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Poly[tri(ethylene glycol) ethyl ether methacrylate]-Coated Surfaces for Controlled Fibroblasts Culturing

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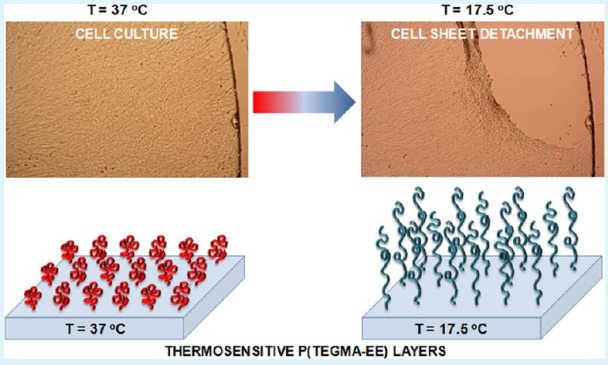
Supporting Information

ABSTRACT: Well-defined thermosensitive poly[tri(ethylene glycol) monoethyl ether methacrylate] (P(TEGMA-EE)) brushes were synthesized on a solid substrate by the surface-initiated atom transfer radical polymerization of TEGMA-EE. The polymerization reaction was initiated by 2-bromo-2-methylpropionate groups immobilized on the surface of the wafers. The changes in the surface composition, morphology, philicity, and thickness that occurred at each step of wafer functionalization confirmed that all surface modification procedures were successful. Both the successful modification of the surface and bonding of the P(TEGMA-EE) layer were confirmed by X-ray photoelectron spectroscopy (XPS) measurements. The thickness of the obtained P(TEGMA-EE) layers increased with increasing polymerization time. The increase of environmental temperature above the cloud point temperature of P(TEGMA-EE) caused the changes of surface philicity. A simultaneous decrease in the polymer layer thickness confirmed the thermosensitive properties of these P(TEGMA-EE) layers. The thermosensitive polymer surfaces obtained were evaluated for the growth and harvesting of human fibroblasts (basic skin cells). At 37 °C, seeded cells adhered to and spread well onto the P(TEGMA-EE)-coated surfaces. A confluent cell sheet was formed within 24 h of cell culture. Lowering the temperature to an optimal value of 17.5 °C (below the cloud point temperature of the polymer, T_{CP}, in cell culture medium) led to the separation of the fibroblast sheet from the polymer layer. These promising results indicate that the surfaces produced may successfully be used as substrate for engineering of skin tissue, especially for delivering cell sheets in the treatment of burns and slow-healing wounds.



KEYWORDS: *poly[tri(ethylene glycol) monoethyl ether methacrylate], thermosensitive surfaces, surface-initiated atom transfer radical polymerization, fibroblasts culture, surface modification, cell sheet engineering*

1. INTRODUCTION

Thermosensitive polymer brushes (polymer layers) on solid supports have received great attention and have been explored for a wide variety of applications.[1−4] Such layers are able to change their properties in response to temperature. At a certain temperature (the cloud point temperature (T_{CP})), polymer brushes undergo a phase transition.[4] Below T_{CP}, polymer chains on the surface are hydrated by water molecules and swollen, whereas raising the temperature above T_{CP} leads to their dehydration and shrinkage.[4] This behavior results in a fast switch in surface philicity from hydrophilic to hydrophobic in a manner that is completely reversible. The ability to switch the surface properties by a simple thermal stimulus offers possibilities for the fabrication of smart materials.[2]

The synthesis methods used to obtain thermosensitive polymer brushes on a surface can be divided into two main categories: grafting to and grafting from the surface.[5,6] Grafting to the surface involves the application of effective reactions between functional groups present on the surface and the end-functionalities of the polymer chains. This method can be performed relatively easily, but only a limited amount of polymer can be tethered onto the substrate. The chains that are already attached begin to overlap, thereby forming a barrier that prevents other polymer chains from binding to the support. Grafting from the surface utilizes the polymerization of monomers initiated by an initiator that is attached to the surface. In this method, a high grafting density can be reached. The small molecules of the monomer can easily penetrate the already grafted polymer layer. To obtain well-defined polymer brushes via this method, living/controlled polymerization processes, such as reversible addition−fragmentation chain transfer polymerization (RAFT), atom transfer radical polymer-

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ization (ATRP), and nitroxide-mediated radical polymerization (NMP), have been used.[3,7]

During the past few years, many thermosensitive polymeric materials were designed for biomedical applications so that a variety of novel monomers yielding biocompatible polymers were developed and investigated. Among these monomers, the oligo(ethylene glycol) methacrylates (OEGMA) gained increasing interest.[8,9] Due to the presence of a hydrophobic methacrylate backbone and hydrophilic oligo(ethylene glycol) side chains, poly(oligo(ethylene glycol) methacrylates) (POEGMA) possess amphiphilic character what leads to their thermosensitivity.[9] Oligo(ethylene glycol) side chains are responsible for the solubility and formation of hydrogen bonds with water molecules, whereas the main chain leads to competing hydrophobic interactions. The main advantage of the thermoresponsive mechanism of POEGMA over that of the most commonly investigated poly(N-alkyl acrylamides), such as PNIPAM, is the good reversibility of the process. The heating and cooling behaviors of POEGMA are similar, whereas PNIPAM solutions exhibit hysteresis.[10] Additionally, the T_{CP} of POEGMA in aqueous solutions can be tuned over a wide range of temperatures (20−90 °C) by simply changing the length of the oligo(ethylene glycol) (OEG) side chains or by the copolymerization of different OEGMA monomers.[11,12] These polymers have been grafted successfully onto solid substrates, such as silicon, glass, gold, titanium, iron, and polymer supports, thereby leading to surfaces with temperature-switchable properties.[3,13−18] Surface-initiated ATRP (SI-ATRP) is mainly used to obtain POEGMA brushes on solid substrates.[3]

Both the biocompatibility and the temperature-sensitive nature of POEGMA brushes broaden the scope of their biomedical applications. Below T_{CP}, the hydrophilic nature of oligo(ethylene glycol) side chains is one of the factors preventing the adsorption of proteins, cells, bacteria, and other biological species (for side chains at least five ethylene glycol units in length). This property as one of many makes these polymers suitable for preparing antibiofouling surface coatings.[14−17,19,20] However, above T_{CP}, POEGMA exhibit cell-attractive properties, which lead to the adhesion of cells. This phenomenon was investigated for gold surfaces covered with poly(2-(2-methoxyethoxy)ethyl methacrylate-co-oligo(ethylene glycol) methacrylate) (P(MEO_2MA-*co*-OEGMA)) obtained by the grafting to technique (adsorption of the disulfide-functionalized P(MEO_2MA-*co*-OEGMA) on gold)[21−23] or by grafting from method (polymerization of MEO_2MA and OEGMA comonomers initiated with the macroinitiator layer on an alternating polyelectrolyte interlayers on gold).[24,25] Seeded mouse fibroblast cells and breast cancer cells became adherent to and spread onto this surface as single cells within 2−3 days of cell culture. Afterward, a reduction in the temperature of the cell culture medium led to rounding of the cells, thus indicating the minimization of contact between the surface and single cells.

