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Endothelial, Smooth Muscle and Fibroblast Cell Sheet Fabrication from Self-assembled Thermoresponsive Poly(glycidyl ether) Brushes

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Keywords

Cloud point temperature, benzophenone-driven aggregation, block-copolymer adsorption, UV-induced C, H-insertion, pancake-to-brush transition, switchable surfaces

Materials

Materials for surface modification. All chemicals and solvents were purchased from Sigma Aldrich (Steinheim, Germany) and used as received unless stated otherwise. Acetone and ethanol applied for surface modification and cleaning were distilled under reduced pressure before use to remove impurities. Bare Si wafers with a 2 nm SiO_2 layer were supplied by Silchem GmbH (Freiberg, Germany), cut into quadratic pieces (11 x 11 mm), washed with ethanol and dried under a stream of N_2. Gold-coated QCM-D sensor chips were obtained from Q-Sense LOT-QuantumDesign (Darmstadt, Germany).

Materials for cell culture. Falcon® PS culture dishes (d = 35 mm) were purchased from Th. Geyer GmbH & Co. KG (Berlin, Germany). Tissue culture PS dishes (d = 35 mm), 24-well TCPS plates and thermoresponsive 24-well culture plates with a hydrogel-like PNIPAM coating (UpCell™) were supplied by VWR International (Leuven, Belgium) and used as received. 24-well PS plates and Cellstar TCPS culture flasks were purchased from Greiner Bio-One GmbH (Frickenhausen, Germany). Dulbecco's modified Eagle medium (DMEM 4.5 g/L glucose #31966-021 and DMEM 1 g/L glucose #21885108), dispase II (#17105-041), Penicillin-Streptomycin (#15140122), and trypsin/EDTA solution (#15400054) were purchased from Thermo Fisher Scientific (Darmstadt, Germany). Accutase® solution (#A6964), propidium iodide (#P4170) and fluorescein diacetate (#F7378) were supplied by Sigma Aldrich (Steinheim, Germany). Fetal bovine serum was purchased from PAN-Biotech GmbH (#P30-3306, Aidenbach, Germany) and from Biochrom (#S 0615, Berlin, Germany). Collagenase NB4 (#17454) was supplied by Serva GmbH (Halle, Germany). HAoSMCs (#FC-0015), VascuLife SMC medium (#LL-0014), HUVECs (#FC-0003) and VascuLife VEGF medium (#LL-0003) were derived from CellSystems Biotechnologie Vertrieb GmbH (Troisdorf, Germany). All other expendable materials for cell culture were purchased from Sarstedt (Nümbrecht, Germany).

Methods

Characterization of thermal properties of PGE. Dynamic light scattering (DLS) was performed on a Zetasizer Nano-ZS analyzer (Malvern Instruments, Malvern, United Kingdom) equipped with a 50 mW frequency doubled DPSS Nd:YAG laser (λ=532 nm) at concentrations of 2.5 and 0.25 mg mL^{-1} in Milli-Q water at 10, 20 and 37 °C. Measurements were performed at least in triplicate using Quartz cuvettes supplied by Hellma Analytics GmbH (Müllheim, Germany) and each sample was equilibrated for at least 120 s. Turbidity measurements were performed on a Lambda 950 UV-Vis spectrometer at λ = 500 nm with a PTP 6 Peltier Temperature Programmer from Perkin Elmer (Waltham, MA, USA). Cloud point temperatures (CPTs) were determined at concentrations between 1 and 20 mg mL^{-1} in Milli-Q water employing heating/cooling rates of 0.5 °C min^{-1} with a data point recording every 0.2 °C. The temperature-dependent transmittance of the aqueous polymer solutions was measured for at least three up- and down cycles and the CPT was defined as the temperature at the inflection point of the normalized transmittance versus temperature curve.

Surface Modification and Characterization. Spin-coating was performed using a WS-650-23 spin-coater from Laurell Technologies Corporation (North Wales, PA, USA). Si wafers (11 x 11 mm) were coated using a 0.5% (w/w) solution of PS (30 µL) in toluene at 3000 rpm for 60 s. Solutions were prepared using PS (M_n=132 kg mol^{-1}, PDI=1.9) from Falcon® culture dishes supplied by Th. Geyer GmbH & Co. KG (Berlin, Germany). Static water contact angles (CAs) were measured with an OCA contact angle system from DataPhysics Instruments GmbH (Filderstadt, Germany) and fitted with the software package SCA202 (version 3.12.11) using the sessile drop method. CAs were determined before and after surface functionalization. A drop of MilliQ water (2 µL) was placed onto the respective surface and CAs were determined with an elliptical fitting model. For each substrate, CAs were measured on at least three different spots to test for the homogeneity of the sample and ten independent substrates (n = 10) to test for reproducibility. The dry layer thickness of the polymer coatings was determined by multi-

