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Thermoresponsive Substrates used for the Expansion of Human Mesenchymal Stem Cells and the Preservation of Immunophenotype

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Abstract

The facile regeneration of undifferentiated human mesenchymal stem cells (hMSCs) from thermoresponsive surfaces facilitates the collection of stem cells avoiding the use of animal derived cell detachment agents commonly used in cell culture. This communication proposes a procedure to fabricate coatings from commercially available pNIPAm which is both affordable and a significant simplification on alternative approaches used elsewhere. Solvent casting was used to produce films in the micrometer range and successful cell adhesion and proliferation was highly dependent on the thickness of the coating produced with 1µm thick coatings supporting cells to confluence similar to conventional tissue culture plastic (TCP). 3T3 cell sheets and hMSCs were successfully detached from the cast coatings upon temperature reduction.

Furthermore, results indicate that the hMSCs remained undifferentiated as the surface receptor profile of hMSCs was not altered when cells were detached in this manner.

Keywords Thermoresponsive polymers; human mesenchymal stem cells; cell expansion and regeneration; flow cytometry; stem cell differentiation

Disclosures

The authors indicate no potential conflicts of interest.

Introduction

As evidenced by the literature, the generation of cells for the repair of damaged tissues using stem cell therapy holds much promise due to the pluripotent nature of stem cells. *For clinical and therapeutic purposes it is desirable to eliminate the use of any animal derived products such as trypsin which is routinely used in cell culture, and the use of thermoresponsive platforms capable of hosting and releasing stem cells offers an alternative route for stem cell regeneration. This is becoming increasingly desirable as the regulatory biomedical landscape suggests a shift away from the use of animal derived products and the evolution of new techniques that avoids their employment.*

A number of factors are capable of influencing or inducing stem cell differentiation including; cell-material interactions, material-surface properties such as chemical composition and energy and cell growth factors [1-2]. While the ability to differentiate MSCs holds the key to the promise of stem cell therapy, it is also important to be able to culture them for large scale applications in an undifferentiated state in a reproducible and reliable fashion so that they can be differentiated when desired. There has been extensive research on the use of biomaterials with a view to preserving and differentiating not only hMSCs, but also embryonic stem cells and hematopoietic stem cells [3]. The mostly extensively investigated biomaterials used for this

purpose are collagen, fibrinogen, hyaluronic acid, glycosaminoglycans (GAGs), hydroxyapatite (HA) in terms of natural materials and polyglycolic acid (PGA), polylactic acid (PLA), poly(ethylene glycol) (PEG) and the copolymer polylactide-co-glycolide (PLGA) in terms of synthetic materials. These can take the form of 2-D and 3-D scaffolds, microparticles, hydrogels and coatings *etc.* [3-5]. Modifications to the cell substrate used to guide hMSC differentiation *in vitro* indicate that -NH₂ and -SH modified surfaces promote osteogenesis and -OH and -COOH modified surfaces promote and maintain chondrogenesis [2]. Thermoresponsive polymers were also used for hMSC proliferation and multi-differentiation potentials, for example pNIPAm-grafted polydimethylsiloxane (PDMS) and NIPAm-based polyelectrolyte multilayer films, hMSC cells reserved their potential for differentiation and showed better viability on NIPAm based polymer films than that on the plates surface coated with gelatine [6-7]. When cells are detached from thermoresponsive polymer coatings the excreted extracellular matrix proteins are preserved which has been shown to facilitate the maintenance of important cell membrane proteins around rat bone marrow MSCs and human adipose tissue MSCs [8]. However, the film fabrication procedures are complicated and how the cells behave post detachment hasn't been reported.

With this in mind, the aim of the present study was to develop a simple method to produce pNIPAm coatings with a view to cell regeneration, particularly non-differentiated stem cell regeneration. Recently we published on the fabrication of ultra-thin poly-N-isopropylacrylamide (pNIPAm) based films for cell and cell sheet regeneration via the spin coating method [9-11]. In one of these studies commercially sourced polymer was used to produce pNIPAm solutions from which the films were prepared. A number of different cell lines including 3T3 fibroblasts and hMSCs were successfully grown under standard physiological conditions and were subsequently gently detached upon temperature reduction. [10]

Studies have shown that cells attach and therefore proliferate poorly on thick or bulk pNIPAm coatings and many studies report a coating thickness limitation above which there is a

dramatic reduction in the number of cells attaching to the polymer surface [12-16]. In studies where pNIPAm was covalently grafted for this purpose, this thickness limitation is in the order of tens of nanometers with the exception of coatings formed via plasma polymerization [12, 17-19]. Our previous studies using spin coated commercially sourced polymer found that there was no relationship between the thickness of the film produced and successful cell attachment and proliferation within the thickness-scale employed (> 30 nm to < 2000 nm) [10].

