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A new quantitative experimental approach to investigate single cell adhesion on multifunctional substrates

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ABSTRACT

Cell adhesion is fundamental for the organization of cells in multicellular organisms since it has a key role in several physiological functions that drive tissue formation and development. A better knowledge of the affections that influence the adhesion capability of cells in several pathologies, such as cancer diseases or multiple sclerosis could enable the development of new therapeutical strategies. Whereas the optimal control of cell adhesion and growth on new technological materials is a primary issue in modern tissue engineering, few techniques are able to provide quantitative and reliable results on cell adhesion. We present a method that enables the investigation of cell adhesion at the single cell level and provides the capability to test the adhesion of a single cell on multifunctional substrates. To reach this goal we applied single cell force spectroscopy (SCFS) on custom designed patterns of molecules prepared on a rigid substrate by using a cantilever based molecule deposition tool, and we tested the adhesion of Chinese Hamster Ovary cells and Human Embrionic Kidney cells on two polyelectrolytes that are widely used as adhesive factors for cells growth: Polyethylenimine and Poly-D-Lysine. Our results confirm the common hypothesis on the mechanism of adhesion promotion by protonated molecules. Optimizations of the experimental settings of SFCS experiment are introduced here. The presented technique offers the unique opportunity to be extended to the study of cell adhesion on an unlimited number molecular species.

1. Introduction

The study of the adhesion properties of cells has a great interest in biomedical and biotechnological research, since cell adhesion regulates the mechanisms of cell cycle in multicellular organisms, and is also strictly related to the development of several pathological states. Pathological mechanisms in cancer diseases severely affect cell adhesion properties; this alteration contributes to cancer propagation through tissues (Shibue and Weinberg, 2011; Rodrigues-Ferreira et al., 2012). Cell-adhesion molecules (CAMs) have a primary role in the determination of neural plasticity, as well as in the development of neurodegenerative pathologies such as Alzheimer's disease and multiple sclerosis (Cotman et al., 1998).

From a technological point of view the adhesion of cells is the pivotal issue in tissue engineering (Bacakova et al., 2004; Nikolovski and Mooney, 2000). The guidance of cell growth on the surface of microelectrodes arrays (MEA) obtained by modulating the deposition of molecules that promote cell attachment increases the quality of extracellular recording of the neuronal activity (Jungblut et al., 2009). In planar patch clamp chemical modification using adhesion molecules is used to place cells on the detection aperture and to promote the growth of a high quality cell to aperture seal (Py et al., 2011). All these topics confirm the critical value that a perfect control of cell adhesion properties on materials, other cells or tissues assume for new developments in biomedical and biotechnological fields. In spite of this, few techniques allowing for the direct and quantitative measurements of cell adhesion are currently available. The first attempt to study cell adhesion was based on simple washing assay (Klebe, 1974). More sophisticated techniques based on the application of shear forces have been proposed recently. The portion of adherent cells within a population can be determined by means of shear force applied via centrifugation, providing valuable statistical information (Burdsal et al., 1994; McClay and Hertzler, 2001). Flow cytometric assay has been recently used to study the adhesion of human colon adenocarcinoma cells on endothelial cells derived from different human tissues (Paprocka et al., 2008). Thickness Shear Mode (TSM) sensors as well as Quartz Crystal Microbalances (QCM) have been used to obtain an indirect evaluation of cell adhesion and cell kinetic reactions (Starly and Choubey, 2008; Wegener et al., 2001). However, all these techniques only provide

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qualitative results on cellular adhesion strengths, and are hardly reproducible due to the fact that the resistance of cells to mechanical detachment depends not only on the number, distribution and strength of adhesion bonds but also on area of contact and cell shape parameters, which are intrinsically variable within cell population. More recently, impedance based sensors have been applied to study cell adhesion, since the effective contact area between cell and substrate affects the sensor impedance and is strictly related to both number of adherent cells and their spreading state (Asphahani et al., 2008; Wegener et al., 2000).

Single cell force spectroscopy (SCFS) has been developed and successfully used in the investigation of cell adhesion on functional surfaces or on other cells (Bertoncini et al., 2012; Elter et al., 2012; Krieg et al., 2008). The adhesion kinetic of single cells has been characterized using Atomic Force Microscopy (AFM) or micropipette based setup such as Biomembrane Force Probe (BFP). BFP allows achieving higher force sensitivity, whereas AFM allows accessing a larger range of forces. The advantage of single cell approach is to obtain consistent results not affected from cell–cell inherent variability, and to provide the unique opportunity to observe individual adhesion states, not averaged from a population of cells.

Our purpose is to develop a method that opens the way towards the quantitative measurement of the adhesion properties of a single cell on different molecular species in a single experiment. To this purpose the use of a dedicated setup combining AFM and micropipette-based actuator has been proposed (Ounkomol et al., 2010). On the other hand, the same aim can be addressed simply by working on sample preparation, making the different molecular species simultaneously accessible to a living cell previously attached to an AFM cantilever. A micro-contact printing based method has been recently applied to create patterned substrates with distinct stripes of laminin and collagen (Dao et al., 2012). In that work (Dao et al., 2012) an array of laminin stripes on a mica substrate was obtained by a modification of the lift-off method (von Philipsborn et al., 2006), and the remaining bare mica areas were backfilled by adding a solution with collagen, thus obtaining a bi-functional substrate. The main drawback of this method is related to the non-trivial assumption that the two different molecular species are not interacting with each other, giving rise to an intermixed area on the sample. Furthermore, this technique is suitable to test a maximum of two molecular species only.

