Insights on Amyloid Spherulites Structure at Molecular Level

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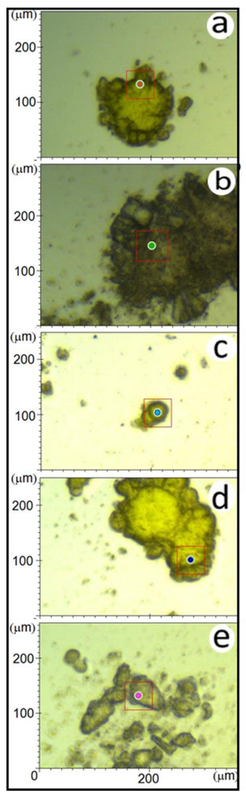
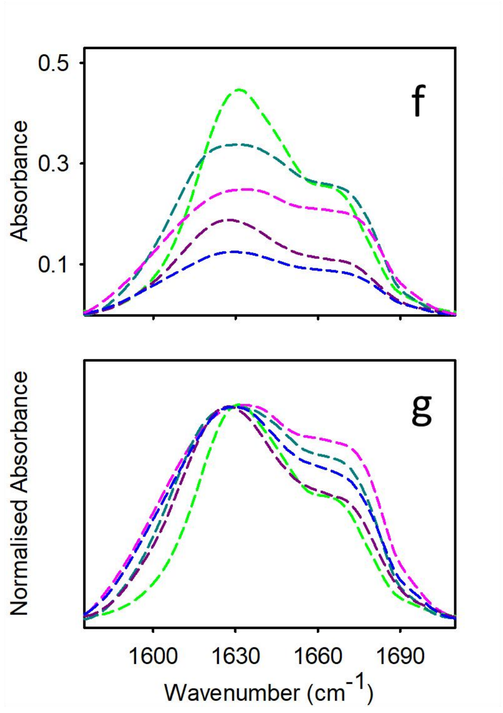
Large scale arrangements of amyloid-like structures can be formed packed into 3D well defined superstructures which conserve their basic structural arrangement of cross β-sheet. In vitro, at high temperature and in solution at pH far from the isoelectric point of the originating protein, amyloid spherulites can be prepared. They are fascinating aggregates characterized by spherical shape formed by a dense core and a low-density hedgehog corona containing amyloid structures. Their characteristic radius ranges from few μm up to mm and this makes these structures relevant for application as scaffold or drug releasing matrices. For this reason, profound knowledge of their structural properties is of utmost importance. In this study we use Micro-FTIR to analyze Human Insulin spherulites aiming at elucidating a relation between molecular structures and mesoscopic properties. The coupling between IR spectroscopy and optical imaging enables the analysis of such spatially heterogeneous sample allowing revealing structural details which are not accessible with bulk methods. Experimental results show that the large morphological heterogeneity of spherulites is underlined by common properties at secondary structure level. A broad peak at 1620 cm^{-1} which is characteristic of intermolecular β-sheets in amyloids is present together with a large peak at 1660–1680 cm^{-1} attributed to native-like structures and disordered structures. These peaks contribute in different extent to the spectrum of different spherulites. Moreover, data systematically show changes in density within the single spherulite and in the lower density regions show the presence of increasing content of intermolecular β-structures with progressively stronger H-bonds moving away from the center of the aggregate. This result is corroborated by 3D fluorescence lifetime imaging on Thioflavin T (ThT) stained samples which reveal spatial distribution of fluorescent lifetime that gradually changes from the inner to external regions of the spherulite. It is immediately evident that beta structure strength relates to ThT lifetime differences this offering insights on both the dye and the sample in analysis.

