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The Role of Surface Receptor Density in Surface-Initiated Polymerizations for Cancer Cell Isolation

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Abstract

Fluid biopsies potentially offer a minimally invasive alternative to traditional tissue biopsies for the continual monitoring of metastatic cancer. Current established technologies for isolating circulating tumor cells (CTCs) suffer from poor purity, yield, and require fixatives that preclude the collection of viable cells for longitudinal analyses of biological function. Antigen Specific Lysis (ASL) is a rapid, high-purity method of cell isolation based on targeted protective coatings on antigen-presenting cells and lysis depletion of unprotected antigen-negative cells. In ASL, photoinitiators are specifically labeled on cell surfaces that enable subsequent surface-initiated polymerization. Critically, the significant determinants of process yield have yet to be investigated for this emerging technology. In this work, we show that the labeling density of photoinitiators is strongly correlated with the yield of intact cells during ASL by flow cytometry analysis. Results suggest ASL is capable of delivering ~25% of targeted cells after isolation using traditional antibody labeling approaches.. Monomer formulations of two molecular weights of PEG-diacrylate ($M_n \sim 575$ and 3500) are examined. The gelation response during ASL polymerization is also investigated via protein microarray analogs on planar glass. Finally, a density threshold of photoinitiator labeling required for protection during lysis is determined for both monomer formulations. These results indicate ASL is a promising technology for high yield CTC isolation for rare-cell function assays and fluid biopsies.

Graphical abstract

Associated Content:

Supporting Information: UV-Vis spectra of SA-EITC, SA-Cy3, and SA-PE conjugates. Calibration equations of molar ratios for SA:EITC and SA:Cy3. Regression analysis of mean fluorescence intensity of eosin loading during competitive cell binding experiments. Photograph of the photopolymerization experimental set up. Negative controls of photoinitiator labeling of ASL.

Introduction

Metastatic progression plays a central role in the often fatal nature of cancer, and directly accounts for an estimated 90% of all cancer related deaths.^{1, 2} Although the underlying mechanisms that drive the metastatic process remain an area of intense research, the link between metastasis and the presence of CTCs in peripheral blood has long been observed. Many have reported that the detection of high numbers of CTCs in cancer patients is correlated with poorer prognosis.^{3, 4, 5, 6} These findings have prompted much effort in the detection, isolation, and characterization of CTCs in patient peripheral blood samples for clinical use as a “fluid biopsy”, with the hope of establishing a reliable prognostic indicator. Critically, the poor performance in both sensitivity and specificity of CTC isolation strategies has prevented their incorporation into standard therapy practices, owing to insignificant improvements in clinical outcomes.⁷ To date, only one CTC isolation technology is clinically validated by the FDA called CellSearch, designed by Veridex (Raritan, NJ), which involves enrichment with immunomagnetic beads against EpCAM followed by immunofluorescent labeling and analysis to further confirm the epithelial phenotype of the enriched population.⁸ This system is the most commonly employed CTC isolation and enumeration approach. CellSearch is not a part of standard treatment regimens because of low sensitivity resulting in considerable false negatives in metastatic patients.² Other CTC isolation approaches are currently in early stages of development that isolate cells based on marker recognition similarly to CellSearch, as well as physical properties such as size and stiffness.^{7, 9, 10, 11, 12} Typically, these techniques are based small scale, microfluidic devices that inherently limit throughput rates.¹³

Further confounding the development of a reliable fluid biopsy is a recently described process known as epithelial-mesenchymal-transition (EMT) shown to cause a reversible and dynamic shift in the tumor cell phenotype as epithelial markers are downregulated.^{14, 15} This suggests that CTC detection strategies based on recognition of epithelial markers may not “see” a large proportion of the most highly metastatic CTCs.^{16, 17, 18} These cells represent significant false negative events in current isolation approaches, contributing to poor assay sensitivity (i.e. cell yield), specificity, and ultimate utility. Therefore, the development of a rapid and versatile assay that delivers functional cells is warranted to better understand the biological relationship between metastatic drivers and the phenotype of observed CTC populations.

Recently, we described a fundamentally novel approach to cell sorting based on photopolymerization of hydrogel coatings on cells in suspension.¹⁹ Cells are sorted by a process of Antigen Specific Lysis (ASL), where antigen-positive species are coated and protected in harsh environments that rapidly lyse virtually 100% of all antigen negative cells. The polymer coatings are later removed through *ortho*-nitrobenzyl photocleavable linkages. This versatile and rapid processing method is easily scalable and fundamentally capable of sorting large cell numbers while providing a facile and relatively inexpensive isolation method with exceptionally high purities that rival fluorescent sorting. Viable cells are selectively encapsulated based solely on surface marker expression and subsequent antibody incubations that tether eosin molecules (type-II photoinitiator) to the surface of target cellular species.^{20, 21} Upon exposure to visible light (530 nm) in the presence of a tertiary

amine coinitiator and PEG-diacrylate monomer, a free radical polymerization proceeds in the immediate vicinity of eosin functionalization, crosslinking a protective coating around target species.^{22, 23} The entire mixture is then exposed to surfactants that lyse antigen-negative cells.

