



Cite this: *Biomater. Sci.*, 2019, 7, 3204

## Immobilization approaches can affect protein dynamics: a surface-enhanced infrared spectroscopic study on lipid–protein interactions†

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The intrinsically disordered Parkinson disease protein  $\alpha$ -synuclein ( $\alpha$ S) performs conformational changes induced by intermolecular protein–protein as well as by protein–membrane interactions. Aggregation of  $\alpha$ S is a hallmark for the disease, however the role of the membrane in the aggregation process still needs to be clarified. We used a surface-enhanced infrared absorption (SEIRA) spectroscopic approach to investigate the effect of lipid interactions on  $\alpha$ S conformation. The near-field detection of SEIRA allows to study exclusively structural changes of immobilized  $\alpha$ S with the advantage that the supernatant remains undetected and thus does not interfere with the spectral read-out. self-assembled monolayer (SAMs) of mixed NHS-PEG-SH linker and MT(PEG)<sub>4</sub> spacer molecules were utilized to immobilize  $\alpha$ S. The linker/spacer composition of the SAM was adjusted to prevent  $\alpha$ S– $\alpha$ S interactions. Two different methods were applied for site-specific (C-terminal and N-terminal)  $\alpha$ S immobilization. The immobilized protein was then exposed to lipid vesicles and SEIRA difference spectra were recorded to monitor the  $\alpha$ S conformation over time. Irrespective of the used immobilization method,  $\alpha$ S tethering hindered lipid-induced conformational changes. The spectra also indicate that a fraction of the immobilized  $\alpha$ S eventually desorbs from the surface into the supernatant solution. Desorbed  $\alpha$ S performs conformational changes and formation of  $\beta$ -structured aggregates is observed upon interaction with either lipid vesicles or supplementary  $\alpha$ S. Our study demonstrates that  $\alpha$ S aggregates only when the protein is free in solution and that surface immobilization procedures, commonly used in many analytical applications, can change the dynamic behavior of proteins thereby affecting protein structure and function.

Received 27th January 2019,  
Accepted 13th May 2019

DOI: 10.1039/c9bm00140a  
rsc.li/biomaterials-science

## Introduction

The intrinsically disordered  $\alpha$ -synuclein ( $\alpha$ S) is a physiologically abundant protein. However, aggregates of this 140-amino-acid protein are major components of inclusions (Lewy bodies) found in the brain of patients who show symptoms of Parkinson's disease (PD). The presence of these  $\alpha$ S- and lipid-rich Lewy bodies suggests that aggregation of  $\alpha$ S is driven by interactions with membranes. It has been shown that  $\alpha$ S interacts with synaptic vesicles *in vivo* and its over-expression inhibits neurotransmitter release.<sup>1</sup> Moreover, several *in vitro* studies revealed that  $\alpha$ S interacts with lipids and membranes.<sup>2–6</sup> It was found that the N-terminus of  $\alpha$ S binds to negatively charged membranes thereby adopting  $\alpha$ -helical structure,<sup>3,4,5,7–9,10,11</sup> that  $\alpha$ S aggregates in presence

of membranes,<sup>4,9,12</sup> and that membrane interactions accelerate the formation of  $\beta$ -structured aggregates compared to protein aggregation in solution.<sup>9,13</sup> However, formation of aggregates and fibrils were also observed at high protein concentrations, even in the absence of membranes, due to enhanced  $\alpha$ S– $\alpha$ S intermolecular interactions.<sup>14,15</sup> Fourier transform infrared spectroscopy (FTIR) is a technique highly suitable to monitor conformational changes of amyloid proteins in aqueous solution and in interaction with lipids.<sup>14,16–19</sup> Most often, attenuated total reflection (ATR)-FTIR is applied for *in vitro* analysis of protein conformation.<sup>20–23</sup> With this technique, detection of the IR-active sample is feasible up to several hundred nanometers penetration depth ( $d_p$ ) of the IR evanescent field into the sample placed on the internal reflection element (IRE) of the ATR unit.<sup>16</sup> Recently, we demonstrated successful application of ATR-FTIR spectroscopy to study the interactions of  $\alpha$ S with solid supported lipid bilayers (SSLB) as biomimetic membranes.<sup>9</sup> A SSLB was formed on the IRE (also referred to as ATR crystal), and subsequent exposure of the SSLB to  $\alpha$ S in solution facilitated the investigation of  $\alpha$ S–membrane inter-

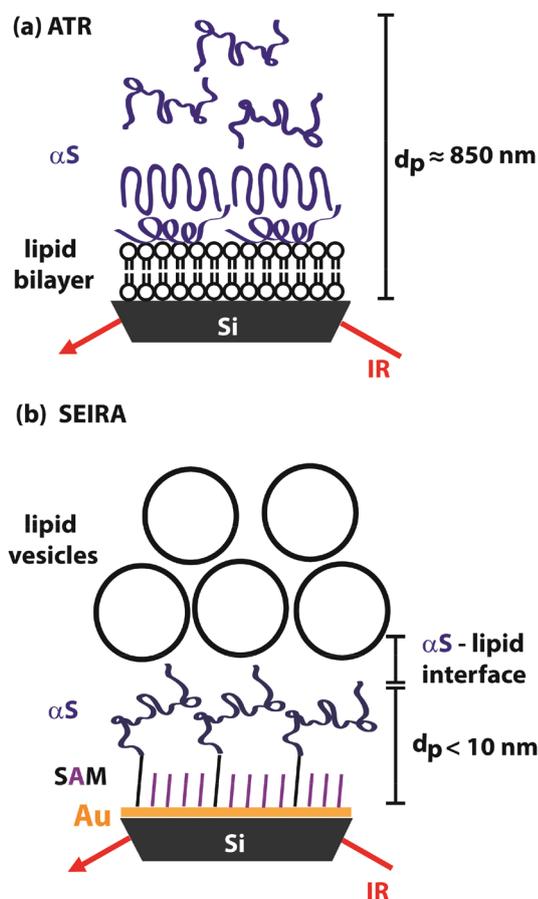
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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c9bm00140a



