The use of gas plasma treatment to improve the cell–substrate properties of a skin substitute made of poly(ether)/poly(ester) copolymers

G. J. BEUMER, C. A. VAN BLITTERSWIJK*, M. PONEC‡
*Department of Dermatology, *Laboratory for Otobiology and Biocompatibility, ENT Department, and ‡Department of Dermatology, Biomaterials Research Group, University Hospital, Leiden, The Netherlands*

The objective of this study was to improve the cell–substrate interactions between skin cells and a biodegradable elastomeric matrix, part of a cell-seeded skin substitute for the treatment of large-scale deep dermal skin defects (i.e. burn wounds). Polyactive, a synthetic biodegradable elastomeric copolymer, was used as the constituent of the bilayered matrix. It consists of a dense toplayer seeded with epidermal keratinocytes and a macroporus underlayer, which may be seeded with dermal fibroblasts. Although former studies demonstrated the suitability of the copolymer as a substrate for these skin-derived cell types, we aimed to improve the bilayered matrix' seeding efficiency. Using radio frequency glow discharge (RFGD) pretreatment significantly improved the adherence and growth of SVK14 epithelial cells seeded on the dense copolymeric toplayers and on non-tissue grade plastics, approximating tissue culture polystyrene values. With scanning electron microscopy (SEM), early epithelial cell–substrate interactions were investigated. Seeding efficiency and growth of dermal fibroblasts into the porous underlayers was improved as was visualized with the SEM and confocal scanning laser microscopy. It is concluded that RFGD pretreatment is a cost-effective measurement for improving cell–substrate properties of the investigated copolymers.

1. Introduction

For the treatment of third-degree large body surface area skin defects, grafting of cultured epithelial sheets is currently clinically used [1]. To improve graft take and functional and cosmetic results, the use of a dermal analogue has been widely advocated [2–4]. As such, a matrix consisting of a dense top- and porous underlayer, made of a synthetic biodegradable elastomer is under investigation in our laboratories [5]. This elastomer, Polyactive, is a poly(ethylene oxide): poly(butylene terephthalate) (PEO:PBT) copolymer, especially developed for biomedical purposes [6]. To improve wound healing, the porous underlayer of this matrix may be populated with dermal components, i.e. fibroblasts, prior to grafting.

To reduce the biopsy–transplantation interval, needed to grow sufficient surface area of sheets of keratinocytes, and to improve seeding efficiency and fibroblast growth in the porous underlayer, we searched to optimize the substrate characteristics of the matrix.

Several biochemical methods as well as physical approaches were assessed, including serum preincubation, collagen precoating and gas (argon) plasma surface etching of the biomaterial. Pilot experiments revealed gas plasma etching to be the most practical alternative in terms of effectiveness, costs and reproducability. The treatment is reported to cause an increase in the hydrophilicity of most material surfaces by etching and incorporation of hydroxyl groups in the most superficial layers of the exposed materials through a cascade of chemical reactions [7]. The hydrogel-like character of the copolymeric matrices however, prevented direct assessment of the treatment on surface hydrophilicity [8]. Therefore, short-term effects were assessed by directly visualizing the early cell–biomaterial attachment events with scanning electron microscopy (SEM). This paper reports the early and long-term effects of radio frequency glow discharge (RFGD) pretreatment on cell–biomaterial interactions. Quantification and visualization of early cell–substrate attachment and long-term growth of dermal and epidermal cell types on both dense and porous biomaterials, were performed, using confocal scanning laser microscopy (CSLM) and SEM.

2. Materials and methods

2.1. Cell culture

SV-40 transformed epidermal keratinocytes (SVK14) were cultured in a mixture of Dulbecco's Modified Essential Medium (Gibco) and Ham's F-12 (Gibco) (ratio 3:1) supplemented with 5% Foetal Bovine Serum (FBS) (Gibco), 10^{-6} M isoproterenol (Sigma), 0.4 $\mu g\,ml^{-1}$ hydrocortisone (Sigma), 80 $U\,ml^{-1}$ penicillin and 80 $\mu g\,ml^{-1}$ streptomycin [9]. The cultures

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were incubated at 37 °C in a humidified, 10% CO_2-containing atmosphere. The medium was renewed twice a week.

