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Optimization of 3-aminopropyltriethoxysilane functionalization on silicon nitride surface for biomolecule immobilization

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ABSTRACT

The 3-aminopropyltriethoxysilane (APTES) is a common method for biomolecule immobilization on silicon and silicon derivatives such as silicon nitride (Si_3N_4). However, there are many parameters which impact the efficiency of APTES modification such as APTES concentration and reaction time. Thus, various APTES concentrations (0.1%, 0.5%, 1%, 2%, 5%, and 10%) under different reaction times (15, 30, 60 and 120 min) were compared to achieve the optimal APTES modification condition which produced a thin and stable APTES layer on Si_3N_4 surface. The modified surfaces were characterized by contact angle (CA) measurement, Fourier transform infrared (FTIR) spectroscopy and spectroscopic ellipsometry to determine the wetting property, chemical bonding composition and surface thickness, respectively. In addition, biotin was used as a model to determine the effectiveness of APTES modification condition by coupling with glutaraldehyde (GA). The Alexa Flour 488 conjugated streptavidin was performed to visualize the presence of biotin using fluorescence microscopy due to the specifically binding between biotin and streptavidin. The atomic force microscopy (AFM) was utilized to determine the surface topology which was an indicator to demonstrate the agglomeration of APTES molecule. Moreover, ion sensitive field effect transistor (ISFET) was employed as a biosensor model to demonstrate the effect between surface thickness and sensitivity of biosensor. The results show that the APTES thickness is directly correlated to the APTES concentration and reaction time. Since the importance parameter for ISFET measurement is the distance between biomolecule and sensing membrane of ISFET, the thicker APTES layer negatively impacts the sensitivity of ISFET based biosensor because of the ion shielding effect. Therefore, these results would be valuable information for development of Si_3N_4 biosensor, especially ISFET based biosensor.

1. Introduction

Silicon nitride (Si_3N_4) is a robust material which is commonly used as the insulator for microelectronics and microsystem devices [1,2]. It has also played an important role in integrated circuit technology, thin film transistors, optics and optoelectronics [3–5]. Nowadays, the integration of microelectronic and biochemistry has been developed to miniaturize silicon nitride-based biosensors, Ion Sensitive Field Effect Transistor (ISFET) [6] or Electrolyte Insulator Semiconductor Field Effect Transistor (EISFET) [7]. The efficiency of biosensor performance was based on biomolecule immobilization method and type of substrate [7]. Thus, the substrate materials need modification and functionalization in order to bind with biomolecules. However, it was a complicate process to immobilize biomolecules onto Si_3N_4 surface because it was electrically neutral and non-porous. Therefore, immobilization methods based on physical adsorption, ionic interaction and entrapment were not effective [8]. On the other hand, many immobilization methods based on covalent immobilization on silicon and silicon derivatives, such as Si_3N_4, has been widely proposed such as silanization using organosilane [9], hydroxylation using terminal alkene/alkyne under thermal [3] or ultra-violet light activation [10] and plasma activation process using inductively coupled plasma reactive ion etching (ICP-RIE)

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technique [11]. Silanization of silane compounds (e.g. amino, epoxy, thiol, etc.) was commonly used to prepare organic monolayer on any substrates in several applications such as surface coating, medical devices, and micro- and nano-fabrication technologies [9,12]. A popular choice of organosilane was 3-aminopropyltriethoxysilane (APTES) which was highly reactive toward various functional groups for immobilization of DNA, antibodies, and enzymes [9,13–15]. However, there were many factors which affect the silanization process such as silane concentration, silanization time, moisture (humidity) and temperature of the solution [16,17]. Hereby, the optimal silanization for any solid substrates was one of critical parameters to create controllable APTES-modified surfaces.

The aim of this study was to achieve the optimal silanization condition for APTES modification on Si_3N_4 surface which could create a thin and stable APTES layer with high density of amine groups. These surface amino groups are key issues for biomolecule immobilization in the construction of biosensor development. The Si_3N_4 surface was selected to represent a sensing membrane of Si_3N_4 based biosensors including ISFET and EISFET. Different APTES modification conditions were studied and characterized by contact angle, Fourier transform infrared (FTIR), and spectroscopic ellipsometry to determine wetting property, chemical bonding composition, and surface thickness, respectively. In addition, biotin was used to demonstrate the efficiency of APTES modified Si_3N_4 surface using glutaraldehyde (GA) as a coupling agent. The presence of biotin on the surface was visualized by Alexa Flour 488 conjugated streptavidin through fluorescence microscopy. The atomic force microscopy (AFM) was also used to determine the surface topology which is attributed to the agglomeration of APTES molecule on the Si_3N_4 substrate. In addition, ISFET based biosensor was employed to demonstrate the effect between APTES layer thickness and the sensitivity of ISFET measurement which would be beneficial for development of ISFET based biosensor.