While we have shown ASL delivers viable (~90%) and highly pure (>97%) populations of marker-presenting cells, cell yield has yet to be fully investigated for this process. For CTC sorting, target cells exist in extreme rarity (~1 per 10⁶ nucleated cells). Increasing isolation yield will minimize the occurrence of false negatives and potentially provide the key difference for an accurate diagnosis. Further, for any antigen-based isolation platform, successful positive capture of cells is inherently dependent on the density of surface markers available for targeting. For ASL, the surface density of markers dictates the concentration of eosin photoinitiators present at the cell surface. A distinct threshold eosin density has been previously reported for surface-mediated polymer amplification assays, below which radical generation is not sufficient to reach the gel point.^{24, 25} Therefore, we hypothesize a minimum density of antibody-photoinitiator binding is required to form complete protective coatings.

Herein, we investigate the effect of photoinitiator loading on polymerization and lysis-based negative depletion of cells. (Figure 1) A non-small cell lung cancer line (A549) serves as a model CTC. To eliminate heterogeneity in surface marker clustering, our investigation first nonspecifically biotinylates the cell surfaces for polymer protection studies. This approach allows for more consistent biotin functionalization and limits the potential for signaling activation that targeting a specific marker might enact, which could influence our results. We then shift to the more clinically relevant antibody-based strategies, targeting either EpCAM or a panel of metastatic markers for comparison to our covalent labeling approach. Finally, we relate the density dependent protection to thin film polymerization behavior on an analogous system of biotin-functionalized protein microarrays. These glass microarrays afford a controlled environment to quantitatively determine the gelation response to the photopolymerization conditions used in the cell studies.

The impact of this study extends beyond our new lysis isolation process to the field of immunoprotective coatings on cells. Immunoprotection requires a complete polymer barrier to isolate the non-native cellular material from a host's immune response. Several groups are utilizing nano- and micro-scale polymers to isolate individual cells from immune response.^{20, 26, 27, 28, 29, 30} As the thickness of these materials decreases, the diffusive flux of oxygen and low molecular weight nutrients increases. Critically, as the coating thickness approaches the diffraction limit of light, it is increasingly challenging to visually verify the completeness of the coating. This study uses surfactant lysis as a new approach to cheaply and quickly verify the completeness of the coating.

Experimental Section

Materials and Reagents

The A549 cell line (non-small cell lung cancer) was purchased from American Type Culture Collection. RPMI-1640 culture media, penicillin/streptomycin (1%), and trypsin/EDTA

(0.25%) were supplied by Gibco™. Advantage-grade fetal bovine serum (FBS) was purchased from Atlanta Biologicals. Lyophilized streptavidin, streptavidin-Cy3 conjugate, biotinylated bovine serum albumin (b-BSA), sulfo-NHS-LC-biotin, CellTracker™-Deep Red dye, Hoechst 33342 stain, and yellow-green FluoSpheres™ nanoparticles (0.02µm, carboxylate-terminated, 505/515nm excitation/emission) were purchased through Thermo Fisher Scientific. Bovine serum albumin (BSA), Polyethylene glycol diacrylate ($M_n \sim 575$), Polyethylene glycol ($M_n \sim 3400$), monomer MEHQ dehibiting columns, 1-vinyl-2-pyrrolidinone, acryloyl chloride, triethylamine, Eosin-5-isothiocyanate (EITC), sodium dodecyl sulfate (SDS) were all supplied from Sigma Aldrich. Triethanolamine (TEA), dichloromethane, and diethyl ether were supplied by Fisher Chemical. DNase I (>500U/mg, from bovine pancreas) was supplied by Biomartik. Monoclonal antibodies against EpCAM (IgG2b, clone 9C4), CD44 (IgG1, clone BJ18), and HER1/EGFR (IgG1, clone AY13) were purchased from BioLegend, and anti-ICAM-1 (IgG1, clone HA58) was purchased from eBiosciences. Biotinylated goat anti-mouse IgG antibody was purchased from Vector Labs. Streptavidin R-phycoerythrin conjugate was purchased from Anaspec. The QuantiBRITE phycoerythrin (PE) fluorescence flow cytometry quantitation kit was supplied by BD Pharmingen.

