Accepted Manuscript

Title: Detection of β- amyloid peptide (1-16) and amyloid precursor protein (APP770) using spectroscopic ellipsometry and QCM techniques: a step forward towards Alzheimers disease diagnostics.

Authors: M.K. Mustafa, A. Nabok, D. Parkinson, I.E. Tothill, F. Salam, A. Tsargorodskaya

PII: S0956-5663(10)00417-3
Reference: BIOS 3923

To appear in: Biosensors and Bioelectronics

Received date: 19-4-2010
Revised date: 2-7-2010
Accepted date: 13-7-2010

Please cite this article as: Mustafa, M.K., Nabok, A., Parkinson, D., Tothill, I.E., Salam, F., Tsargorodskaya, A., Detection of β- amyloid peptide (1-16) and amyloid precursor protein (APP770) using spectroscopic ellipsometry and QCM techniques: a step forward towards Alzheimers disease diagnostics., Biosensors and Bioelectronics (2010), doi:10.1016/j.bios.2010.07.042

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.
Detection of $\beta$- amyloid peptide (1-16) and amyloid precursor protein (APP$_{770}$) using spectroscopic ellipsometry and QCM techniques: a step forward towards Alzheimers disease diagnostics.

M.K.Mustafa$^1$, A.Nabok$^1$, D.Parkinson$^2$, I.E.Tothill$^3$, F.Salam$^3$, A.Tsargorodskaya$^1$

1 Materials and Engineering Research Institute, Sheffield Hallam University, Howard Street, S1 1WB, UK
2 Biomedical Research Centre, Sheffield Hallam University, Howard Street, S1 1WB, UK
3 Cranfield Health, Cranfield University, Bedfordshire, MK43 0AL, UK

*Corresponding author, E-mail address: m.k.mustafa@shu.ac.uk, fax: +44 1142253501
Abstract
A highly sensitive method of spectroscopic ellipsometry in total internal reflection mode (TIRE) was exploited for detecting β- amyloid peptide (Aβ1-16) in the direct immune reaction with monoclonal DE2 antibodies (raised against Aβ1-16) electrostatically immobilised on the surface of gold. A rapid detection of Aβ1-16 in a wide range of concentrations from 5 μg/ml down to 0.05 ng/ml was achieved using a cost-effective and label-free direct immunoassay format. TIRE dynamic spectral measurements proved that the immune reaction between DE2 monoclonal antibodies and Aβ1-16 is highly specific with the affinity constant $K_D = 1.46 \cdot 10^{-8}$ (mol/l). The same DE2 antibodies were utilised for detection of amyloid precursor protein APP$_{770}$, a larger protein containing Aβ$_{1-16}$ domain, using the quartz crystal microbalance (QCM) measurements in liquid. A combination of QCM and TIRE kinetics results allowed the evaluation of the originally unknown concentration of APP$_{770}$ in complete medium solution containing other proteins, salts, and amino-acids.

Keywords: Alzheimer's disease, β-amyloid peptide 1-16, amyloid precursor protein (APP$_{770}$), direct immunoassay, total internal reflection ellipsometry, quartz crystal microbalance
1. Introduction

One of the major neuropathological hallmarks of Alzheimer’s disease (AD) is the deposition of β amyloid (Aβ) in the form of senile plaques (SPs) and often in the walls of cerebral and meningeal blood vessels. The central core of SPs consists of an insoluble deposit of 1-42 amino acid peptide known as β-Amyloid peptide or Aβ (Clark, 1985; Glenner, 1984). Until recently it was believed that aberrant processing gives rise to deposition of Aβ and hence AD, but it has recently been shown that Aβ is produced under normal circumstances in healthy cells in vitro (Haass, 1992; Hyman, 1992; Seubert, 1992) and is detected in the cerebrospinal fluid of healthy individuals (Seubert 1992; Ramakrishna, 1996). Aβ is derived from a larger transmembrane spanning protein known as the amyloid precursor protein (APP) the role of which is not clearly identified. APP consists of a single trans-membrane spanning domain, a large extracellular N terminus and a short intracellular C terminus (Kang et al., 1987). The Aβ region of APP corresponds to amino acids 11-15 of the transmembrane domain and 28 amino acids of the extracellular domain (Kang et al., 1987). Alternative splicing of the APP gene gives rise to at least 10 protein isoforms. Three of the APP isoforms known to contain Aβ are APP\textsubscript{770} (full length APP), APP\textsubscript{751} (minus exon 8) and APP\textsubscript{695} (minus exons 7 and 8).

