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Dimeric structures of α-synuclein bind preferentially to lipid membranes

Eleni Giannakis [a], Jessica Pacífico [b,1], David P. Smith [c,2], Lin Wai Hung [c,d,e], Colin L. Masters [c,d], Roberto Cappai [c,d,e,f], John D. Wade [a], Kevin J. Barnham [c,d,e,*]

[a] The Howard Florey Institute of Medical Research, Australia
[b] Department of Chemistry, The University of Melbourne Victoria, 3010, Australia
[c] Department of Pathology, The University of Melbourne, Victoria, 3010, Australia
[d] The Mental Health Research Institute of Victoria, Parkville, Victoria, 3052, Australia
[e] Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, Australia
[f] Centre for Neuroscience, The University of Melbourne, Victoria, 3010, Australia

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Abstract

There is substantial evidence which implicates α-synuclein and its ability to aggregate and bind vesicle membranes as critical factors in the development of Parkinson's disease. In order to investigate the interaction between α-synuclein wild type (Wt) and its familial mutants, A53T and A30P with lipid membranes, we developed a novel lipid binding assay using surface enhanced laser desorption/ionisation-time of flight-mass spectrometry (SELDI-TOF MS). Wt and A53T exhibited similar lipid binding profiles; monomeric species and dimers bound with high relative affinity to the lipid surface, the latter of which exhibited preferential binding. Wt and A53T trimers and tetramers were also detected on the lipid surface. A30P exhibited a unique lipid binding profile; monomeric A30P bound with a low relative affinity, however, the dimeric species of A30P exhibited a higher binding ability. Larger order A30P oligomers were not detected on the lipid surface. Tapping mode atomic force microscopy (AFM) imaging was conducted to further examine the α-synuclein–lipid interaction. AFM analysis revealed Wt and its familial mutants can penetrate lipid membranes or disrupt the lipid and bind the hydrophobic alkyl self-assembled monolayer (SAM) used to form the lipid layer. The profile of these studied proteins revealed the presence of 'small features' consistent with the presence of monomeric and dimeric forms of the protein. These data collectively indicate that the dimeric species of Wt and its mutants can bind and cause membrane perturbations.

Keywords: Parkinson's disease; α-Synuclein; Lipid membrane; SELDI-TOF MS; AFM

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disorder, which is characterized by a loss of dopaminergic neurons in the substantia nigra, resulting in resting tremors, slow movement and rigidity [1]. Prevalence rates of PD are ~1% amongst individuals aged between 65–69 and increase in those aged 80 years and older to ~3% [2].

α-Synuclein is a 14.5 kDa presynaptic protein believed to play an important role in the pathogenesis of PD [3]. Evidence supporting this hypothesis includes the finding that Lewy bodies, cytostolic inclusions characteristic of PD, consist largely of fibrillar α-synuclein [4]; triplication or point mutations (A53T and A30P) in the α-synuclein gene have also been linked to PD [5–7]. Furthermore, expression of human α-synuclein in transgenic mice produces a PD phenotype, which is both age and dose-dependent [8].

The mechanisms by which α-synuclein induces toxicity in PD pathogenesis remain unclear, although there is substantial evidence which implicates lipid membrane binding and the ability of α-synuclein to aggregate as important factors. Fibril formation maybe initiated by a number of factors; (i) misfolded intermediates, which exhibit non-polar patches, binding to other

* Corresponding author. Department of Pathology, The University of Melbourne, Victoria, 3010, Australia. Tel.: +61 3 8344 2555; fax: +61 3 9347 6750.
E-mail address: kbarnham@unimelb.edu.au (K.J. Barnham).
[1] Current address: Departamento de Química Física, Universidade de Vigo, Campus de Marcosende, Vigo 36310, Pontevedra, Spain.
[2] Current address: Institute of Molecular and Cellular Biology, University of Leeds, Leeds, UK.

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intermediates via hydrophobic–intermolecular interactions [9], (ii) increased protein concentration arising from triplication of the α-synuclein gene [5], (iii) exposure to oxidative factors [10], or (iv) interactions with lipids [11]. Point mutations can also accelerate α-synuclein fibrilisation, supporting the premise that this event may be a critical factor in disease pathogenesis [9,12]. The process of fibril formation is often preceded by oligomeric intermediates termed protofibrils, these are consumed as more stable fibrils are formed. It is these intermediate products, rather than the fibrils, which are believed to be the toxic species responsible for the neurodegeneration associated with the disease [13].

The α-synuclein–membrane interaction is considered to be a potential cytotoxic event, resulting in the permeabilisation of the cell membrane [14]. Upon interactions with lipid membranes, α-synuclein undergoes a conformational change from a random coil to an α-helical structure [15]. The N-terminus of α-synuclein contains a series of 11 amino acid repeats with a conserved hexameric motif (KTKEGV), which is characteristic of lipid binding α-helical domains of apolipoproteins and facilitates this inducible structural change. The acidic C-terminus of the protein, however, remains essentially unstructured [15–17]. Examination of the familial point mutations revealed that A53T binds to membranes with a similar affinity to α-synuclein wild type (Wt), whilst A30P exhibits weaker binding [18–21]. Interestingly, it is the intermediate oligomers which bind to membranes with a high affinity and cause more rapid permeabilisation of cell membranes than the monomeric species [13,14,22]. Ascertaining the precise oligomeric species that associate and disrupt membranes is thus critical for further understanding of the pathogenesis of the disease process. Towards this aim, we developed a novel assay using surface enhanced laser desorption/ionisation-time of flight-mass spectrometry (SELDI-TOF MS) to measure the ability of α-synuclein Wt, A53T and A30P to bind "on-chip" to synthetic lipid membranes. The α-synuclein–lipid interaction was further examined by atomic force microscopy (AFM) imaging.

