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Upper Critical Solution Temperature Thermo-Responsive Polymer Brushes and a Mechanism for Controlled Cell Attachment

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We report the synthesis of thermo-responsive polymer brushes with Upper Critical Solution Temperature (UCST)-type behaviour on glass to provide a new means to control cell attachment. Thermoresponsive poly(*N*-acryloyl glycinamide)-*stat*-poly(*N*-phenylacrylamide) (PNAGAm-PNPhAm) brushes with three different monomer ratios were synthesized to give tunable phase transition temperatures (T_p) in solution. Surface energies of surface-grafted brushes of these polymers at 25, 32, 37 and 50 °C were calculated from contact angle measurements and atomic force microscopy (AFM) studies confirmed that these polymers were highly extended at temperatures close to T_p in physiologically-relevant media. Importantly, NIH-3T3 cells were attached on the collapsed PNAGAm-PNPhAm brush surface at 30 °C after 20 h incubation, while release of cells from the extended brushes was observed within 2 h after the culture temperature was switched to 37 °C. Furthermore, the changes in cell attachment followed changes in the Lewis base component of surface energy. The results indicate that, in contrast to the established paradigm of enhanced cell attachment to surfaces where polymers are above a Lower Critical Solution Temperature (LCST), these novel substrates enable detachment of cells from surfaces at temperatures above a UCST. In turn these responsive materials open new avenues for the use of polymer-modified surfaces to control cell attachment for applications in cell manufacture and regenerative medicine.

Introduction

Polymer brushes form an important class of functional surfaces, with numerous applications in the biomedical field.[1-5] Many of the reported polymer brushes so far have been developed for use as antifouling surfaces, due to the ability to tune polymer chemistry to resist short-term protein adsorption and subsequent cell adhesion.[6-9] Thermo-responsive polymer brushes have been considered as functional substrate modifiers as their surface wettability can be easily adjusted by changing temperature, enabling application in controlled cell adhesion and detachment.[10, 11] The most widely studied thermo-responsive polymer is poly(*N*-isopropylacrylamide) (PNiPAm), which exhibits a lower critical solution temperature (LCST) phase transition in water at 32 °C. This polymer has been prepared in the form of surface-displayed brushes which can be used to facilitate cell sheet generation by incubating cells at the polymer brush surface at physiological temperatures, followed by recovery of cells in increased numbers after a culture stage and subsequent cooling of the surfaces below the polymer phase transition temperature to enable cell detachment and recovery.[12-14] Thermoresponsive co-polymers from poly(ethyleneglycol)methacrylate monomers have also been attached to, or grown from, surfaces in the form of polymer brushes, allowing control of cell attachment and detachment at temperature close to 37 °C.[15-17] Similar approaches have been adopted to control cell attachment with polypeptide brushes based on L-alanine derivatives with LCST-type thermo-responsive behaviour, for which capture and release of MCF-7 cells over the temperature range from 37 °C to 20 °C was demonstrated.[18]

However, there has been relatively little work focused on polymer brushes with Upper Critical Solution Temperature (UCST)-type phase transition behaviour which might allow controlled cell attachment at temperatures below 37 °C but cell release above this temperature. The limited evaluation of UCST transitions in practical applications to date is probably because relatively few polymers exhibit UCST behaviour in aqueous media.[19-22] Poly(*N*-acryloyl glycinamide) (PNAGAm) and related materials (e.g. poly(N-acryloylasparaginamide), PNAAAm) have been the most studied reversible hydrogen bonding based UCST polymers, as a result of their phase transitions being adjustable to body temperature in ionic solutions.[20, 23] For biomedical applications, examples have been demonstrated for PNAGAm based hydrogels as thermoresponsive drug delivery systems,[24] but not for controllable cell culture and delivery.

Accordingly, we have investigated surfaces to which cells attach at temperatures below that in normal tissue but which detach promptly when the surrounding temperature is raised above the normal physiological value (37 °C). (Figure 1) UCST polymer brushes were therefore prepared by polymerizations from initiator-modified silicon surface via surface-initiated controlled radical polymerisation (SI-CRP).

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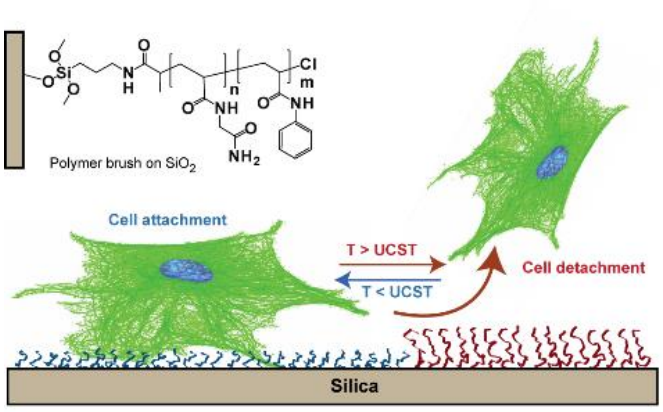

Figure 1. Cell attachment on UCST-type thermo-responsive polymer brushes at far below T_p, and detachment at close to/above T_p; The structure of the copolymer grafted to glass is also shown..

