Nanoscale Diffusion Regimes of Amyloid-β Studied by Liquid-Phase TEM

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Abstract: This project studies the diffusion regimes of misfolded proteins amyloid-β-42 (Aβ-42) aggregates observed by liquid-TEM. Videos acquired using this technique were analysed to reconstruct particle trajectories and thus quantify their dynamic behaviour at the nanometric scale. Due to the low contrast of these protein samples, the videos were preprocessed by normalisation, clipping, Gaussian filtering, and averaging of consecutive frames. Then, regions of interest were selected, and particle tracking was performed to obtain their trajectories. From these trajectories, the mean squared displacement (MSD) was calculated and fitted using a power law to determine the diffusion exponent α. Large aggregates of diameters circa 100nm yielded $\alpha \sim 0$ and therefore they showed negligible translational mobility while for small oligomers of diameter circa 8nm the exponent $\alpha \sim 1$ showing a behaviour compatible with normal Brownian diffusion.
Keywords: Amyloid-β, Liquid-phase TEM, protein aggregation, particle tracking, diffusion.
SDGs: Health and well-being.

I. INTRODUCTION

This project aims to investigate the diffusion regimes of misfolded amyloid-β-42 (Aβ-42) assemblies imaged by liquid-phase transmission electron microscopy (liquid-TEM) with nanometer spatial and millisecond temporal resolution.

Aβ is a key protein that in brains affected by Alzheimer's disease (AD) misfolds and aggregates in the brain, strongly influenced by its transient structure and nanoscale dynamics in liquid environments [1]. Therefore, understanding and characterising the physico-chemical properties of this protein could help elucidate its mechanisms of action and be highly valuable in providing new perspectives on the disease. In this day and age, neurodegenerative diseases, including AD, are one of the most common causes of death around the world [2]. This makes the study Aβ a major research priority, given that no known cure is currently available [1]. What we do have is substantial evidence that AD begins with an incorrect folding in the brain of a series of proteins, among them Aβ, which leads to the formation of aberrant toxic aggregates[1]. These aggregates induce neuronal damage that, over time, leads to neuronal death and to the deterioration of the brain's capacities and functions.

Aβ can present different morphologies, including monomers, oligomers, fibrils and plaques. Historically, scientists thought that mature protein aggregates, such as fibrils, were the distinctive hallmark of AD [3]. However, over the last two decades, numerous studies have shown that aggregates composed of small numbers of Aβ protein monomers, called oligomers, are highly neurotoxic [1, 3]. Oligomers are conglomerates of monomers, the smallest protein component, that can differ considerably in size, shape, flexibility, structure, composition, and ability to cause neuronal damage. The heterogenous nature of oligomers makes it imperative to develop methods that characterize their properties to better understand their molecular origin [1].

The present project focuses on investigating the diffusive behaviour of Aβ-42 aggregates in solution at the nanoscale; their movement trajectories will be analysed from liquid-TEM videos by tracking individual Aβ-42 particles and subsequently characterising their stochastic motion in the liquid media. Liquid-TEM is an innovative technique that combines a TEM microscope with a special liquid holder that allows the direct visualisation of objects at the nanoscale in liquid media. In order to preserve the structural integrity of all the imaged proteins, it is extremely important that the images and videos are obtained under ultralow electron dose conditions [4]. The videos obtained through this technique are processed using an image analysis routine to extract first the Aβ-42 particles that will be used for the investigation. Then the individual trajectories of each Aβ-42 particle are extracted and the mean squared displacement (MSD) is calculated as a function of time, making it possible to characterize the different diffusion regimes. Therefore, by analysing the behaviour of the MSD, we will be able to distinguish between different types of particle movement: normal Brownian diffusion and anomalous diffusion. This way, one can provide information on the dynamics of this protein's aggregates at the nanoscale under near-native conditions.

II. METHODS

A. Video acquisition

The Liquid-TEM videos were acquired using a JEOL F200 NEOARM TEM. The Aβ-42 samples were prepared

at a concentration of 0.5 mg/mL and for image acquisition, a sample volume of between 1.0 and 2.5 μL was deposited in the liquid cell [5].

