Macromolecular Bioscience
www.mbs-journal.de

Synthetic, Chemically Defined Polymer-Coated Microcarriers for the Expansion of Human Mesenchymal Stem Cells

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Mesenchymal stem cells (MSC), also called marrow stromal cells, are adult cells that have attracted interest for their potential uses in therapeutic applications. There is a pressing need for scalable culture systems due to the large number of cells needed for clinical treatments. Here, a tailorable thin polymer coating—poly(poly(ethylene glycol) methyl ether methacrylate-*ran*-vinyl dimethyl azlactone-*ran*-glycidyl methacrylate) [P(PEGMEMA-*r*-VDM-*r*-GMA); PVG]—to the surface of commercially available polystyrene and glass microcarriers to create chemically defined surfaces for large-scale cell expansion is applied. These chemically defined microcarriers create a reproducible surface that does not rely on the adsorption of xenogenic serum proteins to mediate cell adhesion. Specifically, this coating method anchors PVG copolymer through ring opening nucleophilic attack by amine residues on poly-L-lysine that is pre-adsorbed to the surface of microcarriers. Importantly, this anchoring reaction preserves the monomer VDM reactivity for subsequent functionalization with an integrin-specific Arg-Gly-Asp peptide to enable cell adhesion and expansion via a one-step reaction in aqueous media. MSCs cultured on PVG-coated microcarriers achieve sixfold expansion—similar to the expansion achieved on PS microcarriers—and retain their ability to differentiate after harvesting.

1. Introduction

Human mesenchymal stem cells (hMSCs), also referred to as marrow stromal cells, are adult cells capable of differentiation down multiple cell lineages[1–5] and have demonstrated immunosuppressive and anti-inflammatory properties.[6] These traits make hMSCs a target for potential therapeutic applications, with 500 ongoing or completed clinical trials as of 2016.[7] Millions of hMSCs per kilogram of patient are generally used for clinical applications,[8] underscoring a pressing need for scalable culture systems. In order to reduce the time and cost associated with the expansion of hMSCs for clinical applications, innovation on the traditional, 2D cell culture flasks has resulted in improved culture systems such as cellstack plates, bioreactors, and microcarriers.[9] Microcarriers are one system currently used for hMSC expansion, 100–300 μm beads provide a surface for cell adhesion and are subsequently kept in suspension in a bioreactor. The constant mixing in the bioreactor provides gas and nutrient exchange that improves upon the diffusion limits that plague traditional cell culture. Microcarrier systems can produce relevant cell numbers—hundreds of millions of cells in a culture system—and show promise for scale-up to meet industrial lot size requirements, and offer improvements over traditional, 2D cell culture including a high surface area, uniformity, and lower media and resource requirements.[10]

Microcarriers for cell culture may exist as a bare material or have a functional coating. Bare microcarriers are mainly made of polymers including poly(hydroxyethyl methacrylate),[11] polystyrene (PS),[12–14] polyacrylamide,[15,16] poly(L-lactic acid),[17] and poly(lactic-*co*-glycolic acid).[18] Coatings for microcarriers consist of charged molecules, peptides (e.g., Corning Synthemax, CellBIND), or proteins (e.g., collagen, gelatin, Cultispher-S) that facilitate cell adhesion, typically with no potential for further customization or tailoring. Additional commercially available microcarriers include dextran-based Cytodex I, II, and III (GE Healthcare), Sigma-Solohill (collagen- or recombinant protein-coated), and Cultispher (Percell Biolytica AB).

Commercially available versions of microcarriers are effective at up to tenfold expansion of hMSCs.[8,12,19–21] Despite the improvements they represent over tissue culture flasks, currently commercially available microcarriers suffer from drawbacks similar to those that plague traditional cell culture

