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Straightforward, One-Step Fabrication of Ultrathin Thermoresponsive Films from Commercially Available pNIPAm for Cell Culture and Recovery

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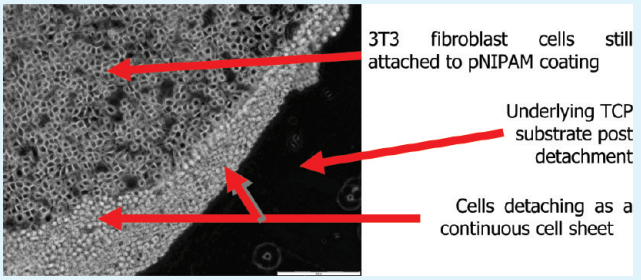
Supporting Information

ABSTRACT: The use of thermoresponsive surfaces as platforms for cell culture and cell regeneration has been explored over the last couple of decades. Poly-*N*-isopropylacrylamide (pNIPAm) is a well characterized thermoresponsive polymer which has an aqueous lower critical solution temperature (LCST) in a physiologically useful range, which allows it to reversibly attract (T < 32 °C) and repel water (T > 32 °C). It is this phenomenon that is exploited in temperature-controlled cell harvesting. pNIPAm coatings are generally poorly cell compatible and a number of complex or expensive techniques have been developed in order to overcome this issue. This study seeks to design a simple one-step system whereby commercially sourced pNIPAm is used to achieve similar results. Films were deposited using the operationally simple but rheologically complex spin coating technique. Reversible temperature modulated cell adhesion was achieved using a variety of different cell lines. This system offers a simplistic and cheaper alternative to methods used elsewhere.



KEYWORDS: poly-*N*-isopropylacrylamide, cell recovery, spin coating, surface properties

INTRODUCTION

The sustained interest in developing thermoresponsive cell delivery platforms is evidenced by the multiple journal publications detailing ongoing developments in this area per annum. The design of thermoresponsive polymer based cell culture carrier and delivery systems has been ongoing since the early 1990′s with numerous methods of thermoresponsive substrate fabrication reported. Traditional methodologies employed for the harvesting of anchorage-dependent cells using proteolytic enzymes or mechanical scraping degrade cell-to-cell interconnector junctions and culturally deposited extra cellular matrix (ECM). Such conventional techniques can be detrimental to cell viability and cell surface receptor and transmembrane protein integrity, which in turn may impair subsequent cell functionality.[1−5] A cell harvesting technique, using thermoresponsive polymer films as a substratum for cell growth, from which cells and cell sheets can be detached, offers a viable and gentle alternative for recovering viable cells and cell sheets.[6−9] The recovery of highly viable cells is especially important in areas of biomedical science, where it is imperative that undamaged cells are retained or isolated and therefore enzymatic or mechanical disaggregation is unfavorable. Additionally, using temperature as a means of detaching cells in this manner with cell to cell junctions and ECM maintained allows for the garnering of contiguous cell sheets. This cell sheet may then be used for tissue engineering purposes such as for 2D or 3D biomedical constructs or for tissue damage repair.[2,10−13]

Because of its physiologically proximal lower critical solution temperature (LCST) of 32 °C, poly-*N*-isopropylacrylamide (pNIPAm) is the thermoresponsive polymer predominantly used in cell and cell sheet regeneration. Unfortunately it has been shown that pNIPAm surfaces are generally nonconducive to reasonable cell growth and a number of approaches been have taken in order to overcome this significant obstacle.[11,14,15] To date, the most successful of these approaches have been through the immobilization of pNIPAm via the electron beam polymerization (EBP) method and plasma polymerization. While these techniques have generated successful outcomes in cell and cell sheet regeneration there is a need to develop an accessible means of thermoresponsive dish fabrication as the technology or expertise needed for these methods of preparation is outside

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the financial and technological scope of most tissue culture laboratories.[16] Okano et al. have been the chief proponents of cell sheet regeneration via thermoresponsive delivery systems using EBP and have expanded the production of these surfaces for global retail sale. At the time of writing, the retail price quoted for one 35 mm thermoresponsive dish was more than €20 (when bought in a pack of 30) and therefore the use of these commercially sourced dishes is impractical for routine cell culture experiments or for long scale investigations. One of the first reported uses of pNIPAm based conventional nongrafted films, with a view to cell recovery, was by Takezawa et al. Cells were cultured on pNIPAm conjugated with collagen and a cell monolayer was reached after a suitable period of incubation and detachment was achieved under cooling conditions, cell growth on pNIPAm alone was comparatively very poor.[15] A similar approach has been taken by other groups via the addition of an overlayer of cell adhesion promoting proteins onto thick solvent cast films, thus negating the adverse effects of pNIPAm on cell adherence.[6,14,17] The nongrafting techniques outlined use the solvent casting technique to deposit the polymer layer which is an inexpensive and simple method of deposition, but conjugation with collagen or the addition of an overlayer of proteins is time-consuming and adds additional expense and complications. Additionally, incorporation of animal sourced proteins introduces a risk of disease transfer, which would clearly be undesirable if the cells were to be used for therapeutic tissue engineering purposes.[18] Thus, there has been continuing impetus to refine the techniques used to produce suitable thermoresponsive platforms with several publications per annum aiming to improve on established techniques or offer alternative approaches without the need for animal based products such as Schmidt et al.'s recent study into using pNIPAm microgels as a platform for switchable cell culture and Wischerhoff et al.'s study into using PEG based platforms as an alternative to pNIPAm.[19,20] The development of a system that would allow for the routine and reproducible production of such surfaces is desirable and is investigated in this study.

Films were deposited using the operationally simple spin coating method from polymer solutions prepared from commercially sourced pNIPAm, thus avoiding the need for expensive equipment or synthesis capabilities. Spin coating is a common tool used in the microelectronics industry but its application in producing thermoresponsive surfaces for cell regeneration is in its infancy. The films produced via the spin coating method were uniform and reproducible. Ultimately, this research seeks to develop a one-step simplistic method for thermoresponsive culture dish fabrication that can be easily developed in mainstream laboratories for "in-house" cell harvesting purposes.

The thickness of the pNIPAm coatings is often quoted as being a limiting factor for successful cell adhesion and proliferation outcomes with pNIPAm coatings of over 30 nm thickness showing low affinity for cell adhesion;[21−23] therefore, it was imperative that this parameter could be evaluated and the thickness of the spin-coated pNIPAm films was measured using atomic force microcopy (AFM), scanning electron microscopy-3D-MeX (SEM-3D-MeX) and profilometry analysis. Further characterization was achieved using contact angle, AFM, Fourier transform infrared spectroscopy (FTIR) and X-ray photoelectron spectroscopy (XPS) analysis. Cell growth and cell detachment was monitored using phase contrast microscopy and time-lapse microscopy. Quantitative assessment of cell growth on the polymer films was achieved through the alamarBlue metabolic activity assay and the PicoGreen total DNA quantification assay.

EXPERIMENTAL SECTION

Poly-N-isopropylacrylamide (M_n 20 000−25 000), anhydrous Ethanol, EtOH (200 proof, 99.5%), Dulbecco's modified Eagles medium (DMEM), Hanks' balanced salt solution (HBSS), p-streptomycin, fetal bovine serum (FBS), phosphate buffered saline solution (PBS), Trypan blue stain, trypsin, trypsin-EDTA, were purchased from Sigma Aldrich and used as received. For stem cell culture non heat inactivated fetal bovine serum from Hyclone was used to supplement the growth media. 3T3Mouse embryo fibroblast-like cells were kindly provided by University College Cork, human mesenchymal stem cells (hMSCs) were kindly provided by the Regenerative Medicine Institute (REMEDI) group in the National Centre for Biomedical Engineering Science (NCBES), NUI Galway. C33A cervical carcinoma cells, CaSki cervical carcinoma Cells, SW480 colorecatal adenocarcinoma cells, SiHa cervical squamous carcinoma cells, HeLa cervical adenocarcinoma cells, A549 lung carcinoma cells were purchased from the American Type Culture Collection and HaCaT normal human keratinocytes were purchased from Cell Line Service, Germany. Quant-iT PicoGreen dsDNA assay from Invitrogen, alamarBlue assay from Biosource, Thermanox plastic 25 mm discs from NUNC, all other plastic consumables from Sarstedt. Fused silica glass disk, 20 mm in diameter from UQG optics.

