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Surfaces that Adhesively Discriminate Breast Epithelial Cell Lines and Lymphocytes in Buffer and Human Breast Milk

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Abstract

We report new surface coatings that adhesively distinguish three breast epithelial cell lines (MCF-10A, MCF-7, TMX2–28) when cell suspensions in buffer or breast milk are flowed over the coatings. We also report selective capture of epithelial cells and rejection of Jurkat lymphocytes, with average selectivities exceeding 60 and captured cell purities of often exceeding 99+ %. The surfaces achieve the dual goals of selective cell capture and resistance to fouling by proteins and other components of breast milk. The coatings do not rely on antibody targeting of cell-surface markers but instead contain polycation chains embedded within a layer of end-tethered polyethylene glycol (PEG) chains. The PEG, somewhat shielding the polycations, prevents surface fouling by proteins, non-desired cells, and other milk components, while the polycations produce electrostatic attractions that are heterogeneous on nanoscopic length scales. These electrostatic heterogeneities on the engineered coating, shown to produce curvature-selective particle capture in other studies, produce the cell selectivity here. The ability of the engineered surfaces to discriminate these cell lines via an electrostatic driving force is remarkable, as the cells are of very similar surface charge as evidenced by their nearly identical zeta potentials. The current surfaces, which likely distinguish cells based on their electrostatic surface landscape combined with other factors, adhesively distinguish cell lines that may differ only slightly in their expression of a surface marker, or cancer cells that minimally express EpCAM but which have different distributions of electrostatic charge on their surfaces. These surfaces are among the first to be documented for the compatibility of a polymer brush with human breast milk and may find use in technologies that capture cells from human breast milk or other complex fluids for cancer risk assessment.

Keywords

buried functionality; buried adhesive groups; cell capture; cell sorting; cell collection; breast milk biomaterial compatibility; electrostatic interactions; steric interactions

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Introduction

The ability to capture specific types of flowing cells from blood or other complex fluids remains a cross-disciplinary technological challenge. Access to rare circulating tumor cells may enable assessment of disease progression¹ and treatment effectiveness, while capture of stem cells or specific populations of leukocytes and Tcells from blood facilitates cell-based therapies.² In contrast to the expansive literature for cell capture from blood,^{3–5} cell capture from breast milk is little-studied even though breast cancer is the most frequent cancer diagnosis among women of reproductive age⁶ and giving birth is associated with an increased risk of developing breast cancer that can last more than 20 years.⁷ Access to breast epithelial cells from breast milk may form the basis for a non-invasive, easy-access cancer screening for women of childbearing age.^{8–11} Ongoing research targets analysis of breast cells from milk with the goal of predicting breast cancer risk years after lactation and of detecting existing cancers earlier than possible by mammography of breast exam. A screen based on breast milk would be relevant to many women. In 2017 in the US, there were 12770 new breast cancer diagnoses of women less than 40 years old, and 49360 new diagnoses for women in their 40's,¹² a substantial fraction of the 240,000 total new diagnosed cases annually.¹³ At the same time the age of women giving birth is increasing with substantial number of women having children in their 40's.¹⁴

Breast milk is a complex fluid, containing sloughed epithelial cells and lymphocytes, along with proteins, fats, and other molecules.^{9, 15} In contrast to blood, the proportion of cell types and other molecules in human milk varies greatly from woman to woman and across multiple samples from the same woman, presenting a challenge in specimen manipulation. Presently, isolation of epithelial cells from milk involves multiple labor-intensive processing steps including: concentrating the cells in buffer, counting the total cell population, incubating with antibody-coated paramagnetic beads targeting an epithelial surface marker such as EpCAM (epithelial cell adhesion molecule), running the cells over a magnetic column to collect the negative fraction, and finally collecting the captured cells from the column.⁹ In addition to being labor-intensive, this protocol is expensive and does not capture those epithelial cells that have a low expression of the selected surface marker.

Here we demonstrate the cell capture performance of engineered antibody-free polymer-based coatings that address goals specific to breast milk: capture of epithelial cells from a complex fluid, adhesive discrimination of different cell types, and an ability to tolerate dissolved proteins and fats without fouling, loss of coating, or interference with cell capture. In this study, polymer-coated surfaces impose nanometric precision over electrostatic attractions and steric repulsions with cells, and accomplish adhesive discrimination of different cell types.

The coated surfaces, shown schematically in Figure 1, contain polycation chains, here homopolymer PLL (poly-L-lysine), physisorbed to the negative silica surfaces of microscope slides. Flow deposition enables precise tuning of homopolymer PLL deposition at a desired level, typically below that of surface saturation. Accessible cationic charge is concentrated only in the vicinity of the adsorbed polycation chains, which are randomly distributed on the surface.¹⁶ Thus the surfaces contain electrostatic heterogeneity, with clustering of positive

charge in the regions near the polycation chains. The polycation adsorption is relatively low and insufficient to reverse the negative overall zeta potential of the surfaces.