2. Materials and methods

2.1. Expression and purification of α-synuclein Wt, A30P and A53T

α-Synuclein Wt and its mutants were prepared as described by Cappai et al. [23]. Briefly, human α-synuclein Wt was cloned into pRSETB (Invitrogen, Carlsbad, CA, USA) and expressed in *Escherichia coli*, BL21 (DE3). The Quikchange mutagenesis kit (Stratagene, La Jolla, CA, USA) was used to generate A30P and A53T. Purification was performed as previously described [23].

2.2. Preparation of α-synuclein Wt, A30P and A53T for functional studies

α-Synuclein Wt, A30P and A53T were initially filtered (0.2 μm Minisart RC4 filters, Sartorius, Goettingen, DEU) to remove pre-formed aggregates and prepared in 10 mM phosphate buffer, pH 7.4 (PB) at a final concentration of 200 μM.

2.3. Preparation of vesicles

Equal amounts of *1*-palmitoyl-2-oeoyl-*sn*-glycero-3 [phospho-L-serine] (POPS) and *1*-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) (Avanti Polar Lipids, Inc., Alabaster, AL, USA) (16 mg) were dissolved in 2 ml of ethanol-free chloroform. The solvent was evaporated and lipids were re-suspended in 1 ml of PB by mixing (200 rpm) with glass beads for 1 h at 37 °C. The lipids were sonicated for 15 min and subjected to several freeze thaw cycles in liquid nitrogen. The lipids were finally passed through a 0.05 μM filter (Millipore, Bedford, MA, USA) in an Avanti "mini extruder" apparatus until the solution was translucent.

2.4. Formation of a lipid monolayer on H50 ProteinChip arrays

Hydrophobic H50 ProteinChip arrays (Bio-Rad Laboratories, CA, USA) with functional C8 groups were placed in a humidity chamber. Each spot was loaded with 5 μl of CHAPS (30 mg/ml), which was immediately wicked off, followed by three 2 min washes with 5 μl of PB on a shaking platform. The POPC/POPS mixture or PB (5 μl) was applied to each spot and incubated for 2 h at 37 °C. Arrays were washed twice with PB to remove unbound lipid.

2.5. Protein binding to the lipid coated H50 ProteinChip arrays

BSA (Sigma Aldrich, Munich, DEU), melitin (Sigma Aldrich), α-synuclein Wt, A30P or A53T (5 μl, 50 μM) were loaded onto spots coated with either the POPC/POPS lipid mixture or PB and incubated for 5 min with agitation. Arrays were washed twice with PB for 2 min, followed by two 1 min washes with 1 mM HEPES, pH 7.2. The arrays were air dried and l μl of 50% saturated sinapinic acid in 50% (v/v) acetonitrile, 0.5% trifluoroacetic acid was applied onto each spot twice, arrays were air dried between each application. Sinapinic acid was chosen since high mass proteins were to be assessed. Arrays were finally analysed by SELDI-TOF MS (PBSIIc) and subsequent spectra was examined using ProteinChip software® (Bio-Rad Laboratories). Every 5th position on the spot was scanned, 5 transients per position (65 total) were collected.

2.6. SELDI-TOF MS analysis of α-synuclein Wt, A53T and A30P on NP20 ProteinChip arrays

1 μl of α-synuclein Wt, A30P and A53T was loaded onto spots of a NP20 ProteinChip array (Bio-Rad Laboratories) and air dried. Matrix was prepared and applied as described above. Arrays were analysed by SELDI-TOF MS and spectra was examined using ProteinChip software®.

2.7. Preparation of samples for AFM Imaging

The surfaces used were prepared via a 3 step procedure. Glass slides were cleaned with piranha solution (sulphuric acid: H_2O_2 (3:1, v:v) for 1 h and then rinsed with MilliQ water. A mask was applied to the surface in order to generate a spherical surface on the glass substrate allowing improved localization of the sample. 5 nm of chromium (Proscitech, Thuringowa, QLD, AU) and 50 nm of gold (Proscitech) were sequentially sputter coated on the substrates with an EMITECH K575 sputter-coater apparatus (Emitech Ltd., Ashford Kent, UK). Finally, a SAM of octadecanethiol was organized on the gold spherical surface of the substrate by leaving the substrate in a 1 mM solution overnight. The samples were then ready for lipid adsorption. The contact angle for the SAM was measured at 108°±4° with a Dataphysics Contact Angle System OCA 15 plus, at ambient temperature with a CCD camera (Teli) and the computer software 'SCA 20'.

