

Controlled surface initiated polymerization of *N*-isopropylacrylamide from polycaprolactone substrates for regulating cell attachment and detachment

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Abstract: Poly(ϵ -caprolactone) (PCL) substrates were modified with thermoresponsive poly(*N*-isopropylacrylamide) (PNIPAM) brushes to direct and control cellular attachment and detachment. Prior to brush growth, the surface of PCL was activated by a diamine to allow for initiator coupling. Infra-red spectra taken before and after cell culturing demonstrated the covalently attached nature of the PNIPAM brushes. PCL is a biocompatible polymer and to prove that the modifications described above did not change this characteristic property, a cell attachment / detachment study was carried out.

Keywords: polycaprolactone • poly(*N*-isopropylacrylamide) • ATRP • polymer brushes • cell response

The modified substrates showed a lower cell attachment when compared to PCL alone and to PCL films modified with the initiator. The possibility to detach the cells in the form of a sheet was proved using PNIPAM-modified PCL films by lowering the temperature to 25°C. No relevant detachment was shown by the unmodified or by the initiator modified surfaces. This confirmed that the detachment was temperature dependent and not connected to other factors such as polymer swelling. These functionalized polymeric films can find applications as smart cell culture systems in regenerative medicine applications.

1. Introduction

Atom Transfer Radical Polymerization (ATRP), pioneered by Krzysztof Matyjaszewski, is one of the most successful methods to create polymer brushes with control over molar mass and molar mass distributions.^[1-8] The discovery of ATRP initiated more and more research activities in many different areas in the field of polymer technology, ranging from understanding the underlying mechanism and kinetics of ATRP,^[1-2] and the surface-initiated polymerization of polymer brushes,^[9-12] to the growth and use of thermoresponsive polymers.^[13-14] In recent years, studies of cells and bacteria behavior on polymers grown by ATRP were also performed to control cell adhesion, proliferation, migration and growth.^[15-19] Surface-initiated ATRP was used with great success to obtain designed surfaces exhibiting coatings with thicknesses in the nanoscale with targeted and controlled properties.^[1-4] Gold and silicon were most often employed as substrates for polymerization^[3-4, 20-28], although tissue culture polystyrene (TCPS)^[29-31] or titanium^[32-36] were also investigated as substrates for biomedical applications. In the specific case of polymer-based scaffolds for tissue engineering, the use of biocompatible polymer substrates, such as poly(ϵ -caprolactone) (PCL) or other polyester based polymers, is desirable.^[37-39] In these settings, the aim of this work is to provide a comprehensive introduction to the latest literature regarding cell behavior on different types of polymer brushes obtained by ATRP, e.g. non-biofouling or cell adhesive brushes, combined with new results obtained

by us on PCL films modified by thermoresponsive poly(*N*-isopropylacrylamide) (PNIPAM) brushes.

Non-biofouling or cell adhesive surfaces derived from functionalization with polymer brushes are an advancing research field, which can be used for biomedical implants, biosensors, and as carriers for targeted drug delivery.^[40-42] These surfaces are based on minimizing the intermolecular interaction forces between the extracellular matrix and the surface,^[41] thereby facilitating removal of potentially adherent cells. The most frequently studied non-biofouling polymers obtained by ATRP are polyethylene glycol (PEG) based^[4-5, 43] or zwitterionic brushes.^[24, 44] The hydrophilic nature of these types of brushes creates a hydration layer onto the surface that prevents protein adsorption and consequently cell adhesion. Therefore, these brushes are

