Microcontact Printing: How the Reusability of Stamps Without Reinking Affects Cell Adhesion

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Abstract

Microcontact printing is a method that utilizes a polydimethylsiloxane (PDMS) stamp to pattern extracellular matrix (ECM) onto a substrate which can be used to adhere to biological substances such as proteins and cells. This technique is effective for studying, maintaining, and isolating biological variables. Specifically, it has been used for creating neural networks and understanding cell adhesion and differentiation. Stamps are often reinked with the ECM substrate before each use, a time-consuming process. Others continue to reuse the stamp without reinking to shorten the process. Thus, it is necessary to understand the effects that stamping without reinking has on cell adherence. This was investigated by fabricating three replicate PDMS stamps using the columns on pennies as a mold. Each stamp was used to stamp gelatin into three separate well plates without reinking the gelatin between uses. Cells were then seeded onto the stamped ECM and fixed after 24 hours. The cell cytoskeletons were dyed with a DAPI/TRITC-phalloidin/ PBST-T solution and imaged using a fluorescent microscope where the cell adhesion was quantified by calculating the confluency using ImageJ software. There was no statistical difference between the cell adhesion of stamps and the number of repeated uses. However, stamp 1 had cell adhesion for each repeated use with higher confluency values and the other stamps had little or no cell adhesion. Small sample size and variability in the stamps during fabrication could have led to insignificant statistical results. A larger sample size and high-quality stamps in future iterations could statistically support that cell adhesion decreases as the stamp is used repeatedly without reinking. Thus, it is necessary to reink the stamp before each use when using it in applications such as studying cell adhesion, proliferation, and differentiation.

Introduction

Microcontact printing (μCP) is a procedure that utilizes soft lithography to pattern molecular assemblies arranged on surfaces by adsorption of ink onto a substrate using a master PDMS stamp [1]. Surface patterning using μCP has prompted research for a wide range of biomedical engineering applications, such as to pattern proteins and cells for biomimetic sensors, lab-on-a-chip, and microarrays [6]. With the ability to manipulate surface chemical functionalization and influence cells, μCP has shown to be an advantageous technique for studying things such as cell adhesion and differentiation, and protein interaction in a biological system [1,6]. Surfaces with micropatterns of biomolecules have attracted a lot of attention in recent years. Hu et al. demonstrated the importance of cells adhering to the ECM protein. Microcontact printing is dependent on cell adhesion and it is required for a variety of physiological functions. Cell adhesion controls apoptosis, mitogenesis, cell differentiation, and cell migration at the cellular level. In the study, the adhesion between the protein and the substrate was able to regulate the cell behavior [4]. Allowing the cells, proteins, or other biological material to adhere to restricted areas promotes the ideal conditions for isolating the biological variable being studied [7]. Filipponi et al. used μCP to pattern proteins and biomolecule microarrays. They analyzed how the mold of the PDMS stamp was directly related to how proteins will be labeled in an array format in the shape of the mold [5]. These examples demonstrate how studying the patterning and adhesion of cells on a substrate can help

researchers better understand cell migration, differentiation, cell-to-cell interactions, and protein interactions which are crucial components of complex biological systems.

Microcontact printing has many different applications, which lead to a plethora of different methods for stamp fabrication and stamping. Many researchers reink their stamps before each use; however, this process can be lengthy due to the time required for the ECM to dry on the stamp before its use. Others may attempt to continue to use the stamp without reinking to reduce steps and ultimately cut down on time. Hou et al. further elaborates that many successive uses of the same stamp leads to ink depletion on the stamp, while reinking before each use causes many interruptions [10]. Thus, it is necessary to evaluate how microcontact printing without reinking can affect the adherence to the ECM.

The main quantitative parameter observed was the rate of cell adhesion quantified by cell confluency. A normal rate of cell confluency is 80% [2]. However, in this experiment, a higher rate of cell adhesion on the stamp (90-100% confluency) with much less adhesion outside the borders of the stamp (0-10% confluency) would be ideal. In this study, three replicate stamps were fabricated and used three times without reinking. The overall goal of this study is to see major differences in cell confluency between each subsequent use of the PDMS stamp.