The grafting techniques used to produce thermoresponsive coatings are technologically expansive and economically expensive and many studies seek to investigate less complex and cheaper alternatives. Alternatively there are proprietary thermoresponsive plates available on the retail market but these are expensive and therefore impractical for routine cell culture use.

Our previous investigations include using cell adhesion promoting agents as an over layer on top of thick solvent cast pNIPAm films to improve cell adhesion as well as the aforementioned spin coating approach [10, 20]. This study seeks to offer a significant simplification on methods employed elsewhere by investigating the possible thickness limitation of films produced using the simple solvent casting method. Solvent casting is a very simple, routine and inexpensive technique used to produce polymer films mainly in the micrometer range where a solution of the polymer is prepared in a suitable solvent and is spread around the substrate surface evenly, after which it is dried to remove the solvent. Moreover, solvent casting is a common method used in cell culture protocols for example when depositing cell adhesion promoting layers such as fibronectin or poly-L-lysine and therefore is a method familiar to most cell culture operators making it a widely accessible approach for film production compared to methods used elsewhere [21-22]. Previous studies have shown that films produced in this manner are poor cell hosts compared to conventional tissue culture plastic (TCP) substrates but this study indicates that the thickness of the solvent cast films is critical to the cell response with cells attaching and proliferating on thin ($1\text{ }\mu\text{m}$) films similar to TCP with the numbers of cells attaching to films decreasing with increasing film thickness [23-24]. A schematic illustrating the

experimental design is given in figure 1. To further simplify the method; commercially sourced pNIPAm was used thus avoiding the expense, equipment and expertise needed for polymerization or grafting procedures which makes the method accessible to all laboratories where non-invasive cell or cell sheet detachment is desirable. While using non-grafting techniques to produce films may lack the elegance of the grafting techniques due to polymer dissolution upon temperature reduction, studies show that pNIPAm is not cytotoxic to cells [24-25]. We believe therefore, that this method provides a viable and affordable alternative for cell and cell sheet regeneration.

Figure 1: Schematic illustrating the simple strategy employed for cell sheet regeneration.

The initial model cell line used was 3T3 fibroblasts for preliminary proof of concept, followed by hMSCs which were characterized post detachment according to the guidelines and standards established by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy.

Experimental Section

Materials

pNIPAm (Mn 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, Fluorescence-activated cell sorting (FACS) buffer, phosphate buffer saline (PBS) with 2 %FCS and 0.01% sodium azide were purchased from Sigma Aldrich, phycoerythrin (PE) labeled CD19, CD334, CD45 from Biosciences and used as received. For stem cell culture non heat inactivated fetal bovine serum from Hyclone was used to supplement the growth media. 3T3 mouse embryo fibroblast-like cells were kindly provided by University College Cork, hMSCs were kindly provided by the Regenerative Medicine Institute (REMEDI) group in the National Centre for Biomedical Engineering Science (NCBES), NUI Galway. 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 and Film Characterization

Films were prepared using the solvent casting method from a 4% (w/v) of pNIPAm in EtOH on 35 mm Petri dishes, after which they were dried slowly overnight in an EtOH soaked atmosphere desiccator before drying completely in a vacuum oven set to 40 °C and 600 mBar overnight to ensure any residual solvent is eliminated. Films were sterilized under mild UV light for 2 h prior to cell culture experiments. For Fourier transform infrared spectroscopy (FTIR) analysis films

were deposited onto aluminum stubs. Samples were stored at room temperature and routinely used within a month of preparation.

Film thickness measurements were performed using the simple scratch test method. Dried polymer films prepared as described above and a sharp blade was used to scratch the surface of the polymer from the underlying substrate and atomic force microscopy (AFM) analysis was used to assess to z height difference between the underlayer and the polymer surface. 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 and a matrix of 512 x 512 data points along the x-y plane were analyzed in a single scan. Four 100 μm x 100 μm scans were recorded at a scan rate of 1 Hz on each ablated area to ensure statistical accuracy. The thickness of the films can also be predicted via a convenient formula, eq. 1

$$H = \frac{CV\rho}{S} \quad (\text{eq. 1})$$

Where H is the thickness of the final film; C, V and ρ are the concentration, volume and density of polymer solution, respectively; S is the surface area of the substrate upon which the film was coated on. As can be seen in Table 1 the predicted thickness values and the measured thickness values are in good agreement.

