



Elucidation of the multistep process of amyloid fibril formation

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Doctoral Dissertation

**Elucidation of the multistep process of
amyloid fibril formation**

(アミロイド線維の多段階的形成プロセスの機構解明)

January, 2024

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Abbreviations

AFM, atomic force microscope

AUC, analytical ultracentrifugation

CD, circular dichroism

DLS, dynamic light scattering

FTIR, Fourier transform Infrared

PEG, polyethylene glycol

SAXS, small-angle X-ray scattering

SVD, singular value decomposition

TEM, transmission electron microscope

ThT, thioflavin T

UV, ultraviolet

Chapter 1

General Introduction

Amyloid fibrils

Amyloid fibrils are a form of abnormal protein aggregates formed by protein misfolding. Amyloid fibrils were discovered when their deposits were extracted from the brain by Virchow et al. in the mid-19th century (1). Initially, amyloid fibrils were thought to be polysaccharides because they exhibited an iodo-starch reaction, but about 100 years later it became clear that their major component was protein. About 50 years ago, it was revealed that amyloid fibrils have a highly ordered structure.

Amyloid fibrils typically exhibit an elongated needle-like morphology without branches (Figure 1-1) (2-4). Thickness and length of amyloid fibrils varies slightly depending on the protein species and formation conditions, but they showed a width of several to several tens of nanometers and a length of several micrometers. In addition, amyloid fibrils exhibit a hierarchical structure with multiple bundles of protofibrils, and a more microscopic level, a cross- β structure with β -strands stacked perpendicular to the long axis of the fibril was identified in the 1950s by studies of X-ray diffraction and electron microscopy (4, 5).

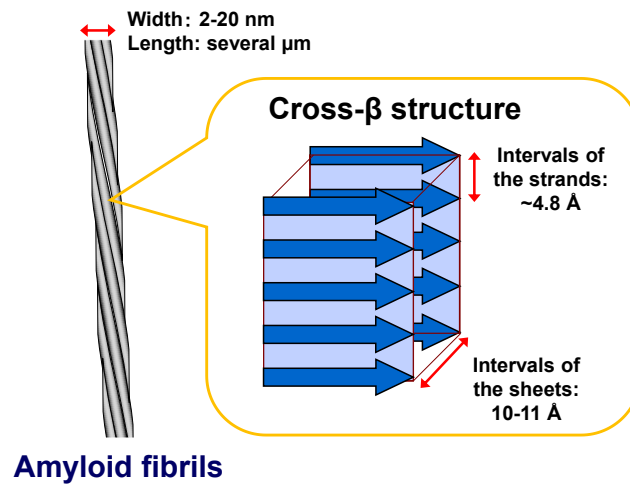


Figure 1-1. Basic structure of amyloid fibrils. Amyloid fibrils consist of several protofilaments, and each protofilament is composed of cross- β structure where β -strands stacked perpendicular to the long axis of the fibril.

In biological system, amyloid fibrils have been reported to exhibit cytotoxicity by interacting with biological membrane. Indeed, it has been reported that amyloid fibrils are involved in various neurodegenerative diseases and amyloidoses (6). The correspondence between diseases and amyloidogenic proteins is summarized in Table 1-1. For example, amyloid- β peptide is responsible for Alzheimer's disease, α -synuclein for Parkinson's disease, and transthyretin for senile systemic amyloidosis. In this content, the structure and formation process of amyloid fibrils are currently being studied intensively to develop methods for the prevention and treatment of diseases.

Table 1-1. Representative diseases and associated amyloid protein/peptides.

Disease	Amyloid Protein
Alzheimer's disease	Amyloid- β peptide
Parkinson's disease	α -Synuclein
senile systemic amyloidosis	Transthyretin
AL amyloidosis	Light chain of immunoglobulin
AA amyloidosis	Serum amyloid A
Huntington's disease	Huntingtin fragments
Frontotemporal dementia	Tau protein
Type II diabetes	Islet amyloid polypeptide
Creutzfeldt-Jakob disease	Prion protein
Dialysis-related amyloidosis	β_2 -Microglobulin
amyotrophic lateral sclerosis	Fused in sarcoma

Formation scheme of amyloid fibrils

Amyloid fibril formation is progressed by nucleation and subsequent growth (Figure 1-2). Amyloid nucleation is hampered by a high energy barrier and is unlikely to occur spontaneously, and is therefore the rate-limiting step in the overall amyloid fibril formation reaction. However, once nucleus is formed, explosive growth of amyloid fibrils occurs by propagating the amyloid structure by successive association of protein monomers to its own end structures, which serve as template. This reaction scheme, which is called nucleation-dependent polymerization mechanism, is the general framework of amyloid fibril formation (7).

Amyloid nucleation, which is achieved by the early association of protein

molecules, is thought to be triggered by destabilization or partial denaturation of the protein native structure. In addition, the effect of enhancing the intramolecular interaction of the protein are also expected to promote amyloid nucleation. For example, changes in salt concentration or pH of solvents, mutations in amino acid sequence, and aggregation-promoting additives contribute to the initiation of amyloid nucleation.

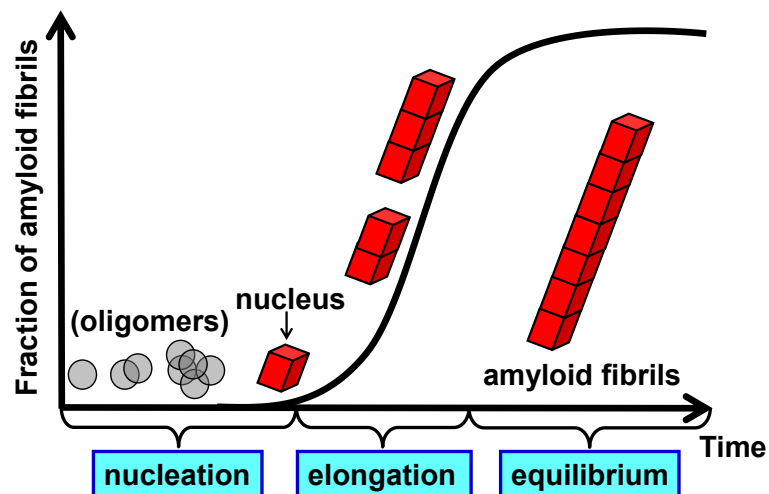


Figure 1-2. Formation scheme of amyloid fibrils. Amyloid fibril formation is progressed by nucleation and subsequent growth.

Multistep formation process of amyloid fibrils

Recently, research focusing on the formation mechanism of amyloid fibrils has been vigorously pursued, and the details are gradually becoming clear. On that occasion,

it has been suggested that the formation process of amyloid fibrils is a multistep reaction that involves several metastable states. Here, I introduce the possibility of the multistep nature of amyloid fibril formation using the amyloid nucleation step as an example. Amyloid nucleation is often explained by the classical nucleation theory of crystallography (8, 9). According to this theory, the energy barrier of nucleation is described by a convex landscape, and there is no thermodynamically stable intermediate state. On the other hand, it has recently been shown that prefibrillar aggregates such as amyloid oligomers and protofibrils are identified during the amyloid nucleation (10-12) (10-12). Furthermore, they have been reported to act as on-pathway intermediates of amyloid nucleation. In light of the above findings, it is suggested that amyloid nucleation is not a one-step scheme but a multistep reaction via intermediate metastable states depending on the reaction conditions. This multistep nature may be a property not only of amyloid nucleation but also of amyloid fibril formation in general, including fibril elongation and structural development. However, few reports have experimentally identified the formation of metastable states in amyloid formation due to its short lifespan and polydispersity, and much remains unknown about the multistep nature of amyloid fibrils.

Contents of this thesis

In this thesis, I have investigated the formation process of amyloid fibrils focusing on the multistep nature of amyloid fibril formation, in terms of early aggregation process (Chapter 2), structural propagation process (Chapter 3), and amyloid formation process inside condensate (Chapter 4).

In Chapter 2, I have investigated the early aggregation process of bovine and human insulin by static and dynamic light scattering in combination with thioflavin T (ThT) fluorescence and Fourier transform infrared (FTIR) spectroscopy. The time dependence of light scattering has revealed that oligomers and protofibrils form in bovine insulin, in contrast to no significant aggregation in human insulin. By focusing on bovine insulin for kinetic analysis, it has been revealed that the protofibril formation process was divided into two steps with reference to fractal dimension. When modeled the experimental data of static and dynamic light scattering based on the Smoluchowski aggregation kinetics with fractal aggregation and end-to-end association, I found the initial formation of spherical oligomers and their subsequent uniaxial docking. Furthermore, the analysis of temperature and salt concentration dependence revealed that the end-to-end association is the rate-limiting step, where structure organization occurred with dehydration. The established model for protofibril formation where oligomers are incorporated as a precursor provides insight into the molecular mechanism how protein molecules assemble during the early stage of amyloid formation.

In Chapter 3, I have investigated the behaviors of self- and cross-seeding of amyloid fibrils of human and bovine insulin in terms of thioflavin T fluorescence, morphology, secondary structure, and iodine staining. Insulin amyloid fibrils exhibited different structures depending on species, and each of which replicated in self-seeding.

In contrast, gradual structural changes were observed in cross-seeding, and a new-type amyloid structure with distinct morphology and cytotoxicity was formed when human insulin was seeded with bovine insulin fibrils. Remarkably, iodine staining tracked changes in amyloid structure sensitively, and singular value decomposition (SVD) analysis of the ultraviolet-visible (UV-Vis) absorption spectra of the fibril-bound iodine has revealed the presence of one or more intermediate metastable states during the structural changes. From these findings, I propose a propagation scheme with multistep structural changes in cross-seeding between two heterologous proteins, which is accounted for as a consequence of the rugged energy landscape of amyloid formation.

In Chapter 4, I focused on optical trapping as a method for spatiotemporal control of protein droplet formation and subsequent amyloid fibril formation. Droplet-like protein condensates have recently attracted much attention due to their critical roles in biological phenomena. They typically exhibit high fluidity and reversibility, but otherwise, they become reaction sites for the formation of amyloid fibrils. To understand the functionality and pathogenicity of protein condensates, it is critical to investigate their physicochemical properties at the single droplet level; however, this is currently difficult due to the lack of applicable methods, and thus the molecular details behind these processes are largely unknown. Here, we demonstrate that optical trapping is capable of inducing the formation of a single liquid-like condensate of α -synuclein in a spatiotemporally controlled manner. The irradiation of tightly focused near-infrared laser at an air/solution interface formed a condensate in the coexistence of polyethylene glycol. The fluorescent dye-labeled imaging showed that the optically induced condensate has a gradient of protein concentration from the center to the edge, suggesting that it is fabricated through the mechanism of optical evolved assembling. Furthermore, Raman spectroscopy and

thioflavin T fluorescence analysis revealed that continuous laser irradiation induces transition inside the condensate to β -sheet rich structure, ultimately leading to the formation of amyloid fibrils. These observations indicate that optical trapping is a powerful technique for tracking the molecular mechanisms of condensate appearance and growth, and furthermore, subsequent aging leading to amyloid fibril formation.

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Chapter 2

Mechanistic modeling of amyloid oligomer and protofibril formation

Introduction

Amyloid fibrils are fibrillar protein aggregates, the deposition of which is a hallmark of pathology in amyloidoses and neurodegenerative diseases (1). They commonly consist of an ordered cross- β structure and needle-like morphology (2-4), and their formation follows a nucleation-dependent polymerization mechanism. The simplest mechanism of nucleation assumes one-step, where no thermodynamically stabilized assembly species populate (5,6). Albeit this theoretical assumption, however, a variety of early aggregates are often identified in various proteins. While the size and structure of early aggregates vary depending on proteins and reaction conditions, annular oligomers and rod-like protofibrils are experimentally captured before nucleation (7-9). The oligomers and protofibrils are smaller in size and have more hydrophobic surface properties compared to those of mature amyloid fibrils. Therefore, it has been suggested that they are prone to induce neurotoxicity through the damage of plasma membrane and the binding to signaling receptors (10). The deep involvement of these aggregates is prominent in particular in the pathology of Alzheimer disease (AD), where oligomeric aggregates of amyloid- β have been implicated as a primary agent in neuronal dysfunction (11,12). Indeed, it is recently worth noting that targeting these species is leading to breakthroughs in immunotherapy of AD (13,14).

In this context, elucidating the mechanism of protein assembly at the early stages of amyloid formation has become an important issue from the perspective of not only protein chemistry but also pathology and medicine, and emphasis has been placed on mechanistic studies at the protein molecular level. Nevertheless, details of the formation of oligomers and protofibrils remain elusive because these species are often heterogeneous, and furthermore, their population is small. In addition, some species act as on-pathway

intermediates (15-18), while others do off-pathway (19,20), and these features taken together make the time-dependent evolution of early aggregates difficult to be identified. Although many studies have employed schemes where protofibrils are formed via oligomers, it is often speculative and not supported by experimental evidence sufficiently. Quantitative elucidation of molecular mechanism of the early stages of amyloid formation is required for obtaining clues for comprehensive understanding of protein assembly during the formation of oligomers and protofibrils.

To address a more exact mechanism for the formation of amyloid oligomers and protofibrils, we have investigated the time-dependent process of early aggregation using insulin as a representative amyloidogenic model mainly based on static and dynamic light scattering, and in addition, thioflavin T (ThT) fluorescence and Fourier transform infrared (FTIR) spectroscopy. Insulin, a hormone protein essential for glucose metabolism, is one of the best model proteins for studying amyloid formation mechanism as it shows high kinetic quantitativity in amyloid fibril formation in vitro (21-23). Insulin is a 51-residue protein consisting of A-chain with 21 residues and B-chain with 30 residues that are connected by two interchain disulfide bonds. We previously identified in the fibrillation process of insulin from bovine under high NaCl concentration and at high temperature, where protofibril-like aggregates populate transiently before the formation of amyloid fibrils (16,24). Given that the transformation of protofibrils to amyloid fibrils is enhanced with supply of heat as external energy, they are suggested to serve as an on-pathway intermediate of amyloid fibrils.

By focusing on the protofibril formation of bovine insulin, an early-stage aggregation model has been constructed in this study based on the time course of association number and hydrodynamic radius estimated from the light scattering data. The early aggregation

of bovine insulin exhibited size development while maintaining a monomodal distribution, which has enabled the model construction based on Smoluchowski's scheme applied for colloidal aggregation (25,26). The whole reaction was divided into two types of assembly, i.e., an isotropic protein assembly to form oligomers, which is then followed by an anisotropic end-to-end association to form protofibrils. Interestingly, insulin from human, which has been investigated in parallel with bovine, did not exhibit early aggregation, from which the sensitivity of early aggregation to amino-acid sequence differences has also been suggested.

Experimental procedures

Materials. Bovine insulin was purchased from Sigma-Aldrich Co. Ltd. (St. Louis, MO). Recombinant human insulin was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). ThT was purchased from Wako Pure Chemical Industries, Ltd. HCl solution and NaCl were purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

Formation of amyloid fibrils. Bovine and human insulin were dissolved in 30 mM HCl and whose concentrations were determined using an absorption coefficient of $1.08 \text{ (mg/mL)}^{-1} \text{ cm}^{-1}$ at 276 nm (27). The spontaneous fibrillation of bovine and human insulin was performed by heating under acidic conditions; various concentrations of insulin from 1.0 to 20 mg/mL in 25 mM HCl containing NaCl at different concentrations ranging from 0 to 1.0 M, were heated at 75 °C with a thermo-regulated heat block (Dry Thermo Unit DTU-1B; TAITEC, Nagoya, Japan).

ThT fluorescence assay. To monitor the formation of amyloid fibrils, ThT fluorescence assay was performed. At different time points, a 4.5 μL aliquot of the sample solution was mixed with 1.5 mL of 5 μM ThT in 50 mM Gly-NaOH buffer (pH 8.5), and the fluorescence intensity at 485 nm was measured using an excitation wavelength of 445 nm with a RF-5300 PC spectrofluorometer (Shimadzu Corporation, Kyoto, Japan).

FTIR absorption spectroscopy. FTIR spectra were measured with a Jasco FT/IR-4700ST spectrometer equipped with a DLATGS detector (Jasco, Tokyo, Japan). Prior to the sample preparation, native insulin was preincubated in 25 mM DCl and at 37 °C for 24 h to complete the H/D exchange of amide protons, and 5.0 mg/mL of insulin solution

dissolved in 25 mM DCl containing 0.5 M NaCl was heated at 75 °C. At different time points, an approximately 50 μL aliquot of the sample, which was air-cooled to room temperature, was sealed with a CaF_2 window cell and a 50 μm polytetrafluoroethylene spacer embedded inside the water-circulating system connected to a thermoregulated water bath. FTIR spectra were then measured at 25 °C by collecting 256 interferograms at a resolution of 2 cm^{-1} . The obtained spectra exhibited the amide I' bands, which was slightly shifted to lower frequencies than the amide I bands due to the substitution of amide protons by deuterium.

Static and dynamic light scattering. To probe the evolution of protein aggregates, static and dynamic light scattering measurements were performed using a Zetasizer Nano S (Malvern Instruments Ltd., Worcestershire, U.K.) with $\theta = 173^\circ$ and $\lambda = 632.8 \text{ nm}$. The heat-induced aggregation of insulin was initiated instantaneously by mixing an insulin solution with preheated other components, and the sample solution was immediately transferred to a preheated quartz microcuvette embedded inside the thermo-regulating system. The temperature jump of the sample solution to the reaction temperature was completed within approximately 60 sec. Each light scattering data during the time course measurement was collected using an acquisition time of 5 sec.

The normalized time auto correlation function of the scattering intensity for a given correlation time τ , $g^{(2)}(\tau)$, was defined by

$$g^{(2)}(\tau) = \langle I(t)I(t+\tau) \rangle / \langle I(t) \rangle^2 \quad [1]$$

where $I(t)$ and $I(t + \tau)$ are the scattering intensities at time t and $t + \tau$, respectively. The angle brackets indicate averaging over time. $g^{(2)}(\tau)$ is related to the normalized electric field-field time autocorrelation function, $g^{(1)}(\tau)$, via the following equation:

$$g^{(2)}(\tau) = B + \beta[g^{(1)}(\tau)]^2 \quad [2]$$

where B is the long-time value of $g^{(2)}(\tau)$ ($B \sim 1$) and β is an instrument parameter. The moment method by Frisken (28), which is a reformulated version of the method of cumulants gives

$$g^{(1)}(\tau) = \exp(-\Gamma\tau)(1 + (\mu_2 / 2!)\tau^2 - (\mu_3 / 3!)\tau^3) \quad [3]$$

where Γ is the mean decay rate, and μ_m are the m th moments about the mean, which correspond directly to the m th cumulants in the method for cumulants (28). The functions of eqs. 2 and 3 with terms up to the second moment about the mean, μ_2 , were used for the fitting analysis. Γ is given by

$$\Gamma = D_{\text{dif}} q^2 \quad [4]$$

where D_{dif} is the diffusion coefficient of the particles. q is the magnitude of the scattering vector defined as

$$q = 4\pi n \sin(\theta / 2) / \lambda \quad [5]$$

where n is the refractive index of the solvent, θ is the scattering angle, and λ is the wavelength of the light. The diffusion coefficient, D_{dif} , is rationalized to the hydrodynamic radius of the particle, R_H , via the Stokes-Einstein relation:

$$R_H = k_B T / 6\pi\eta D_{\text{dif}} \quad [6]$$

where k_B is the Boltzmann constant, T is the thermodynamic temperature, and η is the viscosity of the solvent. Size distribution weighted by light scattering intensity was also obtained by the non-negative least-squares (NNLS), a regulation method for numerical inversion of the Laplace transform implemented in Zetasizer software version 7.13 (Malvern Instruments Ltd.).

The excess static light scattering from the protein particles, I_{ex} , is determined by

$\langle I(t) \rangle_{\text{sol}} - \langle I(t) \rangle_{\text{solv}}$, where subscripts “sol” and “solv” denote a protein solution and a solvent, respectively. The relative excess light scattering intensity, I/I_0 , is calculated by

$$I / I_0 = (n^2 I_{\text{ex}} / c_p) / (n_m^2 I_{\text{ex,m}} / c_{p,m}) \quad [7]$$

where c_p , $I_{\text{ex,m}}$, $c_{p,m}$, and n_m are the mass concentration of the particle, the excess light scattering intensity from the primary (monomeric) particle, its mass concentration, and the refractive index of the dissolving solvent, respectively. For Rayleigh scattering, the excess light scattering intensity is rationalized by the Rayleigh ratio, $R(\theta)$:

$$I \propto R(\theta) \quad [8]$$

$R(\theta)$ is expressed as (29)

$$R(\theta) \propto M_{\text{eff}} c_p P(q) S(q) \quad [9]$$

where $P(q)$ is the form factor, i.e., the intraparticle or the intracluster interference, and $S(q)$ is the structure factor, i.e., the interparticle or the intercluster interference. M_{eff} is the effective molecular weight of the aggregate, given as

$$M_{\text{eff}} = i_{\text{eff}} M_0 \quad [10]$$

where i_{eff} is the effective number of the primary (monomeric) particles in the aggregate and M_0 is the molecular weight of the monomer. Here, we assumed $P(q) = 1$, which is a reasonable approximation when the size of a particle is smaller than q^{-1} , i.e., $qR_H < 1$ (29,30). q^{-1} was 38 nm in the present experimental setup. The present experimental conditions under which the protein was sufficiently diluted and satisfies $S(q) = 1$., where the interparticle effect on the scattering is negligibly small. Accordingly, the excess light scattering intensity is proportional to the molecular weight: eqs. 7-10 give

$$I / I_0 = (M_{\text{eff}} / M_0) \quad [11]$$

Results

Amyloid fibril formation of bovine and human insulin. I first characterized the fibrillation process of bovine and human insulin, the amino acid residues of which differ by only three out of 51 residues, by using ThT fluorescence assay. In our previous study, the formation of protofibrils showing high ThT fluorescence intensity has been identified in bovine insulin at high NaCl concentration and high temperature (16,24). Indeed, an explosive increase and subsequent decrease in ThT fluorescence were observed when bovine insulin solution at 5.0 mg/mL was heated in the presence of various concentrations of NaCl (Figure 2-1A). The protofibrils accumulated in a larger amount and with a longer lifetime under 0.5 M NaCl than 0.1 and 0.2 M NaCl conditions, suggesting that the protofibrils were more favorably produced at higher NaCl concentrations. Unexpectedly, a sigmoidal pattern with a lag time in ThT fluorescence, which is attributed to a typical nucleation-dependent polymerization mechanism, was observed in human insulin when measured at the same protein and NaCl concentrations (Figure 2-1B). These results indicate that the fibrillation process via protofibrils is intrinsic to bovine insulin, albeit the amino acid sequences between bovine and human insulin differ only slightly. The bovine insulin-specific formation of protofibrils was also identified by the construction of phase diagrams (Figure S2-1) and small-angle X-ray scattering (SAXS) (Figure S2-2).

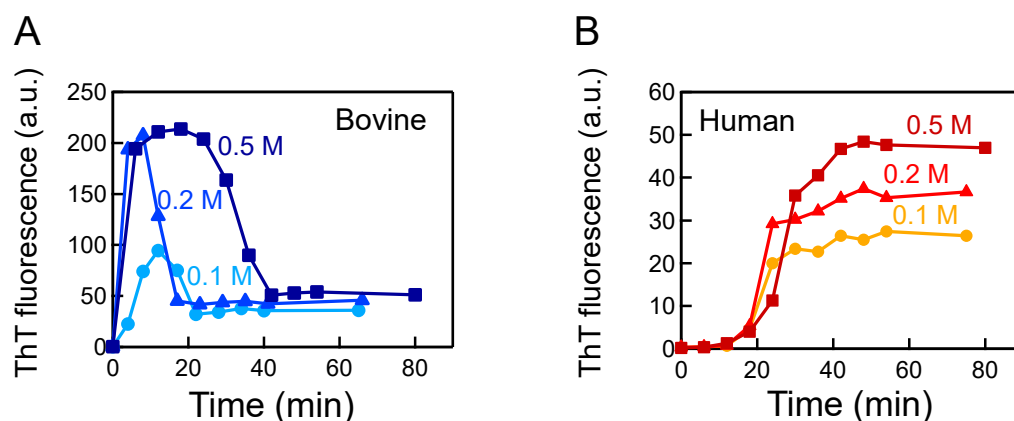


Figure 2-1. Time courses of ThT fluorescence intensity in amyloid fibril formation of bovine and human insulin. Amyloid fibril formation was induced by heating at 75 °C under the conditions of 5.0 mg/mL insulin and 0.1-0.5 M NaCl.

I also performed time-resolved FTIR measurement to verify the difference in the fibrillation process between bovine and human insulin, where deuterated conditions were used to observe the amide region without any interference of water absorption. The basic ThT fluorescence pattern of time course was conserved in both insulin species even in deuterated solution, although the fibrillation rate became slower (Figure S2-3). In both types of insulin, a sharp β -sheet peak was observed at around 1620-1630 cm^{-1} in the amide I' region as fibrillation progressed (Figure 2-2A, B). As observed in the second derivative spectra, the β -sheet peak at 1618 cm^{-1} appeared abruptly and another β -sheet peak at 1628 cm^{-1} developed thereafter for bovine insulin (Figure 2-2C), supporting the appearance of protofibrils prior to the formation of mature fibrils (16,24). In contrast, the spectral shape was unchanged significantly during the lag time and only a single β -sheet peak at 1623 cm^{-1} developed cooperatively with ThT increase for human insulin (Figure 2-2D), supporting the formation of mature fibrils without any prefibrillar aggregates.

Furthermore, the differences in β -sheet peak of bovine and human insulin suggested their structural difference consistent with our previous study (31).

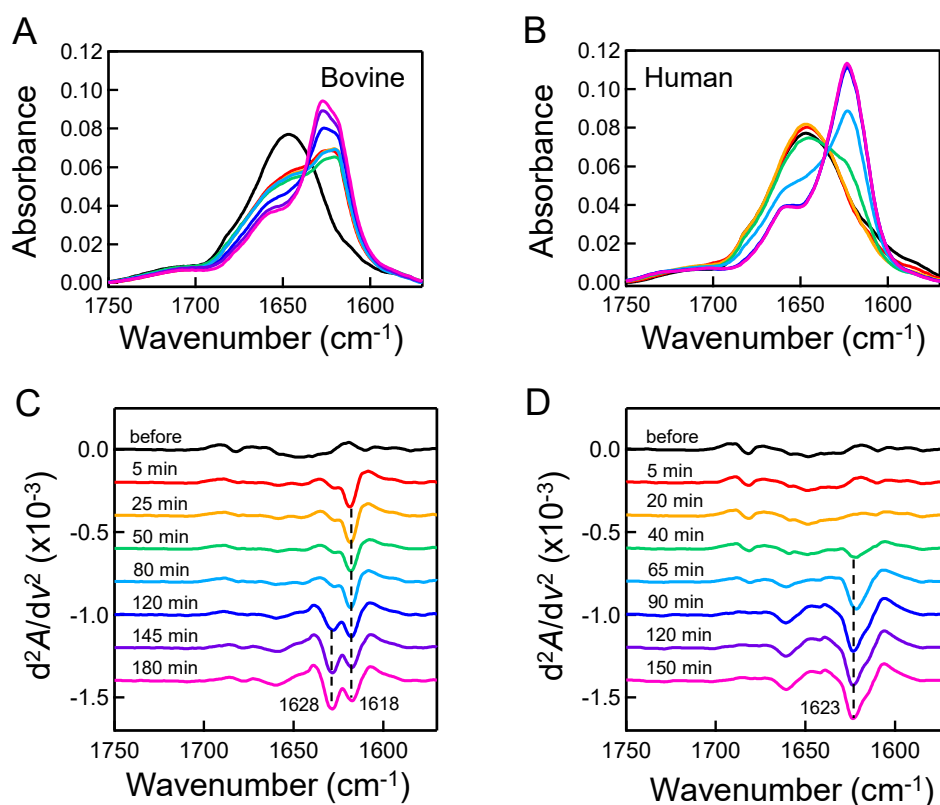


Figure 2-2. Time dependence of FTIR spectra at the amide I' region. (A, B) FTIR spectra of bovine (A) and human insulin (B). Amyloid fibril formation was performed under the conditions of 5.0 mg/mL insulin and 0.5 M NaCl at 75 °C. At different time points, an aliquot of the reaction solution, which was air-cooled to room temperature, was sealed with a cell and measured at 25 °C. Spectra were normalized so that the integrated intensity of the amide I' band ranging from 1580 to 1750 cm⁻¹ was set to be equal. (C, D) Second derivative FTIR spectra of bovine (C) and human insulin (D). The dashed black lines indicate the positions of 1618 cm⁻¹ and 1628 cm⁻¹ for bovine insulin and 1623 cm⁻¹ for human insulin.