Current clinical practice of AD diagnostics is based upon detection of Aβ\textsubscript{1–42} in ELISA sandwich immunoassay (Ibach, 2006), quite reliable but expensive and laborious procedure; such procedure is not suitable for early diagnostics of AD. Therefore, the development of express and cost-effective methods AD diagnostics (particularly at early stages of AD) is in great demand nowadays, considering ageing population in developed countries. Several attempts in this direction were made recently using different biosensing technologies of scanning tunneling microscopy (Kang, 2009), and surface plasmon resonance (SPR) (Hegnerova et al., 2009; Skerget, 2010; Haes et al., 2005). A well-established method of SPR has attracted great deal of attention because of its high sensitivity in bio-chemical analysis.

A relatively new method of total internal reflection ellipsometry (TIRE) (Westpal, 2002; Arwin, 2004), which is closely related to SPR but offers even higher sensitivity, was successfully implemented recently as an analytical tool in different bio-sensing
projects, for example for detection of low molecular weight toxins (Nabok et al., 2005, Nabok et al., 2007a, Nabok et al., 2007b, Nabok et al., 2008). The sensitivity of TIRE was sufficient for detection of mycotoxins and alkylphenols in sub-ng/ml concentrations in the direct immunoassay format.

Although Aβ_{1-42} is the actual marker for AD, the detection of a smaller water soluble peptide Aβ_{1-16} will be a step forward in the development of a rapid and cost-effective bioanalytical tool for AD diagnostics. The immunoassay with specific monoclonal DE2 antibodies raised against Aβ_{1-16} (Newton and Parkinson, 2006) was utilized in this work in conjunction with the method of TIRE. In addition, TIRE dynamic spectra measurements were performed to study the kinetics of the immune reaction and subsequent evaluation of the affinity constant.

The proposed method of detection (i.e. TIRE immunoassay with DE2 antibodies) was tested recently using much larger antigen molecules of amyloid precursor protein APP_{770} containing Aβ_{1-16} target domain (Nabok et al., 2010). The APP_{770} molecules in rather small concentrations (from few nmol/l down to tens of pmol/l) were detected in a complex solution, e.g. complete medium (CM). In this work QCM microfluidic measurements were performed to monitor in-situ the immune reaction between APP_{770} and DE2 antibodies in complete medium (CM), a complex mixture of different salts, amino-acids and proteins (other than APP). The latter factor constitutes an additional challenge and brings the procedure closer to a real biomedical analysis.

2. Experimental details
2.1 Samples preparation and immunoassay details

β-amyloid peptide 1-16 was synthesized by automatic solid phase peptide synthesis with standard Fmoc precursors on a PTI Symphony synthesizer. The cleaved peptide was purified by reverse-phase HPLC. Monoclonal antibody against β-amyloid peptide was produced by immunisation of mice with a conjugate of synthetic Aβ_{1-16} linked to keyhole limpet haemocyanin (KLH) with glutaraldehyde. The clone DE2 was selected on the basis of stable production of IgG₁ that recognised both synthetic Aβ_{1-16} and natural
APPsα derived from human cell lines by immunoblotting. Screening with peptides taken from within Aβ1-16 was used to show the epitope recognized by DE2 is within the sequence Aβ9-14. Bulk DE2 was produced by culturing hybridoma cells in DMEM medium supplemented with 5% fetal bovine serum to exhaustion (Campbell, Pearson and Parkinson, 1999).

A human APP770 expression vector was produced by reverse-transcriptase PCR of a cDNA library prepared from mRNA from HeLa cells. The full-length transcript was subcloned into a pCI-neo expression vector (Promega Corp.) which was used to transfect Chinese hamster ovary (CHO) cells by calcium phosphate precipitation. Transfected cell were selected by culturing in medium containing 0.3 mg/ml G418. G418-resistant cells were expanded into DMEM medium supplemented with 10% FBS and selected for APP770 secretion by immunoblotting with DE2. The technology of production of APP770 was described in detail in (Newton and Parkinson, 2006).

