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Review

ATR-FTIR: A "rejuvenated" tool to investigate amyloid proteins ☆

Rabia Sarroukh, Erik Goormaghtigh, Jean-Marie Ruysschaert *, Vincent Raussens

Center for Structural Biology and Bioinformatics, Laboratory of Structure and Function of Biological Membrane, Faculté des Sciences, Université Libre de Bruxelles (ULB), Campus de la Plaine CP 206/2, Boulevard du Triomphe, B-1050, Brussels, Belgium

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ABSTRACT

Amyloid refers to insoluble protein aggregates that are responsible for amyloid diseases but are also implicated in important physiological functions (functional amyloids). The widespread presence of protein aggregates but also, in most of the cases, their deleterious effects explain worldwide efforts made to understand their formation, structure and biological functions. We emphasized the role of FTIR and especially ATR-FTIR techniques in amyloid protein and/or peptide studies. The multiple advantages provided by ATR-FTIR allow an almost continuous structural view of protein/peptide conversion during the aggregation process. Moreover, it is now well-established that infrared can differentiate oligomers from fibrils simply on their spectral features. ATR-FTIR is certainly the fastest and easiest method to obtain this information. ATR-FTIR occupies a key position in the analysis and comprehension of the complex aggregation mechanism(s) at the oligomer and/or fibril level. These mechanism(s) seem to present strong similarities between different amyloid proteins and might therefore be extremely important to understand for both disease-associated and functional amyloid proteins. This article is part of a Special Issue entitled: FTIR in membrane proteins and peptide studies.

Contents

Abbreviations: AD, Alzheimer's disease; ADDLs, amyloid-beta derived diffusible ligands; ANS, 8-anilino-1-naphthalenesulfonic acid; ATR, Attenuated total reflection; Aβ, Amyloid beta; CD, circular dichroism; EM, electron microscopy; EPR, electron paramagnetic resonance; FTIR, Fourier-transform infrared spectroscopy; HETs, prion of the filamentous fungus P. anserine; hIAPP, human Islet Amyloid Polypeptide; IAPP, Islet Amyloid Polypeptide; IRE, internal reflection element; PrP, prion protein; PrP^{C}, cellular prion protein; PrP^{Sc}, pathological (scrapie) isoform of the prion protein; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; SH3 domain, Src homology 3 domain; ssNMR, solid-state Nuclear magnetic resonance; ThT, Thioflavine T; TTR, Transthyretin; WB, Western Blot

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* Corresponding author. Tel.: +32 2 650 5377.
E-mail addresses: rsarrouk@ulb.ac.be (R. Sarroukh), egoor@ulb.ac.be (E. Goormaghtigh), jmruyss@ulb.ac.be (J.-M. Ruysschaert), vrauss@ulb.ac.be (V. Raussens).

1. Introduction

Amyloid refers to insoluble protein aggregates that are responsible for amyloid diseases (e.g. Alzheimer's, Parkinson's, Huntington's, prion's diseases, type II diabetes, and also some cancers) affecting large populations worldwide [1,2]. But they are also implicated in the binding and storage of peptide hormones in the brain, formation of bacterial biofilms, melanin formation and initiation of antiviral innate immune response (this latter category is nowadays referred to as "functional amyloids" and the number of proteins involved in these is

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expected to increase rapidly in the near future) [3–7]. The widespread presence of protein aggregates but also, in most the cases, their deleterious effects explain worldwide efforts made to understand their formation, structure and biological functions [8].

Amyloid fibrils present an unbranched filamentous morphology [9] and are characterized by β-strand repeats running perpendicular to the fiber axis. This structure named cross-β is a unique motif among protein folds [2,10]. Whether any protein is able to form amyloids is still a matter of debate and reassesses our current knowledge underlying the principles of protein folding, misfolding and aggregation. Two apparently contradictory theories are currently proposed. The first one postulates that fibril formation is a general property occurring for any polypeptide chain in some circumstances (sometimes far from any physiologically relevant conditions) [11], while the second restricts their formation to specific proteins collectively classified as the 'amylome' [12].

Extensive efforts have been carried out during the past few years to understand the pathways leading to this structural conversion [13]. Fibril formation is presently described by a two-step process. The *nucleated conformational changes* conversion of the native polypeptide chain promotes oligomer species (Fig. 1). This first step is often subdivided into a lag phase and a nucleation phase. The *elongation* process leads to mature fibrils by a self-template growth mechanism [14–16]. Fibrils can also undergo fragmentation which enhances the kinetics of the aggregation process by increasing their number and suppressing the limiting step of misfolded conformer's production [17–19]. Those two misfolding processes are not exclusive and may occur concomitantly.

The common structural motif shared by fibrils for all amyloidogenic proteins have suggested a common mechanism of aggregation and toxicity [20–22]. Why and how protein aggregates is largely not understood. Moreover, during the aggregation several species are formed and do not contribute in the same way to cell toxicity. It is not clear whether specific species (fibrils [23], oligomers [24,25]) or membrane-associated conformational changes [26,27] lead to cell death. This raises the question of a relationship between structure and cytotoxicity [28–34].

Fibrils have been characterized using multiple biophysical techniques such as Electron Microscopy (EM) [9,35,36], Atomic Force Microscopy (AFM) [37–40], Electron Paramagnetic Resonance (EPR) [41–44] and at higher resolution using solid state Nuclear Magnetic Resonance (ssNMR) [45–50] and X-ray diffraction [9] (for reviews see [10] and [2]). On the other hand, little information on the structure and properties of oligomers is available and no real agreement has been currently reached to describe their function in the whole aggregation process [10,45,51–53] (Fig. 1).