Human foreskin fibroblasts (fourth passage) were cultured in Eagle's (Gibco) culture medium, to which 10% New Born Calf Serum (Gibco), and penicillin (80 U ml^{-1})/streptomycin (80 μg ml^{-1}) were added. The medium was renewed twice a week. Cells were inoculated at 8000 cells cm^{-2}. This method usually rendered confluent cultures of fibroblasts within 1 week which were then trypsinized (0.25% trypsin in phosphate-buffered saline (PBS), supplemented with 0.05% EDTA and 0.1% glucose, pH 7.6) and subcultured.

2.2. Experimental substrates

Culture dishes of both tissue and non-tissue culture grade (tissue culture polystyrene, TCPS) were used as reference substrate materials, representing standard (tissue culture, TC) and poor (non-tissue culture, n-TC) substrate qualities. As experimental substrates we used dense and porous Polyactive™ films (CAM Implants B.V., Leiden, The Netherlands). Polyactive is an elastomeric polyether/polyester segmented block copolymer with variable weight ratios of the soft (polyethylene oxide, PEO) and hard (polybutylene terephthalate, PBT) segments. For the experiments, dense films (from 50–100 μm thick) and bilayered matrices were used, made of the 55:45 type Polyactive (PEO:PBT weight ratio). Matrices typically had a thickness of approximately 250 μm, with pore sizes ranging from 100–200 μm. For culture purposes, dense copolymeric films were glued to the culture dishes with a silicon type A-based medical-grade adhesive (Dow Corning), rinsed and sterilized overnight by UVB-irradiation.

Bilayered films were kept submerged using a stainless steel ring.

2.3. RFGD treatment

Experimental substrates were placed in a vacuum chamber and at a vacuum of 10^{-4} torr (1 torr = 133.322 Pa), air was replaced by argon in three or more gassing–pumping cycles, using an Edwards E_2M_2 oil-diffusion pump unit. With a Harrick XG-2 radio frequency glow discharger unit (Harrick Inc., Ossining, New York), electrodeless discharge (radio frequency $1–10^2$ MHz) was used to create a gas plasma, at 10^{-2} torr. The substrates were exposed to and sterilized by this plasma for 3 min. Immediately thereafter, cells were inoculated on to the respective substrates.

2.4. Proliferation and cell attachment assay

Biomaterials and tissue (TC) and non-tissue (n-TC) grade tissue culture dishes, all with and without RFGD pretreatment were inoculated with 4000 keratinocytes or 10,000 fibroblasts per square centimeter. At 4 and 24 h and 2, 3, 5 and 7 days incubation, cells were harvested by trypsinization and each sample was counted in triplicate, using a Buch counting chamber. Statistical comparison of cell numbers between experimental (RFGD treated) and non-treated substrates were performed at day 3, 5 and 7, using a student's t-test after estimating the variance, (σ^2), with ANOVA. A significance level of 5% has been employed.

To assess early attachment events, epithelial cells were seeded on pretreated and non-pretreated dense copolymer films at a seeding density of 6000 cells cm^{-2}. Cells were fixed after 10, 30, 60 and 240 min and their morphology was evaluated by SEM.

Human dermal fibroblasts were seeded into the pretreated porous underlayers at a seeding density of 20000 cm^{-2} and incubated for 1, 3 and 5 weeks with medium renewals twice a week. Subsequently, growth and morphology of the cells were assessed using SEM and CSLM.

2.5. Microscopy

For SEM, the medium was carefully replaced by PBS (pH 7.5) and subsequently fixed in 1.5% Glutar-dialdehyde in 0.14 M cacodylate buffer PH 7.4 for 1 h at room temperature and subsequently overnight at 4 °C and thereafter dehydrated in ethanol series. For SEM, the specimens were then critical-point dried (Balzers CPD030), gold sputter-coated and examined on a Philips SEM 525 at 15 keV.

For CSLM, specimens were washed twice in PBS, pH 7.5, fixed in 1.5% GA in 0.14 M cacodylate and washed in tris-buffered saline (TBS), incubated (1 h, room temperature) with primary anti-body (monoclonal mouse–α-human Vimentin) and washed with TBS (20 min). Secondary antibody (rat–α-mouse-FITC) incubation at room temperature in the dark for 2 h was followed by 10 min TBS washing (in the dark). Specimen were mounted and examined, using a Zeiss CSLM.