Monitoring bovine insulin-specific early aggregation by dynamic light

scattering. I next carried out dynamic light scattering measurements to track the formation of prefibrillar aggregates of bovine insulin in a comparative manner with human insulin. When the time-dependent evolution was monitored after temperature jump, the decay of autocorrelations, $g^{(2)}(\tau)-1$, of both bovine and human insulin exhibited time-dependent increase, indicating that the size of protein aggregates evolved with the progression of time (Figure 2-3A, B). Interestingly, the autocorrelations of bovine insulin developed rapidly maintaining a single exponential pattern, and the data analysis by NNLS method exhibited a rapid growth of aggregates with a monomodal distribution (Figure 2-3C). On the other hand, the autocorrelations showed a slow evolution in human insulin. Although they apparently showed multiple exponential patterns, the data analysis by the NNLS method clarified that this evolution is attributed to the formation of a small fraction of large aggregates. The distribution of the aggregates became almost negligible when considering it as a volume fraction, and therefore it was concluded that early aggregation did not proceed in human insulin (Figure 2-3D).

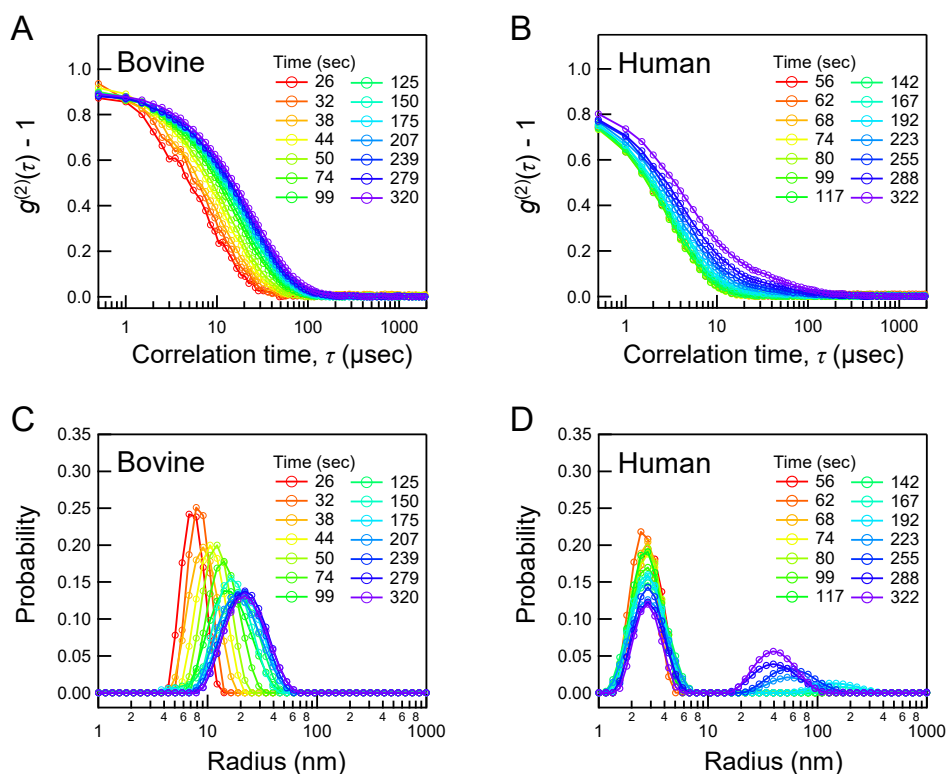


Figure 2-3. Time dependence of dynamic light scattering during the early aggregation phase of amyloid fibril formation. The heat-induced aggregation of insulin was performed under the conditions of 5.0 mg/mL insulin and 0.5 M NaCl, and monitored for approximately 5 min after initiation of the reaction by temperature jump to 75 °C. (A, B) Time dependence of the normalized time autocorrelation functions of bovine (A) and human insulin (B). (C, D) Intensity-weighted distribution of the size of bovine (C) and human insulin (D) determined by NNLS analysis on the autocorrelations.

These results demonstrate that bovine insulin forms protofibrils in the early stage of fibrillation, in contrast to typical one-step nucleation without any thermodynamically metastable aggregates. Furthermore, the aggregates kept monomodal and relatively narrow size distribution during the measurement time period in Figure 2-3C, which has indicated that the data are suitable for more quantitative analyses. The gradual and

monomodal size development was specific to bovine insulin, which was also supported by analytical ultracentrifugation measurements (Figure S2-4). I hereafter focused on the time-dependent process of early aggregation of bovine insulin in the current reaction conditions using dynamic and in addition, static light scattering.

Estimating the number of phases in the aggregation of bovine insulin.

I analyzed data from concurrent monitoring of dynamic and excess static light scattering to grasp the framework of the aggregation kinetics of bovine insulin. The time dependence of hydrodynamic radius R_H , a parameter determined from the autocorrelation function of dynamic light scattering according to eqs. 4-6, showed explosive growth and subsequent gradual development (Figure 2-4A), indicating that the early aggregation proceeds in two steps. The relative light scattering intensity, I/I_0 obtained from static light scattering according to eqs. 7-10, which corresponds to effective association number of protein particles in an aggregate, also showed a similar pattern of size evolution to that of R_H (Figure 2-4B). In the first step, as the protein concentration decreased, a slight delay became more prominent by approximately 30 sec after initiating the reaction (Figure 2-5). This result suggests that the first step involves a lag phase before the explosive growth.

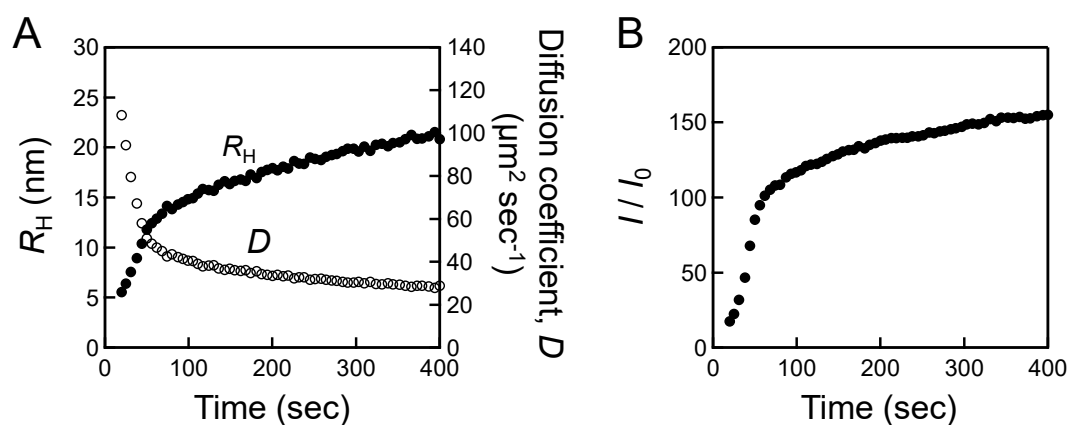


Figure 2-4. Aggregation kinetics during the early aggregation phase of bovine insulin. (A) Time dependence of the diffusion coefficient and the hydrodynamic radius. (B) Time dependence of the relative excess static light scattering intensity.

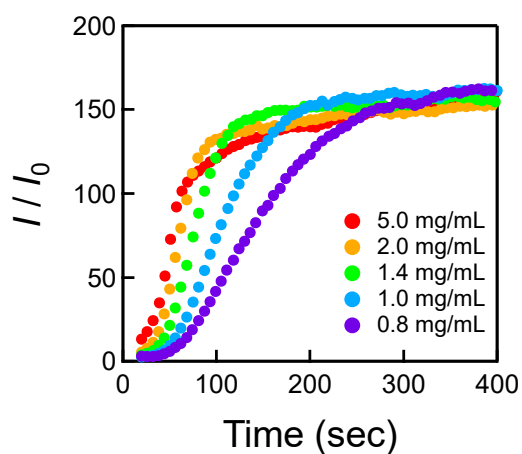


Figure 2-5. Protein concentration dependence on the early aggregation kinetics. Time dependence of the relative excess static light scattering intensity was measured in the range of 0.8-5.0 mg/mL.

To further explore the character of aggregates formed in each step, I examined the time dependence of fractal dimension, D_f , which reflects the packing of primary particles in the aggregate, i.e., compactness or morphology of the aggregates (32). D_f obeys the following relationship: (33,34)

$$i = (R_i / R_0)^{D_f} \quad [15]$$

where i is the number of a primary particle in a cluster, R_i is the radius of the cluster of an i -mer, and R_0 is the radius of a primary particle. Here, the primary particle is regarded as a monomer, and the hydrodynamic radius R_H corresponds to R_i in this equation (26). According to eqs. 10 and 15, the effective number of primary particles, i_{eff} , in a cluster is written as

$$i_{\text{eff}} = (M_{\text{eff}} / M_0) = (R_H / R_0)^{D_f} \quad [16]$$

Thus, the power-law relationship between the relative excess light scattering intensity and relative hydrodynamic radius is obtained as

$$\log(I / I_0) = D_f \log(R_H / R_0) \quad [17]$$

Here, the fractal dimension obtained from the slope of $\log(I/I_0)$ against $\log(R_H/R_0)$ was $D_f = 2.15 \pm 0.04$ in the range up to approximately 60 sec, corresponding to the boundary of the first and second steps. This value is closer to that for reaction-limited cluster aggregation ($D_f = 2.05$) (34,35) than that for diffusion-limited cluster aggregation ($D_f = 1.75$) (36).

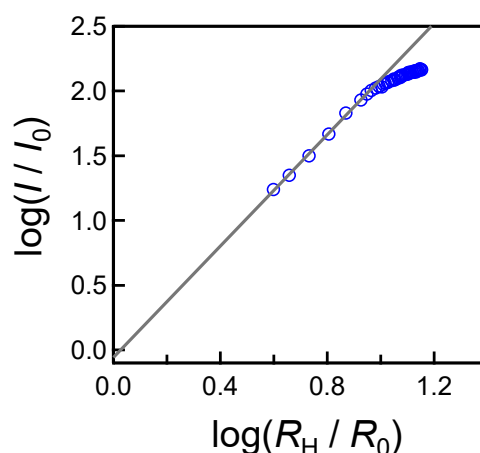


Figure 2-6. Power-law relationship between excess static light scattering intensity and hydrodynamic radius. I applied the fitting analysis with eq. 17 to the data region up to approximately 60 sec after initiating the reaction, which satisfies $R_H < 38$ nm ($qR < 1$). The solid line represents the fitting line with a slope of 2.15 and an intercept of -0.06 ± 0.03 .

However, the time dependence of $\log(I/I_0)$ against $\log(R_H/R_0)$ gradually deviated from the original slope in the second step (Figure 2-6). In light of our previous finding that bovine insulin forms protofibril-like prefibrillar aggregates before the formation of amyloid fibrils (24), it was presumed that the association mode of the clusters changed from spherical to rod-like. Based on this result, I presumed that fractal aggregation-like spherical association proceeds in the first step, which is followed by anisotropic association towards a rod in the second step.

Establishment of an aggregation model of bovine insulin. Based on the above-mentioned characterization that the aggregation of bovine insulin consists of two different

association modes, the aggregation process of bovine insulin was modeled based on Smoluchowski aggregation kinetics. In this theory, aggregation is formulated by two diffusing particles colliding and irreversibly coagulating with a given probability (25), as represented by the following equation of the time dependence of the concentration (the fraction or the population) of all i -mers:

$$\frac{dn_i}{dt} = \frac{1}{2} \sum_{j=1}^{i-1} k_{i-j,j} n_{i-j} n_j - \sum_{j=1}^{\infty} k_{i,j} n_i n_j \quad [18]$$

where n_i is the concentration of i -mer. The concentration is normalized to the initial primary particle concentration ($\sum_i i n_i = 1$). t is time, which was set to zero when heating of protein solution was initiated. $k_{i,j}$ is the rate constant at which an i -mer and a j -mer collide by diffusion per unit time. $k_{i,j}$ is given as

$$k_{i,j} = \frac{c_{0,1st} k_s}{W} A_{i,j} \quad [19]$$

where $c_{0,1st}$ is the initial concentration of the primary particle (the initial number density of the monomer) in the first step, i.e., $c_{0,1st} = 5.25 \times 10^{23}$, k_s is the Smoluchowski rate constant ($k_s = 8k_B T / 3\eta$), and W is Fuchs stability ratio, which describes the interparticle interaction between two particles (37). $A_{i,j}$ is the correlation term of the probability that an i -mer and a j -mer collide by diffusion per unit time. For fractal-like aggregation of spherical aggregates in the first step (Figure 2-7A), $A_{i,j}$ is described as:

$$A_{i,j} = \frac{1}{4} \left(i^{1/D_f} + j^{1/D_f} \right) \left(i^{-1/D_f} + j^{-1/D_f} \right) \quad [20]$$

which is a modified description with the concept of the fractal to the original formula by Smoluchowski (34,38,39). Here, two different W values were adopted to reproduce the lag phase and the subsequent rapid aggregation, respectively, in the first step as observed in Figure 2-5. For end-to-end aggregation of rod-like aggregates in the second step (Figure

2-7B), A_{ij} in eq. 19 is replaced by another correlation term, $B_{i',j'}$, which describes the probability that an i' -mer and a j' -mer collide by diffusion per unit time (40):

$$B_{i',j'} = \frac{1}{8} \frac{\delta^2 \omega^2}{L_{i'} + L_{j'}} \left(\frac{\ln(L_{i'}/b) + \nu_{i'}}{L_{i'}} + \frac{\ln(L_{j'}/b) + \nu_{j'}}{L_{j'}} \right) \quad [21]$$

$$\nu_{i'} = 0.312 + 0.565(L_{i'}/b)^{-1} - 0.1(L_{i'}/b)^{-2}$$

Here, it should be noted that i' and j' were defined as the association number of spherical aggregates formed in the first step. Therefore, $k_{i',j'}$ in the second step is given as,

$$k_{i',j'} = \frac{c_{0,2nd} k_s}{W} B_{i',j'} \quad [22]$$

Where $L_{i'}$ is the length of rod of an i' -mer. δ and ω are the minimum allowed distance and angle between two colliding rods, respectively. $\delta\omega$ was defined as $\delta\omega = d(i'+j')/2i'j'$ as described previously (41), while in the present work, I considered that δ and ω change depending on the length of the rod. These distinct aggregation modes were switched at a certain threshold cluster size, and the size of fractal aggregated clusters was set to the threshold before the start of end-to-end aggregation; therefore, the initial concentration of the primary particle in the second step, $c_{0,2nd}$, was set to 2.38×10^{21} .

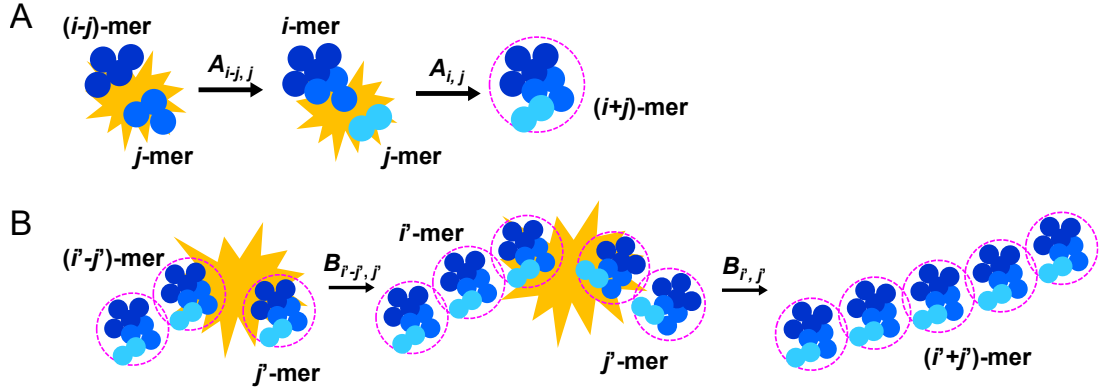


Figure 2-7. Schematic illustration of the present aggregation model for fractal (A) and subsequent end-to-end aggregation (B).

The aggregation equations were numerically solved considering the clusters with $i < 7500$, which was enough to conserve the total mass of the system in eq 18 approximating to an infinite system. Based on this, the excess light scattering intensity was calculated according to (39)

$$I / I_0 = \sum_i i^2 n_i \quad [23]$$

The Smoluchowski equation describes the time dependence of the population of the i -mer, in_i . Then, the diffusion coefficient of a system composed of an ensemble of aggregates was calculated by

$$D_{\text{dif}} = \sum_i p_i D_i \quad [24]$$

$$p_i = i^2 n_i / \sum_i i^2 n_i$$

where D_i is the diffusion coefficient of the i -mer, and p_i is the normalized intensity-weighted distribution of the i -mer.

The theoretical time dependence of I/I_0 and diffusion coefficient defined by eqs.

23 and 24 successfully reproduced the experimental data (green lines in Figure 2-8A, B). Either fractal or end-to-end aggregation model alone could not reproduce the data (red and blue lines, respectively, in Figure 2-8A, B), verifying that the current aggregation requires the combination of these two aggregation mechanisms. Through fitting the model curve to the experimental plots, the thresholds of the lag phase in the fractal aggregation and of the shift to the end-to-end association mechanism have been predicted to be ~ 30 mer ($R_{30} \sim 6.6$ nm) and ~ 150 mer ($R_{150} \sim 13.7$ nm), respectively. The values of W have also been predicted to be $4.6 (\pm 0.1) \times 10^7$ (lag phase) and $3.5 (\pm 0.1) \times 10^6$ (growth phase) for the first fractal aggregation and $1.7 (\pm 0.1) \times 10^9$ for the subsequent end-to-end association. These values are clearly larger than 1, indicating that the early aggregation of bovine insulin progresses in a reaction-limited manner in all steps.

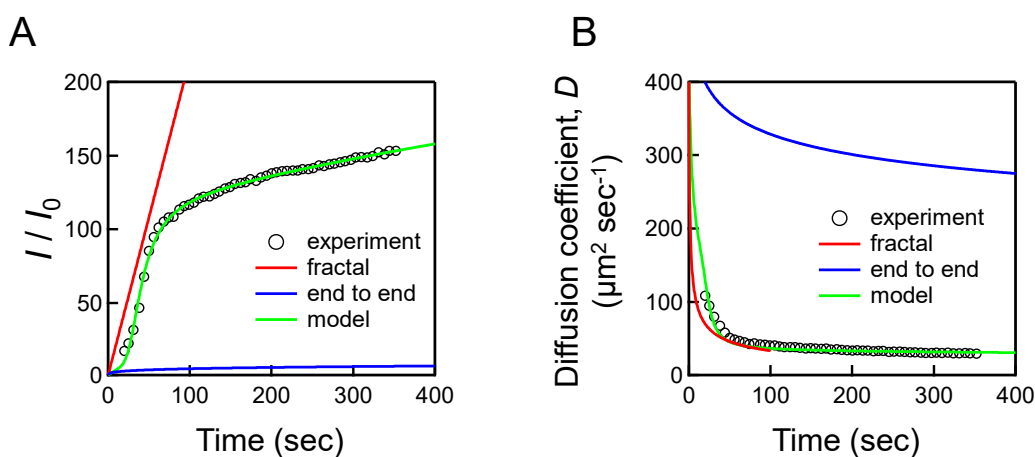


Figure 2-8. Time dependence of the relative excess static light scattering intensity (A) and the diffusion coefficient (B) simulated by the Smoluchowski aggregation equation. The black circles indicate experimental data. The red, blue, and green lines indicate the curves simulated by fractal

aggregation, end-to-end aggregation, and the present multistep model, respectively.

Effect of temperature and salt concentration on the early aggregation.

I further measured the dependence of temperature and NaCl concentration to examine detailed characteristics of the aggregation. When I/I_0 was monitored in the range of 70-80 °C, the time course showed significant acceleration with the increase of temperature (Figure 3-9A). To examine activation enthalpy, ΔH^* , in each aggregation step, I analyzed an Arrhenius plot of an effective rate constant of aggregation, $k_{\text{eff}} = k_s/W$, where the specific probability of the reaction is taken into account by combining W with k_s . The values of W and k_{eff} obtained by fitting analysis are shown in Table S2-1. ΔH^* is expressed as (38)

$$k_{\text{eff}} = k_{\text{eff},0} \exp\left[-\Delta H^* \left(1/RT - 1/RT_0\right)\right] \quad [25]$$

where $k_{\text{eff},0}$ is k_{eff} at the reference temperature, T_0 , and R is the gas constant. ΔH^* was obtained from the slope, the value of which were 133 ± 8 kJ/mol (lag phase) and -14 ± 14 kJ/mol (growth phase) for the initial fractal aggregation, and 177 ± 24 kJ/mol for the end-to-end association (Figure 3-9B). It has been revealed that the pathway consists of two dominant energy barriers for the progression of aggregation, and the latter end-to-end association acts as a rate-limiting step in the whole reaction.

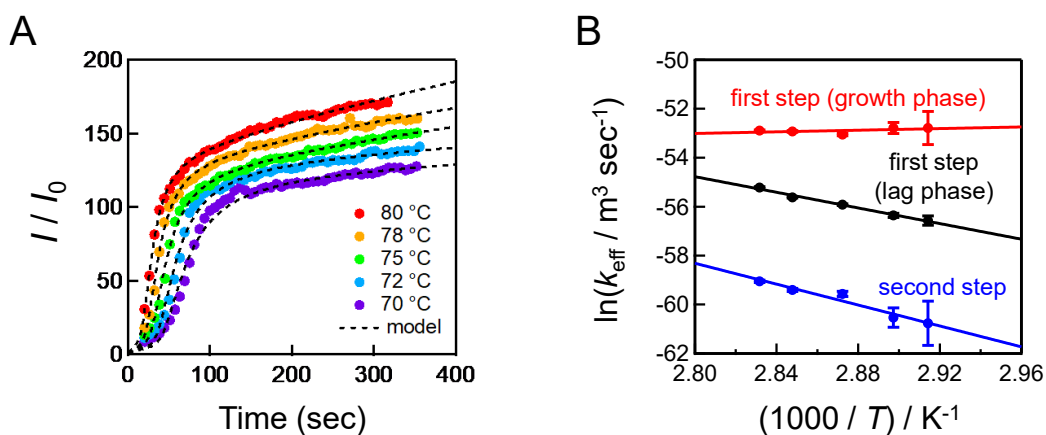


Figure 2-9. Temperature dependence on the early aggregation of bovine insulin in the range of 70 to 80 °C. (A) Time dependence of relative excess static light scattering intensity. (B) Temperature dependence on the effective aggregation rate constant, k_{eff} , for first (lag and growth periods) and second steps. k_{eff} was determined by the relative excess light scattering intensity, I/I_0 . The solid lines indicate the fitting line by eq. 25 in the range between 70 to 80 °C.

The concentration dependence of NaCl was also monitored in the range of 0.40-0.55 M. When I/I_0 was monitored, its time evolution showed selective acceleration in the end-to-end association (Figure 2-10A), which has been clearly demonstrated by k_{eff} determined by curve fitting (Figure 2-10B and Table S2-2). Considering that the NaCl concentrations used for the analysis are high enough to electrostatically shield protein molecules (Figure S2-5), it is expected that the salt-induced acceleration is related to the hydration state, the properties of which tend to be modulated by ionic interactions with protein molecules and water. In agreement with this idea, k_{eff} of the end-to-end association

showed relationship to Hofmeister series when NaCl was substituted for other salts with different cations (Figure 2-11A, B and Table S2-3).

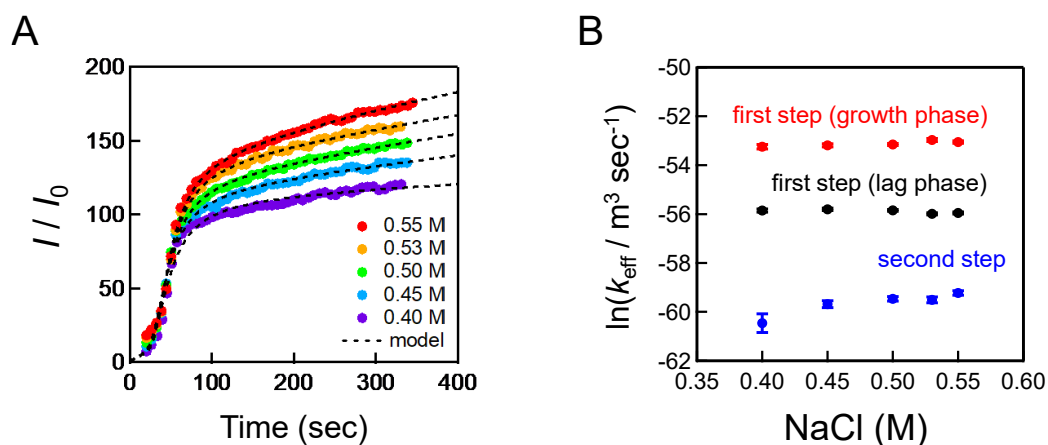


Figure 2-10. Salt concentration dependence on the early aggregation of bovine insulin. (A) Time dependence of relative excess static light scattering intensity in the range of 0.4 to 0.55 M NaCl. (B) Salt concentration dependence on the effective aggregation rate constant for first (lag and growth periods) and second steps.

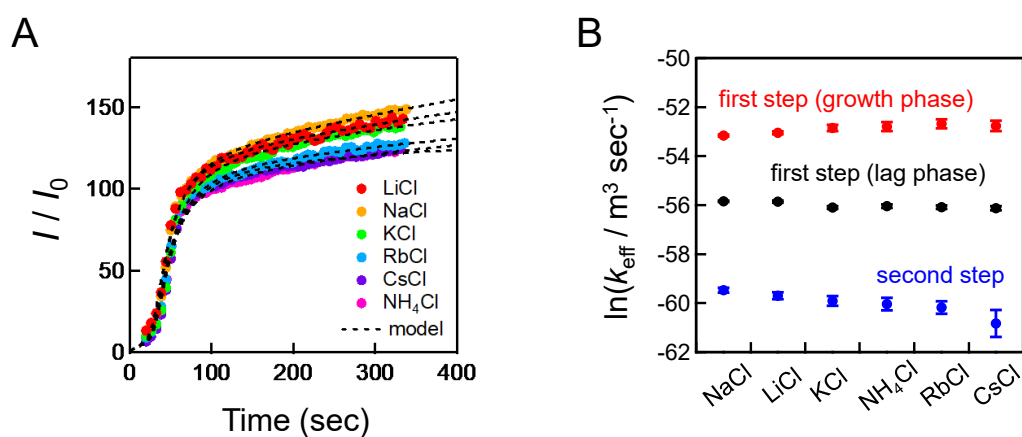


Figure 2-11. Hofmeister salt dependence on the early aggregation of bovine insulin. (A) Time dependence of relative excess static light scattering intensity of various salts with different cations is shown. (B) Hofmeister salt dependence on the effective aggregation rate constant for first (lag and growth periods) and second steps.

Discussion

Early aggregation process of bovine insulin. In this study, I have achieved to describe the protofibril formation mechanism of bovine insulin based on Smoluchowski aggregation kinetics. Protofibrils have attracted attention as a precursor of amyloid fibrils, and recently, they have also been focused on as pathologically important aggregates that exhibit cytotoxicity along with oligomers (12). However, the detailed process of protofibril formation has been poorly tracked, often due to the low and heterogeneous distribution of aggregates in the early stage. As a breakthrough for the difficulty, I have found an almost monodisperse development of early aggregates in bovine insulin (Figure 2), which has allowed for the establishment of a mechanistic model of oligomer and protofibril formation.

The Smoluchowski aggregation equation or population balance equation is an attractive basis for analyzing protein aggregation because of its simple assumption of irreversible colliding of two particles. Indeed, it has been applied when analyzing aggregation processes of many peptides and proteins (26,38-40,42-47). The examples include studies on amyloid fibril formation (42-45,47); however, they mainly describe the elongation process of amyloid fibril formation by applying only one type of aggregation mode. Here, I have revealed that the early aggregation of bovine insulin could be divided into two steps based on changes in fractal dimension, and successfully reproduced the experimental data by assuming isotropic fractal aggregation, followed by anisotropic end-to-end elongation. Recently, the development of AmyloFit has greatly advanced our understanding of microscopic steps during amyloid fibril formation, which has been extended to kinetic modeling of oligomer dynamics for therapeutic strategies for their inhibition (48,49). At present, however, it is limited to revealing the distribution of

oligomers with a fixed association number. The schematic model presented in this study is based on the molecular weight and diffusion coefficient of aggregates, and has seamlessly provided time dependence of aggregates not only in the size but also in the shape.

