Fibronectin patterning of viscous polymer films by microcontact printing

Sandipan Dawn, Jacob John, Kenneth Carter, Harry Bermudez, Andreas P Kourouklis
Polymer Science and Engineering, University of Massachusetts, Amherst; Bioengineering, University of Illinois Urbana-Champaign

Abstract
In working toward the goal of mimicking multiple features of the extracellular matrix (ECM), we developed a strategy to create adhesive protein patterns on polymer films with tunable viscous characteristics. The block copolymer films are generated by interfacial self-assembly with the presentation of dopant homopolymer, since the concentration of the latter is known to affect the lateral mobility of the film. The supported polymer films are subsequently surface-modified by microcontact printing using fibronectin (FN), yielding material surfaces which can potentially display independent control over mechanical and adhesive properties. This work demonstrates a new method for the design of materials with the potential to recapitulate the viscous component of the ECM in future in vitro studies.

Objective
Our work pertains to the concept and realization of soft patterned material - specifically, to the fabrication of supported block copolymer films and their patterning with adhesive proteins (as a prototype proof-of-principle). In contrast to most previous studies using bulk and static (e.g., cross-linked) materials, our patterned and mobile films show the potential to decouple adhesive and mechanical properties.

Introduction
The design of artificial substrates that exhibit physical and biochemical characteristics of the extracellular matrix (ECM) is commonly used to investigate cell-ECM interactions. Factors that demonstrate significant influence on cell adhesion include substrate mechanical properties [1] [2] [3], the type of adhesive ligands [4] [5], and the spatial characteristics of ligand presentation [6]. Control over multiple design characteristics is required in order to reach the complexity of the natural and three-dimensional ECM. One route toward achieving this goal is to merge different fabrication techniques. At the nanoscale, patterning techniques such as nanoimprint lithography, e-beam lithography and other approaches based on self-assembly are widely used [7] [8]. At the micron-scale, patterning methods tend to be top-down approaches: photolithography [9] [10], colloidal templating [11] [12] [13], and microcontact printing [14] [15] [16]. Thus the combination of both nano- and micro-scale features has promise to build increasingly complex materials that more closely mimic the native ECM. The selection of a cell-adhesive protein or ligand is another significant part of biomimetic materials design. Fibronectin (FN) has been widely used to decorate solid substrates for the investigation of anchorage-dependent cell behavior, mainly due to the different domains of FN that participate in the binding of growth factors or cell-adhesion receptors [17]. Perhaps, the best-known domain of FN is the RGD sequence, which is recognized by several transmembrane integrin receptors [18]. An additional complication in the design of artificial substrates is the recognition that the ECM (and even FN-coated substrates) is subject to cell-induced remodeling. Cells apply force to their environment, unfolding proteins such as FN to expose so-called cryptic sites [19], and causing focal adhesion reorganization [20] [21]. Furthermore, the mechanical properties of the underlying substrate can indirectly influence cell responses, by modulating the effect of protein surface density [2]. This interplay of mechanical and biochemical signals becomes increasingly complex under physiological conditions [22]. In particular, various biological processes such as cell-induced remodeling [23] and enzymatic and cross-linking [24] result into a natural ECM whose mechanical properties demonstrate viscous and elastic characteristics [25] [26]. However, there is still limited understanding on the viscous component of the ECM and its effects on cell mechano-sensing. Thus, the creation of substrates with viscous-
like components, like the mobile polymer films, aims to activate the mechanisms that partly regulate cell adhesion mechano-sensing in response to the viscous component of the ECM.
Figure Legend

Figure a) (A) Self-assembly of poly(butylene)-b-poly(ethylene oxide) (PB-PEO) monolayer on glass coverslip by Langmuir-Blodgett technique (PB: red, PEO: blue). (B) Self-assembly of two-molecules thick polymer film by Langmuir-Schaefer deposition of PB-PEO with NHS-ester modified PEO ends (NHS-ester: black stars). The chemical structures of the used block copolymers polymers are presented in Supplemental information S3. (C) Drop-casting of fluorescent fibronectin (green color) on PDMS. (D) Microcontact printing of fluorescent FN dots onto the supported polymer film. The cartoon is out of scale.

Figure b) Fluorescence images of films following microcontact printing of FN. (A) Example of unsuccessful pattern transfer. Bright green areas indicate the presence of fluorescent FN and the dark areas indicate removal of the film due to tear-out. Example of successful pattern transfer on (B) neat and (C) doped films. Bright green areas indicate FN patterns. Scale bar is 25 μm.

Figure c) Average diameter of FN dot under PBS solution for neat (black column) and doped (gray column) films.

Results & Discussion

We started the construction of this new hybrid material with the creation of supported thin films from amphiphilic block copolymers using Langmuir-Blodgett (LB) and Langmuir-Schaefer (LS) techniques [27] [28]. As depicted in Figure a.A and a.B, the bottom layer was formed by an LB step: pulling a glass coverslip through PB-PEO block copolymer film at the air/water interface. The top layer was self-assembled onto the bottom layer by an LS step: horizontally transferring a monolayer through a second PB-PEO film at the air/water interface. In order to pattern the polymer film with proteins, a reactive NHS-ester group was incorporated at the end of the PEO block. Five mole percent of ester-modified polymer was introduced at the top layer of the film to chemically react with amines of fibronectin during the microcontact printing procedure.

