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Research Paper

Electrogenerated chemiluminescence biosensor array for the detection of multiple AMI biomarkers

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ABSTRACT

An electrogenerated chemiluminescence (ECL) biosensor array for the individual and simultaneous detection of multiple biomarkers has been developed at first time on the basis of the aptamer-based biosensor incorporating a versatile signal probe. As principle-of-proof, the acute myocardial infarction (AMI) biomarkers, myoglobin(Myo), cardiac troponin I(cTnI) and cardiac troponin T(cTnT) were chosen as model analytes. The biosensor array was fabricated by self-assembling thiolated specific ssDNA aptamers on the surface of gold electrodes, respectively. After each target analyte was bound to the capture probes and then to each corresponding biotinylated antibody, and finally to the versatile ECL signal probe, ECL signals were recorded using PMT or CCD as a detector. For PMT model, the developed method showed extremely low detection limits for 0.30 ng/mL cTnT, 31 pg/mL Myo, 0.79 pg/mL cTnI. For ECL image model, a biosensor array containing three target biosensors and a control biosensor was found to be highly sensitive, no cross-talking, and accurate towards simultaneous detection of the AMI biomarkers. This work demonstrates that the aptamer-based biosensor array with a versatile signal probe is a promising for ECL simultaneous detection of multi-biomarkers.

1. Introduction

Design and development of simple, sensitive, and selective biosensing methods for the detection of a group of specific protein biomarkers have received more and more attention in clinical tests since the information of a group of specific biomarkers can provide more efficient clinical diagnosis index [1]. A variety of the biosensing platforms for simultaneous detection of multiple biomarkers have been established, such as fluorescent biosensor array [2,3], electrochemical biosensor array [4,5,6], surface acoustic wave biosensor array [7] and electrogenerated chemiluminescence (ECL) immunoassay microarrays [8]. From readout approaches, fluorescent labels which are commonly used in fluorescence assays suffer from photobleaching; furthermore, spectral overlap of the reporter dyes may limit the degree of multiplexing, and luminescent impurities can interfere with the interpretation of results [8]. Electrochemical methods are suffered from the low sensitivity. ECL method has attracted considerable interest for its intrinsic advantages, such as high sensitivity and good stability [8,9].

Extensive efforts have been devoted to improving the ECL methods for simultaneous detection of multiple biomarkers in one run, including developing high-resolution strategies and electrode platforms, exploring new molecular recognition elements and searching signal-enhanced approaches. In resolution strategies, there are two models using photomultiplier tube (PMT) or charged coupled device (CCD) detectors. One is potential resolution using different ECL signal compounds [10,11,12] while another is spatial resolution using the same ECL signal compound. Potential resolution involves more than one ECL probe preparations and conflict reaction between hydrogen peroxide luminol and $Ru(dcbpy)_3^{2+}$ [13]. Spatial resolution involves using ruthenium complex-labeled antibodies as signal probes and PMT as a detector [14,15]. The ECL signals from the immunosensor array were sequentially detected by a photomultiplier with the switch on/off of each biosensor in the array. This approach can offer a high sensitivity but that is not simultaneous detection at the same time using PMT. The spatial-resolved model is expected to be more widely applied in ECL biosensor array since a large number of the ECL biosensors can be used in this model using CCD.

In the design of biosensors, antibodies were widely utilized as molecular recognition elements for proteins due to their high affinity and specificity. However, antibodies have some limitations such as their production in vivo, limited self-lives and

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temperature-sensitive to undergo denaturation. Aptamers, which are artificially synthesized by oligonucleotides, have high specificity and affinity for various target compounds such as small drugs and proteins [16,17]. Thus, aptamers as molecular recognition elements have been applied in biosensors [18,19,20]. Recently, our group [21] developed an ECL method for the detection of multiple protein kinases based on spatial-resolved mode using a ruthenium complex-labeled protein A as a versatile ECL probe for bioassay of multiple protein kinases. However, compared with biotin-streptavidin conjugate ($K_d = 10^{-15}$ M) which represents one of the strongest known noncovalent interactions [22], weak bioaffinity of protein A with a variety of antibodies is limited to its applications. Biotin-streptavidin interaction has been used to electrochemical immunosensor array [23] and a label-free ECL immunosensor [24]. Ii is expected that biotin-streptavidin interaction is promising approach for construction of the probes.

Acute myocardial infarction (AMI), a severe cardiovascular disease, is one of the most common reasons for unexpected sudden death and may cause irreversible tissue injury or necrosis in the myocardium [25], and one of the leading causes of death in both low- and middle-income nations [26]. It is reported that many cardiac biomarkers, such as myoglobin (Myo) [27], cardiac troponin I (cTnI) [28] and cardiac troponin T (cTnT) [29], have been employed to diagnose AMI. The method commonly used for individual detection of AMI biomarkers is an immunoassay, such as radioimmunoassays for Myo [30], enzyme-linked colorimetric immunosorbent assays for cTnI [31], chemiluminescence immunoassay for Myo, creatine kinase MB, cTnI and fatty acid-binding protein [32], electrochemical immunoassay for cTnI [33], ECL immunoassay for cTnI [34], fluorescent immunoassay for cTnI [35], photoelectrochemical immunosensing of cTnI [36]. However, ECL biosensor array for the detection of multiple AMI biomarkers has not been reported.

