Exploring the anomalous cytotoxicity of commercially-available poly(N-isopropyl acrylamide) substrates

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Poly(N-isopropyl acrylamide) (pNIPAM) is a stimulus-responsive polymer that has been of great interest to the bioengineering community. When the temperature is lowered below its lower critical solution temperature (~32 °C), pNIPAM rapidly hydrates, and adherent cells detach as intact cell sheets. This cell-releasing behavior in a physiologically relevant temperature range has led to NIPAM’s use for engineered tissues and other devices. In a previous study, however, the authors found that although most techniques used to polymerize NIPAM yield biocompatible films, some formulations from commercially-available NIPAM (cpNIPAM) can be cytotoxic. In this work, the authors investigate the reasons underlying this anomaly. The authors evaluated the response of a variety of cell types (e.g., bovine aortic endothelial cells, BAECs; monkey kidney epithelial cells, Vero cells; and mouse embryonic fibroblasts, 3T3s) after culture on substrates spin-coated with sol-gel (spNIPAM) and commercially-prepared (cpNIPAM). The relative biocompatibility of each cell type was evaluated using observations of its cell morphology and function (e.g., XTT and Live/Dead assays) after 48 and 96 h in culture. In addition, the substrates themselves were analyzed using NMR, goniometry, and XPS. The authors find that all the cell types were compromised by 96 h in culture with cpNIPAM, although the manner in which the cells are compromised differs; in particular, while Vero and 3T3 cells appear to be undergoing cytotoxic death, BAECs undergo apoptotic death. The authors believe that this result is due to a combination of factors, including the presence of short chain oligomers of NIPAM in the commercially-available preparation. This work will provide valuable insights into the cytotoxicity of commercially-prepared polymer substrates for this type of bioengineering work and therefore into the applicability of cells grown on such surfaces for human subjects. Published by the AVS. https://doi.org/10.1116/1.5045142

I. INTRODUCTION

Poly(N-isopropyl acrylamide) (pNIPAM or pNIPAAm) is a thermoresponsive polymer that experiences a phase change when exposed to its lower critical solution temperature (LCST) of ~32 °C. Above the LCST, pNIPAM is relatively hydrophobic, and the chains of the polymer are collapsed; below the LCST, pNIPAM is relatively hydrophilic, and its chains are extended. Although the magnitude of the change to the polymer’s thickness and wettability is relatively small, it has great impact on adherent mammalian cells: while cell sheets normally require enzymatic digestion or mechanical scraping to remove them from the substrates to which they adhere, they will spontaneously “pop off” from NIPAM substrates as contiguous sheets—similar to a Post-It™ note—capable of adhering and proliferating elsewhere. Due to this property occurring at relevant physiological temperatures, pNIPAM has attracted a great deal of attention in cell sheet engineering, drug delivery, formation of gold nanoparticles, and the development of a thermoresponsive membrane. However, before pNIPAM can be extensively used for human applications, it must be demonstrated that pNIPAM is benign, especially considering that the NIPAM monomer itself is known to be toxic. Although hundreds of papers describe the use of pNIPAM with mammalian cells, few papers assess its cytotoxicity. In a recent study, we performed the first comprehensive study of NIPAM cytotoxicity. In that work, NIPAM monomer and the most commonly-used NIPAM-derivatized substrates were created using a variety of techniques, including free radical polymerization, sol-gels spin-coated onto glass, plasma polymerization, and atom transfer radical polymerization. Using the NIPAM substrates, endothelial cells, epithelial cells, smooth muscle cells, and fibroblasts were cultured. The effects of direct contact with monomeric and pNIPAM substrates were evaluated. Monomeric and commercially-available pNIPAM (cpNIPAM) was demonstrated to be the only formulations that were cytotoxic toward cell types, with as little as 18% viability for MTS assay concentration gradient results. In this work, the reason behind this anomaly was explored. The pNIPAM-coated surfaces were first evaluated for their thermoresponse and surface chemistry using contact
angle measurements, cell detachment, and x-ray photoelectron spectroscopy (XPS). Having observed that different cell types experienced slightly different levels of cytotoxicity by Cooperstein et al., we evaluated the relative biocompatibility of the substrates toward several cell types: bovine aortic endothelial cells (BAECs); monkey kidney epithelial cells (Vero); and mouse embryonic fibroblasts (3T3s). Cellular response was evaluated by observing their cell morphology as well as through Live/Dead and XTT assays. Finally, the leachate (or extract) from the polymerized NIPAM surfaces was evaluated using nuclear magnetic resonance (NMR). We found that the viability of 3T3 (fibroblast) cells exposed to cpNIPAM substrates was the lowest, indicating that the surfaces were cytotoxic and resulted in more lytic death. However, we also found that BAECs—which appeared to have reasonable viability using Live/Dead assays—had unusual cell morphology indicative of apoptic death. We hypothesize that the diminished cell viability is caused by a combination of factors, including the inclusion of short chain length oligomers/NIPAM monomer that disrupts cellular behavior. Given the interest of researchers in this field, this work provides valuable insights into the appropriateness of using as-is commercially-prepared polymer substrates without any postprocessing or preprocessing for this type of bioengineering work and therefore into the applicability of this type of NIPAM-derived engineered tissues for human use.