In this study, we investigated how using the same PDMS stamp without reinking of ECM (gelatin) affects cell adherence. As mentioned in the background, cell patterning and adherence are vital parameters for cells due to their role in controlling biological factors such as apoptosis and cell migration. Thus, the logical question is what factors alter the replicability of stamps which leads to more precision with cell adhesion. We hypothesize that increasing the stamp usage without reinking will decrease cell adhesion.

Methods

A PDMS stamp was consecutively used three times without reinking to test the reusability of the stamp. Thus, the independent variable being tested was microcontact printing without ECM reinking and the dependent variable was cell adherence. Three stamp replicates were used to ensure that the data collected was accurate and each stamp was used three times without reinking to show a general trend in cell coverage. Additionally, a positive control and negative control were created by using no stamp. The former used only the ECM and the latter used nothing. All other variables remained constant to control variability including the stamp shape, size, design, gel concentration, cell density, and amount of time left in the medium before fixing.

(i) PDMS Stamp Fabrication

First, a PDMS solution was made using a curing agent and PDMS base in a 10:1 weight-to-weight ratio. The PDMS solution was centrifuged for 5 minutes and subsequently poured into a mold containing three sanitized pennies with the column design facing up. The dish containing the molds was placed in a vacuum desiccator for 15 minutes to remove possible air bubbles and then the PDMS was cured at room temperature for 48 hours.

(ii) Microcontact Printing

A non-tissue culture 12-well plate was sterilely pretreated with 750 μL of Pluronic F-127. The Pluronic F-127 treatment was put into 11 wells and was absorbed under the biosafety cabinet for 30 minutes. There was one positive control containing ECM and one negative control containing nothing. Nine wells were used for the 3 ECM protein samples and their respective

replicates. Simultaneously, the stamp was prepared by gently peeling the PDMS off the pennies. A scalpel was used to cut a stamp that was no larger than 1 cm^2 from the patterned columns of the PDMS penny molds. From the molds, 6 stamps were cut, but only 3 were used. The stamps were placed on foil with the patterned side up to be placed in the plasma treatment chamber for 10 minutes. The plasma treatment will make the PDMS more hydrophilic for the adsorption of ECM proteins to the stamp. After plasma treatment, they were placed patterned side up in a clean petri dish. Gelatin (2%) was pipetted to completely cover the entire pattern and the protein was absorbed for 30 minutes at room temperature. The Pluronic solution was then aspirated off the wells and rinsed twice with PBS+anti-anti solution. Afterward, the wells were allowed to dry in the biosafety cabinet. Following incubation of the stamps with ECM, the excess solution was removed, being careful to not touch the surface of the stamp. Under a stream of controlled air, the PDMS stamps were thoroughly dried. Each of the three stamps was firmly pressed for 1 minute into the wells using forceps and the cap of a centrifuge tube. This was repeated for each of the three stamps twice more. The well plate was then stored in the fridge with PBS+anti-anti solution.

(iii) Cell Seeding

Cells were removed from the incubator and viewed after a microscope. In a 37º water bath, 15 mL of cell medium, sterile PBS, and trypsin were warmed. Old media was aspirated off the flask and 2 mL of PBS was added. Once the PBS was aspirated off and 2 mL of trypsin solution was added, the well plate was placed in the incubator for 4 minutes. Then, the trypsin was deactivated using 2 mL of medium. The medium/cell solution was put into a sterile 15 mL conical tube and centrifuged for 3 minutes. After centrifugation, the cells were resuspended in 6.5 mL of medium and mixed thoroughly. The PBS+anti-anti solution was removed from the stamped well plate and 0.5 mL of the cell suspension was added to each of the wells containing the replicate ECM patterns. An additional 0.5 mL of medium was added to have 1 mL total in each well. The cells were attached for 45 minutes and the medium was removed. The wells were then thoroughly washed with sterile PBS to remove any cells that did not adhere to the patterned surface. The cells were fixed 24 hours after seeding.