Figure 6 shows the scheme of oligomer and protofibril formation of bovine insulin revealed in this study. Fractal-like spherical aggregation proceeds in the first step, and their end-to-end association initiates in the second step to form rod-like aggregates when the spherical clusters reach a certain size. This scheme shows that oligomer and protofibril formation essentially progresses by a mechanism close to colloidal aggregation, distinct from the nucleation-dependent growth of amyloid fibrils. The switching of aggregation mode occurs after the size of spherical clusters develops to ~ 13.7 nm in hydrodynamic radius (Figure 2-8). Given that the first step corresponds to oligomer formation, the present scheme describes the protofibril formation mechanism using oligomers as building blocks. This two-step scheme has not been considered in our previous tracking of protofibril formation of bovine insulin by SAXS (24), just by approximating aggregate shape to an elliptical cylinder over the whole measurement time range. However, this analysis also showed initial thickening of the elliptical cylindrical aggregates prior to subsequent elongation, presumably corresponding to the initial formation of spherical clusters.

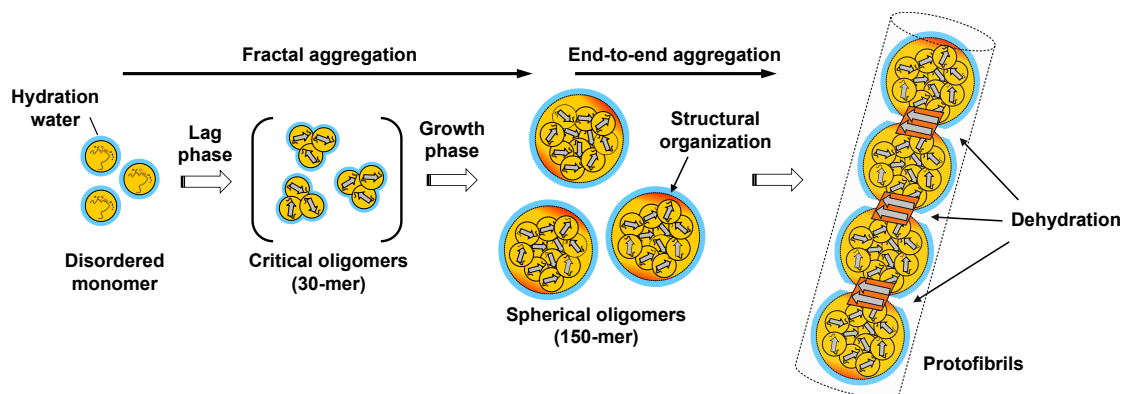


Figure 2-12. Schematic illustration representing the protofibril formation mechanism of bovine insulin.

Proposed mechanism of aggregation scheme switching. The proposed scheme involves switching of the two aggregation schemes at a threshold of cluster size. Since the formation of spherical and rod-like aggregates obey different correlation terms, A_{ij} and B_{ij} , respectively, their formation pathways are theoretically different. However, the present observation suggests that they approach each other as aggregation proceeds, making it possible to switch at the point of closest proximity. Because protofibrils contain some amount of β -sheet structure (Figure 2-2A, C), their critical free energy is expected to be higher than that of oligomers resulting in an initial kinetic preference for oligomer formation. As aggregates develop in size, however, protofibrils gain more pronounced stability and eventually exceed the stability of oligomers, which may change the aggregation pathway from oligomers to protofibrils. Since hydrophobic interactions are considered to drive oligomer formation, the growth of oligomers will become unfavorable with a threshold where the number of hydrophobic residues is insufficient to fill the interior of the cluster and some of the hydrophilic residues must be buried; this may

determine the timing of the switch from spherical oligomers to anisotropically shaped protofibrils with a smaller relative internal volume.

It is also important to clarify what is the driving force for the end-to-end assembly. Although no structural details were obtained directly in this study, the presence of a lag phase in the oligomer formation (Figure 2-5) indicates that oligomers contain some degree of ordered structure. Considering that oligomers serve as building blocks for protofibril growth, they are expected to acquire some specific structure that plays a role as binding sites at two ends. Interestingly, a strong salt-induced enhancement was observed in the second step even at high salt concentrations where the charge shielding is fully completed (Figure S2-5). In addition, the Hofmeister series (50) was highlighted when analyzing various salts (Figure 2-11A, B), strongly implicating the involvement of water molecules in the end-to-end association. There are several studies proposing interplays between water and the structural dynamics of proteins, where dehydration of polar groups of proteins has been revealed to undergo in the rate-limiting step of protein folding, protofilament association and elongation of amyloid fibrils (51-53). Given that the hydrogen bonds formed by polar groups provide directional specificity, it is presumed that water molecules, which act as hydrogen bond-forming groups, overcome the high dehydration energy to acquire interactions with orientational specificity important for organizing ordered structure. Likewise, aggregate structure becomes more intensely ordered in the end-to-end association, presumably forming a β -structure, through expulsion of water from polar groups at the interface, by paying the cost of dehydration energy barrier.

Differences in aggregation scheme between bovine and human insulin.

Another notable observation in this work is that human insulin showed the simplest one-step nucleation scheme without any significant formation of oligomers and protofibrils (Figure 2-3B, D). The amino acid sequence differs between bovine and human insulin only by 3 of 51 residues. The disappearance of early aggregates in human insulin despite such high sequence homology implies that the nucleation mechanism is highly dependent not only on the reaction conditions but also on the amino acid sequence. This is quite in analogy to observations that the Δ E22 and E22G mutant of amyloid β produces markedly larger amounts of oligomers than the wild type (54,55) and that the E46K and A30P mutants of α -synuclein promote and delay oligomer formation, respectively (56). Both of these are considered to be representative examples in living systems in which even slight residue differences affect aggregation pathway.

In our previous study, I identified structured regions of bovine insulin protofibrils by protease digestion. The result demonstrated that the 8th and 10th positions of the A chain were structured in protofibrils (16). Combining this finding with another observation that porcine insulin, in which only the 30th residue of the B-chain is bovine type, showed a one-step nucleation similar to human insulin (data not shown), it is suggested that two residues of the A-chain contribute to oligomer and protofibril formation. The initiation of oligomer and protofibril formation is expected to be driven by hydrophobic interactions and formation propensity of β structures; however, no clear differences between bovine and human insulin have been so far found even using algorithms (57), implying that very slight sequence characteristics may cause variations in amyloid nucleation scheme.

Supporting information

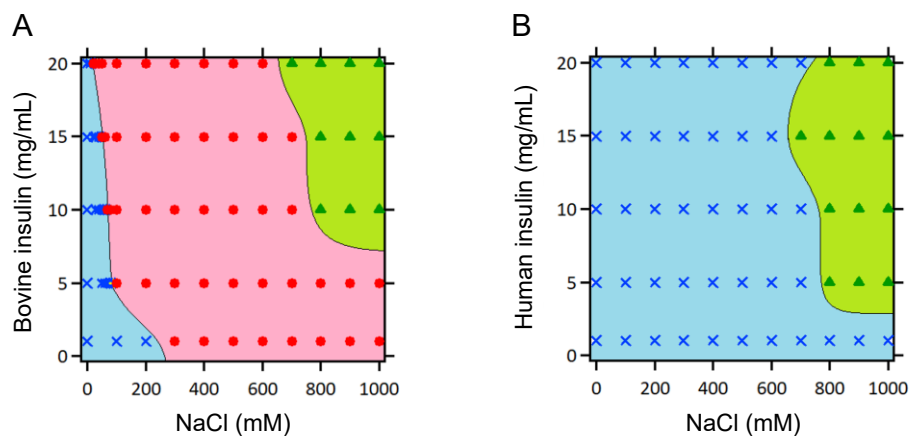


Figure S2-1. Phase diagram of amyloid fibril formation pathway of bovine (A) and human insulin (B). The blue and red areas indicate amyloid fibril formation pathway without and with prefibrillar aggregates, respectively. The green area indicates amorphous aggregation pathway. The phase diagrams were constructed based on the results of ThT fluorescence assay.

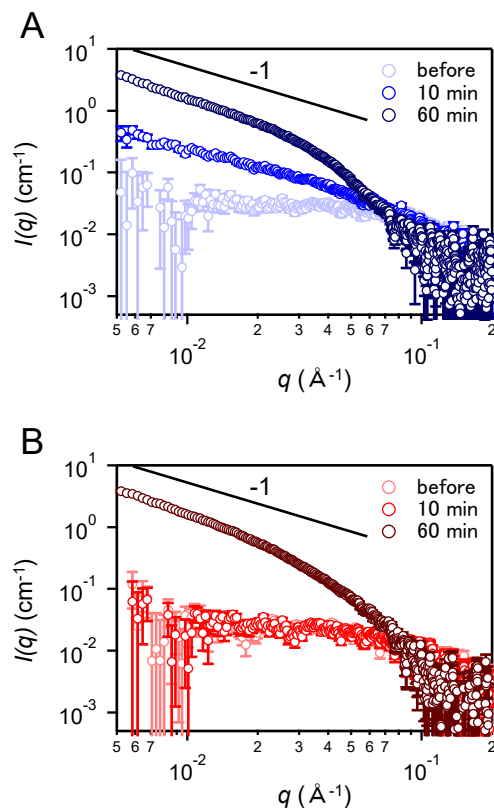


Figure S2-2. SAXS profiles of aggregated species of bovine (A) and human insulin (B).

Samples of 5.0 mg/mL insulin dissolved in 25 mM HCl containing 0.1 M NaCl was heated at 75 °C for 10 and 60 min, and the reaction was instantly stopped with cooling on ice. SAXS measurements were conducted with a laboratory-based instrument NANOPIX (Rigaku, Japan). The sample-to-detector distance (SDD) was set to be 1330 mm, with which the covered q -range was $0.005 \text{ \AA}^{-1} \leq q \leq 0.20 \text{ \AA}^{-1}$. All measurements were performed at 25 °C. One-dimensional scattering profiles $I(q)$ were obtained by the radial averaging of two-dimensional scattering patterns using SAngler software (58). After correction by the transmittance and subtraction of buffer scattering, the absolute scattering intensity was obtained using the standard scattering intensity of water ($1.632 \times 10^{-2} \text{ cm}^{-1}$) (59).

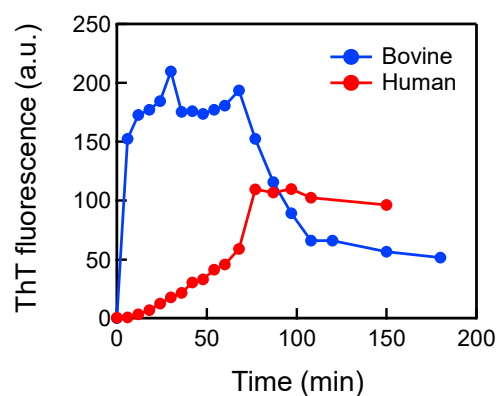


Figure S2-3. Time courses of ThT fluorescence intensity of amyloid fibril formation of bovine and human insulin under deuterated conditions. Prior to the sample preparation, bovine insulin was preincubated in 25 mM DCl and at 37 °C for 24 h to complete the H/D exchange of amide protons, and 5.0 mg/mL of insulin solution dissolved in 25 mM DCl containing 0.5 M NaCl was heated at 75 °C. At different time points, a 4.5 μ L aliquot of the sample solution was mixed with 1.5 mL of 5 μ M ThT in 50 mM Gly-NaOH buffer (pH 8.5), and the fluorescence intensity at 485 nm was measured using an excitation wavelength of 445 nm with a RF-5300 PC spectrofluorometer (Shimadzu Corporation, Kyoto, Japan).

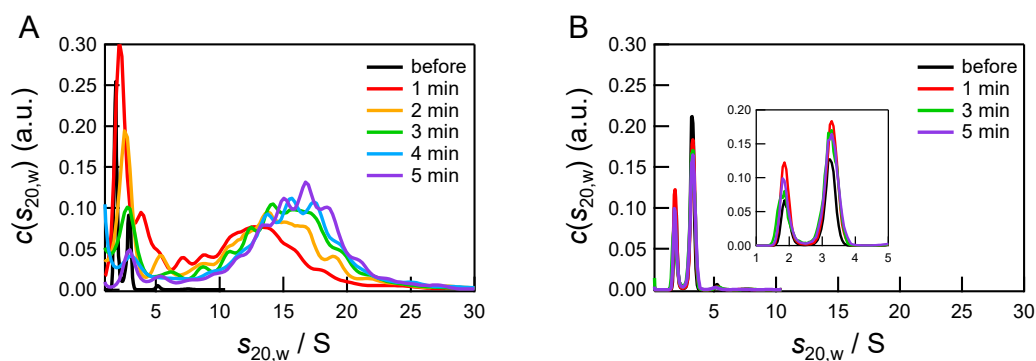


Figure S2-4. Sedimentation coefficient distributions of early aggregated species of bovine (A) and human insulin (B). Analytical ultracentrifugation (AUC) was conducted with a ProteomeLab XL-I analytical ultracentrifuge (Beckman Coulter, Brea, CA, USA). A sample of 5.0 mg/mL insulin dissolved in 25 mM HCl containing 0.5 M NaCl was heated at 75 °C for different times ranging from 1 to 5 min, and the reaction was instantly stopped with cooling on ice. For bovine insulin, sample solutions were diluted 2-fold with 25 mM HCl to suppress further flocculation of aggregates induced by cooling. Sedimentation velocity analytical ultracentrifugation (SV-AUC) measurements were performed using Rayleigh interference optics at 40,000 rpm at 20 °C with a 1.5 mm path-length cell. The experimental data of SV-AUC were analyzed with SEDFIT software which executed the fitting with Lamm formula (60). For the analysis, the density and viscosity of solvents, and partial specific volumes of each protein were calculated from their amino acid sequences with SEDNTERP software (61).

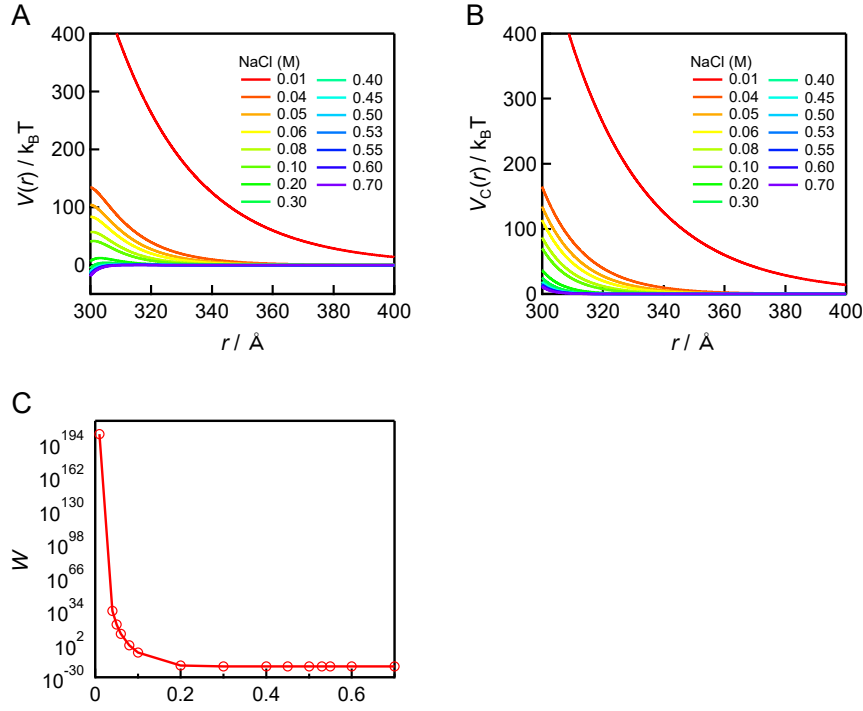


Figure S2-5. Salt concentration dependence of intermolecular potential of a protein molecule, $V(r)$ (A), screened repulsive Coulomb potential, $V_C(r)$ (B), and Fuchs stability ratio, W (C). Based on the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory, $V(r)$ is expressed as

$$V(r) = V_C(r) + V_{AY}(r)$$

where r is the distance between the protein molecules. σ is the diameter of the protein. The terms, $V_C(r)$ and $V_{AY}(r)$ are the screened repulsive Coulomb potential and the attractive potential, respectively. Here, $V_{AY}(r)$ is assumed to be Yukawa-type potential:

$$V_C(r) = \frac{Z^2 e^2}{\epsilon(1 + 0.5\kappa\sigma)^2} \frac{\exp[-\kappa(r - \sigma)]}{r}$$

$$V_{AY}(r) = -J \left(\frac{\sigma}{r} \right) \exp \left[\frac{-(r - \sigma)}{d} \right]$$

Z is the net charge on the protein, e is the elementary charge, ε is the dielectric constant of the medium, κ is the reciprocal Debye-Hückel screening length, J is the depth of attractive potential at $r = s$, and d is the range of the attractive potential. In the present calculation, I used $J = 30 k_B T$, $d = 3 \text{ \AA}$, $Z = +900$ ($+6 \times 150$ mer), and $\sigma = 300 \text{ \AA}$ (30 nm).

Table S2-1. Effective aggregation rate constant for the temperature dependence on early aggregation of bovine insulin obtained by fitting analysis

Temperature (°C)	threshold association number for the lag phase in the first step	threshold association number for the second step	$k_{\text{eff}} (\text{m}^3 \text{sec}^{-1})$		
			first step (lag phase)	first step (growth phase)	second step
70	30	135	$2.7 (\pm 0.5) \times 10^{-25}$	$1.2 (\pm 0.8) \times 10^{-23}$	$4.1 (\pm 3.7) \times 10^{-27}$
72	30	145	$3.4 (\pm 0.3) \times 10^{-25}$	$1.2 (\pm 0.3) \times 10^{-23}$	$5.1 (\pm 2.0) \times 10^{-27}$
75	30	150	$5.2 (\pm 0.2) \times 10^{-25}$	$9.0 (\pm 0.5) \times 10^{-24}$	$1.4 (\pm 0.1) \times 10^{-26}$
78	30	160	$7.0 (\pm 0.2) \times 10^{-25}$	$1.0 (\pm 0.04) \times 10^{-23}$	$1.6 (\pm 0.1) \times 10^{-26}$
80	30	170	$1.0 (\pm 0.04) \times 10^{-24}$	$1.0 (\pm 0.03) \times 10^{-23}$	$2.3 (\pm 0.1) \times 10^{-26}$

Table S2-2. Effective aggregation rate constant for the salt concentration dependence on early aggregation of bovine insulin obtained by fitting analysis

NaCl (M)	threshold association number for the lag phase in the first step	threshold association number for the second step	$k_{\text{eff}} (\text{m}^3 \text{sec}^{-1})$		
			first step (lag phase)	first step (growth phase)	second step
0.40	30	130	$5.5 (\pm 0.4) \times 10^{-25}$	$7.5 (\pm 0.7) \times 10^{-24}$	$5.5 (\pm 2.1) \times 10^{-27}$
0.45	30	140	$5.8 (\pm 0.3) \times 10^{-25}$	$8.0 (\pm 0.5) \times 10^{-24}$	$1.2 (\pm 0.2) \times 10^{-26}$
0.50	30	150	$5.5 (\pm 0.2) \times 10^{-25}$	$8.2 (\pm 0.4) \times 10^{-24}$	$1.5 (\pm 0.1) \times 10^{-26}$
0.53	30	160	$4.8 (\pm 0.2) \times 10^{-25}$	$9.9 (\pm 0.6) \times 10^{-24}$	$1.4 (\pm 0.2) \times 10^{-26}$
0.55	30	170	$5.0 (\pm 0.2) \times 10^{-25}$	$9.1 (\pm 0.5) \times 10^{-24}$	$2.0 (\pm 0.2) \times 10^{-26}$

Table S2-3. Effective aggregation rate constant for the Hofmeister salt dependence on early aggregation of bovine insulin obtained by fitting analysis.

Salt	threshold association number for the lag phase in the first step	threshold association number for the second step	$k_{\text{eff}} (\text{m}^3 \text{sec}^{-1})$		
			first step (lag phase)	first step (growth phase)	second step
NaCl	30	150	$5.8 (\pm 0.3) \times 10^{-25}$	$8.0 (\pm 0.5) \times 10^{-24}$	$1.5 (\pm 0.2) \times 10^{-26}$
LiCl	30	145	$5.5 (\pm 0.2) \times 10^{-25}$	$9.1 (\pm 0.6) \times 10^{-24}$	$1.2 (\pm 0.2) \times 10^{-26}$
KCl	30	140	$4.3 (\pm 0.3) \times 10^{-25}$	$1.1 (\pm 0.1) \times 10^{-23}$	$9.6 (\pm 1.9) \times 10^{-27}$
NH ₄ Cl	30	125	$4.6 (\pm 0.4) \times 10^{-25}$	$1.2 (\pm 0.2) \times 10^{-23}$	$8.5 (\pm 2.2) \times 10^{-27}$
RbCl	30	130	$4.4 (\pm 0.3) \times 10^{-25}$	$1.3 (\pm 0.2) \times 10^{-23}$	$7.4 (\pm 1.9) \times 10^{-27}$
CsCl	30	130	$4.2 (\pm 0.4) \times 10^{-25}$	$1.2 (\pm 0.3) \times 10^{-23}$	$3.8 (\pm 2.1) \times 10^{-27}$

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Chapter 3

Multistep changes in amyloid structure induced by cross-seeding on a rugged energy landscape

Introduction

Protein misfolding induces aberrant aggregation, and often results in the formation of amyloid fibrils that are associated with more than 40 amyloidoses and neurodegenerative diseases, including Alzheimer's and Parkinson's diseases (1-4). Although needle-like morphology and cross- β structure are common basic properties of amyloid fibrils, it has been revealed that amyloid fibrils show microscopic structural diversity (4-6). Differences in amyloid structure are observed even when amino acid sequence is identical or very similar, and details of these different structures have recently been clarified at an atomic level owing to the progress of cryo-electron microscopy (cryo-EM) (7, 8) and solid-state NMR spectroscopy (9, 10). Variations of amyloid structure often lead to various physicochemical properties such as morphology (11, 12) and structural stability (13). Moreover, it has also been suggested that such structure-dependent properties of amyloid fibrils exert diverse pathological effects in vivo (7, 8, 14-16) and researches on relationships between amyloid fibril structure and its phenotype have been conducted vigorously. In prion disease, which has been revealed as an infectious amyloidosis, it is proposed that the diverse structures of prion fibrils result in different transmissibility (13). Amyloid conformations are thus an important factor for determining various physiological phenotypes, and now attract much attention for understanding pathology based on protein structure.

According to a nucleation-dependent polymerization, which is the basic model of amyloid assembly, the growth of amyloid fibrils is accelerated by the presence of fibril seeds (17). With this mechanism, diverse structures of amyloid fibrils are proposed to proliferate in vivo and therefore to play an important role in the transmission and progression of diseases. Typically, seed-dependent elongation with the same proteins, i.e.,

self-seeding, occurs most preferably, and in this case, the structure of seeds is replicated robustly during elongation (18). As a detailed molecular picture, the dock-lock model has been provided (19). On the other hand, cross-seeding is less likely to proceed than self-seeding, although it occurs when proteins can cross a species barrier, as seen between variants of the same protein (20-22), and additionally between heterologous proteins in some cases (23, 24). Several studies about the propagation of amyloid structures in cross-seeding have been conducted so far, and in an early report on prion protein variants by Jones and Surewicz, it was demonstrated that the morphology and secondary structure of seed fibrils were inherited exactly when they were used as a template in the cross-seeding of proteins from other species (20). In contrast, recent studies have revealed that cross-seeding sometimes produced amyloid fibrils different from those used as seeds with respect to secondary structure and cytotoxicity (25-27). The latter observations suggest that the complete replication of seed fibrils is not always achieved in cross-seeding, although detailed mechanisms remain unclear.

In this study, we investigated self- and cross-seeding of human and bovine insulin. Insulin is a hormone protein associated with glucose metabolism and consists of two polypeptide chains, A-chain (21 residues) and B-chain (30 residues), which are cross-linked by two interchain disulfide bonds. It is one of the best models for studying amyloid formation mechanism because it forms amyloid fibrils readily in vitro (28). Between human and bovine insulin, amino acid sequences differ in three residues (two in A-chain and one in B-chain) (Figure 3-1), while their native structures are almost the same (21). Since these amino acid sequences are close enough to cross species barriers with each other, they are good models for investigating interspecies cross-seeding. We tracked preservation or change of amyloid fibril structure in the process of repetitive self- or

cross-seeding of human and bovine insulin by using thioflavin T (ThT) fluorescence, atomic force microscope (AFM), Fourier transform infrared (FTIR) spectroscopy and iodine staining. In addition, the cytotoxicity of amyloid fibrils was also analyzed using cell lines as one of the indicators of phenotype.

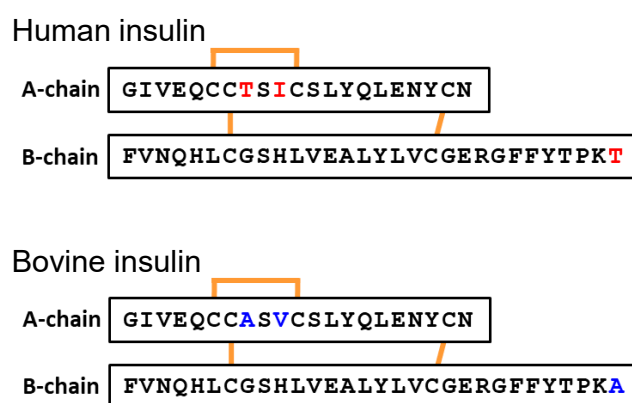


Figure 3-1. Amino acid sequences of human and bovine insulin. The red and blue characters represent different residues between human and bovine insulin, respectively.

The results of the measurements with several different techniques have demonstrated that human and bovine insulin amyloid fibrils adopted different structures even though only three residues are different. While both of these amyloid structures were replicated by self-seeding, they changed gradually when cross-seeding was performed. Notably, a new-type fibril structure with distinct morphology and cytotoxicity was observed when human insulin was cross-seeded with bovine insulin seeds. Here, iodine staining, a coloring reaction through the formation of polyiodide ions on the surface of amyloid fibrils (29), worked as a sensitive probe for reporting changes in amyloid structures. The ultraviolet-visible (UV-Vis) absorption spectra of iodine-stained amyloid

fibrils showed complicated changes, and by introducing singular value decomposition (SVD) analysis, intermediate metastable states have been identified in the process of cross-seeding.

Materials and Methods

Materials. Recombinant human insulin was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Bovine insulin was purchased from Sigma-Aldrich Co. Ltd. (St. Louis, MO). Iodine, potassium iodine, and ThT were purchased from Wako Pure Chemical Industries, Ltd. HCl solution and NaCl were purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

Formation of insulin amyloid fibrils. Human and bovine insulin were dissolved in 25 mM HCl and whose concentrations were determined using an absorption coefficient of $1.08 \text{ cm}^{-1} (\text{mg/mL})^{-1}$ at 276 nm (30). Spontaneous fibrillation of human and bovine insulin was performed by heating; a sample solution containing 2.0 mg/mL insulin, 25 mM HCl and 100 mM NaCl was incubated at 65 °C for 24 h without agitation. For seeding experiments, seeds were obtained by sonicating pre-formed fibrils with 20 pulses with 1 sec pulse length at a power level of 2.0 W. Seeding reaction was essentially performed under the same solvent conditions as used for the spontaneous fibrillation except for the addition of 5% (v/v) seeds. The reaction temperature was set at 37 °C to prevent spontaneous fibrillation of native insulin and the incubation time was set for 18 h for each reaction.

ThT fluorescence assay. To assay the formation of amyloid fibrils, ThT fluorescence assay was performed with fundamentally the same protocol as previously reported (31). In the assay, 3 μL of a sample solution was mixed with 1.5 mL of 5 μM ThT in 50 mM Gly-NaOH buffer (pH 8.5). After 1 min incubation, ThT fluorescence intensity at 485 nm was measured using an excitation wavelength of 445 nm with a RF-5300 PC

spectrofluorometer (Shimadzu Corporation, Kyoto, Japan).

Attenuated total reflectance (ATR)-FTIR spectroscopy. ATR-FTIR spectra were recorded with a Nicolet iS5 FT-IR equipped with an iD5 ATR accessory (Thermo Fisher Scientific, Tokyo, Japan). Each fibril sample was sonicated before measurements with the same protocol as used for the preparation of seeds to improve dispersibility. 3.5 μL of a sample solution was spotted onto the surface of a diamond crystal and then dried using compressed air. FTIR spectra were measured by collecting 128 interferograms with a resolution of 4 cm^{-1} . A spectrum of atmospheric air was used as a reference spectrum to subtract the contributions of water vapor and carbon dioxide.

Iodine staining of amyloid fibrils. Iodine staining of amyloid fibrils was performed as previously reported (29). Iodine solution containing 24 mM KI and 3.2 mM I_2 was initially prepared, and each sample of 2.0 mg/mL insulin fibrils was sonicated with the same protocol as used for the preparation of seeds to improve dispersibility. Then 0.50 mg/mL insulin fibrils containing 0.9 mM KI and 0.12 mM I_2 in 25 mM HCl were prepared by mixing the fibril sample and the iodine solution. After 1 min incubation, the color of the iodine-stained insulin fibrils was analyzed by UV-Vis spectroscopy. UV-Vis absorption spectra were recorded with a Jasco V-650 spectrometer (JASCO, Tokyo, Japan) using a quartz cell with a 1 cm optical length.

