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Article

Polymer-Coated Magnetic Microspheres Conjugated with Growth Factor Receptor Binding Peptides Enable Cell Sorting

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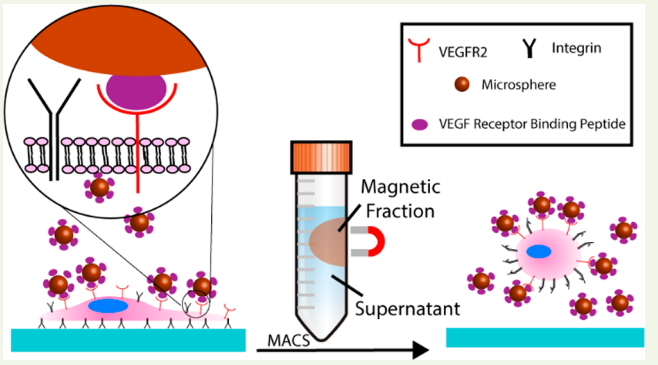
ABSTRACT: The separation and sorting of human cells is an important step in the bioprocessing of cell-based therapeutics. Heterogeneous mixtures of cells must be sorted to isolate the desired cell type and purify the final product. This process is often achieved by antibody-based sorting techniques. In this work, we demonstrate that magnetic microspheres may be functionalized with peptides that selectively bind to cells on the basis of their relative concentration of specific surface proteins. Five-micrometer-magnetic microspheres were coated with the synthetic copolymer PVG (poly(poly(ethylene glycol)methyl ether methacrylate-*ran*-vinyl dimethyl azlactone-*ran*-glycidyl methacrylate) and functionalized with the vascular endothelial growth factor receptor binding peptide (VRBP), which binds to the vascular endothelial growth factor receptor (VEGFR). These microspheres exhibited low cytotoxicity and bind to cells depending on their relative surface protein expression. Finally, coated, magnetic microspheres were used to separate heterogeneous populations of cells dependent on their VEGFR expression through magnetic-assisted cell sorting (MACS), demonstrating that peptide-based cell sorting mechanisms may be useful in the bioprocessing of human-cell-based products.



KEYWORDS: *biomaterials, cell sorting, growth factor receptor binding peptides*

1. INTRODUCTION

In vitro cell culture for research or therapeutic applications relies on the precise isolation and analysis of cell types and subtypes, which often require different growth media, surfaces, and techniques. Cell sorting techniques typically use antibodies to bind and isolate cells expressing certain surface markers from heterogeneous populations. Fluorescent assisted cell sorting, which employs fluorescently tagged antibodies,[1,2] and magnetic activated cell sorting (MACS),[3] which uses antibody-functionalized magnetic microbeads, are the two most prevalent methods used to isolate subpopulations of cells on the basis of their surface protein expression. Antibody-based sorting methods have achieved such widespread use due to their high specificity, broad range of potential targets, and ease of use.[4,5]

Despite being generally well-suited to the task of separating mixed cell populations, there are some limitations to antibody-mediated cell sorting. The development and characterization of antibodies for cell sorting applications is time-, labor-, and cost-intensive, in comparison to other biologically active molecules.[6,7] Additionally, the physical size of antibodies may restrict the ability to copresent and multiplex antibodies on a single bead or fluorophore and can lead to unwanted side reactions and cross-reactivity.[8] The limitations of antibodies as capture ligands has led to the evaluation of other potential ligands, including protein scaffolds,[9,10] nucleic acid scaffolds, and peptides.[11] In particular, there is an expanding library of bioactive peptides with the capacity to bind to cell surface molecules.[12,13] For example, growth factor mimicking peptides have been shown to bind to growth factor receptors, including vascular endothelial growth factor receptor (VEGFR),[14] epidermal growth factor receptor, fibroblast growth factor receptor,[15] and transforming growth factor β receptor,[16] among others.[17] Previous studies have shown that the VEGFR-binding peptide Cys-Gly-Gly-Lys-Leu-Thr-Trp-Gln-Glu-Leu-Tyr-Gln-Leu-Lys-Tyr-Lys-Gly-Ile-amide (CGGKLTWQELYQLKYKGI-NH_2; "VRBP"; otherwise known as "QK") can enhance VEGF-dependent endothelial cell proliferation when immobilized at a high density and can block VEGF-dependent proliferation when immobilized at a lower density.[18,19] Peptides that bind to cell surface receptors may be rapidly developed at a low cost to address some of the drawbacks associated with antibody-based cell sorting.

