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Single molecule characterisation of the interactions between extracellular chaperones and toxic α -synuclein oligomers

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Summary

The aberrant aggregation of α -synuclein is associated with several human diseases, collectively termed the α -synucleinopathies, which includes Parkinson's disease. The progression of these diseases is in part mediated by extracellular α -synuclein oligomers which may exert effects through several mechanisms, including prion-like transfer, direct cytotoxicity and pro-inflammatory actions. In this study, we show that two abundant extracellular chaperones, clusterin and α_2 -macroglobulin, directly bind to exposed hydrophobic regions on the surface of α -synuclein oligomers. Using single-molecule fluorescence techniques we found that clusterin, unlike α_2 -macroglobulin, exhibits differential binding to α -synuclein oligomers which may be related to structural differences between two previously described forms of α S oligomers. The binding of both chaperones reduces the ability of the oligomers to permeabilize lipid membranes and prevents an oligomer-induced increase in ROS production in cultured neuronal cells. Taken together, these data suggest a neuroprotective role for extracellular chaperones in suppressing the toxicity associated with α -synuclein oligomers.

Introduction

The α -synucleinopathies are a group of progressive and ultimately fatal neurodegenerative disorders including Parkinson's disease (PD), dementia with Lewy bodies (DLB) and multiple

system atrophy (MSA). The pathological hallmark of these disorders is the selective loss of neurons and the aberrant accumulation of α -synuclein (α S) within protein inclusions in neuronal or glial cells (Chiti and Dobson, 2017). How the aggregation of α S causes disease is still unclear, however, a body of data implicates the direct cytotoxicity of α S oligomers (Chen et al., 2015; Chiti and Dobson, 2017; Ingelsson, 2016; Winner et al., 2011). During the aggregation process, α S oligomers undergo a structural conversion from a relatively unstable species to more stable and compact oligomers that have increased cytotoxicity and resistance to proteinase-K degradation compared to the preceding oligomers (Cremades et al., 2012; Horrocks et al., 2015; Iljina et al., 2016). This conversion occurs before the oligomers are incorporated into fibrillar structures and is a critical step in the aggregation pathway of α S.

α S can account for up to 1% of all cytosolic proteins in neurons, but it is also present in extracellular fluids, including cerebrospinal fluid (CSF) and blood plasma (El-Agnaf et al., 2003). Recent evidence suggests that this extracellular α S significantly contributes to the onset and spreading of disease within the affected brain (Lee et al., 2014). Indeed, as with a growing number of neurodegenerative diseases, it appears that the local spread of pathology may be due to a prion-like propagation process (Aulić et al., 2014; Chiti and Dobson, 2017; Emmanouilidou and Vekrellis, 2016; Marques and Outeiro, 2012). Direct neurotoxicity of extracellular α S has also been observed, which could be caused by the unregulated insertion of α S aggregates into cell membranes and/or neuroinflammatory responses such as microglia activation and generation of intracellular reactive oxygen species (ROS) (Cremades et al., 2012; Fusco et al., 2017; Reynolds et al., 2011; Zhang et al., 2005).

Extracellular chaperones (ECs) are a small class of proteins that act efficiently to enhance the clearance of misfolded proteins from extracellular body fluids. Clusterin (CLU) was the first mammalian EC discovered (Wilson and Easterbrook-Smith, 2000), and has been shown to inhibit the aggregation of a very broad range of proteins, including α S (Yerbury et al., 2007). Thus, since both CLU and aggregates of α S can be present together outside cells, a direct *in vivo* interaction between the two proteins is feasible and likely. Similarly, another well characterized EC, α_2 -macroglobulin (α_2 M) (Wyatt et al., 2014), may also interact with α S. A polymorphism in the α_2 M gene has been linked with PD, although this link cannot be established for all populations (Kruiger et al., 2000; Nicoletti et al., 2002; Tang et al., 2002).

In addition to a possible extracellular interaction, CLU and α S may interact within the cellular environment. Under conditions of endoplasmic reticulum (ER) stress, the secretion of CLU to the extracellular environment is inhibited, and the protein is retrotranslocated from the endoplasmic reticulum/Golgi to the cytosol (Nizard et al., 2007; Zhang et al., 2014). We have recently shown that this process is sufficient to protect cultured neuronal cells and *Drosophila melanogaster* from proteotoxicity associated with the aggregation of the amyotrophic lateral sclerosis (ALS)-linked protein TDP-43 (Gregory et al., 2017). ER stress has been linked to both ALS and PD pathologies and, moreover, the overexpression of mutational variants of α S is sufficient to induce ER stress (Gallegos et al., 2015). These observations suggest that a cytosolic interaction between aggregated α S and CLU is also possible. Indeed, CLU has been found co-localized with intracellular α S in patients with a variety of diseases, including cortical Lewy bodies in DLB, brain stem Lewy bodies in PD and DLB, and in glial cytosolic inclusions in MSA (Sasaki et al., 2002).

Despite the potentially critical importance of the binding between ECs and α S oligomers, our understanding of the nature of the interaction is limited. Previous work has shown that both CLU and α ₂M bind to misfolded proteins to inhibit their aggregation (Wyatt et al., 2013). However, very limited information is available regarding whether specific sizes or structures of oligomers are bound preferentially, or on the stoichiometries of binding of chaperone to misfolded client proteins. CLU is known to interact with oligomers of the 40 amino acid isoform of amyloid- β ($A\beta$) ranging from dimers up to 50mers (Narayan et al., 2012). CLU also forms stable high-molecular weight complexes with amorphous aggregates of proteins with a mass ratio of 1:2 (CLU:client) (Wyatt et al., 2009), however the stoichiometry of complexes formed between either CLU or α ₂M and amyloid-forming proteins is not known.

In this report, we used a single-molecule fluorescence technique, termed two-color coincidence detection (TCCD) (Orte et al., 2008a), to show that both CLU and α ₂M interact directly with α S oligomers. TCCD allows the properties of two individual proteins, each labelled with one of two spectrally distinct fluorophores, to be studied with high sensitivity (Horrocks et al., 2015). This approach allows the detection of individual species by avoiding measurements of ensemble averages and has been used previously to study the kinetics of α S aggregation (Cremades et al., 2012; Horrocks et al., 2015). In the present study we demonstrate that the interactions between the chaperones and α S oligomers are inhibited by 4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid (bisANS), suggesting that the binding involves exposed hydrophobic groups on the surface of the oligomers. Additionally, we show that the chaperones specifically inhibit both an α S-induced increase in lipid membrane permeability and the α S-induced induction of ROS production in neuronal cells.