actions. Since we used a silicon (Si) ATR crystal ( $d_p \approx 850$  nm at  $1000$   $\text{cm}^{-1}$ ), the IR evanescent field detected the protein–membrane interactions at the interface, but in addition the protein–protein interactions in the supernatant (Fig. 1). Hence conclusive detection of conformational changes, induced solely by protein–lipid interactions, becomes illusive. Moreover, sedimentation of  $\alpha$ S on the lipid bilayer increases the protein concentration close to the SSLB, which in turn leads to more protein–protein interactions, and thus the  $\alpha$ S– $\alpha$ S interactions in the supernatant will contribute to the aggregation of  $\alpha$ S. One can speculate that under physiological conditions (low  $\alpha$ S concentration) conformational changes of  $\alpha$ S are mainly driven by the membrane, but this hypothesis is

difficult to examine with conventional ATR because the protein in the supernatant and the protein in direct lipid contact will both contribute to the IR absorbance signal. To investigate exclusively the contribution of lipid interaction to the aggregation process of  $\alpha$ S, intermolecular  $\alpha$ S– $\alpha$ S interactions have to be prevented. Therefore, we explored here a novel methodological approach, namely SEIRA detection combined with site-specific (N- or C-terminal) immobilization of  $\alpha$ S on a gold surface and subsequent exposure to lipid vesicles. The near-field effect of SEIRA offers the advantage to monitor solely the interactions between the immobilized  $\alpha$ S monomers and the membrane without interference from  $\alpha$ S in the supernatant solution (bulk  $\alpha$ S). As compared to conventional ATR, the penetration depth of the IR beam into the sample is considerably less for SEIRA ( $d_p < 10$  nm) and thus does not or only negligibly detect the supernatant solution. In addition, the signal is enhanced in the vicinity of the metal surface which allows to detect samples with low concentrations.<sup>24,25</sup> Lipid-induced conformational changes of immobilized  $\alpha$ S were probed by adding POPG lipid vesicles to the supernatant. It has been shown that the affinity of  $\alpha$ S to POPG vesicles is even higher compared to its affinity to lipid mixtures present under pathophysiological conditions<sup>26</sup> and thus POPG vesicles are well suited for our proof-of-principle study. The high sensitivity of the SEIRA technique assures the detection of slightest changes in the structure of the immobilized  $\alpha$ S monolayer induced by lipid interactions, while the lipid vesicles and the supernatant are not detected.



**Fig. 1** Schematic comparison of ATR and SEIRA experiments for investigation of  $\alpha$ S–membrane interactions. (a) ATR experiment with a lipid bilayer formed on the surface of a Si IRE, and addition of  $\alpha$ S in aqueous solution; a higher penetration depth ( $d_p$ ) of the evanescent IR field leads to simultaneous detection of protein–membrane interactions at the interface and  $\alpha$ S– $\alpha$ S interactions in the supernatant solution. (b) SEIRA experiment with  $\alpha$ S immobilized on a SAM of mixed PEG linkers (black) and spacers (violet). The lower penetration depth ( $d_p$ ) of the IR signal in the vicinity of the gold film results in the exclusive detection of the immobilized protein while the bulk solution is practically outside of the evanescent field. High surface sensitivity of SEIRA experiments allows for observation of slightest  $\alpha$ S conformational changes. Size scales and proportions are not accurate and do not represent the reality.

## Experimental section

### SEIRA experiments

SEIRA measurements were performed by utilizing a single reflection unit using a Si prism IRE as reported before.<sup>27</sup> A thin SEIRA-active film with gold nanostructures was deposited on a Si crystal by application of a deposition method described in details elsewhere.<sup>27–29</sup> In brief, the surface of the Si prism was polished and covered with 40% w/v  $\text{NH}_4\text{F}$  for 1 min to remove the residual oxide layer and to terminate the surface with hydrogen. Afterwards the Si prism was rinsed with water and tempered at  $60$   $^\circ\text{C}$  for 20 minutes. A 1 : 1 : 1 mixture of  $\text{NaAuCl}_4$  (0.03 M), and  $\text{Na}_2\text{SO}_3$  (0.3 M) +  $\text{Na}_2\text{S}_2\text{O}_3$  (0.1 M) +  $\text{NH}_4\text{Cl}$  (0.1 M), and HF (2% w/v) was deposited on the surface of the Si crystal for 1 minute. The crystal was rinsed with water. Possible contamination from plating was electrochemically removed by applying a dc voltage of +1.5 V between the gold thin film and a Pt counter electrode for 1 min in  $\text{H}_2\text{SO}_4$  (0.1 M). SEIRA measurements were performed with a Vertex 80v FTIR spectrometer (Bruker). IR spectra were recorded with 100 scans, a resolution of  $4$   $\text{cm}^{-1}$  and atmospheric compensation was applied. Fourier transformation was performed with a Mertz phase correction and a Blackman-Harris 3-term apodization. All SEIRA experiments were performed as difference measurements and thus are indicated as surface-enhanced infrared difference absorption (SEIDA) spectra.



### SEIDA spectra of $\alpha$ S in solution on a non-modified gold film

SEIDA spectra of  $\alpha$ S in solution ( $1 \text{ mg mL}^{-1}$ ) were monitored on a bare gold film in order to investigate the potential influence of the gold film on the vibrational modes. The spectra were used as reference for band assignment of the SEIDA spectra with immobilized  $\alpha$ S.

