



Study on the binding and coloration of iodine to the amyloid fibril structures

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Doctoral Dissertation

Study on the binding and coloration of iodine
to the amyloid fibril structures

アミロイド線維構造に対するヨウ素の
結合性及び呈色特性に関する研究

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Abbreviations

A β	amyloid β
ACN	acetonitrile
AD	Alzheimer's disease
AFM	atomic force microscopy
ALS	amyotrophic lateral sclerosis
ANS	8-anilino-1-naphthalenesulfonic acid
ATR	attenuated total reflection
BSA	bovine serum albumin
β 2m	β 2-microglobulin
CAA	cerebral amyloid angiopathy
CD	circular dichroism
Calcein	bis[<i>N,N</i> -bis(carboxymethyl)aminomethyl]fluorescein
CMC	critical micellar concentration
CR	Congo Red
DLS	dynamic light scattering
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
ESI	electrospray ionization
H/D	hydrogen/deuterium
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high pressure liquid chromatography
i.d.,	inner diameter
FTIR	Fourier transform infrared spectroscopy
LC/MS	liquid chromatography/Mass spectrometry
MAS	magic angle spinning
MS	mass spectrometry
MS/MS	mass spectrometry/mass spectrometry
M.W.	molecular weight
NMR	nuclear magnetic resonance

PG	phenylglyoxal
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
POPG	1-palmitoyl-2-oleoyl-sn-glycero-3-[phosphor-rac-(1-glycerol)]
rpm	rotation per minutes
SDS	sodium dodecyl sulfate
ssNMR	solid-state NMR
TEM	transmission electron microscopy
Tris	tris(hydroxymethyl)aminomethane
TFA	trifluoroacetic acid
ThT	thioflavin T
TOF	time of flight
Triton X-100	polyoxyethylene(10) octylphenyl ether
TSEs	transmissible spongiform encephalopathies
UV-vis	ultraviolet-visible region

Chapter 1.

General introduction

1-1. Amyloid fibrils and its association with protein misfolding diseases

In the 20th century, the average life span of human had lengthened to a great extent due to advances in science and medical technology. We have come to be able to cure many diseases which were once lethal for human. However, there are still a number of diseases for which treatment methods have not been established. One of the diseases is protein misfolding disease or amyloidosis which is induced by protein aggregates called amyloid fibrils. Up to now, it is reported that more than 50 disorders are associated with protein misfolding and subsequent conversion into amyloid fibrils¹ (Table 1). For example, Alzheimer's disease (AD) is the most famous one. This disorder is induced by an aggregation of A β peptide in the brain, and it has been labelled the 'twenty-first century plaque' because of its difficulty in therapy and an explosive increment of patients all over the world. Aiming to treat amyloidosis, many researches have been conducted, and we have got important knowledge about amyloid fibril structure, mechanisms of fibril formation, and onset of the disease¹. However, there are no curative treatments and reliable diagnostics for any disorders, so that further researches are necessary.

Table 1-1. Several human disorders associated with amyloid fibril formation.

Disease	Aggregating protein or peptide
Alzheimer's disease	A β peptide
Spongiform encephalopathies	Prion protein or its fragments
Parkinson's disease	α -Synuclein
Huntington's disease	Huntingtin fragments
Familial amyloidotic polyneuropathy	Transthyretin mutants
Type II diabetes	Amylin
Haemodialysis-related amyloidosis	β_2 -Microglobulin

1-2. Protein folding and misfolding

According to the Anfinsen's dogma, protein folds into a stable unique structure which is determined only by its amino acid sequence². This postulate had been widely accepted because it can be applicable to many proteins. However, now it has been known that several proteins and peptides possess two types of stable conformations, native structure and amyloid fibril in spite of the same amino acid sequence³. From the point of view of free energy, it is thought that there are several stable conformations of proteins such as native state, amyloid fibrils, and metastable intermediates such as partially folded states and oligomers³ (Figure 1-1). Processes of protein folding and misfolding can be described as a diffusional search on a free energy surface⁴. Unfolded proteins tend to fold into native states via folding intermediates. Because of its position in the energy funnel, native states are thermodynamically stable. But they stochastically change into amyloid fibrils via partially folded state and oligomeric state by overcoming high energy barrier.

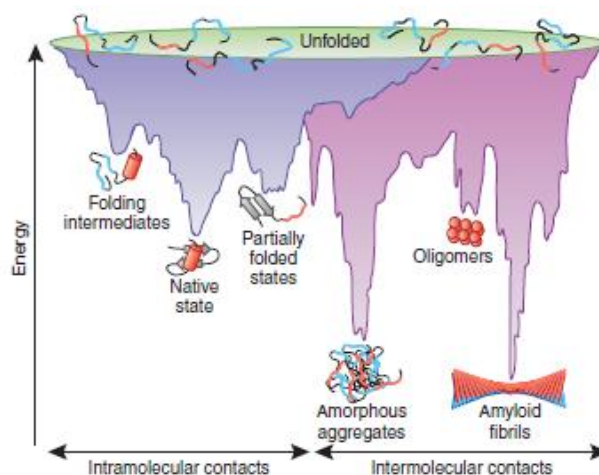


Figure 1-1. Schematic illustration of energy landscape of protein folding and misfolding³. The purple area corresponds to folding process of monomeric proteins into native state via intramolecular contacts, and pink area corresponds to misfolding process of oligomeric proteins into amorphous aggregates and amyloid fibrils via intermolecular contacts. Both parts of the energy surface overlap. Formation of aggregates can occur from intermediates populated during *de novo* folding or partially folded states induced by destabilization of native state.

1-3. Proposed model of amyloid fibril formation

Details of the mechanism of amyloid fibril formation are still not understood, but it is suggested that fibril formation generally proceeds via a nucleation-dependent model (Figure 1-2)⁵. According to this model, formation of amyloid fibrils can be divided into two phases, nucleation phase and elongation phase. In the nucleation phase, a protein or peptide which initially exists as a monomeric state assembles into oligomeric state. Subsequently, the oligomer changes into a nucleus which has an ability of structural propagation. In the elongation phase, the nucleus converts protein monomers into amyloid forms and rate of aggregation becomes greater. In general, the population of the oligomers is very low because of its instability so that structural transition from oligomers to nucleus hardly occurs. Thus the conversion from oligomers to nucleus is rate-limiting step in the overall reaction and the time until the formation of nucleus is defined as a 'lag time'. On the other hand, when seeds which are fragments of fibrils obtained by sonicating fibrils are added to a protein or peptide solution, fibril formation proceeds without lag time due to the ability of structural propagation of seeds. This reaction is called 'seeding reaction' against the 'spontaneous reaction' (Figure 1-2).

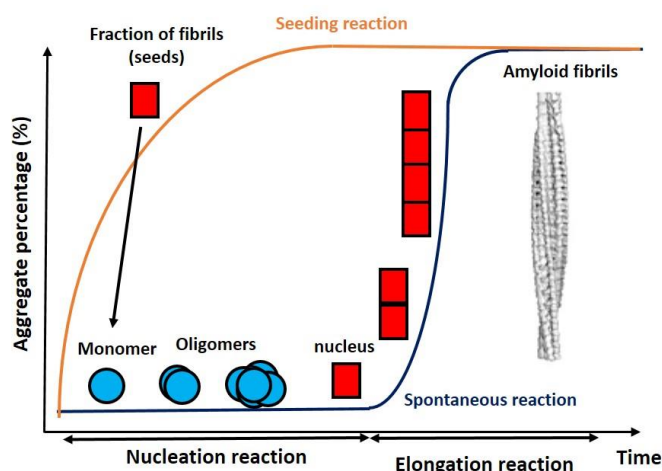


Figure 1-2. Schematic illustration of nucleation-dependent mechanism of amyloid fibril formation⁵.

1-4. Common features of amyloid fibrils

Due to wealth of reports, we have many pieces of knowledge about the features of amyloid fibrils. From macroscopic point of view, amyloid fibrils exhibit unbranched needle like morphology and are 30-120 Å in diameter⁶. The fibrils contain several protofilaments which form long-range coiling along to a direction of fibril elongation (Figure 3A). From microscopic point of view, there is a common characteristic structure called cross- β structure in which β -sheets stack along to a long axis of fibrils and β -strands

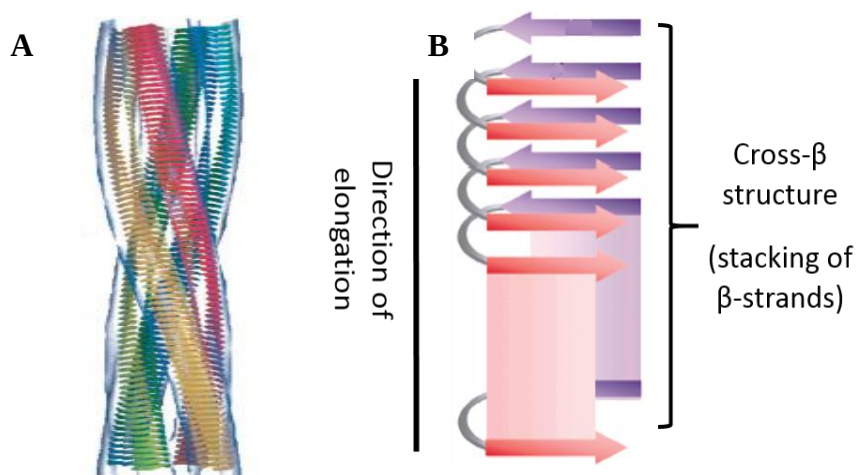


Figure 1-3. Structural model of (A) amyloid fibril composed of several protofilaments⁷ and (B) cross-β structure composed of β-strands.

orient vertically to the axis (Figure 1-3B)⁷.

About the cytotoxicity of amyloid fibrils, a variety of mechanism have been suggested and we still lack general understanding⁸. Amyloid toxicity can result from the loss of the function of a protein or from sequestering other proteins by taking them in aggregates⁹. For example, Olzscha et al. reported that the toxicity of amyloidogenic protein correlates with the capacity of their aggregates to promote aberrant protein interactions and to deregulate the cytosolic stress response¹⁰. However, for most amyloid diseases, a gain of cytotoxicity by aggregation remains a favored hypothesis. As the simplest explanation, amyloid fibrils can disrupt tissue, organ, and membrane via mass action. The cytotoxicity due to mass action might be the most crucial one for most systemic amyloidosis and for the cerebral amyloid angiopathy (CAA)¹¹⁻¹². Other classes of cytotoxicity which do not depend on mass of fibrils have also been reported. About Aβ toxicity, two general classes of interaction between Aβ and membrane may occur. Prefibrillar oligomeric species of Aβ peptide with low molecular weight can induce membrane disruption through the insertion into lipid bilayer¹³⁻¹⁴. On the other hand, many membrane receptors which can react with Aβ peptide have been identified and oligomeric and fibrillar forms of Aβ may act either as agonists or antagonists^{13, 15}. Furthermore, it has been currently known that there are several types of functional amyloid fibrils without any toxicity in fungi, bacteria, insects, and human body¹⁶⁻¹⁷. These fibrils are used for storing protein or peptide as

fibrils¹⁸⁻¹⁹, modulation of water interface tension²⁰, and synthesis of melanin²¹. Though there is not a comprehensive theory which can explain these differences in properties and function of fibrils, many research progresses suggest that structural variation of fibrils can be a clue to solve this problem²².

1-5. Polymorphism; structural diversity of amyloid fibrils

Recent studies have reported that polymorphism, i.e., structural variation of amyloid fibrils at a molecular level can be induced by several perturbation provided by alternating reaction conditions such as salt or other additives, pH, temperature, agitation, and other factors²².

Polymorphism of fibrils was indirectly recognized long before as pathological differences in prion diseases or transmissible spongiform encephalopathies (TSEs) and called ‘prion strains’²³ at first. Degree of progress and pathology of prion diseases dramatically change depending on source of infection even though the same causal protein. Clinical and biomedical characteristics such as period of incubation and seriousness of the disease tend to be transmitted from host and be furthermore maintained through several passages. In addition, it is reported that causal agents responsible for the TSEs are infectious to some species but not to others²³. These ‘prion strains’ and ‘species barrier’ are similar to the characteristics of virus infection, supporting a hypothesis that amyloid deposits might contain nucleic acids as transmitters of pathological information. However, it has been currently known that not nucleic acids but conformations of proteins or fibrils act as transmitters. Tanaka et al. demonstrated that Sup35 prion proteins in yeast form two types of amyloid fibril structures with different physical properties by changing temperature²⁴. Prion strains are attributed to polymorphic differences among infectious agents²⁵⁻²⁶ and the species barrier could be due to differences in amino acid sequence and conformation of prion proteins between an infectious prion donor and an acceptor²⁷⁻²⁹.

In the past, polymorphism was recognized as morphological differences or variations of amino acid region which forms ‘core-region’ of amyloid fibrils and was discussed by electron microscopy³⁰, patterns of proteolysis³¹, resistance to denaturation by chaotropic agents³², and sedimentation³³. Recently, due to advances in analytical methods such as solid-state NMR (ssNMR)³⁴⁻³⁵, cryo-TEM³⁴⁻³⁵, X-ray crystallography³⁶, Raman

Spectroscopy³⁷, and electron paramagnetic resonance (EPR)³⁸⁻³⁹, we can investigate amyloid fibril structures with atomic resolution. Petkova et al. reported that by combining ssNMR and cryo-TEM, they succeeded in determining cross-sectional diagrams of A β amyloid fibrils³⁴. In addition, they also succeeded in demonstrating that A β amyloid fibrils formed with agitation exhibited different structure (Figure 1-4A) with higher cytotoxicity than fibrils formed under a quiescent condition (Figure 1-4B), suggesting that pathological differences among AD patients may be derived from structural variations of amyloid fibrils at a molecular level. About A β fibrils, other structural polymorphs have also been reported. Lu et al. reported that two types of amyloid fibril structure formed in the presence of seeds derived from different AD patient's brains with distinct pathology showed structural differences in the point of view of structural symmetry of cross-sectional diagrams. A fibril possessed structure with 2-fold symmetry

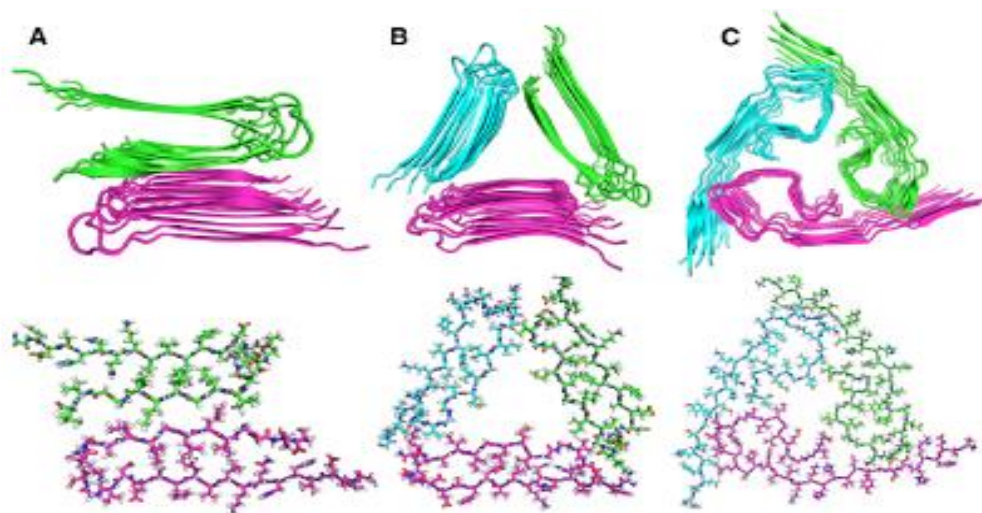


Figure 2-4. Molecular structural models for three A β fibril polymorphs, based on data from ssNMR and cryo-TEM²². (A) Cross-sectional diagram of A β amyloid fibrils with 2-fold symmetry formed under agitated condition in vitro. (B) Cross-sectional diagram of A β amyloid fibrils with 3-fold symmetry formed under quiescent condition. (C) Fibrils with 3-fold symmetry made from seeds which derived from brain tissue of a patient with AD. In each case, the direction of fibril growth is approximately perpendicular to the page. Upper parts are cartoon representations. Eight A β molecules are shown in each subunit. Lower parts are atomic representations, with one molecule in each subunit. Residues 9-40 are shown in (A) and (B). Residues 1-40 are shown in (C). Models in (A)-(C) are based on PDB:2LMN, 2LMP, 2M4J.

which was almost the same as that in Figure 1-4A, and another fibril exhibited cross-sectional diagrams with 3-fold symmetry (Figure 1-4C), slightly different from that in Figure 1-4B.

Polymorphism of α -Synuclein (α -syn) fibrils which causes Parkinson's disease (PD) has been studied with NMR and EPR. These data indicate that there are both rigid protein segments which are primarily β -strands and disordered segments with high flexibility, and both the locations and lengths of the rigid segments can vary significantly among different polymorphs³⁸⁻³⁹.

Now, the formation of structurally distinct, polymorphic amyloid fibrils is recognized as a common feature of amyloidogenic proteins and a considerable attention is paid to polymorphism for understanding correlation between fibril structures and properties such as cytotoxicity. However, the number of reports studying polymorphism at molecular level is still small because of difficulties in sample preparations and measurements. Now, ssNMR is the most powerful tool for investigating fibril structures but it requires isotope-labeling with ^{13}C and ^{15}N . In addition, to achieve very high homogeneity of fibrils, samples must be prepared by performing seeding reaction several times³⁴⁻³⁵ so that it takes a lot of time and costs. Moreover, the most serious problem is that these methods can not be applied to fibrils derived from human body. To accumulate molecular insight into structural variation of amyloid fibrils and its pathological significance efficiently, a method which can readily analyze fibril structure covering a wide range of samples is necessary.

1-6. Existing molecular probes for amyloid fibrils

Extrinsic molecular probes are very valuable for detection, diagnosis, and examining kinetics of amyloid fibril formation. Many probes such as ANS have been reported and among them, thioflavin T (ThT) and Congo Red (CR) are frequently used⁴⁰. CR has been used for identification of amyloid fibrils since the 1920s. Upon binding to amyloid fibrils, CR exhibits a characteristic apple green birefringence, and a remarkable shift of absorbance maximum from 490 nm to 540 nm is observed⁴¹. CR is mainly applied for *in vitro* diagnosis of amyloid fibrils in tissue sections using polarization microscopy. But it lacks high sensitivity and is not a good probe for *in vivo* detection in a complex body

system because it may interact with proteins in native and partially folded states and effects of either inhibition and acceleration of fibril formation are reported for some proteins⁴²⁻⁴³.

ThT was firstly reported in 1959 as a fluorescent dye that exhibits enhanced fluorescence upon binding to amyloid fibrils in tissue sections⁴⁴. When ThT is in non-binding state, it emits little fluorescence, and upon interaction with amyloid fibrils, ThT fluorescence is considerably enhanced with excitation and emission maxima at about 450 nm and 480 nm, respectively⁴⁵. It possesses higher sensitivity at low amyloid concentration than CR. In addition, effects of ThT on fibril formation are very limited⁴⁶⁻⁴⁷. Thus ThT has been used for many application such as direct observation of amyloid fibrils growth with total internal reflection fluorescence microscopy⁴¹ and diagnosis of fibrils in tissue sections under appropriate conditions⁴⁴. The fluorescence intensity of ThT is proportional to the weight concentration of amyloid fibrils⁴⁸⁻⁴⁹ and is not independent on the number or length distribution of fibrils⁴⁹, so that by using ThT we can trace whole reaction of fibril formation as shown in Figure 1-2 and examine the fibrillation kinetics.

Though the binding modes of ThT and CR to amyloid fibrils have extensively been studied, it is not fully understood because of a lack of the binding model with high resolution due to heterogeneity of amyloid fibril samples. When ThT and CR are in bound states, they exhibit anisotropy, indicating that they orient along to a long axis of a fibril⁵⁰. About ThT, it is suggested that binding site of ThT is in a cavity with a diameter of 8-9 Å on surface of cross-β structure⁵¹. As for CR, plausible binding locations are grooves formed by β-strands along the side of the β-sheet extension direction⁴⁰. A combination of hydrophobic interactions and specific interactions such as salt bridges contributes to the binding. The shift of absorbance maximum may be derived from formation of a more conjugate system made of CR molecules⁴⁰. Besides CR and ThT, several molecules which are derivatives of both CR and ThT have been designed⁵²⁻⁵³. For example, Pittsburgh compound-B (PiB) which is a derivative of ThT has a high affinity toward amyloids due to a modification of benzothiazole group neutral⁵⁴. Radioactively labeled PiB is already applied to living human for non-invasive diagnosis and can provide quantitative information on amyloid deposits⁵⁵.

ThT, CR, and other existing probes are suffice for detection or examining kinetics.

