Surface Characterization of the Extracellular Matrix Remaining after Cell Detachment from a Thermoresponsive Polymer

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The temperature-responsive behavior of poly(N-isopropyl acrylamide) (pNIPAM) directly affects the attachment and detachment of cells cultured on these surfaces. At culture temperatures, cells behave similarly to those on tissue culture polystyrene (TCPS), while at room temperature, cells cultured on pNIPAM spontaneously detach as a confluent sheet. In comparison, cells grown on TCPS remain attached indefinitely after the temperature drop, requiring enzymatic or mechanical removal. In this work, we present an examination of the response of bovine aortic endothelial cells (BAECs) and extracellular matrix (ECM) proteins to plasma polymerized NIPAM (ppNIPAM) surfaces using X-ray photoelectron spectroscopy (XPS), time-of-flight secondary ion mass spectrometry (ToF-SIMS), and immunostaining. Immunoassay results reveal that, although fibronectin, laminin, and collagen closely associate with the cell sheet, some collagen may be associated with the surface, as well. Our XPS results indicate that ppNIPAM surfaces after cell liftoff differ from their blank counterparts, the primary distinction being the presence of amide and alcohol species on ppNIPAM surfaces used for cell culture, possibly owing to the presence of a proteaceous film. Finally, a comparison between ppNIPAM-treated surfaces used for cell culture versus control surfaces by principal component analysis of the ToF-SIMS data confirms that the surfaces differ; the presence of molecular ion fragments from amino acids (e.g., alanine, glycine, and proline) is the chief reason for this difference. Therefore, from our surface characterization of ppNIPAM-coated TCPS after cell liftoff, we conclude that although low-temperature liftoff of the BAEC monolayer is accompanied by the majority of the components of the ECM, some of the ECM proteins still remain at the surface.

Introduction

Using poly(N-isopropyl acrylamide) (pNIPAM), it is possible to rapidly recover intact cell sheets from culture surfaces using only a modest temperature drop as the means of detachment. At normal cell culture temperatures (37 °C), cells will respond to a pNIPAM-coated surface similarly to those cultured on tissue culture polystyrene (TCPS). After being cooled to room temperature, cells cultured on pNIPAM spontaneously detach as a contiguous sheet, while those grown on TCPS will remain attached for hours or days, requiring enzymatic digestion or physical scraping to detach them.

This sharp property change due to a thermal transition around physiological temperatures has piqued the interest of many in the biomaterials community. Many cell types have been found to respond to pNIPAM by exhibiting confluent monolayer detachment, (e.g., urothelial, vascular smooth muscle, liver, lung, and spleen cells). Additional uses for pNIPAM include “smart” coatings to release biofouling films, for cell patterning, controlled drug release, for fluid control in microfluidic devices, and as a microfluidic affinity chromatography matrix for the separation of complex biological mixtures. Recently, pNIPAM has shown promise for the fabrication of...
functional, three-dimensional tissue, and envisioned applications of pNIPAM research include skin grafts, heart tissue, and other engineered tissues. This field has been named "cell sheet engineering".

There are many techniques to prepare N-isopropyl acrylamide (NIPAM) surfaces. Initially studied as a hydrogel, pNIPAM has been immobilized on surfaces by co-grafting pNIPAM with other polymers, by immobilizing pNIPAM by photolithography, by polymerizing pNIPAM with previously activated surfaces, and by vapor-phase deposition of plasma polymerized NIPAM (ppNIPAM).

In a recent study by the Okano group, it was demonstrated that cell sheets detached from pNIPAM surfaces appear to retain their function upon transfer to another growth surface, possibly because of the concurrent detachment of an extracellular matrix (ECM) protein, fibrinectin (FN). Despite the widespread interest in cell-surface interactions, nondestructive harvest of tissues, and the ECM, we are not aware of other work studying the behavior of the ECM (aside from the pioneering effort by the Okano group) or the extent of ECM detachment on pNIPAM upon cell detachment.

