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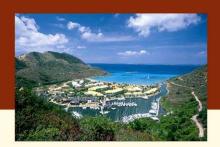




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Ellipsometric Immunosensor for Detection of Amyloid Precursor Protein with a View of Alzheimer's Disease Diagnostics

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Abstract: The detection of amyloid precursor protein isoform 770 (APP770) was achieved with the use of total internal reflection ellipsometry (TIRE) in a direct immunoassay format with DE2 monoclonal antibodies raised against the β amyloid peptide 1-16 (Aβ 1-16) which is a part of APP770. DE2 antibodies were immobilised on the surface of gold by electrostatic binding to a layer of (poly)allylamine hydrochloride (PAH) via an intermediate layer of Protein G molecules. TIRE spectra were recorded after adsorption (binding) of every molecular layer in a sequence of PAH, Protein G, DE2, and APP770. A noticeable increase in the adsorbed layer thickness was obtained upon binding of APP770 molecules from its solution of unknown concentration in Complete Medium, a complex mixture containing other proteins. For a purpose of TIRE biosensor calibration, complementary quartz crystal microbalance (QCM) measurements were utilised and allowed the evaluation of surface concentrations of DE2 and APP770 of 1.08·10¹¹ cm⁻² and 4.73·10¹² cm⁻², respectively. *Copyright* © 2010 IFSA.

Keywords: Alzheimer's disease, Amyloid precursor protein, Amyloid peptide, Direct immunoassay, Total internal reflection ellipsometry, Quartz crystal microbalance.

1. Introduction

One of the most noticeable pathological characteristics of Alzheimer's disease (AD) is the deposition of senile plaque in brain tissue [1]. The main constituent of the senile plaque is beta amyloid peptide

containing 39 to 42 amino-acids, denoted as A β (1-42) [2, 3]. It is believed that this peptide is cleaved from a large trans-membrane protein known as amyloid precursor protein (APP) due to disrupted enzymatic processes, although the presence of A β 1-42 was recently detected in healthy individuals [4, 5]. As shown in Fig. 1, APP consists of a single transmembrane spanning domain, a large extracellular N terminus and a short intracellular C terminus [6]. The A β region of APP corresponds to amino acids 11-15 of the transmembrane domain and 28 amino acids of the extracellular domain [6]. Alternative splicing of the APP gene gives rise to at least 10 protein isoforms. Three of the APP isoforms known to contain A β are APP770 (full length APP), APP751 (minus exon 8) and APP695 (minus exons 7 and 8).

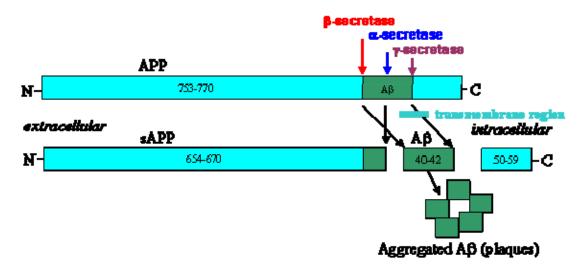


Fig. 1. Biochemistry of amyloid precursor protein.

The interest in all mentioned above APP isoforms as potential sources of $A\beta$ (1–42) is still great; and the development of a rapid method for detection of the above isoforms would be a step forward in AD research. The detection of $A\beta$ (1-42), an actual marker for AD, is a daunting task because of its relatively low molecular weight and poor solubility in water. That is why much larger and thus easier detectable molecules of APP seemed to be more suitable for this concept-proof work.

The detection of APP770 isoform was carried out in direct immunoassay with specific monoclonal antibodies DE2 raised against β amyloid peptide (1-16) [7]. An optical method of total internal reflection spectroscopy (TIRE) which was recently established as a very sensitive bioanalytical tool [8-13] was utilised in this work for detection the immune reaction between APP770 and DE2 antibodies immobilised on the surface of gold. Additional challenges lied in the use of stock solutions of DE2 and APP770 of unknown concentrations in complete medium (CM), a complex solution containing other proteins, amino-acids, salts, and vitamins. Therefore, complementary quartz crystal microbalance (QCM) measurements were performed in order to evaluate the concentrations of molecules DE2 and APP770 adsorbed on the surface and subsequently calibrate the TIRE method.