Monomer Synthesis and Preparation

Polyethylene glycol diacrylate ($M_n \sim 575$, PEGDA-575) was twice flowed by gravity through a dehibiting column to remove the polymerization inhibitor monomethyl ether hydroquinone, and stored for use protected from light at room temperature. Polyethylene glycol ($M_n \sim 3400$) was dissolved in DCM and reacted with acryloyl chloride and triethylamine according a previously published protocol.¹⁹ Monomer product purity (~90% acrylation yield) was confirmed by ¹H-NMR and was stored at 80% (w/v) in water protected from light. Co-monomer 1-vinyl-2-pyrrolidinone was used as received.

Photoinitiator-Protein Conjugation

The conjugation reaction to label eosin-5-isothiocyanate to streptavidin primary amines was adapted from a previous protocol,³¹ with only a minor protocol modification of reacting at pH 10 in 0.1 M carbonate/bicarbonate buffer. Conjugates were purified with a 5 mL 7 KDa MWCO Zeba desalting column and the molar ratio confirmed with UV-vis analysis of absorbances at 280nm and 530nm. (SI Figure 1)

Cell Culture and Fluorescent Labeling

A549 cells were cultured in RPMI-1640 supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C and 5% CO₂ for approximately 48 hours. Cell flasks were 75–90% confluent at the time of experimentation. To harvest the A549 cells, the culture media was removed and the cells were washed with 37°C sterile phosphate buffered saline (PBS 1×). Trypsin/EDTA (0.25%) was added to each flask and incubated for 3–5 minutes. Fresh culture media was added to the flask and gently rinsed by pipetting to detach cells. The cell suspension was then centrifuged at ~500g for 3 minutes and the supernatant discarded. The cell pellet was resuspended in fresh media and immediately stored on ice. To begin fluorescent labeling cell samples, cells were first centrifuged at 500g for 1.5 minutes at 4°C and resuspended in cold PBS by gentle vortexing. Initial cell counts from culture were

obtained by flow cytometry using an Accuri C6 cytometer and adjusted to 1×10^6 per sample tube. Cells were rinsed in cold PBS twice more, then resuspended by vortexing in $0.5 \mu\text{M}$ CellTracker™-Deep Red in cold PBS and incubated for 30 minutes on ice. Samples were then rinsed three times with cold PBS as before. Experiments investigated two groups: covalently biotinylated cell samples and antibody immunolabeled samples.

For covalent biotinylation, cells were incubated with 1mM sulfo-NHS-LC-biotin prepared in cold PBS for 30 minutes then rinsed three times with cold PBS. Cells were then rinsed once more with PBS supplemented with 3% FBS in PBS (PBS/FBS) and incubated for 10 minutes on ice. To predictably and incrementally vary photoinitiator loading while keep covalent biotinylation conditions constant, streptavidin-EITC was combined with unconjugated streptavidin at known and varied ratios with a total streptavidin concentration of $25 \mu\text{g/mL}$. Upon contacting with cells for 30 minutes, the proteins competitively bound to biotin sites, allowing for photoinitiator (eosin) to be reliably loaded at desired levels. After rinsing twice with cold PBS, cell count was obtained by cytometry and adjusted to 3×10^5 cells per sample tube to ensure consistent cell density during cell photopolymerization.

For antibody labeling of samples, cells were incubated with primary antibodies (anti-EpCAM or a “metastatic” cocktail of anti-CD44, anti-ICAM-1, and anti-EGFR) consisting of $0.5 \mu\text{g}$ of each antibody in $100 \mu\text{L}$ PBS/FBS for 40 minutes, then rinsed three times in PBS/FBS. Cells were then resuspended in 1:400 dilution of biotinylated goat anti-mouse IgG antibody in PBS/FBS for 40 minutes, and rinsed again three times with PBS/FBS. After the biotinylation by immunolabeling, samples were incubated with $25 \mu\text{g/mL}$ SA-EITC for 30 minutes protected from light. After rinsing twice with cold PBS, cell count was obtained by cytometry and adjusted to 3×10^5 cells per sample tube to ensure consistent cell density during cell photopolymerization. A negative labeling control was also performed for each monomer formulation (SI Figure 6) that consisted of A549 cells incubated with $25 \mu\text{g/mL}$ SA-EITC for 30 minutes without prior incubation with biotinylating agents, followed by the polymerization and lysis procedures detailed for biotinylated samples.