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often used on surfaces that require the prevention of non-specific adhesion of proteins, e.g surface-based diagnostic devices or implanted biomaterials.^[44-46] Non-specific protein adsorption onto these surfaces can otherwise trigger undesirable events.^[5] In the case of PEG and other hydrophilic polymers, a hydration layer is formed due to hydrogen bonding with water. In the case of zwitterionic brushes, hydration occurs as a result of electrostatic forces.^[5, 44] Other polymers used for growing brushes can be either cell adhesive such as poly(methacrylic acid) (PMAA) or non biofouling like poly(2-hydroxyethyl methacrylate) (PHEMA) and poly(poly(ethylene glycol) methacrylate) (PPEGMA). This behavior depends on the relative hydrophobicity of the polymer. However, to further tune the cellular adhesion and behavior the acrylate groups in these polymers are often used to modify the brushes with peptides or proteins.^[4, 47] Peptides and proteins that are investigated for their cell adhesive properties include RGD (*Arginine-Glycine-Aspartic Acid*) or RGD containing sequences (e.g. GRGD, RGDS, GRGDS or fibronectin), collagen and collagen-mimetic peptides like GFOGER (*Glycine-Phenylalanine-Hydroxyproline-Glycine-Glutamate-Arginine*).^[4, 29-30, 36, 43, 48-50] An example is the modification of PMAA with RGD investigated by Navarro *et al.*^[51] They showed that cells morphology was dependent on the position of the RGD on the brush. When RGD was coupled on the top surface of the PMAA brush, cells spread well with marked focal adhesion points at the periphery of the cytoplasm.^[51] However, if the RGD was attached to the middle section of the PMAA brush, cells were found to adopt a rounded morphology and focal adhesions concentrated toward the internal part of the cell.^[51]

Besides the already mentioned non-biofouling and cell adhesive brushes, there are also brushes that can be used to tune the cellular behavior, for instance by employing thermoresponsive polymers such as, PNIPAM^[4-5, 52-56] and poly(2-(2-methoxyethoxy)ethyl methacrylate-co-oligo(ethylene glycol) methacrylate) (P(MEO₂MA-co-OEGMA)).^[52-54, 57-63] These two polymers trigger a different cellular response above and below their critical solution temperature (LCST) due to a change in chain conformation and variations in intra- and intermolecular interactions around the LCST in these polymers. Above the LCST, the polymer assumes a dehydrated collapsed and more hydrophobic state resulting in a cell adhesive brush. When lowering the temperature below the LCST, these polymers assume a highly hydrophilic state resulting in swelling and non-biofouling. The effect of LCST has been well studied and explained in terms of cell detachment and cell sheet harvesting from different substrates (e.g silicon, glass or TCPS).^[27, 52, 64-71] Cell sheet harvesting is a branch within the tissue engineering field, in which cells are expanded at 37 °C on top of PNIPAM grafted culture dishes.^[52, 69] After reaching confluency, cells are harvested as intact sheets by simply reducing the temperature to below the LCST. The

normal procedure for detaching cells from a culture plate, or any other surface that serves as substrate for cell growth, is based on the use of a scraper or an enzyme able to digest the proteins produced by the cells during proliferation (e.g. trypsin). Both techniques lead to a loss of cells. During cell scraping, part of the cells are at risk of being destroyed due to the mechanical stress applied to them by the scraper. Enzymes such as trypsin digest surface proteins, resulting in a release of cells from the surface and from each other. Additionally, this enzymatic digestion is not specific, thus all cell surface proteins are affected. Evidence for trypsin-induced changes in cultured cells has been provided for keratinocytes (Umegaki *et al.* 2004),^[72] epithelial (Reiners *et al.* 2000)^[73] and endothelial cells (Lopes *et al.* 2001).^[74] The use of PNIPAM as a harvesting technique gives the advantage of not affecting cell integrity and, as previously mentioned, the possibility to collect the cells in a ready to use form.

In 1995, Okano *et al.*^[75] proposed a mechanism for cell detachment on PNIPAM surfaces upon lowering the working temperature below LCST. It was shown that the metabolism of the cultured cells plays an important role in the detachment. By lowering the temperature to 20°C, PNIPAM becomes hydrated and cell metabolic activity is correspondingly decreased. A subsequent increase of the temperature to an optimal value but still below the LCST, will enhance the detachment as a result of increased cell metabolic activity. As the cellular metabolism is different for every cell type, this optimum temperature will change, as well. Later, other researchers also studied the detachment mechanism of cells on thermoresponsive brushes.^[61, 64, 76] Using Total Internal Reflection Fluorescence (TIRF) microscopy, Uhlig *et al.*^[61] confirmed that the detachment mechanism was cell type dependent and that cells play an active role in this mechanism. Cooperstein *et al.* also discussed this mechanism.^[64] They proposed a two-step detachment mechanism from PNIPAM brushes. Their model includes a passive step involving the hydration of polymer chains and an active step involving cell shape change and detachment from the surface driven by cytoskeletal action and metabolic processes.