As previously mentioned, biophysical tools have been nicely exploited to provide important information regarding the 3D structure of fibrils [35,54–56]. The main limitation of these high resolution techniques is the important amount of protein material required, the recording time and the necessity to deal with insoluble (or at least hydrophobic or amphipathic) aggregates which above all are in constant equilibrium between different aggregated states. Fourier-transform infrared spectroscopy (FTIR) offers a unique opportunity to overcome those limitations and allows the characterization of the aggregates formed during the aggregation. Recording time is short, preventing time dependent structural changes. A few μg of protein is sufficient to evaluate the secondary structure of aggregates. Moreover, contrary to circular dichroism (CD), in FTIR β-sheet contribution has the highest absorption coefficient; therefore FTIR is particularly well-adapted to analyze β-sheet-rich proteins. Keeping all the advantages of "classical" FTIR, attenuated total reflection (ATR) (sometimes also referred to as attenuated total reflectance) FTIR provides additional extremely useful features when studying amyloid proteins. ATR-FTIR overcomes the problem of insolubility because the sample is analyzed as a thin film on the surface of an internal reflection element (IRE). Moreover, proteins (and more generally molecules) in this thin film can be oriented; therefore, linear dichroism experiments can provide useful information about the relative orientation of the corresponding dipoles which could then be related to the orientation of secondary structures. Recent ATR devices require less than 100 ng of proteins to record good quality

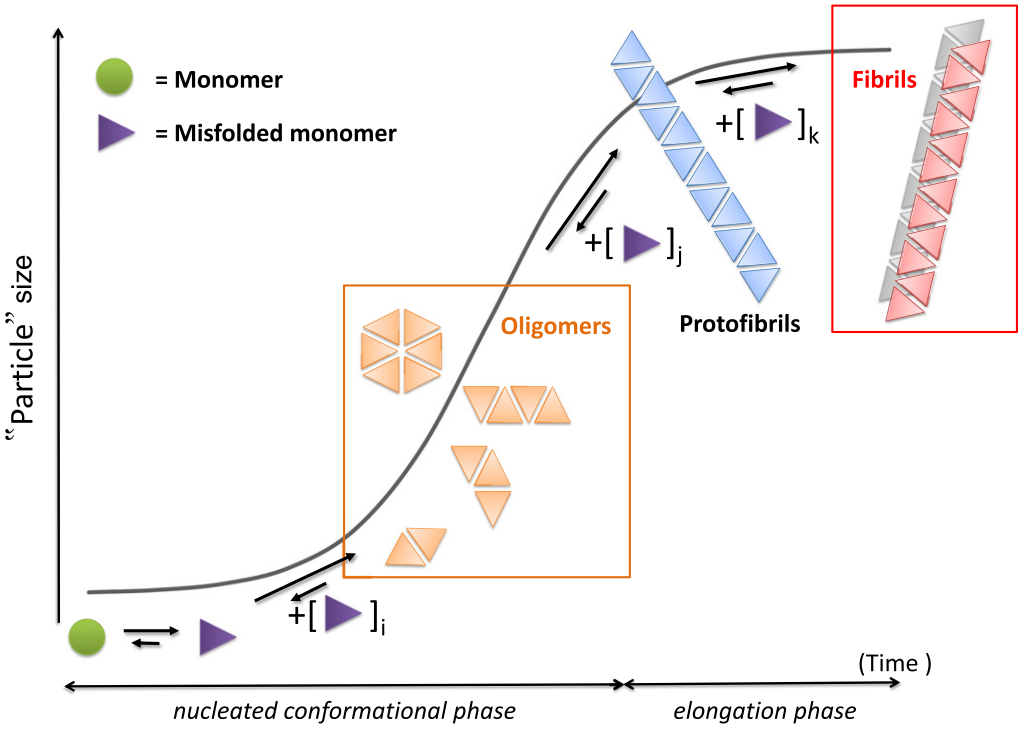

Fig. 1. Schematic representation of the aggregation process of amyloid proteins. This scheme displayed different entities or 'particles' observed along the process including oligomers at the end of the nucleation phase and before the elongation one. During the nucleation phase, native monomers are transformed into misfolded ones and then started to aggregate into bigger particles (collectively called oligomers). During the elongation phase, addition of misfolded monomers and/or oligomers at the extremities of protofibrils and association of protofibrils aimed at fibril formation.

spectra. Many excellent reviews have been published over the years describing all the properties and advantages of ATR-FTIR [57–64].

2. Structural features of amyloid fibrils highlighted by (ATR)-FTIR spectroscopy: a brief survey

Fibrils are currently well-characterized structurally. The diffraction pattern of cross-β fibrils was first observed by Astbury in 1935 [65], and next modeled by Corey and Pauling [66]. Fibril structures are made of proteins/peptides or protein segments stacked along the fiber axis. The packing results in protein sheets running parallel to each other.

2.1. FTIR and amyloid fibrils: a tentative clarification of a historical putative mistake

While FTIR spectroscopy was used since early 1970 to investigate amyloid fibrils [67,68], the apparent discrepancy between the IR spectral analysis and later NMR findings about fibrils [47,55,69] have for a long time put FTIR in the shade when coming to fibril analysis. Indeed, the first results obtained demonstrated that fibrils displayed a typical anti-parallel β-sheet structure characterized by two bands in the amide I: a low frequency, high intensity band around 1620–1630 cm^{-1} and a high frequency, low intensity band located around 1685–1695 cm^{-1}. Extensive theoretical and experimental works have demonstrated that the simultaneous presence of these two bands is assigned to anti-parallel β-sheet while the presence of only the low frequency band around 1630–1640 cm^{-1} is attributed to parallel β-sheet [70–75]. Unfortunately, latter NMR studies [47,55,69] and nowadays high resolution structure [76–79] all pointed to parallel β-sheet structure in the vast majority of amyloid fibrils.

Therefore, FTIR was supposedly wrong and it was stated that fibrils had specific and non-typical β-sheet spectral features. Since then FTIR was only used to prove the presence and especially the increase of β-sheet content upon fibrillization without further assignment of the relative orientation of the strands in the sheet. It is true that in some cases, especially with ATR-FTIR, special care should be taken when recording and analyzing protein spectra, as elegantly demonstrated by Keith Oberg while in Tony Fink's lab [80]. Later on, the late and highly regretted Tony Fink also demonstrated that specific supersecondary structures like beta-helices could lead to unexpected spectral features [81].

