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Behaviors of keratinocytes and fibroblasts on films of PLA_{50}–PEO–PLA_{50} triblock copolymers with various PLA segment lengths

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Abstract The growth of human primary keratinocytes and fibroblasts on PLA–PEO–PLA copolymer films was investigated as an intermediate stage of a strategy aimed at making implantable dermo-epidermal substitutes. Four PLA–PEO–PLA triblock copolymers with the same PEO block and different DL-lactic acid/ethylene oxide molar ratios (LA/EO) (0.8, 1.4, 1.8 and 2), were synthesized and characterized by ^{1}H-nuclear magnetic resonance and infrared spectroscopy. The films made of these copolymers were more hydrophilic than PLA_{50} and than tissue culture polystyrene controls according to contact angles with water. Proliferation and adhesion of human skin cells were evaluated by MTT assay and by scanning electron microscopy. The presence of PEO in the triblock copolymers influenced cell adhesion and proliferation of fibroblasts, whereas keratinocyte adhesion and proliferation were not affected. These features emphasize the interest of PLA–PEO–PLA triblock copolymers to serve as better compounds than the racemic PLA previously investigated to make supports for human skin primary cells and scaffolds for skin engineering.

1 Introduction

Since the early eighties, reconstructive skin surgery for severe burns, and other chronic wounds, has been based on the grafting of autologous cultured epithelial sheets (cultured epithelial autografts [CEA]) [1]. This technique is flawed by the fragility of epidermal sheet, the incomplete differentiation of keratinocytes and the absence of dermal replacement. However, it remains the unique solution in the case of large burnt surfaces [2, 3]. The development of skin substitutes that include both dermal and epidermal compartments is therefore a major goal in the field and it should greatly improve skin reconstruction and surgical procedures. Accordingly, various approaches have been proposed to create full-thickness skin equivalents using different dermal substitutes based on biological materials, e.g. fibrin [4], collagen [5], de-epidermized dermis (DED) [6]. The use of bioresorbable synthetic polymers to grow tissues is of interest because it limits the risks of virus- and protein-related disease transmissions, ensures better batch-to-batch control, and allows adjustments of mechanical property to match clinician and treatment requirements. Last but not least, the degradability allows elimination of the foreign support material during regeneration of the neotissue. Among polymers having the reputation of being degradable, polycaprolactone [7] and poly(hydroxyl butyrate) [8] have been tested for skin reconstruction. However, these polymers degrade very slowly in the animal body [9] and are not optimal to elaborate skin substitutes and to restore flexibility to the implanted site. In contrast to these materials, poly(α-hydroxy acid)s derived from lactic and glycolic acid (PLAGA) degrade much more rapidly in vivo according to what was shown many years ago [10]. Polymers derived from lactic acid have been tested to create skin equivalents, e.g. PLA/PLAGA [11], and proved their great potential as temporary therapeutic devices in human bone surgery [12] and as drug release systems in pharmacology [13].

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A couple of years ago, a program in our laboratory aimed at developing a skin substitute based on bioresorbable lactic acid-based polymers was initiated. Our objective is to achieve a porous scaffold based on bioresorbable polymer, colonized by dermal fibroblasts and covered with an epidermal sheet that could be effectively applicable to skin surgery. From this purpose, we first tested the potential of a lactic acid-co-glycolic acid copolymer ($PLA_{37.5}GA_{25}$, composed of 37.5% L-lactic acid, 25% glycolic acid and the 37.5% remaining being the stereoisomer of L-lactic acid, i.e. D-lactic acid). Fibroblasts adhered and grew well on the corresponding films, whereas the presence of glycolic acid had deleterious effects on keratinocyte proliferation. In parallel, it was found that keratinocytes grew well on glycolic acid-free poly(DL-lactide) films despite of a slight delay with respect to standard tissue culture polystyrene (TCPS) [14]. Modifications of racemic PLA surfaces by NaOH treatment improved cutaneous cell growth but films lack flexibility [15].