Spherulites strucutral charaterisation – sample heterogeneity

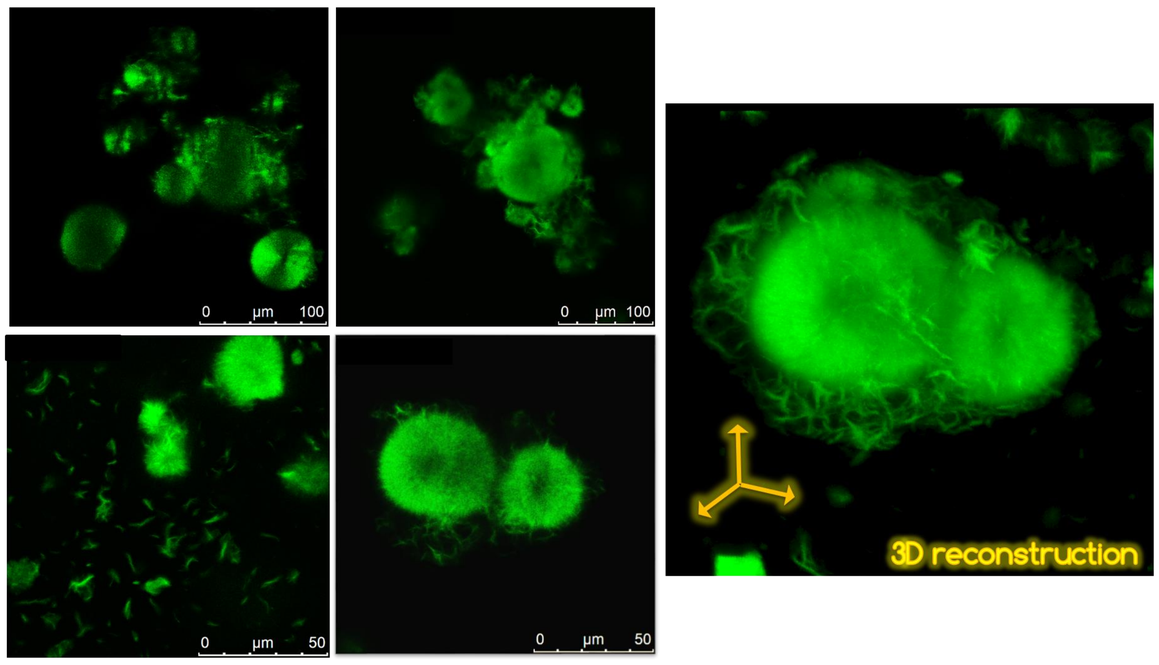




"Different amount of secondary structures contribute in different extent to the spectra."

The 1620 cm^{-1} component appears to have different widths in each spectra.

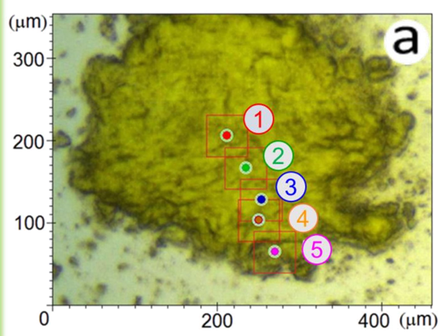
This peak shifts toward lower wavenumbers when hydrogen bond strength in β-aggregate structures increases.

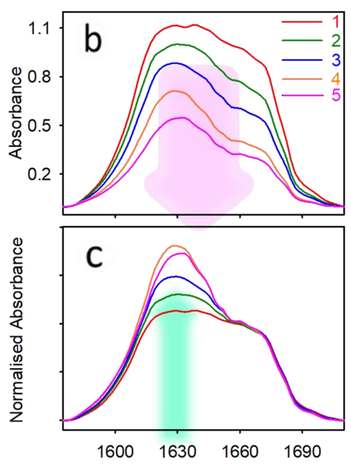


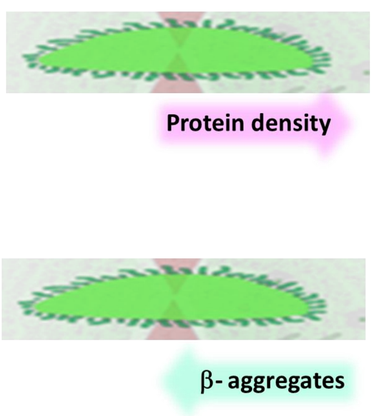
Micro-FTIR a)-e) Representative optical images of HI spherulites obtained incubating a 5mg/ml HI insulin solution in HCl (pH 1,85) and NaCl (0,25 mg/ml) a 60°C for 24 hours. The colored 50 μm x 50 μm squares represent the ROIs where FTIR spectra were acquired. Light path is 17 um. f) FTIR spectra in the Amide I' region (1575 cm^{-1} – 1710 cm^{-1}) acquired in each ROI of each image distinguished with the same color code g) FTIR spectra normalised at their maximum value. ime evolution of Con A (8± 0.1 mg/ml, pD ~ 2.5) FTIR spectrum in the Amide I' region during thermal incubation at 60 °C. The arrows indicate the direction of changes as a function of time.

Confocal Microscopy: 1024×1024 representative laser scanning fluorescence confocal microscopy measurements of insulin amyloid spherulites sample stained with Thioflavin T. The sample is obtained by incubating a 5mg/ml HI solution in HCl (pH 1,85) e NaCl (0,25 mg/ml) a 60°C for 24 hours- The diameter of observed structures ranges from few to hundreds micrometers, their morphology share an evident radial simmetry of fibril distribution. ThT fluorescence intensity presents a minimum (nearly vanishing fluorescence) in the core of the structure to the external part. Sparse britght fibrils are also present.

