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On-chip detection of myoglobin based on fluorescence

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ABSTRACT

A disposable immunosensor cartridge was developed that allows antibodies to be immobilized on the surface for the detection of myoglobin, a marker for the early assessment of acute myocardial infarction (AMI) using fluorescence techniques. The anti-myoglobin antibody was immobilized on a polystyrene substrate based on covalent bonding *via* silanization. The immunosensor chip layers were fabricated from sheets by CO_2-laser ablation. The functionalized polystyrene surfaces were characterized by contact angle measurement, X-ray photoelectron spectroscopy (XPS), and atomic force microscopy (AFM). After the antigen–antibody reaction as a sandwich enzyme-linked immunosorbent assay (ELISA) with a horseradish peroxidase-conjugated secondary antibody (HRP-anti-myoglobin), addition of fluorogenic substrate produced a fluorescent dye which was quantified on-chip using fluorescent technique. The immunosensor response was linear for myoglobin concentrations between 20 and 230 ng/ml ($r = 0.991$, $n = 3$). The detection limit was found to be 16 ng/ml, which is lower than the clinical cut-off value for myoglobin in healthy patients. This protocol could be extended to the detection of other important cardiac markers simultaneously in microchannels.

1. Introduction

Cardiovascular disease is the leading cause of death all around the world. Cardiac biomarkers are a useful diagnostic tool for ancillary testing (Adams and Miracle, 1998). One of such tests is to determine the changes in the relative concentrations of 4 critical cardiac markers: myoglobin (Mb), CK-MB, troponin I, and troponin T (Plebani and Zaninotto, 1999). Myoglobin (Mb, ~17.8 kDa) although not cardiac-specific, is one of the very early markers that increases after AMI (Montague and Kircher, 1995). Its concentration in blood rises quickly at about 1 h after the onset of AMI, reaching the maximum between 6 and 15 h after the onset of symptoms (Christenson and Azzazy, 1998). Myoglobin serum concentrations return to baseline within 24 h (Brogan et al., 1994). These characteristics allow the early confirmation of AMI. Generally, the diagnosis involves the extraction of blood from the patients to be sent to a laboratory for testing. The test results will usually be known a few hours later. In this critical situation, it will be useful if these tests can be done at the point-of-care or at the patient's bed side with the results known within minutes rather than hours (Stubbs and Collinson, 2001; Lode, 2005). Therefore, it is necessary to develop a disposable, low-cost, simple, and fast device that enables the determination of the relative concentrations of this marker. Such a device, cobas h 232, has been developed and marketed by Roche for point-of-care diagnosis of cardiac markers (http://www.roche.com/prod_diag_roche-cardiac-t.htm). But the marketed system performs only single assay at a time. Thus, despite the availability and active use of the cobas h 232 system, it is still necessary to develop a system that has the potential for multiplexing multiple assays better than the above system.

The immunoassay is one of the most important analytical methods for clinical diagnosis and biochemical studies because of its extremely high specificity (Chan et al., 2003). Of the various solid-phase immunoassay formats, the sandwich ELISA typically carried out in polystyrene microtiter plates is superior to other types of heterogeneous solid-phase immunoassays with respect to sensitivity, specificity and kinetics (Diamandis and Christopoulos, 1996). However, this method requires large volumes of reagents and long incubation times due to the high volume-to-surface ratio. The use of microfluidic devices to perform sandwich ELISA permits one to specifically address these drawbacks. The low scale of these devices reduces the volumes and diffusion distances inside the microchannels, therefore decreasing the duration of the successive incubation periods (Herrmann et al., 2006; Yager et al., 2006).

To date, most of the microfluidic immunoassay systems have been fabricated using silicon, metal, and glass as the substrate material (Bange et al., 2005). However, machining these materials

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and fabrication is often a time-consuming and expensive process that presents a number of problematic issues that may ultimately hinder their widespread use in commercial applications. Polymers are low-cost alternative substrate materials for biochip and/or biomedical applications (Mathieu et al., 2003). The potential of polymers and plastic as substrate materials in microfluidic applications has been reviewed (Mello, 2002). Of the available polymers, polystyrene (PS) has been widely employed in many industrial and medical fields because of its low-cost and high workability. PS is usually utilized in the domain of diagnostics due to its optical transparency, good mechanical properties, durability, and non-toxicity. However, it also presents several general disadvantages – poor chemical resistance, difficulty in controlling surface chemistry, background fluorescence and hydrophobicity – that may pose problems in some applications. Surface modification to improve wettability, adhesion, biocompatibility and topography is thus necessary. A non-treated PS surface is by nature hydrophobic; however, it can be easily modified by ion beam treatment, plasma treatment, UV/ozone treatment and graft polymerization that can alter the chemistry of surface. Moreover, biomolecules attach to surfaces *via* a variety of mechanisms. The PS can be utilized through chemical reactions allowing covalent bonding of a variety of reactive groups that can be used for subsequent covalent immobilization of biomolecules. Furthermore, optical biosensors represent one of the major families of biosensors which have been exploited for bioassay applications, because of some advantages, such as sensitivity, simplicity and immunity to electromagnetic interference (Ligler and Rawe-Taitt, 2002). Of the variety of optical techniques that have been successfully demonstrated in microfabricated structures, fluorescence is a popular technique as it provides powerful detection tools, increasing sensitivity and gives the possibility of imaging sites on a restricted area for detection of on-chip reaction (Chabinyc et al., 2001; Schilling et al., 2002).

