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Supported bilayer membranes for reducing cell adhesion in microfluidic devices [post-print]

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TECHNICAL NOTE

Supported Bilayer Membranes for Reducing Cell Adhesion in Microfluidic Devices

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The high surface area-to-volume ratio of microfluidic channels makes them susceptible to fouling and clogging when used for biological analyses,including cell-based assays. We evaluated the role of electrostatic and van der Waals interactions in cell adhesion in PDMS microchannels coated with supported lipid bilayers and identified conditions that resulted in minimal cell adhesion. For low ionic strength buffer, optimum results were obtained for a zwitterionic coating of pure egg phosphatidylcholine; for a rich growth medium, the best results were obtained for zwitterionic bilayers or those with slight negative or moderate positive charge from the incorporation of 5-10 mol% egg phosphatidylglycerol or 30 mol% ethylphosphocholine. In both solutions, the presence of 10 g/L glucose in the cell suspension reduced cell adhesion. Under optimum conditions, all cells were consistently removed from the channels, demonstrating the utility of these coatings for whole-cell microfluidic assays. These results provide practical information for immediate application and suggest future research areas on cell-lipid interactions.

Introduction

Microfluidic devices have emerged as a critical analytical tool to study cells and tissues using small quantities of reagents to produce high resolution data. Microchannels also recreate the scale, geometry, and flow conditions of biological structures, such as capillaries, *in vitro* yielding more accurate results and a superior understanding of *in vivo* conditions. Their small size makes them amenable to studies of limited volume samples, including individual cells and organelles.[1] Narrow channels also improve separation and detection steps. Additionally, microfluidic devices often have lower costs associated with fabrication, experimentation, and disposal than other analytical tools with similar capabilities due to reduced reagent consumption.[2] Despite these advantages, the small size and high surface area-to-volume ratio of microfluidic channels also increase the likelihood of fouling and clogging, particularly when handling biological samples such as intact cells.[3]

Among the many strategies for coating microfluidic channels,[4] supported phospholipid bilayers present a simple, biocompatible solution to the undesired adhesion of cells and proteins. Conveniently, small unilamellar vesicles formed from phospholipids spontaneously fuse and rupture along hydrophilic glass and PDMS channel walls, generating a continuous supported bilayer membrane that coats the channel and generates a uniform zeta potential inside the microfluidic device.[5,6] As phospholipid bilayers are the main structural component of biological membranes, this coating also provides a biocompatible environment that mimics *in vivo* conditions. Finally, supported phospholipid bilayers can be tailored to specific functions by judicious selection of the lipid head groups. This tailoring had been used previously to tune electroosmotic flow[6,7] or to enhance cell adhesion.[8] Cell adhesion has also been promoted by chemical modification of the lipid bilayer to incorporate peptides or proteins that promote cell attachment.[9,10]

While cell adhesion is desirable for on-chip cell culture, it is undesirable for flow-through assays, such as microfluidic flow cytometry or chemical cytometry. A common challenge in these assays is that cells adhere to channel walls and eventually block flow, preventing further data collection. Supported lipid bilayers of pure phosphatidylcholine and PEG-grafted lipids have been used to reduce cell adhesion;[11–13] but a study of the effect of bilayer composition on cell adhesion has not been undertaken previously. The goal of this work was to minimize cell attachment by determining the optimal lipid mixture. Because electrostatic interactions were expected to play a key role, we focused on the net charge of the bilayer and investigated differences in results for a low ionic strength buffer versus a high ionic strength, biomolecule-rich media. By better characterizing how channel coating composition prevents or encourages cell adhesion, microfluidic devices may be optimized for flow-through cellular analysis.

Methods

Cell culture

Dictyostelium discoideum cells, strain KAX3 (DBS0236487), were obtained from the Dicty Stock Center[14] and maintained in

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axenic liquid culture at a density of 10,000-4,000,000 cells/mL at 22 °C with shaking at 180 rpm. HL-5 media was prepared in house and was composed of 14 g/L proteose peptone, 7 g/L yeast extract, 3.5 mM Na_2HPO_4, 11 mM KH_2PO_4 at pH 6.3-6.7, supplemented with 13.5 mg/mL glucose, 100 µg/mL ampicillin, and 300 µg/mL streptomycin.