Atomic force microscopy (AFM). 10 μL of a sample solution was loaded to a freshly cleaved mica plate, left for 1 min, and then rinsed using 200 μL of distilled water. Residual solution was removed with filter paper and left to dry overnight. AFM images

were obtained using a dynamic force mode with Probestation NanoNavi II/IIe (Hitachi High-Tech Science, Tokyo, Japan). The scanning tip used was an OMCL-AC160TS-C3 micro cantilever (Olympus Corporation, Tokyo, Japan; spring constant = 21–37 N/m, resonance frequency = 270–340 kHz), and the scan rate was set to 0.5 Hz with the recording of 256×256 points per image.

Transmission electron microscopy (TEM). Samples were diluted 10-fold with distilled water and 5 μ L of a sample solution was spotted onto a collodion-coated grid (Nisshin EM Co., Ltd., Tokyo, Japan). After 1 min, the solution on the grid was removed with filter paper. Then 5 μ L of 5% (w/v) hafnium chloride was spotted onto the grid, left for 1 min, the solution was removed in the same manner. TEM images were obtained using an H-7650 transmission microscope (Hitachi High-Technologies Corporation, Tokyo, Japan) with a voltage of 80 kV.

Cytotoxic assay. Cell viability was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell count kit (Nacalai Tesque, Kyoto, Japan), which is based on the conversion of tetrazolium salt by mitochondrial dehydrogenase to a formazan product. PC12 cells were maintained in Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 5% horse serum, penicillin (100 U/mL), and streptomycin (100 μ g/mL) in 5% CO₂ at 37 °C. The cells were plated in 96-well plates at a density of 20,000 cells/well and grown overnight. The cells were incubated in 110 μ L medium in the absence and presence of insulin samples diluted with phosphate buffered saline (PBS) to indicated concentrations. After 24 h incubation, 10 μ L of MTT reagents were added and incubated for 4 h in 5% CO₂ at 37 °C. The reaction was stopped

by adding 100 μ L of solubilization solution (40 mM HCl in 2-propanol). The plates were read with a Multiskan FC microplate reader (Thermo Fisher Scientific Inc., Waltham, MA) at 570 nm. Each sample was assayed in triplicate and the cell viability was calculated using the value obtained by the addition of PBS alone as a reference.

Results

Formation of human/bovine insulin amyloid fibrils. Human and bovine insulin amyloid fibrils were first prepared by heating under acidic conditions, as used in earlier studies (32-34). After the completion of the reaction, more than 99 % of insulin protein was converted to fibrils for both species, which was confirmed by measuring the concentration of residual insulin monomer in the supernatant of the samples after centrifugation. When amyloid fibrils were mixed with ThT, human insulin fibrils showed a higher and slightly red-shifted fluorescence spectrum compared to bovine insulin fibrils (Figure 3-2). In light of the previous suggestion that the ThT fluorescence properties vary depending on the binding mode of ThT on amyloid fibrils (35), the difference in ThT fluorescence spectrum implies that the structures of amyloid fibrils are different between these two species.

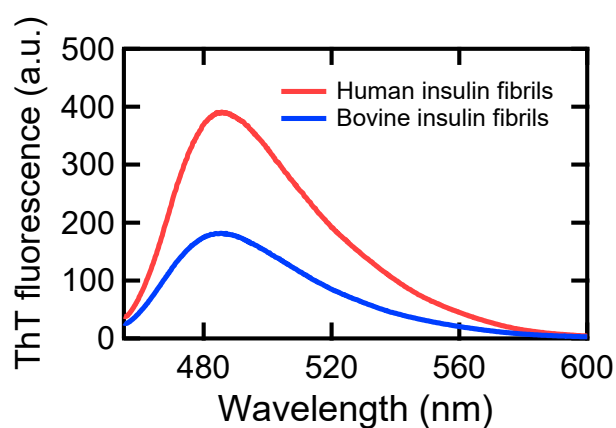


Figure 3-2. ThT fluorescence spectra. The red and blue solid lines represent the spectra of human and bovine insulin amyloid fibrils, respectively.

I further analyzed the morphology and secondary structure of these amyloid fibrils. TEM images exhibited needle-like morphology in both fibrils and there was no

significant difference in their appearance (Figure 3-3). On the other hand, AFM images revealed a difference in thickness between human (12.5 ± 2.2 nm; $n = 20$) and bovine (7.2 ± 1.4 nm; $n = 20$) insulin fibrils (Figure 3-4). Furthermore, ATR-FTIR spectra in amide I region showed a subtle but significant difference in shape between the two types of insulin fibrils (Figure 3-5; upper panel). The second derivative spectra showed the difference more clearly in the wavenumber of minima at around 1630 cm^{-1} corresponding to β -sheet structure (36), in agreement with the previous report by Surmacz-Chwedoruk et al (21). In addition, differences in the position of minimum at around 1670 cm^{-1} corresponding to β -turn (37) were observed (Figure 3-5; lower panel). Far-UV CD spectra also showed slightly different spectral shapes between human and bovine insulin fibrils while those in native structure were quite similar (Figure S3-1 in the Supporting Material). It was suggested that, while both of the human and bovine insulin amyloid fibrils are constructed from cross- β structure in common, there are subtle conformational differences in β -sheet and β -turn.

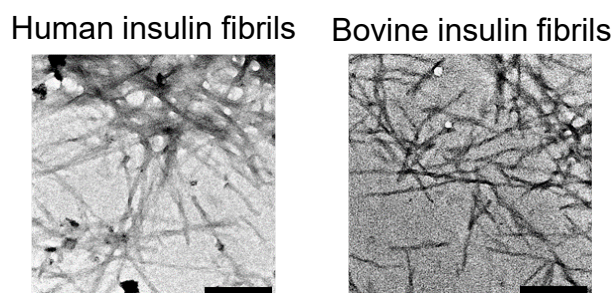


Figure 3-3. TEM images. The black scale bars represent 200 nm.

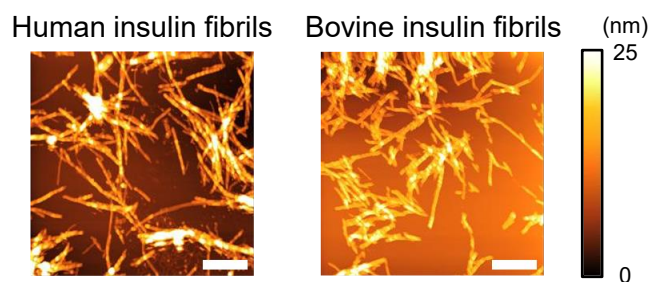


Figure 3-4. AFM images. The white scale bars represent 1 μm. The color scale on the right represents the height of the sample.

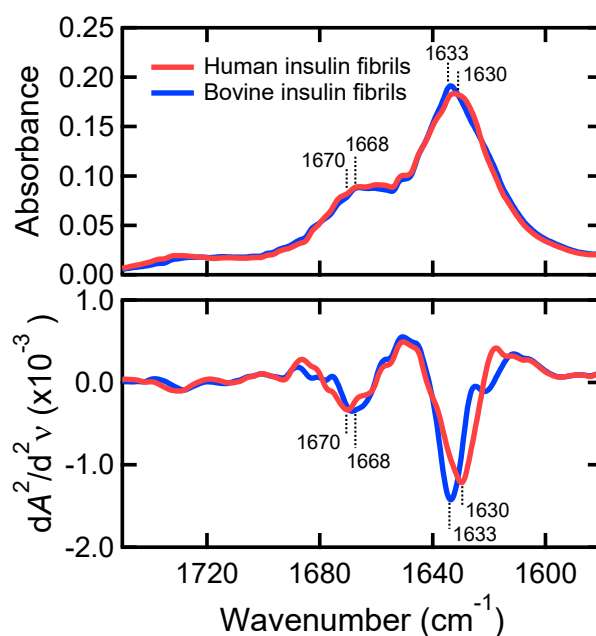


Figure 3-5. ATR-FTIR absorption spectra (upper panel) and their second derivatives (lower panel) in amide I region. The positions of two main peaks of β -sheet and β -turn structures, which were determined from the analysis of local minima of the second derivatives, are indicated on each spectrum.

Color formation of human/bovine insulin amyloid fibrils by iodine staining.

I next investigated the color formation of insulin fibrils by iodine staining, whose binding modes have been proposed to vary depending on surface topology of β -pleated sheets and/or their edges (29). The iodine solution itself has yellowish color, showing two major peaks from I_3^- at 288 nm and 352 nm and a minor broad peak from I_2 at around 425 nm (Figure S3-2) (29). When mixed with an iodine solution, human and bovine insulin fibrils exhibited different colors (Figure 3-6), giving different shapes in the UV-Vis absorption spectra mainly in a visible region (400–750 nm) (Figure 3-7). Iodine-stained human insulin fibrils had a major absorption at around 560 nm, whereas iodine-stained bovine insulin fibrils had one at around 480 nm. This observation indicates that iodine staining can detect differences in fibril structure sensitively. Each specific structure of the human and bovine amyloid fibrils was highly reproducible, which was confirmed by observing quite similar UV-Vis spectra after iodine staining of three independent amyloid fibrils (data not shown).

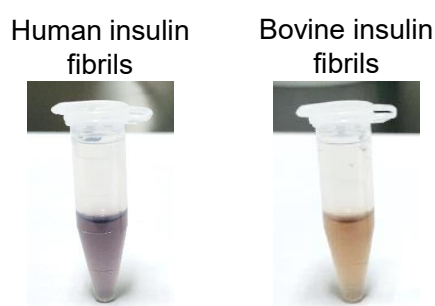


Figure 3-6. Photographs of iodine-stained human and bovine insulin fibrils.

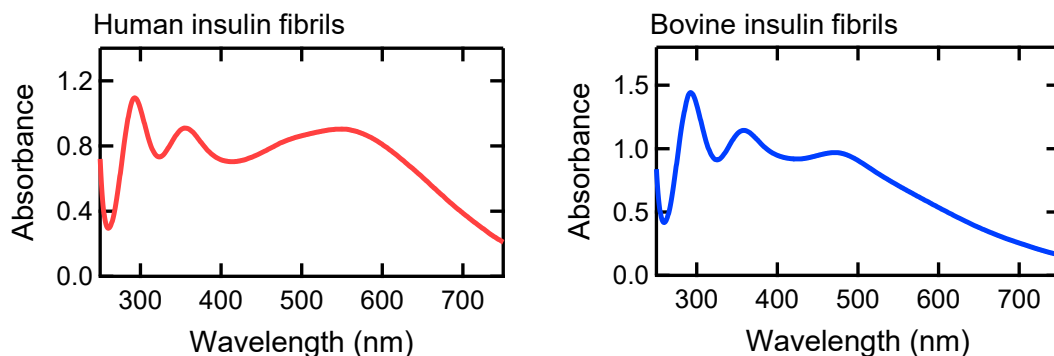


Figure 3-7. UV-Vis absorption spectra of iodine-stained human and bovine insulin fibrils. For each absorption spectrum, the spectrum of unstained fibrils was subtracted to obtain net spectrum derived from iodine molecules.

To evaluate the spectral difference in more detail, I performed spectral deconvolution and compared absorption bands constructing the experimental spectra. Each spectrum was replotted against wavenumber, and spectral deconvolution was performed using a sum of Gaussian distributions according to the following model function;

$$A(\nu) = \sum_{j=1}^n A_j \exp \left\{ -\frac{(\nu - \nu_j)^2}{2\sigma_j^2} \right\} \quad (1)$$

where A_j and ν_j represent amplitude and center wavenumber of the j th absorption band, respectively, and σ_j is a coefficient related to the full width at half-maximum (FWHM) of the j th absorption band, as is described as $2\sigma_j\sqrt{2\ln 2}$. For stable and accurate convergence, two center wavenumbers and one FWHM of the absorption band parameters were fixed with reference to the local minimum and its distance to the inflection point in the second derivative spectrum.

As a result, the absorption spectra were reproduced with six Gaussians and the fitted curves reproduced the experimental spectra well (Figure 3-8). Our previous study

revealed that three absorption bands in a higher wavenumber at around 34×10^3 , 28×10^3 and $24 \times 10^3 \text{ cm}^{-1}$ are assigned to I_3^- and I_2 molecules (dashed lines in Figure 3-8), and the remaining three absorption bands in a lower wavenumber at around 21×10^3 , 18×10^3 and $15 \times 10^3 \text{ cm}^{-1}$ are assigned to polyiodide ions larger than I_3^- (filled bands in Figure 3-8) (29). Since the I_3^- and I_2 absorption bands are observed in the iodine solution itself (Figure S3-2), the color formation by the iodine staining could be explained by the appearance of the latter three absorption bands, which, in order from the highest wavenumber, I refer to as band I (orange filled area), band II (green filled area), and band III (blue filled area), respectively. The center wavenumbers of the bands I–III were almost the same, but the relative intensity ratio was significantly different between human and bovine insulin fibrils. This observation suggests that the constituent iodine binding sites have very similar properties in both insulin fibrils but the number of binding site and/or the binding constants of polyiodides are different, which resulted in different colors.

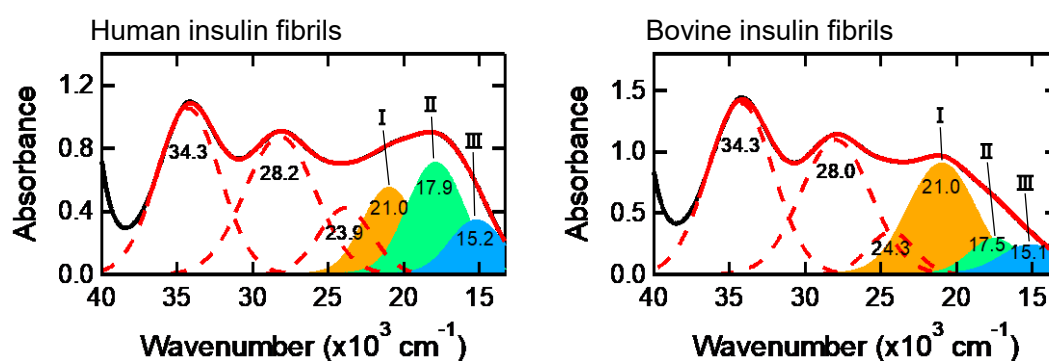


Figure 3-8. Deconvolution of UV-Vis absorption spectra. Note that the spectra were replotted against wavenumber and the deconvolution was performed by using eq. 1. Black and red solid lines represent the experimental and reproduced spectra, respectively, and the experimental spectra are the same as shown in Figure 2-7. Six Gaussian peaks are represented with red dashed lines and filled

areas, which correspond to absorption components obtained by the deconvolution.

Structural propagation of insulin amyloid fibrils in self-seeding. I carried out self-seeding experiments to examine the propagation characteristics of amyloid structure. For the seeding reaction, 37°C was selected as reaction temperature to promote only seed-dependent elongation without co-occurrence of the spontaneous fibrillation. The spontaneously formed insulin fibrils analyzed in Figures 3-2 – 3-8 were defined as the 1st fibrils and used as seeds for the first run of self-seeding reaction. Afterwards, the seeding reactions were repeated using amyloid fibrils of the previous generation as seeds. As a result, ThT fluorescence intensity was preserved even after the repetitive self-seeding reactions in both species (Figure 3-9), implying each fibril structure was replicated. The 1st fibrils showed lower intensity than the 2nd–5th fibrils, which suggests smaller number or lower affinity of ThT binding probably because the 1st fibrils tended to contain diverse fibril morphology (38) and/or fibril clumps with buried surfaces due to the formation at high temperature. However, the results of ATR-FTIR spectra and their second derivatives also showed the preservation of secondary structure even after the repetitive self-seeding reaction (Figure 3-10).

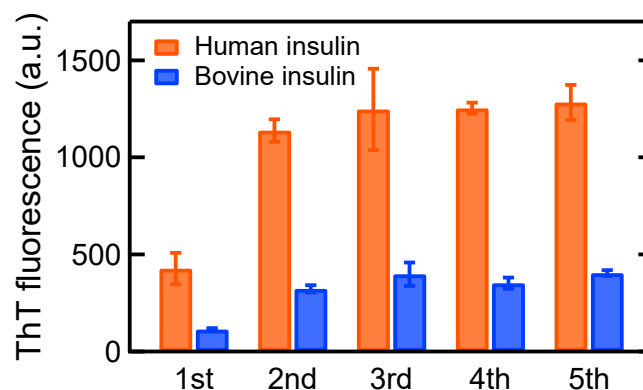


Figure 3-9. ThT fluorescence intensity of the 1st-5th amyloid fibrils formed by repeats of self-seeding. The 1st fibrils correspond to the spontaneously formed amyloid fibrils analyzed in Figures 2.2 - 2.8 and were used as original seeds in this analysis. All samples were assayed in triplicate and error bars depict S.D.

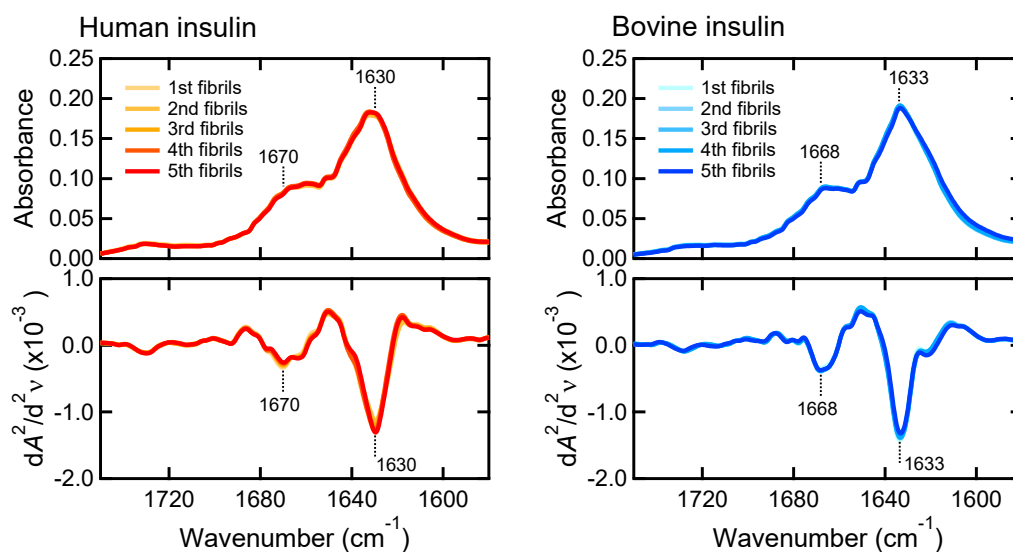


Figure 3-10. ATR-FTIR absorption spectra of the 1st-5th amyloid fibrils (upper panel) and their second derivatives (lower panel) in amide I region of human and bovine insulin. The spectra of the original seeds (i.e., 1st) are also shown. The positions of two main peaks of β -sheet and β -turn structures are indicated in the graphs.

The replication of amyloid structure was further verified by iodine staining, which is expected to detect even slight structural differences with high sensitivity (29). The self-seeded 2nd–5th fibrils consistently exhibited the same shape of UV-Vis absorption spectra throughout all generations (Figure 3-11), and as a consequence, the spectral deconvolution of the UV-Vis absorption spectra of the 5th fibrils reproduced almost the same absorption bands as those in the 1st fibrils (Figure 3-12). The spectral intensity of the 1st fibrils was slightly lower than those of the 2nd–5th fibrils, especially in human insulin, although it was not so prominent as that in the case of ThT fluorescence intensity.

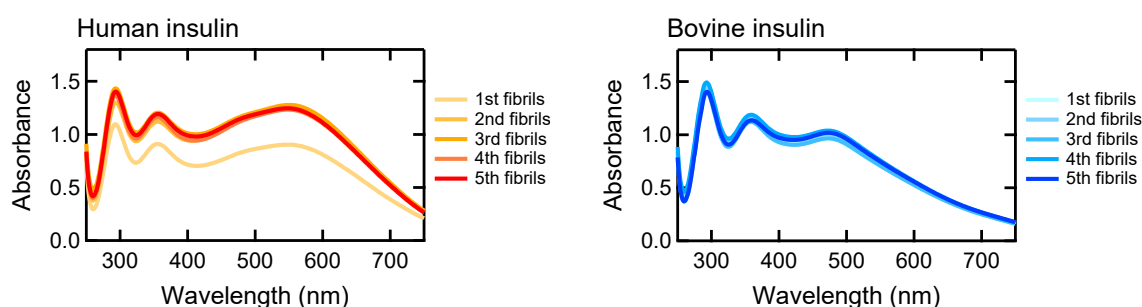


Figure 3-11. UV-Vis absorption spectra of iodine-stained human and bovine insulin fibrils formed by self-seeding. For each absorption spectrum, the spectrum of unstained fibrils was subtracted to obtain net spectrum derived from iodine molecules.

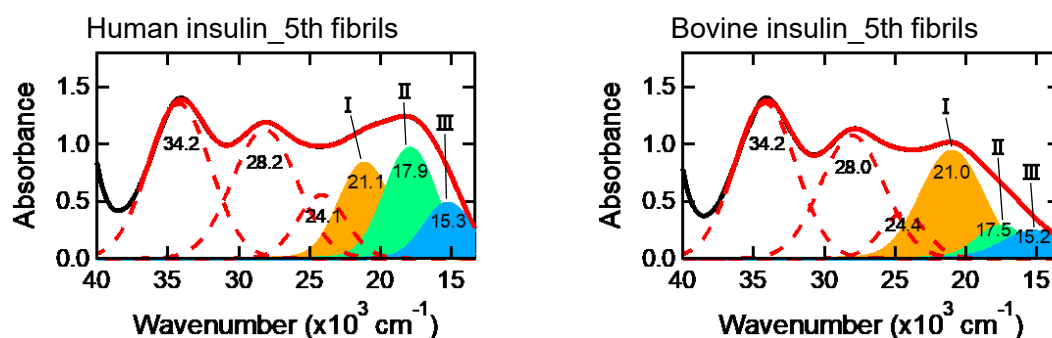


Figure 3-12. Deconvolution of UV-Vis absorption spectra of iodine-stained final products (i.e., 5th) of human and bovine insulin fibrils obtained by self-seeding. Black and red solid lines represent the experimental and reproduced spectra, respectively. Six Gaussian peaks are represented with red dashed lines and filled areas, which correspond to absorption components obtained by the deconvolution.

Structural propagation of insulin amyloid fibrils in cross-seeding. I further carried out cross-seeding experiments to examine the propagation characteristics of amyloid structure between proteins with different amino acid sequences. In this work, human/bovine insulin proteins were cross-seeded with bovine/human insulin seeds. For the first cycle of the cross-seeding, the 2nd fibrils obtained in the self-seeding were used as seeds to ensure the structural homogeneity (see Discussion for details), considering that ThT fluorescence intensity and UV-Vis absorption spectrum of the iodine-stained form were smaller in the 1st fibrils compared to the subsequent generations. As a result, unlike self-seeding, ThT fluorescence intensity of the cross-seeded insulin fibrils gradually changed as generation progressed. Although the 1st fibrils showed similar intensity to that of the original seeds, ThT fluorescence intensity was gradually decreased in bovine insulin seeded with human insulin seeds, (Figure 3-13, orange bars), meanwhile it was gradually increased in human insulin seeded with bovine insulin seeds (Figure 3-

13, blue bars). It was suggested that the templating ability of the original seeds is weak upon cross-seeding and thus allows fibril structure to change towards a more thermodynamically stable one.

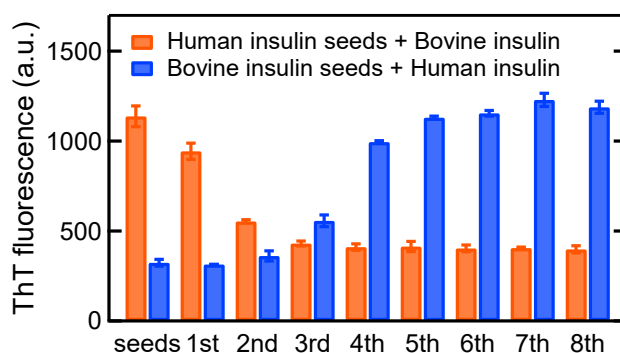


Figure 3-13. ThT fluorescence intensity of the 1st-8th amyloid fibrils formed by repeats of cross-seeding. The intensities of the original seeds are also shown. All samples were assayed in triplicate and error bars depict S.D.

I next analyzed changes in secondary structure by ATR-FTIR spectroscopy. Similar to the ThT result, the spectra were gradually changed as the reaction was repeated (Figure 3-14). When bovine insulin was seeded with human seeds, the spectral shape became similar to that of the bovine fibrils (Figure 3-14, see also Figure 3-5), suggesting that fibril structure changed to the intrinsic fibril structure of bovine insulin. Interestingly, when human insulin was seeded with bovine seeds, fibril structure did not change to the intrinsic human fibril structure. The spectral shape of the 8th fibrils did not coincide with that of human fibrils, and it showed a deeper second derivative minimum at 1630 cm^{-1} compared to that of human insulin fibrils and the position of the β -turn shoulder was shifted to 1662 cm^{-1} (Figure 3-14, see also Figure 3-5). It is estimated that a new-type of

insulin fibrils with distinct β -sheet structure was formed. Far-UV CD analysis also showed a new spectral shape particularly when human insulin was seeded with bovine seeds (Figure 3-3).

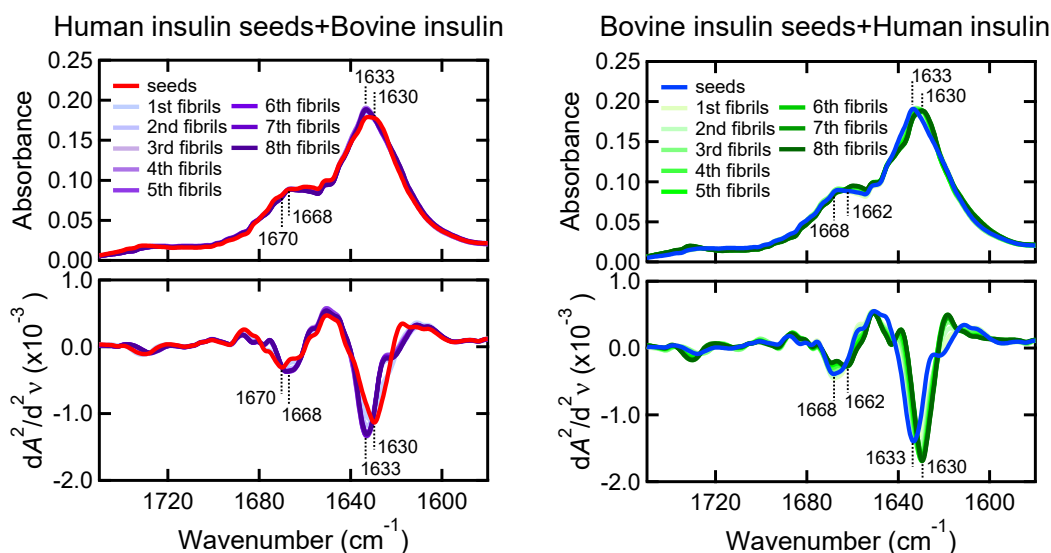


Figure 3-14. ATR-FTIR absorption spectra of the 1st-8th amyloid fibrils (upper panel) and their second derivatives (lower panel) in amide I region of bovine and human insulin. The spectra of the original seeds are also shown. The positions of two main peaks of β -sheet and β -turn structures are indicated on the spectra of the seed fibrils and the 8th fibrils.

I further tracked structural changes during the repeated cross-seeding by using iodine staining. The color of iodine staining detected structural changes successfully, and the UV-Vis absorption spectra of iodine-stained fibrils showed clear spectral changes in a longer wavelength region in both types of cross-seeding (Figure 3-15). In agreement with the FTIR result, the absorption spectrum reached a distinct shape that was neither human nor bovine fibrils upon seeding of human insulin with bovine seeds, while it reached a shape similar to that of the bovine fibrils upon seeding of bovine insulin with

human seeds. The spectral deconvolution of the 8th fibrils further verified differences in the composition of the bands I–III from those of the original seeds. When bovine insulin was seeded with human seeds, almost the same component of the absorption bands as those of bovine insulin fibrils was demonstrated (Figure 3-16). When human insulin was seeded with bovine seeds, the relative intensity ratio was clearly different from those of human and bovine fibrils while the center wavenumbers were similar (Figure 3-16).

