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Modified polyglycidol based nanolayers of switchable philicity and their interactions with skin cells

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ABSTRACT

Temperature-responsive polymer surfaces based on modified high molar mass polyglycidol were obtained via the "grafting onto" technique. The solid support (glass or silica) was modified to introduce reactive anhydride groups. The covalent bonding of the temperature-responsive poly(glycidol-co-ethyl glycidyl carbamate) to the base support was obtained by the chemical reaction between reactive functional groups on the support and on the polymer. The surface properties, such as the composition, morphology, thickness and wettability, and their changes with the temperature were investigated using Fourier Transform Infrared Analysis, Atomic Force Microscopy, ellipsometry and contact angle measurements, respectively. The attachment of the hydrophobically modified polyglycidol derivatives produced a polymer layer with a thickness of 20–60 nm and a changeable philicity due to the temperature alterations. This unique behavior was applied to the investigation of the interaction of polymer nanolayers with skin cells (fibroblasts and keratinocytes). The skin cells adhesion to the poly(glycidol-co-ethyl glycidyl carbamate) surface was possible at temperatures above the phase separation temperature, when the polymer layer was hydrophobic.

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1. Introduction

Temperature-responsive polymer materials, such as gels, micro- and nanogels, micelles and surfaces, have gained a significant interest in recent decades [1]. Such systems recognize changes of temperature as a signal and undergo large changes of their properties over a narrow range of temperatures [2]. The temperature-dependent conformational changes of these polymer systems appear above a certain value, called the cloud point (T_{CP}) (or phase separation temperature), and are reversible.

Recently, attention has been directed toward smart, temperature-responsive surfaces. Covalently attaching a thermo-responsive polymer to a solid substrate leads to stable polymer layers with temperature-dependent properties. Below the T_{CP} of the thermo-responsive polymer, the layer formed by this polymer exhibits hydrophilic properties – it is hydrated and its polymer chains adopt an expanded state. However, phase separation occurs as the temperature increases. Above the T_{CP}, the polymer chains collapse due to dehydration and the surface becomes hydrophobic [3].

Temperature-responsive *N*-substituted acrylamide surfaces have been the most widely investigated system. Studies have focused mainly on poly(*N*-isopropylacrylamide) (PNIPAM) [4–7] and its copolymers with *N*-tert-butylacrylamide [8], ethylene glycol [9], dimethylaminoethyl methacrylate [10], *n*-butylmethacrylate [11], acrylic acid [12], *N*-acryloxysuccinimide [13], 1-vinylimidazole [14] and vinylidenefluoride [15]. Temperature-responsive surfaces based on oligo(ethylene glycol) methacrylates [16,17], copolymers of ethylene oxide and propylene oxide [18] as well as poly(2-oxazolines) [19] have also been explored.

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Different approaches have been used to obtain temperature-responsive surfaces. In particular, the majority of studies use the "grafting from" and "grafting to" methods [20]. In the "grafting from" (also referred to as "surface-initiated polymerization"), the polymerization is initiated from the surface by attached initiating groups. Surface-initiated polymerization is compatible mostly with a radical polymerizations [16,21,22] but also with cationic polymerization [23]. The "grafting from" method leads to a surface made of functional polymer brushes of large thickness (up to 100 nm) and high density in a controllable manner [24]. However, this approach is technically demanding and the obtained polymer brushes can be quite inhomogeneous due to differences in the polymer chain lengths.

In the "grafting to" approach, a previously synthesized polymer reacts with the appropriate functional groups located on the surface. This reaction can be conducted by the surface-termination of living polymer chain ends [20]. The resulting brushes are frequently more homogeneous compared with those obtained by the "grafting from" approach, if a polymer with low molar mass dispersity is used [24]. A high grafting density can be difficult to obtain due to the steric screening of the surface reactive sites by the already bonded polymer chains. Furthermore, only thin layers (approximately 10 nm) can be obtained [20]. Alternatively, a simpler method for a "grafting to" surface preparation is a temperature-induced chemical reaction between the polymer and the surface, called a "melt reaction" [25,26]. Here, functionalized polymer reacts with complementary functional groups located on the surface. This method can be performed relatively easily because casting the polymer solution or spin coating is only required to cover the substrate. The covalent bonds between polymer chains and the surface are formed merely under the influence of temperature, what greatly simplifies the protocol for polymer layer preparation. The simplicity of this coating method is greatly appreciated. This process was used by Yang et al. [27] to develop temperature-responsive nanofilms of *N*-isopropylacrylamide copolymers onto hydroxylated glass surfaces. Following this approach, Montagne et al. [28] reported PNIPAM films grafted onto modified gold wafers and showed dependence of polymer chains conformation on the surface with the temperature. Using the "melt" process, Yim et al. [29] prepared temperature-responsive surfaces through the reaction of COOH-terminated PNIPAM with the hydroxyls on the substrate. Hirata et al. [30] applied temperature-induced immobilization of PNIPAM copolymers to hydroxylated wafers through a urethane bond to obtain thermoresponsive surfaces of different thicknesses.

To date, the interesting behavior of thermoresponsive surfaces has been exploited in a wide range of applications, including drug delivery systems [31], bioseparation [32], immobilization of biomacromolecules [33], antibacterial systems [34] and cell sheet engineering [35].

In this work, we report an easy method for obtaining temperature-responsive surfaces based on polyglycidol derivatives with well-defined surface properties and describe their interactions with skin cells. So far, mostly the PNIPAM brushes were well developed for interaction with cells. However, the PNIPAM layers have some disadvantages. The aggregation of PNIPAM chains sometimes irreversible, influencing the transition behavior. Moreover, in order to optimize the properties, especially the surface–cell interactions, the surface may need to be modified, also with biologically active species. As PNIPAM is not a reactive polymer, its chemical modification is challenging. This may set limits to its applications and justifies the search for other biocompatible polymer layers containing reactive functional groups. Polyglycidol, a hydrophilic, water-soluble, nontoxic and biocompatible polyoxirane [36] can be a good candidate. The presence of one primary hydroxyl functional group per monomeric unit enables its hydrophobic modification into temperature-responsive polymers [37]. Temperature-responsive polyglycidol derivatives with different structures and topologies, including block [38], star-block [39] and random copolymers [37] with T_{CP} ranging from 21 to 86 °C were obtained. Temperature-responsive polyglycidol-based materials, including aqueous solutions [37], self-assembled structures [40], superabsorbing hydrogels [41] and cryogels [42] were also studied.

Here, we report the synthesis and characterization of polyglycidol-based surfaces made by the chemical reaction between the polymer and the activated solid substrate. Covalent bonds were formed as a result of the reaction between the polyglycidol hydroxyl groups and the anhydride groups located on the surface at increased temperature. The surface morphology, wettability and thickness, as well as their changes with temperature are described.

The studies concerning the effective support for the skin cell culture are of potential great therapeutic importance for treatment of difficult to heal wounds (burns or diabetes caused injuries). Nowadays the best solution to this problem are skin grafts or skin-cell grafts, as many conventional treatments (hydrogel dressing, allografts or xenografts) are costly, slow and not always effective. However the cell culture procedures used to obtain large-surface grafts have still to be optimized. In this work, the influence of surface properties on the skin cells (fibroblasts and keratinocytes) attachment and growth is examined.

