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Adherence and Proliferation of Endothelial Cells on Surface-Immobilized Albumin-Heparin Conjugate

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ABSTRACT

Small-diameter vascular grafts rapidly fail after implantation, due to occlusion caused by thrombosis. This problem cannot be overcome using medication. A promising improvement of graft patency is the seeding of endothelial cells (EC) on the luminal surface of the vascular graft. Conjugates of albumin and heparin, which were developed to obtain nonthrombogenic coatings, could form an ideal coating for vascular grafts. Besides presenting anticoagulant function, heparin will bind proteins with cell adhesive properties, thus facilitating adherence of EC to the graft surface. EC were able to grow to confluency on CO_2 gas plasma–treated polystyrene (PS-CO_2) coated with albumin-heparin conjugate. CO_2 gas plasma treatment resulted in the introduction of functional groups at the surface (e.g., hydroxyl, aldehyde, carboxylic acid, and epoxide groups). Addition of albumin-heparin conjugate to the functionalized surface in an aqueous solution with pH 8.2 yielded a stable monolayer of covalently bound conjugate. The number of cells adhering and proliferating on this surface was comparable to the number of cells on fibronectin-coated PS-CO_2. However, the structure and size of EC proliferating on surface-immobilized albumin-heparin was more irregular. Long-term adherence might be improved by adding fibronectin to the albumin-heparin surface, either as a mixture with albumin-heparin or in a separate incubation step.

INTRODUCTION

SYNTHETIC VASCULAR GRAFTS OF DACRON OR EXPANDED TEFLON, with a diameter exceeding 5 mm, placed in high-flow, low-resistance locations such as the aortoiliac position, have been successfully used in vascular surgery for some decades.[1–3] However, small-diameter grafts inserted in low-flow blood vessels fail rapidly, due to occlusion caused by thrombosis and fibrotic hyperplasia.[4–11] Although anticoagulant treatment and administration of platelet inhibitors may improve the patency of larger diameter vascular grafts,[12] a similar effect has not been reported for small-diameter grafts.

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In contrast to a number of animal species, spontaneous outgrowth of endothelium on vascular prostheses implanted in humans does generally not occur,[3] although recently spontaneous endothelialization of a Dacron axillofemoral bypass graft in a patient was reported.[13] Endothelial cells (EC), which form the inner lining of the natural vessel wall, display anticoagulant and procoagulant activities, thereby creating a delicate balance between anticoagulation and haemostasis.[14,15] Seeding of endothelial cells on the luminal surface of the vascular graft would therefore appear to be a promising method to avoid occlusion of small-diameter prostheses.[16–25]

There are, however, several requirements to be fulfilled to allow the preparation of seeded prostheses. EC used for seeding need to be autologous, and, depending on the size of the vascular graft, relatively numerous EC are required to allow the formation of a confluent layer. Furthermore, the material used for vascular grafting should be able to support both adhesion and proliferation of endothelial cells. Finally, because the layer of seeded endothelial cells will, in most cases, be incomplete immediately after seeding, the prosthetic material should display antithrombogenic properties.

A coating consisting of albumin and heparin combines anticoagulant activity of heparin with passivation of the surface by albumin. Coating of polyurethane catheters and poly(tetrafluoroethylene) sheets with conjugates of albumin and heparin decreases the *in vitro* deposition, aggregation and activation of platelets.[26,27] Albumin-heparin conjugate is also effective in reducing complement activation.[27] Moreover, a number of adhesive proteins excreted by endothelial cells have binding sites for heparin. Conjugates of albumin and heparin could therefore constitute an ideal coating for vascular grafts.

In the present study, polystyrene was modified by gas plasma treatment to generate chemical groups at the surface which were used to covalently immobilize albumin-heparin conjugate to the surface. This substrate was subsequently tested for its capability to allow adhesion and proliferation of endothelial cells (Fig. 1). Human umbilical vein endothelial cells (HUVECs) were used as a model for vascular endothelial cells.

MATERIALS AND METHODS

Materials

Albumin-heparin conjugate, 11.6% (w/w) heparin, was obtained from Holland Biomaterials Group (Enschede, The Netherlands). ^{14}C-formaldehyde (55 mCi/mmol) and Na^{125}I (17.4 mCi/μg) were bought from Amersham (Amersham, U.K.). $NaCNBH_3$ was from Janssen Chimica (Beerse, Belgium). Iodobeads were

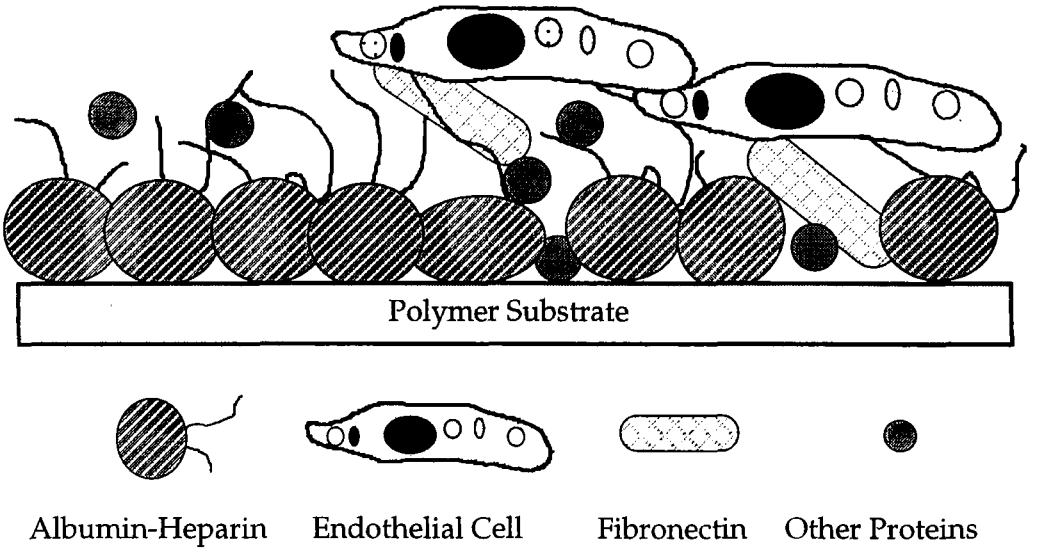

FIG. 1. Schematic representation of endothelial cells using heparin-binding adhesive proteins to adhere to albumin-heparin conjugate immobilized on a polymer substrate.

obtained from Pierce (Rockford, IL). Optiphase HiSafe-3 was from Wallac (Milton Keynes, U.K.). PD10 columns and gelatin-sepharose were from Pharmacia (Uppsala, Sweden). Phosphate-buffered saline (PBS, pH 7.4) was purchased from NPBI (Emmercompascuum, The Netherlands). Polystyrene discs (22 mm diameter) were obtained from W. S. V. Kunststoffen (Utrecht, The Netherlands). Polystyrene (PS) and tissue culture polystyrene (TCPS) petri dishes (35 mm diameter) were from Greiner GmbH (Frickenhausen, Germany). TCPS 12-well plates were obtained from Costar (Cambridge, U.K.). Argon (Ar) and carbon dioxide (CO_2) were from Hoekloos (Schiedam, The Netherlands). 1,3-Di-tert-butylcarbodiimide was purchased from Aldrich Chemie (Brussels, Belgium). Pig mucosal heparin sodium (195 U/mg) was from Bufa Chemie (Castricum, The Netherlands). Medium M199, Medium RPMI 1640, glutamax-1, penicillin, streptomycin, fungizone, trypsin-EDTA, and basic fibroblast growth factor (bFGF) were purchased from Life Technologies (Paisley, U.K.). Collagenase I was from Sigma Chemical Company (St. Louis, MO). A plasma fraction containing human fibronectin was a gift from the Central Laboratory of the Blood Transfusion Service (CLB, Amsterdam, The Netherlands). All other chemicals were of the highest purity available from Merck (Darmstadt/Hohenbrunn, Germany).