In our work a nano-drop patterning tool based on a cantilever system has been used to control the spatial distribution of different adhesion molecules. This approach provides significant advantages with respect to the other methods previously reported in literature. First, the different molecular species are deposited in different areas of the substrate and are not interacting with each other during sample preparation, avoiding the contamination and mixing between different components. Second, the inkjet technique used in this work can overcome the limitation on the number of molecular species. Third, by patterning the adhesion molecule on an agarose coated substrate we also introduce a cell repulsive area on the substrate, thus reinforcing the selective effect of positive adhesion cell cues with an additional negative cue. This solution has been adopted aiming to increase the efficiency of the cell capture on the AFM cantilever.

We applied our method to the study of the mechanism of adhesion of Chinese Hamster Ovary cell (CHO) on two different poly-electrolytes, Poly-D-Lysine (PDL) and Polyethylenimine (PEI). These are two of the most extensively used and characterized polycations in biomedicine, exploited for example in gene delivery and in cell patterning (Hwang et al., 2005; Picart et al., 2002; Royce Hynes et al., 2007; Vodouhe et al., 2006). In particular, an higher efficiency of PEI with respect to PDL has been demonstrated in the development of ordered arrangement of cells, as well as the increasing of signal to noise ratio in recording extracellular activity (Liu et al., 2008; Soussou et al., 2007).

Since it has been demonstrated that not only the chemical composition but also other properties of the substrate, such as roughness and stiffness, can influence cell adhesion and differentiation (Gentile et al., 2010; O'Connor et al., 2012), we characterized the morphological and mechanical properties of the patterned substrates. In order to evaluate the electrostatic contribution to cell adhesion, we have also measured the surface potential of the PDL and PEI stripes by using Kelvin probe force microscopy (KPFM), a non invasive scanning probe microscopy technique that provides the topographical and electrical characterization of the sample with a high spatial (nm) and electrical (mV) resolution (Jacobs et al., 1998). The kinetics of cell spreading on PEI and PDL was investigated using fluorescence optical microscopy and an impedance sensor system, which provided a first characterization of the adhesion properties of CHO cells on the above-mentioned polyelectrolytes.

The early stage of cell adhesion has been deeply investigated by using SCFS. Single cells were detached from the substrate after allowing the cell to come into contact with it for different spans of time (referred to as the 'adhesion time' in the rest of the text); the analysis was limited to the early stage of adhesion (adhesion time ≤60 s). Considerations on the molecular species that trigger the initial stage of adhesion have been deduced by suppressing the activity of particular adhesion molecule species. The obtained results allowed us to gain a better understanding of the different response of cells when incubated on these two molecular substrates. The optimization of the technique here presented opens the way to the extensive use of SCFS to test the effect of different molecules on cell adhesion.

2. Materials and methods

2.1. Surface coating patterning with adhesion proteins

Agarose 0.15% w/w (Sigma,USA) aqueous solution was prepared by dissolving the agarose powder in Milli-Q water brought to its boiling point in a microwave oven for 2 min. The solution was freshly prepared before use and kept warm on a hot plate set at $T=80\ ^{\circ}C$ to keep agarose in its fluid state. PDL and its fuorescence labeled analog (FITC-PDL MW 70,000, Sigma, USA) were dissolved in Milli-Q water at a concentration of 10^{-2}M; 25 μL aliquots were frozen and kept at −20 °C. PEI (MW 7,50,000, 10^{-2} M in Milli-Q-water) was purchased from Sigma, USA. PEI was labeled with the fluorescent dye Alexa 555 (Life Technologies, USA) was carried out as follows. A sodium bicarbonate buffer was prepared. $NaHCO_3$ 2% in Milli-Q water with pH=9. Alexa 555 dye was added to the PEI solution (4 mg/mL in $NaHCO_3$) under stirring, dark incubated for an hour and subsequently dialyzed in the dark for 5 days until the excess fluorophore was removed.

Etched Glass coverslips (Menzel-Glaser, Germany) were used as substrates and were cleaned with Hellmanex aqueous solution 1 vol% at 60 °C in ultrasonication bath for 15 min, rinsed at least three times in Milli-Q water and dried in an oven at 80 °C. The glass coverslips were then treated with UV/Ozone Cleaner (Bioforces, USA) for 45 min to increase hydrophilicity and to facilitate the agarose spreading.

Agarose solution was placed on the substrates before cooling happened, causing gelification. 150 μL of solution was placed on each coverslip under sterile hood and spin coated at 1000 rms for 30 s (Laurell Technologies Corporation, USA). The resulting agarose layer had a thickness of about 20 nm (Marconi et al., 2012).

The agarose layer was let to dry at room temperature (RT) overnight. Lines of PDL and PEI on the agarose coated substrates