The effect of polymeric membrane surface on HaCaT cell properties

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\section*{ABSTRACT}

The control of the surface properties is an important issue for applicability of polymer membranes interacting with cells. In this work, the influence of surface roughness and stiffness of two polymer membranes on viability and mechanical properties of keratinocytes was studied. Terpolimer polyglycolide, polycaprolactone and polylactide, (PGA-PCL-PLA) and copolymer polycaprolactone, polylactide (PGA-PCL) substrates were used for membranes fabrication. Surface modification – the hydrolysis of the obtained membranes was carried out. The analysis of membranes’ surface properties revealed that RMS surface roughness and roughness factor of PGA-PCL-PLA membrane decreased after hydrolysis while its stiffness increased. In contrast, the PGA-PCL membrane stiffness was only slightly affected by NaOH treatment. Immortalized human keratinocytes (HaCaT) were grown under standard conditions on the surface of the studied membranes and characterized by means of atomic force microscopy and fluorescence microscopy. The results showed the substrate-dependent effect on cells’ properties.

\section*{1. Introduction}

Search for the biocompatible and biodegradable polymer-based biomaterials becomes of a great importance for biotechnology and regenerative therapies including tissue engineering. Particularly important are polymer surface properties determining the cell-scaffold and cell-artificial membrane interaction (Chen et al., 2013; Gogolewski, 2000; Shirilff and Hench, 2003). Investigations of these parameters have been performed by many analytical techniques including evaluation of morphological and biological as well as mechanical properties of the cells growing in the presence of various polymeric biomaterials.

Biomaterials for implantation purposes have to mimic physical properties of the tissue being replaced, with a minimum toxic response of the host, due to the lack or minimal release of toxic substances during degradation in the body (Kennedy and Panesar, 2006).

The polymers commonly used in tissue engineering belong to polyesters like polylactide, polyglycolide, polycaprolactone and copolymers thereof. They have been widely adopted for biomedical application because of their high biodegradability and controlled rate of biodegradation, as well as their good biocompatibility due to mechanical and thermoplastic characteristics (Xu et al., 2010).

Biocompatibility, biodegradation and functionality of natural and artificial membranes are determined by the materials surface properties, particularly mechanical parameters. It is well recognized, that cells receive their surviving, proliferation, differentiation and death signals from environment through bioactive signaling molecule receptors and mechanical interactions as well (Georges and Janmey, 2005). It has been proven, that cells will not grow properly in an environment not similar to their native tissue mechanical milieu (Engler et al., 2004). Thus it is necessary to optimize the biopolymer substrate properties to match the target cell requirements (Sanabria-DeLong et al., 2007). It has been shown, that stiffness of the substrate effects the cell proliferation and different cell types respond to different ranges of stiffness (Discher et al., 2009), with relatively small changes in substrate elasticity triggering the strong cellular changes (Mammoto et al., 2009). Surface probe measurements of local elasticity allow a relatively accurate assessment of the mechanical properties of various materials, including thin films (biomembranes). Substrate stiffness cannot be predicted from the concentrations of biopolymers or synthetic polymers used to prepare substrates, but need to be tested directly by various techniques (Levental et al., 2010). Synthetic polymers like polyglycolide (PGA), copolymers of polylactide (PLA) and polycaprolactone
(PCL) are frequently used for the production of biomembrane mimics. In regenerative medicine polyesters are used for making dressings for hard-healing wounds or as skin-substitute material. One possible approach is to isolate of patient’s cells and after culturing in vitro on polymer matrices, implanting the obtained engineered tissue construct into target location (Dhandayuthapani et al., 2011; Liu et al., 2007). The advantage of such a construct is due to the possibility of obtaining reproducible, well-defined chemical composition with controlled mechanical properties and dimensions and optimal rate of biodegradation. In order to improve their biocompatibility, they often require additional surface modification. In presented study membranes constructed from terpolymer polyglycolide – polycaprolactone – polylactide (PGA-PCL-PLA) and copolymer polycaprolactone – polyglycolide (PGA-PCL) were used as substrates. Polymeric membranes were prepared by the phase inversion method. Membrane surface modification was performed by hydrolysis in the presence of NaOH. The quantitative estimation of carboxyl groups in the studied membranes was performed by the dye interaction method (Kang et al., 1993). To analyze the modified and unmodified surface morphology the scanning electron microscope, nano-mechanical test instrument and the atomic force microscope (AFM) were applied. According to our knowledge, no data concerning of the surface properties of both membranes are yet available. The effect of substrate surface properties on keratinocytes was estimated by evaluation of the viability and mechanical characteristic of cells grown on investigated polymer membranes.

