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# Culture Conditions can Counterbalance the Thermosensitive Properties of Poly (N-Isopropylacrylamide) during Cell Harvesting

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## Abstract

Poly (N-isopropylacrylamide) (PNIPAM) has been widely referenced as an alternative solution for proteolytic-enzyme-based cell harvesting. However, the reversibility (i.e., consecutive attachment-detachment cycles) of PNIPAM for cell harvesting has not been studied. In this work, we synthesized PNIPAM-grafted glass surfaces to study the efficiency of fibroblastic cell detachment after iterative use. For robust quantification of thermally triggered cell detachment, an innovative wide-field lens free video microscope was introduced. The cell detachment efficiency was shown to decrease significantly after each cell culture experiment due to the loss of polymer brush thermosensitivity. The influence of the temperature variation rate on cell detachment from the re-used PNIPAM-grafted surfaces was further characterized. The cell detachment behavior on these substrates was shown to be quite similar to that on bare glass substrates. Furthermore, it was demonstrated that non-negligible cell detachment occurs and is modulated according to the temperature variation rate. This study aims to inform scientists using PNIPAM-grafted substrates for cell detachment about possible issues, to help scientists to more carefully monitor culture experiments, and to aid scientists in the interpretation of results obtained from cell detachment experiments performed on PNIPAM-grafted surfaces.

**Keywords:** Biomaterials; Poly (N-isopropylacrylamide); Cell detachment; Thermosensitivity; Lens free video microscope

## Introduction

Adherent cell culture often involves harvesting cells via detachment from culture surfaces. Cell detachment is usually achieved through the use of enzymes (e.g., trypsin, accutase, or collagenase) that degrade the Extracellular Matrix (ECM), cell-cell junctions and membrane surface protein receptors [1]. Poly (N-isopropylacrylamide) (PNIPAM) is an interesting alternative to enzymes for use in cell harvesting, as it enhances the preservation of the extracellular matrix compared to enzymatic methods [2]. Cell detachment is mediated by temperature, which modulates PNIPAM [3]. Above a Lower Critical Solution Temperature (LCST), usually approximately 32°C, PNIPAM chains are in a poor solvent state and exhibit hydrophobic interactions, enabling cell adhesion. Below the LCST, PNIPAM chains behave in an opposite manner. When PNIPAM chains are in a good solvent state and show hydrophilic interactions, cell detachment is triggered.

Theoretical and experimental studies have attempted to elucidate the mechanism of detachment using PNIPAM brushes [3-6]. According to the literature, the following two PNIPAM brush parameters should be considered: the thickness and the density of the polymer chains [4]. The effect of chain length may be negligible, depending on the mode of adsorption of the anchoring proteins [5]. A balance must be found among these parameters. In this case, complete cell detachment is achieved when the polymer swells through a loss of equilibrium between tensile forces from the cellular cytoskeleton and the tight anchorage of ECM proteins [3,6].

PNIPAM-grafted surfaces have been demonstrated to exhibit reversible responses to temperature [7]. Nevertheless, the reversibility of cell detachment on these surfaces (as described above) has not yet been studied. In this work, we synthesized PNIPAM-grafted glass surfaces to study the efficiency of fibroblastic cell detachment after iterative use. To obtain more information on these surfaces, they were further characterized. Their thermosensitivity was analyzed, and cell detachment was compared with that on bare glass surfaces under different temperature variation rates.

Additionally, both the protocols employed for cell detachment and the methods used to characterize this process may present variability. To assess the cell detachment efficiency of PNIPAM, some authors consider the area and circularity of the cells [5], while other authors withdraw the medium supernatant to count the number of detached cells [2]. Using the former method, no concrete evidence of detachment has been obtained. In the latter method, the protocols for counting the cells and for withdrawing the cooled media containing the detached cells can be user-dependent. Furthermore, none of these methods enable dynamic studies. To overcome the aforementioned drawbacks in the quantification of cell detachment, we introduced a novel custom-built lens free video microscope coupled with a pattern recognition algorithm. The lens free video microscope offers a wide field of view, along with the ability to differentiate detached cells from a cell population dynamically without the need for labeling or intervention from the user. These methods have been previously described [8,9]. Use of this method for quantifying cell detachment ensures a greater statistical significance due to the wide field of observation (24 $mm^2$;

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a thousand cells are visible at a glance). Further, manipulation of the culture surfaces is reduced, and potential mishandling by the operator is minimized.

## Materials and Methods

### Chemicals and substrates

Dulbecco's modified Eagle's medium (DMEM), DMEM phenol red, newborn calf serum, penicillin-streptomycin solution, trypsin-EDTA, Bovine Serum Albumin (BSA), Triton X-100, phosphate-buffered saline supplemented with $Mg^{2+}$ and $Ca^{2+}$ (PBS+) and sucrose were purchased from Life Technologies™.

N-isopropylacrylamide (NIPAM, 97%), copper(I) chloride, N,N,N',N'',N''–pentamethyldiethylenetriamine (PMDETA, 99%), triethylamine (TEA, ≥99%), 3-aminopropyltriethoxysilane (APTES, 99%), α-bromoisobutyrylbromide (BIBB, 98%), potassium chloride, ethanol (anhydrous, ≥99.5%), dichloromethane (anhydrous, ≥99.8%), n–hexane (anhydrous, 95%), Tween20, $NH_4Cl$, vinculin (Ref. V9131) and phalloidin tetramethylrhodamine B isothiocyanate (Ref. P1951) were obtained from Sigma Aldrich. Anti-mouse FITC (Ref. 115-095-146) was purchased from Jackson ImmunoResearch. A fluorescent mounting medium kit (Ref S3023) was procured from Dako. All aqueous solutions were prepared in deionized water obtained using a Synergy® water purification system from Millipore. Glass microscope slides (20 mm × 20 mm) were provided by Dominique Dutscher. Silicon wafers with native oxide were diced into 10 mm × 20 mm pieces.

### Poly (N-isopropylacrylamide) grafting

The grafting of PNIPAM brushes onto glass microscope slides and diced silicon wafers proceeded as depicted in Figure 1. i) The silanization of the glass and $SiO_2$ surfaces was followed by ii) the covalent attachment of a surface initiator, which eventually promoted iii) PNIPAM grafting via Atom Transfer Radical Polymerization (ATRP). For each synthesis, a set of glass chips and $SiO_2$ substrates was grafted under the same conditions for further physical and biological characterization.

