Formation of neutral peptide aggregates studied by mass selective IR action spectroscopy

Sjors Bakels, Sebastiaan B.A. Porskamp and Anouk M. Rijs*

Abstract: The spontaneous aggregation of proteins and peptides is widely studied due to its relation to neurodegenerative diseases. To understand the underlying principles of peptide aggregation, elucidation of structure and structural changes upon their formation is key. This level of detail can be obtained by studying the peptide self-assembly in the gas phase. Structural characterization of aggregates is mainly done on charged species, as adding charges is an intrinsic part of the technique to bring molecules into the gas phase. Studying neutral peptide aggregates will complement the existing picture. These studies are restricted to dimers due to experimental limitations. Here, we present advances in laser desorption molecular beam spectroscopy to form neutral peptide aggregates in the gas phase consisting of up to fourteen monomeric peptides. The combination with IR-UV spectroscopy allowed us to select each aggregate by size and subsequently characterize its structure.

Self-assembly of proteins and peptides into distinct ordered structures has triggered interest in a wide variety of research fields ranging from biology[1] to the development of smart materials[2] and life-like nanosystems[3]. Amongst these examples, protein and peptide aggregation is mostly studied in relation to human disorders such as the neurodegenerative diseases Alzheimer’s and Parkinson’s Disease, where the formation of aggregates into amyloid fibrils is observed.[4] Multiple techniques have been used to provide insight in the aggregation process, such as Transmission Electron Microscopy[5], cryo-EM[6], Circular Dichroism[7], NMR[8] and FT-IR spectroscopy[9]. These methods revealed the structure of full-grown fibrils, but lack the ability to study the structure of the toxic early stage, such as dimers, trimers and small oligomers, due to complexity and heterogeneity.

Mass spectrometry coupled with techniques such as infrared spectroscopy and/or ion mobility can shed light on the structure of these oligomers.[10] Electrospray ionization is commonly used to bring these aggregates as charged species into the mass spectrometer. The initial steps of aggregate formation have been probed by mass spectrometry combined with IR spectroscopy and ion mobility focusing on the amyloid prone peptides VEALYL (insulin) and NFGAIL (human islet amyloid polypeptide).[11] An increase in beta-sheet character, indicative for the formation of fibrils, in the IR signatures was observed in the more extended oligomers. Charge plays an important role, since it can induce unfolding and consequently alter the structure of the peptide. Studying neutral peptides will bring insights into non-charge driven structural preferences upon aggregation. However, the formation of aggregates of neutral peptides is not straightforward. There are only a handful of studies on peptide dimers, using either a heatable source or laser desorption to bring them into the gas phase. The groups of Gerhards and Rijs/Gaigeot have studied the structure of neutral (Ac-Phe-OMe)₂ using IR-UV spectroscopy.[12] They showed that the charge on the lysine prevents the formation of extended peptide backbone segments as observed in amyloid fibrils. In contrast, the studied neutral dimer did form a beta-sheet structure in the gas phase. Recently, we have studied the competition between intra- and intermolecular interactions upon dimer formation of alanine containing peptides.[14]

In this work, we show for the first time the formation of stable higher order clusters of neutral peptides in the gas phase using laser desorption. The experiment is described in the experimental section and SI.1. The peptide used in our experiments is the capped dipeptide Ac-Ala-Ala-OBn (BioMatik, >95% purity, molecular weight of 292.31 amu). The mass spectrum presented in Fig.1 is recorded at the resonant ionization wavelength of the trimer (37460 cm⁻¹). The numbers indicate the amount of monomeric peptide units involved in each aggregate.

Figure 1. Mass spectrum and chemical structure of the studied peptide Ac-Ala-Ala-OBn obtained at the trimer resonant ionization wavelength of 37460 cm⁻¹. The numbers indicate the amount of monomeric peptide units involved in each aggregate.

