Tuning reversible cell adhesion to methacrylate-based thermoresponsive polymers: Effects of composition on substrate hydrophobicity and cellular responses

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Abstract: Thermoresponsive polymer (TRP) cell culture substrates are widely utilized for nonenzymatic, temperature-triggered release of adherent cells. Increasingly, multicomponent TRPs are being developed to facilitate refined control of cell adhesion and detachment, which requires an understanding of the relationships between composition-dependent substrate physicochemical properties and cellular responses. Here, we utilize a homologous series of poly(MEO2MAx-co-OEGMAY) brushes with variable copolymer ratio (x/y) to explore the effects of substrate hydrophobicity on L-929 fibroblast adhesion, morphology, and temperature-triggered cell detachment. Substrate hydrophobicity is reported in terms of the equilibrium spreading coefficient (S), and variations in copolymer ratio reveal differential hydrophobicity that is correlated to serum protein adsorption and initial cell attachment at 37°C. Furthermore, quantitative metrics of cell morphology show that cell spreading is enhanced on more hydrophobic surfaces with increased (x/y) ratio, which is further supported by gene expression analysis of biomarkers of cell spreading (e.g., RhoA, Dusp2). Temperature-dependent cell detachment is limited for pure poly(MEO2MA); however, rapid cell rounding and detachment (<20 min) are evident for all poly(MEO2MAx-co-OEGMAY) substrates. These results suggest that increased MEO2MA content in poly(MEO2MAx-co-OEGMAY) substrates elicits enhanced protein adsorption, cell adhesion, and cell spreading; however, integration of small amounts of the more hydrophilic OEGMA unit facilitates both cell attachment/spreading and detachment. This study demonstrates an important role for the composition-dependent control of surface hydrophobicity in the design of multicomponent TRPs for desired biological outcomes. © 2017 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 105A: 2416–2428, 2017.

Key Words: thermoresponsive polymers, oligo(ethylene glycol) methacrylates, polymer brushes, wettability, spreading coefficient, protein adsorption, cell adhesion, cell spreading

INTRODUCTION

There is a great interest in developing novel polymer systems with tunable physical and chemical properties that dictate cell–biomaterial interactions and facilitate the control of subsequent cell behavior.1 In the context of biomedical implants, poly(ethylene glycol) (PEG)-based systems have been used extensively to render biomaterial surfaces resistant to cell adhesion and limit inflammatory responses.2,3 Conversely, an array of polymeric systems has been fabricated to provide a platform that promotes cell adhesion,4 directed cell migration,5 or differentiation toward a tissue-specific cell phenotype.6 “Smart” polymers have been proposed that exhibit physicochemical changes in response to external stimuli, which provides another avenue to regulate the cell–biomaterial interface. To this end, thermoresponsive polymers (TRPs) have been used in diverse biomedical applications due to their temperature-dependent hydrophobicity and conformation.7 TRPs in an aqueous environment exhibit a reversible phase change at their respective lower critical solution temperatures (LCST). Below the LCST, polymer chains are hydrophilic and well-hydrated; while polymer chains above the LCST become more hydrophobic and precipitate from solution as intermolecular interactions between/within polymer chains dominate polymer–water interactions. TRPs immobilized on solid
substances have garnered widespread utility in cell-culture applications, as polymers exhibit a collapsed conformation above the LCST that facilitates protein adsorption and cell adhesion. As the TRP is cooled below the LCST, the hydrophobic-to-hydrophilic phase change mechanically lifts cultured cells. Importantly, the use of TRPs preserves extracellular matrix (ECM) and cell surface proteins that could be degraded during traditional protease-mediated cell detachment. Consequently, TRPs have emerged as a promising tool in tissue engineering and regenerative medicine for the mechanical release of adherent cells and the preservation of cell phenotype in culture.

The use of the thermoresponsive poly(N-isopropylacrylamide) (PNIPAM) is ubiquitous in biomedical applications. PNIPAM displays an LCST of 32°C in water and is relatively insensitive to other environmental conditions; hence, it has been used to culture diverse mammalian cell types at 37°C. Furthermore, copolymers of PNIPAM have been used to further refine control of cell morphology and phenotype or for biofunctionalization. The success of PNIPAM and its copolymers has prompted the exploration of myriad copolymer systems that exhibit thermoresponsive behavior and enable controlled cell adhesion and detachment. A specific class of methacrylate-based TRPs has been developed that incorporates short oligo(ethylene glycol) side chains into the macromolecular structure to impart the non-toxic and nonimmunogenic properties of the widely used PEG, while maintaining temperature-dependent “switching” of physical properties. Poly(MEO2MA-co-OEGMA) has been fabricated via surface-initiated atom transfer radical polymerization (SI-ATRP) of random copolymers of 2-(2-methoxyethoxy)ethyl methacrylate (MEO2MA) and oligo(ethylene glycol) methacrylate (OEGMA). The LCST of poly(MEO2MA-co-OEGMA) varies between 28°C and 90°C in water and can be finely tuned by altering the comonomer ratio during polymer synthesis.

Poly(MEO2MA-co-OEGMA) with physiologically relevant LCST values of 34°C assembled on gold or glass substrates have been shown to support cell adhesion at 37°C and detachment upon cooling. More recently, poly(MEO2MA-co-OEGMA) biocompatibility and serial cell culture with repeated thermal switching cycles in serum-containing media has been demonstrated. However, these studies have focused on poly(MEO2MA-co-OEGMA) substrates with fixed composition, and the effects of variable copolymer composition on temperature-dependent cell attachment and detachment are largely unexplored. The utility of poly(MEO2MA-co-OEGMA) substrates in tissue engineering applications, such as “cell sheet” engineering of confluent monolayers or the propagation/harvesting of stem cells with intact phenotypic cell membrane markers, would be enhanced by insight into the relationships between composition-dependent physicochemical properties and temperature-dependent cell behavior.