The aim of this work is to design and to develop an ECL biosensor array for the detection of multiple biomarkers, using the aptamers as capture probes and ruthenium complex (Ru1)-labeled streptavidin (SA) as one versatile ECL signal probe (Scheme 1). As principle-of-proof, AMI biomarkers, Myo, cTnI and cTnT, were chosen as model analytes, while the thiolated specific aptamers including Myo ssDNA aptamer [37], cTnI ssDNA aptamer [38] and cTnT ssDNA aptamer [39] were chosen as the capture probes. The aptamer was self-assembled on the surface of gold electrode, respectively and then bound to the individual target, further to biotinylated antibodies commercially available and finally to versatile ECL signal probe, as shown in Scheme 1. In this paper, the synthesis and characterization of a versatile ECL signal probe (Ru1-SA), fabrication and performance of the ECL biosensor array for multi-target detection with photomultiplier tube (PMT) and charged coupled device (CCD) as detectors are presented.

2. Experiment section

2.1. Reagents and apparatus

Bis(2,2′-bipyridine)-4′-methyl-4-carboxybipyridine-ruthenium N-succinimidylester-bis(hexa-fluorophosphate) (abbreviated as $Ru1PF_6$), human myoglobin (Myo), streptomyces avidinii (SA) were purchased from Sigma-Aldrich (USA). Human cardiac troponin I (cTnI), human cardiac troponin T (cTnT), and biotinylated anti-cTnT antibody were obtained from Abcam Trading Company Ltd (USA). Biotinylated anti-Myo antibody was obtained from Fitzgerald (USA). Biotinylated anti-cardiac troponin I antibody was obtained from GeneTex (USA). Myo ssDNA aptamer (Myo 40-7-27, 5′-HS-$(CH_2)_6$-CCCTCCTTTCCTTCGACGTAGATCTGCTGCGTTGTTCCGA-3′, bp = 40), cTnI ssDNA aptamer (Tro4, 5′-HS-$(CH_2)_6$-CGTGCAGTACGCCAACCTTTCTCATGCGCTGCCCCTCTTA-3′, bp = 40) and cTnT ssDNA aptamer (AraHH001, 5′-HS-$(CH_2)_6$-ACGTACCGACTTCGTATGCCAAC AGCCCTTTATCCACCTC-3′, bp = 40) were synthesized by Sangon Biotech Co., Ltd (China). ECL measurements were performed on MPI-E ECL detector (Xi'an Remax Analysis Instruments Co. Ltd, China) and ECL images were obtained on multi-functional ECL imaging analyzer using PMT and EMCCD as the detectors, set up in our Lab [40]. Other reagents and the apparatus used in this work are provided in Supplementary data.

2.2. Synthesis of Ru1-SA

The ECL versatile probe, ruthenium complexes-labeled streptavidin (abbreviated as Ru1-SA), was synthesized according to the reference [8] with some modifications. Briefly, 2.0 mg $Ru1PF_6$ (1.97 μmol) was dissolved in 200 μL of DMSO and then added into 2.0 mL of 1.0 mg/mL SA prepared by 10 mM PBS (pH 7.4), and incubated at 4 °C for 24 h. Ru1-SA was purified by dialysis cassettes (cut MW, 10 KDa), and then by gel filtration chromatography using and a Sephedex G-25 column (φ = 1.0 cm, length = 20.0 cm) and 10 mM PB (pH 7.4) as elution solvent at a flow rate of 0.4 mL/min. The characterization of Ru1-SA collected is provided in Supplementary data (Figs. S1-S6).

2.3. Fabrication of the aptamer-based biosensors and the biosensor array

For the fabrication of the aptamer-based biosensors, cTnT biosensor as a model, typically, 10 μL of 10 μM cTnT ssDNA aptamer was drop-coated on the surface of a gold electrode (diameter 2 mm) pretreated according to the previous method [41] and then stayed in incubation under a centrifuge tube for 4 h at room temperature. After thoroughly washed, the resulted electrode was immersed in 1.0 mM cysteamine prepared using 10 mM PBS (pH 7.4) for 40 min. After washed with 10 mM PB (pH 7.4) and dried under a stream of N_2 gas, the fabricated biosensor was stored at 4 °C.

For the fabrication of the biosensor array, a four gold electrode array was designed in this work, as shown in Scheme 1. Typically, 2.5 μL of 10 μM ssDNA aptamer was attentively drop-coated on the surface of the corresponding gold working electrode (diameter 1 mm), respectively. The pretreatment of gold electrodes and other processes were the same as described above.

2.4. ECL measurement

For PMT model, an aptamer-based biosensor fabricated was incubated in 100 μL of one target solution (Myo, cTnI, cTnT) or sample for 60 min, and then 10 μL of the premixed conjugate solution of biotinylaed antibody and Ru1-SA (mole ratio 1:1, 5×10^{-8} M) was drop-coated on the surface of the target-bound biosensor and allowed to stay for 60 min. After each incubation step, the resulting electrode was washed with 10 mM PB (pH 7.4). The electrodes were put into 2.0 mL of 0.1 M PBS (pH 7.4) containing 50 mM TPA and ECL measurements were performed with a scan rate of 50 mV/s. The concentration of the target protein (Myo, cTnI, cTnT) was quantified by the increased ECL intensity ($\Delta I = I - I_0$), where I_0 is the ECL peak intensity in the absence of the target antigen and I is the ECL peak intensity in the presence of the target antigen.

For CCD imaging model, typically, 2.5 μL of the individual target protein solution (Myo, cTnI, cTnT) or target protein mixture solution was carefully drop-coated on the surface of the corresponding biosensor in the array, respectively, allowed to incubation for 60 min. And then, 2.5 μL of the premixed conjugate solution of biotinylated antibody and Ru1-SA (mole ratio 1:1, 5×10^{-7} M) was drop-coated on the surface of the corresponding biosensor