Detection of Neuron Specific Enolase (NSE) with the Protein Biosensor Based on Imaging Ellipsometry

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Abstract: Tumor markers can provide convincing evidence for tumor angiogenesis in early-stage, so that the need for novel and effective methods which can detect tumor markers rapidly, sensitively and reliably is consequently being subjected to extensive interest. The biosensor based on imaging ellipsometry (BIE) is developed for the detection of Neuron specific enolase (NSE) as a trial and its diagnosis performance is evaluated. Anti-NSE antibody as ligand is immobilized on protein A modified silicon substrate to form NSE sensing surface. Then, NSE test is carried out with the setup of a calibration curve for clinical quantitative detection purpose. The relationship between BIE signal y (grayscale value) and NSE concentration x (ng/ml) is y=19.6 lg(x) + 70.1 and the limit of detection achieves 2 ng/ml. The specificity, reproducibility and accuracy for NSE detection with BIE are all adequate to clinical diagnosis requirements. 149 serum samples have been detected quantitatively with BIE and their results are in agreement with a commercial ELISA immunoassay.

Keywords: Neuron specific enolase (NSE), tumor markers, protein microarray, biosensor, imaging ellipsometry, calibration curve, limit of detection, specificity, reproducibility, accuracy.

1. INTRODUCTION

The detection of tumor markers is an efficient approach for cancer diagnosis in early stage, providing a possibility for carrying out essential therapy [1, 2]. Since its overwhelming contribution to cancer cure, the need for novel and effective methods which can detect tumor markers rapidly, sensitively and reliably is consequently being subjected to extensive interest.

The concept of biosensor based on imaging ellipsometry (BIE) for visualization of biomolecular interactions has been proposed for more than 10 years [3, 4]. Imaging ellipsometry with the advantages of ellipsometry and microscopy simultaneously [5] can reflect the surface concentration of protein adsorbing layer accurately and sensitively without any labeling [4, 6]. It provides possibility to apply BIE's function in a broad range of various biological systems, especially for the detection of protein interactions. So far, several bio-targets, such as five markers of hepatitis B [7], castor toxin ricin [8], phage M13KO7 [9], and SARS virus [10], have been successfully tested with the biosensor.

Neuron specific enolase (NSE), encoded by the ENO2 gene [11], is an enzyme in humans and its amount increases remarkably in patients with small cell lung cancer [12]. Thus, NSE is identified as a tumor marker in clinic which concentrates on patients with small cell lung cancer. Measurement of NSE levels can provide information not only about the extent of the disease but also about the patient's response to treatment [13]. In this investigation, NSE is chosen to be a model of tumor markers and then tried to detect quantitatively with BIE.

2. MATERIALS AND METHODS

2.1. BIE and its Detection Principle

BIE which has been developed in our laboratory [3, 4] is to perform molecular interaction as the high throughput immunoassays [6, 14, 15]. Now, it is mainly composed of a micro-fluidic reactor array system [5] and an imaging ellipsometry reader [16, 17].

A micro-fluidic reactor array system including 48 independent flow channels is used to fabricate protein micro-array for high throughput detection by a series of continuous processes, including surface patterning, solution delivery, ligand immobilization, blocking, target capture, rinsing, etc. The micro-fluidic array system is composed of four main parts: sample reservoir, multi-cell array, multi-channels and pumps. When a silicon wafer substrate is placed on the multi-cell array to form closed paths, a patterned microarray can be fabricated because each cell has an independent inlet and outlet for solution delivery. The inlet micro-channels are

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connected to the sample reservoir and the outlet micro-channels are connected with pumps offering negative pressure. Using the micro-fluidic reactor system, various ligands are delivered to different cells to form a sensing array, and each sensing surface can be prepared homogeneously.

Imaging ellipsometry which combines the power of ellipsometry with microscopy and works in the off-null mode [3, 4] is an enhancement of standard single-beam ellipsometry, acting as the data acquisition of the microarray. Upon the high sensitivity requirement for weak affinity biomolecular interactions, its sensitivity has been improved by the polarization setting optimization, the wavelength optimization and an imaging device with low noise [6, 18]. It can be utilized for the visualization of ultra-thin films and slight surface concentration change. Here, it is used to quantify the thickness distribution of protein layers [6, 14]. A slight variation of layer thickness can be remarkably distinguished by imaging ellipsometry and the result is represented by images in grayscale format (8 bits, 256 grayscale or 10 bits, 1024 grayscale). The grayscale value is approximately linearly proportional to the thickness corresponding to the surface concentration [6].