It is well known that cellular responses to polymeric substrates are influenced by a host of factors, including surface chemistry, topography, wettability, charge, and elasticity, any of which has the potential to act synergistically or antagonistically with others in mediating cell function. Surface wettability is increasingly recognized as a key factor in the design of multicomponent TRPs for specific biomimetic applications. Precise control of surface wettability and cell–material interactions are enabled by the composition of components with desired temperature-dependent hydrophobicity and biological properties. To this end, the manipulation of poly(MEO2MA-co-OEGMA) composition educes variable surface properties and LCSTs that present an avenue for optimizing cell–substrate interactions. Previously, we extensively characterized the surface physical properties of poly(MEO2MA-co-OEGMA)-coated glass substrates, including both the kinetics and temperature-dependence of surface wettability, as analyzed by atomic force microscopy (AFM). We showed that incremental increases in the hydrophilic OEGMA monomer led to consistently lower contact angles, while there were no observed differences in surface morphology or brush height. To provide a preliminary assessment of the impact of composition-dependent wettability on cell function, we showed that increased OEGMA content correlated with decreased initial cell attachment after 6 h. In another study, we grafted poly(MEO2MA-co-OEGMA) to gold nanoparticles and showed that increased OEGMA content elicited decreased cellular uptake. Together, these studies suggest an important role for composition-dependent surface properties in governing cellular interactions with poly(MEO2MA-co-OEGMA); however, the cellular responses to poly(MEO2MA-co-OEGMA)-coated cell culture substrates remain limited. A critical aspect of the cell–substrate interface in a biological environment is the adsorbed protein layer, which provides a scaffold for cell adhesion and essential biochemical cues that regulate cell function. Protein adsorption to solid substrates is thermodynamically driven and significantly influenced by surface hydrophobicity, among other factors. A plethora of studies have reported on the essential role of protein adsorption in mediating cell adhesion, and regulating subsequent cell spreading and the regulation of gene expression. Additionally, the primary appeal of the utility of TRPs in cell culture is temperature-triggered cell detachment; however, the effects of poly(MEO2MA-co-OEGMA) composition on changes in cell morphology and adhesion below the LCST have not been investigated. The nature of the cell–substrate interface is complex, and the rational application of poly(MEO2MA-co-OEGMA) TRP substrates requires an understanding of how the surface physicochemical properties and the extent of protein adsorption collectively govern diverse aspects of cell function.

In this study, we used a homologous series of poly(MEO2MA-co-OEGMA) substrates with 0–8% OEGMA to rigorously investigate the relationships between surface hydrophobicity, protein adsorption, quantitative metrics of cell spreading/morphology, and temperature-dependent cell attachment and detachment. Variation of the OEGMA content between 0% and 8% elicits copolymer LCST values ranging from 28°C to 37°C; hence, all the substrates utilized in this study exhibit a hydrophilic- to-hydrophobic switch at temperatures that facilitate cell culture at 37°C. Surface hydrophobicity is quantified with the thermodynamic parameter,
spreading coefficient (S), which represents the negative free energy change associated with the wetting of a liquid over a solid surface.\textsuperscript{43} The magnitude of the spreading coefficient is determined by the macroscopic energy balance between liquid–solid adhesion and liquid–liquid cohesion and has been used extensively to characterize the wettability of diverse engineered materials.\textsuperscript{43,44} In the context of biological systems, the spreading coefficient and physics of liquid wetting has been used to describe cellular and tissue spreading dynamics in embryonic morphogenesis and cancer propagation, where the extent of spreading is influenced by the competition between cell–substrate adhesion and cell–cell cohesion.\textsuperscript{45,46}

Here, we relate the equilibrium spreading coefficient to serum-protein adsorption, cell attachment, the kinetics of cell spreading, and the regulation of genes implicated in cell spreading (e.g., RhoA and Dusp2). Furthermore, the effects of copolymer composition on temperature-induced cell detachment below the LCST are explored. In this investigation, we utilize a single-cell dispersion of L929 fibroblasts so that morphological and phenotypical changes are primarily influenced by cell–protein–substrate interactions and, specifically, the composition dependent changes in substrate wettability. The results of this study suggest a composition-dependent correlation between the poly(MEO\textsubscript{2}MA-co-OEGMA) substrate spreading coefficient and protein adsorption, cell spreading, and temperature-dependent cell attachment and detachment. These results can be broadly applied to the design of multi-component thermoresponsive systems that can be tuned to program essential physical characteristics to direct cellular behaviors. Furthermore, these results may inform the application of poly(MEO\textsubscript{2}MA-co-OEGMA) brushes designed for selective adsorption of cell function mediating proteins or enhancing cell culture and temperature-dependent detachment of specific cell types.