Gas Plasma Treatment of Polystyrene Discs and Petri Dishes

Polystyrene surfaces used for gas plasma treatment were rinsed ultrasonically with hexane, ethanol, and demineralized water (four times 15 min for each solvent) and subsequently dried *in vacuo*.

The plasma reactor consisted of a glass tube with an internal diameter of 6.5 cm and a length of 80 cm. The reactor was equipped with three externally placed, capacitively coupled electrodes. The hot electrode was placed in the centre of the reactor and the cold electrodes at 10 cm (Ar treatment) or 30 cm (CO_2 treatment) in distance at both sides. One side of the reactor was connected to a gas inlet system with flow control. The reactor was evacuated by a two-stage rotary vane pump. The electrodes were powered through a matching network by a 13.56-MHz radio frequency generator. The plasma treatment time was computer controlled. The plasma treatment procedure was as follows: 12 polystyrene discs or six polystyrene petri dishes were placed between the powered and grounded electrodes on a glass substrate holder. The reactor was evacuated until a pressure of 0.01 mbar, before an Ar or a CO_2 flow of 20 cm^3/min (STP) was established through the reactor. After 5 min, the plasma was started (0.10 mbar, 41 Watt). After 1 min, plasma treatment was terminated and the gas flow was maintained for 2 min. The reactor was brought to atmospheric pressure with air. The discs were turned, and the procedure was repeated. To prevent surface rearrangement processes, treated surfaces were stored at a temperature −20°C after treatment until characterization or use.

XPS Analysis

Atomic percentages of incorporated oxygen were determined by means of x-ray photoelectron spectroscopy (XPS) using a Kratos XSAM-800. For argon-treated surfaces, incorporated carboxylic acid groups were determined by means of derivatization XPS, using a derivatization reaction with 2,2,2-trifluoroethanol (TFE), according to the method of Chilkoti et al.[28] and modified by Terlingen et al.[29] In short, plasma-treated samples were placed vertically in a glass vial on glass beads. After TFE (0.45 ml), pyridine (0.2 ml), and 1,3-di-tert-butylcarbodiimide (0.15 ml) were injected between the glass beads, the vial was closed and the reaction was allowed to proceed for 24 h at room temperature. Subsequently, atomic percentages of incorporated fluor were determined with XPS.

^{14}C Labeling of Albumin-Heparin Conjugate

Albumin-heparin was labeled by reductive methylation using ^{14}C-fomaldehyde in the presence of $NaCNBH_3$.[30] To 500 mg (~7 μmol) of albumin-heparin conjugate in 10 ml of PBS, 6.1 μmol of ^{14}C-formaldehyde (55 mCi/mmol) was added. Subsequently, 60 μmol $NaCNBH_3$ was added in six aliquots with 15-min intervals. After 3 h at room temperature, the labeled conjugate was dialyzed for 48 h against 5 L of demineralized water, which was replaced every 12 h. Finally, the conjugate was lyophilized. The specific activity of ^{14}C–albumin-heparin conjugate was 1,502 ± 22 dpm/μg.

Surface Immobilization of Albumin-Heparin Conjugate

^{14}C–albumin-heparin conjugate was bound to PS-Ar and PS-CO_2 discs (9.0 cm^2) by adding a conjugate solution to the surface either directly or after preactivation of carboxylic acid groups at the surface using *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide (EDC) and *N*-hydroxysuccinimide (NHS). Preactivation of carboxylic acid groups was achieved by immersing the discs for 30 min in 5 ml of MES buffer (5 mM, pH 4.6) to which EDC (1 mM) and NHS (0.2 mM) were added. After rinsing with cold MES buffer, followed by rinsing with borate buffer (5 mM, pH 8.2), preactivated discs were placed in borate buffer containing 15 mg/ml albumin-heparin conjugate. Discs that were not preactivated were stored in MES buffer before being placed in conjugate solution. After 1 hr of incubation with albumin-heparin conjugate, the discs were rinsed using PBS (three times), a phosphate buffer (12 mM) containing 1 M NaCl with a pH of 3.5 (three times), and a similar solution with a pH of 10.5 (three times). Finally, the surfaces were rinsed twice with PBS.

^{14}C–albumin-heparin conjugate was coated or immobilized on PS, TCPS, and PS-CO_2 petri dishes (10.9 cm^2) in a similar way, except that 1 ml of MES buffer (with or without EDC/NHS) and subsequently 1 ml of albumin-heparin conjugate was pipetted into the petri dish instead of immersing the surface in solution. The reaction was carried out for 45 min.

Stability of Albumin-Heparin Conjugate–Coated Surfaces

^{14}C–albumin-heparin–coated discs were placed at 37°C in vials containing 8 ml of complete cell culture medium (CM) supplemented with 20% (v/v) pooled human serum (CMS). CM consisted of Medium M199 (50% v/v), Medium RPMI 1640 (50% v/v), glutamax-1 (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and fungizone (2.5 μg/ml). At selected times, the supernatant was replaced by fresh CMS. After addition of 13 ml of HiSafe-3, radioactivity in the supernatant was measured. At day 17, the radioactivity of the surfaces was determined as well. Therefore, petri dishes or discs were incubated twice with 2 ml of 1 M NaOH for 24 h. Subsequently, the surfaces were broken into pieces, and radioactivity in the NaOH solutions and at the surface were determined after the addition of 18 ml of HiSafe-3.

The stability of albumin-heparin–coated surfaces in SDS was determined by measuring radioactivity in the supernatant and on the surface after vigorous shaking of the discs in a 3% SDS solution at 37°C for 48 h.

Surface concentrations were calculated by dividing the surface-bound radioactivity by the specific activity of the conjugate and the surface area.

^{125}I Labeling of Fibronectin

Human plasma fibronectin, purified on a gelatin-sepharose column, was labeled with ^{125}I employing Iodobeads according to the method of Markwell.[31] A total of 500 μCi Na^{125}I (15.5 mCi/μg) was added to three Iodobeads that were rinsed with PBS. After 5 min of incubation at room temperature, 5 mg of fibronectin in PBS was added. After 12 min, free iodine was removed from the reaction mixture, using a series of 3 PD10 columns, with PBS as eluent. The specific activity of ^{125}I-fibronectin was 30.5 $\pm$ 0.4 dpm/ng, with $<$1% unbound ^{125}I (PD10-column).