angle spectroscopic ellipsometry at 70° with a SENpro spectroscopic ellipsometer from SENTECH Instruments GmbH (Berlin, Germany). The thickness of the SiO_2 layer before spin-coating and the additional thickness of the spin-coated PS layer were determined separately using a Cauchy layer for modelling and respective average values of at least three different spots on the surface were taken as fixed values for the subsequent modeling of the adsorbed PGE layer. The PGE thickness was measured at wavelengths from 370 nm to 1050 nm and was fitted using a model consisting of the previously measured SiO_2 and PS layers with fixed parameters, a PGE layer with a fixed RI of n=1.5 and air as the surrounding medium. For photo-immobilization, samples with adsorbed PGE layers were irradiated with UV light using a UV-KUB 2 from KLOÉ (Montpellier, France) with a wavelength of 365 nm and a radiant exposure of 4.0 J cm^{-2} for 160 s, which corresponds to an irradiance of 25 mW cm^{-2} (100%). QCM-D measurements were performed on Q-Sense E1 system from LOT-QuantumDesign (Darmstadt, Germany) with a standard flow module and a Reglo Digital peristaltic pump from Ismatec (Wertheim, Germany). The software QSoft401 version 2.5.22 was used for data acquisition and QTools 3 version 3.1.25 from Biolin Scientific AB 2000-2014 (Stockholm, Sweden) was used for data analysis. AFM measurements were performed on an AFM Nanoscope MultiMode 8 equipped with a fluid cell and a thermal application (TA) controller from Bruker (Billerica, MA, USA). The morphology and material properties of the PGE-3.0 coatings was measured in PeakForce QNM (Quantitative NanoMechanics) mode. Coated Si wafers were mounted on the AFM head, degassed Milli-Q water was inserted into the liquid cell and the TA controller was set to the desired temperature (37 or 20 °C) and equilibrated for at least 10 min prior to each measurement. To obtain high resolution images with reduced sample damage, SNL-10 cantilevers from Bruker (Billerica, MA, USA) with a nominal spring constant of 0.35 N m^{-1} and a tip radius of 2-12 nm were used, and images were recorded with a loading peak force of 500 pN or 1 nN, 512 points per line and scan rates of 0.7 Hz. Obtained images were analyzed with the Nanoscope analysis software (version 1.4) and

processed using 1st order flattening. Roughness and depth analysis tools were used to obtain the surface parameters. Microscopic images of HDFs, HAoSMCs and HUVECs were taken on a Zeiss Observer Z1 from Carl Zeiss Microscopy GmbH (Jena, Germany) and evaluated with the software Zen 2 Version 2.0.0.0 from Carl Zeiss Microscopy GmbH (Jena, Germany). Macroscopic photographs were taken with a Nikon D3100 from Nikon GmbH (Düsseldorf, Germany).

Dynamic light scattering (DLS) measurements

To investigate the temperature- and concentration-dependent hydrodynamic radius of PGE in Milli-Q water, DLS measurements were performed at 10, 20 and 37 °C and 2.5 as well as 0.25 mg mL^{-1} (10 µM). At moderate concentration (2.5 mg mL^{-1}), PGE forms 30-50 nm sized aggregates at 10 and 20 °C and slightly bigger particles at 37 °C, as evident from both number and volume distributions (Figure S1a, c). It was previously reported that poly(GME-*ran*-EGE) copolymers with comparable molecular weight (24 kDa) and no benzophenone (BP) block form up to 1 µm sized aggregates at 20 and 37 °C, whereas their particle size decreases to about 10 nm upon cooling to 10 °C (measured at 10 mg mL^{-1}).[1] This rather uniform aggregation behaviour of the PGE block copolymer indicates the hydrophobically-driven association of the BP anchor-blocks in aqueous solution. At dilute concentration (0.25 mg mL^{-1}), however, the hydrodynamic radius of PGE decreases to about 10 nm at 10 °C (Figure S1b, d), which is in the same range as it was reported before for aqueous and ethanolic poly(GME-*ran*-EGE) solution (10 mg mL^{-1}) at 10 and 20 °C, respectively[1], as well as ethanolic PGE solutions (20 mg mL^{-1}) at 20 °C.[2] Due to the negligible aggregation of PGE under dilute conditions and lowered temperature, PS substrates were coated with PGE in Milli-Q water at 10 °C and 10 µM, in order to attain the homogeneous adsorption of PGE-3.0 as monolayers.

