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Endothelial cell compatibility testing of various prosthetic surfaces

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Numerous methods are proposed to reduce the surface thrombogenicity of vascular prostheses, among them endothelialization of the lumen which has had clinical application since 1985. One of the problems is to collect enough cells to rapidly obtain a complete monolayer at the time of implantation. Thus, material improvements are necessary to enhance cell adhesion and spreading. A collaboration with the Bakoulev Institute (Moscow) gave us the opportunity to study the cytocompatibility of carbon coated materials (PAN and Vitlan®), polyester coated with albumin and/or synthetic polysaccharide. Studies were carried out with cultured human umbilical vein endothelial cells (HUVEC). Two steps are distinguished: indirect tests (medium added with materials extracts), then direct tests (cells directly seeded onto materials). Neither PAN, Vitlan® nor polyesters extracts have provoked a toxic effect on HUVEC. Concerning attachment on materials, a maximum of 60% of seeded cells is reached. Cells could proliferate and confluency is obtained between days 5 and 10 for the best materials. SEM corroborated these results. On polysaccharide-coated polyester (M. 11), HUVEC produced significant levels of vWF after thrombin stimulation (ELISA assay): vWF was functional (ristocetin cofactor activity). In conclusion, PAN and M. 11 gave encouraging results and further studies remain to be investigated.

1. Introduction

Human vascular endothelial cells have been obtained in culture for 20 years since the original publication of Jaffe [1]. The use of cultured endothelial cells provides a useful model to study fundamental mechanisms of cellular function and alterations of endothelial integrity which are involved in disorders such as inflammation, thrombosis and atherosclerosis. In addition, this cell system can be used in the biomaterial field to study endothelial cell behaviour at the cell–biomaterial interface. Indeed, because very small diameter vascular grafts are particularly at primary risk of occlusion and because they do not spontaneously endothelialize in humans, *in vitro* lining of prosthetic implants with cultured endothelial cells which could prevent thrombus formation became an attractive, then feasible and promising concept with Herring *et al.* [2] who first reported successful cell seeding of a vascular graft. Thus, the vascular endothelium may well be "a key for the future" [3] to definitive success in the treatment of cardiovascular disorders even though numerous methods have been proposed in order to reduce the surface thrombogenicity of vascular prostheses: improvement of physico-chemical properties, pretreatment with proteins, incorporation of negative charges, application of anticoagulant and antiplatelet agents. In order to select candidates for endothelial cell seeding and to develop less-thrombogenic graft materials, our laboratory is involved in cytocompatibility testing studies with cultured endothelial cells isolated from umbilical cord veins [4–6] because of their ample availability and ease of isolation and culturing, compared with cells of various other origins which behave qualitatively in remarkably similar manner. One of the main problems in the technique of endothelialization of vascular prosthesis is to collect enough endothelial cells in order to rapidly obtain a complete monolayer at the time of implantation. For that purpose, material improvements seem to be necessary to enhance cell adhesion and spreading. In September 1992, a collaboration with the Bakoulev Institute of Cardio-vascular Surgery in Moscow started, and gave us the opportunity to study the cytocompatibility of carbon coated materials (PAN and Vitlan®), since a carbon coating was proved to enhance hemocompatibility, cell adherence and spreading [7], and a polyester coated with albumin and/or synthetic polysaccharide. Studies were thus carried out with human umbilical vein endothelial cells (HUVEC) in culture. Two steps can be distinguished: indirect tests, using the medium added with materials extracts, then direct tests in which cells

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were directly seeded onto materials in order to check if these could be selected as candidates for endothelialization before implantation.

2. Materials and methods

2.1. Endothelial cell culture

HUVEC were isolated and cultured as previously described [4–6] according to the modified original method of Jaffe *et al.* [1]. At confluency, cells were harvested for experiments by trypsinization [4–6], and HUVEC were seeded either in 96-well microtiter plates or 15.5 mm 4-well plates from Nunc (Roskilde, Denmark). Experiments were carried out with 1–3 days post-confluent cultures at passage 1 or 2.

2.2. Materials

Different knitted Polyacrylonitriles (PAN1, PAN2) coated with carbon, carbon radio-grafted polypropylene (Vitlan®), polyesters coated with albumin and heparin (M. 2), with albumin (M. 10) or with albumin and hemi-synthetic polysaccharide (M. 11) and non-coated polyester (M. UnC) were provided by the Bakoulev Institute as non-sterilized pieces. PAN1, PAN2 and Vitlan® were sterilized by 30 min boiling. Polyesters were sterilized by ethylene oxide treatment, and were allowed to stand for 21 days at room temperature in order to promote the release of adsorbed and absorbed ethylene oxide.