Nevertheless, at the turn of the century, a few works on some well-defined amyloid fibrils indicated that the remaining high frequency band (1685–1695 cm^{-1}) could be due to residual other structures in the samples. Bouchard et al. in Dobson's and Robinson's lab [82] showed that insulin at pH 2.3 aggregates upon heating at 70 °C. While the FTIR spectrum once more showed the two typical bands for anti-parallel β-sheet for short incubation times, upon longer incubation (18 h) the high frequency component at 1690 cm^{-1} completely vanished consistent with a parallel β-sheet organization. Zurdo et al. [83] incubated at low pH a Src homology 3 domain (SH3) domain of the β subunit of bovine phosphatidylinositol-3′-kinase. To demonstrate that no other structure than fibrils is present in the sample, they add pepsin to digest any non-fibrillar structure (amyloid fibrils are highly resistant to proteolysis). During the pepsin treatment, FTIR spectra showed the disappearance of the high frequency band of the β-sheet contribution. A nine residue peptide from Sup35 forming amyloid-like crystals has been studied by Balbirnie et al. in Eisenberg's lab [84]. Extremely pure and well-characterized, this crystal was submitted to FTIR and showed only the low frequency band (1633 cm^{-1}) typical of parallel β-sheet.

This could explain the discrepancy between early works and more recent applications of FTIR in fibril studies. Moreover, if mature fibrils made by full length amyloid proteins are essentially in a parallel β-sheet conformation, we think it is now well-accepted that shorter peptides (usually encompassing only one strand) have a higher propensity to adopt an anti-parallel conformation. Also, for decades, nobody in the field had imagined that other structures like oligomers (see below) could even exist and can be in a dynamic equilibrium with fibrils.

Despite this historical "glitch", FTIR played an important role in the study of amyloid fibrils, as we will briefly survey here with a specific emphasis on ATR-FTIR when possible. We refer the reader to the more exhaustive and excellent review on this topic by P.H. Axelsen in this same issue.

2.2. Studying the β-sheet structure formation

In the early 1990s, the pioneering work on amyloid fibrils carried out by Gasset and Baldwin in Stanley Prusiner's group [85,86] reported for the first time the β-structure signature of amyloids using ATR-FTIR with bands at 1685–1695 and 1623 cm^{-1} in the amide I band. Conversion of the cellular prion protein (PrP^{C}) to its infectious form PrP^{Sc} is associated with a decrease in the α-helical and turn structures and an increase in the β-sheet structure [87]. More importantly, a quantification of the secondary structure demonstrates that increasing β-sheet content in prion rods is accompanied by an assembly into fibrils and an increase in infectivity [85,86]. This was extremely important information to reinforce the Prusiner's prion theory.

Later, comparing globular proteins (with at least 30% of β-sheet structure) to amyloid fibrils revealed a major difference in the band position assigned to β-structure [88]. For example, transthyretin (TTR) in its globular state displays an absorption maximum in the Amide I band located at 1630 cm^{-1} and at 1615 cm^{-1} while in fibrils.

In conclusion, amyloid fibrils have a spectral signature clustering between 1611 and 1630 cm^{-1}, while for native β-sheet proteins it extends from 1630 to 1643 cm^{-1}. These differences are correlated to inherent β-sheet properties like the assemblage in longer sheet, longer β-strand formation as well as more planar sheet formation [89,90].

More recently, we used ATR-FTIR to study well-characterized and well-defined amyloid fibrils made with Alzheimer's amyloid-β peptides (Aβ), Aβ 42 [29], Aβ 40 [91] (Fig. 2A, a–d), α-synuclein [33], and lysozyme [92]. In all cases, we could observe only the low frequency peak (around 1626–1630 cm^{-1}) characteristic of parallel β-sheet demonstrating that ATR-FTIR is also a convenient and versatile tool to study amyloid fibril structures.

2.3. Conformational changes promoting fibril formation

Highlighting and understanding the initial conformational changes favoring the structural formation of fibrils is a challenging process in protein folding and misfolding. Due to the repeated structure motif observed in fibrils, studies started to investigate molecular events that might influence β-hairpin formation and/or stabilization like secondary structure conversion, β-turn formation and possible side-chain interactions stabilizing β-sheets.

Independent detection of secondary structures on IR spectrum allows demonstrating that β structure (1637–1613 cm^{-1}) is promoted by the destabilization of the α-helices (1662–1645 cm^{-1}) for various systems like Aβ [93], the insulin protein [82,94] and β lactoglobulin [95]. Janek et al. demonstrated for Aβ 42 that a substitution of L-amino acids by their D-enantiomers directed formation of fibrils (increase in 1634 cm^{-1} peak) by destabilizing α-helices (1654 cm^{-1}) along the Aβ 42 peptide [96]. They highlighted that substitution located in a region encompassing residues 11–24 in monomers promoted β-sheet structures to a larger extent compared to substitution in other regions [96].

Those data raised the link between α-to-β conformational changes and amyloidogenicity of proteins as earlier proposed by Prusiner on fragments of PrP [87]. In line with this observation, Murakami et al. [97] provide the first predictions of secondary structure content by analyzing ATR-FTIR spectra of Aβ monomers and fibrils of various Aβ

