Effects of Methacrylate-Based Thermoresponsive Polymer Brush Composition on Fibroblast Adhesion and Morphology

CHRISTOPHER R. ANDERSON,¹ CARA ABECUNAS,¹ MATTHEW WARRENER,¹ ANDRÉ LASCHEWSKY,²
and ERIK WISCHERHOFF²

¹Department of Chemical and Biomolecular Engineering, Acopian Engineering Center, Lafayette College, 740 High Street, Easton, PA 18042, USA; and ²Fraunhofer Institute for Applied Polymer Research, Geiselbergstr. 69, 14476 Potsdam-Golm, Germany

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Abstract—Thermoresponsive polymers are being used increasingly in cell culture applications due to their temperature dependent surface properties. Poly(MEO₂MA-co-OEGMA) (PMO) brushes offer tunable physical properties *via* variation in the copolymer ratio, but the effects of composition on cell-substrate interactions is unclear. To this end, a series of PMO brushes (0–8% OEGMA) was fabricated and L-929 fibroblast adhesion and morphology was quantified in the presence of serum (FBS) or after functionalization *via* the adsorption of fibronectin (FN) and vitronectin (VN). Quantification of the adsorption of model proteins, bovine serum albumin and FN, revealed that the extent of adsorption was correlated to the amount MEO₂MA content, which represents the more hydrophobic component in PMO brushes. Cells exhibited delayed attachment and spreading on all PMO substrates in the presence of FBS. After 24 h, cell attachment was comparable; however, increased spreading was correlated with increased MEO₂MA content. Adsorption of FN significantly increased initial cell attachment to all PMO surfaces after 2 h. This was not observed with VN; however, both FN and VN increased cell spreading/decreased cell circularity for all PMO substrates relative to FBS. Pure MEO₂MA brushes with FN exhibited increased cell spreading/decreased cell circularity relative to other PMO substrates after 2 h, and elicited the highest cell density after 24 h. These results demonstrate that increased MEO₂MA content in PMO substrates facilitates cell attachment and spreading, which can be further enhanced by adsorbing FN in the absence of other proteins.

Keywords—Smart polymers, Stimulus-responsive, Oligo(ethylene glycol) methacrylates, Biomaterials, Protein adsorption, Fibronectin, Vitronectin.

ABBREVIATIONS

BSA	Bovine serum albumin
ECM	Extracellular matrix
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FN	Fibronectin
LCST	Lower critical solution temperature
MEO ₂ MA	2-(2-Methoxyethoxy)ethyl methacrylate
NTBAM	<i>N</i> -tert-butylacrylamide
OEGMA	Oligo(ethylene glycol) methacrylate
PDADMAC	Poly(diallyl dimethyl ammonium chloride)
PEG	Poly(ethylene glycol)
PEI	Poly(ethyleneimine)
PMO	Poly(MEO ₂ MA-co-OEGMA)
PNIPAM	Poly(<i>N</i> -isopropylacrylamide)
PSS	Poly(styrene sulfonate)
TRP	Thermoresponsive polymer
VN	Vitronectin

INTRODUCTION

Thermoresponsive polymers (TRP) have garnered significant interest in diverse biomedical applications due to their temperature dependent physico-chemical properties. TRPs have been used extensively as cell culture substrates as their “switchable” properties enable control of the cell-polymer surface interface.¹⁰ TRPs that are utilized for cell culture exhibit a change in molecular conformation in an aqueous environment at a lower critical solution temperature (LCST). Specifically, the TRPs are miscible in water below the LCST and precipitate from solution as the temperature is raised above the LCST. For TRPs with an LCST

Address correspondence to Christopher R. Anderson, Department of Chemical and Biomolecular Engineering, Acopian Engineering Center, Lafayette College, 740 High Street, Easton, PA 18042, USA. Electronic mail: anderscr@lafayette.edu

below 37 °C, the polymer exhibits a collapsed, hydrophobic conformation that facilitates cell adhesion and proliferation in standard cell culture environments.^{10,13} However, when TRP substrates are subsequently introduced to a temperature below the LCST, the polymers extend as they switch to a miscible state and adherent cells are mechanically detached from the polymer substrate.^{5,52,55} The detachment of cells from TRP substrates preserves cell–cell and cell–matrix interactions that would be otherwise compromised during protease-mediated chemical detachment (e.g. trypsin) from traditional tissue culture plastic.^{6,7,23}