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DOI: 10.1002/mabi.201800299

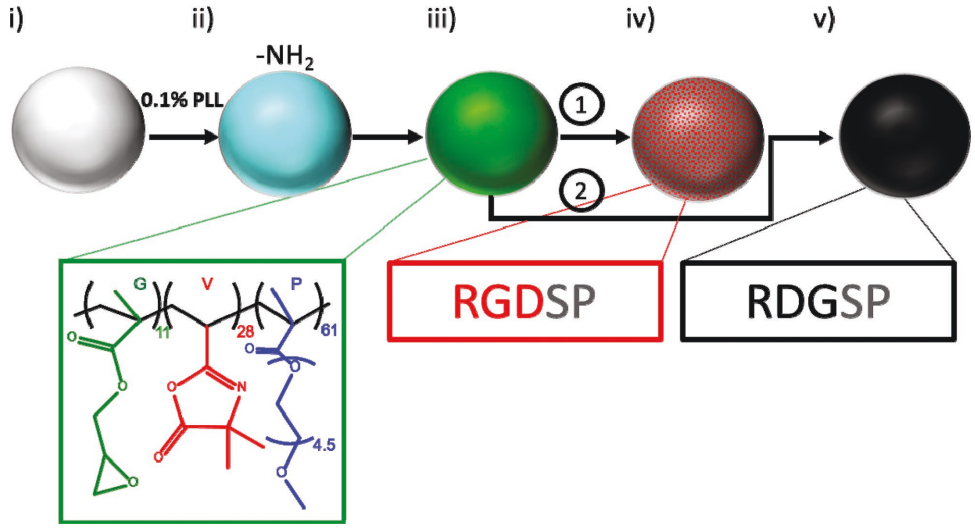

Figure 1. Schematic of coating process. i) Polystyrene microcarriers are coated in ii) PLL and iii) PVG. Desired peptides can be applied to this coating, in this example, a peptide functionalized with iv) Arg-Gly-Asp (RGD) and v) a scrambled version of the peptide.

methods, including the inability to provide a chemically defined surface for cell culture. A chemically defined surface can create a cell culture environment with homogeneous presentation of adhesive signals and repeatability that does not rely on the batch-to-batch variability[22] of natural protein matrices or adsorbed serum proteins, which can affect cell behavior and lineage determination.[23–25] A microcarrier that presents a cell-adhesive peptide on a chemically defined surface would potentially provide cell adhesion and expansion seen in commercially available microcarriers while also minimizing nonspecific adsorption of proteins present in cell culture media. This innovation represents a step toward the creation of a xeno-free cell culture system for the expansion of hMSCs. Xenogenic components of cell culture media, like fetal bovine serum (FBS), can introduce variability to the cell culture media.[26,27] Additionally, the use of cells that have been cultured in media containing FBS has been reported to create an immunological response to FBS proteins.[28,29]

Here, we present a method to convert existing microcarriers into chemically defined microcarriers by coating them with a copolymer. We recently developed and validated the chemically defined synthetic copolymer coating, poly(polyethylene glycol methyl ether methacrylate-*ran*-vinyl dimethyl azlactone-*ran*-glycidyl methacrylate) [P(PEGMEMA-*r*-VDM-*r*-GMA), hereafter referred to as PVG] in 2D, spin-coated onto flat substrates. When functionalized with the cell-adhesive peptide sequence Arg-Gly-Asp (RGD) this surface is suitable for hMSC adhesion and expansion.[30–32] Peptide conjugation through ring opening of the VDM monomer was optimized to 1 mM in phosphate buffered saline (PBS) solution at room temperature. The reported reaction efficiency is ≈70%.[31,32] We present a method here to covalently anchor the PVG copolymer to amine groups presented on the surface of existing PS-based microcarriers to create a chemically defined, customizable microcarrier. These PVG-coated microcarriers resist nonspecific protein adsorption and prevent the adhesion of cells. The coated microcarriers can then be functionalized with integrin-specific RGD peptide to restore adhesion and expansion of hMSCs.

2. Results

2.1. PVG Coating of Microspheres

In our previous work, we have shown that the PVG copolymer can be cross-linked to create a coating on a range of planar substrates including PS and the coating is stable under typical cell culture conditions.[30–32] On the planar substrate, control of the coating thickness and uniformity was achieved through spin rate and polymer concentration during spin coating. However, spin coating is not a viable option on the 3D microcarrier surfaces. Hence, to have a uniform coating on spherical microcarriers, we developed a process that used an anchoring layer to react with the epoxy groups in the PVG. The anchoring layer is a poly-L-lysine (PLL) layer which presents primary amines that readily react with the epoxy groups in the PVG. Using this method, the PVG copolymer coating was applied to both 2D and 3D PS substrates as described in the Experimental Section (**Figures 1** and **2**a). We prepared the analogous 2D surfaces in order to analyze the elemental content and reactivity of the anchored PVG. PVG layer was applied to a silicon wafer via the sequential anchoring process (Figure 2a). The silicon from the substrate provides an internal standard for absolute quantification of the elemental nitrogen content resulting from the overlying coating. After each stepwise addition of a nitrogen-containing material (PLL, PVG, RGD Peptide), the ratio of [atomic% nitrogen/atomic% silicon] increased, which confirms the successful addition of a nitrogen-containing layer (Figure 2b). Monitoring the water contact angle of the surface after each layer addition also provides a second means of verifying the chemistry. Starting with a planar PS surface, the water contact angle measurement decreased after PLL and PVG were added to the coating, which is representative of a more hydrophilic surface (Figure 2c, Figure S1, Supporting Information). The average water contact angle of 52.7° on a PVG-coated surface was consistent with the previously reported value of 59° for a 30 nm thick PVG coating, and 52° for 6 nm thick PEG brushes.[32] Finally, we used polarization modulation-infrared

