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Potential control of horseradish peroxidase immobilization on gold electrode

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GE Cunwang[1,2], YU Wei[1], LI Changan[1], WANG Nanping[2] & GU Ning[1]

1. Key Laboratory of Molecular and Biomolecular Electronics (Southeast University), Ministry of Education, Research Center for Nano-scale Science and Technology, Southeast University, Nanjing 210096, China;
2. Department of Chemistry, Nantong Teachers College, Nantong 226007, China

Correspondence should be addressed to Gu Ning (email: guning@seu.edu.cn)

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Abstract A new approach based on potential control was firstly used for the immobilization of horseradish peroxidase (HRP) as the model protein. The self-assembly monolayer (SAM) was prepared with 2-aminoethanethiol (AET) on the gold electrode. The charge on HRP was adjusted by means of the acidity of the phosphate buffer solution (PBS) for dissolving the HRP. The influence of electric potential on HRP immobilization was investigated by means of colorimetric immunoassay of enzyme-substrate interaction (CIESI) using an automatic plate reader. The HRP modified electrodes were characterized with X-ray photoelectron spectroscopy (XPS) as well as atomic force microscope (AFM) on template-stripped gold surface. The potential for maximum immobilization of HRP was near the zero charge potential. The result indicates that controlled potential can affect the course of HRP immobilization without the loss of enzymic activity. It is of great significance for the control of biomolecular self-assembly and the intrinsic electric device.

Keywords: electric potential control, horseradish peroxidase, immobilization, gold electrode, self-assembly.

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Based on the self-assembled monolayer (SAM) technique, a number of methods for immobilizing protein onto electrodes have been recently reported, such as entrapment method in which the protein was wrapped with regenerated silk fibroin[1], self-assembled monolayer[2] and silica sol-gel[3], layer-by-layer self-assembly method in which the protein was adsorbed to opposite charged macromolecules due to electrostatic attraction[4], reversed micelle[5], cross-linked method[6] and surface spin-coating method[7]. In addition, self-assembled monolayer of protein is an exciting field, in which the response of enzyme sensor can be further improved. However, the enzyme must be modified, accompanying the loss of activity and interference to measurement.

Electric potential was basically applied to controling the electrophoretic mobility of protein. And it was applied to enriching the molecule so as to improve the sensitivity in electrochemical measurement[8,9]. The adsorption of human serum albumin (HAS) was enhanced under the electric field [10] on polysulfone microfiltration membrane, in which an increase in electric-field intensity led to a slight increase in adsorbed HAS at the pH above isoelectric point (IP). Zapien et al.[11] reported that ferritin adsorbed at fairly negative potentials via electrostatic interaction. Although there have been numerous studies concerning protein immobilization, there is no investigation to date virtually on controlled fabrication of protein. Herein, we present a novel protocol to immobilize

protein under the control of electric potential based on our potential control of DNA self-assembly[12,13]. The electrode was firstly derived with the SAM of AET with the amino-group stretching outside. HRP was immobilized on AET through the interaction between amino-group and HRP, which was adjusted by the charge on HRP through the acidity of the PBS for dissolving the HRP. With an automatic plate reader, the quantity of immobilized HRP on gold was evaluated by CIESI under different potentials and acidities. The influence of electric potential on HRP immobilization was investigated by means of XPS as well as AFM on template-stripped gold foil. And potential mechanisms were interpreted as well.

1 Materials and methods

1.1 *Materials and apparatus*

Chemicals used in the present paper include HRP (300 U/mg, IP 7.2, Shanghai Lizhu Biotechnical Co.), 2-aminoethanethiol (AET) (Sigma) and the substrate of 3, 3′, 5, 5′-tetramethylbenzidene (TMB)/ hydrogen peroxide (H_2O_2) (Sigma). The electrodes for CIESI measurement with the diameter of 0.5 mm wire (>99.99%) were sealed in a neutral glass tube. The gold foils for XPS measurements were obtained by vacuum evaporation of high-purity gold onto clean single-crystal silicon wafers, which had been precoated with chromium to improve adhesion. Flat gold foils for AFM experiments were prepared with template-stripped technique[14] with minor modifications. All other chemicals were of analytical purity. Millipore water was distilled twice, and sterilized in Milli-Q plus (Millipore Corp., MA). All solutions were deaerated with the purge of nitrogen at room temperature, unless otherwise stated. Potential controls were performed on a CHI 660A workstation (CH Instrument Co., Austin, TX). The electrochemical cell consisted of three electrodes with a gold or modified gold electrode as the working electrode, and a saturated calomel electrode (SCE) and a platinum wafer as the reference and the counter electrode, respectively. The optical density (OD) of assay was read with Stat Fax 2100 automatic micro plate reader (Stat fax-2100, International Immuno-Diagnostics Beiken Co.). XPS was performed with ESCALab MK2 spectrometer (VGScientific Ltd. UK) using an Mg Kα monochromatic X-ray source ($h\nu$ = 1253.6 eV). All spectra were calibrated in reference to the unfunctionalized aliphatic carbon C1s at binding energy of 284.6 eV with constant analyser energy of 20 eV. AFM images were collected with a Nanoscope IIIa microscopy (Digital Instruments, Santa Barbara, CA) with a tip of silicon nitride in tapping mode at room temperature. The scanning rate was 1 Hz.

