Cell sheet detachment affects the extracellular matrix: A surface science study comparing thermal liftoff, enzymatic, and mechanical methods

Heather E. Canavan,1,3 Xuanhong Cheng,2,3 Daniel J. Graham,1,3 Buddy D. Ratner,1,2,3,4 David G. Castner1,2,3,4

1 National ESCA and Surface Analysis Center for Biomedical Problems, Box 351750, University of Washington, Seattle, Washington
2 University of Washington Engineered Biomaterials, Box 351720, University of Washington, Seattle, Washington
3 Department of Bioengineering, Box 351750, University of Washington, Seattle, Washington
4 Department of Chemical Engineering, Box 351750 University of Washington, Seattle, Washington

Received 27 September 2004; accepted 23 November 2004
Published online 5 August 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jbm.a.30297

Abstract: This work compares the removal of bovine aortic endothelial cell (BAEC) monolayers via 1) low-temperature liftoff from a “smart polymer,” plasma polymerized poly(N-isopropyl acrylamide) (ppNIPAM), 2) enzymatic digestion, and 3) mechanical dissociation from ppNIPAM surfaces. We examine the surfaces after cell removal by using X-ray photoelectron spectroscopy (XPS), time-of-flight secondary ion mass spectrometry (ToF-SIMS), immunostaining, and cell adhesion assay. Immunoassay results indicate that low-temperature liftoff nondestructively harvests the cell sheet and most of the underlying extracellular matrix (ECM), whereas enzymatic digestion and mechanical dissociation leave behind surfaces that better promote cell adhesion. We conclude that the removal of BAEC cells via low-temperature liftoff from ppNIPAM-treated surfaces is less damaging to the ECM proteins remaining at the surface than the other methods. © 2005 Wiley Periodicals, Inc. J Biomed Mater Res 75A: 1–13, 2005

Key words: cell removal; ECM; N-isopropyl acrylamide; ToF-SIMS; XPS

INTRODUCTION

The removal of cells cultured on tissue culture polystyrene (TCPS) usually requires harsh enzymatic or mechanical methods that have deleterious effects on the morphological appearance of the cells being harvested.1–9 For instance, removal by enzymatic digestion tends to yield disaggregated cells with a rounded appearance,3,4,8 whereas removal by mechanical dissociation yields a few cells surrounded by a crystalline matrix. These morphological changes have recently been attributed to changes in the disruption of the cellular membranes and glycolalix, often leading to a loss of cellular activity.10,11 Furthermore, it has been observed that these methods affect the behavior and chemical makeup of the cells themselves, as observed by infrared spectroscopy and lactate dehydrogenase assay.10,11

It naturally follows that such readily measured effects on the cells harvested must be concurrent with damage to the extracellular matrix (ECM) underlying the cells, as well. Because the ECM is known to be vital to the adhesion, proliferation, and differentiation of cells,12,13 this has important implications for cell culture. Cell release from poly(N-isopropyl acrylamide) (pNIPAM)-treated surfaces is being investigated as a nondestructive cell harvest alternative. By culturing cells on pNIPAM, it is possible to rapidly recover intact cell monolayers from culture surfaces by using a
modest temperature drop as the sole stimulant for
detachment.14 Discussion of the hypothesized mecha-
nism behind this behavior can be found in articles by
Cheng et al.15 and Okano et al.16

The fact that pNIPAM undergoes a sharp property
change in response to a moderate thermal stimulus
near physiological temperatures has generated great
interest in the biomaterials community. Many cell
types detach from pNIPAM as confluent monolayers.17–21 In addition, pNIPAM is being investigated as a
“smart” biofouling and release coating, to create cell
patterns, for controlled drug release, and as a mi-
crofluidic affinity chromatography matrix for the sepa-
ration of complex biological mixtures.15,22–26 Clearly,
the use of this apparently nondestructive technique
for the harvest of cells would be of interest to those
concerned with the observations of individual cells,16
because it is to those interested in obtaining whole
sheets for the fabrication of functional, three-dimen-
sional tissue.27 However, to further develop the tech-
nique, its effects on the harvested cell sheets and the
underlying ECM must be compared with other, more
traditional cell removal methods.