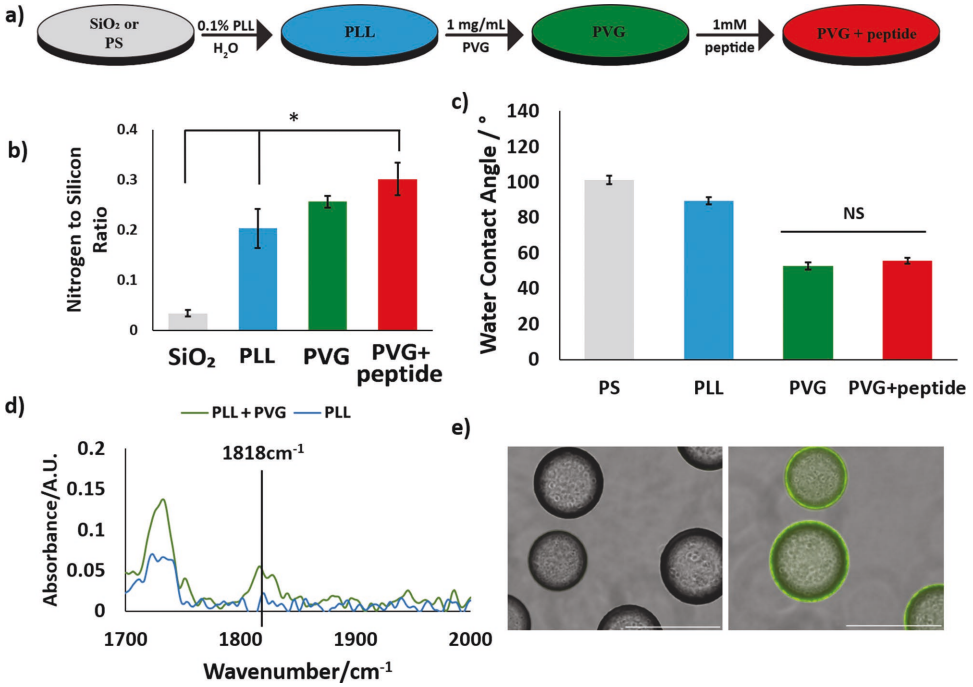

Figure 2. Characterization of the sequential anchoring PVG coating method. a) Schematic representation of sequential anchoring process on 2D surfaces. b) X-ray photoelectron spectroscopy (XPS) N/Si ratio increases in each step of the polymer coating process in 2D. *$p < 0.05$ by one-way ANOVA. c) Contact angle measurements of coated and uncoated PS samples. Significance of $p < 0.05$ except where noted. d) PM-IRRAS of PVG-coated slides shows an intact peak at 1818 (oxazoline ring). e) Fluorescence micrograph of uncoated (left) and PVG-coated (right) microcarriers. Scale bar = 200 µm.

reflection absorption spectroscopy (PM-IRRAS) to determine the mode of anchoring between the PVG and PLL layers. The two possibilities are that the reaction between PVG and PLL occurs by nucleophilic ring opening of the VDM ring by the primary amines in PLL, or the VDM stays largely intact and the anchoring occurs by ring opening to the GMA groups in PVG (Figure 2d). The PM-IRRAS studies require a gold-coated glass substrate, on which PLL does not readily adsorb. Hence, we first deposited a carboxylic acid–terminated self-assembled monolayer (SAM) of hexa(ethylene glycol) dodecane thiol (See Experimental Section). These SAMs then reacted with PLL through *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC)/*N*-hydroxysuccinimide (NHS) chemistry to bind PLL to the surface. The intact oxazoline ring present in VDM shows an absorbance at 1818 cm^{-1}. PLL-functionalized SAMs (blue line) did not have a peak at 1818 cm^{-1}, while PVG-functionalized surfaces (green line) showed a peak centered at 1818 cm^{-1}. This suggests that the PLL-PVG reaction is predominantly occurring through the ring opening of the epoxide in the GMA side chain under the conditions employed, leaving the VDM ring largely intact for further functionalization with peptides. Additionally, SAMs that were reacted with PVG but not PLL showed no detectable peak at 1818 cm^{-1}, which provided further evidence that the anchoring of PVG to the surface is PLL dependent. The lack of a peak at 915 cm^{-1} indicates that there is no detectable intact epoxide on these surfaces,[33] providing further evidence for the proposed mechanism. (full spectra and 800–1200 cm^{-1} inset in Figure S2, Supporting Information). Next, PVG-coated microcarriers were prepared and reacted with a fluorescent 5-sulfodichlorophenol (SDP) ester, to confirm the presence of the PVG coating via fluorescence microscopy (Figure 2e). In the absence of the PLL layer, the PVG coating did not attach to the microcarrier and hence did not fluoresce (Figure S3, Supporting Information). These results also suggest that the presence of PVG was not due to adsorption of the PVG layer to the microcarrier since the microcarriers did not fluoresce in the absence of the anchoring layer. Finally, we analyzed whether PLL desorbs from the surface of PVG-coated microcarriers. We prepared microcarriers using a fluorescently tagged PLL and incubated them in Minimum Essential Medium—Alpha Modification (αMEM) + 10% FBS for 7 days. The fluorescence of media incubated with fluorescently coated microcarriers did not increase above that of normal cell culture media, indicating no detectable desorption of PLL from the microcarrier surface (Figure S4, Supporting Information).

