Interactions between a biodegradable polymer, poly(hydroxybutyrate-hydroxyvalerate), proteins and macrophages

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SUMMARY : The effect of the chemical and morphological modifications of the surface of poly(hydroxybutyrate-hydroxyvalerate) (92/8) (PHB/HV) films induced by an alkaline hydrolysis on the adsorption of three proteins known to modify cell behavior, namely albumin, collagen and fibronectin and on the adhesion and proliferation of monocytes-macrophages of the J774 cell line, has been studied.
Before treatment, the water contact angle θ of the face that went through a corona pre-treatment was higher than the other one. It increases with hydrolysis to equal the value of θ angle of the other face after 2 hours. The XPS analysis revealed that all the impurities present on the native material have been eliminated after hydrolysis leading to a similar chemical composition on both faces. The surface concentrations of -COOH functions accessible to [^{3}H]-lysine increases sharply with the duration of hydrolysis. The sharpest increase is observed at the very beginning of hydrolysis. The surface morphology of the polymer is also modified by hydrolysis.
The adsorption of the three proteins increases when the polymer is hydrolyzed as well as the adhesion and proliferation of the monocytes-macrophages, no matter which protein was adsorbed. There are some evidence suggesting that the protein conformation is different on both substrates. Collagen has a drastic repulsive effect on the cells on the native polymer but this repulsive effect disappeared on the hydrolysed one. Accordingly, it clearly appears that it is possible to modulate the biocompatibility of PHB/HV by either chemical hydrolysis or protein adsorption.

Introduction

Poly(β-hydroxyalkanoates) are biodegradable aliphatic polyesters that show good biocompatibility and have been investigated as potential biomaterials [1-3].

Host cell adhesion plays a very important role in the biocompatibility of materials. Many studies of cell adhesion on different substrates have already been reported [4] but most of them have been focused on the adhesion of fibroblasts or endothelial cells [5]. Only a few have been dedicated to the attachment of monocytes-macrophages [6]. Monocytes are involved in the response of the body to a polymeric implant. They migrate to the implantation site where they differentiate into macrophages that play a major role in the resorption process by phagocytozing fragments of the implant [7] and are therefore involved in the biodegradation of foreign materials [8].

It has been shown that cell adhesion strongly depends on the surface chemistry of the implant and, in particular, on hydrophilicity [4,9-11], electrical properties [12] and the chemical

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composition [13-16]. Surface morphology of the biomaterial also plays an important role to modulate cell adhesion [17].

It is generally accepted that plasma proteins instantly adsorb on the biomaterial when it comes in contact with biological fluids [4,13] and that the biocompatibility of a polymeric material is largely determined by specific interactions between adsorbed proteins and receptors located on the surface of cells [18]. Actually, many authors have shown that adsorbed proteins like fibronectin, albumin, laminin, collagen and vitronectin are involved in cell adhesion [4]. Fibronectin preadsorption is known to greatly increase cell attachment and spreading while albumin - the major plasmatic protein - prevents it [13]. Collagen - the major constituant of the connective tissue - is known to enhance the attachement and growth of most anchorage-dependent cells [5,19]. The differences in protein behavior on different substrates are thought to come from conformational changes in the adsorbed proteins that modulate the availability of the cell binding domains. These observations led to the development of strategies to modify the surface characteristics of materials in order to promote the adsorption of proteins stimulating cell adhesion. Efforts to improve biocompatibility are thus focused on selecting and modifying the interactions between proteins and cells.

The aim of this work was to evaluate the effect of an alkaline hydrolysis applied on poly(hydroxybutyrate-hydroxyvalerate) films. We have investigated its influence on the adsorption behavior of three proteins known to modify cells behavior (albumin, collagen and fibronectin) and on the adhesion and proliferation of monocytes-macrophages from the J774 cell line. Protein adsorption was monitored by radiochemical and immunoenzymatic assays. Different surface properties were examined : hydrophilicity evaluated by the static water contact angle, surface chemical composition determined by X-Ray Photoelectron Spectroscopy (XPS), number of accessible carboxylic functions to determined by chemical grafting and radioassay [20, 24], surface morphology analysed by Scanning Electron Microscopy (SEM) and by Atomic Force Microscopy (AFM).

Material and methods

Material

The polymer of interest is a copolymer of poly(hydroxybutyrate-hydroxyvalerate) (92/8) (PHB/HV) under the form of 50 µm thick films from ICI (BIOPOL, Cleveland, England). During the manufacturing process, one of the faces was modified by a corona pretreatment.

To modulate its surface properties, the polyester film was submitted to an alkaline hydrolysis at room temperature with 2.5 M KOH in methanol/water 50/50 (v/v) for different periods of time.