Until now, however, no study has compared the
effect that cell removal methods have on the underly-
ing ECM. We previously found that fibronectin (FN)—
and possibly most of the ECM—is recovered concur-
rently with the cell sheets upon detachment from
ppNIPAM-coated surfaces.28 In this work, we deter-
nine the efficacy of this removal method and its effect
on the underlying ECM layer by comparing the re-
moval of BAECs monolayers by low-temperature lift-
off to more traditional methods, including enzymatic
digestion and mechanical dissociation. Using X-ray
photoelectron spectroscopy (XPS), we characterize the
outer layer of the substrate after cell liftoff and dem-
onstrate the ability of XPS to distinguish the effect that
each treatment has on the surface underneath the cell
sheet it removes. We use principal component analysis
(PCA) to aid in the interpretation of time-of-flight
secondary ion mass spectrometry (ToF-SIMS) data to
demonstrate that three cell removal methods yield
distinctly different “footprints” remaining on the
surfaces after cell harvest. Using immunoassay, we char-
acterize the effect that each of the three cell removal
techniques has on the ECM proteins associated with
the cell sheet versus those retained on the substrate.
Finally, we perform an adhesion assay to determine
the viability of any remaining ECM proteins left at the
surface after the three removal methods. From this
surface characterization by immunoassay, cell adhe-
sion assay, XPS, and ToF-SIMS, we conclude that the
removal of BAEC cells from ppNIPAM-treated sur-
faces is less damaging to the ECM proteins remaining
at the surface than are enzymatic or mechanical de-
tachment methods.

MATERIALS AND METHODS

Materials

Cell culture supplies were purchased from Gibco Invitro-
gen Corporation (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). N-isopropyl acrylamide (NIPAM, 97%)
monomer was purchased from Aldrich (Sigma-Aldrich,
St. Louis, MO) and used as received.

Mouse polyclonal anti-bovine FN antibody was from
Sigma (St. Louis, MO); rabbit polyclonal anti-bovine laminin
(LN) antibody was from Novus Biologicals, Inc. (Littleton,
CO); goat polyclonal anti-bovine collagen type I (Coll I) and
type IV (Coll IV) antibodies were from Chemicon Interna-
tional (Temecula, CA); and Hoechst 33342 trihydrochloride,
trihydrate, [2,5′-bi-1H-benzimidazole, 2′-(4-ethoxyphenyl)-
5-(4-methyl-1-piperazinyl)] was from Molecular Probes, Inc.
(Eugene, OR). Fluorescein isothiocyanate (FITC)-conjugated
donkey anti-mouse IgG antibody, sulforhodamine 101
(Texas Red)-conjugated donkey anti-rabbit IgG antibody,
Texas Red-labeled donkey anti-goat IgG antibodies, and
tetramethylrhodamine isothiocyanate (TRITC)-labeled don-
key anti-goat IgG were from Jackson ImmunoResearch Lab-
oratories (West Grove, PA).

Methods

To minimize sample variance, samples for all analytical
techniques (immunoassay, XPS, and ToF-SIMS) were all ob-
tained from ppNIPAM substrates that were prepared in one
batch. For cell removal by enzymatic digestion and low-
temperature liftoff, the substrates used were 48-well TCPS
plates. In mechanical dissolution, larger 12-well plates had
to be used to accommodate the scraper.

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 in water for at least 24 h to
reduce free ions remaining from the buffer. Next, the well
bottoms were harvested: well bottoms from 48-well plates
were punched out, whereas 12-well plates were sliced by
using a heated NiCr wire. The surfaces were then packed in
sealed, inert containers backfilled with N2 and stored until
analysis.

pNIPAM deposition

pNIPAM surfaces have been fabricated in a variety of
forms, including as hydrogels,29–32 immobilized on surfaces
by cografting it with other polymers,33 by photolithogra-
phy,34 and by polymerization with previously activated sur-
faces.35,36 The method we chose was by vapor-phase depo-
sition of plasma polymerized NIPAM (ppNIPAM), as previously described in an article by Pan et al.36

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 non-essential amino acids, 1 mM MEM sodium pyruvate, 100 U/mL penicillin, and 100 µg/mL streptomycin. BAECs cells used in the experiments were between passages 7 and 15. Cell incubation was performed at 37°C in a humidified atmosphere. The cells were dissociated from the culture flasks with trypsin/ethylenediaminetetraacetic acid (EDTA), washed with Dulbecco’s phosphate-buffered saline (DPBS) before seeding onto 48-well or 12-well plates.

Cell sheet detachment

BAECs were plated from complete media containing 10% FBS into either a 48-well plate at the cell density of 2.5 × 10⁴ cells/well (for trypsinization and liftoff) or a 12-well plate at the cell density of 5 × 10⁴ cells/well (for mechanical dissociation) and cultured at 37°C. The wells were either ppNIPAM coated or bare TCPS. BAECs were cultured until confluence. Cell removal took place by one of three methods: low-temperature liftoff, enzymatic digestion, or mechanical dissociation using a silicone scraper.

Low-temperature liftoff

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.

Enzymatic digestion

To test the effect of enzymatic digestion with trypsin, BAECs were washed with prewarmed DPBS and incubated with 0.25% trypsin–1 mM EDTA (Gibco-Invitrogen) at 37°C for 5 min. The wells were rinsed three times with DPBS to wash off the cells.