2.2. Cell Attachment to Microspheres

We used the sequential anchoring method to create PLL- and PVG-coated PS microspheres. The PVG-coated microcarriers were then split into three groups: unmodified PVG, PVG functionalized with an integrin-binding RGD, and PVG functionalized with a scrambled version of the RGD peptide (RDG, scram). hMSCs seeded onto microcarriers at a density of 10 000 cells cm^{-2} attached and grew for 24 h, at which point they were either fixed and fluorescently stained or lysed and analyzed for DNA content using the CyQuant Proliferation Assay kit. Representative images of the prepared microcarriers (**Figure 3**a), stained for actin and nuclei, showed adhesion to the surface of

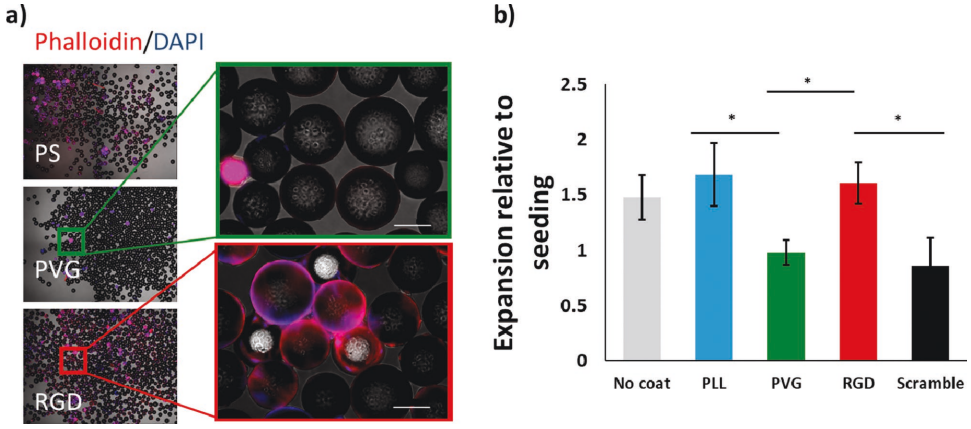

Figure 3. hMSCs attachment to microcarriers after 24 h. a) Representative images of PS, PVG-coated, and PVG + RGD microspheres at 4× (left) and 20× (right). Scale bar = 100 µm. b) hMSC expansion relative to seeding after 24 h as measured by DNA quantification. *$p < 0.05$ by ANOVA with a post hoc Tukey's test.

the PS, PLL (not shown), and RGD-functionalized microcarriers, while the PVG and scramble RDG-functionalized (not shown) conditions showed aggregates of cells adhered to one another but were not associated with a microcarrier.

hMSCs grown for 24 h in the PS, PLL, and RGD-functionalized conditions exhibited a significantly higher cell number than cells grown on the PVG and scramble-functionalized conditions (Figure 3b). The non-adherent PVG and scramble conditions maintained a DNA content similar to that of the number of cells that were seeded.

2.3. Cell Expansion and Differentiation on Microspheres

To study the potential for expansion of hMSCs in microcarrier culture, we seeded hMSCs onto PS, PLL-coated, and PVG-coated microcarriers functionalized with RGD. The number of cells increased in these conditions, as opposed to the PVG-coated and scramble peptide-coated microcarriers, in which the number of cells did not change over the course of 7 days (**Figure 4**a). PS, PLL-coated, and PVG-RGD microcarriers all achieved 6× expansion by 4 days, at which point the cell density was high enough to form cell-and-microcarrier aggregates—large clumps in which multiple microcarriers were held together by a mass of hMSCs (Figure 4b).