Microfabrication

Hybrid PDMS-glass microfluidic devices were prepared using standard photolithography and soft lithography methods. Briefly, masters were prepared by spin-coating SU-8 2015 (Microchem) onto silicon wafers for 10 s at 500 rpm followed by 30 s at 2000 rpm, soft baking for 5 min at 95 °C, and exposing through a transparency photomask (Fineline Imaging, 32,000 dpi) to a total of 150 mJ/cm^2 using an OAI Model 200 mask aligner. Wafers were hard baked for 4 min at 95 °C, developed with orbital shaking in SU-8 developer for 3 min, rinsed with isopropyl alcohol, then post-exposure baked for 65 min at 95 °C. Finally, masters were silanized under reduced pressure in a vacuum desiccator containing a small quantity of trichlorooctylsilane overnight.

PDMS molds were prepared by mixing Sylgard 184 (Dow Corning) in a 10:1 ratio, degassing, and baking for 15 min on a hotplate until cured. Access holes were made using a 1 mm biopsy punch. Assembled devices were prepared by treating the PDMS mold and a coverglass (#1, Fisherbrand) for 2 min in oxygen plasma (Harrick, PDC-001). Reservoirs to hold solutions were cut from silicone tubing (Masterflex, EW-96440-16) and plasma sealed over the access holes.

For coatings, egg phosphatidylcholine (PC), egg phosphatidylglycerol (PG), 1-palmitoyl-2-oleoyl-sn-glycero-3-ethylphosphocholine (chloride salt) (EPOPC), and phosphatidic acid (PA) were obtained from Avanti Polar Lipids. Lipid solutions in chloroform were prepared in varying mole ratios, vortexed, and then stored as dried thin films under nitrogen at -20 °C until use. Vesicles were formed by sonicating 2 mg dried lipids with 2 mL of 10 mM Tris-HCl (pH 7.5), 150 mM NaCl buffer using a microtip (Branson 250) for 15 min with a duty cycle of 30% at power level 2. The resulting vesicle solution was centrifuged for 5 min at 12,000×g and stored at 4 °C for at least 2 h but no more than 2 weeks before use. Supported bilayer coatings formed spontaneously when small unilamellar vesicles were added to the freshly sealed devices.[6] Bilayer formation was periodically confirmed by electroosmotic flow measurements using gated injections of fluorescein dye on a cross channel chip. For adhesion experiments, devices were single straight channels, 56 µm wide at the top, 80 µm wide at the bottom, 17 µm deep, and 2 cm long. These dimensions ensured that >100 cells were in the channel at the start of each assay, ensuring that the percentage of cells removed during the wash step was measured with sufficient precision.

Adhesion assay

Adhesion assays were conducted in either 20 mM phosphate buffer (pH 7.0) or low fluorescence media. Low fluorescence media was composed of 50 mM $FeCl_2$, 10 g/L glucose, 5 mM K_2HPO_4, 5 g/L casein peptone, 0.5 mM NH_4Cl, 0.2 mM $MgCl_2$, 10 µM $CaCl_2$, 13 µM $Na_2EDTA{\cdot}2H_2O$, 13 µM $ZnSO_4{\cdot}H_2O$, 18 µM H_3BO_4, 2.6 µM $MnCl_2{\cdot}4\ H_2O$, 0.7 µM $CoCl_2{\cdot}6H_2O$, 0.6 µM $CuSO_4{\cdot}5H_2O$, 81 nM $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, pH 6.5. Cells were centrifuged for 2 min at 1000×g, washed once and resuspended in phosphate buffer or low fluorescence media before loading onto the microfluidic device at a density of 2×10^7 cells/mL. Gentle vacuum (-50 kPa) from a diaphragm pump was used to load cells into the device for 1 min. Once the cell suspension had filled the entire channel, flow was stopped by equalizing the fluid levels in the two reservoirs, and cells incubated in the device at room temperature for 15 min. During the incubation step, the number of cells in the channel was determined using a microscope to count individual cells twice. Next, the reservoirs were flushed three times with cell-free buffer or media until all cells in the reservoirs were removed, and the cell-free solution was used to rinse the channel using hydrostatic flow by placing the channel upright for 5 min. After this rinse, the outlet reservoir was rinsed to remove cells that had exited the channel, flow was stopped, and cells that remained in the channel were counted twice.

Results and discussion

For the adhesion assays, we used the social amoeba *D. discoideum*, a unicellular eukaryotic model organism. *D. discoideum* is similar in size and cell membrane composition[15] to non-adherent mammalian cells and is commonly used in studies of cell adhesion.[16,17] Individual cells were easily identified and counted before and after rinsing the device (Figure 1).