The substrates for TIRE measurements were prepared by consecutive thermal evaporation of chromium (3±1 nm) and gold (30±3 nm) layers on standard microscopic 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. Prior immobilisation of antibodies, the surface of gold was modified with the layer of mercaptoethyl sodium sulfonate to enhance the negative surface change on sensing surface (Surjaya et al., 2008). Similar treatment was used to modify the surface of gold electrodes of quartz resonators.

The method of electrostatic layer-by-layer deposition (Lvov and Decher, 1994; Nabok, 2005) was used for immobilisation of proteins on the surface. DE2 antibodies were electrostatically attached to the surface via the polycationic layer of poly(allylamine hydrochloride) (PAH, 2 mg/ml). An intermediate layer of Protein G (0.02 mg/ml) molecules at pH 7.5 (charge = -2.2) having a binding site to the second domain of IgG was used to orient DE2 with their Fab-fragments towards the solution; such procedure improved the sensitivity in about 3 times as compared to randomly adsorbed antibodies (Starodub et al., 2005). The structure of sensing layer is schematically shown in Figure 1 (lower inset). All the chemicals used (apart from DE2, Aβ1-16, and APP770), i.e. Triz-HCl buffer, protein G, PAH, were acquired from Sigma-Aldrich.
2.2 Experimental methods

The method of total internal reflection ellipsometry (TIRE) used in this work combines the advantages of highly sensitive spectroscopic ellipsometry with experimental conveniences of Kretschmann surface plasmon resonance (SPR) (Kretschmann, 1971). The TIRE experimental set-up in Figure 1 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 of 68° trapezoidal prisms allows coupling of the light beam into a thin gold film deposited on the BK7 glass slide at the conditions close to total internal reflection. 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 the injection of solutions into the cell and thus performing molecular adsorption and different biochemical reactions on the gold surface.

Figure 1

In contrast to the conventional biosensing analytical tool of SPR based upon monitoring the intensity of reflected $p$-polarised light, the method of TIRE detects two parameters $\Psi$ and $\Delta$ 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:

$$m\Psi = \frac{A_p}{A_s}, \quad \Delta = \varphi_p - \varphi_s .$$

In this work the $\Delta(\lambda)$ spectra were selected for further analyze because of superior sensitivity as compared to $\psi(\lambda)$ spectra (Nabok et al., 2005). Recording of single TIRE spectroscopic scans was performed in a standard Trisma/HCl buffer solution (pH 7.5) after completing each adsorption (or binding) step. The cell was rinsed between adsorption steps by purging the same buffer solution ten times of the cell volume.

Upper inset of Figure 1 shows typical of $\psi(\lambda)$ and $\Delta(\lambda)$ spectra recorded in such TIRE measurements. A phase drop of $\Delta$ from $270^0$ down to $-90^0$ is characteristic to the particular ellipsometric instrument used, while in theory $\Delta$ is varied between 0-360. The correction of negative values of $\Delta$ by adding 360$^0$ transforms $\Delta$ spectrum into a
monotonically increased curve (see inset Fig. 1) and eventually improve the resolution of TIRE measurements.

The kinetic of molecular adsorption (binding) has been monitored in-situ during the incubation period for each reagent using dynamic TIRE scans, i.e. recording a number of spectra after a certain time interval (typically 15 - 20 minutes). Then, the time dependencies of either $\Psi$ or $\Delta$ at a selected wavelength were extracted for the study of kinetics of molecular adsorption or binding.

The emphasis of the QCM measurements was to prove a possibility of detection of APP$_{770}$ in a complex CM solution in immune assay with monoclonal DE2 antibody specific to the A$\beta_{1-16}$ domain, a part of a large trans-membrane protein APP770. A fully automated QCMA-1 biosensor instrument and sensor chips from Sierra Sensors GmbH were used. QCMA-1 chips combine two sensing spots on the same AT-cut quartz crystal with a fundamental frequency of 19.5 MHz enabling in-situ monitoring of both the active and control sensor channels simultaneously. The operating temperature of the QCM sensor chip was maintained at 25ºC throughout the experiments.