2.3. Extracts

For the indirect method, the first step in the assay involved the collection of extractables which were leached or released from the materials. In order to obtain these extracts, according to the AFNOR standard [8], fragments of sterilized materials were immersed in non-supplemented culture medium (IMDM) for 120 h, without agitation, in borosilicated glass tubes at 37 °C in a humidified atmosphere containing 5% CO_2. The ratio of the surface sample to the volume of extraction vehicle was 6 cm^2/ml. At the end of the extraction period, the liquid was recovered and the so-called extracts were ready to use for indirect cytocompatibility. A borosilicated glass tube containing the same extraction vehicle with no material was processed according to the same conditions and the corresponding extract provided the control for the testing procedure.

2.4. Indirect cytocompatibility

Viability and metabolic activity of confluent HUVEC cultured in supplemented pure or diluted (50%) extracts for 24 h in 96-well microtiter plates were tested by the neutral red assay [9] and the MTT [10] test, respectively. Six replicates were performed for each concentration of extracts.

2.4.1. Neutral red assay

Cell layers were rinsed in PBS and neutral red was added (0.4% w/v in distilled water, then diluted 1/80 in IMDM - fetal calf serum 2%). After 3 h at 37 °C, the solution was removed and acetic acid (1% in ethanol 50% v/v) added for 5 min. The absorbance was measured at 540 nm, using an enzyme-linked immunosorbent assay microtiter plate spectrophotometer (Laboratoires Dynatech, Saint-Cloud, France).

2.4.2. MTT assay (methyl tiazol tetrazolium)

Cell layers were rinsed in PBS and MTT in PBS (5 mg/ml) was added for 3 h at 37 °C. Then, MTT solution was removed and the insoluble formazan crystals formed were dissolved in DMSO. The absorbance was measured at 540 nm as above.

2.5. Direct cytocompatibility

In order to avoid cell adhesion to the plastic of wells, which could occur through material meshes during seeding, an agarose layer was prepared and poured into the well as previously described [11]. Circular patches (15.5 mm in diameter) were directly stamped out of sheets of the material: they had a surface area of approximately 2 cm^2 to fit 4-well tissue culture polystyrene plates and after sterilization were deposited on agarose layer. If necessary, materials (PAN2) were fixed by means of Teflon rings (PolyLabo, Paul Block, Strasbourg, France). Finally cells were seeded on control (plastic wells) and on material surfaces.

Attachment test: cell attachment was measured at 3 and 6 h (four culture wells were studied per series and time) after seeding at a density of 4×10^4 cells/cm^2. At the end of the incubation period, HUVEC were detached by trypsin treatment and counted using a calibrator Malassez cell.

Proliferation test: *DNA synthesis*: seeding was performed at 4×10^4 cells/cm^2. Cells were cultured on materials for about 25 days and the DNA synthesis was periodically determined by incorporation of [^{3}H]-thymidine: cells were deprived of FCS for 7 h in medium containing 0.5% BSA in order to prevent cell detachment, and then incubated with [^{3}H]-thymidine (185 kBq/2 cm^2 well) for 17 h. Trichloracetic acid precipitate was solubilized in NaOH 0.3 N and the radioactivity was determined in a liquid scintillation counter.

Scanning electron microscopy (*SEM*): at chosen periods, materials covered by HUVEC were classically fixed and observed [4].

Endothelial cell characterization: the persistence of HUVEC differentiation on endothelialized materials, was checked in cell supernatants by measuring von Willebrand factor (vWF) release of confluent HUVEC after human thrombin stimulation (Diagnostica Stago, Asnières, France, 414 units/mg protein), by ELISA assay (Asserachrom, Diagnostica Stago) and by the ristocetin cofactor activity (Hoechst-Behring, Rueil-Malmaison, France). Confluent HUVEC on control or M. 11 material surfaces were treated with

