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Label-free impedance detection of low levels of circulating endothelial progenitor cells for point-of-care diagnosis

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ABSTRACT

This paper presents a novel microfluidic system for rapid label-free detection of endothelial progenitor cells (EPCs) from small volumes of white blood cells samples, to obtain a bedside cardiovascular diagnostic solution. The system was built on a single 1 cm^2 microelectrode array silicon chip, integrated with negative dielectrophoresis for cell trapping, surface immunochemistry for selective cell capture, and fluidics for cell washing and impedance detection. The level of circulating EPC level in blood is a biomarker of clinical interest, linked to the assessment of risk factors in cardiovascular diseases which are a major global concern. Rare EPCs are usually detected through *in vitro* culture or flow cytometry, which are too time-consuming to bring timely reports in acute diseases. Although microfluidics approaches have enabled reduced processing time and enhanced portability, their sensitivity and processing volumes are still inadequate for rare cell detection at a bedside setting. Using small highly sensitive microelectrodes, our novel integrated system achieved the detection of 720 EPCs in a small 12 μl sample of 72,000 peripheral blood mononuclear cells (PBMC), i.e. equivalent to a concentration of EPCs of 0.1% of 100 μl blood. This demonstrated that clinically significant level of EPCs (<0.5% of PBMC) could be detected for the first time on a detection system at bedside set-up, showing great potential in applications for point-of-care diagnosis.

1. Introduction

Heart diseases are the number one cause of death worldwide, claiming the lives of an estimated 17.5 million people every year (Mackay and Mensah, 2004). Almost 50% of the cases are caused by the coronary circulation failing to supply adequate blood to the cardiac muscles due to atherosclerosis – the building up of plaque on the walls of arteries, narrowing the flow passage. A portable tool to monitor the evolution of specific biomarkers or help make critical decisions to choose the appropriate therapy in acute conditions at the point-of-care is highly demanded by cardiologists and patients worldwide.

Circulating endothelial progenitor cells (EPCs) have shown very promising potential to inform on cardiovascular risks and therapy efficiency (Rosenzweig, 2005). EPCs originate from the bone marrow and are circulating in the blood with the ability to differentiate into vascular endothelial cells that help in the repair of blood vessel linings. In cases of endothelial injury in healthy people, it was suggested that insufficient circulating EPCs might influence the onset of cardiovascular disease (Hill et al., 2003). The risk of death from cardiovascular causes was also shown to decrease with elevated levels of circulating EPCs (Werner et al., 2005; Boilson et al., 2008).

More particularly, occurrence of restenosis from stenting in patients suffering from atherosclerosis has been a major concern (Indolfi et al., 2003). Intravenously transfused EPCs have been shown to enhance reendothelization and inhibit restenosis (Werner et al., 2003). In addition, stents coated with CD34+ antibodies to capture EPCs showed promising results with lower incidence of restenosis. Elevated levels of circulating EPCs increase the efficiency of these stents (Aoki et al., 2005). Hence EPC levels can be used to help cardiologists in the assessment of the feasibility of deploying these CD34+ coated stents. Finally, the level of circulating EPCs also can aid in determining the efficacy and efficiency of statin treatment in patients with coronary artery disease (Vasa et al., 2001). Consequently, a point-of-care diagnostic test detecting the level of circulating EPCs in the blood of cardiovascular patients may be very helpful to cardiologists.

In vitro cell culture and flow cytometry (fluorescence-activated cell sorting, or FACS) have been widely used for the quantification of circulating EPCs (Siena et al., 1991; Khan et al., 2005; Van Craenenbroeck et al., 2008). Although these methods offer high sensitivity and accuracy, they are time-consuming (a complete FACS

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experiment takes around 4–5 h) and have to be performed in the laboratory by skilled personnel on bulky equipments. In addition, large sample volumes (few millilitres of blood) are usually required as the level of EPCs in blood is low, around 0.01–1% of peripheral blood mononuclear cells (PBMC) (4000–11,000 PBMC/μl of blood), necessitating trained personnel to draw the sample and aggravating patient discomfort.

In recent years, an increased interest has been seen in the development of total analysis solutions in microfluidic or lab-on-chip devices for integrated cell-based detection (Andersson and Van den Berg, 2003) which enabled a faster detection. Techniques integrated with microfluidic systems include optical detectors, electrochemical detectors, mass spectrometric detectors and nuclear magnetic resonance detectors (Yi et al., 2006), optical and electrochemical detectors being the most widely used for cell detection. In contrast to optical detection, which usually requires bulky light sources or optical detectors, electrical devices can be readily miniaturized and integrated on-chip, while electronic detection signals are most convenient for recording and processing. Though label-free detection methods, such as electrochemical methods (Lin et al., 2008) or polymerase chain reaction (Belgrader et al., 1998) were used in faster procedures, they still had to rely on a supplementary sample purification step to overcome their limitation in specificity for EPC detection (Steurer et al., 2008).