**Silanization:** Glass slides and silicon chip surfaces were hydroxylated and cleaned in an $O_2$ plasma reactor (AST products, Inc.) under a flow of 20 sccm for 5 min with 80 W RF power. Hydroxylated surfaces were immersed for 1 min into a $5 \times 10^{-4}$ M aqueous solution of APTES filtered through a 0.22 µm filter (Acrodisc® PSF syringe filter, Pall® Life Science). The time of immersion determined the PNIPAM density. Slides and chips were rinsed with deionized water and dried.

**Polymerization initiator grafting:** After silanization, the substrates were immersed for 30 s in 25 ml of a dichloromethane solution containing 1.25 ml of TEA and 250 µl of BIBB (a surface initiator). The substrates were then rinsed in dichloromethane. Subsequent rinsing was performed in ethanol followed by deionized water, and the substrates were eventually dried.

**PNIPAM grafting:** NIPAM was recrystallized twice before being dissolved in *n*-hexane. A 20 ml aqueous solution of 0.5 g of NIPAM and 150 µl of PMDETA was stirred and bubbled in a balloon under argon for 30 min. The solution was then transferred to another balloon containing 12 mg of CuCl, also in an argon atmosphere, and stirred. Eventually, the substrates were immersed in this mixture for 3 min. The time of immersion determined the chain length of the polymer brushes.

### Physical characterization of poly (N-isopropylacrylamide)

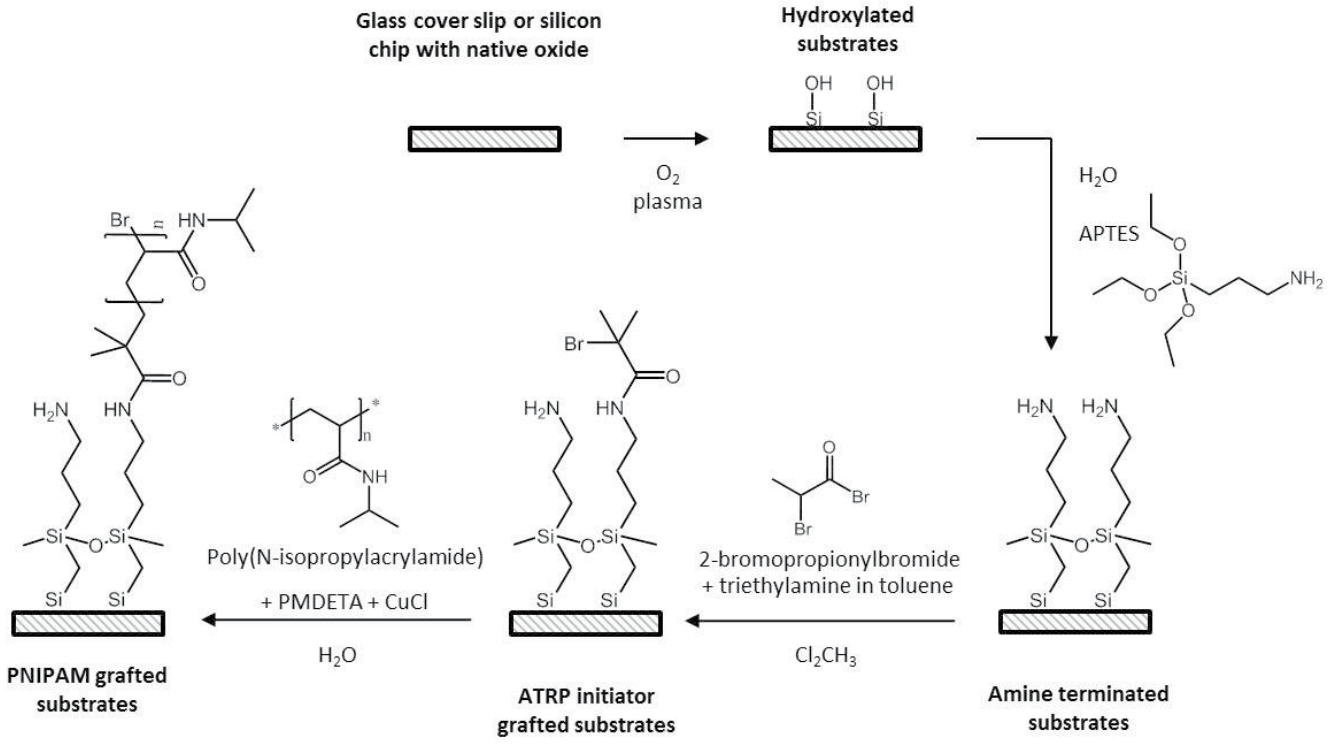



**Figure 1:** Stepwise process of PNIPAM grafting.

**Ellipsometry:** To obtain the dry thickness of PNIPAM, which is the thickness of the collapsed chain above the LCST, the grafted silicon chips were analyzed using a custom-built rotating compensator ellipsometer [10] with a 632 nm wavelength laser and a 70° angle of incidence. Refractive indexes of 1.46 for $SiO_2$ and 1.47 for PNIPAM were considered [10]. A Si/$SiO_2$/PNIPAM multilayer was assumed. The measurements were performed at 3 different locations on the chips.

**Contact angle:** To characterize the thermoresponsive behavior of the PNIPAM-grafted glass slides, static contact angle measurements were performed using a drop shape analyzer (DSA 100, Krüss). A temperature control engineered system was used to set the temperature of the substrates. The system was composed, from top to bottom, of a thermal sensor (Pt 100, DM-314, Lab facility) glued to a Peltier element (121 W, MCHPE-200-14-11-E, Multicomp) that was bound with thermal conductive tape (BOND-Ply 100, The Bergquist Company) to a radiator coupled to a fan (109P5412H2026, Sanyo Denki). The thermal sensor and the Peltier element were wired to a thermo-controller (TEC SourceMeter® 2510, Keithley) for control engineering of the temperature (Figure S1). For each glass slide, measurements were carried out from 40°C to 20°C, with 5°C steps. Triplicates were used for grafted and non-grafted substrates. For each set temperature, deionized water droplets (3 µl) were deposited at 9 different points on the glass slides. The average contact angle between the right and the left angle of each droplet was evaluated.

## Cell culture

Mouse embryonic fibroblast cells (NIH 3T3, ATCC) were grown in a 5% $CO_2$ incubator (MCO-20AIC, Sanyo) in 75 $cm^2$ tissue culture flasks (BD Falcon) with phenol red DMEM (Ref 41966-019) containing 1 mM sodium pyruvate (pH 7.87). The medium was supplemented with 10% (v/v) Newborn Calf Serum (NBCS) and (100 U/ml)/(100 µg/ml) Penicillin/Streptomycin (P/S). The medium was changed every second day. Once the cells reached 70% confluence, they were sub-cultured using a Trypsin-EDTA (0.025%/0.01%) solution for 4 min at 37°C.