Results

We first performed TCCD measurements to explore the interaction of the ECs with α S during the aggregation process. To achieve this we made use of the A90C mutational variant α S which allows the conjugation of a fluorophore through a single thiol group; previous studies have shown that the conjugation does not significantly change the behavior of the protein from that of wild-type α S (Cremades et al., 2012). α S^{A90C}-AF488 (70 μ M) was incubated under aggregation-inducing conditions in the presence of CLU-AF647 (0.7 μ M). We took aliquots from the aggregation reaction and monitored via TCCD the stoichiometry of any CLU: α S complexes formed. The number of monomers in an oligomer is estimated based on the total fluorescence intensity of the oligomer compared to that of the monomer. Since this is not a direct measurement of the oligomer size, and is approximate, we refer to this as the apparent size. Wherever the term 'monomer' is used regarding CLU and α ₂M we are referring to the physiological heterodimer and tetramer, respectively. Under these conditions both CLU and α ₂M were found to greatly inhibit the aggregation of wild-type α S (Figure S1). Previous fluorescence lifetime experiments have shown that AF488 conjugated to α S is not quenched during the oligomerisation of the protein (Cremades et al., 2012). Additionally, since CLU-AF647 did not show any evidence of quenching when bound to unlabeled α S fibrils (Figure S1) we assumed that fluorescence quenching is not a significant factor, particularly when bound to the small oligomers. We were interested to see to which species CLU was bound, and so searched for coincidence events between the two different fluorophores. After 6 h of aggregation we observed coincident events showing that CLU was

predominantly bound to small oligomers (approximately tetramers) with an equimolar stoichiometry to α S. For larger oligomers, CLU was found to bind to α S at substoichiometric ratios, with an average CLU/ α S = 0.1 calculated for the largest oligomers detected (Figure 1a and c). As expected, after 48 h of aggregation a larger number of α S oligomers were detected than after 6 h (Figure 1b and d, additional time-points shown in Figure S2). At this later time point, as at 6 h, a population of larger α S oligomers with a low CLU/ α S ratio (approximately 0.06) was detected (Figure 1b and d). In addition, a population of 'CLU-rich' oligomers were also detected (Figure 1b). These CLU-rich oligomers had an average CLU/ α S ratio of 0.8 and generally contained less than 15 α S monomers. Overall these data show that CLU binds to a wide range of α S species, from at least dimers to oligomers containing 30 α S molecules.

We next studied the formation of α_2 M- α S complexes in a similar manner to that used above for CLU. α S^{A90C}-AF488 (70 μ M) and α_2 M-AF647 (0.7 μ M) were co-incubated under conditions that facilitated aggregation and the resulting oligomers were examined using TCCD at various time points. Similarly to CLU, α_2 M significantly inhibited the aggregation of α S under these conditions (Figure S1) and again after 6 h of incubation the resulting small α S oligomers showed a broad distribution centred around an equimolar stoichiometry with α_2 M (Figure 2a and c). The ratio tended to decrease as increasing numbers of α S monomers were present in the oligomer. The largest oligomers detected (consisting of \sim 30 α S molecules) had an approximate ratio of α_2 M/ α S = 0.03. After α S^{A90C}-AF488 had been incubated with α_2 M-AF647 for 48 h, oligomers were more abundant, and tended to contain more α_2 M than similarly-sized oligomers after 6 h of incubation (Figure 2b and d, additional time-points shown in Figure S2). Small oligomers detected in the aggregation reaction after 48 h still had an approximate equimolar ratio of α_2 M: α S. However, in the largest oligomers detected at 48 h (containing \sim 30 α S monomers), the ratio of α_2 M/ α S was around 0.1, approximately three times greater than at the 6 h time point. In order to further compare the differences in binding stoichiometry at various times during the aggregation reaction, the average numbers of apparent α_2 M and α S monomers in different oligomers were plotted (Figure 2e,f). This reveals that (i) at all time points, there was a linear dependence of the α_2 M: α S ratio on oligomer size (Figure 2e), and (ii) the α_2 M: α S ratio increased linearly over time for oligomers of all sizes (Figure 2f).

To further investigate the binding of the ECs to the oligomers, we examined whether or not this binding was a result of hydrophobic interactions by exploiting the effects of the presence of bisANS, a well-established probe of solvent-exposed hydrophobicity (Bothra et al., 1998; Poon et al., 2002; Sheluho and Ackerman, 2001). We first incubated wild-type α S for 9 h under aggregating conditions and examined the species formed at this time-point by super-resolution microscopy using Nile red; we found that they matched the characteristics observed previously for oligomers formed under these conditions (i.e. approximately spherical and < 200 nm in diameter; Figure S3). The mixture of monomeric and oligomeric α S was adsorbed to a microplate and next blocked with BSA and treated with a range of concentrations of bisANS to block any exposed hydrophobic regions. Following subsequent incubation with either CLU or α_2 M, the extent of chaperone binding was assessed by an enzyme-linked immunosorbent assay (ELISA). The bisANS dose-dependently reduced the binding of both CLU and α_2 M to the α S oligomers (Figure 3a and b), suggesting that the hydrophobic regions exposed on the surface of the

oligomers mediates the binding. Neither of the chaperones were found to bind to the BSA blocker or to monomeric α S (Figure S3).