Cell sheet engineering is a rapidly developing research area.[26,27] The idea of cell sheet engineering assumes culturing of cells in a form of monolayers/sheets on surfaces covered with a thermosensitive polymer and their detachment by cooling the system below the phase transition temperature of the polymer. This idea was initiated and developed by Okano.[26] Until now, although a large group of thermosensitive synthetic polymers has been developed, only poly(N-isopropylacrylamide) (PNIPAM) and its copolymers with oligoethylene glycol methacrylates were used for preparation of polymer layers efficient in cell sheet engineering.[28−32] Mainly, electron beam and plasma polymerization were used to fabricate cross-linked-layers from these polymers on a solid support.[30,31,33,34] However, both ATRP and RAFT polymerization were also used to prepare PNIPAM polymer chains tethered to the surface.[35,36] The obtained PNIPAM surfaces were applied to prepare a variety of cell sheets from different cell types, such as bovine aortic endothelial cells, fibroblasts, muscle cells, kidney cells, cardiac myocytes, urothelial cells, epithelial cells, hepatocytes, and chondrocytes.[33,34,37] However, both the PNIPAM surface preparation techniques and the polymer layer itself possess certain limitations that must be considered when thinking about applications in cell sheet engineering. For example, the e-beam method used to obtain the polymer surface is an expensive and hazardous technique. Moreover, it has been reported that PNIPAM has a tendency to interact with biological compounds such as amino acids or proteins via hydrophobic or hydrogen-bonding interactions.[38,39] Additionally, its phase transition curves show a hysteresis during the cooling process caused by an irreversibility in the coil-to-globule transition due to the incomplete rehydration of PNIPAM chains.[40] Winnik et al. reported recently the relation between the T_g of polymer and its phase transition.[41] According to their findings, polymers showing hysteresis have a T_g above 100 °C. In such cases, the polymer concentration and the rate of heating/cooling affect the hysteresis. Such behavior can however influence interactions with cells because both cell adhesion and detachment are regulated, among others, by the hydration rate of polymer chains on the surface. For poly[tri(ethylene glycol) monoethyl ether methacrylate], the subject of this study, no significant hysteresis of phase transition was reported.

Keeping in mind the issues considered above, in this work, as an alternative for now existing polymer substrates used in cell culture, poly[tri(ethylene glycol) monoethyl ether methacrylate] (P(TEGMA-EE)) brushes on solid support were prepared for formation of separated human skin cell sheets. Effective supports for skin cell culture are of great potential therapeutic importance for the treatment of wounds that are difficult to heal (e.g., burns or diabetes-related injuries). This is an obvious need for developing such active surfaces.

In this work, P(TEGMA-EE) layers were obtained by surface-initiated atom transfer radical polymerization (SI-ATRP) from silicon/glass wafers modified with α-bromoisobutyrate groups. Several surface characterization techniques, such as atomic force microscopy (AFM), contact angle, ellipsometry, and X-ray photoelectron spectroscopy (XPS), were used to monitor the modification steps and to analyze the polymer brushes formed. Culturing of human fibroblasts on P(TEGMA-EE) substrates led to the formation of confluent cell sheets that can be easily and quickly separated as monolayers by simple decreasing the temperature below the T_{CP} of the polymer.

2. EXPERIMENTAL METHODS

2.1. Materials. Ethyl 2-bromo-2-methylpropionate (EBiB, 98%), 2-bromo-2-methylpropionyl bromide (BiBr, 98%), (3-aminopropyl)-triethoxysilane (APTES, >98%), triethylamine (TEA, >99%), tri(ethylene glycol) monoethyl ether methacrylate (TEGMA-EE, M_n = 246 g/mol), copper(I) chloride (CuCl, 99.999%), and 2,2′-bipyridyl (Bpy, 99%) were purchased from Sigma-Aldrich (Poznan, Poland) and used as received. Sulfuric acid (H_2SO_4, 95%, pure p. a.), hydrogen

peroxide (H_2O_2, 30%, pure p. a.), dichloromethane (DCM, 99.8%, pure p. a.), acetone (99.5%, pure p. a.), ethanol (EtOH, 99.8%, pure p. a.), methanol (MeOH, 99.9%, super gradient grade), and water (super gradient grade) were purchased from POCH S.A. (Gliwice, Poland) and used as received. Silicon wafers (p-doped, ⟨100⟩-oriented, 10−20 Ω·cm resistivity) with a thickness of 505−545 μm were purchased from Cemat Silicon S.A. (Warsaw, Poland) and cut into 10 × 10 mm pieces for further modification. Glass wafers (pure white borosilicate glass; diameter, 13 mm; thickness, 0.13 mm) were purchased from VWR International (West Chester, USA). Tissue-culture polystyrene 24-well plates (TCPS) (TPP AG), Dulbecco's modified eagle medium advanced therapy medicinal product (DMEM-ATMP) ready high glucose (4.5 g/L) (+10% fetal bovine serum advanced therapy medicinal product (FBS-ATMP), + 1% L-Glutamina) (PAA Laboratories GmbH), trypsin in ethylenediaminetetraacetic acid (EDTA, PAA Laboratories GmbH), and AlamarBlue (Invitrogen Corporation) were used as received. Silica wafers coated with layers of P(TEGMA-EE) were used for surface characterization with atomic force microscopy (AFM), contact angle, ellipsometry, and X-ray photoelectron spectroscopy (XPS), whereas the glass wafers were used to investigate cell culture and cell detachment.

2.2. Wafer Modification. Silicon/glass wafers were modified in order to introduce reactive amine groups onto the surface, and for that purpose, the method similar to that described in the literature was used.[42−44] The wafers were cleaned in the following steps: degreasing with acetone and rinsing several times with deionized water and then immersing into piranha solution (1:3 v/v mixture of 30% H_2O_2 and 95% H_2SO_4) at 100 °C for 2 h, followed by washing with deionized water, acetone, and deionized water. (*CAUTION*: "Piranha" solution reacts violently with organic materials; it must be handled with extreme care). The freshly prepared hydroxyl-terminated wafers were then immersed in 40 mL of ethanol containing 2 vol % of (3-aminopropyl)triethoxysilane. The solution was stirred at room temperature for 2 h. Next, the wafers were rinsed three times with ethanol and heated at 120 °C. After 24 h, cooled plates were rinsed again three times in ethanol for 15 min and dried under vacuum.

2.3. Preparation of Initiator-Functionalized Wafers. The coupling of the bromide SI-ATRP initiator onto amino-functionalized wafers was performed similarly to the procedure described in ref 45. Briefly, amino-functionalized wafers were dipped into 40 mL of dichloromethane containing 0.41 mL (3.00×10^{-3} mol) of triethylamine. Then, 0.37 mL (3.00×10^{-3} mol) of 2-bromo-2-methylpropionyl bromide was added under argon flow, and the resulting solution was stirred for 4 h at room temperature (20 °C). Afterward, the wafers were rinsed two times in dichloromethane for 15 min and then rinsed two times with ethanol. Finally, the initiator-functionalized wafers were dried under vacuum and stored under an argon atmosphere before use.

2.4. Surface-Initiated ATRP of Tri(ethylene glycol) monoethyl ether methacrylate. The ATRP reaction was performed using a molar ratio of [TEGMA-EE]/[EBiB]/[CuCl]/[Bpy] of 800/1/1/2, respectively. A mixture of methanol and water (24 mL, methanol/water 2:1 v/v) was placed in a Schlenk flask equipped with a stirrer and an argon/vacuum inlet valve. The monomer (TEGMA-EE, 16 mL, 6.63×10^{-2} mol) was dissolved in this solution followed by the addition of the ligand (Bpy, 25.9 mg, 1.66×10^{-4} mol) and the catalyst (CuCl, 8.2 mg, 8.29×10^{-5} mol). The final mixture was degassed by three freeze−vacuum−thaw cycles. Initiator-functionalized wafers were simultaneously placed in the holder, transferred into a separate reactor (100 mL volume), and degassed by three vacuum-argon filling cycles. After the initiator (EBiB, 12.2 μL, 8.29×10^{-5} mol) was introduced to the polymerization reaction mixture, the polymerization solution obtained was quickly transferred under an argon atmosphere to a separately prepared reactor containing functionalized wafers. Surface-initiated ATRP was carried out at room temperature. Reaction mixture samples were taken out for GPC-MALLS analysis after various polymerization times (1, 2, 3, 4, 6, and 21 h). Wafers with polymer layers (taken out after the same time of polymerization) were characterized and used for cell culture studies. Before surface characterization, the wafers were rinsed three times in acetone for 30 min, then rinsed three times with ethanol, and dried under vacuum.