(iv) Cell Staining

For each well plate, the actin cytoskeleton of the cells was stained to allow for image analysis. A 1X PBS solution was used to gently wash the fixed cells in the well plate twice. The remaining PBS was removed from the wells and 600 uL of a DAPI/TRITC-phalloidin/ PBST-T solution was pipetted into each well. The well plate was immediately covered with aluminum foil to protect the dye from light and then placed on a shaker for 35 minutes. Then, the dye was removed from each well and the wells were washed with PBS three more times. Each well was covered with PBS solution before being viewed under a fluorescent microscope where the cells' cytoskeletons fluoresced a bright red color. High-resolution micrographs were taken and ImageJ was used to measure the area of cell coverage for each stamp to assess cell adherence with repeated stamping. Thus, the experimental endpoint was acquiring the high-resolution micrographs.

(v) Statistical Analysis

A Kruskal-Wallis one-way ANOVA was performed for statistical analysis to determine if repeated uses of stamps led to lower cell adhesion. GraphPad was used to perform the analysis,

and a *p*-value less than 0.05 indicated statistical significance. A one-way ANOVA was chosen because even though there were three repetitions of stamping, there was only one independent variable. In addition, the data was non-parametric. Then, a Dunn's multiple comparisons test was completed to find statistical differences between each experimental group resulting in three additional *p*- values.

Results and Discussion

Six PDMS stamps were successfully fabricated and underwent plasma treatment as seen in Figure 1. The best three stamps were selected for use in microcontact printing based on their defined columns.

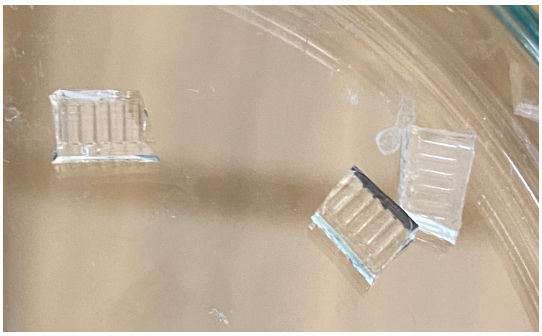

Figure 1: Representative fabricated PDMS stamps using penny columns as a mold.

As shown in Table 1 and Figure 3, Stamp 1 provided the most conclusive results. Stamp 1 shows a clear downward trend in confluency with each repeated use, 90% on use one, 65% on use two, and 42% on use three. Stamp 2 showed small levels of cell adherence with the original first use of the stamp (20% confluency), but no cells were visible on the following uses. Stamp 3 shows no cell adherence on use one, two, or three.

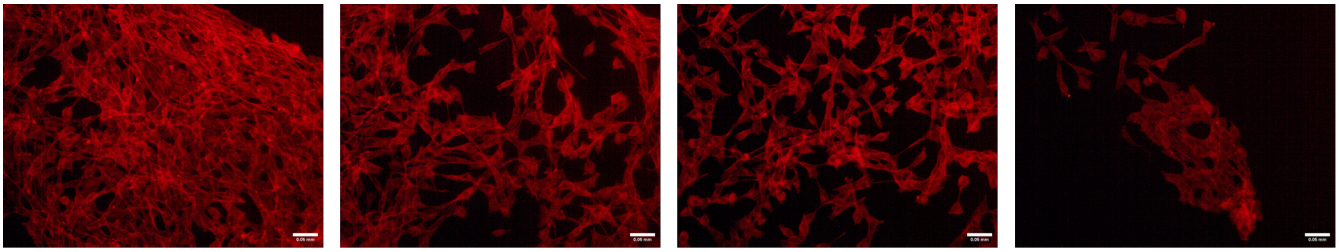

Figure 2: Representative high-resolution fluorescent micrographs of cell cytoskeletons stained with DAPI/TRITC-phalloidin for: (a) first use of Stamp 1, (b) second use of Stamp 1, (c) third use of Stamp 1, and (d) first use of Stamp 2.

Table 1: Cell confluency for different stamps and repeated uses.