Having shown that the chaperones bind to exposed hydrophobic regions present on α S oligomers, we hypothesized that this binding could act to reduce the cytotoxicity of the oligomers. For this purpose we first employed a methodology that enables the quantification of aggregate-induced toxicity by measuring their effects on the permeability of a lipid bilayer (Flagmeier et al., 2017). In this assay, the Ca^{2+} -sensitive dye Cal-520 is incorporated into surface tethered vesicles and Ca^{2+} present in solution can enter the vesicles when the lipid membrane is permeabilized (by, for example, a protein oligomer). The resulting increase in fluorescence intensity can be quantified by total internal reflection (TIRF) microscopy and used to determine the extent of membrane permeability. In contrast to a non-EC control protein (BSA), incubation of α S oligomers with both CLU and α_2 M significantly reduced the ability of the oligomers to permeabilize the membranes ($96\pm 4\%$ and $69\pm 7\%$ protection at a 1:10 substoichiometric ratio of chaperone to monomer, respectively; Figure 3c and d). BSA did not reduce the Ca^{2+} influx even when present at an equimolar ratio of BSA: α S (Figure S3). The effect of the chaperones was dose-dependent, with both chaperones providing $>95\%$ protection at a concentration equimolar to monomeric α S. Additionally, both CLU and α_2 M reduced the α S-induced permeabilization when α S was aggregated in the presence of the chaperone in a 1:100 substoichiometric ratio of chaperone to α S monomer (Figure S3).

To determine whether this effect was sufficient to alter the cellular response to α S oligomers, we measured the effect of both ECs to on the cellular production of reactive oxygen species (ROS) (Cremades et al., 2012). Intracellular ROS has previously been shown to activate apoptosis in neurons (Jenner, 2003) and is typically one of the first aberrant cellular responses induced by exposure to toxic protein oligomers (Canevari et al., 2004; Zampagni et al., 2011). The oxidation of dihydroethidium (DHE) to ethidium was used to measure the rate of ROS production immediately after the addition of α S oligomers to Neuro-2a cells, in the presence or absence of the ECs. The addition of pre-formed oligomers alone, or of oligomers preincubated with BSA resulted in an approximately 2.5-fold increase in the rate of ROS production. Preincubation of the oligomers with CLU or α_2 M ameliorated their ability to induce ROS formation in cells (Figure 3e and f). As neither chaperone alone had any effect on the rate of ROS production, the protective effect is not the result of the chaperones inhibiting the ability of the cells to produce ROS, but rather indicates a protective effect conferred by the ECs binding to the oligomers.

Discussion

In the present study we investigated the direct interaction of ECs with α S under conditions where aggregation occurs. We first used TCCD to examine the stoichiometry of the EC/ α S complexes. Using this approach, we have shown that the nature of the binding between the chaperones and α S oligomers is specific for each chaperone, and in the case of CLU, appears to depend on subtle differences in the oligomeric structures. In aliquots taken from aggregation reactions after 6 h, the stoichiometry of the complexes formed between each EC and α S were similar. Both chaperones tended to bind small oligomers (< 5 α S monomers) in an approximate equimolar ratio. Oligomers containing > 5 α S monomers were observed to be associated with proportionally less chaperone, suggesting that the number of chaperone-accessible binding sites on the α S

oligomer surface does not increase linearly with the number of α S monomers in an oligomer. These data also indicate that on average slightly fewer α_2 M molecules are associated with the oligomers of all sizes when compared with CLU. This is likely to be the result of the significant difference in size between the two chaperones - α_2 M is much larger than CLU (approximately 720 kDa for the α_2 M tetramer and 80 kDa for the CLU heterodimer). CLU is known to exist in solution as a polydisperse mixture of oligomers of the heterodimer (Hochgrebe et al., 2000; Poon et al., 2002). It is not known whether these species exhibit variable chaperone activity; however even a CLU tetramer is approximately half the size of α_2 M. As a result, the binding of α_2 M to α S is likely to be sterically limited by previously bound chaperones to a greater extent than for CLU. This could potentially help explain the observation that dimeric α_2 M is a more efficient chaperone than the native tetramer (Wyatt et al., 2014). The biological function of CLU oligomers is not known, although it has been suggested that they act as reservoirs of a more chaperone-active CLU heterodimer that is released when required (Poon et al., 2002). Unfortunately, the role of these oligomers is difficult to investigate experimentally using single-molecule techniques due to the rapid dissociation of the oligomers upon dilution.

Interestingly, compared to the complexes observed after 6 h of incubation, a distinct population of CLU/ α S complexes emerged at later time points that were relatively large (up to ~ 15 α S monomers) and CLU-rich (CLU/ α S = 0.8). One possible explanation for the appearance of this population may be a time-dependent association of additional α S monomer with already formed CLU/ α S complexes, however the clear delineation between this population and those observed at 6 h suggests this is not the case. It was recently shown that α S oligomers exhibit a broad distribution of structure-dependent surface hydrophobicity (Bongiovanni et al., 2016). The data presented here indicate that the binding of CLU to the oligomers is mediated by hydrophobicity, suggesting that the ability of CLU to bind to the oligomers is dependent on some aspect of the oligomer structure. Furthermore, the observed time-dependence of the differential binding of CLU to α S oligomers cannot be explained by differential binding of CLU monomers and oligomers which are both present at the start of the experiment before dilution for single molecule measurements. This can, however, be explained by CLU binding to α S oligomers of different structures.

Both α_2 M and CLU have previously been shown to reduce the toxic effects of A β oligomers (Fabrizi et al., 2001; Narayan et al., 2014), and reduce the toxicity of CSF from Alzheimer's disease patients and healthy controls (Drews et al., 2017; Yerbury and Wilson, 2010). This is believed to be a result of the endocytic clearance of the oligomer from the extracellular space through the formation of oligomer-chaperone complexes such as those directly observed in this report. This process is thought to be one of the central systems acting to maintain extracellular proteostasis (Wyatt et al., 2013). Additionally, the work presented here indicates that the binding of ECs to oligomers directly inhibits the latter from aberrantly interacting with lipid membranes. Similarly to other client proteins (Poon et al., 2002), the association between ECs and α S oligomers appears to be mediated by regions of exposed hydrophobicity, evidenced by the inhibition of binding by the hydrophobic probe bisANS. Thus, by interacting with the oligomers, chaperones appear to shield the surface hydrophobicity present on the oligomers. Although the precise mechanism by which α S oligomers confer cytotoxicity is unknown, direct membrane disruption appears to be one of the contributing factors (Poon et al., 2002). Given that hydrophobicity

contributes to the interaction between α S and lipid membranes (Pfefferkorn et al., 2012; van Rooijen et al., 2009) it is not surprising that the chaperones inhibited the permeabilisation of lipid vesicles caused by α S oligomers. This provides a feasible mechanistic explanation for the observation that the chaperones prevent an increase in ROS production elicited by the oligomers.