Mechanical dissociation

To test the effect of mechanical dissociation, BAEC layers were removed from the surfaces with a rubber blade (Corning, Corning NY) in DPBS buffer and rinsed with DPBS.

Immunostaining

The detached cells from ppNIPAM and TCPS were fixed at 37°C with 4% paraformaldehyde in DPBS for 5 min. Afterward, they were rinsed and incubated with 5% donkey serum (DS) in DPBS for 60 min at room temperature for blocking. The cells 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 among the major ECM protein types secreted by endothelial cells.12,37 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. After 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 laminin 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 IV were stained by goat polyclonal anti-bovine Coll 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 cells were rinsed again before observation using DPBS and stored in DPBS during observation. All stained dishes were observed under an inverted microscope with fluorescence equipment (TE300; Nikon, Melville, NY) or a phase-contrast microscope. The double-stained images were superimposed with software (Metamorph Images; Universal Imaging Corporation, Downingtown, PA) to yield the final images. At least four replicates were prepared for each sample. Figures 1–4 present representative results from each condition.

Cell adhesion assay

After cell removal by the three methods, the surfaces from which the cells were detached were tested by using an adhesion assay. To achieve this, 1 mL of BAEC cells in serum-free media were seeded on the 48-well surfaces and 2 mL of BAEC cells were seeded on the 12-well plates. Both 48-well and 12-well plates were plated at a cell density of 5 × 10⁴/mL. The plates were cultured at 37°C for 2 h. The cell morphology was observed under a phase-contrast microscope. The number of attached cells was determined by using lactate dehydrogenase (LDH) (Roche Diagnostics, Basel, Switzerland) assay, which is based on the principle of releasing the cytoplasmic enzyme LDH and measuring its activity in the supernatant. Briefly, the samples with adhered cells were washed twice with DPBS, and cells were permeabilized with 500 µL of 1% Triton-X100 for 30 min; 100 µL of the supernatant was transferred to a 96-well TCPS plate and mixed with the reaction solution for 30 min at room temperature. The absorbance of the solution was measured at 490 nm by using a SpectraCount™ plate reader (Packard, PerkinElmer, Boston MA) and fit to a standard curve to determine the number of cells on each surface. The experiments were performed with six replicas at each test condition.
Statistical analysis

Adhesion assay results obtained from secondary cell cultures on treated surfaces were compared with suitable primary culture adhesion controls. To determine the statistical significance of differences observed between the sample and control means, two-tailed student t tests were performed by using SPSS (SPSS, Inc., Chicago IL).

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 regions were conducted on all samples.

Data treatment was performed on the Service Physics ESCAVB data reduction software. 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. Core-level spectra were peak-fit by using the minimum number of peaks possible to obtain random residuals. The binding energy shift of the C-N/C=O peak was constrained to +1.5 eV from that of the C-H peak. A 100% Gaussian line shape was used to fit the peaks, and a Shirley function was used to model the background. At least five replicates were prepared for each sample, with three composition and one high resolution spectra acquired on each replicate.

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 (MCP), and a time-to-digital converter (TDC). Positive secondary ions mass spectra were acquired over a mass range from m/z = 0–450. 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. The area of analysis for each spectrum was 100 μm × 100 μm, and the total ion dose used to acquire each spectrum was <2 × 10¹² 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 by using the CH₃⁺, C₃H₅⁺, C₆H₁₀⁺, and C₇H₇⁺ peaks before further analysis. At least five replicates were prepared for each sample type, with three spectra acquired on each replicate.

Figure 1. Representative FN immunostaining results of cell sheets and the surfaces they were removed from with use of the following methods: low-temperature liftoff from ppNIPAM-treated TCPS (top), mechanically dissociating cells from TCPS (middle), and enzymatic digestion with trypsin/EDTA from TCPS (bottom). Images are bright-field images (left of each column) and immunostained images (right of each column). FN stained green with FITC-labeled secondary antibody, and cell nuclei stained blue with Hoechst 33342 dye. Bar = 100 μm.

Figure 2. Representative LN immunostaining results of cell sheets and the surfaces they were removed from with use of the following methods: low-temperature liftoff from ppNIPAM-treated TCPS (top), mechanically dissociating cells from TCPS (middle), and enzymatic digestion with trypsin/EDTA from TCPS (bottom). Images are bright-field images (left of each column) and immunostained images (right of each column). LN stained red with Texas Red-labeled secondary antibody, and cell nuclei stained blue with Hoechst 33342 dye. Bar = 100 μm.
Principal components analysis (PCA)