However, structural information at a molecular level obtained by existing probes is quite poor. For example, ThT binds to not only amyloid fibrils but also amorphous aggregates, suggesting that specificity of ThT is not so high and recognize a wide range of structures⁵⁶. Although fluorescence intensity of ThT changes depending on types of fibrils, fluorescence spectral shapes are very similar despite of a variety of fibril structures and the correlation between fluorescence intensity and fibril structure is unclear. The same is true with respect to the CR probe, and thus they are inadequate for discussing polymorphism of amyloid fibrils. A new probe which can provide structural information at a molecular level about fibril structures is necessary.

1-7. Iodine staining

In this study, we focus on iodine staining as a new molecular probe for amyloid fibril structures. Iodine staining of amyloid fibrils in vivo was firstly reported by Virchow in 1850s⁵⁷⁻⁵⁸. Because it was reminiscent of the starch-iodine reaction, the effect was attributed to the presence of a “carbohydrate moiety” by mistake and, unfortunately, a mechanism underlying color reaction between amyloid fibrils and iodine molecules have hardly been studied and details of the mechanism are still unknown. On the other hand, there are many reports about iodine-stained polysaccharide and polymers such as amylose⁵⁹, polyvinylalcohol⁶⁰, and nylon-6⁶¹. About starch including amylose, it was reported that absorption spectra of iodine-stained amylose significantly change depending on degree of polymerization and other structural properties^{59, 62-63}. It has been shown in many reports that the polyiodides I_n^- ($n=3, 5, 9\dots$) in the iodine-amylose complex are occluded in the helical cavity of the amylose and arranged in a linear array, parallel to helical axis^{62, 64-67}.

As for synthetic polymers, poly(vinyl acetate) (PVA), poly(vinylpyrrolidone) (PVP), nylon-6, and polyaniline are reported to form complex with iodine molecules^{60, 68-70}. Because iodine-doped polymers can be applied to polarizer or conductor⁷¹⁻⁷², these complexes have been extensively studied by using resonance Raman spectroscopy⁷³, Mossbauer spectroscopy⁷⁴, and X-ray diffraction⁷⁵. About nylon-6 doped with iodine molecules, I_5^- ions thought to be in form of $H^+ I_5^-$ interact strongly with nylon-6 chains, whereas I_3^- ions are thought to be in form of $K^+ I_3^-$ are weakly bound to nylon chains⁶¹. The species I_3^- are thought to exist in the lamellar crystalline of the polymer and easily

desorb, whereas the I_5^- species exist in the lattice formed between I_5^- ions columns and nylon-6 chains. In addition, a wealth of research about other polymers stained with iodine accumulates.

Indeed, Dzwolak reported iodine staining may respond to surface structures of amyloid fibrils⁷⁶. Thus it is expected that by using wealth of knowledge, iodine staining can be a powerful tool for structural analysis of amyloid fibrils.

1-8. Amyloidogenic proteins and peptides used in this work

Insulin is a hormone protein associated with glucose metabolism and consist of two polypeptide chains (ie, A chain of 21 amino acid residues and B chain of 30 amino acid residues) linked by two intermolecular disulfide bonds (Figure 1-5). Insulin is one of the best model for amyloid research because it readily forms amyloid in vitro⁷⁷. In addition, there are a number of reports about polymorphism of insulin amyloid fibrils⁷⁸⁻⁷⁹. For example, Zako et al. reported that by adding reductive TCEP, insulin forms noodle-like amyloid fibrils with low cytotoxicity instead of needle-like fibrils with high cytotoxicity⁷⁸.

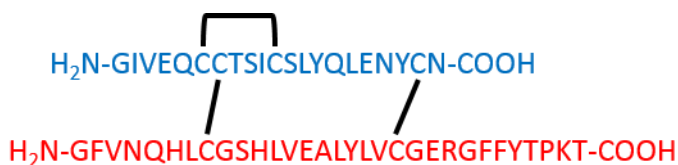


Figure 1-5. Amino acid sequence of human insulin hormone peptide. A chain and B chain are represented in blue and red, respectively. Three disulfide bonds between cysteine residues are represented in black line.

Insulin B chain

It is reported that each polypeptide chain contained in insulin can also form amyloid fibrils⁸⁰. Especially for B chain, fibrillation reaction proceeds with a characteristic feature. As shown in Figure 1-2, prefibrillar oligomers are generally unstable so that the population of them is quite low. However, for B chain, an accumulation of oligomers is observed at a specific condition (unpublished data; see Results in chapter 3). Thus the B chain of insulin is a promising model for understanding a mechanism of structural transition from oligomers to fibrils.

Amyloid β ($A\beta$)

$A\beta$ peptide is proteolytic product of amyloid precursor protein and there are several types of $A\beta$ peptide with different number of residues from 39 to 43⁸¹. Among them, $A\beta_{1-40}$ and $A\beta_{1-42}$ have extensively been studied because they are main components of deposits in AD patient's brain⁸². In this study, we use $A\beta_{1-40}$ (Figure 1-6) of which there are several reports studying polymorphic structures⁸³.

H₂N-DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV-COOH

Figure 1-6. Amino acid sequence of $A\beta_{1-40}$ peptide.

Lysozyme

Lysozyme is hydrolytic enzyme reacting with peptidoglycans in cell walls of bacteria and consist of 129 amino acid residues (Figure 1-7). It is reported that variants of hen egg white lysozyme are unstable and tend to form amyloid fibrils⁸⁴ and lysozyme have been extensively studied as a model of amyloidogenic protein in vitro⁸⁵⁻⁸⁶. Wild type of lysozyme can form amyloid fibrils under acidic condition at high temperature⁸⁷.

H₂N-KVFGR CELAA AMKRH GLDNY RGYSL GNWVC AAKFE SNFNT QATNR NTDGS
TDYGI LQINS RWWCN DGRTP GSRNL CNIPC SALLS SDITA SVNCA KKIYS
DGNGM NAWVA WRNRC KGTDV QAWIR GCRL-COOH

Figure 1-7. Amino acid sequence of hen egg white lysozyme.

β 2-microglobulin (β 2m)

β 2m is an amyloidogenic protein associated with dialysis-related amyloidosis (Table 1-1). Native β 2m consists of 99 amino acid residues (Figure 1-8) and is a component of type-I major histocompatibility antigen. In blood, the concentration of β 2m increase for a long period, a risk of amyloidosis significantly become high. Though a molecular mechanism of β 2m fibrillation remain unknown, recent studies reported that minimal sequence essential for amyloid formation was identified and physicochemical studies for addressing the mechanism of fibril formation in the context of protein conformation were performed⁸⁸⁻⁹⁰.

H₂N-MIQ RTP KIQVY SRHPA ENGKS NFLNC YVSGF HPSDI EVDLL KNGER IEKVE HSDLS
FSKDW SFYLL YYTEF TPTEK DEYAC RVNHV TLSQP KIYKW DRDM-COOH

Figure 1-8. Amino acid sequence of β 2m protein.

1-9. Scope and outline of this work

In this work, we tried to verify the utility of iodine staining as a new probe for amyloid fibril structures. We focused on an ability of structural recognition of iodine staining for amyloid fibrils, which is described in Chapter 2. Color reactions between iodine molecules and fibrils change depending on types of fibrils, indicating that iodine staining can distinguish polymorphism of amyloid fibrils. Then, we discuss the molecular mechanism of iodine staining and propose a model of iodine binding to amyloid fibrils. In Chapter 3, we discuss the utility of iodine staining for medical diagnosis. First, we investigated whether iodine staining can detect prefibrillar intermediates with low population. Second, we investigated the correlation between colors of fibrils stained with iodine and extent of their cytotoxicity. In chapter 4, we discuss the utility of iodine staining.

Chapter 2.

Utilizing iodine staining for distinguishing nanostructures of amyloid fibrils

2-1. Introduction

Amyloid fibrils are protein aggregates associated with many neurodegenerative diseases including Alzheimer's and Parkinson's diseases¹. Though amyloid fibrils have common structural features such as unbranched fibrillar morphology and cross- β structures, recent researches have revealed that amyloid fibrils exhibit structural variations at a molecular level^{22, 34-35, 37}. These polymorphic properties attract remarkable attentions for understanding molecular mechanism of amyloid diseases and developing curative treatments. However, reported instances of structural elucidation at a molecular level are still limited because of difficulties in sample preparations. In many cases, amyloid fibrils are heterogeneous and are not suitable for spectroscopic methods such as ssNMR and EPR, which are used for structural elucidation today.

To solve this problem, we tried to develop a new extrinsic molecular probe and focused on iodine staining⁵⁷⁻⁵⁸. In this chapter, we show iodine staining can recognize structural differences of amyloid fibrils and can be applied to a wide range of amyloid samples which are not suitable for existing methods for structural analysis as those mentioned above. Firstly, we present basic properties of iodine staining of amyloid fibrils. Iodine molecules can bind to a wide range of amyloid fibrils and can distinguish polymorphic properties of insulin and A β ₁₋₄₀ fibrils. Colors of iodine-stained amyloid fibrils clearly change depending on types of amyloid fibrils even if they are made from same protein. Absorption spectral shape and amplitude of iodine-stained fibrils in visible region depends on the fibril structures and the amount of fibrils, indicating iodine staining can be used for both qualitative and quantitative analysis of amyloid fibrils. Further titration experiments and spectral deconvolution reveal there are at least three types of iodine-binding sites on the surface of amyloid fibrils. Besides, resonance Raman spectroscopy suggest that a few types of iodine complexes exist as a fibril-bound form. These differences in binding sites and types of iodine molecules may be derived from surface properties of fibrils characterized by amino acid residues which are exposed to solvent, and we tried to identify the amino acid by modifying them with chemical methods. Binding patterns of iodine molecules to amyloid fibrils are highly controlled and iodine molecules orient and polymerize along to long axis of fibrils, which are suggested by observation of spherulites with polarized microscopy.

2-2. Materials and methods

Reagents

Recombinant human insulin was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Concentrations of insulin dissolved in 25 mM HCl were determined using an absorption coefficient of 1.08 for 1.0 mg/ml at 276 nm⁹¹ and we prepared insulin solution at a concentration of 10.0 mg/ml as a stock solution. A β ₁₋₄₀ peptide powder was purchased from PPEPTIDE INSTITUTE, INC (Osaka, Japan). Iodine, potassium iodide, trifluoroacetic acid (TFA), NaOH, Na₂HPO₄, NaH₂PO₄, α -cyclodextrin, thioflavin T (ThT), dithiothreitol (DTT), bovine serum albumin (BSA), lysozyme, from Egg White, chloroform, dimethyl sulfoxide (DMSO), polyoxyethylene(10) octylphenyl ether (Triton-X100), ascorbic acid (AsA), and V8 protease were also purchased from Wako Pure Chemical Industries, Ltd. HCl solution at a concentration of 100 mM and 1 M, 28 % (v/v) NH₃ aq, NaCl, SDS, glycine (Gly), Tris, acetonitrile (ACN), NaHCO₃, and NH₄HCO₃ were purchased from NACALAI TESQUE, INC (Kyoto, Japan). Calcein and HEPES were purchased from DOJINDO LABORATORIES (Kumamoto, Japan). *N*-succinimidyl acetate, *N*-acetylimidazole, and phenylglyoxal (PG) were purchased from Tokyo Chemical Industry CO., Ltd (Tokyo, Japan). 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-[phosphor-rac-(1-glycol)] (POPG) were purchased from NOF CORPORATION (Tokyo, Japan).

Formation of insulin and A β ₁₋₄₀ amyloid fibrils

Spontaneous formation of amyloid fibrils was performed by heating under acidic conditions. Human insulin dissolved in 25 mM HCl at a concentration of 2.0 mg/ml was used as a sample. Fibrillation reaction was performed by incubating this solution at 65 °C for 24 hours. For inducing polymorphism of amyloid fibrils, two types of additives, 100 mM NaCl or 100 μ M SDS were used in addition to the non-additive solvent condition. After the formation of amyloid fibrils, which were referred to as parent fibrils, seed-dependent fibril formation was performed. For fragmentation, the parent fibrils were subjected to pulsed sonication using XL2000 sonicator (Misonix, San Diego, California, USA) operating for 1 sec with power level at 2.0 W. Sonication was performed in intervals to ensure that amyloid samples are not excessively heated and total time of sonication

was 40 sec. Then the fragments of fibrils were added as seeds to 2.0 mg/ml insulin dissolved in 25 mM HCl at a final concentration of 5 %. It should be noted that the seed-dependent fibrillation reaction was performed in the same solvent condition without any additives. The samples were incubated at 37 °C for 24 hours to obtain daughter fibrils. In the case of granddaughter fibrils, seeds were derived from daughter fibrils and the condition of fibril formation was the same as that used for the formation of the daughter fibrils.

For the formation of A β ₁₋₄₀ amyloid fibrils, lyophilized powder of A β ₁₋₄₀ peptide was initially dissolved in 0.05 % (v/v) NH₃ aq at a concentration of 1 mM. By diluting this A β ₁₋₄₀ solution, A β ₁₋₄₀ at 50 μ M in 50 mM phosphate buffer (pH 7.5) containing 100 mM NaCl was prepared. According to previous findings which demonstrate that agitation is an effective factor to induce polymorphism of A β ₁₋₄₀ amyloid fibrils³⁴, sample solution was incubated at 37 °C under two different conditions; i.e., with or without agitation (1,300 rpm) for 100 hours to obtain two different types of polymorphism. For the condition of agitation, we used Thermomixer C (Eppendorf, Deutschland). Two types of parent fibrils were sonicated as the same procedure for insulin fibrils to obtain seeds. For both types of the parent fibrils, seeding reaction was performed at a concentration of 50 μ M A β containing 5 % of seed in 50 mM phosphate buffer (pH 7.5) with quiescent condition at 37 °C for 100 hours to get two types of daughter fibrils. After the formation of A β ₁₋₄₀ amyloid fibrils, the solution was centrifuged at 12,000 g for 20 min to precipitate the fibrils. After removing supernatant, the A β ₁₋₄₀ fibrils were resuspended by a small volume of 25 mM HCl for solvent exchange. Though there must have been some loss of A β peptide through the preparation procedure, we assumed that a final concentration of A β ₁₋₄₀ fibrils was ideally 83 μ M based on the assumption that all of the A β peptide could be collected.

Formation of lysozyme and β 2m amyloid fibrils

For fibrillation of lysozyme, lysozyme was dissolved in 25 mM HCl at a concentration of 14.7 mg/ml (1 mM) and then pH of the solution was adjusted to 2.0 by 100 mM HCl⁸⁷. The solution was incubated at 65 °C for 48 h.

For fibrillation of β 2m, β 2m was dissolved in water and the concentration of it was determined using an absorption coefficient of $\epsilon_{280}=19,300 \text{ cm}^{-1}\text{M}^{-1,92}$. Then the solution

was diluted to 0.3 mg/ml in 3.2 mM HCl containing 40 mM NaCl. Fibril formation of this sample was performed under agitated condition with 1,200 rpm at 37 °C for 120 h.

ThT assay for insulin and A β ₁₋₄₀ amyloid fibrils

The ThT assay was performed according to the method of Naiki et al.⁴⁵ with a slight modification of solution; 4.5 μ l aliquots of sample solution was mixed with 1.5 ml of 5 μ M ThT in 50 mM Gly-NaOH buffer (pH 8.5) at room temperature. After 1 min for equilibrium, fluorescence intensity at 485 nm was measured with an excitation wavelength of 445 nm to assay the fibril formation for insulin amyloid fibrils with RF-5300 PC (Shimadzu CO., Kyoto, Japan). For A β ₁₋₄₀ amyloid fibrils, fluorescence intensity at 482 nm was measured by exciting with 450 nm and. Time course monitoring of the fibril growth was conducted in a same way as that mentioned above and measurements were performed every one hour.

Atomic force microscopy (AFM)

Samples of insulin amyloid fibrils were diluted 250 times with 25 mM HCl. 10 μ l aliquots of diluted sample was deposited onto a freshly cleaved, dry mica surface. In the case of the amyloid A β ₁₋₄₀ fibrils, undiluted solution was deposited onto the mica. The solution was adsorbed for 1min and then the mica surface was washed once with 100 μ l of water. The solution was blotted off by tissue paper and the mica plate was air-dried. AFM images were obtained using SPA-400 and nano navi (Hitachi High-Tech Science, Tokyo, Japan). The scanning tip was a OMCL-AC160TS-C3 micro cantilever (OLYMPUS, Tokyo, Japan; spring constant = 21-37 N/m, resonance frequency = 270-340 kHz), and the scan rate was 1.0 Hz.

Attenuated total reflection Fourier transform infrared (ATR-FTIR) absorption measurement

ATR-FTIR spectra were measured with a FT/IR-6100 (JASCO Corporation, Tokyo, Japan) at room temperature. 2 μ l of sample solution was blotted onto a Ge prism and then dried with dry air for removing water molecules. The number of scan, spectral resolution, and a range of spectral measurement were set to be 128 times, 4 cm⁻¹ and from 4000 to 600 cm⁻¹, respectively. As a control, we measured spectrum of air in a room which contain

CO₂ and H₂O and used it for suppressing the contribution of H₂O molecules to spectral shape of fibril sample.

Circular dichroism (CD) spectroscopy

For CD measurements, fibril solution at a concentration of 0.25 mg/ml in 25 mM HCl was prepared. CD spectra were measured with a quartz cell with 1 mm optical length and a J-1100 CD Spectrometer (JASCO Corporation, Tokyo, Japan) at 25 °C. A range of spectra, number of scan, attenuation, and spectral resolution were set to be from 250 to 190 nm, 8 times, 20 mdeg, and 0.5 nm, respectively.

Iodine staining of amyloid fibrils

Iodine and potassium iodide were dissolved in water at final concentrations of 24 mM KI and 3.2 mM I₂. In fact, several iodine molecular species, I⁻, I₂, and I₃⁻ are in equilibrium in the solution so that concentrations represented above are do not represent the actual concentrations of these species inside the solution. It was also assumed that equilibrium constants among iodine molecules should change by perturbations such as pH, temperature, and binding to amyloid fibrils. Thus we use the values of KI and I₂ used for the preparation of the iodine solution for sake of simplicity in this study.

For insulin amyloid fibrils, fibril solution was mixed with the iodine solution at concentrations of 0.25 mg/ml insulin (or fibrils), 0.3 mM KI, and 0.04 mM I₂ in 25 mM HCl. The reaction between fibrils and iodine molecules immediately finished within 1 min. On the other hand, colour of iodine tended to be faded by light so that we performed spectral measurements within 10 min after preparing the samples. UV-vis spectra were measured with a quartz cell with 1 cm optical length and UV-2400PC (SHIMADZU, Kyoto, Japan) or Jasco V-650 spectrometer (JASCO Corporation, Tokyo, Japan) at room temperature. A range of spectra was from 750 to 250 nm and spectral resolution was set to be 0.5 nm. As controls, fibril 0.25 mg/ml fibril solution without iodine, native insulin solution mixed with iodine, and iodine solution, all of which were in 25 mM HCl, were also measured.

For seeding-dependent fibrillation experiments, spectral measurements were performed every one hour under same measurement conditions as those mentioned above.

For titration experiments, an iodine solution containing 18 mM KI and 2.4 mM I₂ in 25 mM HCl was prepared. First, 1 ml of insulin fibril solution at a concentration of 0.5 mg/ml in 25 mM HCl was prepared and its UV-vis absorption spectrum was measured as a baseline. Next, 4 µl of iodine solution was added. After mixing a sample with a micropipette, UV-vis spectrum was measured within 10 min. A series of operations were repeated until absorbance from 750 to 400 nm saturated.

For Aβ amyloid fibrils, sample solution was mixed with iodine solution at final concentrations of 80 µM Aβ, 0.04 mM I₂, and 0.3 mM KI in 25 mM HCl. For lysozyme amyloid fibrils, sample solution was mixed with iodine at final concentrations of 125 µM lysozyme 0.04 mM I₂, and 0.3 mM KI in 25 mM HCl. For β2m, the sample solution was mixed with iodine solution at final concentrations of 0.15 mg/ml β2m, 20 mM NaCl, 0.064 mM I₂, 0.48 mM KI in 1.6 mM HCl.

Verifying effects of residual salts on iodine staining

Insulin fibril formation with the non-additive condition was performed in the same way as those mentioned above. An aliquot of the sample was stained with iodine and the remaining solution was subjected to pulsed sonication for 1 sec with power level at 2.0 W. The total time of sonication was 20 sec. Then, NaCl or SDS salt was added to the sample at final concentration of 5 mM or 5 µM, respectively. After incubation for 24 h at 37 °C, the sample was stained with iodine and subjected to UV-vis spectroscopy.

Reduction of iodine molecules bound to amyloid fibrils

Iodine-stained amyloid fibril samples were prepared by the same procedure as those mentioned above. In addition, daughter fibrils were formed in the presence of 2.4 mM KI and 0.32 mM I₂. After the formation of fibrils, fibril solution was diluted 8 fold with 25 mM HCl at final concentrations of 0.25 mg/ml fibril, 0.3 mM KI, and 0.04 mM and we performed UV-vis measurement of it. Then, both fibril samples were mixed with small aliquots of AsA solution dissolved in water at a final concentration of 30 µM. The reaction between iodine molecules and ascorbic acid molecules finished within 1min and we measured UV-vis spectra. Among all measurements, the parameters were set to be the same as those mentioned above.