Overall, despite evidence that the binding of both α_2 M and CLU to α S oligomers is mediated by surface exposed regions of hydrophobicity on the oligomers, the binding of each chaperone shows unique characteristics. The data suggest that the interaction between the chaperones and α S oligomers appears to depend on both the identity of the chaperone as well as the structural properties of the oligomer. When considered alongside the chaperone-mediated reduction in α S-induced membrane permeability and ROS production, these data provide evidence to support a neuroprotective role for ECs in the α -synucleinopathies and suggest a mechanism by which these chaperones may operate within other disease contexts.

Experimental Procedures

Protein purification and labelling

α S (wild-type and A90C; α S^{A90C}), CLU and α_2 M were purified as described previously – see (Cremades et al., 2012; French et al., 2008; Poon et al., 2002) respectively. α S^{A90C} was labelled with either Alexa Fluor 488 (AF488) C₅ maleimide or Alexa Fluor (AF647) C₂ maleimide (Invitrogen). The α S was first incubated for 15 min with 10 μ M DTT at room temperature (RT) to ensure reduction of the engineered cysteine residue. The reduced α S^{A90C} was concentrated to approximately 400 μ M using a Vivaspin 500 (10,000 MWCO) and buffer exchanged through a PD-10 column (GE Healthcare Life Sciences) into degassed PBS. The protein was then added to a 1.5-fold molar excess of the functionalized fluorophores, and the tube was flushed with nitrogen to prevent oxidation of the cysteines. The protein was incubated at 4 °C overnight with shaking followed by purification from unreacted fluorophore using a PD-10 column equilibrated in PBS, pH 7.4. The fluorescent labelling of α S^{A90C} has previously been shown to have minimal influence on the aggregation of the protein (Cremades et al., 2012; Horrocks et al., 2015). Similarly, CLU and α_2 M (Sigma Aldrich) were individually labelled with N-hydroxysuccinimidyl ester forms of either AF488 or AF647 (Invitrogen). To achieve this, the proteins (each at approximately 2 mg.ml⁻¹) were incubated with a 10-fold molar excess of the functionalized fluorophore for 1 h at RT or overnight at 4 °C. Unconjugated dye was removed by buffer exchange into PBS (or PBS/azide in the case of α_2 M) using a PD-10 column. The final protein concentration and labelling efficiency was determined according to the manufacturer's instructions.

Aggregation of α S

Any pre-aggregated material present in the monomer stock was first removed from the monomer population by ultra-centrifugation at 90,000 rpm for 1 h at 4 °C. Then, α S was aggregated in the presence or absence of the ECs. When present, the chaperone was used at a molar ratio to α S of 1:100. All aggregations were performed using 70 μ M α S in PBS (pH 7.4) with shaking at 200 rpm, 37 °C in an Innova43 Incubator Shaker Series (New Brunswick Scientific). Protein LoBind tubes (Eppendorf) were used to minimize protein adsorption; time point samples were flash frozen in liquid nitrogen for storage before use.

Microfluidics

Single-channel microfluidic devices were used to increase the rate of data acquisition through sample flow and remove the bias for preferentially measuring smaller species that occurs as a result of diffusion. These devices were made of PDMS, patterned using a silicon wafer, and bonded to borosilicate glass cover slides by exposure to oxygen plasma. The construction and use of these devices for examining α S aggregation has been described previously (Horrocks et al., 2015).

Single-molecule two-color coincidence detection

TCCD measurements were performed with fluorescent proteins present at 50 pM. Dilutions, in freshly filtered (0.02 μ m) PBS, were performed immediately before analysis. TCCD measurements were made using a custom-built confocal microscope. Briefly, the intensities of a 488 nm laser (Spectra Physics, Cyan CDRH) and 633 nm laser (Melles Griot, 25LHP151 HeNe) were first attenuated using neutral density filters. The beams were expanded and collimated by passage through a spatial filter (488 nm laser) or telescopic lenses (633 nm laser) before being made concentric with a dichroic mirror (505DRLP Omega Filters). The beams were then directed into the back port of an inverted microscope (Nikon Eclipse TE2000-U) and focused 10 μ m into the sample by a Fluor 100X, 1.30NA oil-immersion objective (Nikon). The emitted fluorescence was collected by the same objective and passed through a 50 μ m pinhole (Melles Griot) before being separated into two channels by a further dichroic mirror (585DRLP, Omega Filters). Emission in each channel was passed through long-pass and band-pass filters (535AF45 and 510ALP Omega Filters for blue-green channel, 696AF55 and 565ALP Omega Filters for red channel) and focused on avalanche photodiodes (Perkin-Elmer Optoelectronics, SPCM-14) for quantification.

The apparent size of an oligomer measured by confocal microscopy was calculated by first determining the fluorescence intensity of the monomer. This was typically given by the average intensity of non-coincident events before the sample was incubated under conditions to promote aggregation. The number of monomers in each coincident burst was then characterized as:

$$N_{Monomers} = \frac{I_D}{I_{MD}} + \frac{I_A}{I_{MA}}$$

where I_D and I_A are the intensities of the coincident burst in the donor and acceptor channels respectively, and I_{MA} and I_{MD} are the mean monomer intensities in the donor and acceptor channels respectively (Orte et al., 2008a). In a similar fashion, the natural logarithm of the apparent ratio of chaperone to client in each oligomer (Z) was calculated according to the equation:

$$Z = \ln \left(\frac{\left(\frac{I_{chaperone}}{I_{m_{chaperone}}} \right)}{\left(\frac{I_{client}}{I_{m_{client}}} \right)} \right)$$

where I refers to the intensity of a peak above the threshold from fluorophores conjugated to the chaperone or client protein, and I_m refer to the intensity of the monomer. These Z values are used to display the ratio of chaperone to client, such that the scale is visually symmetrical around

a 1:1 stoichiometry ($Z = 0$). It should be noted that in these experiments the apparent monomer intensity determined from any given data set is dependent upon the value used to threshold that data. However, since the threshold was determined automatically as described previously (Clarke et al., 2007; Orte et al., 2008b), the error in the calculated number of monomers labelled with each fluorophore is the same when comparing the two labelled species. Thus, all references to the number of monomers comprising an oligomer refers to apparent monomers, and although the given values scale correctly with each other they may differ from the true value.