The attachment and detachment of a cell from a thermoresponsive substrate is not only affected by the type of cell, but also by the length and the grafting density of the polymer brush. Both the length and density of brushes determine the extent and rate of hydration. For example, Xu *et al.* grew PNIPAM brushes on silicon substrates with a variable length between 3 and 31 nm and showed that thicker brushes resulted in enhanced fibroblast attachment and growth.^[77] However, Akigama *et al.*^[78] found an optimum brush length around 20 nm for bovine endothelial cells when PNIPAM brushes were grown on TCPS. This difference in results might be due to the more hydrophobic nature of TCPS that enhances the hydration.^[78] Mizutani *et al.*^[31] showed a decrease in endothelial cell attachment for

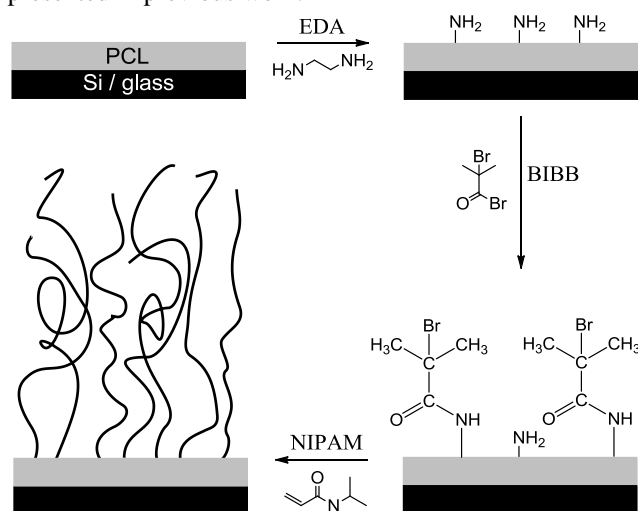
PNIPAM brushes grown on TCPS. When the brush length exceeded 60 nm, cell adhesion was negligible. PNIPAM grown on glass substrates showed an optimal cell attachment and detachment of fibroblasts with brush length estimated to 11-13 nm.^[79] These studies all reported that cell attachment occurred at short brush lengths. However, Sui *et al.*^[80] has recently shown that cell attachment can occur for brush lengths up to 220 nm. Whereas low density polymer brush with a dry thickness of ~ 10 nm showed the highest cell adhesion with elongated morphology, increasing the brush density and dry thickness resulted in decreased cell density and elongation. A general consensus on the effect of brush length on cell adhesion is still missing. The results we obtained suggest a way to modulate cell adhesion and shape by changing the length of the brushes and opening new application of this surface modification.

PNIPAM brushes were also modified with different polymers and peptides to influence cell attachment and detachment. Ebara *et al.* modified PNIPAM with acrylic acid (Aac) and 2-carboxyisopropylacrylamide (CIPAM) to introduce a functional carboxylic acid group.^[81] It was shown that Aac moieties shifted the LCST to a temperature higher than 37 °C while CIPAM kept the LCST around 32 °C, thus below the physiological temperature. In a later study, Ebara modified this carboxylic acid with a variety of peptides (RGD, RGDS, GRGD, GRGDS).^[29-30] The cell densities of either bovine aortic endothelial cells^[29] or human umbilical vein endothelial cells^[30] increased according to the trend: RGD < GRGD < RGDS < GRGDS. This trend was related to the relatively higher biospecificity to integrins of longer peptide sequences.^[30] Xu *et al.* copolymerized PNIPAM with poly(ethylene glycol) monomethacrylate (PEGMA) to tune the amount of attached cells and the detachment rate.^[77] They showed that by increasing the PEGMA amount from 0 to 1%, cell attachment and detachment rates were significantly decreased. Kong *et al.*^[79] showed that modification of PNIPAM with Poly((3-(methacryloylamino)propyl)-dimethyl(3-sulfopropyl)-ammonium hydroxide) (PMPDSAH) resulted in an optimal attachment and detachment of fibroblasts on films with thickness of 3–4 nm approximately and a 75:1 mole ratio of NIPAAm and MPDSAH.