2. Experimental

2.1. Materials

Ethoxyethylglycidyl ether (EEGE) was obtained by reacting 2,3-epoxypropanol-1 (glycidol) (96%, Aldrich) with ethyl vinyl ether (Aldrich) catalyzed with *p*-toluenesulfonic acid (98.5%, Aldrich), according to the procedure described in [43]. The obtained product was fractionated under reduced pressure. Fractions of 99.8% (GC) purity were used for the polymerizations. DMF (POCH, Poland) was dried over CaH_2, distilled under a dry argon atmosphere and was then dried over P_2O_5 and distilled again. Ethyl isocyanate (98%, Aldrich) was distilled under argon atmosphere. Diethyl zinc (1 M solution in hexanes, Aldrich), dibutyltin dilaurate (DBTL) (95%, Aldrich), H_2SO_4 (95%, POCH, Poland), hydrogen peroxide (30%, Chempur, Poland) and (3-aminopropyl)triethoxysilane (APTES) (99%, Aldrich) were used as received. Poly(ethylene-

alt-maleic anhydride) (PE-MA), typical M_w = 100 000–500 000 g/mol (Aldrich) was dissolved in acetone, precipitated in hexane and then dried at 120 °C for 24 h. Ethanol (99.8%, POCH, Poland), acetone (99.5%, POCH, Poland) and methanol (POCH, Poland) were filtered before use. Polished prime silica wafers (Cemat Silicon S.A, Poland) with a thickness of 500–550 μm and ϕ = 100 mm were cut into 1 × 1 cm pieces. Borosilicate cover glasses with a thickness of 0.13 – 0.16 mm and ϕ = 13 mm were obtained from VWR International (Poland).

2.2. Synthesis and biocompatibility of temperature-responsive poly(glycidol-co-ethyl glycidyl carbamate) (mPGl)

The synthesis of high molar mass polyglycidol and its hydrophobic modification [37] has been described previously. Briefly, ethoxyethylglycidyl ether (EEGE) was polymerized in bulk with $ZnEt_2/H_2O$ (1:0.8) for 24 h at 55 °C. Acidic hydrolysis with 3 M HCl removed the protective ethoxyethyl groups, resulting in linear polyglycidol. To achieve hydrophobic modification polyglycidol was reacted with ethyl isocyanate, in the presence of DBTL as a catalyst and DMF as the solvent. The molar ratio of ethyl isocyanate and DBTL per 1 mol of polyglycidol OH groups was [0.4]:[0.02]:[1], respectively. The reaction was carried out for 24 h at 48 °C. After the reaction, the poly(glycidol-co-ethyl glycidyl carbamate) (mPGl) was precipitated into diethyl ether and dried under reduced pressure. The biocompatibility test of poly(glycidol-co-ethyl glycidyl carbamate) in culture medium solution (32% of modification, T_{CP} = 46 °C) has been described previously [42]. Briefly, the biocompatibility test was performed in culture medium with different concentrations of mPGl (0.001, 0.01, 0.1, 1 and 10 mg/mL) and without the polymer as a control sample. After certain time, the cells survival was determined using a metabolic test.

2.3. Synthesis of temperature-responsive surfaces

2.3.1. Preparation of wafers

The silica and glass wafers were ultrasonically cleaned in freshly distilled water and subsequently in ethanol for 30 min to remove any residual contaminants. After threefold rinsing of the wafers in distilled water and blow-drying under a stream of argon, the wafers were immersed in a mixture of 30% hydrogen peroxide and 95% sulfuric acid (1:3) (piranha solution) for 2 h. The wafers were then rinsed five times in deionized water for 15 min and held for 24 h at 120 °C in a dust-free vacuum atmosphere. The cleaned wafers were immersed in a 1% solution of (3-aminopropyl)triethoxysilane in ethanol. The reaction was carried out under argon atmosphere. After 2 h, the wafers were rinsed three times with filtered ethanol, blow-dried in a stream of argon and placed in the vacuum oven for 24 h at 120 °C. Poly(ethylene-alt-maleic anhydride) layers were obtained according to the procedure described elsewhere [44,45]. Briefly, a 0.15% solution of poly(ethylene-alt-maleic anhydride) in a mixture of acetone/THF (2:1) was spin-coated onto aminosilylated wafers (time of rotation – 30 s and speed of rotation – 4000 rpm). The wafers were then annealed at 120 °C for 4 h. Afterward, they were rinsed three times in acetone for 15 min to remove any residual contaminants and unreacted polymer and were annealed again for 24 h at 120 °C. The prepared wafers were stored in a vacuum desiccator and were annealed at 120 °C before use.

2.3.2. Immobilization of temperature-responsive poly(glycidol-co-ethyl glycidyl carbamate)

Poly(glycidol-co-ethyl glycidyl carbamate) solutions in methanol (0.25%, 0.5%, 1% and 10%) were spin-coated onto functionalized wafers. The time of rotation (30 s) was the same for all samples, while the speed of rotation varied between 1000 and 5000 rpm. The surfaces were then annealed for 2 h at 120 °C under vacuum. Afterward they were rinsed three times in methanol for 15 min to remove any residual contaminants and unreacted polymer and were annealed again for 2 h at 120 °C. The prepared surfaces were stored in a vacuum desiccator.

2.4. Cell culture

Human skin fibroblasts (FK1) derived from dermis of the 25-year-old white male were cultured in DME-M_ATMP-Ready high glucose (4.5 g/L) medium (PAA Laboratories, Austria) supplemented with 10% FBS ATMP-Ready (PAA Laboratories, Austria), 1% L-glutamine (PAA Laboratories, Austria) and 1% antibiotics (penicillin, streptomycin, amphotericin B, PAA Laboratories, Austria) and were used at the 5th to 6th passage. Normal Human Epidermal Keratinocytes (NHEK) (Lonza Walkersville, Switzerland) were cultured in KGM-2 with SingleQuots of growth supplements (Lonza Walkersville, Switzerland). Cell cultures were incubated in a humidified 5% CO_2/95% air controlled atmosphere at 37 °C. The temperature-responsive mPGl surfaces were placed into the wells of 24-well TCPS (tissue culture polystyrene) plates (TPP Techno Plastic Products, Switzerland) and were sterilized with 70% ethanol for 4 h. The sterilized surfaces were rinsed twice with 2 mL of the appropriate culture medium and were incubated in this medium at 25 °C for 3 h. The temperature was then raised to 37 °C and the surfaces were incubated at that temperature for 3 h. Laminin (Aldrich) was added at a concentration of 650 ng/cm^2 dissolved in TBS (final concentration 2.42 μg/mL) and the surfaces were incubated at 37 °C overnight. FK1 cells were washed with PBS ATMP-Ready medium (PAA Laboratories, Austria) followed by detachment using 0.25% trypsin in 1 mM EDTA (PAA Laboratories, Austria). NHEK cells were detached using the Reagent Pack™ Subculture Reagent Kit containing Trypsin/EDTA, HEPES Buffered Saline Solution (HBSS) and Trypsin Neutralizing Solution (TNS) (Lonza Walkersville, Switzerland). Suspended cells were seeded onto pre-conditioned mPGl surfaces or TCPS (control wells) at a density of 1 × 10^4 - cells/cm^2 in 1 mL of medium. Cell attachment, proliferation, viability and morphology were assessed after 2.5, 4, 8, 12, 48 and 72 h of culture at 37 °C. Growth medium containing unattached cells was removed and the attached cells were rinsed once with culture medium and were incubated for 4 h in 400 μL of the appropriate fresh medium supplemented with 10% AlamarBlue (Invitrogen Corporation, Poland). An aliquot (200 μL) of medium from

each well was spectrophotometrically analyzed and the cell count was estimated as a percent of the control. Experiments, for each time of cell culture, were performed for four samples and statistically analyzed.