2. Materials and methods

2.1. Chemical reagents

Ammonium hydroxide (NH_4OH), hydrochloric acid (HCl), 3-Aminopropryltriethoxysilane (APTES), glutaraldehyde, bovine serum albumin (BSA) and Tween 20 were obtained from Sigma-Aldrich (St. Louis, MO, USA). Hydrogen peroxide (H2O2), ethanol, sodium cyanoborohydride ($NaBH_3CN$) and 10 mM phosphate buffer saline (PBS) tablet were supplied by Merck Ltd. (Darmstadt, Germany). Alexa Flour 488 conjugated streptavidin, amine conjugated biotin (biotin-NH_2) and amine conjugated poly ethylene glycol (PEG-NH_2) were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Washing buffer was prepared by mixing 0.05% Tween 20 and 10 mM PBS. Deionized water was purified by a Millipore Milli Q system (resistivity ≥ 18 MΩ cm). All chemical reagents were of analytical grade and were used without further purification. The Si_3N_4-ISFET based biosensors were designed and fabricated at Thai Microelectronics Center (TMEC), Thailand. Ag/AgCl reference electrode and readout circuit were obtained from Winsense Co., Ltd. (Thailand).

2.2. Surface modification

The Si_3N_4 deposited silicon (Si) wafer was cut into small pieces. These samples were then cleaned according to Ref. [18]. Briefly, they were immersed in an oxidizing solution which was composed of H_2O, H_2O_2, and NH_4OH at a ratio of 5:1:1 at room temperature for 5 min to remove any organic contaminants. The excess solution was washed by deionized water. Then, they were activated in a mixture solution of HCl and H_2O at a ratio of 1:7.5 at room temperature for 10 min. After rinsing with deionized water, the cleaned surfaces were separately immersed in different APTES concentrations (0.1%, 0.5%, 1%, 2%, 5%, and 10%) and incubated at room temperature under various reaction times from 15 to 60 min. The excess solution was thoroughly rinsed with ethanol and deionized water. Next, these surfaces were subsequently cured at 100 °C for 30 min to remove the moisture and to allow siloxane (Si-O-Si) bonding. Finally, the silanized surfaces were coupled with 2.5% GA at room temperature for 1 h and washed with PBS to produce aldehyde-terminated surfaces.

2.3. Surface characterization

The modified surfaces were characterized by contact angle (CA) measurement, Fourier transform infrared (FTIR) spectroscopy, and spectroscopic ellipsometry to determine the wetting property, chemical bonding information, and surface thickness, respectively.

2.3.1. Contact angle measurement

A contact angle (CA) goniometry from Rame-Hart Instrument co. (Succasunna, NJ, USA) was performed in static mode at room temperature. A drop of deionized water was gently placed onto the surface and analyzed by drop analysis software. The measurement was made in three different regions on the same surface with three replications.

2.3.2. Fourier transform infrared (FTIR) analysis

The Nicolet 6700 FTIR spectrometer from Thermo Fisher Scientific Inc. (Waltham, MA, USA) was employed to demonstrate the chemical bonding on the modified surface. It was performed in attenuated total reflectance (ATR) mode. Each spectrum was measured in the range from 4000 to 625 cm^{-1} at a resolution of 2 cm^{-1} with 64 scans. The untreated surface was recorded and set as a reference.

2.3.3. Surface thickness measurement

The thickness of the APTES modified Si_3N_4 surface was measured using an Ellipsometer VB-400 from J. A. Woollam Co., Inc. All measurements were performed at an incidence angle of 70° in air at room temperature with the wavelength of 550 nm. The ellipsometric parameters were fitted using Cauchy equation to build up the model of APTES layer [19].

2.4. The efficiency of APTES modification

The efficiency of APTES modified Si_3N_4 surface was evaluated using biotin-NH_2 as a model. The Alexa Flour 488 conjugated streptavidin was utilized to visualize the presence of biotin-NH_2 on the surface through fluorescence microscopy. In addition, ISFET was used to demonstrate the successfully APTES modification on Si_3N_4 surface for development of biosensor.

2.4.1. Electrical measurement of ISFET

The source and drain terminals of ISFET and a reference electrode were connected to the readout circuit. All measurements were performed in a light shielded bottle containing 0.1 mM PBS, pH 7.4 to prevent the electrical disturbance from the ambient light. This assay relies on a different surface potential induced by the charged molecules on the modified surface of ISFET when applying constant drain current at 50 μA.