Our laboratory is developing tools for point-of-care instrumentation for monitoring cardiac makers in small blood samples (Irawan et al., 2007, 2005; Garcia et al., 2004). One aim is to fabricate polymeric devices that contain primary antibody covalently immobilized onto polystyrene. The main issue addressed in this paper is, therefore, the development of (1) strategies for simple and highly efficient in-channel immobilization of antibody onto polystyrene for plastic based microfluidic chip, (2) sandwich immunoassay for the detection of human heart myoglobin. Our approach is to explore the imaging of fluorescence light emission generated from sandwich ELISA in a microchannel using an epifluorescence microscope and a cooled CCD camera. Rapid prototyping of PS, covalent immobilization of antibody onto the microchannel and epifluorescence microscopy have been combined in to achieve the detection of myoglobin.

2. Experimental

2.1. Materials

3-Aminopropyltriethoxysilane (APTES, 99%), glutaraldehyde (50% in water), 30% H_2O_2 were purchased from Sigma–Aldrich, USA. Amplex Red was purchased from Invitrogen, USA. Bovine serum albumin (BSA) and human heart troponin T were purchased from Sigma (USA). Human heart myoglobin, its monoclonal antibody (mouse host), and HRP-anti-myoglobin were purchased from Fitzgerald, USA. Phosphate buffered saline (PBS, pH 7.4) was used as the primary buffer (Sigma). Deionized (DI) water having 18 MΩ cm resistivity was used in making all aqueous solutions.

2.2. Apparatus

The individual layers of the microfluidic devices were fabricated using a CO_2-laser cutting tool (Epilog Laser, Legend 24TT, USA). Contact angle measurements were performed using a FTA200 Dynamic Contact Angle Analyzer (First Ten Ångstroms, USA) combined with a program, FTA 32 utilizing the sessile drop. Contact angle values were calculated using software provided by the manufacturer. The AFM measurements were performed using a Nanoscope AFM (dimension 3100 scanning probe microscope, Digital Instruments, USA) in tapping mode. XPS analysis was performed using a Kratos AXIS spectrometer (Kratos Analytical Ltd. UK) with monochromatic Al Kα (1486.71 eV) X-ray radiation. The fluorescence signal was detected using a Zeiss upright epifluorescence microscope (Axioskop 2 mot plus, Germany). A mercury arc lamp was used as the light source with the appropriate set of filters for rhodamine excitation and emission wavelengths. A Zeiss 12-bit cooled CCD camera (AxioCam MRc5, Germany) operated with Axiovision software was used to acquire images of the channels at an exposure time of 2 s.

2.3. Device fabrication, assembly, and microfluidic system

Before fabrication, device design, width (0.2 mm) and the lengths (6.2 mm) of channels and the individual layers were drawn using a computer-aided drawing program, and then transferred to the computer-controlled CO_2-laser cutting machine. The channel layout is shown in Fig. 1. The device consisted of 3 layers. Layer 1, was cut from 1 mm thick sheets of PS (Professional Plastics, USA). Layer 2 is a Mylar channel layer (0.051 mm, adhesive layer on both sides, Fralock Div. of Lockwood Ind., USA), which is

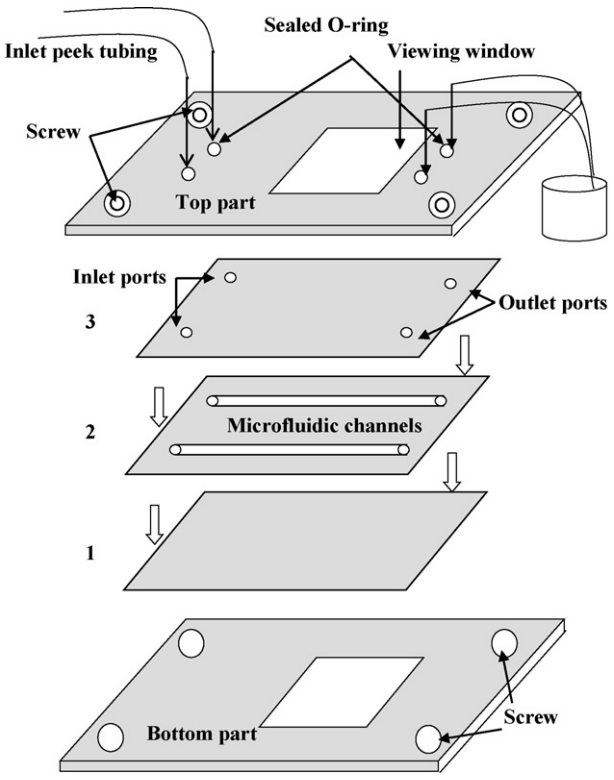

Fig. 1. Microfluidic device fabrication (not in scale). (1) PS substrate modified with APTES, (2) mylar channel layer with adhesive on both sides, and (3) channel-capping layer (optically clear PMMA) forming the top of microfluidic channels. The top part (with O-ring to hold connecting tubing in place) and bottom part of the manifold are sealed with screws and nuts.