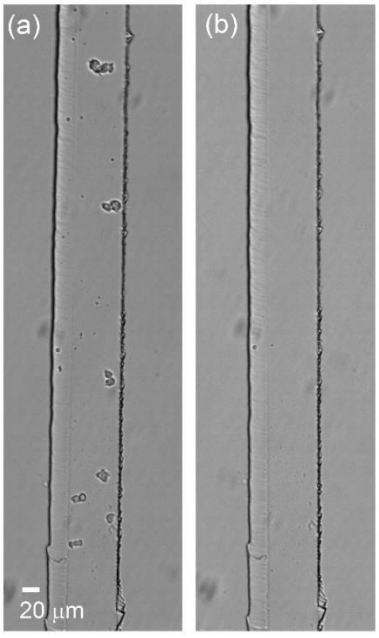

Figure 1. White light micrographs of cells in low fluorescence media in a microfluidic channel coated with 100 mol% PC (a) before and (b) after rinsing.

As a control, we first tested cell adhesion in bare (uncoated) hybrid PDMS-glass chips after a 15 min incubation. Past studies have suggested that cell adhesion increases steadily for the first 10-40 min of surface contact,[18,19] and this seemed a reasonable period of time during which flow may be stopped during device set-up for on-chip assays. In uncoated devices, most cells remained in the channel after rinsing (Figure 2). This was not surprising since cell adhesion to PDMS and glass is well-characterized.[20] Our tests were conducted in both low ionic strength phosphate buffer and in high ionic strength, protein-rich, low fluorescence media. The 20 mM phosphate buffer (pH 7.0) used in these experiments has been used previously to study metabolism in *D. discoideum*.[21] Low fluorescence media is a defined growth medium for *D. discoideum* designed to reduce background fluorescence for imaging.[22] These two solutions represent common aqueous environments for *D. discoideum* cells that vary substantially in their ionic strength and composition. Despite these differences, similarly high levels of cell adhesion were observed in the bare PDMS-glass channels with both solutions.

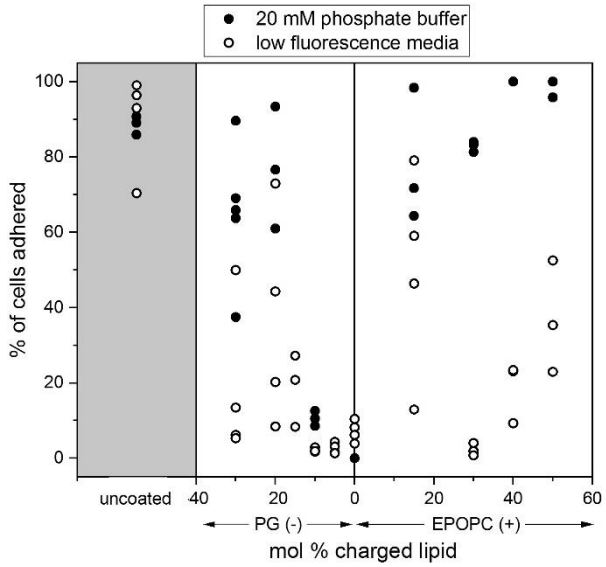

Figure 2. Percent of cells left in the channel after the wash step for various coatings and buffers. Mol% of negatively charged PG increases from right to left; mol% of positively charged EPOPC increases left to right. The remainder of each bilayer was composed of zwitterionic PC, such that the zero point on the *x*-axis represents a pure PC coating. Each data point represents a unique microfluidic device with *N* = 3-5 replicates on different devices for each condition.

Next we tested cell adhesion as a function of supported lipid bilayer charge by varying the mole percent of zwitterionic PC and either negatively charged PG or positively charged EPOPC. We selected PC, PG, and EPOPC because these lipids are readily available, inexpensive, and have commonly been used in supported lipid bilayer coatings of microfluidic devices. Additionally, EPOPC is one of the only commercially available, positively charged phospholipids, since most positively charged lipids are synthetic lipids designed for transfection that do not include the phospho-head group. We confirmed the presence of lipid bilayers of the expected charge by measuring the electroosmotic mobility in devices coated with PC only, EPOPC/PC, and PG/PC. As expected, we found that EOF was suppressed by pure PC coatings, reversed with EPOPC-containing coatings, and varied in magnitude but remained cathodic for PG coatings (data not shown). In general, supported lipid bilayer coatings reduced cell adhesion compared to bare PDMS, but the reproducibility and magnitude of this effect depended on the bilayer composition.

