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C. James Kirkpatrick
Technical University of Aachen (RWTH)

Tillmann Otterbach
Technical University of Aachen (RWTH)

Dirk Anderheiden
Technical University of Aachen (RWTH)

Johannes Schiefer
Technical University of Aachen (RWTH)

Horst Richter
Technical University of Aachen (RWTH)

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QUANTITATIVE SCANNING ELECTRON MICROSCOPY (SEM) TO STUDY THE ADHESION AND SPREADING OF HUMAN ENDOTHELIAL CELLS TO SURFACE-MODIFIED POLY(CARBONATE URETHANE)S

C.James Kirkpatrick*, Tillmann Otterbach, Dirk Anderheiden[1], Johannes Schiefer, Horst Richter, Hartwig Höcker[1], Christian Mittermayer, Albert Dekker

Institute of Pathology and [1]Institute of Macromolecular and Textile Chemistry, Technical University of Aachen (RWTH), Federal Republic of Germany

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Abstract

The polymers currently in use as vascular prostheses are, in the native state, poor substrates for endothelial cell (EC) adhesion and growth. This has a negative effect on the success of pre-seeding regimes. One attempt to improve the polymer substrate is to covalently couple reactive molecules to the surface, which can be used as anchorage points for EC or serve as spacer molecule to couple biological signal molecules such as oligopeptides. We have used a digitized image analysis system coupled to a scanning electron microscope to study the adhesion and spreading of human venous EC to unmodified poly(carbonate urethane) (uPCU), hydroxyl-functionalized PCU (mPCU) and to mPCU surfaces with succinyl or adipoyl dichloride coupled to it. Although the uPCU did not promote EC growth over a 5 day period, the early (30 minutes) adhesion and spreading behaviour on this surface was comparable to that of some modified surfaces which gave good promotion of EC growth in the longer term studies. Adhesion, but not spreading data at 4 hours gave a good correlation with the longer term studies of EC growth on the polymer surfaces. This study not only presents new directions in polymer development for vascular grafts, but also indicates the dangers associated with using early cell adhesion behaviour as a parameter of bifunctionality of biomaterial surfaces.

Key Words: Endothelial cell seeding, vascular graft, poly(carbonate urethane), surface modification, cell adhesion, cell spreading.

*Address correspondence to:
C. James Kirkpatrick,
Institute of Pathology,
Klinikum der RWTH Aachen,
Pauwelsstraße 30,
D-5100 Aachen,
Federal Republic of Germany
Telephone Number: (49)-241-8089298
Fax Number: (49)-241-8089284

Introduction

Vascular prostheses in current medical practice are made of polymers which, despite acceptable bulk structural properties, are poor substrata for the support of endothelial cell (EC) growth. This topic has recently been reviewed in detail (Kirkpatrick *et al.*, 1991). This has led to a search both for suitable surface modifications of these polymers (Williams *et al.*, 1985; Curti *et al.*, 1989; Van Wachem *et al.*, 1989; Vohra *et al.*, 1990; Dekker *et al.*, 1991) and for new genera of polymers. Among those undergoing development are various species of polyurethanes (Teijeira *et al.*, 1989; Brothers *et al.*, 1990).

Recently, Anderheiden *et al.* (1991) described the development of various poly(carbonate urethane)s (PCU). Of particular interest was a PCU, based on poly(hexamethylene carbonate) diol and 4, 4'-diphenylmethane diisocyanate (MDI), with 1,4-butanediol as chain extender. In order to increase the biocompatibility of this PCU, this group successfully used photo-initiation of the polymerization of 4-hydroxybutyl acrylate in the presence of the PCU to form an inter-penetrating network, containing hydroxyl groups. The successful introduction of hydroxyl groups into the polymer was shown by analytical techniques, including XPS (X-ray photoelectron spectroscopy), FTIR-ATR- (Fourier transformed infra-red spectroscopy in attenuated total reflection) and ^{13}C-FT-NMR- (Fourier transformed nuclear magnetic resonance) spectroscopy. The principal aim in synthesizing a polymer with superficially localized hydroxyl groups was to use these groups to covalently couple short-chain bifunctional aliphatic spacer molecules, to which biological signal molecules, such as cell-binding peptide fragments of known basement membrane or extracellular matrix components (ECM), could be coupled (Breuers *et al.*, 1988).

In the present study we have used cultured human venous endothelial cells (EC) to study and compare the ability of these newly developed PCU to promote adhesion, spreading and growth of EC. Both adhesion and spreading were measured in the early phase of cell-material interaction using quantitative scanning electron microscopy (SEM) with an image analysis system.

