FUNCTIONAL COATING OF HETEROGENEOUS MICROSTRUCTURE SURFACES WITH SELF INTERACTING BIOMOLECULES

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ABSTRACT

This paper presents useful techniques for applying coatings of the extracellular matrix (ECM) proteins fibronectin (FN) and basement membrane extract (BME) to small, defined polymeric microstructures. We found that several cell types, such as fibroblastic and epithelial, have preferential adherence to these ECM component coatings, but that the coatings were ineffective when applied using standard coating protocols. Thus we developed strategies to effectively apply FN and BME, overcoming inherent difficulties with their use in applications such as the micropallet array technology. These results would be of consequence for numerous bioMEMS devices that involve cell interaction with similar microstructured polymer surfaces.

KEYWORDS: Microstructures, Extracellular Matrix, Cell Adhesion

INTRODUCTION

Traditional laboratory culture of adherent cells has utilized flat growth surfaces made of oxidized polystyrene (i.e., tissue culture plastic) or other surfaces such as glass that are derivatized or coated (i.e., with extracellular matrix (ECM) components such as collagen or fibronectin (FN)) to promote cellular adhesion. Recently, several groups have reported success in more advanced culture configurations such as three dimensional cell culture within microscale bioscaffolding and gelled substrates of collagen, basement membrane extract (BME), i.e., Matrigel, or other polymeric hydrogel materials [1]. However, as the field of bioMEMS progresses there is increasing need to establish conditions for the culture of adherent cells upon defined, non-organic three dimensional microstructures for applications in biomedical and bioengineering research. A significant determinant on which the success of such systems rests is the ability of the microstructures to adequately and appropriately support cell adhesion, while also, in some cases, preserving regions of the device so that they remain unfavorable to cell adhesion.

The techniques presented in this paper to apply ECM biomolecules to heterogeneously wetted microstructures were developed for use with micropallet arrays, which are composed of thousands of microscale polymer pedestals arrayed on a glass substrate [2], Figure 1A. Each pedestal, or micropallet, holds sequestered cells in culture. A silane monolayer applied to the surfaces of the array renders them highly hydrophobic, causing air to become trapped between the micropallets when the array is wetted, Figure 1B. These air barriers effectively constrain cells to the top surfaces of the pallets, but create conditions that make proper coating of the micropallets with FN or BME difficult. Similar heterogeneously structured surfaces are found in many bioMEMS-based cell handling systems and the findings of this work are applicable to many such devices.

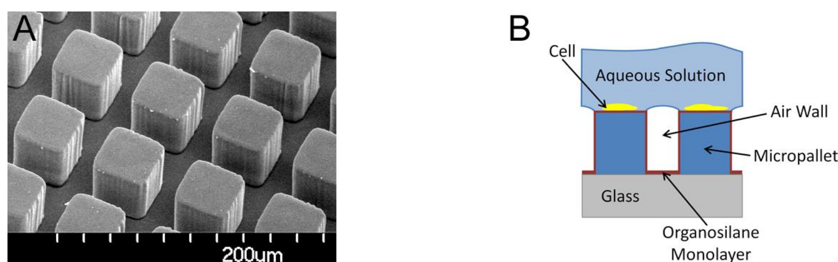

Figure 1: A) SEM micrographs of micropallet arrays. Micropallets in all figures are 40 x 40 microns and 50 microns tall. B) Mechanism of cell sequestration to top surfaces of micropallets. The hydrophobic surfaces and geometry of the micropallet array causes air to become trapped, preventing cells from falling between the micropallets.

THEORY

Fibronectin and BME are ECM components commonly used to coat cell culture surfaces for promotion of cell adhesion and proliferation. However, both components posses self-interacting natures that make them ill-suited for application to microstructured and heterogeneously-wetted surfaces using traditional application techniques.

Fibronectin's structure makes it susceptible to self-polymerization, especially when subjected to shearing forces that alter its molecular conformation and expose specific FN-FN binding sites [3]. When in suspension, the FN dimer molecule adopts a compact conformation that shields its FN binding sites from interactions with other FN dimers, Figure 2A. However, when subjected to shearing forces, the FN molecule is elongated, Figure 2B, and the FN binding sites are able to interact resulting in the polymerization of FN into large, insoluble structures capable of supporting cell adhesion, Figure 2C. This mechanism makes the application of effective FN coatings to microstructured surfaces difficult, especially for cases in which shearing forces are introduced during the application process. Therefore, it was necessary to develop new techniques to effectively coat the growth surfaces of the micropallet array with FN.