II. EXPERIMENTAL METHODS
A. Materials
PNIPAM, with a molecular weight of ~40 000 grams/mol, was purchased from Polysciences, Inc. (Warrington, PA). N-isopropyl acrylamide (99%) was purchased from Acros Organics (Geel, Belgium). Tetraethyl orthosilicate (TEOS) and isopropanol were purchased from Sigma-Aldrich (St. Louis, MO). HPLC-grade methanol, HPLC-grade dichloromethane, acetone, 200 proof ethanol, and hydrochloric acid (1N) were purchased from Honeywell Burdick and Jackson (Deer Park, TX).

Square glass coverslips were purchased from Fisher Scientific (Pittsburgh, PA). Round glass coverslips were purchased from Ted Pella (Redding, CA). The silicon chips were obtained from Silitec (Salem, OR).

Dulbecco’s modified eagle’s medium (DMEM), minimum essential medium with alpha modification (αMEM), and Dulbecco’s phosphate buffered saline without calcium or magnesium were purchased from HyClone (Logan, UT). BAECs were from Genlantis (San Diego, CA). Fibroblasts (MC3T3-E1, 3T3s) and Vero cells (CCL-81) were purchased from ATCC (Manassas, VA). Fetal bovine serum (FBS), fungizone, and penicillin/streptomycin were from HyClone (Logan, UT). Minimum Essential Medium NonEssential Amino Acids solution (MEM NEAA) and 0.25% trypsin/ethylenediamine tetra-acetic acid (EDTA) were purchased from Gibco (Grand Island, NY). Ethidium Homodimer I was purchased from Invitrogen (Grand Island, NY). Calcein AM was purchased from Biotium (Fremont, CA).

1. Surface preparation
For later surface analysis, silicon chips were cut into 0.8 cm × 3 cm rectangles for goniometry and 1 cm × 1 cm squares for XPS. The surfaces were cleaned using an ultrasonic cleaner from Magnasonic ultrasonic cleaner (Toronto, Canada) for 15 min: dichloromethane, acetone, methanol (sequentially). The chips were then rinsed with deionized water, dried with nitrogen, placed in a Petri dish, wrapped in Parafilm, and stored in a dessicator until used for deposition or goniometry.

Glass cover slides were cleaned for 30 min in an acid wash (1:1 solution by volume of methanol: hydrochloric acid), rinsed with deionized water, dried with nitrogen, placed in a Petri dish, sealed with Parafilm under nitrogen, and stored in a dessicator until used for cell culture or solution deposition.

2. cpNIPAM solution preparation
Isopropanol was poured into a 15 ml centrifuge, and its weight was determined. The amount of cpNIPAM to be dissolved into the isopropanol was calculated based on the weight of the isopropanol to achieve 1% of pNIPAM by weight or 2% of pNIPAM by weight.

3. spNIPAM solution preparation
Solution preparation using sol-gel (spNIPAM) was performed following a method previously described.20 35 mg of pNIPAM, 5 ml of deionized water, and 200 μl of hydrochloric acid were mixed in a 15 ml centrifuge tube, and a weight percentage of pNIPAM was determined. In a separate 15 ml centrifuge tube, 250 μl of TEOS solution (1 TEOS:3.8 ethanol:1.1 water:0.0005 HCl), 43 μl of deionized water, and 600 μl of ethanol were mixed and weighed. The appropriate amount of the pNIPAM solution was added to achieve the final weight percentage of pNIPAM of 0.35%.

4. spNIPAM and cpNIPAM solution deposition
To spin coat surfaces with pNIPAM, 100 μl of either the spNIPAM or cpNIPAM solution was evenly distributed onto clean glass slides and Si chips placed on the spin coater, an H6-23 spin coater from Laurel Technologies (North Wales, PA). The surfaces were spun at 2000 rpm for 60 s. The surfaces were placed in a Petri dish, sealed with Parafilm under nitrogen, and stored in a desiccator until used for cell culture or surface analysis.

B. Surface characterization
1. Goniometry
Contact angle measurements were performed with an Advanced Goniometer model 300-UPG from ramé-Hart Instrument Co. (Mountain Lakes, NJ) with an environment chamber and the DROPimage Standard program. Inverted bubble contact angles were taken in Milipore water (18 MΩ). Angles were obtained at room temperature (21 °C).
samples were run as insulators meaning the electron gun was used. Pressure in the analytical chamber during X-ray spot size for these acquisitions was 800 × 800 μm². When confluent, the cells were lifted from the cell culture flasks with 0.25% trypsin/EDTA and passaged into new flasks from passage 2 up to 10.