Film Preparation. Spin coated pNIPAm films were fabricated by initially depositing a 150 μL aliquot of an ethanolic polymer solution onto a slowly spinning Thermanox disk, (150 rpm) on a Laurell Technologies WS-400B-6NPP/LITE spin coater. Rapid acceleration followed to a preprogrammed speed. For all studies, the following parameters were used, unless otherwise stated; concentration 2% w/v pNIPAm in EtOH, final spin speed 6000 rpm, final spin time 30 s. All Thermanox discs were housed in 35 mm Petri dishes and were dried slowly overnight in an EtOH soaked atmosphere before drying completely in a vacuum oven set to 40 °C and 600 mBar for 4 h to ensure any residual solvent is eliminated. Films were sterilized under mild UV light for 2 h prior to cell culture experiments. For film thickness evaluation pNIPAm films were prepared as described above, onto high-quality fused silica glass substrates and for FTIR analysis films were deposited onto aluminum stubs. Samples were stored at room temperature and routinely used within a month of preparation.

Thin Film Measurement. Spin coated film thickness on fused silica glass discs was assessed using a combination of analytical techniques; AFM, profilometry and SEM-3D-MeX. Before analysis, the polymer film was subjected to laser ablation in nine distinct regions of the film and the height difference between the remaining polymer and the underlying substratum was assessed. The fused silica glass substrates were used instead of the Thermanox discs as the ablation process interacts with the Thermanox polymer based discs as well as the film of interest during ablation, which would render the film measurement process useless.

An ArF excimer laser (ATL Atlex, Wermelskirchen, Germany) was used in conjunction with a machining center (Optec MicroMaster, Frameries, Belgium) to ablate selected areas on the thin film surface. The excimer laser operates at a wavelength of 193 nm with a pulse length of a few nanoseconds. At this wavelength the photon energy is high enough to break chemical bonds on the polymer surface and photochemical ablation is achieved. Laser parameters included a pulse repetition frequency of 200 Hz at a fluence of 66 mJ/cm^2. A standard mask projection machining approach was used to shape the laser beam. An optical demagnification of 5X was employed to produce 9 ablated areas of approximately 400 μm × 400 μm in size of which four random windows were chosen for thickness evaluation.

SEM analysis samples were gold coated and then examined using a Hitachi S-4700 SEM in low magnification mode at an accelerating voltage of 10 kV. MeX software was used to compile a 3D simulation of the surface topography by combining 3 conventional SEM stereo images taken at −5, 0, and +5°. By inputting relevant specifics, such as the scan bar size and the magnification into the MeX software, it is possible to measure topographical specifics, such as the z height difference between the polymer surface and the underlying substratum (exposed post ablation). Four measurements were taken to ensure statistical accuracy.

A Zygo Newview 100 surface profiler was also utilized to measure the depth of the thin films. This is a scanning white light interferometer and provides surface topography and film thickness measurements to an accuracy of 0.1 nm. It has a field of view which is variable from 6 mm ×4.5 mm down to 0.18 mm ×0.14 mm, has a vertical range of 100 μm and it can measure roughness and step-heights in the range 0.1 nm up to 10′s of micrometers. Four 10 μm scans were recorded to ensure statistical accuracy. The objective used for all measurements was a 20X Mirau, with zoom set at 0.5×.

AFM images were obtained in tapping mode in air using a Dimension 3100 AFM (Digital Instruments, Santa Barbara, CA, USA) and Veeco 1−10 Ohm-cm phosphorus (n) doped Si tips a matrix of 512 × 512 data points along the $x-y$ plane were analyzed in a single scan. Four 100 μm × 100 μm scans were recorded in at a scan rate of 1 Hz on each ablated area to ensure statistical accuracy and the z-height difference between the polymer surface and the underlying substratum, (exposed post ablation) was measured.

Other Surface Characterization. AFM was also used to assess the roughness of the deposited pNIPAm coatings using 10 μm × 10 μm scans at a rate of 1 Hz and a matrix of 512 × 512 data points along the $x-y$ plane were analyzed in a single scan. The roughness of the films was reported as root-mean-square (rms) roughness values, where rms denotes the standard deviation of the Z-values along the reference line. FTIR and XPS spectra were acquired using the Hitachi FTIR-8300 in transmission mode and the AXIS 165 X-ray photoelectron spectrometer respectively.

Contact Angle Measurements. Advancing contact angle measurements were performed using the advancing drop method on a home-built goniometer. The goniometer was assembled on an optical rail from Newport Optics with opto-mechanical components from Newport Optics and Edmund Optics. DROPimage software marketed by Rame Hart and developed by F. K. Hansen was applied for determining contact angles. Polymer samples were placed in a temperature-controlled environmental chamber mounted on a tilt stage and the temperature on the surface was monitored using a thermocouple attached to the chamber stage. Continuous water circulation through the chamber from a heated water bath ensured that the temperature was maintained above the polymer LCST while measurements were taken. In a typical experiment, a drop was deposited on the surface with an initial radius of about 3 mm. For the advancing contact angle experiment, a thin stainless steel needle (gauge 22) was inserted in the center of the drop from above. The volume of a drop was increased by pumping liquid into the drop using a syringe pump. The pumping speed was adjusted to maintain the rates of advancing below 0.5 mm/min.

Cell Culture. 3T3 cells were maintained in Dubecco's Modified Eagles Medium (DMEM), supplemented with 10% fetal bovine serum and (FBS) 1% penicillin streptomycin antibiotics. The human hMSCs used in these studies were isolated from human bone marrow. For experimentation, the hMSCs used were passaged no more than 5 times. All the other cell lines used were maintained and cultured according to their company prescribed protocols. When cells reached 80−90% confluence, cells were harvested and used for reseeding or for experimentation where appropriate. For experimentation, 3T3 cells were seeded in triplicate at a density of 40,000 cells/cm^2 on the pNIPAm films and on tissue Thermanox controls and incubated for 24/48 h. For single cell, higher magnification time-lapse microscopy cell detachment investigations, cells were seeded at a density of 10 000 cells/cm^2 and incubated for a 24 h time period. For experimentation hMSCs were seeded in triplicate at a density of 6,000 cells/cm^2 and incubated for 48 h. For all other cell lines used the incubation period was 24 h and the cell seeding density was 25 000 cells/cm^2. Incubation conditions were a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. In all cases the samples were placed on a thermoplate set to 37 °C to maintain a working temperature above the polymer LCST. Similarly, care was taken when handling samples during the incubation duration to ensure that the temperature was maintained above the LCST to prevent premature cell detachment.

Table 1. Summary Table of the Thickness of Spin-Coated Films (2% w/v pNIPAm in EtOH) as Measured by Profilometry, AFM, and 3D-SEM & MeX Analysis (see the Supporting information for associated images of the results.)

technology	thickness (nm)	standard deviation (nm)
profilometry	104	±9
AFM	106	±6
3D-SEM & MeX	102	±5

Cell Activity Assays. The metabolic activity of the cells grown on the prepared samples and the control was assessed using the alamarBlue assay 24/48 h after cell seeding.[24] Total DNA content was also quantified at 24/48 h by the Quant-iT PicoGreen dsDNA assay kit.[25]

Cell Imaging Techniques. After incubation films were microscopically observed for cell growth using an Olympus BX51 with Image Pro-Plus analysis system phase contrast microscope. To initiate cell detachment, we removed warm cell media, added cold HBSS, and placed the seeded samples on a digitally controlled thermal/cooling plate set to 4 °C. Micrographs of the plates were captured frequently on the phase contrast microscope to monitor cell detachment. Additionally, an Olympus IX81-ZDC time-lapse microscope with Andor IQ software was used to image cell and cell sheet detachment upon initiation of cold treatment.

RESULTS AND DISCUSSION

Surface Characterization. Spin coating, though practically simple, is an extremely complex and dynamic process and many variables determine the final film properties. Submicrometer film thickness cannot be evaluated trivially and a system of thin film appraisal was developed using SEM-3D-MeX, AFM and profilometry analysis. Initially, films were ablated carefully in nine areas of the sample film using a femtosecond laser. Using 3D-SEM MeX software, a 3D construct of a deposited film was simulated in an area where the film had been ablated and the z height difference between the polymer plane and the substrate plane calculated. The results were validated using AFM and profilometry techniques. The results are in very good agreement with film thickness predicted to be between 100 and 110 nm, Table 1.