Stamp	Number of Times Stamped	Confluency (%)
1	1	90
	2	65
	3	42
2	1	20
	2	0
	3	0
3	1	0
	2	0
	3	0

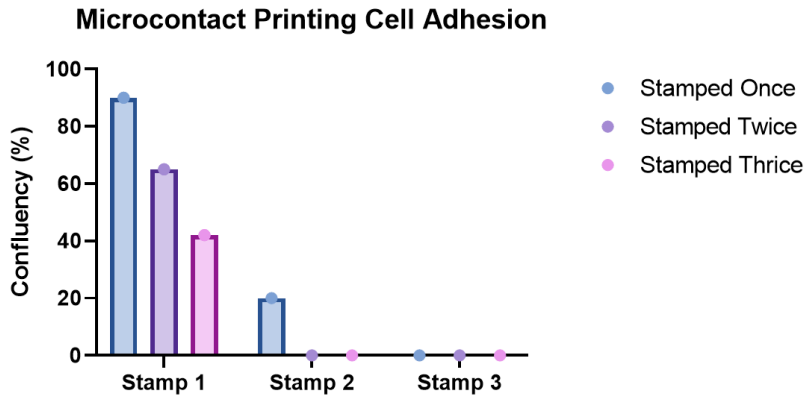

Figure 3: The cell adhesion 24 hours after microcontact printing is shown by the stamp number on the x-axis with the use indicated by color and the confluency percent on the y-axis.

Instead of fully replicating a biological system, cell patterning allows for better comprehension of cell morphology and its interactions with ECM similar to what Hu et al. demonstrated in their study about the importance of cell adherence for studying cellular interactions. In this study, Stamp 1 gives an ideal representation of how cell confluency decreases as the stamp is reused without reinking. Contrastingly, Stamp 2 does not follow an ideal trend and is inconclusive because although there appeared to be cell adherence in the first well, there were no cells in the subsequent uses of that stamp. The effect that repeated uses without reinking have on cell confluency also cannot be concluded from Stamp 3 because it showed no cell adherence in any uses of the stamp. The lack of amount of stamps represents a limitation of the experiment, especially when aiming to study confluency without reinking between each stamp. To address this issue and to improve experimental results, more PDMS stamps would need to be fabricated. Although the microcontact stamps were designed to simulate cell interactions and adhesion, the lab setting's artificiality may result in abnormal behavior that does not represent the complexity of biological systems. Additionally, discrepancies between a professional lab that specifically studies microcontact printing and our undergraduate lab that does not could lead to differences in results. In Figure 4, we can see the degree of success that can be obtained in a professional lab setting.

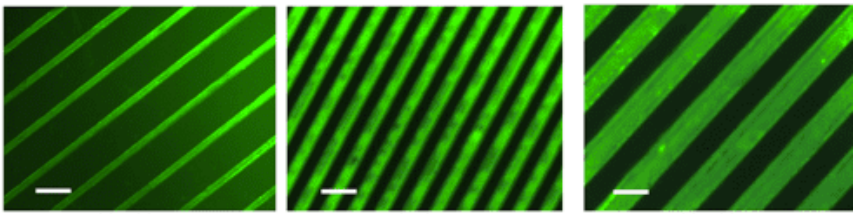

Figure 4: Images from [3] showing successful cell cultures using microcontact printing in a professional lab.

While our experiment provided minimal results, Figure 4 shows the high rates of confluency and precision that can be achieved with microcontact printing in the laboratory setting.

The one-way ANOVA test produced a p-value of 0.7857 ($p > 0.05$) indicating that there is no statistical difference between the groups. Furthermore, Dunn's multiple comparisons test gave p-values > 0.9999 for all of the group comparisons indicating that there is no significant difference between the number of times a stamp is used without reinking and the cell adherence. Thus, the hypothesis that the cell confluency would decrease as the stamp is used more without ECM reinking is not supported. More replicates of stamps could help increase the probability of seeing statistical differences; however, there were only three replicates and outliers could have affected the statistical analysis greatly. Stamp 1 showed great cell adherence for the first use and still had cellular adherence for the second and third uses. Stamp 2 had little cellular adherence for the first use and had none for the second and third uses. Stamp 3 had no cellular adherence for any uses. This could be caused by variability in the stamp fabrication process which could lead to stamps with different capabilities of transferring the ECM to the well plate for cell seeding which ultimately affects the ability of the stamp to make a pattern for the cells to adhere to.

Conclusion

The overall goal of this study was to see the reusability of the PDMS stamp without reinking of ECM (gelatin) and how that affects cell adherence on the stamp. The general hypothesis was that when the stamp usage increased without reinking, the less the cells adhered which was measured by cell confluency. Based on the results, this hypothesis was not supported by the statistical analysis. Overall, this study was conducted to demonstrate the dependence on cell adherence for microcontact printing and how cell adherence controls cell behavior variability.

