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Optimization of the interaction between ethylene-vinyl alcohol copolymers and human endothelial cells

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To better understand and optimize the fine interactions that occur during adhesion events between human cells and synthetic materials, we seeded human umbilical vein endothelial cells (HUVEC) onto ethylene-vinyl alcohol (EVOH) copolymer films prepared by casting. Different adhesive proteins, e.g. fibronectin and gelatin, and the monoclonal antibody (MoAb) CLB-HEC19 specific for the endothelial cell membrane were used to coat the materials. We used atomic force microscopy (AFM) to analyse the EVOH film structure, to test its planarity and homogeneity, before seeding it with endothelial cells. The metabolic changes induced in the endothelial cells by interactions with the copolymer functional groups and the adhesive proteins were monitored by a micro-electronic pH sensor, positioned close to the HUVEC monolayer. We found that the adhesion of HUVEC onto various substrates was finely modulated by the MoAb CLB-HEC19 and that the endothelial cell metabolic rate was enhanced when cultured onto a CLB-HEC19 coating. Surface roughness seems also to play a role in the interaction with HUVEC. The AFM measurement analysis demonstrated that L6 surface is rougher than R20. These surface characteristics could favour cell adhesion; in fact HUVEC adhesion results on R20 were significantly lower than on L6.

1. Introduction

One of the major problems in the use of synthetic materials as biomaterials is to make sure that they are biocompatible and eventually to improve their biocompatibility. To be biocompatible a material should be both not cytotoxic and cytocompatible, i.e. able to support cell adhesion and growth. A new idea of biocompatibility [1] involves the concept of bioactivation, which includes positive stimulatory effects of the biomaterials on the host tissues.

Several factors can affect the interaction between cells and the surface of the material, such as the degree of material wettability, the surface roughness, the presence of functional groups, and coating with adhesive proteins.

The aim of this study was to better understand and optimize the fine interactions that occur during adhesion events between human endothelial cells and ethylene-vinyl alcohol (EVOH) copolymers (Clarene, Solvay, Italy). EVOH copolymers are thermoplastic resins with a semicrystalline structure [2] whose properties are closely linked with their composition and processing method. Potential applications of EVOH copolymers have been reported in the field of contact lenses [2] and vascular grafts [3].

A new development in studying the boundary between cells and biomaterials is the availability of nanotechnologies able to record and process micro-information at both structural and physiological levels. We used atomic force microscopy (AFM) to analyse Clarene film structure, to test its planarity and homogeneity, prior to seeding it with endothelial cells. The metabolic changes induced in the endothelial cells by the interactions with the copolymer functional groups, adhesive proteins and a monoclonal antibody (MoAb) specific for the endothelial cell membrane (CLB-HEC19) [4] were monitored by a microelectronic pH sensor [5], positioned close to the endothelial cell monolayer.

2. Materials and methods

2.1. EVOH film preparation

EVOH copolymer films were obtained by solution casting on ultrasonically cleaned glass microscope slides. Two types of Clarene, R20 and L6, having an

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ethylene molar content of 40 and 29%, respectively, were used. Solutions containing 2% R20 and 1.25% L6, respectively, were prepared in dimethyl sulphoxide (DMSO), and 350 µl of each solution were dropped onto glass slides. The slides were dry heated in a stove at 120 °C for 25 min. This whole procedure was repeated twice. The slides were then placed in a vacuum oven at 50 °C for 5 days to obtain complete evaporation of the solvent.

2.2. Coating with adhesive proteins

The adhesive proteins fibronectin (Fn) and gelatin (Ge), and the MoAb CLB-HEC19 were used to coat glass slides with and without EVOH films and 96-well plates. The glass slides were cut to fit into 6-well culture plates. They were dry heat sterilized (150 °C for 1 h) and then coated with 500 µl of Fn (10 µg ml^{-1}) (Gibco), 500 µl 2% Ge (Sigma) or 200 µl immunoglobulin G CLB-HEC19 (0.05 mg ml^{-1}), prepared and characterized at the CLB, Amsterdam [6]. The coating was performed overnight at 4 °C.