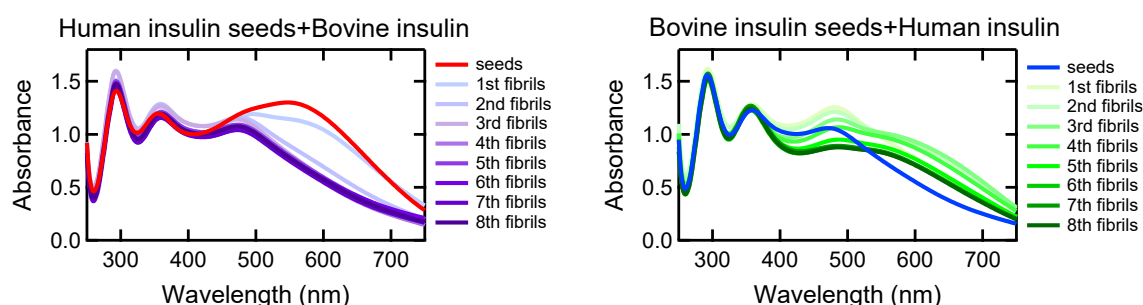


Figure 3-15. UV-Vis absorption spectra of iodine-stained bovine and human insulin fibrils formed by cross-seeding. For each absorption spectrum, the spectrum of unstained fibrils was subtracted to obtain net spectrum derived from iodine molecules.

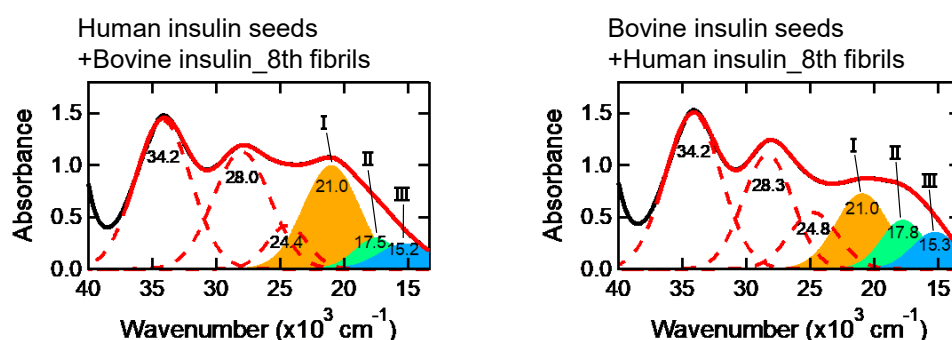


Figure 3-16. Deconvolution of UV-Vis absorption spectra of iodine-stained final products (i.e., 8th) of bovine and human insulin obtained by cross-seeding. Black and red solid lines represent the experimental and reproduced

spectra, respectively. Six Gaussian peaks are represented with red dashed lines and filled areas, which correspond to absorption components obtained by the deconvolution.

Morphology and cytotoxicity of the final fibril product after repeated self-seeding or cross-seeding. To further demonstrate the above-mentioned structural replication or change upon repeated self-seeding or cross-seeding, I performed morphological observation by TEM and AFM and cytotoxic assay. I selected the final generation of insulin fibrils formed by self-seeding (5th fibrils) and cross-seeding (8th fibrils) as samples. As a result, although it was difficult to identify any differences in appearance from TEM images (Figure 3-17), AFM observation revealed the inheritance or change of fibril thickness successfully (Figure 3-18). The thicknesses of self-seeded human and bovine insulin were 12.1 ± 2.0 nm and 6.9 ± 1.2 nm, respectively ($n = 20$), both of which were almost the same as those of the spontaneously formed human and bovine insulin fibrils. The thicknesses of the cross-seeded human and bovine insulin, on the other hand, were 15.1 ± 1.9 nm and 6.9 ± 1.3 nm, respectively ($n = 20$). The value of the cross-seeded bovine insulin supports the deviation to bovine-like fibril structure, and the thickest value of the cross-seeded human insulin strongly supports the formation of a new-type fibrils.

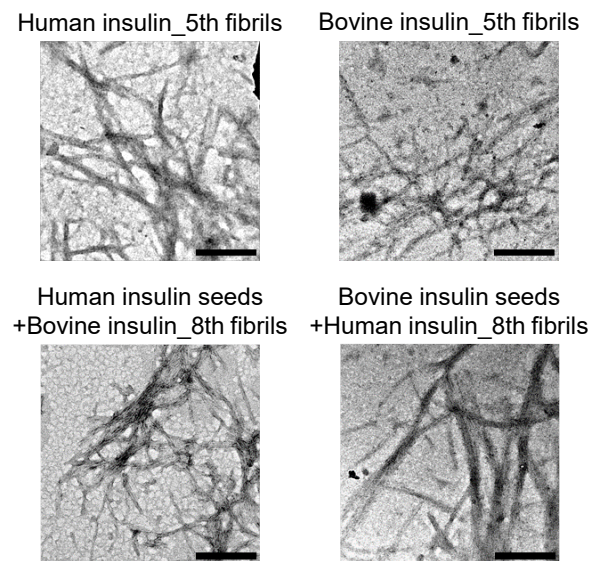


Figure 3-17. TEM images. The black scale bars represent 200 nm.

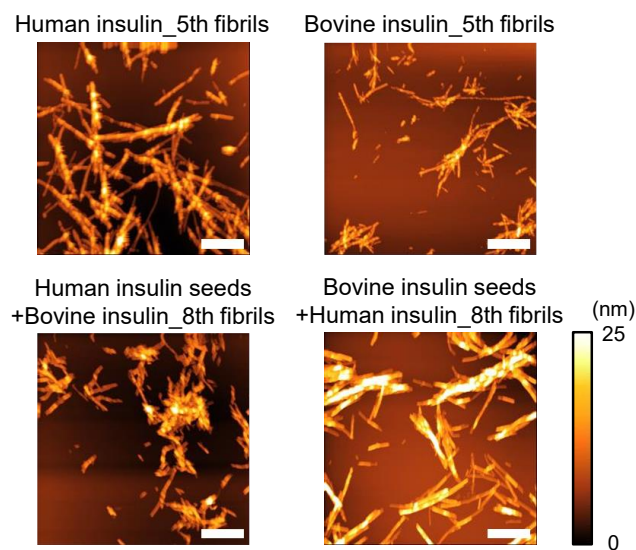


Figure 3-18. AFM images. The white scale bars represent 1 μm . The color scale on the right represents the height of the sample.

I next examined cytotoxicity against PC12 cells by MTT assay. As a result, all of the four types of insulin fibrils showed decrease in cell viability in a dose-dependent

manner (Figure 3-19). The cytotoxicity varied with the type of insulin fibrils, and the self-seeded human insulin yielded significantly lower values of cell viability than the self-seeded bovine insulin (Figure 3-19, red and blue plots). This result indicates that the different structures of insulin fibrils have different cytotoxicity. The cross-seeded bovine insulin yielded almost the same cytotoxicity as the self-seeded bovine insulin (Figure 3-19, purple plot), supporting the formation of bovine-like fibril structure. Interestingly, the cross-seeded human insulin yielded the lowest cell viability that was distinct from human or bovine fibrils (Figure 3-19, green plot). This result indicates that the new fibril structure emerged by cross-seeding has a potency to show a new phenotype.

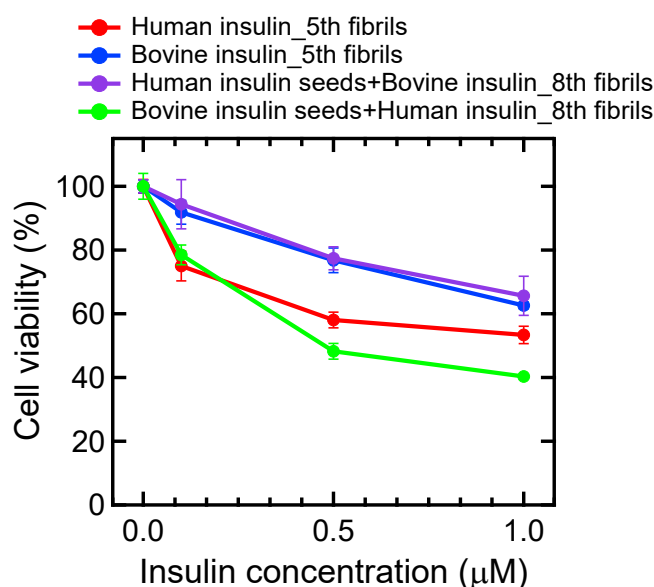


Figure 3-19. Cell viability obtained from MTT assay of insulin fibrils at different concentrations using PC12 cells. Each sample was assayed in triplicate and the error bars depict S.D.

Investigation of the process of the structural changes during the repeated cross-seeding. To gain a detailed picture regarding the pathway of the structural

changes induced by cross-seeding, SVD analysis was performed with the UV-Vis absorption spectra of iodine staining in the repeated cross-seeding shown in Figure 3-15.

A set of the UV-Vis spectra, $\mathbf{M}$, was decomposed as follows;

$$\mathbf{M} = \mathbf{U}\mathbf{W}\mathbf{V}^T \quad (2)$$

where $\mathbf{U}$, $\mathbf{W}$, $\mathbf{V}^T$ represent the left-singular matrix, singular matrix, and right-singular matrix of the data-set matrix $\mathbf{M}$, respectively, assuming that $\mathbf{M}$ is a $m \times n$ matrix, $\mathbf{U}$ is a $m \times m$ unitary matrix, $\mathbf{W}$ is a $m \times n$ diagonal matrix, and $\mathbf{V}^T$ is a $n \times n$ unitary matrix. In the analysis, I constructed $\mathbf{M}$ containing all the 18 spectra shown in Figure 3-15 in a manner where each column was composed of a single spectrum. In this case, each column of $\mathbf{U}$ is interpreted as a normalized diagonalized wave. The weights and proportions of the waves are described by the corresponding diagonal element of $\mathbf{W}$, which is called singular values, and corresponding row element of $\mathbf{V}^T$, respectively.

As a result, I obtained two dominant singular vectors, whose singular values satisfied accumulating contribution ratio of 0.94 (Figure 3-20A, B). This means that all absorption spectra observed in the cross-seeding are almost reproducible by the sum of the two diagonalized waves multiplied by the proportions. With these singular vectors, which were referred to as SV1 and SV2 in contribution order, I carried out transition diagram analysis by plotting the $\mathbf{V}^T$ values of SV1 versus those of SV2. Here, the result of the analysis using the whole wavelength range of the experimental data is shown. The transition diagrams obtained gave curved plots in the both types of cross-seeding, and they were explained by more than one line segment (Figure 3-20C, D). A single line segment represents a two-state transition, and the points on the line segment can be considered to be a mixture of two fibril structures represented at the both ends of the line segment (39, 40). The number of the lines thus corresponds to the number of transitions,

and the line intersections in the diagrams correspond to intermediate states. The plots in the diagrams were explained by assuming two line segments and one intersection in the cross-seeding of bovine insulin with human insulin seeds (Figure 3-20C), and four linear segments and three intersections in the structural change in cross-seeding of human insulin with bovine insulin seeds (Figure 3-20D). These results suggest that these structural changes are multistep, and intermediate metastable states are routed in the process of cross-seeding. The multistep changes were reproduced when another dataset of the repeated cross-seeding was subjected to the SVD analysis, although the route did not seem exactly the same in the case of bovine insulin seeded with human seeds (Figure S3-4); nevertheless, the plots showed two line segments and finally reached the bovine-type fibril structure, supporting the multistep structural changes in cross-seeding. It was also confirmed that a similar result was obtained even when the wavelength range was limited to 400-750 nm, supporting that spectral changes in the visible region contribute importantly to this analysis.

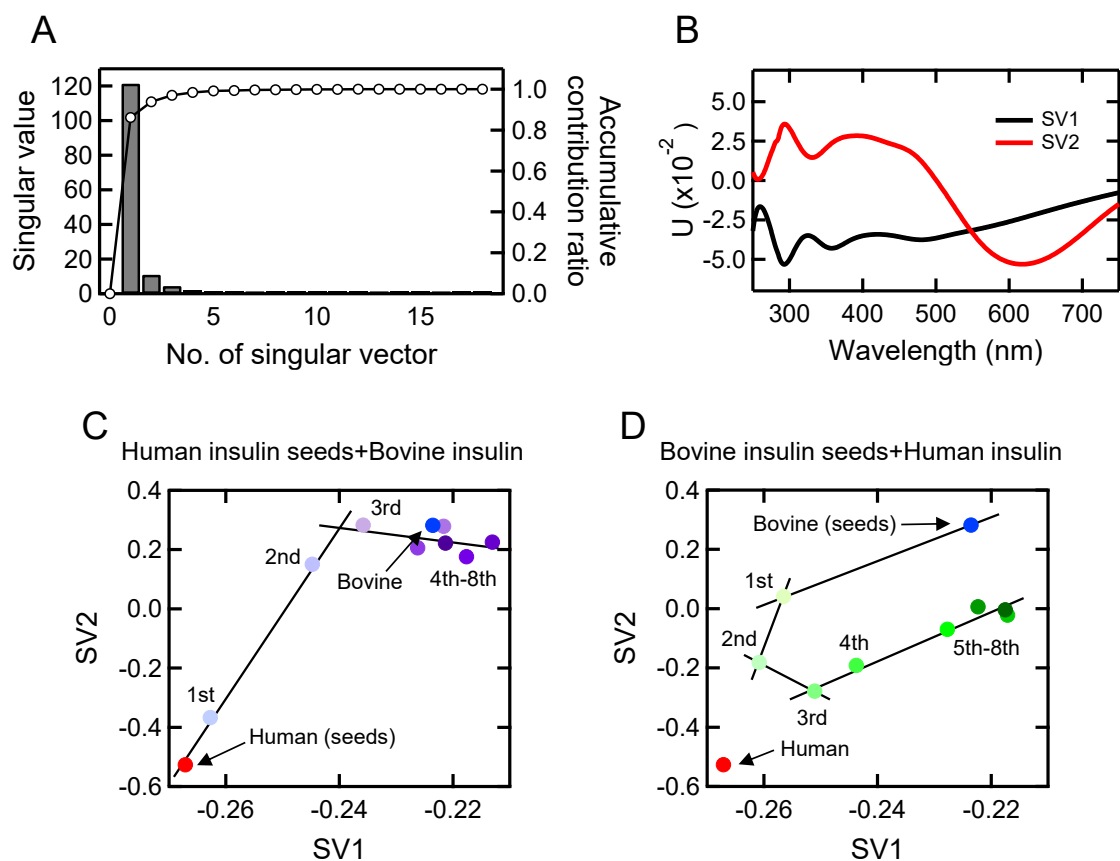


Figure 3-20. SVD analysis of UV-Vis absorption spectra obtained from iodine-stained insulin fibrils formed by cross-seeding. (A) The diagonal element of the singular matrix. (B) The 1st (SV1) and 2nd (SV2) columns of the left-singular matrix. (C, D) Transition diagrams obtained by plotting scores of the 1st (SV1) and 2nd (SV2) rows of the right-singular matrix. Solid lines, each of which represents a two-state transition, were superimposed to show that both of the plots can be described as a multistep transition.

Discussion

Structural changes induced by cross-seeding of human and bovine insulin.

It was revealed that the cross-seeding of human and bovine insulin caused gradual structural changes from the original seeds, in contrast to exact replication in self-seeding. The structural changes occurring in the repeated cross-seeding were successfully tracked by FTIR spectra and iodine staining, and in particular, the changes in visible color of iodine staining were obvious and clearly demonstrated complex changes. Iodine develops color by forming polyiodide ions in complex with crystalline compounds or polymers, and iodine-starch reaction is one of the best-known reactions in which iodine shows blue color by complexing with starch (41). Similarly, amyloid fibrils develop visible color with iodine, which is mechanistically explained by the formation of linear polyiodide ions composed of I_3^- and I_5^- on the fibril surfaces (29, 42). Although the use of iodine staining in amyloid researches has not progressed so far due to the discovery of fluorescent dyes such as Congo Red and ThT, I have recently found that iodine staining could be used as a sensitive probe of structural polymorphism of amyloid fibrils (29). In this study too, iodine staining worked as a good strategy for evaluating structures of amyloid fibrils and their descendant generations formed by repeated self- or cross-seeding in detail. Considering that different color could be obtained even when the secondary structure was almost the same in our previous study, it is expected that the color of iodine staining senses higher-order structural differences like packing of protofilaments sensitively.

The most important finding obtained in this study is that the process of the repeated cross-seeding goes through one or more intermediate states. SVD analysis of the UV-Vis spectra obtained from the iodine-stained insulin fibrils revealed structural changes consisting of two or more structural transitions (Figure 3-20). Intriguingly, the

number and types of intermediates varied depending on the direction of cross-seeding, and furthermore, a new fibril structure with distinct secondary structure, iodine color tones, morphology and cytotoxicity appeared in a pathway-specific manner when human insulin was cross-seeded with bovine insulin seeds (Figure 3-15, 3-16). The new fibril structure is not described as a mixture of the intrinsic bovine and human fibril structures, as its position in the transition diagram in Figure 3-20D is clearly apart from the line connecting the plots of human and bovine fibrils (i.e., red and blue plots, respectively). The existence of metastable intermediate states is a strong experimental indication of the rugged energy landscape of amyloid fibrils, and the multistep structural changes as well as the pathway dependency of the convergent structure of amyloid fibrils are likely to be a natural consequence arising from the rough energy surface of the amyloid landscape that contains a number of local minima.

Predicted scheme for propagation of amyloid fibrils in cross-seeding. On the basis of the present results, I propose a possible scheme of structural propagation induced by self-seeding and cross-seeding of human and bovine insulin. Figure 3-21A shows the case of bovine insulin. When bovine insulin is self-seeded, native bovine insulin reaches the inherent bovine-type fibril structure, which is presumed the most thermodynamically preferable fibril state of bovine insulin (Figure 3-21A, blue arrow). In contrast, upon cross-seeding, native bovine insulin first falls into the state of human-type fibril structure by templated with human insulin seeds (Figure 3-21A, red arrow). However, stepwise structural changes via an intermediate metastable state accompany and the fibril structure eventually adapts to the bovine-type fibril structure. Such multistep structural changes in cross-seeding is probably explained by conformational shift of

amyloid structures due to the stepwise generation of more thermodynamically advantageous structures by repeated cross-seeding. Here, it should be noted that similar structural transitions may occur through growth competition among different fibrils if initial seeds contain some structural variants (43). However, given that almost no structural change was observed even after the repetitive self-seeding (Figure 3-11, 3-12), it is indicated that the seeds used for cross-seeding are very homogeneous, although there is some variety at the level of morphology (38). Based on the assurance of the fibril homogeneity from the behavior in the repeated self-seeding as well as the use of the 2nd fibrils, the structural changes in cross-seeding are more likely to have occurred by structural adaptation rather than competition among preexisting fibrils.

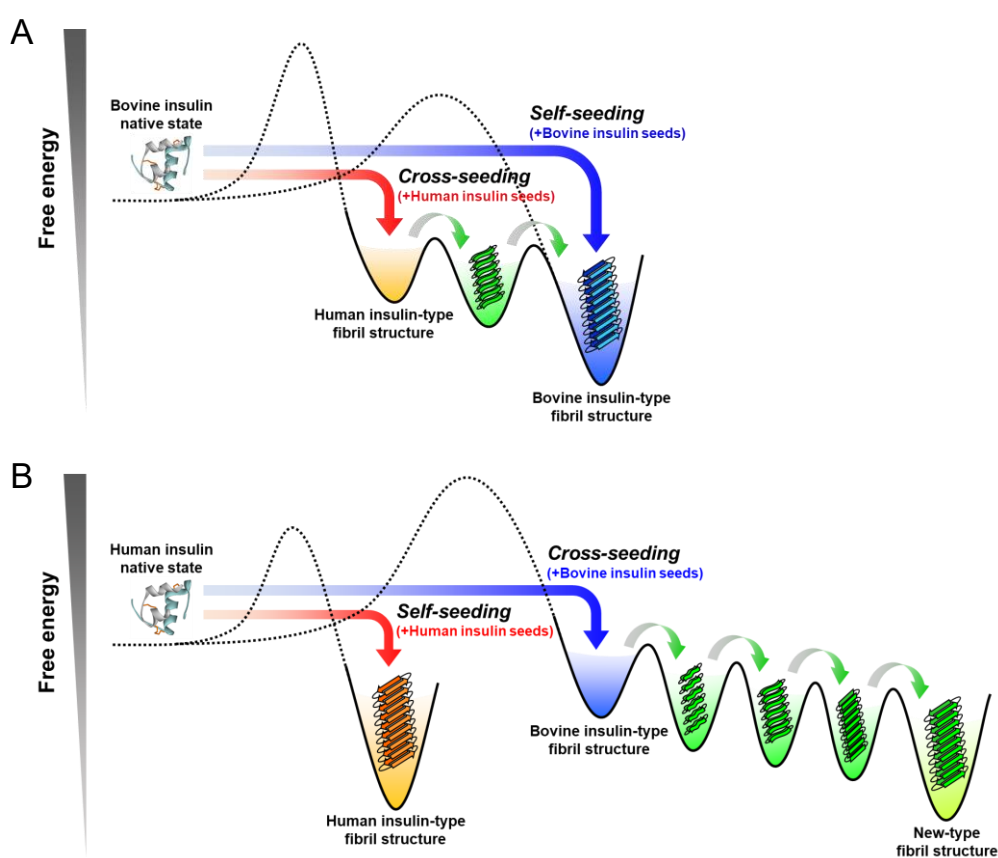


Figure 3-21. Schematic illustration for the energy landscapes of bovine (A) and human insulin (B). See the main text for details. The number of intermediate states was tentatively determined from the SVD results shown in Figure 2-20. It should be noted that the energy level of each structural state and the height of each energy barrier in the illustration do not include quantitative information.

In the case of human insulin (Figure 3-21B), the energy landscape is predicted to have different features, given that its cross-seeding eventually reaches a new-type fibril structure rather than the inherent human-type fibrils. When human insulin is self-seeded, native human insulin selectively reaches the inherent human-type fibril structure (Figure 7B, red arrow). When cross-seeded with bovine insulin seeds, native human insulin would first fall into the bovine-type fibril structure. However, the structure changes through several intermediate metastable states. Interestingly, unlike the cross-seeding of bovine insulin in Figure 3-21A, the pathway leads to the new structure (Figure 3-21B, blue arrow). The intrinsic human fibril structure is presumed to be kinetically blocked by a large energy barrier, resulting in the observation that human insulin finally took the new-type fibril structure.

Regarding the structural characteristics of the intermediate states and the newly emerged fibril structure, they could not be mentioned only from the results of iodine staining because the relationship between its color tone and the property of amyloid structure still remain unclear (29). To gain insights into the structural characteristics of the intermediates, I attempted SVD of ATR-FTIR spectra. Although the plots were more scattered than those of iodine staining, the transition diagram in the cross-seeding of human insulin with bovine seeds gave nonlinear shapes of the plots in agreement with the result of iodine staining (Figure S3-5). Additionally, the major peaks were observed in a

range of 1620-1640 cm^{-1} in the singular vectors SV1 and SV2, from which it was implicated that the intermediate metastable states result from slight differences in β -sheet conformation. If further studies reveal the relationship between iodine color and amyloid structure, detailed structural characteristics will be clarified.

Implication for the manner of fibril propagation in cross-seeding. From previous studies of prion protein and other proteins, the “conformational selection and population shift” model, which has originally been proposed for explaining biomolecular recognition, has so far been recognized as a valid model to explain the molecular mechanism of cross-seeding (44). In this model, each protein is considered to possess a set of thermodynamically permissible structures, and the overlap of one or more of these structures between proteins with different sequences indicates the achievement of cross-seeding across a species barrier. Indeed, it can explain many experimental observations including cross-species prion transmission (45) and cross-talk between A β 42 and A β 40 (22). When this model is adapted to the energy landscape observed in this study, the thermodynamically permissible structures per protein correspond reasonably to a number of energy minima distributing over the landscape. If any of these energy minima coincide with the seed fibril structure, seed fibrils will interact with the counter protein molecules and guide them to fall to the energy minimum of the seed structure by skipping the nucleation barrier.

In addition to a large number of energy minima as candidates of fibril structures to be selected, an additional property worth noting is that some energy minima are positioned in adjacent on the landscape and tend to connect mutually by low energy barriers. This topology allows the deviation of fibril structure, so-called conformational

switching, drift, adaptation, or evolution (25, 46-48). In such cases, it is not so uncommon for fibrils to take a distinct structure from that of the original seeds as observed in the cross-seeding of the present study. Furthermore, it is expected that the energetic pathway to the final state will become rugged with multiple minima, resulting in the transient formation of metastable states. Considering this feature of the energy landscape, multiple cycles of cross-seeding will show dynamic equilibrium with transient accumulation of several different fibril species. If such energetic properties are generalized to a wide range of pathogenic proteins, cross-seeding will produce a variety of fibril structures over a time period of transmission, and thus, will be involved in pathology in a more complicated manner causing time-dependent fluctuation in infectivity and pathogenesis.

The energy landscape of amyloid fibrils is thought to vary with protein sequence (10), which will contribute to determine candidates of the final amyloid structure generated by cross-seeding. In addition to this, a specific interaction of protein molecules with seeds is another important factor, as it decides an initial landing point on the energy landscape (i.e., fibril templating). To investigate the role of the three different amino acid residues between human and bovine insulin in the latter issue, cross-seeding of human/bovine insulin was assessed using amyloid fibrils consisting of human A-chain or B-chain (49, 50) as seeds. However, neither of these fibrils did not function as a fibril template, only accelerating fibril formation by shortening induction period (Figure S3-4). This result suggests that the interaction between the proteins and seeds is not restricted to local amino acid sequence, but more higher-order and complex factors are involved for exerting template ability.

Supporting information

CD spectroscopy. CD spectra were recorded using a J-1100 spectropolarimeter (JASCO, Tokyo, Japan). Sample solutions were prepared at a protein concentration of 0.2 mg/mL in 25 mM HCl for native insulin and 0.2 mg/mL in 25 mM HCl containing 100 mM NaCl for fibril samples, and in the measurement of amyloid fibrils, the sample was sonicated to improve dispersibility. Each scan was performed at 20 nm/min from 250 to 196 nm with a 0.1 cm path-length quartz cell. All spectra were obtained from the integration of five individual scans and expressed as mean residue molar ellipticity $[\theta]$ in monomer unit.

Purification of A-chain and B-chain. A-chain and B-chain were obtained from human insulin. A-chain was purified as described previously (49) with a slight modification. Human insulin was dissolved at a concentration of 5.0 mg/mL in 50 mM sodium phosphate buffer (pH 7.4) containing 1 mM ethylenediaminetetraacetic acid and 10 mM dithiothreitol. After overnight incubation at 25 °C, the solution was centrifuged at $1,800 \times g$ for 30 min and A-chain in the supernatant was separated, and then solvent exchange with Milli-Q water was performed using a PD-10 column. B-chain was purified as described in our previous report (50). The A-chain and B-chain concentrations were determined using absorption coefficients of $1.07 (\text{mg/mL})^{-1} \text{ cm}^{-1}$ and $0.90 (\text{mg/mL})^{-1} \text{ cm}^{-1}$ at 280 nm (49, 50), respectively.

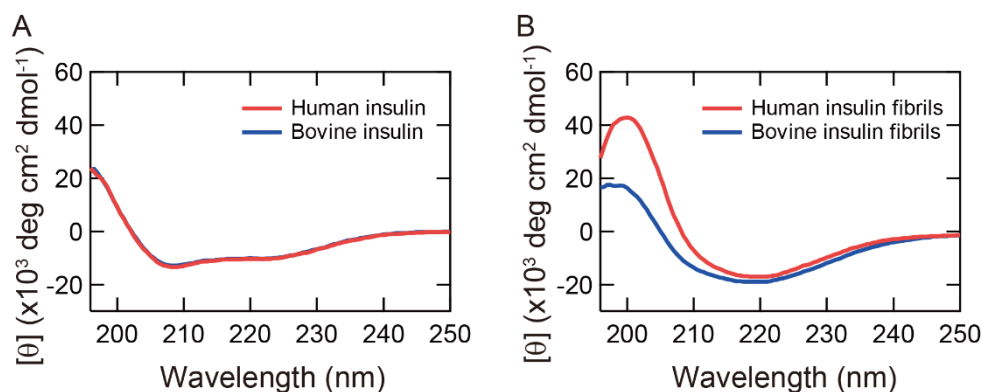


Figure S3-1. Far-UV CD spectra of human and bovine insulin in (A) native and (B) fibrillar states. In panel B, the same amyloid fibrils investigated in Figure 3-5 were subjected to the CD measurements.