2. Materials and methods

2.1. Materials

Copolymer glycolide/caprolactone ~16% mol caprolacton (Alpha) and 84% mol glycolide (PGA-PCL), average molecular mass 130,000 g/mol; dispersion of molecular weight 2.2. Terpolymer L-lactide/glycolide/caprolactone (PGA-PCL-PLA) ~71% mol lactide, 12% mol glycolide, 17% mol caprolactone, average molecular mass 100,000 g/mol, dispersion of molecular weight 2.9 were prepared. Zirconium acetylacetonate, was used as catalyst for the synthesis instead of standard tin catalysts due to the later potential toxicity for living cells.

Polymers were purified from the residual monomers by dissolving them in chloroform (Polish Chemical Reagents, POCh), and then added slowly, dropwise to the cold methanol (POCh), to coagulate. After preliminary drying in air polymers were finally dried in a vacuum oven at 25 °C.

2.2. Membrane preparation

The polymers were dissolved in trichloroethylene for 24 h on a stirrer to obtain a clear solution. For degassing, solution was placed in a vacuum oven for 1 h. For membrane preparation the polymer solution was evenly distributed on the surface of glass plates with a knife collector and plates were dried in oven with forced air circulation at 30–45 °C for 24 h. The first layer of the membrane was performed with the distance of a knife of 0.3 mm, and then dried 2–4 h in an oven at 40 °C, then second layer was applied gently with a focus of blade on the 0.15 mm and dried for 24 h at 40 °C.

2.3. Membrane surface hydrolysis and determination of carboxyl groups

The obtained membranes (4 × 4 cm²) were placed in glass flask with solution of 1 and 2 M sodium hydroxide in water for 1–2 h at 37 °C with mechanical stirring. After hydrolysis, the membranes were washed three times with distilled water.

The amount of carboxyl groups on the membranes was determined by the modified rhodamine-carboxyl interaction method (Kang et al., 1993). 4 mg of Rhodamine 6G was dissolved in 4 ml of PBS (pH 12) and solution was immediately extracted with 100 ml of benzene. The yellow extract was used as a dye reagent for further experiments. The untreated and hydrolyzed membranes were soaked in 5 ml of dye reagent. After dissolution of the membrane the decrease in the absorbance at 486 nm was measured. The concentration of the carboxylic acid groups on the surfaces was calculated from the calibration curve.

2.4. Contact angle

Static contact angle experiments were performed by the sessile drop technique using a Kruss Easy Drop (DSA25) instrument. The measurements were carried out at room temperature. Contact angles were expressed as the average of ten measurements at different spots.

2.5. Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM; Hitachi TM1000) was used for imaging the membrane surface and cross-sectional morphologies. Membranes were poured onto a glass plate of size 10 × 27 cm². The size of obtained membranes was 9 × 22–24 cm². Each membrane was divided into four equal parts, one of them was remained unmodified and the other parts were subjected to hydrolysis, in different conditions (time and NaOH concentration).

Subsequently the edges of the width ~ 2 cm and pieces of size about 2 cm² (3–4 pieces from different areas) were cut. Membrane samples were first immersed in ethanol and fraccuted in liquid nitrogen into about 0.1–0.4 cm² pieces. Membranes were fixed on stubs with carbon self-adhesive tapes. The samples were coated with a 7–10 nm layer of gold using K550X Sputter Coater apparatus. Coated samples were examined at magnification of 300 (at least 4 micrographs were taken for each sample) at an acceleration voltage of 15 kV.

2.6. Morphological analysis by atomic force microscopy (AFM)

Membrane surfaces were evaluated using AFM Park SystemsXE-120 operating in a contact mode. The measurements were carried out using silicon nitride cantilevers (MLCT, Bruker) with the measured spring constant of 0.039 N/m (nominal spring constant of 0.03 N/m) and 0.011 N/m (nominal spring constant of 0.01 N/m) For each group of the studied membranes, topography measurements were carried out in contact mode on randomly chosen regions of the sample surface (more than 10 images for each sample were taken to gain better knowledge of the variations of local structures). All scans were performed under ambient conditions (air). For all images, we started with the same values of scan parameters. The scan rate was 1 Hz and set point was set to 1.0 nN. However, in each case, final optimizations were performed.