2.4.2. Biotin immobilization

The biotin-NH_2 and PEG-NH_2 were prepared at a concentration of 500 nM and employed as a target and negative control molecule, respectively. The APTES-GA modified ISFETs were immersed in 160 mM $NaBH_3CN$ at room temperature for 1 h. The excess solution was rinsed with deionized water and the gate potential was measured as a baseline voltage ($V_{baseline}$). Then, biotin-NH_2 and PEG-NH_2 were separately applied onto the modified surfaces at room temperature for 1 h. The excess solution was washed with 0.1 mM PBS, pH 7.4. The gate potential change responded to biotin-NH_2 and PEG-NH_2 was calculated ($\Delta V = Vb_{iotin\ or\ PEG} - V_{baseline}$). Then, 5% BSA was dropped onto each

modified surface at room temperature for 1 h to block non-specific binding site. The excess solution was rinsed with washing buffer and the gate potential was measured. After that, Alexa Flour 488 conjugated streptavidin was dropped onto the modified surfaces and incubated at room temperature for 1 h. Then, the modified surfaces were washed with washing buffer, 0.1 mM PBS, and deionized water, respectively. The gate potential change corresponded to streptavidin and biotin reaction was measured and compared to the blocked voltage to achieve the gate potential change ($\Delta V = V_{streptavidin} - V_{blocking}$). Finally, the surfaces were blown dry by a stream of nitrogen gas before fluorescence measurement.

2.4.3. Fluorescence detection

The fluorescence signals were obtained from fluorescence microscopy using camera Olympus DP72 (Tokyo, Japan) with excitation at 495 nm and emission at 519 nm, specific to Alexa Flour 488 conjugated streptavidin. The exposure time of sample is 50 μs. Every image was further processed using imageJ to determine the fluorescence pixels intensity. This corresponded to the amount of Alexa Flour 488 conjugated streptavidin and biotin-terminated Si_3N_4 surface. The amount of fluorescence pixels intensity was calculated according to Ref. [20] and presented in terms of the relative fluorescence pixels intensity.

2.4.4. Surface morphology measurement

The surface roughness of APTES modified Si_3N_4 surface was analyzed using Hitachi AFM 5300E from Hitachi, Ltd. (Tokyo, Japan). The topological images of the APTES modified surfaces were achieved using a Si_3N_4 tip in the tapping mode under ambient conditions in the measurement area of 10 μm × 10 μm. The root mean square (RMS) roughness was calculated from the fluctuation of the surface height around the average height in the image [2].

3. Results and discussion

3.1. Contact angle measurement

The contact angle (CA) measurement is a simple indicator to determine the wetting property during the functionalization process. The CA measurement for various APTES conditions is shown in Table 1. The CA value of untreated Si_3N_4 surface was 61.66 ± 3.45° which was slightly hydrophobic due to organic or various particle contaminations. After cleaning step, a CA value was found to be 30.12 ± 2.36°. This finding showed the conversion of wetting property from hydrophobicity to hydrophilicity due to the formation of hydroxyl (-OH) groups or silanol (Si-OH) groups on the surface. These groups are important for surface modification which further reacts with silanol (Si-OH) groups of APTES molecules. The APTES modified Si_3N_4 surface was performed in different APTES concentrations ranging from 0.1% to 10% with various reaction times from 15 to 120 min in liquid phase. All CA values of APTES modified Si_3N_4 surface are higher than cleaned Si_3N_4 surfaces. This could be due to the replacement of the –OH groups by the hydrophobic character of the grafted alkyl chain of APTES molecules [21]. It is correlated to the reports which describe the relation between contact angles and APTES layer [16].

Table 1
The measurements of static contact angle of various silanization condition for APTES modification on Si_3N_4 surface with different reaction time. For each condition, the reported value was an average from three different regions on the same surface with standard deviation (SD) and coefficient of variation (%CV).