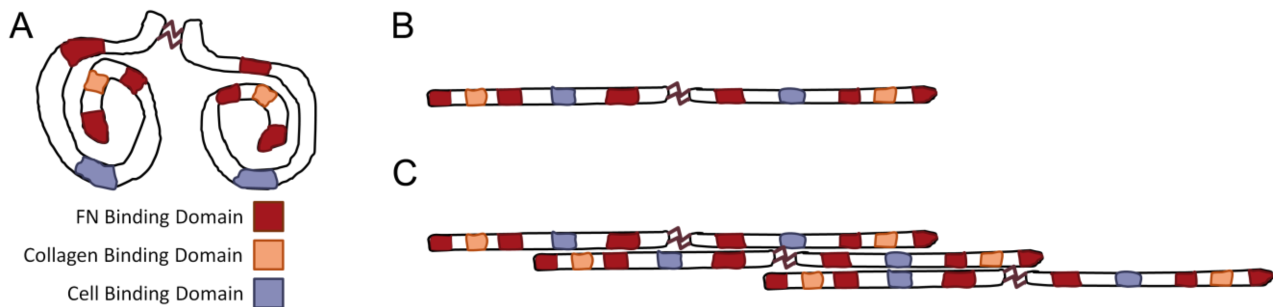

Figure 2: Hypothesized conformal states of FN showing approximate locations of binding domains within the molecule and proposed mechanism of polymerization.[3] A) When in suspension in its soluble form, the FN dimer maintains a compact form that shields FN-FN binding sites from interaction. B) On exposure to appropriate forces, such as fluid shearing forces, the FN dimer becomes elongated, exposing FN-FN binding sites. C) The exposure of binding sites enables FN molecules to polymerize into insoluble structures capable of supporting cellular adhesion.

Basement membrane extract is an anomalous coating for support of cellular adherence as it can be formulated by concentration to form gelled coatings of specified relative thicknesses, thin vs. thick. This property, along with its composition, a mixture of proteoglycans and fibrous matrix proteins such as collagen and laminin, makes it a particularly useful coating choice and perhaps an extremely desirable one, as it has been shown to promote and maintain specific phenotypes in a variety of cells [4]. However, like FN, its physical nature makes its application to small, discrete polymeric growth surfaces, e.g., micropallets, difficult, particularly due to poor adhesion to the surface, a characteristic which is possibly attributed to its gel-like properties. Thus it was again necessary to develop a new technique for coating micropallets with BME.

EXPERIMENTAL

For initial FN coating experiments FN was diluted to 20 µg/ml in phosphate buffered saline (PBS), which was applied to micropallet arrays and incubated at room temperature for 1 h, after which it was removed by washing x3 with PBS. Due to the observations described below, of FN structures spanning the gaps between micropallets, we developed, after investigative experiments, the following protocol to effectively coat each micropallet growth surface with FN: FN at 20 µg/ml in H_2O was applied to the arrays for 1 h at room temperature, serially replaced with H_2O x3 to remove residual non-polymerized FN molecules, followed by serial replacement x3 with 70% ethanol. Both sets of exchanges were half-volume exchanges such that the array's surface was never exposed to air. Due to ethanol's low surface tension, the air barriers collapsed and 70% ethanol filled the inter-pallet spaces. The ethanol was removed from the arrays, which were then allowed to dry completely before use.

For initial BME coating experiments, BME was diluted to 1 mg/ml in PBS which was applied to the micropallet arrays and incubated at 37 °C for 1 h, after which it was removed by washing x3 with PBS. Due to the findings reflecting poor adhesion and similar bridging structures as seen with FN, we developed the following protocol, again, after investigative experiments, to effectively coat each micropallet growth surface with BME: Sulfo-SANPAH, a heterobifunctional crosslinker, was diluted to 0.5mM in 50mM HEPES buffer and micropallet arrays were submerged in the solution and exposed to 365nm UV radiation for 15 minutes, washed with 50 mM HEPES and the process was repeated. BME diluted to 20µg/ml in PBS was then applied to the micropallet arrays, incubated 16 h at 4°C, and washed with PBS. All steps were performed with reagents at 4°C to prevent gelling of the BME and under conditions that did not disrupt the restricting trapped air between micropallets.

For all evaluative experiments, the FN or BME coatings were colorimetrically stained using appropriate primary and isotype matched irrelevant control antibodies and developed with either TrueBlue or Vectastain DAB peroxidase substrates for visualization. NIH/3T3 fibroblast cells, maintained in culture in a cell incubator at 37° C/10% CO_2, were used to evaluate cellular adherence and behavior on the coated micropallet arrays.