Experimental

Materials

Silicon wafers (10 ± 0.3 mm diameter, single side polished, thickness 525 ± 25 µm) were obtained from PI-KEM (UK). Circular glass coverslips (10 mm diameter) were obtained from Scientific Laboratory Supplies Ltd. (UK). Deionized water was obtained from an Elga Pure Nanopore 18.2 MΩ water purification system. All chemicals were analytical reagent grade and were used as received from the manufacturer. Glycinamide hydrochloride (≥99.0%), acryloyl chloride (96%), potassium carbonate (pure) and deuterium oxide (99.9%) were purchased from Sigma-Aldrich (UK). Diethyl ether, acetone, methanol and dichloromethane (DCM, HPLC grade) were obtained from Fisher Scientific (UK). 2-Chloropropionamide (98%), Copper(I) chloride (97%), copper(II) chloride (>99%), tris[2-(dimethylamino)ethyl]amine (Me6TREN) (97%), *N*-phenylacrylamide (PhAm) (99%) and methyl sulfoxide (DMSO) (≥99%) were purchased from Sigma-Aldrich (UK). 3-Aminopropyltriethoxysilane (APTES) (99%) was purchased from AcroSeal (UK). 2-Chloropropionyl chloride (CPC) (97%), triethylamine (99%) were purchased from Sigma-Aldrich (UK). Hydrogen peroxide (>30%), ammonium hydroxide (35%) and ethanol (99.8%, HPLC grade) were obtained from Fisher Scientific (UK). Ethylene glycol (99.8% anhydrous) was purchased from AcroSeal (UK). Diiodomethane (99%) and phosphate buffered saline (PBS) were obtained from Sigma-Aldrich (UK). Aqueous HCl and NaOH solutions were used to adjust the solution pH as desired.

Dulbecco's modified eagle media (DMEM), 10 % foetal calf serum (FCS), 1 % antibiotic/antimycotic solution, 1 % ι-glutamine (2 mM) and 1 % non-essential amino acids were purchased from Sigma Aldrich (UK). Presto blue cell viability reagent was obtained from Invitrogen. Trypan blue solution (0.4%, prepared in 0.81% sodium chloride and 0.06% potassium phosphate, dibasic) was purchased from Sigma Aldrich (UK).

Synthesis of *N*-acryloyl glycinamide (NAGAm)

Glycinamide hydrochloride (20 g, 180.9 mmol) and potassium carbonate (50 g, 361.9 mmol) were dissolved in deionized water (100 mL) in a 1 L three-necked round-bottom flask under stirring in an ice bath. Acryloyl chloride (15 mL, 177 mmol) in dry, cold diethyl ether (200 mL) was added dropwise under vigorous stirring over 30 min in ice bath, and kept stirring at r.t. for another 2 h. The solvents were then removed by rotary evaporation at 35 ^{0}C. The remaining aqueous liquid was removed by freeze drying. The crude brittle solid was extracted with acetone (400 mL) under stirring at 40 ^{0}C for at least 15 min. Insoluble potassium salts were filtered off and the acetone was removed by rotary evaporation at 35 ^{0}C. To remove small amounts of polymeric impurities, the solid was dissolved in warm methanol and dichloromethane (DCM) (v/v = 1/4, 250 mL) and filtered. The methanol was removed by rotary evaporation at 40 ^{0}C. The product was further purified by recrystallization from a mixture of methanol (60 mL) and acetone (120 mL) at -25 ^{o}C.

Preparation of UCST-type thermal responsive polymer brushes by Surface Initiated Atom Transfer Radical Polymerization (SI-CRP)

The glassware and substrates used were cleaned by immersion in 'piranha' solution (*Caution: -a highly reactive 3:7 mixture of hydrogen peroxide and concentrated sulfuric acid*) for at least 2 h, then washed with deionized water a couple of times and dried in 120 ^{o}C oven overnight. Silicon wafers (dia. 1 cm, x 28) were submerged by first washing with acetone, propan-2-ol and deionized water, and then immersed for 30 min in ammonia solution (56 mL), hydrogen peroxide solution (56 mL) and deionized water (184 mL) at 75 ^{o}C. The wafers were then thoroughly rinsed with deionized water and DCM, and dried under a stream of nitrogen gas before use.

3-Aminopropyltriethoxysilan (APTES) in ethanol (2.0 %, v/v) was aged for 5 min at r.t. The cleaned silicon wafers (dia. 1 cm, x 28) were immersed in the above solution for 30 min, then washed with ethanol, dried under a stream of nitrogen gas, and annealed for 30 min in 120 ^{o}C oven. Then, the wafers (diameter 1 cm, x 28) were immersed in a mixture of 2-chloropropionyl chloride (3.0 mmol, 0.30 mL) and trimethylamine (3.0 mmol, 0.42 mL) in DCM (60 mL) for 30 min. The wafers were then removed and washed with ethanol and DCM, and dried under a stream of nitrogen gas before use.