## Monitoring of cell detachment

Thermally induced cell detachment was characterized using an in-house-built lens free video microscope (Figure 2). The lens free video microscope, as demonstrated previously, consists of a 5.7 mm × 4.3 mm 12-bit CMOS RGB sensor (MT9P031, APTINA) with a pixel pitch of 2.2 µm and an LED (dominating wavelength, 525 nm) with a pinhole of 150 µm. In a typical experiment, a Petri dish containing cells is placed on the sensor as shown in Figure 2A. Illumination is provided by the LED, along with the pinhole, from a distance of approximately 5 cm. The light scattered by the sample and the light passing directly from the source to the sensor interferes to form a holographic pattern; this pattern is recorded by the sensor (Figure 2B).

Glass slides (with or without PNIPAM) were sanitized via overnight immersion in 70% ethanol. Harvested cells were seeded into Cytoo™ chambers (Cytoo™) containing 20*20 mm substrates and the medium of interest at a concentration of $10^4$ cells/$cm^2$ (Figure 2C). The cells

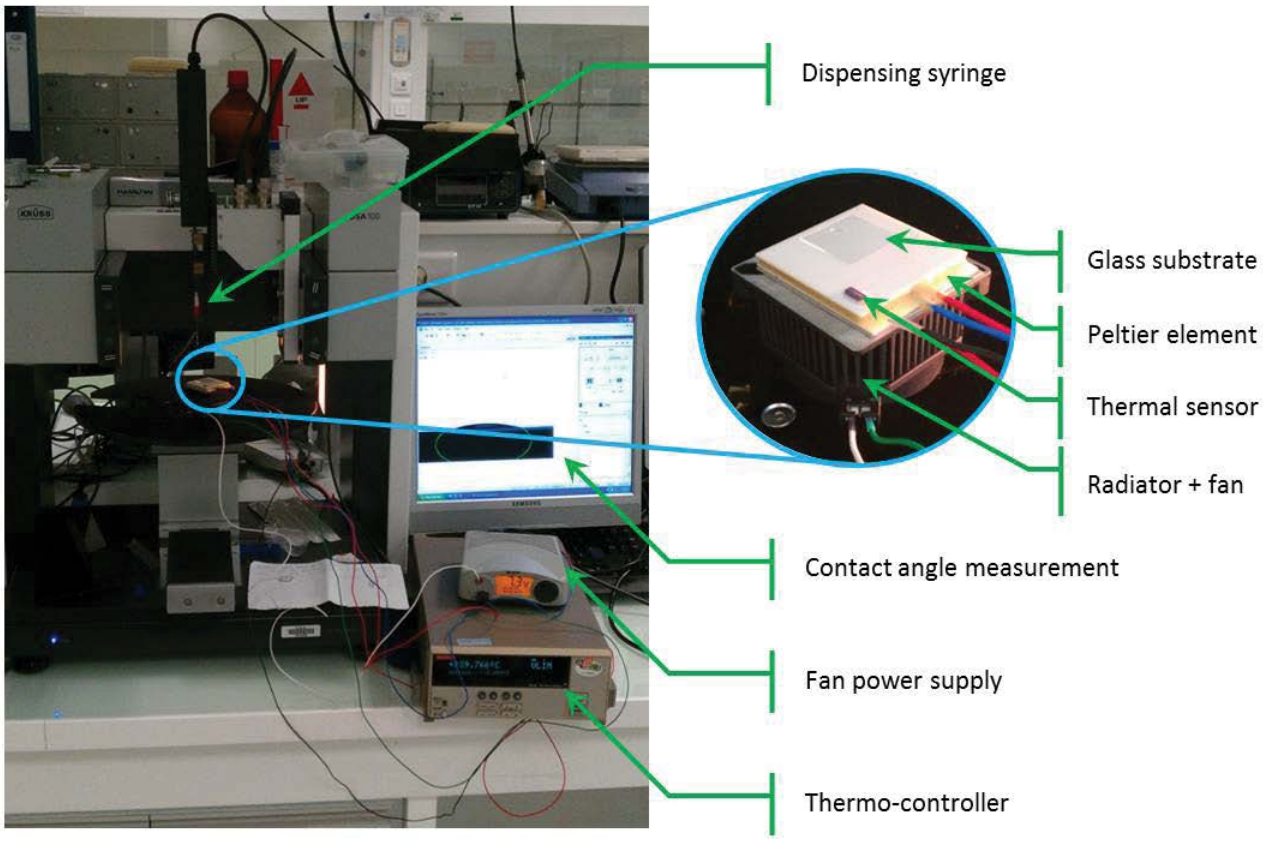



**Figure S1:** Setup used for the static contact angle measurements.

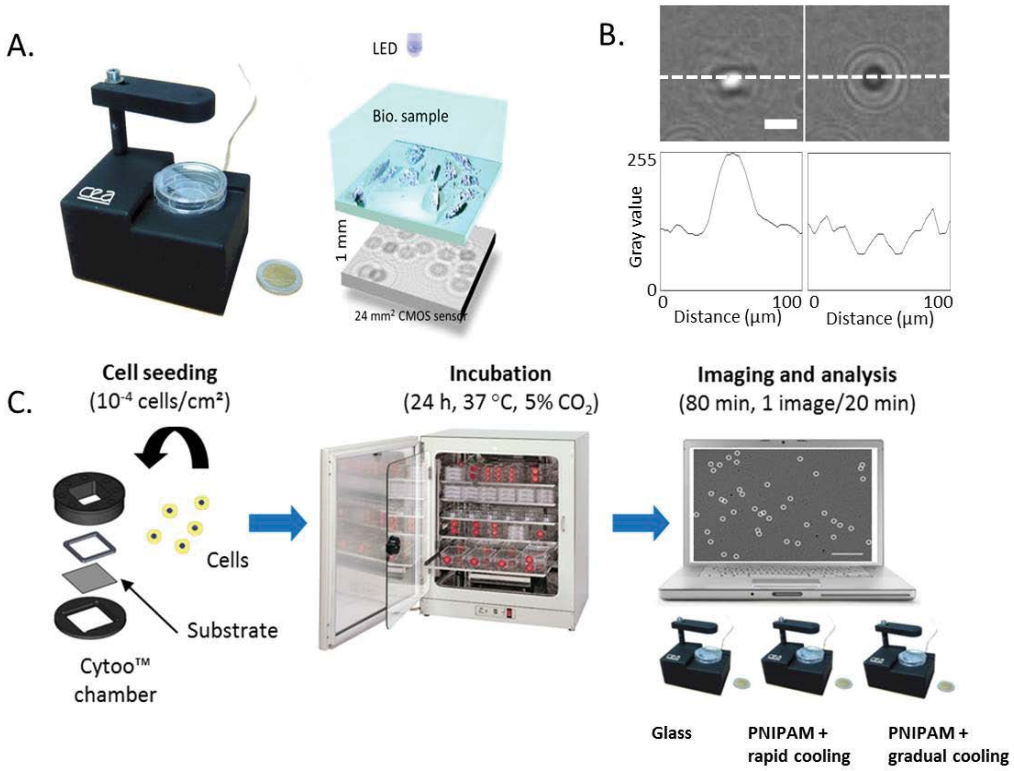



**Figure 2:** Cell detachment monitoring and set-up. A. Photograph of lens free video microscopy (left) and schematic diagram illustrating its underlying principle (right). B. Difference in lensfree holographic patterns obtained from a cell while it is adhered to the substrate (left) and while it is floating (right). The scale bar is 20 µm. The images were acquired at 20-min intervals. When adhered to the substrate, the cell presents a holographic pattern with a bright center with a gray-value greater than 200 gray levels on a scale of 0-255. When the cell detaches from the substrate, there is a sharp change in the center of the holographic pattern, and the gray-value reduces to less than 100 gray levels. C. Protocol schematic of a cell detachment experiment and monitoring using lens free video microscopy.

were then cultured for 24 h in a 5% $CO_2$ incubator at 37°C prior to the cell detachment experiment.

To perform thermal cell lift-off, three lens free video microscopes were placed in the dark at room temperature. One system comprised a Cytoo™ chamber with a glass slide, and the other two contained a PNIPAM-grafted glass slide. A total of five images were acquired at a rate of one image per 20 minutes to observe cell detachment. Immediately before the first image was acquired, the medium was replaced with room-temperature medium in one of the microscopes with a PNIPAM-grafted glass slide; this is referred to as "rapid" cooling. In the other lens free video microscope containing a PNIPAM-grafted glass slide, the medium was allowed to cool to room temperature (20-22°C); this is referred to as "gradual" cooling. Triplicate runs were carried out to obtain triplicate data for each condition. The same protocol was carried out with the bare glass slides; this served as a control experiment. The different substrates were immersed in 1 ml of trypsin-EDTA for 10 min at 37°C to remove the remaining cells, followed by two washes with 1 ml of PBS. Subsequently, 1 ml of trypsin-EDTA was dispensed onto the substrates, followed by 10 min incubation at room temperature. Finally, the substrates were rinsed with 1 ml of PBS. The PNIPAM substrates were stored wet (in PBS) at 4°C between cell culture experiments.