2.8. Formation of a lipid monolayer

The AFM imaging samples were placed in a humidity chamber. Each slide was loaded with 10 μl of CHAPS (30 mg/ml), which was immediately wicked off, followed by five 2 min washes with 10 μl of PB. The POPC/POPS mixture or PB (10 μl) was applied to each spot and incubated for 2 h at 37 °C. Slides were washed with PB three times to remove unbound lipid.

2.9. Protein binding to the lipid coated surfaces

BSA, melitin, α-synuclein Wt, A30P or A53T (10 μl, 50 μM) were loaded onto slides coated with the POPC/POPS lipid mixture or PB and incubated for

5 min. Slides were washed twice with PB for 2 min, followed by two 1 min washes with 1 mM HEPES, pH 7.2. The slides were air dried in preparation for AFM analysis [13].

2.10. Tapping mode AFM imaging

Tapping mode imaging was conducted with a Dimension 3100 Atomic Force Microscope (Veeco Instruments, Santa Barbara, CA, USA) using a Nanoscope III controller and the program Veeco DI v5.18 for data treatment. Budget Sensors cantilevers (Sofia, BGR) with a 300 kHz resonant frequency and spring constant k=40 N/m were used in the analysis. Interpretation was made using the WsXM 4.0v7.5 program from Nanotec Electronica (Madrid, ESP) [24]. The scan rates were set between 0.5–1 Hz, depending on the scan size and the samples per line were set to 512 for 512 lines.

The protein average sizes were determined from 6–10 scanned areas on each sample, up to 4 samples were prepared for each protein.

3. Results

3.1. Oligomeric profiles of α-synuclein Wt, A53T and A30P on NP20 arrays

The oligomeric content of freshly prepared α-synuclein Wt, A53T and A30P was initially assessed on NP20 arrays. Monomers and several oligomeric species, including dimers, trimers, tetramers and pentamers, were clearly detected (Fig. 1). Matrix adducts were detected as noted by the presence of a minor peak with a mass shift of ~224 Da. The area under the curve (AUC) values was subsequently assessed. The monomers were identified to be the most abundant species for α-synuclein Wt, A53T and A30P, followed by dimers and the remaining larger order oligomers.

3.2. Oligomeric profiles of α-synuclein Wt, A53T and A30P on lipid coated H50 arrays

In order to identify the oligomeric species which bind to lipid membranes, a novel solid phase lipid binding assay was developed. Lipid monolayers were formed on H50 ProteinChip arrays using small unilamellar vesicles prepared from a mixture of extruded and sonicated phospho-L-serine/phosphocholine. The ability to create a lipid monolayer on the chip surface was initially assayed by examining the amount of (i) non-specific binding of bovine serum albumin (BSA), which binds with a higher affinity to hydrophobic surfaces in the absence of lipid [25] and (ii) specific binding of melitin, a membranolytic peptide [26]. BSA bound to the hydrophobic surface with an AUC value of 2060. In the presence of lipid; BSA binding decreased ~2 fold with a signal to noise value of 1022 (Fig. 2). Melitin was shown to bind with a ~10 fold increase in signal to the chip surface in the presence of lipid with an AUC value of 506 in comparison to an AUC value of 48 in the absence of lipid (Fig. 2). These control experiments demonstrated that we were able to specifically capture and analyse a lipid binding protein using this system.

Upon establishment of the methodology, we assessed the lipid binding ability of Wt α-synuclein, A53T and A30P. Wt α-synuclein monomer bound specifically to the lipid surface with an AUC value of 8663 compared to 145 in the absence of lipid. A53T monomer also bound specifically to the lipid coated arrays in a similar manner to Wt α-synuclein, an AUC value of 8691 was recorded. A30P monomer exhibited a decreased binding with an AUC value of 1198 being recorded (Fig. 3). Assessment