PCA was performed by 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 Jackson or Wold et al. Briefly, a “complete” peak set was constructed by using all of the major peaks from each sample type (i.e., surfaces after cell removal by low-temperature liftoff, enzymatic digestion, and mechanical dissociation) in the 0–200 m/z region. Any ToF-SIMS spectrum where the sodium ion peak intensity was >1% of the intensity of the spectrum was discarded because of the matrix effects of the sodium ion on the SIMS fragmentation process. 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 most of the variation within the data set. From this, an output of both a “scores” and a “loadings” plot was 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. 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

The desire to separate different cell types from the same culture (e.g., keratinocytes from fibroblasts) or to obtain monodispersed cells for experiments has led to research into the optimal methods for separating cells from their substrate and from each other. It has been observed that these methods often have deleterious effects on cell morphology. Removal by enzymatic digestion tends to yield disaggregated cells with a rounded appearance, whereas removal by mechanical dissociation yields a few cells surrounded by a crystalline matrix. Until now, however, no study has compared the effect that cell removal methods have on the underlying ECM. In this work, we investigate the effect that some common cell removal methods (mechanical dissociation and enzymatic digestion) have on cells and their underlying ECM. In addition, we compare these methods with cell harvest from ppNIPAM, a temperature-responsive polymer used to coat TCPS. Previously, we found that FN—and possibly most of the ECM—is recovered concurrently with the cell sheets upon detachment from NIPAM-coated surfaces. In the following work, we examine the effect of each of the cell removal methods on the cell sheets, as well as the surfaces left behind, by using both traditional cell biology techniques (immuno-
staining and cell adhesion assay) and traditional surface analytical techniques (XPS and ToF-SIMS).

**Immunoassay results**

Immunoassay is an excellent tool for the qualitative location of proteins, and when used in conjunction with bright-field imaging, provides insight into the appearance of the cells themselves. In this study, we have examined the location of several of the major ECM proteins of BAECs: LN, FN, and collagen. Both LN and FN have binding sites for cells, as well as collagen and other ECM molecules. Type IV collagen (Coll IV) is believed to function as a scaffold by which other ECM proteins and constituents bind and align. Although Coll IV is known to be the primary type of collagen in basement membranes secreted from endothelial cells, it has previously been found that endothelial cells may respond to minute differences in their surroundings (e.g., in the media or the type of substrate used) by extruding differing amounts or types of collagen. For this reason, we have chosen to perform immunoassay on both Coll IV and collagen type I (Coll I). Each of these proteins provide the means for diverse biological functions, including cell adhesion, morphology, migration, and differentiation of cells, thereby serving as excellent models for the study of behavior of the ECM upon cell removal from ppNIPAM-coated surfaces.

In this section, we present immunostaining results from cells grown on both TCPS and ppNIPAM-coated TCPS. Images were also obtained from cells retrieved by mechanical dissociation and enzymatic digestion from bare TCPS controls but are not shown here. These control images are available in the Supplementary Material. Figures 1–4 present representative results of immunostaining of cells and the surfaces from which the sheets were harvested. Presentation of the results in this manner allows for the direct comparison of the fluorescence from the cells and surfaces. Because of the large number of images, the results have been grouped by each protein (e.g., FN and LN). Each row represents the technique used for cell removal: low-temperature cell liftoff from ppNIPAM (top), mechanical dissociation from TCPS (middle), and enzymatic digestion from TCPS (bottom). The columns contain either a bright-field image (left column) or a fluorescence image (right column). The ECM proteins stain red or green, depending on the type of fluorophore used. Observation of cell sheets harvested by low-temperature cell liftoff (top) is consistent with previously obtained results: the entire cell sheet has detached, and both the FN and LN stain predominantly with the cell sheet. Because the contrast between the cell sheet and the underlying surface is easily distinguished, we conclude that little (if any) LN or FN remains on the ppNIPAM surface after cell detachment. The staining of Coll I and IV are less conclusive; although the cell sheets are still detached as confluent monolayers, lower contrast between the stained cell sheet and surface is observed.

The results of immunoassay of cell sheets harvested by mechanical dissociation (middle rows) and enzymatic digestion (bottom rows) show a striking difference from low-temperature liftoff. Although all three techniques yielded cells, the technique used to harvest the cells appears to have a great effect on both cell appearance and ECM protein staining. For instance, harvesting by mechanical dissociation appears to affect the appearance of the cells (which are somewhat rounded) and breaks the cell layer into small pieces. However, mechanical dissociation does not appear to be overly damaging to the ECM. Both LN and FN (see Figs. 1 and 2) from scraped cells appear to fluoresce as strongly as do sheets harvested by low-temperature liftoff. Furthermore, physical mechanical dissociation appears to leave interwoven ECM layers more or less intact in the cell patches.