Additionally, hMSCs did not adhere to PVG-coated glass microcarriers but expanded on RGD-functionalized PVG-coated glass microcarriers, demonstrating the versatility of the sequential anchoring application of PVG (Figure S5, Supporting Information). To test whether hMSCs that had been cultured on PVG-RGD-coated microcarriers retained their potential to differentiate down multiple lineages, hMSCs were harvested from microcarriers with trypsin and mechanical agitation after 7 days of culture, then induced to either osteogenic or adipogenic differentiation. The differentiated cells stained positive for mineral deposits or lipid droplets, respectively, suggesting that hMSCs retained their capacity to differentiate to both osteoblasts and adipocytes, respectively, after expansion on microcarriers (**Figure 5**a,c). Cells cultured on all microcarrier conditions showed similar levels of lipid droplet or mineral deposition post differentiation (Figure 5b,d). The ability of hMSCs to differentiate post culture on PVG-RGD microcarriers

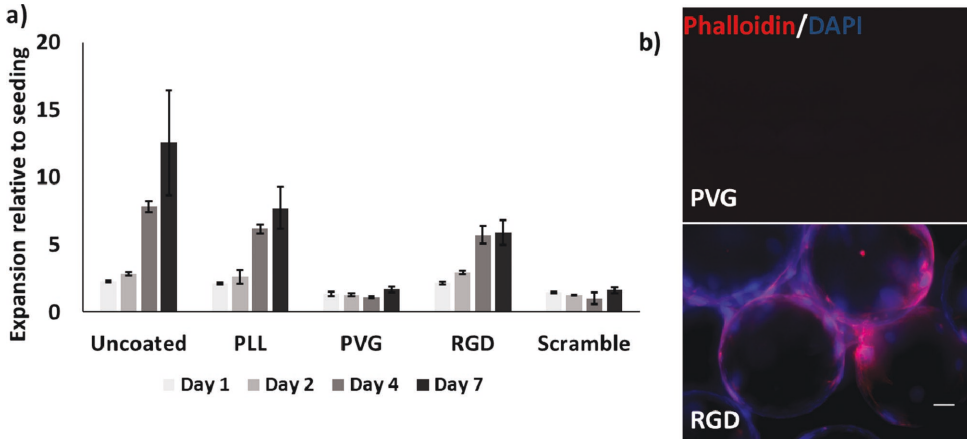

Figure 4. hMSCs readily expand on PVG-coated microcarriers functionalized with RGD. a) DNA content of hMSCs expanded on microcarriers and b) representative fluorescent maximum intensity projection of hMSCs with nuclei stained with DAPI (blue) and rhodamine phalloidin (red) after 7 days of growth on PVG-RGD microcarriers. Error bars are one standard deviation. Scale bar = 16 µm.

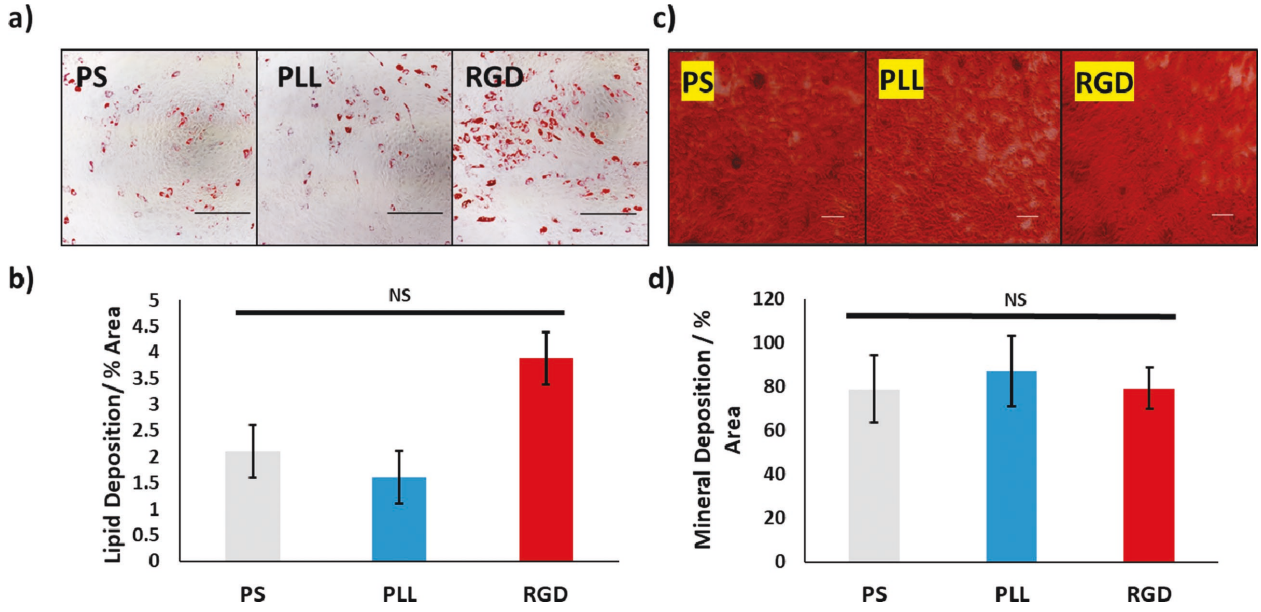

Figure 5. hMSCs expanded on microcarriers for 7 days appear to retain osteogenic and adipogenic differentiation capacity after harvesting. a) Micrograph of Oil Red O staining for lipid droplets on hMSCs differentiated to the adipogenic lineage. b) Quantification of lipid deposition. c) Alizarin Red S staining for mineral deposits on expanded hMSCs that were differentiated to the osteogenic lineage. d) Quantification of mineral deposition. Scale bar = 100 µm.

suggests that PVG-coated microcarrier culture does not cause a substantial loss of multipotency in hMSCs.