4. Cell culture on pNIPAM substrates

pNIPAM surfaces prepared via plasma polymerization, commercial polymerization, and sol-gel were used for culture of mammalian cell lines according to previously established protocols in our group. These glass slides were placed into 6 well plates, and the cells at passage 6 through 9 were seeded at 1.0 × 10⁵ cells directly onto the substrates in regular cell culture media. The time to confluence was about 2 days. From these substrates, cell detachment was performed in cold, 4 °C media without added supplements. To initiate the detachment, the regular cell culture media was replaced with cold nonsupplemented media. The well plates with cells in the cold media were placed on a shaker table. The detachment was allowed to proceed for the desired amount of time (up to 3 h) at 4 °C. Images were obtained using 20x objective on a Nikon Eclipse TS200F inverted microscope with an epifluorescence attachment (Nikon Instruments, Melville, NY) and a SPOT Insight color mosaic digital camera (Diagnostic Instruments, Sterling Heights, MI), before cell detachment began and at 60 min intervals during cell detachment. The cells were counted from the beginning of detachment to the end of detachment using ImageJ software.

5. Preparation of pNIPAM extracts for NMR

Extracts from cpNIPAM and spNIPAM were obtained at room temperature (20 °C) and body temperature (37 °C) according to a previously described protocol. Briefly, to make extracts, the previously mentioned pNIPAM surfaces were incubated in deuterated (surface to liquid volume ratio of 2.16 cm²/ml) for 24 h at room temperature and body temperature. After 24 h, the resulting extracts were transferred to centrifuge tube and used for NMR.

C. Cytotoxicity testing of NIPAM surfaces: XTT assay

XTT cell viability assay was purchased from Biotium (Fremont, CA). The procedure for XTT assay was adapted from the procedures provided by the manufacturer. A working of XTT solution was prepared by adding 50 μl of activated XTT (Biotium XTT solution + activation reagent) to 100 μl of cell media. After 48 and 96 h of incubation on pNIPAM and control glass substrates, BAECs, 3T3s, and Vero were removed from the incubator, and cell media was replaced with working XTT solutions. Cells were incubated for 6 h and then removed for absorbance measurements at 475 and 660 nm (background) with a SpectraMax M5 microplate reader (Molecular Devices, San Jose, CA). A blank control was included in empty wells in every plate, in which XTT working solution was added to empty well plates and incubated for 6 h.

Viability of all cell lines was calculated utilizing this equation:

\[
\% \text{Viable Cells} = \frac{\text{Sample Absorbance} - \text{blank absorbance}}{\text{Control absorbance} - \text{blank absorbance}} 
\]

D. Live/Dead assay

The procedure for Live/Dead assay follows those previously completed by Cooperstein and Canavan. Briefly, a combined live/dead solution was made by adding 1 μl of calcine AM, and 1 μl of ethidium homodimer to 1 ml of DPBS. Cells grown on pNIPAM substrates and control glass were removed from incubation at 48 and 96 h, and cell media were replaced with working live/dead solution. Cells were incubated at 37 °C and 5% CO₂ with live/dead solution for 45 min and then rinsed with sterile DPBS prior to imaging. Fluorescent images were taken on a Nikon Eclipse TS200F inverted microscope at 10x with an epifluorescence attachment (Nikon Instruments, Melville, NY) and a SPOT Insight color mosaic digital camera (Diagnostic Instruments, Sterling Heights, MI).
E. Characterization of extract solution: NMR

NMR was used to confirm successful polymerization of the cpNIPAM and spNIPAM. The NMR spectra of cpNIPAM and spNIPAM were taken at the University of New Mexico (UNM) Nuclear Magnetic Resonance facility with an Advance III NMR spectrometer (Bruker, Billerica, MA). It is a 300 MHz, standard bore, nanobay instrument. Spectra were obtained on a 5 mm broadband/proton probe, at room temperature, using CDC13 as a solvent.