The surface area not covered with polycation chains is later backfilled with a nonadhesive polymer brush, here polyethylene glycol (PEG). Here the PEG brush is created through the adsorption of a PEG-PLL graft copolymer. Notably, the cationic PLL functionality which serves as the anchoring component of the adsorbing copolymer is established to be inaccessible to cells and proteins in solution, so that the homopolymer PLL is the predominant interactive cationic surface functionality.¹⁶

Characterization of surfaces containing cationic species and PEG brushes form the basis for our understanding of the current surfaces. In the direction perpendicular to the engineered surface, the attractions and repulsions are of similar range and therefore competitive. The random in-plane positioning of adsorbed PLL chains produces a random positioning of accessible nanoscopic “clusters” of cationic functionality. The total numbers of these cluster per area is, importantly, quite precise, producing a well-characterized average cluster spacing.^{16, 17} Thus, while charged clusters are arranged randomly, length scales emerge that render adhesion sensitive to curvature^{18, 19} and distributions of interactive features on the cells themselves. The physical²⁰ and protein-interactive^{16, 21, 22} features of the surface libraries in the current work have been well studied: coating molecules remain immobilized on the support over a broad range of ionic strengths beyond the current conditions, the adsorbed polymers have well-characterized surface loadings and thicknesses, the PEG brushes are entirely repellent to an array of proteins, and the polycations are established to lie flat (1–2 nm) at the base of the PEG brush, even when exposed to challenging species (excepting certain additional polycation homopolymers). Further employing synthetic polymers (with control over molecular weight and copolymer composition), the platform offers potential additional advantages over antibody-coated surfaces, including lower cost, scalability, and shelf life. The coatings in this investigation are demonstrated on planar surfaces but could be more broadly applied on microfluidic surfaces¹⁸ or microparticles.²³

This study employs cell line models for cells potentially found in breast milk: MCF-10A (a non-tumorigenic model of healthy human breast cells), MCF-7 (human breast cancer employed as a model breast cancer cell and model circulating tumor cell^{24–26}), and TMX2–28 (a Tamoxifen selected clone of MCF-7 and therefore resistant to this cancer drug), and Jurkat lymphocytes (a model for immune cells present in milk, interfering with epithelial nucleic acid-based risk and cancer assessment²⁷). These cells are suspended in buffer and spiked into human breast milk.

The surfaces in the current work advance our prior surface designs targeting breast cell capture.²⁸ We previously compared the capture of the closely related MCF-7 and TMX2–28 cell lines from buffer on surfaces that presented adhesive cationic charge clusters on a background surface that was anionic and substantially electrostatically repulsive. These surface designs were able to adhesively discriminate the two closely-related cell types, with selectivity that was surprising: There was a 7:3 preference for capture of TMX2–28 cells over the MCF-7 cells, translating to a selectivity, S , of 2–2.5 (S is defined as the ratio of target to nontarget cells on the surface, normalized by the same ratio in solution). This was

an extremely encouraging result, since the basis for cell capture was not affinity (biomolecular recognition)-based. We demonstrated that the undesirable capture of MCF-7 cells was a result of fouling/ non-specific surface interactions, which if could be eliminated, would produce greater discrimination. Therefore the surfaces in the current report employ a PEG brush to provide steric rather than electrostatic repulsion that competes with the cell-attracting clusters of cationic charge. The competition between steric repulsions and electrostatic attractions is a different strategy than the use of PEG brushes to prevent protein adsorption and tethering molecular targets at the brush edge or forward of the brush for efficient capture.²⁹

This work addresses capture of the individual cell lines, compares the behavior of single cell suspensions to cell mixtures, demonstrates the impact of milk on cell capture, and reports sharp selectivity for a mixture of two cell types, all employing an antibody-free surface coating.

Experimental Materials and Methods

Cell preparation.

Three human breast epithelial cell lines (MCF-10A, MCF-7 and TMX2–28), and one human T lymphocyte cell line (Jurkat) were maintained in a 37°C humidified-incubator at 5% CO₂. The MCF-10A, MCF-7 and Jurkat were obtained from ATCC, and the TMX2–28, a Tamoxifen-resistant clone of MCF-7, was a gift from John Gierthy. The culture medium and maintenance protocol for the breast cancer cell lines, MCF-7 and TMX2–28, were previously described.²⁸ MCF-10A was cultured in complete growth medium (MEGM cat # CC-3150 from Lonza/ Clonetics Corp. plus 10 ng/ mL cholera toxin). All 3 breast cell lines were grown as attached cultures in T-75 flasks, re-fed every 3 days and sub-cultured or prepared for experiments when 80 – 90% confluent using enzymatic treatment. Briefly, media was removed, the cell monolayer was rinsed with phosphate buffered saline and 2 mL of 0.25% (w/v) trypsin/ 0.53 mM EDTA was added and the flask was placed in the incubator for 5 minutes after which the trypsin was neutralized with complete growth medium. A cell pellet was obtained by centrifuging for 5 min at 200 x *g*. Jurkat cells were cultured in the complete growth medium (RPMI-1640 plus 10% fetal bovine serum and 2 g/ L sodium bicarbonate) as floating cultures in T-75 flasks, re-fed every 3 days and sub-cultured before reaching 3 million cells/ mL.

When preparing cells for experiments, the cell pellets were resuspended in phosphate buffered saline (PBS), a small aliquot was diluted 1:1 with Trypan Blue and live and dead cells were counted in a hemocytometer to determine cell viability. Only suspensions with greater than 95% viable cells were used in experiments. Cells were brought to a concentration of 1.25×10^6 cells per mL in PBS (unless noted) and used in an experiment within one hour.