Surface Concentration and Stability of Albumin-Heparin and Fibronectin Coatings in Petri Dishes

Using ^{125}I-fibronectin, the quantity of fibronectin coated on selected materials used for cell proliferation studies as well as the stability of these coatings in CMS were determined. For coating with fibronectin, 1 ml of fibronectin solution (0.025 mg/ml in PBS) was added to petri dishes, and after 30 min, each dish was rinsed twice with PBS. Surfaces onto which albumin-heparin was immobilized were also coated with fibronectin (0.05 mg/ml), and a mixture of conjugate (15 mg/ml) and fibronectin (0.05 mg/ml) was immobilized as well.

^{125}I-radioactivity bound at the surface and surface concentrations were determined as described for ^{14}C–albumin-heparin, except for the addition of HiSafe-3.

Isolation of Endothelial Cells

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical veins according to the method of Jaffe et al.[32] and modified by Van Wachem et al.[33] In short, the umbilical vein was rinsed with PBS and incubated for 20 min at 37°C in 0.05% trypsin–0.02% EDTA solution in PBS. CMS was added to inactivate trypsin, and after exchange of the medium by fresh medium, cells were plated in fibronectin-coated TCPS flasks and grown until the third passage. Before seeding, cells were detached from the TCPS surface by incubation with 0.05% trypsin–0.02% EDTA solution in PBS.

Proliferation of Endothelial Cells on Various Substrates

Proliferation of HUVECs was investigated using petri dishes of PS, TCPS, and PS-CO_2, either with or without coating with fibronectin and albumin-heparin conjugate. Albumin-heparin– and/or fibronectin-coated surfaces were prepared as described above.

Before cell seeding, surfaces were placed overnight at room temperature in PBS containing penicillin (100 U/ml) and streptomycin (100 μg/ml).

Third passage HUVECs were seeded at a density of 10,000 cells/cm^2 and cultured at 37°C in 5% CO_2 saturated with water vapor. Culture medium (0.20 ml/cm^2) containing 20% (v/v) human serum, 5 U/ml heparin, and 0.29 ng/ml bFGF was replaced every other day. At selected times, cells were trypsinized, and cell numbers were determined using a Bürker counting device.

RESULTS

Gas Plasma Treatment of Polystyrene Surfaces

The results of the XPS analyses of Ar- and CO_2-treated surfaces (Table 1) demonstrate that during plasma treatment the oxygen content of all surfaces increased. The oxygen content of CO_2-treated surfaces was significantly higher than of Ar-treated surfaces. On Ar-treated surfaces, some nitrogen was incorporated, whereas nitrogen was hardly detectable on CO_2-treated surfaces. Some oxygen was found on untreated polystyrene. Following derivatization with 2,2,2-trifluoroethanol (TFE), argon-treated surfaces contained fluorine, which was not present before this reaction. From this quantity of fluorine, the amount of oxygen atoms being present in carboxylic acid groups was calculated to be 6.4% of the total amount of oxygen present.

TABLE 1. ELEMENTAL ANALYSIS OF UNMODIFIED AND GAS PLASMA–TREATED POLYSTYRENE SURFACES AND OF TISSUE CULTURE POLYSTYRENE

	n	*C* (atomic %)	*O* (atomic %)	*N* (atomic %)	*F* (atomic %)
PS	6	99.6 ± 0.2	0.4 ± 0.2	0.0 ± 0.0	
PS TFE	2	98.4 ± 1.0	0.0 ± 0.0	1.0 ± 0.2	0.0 ± 0.0
PS-Ar	4	87.3 ± 0.9	11.6 ± 0.7	1.0 ± 0.2	
PS-Ar TFE	4	85.8 ± 1.0	11.5 ± 0.5	1.0 ± 0.2	1.1 ± 0.3
PS-CO_2 (disc)	2	79.2 ± 2.0	20.7 ± 2.0	0.1 ± 0.0	
PS-CO_2 (petri dish)	4	79.0 ± 2.0	20.9 ± 1.9	0.1 ± 0.1	
TCPS	2	86.3 ± 0.1	13.7 ± 0.1	0.0 ± 0.0	

TCPS, tissue culture polystyrene; *n*, number of surface samples measured; TFE, surfaces modified with 2, 2, 2-trifluoroethanol to determine the percentage of O-atoms present in carboxylic acid groups.

Immobilization of ^{14}C–Albumin-Heparin Conjugate onto Gas Plasma–Treated Polystyrene Surfaces: Surface Concentration and Stability

^{14}C–albumin-heparin conjugate was immobilized onto native, argon-treated, and CO_2 gas plasma–treated polystyrene surfaces, with or without preactivation of carboxylic acid groups on the surface. Preactivation was performed using *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide (EDC) in the presence of *N*-hydroxysuccinimide (NHS).

Surface concentration of albumin-heparin conjugate to native polystyrene was 0.27 $\mu g/cm^2$; on PS-Ar, the surface concentration was 0.10 $\mu g/cm^2$ (Fig. 2). More albumin-heparin conjugate was immobilized on PS-CO_2 than on PS-Ar and native polystyrene (0.41 $\mu g/cm^2$). Preactivation of carboxylic acid groups increased the surface concentration of albumin-heparin on PS-Ar, whereas on PS-CO_2 preactivation decreased the quantity of immobilized albumin-heparin.

Most of the albumin-heparin bound to PS was released upon incubation in SDS. In contrast, the percentage of conjugate released from gas plasma–modified surfaces after exposure to SDS was much smaller.

When the release of albumin-heparin conjugate was determined upon incubation in cell culture medium (CMS), a small amount of conjugate was released from the gas plasma–treated surfaces within 1 day, after which the surfaces were stable (Fig. 3). In contrast, almost 50% of the initially bound conjugate was released from native PS during the first 24 h, after which the remainder of the coating was stable during >2 weeks.

The conjugate coating was slightly more stable on PS-Ar preactivated with EDC and NHS than on PS-CO_2. On the latter surface, the concentration of conjugate was much higher. Based on these results, further investigations were carried out using PS-CO_2, without preactivation with EDC/NHS.

Surface Concentration and Stability of Fibronectin Coatings

The quantity of fibronectin immobilized onto PS-CO_2 was comparable to the quantity on TCPS (Fig. 4A). On albumin-heparin–coated PS-CO_2, a relatively large quantity of fibronectin was bound in a second step, displacing only a small amount of albumin-heparin from the surface. The surface concentration of albumin-heparin conjugate immobilized on PS-CO_2 petri dishes was higher than on PS-CO_2 discs (cf. Figs. 4B and 2).