The effect of enzymatic digestion with trypsin on cell appearance is far more pronounced; the cells have a rounded appearance, consistent with previous observation of trypsinized cells. In addition, the ECM proteins fluoresce far more weakly than do ECM proteins obtained from liftoff or mechanical dissociation of the surfaces. Furthermore, only the regions directly surrounding individual cell nuclei (stained in blue) appear to fluoresce after trypsinization. These results seem to indicate that enzymatic digestion of the ECM with trypsin is more damaging to the ECM proteins than is liftoff or mechanical dissociation.

From these results, we conclude that the low-temperature liftoff technique nondestructively harvests the cell sheet and most of the underlying ECM, whereas mechanical dissociation and enzymatic digestion are damaging to both the cells and ECM. Furthermore, although most of the ECM proteins are harvested with the cell sheet itself during low-temperature liftoff, the location of cell removal is inconclusive from immunostaining.

**XPS results**

In the preceding section, immunoassay results were presented by showing the effect that cell detachment methods have on cells cultured on two different substrates (TCPS or ppNIPAM). In the following surface analysis results (XPS and ToF-SIMS), we present only the data from cells cultured on ppNIPAM-coated TCPS to highlight the differences arising from the detachment methods, instead of those arising from surface chemistry.
We find that the composition of our ppNIPAM films (78.8% C, 17.8% O, and 10.8% N) differs from that 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 the same method (76.2% C, 11.3% O, and 12.5% N). It is possible that this difference is due to a “shadow effect” from the sides of the well, which prevents the gas species in the plasma from coating the well bottom in an identical manner to that on flat substrates. Whatever the source of this difference, approximately two thirds of the monomer structure is retained in the plasma polymerized film, which is sufficient to yield the temperature-dependant behavior of cells (as discussed in the next section) as well as the surface wettability as determined by contact angle measurements.55

XPS is sensitive to the atomic composition in the outer 20–100 Å of a surface and can usually provide an estimate of the relative film thickness. Unfortunately, because pNIPAM and proteins are both primarily composed of carbon, nitrogen, and oxygen, XPS cannot be used as a reliable indicator of film thickness. Still, we can see obvious differences in the relative C, N, and O atomic percentages in the XPS results obtained of surfaces after cell removal by mechanical dissociation, by low-temperature cell liftoff, and by enzymatic digestion (Table I). We find that ppNIPAM has the highest atomic % carbon of any surface (78.8%) and the lowest amounts of oxygen and nitrogen (14.0% and 7.2%, respectively). On culture with cells and their subsequent removal by any of the three methods, the surfaces are generally observed to be composed of more oxygen and nitrogen and slightly less carbon. In addition, each of these surfaces has some remaining sulfur and phosphorous (from remaining ECM proteins and/or media), as well as sodium, chlorine, and other salts (from the buffers used and/or the trypsin/EDTA solution in the case of that treatment).

The difference between the ppNIPAM and the same surface after low-temperature liftoff is the greatest, with carbon decreasing by 9% (to 70%), oxygen increasing by 4%, and nitrogen increasing by 4%. In addition, trace amounts (<1%) of sulfur, phosphorous, sodium, and chlorine are present. Cell removal by enzymatic and mechanical treatment both yield relatively similar atomic % of the primary components (74% C, 16% O, and 8–9% N). In addition, both of these treatments result in higher amounts of salts (nearly 1% of chloride and sodium) and have larger standard deviations (up to 5.5% for enzymatic digestion) than the low-temperature liftoff samples do.

When we examine the chemical shifts evident in the high-resolution C 1s spectra, the differences between surfaces before and after cell exposure are clearer. Immediately obvious are the 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) in the treated samples (Fig. 5).

In Table II, we again find that the difference between the ppNIPAM substrate and the low-temperature liftoff is the greatest, because the C—H peak decreases by 14%, whereas the C—OH/C—N and N—C═O species are found to increase by 8% and 5%, respectively.
respectively. In comparison, the C—H peak decreases by just 2% in the mechanically dissociated surfaces and by 6% on the enzymatically dissociated surfaces. Meanwhile, the C—OH/C—N species increases by 5% in the enzymatically dissociated surfaces and 2% in the mechanically dissociated surfaces. The N—C=O species actually decreases slightly in the mechanically dissociated surfaces and increases by 2% in the enzymatically dissociated surfaces. Again, the standard deviations are lowest for the ppNIPAM (all < 1.8%) and low-temperature liftoff (all < 1.0%) surfaces. The highest standard deviations are for the mechanically dissociated surfaces, from 2–5%.