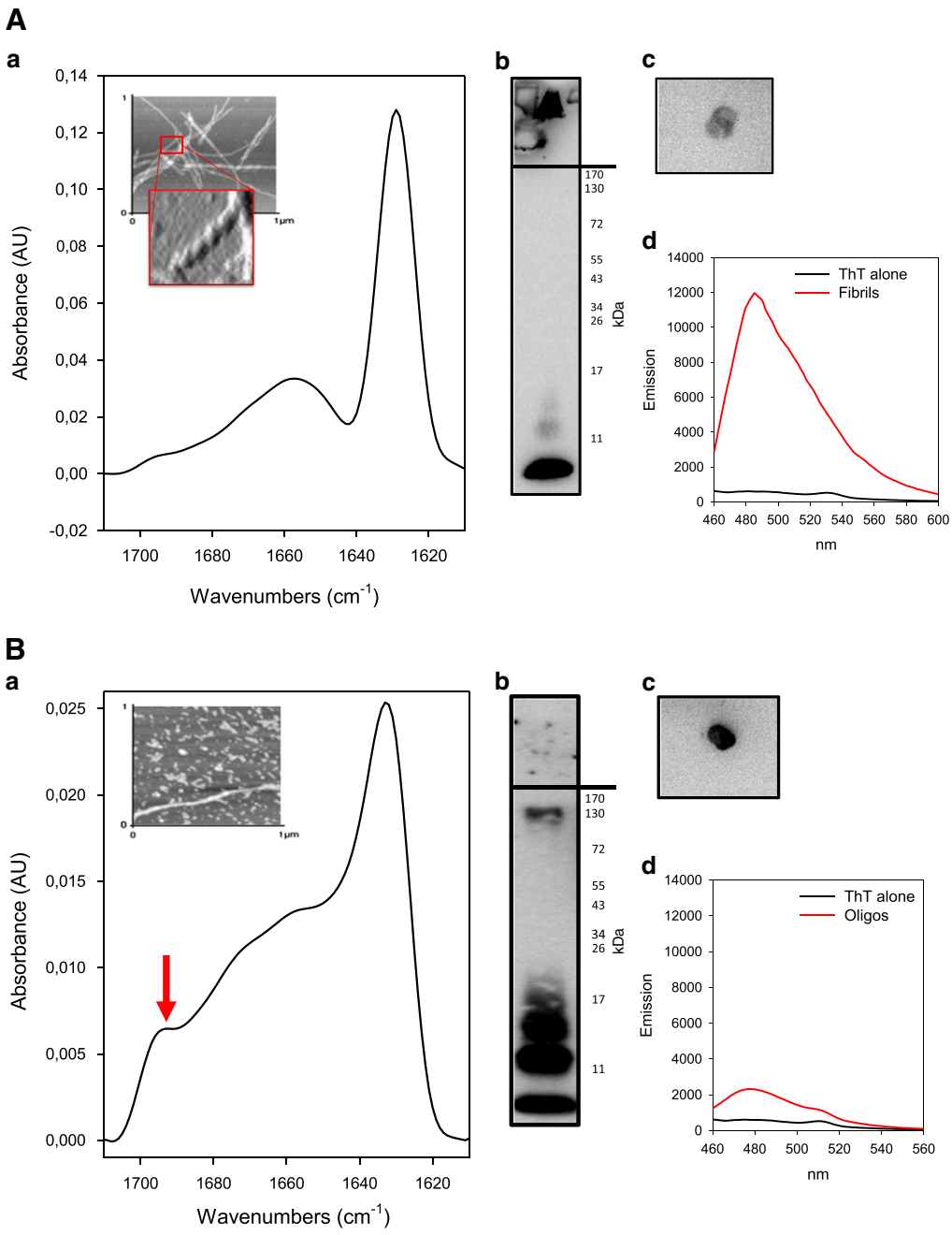

Fig. 2. Assessments of Aβ 42 fibrils (A) and oligomers (B). (a) ATR-FTIR spectra in the amide I region of Aβ 42. In B, a red line is shown at 1695 cm^{-1} to facilitate the identification of the major difference between spectra; (inserts) 1 μm × 1 μm contact mode AFM images of the corresponding Aβ 42. (b) Western blot analysis of the samples with monoclonal antibody 6E10. (c) Dot–blot analysis of Aβ 42 fibrils (panel A) and oligomers (panel B) with the conformation-dependent A11 antibody. (d) Corresponding ThT fluorescence emission spectra.

peptides (natural mutations). Fresh wild-type peptide displays 48% of β structure while mature fibrils contain 58%. All fibril-forming mutants showed an increase in β-sheet content (contribution between 1620 and 1640 cm^{-1}) in monomers correlated to greater amyloidogenicity [97].

Besides the α-to-β conformational changes, it has been argued that a β-turn formation is the critical limiting step leading to β-hairpin observed in fibrils [97–99]. Replacing amino acid pairs in the turn region of Aβ (residues 24–27) with ^{D}ProGly (an effective turn-nucleating motif) modulated fibril formation. β-sheet content increases with a maximum intensity change around 1628 ± 2 cm^{-1} consistent with a cross-β morphology [98].

Finally, influence of aromatic amino acids on β-sheet formation is a matter of debate and raised questions on whether their hydrophobicity or formation of stabilizing π–π interactions can promote peptide self-assembly [100]. A truncated model of Islet Amyloid Polypeptide protein (IAPP) (aa 20–29) containing only one aromatic residue at position 23 (Phe 23) was used to clarify this role because this side-chain was demonstrated not to participate in cross-β structure [69]. Different IAPP variants at position 23 (Tyr, Leu, Phe, pentafluorophenylalanine (F5-Phe), cyclohexylalanine (Cha), α-naphthylalanine (1-Nap), and β-naphthylalanine (2-Nap)) were therefore engineered to clarify the contributions of steric, hydrophobic, and π-π effects on peptide self-assembly. Tyr, Leu, and Phe are aromatic amino acids with different

hydrophobicity (Tyr < Leu<, Phe) but unlike Leu, Phe and Tyr have planar aromatic side chains. The other mutation models result in an increase in hydrophobicity and a change in steric profile (Cha has no planar side chains while 1-Nap and 2-Nap are planar). Fibril growth was controlled by FTIR. The position of the β-sheet at 1626 cm^{-1} on spectra demonstrates that mutants display β structure related to amyloid. Compelling β-sheet studies provided by FTIR, kinetic response to an amyloid specific fluorescent dye (e.g. Thioflavin T, ThT) and TEM images demonstrate that high amyloidogenicity of aromatic amino acids is a function of hydrophobicity and not the ability to form stabilizing or directing π–π bonds. Besides, site-specific analysis of secondary structures at this position 23 was probed by isotope-edited Fourier transform infrared. Isotope labeling by ^{13}C results in a shift in absorption to smaller wavenumbers (by ~35–40 cm^{-1}) and therefore a separation of $^{13}C{=}O$ vibration from the unlabelled $^{12}C{=}O$ is visible allowing specific assignment of secondary structure to the labeled amino acid. Isotope-edited FTIR is an extremely powerful method which has been applied to amyloid local structure determination and also to the respective alignment of residues in fibrils [101,102]; for more information see the excellent review on this specific topic by I. Arkin in this issue. All mutants display the same amide I isotopic shifted band at 1605 cm^{-1} demonstrating that they form the same β-sheet packing [100].

