Amyloidogenic proteins and capillary electrophoresis: from folding to small molecule binding

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1 Introduction
Several disorders commonly known as amyloidoses are predominantly caused by a misfolding process associated with the formation of extracellular toxic protein aggregates and insoluble fibrillar deposits in various tissues and organs [1]. Among the 21 amyloidogenic proteins known to date, each one involved in one type of amyloidosis, we studied β-amyloid (Aβ) peptides, which are related to Alzheimer’s disease (AD); α-synuclein, that has a role in the etiology of Parkinson’s disease; β2-microglobulin, associated with Dialysis Related Amyloidosis. Folding studies of these proteins are important to clarify their pathological aggregation pathway, a dynamic and complex process that implies the formation of different intermediate species at equilibrium, including conformers, non-covalently associated soluble oligomers, protofilaments and protofibrils, before fibril deposition.

Capillary electrophoresis (CE) is a separative technique that enables the separation and the detection of these species by monitoring folding/unfolding/misfolding equilibria and conformational transitions of proteins. Once assessed methods able to study the aggregation process of these proteins until fibril formation, the availability of CE methods is extremely valuable, as they can be used also as a starting point for binding studies with small molecules, to investigate the possible effect exerted on the equilibrium of these intermediate species [2]. Coincubation studies with small molecules were carried out to search ligands that could interact at different levels of the fibrillogenesis process with particular attention to oligomers or partially structured conformers, the most toxic species.

2 β-amyloid peptides
β-amyloid peptides are produced by the cleavage of a larger amyloid precursor protein (APP) and are found in the senile plaques in the brain of AD patients. As suggested by the amyloid cascade hypothesis [3], Aβ aggregates play a significant role in the etiology of AD. Through an oligomerisation process, the pathway of fibril formation features the presence of transient and still soluble oligomers, that seem to differ not only in their number of peptide-building blocks, but also in their overall conformation and biological activity. CE is an excellent complementary tool for the characterization of such a process, that can be only partially elucidated by a variety of spectroscopic techniques.

The most amyloidogenic peptide, involved in AD, is Aβ1−42, a fragment composed of 42-amino acid with a molecular weight of 4514.15 Da. We have previously proved that CE is effective in the monitoring of the nucleation steps that lead to the formation of Aβ1−42 fibrils. A suitable CE method has been set up to separate and isolate the forming intermediate species so to enable, on a neuroblastoma cellular line, an independent MTT assay that has assigned the cytotoxic activity to a specific peak in the electropherogram, corresponding to a >50kDa oligomer [4]. Our results found a sound confirmation in a recent work, where in vivo studies carried out with transgenic mice, reveal that extracellular accumulation of a 56 kDa soluble...
oligomer of Aβ_{1–42} is the responsible for memory impairment and possibly contributes to cognitive deficits associated with AD [5].

Given these premises, we used this CE method as a starting point. First, various standards of Aβ_{1–42} were studied, because the peptides showed different electrophoretic profiles and aggregation kinetics, depending on synthesis, freeze-drying and solubilisation processes. This implicated a careful optimisation of sample preparation in order to avoid microprecipitation, to improve the electrophoretic traces and to ensure reproducibility of the fibrillogenesis kinetics. On this new source, once the CE method was standardised with similar results that we have obtained in the past [4], Flow Field-flow Fractionation (FIFFF) was used to confirm the pattern of oligomer formation already described, and to add further and complementary data on the transient species that precede fibril deposition. This separative technique allows the monitoring of Aβ aggregation while insoluble material and particles with even µmeter-long diameter are forming, and therefore it can explore a time window that is prohibitive by CE. At low aggregation time, FIFFF with UV detection revealed the presence of a peak whose hydrodynamic radius would correspond to a mass of ∼60 kDa, consistent with the globular aggregate dimensions previously assessed by CE and ultrafiltration for Aβ_{1–42}. At aggregation time longer than 48 hours, FIFFF with MALS (Multi Angle Light Scattering) detection evidenced the presence of much larger aggregates with average length of ∼1.5 µm, consistent with fibril dimensions.

We subsequently exploited the CE method to study the perturbation of the described equilibria by co-incubating small molecules. This task was accomplished by integrating the CE information with other techniques such as in vitro fibrillogenesis by Thioflavin T test, toxicological assays on neuroblastoma cells, transmission electron microscopy data.

