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ARTICLE in BIOMACROMOLECULES · JULY 2015

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# Controlling the Crystallinity of Thermoresponsive Poly(2-oxazoline)-Based Nanolayers to Cell Adhesion and Detachment

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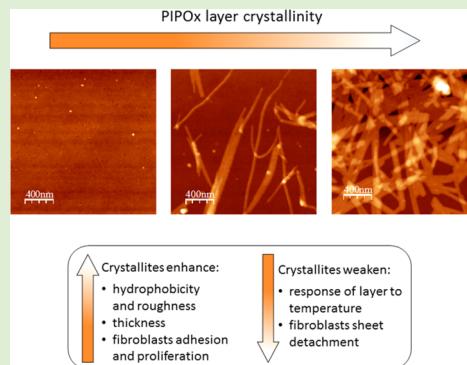
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## Supporting Information

**ABSTRACT:** Semicrystalline, thermoresponsive poly(2-isopropyl-2-oxazoline) (PIPOx) layers covalently bonded to glass or silica wafers were obtained via the surface-termination of the living polymer chains. Polymer solutions in acetonitrile were exposed to 50 °C for various time periods and were poured onto the functionalized solid wafers. Fibrillar crystallites formed in polymerization solutions settled down onto the wafers next to the amorphous polymer. The amount of crystallites adsorbed on thermoresponsive polymer layers depended on the annealing time of the PIPOx solution. The wettability of PIPOx layers decreased with the increasing amount of crystallites. The higher content of crystallites weakened the temperature response of the layer, as evidenced by the philicity and thickness measurements. Semicrystalline thermoresponsive PIPOx layers were used as biomaterials for human dermal fibroblasts (HDFs) culture and detachment. The presence of crystallites on the PIPOx layers promoted the proliferation of HDFs. Changes in the physicochemical properties of the layer, caused by the temperature response of the polymer, led to the change in the cells shape from a spindle-like to an ellipsoidal shape, which resulted in their detachment. A supporting membrane was used to assist the detachment of the cells from PIPOx biosurfaces and to prevent the rolling of the sheet.



## INTRODUCTION

Thermoresponsive polymer layers that are covalently bonded to the solid substrates have attracted significant interest in recent years. At the lower critical solution temperature (LCST) of tethered polymer, the phase transition of the layer occurs,<sup>1</sup> which is accompanied by physicochemical changes in the surface properties.<sup>2–4</sup> The changes are reversible; therefore, these layers are called “intelligent” and said to be able to “switch properties”.

Biocompatible polymer nano- or microlayers that can switch properties are commonly used in cell sheet technology. This concept, introduced by Okano,<sup>5</sup> assumes that cells adhere to, proliferate, and form a sheet on a layer when it is dehydrated (at a temperature above LCST) and detach from a hydrated layer (at temperature below LCST). Changes in the layer properties allow an intact cell sheet to detach with extracellular matrix (ECM) and cell growth proteins.<sup>6,7</sup> The best known and widely described thermoresponsive polymer layers for cell sheet engineering are based on poly(*N*-isopropylacrylamide) (PNIPAM) and its copolymers.<sup>8–10</sup>

Poly(2-isopropyl-2-oxazoline) (PIPOx), a structural isomer of PNIPAM, and copolymers of 2-isopropyl-2-oxazoline can serve as an alternative. Well controlled living polymerization leading to these polymers, their good biocompatibility and easily adjustable

transition temperature, make these materials as promising substrate for cell sheet culture.

Next to the transition temperature of tethered polymer, the morphology, philicity, and presence of adhesion promoters of the substrate plays an important role in culture and detachment of cells or cell sheets and may be controlled.<sup>6,7</sup> In general, cells spread and proliferate fastest when cultured on mildly hydrophobic surfaces.<sup>11</sup> The proteins from culture medium can accumulate in greater amounts at more rough surfaces and strengthen cell adhesion.<sup>12,13</sup>

For inorganic<sup>14–18</sup> or nonthermoresponsive polymeric layers,<sup>19–21</sup> it was observed that the surface crystallinity influences the adhesion and proliferation of cells. The influence of substrate crystallinity on the adhesion and proliferation depends on the cell line. The inorganic, crystalline substrates used for the cell culture are mainly based on hydroxyapatite (HA)<sup>14–17</sup> or silicon carbide.<sup>18</sup> Seydlova et al.<sup>14</sup> observed that fibroblasts proliferated fastest on crystalline HA layers, whereas keratinocytes grew better on an amorphous HA layer. Grover et

Received: June 3, 2015

Revised: July 28, 2015

Published: July 30, 2015

al.<sup>15</sup> noticed that HA surfaces with lower crystallinity facilitated osteoblasts attachment and increased the proliferation rate compared with surfaces with higher crystallinity. The modification of crystalline hydroxyapatite with cell adhesive peptides improved osteoblasts culture.<sup>16,17</sup> Human fibroblasts and keratinocytes grew faster, were better distributed, and flattened on single-crystal silicon carbide than cells cultured on amorphous silicon carbide surfaces.<sup>18</sup> In case of nonthermoreponsive polymeric layers, fibroblasts grew faster on amorphous poly(L-lactide) (PLLA) surfaces than on crystalline PLLA surfaces.<sup>19,20</sup> In contrast, hepatocytes proliferated faster on crystalline surfaces of polycaprolactone and poly(L-lactide) blended with poly(D,L-3-methyl-1,4-dioxan-2-one) than on amorphous ones.<sup>21</sup> Neither the adhesion nor the proliferation differed between osteoblasts cultured on crystalline and amorphous PLLA.<sup>20</sup>

For thermoresponsive polymeric layers, it is difficult to assess the influence of crystallinity on cell adhesion, proliferation, and detachment. Hitherto, semicrystalline thermoresponsive polymeric layers were not used for cell culture, except for poly(2-isopropyl-2-oxazoline) (PIPOx) layer reported in our previous work,<sup>22</sup> where we described the preparation of a thermoresponsive PIPOx-based nanolayer covalently attached to solid silica and glass wafers. Fibrillar structures were observed on the layer. This layer was tested as a support for dermal fibroblasts culture and detachment. Below the phase transition of PIPOx, the confluent cell sheet that formed above  $T_{CP}$  was spontaneously detached from the layer, making it a promising biosurface for further investigations in the field of cell engineering. However, the influence of crystallites on the polymer layer properties, and the consequent influence on dermal fibroblasts adhesion and detachment are unknown.

