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Test system for evaluating the influence of polymer properties on primary human keratinocytes and fibroblasts in mono- and coculture

Karoline Trescher[a], Toralf Roch[a,b], Jing Cui[a,#], Karl Kratz[a,b], Andreas Lendlein[a,b]*, Friedrich Jung[a,b]

[a]*Institute of Biomaterial Science and Berlin-Brandenburg Center for Regenerative Therapies, Helmholtz-Zentrum Geesthacht, Kantstraße 55, 14513 Teltow, Germany*
[b]*Helmholtz Virtual Institute − Multifunctional Biomaterials for Medicine, Teltow and Berlin, Germany*
[#] *Present address: BASF Construction Chemicals GmbH, Dr.-Albert-Frank-Strasse 32, 83308 Trostberg, Germany*

* To whom correspondence should be addressed:
Phone: +49 (0) 3328 352450; Fax: +49 (0) 3328 352452;
E-mail: andreas.lendlein@hzg.de

Interactions of cells with polymer-based biomaterials are influenced by properties of the substrate. Polymers, which are able to induce cell specific effects, gain increasing importance for biotechnology and regenerative therapies. A test system was developed, which allows studying primary human keratinocytes and fibroblasts in mono- and cocultures to analyze and operate the effect of polymer properties. This system offers to identify polymers for keratinocyte cultivation or wound dressings, since adherence, viability and functionality can be analyzed. Especially the coculture system enables the characterization of potential cell specific effects of polymer-based biomaterials. To establish a coculture test system, it is challenging to find a suitable culture medium, to identify initial seeding densities for comparable cell growth and to develop methods to distinguish and characterize both cell types.

Poly(*n*-butyl acrylate) networks (cP*n*BAs) as model biomaterials were used to demonstrate the applicability of our newly developed co-culture screening system for differential cell growth. The apparent Young's modulus of the cP*n*BAs differentially regulated fibroblasts and keratinocytes. Particularly, cP*n*BA73 with an apparent Young´s modulus of 930±140 kPa measured in phosphate buffered saline (PBS) solution at ambient temperature (Yoshikawa et al., 2012) seemed to have favoring properties for keratinocyte adhesion, while fibroblast adhesion was not affected. For keratinocytes the concentration of some pro-inflammatory cytokines was lower on

cP*n*BA73 and a decreased deposition of collagen, elastin and fibronectin was observed in the coculture.

Keywords:
keratinocytes, fibroblasts, coculture, poly(*n*-butyl acrylate) networks, biomaterial characterization

1. Introduction

Polymer-based biomaterials are increasingly explored for biotechnological applications and regenerative therapies. Biofunctional polymeric materials, designed to induce cell specific effects, are proposed as next generation of biomaterials. To obtain such cell specificity, chemical and physical properties of polymers have to be adjusted (Scharnagl et al., 2010; Shastri and Lendlein, 2009). The specificity of materials for different cell types can only be tested in coculture systems, which are for cutaneous cells for example keratinocytes and fibroblasts. *In vivo,* keratinocytes of the epidermis and fibroblasts of the dermis are separated by the basement membrane, but communicate by soluble factors (Kanitakis, 2002; Stark et al., 1999). Furthermore, during wound healing their proper interaction plays a critical role (Werner et al., 2007). To isolate keratinocytes from skin biopsies, the epidermal and dermal layer needs to be separated (Aasen and Izpisua Belmonte, 2010). Despite enormous improvements, the cultivation of keratinocytes is still challenging and a substrate favoring keratinocyte adhesion and proliferation over fibroblasts could overcome the need to separate the cell layers before seeding. Because of promoting paracrine interactions fibroblasts can be used as feederlayer for keratinocyte cultures, but their proliferation has to be limited to prevent overgrowth (Jubin et al., 2011; Rheinwald and Green, 1975, 1977). To evaluate potential cell specific effects of polymers aiming applications for the improved cultivation of keratinocytes and for skin regeneration, an appropriate test system would be useful. Systematic coculture studies for the effect of polymers on primary human keratinocytes and human fibroblasts are missing, but are required to clearly identify cell specific effects of the tested polymers. Therefore, a test system for the evaluation of effects on both cell types in mono- and cocultures was developed in the current study.
Besides the chemical composition of the substrate and the topography (roughness and

geometry) (Jiang et al., 2011; Stoppato et al., 2013), the apparent Young´s modulus can influence cell adherence, viability and function (Georges and Janmey, 2005). Hydrophobic, transparent poly(*n*-butyl acrylate) networks (cP*n*BAs) in medical grade quality have been developed as potential candidate polymers for active regulation of several cell types. Their apparent Young´s modulus can be adjusted in a wide range by variation of its crosslinking density (Cui et al., 2011). In contrast to hydrogel-based biomaterials, the benefit of such hydrophobic polymers is that the apparent Young´s modulus is not influenced by exposure to physiological conditions (Braune et al., 2011). cP*n*BA networks with apparent Young´s moduli of E=120±10 kPa (cP*n*BA04) and E=930±140 kPa (cP*n*BA73) (Yoshikawa et al., 2012) were selected as first model polymers to be analyzed with the new test system (Trescher et al., 2012a). The viability and the density of adherent cells, as well as their ratio in coculture and their functionality in terms of deposition of components of the extracellular matrix (ECM) were analyzed 48 hours after seeding the cells on the polymer samples. Since cP*n*BAs are flexible enough to be aligned to body curvatures and their transparency would enable the monitoring of the healing process, they could be potentially used as wound dressings. Therefore also the secretion of different cytokines and growth factors by the cells cultured on the polymers was studied.