The QCM chips were prepared following the same procedure as described above in TIRE sample preparation. Then the chip was docked to the QCMA-1 instrument and primed with running buffer (10 mM PBS, pH 7.4) at a flow rate of 50 μl min$^{-1}$. In the immobilization stage, PAH (10 mg/ml) was first injected using automated programme injection needle and was kept at both channels for 400 s. The same incubation time and flow rate has been used for subsequent immobilization of Protein G and DE2 antibody. DE2 antibody was not introduced in the control channel.

The response of QCMA-1 sensor to binding of APP$_{770}$ from its solution in CM was recorded simultaneously in both active and control channels for 300 s incubation time and 50 μl min$^{-1}$ flow rates. Every adsorption (binding) steps were ended with the surface regeneration using 0.1 M HCl.

3. Results and discussion

Figure 2 shows typical set of TIRE $\Delta(\lambda)$ spectra recorded in a standard Tris/HCl buffer solution (pH 7.5) after completing every adsorption (or binding) steps in the
following sequence: PAH, Protein G, DE2 antibodies and \( \beta A_{1-16} \) in different concentrations of 0.05 ng/ml, 0.5 ng/ml, 5.0 ng/ml, 50 ng/ml, 500 ng/ml and 5000 ng/ml. The \( \Delta(\lambda) \) spectra presented in this figure were corrected by adding 360° to the negative values, which allowed us to improve the sensitivity of detection: the small spectral shift (smaller than the wavelength increment), which is normally not resolved in original \( \Delta(\lambda) \) spectra, became visible after such alteration.

Figure 2

The parameters of the adsorbed layers such as thickness \( (d) \) and refractive index \( (n) \) were evaluated by fitting the TIRE spectra to the model system using the J.A. Woollam software (Woollam, 2002). A four-layer model for TIRE measurements (Nabok et al., 2008) shown in Table 1 consists of BK7 glass (as an environment), Cr/Au layer, adsorbed layer, and water (as a substrate) was employed.

Table 1

The dispersion functions of refractive indices for BK7 glass and water were taken from WVASE32 library (Woollam, 2002). The effective parameters for Cr/Au layer (the thickness and dispersion of a complex refractive index) were obtained for every sample by fitting the spectra recorded on bare gold surface; the obtained parameters were 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 (Nabok et al., 2007, 2008). The Cauchy dispersion model for the adsorbed layer was used (Woollam, 2002):

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

(2)

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 close to the real situation since all molecular layers used (polymers and proteins) have similar refractive index close to 1.42. The imaginary part of the refractive index (extinction coefficient) was fixed at zero \( (k=0) \) assuming that all molecular layers are optically
transparent in the selected spectral range of 370-1000 nm. In such conditions, all changes in the adsorbed layers are associated with the thickness. The obtained changes in the effective thickness of Aβ1-16 layer in respect to the layer of DE2 antibodies are summarized in supplementary Table S1. The thickness values (d) represent effective thickness of molecular layers adsorbed on the surface; these values together with the mean square error came as result of TIRE data fitting. The increase in the effective thickness (Δd) corresponds to the thickness increment caused by adsorption (binding) of respective molecules. Because the TIRE experiments on Aβ1-16 binding were carried out on the same sample (without surface regeneration) in the sequential increasing of Aβ1-16 concentration starting with the smallest concentration of 0.05 ng/ml, the accumulative concentration of Aβ1-16 was used in the Table S1 as well as in the calibration curve (Figure 3).

The obtained values of thickness correlate with the size (or molecular weight) of adsorbed molecules as well as with their concentrations on the surface. The thickness increment (Δd) increases from 0.756 nm for the smallest PAH molecules (molecular weight for repeated unit is 93.5, molecular weight of the polymer (MW=70 KDa), to 2.112 nm for Protein G (MW=25 KDa), and up to 8.637 nm for large DE2 molecules (MW=120 KDa).