Resonance Raman measurements

Two types of excitation wavelength and spectrometer were used in this study. For 514.49 nm excitation, HR-800-LWR (HORIBA, Kyoto, Japan) was used and for 785 nm excitation, inVia Raman microscope (Renishaw, UK). Both instruments were equipped with a filter for suppressing Rayleigh scattering. Insulin amyloid fibril solution was stained with iodine at concentrations of 0.5 mg/ml fibrils, 0.6 mM KI and 0.08 mM I₂ in 25 mM HCl. Sample solution was taken in a glass calibrated pipet (Drummond Scientific Company, USA) with its inner diameter of 1.13 mm. An excitation light was focused on the interface between the sample solution and a wall surface of the pipet to avoid an attenuation of Raman scattering by self-absorption of the sample solution. Raman scattering was measured with 90° scattering mode from 0 to 500 cm⁻¹ at room temperature. The number of scan was 30 for HR-800-LWR and 10 for inVia Raman microscope. Spectral resolution was set to be 0.17 cm⁻¹. For both instruments, laser power was set to be 300 mW. As controls, native insulin solution mixed with iodine, iodine solution, native insulin solution, and fibril solution, all of the which were dissolved in 25 mM HCl were also measured.

Polarization microscopic observation of spherulites

To prepare spherulites, insulin solution at a concentration of 5 mg/ml in 25 mM HCl was dropped on a glass plate which was sealed with a cover glass, heated to 70 °C and incubated for 2 hours, according to the method reported by Rogers et al⁹³⁻⁹⁴. The spherulite formed was then stained with iodine by adding a small amount of iodine solution containing 24 mM KI and 3.2 mM I₂ in water and observed with a microscopy of ECLIPSE LC100OPL (NIKON CORPORATION, Tokyo, Japan) with a polarizer placed in the optical pathway between the objective lens and the digital camera. To investigate the polarization property of the sample, the polarizer rotated counterclockwise 30 degrees in a stepwise fashion and images were taken with a DS-L3 digital camera (NIKON CORPORATION, Tokyo, Japan). As controls, images of unstained spherulites were also obtained by the same procedures.

Chemical modification of amyloid fibrils

In this study, we focused on three types of amino acid residues: lysine (Lys), tyrosine (Tyr), and arginine (Arg) in the insulin protein and modified their side chains respectively with chemical methods. For modification of Lys, we used *N*-succinimidyl acetate to introduce an acetyl group at an amino group of the side chain (Figure 2-1A). Insulin have one Lys residue and two amino groups at N-terminus (Figure 1-5). Fibril sample which was formed in 25 mM HCl was suspended in 100 mM phosphate buffer (pH 6.0) at final concentrations of 1.0 mg/ml (176 μ M) fibrils and 12.5 mM HCl. Then, *N*-succinimidyl acetate was added to a final concentration of 3 mM⁹⁵ and the sample solution was incubated at 25 °C for 1 h.

For modification of Tyr, *N*-acetylimidazole was used to introduce an acetyl group at a phenyl group of Tyr residue (Figure 2-1B). There are four Tyr residues in insulin protein. Fibrils were suspended in 100 mM Tris-HCl (pH 7.5) at final concentrations of 1.0 mg/ml fibrils and 12.5 mM HCl. *N*-acetylimidazole was added to a final concentration of 40 mM

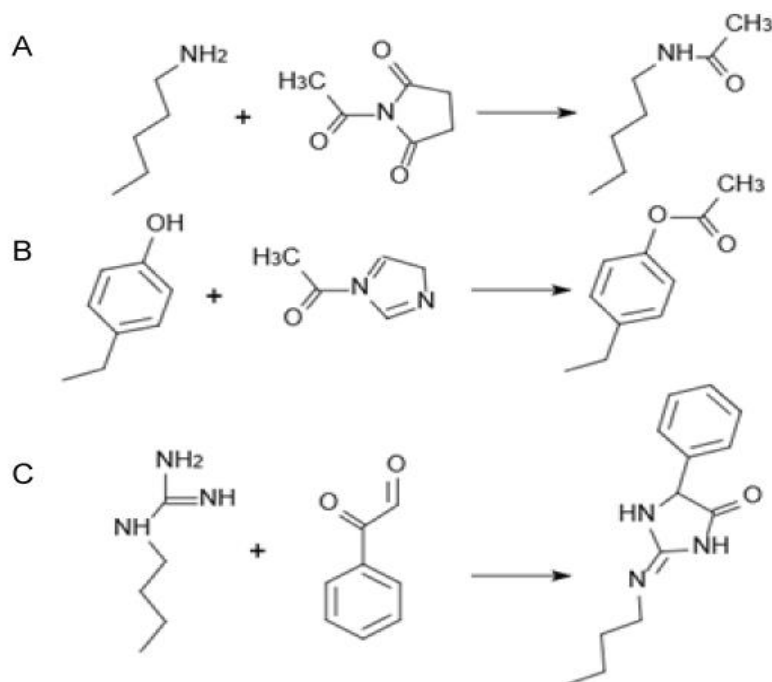


Figure 2-1. Reaction scheme between amino acids and modification reagents. (A) Modification scheme of lysine with *N*-succinimidyl acetate. (B) Modification scheme of tyrosine with *N*-acetylimidazole. (C) Modification scheme of arginine with phenylglyoxal (PG).

and the sample solution was incubated at 25 °C for 1 h⁹⁶.

For modification of Arg, PG was used to introduce it at a guanidine group of Arg residue (Figure 2-1C). There are one Arg residue in insulin protein. Fibrils were resuspended in 125 mM NaHCO₃ buffer (pH 8.6) at final concentrations of 1.0 mg/ml insulin and 12.5 mM HCl. Then, PG was added to a final concentration of 6 mM and the sample solution was agitated with 1,000 rpm at 37 °C for 20 h⁹⁷.

It is noted that all of the amino acid modification reagents were initially dissolved in DMSO, and a final concentration in each sample solution is less than 5 % (v/v). It is reported that DMSO with high concentration can dissolved amyloid fibrils⁹⁸, but under those conditions mentioned above, the dissociation of fibrils do not occur.

After reaching the reactions in equilibrium, all samples were centrifuged with 12,000 g for 30 min to precipitate fibrils, and then supernatant was removed. Pellets of fibrils were resuspended in 25 mM HCl to a final concentration of 1 mg/ml. This value is based on the assumption that all fibrils are completely collected. These samples were subjected to the CD spectroscopy, iodine staining, and UV-vis spectroscopy in the same ways as those mentioned above.

Purification of insulin with amino acid modification by using high pressure liquid chromatography (HPLC)

After the chemical modification as mentioned above, 1,000 µl of fibril samples suspended in 25 mM HCl were centrifuged again with 12,000 g for 30 min to precipitate fibrils, and then supernatant was removed. Pellets of precipitated fibrils were dissolved in 100 µl of 1M NaOH to dissociate fibrils into monomeric state. The solution was diluted with water containing 0.1 % TFA at a final concentration of 200 mM NaOH. To remove insoluble aggregates, the sample solution was filtered with Millex Syringe-driven Filter Unit (Merck Millipore, Germany) and then it was subjected to HPLC.

Purification of insulin with modification was performed by using HP Agilent 1100 Series HPLC (Agilent technologies, USA) and reversed-phase C18 COSMOSIL Protein R HPCL columns (250 mm × 4.6 mm i.d., average particle size of 5 µm, NACALAI TESQUE, INC, Kyoto, Japan) with mobile phase consisted of water/ACN mixed-solvent system containing 0.1 % TFA at a flow rate of 1 ml/min. Separation of modified insulin

was performed with a gradient of 0-50 % ACN in 30 min (Table 2-1) and detection of insulin was conducted based on absorption at 220 and 276 nm. The column eluent was collected and lyophilized with FDU-2200 (TOKYO RIKAKIKI CO. LTD, Tokyo, Japan) and vacuum pump GLD-051 (ULVAC Kiko, Inc. Miyazaki, Japan) after freezing the eluent with liquid nitrogen.

Time (min)	% (ACN)
0	0
5	30
25	50
25.1	80
30	80
30.1	0
35	0

Table 2-1. Time course of ACN percentage in the mobile phase used for purification of modified insulin. According to previous results, monomeric insulin was eluted from 30 to 40 % of ACN and thus we focused on region from 30 to 50 % of ACN and used linear gradient in that region.

Digestion of modified insulin with V8 protease

The lyophilized powder of modified insulin was dissolved in 100 mM NH_4HCO_3 (pH 7.8). Concentration of insulin in each sample was determined using an absorption coefficient of 1.08 at 276 nm. It should be noted that as for the PG modification, PG have an absorption in UV region and we could not determine the concentration of insulin. The solution was mixed with V8 protease solution at final concentrations of 176 μM (1 mg/ml) insulin and 5 μM V8 protease in 100 mM NH_4HCO_3 . The sample was incubated at 37 °C for 20 h. After the digestion, DTT dissolved in water was added at a final concentration of 2 mM and incubated at 37 °C for 2 h.

After reducing the samples, desalting of those were performed by using ZipTip pipette tips (Merck Millipore, Germany) for following electrospray mass spectrometry (ESI-MS). 10 μl of the samples were aspirated and dispensed with 10 cycles for maximum binding of peptide fragments to resin. The peptides bound to the resin were washed with water containing 0.1 % TFA with 5 cycles, and then eluted with 80 % ACN solution which also contain 0.1 % TFA.

Liquied chromatography/Mass Spectrometry (LC/MS)

LC/MS was performed on an LTQ-Orbitrap Discovery mass spectrometer (ThermoFisher Scientific, Bremen, Germany), which were connected to a Dionex UltiMate 3000 pump (Germering, Germany) and an HTC-PAL autosampler (CTC Analytics, Zwingen, Switzerland). The mobile phase consisted of 0.1 % formic acid in water (solvent A) and 0.1 % formic acid in acetonitrile (solvent B). The peptides were fractionated on an L-column Micro C-18 (150 mm length $\times$ 100 μ m i.d., particle size of 3 μ m, CERI, Tokyo, Japan) with a linear gradient of 3-43 % solvent B in 40 min at a flow rate of 500 nL/min. The column eluent sprayed directly into an ion source of the mass spectrometer by using a spray voltages of 1.8 kV. A lock mass function was used to obtain high mass accuracy during the gradient.

The mass spectra were recorded in a range of m/z 300-1500. In mass spectrometry /mass spectrometry (MS/MS), the top seven high-intensity precursor ions on each mass spectrum of eluents were selected automatically for subsequent product ion analysis by a data-dependent scan mode with a dynamic exclusion option.

For identification of fragments of the human insulin, MS/MS data were interpreted through the MASCOT MS/MS ions search (Matrix Science, London, United Kingdom). Peptides were identified with a peptide mass tolerance of 2 ppm and a fragment m/z tolerance of 0.8 Da. "None" was selected as the enzyme used.

All of the experiments using LC/MS were performed by Associate Professor Ken-ichi Yoshino (Biosignal Research Center, Kobe University).

2-3. Results

Formation of insulin amyloid fibrils in the absence and presence of additives

First, we confirmed the formation of insulin amyloid fibrils and investigated polymorphism of prepared fibrils by monitoring intensity of ThT fluorescence. Earlier studies revealed that insulin amyloid fibrils are formed spontaneously at high temperature under acidic conditions⁹⁹⁻¹⁰¹ and insulin amyloid fibrils often exhibit polymorphism induced by solvent conditions such as pH, salts, and other additives⁷⁸. In this study, to obtain polymorphic amyloid fibrils, insulin was dissolved in 25 mM HCl and effects of two types of additives, NaCl and SDS, were examined. After incubation for 24 hours at 65 °C, amyloid fibrils were formed under all solvent conditions as confirmed by ThT fluorescence and these fibrils exhibited different fluorescence intensity (Figure 2-2A). It is reported that an affinity and stoichiometry between ThT and fibrils may change depending on fibril structures⁴⁰, implying the prepared fibrils are polymorphic. However, the possibility that these different ThT fluorescence intensity is ascribed to different degree of contamination of non-fibrillar aggregates such as amorphous aggregates cannot be excluded. To examine this, we performed seeding reaction, utilizing an ability of

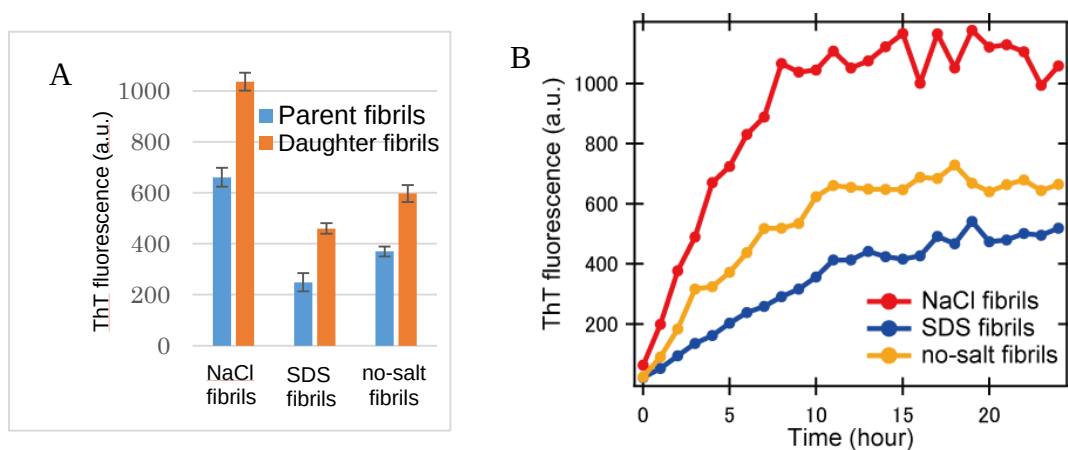


Figure 2-2. Confirming insulin amyloid fibril formation by using ThT assay. (A) ThT fluorescence intensity at 485 nm for each prepared fibrils. Blue and orange bars represent fluorescence intensity for three types of parent and daughter fibrils, respectively. All measurements were performed three times and the error bars depict the S.D. (B) Time course measurements of seed-dependent fibril growth. Color codes are defined in the legends, specifying types of prepared amyloid fibrils. Calculated values of τ^{-1} , which means time constants of growth reactions, for NaCl, SDS, and no-salt fibrils are 0.23 ± 0.02 , 0.09 ± 0.01 , and 0.18 ± 0.01 , respectively.

structural propagation of amyloid fibrils. It should be noted that the seeding reaction was performed in the absence of any additives, only with a small amount of contamination of NaCl (5 mM) or SDS (5 μ M) coming from the solution of parent fibrils. Daughter fibrils were formed in the presence of fragmented parent fibrils as seeds at lower temperature (37 °C) to make seeding reaction dominant. After seeding reaction, the daughter fibrils exhibited higher fluorescence intensity than that of the parent fibrils (Figure 2-2A), indicating that the parent fibrils contained a fraction of non-fibrillar aggregates which did not self-propagate in the seeding reaction. Nevertheless daughter amyloid fibrils still exhibited different fluorescence intensity and especially, fibrils induced by NaCl exhibited clearly higher fluorescence intensity than that of other fibril. From the point of view of growth rate, there are slight differences among fibrils (Figure 2-2B). The growth reaction of seeds induced by NaCl salts saturated in a shorter period of time ($\sim$ 9 h) than those of other two types of fibrils ($\sim$ 11 h). Time constants of each growth reaction were calculated by fitting them with single exponential equation (1) (see Figure 2-2 legends), supporting the difference of reaction rate among each fibril growth.

$$f(x) = y_0 + A\exp(-\tau^{-1}x) \quad (1)$$

These results suggest that we could obtain at least two types of polymorphism. We refer to these amyloid fibrils generated under the conditions in the absence and presence of NaCl and SDS as no-salt fibrils, NaCl fibrils, and SDS fibrils, respectively. Structural information about fibrils obtained by ThT assay is very limited, so we next tried to confirm polymorphism by using other methods.

Morphology and cross- β structure of amyloid fibrils generated under different additive conditions

To examine the morphological differences of fibrils, the three types of amyloid fibrils were subjected to AFM. As a result, AFM images verified needle-like morphology (Figure 2-3, A-C). Furthermore, NaCl fibrils were thicker ($\sim$ 13 nm) compared to SDS ($\sim$ 4.5 nm) and no-salt fibrils ($\sim$ 3nm), supporting the formation of polymorphic amyloid fibrils.

When ATR-FTIR spectra in amide I region were investigated to obtain information about secondary structures (Figure 2-3D, upper panel), all fibrils exhibited similar

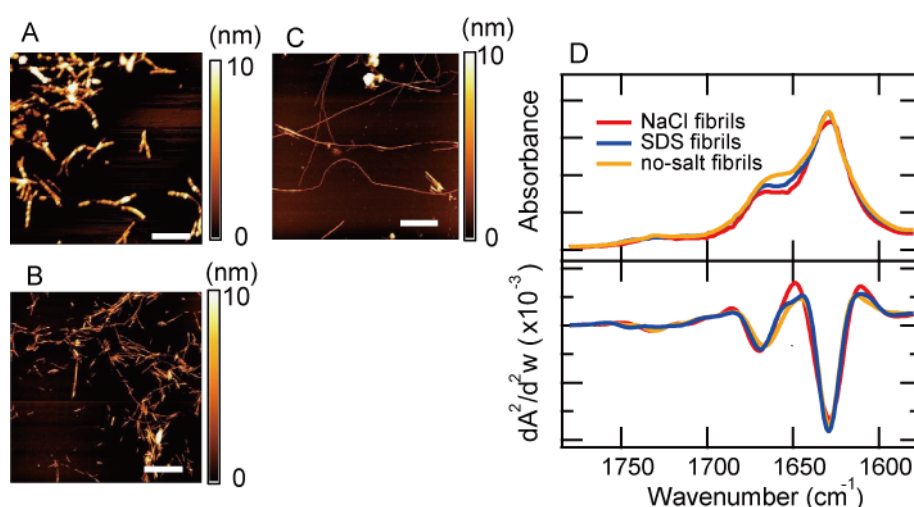


Figure 2-3. Polymorphism of prepared fibrils confirmed by AFM and ATR-FTIR. (A-C) AFM images of NaCl fibrils (A), SDS fibrils (B), and no-salt fibrils (C). The white scale bars in images represent 1 μm . The scale bars on the right represent the height of the sample. The lighter the color becomes, the higher the sample is from the surface of mica plate. (D) ATR-FTIR absorption spectra (upper panel) and second derivatives of the spectra (lower panel). Color codes are defined in the legends, specifying types of prepared fibrils.

spectral shapes and had a peak at $\sim 1630\text{ cm}^{-1}$, which is assigned to β -sheet structure¹⁰². Second derivatives of the spectra also showed that fibrils had very similar spectral shapes (Figure 2-3D, lower panel). Around 1670 cm^{-1} , positions of local minima are slightly different among the three types of fibrils. This difference might be caused by structural differences of β -turn or contributions of water molecules which were not removed completely by spectral analysis. From these results, it is suggested that these fibrils have similar cross- β structures and polymorphism may be derived from tertiary and/or higher-order structure including hierarchical architecture such as protofibril or bundle structures.

Distinguishing polymorphism of insulin amyloid fibrils by iodine staining

To explore the interaction between the prepared fibrils and iodine molecules, we investigated the formation of colors by the addition of I_2/KI solution to the fibril samples. As mentioned in Materials and Methods, in the I_2/KI solution, three types of iodine molecules, I^- , I_2 , and I_3^- , are stably exist⁶¹.

As a result, all fibrils could be stained with iodine solution giving red to blue colors

(Figure 2-4A), demonstrating that iodine molecules bind to amyloid fibrils. Absorption spectra in UV-visible region exhibited distinct spectral shapes especially in long-wavelength region (400 ~ 740 nm) depending on polymorphism of fibrils (Figure 2-4B). It is noted that these spectra are differential spectra obtained by subtracting spectrum of unstained fibrils from that of iodine-stained fibrils to exclude the absorption of protein molecules. NaCl fibrils stained with iodine had absorption band at ~ 550 nm (Figure 2-4B, red) whereas SDS and no-salt fibrils had absorption band at ~ 650 nm (Figure 2-4B, blue and orange, respectively). On the other hand, native insulin mixed with iodine solution had very weak absorption band in that region and only had a few absorption components derived from free iodine molecules in short wavelength region (Figure 2-5). Iodine solution exhibited very similar spectrum even in the case of the presence of native insulin (Figure 2-5A), suggesting that iodine molecules can specifically react with fibril structures. Second derivatives of the spectra verified the differences in peak positions among the three types of fibrils (Figure 2-4C). In long-wavelength region (400 ~ 750 nm), while SDS and no-salt fibrils had local minima at 480, 580, and 660 nm (Figure 2-4C, blue and orange, respectively), NaCl fibrils had those at 490 and 560 nm (Figure 2-4C, red).