In this work, we use both biological and surface analysis techniques to examine the ECM and its release from ppNIPAM. Using immunoassays, we demonstrate the qualitative location and identity of the proteins in the detached cell sheet. Using X-ray photoelectron spectroscopy (XPS), we characterize the outer layer of the substrate after cell liftoff and demonstrate the ability of XPS to distinguish between blank TCPS, ppNIPAM-coated TCPS, and ppNIPAM-coated TCPS after cell liftoff. Finally, we use time-of-flight secondary ion mass spectrometry (ToF-SIMS) and principal component analysis (PCA) to demonstrate that the three surfaces are easily distinguished.

Our surface characterization by immunoassay, XPS, and ToF-SIMS suggests that although low-temperature liftoff of a monolayer of bovine aortic endothelial cells (BAECs) from the NIPAM surface is accompanied by the majority of the components of the ECM, some protein is left at the surface after liftoff.

**Materials and Methods**

**Materials.** The cell culture media were purchased from Gibco Invitrogen Corp. (Carlsbad, CA) and filtered through 0.2-μm filters before use. BAECs were a generous gift from Dr. Cecilia Giachelli (University of Washington, Seattle, WA). TCPS 48-well plates were from Falcon (BD Biosciences, Franklin Lakes, NJ). NIPAM (97%+) monomer was purchased from Aldrich (Sigma-Aldrich, St. Louis, MO) and used as received. NIPAM plasma deposition onto TCPS plates was performed using a procedure previously described in a paper by Pan et al.

**Methods.** To minimize sample variance, samples for all analytical techniques (immunoassay, XPS, and ToF-SIMS) were all obtained from the same 48-well plate. For immunoassay analysis, four replicates were observed for each staining procedure. For XPS and ToF-SIMS analysis, five replicates were used for each analysis. To obtain the samples used for analysis in the XPS and ToF-SIMS high-vacuum chambers, the wells were washed with deionized water three times and soaked with water for at least 24 h to remove free ions remaining in the buffer. Next, the well bottoms were removed from the 48-well plates using a punch and hammer. The surfaces were then packed in inert containers backfilled with N2, sealed, and stored until analysis.

**Cell Culture.** BAECs were cultured in Dulbecco’s modification of Eagle’s medium (DMEM) supplemented with 4.5 g/L glucose, 10% fetal bovine serum (FBS), 0.1 mM MEM nonessential amino acids, 1 mM MEM sodium pyruvate, 100 U/mL penicillin, and 100 μg/mL streptomycin. BAEC cells used in the experiments were between passage 7 and passage 15. Cell incubation was performed at 37 °C in a humidified atmosphere with 5% CO2. Cell incubation was performed at 37 °C in a humidified atmosphere with 5% CO2. The cells were dissociated from the culture flasks with trypsin/ethylenediaminetetraacetic acid and washed with Dulbecco’s phosphate buffered saline (DPBS) before seeding onto 48-well plates.

**Cell Sheet Detachment.** BAECs were plated from complete media containing 10% FBS into a 48-well plate at the cell density of 2.5 × 10^5 cells/well and cultured at 37 °C. The wells were either ppNIPAM-coated or bare TCPS. BAECs were cultured until confluence. To test cell liftoff behavior, the culture media were removed and replaced with serum-free DMEM, and the cells were incubated at room temperature for 2 h for cell sheet detachment.

**Immunoassay.** The detached cell sheets from ppNIPAM and intact cell sheets on TCPS were fixed at 37 °C with 4% paraformaldehyde in DPBS for 5 min. Afterward they were rinsed and incubated with 5% donkey serum in DPBS for 60 min at room temperature for blocking. The cell sheets were stained in the wells that they were released from to detect the location of FN, LN, Coll I, and Coll IV. These proteins are the major ECM protein types secreted by endothelial cells. Immunostaining of FN was performed by reacting with mouse polyclonal anti-bovine FN antibody at a 1:50 dilution for 30 min at room temperature. Following three washes with DPBS, the samples were incubated for 1 h with a 1:35 dilution of FITC-conjugated donkey anti-mouse IgG antibody and again washed three times. Immunostaining of LN was performed by reaction with rabbit polyclonal anti-bovine LN antibody at a 1:100 dilution for 30 min at room temperature, followed by incubation for 1 h with a 1:35 dilution of Texas Red-conjugated donkey anti-rabbit IgG antibody. Coll I and Coll IV were stained by goat polyclonal anti-bovine collagen I and IV antibodies at 1:15 dilution for 30 min at room temperature, followed by Texas Red-labeled donkey anti-goat IgG antibodies and TRITC-labeled donkey anti-goat IgG, respectively, at 1:35 dilution at room temperature. After rinsing with DPBS, Hoechst 33342 dye was added to a final concentration of 10 μg/mL and incubated at room temperature for 30 min. All cell sheets were rinsed again before observation using XPS and stored in DPBS during observation. All stained dishes were observed under an inverted microscope with fluorescence equipment (TE200; Nikon) or a phase contrast microscope. In Figure 2, two double-stained images were superimposed with software.
(Metamorph Images). At least four replicates were prepared for each sample. 