2.5. Cell Assay. Glass wafers for cell culture and detachment studies were sterilized by incubation in 70% ethanol for 4 h. The ethanol was then exchanged with ultrapure deionized water followed by an exchange with cell culture medium. DMEM-ATMP supplemented with glucose (4.5 g/L), 10% FBS-ATMP, 1% L-glutamine, and 1% antibiotics (penicillin, streptomycin, amphotericin B) was used as a culture medium.

Cell Adhesion and Detachment Assay. The wafers were transferred to a 24-well TCPS plate filled with 2 mL of culture medium and were then incubated at 20 °C for 3 h. The plate containing the wafers was then heated to 37 °C in a cell culture incubator for 3 h. Human fibroblasts (derived from the Cell Bank at the Centre for Burn Treatment in Siemianowice Slaskie) were recovered from the TCPS plates using 0.25% trypsin in 1 mM EDTA (during the fifth or sixth passage) and were then seeded on a preheated layer of P(TEGMA-EE) at a concentration of 1×10^4 cells/cm^2. As a positive control, a tissue-culture polystyrene (24-well plate) was used. Cell culture was carried out using a cell culture incubator at 37 °C with the air enriched with 5% CO_2. Cell attachment, viability, and morphology were assessed after 2.5, 4, 8, 12, 24, and 72 h of culture. For these experiments, growth medium containing unattached cells was removed, and the attached cells were rinsed once with culture medium (preheated to 37 °C) and were incubated for 4 h in 400 μL of the appropriate fresh medium supplemented with 10% AlamarBlue. An aliquot (200 μL) of culture medium from each well was spectrophotometrically analyzed, and the cell count was estimated as a percent of the control. Cell detachment from the P(TEGMA-EE) layer was performed for samples on which fibroblasts were cultured for 24 h. After that time, the wafers were then incubated at 17.5 °C, and cell detachment was observed with time. Experiments for each of the cell culture times were performed for four samples and statistically analyzed.

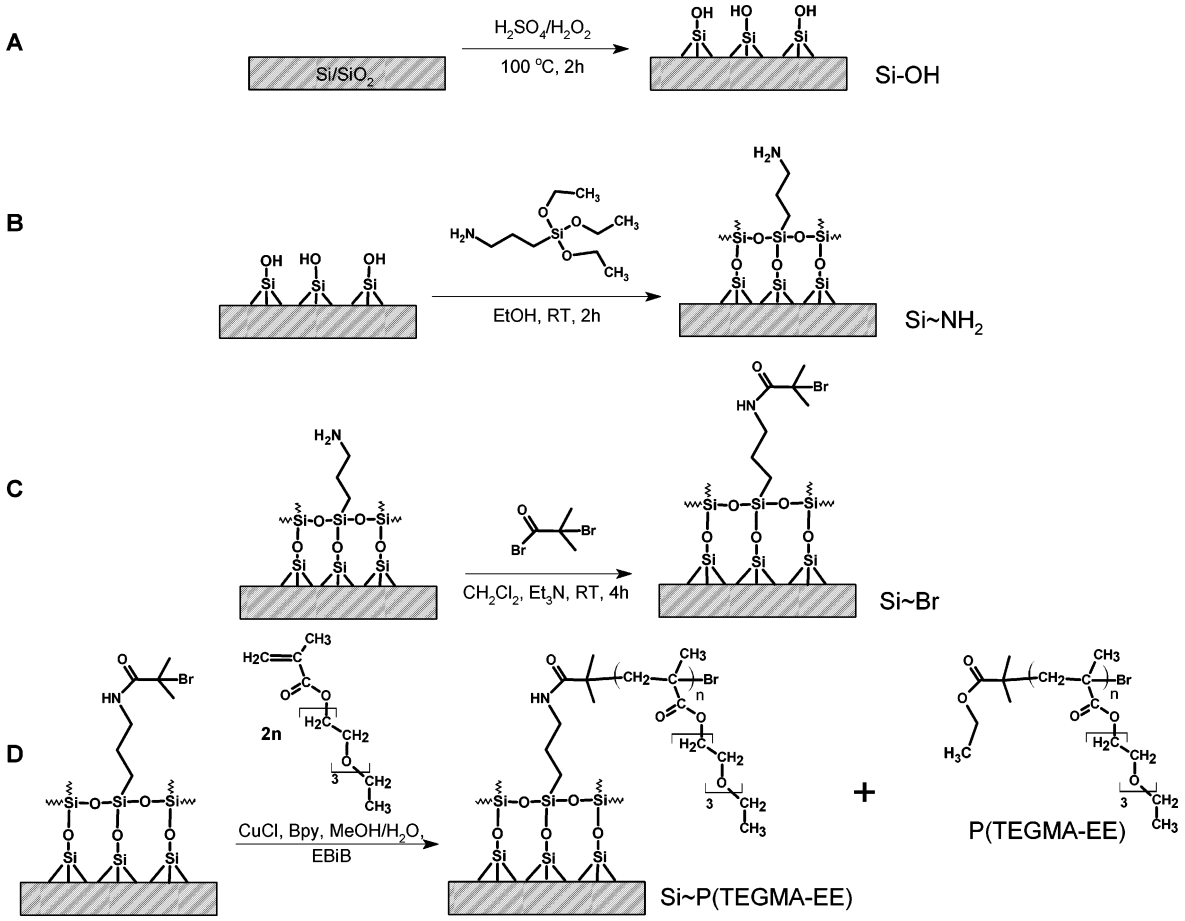
Cell Sheet Formation and Detachment. To prepare and recover a fibroblast sheet, cells were seeded on the P(TEGMA-EE) layer (wafer sterilization and conditioning were performed as described above) with a density of 1.5×10^5 cell/cm^2. Cell culture was performed in cell culture medium at 37 °C using air enriched with 5% CO_2. After reaching the confluent cell sheet (24 h), the wafers were incubated at 17.5 and 21 °C, and cell detachment was observed for various time periods.

2.6. Characterization. *Gel Permeation Chromatography.* Gel permeation chromatography (GPC-MALLS) analysis of P(TEGMA-EE) in *N*,*N*-dimethylformamide (DMF) with 5 mmol/L of LiBr was performed at 45 °C using a multiangle light scattering detector (DAWN EOS, Wyatt Technology, λ = 658 nm), refractive index detector (Dn-2010 RI, WGE Dr. Bures), and column system (100 Å PSS GRAM column from PSS and two PL gel MIXED-C columns from Polymer Laboratories). The nominal flow rate was 1 mL/min. The results were evaluated with ASTRA 5.3.4.10 software from Wyatt Technologies. The refractive index increment (dn/dc) of poly[tri(ethylene glycol) monoethyl ether methacrylate] in DMF was measured independently to 0.0467 mL/g and used for the calculations.

Cloud Point Measurements. The cloud point of the P(TEGMA-EE) polymer in both water and culture medium was measured using a Jasco V-530 UV−vis spectrophotometer equipped with a cuvette thermostatted by Medson MTC-P1 thermocontroller. The transmittance was monitored as a function of temperature at a wavelength λ = 700 nm. The cloud point value was determined as the temperature at which the transmittance of the polymer solution (both water and culture medium, 5 g/L) reached 50% of its initial value.