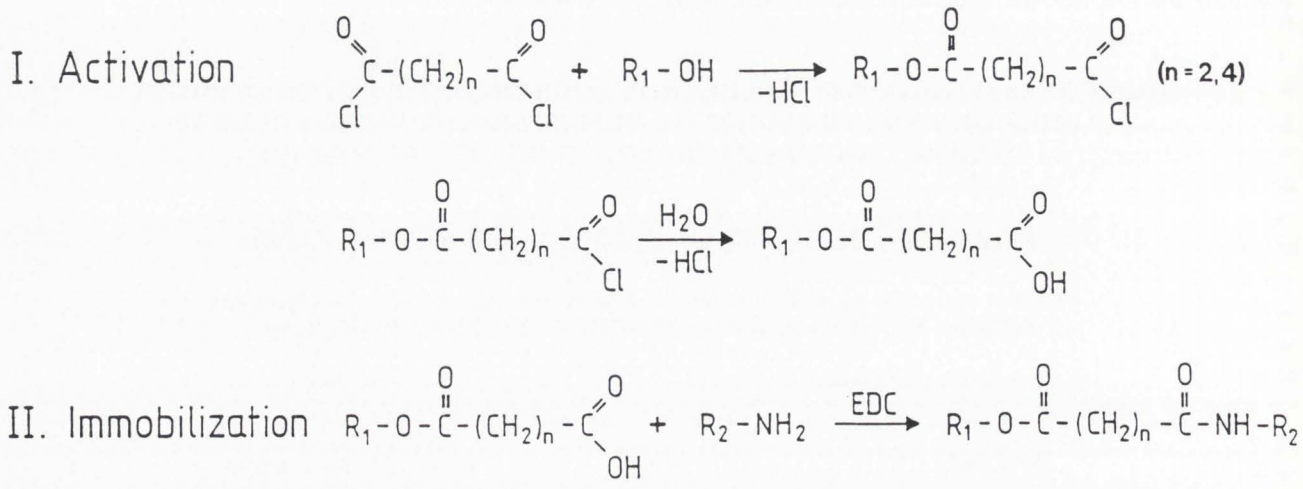


Figure 1. Mechanism of the diacid dichloride activation and immobilization of biomolecules using EDC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide). R1-OH : hydroxyl-functionalized PCU (mPCU); R2-NH_2 : NH_2-containing biomolecule.

Materials and Methods

Synthetic PCU and Modifications

The synthesis and characterization of the unmodified PCU (uPCU) and the hydroxyl-functionalized PCU (mPCU) are described in detail elsewhere (Anderheiden *et al.*, 1991). Activation of the OH-functionalized PCU was performed with succinyl-(C4) and adipoyl dichloride (C6) as bifunctional spacer molecules. The reactions were carried out in dried toluene with polymer film pieces of about 9 cm^2. Two mmol of the carboxylic acid dichloride was added and stirred for 20 minutes at room temperature. Subsequently, 4 mmol dried pyridine and afterwards 1 ml of 12 M hydrochloric acid solution with water were added. The films were then washed in sodium bicarbonate buffer (0.1 M; pH 8.5) and distilled water. All chemicals were purchased from Merck, Darmstadt.

The reaction mechanism is shown in Fig. 1. After esterification of the OH-groups in the first reaction, the second acid dichloride function was hydrolyzed to obtain the carboxylic acid groups. This step was important for the biocompatibility of the spacer-activated material. Further immobilization of biologically active signal molecules can be obtained in the immobilization reaction using water soluble carbodiimide (EDC), which is shown in Fig. 1. The successful activation of the mPCU surfaces can be proven by FTIR-ATR investigations (Anderheiden *et al.*, 1992). Unmodified and OH-functionalized PCU, as well as spacer-activated PCU surfaces were used in this study.

Endothelial Cell Culture

Human adult saphenous vein EC (HASVEC) were cultured from intact sterile remnant veins from coronary artery bypass surgery. Vein segments were cannulated and rinsed with HEPES [N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) buffer (0.137 M NaCl, 4.0 mM KCl, 10 mM HEPES, 10 mM glucose; pH 7.55). EC were isolated using 0.1% collagenase I (Worthington, supplied by Interchem, Munich)/0.01% EDTA (ethylenediaminetetraacetic acid; Sigma; Munich) in HEPES/0.25% bovine serum albumin (Sigma) for 15 minutes at 37 °C. Isolated HASVEC were grown in primary and secondary culture in a mixture of Ham's F-12 and Iscove's modified Dulbecco's medium (Gibco, Karlsruhe) in a ratio of 1 : 1 (volume/volume), supplemented with penicillin-streptomycin (Gibco; 80 U/ml and 80 μg/ml respectively) and L-glutamine (Gibco, final concentration 1.6 mM), in addition to 20% pooled human serum. Cultures also contained endothelial cell growth factor (ECGF) (Boehringer, Mannheim) and heparin (Sigma) at concentrations of 10 ng/ml and 50 μg/ml respectively. Prior to seeding the tissue culture flasks were coated overnight with 0.2% aqueous gelatin (Serva, Heidelberg). Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 and 10% oxygen. Subcultures were prepared by treating intact monolayers with 0.1% collagenase I and replated using a splitting ratio of 1 : 2 to 1 : 6.

To characterize the endothelial phenotype, cells were grown in Lab-Tek culture chambers (Nunc, Wiesbaden), coated with fibronectin (10 μg/ml) (Boehringer, Mannheim). Cells were taken from passage 0 (P0) up to passage 6 (P6) and prepared for SEM (see below), transmission electron microscopy (TEM) and immunocytochemistry. TEM studies were performed on confluent cultures of HASVEC, which were fixed for 30 minutes in cacodylate-buffered 1.5% glutaraldehyde, pH 7.4, for 1 hour, post-fixed in 1% osmium tetroxide, dehydrated and embedded in Epon. Ultrathin sections were stained with 2% uranyl acetate and 0.4% lead citrate, and examined in a Philips 400 TEM. For immunocytochemistry, an indirect immuno-fluorescent method for factor VIII related antigen was employed (Kirkpatrick, 1984).

HASVEC seeding on to polymers

Control materials consisted of glass coverslips (Assistent, Caspar & Co., Aachen) and Thermanox discs, not glued to Thermanox discs. Polymer films were cut in disc form (13 mm diameter) and anchored to Thermanox discs (Nunc, Wiesbaden) of the same diameter using a biocompatible double-sided adhesive (Lohmann, Neuwied). The biocompatibility of this adhesive was proven using L 929 mouse fibroblasts, which adhered and proliferated well on this adhesive with no significant changes compared with standard negative controls (unpublished results). Moreover, the compatibility of this material with human endothelial cells was confirmed by our experience over the last two years. The discs were then placed in the wells of a 24-multiwell (16 mm diameter) tissue culture cluster (Falcon, Becton-Dickinson, Heidelberg). HASVEC taken from the second passage (collagenase treatment; as above) were seeded in a single cell suspension at a plating density of 2 x 10^4 cells/well in 500 μl of complete serum-containing medium as described above. Cells were allowed to adhere and spread on the synthetic polymers for 30 minutes or 4 hours.