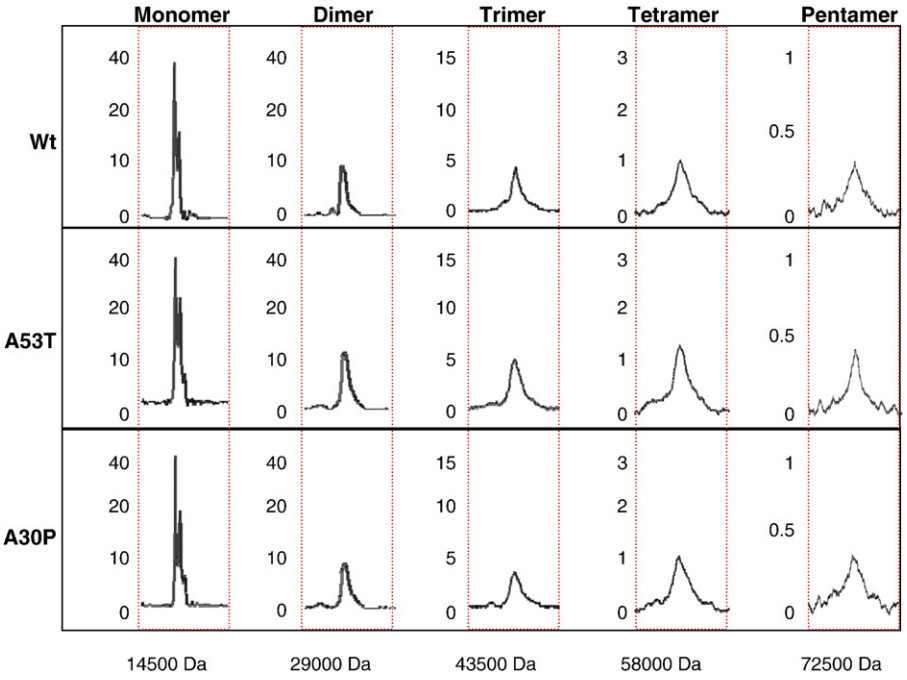

Fig. 1. SELDI-TOF MS profiles of α-synuclein Wt, A53T and A30P on NP20 arrays. Samples were loaded onto arrays and air dried. SPA was applied to each spot and then subjected to SELDI-TOF MS. Resulting spectra analysed in ProteinChip software, revealed the presence of monomeric and several oligomeric species. Peak intensities and molecular weights are shown on the Y and X axis, respectively.

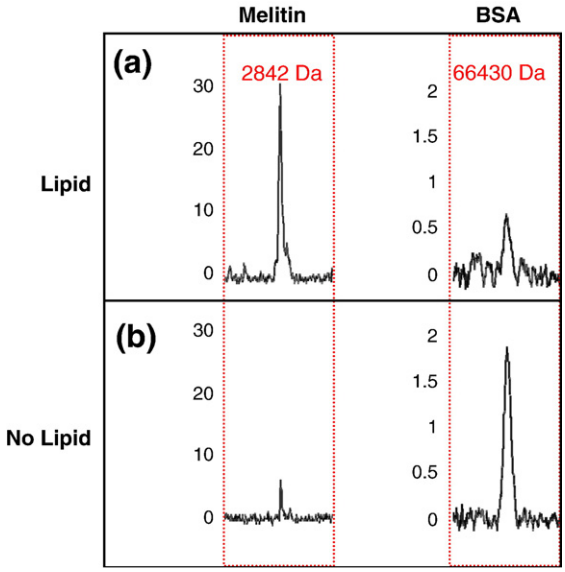

Fig. 2. SELDI-TOF MS profiles of melitin and BSA on lipid coated H50 arrays (panel a) and H50 arrays alone (panel b). Melitin or BSA was loaded on spots coated with either a POPC/POPS lipid monolayer or 10 mM phosphate buffer, pH 7.4 (PB) alone and incubated for 5 min. Spots were washed to remove non-specifically bound components. SPA was applied to each spot and arrays were subsequently subjected to SELDI-TOF MS. Analysis of resulting spectra revealed that melitin binds with a higher affinity to the lipid coated surface, whilst BSA exhibits higher affinity to hydrophobic surface in the absence of the lipid.

of the Wt α-synuclein oligomers revealed the presence of dimers, trimers and tetramers on the lipid surface. The dimer exhibited a higher signal as shown by the AUC, which implies a preferential binding and a higher affinity for the dimer over monomer (AUC value of 12663) (Fig. 3). A53T exhibited an oligomeric lipid binding profile similar to Wt α-synuclein, again dimers, trimers and tetramers were detected on the lipid surface. Dimers exhibited the highest binding ability (AUC value of 12165), preferentially binding to the lipid surface in comparison to the monomer (Fig. 3). Interestingly, A30P exhibited a unique oligomeric lipid binding profile. A30P dimeric species were detected on the lipid surface, however, larger order A30P oligomers were not identified. The A30P mutant dimers exhibited ~7 fold higher AUC value (8986) than the monomeric species (Fig. 3).

3.3. Tapping mode AFM imaging

In order to further examine the ability of Wt α-synuclein, A53T and A30P to interact with lipid membranes AFM analysis was conducted. Lipid monolayers were initially created on a hydrophobic surface of an octadecanethiol SAM. The affinity of sulfur atoms in alkanethiol molecules towards gold surfaces allows SAM formation on the metallic surface [25,27,28]. Changing the functionality of the alkanethiols and their chain length gives surfaces with tunable properties [28]. The hydrophobicity of long chain alkylthiols was used to adsorb a monolayer of lipids on the substrate.

Fig. 4a shows the plain gold surface with the self-assembled monolayer (SAM) (RMS roughness of 1 nm, average height of