**MATERIALS AND METHODS**

**Fabrication of poly(MEO\textsubscript{2}MA-co-OEGMA) substrates**

Thermoresponsive poly(MEO\textsubscript{2}MA-co-OEGMA\textsubscript{x/y}) brushes with variable composition (e.g., x/y ratio) were grafted from polyelectrolyte multilayer structures according to the methods described in detail by Wischerhoff et al.\textsuperscript{24} Briefly, polyelectrolyte structures consisting of polyethylenimine (PEI), polystyrenesulfonate (PSS), and poly(diallyldimethylammonium chloride) (PDADMAC) were built on glass substrates via layer-by-layer deposition and coated with the macroinitiator MA01, synthesized as previously described,\textsuperscript{24} prior to reaction. Substrates were incubated with solutions containing MEO\textsubscript{2}MA (M\textsubscript{x} = 188 g/mol) and OEGMA (M\textsubscript{y} = 475 g/mol), and poly(MEO\textsubscript{2}MA\textsubscript{x/co-OEGMA\textsubscript{y}}) brushes were assembled on the macroinitiator layer by SI-ATRP. The molar ratio of MEO\textsubscript{2}MA\textsubscript{x/co-OEGMA\textsubscript{y}} varied from (x/y) = 100:0 to 92:8, and the SI-ATRP reaction time was 60 min. The final multilayer sequence was as follows: PEI/PSS/PDADMAC/MA01/poly(MEO\textsubscript{2}MA\textsubscript{x/co-OEGMA\textsubscript{y}}). Layer thickness and copolymer surface density were measured previously via ellipsometry. The PEI/PSS/PDADMAC/MA01 layer thickness was 62 ± 2 nm, the macroinitiator MA01 layer thickness was 12.5 ± 1 nm, and the poly(MEO\textsubscript{2}MA\textsubscript{x-co-OEGMA\textsubscript{y}}) layer thickness was 65.5 ± 2 nm.\textsuperscript{24} The copolymer surface density was estimated at 4.0 × 10\textsuperscript{7} mol/m\textsuperscript{2}.\textsuperscript{47} The grafted poly(MEO\textsubscript{2}MA\textsubscript{x-co-OEGMA\textsubscript{y}}) brushes exhibited variable LCSTs ranging from 28°C to 37°C in PBS.\textsuperscript{21} All reagents above were purchased from Sigma-Aldrich (St. Louis, MO).

**Spreading coefficient experiments**

Spreading coefficient (S) at the liquid polymer surface was calculated using the following equation\textsuperscript{44}:

\[
S = \gamma_{\text{WD}}(\cos\theta - 1)
\]

where \(\gamma_{\text{WD}}\) is the water/decane interfacial tension and \(\theta\) is the contact angle with the polymer surface. Both interfacial tension and contact angle were independently measured over a temperature range of 20–45°C with a profile analysis tensiometer (PAT1D, Sinterface Technologies, Germany). A pendant drop of water was formed on a tip inside a cuvette filled with purified decane (Sigma-Aldrich) and the interfacial tension calculated by axisymmetric drop shape analysis using the Laplace equation.\textsuperscript{48} The drop area was maintained constant at a value of 30 mm\textsuperscript{2} via feedback control. With known water in decane interfacial tension values, the drop \(V \sim 20\ \mu\text{l}\) was deposited on the poly(MEO\textsubscript{2}MA\textsubscript{x/co-OEGMA\textsubscript{y}}) surfaces \((x/y) = 100:0\) to 92:8) and the contact angle \((\theta)\) measured in a sessile drop configuration. For each temperature, contact angle values were taken after equilibrium was reached (between 1 and 3 h). Experiments were performed in triplicate for each temperature. Results are presented as averages ± standard deviation.

**Quantification of protein adsorption**

Poly(MEO\textsubscript{2}MA\textsubscript{x-co-OEGMA\textsubscript{y}}) substrates \((n = 4)\) with \((x/y) = 100:0\) to 92:8 were incubated in Dulbecco’s Modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C. After 24 h, media were removed and substrates were washed twice with warm PBS at 37°C. Substrates were then transferred to new 6-well plates. The 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.\textsuperscript{17,37,38} The protein elution buffers were concentrated using 30 kD Microcon centrifugal filters (Millipore, Billerica, MA) at 14000 rcf for 25 min. Residual protein elution buffer was removed by adding 500 µL PBS to the filters and centrifuging at 14,000 rcf for 25 min. Isolated proteins were subsequently resuspended in 50 µL of PBS and the protein concentration was measured with the colorimetric DC Protein Assay kit (Bio-Rad, Hercules, CA) according to manufacturer’s instructions. Results are presented as averages ± 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 culture and imaging**

Murine L-929 fibroblast cells (ATCC, Manassas, VA) were grown in tissue culture polystyrene TCP flasks (Nunc;
Thermo Scientific) in DMEM (Lonza, Walkersville, MD), supplemented with 10% FBS (HyClone, Thermo Scientific), and 1% penicillin/streptomycin (Lonza) at 37°C and 5% CO₂. Cells were grown to confluence prior to experiments. Cells were removed from flasks with trypsin/EDTA (Lonza), centrifuged, and resuspended in complete cell culture media, and seeded at 25,000 cells/cm² for experiments. Phase contrast images of cells were acquired with an inverted optical microscope (Nikon Eclipse TE 100) to observe cell health and morphology.

Quantification of cell adhesion and spreading morphology
At the time of experiment, growth media was aspirated and nonadherent cells were removed from polymer substrates by rinsing twice with warm PBS. Poly(MEO2MAₜ-co-OEGMAₜ)-coated coverslips with (x/y) = 100:0 to 92:8 were transferred to new 6-well plates. Substrates 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 attached cells. Absorbance values were measured at 490 nm with a multimode plate reader (BioTek, Winooski, VT). The percentage of cell spreading was quantified by manually counting the number of cells exhibiting cell protrusions extending from the cell body relative to the total number of cells in phase contrast images (n = 6 images). For each image, the area and perimeter of each cell was also measured with NIS Elements software (Nikon; Basic Research version 3.1). These measurements were used to calculate cell circularity: c = 4π · area/perimeter², 17,37,38 Cell spreading and circularity calculations were based on total cell counts ranging from approximately 20 to 45 cells/image, depending on the time in culture. In a separate set of experiments, the temperature-dependent changes in cell morphology and adhesion were investigated. Cells were cultured for 24 h at 37°C prior to introducing cells to room temperature. At this time, cells remained subconfluent, which enables observation of changes in morphology and adhesion of well-spread single cells, primarily due to temperature-dependent changes in substrate–cell interactions. At the time of experiment, media at 37°C was replenished with media at 20°C to elicit a sharp and immediate change in temperature below the LCST of all poly(MEO₂MAₜ-co-OEGMAₜ) substrates. Adherent cells were imaged immediately after changing the media (t = 0 min) and repeatedly over the course of 60 min. From images taken between 0 and 20 min, when cell morphologies were most dynamic, cell circularity values were calculated as described. Alternatively, cell density at 20°C was measured with the MTS-based assay, and related to cell adhesion controls measured at 37°C after 24 h to determine the percentage of cells that remained adherent. All experiments were performed in triplicate with independent substrates. Results are presented as averages ± 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.

