# Micropatterned Laminin-1 matrices

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## ABSTRACT

We exploited the gentleness of the microcontact printing (μCP) technique to pattern stripes or grids of physisorbed Laminin-1 (Ln-1) onto underivatized glass. We verified the effect of this lithographic technique on the conformation of the laminin molecules and on the morphology of the protein matrices obtained from different concentrations and with or without calcium. Firstly we investigated the obtained pattern by indirect immunofluorescence, which both showed a well-defined printed area and gave a preliminary indication of protein structural integrity. Then, we imaged the patterned regions by Atomic Force Microscopy (AFM), demonstrating both features of physiological matrices, including correct interactions between the protein arms, and recognizable single Ln-1 molecules. Interestingly this result was strongly affected by the composition of the protein solution.

*Keywords:* laminin, self-assembling, micropatterning, atomic force microscopy, cell phenotype

## 1 INTRODUCTION

Laminin-1 (Ln-1), see Figure 1, is a large (900 kDa) heterotrimeric, cruciform-shaped extracellular matrix (ECM) protein having three short arms of about 40 ± 5 nm and one long arm of about 77 nm [1- 3]. Each arm has one or more globular domains separated by rod-like regions. This protein is particularly important for its instructive role on the cellular behaviour, which is due to highly specific interactions between laminin domains and integrin receptors. In particular laminin is involved in cell growth, differentiation, adhesion, polarity and neurite outgrowth [4-6]. All these capabilities are strongly dependent on the morphology of Ln-1 matrices, which are formed by a well-defined process involving the terminal globular domains of the short arms and requiring divalent cations [4- 6]. It has been demonstrated that a Ln-1 flat network with distinguishable polygonal features is better able to promote neuritogenesis and neurite outgrowth in vitro than a largely aggregate matrix [5]. All these Ln-1 biological activities can be shown only on specific cell types such as epithelial cells and neurons, which express the appropriate integrin receptors on their surface.

Both patterned and non patterned laminin-1 is frequently used as a cell adhesion molecule in cell culture applications. We were interested in investigating a gentle technique such as microcontact printing (μCP) for the patterning of laminin, and in particular whether it preserves the laminin structure and the morphology of self-assembled matrices under different laminin concentrations and in the presence/absence of calcium ions. While laminin may function as a non-specific, passive cell-adhesion molecule through weak (electrostatic) interactions, its high-affinity binding to integrin-bearing cells constitutes an active signal, which affects cell behaviour. For this purpose we have tested our laminin patterns on B104 neuroblastoma cell line.

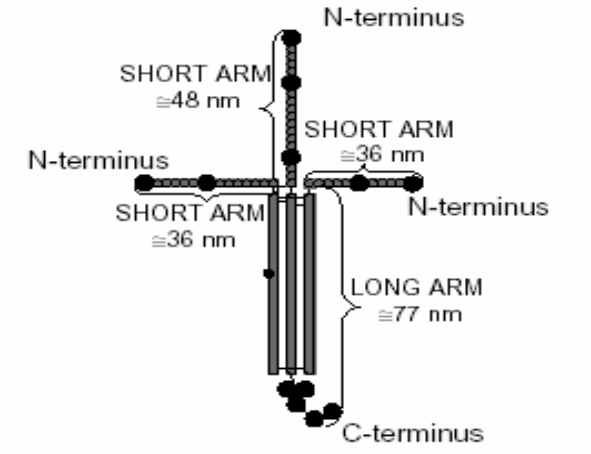


Fig.1. Laminin-1 heterotrimeric morphology. The globular domains of the protein are indicated by black dots

## 2 RESULTS AND DISCUSSION

We have exploited the gentleness of a soft lithography technique, μCP, to pattern both stripes and grids of laminin-1 on glass substrates that were not chemically modified. μCP was carried out by applying a drop of laminin solution onto the surface of a poly(dimethylsiloxane) (PDMS) replica of a master, and then placing the protein-inked side of the stamp in contact with the glass surface. We tested two factors potentially influencing the laminin self-assembly process, i.e. the protein concentration (10 and 30 μg/ml in Phosphate Buffer Solution, PBS) and the presence of calcium (2 mM) in the solution.

First we carried out immunofluorescence microscopy on patterned substrates prepared from different laminin solutions (10 μg/ml and 30 μg/ml). Samples were incubated first with an unlabelled anti-laminin primary

antiserum and then with rhodamine-labelled anti-immunoglobulin. We obtained similar results with both laminin concentrations (Fig.2), with well defined features over the whole printed area. Thus the Ln-1 molecules maintain their antigenicity, and hence likely their structural integrity. In addition to ensuring biocompatibility, μCP is an easy and quick one-step lithographic process. Although the protein is only attached by physisorption, the adhesion is strong, since virtually no protein was released after long incubation times and several washings. This resistance to detachment is probably due to the surface hydroxylation of the glass substrates induced by treatment with the piranha solution ($H_2SO_4$ : $H_2O_2$, 3 : 1) used for cleaning before imprinting.

