



Contents lists available at ScienceDirect

Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy

journal homepage: www.elsevier.com/locate/saa

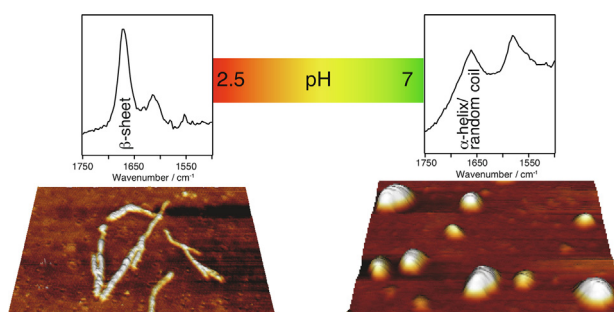
pH-dependent disintegration of insulin amyloid fibrils monitored with atomic force microscopy and surface-enhanced Raman spectroscopy

Erwan Y. Darussalam^a, Orsolya Peterfi^{b,1}, Tanja Deckert-Gaudig^{a,b}, Ludovic Roussille^a, Volker Deckert^{a,b,c,*}^a Leibniz Institute of Photonic Technology (IPHT), Albert-Einstein-Str. 9, 07745 Jena, Germany^b Institute of Physical Chemistry and Abbe Center of Photonics, Friedrich-Schiller-University Jena, Helmholtzweg 4, 07743 Jena, Germany^c Institute of Quantum Science and Engineering, Texas A&M University, College Station, TX 77843-4242, USA

HIGHLIGHTS

- Disintegration of insulin amyloid fibrils by increasing the pH of the environment.
- Topography imaging of individual insulin aggregates at different pH values.
- In-situ monitoring of the pH dependent disassembly of amyloid fibrils with an AFM.
- Tracking of conformational changes in insulin aggregates with SERS.
- Correlation of morphology and conformation of amyloid fibrils as a function of the pH.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:

Received 30 October 2020
 Received in revised form 14 February 2021
 Accepted 1 March 2021
 Available online 8 March 2021

Keywords:

Amyloid fibril
 Insulin
 Atomic force microscopy
 Surface-enhanced Raman spectroscopy

ABSTRACT

Aggregation of insulin into amyloid fibrils is characterized by the conversion of the native secondary structure of the peptide into an enriched β -sheet conformation. In vitro, the growth or disintegration of amyloid fibrils can be influenced by various external factors such as pH, temperature etc. While current studies mainly focus on the influence of environmental conditions on the growth process of insulin fibrils, the present study investigates the effect of pH changes on the morphology and secondary structure of mature fibrils. In the experiments, insulin is fibrillated at pH 2.5 and the grown mature fibrils are suspended in pH 4–7 solutions. The obtained structures are analyzed by atomic force microscopy (AFM) and surface-enhanced Raman spectroscopy (SERS). Initially grown mature fibrils from pH 2.5 solutions show a long and intertwined morphology. Increasing the solution pH initiates the gradual disintegration of the filamentous morphology into unordered aggregates. These observations are supported by SERS experiments, where the spectra of the mature fibrils show mainly a β -pleated sheet conformation, while the amide I band region of the amorphous aggregates indicate exclusively α -helix/unordered structures.

The results demonstrate that no complex reagent is required for the disintegration of insulin fibrils. Simply regulating the pH of the environment induces local changes in the protonation state within the peptide chains. This effectively disrupts the well-ordered β -sheet structure network based on hydrogen bonds.

© 2021 Elsevier B.V. All rights reserved.

* Corresponding author at: Leibniz Institute of Photonic Technology (IPHT), Albert-Einstein-Str. 9, 07745 Jena, Germany.

E-mail address: volker.deckert@leibniz-ipht.de (V. Deckert).¹ Present address: Faculty of Pharmacy, George Emil Palade University of Medicine, Pharmacy, Science, and Technology of Târgu Mureș, Gheorghe Marinescu Street No. 38, 540139 Târgu Mureș, Romania.

1. Introduction

Aggregation of proteins and peptides into misfolded structures is associated with the development of diverse neurodegenerative diseases (e.g. Alzheimer's disease) and metabolic disorders (e.g. diabetes). The morphology of the involved aggregates is polymorph and ranges from nanometer-sized oligomers to intertwined filamentous micrometer long structures [1–5]. It has been established that protein fibrillation involves a very delicate molecular equilibrium and during the self-assembling process, a hydrogen bond-based β -sheet structure of the core develops [6–9]. The molecular origin of fibril stability has been addressed in several studies and reported to additionally involve electrostatic and van der Waals interactions and aromatic π - π stacking. Any mechanical or chemical perturbation during fibrillation growth affects the reaction kinetics as well as the molecular and morphological structures of intermediates and mature fibrils. The interactions between peptide chains control the supramolecular morphology and are specific for each protein. This complicates both the establishment of a generally valid building mechanism and the development of drugs to disintegrate amyloidogenic aggregates.

It has been reported that various parameters can change the properties of a peptide/protein such that the aggregation process is initiated or hampered [2–5,10]. From a chemical perspective, these include protein sequence, pH, net charge of the molecule, excipients, and interfaces. Physical factors are pressure, temperature, agitation and lyophilization, which can influence the stability of the peptides or protein. The simplest chemically induced manipulation of fibrillation is by changing the pH of the growth solution. Since distinct amino acids are prone to protonation (i.e. glu, his, arg, lys) or deamidation (i.e. asn, gln), working under acidic or basic conditions, respectively, affects not only the local structure within the peptide/protein but its net charge state. As a result, electrostatic attractive/repulsive interactions between peptide chains alter. In the experiments this can lead to an acceleration or suppression of the aggregation process, whereby morphologically different aggregates are generated [10–16]. The susceptibility to the introduction or abstraction of protons can be exploited to destabilize the well-ordered structural network in mature fibril [13,16–18].