The details shown in Figure 2 and Figure 3 demonstrates that our original claim that stamp usage will decrease cell adherence was not supported. When looking over the results, it can be seen that only Stamp 1 showed a decrease in cell adherence over each stamp use while the other stamps had a lack of cell adherence. Therefore, only Stamp 1 demonstrated a decrease in cell adherence over usage, which can be seen in Figures 1a - 1c. The issues that were seen in Stamps 2 and 3 could be due to the artificial environment the stamps were in and potential cross-contamination within the experiment. The trend found in Stamp 1 demonstrates the reliance on protein-substrate adhesion for microcontact printing and physiological activities on a cellular level [4]. This can be seen by the decline of confluency in Figure 3 in comparison to the lack of trends in the other stamps. When looking over the p-values using the one-way ANOVA test and Dunn's comparison test showing a high p-value, there is no clear indication of statistical difference in our data.

Potential future experiments that could aid in strengthening our data would include increasing the amount of PDMS stamps or employing different materials such as fibronectin to potentially increase cell adherence. When looking at our overall experiment, the technique of microcontact printing is well-suited for a variety of applications in biomedical engineering and research laboratories. On a larger scale with more and higher quality stamps, it could be particularly beneficial for printing nanoarrays for biosensing, which detects target molecules

using principles similar to those utilized by the immune system. Biosensing used in point-of-care testing can help monitoring health be more accessible [9]. The future outlook of microcontact printing is promising.

References

[1] A. J. Khadpekar, M. Khan, A. Sose, and A. Majumder, "Low cost and lithography-free stamp fabrication for microcontact printing," *Scientific Reports*, vol. 9, no. 1, 2019.

[2] "Cell culture guidelines - ABCAM," *Abcam*. [Online]. Available: https://www.abcam.com/ps/pdf/protocols/cell_culture.pdf. [Accessed: 11-Apr-2022].

[3] I. Elloumi-Hannachi, M. Maeda, M. Yamato, and T. Okano, "Portable microcontact printing device for Cell Culture.: Semantic scholar," *undefined*, 01-Jan-1970. [Online]. Available: https://www.semanticscholar.org/paper/Portable-microcontact-printing-device-for-cell-Elloumi-Hannachi-Maeda/5c822c9f3c88a1329570d4770b7607212dea4688. [Accessed: 25-Apr-2022].

[4] S. Hu, T.-H. Chen, Y. Zhao, Z. Wang, and R. H. Lam, "Protein–substrate adhesion in microcontact printing regulates cell behavior," *Langmuir*, vol. 34, no. 4, pp. 1750–1759, 2018.

[5] L. Filipponi, P. Livingston, O. Kašpar, V. Tokárová, and D. V. Nicolau, "Protein patterning by microcontact printing using pyramidal PDMS stamps," *Biomedical Microdevices*, vol. 18, no. 1, 2016.

[6] M. Lindner, A. Tresztenyak, G. Fülöp, W. Jahr, A. Prinz, I. Prinz, J. G. Danzl, G. J. Schütz, and E. Sevcsik, "A fast and simple contact printing approach to generate 2D protein Nanopatterns," *Frontiers in Chemistry*, vol. 6, 2019.

[7] A. P. Quist, E. Pavlovic, and S. Oscarsson, "Recent advances in microcontact printing," *Analytical and Bioanalytical Chemistry*, vol. 381, no. 3, pp. 591–600, 2005.

[8] M. Brehmer, L. Conrad, and L. Funk, "New developments in soft lithography," *Journal of Dispersion Science and Technology*, vol. 24, no. 3-4, pp. 291–304, 2003.

[9] N. Bhalla, P. Jolly, N. Formisano, and P. Estrela, "Introduction to biosensors," *Essays in Biochemistry*, vol. 60, no. 1, pp. 1–8, 2016.

[10] P. Hou, R. Kumar, B. Oberleiter, R. Kohns, D. Enke, U. Beginn, H. Fuchs, M. Hirtz, and M. Steinhart, "Scanner-based capillary stamping," *Advanced Functional Materials*, vol. 30, no. 25, p. 2001531, 2020.