## pH measurements

pH measurements were performed at different time points to evaluate the stability of the medium under atmospheric conditions. These measurements were also compared with the pH of the stored bulk medium and of the incubated medium (5% $CO_2$, 37°C). Aliquots (2 ml) of the medium were removed for exposure to atmospheric conditions and for incubation, and these aliquots were dispensed in Cytoo™ wells to reproduce the cell culture conditions. A pH meter (InLab® Micro Pro, Mettler Toledo) was used to measure the pH of 1 ml of medium in a 2 ml vial (Eppendorf) for each condition. Triplicate analyses were performed for each condition.

## Thermal characterization

Thermal characterization of the "gradual" cooling of the Cytoo™ chamber was performed via IR imaging (Flir A20). The Cytoo™ chamber was incubated at 37°C for several hours in a 5% $CO_2$ incubator containing 2 ml of culture medium. As soon as the chamber was removed from the incubator, it was placed under the IR camera to monitor its cooling over time. The time between the withdrawal of the chamber from the incubator and the initiation of temperature recording was approximately one minute. The temperature read at any given time point was an average of the temperatures recorded in the area of the medium at that particular time point.

## Staining

Cell adhesion was characterized via the labeling of actin filaments

and immunostaining of focal adhesion points. Phalloidin was used to identify actin filaments. Focal adhesion points were detected by labeling vinculin. Cells were plated at a density of $10^4$ cells/cm$^2$ and incubated for 24 h on bare glass slides (i.e., control substrates) and PNIPAM substrates. Staining was carried out either immediately after cell culture to fix the cells before they sensed a change in temperature or after the medium was replaced with room temperature medium. Then, the cells were incubated for 20 min at room temperature. The cells were pre-permeabilized for 30 s with 0.5% Triton X-100 in Cytoskeleton Buffer (CB) containing 10% sucrose. CB is a deionized water solution with a pH of 6.1 (adjusted with NaOH) containing 10 mM 2-(N-morpholino) ethanesulfonic acid, 138 mM KCl, 3 mM $MgCl_2$ and 2 mM ethylene glycol tetraacetic acid. The cells were then fixed for 20 min using 4% (v/v) Paraformaldehyde (PFA) in CB containing 10% sucrose (solution A). Next, the cells were washed once with solution A, and the PFA auto-fluorescence was quenched in 0.1 M $NH_4Cl$ for 10 min. The cells were subsequently washed 3 times in PBS+, and non-specific sites were blocked via exposure to 10% goat serum with 3% BSA for 1 h. The cultures were incubated with the primary antibodies for 1 h (vinculin diluted 1:800 in 0.1% (v/v) Tween20 and 1% (v/v) BSA in PBS) and subsequently washed 3 times with PBS+. The cultures were then incubated with an anti-mouse FITC-conjugated secondary antibody (diluted 1:500 in 0.1% (v/v) Tween20 and 1% (v/v) BSA in PBS). Actin filaments were stained with phalloidin tetramethylrhodamine B isothiocyanate (diluted 1:800 in 0.1% (v/v) Tween20 and 1% (v/v) BSA in PBS) for 50 min, followed by 3 washes with PBS+. The nuclei were counterstained with Hoechst (diluted 1:1,000) for 5 min. A final wash was performed for 10 min. Then, glass coverslips were mounted on the glass slides using a fluorescent mounting medium kit, and the slides were stored at 4°C before imaging. The cells were finally imaged using a Nikon Eclipse Ti spinning disk confocal microscope with a 40x/1.3 NA objective.

## Cell viability

To determine the cytotoxicity of thermally induced cell detachment, necrosis and apoptosis were assessed via annexin-V and propidium iodide (PI) staining using fluorescein isothiocyanate (FITC) Alexa Fluor® 488 Annexin-V (Dead Cell Apoptosis Kit, Invitrogen) [11,12]. The following 3 sample types were considered in this assay:

- Cells cultured on bare glass, from which cells harvested via trypsin treatment were analyzed; these cells are referred to as the negative control.

