Application note

Determination of lipid protein binding stoichiometry using Microfluidic Diffusional Sizing

Here we assess the stoichiometry of α-synuclein binding to lipid vesicles by microfluidic diffusional sizing (MDS) on the Fluidity One and Circular Dichroism (CD). We find that the results obtained are consistent between the two techniques, but that MDS presents advantages in both speed and simplicity.

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Introduction

The interaction of α-synuclein with lipid membranes is believed to be key in its normal function. However, in some conditions these interactions can result in amyloid fibril formation, a hallmark of Parkinson's disease(1).

Indeed protein-lipid interactions as a whole are widespread and essential, yet little understood. Reviews of the field remark that systematic investigation is still required (2), so suitable tools must be in place to support this.

Here we investigate the interaction of α-synuclein with lipid vesicles by Microfluidic Diffusional Sizing (MDS) and Circular Dichroism (CD) to assess the two techniques as methods for studying protein-lipid interactions.

Methods

Small Unilamellar Vesicle (SUV) preparation

A dry, thin lipid film was prepared by evaporating the chloroform from a 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS) solution under a nitrogen stream. The film was placed under vacuum overnight to remove any final chloroform traces. Phosphate buffer was added to hydrate the film and the solution was stirred at room temperature for 2 hours.

The resulting lipid solution was frozen and thawed 8 times using liquid nitrogen and a water bath set to 37 °C. The solution was then sonicated on ice with a probe sonicator for 3x 5-minute intervals, with 50% pulse cycles at 20% power. The final solution was centrifuged for 30 minutes at 15,000 rpm to remove residues, then stored at 4 °C and used within 2 days.

Sample preparation

50 µL solutions were prepared at varying ratios of α-synuclein to SUV. These were made by combining the stock 2.5 mM vesicle solution and 100 µM α-synuclein solutions, with the remaining volume made up using phosphate buffer. Each sample was incubated for 1 minute before testing.

For the MDS measurements, a 5 µL aliquot was used for each test. All readings were taken on a Fluidity One instrument using the largest size range (2-20 nm).

For the CD measurements, far UV spectra were obtained on 200 µL samples by averaging five individual spectra recorded between 205 and 250 nm with a bandwidth of 1 nm, data pitch of 0.2 nm, scanning speed of 50 nm min⁻¹ and a response time of 1 s. For each sample the CD signal of the buffer used was recorded and subtracted from the CD signal of the protein. The fraction of protein bound to the vesicles was calculated using the signal measured at 222 nm. All readings were taken on a spectropolarimeter JASCO J-810 instrument.

Results

For the MDS readings, the binding of α-synuclein to DOPS vesicles was monitored by directly measuring the change in synuclein size in the presence of increasing concentrations of vesicles.

For the CD readings the change in the protein’s structure, from disordered structure to alpha helical on binding to vesicles, was monitored by following the decrease in ellipticity at 222 nm.
Figure 1: Results from MDS and CD analysis of varying ratios of α-synuclein and vesicles

Figure 2: Illustration of the protein-lipid interactions progressing over changing concentration of lipid, linked to the labelled points on Figure 1.

A At an initially low lipid concentration, very few α-synuclein molecules are bound to vesicles, most are free in solution. This results in the low average ellipticity and size observed by CD and MDS respectively.

B As lipid concentration increases, a greater number of interactions occur, resulting in a greater number of bound α-synuclein molecules. This gives the increase in average size observed in the MDS data, and the decrease in average ellipticity in the CD data.

C At higher still lipid concentration all α-synuclein molecules are bound to vesicles, giving the observed maximum in size and minimum in ellipticity values.

D After further lipid concentration increases, there is little change in observed ellipticity. The slight decrease in size observed by MDS indicates redistribution of the α-synuclein over the greater number of vesicles available.
These results were then plotted against the vesicle concentration, as shown in Figure 1, and we see that the two methods offer comparable results.

The results were fitted (see appendix) to determine stoichiometry, $L$, the average number of lipid molecules bound to each $\alpha$-synuclein at saturation, and $K_d$, the dissociation constant.

In addition to this, we can attribute the changes in size and ellipticity observed to the changing protein-lipid interactions; see Figure 2 which illustrates the labelled points from Figure 1.

This analysis reveals that MDS testing on Fluidity One was able to provide additional insights into the nature of the protein-lipid interactions that CD testing could not provide; the redistribution of the $\alpha$-synuclein molecules across the SUVs present at point D on Figure 1 vs point C is only clear from the MDS data.

Previous data measuring the stoichiometry of binding between lipid molecules and $\alpha$-synuclein found values of 33 (testing by CD (1)) and 78 (testing by diffusive sizing on a PDMS device using pre-labelled protein (3)). In the present work we find 29 for CD and 25 for Fluidity One MDS testing. The closer agreement of the Fluidity One MDS measurement with CD compared to the previous diffusive sizing custom rig reflects the improved performance of the technique after development as a commercial product.

**Conclusion**

MDS analysis as performed on the Fluidity One offers a new method to quantify protein lipid interactions, based on the change in size of the protein vs protein-lipid complex. We observe that this approach offers multiple advantages over CD for this analysis.

Firstly, the well understood structural change of $\alpha$-synuclein upon binding to a lipid (4) (5) (6) was instrumental in obtaining and understanding CD results. Without prior knowledge of this change, or in the case of a protein-lipid interaction which does not generate a structural change, CD analysis would not be possible.