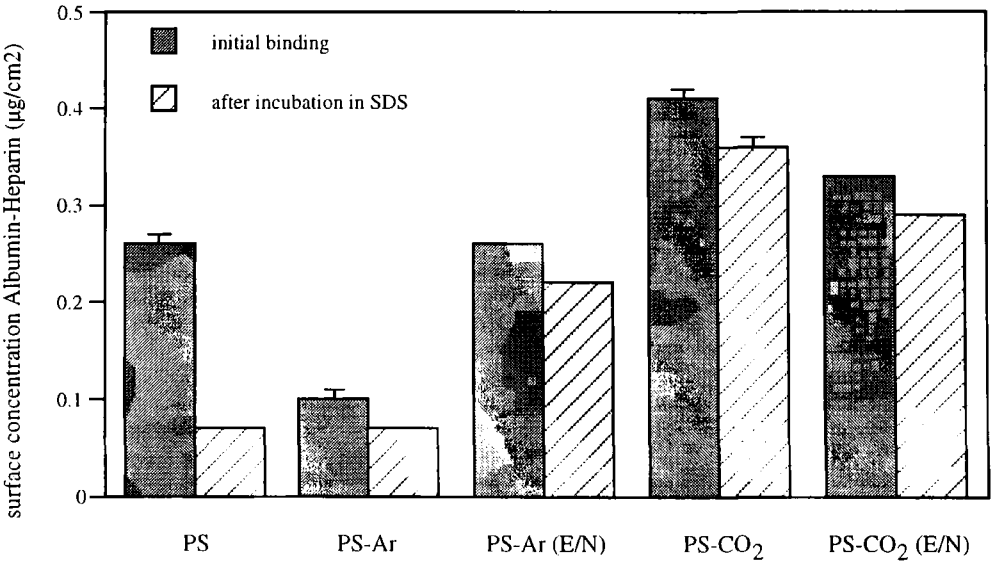

FIG. 2. Surface concentration of ^{14}C–albumin-heparin conjugate immobilized on polystyrene (PS), argon plasma-treated polystyrene (PS-Ar) and CO_2 plasma–treated polystyrene (PS-CO_2) with or without preactivation of surface carboxylic acid groups by EDC and NHS (E/N). Bars represent surface concentrations before and after exposure to 3% SDS solution for 48 h ($n = 3$, ± SD).

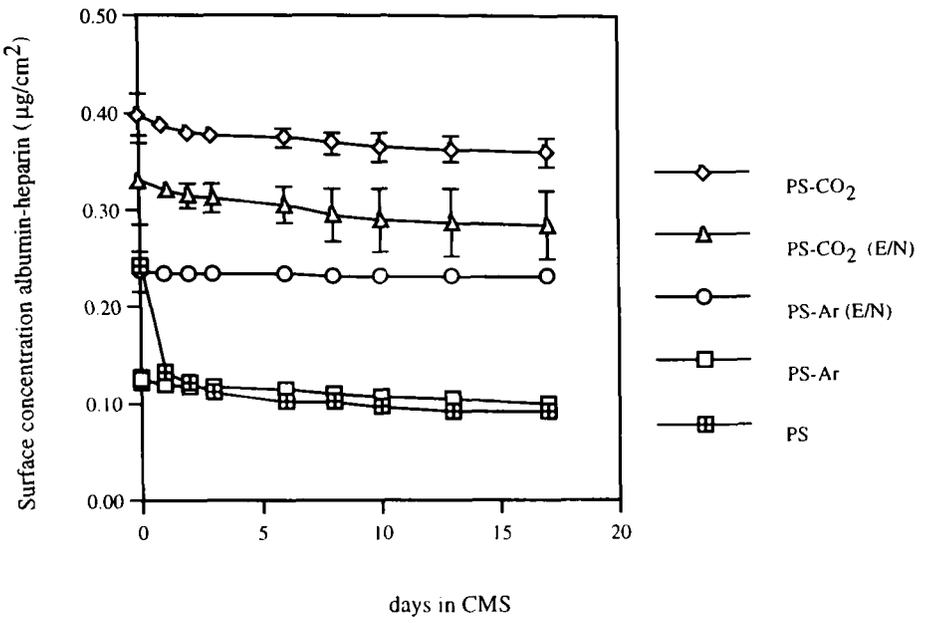

FIG. 3. Surface concentration of ^{14}C–albumin-heparin conjugate immobilized on polystyrene (PS), argon plasma–treated polystyrene (PS-Ar), and CO_2 plasma–treated polystyrene (PS-CO_2) discs with or without preactivation of surface carboxylic acid groups by EDC and NHS (E/N) as a function of storage time in cell culture medium (CMS) at 37°C (n = 3, ± SD).

Part of the protein coatings was released during overnight incubation in a solution of penicillin and streptomycin in PBS. This effect was observed for TCPS as well as PS-CO_2, and for both albumin-heparin and fibronectin. During overnight incubation in cell culture medium, a small percentage of bound fibronectin and albumin-heparin conjugate was released from PS-CO_2 surfaces (Fig. 4). Fibronectin was released from TCPS to the same extent as from PS-CO_2. When bound after immobilization of albumin-heparin conjugate, however, fibronectin was almost completely released in CMS.

Adherence and Proliferation of Endothelial Cells on Albumin-Heparin Surfaces

HUVECs adhered and proliferated on both native PS-CO_2 and on TCPS, but hardly on native PS (Fig. 5A). On PS-CO_2 and TCPS coated with albumin-heparin conjugate, without (Fig. 5B) or with fibronectin coated in a separate incubation (Fig. 5D), cells adhered and proliferated as well. On PS coated with albumin-heparin, cell proliferation was only transient even when fibronectin was added.

During the second week of culturing, cells detached from albumin-heparin coatings and uncovered spots were observed. However, total cell numbers remained constant or increased slightly. After coating of surfaces with fibronectin (Fig. 5C), the differences in cell numbers between the substrates during proliferation disappeared.

On all surfaces except native and albumin-heparin–coated PS, cells had formed a confluent monolayer at day 7. Figure 6 illustrates the morphology of cells cultured on selected surfaces. On all fibronectin-coated surfaces and on native PS-CO_2 and TCPS, HUVECs showed the cobblestone structure, typical for endothelial cells in culture. On albumin-heparin–coated surfaces, the structure and size of endothelial cells were more irregular.

DISCUSSION

Because, in humans, the luminal surface of vascular prostheses is not spontaneously overgrown by endothelium, seeding of endothelial cells has been proposed to obtain endothelialization of vascular grafts, thus preventing thrombotic complications. A prerequisite for successful cell seeding is the optimal adhe-