2. Methodology

2.1. Production of Monoclonal Antibodies (DE2) and Amyloid Precursor Protein (APP770)

Monoclonal antibody against β -amyloid peptide, were generated by immunization of BALB/C mice with a conjugate of synthetic β -amyloid (1-16) linked to keyhole limpet haemocyanin (KLH) with

glutaraldehyde following the procedure described in detail in [7]. The clone DE2 was selected on the basis of stable production of IgG_1 that recognised both synthetic β -amyloid (1-16) and natural APPs α derived from human cell lines by immunoblotting. Bulk DE2 was produced by culturing hybridoma cells in DMEM medium supplemented with 5 % fetal bovine serum to exhaustion.

The procedure of preparation of different APP isoforms was described in detail in [7]. A full-length cDNA for human APP770 was subcloned in pCIneo (Promega). The resultant plasmid was transfected into Chinese hamster ovary cells by the use of the calcium phosphate method. Transfected cells were selected by growth in medium containing 0.3 mg/ml geneticin (G418) and cloned by limiting dilution. Indiviual clones were assessed for APP section by immunoblotting 24hour-conditioned medium with DE2. The clone used in this paper (CHO.F5.E4) was grown in 5 % fetal bovine serum in DMEM at 37 0 C, 5 % CO₂ and the medium collected after 72 hours used as a source of APP770.

Both DE2 antibodies and APP770 isoform were kept in complete medium (CM) solution which provides the essential amino acids, salts and vitamins that required for functioning of the hybridoma and CHO.F5.E4 cells, respectively. The concentration of DE2 and APP770 were therefore unknown.

2.2. Sample Preparations for TIRE and QCM Measurements

The substrates for TIRE measurements were prepared by consecutive thermal evaporation of layers of chromium (Cr) of about 3 nm thick and gold (Au) - 25 nm on standard microscopic BK7 glass slides without breaking the vacuum of about 10⁻⁶ Torr, using the Edwards E306A evaporation unit. The presence of thin Cr layer improves the adhesion of Au layer to glass.

The method of electrostatic layer-by-layer deposition [14, 15] was used for immobilisation of proteins on the surface. Prior immobilisation, the surface of gold was modified with the layer of mercaptoethyl sodium sulfonate to enhance the negative surface change on the surface [16]. DE2 antibodies were electrostatically immobilised on the surface via the polycationic layer of poly(allylamine hydrochloride) (PAH) [14, 15]. An intermediate layer of Protein G molecules having a binding site to the second domain of the DE-2 antibodies was used to orient DE-2 with their Fab-fragments towards the solution; such procedure improved the sensitivity in about 3 times as compared to randomly adsorbed antibodies [15]. All the chemicals used (apart from DE2 and APP770), i.e. Tris-HCl buffer, ammonia buffer, protein G, PAH, peptides, were acquired from Sigma-Aldrich.

2.3. TIRE Method

The method of total internal reflection ellipsometry (TIRE) combines the advantages of highly sensitive spectroscopic ellipsometry with experimental conveniences of Kretschmann surface plasmon resonance (SPR) [16]. The TIRE experimental set-up in Fig. 2 (described earlier in [10-13]) was built on the basis of an automatic spectroscopic J. A. Woollam ellipsometer M2000 operating in the 370-1000 nm spectral range and exploiting the rotating compensator principle. The addition was a 68⁰ trapezoidal prism which allows coupling the light beam to a thin gold film deposited on the BK7 glass slide at the conditions close to total internal reflection (similarly to conventional Kretschmann SPR systems [17]). A cell of 0.2 ml in volume with the inlet and outlet was sealed against the gold coated slide via rubber O-ring to enable performing molecular adsorption and different biochemical reactions on the gold surface.

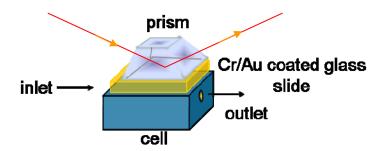


Fig. 2. The cell with the gold coated glass slide and prism.