2. Materials and Methods

2.1 Cultivation of primary human keratinocytes and primary human dermal fibroblasts

Primary human keratinocytes and primary human dermal fibroblasts from adult donors (cryopreserved cells; Provitro, Berlin, Germany) were used for the experiments. Before seeding on the cP*n*BA samples, keratinocytes were cultured in serum free keratinocyte growth medium (KGM adv., Provitro) and fibroblasts in Dulbecco´s modified Eagle Medium (DMEM; LifeTechnologies, Darmstadt, Germany) supplemented with 10 vol-% fetal bovine serum (FBS) superior (Biochrom, Berlin, Germany). To subcultivate keratinocytes and fibroblasts, the cells were rinsed with phosphate buffered saline without calcium and magnesium (PBS-/-; Biochrom) and incubated with a 0.5 wt-% EDTA-solution (ethylenediaminetetraacetic acid in PBS-/-; Sigma-Aldrich, Munich, Germany) for 10 min. Afterwards, the cells were loosen with 0.025 vol-% trypsin/ 0.053

mM EDTA (ATCC/LGC Standards GmbH, Wesel, Germany). The enzymatic activity of trypsin was stopped for fibroblasts and keratinocytes with DMEM+FBS10% and defined trypsin inhibitor (Invitrogen, Darmstadt, Germany), respectively. After centrifugation fibroblasts were resuspended in DMEM+FBS10% and keratinocytes were resuspended in KGM adv.

For the growth curves and the test system, KGM adv. was used as consistent medium for both cell types in mono- and coculture for resuspension and cultivation.

2.2 Determination of initial seeding densities (pilot study)

Growth curves were performed to determine the baseline seeding densities for keratinocytes and fibroblasts to reach comparable cell numbers per ml or cm^2 in mono- and coculture after 48 hours of cultivation.

To determine the optimal growth for monoculture conditions, cells were initially seeded at 1×10^4, 2×10^4 and 3×10^4 cells/cm^2 on glass cover slips (hydrolytic class 1 glass; Ø 14 mm, thickness # 1 = 0.13 – 0.16 mm; Gerhard Menzel GmbH, Brunswick, Germany) in 24 well plates. After 12, 24, 36, 48, and 60 hours, cells were detached from the culture plates and viable cell numbers per ml were determined by the CASY system (Innovatis Systems/Roche Diagnostics, Mannheim, Germany).

To determine the optimal growth for coculture conditions, cells were initially seeded with 5×10^3 cells/cm^2 and 1×10^4 cells/cm^2 of each cell type. After 24, 48, 72, and 96 hours cells were stained for keratin 14 and vimentin (see chapter Material and Methods 2.4. Cell adherence and viability) to clearly identify and count the cell types by ImageJ (National Institutes of Health).

2.3 Polymer networks

Poly(*n*-butyl acrylate) networks (cP*n*BAs) were synthesized by polymerization of *n*-butyl acrylate and a low molecular weight poly(propylene glycol) dimethacrylate (PPGDMA, M_n = 560 g·mol^{-1}) as crosslinker (Cui et al., 2011). For the synthesis of cP*n*BA04 a PPGDMA content of 0.4 wt-% in the starting reaction mixture was applied, and 7.3 wt-% PPGDMA for cP*n*BA73. The obtained cP*n*BAs exhibited similar surface properties with advancing contact angles ranging from $\theta_{advancing}$ = 111° to 123°, a surface roughness (R_q) below 40 nm and an isoelectrical point at pH 3.7 observed in

streaming potential investigations (Hiebl et al., 2012).

The local mechanical properties were determined by an atomic force microscopy (AFM) based indentation method performed in a 10 mM phosphate buffered saline (PBS) solution with pH 7.4 at ambient temperature. Here, apparent Young´s moduli of E = 120±10 kPa for cP*n*BA04 and E = 930±140 kPa for cP*n*BA73 were obtained (Yoshikawa et al., 2012). Before biological tests started, all samples were sterilized by gas sterilization using ethylene oxide.

2.4 Cell adherence and viability

Keratinocytes and fibroblasts were seeded on 13 mm diameter disks of both polymers in 24 well plates, either in monoculture (3×10^4 cells/cm^2) or coculture (1×10^4 cells/cm^2 each type, both cell types were seeded as one suspension). The cells were cultured for 48 hours in KGM adv. (5% CO_2, 37 °C, humidified atmosphere). Glass coverslips (Menzel) were used as control material.

Cell viability was analyzed by live/dead staining as previously described (Trescher et al., 2012b) using fluorescein diacetate (FDA, 25 µg/ml; Sigma, Munich, Germany) and propidium iodide (PI; 2 µg/ml; LifeTechnologies). FDA visualized viable cells in green and PI the DNA of dead cells in red. Thereafter, one image was scanned from each sample by a confocal laser scanning microscope (cLSM Axiovert 200M, Zeiss, Jena, Germany) with 5fold magnification (n=3; see typical image at Figure 1a). From the densities of viable and dead cells, determined using the ImageJ software, the relative cell density (reflects the density of adherent cells on the respective sample related to the density of adherent cells on the glass control) and viability (density of viable cells×100/(density of viable cells + density of dead cells)) were calculated.