Breast milk.

Human breast milk was collected after receiving University of Massachusetts-Amherst Institutional Review Board approval. For these studies frozen milk was thawed and 30 mL

aliquots were centrifuged at 1771 x g for 10 min at 24°C to pellet the cells. After centrifugation the top fat layer was removed and the liquid milk (serum) was carefully collected without disturbing the cell pellet. This cell-free and fat-reduced milk was used in experiments to study 1) the adsorption of milk constituents to the test surfaces, and 2) to study the behavior of MCF-10A and Jurkat cells in milk. In the latter case, cell-free milk was spiked with cells in PBS. In both cases the milk was diluted 1:1 with PBS.

Copolymer synthesis.

We synthesized PEG-PLL graft copolymer, one of the two polymers comprising the coating, in house, following established methods.^{16, 20, 30} Briefly, Poly-L-lysine hydrobromide (PLL) with a nominal molecular weight 15K-30K (Sigma Aldrich) was dissolved in 50 mM pH 9 borate buffer. An appropriate amount of PEG-SVA (polyethylene glycol succinimide valerate, Laysan Bio, Inc.), targeting 35% functionalization of NH₂ groups on the PLL, was added and the solution was stirred for 6 h. The solution was then dialyzed against pH 7.4 phosphate buffered saline for 24 hours and then against DI water for another 24 h before freeze drying and storage at -20°C. The grafting ratio, defined as the number of PLL monomers to PEG side chains, was found to be 3.5 as determined for solutions in D₂O, characterized by ¹H NMR. The polymer composition was determined by the lysine side chain peak (-CH₂-N-) at 2.909 ppm and the PEG peak (-CH₂-CH₂-) at 3.615 ppm.

Coating preparation.

Monolayer coatings were applied in two steps, following established methods,²⁰ to FisherFinest (Fisher Scientific) microscope slides that had been prepared by soaking overnight in concentrated sulfuric acid and then thoroughly rinsed in deionized (DI) water and dried in nitrogen. After sealing in a laminar shear flow chamber, where the slide comprised one wall, flowing pH 7.4 phosphate buffer (0.002 M KH₂PO₄ and 0.008M Na₂HPO₄) was introduced, followed by introduction of a 5 ppm solution of Poly-L-lysine hydrobromide (PLL, MW 15K-30K, Sigma Aldrich) in the same buffer. After a chosen number of minutes of flowing PLL solution, depending on the desired amount of immobilized PLL, the flowing buffer was reintroduced for ~5 minutes. This was followed by introduction of a 100 ppm PEG-PLL solution in the same buffer, to form a saturated brush on regions of the surface on which PLL had not been immobilized, and reintroduction of the flowing buffer. The sequential processes of PLL and PEG-PLL adsorption were tracked using a custom-built near-Brewster reflectometer. Additionally a calibration curve enabling a precise amount of PLL deposition for a controlled PLL solution flow time was established using near Brewster reflectometry,³¹ with resolution and reproducibility as fine as 0.01 mg/m² of PLL chains on the surface.²³ This method of depositing PLL for a controlled amount of time facilitates precise control over the amount of PLL per unit area and also enables complete backfilling of the remaining surface area without displacement of the PLL. The resulting coatings, brushes with embedded PLL molecules randomly positioned about the surface and adsorbed flat to the substrate, have been well characterized²⁰ to establish their monomolecular character, 15–17 nm thickness, complete resistance to removal upon exposure to a range of protein solutions and ionic strength conditions beyond those in the current work. All coating molecules were adsorbed employing a wall shear rate of 5 s⁻¹.

Cell adhesion studies, from milk or buffer.

Cell capture was studied in the same flow chambers in which the coatings were prepared. Following coating deposition, the flow was switched to the buffer of choice for the study, and the wall shear rate was increased to 22 s^{-1} . The buffers included physiological phosphate buffered saline (PBS, 0.002 M KH_2PO_4 , 0.008M Na_2HPO_4 , and 0.15 M NaCl); or buffers with a lower ionic strength but identical osmotic pressure (0.002 M KH_2PO_4 , 0.008M Na_2HPO_4 , and $\sim 0.25 \text{ M}$ sucrose). Then a suspension containing 1.25×10^6 cells/mL in the test buffer of interest (or human breast milk, described above) was flowed through the chamber and cell capture recorded on video using a Nikon Diaphot microscope with a 10x objective to produce a $470 \mu\text{m} \times 350 \mu\text{m}$ field of view. Captured cells were tested with Trypan Blue stain to confirm that, in the minutes following capture, they were still viable. Videos were analyzed using Image J to determine the cells on the surface as a function of time. When reporting the number of cells captured per area in a given run, the cells per area in the test run was normalized by the cells per area captured on a saturated PLL surface. Cell capture on PLL was previously established to occur at a rate proportional to the cell concentration in the suspension. In this way, each test run was calibrated to normalize data to eliminate the impact of modest variations ($\sim 10\%$) in bulk solution cell concentration.

Reflectometry to assess adsorption from milk.