The most well studied TRP is poly(*N*-isopropylacrylamide) (PNIPAM), which exhibits an LCST of 32 °C in water¹⁸ and is relatively insensitive to other environmental conditions such as pH and ion concentration.³⁵ Consequently, PNIPAM has been used extensively in cell culture applications and has enabled culture of endothelial cells,²³ epithelial cells,³¹ and myoblasts.³⁹ However, it has been demonstrated that the NIPAM monomer is cytotoxic and there is increased concern regarding the viability of cells exposed to PNIPAM extracts.^{12,49} An alternative class of TRPs has been described that incorporates short oligo(ethylene glycol) side chains into the macromolecular structure and copolymerization approaches with these monomers elicit variable LCSTs.²⁸ Random copolymers of 2-(2-methoxyethoxy)ethyl methacrylate (MEO₂MA) and oligo(ethylene glycol) methacrylate (OEGMA) exhibit LCST values between 26 and 90 °C in water.^{25–27} The physico-chemical properties of poly(MEO₂MA-co-OEGMA) (PMO) can be finely tuned by altering the composition of MEO₂MA to OEGMA monomers in the PMO polymer synthesis,²⁶ which presents an opportunity for optimizing cell–substrate interactions in cell culture applications. Further, the integration of the oligo(ethylene glycol) chains into the PMO structure imparts the nontoxic and nonimmunogenic properties of the widely used poly(ethylene glycol) (PEG), while maintaining temperature dependent “switching” of physical properties that is independent of other external conditions.²⁵ The advantages of the PMO system have led to its use for cell culture and PMO-coated gold substrates^{46,52} and glass substrates⁵³ have been shown to support cell adhesion above the LCST and detachment upon thermal switching below the LCST. Moreover, a recent study demonstrated the biocompatibility and cell attachment of murine fibroblasts to PMO substrates (LCST of 34 °C) for several successive cell culture cycles in serum-containing media.³⁷

The introduction of polymer substrates to serum containing cell culture media results in rapid adsorption of proteins to the solid surface, which regulates, in

part, the subsequent cellular responses.⁵¹ Adsorbed proteins provide the structural scaffold for cell attachment and transmit essential biochemical signals that mediate cell migration, proliferation, and differentiation. Indeed, the adsorption of serum proteins has been shown to be essential for cell attachment to PNIPAM brushes.^{1,56} PMO brushes with a MEO₂MA:OEGMA molar ratio of 94:6 (LCST of 34 °C) exhibit limited cell attachment after 1 h in culture; however, cell attachment is enhanced significantly by repeated thermal-switching in the presence of serum proteins.³⁷ Further, cells attached to PMO brushes significantly upregulate the extracellular matrix (ECM) protein fibronectin (FN), known to promote cell adhesion and spreading, after 48 h in culture.³⁷ Interestingly, the upregulation of FN is attenuated in cells attached to PMO brushes that were subjected to multiple thermal-switching cycles in the presence of serum proteins. These results suggest that protein adsorption has a key role in mediating initial cell attachment to PMO substrates and adherent cells may regulate gene expression to render the surfaces more conducive to cell adhesion and spreading.

PMO brushes have shown to be viable TRP cell culture substrates; however, the cellular responses to PMO have not yet been fully elucidated. Polymer composition is an important mediator of protein adsorption and subsequent cellular interactions,^{3,45,54} and therefore, may be an important consideration in the utility of PMO substrates for cell culture applications. Further, the effectiveness of PMO substrates may be enhanced by selective adsorption of the extracellular matrix proteins, FN and vitronectin (VN), which are known to promote cell adhesion and growth *in vitro*.^{42,43,48} The objective of this study was to systematically investigate cell attachment and morphology in response to PMO brushes with variable composition in the presence of serum proteins, FN, and VN. A series of PMO substrates with physiologically relevant LCST values ranging from 28 to 37 °C (0–8% OEGMA) was fabricated and the attachment and morphology of murine L-929 fibroblasts was quantified in the presence of serum proteins or after surface functionalization *via* the adsorption of FN and VN. The cellular responses to PMO substrates were correlated to the adsorption of model proteins, BSA and FN. Owing to its widespread use in cell culture applications, commercially available PNIPAM-based substrates were used as a reference. The results of this study reveal the significance of tuning PMO composition to dictate cell–material interactions and suggest that PMO brushes should be functionalized with biologically active proteins that promote cell adhesion, spreading, and growth over time.

MATERIALS AND METHODS

Fabrication of Poly(MEO₂MA-co-OEGMA) Substrates

PMO substrates were synthesized by surface-initiated copolymerization of various ratios of 2-(2-methoxyethoxy)ethyl methacrylate (MEO₂MA; Sigma-Aldrich, St. Louis, MO) and oligo(ethylene glycol) methacrylate (OEGMA, $M_n = 475$ g/mol; Sigma-Aldrich) according to previously described methods.⁵³ Briefly, multilayer sequences of {PEI/PSS}/{PDADMAC/PSS}₄/PDADMAC/MA01} were assembled onto glass coverslips *via* “layer-by-layer” deposition and MEO₂MA-co-OEGMA polymers were grafted to the MA01 macroinitiator layer by atom transfer radical polymerization (ATRP). The MA01 macroinitiator contains sulfonate and ATRP initiator groups and the reaction time was 60 min. PMO substrates were fabricated with variable MEO₂MA:OEGMA monomer ratios in copolymer synthesis, and the OEGMA content ranged from 0 to 8% by mole. Figure 1 outlines the monomer ratios and corresponding LCST in PBS for poly(MEO₂MA-co-OEGMA) substrates used in this study. Substrates are referred to as “PMO” in the text and the variable copolymer composition is indicated by the corresponding LCST (e.g. PMO 28, PMO 31, *etc.*). Thermoresponsive PNIPAM substrates were acquired *via* commercially available Nunc 6-well multiwell dishes with UpCell™ Surfaces (Thermo Scientific, Logan, UT).