III. RESULTS AND DISCUSSION

In a previous study, our group demonstrated that although the cell types, surface deposition methods, and ultimate utility of cellular/NIPAM constructs differ between laboratories, the vast majority of those preparations are biocompatible and therefore suitable for use in bioengineering. An anomalous result was that commercially-prepared NIPAM (cpNIPAM) used as-is from its manufacturer was relatively more cytotoxic than all other polymerized forms in our lab (~20%).16 In addition, we found that endothelial cells (BAECs) had increased sensitivity to commercially-prepared NIPAM after 48 h. These results were consistent with other research in our laboratory indicating that cellular sensitivity varies with cell type, as endothelial cells were more sensitive to polymers than epithelial cells.22

In this work, we further explored these anomalous results. The stimulus-response, chemistry, and biocompatibility of commercially-available NIPAM at two concentrations (1% and 2%) were evaluated alongside silicon controls as well as spNIPAM. Using contact angle goniometry, cpNIPAM-coated surfaces were first evaluated to demonstrate their thermoresponse. The surface chemistry (and presence or absence of any potentially harmful contaminants) was assessed using XPS. The relative biocompatibility of the substrates toward several cell types was then assessed using endothelial cells (BAECs), epithelial cells (monkey kidney epithelial cells, Veros), and fibroblasts (mouse embryonic fibroblasts, 3T3s). Cellular response was evaluated via Live/Dead and XTT assays as well as observations of cellular morphology. Finally, the leachate (or extract) from the polymerized NIPAM surfaces was evaluated using NMR.

A. Polymer thermoresponse

Contact angle measurements were obtained for each of the substrates prepared to determine their thermoresponse. The contact angle of a captive air bubble was measured for each substrate in ultrapure water at either room temperature (~25 °C) or body temperature (37 °C). Three samples were

Fig. 1. Captive bubble contact angle measurements of silicon controls and pNIPAM-coated surfaces measured at room temperature (25 °C) and body temperature (37 °C) in ultrapure water. N = 27.

Fig. 2. (a) Surface chemistry of NIPAM-coated surfaces as determined by XPS. In part (a), the atomic % of carbon, oxygen, nitrogen, and silicon is shown for silicon control (middle left, gray), spNIPAM-coated substrates (middle, blue), cpNIPAM 1%-coated (right middle, light purple), cpNIPAM 2%-coated (right, dark purple). The theoretical composition of NIPAM as calculated from the monomer (left, black) is shown for comparison. N = 24. In part (b), the high-resolution carbon species (C—C/C—H, C—OH/C—N, and N—C=O) are shown for silicon control (middle left, gray), spNIPAM-coated substrates (middle, blue), cpNIPAM 1%-coated (right middle, light purple), cpNIPAM 2%-coated (right, dark purple). The theoretical composition of NIPAM as calculated from the monomer (left, black) is shown for comparison. N = 12.

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analyzed on three spots per sample. Three replicates were performed for each type of pNIPAM-coated substrates, resulting in \( n = 27 \).

Figure 1 shows the results of these measurements. Each of the pNIPAM-coated substrates tested yields higher contact angles at body temperature than at room temperature. On average, there is a 13° change between contact angles at body temperature and room temperature (46.6° vs 31.5° for 1% cpNIPAM, 55° vs 41.6° for 2% cpNIPAM, and 34.6° vs 23.7° for spNIPAM).

Although the contact angles differ depending on the surface characterization technique, previous studies have shown that the specific values at each temperature are not critical to determine the thermoresponse of the substrate—it is the relative change in wettability that is crucial to their thermoresponse.\(^6\) The relative change in contact angles across the LCST indicates the desired result: each of the prepared pNIPAM surfaces is thermoresponsive.

Although the standard deviations are higher (up to 12°) than other preparation methods (e.g., plasma polymerized NIPAM, ±2),\(^{20}\) this analysis confirms that the films are stimulus-responsive and of sufficient quality for later cell response assays. The silicon (negative) controls showed minimal variation between body temperature and room temperature, which is also consistent with expected results.

### B. Surface chemistry

The surface chemistry of these pNIPAM-coated substrates was accessed by XPS. Figures 2(a) and 2(b) show the results of survey and high-resolution C1s spectra for all four types of pNIPAM-coated surfaces, as well as the composition of NIPAM as predicted from the stoichiometry of the monomer.

In Fig. 2(a), it is shown that spNIPAM contains far less C (and far more Si), which is expected, as the TEOS solution used to create the sol-gel contains organosilanes. In comparison, the presence of silicon in cpNIPAM films is detected at low concentrations/not detected, indicating that the films are sufficiently thin (i.e., ≤50–100 nm thick). The cpNIPAM surfaces have an elemental composition (76.9% C, 11.8% N, 11.0% O, and 1.4% Si; and 77.3% C, 11.9% N, and 10.8% O, respectively, for 1% and 2% cpNIPAM) that is consistent with the predicted composition of the monomer (∼75% C, 12.5% O, and 12.5% N). No other elements were detected on the cpNIPAM substrates, ruling out the presence of any potentially cytotoxic contaminants (such as catalysts used to polymerize cpNIPAM).