2.7. Mechanical properties of the membranes

A Hysitron TI 950 TribolIndenter™ nanomechanical test instrument was used to perform nanoindentation on membrane samples. Load controlled quasi-static tests were performed on the samples using a diamond Berkovich probe.

Small pieces (15 mm × 15 mm) of the membrane were attached to a petri dish. Samples were fixed on vacuum stage afterward and measured. Tested positions were chosen using optical microscope which is mounted in nanoindentation instrument. There were performed 16 indenters in matrix of 4 × 4 with a separation of 10 μm between each indent on each sample. Load and unload rates were set to 100 μm/s. The obtained load/unload versus displacement curves were analyzed using Oliver-Pharr method (Oliver and Pharr, 2004) where the Young’s modulus is determined from the unload part of the curve.

2.8. Cell culture

The spontaneously immortalized human keratinocyte cell line
(HaCaT, Deutsches Krebsforschungszentrum Stabstelle Technologietransfer Heidelberg, Germany) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Life technologies, USA) containing 4.5 g/l glucose and supplemented with 10% fetal bovine serum (FBS, Life technologies, USA), 2 mM L-glutamine (Life Technologies, USA) and 1% of antibiotics mixture (10,000 U/ml penicillin – 10,000 μg/ml streptomycin, Life Technologies, USA). Cells were grown at 37 °C in humidified atmosphere of 5% CO₂ and subcultured at approximately 80% of confluence, after treatment with 0.25% trypsin/EDTA solution (Life Technologies, USA), according to producer protocol. For the experiments 50,000 cells were seeded onto membrane attached by Polydimethylsiloxane to 10 mm glass coverslips (Menzel Gläser, Germany), placed into the 24-well plates and incubated at 37 °C in humidified atmosphere of 5% CO₂ for 48 h.

2.9. MTT cell viability test

The MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, Sigma-Aldrich, USA] assay was performed after 48 h of growth in 24 well plates according to the producers’ protocol and used as a viability test for tracking the effect of the membranes on the HaCaT cells growth. The 500 μl/well of MTT Working Solution (0.5 mg/ml in DMEM without phenol red) was added to the cells and the plates were further incubated for 3 h at 37 °C in humidified atmosphere of 5% CO₂. To dissolve the purple formazan product, the MTT Working Solution was replaced by DMSO and plates were incubated for 2 h at 37 °C. The absorbance was measured at 570 nm in a micro plate reader (Synergy 4, BioTek, USA) with a spring constant of 0.01 N/m were used. The cells were dissolved in 0.01 N/m DMSO and plates were incubated for 2 h at 37 °C. The absorbance was measured at 570 nm in a micro plate reader (Synergy H4, Biokom). The results were expressed as a relative viability of cells cultured on investigated membranes and control cells grown on glass coverslips.

2.10. Actin filaments morphology

The cells were fixed in 3.7% paraformaldehyde (Sigma-Aldrich, USA) dissolved in PBS for 10 min at room temperature (RT) and permeabilized with a solution of 0.2% Triton X-100 (Sigma-Aldrich, USA) dissolved in PBS (5 min, RT). The actin filaments were stained with phalloidin labeled with Alexa Fluor 488 (1:200, Life Technologies, USA) dissolved in PBS for 10 min at room temperature (RT) and per- fixed with NaOH solutions (Life Technologies, USA), 2 mML-glutamine (Life Technologies, USA) according to producer protocol. For the experiments 50,000 cells were seeded onto membrane attached by Polydimethylsiloxane to 10 mm glass coverslips (Menzel Gläser, Germany), placed into the 24-well plates and incubated at 37 °C in humidified atmosphere of 5% CO₂ for 48 h.

