Smart surfaces for pH controlled cell staining†

Simona Argenti^{†,*,a} Laura Blasi^{†,*,a} Giuseppe Ciccarella,^a Antonella Cazzato,^b Giovanna Barbarella,^b Roberto Cingolani^a and Giuseppe Gigli^a

Received 16th July 2009, Accepted 8th September 2009

First published as an Advance Article on the web 29th September 2009

DOI: 10.1039/b914277k

In this work, novel smart surfaces for *in situ* cell staining were realized by covalent attachment of pH-responsive microgels on platforms dedicated to microfluidics for lab-on-chip. The poly(methacrylic acid) microgels were firstly synthesized in solution and then covalently immobilized on a glass surface. As they preserve their pH-sensitive nature after the covalent immobilization, microgels were loaded with an oligothiophene-conjugated anti-human CD4 monoclonal antibody, and finally incubated with a Jurkat T-cell suspension. The physiological pH of the extracellular environment induced the pH-triggered release of the labeled antiCD4 antibodies and the selective staining of the CD4-positive subpopulations within the Jurkat cell suspension. The realization of this type of smart surface for the encapsulation of specific monoclonal antibodies and their release in an on-demand way should have an enormous potential in developing fully integrated platforms for cell analysis.

An important challenge for the development of lab-on-chip devices (LOCs) for cell analysis and flow cytometry, is to integrate sample preprocessing with counting and characterization of cells.¹ Indeed, most of the current strategies to realize microdevices for single-cell analysis require extensive sample preparation outside the device, involving time and sample consuming procedures.² Further, several studies report that handling procedures and improper stimuli during blood processing can alter the original immuno-phenotype of the separate cells, thus inducing sample preparation artifacts.^{1a}

Flow cytometric analysis has broad applications in medicine, including AIDS detection and patient monitoring,³ and requires fluorescent staining of cells prior to analysis both in LOC devices and standard equipments.²

To date, staining procedures have been developed “on-chip” by directly loading cells and staining reagents onto the microfluidic chip.⁴ However, these labeling techniques require injections of reagents and buffers by highly trained personnel, as well as the transport of labile staining reagents where the analysis takes place. The precise control of the microenvironment during the staining process of each individual blood cell could overcome these problems,

thus reducing the potential for alterations of the original state of the cells of interest. In this context, the development of a reliable technological platform to retain functional molecules (*e.g.* labeled antibodies) in an active state on the device surface and to release them in an on-demand and reversible way, is strongly desirable. To this aim, hydrogels represent excellent candidates as marker delivery systems because of their properties of hydrophilicity, biocompatibility, and mechanical strength. When applied to surfaces, hydrogels form a soft three-dimensional (3D) matrix able to encapsulate bioactive molecules, ensuring them a protective environment and inhibiting their degradation.⁵ However, in the development of microchip-based diagnostics, the unique property of stimuli-responsive hydrogels to encapsulate and subsequently release macromolecules has not been fully explored. To exploit this property, a decrease in the hydrogel size to the micrometer scale presents many advantages, since the decrease in hydrogel size enhances the hydrogel response time and minimizes the ion diffusion limitations associated with larger hydrogels.^{5,6} In addition, micrometer-sized hydrogels (microgels) combine the favourable properties of a hydrogel with those of colloidal particles, including large surface to volume ratio, low dispersity and versatility of surface functional groups, as well as comparable size to that of microchannels in LOCs.⁷ A functional use of microgels in LOCs requires that they have to be fixed within the active regions of the device channels and not flow out during the analysis.^{7b} Nowadays, microgels have been deposited as close-packed monolayers on a surface using layer-by-layer (LbL) assembly for uptake and release of low molecular weight molecules such as methyl orange or doxorubicin, and small peptides such as insulin.⁸ In these systems, release processes were driven by diffusion or temperature variations. So far, the potential of pH-responsive microgels for uptake and release of macromolecules in soft and responsive coatings, have not been demonstrated.

In this paper, a novel method for *in situ* pH-controlled cell staining by means of covalently immobilized poly(methacrylic acid) (PMAA) microgels is described. For the cell labeling, an oligothiophene (TF) conjugated anti-human CD4 monoclonal antibody (MAb) has been used. The antibody-loaded microgels immobilized on the glass support were incubated with a Jurkat T-cell suspension at the physiological pH of the extracellular environment, leading to the pH-triggered release of labeled antiCD4 antibodies. The highly selective staining of the CD4-positive subpopulations within the Jurkat cell suspension was demonstrated by confocal laser scanning microscopy (CLSM).

The PMAA microgels were firstly synthesized in solution according to the procedure reported in literature,⁶ and then covalently immobilized on a glass substrate, by exploiting the functional groups existing on the microparticles and on the chemically modified solid surfaces.