The acquisition of the videos was carried out under ultra-low electron dose conditions, under the dose of $10e^{-}/\mathring{A}$, with the aim of reducing beam-induced damage to the protein sample. The electrons were accelerated to an energy of 200 keV, and the videos were recorded as image sequences at a frequency of 20 fps using 10% of the electron beam with the beam modulator attached to the microscope.

The sample preparation and video acquisition were done before the analysis developed in this project, and they were performed by Dr. Ruiz-Pérez's group [5].

B. Video processing

The videos obtained through liquid-TEM presented very poor contrast, which made it very difficult to make a precise identification and temporal tracking of the Aβ-42 aggregates in order to perform their statistical treatment. This low-contrast behaviour is very characteristic of biological compounds, in this case proteins, since they are mainly composed of low atomic number elements [4]. In addition, as mentioned in the introduction, with the aim of not producing structural damage in our sample, a low-intensity electron beam is used, making the quality of the obtained image even more limited[4]. Finally, one last limitation must also be taken into account, which is that in our case the sample is found in a liquid medium where it does not remain immobile as in cryo-TEM or conventional TEM. This causes additional fluctuations and a certain defocusing is introduced due to the continuous movement of the particles in the solution, not only laterally but also in the Z direction.

After the videos were acquired, digital image processing was performed. The objective of this procedure was to eliminate noise and improve the contrast in the videos. This way, one can see the oligomeric protein in greater detail and obtain reliable trajectories for subsequent diffusion analysis. The processing consisted of using a Python library specifically designed for this purpose [6], a manual contrast enhancement using Fiji image-processing software [7], and finally a Python-based particle detection and tracking algorithm.

Processing: The first stage of the video processing consisted of the use of the Python library SimplyPyTEM, an open-source library developed to simplify transmission electron microscopy and the analysis of the in situ TEM videos and images[6].

Through this library, the images extracted from the videos i.e. video's frames were processed with different contrast enhancement methods, filters, and temporal averages between consecutive frames. The aim was to make a comparison of the obtained results and thus be able to select the most suitable combination for the later analysis. Specifically, the tested methods were: normalization, the clip method, equalization, and the Gaussian filters. Normalization rescaled the pixel intensity values to a common range, while clipping reduced the effect of extreme bright or dark pixels. Histogram equalization enhanced the contrast by redistributing intensity values, and Gaussian filtering smoothed high-frequency noise in the images. The temporal averages were calculated with 5, 10, 15, 20, 25, 40, 50 and 100 consecutive frames.

After comparing all possible combinations, it was concluded that the optimal configuration to achieve the best signal-to-noise ratio and strike an adequate balance between spatial contrast improvement and the preservation of temporal resolution would consist of averaging 20 consecutive frames. This was then followed by normalisation, clipping, and a Gaussian filter. This configuration was applied to all videos of the dataset.

After the automated preprocessing, the Fiji image processing software [7] was used to select regions of interest from the obtained videos. The priority was to observe a significant number of visible oligomers or clearly distinguishable individual particles within the frames. In addition, a manual contrast enhancement was carried out with the purpose of improving the visualization of the particles of interest and optimizing as much as possible the quality of the videos for their subsequent analysis.

Particle Tracking. Once the regions of interest had been selected, particle tracking was carried out using an algorithm developed in the Molecular Bionics Lab using Python. The goal was to reconstruct the temporal trajectories of the Aβ-42 aggregates over time. Through this code, each preprocessed frame was analysed individually by means of an image segmentation procedure. First, an intensity threshold (minimum threshold) was used in order to separate the regions corresponding to the particles from the background. After that, an area threshold (minimum area) was also applied in order not to take into account detections associated with noise or small intensity fluctuations not related to real aggregates.

The regions that met these criteria were considered as particles, and the position of each of them was determined by calculating their centre of mass. In this way, each of the particles detected in a frame was labeled with a two-dimensional coordinates(x, y), in the image pane. By repeating this process throughout all the frames, it was possible to reconstruct the temporal evolution of the position of the particles i.e. protein oligomers and thus obtain their individual trajectories.

For the videos in which several visible particles appeared simultaneously, it was necessary to label the particles detected in consecutive frames in order to reconstruct multiple trajectories within the same region

of interest. This same label for a particle between successive detections was assigned according to criteria of spatial proximity and temporal continuity. In this way the same particle cannot undergo arbitrarily large displacements between two consecutive frames. Therefore, a maximum travel distance and a maximum number of frames in which the particle could remain undetected were established in order to link detections to the same particle trajectory.