On the other hand, in short-wavelength region (280 ~ 400 nm) fibrils stained with iodine exhibited similar patterns with minima at 300 and 360 nm (Figure 2-4C). These absorption bands were also observed in the case of iodine solution (Figure 2-5A),

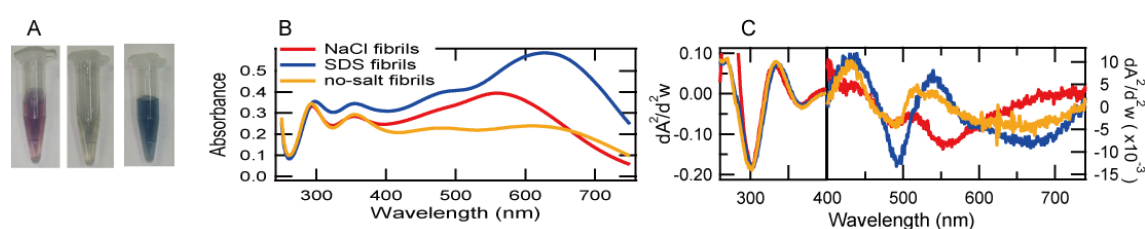


Figure 2-4. UV-vis absorption spectra of iodine-stained insulin amyloid fibrils.

(A) Pictures of NaCl fibrils, no-salt fibrils, and SDS fibrils stained with iodine from left to right. (B and C) Absorption spectra in UV-visible region (B) and their second derivatives (C) of iodine-stained fibrils. Color codes are defined in the legends in the panel B, specifying types of amyloid fibrils. In the panel C, it should be noted that the scales of vertical axes for the region of 400-740 nm and that of 250-400 nm are different.

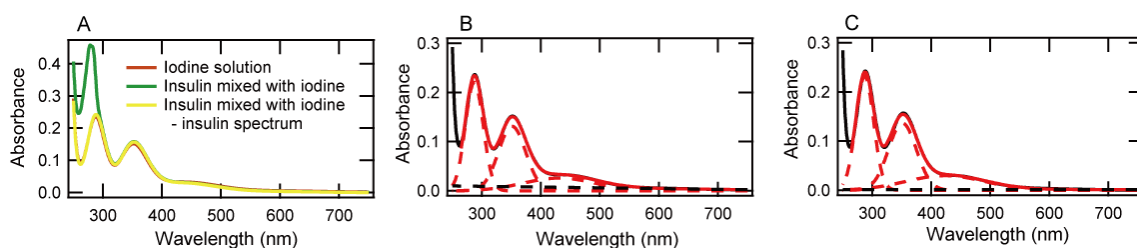


Figure 2-5. Interaction between iodine molecules and native insulin. (A) UV-vis spectra of iodine solution, insulin solution mixed with iodine, and differential spectrum. Color codes are defined in the legend in panel A. It should be noted that yellow line represents differential spectrum obtained by subtracting spectrum of native insulin solution from the spectrum of insulin solution mixed with iodine represented with green line. (B and C) Results of spectral deconvolution of iodine solution (B) and differential spectrum (C). Black and red solid lines represent the experimental or differential spectra and reproduced spectra, respectively. Three Gaussian peaks are represented with dotted red lines and baseline is represented with dotted black line.

suggesting that they may be due to I_3^- ions which can exist stably in water solution⁶¹.

In long-wavelength region (400-750 nm), only a weak absorption band at ~ 430 nm was observed in the absence of fibrils, as shown in Figure 2-5, which is conceived to be assigned to I_2 molecules according to a previous study⁶¹. On the basis of this, as for the absorption in long-wavelength region in Figure 2-4B, we attribute it to new molecular species of iodine bound to fibrils because of the non-existent of it in the spectrum of iodine solution, and previous studies have revealed that absorption bands in that region are derived from complexes of iodine molecules such as I_2 - I_2 , I_2 - I_3^- , and I_3^- - I_3^- ⁶¹.

Quantitative analysis of the absorbance by spectral deconvolution

We next performed spectral deconvolution to discuss absorbing components constructing the experimental spectra quantitatively. Each experimental spectra from 750 to 270 nm were fitted by a sum of Gaussian distributions to reproduce the absorption spectra according to a following model function;

$$A(x) = \sum_{j=1}^n A_j \exp\left(-\frac{(x-x_j)^2}{c_j}\right) + b_0 + b_1x \quad (2)$$

where A_j , x_j , and c_j represent an amplitude, center wavelength, and bandwidth of absorbing components respectively, and in this equation we assumed a baseline slope

with b_0 and b_1 to consider Rayleigh scattering originating from the fibrils. As for the number of absorbing components and their positions, they were determined by extracting the local minima of the second derivatives, except for the component around 430 nm; although any minima are not observed around this wavelength obviously in the second derivative spectra, this component derived from I_2 molecules could be observed clearly in the spectrum of iodine solution (Figure 2-5, B and C) and an additional installation of it certainly improved the reproduction of the experimental data. And for NaCl fibrils, we assumed an absorbing components at 625 nm. This band could be observed in the second derivative spectrum but its position could not be determined precisely because of its broadness. Thus we fixed its position to make results of curve fitting convergent. In curve fitting, the positions of bands, which were represented by parameter x_j , were fixed except for the band around 430 nm because of its uncertainty and weakness. All other parameters such as A_j and c_j were unrestricted, and a range of curve fitting was set to be from 750 to 270 nm for suppressing a contribution of absorbing component in far-UV region (< 250 nm).

As a result, all of the absorption spectra were reproduced successfully by approximating with six Gaussians and a linear baseline (Figure 2-6). The three absorbing components in short-wavelength region (270~450 nm) represented with dotted red lines corresponded to those of free iodine molecules (Figure 2-5A) so that these components may be mainly

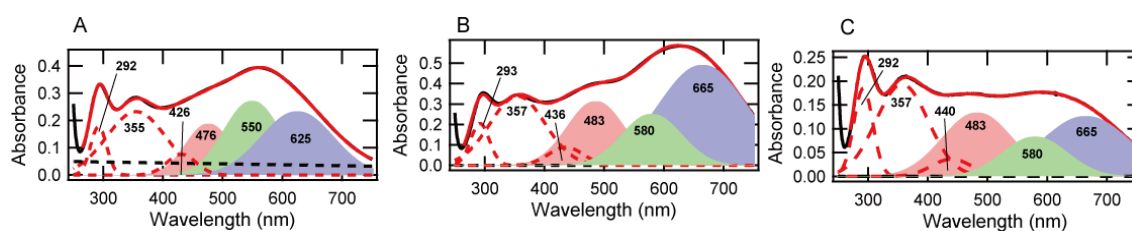


Figure 2-6. Spectral deconvolution of iodine-stained insulin amyloid fibrils.

Results of spectral deconvolution of iodine-stained NaCl fibrils (A), SDS fibrils (B), and no-salt fibrils (C) with model function. Among all figures, black and red lines represent the experimental differential spectra and reproduced spectra, respectively. Six Gaussian peaks represented with dotted red lines and filled areas correspond to absorbing components obtained by the deconvolution and dotted black line represents a baseline factor derived from Rayleigh scattering. Each positions of the components are represented in the legends. The area ratios of filled area with red, green, and blue are 1.0:1.8:2.0 (A), 1.2:1.0:2.5 (B), and 1.7:1.0:2.1 (C), respectively.

derived from an excess free I_2/I_3^- molecules. However, there are slight differences especially in the parameters c_j between iodine solution and iodine-stained fibrils (Table 2-2). Especially about bands around 360 nm, the bandwidth of those components became bigger upon binding to fibrils. These differences may indicate that in the case of fibril samples, absorption in the short-wavelength region is attributable to iodine complexes bound to fibrils, or to combination of free I_3^- ion and fibril-bound iodine complexes. I^- ion has absorption in far-UV region so that it does not affect the experimental spectra⁶¹.

	Component 1 (~290nm)	Component 2 (~360 nm)
Iodine solution	453	1054
NaCl fibrils	450	3656
SDS fibrils	569	4182
no-salt fibrils	513	3819

Table 2-2. Comparing bandwidth of the bands in short-wavelength region. The parameters c_j which correspond to bandwidth of band around 290 and 360 nm are represented.

About the three components in long-wavelength region represented with filled areas (Figure 2-6) are assigned to iodine molecular complexes, suggesting that there are a few types of iodine complexes which bind to amyloid fibrils. Details of this topic will be discussed in titration and resonance-Raman measurements. Comparing the results of NaCl fibrils to those of SDS and no-salt fibrils, the parameters of absorbing components are clearly different (Table 2-3). From these results it is suggested that iodine molecules can specifically react with amyloid fibrils and spectral patterns of stained fibrils can depend on their polymorphic structures. On the other hand, when the results of SDS fibrils

Table 2-3. Comparing parameters of absorbing components in long-wavelength region. The parameters x_j , A_j , and c_j are correspond to position, amplitude, and bandwidth of absorbing component.

	x_1	A_1	c_1	x_2	A_2	c_2	x_3	A_3	c_3
NaCl fibrils	476	0.128	3670	550	0.126	4694	625	0.098	8308
SDS fibrils	483	0.203	5878	580	0.122	5031	665	0.211	10236
no-salt fibrils	483	0.133	6438	580	0.083	5464	665	0.126	10125

and no-salt fibrils were compared, center wavelength and bandwidth of the absorption band were very similar in long-wavelength region only with slight differences in amplitude (Table 2-3). This suggests that fundamental structure of the no-salt fibrils is almost analogous to that of SDS fibrils, and the weaker color formation of the no-salt fibrils even under the same iodine concentration would indicate their smaller accessible area for binding of iodine molecules or lower affinity for iodine than that of SDS fibrils.

Effects of residual salts on iodine staining

There was a concern over effects of residual amount of NaCl or SDS salts derived from the parent fibril solution on the spectral shape. Thus we verified effects of residual salts on iodine staining. First, we prepared daughter no-salt fibrils and an aliquot of the sample was stained with iodine. The remaining fibril solution was sonicated and then added NaCl or SDS salts to facilitate permeation of salts into inner fibril structures. After the incubation for 24 h for fibril reassembly, the samples were stained with iodine. As a result, the spectral shapes and positions of bands were conserved even after the addition of two types of salts to the no-salt fibrils (Figure 2-7, A and B), supporting that the spectral differences in the long-wavelength region are attributable to fibril conformation.



Figure 2-7. Effects of residual salts on iodine staining. (A) UV-vis absorption spectra of no-salt fibrils (orange) and no-salt fibrils containing NaCl (red) or SDS (blue) salts stained with iodine. (B) Second derivatives of the spectra in a range from 400 to 740 nm. Color codes are defined in the legends in the panel A, specifying types of fibrils.

Reducing iodine molecules bound to fibrils

Dzwolak demonstrated that iodine molecules bound to insulin amyloid fibrils exist in solvent-accessible surface area of fibrils by reducing iodine molecules with AsA⁷⁶. This experiment is based on the concept that if iodine molecules existed in inner structures of

fibrils, AsA could not access or react with iodine molecules. According to the experimental data, fibril-bound iodine molecules were reduced by AsA and thereby absorption in long-wavelength region disappeared. To verify whether the similar phenomenon could be observed in our system, we prepared fibril samples stained with iodine and added AsA solution to them. In addition, we performed daughter fibril formation in the presence of iodine molecules for facilitating permeation of iodine molecules in inner structures of fibrils. After the formation of fibrils, AsA was added and subjected to UV-vis spectroscopy. As a result, by reducing iodine-fibril complexes, absorption in long-wavelength region completely disappeared among all samples except for Rayleigh scattering derived from fibrils (Figure 2-8). These results indicate that iodine complexes exhibiting absorption in long-wavelength region exist only in solvent-accessible surface area of fibrils. Interestingly, absorption spectra of fibrils formed in the presence of iodine molecules are different from those of fibrils formed in the absence of iodine. Especially about SDS and no-salt fibrils, compared to samples mixed with iodine after finishing fibril formation (Figure 2-8, B and C), peaks around 660 nm became weak, but peaks around 490 nm became strong. (Figure 2-8, E and F). These differences may be due to new polymorphism of fibrils induced by iodine molecules.

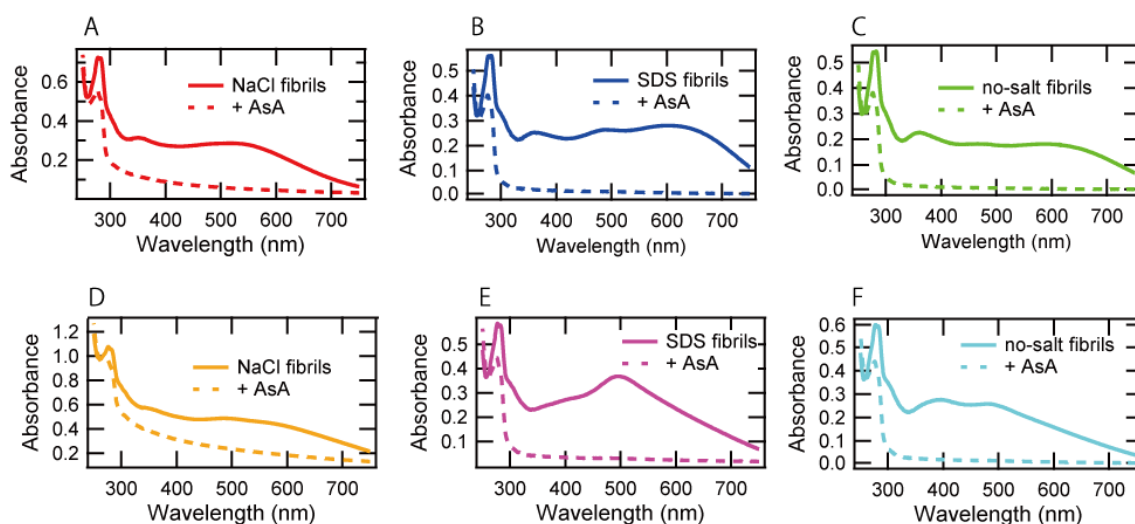


Figure 2-8. Reduction of fibril-bound iodine molecules by using AsA. (A-F) UV-vis spectra of iodine-stained fibrils before (solid lines) and after reduction (dotted lines) by AsA. Color codes are defined in the legends in each panel. It is noted that the samples represented in upper line (A-C) or lower line (D-F) were formed in the absence or presence of iodine molecules.

From these results, SDS and no-salt fibrils exhibited similar spectral patterns both in the absence and presence of iodine molecules when fibrils were formed, supporting our postulation that fundamental structures of SDS and no-salt fibrils are analogous. Although the possibility that these slight differences in absorption reflects the polymorphism of fibrils cannot be denied, in this case the differences were thought to be very small and thus we limited the discussion to NaCl and SDS fibrils hereafter.

Tracking structural propagation of amyloid fibrils by using iodine staining

One of the most common characteristics of amyloid fibrils is the ability of structural propagation which is so-called seeding ability. If the spectral differences observed above depends on the polymorphism, spectral shapes are expected to be inherited after seeding

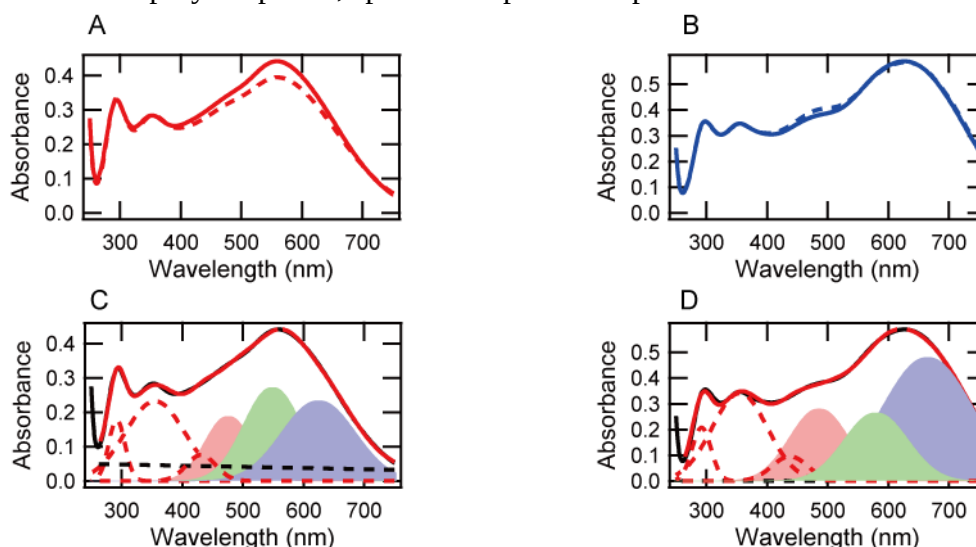


Figure 2-9. Effects of seeding reaction on iodine staining. (A and B), UV-vis absorption spectra of iodine-stained daughter and granddaughter fibrils of NaCl (A) and SDS fibrils (B). In both panels, dashed lines and solid lines represent the spectra of daughter fibrils and those of granddaughter fibrils, respectively. (C and D), Results of spectral deconvolution of NaCl (C) and SDS (D) granddaughter fibrils. In both panels, black and red lines represent the experimental differential spectra and reproduced spectra, respectively. Six Gaussian peaks represented with dotted red lines and filled areas correspond to absorbing components obtained by the deconvolution and dotted black line represents a baseline factor derived from Rayleigh scattering. The area ratios of filled area with red, green, and blue are 1.0:1.8:2.0 (A), 1.2:1.0:2.5 (B), respectively.

reaction. Thus we investigated the effects of seeding reaction on iodine staining. In this analysis we prepared granddaughter fibrils by using the daughter fibrils as seeds and UV-visible absorption spectra of stained granddaughter fibrils were compared with those of daughter fibrils. The granddaughter fibrils exhibited very similar spectral patterns to those of daughter for both NaCl and SDS fibrils (Figure 2-9, A and B). Results of spectral deconvolution of the granddaughter fibrils also showed similar pattern to those of daughter fibrils shown in Figure 2-6. Especially about area ratio of the bands in long-wavelength region (represented with filled areas), they shared almost the same values,

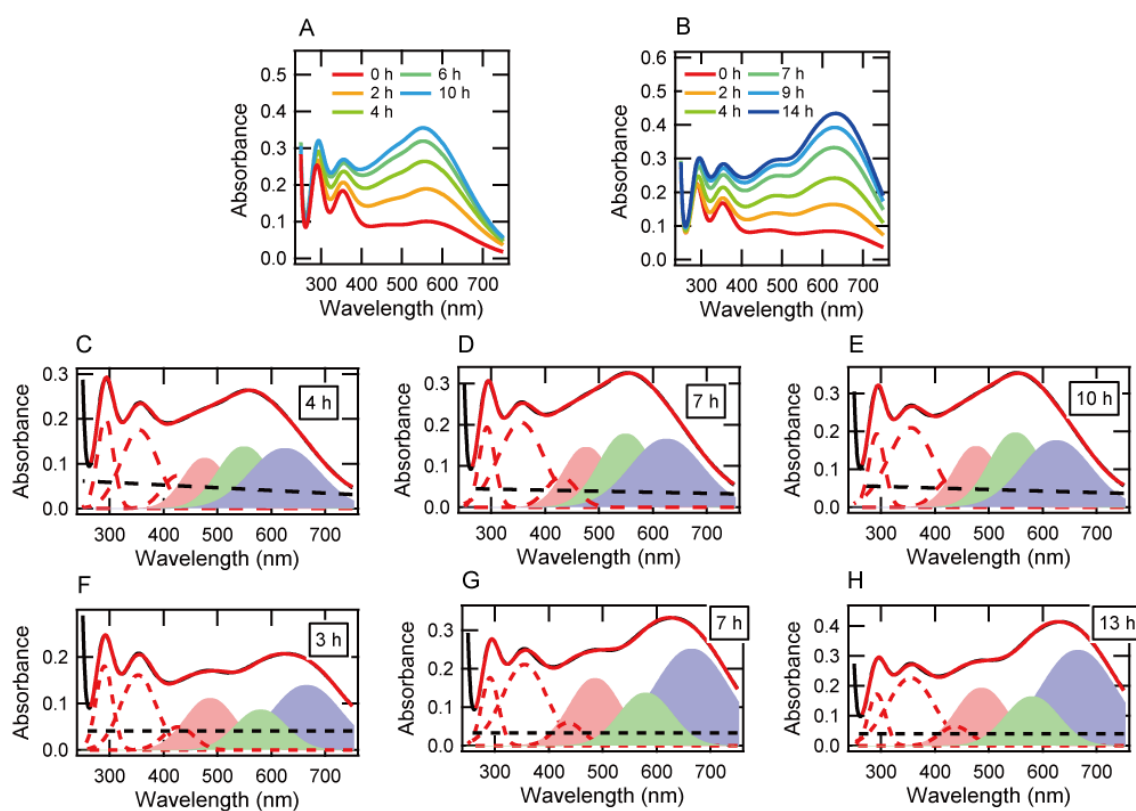


Figure 2-10. Time course of UV-vis spectra monitoring seed-dependent fibril growth by using iodine staining and spectral deconvolution. (A and B) Time course of UV-vis spectroscopy of NaCl (A) and SDS fibrils (B) stained with iodine. Color codes are defined in the legends, specifying time points when the spectra were measured. (C-H) Results of spectral deconvolution of NaCl (C-E) and SDS fibrils (F-H). In these panels, black and red lines represent the experimental differential spectra and reproduced spectra, respectively. Six Gaussian peaks represented with dotted red lines and filled areas correspond to absorbing components obtained by the deconvolution and dotted black line represents a baseline factor. In upper right in each panel, time points are represented.

suggesting binding patterns of iodine molecules to fibrils were mostly conserved after seeding reaction. From these results, we conclude iodine staining can distinguish polymorphism of amyloid fibrils.