### Self-assembled monolayers (SAMs) for $\alpha$ S immobilization

One possibility to assure that  $\alpha$ S is in the vicinity of the SEIRA metal film is to tether the biomolecule to the surface. Immobilization can be achieved by modification of the gold layer with self-assembled monolayers (SAMs) of linker molecules.<sup>27,30–32</sup> SAMs are considered to provide a stable and reproducible approach for the immobilization of molecules. SAMs of polyethylene glycol (PEG) have been utilized in several studies to modify a surface for specific protein immobilization.<sup>31,33</sup> Thiol tail groups are suitable to link SAMs on gold films whereas functional head groups of SAMs can be applied for the selective immobilization of biomolecules.<sup>34–37</sup> In a previous SEIRA study, we have devised SAMs of mixed NHS-PEG-SH (10 kDa) (NHS: *N*-hydroxysuccinimide) and MT(PEG)<sub>4</sub> (methyl- and sulfhydryl-terminated PEG) for immobilization of poly-L-lysine (PLL) to gold.<sup>27</sup> In this work, we used SAMs of 5 kDa NHS-PEG-SH (Nanocs) and MT(PEG)<sub>4</sub> (Thermo Scientific), thereby we reduced the length of the NHS-PEG-SH linker molecules to  $\approx 30 \text{ nm}$  and facilitated protein immobilization closer to the gold surface leading to more enhancement of the absorbance signal. MT(PEG)<sub>4</sub> are only 1.58 nm long, and were applied as inactive spacers to passivate the gold surface against non-specific interactions between  $\alpha$ S and the gold film. In order to avoid uncontrolled  $\alpha$ S aggregation caused by intermolecular interactions among the immobilized  $\alpha$ S monomers, we reduced the number of NHS sites, available for protein immobilization, by adjusting the relative concentration of NHS-PEG-SH (linker) and MT(PEG)<sub>4</sub> (spacer). Increasing the concentration of the MT(PEG)<sub>4</sub> spacers in the mixed PEG SAM leads to an increased distance between the immobilized  $\alpha$ S monomers which in turn decreases intermolecular interactions. Formation of mixed SAMs succeeded by exposure of the gold film to a 1 mM aqueous solution of mixtures of NHS-PEG-SH and MT(PEG)<sub>4</sub> for  $\approx 24 \text{ h}$  with different linker:spacer compositions, *i.e.* 1:1, 1:10, and 1:100. Thus the SAM composition was adjusted by utilizing short spacers to passivate the gold surface and furthermore to create sufficient spacing among the longer linker molecules. The spacing prevented intermolecular interactions of the immobilized  $\alpha$ S and subsequent exposure to lipid vesicles facilitated the investigation of  $\alpha$ S conformational changes specifically induced by the lipids.

### Immobilization of $\alpha$ S

The SAM of mixed PEGs was modified for protein immobilization. We used human  $\alpha$ -synuclein recombinantly expressed in *Escherichia coli* and N-terminally tagged with histidine (Sigma-Aldrich). The protein was tethered at one of its two termini,

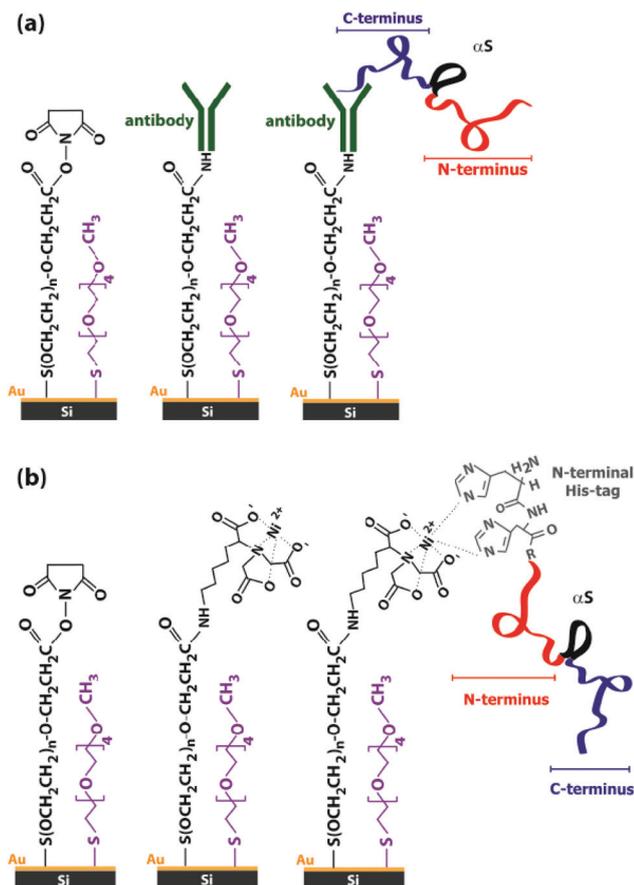
either by modification of the SAM with an  $\alpha$ S antibody (*anti*-synuclein- $\alpha$  antibody from rabbit, Sigma Aldrich) for C-terminal immobilization or with aminonitriilotriacetic acid (ANTA) for N-terminal tethering. Both procedures do not use covalent binding of  $\alpha$ S to the surface and should allow for less surface confinement.<sup>38</sup> For C-terminal immobilization, the SAM was exposed to an antibody solution ( $0.1 \text{ mg mL}^{-1}$  in PBS buffer with 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2). The NHS ester head group of NHS-PEG-SH linker reacts with the amino group of the antibody. A similar procedure has been reported before for the immobilization of the A $\beta$  peptide with an antibody, using a Germanium surface modified with triethoxysilanes and *N*-hydroxysuccinimidyl ester (NHS) linkers.<sup>22</sup> After the reaction, the supernatant was removed and the surface was rinsed substantially with H<sub>2</sub>O to remove remaining free antibody. The PEG SAM was then modified with covalently bound antibodies. A background spectrum was recorded. Afterwards,  $\alpha$ S ( $1 \text{ mg mL}^{-1}$ ) was added to the supernatant and SEIRA difference spectra (SEIDA spectra) were taken for 2 hours following the (non-covalent) immobilization process of  $\alpha$ S to the antibody. The surface was again rinsed with H<sub>2</sub>O to remove non-specifically adsorbed  $\alpha$ S and  $\alpha$ S in solution. SEIDA spectra were recorded over time to ensure the stability of the immobilized  $\alpha$ S. Fig. 2a sketches the different steps of  $\alpha$ S immobilization *via* the C-terminus.