Initiator-modified silicon wafers or glass coverslips (dia. 1 cm) were placed in Schlenk tubes (one wafer in each tube, five reaction per time) in a Carousal 12 Reaction Station (Radley, UK), degassed through three vacuum/refill cycles with dry nitrogen gas, and filled with dry nitrogen (*Caution: do not overfill, these tubes are not suitable for high pressure reactions*). NAGAm (1.1 g, 8.59 mmol), PhAm (141.8 mg, 0.95 mmol), Me6TREN (22.7 mg, 0.10 mmol) and $CuCl_2$ (6.4 mg, 0.05 mmol) were dissolved in DMSO/D_2O (4:1, v/v, 11.0 mL) at 20 ^{o}C in a 25 mL two-necked round-bottom flask, and degassed with dry nitrogen gas for 30 min. CuCl (4.9 mg, 0.05 mmol) was then added to the above solution, and degassed for another 15 min. The SI-CRP of NAGAm and PhAm was then initiated by transferring the mixed solution (2.0 mL) into each Schlenk tube preheated to 45 ^{o}C. After 4 h, the reactions were quenched by

air, and the wafers were washed with deionized water, sonicated in methanol, and then dried under a stream of dry nitrogen gas.

Polymer dry brush thickness measured by ellipsometry

Ellipsometry measurements were performed with a J.A. Woollam Co. Alpha-SE spectroscopic ellipsometer at a wavelength range of 380–900 nm and 70^0 incident angle. A refractive index of 1.50 was assumed for dry PNAGAm-PPhAm brushes. Ellipsometric data were fitted to Cauchy model. Three measurements were recorded for each sample and the mean and standard deviation were determined in each case.

Characterization of polymer brush surface by X-Ray Photoelectron Spectroscopy (XPS)

XPS measurements were acquired on an Axis Ultra spectrometer (Kratos Analytical, UK) with a monochromatized Al Kα radiation operating at 150 W with an emission current of 8 mA. The base pressure was typically 10^{-8} to 10^{-10} mbar. The survey spectra for the determination of the element composition were recorded with a pass energy of 160 eV, and for the high resolution spectra that was 20 eV. The energy resolution was chosen to be 1.0 eV for survey spectra and 0.10 eV for high resolution spectra. The data were peak-fitted using CasaXPS software and all binding energies were referenced relative to the main hydrocarbon C1s signal centred at 285 eV.

Determination of surface energy of the polymer brush glass coverslips at different temperatures

Static contact angle using three different liquids water, ethylene glycol (EG) and diiodomethane (DIM) were measured on polymer brushes glass coverslips at 25, 37 and 50 ^{0}C using a CAM 200 Optical Contact Angle Meter (KSV Instruments Ltd, UK) fitted with a thermostated mental cell, connected to a refrigerated/heated bath circulator (Fisherbrand, UK) to maintain the temperature of the sample. Liquid surface energy at determined temperatures was also measured using the same instrument. At least three measurements were recorded for each sample and the mean and standard deviation were determined in each case.

Polymer brush thickness measured by Atomic Force Microscope (AFM) in air and PBS buffer (100 mmoL, pH 7.4) at 25 and 37 ^{0}C

AFM studies were carried out in Peak Force Tapping Mode using FastScan Dimension Icon AFM (Bruker, UK) equipped with digital instruments NanoScope Heater Controller (Veeco Metrology Group, USA). Silicon nanoprobes (Model: RTESPA) (Bruker, UK) with nominal force constants of 40 Nm^{-1} were used. Topographic imaging was performed in air, and also in PBS buffer (100 mM, pH 7.4). Prior to AFM measurements, the heater controller was set up to the determined temperature (25 or 37 ^{o}C). A water column was then generated by adding drops of PBS buffer (100 mM, pH 7.4) between the cantilever holder and the to-be-measured surface area, and left for at least 5 min to reach equilibrium. Brush heights are reported as the mean ± standard deviation.

3T3 Cell attachment (30 ^{0}C) and detachment (37 ^{0}C) on polymer brushes – Presto Blue (PB), Trypan Blue, and microscopy analysis

Polymer brush coverslips (diameter= 1 cm) and unmodified glass coverslips (the same diameter 1 cm, P-Ctrl) were placed in nTCP wells. Coverslips were sterilized with antibiotics for 20 min and dried in air before use. 1 x 10^5 Cells in 500 µL media were seeded in each well and incubated at 30 ^{0}C for 20 h. Standard curves of metabolic activity of cells on unmodified glass coverslips (diameter 1.5 cm) at both 30 ^{0}C and 37 ^{0}C were performed via Presto Blue assays. After 20 h incubation, the coverslips were transferred to new wells with 400 µL fresh media in each well and microscope images were taken. 100 µL of PB was then added to each well and incubated at 30 ^{0}C for another 2 h before read. After reading, the coverslips were incubated at 37 ^{0}C for another 2 h to release the cells. The viabilities of released cells in supernatant were tested with Trypan Blue assay. The coverslips were then transferred to new wells, and filled with 400 µL of fresh media in each well. The microscope images were taken at this time. 100 µL of PB was then added to each well and incubated at 37 ^{0}C for another 2 h before being read.

