation by using fluorescence intensity at 482 nm. Based on Table 1, it was shown that the fluorescence absorption intensity in samples containing copper II is more intense compared to the samples that does not contain any copper II There were also notable increase in the absorbance of protein samples with longer incubation period which might signify larger amount of protein fibrils. It can be concluded from this part of study that the criteria for maximum HSA fibrillation at pH 7.4 were as follows; (i) higher temperature (ii) presence of copper II and (iii) longer incubation period. This outcome is consistent with study that claimed copper (II) promotes HSA fibrillation [6].

Table 1
Fluorescence absorbance intensity in the presence and absence of copper II

	Absorption Intensity (a.u)	
Catalyst / Incubation time	(+) copper II	(-) copper II
30 days	0.140 a.u	0.070 a.u
60 days	0.165 a.u	0.074 a.u
90 days	0.180 a.u	0.077 a.u

3.2 Fourier Transform Infrared Spectroscopy (FTIR)

Since the beginning of 1970, FTIR spectroscopy was used to examine protein and amyloid fibrils. A study from Kanapathy *et al* showed that there was a presence of amino acid, which in turn proves the presence of protein at wavelength range of 1600 to 1700 cm^{-1} observed in all four spectrums of four different samples [14]. However, because it has long been believed that IR spectrum study and later NMR findings on fibrils contradict, FTIR has been relegated to the background in terms of fibril analysis [15]. As a matter of fact, the initial results demonstrated that fibrils exhibited a characteristic anti-parallel β-sheet structure, which is symbolised in the amide I band that is present between 1600 cm^{-1} to 1700 cm^{-1} [16,17]. The formation of the cross-β structure in HSA samples due to fibrillation is further supported by FT-IR results as reflected in Figure 2 (a) and (b)