Existing amyloid probes such as ThT can evaluate relative amount of fibrils in the case of fibrils with the same structure, and thus they can track time-dependent fibril formation, making kinetic discussion possible⁴⁰. To investigate whether iodine staining can be used for this purpose, we performed time course measurements of color reaction for NaCl and SDS fibrils growing in the presence of seeds with UV-vis spectroscopy (Figure 2-10 and 2-11). After the acquisition of spectra, we performed spectral deconvolution (Figure 2-10) with the same procedures as those mentioned above and plotted areas of each three absorbing components in the long-wavelength region against the time (Figure 2-11, A and B). Time course of ThT which was measured concomitantly is also plotted (Figure 2-10, A and B, black points and lines) as a control. As for the growth reaction of NaCl fibrils (Figure 2-11A), areas of each absorbing components at 476 (red), 550 (green), and 625 nm (blue) gradually became large and saturated around 8 h in agreement with the time

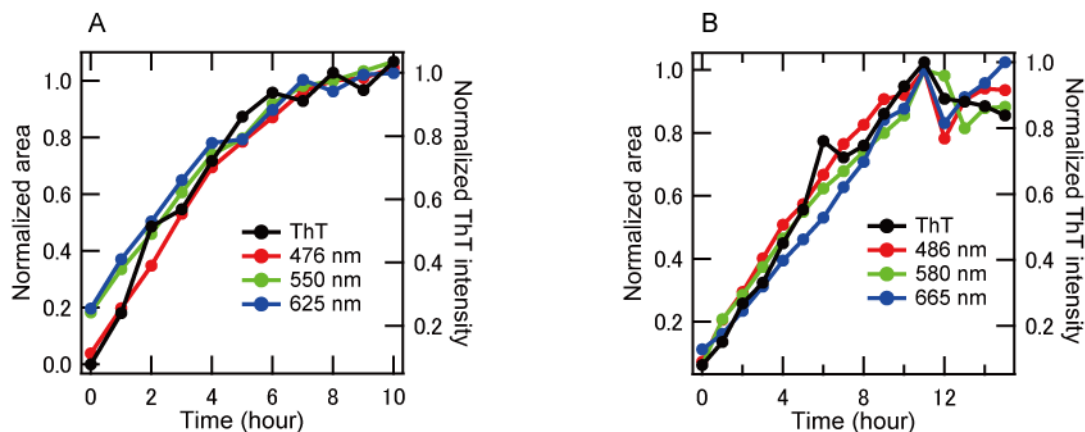


Figure 2-11. Tracking fibril growth of seeding reaction by using ThT assay and iodine staining. (A) Time course of area of three absorbing components at 476 (red), 550 (green), and 625 nm (blue) for NaCl fibrils. (B) Time course of area of three absorbing components at 483 (red), 580 (green), and 665 nm (blue) for SDS fibrils. In both panels, left and right axis represent values of normalized area and ThT intensity, respectively. Black points and lines represent time course of ThT intensity. The values of area were calculated by integrating bands approximated with Gaussian. It is noted that between panel A and B, horizontal axes are different from each other because of the differences in saturation time of the reaction.

course of ThT, indicating that iodine staining can track fibrillation reaction quantitatively. As for the case of SDS fibril growth, areas of three components at 483 (Figure 2-11B, red), 580 (green), and 665 nm (blue) also exhibited similar tendency of growth with each other and almost saturated around 12 h, in analogy with time course of ThT. Thus iodine staining could be applied to track fibril growth without regarding their types.

Stoichiometry between fibrils and iodine molecules.

From the results of spectral deconvolution (Figure 2-6), it is suggested that there is a few types of iodine molecular species binding to amyloid fibrils. If several iodine molecular species bound to amyloid fibrils exist, it is expected binding motif such as binding constants and stoichiometry between fibril and iodine species will be different from each other. Thus we performed titration experiments until the absorption in the long-wavelength region saturated to further investigate the concentration dependence of iodine molecules. As a result, gradual development of the absorption spectrum was observed for both NaCl and SDS fibrils with an increase of iodine concentration (Figure 2-12, A and B). The positions of the six absorption components were extracted from the second derivative spectra (Figure 2-12, C and D) for spectral deconvolution. In light of the observation that there were no prominent shifts in their positions of components in long-wavelength region (550~750 nm) (Figure 2-12, C and D), suggesting that each component was identical through the titration experiments. And thus we tentatively assumed the parameter c_j representing the bandwidth of the j th absorbing component to be constant.

On the other hand, it is noted that red-shift of an absorbing component between 450 and 500 nm was observed both for NaCl (Figure 2-12C) and SDS fibrils (Figure 2-12D), suggesting that this absorption could be attributed to combination of several components and ratio of contribution of each component may change through the titration experiments. However, in the second derivatives of the spectra, only one local minimum was observed so that we assumed it as one component around 500 nm, but with a variable c_j throughout the all spectra. In addition, as for the rest of three components in shorter wavelength, especially about that around 360 nm, there might be two types of absorption derived from free iodine species and fibrils-bound ones (Table 2-3), thus the parameters c_j of these

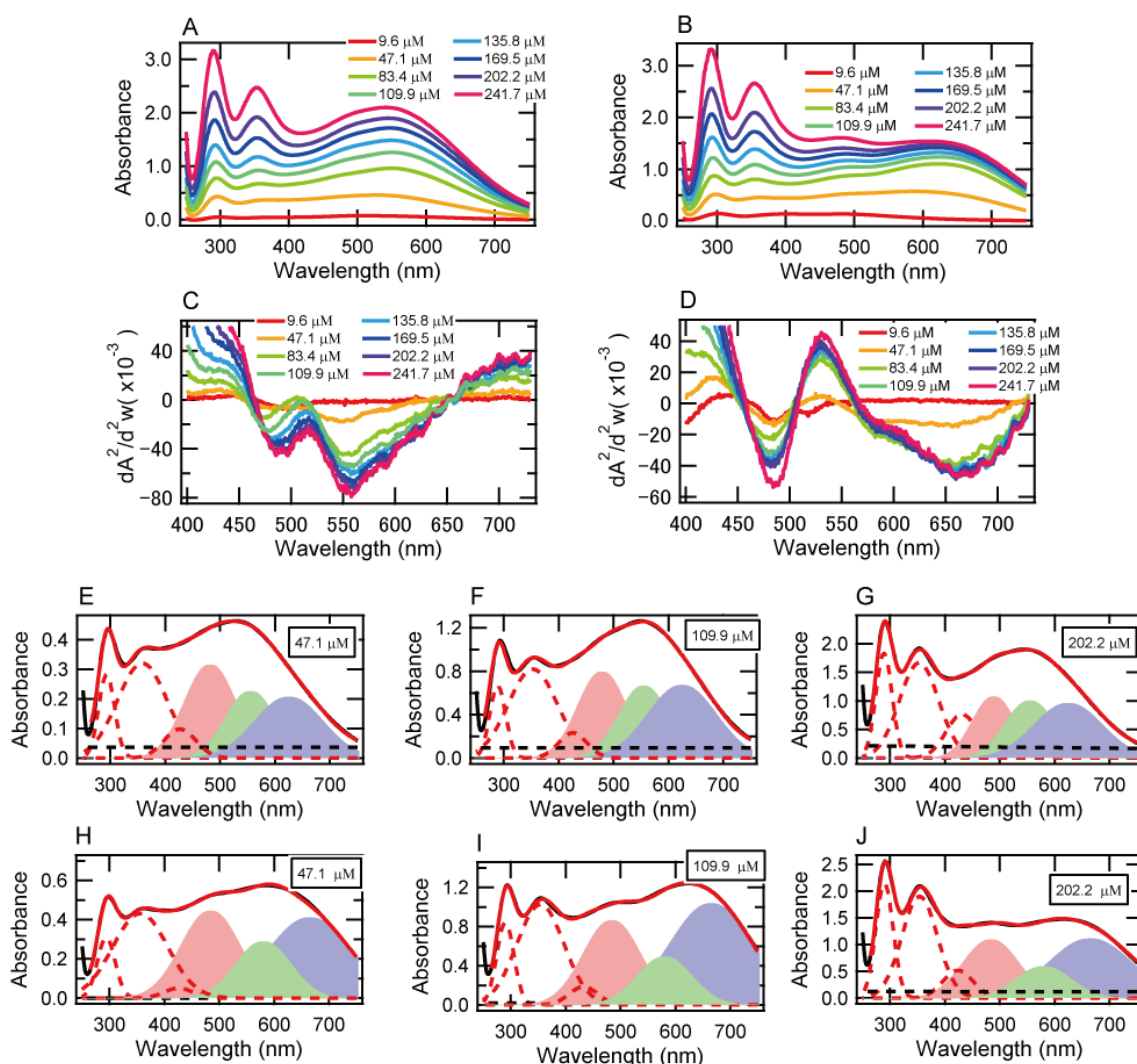


Figure 2-12. Iodine concentration dependence of UV-vis absorption spectra derived from fibril-iodine complexes. (A and B) Concentration dependence of UV-vis spectroscopy of NaCl (A) and SDS fibrils (B) stained with iodine. Color codes are defined in the legends, specifying concentration of I_2 molecules. (C and D) Second derivatives of the spectra of NaCl (C) and SDS fibrils (D). Colors in each panel are the same as those in panel (A) and (B), respectively. (E-J) Results of spectral deconvolution of NaCl (E-G) and SDS fibrils (H-J). In these panels, black and red lines represent the experimental differential spectra and reproduced spectra, respectively. Six Gaussian peaks represented with dotted red lines and filled areas correspond to absorbing components obtained by the deconvolution and dotted black line represents a baseline factor. In upper right in each panel, time points are represented.

components were set to be variable. Under these conditions, we performed spectral deconvolution with equation (2) (Figure 2-12, E-J).

After the deconvolution, the areas of each absorbing component in long-wavelength region were calculated by integrating Gaussian and plotted against iodine concentration. Then, saturation curves were fitted with Hill equation¹⁰³ which is used for evaluation of ligand binding to allosteric proteins such as hemoglobin

$$G(x) = \frac{max}{\left\{1 + \left(\frac{x_{half}}{x}\right)^{rate}\right\}} \quad (3)$$

where *max*, *xhalf*, and *rate* mean maximum of area, binding constant, and cooperativity of binding, respectively. About SDS fibrils, each component exhibited different saturation curves and parameters (Figure 2-13, A-C). Comparing absorbing component at 483 nm (Figure 2-13A) with that at 580 nm (Figure 2-13B), *rate* is similar value but *xhalf* is clearly different, indicating absorbing component at 580 nm reflects strong binding to fibrils. About a component at 665 nm (Figure 2-13C), *xhalf* is similar to that at 580 nm, but *rate* is the highest value, indicating that this component increases cooperatively. These

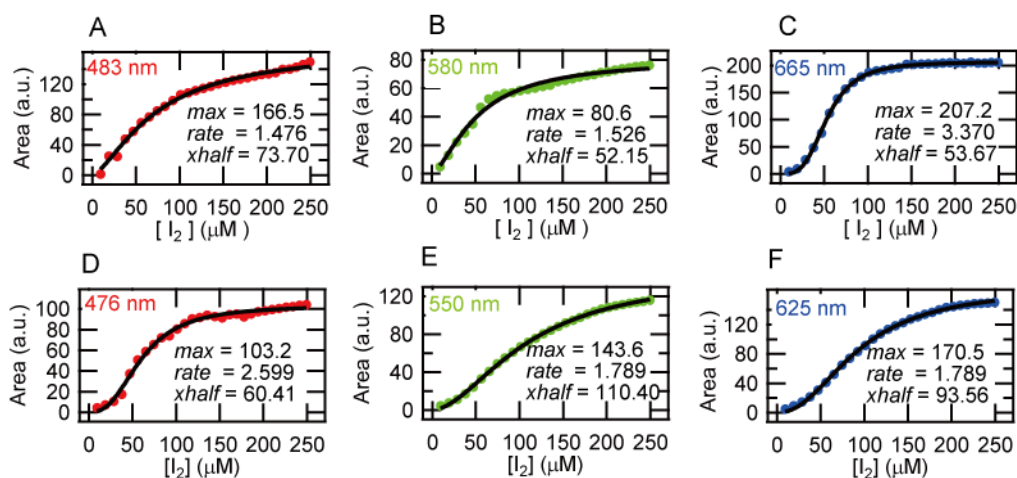


Figure 2-13. Characterization of each absorbing components derived from complexes of iodine molecules. (A-C) Concentration dependence of each absorbing components at 483 (A), 580 (B), and 665 nm (C) of iodine-stained SDS fibrils. (D-E) Concentration dependence of each absorbing components at 476 (D), 550 (E), and 625 nm (F) of iodine-stained NaCl fibrils. Among all panels, each parameters concerning binding to iodine molecules calculated by the fitting of Hill equation are represented insets. Position of each component is represented in upper left in each panel.

results suggest that there are at least three specific iodine-binding sites. Iodine molecules which bind to fibrils may exist in solvent accessible surface (Figure 2-8), thus the differences among three iodine-binding sites would be derived from surface properties of fibrils. As for NaCl fibrils, they also have three different specific binding sites (Figure 2-13, D-F) similarly with SDS fibrils but their binding constants tended to be weaker than those of SDS fibrils. These results suggest that there are at least three types of iodine-binding sites on the surface of amyloid fibrils and the properties of binding sites change depending on types of amyloid fibrils. In other words, iodine molecules can recognize specific structures on the surface of amyloid fibrils. Furthermore, all of the binding sites exhibited that the value of rate is larger than 1, suggesting that binding mode between fibrils and iodine molecules cannot be so simple model but should be explained by more complicated and cooperative mechanism associated with three or more binding species. Because absorption in visible region is due to the complex of iodine molecules, amyloid fibrils may act as a scaffold for polymerization of iodine molecules as the same case as amylose and polymers.

Characterization of fibril-bound iodine species

To characterize molecular species of iodine which bind to amyloid fibrils, we measured Raman spectra of them. Previous researches reported that iodine species such as I_2 , I_3^- , and $I_2-I_3^-$ can be distinguished from each other by using Raman spectroscopy⁶¹. For example, two types of iodine molecules, I_3^- and $I_2-I_3^-$, which bind to nylon-6 polymer have been identified by using resonance Raman spectroscopy⁷³. In our system, iodine-stained fibrils have absorption in visible region so we expected that scattering intensity become large due to resonance Raman effects by using laser with visible wavelength. In this study, we used two types of laser with different wavelength, 514.49 and 785 nm, for selective enhancement of each component in visible region; by exciting iodine-fibril complexes with 514.49 nm laser, filled areas with red and green (Figure 2-6) are expected to be mainly enhanced, and thus molecular information of those components can be extracted. Besides, by exciting with 785 nm laser, we will obtain molecular information about the component represented with filled blue area.

As a result, by exciting with 514.49 nm laser, both samples of NaCl and SDS fibrils

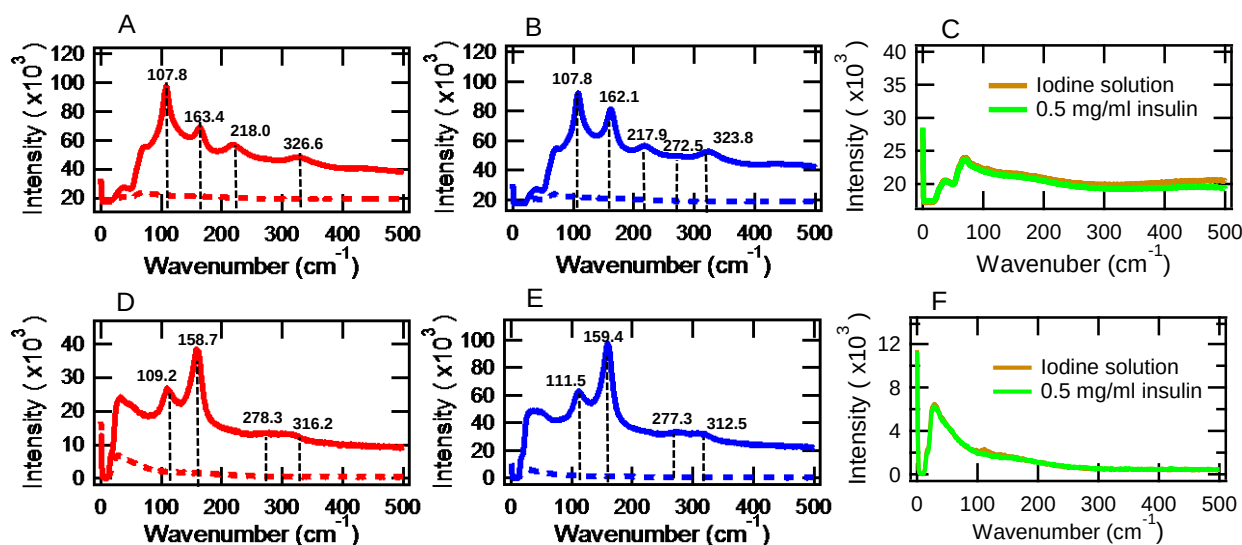


Figure 2-14. Characterization of iodine molecular species by resonance Raman spectroscopy. (A-C) Resonance Raman spectra of NaCl fibrils (A), SDS fibrils (B) and controls obtained by exciting them with 514.49 nm laser. (D-F) Resonance Raman spectra of NaCl fibrils (D), SDS fibrils (E), and controls (F) obtained by exciting them with 785 nm laser, respectively. In panel A, B, D, and E, dotted and solid lines represent spectra of unstained and iodine-stained fibrils, respectively. Among all spectra, solid line represents spectra of iodine-stained fibrils and dotted line represents the spectra of unstained fibrils. Positions of peaks are represented in the legend in each panel. In panel C and D, color codes are defined in the legends, specifying types of control samples.

stained with iodine (Figure 2-14, A and B, solid red and blue lines) exhibited five times larger scattering intensity than did fibrils only (Figure 2-14, A and B, dotted red and blue lines) and controls (Figure 2-14C). Iodine solution without fibrils exhibited similar spectral pattern to those of unstained fibrils (Figure 2-14C), suggesting that resonance Raman effects are dominant factors for spectral shapes and intensity as expected. In other words, we can observe only iodine species bound to fibrils. Both NaCl and SDS fibrils have strong peaks at $\sim 100 \text{ cm}^{-1}$ and $\sim 160 \text{ cm}^{-1}$ (Figure 2-14, A and B) but the positions of the peak around 160 cm^{-1} were slightly different, suggesting environmental differences of iodine-binding sites in the amyloid surface. The peak at $\sim 100 \text{ cm}^{-1}$ is assigned to I_3^- and the peak at $\sim 160 \text{ cm}^{-1}$ is assigned to $\text{I}_2\text{-I-I}_2$ complex⁷³ (Table 2-4). For both fibrils, two weak peaks are observed at $\sim 200 \text{ cm}^{-1}$ and $\sim 320 \text{ cm}^{-1}$, which are assigned to be overtones of two strong peaks. In addition, for SDS fibrils a weak peak around 270 cm^{-1} could be

observed, which could be assigned to combination band. Selective rule of resonance Raman scattering including overtones and combinations depends on symmetric group of molecules, suggesting that between NaCl and SDS fibrils, there are a few types of iodine molecules with different symmetric property.

In the case of 785 nm excitation, resonance Raman effects were observed for both NaCl and SDS fibrils (Figure 2-14,D and E), similar to those obtained by 514.49 nm excitation, and both samples exhibited two strong peaks at 110 and 160 cm^{-1} . In these spectra, positions of the peaks are slightly shifted, suggesting the differences in iodine-binding sites and we assigned those peak at 110 and 160 cm^{-1} to be I_3^- and $\text{I}_2\cdots\text{I}_3^-$, respectively (Table 2-5).

Peak positions	Assignment
107 cm^{-1}	$\text{I}_3^-(\nu_1)$
162 cm^{-1}	$\text{I}_2\cdots\text{I}^-\cdots\text{I}_2(\nu_2)$
218 cm^{-1}	Overtone ($2\nu_1$)
273 cm^{-1}	Combination ($\nu_1+\nu_2$)
325 cm^{-1}	Overtone ($2\nu_2$)

Table 2-4. Assignment of resonance Raman peak obtained by exciting 514.49 nm. Peak positions are averaged between results of NaCl and SDS fibrils. It is noted that the peak at 272 cm^{-1} was observed only in the case of SDS fibrils.

