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Biomarkers of liver fibrosis detecting with electrochemical immunosensor on clinical serum

Yao Yao[a,1], Jianfeng Bao[b,1], Yanli Lu[a], Diming Zhang[a], Senbiao Luo[c], Xing Cheng[a], Qian Zhang[a], Shuang Li[a], Qingjun Liu[a,*]

[a] *Biosensor National Special Laboratory, Key Laboratory for Biomedical Engineering of Education Ministry, Department of Biomedical Engineering, Zhejiang University, Hangzhou 310027, PR China*
[b] *Xixi Hospital of Hangzhou, Hangzhou 310023, PR China*
[c] *Shangyu People's Hospital of Zhejiang Province, Shangyu 312000, PR China*

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ABSTRACT

Diagnosing hepatic fibrosis at an early stage with sensitive and specific monitoring approach is crucial for patient therapeutics and survival. In this study, an electrochemical immunosensor was established to detect representative biomarkers of liver fibrosis, such as hyaluronate acid (HA) and transforming growth factor beta 1 (TGFβ1). Through a self-assembled monolayer of polyethylene glycol (PEG), antibodies against HA and TGFβ1 were successfully immobilized on interdigitated electrodes. It produced a robust and sensitive membrane by improving the uniformity, density, and distribution of the antibodies for the biomarkers. Based on impedance sensing, HA and TGFβ1 were sensitively detected in the ranges of 1–1000 ng/ml. The detection limits of HA and TGFβ1 reached 0.586 ng/ml and 0.570 ng/ml, respectively. In addition, for the detection of clinical serum samples, the results were in excellent agreement with the tests of HA, type III pre-collagen (PCIII), IV collagen (IV-C), and laminin (LN) that conducted by radioimmunoassay for liver fibrosis. The research indicated that the approach provided a valuable, universal, and label-free strategy in evaluating liver fibrosis and other chronic diseases for point-of-care diagnostics.
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1. Introduction

Chronic hepatic disease causes high morbidity and mortality worldwide, which can lead to liver fibrosis and the subsequent development of cirrhosis and even hepatocellular carcinoma [1–3]. For chronic liver injuries of many etiologies, including viral hepatitis, alcohol abuse, metabolic diseases, autoimmune diseases, and cholestatic liver diseases, they could produce fibrosis as a result of deregulation of the normal healing process with massive accumulation of extracellular matrix (ECM) [2,4]. Therefore, the early diagnosis of liver fibrosis is vital for therapeutic decisions and prognostic evaluations. Among the various diagnostic approaches, needle biopsy is considered as the 'gold standard' [3,5–7]. However, it was invasive, confounded by high sampling heterogeneity and carried a finite risk of complications. Especially, it is not suit for frequent evaluations of this chronic disease.

In clinical diagnosis, simultaneously detecting a number of fibrosis biomarkers in serum directly was an alternative and helpful approach to evaluate liver fibrosis [8–11]. The conventional fibrosis biomarkers, including hyaluronate acid (HA), type III pre-collagen (PCIII), IV collagen (IV-C), and laminin (LN), have been used in clinical to analyze conditions of liver fibrosis. In addition, some novel biomarkers, such as human transforming growth factor beta 1 (TGFβ1), also showed significant promise for monitoring hepatic fibrosis [8,12,13]. However, there were lots of disadvantages in common approaches, such as radioimmunoassay (RIA), enzyme immunoassay (EIA), and fluoroimmunoassay (FIA) to detecting those biomarkers [14–16]. For instance, isotope used in RIA is dangerous and difficult for treatment of waste from the detection procedures and storage of reagents, while labeled molecules used in EIA and FIA also made them complicated and time-consuming. Thus, it is urgent for developing more reliable, efficient, and simple approaches to evaluate liver fibrosis with serum biomarkers.

Compared to the common immunoassays, electrochemical sensing had attracted more interests due to their inherent advantages, including high sensitivity, label-free, time-saving, and eases of operating. It exhibited great potential in point-of-care diagnostics for early detection of various diseases. The previous study

* Corresponding author.
E-mail address: qjliu@zju.edu.cn (Q. Liu).
[1] These authors contributed equally to this work.

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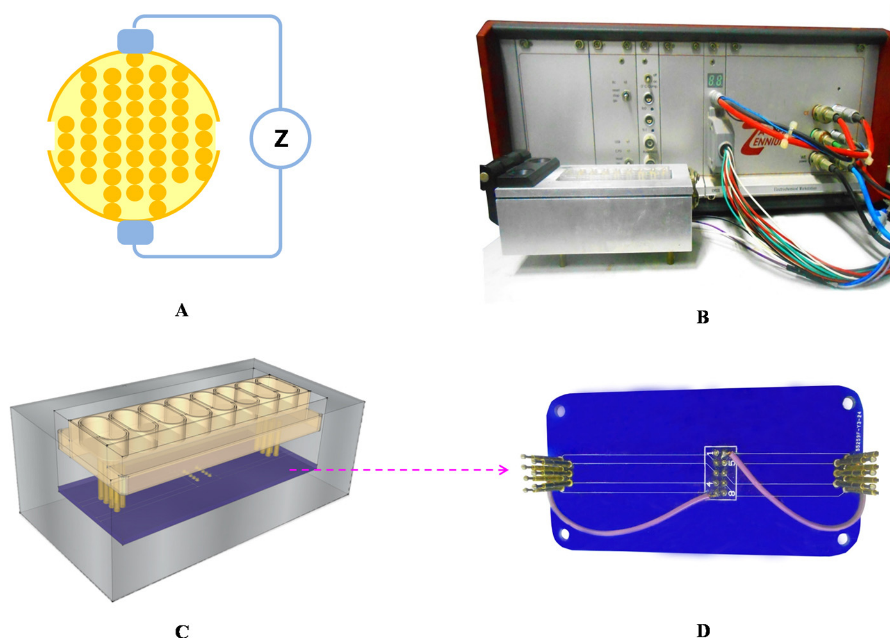