APTES concentration (%)	Contact angle (°)			
	Reaction time (min)			
	15	30	60	120
0.1	49.43 ± 1.55	53.04 ± 5.15	55.01 ± 1.28	64.45 ± 1.95
0.5	56.22 ± 5.67	62.22 ± 1.48	64.27 ± 6.43	67.69 ± 3.11
1	62.15 ± 1.80	70.92 ± 2.94	71.33 ± 2.87	78.20 ± 2.33
2	73.93 ± 3.44	74.42 ± 4.84	77.45 ± 4.83	83.78 ± 4.14
5	71.75 ± 2.99	71.91 ± 1.93	78.82 ± 1.61	82.87 ± 4.37
10	77.90 ± 1.84	78.78 ± 2.04	80.78 ± 2.40	88.20 ± 1.45

3.2. Fourier transform infrared (FTIR) analysis

The FTIR characterization was performed to demonstrate the chemical composition of APTES modified surface. The chemical bonding of APTES modified Si_3N_4 substrate was studied in the range of 4000–625 cm^{-1}. The spectra of all APTES modified surfaces are similar which is shown in Fig. 1. The important peak which is assigned to NH_2 bending occurs at 1700-1400 cm^{-1}. This was correlated to the terminal amine group of APTES modified surface which further facilitates the conjugation between biomolecule and Si_3N_4 substrate [15]. The peak corresponding to the carbon backbone of APTES molecule was found at 2900–2800 cm^{-1} [13,17]. The peak of Si-O-Si stretching occurs at 1140 and 1020 cm^{-1}. These peaks suggest that APTES is successfully immobilized on the surface. However, the peak of Si-O-C might be found at 1108 cm^{-1} when the hydrolysis of APTES is incomplete. This group indicates the existence of ethoxy groups of APTES molecule. The result shows that the FTIR spectra became very weak when the APTES concentration was decreased due to the presence of a thin silane layer which was described in the following section.

3.3. Surface thickness

The thickness of APTES layer at a concentration of 0.1%, 0.5%, 1%, 2%, 5%, and 10% on Si_3N_4 surfaces were measured for using spectroscopic ellipsometer which is presented in Fig. 2. According to APTES reaction, the ethoxy groups of APTES can be hydrolyzed prior to react with hydroxyl groups after being dissolved in water. However, these groups are able to link to neighboring APTES molecule resulting in the polymerization of APTES molecule. This phenomenon depends on its concentration and reaction time as described in Ref. [22]. The result shows that the lowest APTES layer was obtained from the lowest APTES concentration with the shortest reaction time (0.1% APTES and 15 min). This is due to the reaction between APTES molecules and hydroxylated surface which could be faster than APTES polymerization in the solvent. In contrast, the thickest APTES layer was achieved at a condition of 10% APTES and 120 min which is the highest APTES concentration and the longest reaction time. This is due to self-polymerization of APTES molecule resulting to an agglomeration of APTES layer. However, a multilayer of APTES under low APTES concentration can occur when reaction time increases [13]. This is correlated to the previous studies which explained the relation between silanization reaction and APTES concentration in the solvent [22,23].

3.4. The efficiency of surface modification

3.4.1. Fluorescent detection

The Biotin-NH_2 was used as a model to demonstrate the success of biomolecule immobilization process whereas the PEG-NH_2 was employed as a negative control to determine non-specific binging background. The Alexa Flour 488 conjugated streptavidin was employed to demonstrate the efficiency of different APTES modified Si_3N_4 surface conditions due to the specificity between biotin and streptavidin. The relative fluorescence pixel intensity was calculated according to Ref. [20] and presented in Fig. 3. There is no fluorescence presented on bare Si_3N_4 surface whereas small amount of fluorescence pixel intensity could occur on the APTES modified surface with PEG-NH_2 because of the unspecific binding between Alexa Flour 488 and the remaining reactive aldehyde groups on the modified surface. However, this is lower than the fluorescence pixel intensity which was obtained from APTES modified surface with biotin-NH_2. The result shows that the

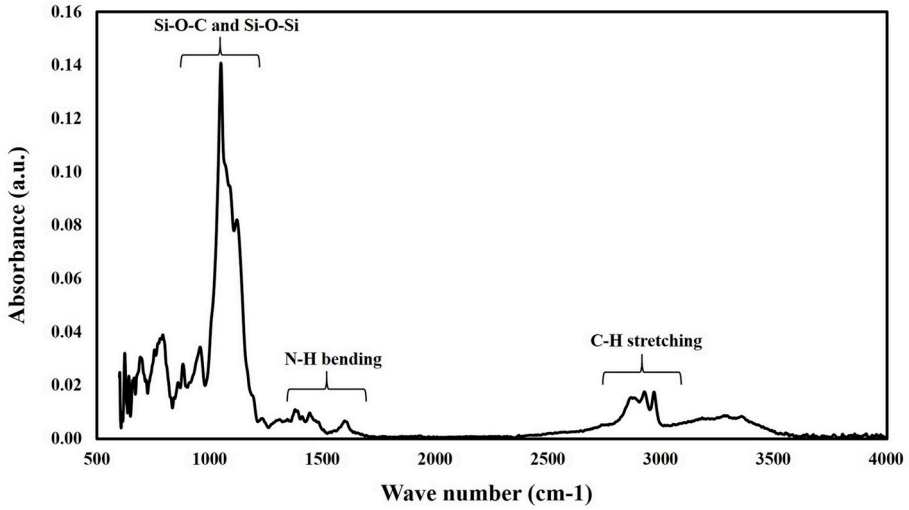

Fig. 1. The FTIR spectrum of APTES modified Si_3N_4 surface in ATR mode [15]. For each condition, the reported value was an average from three different samples (n = 3).