The roughness of the deposited films was assessed using AFM. The rms average roughness of the spin coated films was 4.9 ± 4.4 nm and therefore the films were assessed to be smooth. FTIR and XPS analysis confirmed pNIPAm film deposition using both methods of preparation. Typical FTIR acyl C═O absorption peaks were observed at approximately 1650 cm^{-1}and 1550 cm^{-1}, while peaks observed at around 2970 cm^{-1} can be attributed to the stretching vibrations of C−H in the methyl groups, peaks observed at around 1460 and 1370 cm^{-1} arise

because of the asymmetric and symmetrical bending of the C−H bonds of a methyl group, respectively, and the peaks observed at approximately 3300 cm^{-1} can be attributed to the stretching of N−H groups.[26,27] XPS was used to confirm pNIPAm film deposition by analyzing the atomic composition of the outer 10 nm of the deposited films. The stoichiometry of the NIPAm monomer is 75.0% C, 12.5% N and 12.5% O. The composition of the spin coated pNIPAm films was predicted to be 78.3, 11.0, and 10.6% for C, N, and O, respectively. As can be seen the atomic composition of the monomer structure is well retained in the deposited polymer films, thus confirming successful film deposition. Three replicates of each sample type were analyzed.

It is well-documented that cells exhibit resistivity to attachment and growth on ultra hydrophobic substrates such as untreated polystyrene and such surfaces are treated through a variety of means to attain more wettable surfaces on which cells will grow.[28−30] Cells are also resistant to attachment on very hydrophilic substrates, as water association with the hydrophilic substrate impedes the protein adsorption which precedes and mediates cell attachment.[31,32] Studies have shown that although neither extreme is conducive to cell growth, a balance between the two can achieve optimum cell attachment. Lee et al. investigated the correlation between substrate wettablilty and cell adhesion and growth by oxidizing polymer surfaces using plasma treatment, thus creating a wettability gradient and monitoring seeded cell interactions. The results indicated that moderately hydrophilic substrates with a water contact angle of approximately 55° achieved optimum cell attachment and growth.[32] There is a large disparity in the contact angle results reported for pNIPAm coatings which have successfully supported reversible cell adhesion; this is most likely due to differing modes of measurement (e.g., sessile/advancing/humidity controlled), or the manner in which the polymer is deposited. The contact angle reported for EBP polymerized and plasma polymerized pNIPAm surfaces which were bioadhesive were 78 and 40°, respectively (both measured using static contact angle techniques above the polymer's LCST).[33,34] Da Silva et al. in their thorough review of thermoresponsive surfaces used for cell sheet regeneration, highlight that the disparity in contact angles observed for pNIPAm surfaces generated by different methods indicates that there are factors other than wettability which influence the cell outcome, as surfaces generated that are not bioadhesive display contact angles that indicate that the surfaces would be conducive to cell adhesion according to wettability factors alone.

Even though pNIPAm shows a dramatically increased hydrophobicity over its LCST, compared with below the LCST, a degree of water is still retained within the polymer matrix.[5,35] This is verified empirically by the contact angle results attained here. We believe advancing contact angle offers a true representative indicator of real water-polymer interaction, as it allows us to view water-polymer interaction over time, thus giving a more realistic interpretation of how water based cell culture medium interacts with the polymer. The average Thermanox control contact angle was 51.86 ± 0.78°, which is close to the optimal contact angle reported to be ideal for cell growth by Lee et al. The advancing contact angle results attained for the spin coated pNIPAm coatings indicate that the water droplet interacts with the polymer films over time and hence a slight reduction in the advancing contact angle is observed. The average contact angle observed for spin coated films over time was 45.94 ± 1.34°, which is relatively close to contact angle reported for plasma polymerized pNIPAm surfaces. The contact angle measurements were repeated 3 times with almost identical results observed.

Biocompatibility Assessment. This study sought to investigate the efficacy of deposited planar pNIPAm films for use in cell and cell sheet harvesting. 3T3 cells were seeded on the prepared films and Thermanox positive controls at a density of 40 000 cells/cm^2 and incubated for 48 h and 5% CO_2. 3T3 cells seeded on the 100 nm thick spin coated films grew to confluence, similar to the Thermanox controls and assumed an elongated morphology, as is expected for this type of adherent cell line.[36] Quantification of cell growth was achieved via the PicoGreen Total DNA quantification assay and the alamarBlue metabolic activity assay. The PicoGreen results, Figure 1A indicate that the DNA quantity on the 100 nm thick pNIPAm films is practically equivalent to that on the Thermanox controls. The alamarBlue results indicated that the metabolic activity of cells grown on the 100 nm pNIPAm films was almost identical to that of cells grown on the Thermanox controls, Figure 1B.

Previous reports indicate that pNIPAm coatings of thickness greater than 30 nm are nonconducive to cell adhesion and cell growth is generally poor.[8,11,33,34,37,38] It is hypothesized that the low concentration of polymer density in ultrathin grafted films allows for the seeded cells to interact with the underlying bioadhesive substratum.[5,35] This is further confirmed by publications released by the Okano group, which concluded that the relative hydrophobicity/hydrophilicity of the grafted polymers was influenced by the hydrophobicity of the basally located TCP (tissue culture plastic) when the coatings are sub 30 nm in thickness. They infer that at thicknesses higher than this threshold, this influence is negligible.[21,39,40] In the present study, the results indicate there is no such dependency or influence from the underlying substratum as almost all of the spin coated pNIPAm films used were well in excess of the hypothesized thickness threshold for successful cell growth. There is some literature inconsistency in this regard with some reports asserting successful cell adhesion and growth on pNIPAm or pNIPAm copolymer coatings with a higher density/thickness than is reported to be ideal for cell attachment and growth.[5,41,42] To the best of the authors' knowledge, in all of the cases reported, where cells successfully adhered to pNIPAm coatings of >30 nm, EBP was not the polymer coating fabrication method used. Cole et al. in their comprehensive review of stimuli responsive interfaces surmise that the coating microstructure may be influential on the cell response and that further investigations into pNIPAm coatings generated using different methods must be undertaken in order to understand pNIPAm-cell interfacial behavior.[5]

Why spin-coated pNIPAm films are bioadhesive and other pNIPAm films are not is not obvious. The dynamic spin coating technique differs from the solvent casting technique (which is commonly used to deposit nongrafted films of pNIPAm), in that a rapid liquid to solid phase transition occurs due to the rapid solvent evaporation associated with the spinning process. This rapid solvent evaporation may lead to a type of polymer chain deformation or stress thus trapping the chains in a type of organized orientation. Differences in the mode of evaporation between the two polymer deposition processes probably lead to films that differ considerably in their micromechanical and microstructural characteristics and therefore differ in cellular interfacial response.

Cell Detachment. To initiate cell detachment warm cell media was removed from the tissue culture dishes and cold HBSS was added in its stead and the dishes were placed on a

