Biosensors and Bioelectronics 67 (2015) 134–138

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Surface modification of silicon dioxide, silicon nitride and titanium oxynitride for lactate dehydrogenase immobilization

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ARTICLE INFO

Article history:
Received 9 April 2014
Received in revised form
4 July 2014
Accepted 22 July 2014
Available online 30 July 2014

Keywords:
Surface modification
Silanization
Immobilization
Lactate dehydrogenase
Silicon dioxide
Silicon nitride
Titanium oxynitride

ABSTRACT

Three different types of surface, silicon dioxide (SiO_2), silicon nitride (Si_3N_4), and titanium oxynitride (TiON) were modified for lactate dehydrogenase (LDH) immobilization using (3-aminopropyl)triethoxysilane (APTES) to obtain an amino layer on each surface. The APTES modified surfaces can directly react with LDH via physical attachment. LDH can be chemically immobilized on those surfaces after incorporation with glutaraldehyde (GA) to obtain aldehyde layers of APTES-GA modified surfaces. The wetting properties, chemical bonding composition, and morphology of the modified surface were determined by contact angle (CA) measurement, Fourier transform infrared (FTIR) spectroscopy, and scanning electron microscopy (SEM), respectively. In this experiment, the immobilized protein content and LDH activity on each modified surface was used as an indicator of surface modification achievement. The results revealed that both the APTES and APTES-GA treatments successfully link the LDH molecule to those surfaces while retaining its activity. All types of tested surfaces modified with APTES-GA gave better LDH immobilizing efficiency than APTES, especially the SiO_2 surface. In addition, the SiO_2 surface offered the highest LDH immobilization among tested surfaces, with both APTES and APTES-GA modification. However, TiON and Si_3N_4 surfaces could be used as alternative candidate materials in the preparation of ion-sensitive field-effect transistor (ISFET) based biosensors, including lactate sensors using immobilized LDH on the ISFET surface.

1. Introduction

The integration of biomolecules and microelectronics is being actively developed to achieve miniaturized devices (Sakata and Miyahara, 2005) for biomarker detection. Microelectronic devices using silicon dioxide (SiO_2), aluminum oxide (Al_2O_3), silicon nitride (Si_3N_4), tantalum pentoxide (Ta_2O_5), and tin oxide (SnO_2) have been investigated for their ability to incorporate protein (Lenci et al., 2011; Lue et al., 2011). Recently, titanium oxynitride (TiON) has been considered a promising candidate due to its prominent properties of high refractive index, high dielectric constant, chemical stability, and water insolubility (Bunjongpru et al., 2013). Because the permanent attachment of biomolecules onto solid substrates is a crucial factor for biosensor development, silanization has great potential as an approach to introduce reactive moieties of biomolecules onto inorganic surfaces using a surface treatment of organosilane. (3-aminopropyl)triethoxysilane (APTES) is extensively employed to functionalize surfaces with an amine layer (APTES surface) for immobilization of DNA, antibodies, and enzymes (Lenci et al., 2011; Yadav et al., 2014). There are three ethoxy ($-OCH_2CH_3$) groups in an APTES molecule. They can be hydrolyzed in aqueous environments or anhydrous organic solvents to form silanol groups (Si–OH) (Xie et al., 2010; Yadav et al., 2014). This group undergoes condensation with a hydroxylated surface via the hydrogen bond and then forms a siloxane (Si–O–Si) linkage over the treated surface. In addition, the condensation of neighbor APTES molecules also forms a polymer matrix linked by siloxane (Si–O–Si) bonds with a subsequent loss of water molecules by curing, leading to amino-terminated ($-NH_2$) surfaces (Gunda et al., 2014; Yadav et al., 2014). These amino ($-NH_2$) groups on APTES surface react with biomolecules via physical adsorption e.g., electrostatic force, hydrogen bonding, etc. (Lee et al., 2009). APTES-modified surfaces are convenient for protein immobilization on substrate surfaces; however, the immobilized protein may

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http://dx.doi.org/10.1016/j.bios.2014.07.057
0956-5663/

denature and be easily removed from the surface during continuous processes. To overcome these problems, covalent immobilization by coupling with glutaraldehyde (GA) is preferable to produce functionalized aldehyde groups (APTES-GA-modified surface) for direct chemical interaction with biomolecules via Schiff's base (C=N) formation (Diao et al., 2005).