SEM quantitation of EC adhesion and spreading

Seeded discs (test and control) were harvested by removal from the multiwells and dipping 5 times through an air/liquid interface to remove non-adherent cells. The Gey's balanced salt solution (GBSS, Gibco) was used at 37 °C. The cells were fixed in 1.5% glutaraldehyde in GBSS for 1 hour, followed by transfer to GBSS and post-fixation for 12 hours at 4 °C in 3% glutaraldehyde in GBSS. Specimens were prepared for SEM by dehydrating in graded alcohols, each for 5 minutes, followed by air drying. This preparative technique was found to give the best structural integrity of EC, when compared with critical point drying, which tended to cause tear artifacts in the extremely flat EC in the spread state. Specimens were mounted on aluminium stubs using double-sided adhesive and sputtered with gold using a Balzers SCD-030 sputter coating device (3 minutes, 20 mA) to yield a coating thickness of 30 nm. Specimens were examined in a Philips SEM 515 using a tilt angle of 0 degrees and accelerating voltage of 20 kV.

Quantitation of adhesion and spreading of EC was performed using an image analysis system connected to the video outlet of the SEM. Briefly, the SEM picture was transferred to a color monitor (Sony, Trinitron colour, PVM-1370 QM). This was connected to a personal computer (PC) (AT-286), containing an analog-digital converter, which digitizes the analog picture. With the help of a graphic tablet (Summagraphics, MM 120 II) cell number and cell area were marked on the monitor. Cell area was calculated by vector addition in the computer. Further data processing was carried out using a programme that stored the data in an ASCII file, which enabled processing using an Excel statistics programme.

The software specially designed for this system displayed 21 rectangular fields on the computer monitor, each field corresponding to certain coordinates on the specimen stage. Using a low magnification (x 150) the number of adherent cells was counted in each of the 21 fixed fields. In the same mode, i.e., during the adhesion quantification, cells were "typed" according to their morphology, as shown in Fig. 2. This latter study documented the percentage of EC in each of the three morphological groups, corresponding to a certain spread state. Type 1 refers to an early stage of interaction with the material and shows an approximately spherical EC with negligible cytoplasmic spreading. A further stage of spreading, designated as type 2, shows an EC with considerably spread cytoplasm, yet with a raised nucleus. Type 3 refers to a fully spread EC with flattened nucleus and extensively spread cytoplasm.

EC spreading was quantified at a magnification of x 1,200. Each of the 21 fields was taken and one cell randomly chosen for assessment. Using the graphics tablet the cursor was moved around the circumference of the chosen EC. The enclosed area was measured electronically and the value stored in the PC for later data processing and statistical testing.

The measurements were performed by a technician who was not informed of the different modifications. Using a student's t-test, differences were considered to be significant at a 5% level.

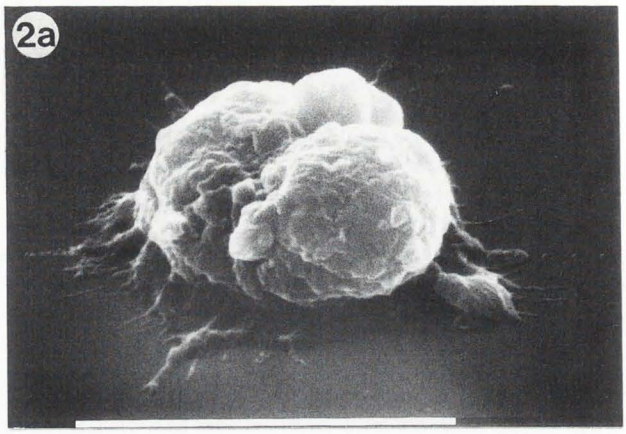
SEM assessment of EC monolayer formation

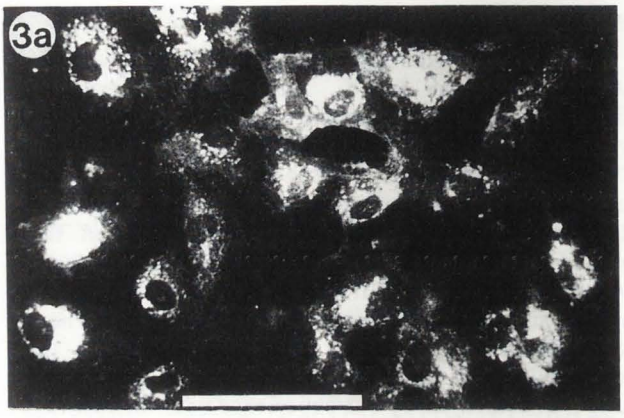
Specimens were prepared, sterilized and placed in wells of a 24-multiwell chamber as described above. To study the ability of these materials to promote the growth of an EC monolayer, a single cell suspension of HASVEC at a plating density of 4 x 10^4 cells/well was seeded in a total volume of complete medium (as above) of 0.8 ml. The cells were then cultured under standard conditions (as above) for 5 days, changing the medium after 2 days. At the end of the incubation period, at which time the glass control cultures were confluent, cells were harvested by rinsing cultures twice with GBSS and fixing for 30 minutes in 1.5% glutaraldehyde. Specimens were prepared for SEM as described above and examined at 20 kV and a tilt angle of 45 degrees.