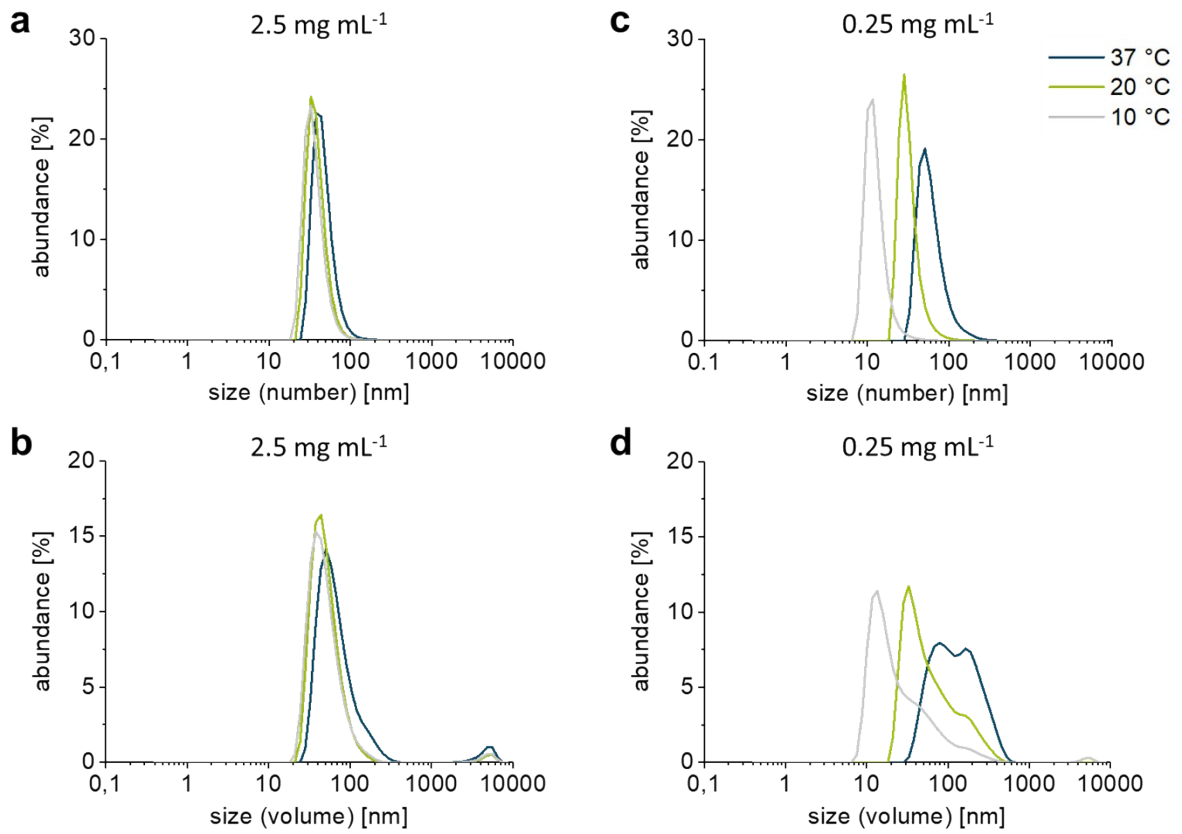

Figure S1. Temperature-dependent number and volume distributions of the hydrodynamic radii of PGE in Milli-Q water at moderate concentrations of 2.5 mg mL^{-1} (a-b) and at dilute concentration of 10 µM (0.25 mg mL^{-1}) (c-d) measured by DLS.

UV-Vis turbidimetry measurements

The concentration-dependent cloud point temperature (CPT) of PGE in Milli-Q water was determined by UV-Vis transmittance measurements in the range between 1 and 20 mg mL^{-1}. In accordance to previous reports of a poly(GME-*ran*-EGE) copolymer with comparable molecular weight (24 kDa) and without a BP block[1], the CPTs of PGE are only slightly dependent on the concentration between 1 and 5 mg mL^{-1} and show only small hysteresis (Figure S2a). However, at higher concentration (10 - 20 mg mL^{-1}), CPTs decrease markedly and a significant hysteresis occurs (Figure S2b), which is presumably caused by the hydrophobic, supramolecular association of the BP anchor blocks. In addition, the CPTs of PGE in the concentration range between 1 and 5 mg mL^{-1} is shifted to higher temperatures (20-22 °C)

and phase transition regimes are significantly broadened (> 10 °C) as compared to poly(GME-*ran*-EGE) with a similar GME/EGE ratio of 1:3, which exhibits lower CPTs (16-18 °C) and much sharper transition regimes (~2-3 °C).[1] This can be explained by the benzophenone-driven intermolecular aggregation of PGE, which is already prevalent at 10 °C below the temperature-triggered phase transition and hence, shifts the CPTs as well as broadens the phase transition.

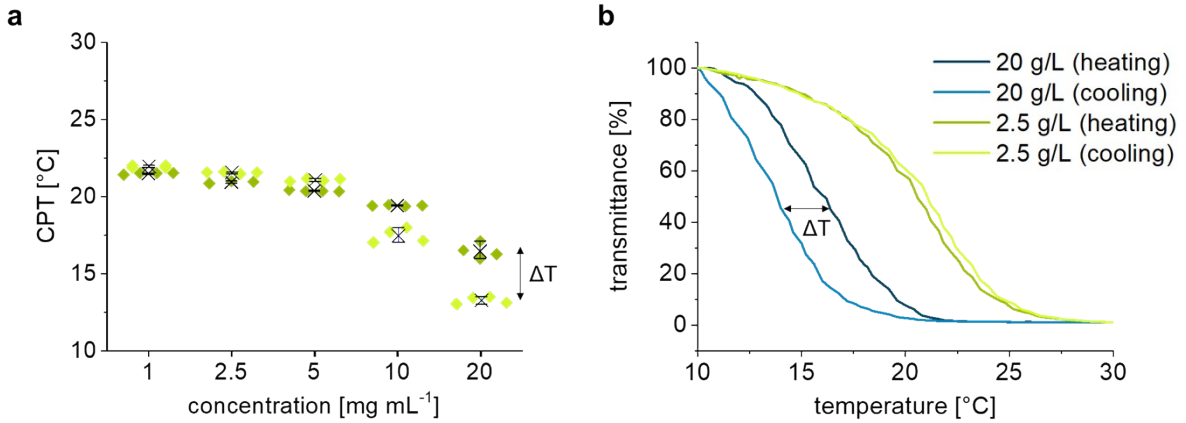

Figure S2. (a) Concentration-dependent CPTs of PGE measured by turbidimetry in Milli-Q water at concentrations between 1 and 20 mg mL^{-1}; (b) Representative normalized transmittance curves (heating and cooling) of PGE in Milli-Q water at high (20 mg mL^{-1}) and moderate (2.5 mg mL^{-1}) concentration.