1.2 Electric potential control of HRP immobilization *on gold modified with AET*

HRP is composed of 18% of glycoprotein with the active center of ferrous porphyrin[15]. The autoxidizations of the carbohydrate ring of glycoproteins to form the aldehyde group were studied by Beswick et al.[16] and Wolff et al.[17]. And HRP is abundant of carboxyl group and aldehyde group, which would possibly conjugate to the amino group of AET. The association of the HRP to AET surface is possibly due to electrostatic attraction and formation of Schiff base, respectively. The process of HRP immobilization is shown in fig. 1. The pretreated gold electrodes[13] were initially immersed into 1 mmol/L AET ethanol solution for 10 h. After immersion in ethanol for 4 h to desorb free AET, the electrodes were thoroughly washed with ethanol and distilled water, which were subsequently stored in distilled water till use and denoted as AET/Au. The electrodes were subsequently used as the working electrode under the controlled potential for a period of time in 0.01 mol/L PBS containing 0.1 mg/mL of HRP. The electrodes modified with HRP were then reined manually with 0.5% (volume percent) Tween 20 in 0.01 mol/L PBS (pH 7.4) for 15 min twice followed by soaking in aforementioned buffer for 30 min to remove any non-specifically adsorbed HRP. Finally, the electrodes were dried in a stream of pure nitrogen and kept at 4℃ for assay. The immobilization of HRP on open circuit is the same as the above except absence of applied potential. Likewise, all electrodes for XPS and AFM measurement were prepared similarly above, accompanying the control of the electrode area of about 45 mm^2.

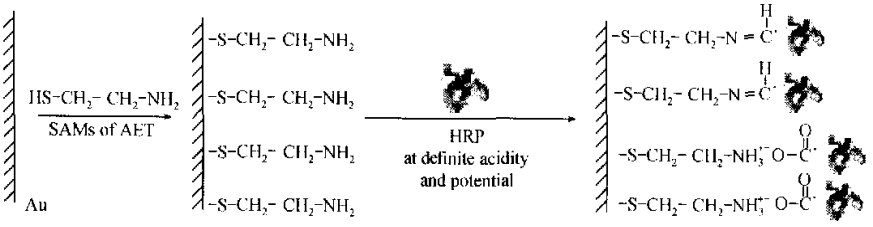

Fig. 1. Schematic diagrams of HRP immobilization under potential control.

1.3 Colorimetric assay of interaction between HRP and its specific substrate

The electrodes immobilized HRP were dipped into 5 mL test tube containing 0.2 mL substrate of TMB and 0.2 mL of hydrogen peroxide, followed by incubating at 37℃ for 20 min. After colour reaction was stopped by adding 0.05 mL terminator of 2 mol/L sulfuric acid, the substrate mixture of 200 μL was distracted into polystyrene microwell and then read the optical density (OD) at 450 nm in the reference wavelength of 630 nm with an automatic plate reader. All samples were tested duplicately, and arithmetical means and standard deviations of absorbance were calculated. The OD was applied to addressing the activity of enzyme.

2 Results and discussion

2.1 Selection of assembly time and buffer for electric potential control of HRP

To investigate the influence of time on HRP immobilization, HRP was dissolved in a pH 8.0, 0.01 mol/L PBS to the concentration of 0.1 mg/mL. The HRP-modified electrodes were prepared with different assembly times under open circuit. The property of the modified electrode was examined by CIESI with the result of OD. The OD with the assembled time of 30 min reached to about 95% height of that within 60 min, suggesting that potential controlled time of 30 min was used for the control potential. And for convenience, the concentration of HRP and ionic strength of buffer were set as 0.1 mg/mL and 0.01 mol/L phosphate buffer, respectively.