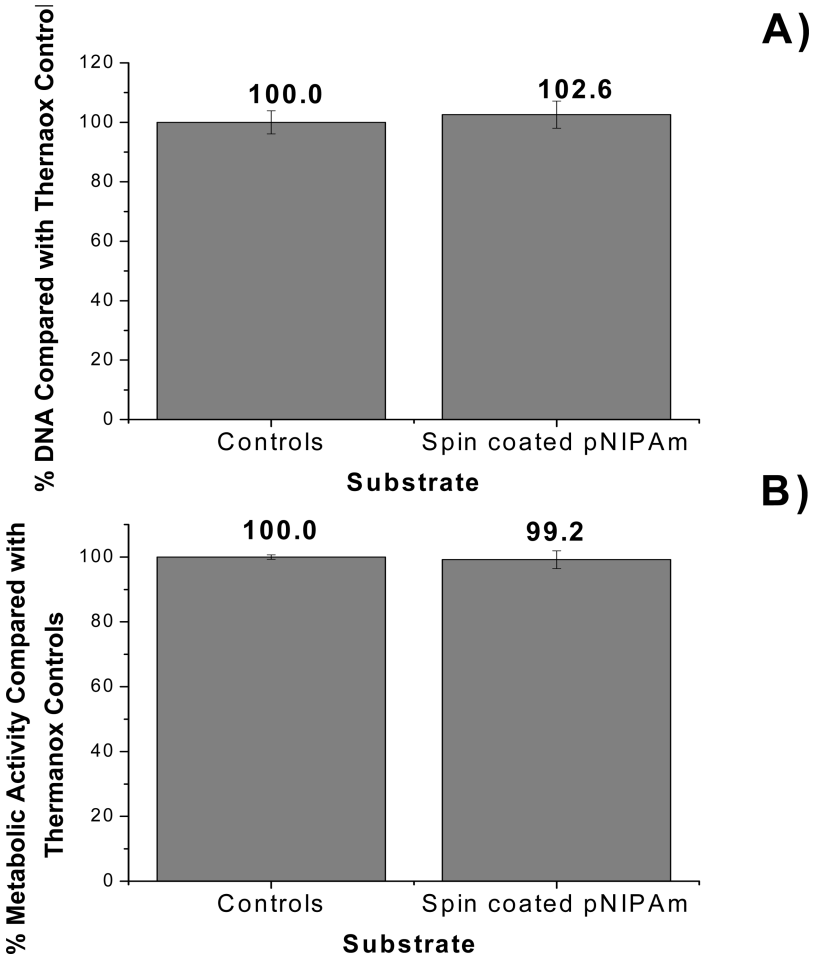

Figure 1. (A) PicoGreen total DNA assay of 3T3 cells grown on: Thermanox controls and spin coated pNIPAm films, (B) alamarBlue assay test results comparing cell metabolic activity on Thermanox controls and spin coated pNIPAm films. Assays were performed on the samples after 48 h incubation with an initial seeding cell density of 40 000 cells/cm^2. Error bars refer to standard deviation where 3 separate samples of each type of substrate were used and assays were performed in triplicate.

digitally controlled thermal/cooling plate set to 4 °C. This reduction in ambient temperature below the polymer's intrinsic LCST induces the polymer surface to switch from hydrophobic to hydrophilic, which initiates cell detachment.[6,7,11,17] Previous reports by Okano et al. have described how this surface change from hydrophobic to hydrophilic encourages passive cell detachment.[7] Active cell detachment follows and plays a significant role in the complete cell detachment.[7,8] Additionally, they report that a significant reduction in temperature impairs the detachment rate as the cellular metabolic activity that drives active detachment decreases. Therefore, cooling to a low temperature is recommended to hydrate the pNIPAm initially, prior to raising the temperature to approximately 25 °C to reactivate cell metabolism and therefore achieve optimum detachment.[7,43] In this experimental system, the ambient temperature is reduced to 4 °C, but this is not followed with a temperature ramp. A spin-coated pNIPAm polymer is not covalently attached to the underlying substrate, as is the case with grafted pNIPAm, and polymer dissolution into the culture medium causes the cells to disassociate and detach, as there is no longer a film to attach to; therefore, only cell−substrate contact is broken and cell-to-cell contact remains. Therefore, the cell detachment process occurs passively rather than actively as is the case with cell detachment from thermoresponsive grafted surfaces. It is consequently not necessary to increase the temperature after the initial cooling stage as detachment is not governed by the metabolic processes of the cells.[14,17] The thinness of the polymer coating means that the amount of polymer that dissolves into the culture medium is minimized when compared with cell detachment from conventionally prepared thicker solvent cast pNIPAm films (for a 100 nm film, the amount of free polymer that dissolves was calculated to be approximately 0.049 mg).[14,17] The toxicity of pNIPAm has been investigated elsewhere. Malonne et al. performed oral toxicity profiles using pNIPAm at a concentration of 2000 mg/kg body weight in mice and found no detectable toxicity after 28 days, whereas Takezawa et al.'s studies suggest no cytotoxicity issues under cell culture conditions.[15,44] Although the amount of free polymer is small, it could be regarded as a contamination for clinical purposes, but cell sheets recovered in this way could be very useful for laboratory investigations. Cells grown on Thermanox failed to detach with temperature reduction as expected. The cell sheets began detaching from the 100 nm thick pNIPAm films in between 30 s to 5 min upon cold treatment, with complete sheet detachment achieved in less than 12 min. When the polymer film is subjected to cold treatment in the absence of cells, the integrity of the film is lost

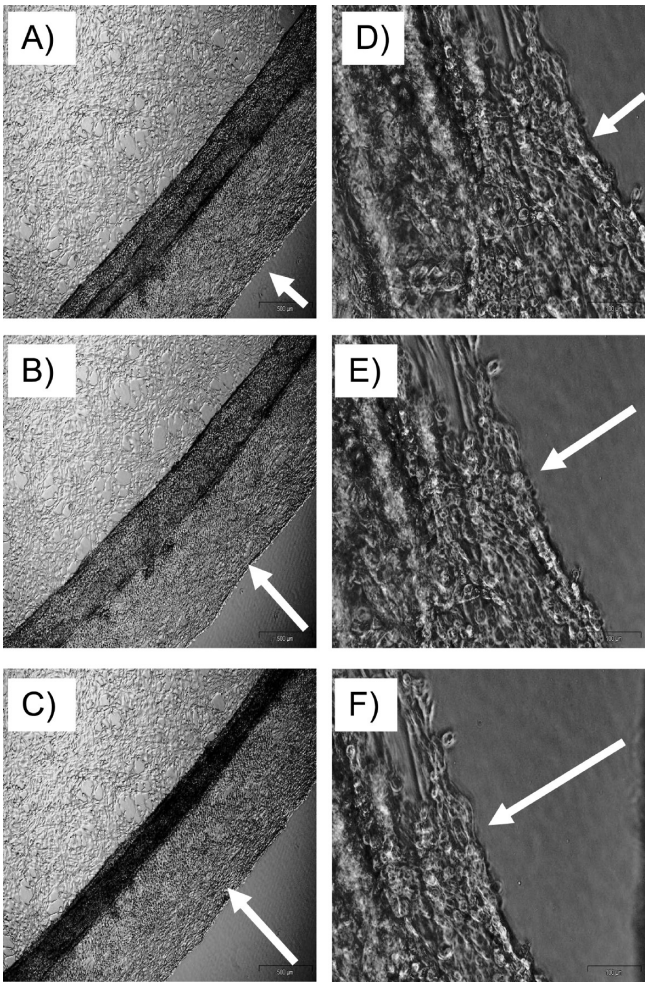

Figure 2. (A−C) Bright-field images taken sequentially (scale bar 500 μm) of a 3T3 cell sheet lifting off from a 100 nm thick pNIPAm film spin coated onto Thermanox. (D−F) Bright-field images taken sequentially (scale bar 100 μm) of a 3T3 cell sheet lifting off from a 100 nm thick pNIPAm film spin coated onto Thermanox. Cell density 40 000 per cm^2, incubation time 48 h.

immediately but in the presence of a cell sheet and ECM the cold media is slower to penetrate to the polymer layer through the tight cell to cell junctions. This process has been repeated in excess of 50 times with consistent detachment as above resultant. It is usual to remove a full cell sheet in this way but if the cell layer is not completely confluent, it is observed that localized large clusters, typically of a few millimeters in scale, detach. Cells detached with cell-to-cell junctions intact and culturally deposited ECM preserved, Figures 2−4.

Time lapse microscopy was also used to monitor the detachment of nonconfluent cells, after 10 000/cm^2 3T3 cells were seeded on the spin-coated pNIPAm films and incubated for 24 h. This sequential imaging technique tracked the transition of individual cells and small groups of cells from their flattened elongated form, through transformations in shape, as the elongated cells contract and a more rounded form begins to emerge, until complete detachment is achieved, Figure 5. Individual cells detached quicker than cell groups. In many of the cells imaged, lamellipodian projections can be seen clearly retracting over time. The cells with these protrusions tended to be the slowest to detach from the substrate. In the time lapse video, cells can be seen clearly floating once detached, this is not discernible on the images included here as the stage on which the samples are housed remains static throughout. Single cells or small numbers of cells, take longer to detach than a cell sheet, as the amount of basally deposited ECM laid down is reduced and the contractile forces that increase the tension as a sheet detaches is therefore absent. Additionally, as a cell sheet detaches, the cells contract simultaneously leading to a high level of cooperative behavior, which leads to accelerated sheet detachment compared with single cells or small cell clusters. Focusing on an individual cell and a cell doublet it is possible to track the retraction of the cells with time after cooling, Figure 5B−C. The doublet cell−cell interaction is maintained throughout the detachment process.