In the present contribution, we focus on the behavior of mature insulin fibrils after suspension in solutions with different pH values in the range of 2.5–7. To the best of our knowledge, only two earlier studies approached the effect of pH changes on insulin amyloid fibrils [16,18] but no detailed information about molecular structure changes and morphology on the single particle level are available. Insulin is a 51 amino acids peptide that contains two chains (A chain with 21 residues and B chain with 30 residues) that are linked via disulfide bridges. It has been demonstrated that the S-S bonds between the cysteine residues connecting the two strands remain intact during fibrillation [19] and the natively predominant α -helical structure converts to a highly ordered β -sheet rich conformation. This drastic conformational change is associated with the formation of multiple intertwined filamentous structures. As for other peptides and proteins, it has been observed that the insulin fibrillation process is highly susceptible to environmental conditions [14,20–23]. The profound morphological transformation of insulin during fibrillation can be well studied in-situ by scanning probe microscopy on individual species. With an atomic force microscopy (AFM) the height, length, elasticity/stiffness and adhesion of amyloid fibrils can be determined not only for dry samples, but also in a liquid environment. This permits to follow the dynamic aggregation process directly and to visualize morphological and mechanical details [24–28].

When the chemical structure is of interest, Raman spectroscopy, among other techniques, is well suited to characterize amyloidogenic species not only in the bulk sample [29–32], but also at the single fibril level when applied in the near-field [33–36]. In Raman spectroscopy no labeling is required which might interfere with the chemical structure of the probed sample and the sample does not need to be specifically pre-treated. The drawback of low sensitivity of conventional Raman spectroscopy in the visible range can be overcome by using surface-enhanced Raman scattering (SERS) where the electromagnetic field can be enhanced by surface plasmons in rough nanostructured surfaces, such as silver or gold when irradiated with the appropriate laser wavelength. This phenomenon enables the chemical characterization down to the single-molecule level. See for instance ref. [37–39] and literature cited therein. In addition to its high sensitivity, this label-free technique (like standard Raman spectroscopy) also provides specific information about the chemical composition of the probed sample. As a result, SERS has become an extensively applied technique in surface and biological analysis [40,41]. When applied to amyloid fibrils, the conversion of the peptide's native structure to a β -sheet rich conformation can be identified by considering the amide I band region, which provides specific information about the environment of a peptide bond. While for α -helix and random coil structures in Raman spectroscopy bands at 1640–1664 cm^{-1} are detected, bands at 1665–1679 cm^{-1} can be assigned to β -sheet structures [33–36].

In the present study, insulin from bovine pancreas was fibrillated at pH 2.5 and the obtained gel was suspended in pH 4–7 solutions. The AFM topography images of the dried samples indicated the degradation of mature fibrils to amorphous aggregates. The subsequent SERS experiments confirmed the gradual disintegration of the well-ordered β -pleated sheet structure of mature fibrils to a conformation dominated by α -helix/unordered structures.

2. Material and methods

2.1. Sample preparation

Insulin from bovine pancreas (Sigma-Aldrich, Germany) was dissolved (60 mg/mL) in HCl (pH 2.5) and heated at 70 °C for 2.5 h without stirring. After cooling to room temperature, 10 μL of the fibrillar gel was suspended in aqueous solution (pH 2.5, 4, 5, 6 and 7), 1:100 (V/V) respectively. pH values from 4 to 7 were adjusted by adding a 20 mM Tris (tris(hydroxymethyl)aminomethane) solution. 10 μL of the sample was dropped onto cleaned (ethanol) glass cover slips. After incubating for 5 min, the drop was gently removed from the glass surface with a pipette and washed twice with 10 μL of the respective solution. The samples were dried at room temperature for the AFM measurements in the dry state. For in situ AFM imaging in solution, 10 μL of the pH 2.5 sample was incubated, rinsed and transferred to an AFM liquid cell. After injecting 700 μL of a pH 4 solution AFM images were immediately acquired.

2.2. Atomic force microscopy

AFM measurements were performed with a JPK NanoWizard I (Bruker-JPK BioAFM, Germany) AFM in intermittent contact mode. For dry state measurements Tap 190Al-G cantilevers (Budget Sensors, Bulgaria) were used. Liquid measurements were performed with NSG10 (NT-MDT, Russia) cantilevers. 1–10 μm images were scanned with a 256 $\times$ 256 pixels resolution. On each sample at least 10 different scan regions were investigated for each pH condition. To follow the dynamic degradation process in liquid, the

same sample area was scanned continuously for approx. 80 min. Topography images were offset corrected applying a line by line polynomial fit procedure (JPK imaging processing software, Bruker-JPK, Germany)

2.3. Surface-Enhanced Raman spectroscopy

A silver colloidal SERS substrate was prepared following the procedure from Leopold et al. described in ref. [42]. 10 μ l of the colloid was applied to a pre-cleaned glass slide, which was previously treated with $\text{HNO}_3/\text{H}_2\text{O}_2$ 3:1 for 2.5 h and dried under argon. 10 μ l of the same insulin fibril suspensions (pH 2.5, 4, 5, 6, 7, $c = 1 \cdot 10^{-4}$ mol/L) used for AFM imaging was drop-cast onto the silver nanoparticles. After incubating for 5 min the solution was gently sucked off and the sample was rinsed with 10 μ l of the respective solution. After drying under argon, SERS spectra were immediately acquired. The Raman system was equipped with a 40x (N. A. 1.35) microscope objective. For excitation laser light at $\lambda = 532$ nm with a power on the sample $P = 50$ μ W was used. Spectra were acquired at $t_{\text{acq}} = 3$ –10 s with 6 acc. on 20–30 different areas on each sample. All raw spectra were imported in Igor-Pro 7.08 32 bit (WaveMetrics Inc. USA) and arranged with a y-offset for better visualization. No further data processing was applied.