either control medium alone or medium plus thrombin (2 NIH units/ml) for 1 h at 37 °C. Basal production and stimulus-induced release of vWF were studied. Three sets of four wells (8 cm^2) were treated for each of the stimulated and unstimulated HUVEC series. At the end of the incubation time, the supernatants were collected and centrifuged at 800 g for 10 min, whereas the number of cells was determined by enumeration in a Malassez cell after trypsin treatment. The resulting supernatants were precipitated overnight using one volume of saturated ammonium sulfate in 0.1 M PBS (pH 7.4) at 4 °C. Precipitates were collected by centrifugation at 20 000 g for 15 min and resuspended in 0.1 M PBS (pH 7.4) in order to concentrate vWF before dialysis overnight against the same buffer. vWF was measured in the concentrated cell supernatants (7-fold) by ELISA assay. Standard curves were obtained using dilutions of normal human plasma provided with the kits. Normal (1 ml) plasma is defined to contain 1 unit. The production of vWF was obtained as milliUnits/ml and then converted in mU/10^6 cells. To ensure that released vWF was functional, we performed the determination of the ristocetin cofactor activity of vWF in concentrated supernatants using the platelet agglutination method according to the following principle: stabilized platelets are agglutinated in the presence of vWF and the antibiotic ristocetin A.

2.6. Data analysis

Statistical analysis was performed using the student's *t*-test, and $p < 0.05$ was considered to be significant. The results are shown as mean ± SD.

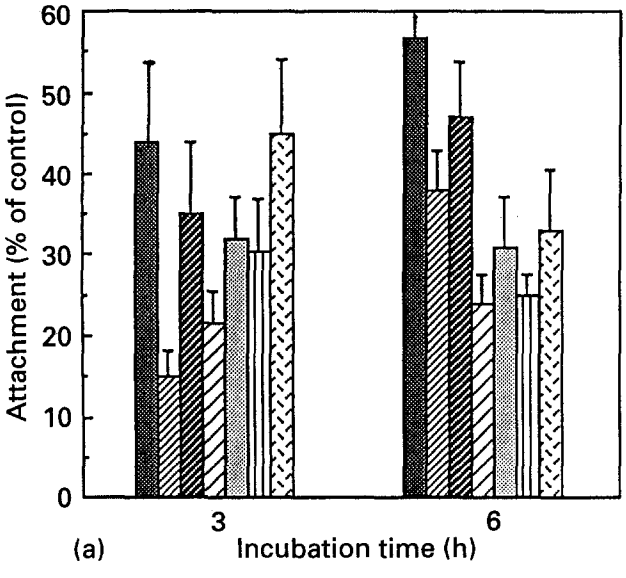
3. Results and discussion

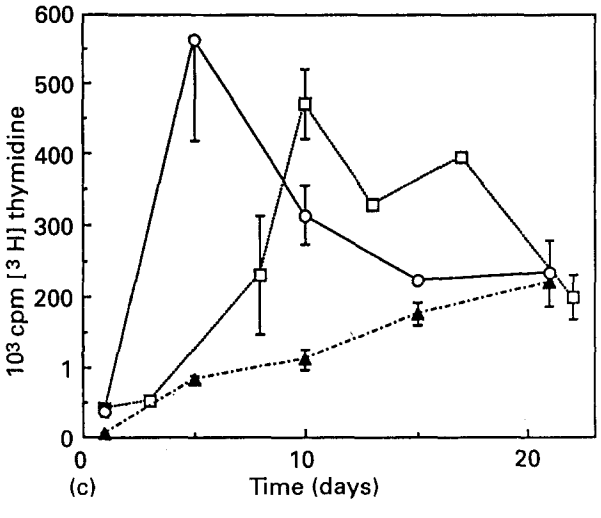
3.1. Indirect cytocompatibility

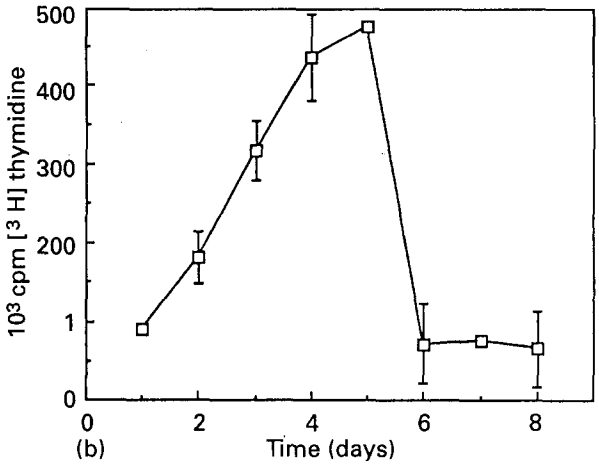
Neither PAN, Vitlan® nor polyesters extracts provoked a toxic effect on cultured HUVEC, as proved by no significant difference (data not shown) between absorbance as far as a cell viable percentage over 75% is required in the standard [8] to rule out any cytotoxic effect of the extracts on the cultured cells. No cell morphological alteration could be observed during the 24 h incubation with the different extracts.