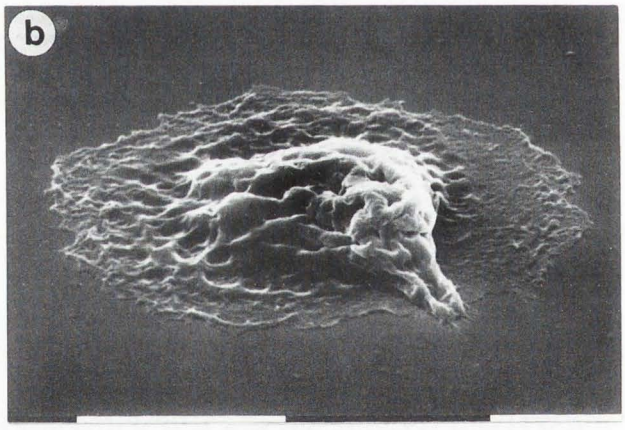
Results and Discussion

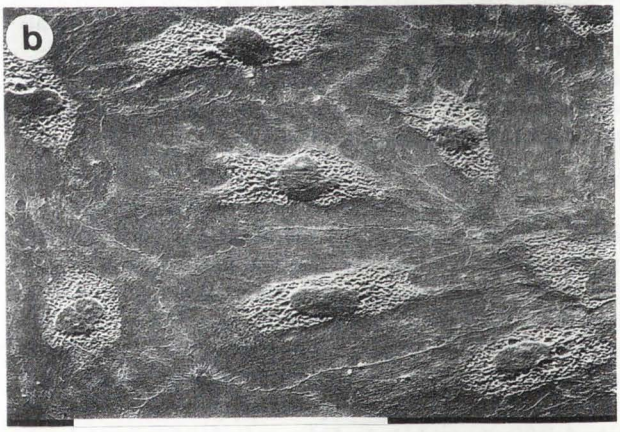
HASVEC characterization

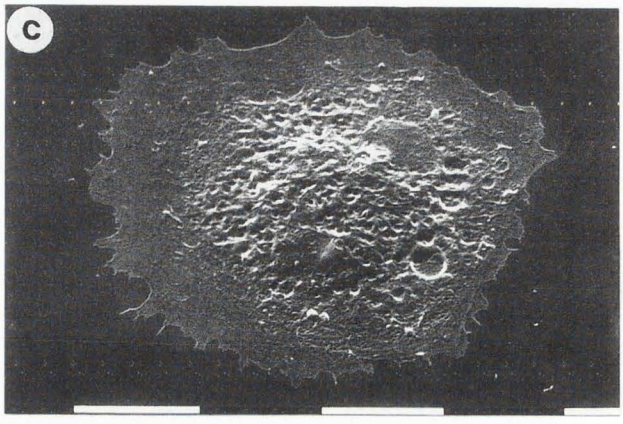
Immunocytochemical studies, performed on cells in passages P0 to P5 showed a strongly positive reaction for factor VIII RA (related antigen) (Fig. 3a). Cultures studied by SEM up to P6 revealed polygonal cells with clearly demarcated cell boundaries (Fig. 3b). Similarly, TEM studies showed the presence of intracytoplasmic Weibel-Palade bodies up to P6 (Fig. 3c). These results clearly show that the cells used in this study are endothelial cells, which kept their typical endothelial characteristics up to passage 6. To avoid possible phenotypical changes of the culture in time, we only used cells of the second passage.











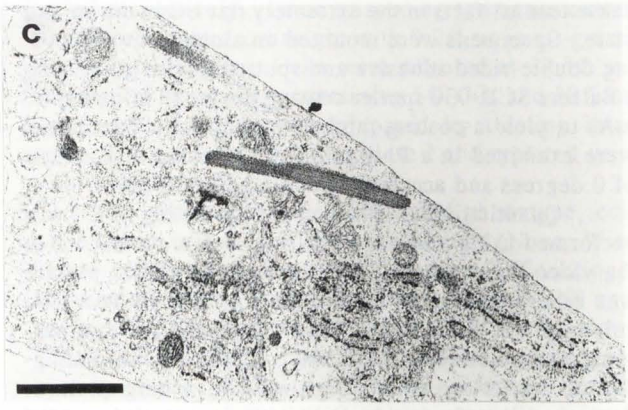
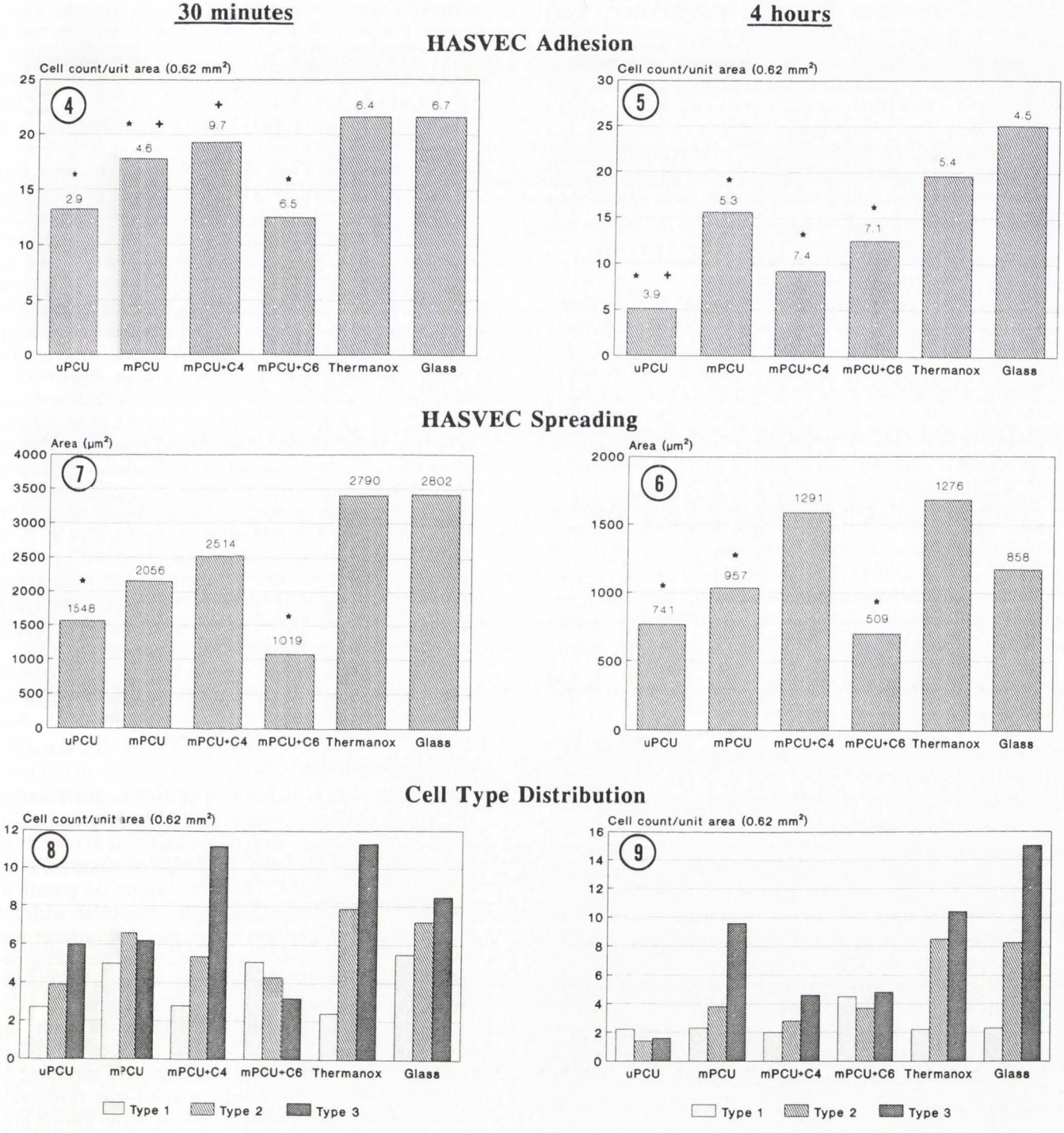