3. Results and discussion

From two earlier studies it is known that insulin fibrils degrade in an aqueous suspension at $\text{pH} > 8$ yielding amorphous aggregates/oligomers, which can be imaged with an AFM or a transmission electron microscope (TEM) [16,18]. These data provide information about the bulk samples, but individual structures or

the dynamics of the disintegration process were not studied. In our experiments, the morphology of disintegrated insulin mature fibrils after suspension in pH 4–7 solutions was first investigated using an AFM. In the next step, SERS experiments followed, focusing on changes in the secondary structure of the generated specimen.

The initially long intertwined insulin mature fibrils grown at pH 2.5 in Fig. 1a show the typical height of ~ 5 nm with a length of several micrometers. The observations are consistent with previous results that have shown that insulin favorably forms fibrils in low pH solutions, which are remarkably stable in their growing medium [22,32,34,43,44].

When mature insulin fibrils grown at pH 2.5 were suspended in a pH 4 solution, the morphology became less defined with structures that were typically shorter and thicker (height ~ 10 –15 nm, see Fig. 1b), which indicated the beginning of disintegration. Although the majority of the imaged structures was filamentous, already a few irregularly structured aggregates (height ~ 17 –25 nm) were detected. In the further experiments, mature fibrils were suspended in solutions with a pH of 5–7. The corresponding AFM topographies are given in Fig. 1c–e. The gradual degradation process when the pH of the medium was increased to 5 and 6 (Fig. 1c,d) is clearly visible. Similar to pH 4, at pH 5 the majority of the detected structures were filamentous, while the appearance of pH 6 particles was already more similar to the situation at pH 7. At pH 7, elongated structures could hardly be detected, and the sample was dominated by more or less spherical particles with a height ~ 6 –17 nm (see Fig. 1e). In Fig. 1f profile lines across selected areas on the samples show the height differences of fibrils and disintegrated species. Ten different areas were investigated on each sample, from which a selection of additional five images for each

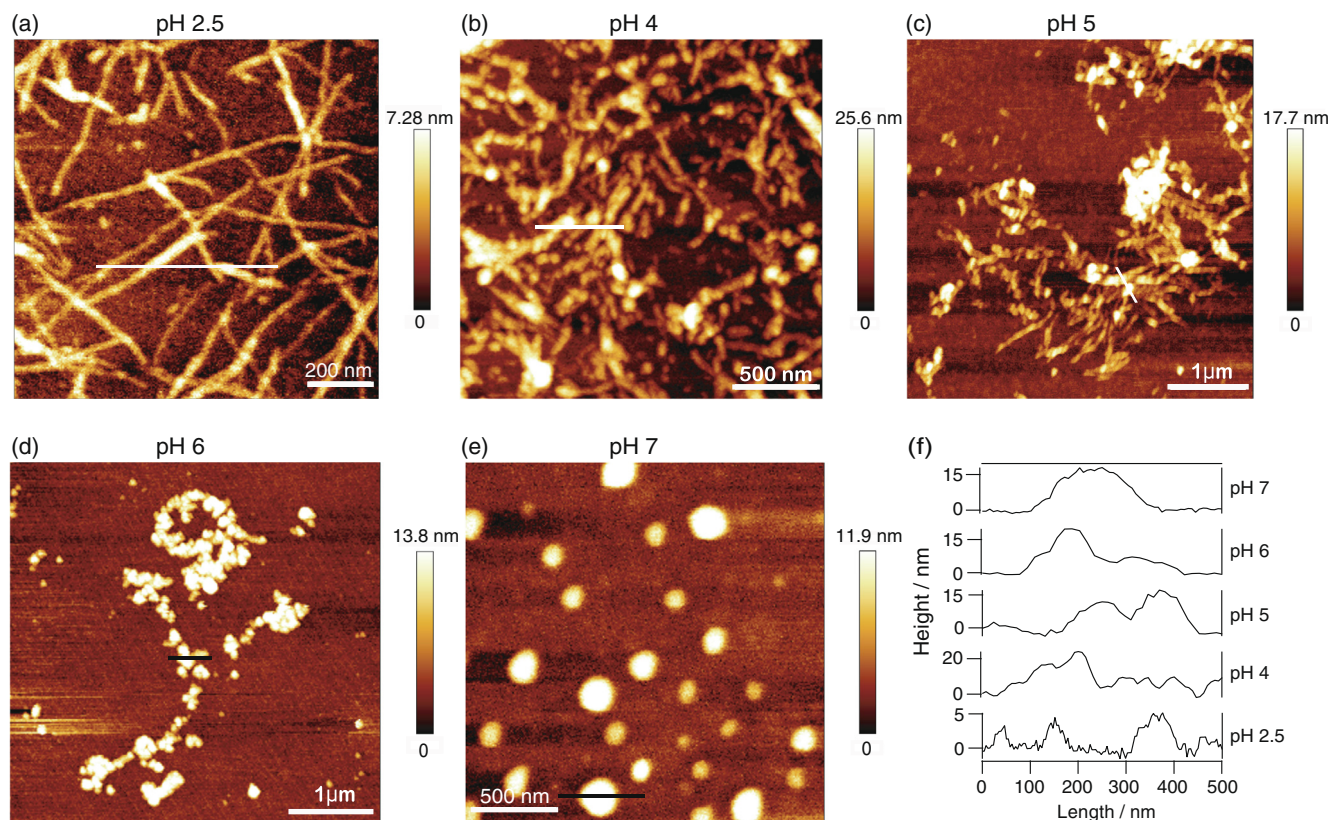


Fig. 1. AFM topographies of dried aggregates obtained after suspending mature insulin fibrils in solutions with a different pH. (a) Mature insulin fibrils suspended at pH 2.5; (b) mature fibrils suspended at pH 4; (c) mature insulin fibrils suspended at pH 5; (d) mature insulin fibrils suspended at pH 6; (e) mature insulin fibrils suspended at pH 7; (f) profile lines across representative structures of the samples in (a)–(e). White and black lines, respectively, in (a)–(e) indicate measured profile lines. The images in (a)–(e) were offset-corrected applying a line by line polynomial fitting procedure.