In this paper, we report the application of semicrystalline, thermoresponsive PIPOx nanolayers for cell sheet engineering. The synthesis of a series of thermoresponsive PIPOx layers of different crystallite contents and the control of the surface crystallinity are demonstrated. The influence of crystallites on the thermoresponsive behavior and volume transition of PIPOx surfaces is presented. The study shows dependence between PIPOx surface crystallinity and human dermal fibroblast adhesion, proliferation and detachment. A method to prevent the rolling of a detached cell sheet is shown.

## MATERIALS AND METHODS

**Materials.** Chemicals. 2-Isopropyl-2-oxazoline (IPOx) was synthesized according to procedure described by Witte et al.<sup>23</sup> Raw 2-isopropyl-2-oxazoline was dried over KOH, distilled under reduced pressure, then dried over  $\text{CaH}_2$  and distilled again. Acetonitrile (for HPLC super gradient, min. 99.9%, POCH Gliwice) was dried over  $\text{CaH}_2$  and distilled under a dry argon atmosphere before use. Ethanol (min. 96%, POCH Gliwice), methanol (for HPLC, min. 99.9%, POCH Gliwice), and acetone (99.5%, POCH Gliwice) were filtered before use. Methyl 4-nitrobenzenesulfonate (99%, Aldrich),  $\text{H}_2\text{SO}_4$  (95%, POCH Gliwice), hydrogen peroxide (30%, Chempur), and (3-aminopropyl)-triethoxysilane (APTES; 99%, Aldrich) were used as received.

**Solid Wafers.** Borosilicate cover glasses ( $\phi = 1.3 \text{ cm}$ ) were obtained from VWR International. Silica wafers ( $\phi = 10 \text{ cm}$ , Cemac Silicon,  $\text{SiO}_2 = 3 \text{ nm}$ ) were cut into  $1 \text{ cm}^2$  pieces.

**Cell Culture.** Human dermal fibroblasts (HDFs) were derived from the Cell Bank at the Centre for Burn Treatment in Siemianowice Slaskie. Dulbecco's modified eagle medium advanced therapy medicinal product (DMEM; PAA Laboratories GmbH), trypsin in EDTA (PAA Laboratories GmbH), AlamarBlue (Invitrogen Corporation), PBS (PAA Laboratories GmbH), and cell dissociation reagent TrypLE (Life Technologies) were used as received. Tissue culture polystyrene plates (TCPS) containing four wells of diameter  $\phi = 2 \text{ cm}$  were used to

store and transport grafted polymer wafers. Skin substitute membrane Suprathel (PolyMedics Innovations GmbH) was used to assist the detachment and transfer of human dermal fibroblast sheets.

**Synthesis of Poly(2-isopropyl-2-oxazoline).** Poly(2-isopropyl-2-oxazoline) was synthesized using a procedure described elsewhere.<sup>22</sup> The theoretical degree of polymerization (DP) was 180. 2-Isopropyl-2-oxazoline (29.35 g, 0.257 mol) and 0.32 g ( $1.48 \times 10^{-3}$  mol) methyl 4-nitrobenzenesulfonate were dissolved in acetonitrile, resulting in a total monomer concentration of 30% (v/v). The mixture was degassed, and the polymerization was carried out at 50 °C for 5 days until full conversion of the monomer (as verified by gas chromatography). After this time, a sample of 10 mL was withdrawn from the reaction mixture, and the living polymer chains were terminated by the addition of KOH solution in methanol (equimolar to the initiator). Acetonitrile was evaporated, polymer was dissolved in water, and lyophilized. The obtained polymer was designated PIPOx0.

The solution of living PIPOx remaining in the reactor (80 mL) was continuously annealed at 50 °C for 7 days. This solution was then used to prepare polymer layers on the solid wafers.

**Preparation of PIPOx Layers on Solid Wafers.** The glass and silica wafers were treated to introduce the amine groups onto their surface, as described elsewhere.<sup>22</sup>

The living PIPOx chains in the solution remaining in the reactor were grafted using the amine groups of the glass or silica wafers. Four portions of the solution of living PIPOx (4 × 20 mL) were poured into the reactors with functionalized solid wafers. The living PIPOx was grafted to the surface via amines immediately after the monomer was fully converted (surface named sPIPOx0) and after two (sPIPOx2), four (sPIPOx4) and seven (sPIPOx7) days of annealing of the PIPOx solution at 50 °C.

Acetonitrile was added after pouring the living PIPOx solutions into the reactors containing solid wafers, resulting in a total PIPOx concentration of 50 g/L. The reactions were carried out at room temperature for 1 day under constant stirring. The wafers were then removed from the reactor and rinsed once in acetonitrile, three times in distilled water, and once in ethanol (under ultrasonication for 30 s) to remove polymer that was not bound to the surface. Wafers covered in PIPOx layers were dried under reduced pressure and stored in a dust-free vacuum.

**Measurements.** The  $M_n$  and  $M_w/M_n$  of PIPOx0 were determined using a size exclusion chromatography (GPC-MALLS) system equipped with a multiangle light scattering detector (DAWN EOS, Wyatt Technologies,  $\lambda = 658 \text{ nm}$ ) and a refractive index detector ( $\Delta n$ -1000 RI WGE DR Bures,  $\lambda = 620 \text{ nm}$ ). 2xPL gel MIXED-C (Polymer Laboratories) columns were used. PIPOx0 was dissolved in DMF. Measurements were performed at 45 °C at a nominal flow rate of 1 mL/min. The refractive index increment ( $dn/dc$ ) of PIPOx0 was 0.065 mL/g, as we reported previously.<sup>22</sup> The results were evaluated using the ASTRA software from Wyatt Technologies.