2.4. Polarized FTIR methods, an alternative method to X-ray diffraction powder patterns to study fibrils

Linearly polarized ATR-FTIR method is a spectroscopic technique used to determine the orientation of functional groups within the proteins in highly ordered systems, like membranes. [58,59,61]. This method is based on the property that the intensity of IR light absorption depends on the relative orientation of the transition dipole moment and the exciting electric field of the incident light. Absorption becomes maximal when the transition dipole moment is parallel to the electric field of the incident light. Therefore, recording two IR spectra of an oriented sample with light polarized perpendicular to each other allows the determination of the relative orientation. A difference spectrum (dichroic spectrum) is calculated and its analysis allows both qualitative and quantitative estimation of the orientation of dipoles related to specific vibrations (for a complete description of this method sees the following references [103,104]).

Given the known conformation of fibrils, a large body of data demonstrates that the polarized FTIR method could be quickly use to verify if aggregate preparations are in cross-β spine conformation. For that purpose, fibrils are aligned by air-blowing procedure on a surface: IRE in ATR mode or CaF_2 windows for transmission mode. Fibrils are forced to align by moving along the surface by the air-jet. Orientation study is next performed by analyzing three important bands in the dichroic spectrum. Amide A (3000–2840 cm^{-1}, N – H stretching mode of vibration of the peptide bond), Amide I (1600–1700 cm^{-1}, C═O stretching of the peptide bond) and Amide II (1600–1500 cm^{-1}, mainly N – H bending mode of vibration). Amide A and I are expected to have a resultant dipole moment perpendicular to the backbone but aligned with the fiber axis. Amide II's dipole moment on the contrary, is parallel to the backbone [105–110]. Recently, such a linear dichroic analysis was performed showing that even with non-oriented fibrils cross-β patterns could be determined [110]. This analysis is only valuable for fibrils and not for oligomers which are globular shaped and displayed high anisotropy. Even if X-ray diffraction remains the 'golden' technique to characterize the cross-β conformation, the polarized ATR-FTIR method on aligned samples is also a quick and suitable complementary method.

3. ATR-FTIR and the new amyloid cascade: a structural breakthrough

In the last ~15 years, the amyloid cascade hypothesis has been revisited to include new "player(s)" in the field: the oligomers. Unfortunately, the term oligomer refers to any aggregated but still soluble particle of the amyloid protein or peptide involved, no matter its size or other properties. Oligomers in the literature are also called globulomers, amyloid-beta derived diffusible ligands (ADDLs),…No single definition or precise nomenclature exists so far [111].

Due to the emerging role of these species in amyloidosis' aetiology, the new amyloid cascade hypothesis was enounced. The fibrils are not the most important structure anymore at least in terms of toxicity. Indeed, clear observations especially in Alzheimer's disease (AD) demonstrated that the disease symptoms were not correlated with plaque burden (number or size) but more likely with oligomer formation. In some diseases (e.g. AD), oligomers might even be present decades before any visible cognitive defects [112]. Other studies on cellular systems clearly demonstrated the high toxicity of the oligomers compared to fibrils [113]. An emerging idea is that amyloid fibrils might be a sort of final, less toxic, storing element for these proteins or peptides in their detrimental conformation [114]. While oligomers have been identified in almost all disease-associated proteins/peptides, it seems they also exist for functional amyloid [3].

As soon as researchers demonstrated the highly toxic character of oligomers, intensive research on their topology, structure and of course on their toxic mechanism(s) were carried out.

From a topological and size point of view, nothing has been formally demonstrated yet. For Aβ, some groups assume that toxicity is already associated with dimers [115] while others think at least tetramers (or a multiple of these) are required [116]. Just like fibrils, oligomers are SDS-resistant and can therefore be observed by SDS-PAGE (being particularly cautious and using other techniques to confirm the observed results as carefully demonstrated by Bitan and Teplow in their seminal paper: "What you see is not always what you get" [111]). First cognitive impairments were even associated with the apparition of a dodecamer of Aβ (Aβ*56) in the brain of model mice [117]. Nevertheless, so far no conclusive answer has been given to which oligomer in particular is the most toxic. Most probably, the intrinsic polymorphism due to highly dynamic reorganization in oligomer populations is required to obtain "full" toxicity [27].

Moreover, the exact mechanism(s) of toxicity is still far from being completely understood (for review see [118]). Interaction with membranes is considered as one of the most probable mechanisms or at least as a first step [119–124]. But other (non exclusive) possibilities have been suggested such as impairment in calcium homeostasis, oxidative stress, mitochondrial impairment, activation of the innate immune system…

From a structural point of view, it was rapidly demonstrated that oligomers were rich in β-sheet. It was therefore assumed they adopted the same cross-β structure as fibrils and were just "nascent" (proto)fibrils [125]. Due to their smaller size, they could interact more rapidly and easily with cells and exert more toxic effects.

Like fibrils, oligomers formed from different amyloidogenic proteins or peptides showed some singular similarities in their topology. Described as globular or spherical, most of the oligomers from different proteins/peptides studied can form round- or ring-shaped structures as identified by EM or AFM [37]. All these elements pointed to the same conclusion already obtained for fibrils: oligomers share the same structure and mechanism(s) no matter the protein/peptide they originated from.