The issue of specificity was addressed by functionalising surfaces of the microsystem with specific capture probes. For example, Wang et al. (2008) immobilised specific antibodies onto the solid surface of an electrical sensor for specific capture of analytes like cells and proteins. Immunochemical interaction occurring at the surface of the electrode causes the captured analytes to change the impedance of the electrode which can be subsequently measured. Furthermore, in a previously reported work (Chen et al., 2008a), enhancement of the microelectrode array (MEA) sensitivity towards "few cells" detection was demonstrated by using small microelectrodes (60–100 μm in diameter), opening the potential to detect low levels of EPCs.

In addition to speed and sensitivity, point-of-care devices should also be able to process small samples collected in minimally invasive procedures such as a finger prick. In the case of rare circulating cells, the processing of these small volumes with microfluidics in tubings and channels can lead to critical sample loss (by adsorption to the side wall for example), drastically reducing sensitivity. For example, Nagrath et al. (2007) reported a capture efficiency of 65% for cancer cells spiked in PBS. The device presented here used a batch approach, where the sample is loaded directly into a plastic chamber located on top of the MEA detection surface. There was no flow in tubings or channels to bring the cells to the detector, to minimise cell loss. Dielectrophoresis was used to concentrate the cells on the active detection area, the microelectrodes. Dielectrophoresis (DEP) is a valuable tool often used in microfluidic devices for rapid cell trapping to specified locations, without the use of bulk fluidics. Positive DEP has been shown to successfully trap bacteria cells to localised electrodes for detection (Suehiro et al., 2003). However positive DEP requires a low conductivity media which is different from the natural cell environment (Huang and Pethig, 1991), thus requiring an additional washing step, which increases the risk of cell loss. Negative DEP, which is effective for standard cell culture buffers and has recently been shown to successfully trap single cells (Thomas et al., 2009), was therefore employed in our detection system to position the cells on the sensitive area of the MEA, without the use of fluidics.

In this paper, we demonstrate the rapid, label-free and highly sensitive detection of EPCs (CD34+ cells) from a small sample volume of PBMC (4 μl) on our novel microfluidic system. We coated a layer of an antibody recognising EPCs on the surface of the working electrodes on our MEA chip for specific capture of the EPCs. Negative DEP concentrated and trapped the cells at the centre of the working electrodes of the MEA chip. Label-free impedance detection results showed that high sensitivity was achieved within small localised electrode areas, resulting in the detection of 720 EPCs in a small 12 μl sample of 72,000 PBMC, which is equivalent to a concentration of EPCs of 0.1% of 100 μl blood. This demonstrated for the first time that clinically significant level of EPCs (<0.5% of PBMC) (Hill et al., 2003) could be detected on a microsystem in a bedside set-up, where PBMC samples can be easily obtained off-chip. To realise the great promise it holds in applications for point-of-care diagnosis, the detection capability shown here will have to be integrated with sample preparation methodologies to obtain PBMCs from a small whole blood sample.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

2-[Methoxy(polyethyleneoxy) propyl]trimethoxysilane (Mw = 460–590, silane-PEG) was from Gelest (Morrisville, PA). All the solvents, including toluene and triethylamine, were HPLC grade and purchased from Sigma–Aldrich, along with 11-mercaptoundecanoic acid 95% (11-MUA, cat# 450561), 3-mercaptopropionic acid 99% (3-MPA, cat# M580), N-hydroxysuccinimide 97% (NHS, cat# 56480), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC, cat# E6383) and albumin bovine serum (BSA, cat# A3294). Phosphate buffer solution (PBS, pH 7.4, calcium chloride and magnesium chloride ions non-present) was from Invitrogen and the CD34 clone 4H11[APG] antibody (cat# MA1-19229) was from Affinity BioReagents Pte Ltd.

2.1.2. Cells

CD34+ cells purified from the bone marrow were purchased from StemCell Technologies Inc. (Vancouver, Canada) and received as frozen vials of 0.2 million cells (cat# ABM016F). They were cultured according to standard non-adhering cell culture and could remain viable for 5 days without substantial loss of the CD34 expression (79 ± 10%). PBMC samples from StemCell Technologies Inc. (cat# PB003F) were received as frozen vials of 15 million cells and cultured according to standard non-adhering cell culture, except for the fact that they could not be kept in culture for over 5 days.

2.1.3. Mixture cells sample

Mixtures of CD34+ cells and CD34− cells (Jurkat cells or PBMC) were prepared by spiking the necessary amount of CD34+ cells into a CD34− cell suspension in PBS.

2.2. Methods

2.2.1. Microchip design and fabrication

MEA chips are 1 cm × 1 cm and were fabricated using the process described in a previous work (Chen et al., 2008b), by applying standard silicon microfabrication technologies. The electrode design on the MEA chip was evaluated and optimized through simulations (Vector Fields) to enable negative DEP, while accounting for the requirement for small individually controlled working electrodes to achieve high sensitivity. To carry out the dual functions of DEP and impedance spectroscopy, the chip was designed to contain an array of 24 (4 × 6) small working Au disk electrodes of 100 μm diameter, each surrounded with a Au horse-shoe shaped electrode, as shown in Fig. 1(a). Each of the working electrodes leaded out individually to a connection pad. The horse-shoe shaped electrodes