- Cells cultured on bare glass undergoing Thermal Treatment (TT), from which only cells collected from the supernatant were analyzed. The conditions for thermal treatment were analogous to the "rapid" cooling treatment described above; the medium was replaced with room temperature medium immediately before the cell detachment experiment. This "rapid" cooling assay was applied in the viability tests; in this assay, the cells were placed under the most stringent conditions for the evaluation of their thermal resistance properties. After the "rapid" cooling treatment, the cells were kept at room temperature for either 20 min or 80 min.

For the staining procedure, the harvested cells were washed twice in cold PBS and re-suspended in 1× annexin-V binding buffer to obtain a 100-µl aliquot. Then, 5 µl of annexin-V-FITC and 1 µl of PI were added, and the aliquots were incubated for 15 min at room temperature in the dark. Next, binding buffer (400 µl) was added, and the cells were immediately analyzed using a BD LSR II flow cytometer (Becton Dickinson). Unstained cells were washed twice in cold PBS and re-suspended in 1× annexin-V binding buffer to obtain an aliquot of 500 µl. The harvesting of these cells was synchronized such that flow cytometry analysis of the stained cells could be performed simultaneously. Triplicate samples were used, and at least 1000 events were considered. A viability assay was carried out at 20 min and 80 min after the initiation of thermal-mediated cell detachment; these times correspond to the first and last time point, respectively, after which the cells were exposed to room temperature during lens free observation. Overall, a total of 4 viability assays were performed.

## Results

### Validation of PNIPAM grafting

The thickness of the polymer layer on the PNIPAM-coated surfaces was determined to be 38 nm ± 5 nm through ellipsometry. The thermal behavior of the grafted substrates was investigated by measuring the static contact angle on the PNIPAM glass chips. These data were compared with those obtained from a bare glass slide (Figure 3A).

Before the substrates were used for cell culture, the mean contact angle slowly decreased with an increase in temperature on the glass slide; a characteristic "S curve" was observed for the PNIPAM glass chips, with an LCST between 32-33°C (Figure 3A). The measured value for the LCST was consistent with the literature and confirmed that PNIPAM grafting was achieved.

### PNIPAM behavior under consecutive cell culture detachment cycles

The PNIPAM substrates used after three cell culture detachment experiments were characterized via measurement of the contact angle. This characterization revealed that the PNIPAM substrates no longer responded to temperature and behaved similarly to bare glass slides (Figure 3B).

Cell detachment could be followed via lens free microscopy on one PNIPAM-coated glass slide during consecutive cell detachment experiments. An example that illustrates the counting protocol performed via the lens free microscopy approach is provided in Figure 4A and 4B. Figure 4C shows the detachment rate over time in these consecutive cell detachment experiments. A large population of 1851 ± 590 cells was observed for each condition. The results clearly revealed a significant reduction of the cell detachment efficiency starting from the second use, from 75% efficiency 20 min after initiating thermal cell detachment during the first experiment to 13% in the second experiment. This low cell detachment efficiency was also observed in the subsequent (third) experiment.

### Influence of the temperature variation rate on thermally triggered cell detachment

Further cell culture detachment experiments using lens free microscopy were performed on PNIPAM-coated glass slides and bare glass slides to study the influence of the rate of variation in the medium temperature ("rapid" cooling or "gradual" cooling).

The results of the lens free acquisition data analysis are plotted in Figure 5A for the PNIPAM-grafted glass slides and Figure 5B for the bare glass slides (i.e., the control substrates without PNIPAM). Each condition resulted in high variability in the cell response, as shown by the large error bars. The initial detachment may correspond to either dividing cells or dead cells. For each type of substrate, we observed that "gradual" cooling induced a linear rate of cell detachment over time. By contrast, "rapid" cooling induced an increased rate of cell detachment

within the first 20 min (20%), which was followed by either a plateau or a slight reduction in the number of detached cells (Figure 5A and 5B). With "rapid" cooling, the percentage of detached cells within the first 20 min was equivalent to the percentage of detached cells observed after 80 min of gradual cooling. The same trends and levels of cell detachment were observed with both substrates (PNIPAM and bare glass slides) as the temperature variation rate changed.

## pH and thermal variation

The thermal and pH variation that cells were exposed to during the previous cell detachment were investigated and quantified.

For the thermal variation, an IR camera was used to monitor the temperature variations in a Cytoo™ well exposed to "gradual" cooling over time. Figure 6A shows the variation in the Cytoo™ chamber

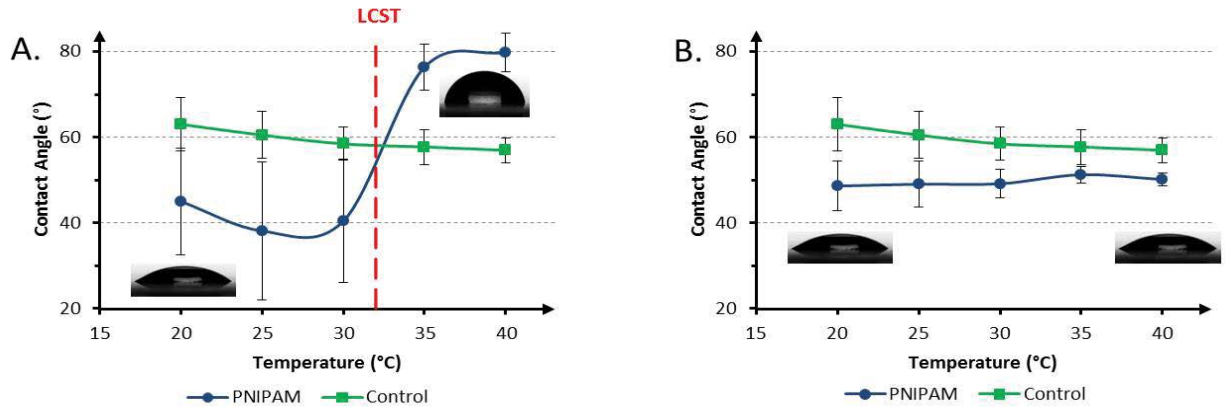



**Figure 3:** Static contact angle measurement versus temperature after first use (A) and after several uses (B) of the PNIPAM-grafted chips for cell detachment. For each curve, 3 substrates were used, and 9 measurements were performed on each substrate in different locations for any given temperature. Thermal behavior of PNIPAM-grafted (green) and bare glass slides (Ctrl, blue). LCST: Lower critical solution temperature.