Cell Suspension Photopolymerization and Lysis

Polymerization formulations were prepared in PBS consisting of 21 mM TEA, 35 mM VP, with 25% (w/v) of either PEGDA-575 or PEGDA-3500, and 0.05% (w/v) Fluosphere nanoparticles. All formulations were adjusted to $\text{pH} \sim 7.5$ with 1.2 M HCl and purged with humidified, ultra-pure N_2 for 15 minutes just prior to combining with cells. Each sample was polymerized in a sterile cover glass-bottom 35 mm culture dish placed inside a 150 mm culture dish outfitted with purging inlet and outlet ports. (SI Figure 5) The entire system was purged with N_2 for 5 minutes, at which point irradiation light was introduced while continuing to purge, consisting of 30 mW/cm^2 collimated 530 nm light (ThorLabs LED lamp) for 10 minutes. Light intensity was measured with a visible spectrum radiometer probe at the sample surface. Lysing solutions were prepared in two parts: (1) DNase I solution of 5 mM MgCl_2 , 1 mM CaCl_2 and 500 U DNase I in PBS, (2) 10% SDS in PBS. Lysing in the presence of DNase minimizes cell clumping due to free DNA in solution. After polymerization and rinsing, each cell pellet was gently resuspended in $500 \mu\text{L}$ of solution (1)

and incubated for 5 minutes at room temperature, followed by addition of 500 μL of solution (2).

Flow Cytometry and Cell Fluorescence Analysis

Cell samples were analyzed during the isolation process just before addition of polymerization solution and after the lysis procedure to calculate the percent yield. To obtain a cell count before polymerization, each sample was redistributed evenly in 10 mL of cold PBS, and then 150 μL of the suspension was pipetted off for analysis. Cytometer fluidics were set to analyze 100 μL of the sample volume. “Positive” cell counts were defined from controls as FL4+ (CellTracker-Deep Red) and FSC 2×10^6 (intact cell). (see Figure 2) Additionally, the FL1 channel was monitored in this gated population to determine the SA-EITC loading corresponding to each sample. Identical run constraints and gating were imposed after lysis to monitor yield. In parallel experiments, streptavidin-PE conjugates were also reacted with covalently biotinylated A549 populations at identical conditions to streptavidin-EITC binding experiments at various loading levels to calibrate FL2 of streptavidin-PE loaded samples vs. FL1 of SA-EITC loaded samples. Samples of 10,000 events were analyzed by flow cytometry and the FL2 mean fluorescence of SA-PE tagged samples was plotted vs. FL1 of identically biotinylated SA-EITC samples, shown in Figure 3A. BD QuantiBRITE PE quantitation beads were then used to quantify PE fluorescence by vortexing in 500 μL PBS and analyzing in the FL2 channel to calibrate PE binding on biotinylated A549 cells, as shown in Figure 3B. The degree of substitution (DOS) of SA-PE and SA-EITC was also determined for fluorescent analysis. The mean DOS of SA-PE was provided by the manufacturer (Anaspec) to be 1.07 phycoerythrin per streptavidin, with the UV-vis spectrum of this conjugated provided in SI Figure 3. The DOS of the SA-EITC conjugate was determined by UV-Vis analysis. Standard curves of unconjugated streptavidin and EITC were prepared, allowing for SI Equation 1 to be generated that outputs the molar ratio of EITC to SA. The UV-vis of the conjugate used in this study is shown in SI Figure 1, and the absorbance values at 280 nm and 530 nm were used to calculate a mean DOS of 4.05 eosin groups per streptavidin. Finally, an average cell diameter of 17 μm was determined through automated bright field image analysis with a Cellometer cell counter (Nexcelcom) and was used to calculate average cell surface area of 908 μm^2 , assuming a perfect spherical cell geometry. Using these data with the fluorescence cytometry calibrations listed above, Equation 1 was developed to determine eosin molecule density from the mean FL1 obtained for each yield experimental trial.

$$\frac{\text{\#EITC molecules}}{\mu\text{m}^2} = \frac{3.78[36.1(\text{FL1}) - 114929] + 111}{908\mu\text{m}^2}$$

Equation 1: Calibration equation to relate photoinitiator cell surface density to mean FL1 of individual cell samples.

Cell Fluorescent Imaging

A549 cells were imaged after exposure to lysing conditions with a Nikon Ti-U inverted epifluorescent microscope. Cells were incubated with 5 $\mu\text{g}/\text{mL}$ Hoechst 33342 stain for 10

minutes, rinsed once with cold PBS and pipetted onto a coverslipped microscopy slide for imaging with a 20× objective. Hoechst DNA staining was imaged with UV excitation, followed by an overlay image of blue excitation to visualize the yellow-green imaging nanoparticles loaded in polymer coatings.