Peak positions	Assignment
111 cm^{-1}	$\text{I}_3^-(\nu_1)$
159 cm^{-1}	$\text{I}_2\cdots\text{I}_3^-(\nu_2)$
278 cm^{-1}	Combination ($\nu_1+\nu_2$)
314 cm^{-1}	Overtone ($2\nu_2$)

Table 2-5. Assignment of resonance Raman peak obtained by exciting 785 nm. Peak positions are averaged between results of NaCl and SDS fibrils.

Analysis of iodine-binding modes by using spherulites

It is reported that iodine molecules can stain amylose, polyvinyl alcohol, and Nylon-6⁷³. Additionally, iodine molecules which bind to them are oriented in a specific manner⁷¹. Thus it is anticipated that fibril-bound iodine molecules have some kind of orientation. To verify this, we tried to observe a polarization property of stained fibrils. It has been

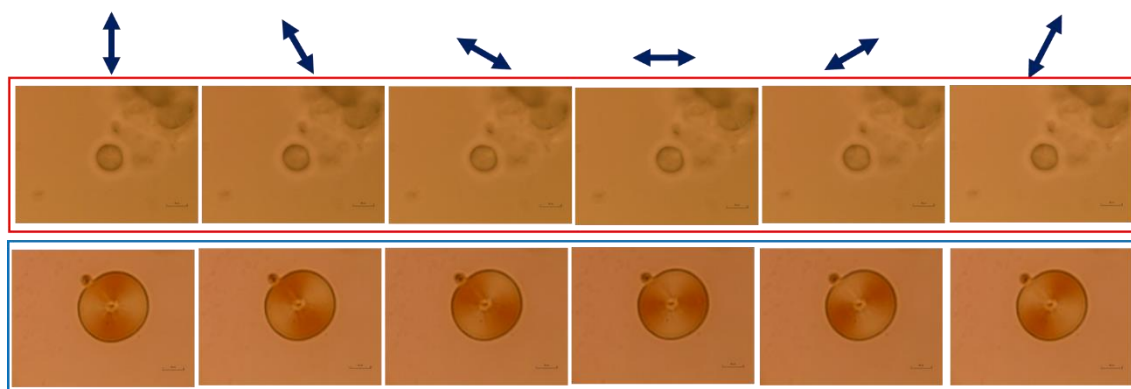


Figure 2-15. Orientation of iodine molecules bound to amyloid fibrils. Images of unstained and stained spherulites are shown in upper and lower stages, respectively. Arrows which are above the images indicate the direction of polarizer.

reported that iodine molecules absorb polarized light parallel to the long-axis of them and used for polarizing materials⁷¹. If iodine molecules are oriented in a specific manner, a polarization property of iodine will provide valuable information regarding the fundamental pattern of iodine binding. As a beneficial structure, we used spherulites instead of amyloid fibrils. Spherulite is a spherical protein aggregates and constructed two components, central nucleus regions and amyloid fibrils⁹³⁻⁹⁴. In the spherulite, fibrils extend radially from the nucleus regions like chestnuts in burrs. Because the orientation of amyloid fibrils are highly controlled in the spherulite, we suppose it the best model for evaluating the orientation of fibril-bound iodine molecules. It should be noted that to form spherulite, high concentration of protein is necessary so that fibrillation condition was quite different from those used for forming fibrils. Spherulites stained with iodine were observed by using a polarized microscopy. Emitting an unpolarized light stained spherulites and then a transmitted light were observed through a polarizer. As a result, iodine-stained spherulites exhibited different images respectively depending on the direction of the polarizer (Figure 2-15). On the other hand, images of unstained spherulites were independent of the direction of polarizer, suggesting that the anisotropy of stained spherulites are derived from iodine molecules. The directions of amyloid fibrils in the dark region of stained spherulites were coincided with the direction of polarizer, and considering the structure of spherulite, iodine molecules such as I_3^- exist as linear molecules are oriented along the fibril axis, absorbing polarized light parallel to the long

axis of iodine with high efficiency.

Iodine staining of A β and other amyloid fibrils

The results so far indicate iodine staining can provide structural information of insulin amyloid fibrils. So we investigated that iodine staining can be applied to other amyloidogenic proteins or peptides. In this study, A β_{1-40} peptide were used as a model because there are many reports about polymorphism of A β_{1-40} amyloid fibrils³⁴⁻³⁵. It was reported that A β_{1-40} amyloid fibrils which were formed with agitation exhibited higher cytotoxicity than fibrils formed without agitation³⁴. We prepared these two types of A β_{1-40} amyloid fibrils and verified whether iodine staining can recognize these fibrils. Preparation of fibril solution was performed by seeding reaction as same as insulin fibril preparation. It is noted that A β_{1-40} fibrils formed in a neutral buffer were transferred in acidic 25 mM HCl solution because iodine staining can be used at low pH condition (data not shown), while the reason is not known at this time. Fibril formation was confirmed by observing with AFM (Figure 2-16, A and B)

As a result, two types of A β_{1-40} amyloid fibrils exhibited different absorption spectra by staining with iodine (Figure 2-16C). This results suggest that iodine staining can be used with a wide range. Considering that these fibrils exhibit different cytotoxicity, it might be expected that iodine staining can be used as an index of amyloid cytotoxicity. However, UV-vis spectra of A β_{1-40} amyloid fibrils stained with iodine exhibited spectral shape for each experiment. Though the difference between two types of fibrils might be exist, at

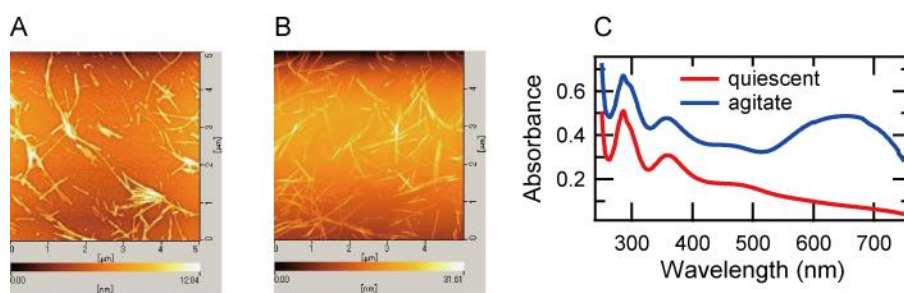


Figure 2-16. UV-vis spectra of two types of iodine-stained A β_{1-40} amyloid fibrils. (A and B) AFM images of A β_{1-40} amyloid fibrils formed under quiescent (A) or agitated (B) conditions. (C) UV-vis spectra of iodine-stained A β_{1-40} amyloid fibrils. Color codes are defined in the legend, specifying the condition under which each fibril was formed.

this time we lack a precise reproducibility of the experimental results.

In addition to the case of A β ₁₋₄₀ fibrils, we tested two types of proteins, lysozyme and β 2m. These proteins are famous as amyloidogenic protein and the formation of fibrils have been studied well^{84-85, 88-90, 92}. According to those research, we performed fibrillation of these proteins and confirmed it by ThT assay (data not shown). Then they were stained with iodine. As a result, both fibrils could be stained with iodine and exhibited absorption in long-wavelength region (Figure 2-17A), indicating that iodine staining can be applied to a wide range of samples. Between the spectra of lysozyme and β 2m, positions of local minima were different from each other (Figure 2-17B), supporting that iodine staining can recognize structural differences of amyloid fibrils.

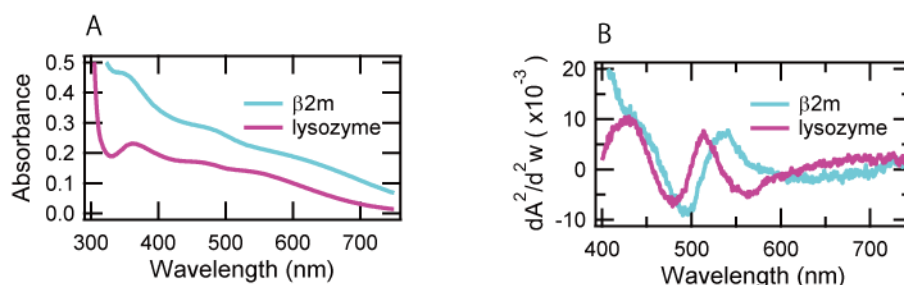


Figure 2-17. Iodine staining of lysozyme and β 2m amyloid fibrils. (A) UV-vis spectra of iodine-stained lysozyme and β 2m amyloid fibrils. (B) Second derivatives of the spectra in long-wavelength region (> 400 nm). Color codes are defined in the legends, specifying types of amyloid fibrils.

Chemical modification of amino acid residues in amyloid fibrils

From these results, it is suggested that iodine molecules bind to specific sites on the surface of amyloid fibrils. However, factors that characterize binding sites are still unknown. We suspected that those differences might be derived from side chains of amino acids which cover the surface of cross- β structure, and to verify this hypothesis, we performed chemical modification of several specific amino acid residues conceived to be react with iodine molecules. If any of three residues have an affinity to iodine molecules, patterns of binding of iodine will be affected, resulting in the color change of iodine-fibril complexes.

As candidates for chemical modification, we focused on three amino acid residues, Lys, Arg, and Tyr. Iodine molecules have negative charge so that electrostatic interaction between iodine molecules and the positively charged Lys and Arg may occur. As for the

Tyr residues, it is reported that it possesses high affinity to iodine molecules and form a covalent bond between them¹⁰⁴.

As a result, after modifying them, absorption spectra in long-wavelength region obviously changed (Figure 2-18, A and B). It should be noted that all of the absorption spectra in Figure 2-18 are represented as differential spectra which was obtained by subtracting spectra of unstained amyloid fibrils with modification. Especially about Arg modification, PG reagent have absorption around 350 nm and thus the spectral shape in that region cannot be discussed exactly. That's why we discuss only in long-wavelength region (> 450 nm). As for NaCl fibrils (Figure 2-18A), absorption in visible region decreased when Arg residues were modified. As for Lys and Tyr modification, the positions of peak top shifted to shorter wavelength. In the case of SDS fibrils, decrease of absorption around 650 nm was observed after amino acid modification among all of the samples (Figure 2-18B). In general, the large number of iodine molecules polymerized, the lower energy they absorb, which is similar to conjugation of benzene. Based on this concept, chemical modification inhibits polymerization of iodine molecules with high degree, indicating contribution of these amino acids to iodine binding.

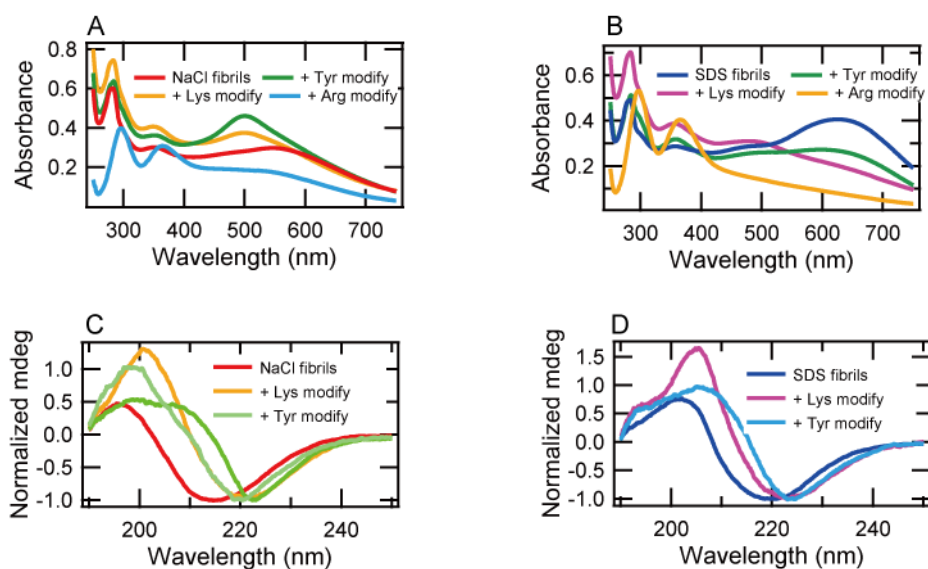


Figure 2-18. Effects of amino acid modification on iodine staining and secondary structures of fibrils. (A and B) Effects of modification on absorption spectra of NaCl (A) and SDS fibrils (B). (C and D) Effects of modification on secondary structures of NaCl (C) and SDS fibrils (D). Color codes are defined in the legend, specifying modification reagent mixed with fibrils.

Considerations represented above were based on that fibril structures did not change through chemical modification. Thus we confirmed secondary structures of fibrils by CD spectroscopy (Figure 2-18, C and D). Although PG have absorption in far-UV region and thus we could not obtain reliable data, as for NaCl (Figure 2-17C) and SDS fibrils (Figure 2-17D), spectral shapes assigned to β -sheet structure were conserved after the chemical modifications. However, the positions of local minima might be shifted. Modification reagents have absorption in far-UV region, and thus the possibility that these spectra do not reflect fibril structure cannot be denied. Examining fibril structures by using other methods such as ATR-FTIR are necessary.

Identification of chemically modified insulin by using ESI-MS

To confirm that amino acid modification proceeded as planned, we examined a molecular weight of modified insulin by ESI-MS. Toward mass spectrometry, two types of fibril samples, NaCl and SDS fibrils, suspended in the reaction solution were precipitated and the pellets of fibrils were dissolved in strong base condition. After dilution and filtration, these samples were subjected to HPLC for purification. In this experiment, we used C18 reverse-phase column and mobile phase consisted of water/ACN mixture containing 0.1 % of TFA, and detection of insulin was performed by measuring absorption at 220 and 276 nm because insulin protein exhibit absorption at both positions.

As a result, all of the chromatogram for each sample exhibited many peaks (Figure 2-19), indicating heterogeneity of the samples. According to the previous study (data not shown), fraction of intact insulin is observed around 40 % of ACN (~ 15 min), thus the fraction from 10 to 25 min were conceived to be elution of insulin and its derivatives. Fractions eluted within 10 min were conceived to be residual modification reagents and those eluted around 30 min might be impurity with high hydrophobicity. In general, inner structures of amyloid fibrils densely pack, and the chemical modification might occur with insulin protein exist in solvent-accessible surface. In addition, insulin protein contain several functional group which can react with modification reagents, and there might be distribution of degree of modification, resulting in the heterogeneity. As for the Arg modification, there is only one residue in insulin, but it is reported that two PG molecules

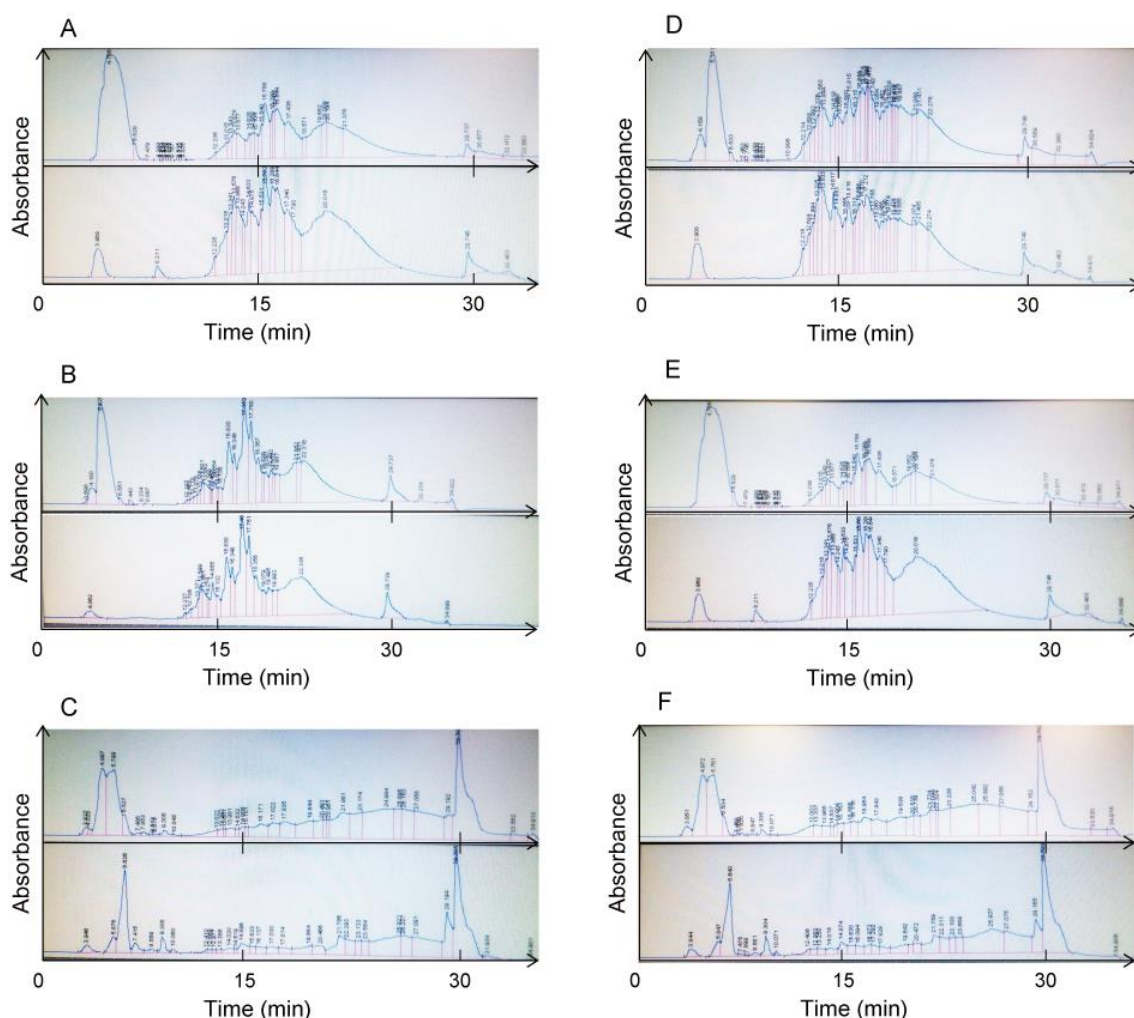


Figure 2-19. Chromatograms of chemically modified insulin obtained by using reverse-phase HPLC. (A-C) Chromatograms of insulin derived from NaCl fibrils with chemical modification of Lys (A), Tyr (B), and Arg (C). (D-F) Chromatograms of insulin derived from SDS fibrils with chemical modification of Lys (D), Tyr (E), and Arg (F). In each panel, upper and lower panel represent chromatograms detected by an absorption at 220 (upper panel) and 276 nm (lower panel).

are introduced to Arg residue[.]. Off course, possibility that side reactions which we did not assume occur through the preparation procedure containing chemical modification and dissociation of fibrils cannot be denied. In these experiments, we could remove modification reagent which remain intact, but could not separate insulin with different degree of chemical modification or with side reaction. We collected fraction eluted from 15-20 min and lyophilized them.

Before LC/MS analysis, we performed enzymatic digestion and reduction of insulin with

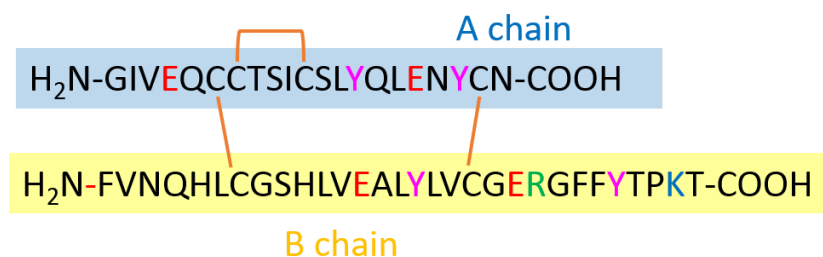


Figure 2-20. Amino acid sequence of insulin protein. Red letters correspond to Glu (E) and the V8 protease digests peptide bonds at the carboxylic side of it. Lys (K), Tyr (Y), and Arg (R) are represented with blue, pink, and green letters. Three disulfide bonds are represented with orange lines.

modification for discussing site specificity of chemical modification (Figure 2-20). In this experiment, we used V8 protease which hydrolyzes peptide bond at the carboxylic side of Glu (E) in ammonia-based buffer¹⁰⁵. In addition to reduction of disulfide bonds, position of chemically modified residues, especially about Tyr residue, can be identified. After desalting by using ZipTip, the samples were subjected to LC/MS analysis.