Theoretically modelled surface structure of PGE-3.0 in comparison to PGE-0.7

To compare PGE-3.0 brush conformation with ultrathin coatings, theoretical modelling was applied to PGE-0.7 layers. As illustrated in Figure S3, average anchor distances l is markedly higher and values for the packing parameter $2R_f l^{-1}$ are significantly lower for PGE-0.7 coatings under both bad as well as theta solvent conditions.

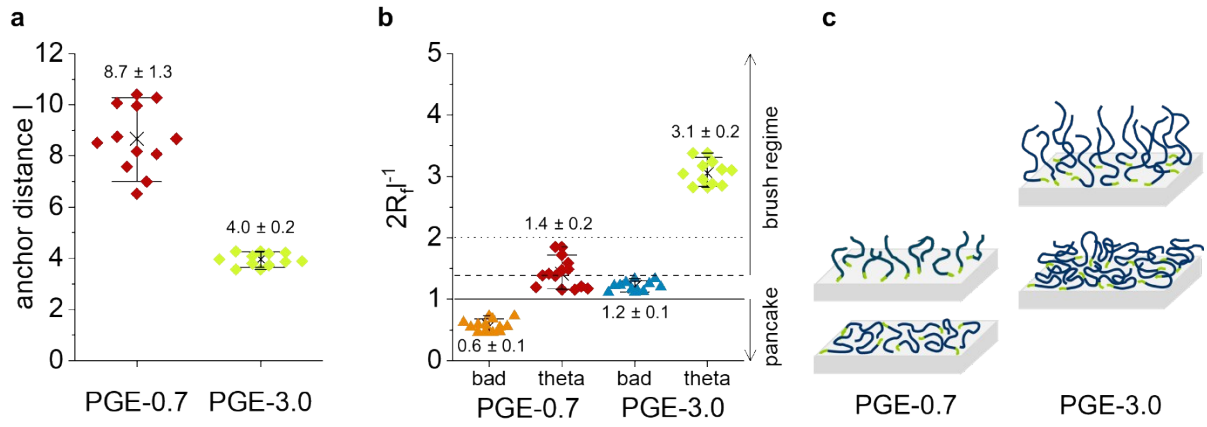

Figure S3. Theoretically estimated degree of chain overlap ($2R_f\ l^{-1}$) on PS-coated silicon wafers. (a) Anchor distance l calculated from the dry layer thickness of PGE-0.7 and PGE-3.0 coatings determined by ellipsometry. (b) Degree of chain overlap $2R_f\ l^{-1}$ calculated from the anchor distance l and the estimated Flory Radius R_f under bad (orange and blue triangles) and theta (red and green diamonds) solvent conditions. $2R_f\ l^{-1}$ values are shown for each replicate together with their mean value (black cross) and their 90% confidence interval (whiskers). (c) Schematic illustration of the temperature-dependent structure of the PGE-0.7 and PGE-3.0 coatings.

Investigation of the adhesive interaction between PS and poly(GME-*ran*-EGE)

To investigate the affinity of thermoresponsive GME/EGE copolymers towards PS and to therefore deduce their chain conformation on PS substrates, adsorption experiments of a poly(GME-*ran*-EGE) copolymer were conducted using quartz crystal microbalance with dissipation (QCM-D). Gold-coated QCM-D sensor chips were used as received and spin-coated as described above, applying a solution of PS in toluene (1.0% (w/w), 30 µL). The PS-coated sensor chips were dried at ambient conditions overnight, rinsed with ethanol and dried under a stream of N_2 before use. Adsorption of a poly(GME-*ran*-EGE) (M_n = 43 kDa, PDI = 1.45, GME:EGE = 1:3) copolymer without a surface reactive anchor group was performed in a QCM-D to determine the adsorbed mass by online-monitoring of the change in resonance frequency *(Δf)* and dissipation *(ΔD)* of a piezoelectric quartz crystal over time. Changes in the

fundamental frequency (4.95 MHz) and in overtones 3 to 13 were measured. For calculation of the layer thickness, the Sauerbrey relation was chosen, as it sufficiently describes thin layers with negligible viscoelasticity. Calculations were conducted using the software package QTools® considering the 3rd overtone. Start and end of all measurements were performed in ethanol to generate a reliable and comparable baseline. Therefore, fluid density was set to 789 kg m^{-3}, fluid viscosity to 0.0012 kg m^{-1} s^{-1}, and the layer density was estimated to be 1000 kg m^{-3}. Ethanol was distilled before use and degassed for 20 min in an ultrasonic bath. PS-coated sensor chips were inserted into the flow chamber and were equilibrated under ethanol flow (0.1 mL min^{-1}) until the baseline was constant. A solution of the polymer in ethanol (0.01 mM) was flown over the sensor chips for 8 min (0.1 mL min^{-1}), the flow was stopped to adsorb poly(GME-*ran*-EGE) under static conditions for 30 min, and the surfaces were flushed with ethanol to remove loosely adsorbed polymer. The dry thickness of the adsorbed films was measured by ellipsometry on PS-coated Si wafers and determined to be 0.4 ± 0.1 nm and the solvated thickness calculated from QCM-D measurements was 1.2 ± 0.2 nm, indicating a degree of solvation of the adsorbed films of about 67% (Figure S4a). This is roughly in the range of what was determined for ultrathin PGE coatings.[2] In addition, the water CA significantly changes from 90.3 ± 0.5° to 69.8 ± 0.3° (Figure S4b), which was also reported for ultrathin PGE coatings. The reproducible formation of stable, ultrathin poly(GME-ran-EGE) layers with an average adsorbed areal mass of 124 ± 18 ng cm^{-2} (n = 3) reached saturation after one adsorption cycle and did not significantly change upon performing multiple cycles (Figure S4c). This indicates a rather strong interaction of the PS surface with the thermoresponsive polymer and suggests the formation of a pancake-like conformation of poly(glycidyl ether)s on PS substrates.