Immunohistochemistry
After 24 h, cells were fixed on poly(MEO₂MAₜ-co-OEGMAₜ)-coated coverslips (n = 4; x/y = 100:0 to 97:3) at 37°C to prevent the mechanical release from thermobrushes during the staining process. Cells were fixed with 4% paraformaldehyde/PBS for 20 min and permeabilized with 0.1% Triton X-100/PBS for 5 min. Cells were subsequently washed in PBS 2×5 min, blocked with 1% BSA/PBS for 30 min, and washed again with PBS 2×5 min. Each cell sample was then immunolabeled for vinculin using FITC-conjugated monoclonal antivinculin (Sigma) diluted 1:100 in BSA/PBS for 1 h at room temperature. Samples were then washed with BSA/PBS 2×5 min and stained with TRITC-conjugated rhodamine phalloidin (Invitrogen) diluted 1:160 in BSA/PBS for 20 min at room temperature to enable visualization of F-actin. Samples were again washed with BSA/PBS 2×5 min and mounted using VECTASHIELD Antifade Mounting Medium with DAPI (Vector Laboratories).

Confocal microscopy and quantification of cell height
Immunolabeled cells were imaged using a Nikon C1Plus Confocal on the Ti-E Motorized Inverted Microscope. Representative images were acquired using a CFI Plan APO 60X/1.4 NA Nikon oil immersion objective and Nikon Basic Research Software. Cell height was measured using the live volume screen obtained from a complete z-stack. A minimum of ten cells were measured per condition.

Cell harvest and RT-PCR
Cells were harvested from control TCP surfaces with Trypsin-Versene (Lonza) and collected in sterile PBS. Cells cultured on poly(MEO₂MAₜ-co-OEGMAₜ) surfaces (n = 4) with x/y = 100:0 to 97:3 were incubated in 1 mL PBS for 30 min to allow for temperature-controlled detachment and then collected. All cell samples were centrifuged at 4000 rpm for 5 min and cell pellets were resuspended in 350 µL Buffer RLT (QIAGEN). Total RNA was isolated following the manufacturer’s protocol (QIAGEN RNeasy Mini Kit) and quantified using a spectrophotometer (Thermo Scientific NanoDrop 2000). Reverse transcription to cDNA was performed using the High Capacity cDNA RT Kit from Applied Biosystems. Polymerase chain reaction (PCR) amplification was performed using the StepOne Plus Real-Time PCR system (Applied Biosystems) with Fast SYBR Green reagent for DNA detection. Cycle parameters were: 95°C for 20 s, 40 cycles of 95°C for 3 s, and 60°C for 30 s. Primers were designed using Primer Express Software v.3.0 with a target melting temperature of 60°C and 20 bp target length, validated, and optimized. 18sRNA served as the housekeeping gene. Primer sequences and concentrations were used as previously described. 25

RESULTS
Temperature-dependent spreading coefficients
Poly(MEO₂MAₜ-co-OEGMAₜ) brushes were grown on polyelectrolyte multilayer structures by SI-ATRP 24 and the molar ratio of MEO₂MAₜ to OEGMAₜ varied from (x/y) = 100:0 to 92:8, which corresponds to LCSTs ranging from 28°C to
37°C in solution. To classify the different wetting states on poly(MEO2MA-co-OEGMA) substrates, the spreading coefficient (S) was calculated from equilibrium contact angle (θ) and interfacial tension measurements of water in decane. The spreading coefficient (S) measures the difference between the surface free energy and its value in the case of complete wetting:

\[ S = \gamma_{SD} - (\gamma_{WD} + \gamma_{SW}) = \gamma_{WD} (\cos \theta - 1) \]  

(eq. 2)

Here, \( \gamma_{SD} \), \( \gamma_{WD} \), and \( \gamma_{SW} \) are the solid/decane, water/decane, and solid/water surface energies, respectively. If \( \gamma_{SD} = \gamma_{WD} + \gamma_{SW} \), then \( S = 0 \) and the system will be in equilibrium when a uniform liquid layer covers the whole solid surface (complete wetting). On the other hand, if \( \gamma_{SD} < \gamma_{WD} + \gamma_{SW} \), a droplet with a finite contact angle minimizes the free energy of the system and \( S < 0 \), which is indicative of partial wetting behavior. Decreased spreading coefficients are correlated with increased hydrophobicity, and thus, less wettability of solid surfaces. The spreading coefficient for poly(MEO2MA-co-OEGMA) substrates as a function of temperature is shown in Figure 1A. All poly(MEO2MA-co-OEGMA) substrates show a decrease in spreading coefficient with increased temperature. Surface hydrophobicity is greatest for \( (x/y) = 100:0 \) (e.g., pure poly(MEO2MA)), as the spreading coefficient is significantly lower than all other copolymer compositions for all temperatures. Interestingly, variation in the copolymer composition between 97:3 < (x/y) < 92:8 shows relatively small changes in the spreading coefficient relative to pure poly(MEO2MA). The temperature sensitivity of surface hydrophobicity is further illustrated in Figure 1B, where the first derivative of the spreading coefficient (dS/dT) as a function of temperature is reported. Here, a composition-dependent trend is evident as \( (x/y) = 100:0 \) substrates are the most responsive to changes in temperature, and incremental increases in the OEGMA content attenuate temperature-dependent changes in the spreading coefficient. Moreover, there is a shift toward increased temperature in dS/dT minima, which reflects the increased poly(MEO2MA-co-OEGMA) brush LCST associated with increased OEGMA content in the copolymer composition.