Cell Culture

Murine L-929 fibroblast cells (ATCC, Manassas, VA) were grown in polystyrene flasks (Nunc; Thermo Scientific) in Dulbecco's Modified Eagle's medium (DMEM) (Lonza, Walkersville, MD), supplemented with 10% fetal bovine serum (FBS) (HyClone, Thermo Scientific), and 1% penicillin/streptomycin (Lonza) in a 5% CO₂ environment at 37 °C. Cell culture media was replaced every 48 h. Cells were removed from flasks by trypsinization with trypsin/EDTA (Lonza) and centrifuged before resuspension in cell culture media.

Cell Seeding

Glass coverslips grafted with PMO brushes were placed in 6-well plates and incubated with pure ethanol for 15 min and subsequently washed with sterile water three times for 5 min at room temperature. Sterilized PMO brushes were allowed to dry and were transferred into new 6-well plates. L-929 fibroblasts were seeded on either PMO or PNIPAM substrates at a density of

25,000 cells/cm² and maintained in complete growth media at 37 °C for 2, 6 or 24 h. Alternatively, PMO and PNIPAM substrates were pretreated with DMEM supplemented with 10% FBS, recombinant murine fibronectin (40 µg/mL; R&D Systems, Minneapolis, MN), or recombinant murine vitronectin (2 µg/mL; R&D Systems) for 2 h at 37 °C to enable equilibrium protein adsorption to the substrates prior to cell seeding. After 2 h, solutions were aspirated and substrates were rinsed with warm PBS. Cells were subsequently seeded at a density of 25,000 cells/cm² and maintained in serum-free DMEM at 37 °C for 2, 6 or 24 h.

Quantification of Protein Adsorption

PMO and PNIPAM substrates ($n = 4$) were incubated in Dulbecco's Modified Eagle's medium (DMEM) supplemented with either fluorescein isothiocyanate (FITC) labeled bovine serum albumin (BSA; 500 µg/mL, Sigma) or HiLyte Fluor™-488 labeled fibronectin (40 µg/mL; Cytoskeleton, Inc., Denver, CO) for 2 h at 37 °C. After 2 h, media was aspirated and substrates were washed twice with PBS. PMO substrates were then transferred to new 6-well plates. Adsorbed proteins were desorbed from polymer surfaces by incubation with a protein elution buffer consisting of SDS (3 wt%) and DTT (1 mg/mL) in urea (8 M) overnight at 37 °C (all reagents from Sigma). The fluorescence intensity of desorbed protein solutions was measured with a multimode plate reader (BioTek Synergy), and protein concentrations were calculated from standard curves obtained from serial dilutions. Results are presented as average protein density (µg/cm²) ± standard deviation. Statistical analysis was done with the one-way analysis of variance (ANOVA) test followed by a *post hoc* Tukey's test with a 95% confidence interval.

Cell Imaging and Quantitative Analysis of Cell Morphology

At the time of experiment, phase contrast images of cells were acquired with an inverted optical microscope (Nikon Eclipse TS100) to observe cell morphology. Images were analyzed with NIS Elements software (Nikon; Basic Research version 3.1) to quantify cell spreading and cell circularity. The total number of cells (≈ 50 -150) in each image ($n = 6$) was counted manually with the software, as well as the number of non-circular cells that exhibit observable protrusions extending from cell bodies. The percentage of spread cells was calculated as follows:

FIGURE 1. Schematic of cell culture on thermoresponsive poly(MEO₂MA-co-OEGMA) substrates. (a) PMO substrates with varying co-monomer ratios exhibit a higher LCST and lower relative hydrophobicity as the OEGMA content increases. (b) Substrates below the LCST at room temperature are hydrophilic and are protein/cell resistant. (c) Substrates above the LCST at 37 °C are hydrophobic and facilitate protein adsorption and cell adhesion.

$$\% \text{ Cell Spreading} = \frac{\text{Cells with Protrusions}}{\text{Total Cells}} \times 100\%$$

The area and perimeter of each cell was also measured with the software. The circularity of each cell was calculated with the following equation:

$$\text{Cell Circularity} = \frac{4\pi * \text{Area}}{\text{Perimeter}^2}$$

Cell circularity values range from 0 to 1. A cell circularity value of 1 indicates a round morphology, and circularity values approach 0 as cells are more spread and elongated. Results are presented as averages $\pm$ standard deviation. Statistical analysis was done with the one-way analysis of variance (ANOVA) test followed by a *post hoc* Tukey's test with a 95% confidence interval.

Quantification of Cell Attachment

At the time of experiment, growth media was aspirated and nonadherent cells were removed from polymer substrates by rinsing twice with warm PBS. PMO-coated coverslips were transferred to new 6-well plates. PMO and PNIPAM substrates ($n = 4$) were then incubated with fresh DMEM supplemented with 2% FBS and the MTS-based CellTiter 96[®] Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI) was used according to manufacturer's instructions to quantify the number of adherent cells. Absorbance values were measured at 490 nm with a multimode plate reader (BioTek Synergy). Statistical analysis was done with the one-way analysis of variance (ANOVA) test followed by a *post hoc* Tukey's test with a 95% confidence interval.