Additional samples (n=3) were stained for keratin 14, vimentin, and DNA as described previously (Trescher et al., 2012b), as well as keratin 10 (monoclonal rabbit antibody to human keratin 10, ab76318, 1:250 (v/v), Abcam, Cambridge, UK and IgG anti-rabbit Cy5, Jackson Immuno Research, Newmarket, UK, 1:200 (v/v)) in addition. From each sample 5 fields of view were randomly chosen and scanned with the cLSM in 10-fold magnification. Keratin 14 was used as a specific marker of undifferentiated (basal) keratinocytes and vimentin was used to detect fibroblasts (Lammers et al., 2009). Thus both cell types could be clearly distinguished on coculture samples (see typical image at

Figure 1b; both cell types stained on glass) and counted with the ImageJ-Software in order to calculate the ratio of keratinocytes to fibroblasts types. In the monocultures the purity of cell population was confirmed by the keratin14/vimentin stain.

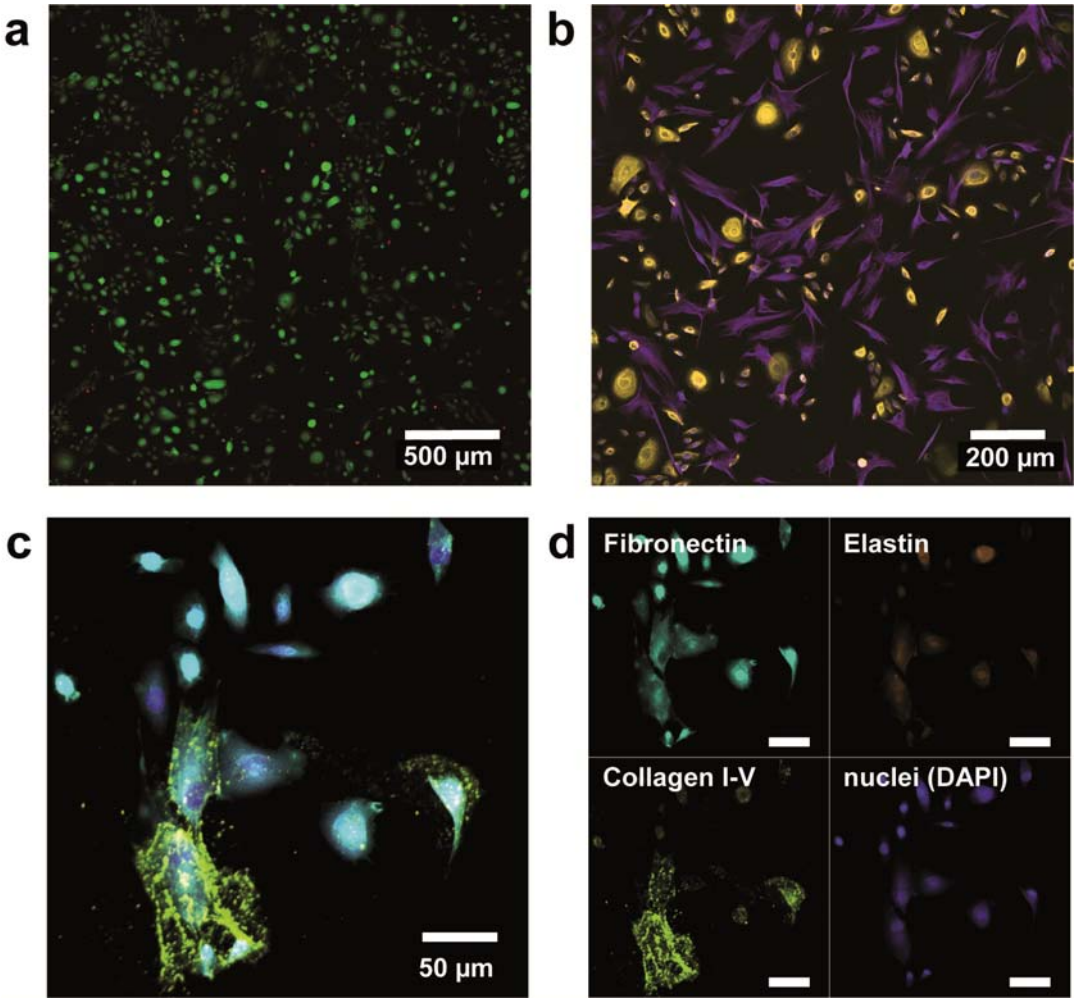

Figure 1: Representative images of cocultured keratinocytes and fibroblasts on glass after 48 hours culture.
(a) Viability staining; viable cells were labeled by fluorescein diacetate shown in green and the DNA of dead cells was labeled by propidium iodide shown in red.
(b) Immunocytochemical labeling of keratin 14 for keratinocytes, shown in yellow, and vimentin for fibroblasts, shown in violet. Cells could be clearly distinguished and counted with ImageJ.
(c) ECM staining; overlay image of immunocytochemical labeling of fibronectin, elastin, collagen I-V and the DNA (nuclei).
(d) Single channels of ECM staining with indicated stained components, bar = 50 µm. Images shown in pseudocolour.