2.2 Influence of controlled potential on immobilization of HRP

To further investigate the influence of charge on electric potential-controlled immobilization of HRP, a charged prosthetic group was taken into consideration. Depending on the pH of the solution, charged prosthetic groups are changed according to the protonation-deprotonation equilibrium. Consequently, the charges on HRP were adjusted by pH of the buffer. The IP of HRP is 7.2, at which there is no net electric charge on a protein. Below the IP pH, the molecule is positively charged. While above the isoelectric pH, it is negatively charged. The HRP-modified electrodes were prepared at different controlled potentials and acidities of buffer, and compared with that at open circuit. Subsequently, the electrodes were evaluated by CIESI with the result of ODs. Fig. 2(a), (c) and (e) show the OD versus the controlled potential in pH 7.2, 6.4 and 8.0 PBS, respectively. The total charges of different potential-controlled immobilization were derived from the integration of curve of amperometric response versus controlled time (*i-t*) under potentiostatic mode with the software of electrochemistry workstation. Fig. 2(b), (d) and (f) show the OD versus the total charge in PBS of pH 7.2, 6.4 and 8.0, respectively. The current polarity is determined by the polarographic, positive reduction current. More positive current is corresponding to more reduce current and negative potential. The more negative the potential is, the more positive the current will be. Comprehensively, the following features are observed.

(i) Analyses of maximum OD. By comparing the controlled immobilization as HRP differently charged, the maximum OD appears at controlled po-

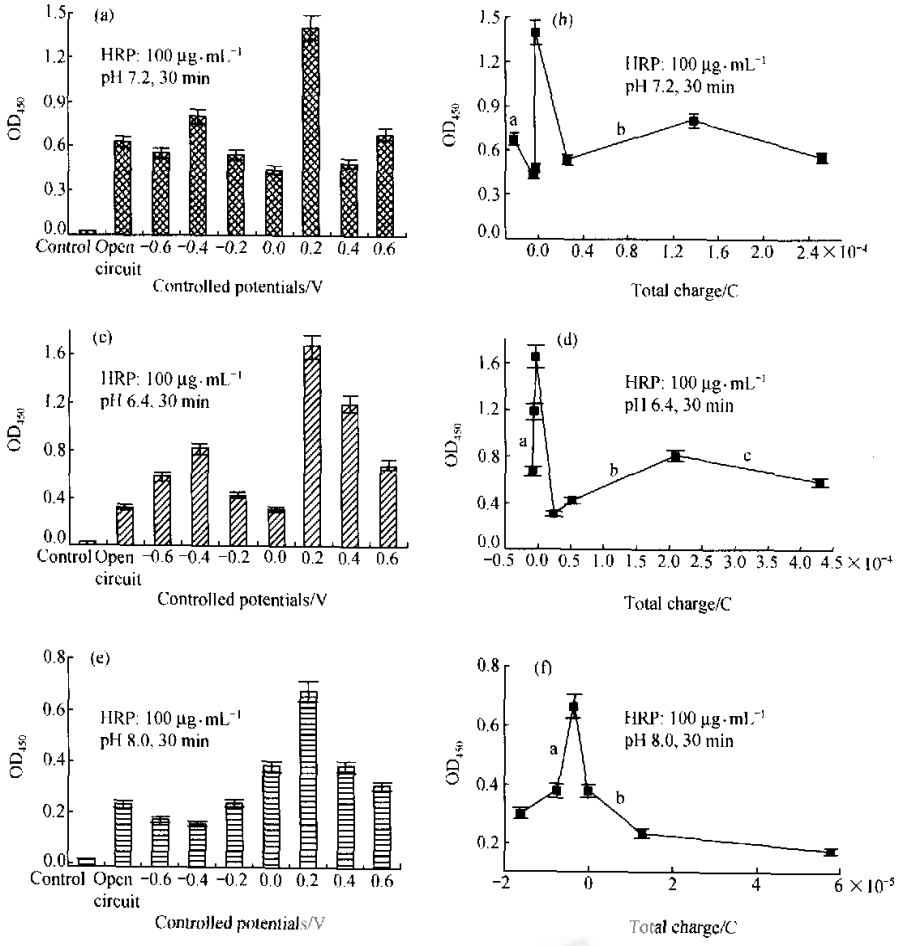

Fig. 2. Comparison of optical density for measuring of HRP electrode versus the controlled potential and total charge as HRP uncharged in pH 7.2 ((a) and (b)), positively charged in pH 6.4 ((c) and (d)) and negatively charged in pH 8.0 ((e) and (f)) phosphate buffer.