Phase contrast microscopy was used to monitor cellular growth in the case of cell-seeding on the dense, translucent copolymers.

3. Results

3.1. Attachment of eptihelial cells on to the dense toplayer

Epithelial cells, when seeded on non-treated copolymer as a substrate, showed a delayed spreading compared to cells seeded on RFGD-treated copolymer substrates. Ten minutes after inoculation, cells adhering to the RFGD-treated substrates already showed extensive "ruffled" outer membranes, whereas with the non-pretreated substrate this phenomenon is first noticed 30 min post-inoculation. Cells adhering to the pretreated copolymer spread out quicker and appeared more flattened as compared to cells adhering to the non-treated copolymeric substrate (Fig. 1d and h)

3.2. Proliferative behaviour of keratinocytes seeded on the dense toplayer

To allow the investigation of individual epithelial cell–substrate interactions we could not use fresh

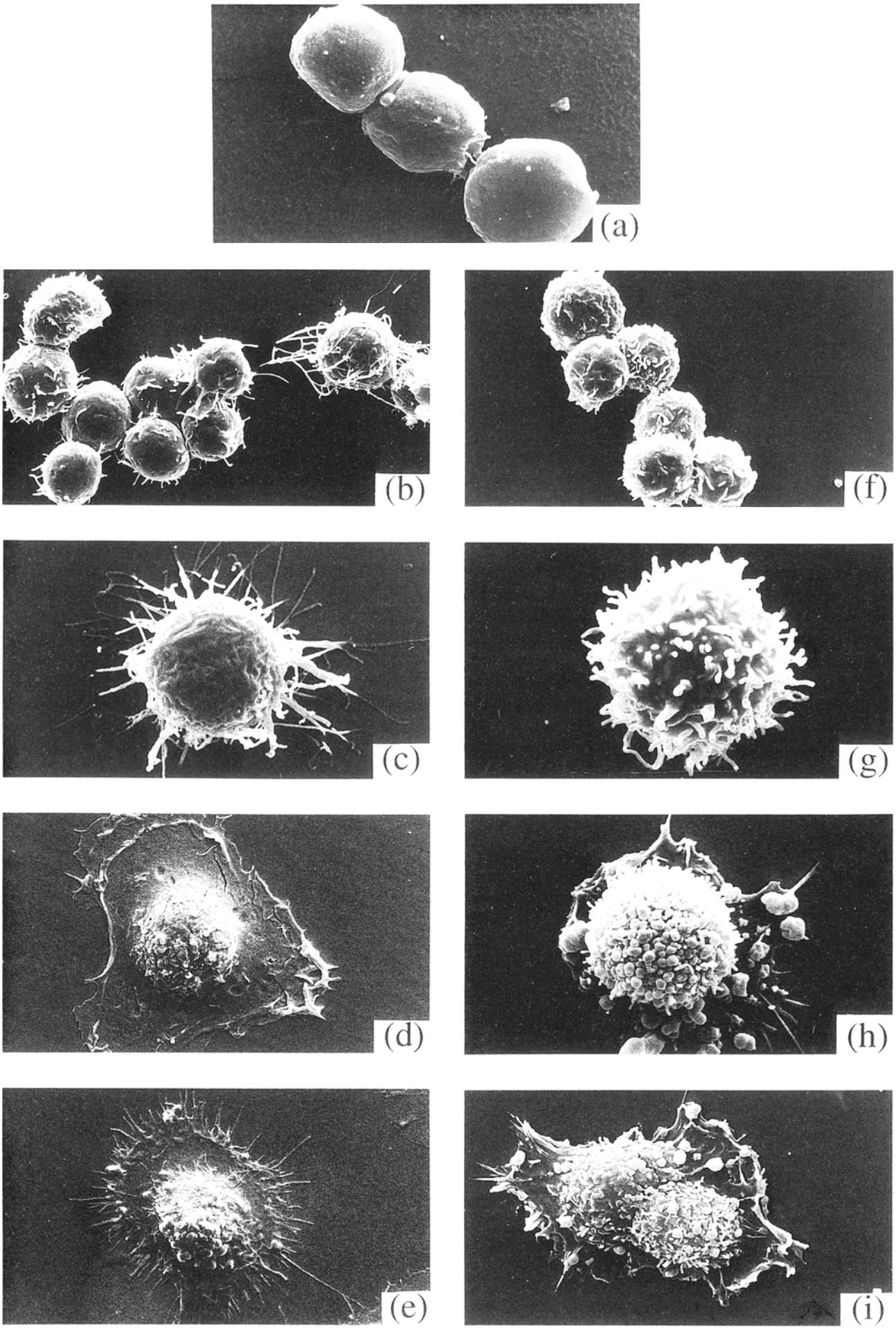

Figure 1 Scanning electron micrograph showing SVK 14 cells, (a) 0, (b,f) 10, (c,g) 30, (d,h) 60 and (e,i) 240 min after inoculation on RFGD pretreated copolymer (b–e) or non-pretreated copolymer surfaces (f–i).