2.3. Silanization

Glass slides were amino-silanized by immersion in a solution of 90% 2-propanol, 5% water and 5% aminopropyltriethoxysilane (APTS) (Fluka) for 1 h, then rinsed and dried in a vacuum oven (1 h at 120 °C). The presence of the amine groups on the slides was confirmed by the Rimini test. The silanized slides were put into 6-well culture plates and coated with MoAb CLB-HEC19, as described in Section 2.2 above.

2.4. Human endothelial cell adhesion and proliferation tests

Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords as previously described [7] and cultured in equal parts of media M199 and RPMI 1640 (Gibco), 20 mM Hepes buffer, 2 mM L-glutamine, 100 U ml^{-1} penicillin-streptomycin, 5 µg ml^{-1} fungizone with 20% pooled human serum. The HUVEC were cultured to confluence in 80 cm^2 polystyrene (TCPS) flasks (Greiner) in an atmosphere of air with 5% CO_2 at 37 °C. Second to third passage HUVEC were used for the experiments.

The coated slides and the 96-well plates were rinsed three times with phosphate buffer solution (PBS) (Gibco) and then seeded with HUVEC at a density of 2.5×10^4 cells cm^{-2}. Four hours after seeding the HUVEC were trypsinized. Cells from the 6-well plates were counted by means of a haemocytometer, while the total protein content of the 96-well plates was determined by the colorimetric test Kenacyd Blue [8]. HUVEC seeded onto silanized slides were trypsinized 3 days after seeding.

2.5. Contact angle measurements

The underwater air-to-glass contact angles were measured by a Rame & Hart goniometer.

2.6. AFM analysis

The AFM used was a Park Scientific Instruments (PSI) Autoprobe CP, equipped with a 100 µm scanner and ScanMaster^{Tm} piezoelectric non-linearity correction system for the x and y directions. The tips used were 2 µm thick, 85 µm long Silicon Ultralevers (PSI), with a typical resonance frequency of 300 kHz. Images were acquired both in non-contact (NC) mode and intermittent contact (IC) mode on two batches of Clarene R20 and Clarene L6 films deposited onto glass slides using different tips. For each of the four samples, a set of 20 images consisting of 512×512 pixels on a 10 µm by 10 µm area with a 16-bit resolution in height was acquired at random positions on the surface.

To derive the surface roughness, all images were subjected to the following processing. At first, a second order fit in the x and y directions was subtracted from the image to correct the z direction piezoelectric non-linearity. The surface roughness was then calculated as the mean of the differences between the sample height at each pixel position and the average height of the whole image.

2.7. HUVEC metabolism measurements

Cell metabolism measurements were carried out using a microelectronic pH sensor (ISFET) close to the HUVEC monolayer. The core of the measuring system consists of a flow-through microchamber. The top of the chamber is made of an ad hoc encapsulated ISFET sensor, and the slide with the cell population on it forms the bottom of the chamber. The depth of the microchamber, i.e. the distance between the cell population and the microsensor, is obtained by means of a calibrated Teflon spacer of 300 µm.

The slides were pretreated with Ge + MoAb CLB-HEC19 before seeding the HUVEC at a density of 2.5×10^4 cells cm^{-2}. The cells were incubated for 24 h at 37 °C, 5% CO_2, before performing the measurements.

The microchamber was subjected to ON-OFF cycles of culture medium fluxes. The cell-induced acidification of the microvolume was measured during the OFF condition (3 min). Standard pH values were restored during the ON condition (3 min).

3. Results

Preliminary experiments were carried out to compare the effects of Fn and MoAb CLB-HEC19 on HUVEC adhesion. CLB-HEC19 stimulates HUVEC adhesion in a manner comparable to Fn (Fig. 1). We found that the covalent binding of the MoAb by means of aminosilane treatment enhanced HUVEC adhesion (Fig. 2). These results were subsequently confirmed when we tested HUVEC adhesion onto R20 and L6 copolymer films (Fig. 3). We found that CLB-HEC19 improves cell adhesion on both the materials, even when compared with Fn and Ge coatings.