In this article, we wish to consider the growth of keratinocytes and fibroblasts on more hydrophilic films made from PLA–PEO–PLA triblock copolymers. Poly(ethylene oxide) is a hydrophilic polymer that can be eliminated from the body provided its molecular weight is low. Four PLA–PEO–PLA triblock copolymers were synthesized that had different DL-lactic acid/ethylene oxide (LA/EO) molar ratios, namely 0.8, 1.4, 1.8 and 2. Films were manufactured by solvent evaporation and carefully characterized by spectrometric techniques and contact angle measurements. Cells were then seeded onto the various films and the influence of the presence of the PEO segment on the adhesion, the proliferation and the morphology of the grown cells was evaluated.

2 Materials and methods

2.1 Materials

2.1.1 *Chemicals*

DL-lactide obtained from Purac (Gorinchem, The Netherlands) was recrystallized once in acetone and carefully dried prior to polymerization. Poly(ethylene glycol) (20,000 g mol^{-1}) from Fluka (Geneva, Switzerland) and zinc lactate from Sigma were dried under vacuum prior to use.

(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), (MTT), was purchased from Sigma (L'Isle d'Abeau, France).

Poly(DL-lactic acid) was synthetized as previously [16]. Freshly recrystallised DL-lactide was mixed with zinc lactate in a 100-mL round bottom flask (molar ratio initiator/monomer = 1,200). Typically the flask was sealed under dynamic vacuum after careful degassing through vacuum/argon cycles. The feed was allowed to polymerize at 130 °C for 200 h. The resulting material was dissolved in acetone and the solution was filtered through a 5G sintered-glass filter. The polymer was precipitated by addition of methanol and then washed several times with small amounts of methanol. The recovered compound was allowed to dry under vacuum for 10 days at 40 °C.

PLA–PEO–PLA triblock copolymers were prepared as described previously [17]. Typically, 5 g of PEO 20,000 and various amounts of D,L-lactide (8.2, 16.4, 24.5 and 32.7 g) were introduced into four flasks, the initial molar ratio of ethyl oxide to lactate units (LA/EO) being respectively 1/1, 2/1, 3/1 and 4/1. Zinc lactate (7 mg) was then added. After degassing, the flasks were sealed under vacuum and the polymerization was allowed to proceed at 140 °C. After 7 days, the products were recovered by dissolution in CH_2Cl_2 and precipitated in diethyl ether. Finally, the products were dried under reduced pressure up to constant weight.

2.1.2 *Cells*

Human keratinocytes (HK) were isolated from foreskin following the Rheinwald and Green method [18]. They were cultured in serum-free medium (SFM) supplemented with epidermal growth factor (EGF, 5 ng/mL) and bovine pituitary extract (BPE, 50 µg/mL, Invitrogen, Cergy Pontoise, France).

Human dermal fibroblasts (HDF) were obtained using the explant technique from mammoplasty pieces and were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum (BioMedia, Boussens, France), penicillin (100 U/mL), streptomycin (100 µg/mL) and amphotericin B (250 ng/mL, Invitrogen, Cergy Pontoise, France).

2.2 Methods

2.2.1 *Polymer synthesis and characterization*

Polymer films were made by the solvent evaporation method after the casting of acetone solutions into flat-bottom stainless steel wells. Disks (weight 50 mg, diameter 1 cm, thickness 150 µm) were then cut by stamping. The disks were sterilised by dipping into an ethanol bath twice for 4 h before being allowed to stand in a bath of sterile water containing penicillin and streptomycin for 12 h.

Nuclear magnetic resonance (^{1}H NMR) spectra were recorded at 30 °C with a Bruker spectrometer operating at 360 MHz by using $CDCl_3$ as solvent.

Infrared spectra were recorded on a Perkin-Elmer1760 spectrometer. Films of the copolymers were prepared by casting chloroform solutions onto a NaCl plate.

Contact angle measurements were made using a Krüss drop shape analysis system.