Furthermore, analysis by MDS presents significant advantages in simplicity and sample volume requirements over CD tests. For the CD analysis, 200 µL of sample was required and care was required to ensure the buffer was of high purity and optically transparent, the sample was sufficiently degassed and the concentration of the synuclein was determined prior to beginning the experiment. In contrast the Fluidity One MDS analysis required just 5 µL of sample with no special requirements, and with the benefit of concentration being measured alongside size in the same test. MDS testing also presents a significant cost advantage, with the cost of the instrument being around half that of the CD spectrometer.

Finally, the MDS analysis was able to provide greater insights into the precise nature of the protein-lipid interaction than CD could, as further changes that did not alter ellipticity were observed.

With these advantages in sample requirements, generality of the method, and the ability to observe changes which were not detected by CD, MDS analysis on the Fluidity One presents a significantly improved method to observe protein-lipid interactions.
References


Appendix

Fitting equation for results in Figure 1

<table>
<thead>
<tr>
<th>Method</th>
<th>Term</th>
<th>Value</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD</td>
<td>$K_D$</td>
<td>$2 \times 10^{-6}$</td>
<td>$1 \times 10^{-6}$</td>
</tr>
<tr>
<td>CD</td>
<td>$L$</td>
<td>29</td>
<td>-</td>
</tr>
<tr>
<td>MDS</td>
<td>$K_D$</td>
<td>$&lt; 3 \times 10^{-6}$</td>
<td>-</td>
</tr>
<tr>
<td>MDS</td>
<td>$L$</td>
<td>25</td>
<td>2</td>
</tr>
</tbody>
</table>

CD fitting equation:

$$y = -1 \times \left( \frac{\alpha_{syn} + \frac{\text{DOPS}}{L} + K_D}{2 \alpha_{syn}} - \sqrt{\left( \frac{\alpha_{syn} + \frac{\text{DOPS}}{L} + K_D}{2 \alpha_{syn}} \right)^2 - 4 \times \frac{\text{DOPS}}{L} \times \alpha_{syn}} \right) \times \maxSig + \Sig0$$

Where:

- $y$ is ellipticity at 222 nm (mdeg)
- DOPS is concentration of lipid in M
- $L$ is number of lipid molecules bound to each protein molecule
- $K_D$ is the dissociation constant
- $\alpha_{syn}$ is the concentration of $\alpha$-synuclein used $= 2 \times 10^{-5}$ M
- $\Sig0$ is ellipticity at 222 nm in absence of lipid $= -4.16$ mdeg
- $\maxSig$ is minimum ellipticity measured $= -52 \pm 3$ mdeg (fit)
Appendix continued

MDS fitting equations:

\[
\text{Conc}_{\text{asynbound}} = \left( \frac{(\text{asyn} + \frac{\text{DOPS}}{L} + K_v) - \sqrt{(\text{asyn} + \frac{\text{DOPS}}{L} + K_v)^2 - 4 \times \frac{\text{DOPS}}{L} \times \text{asyn}}}{2} \right)
\]

\[
R_{av} = \left( 1 - \frac{\text{Conc}_{\text{asynbound}}}{\text{asyn}} \right) \times R_{\text{asyn}} + \frac{\text{Conc}_{\text{asynbound}}}{\text{asyn}} \times \left( R_{\text{ves}} + \frac{\text{Conc}_{\text{asynbound}} \times \text{DOPS}}{R_{\text{complex}} - R_{\text{ves}}} \right)
\]

\[
R_{\text{meas}} = \left( \frac{R_{av} \times \text{asyn} + \alpha \times \frac{\text{DOPS}}{L} \times \left[ R_{\text{ves}} + \frac{\text{Conc}_{\text{asynbound}} \times \text{DOPS}}{R_{\text{complex}} - R_{\text{ves}}} \right]}{\left( \text{asyn} + \alpha \times \frac{\text{DOPS}}{L} \right)} \right)
\]

Where:

- \( R \) is hydrodynamic radius
- \( R_{av} \) is average hydrodynamic radius of \( \alpha \)-synuclein containing species
- \( R_{\text{meas}} \) is measured hydrodynamic radius accounting for weak contribution of vesicles
- \( R_{\text{ves}} \) is hydrodynamic radius of free vesicles = 10.2 ± 0.7 (fit)
- \( R_{\text{complex}} \) is radius of the protein-lipid complex when the vesicle reaches maximum coverage = 12.3 ± 0.5 (fit)
- \( R_{\text{asyn}} \) is the hydrodynamic radius of free \( \alpha \)-synuclein = 3.4 nm
- DOPS is concentration of DOPS in M
- \( \alpha \) is the relative signal of DOPS compared to \( \alpha \)-synuclein = 0.2
- asyn is the concentration of \( \alpha \)-synuclein used = 2 x 10^{-5} M
- \( L \) is number of lipid molecules bound to each protein molecule
- \( \text{Conc}_{\text{asynbound}} \) is the concentration of \( \alpha \)-synuclein bound to DOPS

In the second equation we assumed that the average radius of the protein-lipid complex varies linearly with the average number of \( \alpha \)-syn molecules per vesicle, between the radius of the free vesicles and that of the vesicle at maximum coverage. This expression is then accurate as long as there are one or more \( \alpha \)-syn molecules per vesicle.

In the third equation we account for the fact that there is a weak signal from DOPS itself.