Figure 2 (at left). Spread state of HASVEC. Bars = 10 μm. **a**. Type 1. Early spreading with rounded nucleus and minimal expression of cytoplasm. **b**. Type 2. Later stage of spreading with clearly spread cytoplasm, yet with raised nucleus. **c**. Type 3. Advanced spreading with flattened nucleus and extensive cytoplasm.

Figure 3a (at right). Strong reaction of HASVEC for factor VIII RA in indirect immunofluorescence. Bar = 100 μm. **3b**. SEM of HASVEC from passage 5, showing polygonal morphology. Bar = 0.1 mm. **3c**. TEM of HASVEC from passage 6 with Weibel-Palade bodies. Bar = 1 μm.



Figures 4 and 5. HASVEC adhesion to substrates after 30 minutes (**Fig. 4**) and 4 hours (**Fig. 5**). Values given as mean cell count/unit area (0.62 mm^2). In addition, standard deviation (SD) of the mean is shown above the bars for each substrate. **Fig. 4**: * : $p < 0.05$ compared with Thermanox; + : $p < 0.05$ compared with uPCU. **Fig. 5**: * : $p < 0.05$ compared with Thermanox; + : $p < 0.05$ compared with mPCU, mPCU+C4 or mPCU+C6.

Figures 6 and 7. HASVEC spreading on substrates after 30 minutes (**Fig. 6**) and 4 hours (**Fig. 7**). Values are given as cell area (μm^2) + SD of mean. *: $p < 0.05$ compared with Thermanox.

Figures 8 and 9. Cell type distribution on various substrates after 30 minutes (**Fig. 8**) and 4 hours (**Fig. 9**). Values are given as cell count/unit area (0.62 mm^2) for each of the 3 cell types.

HASVEC interaction with synthetic polymers

In clinical practice, successful seeding of vascular grafts with endothelial cells will depend upon a good interaction of the cells with the luminal surface of the prosthesis. This interaction has to be established within a relatively short time before blood flow is restored. In the long-term, the relatively small amount of adherent endothelial cells has to proliferate to cover the luminal surface. Therefore, both adhesion and spreading of endothelial cells after 30 minutes and 4 hours, as well as the monolayer formation after 5 days were studied.

Adhesion

Fig. 4 shows the results of HASVEC adhesion after 30 minutes of interaction with the various substrates. Two positive controls were included, glass and Thermanox, the latter being a commercially available polystyrene, surface-modified to promote cell growth. No details are obtainable concerning the type of modification. HASVEC adhesion to the unmodified PCU (uPCU) was statistically significantly lower ($P < 0.05$) than the controls. The OH-functionalized PCU (modified PCU, mPCU) gave a significantly higher cell adhesion than the uPCU, but was also significantly lower than the two positive controls. However, the addition of the C4 spacer to the mPCU (mPCU+C4) resulted in a promotion of HASVEC adhesion of the order of both positive controls (no statistical significance between mPCU+C4 and Thermanox or glass). The addition of the C6 spacer (mPCU+C6) did not result in any significant improvement of cell adhesion, compared with uPCU.

HASVEC adhesion after 4 hours of interaction with the substrates gave a different spectrum of behaviour. Comparing Fig. 4 and Fig. 5, it can be seen that between 30 minutes and 4 hours, the positive controls showed altered adhesion dynamics. While HASVEC adhesion to glass continued to increase, adhesion to Thermanox appeared to have reached a saturation value already after 30 minutes. One of the most important findings is the extremely poor performance of the uPCU. This was much less apparent on examination of the data for 30 minutes adhesion. At 4 hours, cell adhesion to uPCU was statistically significantly reduced ($P < 0.05$), compared to all three modified surfaces (mPCU, alone and with the spacers C4 and C6). Nevertheless, all three modifications did not achieve the high adhesion promotion of either Thermanox or glass.

Spreading

The high standard deviation values in Figs. 6 and 7 indicate that there is very large variation in the areas occupied by cells adhering to the various substrates. Even at 30 minutes there was a subpopulation of HASVEC which spread very rapidly. This large variation makes the statistical study less sensitive than if greater homogeneity in cell size had occurred. Nevertheless, analogous to the early (30-minute) adhesion study (Fig. 4), cell spreading on the mPCU+C4 surface gave a mean value of 1590 μm^2, which did not show statistical significance from the mean value on Thermanox (1681 μm^2) (Fig. 6). Despite the high mean value for mPCU+C4, none of the three surface modifications gave statistically significantly higher values for cell spreading than the uPCU, although the value for mPCU+C4 was marginally below statistical significance ($P < 0.05$).