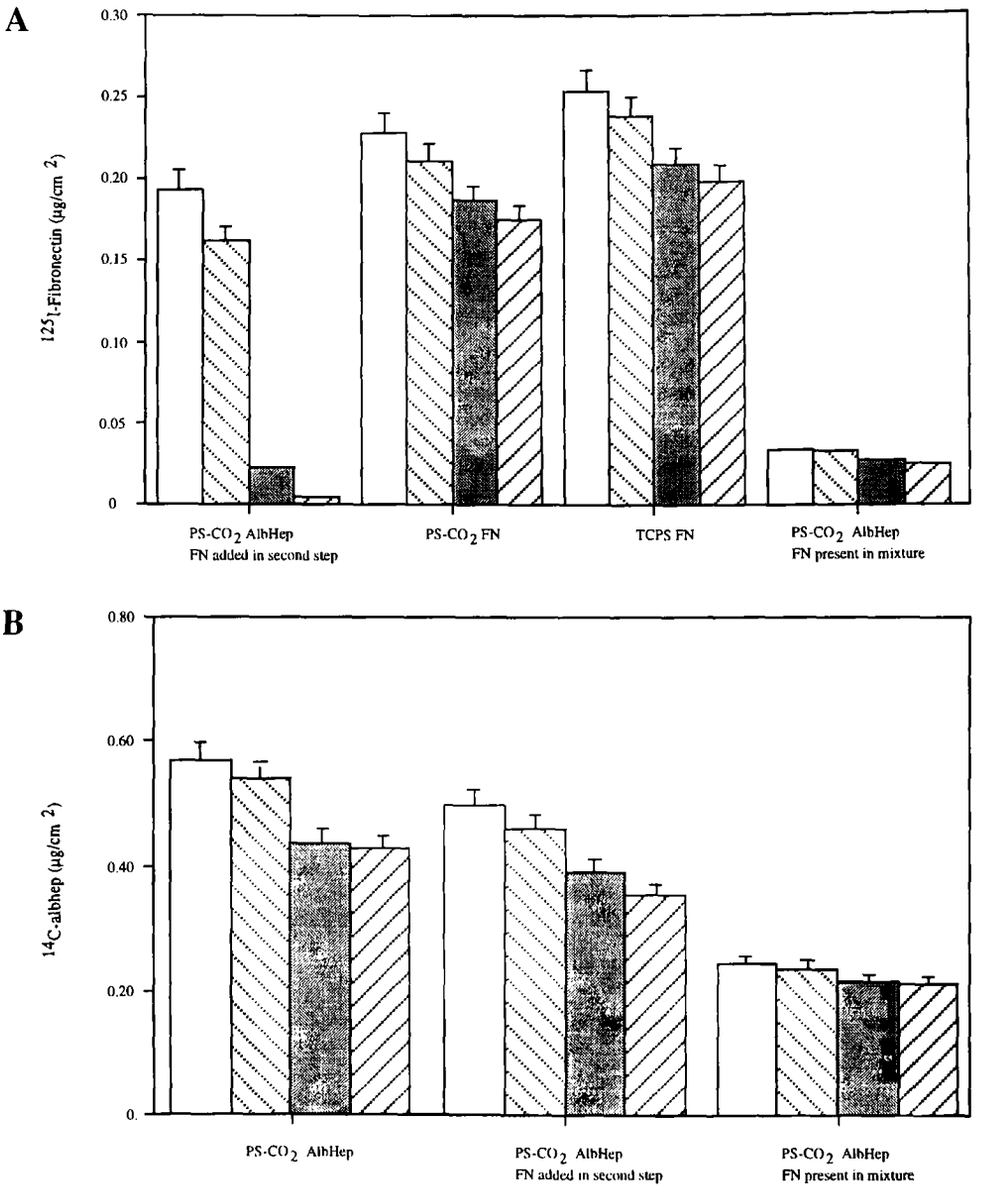

FIG. 4. Surface concentration of fibronectin (**A**) and albumin-heparin conjugate (**B**) on a series of surfaces after coating and rinsing □, after overnight incubation in penicillin-streptomycin solution ▨, and after 1 □ and 4 days ▨ incubation in CMS at 37°C. PS-CO_2 AlbHep/FN added in the second step represents an albumin-heparin coating onto which additional fibronectin was bound in a second step. In contrast, PS-CO_2 AlbHep/FN present in mixture represents a coating of a mixture of albumin-heparin and fibronectin (n = 3, ±SD).

sion of endothelial cells to the graft surface. In addition, the graft surface should have antithrombogenic properties because it is not expected that the layer of seeded endothelial cells is confluent immediately after seeding. The present study was undertaken to investigate whether or not albumin-heparin conjugate, which displays anticoagulant activity, could function as a substrate for endothelial cells.

To prevent displacement of albumin-heparin from the surface by cells and proteins, gas plasma treatment was used to generate functional groups at the surface, which subsequently were used to covalently bind the conjugate. Argon plasma treatment of polystyrene resulted in the introduction of oxygen-containing groups at the surface. In the analyzed top layer of 100 Å of PS-Ar, ~6.5% of the incorporated oxygen was determined as carboxylic acid groups, which agrees with data reported by Van Delden et al.,[34] who studied covalent binding of albumin-heparin to argon-treated polystyrene. Using EDC and NHS, sufficient carboxylic acid groups at the surface can be activated to covalently bind ±0.22 μg/cm^2 of albumin-heparin conjugate, which is in the order of a theoretical side-on monolayer. As not all conjugate immobilized to PS-Ar with-

out EDC and NHS was desorbed from the surface in SDS solution, it is possible that some conjugate was immobilized via aldehyde and epoxide groups, which were introduced at the surface by argon treatment, or that SDS desorption did not remove all physically adsorbed conjugate.

CO_2-plasma treatment of polystyrene introduced more oxygen-containing groups at the surface than treatment with argon, which agrees with the results of Takens,[35] who introduced 22% oxygen on solution-casted polystyrene films in a similar way. Takens[35] showed that CO_2 plasma introduces hydroxyl, aldehyde, carboxylic acid, and epoxide groups at the surface of polystyrene.

In the present study, more albumin-heparin conjugate was immobilized on CO_2-treated polystyrene surfaces than on argon-treated polystyrene, which might be the result of differences in chemical composition of the surface or of the higher oxygen content of PS-CO_2. Surprisingly, without preactivation of surface carboxylic acid groups by EDC and NHS, more albumin-heparin was immobilized on PS-CO_2 than with preactivation. It is probable that covalent immobilization of the conjugate on CO_2-treated polystyrene is established by reactive epoxide groups; binding of conjugate NH_2-groups to surface aldehyde groups might also play a role. Incubation with EDC and NHS deactivates some of these groups—for instance, through hydrolysis.