A custom-built reflectometer, described previously³¹ and used frequently by our lab to quantify polymer and protein adsorption from flow with a resolution of 0.01 mg/m^2 ,^{16, 32} was employed to quantify adsorption of proteins and other species from milk on test surfaces. The same flow chambers employed to deposit the coatings were employed in studies of adsorption of molecules and, potentially fat globules from human breast milk that was diluted by half in phosphate buffered saline. As the diluted breast milk was flowed through the chamber, the change in intensity of a laser beam, reflected inside the substrate, was monitored in time. The laser beam was parallel polarized and with its incident angle at the Brewster angle, the only back reflected intensity was due to the coating itself, which was well quantified. Additional increases in signal indicated adsorption of unknown species, likely to include proteins, from milk. Since the identity of the adsorbing compounds was unknown, the adsorbed mass per area was estimated using a calibration constant for fibrinogen^{32, 33} which assumes that the material adsorbing from milk has a similar refractive index. We have found that all the proteins and polymers we have studied have layer refractive indices within about 10% of each other,^{16, 33} so that this approach provides a reasonable estimate here.

Distinguishing epithelial cells on surfaces containing multiple cells types.

In studies to quantify selective cell capture, the numbers of MCF-10A cells on surface that also contained Jurkat cells was determined by the application of a fluorescent antibody against EpCAM. After cells were captured from a run using a mixed suspension of MCF-10A and Jurkat cells, buffer was flowed through the chamber until cells were cleared from the free solution in the chamber and the lines. Video monitoring during this step ensured that adhered cells were retained. The chamber was then opened, the slide was removed, and cells were fixed on the surface using a standard method of cold acetone,

methanol and buffer. 2 μ L of FcR blocking reagent (Miltenyi Biotech), followed by 50 μ L of FITC- antibody to EpCAM (CD326, Miltenyi Biotech) was added to each cell. After additional processing with phosphate buffered saline and application of Fluoromount G mounting media medium (Southern Biotech) and drying, slides were imaged via fluorescent microscopy with excitation at 488 nm.

Results

Characterization of cells

Expecting electrostatic interactions to play a significant role in cell capture, we measured the zeta potentials of the different cell types in PBS and in phosphate buffer, where the latter lacks the NaCl component of PBS and therefore has a lower ionic strength. As a result of the greater Debye length ($\kappa^{-1} = 2$ nm) for the phosphate buffer compared with the PBS ($\kappa^{-1} = 1$ nm), the zeta potentials of all the cell lines are slightly more negative in phosphate buffer. This effect results from the impact of added salt on the near-surface the ion distribution relative to point of zero shear. Additionally depending on the groups present, a secondary impact of added ions may shift the underlying surface potential due to the impact of ions on the dissociation of weak acid groups.

Additionally, cell viability was assessed with Trypan Blue staining. We found no impact of processing in in flow on the cell viability, which was always in excess of 95% and captured cells were additionally viable at the short times of this study, within minutes of their adhesion to capture surfaces.

Electrostatic vs Steric Brush Competition in Cell Capture

Cell capture studies were conducted by flowing suspensions containing $\sim 1.25 \times 10^6$ cells/mL over the test surfaces, which were mounted as one wall of a laminar slit shear flow chamber. The wall shear rate was 22 s^{-1} .

Figure 2 shows, as an example, raw data for 2 cell capture runs with MCF-10A cells in phosphate buffered saline. In one run the surface contains an adsorbed PEG-PLL layer which forms a non-adhesive PEG brush, and in the other, the surface contains a cell-adhesive layer of adsorbed homopolymer PLL. Both cases serve as controls. The cell counts from the Image J analysis of video data comprise the main figure, while 3 video frames of cell accumulation are included to provide perspective as to the appearance of the surface as cells are captured. A lack of cell accumulation on the PEG-PLL-based PEG brush surface confirms the intended non-adhesive character of this surface and further validates that, in this study, as was the case in previous studies, PEG-PLL adsorbs to form a cell-repulsive PEG brush. The PLL-coated surface, intended to be substantially electrostatically adhesive, captures cells in a linear fashion as a function of time. Besides confirming the intended adhesive nature of the PLL coating, these data demonstrate that in this range of cell numbers on the surface, the adhered cells negligibly affect the capture of additional cells. Thus the rate of cell accumulation, for instance on the PLL coating, provides a means of assessing cell-surface interactions without interference from cell-cell-interactions. Also, Figure 2 establishes that, at least up to 200 cells in the field of view, cell capture is controlled

exclusively by pairwise cell-surface interactions. This result defines a quantitative working range for further studies, below.

Role of the Density of Electrostatic Features and Range of Electrostatics in Cell Capture

The surfaces in this work capture cells through electrostatic attractions between PLL chains on the engineered surface and negatively charged groups on the surfaces of flowing cells, as evidenced in the experiments of Figure 3. Here, for single cell-line suspensions of MCF-10A cells in part A and for Jurkat cells in part B, the rate of cell capture is summarized for a series of up to 18 different surface types, varying the numbers of PLL molecules per area on the x-axis, and for different concentrations of electrolyte, for the different data sets. Figure 3C summarizes the capture of cells from 4 different lines on these different surfaces, in PBS. All surfaces are backfilled with a PEG brush following adsorption of the different PLL amounts. The y-axes report cell capture rates, calculated from the slopes of the cell accumulation data like those in Figure 2.

