Analytical Technique of Spectroscopic Ellipsometry for Studying Protein-Protein Interaction

A. Nabok[1], V. Kriechbaumer[2], M.K. Mustafa[1], B.Abell[2], D.Smith[2], A.Tsargorodskaya[1]
[1] Materials and Engineering Research Institute,
[2] Biomedical Research Centre,
Sheffield Hallam University, Sheffield, S1 1WB, UK,
e-mail: a.nabok@shu.ac.uk

Abstract—**The method of total spectroscopic ellipsometry in its total internal reflection (TIRE) mode was utilized in this work for the study of protein-protein interaction. Two different systems were studied here: (i) the interaction of chaperones, such as heat shock proteins Hsp70 and Hsp81 with different chaperone receptors including the new one OEP61-TM; (ii) the aggregation of alpha-synuclein monomers and oligomers (involved in Parkinson's diseases) in phospholipid membranes. The method of TIRE provides unique quantified information on such protein interaction. Changes in the effective thickness of adsorbed molecular layer obtained by fitting the single TIRE spectra allows us to quantify the process of binding of chaperons to receptors or aggregation of alpha-synuclein in the lipid membrane. TIRE dynamic measurements allow monitoring protein interaction in-situ and evaluate the association (affinity) constants of such interaction. TIRE study revealed a clear separation between specific and non-specific interaction of proteins; such data were reported for the first time. A complementary method of atomic force microscopy (AFM) was used in this work to visualize the protein build-up on the surface.**

Keywords- ***chaperone, receptors, alpha-synuclein, spectroscopic ellipsometry, total internal reflection ellipsometry, electrostatic layer-by-layer deposition; AFM***

I. INTRODUCTION

This work is dedicated to the study protein-protein interaction, a problem which is far from being extensively studied and fully understood, as compared to more established fields of immune and enzyme reactions. Two examples are studied in our work: first, the interaction of chaperone proteins with respective receptors extracted from plants, and second, the study of aggregation of α-synuclein, a peptide involved in Parkinson's disease.

Chaperone proteins play an important role in cells protecting proteins from high temperatures and other cellular stresses, stabilizing protein structure and preventing them from aggregation and degradation. It was recently suggested that molecular chaperones, such as heat shock proteins Hsp70 and Hsp81, can form complexes with freshly translated proteins and thus prevent their aggregation [1]. Furthermore, the recent finding of chaperone receptors in plants [2] indicates more specific involvement of molecular chaperones in protein targeting. The study of the mechanisms of protein targeting may have a substantial impact in a number of applications including the origin of neurological diseases.

The problem of aggregation of α-synuclein in lipid membranes was identified as the main mechanism of Parkinson's disease development. These α-synuclein oligomers are thought to be the toxic entities responsible for bring about cell death, and similar oligomers have also been implicated in a range of other disease states. One of the possible mechanisms of α-synuclein build-up is the penetration of oligomeric α-synuclein into the membrane as opposed to resting on the surface. This is a novel mechanism that indicates cellular toxicity can be caused by punching holes through the membrane.

Physical methods such as total internal reflection ellipsometry (TIRE) can compliment traditional biological methods of studying protein-protein interaction and provide necessary quantification of such interactions. The method of (TIRE) was selected here because of its high sensitivity to molecular adsorption [3] and its recent successful applications in bio-sensing [4-6]. The results of TIRE study of interaction of chaperones (Hsp70 and Hsp81) with the novel plant chaperone receptor OEP61 are presented here for the first time. The method of atomic force microscopy was exploited in this work as a complimentary technique of visualizing protein aggregates on the surface; it was successfully implemented here for direct observation of α-synuclein oligomers in lipid membranes.