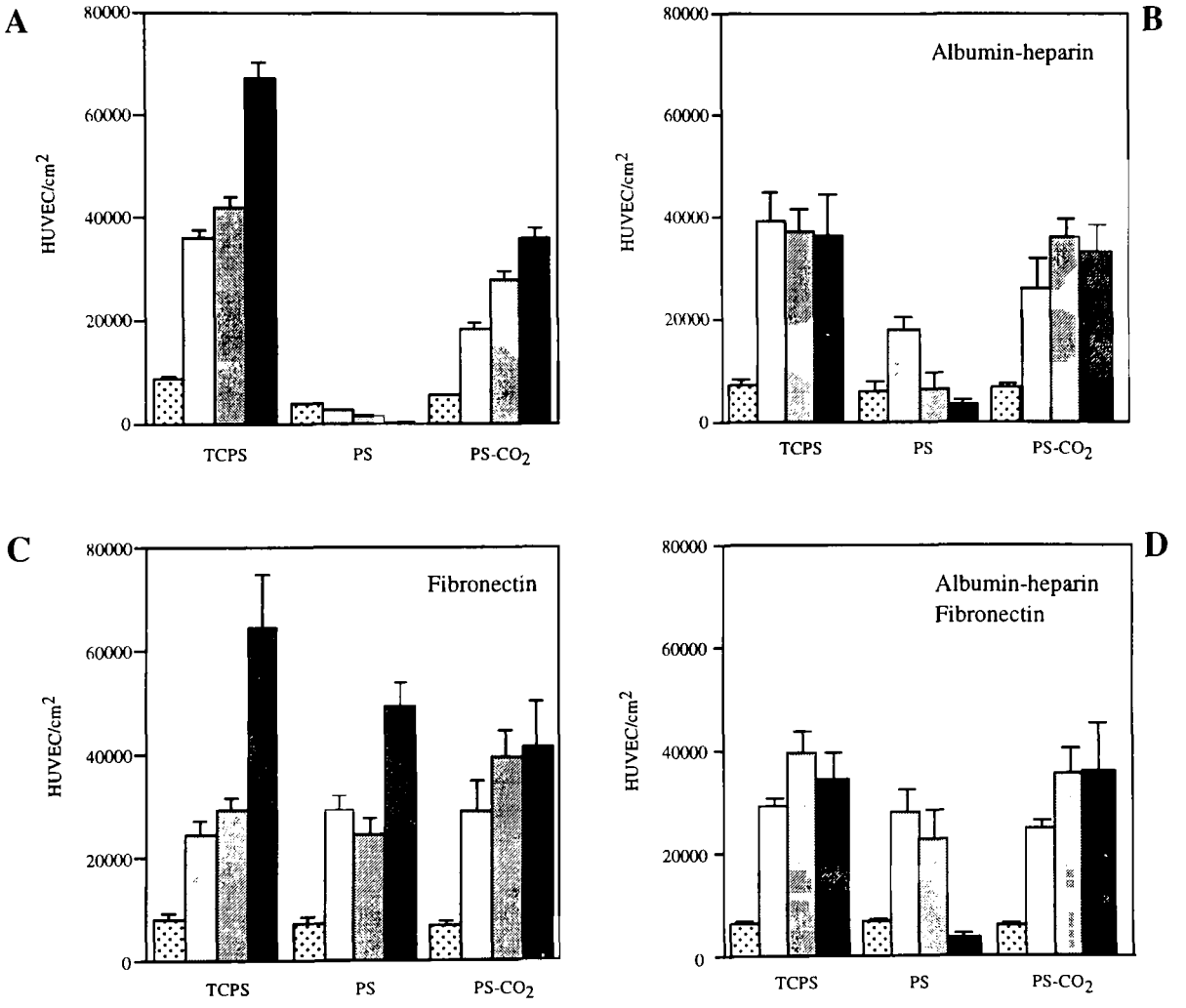

FIG. 5. Number of endothelial cells on TCPS, PS and PSCO_2, either without (**A**) or with immobilized albumin-heparin (**B**), fibronectin (**C**), or albumin-heparin with fibronectin added during a second incubation (**D**), at different proliferation times: ▨ day 1, □ day 4, ■ day 7, and ■ day 13. Third passage HUVECs, seeded in a density of 10,000/cm^2, were cultured in cell culture medium containing 20% (v/v) human serum, 5 U/ml heparin, and 0.29 ng/ml bFGF (n = 3, ±SD).

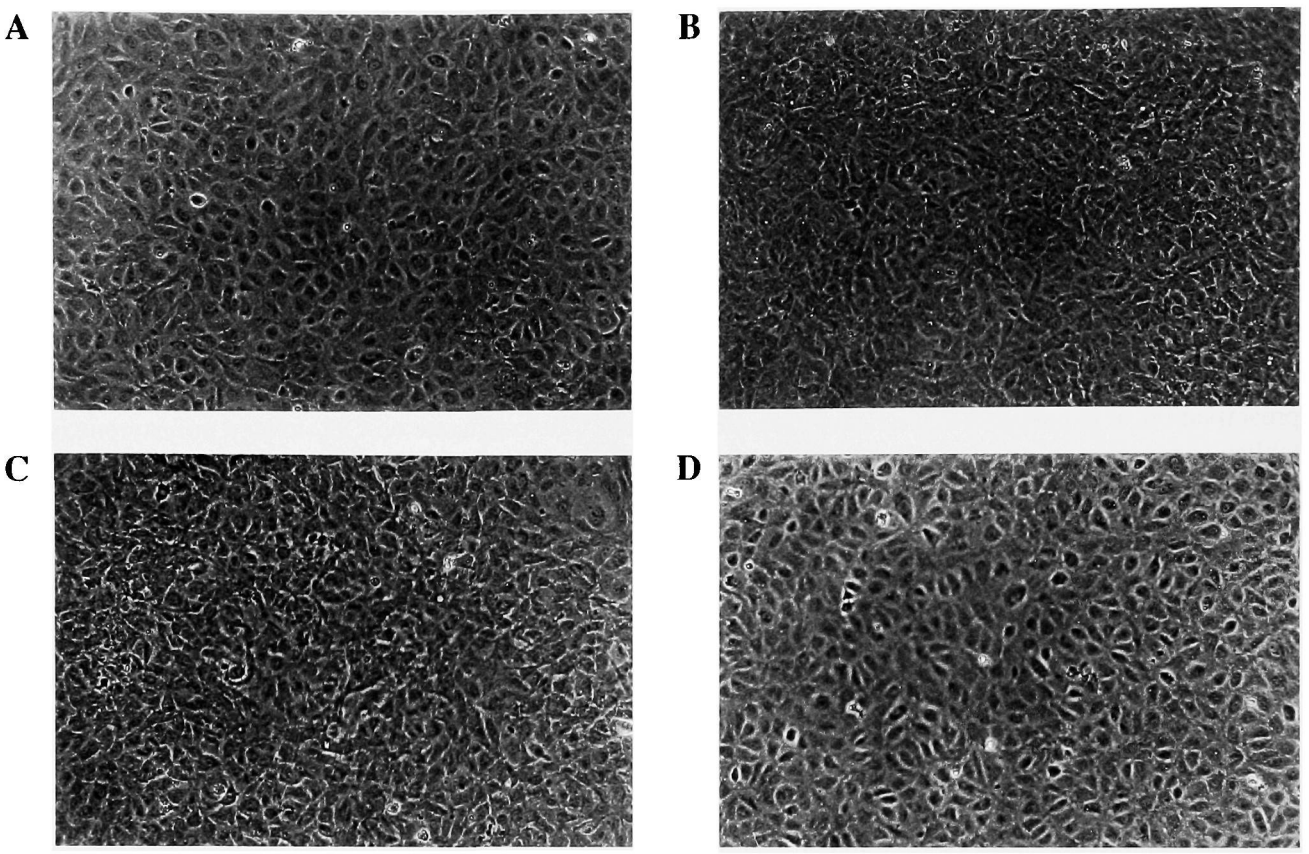

FIG. 6. Light-microscopic images of HUVECs grown for 7 days on TCPS-FN (**A**), on PS-CO_2 (**B**), and on albumin-heparin immobilized on PS-CO_2 without (**C**) or with (**D**) fibronectin coating after immobilization of albumin-heparin. Third passage HUVECs, seeded in a density of 10,000/cm^2, were cultured in cell culture medium containing 20% (v/v) human serum, 5 U/ml heparin, and 0.29 ng/ml bFGF (original magnification, ×128).

Besides the stability of the conjugate coatings in SDS solution, release of immobilized conjugate in cell culture medium (CMS) was also determined. Initially, some albumin-heparin was released from the surface, after which the residual immobilized conjugate was stable. The quantity of conjugate still present on the surfaces after contact with CMS was within the experimental error equal to the quantity present on the surface after SDS desorption.

During cell culture also, part of the conjugate may be displaced by other proteins, which may influence adherence of HUVECs to the substrate. Several authors have postulated that, in order to be able to adhere, endothelial cells must deposit fibronectin, which displaces proteins previously adsorbed to the surface from the serum-containing medium.[36–39]

Our studies demonstrate that endothelial cells were able to adhere to and proliferate on albumin-heparin conjugate covalently immobilized onto CO_2 plasma–modified polystyrene. Cells also proliferated on uncoated PS-CO_2, indicating that sufficient adhesive proteins adsorbed from the serum-containing medium during adhesion or that fibronectin released by the cells displaced proteins already present at the surface.