The SiO_2, Si_3N_4, and TiON layers were chosen as the surface materials for the ion sensitive field effect transistor (ISFET) for the development of a biomarker detection device. Each type of ISFET surface material was investigated for the proper LDH immobilization method. The achievement of LDH incorporation into the ISFET surface will ensure the further development of a lactate sensor based on the ISFET device as the immobilization method affects the ability to incorporate target biomolecules. Therefore, each surface material treated with APTES or APTES coupled with GA was chosen to obtain a reactive surface for the incorporation of LDH molecules. The surface properties and functional groups of each modified surface were characterized using CA measurement, FTIR analysis, and SEM. The efficiency of LDH incorporation on each surface was determined for both the amount of protein and LDH activity. All these experimental results will be invaluable information regarding appropriate materials and methods in lactate sensor fabrication.

2. Experimental

2.1. Chemicals and reagents

Si_3N_4, SiO_2, and TiON surfaces were obtained from Thai Microelectronic Center (TMEC). The organosilane reagent 3-aminopropyltriethoxysilane (APTES), the cross-linker glutaraldehyde (GA), lactate dehydrogenase (LDH), pyruvate, and β-nicotinamide adenine dinucleotide reduced dipotassium salt (NADH) were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). The Bradford reagent for protein determination was purchased from Bio-Rad Laboratories Inc. (Hercules, CA, USA). All other reagents were commercially available, analytical reagent grade.

2.2. Surface modification

All surface materials were cut into small pieces (approximately 0.5 cm × 0.5 cm) and then cleaned with piranha solution (30% H_2O_2:H_2SO_4=1:3 v/v) at room temperature for 30 min. Then, the surfaces were thoroughly rinsed with deionized water and dried before being subjected to the silanization process. This reaction was carried out in 5% (v/v) APTES in ethanol at room temperature for 2 h. The samples were then rinsed with ethanol and dried at 60 °C overnight to obtain the APTES modified surfaces. The APTES-GA modified surfaces were prepared by immersing the APTES modified surfaces into a 2.5% GA solution in phosphate buffered saline (PBS) at room temperature for 1 h and washed with PBS. This APTES-GA modified surface contains aldehyde-functionalized layer. All different modified surfaces were evaluated for their wetting property, chemical bonding composition, and surface morphology by CA measurement, FTIR analysis, and SEM, respectively.

2.3. Contact angle (CA) measurement

The CA was measured using a goniometer, Model 250 from Rame-Hart Instrument Co. (Succasunna, NJ, USA) at room temperature using the static sessile drop method and image analysis of the drop profile. A droplet of deionized water was gently placed onto each surface and measured for its CA. An average value was obtained for each surface for three different regions on the same surface.

2.4. Fourier transform infrared (FTIR) analysis

The chemical bonding on each modified surface was analyzed by the Nicolet 6700 FTIR spectrometer from Thermo Fisher Scientific Inc. (Waltham, MA, USA) operating in attenuated total reflectance (ATR) mode. Each spectrum was recorded in the range 4000–625 cm^{-1}at a resolution of 2 cm^{-1} with 64 scans. The spectra were measured in three different regions on the same surface, and the untreated surface was recorded as a sample blank.

2.5. Scanning electron microscopy (SEM) observation

The surface morphology of the modified surface was observed by Hitachi S-4700 SEM from Hitachi High-Technologies Corp. (Tokyo, Japan) with an acceleration voltage of 5 kV. All surfaces were coated with gold to increase the conductivity prior to measurement. Each surface was measured in three different regions, and the untreated surface was recorded as a sample blank.