The cloud points ( $T_{CP}$ ) were determined for PIPOx0 dissolved in water and in culture medium (5 g/L). A Jasco V-530 UV-vis spectrophotometer equipped with a programmable Medson MTC-P1 thermocontroller was used. The measurements were performed at a constant transmittance wavelength of  $\lambda = 700 \text{ nm}$ . The solutions were heated from 25 to 50 °C at a heating rate of 1 °C/min. The samples were then cooled to 25 °C at cooling rate of 1 °C/min. The cloud point was determined from transmittance–temperature curve as a temperature at which the transmittance reaches 50% of its initial value.

A Multi-Mode AFM microscope equipped with a NanoScope 3D controller (Veeco Instruments Inc., CA, U.S.A.) and piezoelectric scanner was used to visualize the morphology of PIPOx layers. Phosphorus-doped silicon cantilevers (Model RTESP, Veeco Instruments Inc., U.S.A.) with a length of 125  $\mu\text{m}$  and a tip height of 15–20  $\mu\text{m}$  were applied. Micrographs were recorded in tapping mode. The coverage of the surface with crystallites was calculated with a “bearing analysis” of micrographs. Objects (fibrils adsorbed on the polymer layer) were marked using the NanoScope V531r1 software. The area of marked objects was calculated to obtain the content of PIPOx fibrils on the surface. Roughness parameter for the surface ( $R_a$ ) was calculated using WSxM software.<sup>24</sup>

The thickness of the PIPOx layer grafted to the silica substrates was determined using a Sentech SE 850 ellipsometer equipped with a xenon lamp and thermostatically controlled chamber. Dry layers were measured at ambient temperature. Surface maps were generated by studying 225 measuring points per 1 cm<sup>2</sup> sample area. The surfaces were then incubated in water at 20 °C for 2 h. The PIPOx layers were then dried with a stream of argon, and 10 points of the surface were measured in a thermostatic chamber at 20 °C. Analogous measurements were carried out for layers incubated at 40 °C. The results were fitted to the mathematical model using the Cauchy method and evaluated with the SpectraRay 3 Software.

The quantitative surface composition (atom %) was determined with an X-ray (VGScienta) Photoelectron Spectroscopy (XPS) system (Prevac, Poland) equipped with a R3000 energy analyzer. The photoelectron spectra were recorded with a pass energy of 200 eV using monochromatized Al K $\alpha$  radiation ( $h\nu = 1486.6$  eV). All XPS data were acquired at a nominal photoelectron takeoff angle of 45°, and the takeoff angle is defined as the angle between the surface normal and the axis of the analyzer lens.

The water contact angles ( $\Theta$ ) of surfaces were measured using a CAM101 goniometer equipped with a temperature control unit (Intelligent digital controller OMRON SEGN) connected to a thermostatically controlled chamber. The measurements were performed following the sessile drop method. Dry layers at ambient temperature were measured first. The layers were then placed in water at 20 °C and incubated for 2 h. Subsequently, the swollen PIPOx layers were dried with a stream of argon, and the contact angle was measured in a thermostatic chamber at 20 °C. Analogous measurements were carried out for layers incubated at 40 °C.

#### Human Dermal Fibroblasts (HDFs) Culture and Detachment.

Human dermal fibroblasts were cultured according to a procedure reported in our previous paper.<sup>25</sup> Briefly, sterilized wafers were placed in four-well plates and incubated for 3 h in DMEM culture medium at 25 °C. The temperature was then increased to 37 °C, and the wafers were conditioned for another 3 h. Human dermal fibroblasts were then seeded on the wafers at a density of  $1 \times 10^5$  cells/1 cm<sup>2</sup> ( $3 \times 10^5$  cells/well). The cells were cultured in an incubator at 37 °C in 5% CO<sub>2</sub>. Cells adhesion, proliferation, and morphology were assessed after 2.5, 4, 8, 12, and 24 h. The number of cells was calculated using the AlamarBlue test.

When a confluent fibroblasts sheet had formed on the surfaces after 24 h of culture, the sheets were detached by lowering the temperature to 20 °C. The cells that exhibited morphological changes (ellipsoidal cells) at decreased temperatures were marked using the GIMP 2.8.2 software, and their concentration was calculated.

Suprathel skin substitute membrane was used to assist the detachment and transfer of fibroblast sheets from wafers.<sup>26</sup> This membrane was placed onto the confluent cell sheet at 37 °C, and the temperature was then decreased to 20 °C. After 15 min, the membrane was removed with stuck cells that had detached from the wafer. The membrane with a continuous sheet of detached cells was transferred to another culture dish. The wafers were photographed after the removal of membrane, and cells that remained on the surface were counted.

When the detached cells adhered to the membrane were transferred to a new culture dish, fresh culture medium was added immediately and the temperature was increased to 37 °C. The culture was continued at 37 °C in 5% CO<sub>2</sub>. The culture medium was removed and replaced by the fresh portion after every 48 h periods. After 10 days of culture the membrane was removed. The culture medium was then aspirated, and 5 mL of the cell dissociation reagent TrypLE was added to the cells. After 3 min, the mixture of cells and enzyme was removed from the well and centrifuged (1500 rpm, 10 min). The supernatant was then decanted, and the cells were suspended in 1 mL of PBS. A 25  $\mu$ L sample was withdrawn from the suspension and transferred to a Tali Image-Based Cytometer to count the cells.