N-Terminal immobilization of  $\alpha$ S was performed with an alternative and widely applied approach, namely affinity binding of the  $\alpha$ S histidine-tags to surfaces modified with aminonitriilotriacetic acid (ANTA).<sup>39,40</sup> A SAM of mixed PEGs was formed on the thin gold film. Afterwards the NHS-PEG-SH linker molecules were modified by exposure of the PEG SAM to ANTA as described in details elsewhere.<sup>39</sup> In brief, the PEG SAM was rinsed with water and consecutively with potassium carbonate buffer (500 mM, pH 9.8). Then 2 mM ANTA solution (500 mM potassium carbonate buffer, pH 9.8) was added. The SAM was modified with ANTA overnight. Afterwards, the surface was rinsed with H<sub>2</sub>O and the supernatant was exchanged with protein-binding buffer (50 mM Tris, pH 7.4, 100 mM NaCl, 1 mM NiCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>). A background spectrum was recorded,  $\alpha$ S ( $1 \text{ mg mL}^{-1}$ ) was added and the  $\alpha$ S immobilization process was monitored with SEIDA spectra. Fig. 2b schematically depicts the steps of  $\alpha$ S immobilization *via* the N-terminus.

### Addition of lipid vesicles

To investigate the interactions of immobilized  $\alpha$ S with the membrane, the protein was exposed to small unilamellar vesicles (SUVs) of POPG (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphorac-(1'-glycerol)) and SEIDA were recorded over time. POPG lipids (Avanti Polar Lipids) were used without further processing. The desired amount was taken from the lipid stock solution to obtain a vesicle concentration of  $2.5 \text{ mg mL}^{-1}$ . Chloroform was removed by placing the sample under a gentle stream of nitrogen for about 10 minutes. The resulting lipid film was placed in a vacuum chamber for two hours, resuspended in 1 mL Tris-HCl buffer (10 mM, pH 7.4), mixed





**Fig. 2** Immobilization of  $\alpha$ S to a NHS-PEG-SH:MT(PEG)<sub>4</sub> SAM on a gold surface. Two different methods were applied for affinity-based, non-covalent immobilization of  $\alpha$ S (a) C-terminal tethering of  $\alpha$ S to an antibody modified SAM. (b) N-Terminal tethering of  $\alpha$ S via His-tags to an ANTA modified SAM.

thoroughly, and incubated at room temperature for 20 minutes. The SUVs were prepared by extrusion with a hand-held extruder (Avanti Polar Lipids) through a filter-supported 30 nm polycarbonate membrane. When added to the supernatant, the lipid vesicles were close enough to interact with the immobilized  $\alpha$ S as we verified by the rise of lipid signals at  $\sim 2850\text{ cm}^{-1}$  and  $\sim 2930\text{ cm}^{-1}$  in the spectra.

### Control experiments with SEIRA and ATR

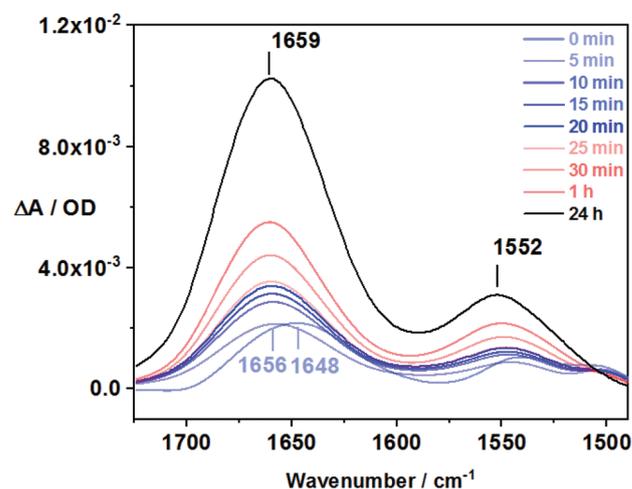
We performed SEIRA control experiments with supplementary  $\alpha$ S ( $1\text{ mg mL}^{-1}$  in Tris-HCl buffer) which was added to the supernatant. Supplementary  $\alpha$ S results in an increase of the local protein concentration in proximity of the immobilized  $\alpha$ S and thus enhanced protein-protein interactions. Spectra were recorded over time. As mentioned above, the near-field nature of SEIRA restricts the detection of conformational changes to the immobilized  $\alpha$ S monolayer, while the supernatant with  $\alpha$ S in solution remains practically undetected. Thus the effect of additional protein interactions on the immobilized monomers could be tracked. Furthermore, ATR-FTIR control measurements of the supernatant solution were performed with a

multi-reflection silicon ATR-cell (Bio-ATR II with 7–9 reflections, Bruker) and a Vertex 70v FTIR spectrometer (Bruker) equipped with a mercury cadmium telluride (MCT) detector. IR spectra were recorded with 32 scans and a resolution of  $4\text{ cm}^{-1}$ . Atmospheric compensation was applied to the spectra. The supernatant was removed after each SEIRA experiment and placed on the ATR crystal. Depending on the experiment, the supernatant comprised desorbed  $\alpha$ S together with POPG SUVs or desorbed  $\alpha$ S together with supplementary  $\alpha$ S. The ATR control measurements provided insights into conformational changes and aggregation behavior of  $\alpha$ S in the supernatant solution not detectable by SEIRA.