XPS Analysis. XPS spectra were acquired on Surface Science Instruments X-Probe and S-Probe instruments. Each of these systems is equipped with a monochromatized aluminum Kα X-ray source, an electron flood gun for charge neutralization, and a hemispherical electron energy analyzer. All survey scans for compositional analyses were acquired at a pass energy of 150 eV, and all high-resolution scans were acquired at a pass energy of 50 eV. Compositional analyses (0–1100 eV) and high-resolution scans of the C(1s) region were carried out on all samples. Binding energies for high-resolution spectra were referenced to the C(1s) (C–C–C–H) peak at 285.0 eV to account for binding energy shifts inherent to insulator samples. At least five replicates were analyzed of each sample, with three survey spectra and one high-resolution carbon spectrum acquired on each replicate.

Data treatment was performed on the Service Physics ESCAVB data reduction software. Core-level spectra were peak fit using the minimum number of peaks possible to obtain random residuals. A 100% Gaussian line shape was used to fit the peaks, and a Shirley function was used to model the background.

ToF-SIMS Analysis. A model 7200 Physical Electronics instrument (PHI, Eden Prairie, MN) was used for static ToF-SIMS data acquisition. The instrument has an 8 keV Cs+ ion source, a reflectron time-of-flight mass analyzer, chevron type multichannel plates, and a time-to-digital converter. Positive secondary ions mass spectra were acquired over a mass range from m/z = 0 to m/z = 450. Negative ion ToF-SIMS spectra were not considered in this study because of their lower information content and lack of unique peaks for different amino acids.24 The area of analysis for each spectrum was 100 µm x 100 µm, and the total ion dose used to acquire each spectrum was less than 2 x 10^12 ions/cm². The mass resolution (m/δm) of the secondary ion peaks in the positive spectra was typically between 4000 and 6000. The ion beam was moved to a different spot on the sample for each spectrum. Positive spectra were calibrated using the CH₃⁺, CH₂⁺, CH⁺, and CH⁻ peaks before further analysis. At least five replicates were prepared for each sample type, with three spectra acquired on each replicate.

PCA. PCA was performed using PLS Toolbox version 2.0 (Eigenvector Research, Manson, WA) for MATLAB (MathWorks, Inc., Natick, MA). All spectra were mean-centered before running PCA. Although a detailed description of PCA is not warranted here, the interested reader is referred to the more complete discussion of PCA by Jackson25 or Wold et al.26 Briefly, a “complete” peak set was constructed using all of the major peaks from each sample type (blank TCPS, ppNIPAM-coated TCPS, and ppNIPAM-coated TCPS after cell lift-off) in the 0–200 m/z region. Any ToF-SIMS spectrum where the sodium ion peak intensity was >1% of the total intensity of the peak set was discarded because of the matrix effects of the sodium ion on the SIMS fragmentation process.24 Selected peaks were then normalized to the total ion intensity to account for fluctuations in secondary ion yield between different spectra. PCA was then used to capture the linear combination of peaks that described the majority of the variation within the dataset. From this, an output of both a “scores” and a “loadings” plot were obtained. In previous work by our group, ToF-SIMS has been used to identify proteins present at a surface through the identification of the unique amino acid fragmentation patterns in the ToF-SIMS positive ion spectra.34 In this work, we use ToF-SIMS to detect the characteristic molecular ions of ppNIPAM, TCPS, and amino acids to distinguish between different samples.