Comparing Figs. 6 and 7, it can be seen that between 30 minutes and 4 hour, HASVEC spreading on glass proceeded with greater speed than on Thermanox, with the result that at 4 hours, Thermanox and glass surfaces were indistinguishable from the point of view of promotion of cell spreading. As with the 30-minute values, spreading at 4 hours gave a similar trend, with the highest mean value on the modified surfaces being achieved on mPCU+C4, albeit without statistical significance, compared with the other modifications (mPCU, mPCU+C6) or uPCU (Fig. 7). Of particular importance is the good performance of the unmodified surface, uPCU, a deceptive result on consideration of the poor performance of this surface in the monolayer growth assay (see below).

An analysis of the distribution of cell types at 30 minutes (Fig. 8) underlines the high proportion of cells in the late stage of spreading (type 3) on both mPCU+C4 and Thermanox. This parallels the high mean value for cell spreading on these two surfaces. The relatively high value of type 3 cells on uPCU is surprising in light of the poor growth promotion of this surface.

Cell type distribution at 4 hours (Fig. 9) reveals a high proportion of type 3 cells on mPCU, as well as on Thermanox and glass. Cell spreading on uPCU was shown by this analysis to be extremely poor.

Monolayer growth

After 5 days *in vitro*, HASVEC-seeded Thermanox gave a confluent monolayer with well-spread, polygonal EC (Fig. 10a). Despite the ability of EC to adhere to and spread on the unmodified PCU substrate (30 minutes, 4 hours), the uPCU failed to support the growth of a HASVEC monolayer (Fig. 10b). Compared with the uPCU surface, EC growth on the modified surfaces was good, with considerable areas of confluence being observed in the mPCU (Fig. 10c), as well as in the spacer-modified cases, mPCU+C4 (Fig. 10d) and mPCU+C6.

The data for HASVEC interaction with the hydroxy-functionalized PCU with the succinyl spacer (mPCU+C4), showing good adhesion and spreading in the early phase (30 minutes), coupled with favourable longer-term effects, evidenced by monolayer growth promotion, suggest that this surface holds promise for EC seeding technologies. One of the important aspects of the present data concern the predictability of the short-term adhesion and spreading assays with respect to longer-term cell-material interactions. The early adhesion studies (30 minutes) taken in isolation might suggest that uPCU is a suitable substrate for EC seeding. This is, however, deceptive and was not borne out by the investigation to determine cell proliferation on the surface. Of the early studies, i.e., adhesion and spreading at 30