II. TEXPERIMENTAL METHODS AND SAMPLES PREPARATION

The experimental set-up for total internal reflection ellipsometry (TIRE), schematically shown in Fig. 1a, is based upon commercial M2000 J.A. Woollam spectroscopic ellipsometer with the addition of a 68^0 glass prism which provides the coupling of light into a thin metal (gold) film [3,4]. By its geometry, the method of TIRE closely resembles the technique of surface plasmon resonance (SPR) with one crucial difference of detecting two parameters of Ψ and Δ in comparison to only one parameter of reflected light

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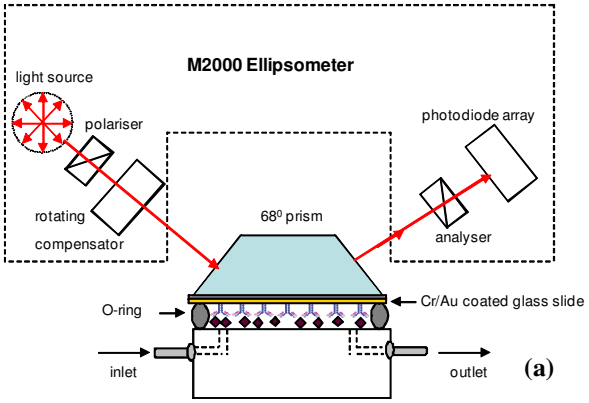
intensity in SPR. Parameters of Ψ and Δ can be defined, respectively, as the ratio of amplitudes and the phase shift between p- and s- components of polarized light:

$$tn\Psi = \frac{A_p}{A_s}, \ \Delta = \varphi_p - \varphi_s \qquad (1)$$



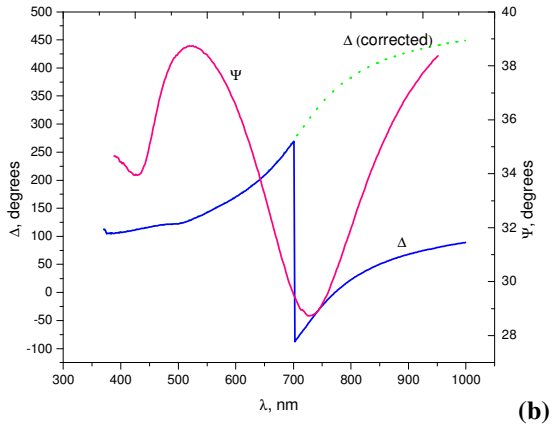


Fig. 1. (a) TIRE experimental set-up. (b) Typical TIRE spectra of Ψ and Δ measured on bare surface of gold. Dotted line shows corrected (by adding 360° to negative values) of Δ(λ) spectrum.

Fig. 1b shows typical spectra of Ψ and Δ; the spectrum of Ψ is very much similar to a traditional SPR curve, while the spectrum of Δ provides a new phase related quantity not available in the SPR method. The method of TIRE often called as "phase SPR" was proved to be much more (at least 10 times) more sensitive than traditional SPR [3].

Two types of TIRE measurements were performed: (i) Single spectroscopic scans which were carried out in the same Trizma-HCl buffer solution (pH 7.5) after completion every adsorption (binding) steps. These measurements were followed by fitting of TIRE spectra to the four-layer model and evaluation of the thickness of the molecular layer adsorbed on the surface of gold. The details and limitations of the fitting procedure were outlined in detail in our early publications, for example [3]; (ii) Dynamic spectral measurements during in which a number of Ψ and Δ spectra were recorded *in-situ* during all adsorption (or binding) stages after a certain time interval; then the time dependences of Ψ and Δ at a selected wavelength were presented and further analyzed for the purpose of studying the kinetics of adsorption and binding processes. Following the procedure outlined in detail in [4-6] the rates of adsorption r_a and de-sorption r_d were found, and the association K_A and affinity K_D constants were found as their ratio:

$$K_A(l/mol) = \frac{k_a}{k_d}, \ K_D(mol/l) = \frac{1}{K_A} = \frac{k_d}{k_a} \qquad (2)$$

The method of atomic force microscopy (AFM) in tapping mode was utilized here for the study of morphology of adsorbed molecular layers and evaluation of the surface roughness of the surface. Nanoscope IIIa instrument (from Digital Technology) was used in a tapping mode with the mean oscillation frequency of 300 kHz, and scanning rate of 1s. The radius of silicon "super sharp" tips (from Veeco) was about 2 nm.