Total internal reflection microscopy

Borosilicate glass cover slides (24 x 50 mm, thickness number 1; VWR International) for use in TIRF microscopy were cleaned by exposure to oxygen plasma for 30 min (Plasma system FEMTO, Diener Electronic). Frame-Seal incubation chambers (Bio-Rad) were attached to the surface of the slides to create wells which were then coated in aspartic acid (1 mg/ml; Sigma Aldrich) for 15 min. The aspartic acid was removed and the slide was rinsed with PBS before use. Samples were analyzed at approximately 3 μM monomer equivalents – a concentration that allowed individual aggregates to be resolvable on the surface of the slide. Either 5 μM thioflavin T or 5 nM Nile red was added to the sample before imaging to visualize αS aggregates (Bongiovanni et al., 2016). Measurements were performed on a custom built inverted optical microscope. The intensities of 405 nm, 532 nm and 641 nm lasers were attenuated using neutral density filters, after which the beams were circularly polarized using quarter-wave plates specific to each wavelength. The beams were then expanded and collimated using Galilean beam expanders and made concentric using dichroic mirrors before being passed through the back aperture of an inverted microscope and focused using an oil immersion TIRF objective (APON60XO TIRF, Olympus). Fluorescence emission was separated from excitation light using dichroic mirrors (Di02-R532 and Di01-R405/455/561/635 for 532 nm and 405/641 nm excitation respectively, Semrock) and passed through appropriate filters (BLP01-488R-25, LP02-568RS-25 and BLP01-635R-25 for 405 nm, 532 nm and 641 nm excitation respectively, Semrock). The fluorescence was then expanded and focused on an electron multiplying charge coupled device (Evolve 512, Photometrics) for imaging.

Super resolution images were reconstructed using the Drift Calculator and Peak Fit package (GDSC SMLM, University of Sussex) in ImageJ using gain = 37.7 ADU/photon, minimum photons > 30 and precision < 30 nm. Cluster analysis was performed to remove random localisations using DBSCAN (sklearn v0.18.1, Python 2.7) with minimum points threshold = 10 and epsilon = 3.

Enzyme-linked immunosorbent assay

αS was aggregated for 9 h as described above, centrifuged at 13,000 $\times g$ for 10 min to remove any large aggregates and then diluted to 2.5 μM . The protein was then adsorbed to a high-binding 96 well microplate (Corning) for 2 h at RT with gentle shaking. After the incubation, the plate was rinsed with PBS and blocked with 150 μM BSA as above. Some wells were then incubated with bisANS (Sigma) in a concentration range from 0 – 100 μM to block solvent-exposed hydrophobic regions (Poon et al., 2002). Following this the wells were incubated with CLU or $\alpha_2\text{M}$ for 1 h at RT (each at 10 $\mu\text{g}\cdot\text{ml}^{-1}$ diluted into the BSA blocking solution). The wells were then rinsed five times with PBS before the amount of bound chaperone was quantified using HRP/chromogen detection of appropriate antibodies according to the manufacturer's instruction (antibodies and reagents from CLU and $\alpha_2\text{M}$ ELISA kits ab174447 and ab108883; Abcam).

Single vesicle assay

A quantitative vesicle assay was used to measure the ability of α S oligomers to permeabilize membranes as described previously (Flagmeier et al., 2017). α S was aggregated as described above, aliquots were removed and diluted such that the final concentration of α S added to the vesicles was 50 nM. The diluted samples were preincubated for 5 min at RT in the presence or absence of α_2 M, BSA or CLU (concentrations indicated in the figure legend) before being added to the solution above POPC lipid vesicles containing Cal-520 (100 μ M; Stratech Scientific Ltd.) tethered to the surface using biotin/neutravidin linkage. A change in the fluorescence as a result of Ca^{2+} (present at 1.3 mM in L-15 buffer, Thermo Scientific) entering the vesicles was quantified by means of TIRF microscopy using a 488 nm laser for excitation (Toptica Photonics) and emission filters BLP01-488R-25 and FF01-520/44-25 (Semrock). The fluorescence intensity of each vesicle was then normalized to the maximum possible fluorescence intensity of the vesicle measured following incubation with ionomycin (1.4 μ M; Sigma). For each sample the acquisition of 9 fields of view (3x3 grid) was automated to prevent user bias.

Dihydroethidium assay

Dihydroethidium (DHE) was used to measure the intracellular rate of ROS production in Neuro-2a cells using a method similar to that previously described (Cremades et al., 2012). The cells were cultured in Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F-12 (ThermoFisher Scientific) supplemented with 10 % (v/v) foetal bovine serum (Bovagen Biologicals) and incubated in a Heracell 150i CO_2 incubator (ThermoFisher Scientific) under 5% (v/v) CO_2 at 37 °C. Cells to be analysed were seeded in 24 well plates and left to grow to approximately 50% confluency. The cells were rinsed with PBS before DHE (2 μ M in PBS) was added. An epifluorescence microscope was used to quantify both the oxidized (ethidium; excitation 405 - 435 nm, emission 440 - 480 nm) and reduced (DHE; excitation 502 - 560 nm, emission 590 - 630 nm) forms of DHE. Measurements were taken every 30 s for 15 min before the addition of aggregated α S (30 μ M monomer equivalent) with and without preincubation with BSA, CLU or α_2 M (all 3 μ M, 5 min at RT). DHE (2 μ M) was present in any sample added to the cells to prevent the dilution of the fluorophore. Measurements were then taken of the same cells for a further 15 min. The ratio of the mean ethidium intensity to the mean DHE intensity before and after the addition of the sample was calculated; a linear regression was fitted to the data and the gradient of the slope used to determine the change in the rate of oxidation of DHE within cells. In each experiment, the first two data points collected after the addition of the DHE and sample (i.e. the 0, 0.5, 15.5 and 16 min time points) were excluded from the analysis, as the sample addition briefly disturbed the fluorescence measurement.

Author contributions: The study was conceived and designed by M.R.W., D.K. and D.R.W. Experiments were performed by D.R.W., D.C., M.H.H., S.D., P.F., C.G.T. and L.T. Data were analysed by D.R.W., M.H.H. and S.D. All authors interpreted results, contributed to the manuscript and approved the final version.