## Results and discussion

### Effect of the gold surface

Protein immobilization on gold surfaces might influence IR band frequencies when SEIRA spectra are compared to conventional ATR measurements.<sup>27,41</sup> Thus we used SEIRA spectra of  $\alpha$ S in solution placed directly on a non-modified gold film (Fig. 3) as reference for our studies with immobilized  $\alpha$ S. A spectrum of H<sub>2</sub>O was recorded before addition of the protein solution. Difference spectra were taken and show the presence of the protein by the rise of the amide I ( $1648\text{ cm}^{-1}$ – $1659\text{ cm}^{-1}$ ) and amide II ( $\sim 1550\text{ cm}^{-1}$ ) bands. The amide I band at  $1648\text{ cm}^{-1}$  (Fig. 3, 0 min) indicates a disordered conformation of  $\alpha$ S in solution at the beginning of the experiment. Protein sedimentation on the gold surface and possible formation of  $\alpha$ S multilayers leads to an intensity increase and a shift of the amide I band to  $1656\text{ cm}^{-1}$  within a few minutes



**Fig. 3** SEIRA spectra of  $\alpha$ S in aqueous solution on a non-modified bare gold film. Upon exposure to a gold layer, the spectrum shows a broad amide I band with a peak at  $1648\text{ cm}^{-1}$  tentatively assigned to the disordered structure of  $\alpha$ S. The spectrum was recorded a few seconds after  $\alpha$ S addition and is depicted as 0 min. The amide I peak shifts to  $1656\text{ cm}^{-1}$  and finally to  $1659\text{ cm}^{-1}$ . This shift indicates  $\alpha$ S conformational changes from a disordered structure to a predominantly  $\alpha$ -helical structure over time.



(Fig. 1, 5 min) and to  $1659\text{ cm}^{-1}$  after hours. The band shift indicates that  $\alpha$ S in solution performs a conformational change and adopts a more  $\alpha$ -helical structure on the gold surface over time. The significant influence of surface interaction on protein conformation becomes obvious. It is most likely so prominent for  $\alpha$ S, because the intrinsically disordered structure maximizes the available contact surface.

### Optimization of SAM composition with linker and spacer molecules

SAMs of mixed PEGs were utilized for a controlled  $\alpha$ S immobilization on the gold film.<sup>27,38,42</sup> Our objective was to hinder intermolecular interactions among the immobilized  $\alpha$ S monomers. Intuitively, lower relative concentration of linker molecule NHS-PEG-SH compared to the unreactive MT(PEG)<sub>4</sub> spacer leads to less available binding sites for  $\alpha$ S. We performed experiments with mixed SAMs and varied the linker: spacer compositions, 1:1, 1:10, and 1:100. Our experiments show that a lower linker concentration leads to immobilization of fewer  $\alpha$ S molecules on the SAM as depicted exemplarily by Fig. S1, ESI†. This is manifested by the reduced intensity of the amide I and amide II bands of the  $\alpha$ S immobilized to the NHS-PEG-SH:MT(PEG)<sub>4</sub> 1:100 SAM compared to the 1:1 SAM. SEIDA spectra demonstrate that a decrease of available binding sites reduces the protein binding. However, the SEIDA signal for  $\alpha$ S immobilized on 1:1 SAM (Fig. S1a†) is only ~3 times higher than for 1:100 SAM (Fig. S1b†). A possible explanation could be that the comparatively large sequence length of immobilized  $\alpha$ S monomers cover the SAM substantially, and block a fraction of available linker molecules as depicted schematically in Fig. S2.† Thus, not all available linker molecules can bind  $\alpha$ S monomers. As result from our experiments, we observe that the correlation between the number of available linker molecules and the effectively used immobilization sites is not linear. Also other effects might contribute to this nonlinearity, as the composition of the deposited SAM strongly depends on the surface affinity of the involved molecules and on the deposition time. Since  $\alpha$ S immobilization to a 1:100 SAM is stable and reproducible, and intermolecular  $\alpha$ S- $\alpha$ S interactions are reduced due to sufficient spacing between the immobilized  $\alpha$ S monomers, we have chosen the 1:100 mixed SAM as the default SAM composition for all measurements in this work.

### C-Terminal immobilized $\alpha$ S

For C-terminal immobilization, the 1:100 SAM was first functionalized with an  $\alpha$ S C-terminal antibody as explained in the Experimental section. A SEIDA spectrum of the PEG SAM functionalized with the covalently bound antibody is shown in Fig. S3a (ESI†). Successful C-terminal immobilization of  $\alpha$ S was monitored in Fig. S4 (ESI†) and the amide I band ( $\approx 1652\text{ cm}^{-1}$ ) hints a predominant disordered protein for the immobilized  $\alpha$ S. The absorbance of the antibody does not contribute significantly to the total amide I signal of the immobilized protein (Fig. S5†). Membrane interactions were investigated by exposure of the immobilized protein to POPG vesicles,

and SEIDA spectra were recorded over 24 hours. As depicted in Fig. 4a, the interaction of POPG SUVs with immobilized  $\alpha$ S doesn't result in any significant spectral changes. Thus POPG vesicles did not induce any conformational changes to the  $\alpha$ S monolayer immobilized at the C-terminus, in contrast to  $\alpha$ S in solution where N-terminal conformational changes to  $\alpha$ -helical structure have been observed after interaction with POPG SUVs.<sup>4,9,26</sup> This observation is surprising since the immobilization at the C-terminus seems not to restrict the conformation and  $\alpha$ S remains disordered after C-terminal immobilization similar to  $\alpha$ S in solution. Thus the N-terminus should be accessible for vesicle interaction. The N-terminal amino acids 6–97 were reported to be involved in lipid interactions.<sup>26</sup> Since the C-terminal antibody utilizes amino acids 91–140 (immunogen range) for binding the protein, most of the N-terminal amino acid residues (1–90) remain accessible after immobiliz-

