Published in final edited form as:

RSC Adv. 2012 ; 2: 7673–7676. doi:10.1039/C2RA20938A.

Accurate and Effective Live Bacteria Microarray Patterning on Thick Polycationic Polymer Layer Co-Patterned with HMDS

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Abstract

A new bacteria microarray patterning technique is developed by patterning thick polycationic polymers on glass surface, which generates high-coverage and high-precision *E. coli* cell patterns. Cell immobilization efficiency is greatly improved, compared to conventional monolayer surface patterning approach. Cell viability tests show very low cytotoxicity of polyethyleneimine (PEI). This advancement should further accelerate biomedical and bacteriological researches in the micro scale.

Intersection between micro/nano technology and microbiology in the recent decade has made a significant impact on the research and applications of microorganisms with high spatial resolution.¹ Within this context, site-specific immobilization of bacteria is still a subject under substantial interest.^{1, 2} Its key importance in many biomedical and bacteriological research and applications, can be found in fundamental studies in cell-cell and cell-surface interactions towards biofilm formation involving quorum sensing,³ fabrication of high-throughput biochemical and drug screening platforms,⁴ functional biosensors,⁵ and energy harvesting from motile bacterial biomotors for nano-electromechanical devices.⁶

These technologies require highly accurate spatial placement of live bacterial cells at specific location, such as transducer electrodes or bacterial microarrays, with microscale or even single-cell resolution. While direct printing of bacteria has been demonstrated using microcontact printing with hydrogel⁷ or elastomer stamps,⁸ thermal inkjet,⁹ and bubble-jet¹⁰ with good viability on agar surfaces, these methods suffer significantly from their low pattern resolution (hundreds of microns). On the other hand, a majority of the high resolution bacteria patterning platforms are constructed on chemically modified surfaces fabricated using micro/nano surface modification and patterning techniques.⁴ Along this approach, surface immobilizations of bacteria have been demonstrated on chemically modified surface patterns or microwells through hydrophobic interaction via alkanethiol,¹¹ electrostatic interaction via poly-L-lysine,¹² bio-specific interactions via conjugations between antibody-virus,¹³ antibody-antigen,¹⁴ or streptavidin-biotin,¹⁵ or chemical attachment via amine- and carboxylic acid-based conjugation.¹⁶ In addition, to site-

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[‡]Electronic Supplementary Information (ESI) available: Full experimental details. See DOI: 10.1039/b000000x/

specifically immobilize bacteria at the desired location, area where cell adhesion is not desired is commonly passivated with a layer of poly(ethylene glycol) (PEG). Such patterned surfaces have been fabricated on glass and silicon surfaces using micro/nano fabrication technologies, such as microcontact printing on PDMS,¹⁷ capillary micromolding,¹³ focused Ga⁺ ion beam etching,¹⁸ photocatalytic oxidation of silane,¹⁶ photolithography,¹⁹ and low-energy electron beam lithography.²⁰

Recently, the efficiency and reproducibility of surface modification-based bacteria patterning approach have been questioned.¹⁸ Such issues, which were not found in eukaryotic cell patterning, may be attributed to the rigid cell wall of the bacteria allowing only a small contact area with the substrate surface, or the pili and extracellular polymeric substances preventing a direct contact to the substrate surface.¹⁸ These issues were previously addressed elegantly where bacteria immobilization efficiency could be enhanced through careful selection of affinity-purified antibody which targets surface antigens at the outer cell wall.¹⁸ However, this approach could be complicated by the extra effort, cost, and time required for obtaining the purified antibody, as well as prior knowledge on the genetic and physiological information of the test strains.

Another reason for the low patterning and immobilization efficiency, as have been pointed out recently,²¹ could be due to the fact that most surface modification methods are based on modifying surfaces with only ultrathin or monolayers of tethering material for bacteria immobilization purpose.²¹ According to the strains we tested, monolayers of tethering materials, e.g. poly-L-lysine (PLL), do not provide enough attachment force, such as positive electrostatic charge, for bacteria immobilization (data not shown). In fact, in most bacteria surface adhesion force measurement studies using Atomic Force Microscopy (AFM), where secure bacteria attachment on the AFM tips is critical, it is a common practice to coat a thick layer of PLL by completely air-drying PLL drops on the AFM tips without any water rinsing.²¹

