Protein fibrillation and particle interaction by Fluorescence Correlation spectroscopy.

Purpose of the visit

Many proteins are able to form aggregates under the appropriate conditions. Several important diseases such as Alzheimer's, Parkinson's, Haemodialysis related amyloidosis, etc are related to the formation of protein aggregates. Fibrillation of amyloidogenic proteins is a nucleation process with a lag phase corresponding to the time required to form the critical nuclei. After nucleation, elongation step occurs. Several reports suggest that the most noxious aggregated form is small oligomers rather than long fibrils. This process has been previously study by means of Fluorescence Correlation spectroscopy. In this project the aim is to use that technique to analyze the effect of nanoparticles on the fibrillation process. Previous results show that for example in the case of b-amyloid polymeric particles inhibit the formation of fibrils, but little can be obtained regarding the precise mechanism of interaction. FCS is a powerful tool that cold help to understand the evolution from monomers to fibrils in presence of particles. The possibility of using different dyes for fibrils and nanoparticles would in principle allow us to analyze the cross correlation function and therefore interpret results in terms of co-localization, i.e, study the binding of the different aggregated states of protein o the surface of the particles
The short visit motivation is to establish the framework and experimental conditions to perform future experiments regarding this project.

Description of the work carried out during the visit

In order to establish the best experimental conditions and for future experimental planning, we focus our experiments in the fibrillation of beta amyloid protein 1-40 and the effect that Dark Red fluorescence polystyrene particles have in the aggregation process of this protein.
In order to observe the protein aggregates, a dye specific for amyloid aggregates is used. Thioflavin T (ThT) binds specifically to amyloid aggregates and its emission wavelength changes after this binding. ThT has an emission band located at 455 nm when bound to protein aggregates. When free, the emission band is shifted towards shorter wavelengths.
The Polystyrene nanoparticles used have a nominal size of 40 nm and are loaded with a dark red dye with emission wavelength 630 nm.
Each experiment was analyzed using two different microscope channels, one at 458 nm, that correspond to the emission signal of the Thioflavin T dye and other at 633 nm that correspond to the emission of the fluorescence nanoparticles.
Different experiments were performed to identify the correct concentration of dye (ThT), protein and particles required for each experiment.
After this first approach we need to explore if the fibrillation process can be followed by this technique both in absence and presence of fluorescence particles.
In the following section the relevant experiment results are summarize.

Description of the main results obtained
Results:

1. Concentration of nanoparticles and particle stability in relevant buffer:

Correlation curves for the fluorescence nanoparticles in phosphate buffer were obtained for different nanoparticle concentrations (figure 1)

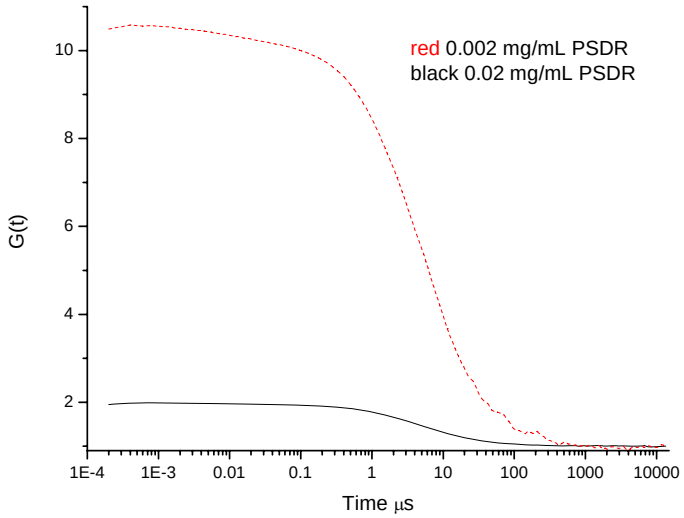

Figure 1: Correlation curve for two different particle concentrations using the 633 nm channel

The values in total count rate indicate that the appropriate particle concentration range is in the order of 0.002 mg/mL.
The analysis of the correlation curves gives us information about the average size of the particles. The results indicate the presence of aggregates of nanoparticles of average size 200nm (table 1). Further experiments were the beads were sonicated before the measurements give closer values to the expected size. Therefore sonication previous to experiment is needed to obtain the best results.