From the reconstructed trajectories, and using the same Python code, the diffusion analysis was carried out by calculating the MSD.

C. Diffusion theory and analysis using MSD

The motion of Aβ-42 aggregates in a liquid medium can be studied within the general framework of Brownian motion [8]. This is possible because this type of motion appears when a particle that is suspended in a fluid experiences random and continuous collisions with the molecules that are in that medium. This results in the particle obtaining a stochastic trajectory.

If there are no external forces acting on the particle or a biased motion direction, the mean displacement of a Brownian particle tends to 0, as all directions of motion are equally probable but the square of this displacement increases with time, thus allowing particle diffusion to be characterized.

Through the Langevin equation [8], a classical microscopic description of Brownian motion is obtained. There, the dynamics of a given particle are the result of the balance between a viscous friction force and a fluctuating force associated with thermal collisions of the given particle with the particles in the medium.

$$\frac{dv}{dt} = -\gamma v \; + \; \theta(t) \tag{1}$$

Where in this expression v is the velocity of the particle, γ is the viscous damping constant, and θ is a random fluctuating term associated with thermal collisions of the particle of interest with the particles in the medium responsible for its irregular motion.
As already mentioned, each protein particle detected in the videos is represented by a two-dimensional trajectory in the image plane, $r(t) = (x(t), y(t))$.
From these trajectories, the MSD is used to quantify the particle dynamics. For an individual particle tracked over time, the MSD is defined as:

$$MSD(\tau) =< |r(t+\tau) - r(t)|^2 > \tag{2}$$

where τ is the lag time defined as the time that has passed between two positions of the same particle, and the average is performed over all pairs of positions separated by that temporal interval.

The lag time is calculated as:

$$\tau = k\Delta t \tag{3}$$

where k is the number of separation frames and Δt is the temporal interval between two consecutive frames (1/fps).

For a trajectory of a single particle formed by N positions, the temporal MSD is calculated as[9]:

$$MSD(\tau) = \frac{1}{N-k} \sum_{i=1}^{N-k} [(x_{i+k} - x_i)^2 + (y_{i+k} - y_i)^2] \tag{4}$$

If several trajectories are available, the combined MSD can be obtained as an average of the MSDs of each individual particle. This is an approximation given the heterogeneity of the Aβ-42 aggregates.

If a particle diffuses freely through a homogeneous medium, Brownian theory predicts that the MSD grows linearly with time. This would be given by $MSD(\tau) = 2dD_\tau$, where d is the number of dimensions of space, D the diffusion coefficient, and τ the lag time[8]. In our case, the dimensionality of the space is two and, if we were in a regime of normal Brownian diffusion, the diffusion coefficient would be obtained from the slope of the MSD(τ) curve as $D = \frac{1}{4}\frac{d(MSD)}{d\tau}$ where by quantifying the mobility of the particle in the medium, a high value of D would indicate that the particle moves large distances, and a low value would indicate reduced mobility.

Since the studied system may deviate significantly from the ideal case of Brownian motion as the protein particles in this study are in confinement in a liquid cell and therefore not resemble a linear model, a more general power-law was considered.

$$MSD(\tau) = D\tau^\alpha + C \tag{5}$$

In this expression, D is a prefactor related to the mobility of the particle, α is the diffusion exponent, and C is a constant term that accounts for possible contributions to the error (localization, noise, offset, ...)

Through the value of α, we can classify the observed diffusive regime [9].

- $\alpha = 1$. The particle is in the regime of normal Brownian diffusion, in which the MSD grows linearly with time.
- $\alpha < 1$. The particle is in a subdiffusive regime, and therefore the MSD grows more slowly than in normal Brownian diffusion.
- $\alpha > 1$. The particle is in the superdiffusive regime, in which the MSD grows more rapidly than in normal diffusion.

Therefore, we will obtain normal Brownian diffusion for $\alpha = 1$ and anomalous diffusion for $\alpha \neq 1$.

III. RESULTS

In this section, two different Aβ-42 experiments are presented: one corresponding to large oligomers and the other to small oligomers.

