Elasticity-Modulated Microbeads for Classification of Floating Normal and Cancer Cells Using Confining Microchannels

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ABSTRACT: Engineered microbeads have a wide range of applications in cancer research including identification, characterization, and sorting of cancer cells. In particular, the microbead-based cancer identification techniques are mainly based on the known genetic or biochemical biomarkers; and detection specificity is yet to be improved. On the other hand, it has been discovered that biomechanical properties of cancer cells such as cell-body elasticity can be considered as cancer biomarkers. Here, we report a straightforward microfluidic classification scheme for floating/dissociated normal and cancer epithelial cells using a confining microchannel device together with calcium-alginate hydrogel microbeads. The hydrogel microbeads are generated based on the microfluidic emulsion process, with characterization on the process parameters (e.g., liquid driving pressure and cross-linking duration) in order to specify the resultant bead diameter and elasticity. These engineered microbeads are first mixed with a cell mixture of dissociated human nasopharyngeal epithelial cells (NP460) and nasopharyngeal carcinoma cells (NPC43). The cell elasticity can then be reflected from the locations of captured cells in the device. Experiments further demonstrate that the cell classification has a success rate of >95%. Furthermore, we performed the microbead-based cell classification on a whole blood sample containing floating human breast epithelial cells (MCF-10A) and breast cancer epithelial cells (MDA-MB-231) with a success rate of >75%, revealing its directly applicability to identification of circulating tumor cells in human blood. Together, this research demonstrates a new application of engineered hydrogel microbeads for classification of cells based on their mechanical properties.

KEYWORDS: microfluidic, microbead, elasticity, cell classification, cancer

INTRODUCTION

Engineered microbeads have been widely adopted in cancer diagnosis and other biomedical applications such as drug delivery and tissue regeneration. They can function as micro/nanoparticles as affinity-based cell carriers or cell-position indicators. Their biomaterials are often engineered to integrate with biochemical biomarkers such as antibodies and aptamers. The microbeads are typically applied as an imaging accessory to enhance the biomarker signals to observe locations of target cancer cells, and to isolate cancer cells. While very effective, there are still great demands on further development of functional microbeads for extended cancer cell characterization capability.

Considering the heterogeneous genetic and phenotypic properties of cancer cells, cancer diagnosis can be achieved in the manner of single-cell analysis, in which the single-cells are obtained from chemical-dissociation of a resected tumor portion or directly from human blood. Tissue dissections have been widely used in cancer diagnosis, treatment, as well as the mechanistic study of cancer pathology, with...
various techniques recently developed such as arginine-glycine-aspartic acid magneto-optical nanoparticles and indocyanine green fluorescence. On the other hand, cancer cell properties including their metastatic tendency can be examined using the circulating tumor cells (CTCs). The presence of CTCs indicates the higher potential of establishing new colonies at other body sites through extravasation. Further, the density of CTCs in cancer patients’ blood is considered a prognostic marker, especially after surgical resection.

Although there has been a wide range of genetic and biochemical biomarkers for cancer cells, the specificity for cancer identification is yet to be improved. Importantly, it has also been discovered that the biophysical cell properties related closely to the invasiveness of cancer cells, and the cancer metastatic potential depends also on the mechanical properties of the tumor cells. It has been shown that the elasticity of cancer cells is often significantly lower compared to the normal cells. In particular, elasticity can also be considered an indicator for fluidization of malignant cancer cells transited from tumor cells. Cell-body elasticity can directly reflect deformation of a cell when it squeezes through a narrow region. For instance, breast cancer cells require a shorter time than normal breast cells to deform and enter a microchannel. To date, elasticity has already been considered as a biomarker for cancer types such as ovarian cancer and prostate cancer. Recently, it has been demonstrated that hydrogel microbeads can be applied for cell-stress sensing, which has a close correlation with the cell elasticity. Though very effective, the throughput is largely limited by the nanoindentation-based cell-by-cell probing manner and the measurement is constrained to only adherent cells. Together, development of high-throughput microbead-based techniques classifying cancer cells for their distinct elasticity, as an additional step of cancer cell identification, would help to further enhance specificity of cancer detection and characterization.