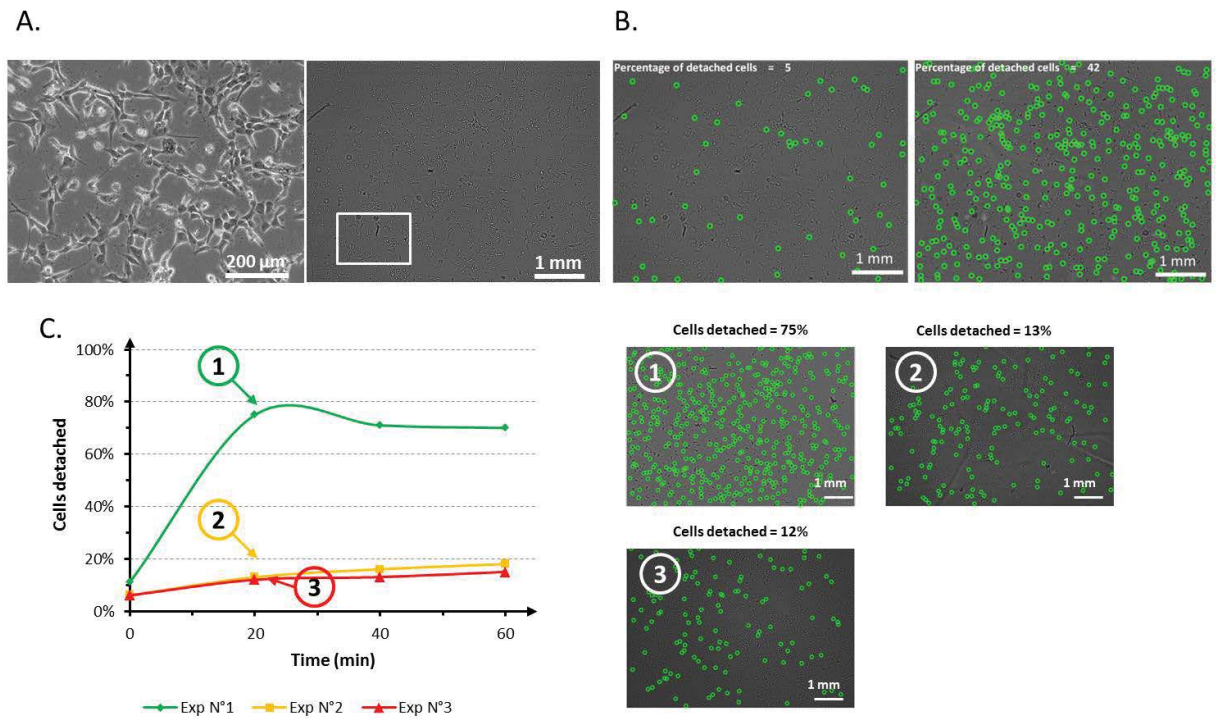



**Figure 4:** Lens free imaging of cell detachment. A. Comparison of the field of view between 10X-magnified standard microscopy (left) and lens free video microscopy (right). The white square represents the size of the field of view of standard microscopy. B. Cell detachment quantification based on the data recorded via lens free video microscopy. Detached cells were identified using pattern recognition and are encircled in green. The percentage of detached cells was calculated at t=0 (left) and t=20 min (right). C. Cell detachment efficiency on a PNIPAM substrate with successive thermal detachment experiments. Cells were cultured in "red" media and were subjected to a "rapid" cooling. Images show the number of detached cells after 20 min of thermal-induced detachment for experiments n°1 (1), n°2 (2) and n°3 (3). Number of cells analyzed per experiment: 1851 +/-590.

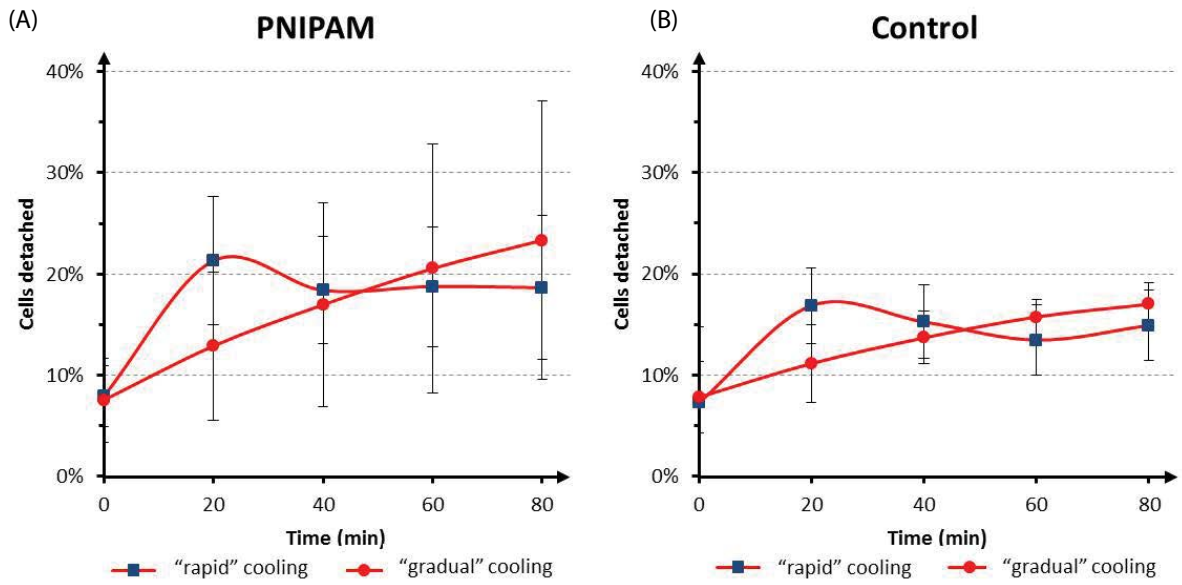



**Figure 5:** Influence of the temperature variation rate during cell detachment. Cell detachment over time for PNIPAM-grafted glass slides (A) and for control slides (i.e., bare glass) (B). N=3. Number of cells analyzed per experiment: 1851 +/-590.

temperature over time. The Cytoo™ chamber reached a temperature of 30°C in approximately 4 min (1 min had to be added to account for the time required to remove the chamber from the cell culture room, place it under the IR camera and start recording). In less than 20 min, the chamber reached room temperature. In the case of "rapid" cooling, the temperature of the cell medium dropped from 37°C to 20-22°C in less than one minute.

Regarding the pH, it can be observed from Figure 6B that the cells were cultured in a more basic environment compared with the recommended pH range for cell culture (7.2-7.4). During the cell detachment experiments, the pH increased over time as $CO_2$ escaped from the culture media. Under "gradual" cooling, the pH variations were approximately 0.4 pH units in 20 min and 0.8 pH units in 1 h 30 min. During "rapid" cooling, the medium exhibited slightly lower pH variations over time: 0.3 pH units in 20 min and 0.7 pH units in 1 h 30 min.