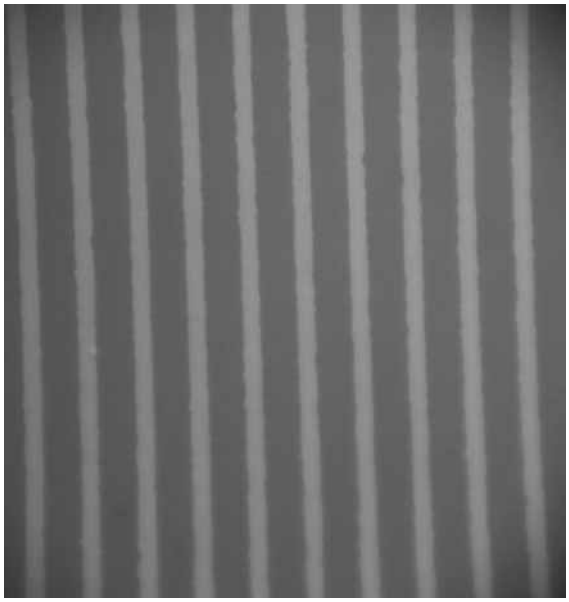

Fig.2. Immunofluorescence micrograph of the obtained Ln-1 pattern. The printed stripes are about 11 μm-wide

Next we examined the structure of the exposed surface of the printed regions by Atomic Force Microscopy (AFM). In the case of the 10 μg/ml laminin solution without calcium, we found that Ln-1 self-assembles in polygonal networks that resemble physiological matrices and result from an interaction between the terminal domains of the arms (Fig 3). In these structures it is also possible to identify single laminin molecules with recognizable globular domains and rod-like regions.

Interestingly, a completely different result was obtained using a solution with the same protein concentration but containing 2 mM calcium, the ion capable of inducing the polymerization of laminin. The AFM image reveals a layer with a chaotic distribution of molecules in which neither single molecules nor polygonal features may be identified (Fig. 4). However, at a closer look, this distribution is only apparently chaotic. We rather believe that it corresponds to

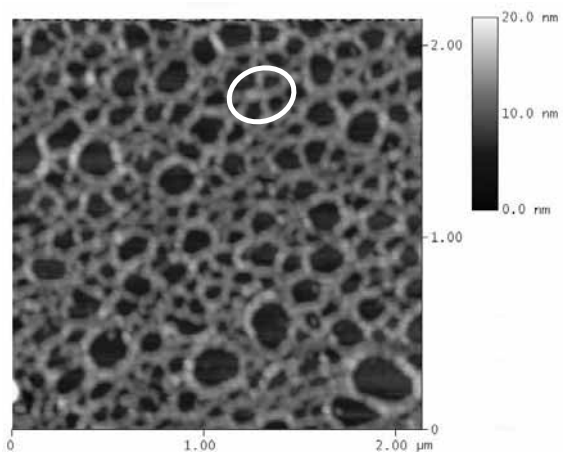


Fig. 3. AFM image of the self-assembled protein network in a printed region of Ln-1 from a 10 μg/ml PBS solution without calcium. The white circle marks a single laminin molecule

superimposed layers of polygonal networks (Fig 4). Two facts support this interpretation. First, regions printed from solutions with calcium have a thickness of 13 to 19 nm, compared to 4 nm for regions printed from solutions without calcium. Second, in one case the pattern obtained from a solution with calcium was disuniform, showing isolated areas with a clear polygonal network (Fig 5). It is likely that these areas resulted from an imperfect and weak contact between the protein-inked stamp and the glass substrate, whereby less protein was released onto the glass, yielding a monolayer.

Interestingly, the physiological organization of the physisorbed Ln-1 network does not occur with the more concentrated solution (30 μg/ml), both with and without calcium. Again, laminin polymerization is apparently random and single Ln-1 molecules may no longer be recognized (data not show), notwithstanding the positive result with the immunofluorescence assay.

