Int. J. Electrochem. Sci., 8 **(2013)** 2459 - 2467

International Journal of
ELECTROCHEMICAL
SCIENCE
www.electrochemsci.org

Probing of EDC/NHSS-Mediated Covalent Coupling Reaction by the Immobilization of Electrochemically Active Biomolecules

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Received: 17 December 2012 / *Accepted:* 3 January 2013 / *Published:* 1 February 2013

1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide / *N*-hydroxysulfosuccinimide (EDC/NHSS)-mediated two-step amine coupling reaction is one of most attractive approaches for the elaboration of affinity biosensors. In this work, we investigated the EDC/NHSS-mediated reaction on 3-mercaptopropionic acid (MPA)-covered electrode by the immobilization of electrochemically active biomolecules (dopamine or DA, amyloid-β–Cu^{2+} or Aβ–Cu^{2+} and Laccase). The amino groups of DA molecules and lysine residues of Aβ/Laccase make these analytes easy for the reaction with EDC/NHSS-activated MPA self-assembled monolayer (SAM). Effects of EDC/NHSS concentration ratio, pH value, activation/coupling time and analyte concentration on the coupling efficiency were investigated by cyclic voltammetry. The optimal conditions for the EDC/NHSS-mediated amine coupling reaction were reported. We believe that this work will be valuable for the immobilization of biomolecules and the fabrication of affinity biosensors.

Keywords: affinity biosensors; carbodiimide; *N*-hydroxysulfosuccinimide; self-assembled monolayer; cyclic voltammetry

1. INTRODUCTION

The elaboration of affinity biosensors is probably one of the most promising ways to solve some of the problems concerning sensitive, fast and low-cost measurements. Attachment of biomolecules on different surfaces is the pivotal part of research for the development of affinity biosensors. Self-assembled monolayer (SAM) is an excellent platform to develop biosensors because it offers a good control at molecular level [1,2]. The exposed functional terminus of SAM is utilized for

the attachment of biomolecules through covalent bond formation or non-covalent interaction. Comparatively, covalently attached molecules survive such repetitive operations over a longer period of time. Among kinds of covalent coupling reactions, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide / *N*-hydroxysulfosuccinimide (EDC/NHSS)-mediated reaction is one of most attractive approaches and has been widely applied in the conjugation of biomolecules onto different substrates because of its high conversion efficiency, mild reaction condition and excellent biocompatibility with little influence on the bioactivity of target molecules [2-10]. However, most researchers accepted the general rule that carboxylic acids were activated to NHSS-esters by EDC/NHSS without considering the reaction conditions; the results clearly presented some differences depending on the experimental conditions used for the activation treatment. For example, the concentrations of EDC and NHSS used in the references span a wide range (from a few 0.1 M down to the mM range) [8,9,11-14], the relative concentrations of EDC and NHSS strongly vary from one study to another one [8,9,13,15,16], and the temperature as well as pH values seem to influence the coupling efficiency [8,9]. In fact, the amine-reactive *O*-acylurea intermediates resulting from the activation of carboxylic acids by EDC not only react with amine groups, but also undergo hydrolysis to regenerate acid groups, dehydration with neighboring carboxylic acids to produce anhydride and intramolecular acyl rearrangement to produce *N*-acylurea (Fig. 1) [8,9].

Previously, several analytical techniques, such as quartz crystal microbalance (QCM), infrared (IR) spectroscopy, electrochemistry, UV-vis absorption spectroscopy, ellipsometry, surface plasmon resonance (SPR), transmission electron microscopy (TEM), atomic force microscopy (AFM) and scanning tunneling microscopy (STM), have been used to characterize SAM on different kinds of substrates, determine the thickness of the layer and visualize the thin film in molecular resolution [1]. Among these techniques, voltammetric technique is simple, sensitive and inexpensive to implement [17-19]. Biomolecules (protein, peptide, nucleic acid or small molecules) binding on the SAM will cause the generation or consumption of an electrochemically active molecule, or change the resistive or capacitive properties of the thin film. As a result, the signal can be detected electrochemically and the signal intensity depends strictly on the amounts of immobilized/captured molecules. In this work, we investigated the immobilization of different molecular size of biomolecules on 3-mercaptopropionic acid (MPA)-covered gold electrode through EDC/NHSS-mediated two-step amine coupling reactions. Dopamine (DA) is an electrochemically active monoamine compound. The amino group of DA makes it easy for the reaction with NHSS ester. We first investigated the effects of EDC/NHSS concentration ratio, pH value, activation/coupling time and DA concentration on the coupling efficiency by monitoring the current change of DA-modified electrode. Furthermore, the efficiency for the immobilization of large biomolecules was addressed.