In contrast to the conventional biosensing method of SPR based upon the monitoring of the intensity of reflected *p*-polarised light, the method of TIRE detects two parameters Ψ and Δ related, respectively, to the amplitude ratio (A_p/A_s) and the phase shift $(\varphi_p - \varphi_s)$ of *p*- and *s*- components of polarised light:

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

The sensitivity to molecular adsorption of Δ was shown to be about 10 times higher than that of Ψ [11], that is why $\Delta(\lambda)$ spectra were used in our study. Recording of TIRE spectra was performed in a standard Trisma/HCl buffer solution (pH 7.5) after completing each adsorption (or binding) stage.

2.4. QCM Measurements

AT-cut quartz crystals (from Euroquartz) with the nominal frequency of 10 MHz were used in this study. QCM experiments were carried out using a batch of 10 crystals. The adsorption sequence was the same as that described earlier in TIRE experiments, i.e. PAH, Protein G, DE2, and APP770 (stock solution). After each adsorption step the crystals were thoroughly rinsed in ammonia bicarbonate buffer and water in order to avoid any salt residues on the surface and then dried in a gentle steam of nitrogen gas. The frequency was recorded on dry crystals after completing each absorption stage. The added mass (Δ m) caused by adsorption of molecules was calculated from the frequency shift (Δ f) from a nominal frequency of the quartz crystal (f_0) at every adsorption (binding) stage using Sauerbrey equation [18]:

$$\Delta m \left(g / cm^2 \right) = \frac{\Delta f}{2.26 x 10^{-6} f_0^2} \tag{2}$$

3. Results and Discussion

3.1. TIRE Measurements

Fig. 3 shows typical set of TIRE $\Delta(\lambda)$ spectra recorded in a standard Tris/HCl buffer solution (pH 7.5) after completing every adsorption (binding) step in the following sequence: uncoated gold surface, PAH, Protein G, DE2 antibodies, APP770 in different concentrations starting from 1:16 diluted stock solution of APP.

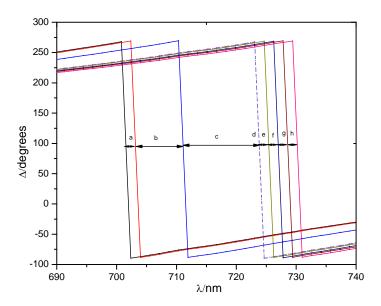


Fig. 3. A set of TIRE $\Delta(\lambda)$ spectra measured on bare gold surface (a); after adsorption of PAH (b); Protein G (c); antibodies DE2 (d); and after binding APP770 of different dilution: 1:16 (d); 1:8 (e);1:4 (f); 1:2 (g); 1:1 (h).

Parameters of the adsorbed layers such as thickness (d) and refractive index (n) can be evaluated by fitting TIRE spectra to the model system using the J. A. Woollam software [19]. A four-layer upside down model for TIRE measurements, shown in the Table 1, consists of BK7 glass (as an environment), Cr/Au layer, adsorbed layer, and water (as a substrate) [11].

Layer	Parameters	Comments
3. BK-7 glass	n, k dispersions from WVASE32 library;	fixed during fitting
(ambient)	<i>n</i> =1.515, <i>k</i> =0 at 633 nm	
2. Cr/Au film		The values of d, and dispersions
	$n = 0.359 \pm 0.078$; $k = 2.857 \pm 0.114$	for n and k were obtained by
	at 633 nm	fitting TIRE data for every new
	d is varied in the range of 25-28 nm	sample, then kept fixed in further
		fittings
1. Adsorbed	Cauchy model: A=1.396, B=0.01, C=0	parameters of Cauchy model A, B,
layer	giving $n = 1.42$, $k = 0$ at 633nm	and C are fixed; d - variable
0. Water	n, k dispersions from WVASE32 library,	fixed during fitting
(substrate)	n=1.33, k=0, at 633nm	

Table 1. The four-layer model for TIRE data fitting.