AFM was also used to assess the roughness of the deposited pNIPAm coatings using 10 μm x 10 μm scans. 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 X-ray photoelectron spectroscopy XPS spectra were acquired using the Hitachi FTIR-8300 in transmission mode and the AXIS 165 X-ray photoelectron spectrometer respectively.

Advancing contact angle measurements were performed using the advancing drop method on a home-built goniometer as previously described [10].

Cell Culture, Imaging Techniques and Flow Cytometry of detached hMSCs

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. When cells reached 80-90 % confluence, cells were harvested and used for reseeding or for experimentation where appropriate.

For experimentation, cells were seeded in triplicate at a density of 40,000 and 20,000 cells/cm² for 2 and 5 days on the pNIPAm films and on TCP controls in the cases of 3T3 and hMSC cells respectively. Incubation conditions were a humidified atmosphere of 95 % air and 5 % CO₂ 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 lower critical solution temperature (LCST). The metabolic activity of the cells grown on the prepared samples and the control was assessed using the alamarBlue assay. Total dsDNA content was also quantified using the Quant-iT PicoGreen dsDNA assay kit.

Cell proliferation and cell detachment was microscopically observed using an Olympus BX51 with Image Pro-Plus analysis system phase contrast microscope. To initiate cell detachment, the warm cell media was removed after which cold HBSS was added and the samples were then placed 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.

For flow cytometry analysis, cells were detached from the thermoresponsive films as described followed by gentle and repetitive pipetting to yield singular cells. Cells were detached from TCP controls via conventional trypsinization. The cells were stained with CD90 (also known

as Thy-1), CD73 (known as ecto 5' nucleotidase), CD105 (known as endoglin), CD19 (a marker of B cells that may also adhere to hMSCs in culture and remain vital through stromal interactions), CD34 (a primitive hematopoietic progenitor marker) and CD45 (a pan-leukocyte marker) antibodies, all PE-labeled, as per the manufacturer's instructions and were analyzed by the flow cytometry using BD FACSCanto from Bioscience and the results were analyzed by Flowjo™ software [26]. Additionally, half the cells detached from thermoresponsive surfaces were reseeded on TCP and incubated for a further 3 days after which the same marker expressions were checked to define the post-detachment affects. All FAC experiments were performed in triplicate and for positive controls hMSC cells were mixed with 10% CD45-positive haematopoietic cells (HC) before seeding on TCP to induce MSC differentiation and were incubated for 5 days.

Results and Discussion

The relative roughness of the films was also assessed via AFM analysis; the results of which can be seen in Table 2 and all of the films were assessed to be relatively smooth.

The relative hydrophilicity/hydrophobicity of the polymer films was measured by advancing contact angle above the polymer's transition temperature. Distinct stick/slip behavior was clearly evident as the water droplet interacts with the film surface. This type of behavior is described in detail by Gilcreest *et al.*. The stick angle is generally reported as close to the real contact angle of the films in cases where stick/slip behavior is observed, which in this case was an average of 68 ± 2 degrees for 1 μm films, 72 ± 1 degrees for 2 μm and 57 ± 1 degrees for 4 μm films from 500 s to 1800 s (allowing for the initial calibration of the advancing drop)[27]. While the stick angles of the 1 μm and 2 μm are relatively close in value the 4 μm films are significantly more hydrophilic. FTIR and XPS analysis confirmed film deposition. FTIR spectra displayed peaks at 1500 cm^{-1} , 3300 cm^{-1} , 3000 cm^{-1} , 1450 cm^{-1} , 1300 cm^{-1} , 1730 cm^{-1} corresponding to a N-H bending peak, a N-H stretching vibration, a C-H stretching vibration, a C-

H asymmetric stretching vibration, a C-H symmetric stretching peak and a C=O stretching peak respectively which correspond to the fingerprint peaks of pNIPAm. The stoichiometry of the NIPAm monomer is 75.0 % C, 12.5 % N and 12.5 % O and the elemental composition of the solvent cast pNIPAm coatings measured by XPS was 77.4 %, 11.8 % and 10.7 % for C, N and O respectively which is quite close to the monomer stoichiometry thus confirming pNIPAm film deposition.