3. Discussion and Conclusion

The large number of clinical trials focused on using human cells in general and hMSCs in particular[8] underscores the need for cost effective, scalable, chemically defined, and reproducible cell culture methods. Previously, we have reported on a PEG-based, functionalizable coating for 2D surfaces, PVG.[30–32] In order to meet the needs of scalable cell manufacturing, here, we have developed a sequential anchoring mechanism to create PVG-coated microcarriers. The PVG coating permits on-demand functionalization with desired peptides in a one-step, aqueous reaction. We functionalized PVG-coated microspheres with an RGD-containing peptide and a scrambled version of the peptide. We have shown evidence for the mechanism of this application using fluorescence microscopy, X-ray photoelectron spectroscopy (XPS) and PM-IRRAS, which together suggest that the PVG is present on the surface of the microcarriers, chemically bound to the surface, and presents intact VDM rings for efficient conjugation of peptides.

hMSCs attached readily to RGD-functionalized microcarriers at levels similar to the commercially available PS microcarriers, but did not attach to PVG-coated microcarriers functionalized with a scrambled version of this peptide. Twenty-four hours post seeding, the DNA content in these non-adherent conditions indicated that the cells that had been seeded remained in culture (Figure 3b). However, cells in these conditions attached poorly and formed aggregates that were not attached to microcarriers (Figure 3a). These aggregates would be expected to have the same amount of DNA as the number of seeded cells, but would not be expected to survive and grow. As expected, the cells grown on blank PVG-coated and PVG-coated + RDG (scramble) functionalized microcarriers did not expand over time (Figure 4). Overall hMSC expansion on PVG-coated + RGD functionalized microcarriers was less than expansion on the uncoated PS microcarriers after 7 days, but expansion on the PS, PLL-coated, and PVG-coated + RGD functionalized microcarriers were comparable after 4 days. The decrease in hMSC proliferation on PVG-coated + RGD and PLL-coated microcarriers after 4 days can potentially be attributed to higher levels of microcarrier aggregation (Figure 4b). This decrease in cell expansion across all conditions could be due to aggregation of the microcarriers, which was substantial starting at day 4, especially in PVG-coated microcarriers compared to PS or PLL microcarriers. This increased aggregation may be driven by cells adhering to multiple microcarriers at once, forming a cluster. These clusters potentially reduce the amount of available surface area for cell growth, increasing the contact between cells leading to contact inhibition of proliferation, which halts the cell cycle and reduces cell division. To overcome this plateau in expansion, future studies will seek to optimize factors including microcarrier diameter, PVG concentration, and adhesion peptide density to maintain cell adhesion while reducing aggregation.

The ability to functionalize PVG-coated microcarriers with the desired peptides opens opportunities for driving growth of multiple cell types through the incorporation of peptides that react with different cell receptors. For example, microcarriers can be tailored for specific cell types using attachment peptides (e.g., Ile-Lys-Val-Ala-Val, IKVAV) or growth factor sequestering peptides to drive cell proliferation. Additionally, the incorporation of biologically active peptides to the PVG surface is simple and does not require harsh solvents—the reaction consists of an hour-long incubation with a cysteine-containing peptide at room

temperature—lending to its ease of use and eliminating the need for activation steps or harsh solvent conditions. The concentration of peptides needed for functionalization is very low, 1 mM, which will keep the cost of production low for large-scale applications such as those required for clinical applications. PVG-coated microcarriers also represent a chemically defined surface that does not rely on the adsorption of serum proteins to enable cell adhesion. While hMSCs were cultured in media containing FBS for this work, this surface represents a xeno-free culture surface and a step toward a completely xeno-free culture system. We are not aware of any other microcarrier for cell culture that is tailorable to unique applications in an aqueous, one-step process. As such, this development represents an important innovation in the field which could increase the ease with which this technology is adopted. Additionally, these PVG-coated microcarriers remain capable of facilitating hMSC adhesion after 1 month of storage, making them suitable for long-term use (Figure S6, Supporting Information). In this work, we demonstrated the addition of cell attachment functionality which supported hMSC multilineage differentiation capacity after expansion, suggesting that these microcarriers are a relevant platform for expanding cells while maintaining hMSC functionality.