The viability of the recovered cells was assessed using Trypan dye exclusion assay and >95% of the detached cells were viable. Additionally, 3T3 cells detached using temperature control were centrifuged repeatedly at a temperature below the polymer's LCST and resuspended to ensure that any dissolved polymer was removed and the cells were then reseeded in culture flasks. Cells grew to confluence further confirming detached cell viability (see the Supporting Information for associated image).

Cells seeded on 100 nm thick pNIPAm films spun onto borosilicate glass coverslips, nontreated polystyrene suspension culture dishes and tissue culture plastic treated polystyrene dishes also grew to confluence similar to Thermanox controls and detached upon cold treatment. PicoGreen assay results indicate that cells grew between ±10% compared with Thermanox controls confirming that pNIPAm films spun onto a variety of substrates can successfully host cells to monolayer. (The methodology used to spin onto the dishes is the same as described for Thermanox with the base of the dished used as the substrate.)

Further Cell Culture on Spin-Coated Temperature Responsive Films. Once it was established that 3T3 cells could successfully be grown on and recovered from spin-coated pNIPAm films we repeated the procedure using a variety of different cell types. Cells were seeded on the 100 nm thick spin coated samples and Thermanox controls for 24 h and detachment was initiated upon cold treatment. Human mesenchymal stem cells (hMSC) grew on the spin coated pNIPAm films and assumed a morphology which mimicked that seen on control dishes, Figure 5A. Complete cell detachment was achieved in 20−30 min, Figure 6B. A PicoGreen DNA quantification assay estimated the DNA quantity and therefore the cell growth to be slightly higher on the spin-coated samples compared with the Thermanox controls, Figure 5C.

A variety of immortalized cell lines were similarly investigated and observed microscopically and the results observed are summarized below, Table 2. As can be seen, not all of the cell lines showed comparable growth to the controls. The HaCaT human keratinocytes in particular did not attach and proliferate similar to the controls, with many remaining in suspension. The CaSki cervical cancer cells grew on the pNIPAm samples similar to the controls but were slow to detach compared with other cells with 70% remaining after 60 min of cold treatment; this may be due to a tight network of cells inhibiting the penetration of water, which initiates the cell lift off. These results suggest there may be a cell line dependency in the use of this system, though 7 out of the 9 cell lines tested including the 3T3 fibroblasts and hMSCs were capable of hosting cells similar to the controls.

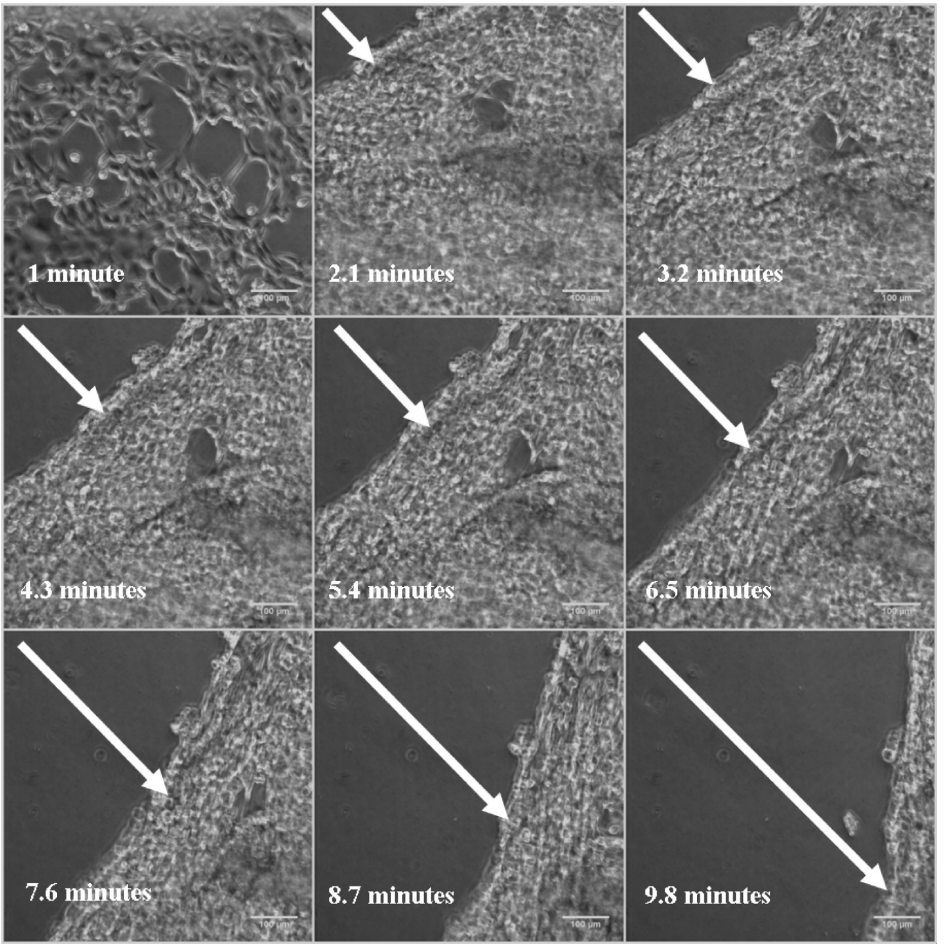

Figure 3. Time lapse microscopy image montage (scale bar 100 μm) of a 3T3 cell sheet detaching from a 100 nm thick pNIPAm coating which was spin coated onto Thermanox. Images are taken sequentially from left to right and downward. Images map sequentially the cell layer detaching from the polymer surface. Cold treatment was initiated approximately 1 min prior to the time lapse video commencing, and full detachment was achieved in this instance in less than 10 min. The cell sheet contracts upon detaching from the thermoresponsive layer. A total of 100 images were taken, 1 every 6 s, and the 9 above represent the full time spectrum.

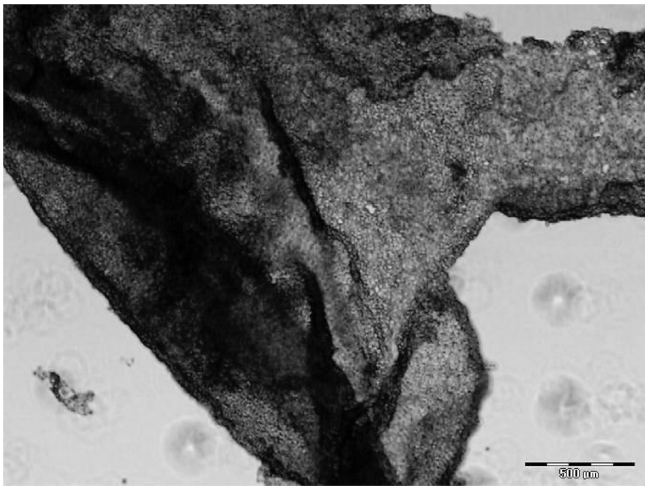

Figure 4. Bright-field micrograph of a full 3T3 cell sheet detached from a 100 nm thick spin-coated pNIPAm substrate. (scale bar 500 μm).

Process Refinement. Finally, to further refine the spin-coating parameters needed to achieve a satisfactory cell outcome a number of samples were prepared varying the three main parameters that contribute to uniform film deposition. As previously referred to the process of spin-coating films is relatively simple to perform but is itself a complex process and there have been many attempts to model the process behavior though none completely satisfy all of the critical variables which control the rheological and evaporation processes which occur during the spinning of a film. The most important and basic behavior components that contribute to the process are summed in eq 1 below

$$\frac{dh}{dt} = \frac{2\rho\omega^2 h^3}{3\eta_0} \quad (1)$$

where h = film thickness, t = spinning time, ρ = density of fluid, ω = spin speed, and η_0 = initial viscosity of solution.[45] The viscosity and density of the polymer coating are dependent on the concentration of the polymer in solution and the thickness of the deposited films is determined by this and the spinning time and speed. Therefore, we investigated the effects that varying final spin speed, spin time and polymer concentration has on the biocompatibility of spun pNIPAm films to gain a better insight into whether the thickness of the spin coated film is a crucial