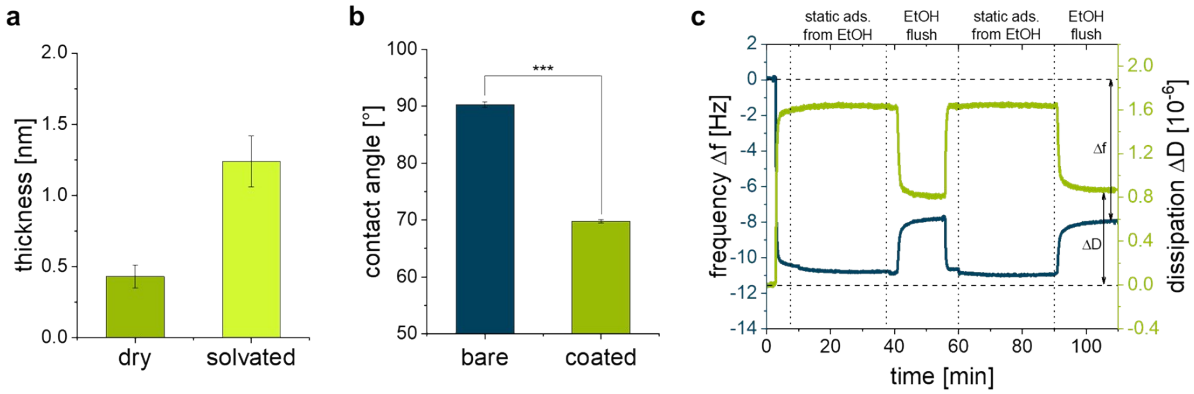

Figure S4. (a) Dry and solvated (EtOH) layer thickness of a poly(GME-*ran*-EGE) copolymer adsorbed onto PS from dilute ethanolic solution (10 µM) measured by ellipsometry and QCM-D, respectively (n = 3, error bars indicate SD) (b) Static water contact angle of bare PS and PS coated with poly(GME-*ran*-EGE) (n = 3, error bars indicate SD) (c) Representative frequency and dissipation curves of two consecutive online adsorption cycles of poly(GME-*ran*-EGE) measured by QCM-D.

Atomic force microscopy (AFM) quantitative nanomechanical mapping (QNM)

AFM measurements were performed to investigate the morphology and material properties of PGE-3.0 coatings. To obtain high resolution images with reduced sample damage, SNL-10 cantilevers from Bruker (Billerica, MA, USA) with a nominal spring constant of 0.35 N m^{-1} and a silicon nitride (Si_3N_4) tip radius of 2-12 nm were used, and images were recorded with a loading peak force of 500 pN. Prior to each measurement, calibration of the cantilevers was carried out on hard clean mica substrates and the spring constants of the cantilevers were extracted with the thermal noise method.[3-4] The surface morphology was measured in Milli-Q water at 37 and 20 °C. As shown in Figure S5, laterally homogeneous and smooth coatings were obtained. The increased roughness at 20 °C illustrates the temperature-triggered rehydration of the thermoresponsive PGE-3.0 coatings on the nanometer scale and shows characteristic island-like domains, which were previously reported for similar thermoresponsive brush coatings based on poly(N-isopropyl acrylamide) (PNIPAm).[5]

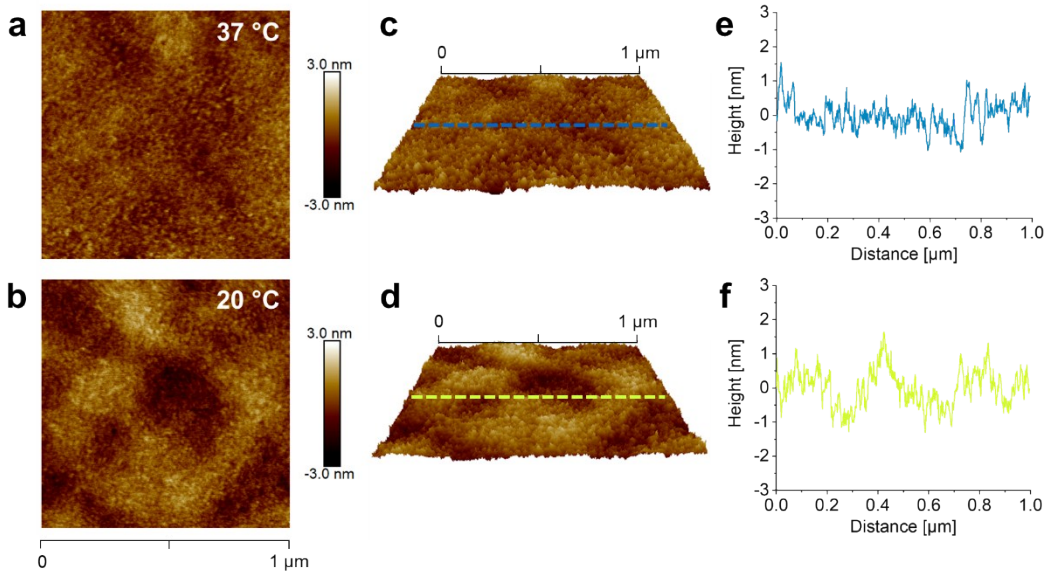

Figure S5. 2D and 3D surface topography and cross section profiles of 3 µm images of PGE-3.0 on PS-coated silicon wafers in Milli-Q water at 37 °C (a, c, e) and 20 °C (b, d, f) measured by AFM in PeakForce QNM mode with an applied loading peak force of 500 pN.