Keratin 10 is a marker for suprabasal keratinocytes. Therefore keratin 10 positive cells were counted to detect potential influences of the polymers to the differentiation of keratinocytes.

2.5 Analysis of deposited extracellular matrix (ECM) components

To analyze the deposition of fibronectin, collagen (I-V) and elastin by the cells seeded on the polymers (cP*n*BA04 and cP*n*BA73), samples (n=3) were stained with corresponding primary (rabbit antibody to human collagen I-V, ab36064, 1:20 (v/v), Abcam; sheep antibody to human fibronectin, AF1918, 1:20 (v/v), R&D Systems, Minneapolis, USA; guinea pig antibody to human elastin, ab52115, 1:40 (v/v), Abcam) and secondary antibodies (IgG anti-rabbit Cy5 and IgG anti-sheep DyLight488, both Jackson Immuno Research and IgG anti-guinea pig Alexa555, Invitrogen, all 1:200 (v/v)) as well as 4',6-diamidino-2-phenylindole (DAPI; Carl Roth GmbH, Karlsruhe, Germany). Staining and embedding were performed as reported previously (Trescher et al., 2012b), except that 5 wt-% BSA + 0.3 vol-% Triton were used for blocking and antibodies were diluted in 1 wt-% BSA + 0.3 vol-% Triton. From each sample 5 fields of view were randomly chosen and scanned with the cLSM in 40-fold magnification after excluding background and unspecific signals (see typical image at Figure 1c and 1d). From the images the stained area for each channel (fluorescence intensity above a given threshold) was measured as a value in μm^2 with the LSM Image Browser Software 4.2.0.121 (Zeiss, Jena, Germany). DAPI stained DNA was counted to represent the cell number. Afterwards the quotient of stained area per cell number (μm^2/cell) was calculated from each picture. Quotients of the 5 pictures were averaged for each sample. The mean and standard deviations were calculated from 3 samples.

2.6 Secretion of cytokines and growth factors

Supernatants of the cells seeded on the polymers (n=6) were harvested and analyzed for interleukin(IL)-1α, IL-1β, IL-6, IL-8, IL-10, IL-12, tumor necrosis factor alpha (TNFα), granulocyte macrophage colony stimulating factor (GM-CSF), vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) using the multi-plex System (Bio-Plex200, BioRad, Munich, Germany) according to manufacturer's instructions. Keratinocyte growth factor (KGF) concentrations were analyzed by an enzyme-linked immunosorbent assay (ELISA; R&D, Minneapolis, USA). A plate was prepared according to supplier's instruction and absorbance was measured in an ELISA reader at 450 nm (Tecan, Männedorf, Switzerland) after 20 min incubation.

2.7 Statistics

Gaussian distribution of the samples was tested using Kolmogorov and Smirnov test. For the statistical analysis of soluble factors (n=6), unpaired t-test using Prism5 was used. To analyze growth curves a two-way ANOVA with a Bonferroni post-test was performed. The null hypothesis was rejected with a probability α of less than 0.05.

For viability, selectivity and ECM components no statistical analyses were performed because of the low sample size (n=3). Tendencies were stated from the values.

3. Results and Discussion

In the current study, a coculture test system for evaluating the effect of polymer properties on primary human keratinocytes and primary human dermal fibroblasts at the same time was established. Such a test system should allow the identification of polymers favorable for keratinocyte cultivation or applications in skin regenerative medicine. Since the isolation and cultivation of keratinocytes is still challenging, e.g. a polymer, which supports keratinocyte adhesion but limits fibroblast adhesion could improve the keratinocyte isolation and culture. Since keratinocytes and fibroblasts interact with each other via soluble factors, it is especially important to determine, how the cells are influenced by polymer properties in coculture.

