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Isolation of cancer cells by "in situ" microfluidic biofunctionalization protocols

Stefania De Vitis [a], Giuseppina Matarise [b], Francesca Pardeo [b], Rossella Catalano [b], Natalia Malara [a], Valentina Trunzo [b], Rossana Tallerico [b], Francesco Gentile [b], Patrizio Candeloro [b], Maria Laura Coluccio [b], Alessandro Massaro [f], Giuseppe Viglietto [b], Ennio Carbone [b,d], Jörg P. Kutter [c], Gerardo Perozziello [b,*], Enzo Di Fabrizio [e]

[a] Italian Institute of Technology IIT, Genova, Italy
[b] University of Catanzaro "Magna Græcia", 88100, Catanzaro, Italy
[c] Department of Pharmacy, University of Copenhagen, Universitetsparken 2, 2100 Copenhagen, Denmark
[d] Department of Microbiology Tumor and Cell Biology (MTC), Karolinska Institutet, 17177, Stockolm, Sweden
[e] King Abdullah University of Science and Technology (KAUST), 23955-6900, Thuwal, Saudi Arabia
[f] Istituto Italiano di Tecnologia (IIT), Center for Biomolecular Nanotechnologies (CBN), Via Barsanti, 73010 Arnesano (LE), Italy

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ABSTRACT

The aim of this work is the development of a microfluidic immunosensor for the immobilization of cancer cells and their separation from healthy cells by using "in situ" microfluidic biofunctionalization protocols. These protocols allow to link antibodies on microfluidic device surfaces and can be used to study the interaction between cell membrane and biomolecules. Moreover they allow to perform analysis with high processing speed, small quantity of reagents and samples, short reaction times and low production costs. In this work the developed protocols were used in microfluidic devices for the isolation of cancer cells in heterogeneous blood samples by exploiting the binding of specific antibody to an adhesion protein (EpCAM), overexpressed on the tumor cell membranes. The presented biofunctionalization protocols can be performed right before running the experiment: this allows to have a flexible platform where biomolecules of interest can be linked on the device surface according to the user's needs.

1. Introduction

Cancer is a leading cause of mortality in the developed world [1] and incidence rates for many cancers are increasing [2]. A method for an early diagnosis of this disease is the detection and isolation of CTCs (Circulating Tumor Cells) in the patient's blood. Studies revealed an important and growing role of microfluidics in this field [3–5].

In the last years the use of microfluidic systems in medicine and biology [6–8] and in particular in cancer research [9–11] is increasing enormously and novel and interesting approaches have been developed [12–14]. The advantages of using these devices in biological and medicine research are due to the capacity to obtain the same results of standard methods of analysis but with high processing speed, small quantity of reagents and samples, short reactions times, low production cost [15] enhanced sensitivity, real-time analysis and automation [16–19].

Since the first application of microfluidics systems [20] many studies have been made to isolate specific molecules from complex samples [21–23]. However, in the last years many works have been focused on the isolation of CTC, malignant cancer cells identified in the peripheral blood used as a biomarker for cancer diagnosis, to monitor the patient's response to different treatments [24–26] and to study the metastasis diffusion process [27].

In this work we present a method for "in situ" bio-functionalizing microfluidic systems allowing to link antibodies in microfluidic channel surfaces. In particular we demonstrate to biofunctionalize our devices with specific antibodies which bind specific proteins present on the membrane surface of cancer cells: our target protein was Ep-CAM, a glycosylated membrane protein located in low levels on surface of many healthy epithelial tissues but overexpressed also in most primary and metastatic malignant tumors [28–30]. Biofunctionalized microfluidic devices were used to sort colon cancer cells HCT-116 from healthy cells.

* Corresponding author. Tel.: +39 09613694380; fax: +39 09613694147.
E-mail address: gerardo.perozziello@unicz.it (G. Perozziello).

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2. Materials and methods

2.1. Materials

Methanol was purchased from Sigma–Aldrich (St. Louis, MO). For the chamber fabrication, SU-8 photoresist and developer were supplied by MicroChem (Newton, MA); silicone elastomer and curing agent were purchased from Dow Corning (Midland, MI). Phosphate buffered saline (PBS) was from Sigma–Aldrich (St. Louis, MO). Glass slides (25 × 75 mm and 1 mm thick) were purchased from Knittel Glaser (Bielefeld, Germany). For the microfluidic chamber biofunctionalization, 3-aminopropyltrimethoxysilane (APTMS), biotin, N-hydroxysuccinimide (NHS, 98%), and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) were purchased from Sigma–Aldrich (St. Louis, MO). Streptavidin were from Polysciences, Inc. (Eppelheim, Germany). Human serum was obtained from healthy donors' buffy-coat cells by Ficoll-Paque gradient centrifugation. Biotinylated anti-Human Ep-CAM/TROP1 antibody and Fluorescein conjugated Ep-CAM/TROP1 were purchased from R&D Systems. HCT-116 and cardiomyocytes were purchased from ATCC. The healthy cells lymphocytes derived from human blood. All experiments were performed according to ethics guidelines and approvals.