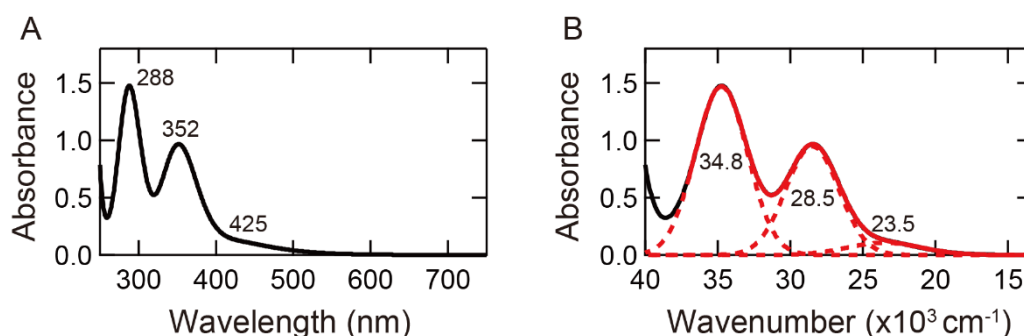


Figure S3-2. UV-Vis spectrum (A) and its deconvolution (B) of an iodine solution. An iodine solution containing 0.9 mM KI and 0.12 mM I₂ in 25 mM HCl was used for the measurement. A black line represents the experimental spectrum, and a red line represents the fitted spectrum using eq. 1 assuming three Gaussian bands (i.e., $n = 3$). Three Gaussian peaks are represented with dashed red lines, which correspond to absorption components obtained by the deconvolution. The Gaussian bands at 288 nm ($35 \times 10^3 \text{ cm}^{-1}$) and 352 nm ($29 \times 10^3 \text{ cm}^{-1}$) were assigned to I₃⁻, and that at 425 nm ($24 \times 10^3 \text{ cm}^{-1}$) was assigned to I₂ (29).

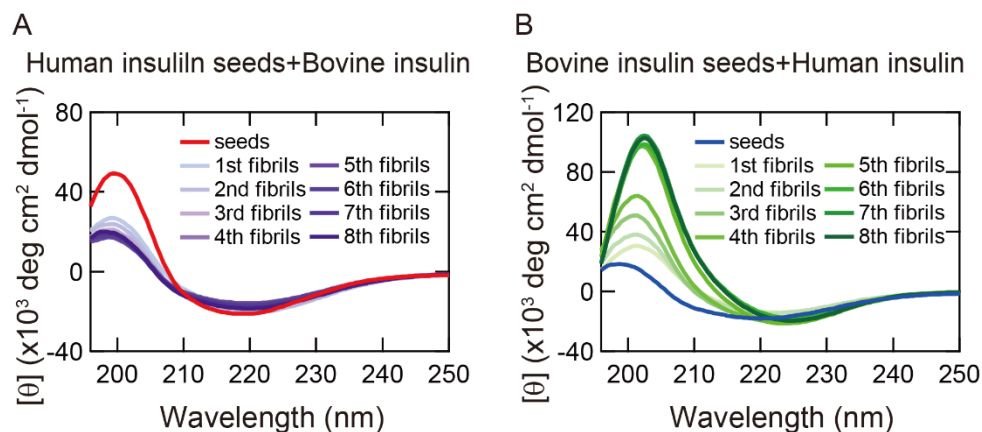


Figure S3-3. Far-UV CD spectra of (A) bovine insulin cross-seeded with human seeds and (B) human insulin cross-seeded with bovine seeds. The same amyloid fibrils investigated in Figure 3-14 were subjected to the CD measurements.

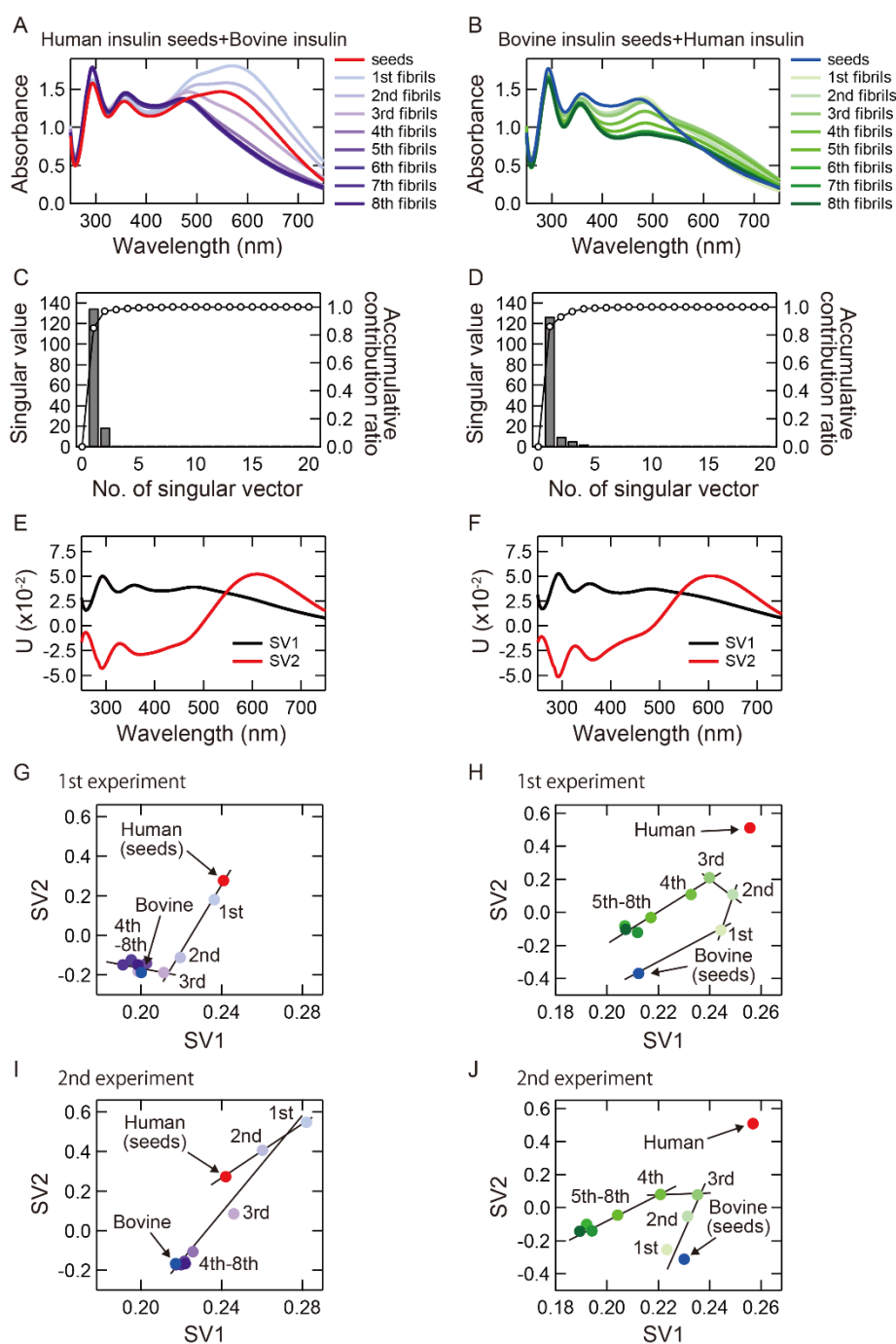


Figure S3-4. Reproducibility of SVD analysis of UV-Vis absorption spectra obtained from iodine-stained insulin fibrils formed by cross-seeding. To examine the reproducibility of the placement of points and the line segments on the SVD diagram, an additional SVD analysis was performed for each type of cross-seeding using a newly obtained dataset in addition to the original one. (A,

B) UV-Vis absorption spectra of iodine-stained bovine (A) and human insulin fibrils (B) formed by cross-seeding. The datasets in panels A and B, respectively were subjected to SVD analysis together with the original ones shown in Figure 2-15, respectively. Before the analysis, the intensity of the two independent datasets was normalized based on the peak intensity around 295 nm of the seeds. (C, D) The diagonal element of the singular matrix for the datasets of bovine insulin seeded with human seeds (C) and those of human insulin seeded with bovine seeds (D). (E, F) The 1st (SV1) and 2nd (SV2) columns of the left-singular matrix of bovine insulin seeded with human seeds (E) and human insulin seeded with bovine seeds (F). Both of the results showed two dominant singular vectors SV1 and SV2 similar to those shown in Figure 3-20B. (G-J) Transition diagrams obtained by plotting scores of the 1st (SV1) and 2nd (SV2) rows of the right-singular matrix of bovine insulin seeded with human seeds (G, I) and human insulin seeded with bovine seeds (H, J). Solid lines were superimposed in all panels to estimate the number of transitions.

As shown in panels H and J, the transition diagram of human insulin seeded with bovine seeds successfully reproduced the characteristic U-shaped route with similar placements of the plots, and the formation of the same new-type fibrils was also confirmed. However, regarding the number of line segments, only three line segments were identified in panel J and it was difficult to conclude the exact number of intermediate metastable states that pass through during the cross-seeding process.

In the case of bovine insulin seeded with human seeds, significant differences in the placement of the plots were observed between panels G and I, failing to reproduce the same route of the structural transitions. Nevertheless, the plots were composed of two line segments. It was also demonstrated that fibril structure changed to the intrinsic fibril structure of bovine insulin after multistep structural changes. Although the pathways were diverse, it was finally concluded that the structural changes proceed with a two-step structural transition through an intermediate metastable state.

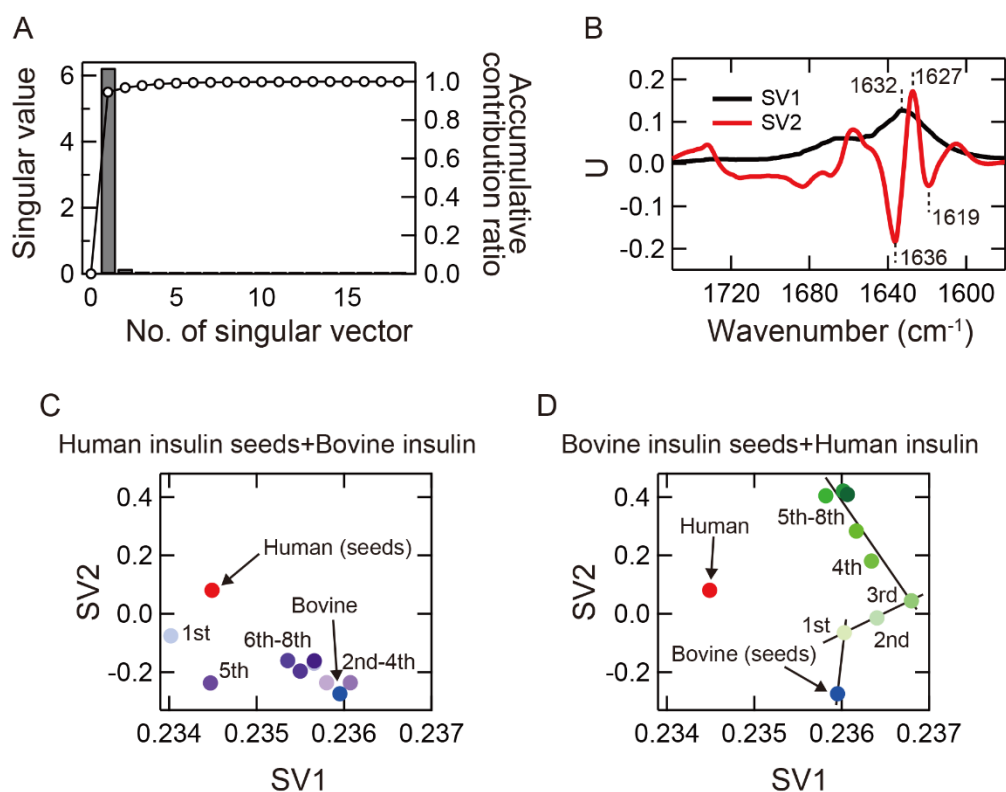


Figure S3-5. SVD analysis of ATR-FTIR spectra obtained from insulin fibrils formed by cross-seeding. The SVD analysis was performed with the FTIR spectra observed in the repeated cross-seeding shown in Figure 3-14. (A) The diagonal element of the singular matrix. (B) The 1st (SV1) and 2nd (SV2) columns of the left-singular matrix. The positions of main peaks at around 1630 cm⁻¹ indicative of β -sheet structures are labeled in the graphs. (C, D) Transition diagrams obtained by plotting scores of the 1st (SV1) and 2nd (SV2) rows of the right-singular matrix. The plots in the transition diagrams were more scattered than those of iodine staining due to small changes in FTIR spectra, making it relatively difficult to capture intermediate states especially in the cross-seeding of bovine insulin with human seeds (C). Nevertheless, the transition diagram of the cross-seeding of human insulin with bovine seeds showed systematic changes in plots which could be represented by the combination of multiple linear segments (D). In this plot, the most of the intermediates identified in Figure 3-20 were found, except for one in the 1st–3rd generations. The partial success in the

SVD analysis of the FTIR spectra suggests that the multistep structural transitions could also be detected at the secondary structure level. It is tempting to find a clear correlation in singular vectors between FTIR spectra and iodine staining; however, this could not be achieved only with the present experimental data.

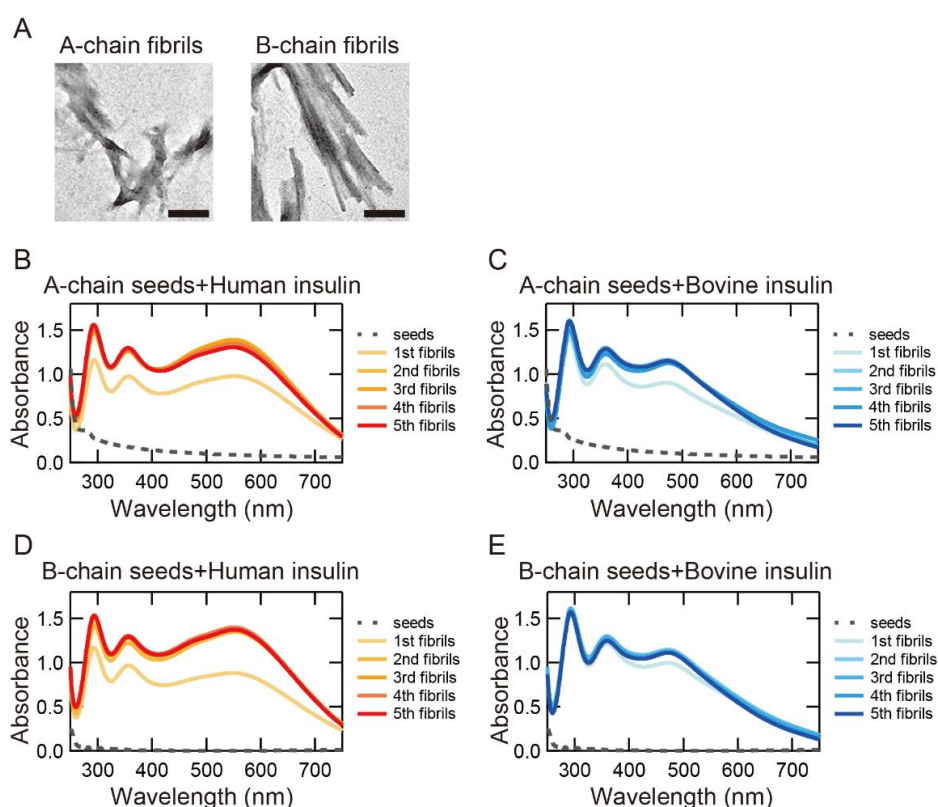


Figure S3-6. Cross-seeding of human/bovine insulin using human A-chain and B-chain fibrils as seeds. (A, B) TEM images of A-chain (A) and B-chain fibrils (B). The black scale bars represent 200 nm. (C–F) UV-Vis absorption spectra of iodine-stained human and bovine insulin cross-seeded with A-chain (C, D) or B-chain fibrils (E, F). Seeding reaction was performed under the same conditions as used for the cross-seeding with human or bovine insulin fibrils as seeds. The incubation time was set to 18 h for each cycle of cross-seeding except for the first cycle, where the incubation time was changed to 100 h because the reaction speed was much slower. Iodine staining was performed by using the same protocols as used for the self- and cross-seeding with human or bovine insulin fibrils as seeds. As shown by dashed lines in (C) and (D), A-chain and B-chain fibrils themselves showed little absorption even after mixing with iodine solution, probably due to the disruption of polyiodide ions by their reduction by free thiol groups of cysteine residues in A-chain and B-chain fibrils. The UV-Vis absorption spectra showed a similar shape to those of the self-seeding of human and bovine insulin, respectively (see Figure 3-11), demonstrating that A-chain and B-chain fibrils did not work as a template.

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Chapter 4

Spatiotemporal formation of a single liquid-like condensate and amyloid fibrils of α -synuclein by optical trapping at a solution surface

Introduction

Proteins are one of the most important biomacromolecules that constitute living systems. The majority of the functional proteins in cytoplasm or organelles have been thought to function typically as a dissolved state. However, it has recently been found that some proteins tend to form droplet-like condensates (1-4). They appear to have a role of compartmentalizing proteins like organelles, and therefore are often referred to as “membrane-less organelles”. Many types of protein droplets have been identified, and various physiological functions, such as intracellular metabolism, dynamic assembly in cytoskeletons, and the initiation of autophagy, have been reported. The condensed state of protein molecules contributes to co-localize proteins and their specific substrates to conduct efficient chemical reactions or to protect proteins when exposed to cellular stress.

Protein droplets typically exhibit high fluidity and reversibility, which make them to form and dissociate rapidly in response to change in environment (1-4). The most fundamental mechanism of protein droplet formation is suggested to be liquid-liquid phase separation (LLPS), where an excluded volume effect caused by intracellular macromolecular crowding as a key factor that enhances to form the weak multivalent interactions intermolecularly (5-7). The protein droplets are formed by several types of weak intermolecular interactions, mainly ionic, cationic- π , and π - π interactions (3,8). They act in a multivalent manner, and it has recently been found that low complexity domains (LCDs), sequence regions consisting of low amino acid residue diversity, have a high propensity to form such interactions and are importantly involved in droplet formation (9,10). However, droplets undergo gelation or solidification over time and lose their inherent fluidic and reversible feature. Furthermore, they occasionally play an adverse role as a reaction site for the nucleation of amyloid fibrils (4,5,7,11). It is evident

from these observations that protein droplets contribute deeply not only to their own cellular functions but also disrupt living systems and then cause neurodegenerative diseases. Faced with the dual nature of protein droplets as a potential double-edged sword, understanding their properties and the subsequent processes of solidification and amyloid fibril formation within individual protein droplets becomes crucial. Although many conventional methods, such as the addition of polyethylene glycol (PEG) and overexpression of target proteins in cells, effectively induce droplet formation (5, 7, 11), methods for spatiotemporal control of droplet formation have yet to be developed.

In this study, we have aimed to form protein droplets spatiotemporally by optical trapping in order to characterize the properties and behavior of protein droplets at the single droplet level. The optical trapping is a technique for manipulating micro- and nanoparticles by mechanical force generated when light interacts with dielectric materials. In 1986 Ashkin proposed optical tweezers, in which a single microparticle (MP) is trapped at a tightly focused laser beam spot in solution in non-destructive manner without any contact (12). Since then, optical trapping of MPs and nanoparticles (NPs) in solution has attracted attention in the various research fields, ranging from optics and physics to biology (13-25). As a basic principle of optical trapping, a gradient force is exerted on an object by focusing a trapping laser, and the object is stably trapped when its force is larger than the scattering and absorption forces. The total sum of gradient, scattering, and absorption forces determines the optical force exerted on the target object, and when it suppresses the Brownian motion and compensates the gravity of micro- or nanoparticles, they are trapped and confined inside optical potential. However, trapping protein molecules is typically challenging because optical forces available in solution are too weak. This is due to the fact that protein molecules are nanometer-sized and their

refractive index is almost the same as that of aqueous solvents.

Nonetheless, we have discovered a strong trapping when the trapping laser is focused on surface or interface (26-33). When plural NPs are trapped in a laser focus at the interface and form an aggregate, the trapping extends beyond the focus to give a large assembly. This phenomenon is referred to as “optically evolved assembling”. Although exact mechanisms are complex and appear to vary depending on targets, we have explored various types of optical trapping, such as assembling of polystyrene NPs at air/solution interface (32,33), swarming of gold NPs at glass/solution interface (26,27), and furthermore, crystallizing amino acids beyond nanoscale at air/solution interface (28,29). As for the assembly of protein molecules, lysozyme formed a disk-like assembly in a supersaturated solution, and the assembly size reached a submillimeter diameter and a few ten micrometers in depth (30,31).

Utilizing the characteristic assembling behavior observed at air/solution interface, this study has discovered an observation that a single droplet-like condensate of α -synuclein is formed upon trapping through the assistance of polyethylene glycol (PEG). α -Synuclein is a pathogenic protein associated with synucleinopathies. It is a natively unfolded protein composed of N-terminal, NAC, and C-terminal domains, and has a molecular weight of 14460. It has been shown that this protein tends to form droplets by LCDs embedded in the N-terminal and NAC domains by the addition of PEG (7,34). The condensate was formed after an induction period, and the rate of the droplet appearance and growth showed a dependence on laser power, α -synuclein concentration, and PEG concentration. Through fluorescence observation using dye-conjugated α -synuclein, structural properties of the condensate, such as the shape of the condensate and the distribution/homogeneity/fluidity of protein molecules within the condensate, have

been characterized. The condensate shrank and disappeared almost completely after the laser was switched off in the early stages. However, prolonged trapping led to the condensate deformation, and furthermore, the emergence of amyloid-like aggregates from the laser focus, as revealed by Raman light scattering spectroscopy, thioflavin T fluorescence imaging, and the analysis of electron microscopy. The results demonstrate that optical trapping serves as an effective inducer not only for condensate formation and growth but also for subsequent amyloid fibril formation, enabling the tracking of the detailed time-dependent process at a single condensate level.

Materials and Methods

Materials

D₂O was purchased from Eurisotop (Saint-Aubin, France). Tris and sodium chloride were purchased from Nacalai Tesque (Kyoto, Japan). PEG-8000 was purchased from Sigma-Aldrich (St. Louis, MO). NHS-rhodamine was purchased from Thermo Fisher Scientific (Waltham, MA).

Expression and purification of α -synuclein

Recombinant human α -synuclein gene was subcloned into NdeI/BamHI sites of the vector pET19b (Novagen). *Escherichia coli* BL21(DE3)pLysS cells were transformed with the plasmids and grown in LB medium containing 100 μ g/mL ampicillin. Expression of α -synuclein was induced with 0.5 mM isopropyl-1-thio- β -D-galactopyranoside at 37°C for 5 h and the cells were harvested by centrifugation at 8,100 $\times$ g for 7 min. The cells were suspended in 100 mM Tris-HCl (pH 8.0) containing 300 mM NaCl and 1 mM EDTA, and disrupted by sonication. The cell lysate was boiled at 98°C for 10 min and centrifuged at 48,000 $\times$ g for 15 min. The supernatant was precipitated by shifting pH to 3.5 with HCl and centrifuged at 17,000 $\times$ g for 15 min. The supernatant was dialyzed against 20 mM Tris-HCl containing 1 mM EDTA and 100 mM NaCl (pH 8.0), and loaded onto HiTrap™ Q HP column (Cytiva). Proteins were eluted with a linear gradient of 0.10-0.35 M NaCl. The pure fraction of α -synuclein was dialyzed against Milli-Q water and lyophilized. The concentration of α -synuclein was determined using the extinction coefficient of 5,120 M⁻¹ cm⁻¹ at 280 nm (35).

Fluorescent labeling of α -synuclein

N-Hydroxysuccinimide (NHS)-rhodamine labeling of α -synuclein was performed according to the manufacturer's instructions (ThermoFisher Scientific). NHS-rhodamine was dissolved in DMSO at 10 mg/mL. A 10-fold molar excess of NHS-rhodamine was mixed with α -synuclein in D₂O conjugation buffer (0.1 M sodium phosphate, 0.15 M sodium chloride; pH 7.2) and incubated at room temperature for 1 hour. Excess dye was removed with the Thermo Scientific™ Fluorescent Dye Removal Columns. The concentration of NHS-rhodamine labeled α -synuclein was determined according to the equation provided by the manufacturer. Buffer exchange of the labeled protein solution to 25 mM Tris-DCI (pH 7.4) was performed using 3 kDa Amicon® Ultra Centrifugal Filter Devices (Millipore) and then stored at 4°C protected from light.

Sample preparation for optical trapping

Lyophilized α -synuclein powder was dissolved in 25 mM Tris-DCI (pH 7.4) at ~30 mg/mL. The protein solution was passed through 100 kDa Amicon® Ultra Centrifugal Filter Devices (Millipore) to remove undissolved protein aggregates and then α -synuclein concentration was determined using the extinction coefficient of 5120 M⁻¹ cm⁻¹ at 280 nm (35). For optical trapping experiments, a sample solution containing 200 μ M α -synuclein, 25 mM Tris-DCI, 100 mM NaCl, and 7.5% PEG, was used as basic conditions. For fluorescence imaging, 1% NHS-rhodamine labeled α -synuclein, 0.00225 % rhodamine B-labeled PEG, or 5 μ M ThT was added to the sample as a probe.

Optical setup

The experimental setup was configured on an inverted microscope (Olympus, IX71) and comprised optical trapping, bright field imaging, and confocal fluorescence imaging

systems (Figure S4-1 and S4-2). A continuous wave laser with a wavelength of 1064 nm (Coherent, MATRIX 1064-10-CW) was tightly focused onto the solution/air interface through an air-immersion objective lens (Olympus, UPLFLN60X, Semi-Apochromat Objectives) with a numerical aperture of 0.9 and a magnification of 60. The use of conditions enabled the trapping of protein clusters and the formation of LLPS. The laser powers throughout the objective lens were adjusted to 0.8, 1.0, or 1.2 W using a combination of a half-wave plate and a polarizing beam splitter for precise power control. Localization of the surface position was achieved using a focused 633 nm laser. Bright field imaging was achieved using a halogen lamp, which illuminated the sample through a condenser (NA 0.3). The transmitted light from the halogen lamp was collected via the same objective lens as the trapping laser and recorded using a charge-coupled device (CCD) camera (Watec France, WAT231S2), generating transmission images.

Confocal Fluorescence Imaging and Analysis

To assess the concentration increase and distribution of α -synuclein condensates, confocal laser scanning microscopy (Olympus, Fluoview FV300,) was employed. For the NHS-rhodamine labeled α -synuclein and the rhodamine B-labeled PEG, a 532 nm laser served as the excitation source, with powers of 1 mW after the objective lens for imaging. The pinhole size of the confocal aperture was selected to be 300 μ m. Fluorescence emission light was recorded as fluorescence images, with scanning times of 5.42 s and 1.12 s for concentration distribution and FRAP measurement, respectively.

Depth Scanning and FRAP Analysis

The objective lens stage was motorized and computer-controlled, enabling stepwise

depth-scanning. For FRAP measurement, an intense excitation laser was utilized with the wavelength and the power of 532 nm and 10 mW respectively, distinct from the imaging laser. Unlike the excitation laser for fluorescence imaging, the photobleaching excitation laser followed a separate optical path and remained stationary during the procedure. Thus, the dual functions of photobleaching and fluorescence imaging scanning were facilitated by a shared laser source, functioning synchronously.

Amyloid Fibril Formation and Raman Microspectroscopy

Characterization of amyloid fibril formation utilized the dye of ThT to observe the emission in fluorescence images, excited by a laser with a wavelength at 405 nm with a power of 1 mW after the objective lens. Raman microspectroscopy was conducted to evaluate the state of protein under optical trapping. The protein sample was irradiated by an excitation laser with a wavelength of 532 nm and a power of 50 mW. Signals from the sample were collected through a pinhole of 100 μm and resulting in spectra using a polychromator (Andor Technology, SR-303i-A) equipped with a CCD camera (Andor Technology, DU920P-BV). The acquisition conditions comprised an exposure time of 15 s and an accumulation of 2 times which were the best for detecting the Raman spectra with the trapping laser simultaneously. Spectra were measured before, during, and after irradiation of the trapping laser.

Transmission electron microscopy (TEM)

The sample solution after optical trapping experiment was collected and sonicated for 1 min using a s bath. A 5 μL of the sample solution was then placed on carbon-coated copper grids for 1 min and the excess solution was removed with filter paper. The fibrils

absorbed on the grid were negatively stained with a 5 μ L of gadolinium acetate solution (EMstainer, NEM Inc., Tokyo, Japan) for 1 min and the excess solution was removed with filter paper. TEM observation was performed using a JEM-1010 (JEOL, Tokyo, Japan) at 100 kV with a magnification of $\times 150000$.

Results

Observation of the PEG-assisted optical trapping of α -synuclein

I first attempted optical trapping of α -synuclein under various PEG concentration conditions. D₂O was used as a solvent to prevent temperature increase of the sample solution due to 1064 nm laser irradiation. When the 1064 nm laser was focused on the air/solution interface of the α -synuclein solution in the presence of PEG, a circular condensate with a clear contour was formed and its size developed with time (Figure 4-1). In contrast, no clear formation of condensate was observed in the absence of PEG, suggesting that PEG is prerequisite for the α -synuclein condensate formation. Given that laser trapping requires targets to be large enough to receive optical force, these observations suggest that PEG contributes to the formation of α -synuclein clusters. In fact, it was recently discovered that α -synuclein forms clusters under conditions near the boundary of the LLPS phase diagram (36). In accordance with the above idea, the PEG concentration required for laser trapping depended on the protein concentration, with higher protein concentrations leading to droplet formation at lower PEG concentrations, and vice versa (Figure S4-3 and S4-4). In addition, the size development of the laser-induced condensate was accelerated at higher PEG concentrations, indicating that the optical trapping efficiency increases with higher cluster concentration. It was also noteworthy that the condensate formation was specific to the air/solution interface, and no condensates were formed even in the presence of PEG when the laser was focused inside the solution.