condition is provided in the Fig. S1–S5. It should be mentioned that tris(hydroxymethyl)aminomethane (Tris) was used instead of NaOH to adjust the pH. Thus, the sample-tip interactions in the subsequent *in-situ* AFM measurements in liquid were better controllable [45]. According to a previous study [18], the usage of Tris instead of NaOH does not affect the results. To obtain a deeper insight into the dynamic pH-dependent amyloid fibril dissociation process, *in-situ* AFM measurements were performed at pH 4. Immediately after suspending the fibrillar gel in pH 4 solution, the sample was transferred to a liquid AFM cell and investigated. Fig. 2a–d shows four topography images of a repeatedly scanned area from a period of 34 min. The complete data set is available in the Movie 1 in the Supplementary Material. In Movie 2 and Movie 3 in the Supplementary Material two more datasets are provided showing the gradual disassembly of mature fibrils after suspending in pH 4 solution.

The images in Fig. 2a–d show that the fibril first split before the right end continuously shortened and finally dissolved within 18 min. In contrast, the left end of the fibril initially showed a similar behavior, but then remained stable. Further dynamic experiments demonstrated that not all fibrils were equally susceptible to the pH change within the examined time period (see Movie 2, 3 in the Supplementary Material). This is in agreement with the image in Fig. 1b where filamentous structures dominate the overall topography while unordered aggregates were only randomly detected. With regards to previous studies in this field [16,18], it was supposed that in case of disintegration, soluble particles were formed instead of insoluble aggregates that detached from the glass surface. Shammass et al. observed morphological changes at pH > 4 in terms of fibril broadening and shortening [18], which is in agreement with our results in Fig. 1b. On the other hand, in their experiments, the dissolving of filamentous structures was found only above pH 8, with complete dissociation at pH 12. The reason why a partial disaggregation could already be observed at pH 4 in the current experiment is probably due to the different sensitivity of the detection methods used. While AFM allows analysis on the single particle level, Shammass et al. measured the absorbance of the solution to determine the concentration of soluble insulin produced.

Naturally, the pH-dependent dissolution of the fibrillar structure must be associated with a change in conformation. In previous

studies the (partial) unfolding of the β -sheet structure in the bulk samples was detected using infrared spectroscopy and the thioflavin T (ThT) binding assay [16,18]. In the present study SERS was employed to sensitively determine conformational changes.

In the first step, SERS spectra from insulin fibrils generated at pH 2.5 were acquired on silver colloids (see Fig. 3a). Although silver nanoparticles are sensitive to hydrochloric acid solutions, band positions are not affected as has been demonstrated in a work on silver island films [46]. Since SERS is a surface sensitive detection method the spectral information is limited to the surface of the probed particles. Consequently, the relative orientation of molecules directly interacting with the SERS substrate affects the spectra. In the experiments slight heterogeneities across the samples in terms of varying band position, intensity and width are often observed and SERS spectra can differ significantly from far-field Raman spectra. In the present experiment, the SERS spectra of fibrils differ from the standard Raman spectrum in Fig. S6, in which the conformation of the bulk sample is probed. The spectra in Fig. 3a are characterized by intensity fluctuations and heterogeneity but the characteristic bands of aromatic and nonaromatic side chains of amino acid residues are detected at 998 cm^{-1} (phenylalanine, phe), $825, 856\text{ cm}^{-1}$ (tyrosine, tyr) [34], and at $656, 759\text{ cm}^{-1}$ (cysteine, cys) [19,29,47]. Amide I bands at $1668\text{--}1676\text{ cm}^{-1}$ (red bar) indicate a β -sheet structure, which is generally considered as marker band for amyloids [29]. Additional bands at $1634\text{--}1648\text{ cm}^{-1}$ point to a contribution from α -helix/random coil structures. The detection of mixed secondary structures was expected and is in agreement with results from near-field Raman experiments [48,49]. It should be noted that contributions from native insulin could be excluded because soluble components were removed during sample preparation.

An aliquot of the mature insulin fibril suspension at pH 4 and pH 7, respectively, was used for the SERS experiments. To exclude contribution from non-fibrillar insulin, samples were rinsed after incubation on the SERS substrate. It should be noted that due to the molar concentration of insulin in the sample, standard Raman spectra were dominated by signals from Tris, which obscured any insulin bands and did not provide useful information. Fig. 3b and c show six out of 20 spectra each, which were acquired on different locations across the samples. When looking at the spectra in Fig. 3a–c, it is apparent that the band at 759 cm^{-1} assigned to cys is sig-