2.11. Atomic force microscopy (AFM) analysis of cell stiffness

Measurements of the HaCaT cells stiffness were performed using a commercial microscope (XE120 model, Park Scientific Instruments, South Korea). The gold-coated silicon nitride cantilevers (MLCT, Bruker, USA) with a spring constant of 0.01 N/m were used. Membranes with cells were washed 2 times with DMEM buffer before each measurement. All measurements were performed in DMEM buffer at room temperature. In order to locate a cell and control the AFM cantilever position, an optical microscope was used. Force curves were collected from randomly chosen keratinocytes (around 20 cells in each repetition). Regions around cell center were selected for the measurement. At each selected region, a grid of 4 × 4 points was created. The set point was 0.5 N and force limit was set up to 4.1 N. Force curves were recorded at the scan velocity equalized to 9 μm/s. Determination of the Young’s modulus was based on a subtraction of two force curves: the calibration curve recorded on a hard surface (in our case it was a part of the membrane without cells) and the other on a given cell (Kobieła et al., 2013). We estimated the modulus by fitting force-indentation data for indentation depth of 300 nm, where we could expect the mechanical response originating from actin cytoskeleton (Sobiepanek et al., 2016). The obtained force versus indentation curve can be fit with the Sneddon extension of the Hertz model (Sneddon, 1965) assuming that the AFM tip is an infinitely stiff indenter of defined geometry. In our case the best fit was achieved when force-indentation curves were analyzed assuming that the shape of the AFM tip is a parabola. Using the paraboloid approximation of the indenting probe, the elastic modulus can be calculated:

\[
F(\delta) = \frac{4}{3} \sqrt{E_{\text{eff}} \pi \delta^3}
\]

where \(R\) is the radius of the AFM tip, \(\delta\) is the indentation depth, and \(E_{\text{eff}}\) is the reduced Young’s modulus, which describes both the cell and AFM cantilever stiffness linked in series. Since the Young’s modulus of the AFM cantilever is much larger than that for cells, the \(E_{\text{eff}}\) can be re-written as follows:

\[
E_{\text{eff}} = \frac{E_{\text{cell}}}{1 - \nu_{\text{cell}}^2}
\]

where \(\nu_{\text{cell}}\) is the Poisson coefficient set to 0.5 since cells can be treated as an incompressible material (Lekka et al., 1999).

3. Results and discussion

3.1. Membrane modification with NaOH solutions

The prepared membranes were chemically modified with NaOH solutions to improve surface availability for cell adhesion. To optimize the amount of the carboxyl group on the membrane surface, the modification was carried out under two different concentrations of NaOH (1 M and 2 M) for two different reaction times (1 h and 2 h).

The results showed in Table 1 indicate that the degree of the conversion of –COOR group into –COOH is dependent on the concentration of NaOH and the reaction time. The highest conversion of –COOR into –COOH was reached with 2 M NaOH and 2 h incubation. These membranes were selected for further experiments. The change of the hydrophilicity of the selected membranes was indicated by the water contact angles listed in Table 2. Although, the surface density of the carboxyl groups on hydrolyzed PGA-PCL-PLA (111.5 μmol/cm²) was almost the same that on the PGA-PCL membrane (113.5 μmol/cm²) the hydrophilicity indicated by the water contact angle was different. The water contact angle of PGA-PCL-PLA membrane decreased with the introduction of carboxyl groups from almost 77° to 54°, while for PGA-PCL membrane the water contact angle decreased only from 75° to 64°. It is well known that the wetting characteristic is correlated with surface roughness (Israelachvili, 1992), therefore a study of the surface morphology and mechanical properties of the selected membranes was carried out.

<table>
<thead>
<tr>
<th>Membrane</th>
<th>Time [h]</th>
<th>Amount of carboxyl group [μmol/cm²]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 M NaOH</td>
<td>2 M NaOH</td>
</tr>
<tr>
<td>PGA-PCL-PLA</td>
<td>0</td>
<td>53.8 ± 0.1</td>
</tr>
<tr>
<td>PGA-PCL-PLA</td>
<td>1</td>
<td>68.1 ± 0.5</td>
</tr>
<tr>
<td>PGA-PCL-PLA</td>
<td>2</td>
<td>107.2 ± 1.2</td>
</tr>
<tr>
<td>PGA-PCL</td>
<td>0</td>
<td>50.3 ± 0.5</td>
</tr>
<tr>
<td>PGA-PCL</td>
<td>1</td>
<td>52.2 ± 2.2</td>
</tr>
<tr>
<td>PGA-PCL</td>
<td>2</td>
<td>60.3 ± 0.3</td>
</tr>
</tbody>
</table>
3.2. Characterization of membrane morphology and stiffness