As mentioned previously, the effect of brushes on cell behavior has been well studied. However, polymeric scaffolds have seldom been used as substrate.^[37-39, 82-83] The possibility to modify polymeric materials with brushes in order to improve their properties, is an appealing strategy to expand their applications in different fields of tissue engineering. Therefore, in this study the thermoresponsive effect of PNIPAM grown from PCL substrates with respect to film and cell behavior was investigated.

2. Results and discussion

PCL films were prepared by spin-coating a 5 wt% polymer solution onto silicon or glass substrates with a thickness in the order of 1 μm. After spin-coating, the PCL films were annealed to release the stress in the film. The spin-coated PCL film was subsequently modified in three steps with PNIPAM brushes, as shown in figure 1. First, PCL was aminolysed with ethylenediamine (EDA), after which the ATRP initiator (BIBB) was attached to the film. NIPAM was subsequently polymerized according to a procedure presented in previous work.^[80]



Scheme 1. Reaction diagram for the modification of PCL films using aminolysis and surface-initiated-ATRP of NIPAM to produce surface tethered PNIPAM brushes.

The roughness of the modified substrates was determined at each different step by capturing Atomic Force Microscopy (AFM) images (data not shown). AFM is a powerful analytical tool for both the characterization and the fabrication of polymer brush structures. The application of AFM in the field of polymer brushes is described in detail by Sui *et al.*^[84] From these AFM images it was clear that the roughness did not change significantly by modifying PCL with either EDA, the initiator or PNIPAM brushes. This implied that modification of PCL with EDA - i.e. breaking the ester bonds of the polymer chains - was sufficient enough for the formation of polymer brushes without introducing significant surface roughness.

The presence of PNIPAM on the surface of the PCL films was also confirmed by Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR) measurements (fig. 2). The spectra of unmodified PCL clearly showed the characteristic ester peak at 1721 cm⁻¹. After the modification with PNIPAM, this peak was significantly reduced and the two characteristic amide peaks at 1637 cm⁻¹ and 1535 cm⁻¹ of PNIPAM were present. The spectra after cell culturing showed the same characteristic peaks for unmodified PCL as well as for PNIPAM, indicating the stable and covalent attachment of PNIPAM to the PCL film. However, ATR-FTIR spectra of PCL films modified with either EDA or the initiator did not show any significant difference, due to

modification of only a thin surface layer. After subjecting these two films to the polymerization mixture, no amide peaks were shown in the FTIR spectra, indicating that PNIPAM could have only grown due to the coupling of initiator to the PCL films by the EDA modification. Also static contact angle measurements on these samples showed a significant decrease in contact angle values from 83° for unmodified PCL to 69° for PCL modified with EDA, due to the formation of extra amine and alcohol groups on the surface.

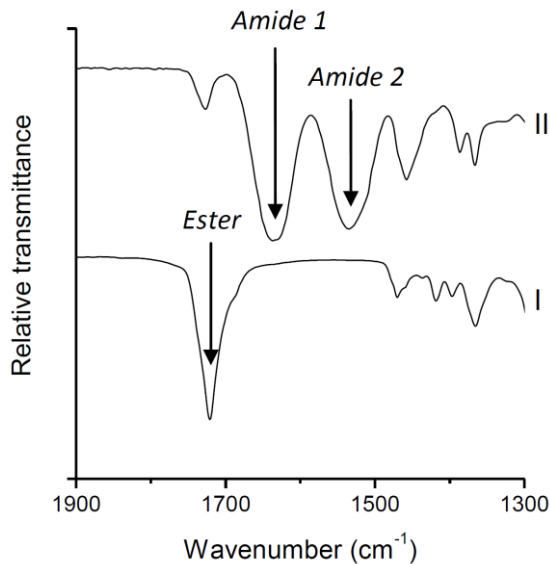


Figure 2. ATR-FTIR analysis before cell culturing of pure (I) and PNIPAM (II) modified free-standing PCL films, respectively.