In general, results were most reproducible between devices for coatings with lower net charge. As the mole percent of charged lipid in the bilayer increased, more variability between devices was observed (Figure 2). This may be due to differences in the stability and uniformity of the coatings as a function of surface charge. A previous study of microchannels coated with supported lipid bilayers found that 100 mol% EPOPC coatings were not stable over time and that charged lipid coatings resulted in higher variability in electroosmotic mobility than a zwitterionic PC coating.[6] The 15 mol% EPOPC coating was an exception to this trend, as it showed higher variability between devices than did coatings with greater amounts of EPOPC. This was true in both buffers and across two batches of lipid.

Cell adhesion in low ionic strength phosphate buffer

In the phosphate buffer, the pure zwitterionic coating resulted in the lowest cell adhesion (Figure 2). Statistically there was no difference between the PC bilayer and the 10 mol% PG bilayer (one-way ANOVA, Tukey test, $p>0.05$); however, for the zwitterionic buffer all cells were consistently removed from the channel, while cell removal was 89±2% for 10 mol% PG coatings. This suggests a practical difference despite the lack of statistical significance in this study.

As the mole percent of charged lipid increased, cell retention increased as well, then plateaued at high concentrations of charged lipid (Figure 2). This was true for both negatively charged and positively charged bilayers. Cell membranes typically have a negative zeta potential at physiological pH, so electrostatic attraction likely increased cell adhesion as the bilayer became more positive with increasing amounts of EPOPC. Indeed, in phosphate buffer, for high levels of EPOPC (40 mol% and 50 mol%), very few to no cells were removed from the channel during the wash steps.

We expected that increasing the negative charge of the bilayer with PG would decrease adhesion by promoting electrostatic repulsion, but this was not observed. Instead, cell adhesion increased with increasing amount of PG in the bilayer, particularly as PG content increased from 10-20 mol%. There are several possible explanations for this observation. Mammalian cells are known to adhere better to polystyrene tissue culture dishes that have undergone treatment that makes them more hydrophilic, increases the density of surface hydroxyl groups, and increases the negative charge.[23–25] A similar effect may be responsible here. Alternatively, acidic phospholipids are known to interact with basic residues of membrane proteins.[26] A previous study found that negatively

charged phosphatidylserine (PS) promotes cell adhesion, although the mechanism for this was not discussed.[8] To further explore this trend, we compared cell adhesion for PG coatings to adhesion on coatings containing phosphatidic acid (PA), another negatively charged phospholipid. We found uniformly high adhesion (>80%) for 10 mol% and 30 mol% PA coatings, suggesting that the trends observed for PG coatings may vary with lipid identity as factors beyond charge play a role in cell adhesion. For example, the small head group of PA can result in spontaneous curvature of supported membranes, and past studies have documented changes in the structure and distribution of PA-containing membranes with changes in the surrounding ionic strength.[27,28] PG, PS, and PA are the most readily available negatively charged phospholipids for coatings. (Phosphoinositides and cardiolipin are also negatively charged, but more costly.) Based on past results and those presented here, PG is the best choice for a negatively charged coating that resists cell adhesion.

Cell adhesion in low fluorescence media

In general, cells were more easily removed from the channel when suspended in low fluorescence media than in phosphate buffer (Figure 2). There are three main chemical differences between low fluorescence media and the phosphate buffer. Unlike phosphate buffer, low fluorescence media contains (1) multivalent cations, (2) casein peptone, and (3) glucose. The presence of divalent and trivalent cations in low fluorescence media would be expected to increase, rather than decrease, cell adhesion. Divalent cations stabilize the gel form of the lipid bilayer and make it more rigid.[29,30] Previous research has shown that cells attach preferentially to lipid bilayers in the more ordered gel phase, and not to the disordered liquid crystalline phase.[31] The phase transition temperatures of the lipid bilayers used in these studies were well below room temperature,[32,33] so all coatings were in the liquid crystalline phase. For this reason, the stabilizing effect of divalent cations would be expected to increase cell adhesion in low fluorescence media compared to phosphate buffer, but this was not observed. Di- and trivalent cations also change the zeta potential of the bilayer, making it more positive.[7] This effect would also be expected to increase, not decrease, cell adhesion.