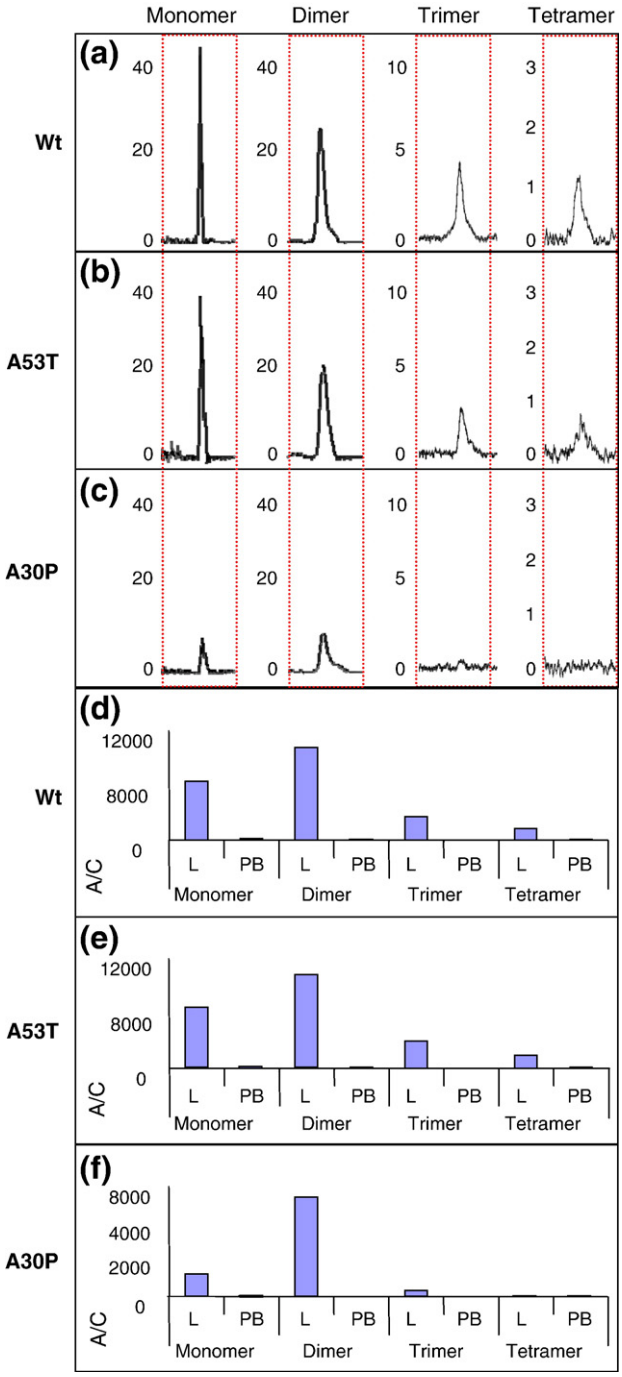

Fig. 3. SELDI-TOF MS profiles of α-synuclein Wt, A53T and A30P on lipid coated H50 arrays. α-Synuclein Wt or its mutants were loaded on spots coated with either a POPC/POPS lipid monolayer or PB and incubated for 5 min. Spots were washed to remove non-specifically bound components. SPA was applied to each spot and arrays were subsequently subjected to SELDI-TOF MS. Analysis of resulting spectra revealed that the Wt (panel a) and A53T mutant (panel b) bound to the surface with a high affinity and exhibited a similar binding pattern, with the dimeric species exhibiting preferential binding. Interestingly, the A30P mutant exhibited weaker binding (panel c); however, again the dimeric species exhibited preferential binding in comparison to the monomer. AUC values of α-synuclein Wt (panel d), A53T (panel e) and A30P (panel f) on lipid coated H50 arrays and H50 arrays alone are also displayed, demonstrating the specific binding patterns of the monomers and oligomers.

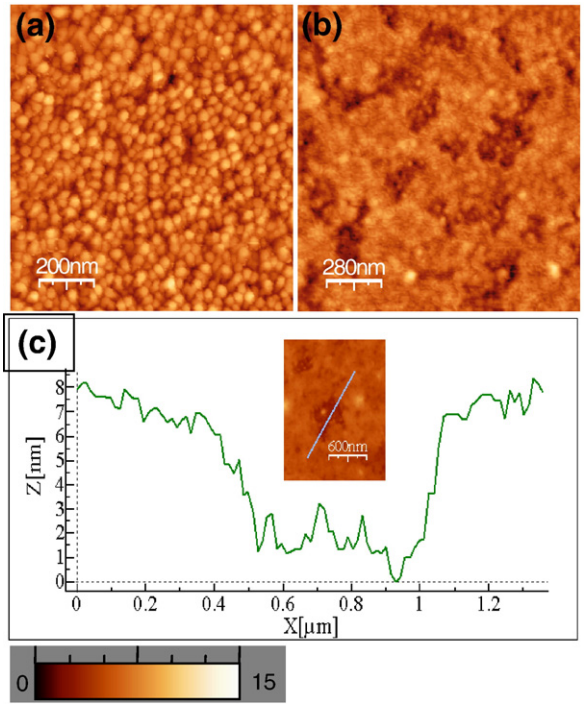

Fig. 4. AFM image of a gold-SAM substrate (panel a), lipid monolayer on a gold-SAM substrate (panel b) and profile studies of the lipid monolayer (panel c). Glass slides were initially cleaned with piranha solution and sequentially sputter coated with chromium and gold. A SAM of octadecanethiol was organized on the gold surface followed by incubations with either PB or POPC/POPS to create a synthetic lipid monolayer. The lipid layer was comparatively smooth, small areas with no lipid allowed the thickness of the lipid film to be ascertained.

3.6 nm calculated on a 0.5 μm×0.5 μm area) and comparatively the smooth lipid monolayer adsorbed on the substrate (RMS roughness of 1.5–2 nm, average height of 5.6 nm on a 3 μm×3 μm scan, Fig. 4b). A profile study of the lipid monolayer on the SAM substrate shows small areas with no lipid, allowing the measurement of the thickness of the lipid film (Fig. 4c). The average height of the lipid layer measured was determined to be thicker than a long chain lipid monolayer [29], which is due to the (a) cumulative effect of gold, alkyl and lipid roughness (b) tapping mode imaging (amplitude can create 1 nm profile difference) (c) furthermore, a 1–1.5 nm error in height can be generated due to the experimental calibration error.