The main roughness parameters of PGE-3.0 coatings were extracted from 3 different 1 µm pictures and are summarized in Table S1 and are compared to those of ultrathin PGE-0.7 coatings as well as to a bare PS substrate.

Table S1. Main roughness parameters obtained from 1 µm PeakForce QNM images measured by AFM with an applied loading peak force of 500 pN. Values for the root-mean squared roughness R_q, mean roughness R_a, and maximal roughness R_{max} (peak to valley vertical variation) for bare PS, PGE-0.7 and PGE-3.0 measured in Milli-Q water at 37 and 20 ºC (n = 3).

Sample	R_q ± SD [nm]	R_a ± SD [nm]	R_{max} ± SD [nm]
PS (20 °C)	0.42 ± 0.02	0.33 ± 0.01	4.20 ± 0.85
PGE-0.7 (37 °C)	0.55 ± 0.13	0.43 ± 0.10	5.44 ± 1.27
PGE-0.7 (20 °C)	0.64 ± 0.12	0.50 ± 0.10	5.85 ± 0.87
PGE-3.0 (37 °C)	0.49 ± 0.09	0.39 ± 0.08	4.67 ± 0.60

PGE-3.0 (20 °C)	0.70 ± 0.11	0.57 ± 0.10	5. 27 ± 0.67

The material properties of PGE-3.0 coatings on PS-coated Si wafers were determined by AFM in PeakForce QNM mode in Milli-Q water at 37 and 20 °C using a loading peak force of 1 nN. Like evident temperature-dependent changes in surface morphology and roughness (Figure 4, Figure S5, Table S1), the elasticity (DMT modulus), deformation and adhesion of the PGE-3.0 coatings slightly differ between 37 and 20 °C (Figure S6). DMT moduli extracted from AFM retraction curves using the Derjaguin-Muller-Toporov (DMT) model revealed the presence of soft PGE films with moduli around 100 MPa, which is about 2 orders of magnitude lower than expected for bare PS.[6-7] The slightly lower DMT moduli at 20 °C indicate a more hydrated brush layer with softer, swollen PGE chains, whereas the PGE brush layers are stiffer in their collapsed, more dehydrated state a 37 °C (Figure S6a, d, g). Accordingly, the deformation of the PGE brushes is slightly higher (Figure S6b, e, h) and the adhesive interaction between the AFM tip and the PGE brushes (Figure S6c, f, i) is slightly lower when the PGE chains are more hydrated at 20°C than in their collapsed state at 37°C.