Proliferation of cells on Brush 3 and subsequent release

3T3 cells 1 x 10^5) were seeded on to sterilized Brush 3 coverslip and cultured at 30 °C for 5 days. Proliferation of cells on the Brush 3 surface was calculated based on Presto Blue measurements taken on day 1, 3 and 5 in comparison with the standard curve measurement at 30 °C. After culturing for 5 days, the cells were released by changing the temperature to 37 °C.

Release of cells as a monolayer cell sheet

3T3 cells (1 x 10^6) were seeded on to sterilized Brush 3 glass coverslips in 100 µl growth media and incubated at 30 °C overnight. After confirming the attachment of cells on the Brush 3 surface under the light microscope, 500 µl growth media was added and the cells were cultured for 24 hours. After this time, the culture plates were raised to 37 °C to release the cells from the brush surface. Viability of the released cell sheets was determined by staining with Live/Dead viability kit (Thermo Scientific) as per manufacturer's protocol. The cells were imaged under a fluorescence microscope and then viability was quantified using ImageJ software.

Results and discussion

For the UCST polymers we synthesised poly(*N*-acryloyl glycinamide) (pNAGAm) which shows UCST-type behaviour with a phase transition temperature (T_p) around 22 °C in aqueous solution,[22] and the resultant material was further characterized by ^{1}H-NMR, FT-IR and DSC (Figure S1-S3). The commercially available hydrophobic monomer *N*-phenylacrylamide (NPhAm) was copolymerized with NAGAm to increase the T_p of the polymer using conditions modified from prior literature for polymerisation of NAGAm.[22] Chloropropionamide (CPA) with CuCl/$CuCl_2$/Me_6TREN (molar ratio 1:1:1:2) was used as a

catalyst system due to its demonstrated high efficacy in polymerization of (meth)acrylamides.[25] The reaction temperature was maintained above the expected phase transition temperature of the (co-)polymers, and in all cases was kept at 45 °C to prevent any aggregation or chain-collapse of the polymer brushes. NAGAm concentration was fixed to be 0.78 M (10 wt %) in DMSO/diH_2O (4:1, v/v) mixed solvent to enhance the rate of polymerization and maintain control of molar mass dispersity (Tables S1 and S2). Kinetic studies of controlled polymerisation for PNAGAm-PNPhAm were carried out prior to the SI-CRP (Figure S4) and it was found that the polymer chain grew fast in the first hour, however, no significant change could be observed after reacting for 2 h.

Temperature-turbidity curves were obtained for the 3 polymer compositions in buffer solutions. As apparent from Figure 2a, the non-linear increase in transmittance, as a proxy for Upper Critical Solution Temperature, occurred at ~24, 28 and 34 °C for the PNAGAm, PNAGAm-PNPhAm (5%) and PNAGAm-PNPhAm (10 %), respectively. We subsequently prepared 2-chloropropionyl chloride modified silica surfaces,[7] which were used to grow the corresponding PNAGAm-PNPhAm brushes via SI-CRP under the above optimized condition (Scheme S1).