4. Experimental Section

PVG Coating of Microcarriers: Untreated polystyrene microcarriers with diameter of 125–212 µm (Corning, Corning, NY) were weighed and incubated in 0.1 wt% 70 000–150 000 Da PLL (Sigma-Aldrich, Milwaukee, WI) for 1 h. PLL adsorbs to PS largely through hydrophobic interactions and its use was common in cell culture applications.[34–36] Microcarriers were then washed twice with deionized water and once with 200 proof ethanol (EtOH) (Pharmco-Aaper). Microcarriers were placed in a 10 mg mL^{-1} solution of PVG polymer in EtOH and allowed to react overnight. Microcarriers at this state were stored in EtOH at –20 °C for up to 1 month.

Stability of PLL: First, PLL hydrobromide (MW 70 000–100 000) (Sigma-Aldrich) was dissolved in 0.1 M bicarbonate buffer at a concentration of 10 mg mL^{-1}. Alexa Fluor 488 SDP Ester (Thermo Fisher, Waltham, MA) was added to the PLL solution according to manufacturer recommendations. Briefly, SDP ester was dissolved at 10 mg mL^{-1} in dimethyl formamide. The SDP ester solution was slowly added to the PLL solution to achieve a final concentration of 1 mg mL^{-1}. The reaction was allowed to proceed overnight under dark conditions. The reaction products were purified using a Slide-a-Lyzer dialysis kit with a pore size of 2000 molecular weight cutoff, dialyzed against distilled water overnight. The purified product was dissolved in water to create a 0.01 wt% solution. A dilution series of 0.01 wt% solution was used to measure the fluorescence intensity and construct the standard curve. The 0.01 wt% solution was used to create PVG-coated microcarriers as described previously. These microcarriers were incubated at 37 °C and 5% CO_2 for 1, 2, 4, or 7 days in αMEM + 10% FBS, at which point samples of the supernatant were taken and analyzed for fluorescence at an excitation/emission of 485/528 using a BioTek Synergy HTX (BioTek, Winooski, VT) plate reader.

Peptide Immobilization: PVG-coated microcarriers were washed twice with PBS and reacted with Cys-Gly-Gly-Gly-Arg-Gly-Asp-Ser-Pro (CGGGRGDSP, "RGD"), Cys-Gly-Gly-Gly-Arg-Asp-Gly-Ser-Pro (CGGGRDGSP, "scramble") peptides (Genscript). Microcarriers were incubated in 1 mM peptide solutions in 1× PBS (Fisher Scientific) for 1 h at room temperature according to the procedure in Schmitt et al. (2015, 2016). The microcarriers were then rinsed twice with PBS and sanitized in 70% ethanol for 30 min before use in cell culture.

Water Contact Angle: Polystyrene substrates were modified with PVG copolymer according to the procedure noted in the *PVG coating of microcarriers* section above. Water contact angle measurements were used to confirm the change in surface properties after modifying PS surfaces with PLL, PVG, and the RGD peptide. Measurements were taken using a Dataphysics OCA 15 Plus instrument with an automatic liquid dispenser. Static water contact angles were measured using 5 µL of deionized water in four different places on each sample. Two angles were taken for each droplet and were reported as the average plus or minus the standard deviation.

XPS: Elemental analysis of PVG functionalization of 2D surfaces was done using XPS. Measurements were taken using a Thermo Scientific Model K-Alpha XPS instrument with monochromatic Al Kα radiation (1486.7 eV). Survey spectra and high-resolution spectra were acquired using analyzer pass energies of 200 and 50 eV, respectively. Single point analysis was done on three separate points with a spot size of 400 µm for each point. Data was collected and analyzed in the Avantage XPS software package. Peak fitting was done with Gaussian/Lorentzian peak shapes and a Shirley/Smart background.

PM-IRRAS: SAMs of carboxylic acid–terminated hexa(ethylene glycol) dodecane thiol at a concentration of 1 mM in EtOH (ProChimia Surfaces, Gdansk area, Poland) were formed on gold substrates (1000 Å, EMF Corporation, TA134), 1 × 1″. The carboxylic acid was activated into a reactive ester using a solution of EDC, 100 mM (Sigma-Aldrich) and NHS, 250 mM (Sigma-Aldrich). The 0.1 wt% PLL in distilled water was covalently bound to the surface through its *N*-terminal amine. PVG (10 mg mL^{-1} in EtOH) was placed on the surface and allowed to react overnight. Samples were rinsed three times with EtOH and dried under N_2. The samples were then placed at an incident angle of 83° in a Nicolet iS50 Fourier transform IR spectrophotometer equipped with a photoelastic modulator (PEM-90, Hinds Instruments, Hillsboro, OR), a synchronous sampling demodulator (SSD-100, GWC Technologies, Madison, WI), and a liquid nitrogen cooled mercury-cadmium-telluride detector. The modulation was set at 1600 cm^{-1} and 1000 scans were obtained for each sample with a resolution of 8 cm^{-1}. The aperture was set to a size of 10 in the OMNIC software, corresponding to a spot size less than 5 mm. The differential reflectance IR spectra were then normalized and converted to absorbance spectra using OMNIC software.