The calibration curve in Figure 3, i.e. the dependence of the increase in the effective thickness of adsorbed layer (Cauchy layer) vs the accumulative concentration of Aβ1-16, appeared as a classical sigmoid curve typical for immune reactions. The linear range stretches from 2 to 500 ng/ml; and the trend to saturation is observed at concentrations higher than 5 μg/ml. The minimal detected concentration of Aβ1-16 was 0.05 ng/ml, which is an excellent result for direct immunoassay. Such high sensitivity of TIRE method is very promising for future diagnostics of AD.

Figure 3

Typical time dependencies of Ψ and Δ at a selected wavelength of 500ng/ml (shown as inset in Figure 4) were extracted from TIRE dynamic spectral measurements during binding of Aβ1-16 molecules to DE2 antibodies immobilised on the surface. Such kinetic
curves were recorded for different concentrations of the antigen (Aβ1-16). Typical kinetic curves for low concentrations of Aβ1-16 given in supplementary Figure S1 demonstrated that 20 min incubation time was sufficient to reach the saturation even at the lowest concentration of Aβ1-16 of 0.05 ng/ml. Then a well-developed procedure of the evaluation of the rates of adsorption (k_a) and desorption (k_d) of the immune reaction was applied (Liu, 2003; Nabok et al., 2007a, Nabok et al., 2007b, Nabok et al., 2008). The characteristic time constant (τ) of the immune reaction was evaluated by fitting the data to the exponential function, \( a \cdot \exp(-t / \tau) + b \). The inverse value of time constant, \( S = \frac{1}{\tau} = k_a C + k_d \) was plotted against the concentration of the antigen (C) in Figure 4, and the values of \( k_a \) and \( k_d \) were found, respectively, from the gradient and intercept of the linear graph. The values of \( k_a = 3.85 \cdot 10^5 (l \cdot mol^{-1} \cdot s^{-1}) \) and \( k_d = 5.64 \cdot 10^{-3} (s^{-1}) \) obtained from the graph in Figure 4 allowed the calculation of the association and affinity constants as \( K_A = \frac{k_a}{k_d} \) and \( K_D = \frac{1}{K_A} \), respectively. The obtained values of \( K_A = 6.83 \cdot 10^7 (l/mol) \) and \( K_D = 1.46 \cdot 10^{-3} (mol/l) \) are typical for highly specific immune reaction with monoclonal antibodies.

Figure 4

The immune reaction between APP770 and DE2 antibodies immobilised on the surface was studied with the QCM micro-fluidic method. Supplementary Figure S2 (a) shows frequency responses during adsorption of layers of PAH, Protein G and DE2 antibody in active channel. The optimal incubation time during these adsorption stages was 400 s. The effect of CM was tested separately, and the results are shown in supplementary Figure S2 (b). The comparison of responses to injection of CM in the active channel (coated with DE2) and the reference channel (without DE2) showed practically no difference. This proves the insignificance of non-specific binding of other proteins present in CM.

Figure 5 shows changes in the resonance frequency in the active channel of QCMA-1 for 2, 4, 8, and 16 times dilution of APP770 in CM. As one can see 300 s is sufficient time
to achieve the saturation of responses. The response is proportional to the concentration of APP\textsubscript{770} (MW = 115 kDa). The baseline was recorded during injection of pure PBS buffer solution.

The value of $K_D = 1.46 \cdot 10^{-4}$ (mol/l) obtained here for A\textsubscript{B}\textsubscript{1-16} allows the correction of our previously reported concentration of APP\textsubscript{770} in stock solution (Nabok et al, 2010). Assuming that the APP\textsubscript{770} (which contains A\textsubscript{B}\textsubscript{1-16} domain) have the same affinity to DE2 antibodies as A\textsubscript{B}\textsubscript{1-16}, and using the concentrations of DE2 ($N = 4.73 \cdot 10^{12}$ cm$^{-2}$) and APP\textsubscript{770} ($n = 1.08 \cdot 10^{11}$ cm$^{-2}$) from our previous work (Nabok et al, 2010), the initial concentration of APP\textsubscript{770} in stock solution in CM can be precisely calculated as $C_0 = \frac{n}{2N - n} \approx 1.7 \cdot 10^{-10}$ (mol/l). The minimal concentration of APP\textsubscript{770} detected in QCM experiments was therefore $C_0/16 = 1.05 \cdot 10^{-11}$ (mol/l). The results showed that our previous estimation (Nabok et al, 2010) was not far away from reality.