## RESULTS AND DISCUSSION

**Poly(2-isopropyl-2-oxazoline) Synthesis.** Poly(2-isopropyl-2-oxazoline) was obtained via a cationic ring opening polymerization, which was carried out in acetonitrile at 50 °C. A

portion of the polymerization mixture withdrawn immediately after the monomer was fully converted was terminated, and the acetonitrile was evaporated. This sample was labeled PIPOx0.

The molar mass, molar mass dispersity, and cloud point temperature of the obtained PIPOx0 are presented in Table 1.

**Table 1. Molar Mass, Molar Mass Distribution, and Cloud Points of the Obtained PIPOx0**

$M_{\text{theor}}$ (g/mol)	DP <sub>theor</sub>	$M_n$ (g/mol)	$M_w/M_n$	DP	$T_{\text{CP water}}$	$T_{\text{CP culture medium}}$
GPC-MALLS				UV-vis, °C		
20000	180	20800	1.01	180	37	35

The absolute molar masses (GPC-MALLS) and the values calculated from the feed were similar and, together with the low molar mass dispersity, confirmed the living nature of the polymerization. The cloud point temperature of PIPOx0 in water is 37 °C, while it decreases to 35 °C in DMEM-ATMP due to a weak salting-out effect of the cell culture medium. The transmittance–temperature curves are sharp, and hysteresis was not observed (data not shown).

The remaining solution of living PIPOx was used to prepare polymer layers on the solid wafers.

**PIPOx Semicrystalline Layers on Solid Wafers.** According to our previously reported observations,<sup>27</sup> the annealing of a solution of PIPOx in nonaqueous, polar solvents with dipole moments higher than that of water (approximately 4 D), such as acetonitrile, dimethyl sulfoxide, or propylene carbonate, leads to PIPOx crystallization. The objects possessing a fibril-like morphology with a length of several microns and a width of approximately 50 nm were formed.<sup>27</sup> As we showed,<sup>27</sup> the amount of crystallites can be controlled by the time of annealing.

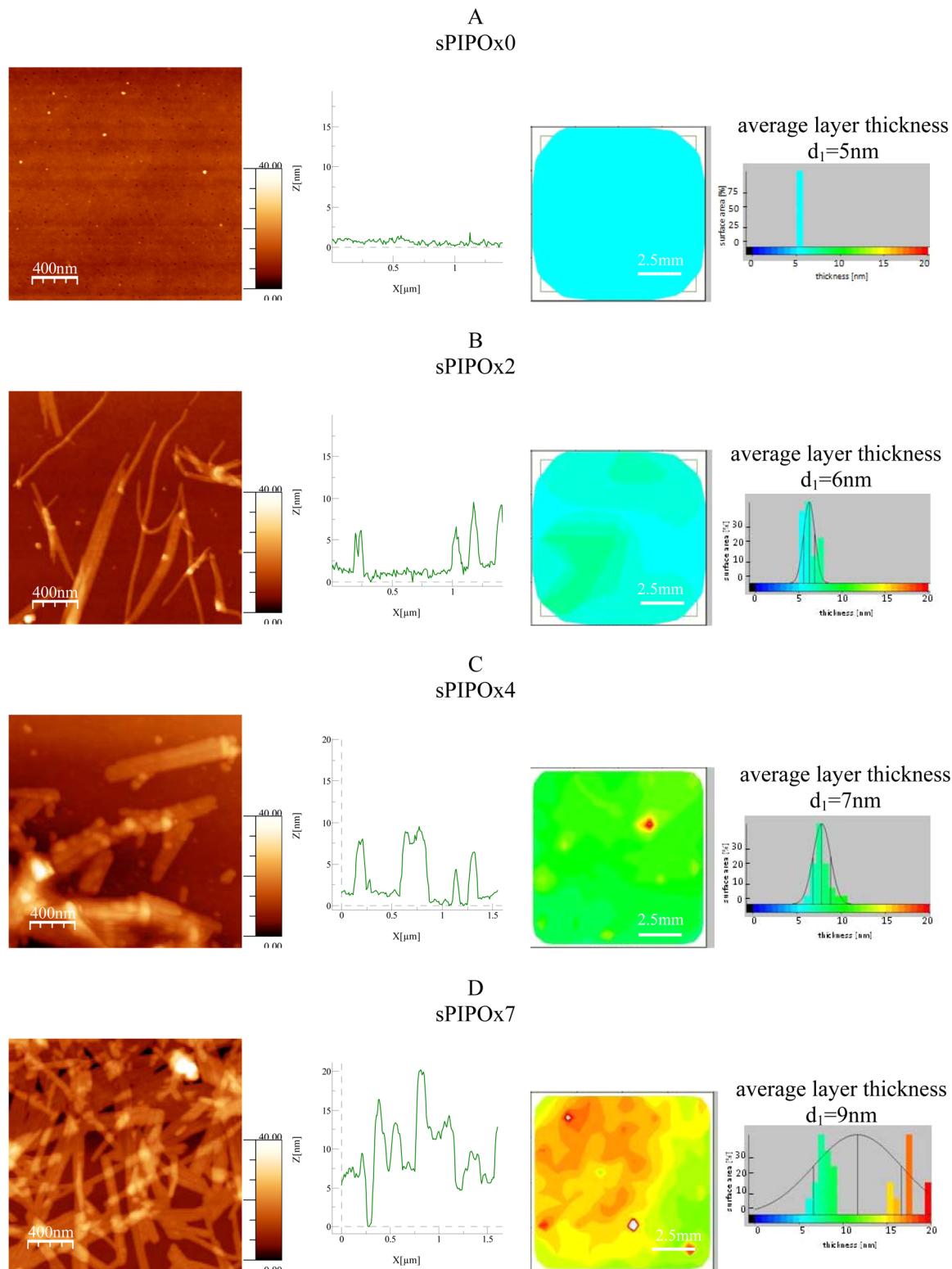
Solutions of living PIPOx in acetonitrile that were annealed for various times were used to prepare the layers. In the first solution used for the preparation of layer immediately after the full conversion of the monomer, crystallites were not observed (surface named as sPIPOx0). The subsequent “grafting to” reactions were performed after two (sPIPOx2), four (sPIPOx4), and seven (sPIPOx7) days of annealing of the living PIPOx solution at 50 °C. Because the crystallites content in the solution increases with the annealing time, therefore, solutions containing different amounts of crystallites were used to prepare these layers.