Microarray Fabrication and Hydrogel Film Formation

Epoxy-functionalized slides were washed with ethanol and loaded into an Affymetrix 417 Arrayer. Serial dilutions of bio-BSA and BSA were prepared in PBS, keeping the total protein concentration in solution constant at 1 mg/ml. Biotinylated-BSA concentration was varied to print 12 concentrations (1000, 400, 160, 64, 25.6, 10.2, 4.1, 1.64, 0.66, 0.26, 0.1, 0 µg/ml). Each slide was printed with an array of 24 spots with each concentration duplicated. Slides were allowed to dry overnight before experimentation. Each array slide was loaded into a Whatman Chip Clip slide incubation apparatus, and blocked for 1 hour with 1 mg/ml BSA in PBS. Slides were then incubated for 30 minutes in 25 µg/ml SA-EITC in 1 mg/ml BSA in PBS. During incubation, the slides were protected from light to prevent photobleaching during photoinitiator loading at biotinylated sites. Slides were then rinsed 3 times with PBS, and photopolymerized with either PEGDA-575 or PEGDA-3500 monomer formulations at identical conditions as detailed above for live cell coatings. Slides were then washed once with PBS, 3 times with deionized water, and allowed to dry for 1 hour. To measure thickness response, a Dektak 6M stylus profilometer was used with a 1 mg stylus force and a scan rate of 120 µm/s. Average height of each spot was measured, with n=6 spots for each measurement.

Cy3 Calibration of Slide Fluorescent Labeling

Array fluorescence was measured using an Affymetrix 428 Array Scanner. Streptavidin-Cy3 conjugates were contacted with array slides at identical conditions as for SA-EITC conjugates detailed above (n=6 spots at each concentration analyzed for Cy3 binding). Both SA-Cy3 and SA-EITC fluorescence was then measured with 532 nm laser excitation, detection with a PMT with a bandpass filter centered at 570 nm, and the gain set at 30 db. Array spot binding density was then calculated by calibrating at 30 db gain with a Cy3 scanner calibration slide from Full Moon Biosystems. Calibration slides contain 12 replicate spots of 28 different concentrations arranged at a 2-fold dilution. By UV-Vis spectroscopy analysis of SA-Cy3 and SA-EITC conjugates (SI Figures 1 and 2, SI Equation 1 and 2), the ratio of EITC to Cy3 functionalization was calculated to be ~2.1, which allowed for photoinitiator surface density to be calculated for each printed array spot.

Results and Discussion

The overall goal of this work was to determine the effect of photoinitiator labeling density on the gelation response and cell protection efficiency (i.e. yield) in lysing conditions. A549 lung cancer cells are biotinylated either covalently or with antibody targeting, then coated by visible light photopolymerization. The protection afforded by the coating was determined by exposing the coated cells to lysing conditions and interrogating the remaining cells by both fluorescent microscopy and flow cytometry. In parallel, an analogous planar microarray system is biotinylated and exposed to identical photopolymerization conditions to provide

hydrogel film thickness information about our system by profilometry analysis. Photoinitiator binding density is quantified fluorometrically in both experimental scenarios for comparison.

We first confirm the structural integrity of polymer coated cells in a 5% SDS lysis solution. Figure 4 shows an overlay of fluorescent micrograph images of A549 cells encapsulated with crosslinked PEGDA-3500 just after exposure to 5% SDS lysis solution. Here, cells were biotinylated with the “metastatic” antibody cocktail (anti-CD44, Ant-ICAM-1, anti-EGFR). Panel A shows a fluorescent image of yellow-green fluorescent imaging nanoparticles previously shown to be physically entangled in the crosslinked film mesh in similar systems,³² showing visible fluorescent signal completely surrounding the cell perimeter. Panel B, taken at the same stage position, is Hoechst 33342 DNA staining, which exhibits fluorescence confined to a smaller region than the polymer coating. The smaller localization of the DNA is consistent with a structurally-intact nuclear envelope. Frame C shows the blue and green channel overlaid, suggesting the cells have remained protected and intact and nuclear DNA localization has remained largely unaltered. We were unable to image any distinct structures in the cellular lysate of unprotected cells. Further, we have previously shown polymer coated cells are viable and proliferate after removal of the coating. In all, fluorescent imaging of the polymer supports the formation of a conformal coating, while nuclear staining supports the integrity of polymer coated cells in lysis conditions.