2. EXPERIMENTAL

2.1 Chemicals and materials

EDC hydrochloride, NHSS, Laccase and MPA were acquired from Sigma-Aldrich. Dopamine hydrochloride was obtained from Sangon Biotech. Co., Ltd. (Shanghai, China). Peptide Aβ(1–16)

(DAEFRHDSGYEVHHQK), the copper-binding fragment of amyloid-β (Aβ), was synthesized by ChinaPeptides Co., Ltd (Shanghai, China). The Aβ(1–16)–Cu^{2+} complex was prepared by mixing Aβ(1–16) with 2-fold excess Cu^{2+} to ensure that all of Aβ(1–16) is in the Aβ(1–16)–Cu^{2+} state. Other chemicals were analytical-grade reagents and were used without further purification. All stock solutions were prepared daily with deionized water and saturated by N_2.

2.2 Procedures

Prior to each measurement, the gold disk electrodes were polished with diamond pastes down to 3 μm and alumina pastes down to 0.3 μm and subsequently sonicated in water. The MPA SAM was formed by immersing the cleaned electrode in a 10 mM MPA solution in the dark for 12 h. Then, the electrode was flushed with excess water and stored in a refrigerator at 4 °C for use. For the EDC/NHSS-mediated amine coupling reaction, the MPA-covered electrode was soaked in EDC/NHSS solution, followed by rinsing the electrode with excess water. Then, the electrode was allowed to react with target molecule (DA, Aβ(1–16)–Cu^{2+} or Laccase). After the electrode had been rinsed with water, voltammetric determination in phosphate-buffered saline solution (PBS buffer, 10 mM, pH 7.0) containing 50 mM Na_2SO_4 was performed on a DY2013 electrochemical workstation (Digi-Ivy, Inc., Austin, TX) using a homemade plastic electrochemical cell. A platinum wire and a Ag/AgCl electrode were used as the auxiliary and the reference electrodes, respectively.

3. RESULTS AND DISCUSSION

3.1 Principle of probing of the covalent coupling reaction

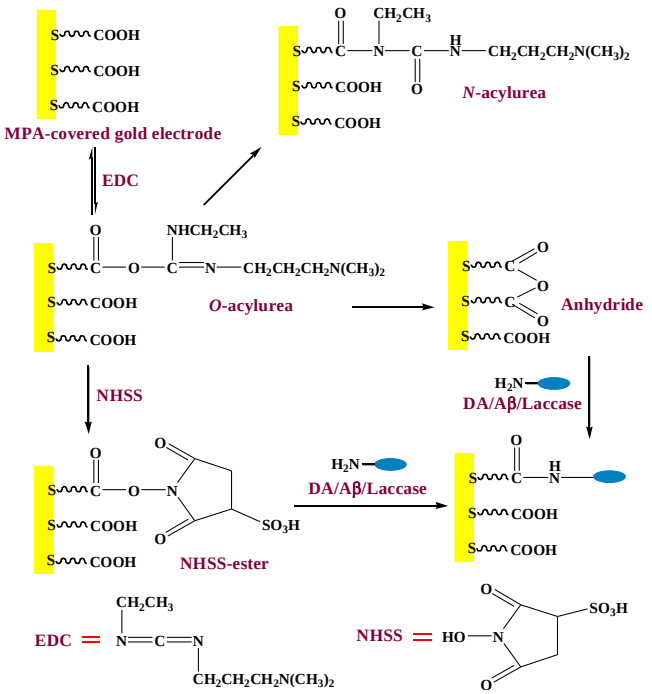

Figure 1. Scheme of EDC/NHSS-activated amine coupling reactions.