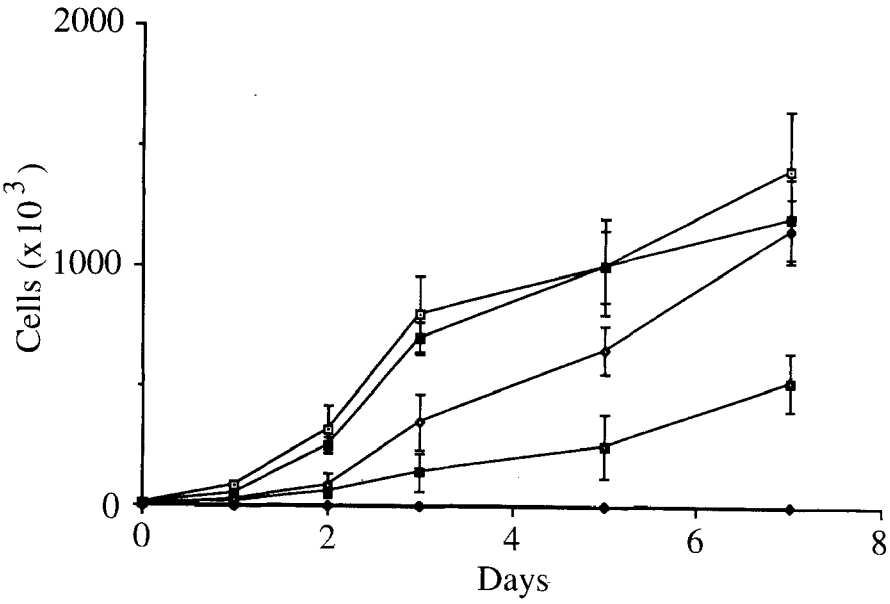

Figure 2 Proliferative activity of SVK 14 cells on RFGD-treated routine culture substrates and dense copolymer films. (⊡) TCPS, (◆) BACT − glow, (■) BACT + glow, (◇) PA − glow, (◘) PA + glow.

biopsy keratinocytes, cultured with the feeder layer technique of Rheinwald and Green [10]. We therefore chose to use the frequently used SVK 14 cell line. Fig. 2 shows the effect of the RFGD treatment on the proliferation of SVK 14 cells on the experimental and control substrates. When these keratinocytes were seeded on the non-treated n-TC grade substrates, few cells were found to adhere and no cell growth could be observed. In contrast to this, keratinocytes appeared to be able to adhere and grow on RFGD treated n-TC grade culture dishes. Although non-treated copolymer appeared to be a suitable substrate allowing keratinocyte attachment and outgrowth, RFGD treatment improved cell attachment as well as subsequent proliferative activity of the keratinocytes (Fig. 2). At day 5 and 7 no significant differences in cell numbers could be found between TC grade culture dishes and RFGD treated biomaterial.