3.2. Direct cytocompatibility

Concerning attachment (Fig. 1a), a maximum of 45% of seeded cells is reached for materials at 3 hours compared to the control, and does not go over 60% at







Surfaces covered by HUVEC	Control		M. 11
Basal production of vWF	1.32±0.6	$p<0.001$	7.6±2.5
	$p<0.01$		$p<0.02$
Stimulus-induced vWF release	3.24±0.9	$p<0.001$	11.6±2.3

(d)

Figure 1(a) Quantitative HUVEC attachment at 3 and 6 h onto the seven tested materials expressed as a percentage of control (■ Vitlan; ▨ PAN1; ▨ PAN2; ▨ M. UnC; ▨ M. 2; ▨ M. 10; ▨ M. 11). (b) [^{3}H]-thymidine incorporation by HUVEC as a function of time on control surface (cell seeding density: 2.10^4 HUVEC/cm^2). (c) [^{3}H]-thymidine incorporation by HUVEC as a function of time on PAN2 (□); M. UnC (▲); M. 11 (○) (cell seeding density: 4.10^4 HUVEC/cm^2). (d) Von Willebrand (vWF) factor release quantified as milliUnits/10^6 HUVEC in cell culture supernatants using an ELISA assay (Asserachrom) after thrombin stimulation (2 NIH units/ml for 1 h at 37 °C).