2.2.2 *Cell adhesion and proliferation*

For cell adhesion determination, circular pieces of PLA_{50} and PLA_{50}–PEO–PLA_{50} specimens stamped to fit the size of the wells of 24-well cell culture plates were seeded with cell suspensions (50,000 cells/cm^2). The deposited cells were let to adhere for various times, namely 15 and 60 min. The wells were then washed once by dynamic dipping into PBS bath before MTT test.

For cell proliferation determination, circular PLA_{50} and PLA_{50}–PEO–PLA_{50} specimens were placed in 24-well culture plates. Keratinocytes were seeded at 12,000 cells/cm^2. For human dermal fibroblasts, the seeding density was 5,000 cells/cm^2 because these cells grow faster than keratinocytes. In all cases the seeded specimens were allowed to stand 30 min before the addition of the appropriate culture medium. MTT assays were performed at day 2, 7 and 11 post-seeding. The proliferation of each cellular type was tested in triplicate.

MTT assays were performed according to literature [19, 20]. Briefly, 500 µl of an MTT solution (1 mg/mL in PBS) was added per well. After 3 h, the MTT solution was removed and the formazan crystals were dissolved in isopropanol. The concentration of formazan was then quantified colorimetrically using a multiwell plate reader (SLT Spectra) at 570 nm.

2.2.3 *Scanning electron microscopy*

For scanning electron microscopy (SEM), cells were first washed with PBS prior to fixation with 3.3% glutaraldehyde in Milloning phosphate buffer, pH 7.2 for an hour at room temperature. They were finally washed in Milloning buffer. Fixed samples were dehydrated in graded ethanol series (30–100%), followed by a final drying with hexamethyldisilazane. Subsequently, the samples were coated with an approximative 10 nm-thick gold film and then examined under a scanning electron microscope (Hitachi 400) using a lens detector with an acceleration voltage of 10 kV at calibrated magnification.

Data point and standard deviation resulted from triplicate. For cell adhesion and proliferation studies, one-way variance analysis (ANOVA) was performed followed by the comparison of the means using multiple range test of Bonferroni). Statistical significance was considered for $p < 0.05$.

3 Results and discussion

3.1 Synthesis and characterization of PLA_{50}–PEO–PLA_{50} triblock copolymers and PLA_{50} polymers

The molecular weights and the LA/EO molar ratios of the four PLA–PEO–PLA triblock copolymers obtained by polymerization of *rac*-lactide in the presence of PEO 20,000 and zinc lactate are shown in Table 1. The resulting triblock copolymers were named as TB1, TB2, TB3 and TB4, respectively. Zinc lactate was used as catalyst instead of stannous octoate or other catalysts whose cytotoxic effects are still under debate [21].

The copolymers were also characterized by combining ^{1}H NMR and IR analysis. Four signals were observed in the ^{1}H NMR spectrum of triblock copolymers. The quadruplet at 5.2 and the doublet at 1.5 ppm were assigned to the methine and methyl protons of PLA blocks, respectively, whereas the complex resonance at 3.6 ppm was assigned to main chain methylene units in the PEO blocks. The methylene proton located in the junction unit between PEO and PLA appeared at 4.3 ppm, together with the CH protons from hydroxylated lactyl end units. The presence of the 4.3 ppm resonance confirmed the triblock structure. The LA/EO ratio was determined from the integrations of

Table 1 PLA_{50}–PEO–PLA_{50} triblock copolymers obtained from the polymerization of *rac*-lactide in the presence of PEO 20,000 using Zinc lactate as co-initiator

Copolymer PLA_{50}–PEO–PLA_{50}	LA/EO in feed	LA/EO in copolymer[a]	$\overline{DP}_{PLA}$ [b]	$\overline{DP}_{PEO}$ [c]	$\overline{Mn}$ [d]
TB 1	1	0.8	174	454	45,000
TB 2	2	1.4	319	454	66,000
TB 3	3	1.8	417	454	80,000
TB 4	4	2	451	454	85,000

[a] Determined by using the integration ratio of resonances due to PEO blocks at 3.6 ppm and to PLA blocks at 5.19 ppm in the ^{1}H NMR spectra.
[b] $\overline{DP}_{PEO} = \overline{Mn}_{PEO}/44$, [c] $\overline{DP}_{PLA} = \overline{Mn}_{PEO} \times (LA/EO)/2$, [d] $\overline{Mn} = 44 \times \overline{DP}_{PEO} + 2 \times 72 \times \overline{DP}_{PLA}$

NMR resonances belonging to PEO blocks at 3.6 ppm and to PLA blocks at 5.2 ppm.