The active cationic chain ends of living PIPOx reacted with the amine groups introduced onto the surface of glass or silica wafers, resulting in the formation of covalent bonds between the polymer and the solid substrates, as we previously proved.<sup>22</sup> Fibrillar crystallites that formed in the polymerization solutions adhered to the wafers, as discussed below.

The surface of sPIPOx0 layers was smooth, as evident in the AFM micrograph and ellipsometry map, confirming the lack of crystallites (Figure 1A). The roughness parameter ( $R_a$ ) for this layer, defined as the centerline average between the highest and the lowest point of the surface irregularities, was 0.4 nm. The average thickness of the polymer layer ( $d_1$ ) was 5 nm.

The other surfaces contained different amounts of fibrillar structures, making surface more rough and inhomogeneous (Figure 1B–D).

A total of 20% of the surface of the sPIPOx2 layer (Figure 1B) was covered with the fibrils of the length of up to 3  $\mu$ m. The average layer thickness ( $d_1$ ) was 6 nm. The roughness increased comparing with sPIPOx0,  $R_a$  was equal to 2.9–3 nm. The surface

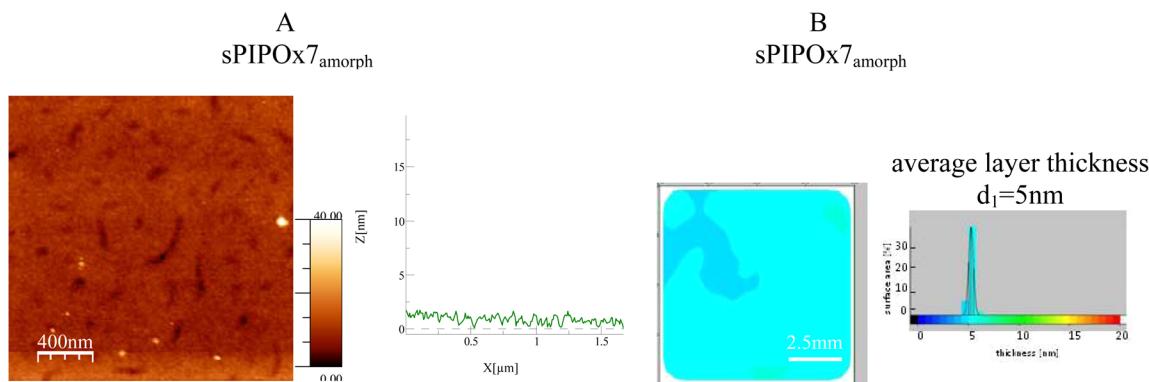


**Figure 1.** AFM micrographs with roughness profile (left) and ellipsometric maps with thickness distribution (right) of sPIPOx0 (A), sPIPOx2 (B), sPIPOx4 (C), and sPIPOx7 (D) layers.

of the sPIPOx4 layer contained significantly more fibrils (40%; Figure 1C) that were several microns long. The thickness of the layers ranged from 7 to 10 nm. Wider thickness distribution and more rough surface ( $R_a$  4–6 nm) could be seen. Most of the sPIPOx7 (Figure 1D) was covered with fibrils (70% of the entire area), showing the greatest inhomogeneity ( $R_a$  5.7–12.15 nm).

The majority of the layer was 9 nm thick, whereas clusters of fibrils reached heights of up to 20 nm.

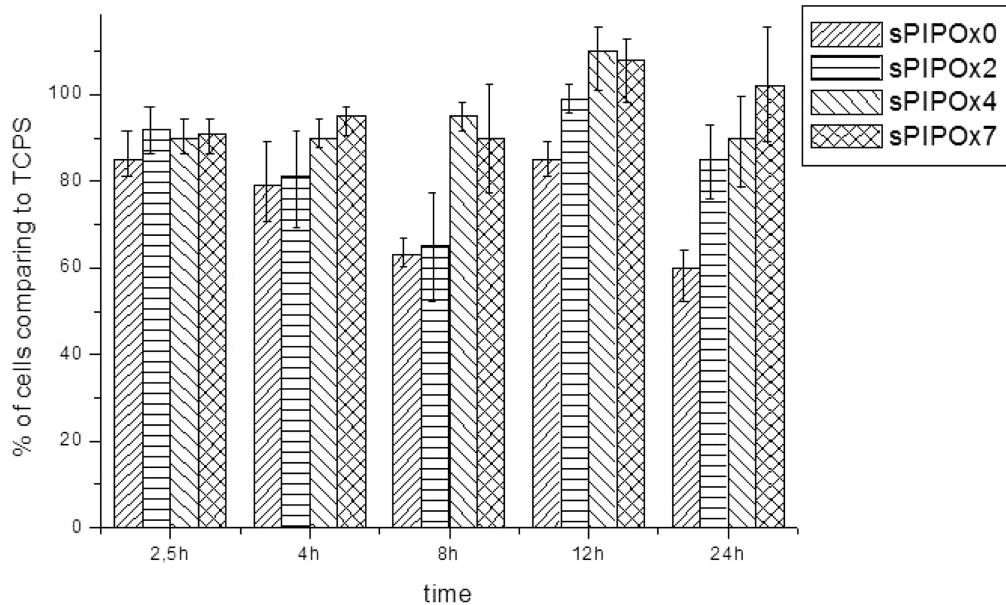
Annealing the wafers above the  $T_m$  of PIPOx<sup>27</sup> and subsequent quenching should destroy the crystalline structures on the surface. This procedure melts the crystallites to yield an amorphous structure, while further washing with water causes



**Figure 2.** AFM micrograph with roughness profile (A) and ellipsometric map with thickness distribution (B) of sPIPOx7<sub>amorph</sub> layer.