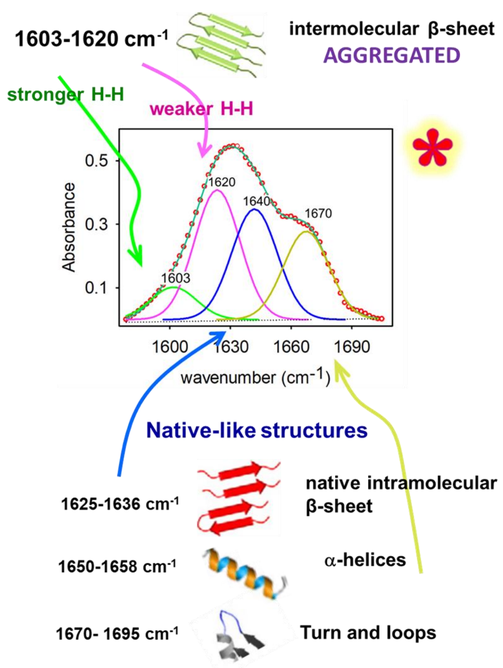
Micro-FTIR single spherulite





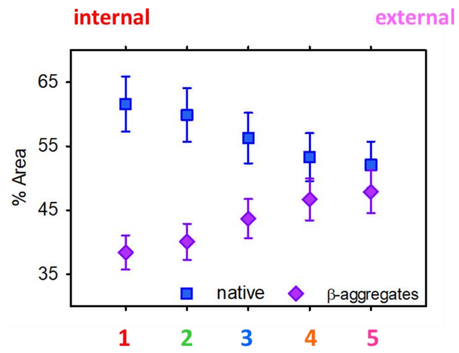


Micro-FTIR: a) 460 μm x 350 μm optical image of a large spherulite. 50 μm x 50 μm red squares are the ROIs where FTIR spectra are acquired. Light path is 17um. b) FTIR spectra in the Amide I' region (1575 cm-1 – 1710 cm-1) acquired in the ROIs c) FTIR spectra normalised at 1670 cm^{-1}.



Spectral deconvolution in 5 Gaussian components of FTIR spectrum.

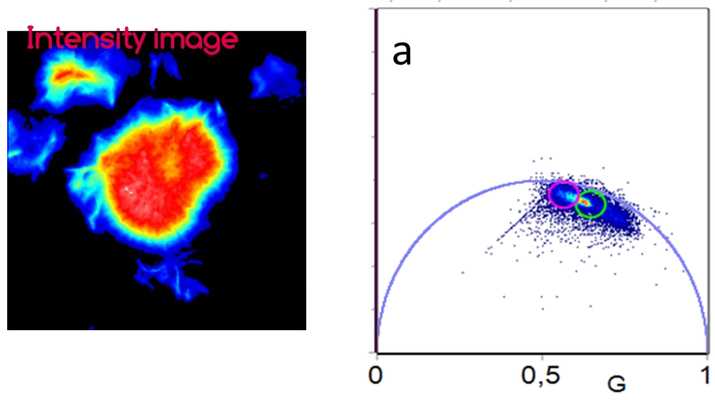
cm^{-1}	1	2	3	4	5
Aggregated 1603	4,94	5,78	7,22	7,91	9,02
1620	33,45	34,3	36,45	38,77	38,94
Native 1640	32,14	32,61	31,02	29,69	28,75
1670	29,47	27,3	25,3	23,63	23,29

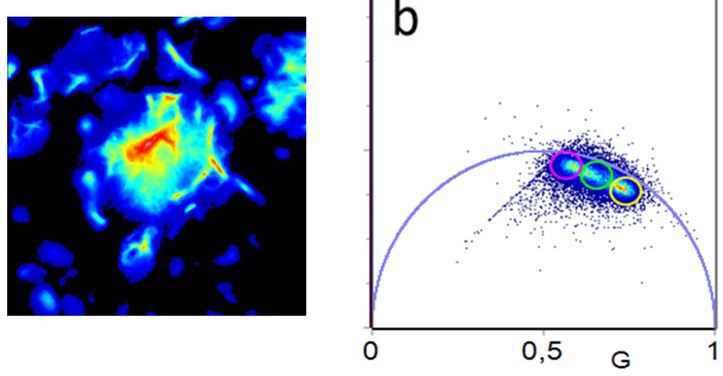


Fractional area of native and aggregate compoente with respect to the total amide I' band

FLIM - Thioflavin T

Phasor plot





Phasor analysis: Phasor analysis of 256 x256 pixel FLIM measurements on ThT signal at about the equatorial plane of a single spherulite a) and at the bottom of the spherulite b) fibrillary structures are also evident. In the Phasor color map each pixel is colored according to the color of the corresponding cursor in the phasor plot.

Pixels whit shorter lifetime distributions are located in the inner part of the spherulites. ThT lifetime increases moving away from the center

Fibrillar structures in the sample are charaterised by the shortest lifetime distribution

Piccirilli et al. (2015) Biophysical Chemistry, 199, pp. 17-24.
Vetri et al. (2018) J Phys Chem B. 122 pp. 3101-3112

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