# Cell behavior by the controlled immobilization of biotinylated proteins in a gradient fashion: non-linear concentration effects produced by unnoticed ligand nanoclustering

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Cell behavior onto bioengineered surfaces, in terms of adhesion, morphology, proliferation and differentiation, is affected by a number of variables including the former substrate derivatization process [1,2]. In this context, it is crucial to avoid uncontrolled exogeneous stimuli as far as possible by using surfaces with immobilized factors presented to the cell in a controlled way. Several examples of biomolecule immobilization strategies onto biomaterials have been described, involving both physical adsorption and chemical binding methods [3,4]. In general, chemical immobilization methods are preferred, since they provide a more stable link between the biomolecules and the biomaterial surface, thus avoiding uncontrolled desorption under physiological environments. However, strong and irreversible immobilization of sensitive biomolecules such as cytokines on surfaces often diminishes their biological functionality. Therefore, chemical immobilization has to ensure that the protein retains its biological activity. Moreover, it has been proven that cell response to many biomolecules is also dose-dependent, thus the biomolecule amount should be the optimal for maintaining its effective local concentration and extend its availability without a systematic risk of high dose. Therefore, it is of primary interest to find strategies that permit the correlation between the concentration of surface-bound growth factors and cell behavior.

One of the strategies proposed for the systematic screening of the effects of surface-immobilized biomolecules is the use of the so called surface gradients, surfaces with a gradually varying composition along their length. They can be generated by different methodologies such as controlled diffusion, gradual immersion of the substrate in a reactive solution or microfluidic devices [5,6,7]. However, surface modification for biomolecule anchoring is often accompanied by changes of very relevant surface properties such as wettability, roughness or stiffness, making their effects very difficult to decouple from those coming from the bioactive motives introduced.

This work describes a simple method for the construction of universal surface chemical gradient platforms based on the biotin/streptavidin model and its application in cell adhesion and differentiation studies [8]. In this approach, surface chemical gradients were prepared in poly(methyl methacrylate) (PMMA), a biocompatible polymer, by a controlled hydrolysis procedure. The resulting modified surfaces were extensively characterized in their physico-chemical properties. Chemical analysis carried out with time-of-flight secondary ion mass spectrometry (ToF-SIMS) and X-Ray Photoelectron Spectroscopy (XPS) showed the formation of a smooth, highly controllable carboxylic acid increasing concentration gradient along the sample surface. Atomic Force Microscopy (AFM) and contact angle (CA) results point out that, in contrast with most of the chemical gradient methods published in literature, the chemical modification performed on the polymer surface barely affects its physical properties. The introduction of carboxylic acid functionality along the surface was then further used for biomolecule anchoring. For this purpose, the surface was allowed the subsequent activation and derivatization with biotin and finally, with streptavidin (SAV) in a directed orientation fashion. SAV gradient was qualitatively assessed by fluorescence microscopy analysis and, later on, quantified by Surface Plasmon Resonance (SPR) technique in order to establish a quantitative relationship between SAV surface densities and surface location.

Such a gradient platform was first used to investigate the correlation between cell adhesion and cell-adhesive ligand surface concentration and organization due to substrate modification [9]. For this purpose, RGD gradient surfaces were created (Fig. 1). Cell culture shows that adhesion behavior changes in a non-linear way within the narrow range of RGD surface densities assayed (2.8 to 4.4 pmol/cm$^2$) with a threshold value of 4.0 pmol/cm$^2$ for successful cell attachment and spreading (Fig. 2). This non-linear dependence may be explained by a non-homogeneous RGD surface distribution at the nanometer scale, conditioned by the stochastic nature of the hydrolysis process. Atomic Force Microscopy analysis of the gradient surface shows an evolution of surface morphology compatible with this hypothesis.