hMSC Culture and Cell Quantification: hMSCs were cultured in Minimum Essential Medium—Alpha Modification (Corning, Corning MA) plus 10% FBS (Gibco, Cat. #16000-044, Dublin, Ireland). To evaluate hMSC attachment to PVG-coated and functionalized microcarriers, passage 4–6 hMSCs were seeded (10 000 cells cm^{-2}) on PS, PLL-coated, PVG-coated, RGD functionalized, and scramble functionalized microcarriers, prepared as previously described. After 24 h, the cells were either fixed, stained, and imaged or lysed for total DNA quantification using a CyQUANT Cell Proliferation Assay Kit (Thermo Fisher Scientific, Waltham, MA). Fluorescence from the CyQUANT assay was read at an emission of 527 nm using a BioTek Synergy HTX plate reader. Cell standards were normalized to the intensities of known numbers of hMSC.

To study hMSC expansion on functionalized microcarrier surfaces, hMSCs were grown for up to a week in each of the different coating conditions. At desired time points (1, 2, 4, and 7 days), cells were lysed, and total DNA was quantified using a CyQUANT Cell Proliferation Assay Kit, per kit instructions. Cell expansion was calculated by dividing the cell number on each day by the number of cells initially seeded on the microcarriers.

Fluorescent Imaging: hMSCs: hMSCs were washed with 1× PBS and fixed in 10% buffered formalin 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 (BSA) (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) for 30 min each, washing in between with PBS. Cells were imaged on an inverted microscope with DAPI, fluorescein isothiocyanate (FITC), and Far-Red filter cube sets.

PVG-Coated Microcarriers: PS microspheres were prepared by incubating in PBS or 0.1% PLL for 1 h at room temperature. PVG (10 mg mL^{-1}

in EtOH) reacted with the surface overnight. Microcarriers were then stained with Alexa Fluor 488 SDP ester for 1 h and imaged on an inverted microscope with a FITC filter cube set.

hMSC Differentiation and Analysis: To evaluate differentiation capacity after expansion on coated microcarriers, hMSCs were differentiated to osteoblasts and adipocytes based on established protocols. For differentiation, hMSCs were seeded at 5000 cells cm^{-2} on collagen-coated plates (Corning, Corning, NY) in 10% FBS in αMEM, and permitted to grow to confluence for 3 days. Osteogenic (OS) medium and adipogenic induction medium (AIM) were prepared. OS medium consisted of 10% FBS in αMEM with 0.1 μM dexamethasone, 10 mMβ glycerol phosphate, and 50 μM ascorbic acid 2-phosphate. AIM consisted of 10% FBS in Dulbecco's Modification of Eagle's Medium (DMEM) high glucose with penicillin (100 U mL^{-1})/streptomycin (100 μg mL^{-1}), 1 μM dexamethasone, 10 μg mL^{-1} insulin, and 500 μM isomethyl isobutyl xanthine (IBMX). Media was changed every 3–4 days, and analysis was performed after 21 days of differentiation. As negative controls, cells were grown for 21 days in 10% FBS in αMEM.

Alizarin Red S stained mineral deposits from osteoblasts, and Oil Red O stained lipid droplets in adipocytes. To perform staining, cells were fixed in 10% buffered formalin solution and incubated Alizarin Red S (40 mM, pH 4.1–4.3) and washed with water three times or Oil Red O working solution for 20 min and washed with water until washings were clear. Working Oil Red O solution was prepared by mixing three parts stock Oil Red O solution (3 mg mL^{-1} in 99% isopropanol) with two parts distilled water and filtering with a 0.2 μm syringe filter.

Statistical Analysis: Experiments were carried out and repeated a total of two to three trials, with $n = 4$ replicates per trial. Except where noted, a one-way ANOVA was conducted to determine significance, as there are multiple groups with one independent variable. A post hoc Tukey's test was then used to determine significance between groups.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

This research was funded by the National Science Foundation (NSF DMR 1709179). The authors would like to acknowledge support from staff and the use of equipment at the Materials Science Center at UW–Madison DMR-1121288 and DMR-1720415.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

cell culture, chemically defined, mesenchymal stem cells, microcarriers, polymer

Received: August 7, 2018
Revised: October 12, 2018
Published online: November 22, 2018

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