RESULTS AND DISCUSSION

Based on our own observations and work published by others [5], it is hypothesized that the FN molecules in solution adsorb onto the top surfaces of the micropallets as well as congregate at the air-liquid interface of the air walls between micropallets during the incubation with FN solution. In initial experiments the FN solution would be drawn off of the array after incubation and the shearing effects of this process would draw the FN into insoluble fibers bridging neighboring micropallets as well as leave large sheet-like structures of FN intact, Figure 3A. It was shown that these FN structures supported cellular adhesion, thus allowing single cells to adhere beyond the boundaries of each micropallet and occupy multiple micropallets. Using the developed method to collapse the air wall network before exposing the array's surface to air resulted in properly coated micropallets with no bridging structures. In each experiment using the developed method, consistently no bridging structures were observed, Figure 3B, and cells were firmly adhered to and restricted to the top surfaces of the micropallets, Figure 3C.

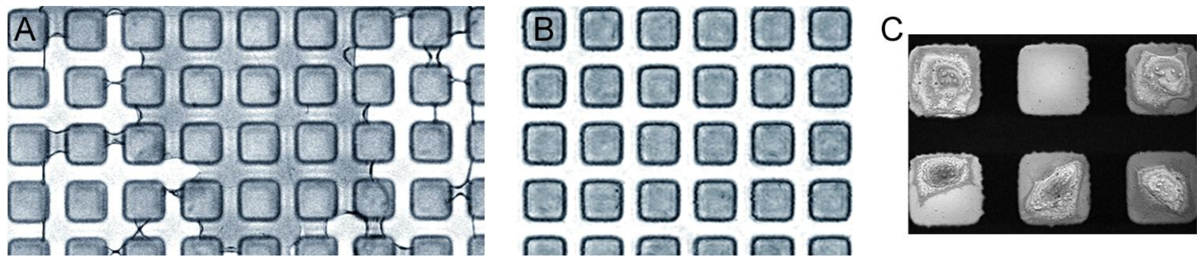

Figure 3: A) When applied using standard methods, FN forms fibrils and large sheet structures that span several micropallets. These structures support cellular adhesion. B) Using the developed technique, uniform coatings of FN can be created on each micropallet, with no FN bridging structures. C) 3T3 fibroblast cells adhered firmly and were constrained to individual micropallets coated with FN using the developed protocol.

BME is a protein mixture that forms a gelled thin film coating when incubated at 37°C. BME had poor adhesion to the micropallets when applied in a traditional manner, Figure 4A, including an apparent capacity of adherent cells to pull the coating from the surface of the micropallets, and formed bridging structures similar to those seen with FN, Figure 4B. The relative thick gelled nature of the coating may have contributed to these phenomena; however, we were unable to overcome them by only lowering the BME concentration. We developed a new method to uniformly coat the micropallets with BME with dramatically increased adhesion by combining two strategies: improving the adhesion to the polymer micropallets by employing a heterobifunctional crosslinker, Sulfo-SANPAH, to bind molecules present in BME to the micropallets; and minimizing the gelling of the BME during coating by using an order of magnitude lower concentration and performing the coating process at 4°C to prevent gelling, which occurs at higher temperatures. The adhesion of the BME coating to the micropallets was significantly improved, Figure 4C, and supported cellular adherence, Figure 4D, without the loss of adhesion to the micropallets due to cellular tractional forces.

Figure 4: A) Stained BME showing poor adhesion to micropallets. B) 3T3 fibroblast cells on micropallets coated with BME using traditional application methods. Cell tractional forces pull BME from the micropallet surfaces and cells are spherical and poorly adhered. Fibrillar bridging structures of BME can also be seen. C) Stained BME on micropallets. Using the developed technique, BME is well-adhered to the individual micropallets and has good uniformity. D) 3T3 cells are well adhered and fibroblastic in appearance on arrays coated using the developed method.

CONCLUSION

The methods described herein provide direction for the capacity to effectively generate FN or BME coatings on restricted surfaces of microfabricated bioMEMS devices. The importance of effectively eliminating bridging structures and, in the case of BME, establishing firm adhesion of the BME to the microstructure cell surface is self evident for bioMEMS structures or systems that involve adherent cells. Thus these methods are likely to be able to be adapted and useful in a wide range of bioMEMS device and bioengineering research strategies.

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