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Declaration of interests: The authors declare competing interests.

References

- Aulić, S., Le, T.T., Moda, F., Abounit, S., Corvaglia, S., Casalis, L., Gustincich, S., Zurzolo, C., Tagliavini, F., and Legname, G. (2014). Defined alpha-synuclein prion-like molecular assemblies spreading in cell culture. *BMC Neuroscience* *15*.
- Bongiovanni, M.N., Godet, J., Horrocks, M.H., Tosatto, L., Carr, A.R., Wirthensohn, D.C., Ranasinghe, R.T., Lee, J.-E., Ponjavic, A., Fritz, J.V., *et al.* (2016). Multi-dimensional super-resolution imaging enables surface hydrophobicity mapping. *Nature Communications* *7*.
- Bothra, A., Bhattacharyya, A., Mukhopadhyay, C., Bhattacharyya, K., and Roy, S. (1998). A fluorescence spectroscopic and molecular dynamics study of bis-ANS/protein interaction. *Journal of Biomolecular Structure and Dynamics* *15*, 959.
- Canevari, L., Abramov, A.Y., and Duchon, M.R. (2004). Toxicity of amyloid beta peptide: tales of calcium, mitochondria and oxidative stress. *Neurochemical Research* *29*, 637-650.
- Chen, S.W., Drakulic, S., Deas, E., Ouberai, M., Aprile, F.A., Arranz, R., Ness, S., Roodveldt, C., Guilliams, T., De-Genst, E.J., *et al.* (2015). Structural characterization of toxic oligomers that are kinetically trapped during α -synuclein fibril formation. *Proceedings of the National Academy of Sciences, USA* *112*, 1994-2003.
- Chiti, F., and Dobson, C.M. (2017). Protein misfolding, amyloid formation, and human disease: a summary of progress over the last decade. *Annual Review of Biochemistry* *86*, 27-68.
- Clarke, R.W., Orte, A., and Klenerman, D. (2007). Optimized threshold selection for single-molecule two-colour fluorescence coincidence spectroscopy. *Analytical Chemistry* *79*, 2771-2777.
- Cremades, N., Cohen, S.I.A., Deas, E., Abramov, A.Y., Chen, A.Y., Orte, A., Sandal, M., Clarke, R.W., Dunne, P., Aprile, F.A., *et al.* (2012). Direct observation of the interconversion of normal and toxic forms of alpha-synuclein. *Cell* *149*, 1048-1059.
- Drews, A., De, S., Flagmeier, P., Wirthensohn, D.C., Chen, W.-H., Whiten, D.R., Rodrigues, M., Vincke, C., Muyltermans, S., Paterson, R.W., *et al.* (2017). Inhibiting the Ca^{2+} influx induced by human CSF. *Cell Reports* *21*, 3310-3316.
- El-Agnaf, O.M., Salem, S.A., Paleologou, K.E., Cooper, L.J., Fullwood, N.J., Gibson, M.J., Curran, M.D., Court, J.A., Mann, D.M., Ikeda, S., *et al.* (2003). Alpha-synuclein implicated in Parkinson's disease is present in extracellular biological fluids, including human plasma. *FASEB Journal* *17*, 1945-1947.
- Emmanouilidou, E., and Vekrellis, K. (2016). Exocytosis and spreading of normal and aberrant alpha-synuclein. *Brain Pathology* *26*, 398-403.
- Fabrizi, C., Businaro, R., Lauro, G.M., and Fumagalli, L. (2001). Role of alpha2-macroglobulin in regulating amyloid beta-protein neurotoxicity: protective or detrimental factor? *Journal of Neurochemistry* *78*, 406-412.
- Flagmeier, P., De, S., Wirthensohn, D.C., Lee, S.F., Vincke, C., Muyltermans, S., Knowles, T.P.J., Gandhi, S., Dobson, C.M., and Klenerman, D. (2017). Ultrasensitive measurement of Ca^{2+} influx into lipid vesicles induced by protein aggregates *Angewandte Chemie International Edition* *56*, 7750-7754.

French, K., Yerbury, J.J., and Wilson, M.R. (2008). Protease activation of alpha2-macroglobulin modulates a chaperone-like action with broad specificity. *Biochemistry* 47, 1176-1185.

Fusco, G., Chen, S.W., Williamson, P.T.F., Cascella, R., Perni, M., Jarvis, J.A., Cecchi, C., Vendruscolo, M., Chiti, F., Cremades, N., *et al.* (2017). Structural basis of membrane disruption and cellular toxicity by α -synuclein oligomers. *Science* 358, 1440-1443.

Gallegos, S., Pacheco, C., Peters, C., Opazo, C.M., and Aguayo, L.G. (2015). Features of alpha-synuclein that could explain the progression and irreversibility of Parkinson's disease. *Frontiers in Neuroscience* 9.

Gregory, J.M., Whiten, D.R., Brown, R.A., Barros, T.P., Kumita, J.R., Yerbury, J.J., Satapathy, S., McDade, K., Smith, C., Luheshi, L.M., *et al.* (2017). Clusterin protects neurons against cytoplasmic proteotoxicity. *Acta Neuropathologica Communications* 5.

Hochgrebe, T., Pankhurst, G.J., Wilce, J., and Easterbrook-Smith, S.B. (2000). pH-dependent changes in the in vitro ligand-binding properties and structure of human clusterin. *Biochemistry* 39.

Horrocks, M.H., Tosatto, L., Dear, A.J., Garcia, G.A., Iljina, M., Cremades, N., Serra, M.D., Knowles, T.P.J., Dobson, C.M., and Klenerman, D. (2015). Fast flow microfluidics and single-molecule fluorescence for the rapid characterization of alpha-synuclein oligomers. *Analytical Chemistry* 87, 8818-8826.

Iljina, M., Garcia, G.A., Horrocks, M.H., Tosatto, L., Choi, M.L., Ganzinger, K.A., Abramov, A.Y., Gandhi, S., Wood, N.W., Cremades, N., *et al.* (2016). Kinetic model of the aggregation of alpha-synuclein provides insights into prion-like spreading. *Proceedings of the National Academy of Sciences, USA* 113, 1206-1215.