Atomic Force Microscopy. Atomic force microscopy (AFM) was performed in atmospheric air, in tapping mode with a MultiMode AFM microscope equipped with a NanoScope 3D controller (Veeco Instruments, Inc., USA) with 125 nm single-crystal silicon cantilevers (Model TESP, Veeco Instruments Inc., USA). In each case, areas of different scan sizes (from 500 nm × 500 nm up to 10 μm × 10 μm) were investigated. Images were recorded at different surface points using NanoScope Software. Root mean square roughness (RMS)

Scheme 1. Four-Step Synthesis of Poly[tri(ethylene glycol) monoethyl ether methacrylate] Layers on Glass and Silicon Substrates[a]



[a](A) Hydroxylation, (B) aminosilylation, (C) formation of initiator-functionalized wafers, and (D) surface-initiated ATRP of TEGMA-EE.

values for each sample were averaged from three measurements of different cross-sections of the images.

The "scratching" experiment was performed by scanning the wafers with the tip in contact mode under relatively high load, followed by tapping mode imaging of the surrounding area. A 1 μm × 2 μm area of the P(TEGMA-EE) film was scanned in contact mode at a scan angle of 45°, a scan rate of 0.5 Hz, and a scan resolution of 64 × 128 lines. The AFM cantilever was deflected by controlling the deflection set point value of the AFM control system. The optimal value was found to be approximately 6.0 V. After the experiment was accomplished, the AFM mode was changed to tapping, and a 5 μm × 5 μm area of the P(TEGMA-EE) film with a wear field was scanned at a scan angle of 0°, a scan rate of 1 Hz, and a scan resolution of 256 × 256 lines. A single topographical cross-section was measured over the wear region to give an approximate P(TEGMA-EE) film thickness.

Ellipsometry. Ellipsometry measurements of the polymer surfaces were carried out on an EP3 Nanofilm spectroscopic imaging ellipsometer with a He−Ne laser (λ = 658 nm), equipped with a xenon lamp for spectroscopy (λ = 360−1001.3 nm). Measurements were performed for dry and wet polymer surfaces. The thickness and optical properties (refractive index) of the polymer film attached to the surface were calculated by the fitting of an appropriate optical model to the measured values of Ψ and Δ. The refractive index was found using multiple-angle-of-incidence analysis (50°−80° for λ = 510.3 nm). The thickness of each layer deposited on the surface was then determined at a 75° angle (for silica wafers) of the incident beam in the wavelength range of 350−1100 nm. Measurements of the wet surfaces were performed at both 20 and 37 °C in a thermostatted chamber. Prior to measuring, the surfaces were incubated in water at the desired temperature for 30 min. The measurements were carried out at an incident angle of 60° with a wavelength of 510.3 nm. The results reported are the average of 20 measurements. Each value of thickness reported was accurate to 0.4−0.7 nm. Analysis of the layer thickness was performed using a Cauchy relation. The final thickness of the polymer layer after grafting was calculated using a multilayer model consisting of a silica wafer, hydroxylated layer, aminosilylated layer, and initiator layer.

Contact Angle. Contact angles (CAs) for modified wafers and P(TEGMA-EE) layers were measured statically using a goniometer CAM101 with a temperature controller (Intelligent digital controller OMRON 5EGN, accuracy ±1 °C). A series of images for the water drop (~3 μL) were acquired during 30 s, and the average CA value was calculated. Measurements were performed for dry and wet surfaces (at 20 and 37 °C). Prior to measurement of the CA of the wet surfaces, the samples were incubated in water for 3 h at the desired temperature and then transferred to a thermostatted chamber, which was connected to a temperature controller. The average of six values of the contact angle from different parts of the sample was used.

X-ray Photoelectron Spectroscopy. The X-ray photoelectron spectroscopy (XPS) measurements were performed on a PHI5700/660 spectrometer using an Al Kα monochromatic X-ray source with an energy of 1486.6 eV and an energy resolution of 0.3 eV. All photoelectron spectra were calibrated against the peaks of Au 4f7/2 at 83.98 eV, Ag 3d5/2 at 368.27 eV, and Cu 2p3/2 at 932.67 eV of binding energy. The quantitative surface composition (atom %) was calculated for each element using the areas of the peaks.

Phase Contrast Fluorescence Microscopy. Fibroblasts were photographed at predetermined time periods using a phase contrast

fluorescence inverted microscope (IX71, Olympus, Japan) equipped with a digital camera (XC50, Olympus, Japan).

3. RESULTS AND DISCUSSION

The grafting from strategy has been applied to obtain thermosensitive poly[tri(ethylene glycol) monoethyl ether methacrylate] brushes. To enable the surface-initiated atom transfer radical polymerization (SI-ATRP), wafers were subjected to chemical modification followed by immobilization of a bromide initiator. The properties of the P(TEGMA-EE) layer were studied by XPS, AFM, contact angle goniometry, and ellipsometry. Afterward, the skin cell (fibroblast) culture and cell detachment from the prepared polymer layer were examined.

3.1. Synthesis and Properties of P(TEGMA-EE) Surfaces. A four-step method for the synthesis of P(TEGMA-EE)-grafted wafers was applied. The general procedure is depicted in Scheme 1.

The modification of glass/silica substrates (steps A, B, and C in Scheme 1) was carried out through hydroxylation, aminosilylation, and the introduction of initiator according to similar methods described in the literature.[42−45] Briefly, the wafers were first treated by "piranha" solution, which removed all organic residue from the wafers and produced hydroxyl-terminated surfaces (abbreviated as Si−OH surfaces, Scheme 1A). Next, the wafers were subjected to an aminosilylation process using a (3-aminopropyl)triethoxysilane (APTES) coupling agent (Si∼NH_2 interlayer, Scheme 1B). The presence of amine groups on the surface enabled the immobilization of the ATRP initiator (2-bromo-2-methylpropionyl species, Si∼Br interlayer, Scheme 1C) by reaction of the amines with acyl bromide groups. Wafers with coupled bromide initiator were used to initiate the ATRP of TEGMA-EE (the Si∼P(TEGMA-EE) layer, Scheme 1D).

It is known that in the ATRP initiated from flat surfaces the very low concentration of initiator (and thus dormant radicals) on the surface leads to uncontrolled chain growth.[46] This problem can be overcome by the addition of a "free" initiator (sometimes called a sacrificial initiator), not bounded to the surface, which increases the overall concentration of initiator (also the dormant radical concentration) to the required level. As a result, polymerization occurs both on the wafer surface and in solution. According to the literature, the molar masses of polymer chains obtained in solution are comparable with those of polymers obtained on the surface.[47]

In this work, SI-ATRP of TEGMA-EE was conducted in the presence of ethyl 2-bromo-2-methylpropionate (EBiB) as a "free" initiator added to the polymerization solution (Scheme 1D). The monomer concentration and the molar ratio of [TEGMA-EE]/[EBiB]/[CuCl]/[Bpy] (800/1/1/2) were constant in all experiments. After certain reaction times, the P(TEGMA-EE) samples from the solution were taken for GPC-MALLS analysis, and at the same time, the Si∼P(TEGMA-EE) wafers were removed from the reaction solution for surface analysis.

As seen in Table 1, the molar masses of P(TEGMA-EE) formed in solution during polymerization increased with increasing reaction time. The chromatogram peaks of P(TEGMA-EE) homopolymers were narrow (Figure 1). Depending on the polymerization time, the number average molar masses of P(TEGMA-EE) ranged from 23 000 to 189 000 g/mol, meaning 90 to 770 TEGMA-EE units in the polymer chains. The molar mass dispersities (M_w/M_n) of

Table 1. Molar Masses and Dispersities of P(TEGMA-EE) Homopolymers Formed in Solution

polymerization time (h)	M_n (g/mol)	M_w/M_n
1	23 000	1.23
2	30 000	1.24
3	42 000	1.24
4	50 000	1.24
6	76 000	1.28
21	189 000	2.43

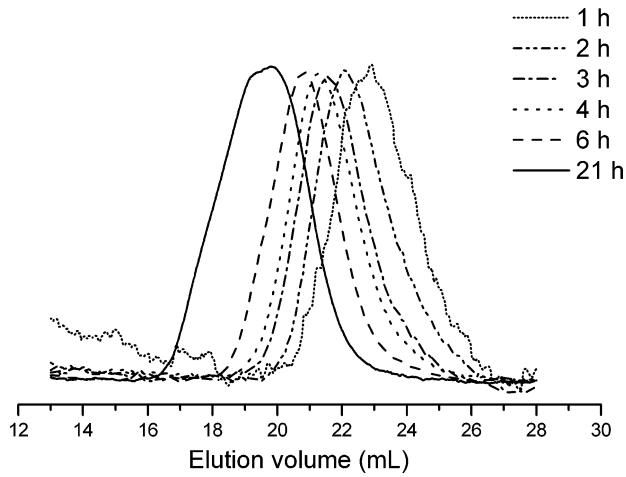


Figure 1. GPC-MALLS chromatograms (RI traces) of P(TEGMA-EE) formed in solution after different polymerization times.