When a solution of fibronectin was brought into contact with albumin-heparin–coated PS-CO_2, a relatively large amount of fibronectin was immobilized, but only a small amount of albumin-heparin was displaced. It is postulated that most of the fibronectin binds to the conjugate via the heparin moiety, which is supported by the observation that, when fibronectin was added to the surface together with albumin-heparin conjugate, significantly less fibronectin and conjugate bound to the surface. During overnight incubation in penicillin/streptomycin solution, part of the fibronectin and albumin-heparin was released from the surfaces. Upon incubation in CMS, fibronectin was removed from a layer of albumin-heparin conjugate through exchange with other proteins, in contrast to the conjugate itself, which was not released from the surface.

Differences in cell morphology rather than in cell numbers were observed. Cells were more spread on all substrates that had been coated with fibronectin than on albumin-heparin surfaces. Moreover, adding fibronectin to the albumin-heparin surfaces clearly improved adherence and spreading of endothelial cells to these surfaces.

The immobilization of albumin-heparin has no beneficial effect in terms of cell adherence and proliferation when compared to fibronectin-coated PS-CO_2. However, besides the inhibiting effect on coagulation and complement activation, immobilized albumin-heparin has been shown to decrease the *in vitro* deposition, aggregation, and activation of platelets.[26,27] In contrast, coating of surfaces with fibronectin enhances adhesion of platelets to the surface.[40–42] Therefore, the quantity of fibronectin added to albumin-heparin surfaces needs to be optimized with respect to endothelial cell adherence and platelet interaction.

Because polystyrene cannot be used in vascular graft surgery, the present model system needs to be evaluated using Dacron or Teflon instead of the polystyrene surface. Unpublished results of Klomp et al.[43] demonstrate that proteins in an aqueous solution with pH 8.2 were immobilized on CO_2 gas plasma–modified polyethylene terephthalate, the base material of Dacron. This layer of proteins immobilized on the Dacron fibers could form the basis of a stable, cross-linked albumin-heparin matrix that fills the pores of the Dacron graft. It is therefore anticipated that albumin-heparin might be useful as a vascular graft coating to prevent thrombus formation and to promote endothelialization of the graft.

CONCLUSIONS

HUVECs are able to adhere to and subsequently proliferate on CO_2 plasma–modified polystyrene. Confluent monolayers were formed, although cell numbers were lower than on TCPS. Immobilization of albumin-heparin conjugate onto CO_2-treated polystyrene resulted in increased cell numbers, as was anticipated. On these surfaces, the number of cells found was determined to be the same as the number on fibronectin-coated PS-CO_2. However, because cell adherence is not optimal on albumin-heparin-coated PS-CO_2, addition of a small amount of an adhesive protein like fibronectin probably is essential if cells are to adhere to the surface under flow conditions. Endothelial cell adhesion versus the blood compatibility of such a coating needs to be evaluated.

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REFERENCES

1. Schmidt, S.P., Sharp, W.V., Evancho, M.M., and Meerbaum, S.O. Endothelial cells seeding of prosthetic vascular grafts—current status. In: Szycher, M., ed. High-Performance Biomaterials. Lancaster, PA: Technomic, 1991, p. 483.
2. Szilagyi, D.E., Elliot, J.P., Smith, R.F., Reddy, D.J., and McPharlin, M. A thirty-year survey of the reconstructive surgical treatment of aortoiliac occlusive disease. J. Vasc. Surg. **3,** 421, 1986.
3. Antiplatelet Trialist's Collaboration. Collaborative overview of randomised trials of antiplatelet therapy. II. Maintenance of vascular graft or arterial patency by antiplatelet therapy. B.M.J. **308,** 159, 1994.
4. Darling, R.C., and Linton, R.R. Durability of femoropopliteal reconstruction. Am. J. Surg. **123,** 472, 1972.
5. Bergan, J.J., Veith, F.J., Bernhard, V.M., Yao, J.S.T., Flinn, W.R., Gupta, S.K., Scher, L.A., Samson, R.H., and Towne, J.B. Randomization of autogenous vein and polytetrafluoroethylene grafts in femorodistal reconstructions. Surgery **92,** 921, 1982.
6. Yeager, A., and Callow, A.D. New graft materials and current approaches to an acceptable small-diameter vascular graft. Trans. Am. Soc. Artif. Intern. Organs **34,** 88, 1988.
7. Lyman, D.J., Albo, D., Jr., Jackson, R., and Knutson, K. Development of small-diameter vascular prostheses. Trans. Am. Soc. Artif. Intern. Org. **23,** 253, 1973.