As a result, six patterns of LC/MS chromatograms were obtained (Figure 2-21). As for insulin derived from NaCl fibrils, similar chromatograms were obtained in the case of Lys (Figure 2-21A) and Tyr (Figure 2-21B) modification. Same mass-to-charge (m/z) values such as 494.9, 558.7, and 579.8 were observed, suggesting that there are same peptide fragments despite of the different modification. It should be noted that the vertical axis correspond to relative abundance of each peptide fragment do not reflect actual amount of that because the value highly depends on ionization tendency of each fragment. Considering charge differences among the fragments, assignment of these fragments were performed (Table 2-6). From these result, by using *N*-succinimidyl acetate modification of Lys and N-terminus in B chain proceeded as planned. In contrast, modification of Tyr residues by using *N*-acetylimidazole did not proceed and the modification of Lys residue occurred. It is reported that *N*-acetylimidazole can acetylate both phenyl and amino group, but reactivity of it to amino group is lower than that to phenyl group¹⁰⁶. These results suggested that all of the Tyr residues in insulin protein are buried in fibril structures. Similar patterns of chemical modification were also observed for the case of SDS fibrils (Figure 2-21, D and E), suggesting that in both fibrils Tyr residues were in dense core structures of amyloid fibrils with high hydrophobicity. As for Arg modification,

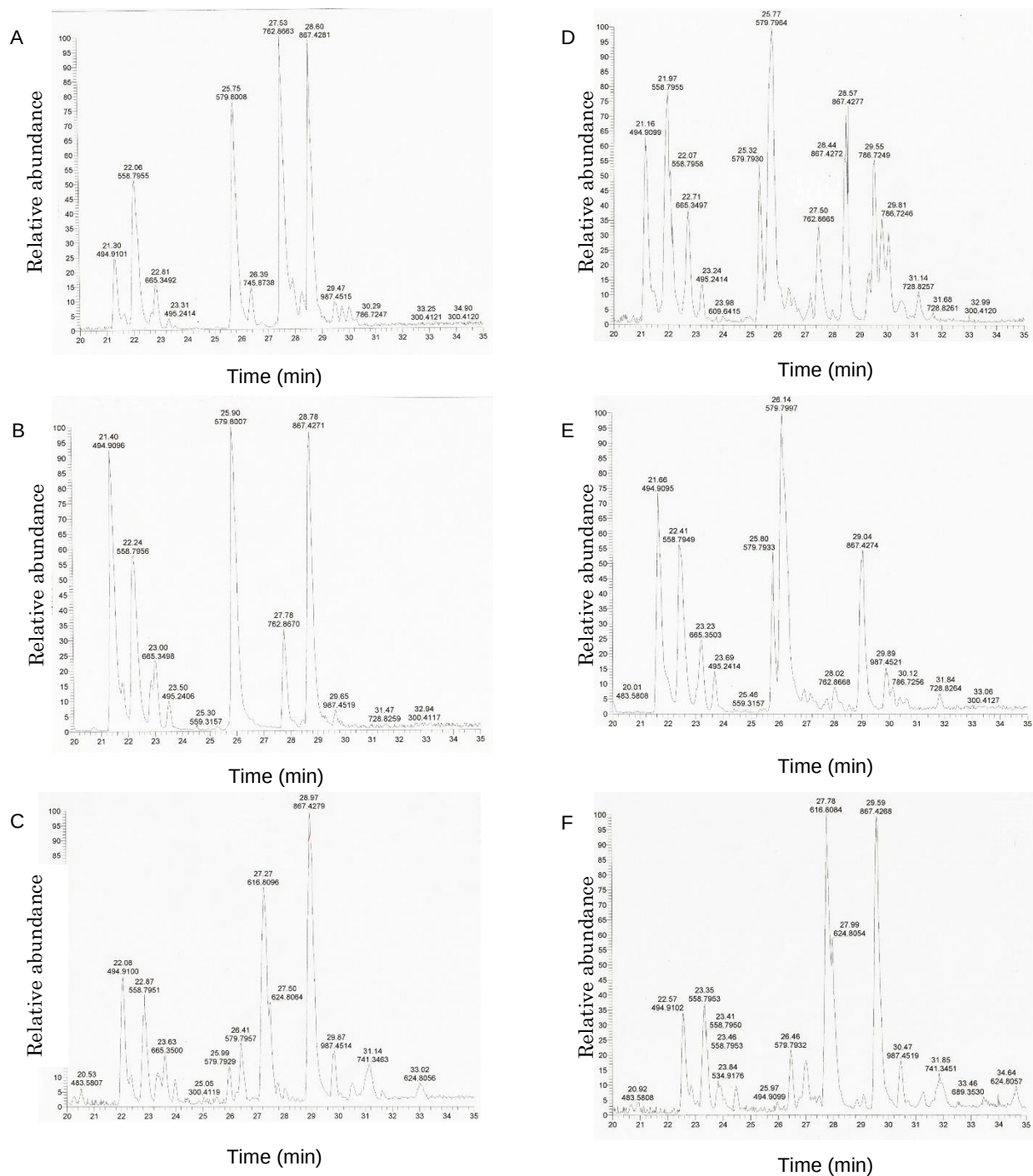


Figure 2-21. LC/MS chromatogram of fragmented insulin with modification. (A-C) LC/MS chromatograms of fragmented insulin derived from NaCl fibrils with chemical modification of Lys (A), Tyr (B), and Arg residue (C). (D-F) LC/MS chromatograms of fragmented insulin derived from SDS fibrils with chemical modification of Lys (D), Tyr (E), and Arg residue (F). In each panel, elution time and m/z values of insulin fragments are represented above peaks.

assignment of m/z values was also performed (Table 2-6). The m/z value of 616.8096 was assigned to fragment of B chain from 22-30 residues, in which Arg residue was modified as expected. From these results, chemical modification of three types of amino acids were proceeded as planned except for Tyr residue, indicating that color changes of fibril-iodine complexes are derived from environmental change of binding sites of iodine characterized by Lys and Arg side chains. However, there was a significant heterogeneity in the modified samples and there were several peaks such as m/z value of 786.7249 (Figure 2-21D) which could not be assigned. Side reactions we do not assume might occur. Besides, from these experiments we could not obtain information about A chain because of its low ionization tendency. Thus the effects of chemical modification, especially about Tyr residues, is still unknown and further investigation is necessary.

Table 2-6. Assignment of m/z values observed in Figure 2-21A, B, E, and F. In parentheses, number of residues, types of peptide (A or B chain), and modification site are represented.

m/z (observed)	Monoisotopic mass (Da)	Amino acid sequence (number of residue, peptide, modification site)
494.9100	1481.7082	FVNQHLCGSHLVE (1-13, B chain)
558.7950	1115.5754	RGFFYTPKT (22-30, B chain)
579.8007	1157.5868	RGFFYTPKT (22-30, B chain, acetyl-K)
616.8096	1231.6036	RGFFYTPKT (22-30, B chain, PG-R)
665.3492	2005.9936	ALYLVCGERGFFYTPKT (14-30, B chain, acetyl-K)
762.8663	1523.7180	FVNQHLCGSHLVE (1-13, B chain, acetyl-N term)
867.4271	866.4200	ALYLVCGE (14-21, B chain)

Discussion

In this chapter, we focused on iodine staining as a new molecular probe for amyloid polymorphism and examined basic properties of iodine staining of amyloid fibrils. Iodine staining can distinguish polymorphism of insulin and A β amyloid fibrils and can be applied other amyloidogenic proteins such as β 2-microglobulin. Reduction experiments of fibril-bound iodine molecules revealed that iodine molecules exist in solvent-accessible surface area. Spectral deconvolution and titration experiments revealed that there are several iodine-binding sites on the surface of amyloid fibrils. In addition, resonance Raman spectroscopy suggested that several iodine molecular species exist in fibril-bound form and by chemical modification of amino acid residues, we revealed that Lys and Arg, which possess positively charged side chain in acidic pH, contribute to the characterization of iodine-binding sites on the surface of cross- β structures. Though there are still unsolved problems, we propose iodine-binding model to amyloid fibrils. From the observation of iodine-stained spherulite, it is suggested that iodine molecules orient along to fibril axis. According to the previous studies about iodine-doped amylose, α -cyclodextrin, polyvinyl alcohol, iodine molecules tend to be encapsulated or to be buried in gap structures of polymeric molecules. As for amyloid fibrils, there are pleated structures formed by β -sheet structures on the surface of amyloid fibrils. We suspect that iodine molecules may be buried in these structures, and the specificity of binding is characterized by side chains of amino acid residues exposed to solvent. Results from chemical modification, electrostatic interactions between iodine molecules and side chains may play a key role for binding. The absorption of iodine-fibril complexes are thought to be derived from polymerization of iodine molecules on the surface of fibrils. In water solution, three types of iodine molecular species, I⁻, I₂, and I₃⁻ stably exist. Other molecular species such as I₅⁻ or I₇⁻ are generally unstable in water, but if, amyloid fibrils act as scaffold of iodine complexation reaction similarly with other amylose and polymers, iodine molecules polymerize on the surface of fibrils. And the differences in peak positions of absorbing components, discussed by spectral deconvolution, may be characterized by differences in degree of polymerization. Molecular species suggested by resonance Raman spectra might show species corresponding to one unit in the iodine polymers. Unfortunately, we could not determine the iodine molecular species precisely,

we will attempt to other approaches such as calculating electronically-excited state and standard vibration analysis of Raman-active modes. If the hypothesis represented above is true, we can explain the sigmoidal curves obtained by curve fitting with Hill equation. One iodine molecular species which possess high affinity to fibrils initially bind to amyloid surface and stabilize binding of other iodine species. These possibilities could be discussed by using stopped-flow monitoring absorption spectra.

Today, many amyloid probes which can detect amyloid fibrils or determine the quantity of fibrils have been reported. For example, ThT which is the most famous one can bind cross- β structures of amyloid fibrils and emit strong fluorescence at 485 nm. Compared to these amyloid probes, iodine staining is thought to be inferior from the point of view of sensitivity because almost all probes emit fluorescence upon binding to fibrils and iodine staining are based on absorption. But in the case of fluorescent probe reported so far, their specificity of binding are not so high that they can't detect specific structure of amyloid fibrils. To discuss fibril structures, iodine staining is highly sensitive for structural diversity of amyloid fibrils and can be applied to a wide range of samples.

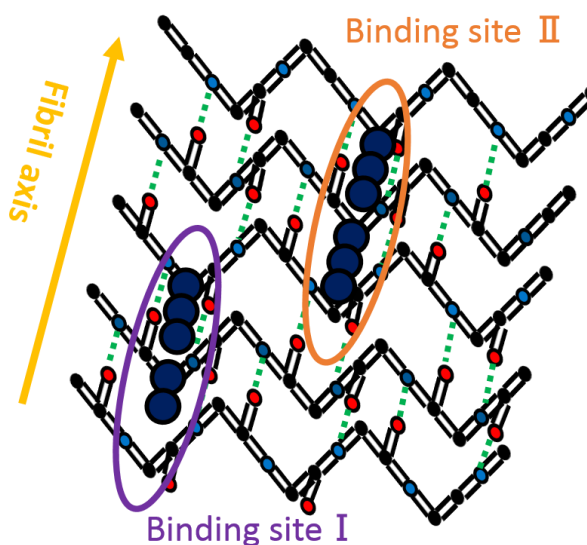


Figure 2-22. Proposed model of iodine binding to the surface structures of amyloid fibrils.

Chapter 3.

Utilizing iodine staining for medical diagnosis

3-1. Introduction

Amyloid fibrils are aberrant protein aggregates implicated in neurodegenerative and systemic diseases such as TSEs and type II diabetes¹. Due to extensive efforts in biological and medical sciences, several medicines retarding development of some amyloidosis such as Alzheimer's disease are currently available¹⁰⁷. However, these disorders are still serious for human health because of their difficulties in therapy and diagnosis.

Especially in diagnosis, there are many problems. In general, formation of amyloid fibrils proceeds via nucleation-dependent model⁵. According to this mechanism, native proteins with function change into fibril forms via oligomerization. These oligomeric species are called 'prefibrillar intermediates' and the population of them is usually quite low because of their unstability from the point of view of free energy³. Thus a detection of causal agents during the incubation period called 'lag time' is almost impossible, making prevention of amyloidosis significantly difficult¹⁰⁷. Until now, there is no reliable diagnostic method applied in vivo. In vitro, there are a few types of molecular probes which can detect oligomeric species. 8-anilino-1-naphthalenesulfonic acid (ANS), which is a fluorescent dye, is one of the most famous molecular probe for oligomeric species. ANS can bind to hydrophobic surfaces of protein, resulting in blue shift of its fluorescence. Thus ANS are often used for detecting structural transition of protein and for examining kinetics of amyloid formation during lag time⁵⁶. But it can also bind to amorphous protein aggregates so that applying it in vivo is distant. In addition, several oligomers of amyloidogenic proteins such as structural information obtained by ANS fluorescence is so limited that we can not determine what type of amyloidosis will occur. Furthermore, several oligomeric species such as A β and α -Synuclein exhibit higher cytotoxicity than fibril forms, thus the detection of prefibrillar intermediates is very important for both diagnosis and therapy.

As for diagnosis after the onset of diseases, identifying protein or peptide forming amyloid fibrils is also quite troublesome¹⁰⁷. Immunohistochemical stain and LC-MS/MS analysis to identify types of amyloidogenic protein and peptide are mainly used for diagnosis¹⁰⁷ but tissue fragments extracted from patients are so contaminated that they lack a degree of accuracy. Furthermore, recent studies have revealed that amyloid fibrils

exhibit polymorphism with distinct pathology despite of same components²² and some deposits in human body are composed of several types of proteins and peptides¹⁰⁸⁻¹¹⁰. These instances make the diagnosis of amyloidosis more complicated.

To address these issues, we tried to apply iodine staining to medical diagnosis. First, we verified that iodine staining could detect oligomeric species of amyloid fibrils. Interactions between iodine molecules and prefibrillar intermediates were slightly observed, suggesting that iodine staining could be used for detecting species with low population. Next, we verified the correlation between colors of fibrils stained with iodine and their cytotoxicity from the point of view of membrane disruption. If the correlations are confirmed, iodine staining could become a powerful probe to determine types of fibrils and their pathology.

3-2. Materials and methods

Reagents

See 'Materials and methods' in chapter 2.

Formation of insulin amyloid fibrils

See 'Materials and methods' in chapter 2. For spontaneous formation of insulin amyloid fibrils with long incubation period, insulin dissolved in 25 mM HCl at a concentration of 2 mg/ml was incubated at 37 °C under agitation (750 rpm) for 300 h.

Formation of insulin B chain amyloid fibrils

Insulin B chain dissolved in NaOH was diluted with Tris-HCl buffer (pH 8.6) at final concentrations of 1.4 mg/ml B chain, 10 mM NaCl in 50 mM Tris-HCl. For fibrillation reaction, this sample was incubated at 25 °C under agitated condition (1,200 rpm) for about 2 h.

ThT assay for insulin, B chain, and A β ₁₋₄₀ amyloid fibrils

See 'Materials and methods' in chapter 2. As for the fibrillation of B chain, the same procedure was applied.

Iodine staining of amyloid fibrils and prefibrillar species

See 'Materials and methods' in chapter 2 for the case of insulin amyloid fibrils. As for iodine staining of B chain, the sample solution of B chain was diluted with HCl solution at final concentrations of 0.14 mg/ml B chain, 1 mM NaCl, 5 mM Tris HCl, 0.6 mM KI, and 0.08 mM I₂ in 90 mM HCl and was subjected to UV-vis spectroscopy. As a control, sample without B chain was also prepared and measured.

Preparation of liposome solution

All unilamellar liposomes used in this study were prepared by the exclusion methods¹¹¹. 10 mg of POPC and POPG were dissolved in 1 ml of CHCl₃ and then the solvent was completely removed by a rotary evaporator. Dried lipid film was resuspended in 10 mM HEPES (pH 7.4) containing 200 mM calcein and the solution was subjected five freeze-

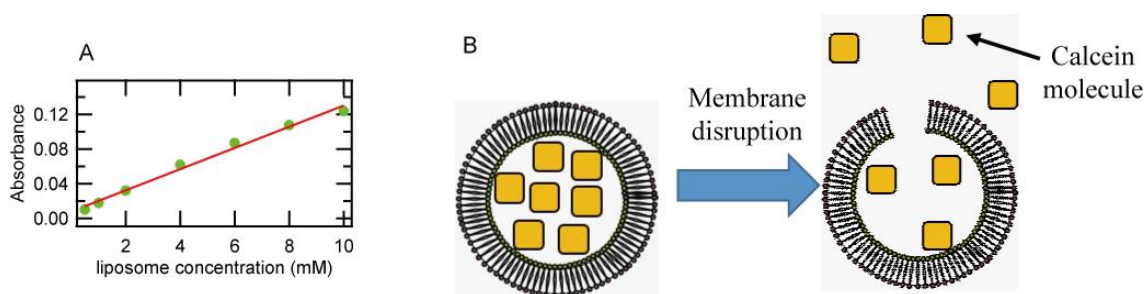


Figure 3-1. Preparation of liposome. (A) Calibration curve constructed by measuring liposome solution not containing calcein with known concentrations of lipid molecules. (B) Schematic illustration of calcein release assay. In the initial state, calcein molecules are contained inside the lipid bilayer and self-quench. By disrupting membrane, calcein molecules are released from liposome, resulting in enhancement of calcein fluorescence.

thawed cycles using liquid nitrogen and a water bath. The solution was subsequently extruded 21 times through 100 nm pore size filters (Whatman, UK) with mini-extruder (Avanti Polar Lipids, Inc., USA) and finally unencapsulated calcein molecules were removed using PD-10 columns (GE Healthcare, USA). The purification of calcein-encapsulated liposome was repeated 5 times. To determine the concentration of liposome containing calcein, liposome solution which do not contain calcein was prepared and calibration curve was constructed (Figure 3-1A). The diameter of the liposome was confirmed by measuring DLS with Zetasizer Nano S (Malvern, UK).

Calcein release assay

Prepared liposome solution containing 200 mM calcein inside the liposome was diluted by 10 mM HEPES (pH 7.4) at a final concentration of 500 μ M in 10 mM HEPES. Fluorescence spectrum of this sample was measured in the range from 450 to 600 nm by exciting at 490 nm as an initial state with a quartz cell. Then 10 μ l of fragmented fibrils prepared as the same procedure as seed preparation was mixed with 200 μ l of the liposome solution and measured fluorescence spectrum. After the mixing, fluorescence spectra were measured at different time points. It should be noted that during the interval of measurement the sample was shielded from light for preventing bleaching of calcein molecules. At last, 5 μ l of 5 % Triton-X100 was added to disrupt liposome completely and measured spectrum as a reference.

As control experiments, 10 μ l of 25 mM HCl and 2 mg/ml native insulin in 25 mM HCl, was added instead of fibril solution and measured time course of fluorescence spectra as the same way. Time course of calcein fluorescence without additives was also measured.

3-3. Results

Iodine staining for prefibrillar oligomeric species during lag time

In chapter 2, we verified that iodine staining can distinguish polymorphism of amyloid fibrils and their amplifications in the elongation phase. In this chapter, we examined whether iodine staining can detect prefibrillar intermediates with low population and furthermore provide structural information about them. In this experiment, insulin dissolved in 25 mM HCl at a concentration of 2 mg/ml containing 100 mM NaCl was incubated at 65 °C. Previous studies revealed that insulin protein readily form amyloid fibrils in acidic condition with high temperature^{99, 101}.

As a result, the reaction finished within 2 hours, as shown by time course of ThT fluorescence intensity (Figure 3-2A). In parallel, we performed iodine staining and UV-vis spectroscopy at the same time points (Figure 3-2A, insert). It should be noted that in the chapter 2, we performed similar experiments monitoring fibrils growth for seeding reaction (Figure 2-10 and 2-11), in which we focused on the elongation phase of fibrils. In contrast, in this experiments we focused on prefibrillar oligomers and monitored

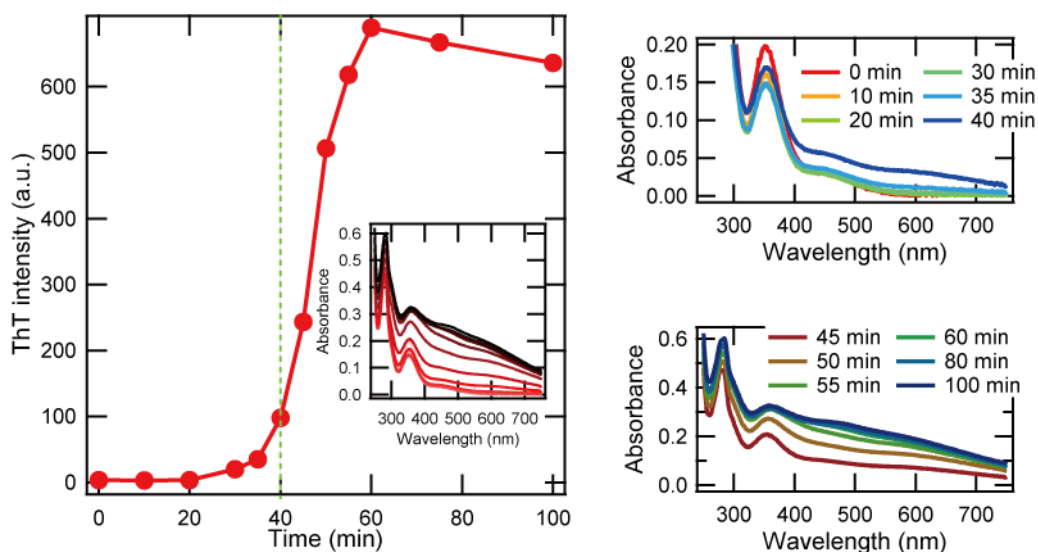


Figure 3-2. Time course monitoring of spontaneous fibril formation of insulin at high temperature. (A) Time course of ThT fluorescence intensity monitoring spontaneous fibril formation. The insert shows UV-vis spectra of iodine-mixed samples obtained at the same time points. The deeper the color became, the later the spectrum was measured. (B and C) Time course of UV-vis spectra of iodine-mixed samples at different time points. Color codes are defined in the legends, specifying time points at which the spectra were measured.