Contact angle measurements and AFM roughness evaluation are shown in Table I and Table II, respectively. Contact angle measurements indicate that there

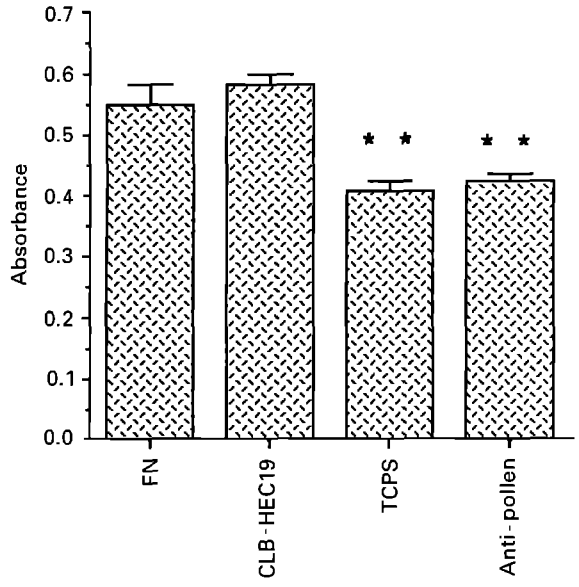


Figure 1 HUVEC adhesion on TCPS as a function of the coating used. Adhesion was measured 4 h after seeding. Statistical significance is calculated with reference to Fn (paired t-test, $^{**}P < 0.01$).

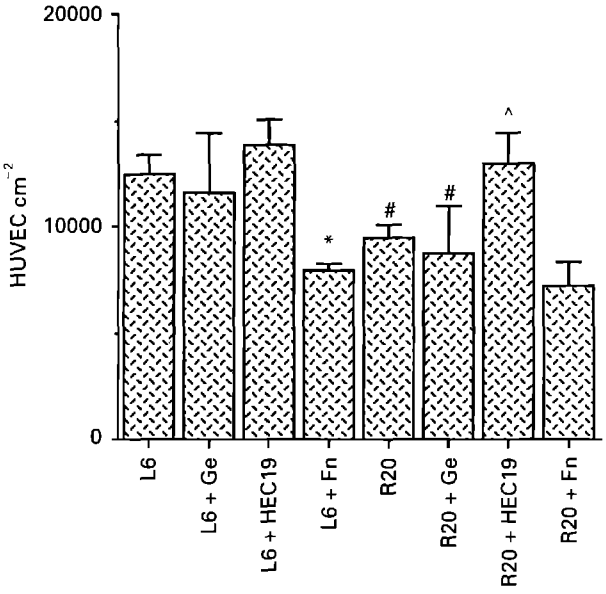


Figure 3 HUVEC adhesion onto L6 and R20 Clarene films as a function of the coating. Statistical analysis (paired t-test) was performed by comparing uncoated with coated L6 films ($^{*}P < 0.05$), uncoated with coated R20 films ($^{\wedge}P < 0.05$) and homologous films of the two L6 and R20 series ($\#P < 0.05$).

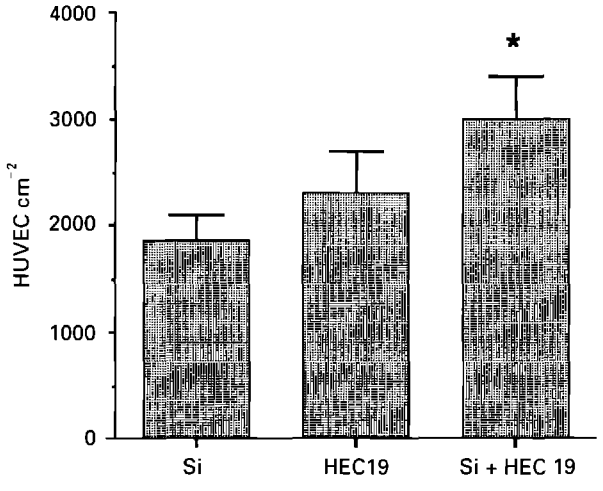


Figure 2 HUVEC adhesion onto untreated and amino-silane (Si) treated glass slides coated with the MoAb CLB-HEC19. Statistical significance is calculated with reference to Si (paired t-test, $^{*}P < 0.05$).

