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Combined detection of C-reactive protein and PBMC quantification from whole blood in an integrated lab-on-a-disc microfluidic platform

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ABSTRACT

There is an increasing need for portable and low-cost diagnostic devices for detecting inflammatory/infectious diseases in a rapid and user-friendly fashion. Here, we present a lab-on-a-disc solution, which performs automated sample pre-treatment and combinedly detects small molecules and counts cells in a whole blood sample with a volume of 8.75 µL with a sample to answer time of 14 min. It is used to detect two common inflammation/infection biomarkers, C-reactive protein (CRP) and peripheral blood mononuclear cell (PBMC) count. The whole blood sample was separated into plasma and PBMC fractions using density gradient centrifugation and centrifugo-pneumatic valving. On-disc CRP detection was performed in the extracted plasma using a CRP-antibody-functionalized magnetic nanobead (MNB)-based agglutination assay and a Blu-ray-based optomagnetic detection unit. On-disc PBMC scanning and quantification was performed using an optical imaging unit. Both detection units were integrated on the centrifugal platform and the entire study was automated in order to ensure reliability of the assay and user-friendliness of the method. We measured the CRP level of subjects with different CRP levels and obtained approximately 73% PBMC extraction efficiency compared to hospital results. The concurrent/combined detection of these two common biomarkers in an automated microfluidic platform with integrated detection units and with a low sample-to-answer time is a significant step forward towards a low-cost, out-of-lab, and portable tool to detect multiple biomarkers of significantly different nature (molecules and cells).

1. Introduction

CRP is a protein found in blood plasma and its level increases significantly in response to most inflammations. It is a widely used biomarker for detecting acute-phase inflammation, infection and tissue damage [1–3]. Additionally, an increase in plasma CRP level indicates a higher risk of myocardial infarction, atherosclerosis or other coronary diseases [4]. The median value of CRP in plasma from healthy adults is 0.8 mg/L [1], concentrations above 3 mg/L indicate a high risk of cardiovascular diseases [4] and in the case of infections, the level can rise to more than 500 mg/L [1]. However, a CRP test is non-specific in nature [5]. Therefore, it is necessary to measure other relevant biomarkers along with CRP to perform a specific diagnosis or an advanced prognosis. One of such biomarkers is the white blood cell (WBC) count or peripheral blood mononuclear cell (PBMC) count, which are also known to increase in response to most inflammations or infections [6,7]. WBCs include five different cell types: lymphocytes, monocytes and granulocytes (eosinophil, basophil and neutrophil), where the former two mononuclear cells (lymphocytes and monocytes) are the PBMCs. The total WBC count, the PBMC count, and the count of each of the individual type of WBCs are markers for different diseases and medical conditions. In addition to inflammation/infections, elevated levels of WBCs have been associated with increased risk of mortality among patients with coronary heart disease or patients undergoing haemodialysis. Furthermore, type-2 diabetes, HIV, different types of cancer, immune system disorder and even hypertension have been reported to increase the WBC count [8–14].

Studies have found positive correlations between the WBC or PBMC counts and different inflammatory biomarkers including CRP [10,13] for most inflammations and certain bacterial infections such as pneumococcal pneumonia [15]. It has also been suggested that PBMCs themselves may participate in the production of CRP in blood [16], hence indicating a strong correlation between the PBMC count and CRP concentration for the prognosis of certain diseases. Furthermore, epidemiologic studies have shown that an elevated CRP level in apparently healthy individuals can be a marker of high potential risk of certain types of cancer and that a high CRP level in cancer patients indicates a risk of earlier death by cancer [17]. PBMCs have also been linked to

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cancer prognosis [18]. Thus, while CRP tests act as the first-line screening for various infectious and inflammatory diseases as well as cancer and cardiovascular conditions, combining CRP detection with WBC or PBMC count and further cell analysis may lead to an improved diagnosis and prognosis.

In clinical laboratories, the CRP concentration is determined using a latex-enhanced immunoturbidimetric assay [19,20], on a human serum sample exposed to a number of sample pre-treatment steps. In the literature, different approaches to determine the CRP concentration in human serum have been presented including a magnetic bead-based agglutination assay [21–23], quantum dots and immunofiltration [24], quantum dots and immunochromatography [25], electrochemical aptamer-based sandwich assay [26], SPR-based immunosensing using CRP antibodies [27], nanoparticle-enhanced SPR using aptamer-antibody sandwich assay [28] as well as fluorescence-based biosensing [29]. Furthermore, Gyros Protein Technologies (Sweden) uses microfluidic discs (Gyrolab CD) to integrate sample pre-treatments minimizing manual interventions to quantify CRP using a nanoliter-scale sample volume. However, separate microfluidic discs are needed to detect different biomarkers and it does not combine an immunoassay with WBC count.