tential of +0.2 V versus SCE without exception. From fig. 2(b), at pH 7.2 as HRP naturally charged, the maximum OD appears at the total charge of -8.09×10^{-7}C, which is minimum point of absolute value of total charge of the differently controlled potential in the acidity. From fig. 2(d), when HRP is positively charged, the maximum OD appears at the absolute minimum for total charge of -1.53×10^{-6}C. Similarly, from fig. 2(e) as HRP negatively charged, the maximum OD appears at the absolute minimum for total charge of -3.40×10^{-6}C. Accordingly, the maximum OD appears at the absolute minimum of total charge as HRP differently charged. In the regime of present solution, the +0.2 V vs. SCE may be near the potential of zero charge (PZC), which is near the PZC of +0.5 V vs. NHS for similar electrode of Climent reported[18]. Although PZC is difficult to be measured for solid electrode with the adsorption complicated to interpreted oppositely to the dropping-mercury one, the maximums of OD may be interpreted according to absorption theory of organic molecule on double-layer[19]. The amount of adsorption rises with the decrease of boundary tension near PZC and maximum adsorption

appears at PZC. Amino group of AET will contact more carboxyl group and aldehyde group, which leads to more HRP immobilized on AET. On the other hand, as the charged course of double layer is neglectable compared with total one of controlled immobilization, the more the HRP immobilized on electrode, the more the prohibitive faradic current will be on electrode surface. The total charge will diminish consequently.

Comparing OD in fig. 2(c) and (e), we can also find that the maximum OD, as HRP positively charged, is more than that as HRP negatively charged. This can be interpreted with the status of amino group on AET. The amino group could be protonized to form $-NH_3^+$ in the acidic solution and associated with carboxyl on HRP via electrostatic interaction. While at IP (pH 7.2) and above IP (pH 8.0), the protonation of $-NH_2$ cannot be a patch on that at acidic solution. Hence, the maximum OD is smaller than that at acidic solution.

(ii) Effect of electrostatic interaction on HRP immobilization. In the regime of the potential control immobilization, the HRP were influenced by the electric field apart from the thermal motion. The double-layer between an electrode and an electrolyte solution could be modeled by Gouy-Chapman-Stern theory[19]. The excess charges in electrode phase were distributed on the surface of electrode and diffusion layer of charge present in electrolyte solution, which is thought as parallel-plate capacitor with the interval of d between two plates. At the distance of d, the surface charge of metal is completely balanced by oppositely charged ions in solution. By increasing the electrode surface potential or the electrolyte concentration, the diffuse layer thickness and the distance over which the double-layer electric field extends are reduced.

The electrostatic potential (Φ) at a distance x from an electrode surface can be related to the electrode surface potential (Φ_0) by $\frac{\tanh(ze\phi/4kT)}{\tanh(ze\phi_0/4kT)} = \exp(-\kappa x)$, where κ is defined as $\kappa = \left[\frac{2n^0 z^2 e^2}{\varepsilon\varepsilon_0 kT}\right]^{1/2}$. Here, n^0 is the bulk electrolyte concentration, Z is the charge of an electrolyte ion, e is the electronic charge, ε is the solvent dielectric constant, ε_0 is the permittivity of free space, k is the Boltzmann constant, and T is the absolute temperature. The reciprocal of κ is effectively the diffuse layer thickness. The expression of k reveals that this thickness could be controlled by adjusting the bulk electrolyte ion concentration. An ideal diffuse layer thickness is accurately defined by k, which may be applied during the initial stages of self-assembly. Increasing Φ_0 applied to an electrode will magnify $\Phi(x)$ experienced by a dipolar molecule and, thus, will amplify the field-induced molecular aligning process. Worley[120] once calculated the torque of polypeptide poly(γ-benzyl *L*-glutamate) in the electric field and found that the voltages larger than 200 mV did not significantly increase the field strength and, therefore, the torque on polypeptide molecule. Thus +0.2 V was nearly the optimal bias to provide the maximum orientational energy. The alignment of HRP will be regular with maximum activity. In addition, applying much larger voltages might induce molecular chemical changes as the result of redox process.

