Amyloid fibrils formation of concanavalin A at basic pH

<table>
<thead>
<tr>
<th>Journal:</th>
<th>The Journal of Physical Chemistry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manuscript ID:</td>
<td>Draft</td>
</tr>
<tr>
<td>Manuscript Type:</td>
<td>Article</td>
</tr>
<tr>
<td>Date Submitted by the Author:</td>
<td>n/a</td>
</tr>
<tr>
<td>Complete List of Authors:</td>
<td>Carrotta, Rita; Italian National Research Council, Institute of Biophysics Vetri, Valeria; University of Palermo, Dep. of Physics and Astronomy Librizzi, Fabio; University of Palermo, Dep. of Physics and Astronomy Martorana, Vincenzo; Italian National Research Council, Institute of Biophysics Militello, Valeria; University of Palermo, Dep. of Physics and Astronomy Leone, Maurizio; University of Palermo, Dep. of Physics and Astronomy</td>
</tr>
</tbody>
</table>
Amyloid fibrils formation of concanavalin A at basic pH

Carrotta R.*, Vetri V.°, Librizzi F.°, Martorana V.*°, Militello V.*°* and Leone M.°*°

* Istituto di Biofisica CNR, Via Ugo La Malfa 153, 90146 Palermo
° Dipartimento di Scienze Fisiche e Astronomiche, Università di Palermo, Via Archirafi 36, 9012
Palermo

ABSTRACT

Mechanisms of partial unfolding and aggregation of proteins are of extreme interest in view of the fact that several human pathologies are characterized by the formation and deposition of protein insoluble material, mainly composed of amyloid fibrils. Here we report on an experimental study on the heat induced aggregation mechanisms, at basic pH, of Concanavalin A (ConA), used as a model system. Thioflavin T (ThT) fluorescence and multi-angle light scattering allowed us to detect conformational changes and growth of ConA fibril-like aggregates. All the data are consistent in demonstrating that the aggregation process develops in different steps. In particular, ThT binding, according to previously published CD data, indicates the occurrence of secondary structure changes that appear to be the rate limiting step for the early stage fibril formation. The intertwining between conformational changes, correlated to the formation of intermolecular β-structures, and the whole aggregation process is discussed as a function of protein concentration: a coagulation process produces the same kind of aggregates at the different concentrations studied. Multi-angle light scattering data highlight the onset of the condensation process having as a result the formation of compact fractal aggregates. AFM microscopy supports this conclusion showing thin fibrils of ConA, formed in the early stage of aggregation, which further interact to form larger structures with a net-like spatial organization.

KEYWORDS

Concanavalin A, amyloid aggregation, ThT binding, conformational changes, light scattering, aggregates structure, AFM.
INTRODUCTION

Protein aggregation understanding is a fundamental target in many research areas, from physics to medicine, from biotechnology to pharmaceutical industry. Many human protein disorders are correlated with formation of proteinaceous deposits, often containing ordered aggregates, called amyloid fibrils. Amyloid fibrils formation can be characterized by selected experimental features, such as green birefringence upon staining with Congo Red dye, fluorescence emission of the Thioflavin T dye, increase of \( \beta \)-sheet secondary structure content with respect to the native protein as detected by infrared absorption and/or circular dichroism spectroscopy and typical fibrillar morphology seen by microscopy techniques.

\textit{In vitro} studies of different model proteins, involved or not in pathological events, showed up the ability of any polypeptide chain to aggregate forming fibrils, under suitable conditions. The amyloid final structure is characterized by high stability and difficulty to be dissolved back to the native state. In the last decades, it has become evident the major role played by destabilizing conditions (choice of pH, increase of temperature, addition of cosolvents, sequence mutations) in prompting amyloid aggregation mechanisms, to favour both intermolecular interactions and partially unfolded protein states prone to associate. The presence of common structural features in amyloid fibrils, no matter which protein they are made of, suggest that in the process to find the highly stable amyloid super-structure rich of intermolecular beta structures, conformational changes at the level of tertiary and secondary structure are strongly implicated.

Light scattering is a widely used technique to monitor protein stability and protein coagulation processes, as well as liquid-liquid phase transitions and crystallization regimes. Time course static light scattering from an aggregating solution gives an estimate of the weight averaged molecular mass growth during the process. Simultaneous time course dynamic light scattering allows following also the growth of the average hydrodynamic radius of the aggregates in solution. Moreover, time evolution of the solution structure factor adds information about the aggregates structural properties.