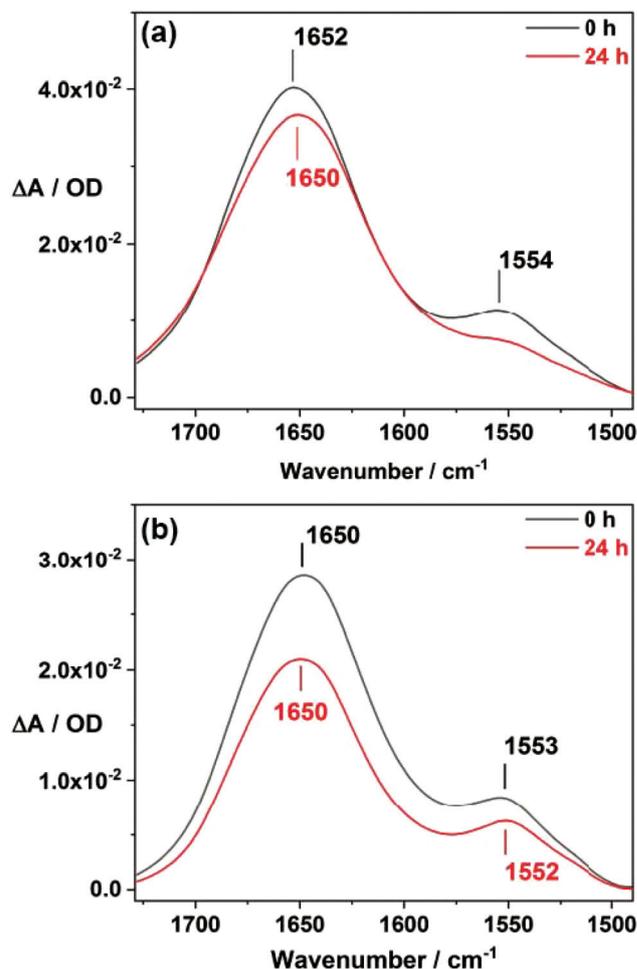


Fig. 4 Conformation of C-terminal immobilized  $\alpha$ S monitored by SEIDA. A decrease in amide band intensities indicates desorption of immobilized  $\alpha$ S from the surface over time. (a) Interaction with POPG SUVs. The disordered structure remains conserved for 24 h, lipid-induced conformational changes were not observed. (b) Effect of supplementary  $\alpha$ S. No conformational changes were detected upon increase of protein–protein interactions.



ation. One could hypothesize that direct interactions with the gold surface might have hindered conformational changes. However, we exclude  $\alpha$ S interactions with gold since the MT (PEG)<sub>4</sub> spacer molecules passivate the gold film and prevent protein interactions. The non-changing amide I band of the immobilized protein at  $\approx 1650\text{ cm}^{-1}$  indicates that  $\alpha$ S remains disordered even after long exposure to POPG SUVs (Fig. 4a). Thus, even carefully designed C-terminal immobilization of  $\alpha$ S seems to constrain the conformational dynamics required for membrane interaction.

Since the space between the monomers was adjusted to avoid protein interactions, immobilized  $\alpha$ S monomers are not expected to aggregate. In order to validate the effect of  $\alpha$ S- $\alpha$ S intermolecular interactions, supplementary  $\alpha$ S was added to the supernatant solution. An increase of intermolecular interactions in the vicinity of the immobilized protein was expected since  $\alpha$ S in solution sediments and interacts with the immobilized protein. SEIDA spectra were recorded to monitor this process (Fig. 4b). Again we have to emphasize that the immobilized  $\alpha$ S predominantly contributes to the SEIDA signal whereas  $\alpha$ S in the supernatant is detected only marginally. The spectra reveal that even increased intermolecular interactions do not trigger any significant conformational changes of the C-terminal immobilized  $\alpha$ S. The amide I band at  $\approx 1650\text{ cm}^{-1}$  hints that the immobilized  $\alpha$ S remains disordered after several hours of interaction with supplementary  $\alpha$ S. The spectra of C-terminal immobilized  $\alpha$ S, before addition of either POPG vesicles or supplementary  $\alpha$ S in the supernatant solution, are shown Fig. S6† and do not change compared to the spectra measured directly after addition (Fig. 4, 0 h).

### N-Terminal immobilized $\alpha$ S

Immobilization of  $\alpha$ S *via* N-terminus was achieved by utilizing the reaction of N-terminal histidine tags of the  $\alpha$ S with the ANTA modified NHS-PEG-SH:MT(PEG)<sub>4</sub> 1:100 SAM. Fig. S7 (ESI†) shows the SEIDA spectra of N-terminal immobilized  $\alpha$ S. After protein immobilization, the surface was rinsed with H<sub>2</sub>O to remove unspecifically adsorbed protein. The peak of the absorbance spectra at  $1661\text{ cm}^{-1}$  shows a considerable shift to higher wavenumbers, compared to the C-terminal immobilized  $\alpha$ S. This indicates that  $\alpha$ S adopts a more  $\alpha$ -helical structure after N-terminal immobilization, similar to the observed  $\alpha$ -helical structure when  $\alpha$ S is adsorbed to a non-modified gold film. We conclude that not only the interaction with membranes, but also the interaction of the  $\alpha$ S N-terminus with surfaces can induce conformational changes.