TABLE I

Substrates	Contact angle (Mean ± SE)
L6	39.36 ± 0.7
L6 + Fn	40.56 ± 1.32
L6 + Ge	41.46 ± 0.97
L6 + HEC19	28.87 ± 0.5***
R20	37.78 ± 0.42
R20 + Fn	41.25 ± 0.71*
R20 + Ge	43.81 ± 0.71***
R20 + HEC19	26.70 ± 2.03***

Statistical significance is calculated with reference to respectively uncoated L6 and R20 (paired t-test, $^{*}P < 0.05$, $^{***}P < 0.001$)

TABLE II

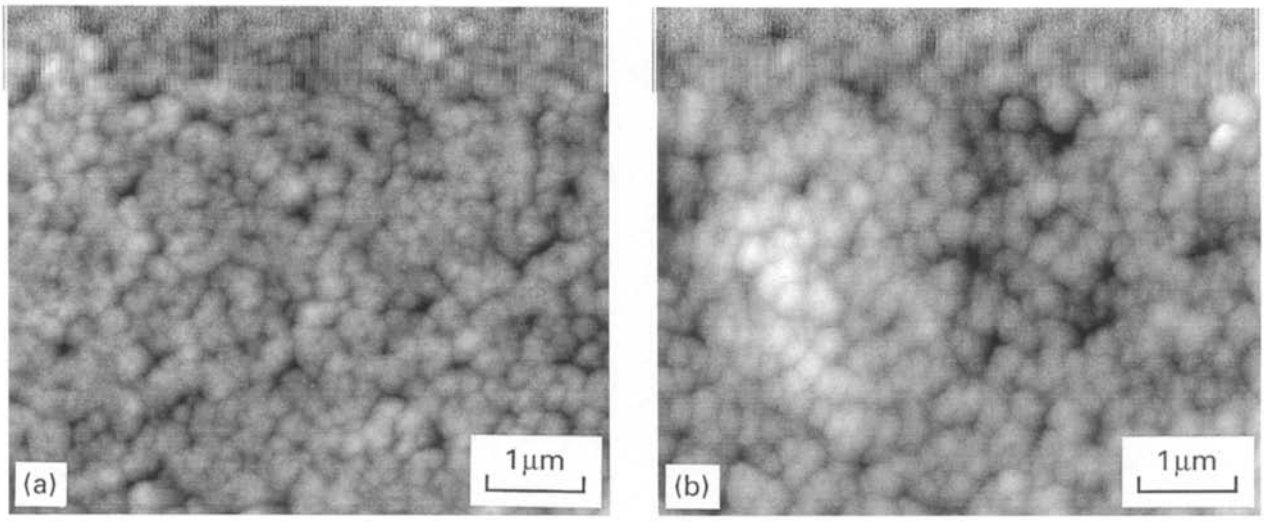
Sample	Roughness (nm)	Stand. Dev. (nm)
Clarene R20	64.2	2.1
Clarene L6	78.9	9.5

is no significant difference between L6 and R20. In both cases Fn and Ge coatings induce a slight increase in the hydrophobicity, while the presence of MoAb CLB-HEC19 significantly enhances the surface hydrophilicity (Table I). Measurements performed using the AFM in IC mode have shown that Clarene L6, reproducibly, exhibits a higher surface roughness than Clarene R20 (Table II). In Fig. 4, typical 10 μm by 10 μm images of the Clarene R20 (Fig. 4a) and Clarene L6 (Fig. 4b) surfaces, alongside with the height histograms (Fig. 4c), are shown.