Results and Discussion

pNIPAM has been immobilized as a cell culture surface using many techniques.18–20 In this work, we use the vapor-phase deposition of ppNIPAM on TCPS 48-well plates following a procedure previously described in a paper by Pan et al.21 This technique affords a one-step, solvent-free, vapor-phase method for the deposition of a film that is sterile and pinhole-free, with excellent surface coverage.

Surfaces treated with pNIPAM have been observed to undergo a transition above and below the lower critical solution temperature (LCST) of ~31 °C. Above the LCST (i.e., at the cell culture temperature of 37 °C), the surfaces are observed to become more hydrophobic (as observed by the water contact angle), and many cell types will adhere and proliferate. Below the LCST (i.e., at room temperature) the surface rapidly hydrates, and cell monolayers will spontaneously detach as a sheet. The mechanism is not entirely understood and is outside the scope of this current manuscript. A more complete characterization of the behavior of ppNIPAM will be presented in an upcoming publication.

In Figure 1, phase-contrast microscopy images are shown of BAECs on regular TCPS (Figure 1a) and ppNIPAM-coated TCPS at cell culture temperatures (37 °C; Figure 1c), as well as the two surfaces after removal from heat (Figure 1b,d). Note that, at cell culture temperature, the cell sheets on the two surfaces are identical, having formed confluent monolayers as evident by their cobblestone appearance. After their removal from heat, those cultured on the ppNIPAM-coated surface detach as a contiguous sheet (see Figure 1d), whereas those grown on TCPS remain attached (Figure 1b). In fact, cells cultured on TCPS will only detach when treated by enzymatic or mechanical removal methods. (A time-lapsed series of images illustrating the detachment of a cell monolayer from ppNIPAM in .mov format is available.)

Immunostaining. In the work presented by Kushida et al.,22 it was demonstrated that when cell sheets and the NIPAM-coated surfaces from which they were recovered were immunoassayed, FN stained only with the cell sheet, not the surface. As FN is believed to be the
primary ECM protein that mediates cell adhesion, the authors concluded that this implied that the ECM is recovered concurrently with the cell sheets upon detachment from NIPAM-coated surfaces.

In this study, we have examined the location of three prominent proteins of the ECM of BAECs: LN, FN, and collage. LN and FN both contain binding sites for cell-surface receptors, collagen, and other ECM molecules. Coll IV is believed to serve as the scaffold to which other ECM proteins and constituents bind and align. Although Coll IV is known to be the primary collagen type in endothelial cell basement membranes, it has previously been demonstrated that identical endothelial cells may respond to differences in media or substrate by extruding differing amounts or types of collagen; for this reason we have chosen to analyze both Coll I and Coll IV. All three proteins provide the means for diverse biological functions, including cell adhesion, morphology, migration, and differentiation of cells. Thus, these proteins are excellent models for the study of the behavior of ECM upon cell detachment from NIPAM-coated surfaces.

Figure 2 presents representative results of ECM protein immunostaining of cell sheets, and the ppNIPAM-coated TCPS from which the sheets were harvested. On the left is the bright field image of the cell sheet and surface. On the right are the doubly stained images; the cell nuclei were stained using Hoechst 33342 dye and appear blue. The ECM proteins stain red or green, depending on the type of fluorophore used.

In Figure 2a, we find that the cell nuclei (as indicated by the blue regions) are co-localized with the LN protein (as indicated by the red color of the Texas Red dye). The contrast between the cell sheet and the underlying surface is easily distinguished; therefore, we conclude that little (if any) LN remains on the ppNIPAM surface after cell detachment. Upon examination of the FN staining results, we find that again the cell nuclei (as indicated by the blue regions) are co-localized with the FN protein (as indicated by the green color of the FITC dye). Furthermore, FN fibrils are easily detected between the cells. (See Figure 2b).