According to Table 1, the LA/EO ratios of the triblock copolymers were lower than in the initial feed ratio. This feature was assigned to the incomplete lactide conversion.

The IR spectra of the four TB1–TB4 triblock copolymers and the control PLA_{50} are shown in Fig. 1. The band at 2,880 cm^{-1} and the band at 1,750 cm^{-1} were assigned to the PEO specific methylene groups and to the PLA specific carbonyl groups, respectively. The band at 2,880 cm^{-1} appeared in the copolymer spectra only, depending on the length of the PLA blocks, the greater its intensity, the smaller the LA/EO ratio. In parallel, the 1,750 cm^{-1} band decreased when LA/EO decreased. Therefore, one could conclude from ^{1}H NMR and IR data that the synthesized compounds were effectively triblock copolymers composed of the same PEO block surrounded by PLA segments of various lengths, and characterized by different LA/EO ratio (Table 1).

The TB1–TB4 triblock copolymers were processed as thin films by solvent-casting in Petri dishes. The hydrophilicity of the various films was characterized by the contact angle between the film surfaces and water (Fig. 2). Tissue Culture Polystyrene Standard (TCPS) and PLA_{50} supports were rather hydrophobic ($\alpha_{TCPS} = 72°$ and $\alpha_{PLA50} = 62°$). The contact angles of the films made of the triblock copolymers depended on the value of the LA/EO ratio of the surface, the lower the ratio, the lower the contact angle, as shown in Table 1. We confirmed by confocal Raman spectroscopy that this latter ratio varies proportionally to the ratio of the bulk polymer for each triblock polymers (Data not shown). In other words, the hydrophilicity of the film surfaces decreased with the LA/EO ratio.

3.2 Behaviors of human skin on the PLA_{50}–PEO–PLA_{50} triblock copolymers

The adhesion and the proliferation of human keratinocytes and fibroblasts on the PLA–PEO–PLA films were compared to Tissue Culture Polystyrene Standard (TCPS) and to PLA_{50} films used as control. Human keratinocytes and fibroblasts were selected because these cells represent the main populations of skin cells. The culture medium used for keratinocytes did not contain serum whereas that used for fibroblasts contained 10% serum. Therefore, the behavior of fibroblasts on the various surfaces might be affected by the early adsorption of serum proteins, and, thus, could depend on the specific interaction of these proteins with the various surfaces.

3.2.1 Cell adhesion

The adhesion data for keratinocytes and fibroblasts in contact with the various surfaces are shown in Fig. 3. Cell adhesion was quantified using the MTT assay that reflects the number of living cells present on a surface at a given time point [19, 20].

The adhesion of keratinocytes was assessed after 15 and 60 min (Fig. 3A). Data at 15 and 60 min on the two controls were not significantly different. However, after 15 min keratinocytes adhered less to PEO-containing polymers than PLA_{50} and TCPS controls. Adhesion decreased according to the decreased of LA/EO ratio. Finally, after 60 min, keratinocyte adhesions on the various triblock copolymers and on the controls appeared comparable. According to these data and the absence of proteins in the culture medium, one could conclude that the triblock

