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Activity analysis of the carbodiimide-mediated amine coupling reaction on self-assembled monolayers by cyclic voltammetry

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ABSTRACT

Carbodiimide provides one of the most popular and versatile methods for the covalent attachment of proteins, nucleic acids and small-molecule organic compounds to self-assembled monolayers (SAMs). In this work, we investigated the carbodiimide-mediated amine coupling reaction on SAMs with cyclic voltammetry. The negatively charged 11-mercaptoundecanoic acid (MUA) SAMs on gold electrodes hindered access of the $[Fe(CN)_6]^{3-/4-}$ redox probe to the electrode surface. Once the carboxyl groups of MUA were activated by carbodiimide to form *O*-acylisourea intermediates, the electrostatic interaction between the positively charged intermediates and the negatively charged $[Fe(CN)_6]^{3-/4-}$ probes decreased the electron-transfer resistance, resulting in the occurrence of reduction and oxidation reactions of the probe. Hydrolysis and aminolysis of the intermediates induced the loss of the positively charged carbodiimide moieties, causing non-conductive SAMs. Moreover, when the carboxyl groups were converted to amine-reactive *N*-hydroxysulfosuccinimide (NHSS) ester intermediates, the negatively charged NHSS esters separated from the negatively charged $[Fe(CN)_6]^{3-/4-}$ probes and formed a barrier for the electron transfer. The introduction of positively charged amine groups through the amine coupling reaction between an NHSS ester and ethanediamine would facilitate the electron transfer again. With this method, the activity of carbodiimide-mediated activation and acylation reactions and the stability of the resulting *O*-acylisourea/NHSS ester intermediates in an aqueous system were addressed. The effects of the solution pH and amine concentration on these reactions were also investigated. We believe that the results will be valuable for the immobilization of biomolecules and the fabrication of biosensors.

1. Introduction

Adsorption biosensors take advantage of different biological recognition elements, such as antibodies, natural or synthetic receptors and nucleic acids. The preparation of adsorption biosensors often relies on the controlled chemical modification of surfaces [1]. Self-assembled monolayers (SAMs) are an inexpensive and versatile surface coating for molecular recognition for sensors, and, in particular, alkanethiol. SAMs on gold have been used frequently for controlling the adsorption of biomolecules [2–5]. Carbodiimide, a zero-length crosslinking agent, provides the most popular and versatile method for the covalent attachment of proteins, nucleic acids and small-molecule organic compounds to SAMs [4,6,7]. Carbodiimide reacts with a carboxyl to form an amine-reactive *O*-acylisourea intermediate. It is well known that this intermediate may react with an amine group to join two molecules by a stable amide bond but is also susceptible to hydrolysis, making it unstable and short-lived in aqueous solution [8]. To increase the efficiency of carbodiimide-mediated coupling, *N*-hydroxysulfosuccinimide (NHS) or *N*-hydroxysulfosuccinimide (NHSS) was most frequently used to react with the *O*-acylisourea intermediate to produce an amine-reactive NHS-ester that is less susceptible to hydrolysis than *O*-acylisourea for the two-step amine coupling reaction [9–18]. On the other hand, competitive paths are the formation of anhydride by dehydration of *O*-acylisourea with a neighboring carboxylic acid and the generation of *N*-acylurea via an intramolecular acyl rearrangement [19,20]. Anhydride can undergo further reaction with NHS to produce the NHS-ester or primary amine to produce the amide accompanied by the regeneration of an acid group. *N*-acylurea is a stable product and cannot react with amino compounds, which form at extreme conditions such as high temperatures and high concentrations [20]. Therefore, a fundamental understanding of carbodiimide-mediated surface-reaction kinetics and mechanisms is essential to make progress on the rational design of complex surface architectures

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used in applications as varied as biosensor devices. Analytical techniques, such as surface plasmon resonance (SPR), surface-enhanced Raman spectroscopy (SERS), quartz crystal microbalance (QCM), Fourier transform infrared spectroscopy (FTIR) and laser scanning confocal microscopy (LSCM), have been used to characterize the carbodiimide-mediated amine coupling of ligands and biomolecules onto carboxyl-terminated Au or Si substrates [1,20–27]. However, most researchers accepted the general rule that carboxylic acids were activated to NHS-esters by EDC/NHS without questioning the truth; the results clearly presented some differences depending on the experimental conditions used for the activation treatment. Moreover, the conditions of the surface reaction are more stringent than when the reaction is carried out in solution because on a surface, the reaction products cannot be purified, and unwanted reactions can lead to irreversible surface contamination [28]. Therefore, there remains significant room to understand carbodiimide-mediated surface-reaction kinetics and mechanisms [19]. Recently, infrared spectroscopy was used by several groups to investigate the 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC)/NHS activation details of carboxy-terminated groups on a silicon surface [19,28–30]. Their results indicated that the amount of surface products depends on the types of substrates and the activation conditions.