Microfluidic techniques have demonstrated its outstanding microflow control and microdroplet/microbead generation capability. Emulsion of two immiscible liquids can generate dispersing microdroplets. For example, oil and water are two immiscible phases and water droplets can be formed by the emulsion process. Microfluidics can offer precise flow control and generate a very consistent size of the emulsified microdroplets, which is a significant improvement from the conventional macroscale emulsion technique. The microdroplets can include defined compositions or be embedded with nanoparticles (e.g., quantum dots). It has also been reported that microbeads with regulated porosity can then be solidified from the microdroplets by either solvent evaporation. Alternatively, microbead solidification can be achieved via internal gelation of polymers by chemical reactions, such as ionic cross-linking of sodium alginate with the liberated Ca ions. Cross-linking of ethylene glycol dimethacrylate-glycidyl methacrylate, and gelation of aqueous pregel mixture of N-isopropylacrylamide, bis-acrylamide, and ammonium persulfate.

On the other hand, there are a number of microfluidic platforms developed for biomechanical phenotyping of floating cells. Generally, microfluidics offer rapid and high-throughput cell characterization comparing to the traditional methods such as micropipet aspiration and atomic force microscopy. For example, an acoustic trapping force has been applied to measure the drag viscous effect and estimate the cell stiffness.

The design of microchannels can help characterizing and sorting cells based on their deformability. Recently, we have reported on a confining microchannel device for characterizing normal and cancerous human epithelial floating cells of their mechanical properties such as elasticity and cytoplasmic viscosity. Further cell characteristics such as surface protein expression and migration along constrictions can also be quantified. Despite the characterization, specificity can be improved by utilizing a more representative model to convert the measured parameter to cell elasticity, and the elasticity estimate is sensitive to any variations in the experimental configuration. Hence, addition of microparticles with a reference elasticity level as the cutoff element to reveal the softer cancer cells would provide a high level of applicability in identification and classification of the cancer cells in an unknown cell extract from cancer patients.

In this work, we, for the first time, utilize engineered hydrogel microbeads as an indicator for elasticity-based classification of floating human normal and cancer cells. We examine the operation parameters of microfluidic emulsion in order to produce the microbeads with desired properties. These microbeads are then mixed with the cell samples, following by injecting the mixture into a confining microchannel device for cell classification. The lower elasticity of the cancer cells can then be reflected from locations of the captured cells. We investigate micromanufacturing of the microbeads with defined size and elasticity for classification of normal and cancerous nasopharyngeal epithelial cells. We further apply this technique for classifying normal and cancerous breast epithelial cells in whole-blood, in order to demonstration its applicability in the direct cell classification of blood, including identification of CTCs.

**METHODS AND MATERIALS**

**Fabrication.** The confining microchannel device and the micro-fluidic microbead generator used in this work were fabricated based on soft photolithography. The mold for the emulsion device was fabricated by patterning a 20 μm-thick layer of negative photore sist (SU-8 2010, Microchem) on a silicon wafer by photolithography. For the confining microchannel mold, a layer of 50 μm thick positive photore sist (AZ50XT, AZ Electronic Materials) was patterning on a silicon wafer by photolithography. Then, deep reactive ion etching was applied to etch the uncovered silicon regions for a depth of 50 μm, followed by removing all the residual photoresist using acetone. Both molds were then deposited with a molecular layer of trichloro (1H,1H,2H,2H-perfluoro-octyl) silane (Sigma-Aldrich) to facilitate substrate release from them in the later process.

In the later fabrication steps, both devices basically shared the same fabrication process. Polydimethylsiloxane (PDMS) prepolym er (Sylgard-184, Dow Corning) was prepared by mixing the PDMS monomer with a 10% volumetric ratio of the curing agent. After degassing in a vacuum chamber and pouring the prepolymer onto the mold masters with a thickness of 3 mm, the masters were baked in an oven at 80 °C overnight for thorough cross-linking of PDMS. The cured PDMS substrate was torn from the mold, and the unwanted PDMS outside the device boundaries was chopped away. We then punched holes at the liquid inlets and outlets. The diameters of these holes were typically 1 mm, except that the outlet of the microbead generator was 5 mm, acting as the microbead collection chamber. Afterward, the PDMS substrate was bonded onto a glass slide using oxygen plasma (Plasma Prep II, SPI Supplies) treatment (energy, 10 kJ). Finally, each fabricated device was baked in an oven for another 6 h to enhance the plasma bonding strength. Stainless steel adaptors (New England Small Tube) and Tygon tubing (Cole-Parmer) were then inserted into the device inlet/outlet for fluidic connections. Before experiments, the device with tubing was exposure under...
ultraviolet light for >2 h in a tissue culture hood for sterilization. Surface treatment was then applied to avoid unwanted cell adhesion by injecting pluronics F-127 into fabricated device for 30 min before experiments.