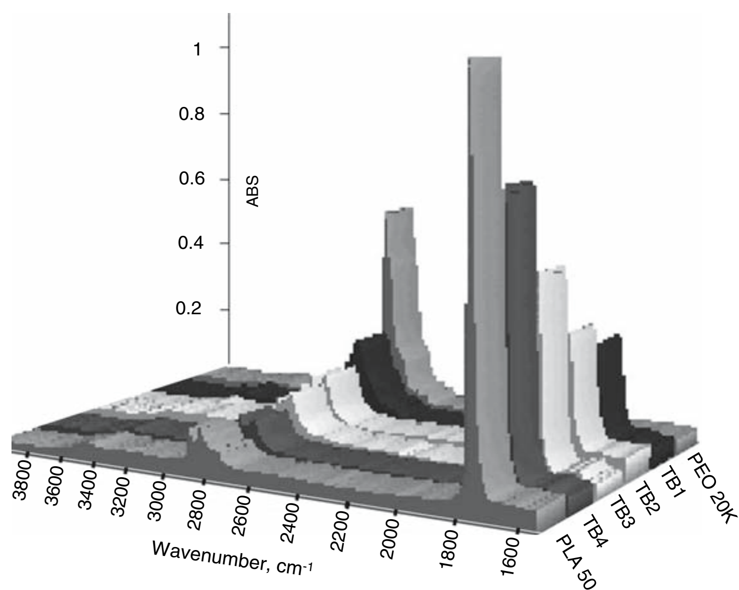

Fig. 1 IR spectra of PEO 20,000, PLA_{50} and various PLA_{50}–PEO–PLA_{50} triblock copolymers (TB4–TB1)

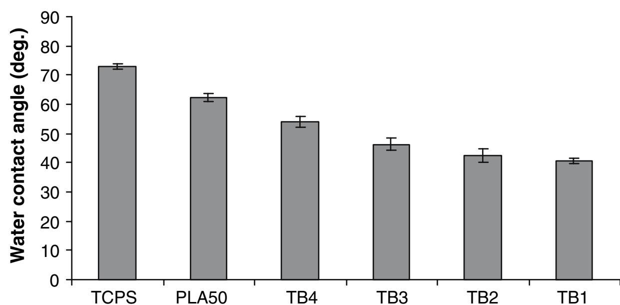

Fig. 2 Water contact angle of polystyrene (TCPS), PLA_{50} and various PLA_{50}–PEO–PLA_{50} triblock copolymers (TB4–TB1). All data points and standard deviations are the result of triplicate experiments

copolymers are compatible with human keratinocyte adhesion.

The adhesion of fibroblasts was also assessed after 15 and 60 min (Fig. 3B). Like for keratinocytes, data at 15 and 60 min on the two controls were not significantly different. In contrast, the adhesion of fibroblasts was drastically influenced by the presence of PEO. Indeed, adhesions on TB1, TB2 and TB3 were significantly lower than those found for the PLA_{50} and TCPS controls. However, the adhesion of human fibroblasts to the TB4 triblock copolymer was comparable to the ones observed with PLA_{50} and TCPS.

3.2.2 *Cell proliferation*

Data on the proliferation of keratinocytes and fibroblasts on the various PLA_{50}–PEO–PLA_{50} films in comparison with the TCPS and PLA_{50} controls, collected at day 2, 7 and 11, are plotted vs. time in Fig. 4A. The populations of keratinocytes at day 2, 7 and 11 days did not differ significantly from those observed for the two controls. From adhesion and proliferation data, one can conclude that human keratinocytes behaved similarly regardless of the film surfaces in the case of the considered series of polymers.

There was no significant difference in fibroblast proliferations at day 2 and 7 on the two controls. At day 11, proliferation on the PLA_{50} control appeared slightly delayed compared to TCPS. After 2 days on the TB1, TB2 and TB3 copolymers, the fibroblast proliferations were significantly slower than on controls. The slow proliferation was consistent with the poor adhesion of fibroblasts to these triblock copolymers. After 11 days, cell