Cyclic voltammetry, the most popular electrochemical technique used to study redox processes at SAM-modified electrodes, is simple and fast to implement [31–33]. The terminal groups of SAMs have a great impact on the redox response and electron-transfer resistance of redox probes in aqueous solutions due to the electrostatic interaction between the terminal groups and ionic redox species [34]. For example, the voltammetric response of $[Fe(CN)_6]^{3-/4-}$ at the SAM-modified electrode is decreased in the order of the terminal group $NH_2 > OH > COOH$, while the response of $[Ru(NH_3)_6]^{3+/2+}$ is increased in the order of $NH_2 < OH < COOH$ [34]. Based on this concept, the EDC-mediated amine coupling reaction on SAMs was investigated here by cyclic voltammetry in the presence of the $[Fe(CN)_6]^{3-/4-}$ couple redox as a probe. To our knowledge, it is the first time that the EDC/NHS activation reactions were studied with an electrochemical technique. Specifically, we aimed to answer the following questions using cyclic voltammetry: How long will carboxyl groups on gold SAM surfaces be saturated by EDC? How long will it take to complete hydrolysis and aminolysis reactions of the *O*-acylisourea intermediates on SAMs? How long will it take for the coupling reaction between NHSS esters and amines to be completed? How do pH and amine concentrations affect the coupling reaction on SAMs? Our results will be valuable for the immobilization of biomolecules through the EDC-mediated amine coupling reaction and the film assembly on different types of substrates.

2. Experimental

2.1. Chemicals and materials

1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride, NHSS and 11-mercaptoundecanoic acid (MUA) were acquired from Sigma. Other reagents were of analytical grade and obtained from Beijing Chemical Reagent Co. (Beijing, China). All stock solutions were prepared daily with deionized water treated with a water purification system (Simplicity Plus, Millipore Corp.). For the EDC-mediated activation reaction, hydrochloric acid (HCl), sodium hydroxide (NaOH) and 2-(*N*-morpholino)ethanesulfonic acid (MES) were used to adjust the pH of the solution. For the hydrolysis and aminolysis of the *O*-acylisourea or NHSS ester intermediate, phosphate buffer solution (PBS) was used to adjust the pH.

2.2. Electrochemical measurements

Cyclic voltammograms (CVs) were collected on a DY2013 electrochemical workstation (Digi-Ivy, Inc., Austin, TX) in a homemade plastic three-electrode cell. The three-electrode system consists of a gold disk electrode with a diameter of 2 mm, a platinum wire auxiliary electrode, and an Ag/AgCl reference electrode. Potential scanned from 0.7 to −0.1 V with a scan rate of 200 mV/s. Electrochemical impedance spectroscopy measurements were performed on a CHI 660D electrochemical workstation (CH Instruments), and the redox mediator used was 1 mmol dm^{-3} $[Fe(CN)_6]^{3-/4-}$ (1:1) solution containing 0.1 mol dm^{-3} KCl.

2.3. 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 MUA SAMs were formed by immersing the cleaned electrodes in ethanol solutions containing 10 mmol dm^{-3} MUA in the dark for 12 h. Then, the electrodes were flushed with excess ethanol and stored in a refrigerator at 4 °C for use. The EDC/NHSS solution was prepared freshly by mixing 50 mmol dm^{-3} EDC with 50 mmol dm^{-3} NHSS in 0.1 mol dm^{-3} MES buffer (pH 6.0) before the MUA film activation step [19].

3. Results and discussion

3.1. Principle of activity analysis of EDC-mediated amide formation

The analysis principle is shown in Fig. 1. It is based on measurements of the electrochemical response of MUA-covered gold electrodes in the presence of the negatively charged $[Fe(CN)_6]^{3-/4-}$ as a redox probe. Because the negatively charged MUA SAMs hinder the access of the redox probe to the electrode surface, no oxidation and reduction peaks are observed at the MUA-covered gold electrode in the $[Fe(CN)_6]^{3-/4-}$ solution (Sample 1). Once the carboxyl groups of MUA SAMs are activated by EDC, the electrostatic interaction between the positively charged *O*-acylisourea intermediate (Sample 2) and the negatively charged $[Fe(CN)_6]^{3-/4-}$ will decrease the electron-transfer resistance and result in the occurrence of the reduction and oxidation reactions of the redox couple. The magnitude of the increase/decrease in electron-transfer resistance is related to the amount of *O*-acylisourea intermediate. Further, hydrolysis and aminolysis of the intermediates will induce loss of the positively charged EDC moieties, leading to negatively charged (Sample 1) and neutral (Sample 3) SAMs, respectively. Thus, the process of formation and hydrolysis/aminolysis of the *O*-acylisourea intermediate can be monitored. Moreover, when carboxyl groups are converted to amine-reactive NHSS esters (Sample 4), the negatively charged NHSS ester intermediate will be excluded from the negatively charged $[Fe(CN)_6]^{3-/4-}$ probe and form a barrier for the electron transfer. However, the introduction of positively charged amine groups through the amine coupling reaction between the NHSS ester and ethanediamine will facilitate the electron transfer (Sample 5). Therefore, the aminolysis of the NHSS ester can also be monitored.

3.2. CVs of EDC-activated SAMs

As shown in Fig. 2A, CV collected at the MUA-covered electrode shows no redox wave in the $[Fe(CN)_6]^{3-/4-}$ solution (black curve), indicating that the MUA SAMs hindered electron transfer between the $[Fe(CN)_6]^{3-/4-}$ probe and the electrode surface. However, after the carboxyl groups of MUA were activated by EDC,