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Influence of surface energy distribution on neuritogenesis

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ABSTRACT

PC12 cells are a useful model to study neuronal differentiation, as they can undergo terminal differentiation, typically when treated with nerve growth factor (NGF). In this study we investigated the influence of surface energy distribution on PC12 cell differentiation, by atomic force microscopy (AFM) and immunofluorescence. Glass surfaces were modified by chemisorption: an aminosilane, *n*-[3-(trimethoxysilyl)propyl]ethylendiamine ($C_8H_{22}N_2O_3Si$; EDA), was grafted by polycondensation. AFM analysis of substrate topography showed the presence of aggregates suggesting that the adsorption is heterogeneous, and generates local gradients in energy of adhesion. PC12 cells cultured on these modified glass surfaces developed neurites in absence of NGF treatment. In contrast, PC12 cells did not grow neurites when cultured in the absence of NGF on a relatively smooth surface such as poly-L-lysine substrate, where amine distribution is rather homogeneous. These results suggest that surface energy distribution, through cell–substrate interactions, triggers mechanisms that will drive PC12 cells to differentiate and to initiate neuritogenesis. We were able to create a controlled physical nano-structuration with local variations in surface energy that allowed the study of these parameters on neuritogenesis.

1. Introduction

Neuronal differentiation is critical to nervous tissue regeneration after injury. The initiation and guidance of a neurite rely on extracellular signals, especially on cell adhesion substrates. Hence, it is of particular interest to unveil the substrates characteristics that are effectively sensed and translated into neurite extension. The pioneering studies of Letourneau and others showed that adhesion on a substrate is critical for neurite extension [1–3]. These studies gave rise to a model in which interaction of transmembrane proteins with molecules of the extracellular matrix (ECM) is translated, through a set of actin-binding proteins, into effects on the microfilamentous cytoskeleton. This molecular mechanism leads to the generation of a tension exerted against the cell membrane, which allows neurite outgrowth with the formation and stabilization of point contacts in the growth cone of primary neurons [4] and of PC12 cells [5].

PC12 cells, though not being primary neuronal cells, express the transmembrane TrkA and p75 receptors to nerve growth factor (NGF) [6], and differentiate into a neuronal phenotype when challenged by appropriate NGF concentrations [7]. This ability makes them a good model to study neuronal differentiation mechanisms, and thus axonal regeneration. Different kinds of stimuli can trigger PC12 cell differentiation. First, NGF-addition to the culture medium elicit differentiation either by activating the synthesis of proteins, which associate with the actin/microtubule cytoskeleton, including Tau [8,9] and MAP1B [9], or by activating a signalling cascade pathway, including IκB kinase complex [10]. Second, in NGF-free medium: ECM proteins used as culture substrates induce differentiation, either a combination of different collagen types associated with proteoglycans, glycosaminoglycans, fibronectin and laminin [11] or ECM derived from astrocytes [12]. Third, in NGF-free medium as well, PC12 cells were observed to grow neurites either after electric stimulation [13] or when cultured on electroactive surfaces [14].

Gradients of soluble molecules, including calcium [15] and neurotrophic factors [16], influence neurite outgrowth through the growth cone, which recognizes and transduces a combination of signals into a specific trajectory towards target cells. Yet the contribution of the physical cues on PC12 cell differentiation remains poorly understood and few studies addressed substratum physical influence. The influence of a gradient at large scale (4.24 mm × 4.24 mm) in surface energy was studied by Murnane et al. [17], and showed that neurites of PC12 cells are preferentially initiated in directions of changing adhesion, under NGF treatment. Other studies showed that the topography of the underlying culture substrate, at smaller scales (≤1 μm), acts in cooperation with NGF to modulate neuritogenesis in PC12 cells [18,19]. In addition,

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biomaterials, such as modified silicon nanoporous membranes, induce changes in PC12 cell morphology, in presence of NGF [20]. Thus, PC12 cells seem spatially aware of nanoscale structures onto which they are plated. It has been suggested that filopodia may be the "sensors" of the substrate nanotopography [19].

In our study PC12 cells were cultured on physically modelled surfaces, by modifying chemically glass coverslips using NH_2- and CH_3-terminated trialkoxysilanes. These molecules form covalent bonds with the silica surface [21] thus providing relatively stable surfaces, known as self-assembled monolayers (SAMs) or silanized surfaces. These surfaces have proved [22] to be an alternative to biopolymers like poly-L-lysine (PLL), a standard neuronal cell-adhesion substrate [23]. PLL is adsorbed on glass coverslips by physisorption and it is generally assumed to promote a "non-specific" interaction with the external surface of the cells, since specific lock-and-key mechanisms are absent. SAMs form a class of surface whose properties can be monitored at the molecular scale, and thus serve as model surfaces for cell–surface and protein–surface interactions. For example NH_2-terminated SAMs modulate morphological development of hippocampal neurons [24] and of endothelial cells [25].

Here, we present a new kind of stimulus that triggers PC12 cell differentiation: specific physical properties of the substrate, at sub-micrometer scale. We compare surface properties of biopolymers-coated and of silanized glass coverslips and we show that, beyond surface chemistry, the distribution of physical cues has a clear impact on neuritogenesis in PC12 cells in NGF-free medium. In addition, immunofluorescence was conducted to assess the changing effects of the different substrates on PC12 cell cytoskeleton. The strength of the adhesion that PC12 cells established with the substrates was evaluated by interferometry, to characterize cell–substratum interfaces in cell culture conditions. Then we evaluated the possible influence of serum proteins adsorption on surface properties, using the fluid mode of the atomic force microscope (AFM).