An important structural breakthrough came from Charles Glabe's group. They devised a conformational antibody, A11, directed against Aβ oligomers (at least against species bigger or equal to heptamers). This antibody was not reactive against Aβ fibrils. More importantly, when applied to oligomers formed by other amyloidogenic proteins (see below), A11 was reactive showing that indeed oligomers shared a common structure but different from fibrils [21]. Soon after, other conformational anti-oligomers antibodies were obtained aiming at the same results [126].

Due to their aggregated state, their relatively high hydrophobicity (e.g. ANS binding), their heterogeneity in size and their high content in β-sheet, ATR-FTIR is well-indicated to evaluate oligomer's secondary structure(s).

Very first reports displaying FTIR spectra of oligomers indicated that they were compatible with a high content in β-sheet.

3.1. Anti-parallel β-sheet is the spectral feature of oligomers

Starting with Aβ 42 and using well-defined protocols, we formed oligomers devoid of any fibrils as assessed by Western blot, AFM, ThT fluorescence and A11 dot blot analyses. An ATR-FTIR analysis of these well-characterized oligomers demonstrated an anti-parallel orientation of the β-strands in the peptide due to the presence of both low and high frequency bands of the β-sheet. As previously mentioned, Aβ 42 well-characterized fibrils displayed only the low frequency band characteristic of parallel β-strands. The respective intensity of the two bands is in perfect agreement with a 100% anti-parallel alignment when compared to spectra of all anti-parallel β-sheet proteins [29]. Furthermore, the Aβ 42 oligomer spectrum displayed striking similarities with membrane β-barrel porin spectrum. Based on this fact and that A11 conformational antibody developed by Kayed et al. [21] recognizes a wide variety of amyloidogenic oligomers (Aβ, hIAPP, α-synuclein, lysosyme, insulin) as well as pore-forming proteins (α-hemolysin, …) [127], we suggested that Aβ 42 oligomers might adopt a porin-like β-barrel conformation.

Applying similar procedures and ATR-FTIR to other amyloid protein oligomers, we demonstrated that Aβ 40 (Fig. 2), Aβ 40 arctic mutant, α-synuclein, all displayed characteristic anti-parallel orientation of their β-strands. The presence of this specific structure was observed in other amyloid proteins/peptides (see Table 1) like β_2-microglobulin [128], prion-related peptide $PrP_{82\text{-}146}$ [129], transthyretin (TTR) [130], lysozyme [131,132], HETs prion protein (prion of the filamentous fungus P. anserine) [30] and SRC Homology 3 Domain (SH3 domain) [83]. Most of these articles barely mentioned that their oligomeric preparations have FTIR spectral features compatible with an anti-parallel β-sheet conformation, most if not all of these did not use any of this information in their conclusions. Altogether this demonstrated that anti-parallel β-sheet is the major conformational contribution in oligomers. This might, especially when considering the potential formation of porin-like β-barrel for these proteins, be important on the one hand for the differentiation between oligomers and fibrils and on the other hand for the comprehension of the huge difference observed in cytotoxicity for both species (oligomers vs. fibrils).

Early 2012, this conclusion has been further strengthened by X-ray derived atomic structure of a segment of αB crystallin sharing sequence homology with Aβ peptide and forming A11-positive oligomers. The high-resolution structure of these αβ crystallin oligomers demonstrated the formation of an anti-parallel β-barrel made of six strands [53,125]. The anti-parallel alignment of the β-strands in oligomers have also been evidenced by time-resolved hydrogen exchange mass spectrometry on Aβ oligomers [133], and an exemplar analysis by NMR of a very low percentage of oligomers of Fyn SH3 in the presence of the correctly folded protein [134].

Therefore, ATR-FTIR is a handy, rapid and versatile method to evaluate the secondary structure of various amyloid aggregates and to shed light on oligomer presence in a sample (Fig. 2).

3.2. Is the anti-parallel β-sheet structure a signature of amyloid cytotoxicity?

Oligomers can exert several harmful effects on the viability and functions of cells [28,135–141]. Today, even if no consensus on oligomer targets is reached, the hypothesis of a unique mechanism by which oligomers might be toxic has been proposed. As postulated by Bucciantini et al. [135], this mechanism may be linked to common conformational features rather than specific properties of proteins or peptides. Considering the increasing number of polypeptide chains adopting anti-parallel structure in their oligomeric conformation, we may interrogate if anti-parallel β-sheet is the signature for cytotoxicity [21,28,135].

Our observation as well as literature data [142] and the previously mentioned fact that A11 conformational antibody raised against oligomers also recognized pore-forming proteins, suggest that oligomers may adopt a pore-like morphology, an especially favorable conformation to interact in a detrimental way with lipid membranes. This hypothesis corroborates many experimental evidences nicely reviewed in [137].

A correlation between cytotoxicity and the anti-parallel β-sheet structure was recently demonstrated by two different studies [30,31]. First, Sandberg and coworkers in Härd's laboratory [31] developed an extremely elegant approach by mutating A21 and A30

Table 1
Infrared and species assignments for several amyloid proteins/peptides.