The preliminary results of the cell metabolism measurements (Fig. 5) indicate that the metabolic rate of confluent HUVEC cultured on CLB-HEC19 is significantly higher than the control on Ge and shows a rather constant trend with time.

4. Discussion

We have demonstrated that the adhesion of human endothelial cells onto various substrates is finely modulated by the MoAb CLB-HEC19 [4, 7] (Figs 1, 2 and 3), which is specific for the endothelial cell membrane, and that the endothelial cell metabolic rate is enhanced (Fig. 5) when cultured onto a CLB-HEC19 coating. Our data on HUVEC adhesion onto glass slides (Fig. 1) confirm previous data [6] on polyethylene. Covalent binding of the antibody to the glass substrate using an amino-silane proved to ameliorate HUVEC adhesion and growth, compared with amino-silane or CLB-HEC19 alone (Fig. 2). Amino-silanized glass improves the stability of the antibody surfaces and could be combined with the Langmuir–Blodgett method to produce densely packed



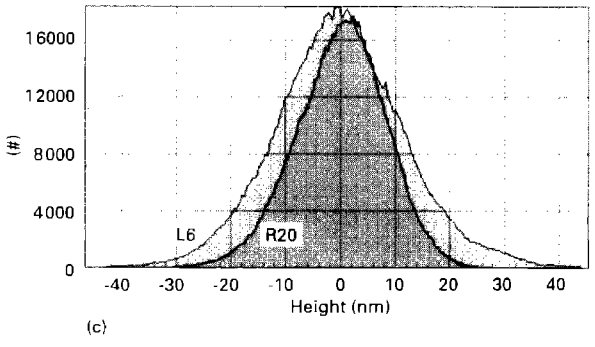


Figure 4 AFM images (10 μm by 10 μm field of view) of (a) Clarene R20 and (b) Clarene L6 films deposited onto glass slides. In (c) the height level histograms for both images are shown, indicating that the Clarene L6 film has a higher surface roughness than the Clarene R20 film.

antibody films [9] to further improve cell adhesion [10]. HUVEC adhesion onto L6 and R20 films precoated with MoAb CLB-HEC19 is also enhanced in comparison with the same films untreated or treated with Fn and Ge (Fig. 3). R20 has a higher ethylene molar content (40%) than L6 (29%), therefore R20 was supposed to be more hydrophobic than L6. In spite of these considerations, L6 and L20 surfaces have contact angles not statistically dissimilar from each other (Table I) over a range of wettabilities that is considered optimal for endothelial cell adhesion [11]. The coating with Fn and Ge makes the surfaces slightly but significantly (in the case of R20) more hydrophobic. The decreased cell adhesion on films treated with Fn and Ge (Fig. 3) could possibly be attributed to this enhanced hydrophobicity. In