2.5. Measurements

The ^{1}H NMR spectra of polyglycidol and poly(glycidol-co-ethyl glycidyl carbamate) were recorded at 25 °C on a Bruker Ultrashield spectrometer at 600 MHz in D_2O.

The molar mass and molar mass dispersity of the obtained polyglycidol were determined using a size exclusion chromatography system containing a multiangle light scattering detector DAWN EOS (λ = 658 nm) (Wyatt Technologies) and a Δn-1000 RI refractive index detector (λ = 620 nm) (WGE Dr. Bures). The measurements were performed at 45 °C in DMF with 5 mmol/L LiBr. The column set used for the SEC measurements consisted of a PL gel guard column, two PL gel MIXED-C columns and a 100 Å PSS GRAM column. Chromatograms were collected and evaluated using the ASTRA software (Wyatt Technologies). The refractive index increment (d*n*/dc) of polyglycidol in DMF was measured independently and was found to be 0.054 mL/g.

The cloud point measurements were performed using a UV–Vis spectrophotometer (Jasco V-530) equipped with a cuvette thermostated by a MTC-P1 thermocontroller. The transmittance was monitored as a function of temperature at a wavelength λ = 500 nm. The cloud point values were recorded as the temperature at which the transmittance of the polymer solution (5 g/L) reached 50% of its initial value.

Atomic force microscopy studies (AFM) were performed on a Multi Mode AFM microscope with a NanoScope 3D controller (Veeco Instruments Inc.) operating in tapping mode in air with standard 125 μm single-crystal silicon cantilevers (Model TESP). Images of different scan sizes from 500 nm to 10 μm were obtained using a piezoelectric scanner. Micrographs were recorded using the NanoScope Software V531r1 and the most representative images for each sample were selected from three measurements at different surface points. The root mean square values of surface roughness (RMS) for each sample were averaged from three measurements.

Contact angle measurements were performed using a contact angle goniometer CAM101 (KSV Instruments) with a temperature control unit (Intelligent digital controller OMRON 5EGN) with an accuracy of ±1 °C. The water contact angles were determined in air using the sessile-drop method. From a micropipette, a water droplet of 4 μL was placed on the surface and was registered during 30 s. The contact angle measurements were performed for dry and wet surfaces. The measurements for wet surfaces were performed at 20 and 40 °C. Before measurement, surfaces were incubated in water at the desired temperature for 3 h and were then transferred to a thermostated ceramic chamber connected to a temperature control unit. The contact angle value was taken as an average of five measurements at different parts of two surfaces fabricated under the same experimental conditions.

The FT-IR spectra of poly(glycidol-co-ethyl glycidyl carbamate) and poly(ethylene-alt-maleic anhydride) were recorded using FTIR 6700 spectrometer (Nicolet, Thermo Scientific) equipped with a diamond crystal Smart OrbitTM accessory working in attenuated total reflection mode (ATR). The FT-IR spectra of the surfaces were obtained using the same spectrometer equipped with a Specular accessory. All spectra were acquired between 4000 and 500 cm^{-1} with 64 accumulations. After measurement, the spectra were evaluated by the OMNIC™ software. A spectrum of a freshly cleaned silica plate was recorded before the measurements and was used as a background reference.

The thickness of each layer of the polymer films was determined using a spectroscopic imaging ellipsometer (Nanofilm EP3) with a He–Ne laser (λ = 658 nm) and a xenon lamp (λ = 360–1001.3 nm). The 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 fitting 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). Thickness of each layer deposited on the surface was then determined at 5° (for glass wafers) or 75° (for silica wafers) angle of the incident beam in the wavelength range 350–1100 nm. The measurements of wet surfaces were performed at 20 and 40 °C in a thermostated chamber. Before measuring, the surfaces were incubated in water at the desired temperature for 30 min. The measurements were carried out at incident angle of 60° for the wavelength 510.3 nm. The reported results are the average of 20 measurements. Each thickness value reported was accurate to 0.4–0.7 nm. The analysis of layer thickness was done using a Cauchy relation. The final thickness of the polymer layer after grafting was calculated using the multilayer model consisting of silica/glass wafer, hydroxylated layer, aminosilylated layer and PE-MA layer.

3. Results and discussion

3.1. Synthesis and characterization of poly(glycidol-co-ethyl glycidyl carbamate) (mPGl)

Both cationic [46] and anionic [47] polymerization of glycidol (2,3-epoxypropanol-1) lead to highly branched structures with low molar mass. In order to obtain linear polyglycidol of controlled molar mass, protection of hydroxyl groups of the monomer before polymerization with the ethoxyethyl groups was applied [48].

In the present study, anionic-coordination polymerization of glycidol with protective ethoxyethyl groups using $ZnEt_2/H_2O$ as a catalyst was applied. A linear high molar mass polyglycidol (M_n = 2 200 000 g/mol) with a narrow molar mass distribution (M_w/M_n = 1.3) was obtained. According to our previous study [37], polyglycidol subjected to hydrophobic modification with ethyl isocyanate yielded a temperature-responsive copolymer of glycidol and ethylglycidyl carbamate (mPGl) (Fig. 1a). Copolymer with 40% of hydrophobic ethyl glycidyl carbamate groups

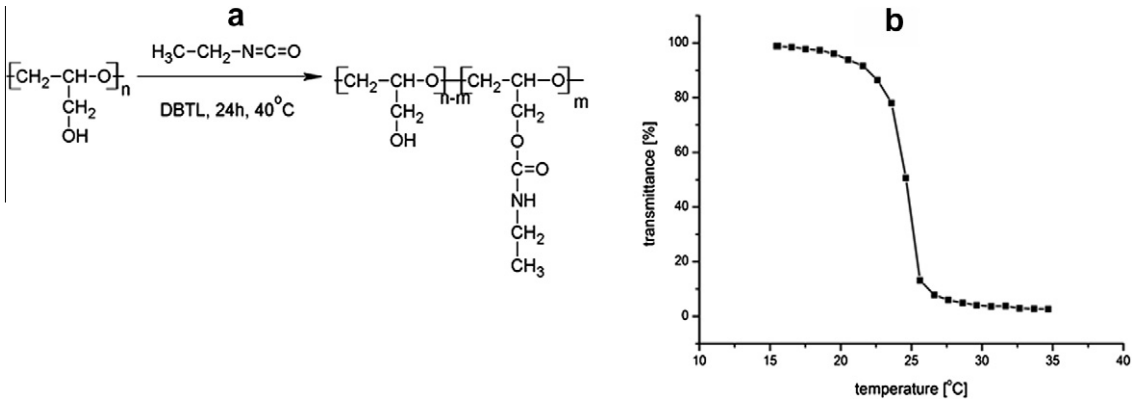

Fig. 1. (a) Hydrophobic modification of polyglycidol and (b) transmittance–temperature curve of poly(glycidol-co-ethyl glycidyl carbamate) (40% of ethyl glycidyl carbamate groups, 5 g/L in H_2O, λ = 500 nm).

(as checked with ^{1}H NMR) and T_{CP} of 25 °C (Fig. 1b) was used in this study.