A. Analysis of large oligomers

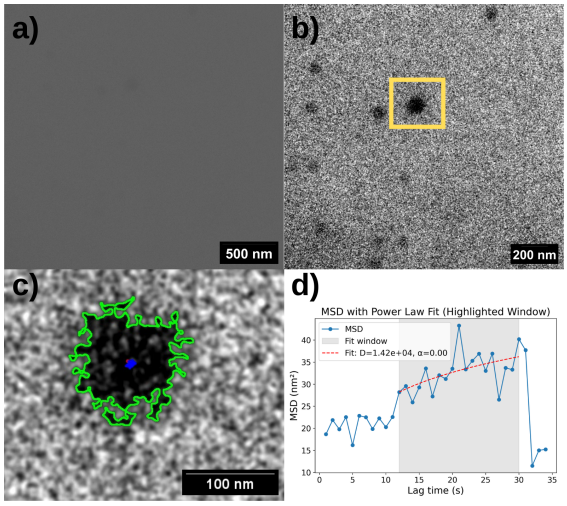

FIG. 1: Analysis of large Aβ-42 oligomers in liquid-TEM. **a)** Representative raw frame from the acquired liquid-TEM video. **b)** Preprocessed frame with a selected region of interest containing an individual oligomer. **c)** Cropped region of interest from b), showing the segmented contour of the selected oligomer (in green) and the trajectory (in blue) of its centroid (a red dot). **d)** Plot of the MSD vs lag time with the experimental calculations and its parameters for best fits.

In this first experiment, oligomers with approximate sizes of 100 nm, we call them large oligomers, were analyzed, recorded for 34s at 20 fps with a pixel size of 0.64nm. Figure. 1a shows a frame of the obtained raw video. This frame was preprocessed using the methodology already mentioned in the methods section, thus making it possible to obtain a video with improved contrast, as can be observed in the frame shown in Figure. 2b, where a selected region isolating a single oligomer is also visible. Figure. 1c shows the cropped image of the isolation region of a single oligomer selected in Figure. 1b in a yellow box. The particle-tracking methodology has been applied to the oligomer in Fig. 1b, based on particle identification using the image segmentation criteria described in the methods; its trajectory is also shown by the movement of its centre of mass, depicted as a red dot in Figure 1c. Finally, the MSD was calculated from the trajectory of this representative oligomer, showing its result in Figure. 1d.

The result from the best linear region was $\alpha = 0$, which means that this particle remains immobile in the same position in space, although it may be changing in shape and size. This result was already expected only from the observation of the processed videos, since, as no translation movement was seen in the particle, the diffusion exponent was expected to be zero.

The value equaling zero for the diffusion exponent could be associated mainly with the size of the oligomer because the particles with bigger size seem to have less mobility in a viscous fluid. One could also hypothesize that this is due to oligomers' interaction with the liquid cell walls which would account for the particle to be immobilised in the x,y plane. Another possibility is that those oligomers are localised in a potential well and that a higher electron dose would be needed to make them getting out if equilibrium and escape.

As a verification step, it was checked that the same results were obtained for all possible oligomers' sampling in this video. This way, it was verified that all the Aβ-42 aggregates in this sample presented the same results regarding the value of the diffusion exponent.

B. Analysis of small oligomers

A second experiment consisted of the analysis of smaller oligomers, with approximate sizes of 8nm in diameter, recorded for 62s at 20 fps with a pixel size of 0.127nm. The same procedure was followed as in the experiment for 100nm sized oligomers, but now obtaining results both for individual particles, groups of particles, and for the complete set of small oligomers present in Video 2.

After the image processing step, the following aspects were studied separately: (*i*) the complete set of circa 100 oligomers present in the video 2, (*ii*) the set of circa 30 particles that were in the region of interest of Video 2, and (*iii*) a single small oligomer individually, as can be observed in Figures. 2a, 2b, and, 2c, respectively. The aim was to evaluate whether the MSD results varied considerably or were similar across different data volumes.

As shown in Figure. 2d for the case of 100 oligomers ($\alpha = 0.919$), Figure. 2e for a population of 30 oligomers ($\alpha = 1.191$), and 2f for a single selected oligomer($\alpha = 0.97$), the value of the diffusion exponent α is approximately 1 in the three cases.