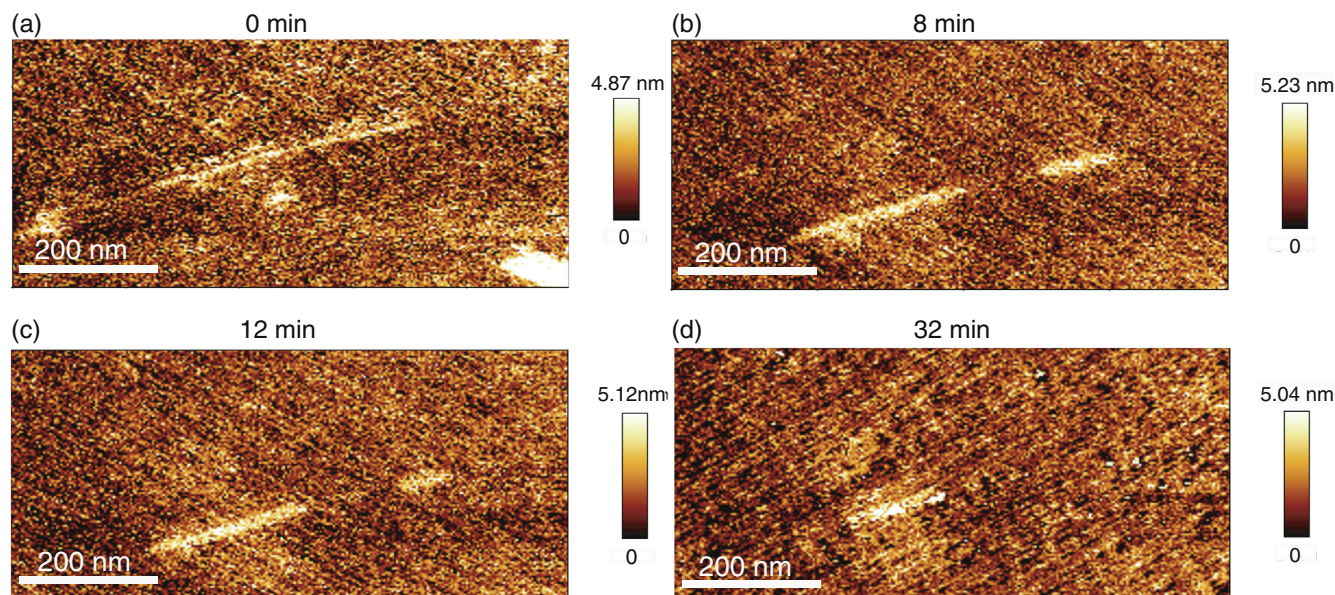


Fig. 2. In situ monitoring the disintegration of a mature insulin fibril after suspension at pH 4 using AFM in liquid. (a) Topography image immediately after suspending the fibrillar gel in pH 4 solution, (b) topography after 8 min, (c) topography after 12 min, (d) topography after 32 min. Scan rate 2.5 Hz, 256×256 px.

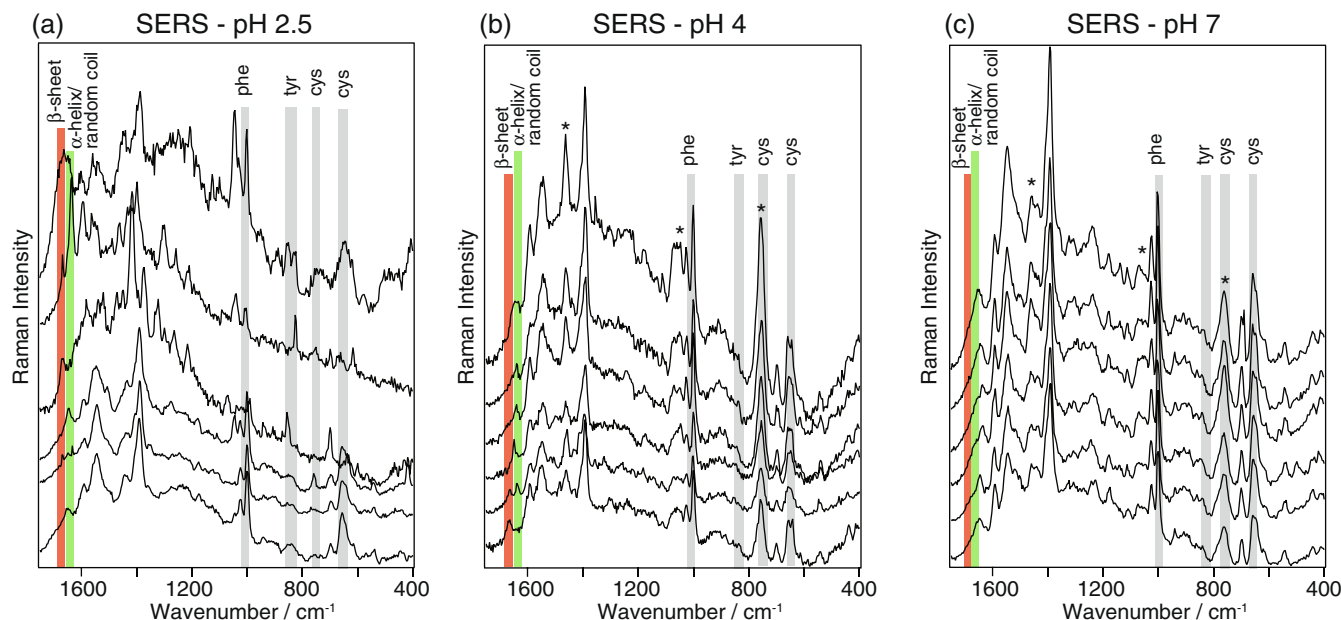


Fig. 3. SERS spectra of dried insulin aggregates on silver colloids, (a) SERS spectra of insulin fibrils after suspension at pH 2.5, $t_{\text{acq}} = 3\text{--}10$ s, 6 acc, $P = 50\text{ }\mu\text{W}$; (b) SERS spectra of insulin fibrils after suspension at pH 4, $t = 5$ s, 6 acc, $P = 50\text{ }\mu\text{W}$; (c) SERS spectra of insulin fibrils after suspension at pH 7, $t = 5$ s, 6 acc, $P = 50\text{ }\mu\text{W}$. Spectra in (a)–(c) were acquired on different areas on the samples. Amide I band regions indicating α -helix/unordered structures (green) and β -sheet structures (red) are highlighted. The marked bands (*) might contain some additional contributions from Tris. For better visibility, the spectra are arranged with a y-offset.

nificantly enhanced in the SERS spectra compared to the Raman spectrum in Fig. S6. This can be attributed to the high affinity of the silver substrate for sulfur in cysteine and was already earlier reported [50]. The fact that no S-S modes were detected in Fig. 3-a-c suggests that the disulfide bridges were broken by binding of the cys residue to the silver substrate via Ag-S bonds.