2.2. Cell culture

In this work HCT-116 cell line, lymphocytes and H9C2 cardiomyocyte cell line were used. We selected these cell lines among the others grown in our laboratories for their analogous sizes: HCT-116 are colon cancer cells with a diameter of about 10 μm and were cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS), 2 mmol L^{-1} L-glutamine, 10 mol L^{-1} thioglycerol, 12.5 U insulin, 0.5 mg hydrocortisone, 30 mg penicillin G and 0.05 g streptomycin. H9C2 cardiomyocytes derived from rat ventricles (about 14 μm in diameter) and were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), 2 mmol L^{-1} L-glutamine, 30 mg penicillin G and 0.05 g streptomycin. Lymphocytes (7 μm in diameter) were obtained from human blood through buffy-coat protocol. The cell were cultured in Petri Dish (100 mm) and, at the time of their optimal confluence, were incubated with Trypsin–EDTA to remove cells from the culture substrate. Later, cells were centrifugated and harvested in phosphate buffered saline (PBS).

2.3. Device fabrication and surface biofunctionalization

The microfluidic devices consisted of a microchamber having dimensions of 10 mm in width, 35 μm in height and 30 mm in length. The devices were fabricated in polydimethylsiloxane (PDMS) by soft-lithography and bonded to the glass slides, permanently, by plasma bonding. Just after plasma surface activation, the microchambers were treated with a solution of APTMS 30% in methanol for 1 h to obtain amino groups on the glass slide. For the device biofunctionalization, the following reagents and molecules were injected and incubated: (1) Biotin (2 mg/mL), EDC (10 mg/mL) and NHS (15 mg/mL) for 2 h. (2) Streptavidin (2 mg/mL) for 1 h. (3) Biotinylated anti-Ep-CAM (100 μg/mL) for 45 min. In between the different steps, the devices were washed with PBS (phosphate buffer saline) for 1 min (Fig. 1b)

2.4. Microfluidic setup

Inlet and outlet of the system were connected to the tip of two pipettes. Silicone tubes were used to connect the syringe pump to the sample reservoir, the sample reservoir to the microfluidic chamber and the latter to the recovery reservoir. The cells in the microfluidic devices were monitored on an optical microscope (Olympus BX51) that was equipped with a CCD camera (Nikon DS-2Mw) connected to a PC (Fig. 1a).

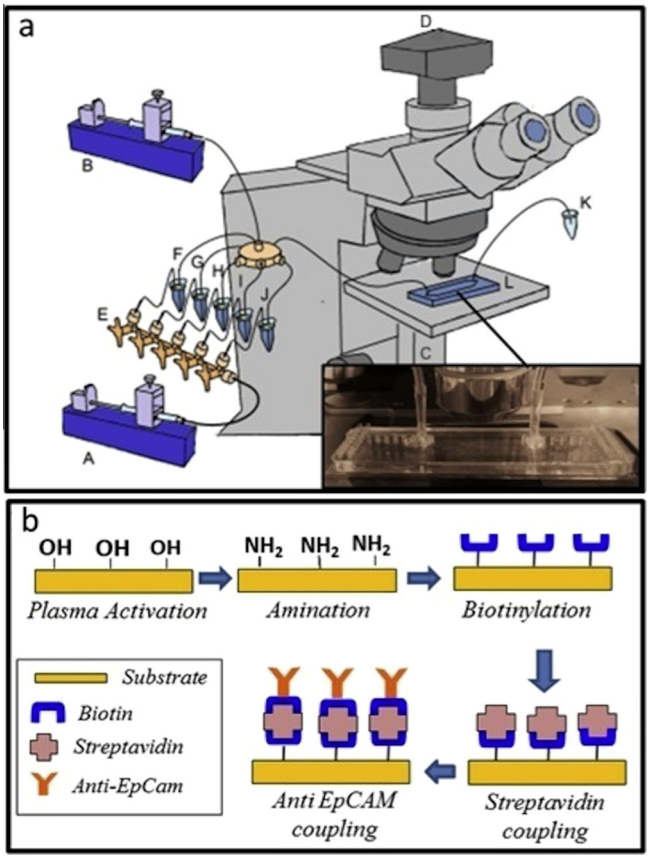

Fig. 1. (a) Schematic representation of the "in situ" biofunctionalization protocol. (b) Schematic representation of the experimental set-up. A: Syringe pump pushing air; B: syringe pump pushing the biological sample; C: optical microscope; D: camera; E: multi port valve; F: PBS vial; G: DI water vial; H: biotin vial; I: streptavidin vial; J: biotinylated antibody vial; K: waste; L: microfluidic device.

2.5. Characterization of the devices

The presence of amino groups on the glass surface was estimated through contact angle and fluorescence measurements. For the contact angle measurements, the characterization of the devices was performed on the glass slides detached from the top PDMS layers. In order to do this, a DI water drop was placed on top of the glass surface after the treatment with APTMS 30% in methanol and the contact angle was measured and compared to the contact angle of a drop placed on top of a native glass. Moreover, aminated devices were incubated with rhodamine isothiocyanate (4 mg/mL) in DMSO for 30 min and after washed with distilled water. This fluorochrome present isothiocyanate groups that form stable amide bonds with NH_2-groups of aminated surface and the reaction can be estimated by fluorescence measurements at the optical microscope (Nikon Eclipse TE2000-U).

The presence of biotin on the device surface was investigated by fluorescence microscopy. Therefore, the chips were incubated for 2 h with fluorescent streptavidin (2 mg/mL) in phosphate buffer saline and then washed with PBS. The streptavidin bonds the biotin molecules present on the chips surface and the complex biotin–streptavidin was detected at the microscope.