2.6. Enzyme immobilization

LDH (1 mg/ml) was prepared in 10 mM phosphate buffer saline (PBS, pH 7.4) and applied onto each modified surface at room temperature for 1 h. It was then gently washed with PBS to remove free LDH. The immobilization efficiency was evaluated in terms of protein content and the LDH activity presented on APTES modified surfaces and APTES-GA modified surfaces.

2.7. Protein determination

The amount of LDH attached on each modified surface was indirectly quantified using the Evolution 600 UV–Vis Spectrophotometer from Thermo Fisher Scientific Inc. (Waltham, MA, USA) by measuring the difference in protein content between the original LDH solution and the remaining supernatant after immobilization. This measurement was a colorimetric method at 595 nm using Bradford protein assay reagent with bovine serum albumin as the protein standard. The amount of immobilized LDH was calculated according to the previously reported by Bradford (1976). The immobilization efficiency of each surface in terms of relative immobilized LDH (%) was calculated using the difference in protein content between the original LDH solution and the immobilized LDH on the surface.

2.8. Enzyme activity determination

The LDH activity was determined by continuously monitoring the decrease of light absorption at 340 nm resulting from the oxidation of NADH by pyruvate using the Evolution 600 UV–Vis Spectrophotometers from Thermo Fisher Scientific Inc. (Waltham, MA, USA). The reaction mixture contained 10 mM PBS, pH 7.4, 28 mM of pyruvate, and 5.58 mM of NADH. Finally, the immobilized LDH on the surfaces was added and measured for enzyme activity every 30 s for 5 min, according to the method described by Henry (1974). The immobilized LDH activity yield was calculated in terms of relative immobilized LDH activity on the surfaces (%) using the difference in LDH activity between free and immobilized forms.

3. Results and discussions

3.1. Contact angle (CA) measurement

The measurements of static contact angle on SiO_2, Si_3N_4 and TiON surface modification by APTES and APTES-GA treatment are shown in Table 1. The untreated SiO_2, Si_3N_4, and TiON surfaces had CAs of $65.26 \pm 0.11°$, $73.42 \pm 0.48°$, and $77.98 \pm 0.77°$, respectively. Theoretically, the wetting property of a solid surface depends on the relationship between the interfacial tensions of water/air, water/solid, and solid/air (Ni et al., 2013). The initial CA values of all surfaces in this experiment imply a slightly hydrophobic surface due to organic or various particle contaminations. The CAs of piranha treated surfaces obviously presented hydrophilic properties with the decreased CA values of $21.78 \pm 0.25°$, $30.12 \pm 2.36°$, and $27.40 \pm 2.09°$. This piranha solution treatment allows an increase of the number of hydroxyl (–OH) groups or silanol (Si–OH) groups on the surface (approx. 10^{15} cm^{-2}) (Diao et al., 2005; Aswal et al., 2006). Large changes of hydrophobicity were found on all APTES-modified surfaces, with CA values of $85.54 \pm 0.32°$, $86.42 \pm 0.61°$, and $88.72 \pm 1.64°$. These changes were due to the replacement of the –OH groups by the amine ($–NH_2$) groups and the carbon backbones of the APTES molecule. There was no significant change of CA after additional treatment of the APTES modified surface with GA. This was also previously reported by Diao et al. (2005). The change of CA after modification can be considered an indicator of the change in surface property during the functionalization process (Gunda et al., 2014).