Ingelsson, M. (2016). Alpha-synuclein oligomers — neurotoxic molecules in Parkinson's Disease and other Lewy body disorders. *Frontiers in Neuroscience* 10.

Jenner, P. (2003). Oxidative stress in Parkinson's disease. *Annals of Neurology* 53, 26-38.

Kruger, R., Menezes-Saecker, A.M.V., Schols, L., Kuhn, W., Muller, T., Woitalla, D., Berg, D., Berger, K., Przuntek, H., Epplen, J.T., *et al.* (2000). Genetic analysis of the alpha2-macroglobulin gene in early- and late-onset Parkinson's disease. *NeuroReport* 11, 2439-2442.

Lee, H.-J., Bae, E.-J., and Lee, S.-J. (2014). Extracellular α -synuclein—a novel and crucial factor in Lewy body diseases. *Nature Reviews Neurology* 10, 92-98.

Marques, O., and Outeiro, T.F. (2012). Alpha-synuclein: from secretion to dysfunction and death. *Cell Death and Disease* 3.

Narayan, P., Holmstrom, K.M., Kim, D.-H., Whitcomb, D.J., Wilson, M.R., George-Hyslop, P., Wood, N.W., Dobson, C.M., Cho, K., Abramov, A.Y., *et al.* (2014). Rare individual amyloid- β oligomers act on astrocytes to initiate neuronal damage. *Biochemistry* 53, 2442-2453.

Narayan, P., Orte, A., Clarke, R.W., Bolognesi, B., Hook, S., Ganzinger, K.A., Meehan, S., Wilson, M.R., Dobson, C.M., and Klenerman, D. (2012). The extracellular chaperone clusterin sequesters oligomeric forms of the amyloid- β 1-40 peptide. *Nature Structural & Molecular Biology* 19, 79-83.

Nicoletti, G., Annesi, G., Tomaino, C., Spadafora, P., Pasqua, A.A., Annesi, F., Serra, P., Caracciolo, M., Messina, D., Zappia, M., *et al.* (2002). No evidence of association between the alpha-2 macroglobulin gene and Parkinson's disease in a case-control sample. *Neuroscience Letters* 328, 65-67.

Nizard, P., Tetley, S., Le Dréan, Y., Watrin, T., Le Goff, P., Wilson, M.R., and Michel, D. (2007). Stress-induced retrotranslocation of clusterin/ApoJ into the cytosol. *Traffic* 8, 554-565.

Orte, A., Birkett, N.R., Clarke, R.W., Devlin, G.L., Dobson, C.M., and Klenerman, D. (2008a). Direct characterization of amyloidogenic oligomers by single-molecule fluorescence. *Proceedings of the National Academy of Sciences, USA* 105, 14424-14429.

Orte, A., Clarke, R.W., and Klenerman, D. (2008b). Fluorescence coincidence spectroscopy for single-molecule fluorescence resonance energy-transfer measurements. *Analytical Chemistry* 80, 8389-8397.

Pfefferkorn, C.M., Jiang, Z., and Lee, J.C. (2012). Biophysics of α -synuclein membrane interactions. *Biochimica et Biophysica Acta* 1818, 162-171.

Poon, S., Rybchyn, M.S., Easterbrook-Smith, S.B., Carver, J.A., Pankhurst, G.J., and Wilson, M.R. (2002). Mildly acidic pH activates the extracellular molecular chaperone clusterin. *Journal of Biological Chemistry* 277.

Reynolds, N.P., Soragni, A., Rabe, M., Verdes, D., Liverani, E., Handschin, S., Riek, R., and Seeger, S. (2011). Mechanism of membrane interaction and disruption by α -synuclein. *Journal of the American Chemical Society* 133, 19366-19375.

Sasaki, K., Doh-ura, K., Wakisaka, Y., and Iwaki, T. (2002). Clusterin/apolipoprotein J is associated with cortical Lewy bodies: immunohistochemical study in cases with α -synucleinopathies. *Acta Neuropathologica* 104, 225-230.

Sheluhu, S., and Ackerman, S.H. (2001). An Accessible Hydrophobic Surface Is a Key Element of the Molecular Chaperone Action of Atp11p. *Journal of Biological Chemistry* 276, 39945.

Tang, G., Zhang, M., Xie, H., Jiang, S., Wang, Z., Xu, L., Hao, Y., Lin, D., Lan, H., Wang, Y., *et al.* (2002). Alpha-2 macroglobulin I1000V polymorphism in Chinese sporadic Alzheimer's disease and Parkinson's disease. *Neuroscience Letters* 328, 195-197.

van Rooijen, B.D., Claessens, M.M.A.E., and Subramaniam, V. (2009). Lipid bilayer disruption by oligomeric α -synuclein depends on bilayer charge and accessibility of the hydrophobic core. *Biochimica et Biophysica Acta* 1788, 1271-1278.

Wilson, M.R., and Easterbrook-Smith, S.B. (2000). Clusterin is a secreted mammalian chaperone. *Trends in Biochemical Sciences* 25, 95-98.

Winner, B., Jappelli, R., Maji, S.K., Desplats, P.A., Boyer, L., Aigner, S., Hetzer, C., Loher, T., Vilar, M., Campioni, S., *et al.* (2011). In vivo demonstration that α -synuclein oligomers are toxic. *Proceedings of the National Academy of Sciences, USA* 108, 4194-4199.

Wyatt, A.R., Kumita, J.R., Mifsud, R.W., Gooden, C.A., Wilson, M.R., and Dobson, C.M. (2014). Hypochlorite-induced structural modifications enhance the chaperone activity of human α -2-macroglobulin. *Proceedings of the National Academy of Sciences, USA* 111, 2081-2090.

Wyatt, A.R., Yerbury, J.J., Ecroyd, H., and Wilson, M.R. (2013). Extracellular chaperones and proteostasis. *Annual Review of Biochemistry* 82, 295-322.

Wyatt, A.R., Yerbury, J.J., and Wilson, M.R. (2009). Structural characterisation of clusterin-chaperone client protein complexes. *Journal of Biological Chemistry* 284, 21920-21927.