^aNational Nanotechnology Laboratories of CNR-INFM, Lecce, Italy. E-mail: simona.argenti@unisalento.it; Fax: +39 0832 298386; Tel: +39 0832 298368

^bCNR, Area di ricerca, ISOF, I-40129 Bologna, Italy

† Electronic supplementary information (ESI) available: Details for synthesis and covalent immobilization of PMAA microgel, as well as for AFM and CLSM characterization. See DOI: 10.1039/b914277k

‡ The first two authors contributed equally to this work.

Fig. 1 (A) Schematic representation of the glass substrate functionalization procedure: (1) oxygen-plasma etching, (2) silanization with 3-triethoxysilylpropyl isocyanate (TESPI) in toluene, (3) incubation with O,O'-bis(2-aminoethyl)polyethylene glycol (diamino-PEG) in THF and (4) incubation with an aqueous suspension of PMAA microgel activated by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC). (B) Uptake and release of TF-antiCD4 into and from the immobilized PMAA microgels.

PMAA microgels were attached to the glass surface according to an innovative procedure described in Fig. 1A. After increasing the hydroxyl group content on the glass surface by oxygen-plasma treatment, 3-triethoxysilylpropyl isocyanate (TESPI) reacted with hydroxyl groups, leaving free isocyanate terminals for further reactions with nucleophilic groups. The amine-terminated PEG (diamino-PEG, average MW = 2000) was reacted with the isocyanate-functionalized glass surface to form urea linkages. A large excess of diamino-PEG spacers was used to link only one terminal amino group to the surface. Subsequently PMAA microgels were covalently attached by carbodiimide chemistry.⁹

After immobilization, the microgels were studied by Atomic Force Microscopy (AFM, XE-100 PSIA) in a dry state and buffered solutions with pH (a) 5.0 and (b) 8.0 (Fig. 2). The microgel on solid supports imaged in air shows a mean diameter of $10 \pm 2 \mu\text{m}$ and a height of $200 \pm 50 \text{ nm}$. (Fig. 2 A–C). The comparison between Fig. 2 A–B and Fig. 2 C–D suggests that swelling of the microgel upon adding buffer solution at pH 5.0 is accompanied by the water ingress into the microgel network.

Upon switching from pH 5.0 (Fig. 2 C–D) to 8.0 (Fig. 2 E–F) the microgel height increases from 500 nm up to 1.2 μm , showing that the immobilized PMAA microgels preserved their ability to undergo reversible volume phase transitions in response to environmental pH, due to the carboxylic groups bound to their polymeric chains.⁶ In particular, the microgel swelling can be ascribed to the Coulombic repulsion between the deprotonated carboxylic groups.

The pH-responsivity of the PMAA microgels was exploited to induce the pH-controlled uptake and release of the TF-labeled anti-CD4 antibody, according to the scheme in Fig. 1B. In the uptake

Fig. 2 (A) Air Non Contact-AFM (NC-AFM) image of immobilized microgel on the glass support. (B) Cross-section of the corresponding AFM images. Fluid Non Contact-AFM images at (C) pH 5.0 and (E) pH 8.0 (D) and (F) cross-sections of the corresponding AFM images.

process the labeled antibodies and the covalently immobilized microgels were incubated at pH 5.0, to ensure that the proteins would load *via* an ion exchange mechanism. Indeed, at this pH the proteins have a net positive charge (pI 5.9) whereas the microgels are negatively charged.⁶ The uptake process was characterized by confocal microscopy (FV-1000, Olympus), to test the ability of the immobilized microgels to encapsulate (Fig. 3) and release (data not shown) the labeled antibody under pH variations. Further, any variation in the maximum emission wavelength of the conjugated TF was observed in the pH range 5.0–7.8.

To release the loaded antibodies, the immobilized microgels were incubated with a Jurkat cell suspension with a pH of 7.8. This pH value is greater than the isoelectric point of the antibodies and the pK_a of the PMAA microgel, therefore both labeled antibodies and PMAA microgels are negatively charged. Under these conditions, the antibody release into the external solution was induced by the increased permeability of the microgel network, as well as by the

Fig. 3 (A) The uptake of TF-antiCD4 antibody into the covalently immobilized microgels was analysed by detecting the fluorescence arising from the TF fluorophore. (B) Fluorescence spectra of the immobilized microgels after the uptake process, assessing the location of the fluorescent biomolecules inside the microgel (1,3,4,5) and the background (2). The scale bar was 10 μm .