Despite the fact that, according to the theory, the normal Brownian diffusive regime is only considered valid for $\alpha = 1$, we consider that these oligomers of $\sim$ 8nm size are found in that regime due to the great similarity of the results obtained with the value equal to 1. This consideration comes from the high improbability of an exact value of 1, and our discrepancy with that value can come from experimental errors with the sample or the process treatment given. It has also been proven that the results obtained for different amount of particles in

the calculations obtain similar values. This leads us to think that oligomers with similar sizes present the same type of diffusion.

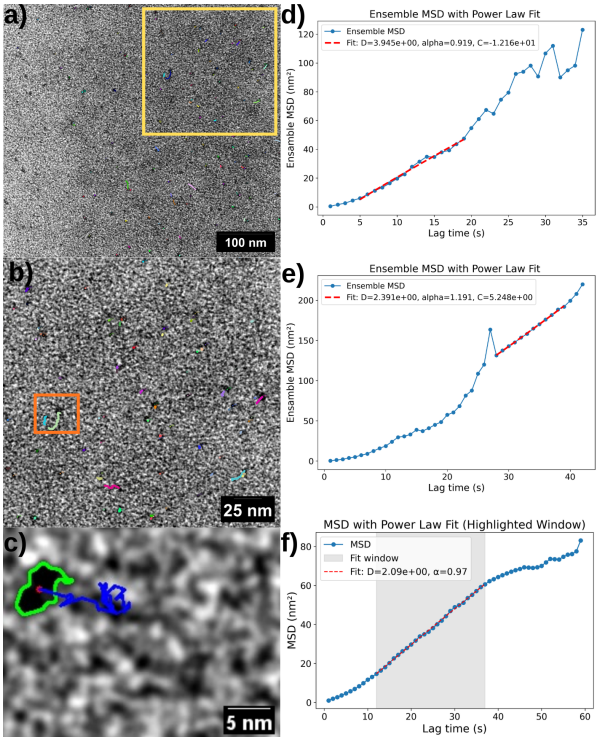

FIG. 2: Analysis of small Aβ-42 oligomers in liquid-TEM. **a)** Representative preprocessed frame showing the full field of view analysed. **b)** Preprocessed region of interest of the full field of view in a) in a yellow box. **c)** Individual tracked oligomer selected in an orange box in b), showing the detected oligomer contour in that specific time point and its trajectory. **d)** Ensemble MSD obtained for all tracked oligomers in a). **e)** Ensemble MSD for the particles of the selected region of interest in b). **f)** MSD obtained for an individual particle in c).

IV. CONCLUSIONS

In this project, the diffusive behaviour of Aβ-42 aggregates has been studied by using Liquid-TEM. The study was based on video processing, particle detection and subsequent tracking, and the extraction of particle trajectories for calculating the MSD and, therefore, the diffusion exponents.
Very different results were obtained for two different samples; for oligomers of size $\approx$ 100nm no displacement was observed ($\alpha = 0$), while it was considered that oligomers which measured sizes $\approx$ 8nm presented normal Brownian diffusion ($\alpha \sim 1$). Therefore, this indicates that the diffusion behaviour of the investigated aggregates is not uniform and strongly depends on their size, making their study complex given the heterogeneity of this protein. Extending this investigation to larger datasets would allow for better statistics. Nonetheless it has been possible to verify that the methodology followed is useful and works for the study of the dynamics of other nanometric objects in solution, not only proteins. What would be interesting is to extend the analysis with oligomers of other characteristics such as oligomers of other misfolded proteins such as tau. The same analysis can be done to evaluate the effect of potential AD drugs on the diffusion behaviour of Aβ-42.