P(TEGMA-EE) were similar and lower than 1.3 for low polymerization times and increased up to 2.4 for polymer obtained after 21 h of polymerization. On the basis of ref 47, we assumed that the molar mass of polymer formed on the surface was similar to that of polymer formed in solution.

The surface composition, morphology, wettability, and thickness were investigated after each modification step (Si−OH, Si∼NH_2, Si∼Br, Si∼P(TEGMA-EE)). Surface composition data obtained by X-ray photoelectron spectroscopy are presented in Table 2.

Table 2. Surface Composition of Modified Wafers As Determined by X-ray Photoelectron Spectroscopy

	elements (atom %)				
modification step	C	N	O	Si	Br
Si−OH	17.2	0.7	57.4	24.7	0.0
Si∼NH_2	28.8	2.7	47.7	20.8	0.0
Si∼Br	25.9	2.2	51.3	20.5	0.1
Si∼P(TEGMA-EE)-21h[a]	72.5	0.0	26.2	1.23	0.0

[a]P(TEGMA-EE) layer obtained after 21 h of synthesis.

Quantitative analysis of the surface composition revealed that the surface of the wafers after "piranha" solution treatment (Si−OH) is mainly composed of O and Si derived from surface oxides. These results are in good agreement with those reported in the literature.[48] The aminosilylation process leading to the Si∼NH_2 interlayer increased the content of carbon on the surface. It is connected with the presence of propyl groups that are derived from (3-aminopropyl)triethoxysilane. The surface concentration of nitrogen also increased, thus confirming the introduction of amine groups onto the surface.

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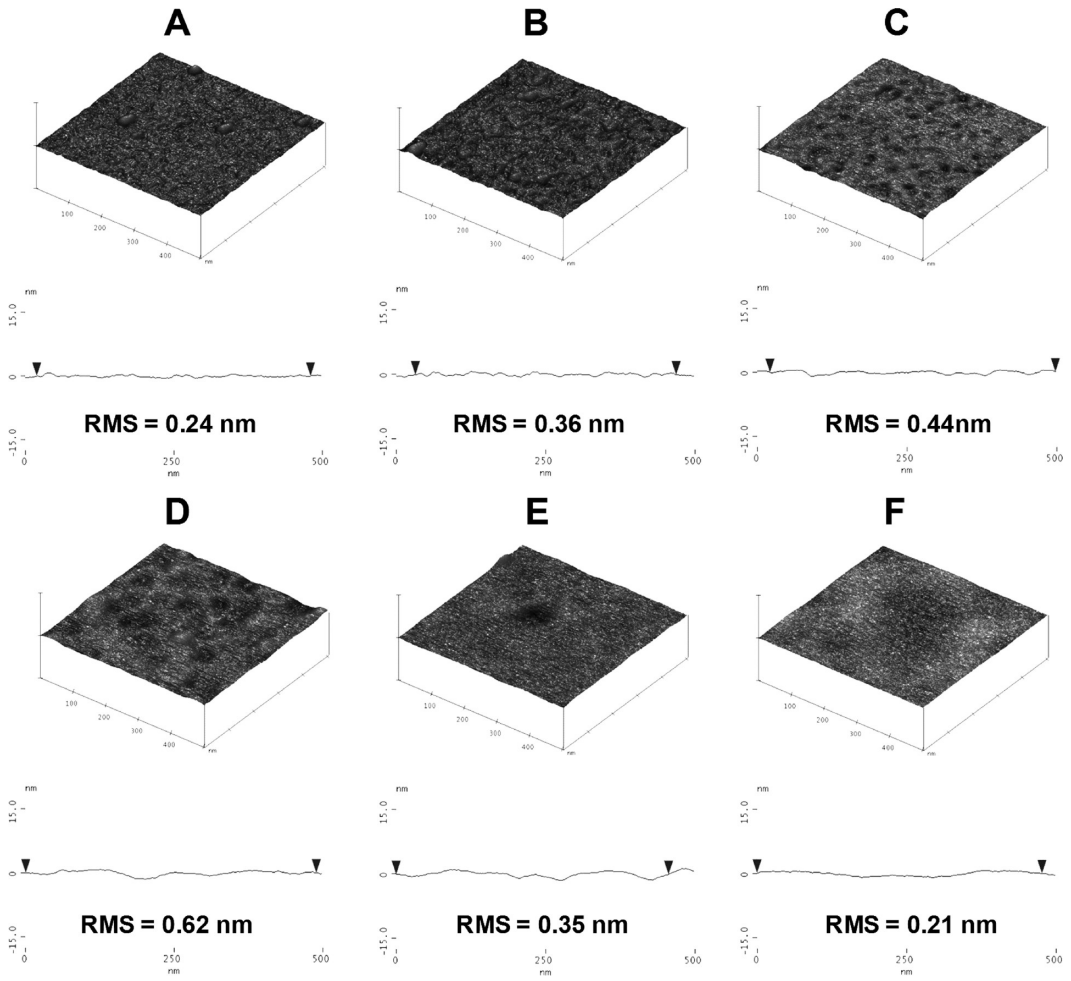

Figure 2. AFM images of Si~P(TEGMA-EE) layers and the root-mean-square values of their roughness obtained after (A) 1, (B) 2, (C) 3, (D) 4, (E) 6, and (F) 21 h of polymerization time.

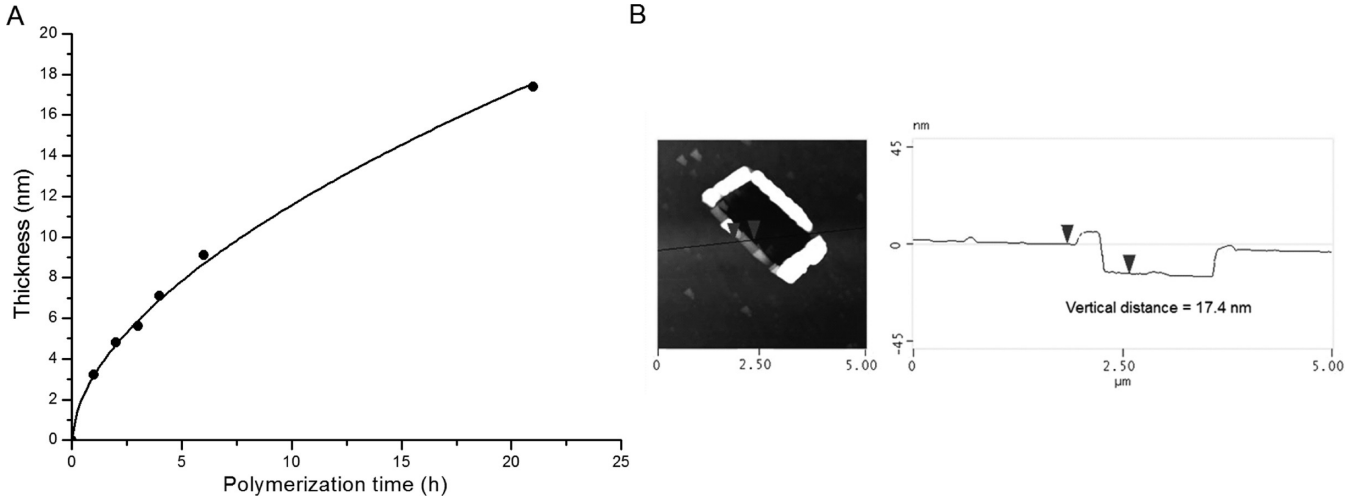

Figure 3. (A) Evolution of film thickness as a function of polymerization time for the SI-ATRP of TEGMA-EE (the line through the data points serves as a guide to the eye only). (B) AFM image of the wear area made on Si~P(TEGMA-EE)-21h film and its cross-section.