Relationship Between Initiator Density and Cellular Protection

Our seminal work in ASL work describes a binary relationship between specific photoinitiator labeling with antibodies and protection from surfactant lysis. On a fundamental level, any antibody-based labeling is a contrast between specific and nonspecific labeling. As a result, our prior work implies that the specific labeling is sufficient to form a protective coating and nonspecific labeling is insufficient. In reality, the marker density of different surface receptors varies by several orders of magnitude within a single cell type. As such, even for a single cell type and a single marker, one would expect some cells to have high marker density and some to have low marker density. We hypothesize that this heterogeneity in marker density will translate to a comparable heterogeneity in photoinitiator density on the surface. In this same population, only a given fraction of cells will have sufficient photoinitiator density to completely form a protective coating on the given cell. Thus, only a fraction of the cells are expected to remain intact after surfactant exposure. The fraction of intact cells following surfactant exposure is expected to increase with an increase in the mean photoinitiator density for the population. In antigen labeling experiments, this equates to higher yields for higher surface densities of the targeted marker.

Figure 5 shows the results of the flow cytometry analysis of process yield dependence on mean photoinitiator labeling density of each sample. The presence of an intact cell was defined by both size and fluorescent intensity. A549 cells were labeled intracellularly with CellTracker Deep Red at the outset of each experiment to enable flow detection with the 630nm laser and 675/25 filter combination in the FL4 channel. The excitation-emission

spectra for this dye was specifically chosen because of the spectral separation at higher wavelengths from the photoinitiator, imaging nanoparticles, and irradiation lamp used in this study to provide a distinct and reliable fluorescent indicator of an intact cell event. Additionally, intact A549 populations consistently show forward scatter above $\sim 2,000,000$ for our instrument. Thus, a “positive” intact cell event was defined based on live stained A549 controls by quadrant gating FSC vs FL4 as $FSC > 2,000,000$ a.u. and $FL4 > 30,000$ a.u. (Figure 2). The yield of intact cells was calculated as the cell count per 1 mL after lysis divided by the cell count per 1 mL just before polymerization. The mean photoinitiator density (fluorophores/ μm^2) for each data point was determined by calibrating overall streptavidin-photoinitiator binding to A549 with a QuantiBRITE fluorescence quantitation assay, and then normalizing with the calculated spherical model surface area.

Figure 5A shows a summary plot of A549 yield versus photoinitiator density for polymer coating with 25 wt% PEGDA-575. Each data point represents an independent isolation experiment. Covalently biotinylated samples were incrementally loaded by competitive binding of varied ratios of streptavidin-photoinitiator and unconjugated streptavidin, which showed very predictable and linear eosin fluorescence after binding, as shown in SI Figure 4. As expected, the yield of intact cells after lysis depletion was strongly and directly dependent on photoinitiator surface density. At the lowest loading density investigated ($\sim 1,600$ eosin molecules per μm^2), the yield was $<1\%$, while at the highest bound of our PEGDA-575 study of above 54,000 eosin molecules per μm^2 showed nearly 50% yield of intact cells. Figure 5B shows a similar summary plot of isolation yield dependence on photoinitiator loading for PEGDA-3500 photopolymerization. As before, A549 cell samples were biotinylated covalently by succinimide-amine chemistry indiscriminately on surface proteins, then loaded with streptavidin eosin at incrementally varied levels by competitive binding with unconjugated streptavidin. At the lowest level of eosin loading of $\sim 1,300$ molecules per μm^2 , yield was again below 1%, while for the highest bound of eosin functionalization at $\sim 43,000$ molecules per μm^2 , the yield here for PEGDA-3500 coated samples was as high as 68%. For both monomer formulations, there is a distinct trend for a larger fraction of cells remaining intact when protected by a coating formed with a higher mean surface density of the eosin photoinitiator. This qualitatively agrees with our hypothesis that more cells in the population are above a threshold photoinitiator density required for protection.

With a protection trend established with nonspecific biotin functionalization of cell surfaces, we sought to verify this trend in an antibody labeling system. In separate experiments, cancer cell targeting scenarios were also investigated using antibodies. We have previously shown that A549 exhibit significantly lower surface density of epithelial markers (EpCAM and E-cadherin) than stem-like markers such as CD44 and metastasis markers like EGFR and ICAM-1.³³ Figure 5A shows that EpCAM targeting resulted in low photoinitiator loading density of below 1,200 eosin molecules per μm^2 . This is in good agreement with our previous findings as well as estimated EpCAM surface densities on tumor cell lines in the literature.^{33, 34} The low eosin surface concentration using EpCAM resulted in low isolation yield near 1% for a PEGDA-575 monomer solution. For PEGDA-3500 in Figure 5B, eosin loading was also below 1,200 molecules per μm^2 and delivered correspondingly yield of

intact cells at around 1%. The yield of intact cells for both monomers are in close agreement with predictions based on the covalent labeling studies.