Reagents

Most reagents were of analytical grade and purchased from Merck (Darmstadt, Germany). Methanol (HPLC grade) was obtained from Lab Scan-CRB (Brussels, Belgium) and H_2O_2 from Aldrich (Gilligham, England). Ultrapure water was prepared with a MilliQ-system (Millipore, Bedford, MA). MES (2-[N-morpholino]-ethanesulfonic acid) and CDI (1-[3-dimethylaminopropyl]-3-ethyl-carbodiimide hydrochloride) were purchased from Janssen-Chimica (Geel, Belgium); soluene 350 TM and Picofluor 40 TM from Packard (Meridan, CT); Triton X-100, Tween 20 (poleoxyethylenesorbitan monolaurate), porcine skin gelatin, L-lysine, OPD (1,2-phenylendiamine dihydrochloride), human albumin and fibronectin were obtained from Sigma (St Louis, MI). Collagen S was purchased form Boeringher Mannheim Biochemica (Mannheim, Germany). L-[4,5-^{3}H]lysine monohydrochloride (specific activity 92,2 Ci/mmole) and NaB^3H_4 were obtained from Amersham (Little Chalfont, UK). The peroxidase conjugated rabbit antibodies to human fibronectin or albumin were purchased from Dako (Glastrup, Denmark).

The phosphate buffer saline (PBS) was adjusted at pH 7.3 and consisted in NaCl (140 mM), KCl (3 mM), KH_2PO_4 (10 mM) and $Na_2HPO_4.2H_2O$ (10 mM). The 0.1 M MES buffer was adjusted at pH 3.5. The PBS-Tween was a 0,1% (v/v) solution of Tween 20 in PBS and the PBS-Triton was a 0.01% (v/v) of Triton X-100 in PBS. The citrate-phosphate buffer at pH 5.5 was obtained by mixing 48 ml of citric acid 98 mM and 50 ml of $Na_2HPO_4.2H_2O$ 200 mM.

Dulbecco's modified Eagle's medium (DMEM) containing 1.5 g/l of glucose and 2 mM of L-glutamine was purchased from Bio Whittaker (Verviers, Belgium) and supplemented with 10% (v/v) of fetal bovine serum (FBS) (Bio Whittaker).
The staining solution was prepared from 0.25 % (p/v) of Coomassie Blue (Serva, Heidelberg, Germany), 45 % (v/v) of methanol and 9% (v/v) of glacial acetic acid.

Protein labelling
Radiolabelling of collagen, human albumin and fibronectin was accomplished by reductive methylation with NaB^3H_4 in the presence of formaldehyde as reported previously [20]. The specific radioactivity of the radiolabelled preparation was respectively 0.015, 0.168 and 0.0127 μCi/μg.

Surface characterization

X-Ray Photoelectron Spectroscopy (XPS)

The surface chemical composition of the samples was determined by XPS using a SSX100/206 spectrometer (Fisons) equipped with an aluminum anode (10kV) and a quartz monochromator. The direction of photoelectron collection made angles of 55° and 71° with the normal to the sample and the incident X-ray beam, respectively. The electron flood gun was set at 6 eV.

For each sample, the O_{1s} and C_{1s} bands were recorded with a 600 µm spot, an emission current of 12 mA and a pass energy of 50 eV, followed by a wide scan collected with a 1 000 µm spot, an emission current of 20 mA and a pass energy of 150 eV. The binding energies of the analyzed peaks were determined by fixing the C_{1s} component due to carbon only bound to carbon and hydrogen at 284.8 eV. The peak area was determined using a linear background and a Gaussian/Lorentzian (85/15) function [21-23].

Water contact angle measurements

The water contact angle was measured with the sessile drop method and an image analysis system [22]. The instrument was equipped with a CCD type MXR 5010 camera and was provided by Electronisch Ontwerbureau de Boer (The Netherlands). The measurements were performed at room temperature. The water was always freshly taken from the purification system just before the experiments. Each reported value is the average of, at least, 10 measurements ± standard deviation (SD).

Radioassay of the surface-accessible carboxylic functions

The radioassay consisted in the activation of the carboxylic functions accessible at the surface of the polymer as in 24. In brief, the sample (disk of 13 mm diameter) was immersed in 1.5 ml of a solution of 0.1% (w/v) of CDI in 0,1M MES buffer (pH 3.5) for 1h at room temperature under shaking. It was then washed once with 0.1M MES buffer solution and twice for 10 min in ultrapure water at room temperature under shaking. The activated sample was immersed in 1 ml of 1 mM L-lysine in PBS (10 µM [^{3}H]-lysine and 990 µM L-lysine *i.e.* 10^{-3} µCi/ml) for 2h at room temperature under shaking. The sample was then washed 3 times for 10 min with PBS-Triton and once for 10 min with water and left in 0.5 ml of Soluene™ for dissolution overnight. The amount of lysine fixed to the polymer was measured by liquid scintillation counting after the addition of 1.5 ml of Picofluor™. For each sample, the measurement was also carried out without the CDI activation in order to determine the amount retaining by physical adsorption.

A Tri-Carb 1600 TR liquid scintillation analyzer (Packard, Meriden, CT) was used. The count rate (cpm) was automatically converted into an absolute value of desintegrations per minute (dpm) using the relationship dpm = cpm/counting efficiency. These values were converted in ng/cm^2 of accessible surface by comparison with standard of [^{3}H]L-lysine. The values reported are the average of three measurements ± SD.