For a given cell type and a given electrolyte concentration in solution, increasing the surface content of PLL increased the cell capture, demonstrating that, in general, cell capture occurred as a result of interactions with PLL. The impact of PLL surface loading on cell capture, for fixed cell type and solution conditions, exhibited a characteristic sigmoidal shape. Without any PLL chains on the surface or with a low PLL loading, cells were sterically repelled from a PEG-PLL-dominated surface, as shown on the left-most side of data in Figure 3 and in the example in Figure 2. At the opposite extreme, with large amounts of PLL and proportionately smaller amounts of PEG-PLL, the rate of cell capture reached a common maximum level, associated with the transport-limited rate, observed to be about 30 cells per minute in the area of observation. At intermediate surface compositions the rate of cell capture is controlled by the surface composition.

A notable feature for a series of surfaces studied with a given cell line and electrolyte concentration is the presence of an adhesion threshold, or a minimum PLL surface loading required for cell capture. This is highlighted in Figure 3B but occurs for all cell lines and solutions studied. Surfaces having PLL loadings below the threshold were ineffective in capturing cells. The presence of adhesion thresholds in the summary curves of Figure 3 suggests that individual immobilized PLL homopolymer chains, within the PLL-PEG backfill, act as clusters of cationic charge that are too weakly binding to adhere cells from flowing suspensions. (If individual PLL chains could capture cells, we would have seen that any surface containing PLL chains could capture cells, albeit at very slow rate. In this case data would trend towards the origins in Figure 3 rather than the x-axes.) Instead, when cells are captured, it is through the action of multiple PLL chains on the engineered surface engaging a target cell in the initial contact.

While increased cell capture with greater PLL surface content demonstrated that immobilized PLL was responsible for cell capture, the influence of electrolyte concentration, which varied among the different curves in Figure 3 A and B, establishes that the attractions are electrostatic in nature. Cell accumulation is more rapid from suspensions of low electrolyte concentration (and large Debye length, $\kappa^{-1} = 4$ nm,) where electrostatic interactions are longer range. In Figure 3 A and B a few nanometers difference in the range

of electrostatic interactions has a substantial influence on cell capture rate, and the electrolyte variations also produce different adhesion thresholds, providing a means to tune the onset of cell adhesion. Worth noting in PBS, NaCl is present in addition to the buffering ions. In phosphate buffer without the additional NaCl ($\kappa^{-1} = 2$ nm) or with a more dilute solution of the buffering ions ($\kappa^{-1} = 4$ nm), the pH is still fixed at 7.4. To maintain a fixed osmotic pressure of ~250 mOsm for the cells, sucrose has been added to the phosphate buffers but not the PBS.

Evident in Figure 3C for the four cell lines MCF-10A, MCF-7, TMX2-28, and Jurkat, the general sigmoidal shape is always seen, but there is a spreading of the capture rate data depending on the cell line. In particular the different cell lines exhibit different adhesion thresholds but the ultimate maximum capture rate is always near 30 cells/min in the field of view, a result of the nearly similar cell sizes. In general the epithelial cancer cell lines were more adhesive to a given surface than were cells of the non-tumorigenic MCF-10A line and the Jurkat cells. Also capture rates of the related two cell lines, TMX2-28 and MCF-7 were more nearly similar than the capture rates of the other cell lines over a broad range of surface compositions, possibly a result of the similarities of the cell lines, with TMX2-28 a tamoxifen-selected clone of MCF-7.

Adsorption of milk components.

Towards capture of cells from breast milk, we employed optical reflectometry to estimate the adsorption of molecular components from milk onto engineered surfaces and to identify engineered surfaces that were repellant to molecular components of breast milk. This study employed human breast milk from which cells were removed by centrifugation.

Additionally, the layer of fat that remained on top of the main fluid was skimmed away to avoid clogging of microfluidic lines. The milk was then diluted 50% in PBS to fix the pH at 7.4, in anticipation of cell capture studies from milk that could be compared to those from buffer. Figure 4 summarizes adsorption from milk on a series of surfaces over which the PLL loading has been varied and the remaining surface backfilled with PEG-PLL. While Figure 4 has the appearance of Figure 3, it should be noted that the y-axis in Figure 4 is an adsorbed mass per unit area, employing a protein-based calibration constant (fibrinogen/albumin) to convert the raw signal to an adsorbed mass. By contrast the y-axis in Figure 3 corresponds to the accumulation rate of captured cells, a fundamentally different quantity that is not readily compared. As milk contains proteins, predominantly casein, in addition to fats, lipids and other molecules, the adsorbed layer may be complex and reporting the adsorbed mass in equivalent protein mass units provides an estimate.