The spNIPAM composition differs significantly from the theoretical composition (8.8% C, 0% N, 36.6% O, and 54.6% Si). As previously stated, the increased concentration of silicon and oxygen is consistent with the presence of

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**Fig. 3.** Live/Dead assay of BAECs (blue), Veros (orange), and 3T3s (gray) incubated on glass (control, left), spNIPAM-coated substrates (left middle), 1% cpNIPAM-coated substrates (right middle), and 2%-cpNIPAM-coated substrates (right) after 48 h in culture. Red = dead; green = live. Scale bar indicates 200 \( \mu \text{m} \). N = 9.
TEOS to create the sol-gel (indicating that the NIPAM and TEOS components have phase separated) but could also arise from the underlying silicon surface (glass, indicating patchy coverage of the film). The trace amounts of nitrogen indicate a very small amount of NIPAM. Previous studies done by Bluestein et al. have shown that storage conditions such as time after deposition, temperature, and humidity all significantly affect spNIPAM film stability. Specifically, over time, regardless of storage conditions, spNIPAM surfaces begin to lose thermoresponse characteristics and delaminate as spNIPAM is physisorbed and not chemisorbed onto surfaces. Furthermore, storage temperatures greatly affect this delamination effect; spNIPAM stored at 25 °C in low humidity experiences the smallest amount of delamination, while spNIPAM stored at 37 °C in low humidity experiences the largest amount of delamination. As our samples are sent from the University of New Mexico to the University of Washington, and often awaits one week prior to XPS, it is likely that our XPS results of spNIPAM are associated with delamination. It is important to note that other than XPS, all other experiments utilizing spNIPAM are completed immediately after fabrication.

Figure 2(b) shows the high-resolution C1s spectra for spNIPAM, 1% cpNIPAM, and 2% cpNIPAM, as well as the silicon control and the theoretical composition of the monomer. The high-resolution analysis shows that the spNIPAM films have a thin amount of NIPAM as the expected carbon species is present at appropriate concentrations (72.5% C—C/H, 20.0% C—OH/N, and 7.5% N—O), consistent with those found with spNIPAM. CpNIPAM films have the expected carbon species at appropriate concentrations (65.5% C—C/H, 19.3% C—OH/N, and 15.2% N—O), consistent with the predicted composition of the monomer (∼66.7% C, 16.7% O, and 16.7% N).

Together, these data indicate that the cpNIPAM-coated surfaces consist of NIPAM that is of sufficient quality to achieve cellular adhesion and popoff, and no contaminants are present that would affect its biocompatibility. Representative survey spectra and high-resolution C1s spectra of silicon controls, spNIPAM, 1% cpNIPAM, and 2% cpNIPAM can be found in Figs. S1 and S2 in the supplementary material.

SpNIPAM-coated surfaces warranted further testing; as such, spNIPAM, 1% cpNIPAM, 2% cpNIPAM, and glass controls all underwent cell detachment experiments (results found in Figs. S3 and S4 in the supplementary material) utilizing BAECs. After incubation for 48 h, pNIPAM-derivatized substrates and glass control substrates underwent detachment studies at 4 °C, utilizing serum-free media. Cells were observed and imaged every 60 min over 3 h and then analyzed with ImageJ cell counter. Control glass substrates had difficulties detaching cells as expected (they detached −3%, −0.4%, and 5.6% at 60, 120, and 180 min, respectively), spNIPAM detached 30.5% of cells, 32.3% of cells,

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<td>Glass (control)</td>
<td><img src="blue" alt="Live/Dead assay of BAECs" />, Veros (orange), and 3T3s (gray) incubated on glass (control, left), spNIPAM-coated substrates (left middle), 1% cpNIPAM-coated substrates (right middle), and 2% cpNIPAM-coated substrates (right) after 96 h in culture. Red = dead; green = live. Scale bar indicates 200 μm. N = 9.</td>
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and 34.7% of cells at 60, 120, and 180 min, respectively. 1% cpNIPAM detached 12.2% of cells, 13.4% of cells, and 14.7% of cells at 60, 120, and 180 min, respectively. 2% cpNIPAM detached 9.4% of cells, 14.3% of cells, and 19.1% of cells at 60, 120, and 180 min, respectively.

Although many articles have been published using pNIPAM substrates to achieve cell detachment, rarely do the authors publish the yields they achieved using their method. We previously showed that spNIPAM yields detachment of ~35%, and cells would detach in islands instead of a sheet. In this work, we find that the detachment of cells from spNIPAM and cpNIPAM are consistent with previous results found in our group, which is far greater than those achieved by controls such as glass (5%). As such, we conclude that all surfaces contain a thin layer of NIPAM, enough for cell adherence and detachment and that there are no contaminants in our NIPAM films that could lead to cytotoxicity effects.

C. Cytotoxicity testing

In order to determine the relative biocompatibility of the substrates, Live/Dead and XTT assays were performed on endothelial cells (BAECs), epithelial cells (Vero cells), and fibroblasts (3T3s) that were cultured on NIPAM substrates for either 48 h or 96 h (see Figs. 3 and 4). Live/Dead assays are considered a semiquantitative assay that stain the cell membrane of live cells green, whereas nonviable cells are dyed red due to the breakdown of their membrane.