Thioflavin T (ThT) is one of the most used dyes for detection and kinetic characterisation in amyloid fibrils formation. Under binding to amyloid structures, ThT fluorescence properties specifically change.
Few studies, focusing on structural modelling, try to explain the specific fluorescence coming from ThT bound to fibrils. Major points are the presence and the accessibility of cavities or surfaces where ThT can bind. While ThT emission is related to conformational properties of the aggregates, light scattering is sensitive to the average mass and the hydrodynamic size and shape of the solute molecules. The combination of the two methods is a very useful tool to highlight different features of amyloid growth.

Concanavalin A (ConA) is a 26KDa lectin protein, extracted from jack bean and constituted by 237 aminoacid residues. Above pH 7, the protein quaternary structure is characterized by a tetramer, made of two identical dimers, perpendicularly arranged. Each monomer presents 12 beta-sheets organised in a jellyroll motif. ConA shows a large structural homology to the human serum amyloid protein, SAP, a two-pentameric subunits protein, which binds to all forms of amyloid fibrils and is generally present in all the in vivo fibrillar deposits. ConA aggregation has been studied as a function of pH, showing the occurrence of two different pathways leading to the formation of amyloids or amorphous aggregates. The relative weight of these two pathways is modulated by pH: amorphous aggregates are preferentially formed at low pH values, while fibrils formation is favoured at high pH values. To focus on the mechanism of fibril formation, in the present study ConA aggregation process at basic pH (pH 8.9) and T=37°C has been monitored at different protein concentrations, by dynamic and multi-angle static light scattering, and by in situ ThT binding. The results obtained with both the techniques allow detecting conformational changes and growth processes in the ConA aggregation. ThT binding, according to previously reported CD data, indicates the occurrence of secondary structure changes that appear to be the rate-limiting step for the early stage fibril formation.

EXPERIMENTAL METHODS

Sample preparation

ConA (type IV, L7647) and ThT were purchased from Sigma Aldrich and used without further purification. All the measurements were performed in phosphate buffer 0.1 M pH 8.9. Protein concentration was determined by UV absorption at λ=280nm, using ε=33280 cm⁻¹M⁻¹ and ranged from...
about 0.1 mg/ml to 1.4 mg/ml. Each solution was freshly prepared at 6°C and filtered just before the measurement through 0.22 µm filters.

ThT fluorescence

ConA aggregation was studied by monitoring in situ ThT binding from solutions incubated at T=37°C. ThT final concentration in ConA solutions was 13.3 µg/ml. ThT did not change the ConA aggregation process, as checked by monitoring time course turbidity from the aggregating solution, with and without dye. Emission spectra were measured using a Jasco FP-6500 instrument equipped with a Jasco ETC-273T Peltier as temperature controller. Samples were filtered in a 1 cm path cuvette and, after 3 minutes for thermal equilibration, emission spectra were recorded (\(\lambda_{\text{ex}}=440\) nm, emission and excitation bandwidth 3 nm, scan-speed 100 nm/min, integration time 1s). It was verified that hydroxylation processes at basic pH did not affect fibril detection 23.

Static and dynamic light scattering (SLS and DLS). Time resolved light scattering experiments were carried on solutions incubated at T=37°C, immediately after ConA dissolution in 0.1M phosphate buffer pH 8.9 and direct filtration in cuvette. The cuvette was placed in a thermostatically controlled cell compartment of a Brookhaven Instrument BI200-SM goniometer, equipped with a 100 mW solid state laser at \(\lambda_0 = 532\) nm. Temperature was controlled by a circulating bath with a tolerance within 0.05°C.

Scattered light intensity at 90° (q=23µm\(^{-1}\)), \(I_{90°}(t)\), and its time autocorrelation function, \(g_2(t)\), were measured simultaneously by using a Brookhaven BI-9000 correlator. The static scattered light was monitored in order to follow the growth of the weight averaged mass \(M_w\) in the aggregation process of the protein, according to the fact that

\[
I_{90°} \propto k c M_w P(\theta = 90°)
\]

with \(P\) representing the form factor and \(k = \frac{4\pi^2 n^2}{\lambda_0^2 N_A} \left(\frac{dn}{dc}\right)^2\), where \(n\) is the refraction index of the solution at T=37°C, \(N_A\) is the Avogadro number, \(\lambda_0\) is the in vacuo laser wavelength, \(dn/dc\) is the derivative of \(n\)
with respect to the protein concentration (we consider $dn/dc=0.18 \text{ cm}^3\text{ g}^{-1}$), $c$ is the protein concentration.