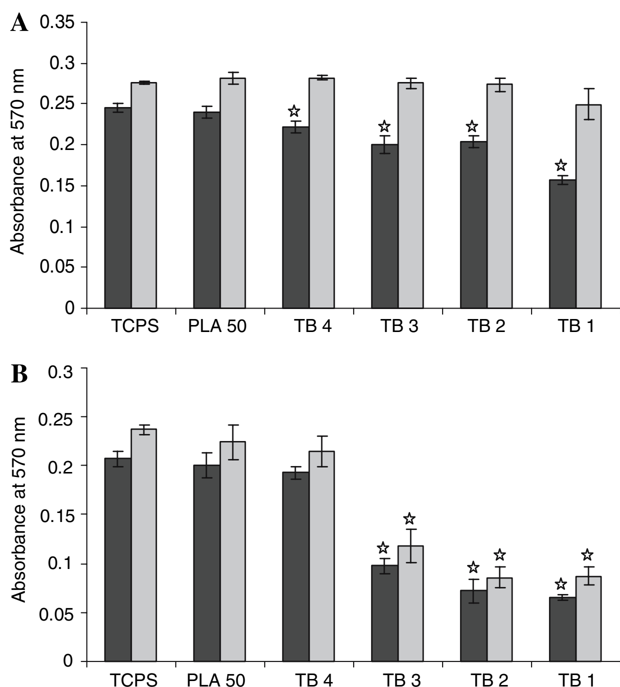

Fig. 3 Adhesion of human keratinocytes (**A**) and human dermal fibroblasts (**B**) after 15 (■) and 60 min (■) on polystyrene (TCPS), PLA_{50} and PLA_{50}–PEO–PLA_{50} triblock copolymers (TB4–TB1). Statistical analysis has been performed with either TCPS or PLA_{50} as reference (☆ represents a p value <0.05). All data points and standard deviations are the result of triplicated experiments

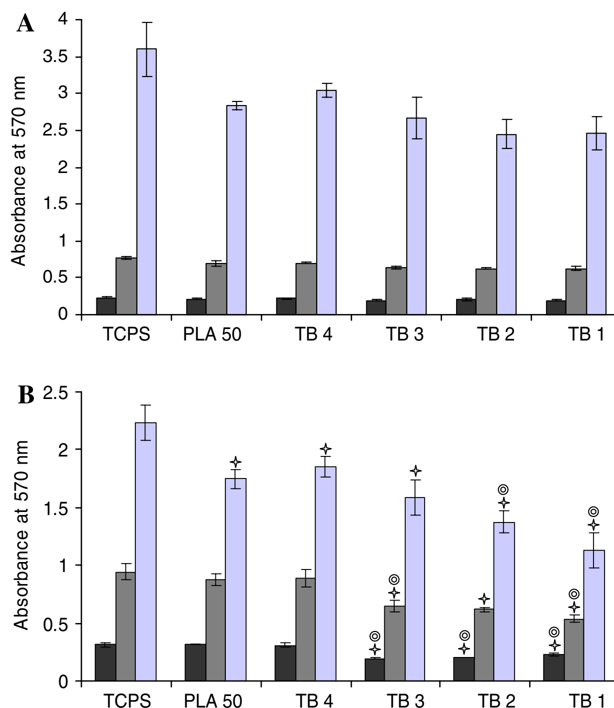

Fig. 4 Proliferation of human keratinocytes (**A**) and human dermal fibroblasts (**B**) at day 2 (■), 7 (■) and 11 (■) on polystyrene (TCPS), PLA_{50} and PLA_{50}–PEO–PLA_{50} triblock copolymers (TB4–TB1). Statistical analysis has been performed with TCPS (✧) and PLA_{50} (◎) as reference (✧ and ◎ represent a p value <0.05). All data points and standard deviations are the result of triplicate experiments

proliferations were still significantly lower than for TCPS. The proliferation on the TB4 surfaces was comparable to those on the PLA_{50} controls at day 2 and 7 and slightly slower at day 11. The proliferation of fibroblasts was thus dependent on the presence of PEO that acted negatively for TB1, TB2 and TB3, but for TB4 that appeared compatible with a polymer supported culture of fibroblast. A determining characteristic seems to be the LA/EO ratio since all copolymers with LA/EO < 2 appeared incompatible.