The substrates for TIRE measurements were prepared by thermal evaporation of a Cr layer (3 nm) on microscopic glass slides followed by a Au layer of (25-27nm in thickness) without breaking the vacuum of about 10^{-6} Torr using BOC Edwards Auto-360 vacuum system. The presence of thin Cr layer improves the adhesion of gold to the glass. Depending on the application, Cr/Au coated slides were treated differently to achieve the best adhesion properties for deposition of different molecular layers.

For immobilization of chaperones receptors the technology of electrostatic layer-by-layer deposition [7] was utilized. In this case, in order to increase the negative surface charge on the surface, the gold layer was treated overnight in the 0.1 M solution of mercaptoethyl sodium sulfonate in methanol. Then the layer of polycations (polyallylamine hydrochloride or PAH) was adsorbed on the surface providing a positive charge required for electrostatic adsorption of chaperone receptors such as OEP61.

For deposition of natural chloroplast membranes containing chaperone receptors the method of Langmuir-Schaefer was used. The monolayers of chloroplast were formed on the water surface in the Langmuir trough (NIMA mini trough) and then compressing it to a surface pressure of 20mN/m. The layer of chloroplast was transferred onto silion and gold coated sample by touching the surface with a sample (method of horizontal lifting or Langmuir-Schaefer).

Finally, the samples for deposition of lipid membranes were prepared by treating gold coated samples in cystamine hydrochloride solution (0.1 M solution in methanol, overnight treatment) to increase the positive surface change.

The samples (solutions) of chaperone receptors (OEP61, HOP, HOP2A, TOC64 , chaperones (Hsp70 and Hsp81), α-synuclein in both monomer and oligomer forms, chloroplast membranes, and phospholipids were supplied by our collaborators from Biomedical Research Centre. The methodology of protein preparation was described earlier in detail.

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III. RESULTS AND DISCUSSON

A. *Interaction of chaperons with receptors immobilized electrostatically*

Typical results of TIRE single scans (e.g. Δ spectra) taken after each step of adsorption (binding) are shown in Fig. 2a. The calibration curve, e.g. the dependence of the thickness of adsorbed layer vs. the concentration of chaperones, which was obtained by fitting the Δ spectra are given Fig. 2b.