When we examine Coll I, we find that although the cell nuclei still are easily distinguished, the contrast between the cell sheet and the surface is not. The same is true for the Coll IV results (See Figure 2c,d, respectively). This observation indicates that the behavior of collagen is different from either LN or FN upon cell detachment from ppNIPAM. However, from immunoassay alone, it is impossible to determine whether this difference arises from a difference in the location of the collagen (e.g., it is evenly split between the cell sheet and the surface) or whether it reflects a difference in the staining ability of collagen itself.

XPS Results. XPS is a quantitative surface analytical tool, sensitive to the atomic composition to the outer 20—100 Å of a material. The analysis of these specimens is complicated by the fact that the composition of NIPAM is extremely similar to that of proteins (i.e., both contain primarily carbon, nitrogen, and oxygen). Therefore, in this case, XPS is unable to provide an estimate of the relative film thickness of the protein layer. Still, we can see obvious differences in the relative C, N, and O atomic percentages in blank TCPS, ppNIPAM-coated TCPS, and ppNIPAM-coated TCPS post-cell liftoff (see Table 1). Upon deposition with ppNIPAM, we find that the relative atomic percents of carbon and oxygen decrease (from 82.3 to 78.8% and 17.6 to 14.0%, respectively) while the amount of nitrogen increases dramatically (from 0.1 to 7.2%).

This result varies from the composition predicted from the stoichiometry of the NIPAM monomer, which is 75.0% C, 12.5% N, and 12.5% O (excluding hydrogen). It also differs from ppNIPAM coatings on silicon chips produced using our method (76.2% C, 11.3% O, and 12.5% N). It is possible that this difference is due to the geometry of the well, preventing the gas species in the plasma from coating the well bottom in an identical manner to that on flat
decreases further to 57.8% after cell harvest. This decrease from 83.9 to 71.5% upon deposition with ppNIPAM and the peaks. Table 2 shows that the C
immediately apparent in Figure 3 because of scaling of hydrocarbon peak at 285 eV, although this loss is not
immediate increases in sulfur (0.4%) and phosphorus (0.3%), and other salts from the buffers.

![Graph](image)

**Figure 3.** High-resolution XPS C(1s) spectra of blank TCPS (top), ppNIPAM-treated TCPS (middle), and ppNIPAM-treated TCPS post-cell liftoff (bottom). Note the steady loss in intensity of the hydrocarbon peak at 285 eV and the concomitant increase of alcohol and amide peaks (shifts of +1.5 and 3.5, respectively). The region containing the π* peak for blank TCPS (top) and ppNIPAM-treated TCPS post-cell liftoff (bottom) are inset.

Table 1. Elemental Compositions Determined by XPS Survey Spectra of Blank TCPS, Blank ppNIPAM-Treated TCPS, and ppNIPAM-Treated TCPS Used for Cell Culture^a

<table>
<thead>
<tr>
<th>description</th>
<th>C</th>
<th>O</th>
<th>N</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td>blank TCPS</td>
<td>82.3</td>
<td>17.6</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>ppNIPAM cell lift</td>
<td>70.2*</td>
<td>17.8</td>
<td>10.7</td>
<td>0.4</td>
</tr>
</tbody>
</table>

^a Relative atomic percent of the major components are presented; trace amounts of Cl, Na, P, Ca, Si, Zn, and K were present in ppNIPAM-treated TCPS used for cell culture (<1%). Standard deviations are <3%, except where noted by an asterisk (ppNIPAM cell lift C atomic % std dev = 3.9%). n = 12 for blank TCPS and blank ppNIPAM-treated TCPS; n = 28 for ppNIPAM-treated TCPS used for cell culture.

is due to the large accumulation of C–N=C–OH and N–C=O species (10.1–25.6%, 0–16.3%) in the ppNIPAM post-cell liftoff sample. As expected, the π* shake-up peak (inset in Figure 3 and appearing at a shift of +6.5 eV) disappears as the aromatic ring of the underlying TCPS is first buried by ppNIPAM. After cell liftoff from the ppNIPAM, the shake-up peak is again detected, most likely because of aromatic rings from the protein layer itself (see Figure 3, inset).