The addition of Wt α-synuclein to the substrate resulted in a clear disruption of the lipid surface surrounding the protein (Fig. 5a). Interestingly, areas were detected which exhibited significant disruption without the presence of protein. These are believed to be areas where the protein was embedded into the lipid layer and removed during the post binding washes subsequently lifting off the surrounding lipid. In Fig. 4b, the lipid monolayer itself presents some defects in its formation, nevertheless the surface sizes of these defects are much smaller than in the presence of the protein (order of magnitude: 10–20 times smaller). The lipid monolayer is therefore considered to be rougher and patchy after treatment with Wt α-synuclein. Further observations indicated that the protein still bound to the lipid layer in fact appears to "push away" the lipid, disrupting the cohesive forces of the monolayer. An interesting characteristic pattern of lipid disruption was identified to surround the protein; disruption was generally limited to one direction, but not surrounding the entire protein as relatively little lipid disruption was observed in the adjacent flanking regions (Fig. 5b). We cannot affirm if the protein is actually embedded in the lipid layer or if it is binding directly to the SAM after lipid disruption. Profile studies of the lipid associated proteins were subsequently conducted, however, since the chemical conformation of these proteins on a surface has not been ascertained, the width and height of the absorbed proteins is unknown. The measurement of protein dimensions on the surfaces was averaged out of 4–6 surfaces and 6–10 spots on each surface. In accordance with SELDI-TOF measurements, we consequently concluded that the "small features", which exhibited an average size of 61–65 nm, were monomeric forms of the protein whilst features double in size were probable dimeric forms of the protein. We observed the same lipid monolayer disruption for the putative monomers and dimers, except the size is roughly doubled in the latter case (Fig. 5c and d, respectively).

It is important to note that the precision on *Z* scan axis versus the *X*–*Y* scan directions is strongly dependent on the scanning parameters, especially the scan size chosen for the sample measurements. Scan sizes of 5–0.5 μm were initially performed which showed large areas of lipid disruption associated with the putative monomers and dimers. Smaller scans (200 nm) were subsequently performed in order to more accurately assess the sizes of the monomers/dimers, but have not been presented due

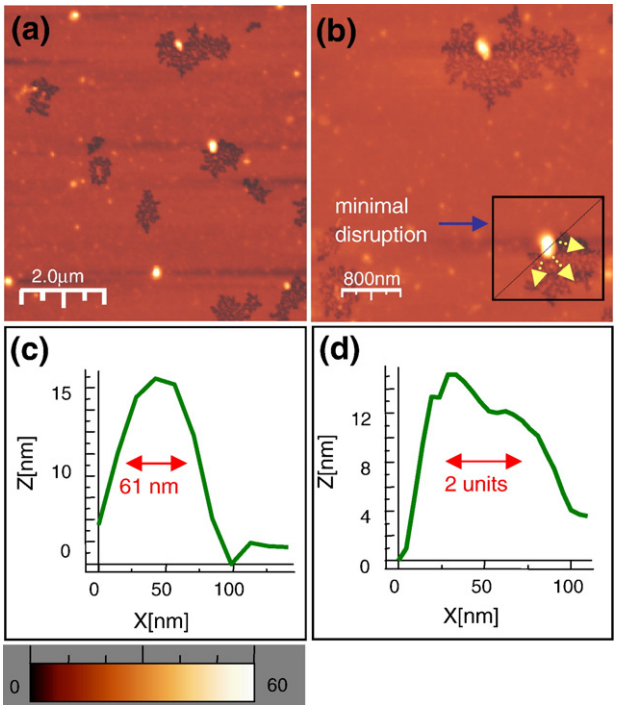

Fig. 5. AFM image of α-synuclein Wt binding to a lipid monolayer. Addition of α-synuclein resulted in a clear disruption of the lipid layer (panel a). A closer view of the associated proteins showed a characteristic pattern of lipid disruption surrounding the embedded protein (panel b). Typical profiles of the associated proteins showed the presence of monomers and oligomers (panels c and d, respectively).

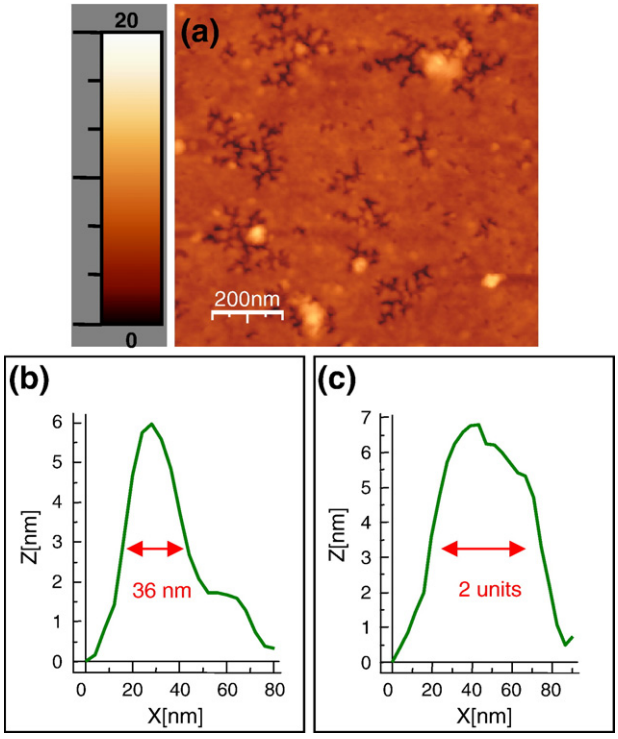

Fig. 6. AFM image of A53T binding to a lipid monolayer (panel a) and typical profiles of the associated proteins, showing the presence of monomers and oligomers (panels b and c, respectively).