The known dispersion functions of refractive indices for BK7 glass and water were taken from WVASE32 library [19]. The effective parameters for Cr/Au (the thickness and complex refractive index dispersion) were obtained for every sample by the data fitting of TIRE spectra measured on bare gold surface and then used as fixed parameters for further TIRE data fitting of the same sample. The TIRE data fitting procedure was described in detail in our previous publications [11-13]. The Cauchy dispersion model for the adsorbed layer was used [19]:

$$n(\lambda) = A_n + \frac{B_n}{\lambda^2} + \frac{C_n}{\lambda^4}$$
 (3)

with the fixed parameters A_n =1.396, B_n =0.01, and C_n =0 yielding a value of refractive index n=1.42 (at 633 nm) for all the molecular adsorbed layers. This approach is not strictly correct but close to the real situation since all molecular layers used (polymers and proteins) have similar refractive index close to 1.42 [20]. The imaginary part of the refractive index (extinction coefficient) k=0 rightly assuming that the adsorbed layers are optically transparent in the selected spectral range of 370-1000 nm. In such conditions, all changes in the adsorbed layer are associated with the thickness. The obtained changes in the effective thickness of adsorbed molecular layers are summarized in Table 2. The values of thickness correlate with the size (or molecular weight) of adsorbed molecules as well as with their concentrations on the surface.

Adsorption stage	D (nm)	∆ d (nm)
PAH	0.462	0.462
Protein G	5.211	4.749
DE2	11.887	6.676
APP770 (1:16)	12.115	0.228
APP770 (1:8)	12.684	0.789
APP770 (1:4)	13.550	0.866
APP770 (1:2)	14.546	0.996
APP770 (1:1)	15.362	0.816

Table 2. The results of TIRE data fitting.

It is clear that the thickness increment (δd) increases from 0.462 nm for the smallest PAH molecules (molecular weight for repeated unit is 93.5, molecular weight of the polymer MW=70,000), to 4.749 nm for larger Protein G (MW=25,000), and up to 6.667 nm for large DE2 molecules (MW=120,000). Decreasing the concentration of APP770 leads to smaller response (e.g. thickness increase), and it is practically disappeared for 1:16 diluted APP770. The latter fact means that concentration of APP770 was rather small. Control TIRE measurements were carried out on adsorption of pure complete medium (CM) solution (e.g. not containing APP770) on top of immobilised DE2 antibodies. No noticeable spectral shift was detected in this test; the TIRE data fitting however revealed a tiny increase in the thickness of about 0.048 nm which can be considered as a noise level for this type of measurements.

The calibration of the TIRE biosensor was not possible at this stage because the concentration of APP770 in stock solution was not known. For the same reason TIRE dynamic spectral measurements could not be used for the evaluation of the association or/and affinity constants of the immune reaction between DE2 and APP770. Additional measurements were therefore required to solve the above problems.

3.2. QCM Measurements

Complementary measurements of the oscillation frequency were performed on dry quartz crystals coated sequentially with PAH, protein A, DE2, and APP770 (from stock solution) in order to evaluate the added mass caused by molecular adsorption. Typical variations of resonance frequency of quartz crystals at different stages of adsorption are shown in Fig. 4. The values of added mass $\Delta m (g/cm^2)$ calculated using Sauerbrey equation (2) and the molar concentration of adsorbed molecules $(M = \Delta m/MW)$ are given in Table 3.

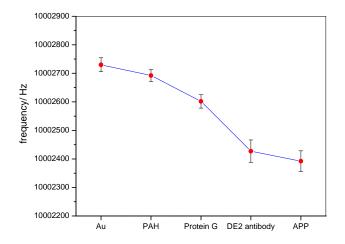


Fig. 4. Resonance frequency of quartz crystals after different stages of adsorption.