8. Hess, F., Jerusalem, C., and Braun, B. A fibrous polyurethane microvascular prosthesis, morphological evaluation of the neo-intima. J. Cardiovasc. Surg. **24,** 509, 1983.
9. Murabayashi, S., Kambic, H., Harasaki, H., Morimoto, T., Yozu, R., and Nose, Y. Fabrication and long-term implantation of semi-compliant small vascular prostheses. Trans. Am. Soc. Artif. Intern. Org. **31,** 50, 1985.
10. Uchida, N., Kambic, H., Emoto, H., Chen, J-F., Hsu, S., Murabachi, S., Harasaki, H., and Nose, Y. Compliance effects on small-diameter polyurethane graft patency. J. Biomed. Mater. Res. **27,** 1269, 1993.
11. Kodama, M. Small-diameter cardiovascular graft. Proc. Jpn. Congr. Mater. Res. **33,** 153, 1990.
12. Sauvage, L.R., Berger, K., Beilin, L.B., Smith, J.C., Wood, S.J., and Mansfield, P.B. Presence of endothelium in an axillary-femoral graft of knitted Dacron with an external velour surface. Ann. Surg. **182,** 749, 1975.
13. Wu, M.H.D., Shi, Q., Wechezak, A.R., Clowes, A.W., Gordon, I.L., and Sauvage, L.R. Definitive proof of endothelialization of a Dacron arterial prosthesis in a human being. J. Vasc. Surg. **21,** 862, 1995.
14. Davies, M.G., and Hagen, P.O. The vascular endothelium—a new horizon. Ann Surg. **218,** 593, 1993.
15. Wu, K.K., Frasier-Scott, K., and Hadzakis, H. Endothelial cell function in hemostasis and thrombosis. Adv. Exp. Med. Biol. **242,** 127, 1988.
16. Herring, M., Gardner, A., and Glover, J. A single-staged technique for seeding vascular grafts with autologous endothelium. Surgery **84,** 498, 1978.
17. Herring, M., Gardner, A., and Glover, J. Seeding human arterial prostheses with mechanically derived endothelium. The detrimental effect of smoking. J. Vasc. Surg. **1,** 279, 1984.
18. Herring, M., Baughman, S., and Glover J. Endothelium develops on seeded human arterial prosthesis. J. Vasc. Surg. **2,** 727, 1985.
19. Herring, M.B., Compton, R., Legrand, D.R., and Gardner, A.L. Endothelial cell seeding in the management of vascular thrombosis. Semin. Thromb. Hemost. **15,** 200, 1989.
20. Graham, L.M., Brothers, T.E., Vincent, C.K., Burkel, W.E., and Stanley, J.C. The role of an endothelial cell lining in limiting distal anastomotic intimal hyperplasia of 4-mm-I.D. Dacron grafts in canine model. J. Biomed. Mater. Res. **25,** 525, 1991.
21. Belden, T.A., Schmidt, S.P., Falkow, L.J., and Sharp, W.V. Endothelial cell seeding of small-diameter vascular grafts. Trans. Am. Soc. Artif. Intern. Org. **28,** 173, 1982.
22. Örtenwall, P., Wadenvik, H., Kutti, J., and Risberg, B. Endothelial cell seeding reduces thrombogenicity of Dacron grafts in humans. J. Vasc. Surg. **11,** 403, 1990.
23. James, N.L., Schindhelm, K., Slowiaczek, P., Milthorpe, B.K., Dudman, N.P.B., Johnson, G., and Steele, J.G. Endothelial cell seeding of small-diameter vascular grafts. Artif. Organs **14,** 355, 1990.
24. Zilla, P., Deutsch, M., Meinhart, J., Puschmann, R., Eberl, T., Minar, E., Dudczak, R., Lugmaier, H., Schmid, P., Noszian, I., and Fischlein, T. Clinical *in vitro* endothelialization of femoropopliteal bypass grafts: an actuarial follow-up over three years. J. Vasc. Surg. **19,** 540, 1994.
25. Meinhart, J., Deutsch, M., and Zilla, P. Eight years of clinical endothelial cell transplantation—closing the gap between prosthetic grafts and vein grafts. Am. Soc. Artif. Intern. Organs **43,** M515, 1997.
26. Engbers, G.H.M., Dost, L., Hennink, W.E., Aarts, P.A.M.M., Sixma, J.J., and Feijen, J. An *in vitro* study of the adhesion of blood platelets onto vascular catheters. Part 1. J. Biomed. Mater. Res. **21,** 613, 1987.
27. Engbers G.H.M. Blood compatibility of albumin-heparin conjugate coated poly(tetrafluorethylene). In: The Development of Heparinized Materials With an Improved Blood Compatibility [Thesis]. Enschede, The Netherlands: University of Twente, 1990, p. 131.
28. Chilkoti, A., Ratner, B.D., and Briggs, D. Plasma-deposited polymeric films from carbonyl-containing volatile precursor: XPS chemical derivatization and static SIMS surface characterization. Chem. Mater. **3,** 51, 1991.
29. Terlingen, J.G.A., Hofman, A.S., and Feijen, J. Effect of glow discharge treatment of poly(acrylic acid) preadsorbed onto polyethylene. J. Appl. Polymer Sci. **50,** 1529, 1993.
30. Dottavio-Martin, D., and Ravel, J.M. Radiolabeling of proteins by reductive alkylation with ^{14}C-formaldehyde and sodium cyanoborohydride. Anal. Biochem. **87,** 562, 1978.
31. Markwell, M.A.K. A new solid-state reagent to iodinate proteins: Conditions for the efficient labeling of antiserum. Anal. Biochem. **125,** 427, 1982.
32. Jaffe, E.A., Nachman, R.L., Becker, C.G., and Miniick, C.R. Culture of human endothelial cells derived from umbilical cord veins. Identification by morphologic and immunologic criteria. J. Clin. Invest. **52,** 2745, 1973.
33. Van Wachem, P.B., Reinders, J.H., van Buul-Wortelboer, M.F., De Groot, P.G., and Van Aken, W.G. Von Willebrand factor in cultured human vascular endothelial cells from adult and umbilical cord arteries and veins. Thromb. Haemost. **56,** 189, 1986.
34. Van Delden, C.J., Lens, J.P., Kooyman, R.P.H., Engbers, G.H.M., and Feijen, J. Heparinization of gas plasma–modified polystyrene surfaces and the interaction of these surfaces with proteins studied with surface plasmon resonance. Biomaterials **18,** 845, 1997.

35. Takens, G.A.J. Carbon dioxide plasma treatment of polystyrene; analysis of the chemical composition and stability of the surfaces. In: Functionalization of Polymeric Surfaces by Oxidative Gas Plasma Treatment [Thesis]. Enschede, The Netherlands: University of Twente, 1997, p. 187.
36. Van Wachem, P.B., Beugeling, T., Feijen, J., Bantjes, A., Detmers, J.P., and Van Aken W.G. Adsorption of fibronectin derived from serum and from human endothelial cells on tissue culture polystyrene. J. Biomed. Mater. Res. **21,** 1317, 1987.
37. Van Wachem, P.B., Beugeling, T., Mallens, B.W.L., Dekker, A., Feijen, J., Bantjes, A., and Van Aken, W.G. Deposition of endothelial fibronectin on polymer surfaces. Biomaterials **9,** 121, 1988.
38. Grinnell, F., and Feld, M.K. Fibronectin adsorption on hydrophilic and hydrophobic surfaces detected by antibody binding and analyzed during cell adhesion in serum-containing medium. J. Biol. Chem. **257,** 4888, 1982.
39. Dekker, A., Beugeling, T., Wind, H., Poot, A., Bantjes, A., Feijen, J., and Van Aken W.G. Deposition of cellular fibronectin and desorption of human serum albumin during adhesion and spreading of human endothelial cells on polymers. J. Mater. Sci. Mater. Med. **2,** 227, 1991.
40. Poot, A.A., Van Aken, W.G., Dekker, A., Beugeling, T., Feijen, J., Bantjes, A., and Van Mourik, J.A. Cell seeding for bioartificial blood vessels. Hybrid Artif. Organs **177,** 285, 1989.
41. Seeger, J.M., and Klingman, N. Improved endothelial cell seeding with cultured cells and fibronectin-coated grafts. J. Surg. Res. **38,** 641, 1985.
42. Allan, B.T., Long, J.A., Clark, R.E., Sicard, G.A., Hopkins, K.T., and Welch, M.J. Influence of endothelial cell seeding on platelet deposition and patency in small-diameter dacron arterial grafts. J. Vasc. Surg. **1,** 224, 1984.
43. Klomp, A.J.A. University of Twente, Section of Polymer Chemistry and Biomaterials, unpublished observations.

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2. Bernard P. Chan, Wenge Liu, Bruce Klitzman, William M. Reichert, George A. Truskey. 2005. In vivo performance of dual ligand augmented endothelialized expanded polytetrafluoroethylene vascular grafts. *Journal of Biomedical Materials Research* **72B**:1, 52-63. [CrossRef]
3. Brian S. Conklin, Huakang Wu, Peter H. Lin, Alan B. Lumsden, Changyi Chen. 2004. Basic Fibroblast Growth Factor Coating and Endothelial Cell Seeding of a Decellularized Heparin-coated Vascular Graft. *Artificial Organs* **28**:7, 668-675. [CrossRef]
4. Alexander M. Seifalian, Alok Tiwari, George Hamilton, Henryk J. Salacinski. 2002. Improving the Clinical Patency of Prosthetic Vascular and Coronary Bypass Grafts: The Role of Seeding and Tissue Engineering. *Artificial Organs* **26**:4, 307-320. [CrossRef]
5. Gert W. Bos, Nicole M. Scharenborg, Andr A. Poot, Gerard H. M. Engbers, Tom Beugeling, Willem G. van Aken, Jan Feijen. 2000. Blood compatibility of surfaces with immobilized albumin-heparin conjugate and effect of endothelial cell seeding on platelet adhesion. *Journal of Biomedical Materials Research* **47**:3, 279-291. [CrossRef]