2.2. Chemicals, Samples and Substrates

Silicon wafers are purchased from Beijing GRINM semiconductor Materials Company. 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), succinic anhydride and aminopropyl-triethoxysilane (APTES) are purchased from Acros Organics. N-Hydroxy-succinimide (NHS), blocking buffer, phosphate buffered saline containing 0.05% Tween-20 (PBST), and bovine serum are purchased from Sigma-Aldrich. Neuron-specific enolase from human brain (NSE), Anti-NSE antibody produced in rabbit, protein A from Staphylococcus aureus, Hepatitis B surface antigen (HBsAg) and Hepatitis B e antigen are also purchased from purchased from Sigma-Aldrich. 149 human serum samples whose the NSE concentration has been detected with the commercial immunoassay kits are collected in the division of Shandong Medical Institute Radioimmunoassay Laboratory, Department of Shandong Medical Academy. Deionized water is produced by ion exchange demineralization, followed by passing through a Milli-Q plus system from Millipore.

2.3. Silicon Wafer Preparation and Surface Modification

Silicon wafer is used as a solid substrate surface for imaging ellipsometry biosensor. Due to the throughput need of 48 independent units, the silicon slides are cut into 25×13 mm^2 rectangular pieces. Then, aiming at washing out the organic and inorganic pollution, silicon wafers are cleaned with a mixture of 30% H_2O_2 and concentrated H_2SO4 (1:3 v/v) for 30 minutes. After being thoroughly rinsed with deionized water and pure ethanol, these silicon wafers are treated with an ethanol solution of APTES (5% APTES and 95% pure ethanol) and incubated for 2 hours at room temperature [16]. Following by intensively rinsing in pure ethanol, the silicon wafers silanized with APTES are reacted with over-saturated succinic anhydride in ethanol for at least 3 hours [17]. After being rinsed with pure ethanol, the silicon wafers are stored in pure ethanol for ligand immobilization.

2.4. Detection of NSE

After surface modification, silicon wafer is placed into the micro-fluidic reactor system to immobilize anti-NSE antibody. Because the anti-NSE antibody is in serum mixture solution, it could not be directly assembled on the surface just depending on covalent immobilization. Therefore, protein A which is commonly used in protein purification is tried to capture anti-NSE antibody to form sensing layer by the interaction between Fc part of antibody and protein A. Firstly, 20 µl of a mixture solution which is prepared with NHS and EDC at the concentration of 0.05 mol/ml and 0.2 mol/ml in deionized water is added to the micro-fluidic reactor system and passed through the surface at a flow rate of 5 µl/min. With NHS and EDC, carboxyl groups on the substrate could react with the amino groups of the protein. Then, 15 µl of protein A at 0.2 mg/ml is adsorbed on the substrate at a flow rate of 1 µl/min. After that, 20 µl of anti-NSE antibody solution as ligand is immobilized specifically to protein A at a flow rate of 1 µl/min. Finally, the silicon surface is blocked by blocking buffer in order to prevent non-specific adsorption of human serum. After these four steps, the sensing surface for NSE detection is prepared in use. When the detection begins, 20µl of NSE standard sample and human serum dilution solutions is individually delivered into different channels to NSE sensing surface by the micro-fluidic system at a flow rate of 1 µl/min. After being rinsed with plenty of PBST and dried under nitrogen, the microarray is sampled and recorded as images in grayscale by the imaging ellipsometry reader.

2.5. Calibration Curve for NSE Quantitative Detection

In order to achieve the goal of quantitative detection, a calibration curve of the signal versus NSE concentration is established with a NSE standard serum sample diluted to 1, 2, 4, 8, 16, 32, 64 and 128 ng/ml with PBST in term of the equal proportion dilution rule. The each of the 8 diluted samples and the original one are measured twice for each to obtain their mean signal in grayscale for the calibration curve, which is used to verify measurement results.

2.6. Quantitative Detection of Human Serum Samples

149 human serum samples are detected by the same procedure to set up calivration curve. Each serum is measured twice, and then NSE concentration results are deduced by the calibration curve. The BIE results are compared with ELISA assay by correlation analysis.