**Protein adsorption and cell attachment**

The effects of temperature-dependent changes in surface wettability on substrate–protein–cell interactions are depicted in Figure 2. The poly(MEO2MA-co-OEGMA) brushes studied exhibit LCSTs ranging from 28°C to 37°C as the copolymer ratio is varied from \( (x/y) = 100:0–92:8 \). Thus, at room temperature \( (T \approx 20°C) \), substrates below their LCST are hydrophilic and relatively resistant to protein adsorption and cell attachment (Fig. 2A). However, as the temperature is raised to standard cell culture conditions \( (T = 37°C) \), poly(MEO2MA-co-OEGMA) substrates above their LCST are more hydrophobic, which promotes protein adsorption and facilitates cell adhesion (Fig. 2B). To investigate the relationship between the spreading coefficient and initial cell attachment to poly(MEO2MA-co-OEGMA) brushes, the number of adherent cells was quantified after 2 h in the presence of serum-containing media at 37°C. Equilibrium spreading coefficients for poly(MEO2MA-co-OEGMA) substrates at 37°C are reported in Figure 3A. For \( (x/y) = 100:0 \), the spreading coefficient is \(-68.2\) mN/m, which is significantly lower than other poly(MEO2MA-co-OEGMA) compositions. This value increased modestly \((59.2\) to \(-57.3\) mN/m) as the \( (x/y) \) ratio ranged from \( 97:3 \) to \( 92:8 \). Figure 3B shows the density of serum proteins adsorbed to poly(MEO2MA-co-OEGMA) substrates after 2 h at 37°C. Poly(MEO2MA) brushes elicit the highest protein adsorption, which reduces progressively as the OEGMA content in the brushes is increased. Poly(MEO2MA) showed a 3.6-fold increase in protein density relative to poly(MEO2MA-co-OEGMA) brushes.

L-929 fibroblasts were used as a model cell line for investigating the cellular behavior on poly(MEO2MA-co-OEGMA) brushes. The extent of cell attachment after 2 h in the presence of serum is shown in Figure 3C. Initial cell attachment to all poly(MEO2MA-co-OEGMA) substrates was modest, as <30% of seeded cells were adherent after 2 h. However, poly(MEO2MA-co-OEGMA) composition has a clear role in the regulation of cell adhesion. The initial cell attachment to poly(MEO2MA-co-OEGMA) brushes decreases as the OEGMA content increases, consistent with the trend seen for protein adsorption.
attachment to poly(MEO2MA) brushes was significantly higher than all other substrates, which suggests that the high adsorbed protein density observed may contribute, in part, to the enhanced cell attachment on the most hydrophobic surface, as indicated by the lowest spreading coefficient. Similar to the magnitude of adsorbed protein density, the number of attached cells decreases as the OEGMA content in the copolymer composition increases.

Cell spreading and quantitative cell morphology
The effect of poly(MEO2MA<sub>x</sub>-co-OEGMA<sub>y</sub>) composition on cell spreading was evaluated by observation of cell morphology with phase contrast microscopy. Figure 4 depicts the progression of cell spreading on poly(MEO2MA<sub>x</sub>-co-OEGMA<sub>y</sub>) substrates over time. At early time points (<6 h), cells exhibit primarily rounded cell morphologies on all surfaces; however, notable differences in the presence of lamellipodia and filopodia at cell peripheries are evident by 6 h. Cell morphologies are highly variable at later time points (24–48 h), as cells are more “star-shaped” on poly(MEO2MA) and increasingly rounded as the OEGMA content increases in the copolymer composition. To further discern the morphologies observed, cell spreading was assessed by quantifying the number of adherent cells that exhibit visible protrusions extending from cell bodies. The kinetics of cell spreading on poly(MEO2MA<sub>x</sub>-co-OEGMA<sub>y</sub>) substrates is shown in Figure 5A. As expected, cell spreading on all substrates increases over time; however, there is a significant lag in cell spreading for (x/y) of 94:6 and 92:8 after 6 and 24 h relative to (x/y) of 100:0 and 97:3. The extent of cell spreading converges for all substrates after 48 h. Cell spreading was further quantified with cell circularity, as shown in Figure 5B, which ranges in value from 0 to 1. Cell circularity values that approach one reflect highly circular cell morphologies, and thus, less cell spreading. Commensurate with cell spreading morphologies (Fig. 5A), cell circularity values are most variable at 6 h on the different substrates. Substrates with (x/y) of 92:8 exhibited the most circular morphology after 6 h, which was statistically greater than (x/y) of 100:0. By 24 h, as spreading increases, circularity begins to
converge on the various substrates. At 48 h, spreading and circularity of cells on all \((x/y)\) substrates are statistically similar.