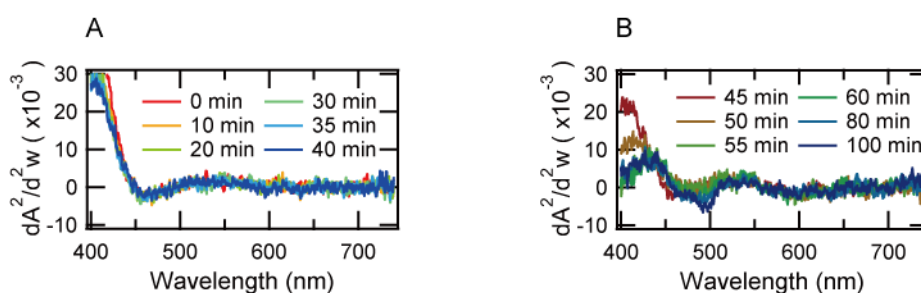


Figure 3-3. Second derivatives of the absorption spectra of iodine-mixed insulin samples. (A and B) Second derivatives of the spectra from 0 to 40 min (A) and 45 to 100 min (B).

spontaneous fibril formation. In general, ThT fluorescence is silent during the lag time because of its low affinity to oligomeric species. The intensity of ThT fluorescence started to increase around 40 min, indicating that formation of nucleus finished within 40 min. In the time domain, UV-vis spectra of iodine-stained samples exhibited small change from (Figure 3-2B). An absorption band around 360 nm became small from 0 to 10 min, suggesting a change in electronic state of I_3^- ions by interactions with prefibrillar oligomeric species. From 30 to 35 min, a slight increase of absorption in long-wavelength region (400-750 nm) was observed. This change might be derived from complexation of iodine molecules by binding to oligomers. After the nucleation phase, absorption in long-wavelength region increased significantly (Figure 3-2C) in accordance with the result of ThT monitoring (Figure 3-2A). To examine the spectral change during lag time more detail, we obtained second derivatives of the spectra (Figure 3-3), but differences in positions of local minima could not be observed, suggesting that differences in absorption spectra may be due to amplitude or baseline derived from iodine-oligomeric species.

These results suggest that iodine staining could recognize prefibrillar species with low population. However, experimental condition used above is an extraordinary one compared to that of physiological one and the formation of fibril finished quickly. To investigate spectral change by iodine staining more detail, we selected a mild condition for slowing insulin fibrillation. Insulin protein dissolved in 25 mM HCl at a concentration of 2 mg/ml was incubated with agitation (750 rpm) at 37 °C. By performing fibrillation reaction at an ordinary temperature for biosystems, we examined whether iodine staining can recognize oligomeric species in long incubation period. If the samples were in

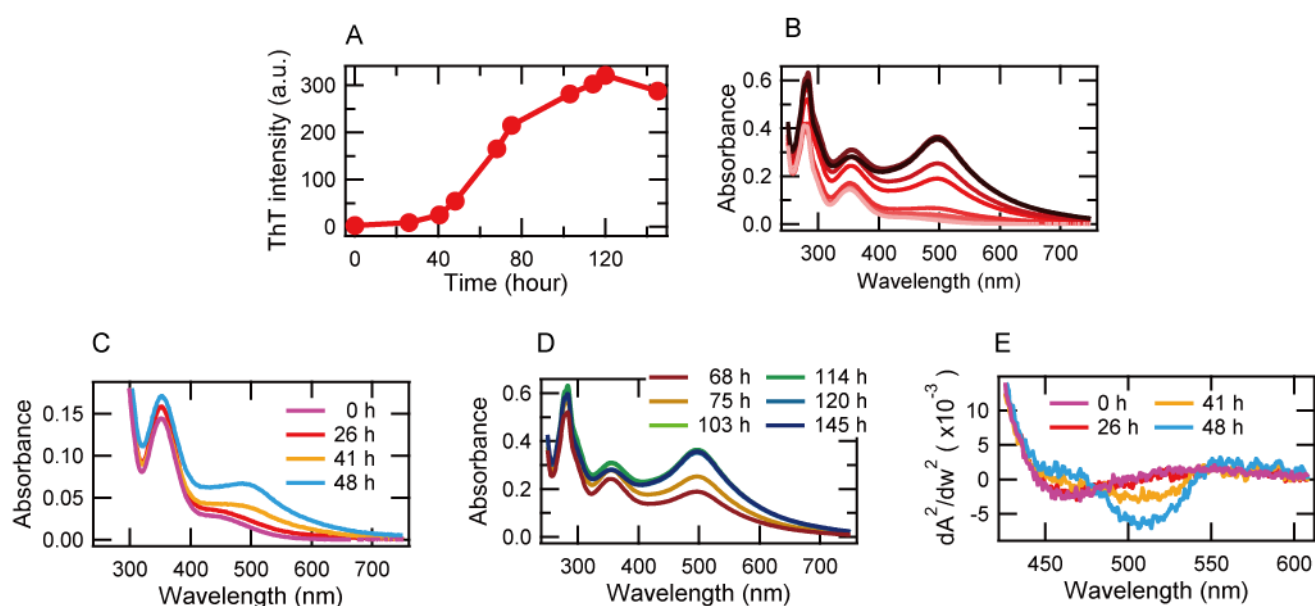


Figure 3-4. Time course monitoring of insulin amyloid fibril formation in a mild condition. (A) Time course of ThT fluorescence intensity monitoring spontaneous fibril formation. (B) Time course of UV-vis spectra of iodine-mixed samples obtained at the same time points. The deeper the color became, the later the spectrum was measured. (C and D) Time course of UV-vis spectra of iodine-mixed samples at different time points. Color codes are defined in the legends, specifying time points at which the spectra were measured. (E) Second derivatives of the absorption spectra shown in panel C.

quiescent condition, it would take several weeks or months for finishing the fibril formation and thus samples were agitated to acceralate the reaction mildly. Fibrillation reaction was monitored by ThT (Figure 3-4A) and iodine staining (Figure 3-4B) as the same procedure as mentioned above. From the result of time course of ThT intensity (Figure 3-4A), whole the reaction and the nucleation phase finished around 120 and 40 hours, respectively. Absorption around 500 nm gradually increased during the lag phase (Figure 3-4C) and became significantly large in the elongation phase (Figure 3D). Furthermore, second derivatives of the spectra during lag phase showed the shift of an absorption maximum derived from iodine molecules, suggesting interactions between fibrils and iodines (Figure 3-4E). These results may suggest that iodine staining could be applied to the detection of causal agents within an incubation period.

Next, we focused on insulin B chain and its unique characteristic. As mentioned above, population of prefibrillar intermediates is quite low because of their unstability, but as for several proteins and peptides, including insulin B chain, an accumulation of those species can be observed (unpublished data). We examined that oligomeric species of B chain can be stained with iodine, and furthermore structural information of those can be extracted by iodine staining.

Results of ThT assay exhibited characteristic pattern in which intensity of ThT increased by two step (Figure 3-5A). First step from 0 to 100 min, corresponds to the formation of oligomeric species. After reaching plateau at 100 min, corresponding to second step, intensity of ThT increased dramatically and fibrils formed. We took fraction of the sample around 80 min and stained it with iodine (Figure 3-5B). It should be noted that the condition of iodine staining is clearly different from those of other fibrils such as insulin and A β fibrils because of the reaction condition of B chain. Fibril formation of B chain was performed in Tris-HCl buffer at pH 8.6, and in our systems acidic condition was suitable for iodine staining. That's why we used this condition to set value of pH less than 2. In addition, high concentrations of iodine molecules were used to compensate decoloration of iodine molecules due to reduction (Figure 2-8) by Cys residues in B chain. In fact, absorption of iodine solution was larger than that of solution mixed with B chain solution (Figure 3-5B) and there was no absorption in long-wavelength region (> 500 nm), suggesting that oligomers of B chain cannot react with iodine. There is also possibility that oligomeric species of B chain dissociate in iodine solution. Up to now, the oligomeric species of B chain was observed under basic pH condition, and thus transferring them into acidic solution might induce dissociation of oligomers.

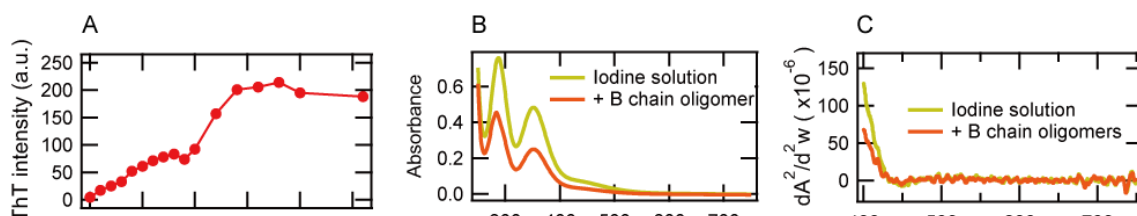


Figure 3-5. Iodine staining of insulin B chain. (A) Time course of ThT fluorescence intensity. Two step reaction from 0 to 100 and 100 to 200 min can be observed. (B and C) UV-vis spectra of B chain oligomers mixed with iodine and their second derivatives (C). Color codes are defined in the legends, specifying conditions of samples.

Correlation between colors of iodine-stained fibrils and their cytotoxicity

The results in chapter 2 indicate that colors of iodine-fibril complexes reflect their surface structures and it is expected there is a correlation between their colors and properties such as cytotoxicity. Thus we examined relationship between cytotoxicity of prepared fibrils and colors. First, we analyzed an ability of membrane disruption of fibrils by calcein release assay. In this experiment, fluorescent calcein molecules are encapsulated in liposomes composed of two types of lipid molecules. In a condensed condition of calcein (above 10 mM), calcein molecules quench fluorescence each other so that quantum yield of them becomes quite little (Figure 3-1B)¹¹² When liposomes containing calcein are disrupted, apparent concentration of calcein become low, resulting in enhancement of fluorescence intensity. Thus the liposome containing calcein can be used as an indicator of the ability of membrane disruption (Figure 3-1B)¹¹³. For this experiment, fibrils were fragmented by sonication to equalize their length and thickness

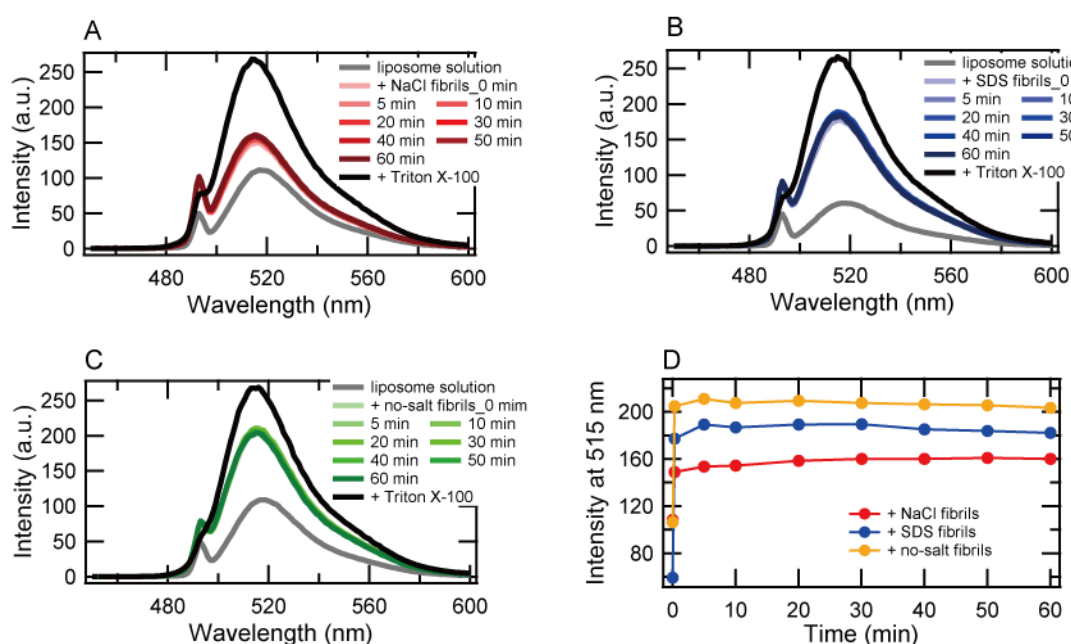


Figure 3-6. Correlation between membrane-disruption ability and colors of fibril-iodine complexes of prepared insulin amyloid fibrils. (A-C) Time course of calcein fluorescence intensity after adding NaCl (A), SDS (B), and no-salt fibrils (C). Color codes are defined in the legends, specifying conditions of the samples. (D) Time course of calcein fluorescence intensity at 515 nm in the case of adding NaCl fibrils (red), SDS fibrils (blue), and no-salt fibrils (orange), respectively.

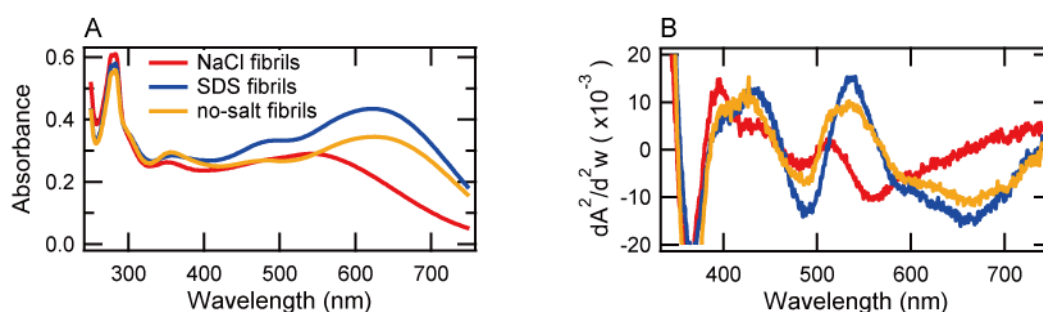


Figure 3-7. Iodine staining of three types of insulin amyloid fibrils. (A and B) UV-vis spectra of iodine-stained fibrils (A) and second derivatives of the spectra (B). Color codes are defined in the legends in panel A, specifying types of fibrils.

for experimental reproducibility.

As a result, all prepared fibrils disrupted liposomes and differences of fluorescence intensity were observed (Figure 3-6, A-C). After disruption of liposomes completely by an addition of Triton X-100, they exhibited similar fluorescence intensity, indicating that comparison among them is reasonable. Among them, no-salt fibrils exhibited the highest membrane-disruption ability (orange) and the percentage of release was about 63 %. The percentage of NaCl (red) and SDS fibrils (blue) were about 38 % and 52 %, respectively (Figure 3-6D).

From those results, SDS and no-salt fibrils exhibited higher cytotoxicity than that of NaCl fibrils. Comparing these fibrils from the point of view of colors of iodine-fibril complexes, SDS and no-salt fibrils stained with iodine exhibited similar spectral shape except for an amplitude, especially about the positions (Figure 3-7, A and B). On the other hand, NaCl fibrils stained with iodine exhibited clearly different positions from those of SDS and no-salt fibrils. Thus the correlation between membrane-disrupting ability and color of iodine-fibril complexes may exist, and based on only these results, amyloid fibrils with high membrane-disruption ability exhibit absorption around 650 nm when they are stained with iodine.

Control experiments of calcein release assay were also performed. First, we monitored time course of calcein fluorescence without any additives (Figure 3-8A) to confirm that liposomes disrupted by time. As a result, the intensity of calcein fluorescence slightly decreased, which were due to bleaching of calcein and after adding Triton-X, the intensity significantly became larger, indicating that liposomes are stable under this condition and

measurement time. Second, we added 10 μ l of 25 mM HCl which was solvent of fibril samples (Figure 3-8B). Similarly with the case of non-additive conditions, fluorescence intensity slightly decreased and became significantly large after adding Triton-X, suggesting that HCl solvent have no effects on liposome. Last, we added 2 mg/ml native insulin (Figure 3-8C). In this case, fluorescence intensity significantly increased immediately after adding insulin. It is reported that native insulin have an affinity to lipid molecules containing palmitoyl group such as POPC and POPG. This results suggested that the differences in membrane-disruption ability among fibrils were derived from not fibril structures but amount of residual native insulin. However, after the formation of insulin amyloid fibrils, there are little native insulin in the solution confirmed by ultracentrifugation. That's why the differences discussed above are conceived to be derived from fibril properties.

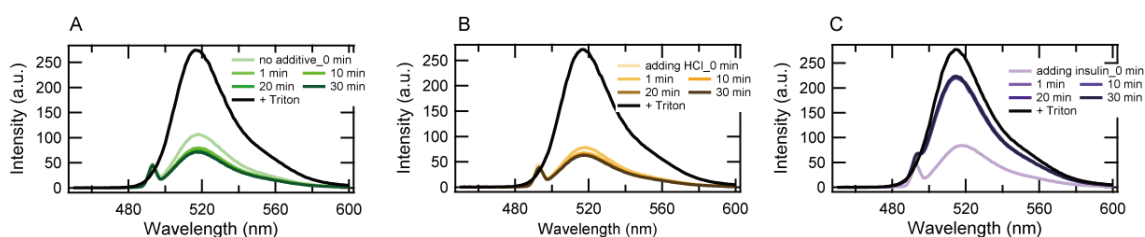


Figure 3-8. Time course of calcein fluorescence intensity in the presence of several types of additives. (A) Time course of calcein fluorescence spectra without any additives. (B and C) Time course of calcein fluorescence spectra after adding 10 μ l of 25 mM HCl (B) and 2 mg/ml insulin solution (C).

3-4. Discussion

As for the detection of oligomeric species by iodine staining during lag time, we tested spontaneous formation of insulin amyloid fibrils under two different conditions, high temperature (65 °C) and low temperature (37 °C) with agitation (750 rpm), and insulin B chain. For both fibrillation conditions of insulin, we could observe spectral change during lag time, suggesting that iodine staining can detect oligomeric species. Fibril formation of these conditions proceed via typical nucleation-dependent model with low population of prefibrillar intermediates. In contrast, as fibrillation reaction of insulin B chain in which the accumulation of oligomeric species during lag time, we could not detect them. As mentioned above, many factors which might cause dissociation of oligomeric species could be thought, thus further investigation is necessary for B chain fibrillation. In addition, it is worth a try with other amyloidogenic proteins such as A β , β 2m, α SN, and others. Especially about A β and α SN, the accumulation of intermediates is reported so that they can be a benefit model. Considering specificity of iodine molecules to amyloid fibrils, iodine could recognize β -sheet or β -sheet like structures. As for A β peptide, it is reported that oligomeric species contain β -sheet structures in the presence of lipid bilayer, expecting that interactions between those molecules may occur.

For the correlation between cytotoxicity and colors of fibril-iodine complexes, we tested three types of insulin amyloid fibrils, NaCl, SDS, and no-salt fibrils. Among them, no-salt fibrils exhibited the highest cytotoxicity, and SDS fibrils possessed higher cytotoxicity than that of NaCl fibrils. Observing absorption spectra of those fibrils stained with iodine, no-salt and SDS fibrils exhibited similar spectral patterns, especially in the positions of the peaks. These results suggest that fibrils exhibiting spectral patterns similarly with those of no-salt and SDS fibrils possess higher cytotoxicity. To confirm this hypothesis, additional experiments with other types of fibrils are necessary.

Chapter 4.

General conclusion

In this work, we addressed basic properties of iodine staining of amyloid fibrils and verified the utility of it in structural analysis of fibrils and medical applications. The most progressive characteristics of iodine staining is that it can clearly distinguish polymorphism of insulin and A β amyloid fibrils despite the same protein. Besides, this method can be applied to other amyloidogenic proteins, suggesting that it can cover a wide range of samples which are not suitable for spectroscopic methods such as ssNMR and EPR. We examined the mechanism of amyloid-iodine interactions and revealed that iodine molecules bind to surface structures of amyloid fibrils. Spectral deconvolution and titration experiments revealed that several types of iodine-binding sites exist on the surface of amyloid fibrils. Observation of spherulites by using polarized microscopy revealed that iodine molecules orient along to fibril axis. By combining these results, we suspect that upon binding to fibrils, iodine molecules are buried in the pleated structure in the surface of cross- β structures and iodine molecules polymerize with the same direction of fibril elongation.

As for an application of medical diagnosis, iodine staining exhibited potential for both early detection of prefibrillar intermediates and diagnosis of fibril cytotoxicity. However, the number of samples is quite inadequate. Further accumulation of data will be necessary.

In conclusion, iodine staining exhibited promising characteristics in distinguishing polymorphism of amyloid fibrils and can be applied to a wide range of samples and purposes such as medical diagnosis. These properties are not observed among existing probes such as ThT, suggesting that iodine staining can be a powerful tool for amyloid research.

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