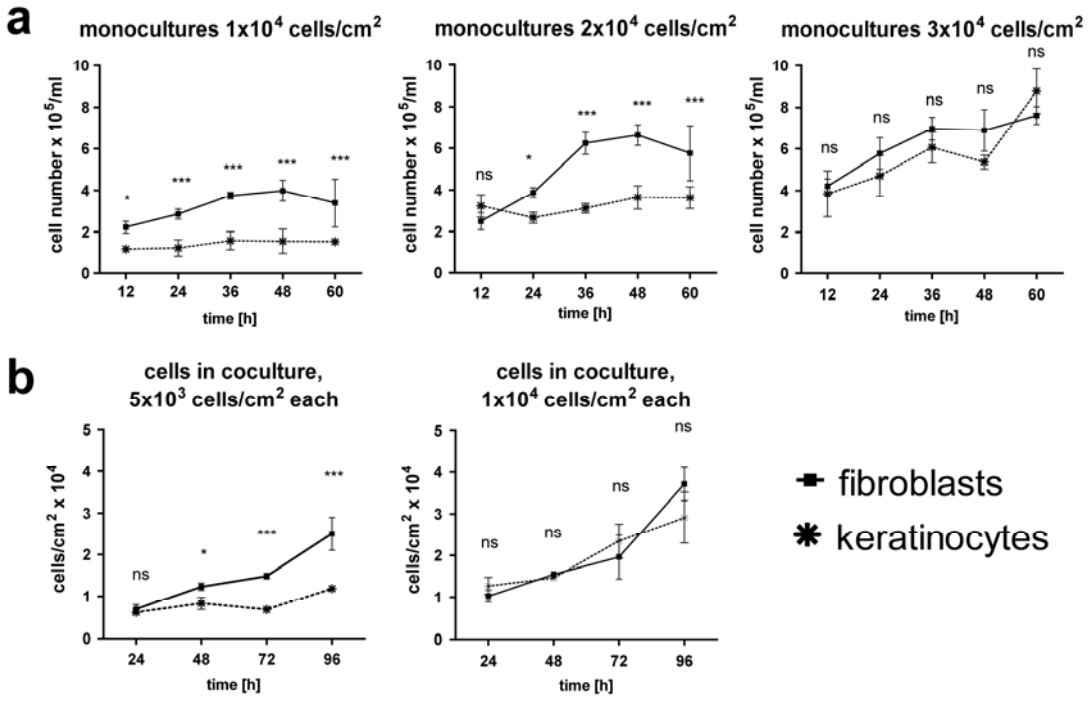

Figure 2: Growth curves of primary human keratinocytes and fibroblasts on glass
(a) Monocultures with initial seeding densities of 1×10^4 cells/cm^2, 2×10^4 cells/cm^2 and 3×10^4 cells/cm^2. Viable cell numbers per ml determined with CASY system at the indicated time points were plotted.
(b) Keratinocytes and fibroblasts were seeded as cocultures with initial seeding density of 5×10^3 cells/cm^2 and 1×10^4 cells/cm^2 each cell type. The cocultured cells were stained for keratin 14 and vimentin so that the cell types could clearly be distinguished and counted with ImageJ.
Data represent mean of 4 samples ± standard deviation. A two-way ANOVA was performed (ns: $p>0.05$; *$p<0.05$, ***$p<0.001$).

Within the pilot study and the test system, both cell types were cultured in KGM adv. as common medium in monocultures as well as in coculture. Keratinocytes did not proliferate in DMEM supplemented with 10 vol-% FBS, which is the regular medium for the fibroblasts (data not shown). Fibroblasts proliferated and showed a physiological morphology during culture in KGM adv. at least for 4 days. A sufficient proliferation and conformance of cell numbers per ml of both cell types was found according to the growth curves determined in the pilot study for an initial seeding of 3×10^4 cells/cm^2 (Figure 2a). Cultures of both cell types reached 90% confluence after 48 hours in the centre of the well. For the cocultured cells, a suitable conformance of cell densities and proliferation for both cell types was found for an initial seeding of 1×10^4 cells/cm^2 (Figure 2b). In the following, an example for testing the influence of polymer properties is described using the established test system. Two poly(*n*-butyl acrylate) networks were chosen as model substrates, to analyze the influence of the substrates apparent Young´s modulus on keratinocytes and fibroblasts. Previous studies for cP*n*BA04 and cP*n*BA73 (Cui et al., 2011) revealed an endotoxin content below the limit set by the US Food and Drug Administration of 0.5 EU/ml (Gorbet and Sefton, 2005) and were tested to be cell-compatible (Cui et al., 2011; Roch et al., 2012). Additionally, it was shown that cP*n*BAs did not activate innate immune mechanisms (Roch et al., 2012) and could serve as a potential substrate for endothelial cells (Hiebl et al., 2012), as well as for monocytes (Mayer et al., 2012).

In the current study, a higher relative cell density was found for keratinocytes in monoculture on cP*n*BA73 with 81%±10% compared to cP*n*BA04 with 57%±8% (Figure 3a). The relative cell density of fibroblasts in monoculture was comparable on both materials (cP*n*BA04: 50%±15%, cP*n*BA73 59%±20%). However, the relative cell density of the coculture was reduced when compared to the monocultures, but did not

substantially differ for the two cP*n*BAs (cP*n*BA04 20%±11%, cP*n*BA73 36%±15%) (Figure 3a). The cell viability on both polymers (Figure 3b) revealed similar levels for adherent keratinocyte monocultures (cP*n*BA04: 94%±2%, cP*n*BA73: 95%±1%). However, monocultured fibroblasts showed a slightly reduced viability on cP*n*BA73 (84%±13%) when compared to cP*n*BA04 (95%±3%).

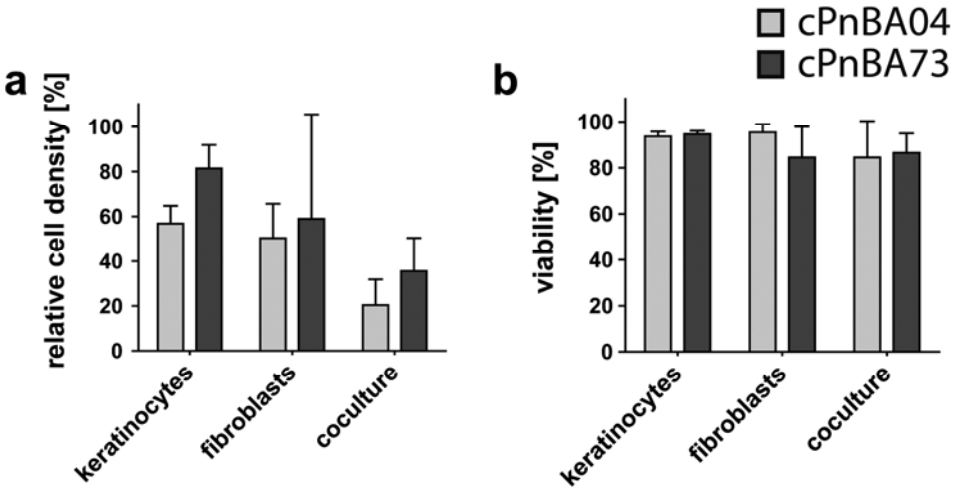

Figure 3: Viability staining of keratinocyte and fibroblast monocultures as well as coculture of both cell types after 48 hours culture on cP*n*BA04 and cP*n*BA73.
(a) Relative cell density (density of adherent cells on the respective sample related to the density of adherent cells on the glass control).
(b) Viability (calculated as density of viable cells×100/(density of viable cells + density of dead cells)).
Data represent mean for three samples ± standard deviation.