The AFM image of typical PGA-PCL-PLA membrane used in our study is shown in Fig. 1a. The surface is characterised by a grain-like structure. The aggregates with mean diameters of about 1 μm × 2.5 μm and heights of 0.5 μm are randomly distributed. Treatment with 2 M NaOH for 2 h resulted in very dramatic changes in surface topography (Fig. 1b). The grain-like structure was transformed into sponge-like surface (compare Fig. 1a, b). The randomly distributed pores achieved a roundish shape and merged together forming bigger holes with an irregular shape. The in-plane diameter of the holes ranged from 0.5 to 1.5 μm and the irregularly shaped holes reached lateral sizes up to 3 μm. It seems, that surface structure of the membrane reacts fast with NaOH and undergoes hydrolysis. On the other hand, the SEM investigations of the cross-section of untreated and NaOH-treated PGA-PCL-PLA membranes do not show changes in the bulk structure of the studied membranes. (compare Fig. 2a, b). It is typical for surface erosion resulting in the membrane thinning over time while maintaining its bulk integrity (Cameron and Kamvari-Moghaddam, 2008; Kohn and Langer, 1996).

Example of typical AFM topography of PGA-PCL membrane obtained in our experiments is presented in Fig. 1c. The membrane topography consists of large aggregates of dimensions 5–10 μm, and pores of dimensions 1–8 μm. After hydrolysis (Fig. 1d) aggregates became smoother and even larger up to 15 μm. Moreover, the pores, visible as dark spots on the surface, increased up to 15 μm. We assume that PGA-PCL membrane degraded through bulk heterogeneous erosion mechanism where the central region of the polymer becomes more degraded than the surface over time. It is clearly visible in SEM micrographs, where completely different cross-section structure was obtained in the membrane after 2 h treatment in NaOH than in the PGA-PCL membrane without treatment (compare Fig. 2c, d).

AFM measurements allowed the calculation of the parameters which characterize the rough surface (Kruszewski and Kobiela, 1999). The following surface parameters were taken into account: (i) RMS – the root mean squared surface roughness, which is the standard deviation of the height value in the selected region and (ii) the roughness factor, which is the ratio of the surface area to the area of the selected region (plane).

In Fig. 1 AFM images from randomly selected 30 × 30 μm² areas of investigated samples are presented. For each surface, several local profiles of various places of the sample were taken. The RMS surface roughness of PGA-PCL-PLA membrane is equal to 212.3 nm ± 32.8 nm (mean ± standard deviation) and the roughness factor is equal to 1.29. After hydrolysis process a significant decrease of the surface parameters was observed. The RMS surface roughness decreased to 148.3 nm ± 19.7 nm. The same trend was observed for the average roughness factor values: from 1.29 down to 1.11.

On the other hand, after hydrolysis process the RMS surface roughness of membrane PGA-PCL increased from 166.8 nm ± 47.3 nm up to 301.3 nm ± 72.1 nm and the roughness factor from 1.01 up to 1.02.

A Hysitron nanomechanical test instrument was used to perform nanoindentation on membrane samples. The mechanical properties of

<table>
<thead>
<tr>
<th>Membrane</th>
<th>Water contact angles [deg]</th>
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<tbody>
<tr>
<td>PGA-PCL-PLA untreated</td>
<td>77.0 ± 0.1</td>
</tr>
<tr>
<td>PGA-PCL-PLA hydrolyzed</td>
<td>54.1 ± 0.2</td>
</tr>
<tr>
<td>PGA-PCL untreated</td>
<td>75.1 ± 0.1</td>
</tr>
<tr>
<td>PGA-PCL hydrolyzed</td>
<td>64.5 ± 0.2</td>
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</table>

Fig. 1. AFM images of a) unmodified PGA-PCL-PLA membrane, b) hydrolyzed PGA-PCL-PLA membrane, c) unmodified PGA-PCL membrane; d) hydrolyzed PGA-PCL membrane.
membranes are quantified by the Young’s modulus values (Table 3). Although there are no differences in Young’s modulus between PGA-PCL-PLA and PGA-PCL membrane, the properties PGA-PCL-PLA was much more affected by the NaOH treatment. Higher loads are required to produce the same displacement for this membrane as compared to the other surfaces. This indicates that hydrolysis induced the significant increase of stiffness only of PGA-PCL-PLA membrane.