Figure 4 demonstrates that (1) there is no detectable adsorption of molecular species from milk on the uniform PEG brushes or PEG brushes containing low amounts of homopolymer PLL; (2) an adhesion threshold of ~4200 PLL molecules / μm^2 or 0.14 mg/ m^2 homopolymer PLL on the collecting surface (for these particular PEG brushes) is required before adsorption from milk is observed; and (3) at high PLL loadings on the surface, the adsorbed amount of material is ~2 mg/ m^2 , which is consistent with a saturated layer of protein such as albumin, fibrinogen, or casein.³³ Indeed, with an estimated protein content of ~1% in human breastmilk,³⁴ the milk in this study contains 5000 ppm protein, exceeding that in many

studies employing buffered solutions of test proteins.^{33, 35, 36} Also, the ability of PEG-PLL brushes, including those with low levels of additional PLL to resist adsorption from milk provides evidence that milk does not remove or damage the polymer brush. Removal of the brush exposes glass and additional PLL chains known to adsorb protein.²⁰ The ability of PLL-containing surfaces to repel milk molecules and to remain robust in milk as shown in Figure 4, is a requirement for a collecting surface that is active in human breast milk.

Adsorption of cells from milk

Figure 5 presents the capture of MCF-10A and Jurkat cells from 50-% diluted milk serum in buffer from which the native cells had been removed by centrifugation. Cells from the MCF-10A and Jurkat cell lines were added, separately, at a concentration of 1.25×10^6 / mL. The fixed pH and known cell concentration was designed to facilitate comparison with Figure 3. In Figure 5, ten PLL loadings on the collecting surface were studied with milk containing either MCF-10A or Jurkat cells, focusing on surface designs (PLL loadings with PEG-PLL brush backfill) below the threshold for milk adsorption in Figure 4. In this way, Figure 5 addresses cell capture onto surfaces that do not allow the adsorption of the molecular species in breast milk.

Figure 4 demonstrates that when cells are added to milk, their capture conforms to the same qualitative features seen for capture from buffers in Figure 3. Critical features, including a lack of adsorption onto uniform PEG-PLL brushes and PEG-PLL brushes containing low levels of PLL homopolymers, are preserved. Above a threshold in the surface loading of PLL homopolymer chains, cells adhere, and the specific PLL loading corresponding to the adhesion threshold is dependent on the cell type.

Given that the molecular components of milk do not adsorb to the collecting surfaces of Figure 4, it is remarkable that the adhesion thresholds observed for MCF-7 and Jurkat cell capture from milk differ from thresholds observed when buffer solutions are employed Figure 3. There is a ~20% lower loading of PLL on the collecting surface needed for capture of either cell type, reflecting the greater adhesion of cells from milk. Possible explanations include differences in osmotic pressure of the milk from that of PBS or depletion forces. Variations in ionic strength giving Debye lengths in the range of 1–2 nm are, however, insufficient to explain the larger differences seen between cell capture from milk and from PBS. An alternate explanation involves the milk proteins themselves potentially adsorbing onto the surfaces of the cells. Cells in breast milk may have milk proteins and other molecular species adsorbed onto the outer surface of the cell, affecting cell capture. This would not be the case for cells grown in culture and suspended in buffer.

Competitive Adsorption from Cell Mixtures: Selective Cell Capture

The cell capture studies in Figures 2, 3, and 5 employed suspensions each containing cells from a single cell line. To address the potential for engineered surfaces to selectively capture one cell type from a cell mixture, we conducted cell capture from 50–50 mixtures of MCF-10A and Jurkat cells with an overall cell concentration of 1.25×10^6 /mL in PBS. This pairing was motivated by interest in separating healthy epithelial cells from lymphocytes, for downstream nucleic acid analysis. In these studies, after exposing test surfaces to flowing

suspensions for 10 minutes, captured cells were identified and counted. To distinguish the types of captured cells, flow chambers were opened after cell capture, and adherent cells were subjected to antibody staining which rendered the MCF-10A fluorescent. Control studies with MCF-10A or Jurkat cells captured on PLL-coated surfaces from single-cell type suspensions confirmed a lack of fluorescence from the Jurkat cells and fluorescence from the captured MCF-10A cells.

For mixtures of targeted MCF-10A cells and competing Jurkat cells, Figure 6 compares the adhesive selectivity of two test surface compositions containing 2500 and 3500 PLL molecules / μm^2 with that of nonselective control surfaces containing a saturated adsorbed layer of PLL. Three surfaces of each type were exposed to buffered cell mixtures and 12 images ($470\text{ }\mu\text{m} \times 350\text{ }\mu\text{m}$) were taken from each. Figure 6 summarizes the resulting selectivity, defined as the ratio of MCF-10A to Jurkat cells counted on the surface normalized by the same ratio (1:1) for the cells in free solution. The selectivity on the two test surfaces is remarkable given that antibody targeting was not employed to capture cells. On the surface functionalized with ~ 2500 PLL molecules/ μm^2 the average selectivity was 61, indicating capture of only one Jurkat cell for every 61 MCF-10A cells captured. For some images, such as the example in Figure 7, the micrographs contained only MCF-10A cells (producing $S = \infty$). Here a selectivity used in Figure 6 was calculated as though the next cell to be captured would be a Jurkat cell, and thus the reported selectivity represented a lower bound for the surface containing a nominal loading of 2500 PLL molecules/ μm^2 . As a consistency check, the nonselective PLL functional surface was examined and found, as expected to have a selectivity of unity, meaning that cells were captured on the surface in proportion to their composition in the bulk solution.