The viability of cocultured cells was 84%±15% on cP*n*BA04 and 86%±9% on cP*n*BA73. Conclusively, the cell viability was between 85% and 95% and was comparable to glass (control culture), indicating that cP*n*BAs did not exert negative effects on keratinocytes and fibroblasts. In order to count the keratinocytes and fibroblasts in the coculture samples unambiguously and not only rely on the different morphologies of the cell types, they were stained for specific markers. Keratin 14 was used as a specific marker of undifferentiated (basal) keratinocytes, keratin 10 as a marker for differentiated (suprabasal) keratinocytes and vimentin was used to detect fibroblasts. The counting revealed a higher density of keratinocytes on cP*n*BA73 (compared to cP*n*BA04), while the density of fibroblasts was comparable on both cP*n*BAs. A keratinocyte/fibroblast ratio of 5.3±2.0 revealed on cP*n*BA73 compared to a ratio of 2.6±0.9 on cP*n*BA04, which indicates a keratinocyte favoring effect. Less than

1% of differentiated keratin 10 positive keratinocytes were found on the samples (monoculture cP*n*BA04 with 0.2%±0.1%; cP*n*BA73 0.8%±0.2%; coculture cP*n*BA04: 0.4%±0.1%; cP*n*BA73: 0.2%±0.2%) as well as on the control (monoculture: 0.37%±0.17%; coculture: 0.04%± 0.04%). No effect of the cP*n*BAs can be stated from that, but it could be shown that proliferative cultures were present. From the mentioned results, both cP*n*BAs could have potential applications as substrates for keratinocytes as well as fibroblasts, while cP*n*BA73 might improve keratinocyte culture.

The deposition of ECM-components was analyzed as an aspect of cell functionality. A comparable deposition of the analyzed ECM components, calculated as area per cell, could be detected for keratinocytes in monoculture. A lower deposition of fibronectin (cP*n*BA04: 47±35 µm^2/cell, cP*n*BA73: 140±155 µm^2/cell) and collagen (cP*n*BA04: 26±10 µm^2/cell, cP*n*BA73: 107±66 µm^2/cell) was observed in comparison to fibroblasts. For the elastin deposition, values of 545±195 µm^2/cell on cP*n*BA04 and 618±18 µm^2/cell on cP*n*BA73 could be detected (Figure 4a). Fibroblasts in monocultures showed comparable values of deposited fibronectin (cP*n*BA04: 379±224 µm^2/cell, cP*n*BA73: 461±397 µm^2/cell) and elastin (cP*n*BA04: 678±372 µm^2/cell, cP*n*BA73: 513±253 µm^2/cell) on both polymers, but the value for collagen was higher on cP*n*BA04 (1328±562 µm^2/cell) compared to cP*n*BA73 (529±139 µm^2/cell) (Figure 4b). In cocultures, a tendency toward higher amounts of ECM deposition on cP*n*BA04 compared to cP*n*BA73 for all three tested proteins was observed: fibronectin (cP*n*BA04: 221±139 µm^2/cell, cP*n*BA73: 64±10 µm^2/cell), collagen (cP*n*BA04: 742±263 µm^2/cell, cP*n*BA73: 428±140 µm^2/cell) and elastin (cP*n*BA04: 1045±327 µm^2/cell, cP*n*BA73: 758±22 µm^2/cell) (Figure 4c). This tendentially reduced deposition of ECM components might be an effect or a cause of the reduced cell density of fibroblasts on cP*n*BA73.

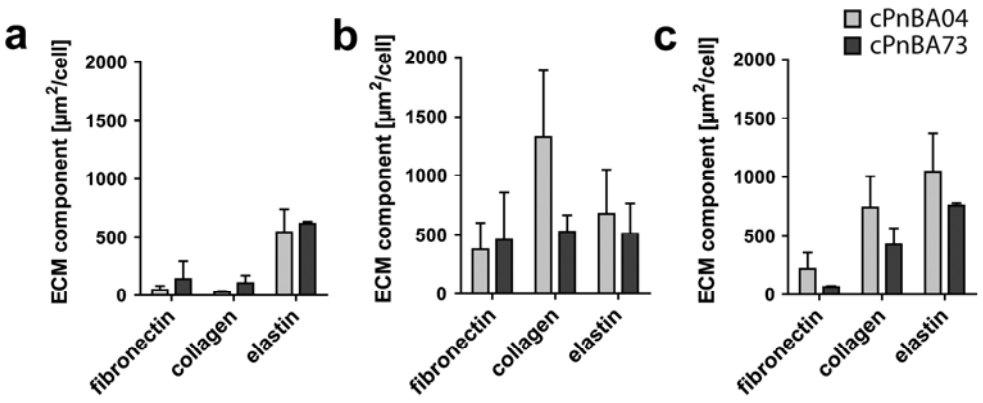

Figure 4: Deposited ECM component per cell in μm^2/cell for keratinocytes (a), fibroblasts (b) and coculture (c) after 48 hours culture on cP*n*BA04 and cP*n*BA73.
Data represent mean for three samples ± standard deviation; 5 fields of view were averaged from every sample.