3T3 cells and hMSCs were seeded on the cast pNIPAm films and observed microscopically for cell proliferation after 48 and 120 hours incubation respectively. 3T3 cells proliferated and grew on 1 μm films similar to conventional TCP controls but the numbers of cells adhering and proliferating reduced as the thickness of the cast films increased with a modest reduction in cell numbers observed on 2 μm films and a marked reduction in cell numbers on thicker 4 μm films. This was confirmed quantitatively via the PicoGreen dsDNA quantification assay with only 25 % of dsDNA compared to TCP controls, Figure 2(A). Metabolic activity alamarBlue assay results displayed a similar trend with the activity of cells on the 4 μm films only 15 % compared to controls, Figure 2(B), which suggests that not only did fewer cells adhere on the thicker substrates, but additionally their metabolic activity was reduced in comparison. hMSC cells were similarly cultured and cell growth was assessed quantitatively and a similar trend was observed for the thickest 4 μm films, but there was a more significant reduction in cell numbers on 2 μm thick films (in comparison to the 1 μm thick films) than in the case of 3T3 fibroblasts, figure 3(C). The alamarBlue assay results reflected this tendency also which suggests that the ideal thickness for cell growth may be cell line dependant, figure 2(D). Additionally these results indicate that while 1 μm thick films were capable of hosting hMSCs to confluence the proliferation is slower than on the TCP controls i.e. 74 % dsDNA compared with TCP controls. The reason why the films become increasingly cell repulsive with increasing film thickness is unclear though it is probably associated with the increased mobility of the polymer chains with increasing film thickness leading to increased hydration which is non-conductive to

protein adsorption which mediates cell attachment, this increased hydrophilicity for the thicker 4 μm thick films was analytically reflected in the advancing contact angle measurements.

Figure 2: (A) PicoGreen total dsDNA assay results comparing 3T3 dsDNA quantity on TCP controls and solvent cast films of differing thicknesses. (B) alamarBlue assay test results comparing 3T3 cell metabolic activity on TCP controls and solvent cast films of differing thicknesses. (C) PicoGreen total dsDNA assay results comparing hMSC dsDNA quantities on TCP controls and solvent cast films of differing thicknesses. (D) AlamarBlue assay test results comparing hMSC metabolic activity on TCP controls and solvent cast films of differing thicknesses. Assays were performed on the samples after 48 h incubation with an initial seeding cell density of 40,000 and 20,000 cells/cm² in the cases of 3T3 cells and hMSCs, respectively. Error bars refer to standard deviation where 3 separate samples of each type of substrate were used and assays were performed in triplicate.

3T3 cells were detached under cold treatment and cell to cell contact was maintained and full cell sheets were recovered within 15 minutes, Figures 3 and 4. hMSCs were similarly detached from the cast pNIPAm surfaces in less than 30 minutes, Figure 5.

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Figure 3: 3T3 cell sheet detaching from a 1 μ m cast pNIPAm film. Cell to cell junctions are maintained as the cell sheet lifts off. Cells began detaching after 1 minute with complete detachment achieved after 15 minutes. This montage follows the progression of cell detachment upon exposure to cold treatment; (A) 0min; (B) 2mins, (C) 4mins; (D) 5mins; (E) 6mins; (F) 8mins; (G) 10mins; (H) 12mins. Scan bar size 500 μ m.

Figure 4: Complete 3T3 cell sheet detached from a 1 μ m thick cast pNIPAm film. Scan bar size 200 μ m.

Figure 5: hMSC cell sheet detaching from a 1 μ m cast pNIPAm film. Cell to cell junctions are maintained as the cell sheet lifts off. Cells began detaching after 5 minutes with complete detachment achieved after 30 minutes. This montage follows the progression of cell detachment upon exposure to cold treatment; (A) 5min, (B) 6mins, (C) 7mins, (D) 9mins, (E) 11mins, (F) 13mins, (G) 15mins, (H) 18mins. Scan bar size 500 μ m.