Related to the mechanism of selectivity and design strategies targeting for suspensions with different bulk solution concentrations and proportions, the results in Figure 6 are consistent with the expected selectivity calculated based on the capture from solutions containing single cell lines in Figure 3C. The proportions of cells captured on selective surfaces is well described by the relative capture rates of cells from suspensions of single cell lines. For instance, on the engineered surface containing 3500 PLL chains / μm^2 , Figure 3C suggests that MCF-10A cells will adsorb at a rate of ~ 11.5 cells/field min while Jurkat cells will adsorb at a rate of 2.5 cells /field min. The ratio of these rates is 4.6, matching the observed selectivity quite well. This error in predicting selectivity becomes large for the more selective surfaces, for instance, that contain 2500 PLL molecules/ μm^2 . Since the selectivity is calculated as the ratio of targeted/nontargeted captured cells, it diverges as the numbers of non-captured cells approach the adhesion threshold. This is not a particular problem as one expects that for most applications selectivities exceeding 50 would constitute excellent performance and the difference between a selectivity of 100 or 1000 would not be of concern.

The consistency of the observed selectivities with the single cell line capture rates indicates that adhering cells do not influence each other in the range we study here, up to at least $450,000\text{ cells/cm}^2$. This behavior is to be expected since the capture of cells is designed to occur through surface-cell interactions. Without interference between cells, as evidenced by the linearity of the capture kinetics, in the example of Figure 2, the results of the summary

plots in Figures 3 and 5 for single cell type suspensions are predictive of behavior of capture of cells from multi-cell type mixtures.

Discussion: Significance and relationship to current technology

Advances in cell capture and in the compatibility with breast milk.

This study demonstrated how coatings can be designed to selectively capture flowing cells based on physicochemical rather than biomolecular interactions. The result comprises a substantial advance for the field of epithelial cell capture and purification: Reliance on adhesion without biomolecular recognition allows capture of targeted cells with low levels of common surface markers such as EpCAM, and the use of synthetic polymers reduces costs, adds scalability, and increases the shelf life and stability of devices with these coatings. The compatibility and non-fouling character of these coatings in breast milk and their ability to adhesively distinguish different cell lines in the presence of breast milk proteins, dissolved fats and other dissolved species is also an important capability. Indeed, this work constitutes the first study of which we are aware, targeting a scalable approach for breast milk analysis for the health of the mother. It is also significant in demonstrating the compatibility of engineered PEG brushes with human breast milk.

Relation to other challenges.

In technologies relying on adhesive specificity for separation of molecular and cellular targets, the design of selective surfaces is paramount; however, other issues can limit overall performance. Separate from the selective adhesion of a surface is the transport of species from solution to the surface itself. Depending on whether the goal is harvesting a large number of rare cells, the selective capture of a purified species, or the purification of the remaining suspension, there is varied importance of contacting every cell with the surface to allow an opportunity for adhesion. Efficient fluid handling and transport schemes are therefore being developed in other research disciplines.

Mechanism.

The surfaces in this work capture cells through electrostatic attractions between cationic surface features and the negatively-charged cell surface, as was established through the ionic strength dependence of cell capture in Figure 3. The zeta potentials in Table I, indicative of the average negative charge on anionic cell surface groups, are identical across cell lines within experimental error. It is therefore unlikely that the cells could be distinguished exclusively based on their average surface charge. The substantial separation of MCF10A and Jurkat cells in Figures 6 and 7 indicates that though electrostatics drive adhesion, additional factors facilitate adhesive discrimination. Further only a slight difference in the adhesive traces (for example in Figure 3C for MCF10A versus Jurkat cells) are needed for a sharp separation. While the adhesion profiles of MCF10A and MCF 7 for instance are closer, there are still sufficient differences between these and other cell line pairings to accomplish adhesion –based separations.

Besides the attractions driven by the immobilized PLL homopolymer, the PEG brush, while preventing surface fouling by proteins and cellular material, provides the additional function

of a long range repulsion (8–10 nm) against approaching cells. With the cationic PLL homopolymer chains immobilized on the substrate and the PEG brush backfilled around PLL molecules, the PLL chains are accessible only when the negative cell surfaces approach within a Debye length of a PLL molecule. This requires compression of the PEG brush or the cell in Figure 1 (or penetration of negative groups protruding from the cells outer surface into the brush, all energetically costly). The competition between the electrostatic attractions and the steric repulsion (PEG brush compression) is a key feature of the surface designs. Discrete attractions (here from clustered polycation functionality on the PLL) working against a uniform repulsive field (here the steric repulsion) have been shown to produce curvature based selectivity,^{18, 37} a behavior which may come into play here.

Conclusions

This study demonstrated a coating design strategy that involved two types of surface chemistry: cationic functionality, attractive to all the cells in the suspension and a PEG brush functionality, which was sterically repulsive to all the components of the suspension. By clustering the cationic functionality on a polyelectrolyte chain immobilized on the surface at random positions and backfilling the remaining surface with a taller PEG brush, cells encountering the surface experienced competing electrostatic attractions and steric repulsions distributed across the area of cell-surface contact. The discrete attractions and more uniform steric repulsions produced net cell-surface interactions that were dependent on cell type and which adhesively discriminated cells. This adhesive discrimination was born out in the different rates of cell capture for different cell types and in a selectivity for one cell type over another when suspensions contained more than one cell type. Linearity in cell capture with time, up to cell-surface loadings of 4×10^5 cells/cm², indicated that capture was governed exclusively by cell-surface interactions (rather than cell-cell interactions on the surface) and so the capture rates for individual cell types were quantitatively predictive of the capture rates of cells in mixed suspensions and of the selectivity itself.