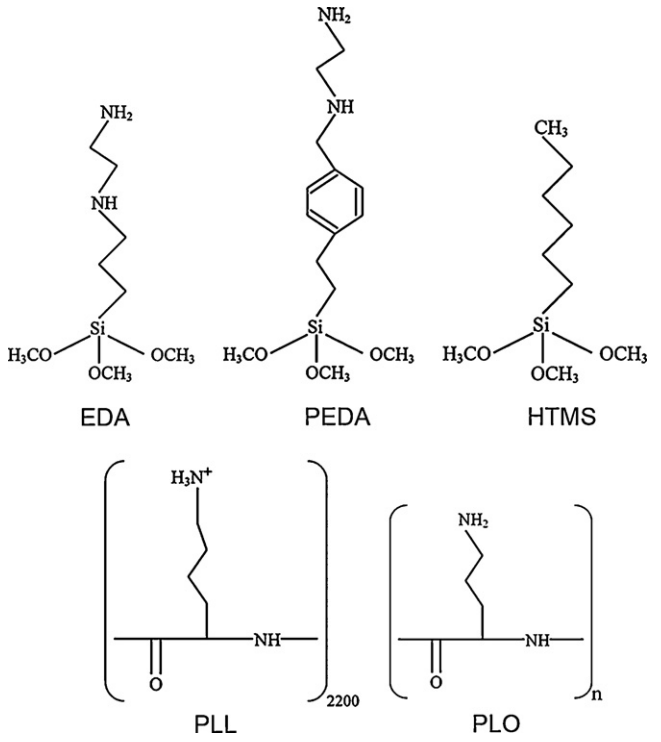

Fig. 1. Sketches of molecules used to modify the surfaces. EDA, PEDA and HTMS were grafted onto clean glass surfaces (coverslips of 35 mm in diameter) by chemisorption in liquid phase. Each of these three molecules contains three hydrolysable functions that allow polycondensation, thus giving the surface a specific physical nanostructure, responsible for a surface energy distribution that is heterogeneous. Contrary to these silanes, PLL and PLO do not form covalent bonds on glass. Hydrogen bonds (plus putative electrostatic bonds for PLL) allow for covering of glass surfaces in a more homogeneous manner.

2. Materials and methods

2.1. Surface modifications

Prior to use, glass coverslips (30 mm-diameter and 100 μm-thick, from Menzel-Glazer) were treated as follow. They were cleaned by ultrasound, 20 min in ultrasonic bath of $CHCl_3$, followed by immersion in piranha solution (3:1 (v/v) concentrated sulphuric acid/40% hydrogen peroxide) (caution: piranha solution is extremely corrosive and can react violently with organic compounds), then thoroughly rinsed with deionized water and dried under a nitrogen stream. Modified surfaces were obtained by immersing clean glass coverslips into a solution 2% *n*-[3-(trimethoxysilyl)propyl]ethylendiamine (EDA) (Acros Organics, 97%), 94% methanol (Carlo Erba Reagents, 99.9%), 4% deionized water, 1 mM acetic acid (Carlo Erba Reagents, 99.9%) [24], during approximately 24 h, at room temperature in an ambient atmosphere. They were then rinsed in methanol and either dried under a nitrogen stream, prior to surface characterisation by atomic force microscopy (AFM), or allowed to dry under a laminar flow hood, prior to cell culture. EDA modifies glass coverslips through chemical bonds. In our hands, surface modification process also leads to a surface on which EDA forms "patches" by self-polymerization, due to an amount of water, here 4% in solution, that is in excess compared to what the reaction between the molecule and the silica surface would require [21]. In addition to EDA, two other trialkoxysilanes, (aminoethylaminomethyl)phenyltrimethoxysilane (PEDA) (ABCR, 90%) and hexyltrimethoxysilane (HTMS) (ABCR, 97%), were used to modify clean glass coverslips, by the same method. Control surfaces were prepared by coating glass coverslips with biopolymers: PLL (PLL solution, 0.01% in water, Sigma) or poly-L-ornithine (PLO) (PLO solution, 0.01% in water, Sigma). Coating was performed on clean glass coverslips, sterilized in a UV chamber, by immersion in PLL or PLO solution, for 1 h at 37 °C. Coated coverslips were then either rinsed in sterile water prior to cell culture, or quickly rinsed in deionized water and dried under a nitrogen stream prior to air-imaging AFM experiments. EDA, PEDA, HTMS, PLL and PLO molecules are represented in Fig. 1. Non-modified clean glass surfaces proved to be unsuitable experimental control as cells did not attach on such surface: although plated at the same density as on silane-modified or biopolymers-coated glass coverslips, PC12 cells adhered poorly and then detached from the surface by 48 h. Therefore, we used as experimental control the standard protocol of PC12 cells seeding on PLL-coated coverslips, treated or not by NGF.

2.2. Surface characterization

2.2.1. Contact angle measurements

To measure the contact angle at a liquid/solid interface, the most direct method is to capture, with a camera, an image of the profile of a drop on a solid surface. Images were captured with a high-resolution black and white video camera mounted on a microscope and monitored by a PC. Then, the images were processed with an edge detection algorithm to determine the profile of the drop. Comparison of the profile with the Laplace equation, which is valid for all free interfaces, allowed to calculate the contact angle.

2.2.2. AFM imaging

All surfaces prepared as described above were analyzed using a Digital Instruments AFM in air tapping mode, with the sur-