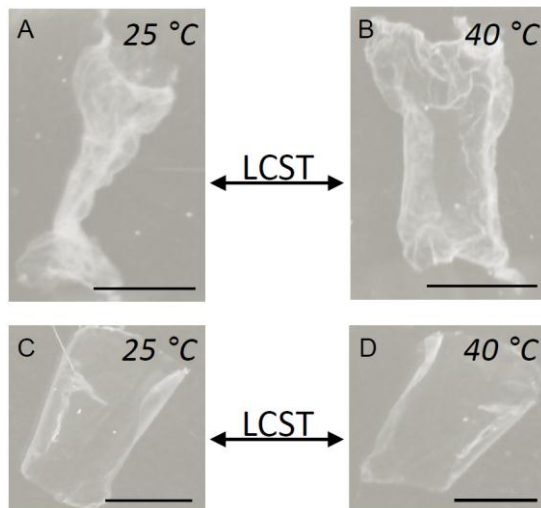


Figure 3. Photographs showing the thermo-responsive effect of PNIPAM modified free-standing PCL films below (25°C) and above (40°C) the LCST (A, B) and the non-responsive non-modified free-standing PCL films (C, D). Scale bar is 5 mm.

To show that PNIPAM was still thermoresponsive, free-standing PCL films that had been modified on one side with PNIPAM were subjected to a temperature shift above the LCST. These films showed a stretched configuration above the LCST and a coiled, shrunken configuration below the LCST. This behavior can be explained by the hydrophobic

nature of PCL and the hydrophobic or hydrophilic nature of PNIPAM depending on the temperature. Below the LCST, PNIPAM assumed a stretched hydrated configuration (hydrophilic state). Above the LCST, PNIPAM assumed a collapsed globular configuration (hydrophobic state). Thus, when keeping the free-standing PCL film below the LCST, the side with PNIPAM assumed the hydrophilic state, folding the hydrophobic PCL side inwards (fig. 3A). Subjecting the film to a temperature above the LCST, both sides of the film became hydrophobic, which led to a stretched configuration (fig. 3B). As a control, an unmodified PCL film was subjected to the same temperature. This film did not show the folded configuration, indicating that the shrunken coiled state shown in fig. 3A was indeed due to the thermoresponsive character of PNIPAM. We note that coiling of a film by polymer brushes was also reported by Zou *et al* for polyvinyl chloride modified with poly(N,N-dimethylacrylamide) (PDMA).^[85]