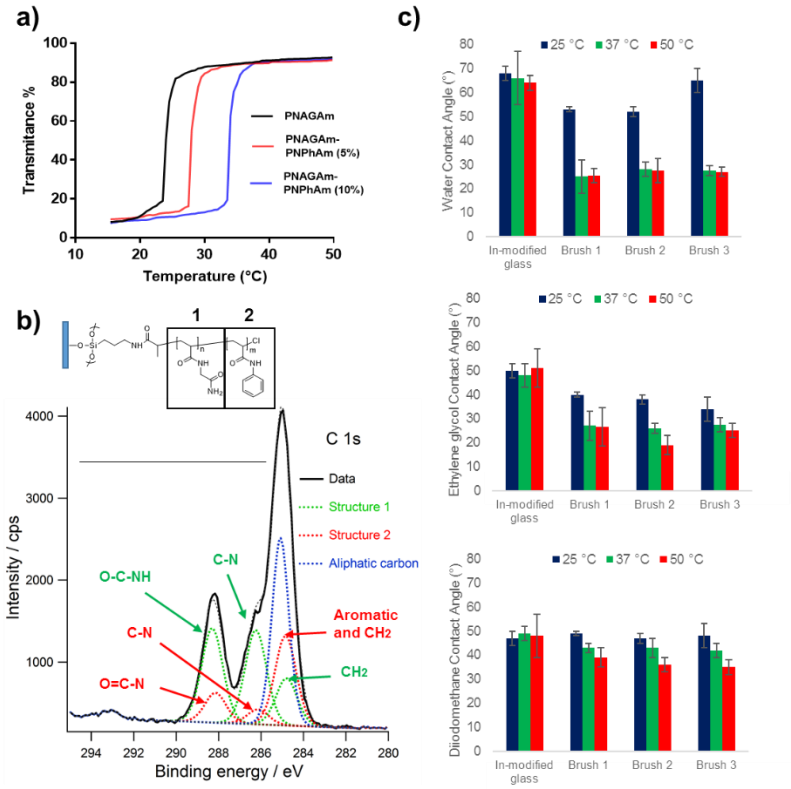

Figure 2. a) Temperature-turbidity curves for PNAGAm and PNAGAm-PNPhAm polymers in aqueous buffer solutions; b) High resolution X-ray photoelectron C 1S spectrum recorded for a PNAGAm-PNPhAm brush with [NAGAm]:[NPhAm]:[CuCl] feed ratio of 180:20:1; c) Contact Angles measured with water, ethylene glycol and diiodomethane on PNAGAm-PNPhAm brushes at 25, 37 and 50 °C; **Key** – In-glass = initiator-modified glass, Brush 1 =[NAGAm]/[NPhAm]/[CuCl] = 200:0:1 ; Brush 2 = [NAGAm]/[NPhAm]/[CuCl] 190:10:1; Brush 3 = [NAGAm]/[NPhAm]/[CuCl] 180:20:1.

Ellipsometric dry brush thicknesses of 22.0 ± 0.2 nm were achieved after 2 h, with a less rapid growth after this time.

High resolution C 1s XPS spectra were obtained for PNAGAm-PNPhAm brushes in order to confirm their chemical structure (Figure 2b). As two monomers were involved, XPS C 1s spectra were fitted with two models using three components for each model, which corresponded to O-C-NH, CN, CH_2 (aliphatic) for NAGAm (Structure **1**), and O=C-N, C-N, CH_2 (aliphatic/aromatic) for NPhAm (Structure **2**). Based on the fitted models, CH_2 for NAGAm (aliphatic) was found at a lower than expected level compared to that for NPhAm (aliphatic/aromatic). The actual molar ratio of NAGAm and NPhAm in polymer brushes was calculated to be lower than the feed molar ratio of 9 : 1, but the presence of underlying substrate peaks in the XPS spectra (Table S1, Figure S5, ESI) suggested that sampling was taking place further into the surface than just the polymer brush. Accordingly, we sought a direct method to evaluate the polymer brush surface behavior in liquids.

Water Contact Angle (WCA) measurements have been widely used to evaluate surface wettability, and surface energy (γ) is a key parameter for characterising the surface interaction with other materials, for example adhesion[26] and friction. We therefore decided to characterise the surfaces via determining the surface energy of these new polymer brush surfaces with three different monomer feed molar ratios: [NAGAm]/[NPhAm]/[CuCl] = 200:0:1 (Brush **1**), 190:10:1 (Brush

2), and 180:20:1 (Brush **3**). We used the method of van Oss *et al*.[27] in which the static contact angles of three liquid components (water, ethylene glycol (EG) and diiodomethane (DIM) in this study) were measured at the surfaces (Figure 2c). The contact angles of water on the three brushes were different from those on initiator-modified glass, and the extent of variation scaled with the monomer ratios in the brushes. Temperature dependent contact angle changes on the three brushes were found for all the three liquids, in contrast to the contact angles observed on the initiator-modified glass (In-glass). Table 1 lists the Liftshitz/van der Waals components calculated from the contact angle data.

Table 1. Surface Energy Data calculated for Three Polymer Brush Surfaces at Three Temperatures.

Surface	T (°C)	γ_S^{LW} (mJ/m²)	γ_S^+ (mJ/m²)	γ_S^- (mJ/m²)	γ_S (mJ/m²)
Brush **1**	25	31.88	0.45	27.94	39.00
	37	33.46	0.06	47.70	36.70
	50	32.82	0.01	49.65	33.86
Brush **2**	25	32.63	0.45	28.68	39.82
	37	33.69	0.04	46.18	36.49
	50	33.44	0.01	48.24	34.60
Brush **3**	25	32.18	1.79	10.19	40.72
	37	34.05	0.06	45.68	37.36
	50	33.86	0.00	47.83	33.94

γ_S: (total) surface energy of a solid; γ_S^{LW}: the Liftshitz/van der Waals component of a solid; γ_S^+: Lewis-acid component of a solid; γ_S^-: Lewis-base component of a solid.

The total surface energies (γ_S) for Brush **1** ranged from 39.00 to 33.86 mJ/m²; for Brush **2** from 39.82 to 34.60mJ/m², and for Brush **3** from 40.72 to 33.94 mJ/m² between 25 to 50 °C, indicating a slight decrease in overall surface energies with the increased temperatures.

As we expected from the analogous polymers prepared in solution (Figure 2a) that Brushes 2 and 3 would have phase transitions closest to physiological temperatures, we carried out additional screening of Brushes 2 and 3 with variable-temperature water contact angle studies. We also conducted additional surface energy component analysis at 32 °C, as we reasoned that Brush 2 would be just above its UCST transition at this temperature but Brush 3 would be just below. The results of these experiments are shown in Figure 3.