3.2. Fourier transform infrared (FTIR) analysis

FTIR spectroscopy was utilized to identify the chemical composition of the modified surfaces. The FTIR spectra of the modified surface after treatment with APTES and APTES-GA are presented in Supplementary material, Fig. S1–S3. The most important structure information regarding APTES films are found between 1800 and 900 cm^{-1}. All FTIR spectra of APTES modified surfaces showed similar features in the range of 1400–1700 cm^{-1} and 2800–3000 cm^{-1}. These are attributed to NH_2 bending and CH_2 stretching mode of the amino and ethoxy groups of APTES films, respectively (Kim et al., 2009; Gunda et al., 2014). In addition, the symmetric and asymmetric of NH stretching mode from amino groups in APTES films also occurred between 3250 and 3350 cm^{-1} but these bands are very weak intensity (Kim et al., 2009). However, the peak at 1195, 1080, and 960 cm^{-1} may be present in all modified surface spectra due to the unhydrolyzed ethoxy ($–OCH_2CH_3$) groups of APTES. In addition, the peak of the Si–O–Si vibration mode occurring around 1140 and 1020 cm^{-1} indicated that APTES had been grafted onto the surfaces (Tan et al., 2011). In all APTES-GA modified surfaces, bands of C=N vibration mode were revealed at 1580 cm^{-1}, resulting from the imine linkage between the surface amine group and one end aldehyde group in GA. In addition, bands of C=O were presented at 1710 cm^{-1} that attributed to the other end of aldehyde groups of GA, yielding aldehyde-terminated surface (Fernandez et al., 2008; Mura et al., 2012).

Table 1
The measurements of static contact angle on SiO_2, Si_3N_4 and TiON surface modification by APTES and APTES-GA treatment. For each surface, the reported value is averaged from three different regions on the same surface with standard deviation (SD) and coefficient of variation (%CV).

Process	Types of surface								
	SiO_2			Si_3N_4			TiON		
	Mean	SD	%CV	Mean	SD	%CV	Mean	SD	%CV
Native	65.26	0.11	0.17	73.42	0.48	0.65	77.98	0.77	0.98
Piranha treatment	21.78	0.25	1.14	30.12	2.36	7.84	27.40	2.09	7.63
Silanization	85.54	0.32	0.38	86.42	0.61	0.70	88.72	1.64	1.85
Cross-linking	85.26	0.67	0.79	80.10	0.23	0.29	81.80	1.04	1.27

3.3. Scanning electron microscopy (SEM)

The SEM micrographs before and after modification of SiO_2, Si_3N_4, and TiON surfaces is presented in Supplementary material, Fig. S4. The surface morphology of the ATPES modified surface showed a horizontal alignment of the APTES layer and packing density without any microscopic cracks or discontinuity in the film structure. This is consistent with the CA measurement and FTIR analysis that showed a larger CA after APTES deposition due to the hydrophobicity of the APTES molecule and the specific vibration mode in the different peaks of APTES molecules. In addition, the APTES-GA modified surface presented an additional aggregation layer after GA treatment.

3.4. The efficiency of LDH immobilization

There are two different methods for LDH immobilization on SiO_2, Si_3N_4, and TiON surfaces, which are APTES and APTES-GA treatments. The immobilization efficiency of LDH on APTES modified surfaces are $51 \pm 7.65\%$, $24 \pm 3.36\%$, and $46 \pm 2.34\%$ whereas the APTES-GA modified surfaces are $72 \pm 1.72\%$, $55 \pm 7.12\%$, and $64 \pm 4.99\%$ as presented in Fig. 1. The major role of LDH immobilization on the APTES modified surface is the electrostatic interaction because of the different isoelectric point (pI) between APTES and LDH. The pI value of APTES is reported to be about 8.7, which indicates that the net charge of APTES is positive at pH 7.4 (Lee et al., 2009). In contrast, the LDH has a negative charge at the same pH because its pI value is 5 (Wittig et al., 2010). Therefore, negatively-charged LDH adsorbed to the APTES-modified surfaces by electrostatic interactions (Lee et al., 2009; Vashist et al., 2014). Despite many advantages of physical adsorption, it also presents weak attachment on the supported surfaces. This drawback leads to poor operational stability (Betancor et al., 2006;