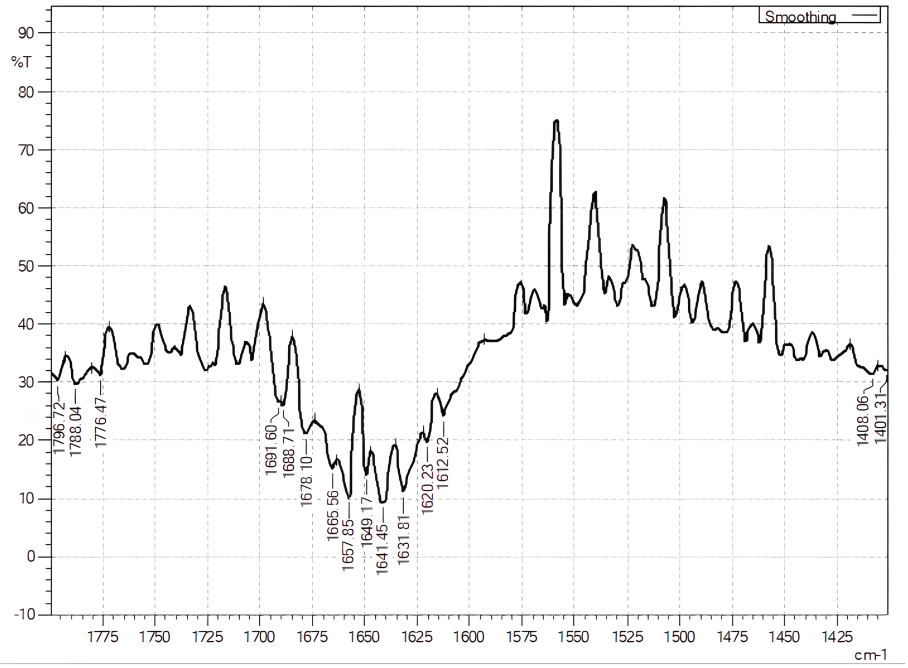

Fig. 2 (a). FTIR spectra of HSA fibrils formed in the presence of copper

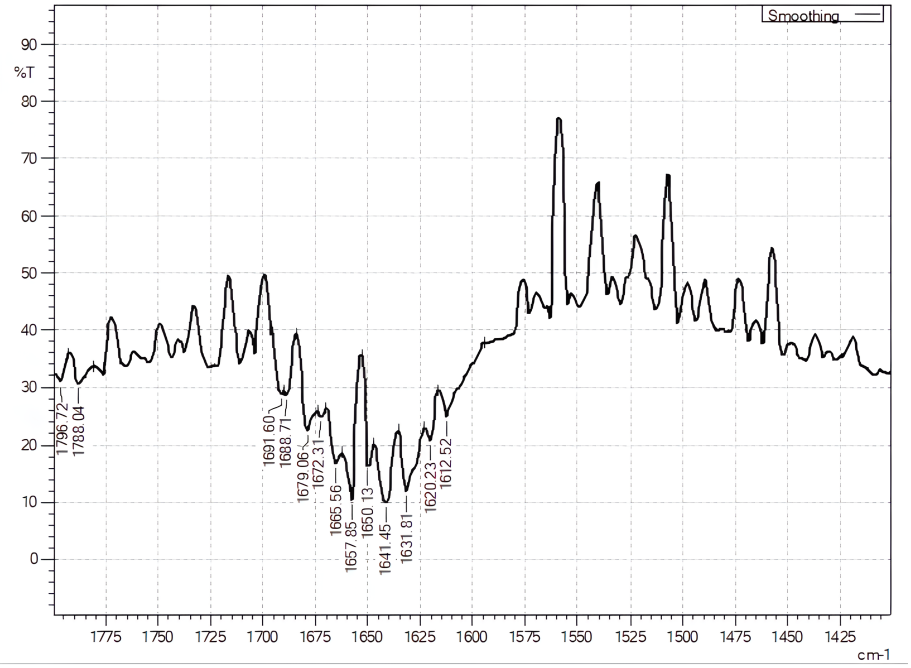

Fig. 2 (b). FTIR spectra of HSA fibrils formed in the absence of copper II

Here, we demonstrate that the amide I region of the infrared spectra of native β-sheet proteins and amyloid fibrils can be used to identify one another. Since FTIR's contribution to β-sheets has the highest absorption coefficient, it is suitable to study β-sheet proteins. The far-IR spectrum (400 cm^{-1}), the mid-IR spectrum (400–4000 cm^{-1}), and the near-IR spectrum are the three wavenumber ranges that make up the IR spectrum (4000-13000 cm^{-1}) [18]. The single bond region of the mid-IR spectrum is comprised of wavelengths between 2500 and 4000 cm^{-1}, the triple bond region is between 2000 and 2500 cm^{-1}, the double bond region is between 1500 and 2000 cm^{-1}, and the fingerprint region is between 600 and 1500 cm^{-1} [15]. As protein fibrils has double bond, the IR spectrum is between

1600-1700 cm^{-1} which is in the range of amide 1 band. The amide I band is used to analyze the secondary structure of proteins, including the distinction between parallel and antiparallel β-sheets because these structures often discriminate between pre-fibrillar structures and fibrils [19].

FTIR results showed that these β-sheets are generally distinct even though both structural types are clearly made up of β-sheet structure. Figure 3 (a) and (b) showed the formation of β-sheets & α-helix structure in the presence and absence of copper. From these data, we can conclude that there is formation of fibrils whether the copper is presence or not. This is because during heating process, there is disruption in the secondary structure of the protein. In summary, the spectral signature of native β-sheet proteins ranges from 1630 to 1643 cm^{-1}, whereas the amyloid fibrils peaks between 1611 and 1630 cm^{-1}. These variations are a result of intrinsic β-sheet properties, such as the tendency to aggregate into longer sheets, the generation of longer strands, and the formation of more planar sheets [20].

3.3 UV–visible Spectroscopy

A small quantity of protein sample is needed for the examination by the quick, non-destructive method of UV-Vis spectroscopy. Due to its adaptability and applicability, it is a frequently used technique found in most laboratories**.** One of the UV-Vis application is particularly for accessing protein conformational changes and aggregation, in combination with novel spectral analysis techniques. Both direct and indirect methods can be used to assess protein aggregation. The most widely used technique, known as direct, involves the straightforward detection of aggregates in solutions. An alternate technique evaluates protein structural changes as they occur during self-assembly [21]. Table 2(a) and (b) below showed the UV-Vis absorbance intensity for HSA sample with and without copper.

Table 2(a)
UV-Vis absorption of samples containing copper II

Incubation time	30 days	60 days	90 days
Absorbance (a.u)	0.0927 a.u	0.1131 a.u	0.3578

Table 2(b)
UV-Vis absorption of samples without copper II

Incubation time	30 days	60 days	90 days
Absorbance (a.u)	0.0402 a.u	0.0789 a.u	0.1185 a.u

Proteins usually show absorption maxima between 180 and 300nm due to almost entirely to π → π* transitions in the peptide bonds. The lateral chains of aromatic residues like tyrosine, tryptophan, phenylalanine, and cysteine absorb light in the near-UV range (240-295 nm). Tryptophan and tyrosine's absorbances are influenced by the microenvironment in which their chromophores are found, and they undergo a modest redshift when moved from a polar to a nonpolar environment [22].