fluorescence pixel intensity increases when APTES concentration and reaction time increase. This correlated to the amount of biotin which is bound on the surface. The lowest and highest fluorescence pixel intensity was present at a concentration of 0.1% and 10%, respectively. This fluorescence pixel intensity is due to a partial APTES coverage; however, the highest fluorescence pixel intensity is due to an agglomeration of APTES molecule. This agglomeration of APTES molecules is correlated to the APTES property which was described in Refs. [22,23]. Thus, the optimal APTES modification condition on Si_3N_4 is 1% with a reaction of 60 min. This condition produces APTES layer which is close to a theoretical report (2 nm) and high density of biotin immobilization.

3.4.2. ISFET measurement

The ISFET measurement was employed to demonstrate the application of biosensors. This measurement depended on the density of immobilized molecule on the sensing membrane of ISFET. Each gate potential change, responding to different APTES condition with excess biotin concentration, was measured and plotted to demonstrate the relationship between gate potential change and APTES concentration. Six APTES concentrations (0.1%, 0.5%, 1%, 2%, 5%, and 10%) at a reaction time of 60 min were selected to demonstrate the impact of APTES thickness for ISFET measurement. The gate potential change at the concentration of 0.1%, 0.5%, 1%, 2%, 5%, and 10% were 3.33 ± 1.53, 4.33 ± 1.53, 9.67 ± 2.52, 8.67 ± 2.08, 4.67 ± 1.15, and 5.00 ± 2.00 mV, respectively, as shown in Fig. 4. The result shows that the maximal gate potential change is obtained at 1% APTES. On the contrary, the gate potential change of 10% is slightly different from 1% due to its fragile structure which could be lost during various steps in biomolecule immobilization process. In addition, Alexa Flour 488 conjugated streptavidin was employed to ensure the binding complex

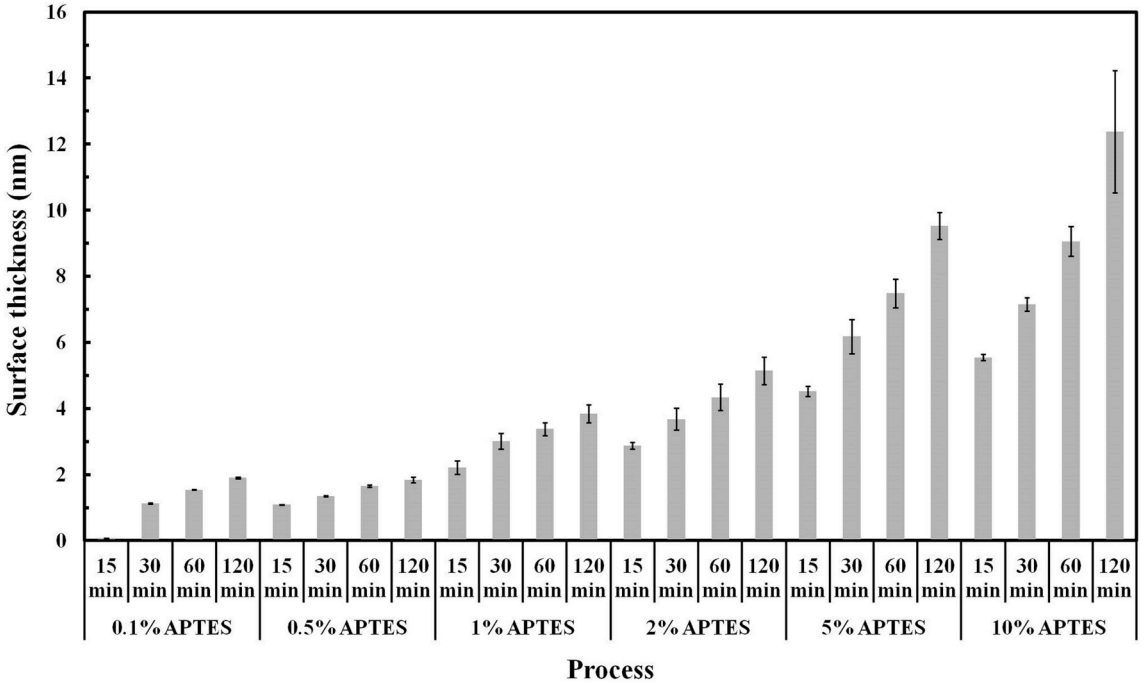

Fig. 2. The measurements of surface thickness of various silanization condition for APTES modification on Si_3N_4 surface. For each condition, the reported value was an average from three different samples (n = 3) with standard deviation (SD).