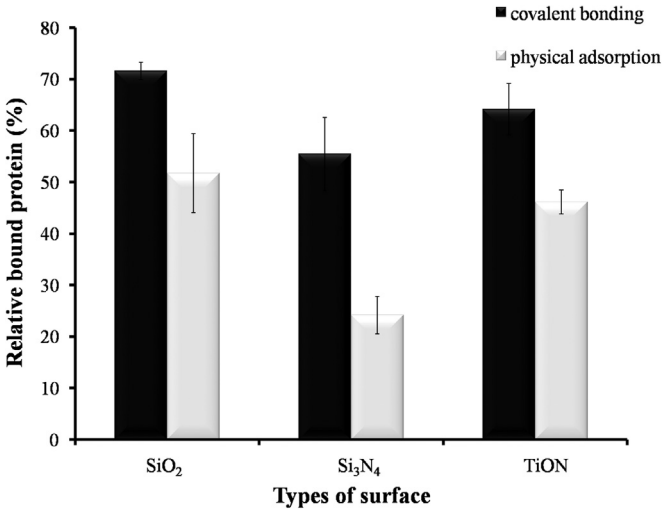

Fig. 1. The comparison of the relative protein content on SiO_2, Si_3N_4 and TiON surface between APTES treated surfaces and APTES-GA modified surfaces. LDH (1 mg/ml) was immobilized onto these modified surfaces and assayed by Bradford Protein Assay using colorimetric method at 595 nm with bovine serum albumin (BSA) as the protein standard. Each data set displayed the averaged of the results obtained for three independent experiments ($n=3$) on the each surface with coefficient of variation (%CV).

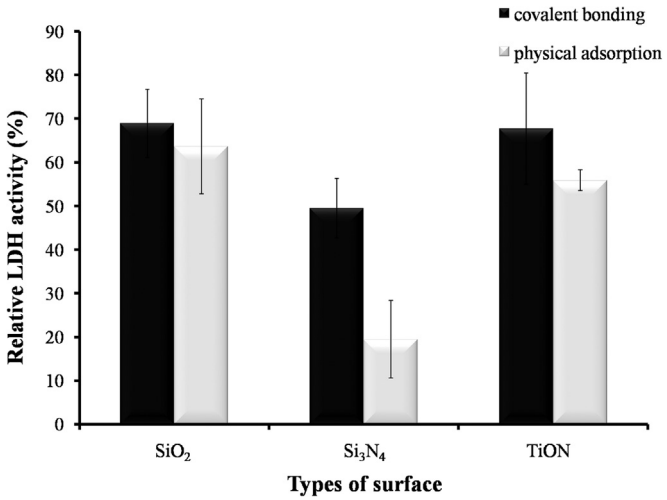

Fig. 2. The comparison of the relative immobilized LDH activity on SiO_2, Si_3N_4 and TiON surface between APTES treated surfaces and APTES-GA modified surfaces. The immobilized LDH activity was measured by a decrease in absorbance at 340 nm resulting from the oxidation of NADH by pyruvate. Each data set displayed the averaged of the results obtained for three independent experiments ($n=3$) on the each surface with coefficient of variation (%CV).