Due to the transparency and elasticity of the cP*n*BAs, applications in the field of skin regeneration might be feasible. To ensure safety and thus enable future medical applications, the polymer networks should not induce the secretion of factors triggering allergy- or skin irritation (Corsini and Galli, 1998; Wilmer et al., 1994). As part of the first barrier of the body, keratinocytes are capable of expressing and releasing several cytokines and growth factors, which can act on keratinocytes but also on other cells of the epidermis as well as on fibroblasts and other cells in the dermis (Grone, 2002; McKenzie and Sauder, 1990). Dermal fibroblasts are also capable of secreting soluble factors (Apte, 1995; Kubo and Kuroyanagi, 2005). Since cP*n*BAs might trigger cytokine release or influence the communication of cells with their environment, the secretion of IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-12, TNFα, GM-CSF, VEGF, HGF, and KGF was analyzed after culturing the cells for 48 h on the samples (Figure 5). Significantly higher concentrations of several pro-inflammatory cytokines and growth factors were observed after culturing keratinocytes on cP*n*BA04 in comparison to cP*n*BA73 (Figure 5a).
In contrast to cP*n*BA73, on cP*n*BA04 a higher concentration of IL-1α was found for keratinocyte monoculture (cP*n*BA04: 813±128 pg/ml vs. cP*n*BA73: 592±30 pg/ml, $p=0.0021$). Keratinocytes contain large amounts of preformed IL-1α, which can be released after cell impairment. This can induce the secretion of IL-6 and IL-8 (Corsini and Galli, 1998), which would partially explain the additionally increased concentration of IL-8 in cP*n*BA04 keratinocytes monoculture (cP*n*BA04: 326±60 pg/ml vs. cP*n*BA73: 254±46 pg/ml, $p=0.0398$). The pro-inflammatory cytokine IL-12 was also higher on cP*n*BA04 in contrast to cP*n*BA73 (266±17 pg/ml vs. 240±22 pg/ml, $p=0.0456$). GM-CSF (127±14 pg/ml vs. 86±10 pg/ml, $p<0.0001$) and VEGF (1680±166 pg/ml vs. 1492±83 pg/ml, $p=0.0321$) showed also higher concentrations in the cP*n*BA04 keratinocytes monoculture in contrast to cP*n*BA73. In contrast, a significantly lower level of IL-6 (153±27 pg/ml vs. 184±16 pg/ml, $p=0.0363$) was found from supernatants of keratinocyte monoculture on cP*n*BA04 in comparison to cP*n*BA73.
For fibroblast monocultures (Figure 5b), no changes in the secretion of cytokines and growth factors were detected, except a significantly higher concentration of IL-6 on

cP*n*BA04 (531±160 pg/ml) in contrast to cP*n*BA73 (379±40 pg/ml, p=0.0476). Also in the coculture supernatants, no significant differences were found between the two polymer networks (Figure 5c). However, the concentrations for IL-6 and IL-8 in the coculture were significantly higher on both cP*n*BAs (p<0.0001 each) compared to the monocultures, which is in good agreement with the literature (Waelti et al., 1992). IL-6 is a pro-inflammatory cytokine, which is reported to stimulate the proliferation of cultured keratinocytes (Grossman et al., 1989). The concentration of the anti-inflammatory cytokine IL-10 in the supernatant did not differ between the polymers, neither in the monocultures nor in coculture. The concentration of the pro-inflammatory factor TNFα in the supernatant was below the detection limit in all cultures. HGF and KGF (for KGF see Figure 5d) are predominantly secreted by fibroblasts. Both factors stimulate the migration as well as the proliferation of keratinocytes (Shirakata, 2010). However, no differences could be detected comparing the supernatants of cell cultures on both polymers. From the results, it could be assumed that cP*n*BA73 might induce less irritant effects to the epidermis when applied as wound dressing.