Table 1: Values obtained from the analysis of the correlation curves in figure 1

[conc] mg/mL	**Total Counts (Hz)**	**Diffusion times (μs)**	**Rh (nm)**
0.02	410	5300	103.68 ± 3.87
0.002	39	5249	102.68 ± 3.83

2. Interaction between Thioflavin T and nanoparticles:

Previous experiments suggest a specific interaction between the nanoparticles and the Thioflavin T. ThT should only emit at 458 nm when bound to protein aggregates in

amyloid form. To confirm these, samples that contained ThT and fluorescence nanoparticles were analyse by means of FCS.
Figure 2 show the correlogram of a solution with nanoparticles and ThT at different concentrations by the 458 nm channel. In this channel if no protein is present, no signal must be observed. But in presence of nanoparticles a signal in the 458 nm channel is observed indicating that, by adsorption of ThT on the particle surface, the emission band of the ThT is shifted in a similar fashion that when ThT binds amyloid aggregates.
This effect could be a drawback in the development of the project

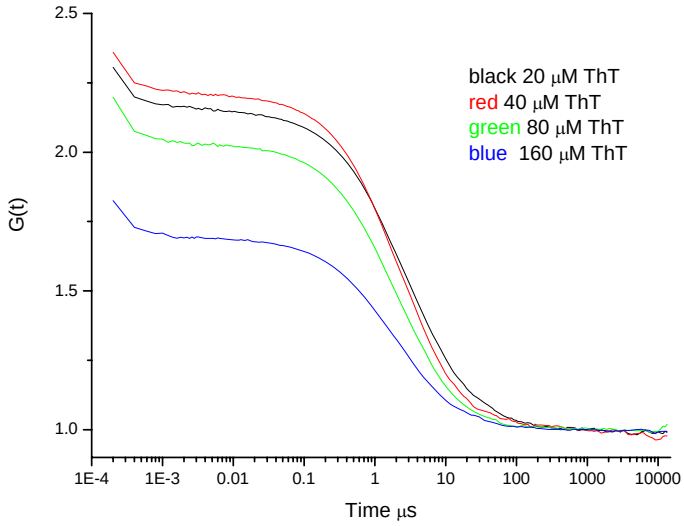

Figure 2: Correlogram for a solution of 0.001 mg/mL nanoparticles and different concentrations of ThT using 458 nm channel.

3. Fibrillation proccess

The fibrillation process from monomeric protein to amyloid fibrils was followed by means of FCS. For this experiment, a fibrillation process was started by incubating monomeric protein in the correct concentration at 37 0C and by continuous shaking at 700 rpm. At selected times and aliquot was removed and freeze in liquid nitrogen to stop the process until the moment of the analysis by FCS. Previous to the analysis, the sample was thawed and equilibrated at room temperature. Figure 3 shows the correlograms that correspond at different time points along this process.

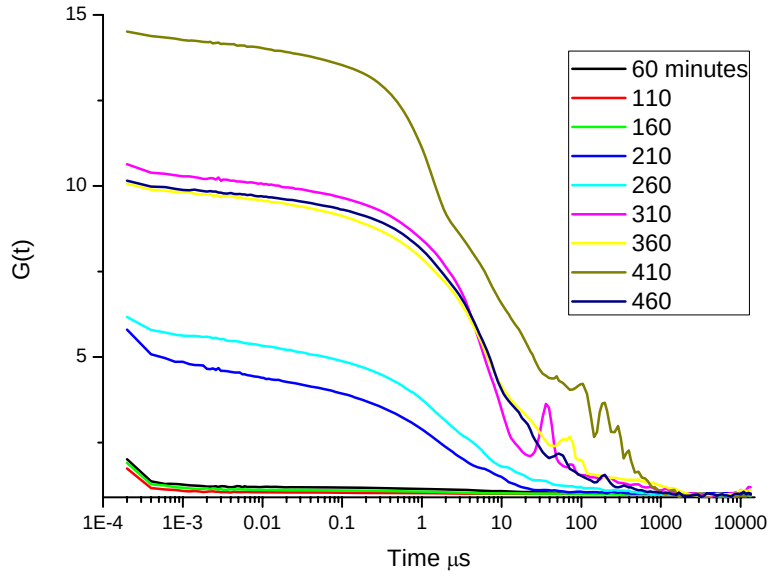

Figure 3: Correlation curves for solution at different time points in a continuous fibrillation process. (458 nm channel)

At early time points when mainly monomeric proteins are present no signal is observed. In this scenario ThT is free in solution and does not emit in the selected channel (458 nm). Once amyloid aggregates start to form (after 210 minutes) ThT binds to the aggregates and the emission is shifted to the correct wavelength and the signal increases.
As the time increases the correlation curve is shifted to longer time indicating the presence of bigger diffusing species.
Table 2 indicates the diffusion times and sizes for the aggregate obtained from the analysis of the correlograms