The biocompatibility of poly(glycidol-co-ethyl glycidyl carbamate) with fibroblasts was previously established [42]. The tests revealed that this polymer is well tolerated by skin cells. mPGl was nontoxic to fibroblasts for a wide range of concentrations (0.001–1 mg/mL).

3.2. Synthesis of temperature-responsive surfaces

It is generally desirable, regardless of the application, for the temperature-responsive polymer to be covalently bound, not merely adhered to the support. For this purpose, wafers often require modification to introduce the desired functional groups. In this work, to bond covalently poly(glycidol-co-ethyl glycidyl carbamate) with a solid silica and glass support, functionalization of solid supports was carried out through hydroxylation, aminosilylation and immobilization of intermediate layer of poly(ethylene-alt-maleic anhydride) (PE-MA) according to procedure described in literature [45]. Modified polyglycidol was then deposited on these modified wafers. The scheme for the surface treatment is shown in Fig. 2. The use of a so-called intermediate layer composed of maleic anhydride and ethylene copolymer allowed for simple and efficient bonding of mPGl by chemical reactions. Upon annealing, covalent ester bonds were formed between the highly reactive anhydride functional groups on the wafers and the hydroxyl groups of mPGl. The surface properties (morphology, wettability and layer thickness) were investigated at each stage of surface preparation.

Hydroxylated wafers were obtained by treating silica wafers with a mixture of 30% hydrogen peroxide and 95% sulfuric acid (piranha solution) (Fig. 2a). The resultant surfaces were very smooth with a surface roughness between 0.11 and 0.19 nm and of hydrophilic nature (Θ = 20°). Subsequently, hydroxylated wafers have been subjected to the organosilane modification using (3-aminopropyl)triethoxysilane (APTES) as a coupling agent. Covalent bonds formed between the hydroxyl groups of wafers and the alkoxysilane groups from APTES introduce reactive amino functional groups to the support (Fig. 2b). The increase of surface roughness (0.2–0.4 nm) and contact angle (75–76°) was observed. The APTES layer thickness determined by ellipsometry was 0.5 nm and remained in good agreement with data from literature [49].

The amino functional groups were then used for grafting an intermediate layer of poly(ethylene-alt-maleic anhydride) copolymer on the support. Upon annealing, the anhydride functional groups of PE-MA were able to form stable five-membered cyclic imide links with the surface amino groups causing the binding of the copolymer to the substrate (Fig. 2c). Before characterization of the wafers they were rinsed several times in acetone to remove the residual contaminants and non-bonded PE-MA copolymer. This should sufficiently help to remove the copolymer that is physically adsorbed onto the surface, if the adsorption is not too strong.

FT-IR confirmed that the poly(ethylene-alt-maleic anhydride) films were covalently bound to the surface. Fig. 3a compares the FT-IR spectra of PE-MA bound to the substrate (dash line) and non-bound to the wafers (solid line). In the spectrum of PE-MA polymer non-bound with the wafer, the following characteristic absorption bands at 1740–1850 cm^{-1} (anhydride C=O stretching vibrations), at 2870 and 2940 cm^{-1} (stretching vibrations of C–H), at 1455 cm^{-1} (scissoring vibrations of C–H), at 1100 and 1220 cm^{-1} (stretching vibrations of C–O) and at 920–960 cm^{-1} (stretching vibrations of C–O–C) are visible. The same adsorption bands can be observed in the spectrum of the PE-MA covalently bound to the wafers. Additionally, a well-separated broad adsorption band is observed at 1710–1750 cm^{-1} that corresponds to the absorption band of stretching vibrations of C=O from the five-membered imide ring. Moreover the intensity of the absorption band at 1100 and 1230 cm^{-1} is increased, confirming the presence of not only the C–O bonds, but also the C–N bonds, and thus the attachment of PE-MA copolymer to the functionalized wafer.

The AFM studies revealed rough layer of PE-MA, comparing with aminosilylated surface and the RMS values for the investigated surfaces were between 2.9 and 3.5 nm. The thickness of the PE-MA layers was determined by ellipsometry. Assuming the refractive index of the copolymer equal to 1.50 [44] the measured thickness of PE-MA was 12 nm. This value is in good agreement with

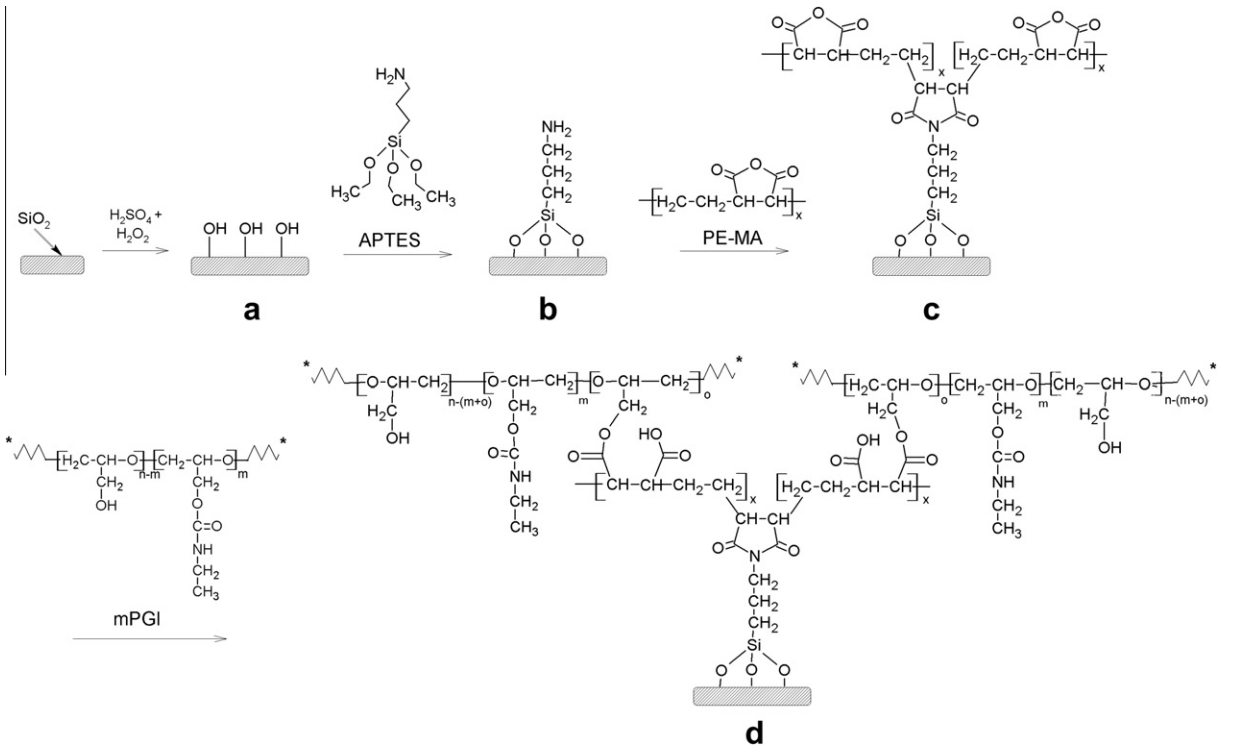

Fig. 2. The general scheme of the mPGl surface preparation: (a) hydroxylation, (b) aminosilylation, (c) deposition of the intermediate layer of poly(ethylene-alt-maleic anhydride) (PE-MA) and (d) layer of poly(glycidol-co-ethyl glycidyl carbamate).