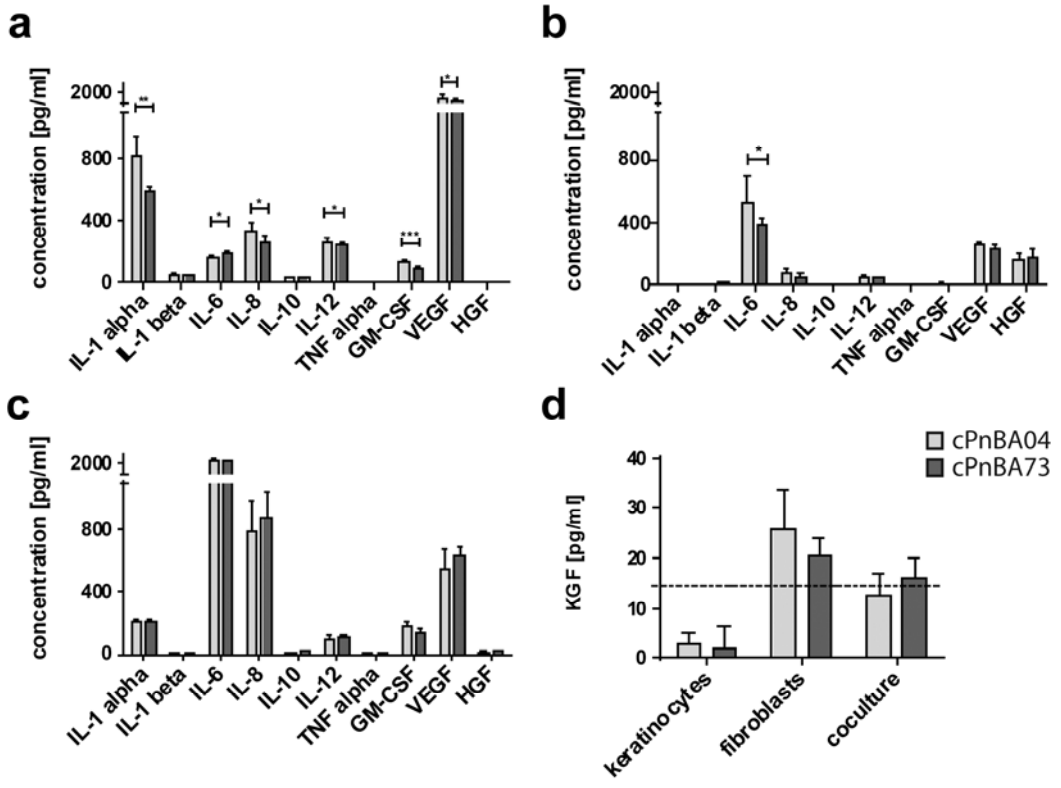

Figure 5: Secretion of cytokines and growth factors by keratinocytes (a), fibroblasts (b) and cocultured cells (c) on cP*n*BA04 and cP*n*BA73 after 48 hours culture. (d) Concentrations of KGF measured in the cell culture supernatant. Dotted line indicates

the detection limit of the ELISA.
Data represent mean for six samples ± standard deviation. An unpaired t-test was performed (*p<0.05, **p<0.005, ***p<0.0005).

Since the surface properties (roughness, zeta-potential and wettability) of both investigated cP*n*BAs were comparable, it can be hypothesized that the different apparent Young´s modulus of the cP*n*BAs induced the different behavior of the cells. Effects of the substrates apparent Young´s modulus were described to be mediated by mechanotransduction, meaning the conversion of mechanical forces into biochemically relevant information. In this process, cell adhesions, the cytoskeleton and adaptor proteins are involved (mainly integrins, connected to actin filaments, e.g. via talin, vinculin, paxilin; also called focal adhesions) (Hoffman et al., 2011). On stiffer polymers stronger forces are generated due to the higher resistance of the substrate, which will be translated into an impact to the cell behavior via intracellular signaling pathways. For cP*n*BAs an influence of apparent Young´s modulus on endothelial cells was reported previously (Hiebl et al., 2012). The cell density and the actin stress fiber formation were lower on the stiffer substrate (cP*n*BA73), likely due to weaker formation of focal adhesions. Besides a large variety of reports about the influence of substrate apparent Young´s modulus to different cell types (Georges and Janmey, 2005; Yeung et al., 2005), recently also the effect to a keratinocyte cell line (HaCaT) was analyzed (Wang et al., 2012). It was demonstrated that the proliferation of the HaCaT cells on a silicon substrate with an apparent Young´s modulus of 200 kPa was higher than on silicon with a 10-fold lower apparent Young´s modulus (16 and 20 kPa) (Wang et al., 2012), which agrees with the results from the current study. The density of primary human keratinocytes increased with rising apparent Young´s modulus (stiffness) of the cP*n*BAs.
Using the established coculture test system, further studies can be performed to increase the knowledge about the influence of different polymer properties on keratinocytes and fibroblasts and offers the chance to identify new biomaterials for keratinocyte cultivation or skin regenerative therapies.

4. Conclusion

In this study, a test system evaluating the influence of polymer properties on primary

human keratinocytes and fibroblasts in coculture including suitable culture medium, initial seeding densities for comparable cell growth, and methods to distinguish and characterize both cell types microscopically was established and it was demonstrated that it can be applied to evaluate cell-specific effects of biomaterials. Therefore, this system represents a useful tool for the selection of suitable polymers for potential applications such as keratinocyte cultivation or regenerative therapies of skin. On hydrophobic polymer networks with adjustable elasticities primary human keratinocytes and dermal fibroblasts adhered and were viable. Interestingly, in the coculture of both cell types a higher keratinocyte/fibroblast ratio, a lower deposition of ECM components and a lower concentration of keratinocyte-secreted inflammatory factors were found for cP*n*BA73 compared to the more elastic cP*n*BA04. Since the chemical and physical surface properties of both cP*n*BAs did not differ, it can be hypothesized that the apparent Young´s modulus of the polymers induced the cell specific effects.

For the future, coculture test systems using other cell types can also be applied for the evaluation of cell-specific effects of polymer-based biomaterials. For example, the knowledge-based improvement of degradable stent-polymers favoring endothelial cell adherence and proliferation, but suppressing the smooth muscle cell proliferation, could be feasible.

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