RESULTS

L-929 murine fibroblasts, widely used in cell adhesion and biocompatibility assays, were used as a model cell line^{20,36,37,53,55} to investigate cell attachment and morphology to PMO brushes with variable composition. PMO brushes with varying MEO₂MA: OEGMA ratios were fabricated as previously described²⁶ and used to investigate cell attachment relative to commercially available UpCell[™] substrates, which are coated with the well-characterized thermoresponsive polymer PNIPAM. Figure 1 illustrates the variable composition and thermoresponsive behavior of the PMO brush system. The ratio of MEO₂MA: OEGMA in the copolymer synthesis can be tuned to manipulate the LCST and other physical properties of the copolymer system (Fig. 1a). Here, we utilized copolymer ratios (0–8% OEGMA) that elicit LCST values between room temperature (≈ 23 °C) and cell culture conditions (37 °C). The LCST is linear with the mole fraction of OEGMA²⁶; hence, an increase in LCST from 28 to 37 °C reflects an increase in OEGMA content. Additionally, OEGMA is less hydrophobic than MEO₂MA; therefore, the relative hydrophobicity of the PMO brushes decreases with increasing the OEGMA content.¹⁵ PMO brushes grafted to glass coverslips enable cell culture on polymer substrates with temperature dependent properties. Polymers below the LCST are hydrophilic, rendering surfaces protein and cell repellent (Fig. 1b), whereas above the LCST, polymer brushes exhibit a hydrophobic, collapsed conformation that facilitates protein adsorption and cell attachment (Fig. 1c). Representative images demonstrating the thermally driven changes in PMO adhesive properties are shown in Supplemental Fig. 1.

Subsequent to cell adhesion at 37 °C, temperature transitions below the LCST causes extension of the polymer brush as it becomes miscible in the aqueous phase, which leads to cell detachment. Images of L-929 fibroblast adhesion to PMO substrates at 37 °C, as well as 30 min after exposing adherent cells to room temperature, are shown in Supplementary Fig. 2.

Cell adhesion and morphology on PNIPAM and PMO brushes was observed with phase contrast microscopy. Figure 2 displays optical images of adherent cells 2, 6, and 24 h after cell seeding in cell culture media with 10% FBS. Cells demonstrated a delayed

initial attachment to PMO brushes of all compositions relative to PNIPAM at 2 and 6 h after seeding. At 24 h, PNIPAM and PMO brushes elicited similar cell attachment; however, an increase in rounded cell morphology was observed as the LCST of PMO brushes increased from 28 to 37 °C. Moreover, PMO 28 showed extensive cell spreading, while PMO 37 showed cells maintained a primarily rounded morphology at 24 h. Proteins adsorbed from serum-containing media impart an intermediate layer to cell culture substrates that provides a scaffold for cell adhesion and spreading.⁵¹ Bovine serum albumin (BSA), the most preva-

FIGURE 2. Phase contrast optical images of L-929 fibroblasts cultured on TRP substrates for 2–24 h in media with 10% FBS. Cells exhibit an initial delay in cell attachment to PMO substrates relative to PNIPAM. Cell attachment to all substrates is comparable at 24 h; however, cell spreading is less prevalent for PMO substrates with increasing OEGMA content and LCST. Scale bar = 100 μ m.

FIGURE 3. Protein adsorption and cell density of L-929 fibroblasts on TRP substrates. (a) Substrates were incubated with fluorescent BSA (500 µg/mL) for 2 h at 37 °C. The extent of BSA protein adsorption is correlated to PMO composition. * indicates the density of adsorbed protein is significantly different from all other substrates ($p < 0.05$). (b) Initial cell attachment to all PMO substrates is significantly less than PNIPAM at 2 and 6 h (** $p < 0.05$); however, there are no statistical differences in cell density between any substrates at 24 h. Initial cell attachment to PMO 28 substrates after 2 h was significantly higher relative to all other PMO substrates (# $p < 0.05$), indicating enhanced attachment to the most hydrophobic PMO surface. Dotted line indicates the cell seeding density (25,000 cells/cm²).

lent serum protein, was used as a model protein to investigate the effects of variable PMO composition on protein adsorption. Figure 3a shows the density of BSA adsorbed to PNIPAM and PMO brushes after 2 h at 37 °C. The extent of BSA adsorption is significantly higher on PMO 28 brushes relative to other substrates, and decreases with increasing LCST for PMO substrates. PMO 37 elicited significantly less BSA adsorption than all other substrates, and was reduced by 82% relative to PMO 28. The cell density on all substrates is quantified over 24 h in Fig. 3b. Initial cell attachment to PNIPAM substrates was substantial, as 88 to 97% of seeded cells were adherent after 2 and 6 h, respectively. The cell densities observed on PNIPAM substrates were significantly higher rela-

FIGURE 4. Quantitative cell morphology of L-929 fibroblasts on TRP substrates after 24 h. (a) Percentage of attached cells showing spreading, as indicated by observable protrusions extending from cell bodies. (b) Average cell circularity. Values range from 0-1, where a circularity of 1 indicates perfect circularity. Increased OEGMA content and LCST in PMO substrates are correlated with less cell spreading and an increase in cell circularity. Experimental groups that share significance are grouped together; otherwise, data are significantly different (* $p < 0.05$).

tive to all PMO brushes at 2 h, which supported attachment of less than 30% of seeded cells. The initial cell attachment to PMO 28 brushes was also significantly higher than all other PMO brushes at 2 h, which demonstrates that cells have the highest affinity for the most hydrophobic PMO surface (100% MEO₂MA: 0% OEGMA). The cell density on all PMO brushes increased after 6 h, although still significantly less than that observed on PNIPAM. After 24 h, cell density was equivalent on PNIPAM substrates and all PMO brushes.