## Cell adhesion on non-coated and PNIPAM-coated substrates

Cell adhesion involves actin filaments and focal adhesion points, which were visualized using staining techniques. The organization and level of expression of vinculin indicated the differences in adherence under different conditions (i.e., different temperatures, substrate types and media), as depicted in Figure 7. When cultured at 37°C, the cells presented the expected elongated morphology on both bare glass and PNIPAM substrates (Figure 7A and 7B). In both cases, vinculin was present at the cell periphery, at the actin filament termini and on the cell surface. Upon exposure to 20°C for 20 min, the cells lost their fibroblastic morphology on all substrate types (Figure 7C and 7D). We observed a dramatic reorganization of vinculin and co-expression of vinculin and actin at cell-cell contact points. The cells that detached from the PNIPAM substrates presented a higher level of vinculin/actin co-expression compared to the cells attached to the glass substrates.

## Effect of thermally mediated harvesting on cell viability

To evaluate the viability of the cells that detached under thermal variation, a cell viability assay was performed (Table 1). Cell viability was decreased by approximately 10% upon thermal treatment compared to the negative control, although it remained high after 20 min at room temperature. Furthermore, the longer the cells were exposed to room temperature, the lower their viability. The results obtained from the glass and PNIPAM substrates were comparable. According to the cell viability test, thermal treatment did not lead to increased apoptosis versus necrosis after 20 min of exposure to room temperature. However, a significant percentage of the cells were found to be necrotic after 80 min of exposure to room temperature.

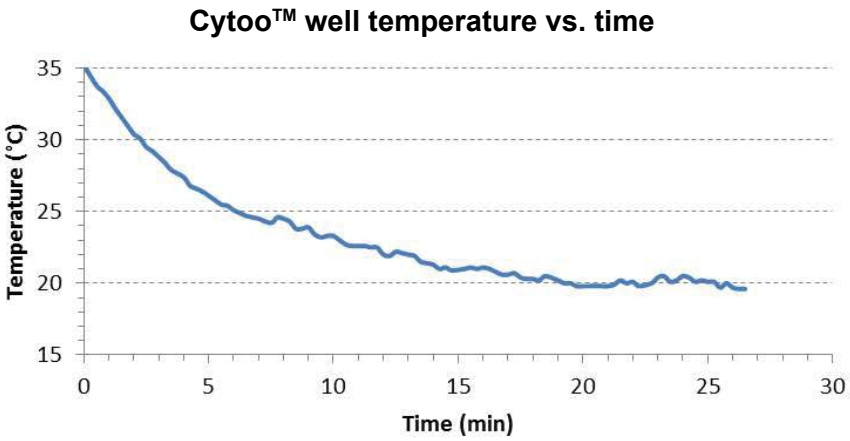
A shorter cell detachment period (i.e., 20 min) was preferable; allowing a higher percentage of viable cells that endured thermally mediated harvesting to be obtained. These cells were impacted by the type of culture media, but not by the substrates.

## Discussion

PNIPAM was developed in 1990 by Okano et al. at the Institute of Biomedical Engineering at Tokyo Women's Medical School. These researchers first demonstrated a novel methodology for harvesting and expanding cells using thermo-responsive polymeric surfaces. Since that time, PNIPAM has been used in diverse fields such as optics [13] and rheology [14]. Thus far, PNIPAM has been mainly utilized in biological applications for drug delivery [15] and cell sheet engineering [16]. However, temperature-mediated cell detachment is still not completely understood and remains mostly qualitative.

Our work focused on the characterization of key conditions for cell detachment on synthesized PNIPAM layers and glass. We studied the influence of these layers in successive experiments and then examined the influence of the temperature variation rate and the buffer regulating

**A.**




**B.**

| | pH | | | |
|---|---|---|---|---|
| **Culture medium** | stock solution, RT | Incubator (5% $CO_2$, 37°C) | 20 min, OA, RT | 1h30, OA, RT |
| DMEM w/ Phenol Red | 7.87 | 7.78 | 8.18 | 8.51 |

**Figure 6:** Evaluation of the temperature and pH variation rate during cell detachment. A. Cooling of the Cytoo™ well was assessed with a thermal infrared camera (FLIR A20). After a 1-h incubation at 37°C, the Cytoo well was left to cool at room temperature. B. pH measurements of the culture media after exposure to open air at different time points. OA: open air, RT: room temperature. For comparison, the pH values of the incubated media and of the stock solution are provided. N=3.

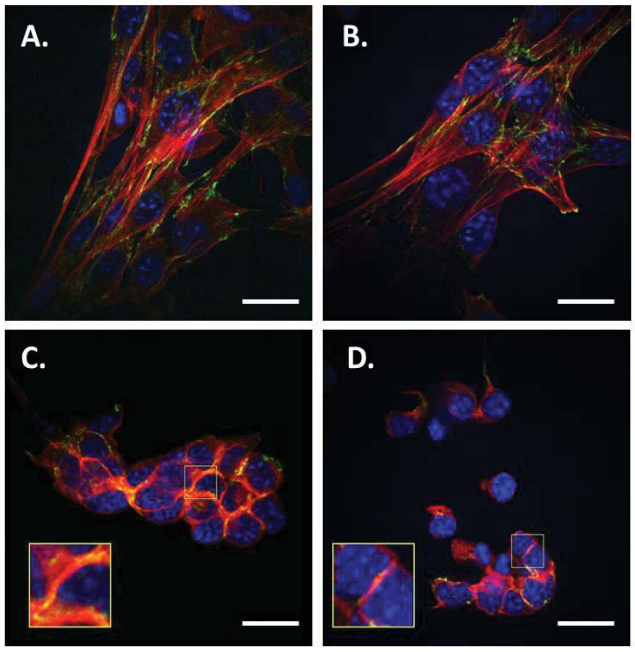



**Figure 7:** NIH-3T3 staining after 24 h of culture: nuclei appear in blue (Hoechst), actin filaments appear red (red-labeled phalloidin) and focal adhesion points appear green (green-labeled vinculin). A,B: Cells were stained immediately after incubation at 37°C. C,D: Cells were stained after a 20-min incubation at room temperature. A,C: Cells cultured on PNIPAM. B,D: Cells cultured on glass, which served as a control substrate. The close-up images (insets) are magnified 2.5 times. The scale bar is 20 µm.