**Cell detachment**

The temperature-dependent changes in cell morphology and attachment to poly(MEO2MA\(_x\)-co-OEGMA\(_y\)) substrates are illustrated in Figure 6. After 24 h in culture at 37°C, cell density is comparable on all substrates; however, cell spreading is attenuated with decreasing \((x/y)\) ratio. After 60 min exposure to 20°C, cell detachment was substantial for \((x/y) = 94.6:92.8\), and almost all remaining adherent cells exhibited a round morphology, independent of substrate composition. The differential effects of poly(MEO2MA\(_x\)-co-OEGMA\(_y\)) composition on cell morphology and attachment are quantified in Figure 7. As expected, lowering the substrate temperature below the LCST led to an immediate increase in cell circularity on all substrates (Fig. 7A). The average cell circularities increased over the following 20 min, reflecting cell retraction and rounding in response to the substrate hydrophobic-to-hydrophilic switch. After 20 min, the average circularity values converged to approximately 90% for all substrates. Cell detachment was most rapid for \((x/y) = 94.6:92.8\), the most hydrophilic substrates, after 10 min (Fig. 7B). Cell attachment ranged from 65% to 50% for \((x/y) = 97.3:92.8\) after 20 min, which was significantly less than that observed for \((x/y) = 100:0\). Furthermore, cell attachment was widespread for \((x/y) = 100:0\) after 60 min, at which point over 90% of adherent cells remained attached to the substrates. In contrast, cell attachment was approximately 50% for \((x/y) = 97.3:92.8\) after 60 min.

**Focal adhesion formation and gene expression**

Poly(MEO2MA\(_x\)-co-OEGMA\(_y\)) substrates with variable composition, and spreading coefficients, elicit differential protein
adsorption, subsequent cell attachment and spreading, and temperature-dependent cell detachment. Moreover, these effects are most profound between (x/y) of 100:0 and 97:3 compared to incremental increases in the OEGMA content seen in (x/y) of 97:3, 94:6, and 92:8. As a result, these two polymer compositions (100:0 and 97:3) were utilized to compare focal adhesion formation and the regulation of cell spreading-mediating genes after 24 h. Confocal microscopy images of cells immunolabeled for the focal adhesion-associated protein, vinculin (green), and the cytoskeletal protein, F-actin (red), are presented in Figure 8A. Cells attached to poly(MEO2MA) substrates reveal more extensive and intense colocalization of vinculin and F-actin (yellow) relative to cells observed for (x/y) = 97:3. Confocal microscopy was used further to investigate the 3D morphology of adherent cells. Cell heights were measured from stacks of images acquired serially throughout the cell bodies. Figure 8B shows significant increase in cell height for (x/y) = 97:3 substrates relative to poly(MEO2MA), which further suggests that the integration of OEGMA in the copolymer composition supports less cell spreading and a rounded morphology. Gene expression analysis was used to investigate the regulation of two genes associated with cell shape and spreading. RhoA is an important intracellular signaling molecule that is known to regulate focal adhesion assembly and induce actin stress fiber formation, which are essential for cell adhesion and spreading.49 Furthermore, changes in RhoA expression have been correlated to surface hydrophobicity mediated changes in the extent of protein adsorption and cell spreading for other copolymer systems.17 In contrast, the expression of the Dusp2 gene has been correlated to cell quiescence and roundedness.50 Differential gene expression was calculated as a fold-change for (x/y) = 97:3 relative to (x/y) = 100:0 using the comparative Ct method.51 Gene expression results show a downregulation of RhoA and an upregulation of Dusp2 for (x/y) = 97:3 substrates relative to Poly(MEO2MA), which taken together, are consistent with the attenuation of cell spreading observed on substrates comprised OEGMA in the copolymer composition.

FIGURE 5. Quantitative cell morphology on poly(MEO2MA-co-OEGMA) brushes as a function of (x/y) ratio and time. (A) Percentage of cells exhibiting spread morphology, marked by the presence of visible protrusions from cell bodies. (B) Average cell circularity. * (p < 0.05) 100:0 and 97:3 are statistically different from 94:6 and 92:8. # (p < 0.05) 100:0 and 92:8 are statistically different. @ (p < 0.05) 97:3 and 92:8 are statistically different.

FIGURE 6. Cell detachment in response to thermal switching of poly(MEO2MA-co-OEGMA) brushes as a function of (x/y) ratio and time.
DISCUSSION
Precise control of cell-substrate interactions is a primary goal in the design and utility of TRPs in cell culture applications. Distinct cell responses to TRPs have been attributed to many substrate properties; however, surface hydrophobicity is increasingly recognized as an essential parameter of multicomponent TRPs for directing cell function.28 To this end, PNIPAM-based thermoresponsive copolymers have been designed to promote cell alignment,10 thermally modulated cell separation,52 and targeted capture of cancer cells53 based on differential hydrophobicity. Poly(MEO₂MA₁₀-co-OEGMA₃) has emerged as an alternative TRP platform.

FIGURE 7. Cellular responses to thermal switching of poly(MEO₂MAₓ-co-OEGMAₙ) brushes as a function of (x/y) ratio and time. (A) Average cell circularity. (B) Percentage of adherent cells released from brushes. *(p < 0.05) 100:0 and 97:3 are statistically different from 94:6 and 92:8. # (p < 0.05) 100:0 is statistically different from all other compositions.