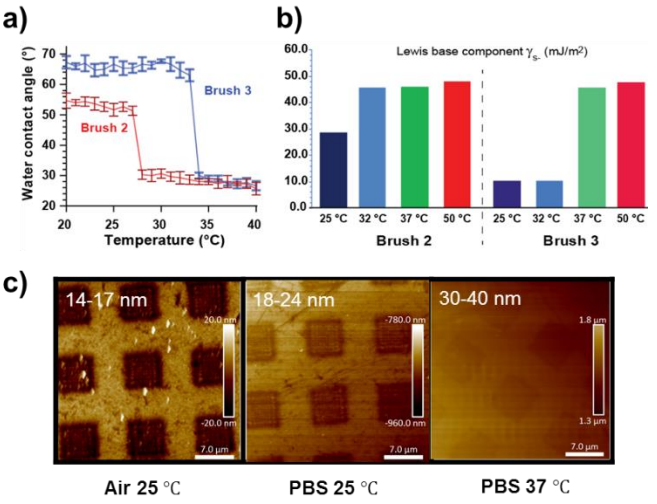

Figure 3 a) Water contact angles for Brush 2 and Brush 3 surfaces between 20 – 40°C; b) Lewis base component of surface energies for Brushes 2 and 3 across the phase transition temperature ranges; c) Peak force tapping-mode AFM images of patterned PNAGAm-PNPhAm brush surface in air at 25 °C and immerged in PBS at 25 and 37 °C. Numbers superimposed on images in (e) indicate height of polymer brushes.

As apparent from the contact angle data, Brush 2 showed a non-linear decrease in contact angle at ~ 28°C, while Brush 3 showed a similar transition at ~ 34 C. However, of most importance were the calculated changes in the Lewis basicity (γ_S^-) for these surfaces with temperature. The Lewis base component is a marker for the capacity to accept hydrogen bonds, and thus the increases in γ_S^- with temperature suggested that these surfaces gained more tightly-bound water. In turn, it might be expected that the enthalpic cost of species adsorbing to the polymer brushes and displacing the bound water would not be offset by entropic gain as water escaped to bulk solvent.[28, 29] As a consequence, the brushes were less likely to adsorb biopolymers and cells as the polymers chains expanded and acquired an associated bound water layer. As shown in Figure 3, a significant increase of Lewis basicity for Brush **2** took place between 25 and 32 °C, while for Brush **3** the value of γ_S^- changed markedly between 32 and 37 °C. These results indicated that polymer Brushes **1** and **2** exhibited phase transitions at temperatures lower than 32 °C, while Polymer Brush **3** retained a hydrophobic and collapsed status until the surrounding temperature reached between 32 and 37 °C.

The UCST-type phase transition of Brush **3** was further studied using atomic force microscopy (AFM) at varied temperatures in air and in PBS. In order to measure the brush thickness by AFM, patterned samples were produced via UV irradiation of initiator modified silicon wafers through a mask. In the exposed area, the C-Cl bond was removed from the initiator, but remained in the masked regions where the brushes could be grown by SI-CRP.[30] As shown in Figure 3c, AFM topographical images of PNAGAm-PNPhAm brushes on silicon wafer were achieved after SI- CRP. Significant height differences were observed between irradiated and non-irradiated regions. These height differences were quantified to evaluate the mean brush thickness. The dry brush thickness measured in air at 25 °C was 14-17 nm, which is

close to the 22 nm thickness evaluated by ellipsometry. When immersed in PBS (100 mM) at 25 °C, the brushes were slightly extended to a thickness of 24 nm, suggesting limited hydration of the brush layer below the UCST. However, on immersion of the brushes in PBS at 37 °C (just above T_p) the polymer chains extended to 30-40 nm (Figure 3cd), demonstrating the hydration of the expanded polymer layer.

The modified glass substrates were used to culture NIH-3T3 cells at 30 °C for 20 h. We observed that while cell attachment was low to Brushes 1 and 2, the NIH-3T3 cells attached and spread well on the Brush 3 (Figure 4a), implying that this surface was bio-adherent at ambient temperatures.

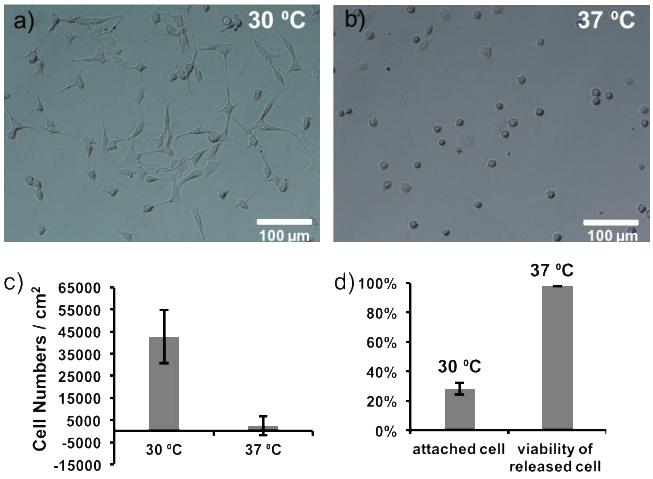

Figure 4. Phase contrast microscopy images of a) cell attachment at 30 °C and b) cell release at 37 °C; c) the cell density of Brush **3** at 30 and 37 °C; and d) the percentage of attached cells on Brush **3** at 30 °C as a proportion of the total seeded (left-hand bar) and the proportion of the cells released at 37 °C which retained metabolic activity (right-hand bar).