Figures 3 and 4 show representative images of those cells cultured in normal media on glass (control, top row), on spNIPAM (top middle), 1% cpNIPAM (bottom middle), and 2% cpNIPAM (bottom). At 48 h (Fig. 3), BAECs grown on each substrate appear to be viable and identical to those grown on the glass coverslip controls. In comparison, the Vero cells and 3T3s grown on cpNIPAM appear to have some cell death (indicated by red). By 96 h (Fig. 4), approximately half of the Vero cells grown on cpNIPAM (1% and 2%) are

![Graph](image)

**Fig. 5.** XTT metabolic assay of BAECs (blue), Veroes (orange), and 3T3s (gray) incubated on glass (control, left), spNIPAM-coated substrates (left middle), 1% cpNIPAM-coated substrates (right middle), and 2%-cpNIPAM-coated substrates (right) after 48 h in culture. Red line indicates 70% viability, below which the substrates are considered cytotoxic. ** indicates $P < 0.05$ in a student t-test between sample vs control. N = 9.
There is also evidence of cell death in the 3T3s grown on these substrates. In contrast, there is relatively little evidence of cell death from BAECs grown on sp- and cpNIPAM substrates; however, where there is cell death (as evidenced by red spots), there is a large zone of inhibition toward growth.

XTT assays are quantitative metabolic assays that determine the percentage of cells that are viable as determined by their ability to reduce tetrazolium salt and produce formazan derivatives. Figures 5 and 6 show the results of XTT assays of all cell types toward the spNIPAM and cpNIPAM films. The red line on Figs. 5 and 6 is set to 70% viability, which is the level below which surfaces are considered to be cytotoxic toward cells. At 48 h (Fig. 5), all three cell types grown on spNIPAM-coated surfaces have >70% viability, indicating that spNIPAM would be considered nontoxic. At the same time point, 3T3 cells grown on cpNIPAM-coated surfaces have 47% and 32% viability (for 1% and 2% cpNIPAM, respectively), indicating that this substrate is cytotoxic toward 3T3 cells.

Interestingly, by 96 h (Fig. 6), it would appear that all of the NIPAM substrates, as well as the glass control, are biocompatible toward all three cell types (as evidenced by viability >70%). However, this could be an artifact of the XTT assay, which is unable to accurately assess cell viability of highly confluent cells, and even some dead cells are capable of reduction.24

D. Cell morphology

In parallel with the cytotoxicity assays, the morphology of the BAEC, Vero, and 3T3 cells grown on the sp- and cpNIPAM substrates was assessed using bright-field microscopy after 48 h (Fig. 7) and 96 h (Fig. 8). The observations of cell morphology results, when considered alongside the differences in cytotoxicity, are particularly striking: from the Live/Dead and XTT assays, it would appear that cpNIPAM is most cytotoxic to 3T3 cells, and that BAECs and Vero cells are relatively unaffected. However, close inspection of the morphology of the cells tells a different story.

The cells grown on control samples (glass coverslips) are shown in the top row for both figures. It can be observed that the Vero cells grow to confluence the most rapidly (center column) on each substrate. Also of interest is that the morphology of the Vero and 3T3 cells grown on spNIPAM and cpNIPAM surfaces is consistent with that of controls (especially at the shorter time frame).

By 96 h, however, distinct abnormalities appear in the cells grown on cpNIPAM substrates (1% and more so on 2%). Close inspection of the BAECs shows that distinct “bubbles” or “holes” have formed within the cell bodies (see Figs. 8 and 9). In an unrelated set of experiments exploring cellular death after exposure to oligomers, our group showed that cells entering apoptic death—rather than lytic death—will first form these “bubbles.” (We hypothesize that they are

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**Fig. 6.** XTT metabolic assay of BAECs (blue), Veros (orange), and 3T3s (gray) incubated on glass (control, left), spNIPAM-coated substrates (left middle), 1% cpNIPAM-coated substrates (right middle), and 2%-cpNIPAM-coated substrates (right) after 96 h in culture. Red line indicates 70% viability, below which the substrates are considered cytotoxic. ** indicates P < 0.05 in a student t-test between sample vs control. N = 9.
neither bubbles nor holes, but are vacuoles containing cellular material.)\textsuperscript{22} Furthermore, other groups have demonstrated similar results from HeLa cells that are undergoing lysis by lysosomes, which appear remarkably similar to those found in our live/dead images of Vero cells and 3T3s.\textsuperscript{25} Apoptosis may explain why the XTT and Live/Dead assays underestimate the cytotoxicity, as those techniques are better suited toward detecting death by cell lysis.