The signals for oxygen and silicon were attenuated by an overlayer of (3-aminopropyl)silane.

Successful anchoring of the initiator is reflected in the presence of a bromine signal on the Si~Br interlayer spectrum. The oxygen content of the Si~Br interlayer increased as a result of the presence of oxygen in the initiator molecule. The nitrogen signal derived from amine groups was attenuated by the attached initiator.

The formation of P(TEGMA-EE) chains on the modified wafers caused a decrease in the Si signal intensity from 20.5 atom % for Si~Br to 1.23 atom % for the P(TEGMA-EE)-21h layer due to coverage of the Si surface with a polymer layer. A

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substantial increase in the C/O ratio from 0.50 for the Si∼Br interlayer to 2.77 for the P(TEGMA-EE)-21h demonstrates the formation of the Si∼P(TEGMA-EE) external layer. The nitrogen signal from the lower interlayer was completely attenuated after surface-initiated ATRP of TEGMA-EE. The signal from Br-end groups disappeared, mostly due to its loss during surface preparation before XPS analysis but also the loss of Br signal can be connected with its exchange to the chlorine atoms coming from the catalyst complex.[49,50] X-ray photoelectron spectroscopy results confirmed that all surface modification steps were successful and led to the formation of P(TEGMA-EE) brushes on the wafers. Atomic force microscopy was used to investigate the morphology of the wafers after modification. The images of Si−OH, Si∼NH_2, and Si∼Br glass surfaces are presented in Figure S1 in Supporting Information. The changes of surface morphology of the Si∼P(TEGMA-EE) layer with increasing polymerization time are shown in Figure 2.

The morphology of the polymer surfaces changed with polymerization time. The RMS value of surface roughness increased from initially 0.24 nm to a maximum value of 0.62 nm for samples obtained after 4 h of polymerization. After exceeding this time, the RMS value started to decrease. These variations, connected with elongation of the grafted P(TEGMA-EE) chains, led to more uniform surface morphology due to the surface coverage with polymer film (Figure 2, wafer after 6 and 21 h).

Ellipsometry was used to measure the thickness of the interlayer after each step of wafer modification and to monitor the increase in polymer film thickness during SI-ATRP of TEGMA-EE. The thickness of the Si∼NH_2 interlayer was 1.6 nm. Immobilization of the ATRP initiator increased the thickness to approximately 2.1 nm. The thickness of P(TEGMA-EE) layer as a function of polymerization time is shown in Figure 3A.

The refractive index value of P(TEGMA-EE) used for the calculations of layer thickness was assumed to be 1.50 and was based on the literature data for P($OEGMA_{300}$) and P(DEGMA-ME-co-$OEGMA_{475}$).[51,52] The P(TEGMA-EE) layer thickness varied between 3 and 18 nm depending upon the polymerization time. The thickness of the immobilized silane/Br-initiator was subtracted from the overall film thickness. Within the first 6 h of polymerization, a rapid increase in the polymer layer thickness was observed. Further increase in the polymer layer thickness with prolonged polymerization time was slowed down due to a decrease in the monomer concentration in the reaction system, loss of the active chain ends, or a reduced rate of monomer diffusion.[53,54]

The thickness of the Si∼P(TEGMA-EE) layer obtained after 21 h of polymerization was also established by AFM. For that purpose, the polymer layer was "scratched" using the AFM tip, and the average depth of the hollow was measured. Figure 3B shows a topographic image of this sample with 1 × 2 μm scratched area and its corresponding cross-section. The height of the layer in the regions surrounding the rectangular wear area increased, thus indicating that the polymer was physically removed by the AFM tip and deposited around the perimeter. The average depth of the hollow was 17.4 nm and is quite similar to the thickness measured by ellipsometry (18 nm).

As mentioned earlier, the molar masses of the "free" polymer chains formed in solution surrounding the wafer can be assumed to be comparable with those formed simultaneously on the wafer surface.[47] Taking it into account, the calculation of the grafting density of poly[tri(ethylene glycol) monoethyl ether methacrylate] chains on wafers was performed using the following equation:[55]

$$\sigma = \frac{h\rho N_A}{M_n}\cdot 10^{-21}$$

where the σ is the grafting density (chains/nm^2), h is the dry layer thickness measured by ellipsometry (nm), ρ is the density of the polymer layer (1.0 g/cm^3 was assumed for P(TEGMA-EE) like for other POEGMAs),[51] N_A is Avogadro's constant, and M_n (g/mol) is the number-average molar mass of the P(TEGMA-EE) chains grafted onto the surface. The calculated grafting density for the Si∼P(TEGMA-EE) layers obtained after 1−6 h of polymerization was approximately 0.1 chains/nm^2. This value is lower than that estimated by the same equation for previously described P($OEGMA_{300}$)[56] and P(DEGMA-ME)[57] brushes obtained by SI-ATRP, which ranged from 0.17 to 0.41 chains/nm^2. This dissimilarity can be explained by the use of a different method to determine the molar mass. In our case, GPC with light scattering detection was applied, which gave the absolute molar masses, whereas in the other studies, polystyrene calibration was used to provide the apparent values of the molar masses. Additionally, the two-step procedure applied in this work of binding the initiator to the surface can lead to a lower concentration of initiator, thereby leading to a lower grafting density.

The contact angle measurements in the sessile drop configuration provided detailed information about the surface philicity. The variation in the philicity of the modified wafers is shown in the Supporting Information (Figure S2A−C). The covering of the surface with P(TEGMA-EE) layer resulted in an increase in the surface hydrophilicity compared with the Si∼Br interlayer (Figure S2D, Supporting Information). The contact angle of the dry Si∼P(TEGMA-EE)-21h equal to 68° ± 1° is lower than that for Si∼Br for about 8−10°. In the literature, the static contact angle of POEGMA polymer brushes on titanium wafers has been observed in a range of 40−52° for oligo(ethylene glycol) (OEG) side chains with 4−23 hydrophilic ethylene glycol units terminated with a methyl group.[58] In our case, only three ethylene glycol units in side chains end-capped by ethyl group are present, which leads to a lower hydrophilicity of the polymer layer and thus higher contact angle values (68° ± 1°).

3.2. Thermosensitive Behavior of P(TEGMA-EE) Polymer and Surfaces. In order to use the prepared polymer surfaces for temperature-induced cell culture and detachment, they have to exhibit thermosensitive behavior upon temperature alteration. The cloud point transition (T_{CP}) of the P(TEGMA-EE) homopolymer in aqueous solution measured by UV−vis was 23 °C (Figure 4, filled circles). Because cell culture is performed in cell culture medium, the thermosensitivity of this polymer dissolved in cell culture medium was also measured (Figure 4, open circles).