FIGURE 8. Confocal microscopy and gene expression analysis of cells attached to poly(MEO₂MA) and poly(MEO₂MA₉₇-co-OEGMA₃) brushes. (A) Confocal microscopy of cells attached to thermoresponsive substrates after 24 h. Cells are labeled with FITC-conjugated monoclonal antivinculin (green) and TRITC-conjugated phalloidin to visualize F-actin (red). Areas of co-localization (yellow) denote focal adhesions. Scale: 10 μm. (B) Cell height measured from stacks of serial confocal microscopy images. * indicates data are significantly different at p < 0.05. (C) Regulation of gene expression after 24 h. Bars represent a fold change for cells cultured on (x/y = 97:3) brushes relative to (x/y = 100:0).
and reversible cell attachment and temperature-triggered detachment below its LCST has been demonstrated; however, substrates fabricated with a fixed composition have been used almost exclusively for such applications. Similar to the array of PNIPAM-based copolymers, an attractive feature of poly(MEO2MA-co-OEGMAx) is the tunable LCST and other physicochemical properties by variation of its copolymer ratio (x/y). Thus, we can manipulate poly(MEO2MAx-co-OEGMAY) brush hydrophobicity by tuning the amount of the hydrophilic OEGMA units in polymers predominantly composed of MEO2MA. Importantly, other essential substrate properties such as chain length and density can be maintained constant via the control of surface-initiated ATRP reaction time and macroinitiator substrate coverage, respectively. Surface hydrophobicity is quantified with the thermodynamic parameter, spreading coefficient (S), which is often used to characterize the wettability of engineered materials. Interestingly, its application in characterizing cell–biomaterial substrate interactions is relatively limited. Brochard-Wyart et al. have invoked the application of the spreading coefficient to model tissue spreading dynamics in tumor cell aggregates where cell–cell cohesion strength is tuned by differential expression of E-cadherin and cell–substrate adhesion is varied by manipulation of fibronectin adsorption. Additionally, they demonstrated that the membrane tension originating from cell spreading is the driving force responsible for the spontaneous formation of transendothelial cell macroapertures. Here, the decreased spreading coefficients with temperature for all poly(MEO2MAx-co-OEGMAY) brushes reflect a decrease in surface wettability as the polymers are subject to a temperature-dependent hydrophilic-to-hydrophobic switch that is required for cell adhesion (Fig. 1a). Independent of temperature, increased OEGMA content in the copolymer ratio led to increased spreading coefficients, and therefore, decreased surface hydrophobicity (Fig. 1b). Many proteins demonstrate a high affinity for adsorption to hydrophobic surfaces due to the strong interaction between hydrophobic functional groups in proteins and the substrates. In contrast, the repulsive hydration forces arising from strongly bound water at the surface generally lead to weak adsorption. For TRP brushes, it is generally thought that the hydrophobic, collapsed polymer chain conformation above the LCST promotes protein adsorption, while hydrophilic and well-hydrated polymers below the LCST are protein repellent. Moreover, the correlation between the adsorption of model proteins and cell adhesion has been demonstrated with PNIPAM brushes fabricated with variable chain length, density, and terminal chemical moieties. We show that the variable hydrophobicity of poly(MEO2MAx-co-OEGMAY) brushes associated with the manipulation of the (x/y) ratio impacts both serum protein adsorption and initial cell attachment to the substrates (Fig. 3). Pure poly(MEO2MA) showed the greatest hydrophobicity, indicated by the lowest spreading coefficient, and also elicited the most protein adsorption and cell attachment after 2 h at 37°C. The magnitude of both protein adsorption and initial cell attachment was attenuated with increasing amount of the hydrophilic OEGMA units (e.g., decreasing (x/y) ratio) integrated into the poly(MEO2MAx-co-OEGMAY) composition. This is consistent with other studies that show a direct correlation between cell attachment and the extent of adsorption of the model protein fibronectin to PNIPAM-based and PEG-based copolymer substrates. Similarly, Joy et al. showed a surface hydrophobicity-dependent adsorption of fibrinogen and 3T3 fibroblast attachment to a library of methacrylate-based terpolymers with variable composition. Our results highlight the influence of surface hydrophobicity in mediating the extent of protein adsorption, which may be an important consideration in the application of poly(MEO2MAx-co-OEGMAY) brushes intended for selective adsorption of proteins that mediate cell adhesion, proliferation, or differentiation of specific cell types.

The adsorption of adhesive extracellular matrix (ECM) proteins from serum-containing media regulates both cell adhesion and subsequent spreading via integrin receptor-mediated signaling. Interactions between ECM proteins and integrin receptors initiate a cascade of signaling events that mediate focal adhesion formation, actin cytoskeletal reorganization, and the generation of contractile forces required for cell spreading. The differential poly(MEO2MAx-co-OEGMAY) brush composition and protein adsorption correlated to marked differences in cell spreading and morphology over time. Specifically, cells exhibited less spread morphologies, indicated by the presence of observable filopodia and lamellipodia, and retained high cell circularity (Figs. 4–5) after 6 h with decreased (x/y) ratio. Further, confocal microscopy image analysis revealed a decrease in cell height on poly(MEO2MA) and a more rounded morphology on the relatively hydrophilic poly(MEO2MAx-co-OEGMAY3) (Fig. 8). In addition to variable structural changes in cytoskeletal organization and cell morphology, differential substrate wettability elicits the regulation of gene expression of receptors and signaling molecules associated with cell motility. Accordingly, we observed a downregulation of RhoA and upregulation of Dusp2 on poly (MEO2MAx-co-OEGMAY) substrates relative to poly(MEO2MA) (Fig. 8). RhoA is an important intracellular signaling molecule that is known to regulate focal adhesion assembly and induce actin stress fiber formation. In contrast, Dusp2 has been described as a negative regulator of integrin-mediated signaling events and its expression has been associated with rounded, quiescent cells. Taken together, these gene expression trends further support the quantitative metrics of cell morphology that suggest increased MEO2MA content in poly(MEO2MAx-co-OEGMAY) substrates facilitates cell adhesion and spreading. Several other studies have reported on the effect of tuning surface hydrophobicity to promote cell adhesion and spreading to a variety of substrates, while other studies have shown that hydrophobicity enhances cell adhesion and spreading, or that superhydrophobic surfaces with high nanoscale roughness prevent cell spreading. However, it is important to note that these diverse outcomes are complicated by variances in substrate surface chemistry, topography, and charge. Previous analysis of poly(MEO2MAx-co-OEGMAY)-coated glass substrates with AFM
revealed comparable surface roughness for poly(MEO2MA) and poly(MEO2MA-co-OEGMA); thus, we attribute the observed differences in cell morphology primarily to the composition-dependent effects of surface wettability on the extent of protein adsorption and subsequent cell-substrate interactions. Our results are most relevant to other studies that have investigated the effect of copolymer composition on cell adhesion and spreading, which show that increased amount of the hydrophobic monomer are correlated with increased cell spreading.