**Table 2. Water Contact Angle and Thickness of sPIPOx<sub>amorph</sub>, sPIPOx0, sPIPOx2, sPIPOx4, and sPIPOx7 Layers at 20 and 40 °C**

layer symbol	crystallite content (%)	contact angle (deg)				thickness (nm)			
		$\Theta_1$ dry ambient	$\Theta_2$ water 20 °C	$\Theta_3$ water 40 °C	$\Delta_\Theta$ ( $\Theta_3 - \Theta_2$ )	$d_1$ dry ambient	$d_2$ water 20 °C	$d_3$ water 40 °C	$\Delta_d$ ( $d_2 - d_3$ )
sPIPOx <sub>amorph</sub>	0	64 ± 0.7	54 ± 0.8	63 ± 0.7	9	5	12	7	5
sPIPOx0	0	65 ± 0.8	54 ± 0.7	64 ± 0.8	10	5	12	7	5
sPIPOx2	20	68 ± 1.0	58 ± 0.9	67 ± 0.9	9	6	12	7	5
sPIPOx4	40	69 ± 0.9	58 ± 1.0	68 ± 0.8	10	7	13	9	4
sPIPOx7	70	73 ± 0.9	60 ± 0.8	64 ± 0.7	4	9	13	11	2



**Figure 3.** Percentage of human dermal fibroblasts onto PIPOx layers of different amount of crystallites comparing to TCPS.

its removal. The sPIPOx7 layer that was incubated at 210 °C for 1 h, cooled with liquid nitrogen, and then washed with water (sPIPOx7<sub>amorph</sub>) was homogeneous and smooth ( $R_a$  approximately 0.5 nm) without fibrils (Figure 2). Traces left by removed crystallites may be seen. The average thickness of this layer after thermal treatment at 210 °C and washing with water was 5 nm, the same as sPIPOx0. The results indicated that crystallites were only embedded on the amorphous PIPOx layer, not covalently bonded, and could be easily removed.

The influence of the crystallinity on the surface wettability was analyzed using contact angle measurements (Table 2). The results indicate that the affinity of the dry PIPOx layers to water at room temperature ( $\Theta_1$ ) depended on the crystallites content

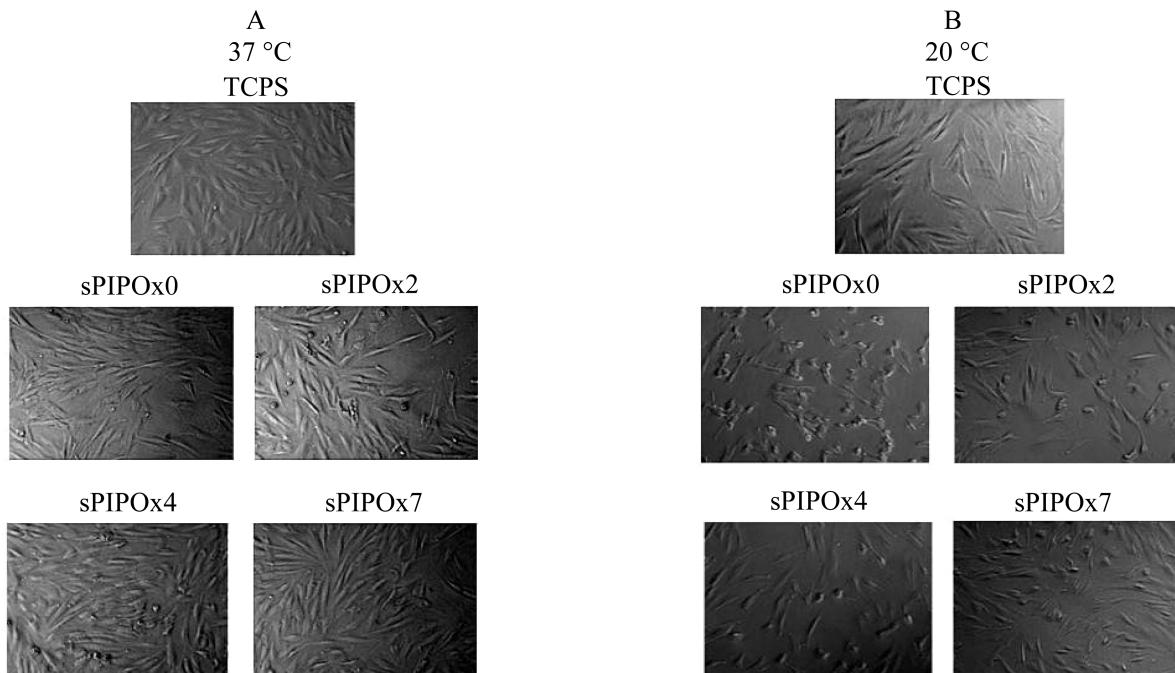
on the surface. Amorphous layers whose surface were free of fibrils (sPIPOx<sub>amorph</sub> and sPIPOx0) showed the greatest affinity to water ( $\Theta_1 = 64^\circ$  and  $65^\circ$ , respectively). The increase in the contact angle with the surface crystallinity confirms the hydrophobic character of PIPOx crystallites.

Amorphous PIPOx is known to be thermoresponsive in water ( $T_{CP} = 37$  °C). The surface philicity and thickness were measured to assess the influence of the nonthermoresponsive crystallites on the behavior of layers incubated in water at different temperatures (Table 2).