Fig. 4 (A) Micrograph of Jurkat cells after the release of TF-antiCD4 MAb from the covalently attached PMAA microgels (objective lens 40x, numerical aperture 0.85). (B) Fluorescence spectrum of TF-antiCD4 antibody recorded on two different cells (region 1 and 2) and background (region 3). The three selected regions are shown in Figure (C) (60x, oil immersion, numerical aperture 1.4). It is noteworthy that the maximum emission wavelength of TF-antiCD4 antibody after binding with CD4 positive Jurkat cells was blue shifted by about 20 nm compared to that of TF-antiCD4 antibody encapsulated into the microgel (Fig. 3 B and Fig. 4 B). The scale bars were 20 μm and 10 μm for Figure (A) and (C), respectively.

electrostatic repulsion between the protein and the hydrogel. The release of the fluorescent antiCD4 antibodies through the enlarged meshes of the microgel network led to *in situ* cellular staining. After the incubation time, the glass supports bearing the cell suspension and the immobilized microgels were analysed by confocal microscopy.

Fig. 4 (A) shows a micrograph of Jurkat cells labeled with fluorescent antiCD4 antibodies released from the PMAA microgels. Different micrographs of Jurkat cells labeled with fluorescent antiCD4 antibodies were analysed cell-by-cell, and the percentages of positive and negative subpopulations were assessed through

a wavelength-specific analysis by CLSM. The experimental results show that the labeled Jurkat T-cells were $\sim 20\%$ of the total. This is consistent with the previously reported observations, indicating that the CD4 antigen was expressed on 20% of the total T-lymphoblastic Jurkat cells.¹⁰ In some cases, it was observed that stained cells were smaller than the unstained cells. The smaller size is compatible with cells in G1- and S-phases of the cell cycle.¹¹

In summary, we realized a simple platform for pH-triggered *in situ* cell staining. Our approach enables the preservation of folded (active) antibody conformations, preventing surface induced denaturation, while the immobilized microgel ensures the antibody to remain hydrated during storage. The platform proposed here provides a significant tool to realize fully integrated sample preparation in single-cell analysis chip and flow cytometry.^{10b}

Acknowledgements

The authors are grateful to Dr Malù Coluccia and Dr Alfredo Ventola for having provided Jurkat T-cells and for useful suggestions in the staining procedure, respectively. Mediteknology srl is also acknowledged for providing us with the labeled antibodies.

Notes and references

- (a) M. Toner and D. Irimia, *Annu. Rev. Biomed. Eng.*, 2005, **7**, 77; (b) D. R. Walt, *Science*, 2005, **308**, 217.
- C. E. Sims and N. L. Allbritton, *Lab Chip*, 2007, **7**, 423.
- D. Barnett, B. Walker, A. Landay and T. N. Denny, *Nat. Rev. Microbiol.*, 2008, **6**, S7.
- (a) C. Lancaster, M. Kokoris, M. Nabavi, J. Clemmens, P. Maloney, J. Capadanno, J. Gerdes and C. F. Battrell, *Methods*, 2005, **37**, 120; (b) S. D. H. Chan, G. Luedke, M. Valer, C. Buhlmann and T. Preckel, *Cytometry, Part A*, 2003, **55a**, 119.
- (a) N. A. Peppas, J. Z. Hilt, A. Khademhosseini and R. Langer, *Adv. Mater.*, 2006, **18**, 1345; (b) D. Guschin, G. Yershov, A. Zaslavsky, A. Gemmell, V. Shick, D. Proudnikov, P. Arenkov and A. Mirzabekov, *Anal. Biochem.*, 1997, **250**, 203.
- G. M. Eichenbaum, P. F. Kiser, S. A. Simon and D. Needham, *Macromolecules*, 1998, **31**, 5084.
- (a) E. Verpoorte, *Lab Chip*, 2003, **3**, 60N; (b) D. S. Peterson, *Lab Chip*, 2005, **5**, 132.
- (a) L. Wang, X. Wang, M. Xu, D. Chen and J. Sun, *Langmuir*, 2008, **24**, 1902; (b) M. J. Serpe, K. A. Yarmey, C. M. Nolan and L. A. Lyon, *Biomacromolecules*, 2005, **6**, 408.
- N. Nakajima and Y. Ikada, *Bioconjugate Chem.*, 1995, **6**, 123.
- (a) K. Kato, M. Toda and H. Iwata, *Biomaterials*, 2007, **28**, 1289; (b) M.-O. Mattsson, E. Lindström, M. Still, P. Lindström, K. H. Mild and E. Lundgren, *Cell Biol. Int.*, 2001, **25**, 901.
- O. Steinberger, Z. Lapidot, Z. Ben-Ishai and D. Amikam, *FEBS Lett.*, 1999, **444**, 125.