Abrupt temperature-induced phase transitions of P(TEGMA-EE) homopolymer were observed in both media. The transitions of P(TEGMA-EE) in water and cell culture medium were narrow and spanned just 1 °C. The transmittance curve drawn in Figure 4 clearly indicates that the phase transition temperature in cell culture medium was shifted to lower values by approximately 3 °C compared to the transition temperature of P(TEGMA-EE) in water. Such behavior is the result of a weak salting-out effect of the cell culture medium. A similar

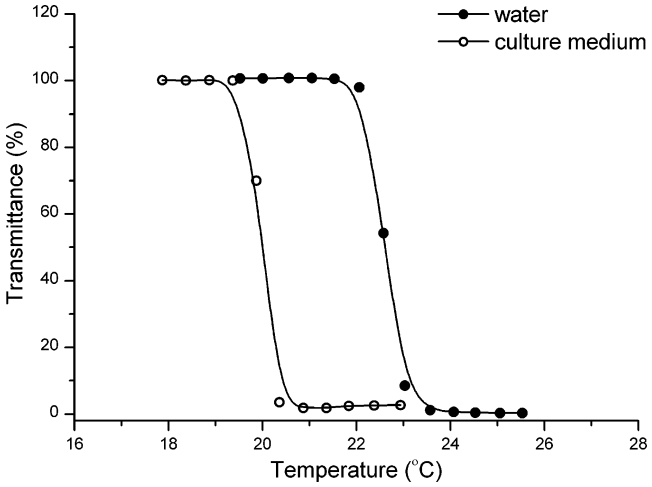

Figure 4. Phase transition temperature of P(TEGMA-EE) measured in water and in cell culture medium (DMEM-ATMP) for polymer concentration equal to 5 g/L (molar mass of the P(TEGMA-EE) was 76 000 g/mol).

shift in T_{CP} was observed for P(MEO_2MA-*co*-OEGMA) in phosphate buffered saline.[25]

Thermosensitive behavior of Si~P(TEGMA-EE) surfaces was observed in water below (20 °C) and above (37 °C) the phase transition temperature of P(TEGMA-EE) using contact angle measurements and ellipsometry. The average contact angle of all wet Si~P(TEGMA-EE) surfaces at 20 °C (below T_{CP} of P(TEGMA-EE)) was 65° ± 2°, indicating a hydrophilic nature of the polymer layer. An increase in temperature above 37 °C was accompanied by an increase in the average water contact angle to 77.5° ± 2°. This behavior reflects temperature-dependent changes in the philicity of all Si~P(TEGMA-EE) layers. As an example, the images of water droplets on the wet Si~P(TEGMA-EE)-21h is given in the Supporting Information (Figure S3). Figure 5 presents the changes of P(TEGMA-EE) layers thickness in response to temperature changes.

An increase of the temperature to 37 °C (that is, above the T_{CP} of the deposited P(TEGMA-EE)) resulted in a decrease of the polymer layer thickness caused by shrinkage of the polymer chains tethered to the solid surface. At 20 °C, the P(TEGMA-EE) chains adopted an extended conformation due to a chain solvation effect with water molecules. Above the T_{CP}, the P(TEGMA-EE) brushes collapsed due to breaking of the hydrogen bonds between water molecules and the polymer chain, thereby leading to a decrease in the polymer layer thickness. This behavior reflects the thermosensitive properties of all prepared polymer substrates.

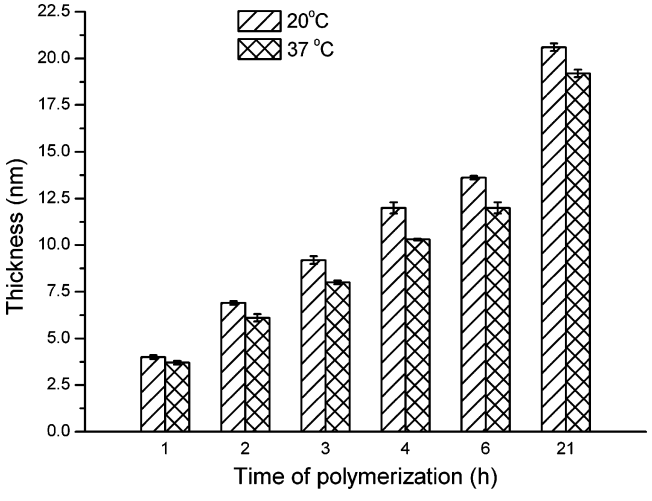

Figure 5. P(TEGMA-EE) layers thickness versus polymerization time for samples incubated in water at 20 and 37 °C.

3.3. Cell Culture and Harvesting. Si~P(TEGMA-EE)-6h and Si~P(TEGMA-EE)-21h were chosen as representative samples to investigate the fibroblast culture and cell detachment from the polymer layers. The thermosensitivity of the polymer film was utilized for temperature-induced detachment of individual skin cells and, more importantly, for the isolation of cell sheets without loss of their integrity.

Cells were seeded at 37 °C on the Si~P(TEGMA-EE)-6h and Si~P(TEGMA-EE)-21h surfaces and on a control surface, tissue culture polystyrene (TCPS). At this temperature, well above the T_{CP} of P(TEGMA-EE), the polymer layers were hydrophobic and favored cell adhesion and proliferation.

Figure 6 shows the adhesion and proliferation profiles of fibroblasts on the Si~P(TEGMA-EE)-21h surface. The number of adherent cells were shown as percentage normalized to the number of cells on the control TCPS samples for each experiment.

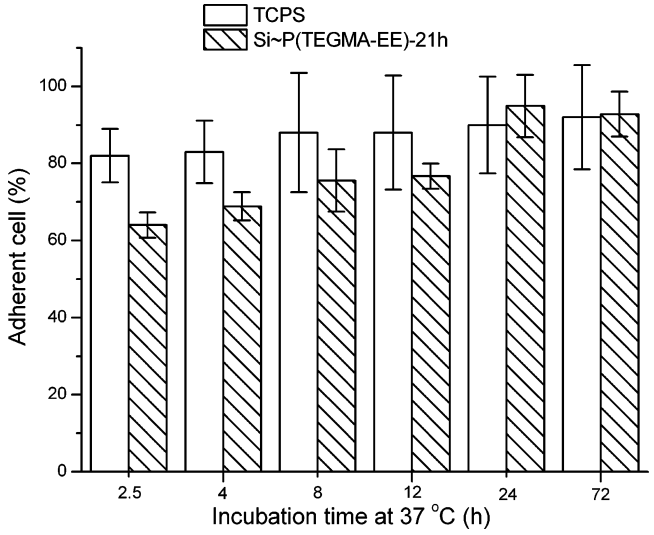

Figure 6. Fibroblasts adhesion/proliferation profiles on the TCPS and Si~P(TEGMA-EE)-21h surfaces. Cells were seeded at a density of 1 × 10^4 cells/cm^2 and then incubated at 37 °C. The number of adherent cells were counted by the use of the Alamar Blue test and averaged over four separate experiments.

As seen in Figure 6, after 2.5 h of cell culture, almost 60% of fibroblasts adhered to the Si~P(TEGMA-EE)-21h layer. When the culture time was prolonged, cell viability increased, and the cells spread and proliferated. The single fibroblast adhesion and spreading is also presented on microscopy images in Figure 7A.

When the samples were cooled, rapid single cell rounding was observed, indicating cell detachment (Figure 7B). The detachment of single cells was performed at 17.5 °C because the phase transition temperature for P(TEGMA-EE) homopolymer measured in cell culture medium was 20 °C (see Figure 4).