In Fig. 3b, low intensity bands at $\sim 1664\text{ cm}^{-1}$ can be assigned to β -sheet structures, while the most of the spectra show a band at $\sim 1639\text{ cm}^{-1}$ indicating α -helix/unordered structures (green). This observation corresponds well with the partial dissolution of the intertwined fibril morphology at pH 4 monitored with the AFM (Fig. 1b, Fig. 2). The SERS spectrum in Fig. 3c was recorded on the amorphous aggregates generated when mature insulin amyloid fibrils were treated with a pH 7 solution. In these experiments, amide I bands were exclusively detected at $\sim 1636\text{--}1652\text{ cm}^{-1}$ (green, α -helix/random coil). In this condition, no spectrum points to the presence of β -sheet structures, which correlates with the AFM results in Fig. 1c, where no more filamentous structures were identified.

It can be assumed that an increased pH changed the protonation state of distinct amino acid residues and thus the net charge of the peptide in amyloid fibril. As a result, the hydrogen bond-based network and the overall fibrillar structure were destabilized. In previous pH-dependent amyloid dissolution experiments and modeling it was suggested that mainly the residues glutamic acid (glu), histidine (his) lysine (lys), arginine (arg) and tyrosine (tyr) were involved in this process [16–18,51]. The pK_s of these amino acids were determined in a model peptide and it was found that glu is partially deprotonated at $\sim$ pH 4 and completely deprotonated at pH 7 [52]. His is protonated at $\sim$ pH 4 and deprotonated at $\sim$ pH 7. Finally, tyr (uncharged) lys, and arg (protonated) remain unchanged up to high pH values. If these findings are applied to the current AFM and Raman results it should be noted that the values can only be considered as estimates, since the model compound had no significant hydrogen bonds. Thus, it was postulated that the deprotonation of glu and his residues was one of the driving forces in the dissolution of the β -sheet secondary structure of insulin fibrils at pH 4–7. Indeed, near-field Raman spectroscopic results

reported a high abundance of COOH groups (17%) on pH 2.5 insulin fibrils[49], which renders the aggregates prone to pH changes. Although, tyr (27%) and $\text{NH}_3^+/\text{NH}_2^+$ (19%) were also frequently present on insulin fibrils [49], they were probably not involved, since the protonation state does not change within the investigated pH range. The role of histidine did not seem to be decisive either, since it was hardly detected ($<1\%$) [49] on the fibrils. Finally, it should be mentioned that the interactions between peptide and solvent can also change depending on the pH and thus contribute to the disintegration [17].

4. Conclusion

In the present contribution mature insulin fibrils grown at pH 2.5 were suspended in pH 4–7 solutions and the produced aggregates were investigated by AFM and SERS. The topography images show that at pH 4 the majority of the detected structures were still filamentous, albeit with greater height and shorter length. As the pH of the medium was raised to 5 and 6, more and more disordered particles were identified until finally at pH 7 no more fibrils were present. The morphological gradual degradation could be followed time-dependently with in-situ liquid AFM measurements at pH 4. During the time period studied, some fibrils disintegrated within minutes, while others remained unaffected. The corresponding SERS experiments confirmed the gradual disintegration of the well-ordered β -sheet structure of the mature insulin fibrils by a shift of the amide I band from 1672 cm^{-1} for the pH 2.5 condition to 1638 cm^{-1} for the pH 7 condition. Accordingly, a mixture of β -sheet and α -helix/unordered conformations was detected in the spectra of mature insulin fibrils after suspension in a pH 4 solution. After considering models estimating the pK_s of individual amino acid residues in proteins, it was postulated that the change in the protonation state of the glutamic acid residue is an important parameter in disrupting the fibril backbone. The results demonstrate that no specifically designed compound is necessary to disrupt the well-ordered β -sheet structure of amyloid fibrils, but a change of the pH can already have a destabilizing effect.

Funding

This work was supported by the Leibniz Science Campus LSC InfectoOptics “HotAim” and DFG - CRC/SFB 1278 Polytarget B04.

CRediT authorship contribution statement

Erwan Darussalam: Investigation, Visualization, Writing - original draft. **Orsolya Peterfi:** Investigation, Visualization. **Tanja Deckert-Gaudig:** Investigation, Formal analysis, Writing - review & editing. **Ludovic Roussille:** Investigation, Visualization. **Volker Deckert:** Conceptualization, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.saa.2021.119672>.