It should also be noted that Brushes 1 and 2 were not sufficiently cell adherent at either 25 °C or 37 °C for any differences to be significant, and incubations of cells for extended periods at temperatures above about 40 °C resulted in cell damage in culture media. Accordingly, we selected Brush 3 for more detailed study. By incubating Brush 3 with 3T3 cells for 20 h, i.e. before cell division started, we were able to quantify surface bound and released cells by a Presto Blue assay. The cell density on Brush **3** surfaces was 42650 ± 275 cm^{-2} (Figure 4c), while the percentage of attached cells (1 x 10^5 cells per well seeded) was 28.2 ± 4.1 % (Figure 4d). The relatively low cell loading efficiency may have been because the brush coverslip did not fully cover the well surface, and cells may have preferred to adhere on the underlying polystyrene rather than the modified surface. When the culture temperature was switched to 37 °C, the cells rounded up from the brushes within 2 h (Figure 4b), and were easily rinsed off by gentle washes with fresh culture media. We found that 94.3 ±10.0 % of the cells were released from the brush (2431 ± 227 cells cm^{-2} left, Figure 4c) on heating up to 37 °C, and 98.1 ± 0.03 % of the released cells were still viable (Figure 4d). These data indicated that the PNAGAm-PPhAm brush modified substrate was able to switch reversibly the attachment and release of cells between ambient temperatures (where the surfaces were 'cell-adhesive') and physiological temperatures ('cell releasing').

Subsequent experiments sought to evaluate if the change in surface properties could still modulate surface attachment over more practically relevant timescales.

3T3 cells were therefore cultured at the lower temperature supportive of attachment on Brush 3 (30 °C) for extended time periods (Figure 5a, b and c). Consistent growth of cells was observed during the culture period implying that Brush 3 surfaces provided stable substrata for attachment and supported cell proliferation. The doubling time of cells cultured on Brush 3 was 1.7 ± 0.1 days, which was slightly lower than the control cells cultured on glass coverslips at 30 °C (1.5 ± 0.3). This might be have been due to the overall reduction in metabolic activity (Figure 5g) typically observed in temperatures lower than normal body temperature.

After 5 days of culture, the cells were released by increasing the temperature to 37 °C (Figure 5 d, e and f). All of the cells were detached after 20 hrs at 37 °C without any external force of rinsing.

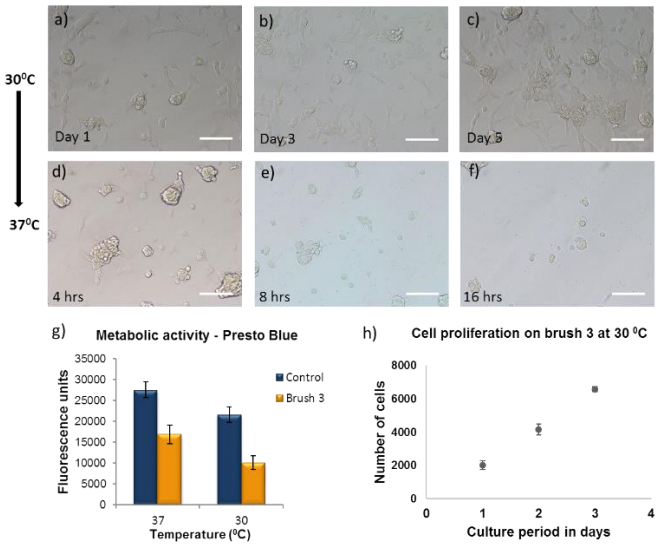

Figure 5. Phase contrast microscopy images of cells cultured on the Brush **3** at 30°C for 1 day (a), 3 days (b) and 5 days (c). The cultured cells were released at 37°C and imaged after 4 hours (d), 8 hours (e) and 16 hours (f). Scale = 50µm. (g) Metabolic activities of 3T3 cells (50,000 cells) cultured on Brush **3** and cell culture-treated plates (control) either at 30 °C or 37 °C for 24 hours. (h) Proliferation of cells cultured on Brush **3** at 30°C measured using presto blue after culturing for 5 days.

In order to form cell sheets on the coverslips, cells were seeded at high density and cultured for 24 hours at 30°C (Figure 6a and c). The cell sheet started to lift off after around 4 hours (Figure 6 b) and completely detached from the coverslip after 16 hours (Figure 6d). The released cell sheet was incubated with Live/Dead stain to check viability. Cells in the centre of the cell sheet showed slightly low viability (77 ± 4%) than on the edges (88 ± 4%). Average viability of the released cell sheet was 84 ± 4%.