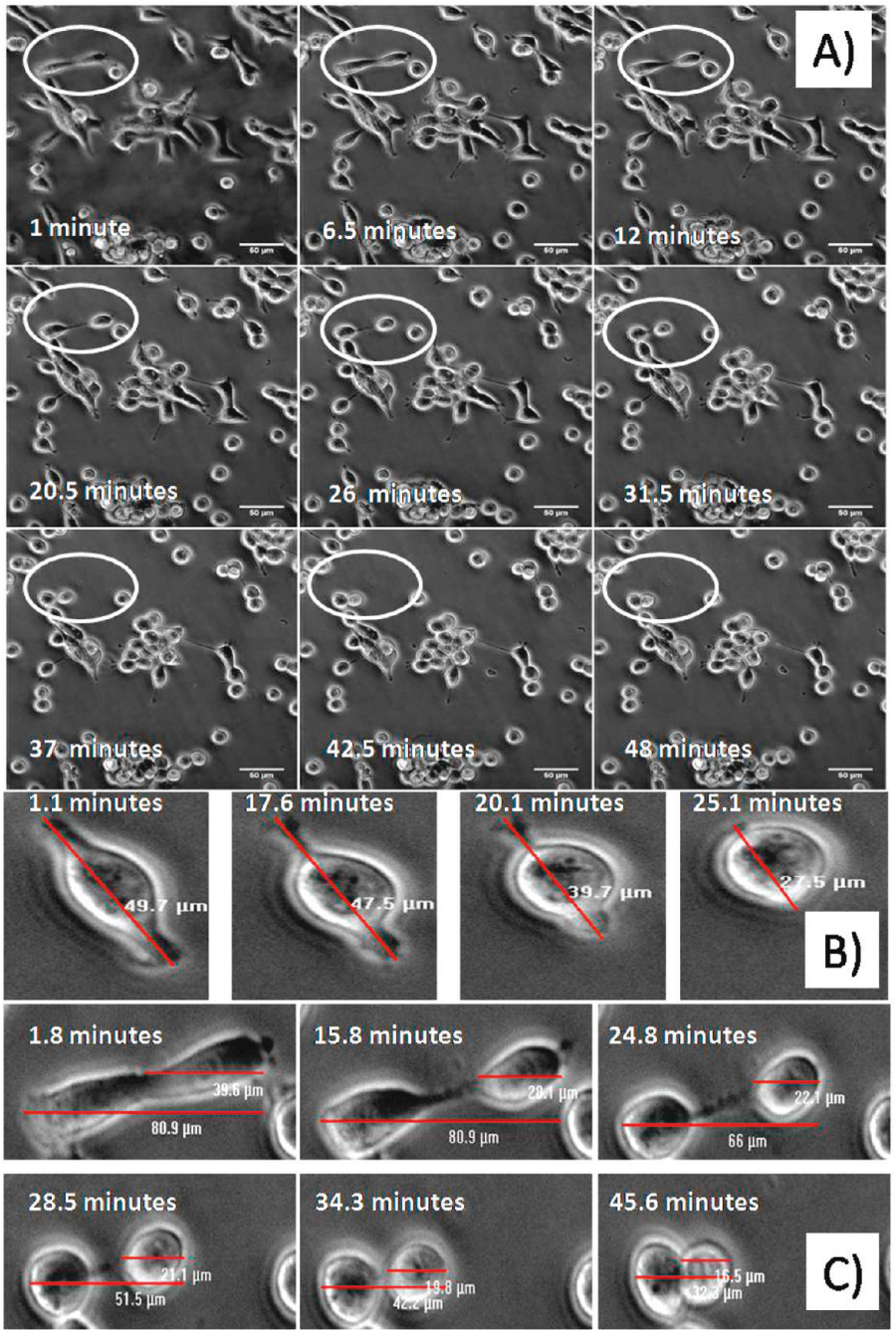

Figure 5. (A) Time lapse imaging sequence proceeding from left do right and downward. This sequential imaging technique tracked the transition of individual cells upon cold treatment from their flattened elongated form, through transformations in shape as the cell's elongated protrusions contract and a more rounded form begins to emerge, until complete detachment is achieved. Cold treatment was initiated approximately 1 min prior to imaging. The images were taken every 10 s over 50 min, with the representative 9 shown above covering this spectrum of this time. (B) Single cell retracting as it detaches from a spin coated pNIPAm substrate upon cold treatment. (C) Cell-doublet detaching from a spin coated pNIPAm film upon cold treatment. The cell-to-cell junction is maintained throughout the detachment process.

determinant for a successful cell growth and proliferation outcome. This approach will also aid in the refinement of the process parameters.

The initial dispensing volume, speed and time of; 150 μL at 150 rpm for 9 s were kept constant, and for all experiments the critical variables of final spin speed, final spin time and polymer concentration were 6000 rpm, 30 s, and 2% w/v pNIPAm in EtOH, respectively, except the variable that was under investigation, i.e., for each measurement, two of the variables were kept constant, while the other was investigated. Cells were seeded at a density of 40 000 cells/cm^2 and alamarBlue and PicoGreen assays performed 24 h after seeding. The relationship between polymer film thickness and concentration was found to be slightly nonlinear when investigated using profilometry, Figure 7.

The concentration of the polymer solution used and consequently the film thickness did not have a very significant effect on

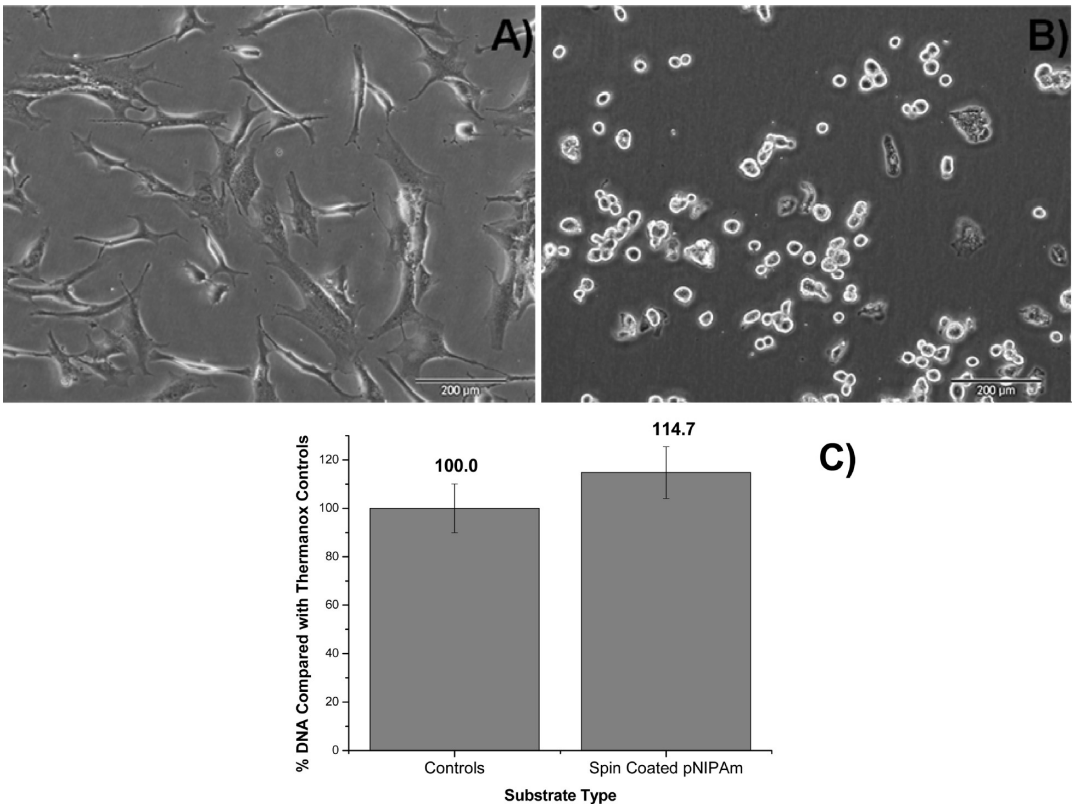

Figure 6. (A) hMSCs seeded and incubated on a pNIPAm spin-coated film for 24 h. Cells grew similar to cells seeded on Thermanox controls. (B) hMSCs completely detached upon cold treatment from the pNIPAm sample in 20−30 min. (C) PicoGreen total DNA assay of hMSCs grown on: Thermanox controls and spin coated pNIPAm films. Seeding cell density of 6 000cells/cm^2, incubation time 24 h. Error bars refer to standard deviation, where 3 separate samples of each type of substrate were used and assays were performed in triplicate.