Moreover, the gradient platform was also used to check effects of the concentration of Bone Morphogenic Factor 2 (BMP2) on the osteoblastic commitment of C2C12 cells in a single experiment. The narrow range of BMP-2 surface densities covered by the gradient allows for the precise tracking of the dose-guided activation of osteogenic markers Osterix (OSX) and Alkaline Phosphatase (ALP). A non-linear dependence of cell differentiation response with BMP-2 surface concentration has been found (Fig. 3). We hypothesize that BMP-2 ligands, pre-clustered on the surface due to the hydrolysis procedure, can favor ligand-receptor interactions, as reported with integrins, thus enhancing cell signaling.

**References**


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**Figures**

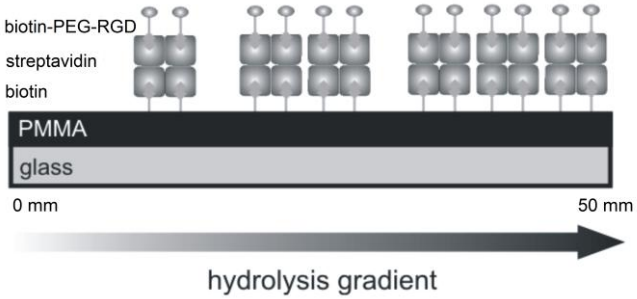


**Figure 1.** Scheme of the functionalization procedure followed to fabricate streptavidin gradients. Here, a biotin-PEG-RGD molecule has been attached, but the platform provides a universal mechanism to create gradients of any biotinylated molecules.

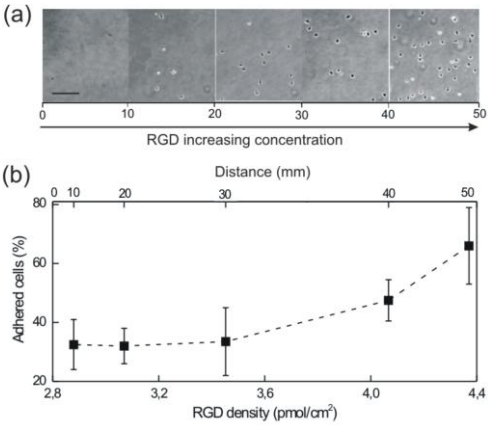


**Figure 2.** Cell adhesion in RGD-modified gradients. (a) Phase-contrast micrographs of NIH/3T3 mouse embryonic fibroblasts adhering to the RGDgradient surface after 1 h of incubation at 37°C (number of seeded cells: $2 \times 10^3$ cells/cm$^2$, n = 3). Scale bar, 500 μm. (b) Number of cells adhering as a function of slide distance, showing a sharp increase in the cell adhesion number for RGD surface densities larger than 4.0 pmol/cm$^2$.

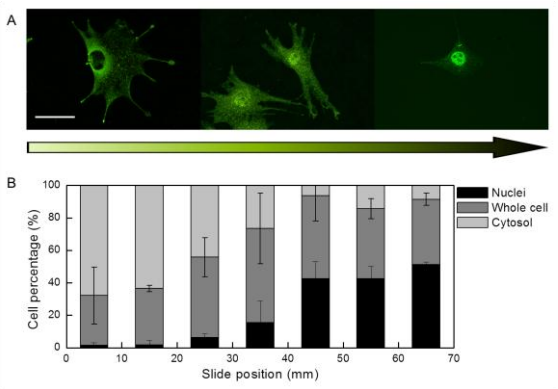


**Figure 3.** Tracking OSX activation and translocation into the cell nuclei as an effect of surface density. (A) Representative immunofluorescent images of C2C12 cells fixed and immunostained for OSX after 24h in culture at different positions on the BMP-2 gradient. Scale bar = 50 μm. (B) Plot of the OSX distribution percentages (selected following the Polak and co-workers criteria) as a function of the slide position (from the less hydrolyzed part of the slide). At least 30 cells were evaluated per selected region in three independent experiments.