We initially hypothesized that the decrease in cell adhesion in low fluorescence media compared to the phosphate buffer resulted from the second difference between these two solutions: the casein peptone content of the low fluorescence media. Protein, especially bovine serum albumin (BSA), is commonly added to buffers to prevent fouling of capillaries and microchannels. The protein forms a dynamic coating on the channel surface, but is less stable than semi-permanent supported bilayer coatings.[4,6] However, addition of 5 mg/mL casein peptone to the 20 mM phosphate buffer did not change cell adhesion for the 30 mol% or 40 mol% EPOPC bilayer or for the 30 mol% PG bilayer ($p > 0.2$ for all conditions tested).

The third major difference between low fluorescence media and the 20 mM phosphate buffer was the presence of a high concentration of glucose. Although we expected cell adhesion to be determined mainly by electrostatic effects, past research has found that van der Waals forces also contribute to cell adhesion and that high sugar content in solution may decrease adhesion of cells by disrupting interactions between the surface and extracellular glycoproteins.[34] To determine whether this effect could account for the decrease in cell adhesion between low fluorescence media and 20 mM phosphate, we tested cell adhesion in 20 mM phosphate, pH 7.0 containing 10 g/L glucose in channels coated with 40 mol% EPOPC. In trials with 20 mM phosphate without glucose, no cells were removed from the channel during the rinse step using this coating (Figure 2). For the same buffer with glucose, 34±1% of cells were removed. Similarly, the addition of glucose to the phosphate buffer reduced cell adhesion for the 30 mol% EPOPC and 30 mol% PG coatings by approximately 20 percentage points. This suggests that van der Waals interactions may play a significant role in cell adhesion in microfluidic channels. The addition of glucose to this buffer also changed the osmotic pressure; however, we do not expect that this change was the cause of the reduced adhesion since addition of a similar amount of casein peptone, which would also affect osmotic pressure, did not have the same effect.

Interestingly, in low fluorescence media, slightly negative (5-10 mol% PG) and moderately positive (30 mol% EPOPC) bilayers were at least as effective at resisting cell adhesion as the pure zwitterionic PC. (The differences between the zwitterionic coating and these charged coatings were not statistically significant [one-way ANOVA, Tukey test, $p>0.05$]). The glucose content of the low fluorescence media may have widened the range of coatings that were effective at reducing cell adhesion. Surprisingly, the 15 mol% EPOPC was less effective at preventing cell adhesion than the 30 mol% EPOPC. As noted above, this coating was an outlier in its chip-to-chip variability as well, which was higher than expected. It remains unclear why this coating did not follow the general trends observed.

Although these studies were conducted with the social amoeba *D. discoideum*, they are likely to be applicable to other cell types. *D. discoideum* is an important model organism for cell migration and adhesion[16,17] that shares many characteristics with mammalian leukocytes,[35–37] which are the object of many flow-through microfluidic studies. *D. discoideum* and leukocytes (which are often referred to as “amoeboid” cells due to their similar behaviours) undergo rapid chemotactic migration in confined spaces that relies on nonspecific attraction to surfaces rather than specific protein binding used by adherent cell types.[38–40] Adherent cells attach to surfaces using a different mechanism, by forming protein networks called focal adhesions that involve integrin binding to specific extracellular matrix components. However, trypsinization of adherent cells is a common step prior to their introduction to a microfluidic device. This procedure temporarily destroys integrins, delaying formation of focal adhesions.[41] Additionally, modelling of cell-

cell adhesion in tissues has suggested that nonspecific chemical interactions, including electrostatic and van der Waals attractions, may play an important role in supplementing focal adhesions.[42] Consequently, the findings presented here should be relevant to other non-adherent cell types, such as leukocytes, and potentially to trypsinized adherent cells as well.

Conclusions

These experiments demonstrate that supported lipid bilayer coatings reduce microfluidic device fouling due to cell adhesion and show how this effect can be tuned by varying the net charge of the lipids. Under optimum conditions, cell adhesion was eliminated, and all cells were consistently removed from the channel. In a low ionic strength buffer, a zwitterionic coating was most effective at preventing cell adhesion. In a richer growth medium, adhesion was much lower and was minimized when the net charge on the bilayer was low, but slightly negative or moderately positive bilayers were equally effective as the pure zwitterionic coating. Lower adhesion in the growth medium was likely due to the high glucose concentration disrupting van der Waals interactions between cells and channel walls. In the future, non-metabolizable glucose analogues could be used to reduce cell adhesion independent of nutrient content. This is particularly important since researchers may wish to modify channel surface charge, for example to control electroosmotic flow, independent of cell adhesion. These results provide practical guidance for researchers who wish to implement these coatings in their devices and suggest avenues of future research on cell-lipid interactions.

Conflicts of interest

There are no conflicts to declare.

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