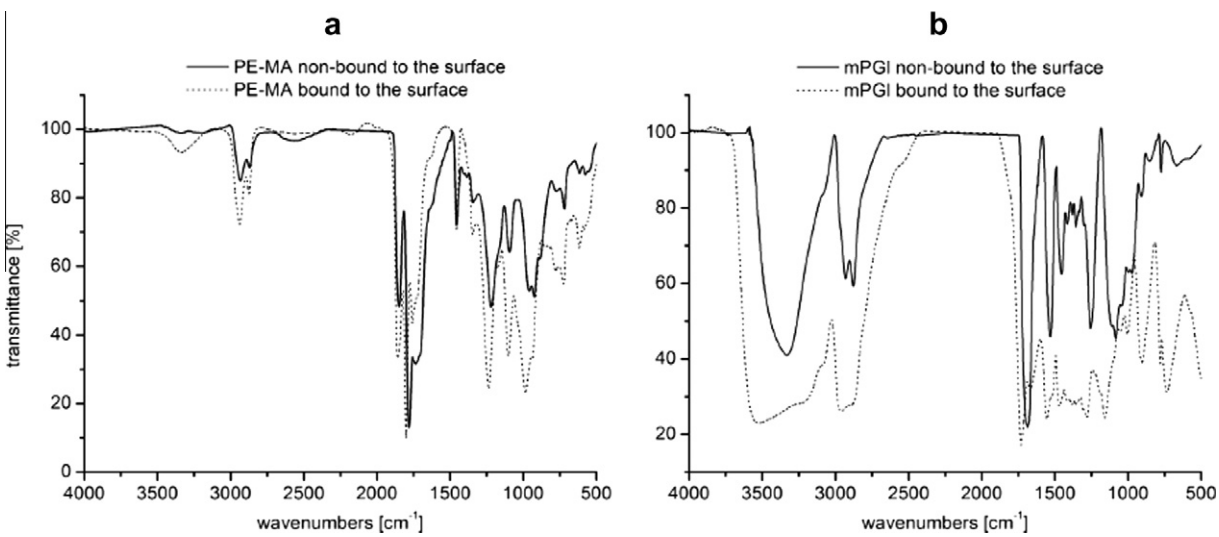

Fig. 3. The FT-IR spectra of (a) PE-MA bound to the surface (dashed line) and PE-MA non-bound to the surface (solid line) and (b) the mPGl bound to the surface (dash line) and non-bound mPGl (solid line).

literature value (8–13 nm) for such surfaces obtained in the same preparation conditions [50]. The contact angle values for the PE-MA wafers were approximately 90°, indicating the hydrophobic nature of the polymer layer. As the surface philicity changed from 90° to 70° when exposed to water, indicating the hydrolysis of the anhydride groups of poly(ethylene-alt-maleic anhydride) into less reactive carboxylic groups, the PE-MA wafers were annealed before use to reconvert the carboxylic groups to the anhydride form.

According to the literature, the functional anhydride groups from poly(ethylene-alt-maleic anhydride) deposited on the surface have been successfully reacted with amines, poly(ethylene glycol) and cellulose [51]. Here,

poly(glycidol-co-ethyl glycidyl carbamate) surfaces were obtained using the "grafting onto" technique. The thermosensitive polymer was covalently immobilized by reacting the hydroxyl groups of the polyglycidol derivative with the complementary anhydride groups of the PE-MA layer on the surface (Fig. 2d). The mPGl was spin coated onto the modified wafer and the reaction was induced merely by annealing at 120 °C, which greatly simplified the protocol for surface coating. The mPGl concentrations were varied between 0.25 and 10% v/v and the rotating speed, from 1000 to 5000 rpm because the spin coating conditions, such as the rotating speed, concentration and viscosity of the solution, have a strong influence on the polymer layer thickness. After reaction, the mPGl surfaces were thoroughly cleaned with methanol to remove any unreacted polymer and residual contaminants.

The presence of a poly(glycidol-co-ethyl glycidyl carbamate) layer covalently bound to the surface was confirmed by FT-IR. Fig. 3b compares the FT-IR spectra of mPGl bound to the surface (dash line) and non-bound with the wafers (solid line). Non-bound polymer means the polymer before spin-coating on the surface, whereas bound mPGl refers to the polymer after reaction with the surface and subsequent washing. In the spectrum of non-bound mPGl, the characteristic absorption bands were visible at 1685 and 1530 cm^{-1} corresponding to the stretching vibrations of the C=O groups from the amide groups and the deformation vibrations of the N–H groups. Moreover, the following absorption bands were also observed at 3300 cm^{-1} (stretching vibrations of O–H and N–H), 2930–2880 cm^{-1} (stretching vibrations of C–H), 1255 cm^{-1} (stretching vibrations of C–N) and 1100 cm^{-1} (stretching vibrations of C–O–C). A similar spectrum was observed for the mPGl on the surface. However, some changes confirm the chemical bonding of the modified polyglycidol to the surface covered with PE-MA. The adsorption band at 1850 cm^{-1} (vibrations of anhydride C=O), seen in the spectrum of PE-MA on surface before reacting with modified polyglycidol (Fig. 3a), is not seen after the reaction (Fig. 3b), thus indicating that most of the anhydride groups have reacted. In the spectrum of modified polyglycidol (Fig. 3b) an adsorption band at 1530 cm^{-1} derived from the NH groups is seen. It was not visible in the PE-MA spectrum (Fig. 3a), thus confirming the presence of covalently bound modified polyglycidol layer.

Fig. 4 compares the surface morphologies of the mPGl deposited on functionalized surfaces by AFM.

The morphology of the resulting surfaces differed when the polymer concentration and speed of rotation were changed. The spin-coating of low concentrated mPGl solution (1%) with rotating speeds from 1000 to 4000 rpm resulted in smooth surfaces (RMS = 0.09–0.18 nm) (Fig. 4a). The increase of rotating speed to 5000 rpm led to irregular surfaces with increased roughness (RMS up to 2.82 nm) (Fig. 4b). Such high rotating speed caused incomplete coverage of the intermediate PE-MA layer by modified polyglycidol. Spin coating with highly concentrated mPGl solution (10%) at low rotating speed (2000 rpm) resulted in a "wavy" surface (Fig. 4c). Conversely, the smooth morphology of mPGl layer was observed when the rotating speed was increased to 5000 rpm (Fig. 4d).

For the investigation of influence of polymer concentration on the polymer layer thickness and wettability the spin coating of polymers with rotating speed equal to 2000 rpm were used. The results of the polymer layer thicknesses and wettability are presented in Table 1.

The contact angle measurements were performed for dry, freshly prepared samples. During 30 s of the measurement droplet was soaking to the dry polymer layer. The most rapid and fastest soaking was observed for the layer made from the highest concentration of mPGl (mPGl_D). In comparison, the permeation of the water droplet into the mPGl_A layer (the lowest polymer concentration) was barely noticeable, implying that the resulting polymer layers have different thicknesses. After incubating the wafers in water at 20 °C for 24 h, the surfaces exhibited hydrophilic properties. The measured mean contact angles varied between 60° and 30°, depending on the concentration of polymer solution used for coating. The water droplet on these surfaces was stable, and soaking was not observed. Such behavior showed that the polymer layer was highly hydrated and swollen and did not absorb more water after this incubation. Increasing the temperature (40 °C) at which wafers were incubated noticeably changed its philicity. The mean contact angle values increased, reflecting a temperature-induced increase in the hydrophobicity of all mPGl layers. For comparison, surfaces coated with non-modified, non-thermosensitive polyglycidol (solution concentration 1%, the same conditions of spin-coating) did not exhibit changes in their surface philicity at elevated temperature. To check the stability of polymer layer after long incubation in water for possible hydrolysis of the ester bond the FTIR and contact angle measurements were performed. No changes of polymer layer properties upon such treatment were detected.