In separate experiments, A549 cell samples were also biotinylated by targeting a panel of markers implicated in metastasis, which included CD44, ICAM-1, and EGFR. In Figure 5A, this antibody targeting strategy allowed for much higher surface densities of between 10,000 and 20,000 eosins per μm^2 , and resulted in higher yield of intact cells of approximately 20% for the PEGDA-575. Similarly, the metastatic marker targeting resulted in ~10% yield of intact cells for the PEGDA-3500. The qualitative trend of higher yields was in fairly good agreement with the percent yield of corresponding covalent biotinylated samples of similar photoinitiator loading. Further, SI Figure 6 shows a negative control representing nonspecific labeling of streptavidin-photoinitiator without biotinylation by antibodies or covalent grafting. For both monomer formulations, photoinitiator loading densities were in the range of 100–200 eosin molecules per μm^2 and yielded <0.1% intact events, indicating that protection during lysis is predominantly due to the specific labeling of streptavidin-photoinitiator at biotinylated sites.

Analysis of Photopolymerization Gelation Response

Quantitative comparison of the lysis protection by PEGDA-575 and PEGDA-3500 indicates a higher yield of intact cells for the PEGDA-3500. Specifically, a PEGDA 575 coating from a photoinitiator density of 35,000 molecules per μm^2 results in ~35% yield, while the same photoinitiator density provides ~60% yield using the PEGDA-3500 monomer. To determine if this discrepancy originates in the sensitivity of polymer formation, we studied the polymerization of each monomer system on protein microarrays. Because of the inherent variability in cell geometry and morphology, direct measurement of nanothin film coating thickness on suspended cell substrates is prohibitively challenging. Protein microarrays on glass offer a reproducible platform to analyze these thicknesses (Figure 6A, 6B, and 6C). Photoinitiator surface densities can be tuned to recapitulate the range seen on cancer cell surfaces during ASL (0 to 20,000 eosin molecules per μm^2). Figures 6D and 6E show plots of measured profilometry thickness of polymerized microarrays at varied photoinitiator densities. Photopolymerization conditions were identical to those used for cell suspensions for both PEGDA-575 and PEGDA-3500 formulations. Plot 6D shows the gelation response for PEGDA-575 reaches average thickness of 85 nm at the high bound of the array experiment at approximately 17,000 eosin molecules per μm^2 . Plot 6E shows the gelation response for PEGDA-3500 which exhibits a lower gel thickness response, reaching approximately 40 nm at the highest eosin density investigated.

Figure 6 also indicates that polymer forms at photoinitiator densities of ~100 and ~1,000 for PEGDA 575 and 3500, respectively. This observation is contrasted with the cell yield data, where comparable densities offers negligible protection (~1% yield, Figure 5). This underscores the requirement of a *complete* coating for protection against lysis. This has dramatic implications for the purity of ASL isolation, where cell with low levels of nonspecific adsorption is unlikely to protect a cell against lysis. Similarly, if large antibody aggregates adhere to a cell surface, it is unlikely to form a complete protective coating around the cell. The higher threshold of the PEGDA-3500 over PEGDA-575 suggests the

3500 system requires a higher antigen density for protection over the PEGDA-575 system. The distinction between the 575 and 3500 systems is not definitively observed in the yield data in Figure 5. Critically, the data in Figure 5 is an analysis based on population means, where cell-level data is obscured.

Determining Photoinitiator Loading Threshold Required for Isolation

The calculated mean photoinitiator density is not a sufficient parameter to determine a required photoinitiator density for the protection of a given cell. To determine a minimum threshold density for protection, we must further examine the distribution of eosin-label density for each population. If an exact threshold exists, we would expect every cell of higher eosin density than the threshold to remain intact through the lysis. As such, we expect a population with X % of events having greater eosin density than the threshold to have a yield of X %. To evaluate this we calculated the yield of each population and drew a line on the surface density histogram where the fraction of events with higher eosin density than the line is equal to that sample's yield. Further, we would expect this threshold to be the same for every trial. For example, if a hypothetical isolation run resulted in 60% yield intact cells, the histogram of eosin labeling for that experiment would be labeled at a density corresponding to 60% of events above the threshold. We then analyzed the surface density corresponding to that line for each population.