### Table 3

<table>
<thead>
<tr>
<th>Membrane</th>
<th>Young’s modulus [MPa]</th>
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<tbody>
<tr>
<td>PGA-PCL-PLA untreated</td>
<td>253.0 ± 1.3</td>
</tr>
<tr>
<td>PGA-PCL-PLA hydrolyzed</td>
<td>482.6 ± 6.3</td>
</tr>
<tr>
<td>PGA-PCL untreated</td>
<td>271.7 ± 2.3</td>
</tr>
<tr>
<td>PGA-PCL hydrolyzed</td>
<td>294.4 ± 2.5</td>
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</table>

3.3. The influence of membrane surface properties on keratinocyte viability

The effect of investigated membranes on HaCaT cell viability was measured by MTT test after 48 h growth (Fig. 3).

The unmodified PGA-PCL-PLA membrane decreased significantly of the keratinocyte viability to 52.4% of the control. The membrane surface modification by hydrolysis resulted in increase of viability up to 61.8% of control. Keratinocytes cultured on unmodified PGA-PCL membrane reveal 77.4% viability which was significantly decreased after hydrolytic modification to 55.6% of control. One-way ANOVA followed by the Tukey HSD post hoc tests showed significant differences of the cell viability on the studied membranes compared with the control on 0.05 significance level (p < 0.01).

3.4. Effect of membrane surface properties on actin cytoskeleton and cells morphology

The actin cytoskeleton staining is widely used for cell morphology visualization. Moreover, it was postulated that in nonmuscle cells actin cytoskeleton defines cells’ mechanical properties (Ognea, 2013).

The structure of actin cytoskeleton in control cells is presented at Fig. 4. The image of the F-actin filaments after staining with phalloidin showed organized microfilaments evenly distributed in the cytoplasm. No more organized structures like microfilament bundles or stress fibers were observed. More visible changes in the cell cytoskeleton were noticed in the cells grown on PGA-PCL-PLA unmodified surface, that appeared to be less biocompatible for the HaCaT than other investigated membranes. In this case, the cells are not well flattened and distances between cells in the colony are clearly visible. On the contrary, the actin cytoskeleton view in the cells grown on modified PGA-PCL-PLA membrane and both PGA-PCL membranes was very similar – the cells are well flattened and the actin microfilaments are organized in regularly distributed mesh network. This suggests good biocompatibility of
hydrolyzed PGA-PCL-PLA and both PGA-PCL membranes.

Cell morphology corresponding to actin cytoskeleton organization was characterized with two morphometric parameters: cell and nucleus surface area. The results are presented in Fig. 5. Measured morphometric parameters revealed that the average area occupied by the nucleus in cells cultured on membranes is between 37.5–47.5 μm² what is comparable to nuclei surface area of the control cells (43 μm²). In the case of cell surface area, significantly different results were obtained for cells cultured on unmodified PGA-PCL-PLA membrane: 116 μm² vs 204 μm² of the control cells.

Statistical analysis of surface areas of cells and nuclei was performed separately for PGA-PCL-PLA and PGA-PCL membranes due to differences in distribution of the data.

In case of PGA-PCL-PLA membranes the distribution of nuclei area was normal with the same variances in all groups (control, unmodified PGA-PCL-PLA and hydrolyzed PGA-PCL-PLA) thus one-way ANOVA analysis was applied to compare the mean values. For the ANOVA test F we obtained p value equal to 0.5657 thus there is no statistically significant difference between the means of cells nucleus area on 0.05 significance level. For the PGA-PCL membranes the distribution of cells area was not normal, hence Kruskal Wallis test was applied to compare control with unmodified and hydrolyzed PGA-PCL membrane. The resulting p value was 0.1533 thus there is no statistically significant difference between the distribution of cells area in the case of PGA-PCL membranes.

3.5. Effect of membrane surface properties on keratinocytes elasticity

The changes in mechanical properties of the HaCaT cells can be quantitatively investigated by their deformability. One option is to use the AFM to determine the elastic modulus since it is a measure of the stiffness of the biological sample in response to an applied load.