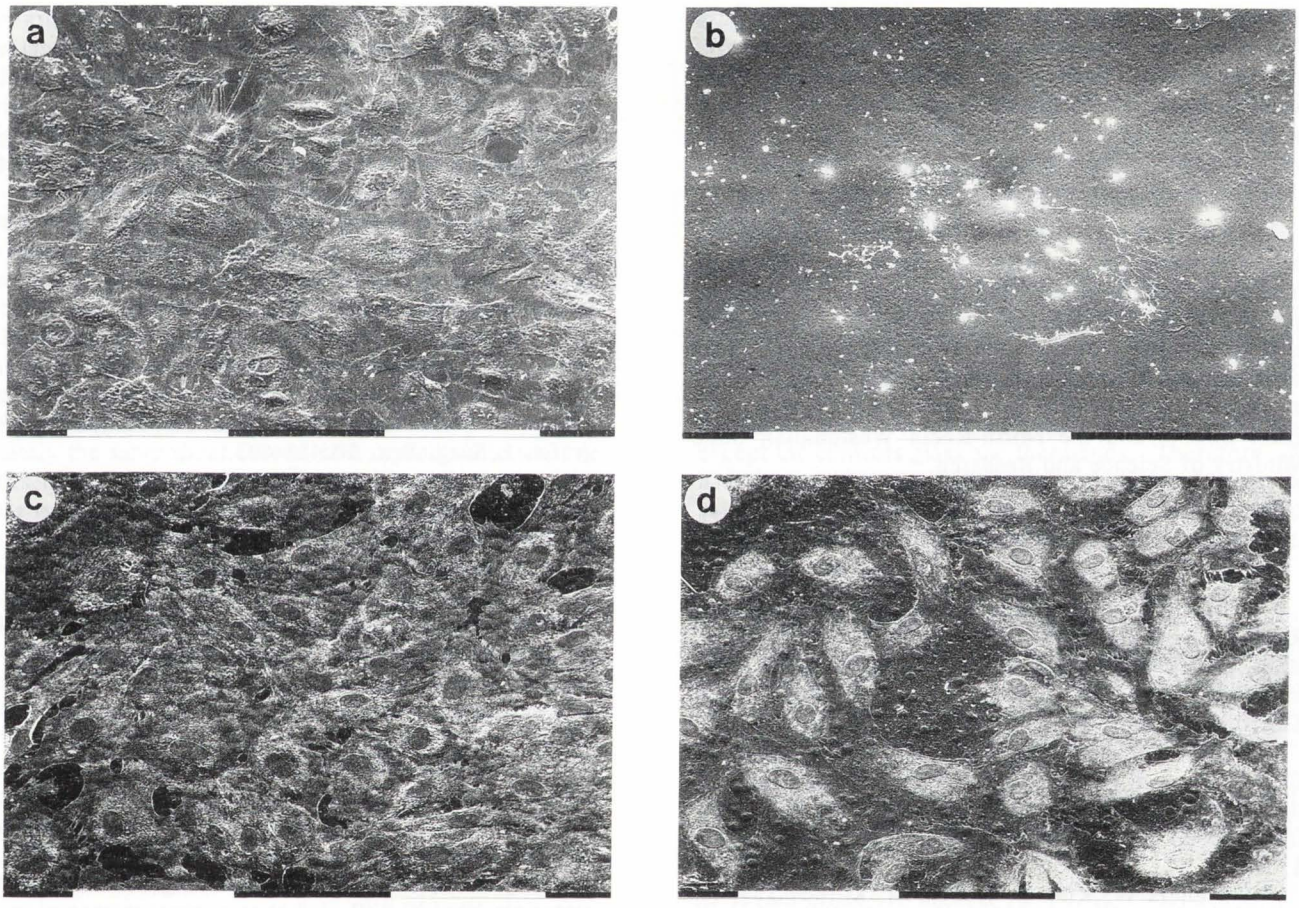


Figure 10. SEM studies of monolayer growth of HASVEC on various polymer surfaces. Bars = 0.1 mm. **a.** Thermanox control, showing excellent coverage of the surface with EC. **b.** uPCU surface, showing no EC growth whatsoever. The surface contains only debris. **c.** mPCU surface with good coverage with EC. Although the entire surface is not covered, the EC demonstrate regular morphology. **d.** mPCU with attached C4 spacer. The result is similar to that on the mPCU surface, that is, although some inter-cellular spaces are present, cells have spread and grown well on the substrate.

minutes and 4 hours, only the mean value for adhesion at 4 hours on uPCU suggested the poor performance of this substrate.

Despite the considerable areas of HASVEC confluence on the modified PCU surfaces (mPCU, mPCU+C4, mPCU+C6), there were foci on which no EC could be found. This could indicate an inhomogeneity of the distribution of OH groups, due perhaps to an imperfect reaction with the hydroxybutyl acrylate. Spreading on the mPCU+C6 surface was surprisingly low, both at 30 minutes and 4 hours, although this surface did support EC growth over the 5 day period studied. This discrepancy cannot be adequately explained at present.

Conclusion

This paper indicates that hydroxy-functionalized poly-carbonate urethanes hold promise as substrata for EC growth promotion. The addition of short-chain aliphatic bifunctional spacer molecules, and, in particular, a 4-carbon spacer give an especially favourable early cell adhesion and spreading. The data presented also underline the unreliability of early cell adhesion studies (30-minute) in predicting the ability of the substrate to promote monolayer growth. The 4-hour adhesion result appears to correlate much better with longer-term growth studies. Spreading, both at 30 minutes and 4 hours is unreliable as a predictor, due to heterogeneous cell type distribution.

Nevertheless, the use of quantitative SEM to determine early cell adhesion and spreading is, in conjunction with assessment of (later) monolayer growth promotion, a useful and valid method to evaluate the suitability of new polymer species for EC seeding purposes. These static *in vitro* studies represent a first step in the testing regimen of polymer suitability for endothelialization. It must, however, in a further stage of

investigation evaluate the behaviour of EC under flow conditions, as has been established by a number of research groups (Kesler *et al.*, 1986; Pratt *et al.*, 1989; Greisler *et al.*, 1990; Miyata *et al.*, 1991).

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References

Anderheiden D, Brenner O, Klee D, Kaufmann R, Richter HA, Mittermayer C, Höcker H (1991). Development and characterization of a biocompatible OH-modified copolymer based on polyurethane. Appl. Macromol. Chem. & Phys. **185/186**: 109-127.

Anderheiden D, Klee D, Heller B, Kirkpatrick CJ, Mittermayer C, Höcker H (1992). Surface modification of a biocompatible polymer based on polyurethane for artificial blood vessels. J. Mater. Sci. Mater. Med. **3**: 1-4.

Breuers W, Klee D, Höcker H, Mittermayer C. (1988). Modification of polyetherurethane for endothelial cell seeding. In: Implant Materials in Biofunction, de Putter C, de Lange GL, de Groot K, Lee AJC (Eds.), Advances in Biomaterials, Vol. 8, Elsevier Science Publ., Amsterdam, 297-303.

Brothers TE, Stanley JC, Burkel WE, Graham LM (1990). Small-caliber polyurethane and polytetrafluoroethylene grafts: A comparative study in a canine aortoiliac model. J. Biomed. Mater. Res. **24**: 761-771.

Curti T, Pasquinelli G, Preda P, Freyrie A, Laschi R, D'Addato M (1989). An ultrastructural and immunocytochemical analysis of human endothelial cell adhesion on coated vascular grafts. Ann. Vasc. Surg. **3**: 351-363.

Dekker A, Reitsma K, Beugeling T, Bantjes A, Feijen J, Van Aken WG (1991). Adhesion of endothelial cells and adsorption of serum proteins on gas plasma-treated polytetrafluorethylene. Biomaterials **12**: 130-138.

Greisler HP, Johnson S, Joyce K, Henderson S, Patel NM, Alkhamis T, Beissinger R (1990). The effects of shear stress on endothelial cell retention and function on expanded polytetrafluoroethylene. Arch. Surg. **125**: 1622-1625.

Kesler KA, Herring MB, Arnold MP, Glover JL, Park H-M, Helmus MN, Bendick PJ (1986). Enhanced strength of endothelial attachment on polyester elastomer and polytetrafluoroethylene graft surfaces with fibronectin substrate. J. Vasc. Surg. **3**: 58-64.

Kirkpatrick CJ (1984). Endothelzellkulturen - ein Modell zur Untersuchung der Pathogenese der Entzündung: Morphologische, biochemische und Biophysikalische Beobachtungen (Endothelial cell cultures: a model to study the pathogenesis of inflammation. Morphological, biochemical and biophysical observations). Habilitation-Thesis, University of Ulm, D-7900, Germany.

Kirkpatrick CJ, Mueller-Schulte D, Roye M, Hollweg G, Gossen C, Richter H, Mittermayer C (1991). Surface modification of polymers to permit endothelial cell growth. Cells & Materials **1**: 93-108.