**Cell Culture.** An immortal human nasopharyngeal epithelial cell line (NP460) and a nasopharyngeal carcinoma cell line (NPC43) were developed by the research team of S. W. Tsao, from cell extracts of nasopharyngeal cancer patients. NP460 cells were maintained in the 50% of complete Eplife medium (Thermo Fisher Scientific), 50% of complete Defined Keratinocyte-SFM (Thermo Fisher Scientific), 100 units/mL penicillin and 100 μg/mL streptomycin. NPC43 cells were maintained in RPMI-1640 (Sigma) added with 10% fetal bovine serum, 4 μM Y27632 dihydrolchoride (Alexis), 100 unit/mL penicillin, and 100μg/mL streptomycin. A human breast cancer cell line MDA-MB-231 was cultured with high-glucose Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA), 10% fetal bovine serum (Atlanta Biological, Atlanta, GA), and 100 units/mL penicillin. A noncancerous breast epithelium cell line MCF-10A was cultured with the Mammary Epithelial Cell Growth Medium SingleQuots Kit (MEGM; CC-4136, Lonza, New York City, NY). All the cell types were cultured in an incubator at 37 °C, saturated humidity and 5% CO2 in air. Cell passage was performed once the cell population reaches ~80% confluence. In the experiments, 0.25% trypsin-EDTA was applied to extract the cells. Each cell type was diluted to a sufficiently low density (10^4 cells/mL) for avoiding any cell aggregation in the microfluidic device in the following experiments.

**Cell Staining.** Vybrant CFDA SE dye (Thermo Fisher Scientific) with a concentration of 1 μL/mL was added in cell culture media for 10 min to stain NPC43 cells or MDA-MB-231 cells, followed by replacing the dye solution with fresh media. Likewise, Hoechst 33342 (Thermo Fisher Scientific) with a concentration of 0.1 μg/mL in phosphate buffered saline (PBS) was applied to stain NP460 cells or MCF-10A cells for 10 min, followed by replacement of fresh media.

**Whole-Blood Sample.** Anticoagulated bovine whole blood samples were purchased from Hongqiang Bio, Guangzhou, China. Image Capture. Microscopic images of the cells and microbeads were captured under an inverted fluorescence microscope (TE300, Nikon) equipped with an sCMOS microscope camera (Zyla 4.2, Andor).

Statistics. All error bars in the plots represent standard errors. p-values are obtained using the Student’s t test. Asterisks represent a significant statistical difference between two groups of data in a plot.

# RESULTS AND DISCUSSION

**Microbead-Based Cell Classification Scheme.** In this work, we consider deformation of microbeads in confining microchannel structures as a cell elasticity indicator for cancer cell classification applications. We should first engineer the microbeads with a comparable size with the cell samples and an elasticity level between the cell types we plan to classify (details are provided in the later sections). On the other hand, the confining microchannel device (Figure 1a) has been developed and configured for human cancer cells based on our previous works. Briefly, its flow region (height, 50 μm) contains two series of 300 μm long confining microchannels, whose width is 30 μm at the channel entrance and reduces to 4 μm at the exit. As the microbeads and cells are smaller than the channel entrance and larger than the channel exit, they can be trapped in the microchannels under adequate pressures.