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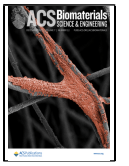

In this work, we employ VRBP bound to the surface of magnetic microspheres to demonstrate a proof-of-concept for peptide-mediated cell sorting. We selected VRBP due to its well-characterized structure and binding characteristics as well as its extensive use in the literature.[14,18−26] We attached VRBP to the surface of magnetic microspheres through coupling to a chemically defined copolymer, poly(poly(ethylene glycol) methyl ether methacrylate-*ran*-vinyl dimethyl azlactone-*ran*-glycidyl methacrylate) [P(PEGMEMA-*r*-VDM-*r*-GMA); PVG]. In this copolymer, synthesized by reversible addition−fragmentation transfer polymerization, PEGMEMA provides a cytophobic background, GMA cross-links the coating, and the VDM acts as a highly reactive unit to efficiently incorporate cell adhesive peptides *via* native chemical ligation chemistry.[27] The use of VRBP-functionalized microspheres in MACS separations demonstrated that interactions between human umbilical vein endothelial cells (HUVECs) and the peptides on the microspheres were specific and capable of separating heterogeneous populations of cells in suspension. Our study provides a proof-of-concept for peptide-based cell sorting using MACS, a technique that may have advantages alone or when used in coordination with antibody-based separation techniques.

2. EXPERIMENTAL SECTION

2.1. PVG Copolymer Synthesis. PVG copolymer was synthesized using reversible addition−fragmentation chain transfer anionic polymerization (RAFT) according to previously reported procedures.[28,29] Briefly, poly(ethylene glycol)methyl ether methacrylate (PEGMEMA), glycidyl methacrylate (GMA), and vinyl dimethyl azlactone (VDM) were added to a 25 mL Schlenk flask. Anisole was added as a solvent to bring the reaction mixture to 13 mL. The chain transfer agent (CTA) 2-cyano-2-propyl benzodithioate and the initiator 2,2′-azobis(2-methylpropionitrile) were added at a monomer/CTA/initiator ratio of 1:1:1. The mixture was degassed using three freeze−pump−thaw cycles. Polymerization was conducted at 70 °C for 15 h and stopped by exposure to the atmosphere. The resultant polymer was precipitated in *n*-hexanes, filtered, and dissolved in tetrahydrofuran (Fisher Scientific). This was repeated three times to remove any unreacted monomer. The resultant polymer P(PEGMEMA-*r*-VDM-*r*-GMA) was dissolved and stored in THF. Gel permeation chromatography yielded a Mn = 47 000 and a dispersity of 2.1. Proton nuclear magnetic resonance spectroscopy was taken using a Bruker Avance-400 400 MHz spectrometer using $CDCl_3$ as the solvent. Proton NMR showed that the final composition of the copolymer was 64% PEGMEMA, 26% VDM, and 10% GMA (Figure S1).

2.2. PVG Coating Characterization. Since direct thickness measurement on the microspheres is not feasible, we created an equivalent planar PS substrate for thickness measurement. A random copolymer containing 96% Styrene and 4% GMA was spin coated on Si/SiO_2 substrate, which was cleaned using piranha acid (3:1 H_2SO_4/H_2O_2; Caution: Reacts violently with organic compounds!) for 30 min, followed by rinsing with copious amounts of deionized water. The sample was then annealed at 160 °C for 3 h under a vacuum to cross-link the PS film and soaked in toluene for 15 min to remove the unreacted polymer. The film was etched under oxygen plasma in a Plasma-Therm 790 ETCH reactive ion etcher for 3 s to create a hydrophilic PS surface for the adsorption of poly-L-lysine (PLL). The substrate was submerged in 0.1% PLL solution in DI water for 1 h, followed by submerging in 10 mg mL^{-1} PVG in 98% EtOH overnight. For control PLL conditions with no PVG coating, substrates were incubated in 98% EtOH. Ellipsometry measurements were performed with a Rudolph AutoElII-VIS-3 three-wavelength ellipsometer. The refractive indexes used for PS were 1.617 at 405 nm, 1.5964 at 546 nm, and 1.5876 at 632 nm. A refractive index of 1.5 was used for the PLL and PVG layers.