Ellipsometry was used to determine the thickness of the mPGl films for dry samples and samples incubated in water at 20 and 40 °C (Table 1). The modified chains of high molar mass polyglycidol grafted to the surface formed layers of approximately 20–60 nm thickness. This thickness may be compared with the dimensions of unperturbed polyglycidol chains in solution to gain some insight into how the chains are connected to the surface. The equation combining the radius of gyration R_g of pure polyglycidol in aqueous solution and its molar mass (weight average) is known from our previous study [52]. Using this equation, we calculated the R_g of polyglycidol used in this work (M_w = 2 900 000 g/mol) and found that its R_g equaled 100 nm, which is much larger than the measured thickness of the PGl layer. One has to keep in mind however that the modified polyglycidol has reactive hydroxyl groups randomly distributed along the chain. These groups may be covalently bound to the surface, thus binding each mPGl chain in more than one place with the surface. This can lead to the flattening of the polymer coil conformation on the surface, as presented schematically in Fig. 5a.

The ellipsometry studies for the mPGl surfaces revealed that coating the wafers with various polymer concentrations led to surfaces with different polymer layer thickness. The thickness of the layers increased with an increasing concentration of the spin coated polymer

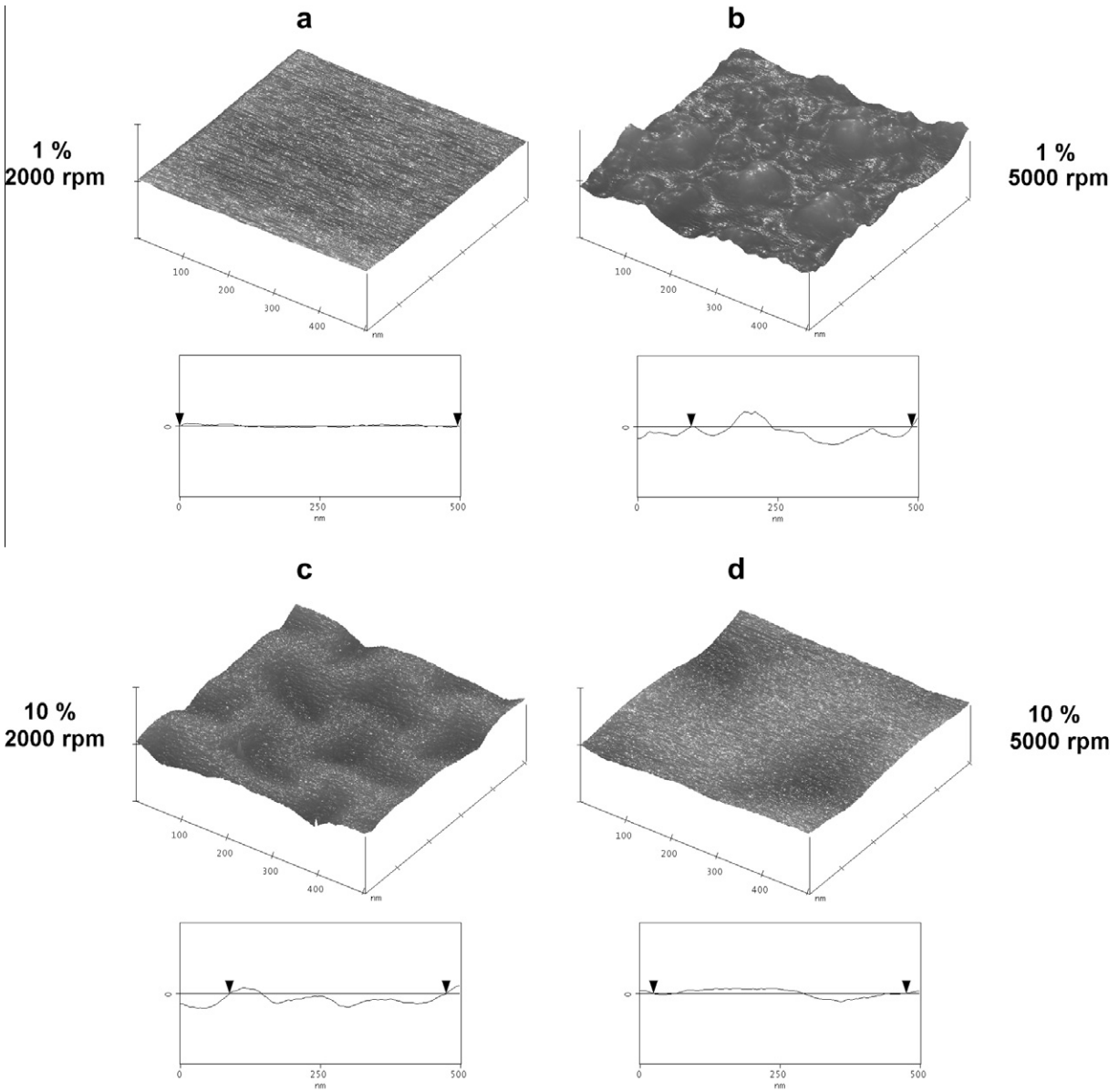

Fig. 4. AFM micrographs of mPGl surfaces obtained using 1% mPGl and 2000 rpm (a) and 5000 rpm (b); and using 10% mPGl and 2000 rpm (c) and 5000 rpm (d).

Table 1
The contact angles and layer thicknesses for mPGl layers.

	Contact angle (°)		Layer thickness (nm)		
	Water/ 20 °C	Water/ 40 °C	Dry sample	Water/ 20 °C	Water/ 40 °C
0.25% (mPGl_A)	60 ± 3	68 ± 2	20 ± 0.7	30 ± 0.5	36 ± 0.5
0.5% (mPGl_B)	55 ± 3	65 ± 4	28 ± 0.5	31 ± 0.4	33 ± 0.4
1% (mPGl_C)	50 ± 3	70 ± 2	34 ± 0.5	38 ± 0.4	41 ± 0.5
10% (mPGl_D)	30 ± 3	55 ± 2	24–60 ± 0.5	27–60 ± 0.5	47 ± 0.5
Non-modified PGl on the surface	50 ± 3	51 ± 3	–	–	–

solution. In the case of the highest polymer concentration applied (10%), film thickness varied in a wide range from 24 to 60 nm. The "wavy" surface of the mPGl_D sample, as seen by AFM (Fig. 4c), could explain the broad range of layer thickness values.

The ellipsometry studies for mPGl surfaces incubated in water (24 h, 20 °C) revealed an increase in the polymer layer thickness. Such behavior can be explained by the swelling of the polymer layer as a result of water penetration.