Taken together, the XPS results indicate low-temperature liftoff of cells yields the most reproducible surface.56 Although the salt ions in PC 1 account for most of the variance in the data, they do not provide insight regarding the variations between the other two cell removal methods. To understand how surfaces after cell removal by mechanical dissociation (o) vary from surfaces after cell removal by low-temperature cell liftoff (+), we must examine the loadings from PC 2 in Figure 6(c). By comparing the PC 2 loadings plot with the PC 2 axis in the scores plot, surfaces after cell removal by low-temperature cell liftoff (+) cluster toward the left hand of the plot, and surfaces after cell removal by enzymatic digestion (x) cluster at the top center of the plot. The ellipses drawn around each group indicate the 95% confidence interval of each grouping.38 To appreciate the reason for these differences, we must inspect the loadings plot for each PC.

For the three different surfaces from which cells were released, Figure 6(a) shows a scores plot of principal component 1 (PC 1), which captures 83.6% of the variance in the data versus principal component 2 (PC 2), which captures 8.7% of the data. Examination of Figure 6(a) shows the grouping for each sample type; surfaces after cell removal by mechanical dissociation (o) cluster toward the bottom of the plot, surfaces after cell removal by low-temperature cell liftoff (+) cluster toward the left hand of the plot, and surfaces after cell removal by enzymatic digestion (x) cluster at the top center of the plot. The ellipses drawn around each group indicate the 95% confidence interval of each grouping.38 To appreciate the reason for these differences, we must inspect the loadings plot for each PC.

Figure 6(b) represents the loadings for PC 1. Each of the peaks that loads negatively in the PC 1 loadings plot corresponds to samples with negative scores on PC 1 in the scores plot. Each of the peaks that loads positively in the PC 1 loadings plot corresponds to samples with positive scores in the PC 1 scores plot. By comparing the PC 1 loadings plot with PC 1 in the scores plot, we find that the largest variance comes from salt ions (e.g., Na+, K+) present on surfaces after cell removal by enzymatic dissociation with trypsin (x). These salt ions most likely arise from salts associated with the trypsin/EDTA solution used to remove the cells from the surface and were present despite repeated and extended rinsing procedures used to try to remove salts from the surfaces. The variation within this group is extremely large. It may be that this variation reflects the fact that treatment with trypsin does not affect the surfaces in a regular, predictable manner, even when those surfaces are prepared at the same time, in the same TCPS culture plate. Another possible reason for this variation is that the presence of salts on the surface induces a matrix effect, cationizing the substrate and affecting the yield of ions from the surface.56

Although the salt ions in PC 1 account for most of the variance in the data, they do not provide insight regarding the variations between the other two cell removal methods. To understand how surfaces after cell removal by mechanical dissociation (o) vary from surfaces after cell removal by low-temperature cell liftoff (+), we must examine the loadings from PC 2 in Figure 6(c). By comparing the PC 2 loadings plot with the PC 2 axis in the scores plot, surfaces after cell removal by low-temperature cell liftoff (+) and enzymatic digestion (x) correspond with peaks that have already been identified as having originated from amino acid residues, such as glycine (Gly, CH$_2$N), lysine (Lys, C$_5$H$_9$N$_2$), alanine (Ala, C$_3$H$_7$N), proline (Pro, C$_4$H$_9$N), and valine (Val, C$_4$H$_9$N).38,41

In comparison, we find that the surfaces after cell removal by mechanical dissociation (O) contain very few molecular fragments from amino acids, and in-

### TABLE II
Composition from High-Resolution XPS C 1s Spectra of ppNIPAM-Treated TCPS and ppNIPAM-Treated TCPS Used for Cell Culture Whereby Cells Were Removed by Enzymatic Digestion, Mechanical Dissociation, and Low-Temperature Liftoff

<table>
<thead>
<tr>
<th>Description</th>
<th>C-H (285)</th>
<th>C-N/C-OH (+1.5)</th>
<th>N-C = O (+3.5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ppNIPAM-treated TCPS</td>
<td>71.5</td>
<td>17.8</td>
<td>10.8</td>
</tr>
<tr>
<td>Cell removal by enzymatic digestion</td>
<td>65.1</td>
<td>22.3</td>
<td>12.6</td>
</tr>
<tr>
<td>Cell removal by mechanical dissociation</td>
<td>70.0*</td>
<td>19.7*</td>
<td>10.3</td>
</tr>
<tr>
<td>Cell removal by low-temperature liftoff</td>
<td>57.8</td>
<td>25.6</td>
<td>16.3</td>
</tr>
</tbody>
</table>