Of all the molecules tested, those that previously showed to interfere with the refolding kinetics of β2-microglobulin, like suramin (Fig. 1a), or to inhibit in vitro fibrillogenesis of the same protein, like a sulfonated compound selected from a chemical library (Fig. 1b) [6], did not produce encouraging results with Aβ_{1–42}. We then tested two structurally related antraquinone derivatives with known anticancer activity. They are both inhibitors of topoisomerase II, an enzyme responsible for uncoiling and repairing damaged DNA. Since a non-covalent ternary complex DNA-drug-enzyme is formed, we hypothesized that similar interactions could play a role also in the block of Aβ oligomerisation. Mitoxantrone (Fig. 1c) and pixantrone (Fig. 1d) showed a striking inhibitory effect on the formation of a transient and toxic oligomer corresponding to peak B of the fibrillogenesis pathway [7-8]. In the presence of mitoxantrone (peptide 100 µM, drug 200 µM) (Fig. 2 left) the toxic species B is depleted within 12 hours (Fig. 2c) while species corresponding to peak A remain stable (Fig 2d). Amorphous aggregates are revealed at Transmission Electron Microscopy (TEM). Pixantrone has the same effect but with a much faster kinetics. The peak is completely depleted after one hour of incubation (t_0).

We also tested the effect of different concentrations of the most active compound pixantrone on Aβ_{1–42} oligomerization and fibrillogenesis (Fig. 3). With pixantrone 50 µM and 100 µM it is evident an inverse correlation between peak B population and pixantrone concentrations. They stabilize peak B for two days while in control peptide peak B area increases over time. With all active concentrations the samples remain visibly clear and at the end of the analysis, where no more peaks are detected in CE, TEM revealed the presence of amorphous aggregates instead of amyloid fibrils.

These drugs could block or reduce the accumulation of large oligomers, disrupting them in favour of lower MW oligomers but it is observed that peak A area is kept constant and no further UV absorbing species are detected at 200 nm, so this hypothesis is very unlikely. We hypothesised that these drugs could form soluble protofibril-oligomer complexes migrating at a time not detectable in the observed electrophoretic run. Finally, the clear solution that is seen at the naked eye in the CE vial does not exclude the presence of precipitated amorphous aggregates, as confirmed by TEM, that would justify the absence of peaks observed in CE.

To support the results obtained we also carried out a classical in vitro fibrillogenesis experiment using Thioflavin T as an indicator of fibril formation. Both compounds completely inhibit the fibrillogenesis (Fig. 4a). To quantify the inhibitory potency of the most active compound pixantrone towards Aβ aggregation,
we also determined, from a typical dose-response curve, an IC$_{50}$ value of 26±4 µM (Fig. 4b). Tests were run in triplicate in three independent experiments.

We thus investigated the effects of the two molecules at different concentrations on the viability of the same neuroblastoma cell line (IMR32) previously used [4], which is sensitive to Aβ induced cell death in a concentration-dependent manner. Cell viability was monitored using the MTT assay. Consistent with their pharmacological and toxicological profiles, both mitoxantrone and pixantrone induced a significant loss of cell viability. Since pixantrone appeared to be overall less toxic compared to mitoxantrone and it was able to interfere with the higher-molecular-weight species of Aβ$_{1-42}$ with a faster kinetics than mitoxantrone, we evaluated the biological role of pixantrone on Aβ$_{1-42}$ induced toxicity. The in vitro experiments performed in this part of the work used concentrations which were diluted 10 fold, since they showed a higher toxicity (data not shown) at the dilutions used in CE. However, in experiments with Aβ the ratio between peptide and analyte has been always maintained as in CE. Consistent with our previous results [4], 10 µM solution of Aβ$_{1-42}$ showed a clear degree of toxicity (cell viability: 59.5 + 1.4 %). Co-incubation of Aβ$_{1-42}$ and pixantrone increased the cell viability when compared to the peptide alone (cell viability Aβ$_{1-42}$ 10 µM/pixantrone 5 µM: 73.9 + 3.9 %; Aβ$_{1-42}$ 10 µM/pixantrone 20 µM: 73.7 + 6.1 %). Values are reported as mean values of at least 4 independent experiments on quadruplicate samples. This reduced vulnerability of IMR32 cells with Aβ in the presence of pixantrone could probably be ascribed to

Fig. 1: Chemical structures of the compounds coincubated with Aβ$_{1-42}$.
Fig. 2: Electropherograms taken at different elapsed times from solubilization, as indicated. Left: 100 µM peptide added with mitoxantrone (peptide/drug ratio 1:2); right: 100 µM control peptide. Wavelength: 200 nm.

Fig. 3: Time course of peak B normalized area (each experiment point is in triplicate) for control peptide and inhibitor-peptide samples.
the fact that pixantrone is engaged in seizing toxic Aβ species and nevertheless it can not be ruled out that Aβ might block the inhibitor intrinsic toxicity.

3 α synuclein

α-synuclein (α-syn) is a natively unfolded protein that constitutes one of the major components of cytoplasmic inclusions, known as Lewy bodies, that are a pathological hallmark of Parkinson’s disease (PD). The mechanism by which α-syn leads to neurodegeneration is unclear, and little is known about the dynamic process of α-syn conversion to fibrils. Recent studies suggest that, as for β-amyloid peptides, intermediate α-syn oligomers and protofibrils, rather than fibrils, might be the true neurotoxic species. Several data support the hypothesis that pathological aggregation arises from a key partially folded intermediate that can either form soluble oligomers or insoluble fibrils [9]. Studies carried out by different techniques including Dynamic Light Scattering, Fourier Transform Infrared Spectroscopy, Fluorescence Resonance Energy Transfer and Atomic Force Microscopy have left unanswered a key question, as still it is not clear if transient oligomers are on the direct pathway to fibrils or are off-pathway. The use of CE to dynamically monitor α-syn aggregation has not yet been explored.