Table 2. Summary Table of Immortalized Cell Lines Seeded and Recovered from Spin-Coated pNIPAm Samples[a]

cell line	cell type	cell proliferation[b]	cell detachment
C33A	cervical cancer	+++	5 min, 100%
CaSki	cervical cancer	+++	60 min, 30%
SiHa	cervical cancer	+++	5 min, 100%
A549	lung cancer	+++	5 min, 100%
HeLa	cervical cancer	+++	10 min, 100%
SW480	colorectal carcinoma	++−	5 min, 100%
HaCat	human keratinocytes	+−−	5 min, 100%

[a] Cell detachment initiated upon cold treatment. Proliferation is in comparison to TCP controls observed microscopically. Cell density 20 000/cm^2, incubation time 24 h. [b] '+++' indicates cell growth on par with positive controls, '++−' indicates that cell growth is moderately impeded compared to positive controls and '+−−' indicates that cell growth observed on the samples is less than 50% compared with positive controls.

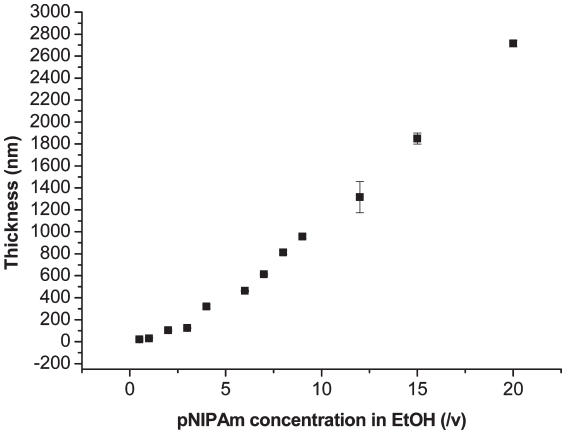

Figure 7. Profilometry results illustrate the near linear relationship between the depositing polymer solution concentrations and spin-coated film thickness.

cell compatibility, with cell growth similar to Thermanox controls in all cases, Figure 8A. The thickness range investigated was from under 30 nm to above 2500 nm. It was not possible to spin polymer concentrations of over 20% w/v as the solutions become too viscous to successfully produce a uniform film. Spin time or spin speed did not have a significant influence on film biocompatibility with the exception of films coated at the very low final spin speed of 300 rpm, panels B and C in Figure . Films spun at this low angular velocity are visibly wet once the spin-cycle is finished (unlike films spun at faster speeds which appear dry), as the spin speed is insufficient to expel the excess polymer solution from the side of the spinning substrate and the resultant films probably differ in their microstructure and surface properties. When films were spun at this low spin speed for the significantly longer time period of 10 min, the films still appeared wet and failed to host 3T3 cells to monolayer level which indicates that low final spin speeds are not suitable in the preparation of these surfaces. The results outlined in Figure 8 indicate that the

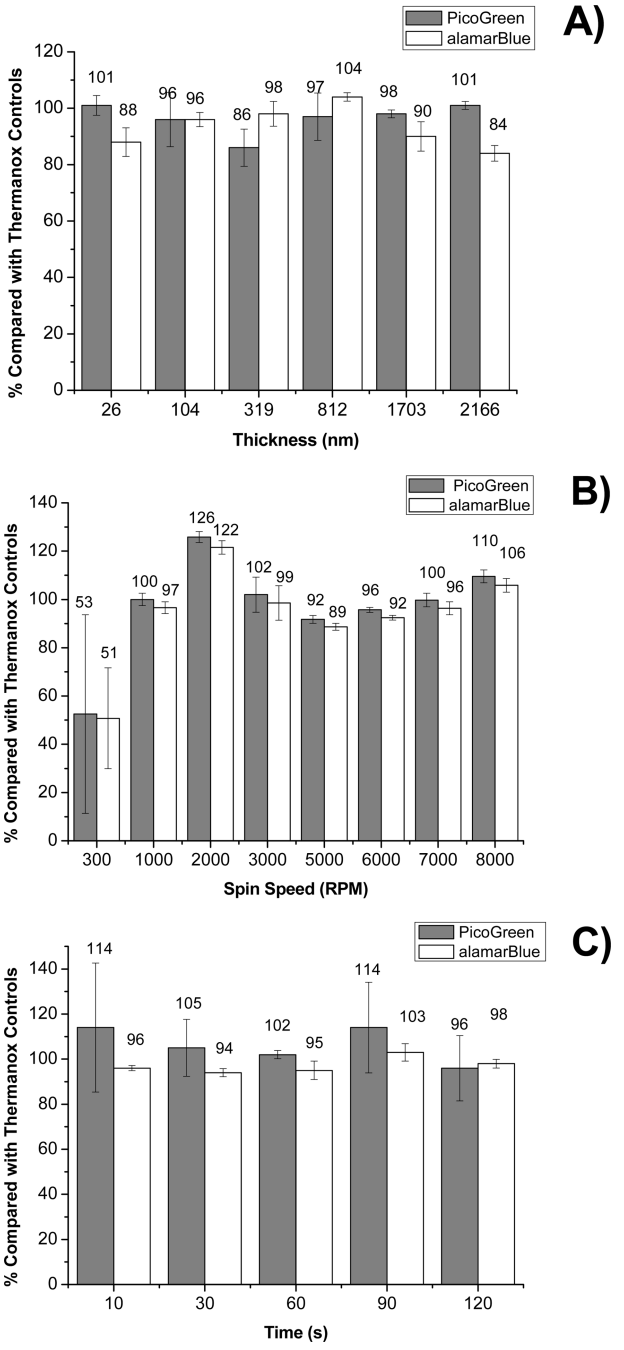

Figure 8. (A) PicoGreen and alamarBlue results of 3T3 cells seeded on pNIPAm spin coated films of increasing thickness from left to right (thickness values approximated from profilometry measurements of similar films deposited on quartz glass). (B) PicoGreen and alamarBlue assay results of 3T3 cells grown on films spun with differing angular velocities, i.e., spin speed increases from left to right. (C) PicoGreen and alamarBlue assay results of 3T3 cells grown on pNIPAm films spun for differing amounts of final spin time. Error bars refer to standard deviation where 3 separate samples of each type of substrate were used. Cell density 40 000 per cm^2, incubation time 24 h.

thickness of the spin coated pNIPAm films is not a limiting factor for successful cell adhesion and proliferation as is observed for other pNIPAm coatings prepared via grafting methods. Additionally, the results indicate that the film process parameters spin speed and spin time do not affect film bioadhesiveness, (with the exception of films spun at a very low spin speed) and therefore the preparation methods can be easily adjusted to optimize the time needed to prepare samples and minimize the amount of polymer product used. Significantly, it demonstrates that thermoresponsive platforms can be simply and routinely prepared using this technology and coupled with the use of a commercially sourced polymer this approach can be employed by laboratories with little or no expertise in polymer chemistry or film preparation techniques. Preliminary investigations into the mechanical properties of the films based on unpublished data suggest that there is not a significant difference in the Young's modulus of films with differing thicknesses. Future work will center on exploring this parameter further in the full range of thicknesses outlined.

CONCLUSIONS

We propose that this approach to fabricating thermoreponsive platforms with a view to cell harvesting is a significant simplification on alternate thermoresponsive dish fabrication techniques. The thickness of the thermoresponsive layer can be simply controlled by varying the spin speed, time, or polymer solution concentration. The fact that cells attached and grew on all spin coated films regardless of spin time, speed or polymer concentration, with the exception of films spun at very low speed, greatly simplifies the replication of this technique by other laboratories. For the retention of undamaged single or small groups of cells, it is necessary to seed a small number of cells initially and incubate for a short time span whereas a cell sheet can be garnered by seeding a high number of cells and incubating for a longer period until confluence is achieved; therefore, it is desirable that the cell seeding protocol is designed correctly to suit the desired purpose, i.e., the number of cells seeded and the incubation time.

A variety of cell lines successfully proliferated on the spin-coated pNIPAm films and detached upon temperature reduction but a slight cell line dependency for a successful outcome is evident.

Preprepared thermoresponsive dishes are now commercially available but are limited in their flexibility for laboratory use, i.e., they are available in limited sizes and on one type of substrate. By employing the protocol described here it is possible to coat much larger substrates easily and different types of substrates can be used. The use of commercially available pNIPAm and the spin-coating deposition technique means that this protocol can be used in any mainstream laboratory with a minimum of investment, as complex polymerization processes, which are expensive and need a high level of expertise, are avoided. This facile, accessible, and inexpensive temperature culture dish fabrication method holds much promise for cell and cell sheet recovery purposes.