3. RESULTS

3.1. Detection of NSE

Protein A can recognize the Fc portion of IgG molecule specifically, so that the Fab portion of IgG with the binding site to recognize antigen can be exposed. By this means, protein A is designed to realize the oriented immobilization of anti-NSE antibody on the modified surface and fabricate NSE sensing surface with good bio-activity. In order to improve the detection performance, several parameters, for instance, the concentration of anti-NSE antibody as the ligand, the reaction time for NSE capture and the ellipsometric setup for data acquisition, should be optimized in advance. Ligand surface concentration is a pivotal factor to decide the amount of analyte capture, further influencing detection sensitivity and detected concentration range. Less ligand results in the scarcity of analyte binding sites; while excess situation affects ligand bio-activity and increases the steric hindrance. Before the NSE detection, a series concentration of anti-NSE antibody has been screened to find out the optimized value to fabricate NSE sensing surface, and its result is shown in Figure **1**. It is obvious that the grayscale value increases with the addition of NSE on anti-NSE antibody surface and the most significant change in grayscale value caused by the capture of NSE occurs at the anti-NSE antibody concentration of 80 μg/ml for all the tested NSE samples. Thus, the concentration is chosen to constitute NSE sensing layer. In addition, the reaction time for NSE capture is also optimized to 20 minutes for achieving its reaction equilibrium and the azimuth angles of polarizer, compensator, and analyzer in ellipsometric setup are fixed at 82°, 45°, and 11°, respectively, for the best of image contrast.

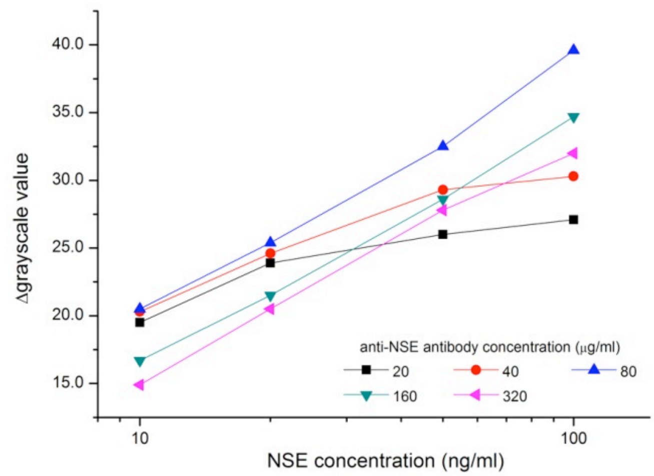
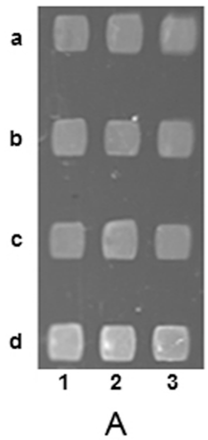


Figure 1: The ligand concentration screening of anti-NSE antibody. Anti-NSE antibody has been diluted at various concentrations (20, 40, 80,160, 320 ng/ml), and then delivered to react with the protein A on the substrate surface to form NSE sensing surface. After the addition of blocking and rinsing buffer, NSE samples whose concentration ranges from 10 to 100 ng/ml have been separately pumped to sensing surfaces for the optimized concentration of anti-NSE antibody by comparing their response signal in grayscale value.

With these optimized parameters, NSE standard sample at the concentration of 17 ng/ml which is the cut-off point in clinic screening has been carried out as a trial for NSE detection. Blank control and negative control are set to ensure detection accuracy and avoid false positive, and the results are demonstrated in Figure **2A** and its corresponding numerical values are listed in Figure **2B**. In the blank control units, grayscale value increases from 70.8 to 71.5 after reacted with NSE standard sample. It is suggested that the non-specific adsorption and other errors may cause an increase of 0.7 grayscale value. In other words, grayscale value increasing beyond 0.7 can be contributed to the specific binding between NSE and its corresponding antibody. The average grayscale value of sample units is 94.3, while the negative control is only 71.9. As a result, it can be concluded that NSE is captured by anti-NSE antibody on the sensing surface for the remarkable rise in grayscale value.



	1	2	3
a	70.8	70.5	71.2
b	72.6	70.7	71.3
c	71.3	72.6	71.8
d	94.7	95.0	93.3

B

Figure 2: Detection of NSE. (**A**) The image in grayscale; (**B**) Average grayscale value in units of image A. Protein A is immobilized in all the 12 units. Then, anti-HBsAg antibody is reacted in row “a” and “b”, while anti-NSE antibody is added in row “c” and “d”. After running with blocking buffer, NSE standard sample is delivered in row “b” and “d”, whereas PBST is added in row “a” and “c”. In that case, row “a” and “b” is the blank control, and row “c” functions as the negative control.