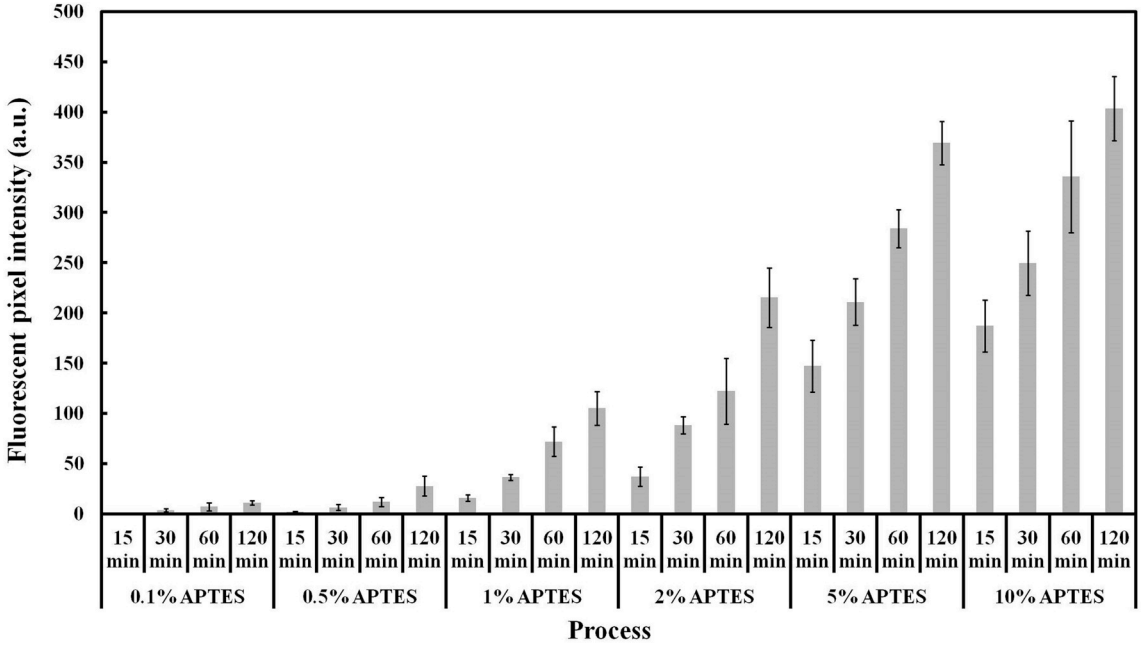

Fig. 3. The comparison of the relative fluorescence pixel intensity of various silanization conditions for APTES modification on Si_3N_4 surface. For each condition, the reported value displayed the average value which was obtained from three different samples (n = 3) with standard deviation (SD).

between biotin and streptavidin. This molecule can also be visualized by fluorescence microscopy. The gate potential change of biotin and streptavidin complex for 0.1%, 0.5%, 1%, 2%, 5%, and 10% were 3.33 ± 1.53, 5.33 ± 0.58, 14.33 ± 3.51, 12.33 ± 2.52, 6.00 ± 1.00, and 5.67 ± 3.06 mV, respectively, as shown in Fig. 4. Although the fluorescence pixel intensity at 10% APTES is higher than 1% APTES, its gate potential change is lower than 1% APTES. This correlated to an aggregation of APTES molecule and distance between biomolecule and sensing membrane of ISFET or Debye screening length (λ_D). This parameter is the distance which the electrical field is screen out by mobile charge carries. Since the Debye screening length (λ_D) is related to the ionic strength of the buffer solution, it is a critical issue which strongly impacts the sensitivity of ISFET measurement for biomolecule detection. The ISFET measurement in this study was performed in 0.1 mM and the Debye screening length was calculated to be 7.4 nM (Fig. 5). The results show that the APTES layer at 10% is higher than Debye length resulting in the ion shielding over short distance. This allows the electrical potential decay over longer distance leading to the decrease of sensitivity. However, Debye screening length can increase by reducing the ionic strength of the buffer solution which can be calculated from Ref. [24]. This might affect the stability of immobilized biomolecule. Therefore, the optimal APTES concentration and reaction time are 1% and 60 min because this condition produces a thin and stable APTES layer with high density of biomolecule.