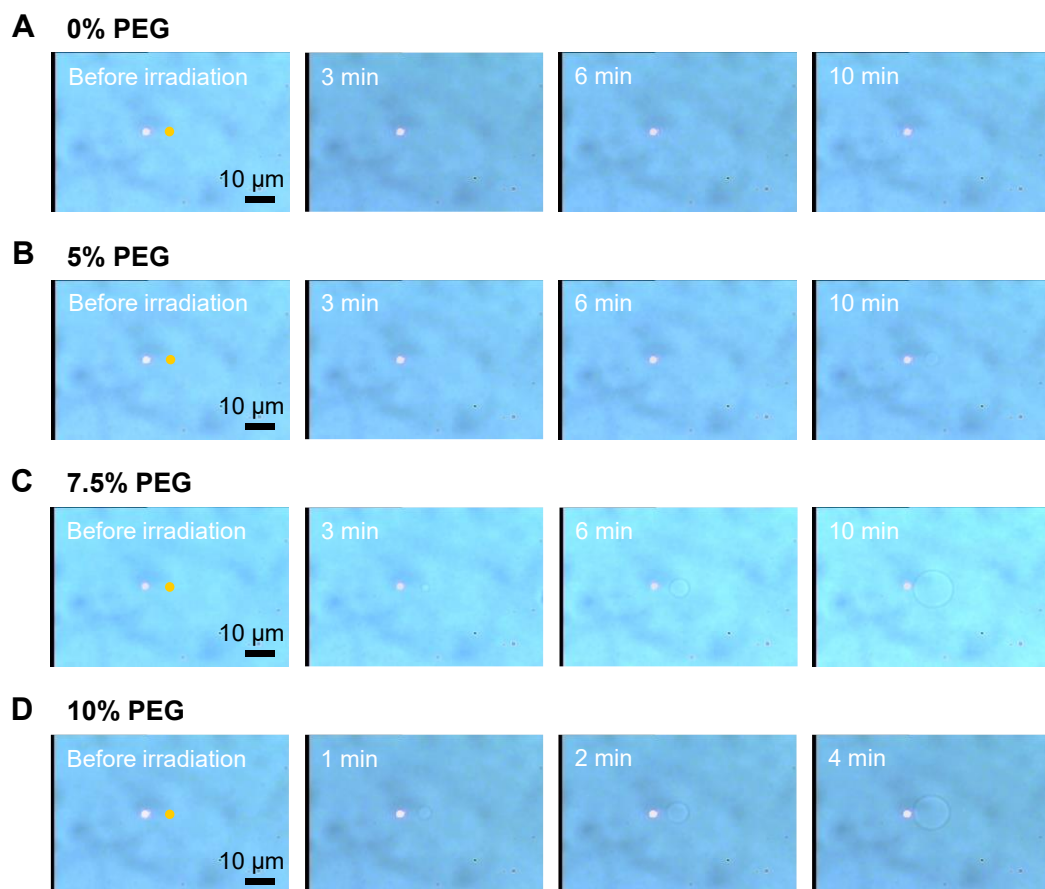


Figure 4-1. Transmission images of α -synuclein condensate induced by optical trapping at the air/solution interface. Sample solutions containing 200 μ M α -synuclein, 25 mM Tris-DCI, and 100 mM NaCl was subjected to optical trapping in the presence of (A) 0%, (B) 5%, (C) 7.5%, and 10% PEG (D). The 1064 nm laser power was set to 1.0 W. The bright red spot is a focused 633 nm laser used to locate the air/solution interface. The focal position of the 1064 nm laser is indicated by a yellow spot.

Evaluation of the shape and growth of the laser-induced droplet

Fluorescence imaging analysis of the laser-induced condensate was performed by mixing a small amount of rhodamine-labeled α -synuclein with unlabeled α -synuclein. An increase in fluorescence intensity was observed inside the condensates, demonstrating

that α -synuclein is concentrated by optical trapping (Figure 4-2A). On the other hand, in contrast to typical LLPS, the PEG concentration in the condensate remained almost constant, as revealed by fluorescence imaging with a rhodamine-labelled PEG (Figure S4-5). The fluorescence image overlapped with the condensate contour in the transmission image, indicating that the edge of the α -synuclein condensate was clear. The fluorescence image quantitatively tracked not only the size development of the condensate but also the internal protein concentration distribution (Figure 4-2B), and both of condensate size and protein concentration at the focus developed synchronously with laser irradiation time (Figure 4-2C). In addition, depth scans performed after condensate formation suggested that the laser-induced condensate has a thick shape with depth below the air/solution interface (Figure S4-6). In Figure 4-2, the α -synuclein concentration after 997 seconds (16.6 minutes) of laser irradiation at the laser focus was determined to be 5.2 mM by comparing the fluorescence intensity inside the condensate to its outside. This concentration indicates that α -synuclein was concentrated 26 times compared to before laser irradiation. Interestingly, the fluorescence intensity showed a gradient towards the laser focus, suggesting a multivalent interaction-assisted optically evolved assembly as a mechanism of condensate formation, which will be discussed later. The trapping rate was further estimated by plotting the total fluorescence intensity of the condensate at the air/solution interface obtained from the confocal fluorescence image at each time point. The plot showed a dramatic increase after the lag time (Figure 4-2D), indicating that the optical trapping accelerates as the condensate grows.

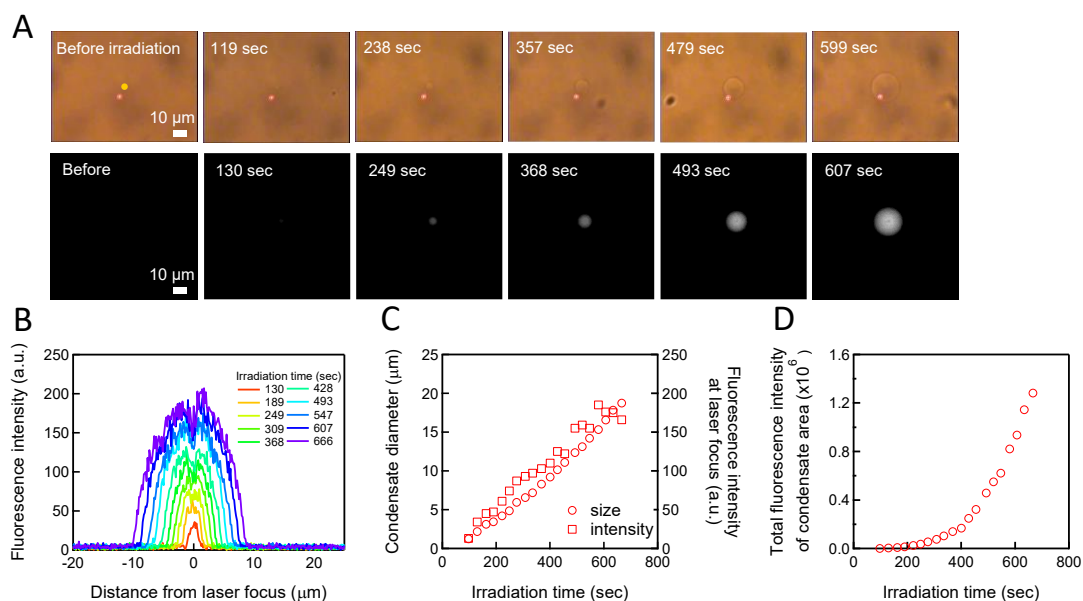


Figure 4-2. Confocal fluorescence imaging of α -synuclein condensate induced by optical trapping. A sample solution containing 200 μ M α -synuclein (1% NHS-rhodamine labeled α -synuclein), 25 mM Tris-DCI, 100 mM NaCl, and 7.5% PEG was subjected to optical trapping. The 1064 nm laser power was set to 1.0 W. (A) Transmission (upper) and confocal fluorescence images (lower) of time dependent formation of α -synuclein condensate. The bright red spot of transmission images is a focused 633 nm laser used to locate the air/solution interface. The focal position of the 1064 nm laser is indicated by a yellow spot. (B) Fluorescence intensity distribution of α -synuclein condensate at the air/solution interface. Fluorescence intensities on a straight line across the laser focus were collected. (C) Time dependence of condensate diameter and fluorescence intensity at the laser focus. The condensate diameter was determined from the width of the cross section across the laser focus. (D) Time dependence of total fluorescence intensity of the condensate at the air/solution interface, which was obtained by integrating fluorescence intensity over the condensate area.

I further performed FRAP analysis to investigate the fluidity of the laser-induced condensate. When photobleaching was performed at different time points during the condensate development process, an instantaneous fluorescence recovery within ~3 sec was observed at all photobleaching timing (Figure 4-3A). This indicates that the condensate has fluidity like droplets produced by PEG-induced liquid-liquid phase separation. It was more fluid than the previously reported α -synuclein droplets formed by PEG-induced LLPS, which showed fluorescence recovery in 10 to several tens of sec (7,37). This is probably because of the effect of the optical trapping. In addition, the reversibility of the droplet was examined by turning off the laser in the middle of the condensate development (Figure 4-3B). When the laser was turned off after 330 sec of irradiation, the condensate shrank with time, although a small clot remained. However, with a shorter laser irradiation time of 150 sec, the condensate almost completely resolved after the laser was turned off. This indicates that the condensate is fluid with high reversibility like LLPS-induced droplets, although it loses its reversibility with continuous laser irradiation.

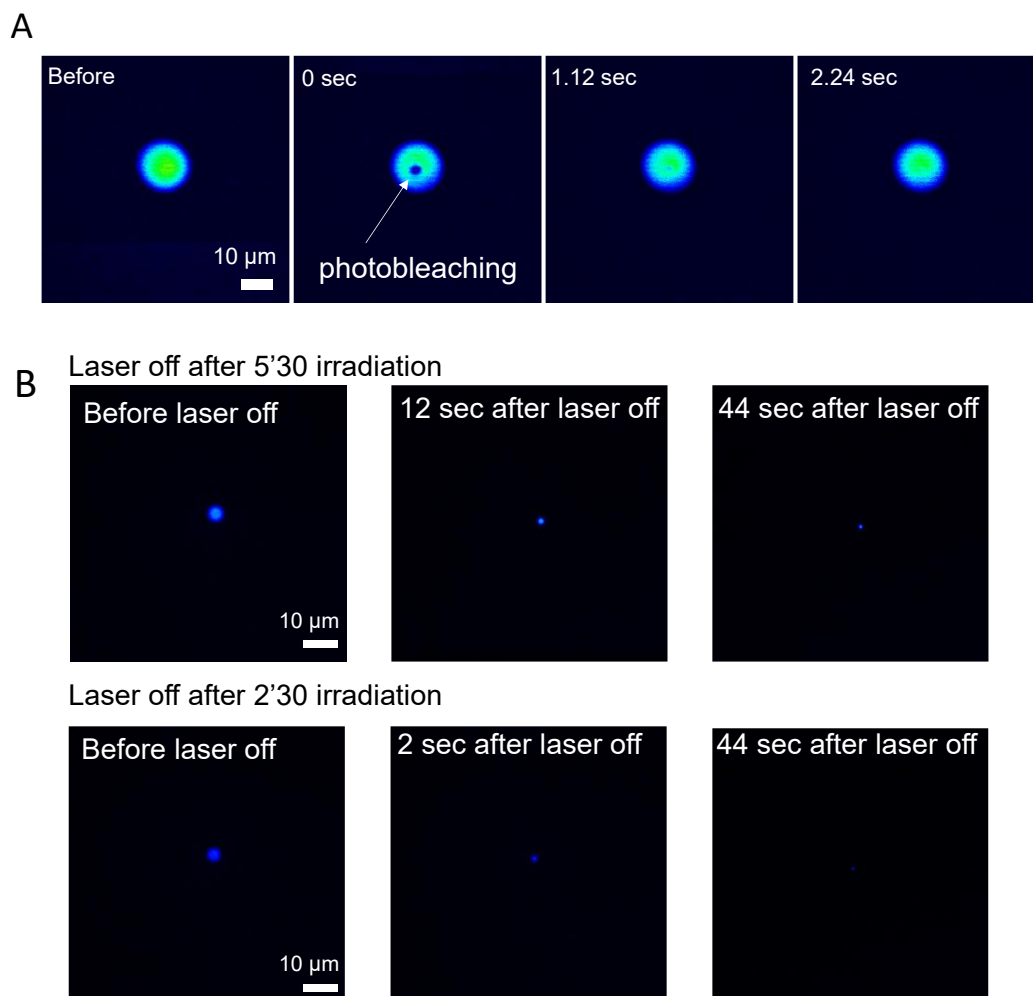


Figure 4-3. Fluidity and reversibility of α -synuclein condensate induced by optical trapping. A sample solution containing 200 μM α -synuclein (1% NHS-rhodamine labeled α -synuclein), 25 mM Tris-DCl, 100 mM NaCl, and 7.5% PEG was subjected to optical trapping. The 1064 nm laser power was set to 1.0 W. (A) FRAP analysis of α -synuclein condensate. Partial photobleaching of condensate was performed at 13 min of the laser irradiation, and subsequent fluorescence recovery was monitored. (B) Reversibility analysis of α -synuclein condensate. The laser was turned off after 330 sec (upper) and 150 sec (lower) of irradiation, and subsequent disappearance of condensate was monitored.

Kinetic characteristics of laser trapping and factors affecting the formation rate of the condensate

To examine factors that affect the rate of condensate formation and growth, I further measured the dependence of laser power, PEG concentration, and protein concentration (Figure 4-4). Overall, the basic behavior was similar regardless of conditions, and the condensate diameter and the fluorescence intensity at the focus increased almost linearly with the laser irradiation time (Figure 4-4A,D,G). As the laser power increased, the lag time for the droplet formation became shorter, and the growth rate after droplet appearance became faster (Figure 4-4A). Similar accelerations were also observed by increasing PEG (Figure 4-4D) and protein concentrations (Figure 4-4G). These results are reasonable based on the mechanism mediated by the α -synuclein clusters, since higher laser power would exert higher optical forces on each cluster, and higher concentrations of PEG and protein would increase the number and/or size of clusters. In all cases, the acceleration of optical trapping was observed as the condensate size grew (Figure 4-4B,E,H). In addition, the relationship between fluorescence intensity at the focus and condensate size was maintained under all conditions (Figure 4-4C,F,I), indicating that laser power, PEG concentration, and protein concentration just alter the rate of laser trapping without changing the mechanism.

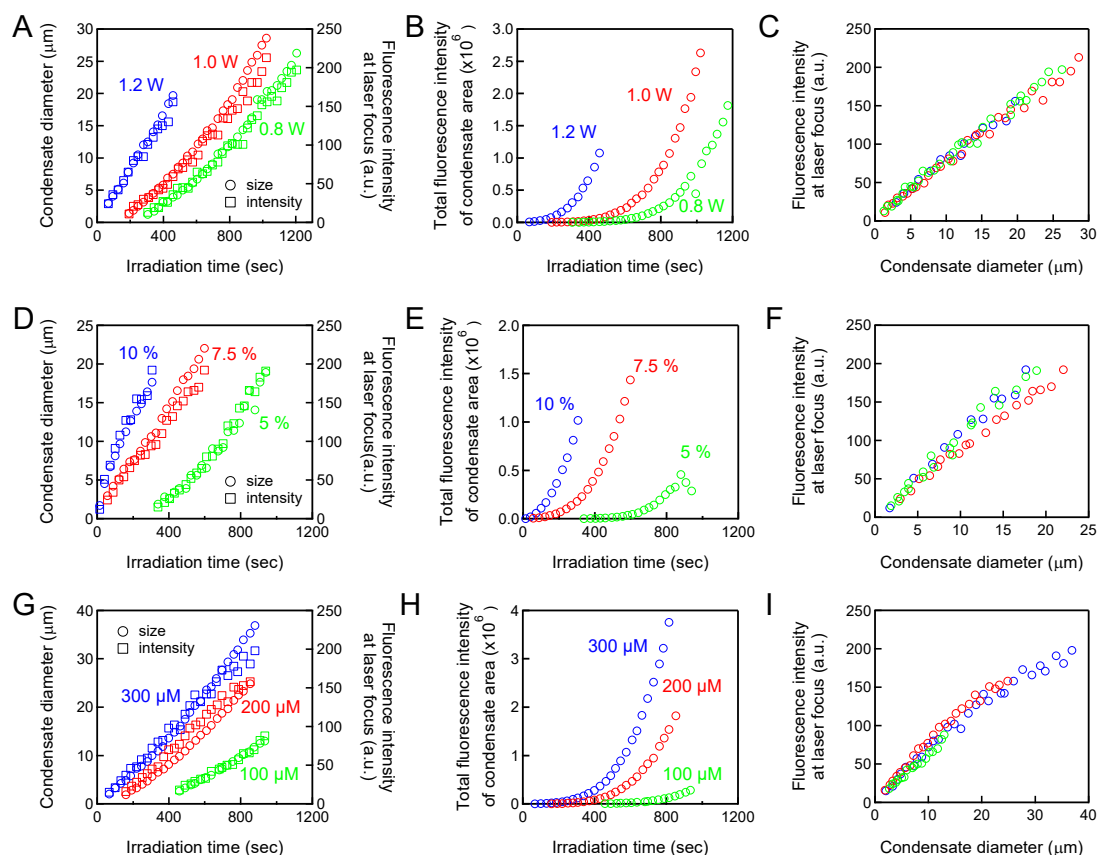


Figure 4-4. Laser power (A, B, C), PEG concentration (D, E, F), and protein concentration dependence (G, H, I) on α -synuclein condensate formation induced by optical trapping. Condensate size and fluorescence intensity was measured by confocal fluorescence imaging, with 200 μ M α -synuclein (1% NHS-rhodamine labeled α -synuclein), 25 mM Tris-DCI, 100 mM NaCl, and 7.5% PEG, and 1.0 W 1064 nm laser power were used as reference conditions. (A, D, G) Time dependence of condensate diameter and fluorescence intensity at the laser focus. (B, E, H) Time dependence of total fluorescence intensity of the condensate at the air/solution interface. (C, F, I) Relationship between condensate diameter and fluorescence intensity at the laser focus.

Condensate aging and amyloid formation by prolonged optical trapping

When laser irradiation was continued after the condensate formation and growth, a dark region of higher refractive index was formed in the center of the condensate and expanded with time. The shape of the condensate was distorted, and eventually, the rod-like structure was extended outward from the laser focus (Figure 4-5A). At this time, many small condensates began to appear around the main condensate, and they moved toward the laser focus and adsorbed to the main condensate. To investigate the structural changes of α -synuclein within the condensate, confocal Raman scattering spectra at the laser focus were measured (Figure 4-5B). Before laser irradiation, no significant amide I band *of protein was observed*, due to low protein concentration. However, the amide I band appeared and developed after laser irradiation (Figure 4-5B, inset). The Raman intensity increased almost linearly similar to the pattern observed in fluorescence imaging analysis (Figure 4-5C); although a slight saturation was observed after 400 sec, this was considered to be related to the deformation of the condensate. Second derivative Raman scattering spectra showed a peak at around 1660 cm^{-1} that shifts to shorter wavenumbers with time (Figure 4-5D). This spectral shift suggested a structural transition of secondary structure from random coil to β -sheet (38), showing that the condensate was aged by prolonged laser irradiation. This observation was more clearly demonstrated at higher protein concentration was as high as $500\text{ }\mu\text{M}$ (data not shown). It is of note that the initiation timing of the peak shift roughly coincided with (or slightly preceded) that of the deformation of the condensate, which has not been achieved at the single condensate level in previous investigations.

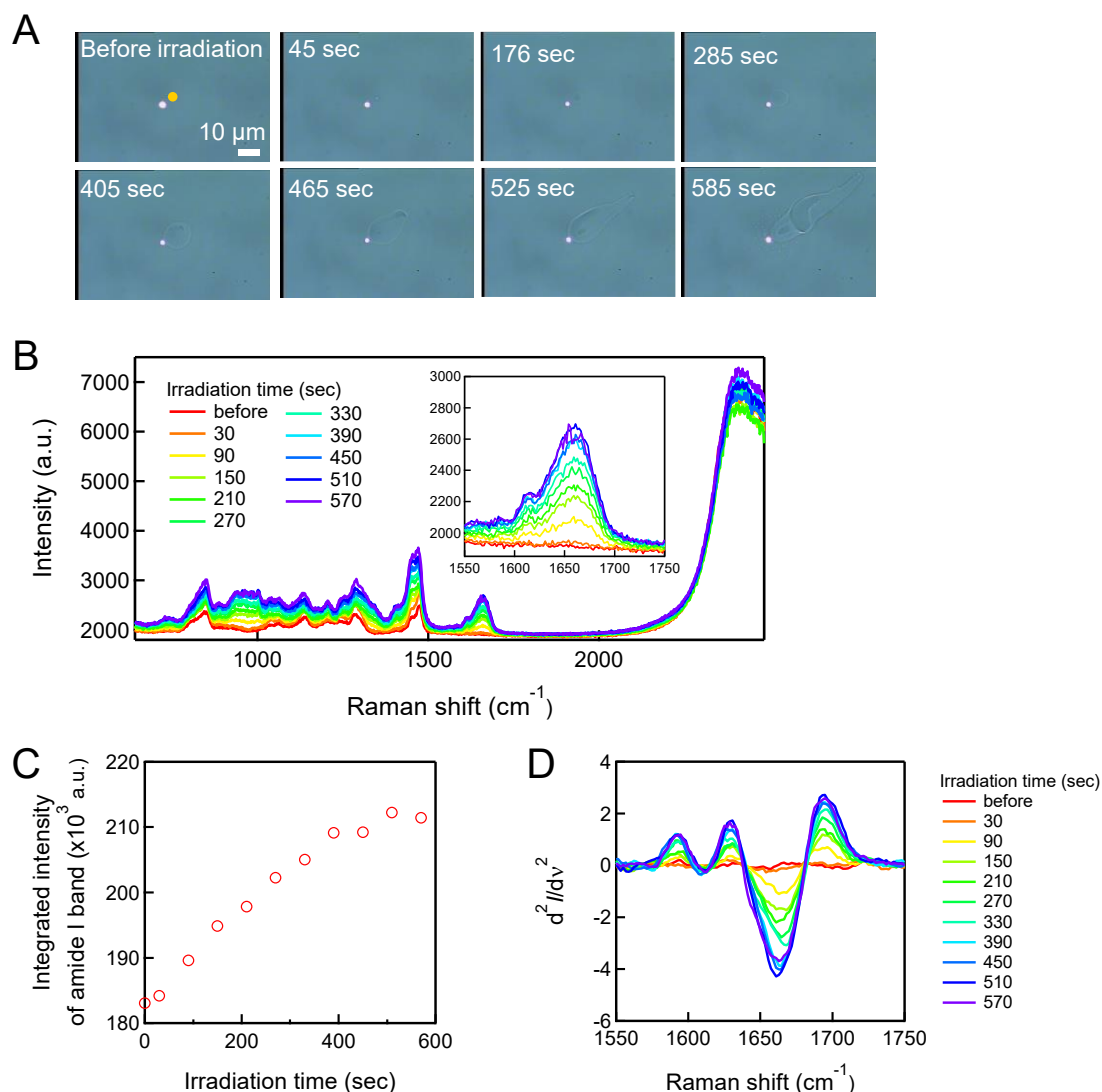


Figure 4-5. Raman scattering measurements of α -synuclein condensate induced by optical trapping. A sample solution containing 200 μM α -synuclein, 25 mM Tris-DCl, 100 mM NaCl, and 7.5% PEG was subjected to optical trapping. The 1064 nm laser power was set to 1.0 W. (A) Transmission images of α -synuclein condensate formation and development. The bright red spot is a focused 633 nm laser used to locate the air/solution interface. The focal position of the 1064 nm laser is indicated by a yellow spot. (B) Time dependence of confocal Raman scattering spectra at the laser focus. The inset represents the spectra in amide I region. (C) Time dependence of integrated Raman scattering intensity of amide I band. (D) Time dependence of second derivative Raman scattering spectra in amide I region.

I presumed that the rod-like structure formed by aging of condensate was amyloid fibrils, and monitored ThT fluorescence during condensate maturation (Figure 4-6). As the droplet developed, ThT fluorescence was detected around the center of the droplet and enhanced as the condensate deforms. The rod-like aggregate developed from the center of the condensate also showed ThT fluorescence, suggesting that the it is composed of amyloid fibrils. In agreement with this, TEM images showed the presence of fibrillar aggregates (Figure 4-7A), which became more evident when analyzing the sample after seeding (Figure 4-7B), supporting that the aging of the laser-induced condensates results in amyloid fibril formation.

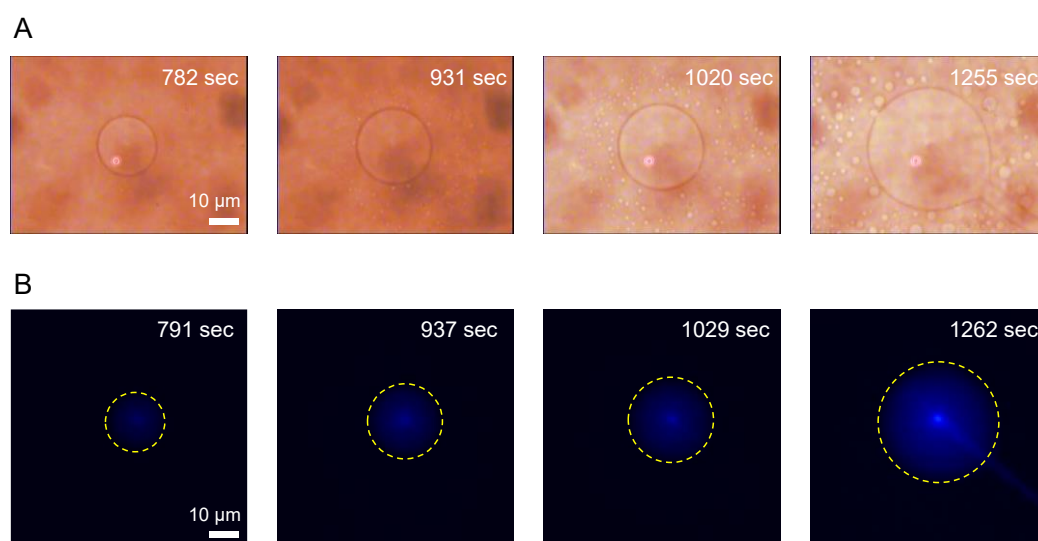


Figure 4-6. ThT fluorescence imaging of α -synuclein condensate induced by optical trapping. A sample solution containing 200 μ M α -synuclein, 25 mM Tris-DCl, 100 mM NaCl, 7.5% PEG, and 5 μ M ThT was subjected to optical trapping. The 1064 nm laser power was set to 1.0 W. (A) Transmission images of α -synuclein condensate development. (B) Confocal fluorescence images of α -synuclein condensate development. ThT fluorescence was monitored with a 405 nm excitation laser. The yellow dashed line indicates the condensate edge in the transmission image in panel A.

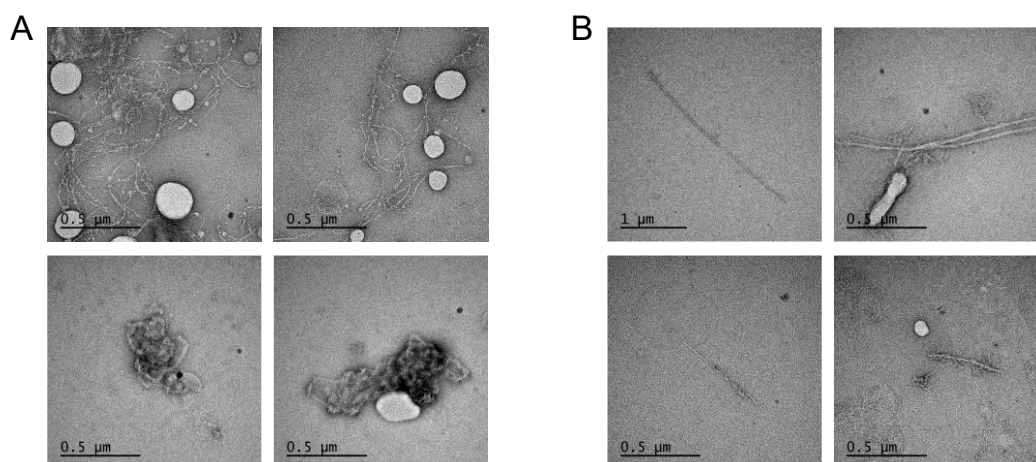


Figure 4-7. TEM observation of α -synuclein aggregates formed by optical trapping. (A) TEM images of α -synuclein aggregates. After optical trapping using the 1064 nm laser at 1.0 W, α -synuclein aggregates was collected and dispersed by ultrasonication, and then subjected to TEM observation. (B) TEM images of α -synuclein aggregates obtained by seeding reaction. α -Synuclein aggregates formed by optical trapping was ultrasonicated and used for seeding reaction as seeds. Seeding reaction was performed under the condition of 200 μ M α -synuclein, 25 mM Tris-DCI, 100 mM NaCl, and 10% (v/v) seed solution at 37°C for 7 days.