The temperature-triggered noninvasive release of adherent cells is paramount to the application of TRPs in cell culture systems; however, the mechanism of cell detachment remains poorly understood. Seminal work by Okano et al. has proposed a two-step mechanism by which lowering the temperature below the LCST elicits rapid polymer hydration, and in response, cells initiate signaling events that induce morphological changes associated with cell rounding and detachment. The requirement for active cellular processes in cell detachment is further supported by studies that have shown that detachment efficiency is impaired at low temperatures sufficient to hinder cell metabolism or by small molecule inhibition of cell signaling and cytoskeletal reorganization. Reflecting the morphological responses to decreased temperature, cell circularity values increased rapidly on all poly(MEO2MA-co-OEGMA) substrates at 20°C (Figs. 6–7). Further, cell detachment was substantial after 20 min for (x/y) = 97:3–92:8, which is consistent with the kinetics of cell detachment from PNIPAM-based substrates. Interestingly, cells remained predominately adherent to poly(MEO2MA) substrates (that is, x/y = 100:0) for up to 60 min. Thus, the most hydrophobic substrate facilitates both extensive protein adsorption and cell adhesion and spreading; however, limited temperature triggered cell detachment. This observation is consistent with recent work by Becherer et al. who developed a novel thermoresponsive glycerol-based copolymer and showed that the mole fraction of the hydrophobic glycyl ethyl ether (EGE) monomer was correlated to both fibrinogen adsorption and fibrinolysis; however, enhanced cell adhesion led to slow detachment rates and homopolymers comprised exclusively of EGE showed negligible cell detachment. Our results point to a necessary role for OEGMA units in the poly(MEO2MA-co-OEGMA) composition to balance cell attachment and spreading above the LCST and cell detachment below the LCST.

Although the mechanisms that promote the retention of adherent cells on poly(MEO2MA) are not yet clear; they are likely influenced, in part, by cellular interactions with residual proteins on the substrates. Some studies have shown that adhesion-mediating ECM proteins such as fibronectin are largely released with cells detached from TRP substrates. However, numerous studies have demonstrated the presence of residual proteins after temperature-triggered changes in TRP conformation and cell detachment. The intensity of vinculin and F-actin co-localization in cells adherent to poly(MEO2MA) substrates suggests robust focal adhesion formation compared to poly(MEO2MA-co-OEGMA) (Fig. 8), which may contribute to enhanced adhesion after temperature induced changes in the polymer conformation if integrin-mediated cell-ECM interactions are preserved. Also, it is important to note that the retention of adherent cells on all poly(MEO2MA-co-OEGMA) substrates is predominately governed by cell-substrate interactions, as cell–cell contacts were not formed for subconfluent cells at the time of analysis. It is likely that detachment from poly(MEO2MA-co-OEGMA) would be further aided by the relative strength of cell–cell cohesion forces as cell–substrate interactions are weakened in the case of confluent monolayers below the LCST. The initial cell responses to poly(MEO2MA-co-OEGMA) substrates are likely governed primarily by substrate physicochemical properties and the nature of proteins adsorbed from the surrounding milieu; however, it is inevitable that cells further modify the cell–material interface over time. Gene expression analysis has revealed widespread changes in the expression of adhesion-mediating proteins, and downstream signaling events required for cell spreading, migration, and proliferation, in response to surface chemistry and hydrophobicity. The convergence of cell spreading and circularity parameters after several days in culture suggests that, indeed, the cell–material interface is changing over time to render the substrate more conducive to cell adhesion and spreading. Thus, further understanding of the long-term cellular adaptation to poly(MEO2MA-co-OEGMA) substrates and mechanistic insight into cell detachment requires a detailed interrogation of the composition and conformation of the initial adsorbed protein layer, and that remaining after cell detachment, and an investigation of the cellular transcriptomic/proteomic responses over time.

CONCLUSIONS

In this study, we present a detailed analysis of the effects of substrate wettability on L-929 fibroblast adhesion and morphology for a series of poly(MEO2MA-co-OEGMA) brushes with variable composition (100:0 < x/y < 92:8). Results suggest that small composition-dependent modifications to the surface hydrophobicity can tune protein adsorption and cellular responses. The equilibrium spreading coefficient was used to characterize the wetting state of poly(MEO2MA-co-OEGMA) brushes and increased OEGMA content in the (x/y) copolymer ratio led to increased surface wettability. Increased hydrophobicity of poly(MEO2MA-co-OEGMA) brushes correlated to increased serum-protein adsorption, initial cell attachment, and the extent of cell spreading, as indicated by morphological metrics and analysis of gene expression. However, the most hydrophobic polymer, pure poly(MEO2MA), showed limited cell detachment below its LCST relative to poly(MEO2MA-co-OEGMA) brushes with (97:3 < x/y < 92:8). This suggests that the integration of small amounts of the hydrophilic OEGMA unit into the polymer composition is necessary to facilitate an appropriate balance between cell attachment/spreading and temperature-dependent cell detachment. This study highlights an important role for the composition-dependent control of surface hydrophobicity and...
cell attachment/detachment, which can be broadly applied to the design of multicomponent thermoresponsive polymers for directed cell function.

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REFERENCES