3.4.3. Atomic force microscopy (AFM)

The atomic force microscope (AFM) was utilized to determine surface topology which was presented in term of root mean square (RMS)

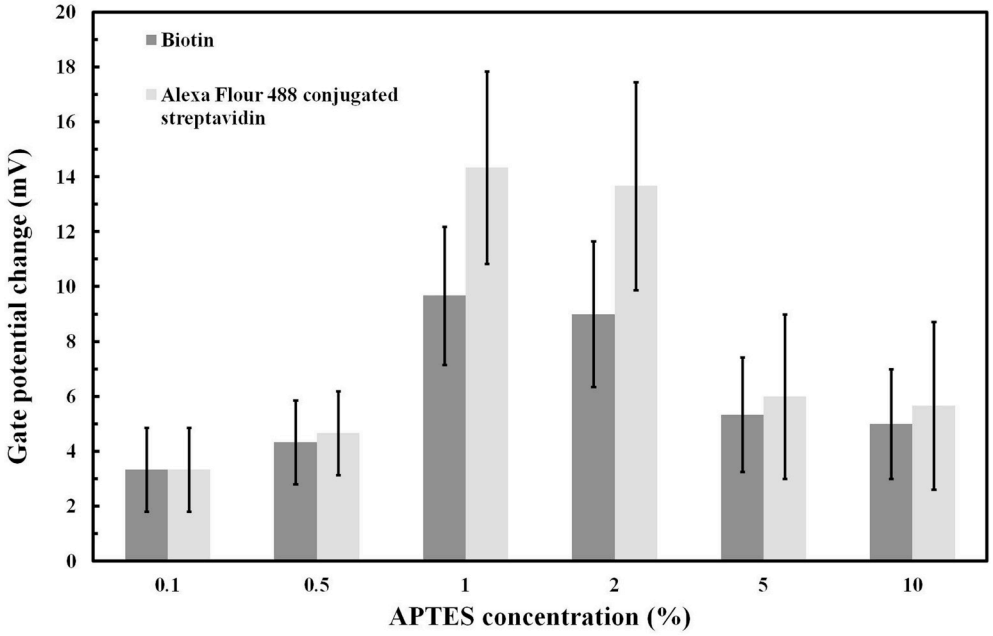

Fig. 4. The relationship between biomolecule immobilization and gate potential change corresponded to APTES concentrations at a concentration of 0.1%, 0.5%, 1%, 2%, 5%, and 10%. For each condition, the reported value displayed the average value which was obtained from three different samples (n = 3) with standard deviation (SD).

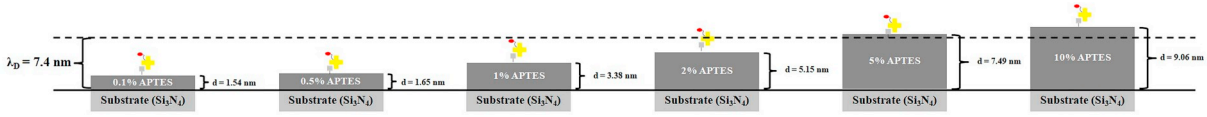

Fig. 5. Schematic representation of the Debye screening length (λ_D) from the ISFET surface at PBS buffer concentration of 0.01× (7.4 nm) in the presence of biotin (grey square) and Alexa Flour 488 (red circle) conjugated streptavidin (yellow cross) under various APTES layer thickness (d). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