The wettability of all layers incubated in water at 20 °C (below  $T_{CP}$ ,  $\Theta_2$ ) increased compared with the dry samples. An increase

**Table 3.** HDFs Cultured and Detached from PIPOx Layers of Different Crystallinity

layer	% of crystallite on layer	a No. of HDFs seeded	b No. of HDFs in sheet (24 h, 37 °C)	c % of HDFs rounded (15 min, 20 °C)	d HDFs detached and transferred	e No. of HDFs after 10 days from detachment
sPIPOx0	0	$3 \times 10^5$	$5.00 \times 10^5$	74	$4.50 \times 10^5$ (90%)	$4.50 \times 10^6$
sPIPOx2	20	$3 \times 10^5$	$6.90 \times 10^5$	48		
sPIPOx4	40	$3 \times 10^5$	$7.38 \times 10^5$	39		
sPIPOx7	70	$3 \times 10^5$	$8.28 \times 10^5$	29	$4.97 \times 10^5$ (60%)	$7.50 \times 10^6$
TCPS		$3 \times 10^5$	$8.19 \times 10^5$	3	0	

**Figure 4.** Morphology of confluent sheet of human dermal fibroblasts on PIPOx layers of different crystallites content and TCPS after 24 h at 37 °C (A) and after 15 min at 20 °C (B).

in the affinity to water is due to the solvation of polymer chains after the penetration of water into the polymer layer.

As the temperature increased to 40 °C (above  $T_{CP}$ ), the layers became more hydrophobic ( $\Theta_3$ ). The response in the surface affinity to water was due to the dehydration of polymer chains. A higher content of crystallites lowered the area of amorphous PIPOx available for dehydration and decreased the response of the surface to temperature. The response was lowest for sPIPOx7 ( $\Delta\Theta = 4$ ), whose surface was 70% covered in crystallites.

The crystallites content also influenced the surface swelling efficiency ( $d_2$ ). Layers free of crystallites (sPIPOx<sub>amorph</sub>, sPIPOx0) and those covered with fibrils by up to 40% (sPIPOx2, sPIPOx4) swelled more than the layer whose surface was 70% covered (sPIPOx7). Crystallites on the surface likely prevent the penetration of water into the polymer layer.

A decrease of the layer thickness related to the volume phase transition of the layer was observed ( $d_3$ ) above the  $T_{CP}$  of immobilized PIPOx. The highest response was for layers that were up to 40% covered with fibrils. In the case of sPIPOx7, nonthermoreponsive crystallites covering 70% of the surface partly interrupted the response of the polymer chains tethered to the solid substrate and weakened the shrinkage of the layer.

#### Fibroblasts Adhesion, Proliferation, and Detachment.

Thermoresponsive, semicrystalline PIPOx layers were used as

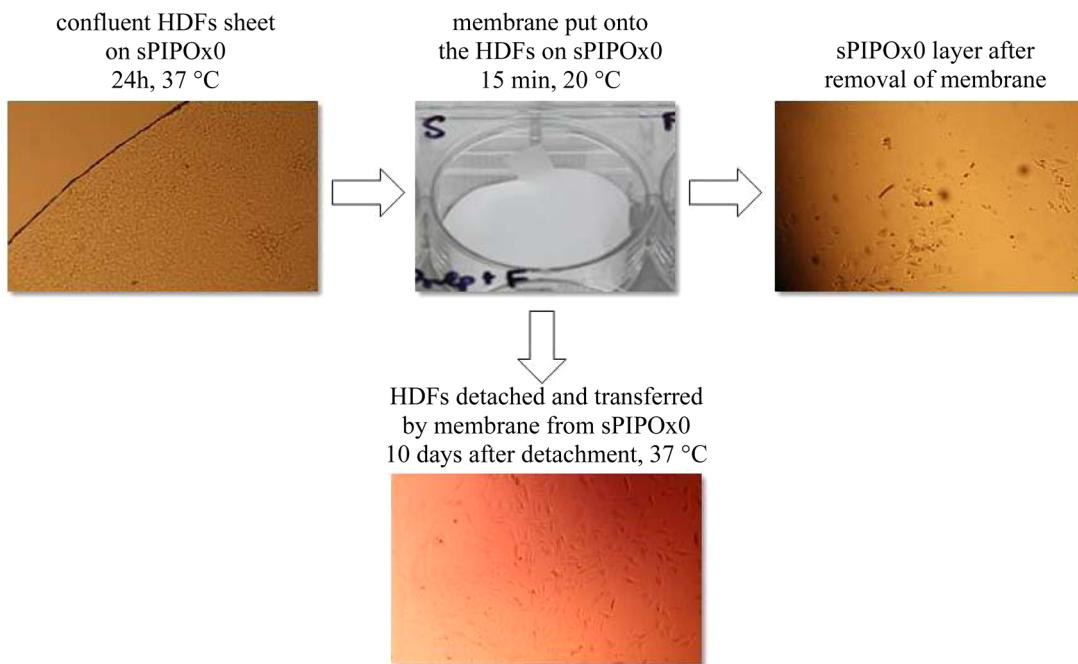
supports for human dermal fibroblasts (HDFs) culture and detachment.

The PIPOx layers and the control sample (TCPS) were immersed in DMEM culture medium at 37 °C. Under these conditions, the PIPOx layers are dehydrated and shrunk (Table 2). The surfaces were seeded with  $1 \times 10^5$  cells/ $1 \text{ cm}^2$  HDFs ( $3 \times 10^5$  cells/well), and the cells began to adhere and proliferate. The numbers of HDFs on PIPOx layers after 2.5, 4, 8, 12, and 24 h compared with the control were calculated from a spectrophotometrical analysis of the AlamarBlue test (Figure 3).

After the first 2.5 h of culture, cells adhered to all PIPOx layers reaching 80–90% of the number of cells on TCPS.

For all studied PIPOx surfaces, the number of cells increased significantly after 24 h of culture (Table 3b). The number of cells on the amorphous sPIPOx0 layer increased at least one and a half times compared with the number of cells at the beginning of the culture. The number of cells at least doubled on semicrystalline sPIPOx2 and sPIPOx4 layers, and almost tripled on sPIPOx7 compared with seeded cells. Notably, a higher content of crystallites on the surface favors HDFs viability and proliferation.