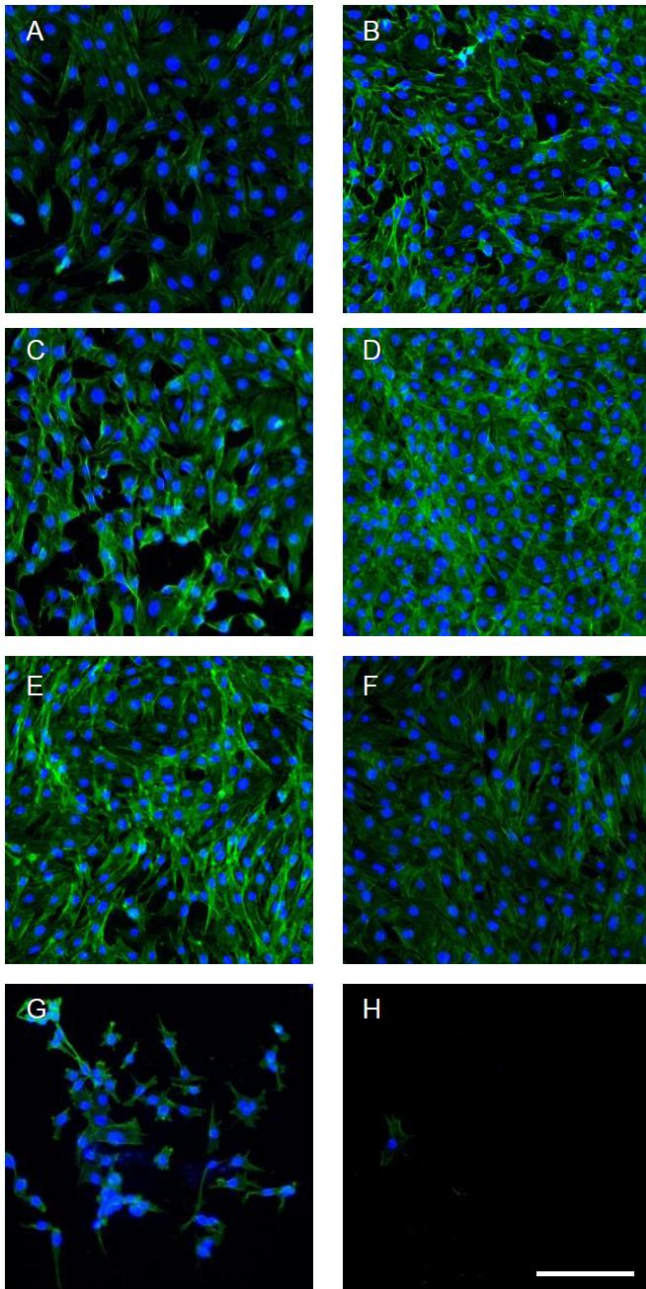


Figure 4. Fluorescent images of cell attachment at 37°C (A, C, E, G) and cell detachment at 25°C (B, D, F, H) on PCL (A, B), PCL modified with EDA (C, D), PCL modified with initiator (E, F), and PCL modified with PNIPAM (G, H). Scale bar is 200 μm .

Cell attachment results showed that all samples sustained cell growth. As already proven by Sui *et al.*^[80], the use of high density brushes can determine a lower cell attachment compared to the PNIPAM non-modified (control) films (fig. 4). Fluorescent pictures of the PCL surfaces (fig. 4) were taken both before and after cell detachment. No significant differences can be seen on EDA-modified, initiator-modified and unmodified PCL films among the pictures taken above and below the LCST. PNIPAM modified samples presented cells attached and spread on the surface, whereas after lowering the temperature no cells could be observed on the films, revealing a complete detachment of the cells. This confirmed that the detachment

was triggered only by the temperature change and not by swelling or any physico-chemical modification of the PCL films.

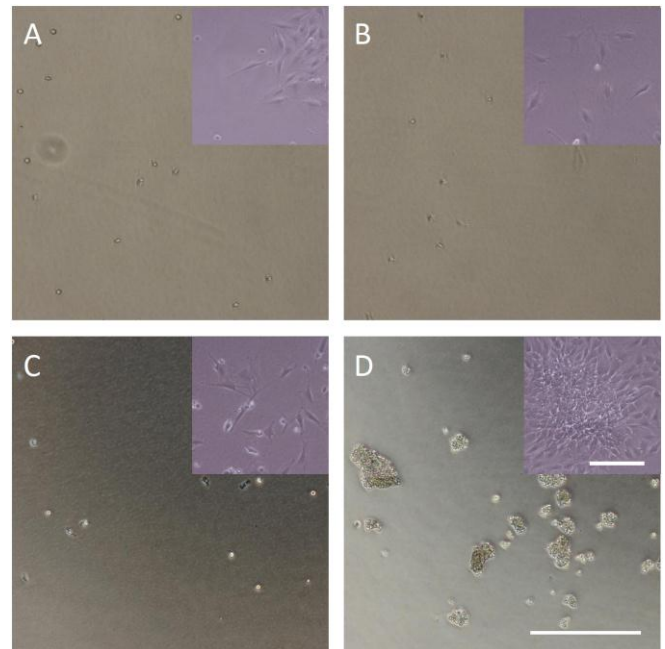


Figure 5. Images of cell reseeding on TCP after cell detachment of (A) PCL, (B) PCL modified with EDA, (C) PCL modified with initiator and (D) PCL modified with PNIPAM. Scale bar is 500 μm . The insets show images after 16 hours of incubation of the detached cells. Scale bar is 200 μm .