Figure 4 shows a striking difference in quantitative cell morphology on PNIPAM and PMO substrates after 24 h. PNIPAM and the most hydrophobic PMO brushes (PMO 28 and PMO 31) exhibit significantly

greater cell spreading, as characterized by the observation of cellular protrusions from cell bodies, compared to less hydrophobic PMO substrates (PMO 34 and PMO 37) comprised of increased amounts of OEGMA (Fig. 4a). Over 90% of attached cells exhibited cell spreading on PNIPAM, PMO 28, and PMO 31 substrates. In contrast, only 72 and 59% of cells were spread on PMO 34 and PMO 37 brushes, respectively. Substrate composition had a more pronounced effect on cell circularity (Fig. 4b), which is an alternative metric that also reflects the extent of cell spreading.^{3,54,56} This metric has been employed to characterize cellular responses to other polymeric substrates with variable composition,³ chain density,⁵⁶ and terminal functional groups.⁵⁴ Circularity values that approach unity are indicative of a round, circular cell morphology, whereas decreased circularities indicate a more elongated, spread morphology. Cell circularity clearly trends with increased OEGMA content and LCST for PMO substrates, which further indicates hindered cell spreading on the less hydrophobic substrates.

The adsorbed protein layer includes essential extracellular matrix proteins that mediate cell signaling events upon interactions with biological systems, and consequently, are a critical determinant of the cellular responses to cell culture substrates. Thus, the initial cell attachment and subsequent spreading on PMO brushes may be enhanced by selective adsorption of bioactive proteins that facilitate these cellular functions. To this end, PMO brushes were preconditioned with FN or VN in the presence or absence of FBS, and cell attachment and morphology was compared to similarly treated PNIPAM substrates. Figure 5 shows the cell density 2 h post-seeding for PNIPAM and PMO substrates functionalized with FN or VN. The initial cell attachment was significantly increased by the adsorption of FN in both the presence and absence of FBS for all PMO brushes (Fig. 5a). Interestingly, cell attachment to PNIPAM substrates incubated with only FN was significantly less than that on PNIPAM treated with FN in the presence of FBS or FBS alone. This attenuation in cell attachment was also observed on PNIPAM substrates incubated with VN in the absence of FBS (Fig. 5b). In contrast to FN, preconditioning with VN did not significantly affect cell attachment to any PMO brushes compared to FBS. As illustrated in Figs. 2 and 5, PMO brushes support less than 40% of the cell attachment observed on PNIPAM in media with serum proteins (Fig. 5c). For PMO brushes preconditioned with either FN or VN, the percentage of attached cells compared to PNIPAM decreases as the OEGMA content increases. PMO 28 and PMO 31, which represent the most hydrophobic surfaces, show statistically comparable cell attachment

FIGURE 5. Initial cell attachment to TRP substrates preconditioned with extracellular matrix proteins after 2 h. (a) Cell density for substrates incubated with 10% FBS, murine fibronectin (FN; 40 µg/mL) or both (FBS + FN) overnight at 37 °C. (b) Cell density for substrates incubated with 10% FBS, murine vitronectin (VN; 2 µg/mL) or both (FBS + VN) overnight at 37 °C. (c) Percentage of cell attachment to PMO substrates relative to PNIPAM for FBS, FN, or VN treated surfaces. Experimental groups that share significance are grouped together; otherwise, data are significantly different (* $p < 0.05$).

relative to PNIPAM treated with FN. For brushes treated with VN, only PMO 28 brushes show the same level of cell attachment compared to PNIPAM.

FIGURE 6. Quantitative cell morphology on TRP substrates pre-conditioned with extracellular matrix proteins after 2 h. A: Percentage of cells spread on substrates incubated with 10% FBS, FN, or VN. B: Average cell circularity for substrates incubated with 10% FBS, FN, or VN. Both * and # indicate data are significant at $p < 0.05$.

The morphology of cells attached to FN and VN treated substrates after 2 h is quantified in Fig. 6. Both FN and VN elicit a significant increase in cell spreading for all substrates; however, the increased cell spreading is most evident for FN treated PMO 28 substrates, as 89% cells are spread relative to 65% and 6% for VN and FBS treated surfaces, respectively. Cell circularity is significantly decreased after 2 h on all PMO substrates incubated with FN or VN compared to FBS. For both PNIPAM and PMO 28 substrates, incubation with FN leads to significantly lower cell circularity values compared to VN. Taken together, these results suggest that FN promotes enhanced cell attachment and spreading on PMO 28 substrates relative to FBS or VN, and has a comparable effect to

VN for substrates with increased OEGMA content (PMO 31-PMO37).