the culture medium pH on the detachment of fibroblastic cells. To evaluate cell detachment, we relied on lens free video microscopy. Kesavan et al. were able to correlate a change in the holographic pattern of cells when they lift off during division [17], and these authors reported an intensity measurement with a significance of 3σ [9]. Using the same principle, cells that lift off during detachment were identified among several thousand cells in an image. A morphological change occurs when cells detach from the substrate [3]: cells that are initially spread out over the surface will become round and eventually lift off from the substrate. In contrast to standard microscopy, lens free video microscopy does not provide detailed morphological information on the imaged cells; however, the observed holographic pattern of the cells allows rapid and direct screening for cell detachment. Lens free video microscopy offers a significant advantage over standard microscopic observations. For example, it provides a wider field of view of 24 $mm^2$, as depicted in Figure 4. Thousands of cells are visualized at a glance, compared with hundreds when using a standard microscope, thus increasing the statistical significance of the results obtained. Moreover, standard microscopic images cannot reveal whether cells are actually detached from the substrate. As a consequence, studies have either measured morphological changes in the cells [5] or counted cells from the harvested supernatant [2]. These methods are not dynamic; the former does not confirm whether the cells detached from the substrate, and the latter is user-dependent, leading to high variability. It has been reported that when detached cells are collected by aspirating the supernatant for quantification, the hydrodynamic shear stress induces the detachment of additional cells that are weakly attached [18]. This results in user-dependent bias and an erroneous increase in the percentage of detached cells. The lens free microscopy approach can detect changes in cell adhesion *in situ* as a result of changes in their holographic pattern. This

detection does not involve personnel intervention, thus overcoming the aforementioned shortcomings of earlier methods. However, the lens free approach to quantifying cell detachment has some limitations. Because dividing cells exhibit a similar pattern to detached cells, the level of cell detachment could be erroneously increased; this limitation is also common in standard microscopic observations. However, dividing cells re-attach to their substrate after cytokinesis. Therefore, the erroneous detection is temporary and is usually not observed in subsequent images. In addition, detached cells may form clusters that are recognized as a pattern, especially at high density, which could lead to an underestimation of the count. This situation is not observed often and can be addressed by optimizing the density of seeded cells, as was performed in this study.

Atom Transfer Radical Polymerization (ATRP), a type of "living" polymerization, can be used to achieve reproducible grafting of polymer brushes with specific thicknesses and densities without the need for expensive laboratory equipment [19]. Using ellipsometry and static contact angle measurements, we demonstrated that PNIPAM was successfully grafted onto glass slides via ATRP and responded to variations in temperature. Ellipsometry measurements confirmed the deposition of a layer with a defined thickness. Contact angle measurements verified that this layer was composed of PNIPAM based on its response to temperature, as previously described in the literature.

The estimation of cell detachment via lens free microscopy demonstrated that these layers were able to undergo relatively efficient cell detachment, with up to 75% cell detachment. Our results are supported by a previous study in which surfaces presenting similar PNIPAM-grafting characteristics, but in association with different cell types, could trigger cell detachment [20]. Additionally, the data on cell viability (Table 1) rule out the possibility that such detachment is related to cell death because the population of detached cells showed high viability after a short thermally triggered cell detachment period of 20 min.

However, based on our measurements using a lens free video microscope, we found that after repeated use, the PNIPAM substrates showed a very low yield, and we did not observe a significant difference between the control and PNIPAM substrates. Due to this insignificant difference in cell detachment between the PNIPAM-grafted and bare glass slides, we hypothesize that the properties of PNIPAM are lost after repeated culture experiments. The contact angle measurements performed on recycled trypsin-treated substrates suggested that the PNIPAM chips lost their thermosensitivity, which is a phenomenon that has not been reported previously. Figure 4C supports our hypothesis that between experiments, the efficiency of cell detachment declined significantly, decreasing from 75% detachment to approximately 12% in the subsequent experiment. Normally, PNIPAM becomes softer as it swells at lower temperatures, and the balance of the forces between the cells and the polymer is lost. In theory, cytoskeletal tension forces prevail over decreased mechanical resistance from the substrate. This loss of equilibrium, along with the pressure exerted on the ventral cytoplasmic membrane due to the swelling of the polymer chains, leads to a combination of ECM protein desorption and disruption of ECM-integrin bonds [4]. These events are supported by the fact that cells detach with the majority of their ECM [21], but not all of it, as some components (i.e., proteins) of the ECM can be found on PNIPAM substrates after cell lift-off [22]. Thus, we believe that biofouling of the PNIPAM surfaces by cell ECM proteins might have occurred, thereby hampering their thermosensitivity. For commercial PNIPAM substrates, users are advised to "not re-use the product as the residual matrix and media components may absorb to the surface, compromising performance." This assertion supports our findings regarding the reuse of PNIPAM substrates.

PNIPAM has been shown to function reversibly in association with temperature under contact angle characterization. It appears that this reversibility is not straightforward when PNIPAM is being exploited for consecutive cell detachment. The grafted glass slides behave similarly to bare glass slides (Figure 5). They exhibit minimal cell detachment, which can be modulated by the culture conditions (Figure 5). Indeed, our experiments demonstrated that as the temperature drops more rapidly, there is a significant increase in the number of cells that detach (at least 2.5-fold) when a rapid temperature variation is applied. The only difference between the re-used PNIPAM surfaces and the bare surfaces is that cells detached with a higher level of vinculin/actin co-expression on PNIPAM (Figure 7C and 7D). This observation might indicate that cells utilize a different mechanism for detachment.

## Conclusion

In this study, we have shown that PNIPAM surfaces can rapidly lose their thermosensitivity when they are used consecutively for cell detachment. As a consequence, the surfaces behave quite similarly to

| | | Culture media | | | | | |
|---|---|---|---|---|---|---|---|
| | | viable | apoptotic | necrotic | viable | apoptotic | necrotic |
| Negative control | Glass | 97.0% | 1.9% | 1.0% | | | |
| | | After 20 min at RT | | | After 80 min at RT | | |
| Thermally treated | Glass | 86.2% | 6.6% | 7.2% | – | – | – |
| | PNIPAM | 88.1% (±2.3%) | 5.6% (±0.9%) | 6.1% (±3.3) | 24.5% (±6.9%) | 12.8% (±3.5%) | 61.9% (±4.7%) |

**Table 1:** Cell viability versus thermal treatment duration. 1000 events were considered for each condition for FACS analysis.

non-modified surfaces and show minimal (but non-negligible) cell detachment (up to 15%). Additionally, we found that the temperature variation rate can modulate this detachment, which is maximized when a sharper temperature drop is applied. Therefore, we have demonstrated that the thermosensitivity of PNIPAM-grafted surfaces can be counterbalanced by the culture conditions (i.e., the number of uses and culture parameters such as the temperature variation rate). Thus, the results of this study will inform researchers of such issues, helping them to better control cell detachment on PNIPAM-grafted surfaces.

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