At pH 7.2, as HRP neutrally charged, the controlled potential shows less effect on the OD, except the maximum immobilization at a bias of +0.2 V. The controlled current is an attribute of moving of the ions and electric transfer of faradic course. In the solution of pH 6.4 as HRP positively charged, from –0.4 to +0.2 V, the OD decreases with the increase of controlled potential and a minimum arises at 0 V. From 0.2 to 0.6 V of the controlled potential, a gradual decrease of OD is also observed. This could be interpreted with the influence of electrostatic interaction. The more negative the controlled potential is, the faster the HRP molecule will approach the AET with HRP positively charged, if the electrostatic interaction is considered. Nevertheless, a decrease was found as the controlled potential negatively shifts from –0.4 to –0.6 V and the maximum OD appears at –0.4 V. This might be interpreted with the influence of homogeneous intermolecular repulsion. If the HRP molecules approach the AET excessively fast under more negative potential, they will rebound and leave the AET with the intermolecular repulsion. Therefore, the intermolecular repulsion should be considered in the

controlled potential immobilization as well as the interaction between electrode and HRP. On segments a and b in fig. 2(d), electrostatic interaction between electrode and HRP has the advantage over the intermolecular repulsion. And intermolecular repulsion is advantaged as the potential negatively to –0.4 V (on the segment c in fig. 2(d)).

Similarly, at pH 8.0 when the HRP is negatively charged, the more positive the controlled potential is, the faster the HRP molecule will approach the surface of AET with HRP negatively charged, if the electrostatic interaction is considered. On segment b in fig. 2(f), electrostatic interaction between electrode and HRP has the advantage over the intermolecular repulsion as controlled potential between –0.4 and +0.2 V. But, as the potential positive to 0.2 V, the decrease in OD is found, in which intermolecular repulsion of HRP should be considered as well. The intermolecular repulsion is advantaged on the segment a in fig. 2(f).

(iii) Other interaction on HRP immobilization. Apart from the electrostatic interaction, intermolecular dipole-dipole interaction and induced dipole moment may play an equally important role when the potential is rather negative[21]. The redox of electrode should also be considered as the electrode potential positive to +0.5 V vs. NHS (in fact 0.275 V vs. SCE), $Au+4OH^- = AuO_2^- + 2H_2O + 3e$. As the alkyl chain of AET is too short to form compact monolayer for protecting the electrode from oxidation, the product of oxidation AuO_2^-, in turn, interacts with negatively charged HRP and influences the immobilization. The segment a in fig. 2(f) could be influenced by repulsion between AuO_2^- and negatively charged HRP.

In addition to the factors above-mentioned, there are many other factors that affect the immobilization of protein under controlled potential, such as hydrophobic interaction, intrinsic electric field of HRP and geometric effects[22]. The intrinsic fields are generated by the charges or by the polar group in apoprotein. These so-called pocket fields can be orders of magnitude stronger than external fields. The geometric effects, in turn, affect the electrostatic effects.

2.3 XPS measurement for HRP-modified electrode under controlled potential at pH 8.0

In order to study the influence of controlled potential on enzymic activity, the samples at controlled potential of +0.2 and –0.4 V, which correspond to maximum and minimum OD, respectively in pH 8.0, were measured by XPS as well as the sample at open circuit control under the control of electrode surface of 45 mm^2. According to the structure of HRP[23], the central iron ion complexes with oxygen atoms on water molecule and nitrogen atoms on imidazole ring. The normalized peak areas of the XPS windows for Au4f, C1s, N1s, O1s, S2p and Fe2p are shown in table 1. Compared with relative oxygen percentages, a minimum content of 10.75% was presented for the electrode controlled at –0.4 V. While that at +0.2 V is as high as 21.23%. The measurement clearly indicates that the coverage for controlled electrode at +0.2 V is the utmost of the three electrodes. From table 1, the iron content of 0.91% for electrode controlled at +0.2 V is higher than that at –0.4 V in the limit of XPS sensitivity, which also indicates the maximum immobilization at +0.2 V. For the electrode controlled at +0.2 V, the minimum content of sulfur and the maximum content of gold are present. All these elementary analyses further indicate the maximum immobilization at +0.2 V. These results also indicate that the amount of HRP is the most at controlled potential of +0.2 V, which is essentially identical to the OD tendency in fig. 2(e). It also suggests that the OD for CIESI can quantify the HRP on electrode. The minimum OD is origin of fewer amount of HRP other than loss of enzymic activity.