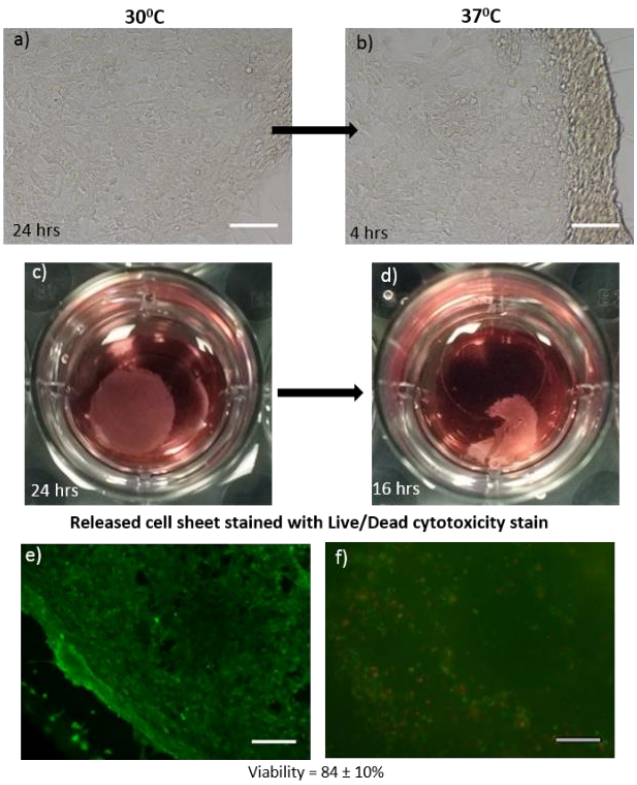

Figure 6. To release the cells as a sheet, 1 x 10^6 3T3 cells were seeded on Brush **3** overnight in 100µl of growth media at 30 ^{0}C and then cultured for 24 hours (a) and (c). Scale = 100µm. The cultured cell sheets were released at 37 ^{0}C (b) and (d). Representative fluorescence images of the released cell sheet stained with Live/Dead viability kit (e) and (f). Scale = 100µm.

The longer-term culture experiments thus demonstrated that the surfaces were able to function as 'reversible attachment' cell support matrices over timescales generally used for cell expansion. The utility of switchable attachment to polymer surfaces for cell culture has been extensively tested over recent years,[31, 32] but the ability to culture cells at lower temperature and then release the cells by heating to body temperature may offer advantages for more thermally sensitive cell types.

Overall, these results are significant also because they demonstrate a mechanism underlying the cell adhesion phenomenon. The change in the Lewis base (γ_S^-) component of these surfaces over a temperature range is an indicator of a change in the ability to accept H-bond donors, such as water, proteins or biopolymers. It has been noted before that bacterial attachment can be correlated, at least in the short term, on the ability of a surface to retain bound water, and the Lewis basicity has been shown to be predictive for the numbers of protein molecules or prokaryotic cells attached.[28] In prior studies, a reduction in Lewis basicity was shown to increase both bacterial and eukaryotic cell attachment for LCST polymers, however, to the best of our knowledge the related but 'inverse' phenomenon of increase in Lewis basicity above UCST has not been demonstrated to affect cell adhesion. The effects were most apparent for Brush 3, which exhibited the greatest overall change in Lewis basicity in pure solvents and the greatest difference between 32 °C and 37 °C: this surface also showed marked changes in cell attachment across this temperature range. The actual change in bound water content of the polymer layer may have taken place at a slightly lower temperature for the polymer brushes in cell culture media, as ionic associations with polymers leading to 'salting-in' can occur.[33, 34] Thus it is conceivable that the Brush 3 polymers chain-extended at a temperature slightly below 34 °C but above 32 °C in media, giving rise to the changes in cell attachment, whereas their Lewis base change occurred in pure solvent just above 34 °C as indicated in Figure 3. Nevertheless, the changes in surface properties around the UCST clearly affected cell adhesion, and the extension of this promising methodology to other cell types is now ongoing in our laboratories. The possibility to modulate further UCST-type changes not only by monomer content but by polymer chain length[35] and/or grafting density at surfaces, as has been demonstrated for LCST-type polymers,[36] offers further means by which cell attachment and behaviour might be controlled.

Conclusions

In conclusion, these new UCST-type thermo-responsive PNAGAm-PNPhAm brushes with adjustable phase transition temperatures allow controlled cell attachment at their lower T_p temperature, and release of cells by heating up to physiological temperature (37 °C). These substrates may allow new ways in which cells can be reversibly associated with surfaces, but with a raised temperature-induced release, rather than a temperature-triggered attachment. In turn, this process should enable modification of carrier surfaces for cell culture and delivery, and placement of cells in externally-addressable dynamic regions for tissue engineering.

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Data access statement

All raw data created during this research are openly available from the corresponding author (cameron.alexander@nottingham.ac.uk) and at the University of Nottingham Research Data Management Repository (https://rdmc.nottingham.ac.uk/) and all analysed data supporting this study are provided as supplementary information accompanying this paper.

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