Fig. 1. The system for the impedance measurement. (A) Schematic of interdigitated electrodes in the bottom of the wells. (B) The detecting system for electrochemical detecting. (C) Perspective drawing of the electrode device and the circuit cavity of the system. (D) Microfabricated circuit array to connect with the electrochemical workstation.

that detected conventional biomarkers, HA, LN, and IV-C, by cyclic voltammetry has laid a foundation to biosensing research for clinical applications [14,17]. Thus, designing electrochemical biosensors to detect typical and candidate biomarkers in clinical samples will provide great benefits for diagnosing and evaluating liver fibrosis.

In this paper, though a self-assembled monolayer of polyethylene glycol (PEG), antibodies of two fibrosis biomarkers, HA and TGFβ1, were immobilized on the interdigitated electrodes for electrochemical detection. The impedance spectra of standard solutions and clinical human serum samples, indicated that the immunosensor could detect both conventional and candidate markers sensitively and selectivity. Compared with results of HA, PCIII, IV-C, and LN, which were tested with conventional immunoassay, electrochemical impedance of this study demonstrated very good consistency.

2. Materials and methods

2.1. Reagents

Antibodies against HA and TGFβ1 were used as sensing membrane for biosensing of the biomarkers. The original concentrations of anti-HA (polyclonal) and anti-TGFβ1 (monoclonal) were 5 mg/ml and 0.5 mg/ml, respectively, obtained from Sigma–Aldrich, USA. Phosphate-buffered saline (PBS, 10 mM, pH 7.4) was prepared by dissolving PBS tablets in ultrapure water and filtering using a 0.22 μm membrane filter. The solvents and reagents of α-thio-ω-carboxy poly (ethylene glycol) (MW 2100, COOH-PEG-SH in short), N-hydroxysuccinimide (NHS), 2-(4-Morpholino) ethanesulfonic acid (MES) for immobilization of the antibodies, and potassium ferricyanide/ferrocyanide ($K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$) for impedance detecting, were all analytical grade and purchased from Sigma–Aldrich.

2.2. Fabrication of the interdigitated electrodes

Interdigitated electrodes, with uniform electric fields and high electrode coverages, were used to measure impedance changes when the biomarkers binding to their antibodies [18,19]. The fabrication of the electrode arrays was based on semiconductor technology. The electrodes device was composed of a four-inch sterilized Pyrex glass 7740. Then, a layer of Cr (20 nm) and a layer of Au (200 nm) were sputtered on the glass. Subsequently, the interdigitated electrodes, interconnects, and pads were patterned from the composited metallic layer through conventional lithography and etching techniques. The working sketch of the electrodes was “circle-on-line” in 8 detecting channels (Fig. 1A) Afterwards, perspex with eight pairs round holes was used as the impedance detecting wells in the experiments. The biosensor detecting system is displayed in Fig. 1B which contained the electrochemical workstation and conversion bracket (Fig. 1C) The conversion bracket consisted of a special circuit and a cavity that were designed for the electrode chip connecting with electrochemical workstation (Fig. 1C) The circuit performed as a transducer through fourteen golden tapers. The cavity provided an excellent environment for preventing sliding of the electrode chips and the circuit.

2.3. Assemblies of the immunosensor

Before immobilization of the antibodies, the gold electrode arrays were rinsed with fresh piranha’s solution (1:3, v/v, H_2O_2 and H_2SO_4) for 2 min, and following thoroughly washed with ultrapure water. Then they were further sonicated in ethanol for 5 min, and dried in nitrogen. Sketch map of bare interdigitated electrodes were demonstrated in Fig. 2A Subsequently, Au-S covalent bonds were formed by immersing the electrodes in 200 μL of COOH-PEG-SH solution (1.5 mg/ml) for approximately 24 h at room temperature. After the unbound COOH-PEG-SH was rinsed several times with ultrapure water and dried with nitrogen, a self-assembled monolayer of PEG could be established on the electrodes (Fig. 2B).

The PEG-modified electrodes were incubated in solution containing antibodies and EDC/NHS. EDC and NHS solutions were prepared in 0.1 M MES buffer (pH = 5, contained 0.5 M NaCl) and 0.1 M PBS buffer (pH = 7.4) respectively. Equal volume of EDC (8 mg/ml) and NHS (16 mg/ml) solutions, were successively coated on the electrodes to activate the carboxyl group of COOH-PEG-SH for 20 min. After that, when pH was increased to 7.2–7.5 with

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