ASSOCIATED CONTENT

Supporting Information. The following images have been included in the associated Supporting Information; AFM topography of spin-coated films, profilometry, AFM ,and SEM-3D-MeX images of the films thickness studies and the results of 3T3 cell reseeding experiments post-cell detachment from a spin-coated pNIPAm film. This material is available free of charge via the Internet at http://pubs.acs.org.

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REFERENCES

(1) Von Recum, H. A.; Okano, T.; Kim, S. W.; Bernstein, P. S. *Exp. Eye Res.* **1999**, *69*, 97–107.
(2) Yang, J.; Yamato, M.; Kohno, C.; Nishimoto, A.; Sekine, H.; Fukai, F.; Okano, T. *Biomaterials* **2005**, *26*, 6415–6422.
(3) Yamatoa, M.; Akiyamaa, Y.; Kobayashia, J.; Yanga, J.; Kikuchi, A.; Okano, T. *Prog. Polym. Sci.* **2007**, *32*, 1123–1133.
(4) Lim, Y. M.; Jeun, J. P.; Lee, Y. M.; Nho, Y. C. *J. Ind. Eng. Chem.* **2007**, *13*, 21–26.
(5) Cole, M. A.; Voelcker, N. H.; Thissen, H.; Griesser, H. J. *Biomaterials* **2008**, *30*, 1827–1850.
(6) Chen, B.; Xu, F. J.; Fang, N.; Neoh, K. G.; Kang, E. T.; Chen, W. N.; Chan, V. *Acta Biomater.* **2008**, *4*, 218–229.
(7) Okano, T.; Yamada, N.; Okuhara, M.; Sakai, H.; Sakurai, Y. *Biomaterials* **1995**, *16*, 297–303.
(8) Da Silva, R. M. P.; Mano, J. F.; Reis, R. L. *Trends Biotechnol.* **2007**, *25*, 577–583.
(9) Okano, T.; Yamada, N.; Sakai, H.; Sakurai, Y. *J. Biomed. Mater. Res., Part A* **1993**, *27*, 1243–1251.
(10) Shimizu, K.; Fujita, H.; Nagamori, E. *Biotechnol. Bioeng.* **2010**, *106*, 303–310.
(11) Matsuda, N.; Shimizu, T.; Yamato, M.; Okano, T. *Adv. Mater.* **2007**, *19*, 3089–3099.
(12) Kumar, A.; Srivatava, A.; Galaev, I. Y.; Mattiasson, B. *Prog. Polym. Sci.* **2007**, *32*, 1205–1237.
(13) Fujita, H.; Shimizu, K.; Nagamori, E. *Biotechnol. Bioeng.* **2009**, *103*, 370–377.
(14) Moran, M. T.; Carroll, W. M.; Gorelov, A.; Rochev, Y. *Journal of the Royal Society Interface* **2007**, *4*, 1151–1157.
(15) Takezawa, T.; Mori, Y.; Yoshizato, K. *Biotechnology* **1990**, *8*, 854–856.
(16) Reed, J. A.; Lucero, A. E.; Hu, S.; Ista, L. K.; Bore, M. T.; Lo pez, G. P.; Canavan, H. E. *ACS Appl. Mater. Interfaces* **2010**, *2*, 1048–1051.
(17) Moran, M. T.; Carroll, W. M.; Selezneva, I.; Gorelov, A.; Rochev, Y. *J. Biomed. Mater. Res., Part A* **2006**, *81A*, 870–876.
(18) Collett, J.; Crawford, A.; Hatton, P. V.; Geoghegan, M.; Rimmer, S. *J. R. Soc., Interface* **2004**, *4*, 117–126.
(19) Schmidt, S.; Zeiser, M.; Hellweg, T.; Duschl, C.; Fery, A.; Möhwald, H. *Adv. Funct. Mater.* **2010**, *20*, 3235–3243.
(20) Wischerhoff, E.; Uhlig, K.; Lankenau, A.; Börner, H. G.; Laschewsky, A.; Duschl, C.; Lutz, J. F. *Angew. Chem., Int. Ed.* **2008**, *47*, 5666–5668.
(21) Nagase, K.; Kobayashi, J.; Okano, T. *J. R. Soc., Interface* **2009**, *3*, 293–309.
(22) Fukumori, K.; Akiyama, Y.; Yamato, M.; Kobayashi, J.; Sakai, K.; Okano, T. *Acta Biomater.* **2009**, *5*, 470–476.
(23) Takahashi, H.; Nakayama, M.; Yamato, M.; Okano, T. *Biomacromolecules* **2010**, *11*, 1991−1999.
(24) Voytik-Harbin, S.; Brightman, A.; Waisner, B.; Lamar, C.; Badylak, S. *In Vitro Cell. Dev. Biol. Anim.*. **1998**, *34*, 239–246.
(25) Ahn, S.; Costa, J.; Emanuel, J. *Nucl. Acids Res.* **1996**, *24*, 2623–2625.
(26) Saitoh, T.; Suzuki, Y.; Hiraide, M. *Anal. Sci.* **2002**, *18*, 203–205.
(27) Wang, L. Q.; Tu, K.; Li, Y.; Zhang, J.; Jiang, L.; Zhang, Z. *React. Funct. Polym.* **2002**, *53*, 19–27.
(28) Harnett, E. M.; Alderman, J.; Wood, T. *Colloids Surf., B* **2006**, *55*, 90–97.
(29) Van Kooten, T. G.; Spijker, H. T.; Busscher, H. J. *Biomaterials* **2004**, *25*, 1735–1747.
(30) Liu, X.; Limb, J. Y.; Donahueb, H. J.; Dhurjatic, R.; Mastrod, A. M.; Vogler, E. A. *Biomaterials* **2007**, *28*, 4535–4550.
(31) Elbert, D. L.; Hubbell, A. *Annu. Rev. Mater. Sci.* **1996**, *26*, 365–294.
(32) Lee, J. H.; Khang, G.; Lee, J. W.; Lee, H. B. *J. Colloid Interface Sci.* **1998**, *205*, 323–330.
(33) Akiyama, Y.; Kikuchi, A.; Yamato, M.; Okano, T. *Langmuir* **2004**, *20*, 5506–5511.
(34) Cheng, X.; Canavan, H. E.; Stein, M. J.; Hull, J. R.; Kweskin, S. J.; Wagner, M. S.; Somorjai, G. A.; Castner, D.; Ratner, B. D. *Langmuir* **2005**, *21*, 7833–7841.
(35) Gilcreest, V. P.; Carroll, W. M.; Rochev, Y. A.; Blute, I.; Dawson, K. A.; Gorelov, A. V. *Langmuir* **2004**, *20*, 10138–10145.
(36) Saad, B.; Abu-Hijleh, G.; Suter, U. W. In *Introduction to Polymeric Biomaterials*; Arshady, R., Ed.; Citus Books: London, 2003; pp 263−299.
(37) Linhui, L.; Yang, Z.; Bo, L.; Changyou, G. *Langmuir* **2008**, *24*, 13632–13639.
(38) Hersel, U.; Dahmen, C.; Kessler, H. *Biomaterials* **2003**, *24*, 4385–4415.
(39) Kobayashi, J.; Okano, T. *Sci. Technol. Adv. Mater.* **2010**, *11*, 1–12.
(40) Elloumi-Hannachi, I.; Yamato, M.; Okano, T. *J. Intern. Med.* **2010**, *267*, 54–70.
(41) Von Recum, H. A.; Kim, S. W.; Kikuchi, A.; Okuhara, M.; Sakuri, Y.; Okano, T. *J. Biomed. Mater. Res., Part A* **1997**, *40*, 631–639.
(42) Xu, F. J.; Zhong, S.; Yung, L. Y. L.; Kang, E. T.; Neoh, K. G. *Biomacromolecules* **2004**, *5*, 2392–2403.
(43) Kikuchi, A.; O., T. *J. Controlled Release* **2005**, *101*, 69–84.
(44) Malonne, H.; Eeckman, F.; Fontaine, D.; Otto, A.; Vos, L. D.; Moes, A.; Fontaine, J.; Amighi, K. *Eur. J. Pharm. Biopharm.* **2005**, *61*, 188–194.
(45) Emslie, A. G.; Bonner, F. T.; Peck, L. G. *J. Appl. Phys.* **1956**, *29*, 858–862.