The cell elasticity classification procedures are straightforward. The microbeads with defined elasticity are first mixed with a cell sample, which might include normal and cancer cells with different elasticity levels. We can then inject the cell–bead mixture into the confining microchannel device with a steady pressure from the inlet. The pressure regulation can be achieved by filling the sample in a syringe without a piston, connecting the syringe to the device tubing, and applying a controlled gage pressure from the rear opening of the syringe. When the sample is driven into the microchannels, some of the cells/microbeads flowing along the microchannels would deform upon compression by the channel walls and stay in the channel with a “penetration distance” from the channel entrance. Such penetration distance depends on both the cell size and elasticity, e.g., a larger or stiffer microparticle has a shorter penetration distance. We have previously derived a theoretical relation that elasticity of a cell can be calculated directly from its corresponding size and penetration distance. It should be mentioned that the estimated elasticity level is also sensitive to the operation parameters such as the inlet pressure; and therefore, our previous theoretical approach has a high technical barrier on the precise pressure control for practical implementation. Here, the added microbeads can eliminate such a technical barrier by offering a reference penetration distance for distinguishing harder and softer cells. That is, a harder or softer cell can be directly observed in the confining channels on whether it has a shorter or longer penetration distance than the microbeads, respectively (Figure 1b). This operation-friendly microbead-based approach provides a direct cell classification result, the population proportion of “softer” cells in a biosample. This approach is highly applicable in cancer diagnosis as the softer cells are likely the malignant cells of many cancer types, such as breast cancer and nasopharynx cancer. Together, the key factor for success of this microbead-based cell classification is on generating microbeads with defined physical properties, functioning as the cutoff levels in size and elasticity for distinguishing normal and cancer cells.

**Microbead Formation.** We have adopted a microfluidic emulsion device to generate hydrogel microbeads with defined physical properties. This device (Figure 2a) includes a layer of flow microchannels with a consistent height of 20 μm. There was an inlet for the oil phase sample and another...
In the experiments, mineral oil (M5904, Sigma-Aldrich) was first injected from both inlets under a positive gauge pressure along in order to remove all air bubbles in the microchannels. We replaced the center microchannel with a solution of 1% (w/v) aqueous alginate (Novamatrix) and 50 mM/mL calcium ethylenediaminetetraacetic acid (Ca-EDTA; Sinopharm Chemical Reagent Co., Ltd.) at another gauge pressure. After the two liquid phases met at the merging channel junction, the emulsified microdroplets were collected at the outlet chamber. Apparently, the droplets were harvested over the chamber base because mineral oil has a lower density (0.8 g/cm\(^3\)) than water (Figure 2c). We then stopped the flows until the collection chamber had a liquid volume of ∼30 µL.

Afterward, we prepared a solution of 0.1% (w/v) acetic acid (Sigma-Aldrich) in mineral oil with vortex mixing and mild agitation (>10 min). It has been reported that hydrophilic acetic acid dissolves in oils.\textsuperscript{45} Its dielectric constant (= 6.2) is much lower than that of water (= 80.4) at room temperature, implicating that acetic acid can dissolve in nonpolar compounds including oils.\textsuperscript{46} Ca-EDTA was initially stable in the collection chamber because EDTA completely chelated the Ca\(^{2+}\) at the neutral pH state. Yet, after pipetting such “acidic oil” with a volume of ∼100 µL into the chamber, acetic acid gradually diffused from the oil phase and reduced the pH of the water phase, triggering the release of Ca\(^{2+}\) from the Ca-EDTA compounds. It has been reported that the G-blocks of alginate should then cross-link with Ca\(^{2+}\) to form hydrogel microbeads.\textsuperscript{47} We kept the microbeads in the collection chamber for target duration to achieve a sufficient level of cross-linking. We then pipetted the microbeads into a 15 mL syringe tube containing >5 mL of phosphate buffered saline (PBS), followed by a brief centrifuge (500 rpm, 3 min) to transfer the microbead into PBS and prevent any further cross-linking of the hydrogel microbeads.

**Bead Size Modulation.** Diameter of the hydrogel microbeads can be modulated by varying the operation parameters with the microbead generator. In particular, we have characterized the microbead diameter as a function of the driving pressures during the microfluidic emulsion. We repeatedly generated the microbeads with the water-phase pressure ranging from 6 to 15 kPa under a fixed oil phase pressure as 17.2 kPa, followed by quantifying them after the acidic oil treatment of 2 h. We have examined that a small portion of the microdroplets might combine together during the acidic oil treatment. This could induce the resultant microbead diameter of ∼5%, compared to the microdroplet diameter before treatment. Results (Figure 2d) indicate that the micro bead size did not significantly vary under the water-phase pressure of 6–8 kPa, but it increased with the pressure range of 8–15 kPa. A water-phase pressure >15 kPa could not induce any microdroplet formation.