Miyata T, Conte MS, Trudell LA, Mason D, Whittemore AD, Birinyi LK (1991). Delayed exposure to pulsatile shear stress improves retention of human saphenous vein endothelial cells on seeded ePTFE grafts. J. Surg. Res. **50**:485-493.

Pratt KJ, Williams SK, Jarrell BE (1989). Enhanced adherence of human adult endothelial cells to plasma discharge modified polyethylene terephthalate. J. Biomed. Mater. Res. **23**: 1131-1147.

Teijeira FJ, Lamoureux G, Tetreault J-P, Bauset R, Guidoin R, Marois Y, Paynter R, Assayed F (1989). Hydrophilic polyurethane versus autologous femoral vein as substitutes in the femoral arteries of dogs: quantification of platelets and fibrin deposits. Biomaterials **10**: 80-84.

Van Wachem PB, Schakenraad JM, Feijen J, Beugeling T, Van Aken WG, Blaauw EH, Nieuwenhuis P, Molenaar I (1989). Adhesion and spreading of cultured endothelial cells on modified and unmodified poly-(ethylene terephthalate): a morphological study. Biomaterials **10**: 532-539.

Vohra RK, Thomson GJL, Sharma H, Carr HMH, Walker MG (1990). Effects of shear stress on endothelial cell monolayers on expanded polytetrafluoroethylene (ePTFE) grafts using preclot and fibronectin matrices. Eur. J. Vasc. Surg. **4**: 33-41.

Williams SK, Jarrell BE, Friend L, Radomski JS, Carabasi RA, Koolpe E, Mueller SN, Thornton SC, Marinucci T, Levine E (1985). Adult human endothelial cell compatibility with prosthetic graft material. J. Surg. Res. **38**: 618-629.

Discussion with Reviewers

S.L. Goodman: I am concerned that only 1 cell/field was measured for area. If the sample size was small (what were the sample sizes?) this could explain the exceedingly large variation between conditions, which could have lead to the observed insignificance.

Authors: The most representative way to sample cells for surface area measurement was to take cells from all fields. In this way for each substrate 42 cells were measured. The high standard deviations of the mean cell area values represent the large variation in areas occupied by cells adhering to the different substrates. The cell type distribution clearly shows that at any time point, the population of adherent cells consists of spherical, medium- and well-spread cells. Therefore, in our

opinion the inhomogeneity of the population of adhering cells is responsible for the high standard deviation and not the sample size.

S.L. Goodman: Non-adherent cells were removed from the test samples by dipping six times through an air-liquid interface. Due to the high surface tension forces produced when dipping in and out of water (buffer), it would be expected that such dipping would remove adherent cells as well as potentially producing an air drying artifact on those cells which remain adherent. Were these possibilities examined?
Authors: The shear forces produced using this dipping method are relatively low. Moreover, using this method to remove non-adherent cells, we could apply approximately the same shear forces, in a reproducible way, to all samples. Drying artifacts were never observed using this method, since the dipping procedure was performed at such velocity that the adhering fluid film could not evaporate between the subsequent dippings.

G. Pasquinelli: The presence of a monolayer endothelium on the polymer surface is not a guarantee in itself for a good patency rate of the implanted graft. In fact, there are several reports indicating that endothelial cells can be perturbed by the synthetic material and, therefore, they can express procoagulant and proinflammatory activities. As far as the modified PCUs are concerned, do you have any data on this issue?
Authors: The functional state of endothelial cells after adhesion to a synthetic surface is also in our opinion a very important issue in endothelial cell seeding of vascular grafts. Recently, we started research on this subject, but as yet no data are available concerning these modified PCU's.

P.W. van Wachem: The mPCU's are to be used for coupling ECM-components (**Introduction**, end of paragraph 2). If this is correct, it is not understood why mPCU+C4 "holds promise for EC technologies". Or are the mPCU's presented as such also of interest for EC seeding technologies? Have the ECM-modified substrates already been processed?
Authors: The original philosophy was to couple a biological signal molecule, e.g., GRGDS (a pentapeptide consisting of aminoacids, glycine, arginine, glycine, aspartatic acid and serine) to a biomaterial surface. This involves the use of a spacer molecule. We decided that we would test each step of the modification procedure to look for a beneficial effect on cell adhesion, spreading and growth. It was an unexpected finding that the spacer-activated surfaces promoted cell adhesion and spreading.

ECM-modified substrates have already been processed, namely PCU surfaces with the cell-binding domain of fibronectin (the pentapeptide GRGDS) coupled to it. The latest experiments showed that PCU with GRGDS coupled to it, sustained proliferation of endothelial cells.

P.W. van Wachem: Are the mPCU's always flat substrates, or can they be made as tubes?
Authors: The experiments presented in this report were all performed with flat substrates. For future application of a surface-modified poly(carbonate urethane) as vascular graft, a tube form is necessary. Therefore, one of our current research efforts is to produce these materials in the form of a tube.

P.W. van Wachem: The use of scanning electron microscopy (SEM) for this type of experiments is not new. Evaluations of both adhering cell numbers and cell spreading can also be made using light microscopy (LM). Why use SEM and not LM?
Authors: The substrates we used were not transparent, except the controls glass and thermanox. Therefore, we had to use SEM as visualization method. Moreover, using SEM, higher magnifications can be achieved, which allowed us to measure the surface areas of the cells more precisely.

P.W. van Wachem: It is disappointing that only 21 cells per substrate were checked, resulting in huge standard deviations! For me, the results on the distribution of cell types, that is, if these concern evaluations of all cells on a certain substrate at a certain time period, are more valuable.
Authors: Per substrate, 42 cells on two separate samples were evaluated. As we already pointed out in the discussion with Dr. Goodman, we are convinced that the large standard deviations are caused by the inhomogeneity of the population of adhering cells. We agree that next to absolute surface area measurements, also cell type distribution is important and must be taken into account.