$P(90^\circ)$ is $\leq 1$. The decrease in intensity is due to the increase of the scatterers average size, $d \geq \lambda_0/10$.

The low concentration regime ($c \leq 1.4\text{mg/mL}$) allows neglecting structure effects, due to protein interactions. Time resolved light scattering for the solution at $c=0.55\text{mg/mL}$ was monitored as a function of the scattering vector $q$, in the range $5$ to $30 \mu\text{m}^{-1}$ (Large Angle Light Scattering) in order to obtain the time evolution of the form factor $P(q)$. Field autocorrelation functions $g_1(t)$ at $90^\circ$, obtained from

$$g_2(t) = 1 + Bg_1^2(t)$$

with $B<1$, were analyzed using a smoothing constrained regularization method (30), in order to obtain the distribution $P(D)$ of the apparent diffusion coefficients $D$:

$$g_2(t) = 1 + B\left| \int P(D)e^{-Dq^2t}dD \right|^2$$

where $B$ is a factor accounting for the experimental setup (ideally $B=1$), $q$ is the scattering vector defined as $q = \frac{4\pi n}{\lambda_0} \sin \frac{\theta}{2}$, with $n$ the medium refractive index at $T=37^\circ\text{C}$ and $\theta$ the scattering angle. We can express the apparent diffusion coefficient as a function of the $z$-averaged hydrodynamic radius $R_h$, by using the Stokes-Einstein relation:

$$D = \frac{kT}{6\pi\eta R_h}$$

where $k$ is the Boltzmann constant, $T$ the absolute temperature and $\eta$ the solvent viscosity. Thus we obtain the distribution $P(R_h)$ of the $z$-averaged hydrodynamic radius. The harmonic mean of the hydrodynamic radius is calculated from the mean value of the distribution $P(D)$:

$$\langle R_h \rangle_{\text{harm}} = \frac{KT}{6\pi\eta\langle D \rangle} = \frac{KT}{6\pi\eta} \left( \int P(D)DdD \right)^{-1}$$

Absolute scale for the scattered intensity was obtained by normalization with respect to toluene, whose Rayleigh ratio at 532 nm was taken as $28\times10^{-6} \text{ cm}^{-1}$. 
Small angle light scattering (SALS)

For the samples at c=0.12mg/mL, 0.55mg/mL and 0.86mg/mL time resolved light scattering was measured as a function of the scattering wave vector \(q\), spanning the range 0.02 to 2\(\mu\)m\(^{-1}\), by using a homemade experimental setup\(^{24}\), equipped with a 20mW helium-neon laser and a charged coupled device Pulnix TM765 camera. Measurable intensities can span a wide dynamical range (from 1 to 33105 a.u.) due to a software integration of multiple exposure times (1/60 to 1/10000sec). SALS data are not in absolute scale and therefore to join LALS and SALS data, low \(q\) data were scaled to the high \(q\) ones by assuming that lower \(q\) SALS data lie on the straight line (in log-log scale) determined by fitting the high \(q\) LALS data. This was done by using the structure factor measured after 320min of incubation at T=37°C. The scaling factor for LALS data was then applied to all sets of data, observing a general good agreement. The analysis of the structure factor data has been performed by assuming a fractal model for the aggregate. We used the expression

\[
S_m(q) = \frac{1}{m} \left[ 1 + \frac{d_f \Gamma(d_f - 1) \sin((d_f - 1) \arctan(q \xi))}{(q r_0)^{d_f} \left( 1 + \frac{1}{q^2 \xi^2} \right)^{(d_f - 1)/2}} \right] \quad \text{for } q r_0 \ll 1 \ll q \xi^{12} \quad (6)
\]

where \(m\) is the number of identical monomers of radius \(r_0\) in the aggregate, \(\Gamma\) is the gamma function, \(\xi\) gives indication of the cluster size and \(d_f\) is the fractal dimension, related to the structural packing of the fractal cluster. The function used is obtained analytically by assuming an exponential cut-off function, with the exponent \(\xi\) giving the size of the fractal aggregate. Late stage structure factors were fit by also considering a hard sphere interaction between the fractal clusters \((I(q) = S_m(q) S_{hs}(q,D/2,\Phi))\), whose fit parameters, \(D/2\) and \(\Phi\), give the hard sphere radius and volume fraction respectively.