To increase the lateral mobility of the polymer film, we introduced a small amount (1 mol %) of polyisobutylene homopolymer during the LS fabrication step [27]; such films are hereafter referred to as “doped.” Films without any added polyisobutylene have lower mobility and are referred to as “neat.” To create well-defined protein patterns on our supported polymer films, we adapted the approach of Polio et al. [29]. The microcontact printing procedure is schematically presented in Figure a.C and a.D. In this study the PDMS stamp has a post diameter of 18 μm and the edge-to-edge distance between
the posts is 9 μm. The surface of the stamp was subjected to oxygen plasma treatment to control the wetting and adsorption of the FN solution [30]. Excessive oxygen plasma treatment made the stamp surface brittle, which impeded the microcontact printing process. On the other hand, insufficient oxygen plasma treatment left the stamp surface hydrophobic and consequently hindered wetting by the protein solution. By monitoring the spreading of the FN solution, we found that an oxygen plasma exposure time of 10 s resulted in adequate surface hydrophilicity. The stamp surface was incubated with the FN solution for 5 min, followed by removal of the solution by gentle inversion of the PDMS stamp and wicking. Excess solution on top of the film or stamp leads to unsuccessful pattern transfer, and therefore the supported films were briefly air-dried prior to stamping. The PDMS stamp was then slowly brought into contact with the surface of the supported polymer film (Figure a.D), allowing terminal NHS-esters to react with FN amines. The microcontact printing was controlled by the weight of the PDMS stamp (having dimensions of 1.0 × 0.5 × 0.5 cm³). That is, the stamp was gently released from the tweezers in the correct orientation on top of the supported film without any external pressure. We note that our initial pattern transfer attempts were unsuccessful, as during the stamp removal the supported film was also removed from the glass coverslip, commonly referred to as “tear-out.” Direct observation of the stamped films revealed dark patches as a result of tear-out (Figure b.A). To optimize the microcontact printing procedure the various interfaces during the stamping procedure were considered. Four different interfaces are candidate locations for material failure or tear-out (Supplementary information S1). These interfaces are: i) glass surface / bottom layer of the polymer film; ii) midplane of the two layers of the polymer film; iii) FN protein / top layer of the polymer film; and iv) FN protein / PDMS stamp surface. Among these four interfaces, the strongest interactions are obviously the covalent bonds between FN amines and the NHS-esters of the top layer of the polymer film. To achieve successful pattern transfer the interaction at the FN protein / PDMS stamp interface should be the weakest, allowing stamp removal without significant perturbation of the underlying polymer film. We therefore explored conditions for stamping by varying the contact time between film and the stamp, as well as the plasma treatment time onto the film (Supplemental information S2). It was found that 5 min contact time produced the best pattern; whereas less than 5 min contact time resulted in partial transfer and more than 10 min contact time did not improve the pattern transfer quality. On the other hand, plasma treatment time was varied between 10 and 30 s to control the surface wetting and FN adsorption strength. It was found that 10 s of plasma treatment along with 5 min contact time produced adequate pattern transfer without tear-out (Figure b.A and b.C). Having achieved a successful pattern transfer we proceeded to examine the stability of the FN patterns over time. Patterned films (both neat and doped) were kept submerged in buffer solution and the patterns were monitored over time by fluorescence microscopy. The dot widths (i.e., diameters) remained constant at ~20 μm in all cases for the entire 18-h time frame, irrespective of whether the films were neat or doped (Figure c). Moreover, it is shown from previous studies that the lateral diffusion coefficients of the neat and doped films are $1 \times 10^{-10}$ and $3.5 \times 10^{-10}$ cm²/s, respectively [27]. Such lateral diffusion of FN-labeled polymer from a single dot of uniform concentration would result in the dot width increasing by more than 50% of its initial size within 3 h [31], which is not observed. This result suggests that the FN proteins are acting as effective crosslinks, prohibiting the redistribution of polymer chains. Given that there are greater than 75 amines per FN and the dimensions of FN (approx. 16 × 9 × 2 nm³) [32] [33] [34], our view of FN-based cross-linking is consistent with the observed dot pattern stability. However, future assays using fluorescently labeled polymer chains will be necessary to assess their mobility shift after patterning with FN dots. We have developed a bottom-up approach for the fabrication of FN patterns on polymer films with tunable lateral mobility. These hybrid substrates were created by assembly of block copolymers chains, followed by microcontact printing of FN spots. This strategy provide useful insight for future efforts toward the design of biomaterials with novel mechanical and bioadhesive properties. The strategy for material fabrication employed in this work initiates the discussion for a number of additional studies focused on material characterization and cell culture. An
Citations


Important assay will be the measurement of polymer chain mobility after FN patterning. This result will reveal critical information for the viscous component of the patterned substrate. In this direction, studies of Atomic Force Microscopy (AFM) can probe the viscous and elastic components of the patterned substrate and provide significant input for its stability over time.

Additional Information

Methods and Supplementary Material
Please see https://sciencematters.io/articles/201601000016.

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Ethics Statement
Not applicable.