Tryptophan and tyrosine, two aromatic amino acids, as well as cystine, are responsible for the absorption that was seen in this study which occurred at 256 nm [23]. From these data, the fibrils absorption has increased along with the increase of incubation time. Moreover, when comparison was made between samples that have the presence and absence of copper II, the absorption is slightly intense in the samples containing copper II. Incubation with copper (II) is generally reported to cause further destabilization in the secondary structure composition [6] which signify that copper accelerated the disruption of the secondary structure.

3.4 THz Time Domain Spectroscopy

Terahertz has a non-ionizing radiation for non-invasive biological applications and biomedical imaging due to its low photon energy which is insufficient to ionise atoms or molecules or to heat materials [24]. The absorption coefficient is a measure of how much light is absorbed by a material at a particular wavelength. In the context of detecting protein fibrils using MATLAB, the absorption coefficient can be used to quantify the amount of protein present in a sample [25]. Png, G., and friends observed THz absorbance of protein fibrils from 0 THz up to 5 THz and found that all graphs showed a large peak at ~3 THz. This was consistent with a study by Novelli *et al.* that discovered protein fibril solutions exhibited better absorption and index of refraction than native protein solutions. Hence, it a very exciting prospect to utilize THz technology to non-invasively diagnose neurological diseases in healthcare setting.