AFM measurements

ConA solution (0.5mg/ml), incubated at 37°C for about 400 minutes was diluted 50 times in bidistilled demineralised water and small aliquots (10µl) were deposited on freshly cleaved mica. The samples were dried by a gentle nitrogen flux and imaged in air. AFM images were acquired in different regions of each
sample in tapping mode using a Veeco Multimode Nanoscope V workstation (Santa Barbara, CA). Etched-silicon probes with a pyramidal-shape tip having a nominal curvature of 8 nm and a nominal internal angle of 25° were used for imaging. The drive frequency was around 300 kHz and the scan rate was between 0.5 and 0.8 Hz. Height and phase images were collected by capturing 512 x 512 points in each scan.

RESULTS

In order to study the mechanism of fibrils formation in solutions of ConA at basic pH, experiments at different protein concentrations have been performed at T=37°C.

It has been reported that ConA undergoes conformational changes and aggregation in the pH range above 8, with a parallel loss of sugar binding activity. However, the instability of ConA at high pH values only recently has been related to amyloid fibril formation. The dimer-tetramer equilibrium is dependent on pH. Below pH 5 (the protein isoelectric point) ConA is a dimer, while for higher values of pH is mainly a tetramer. After dissolution in alkaline conditions protein aggregation starts almost immediately as shown by light scattering results reported later in this section. ConA in its native state, similarly to other all-beta native proteins like beta-lactoglobulin, does not strongly interact with ThT. In alkaline conditions and at 37°C we observe the progressive increase of ThT fluorescence that in turn indicates the formation of binding regions for this dye. The time course of in situ Thioflavin T binding at five different protein concentrations is reported in figure 1a. Continuous lines represent data fits according to the empirical stretched exponential function $A \left[ 1 - e^{-\left( \frac{t}{\tau} \right)^\beta} \right]$, with a constant exponent $\beta=0.7$. The analysis aims at extracting a characteristic time of the ThT binding process ($\tau$) as a function of the concentration. Figure 1 reports also the parameter $\tau$ (b) and the plateau value (c) as a function of concentration. ThT binding rate is independent on concentration (1b) while the final amount of bound ThT is proportional to the concentration (1c). These results are consistent with the occurrence of conformational changes involving the single tetramer of ConA. We have previously studied the early stages of ConA aggregation process at pH 8.9 and we have shown that amyloid formation is triggered by
the transition to a partially unfolded structure; ThT emission increase was found to occur simultaneously with secondary structures changes seen by CD. In particular, the CD absorption (at $\lambda=210\text{nm}$) follows the same trend as ThT fluorescence and the two kinetics showed a time dependence which can be described in terms of stretched exponential.

The intensity decrease in the CD spectrum at $\lambda=210\text{nm}$ is detected in many cases of amyloid fibril aggregation and connected to formation of intermolecular $\beta$-structure in the ordered aggregates. The agreement of the ThT binding data with the CD data confirms that the increase in ThT affinity is correlated to such a conformational change of the protein, at the level of the secondary structure and likely to an increase of intermolecular $\beta$-structures. Moreover, FTIR measurements on ConA aggregates, grown in analogous conditions, have shown the presence of aggregate $\beta$-sheets with strong intermolecular hydrogen bonds usually found in amyloid structures, together with a little amount of antiparallel intermolecular $\beta$-sheets.

ConA aggregation has been also monitored by static and dynamic light scattering at different concentrations with the aim of understanding the pathway leading to aggregates formation. The Rayleigh ratio at $q=23\mu\text{m}^{-1}$ ($\theta=90^\circ$), normalized in order to obtain the weight averaged molar mass of the protein, was read immediately after incubation at $T=37\,^\circ\text{C}$, and results are reported in Figure 2a, divided by the theoretical tetramer molecular mass. Thus, ConA dissolved in pH 8.9 buffer is mainly in the tetrameric form at all the concentrations investigated, although its evident instability under these conditions makes it difficult a mass evaluation. We can infer that ThT fluorescence enhancement is triggered by conformational changes, undergoing during the early stage of the aggregation process and likely involving the tetramer unit. In fact, figure 2b reports on the time evolution of the Rayleigh ratio measured at $q=23\mu\text{m}^{-1}$ (normalized to the Rayleigh ratio calculated for a tetrameric) for samples with different concentrations, incubated at $T=37^\circ\text{C}$. The growth of the scattering intensity indicates that the protein molecules associate forming higher molecular mass scatterers. The process starts with a tetramer and an immediate growth is detected at all the concentrations. It is evident that the aggregation rate, as monitored
by the Rayleigh ratio, does depend on concentration, at difference with the characteristic time $\tau$ extracted by ThT binding experiments. In figure 1a, the ThT fluorescence enhancement reaches a plateau for all the concentrations studied in about 75 minutes and the data normalized to concentration overlap on top of each other, without need of any time scaling. This is not the case for the Rayleigh ratio increase, as evident by figure 2. A scaling of the time-axis taking into account an inverse linear dependence on concentration is reported in the inset of figure 2b, showing a poor scaling result. Simultaneously to the static intensity, also time evolution of the intensity autocorrelation functions is monitored. The average hydrodynamic radius is extracted by the analysis of the intensity autocorrelation function (as reported in the methods section) at different times. Figure 3 shows the growth of the average hydrodynamic radius normalized to the initial tetramer value $R_{h0}=5\text{nm}$. It can be noticed that while the scattering intensity shows the tendency to reach a plateau, the hydrodynamic radius keeps growing. This behavior can be explained considering that at 90° ($q=23\mu\text{m}^{-1}$) the aggregates larger than about 50nm scatter significantly less, due to the form factor effects, and as the aggregates size reaches a critical dimension (of the order of a micrometer) the detection method can become even insensitive to such species. On the other hand, the intensity autocorrelation function can still detect these larger species, although with a low signal to noise ratio.