Increasing the temperature to 40 °C (that is, above the T_{CP} of the deposited mPGl) caused a slight increase in the polymer layer thickness (Table 1). Such behavior is unusual and different in comparison to the behavior of thermoresponsive polymer brushes. The thickness of a temperature-responsive layer usually decreases under heating, which is attributed to the dehydration and shrinking of the polymer chains [53]. In our case, heating the

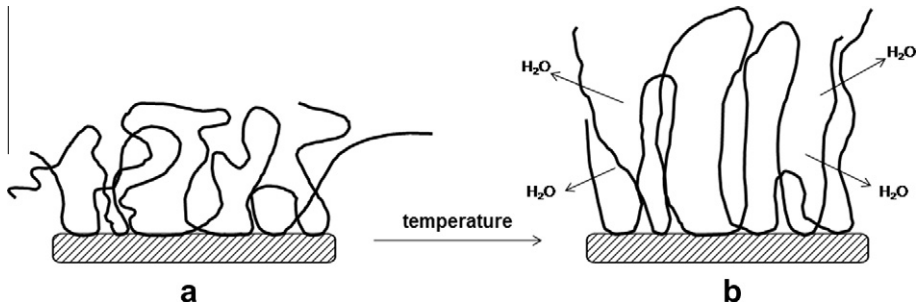

Fig. 5. (a) The scheme of the poly(glycidol-co-ethyl glycidyl carbamate) polymer layer on the surface and (b) possible behavior of the poly(glycidol-co-ethyl glycidyl carbamate) polymer layer at temperature increase.

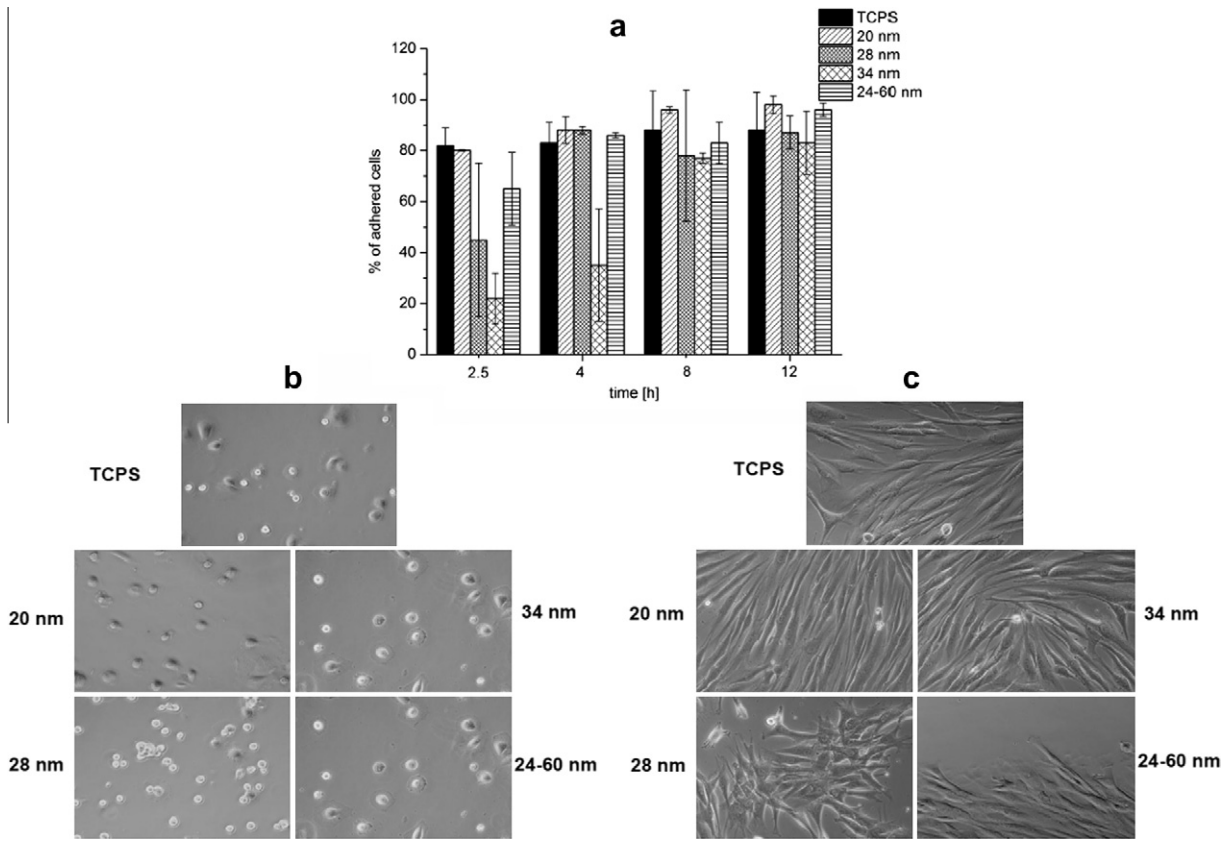

Fig. 6. The percentage of fibroblasts spread onto mPGl surfaces of different thickness and onto the TCPS used as a positive control (a), and microscopy images of fibroblasts adhesion on mPGl surface after 2.5 h (b) and after 72 h of cell culture (c) (cell culture temperature 37 °C).

polymer layer on the surface also caused dehydration and evidently changed the polymer layer's philicity, as confirmed by contact angle studies (hydrophobic nature of the surfaces was observed under increased temperature); however, the layer thickness indicated by ellipsometry either remain constant or slightly increases (Table 1). Such behavior of thermosensitive mPGl on the surface during heating could be explained by the fact that the removal of water from the interior of the polymer layer under the influence of temperature is accompanied by the formation of hydrophobic interaction between polymer chains what cause their stretching (Fig. 5b). This could lead to the situation that the overall decrease of film thickness above T_{CP} was prevented. This finding, although experimentally checked in this case beyond reasonable doubt, is difficult to rationalize in a fully supported, non-speculative way. Also having in mind that the spectroscopic ellipsometry is an indirect, model based method for layer thickness measurement and the fitting models depends on the layer roughness or optical properties of polymer under different external condition, this conclusion still requires more detailed investigations. However, similar behavior of polymer chains under heating has been previously observed for PNIPAM brushes on gold surfaces [54], where the polymer

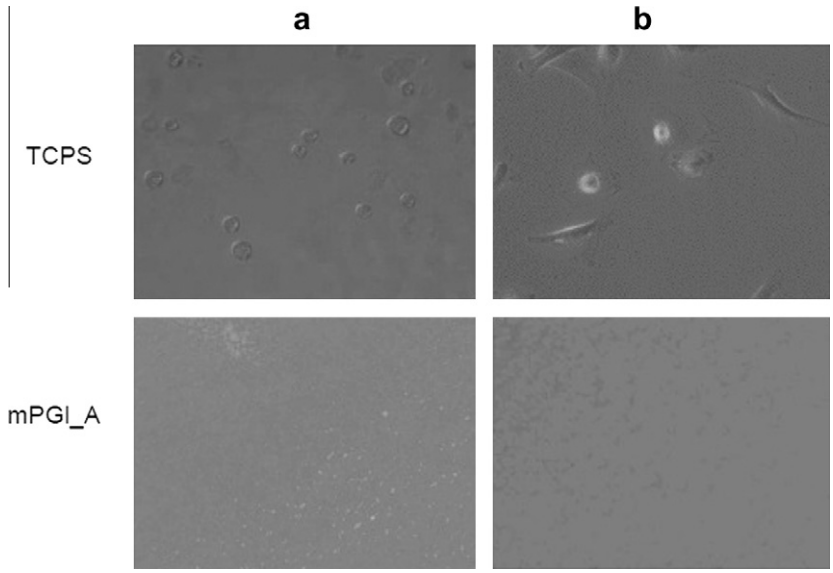

Fig. 7. Fibroblast adhesion to the TCPS control sample and to the mPGl_A surface after 2.5 h (a) and 12 h (b) of cell culture at 20 °C.