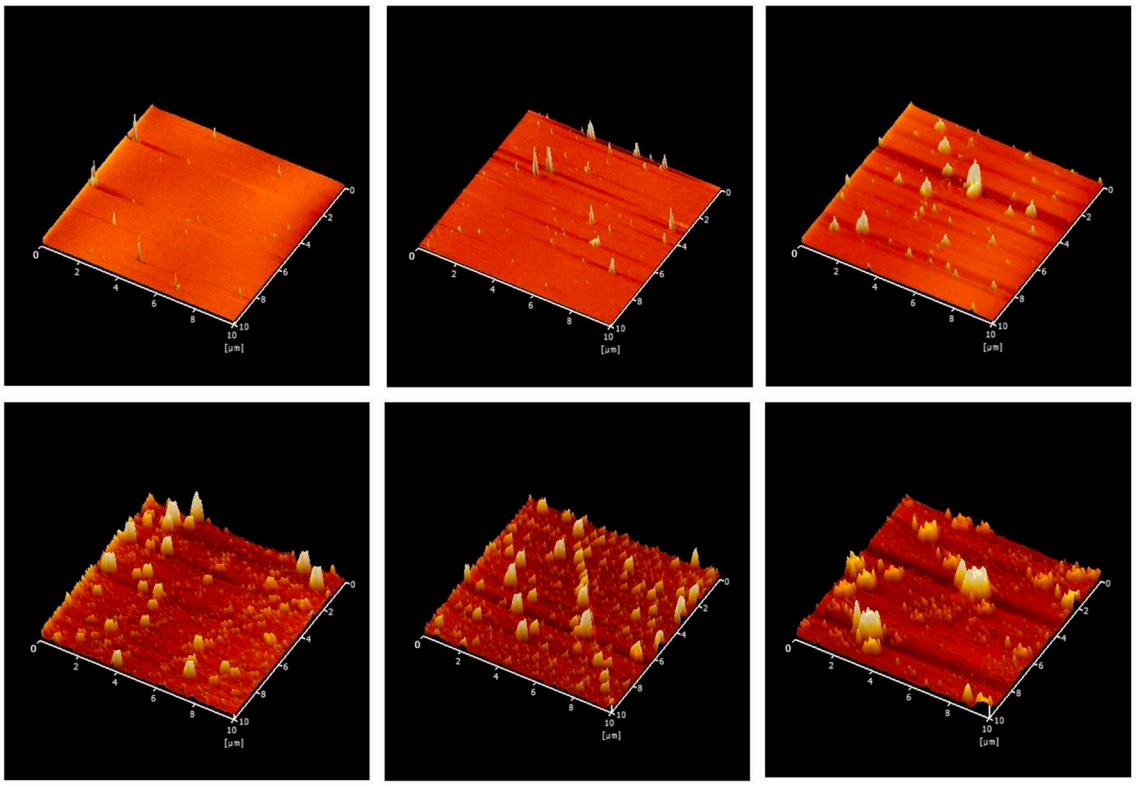

Fig. 6. The AFM surface morphology for six conditions for APTES modification (0.1%, 0.5%, 1%, 2%, 5%, and 10%) on Si_3N_4 surface.

roughness. This value is attributed to the agglomeration of APTES molecule on the Si_3N_4 substrate. This agglomeration of APTES molecule on the surface correlated to the silanization process mechanism which was described in Ref. [22]. The RMS values for 0.1%, 0.5%, 1%, 2%, 5%, and 10% were 0.81, 1.67, 2.45, 3.34, 3.42, and 8.11 nm, respectively. Fig. 6 shows the AFM surface morphology for the different APTES concentration modified on the Si_3N_4 substrate. There are a few agglomerations of APTES molecule on the 0.1% and 0.5% APTES modified Si_3N_4 substrate. A partially APTES agglomeration occurs on the 1% APTES treated on Si_3N_4 substrate. This indicated that the APTES molecules are partially polymerized in the solution prior to functionalize on the surface. There is multi-island of APTES agglomeration presented on 2% and 5% APTES leading to an increasing of their surface thickness. This can be affected to the sensitivity of ISFET measurement for detection of biomolecule. On the contrary, the image of 10% APTES morphology reveals extremely populated fine peaks as a result of the polymerization of APTES. As a result, the 1% APTES at a reaction of 60 min is an optimal condition for attachment of biomolecule on Si_3N_4 surface.

4. Conclusions

The efficiency of biosensor performance depends on the biomolecule immobilization method. The silanization base on APTES modification is a common method for attachment of biomolecule on silicon and silicon substrates, especially Si_3N_4 surface. Consequently, the effect of APTES concentration and reaction time which are the key parameter for APTES modification were studied. The results showed that the lowest and highest fluorescence intensity pixels were obtained at 10% and 0.1% APTES, respectively. The fluorescence intensity pixel also depends on the reaction time of APTES modification. Thus, six conditions for APTES modification which were 0.1%, 0.5%, 1%, 2%, 5%, and 10% at a constant reaction time of 60 min were selected to represent the impact between APTES layer thickness and the sensitivity of ISFET measurement. Although the fluorescence pixel intensity of 10% APTES was higher than of 1% and 0.1%, its electrical signal for ISFET measurement was lower than the others. This is due to the aggregation of APTES molecule and the longer distance between biomolecule and sensing membrane of ISFET or Debye screening length. It could be concluded that there is negative effect on the sensitivity of ISFET measurement with increase in APTES layer thickness. Therefore, the optimal APTES concentration and reaction time for biomolecule immobilization on Si_3N_4 surface were 1% and 60 min, respectively. This condition produces a thin and stable APTES layer with highly biomolecule immobilization which could be useful for ISFET based biosensor development.

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