3.3 Morphology of human skin cells in culture

The cell shapes of human keratinocytes and human dermal fibroblasts grown on the TB1 to TB4 copolymers and on the two controls are shown in Fig. 5. The pictures were taken 2 days after seeding. Human keratinocytes (Fig. 5A) were well spread on all supports, thus proving that keratinocytes adhered correctly and began to proliferate. The colonies formed on TCPS and on PLA_{50} were tightly grouped whereas those formed on the triblock copolymers were not well-defined and seemed to be slightly sparse. The shape of the fibroblasts depended also very much on the material onto which the cells proliferated (Fig. 5B). Cell populations were well spread on TB4, PLA_{50} and TCPS, whereas they remained shrunk on TB3, TB2 and TB1. These pictures confirmed the poor affinity of fibroblasts for the supports with LA/EO < 2.

According to previous data, the influence of PEO on the behavior of keratinocytes and fibroblasts was different. This difference could be somehow linked to the difference in culture medium compositions. Indeed, as mentioned before, fibroblasts were allowed to grow in a medium containing 10% foetal calf serum whereas keratinocytes were cultured in a serum-free medium. It is well known that absorption of serum proteins is reduced when PEO is present on a surface [22]. Therefore, one can assume that the relative increase of the PEO content in the triblock copolymers acted against the protein absorption and consequently limited fibroblast adhesion and proliferation when keratinocyte adhesion is not disturbed by protein coating. Also, the difference in the adhesion structures of the two cell types (adhesion receptors) could explain these different behaviors.

4 Conclusion

In summary, various triblock copolymers (PLA_{50}–PEO–PLA_{50}) with the same PEO block length but different LA/

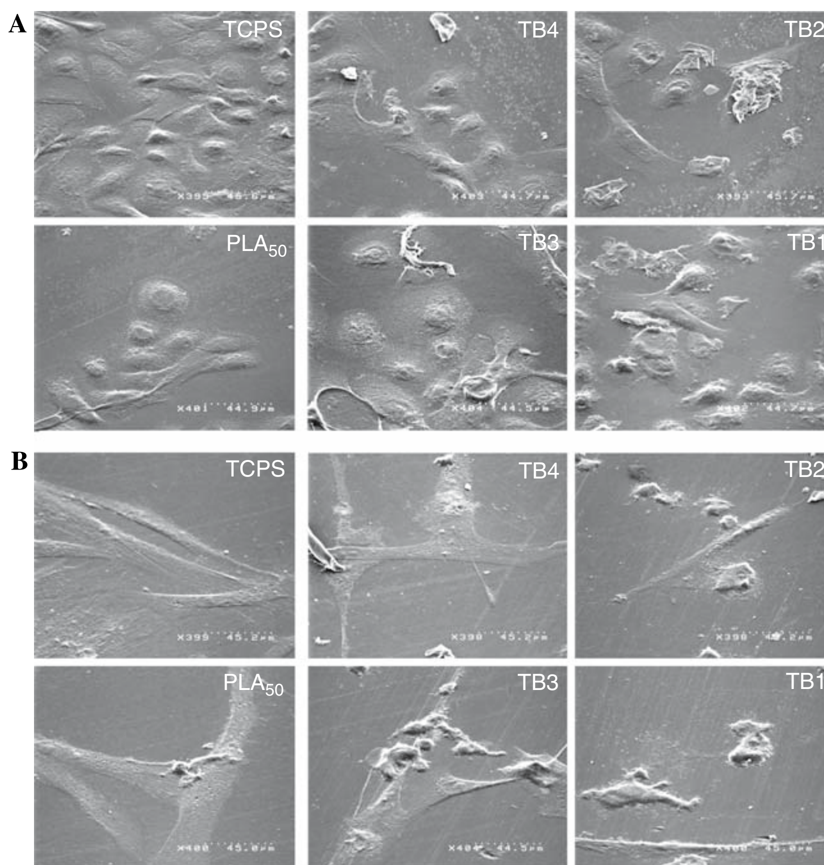

Fig. 5 Electron Microscopy of keratinocytes (**A**) and fibroblasts (**B**) cultivated after 2 days on polystyrene (TCPS), PLA_{50} and PLA_{50}–PEO–PLA_{50} triblock copolymers (TB4–TB1)