4. Conclusions

This work demonstrated the detection of A\textsubscript{B}\textsubscript{1-16} in concentrations down to 0.05 ng/ml using the method of TIRE in direct immune assay format with monoclonal DE2 antibodies. This approach provides the means of rapid, simple, label-free and low cost-effective detection of \textbeta\ amyloid peptide 1-16. The successful TIRE and QCM experiments with amyloid precursor protein APP\textsubscript{770} in a complex solution of CM give a good prognosis for further progress in detection of larger molecules of \textbeta\ amyloid peptide 1-42, the actual marker of Alzheimer's disease, in real biological samples.

Acknowledgements

The corresponding author wishes to thank the Universiti Tun Hussein Onn, Malaysia (UTHM) and Ministry of Higher Education, Malaysia (MoHE) for financial support of his PhD study at Sheffield Hallam University, UK.
References

Lvov Y., Decher G., 1994, Crystallography Reports, 39, 696-616
Nabok A, 2005, Artech House Inc, Boston,
Figure captions:

Figure 1  TIRE experimental setup. Upper inset shows typical $\psi(\lambda)$ and $\Delta(\lambda)$ spectra for uncoated Cr/Au layer recorded in TrisHCl buffer as well as corrected $\Delta(\lambda)$ spectrum; The structure of a sensing layer is given on lower inset.

Figure 2  Zoomed-in section of corrected $\Delta(\lambda)$ spectra recorded on of bare Au (1), after adsorption of PAH (2), Protein G (3), DE2 antibodies (4); and after consecutive binding of $\text{A} \beta_{1-16}$ of different concentrations: 0.05ng/ml (5), 0.5ng/ml (6) 5 ng/ml(7) 50 ng/ml (8), 500 ng/ml (9), and 5 μg/ml (10).

Figure 3  Calibration curve for β-amyloid peptide 1-16.

Figure 4  The evaluation of the rates of adsorption ($k_a$) and desorption ($k_d$) from the kinetics of binding of $\text{A} \beta_{1-16}$ to DE2 antibody. Inset shows typical binding kinetics of $\Psi$ and $\Delta$ in the course from it’s 500ng/ml solution in Tris/HCl buffer) and the evaluation.

Figure 5  Typical QCMA-1 sensor responses to binding of APP$_{770}$ of different concentrations (dilutions) in CM to DE2 antibodies immobilised on the surface.
Figure 1
Figure 2
Figure 3
Figure 4

\[ y = 0.3385 + 0.01816x \]

\[ t = 9.796 \pm 0.106 \text{ (min)} \]

\[ \psi = a \exp(-x/t1) + b \]
Figure 5
Table 1. The four-layer model for TIRE data fitting

<table>
<thead>
<tr>
<th>Layer</th>
<th>Parameters</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>3. BK-7 glass (ambient)</td>
<td>$n, k$ dispersions from WVASE32 library $n=1.515$, $k=0$ at 633nm</td>
<td>fixed during fitting</td>
</tr>
<tr>
<td>2. Cr/Au film</td>
<td>$n = 0.359 \pm 0.078; k = 2.857 \pm 0.114$ at 633nm $d$ is varied in the range of 25-30 nm</td>
<td>The values of $d$, and dispersions for $n$ and $k$ were obtained by fitting TIRE data for every new sample, then kept fixed in further fittings</td>
</tr>
<tr>
<td>1. Adsorbed layer</td>
<td>Cauchy model: $A=1.396$, $B=0.01$, $C=0$ giving $n=1.42$, $k=0$ at 633nm</td>
<td>parameters of Cauchy model $A$, $B$, and $C$ are fixed; $d$ is variable</td>
</tr>
<tr>
<td>0. Water (substrate)</td>
<td>$n, k$ dispersions from WVASE32 library, $n=1.33$, $k=0$, at 633nm</td>
<td>fixed during fitting</td>
</tr>
</tbody>
</table>