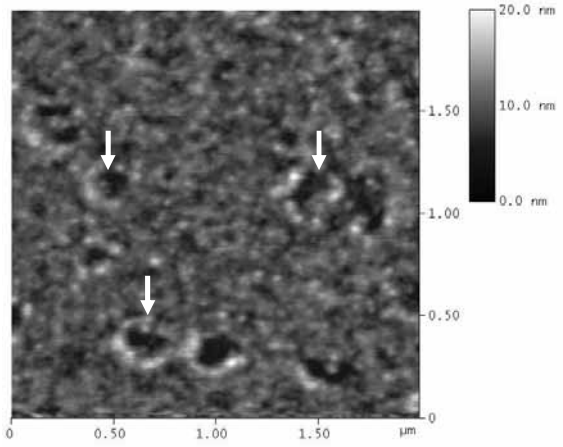


Fig. 4. AFM image of the self-assembled protein network in a printed region of Ln-1 from a 10 μg/ml PBS solution with calcium. The white arrows indicate points at which the underlying layer of molecules may be observed.

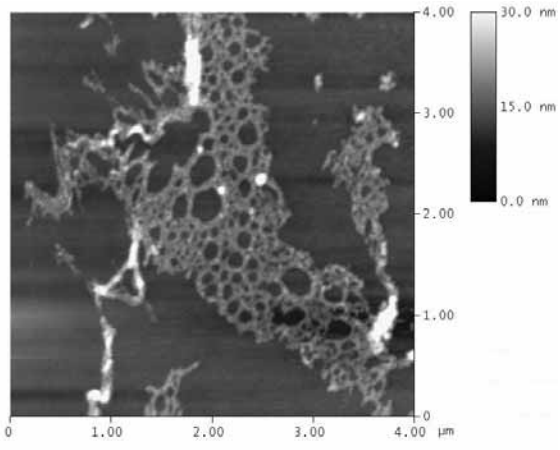


Fig. 5. AFM image of the self-assembled protein network in region obtained with an imperfect print of Ln-1 from a 10µg/ml PBS solution with calcium. It is possible to notice the polygonal structure of the single layer transferred.

We believe that the observed dependence of the morphology on the protein concentration and on the presence of calcium is due to the transfer mechanism by µCP, which deposits the three-dimensional structure of the protein matrix, preformed in solution, onto the two dimensional structure of the substrate. Indeed, high protein concentrations and an optimal calcium concentration can determine a dense cross-linked three-dimensional matrix in solution. This would collapse when the replicas are dried and, upon transfer to the glass, result in a multilayer of superimposed polygonal networks.

Whilst calcium is generally recognised as responsible for laminin polymerization, we have obtained well-defined self-assembly of laminin networks in its absence. We think that an important role in this phenomenon is played by the imprinting process itself. Further studies are under way in our laboratory to support this hypothesis.

Since the patterns generated by this technique will be ultimately used for the fabrication of hybrid devices, we also checked their capability to support the growth of cultured cells. We used the B104 neuroblastoma cell line, which responds to laminin binding in the way typical of neurons, increasing neuritogenesis and neurite outgrowth [4]. This response is due to a specific interaction between laminin domains and the integrin receptors on the cell membrane. Figure 6 shows compliance of B104 cells to the geometry of the patterned region and, more interestingly, well-developed axons. This suggests that patterned laminin retains the ability to promote growth of specific cell types.

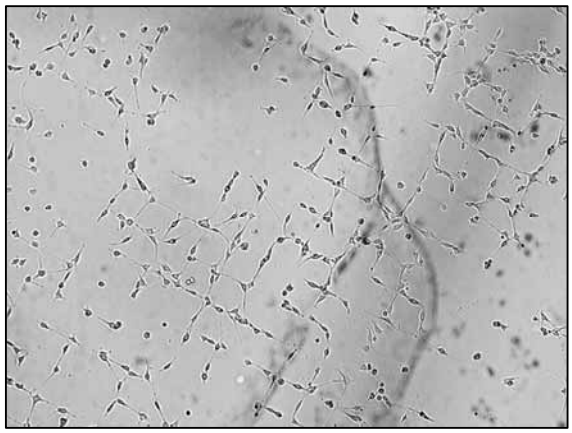

Fig. 6 Micrograph of B104 cells growing on a patterned laminin grid.

## 3 CONCLUSIONS

In conclusion we demonstrate that µCP can generate functional Ln-1 patterns with recognizable laminin molecules having typical quaternary structure and polymerized in mesh-like matrices with the correct involvement of their terminal domains. The formation of these networks was achieved by physisorption, without any functionalization of the substrate. The generation of monolayers was dependent on the laminin concentration and on the presence of calcium. We have also demonstrated that laminin can be used for cell patterning. Based on our data, one-step soft lithography is a powerful technique to realize both micropatterned and self-assembled layers with physiological properties. These layers are biocompatible for cell culture and may find applications for basic biology studies, biosensors, drug discovery and hybrid devices, particularly in those conditions in which a monolayer of adhesive molecule is required.

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