3.2. Calibration Curve for NSE Quantitative Detection

NSE standard sample which is diluted at geometric proportion to 8 grads from 1 to 128 ng/ml as well as the negative control (without NSE addition) has been added to its sensing surface for establishing the calibration curve (shown in Figure **3**). By multi-parameter logistic function fitting, the calibration curve is obtained it appears liner formulation after the logarithmic transformation when NSE concentration ranges from 2 to 64 ng/ml. This calibration curve could be represented by the regression equation: y=19.6 lg(x) + 70.1 (R^2 = 0.98, R^2 is the percentage of total variation in the response that can be explained by

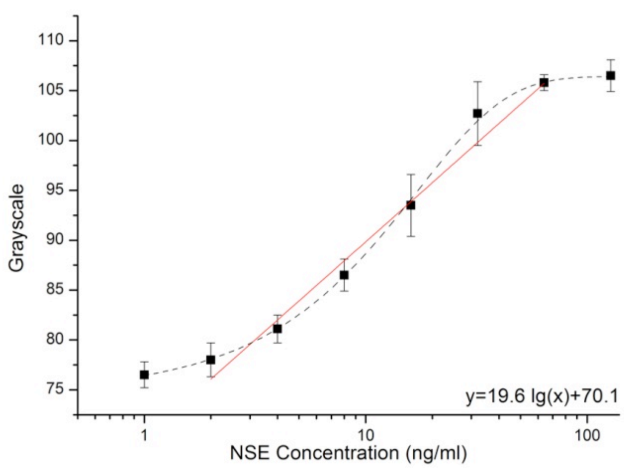


Figure 3: The calibration curve for quantitative detection of NSE.

factors in regression analysis and the higher R^2, the better fitting.), where y is the grayscale value and x presents the NSE concentration. Compared with the negative control, the change of grayscale value caused by the addition of NSE concentration is at 2 ng/ml is 3 times greater than the noise estimated by the blank controls, so that its limit of detection is deduced to 2 ng/ml.

3.3. Specificity, Reproducibility, Accuracy for NSE Detection

Although anti-NSE antibody is immobilized by protein A to keep its bio-activity, its confirmation might be partially different from its original situation in physiological atmosphere, leading to mismatch with non-target antigen. Besides, non-specific adsorption can also induce the binding by non-target molecules and cause false positive results. In that case, it is necessary to check the specificity of the sensing surface for NSE detection. Its specificity is estimated by separately introducing HBsAg, HBeAg and bovine serum on NSE sensing surface, and then their responses in grayscale value are calculated (shown in Figure **4**). Compared with NSE result, the most significant percentage of grayscale value increase is only 6.2%, presenting good specificity for NSE detection.

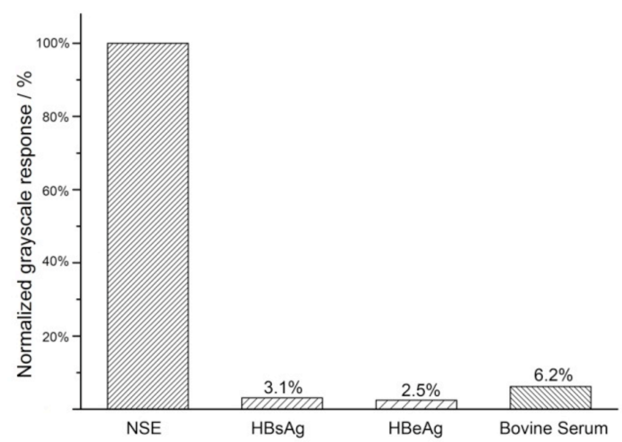


Figure 4: Specificity evaluation in the presence of NSE (50 ng/ml), HBsAg (50 ng/ml), HBeAg (50 ng/ml) and bovine serum (5 times dilution in PBST solution).

The reproducibility is assessed at different NSE concentrations by intra-assay and inter-assay measurements. Each NSE concentration has been tested with 6 duplicates in the same assay; while this measurement has been repeated in 5 independent arrays. The results including the mean and the coefficient of variation (CV) are presented in Table **1**.

Table 1: The Intra-Assay and Inter-Assay Reproducibility for NSE Detection

	Intra-assay			Inter-assay		
Sample concentration (ng/ml)	**10**	**20**	**40**	**10**	**20**	**40**
Duplites	6	6	6	5	5	5
Mean (grayscale value)	89.8	95.1	101.4	89.7	95.6	101.5
Standard deviation (grayscale value)	2.0	1.9	2.2	1.4	2.6	2.6
CV (%)	2.2%	2.0%	2.2%	1.6%	2.8%	2.6%

All the CV% values of intra-slide and inter-slide are less than 3.0%, which demonstrates that BIE has an overwhelming reproducibility and meet the standard of clinical diagnosis.