Standard deviations are ±2%, except where noted by * (mechanical dissociation C-H SD = 5.2%, C-OH = 3.2%). N = 7 for mechanical dissociation and n = 11 for ppNIPAM-treated TCPS, removal of cells by enzymatic digestion, and low-temperature liftoff.
stead correspond primarily to hydrocarbon peaks (e.g., m/z 29, 41, 55, and 69). It is interesting to note that, of all of the fragments traditionally associated with amino acid fragments, \( m/z \) 86 (leucine/isoleucine, \( C_5H_{12}N \)) correlates with mechanical dissociation. However, this fragment may also arise from phospholipids (e.g., phosphatidylcholine), which also contain the \( C_5H_{12}N \) moiety. The presence of high mass fragments (e.g., m/z 166, \( C_5H_{12}NPO_4 \); and 184, \( C_5H_{12}NPO_4 \)) supports the hypothesis that this surface contains lipid fragments. The abundance of hydrocarbons and lipids, and the absence of amino acid fragments, may indicate that the process of mechanical disruption breaks cell walls while removing the cells, liberating lipids that are detected at the surface. From these results, we conclude that, although removal of BAECs from ppNIPAM using enzymatic digestion leaves behind an ECM-like material at the substrate, there is a wide variation in the surfaces obtained. In comparison, removal of cells using low-temperature liftoff leaves behind a substrate rich in both glycine and proline. Because these amino acids are found in high concentrations in collagen, these data give preliminary support of the idea that some collagen remains at the surface after cell liftoff from ppNIPAM-coated surfaces. In an upcoming publication, we intend to more thoroughly determine the identity of proteins remaining at the surface.

Cell adhesion assay results

Although PCA analysis of ToF-SIMS results indicate that low-temperature liftoff of BAECs leaves fragments of amino acids on the ppNIPAM-treated substrate, it is impossible to determine whether the ECM is still functionally active from those results alone. To make this assessment, we next performed a cell adhesion assay, seeding new cell cultures on surfaces previously cultured with BAECs and then subsequently harvested by using the three different methods.

Figure 7 presents representative tabulated results from the cell adhesion assay. In the first five columns are secondary BAEC cells cultured on 1) TCPS from which previous culture was removed by mechanical dissociation; 2) ppNIPAM-treated TCPS from which previous culture was removed by mechanical dissociation; 3) TCPS from which previous culture was removed by enzymatic digestion with trypsin; 4) ppNIPAM-treated TCPS from which previous culture

![Figure 6. PCA of positive ToF-SIMS data including (a) scores plot, (b) PC 1 loadings plot, and (c) PC 2 loadings plot. The scores plot (a) shows that surfaces after cell removal by mechanical dissociation (o) and low-temperature cell liftoff (+) cluster toward the left hand of the plot, whereas surfaces after cell removal by enzymatic digestion (x) cluster largely toward the right-hand side of the plot. The ellipses drawn around each group indicate the 95% confidence interval of each grouping. PC 1 captures 83.6% of the variance in the data. PC 2 captures 8.7% of the variance of the data. PCA scores and loadings plots can be interpreted by looking at the relationship between the scores and loadings for a given PC; for example, each peak that loads negatively in the PC 1 loadings plot (b) corresponds to samples with negative scores in the scores plot (a). Note that PC 1 effectively indicates that the largest variance between the samples comes from salt ions (e.g., Na\(^+\), K\(^+\)) present on surfaces after cell removal by enzymatic dissociation with trypsin (x). PC 2 effectively separates surfaces after cell removal by low-temperature cell liftoff (+) and enzymatic digestion (x), which correspond with peaks that have already been identified as having originated from amino acid residues. The surfaces after cell removal by mechanical dissociation (o) load primarily with hydrocarbon peaks.](image-url)
was removed by enzymatic dissociation with trypsin; and 5) ppNIPAM-treated TCPS from which previous culture was removed by low-temperature liftoff. In the last four columns are the control surfaces, including 6) bare TCPS (no cells previously cultured); 7) ppNIPAM (no cells previously cultured); 8) bare TCPS (no cells previously cultured) treated with trypsin; and 9) ppNIPAM-treated TCPS (no cells previously cultured) treated with trypsin. In each case, the results have been normalized to the total number of cells seeded to allow direct comparison.

Examination of bare TCPS and ppNIPAM-coated TCPS (columns 6 and 7) shows that the control surfaces do not significantly differ in their ability to promote cell adhesion. However, with the exception of trypsin-treated ppNIPAM (column 9), each of the treatments does have a statistically significant impact on adhesion of the secondary cell layer (p < 0.05, 2-tailed t test). The most obvious impact on adhesion is observed when the primary culture is removed by low-temperature liftoff from the ppNIPAM surface (column 5); a significant increase in adhesion is observed, indicating that proteins left at the surface by this method retain their functionality. What was not expected is that the removal of the first culture by mechanically dissociating the cells was equally capable of promoting of adhesion on either ppNIPAM or TCPS surfaces (columns 1 and 2).

PCA results indicated the exposure of ppNIPAM after mechanical dissociation may not have removed all of the ECM proteins from the surface. Pettit et al. found that surface density of adsorbed adhesive proteins can be well below a monolayer and still support full cell adhesion of some cell types. Therefore, even this minimal amount of proteins left after mechanical dissociation may have a positive effect in supporting cell adhesion.

