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Label-free electrochemical immunosensors based on surface-initiated atom radical polymerization

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ABSTRACT

A novel label-free immunosensing strategy for sensitive detection of tumor necrosis factor-alpha antigen (TNF-α) via surface-initiated atom transfer radical polymerization (SI-ATRP) was proposed. In this strategy, the Au electrode was first modified by consecutive SI-ATRP of ferrocenylmethyl methacrylate (FMMA) and glycidyl methacrylate (GMA), and TNF-α antibody was coupled to the copolymer segment of GMA (PGMA) by aqueous carbodiimide coupling reaction. Subsequently, the target TNF-α antigen was captured onto the Au electrode surface through immunoreaction. The whole process was confirmed by scanning electron microscopy (SEM) and surface plasmon resonance (SPR) measurements. With introduction of redox polymer segment of FMMA (PFMMA) as electron-transfer mediator, the antigen-coupled Au electrode exhibited well electrochemical behavior, as revealed by cyclic voltammetry measurement. This provided a sensing platform for sensitive detection of TNF-α with a low detection limit of 3.9 pg mL^{-1}. Furthermore, the "living" characteristics of the ATRP process can not only be readily controlled but also allow further surface functionalization of the electrodes, thus the proposed method presented a way for label-free and flexible detection of biomolecules.

1. Introduction

Recently the controlled radical polymerization, which enables to grow polymer brushes on a wide range of substrates, has already become one of the most versatile approaches for varying interactions with biological systems, and for enhancing their performance in bioapplications through improved hydrophilicity, biocompatibility, conductivity, and adhesive properties (Patten et al., 1996; Hawker et al., 2001; Hawker and Wooley, 2005; Kato et al., 2003; Zhao and Brittain, 2000). Atom transfer radical polymerization (ATRP), of these controlled radical polymerization techniques, has been demonstrated to be a highly versatile and robust synthetic method to prepare well-defined polymers with a controlled molecular weight and a narrow molecular weight distribution (Patten et al., 1996; Kamigaito et al., 2001; Tsarevsky and Matyjaszewski, 2007; Pyun and Matyjaszewski, 2001; Pyun et al., 2003). Indeed, ATRP can be used to build polymerization-based amplification system by using polymer materials as carrier to increase the loading of signal tags due to its easy tuning of the amount of the functional polymer chains and adjustment of the spatial distribution of probe species (Hansen et al., 2008; Sikes et al., 2008, 2009; Yuan et al., 2011a, b).

Electrochemical approaches are useful for detecting protein samples as they have the important advantage of offering label-free and quantitative measurement capability (Qiu et al., 2010). In an attempt to improve the detection limit, indirect electrochemical detection is performed using an auxiliary reaction which involves a labeling (redox active) compound, e.g., monomeric and polymeric ferrocene, for signal generation. For example, Fan et al. have developed the first-generation electrochemical DNA sensor by using ferrocene as label. In their work, the DNA sensor displayed an intense electrochemical response corresponding to ferrocene redox when the ferrocene was in proximity to the Au surface at the initial state, however, the current signals gradual turned off when target DNA was binded to the probe and liberated the ferrocene from the electrode surface (Fan et al., 2003). Ju et al. proposed a novel electrochemical lectin-probe, ferrocene-concanavalin A (Fc-ConA), for in situ monitoring of cell surface glycan by incorporating the specific recognition ability of lectin to glycan and favorable electrochemical property of ferrocenyl group (Xue et al., 2010). The proposed ferrocene-lectin showed good performance for detection of cancer cells with broad detection range, low detection limit, good reproducibility, and simple detection procedure. In addition, a number of ferrocene-containing polymers and polymer brush systems have already been developed (Uhlmann et al., 2009; Ostaci et al., 2008; Brinks and Studer, 2009; Xu et al., 2010a,b; Bo et al., 2010). These ferrocene polymers remained unique redox, catalytic properties,

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as well as good electrochemical responses. Once grafted on solid substrates, the ferrocene polymer brushes have been used for the development of electroactive and electrocatalytic films (Yu et al., 2009), electrochemical DNA sensors (Madeline et al., 2011), and amperometric biosensors (Senel et al., 2010; Zhang et al., 2010). For example, Liu and co-workers reported a novel signal amplification strategy for electrochemical detection of DNA and proteins (Wu et al., 2009). After the capture of target molecules on the electrode surface, activators generated electron transfer for ATRP was used to trigger the growth of long chain polymeric material providing numerous sites for label ferrocene moieties coupling. These ferrocene-containing polymers in turn significantly enhanced the electrochemical signal output. Surinder et al. prepared a copolymer of pyrrole and ferrocene carboxylate modified pyrrole (P(Py-FcPy)) on the indium-tin-oxide (ITO) coated glass surface, and glucose oxidase (GOx) as model enzyme had been entrapped during deposition for fabrication of mediator-less electrochemical biosensor (Netzahualcóyotl et al., 2011). Ferrocene modified pyrrole in copolymer enhanced the redox properties of copolymer, resulted in quasi reversibility with favorable electron transfer and improved electrochemical signal for electrochemical biosensors, indicating the feasibility of fabricating sensitive mediator-less electrochemical biosensors using ferrocene modified polypyrrole film.