EO ratio were successfully synthesized. The copolymers with LA/EO < 2 were not compatible with the culture of fibroblasts. Only the TB4 bioresorbable triblock copolymers with a LA/EO ratio = 2 appeared suitable to grow both keratinocytes and fibroblasts. This study also illustrates that engineering a biocompatible and bioresorbable polymer that allows the culture of two different cell types could be very tricky. Additional investigations on cell-polymer interaction are still necessary in order to match culture conditions for both cell populations. However, a good compromise would include a PLA–PEO–PLA triblock copolymers derived from PEG 20,000 having a LA/EO $\sim$ 2 and would represent an interesting compound to make porous scaffolds for the engineering of dermo-epidermal substitutes.

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References

1. J. M. STILL, H. K. ORLET and E. J. LAW, *Burns* **20** (1994) 539
2. G. G. GALLICO 3rd, *Clin. Plast. Surg.* **17** (1990) 519
3. T. J. PHILLIPS and B. A. GILCHREST, *Epithelial Cell Biol.* **1** (1992) 39
4. L. J. CURRIE, J. R. SHARPE and R. MARTIN, *Plast. Reconstr. Surg.* **108** (2001) 1713
5. J. F. BURKE, I. V. YANNAS, W. C. QUINBY Jr., C. C. BONDOC and W. K. JUNG, *Ann. Surg.* **194** (1981) 413
6. D. Y. LEE, H. T. AHN and K. H. CHO, *J. Dermatol. Sci.* **23** (2000) 132
7. N. T. DAI, M. R. WILLIAMSON, N. KHAMMO, E. F. ADAMS and A. G. COOMBES, *Biomaterials* **25** (2004) 4263
8. A. EL-GHALBZOURI, E. N. LAMME, C. VAN BLITTERSWIJK, J. KOOPMAN and M. PONEC, *Biomaterials* **25** (2004) 2987
9. S. LI and M. VERT, in "Biodegradable Polymers, Principles & Applications", edited by G. Scott and D. Gilead (Chapman & Hall, London, 2003) p. 71
10. D. K. GILDING and A. M. REED, *Polymer* **20** (1979) 1459
11. J. F. HANSBROUGH, J. L. MORGAN, G. E. GREENLEAF and R. BARTEL, *J. Burn Care Rehabil.* **14** (1993) 485
12. F. A. BARBER, B. F. ELROD, D. A. MCGUIRE and L. E. PAULOS, *Arthroscopy* **11** (1995) 537
13. H. SELEK, S. SAHIN, M. T. ERCAN, M. SARGON, A. A. HINCAL and H. S. KAS, *J. Microencapsul.* **20** (2003) 261
14. X. GARRIC, J. P. MOLES, H. GARREAU, C. BRAUD, J. J. GUILHOU and M. VERT, *J. Biomater. Sci. Polym. Ed.* **13** (2002) 1189
15. X. GARRIC, J. P. MOLES, H. GARREAU, J. J. GUILHOU and M. VERT, *J. Biomed. Mater. Res. A* **72** (2005) 180
16. J. LERAY, M. VERT and D. BLANQUAERT, French patent 76 28 183 (Filed on 20 Sept 1976)
17. I. RASHKOV, N. MANOLOVA, S. M. LI, J. L. ESPARTERO and M. VERT, *Macromolecules* **29** (1996) 50
18. J. G. RHEINWALD and H. GREEN, *Cell* **6** (1975) 331
19. T. MOSMANN, *J. Immunol. Methods* **65** (1983) 55
20. M. B. HANSEN, S. E. NIELSEN and K. BERG, *J. Immunol. Methods* **119** (1989) 203
21. M. C. TANZI, P. VERDERIO, M. G. LAMPUGNANI, M. RESNATI and E. STURANI, *J. Mater. Sci. Mater. Med.* **5** (1994) 393
22. A. GOPFERICH, S. J. PETER, A. LUCKE, L. LU and A. G. MIKOS, *J. Biomed. Mater. Res.* **46** (1999) 390