3.3. Proliferation of fibroblasts seeded into the porous underlayers

Seeding of fibroblasts into non-treated porous underlayers resulted in low seeding efficiency and poor population of the underlayer. As was seen with SEM, fibroblasts exhibited poor morphology and formed pseudopodiae and microspikes trying to span the pores (Fig. 3a). Under these circumstances few cells attached and cellular growth was inconsistent. When seeded into RFGD pretreated underlayers, the growth of fibroblasts was considerably improved. First, fibroblasts started to cover the facing surface and the superficial pores of the porous underlayer. Then, 3 weeks post-inoculation, strands of fibroblasts were found to descend into the porous underlayer (Fig. 3b). Five weeks after seeding, the fibroblasts had managed to populate fully both superficial as well as deeper pores (Fig. 3c). With CSLM, the three-dimensional architecture could be visualized and ingrowth of fibroblasts throughout the underlayer was demonstrated (Fig. 4).

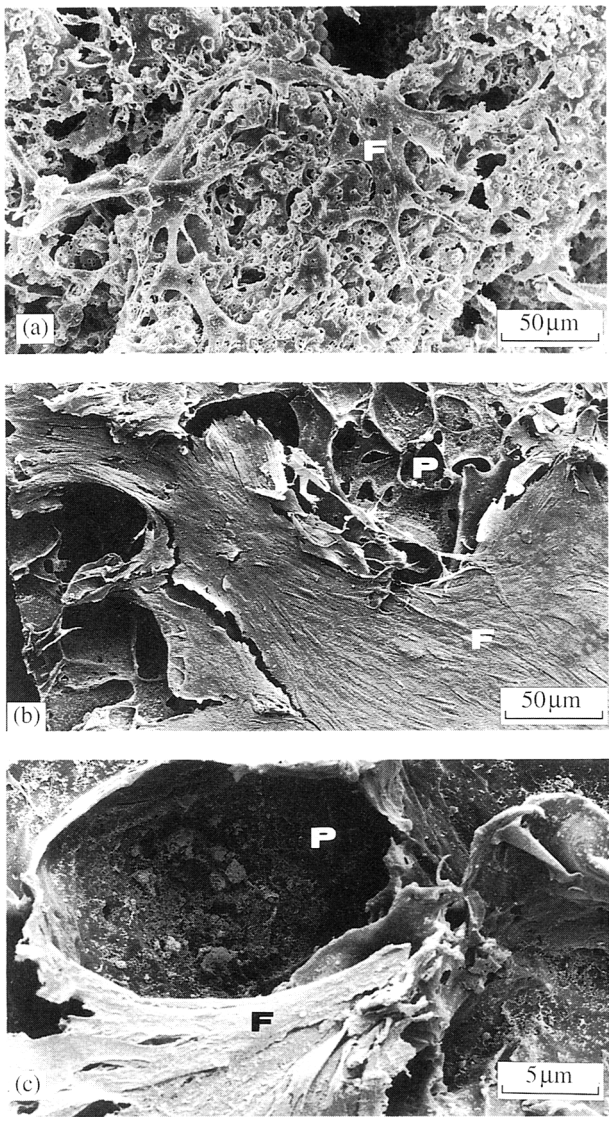

Figure 3 Scanning electron micrograph showing human dermal fibroblasts (a) in the porous underlayer 1 week after inoculation, no RFGD was applied; (b) 3 weeks after inoculation into glow discharge pretreated porous underlayers, and (c) in an RFGD-treated porous underlayer 5 weeks post inoculation.

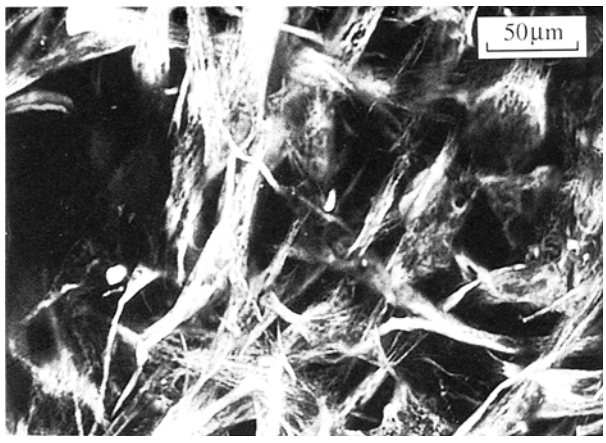

Figure 4 Confocal scanning laser microscopic appearance of α-Vimentin-labelled dermal fibroblasts, after 5 weeks culture in an RFGD-treated porous underlayer of the copolymer matrix.