In contrast, enzymatic digestion with trypsin had a negative impact on the secondary cells cultured: cell adhesion decreased on both ppNIPAM and TCPS when trypsin was used as the method to remove the cultured cells (columns 3 and 4). Trypsin is known to cleave peptide bonds between Arg/Lys and all non-Pro neighbors. Therefore, protein fragments retained on trypsinized surfaces may lack domains such as RGD sequences that are vital to cell adhesion. This partially fragmented protein layer may inhibit the accumulation of new surface ECM proteins in the short term. Thus, the culture surfaces retrieved from

Figure 7. Secondary BAEC adhesion assay results. Columns 1–5 are as follows: secondary BAEC cells cultured on 1) TCPS from which previous culture was removed by physical dissociation; 2) ppNIPAM-treated TCPS from which previous culture was removed by physical dissociation; 3) TCPS from which previous culture was removed by enzymatic dissociation with trypsin; 4) ppNIPAM-treated TCPS from which previous culture was removed by enzymatic dissociation with trypsin; 5) ppNIPAM-treated TCPS from which previous culture was removed by low-temperature liftoff. Columns 6–9 are control surfaces: 6) TCPS (no cells previously cultured); 7) ppNIPAM (no cells previously cultured); 8) TCPS (no cells previously cultured) treated with trypsin; 9) ppNIPAM-treated TCPS (no cells previously cultured) treated with trypsin. Note that the results are normalized to the total number of cells seeded for comparison. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
trypsinization have a negative effect in supporting cell adhesion.

Furthermore, even when bare TCPS surfaces not used for cell culture are treated with trypsin (column 8), there is a slight negative effect on the secondary cells cultured, suggesting that trypsin has adsorbed to the bare TCPS and does not support cell adhesion. This effect is not observed on the ppNIPAM-treated surface (column 9) and may be due to the fact that the trypsin incubation was conducted at 25°C, the temperature at which the ppNIPAM surface is protein resistant.

The cell liftoff on temperature drop from ppNIPAM is proposed to be a direct result of polymer rehydration. However, it has been noticed that adsorbed proteins alone take a long time to detach from ppNIPAM.60 The rapid cell detachment, in contrast to the protein release, reflects the requirement of active cytoskeletal actin for the process, which was previously reported by Akiyama et al.61 However, cells mainly interact with the ECM at focal points. The ECM proteins that do not have strong interactions with cells may be left at the substrate after liftoff to support adhesion of newly seeded cells.

From these results, we conclude that enzymatic digestion with trypsin is damaging to the ECM, whereas both mechanical dissociation and low-temperature liftoff are less destructive alternatives for the removal of the ECM.

CONCLUSIONS

Removing BAEC monolayers from a TCPS substrate usually requires aggressive enzymatic or mechanical methods. ppNIPAM, a “smart polymer,” can be used as a less-destructive method of cell monolayer harvest. In this work, we have demonstrated the efficacy of this removal method and its effect on the underlying ECM layer and compared the removal of BAECs monolayers by low-temperature liftoff to two traditional methods used for cell removal: enzymatic digestion and mechanical dissociation. Our immunoassay results show that the low-temperature liftoff technique removes the cell sheet and most of the underlying ECM without any apparent damage to the ECM. In contrast, both enzymatic digestion and mechanical dissociation are found to be extremely damaging to both the cells and ECM. Adhesion assay results from new cells seeded on surfaces previously cultured with BAECs that were subsequently removed by the three different methods indicate that low-temperature liftoff and mechanical dissociation of the cells from surfaces better promote cell adhesion than the other treatments or bare substrates. Our XPS results indicate that the low-temperature liftoff method leaves behind surfaces containing species more indicative of protein (primarily amide) than the surfaces treated with mechanical or enzymatic digestion. Finally, the molecular ions yielded by ToF-SIMS analysis of the surfaces after cell removal by the three methods can be distinguished by PCA: low-temperature liftoff treatment leaves behind a surface characterized by fragments from amino acids, whereas enzymatic digestion leaves behind a surface containing high levels of salt (most likely from the trypsin and buffer solution). From our analysis of the surfaces left behind, we conclude that the removal of BAEC cells from ppNIPAM-treated surfaces is less damaging to the ECM proteins remaining at the surface than are enzymatic or mechanical detachment methods.

This research was supported by NIBIB grant to the National ESCA and Surface Analysis Center for Biomedical Problems (NESAC/BIO) and NSF ERC grant to the University of Washington Engineered Biomaterials group (UWEB). The authors thank Manuela Almada, Ariana Bramblett, Winston Ciridon, Lu Chen, Janet Cuy, Kip Hauch, Vickie Pan, and Hsieh-Ying Yang for supplies, helpful discussions, and expertise.

References