The accuracy is evaluated by carrying out recovery rate investigation. NSE at various concentrations are separately dissolved into a standard serum sample of known concentration, and detected with BIE to appraise the recovery rate. The test detail is listed in Table **2**. The range of recovery rate is between 96.9% and 103.3%, which has acceptable accuracy according to validation of bioassay in industry perspective.

Table 2: NSE Recovery Rate Investigation for Detection Accuracy Estimation

NSE concentration (ng/ml)			Recovery rate (%)
Sample	**Added**	**Found**	
25.00	10.00	33.90	96.9%
25.00	20.00	44.40	98.7%
25.00	30.00	56.80	103.3%
25.00	40.00	64.70	99.5%
25.00	50.00	74.10	98.8%

3.4. Serum Samples Detection

149 human serum samples including 32 healthy samples and 117 patient samples (23 of them are lung cancer patients) have been tested strictly adhering to the protocol established as above-mentioned as a trial for clinical purpose. Each serum is tested twice and the mean value is accordingly calculated after converting the grayscale into mass concentration. If the NSE concentration exceeds the effective range of the calibration curve, it is diluted advisably by PBST and detected again. The BIE results are compared with a commercial ELISA method regarding as a conventional diagnosis and their difference is evaluated by correlation analysis (shown in Figure **5**). The Pearson correlation coefficient is 0.790, which demonstrates that results of the both tests have significant statistical relevance at the level of 0.01.

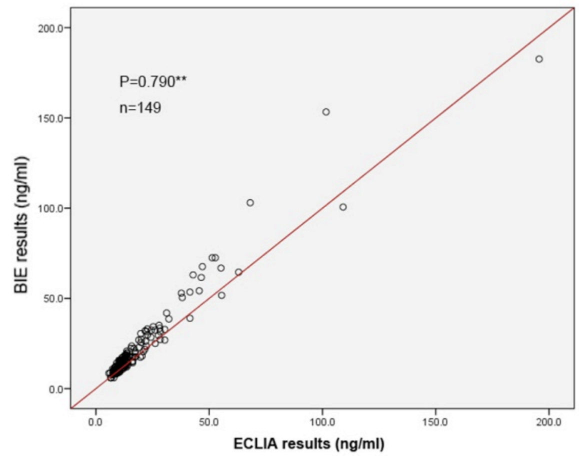


Figure 5: Comparing BIE with ELISA for NSE detection of 149 clinical samples.

CONCLUSIONS

Oriented immobilization method with Protein A has been successfully applied to form NSE sensing surface on silicon substrates. The Fab portion of antibody is set free and exposed outside of the surface; its bio-activity is greatly improved, which lays the first stone for the bio-recognition process between antibody and its target.

NSE is a useful tumor marker for lung cancer screening in an early stage. It is chosen as a model to develop BIE as a protocol and approach for the detection of tumor markers. It is showed evidently that the limit of detection (2 ng/ml) in the present study is more sensitive than the cut-off value to distinguish the normal and the patient. The specificity, reproducibility and accuracy for NSE detection with BIE are all adequate to clinical diagnosis requirements. 149 sera have been tested with BIE, and its results are validated by a commercial ELISA method.

Compared with the conventional method for the detection of tumor markers, BIE maintains several significant advantages as follows. Firstly, BIE is based on non-touch and label-free imaging ellipsometry technology for data acquisition. Non-touch ensures the detection without disturbance to samples, while label-free guarantees the results with less false positive. Secondly, the result of BIE is demonstrated in the form of image, which means the quality of results can be observed and evaluated with results themselves. In that case, results with low homogeneity or unexpected contamination can be easily eliminated in time. What is more, BIE can realize parallel and high throughput analysis at the same time. It provides the possibility to test numerous samples simultaneously. Finally, BIE is of high sensitivity, as the limit of detection for NSE achieves 2 ng/ml much less than the clinical cut-off point. It meets the demand for detection of most tumor markers in clinic test.

To conclude, it is feasible to use BIE as a novel method to detect NSE for clinical purpose. With the oriented immobilization strategy and comprehensive advantages of BIE, the detection shows good specificity, reproducibility and accuracy, as well as acceptable sensitivity. It can be foreseen that BIE has a potential for early-stage detection of tumor markers in clinical and biological application.

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