The adsorption of FN was utilized to promote cell attachment in the absence of other proteins and to further study cell-substrate interactions over time. Figure 7 illustrates the effect of FN adsorption to PNIPAM and PMO substrates on cell attachment and morphology over the course of 24 h in culture. Although cell density is comparable on all substrates after 2 h, PMO brushes with increased OEGMA content show less cell spreading. The cell density and spreading increase after 6 and 24 h in culture on all substrates, however, cells still retain a relatively rounded morphology on the most hydrophilic PMO surfaces (i.e. increased LCST). The extent of FN adsorption and the kinetics of cell growth and morphology on FN-coated substrates are quantified in Fig. 8. As observed with BSA (Fig. 3a), FN adsorption on PMO 28 was significantly greater than other PMO substrates and PNIPAM (Fig. 8a). Similarly, the extent of FN adsorption was attenuated with increasing LCST and OEGMA content. The adsorption of FN to all substrates led to comparable initial cell attachment, as 85–90% of seeded cells were adherent after 2 h (Fig. 8b). PMO composition had a significant effect on cell proliferation, as the cell density on FN-treated PMO 28 substrates increased 1.7 fold after 24 h. This is significantly greater than all other surfaces, which ranged from a 1.5 fold increase for PMO 31 substrates to a 1.2 fold increase for PNIPAM. PMO 28 also exhibited significantly greater initial cell spreading after 2 h relative to other FN treated PMO substrates (Fig. 8c). Cell spreading on PMO 28 and PNIPAM were statistically similar after 2 h. Cell spreading increased over time and was comparable for all substrates after 6 and 24 h. As expected with increased spreading, cell circularity decreased over time for all substrates; however, PMO 28 and PNIPAM showed the lowest circularity values after 24 h (Fig. 8d). For PMO 28 surfaces treated with FN, cell circularity decreased from 0.55 to 0.35 over 24 h. In contrast, PMO 37 surfaces showed a decrease in cell circularity of 0.55 to 0.46.

DISCUSSION

TRPs, such as PNIPAM and MEO₂MA-based copolymers, have received significant interest in cell culture applications and increased efforts are being made to direct cellular responses *via* the design of substrates with specific physical properties.⁴⁷ Cellular responses to polymeric substrates are governed by an array of factors including elasticity, hydrophobicity, charge, surface chemistry, and surface roughness; any

FIGURE 7. Phase contrast images of L-929 fibroblasts cultured on TRP substrates pre-conditioned with fibronectin. Cells exhibit similar cell density and extensive cell spreading on all substrates after 2 h. Cell density increases over time for all substrates; however, cell density and cell spreading are attenuated for PMO substrates with increasing OEGMA content and LCST. Scale bar = 100 μm .

of which may act in concert to mediate protein adsorption and cell behavior.³⁸ Surface hydrophobicity is increasingly recognized as an especially important property of multi-component TRPs that can be manipulated to direct cell function.⁸ For example, selective hydrophobicity of PNIPAM-based TRPs has been utilized to promote cell alignment⁴⁴ and facilitate separation of distinct cell types.³⁰ Further, surface hydrophobicity has an essential role in protein adsorption, which in turn, mediates cellular responses to cell culture substrates.⁵¹ Poly(MEO₂MA-co-OEG-

MA) TRP substrates with a fixed composition (94:6, LCST = 34 °C) have been shown to support cell adhesion,^{52,53} and recent contact angle measurements have shown that small changes in PMO composition affect the substrate hydrophobicity.¹⁵ Specifically, decreasing the MEO₂MA:OEGMA copolymer ratio, that is, an increase in the hydrophilic OEGMA monomer content and LCST, incrementally decreases the surface hydrophobicity.¹⁵ To further characterize the cellular responses to the PMO system, we fabricated a series of PMO brushes with variable compo-

FIGURE 8. Protein adsorption and cellular responses to fibronectin-coated TRP substrates. (a) Substrates were incubated with fluorescent FN (40 $\mu\text{g}/\text{mL}$) for 2 h at 37 $^{\circ}\text{C}$. The extent of FN protein adsorption is correlated to PMO composition. * indicates the density of adsorbed protein is significantly different from all other substrates ($p < 0.05$). (b) Cell density increases over time on FN treated substrates. Cell density is similar on all substrates after 2 and 6 h; however, PMO 28 brushes show a significantly higher cell density after 24 h relative to other TRP substrates (** $p < 0.05$). Dotted line indicates the cell seeding density (25,000 cells/ cm^2). (c) Percentage of cells with observable cell spreading increases over time. PNIPAM and PMO 28 brushes show similar cell spreading at 2 h, which is significantly greater than other substrates (# $p < 0.05$). (d) Average cell circularity decreases with time. PNIPAM and PMO 28 brushes show similar cell circularity after 24 h, which is significantly less than other substrates (# $p < 0.05$).

sition (0–8% OEGMA, LCST = 28–37 $^{\circ}\text{C}$) and investigated protein adsorption, cell attachment and morphology of L-929 fibroblasts in the presence of FBS and adhesion mediating ECM proteins FN and VN.