We have quantified for diameters of NP460 cells and NPC43 cells. We took microscopic images of the trypsinized cells and measured the cell diameters as summarized in Figure 2e, indicating that there is no significant difference on cell size between the two cell types. Hence, we applied a water-phase pressure of 10.3 kPa to prepare the microdroplets (diameter, 13.62 ± SE 0.205 ± SE 0.2 µm) and then the hydrogel microbeads (diameter, 13.93 ± SE 0.205 µm). Furthermore, we have also measured the diameters of MCF-10A (13.16 ± SE 0.35 µm) and MDA-MB-231 (16.05 ± SE 0.46 µm). According to Figure 2e, we configure the water-phase pressure

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**Figure 2.** (a) Photograph of a microfluidic emulsion device. (b) Microscopic image of the microchannel junction at which microfluidic emulsion occurs. Arrows indicate the flow direction. Scale bar: 80 µm. (c) Generated microbeads in the collection chamber. Scale bar: 40 µm. (d) Diameter of microbeads as a function of inlet pressure of the water-phase sample. (N > 30). (e) Average diameters of NP460 cells (N = 90), NPC43 (N = 103) cells, and the microbeads (N = 65) generated with a water-phase pressure of 10.3 kPa. (f) Average diameter of MCF-10A cells (N = 20), MDA-MB-231 (N = 21), and the microbeads (N = 20) generated using a water-phase pressure of 11 kPa. All error bars in the plots are the standard errors.
of 11 kPa to generate microbeads with a diameter (14.11 ± SE 0.3 μm)) between the two cell sizes, as described in Figure 2f.

**Bead Elasticity Modulation.** The bead elasticity is determined by the cross-linking between alginate and Ca²⁺. More specifically, the proportion of cross-linked G-blocks in alginate increases with the material elasticity.⁴⁸ Considering the bead elasticity increases with the treatment duration. On the other hand, we applied the cross-linking between alginate and Ca²⁺. Therefore, we can modulate the bead elasticity by changing the duration of the acidic-oil application.

We performed repeated runs of the microbead generation using different acidic-oil treatment durations from 1 to 5 h. For each group of the treatment duration, we quantified for the corresponding bead elasticities using the confining microchannel device as we previously reported.⁴⁹ Briefly, we injected the microbeads into the confining microchannels under a steady driving pressure, measured the bead diameters and the penetration distance, and converted these measured values to the bead elasticity based on the Hertz and Tatara model. Results (Figure 3a) indicate a clear trend that the bead elasticity increases with the treatment duration. On the other hand, we applied the confining microchannels with the same procedures to quantify the elasticity values of NPC43 (6.43 ± SE 0.11 kPa) and NP460 cells (7.48 ± SE 0.28 kPa). We then chose the oil-acid treatment duration of 2 h such that the hydrogel microbeads would have elasticity (6.76 ± SE 0.033 kPa) between those two cell types (Figure 3b). Likewise, we measured the elastic moduli of MCF-10A (3.62 ± SE 0.10 kPa) and MDA-MB-231 (3.10 ± SE 0.03 kPa) using the same approach (Figure 3c), suggesting that an oil-acid treatment duration of 0.5 h should be configured for distinguishing these two cell types, as shown in Figure 3a.

In addition, we conducted further experiments to examine the variation of the bead elasticity after the microbeads were transferred to the culture media with a natural pH in our experiments. Therefore, it is suggested that the cross-linked microbeads should be maintained in pure mineral oil for storage; and they can be transferred to the biosample right before the cell classification operation.