N-Terminal immobilized  $\alpha$ S was exposed to POPG SUVs to study lipid-protein interaction. SEIDA spectra of the N-terminal immobilized  $\alpha$ S were recorded for 24 hours (Fig. 5a). A slight shift of the amide I absorbance peak to  $1663\text{ cm}^{-1}$  was observed. However, we cannot conclude that the immobilized protein performs significant conformational changes,  $\alpha$ S rather preserves the  $\alpha$ -helical structure after long interaction with POPG SUVs. Thus N-terminal immobilization seems also to hinder further conformational changes of  $\alpha$ S. Similar to the experiments for the C-terminal immobilized  $\alpha$ S,

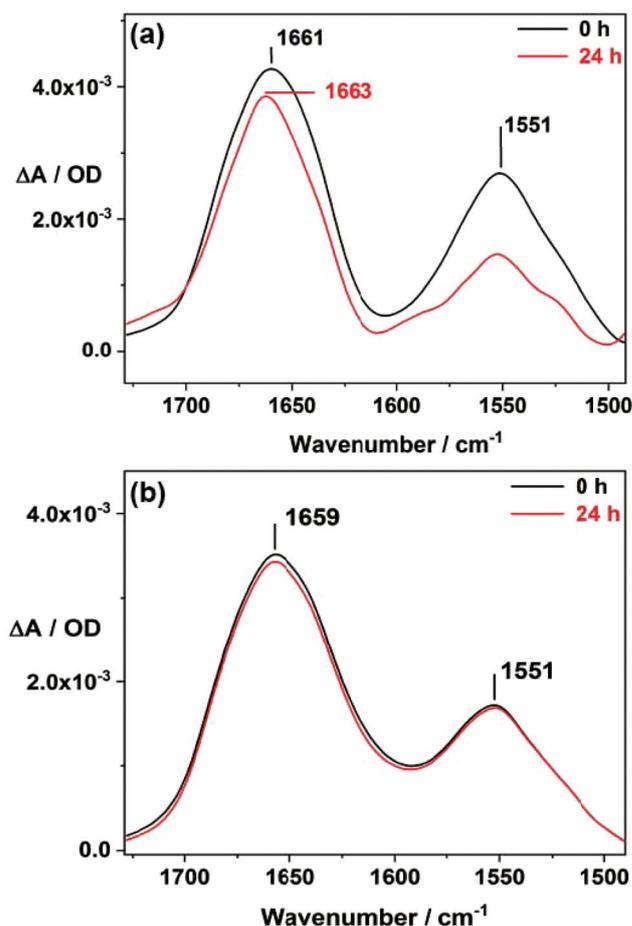


Fig. 5 (a) Conformation of N-terminal immobilized  $\alpha$ S monitored by SEIDA. Partial desorption of immobilized  $\alpha$ S is reflected by the decrease of amide band intensities over time. (a) Interaction with POPG SUVs. After immobilization,  $\alpha$ S adopts already a predominantly  $\alpha$ -helical structure, however lipid interactions do not trigger further conformational changes. (b) Effect of increased protein-protein interactions. A higher  $\alpha$ S concentration in the environment of the N-terminal immobilized protein does not lead to conformational changes.

supplementary  $\alpha$ S was added to the supernatant solution. Again, the aim was to increase the  $\alpha$ S concentration in proximity to the immobilized protein and thus to increase the intermolecular  $\alpha$ S- $\alpha$ S interactions. SEIDA spectra do not indicate any conformational changes for the N-terminal immobilized  $\alpha$ S (Fig. 5b). Hence, the reduced conformational degree of freedom is independent of the immobilization site and of the alternatively applied immobilization approach. Fig. S8† depicts the spectra of N-terminal immobilized  $\alpha$ S before addition of either POPG vesicles or supplementary  $\alpha$ S, and demonstrate again that the spectra do not change if measured directly after addition (Fig. 5, 0 h).

### Interactions of desorbed $\alpha$ S

Utilizing the protein's affinity to an antibody or His-tag for immobilization is not as stable as immobilization performed by covalent binding. Thus, a fraction of the immobilized  $\alpha$ S



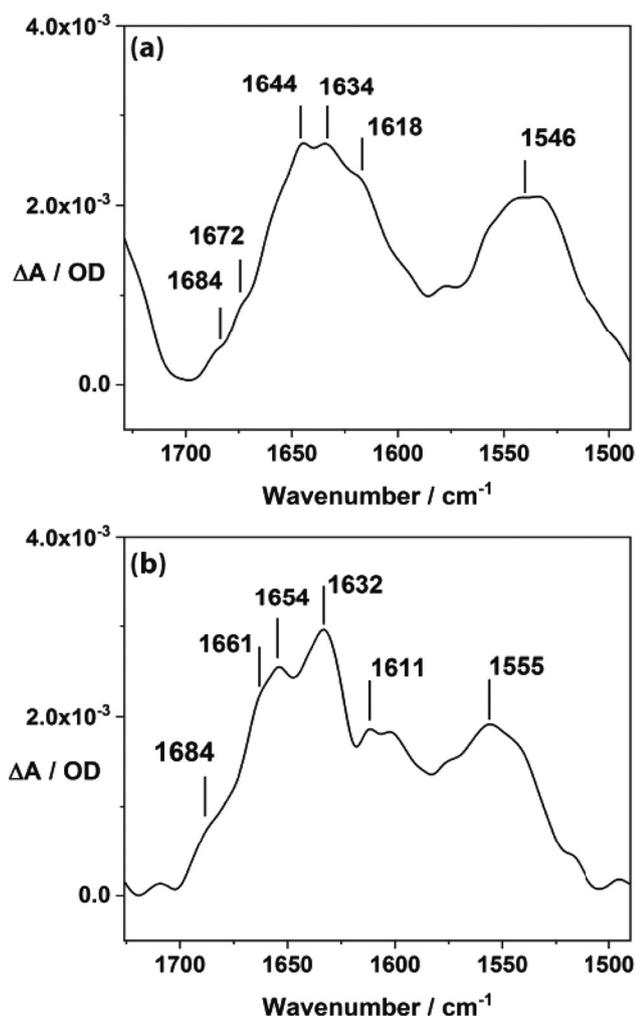
will finally desorb from the surface over the course of the experiment (24 h), as shown in Fig. 4 and 5 by the decrease in band intensities of the amide bands. As a result, the supernatant will comprise desorbed  $\alpha$ S besides POPG SUVs. Since our SEIDA experiment does not probe the supernatant, we analyzed it in a separate ATR-experiment. Therefore, at the end of the SEIDA experiments (after 24 h), 100  $\mu$ L of the supernatant were removed and subsequently used in an ATR (control) experiment. ATR-FTIR spectra of the supernatant solution are shown in Fig. 6 exemplarily for  $\alpha$ S desorbed from the C-terminal immobilized protein. It should be noted that IR band positions might be shifted when SEIRA- and ATR-spectra are compared because of the influence of the metal surface in an SEIRA experiment. However, it becomes obvious that the  $\alpha$ S conformation in the supernatant solution is different from that in the immobilized protein. Several amide I band com-