As defined by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular therapy (ISCT); MSC cells must be plastic-adherent in standard cell culture conditions using tissue culture flasks. Second, ≥ 95 % of the MSC cell surface must express CD105, CD73 and CD90, as measured by flow cytometry. Additionally, these cells must lack the expression (≤ 2 % positive) of CD45, CD334, CD14 or CD11b, CD79 α or CD19 and HLA class II. Third, the cells must be able to differentiate to osteoblasts, adipocytes and chondroblasts under standard in vitro differentiating conditions [26]. With this in mind, we tested the cells for 'positive' cell markers CD73, CD90 and CD105 markers using fluorescence-activated cell sorting, and for 'negative markers' we tested for CD19, CD334, and CD45 markers, as a lack of expression would indicate that the polymer films did not induce hMSC differentiation. Furthermore, half of the detached cells were reseeded and incubated on TCP for 3 days after which the aforementioned marker's expression was checked to define the post-detachment affects. Prior to testing for the membrane markers, cells were detached from controls via conventional trypsinization, by temperature control from thermoresponsive films and again by trypsinization after reseeding and incubation for 3 days. In terms of the 'positive' markers the expression of all 3 was ≥ 95 % which again indicates that no hMSC differentiation had occurred in the cases of cells grown and detached from pNIPAm or the controls and moreover no post detachment affects occurred after reseeding and incubation, Table 3. Furthermore, the results indicated that in all cases the expression of all 3 'negative' cell membrane surface markers was ≤ 2 % positive, Table 3. As these markers are rarely expressed on MSC cells these results indicate

that no hMSC differentiation had occurred in the cases of cells grown and detached from pNIPAm or the controls, furthermore no post detachment effects occurred after reseeding and incubation.

To assess if this method of expansion and propagation is suitable for maintaining hMSCs in an undifferentiated state over serial passages, cells were seeded and detached as previously described from thermoresponsive surfaces and TCP controls, but this time the cells were reseeded and detached through 3 passages. After the final passage the validity pluripotency markers, as described by the ISCT, were assessed by facs canto as previously described. RESULTS
TO BE INCLUDED

As in the case of the dissociation enzyme trypsin, there are similar regulatory concerns surrounding the use of serum which is ubiquitously used to supplement cell culture media. Fetal bovine serum (FBS) is the most commonly used cell medium supplement which, as the name implies, is isolated from cow fetal blood and as such it is highly variable consisting of ill-defined bio-components leading to batch-to-batch inconsistencies. Such variability introduces contamination risks such as the inadvertent exposure to adventitious pathogens, which from a clinical application viewpoint is a risk that should be minimized or eradicated. Taking this into consideration and in light of the results attained using this system to this point, it was decided to seed and incubate hMSCs on 1 μ m pNIPAm films as before, but on this occasion in the absence of supplementary serum RESULTS TO BE INCLUDED

It is important to also highlight that the unimmobilized polymer dissolves upon temperature reduction and is released into the cell culture media. The toxicity of pNIPAm has been investigated by Malonne et al. in mice and their studies suggest no detectable toxicity after 28 days using the relatively high concentration of 2000mg/kg and studies by Takezawa et al. suggests that there are no cytotoxicity issues under cell culture conditions. While in this study the dissolved polymer did not seem to have any deleterious effect on the cells collected it is best to remove as much as possible via media exchange or/and centrifugation before reseeding or

further experimentation. While this did not seem to have any deleterious effect on the cells collected it is best to remove as much as possible via media exchange or/and centrifugation before reseeding or further experimentation.

Conclusions

Solvent cast films prepared from commercially sourced pNIPAm offer a convenient, simple and cheap alternative to produce thermoresponsive surfaces with a view to cell and cell sheet regeneration to methods developed elsewhere, with an optimal film thickness of 1 μm . As the thickness of the cast films increased the surfaces became less bioadhesive and the numbers of cells which attached and consequently proliferated declined. This trend was observed for both 3T3 fibroblast cells and the hMSCs, future work will centre on extending this method for use with a number of different cell lines to investigate if the same tendency is observed for multiple cell types. Cell sheet detachment of 3T3 and hMSC cells was achieved through simple temperature control. Most importantly, hMSC immunophenotypic surface profile FACS analysis indicated that thermoresponsive surfaces do not induce hMSC differentiation and therefore this protocol offers a gentle and non destructive approach for cell detachment therapies where the collection of undifferentiated MSCs is desirable. [COMMENT ON SERIAL PASSAGING AND XENO FREE](#) *Future work will expand on using this type of experimental system for multiple passage expansion in reproducible cell culture conditions with a view to establishing a method which would eliminate the use of any animal derived products (serum, trypsin). Such a protocol would satisfy regulatory safety requirements consistent with clinical compliancy which could be a significant step in the translation of this type of protocol toward practical clinical/therapeutic applications.* Such a protocol would satisfy regulatory safety requirements consistent with clinical compliancy which could be a significant step in the translation of this type of protocol toward practical clinical/therapeutic applications. Finally, this simple method of surface

preparation allows for the preparation of substrates of differing geometries and sizes which allows for greater flexibility in experimental design.