Protein name	Species/entities	Assignment based on	FTIR method	Main β-sheet peak(s) (cm^{-1})		Ref.
				Low freq.	High freq.	
Aβ peptide	Aβ 42 oligomers	ThT fluorescence, A11 response, SDS-PAGE and AFM	ATR-FTIR	1626	1695	[29]
	Aβ 42 fibrils			1630	–	
	$Aβ\ 42_{CC}$ oligomers/protofibrils	ThT fluorescence, A11 response, SDS-PAGE, SEC and EM	FTIR	1630	1691	[31]
	Aβ 40 oligomers	ThT fluorescence, A11 response, SDS-PAGE and AFM	ATR-FTIR	1632	1695	[91]
	Aβ 40 fibrils			1627	–	
	Aβ 40 oligomers	ThT fluorescence, A11 response, SDS-PAGE and AFM	ATR-FTIR	1633	1694	[150]
	Aβ 40 fibrils			1629	–	
α-synuclein	α-Syn oligomers	ThT fluorescence, A11 response, SDS-PAGE and EM	ATR-FTIR	1630	1694	[33]
	α-Syn fibrils			1628	–	
	α-Syn oligomers	ThT fluorescence, SEC, SAXS, EM and AFM	ATR-FTIR	1630	1687	[152,153]
β_2-microglobulin	β_2m short curved structures	Light Scattering and EM	FTIR	1616	1685	[128]
	β_2m fibrils			1620	–	
$PrP_{82\text{-}146}$	PrP_{82-146} oligomers	EM, AFM and Laser scanning confocal fluorescence	FTIR	1623	1690	[129]
	PrP_{82-146} fibrils	microscopy		1626	–	
Transthyretin	TTR soluble aggregates	Light scattering, ANS fluorescence	FTIR	1616	1686	[130]
	TTR fibrils			1625	–	
Lysosyme	Lysosyme oligomers	ThT and ANS fluorescence, EM, SDS-PAGE,	FTIR	1630	1688	[131]
	Fibrillar aggregates at pH 7.5	SDS-PAGE, EM, X-ray diffraction	ATR-FTIR	1628	1692	[132]
	Fibrillar aggregates at pH 2.0			1622	1693	
HETs prion protein	HET-$s_{(218-289)}$ fibers	Turbidity and Congo Red binding	ATR-FTIR	1620	1695	[30]
	HET-$s_{(218-289)}$ fibers			1630	–	
SH3 domain	Amorphous aggregates	EM, SDS-PAGE	FTIR	1612	1684	[83]
	SH3 fibrils			1618	–	

into cysteines in Aβ 40 and 42. These peptides called Aβ 40cc and Aβ 42cc when in oxidative conditions form an intramolecular disulfide bond which stabilized the oligomers and entirely blocked the formation of fibrils. ATR-FTIR demonstrated that these stable oligomers adopted anti-parallel β-sheet structure. More importantly, these oligomers were 50 times more efficient apoptotic agents than fibrils. When suppressing the disulfide bond using reducing conditions, the peptides started to quickly form classical Aβ fibrils and became less toxic.

Later, Berthelot et al. demonstrated that mutagenesis of nontoxic amyloidogenic prion forming domain of HET-s yields to the modulation of β-structure conformation. It correlated to cytotoxicity on yeast cells. Mutations promoting anti-parallel β-sheet structure are toxic to yeast in regard to those promoting parallel and random coil. Moreover, they demonstrate that mutant displaying anti-parallel β-sheet structures interact strongly and disturb the lipid monolayer thickness while non toxic mutants were unable to have such toxic functions [30].

Those data emphasize the emerging hypothesis of the anti-parallel β-sheet conformation as a signature of amyloid oligomers and cytotoxicity. Nevertheless, no consensus is reached so far to describe the molecular mechanism(s) of cytotoxicity.

4. FTIR as a tool to structurally follow the aggregation process

FTIR is an exquisite technique to follow structural changes along the aggregation process. Indeed, as previously mentioned, samples are quickly prepared, spectra are extremely rapid to record, and only minor protein quantities are required. ATR-FTIR has the added advantage to be able to handle hydrophobic and/or insoluble samples. FTIR is therefore one of the rare methods able to give an almost continuous structural view of such processes.

Starting from monomers or oligomers, an intriguing question is how β-strands (re)organize themselves into a different type of β-sheet when fibrils are formed. To investigate this, Decatur's group used isotope-edited (^{13}C-labeled carbonyl) infrared spectroscopic technique on PrP and Aβ short peptides. As mentioned above, introduction of heavier ^{13}C at one residue results in a shift in its absorption to smaller wavenumbers resulting in a separation from unlabeled ^{12}C backbone absorption band. Spectra analysis reveals the local secondary structure of the labeled amino acid according to its band position. The observed band shift is also dependent on residue alignment. When labeled residues are aligned in all the β-strands forming the sheet (i.e. they are in register) an additional shift to lower frequency of maximum ~10 cm^{-1} is observed due to a vibrational coupling effect [143]. Different mutants of $PrP_{109–122}$ labeled with ^{13}C at residues implicated in β-structure (one residue at a time) have been used to probe the organization of strands (i.e. the relative alignment of labeled residues) by following the shift in the maximum of absorption (from 1601 to 1592 cm^{-1}) and stability upon aggregation by quantifying the β-sheet content. They demonstrated that $PrP_{109–122}$ reorganized their β-strands by a dynamic alignment promoted by repeated detachment and annealing of β-strands to finally form stable fibrils [144]. The same behavior was observed for truncated Aβ aa 16–22 [145]. Together with morphology studies by AFM of fibrils (thickness, flexibility and twist), they conclude that a correlation exists between strands (re)organization and aggregation.

Kinetic analysis of aggregation has been next investigated by time-resolved IR spectroscopy [146]. Perálvarez-Marín et al. used pH jump triggered by photolytic release of a caged-proton to promote aggregation of truncated Aβ (aa 1–28). Spectra were recorded on a sub-millisecond time-scale upon aggregation. Data showed that the first step corresponds to a rapid (3.6 s) increase in β-structure intensity at 1623.5 cm^{-1}. This is related to small β-sheet aggregates formation. After that a second slower (48 s) step takes place and leads to formation of large aggregates displaying a major peak at 1619.5 cm^{-1}. Those data surprisingly demonstrate that partially aggregated species absorbing at 1623.5 cm^{-1} corresponding to oligomers are the initial step in fibril formation [146].