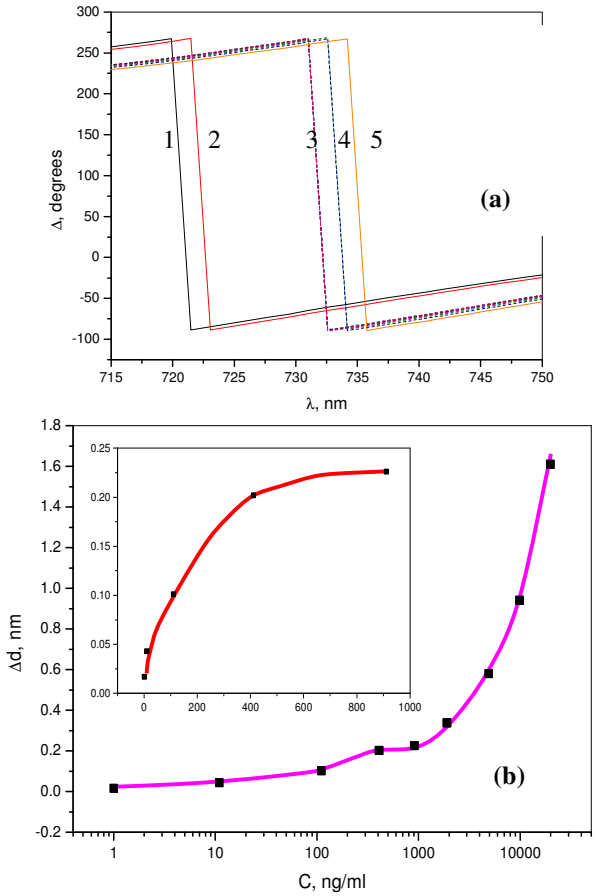


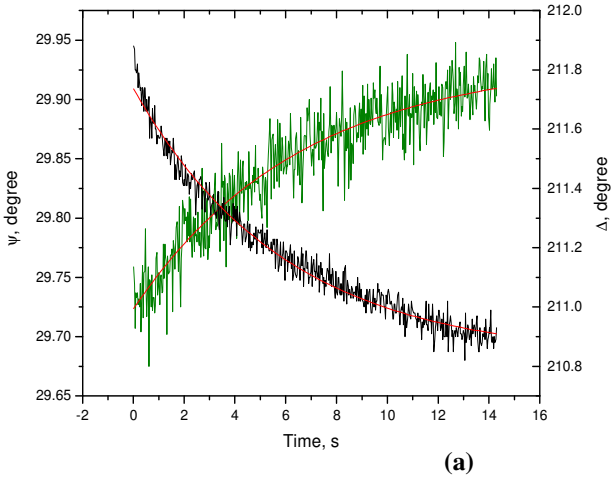
Fig. 2. (a) Typical set of Δ spectra for bare gold surface (1), after adsorption of PAH (2), OEP61 receptors (3) and binding of Hsp70 chaperones of different concentrations: 300 ng/ml (4), and 10µg/ml (5); (b) Time dependence of the thickness increment on the concentration of Hsp70 in solution. Zoomed-in section of the calibration curve at low concentration is given as inset in a linear scale.

The spectral shift in Fig. 2a corresponds to adsorption (or binding) of different molecular layers. Due to a limited resolution of the original Δ spectra, only spectra of two concentrations 300 ng/ml and 10µg/ml of Hsp70 are shown. The correction of Δ spectra allows increasing the resolution; the same effect can be achieved by TIRE data fitting. Typical calibration curve for binding Hsp70 chaperones to OEP61 specific receptors is given in Fig. 2b. As one can see, the effective thickness of adsorbed molecular layer increases and reaches saturation upon binding the chaperones in low concentrations (up to 1 µg/ml) to respective receptors. The maximal values of the response, summarized in Table 1, depend on the type of receptors and chaperones used. It is clear, that OEP61 receptor binds specifically Hsp70 but does not bind non-complementary chaperone Hsp81. At the same time the receptors HOP2A and TOC64 known to be specific to Hsp81 demonstrate binding behavior. However at large concentrations of chaperones exciding 1:1 ratio, a sharp increase of the response is observed for all receptor-chaperone pairs and is most likely caused by non- specific binding.

Table 1. The thickness increment (at saturation) and affinity constants for different pairs of chaperone-receptor.

Receptor	Chaperone	Low concentration		High concentrat.
		δd_{max} (nm)	K_A (l/Mol)	K_A (l/Mol)
OEP61	Hsp70	0.23	$(1.03\pm0.18) \times 10^9$	$(2.08 \pm 0.03) \times 10^6$
	Hsp81	0	n.a.	$(9.47 \pm 1.27) \times 10^4$
HOP2A	Hsp81	0.63	$(4.9 \pm 0.54) \times 10^8$	$(2.46 \pm 0.43) \times 10^4$
TOC64	Hsp81	0.45	$(7.56\pm1.86) \times 10^8$	$(8.34 \pm 0.95) \times 10^5$

The study of binding kinetics using dynamic TIRE measurements allowed the evaluation of the association constant K_A (see an example in Fig. 3). The values of K_A summarized in Table 1 for different receptor-chaperone pairs are typically in the range of 10^8-10^9 (l/mol) which is characteristic for specific receptor-chaperone interaction at low concentrations of chaperones.