2.3. PVG Coating of Microspheres. Untreated iron-modified polystyrene microspheres with diameter of 5 or 10 μm (Sigma-Aldrich Cat #39689) were weighed and incubated in 0.1 wt % 70 000−150 000 Da poly-L-lysine in DI water for 1 h. Microspheres were then washed twice with dH_2O and once with EtOH. The microspheres were placed in a 10 mg mL^{-1} solution of PVG polymer in EtOH and allowed to react overnight. Coated microspheres were washed 2× in PBS and 1× in EtOH before functionalization with peptides.

2.4. Peptide Immobilization. All functionalization reactions on microspheres were conducted in Falcon tubes on a rotating plate to ensure mixing. Peptide functionalization and use in cell culture were conducted on the same day. Immediately before use, microspheres were submerged in sterile 70% EtOH for 20−30 min for sanitization.

PVG-coated surfaces were washed twice with PBS and reacted with the desired peptides. One of either Cys-Gly-Gly-Gly-Arg-Gly-Asp-Ser-Pro (CGGGRGDSP, "RGD") or Cys-Gly-Gly-Lys-Leu-Thr-Trp-Gln-Glu-Leu-Tyr-Gln-Leu-Lys-Tyr-Lys-Gly-Ile-amide (CGGKLTWQE-LYQLKYKGI-NH_2; "VRBP") peptides (Genscript) were first bound to the surface of the PVG-coated microspheres. PVG-coated microspheres were incubated in 1 mM peptide solutions in 1× phosphate buffered saline (PBS) (Fisher Scientific) for 1 h at room temperature according to the procedure by Schmitt et al. from 2015 and 2016.[27,28] Confirmation of the peptide-PVG reaction was done using a fluorescent peptide with the sequence CGGGK(FITC)C and confirmed by fluorescent microscopy.

2.5. *In Vitro* Cell Culture. *HUVECs.* HUVECs (Lonza Cat #C2519A Lot #0000431888, Basel, Switzerland) were cultured in Endothelial Cell Growth Medium 2 (Promocell, Heidelberg, Germany). Cells were thawed from LN2 storage and seeded onto T75 TCPS plates at 3500−7000 cells cm^{-2}. Cells were incubated at 37 °C and 5% CO_2 and manipulated under sterile conditions. Media was changed after 24 h and then every 2−3 days. Cells were passaged at 70−80% confluence using 5 mL TrypLE Select (Invitrogen, Carlsbad, CA) at 37 °C and 5% CO_2 for 5 min. After 5 min, adherent cells were loosened using gentle agitation of the plate.

Neural Progenitor Cells (NPCs). NPCs were derived from WTC-11 induced pluripotent stem cells according to the dual-SMAD inhibition protocol.[30] P6 NPCs were cultured on Matrigel (Corning) coated 10 cm culture dishes in N2B27 media supplemented with 5 ng/mL FGF. Cells were incubated at 37 °C and 5% CO_2 and manipulated under sterile conditions. Media was changed after 24 h and then every 2−3 days. Cells were passaged at 70−80% confluence using 5 mL TrypLE Select (Gibco, Dublin, Ireland) at 37 °C and 5% CO_2 for 5 min. After 5 min, adherent cells were loosened using gentle agitation of the plate

2.6. Fluorescent Imaging. *Live Imaging.* Living and Live/Dead cells were imaged in the appropriate media supplemented with calcein AM (Thermo Fisher, Waltham, MA), ethidium homodimer-1 (Thermo Fisher), or CellTracker living cell fluorescent dyes (Thermo Fisher). Cells were imaged on an inverted microscope with 4′,6-diamidino-2-phenylindole (DAPI), fluorescein isothiocyanate (FITC), and Far Red filter cube sets.

Fixed Cells. Fixed cells were washed with 1× PBS and fixed in 10% buffered formalin solution for 20−30 min. Cells were then permeabilized with 0.1% Triton X-100 (MP Biomedicals, Aurora, OH) in 1× PBS for 20 min. Cells were washed twice with PBS and blocked using 1% bovine serum albumin (Fisher Scientific). Cells were stained for actin cytoskeleton using Alexa-Fluor 647 Phalloidin (Thermo Fisher) and for nuclei using 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich) or Hoechst 33342 for 30 min each, with washing in between with PBS. Cells were imaged on an inverted microscope with DAPI, FITC, and Far Red filter cube sets.