4. Discussion

We report here a method to improve the cell-seeding efficiency of a biodegradable bilayered matrix, to function as a dermal analogue in a full-thickness living skin substitute. The dense toplayer of the matrix should serve as the substrate for epidermal cells, whereas the porous underlayer can be seeded with dermal fibroblasts, to enhance dermal regeneration [5].

In earlier studies we reported the successful seeding and subsequent growth of human and murine epidermal keratinocytes on the dense copolymeric toplayer [11]. Confluent cultures of fourth passage human and rat epidermal keratinocytes were obtained within a 2 week culture period. However, early growth of the keratinocytes was retarded on the biomaterial substrates as compared to the routine cell culture substrate TCPS. In contrast to results obtained with dermal fibroblasts seeded on dense biomaterial substrates, the growth and morphological appearance of dermal fibroblasts seeded into the porous underlayer, was poor.

From several methods to modify cell–biomaterial interactions, RFGD pretreatment of the matrices appeared to be an effective and economic technique for substrate property modification of large quantities of (bio)material. In our hands the pretreatment considerably enhanced dermal and epidermal cell growth on dense and porous copolymeric films. In accordance with reports by Jansen *et al.* [12] Curtis *et al.* [13] and others, RFGD pretreatment even facilitated the growth of epidermal cells on n-TCPS substrates [14, 15].

Schakenraad *et al.* [16] and Curtis *et al.* [17] and others have demonstrated the importance of surface charge density in protein adhesion to surfaces and their influence on cell–substrate interactions. Although epidermal and dermal cells are reported to behave differently with regard to cell–substrate aspects, in our studies RFGD pretreatment improved cell-seeding efficiency on the copolymeric materials with both epidermal and dermal cells [18, 19].

Short-term effects of the treatment were seen when examining early cell attachment events with SEM. The appearance of cell membrane protrusions like blebs, lamellipodiae and microspikes was the initial event in the transition of the cells from their globular shape which they had adopted in suspension towards the flat, polarized, morphology that the cells exhibit when adhered to a solid substrate. When compared with cells seeded on non-treated copolymeric substrates, such alterations in cell-membrane morphology were earlier seen when RFGD treated copolymers were used as a substrate. The cells adhered faster to the RFGD-treated copolymers and exhibited a more flattened morphology compared to cells adhering to the non-treated copolymer, indicating a more extensive spreading of the cells on the pretreated substrates. The results with the porous underlayer show that less suitable substrate characteristics may permanently be ignored by RFGD treatment. RFGD treatment is reported to have a profound effect on cell–substrate interactions by affecting the surface charge density [20]. Results of our experiments are in accordance with these reports. Long-term fibroblast growth into porous copolymer underlayers as well as keratinocyte proliferation on the toplayer, were considerably enhanced on RFGD-treated compared to non-treated materials. Adhesion characteristics of the polymer substrates for proteins may be altered by RFGD, leading to an altered exposure to the cells of the protein layer covering biomaterial surfaces in serum-containing culture media [21, 22]. This altered interface could influence initial cell–biomaterial interfacial behaviour, perhaps also exerting long-term effects on the cultures.

In this study, the CSLM was successfully used as a means to study the three-dimensional architecture of fibroblasts grown in the porous underlayer of the matrices.

In the treatment of large body surface area skin defects, the achievement of permanent wound closure (with autologous transplants) is of great importance [23]. However, regeneration of dermal structures is needed to heal deep dermal skin defects with satisfactory cosmetic and functional results [24]. Therefore, the use of a dermal analogue, to help regenerate the dermis, in addition to the grafting of the epithelial sheets, is necessary. Any means to reduce the time needed to obtain confluent keratinocyte cultures on such a material from fresh biopsies, reduces the risk of infection, hypothermy and dehydration of the patient and is thus of great clinical importance. We consider the use of the RFGD treatment a cost-effective means of improving cell–biomaterial surface interaction.

At present our attention is focused on the necessary reduction of incubation periods needed to achieve population of the porous underlayer with dermal fibroblasts. Furthermore, the intimate association of epithelial cells with connective tissue elements *in vivo*, and stimulation of the deposition of extracellular matrix components by the fibroblasts in the pores of the underlayer require further investigation.

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