References

- [1] G. Malgieri, G. D'Abrascia, L. Pirone, A. Toto, M. Palmieri, L. Russo, M.F.M. Sciacca, R. Tate, V. Sivo, I. Baglivo, R. Majewska, M. Coletta, P.V. Pedone, C. Isernia, M. De Stefano, S. Gianni, E.M. Pedone, D. Miliardi, R. Fattorusso, Folding mechanisms steer the amyloid fibril formation propensity of highly homologous proteins, *Chem. Sci.* 9 (2018) 3290–3298.
- [2] F. Chiti, C.M. Dobson, Protein misfolding, amyloid formation, and human disease: a summary of progress over the last decade, *Annu. Rev. Biochem.* 86 (2017) 27–68.
- [3] R. Riek, D.S. Eisenberg, The activities of amyloids from a structural perspective, *Nature* 539 (2016) 227–235.
- [4] D.S. Eisenberg, M.R. Sawaya, Structural studies of amyloid proteins at the molecular level, *Annu. Rev. Biochem.* 86 (2017) 69–95.
- [5] M.C. Owen, D. Gnutt, M. Gao, S. Warmlander, J. Jarvet, A. Graslund, R. Winter, S. Ebbinghaus, B. Strodel, Effects of in vivo conditions on amyloid aggregation, *Chem. Soc. Rev.* 48 (2019) 3946–3996.
- [6] T.P.J. Knowles, A.W.P. Fitzpatrick, S. Meehan, H.R. Mott, M. Vendruscolo, C.M. Dobson, Welland, Role of intermolecular forces in defining material properties of protein nanofibrils, *Science* 318 (2007) 1900–1903.
- [7] M.R. Sawaya, S. Sambashivan, R. Nelson, M.I. Ivanova, M.I. Apostol, M.J. Thompson, M. Balbirnie, J.J.W. Wiltzius, H.T. McFarlane, A.O. Madsen, C. Riekel, D. Eisenberg, Atomic structures of amyloid cross-beta spines reveal varied steric zippers, *Nature* 447 (2007) 453–457.
- [8] O.S. Makin, E. Atkins, P. Sikorski, J. Johansson, L.C. Serpell, Molecular basis for amyloid fibril formation and stability, *Proc. Nat. Acad. Sci.* 102 (2005) 315–320.
- [9] R. Nelson, M.R. Sawaya, M. Balbirnie, A.O. Madsen, C. Riekel, R. Grothe, D. Eisenberg, Structure of the cross-beta spine of amyloid-like fibrils, *Nature* 435 (2005) 773–778.
- [10] K.L. Zapadka, F.J. Becher, A.L. Gomes Dos Santos, S.E. Jackson, Factors affecting the physical stability (aggregation) of peptide therapeutics, *Interface Focus* 7 (2017) 20170030.
- [11] D. Bernson, A. Mecinovic, M.T. Abed, F. Lime, P. Jageland, M. Palmlof, E.K. Esbjörner, Amyloid formation of bovine insulin is retarded in moderately acidic pH and by addition of short-chain alcohols, *Eur. Biophys. J.* 49 (2020) 145–153.
- [12] E. Smirnova, I. Safenkova, V. Stein-Margolina, V. Shubin, V. Polshakov, B. Gurvits, pH-responsive modulation of insulin aggregation and structural transformation of the aggregates, *Biochim.* 109 (2015) 49–59.
- [13] A. Noormägi, K. Valmsen, V. Tõngu, P. Palumaa, Insulin fibrillation at acidic an physiological pH values is controlled by different molecular mechanism, *Prot. J.* 34 (2015) 398–403.
- [14] D. Kuroski, R.A. Lombardi, R.K. Dukor, I. Lednev, L.A. Nafie, Direct observation and pH control of reversed supramolecular chirality in insulin fibrils by vibrational circular dichroism, *Chem. Commun.* 46 (2010) 7154–7156.
- [15] T. Sneideris, D. Darguzis, A. Botyriute, M. Grigaliunas, R. Winter, V. Smirnovas, pH-driven Polymorphism of insulin amyloid-like fibrils, *PLoS ONE* 10 (2015) e0136602.
- [16] M. Malisauskas, C. Weise, K. Yanamandra, M. Wolf-Watz, L. Morozova-Roche, Liability landscape and protease resistance of human insulin amyloid: a new insight into its molecular properties, *J. Mol. Biol.* 396 (2010) 60–74.
- [17] M.G. Santangelo, V. Foderà, V. Militello, V. Vetri, Back to the oligomeric state: pH-induced dissolution of concanavalin A amyloid-like fibrils into non-native oligomers, *RSC Adv.* 6 (2016) 75082–75091.
- [18] S.L. Shammass, T.P.J. Knowles, A.J. Baldwin, C.E. MacPhee, M.E. Weiland, C.M. Dobson, G.L. Devlin, Perturbation of the stability of amyloid fibrils through alteration of electrostatic interactions, *Biophys. J.* 100 (2011) 2783–2791.
- [19] D. Kuroski, J. Washington, M. Ozbil, R. Prabhakar, A. Shekhtman, I.K. Lednev, Disulfide bridges remain intact while native insulin converts into amyloid fibrils, *PLoS One* 7 (2012) e36989.
- [20] L.J. Domigan, J.P. Healy, S.J. Meade, R.J. Blaikie, J.A. Gerrard, Controlling the dimensions of amyloid fibrils: toward homogenous components for bionanotechnology, *Biopolym.* 97 (2011) 123–133.
- [21] V. Foderà, M. van de Weert, B. Vestergaard, Large-scale polymorphism and auto-catalytic effect in insulin fibrillogenesis, *Soft Matter* 6 (2010) 4413–4419.
- [22] R. Jansen, W. Dzwolak, R. Winter, Amyloidogenic self-assembly of insulin aggregates probed by high resolution atomic force microscopy, *Biophys. J.* 88 (2005) 1344–1353.
- [23] L. Nielsen, R. Khurana, A. Coats, S. Frokjaer, J. Brange, S. Vyas, V.N. Uversky, A.L. Fink, Effect of environmental factors on the kinetics of insulin fibril formation: Elucidation of the molecular mechanism, *Biochem.* 40 (2001) 6036–6046.
- [24] W. Lee, H. Lee, G. Lee, D.S. Yoon, Advances in AFM Imaging Applications for Characterizing the Biophysical Properties of Amyloid Fibrils, 2016.
- [25] W.S. Gosal, S.L. Myers, S.E. Radford, N.H. Thomson, Amyloid under the atomic force microscope, *Prot. Pept. Lett.* 13 (2006) 261–270.
- [26] W. Hoyer, D. Cherny, V. Subramaniam, T.M. Jovin, Rapid self-assembly of alpha-synuclein observed by in situ atomic force microscopy, *J. Mol. Biol.* 340 (2004) 127–139.
- [27] D.P. Allison, N.P. Mortensen, C.J. Sullivan, M.J. Doktycz, Atomic force microscopy of biological samples, *Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol.* 2 (2010) 618–634.
- [28] A. Li, H. Liang, H. Zhao, D. Chen, B. Liu, T. Fuhs, M. Dong, Characterization of inter- and intramolecular interactions of amyloid fibrils by AFM-based single-molecule force spectroscopy, *J. Nanomat.* 2016 (2016) 5463201.
- [29] D. Kuroski, R.P. Van Duyn, I.K. Lednev, Exploring the structure and formation mechanism of amyloid fibrils by Raman spectroscopy: a review, *Analyst* 140 (2015) 4967–4980.
- [30] S. Ma, X. Cao, M. Mak, A. Sadik, C. Walkner, T.B. Freedman, I.K. Lednev, R.K. Dukor, L.N. Nafie, Vibrational circular dichroism shows unusual sensitivity to protein fibril formation and development in solution, *Am. Chem. Soc.* 129 (2007) 12364.
- [31] C. Ortiz, D. Zhang, A.E. Ribbe, Y. Xie, D. Ben-Amotz, Analysis of insulin amyloid fibrils by Raman spectroscopy, *Biophys. Chem.* 128 (2007) 150–155.
- [32] M. Bouchard, J. Zurdo, E.J. Nettleton, D.M. Dobson, C.V. Robinson, Formation of insulin amyloid fibrils followed by FTIR simultaneously with CD and electron microscopy, *Prot. Sci.* 9 (2000) 1960–1967.
- [33] D. Kuroski, A. Dazzi, R. Zenobi, A. Centrone, Infrared and Raman chemical imaging and spectroscopy at the nanoscale, *Chem. Soc. Rev.* 49 (2020) 3315–3347.
- [34] T. Deckert-Gaudig, D. Kuroski, M.A. Hedegaard, P. Singh, I.K. Lednev, V. Deckert, Spatially resolved spectroscopic differentiation of hydrophilic and hydrophobic domains on individual insulin amyloid fibrils, *Sci. Rep.* 6 (2016) 33575.
- [35] S. Rizevsky, D. Kuroski, Nanoscale structural organization of insulin fibril polymorphs revealed by Atomic Force Microscopy, *ÅInfrared Spectroscopy (AFM-IR)*, *Chem. Bio. Chem.* 21 (2020) 481–485.
- [36] S. Bonhommeau, D. Talaga, J. Hunel, C. Cullin, S. Lecomte, Tip-enhanced Raman spectroscopy to distinguish toxic oligomers from Ab1–42 fibrils at the nanometer scale, *Angew. Chem. Int. Ed.* 56 (2017) 1771–1774.
- [37] N.J. Hallas, S. Lai, W.-S. Chang, S. Link, P. Nordlander, *Chem. Rev.* 111 (2011) 3913–3916.
- [38] K. Willets, R.P. Van Duyn, Localized surface plasmon resonance spectroscopy and sensing, *Ann. Rev. Phys. Chem.* 58 (2007) 267–297.
- [39] A. Otto, I. Mrozek, H. Grabhoern, W. Akemann, Surface-enhanced Raman scattering, *J. Phys. Cond. Mat.* 4 (1992) 1143–1212.
- [40] G. Devitt, K. Howard, A. Mudher, S. Mahajan, Raman spectroscopy: an emerging tool in neurodegenerative disease research and diagnosis, *ACS Chem. Neurosci.* 9 (2018) 404–420.
- [41] S.S. Sinha, S. Jones, A. Pramanik, P.C. Ray, Nanoarchitecture based SERS for biomolecular fingerprinting and label-free disease markers diagnosis, *Acc. Chem. Res.* 49 (2016) 2725–2735.
- [42] N. Leopold, B. Lendl, A new method for fast preparation of highly Surface-Enhanced Raman Scattering (SERS) active silver colloids at room temperature by reduction of silver nitrate with hydroxylamine hydrochloride, *J. Phys. Chem. B* 107 (2003) 5723–5727.
- [43] D. Kuroski, R.K. Dukor, X. Lu, L.A. Nafie, I. Lednev, Normal and reversed supramolecular chirality of insulin fibrils probed by vibrational circular dichroism at the protofilament level of fibril structure, *Biophys. J.* 103 (2012) 522–531.
- [44] M. Mauro, E. Craparo, A. Podest, D. Bulone, R. Carrota, V. Martorana, G. Tiana, P.L. San Biagio, Kinetics of different processes in human insulin amyloid formation, *J. Mol. Biol.* 366 (2007) 258–274.
- [45] D.J. Müller, D. Fotiadis, S. Scheuring, S.A. Müller, A. Engel, Electrostatically balanced subnanometer imaging of biological specimens by atomic force microscope, *Biophys. J.* 76 (1999) 1101–1111.