Table 2: Values obtained from the analysis of the correlograms in figure 3

Reaction time (min)	633 nm channel	
	Diff times µs	Rh nm
60	875 23095	17. 12 ± 0.66 451.76 ± 16.80
110	12 1752	0.23 ± 0.03 34.27 ± 1.29
160	61 2214	1.19 ± 0.06 43.31 ± 1.63
210	57 1788	1.12 ± 0.06 34.98 ± 1.32
260	2095 52178	40.98 ± 1.54 1020.69±37.94
310	4381 219460	85.699 ± 3.20 4293.02 ± 159.51

360	187 7910	3.66 ± 0.156 154 .73 ±5.77
410	1843 36361	36.05 ± 1.36 711.28±26.44
460	1292 12668	25.27 ± 0.96 247.81±9.23

Figure 4 shows the variation of aggregates size with time. At the beginning of the reaction few or none amyloid structures are present and the size and abundance increases at the reaction goes to completion. Certain variation can be observed due to the stochastic nature of the process and the experimental set up

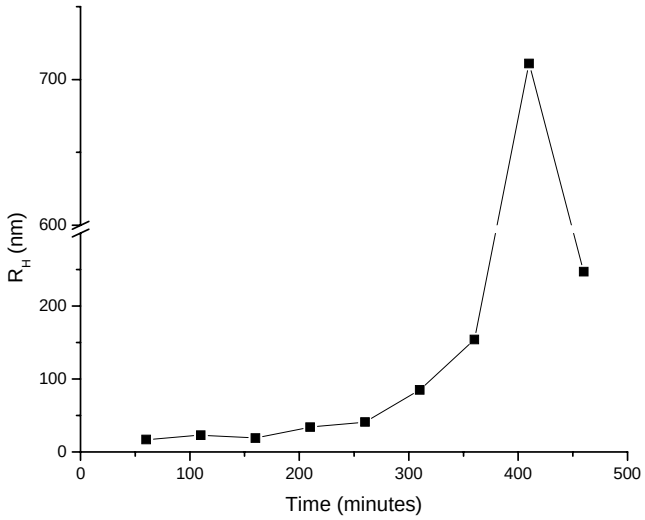

Figure 4: Variation of Hydrodynamic radius versus reaction time

4. Coexistence of fluorescence nanoparticles and amyloid fibrils

When amyloid fibrils and nanoparticles are present in the same solution the amyloid fibrils are detected even though ThT interact with the particles. That means that nanoparticles do not withdraw all the ThT from solution. There is a partition of ThT between amyloid fibrils and nanoparticles. Table 3 resume the results obtained for different ThT concentration in presence and absence of fluorescence beads.

When the 458 nm channel is used two components are observed, low diffusion time corresponding to beads or small amyloid aggregates and a second component with long diffusion times that correspond to the long amyloid fibrils

Table 3: Diffusion times and Hydrodynamic radius obtained from the analysis of samples where amyloid aggregates and nanoparticles coexist using two different channels.

ThT [conc] μM	633 nm channel		458 nm channel	
	Diff times μs	Rh nm	Diff times μs	Rh nm
20 (no beads)			2414 62033	91.66 ± 5.56 2355 ± 141
20	3337 35145	65.28 ± 2.44 687.497 ± 25.562	2589 53618	98.30 ± 5.96 2035.82 ± 141.83
40	3392 3396	66.35 ± 2.48 66.43 ± 2.49	2653 28557	100.73 ± 6.10 1084.286 ± 65.31
80	2265 9114	44.31 ± 1.67 178.29 ± 6.64	2039 27230	77.42 ± 4.69 1033.901 ± 62.28

Conclusions:

a) The fibrillation process of amyloid beta protein can be followed and analyzed using Fluorescence correlation spectroscopy if the specific dye ThT is used. No modification of the protein which could interfere in the fibrillation process is needed.
b) Average size for the growing aggregates can be obtained as the fibrillation process develops.
c) Low concentration of particles is needed to not saturate the detector.
d) In presence of particles, the process can be followed using two different channels. This possibility allows us to confirm the obtained results regarding diffusion time and therefore size.
e) ThT emits at a wavelength that does not allow cross correlation experiments. A dye emitting at longer wavelength is needed.
f) Aggregation of particles must be avoided to be able to easily identify interaction between nanoparticles and protein aggregates.

Future work in collaboration with the institution:

a) Improve nanoparticle dispersion and reduce particle size
b) Modify the experimental plan to use antibody detection with a dye that emits at the correct wavelength to perform cross correlation experiments.
c) Perform fibrillation time course experiment in absence and presence of nanoparticles at different concentration