As shown by fluorescent microscopy, cells adhered to the surface and spread on it when cultured above the LCST (37°C). When the temperature was decreased below the LCST, cells assumed a rounded shape and started to detach from the surface of the PCL films modified with PNIPAM brushes. Conversely, no changes on cell morphology and adhesion could be seen on the control surfaces, where cells maintained their spread shape. In order to harvest the cells, PCL surfaces were rinsed with cell culture media. After rinsing, the same media with suspended cells was used to reseed the cells on a tissue culture plate (TCP). As confirmed in figure 5, the amount of cells obtained from the EDA modified surface and the PCL unmodified surface was negligible compared to the amount of cells obtained from the PNIPAM modified PCL films. Cells detached as sheets from the PNIPAM modified surfaces. After 12 hours of incubation, cells proliferated and covered the bottom of the TCP (insets in figure 5). The cells harvested from the unmodified surfaces were also able to attach and proliferate, but at a much lower density than those harvested from the PNIPAM modified surfaces. These results suggest that the newly functionalized PNIPAM-PCL substrates could find potential application for different areas of tissue engineering such as autologous cartilage implantation or cell-sheet tissue engineering.^[75]

3. Conclusion

In the **present** work, a way to grow brushes on PCL film while maintaining the main properties of the material in terms of biocompatibility and ability of the brush to respond to temperature changes was presented. PCL was modified using aminolysis and ATRP of the thermoresponsive polymer, PNIPAM. The attachment of PNIPAM was confirmed by FTIR and AFM measurements. The thermoresponsive activity was proven by the bending and stretching behavior of unmodified and modified free-standing PCL films. Cells could attach, spread and grow on all surfaces including the PNIPAM modified surface. The possibility to detach cells after cooling the media below the LCST was assessed. Cells were released in the form of sheet from the PNIPAM modified surfaces and their viability after being harvested was evaluated by their ability to grow and expand again on tissue culture plates. The possibility to harvest cells only from the PNIPAM modified samples confirmed that the detachment was triggered only by the temperature change.

4. Experimental section

Materials: *N*-Isopropylacrylamide (NIPAM, Aldrich, 97%) was recrystallized twice from a toluene/hexane solution (50% v/v) and dried under vacuum prior to use. Copper(I) bromide (CuBr, Aldrich, 98%) was purified by stirring in glacial acetic acid, filtering, and washing with ethanol three times, followed by drying in vacuum at room temperature overnight. Hexane (ACS) and ethanol (absolute) were purchased from Merck. Methanol (absolute) and isopropanol (absolute) were purchased from Biosolve. Copper(II) bromide (Sigma-Aldrich, ≥99%), triethylamine (Sigma-Aldrich, 99.5%), *N,N,N',N'',N''*-pentamethyldiethylenetriamine (PMDETA) (Acros Organics, 98%), 2-bromo-2-methylpropionyl bromide (Aldrich, 98%) and (3-aminopropyl)-trimethoxysilane (Aldrich, 97%) were used as received. All water used in the experiments was Millipore Milli-Q grade.

Formation of the PCL substrates: for the fabrication of the supported PCL films, either glass or silicon substrates were first cleaned with Piranha solution, then rinsed extensively with water and ethanol. *Caution: Piranha solution reacts violently with many organic materials and should be handled with great care!* Thin PCL films were obtained by spin-coating PCL from a chloroform solution (5 wt%) at 2000 rpm for 1 minute.

Activation of the polymer films: the spin coated PCL films were immersed into a 50 mL solution of 0.005 mol/L ethylenediamine (ED) in isopropanol (IPA). The reaction was allowed to proceed for 15 minutes under room temperature conditions. Samples were then rinsed with ice-cold water and subsequently soaked in an ice water bath, then dried in a stream of nitrogen and in an evacuated

vacuum oven. The aminated PCL films were immersed into 20 ml of hexane and 0.6 mL of triethylamine (TEA), to which 2 mL of 2-bromoisobutyryl bromide (BIBB) was added dropwise. The reaction mixture was gently stirred for 2 hours at 0 °C to produce the 2-bromoisobutyryl-immobilized PCL surface. The PCL substrates were then washed repeatedly with an ethanol/water (1/1, v/v) mixture and dried under a stream of nitrogen.