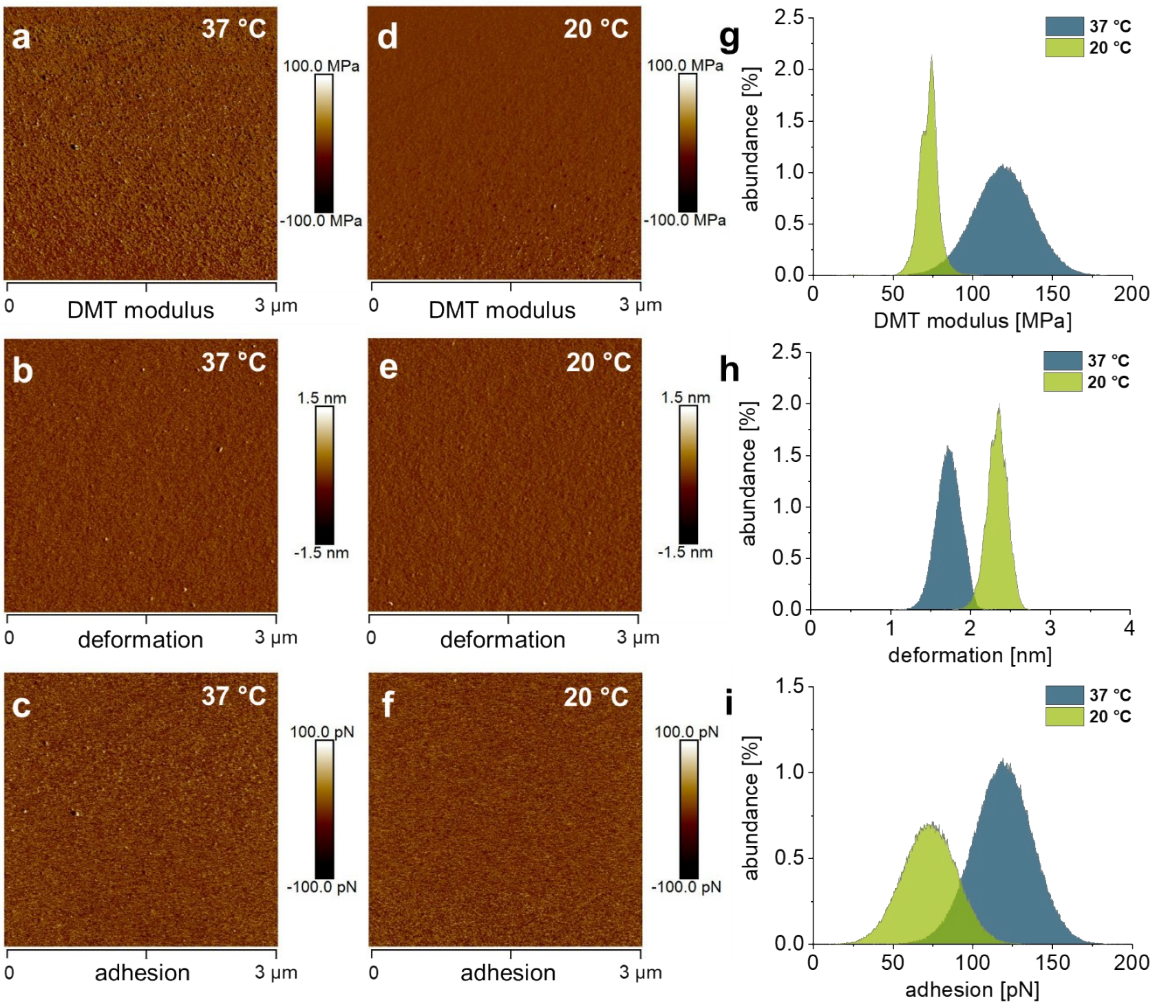

Figure S6. Comparison of PGE-3.0 material properties in Milli-Q water at 37 and 20 °C measured by AFM in PeakForce QNM mode with an applied loading peak force of 1 nN. Comparative 2D maps (a-f) and depth analyses (g-i) of the DMT modulus (a, d, g), deformation (b, e, h) and adhesion (c, f, i).

Adhesion and proliferation of human umbilical vein endothelial cells (HUVECs) on ultrathin PGE-0.7 and brush-like PGE-3.0 coatings

To compare the adhesion and proliferation of HUVECs on ultrathin (PGE-0.7) and brush-like PGE coatings (PGE-3.0), 5.0 x 10^4 cells cm^{-2} cells were seeded on coated 24-well PS plates in culture medium (VascuLife VEGF with 2 % FBS). The coated PS surfaces were sterilized with 70% ethanol for 10 min and washed twice with PBS before cell seeding. 24-well TCPS as well as bare PS plates were used as controls. Cells at the center of the plates were observed

microscopically and pictures were taken after 48 and 72 h. The cell culture medium (Vasculife VEGF, 2% FBS) was exchanged after 48 h. HUVECs only proliferated to confluent monolayers on PGE-3.0 substrates (Figure S7g, h) and TCPS controls (Figure S7a, b), whereas cells did not attach well to bare PS substrates (Figure S7c, d) and did not proliferate to form confluent cell monolayers on PGE-0.7 substrates (Figure S7e, f).

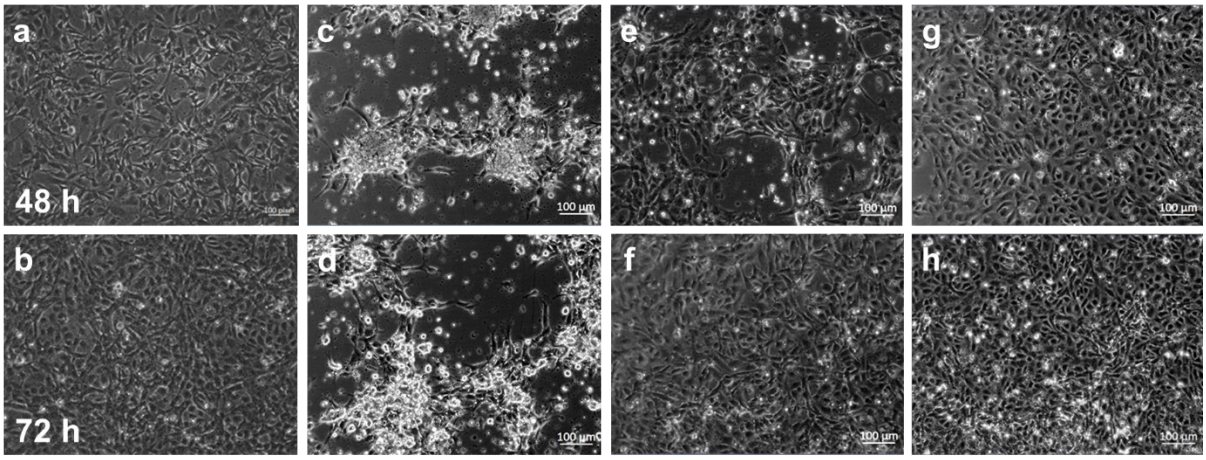

Figure S7. Representative microscopic pictures of HUVECs after 48 and 72 h on TCPS (a-b), PS (c-d), PGE-0.7 (e-f) and PGE-3.0 (g-h). HUVECs (passage 5) were seeded into 24-well plates (PS and TCPS, A = 1.9 cm^{-2}) with a density of 5.0 x 10^4 cells cm^{-2}.