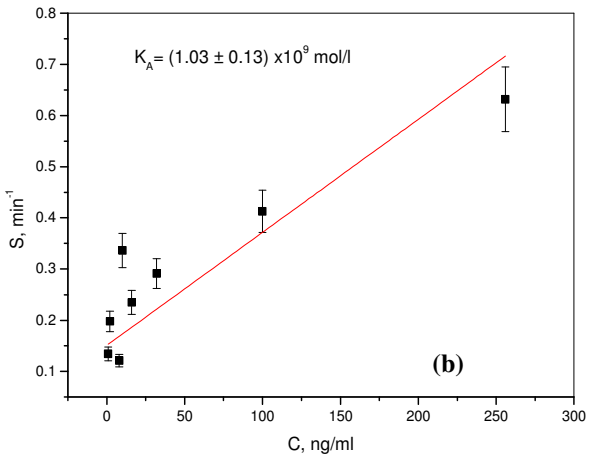


Fig. 3: (a) Typical time dependencies of Ψ and Δ during binding Hsp70 chaperone to OEP61 receptor; (b) Evaluation of the association constant (K_A)

At large concentrations of chaperones, however, much lower values of K_A in the range of 10^4-10^6 (l/mol) were found confirming the earlier suggestion of non-specific interaction. The above quantitative analysis demonstrates a clear separation of specific and non-specific binding.

B. *Interaction of chaperones with receptors naturally present in chloroplast membranes*

Similar type of TIRE measurements and data analysis were performed for binding chaperones to OEP61 receptors present in natural samples of chloroplast membranes. The results obtained are in line with those presented in the previous section. Binding of Hsp70 at low concentrations from 1ng/ml up to 1μg/ml yields the thickness increase reaching saturation at the level of 0.35 to 0.47nm. At larger concentrations (from 5 to 500 μg/ml) however, the thickness increases sharply again which indicate non-specific binding of chaperones. The study of TIRE kinetics also revealed a huge difference in K_A values (from $8.5 \cdot 10^8$ to 10^9 (l/mol) at low concentrations of chaperones and those in the range of 10^5 to $3 \cdot 10^5$ (l/mol) at high concentrations. Such 4 orders of magnitude difference gives clear separation between specific and non-specific binding of Hsp70 chaperons to OEP61 receptors in chloroplast membranes.

It is interesting that to note that blocking OEP61 receptors with specific antibodies diminish binding of low concentrations of Hsp70 chaperones to almost zero values; the association constant can not be calculated in this case. However, at high concentrations of Hsp70 chaperones non-specific binding appeared again yielding the K_A values of about $8.5 \cdot 10^4$ (l/mol) typical for such processes.

Typical AFM image of a sample of chloroplast membrane after binding Hsp70 chaperones in Fig. 4 shows clearly the aggregates with the height of about 6 nm and horizontal dimensions of about 25 nm. At the same time, AFM images of bare chloroplast membranes revealed rather flat and mostly featureless surface with the mean roughness of about 0.5nm. Considering the enlargement of horizontal features due to a finite tip radius of 2-4 nm, these aggregates could correspond to chaperone molecules of on the surface of a sample.

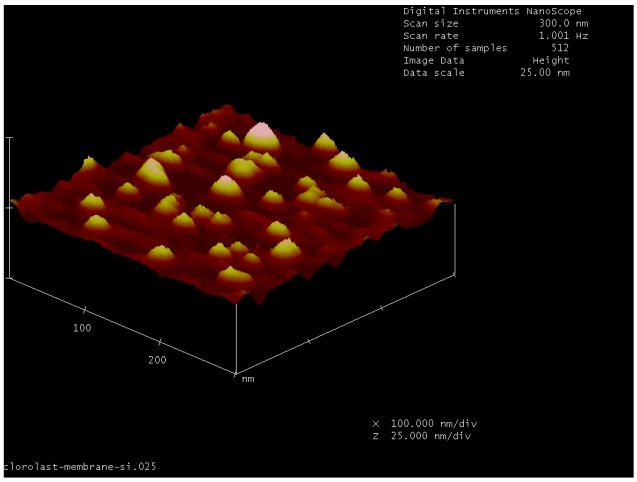
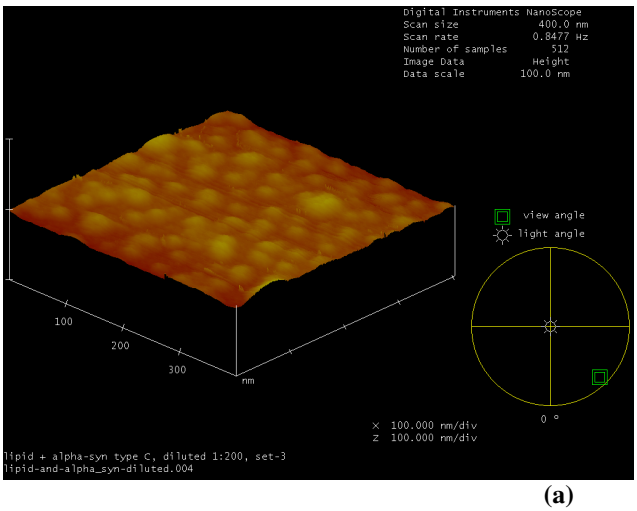