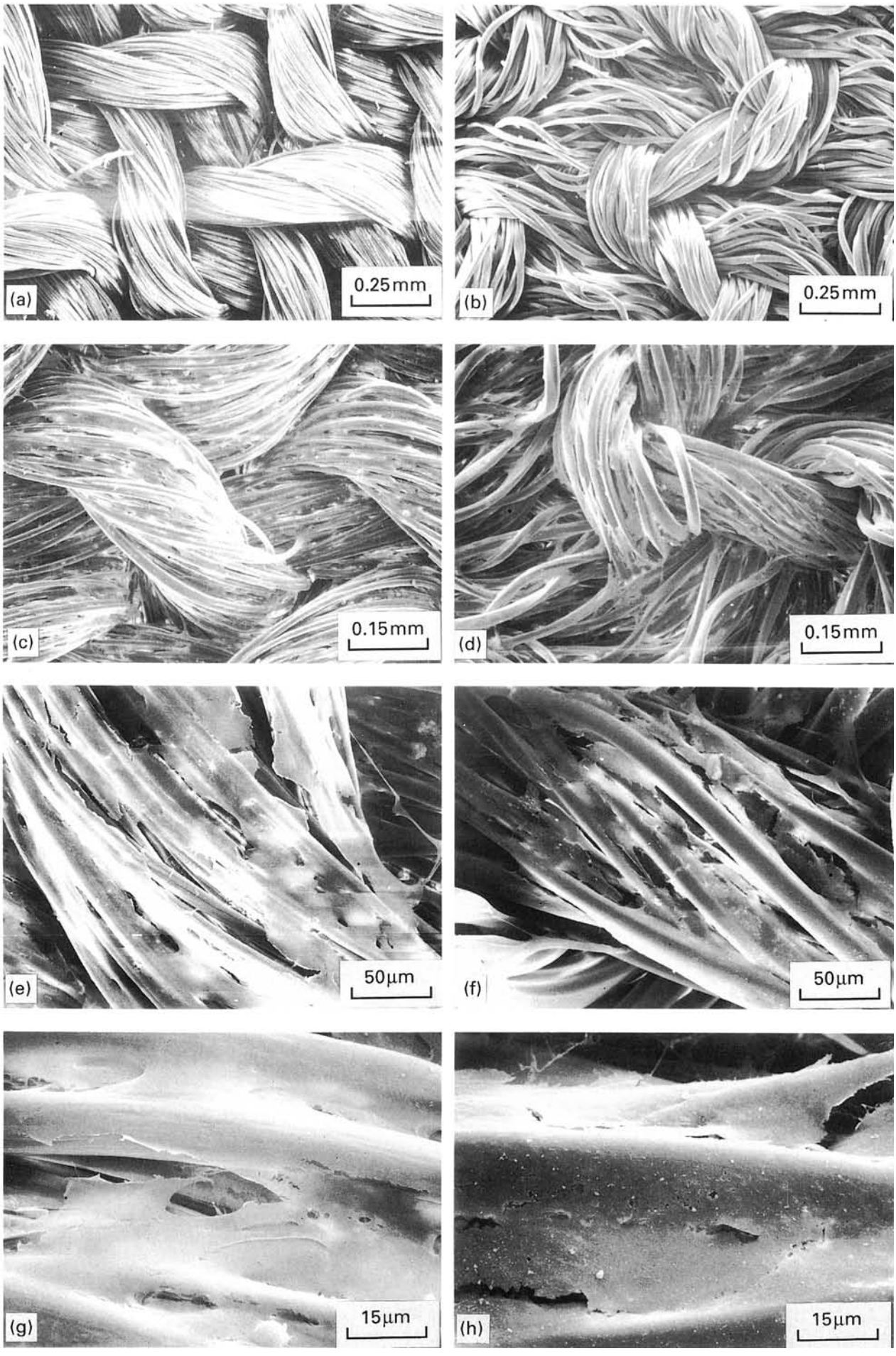


Figure 2 Scanning electron micrographs: (a,b) unseeded PAN2 and M. 2, respectively (original magnification × 60); (c,d,e) seeded PAN2 observed at confluency: original magnifications × 100, × 300, × 1000, respectively; (f, g) seeded M. 2 observed at confluency: original magnifications × 100, × 300, respectively; (h) seeded M. 11 observed at confluency: original magnification × 1000.

6 h. It seems that only carbon-coated materials (Vitlan, PAN1, PAN2) show an improvement in attachment as a function of time. Cell proliferation by the incorporation of [^{3}H]-thymidine is presented in Fig. 1b for HUVEC on control and Fig. 1c for three materials: carbon-coated (PAN2), and polyesters either uncoated (M. UnC) or coated with polysaccharide (M. 11). It is evident, as shown by Fig. 1c, that endothelial cell growth occurred more rapidly with the highest thymidine incorporation value on M. 11. Cell proliferation occurs on the two others but is considerably delayed on M. UnC. Considering the overall results of proliferation for the seven tested materials, they could be classified in four groups according to the peak value of incorporated thymidine, as follows: peak obtained at about 5 days (M. 11); peak obtained at about 10 days (M. 2, M. 10, PAN1, PAN2); peak obtained at about 18 days (Vitlan); peak never obtained (M. UnC).

Thus it appears that whatever the coating of polyester, it improves both HUVEC attachment (Fig. 1a) as well as growth (Fig. 1c). Consequently M. 11 was chosen for the evaluation of the presence of the endothelial marker: vWF. Five days after HUVEC seeding on both control and M. 11, cells were able to produce significant levels of vWF Ag after stimulation with human thrombin, as shown by the results of the ELISA assay (Fig. 1d). Concerning the control, the stimulus-induced vWF release is significantly different from the basal production (3.24 ± 0.9 versus 1.32 ± 0.6 mU/10^6 cells, respectively, $p < 0.01$) and concerning M. 11, the difference is also significant (11.6 ± 2.3 versus 7.6 ± 2.5 mU/10^6 cells, $p < 0.02$). However cells in contact with M. 11 show a higher production of vWF compared with controls in basal conditions (7.6 ± 2.5 versus 1.32 ± 0.6, $p < 0.001$) as well as in stimulated conditions (11.6 ± 2.3 versus 3.24 ± 0.9, $p < 0.001$). The contact between HUVEC and M. 11 could induce a stimulation state in which cells produce an increase of vWF basal production compared with that on the control. Moreover, results show that vWF appearing upon stimulation is accompanied by a marked platelet-agglutinating activity compared to none in the corresponding unstimulated supernatants (data not shown). SEM analysis at confluency of various surfaces (Fig. 2) shows a good cell coverage for PAN2, M. 2 and M. 11.

4. Conclusion

Considering these encouraging results, it seems that the tested materials, and especially PAN and polyester M. 11, could be good supports for endothelialization. Further studies of endothelial cells resistance under physiological flow rates, and dynamic experiments to assess platelet adhesion are necessary and remain to be investigated.

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