Similarly to what done for Aβ1−42, a CE method was set up, to follow in real time α-syn oligomerisation process until fibril formation. In vitro fibril formation of this protein implies an accurate optimisation of various parameters including type and duration of agitation and operative temperature, so to accelerate aggregation within a time frame that is compatible with CE analysis. The most difficult parameter to choose was agitation, as successful formation of fibrils in vitro strongly depends on type and speed of agitation. The aggregation pathway over time is shown in fig. 5, α-synuclein exhibited a main peak and a second species. Over time peak 1 decreased in favour of an increase of peak 2 and formed at longer incubation times new species (peak 3). Spikes in the electropherograms mean the presence of microprecipitation. From the fourth day the sample started to visibly precipitate and TEM showed the presence of fibrils in the pellet and in the suspension that we continued to inject in CE. The sample eventually precipitated at 11th day. Characterization studies on the oligomer size of species revealed a rapid aggregation process because there are already tetramers and esamers after solubilisation.

CE parameters have also been set up and different batches of α-syn samples have been analysed, to standardize the electrophoretic pattern and to make the method suitable for testing, in the future, small molecules known to interfere with the process, namely dopamine, rifampicin or flavonoids.

4 β2−microglobulin

β2−microglobulin (β2-m) is a 99-residue protein which is present on the surface of most nucleated cells. Patients on long-term haemodialysis develop a common and serious complication known as Dialysis Related Amyloidosis, where a systemic deposition of β2-m fibrils occurs mainly in the skeletal muscle system.
and in joints [10]. One $\beta_2$-m conformer, that in the past came to our attention, is an in intermediate of the folding pathway, named $I_2$ [11], which populates the folding reaction at physiological pH for a time sufficiently long to prove its tendency to elongate natural fibrils [12]. Due to its persistence in the reaction, it was possible to separate this species from its native form (N) at equilibrium. This allowed us to consider N and $I_2$ as two independent targets of small molecules and to evaluate the effect of denaturing conditions on the observed equilibrium [12-14]. Here different parameters like temperature and pH have been considered, with particular attention towards those values that have physiological or pathological relevance. Temperatures and pH values like 42 °C and 6.4, respectively, are typical of inflammation and metabolic acidosis that occur in the haemodialysed patient and seem to promote fast and irreversible growth of $\beta_2$-m aggregates [15].

The availability of a CE method that quantifies a protein folding intermediate, that is related to protein stability was a an important stating point, but unfortunately in CE $\beta_2$-m exhibited over time the same electrophoretic profile that the protein has at 25 °C. To observe an increase of the most fibrillogenic species $I_2$, it was necessary to test higher temperatures (60 °C and 80 °C), above the value of transition that indicates the point of an irreversible aggregation. Native form decreases over time in favour of an increase of $I_2$ with a faster kinetics at the increase of temperature (Fig. 6).

No genetic variants of $\beta_2$-m are known and nevertheless several single-site mutants have been evaluated in the past, also by us [13], to investigate their behaviour in relation to folding, misfolding and stability. W60G $\beta_2$-m, where Trp60 is replaced by Gly, was here considered. Data recently published showed that Trp60 has the highest frequency of intermolecular contacts and hence may play a role in the early steps of fibril assembly [16]; also, by using different spectroscopic techniques, it has been established that the presence of Gly at position 60, not only accelerates the refolding kinetics if compared to wild type $\beta_2$-m, but also it stabilizes $\beta_2$-m and abrogates the formation of fibrils [17].

By using CE the refolding kinetics of this $\beta_2$-m species was followed, and the effect that small molecules may exert on this process was evaluated. W60G has a higher kinetic constant and therefore a faster kinetics of refolding than the wild type so the mutant form is more stable than wild type. The most interesting ligand was 573 because it strongly accelerated the refolding process of W60G, confirming to be a potential lead compound able to stabilize native form of both proteins (Table 1).
Fig. 6: Peak N area over time at different values of temperature.

Table 1: Values of kinetic constants for $\beta_2$-m w. t. and W60G.

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<th>$\beta_2$-m wild type</th>
<th>W60G</th>
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<tr>
<td>+ suramin</td>
<td>0.00183 s$^{-1}$ ± 0.00021</td>
<td>0.0056 s$^{-1}$ ± 0.00063</td>
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<td></td>
<td>0.00163 s$^{-1}$ ± 0.00025</td>
<td>0.0022 s$^{-1}$ ± 0.00058</td>
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<tr>
<td>+ 573</td>
<td>0.00201 s$^{-1}$ ± 0.00026</td>
<td>0.015 s$^{-1}$ ± 0.0029</td>
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References