Confocal. Confocal microscopy was conducted on a Zeiss LSM880 confocal microscope with Airyscan. Imaging conditions were selected using the Zeiss-4 color confocal fluorescence setting. Images were generated by creating a 3D rendering of z-stack slices using the Zeiss ZEN software.

2.7. HUVECs Viability in Response to Microspheres. HUVECs were seeded in 96-well plates at a cell density of 5000 cells cm^{-2} and allowed to adhere overnight. Uncoated and PVG-

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coated microspheres were added to the cell culture media after 24 h of culture. Cells were stained using Live/Dead according to the above procedure and imaged at 12 h intervals for 48 h. Living and dead cells were counted on the basis of their respective fluorescence, and the percentage of living cells was determined.

2.8. Magnetic Microsphere Sorting. HUVECs and NPCs were harvested from culture and resuspended at 1 M cells cm^{-2} in 2 mL of EGM-2 media in a 15 mL conical tube. Ten milligrams of PVG +VRBP-functionalized, magnetic microspheres were added to the tube and allowed to bind for 10 min at 8 °C to prevent the uptake of microspheres by cells. After 10 min, a 3" × 1.5" × 0.75" neodymium magnet (K&J Magnetics) was placed on the side of the conical tube, and the supernatant was removed. The magnetic fraction was then washed from the side of the conical tube with 2 mL of fresh EGM-2 and plated for imaging and quantification.

2.9. Statistical Analysis. Experiments were carried out and repeated a total of two to three trials, with $n = 3–4$ replicates per trial. A student's t test or one-way ANOVA was conducted to determine significance, as applicable. In the case that one-way ANOVA was used, a posthoc Tukey's test was then used to determine significance between groups.

3. RESULTS AND DISCUSSION

3.1. PVG Coating of Magnetic Microspheres. In previous work, we applied the PVG copolymer coating to 2D and 3D surfaces to create a chemically defined material, which was then modified with an RGD peptide to enable integrin-specific cell adhesion.[28,29,31] Here, we have used the same sequential anchoring mechanism to apply the PVG coating to 5 and 10 μm diameter iron-modified PS (PS-Fe) beads to create coated microspheres. The PVG coating's high PEG content provides a blank slate background[28,29] that reduces the nonspecific adsorption of serum proteins, ensuring that the surface remains chemically defined and functionalizable (Figure 1).

For enhanced imaging of the microspheres, fluorescein isothiocyanate (FITC)-tagged PLL was used in the sequential anchoring process (Figure 1b,c). The PVG coating rendered the magnetic microspheres inert in the presence of HUVECs. HUVECs that were incubated with uncoated PS-Fe microspheres resulted in significant cell death over 48 h, while PVG-coated microspheres maintained HUVEC viability above 95% (Figure S2). The cell death caused by uncoated PS-Fe microspheres is likely due to internalization of these microspheres by the cells throughout the culture period. The blank-slate PVG coating reduces the nonspecific interactions between HUVECs and the microspheres, which likely reduces internalization and cell death.

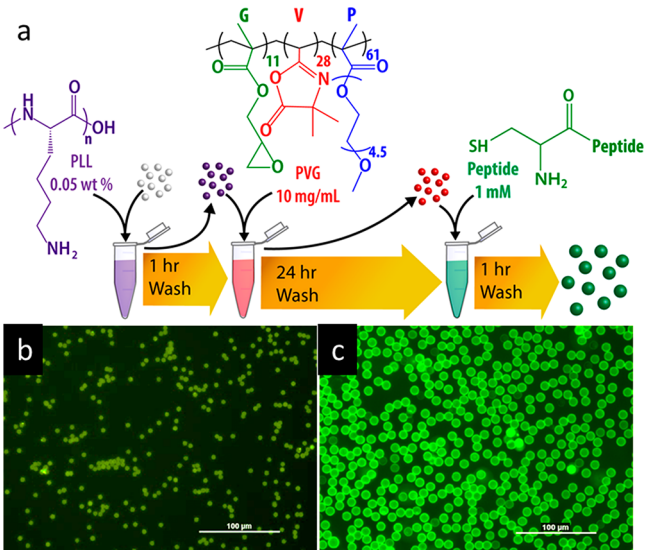

Figure 1. (a) Schematic detailing the process for creating PVG-coated magnetic microspheres. Fluorescent microspheres in either (b) 5 μm or (c) 10 μm diameter microspheres were prepared using FITC-tagged PLL. Scale bar = 100 μm.