to imaging noise issues; during small scans proteins tend to stick to the tips interfering with data interpretation. Tip radius (tip effects) may also complicate AFM imaging and interpretation of distances or sizes.

Upon assessment of the mutant proteins, we observed that A53T and A30P were also able to bind the lipid monolayer and exhibited a similar pattern of disruption to that of α-synuclein Wt (Fig. 6a). A profile study of A53T and A30P proteins associated with the lipid layer revealed the presence of monomers identified to be 46–51 nm and 35–42 nm in diameter, respectively (Figs. 6b and 7b); dimers were also detected (Figs. 6c and 7c). We postulate that the variation in AFM size measurements between Wt and the mutants is likely to be the result of conformational differences between the proteins since (i) both A30P and A53T substitutions have been shown to alter the conformation of α-synuclein [7,30] and (ii) AFM is capable of resolving differences in length, diameter and conformation (stiffness, aggregation, mode of adsorption to a substrate) between features [31].

Control experiments show that BSA embedded in the lipid layer did not perturb the surface monolayer. The size of the protein was ~105 nm (89–118 nm) (Supplementary data). Experiments conducted with melitin revealed total disruption of the lipid monolayer, with small patches of lipid detected after melitin adsorption.

4. Discussion

Common methods employed for the analysis of protein–lipid interactions include (i) ultracentrifugation or size filtration chromatography of vesicles exposed to the lipid binding protein (ii) thin layer chromatography (iii) circular dichroism (CD) spectroscopy and (iv) surface plasmon resonance (SPR) [17,19,25]. Chromatography and ultracentrifugation require several hours; during this period proteins can continue to aggregate complicating data interpretation. CD spectroscopy reveals conformational changes to the protein which are induced upon interactions with lipids [17]; however, quantitative analysis can be difficult. Lipid binding affinities can be assessed by employing SPR, however, isolating the specific oligomers which interact with the lipid membrane cannot be undertaken. As a consequence of these limitations, we developed a novel SELDI-TOF MS assay as a tool for determining the lipid binding profiles of Wt α-synuclein, A53T and A30P with the aim of identifying the key species involved in membrane binding/disruption. During this assay vesicles are coated onto H50 ProteinChip arrays via C8 functional groups creating a supported lipid monolayer. Proteins are subsequently applied to the array for 5 min followed by washing to remove non-specifically bound components. Proteins retained on the chip surface are then resolved by TOF MS which displays the mass-to-charge value and signal intensities of the individual proteins. In contrast to conventional methodologies, this assay allows the rapid detection and relative quantitation of oligomers, which are bound specifically and directly to lipid membranes.

Employing the SELDI-TOF MS-lipid binding assay, we identified that the monomeric species of Wt α-synuclein and A53T bind with a similar ability to the lipid monolayer, whilst A30P binds weakly. This is in agreement with several studies, which have employed vesicle/lipid droplet binding assays combined with ultracentrifugation, sucrose gradient centrifugation

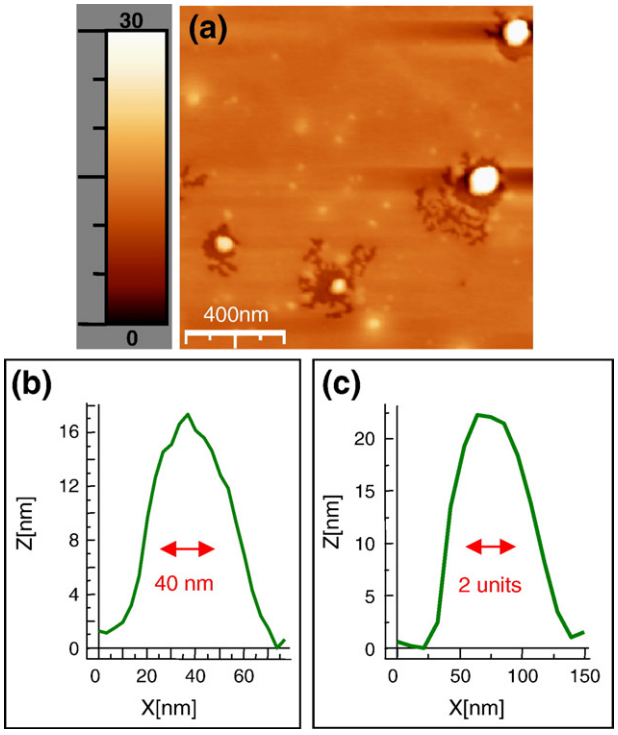

Fig. 7. AFM image of A30P binding to a lipid monolayer (panel a) and typical profiles of the associated proteins, showing the presence of monomers and oligomers (panels b and c, respectively).

or gel filtration, and demonstrated the A53T mutation does not affect lipid binding whilst A30P inhibits but does not completely eliminate binding [18–22,32,33]. Reduced binding of the A30P mutant could be explained by the fact that the proline substitution is in the helical-membrane binding domain, potentially creating a small break in the helix affecting the conformation and decreasing overall helicity [34].