Discussion

Through investigations, I first demonstrated that optical trapping can induce condensate formation of α -synuclein spatiotemporally. The formation of the α -synuclein condensate required a certain induction period. The length of the induction period tended to fluctuate to some extent in each experiment, which indicates that the initiation of the condensate formation has a stochastic nature like crystal nucleation, similar to typical LLPS in crowded environments (39). However, the assembly mechanism is quite different from the PEG-induced LLPS, given that PEG was not expelled from the condensate (Figure S4-5). In the case of optical trapping, PEG is not necessary to thermodynamically stabilize the condensate. Instead, it contributes to cluster formation. α -Synuclein tends to form clusters, and indeed, transient clustering with the aid of PEG near the LLPS boundary has recently been identified (36). They are considered to be essential for optical trapping because the optical force on nano-sized protein molecules is very weak. Clusters are trapped, fused and condensed at the air/solution interface as they are pumped up toward the laser focus through the optical cone (40). The cluster condensation by optical trapping leads to condensate nucleation. As the optical trapping progresses over time, α -synuclein will not only localize to the laser focus, but will eventually overflow to the outside. This phenomenon is not due to photothermal effect because the temperature increase is very small under the present D₂O conditions. As a result, the condensate size grows beyond the laser focus, where multivalent interactions among α -synuclein is expected to contribute to condensate growth by preventing the diffusion of α -synuclein. In fact, the gradient of fluorescence intensity within the condensate is observed (Figure 4-2), which supports this mechanism. Since α -synuclein molecules are very small, the laser scattering as observed in PS MPs (14) is unlikely to promote condensate growth. However, the

condensate has a clear edge, which suggests that multivalent interaction networks that maintain the condensate states of α -synuclein are formed at the focus and persist even after the clusters overflow from the focus. Although it is difficult to address the exact molecular mechanisms at this stage, orientating electrostatic interactions in the laser electric field reminiscent of Kerr effects may contribute to the formation of multivalent interactions.

Interestingly, the acceleration of the condensate growth was observed (Figure 4-2D). Observations after turning off the laser in Figure 4-3B demonstrated that a small clot formed at a fairly early stage. This is due to the high concentration of α -synuclein and possibly its molecular orientation in the laser electric field at the focus, which may contribute to the enhanced trapping efficiency with its high refractive index. This gelation or solidification in the condensate progresses with time, and after prolonged laser irradiation, the condensate begins to deform eventually. This is another interesting observation suggesting the optical force-induced aging of the condensate. Many previous studies have reported that droplets formed by LLPS lose their own fluidic properties by transforming to gel- or solid-like state with time (4, 5, 7, 11). The optically induced α -synuclein condensate also showed time-dependent decrease in reversibility, where α -synuclein underwent conformational changes from random-coil to β -structure with positive ThT fluorescence inside the condensate. This structural change was particularly accelerated at the laser focus, and it eventually led to the formation of a rod-like aggregate composed of amyloid fibrils. Presumably, continued optical trapping increased protein concentration at the focus to supersaturation, providing a reaction field for amyloid nucleation.

Figure 4-8 summarizes the proposed mechanism of the optical evolved assembly

observed in α -synuclein. The α -synuclein condensate formation takes place in two steps; first, α -synuclein clusters are optically trapped at the laser focus, and α -synuclein condensate forms. This trapping is aided by the air/solution interface, which is important for the optical force to overcome thermal fluctuations. Here, it is noteworthy that the rate of incorporation of protein molecules into the condensate was accelerated as the condensate size increased (Figure 4-4). The concentrated clusters are expected to fuse with each other and grow into more concentrated and larger sized clusters by continuous laser irradiation, thereby increasing the refractive index. As a result, the optical trapping is accelerated, and the cluster concentration increases non-linearly with time. In the later stages, many small condensates were observed to be pumped up via the optical cone and eventually fused into the main condensate. The center of the condensate becomes less fluid and the pumped clusters or small condensates are unable to coalesce, so they climb along the edges of the condensate to near the air/solution interface. These small condensates do not pass through the laser focus, and therefore, multivalent interaction networks are not as well developed internally as in the main condensate. Thus, fluorescence intensity, i.e., protein concentration is quite low (data not shown).

While this is the first case of the formation of liquid-like protein condensates using optical force, Walton and Wynne reported the induction of LLPS of a nitrobenzene-decane mixture by focusing 785-nm laser (41, 42). They referred this phenomenon to as laser-induced phase separation (LIPS), and explained its mechanism by the perturbation of the energy landscape through storing electromagnetic energy of the laser. With a similar mechanism, cluster formation may be promoted within the laser focus in combination with the excluded volume effect of PEG. However, a clear difference from the LIP is that the α -synuclein condensate developed beyond the laser focus area. In the

LIPS of the nitrobenzene-decane mixture, a laser intensity was 200 mW, where trapping did not progress significantly. In the present case of α -synuclein, on the other hand, the laser intensity was 1.0 W, where the optical trapping plays a dominant driving force like the observations for lysozyme, PS beads and amino acids.

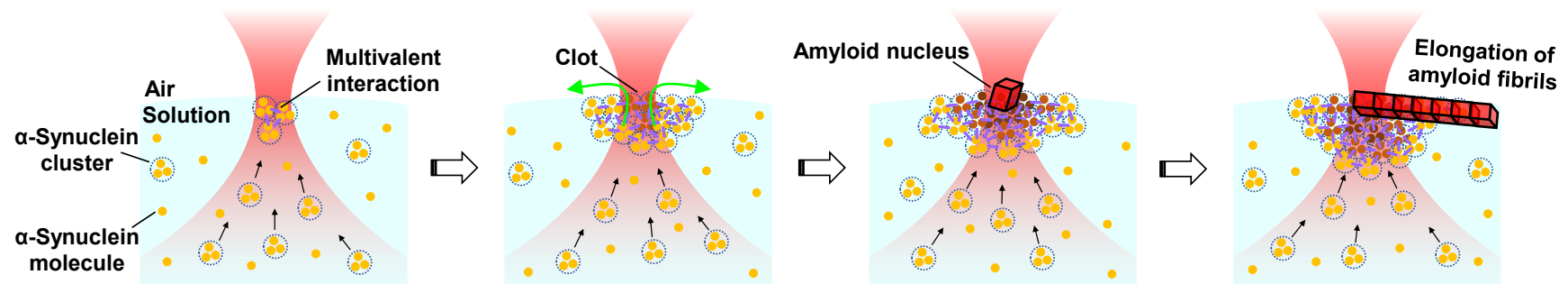


Figure 4-8. Schematic illustration of multivalent interaction assisted optical evoked assembly of α -synuclein.
Proposed process of α -synuclein condensate formation and subsequent amyloid fibril elongation induced by optical trapping is shown.

Supporting information

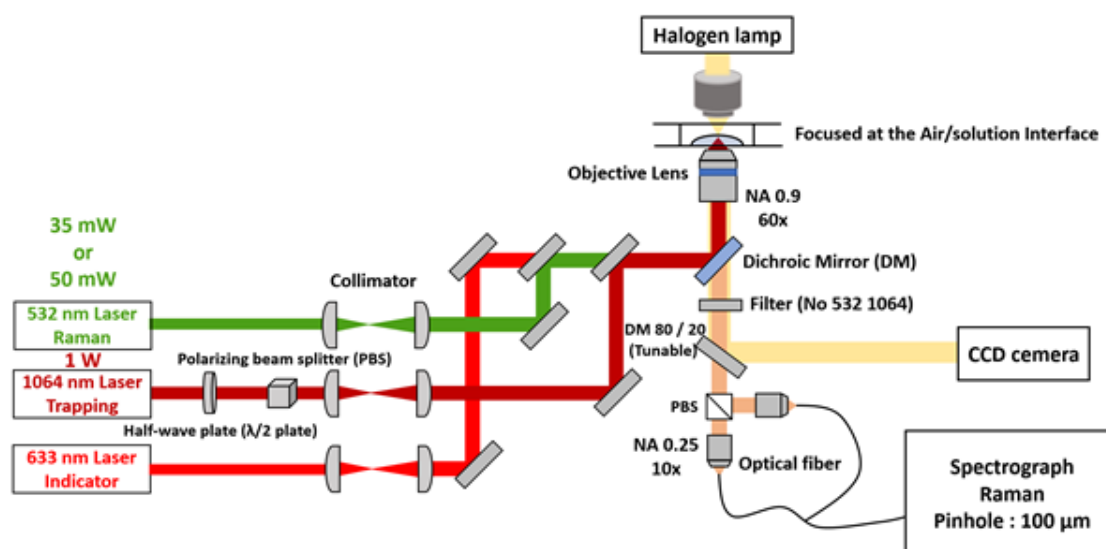


Figure S4-1. The experimental setup includes optical trapping, Raman micro-spectroscopy, and transmission imaging. The sample stage allows movement along the x-y axis, while the objective lens is capable of movement along the z-axis.

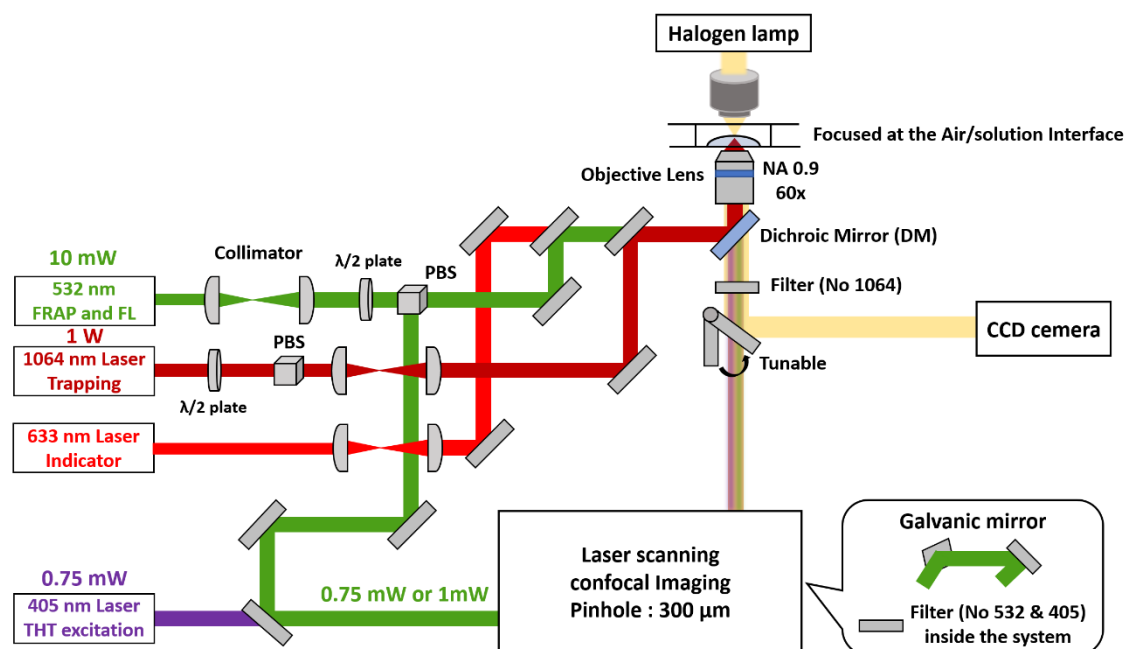


Figure S4-2. The experimental setup includes optical trapping, laser scanning confocal fluorescence imaging, and transmission imaging. The sample stage allows movement along the x-y axis, while the objective lens is capable of movement along the z-axis.

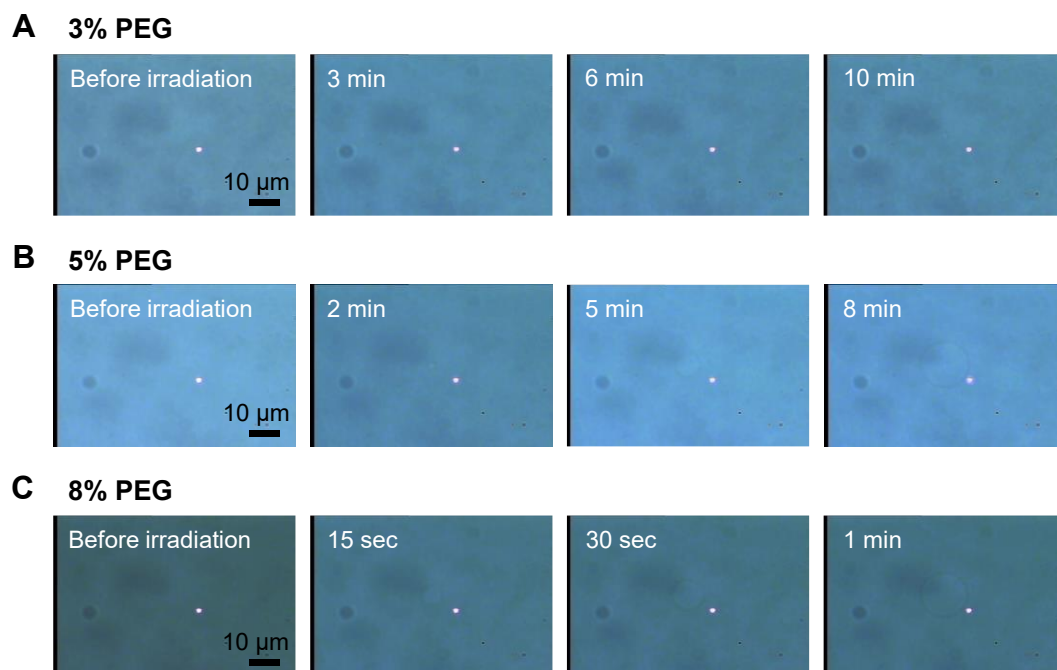
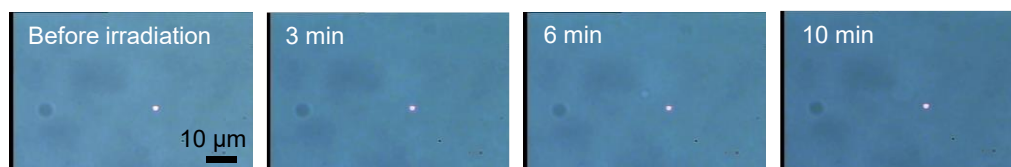
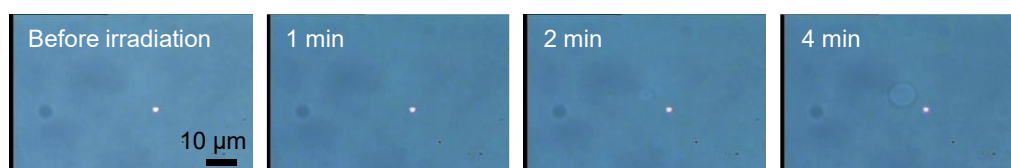


Figure S4-3. PEG-assisted optical trapping of α -synuclein at 500 μ M protein concentration. Laser-induced droplet formation of α -synuclein was examined in the presence of 3% (A), 5% (B), and 8% PEG (C).

A 7.5% PEG



B 10% PEG



C 12.5% PEG

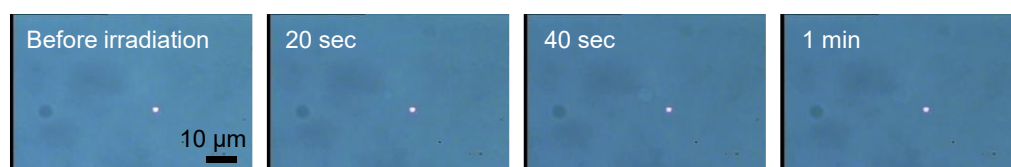


Figure S4-4. PEG-assisted optical trapping of α -synuclein at 100 μ M protein concentration. Laser-induced droplet formation of α -synuclein was examined in the presence of 7.5% (A), 10% (B), and 12.5% PEG (C).

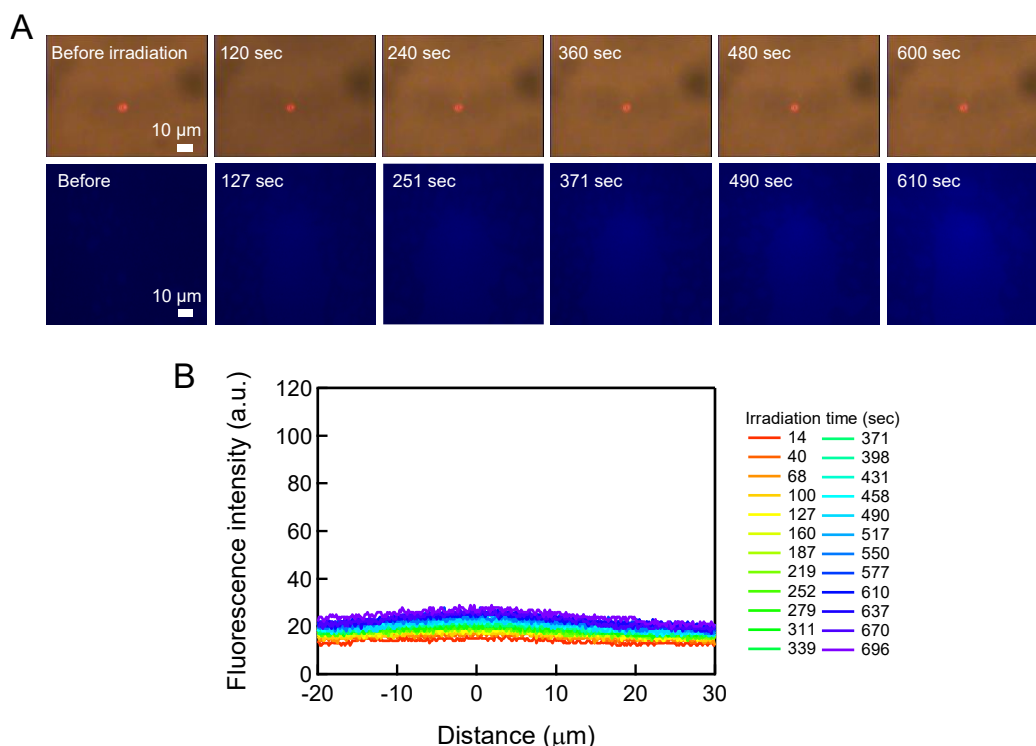


Figure S4-5. Confocal fluorescence imaging of PEG in α -synuclein condensate induced by optical trapping. A sample solution containing 200 μ M α -synuclein, 25 mM Tris-DCl, 100 mM NaCl, 7.5% PEG, and 0.00225 % rhodamine B labeled PEG was subjected to optical trapping. (A) Transmission (upper) and confocal fluorescence images (lower) of time dependent formation of α -synuclein condensate. The bright red spot of transmission images is a focused 633 nm laser used to locate the air/solution interface. The focal position of the 1064 nm laser is indicated by a bright green marker. (B) Fluorescence distribution of PEG in α -synuclein condensate. Fluorescence intensities on a straight line across the laser focus were collected.

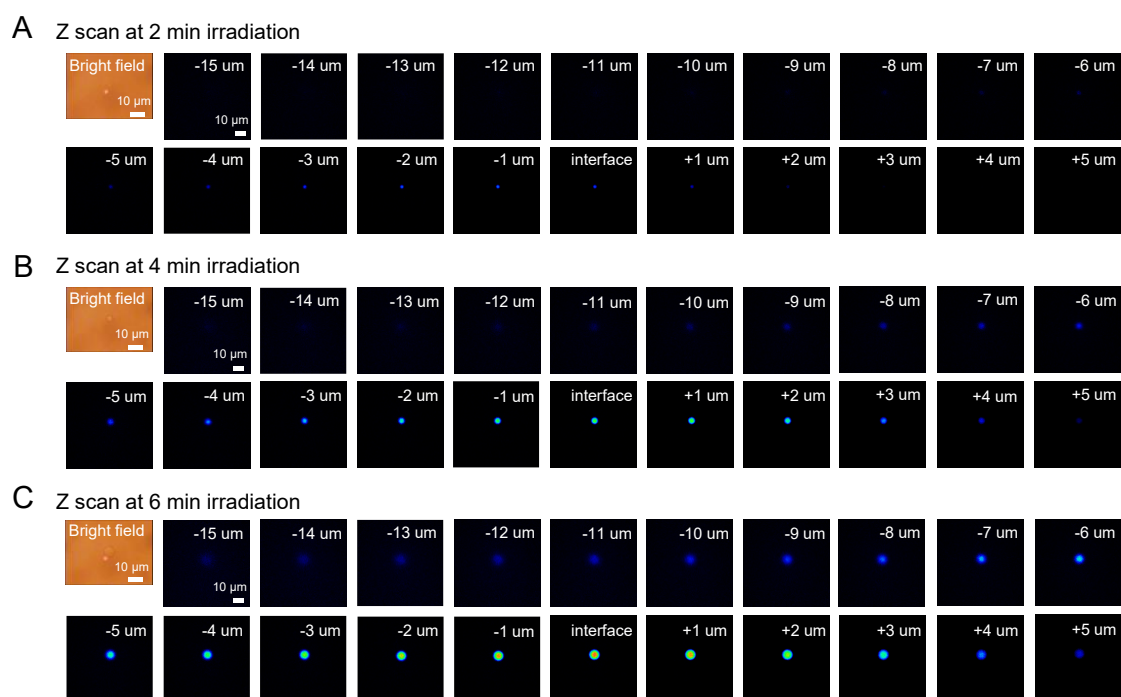


Figure S4-6. Depth scanning of α -synuclein condensate induced by optical trapping. Depth scanning was performed at (A) 2 min, (B) 4 min, and (C) 6 min of the laser irradiation. The objective lens stage was motorized and computer-controlled, enabling stepwise depth-scanning. Confocal fluorescence images were collected from -15 μm to +5 μm at the air/solution interface with a scanning rate of 1.12 sec/ μm . The result indicates that the laser-induced condensate is not a flat thin disk but has a thicker shape. In addition, the decrease in fluorescence intensity with depth in the solution supports the gradient of α -synuclein concentration in the thickness direction.

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Chapter 5

General conclusions

In Chapter 2, the work has shed light on one of the essential schemes of amyloidogenic aggregation, in which oligomers serve as on-pathway intermediates for the formation of protofibrils. This is to our knowledge the first model demonstrating the way of how oligomers are incorporated to form protofibrils based on experimental measurements. Both of the formation of oligomers and protofibrils could be described by simple schemes and the established model has successfully characterized each process. The model analysis of bovine insulin became feasible because only a single pathway was ideally observed, while diverse pathways are predicted to progress simultaneously in most cases of amyloid formation in pathology. Although addressing experimentally the full spectrum of aggregation pathways may not be so straightforward, the current scheme of oligomer and subsequent protofibril formation represent the framework of the pathways of early aggregation. It is also a new discovery that the rate-limiting step of protofibril formation is the association of oligomers where the expulsion of water is involved, indicating that the structural organization progresses considerably during this step.

In Chapter 3, a comprehensive understanding of the propagation mechanism of cross-seeding has been progressed from the perspective of energy landscape. The observation of the multistep structural changes has shed light on molecular details of the conformational deviations and the emergence of a new fibril structure. Remarkably, the presence of the metastable fibril states is a new physicochemical aspect of cross-seeding, and suggests the dynamic feature of amyloid structure during the time course of cross-seeding. The cross-seeding has been reported not only between isoforms of the same protein but also between different proteins, and it is considered to be important for understanding pathology and furthermore, cross-talk between different amyloid diseases. Given that heterologous proteins are actually involved in the pathology of several diseases,

such as Alzheimer's disease, prion disease, Parkinson's disease and type 2 diabetes, accelerating researches on cross-seeding will pave new perspectives on the mechanism of pathology at the level of protein molecules.

In Chapter 4, the study has demonstrated that optical trapping works as a technique to form protein condensates with high fluidity and reversibility. The liquid-like condensate can be produced in a spatiotemporally controlled manner, which is useful for studying structural properties and formation mechanism of protein condensates. Furthermore, this system is expected to elucidate the process of amyloid fibril formation in detail at the single droplet level. Recently, protein condensates have been successfully formed by light using a tag called Cry2 and Corelets, which has opened up a new way for the formation of protein condensates in an optogenetic manner. Comparable to this, optical force has great potential as a powerful methodology for forming liquid-like condensates. Furthermore, whilst the above optogenetic method requires the tag conjugation, the present technique is widely applicable to intact proteins with strong intermolecular interactivity like LCDs. The application of optical force to studies on liquid-like protein condensates has the potential to explore not only future droplet researches of a wide range of proteins but also the manipulation cellular functions via spatiotemporal control of droplets.

Through this thesis, I proposed that amyloid fibril formation is multistep process. By focusing on such multistep process, the elucidation of the detailed mechanism of amyloid fibril formation will be advanced. In the present study, insulin and α -synuclein were targeted for analysis, but the current analytical method can be applied to other proteins as well. Since amyloid fibrils are involved in various neurodegenerative diseases and amyloidosis, there is a need to develop methods for prevention and treatment. In this

context, understanding the intermediate aggregated species present in multistep formation process of amyloid fibrils will help to identify the causes of the diseases, which will also be a target for drug delivery.

List of publications

Main papers

Keisuke Yuzu, Naoki Yamamoto, Masahiro Noji, Masatomo So, Yuji Goto, Tetsushi Iwasaki, Motonari Tsubaki, and Eri Chatani “Multistep changes in amyloid structure induced by cross-seeding on a rugged energy landscape” *Biophys. J.* 120, 284-295 (2021)

Keisuke Yuzu, Hiroshi Imamura, Takuro Nozaki, Yuki Fujii, Shaymaa Mohamed Mohamed Badawy, Ken Morishima, Aya Okuda, Rintaro Inoue, Masaaki Sugiyama, and Eri Chatani “Mechanistic modeling of amyloid oligomer and protofibril formation in bovine insulin” *J. Mol. Biol.* 436, 168461 (2024)

Keisuke Yuzu, Ching-Yang Lin, Po-Wei Yi, Chih-Hao Huang, Hiroshi Masuhara, and Eri Chatani “Spatiotemporal formation of a single liquid-like condensate and amyloid fibrils of α -synuclein by optical trapping at solution surface” submitted to *Proc. Natl. Acad. Sci. USA*, under review

Related papers

Keisuke Yuzu, Mikael Lindgren, Sofie Nyström, Jun Zhang, Wakako Mori, Risako Kunitomi, Terumasa Nagase, Keiichi Iwaya, Per Hammarström, and Tamotsu Zako, “Insulin amyloid polymorphs: implications for iatrogenic cytotoxicity” *RSC Adv.* 10, 37721-37727 (2020)

Takato Hiramatsu, Naoki Yamamoto, Seongmin Ha, Yuki Masuda, Mitsuru Yasuda, Mika Ishigaki, **Keisuke Yuzu**, Yukihiro Ozaki, and Eri Chatani “Iodine staining as a useful probe for distinguishing insulin amyloid polymorphs” *Sci. Rep.* 10, 16741 (2020)

Hamed A Abosharaf, Yuki Sakamoto, Aliaa M Radwan, **Keisuke Yuzu**, Mika Fujimura, Thoria Diab, Tarek M Mohamed, Eri Chatani, Tetsunari Kimura, and Motonari Tsubaki “Functional assembly of *Caenorhabditis elegans* cytochrome b-2 (Cecytb-2) into phospholipid bilayer nanodisc with enhanced iron reductase activity” *Biomolecules* 11, 96 (2021)

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Wakako Mori, **Keisuke Yuzu**, Nadine Lobsiger, Hideo Nishioka, Hisako Sato, Terumasa Nagase, Keiichi Iwaya, Mikael Lindgren, and Tamotsu Zako “Degradation of insulin amyloid by antibiotic minocycline and formation of toxic intermediates” *Sci. Rep.* 11, 6857 (2021)

Yuki Yoshikawa, **Keisuke Yuzu**, Naoki Yamamoto, Ken Morishima, Rintaro Inoue, Masaaki Sugiyama, Tetsushi Iwasaki, Masatomo So, Yuji Goto, Atsuo Tamura, and Eri Chatani “Pathway Dependence of the Formation and Development of Prefibrillar Aggregates in Insulin B Chain” *Molecules* 27, 3964 (2022)

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Reviews

Eri Chatani, **Keisuke Yuzu**, Yumiko Ohhashi, and Yuji Goto “Current Understanding of the Structure, Stability and Dynamic Properties of Amyloid Fibrils” *Int. J. Mol. Sci.* 22, 4394 (2021)

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