3.2. Cell–Microsphere Interactions are Peptide Specific. The creation of an inert surface by the PVG coating ensured that the interactions between cells and microspheres were attributable specifically to the functionalized peptides. The coating thickness measured by ellipsometry was 1.3 nm of PLL and 2.7 nm of PVG, resulting in an overall thickness of 4.0 nm (Table S1), which also confirmed an even coating. The PVG-coated microspheres were reacted with a fluorescently tagged peptide to confirm the presence of the peptide on the microsphere surface (Figure S3). Peptide attachment to the PVG coating at these reaction conditions has been previously measured at 12.6 ± 6.1 pmol cm^{-2}.[28] In order to evaluate the peptide-specific nature of the cell–microsphere interactions, PVG-coated PS-Fe microspheres were functionalized with either an integrin-binding RGD peptide (PVG+RGD), which was not expected to remain bound to cells during cell passaging, or a VEGFR2-binding peptide (PVG+VRBP), which was expected to remain bound. HUVECs cells, which are of therapeutic and research interest for their role in vascular formation, were grown on PVG+RGD-coated polycarbonate slides (according to the procedure outlined by Schmitt et al. in 2016[29]) for 24 h, at which point the functionalized microspheres were added. After incubation for 10 min, cells were harvested from the surface using EDTA.[32] Microspheres and microsphere-bound cells were then isolated using a neodymium magnet and reseeded onto TCPS plates (Figure 2a).

When bare PS-Fe or PVG-coated microspheres were used, cells remained unbound and were not sorted into the magnetic fraction (Figure 2b, left). However, when PVG-coated microspheres were functionalized with VRBP, bound cells were sorted into the magnetic fraction (Figure 2b, right). RGD-functionalized microspheres did not bind and sort cells into the magnetic fraction (Figure 2b, center), as the EDTA-based chelation of Ca^{2+} and Mg^{2+} cations interferes with the divalent cation-dependent integrin receptors that bind RGD[33,34] (Figure 2a, top). The binding and release of RGD-functionalized—but not VRBP-functionalized—microspheres in the presence of EDTA demonstrate that PVG-coated microspheres can be customized with biologically active peptides and that the cell/coating interaction is peptide specific.

3.3. Magnetic Assisted Cell Sorting using Coated Microspheres. VRBP-functionalized microspheres were used to positively sort VEGFR2-expressing HUVECs cells from a mixed population of HUVECs and neural progenitor cells (NPCs), stained green and red, respectively, with CellTracker fluorescent dye. The NPCs were derived from WTC11 induced pluripotent stem cells according to the dual-SMAD differentiation protocol.[30] NPCs are of interest for their ability to differentiate to different neural cell subtypes and have found utility in spinal cord injury[35] and neural organoid models.[36] NPCs express VEGFR at a lower rate than HUVECs and, therefore, were expected to bind less strongly to the PVG +VRBP microspheres (Figure S4). A 50/50 suspension of