Interestingly, the dimeric species of α-synuclein Wt, A53T and A30P all bound with a high relative affinity and were identified to preferentially associate with the lipid surface in comparison to the monomeric species. The role of the dimer in lipid binding has been previously examined by Cole et al. [22] who employed chemical cross linking studies of α-synuclein Wt, A53T and A30P and revealed that principally dimers and trimers associated with lipid droplets and cell membranes. Further to this, Jo et al. [19] treated vesicles with α-synuclein Wt and A30P and revealed that the membrane bound fractions consisted only of dimers. The amount of Wt dimer present in the membrane was higher than the A30P, which is consistent with results herein. Recently, Smith et al. [35] utilised SPR to investigate the interaction of α-synuclein with lipid membranes as a function of its aggregation state. Interestingly, this analysis revealed the presence of a high affinity lipid binder which is an early component from the amyloid aggregation pathway. There has been some debate as to whether (i) the dimer is preferentially binding to the lipid or (ii) the monomeric form of α-synuclein binds to the membrane and subsequently forms dimers. To assess these possibilities Jo et al. [19] measured the α-helicity of fresh and aged α-synuclein upon binding to lipid vesicles, the fresh batch of α-synuclein contained low dimeric content, whilst the aged α-synuclein exhibited higher dimeric content. They identified that the aged sample exhibited higher α-helicity indicating the dimer is preferentially binding. Based on these findings we postulate that the dimer is in fact preferentially binding to the lipid surface.

Examination of the higher order oligomers revealed the presence of Wt and A53T trimers and tetramers on the lipid surface, however, no higher order A30P oligomers were detected. It is important to note that SELDI-TOF MS, like other MS techniques, exhibits lower sensitivity in the higher mass range hence the lack of higher order oligomers does not indicate their absence or failure to bind lipid monolayers, but is a reflection of the limitation of the assay under the described conditions.

AFM imaging analysis using *in situ* tapping mode was conducted to further assess the α-synuclein–lipid interaction. Lipid monolayers were assessed, in place of bilayers, to allow direct comparisons with the SELDI-TOF MS-lipid binding assay, which also employed supported lipid monolayers. It is, however, important to note that AFM is a qualitative approach hence the ratios of oligomer binding cannot be ascertained. AFM averages and images reported in this study were determined from at least 6 positions per sample; scanning probe microscopy techniques do not sample the entire surface. In contrast, SELDI is quantitative; scans are made across the entire spot (every 5th position) and data presented is an average of 65 transients. Employing AFM analysis, we demonstrated that Wt α-synuclein and familial mutants all possess the ability to perturb the lipid surface. Profile studies of proteins associated with the lipid layer revealed the presence of 'small features' consistent with the presence of monomers and those double in size, dimers. AFM studies using lipid bilayers as a model for cell membranes reported similar findings; Jo et al. [36] demonstrated that the association of α-synuclein Wt and A53T resulted in extensive lipid disruption. Further to this, Zhu et al. [13] demonstrated that both α-synuclein Wt monomers and protofibrils (oligomers) disrupt lipid layers, the latter of which causes more rapid disruption.

Interestingly, we noted a characteristic pattern of lipid disruption surrounding the protein on the surface. We cannot determine neither affirm at this stage if the protein is embedded in the lipid layer, but the AFM analysis shows the protein appeared to be pushing the lipid away in one direction, but not surrounding the entire protein as there was no (or relatively little) lipid disruption observed in the adjacent regions above the protein (Fig. 6b). Based on the analysis of the primary sequence, we postulated that the hydrophobic N-terminus buries into the monolayer disrupting the cohesive forces of the surrounding lipid layer, the positive residues in the N-terminus bind the negatively charged lipids [13], whilst the acidic C-terminus exhibits a charge repulsion with the negatively charged membrane surface which "pushes" the lipid away. Interestingly a study by Narayanan and Scarlata [37] demonstrated that changing the pH of the binding buffer actually abrogates this charge repulsion and leads to enhanced lipid binding.

In summary, we employed a novel SELDI-TOF MS-lipid binding assay and AFM to assess the lipid binding profiles of α-synuclein Wt, A53T and A30P. SELDI analysis demonstrated that the dimeric species of α-synuclein Wt and its familial mutants preferentially bind membranes. AFM analysis revealed Wt and its familial mutants can penetrate lipid membranes or disrupt the lipid and bind directly to the SAM used to form the lipid layer. The profile of these studied proteins revealed the presence of 'small features' consistent with the presence of monomers and those double in size, dimers. The information generated from this study will aid to elucidate the role of α-synuclein in PD pathogenesis, which may provide novel therapeutic targets.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamem.2008.01.012.

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