The analysis principle is shown in Fig. 1. It is based on measurements of the electrochemical response of gold electrodes modified with electrochemically active biomolecules. Once the carboxyl groups of MPA SAM are activated by EDC/NHSS to produce NHSS-ester, electrochemically active biomolecules will be immobilized onto the electrode through the formation of covalent bond, resulting in the occurrence of the reduction and oxidation reactions. The magnitude of the increase in peak current depends on the amount of immobilized target molecules which is related to the amount of NHSS-ester intermediates. Since the *O*-acylurea intermediate is prone to undergo hydrolysis to regenerate acid groups, dehydration with neighboring carboxylic acids to produce anhydride and intramolecular acyl rearrangement to produce *N*-acylurea, the activation conditions (e. g. EDC/NHSS concentration ratio, pH value, activation time) will be investigated by monitoring the change of peak current. Moreover, we will investigate the influence of pH and analyte concentration on the reaction between analyte and NHSS ester due to the dependence of NHSS-ester activity on solution pH. Molecule weigh of biomolecules would probably influence the immobilization efficiency. Herein, electrochemically active DA, Aβ(1–16)–Cu^{2+} or Laccase were used as the model analytes with different molecular sizes to study the coupling reaction. The amino groups of DA molecules and lysine residues of Aβ/Laccase make these analytes easy to react with EDC/NHSS-activated SAM.

3.2 Cyclic voltammetry of DA-modified electrode

The reactivity of EDC/NHSS-activated MPA SAM on gold electrode towards the coupling of amine compounds was first investigated with DA. The cyclic voltammetric response of immobilized DA is shown in Fig. 2. No redox peak was observed at EDC/NHSS-activated electrode in PBS solution (blue curve).

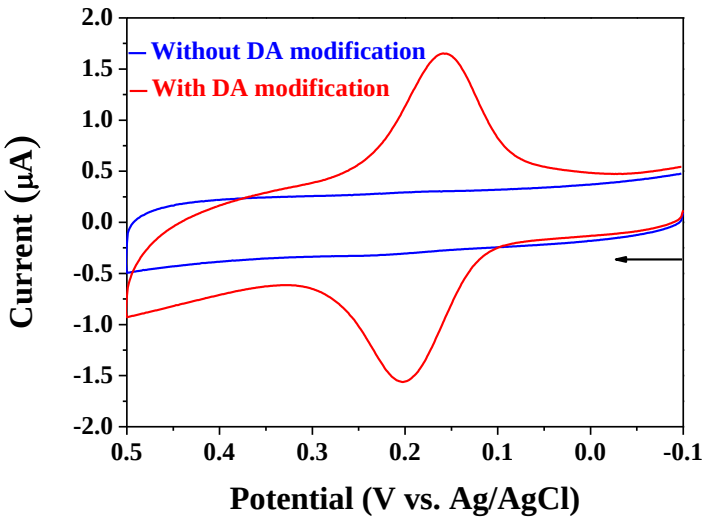

Figure 2. Cyclic voltammograms (CVs) of EDC/NHSS-activated MPA-covered electrode in PBS solution with and without DA modification. The concentrations of EDC, NHSS and DA were 40, 40 and 5 mM, respectively. The electrode was activated by EDC/NHSS for 20 min at room temperature and allowed to react with DA for 30 min in 20 mM PBS (pH 7.5). The solid arrow indicates the scan direction and the scan rate was 100 mV/s.

The redox peaks with Epa = 0.203 V and Epc = 0.158 V in the red curve were attributed to the two-electron, two-proton redox transformation of DA hydroquinone/quinine [20,21], indicating that DA molecules were immobilized successfully on the electrode through the EDC/NHSS-mediated amine coupling reaction.