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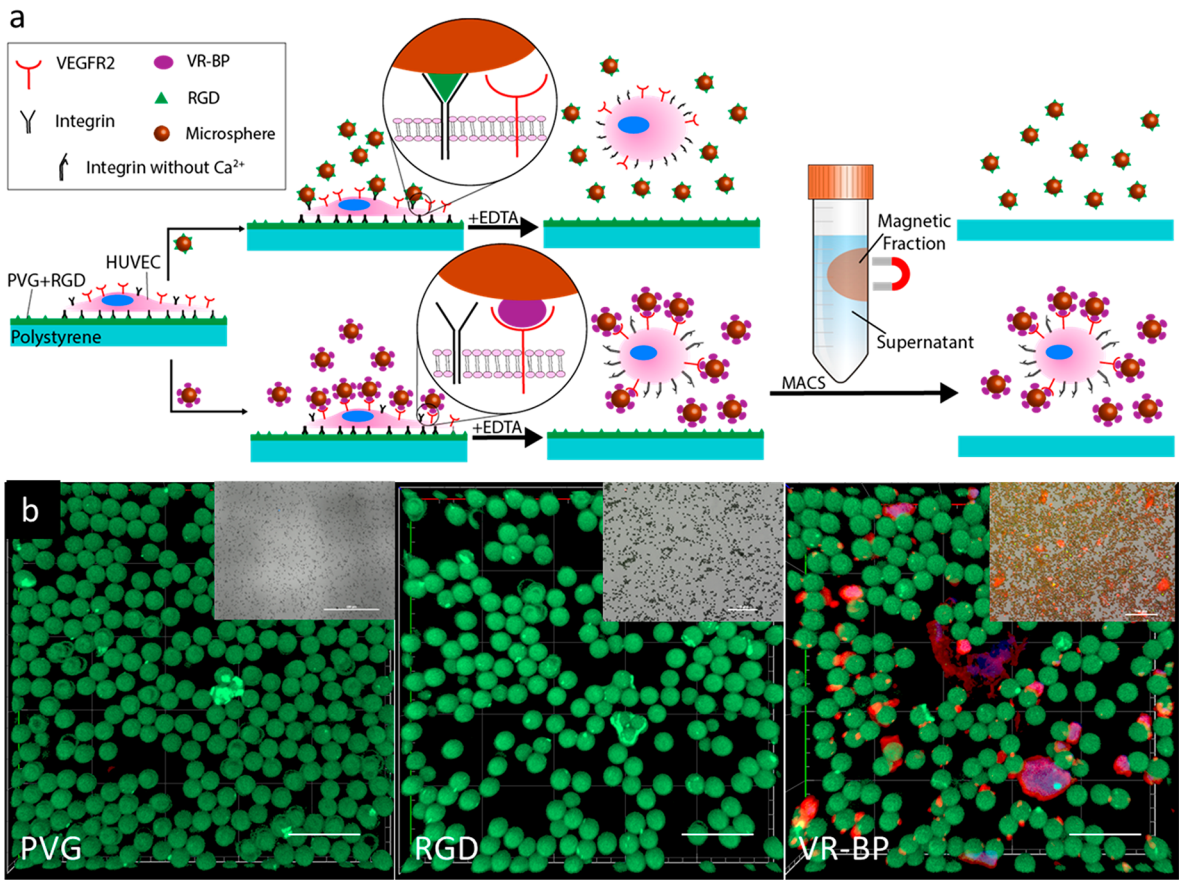

Figure 2. HUVEC receptor binding to microspheres is receptor-specific. (a) HUVECs were cultured on PVG+RGD surfaces and incubated with (top) RGD-functionalized or (bottom) VR-BP-functionalized magnetic microspheres. Cells were harvested using ethylenediamine tetraacetic acid (EDTA), which disrupts integrin binding to RGD by chelating divalent cations. The mixture was then separated into magnetic and supernatant fractions using MACS. (b) Representative images of the magnetic fraction of (from left to right) PVG-coated, PVG+RGD-functionalized, and PVG +VR-BP-functionalized microspheres. HUVEC remain bound to VR-BP microsphere after EDTA treatment and are sorted into the magnetic fraction. Red = CellTracker Red; green = PVG-coated microspheres; blue = Hoechst 33342. Scale bar = 20 μm, inset scale bar = 100 μm