Smith and Chen, 2008; Niu and Jin, 2013). To overcome this problem, a coupling between APTES and GA (Niu and Jin, 2013) is preferred. The GA is a bifunctional cross-linker used to react with the surface amine group, yielding an imine linkage (C=N) with one end aldehyde group in GA. Then, the other end aldehyde group also reacts with the amine group of lysine residues on the exterior of LDH via imine (C=N) linkage. In addition, the type of immobilization also affects the stability of LDH attached onto each surface. Typically, all surfaces were triggered by the piranha treatment to introduce abundant surface hydroxyl groups (–OH) on the samples by hydroxylation process prior to silanization. These groups are very important factors for APTES deposition. As a result, the SiO_2 surface shows a higher level of immobilized LDH protein than the TiON and Si_3N_4 surfaces. This is consistent with the density of silanol groups on its surface. In addition, there are several types of silanol groups that can be formed during the process, as previously reported by Banuls et al. (2013). Therefore, this affects the amount of the available hydroxyl groups presenting on the surfaces that further react with APTES molecules. However, the amine groups of APTES layer can also react with the hydroxyl moieties on the surface via hydrogen bonding that are easily removed from the surface by rinsing in water, leading to the decrease number of available silanol groups and instability of APTES derived layers (Kim et al., 2009). Although LDH covalently linked with a GA-terminated surface via imine linkage shows a maximum level of immobilized proteins in all surfaces, it is not stable (Aissaoui et al., 2013) and could also induce random orientation resulting in a mixture of monomers and dimers. This process is related to the polymerization of GA that may influence the density of reactive groups on the surface. Moreover, the amount of immobilized protein on any surface is different due to the presence of the amine group on the exterior biomolecule, the orientation of the functionalized surface, incubation time, silane concentration, and also temperature (Barbosa et al., 2012).

A high content of LDH on the target surface does not truly indicate the success of the immobilization process. It is also necessary to determine LDH activity, which is the main function of immobilized biomolecules in an enzyme-based biosensor. Therefore, the immobilized LDH activity was also measured to determine the functionality of this enzyme after immobilization. The relative immobilized LDH activities on APTES modified SiO_2, Si_3N_4, and TiON surfaces were $63.64 \pm 10.86\%$, $19.45 \pm 8.87\%$ and $55.84 \pm 2.40\%$, respectively whereas the relative LDH activities of $68.88 \pm 7.83\%$, $49.99 \pm 6.81\%$, and $67.69 \pm 12.74\%$ were found on APTES-GA modified surfaces (Fig. 2). This immobilized LDH activity correlated with the protein content of immobilized LDH on each surface. However, the result for relative LDH activity was generally lower than the LDH protein content in the APTES-GA modified surfaces. This means that there is a partial loss of LDH activity during the chemical reaction in the immobilization process (Yusdy et al., 2009). The results discussed above suggest that these modified surfaces both of APTES and APTES-GA treatment can be used for LDH immobilization. In addition, the functionalized surface relies on the presence of aldehyde groups on APTES-GA treated surfaces may be useful for a wide variety of proteins than APTES treated surface (Diao et al., 2005). This observation is consistent with the results of Fernandez et al. (2008) and Diallo et al. (2013). The result showed the enzyme can functionalize after immobilization onto any solid surfaces. However, the amount of protein content also depends on the surface area because the enhance surface is accommodate more enzyme that previously described in Luo et al. (2004) and Fernandez et al. (2008). In addition, the different saturated protein content on any surfaces is dependent on the density of amino density on the exterior enzyme surfaces (Diao et al., 2005).

4. Conclusions

This study presents a very convenient process for surface modification of SiO_2, Si_3N_4, and TiON. All APTES-GA modified surfaces showed the higher level both of LDH content and activity than all APTES modified surfaces. Therefore, the APTES-GA functionalized surface is a promising option for LDH immobilization, especially on SiO_2 surfaces. SiO_2 surfaces showed higher protein content and LDH activity than both TiON and Si_3N_4 surfaces, but its stability in a harsh environment is limited. This recent result strongly supports that TiON and Si_3N_4 surfaces can be alternative candidate materials for enzyme immobilization for biosensor development. The evidence from this study supports the possibility of using TiON or Si_3N_4 for the surfaces of ISFETs in the core component of the lactate sensors.

Acknowledgments

This work was supported by the Office of the Higher Education Commission and Mahidol University under the National Research University Initiative. We gratefully acknowledge financial support from the Thailand Research Fund (TRF) for providing the Royal Golden Jubilee (RGJ-Ph.D program) Scholarship (PHD/0218/2553). Finally, we also would like to thank TMEC and NECTEC for kindly providing the measurement facilities.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2014.07.057.

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