We observed that the presence of crystallites on poly(2-isopropyl-2-oxazoline) layers, enhancing the surface hydrophobicity and roughness, promotes the proliferation of human dermal fibroblasts. This effect may be observed because fibroblasts spread and proliferate fastest when cultured on mildly



**Figure 5.** HDFs sheet detachment and transfer procedure.

hydrophobic polymeric substrates (wetting angle,  $\Theta$ , of approximately  $70^\circ$ ).<sup>11</sup> In general, cells adhere better to rough surfaces, compared with smooth.<sup>12</sup> However, the scale of roughness of surfaces in this study is only of few nanometer instead of  $10^1 - 10^2 \mu\text{m}$ , observed by other groups,<sup>12,13</sup> thus, is difficult to discuss the impact of such a small change in the roughness on cell adhesion and proliferation. The size, shape, and geometrical distribution of the crystalline PIPOx fibers on the surface seem to be similar to those of the fibrous proteins in the extracellular matrix, such as collagen, elastin, fibronectin, and laminin. Moreover, the fiber-mesh of PIPOx crystallites may facilitate the circulation of fluids that supply oxygen and nutrients to cells as well as the flow of metabolites (including waste) from cells to the ECM, making the culture more efficient.

Contrary to the scraping or enzymatic treatment of a cell sheet, cells can be detached from a thermoresponsive polymer layer by merely lowering the temperature, allowing for the detachment of an intact, undamaged cell sheet and ECM.

Fibroblasts sheets were detached from PIPOx layers of different crystallinity by lowering the temperature to  $20^\circ\text{C}$ . As shown in Figure 4B, fibroblasts changed their morphology at  $20^\circ\text{C}$ .

The morphology of more than 70% of HDFs changed from a spindle-like to an ellipsoidal for sPIPOx0, while this amount was slightly less than 50% for sPIPOx2 and sPIPOx4. Only 29% of HDFs cultured on sPIPOx7 changed their shape (Table 3c).

The change in shape of human dermal fibroblasts, indicating a change in the properties of the substrate, depended on the crystallinity of the layer. The highest number of ellipsoidal cells (74%) was observed on the amorphous PIPOx layer. The higher content of crystallites weakened the response of the layer to temperature, as evidenced by the philicity and thickness measurements (Table 2), which reduced the number of cells that rounded (Table 3c). On TCPS, which is nonthermoresponsive, only 3% of HDFs changed their shape at  $20^\circ\text{C}$ .

After lowering the temperature to  $20^\circ\text{C}$ , an intact HDFs sheet spontaneously detached within 30 min from all semicrystalline

PIPOx layers with crystallite contents of up to 40%. However, the fibroblast sheets rolled during detachment.

To prevent the fibroblast sheet from rolling and assist their detachment, the Suprathel membrane, which is made of artificial skin, was used in this work. The membrane placed on the confluent HDFs sheet cultured on sPIPOx0 and sPIPOx7 layers was removed together with cells, after lowering the temperature. The membrane and adhered cell sheet was transferred to another culture dish (Figure 5).

The cell sheets detached with the membranes from sPIPOx0 and sPIPOx7 did not roll. 90% of HDFs detached from the sPIPOx0 layer, which was free of crystallites, whereas 60% of cells detached from the crystalline sPIPOx7 layer (Table 3d). The cell sheet detached more efficiently from the amorphous sPIPOx0 surface than from the semicrystalline sPIPOx7 surface because the response of the polymer layer to temperature was stronger for layers with smaller amount of crystallites (Table 2). Cells did not detach from nonthermoresponsive TCPS.

Transferred cells were cultured at  $37^\circ\text{C}$  for 10 days, and their viability was assessed as described in Materials and Methods. After 10 days of culture, the number of cells that detached and transferred from thermoresponsive PIPOx layers increased approximately 10-fold (Table 3e). This finding indicates that human dermal fibroblasts cultured and detached from PIPOx layers can further divide and proliferate.

## CONCLUSIONS

Thermoresponsive poly(2-isopropyl-2-oxazoline) layers of different adsorbed crystallites contents were obtained. The amount of crystallites adsorbed on the PIPOx layer depended on the annealing time of its solution. The higher content of crystallites made surface more hydrophobic and weakened the volume transition of the layer above  $T_{\text{CP}}$ . The most pronounced volume transition of the layer, defined as a change in the layer wettability and thickness, was observed for surfaces that were up to 40% covered with crystallites.

The obtained PIPOx surfaces were applied as biomaterials for human dermal fibroblasts culture and detachment. The crystallites on PIPOx layers promoted the proliferation of HDFs. After 24 h in culture, confluent HDFs sheets had formed on all PIPOx layers. The number of cells in the confluent sheet had at least doubled compared with the number of seeded cells.

Lowering the temperature changed the morphology of fibroblasts from spindle-like to ellipsoidal, which was driven by the changes in the physiochemical properties of the layer. These changes led to the detachment of the fibroblast sheet from the surface. Using skin substitute membrane, it was possible to detach and transfer of nonrolled cell sheets.

Because a high crystallites content weakened the volume transition of the layer, detachment from amorphous surfaces was more efficient. Thus, PIPOx crystallites promote HDFs adhesion and proliferation while disturbing cell sheet detachment. The HDFs in the sheet detached from PIPOx remained viable for at least 10 days.

## ASSOCIATED CONTENT

### Supporting Information

XPS data. The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acs.biomac.5b00745](https://doi.org/10.1021/acs.biomac.5b00745).

(PDF)

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### Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

This work was supported by the National Centre for Research and Development, Project POLYCELL PBS1/B9/10/2012. Part of this work was supported by the National Science Centre, 2012/07/N/ST5/00261. The authors thank Andrzej Marcinowski (CMPW PAN, Zabrze, Poland) for AFM measurements and Dr. Jerzy Kubacki (University of Silesia, Katowice, Poland) for XPS measurements.

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