- [46] D. Kurouski, T. Postiglione, T. Deckert-Gaudig, V. Deckert, I. Lednev, Amide I vibrational mode suppression in surface (SERS) and tip (TERS) enhanced Raman spectra of protein specimens, *Analyst* 138 (2013) 1665–1673.
- [47] F.G. Diaz, J.J. Finnerty, M. Campos-Vallette, F. Célis, A.E. Aliaga, C. Fredes, R. Koch, Experimental and theoretical Raman and surface-enhanced Raman scattering study of cysteine, *J. Raman Spectrosc.* 40 (2009) 632–638.
- [48] D. Kurouski, T. Deckert-Gaudig, V. Deckert, I.K. Lednev, Structure and composition of insulin fibril surfaces probed by TERS, *J. Am. Chem. Soc.* 134 (2012) 13323–13329.
- [49] D. Kurouski, T. Deckert-Gaudig, V. Deckert, I.K. Lednev, Structural characterization of insulin fibril surfaces using tip enhanced Raman spectroscopy (TERS), *Biophys. J.* 104 (2014) 49A–49A.
- [50] E. Podstawka, Y. Ozaki, L.M. Proniewicz, Adsorption of S-S containing proteins on a colloidal silver surface studied by surface-enhanced Raman spectroscopy, *Appl. Spectrosc.* 58 (2004) 1147–1156.
- [51] K.W. Tipping, T.K. Karamanos, T. Jakhria, M.G. Iadanza, S.C. Goodchild, R. Tuma, N.A. Ranson, E.W. Hewitt, S.E. Radford, pH-induced molecular shedding drives the formation of amyloid fibril-derived oligomers, *Proc. Natl. Acad. Sci.* 112 (2015) 5691–5696.
- [52] G.R. Grimsley, J.M. Scholtz, C.N. Pace, A summary of the measured pK values of the ionizable groups in folded proteins, *Prot. Sci.* 18 (2009) 247–251.