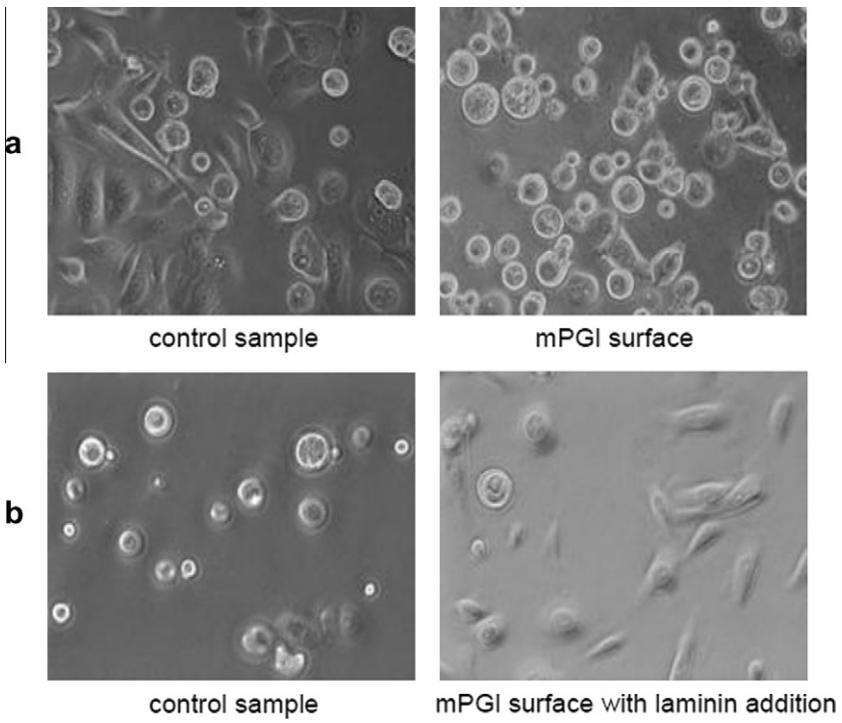

Fig. 8. Keratinocyte adhesion to the TCPS control sample and to the mPGl_B surface: (a) without laminin and (b) after addition of laminin to the culture medium (cell culture temperature 37 °C).

films shrunk and aggregated without any significant difference in height of the layer.

3.3. Cell culture

In previous studies, we have shown the use of cryogels, based on thermosensitive polyglycidol derivatives, as a scaffold for skin cell adhesion [42]. The adhesion and proliferation tests indicated that the cryogels were good supports for cell cultivation. In this work, we investigated the ability of poly(glycidol-co-ethyl glycidyl carbamate) surfaces of different morphologies and thicknesses to interact with human skin cells. Two types of skin cells: fibroblasts and keratinocytes were cultured on mPGl surfaces at 37 °C. At this temperature, the surface exhibited hydrophobic properties, as shown by contact angle

measurements, which could favor cell culture. Cells were also cultured on TCPS under the same conditions as a control. The cell viability was measured after 2.5, 4, 8, 12, 48 and 72 h of cell culture using the AlamarBlue test. The sample results were normalized to the TCPS values and are shown as a percentage of the positive control. The results of the fibroblast (FK1) culture on mPGl surfaces of different thickness and the microscopy images of the fibroblasts distribution on the surface are presented in Fig. 6.

After 2.5 h of culture, fibroblasts started to adhere to the mPGl surface (Fig. 6a and b). The percentage of cells spread on the mPGl surface at this time decreased with the layer thickness. When the culture time was prolonged, the cell viability increased, the cells spread and proliferated. Some deviation was observed for the mPGl_D sample (Table 1) with the highest layer thickness (24–60 nm). The sudden increase in the percentage of attached cells can be explained by the formation of fibroblasts clusters in the pores of the polymer layer (see morphology in Fig. 4c).

A remarkable difference was observed in the distribution of cells on the mPGl surfaces based on the different layer thicknesses after 72 h of cell culture (Fig. 6c). The cells on the mPGl_A and mPGl_B surfaces (polymer layer thicknesses equal to 20 and 28 nm, respectively) formed an integral monolayer, whereas the cells formed clusters on the mPGl_C and mPGl_D surfaces (polymer layer thicknesses equal to 34 and 24–60 nm, respectively). This could be attributed to the differences in surface morphologies. As mentioned before, the wafers with thin mPGl layers (lower concentration of mPGI solutions) formed smooth, regular surfaces (Fig. 4a) that allowed the skin fibroblasts to attach and spread uniformly, comparable to the control samples. For the thicker surfaces (higher concentration of mPGl solution) with a "wavy" morphology (Fig. 4c), the skin fibroblasts grew in clusters, most likely at places where the mPGl layer was thinner.

The hydrophobic properties of the mPGl layer at increased temperature allowed the skin fibroblasts to adhere and proliferate. To verify this statement, skin fibroblast adhesion tests were performed at 20 °C. At this temperature, the surface is hydrophilic. The skin fibroblast cultures at this temperature are shown in Fig. 7.

As can be seen, in contrary to the control sample (TCPS), no adhesion of fibroblasts to polymer surface is observed, when the surface of the modified polyglycidol is hydrophilic (below transition point). The hydrophilic nature of the mPGl layer does not favor the interactions with skin fibroblasts.

The interactions of mPGl surfaces with keratinocytes at 37 °C were also studied. Fig. 8 shows microscopy images after 4 h of cell culture on the mPGl_B surface with and without the addition of laminin compared with the control sample (TCPS).

Keratinocytes barely adhered to the mPGl_B layer after 4 h of cell culture compared to the control sample (Fig. 8a). Keratinocytes adhesion to the mPGl surfaces was much slower than adhesion of fibroblasts. Keratinocytes are environmentally more demanding than skin fibroblasts; therefore, such discrepancies in the culture of these two types of cells can be observed. Adding laminin, a peptide synthesized by epithelial cells in human skin, to the culture improved the adhesion and spreading of the keratinocytes to the mPGl layer (Fig. 8b).

4. Conclusion

Poly(glycidol-co-ethyl glycidyl carbamate), biocompatible with skin cells, was covalently attached to a solid support using the "grafting onto" technique. The polymer was grafted by initiating the reaction between the anhydride groups of the intermediate layer of the glass or silica support and the residual hydroxyl groups of the polymer. The polymer layer thickness ranged between 20 and 60 nm and depended on the concentration of polymer used during the reaction. The resulting polymer surfaces exhibited temperature-responsive behavior when swollen in water and showed a hydrophilic-to-hydrophobic transition in response to temperature changes. Skin cells interaction with the poly(glycidol-co-ethyl glycidyl carbamate) surface was observed at temperature above the phase separation temperature of the immobilized polymer, when the layer was hydrophobic. The fibroblasts are able to form a cell sheet monolayer on the modified polyglycidol surface only at lower polymer layer thicknesses. Although the growth of the keratinocytes is not satisfying, the addition of laminin, improve its interaction with the obtained poly(glycidol-co-ethyl glycidyl carbamate) surfaces.

Acknowledgments

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