ponents occur in the ATR-spectra, in contrast to the SEIDA-spectra in Fig. 4 and 5. The various amide I components reflect the structural heterogeneity of the desorbed  $\alpha$ S in solution after long (>24 h) interaction with POPG vesicles. Amide I band components at 1618  $\text{cm}^{-1}$ , 1634  $\text{cm}^{-1}$ , 1672  $\text{cm}^{-1}$ , and 1684  $\text{cm}^{-1}$  hint that the desorbed  $\alpha$ S forms  $\beta$ -sheets and  $\beta$ -structured aggregates upon interaction with POPG SUVs. This is in agreement with several studies that demonstrate  $\alpha$ S aggregation upon interaction with lipids.<sup>27,43–46</sup> Thus C-terminal immobilization of  $\alpha$ S seems to inhibit lipid-induced protein conformational changes (Fig. 4a), while the desorbed “free”  $\alpha$ S adopts a conformational heterogeneous structure, even to  $\beta$ -structured aggregates (Fig. 5a).

We also probed the effect of increased protein–protein interactions on desorbed  $\alpha$ S. Therefore, supplementary  $\alpha$ S (instead of POPG vesicles) was added to the supernatant of the SEIDA experiment. Afterwards (24 h), 100  $\mu$ L of the supernatant was removed and subsequently analyzed by ATR measurements. The ATR-FTIR spectrum of the supernatant (Fig. 6b) reveals several amide I components at 1611  $\text{cm}^{-1}$ , 1632  $\text{cm}^{-1}$ , and 1684  $\text{cm}^{-1}$ , indicating that  $\alpha$ S in solution also forms  $\beta$ -structured aggregates. Thus our ATR experiments of the supernatant proved that  $\alpha$ S in solution performs conformational changes, with and without membrane interactions, in agreement with several other studies.<sup>8,9,14,44,45,47–50</sup> In contrast, the immobilization of  $\alpha$ S seems to prevent conformational dynamics. Reduced biological activity of covalently bound  $\alpha$ S due to surface confinement was reported before.<sup>38</sup> However, our SEIDA experiments clearly indicate that even non-covalent immobilization has a profound effect on the  $\alpha$ S properties. This is manifested by the hindered ability of the immobilized  $\alpha$ S to adopt  $\alpha$ -helical structure in interaction with POPG SUVs, as well as the hindered conformational changes to form  $\beta$ -structured aggregates after exposure to supplementary  $\alpha$ S. Only after desorption from the surface,  $\alpha$ S formed  $\beta$ -structured aggregates, even without membrane interactions and similar to “free”  $\alpha$ S in solution.

## Conclusion

Application of the SEIRA technique allowed us to differentiate the effects of membrane *versus* protein interactions on  $\alpha$ S aggregation. SEIRA is ideally suited because conformational changes of immobilized  $\alpha$ S after interaction with POPG vesicles can be monitored without interference of the supernatant. We have utilized two different and well-established methods for site-specific immobilization of  $\alpha$ S at both termini. Independent of which terminus of  $\alpha$ S was immobilized or which immobilization procedure was used, no conformational changes were induced, neither after membrane interaction nor after increased protein–protein interactions. The conformational degree of freedom seems to be constrained for immobilized  $\alpha$ S as compared to  $\alpha$ S in solution. However, complementary studies show that  $\alpha$ S aggregates when the protein is free in solution, both with and without membrane



**Fig. 6** ATR-FTIR measurements of desorbed  $\alpha$ S. (a) Interaction with POPG SUVs. (b) Effect of increased protein concentration. Both lipid–protein and protein–protein interactions induce conformational changes with a major fraction of  $\beta$ -sheets and  $\beta$ -structured aggregates. The supernatant solutions were taken from the SEIRA experiments after  $\approx$ 24 h.



interactions. Furthermore, we found that  $\alpha$ S adopts partially  $\alpha$ -helical structure already after N-terminal tethering and upon adsorption to an unmodified gold surface. Hence, conformational changes of  $\alpha$ S to  $\alpha$ -helical structure can also be triggered by surface interactions in general. Our results are of importance for biotechnological applications and protein assays that rely on similar immobilization methods. A thorough understanding of immobilization effects on protein structure and function is crucial for the design of protein *in vitro* studies.

## Conflicts of interest

There are no conflicts to declare.

## Acknowledgements

We gratefully acknowledge financial support by the Deutsche Forschungsgemeinschaft (SFB 969, A2 and SFB 1214, A3).

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