Table 1 Relative HRP film elemental percentages and binding energy determined by XPS

Peak ID	AT(%)			Center/eV		
	Control –0.4 V	Open circuit	Control 0.2 V	Control –0.4V	Open circuit	Control 0.2 V
C 1s	50.67	52.74	48.73	284.6	284.6	284.6
O 1s	10.75	17.08	21.13	532.25	531.9	531.7
Au 4f	27.72	24.08	22.14	83.4	83.2	83.15
S 2p	3.16	2.16	2.30	161.2	161.35	161.25
N 1s	7.70	3.93	4.79	399.8	398.95	399.5
Fe 2p	0	0	0.91	0	0	710.6

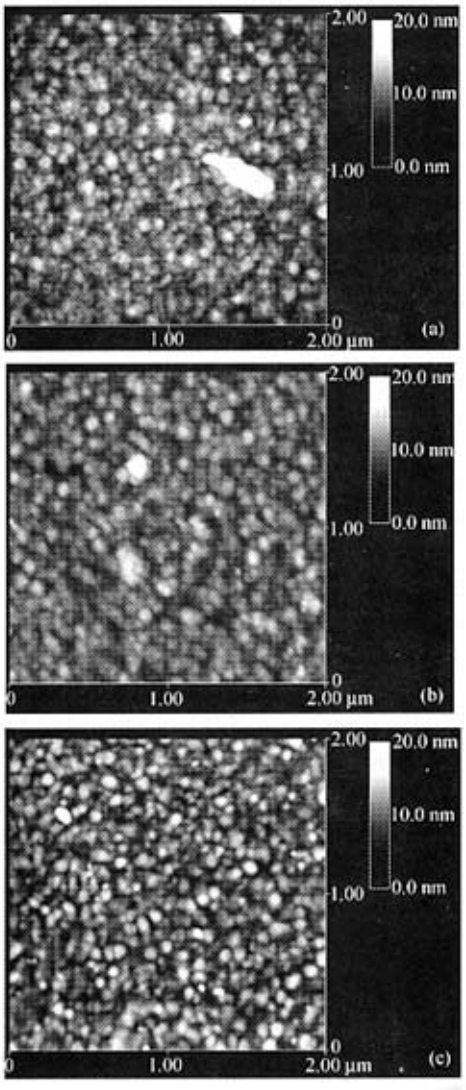

Fig. 3. Tapping mode AFM images for gold foil modified with HRP at +0.2 V(a), open circuit (b) and –0.4 V(c).

2.4 AFM images for HRP modified electrode under controlled potential at pH 8.0

At pH 8.0, as HRP was negatively charged, the maximum optical density appeared for the electrode controlled at +0.2 V, while the minimum at –0.4 V. To correlate with our characterization of OD and XPS, we directly visualized the immobilized HRP on template-stripped gold foils, and more importantly determined the presence of HRP. As shown in fig. 3, atomic force microscope (AFM) was employed to examine gold foil modified with HRP at +0.2 V(a), open circuit (b) and –0.4 V(c). The topology of HRP-modified electrode at +0.2 V is flocky, and compact with the mean roughness (RMS) of 2.420 nm, while that controlled at –0.4 V is of roughness and the grains with the radius of ~20 nm are clearly discernable with the RMS of 1.781 nm, which shows little immobilization of HRP at the controlled potential of –0.4 V. These results are consilient to the XPS ones.

3 Conclusion

This paper demonstrates that the controlled potential, as well as pH of the buffer solution, can affect the immobilization. The potential for maximum immobilization of HRP is near the zero charge potential. The electrostatic interaction should be considered when the controlled potential is between –0.4 and +0.2 V. As the controlled potential positive to 0.2 V, the dipole-dipole interaction, induced dipole interaction and redox interaction should be taken into account. According to the results of XPS and AFM measurement, the optical density of colorimetric immunoassay of enzyme-substrate interaction could be applied to quantifying the HRP on electrode. The immobilization under controlled potential is of great significance for the control immobilization of biomolecule, which may be very useful in the field of biomolecule-based devices studies. Due to the simplicity, practicality, and controllability of this method, it will be widely applied to the development of blood-contacting devices for both medical implants and bioaffinity sensors.

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