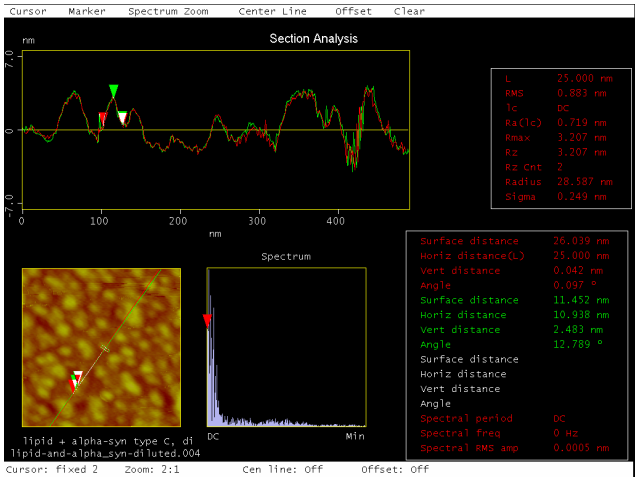
Fig. 4. Typical AFM tapping mode image of chloroplast membrane on silicon surface after adsorption of Hsp70 chaperones.

C. *The study of aggregation of α-synuclein in lipid membranes.*

Preliminary study of the surface morphology of α-synuclein oligomers adsorbed on the surface of lipid membranes deposited on mica was carried out using AFM. Fig. 5 shows typical AFM images of a lipid layer containing α-synuclein. The features with the horizontal dimensions of 20-30nm may correspond to α-synuclein aggregates or oligomers containing 5 to 6 monomer molecules.



(a)



(b)

Fig. 5. Tapping mode AFM images of a lipid layer containing α-synuclein oligomers on mica support: (a) pseudo-3D image; (b) section analysis showing horizontal and vertical dimensions of features along the selected line.

Since the height of these features is in the range of 2 to 3 nm, it can be concluded that α-synuclein oligomers are embedded in the lipid membrane. The roughness analysis of AFM images confirms the above conclusions. It showed that the surface of mica substrates is very flat with the mean roughness of 0.150nm. After deposition of a lipid layer mean roughness has increased to 0.224 nm; the presence of α-synuclein oligomers has resulted in further roughness increase to 1.266 nm.

Further study of α- synuclein monomers and oligomers on the surface of lipids using the methods of AFM and TIRE is currently underway.

IV. CONCLUSIONS AND FUTURE WORK

The optical analytical technique of total internal reflection ellipsometry has found another useful application in the study of protein-protein interaction. The obtained quantified information on the thickness of adsorbed molecular layers and the association constant of binding allows detailed characterization of a novel chaperone receptor extracted from plants in its abilityto bind specifically Hsp70 chaperone. The binding abilities of OEP61 receptors electrostaically adsorbed on the surface and those present naturally in chloroplast membranes were accessed and found to be similar. This confirms one more time that the method of electrostatic layer-by-layer deposition can be successfully used for immobilization of proteins, and elecrostatically immobilized proteins (chaperones this time) retain their functionality. A clear separation of specific and non-specific interactions of proteins was demonstrated in the work for the first time.

A complementary AFM study revealed the presence of proteins (chaperones) on the surface. An important finding of embedding of α-synuclein oligomers into the lipid membrane was confirmed with AFM. Further study of the mechanism of α-synuclein aggregation in lipid membranes using methods of AFM and TIRE is currently underway. This work may shed light into the origin of Parkinson's other neurological diseases.

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