Herein we developed a new surface patterning technique to generate high-coverage bacterial microarrays with high precision, by significantly enhancing bacteria-surface adhesion with thick polycationic layer patterned with a photolithographic lift-off process (Figure 1). Besides, to selectively immobilize bacteria at the desired location, a usual practice is to functionalize the area where bacteria immobilization is not desired with a protein resistant layer, mostly PEG. PEG works for bacteria passivation because bacteria generally adhere to surface through a series of surface interactions which ultimately involve the formation of various protein layers.²² In our work, in contrast to a conventional practice of passivating surface with PEG layer for bacteria repulsion purpose as demonstrated in most previous works,^{13, 17, 18, 23, 24} we exploited a new use of a common photoresist adhesion promoter, hexamethyldisilazane (HMDS), for surface passivation purpose. This unconventional use of HMDS significantly simplifies the fabrication process.

Figure 1 shows the fabrication process of the thick polycationic surface patterning (see ESI). Briefly, oxygen plasma treated glass surfaces were coated with HMDS by evaporation, followed by conventional photolithography process with positive-tone photoresist (AZ5214) to generate 2 μ m holes in the photoresist layer. The samples were then treated with oxygen plasma again to clear off the exposed HMDS layer in the photoresist pattern. This process reveals the bottom hydrophilic silanol groups. Aqueous solution of branched cationic polymers (PEI or PLL) was dispensed evenly onto the sample surface and incubated for 30 min at room temperature, allowing physisorption of the polymer on the negatively charged glass surface. After incubation, excess PEI solution was directly blown dried with a stream of nitrogen gas. A lift-off process in acetone with 10 second ultrasonic pulses was used to remove the photoresist and the polymer layer on it, leaving condensed layer of polymer co-

patterned with HDMS on the glass surface. Water was avoided after polymer was incubated on the surface, due to the high solubility of the hydrophilic polymer in water. AFM scan of the PEI patterned surface revealed a layer of polymer with a thickness of 100 – 200 nm co-patterned with HMDS (Figure 2a). It is worth to note that such high thickness variation is found on almost every single 2 μ m adhesion spot with the center of the spot always thinner due to the lift off process (see Figure 2a). Such polymer patterns can be fabricated consistently across samples, which in turn consistently generate high quality bacteria cell patterns. Therefore, we believe the variation in the cell-adhesion site thickness uniformity might not be a critical factor in affecting the cell attachment. We also believe, theoretically, that the cell adhesion strength could reach a plateau with increasing layer thickness. In our example, it is possible that the adhesion strength has already reached the plateau at a thickness >100 nm, as evidenced by the high quality cell patterns across a large area on the substrate (Figure 3).

In this study, wild type JCL16 *E. coli* strain was used as the biological model to test the patterning methodology. Figure 3 shows the high specificity patterning of bacteria on the PEI and PLL coated spots while no bacteria could be found on the HMDS passivated surface. The results also revealed superior passivation ability of HMDS against *E. coli* binding, in contrast to many protein and mammalian cell patterning studies where HMDS was used to enhance the nonspecific biomolecules physisorption and subsequently used for cell adhesion.^{25, 26} This work also showed its advantage on the pattern fidelity compared to conventional monolayer surface patterning approaches for bacteria patterning, especially with its significant improvements in pattern coverage (99% of the patterned regions occupied by cells). As a control, monolayer PEI and PLL patterns fabricated with an additional water rinsing step to dissolve polymeric materials not directly adsorbed on the glass surface generated bacteria array with much less coverage (data not shown), as we anticipated, mainly due to the weak surface-cell interaction.²¹ Figure 2b shows the AFM scans of the samples patterned with monolayers of PEI with a thickness of about 5nm.

In this work, we unconventionally employed a hydrophobic material, HMDS, as the passivation layer against bacterial cell binding, in contrast to the conventional use of hydrophilic materials, such as PEG. Our study showed that HMDS served as a good candidate material for bacteria repulsion purpose. Indeed, surface hydrophobicity has been found to either enhance or resist surface adhesion depending on the strains of *E. coli*, according to other AFM studies.^{27–29} However, the use of fixated (dead) bacterial cells for force measurements may not represent the same condition where live and motile cells are allowed to dynamically interact with the substrate surface. Moreover, short term force measurements with AFM may not be able to address the mechanisms of relatively long term bacteria-surface interactions during the incubation period (30 min) in our experiment.