3.3 Influence of molar ratios of EDC to NHSS and temperature during activation

As shown in Fig. 1, *O*-acylurea intermediate, the product of EDC-activated carboxylic acid, is susceptible to hydrolysis, dehydration and rearrangement into inert *N*-acylurea. To increase the efficiency of EDC-mediated coupling, NHSS was most frequently used to react with the *O*-acylurea intermediate to produce a relatively stable amine-reactive NHSS-ester for the two-step amine coupling reaction. However, the absolute and relative concentrations of EDC and NHSS used in the literatures vary from one study to another one [8,9,11-13].Therefore, we investigated the effect of concentration ratio of EDC/NHSS on the DA immobilization on MPA SAM. Since EDC at the concentration of 40 mM activates all of the carboxylic acids within 20 min [22], we adapted the EDC concentration at 40 mM and the activation time of 20 min for investigation of the influence of NHSS concentration. Fig. 3A shows the dependence of the oxidation current (I_{pa}) of DA-modified electrode on the EDC/NHSS concentration ratio. It can be seen that the I_{pa} reached a maximum at the ratio of 2:1. The optimal ratio is distinguishing in the previous studies, which is probably due to the difference of SAM substrates used [8,9]. The decrease of activation efficiency at the low ratio such as 1:2 is attributed to the incomplete transformation of *O*-acylurea to NHSS-ester because the residuary *O*-acylurea would be transferred partly into carboxylic acid, anhydride or inert *N*-acylurea. Note that *N*-acylurea is a stable product and cannot react with DA; anhydride can undergo further reaction with NHSS to produce the NHSS-ester or primary amine to produce the amide accompanied by the regeneration of an acid group. Therefore, the generation of anhydride and *N*-acylurea reduced the coupling efficiency. The slight decrease at high ratio such as 3:1 is most probably due to precipitation of reactants on the electrode surface which somehow blocks the surface reaction [8,9].

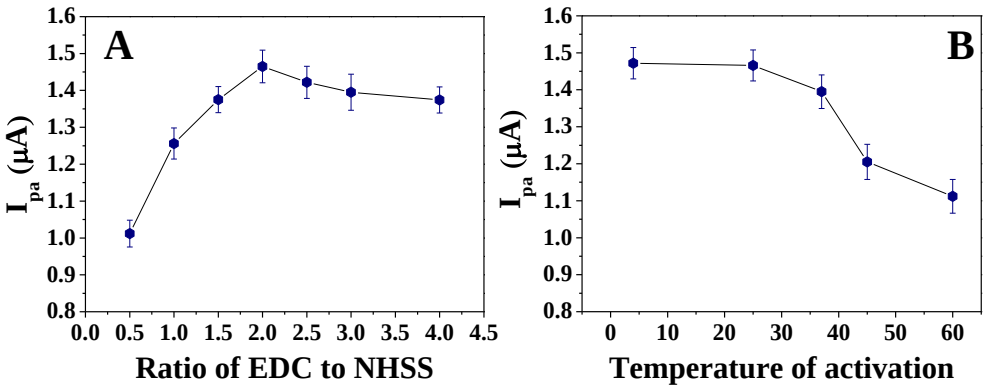

Figure 3. Effects of EDC/NHSS ratio (A) and temperature (B) on the activation efficiency. The temperature was 25 ºC in panel A and the EDC/NHSS ratio was 2:1 in panel B. The other experimental conditions are the same as those in Fig. 2.

Temperature plays an important role in chemical reactions. It is suggested that high temperature probably promoted the hydrolysis of EDC, *O*-acylurea intermediate, NHS-ester, and acyl transfer from *O*-acylurea to *N*-acylurea, decreasing the activation efficiency [9]. We also investigated the effect of temperature on the activation efficiency. Fig. 3B shows the dependence of the oxidation current (I_{pa}) of DA-modified electrode on the activation temperature. The high I_{pa} at 4 and 25 ºC indicates the relatively high activation efficiency. With the increase of activation temperature, the I_{pa} gradually decreases beyond 25 ºC. Therefore, the preferential temperature for activation is in the range of 4 ~ 25 ºC.