**ToF-SIMS Results.** Like XPS, ToF-SIMS is a surface-sensitive analytical tool. ToF-SIMS is considered to be a complementary technique to XPS, because the former yields information regarding molecular species at interfaces, while the latter gives elemental information. Because the fragmentation of the proteins is so extensive, it is not possible to identify large chains of the protein (such as in matrix-assisted laser desorption ionization). Furthermore, the energetic process yields hundreds of peaks in the 0–200 m/z range, making the interpretation of ToF-SIMS spectra difficult, especially when spectra acquired from different substrates or different treatments are compared.

**Table 2. Composition from High-Resolution XPS C(1s) Spectra of Blank TCPS, Blank ppNIPAM-Treated TCPS, and ppNIPAM-Treated TCPS Used for Cell Culture^a**

<table>
<thead>
<tr>
<th>description</th>
<th>C–H</th>
<th>C–N=C–OH</th>
<th>N–C=O</th>
<th>O–C=O</th>
<th>π*</th>
</tr>
</thead>
<tbody>
<tr>
<td>(285 eV)</td>
<td>(+1.5)</td>
<td>(+3.5)</td>
<td>(+4.2)</td>
<td>(+6.5)</td>
<td></td>
</tr>
<tr>
<td>blank TCPS</td>
<td>83.9</td>
<td>10.1</td>
<td>0.0</td>
<td>3.5</td>
<td>2.5</td>
</tr>
<tr>
<td>ppNIPAM- treated TCPS</td>
<td>71.5</td>
<td>17.8</td>
<td>10.8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ppNIPAM cell lift</td>
<td>57.8*</td>
<td>25.6</td>
<td>16.3</td>
<td>0</td>
<td>0.3</td>
</tr>
</tbody>
</table>

^a Standard deviations are <2%, except where noted by an asterisk (ppNIPAM cell lift C–H std dev = 3.1%). n = 6 for blank TCPS and blank ppNIPAM-treated TCPS; n = 14 for ppNIPAM-treated TCPS used for cell culture.

To simplify data interpretation, we used PCA, a multivariate analysis technique that aids in the interpretation of spectra by identifying related variables and focusing on the differences between spectra. Figure 4a shows a scores plot of principal component 1 (PC 1), which captures 37% of the variance in the data, versus principal component 2 (PC 2), which captures 28% of the data. Examination of Figure 4a shows that each type of sample is distinctly grouped from each other; blank TCPS samples (○) cluster toward the bottom left-hand side of the plot, ppNIPAM-treated TCPS samples (+) cluster toward the bottom right-hand side of the plot, and ppNIPAM-coated TCPS after cell liftoff (×) cluster at the top center of the plot. The ellipses drawn around each group indicate the 95% confidence interval of each grouping. To appreciate the reason for these differences, we must inspect the loadings plot for each PC.

Figure 4b represents the loadings from PC 1. Each of the peaks in the PC 1 loadings plot below the origin corresponds to samples in the scores plot that load negatively; each of the peaks in the PC 1 loadings plot above the origin corresponds to samples in the scores plot that load positively. By comparing the PC 1 loadings plot to the PC 1 scores, we find that the TCPS samples (○) are correlated with peaks originating from the TCPS substrate, such as C₆H₅ (m/z = 91). In comparison, the ppNIPAM blank samples (+) are correlated with peaks originating from the ppNIPAM substrate, such as C₆H₅N (m/z = 58). However, the ppNIPAM-coated TCPS after cell liftoff (×) all cluster toward the middle of the origin and are not easily separated. This suggests that the...
The differences between this sample type and the others are not due to the underlying substrate.

To understand how ppNIPAM-coated TCPS samples after cell liftoff vary from the other blank substrates, we must examine Figure 4c, the loadings from PC 2. By comparing the PC 2 loadings plot to the PC 2 scores, we find that the ppNIPAM-coated TCPS samples after cell liftoff load with peaks that have already been identified as originating from amino acid residues, such as glycine (Gly, CH2N), alanine (Ala, C2H6N), proline (Pro, C4H8N), and leucine or isoleucine (Leu/Ile, C5H12N). Because both glycine and proline are found in high concentrations in collagen, these data give preliminary support to the idea that some collagen remains at the surface after cell detachment from ppNIPAM-coated surfaces. In an upcoming publication, we intend to more thoroughly identify proteins remaining at the surface. The other two sample types, blank TCPS and ppNIPAM-coated TCPS, load negatively on the PC 2 scores, because they do not contain amino acid fragments. In fact, there are very few peaks (primarily hydrocarbons) associated with loadings from these samples.