**Classification of Dissociated Cells.** We further demonstrated classification of nasopharyngeal normal (NP460) and cancer (NPC43) cells using the Ca-alginate microbeads, prepared with a water-phase pressure of 10.3 kPa and an oil-acid treatment period of 2 h. In practice, these cells are typically obtained from dissected normal/tumorous tissues. For distinguishing the two different cell types, we first stained the NP460 and NPC43 cells with different fluorescence. We then mixed the designed microbeads (density, 4 × 10⁵ bead/mL) and the NP460 cells (density, 10⁶ cell/mL) and NPC43 cells (density, 10⁵ cell/mL) in a solution containing the same ratio of both culture media. We injected the cell–bead mixture into the confining microchannel device with a driving hydraulic pressure of 0.3 kPa. Next, we took microscopic images at the ratio of both culture media. We transferred the bead mixture to the biosample right before the cell classification test. The results indicated that the bead elasticity was stable in the media for at least 15 min, which should be an ideal time frame for the cell classification test. For the bead suspension of >1 h, we observed that microbeads softened and could no longer be captured in the confining microchannels. The microbeads then became smaller gradually and most of them would degrade thoroughly after 3 days. In fact, it has been also reported that Ca-alginate microbeads would degrade gradually in aqueous solutions such as PBS.⁵¹ The Ca-alginate cross-linking can be lost through calcium exchange with the surrounding solution.⁵² A higher pH environment would allow a faster calcium exchange and CA-alginate degradation;⁴¹ and this may explain why the microbeads would soften in the culture media.
Although NP460 and NPC43 adopted in this work belong to different patients, it has been well proven that cancerous nasopharyngeal cells are generally softer than normal nasopharyngeal cells. In principle, the cancer cells can then be identified by directly observing whether the penetrated position of cells is longer than the microbeads. Additionally, distributions of penetration distance against undeformed cell diameter for the microbeads and the two cell types are analyzed as a scattered plot as shown in Figure 4c, in order to reveal the cell classification performance. The cell diameter $D$ can be further taken into the consideration; and it can be revealed by a simple relation based on the volume conservation: $D = \left[3W(L^2W^2/3)\right]^{1/3}$, where $L$ is the cell length along channel and $W$ is the lateral width of the cell center in the channel. A linear regression line was applied to illustrate basic tendency for the microbeads (hidden line) as reference. Furthermore, we wrote customized scripts with MATLAB R2017a (MathWorks) to implement the Quadratic Discriminate Analysis based on the measured data for cell classification. The computed boundaries of classified regions are shown in Figure 4c, with a separating curve between the regions of NP460 and NPC43 cells. From our results, 95.24% of NP460 cells and 100% of NPC43 cells can be classified correctly, implicating that this cell classification technique should have a success rate of >95%. If necessary, a higher specificity could then be achieved by recollecting all the cells and performing other cell identification tests for the biochemical biomarkers. Here, the objective of adding the engineered microbeads with the cells is to provide a reference cutoff characteristics curve in the penetration distance–diameter plot. Both the fitting line of the microbead data and the separating curve between the two cell types can help the future cell classification test with an unknown nasopharyngeal epithelial cell sample on whether there are present cancer cells.

For the later classification test on an unknown cell sample, the engineered microbeads can be added to the sample, followed by injecting the cell–bead mixture into the confining microchannels. We should then quantify the properties of both the cells and microbeads as a scattered plot of penetration distance against cell diameter. An updated fitting line can be calculated by on linear regression using the new microbead data ($R^2 = 0.58$). By comparing the new regression line with a reference line (hidden line in Figure 4c), we can then obtain proper scaling parameters to rescaling the cell separating curve described in Figure 4c for the new measurement. Notably,
these scaling parameters can eliminate minor variations of experimental configurations (e.g., driving pressure) between different classification tests. The possible existence of cancer cells can then be directly identified by whether there exists any cell with a location above the separating curve in the scattered plot. Future applications of this cell classification scheme is not limited to identifying nasopharyngeal epithelial cells dissociated from the corresponding resected tissue for presence of nasopharyngeal carcinoma, which is relatively more common among southern Chinese areas with to date a death of tens of thousands of people or even more. Dimensions of microbeads and confining microchannels as well as the bead elasticity can be reconfigured to identify other cancer types. Importantly, the captured cells in the device can be recollected using a higher driving pressure (>1 kPa) and the degradable Ca-alginate hydrogel microbeads would allow a wide range of follow-up cell analyses and engineering processes; and the device can be reused after the cell removal by flushing PBS with either a higher forward driving pressure or a reversed flow.

**Classification of Floating Cells in Whole Blood.** We further examined the feasibility of the microbead-based cell classification for whole-blood samples containing multiple epithelial cell types. Importantly, cancer patients may have only the cancer cells found in their blood rather than the noninvasive normal cells, therefore mixing the microbeads with the blood sample can then provide the reference normal-cell properties to identify the softer cells, which are potentially the CTCs. As the whole-blood consists of multiple blood cell types including red blood cells with a relatively high density, direct injection of the whole-blood can lead to clogging of cells (e.g., red blood cells and platelets) along the microchannels. We have tested that the direct blood injection induces aggregation of the blood cells and blocking all the confining microchannels. We first examined a dilution rate of the blood such that cells flowing with the diluted blood would induce the same penetration distance as the case without mixing with the blood. We conducted the experiments with cancerous (MDA-MB-231, at a density of $10^4$ cell/mL) breast cells and