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Figure captions

Figure 1: Schematic illustrating the simple strategy employed for cell sheet regeneration.

Figure 2: (A) PicoGreen total dsDNA assay results comparing 3T3 dsDNA quantity on TCP controls and solvent cast films of differing thicknesses. (B) alamarBlue assay test results comparing 3T3 cell metabolic activity on TCP controls and solvent cast films of differing thicknesses. (C) PicoGreen total dsDNA assay results comparing hMSC dsDNA quantities on TCP controls and solvent cast films of differing thicknesses. (D) AlamarBlue assay test results comparing hMSC metabolic activity on TCP controls and solvent cast films of differing thicknesses. Assays were performed on the samples after 48 h incubation with an initial seeding cell density of 40,000 and 20,000 cells/cm² in the cases of 3T3 cells and hMSCs, respectively. Error bars refer to standard deviation where 3 separate samples of each type of substrate were used and assays were performed in triplicate.

Figure 3: *3T3 cell sheet detaching from a 1µm cast pNIPAm film. Cell to cell junctions are maintained as the cell sheet lifts off. Cells began detaching after 1 minute with complete detachment achieved after 15 minutes. This montage follows the progression of cell detachment upon exposure to cold treatment; (A) 0min; (B) 2mins, (C) 4mins; (D) 5mins; (E) 6mins; (F) 8mins; (G) 10mins; (H) 12mins. Scan bar size 500 µm.*

Figure 4: Complete 3T3 cell sheet detached from a 1µm thick cast pNIPAm film. Scan bar size 200µm.

Figure 5: *hMSC cell sheet detaching from a 1µm cast pNIPAm film. Cell to cell junctions are maintained as the cell sheet lifts off. Cells began detaching after 5 minutes with complete detachment achieved after 30 minutes. This montage follows the progression of cell detachment upon exposure to cold treatment; (A) 5min, (B) 6mins, (C) 7mins, (D) 9mins, (E) 11mins, (F) 13mins, (G) 15mins, (H) 18mins. Scan bar size 500 µm.*

Table 1: Predicted solvent cast film thickness versus thickness measured by AFM analysis.

Predicted Film Thickness (μm)	Measured Film Thickness (μm)
1	1.07 ± 0.07
2	2.04 ± 0.03
4	4.02 ± 0.04

Table 2: Solvent cast film RMS roughness as measured by AFM analysis. 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.

Film Thickness (μm)	RMS Roughness (nm)
1	16.01 ± 0.90
2	24.01 ± 4.88
4	28.85 ± 1.55

Table 3: hMSCs cell surface antigen expression as assessed by FACS

Surface	TCP	Positive Control	1 μm Film	2 μm Film	Post Film Detachment	
Marker	(Trypsinization)				1 μm	2 μm
CD73 (+)	$96.3 \pm 0.5\%$	n/a	$94.9 \pm 0.8\%$	$96.0 \pm 1.0\%$	$95.5 \pm 0.2\%$	$95.2 \pm 0.4\%$
CD90 (+)	$95.0 \pm 0.9\%$	n/a	$95.5 \pm 1.1\%$	$95.9 \pm 0.5\%$	$95.5 \pm 0.5\%$	$94.8 \pm 0.3\%$
CD105 (+)	$99.3 \pm 0.3\%$	n/a	$96.3 \pm 0.7\%$	$94.8 \pm 1.4\%$	$98.2 \pm 0.9\%$	$99.4 \pm 0.3\%$
CD19 (-)	$0.7 \pm 0.2\%$	n/a	$0.3 \pm 0.1\%$	$0.7 \pm 0.2\%$	$1.0 \pm 0.2\%$	$1.1 \pm 0.5\%$
CD34 (-)	$1.0 \pm 0.4\%$	n/a	$1.2 \pm 0.5\%$	$0.9 \pm 0.3\%$	$1.6 \pm 0.2\%$	$1.4 \pm 0.4\%$
CD45 (-)	$0.9 \pm 0.3\%$	$46.7\% \pm 1.9\%(+)$	$1.5 \pm 0.3\%$	$0.9 \pm 0.4\%$	$1.0 \pm 0.4\%$	$1.3 \pm 0.3\%$

+ Positive expression

- Negative expression or extremely low expression