Furthermore, although the results from XTT at 96 h indicate high cell viability (in Fig. 6), it is clear from observation of the live/dead images in Fig. 4 and the morphology images at the same time point in Fig. 8 that there are large areas on cpNIPAM substrates that either have dead cells (such as Vero cells on 1% cpNIPAM) or have no confluence (BAECs on 1% and 2% cpNIPAM).

Conversely, the 3T3 cells grown on cpNIPAM substrates do not appear to have a “bubbled” appearance, although their morphology is also quite abnormal. For example, many of the 3T3s in Figs. 8 and 9 on 1% and 2% cpNIPAM substrates are no longer stellate in appearance but are rounded and without projections. As the 3T3 cells are the ones found to be least viable by XTT assay at 48 h, this may indicate that the 3T3 cells are dying via lysis (rather than apoptosis).

From the collected morphology and viability assays, we conclude that exposure to cpNIPAM affects cell viability for all cell types at shorter time periods (48 h). While some cell types obviously show diminished viability (i.e., 3T3 cells), other cell types undergo changes that will ultimately result in death, potentially by apoptosis. Furthermore, although XTT analysis indicates that cell viability recovers at longer time frames, such results should be viewed with some skepticism, as the appearance of the cells appears compromised (potentially entering apoptosis).

**E. Nuclear magnetic resonance to determine extent of polymerization**

Given that the cpNIPAM-coated substrates appeared to lack any kind of contaminant that would explain the cell death and abnormal morphology results explained above, extracts from the films themselves were analyzed to determine if there was anything leaching from the films that explained diminished cell viability. NMR was used to determine the structure of cpNIPAM and spNIPAM, including the determination of whether any (potentially toxic) NIPAM monomer was present in the films.

Figure 10 shows three NMR spectra: the spNIPAM polymer (top), 1% cpNIPAM extract (middle), and the NIPAM monomer (bottom). The structure of the NIPAM monomer is inset, with the pertinent moieties labeled (alkenes and isopropyl...
FIG. 8. BAECs (left), Vero (middle), and 3T3s (right) cultured directly on glass control substrate (top), spNIPAM-coated glass (top middle), 1% cpNIPAM-coated (bottom middle), and 2% cpNIPAM-coated glass (bottom). Images obtained at 96 h after deposition to observe cell morphology. Scale bar indicates 200 μm. N = 9.

FIG. 9. Enhanced magnification to show differences in cell morphology of BAEC (left), Vero (center), and 3T3 cells (right) on glass control (top) and 2% cpNIPAM (bottom) surfaces after 96 h in culture. Note, in particular, the “bubbled” appearance of BAECs on cpNIPAM.

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amines). The peaks labeled a, b, and c in the region from 5.5 to 6.5 ppm indicate the location where the hydrogen adjacent to double-bonded carbons appears in the NIPAM monomer. These peaks are unique to the NIPAM monomer.20 All three peaks are clearly visible in the NIPAM monomer spectrum but are missing from the NMR spectrum of spNIPAM; the disappearance of these peaks indicates successful polymerization of the polymer. In contrast, the NMR spectrum of cpNIPAM contains the three peaks unique to the NIPAM monomer. Although these three peaks are not of the same intensity in cpNIPAM’s spectrum as they are in the spectrum of the NIPAM monomer, they are present. These results indicate that polymerization of cpNIPAM was incomplete, and some unreacted monomer still exists in the leachate. Given the known cytotoxicity of the NIPAM monomer, this may explain to the increased cytotoxicity of cpNIPAM. These results are consistent with previous results that have demonstrated that short oligomers and monomers of otherwise benign polymers can be quite cytotoxic, as their shorter chains allow the polymer to penetrate into the cell membrane and disrupt cellular processes.