* dr. A.M. Rijs
Radboud University
Institute for Molecules and Materials, FELIX Laboratory
Toernooiveld 7c, 6525 ED Nijmegen (the Netherlands)
E-mail: a.rijs@science.ru.nl

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mildly focused on a graphite sample bar resulting in a beam diameter of about 1 mm and power of 1 mJ/pulse. The sample bar can be varied in height with respect to the axis of the molecular beam (blue arrow). The opening of the nozzle (grey) has a diameter of 0.5 mm (black arrow). After desorption, which takes place inside the gas pulse, the molecules are seeded in a supersonic argon expansion.

![Figure 2](image_url)

Figure 2. a) Schematic view of the desorption set-up. b) Normalized signal of multimers of Ac-Ala-Ala-OBn with respect to the position of the sample bar, where zero marks the nozzle centre. c) Difference between start (at FWHM) of every multimer with respect to the start at FWHM of the monomer. The black arrow indicates the nozzle diameter.

The position of the sample with respect to the nozzle opening is key for optimal performance. Changing the height of the sample bar results in plots as presented in Fig.2b. Here, the normalized signal per multimer is monitored as function of the vertical distance between sample bar and center of molecular beam, such that the opening of the nozzle. The optimal height for monomers is slightly below the center of the nozzle orifice. In contrast, clusters are always found near the lower edge of the nozzle opening. This can be clearly seen in Fig.2c, where the difference between rising edges at FWHM of each aggregate with respect to the monomer (see inset) is monitored. Each point represents the average of multiple measurements taken at three different UV excitation wavelengths. The zero position coincides with the center and 0.25 mm with the lower edge of the nozzle opening.

The longer pathway for the multimers allows for more collisions with present peptide molecules and argon atoms; molecules have more time to cool down and spend more time in a denser environment undergoing collisions required for aggregate formation. This becomes more effective until the sample bar is moved too far away from the molecular beam path, resulting in a sudden drop of signal. The high efficiency of the cooling and clustering process explains why all monomers that are present when partly blocking the nozzle, are completely consumed in the aggregation process when the sample bar is lowered.

The mass-selective IR spectra of the Ac-Ala-Ala-OBn clusters with n=2-7 were measured in the fingerprint region from 1000 to 1800 cm\(^{-1}\), see Fig.3a. The region between 1600 and 1800 cm\(^{-1}\) comprises the C=O stretching vibrations and shows two distinctive peaks. The smaller peak above 1700 cm\(^{-1}\) results from the C=O from the ester moiety present at the OBn-cap, while the peak between 1600 and 1700 cm\(^{-1}\) originates from the peptide C=O groups (amide I). Every peptide monomer has one ester C=O and two peptide C=O groups. The amide II (NH bend) appears as a broad feature located between 1490 and 1570 cm\(^{-1}\). Other distinctive features include the large bands around 1200 cm\(^{-1}\), originating from backbone and amide III motions. Fig.3b and 3c display the normalized IR spectra for aggregates from n=2 to n=7 in the amide I and II region, respectively. These regions are sensitive to hydrogen bonding, thereby providing structural details on intra- and intermolecular interactions. Quantum chemical calculations were performed to structurally assign the experimental IR spectra. For the dimer, eight structural families were determined based on their hydrogen bond patterns (see SI.4). The calculated IR spectrum of the lowest energy structure shows the best agreement with the experiment, and was therefore assigned to the parallel beta-sheet dimer, see Fig.4a. This dimer is formed by two intermolecular hydrogen bonds, where the backbones of the two peptides are aligned in a parallel orientation. The ester C=O groups are not involved in any hydrogen bonding. The individual peptides present in the parallel beta-sheet dimer retain their monomeric structures: The linear and the \(\pi\)-turn conformer are both present in the dimer, where their weak intramolecular hydrogen bonds (C\(\delta\) hydrogen bond and NH-\(\pi\) bond) are replaced by the stronger intermolecular hydrogen bonds. The calculated IR spectrum of an anti-parallel dimer is also compared with the recorded IR spectrum in Fig.4a. This structure, like the parallel dimer, has no ester C=O groups involved in hydrogen bonding, however, was discarded due to a mismatch in the amide II together with its high energy.
The calculations showed that the low energy structures of the trimer were exclusively beta-sheet type structures. Other structural families (globular), were at least 25 kJ/mol higher in energy, but often over 40 kJ/mol. The experimental spectrum only matches to structures where all three monomers are attached via beta-sheet intramolecular hydrogen bonds (see SI.5). Especially, conformers which arise from the attachment of the third peptide to the before assigned dimeric structure P-T-1, are in good agreement. The third peptide can aggregate on both sides of the dimer: at the C7 γ-turn intramolecular or at the weaker C5 intramolecular hydrogen bonded side. Based on energetics and spatial orientation, it is expected that the weaker C5 hydrogen bond is broken to favor the formation of strong intermolecular beta-sheet interactions.[14] Two beta-sheet containing structures remain possible: An all parallel structure and a structure where the third monomer is attached in an anti-parallel fashion to the dimer. The added monomer peptide adopts either the observed linear conformer (for the all parallel structure, Fig.4b left panel) or the γ-turn conformer for the anti-parallel structure (Fig.4b right panel). The anti-parallel structure shows a better overlap in the region around 1200 cm⁻¹; however, the all parallel structure has a considerable (17 kJ/mol) lower energy, and reproduces the double peak in the amide II region better. Overall, the structure of the trimer can be confidently assigned to an all beta-sheet structure, where the peptides are stacked on top of each other. Energetics and spectral features such as the shoulder in the amide I region point to the presence of parallel beta-sheets. The theoretical IR spectra of all beta-sheet tetramer structures show good agreement with the experiment (details in SI.6).