ATRP of PNIPAM brushes: NIPAM (2g, 17.4 mmol) monomer and PMDETA (110μL, 0.35 mmol) were added to a water (6.26 ml) and methanol (0.7 ml) mixture. The solution was purged with nitrogen for 30 min. CuBr (24.9mg, 0.17mmol) and CuBr₂ (3.9mg, 0.017mmol) were added into another reaction flask and flushed with nitrogen. Monomer, ligand and catalyst were then combined and stirred for another 30 minutes to facilitate the formation of the organometallic complex. This solution was then transferred into the flasks containing the substrates covered with SAMs. The flasks were sealed with rubber septa and kept at room temperature under nitrogen. After reaching the desired reaction time of 30 minutes, the substrates were removed from the polymerization solution, exhaustively rinsed with water to remove any unreacted and not surface tethered substances and subsequently dried in a stream of nitrogen.

Preparation of the free-standing PCL films: for the preparation of the free-standing PCL films, a 10 wt% polyvinyl alcohol (PVA) aqueous solution was cast onto the spin-coated PCL film and subsequently dried in vacuum. Then, the PCL and PVA bilayer was peeled off from the solid substrate and immersed into Milli-Q water to dissolve PVA.

AFM measurements: a Dimension D3100 AFM equipped with a hybrid scanner and a NanoScope IVa controller (Digital Instruments, Veeco-Bruker, Santa Barbara, CA) was operated in tapping mode using commercially available silicon cantilevers (PointProbe® Plus silicon probes, PPP-NCH, Nanosensors, Neuchatel, Switzerland) to obtain the surface morphology of the pure and modified PCL substrates.

Static contact angle measurements: an optical contact angle device equipped with an electronic syringe unit (OCA15, Dataphysics, Germany) connected to a charge-coupled device (CCD) video camera was used for static water contact angle measurements. The sessile drop technique was used to determine the change in wettability of the modified PCL films. A drop was deposited onto the PCL surfaces by the syringe, after which the drop contour was fitted by the Young-Laplace method.

Attenuated Total Reflectance FTIR spectroscopy: ATR-FTIR measurements were performed on an Alpha-P FTIR (Bruker Optics, Germany) fitted with the Platinum ATR QuickSnap™ sampling module. The spectra were analysed using Opus 6.5 spectroscopy software (Bruker Optics, Germany).

Cell culture and cell image analysis: A murine osteoblastic cell line MC3T3 was cultured at 37°C in a humidified atmosphere of 5% carbon dioxide, using as culture medium α -MEM supplemented with 10 v/v % FBS, 2 mM L-Glutamine, 1 mM sodium pyruvate, 100 U/mL of penicillin and 100 μ g/mL of streptomycin. The cells were seeded at a density of 20,000 cells/cm² on PCL substrates modified with PNIPAM brushes. Cell attachment and detachment on the PNIPAM modified surfaces was assessed above or below LCST by light microscopy connected with a digital camera (Nikon Eclipse TE 300). Cells were detached by lowering the temperature for 1.5 hrs at 25 °C. The surface was gently rinsed with culture medium and the detached cells collected and re-seeded on tissue culture poly(styrene) for 3 days to confirm their viability. The images of the surfaces taken after detachment were used to confirm that all the cells were removed from the samples. Substrates were washed twice with PBS and fixed with a 3.7 v/v % formaldehyde solution in PBS for 10 minutes at room temperature. Next, the samples were washed two or more times with PBS. Cell membrane was permeabilized by treating the samples with 0.1 v/v % Triton X-100 solution in PBS. Specimens were washed again with PBS and cell nuclei stained with DAPI diluted 1:100 in a 1 v/v % bovine serum albumin solution in PBS for 10 minutes. Cell cytoskeleton was stained with a phalloidin-rhodamine solution diluted 1:200 in PBS for 20 minutes at room temperature. Pictures were taken using a Nikon fluorescent microscope Eclipse E600.

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