3.4 Effect of pH on the activation/coupling reactions

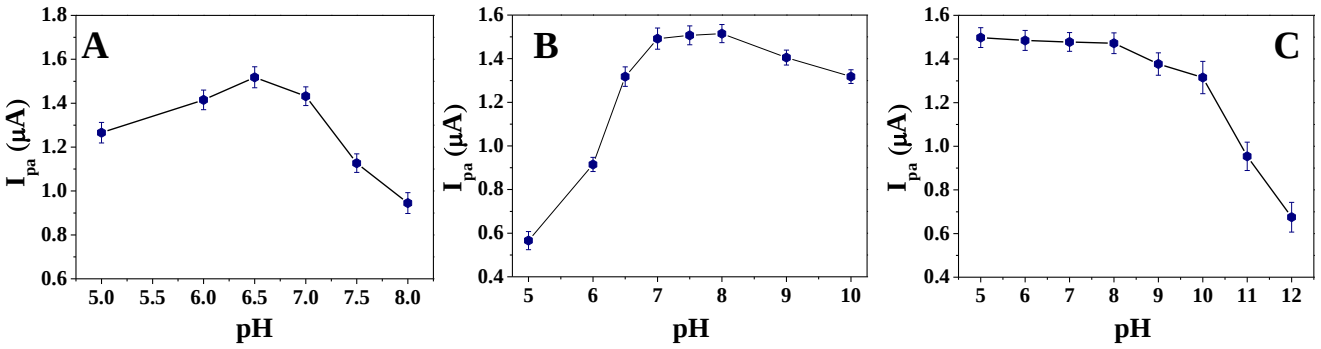

Figure 4. Effects of pH on the EDC/NHSS activation (A) as well as DA coupling (B) reactions and on the stability of NHSS ester (C). The concentrations of EDC, NHSS and DA were 40, 80 and 5 mM, respectively. In panel A, the EDC/NHSS-activated electrode was allowed to react with DA at pH 7.5 for 30 min. In panel B, the EDC/NHSS-activated electrode was activated by EDC/NHSS at pH 6.5 for 20 min. In panel C, the EDC/NHSS-activated electrode was pretreated with PBS for 2 h and then allowed to react with DA at pH 7.5 for 30 min.

It is reported that activity of EDC and stability of *O*-acylurea as well as NHSS-ester depend on solution pH [22-24]. We further investigated the influence of pH on the activation efficiency and DA conjugation. As shown in Fig. 4A, for the activation of carboxyl groups, the optimal pH is around 6.5, which is consistent with that reported previously. For the immobilization of DA, the preferential pH was in the range of 7 ~ 8 (Fig. 4B). The result is understandable since amine group of DA exhibits poor nucleophilic capability to NHSS-ester due to the formation of a protonated amino group at low pH. At high pH, DA has strong nucleophilic capability but NHSS-ester is susceptible to hydrolysis. To prove this hypothesis, we investigated the stability of NHSS-ester by pretreating the NHSS ester-modified electrodes in PBS solutions with different pH for 2 h and then soaking the electrodes in DA solution to couple DA. The results shown in Fig. 4C indicated that the NHSS-ester was stable in acidic and neutral pH solutions but lost the reactivity in basic solutions due to the spontaneous hydrolysis. Moreover, we found that the oxidation peak current decreased by only 13% after pretreatment of the NHSS ester-modified electrode with pH 7.5 PBS solution for 12 h (data not shown).

3.5 Influence of DA concentration and reaction time

For the fabrication of affinity biosensors, biomolecules (e.g. antibody and DNA) are usually immobilized at low concentration (micromolar to millimolar). Therefore, we investigated the effects of DA concentration and reaction time on DA immobilization. Fig. 5 shows the relationship between oxidation current and reaction time in the presence of different concentrations of DA. It is clearly seen that the immobilization efficiency of DA depends on the concentration. The plateaus exhibited by the curves are indicative of the saturation of DA on the electrode surface. When the concentration of DA was 5 mM, the coupling reaction completed within 15 min.