Yerbury, J.J., Poon, S., Meehan, S., Thompson, B., Kumita, J.R., Dobson, C.M., and Wilson, M.R. (2007). The extracellular chaperone clusterin influences amyloid formation and toxicity by interacting with prefibrillar structures. *FASEB Journal* 21, 2312-2322.

Yerbury, J.J., and Wilson, M.R. (2010). Extracellular chaperones modulate the effects of Alzheimer's patient cerebrospinal fluid on A β 1-42 toxicity and uptake. *Cell Stress Chaperones* 15, 115-121.

Zampagni, M., Cascella, R., Casamenti, F., Grossi, C., Evangelisti, E., Wright, D., Becatti, M., Ligurri, G., Mannini, B., Campioni, S., *et al.* (2011). A comparison of the biochemical modifications caused by toxic and non-toxic protein oligomers in cells. *Journal of Cellular and Molecular Medicine* 15, 2106-2116.

Zhang, F., Kumano, M., Beraldi, E., Fazli, L., Du, C., Moore, S., Sorensen, P., Zoubeidi, A., and Gleave, M.E. (2014). Clusterin facilitates stress-induced lipidation of LC3 and autophagosome biogenesis to enhance cancer cell survival. *Nature Communications* 5.

Zhang, W., Wang, T., Pei, Z., Miller, D.S., Wu, X., Block, M.L., Wilson, B., Zhang, W., Zhou, Y., Hong, J.S., *et al.* (2005). Aggregated alpha-synuclein activates microglia: a process leading to disease progression in Parkinson's disease. *FASEB Journal* 19, 533-542.

Figure legends

Figure 1: The ratio of CLU: α S decreases in larger oligomers. α S^{A90C}-AF488 (70 μ M) and CLU-AF647 (0.7 μ M) were co-incubated in PBS (pH 7.4) at 37 °C, with shaking at 200 rpm. Samples were taken from the aggregation reaction after 6 h (a, c) and 48 h (b, d), respectively. The formation of α S-CLU complexes was quantified by single-molecule TCCD. **a and b)** Contour plots of the apparent number of α S monomers comprising an oligomer as a function of the $Z_{\text{CLU}/\alpha\text{S}}$ value, where $Z_{\text{CLU}/\alpha\text{S}}$ represents the logarithm of the apparent ratio of CLU to α S in each oligomer. The data shown are representative of three separate experiments. The numbers in the inserts indicate the number of complexes represented in the plot. **c and d)** Frequency histogram of the number of oligomers at different $Z_{\text{CLU}/\alpha\text{S}}$ (for the data shown in a and b). The dotted lines each indicate a specific CLU: α S^{A90C} stoichiometry (as shown on the upper x-axis). The data are representative of three independent experiments.

Figure 2: Time-dependent changes in the association of α_2 M with α S oligomers. α S^{A90C}-AF488 (70 μ M) and α_2 M-AF647 (0.7 μ M) were co-incubated in PBS (pH 7.4) at 37 °C, with shaking at 200 rpm. Samples were taken from the aggregation reaction after 6 h (a, c) or 48 h (b, d), respectively. The formation of α S- α_2 M complexes was quantified by single-molecule TCCD. **a and b)** Contour plots of the apparent number of α S monomers comprising a given oligomer as a function of the $Z_{\alpha_2\text{M}/\alpha\text{S}}$ value. $Z_{\alpha_2\text{M}/\alpha\text{S}}$ represents the logarithm of the apparent ratio of α_2 M to α S in each oligomer. The numbers in the inserts indicate the number of complexes represented in the plot. **c and d)** Frequency histogram of the number of oligomers at different $Z_{\alpha_2\text{M}/\alpha\text{S}}$ (for the data shown in the upper panels). The dotted lines each indicate a specific α_2 M: α S^{A90C} stoichiometry (as shown on the upper x-axis). Data is representative of three independent experiments. **e)** α S^{A90C}-AF488 (70 μ M) and α_2 M-AF647 (0.7 μ M) were co-incubated in PBS at 37 °C, with shaking at 200 rpm, for the indicated time. For each time point, the average number of apparent monomers of α_2 M and α S per oligomer were calculated. The data were fitted to linear regressions. **f)** The gradients of each linear regression for each timepoint shown in (a). A time-dependent linear increase in the amount of α_2 M found bound to α S can be observed. Data shown are means \pm SEM (n=3).

Figure 3: Hydrophobic shielding reduces the ability of α S oligomers to induce membrane disruption and ROS production. **a and b)** BisANS inhibits the binding of CLU (a) and α_2 M (b) to α S oligomers. The chaperones (present at 10 $\mu\text{g}\cdot\text{ml}^{-1}$) were incubated in an ELISA plate pre-coated with aggregated α S and the amount of bound chaperone was then measured by ELISA. Neither chaperone bound to monomeric α S or the BSA blocker (Figure S3). Data shown are means \pm SD of three independent experiments. **c)** α S oligomers were pre-incubated with CLU, α_2 M or BSA and then added to surface tethered lipid vesicles filled with the Ca²⁺-sensitive fluorophore Cal-520. The extent of membrane permeabilisation was quantified as a percent of maximum fluorescence produced after incubation with the Ca²⁺ ionophore ionomycin. Example images of vesicles after the addition of the indicated sample. The scale bar (bottom right) is 2 μm . **d)** Quantification of the data shown in (c). α_2 M or CLU (0.05-50 nM) was incubated with aggregated α S. The extent of membrane permeabilisation decreased with increasing chaperone concentration. Data shown are means \pm SD of 9 fields of view (at least 800 vesicles) and is representative of two independent experiments. **e)** Aggregated α S preincubated with CLU, α_2 M

or BSA (each present at a 1:10 substoichiometric ratio) was added to N2a cells. The rate of ROS production before and after the sample addition was quantified by measuring the oxidation of DHE to ethidium by epifluorescence microscopy. The change in the rate of ROS generation due to the addition of a sample was calculated by subtracting the gradient of the pre-addition line from the gradient of the post-addition line. Example rates of ROS production in a single cell under the indicated conditions. **f)** Quantification of the data shown in (e). The change in the rate of ROS production produced by each sample relative to the buffer only sample. The values are means \pm SD of approximately 50 cells across three replicate treatments. **, $p < 0.01$; ***, $p < 0.001$, analysed by one-way ANOVA with a Bonferroni post-test.