WBC or PBMC counts are determined in standard clinical laboratories by manual or automatic counting. The manual counting is performed in a haemocytometer using standard microscopy. The automatic counting is commonly done by flow cytometry or automated image cytometry [30]. All these methods all require a number of complex and time-consuming sample pre-treatment steps, including fluorescent labelling, staining and red blood cell (RBC) lysis prior to the actual counting. The widely available point-of-care device HemoCue-WBC (HemoCue AB) can perform WBC counting with high accuracy with no need for sample pre-treatment. However, as the device has no integrated optical imaging unit, it is not possible to investigate physical characteristics of the cells, study cell agglutination or to analyse their morphologic abnormalities. Furthermore, WBC count alone is insufficient for the diagnosis of a disease with full certainty. There are reports on cell analysis and counting in centrifugal microfluidics platform [31–33], but these studies lacked an integrated sample-to-answer platform for cell counting as well as fell short of demonstrating concurrent detection of other relevant biomarker(s) for prognosis of a disease. Similarly, Biosurfit's (Portugal) Spinit® instrument uses surface plasmon resonance to perform an immunoassay and its integrated microscopy module counts WBCs on a microfluidic disc. However, separate microfluidic platforms/discs are needed to perform these two operations and thus it does not perform a combined detection/quantification of the two biomarkers from a single blood sample. Likewise Hemocue-WBC, the Spinit® instrument lacks the imaging display to investigate physical characteristics of cells. Thus, in spite of biosensors having achieved significant success in diagnostics [34], there is an unmet need for a portable and automated diagnostic tool that in a single microfluidic platform combines detection of relevant biomarkers with the WBC/PBMC count for advanced prognosis of inflammatory/infectious diseases.

Here, as a first but significant step forward, we present an integrated platform to combinedly detect the concentration of CRP and quantify PBMCs directly from few microliters of human whole blood, where a single low-cost microfluidic disc is used as the experimental platform onto which all assay steps are integrated and automated. First, the density gradient centrifugation method [35] is used for on-disc plasma and PBMC separation. Then, the separates are transferred to distinct microfluidic chambers for further on-disc processing.

The CRP in the plasma is detected using a sandwich agglutination assay employing magnetic nanobeads (MNBs) [23,36–43]. To promote and accelerate agglutination of MNBs in the presence of CRP, we use a magnetic field incubation protocol [41,44]. Agglomerates are detected using a Blu-ray based optomagnetic readout technique [39–41], which measures the modulation of light transmitted through the sample in response to an applied oscillating magnetic field as function of the frequency of the magnetic field. The individual MNBs are superparamagnetic and substantially spherical and therefore show nominally no signal. Agglomerated MNBs have coupled magnetic and optical anisotropies and therefore show a signal at low frequencies where they can rotate and thereby modulate the intensity of transmitted light in response to the applied oscillating magnetic field. The on-disc PBMC counting is performed on the PBMC separate using an integrated optical imaging unit (oCelloScope, BioSense Aps) [40,45], which is an automated bright-field microscope that uses optical sectioning from confocal microscopy to create a volume image. This makes it possible to capture images of all cells in the sample. The PBMCs are identified and quantified using the instrumental software Uniexplorer 8.0, that employs an object-based image analysis algorithm to segment the cells based on their surface area.

Both detection units (optomagnetic reader and optical volume imaging) are integrated with the automated centrifugal platform facilitating a compact sample-to-answer device for detection of CRP and PBMC quantification directly on a single whole blood sample in a fast, reliable and user-friendly fashion while using a low-cost polymer microfluidic disc consumable. The design and the automated operation of the microfluidic disc to extract PBMCs and a certain amount of plasma to separate microfluidic chambers, the integration of the hardware of the two detection units along with the automated centrifugal microfluidic platform, the on-disc detection of CRP on the extracted plasma sample and on-disc PBMC counting from a single blood sample within a single experiment demonstrate the inherent advantages of the integrated biosensing platform to combinedly detect biomarkers of different sizes or nature.

2. Experimental

2.1. Materials and chemicals

The experimental platform of this study was a Poly(methylmethacrylate) (PMMA)-based microfluidic disc fabricated in-house. The blood samples (EDTA-treated), collected from healthy donors, were purchased from the Copenhagen University Hospital (Rigshospitalet). For efficient separation between the RBCs and the PBMC layer, a density gradient medium (Histopaque-1077, Sigma-Aldrich) was used during blood processing. The MNBs used in this study were carboxy-labelled particles (Millipore Estapor-Merck, Product number: M1-020/50) with an average diameter of 176 nm. These MNBs are nominally spherical and superparamagnetic and thus show no optomagnetic response when they are not agglomerated. The MNBs were functionalized with goat polyclonal CRP antibodies (Midland Bioproducts, USA, Product number: 73307) to enable the agglutination of the MNBs in presence of the CRP antigen in the blood plasma. We have used this affinity-purified CRP antibody because of its well-proven specificity to bind with CRP molecule [21]. CRP-free serum (Hytest Ltd., Turku, Finland) was used for sample dilution and negative control experiments. 10 mM PBS (pH 7.4) buffer was prepared using MilliQ water for MNB suspension and blood dilution.

2.2. Functionalization of magnetic nanobeads with CRP antibodies

The magnetic nanobeads (MNBs) were functionalized with CRP antibodies following a previously described protocol [41]. This protocol defines an anti-fouling surface architecture over the MNBs utilizing bio-orthogonal click chemistry to minimize the formation of non-specific aggregates under the influence of complex biological fluids. Thus, a monolayer of blocking proteins - human serum albumin HSA (Sigma Aldrich, A9511) was firstly immobilized on the MNBs by means of carbodiimide chemistry. 50 mg of HSA was added to 500 µL of MNB sample at a bead concentration of 5% (w/v) followed by overnight incubation. The MNBs were then washed three times and resuspended