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Règims de difusió a nanoescala de l'amiloide-β estudiats mitjançant TEM en fase líquida

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Resum: Aquest projecte consisteix en l'estudi dels règims de difusió d'agregats de proteïnes d'amiloide-β-42 (Aβ-42) mal plegades observats mitjançant TEM en fase líquida. Els vídeos adquirits mitjançant aquesta tècnica es van analitzar per reconstruir trajectòries de partícules i així quantificar el seu comportament dinàmic a escala nanomètrica. Degut al baix contrast d'aquestes mostres proteiques, els vídeos van ser preprocessats mitjançant normalització, *clipping*, filtratge gaussià i promitjos de fotogrames consecutius. A continuació, es van seleccionar les regions d'interès i es va fer un seguiment de partícules per obtenir les seves trajectòries. A partir d'aquestes trajectòries, es va calcular i ajustar el desplaçament quadràtic mitjà (MSD) utilitzant una llei de potències per determinar l'exponent de difusió α, obtenint que els agregats grans de diàmetres al voltant de 100nm van obtenir $\alpha = 0$ i, per tant, van mostrar una mobilitat translacional menyspreable, metre que per oligòmers petits de diàmetre al voltant de 8 nm l'exponent $\alpha \sim 1$ va mostrar un comportament compatible amb difusió browniana normal.
Paraules clau: Amyloide-β, TEM en fase líquida, agregació proteica, seguiment de partícules, difusió.
ODSs: Aquest TFG està relacionat amb l'ODS de Salut i benestar

Objectius de Desenvolupament Sostenible (ODSs o SDGs)

1. Fi de les desigualtats		10. Reducció de les desigualtats	
2. Fam zero		11. Ciutats i comunitats sostenibles	
3. Salut i benestar	X	12. Consum i producció responsables	
4. Educació de qualitat		13. Acció climàtica	
5. Igualtat de gènere		14. Vida submarina	
6. Aigua neta i sanejament		15. Vida terrestre	
7. Energia neta i sostenible		16. Pau, justícia i institucions sòlides	
8. Treball digne i creixement econòmic		17. Aliança pels objectius	
9. Indústria, innovació, infraestructures			

Aquest TFG està relacionat amb l'ODS 3, Salud i Benestar, perquè estudia agregats d'amiloide-β, espècies asociades a procesos neurodegeneratius com la malaltia de l'Alzheimer. On el seu estudi pot contibuir a comprendre la seva dinàmica i per tant al tractament d'aquestes malalties.

Appendix A: Individual MSD curves for multiple particles

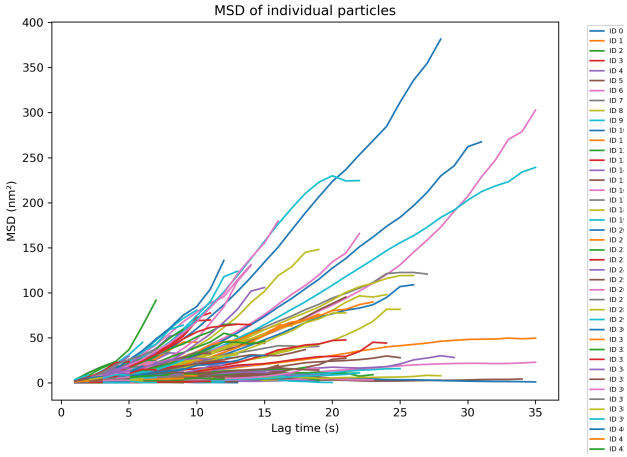

FIG. 3: Individual MSD curves for each particle tracked in Figure 2a), from which the ensemble MSD shown in Figure 2d) was obtained.

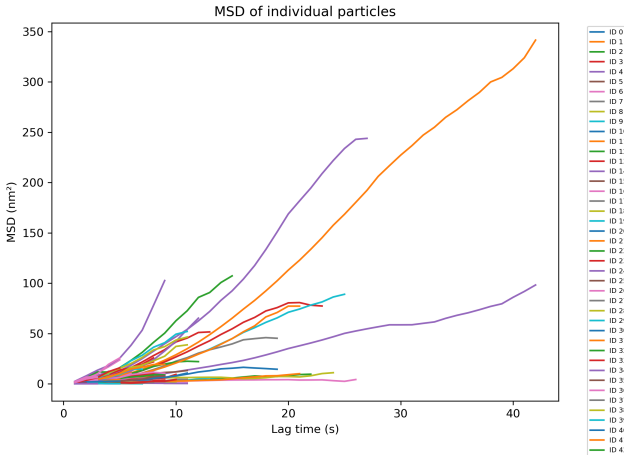

FIG. 4: Individual MSD curves for each particle tracked in Figure 2b), from which the ensemble MSD shown in Figure 2e) was obtained.