To prepare the fibroblast sheet, the cells were seeded on Si~P(TEGMA-EE) layers at 1.5 × 10^5 cell/cm^2 and then were

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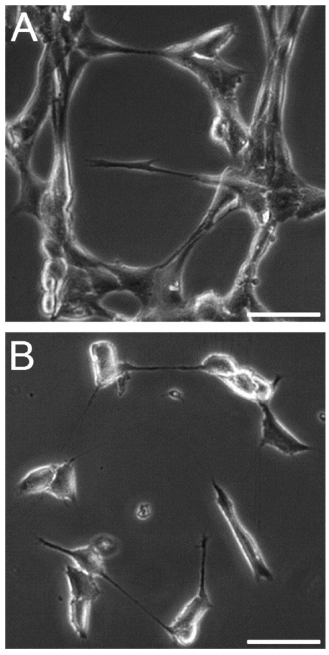

Figure 7. Images of single fibroblasts on the Si∼P(TEGMA-EE)-21h surface after: (A) 24 h of incubation at 37 °C and (B) 20 min after cooling the sample to 17.5 °C. The scale bars are 100 μm.

cultured at 37 °C. After 24 h, fibroblasts proliferated confluently on both samples Si∼P(TEGMA-EE)-6h and Si∼P(TEGMA-EE)-21h, and cell sheet formation was observed (Figure 8B).

Afterward, the temperature was reduced to 17.5 °C, below T_{CP} of the polymer in cell culture medium. Quick detachment of fibroblast sheet in a form of monolayer was observed (Figure 8C−E).

Confluently cultured fibroblasts were detached as a cell sheet from thermosensitive Si∼P(TEGMA-EE) layers by reducing the temperature below T_{CP} of the polymer. A series of experiments showed that the optimal temperature for whole cell sheet detachment was below 18 °C. In the temperature range of 18−21 °C, effective cell detachment was not observed; some of the cells detached as cell clusters, whereas for the rest of the cells, no detachment was detected. This led to the disruption of the cell sheet continuity. These results showed that, in order to obtain a fibroblast monolayer, the detachment temperature should be below the T_{CP} of the P(TEGMA-EE) homopolymer measured in the culture medium.

The entire fibroblast monolayer was detached from both P(TEGMA-EE)-21h and Si∼P(TEGMA-EE)-6h surfaces within 40−60 min of incubation at 17.5 °C. The temperature of cells detachment equal to 17.5 °C is rather low, also in view of the slower cell metabolism at this temperature. However, the P(TEGMA-EE) surfaces can efficiently detach the whole, intact monolayer of cultured fibroblasts at this temperature, what has not previously been observed. In the literature, only the culture of single cells on poly(2-(2-methoxyethoxy)ethyl methacrylate-co-oligo(ethylene glycol) methacrylate) has been described.[21−25] For that polymer surface, cells were adhered for 2 days of incubation in 37 °C, and their detachment was observed as single cell rounding, not as a formed sheet.

Nevertheless, a wide range of available OEG(M)A monomers (acrylates or metacrylates with different length of ethylene glycol units containing different end-functionalities) permits one to adjust, also by copolymerization, the phase transition temperature of the (co)polymers on the surface in a wide range. Therefore, the Si∼P(TEGMA-EE) polymer layer appears to be a good candidate as a substrate to obtain fibroblast sheets, needed in the treatment of wounds and in skin tissue engineering. The detailed studies concerning the dynamics of cell attachment, genotoxicity of the polymer layer,

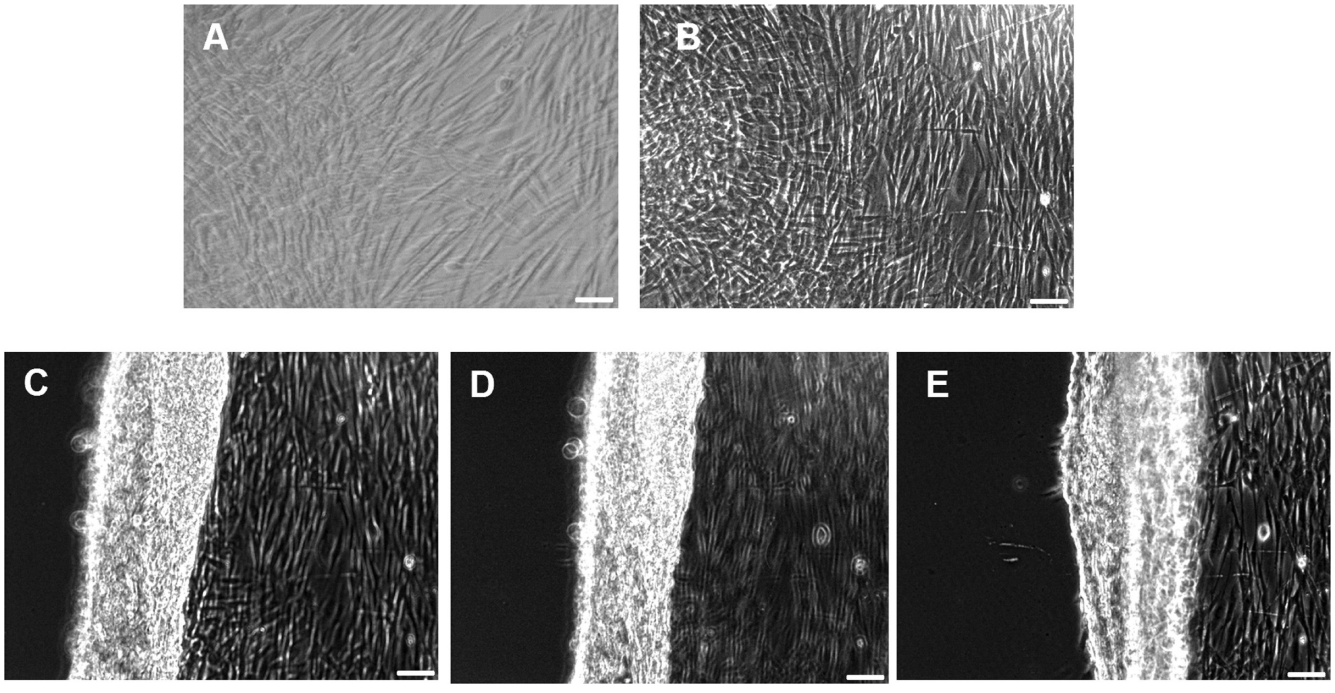

Figure 8. Images of human fibroblasts on (A) TCPS, (B) Si∼P(TEGMA-EE)-21h surface after 24 h of incubation at 37 °C and after (C) 5 min, (D) 10 min, and (E) 20 min of incubation at 17.5 °C. The scale bars are 100 μm.

and cell phenotype after culture on the polymer layer, which are in progress, are promising and will be discussed separately.

4. CONCLUSION

Well-defined poly[tri(ethylene glycol) monoethyl ether methacrylate] brushes grafted onto glass and silicon wafers were obtained by surface-initiated atom transfer radical polymerization. The layers thickness was easily controlled by changes of the polymerization time. The P(TEGMA-EE) brushes obtained were thermosensitive and changed their properties upon temperature alteration. An increase in temperature above the T_{CP} of P(TEGMA-EE) caused shrinking of the polymer chains and changed the surface philicity to hydrophobic. At this temperature, the surface properties of P(TEGMA-EE) make them very effective as a support for human fibroblast adhesion, spreading, and proliferation. Decreasing the temperature below the T_{CP} of P(TEGMA-EE) (that measured in cell culture medium) led to simple cell sheet detachment. Changing the temperature allowed one to switch the properties of the P(TEGMA-EE) surfaces from cell-attractive (hydrophobic) to cell-repellent (hydrophilic). Thus, the P(TEGMA-EE) polymer layers were successfully applied to create and detach confluent fibroblasts sheets without requiring mechanical or enzymatic methods for cell detachment. P(TEGMA-EE) layers may successfully replace the traditionally used supports for cell harvesting. These promising results indicate that the surfaces produced here may be used as a scaffold for tissue engineering.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Results of the AFM and contact angle analysis of modified surfaces. This information is available free of charge via the Internet at http://pubs.acs.org/.

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Notes

The authors declare no competing financial interest.

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