Fibroblasts showed delayed attachment to all PMO brushes in the presence of serum proteins, independent of PMO composition, as few seeded cells (13–30%) were adherent after 2 h (Fig. 2). The initial cell attachment to pure MEO₂MA surfaces (PMO 28) was significantly higher than other PMO copolymer brushes after 2 h (Fig. 3b). In contrast, cell density was comparable on all PMO substrates after 24 h, although cells exhibited markedly different morphologies. Cells exhibited a well-spread, migratory morphology on pure MEO₂MA surfaces (PMO 28) after 24 h and showed an increasingly rounded, circular shape as the ratio of the hydrophilic OEGMA monomer increased in the PMO copolymer brush composition (Fig. 4). Several other studies have

demonstrated a correlation between composition-dependent changes in hydrophobicity and the extent of cell spreading for a homologous series of copolymers.^{3,5,45} For example, Allen *et al.* demonstrated a similar correlation between cell spreading and surface hydrophobicity for PNIPAM copolymerized with varying amounts of the more hydrophobic *N*-tert-butylacrylamide (NTBAM) monomer. Specifically, increases in the NTBAM: PNIPAM ratio led to an increase in cell surface area and a concomitant decrease in cell circularity.³ Similarly, Becherer *et al.* demonstrated a correlation between substrate hydrophobicity and fibroblast adhesion and spreading for a novel glycerol-based thermoresponsive copolymer comprised of varying mole fractions of a hydrophobic glycidyl ethyl ether monomer.⁵

The differential cell adhesion and morphology observed on PMO substrates could be attributed to many surface dependent factors; however, our data points to an important role for the extent of protein

adsorption. Commensurate with enhanced cell adhesion and spreading at early time points relative to other PMO substrates, PMO 28 substrates elicited significantly more adsorption of BSA, which is a primary component of serum proteins (Fig. 3a). It is generally thought that increased surface hydrophobicity promotes protein adsorption,^{17,24,51} and incremental increases in the OEGMA content in PMO brushes led to an attenuation of both BSA adsorption and cell spreading. BSA is used extensively as a model serum protein, and a correlation between BSA adsorption and cell adhesion has been demonstrated for polymer brushes with variable surface chemistry²¹ and grafted polymer chain density.⁵⁶ BSA serves as a useful proxy for modeling serum protein adsorption; however, in the presence of FBS, the composition of the adsorbed protein layer likely varies with polymer composition, as distinct proteins are preferentially adsorbed based on their relative affinities for the surface.^{4,14} Quantitative measurements of adhesion mediating serum proteins such as fibronectin^{3,45} and fibrinogen^{5,21} have revealed a similar correlation between the hydrophobicity of libraries of copolymers with variable composition, the magnitude of adsorbed protein density, and cell adhesion. Interestingly, after 2 and 6 h, all PMO brushes exhibited significantly less cell attachment relative to UpCell (PNIPAM), despite comparable levels of BSA adsorption for PMO 31 and PMO 34. Moreover, PMO 28 brushes elicited a 2.6-fold increase in the density of adsorbed BSA; however, still showed limited initial cell adhesion. By 24 h, cells were adherent and showed comparable cell density on all PMO and PNIPAM surfaces, although cells exhibited less cell spreading on the relatively hydrophilic PMO substrates (PMO 34 and PMO 37). Direct comparisons between PNIPAM and PMO are difficult due to differences in surface chemistry and topology,¹⁵ both of which can influence both protein adsorption and cell behavior. PNIPAM was utilized in this study strictly as a reference, as it is currently the “gold standard” TRP in cell culture applications,¹¹ and we suggest that other series of homologous copolymers are more suitable for direct comparison to the PMO substrates investigated in this study. Here, we attribute the differential cellular responses to PMO substrates primarily to composition-dependent changes in hydrophobicity and protein adsorption, as both surface chemistry and topography are relatively unchanged with PMO composition.¹⁵

The enhanced cell adhesion and spreading initially observed on hydrophobic PMO substrates is likely affected by both the extent and composition of the intermediate protein layer. Specifically, adsorbed ECM proteins provide essential biochemical cues that regulate cell function *via* integrin-mediated cell signaling.^{16,43} The composition of adsorbed proteins is

dynamic due to changes elicited by competitive adsorption and rearrangement.^{4,50} Further, protein bioactivity may change as a function of polymer composition and time due to conformational changes of adsorbed proteins.^{17,24,34} The increased cell attachment and changes in cell morphology observed on PMO substrates over time suggest that the surface properties may be changing with time in culture. While PMO surface properties most likely govern the nature of adsorbed proteins and subsequent initial cellular responses, it is equally likely that cells further modify the cell-substrate interface over time. Specifically, cells may further modify the surfaces *via* deposition of ECM proteins¹⁷ and modulation of cell surface receptors.⁴¹ Indeed, gene expression analysis has revealed widespread changes in the expression of adhesion mediating proteins in response to surface chemistry and hydrophobicity.^{2,3,37} Thus, the cellular responses to variable polymer composition in the presence of serum proteins are complex and the data presented here may reflect many of these factors acting synergistically; however, investigations of these phenomena are outside the scope of this study.