A unique feature of these surfaces was their demonstrated ability to adhesively discriminate different cell lines of similar size and nearly identical zeta potentials, even though the driving force for adhesion was electrostatic. The likely explanation was that the competition between the electrostatic attractions and steric repulsions from the remainder of the surface was different for the different cell types. With purely electrostatic attractions, these cells adhered and discriminated cells without reliance on biomolecular selectivity and could be powerful for capturing cells that lack EpCAM.

The surfaces were shown to be compatible with breast milk: No molecular species adsorbed to the coating surfaces in the surface composition range of interest, a behavior achieved by employing a protein-repulsive PEG brush and by not incorporating cationic functionality that was too dense. The coatings were able to adhere targeted cells and reject the other components of breast milk, most importantly retaining their ability to adhesively discriminate cells and to accomplish cell capture based exclusively on cell-surface interactions in the presence of milk but not subject to interference between different cell types.

This work is the first report of cell capture directly from human breast milk and its significance therefore lies in potential applications that screen for disease based on the captured cells. This work is the first of which we know to demonstrate the compatibility of PEG brush coatings with human breast milk. The use of homopolymers and relatively unsophisticated block copolymers as opposed to biomolecular recognition agents (nucleotides, antibodies) favors economical and scalable production and application of coatings in array of devices.

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Figure 1.

Schematic of cell capture, showing competition between electrostatic attractions and steric repulsions. Engineered surfaces contain homopolymer PLL random coils adsorbed flat to the surface, backfilled with a PLL-PEG graft copolymer to form a PEG brush around the adsorbed PLL homopolymer chains.

Figure 2.

Example data for capture of MCF-10A cells on two control surfaces: on a non-adhesive PEG brush formed by the adsorption of PEG-PLL copolymer on the glass slide and on an adhesive saturated adsorbed layer of PLL (0.4 mg/m^2). For the latter, 3 video frames showing captured cells at different points of the run are included. The dimensions of each micrograph are $470 \text{ } \mu\text{m} \times 350 \text{ } \mu\text{m}$. The cell concentration was $1.25 \times 10^6/\text{mL}$ and the flow rate was 22 s^{-1} .

Figure 3.

Summary of cell capture kinetics on surfaces containing different amounts of PLL homopolymer molecules, on the x-axis, and showing the impact of electrolyte concentration for (A) MCF-10A and (B) Jurkat cells. In (C), cell capture is compared for 4 cell lines, studied individually in PBS (having $\kappa^{-1} = 1$ nm). 4 different cell lines are: Jurkat, MCF-10A, MCF-7, and TMX2-28. Representative x-axis error bars indicate experimental precision for different PLL loadings, and representative y-axis error show the range of data for 2-3 runs. Curves are drawn to guide the eye. The maximum PLL loading of 12000 molecules/ μm^2 corresponds to 0.4 mg/ m^2 .

Figure 4.

Adsorption of molecular components of buffer-diluted (by half) milk onto 8 engineered surface compositions. Error bars show precision for different PLL loadings and a line is drawn to guide the eye.

Figure 5. Capture of MCF-10A cells (green circles) and Jurkat cells (hollow squares) from flowing suspension in human breast milk, diluted 50% in PBS. Error bars indicate experimental precision and curves are drawn to guide the eye.

Figure 6.

Average selectivity values as a function of PLL loading, measured on 3 surfaces of each type and 12 images for each surface. The numbers above the bars represent the calculated selectivities based on the single cell type experiments of Figure 3. Standard deviations for selectivity (which necessarily diverges as selectivity becomes large) are for the 3 surfaces: 2500 PLL/ μm^2 : 85; 3500 PLL/ μm^2 : 1.6; 12000 PLL/ μm^2 : 0.1.

Figure 7.

Example micrographs of two selective surfaces having 2500 and 3500 PLL homopolymer molecules/ μm^2 , respectively, and a control surface having 12000 PLL molecules / μm^2 . Each image measures $470\ \mu\text{m} \times 350\ \mu\text{m}$. Left column for each surface is phase contrast. Middle column is fluorescent, with MCF-10A cells fluorescing and Jurkat cells not fluorescent. Right column superposes phase contrast and fluorescent images.

Table I.

Cell Properties

Cells	Diameter, μm ¹	Zeta Potential in PBS, mV ²	Zeta Potential in phosphate buffer, mV ²
MCF-10A	12.1 – 16.6	-12 ± 3	-16 ± 3
MCF-7	12.4 – 16.9	-12 ± 3	-16 ± 3
TMX2–28	12.3 – 17.0	-11 ± 3	-14 ± 3
Jurkat Tcells	10.4 – 14.8	-11 ± 3	-15 ± 3

¹. Diameters were measured from images of cells based on 100 cells of each type.

². Zeta potentials represent averages of 5 trials per cell type in each PBS or phosphate buffer.