This work was also motivated by the promising applications of ferrocene-containing polymer in biosensing. A label-free electrochemical immunosensor was prepared via an alternative approach of consecutive surface-initiated ATRP (SI-ATRP). The ATRP initiator was immobilized on the Au electrode surface for consecutive surface-initiated ATRP of ferrocenylmethyl methacrylate (FMMA) and glycidyl methacrylate (GMA) or in the reverse order. The poly-GMA (PGMA) has been widely used in advanced biotechnology, such as DNA separation, cell adhesion, enzyme immobilization, and immunological assay, because of the ease in conversion of epoxide groups to a variety of functional of groups, such as –OH, $–NH_2$, and –COOH through a ring-opening reaction (Yuan et al., 2011a, b; Xu et al., 2010a, b, 2005; Ryoko et al., 2008; Wu et al., 2009, 2010). Herein, the redox-active ferrocene moieties in the PFMMA segment was introduced on the Au electrode surface to act as an electron transfer mediator, while the epoxide groups of the PGMA segment was used for coupling of TNF-α, a model protein which indicated an early stage of the inflammatory reaction in response to infection or cancer (Old, 1987). The employed SI-ATRP as well as block polymerization technique in the manufacturing process of this biosensor endowed the immunosensor with flexible turning of the amount of surface functionality FMMA and GMA units and adjustment of the spatial distribution of redox mediators, thus produced a versatile mean for the preparation of electrochemical biosensors with good sensitivity.

2. Experimental

2.1. Materials and reagents

Au substrates (50 Å chrome followed by 1000 Å gold on float glass) were purchased from Evaporated Metal Films (Ithaca, NY). Rabbit tumor necrosis factor-alpha antigen (TNF-α, Ag, 2 μg mL^{-1}) and human anti rabbit TNF-α antibody (anti-TNF-α, Ab, 200 μg mL^{-1}, polyclonal) were obtained from Boster Biological Technology Co. Ltd. (Wuhan, China). Rabbit immunoglobulin G (IgG) was purchased from Signalway Antibody (Nanjing, China). Bovine serum albumin (BSA) was obtained from SunShine BIO (Nanjing, China). Concanavalin A (Con A), thrombin, glycidyl methacrylate (GMA), ferrocenylmethyl methacrylate (FMMA), 2-bromoisobutyryl bromide (BriBuBr, 98%), *N*-hydroxysuccinimide (NHS), N,N,N′,N″,N″-pentamethyldiethylenetriamine (PMDETA, 99%), 1-undecanethiol (1-UDT, 98%), cysteamine, and copper (I) bromide (CuBr, 98%) were purchased from Sigmaaldrich (Shanghai, China). GMA and FMMA were purified in-house, and were passed through a column with activated Al_2O_3 to remove the inhibitors before polymerization. All other chemicals were of analytical grade and were used as received. 0.1 M phosphate buffer solutions (PBS) were prepared by mixing 0.1 M NaH_2PO_4 with Na_2HPO_4. Twice-distilled water was used throughout the study.

2.2. Instruments

All electrochemical experiments were conducted with a CHI 830 electrochemical workstation (Shanghai Chenhua Instrument Co., LTD, China). A conventional three-electrode system was performed in a 5 mL glass cell consisted of a platinum wire as auxiliary electrode, a saturated calomel electrode (SCE) as reference, and a modified Au wire electrode as the working electrode, respectively. All of the experiments were carried out at room temperature (25 ± 1 °C).

The initiator NHS-Br active esters were analyzed by ^1H and ^{13}C NMR spectroscopy on a Avance 400 MHz spectrometer (Bruker, Switzerland). The time-resolved surface plasmon resonance (TR-SPR) spectrometer (DC-TR-SPR, DyneChem HiTech Ltd. China), a single channel and prism coupling-based instrument, was used to investigate the binding performance of a target protein and immmunoreaction of antigen protein onto the polymer films-modified SPR chip. The SI-ATRP process on the Au substrate was characterized by mean of a SEM instrument (JEM-2100, JEOL, Japan) with an acceleration voltage of 5 kV. An UV-2450 UV–visible spectrophotometer (Shimadzu, Japan) was used to monitor polymer and protein-modified Au substrate.

2.3. Preparation of initiator-coupled Au electrode

The *N*-hydroxysuccinmidyl bromoisobutyrate (initiator) was synthesized with our previously reported method (Yuan et al., 2011a, b). The as-prepared initiator was used in the following experiments without further purification (detail synthesis procedures in Supporting information). To prepare the initiator-coupled Au electrode, the Au wire electrode was first pretreated prior to use (see Supporting information).

The cleaned Au wire electrode was soaked in 20 mM deoxygenated cysteamine aqueous solution diluted with 1-undecanethiol (UDT) for 8 h at room temperature in darkness. The UDT with different molar ratio was used as diluent to reduce the density of initiator (from initiator: UDT molar ratio of 1:1 to 0.1:1), which was binded to cysteamine. The resulted mixed monolayer-modified electrode was thoroughly rinsed with water to remove physically adsorption. After dried under N_2, the electrode was immersed in freshly prepared initiator solution (38 mM in DMF) for 1 h at room temperature under stirring. Finally, the initiator-coupled electrode was obtained by a rinse with ethanol and sonication in doubly-distilled water.

2.4. Surface-initiated ATRP

As shown in Scheme 1, two routes were employed for surface functionalization of Au electrodes via successive surface-initiated ATRP to develop the label-free electrochemical immunosensor. In route 1, the redox-PFMMA was first grafted from the Au-initiator surface as the inner block, followed by copolymerization of GMA to obtain the Au-PFMMA-PGMA electrode. In route 2, the PGMA was first grafted from the Au-initiator surface as the inner block,