Additionally, the energetics showed that, as was observed for the dimer and the trimer, the all parallel structure has the lowest energy. The spectra of two possible structures are shown in Fig.4c: an all parallel conformer (6-31G* basis set) and a mixed (anti-)parallel low energy conformer (6-31+G* basis set). Both can originate from the addition of a monomer to one of the two discussed trimers, or by merging two dimers. Spectrally, the overlap for both structures is good in the amide I region, although the all parallel conformer is calculated on a lower level of theory and therefore has a smaller peak around 1800 cm⁻¹ (SI.6). The rest of the spectra shows reasonable overlap for both beta-sheet conformers. In line with the dimer and trimer, the tetramer structure has all peptides stacked via beta-sheets on top of each other. Based on energetics and the dimer and trimer structure, the formation of an all parallel tetramer is expected.

The observed red- and blue-shifts of the amide I and II bands respectively in the IR spectra continue for the higher order clusters (n=4). A similar trend for the aggregation onwards from the tetramer is expected, in which the peptides aggregate into stacked beta-sheet containing structures, where the weak intramolecular hydrogen bonds are broken to favor stronger inter-sheet hydrogen bonds. The observed red shift in the amide I region for the larger aggregates continues towards the amide I band of the solid-state FT-IR spectrum of Ac-Ala-Ala-OBn. This spectrum was recorded using a KBr pellet (SI.7) and shows a clear signature at 1629 cm⁻¹, corresponding to parallel beta-sheets.[18]

In conclusion, we show that it is possible to make aggregates of neutral peptides in the gas phase by allowing the laser desorbed peptides more time to cool down in a higher dense environment. The amount of neutral aggregated peptides (n=14) is unprecedented, as peptide dimers were the highest order clusters created before. IR-UV ion dip spectroscopy and quantum chemical calculations allowed us to assign the structures of the
aggregates (n=2-4) to predominantly parallel beta-sheets, with the peptides stacked on top of each other. Higher order clusters (up to n=7) showed a shift in the amide I towards known beta-sheet signatures observed in the solid phase, indicating that the structural preferences observed in the gas phase are related to those in bulk. The presented results pave the way to complementary studies on neutral biologically active peptides such as the hydrophobic amyloidogenic peptides.

Experimental Section

The neutral peptide aggregates are formed in a laser desorption molecular beam time-of-flight mass spectrometer.11] Conformational and mass selective IR spectra were recorded via IR-UV ion-dip spectroscopy using the free electron laser FELIX. The amber force field and Gaussian 09 were used for our calculations. Details of the experiment are presented in SI.1.

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