Comparison of PGE-3.0 and UpCell™ plates for smooth muscle cell culture

HAoSMCs were seeded on UpCell™ 24-well-plates at a density of 1x10^5 cm^{-2} in VascuLife SMC medium with 10 % FBS as on the PGE-3.0 and TCPS dishes and cultured for 16 h. For live/dead staining of adherent cultures 50 µM propidium iodide and 10 µM fluorescein diacetate were added to the cultures and incubated for 5 min. Samples were imaged on a Zeiss Observer Z1 microscope in fluorescent mode with appropriate filter sets.

HAoSMCs adhered homogeneously on PGE-3.0 and TCPS and stayed viable whereas on UpCell™ surface cell clusters containing several dead cells were observed.

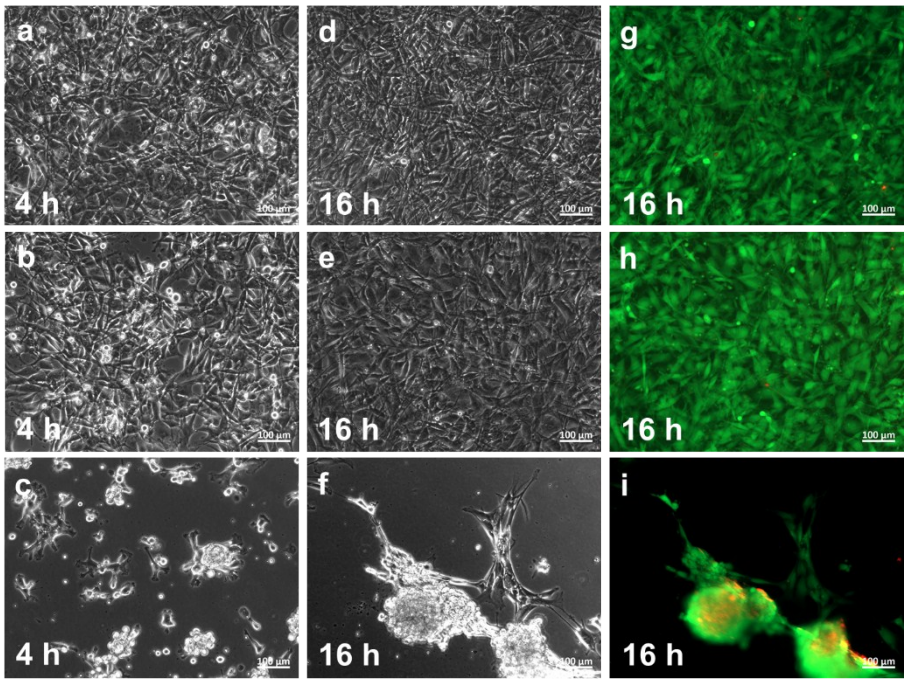

Figure S8. Representative phase contrast images of HAoSMCs 4 and 16 h after seeding on PGE-3.0 (a, d), TCPS (b, e) and UpCell™ (c, f) and respective images of live/dead staining (g, h, i) with fluorescein diacetate (green) and propidium iodide (red).

Impact of serum concentration on cell sheet detachment of HUVECs

Culture conditions of HUVECs on PGE-3.0 were initially optimized to achieve cell sheet detachment. Besides the described culture procedure (24 h in VascuLife VEGF with 2% FBS followed by 48 h in VascuLife with 10% FBS), the culture of HUVEC in VascuLife VEGF medium, containing 2% FBS, for 72 h was tested. HUVECs were also seeded with a density of 8.5 x 10^4 cells cm^{-2} in VascuLife VEGF medium (2% FBS) per dish (d = 35 mm), followed by culture at 37 °C and 5% CO_2 for 72 h with a media exchange after 24 h. For temperature-triggered detachment, confluent cell cultures were incubated in PBS at 20 °C until the cell sheets detached from the surfaces. After the continuous culture in 2 % serum cell sheets could only be detached in fragments whereas complete cell sheets could be detached in the culture with elevated serum concentration after 24 h (Figure S9c, d). Microscopically it was observed that the latter samples exhibited a higher cell density (Fig. S9a, b) which can be explained by a

higher proliferation rate with increased serum concentration.[8] The higher cell density might have resulted in better cohesion of the cell sheet.

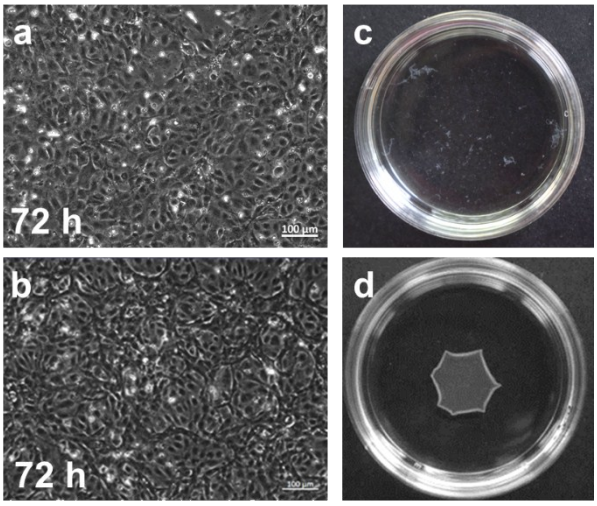

Figure S9. Representative phase contrast images of HUVECs on PGE-3.0 after (a) 72 h culture in VascuLife VEGF medium containing 2% FBS and (b) 24 h in VascuLife VEGF with 2% FBS followed by 48 h in VascuLife with 10% FBS and respective macroscopic images of cell sheet detachment in PBS at 20 °C (c, d).

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