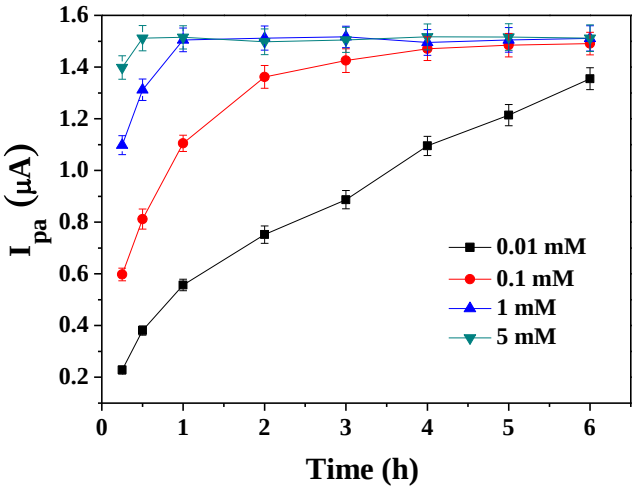

Figure 5. Time-dependence of oxidation current obtained after covering the EDC/NHSS-activated electrode with DA at a concentration of 0.01 (-■-), 0.1 (-●-), 1 (-▲-) and 5 (-▼-) mM. The electrode was activated by EDC/NHSS (2:1) at pH 6.5 for 20 min and then allowed to react immediately with DA (pH 7.5) at room temperature.

3.6 Immobilization of Aβ(1–16)–Cu^{2+} and Laccase

Amine groups of lysine residues of proteins are widely used for anchoring proteins to SAM on surfaces through the formation of stable amide bond. Under the optimal conditions mentioned above, we investigated the immobilization of electrochemically active biomacromolecules, Aβ(1–16)–Cu^{2+} and Laccase. As shown in Fig. 6A, The electrochemical response of a NHSS ester electrode modified with Aβ(1–16)–Cu^{2+} reveals a clear redox peak centred at 93 mV, which is close to that of our previous report [25,26].The couple of redox waves was attributed to the reduction/oxidation of copper center in the Aβ(1–16)–Cu^{2+} complex. The cyclic voltammetric peaks in Fig. 6B are assigned to the redox process of the copper centre embedded in Laccase. The redox potential (130 mV) is close to the reported value [21,27]. The currents increased with the immobilization time and began to level off beyond 9 h, indicating that the SAM surface was saturated by Laccase.

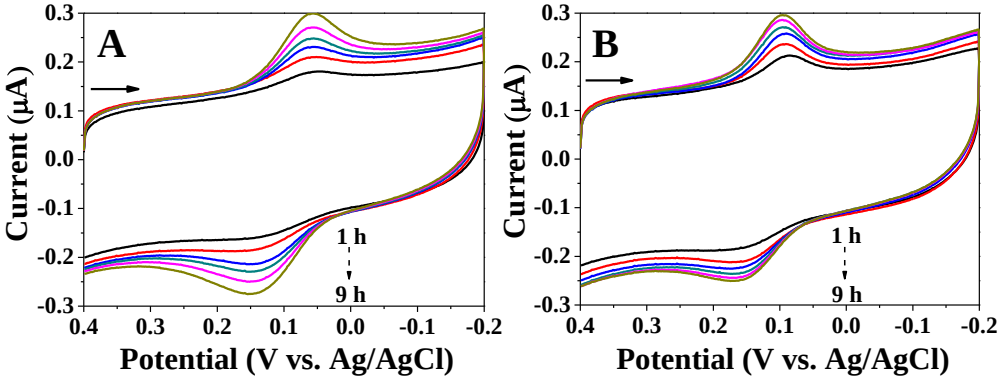

Figure 6. CVs of Aβ(1–16)–Cu^{2+} (A) and Laccase (B) modified electrodes in PBS solution. The concentrations of Aβ(1–16)–Cu^{2+} and Laccase were 10 μM and 1 mg/mL, respectively.

4. CONCLUSIONS

The EDC/NHSS-mediated two-step amine coupling reaction on the MPA SAM was investigated by evaluating the immobilization of electrochemically active biomolecules. The results indicated that the optimal EDC/NHSS concentration ratio and pH for the activation of carboxyl groups of MPA are 2:1 and 6.5, respectively. The temperature ranging from 4 to 25 ºC is the preferential temperature for the activation reaction. The optimal pH for the coupling of amine compounds was in the range of 7 ~ 8. The NHSS-ester was stable in the physiological pH solution for at least 12 h. The reaction rate of NHSS-ester (on gold electrode) with amine-containing biomolecules depended on the concentrations of biomolecules. We believe that this work will be valuable for the immobilization of biomolecules and the fabrication of affinity biosensors.

ACKNOWLEDEMENTS
Partial support of this work by the National Natural Science Foundation of China (21205003) and the Science & Technology Foundation of Henan Province (122102310517) is gratefully acknowledged.

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