Figure 4 shows for all the concentrations studied the correlation between the normalized Rayleigh ratio and the hydrodynamic radius normalized to the tetramer radius in a log-log plot, in order to have indication of the structural properties of the aggregates forming in the early stage of the process. Data overlap indicates that the same kind of aggregates form at all the concentrations studied. For species with hydrodynamic radius below 50nm ($\frac{R_h}{R_{h0}}<10$) no form factor correction is required and data can be fitted with a power law, according to the expression $\frac{M_{ag}}{M_{Te}} = \left[\frac{R_h}{R_{h0}}\right]^{\alpha}$ with $\alpha = 2$.

In order to be able to detect larger aggregated species, up to dimensions on the order of microns, small angle light scattering at $q = 0.42\mu\text{m}^{-1}$ was monitored during incubation at $T=37^\circ\text{C}$, of ConA solutions at
high pH, at three different protein concentrations (0.12mg/ml, 0.55mg/ml and 0.86mg/ml). The time course of the small angle static light scattered at the three different concentrations is reported in Figure 5. In the inset of Figure 5 the two sets of data corresponding to the higher concentrations are scaled to the third one \((c_0=0.12\text{mg/ml})\), by the transformation \(t'=t\left(\frac{c}{c_0}\right)\). A good overlap is obtained, indicating that the process of formation of large aggregates is the same at the different concentrations studied. The continuous line shown in the inset represents an exponential growth according to the expression

\[ I = I_0 \left(1 + e^{-\frac{t}{\tau_{SALS}}}\right) \text{ con } \tau_{SALS} = 2343 \text{ min (for } c = c_0). \]

In order to gain information on the structure of the aggregates during the process, Figure 6a reports some snapshots of the time evolution of the Rayleigh ratio as function of \(q\), for the intermediate concentration (0.55mg/ml). To carry on this experiment twin solutions coming from the same sample have been monitored simultaneously in the LALS and SALS apparatus. LALS and SALS data put together cover a broad region \((0.1<q<30\mu \text{m}^{-1})\) showing structural features of the aggregates, interesting different length-scales from tenth of nanometers (thin fibrils) to microns (fractal aggregates). In the early stages of the aggregation \((t<150\text{ min} \text{ and } qR_g<1)\) data can be analyzed with a Guinier model. Afterwards, data can be well fitted by using a fractal model, according to the expression reported in the methods section \((eq.6)\). The continuous lines in Figure 6a represent the data fitting curves. The best fits were obtained setting the fractal monomer radius \(r_0=5\text{nm}\) \(\text{(see eq.6)}\), corresponding to a tetramer unit. Changing this parameter up to 10nm does not almost influence the fitting quality and yields a variation of the order of 2% in the other fitting parameters. Eventually, data corresponding to the fast increase of the SALS signal \((Figure 5, c=0.55\text{mg/ml}, t>500\text{min})\) were analyzed by adding also an interaction factor in the expression, mimicking a more complicated system. Figure 6b reports on the fitting parameters coming from the combined analysis. The radius of gyration was obtained directly from the Guinier analysis \((t<150\text{min})\) or by using the relation \(R_g = \sqrt{\frac{3d_f}{2}}\xi\) from the cluster size \(\xi\) and the fractal dimension \(d_f\) \((t>150\text{min})\) \(^2\). The increase in the fractal dimension suggests a progressive thickening of the aggregates. The analysis with an
additional interaction term, modeled as hard sphere interaction, gives a volume fraction of the interacting aggregates of about 0.13 in both cases, and a hard sphere radius of about 2.5 µm.