The adsorption of serum proteins has been correlated to cell attachment to PNIPAM brushes,⁵⁶ and numerous studies have demonstrated functionalization of PNIPAM *via* adsorption of specific ECM proteins^{1,9} to promote cell attachment and growth. To enhance cell attachment to PMO brushes, we utilized recombinant fibronectin and vitronectin, which are ECM proteins that promote cell adhesion and migration for diverse cell types.^{32,33} Consequently, these proteins have been widely utilized to coat an array of biomaterials to facilitate cell attachment and spreading.⁴⁰ Similarly, initial cell attachment was markedly enhanced to all PMO brushes after preconditioning with FN (Fig. 5a). The FN-mediated cell attachment was most pronounced for the most hydrophobic surfaces, PMO 28 and PMO 31, and these substrates showed statistically similar cell attachment relative to PNIPAM after 2 h (Fig. 5c). Preconditioning with FN alone or in the presence of FBS did not significantly affect cell attachment to any PMO substrates. Interestingly, preconditioning with FN alone led to a significant decrease in cell attachment to PNIPAM compared to preconditioning with FBS or FBS with FN, which suggests that FN may have a lower affinity for PNIPAM relative to other adhesion mediating proteins in FBS. A similar result was seen with VN-treated PNIPAM, although VN did not significantly enhance cell attachment to any PMO substrates. VN did, however, have a profound effect on the morphology of adherent cells (Fig. 6). Both FN and VN led to a significant increase in cell spreading, and a concomitant decrease in cell circularity, relative to FBS

for all PMO substrates, as well as PNIPAM. For PMO 28 brushes, pretreatment with FN showed significantly more cells spreading and less cell circularity relative to VN.

FN elicited a dramatic increase in initial cell attachment relative to FBS and VN; hence, the kinetics of FN-mediated cell attachment and spreading were assessed over time in the absence of other competing proteins. The primary mechanism of cell attachment to FN involves the binding of $\alpha_5\beta_1$ integrin receptor to exposed RGD-binding sites, and it has been demonstrated that the conformation of FN and related cell attachment is highly sensitive to changes in surface chemistry.^{19,22} Moreover, FN has been shown to preferentially adsorb to hydrophobic surfaces.^{3,17,45,54} Indeed, both the extent of FN adsorption and subsequent cellular responses to PMO brushes are correlated to changes in copolymer composition and hydrophobicity. Similar to the trend observed with BSA, the adsorption of FN is greatest on PMO 28 and diminishes with incremental increases in OEGMA content (Fig. 8a). Initial cell attachment is robust for all FN functionalized PMO substrates after 2 h; however, cell spreading is much more extensive on the hydrophobic PMO 28 substrate relative to other compositions (Figs. 7, 8). Further, cell growth over 24 h is most prolific on PMO 28. The differential cell spreading initially observed converges after 6 h and over 90% of cells exhibit cell spreading after 24 h on all substrates. Concomitantly, cell circularity decreases over time on all PMO substrates; however, this metric is significantly lower for PMO 28 compared to other PMO surfaces. These results suggest that the increased FN adsorption and/or bioactivity is highest on PMO 28, which is pure poly(MEO₂MA). The effect of functionalizing PMO 28 with FN had a much more profound effect on cell growth and morphology over time than subtle changes in the MEO₂MA:OEGMA ratio for other substrates studied (PMO 31-PMO 37). These results are supported by other studies that show FN adsorption to PNIPAM-based³ and PEG-based⁴⁵ copolymer substrates increases with an increase in composition of the more hydrophobic monomer. Additionally, the activity of $\alpha_5\beta_1$ integrin binding sites on adsorbed FN is correlated to hydrophobicity.^{22,29} It is interesting to note that preconditioning with FN led to comparable cell attachment and morphology for PNIPAM and PMO 28 after 2 h; however, cell growth on PMO 28 exceeded that observed on PNIPAM and all other PMO substrates over 24 h, which may be due to the effects of both increased FN adsorption and differences in the availability of integrin binding sites.

Taken together, the results of this study demonstrate that systematic variation in the PMO composition elicits differential cellular responses in the presence

of both FBS and ECM proteins; thus, is an important consideration in the application of PMO TRPs for cell culture. Incremental increases in the MEO₂MA:OEGMA ratio in the PMO brush composition enhances adsorption of BSA and FN, as well as initial cell attachment and spreading. Additionally, FN adsorption to PMO brushes promotes cell attachment, spreading and growth over time in a composition dependent manner. These results suggest that the most hydrophobic substrate, PMO 28, should be functionalized *via* FN adsorption to promote cell adhesion and spreading. Detailed investigations of cell-mediated remodeling of the intermediate protein layer and regulation of gene expression are required to understand the impact of variable copolymer composition on long-term cellular responses to PMO substrates.

ELECTRONIC SUPPLEMENTARY MATERIAL

The online version of this article (doi:10.1007/s12195-016-0464-5) contains supplementary material, which is available to authorized users.

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CONFLICT OF INTEREST

Christopher R. Anderson, Cara Abecunas, Matthew Warrenner, André Laschewsky, and Erik Wischerhoff declare that they have no conflict of interest.

HUMAN AND ANIMAL RIGHTS

No human studies were carried out by the authors for this article. No animal studies were carried out by the authors for this article.

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