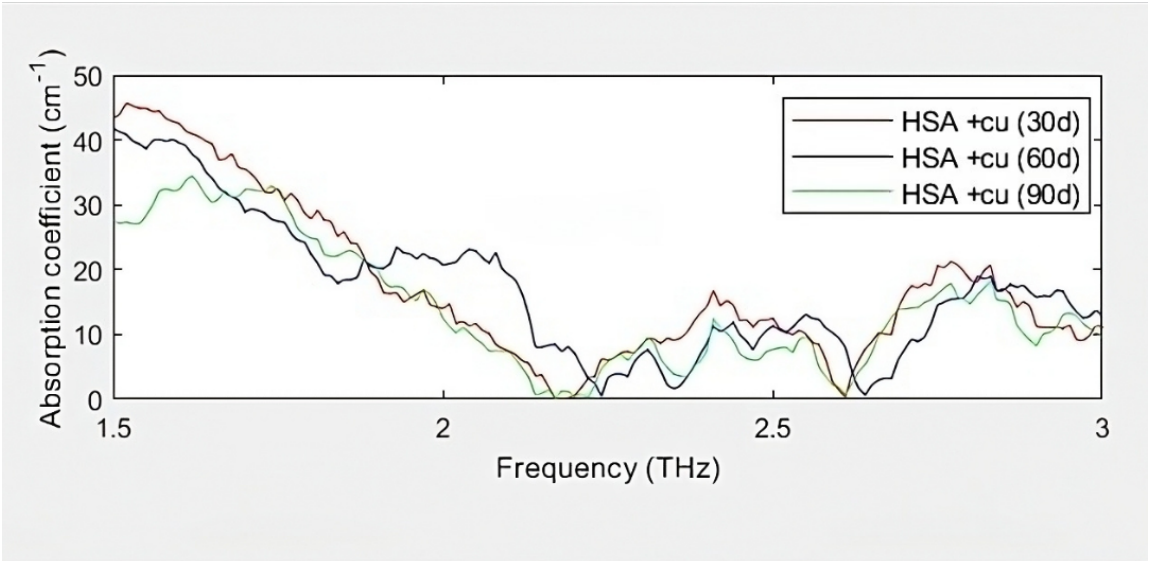

Fig. 3(a). THz absorption coefficient in samples with copper II

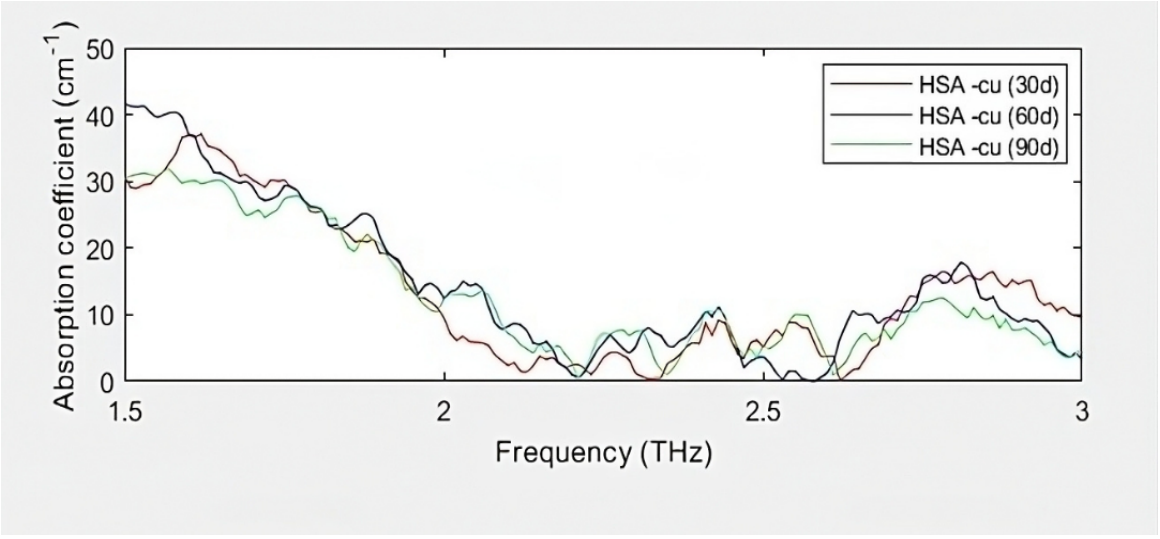

Fig. 3(b). THz absorption coefficient in samples without copper II

Based on the data shown here, it was apparent that from 1.5 to 3.0 THz, there appears to be a slight increase in the THz absorbance of the fibrils containing copper II whereas, there was also

uniform trend of increasing THz absorbance with longer heating time of the protein fibrils. These results were in accordance with other studies that stated was found that the interaction of 6aJL2-R24G with Cu (II) decreases the thermal stability of the protein and accelerates the amyloid fibril formation. This suggests that copper can increase the absorption coefficient of protein fibrils by promoting their formation [6,26].

We have determined how the collective hydration of water changes during the fibrillation process (native → intermediate →fibril) of the protein human serum albumin using terahertz spectroscopy. Terahertz (THz) frequencies (0.1–10 THz) are strongly absorbed by water, making this wavelength a highly sensitive probe for modifications to the hydrogen - bond network [23]. The behaviours of the water molecules close to the surface will be significantly impacted by charges on the surface of the macromolecule, and this will slow down their motion, changing the THz absorption spectra. These are just a few of the common occurrences at the interface of a macromolecule in solution. The contributions of both individual molecules of water and higher domains to the THz absorption spectrum, including the hydrogen bonding network are impacted by protein-water interactions, which go beyond these tightly bound water molecules. [27-29].

4. Conclusions

These spectroscopic techniques have shown that the process of fibrillation is followed by a gradual increase in the β-sheet and unordered configuration at the expense of the α-helical conformation. Protein fibrils samples in this study have been analysed by ThT fluorescence, Fourier Transform Infrared Spectroscopy (FTIR), UV-visible spectroscopy and terahertz time domain spectroscopy. Due to their accessibility and ease of analysis, the spectroscopic techniques described here continues to be the most popular method as the integral study of protein and peptide self-assembly processes. External variables like pH, temperature, salt, and metal ions all had an impact on how the protein aggregated to form protein fibrils. In our case, the fibrillation process was enhanced by the presence of copper II. It can be concluded that this study had contributed towards more in-depth knowledge gained from studying protein fibrillation and aggregation, that further translated in better understanding of significant and rapidly expanding class of neurological disorders that affect life expectancy and quality of life. THz spectroscopy is a technology that is constantly growing. It is one of the cutting-edge and revolutionary new tools for medical diagnosis. Although there has been significant progress in THz detection and tissue imaging, there are still a few problems that prevent its widespread application, including expensive prices, a lack of adequate discriminative accuracy, data analysis, and interpretation. It is safe to expect that these challenges will be overcome in a few years, though, given that THz methods and devices are still in the research and development stage and they are constantly being improved upon. THz spectroscopy has a promising future in medical diagnosis especially in detecting neurodegenerative diseases. Lastly, the implementation of this project will allow the spectroscopic analysis of amyloid fibrils in the far-end infra-red wavelength of electromagnetic spectrum which is the terahertz spectral region.

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