IV. CONCLUSION

Ever since it was demonstrated that intact cell sheets could be harvested from pNIPAM substrates in the mid-1990s, there has been interest in the polymer’s use for applications such as tissue engineering, biological sensors, and drug delivery systems. One of the factors limiting the development of tissues and devices engineered for use with the human body has been the question of whether polymerized NIPAM had the same cytotoxic effects as the monomer itself. The limited number and haphazard comparison studies that had previously been performed using pNIPAM formulations were not sufficient to answer the question of whether pNIPAM was sufficiently benign for human use. Recently, our group published the first, comprehensive study of NIPAM polymerized using a variety of different techniques, exposed to multiple cell types, and assayed with a variety of tests intended to determine biocompatibility (or cytotoxicity). In that study, we identified one anomalous result: commercially-available NIPAM (cpNIPAM) appeared to be cytotoxic. In this work, we performed a study to determine the source behind the cytotoxicity of cpNIPAM in comparison to other commonly-used techniques. Having confirmed that all pNIPAM-derivatized surfaces had the correct thermoresponse (by contact angle goniometry and cell release statistics) and the correct surface chemistry (by elemental analysis via XPS), the pNIPAM substrates were used for cell culture up to 96 h. Using LIVE/DEAD assays, we demonstrated that the cpNIPAM is statistically more cytotoxic than the other polymerized forms studied. Furthermore, we found that
although 3T3 cells appear to be the most sensitive to cpNIPAM toxicity as per Live/Dead and XTT assays, each of the cell types is actually compromised by the substrate and experiences decreased viability. Finally, the absence of contaminants in the pNIPAM leachates (and the presence of unreacted monomer in the commercially-available powder used to coat the surfaces with cpNIPAM) indicates that the source of the cpNIPAM toxicity is the presence of these short, mobile chains interfering with cellular function. These results suggest that the cytotoxicity of cpNIPAM is due to the presence of unreacted monomer. Therefore, for researchers using NIPAM-derivatized substrates, we recommend that they synthesize their own substrates using the methods proven to be nontoxic here and in our previous study. Further studies should evaluate whether additional postprocessing of surfaces (e.g., washing commercial coatings) would improve cell viabilities. Additionally, these results suggest that cell viability assays such as XTT or Live/Dead should always be accompanied by careful observation of the cellular morphology itself, as the assays may underestimate the cytotoxicity of the substrates if apoptic death has been triggered.

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26See supplementary material at https://doi.org/10.1116/1.5045142 for representative XPS spectra and cell images of BAECs pop off from control, cpNIPAM, and spNIPAM surfaces.

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Heather E. Canavan is an associate professor at the University of New Mexico in the Department of Chemical & Biological Engineering. After receiving her Bachelors in Biology from the University of California at Santa Barbara, Canavan worked at the Los Alamos National Laboratory for several years. She then received her Ph.D. in Physical Chemistry from The George Washington University for her work with the late Prof. David Ramaker. Her research was the first to apply x-ray absorption near edge spectroscopy (XANES) in the sulfur K-edge region to observe the structure of proteins under oxidative stress. Her dissertation research was performed at laboratories at the Brookhaven National Laboratory, Naval Research Laboratory, National Institutes of Standards & Technology, and the Food & Drug Administration. After receiving her Ph.D. in Physical Chemistry (with a graduate minor in Forensics), Canavan worked as a postdoctoral fellow at the University of Washington in the National ESCA Surface Analysis Center for Biological Problems (NESAC/BIO) under Dr. David Castner. In a collaborative project with Xuanhong Cheng in the laboratory of Dr. Buddy Ratner, Canavan began using advanced biological and surface analysis methods to understand how poly(N-isopropyl acrylamide) (pNIPAM) is able to detach confluent mammalian cell sheets in response to environmental cues. Canavan joined the University of New Mexico in 2005. Since that time, Canavan has continued to study the applications and uses of pNIPAM for biomedical purposes, such as in engineered tissues and sensors. In a previous publication in this journal, Canavan and Cooperstein explained the mechanism by which cells detach from pNIPAM. In another, they showed that although the NIPAM monomer is cytotoxic toward cells, almost all formulations of the polymerized form are biocompatible, noting that commercially-available pNIPAM (used as-is, without further purification) does cause some cytotoxic effects. In this publication, the authors further explore and explain these effects on mammalian cells. In addition to her research, Canavan is active in both the chemical engineering and biomedical engineering programs at UNM and teaches courses in Biomaterials, Engineering Design for Global Health, and Adaptive Design for the Community. Canavan was elected as an AVS Fellow in 2018 for her research contributions, as well as her contributions to engineering education.

Additional thoughts:
My educational training includes degrees in biology, physical chemistry, and forensics. This interdisciplinary background is what led to my research focus on cell/surface interactions, especially bioactive and stimulus-responsive polymers. As a professor, I have taught courses in thermodynamics, biomaterials, and engineering for global health. More recently, my experience as a breast cancer patient led to my interest in creating adaptive designs for the community to help patients create the devices that will help them live better, more independent lives. Together with a female graduate student, I have started a company that is focused on bringing these adaptive designs to the community at an affordable price.

In the 15 years that I’ve been studying the cytotoxicity of bioactive polymers such as pNIPAM, I’ve been fortunate enough to work with over 50 students and postdocs, 63% are women, and 73% are under-represented minorities. The students who co-authored this paper with me come from such different parts of the world as Poland, Vietnam, and the United States; their educational background spans the Schools of Engineering, Business, Biology, and Psychology; and their ultimate career goals are to work in academia, in industry, and in medicine. I find inspiration that an interest in science and engineering can create connections that not only span a diversity of backgrounds, experiences, and interests, but also recognize that this diversity yields better and more creative results. So, for the young authors out there are asking whether you can find a place in science and engineering, and in this type of research, “sí, se puedes,” and “yes, we can.”