Figure 7A and 7B show representative “low”, “mid”, and “high” photoinitiator labeling examples from our covalent biotinylation study for both PEGDA-575 and PEGDA-3500 polymer formulations. In Figure 8, we plot this threshold gate for all data trials for both PEGDA-575 and PEGDA-3500. There is a general upward trend in threshold photoinitiator density for the PEGDA-575. In the PEGDA-3500 system, the calculated threshold density value is independent of process yield. While the mean photoinitiator density shifts drastically in these systems (~50 fold change), the hypothetical density threshold corresponding to the yield only varies by 4 fold. This suggests that the eventual yield delivered is a strong function of the population distribution of initiator labeling in relation to this threshold. Based on our cytometry labeling calibration analysis described above, these threshold photoinitiator densities are roughly 27,000 eosin molecules per μm^2 and 21,000 eosin molecules per μm^2 for PEGDA-575 and PEGDA-3500 formulations, respectively.

Conclusions

In this work we have investigated the relationship between marker expression density on cancer cells and the yield of intact cells during ASL. A lung cancer cell line (A549) was used as a model CTC and cells were incrementally biotinylated via covalently functionalization and subsequent competitive binding with PI-conjugated and unconjugated streptavidin, which provided a predictable prototypic system to methodically study labeling density effects on yield. We have shown a strong, direct correlation between photoinitiator surface density and yield of protected, intact cells. Based on previous work,¹⁹ we expect these results to translate to heterogeneous cell suspensions and represents a critical first step in validating ASL as versatile platform for CTC isolation.

Further, there was no appreciable difference in the process yields afforded by covalent eosin functionalization and the antibody targeting strategies examined here. Mammalian cell membranes are a complex, dynamic composition of cell proteins and other biomolecules that are distributed over the cell surface into discrete locations, often non-randomly. We realized caution should be taken when selecting biomarkers for photoinitiator targeting because some have been shown to cluster into “islands”,³⁵ which could exaggerate non-uniformity in expression and lead to incomplete coating formation. Because little difference was seen between covalent biotinylation and the markers selected here, we conclude that any surface protein clustering phenomena were not significant enough to preclude successful isolation of the cell/antigen systems studied.

Finally, we purposely chose to investigate an expanded photoinitiator loading range through covalent targeting that far exceeded conventional antibody targeting, and showed elevated yield response at higher photoinitiator loading levels. These results suggest ASL would potentially benefit from the design and implementation of streptavidin-macrophotoinitiators that have been shown to direct several fold greater quantities of photoinitiator molecules to each site of biotin recognition and increase polymer amplification response.³⁶ Moving forward, development of such routes to increased gelation response to biorecognition could conceivably enable robust positive isolation based on targeting poorly expressed proteins that may be of high clinical relevance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Surface Polymerization for Cell Isolation

Figure 1. Schematic of surface polymerization approach for cancer cell isolation. Photoinitiator loading dictates yield of polymerized cells in surfactant lysis conditions.

Figure 2.
Flow cytometry control plot of forward scatter vs. FL4 showing gating definition of an intact cell in upper right quadrant.

Figure 3.
(A) Linear regression of FL2 vs. FL1 for calibration of SA-PE and SA-EITC labeling at varied biotinylation. (B) Cytometry standard curve of FL2 for R-phycoerythrin functionalized beads.

Figure 4.

Fluorescent micrograph overlays of PEGDA coated A549 cells after exposure to 5% SDS lysing conditions. (A) Fluorescent signal from 20 nm yellow-green imaging nanoparticles physically entangled in polymer mesh. (B) Hoechst 33342 blue DNA staining. (C) Overlay of yellow-green nanoparticle and Hoechst 33342 images. Scale bars represent 50µm.

Figure 5. Plots of ASL isolation experiments showing yield intact yells attained after lysis vs. corresponding mean photoinitiator density via either covalent or antibody biotinylation for (A) PEGDA-575 and (B) PEGDA-3500 monomers during photopolymerization.

Figure 6.

(A) Schematic of polymer microarrays to analyze gelation response vs. photoinitiator density. Biotin-BSA protein arrays are printed at serial dilution, contacted with SA-EITC and photopolymerized with cell encapsulation conditions. Panel (B) shows fluorescent array scanner image showing specific SA-EITC binding and fluorescence. (C) Fluorescent microscopy image after photopolymerization showing fluorescent nanoparticle loaded polymer film formation. Scale bar represents 500 μm . Contact profilometry thickness analysis vs. photoinitiator surface density of (D) PEGDA-575 and (E) PEGDA-3500 monomer formulations. Errors bars represent standard error for $n=6$ spot concentrations.

Figure 7. Photoinitiator density threshold analysis. Representative histograms for “low”, “mid”, and “high” photoinitiator loading trials and the FL1 intensity of the gating corresponding to the % yield of each individual experiment for both (A) PEGDA-575 (B) PEGDA-3500.

Figure 8.
The threshold photoinitiator density corresponding to each individual isolation % yield shown for (A) PEGDA-575 and (B) PEGDA-3500 formulations.