Table 3. QCM data on the sequential a	dsorption of PAH	I, Protein G, DE2, and APP770.
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Layer	Added mass	Molar concentration
	$\Delta m \ (\times 10^{-7} \ g/cm^2)$	$C(\times 10^{12} M)$
PAH	1.8229± 1.4488	2.4349 ± 1.3349
Protein G	3.2578 ± 0.8950	13.7823± 2.9740
DE2	7.4337 ± 2.4371	4.73 ± 0.86
APP770	1.0026 ± 0.7544	0.18±0.03

The surface concentrations of DE2 antibodies (*N*) and adsorbed APP770 molecules (*n*) can be found by multiplying the *M* values to Avogadro's number. The resulted values are $N = (4.73 \pm 0.86) \times 10^{12} \, \text{cm}^{-2}$ and $n = (1.08 \pm 0.18) \times 10^{11} \, \text{cm}^{-2}$. In general, molecular adsorption is described by a following differential equation [21]:

$$\frac{dn}{dt} = k_a (N - n)C - k_d n \tag{4}$$

where n and N are the concentrations of adsorbed molecules and binding sites, respectively, C is the concentration of analyte molecules in the environment, k_a and k_d are rates for adsorption and desorption, respectively. The solution of the above is given as [21]:

$$n = N \frac{k_a C}{k_a C + k_d} \left[1 - e^{-(k_a C + k_d)t} \right]$$
 (5)

In our case, N corresponds to the concentration of DE2 antibodies on the surface, n - to the APP770 molecules bound to DE2, and C is the concentration of APP770 in solution. Assuming that binding process was complete after an incubation time of 15-20 minutes (TIRE kinetics measurements provide an evidence of that) the maximal concentration of APP is therefore given by:

$$n = \frac{2N}{1 + \frac{k_d}{k_a C}} = \frac{2N}{1 + K_D / C} \tag{6}$$

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where $K_D = k_d / k_a$ is known as the affinity constant. Factor 2 in eq. (6) appeared because every antibody has two binding sites. Typically for monoclonal IgG-based antibodies such as DE2 the value of K_D is in the range of 10^{-7} (mol/l) or even smaller [22]. Therefore, taking the values of $N = 4.73 \times 10^{12} \, cm^{-2}$ and $n = 1.08 \times 10^{11} \, cm^{-2}$ from QCM measurements, we can estimate the concentration C_0 from eq. (6) for APP770 in stock solution as

$$C_0 = K_D \frac{n}{2N - n} = 1.15 \cdot 10^{-9} \, (mol \, / \, l) \tag{7}$$

This allowed the calibration of TIRE measurements by defining the concentration scale. The minimal concentration of APP770 detected with TIRE biosensor for this particular batch of bio-chemicals DE2/APP770 can be estimated as $C_0 / 16 = 72 \cdot 10^{-12} \, (mol / l) = 72 \, (pmol / l)$.

4. Conclusions

This work demonstrated a possibility of express detection of amyloid precursor protein APP770 in a complex complete medium solution, which typically required 15 minutes considering that all preliminary stages of immobilising DE2 antibodies on the surface and the calibration of the TIRE biosensor are completed. Yet the detection of APP770 was performed using non-expensive and label-free direct immunoassay format. The concentrations of DE2 antibodies and APP770 molecules on the surface were found using QCM measurements which allow the calibration of TIRE experimental data. The concentration of APP770 in CM was found to be quite low (1.15 nMol/l). All the factors and findings mentioned above made the method of TIRE very promising for Alzheimer's disease diagnostics. Further work is therefore required for thorough investigation of immune reactions of DE2 antibodies with β amyloid peptides of different length. These experiments are currently underway.

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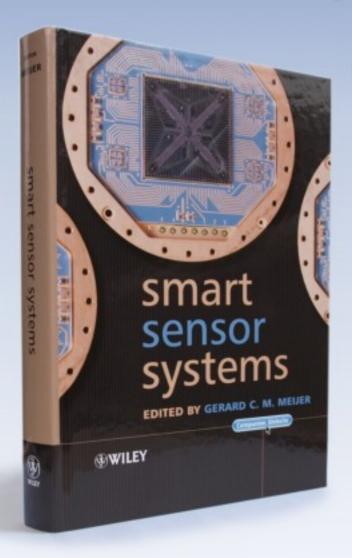
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