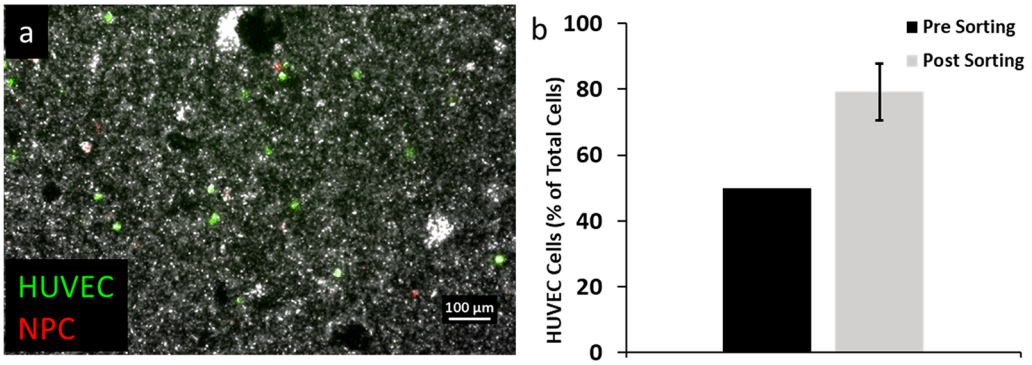

Figure 3. Peptide-based MACS of HUVEC and NPC. A 50/50 suspension of HUVEC and NPC was incubated with PVG+VRBP magnetic microspheres for 10 min and then separated into magnetic and supernatant fractions. (a) Fluorescent and phase micrograph showing that HUVEC cells (green) make up the majority of cells in the magnetic fraction, with a minority of NPC (red). (b) Quantification of the magnetic fraction shows that, after sorting, HUVEC make up 79 ± 8% of the total cells. Scale bar = 100 μm.

HUVECs and NPCs (1 M cells/mL each) were incubated for 10 min with PVG+VRBP microspheres. The microspheres were then removed from suspension using a neodymium magnet, and the magnetic fraction was imaged and quantified. Prior to sorting, 50% of the cells in suspension were CellTracker Green stained HUVECs and 50% were CellTracker Red stained NPCs. After sorting, the proportion of HUVECs in the magnetic fraction increased to 79 ± 8% (Figure 3).

Additionally, the magnetic fraction contained a majority of the HUVECs from suspension, with an average of 77% of HUVECs cells sorted into the magnetic fraction and 23% in the supernatant (Figure S5). The cells bound into the magnetic fraction retained viability 24 h post sorting as shown through a Live/Dead assay, although the presence of

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cellular debris and dead cells implies that optimization of the MACS process may be required to maximize cell viability (Figure S6). The VEGFR-binding peptide VRBP is an ideal testbed for this work due to its history of use in *in vitro* settings and its well-characterized binding to VEGFR2.[18,37,38] However, it is an imperfect peptide for real-world cell sorting applications due to the ubiquity of VEGFR as a cell surface receptor. The ideal cell receptor binding peptide will be very specific, targeting a cell surface protein that can be used to separate cell types from one another at a useful stage in bioprocessing. Examples of ideal targets include the CD31 receptor used to separate endothelial cells and pericytes at the terminal stage of iPSC differentiation or the separation of subpopulations of T cells on the basis of their CD4 and CD8 expression. The expanding library of known peptides, along with the ease and low cost of making new, synthetic peptides, make peptides a promising mechanism for cell sorting and bioprocessing. In future studies, the viability of cells postsorting will need to be improved through the optimization of columns, magnet strength, microsphere type, and incubation time. Given the cost and other drawbacks associated with antibody-based sorting techniques, the ability to rapidly develop, test, and refine peptides as a tool for cell sorting may prove to be a valuable tool for *in vitro* cellular analysis, therapeutic cell production, and bioprocessing.

4. CONCLUSION

In this work, we demonstrated a proof-of-concept that growth factor receptor binding peptides can be used in cell sorting applications. Coated magnetic microspheres with a chemically defined, nonfouling copolymer, PVG, were functionalized using a VEGFR-binding peptide, VRBP. The interactions between these microspheres and HUVECs were shown to be peptide-specific, and finally, the VEGFR-binding microspheres were used to separate a heterogeneous population of HUVECs and NPCs on the basis of their relative receptor expression. The research presented here provides a proof-of-concept for peptide-based cell sorting and may be further applicable in other bioprocessing applications.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsbiomaterials.1c01199.

Figures of NMR spectrum, PVG copolymer structure, HUVEC and cell viability, Live/Dead fluorescent micrographs, quantification of relative VEGFR2 expression, representative phase and fluorescent micrographs, and DNA quantification and table of ellipsometric thickness measurements (PDF)

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Author Contributions

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The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

PVG:poly(poly(ethylene glycol)-*ran*-vinyl dimethyl azlactone-*ran*-glycidyl methacrylate); VRBP:VEGF-receptor binding peptide, also known as the QK peptide

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