Figure 7 reports the AFM images of ConA aggregates formed in solution (0.5mg/ml) after 400 minutes of incubation at T=37°C. Analogous results were found for the lower concentration after 20 hours of incubation, in agreement with SALS results. Long and thin fibrils are present, the latter being organised in a net-like way (see inset). The thin fibrils height is homogeneous in each region of the sample and is about 3nm. This value is comparable to the one previously measured after shorter incubation time (see ref. 22). Furthermore, superimposed over the thin fibrils, tangles formed by thicker and larger structures (whose width varies in the range 30 to 250nm) can be noticed. This evidence is compatible with the hypothesis that thin fibrillar structures, with an intermolecular β-sheets core, which binds ThT, are formed in the first part of the process and then a further supramolecular association occurs, leading to larger and more compact structures.

All these data indicate that the aggregation process develops in several steps. In fact, although the large angle light scattering experiments show a plateau (see Figure 2), further association and supramolecular rearrangement still go ahead with formation of large aggregates up to dimensions of micrometers, as detected by dynamic light scattering, small angle light scattering and by AFM. Comparison of the time scale in Figure 2 and Figure 5 is important to notice that when the LALS stops to increase (for c=0.86mg/ml at about 200min), SALS starts to rise. This indicates that the combined use of the two techniques can give a full picture of the processes going on. Moreover, it is possible to observe that ThT fluorescence probes the formation of β-aggregate structures, involving in this peculiar case the tetramer unit and reasonably the stabilizing of long and thin fibrils. These structures, characterized by intermolecular β-sheets with strong H-bonding typical of amyloid fibrils, orderly interact to form complex supramolecular assemblies.
DISCUSSION

**β-aggregate structure formation and fibrils growth**

The results obtained from light scattering and ThT emission data suggest that these two methods are yielding complementary information on the processes going on. In correspondence with the increase in ThT binding we observe an increase in both, the weight averaged molecular mass $\langle M_w \rangle$ and the $z$-averaged hydrodynamic radius $R_h$, prompting that an association is simultaneously undergoing. This agrees with the AFM data already published that showed the formation of amyloid fibrils during the early stage of the aggregation. In the same paper, the conformational changes detected by ThT binding enhancement are correlated with an increase of β–aggregate structures as can be seen by comparison of fluorescence data with CD data. At the lowest concentration studied, the Rayleigh ratio can be fit by using the expression

$$\frac{R_{90}}{kcM_w^0} = \frac{M_w}{M_w^0} = \left[1 + \frac{t}{zt_0}\right]^z$$

(7)

which describes a coagulation process. Data fit parameters are $z=0.77$ and $t_0=3.4$ min. The curve is shown in figure 2b. In a colloid aggregation framework, the function (7) is obtained by solving the Smoluchowsky equation, assuming a homogeneous kernel ($k_{a,a_i} = a^2 k_{ij}$, for all $a$ values). $k_{a,a_i}$ is the probability of association for clusters with aggregation number $a_i$ and $a_j$ respectively and $k_{ij}$ represents the probability of association for clusters composed by $i$ and $j$ monomers respectively. The parameter $z$ is related to the exponent $\lambda$, according to $\lambda = \frac{z-1}{z}$. The $z$ value obtained by the analysis leads to $\lambda=-0.35$, suggesting a mechanism of aggregation which is governed mainly by clusters diffusion. A fit of the highest concentration data according to the expression (7) in the region where there is no form factor contribution ($t<50$ min), would give $z=2.8$ and therefore $\lambda=0.64$ (about 2/3) (Figure 2b). This result indicates, unlike the low concentration case, that at high concentration larger clusters have a higher
probability to stick together than the small ones. Moreover, by assuming this model at high concentration, the probability of association could be related to the exposed surface of the cluster.