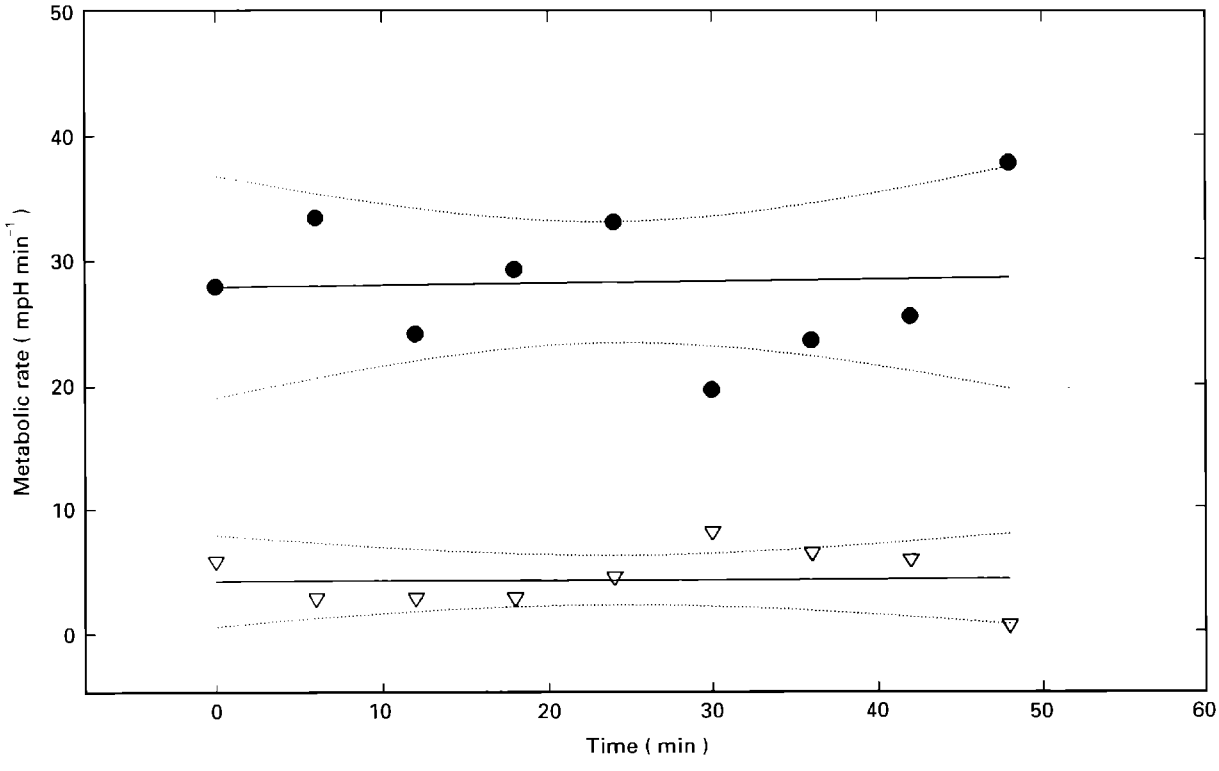


Figure 5 Comparison of output signals obtained from cells with and without CLB-HEC19 MoAbs. Cells at the same density were seeded and grown for 4 days onto two different glass slides precoated with gelatin (●) with and (▽) without CLB-HEC19 MoAbs. Each data point represents the average metabolic rate calculated from the linear regression of the detected sensor signal under closed flow conditions. The solid lines represent the linear regression and the dotted lines represent the 95% confidence interval.

contrast, the presence of CLB-HEC19 shifts the surface wettability of both L6 and R20 towards more hydrophilic figures (Table I) and this might play a role in the enhanced HUVEC adhesion on the materials coated with the antibody.

The observation that there are no significant differences in wettability between L6 and R20 surfaces treated with the same coatings (Table I) seems to suggest that the coatings hide the EVOH surface characteristics from the cells. Surface roughness also seems to play a role in the interaction with HUVEC. The AFM has yielded the best results in image resolution and reproducibility when used in IC mode, and the measurement analysis demonstrates that surface of L6 is rougher than that of R20. These surface characteristics could favour cell adhesion, as demonstrated in Fig. 3, where HUVEC adhesion results on R20 are significantly lower than on L6.

Finally, the acidification measurements shown in Fig. 5 demonstrate that a surface coated with CLB-HEC19 is able to metabolically stimulate the HUVEC. CLB-HEC19 binds specifically to an endothelial cell membrane antigen called endoglin [12]. Endoglin is a major glycoprotein of human endothelium, whose polypeptide sequence is known [13]. An RGD sequence, the key recognition structure found in adhesive proteins like Fn, is located in the exposed region of its extracellular domain. The binding of endoglin with CLB-HEC19 is able to mediate not only endothelial-substrate adhesion, as shown by our results, but also cell-cell adhesion [12]. The function of endoglin is still largely unknown. The results shown in Fig. 5 seem to suggest that endoglin is not only an adhesive structure on the endothelial cell surface, but that it also has a receptor function and is able to trigger cellular metabolic activity. Further investigation is needed to better define the role played by endoglin in the endothelial cell adhesion events. In particular, the clarification of the role of its RGD sequence offers a challenging perspective.

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