More recently, higher resolution techniques (X-ray and ssNMR) demonstrated that soluble, globular, high molecular weight oligomers (i.e. close to fibrils, they are indeed ThT positive) can adopt either a cross-β conformation containing anti-parallel β-sheet [125] or a parallel β-sheet arrangement while still in a globular shape

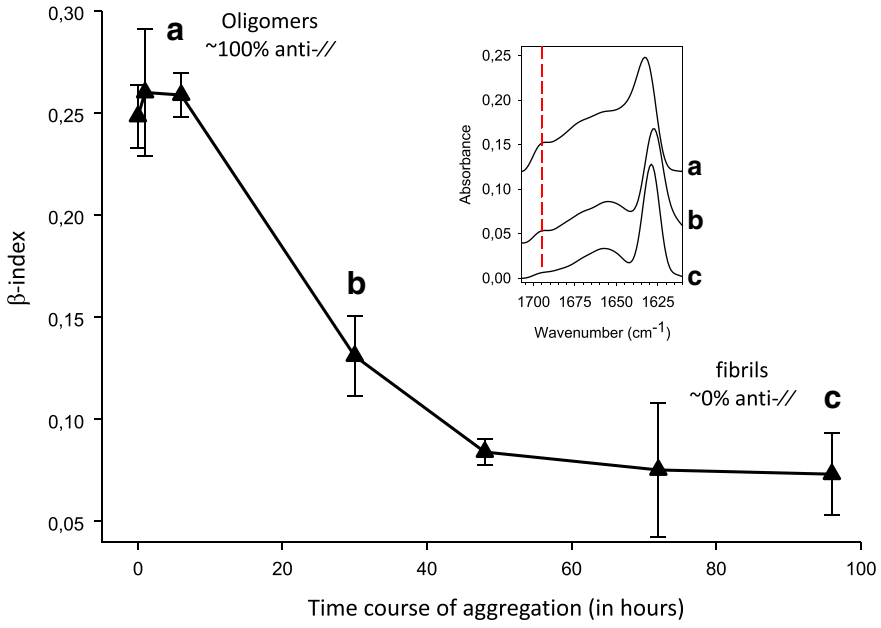

Fig. 3. (ATR)-FTIR as a tool to structurally follow the aggregation process (use of β-index). It was suggested that the intensity ratio between the two spectral contributions of β-structure (I_{1695}/I_{1630}) is proportional to the percentage of anti-parallel vs. parallel organization of the β-strands. We called this ratio the "β-sheet organizational index" or "β-index" for short. We followed the time evolution of this index during the aggregation process of Aβ 42. Insert: Amide I analysis demonstrated that at early stages of aggregation the peptide displayed the 1630 and 1695 cm^{-1} bands characteristic of anti-parallel β-sheet organization (curve a). When fibril formation took place, the 1695 cm^{-1} disappeared resulting in only one major β-sheet spectral contribution around 1630 cm^{-1}, assigned to parallel β-sheet structure (curve c).

[51]. These data demonstrate that structure(s) in-between oligomers and fibrils might adopt a combination of the different structural features of both entities. Therefore, it is tempting to suggest that this conversion is a complex but nevertheless quite progressive and continuous process. New ATR-FTIR results obtained on Aβ are in perfect agreement with this idea (unpublished data). This needs confirmation on other amyloidogenic proteins before drawing a definite conclusion on the involvement of these interesting intermediate species in the aggregation process.

The oligomers-to-fibrils transition was also depicted using ATR-FTIR in our recent Aβ studies [91] (Fig. 3). We demonstrated that the misfolding process for wild type Aβ 42 and Aβ 40 peptide is supported by β-sheet reorganization. Amide I fingerprint analysis demonstrates that at the early stages of aggregation both peptides display the 1630 and 1695 cm^{-1} bands reported for anti-parallel β-sheet organization. When fibril formation takes place, the 1695 cm^{-1} disappears resulting in only one β-sheet contribution around 1630 cm^{-1}, assigned to parallel β-sheet structure. This conversion was suggested to be thermodynamically favorable by molecular dynamics (MD) simulations [147,148]. Nevertheless, evidences for a dynamic equilibrium between oligomers and fibrils during aggregation have been provided [119].

It was suggested that the intensity ratio between the two spectral contributions of β-structure (I_{1695}/I_{1630}) is proportional to the percentage of anti-parallel vs. parallel organization of the β-strands; therefore we tentatively called this ratio the "β-sheet organizational index". Because in amyloid, anti-parallel vs. parallel is also proportional to oligomer to fibril contents we used this index to roughly determine the percentage of oligomers in a sample [91]. The 'β-sheet organizational index' has been applied with success to the study of α-synuclein oligomers [33], or Aβ in the presence of exogenous compounds like other proteins such as apoliprotein E isoforms [149] or ions (Ca^{2+}) [150]. We are convinced this 'β-sheet organizational index' can find many more applications in future amyloid studies.

As first and cleverly suggested by Torleif Härd's group [31,151], and in accordance with our own results [29,91] the "simplest" way to describe the conformational change from anti-parallel to parallel β-sheet could be explained by a 90° rotation of the involved β-strands.

5. Conclusion

We tried to emphasize the role of FTIR and especially ATR-FTIR techniques in the study of amyloid proteins and/or peptides. The multiple advantages provided by ATR-FTIR allow an almost continuous structural view of protein/peptide conversion during the aggregation process. Isotope-edited FTIR can even provide structural information at the amino acid level. Moreover, it is now well-established that infrared can differentiate oligomers from fibrils simply on their spectral features. Today, ATR-FTIR is certainly the fastest and easiest method to obtain this information. ATR-FTIR was indeed the first method to reveal that, contrary to fibrils, oligomers adopt an anti-parallel β-sheet conformation. This specific conformation is now well-correlated with cytotoxic effects of the oligomeric species.

ATR-FTIR is definitely an extremely versatile tool to study amyloids and will continue in the near future to provide new information on these proteins and peptides. It has its own place (in complement to higher resolution methods) in the analysis and comprehension of the complex aggregation mechanism(s) at the oligomer and/or fibril level. These mechanism(s) seem to demonstrate similarities between different amyloid proteins and might therefore be extremely important towards the understanding of both disease-associated and functional amyloid proteins.

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