By scaling the time axis for the concentration, as in the inset of Figure 2b, it can be clearly seen that high concentration samples lag behind during the early stage of the kinetics. This can be interpreted by considering that the conformational change, related to formation of β structures compatible with ThT binding, is a concentration independent rate limiting step for the fibril formation. In fact, the observed ConA aggregation occurs at pH far from the isoelectric point of the protein and in these conditions electrostatic repulsion is likely to reduce the probability of casual collisions, which may lead to disordered assembly, so that the aggregation process requires more specific interactions, like those occurring in the formation of β-aggregates that are stabilized also by hydrophobic interactions\(^{22}\). The formation of these structures from destabilized molecules requires protein structure changes that are concentration independent. As the concentration increases, the association is slowed down by the conformational conversion, needed for the protein-protein interaction, which becomes the limiting step.

The two concentrations studied are not extreme conditions but the different regimes in these two cases are an indication of the connection between the conformational change and the fibrils formation process. At the intermediate concentrations the exponent \(z\) increases, crossing at \(c=0.28\)mg/ml the condition where \(\lambda=0\), that is \(z=1\), meaning a classical mass independent kernel, determined by a balance between the rates of the two processes. Notwithstanding the apparent complexity, all the intensity data at \(q = 23\mu m^{-1}\) plotted as a function of the corresponding average hydrodynamic radius overlap. The line plotted in figure 4 has a slope 2, giving indication of an average fractal dimension \(d_f = 2\). This result agrees with a light scattering study on the Aβ-peptide amyloid aggregation at low pH\(^{9}\).

**Aggregates condensation and structural properties**

The combination of the small and large angle light scattering leads to a deeper comprehension of the aggregation process because, as it can be observed in Figure 5, the process that apparently concludes, as seen by LALS, is clearly *in fieri* when monitored by SALS. Interestingly, as shown in the inset of Figure
5, small angle light scattering data at the three concentrations studied can be overlapped by scaling the
time axis with the concentration. This result states that such kinetic process is the same at all the
concentrations unlike the early step of the coagulation process. The scattered intensity rises exponentially,
suggesting a mechanism similar to a heterogeneous nucleation, where the formation of such large
aggregates is enhanced and maintained by the exposed surface of the same aggregates\textsuperscript{33,34}. The process is
slowed down at some point by aggregates precipitation. In order to gain information on the structural
properties of the aggregates formed, the combination of small and large angle light scattering again can
give some more information. The fractal model applied to the structure factor shows an increase of the
fractal dimension, meaning a compaction of the aggregates, followed by an increase of the aggregates
average size. By imaging the aggregates with AFM it is evident that large aggregates are present in
solution. These are composed by a net of thin fibrils whose formation can be ascribed to the early step
aggregation. The growth of tangles in such a net can explain the increase of the fractal dimension. At
different times, similar aggregates are observed at low and high concentration. It is to be noticed that the
fractal dimension obtained by LALS (see Figure 4) concerns structures of tenths of nanometers, formed in
average during the first 2 hours of the process. At difference, the fractal dimension obtained by kinetic
multi-angle LS data refers to aggregates at much wider length scale.

**CONCLUSIONS**

This work reports that ConA under alkaline conditions presents: 1) a non-nucleated formation of fibril-like
aggregates stabilized by structural reorganization leading to intermolecular $\beta$-structure increase; 2)
amyloid fibrils elongation; 3) a further condensation step with growth of compact aggregates on fibrils
surface. Interpretation of the concentration dependence of the early stage of the aggregation process is
complicated by the intertwining between the conformational changes and the parallel association process.

In the early stage of the process, the formation of intermolecular $\beta$-sheets leading to the formation of long
and thin fibrils represents the rate-limiting step of the observed aggregation. The conformational
instability and the aggregation immediately following the protein solvation, probably arises from a
condition of non-equilibrium, that could be related to protonation/deprotonation of specific residues, like
histidines (for example Hys24 has a pK around 8.45), but also to a demetallization (Mn$^{2+}$ and Ca$^{2+}$ bound
to ConA), which is favoured by metal hydrolisation at high pH$^{35}$. As it is known, the de-metalized protein
is less stable than holo-protein$^{35}$. Both these arguments could be valid and connected, since two
histidines, Hys24 and Hys205, participate in the metal active site.

This study makes it evident how important is the employ of complementary experimental techniques to
investigate a protein aggregation process. Indeed, the analysis of the kinetics independently obtained by
different techniques allowed us to evidence multiple and interconnected processes in ConA aggregation.

ACKNOWLEDGEMENT

We thank Dr. M. Manno for helpful discussion on the theoretical interpretation of data, Dr. P.L. San
Biagio and D. Bulone for their scientific support.

REFERENCES


dimensional structure of concanavalin A. III. Structure of the monomer and its interactions with

structural of concanavalin A. I. Amino acid sequence of cyanogen bromide fragments F1 and F2. *J

1994, 367 (6461), 338-345.