Using a thick layer of the polycationic polymers to enhance bacteria attachment may raise the concern of cytotoxicity on the bacterial cells.^{30, 31} Since the cytotoxicity may adversely affect the patterned cells for subsequent biological studies, we carried out a live/dead assay (LIVE/DEAD BacLight Viability Kit, Invitrogen) to examine the physiological conditions of the patterned microbes.¹⁷ Cell viability condition was indicated by the fluorescent color where live bacterial cells fluoresce green and dead bacterial cells fluoresce red. The incubation time for attaching the cells on the substrate is 30 min, after which the viability test is immediately carried out (see ESI). The viability test is carried out by following manufacturer's protocol which comprises a 15 min incubation of the cell pattern in a viability test assay solution, followed with image acquisition under microscope. Taking into account the image acquisition time, the cells are live on the pattern for at least 1 hr. As presented in Figure 3b and d, more than 95% of cells were alive on the PEI patterns and 75% on the PLL patterns. Results show that the cell viability is much better on PEI patterns

compared to PLL patterns. Studies have shown that PLL has strong cytotoxicity and has been employed as antimicrobial surface coating.³² However, PEI is not found to be an effective antimicrobial agent until it is alkylated.^{33, 34}

Conclusions

In conclusion, we developed a new bacteria patterning technique which can generate high-coverage and high-precision bacteria cell microarray, by enhancing bacteria-surface adhesion with thick polycationic layer patterned on glass surface with a lift-off process. To our knowledge, this is the first work attempting to pattern bacteria on thick layer of polycationic polymer. The thick PEI and PLL layers were found to be very effective in improving cell attachment. The method generated single cells microarrays of wild type *E. coli* with significant improvement in patterning coverage, compared to conventional monolayer surface patterning approach. This work also discovered good passivation ability of HMDS against *E. coli* binding, in contrast to the conventional consensus of hydrophobic material on enhancing nonspecific biomolecules binding. Considering the cytotoxicity possessed by high molecular weight polycationic polymers which may potentially affect the physiological conditions of the patterned bacteria, cell viability tests were carried out and the results show that the cytotoxicity effect was negligible on PEI patterns relative to the PLL patterns. The major advances on bacterial cell immobilization efficiency presented in this work could substantially promote and accelerate biomedical and bacteriological research and applications, which demand high stringency and efficiency on localization of bacterial cells, such as biosensors, high-throughput drug screening, and biofuel cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Prof. James C. Liao at University of California, Los Angeles for providing the *E. coli* cells. This work was supported by the Center for Cell Control (PN2EY018228) through NIH Roadmap for Nanomedicine and the Center for Scalable and Integrated Nanomanufacturing (SINAM) under the National Science Foundation (CMMI-0751621).

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Fig. 1.

Fabrication process of the cell patterning surface. (a) Glass slides coated with HMDS and patterned with photoresist, (b) Polycationic solution incubation, followed by air blown-dry, (c) Lift-off the photoresist in acetone and isopropyl alcohol, and blown-dry, (d) Cells incubated on the patterned PEI chip, (e) Rinsing off non-specific attached cells with PBS. The inset shows the positively charged amine groups in the molecules.

Fig. 2. AFM scans of the chemically patterned surfaces. (a) Thick layer of PEI; thickness approximately 100–200 nm, (b) Monolayer of PEI patterns, fabricated by rinsing the thick PEI layer patterns with DI water to remove the PEI not directly adsorbed on the glass surface; thickness is approximately 5 nm. Images were processed with the Nanoscope Median filter with an order of 3×3 to filter out the high frequency noise.

Fig. 3.

E. coli cells microarray. (a, b): Bacteria adhered to poly(ethyleneimine) (PEI) patterned spots, (c, d): Bacteria adhered to poly-L-lysine (PLL) patterned spots. Each spot was 2 μ m and was occupied by around 2–3 cells. (b, d): Backlight live/dead assay staining of the cells: live cells fluoresce green and dead cell fluoresce red. PEI patterns generated cell array with more than 95% live cells while PLL patterns generated cell array of about 75% live cells. Scale bar is 20 μ m.