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**Figure 5.** (a) Comparison of penetration distances of MDA-MB-231 cells mixed with different diluted bovine blood concentration, under a driving pressure of 0.15 kPa. (b) Microscopic images of captured MCF10A cells, MDA-MB-231 cells, and microbeads. Pathlines of flowing red blood cells (RBCs) have also been observed in the confining channels. Scale bar, 30 μm (inset, fluorescence micrographs). (c) Penetration distance against diameter of the cells and beads mixed in bovine blood with a dilution ratio of 1:8. A fitting line of the microbead distribution is shown as the hidden line. A separating curve between the two cell regions is in red.
noncancerous epithelial cells in bovine whole-blood. As indicated in Figure 3b,c that breast cells are generally softer than nasopharyngeal cells, we applied a lower pressure of 0.15 kPa such that these cells can be trapped in the confining microchannels with measurable penetration distances.39 We repeated the penetration distance measurement of MDA-MB-231 for different dilution ratios of the bovine whole-blood in PBS. The results (Figure 5a) indicate that a blood dilution ratio of at least 1:8 should be adopted for the more representative measured penetration lengths.

In the classification experiments on cells in the whole-blood, we prepared the bovine-whole-blood with a dilution ratio of 1:8 in PBS with addition of MCF-10A and MDA-MB-231 prestained with fluorescence, both at a density of 10^4 cell/mL. We also prepared the Ca-alginate microbeads with a water-phase pressure of 11 kPa and an oil-acid treatment period of 0.5 h to configure the microbead with comparable size and elasticity as the MCF-10A cells. We took both bright-field and fluorescence micrographs of the microchannel regions to capture the positions and dimensions of the cells and the microbeads as shown in Figure 5b, which indicates also the pathlines of flowing blood cells (mainly the red blood cells). The length (L) and lateral width (W) of the cells/beads were converted to the undeformed diameters as described in the previous section. The cell positions are considered as the penetration distance from the channel entrance. Similar to the previous section, a scattered plot of penetration distances and diameters of both the breast cells and the microbeads is shown in Figure 5c. A fitting line is added for the microbead data as reference. The percentages of correct cell classification are 100% for MDA-MB-231 and 77.5% for MCF-10A. It should be noted that Figure 5c is presented for demonstrating the capability of cell classification using the engineered microbeads. The blood samples from cancer patients may include only the cancer cells but not the normal noninvasive normal cells; and therefore, we have to add the microbeads to set the reference biophysical conditions, reflected by the measured penetration distance in the microfluidic device such that we can then consider the cells with a longer penetration distance than the microbeads that is comparable to the potential candidates of CTCs.

Together, these results support that the microbead-based cell classification strategy can be applied to identifying CTCs using the cancer patients’ blood directly, with a reasonable success rate of >75%. Nevertheless, it should be mentioned that although significant differences on the cell properties can still be observed between the normal and cancerous cells from the cancer patients,20,56 there may exist higher heterogeneity on cell properties (i.e., size and elasticity vary among different cells of the same cell type) in the patients’ body, meaning that performance of the cell classification on a particular patient is, to a certain extent, undetermined. Another technical challenge should be related to different levels of the physical properties of cells in different individuals’ body; and hence, the engineered microbeads may not have the physical properties matching very closely to the targeted normal cells for the best success rate in cell classification and cancer cell identification applications.

### CONCLUSION

We report for the first time the application of engineered hydrogel microbeads in elasticity-based classification of floating normal and cancer cells. Fabrication of Ca-alginate microbeads with defined diameter and elasticity is achieved by regulating process parameters in the microfluidic emulsion of microdroplets (driving pressures) and the subsequently solidification process (acid treatment duration). These engineered microbeads are then mixed together with the cell samples and injected into a confining microchannel device for quantifying the cell elasticity. In this work, we have demonstrated configuration of the microbeads for matching the physical properties (diameter and elasticity) close to the target cell types. In essence, we have demonstrated the classification of dissociated normal and cancerous nasopharyngeal epithelial cells with a high success rate of >95% by quantifying the captured cell and bead locations in the confining microchannels. The further cell classification of normal and cancer breast cells in whole blood shows a success rate of >75%, which is lower compared to the case without the whole blood. This microbead-based technique can be further applied not only in cancer cell identification for more cancer types but also as a universal elasticity characterization and classification scheme for human blood and contribute to general cell analysis.

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**Notes**

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