22. Vetri, V.; Canale, C.; Relini, A.; Librizzi, F.; Militello, V.; Gliozi, A.; Leone, M., Amyloid fibrils

Weert, M.; Leone, M., Thioflavin T hydroxylation at basic pH and its effect on amyloid fibril

041401.

25. Olson, M.; Liener, I., The association and dissociation of concanavalin A, the phytohemagglutinin

26. Pfumm, M.; Beychok, S., Alkali and urea induced conformation changes in concanavalin A.

27. Kudou, M.; Shiraki, K.; Takagi, M., Characterization of heat-induced aggregates of concanavalin

28. Kudou, M.; Shiraki, K.; Takagi, M., Stretched-exponential analysis of heat-induced aggregation of


**FIGURE CAPTIONS**

**Figure 1:** ThT emission at $\lambda = 484$nm at different protein concentration (0.2mg/ml; 0.41mg/ml; 0.57mg/ml; 0.96mg/ml; 3mg/ml) (a). The lines represent the fitting curves according to an exponential stretched function. Characteristic time (b) and plateau value (c) obtained by the data analysis.

**Figure 2:** Weight averaged molecular mass normalized to the ConA tetramer mass (aggregation number), obtained by measuring the Rayleigh ratio at the beginning of the incubation at T=37°C, for solutions at different protein concentration (a). Time course of the Rayleigh ratio normalized to the tetramer scattering at different protein concentrations (0.12mg/ml, black; 0.28mg/ml, red; 0.36mg/ml, green; 0.55mg/ml, blue; 0.62mg/ml, yellow; 0.86mg/ml, brown; 1.26mg/ml, orange; 1.38mg/ml, violet) (b). The gray lines represent the fitting curves according to a colloid model with homogeneous kernel (see expression (7) in
discussion section). In the inset the time-axis has been scaled with the transformation \( t = t/c_0 \), where \( c_0 = 0.12 \text{mg/ml} \).

**Figure 3**: Growth of the hydrodynamic radius, normalized to the initial value (ConA tetramer \( R_h^0 \)). Different colors correspond to different protein concentrations (0.12mg/ml, black; 0.28mg/ml, red; 0.36mg/ml, green; 0.55mg/ml, blue; 0.62mg/ml, yellow; 0.86mg/ml, brown; 1.26mg/ml, orange; 1.38mg/ml, violet).

**Figure 4**: Log-log plot of the Rayleigh ratio, normalized to the tetramer scattering, versus the hydrodynamic radius, normalized to the initial value \( R_h^0 \), at different protein concentrations (0.12mg/ml, black; 0.28mg/ml, red; 0.36mg/ml, green; 0.55mg/ml, blue; 0.62mg/ml, yellow; 0.86mg/ml, brown; 1.26mg/ml, orange; 1.38mg/ml, violet). The line represents a power law with exponent 2.

**Figure 5**: Small angle light scattering measured at \( q = 0.42 \mu \text{m}^{-1} \), for three different protein concentrations (0.12mg/ml, black; 0.55mg/ml, blue; 0.86mg/ml, brown). In the inset the data sets at higher concentrations have been scaled (by scaling the time-axis with the concentration) on top to the lowest concentration data set and the line represents a fitting curve according to an exponential function.

**Figure 6**: Time course structure factor for \( c = 0.55 \text{mg/ml} \). Few representative data sets at different incubation times (20min, black; 70min, magenta; 120min, maroon; 170min, blue; 220min, red; 320min, green; 420min, violet; 520min, brown; 760min, orange) (a). The lines represent the fitting curves according to the Guinier model (20, 70 and 120min), the fractal model (170, 220, 320 and 420min) and the fractal model with a hard sphere interaction factor (520 and 760min). (b) Fitting parameters obtained by fitting analysis: Aggregate radius of gyration \( R_g \) (triangles in log-lin scale) and fractal dimension \( d_f \) (circles).

**Figure 7**: Tapping mode AFM image (height data) of ConA aggregates formed at \( \text{pH} \ 8.9 \) from 0.5 mg/ml sample after 400 minutes at 37°C, scan size 3 \( \mu \text{m} \). Long and simple fibrils are present, those structures appear organised in a net-like way. The inset, scan size 1 \( \mu \text{m} \), shows a detail of another area of the same sample.
Figure 1

Figure 2

Figure 3
Figure 4

Figure 5
Figure 6

Figure 7