In this study, we examined the cells cultured on glass substrate as
control and the cells cultured on the investigated membranes. To obtain statistically meaningful results, in total 100–200 individual measurements were performed for each cell within the same region on its surface (around cell center). The mechanical properties of the keratinocytes were determined on the basis of force versus displacement measurements following a method described in the Section 2.11. The results are summarized in boxplots in Fig. 6.

Kerationcytes cultured on all the membranes showed the elastic modulus values larger to that obtained for control measurements. However, the differences in cell stiffness depending on the studied membrane were observed. The relative change in mean values of elastic modulus of cells incubated on hydrolyzed PGA-PCL-PLA membrane was higher (37% of the reference) as compared to unmodified PGA-PCL-PLA membrane (18.6% of the reference). For medians, the changes were 17.4% and 7.9%, respectively. In case of cells cultured on PGA-PCL membranes the relative change in mean values of elastic modulus of cells incubated on hydrolyzed PGA-PCL membrane was lower (22.3% of the reference) as compared to unmodified PGA-PCL membrane (24.9% of the reference). For medians, the changes were 18.8% and 19.5%, respectively. No significant differences were observed.

Statistical analysis of the cell elastic modulus was performed separately for the PGA-PCL-PLA and PGA-PCL membranes due to differences in distribution of the data. In case of PGA-PCL-PLA membranes the distribution of the elastic modulus was normal with the same variances in all groups (control, unmodified PGA-PCL-PLA and hydrolyzed PGA-PCL-PLA) thus one-way ANOVA analysis was applied to compare the mean values. For the ANOVA test F we obtained p value equal to 0.0547 and post-hoc pairwise comparisons using Tukey HSD method showed the statistically significant difference between control and hydrolyzed PGA-PCL-PLA membrane on 0.05 significance level (adj. p value equals 0.0435). For PGA-PCL membranes the distribution of elasticity was not normal, hence Kruskal Wallis test was applied to compare control with unmodified and hydrolyzed PGA-PCL membrane. The resulting p value was 0.0562 and post-hoc pairwise comparisons using Dunn’s method.
showed the significant difference between control and unmodified PGA-PCL membrane on 0.05 significance level (adj. p value equals 0.0325).

Since no significant differences in elastic modulus and roughness factor between native and hydrolyzed PGA-PCL membrane were noticed, it seems that observed changes of investigated parameters did not influence HaCaT cell adhesion. On the other hand, the treatment with NaOH revealed decrease of the water angle making the surface more hydrophilic. In a view of data published by (Arima and Iwata, 2007) it has been suggested, that cell adhesion to monolayer surfaces is mainly determined by surface wettability, but is also strongly affected by the type and density of the surface functional groups and the kinds of cells. Generally, cell adhesion increased with decreasing water contact angle up to 60–70°. Investigated was human umbilical vein endothelial cell (HUVECs) and HeLa cell adhesion on hydrophobic SAMs (alkanethiols) and SAMs pretreated with albumin, that inhibited adhesion, whereas cells adhered to albumin coated hydrophilic SAMs. It shows complex dependence of cell – adhesive surface interactions and allowed conclusion, that moderate wettability provides suitable surface for cell adhesion. Final estimation of particular cell type adhesion on given artificial surface requires specific investigations of interacting functional surface molecules and dynamic changes of plasma cell membrane structure.

4. Conclusions

The objective of the present study was to estimate the role of surface properties of obtained polymer membranes, native and after hydrolytic modification, on the biological and mechanical properties of HaCaT keratinocytes. Modified substrates reveal different effects on HaCaT parameters. The NaOH treatment decreased the water contact angle, RMS surface roughness and roughness factor of PGA-PCL-PLA membrane, whereas significantly increased it stiffness. That changes caused positive effect on significantly higher spread areas and higher relative change in elastic modulus of HaCaT keratinocytes cultured on modified PGA-PCL-PLA membrane, as compared to unmodified. To the contrary, the PGA-PCL membrane stiffens and water contact angle were decreased after hydrolysis, while the RMS surface roughness and roughness factor increased. Under that conditions no significant differences in spread areas and values of elastic modulus of HaCaT cells cultured on PGA-PCL membranes were observed.

Acknowledgments

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References