Conclusions

The temperature-responsive behavior of pNIPAM—and its effect on cells cultured on pNIPAM-treated surfaces—has been well studied. Confluent monolayers of many cell types have been observed to spontaneously detach as a contiguous sheet from pNIPAM upon removal from heat, while their counterparts grown on TCPS remain attached for hours or days, requiring enzymatic or mechanical methods to remove them. Despite this interest in cell behavior on pNIPAM, the behavior of the ECM secreted from the cells (i.e., whether it remains at the surface or detaches with the cell sheet) has not been well studied.

In this work, we have used traditional surface analysis and biological techniques for a thorough examination of the ECM on surfaces coated with ppNIPAM. Our immunoassay results indicate that although FN, LN, and collagen closely associate with the cell sheet, some collagen may be associated with the surface, as well. XPS reinforces the hypothesis that ppNIPAM-treated surfaces are distinctly different after cell culture; the presence of amide and alcohol species indicates that a proteinaceous film exists after cell sheet detachment. Finally, a comparison of ppNIPAM-treated surfaces used for cell culture versus bare ppNIPAM-treated controls by PCA confirms that ppNIPAM-treated surfaces after cell liftoff are not identical to their bare counterparts—the primary reason for this difference is the presence of molecular ion fragments from amino acids (e.g., alanine, glycine, proline) left on ppNIPAM samples after cell liftoff. Therefore, from our surface characterization of ppNIPAM-coated TCPS after cell liftoff, we conclude that although low-temperature liftoff of the BAEC monolayer from the NIPAM surface is accompanied by the majority of the components of the ECM, some protein still remains at the surface.

The presence of molecular ion fragments from glycine and proline of ppNIPAM samples after cell liftoff, both of which are found in high concentrations in collagen, is an interesting preliminary result. Future experiments will further investigate this protein layer remaining after cell liftoff and identify the actual protein species remaining. This remaining protein after liftoff raises questions about the mechanism of liftoff. In the future, we tend to investigate whether the residual ECM proteins at the surface result from the ECM sheet splitting in the middle or whether patches of protein are adhered to small

Figure 4. PCA of positive ToF-SIMS including (a) scores plot, (b) PC 1 loadings plot, and (c) PC 2 loadings plot. The scores plot (a) shows that the TCPS blank samples (○), ppNIPAM-treated TCPS samples (+), and ppNIPAM-treated TCPS used for cell culture (×) all cluster in distinctly different regions. Ellipses around data indicate a 95% confidence interval. PC 1 captures 37.2% of the variance in the data. PC 2 captures 28.2% of the variance in the data. The PCA scores and loadings plots can be interpreted by comparing the relationship between the scores and loadings for a give PC. Peaks that load negatively in the PC 1 loadings plot (b) correspond with samples with negative scores on PC 1 in the scores plot (a). Peaks that load negatively in the PC 2 loadings plot (c) correspond with samples with negative scores on PC 2 in the scores plot (a). Note that PC 1 effectively separates TCPS blank samples (○) from ppNIPAM-treated TCPS samples (+) as a result of peaks originating from the substrates; PC 2 effectively separates ppNIPAM-treated TCPS samples used for cell culture (×) from the blank samples as a result of peaks originating from amino acids in the ECM.
nontemperature sensitive islands of the ppNIPAM surface. Furthermore, we intend to compare the low-temperature liftoff method to other, more traditional cell removal techniques such as enzymatic digestion and physical scraping. Finally, we will investigate whether the remaining ECM layer is itself still active after cell liftoff by performing a cell adhesion assay.

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Supporting Information Available: A time-lapse series of phase-contrast microscopy images of the low-temperature liftoff of a confluent monolayer of BAEC cells from ppNIPAM-coated TCPS. This material is available free of charge via the Internet at http://pubs.acs.org.

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