Scanning Electron Microscopy (SEM)

The surface morphology of the samples was first examined with a scanning electron microscope Hitachi S570 (Tokyo, Japan) equipped with a secondary electron detector. The samples were gold sputter-coated before analysis.

Atomic Force Microscopy (AFM)

The rugosity of the polymer surfaces was measured before and after hydrolysis by AFM in air, using non-contact mode, with an Autoprobe CP microscope (Park Scientific Instrument; Sunnyvale, CA). The tips were 2 µm Ultralevers with an approximate radius of curvature at the apex of 10 nm and a typical resonance frequency of 300 kHz. The parameters determined with this technique were the average roughness (R_{ave}), the rms roughness (R_{rms}) [25] and the specific surface which is the ratio between the surface area and the surface projected. These parameters were measured on 25 different 400 µm^2 spots for each sample.

Protein adsorption

In order to study the behavior of the polymer close to the physiological conditions, the protein concentrations chosen for the adsorption experiments are in the range of the concentration encountered in blood serum.

Radiochemical Assay

All the glassware was gelatin precoated overnight by treatment at 37°C with a 1% (w/v) solution of gelatin in PBS and rinsed 3 times with PBS-Tween to minimize the adsorption of the radiolabelled proteins.

The polymer samples (disks of 13 mm diameter) were incubated for 2h at 37°C in 600 µl of the protein solutions at selected concentrations, ranging from 0 to 1 mg/ml for albumin, from 0 to 50 µg/ml for collagen and from 0 to 10 µg/ml for fibronectin. Each sample was rinsed 3 times with PBS-Tween. It was transferred into scintillation vials, solubilized by overnight treatment at room temperature in 1 ml of Soluene™. Radioactivity was determined after addition of 2 ml of Picofluor™ as above.

The standards used for the conversion of dpm in ng/cm^2 were 20 and 40 µl of the most concentrated solutions for collagen and fibronectin and of the 100 µg/ml solution for

albumin. A sample of polymer had been dissolved in these standards by addition of 1ml of Soluene™ and 2 ml of Picofluor™.
The values are the mean of two independent measurements.

Enzyme-Linked ImmunoSorbent Assay (ELISA)

All the glassware was precoated and rinsed as above.
After incubation for 2h at 37°C in 600 µl of the protein solutions, each sample was rinsed 3 times with PBS-Tween. The sample was then immersed for 1h at 37°C in 500 µl of a 1% (v/v) gelatin solution in PBS and washed 3 times with PBS-Tween. It was then incubated for 1h at 37°C with 300 µl of the appropriate solution of antibody conjugated to peroxidase diluted 2 000 times in PBS-Tween and washed 6 times with PBS-Tween. After transfer into new 24-wells plates, the peroxidase activity was determined by incubation for 20 min at 37°C with a chromogene solution (30 mg of OPD + 30 ml of citrate phosphate buffer, pH 5.5 + 30 µl of H_2O_2) ; the enzymatic reaction was stopped by addition of 500 µl of a 4.5 M H_2SO_4 and the absorbance was read at 510 nm with a Vitalab (Vitalab Scientific, 8550 N, Dieren, The Netherlands) photometer. The results reported on the figures are the mean of two measurements.

Cell culture and determination of cell adhesion and proliferation

The adhesion and proliferation of monocytes-macrophages from the J774 continuous cell line were evaluated by determining the percentage of the polymer surface covered by cells, after inoculation on the native or on the hydrolyzed polymer either without pre-treatment or after a collagen, albumin or fibronectin coating.
Monocytes-macrophages (murine monocyte-macrophage J774 cell line, ECACC, Salisbury, UK) were routinely cultured in 175 cm^2 TCPS culture flasks from Greiner GmbH (Frickenhausen, Germany) in DMEM supplemented with 10% (v/v) FBS, 1 g/l glucose and 2 mM L-glutamine and incubated at 37°C in water saturated air under 5% (v/v) of CO_2.
Disks of 2 cm in diameter of the native and 4h hydrolyzed polymer were sterilized by γ-irradiation (25 kGy) with Co^{60} at Caric-Mediris S.A. (Fleurus, Belgium) and were placed in 12-wells Falcon 3043 plates (Becton Dickinson, NJ). These disks were incubated for 2 h with a solution of either collagen (33 µg/ml), fibronectin (1 µg/ml) or albumin (5 mg/ml) in PBS.
Each well was inoculated with 1 ml of a solution containing 10^5 cell/ml at 37°C in DMEM medium without FBS and examined after 3h for the adhesion study. For the proliferation study, the medium was replaced by DMEM+10% FBS after 3h and the supports were examined after 3 days.

The dishes were then rinsed 3 times with PBS and manual agitation was performed by three circular motions in one direction, three in the opposite one and three linear back and forward motions in order to remove nonadherent cells. The cells were fixed with 5% (v/v) formaldehyde for 5 min. They were then rinsed with water and stained with Coomassie Blue. After sufficient washing, the samples were mounted on glass and observed with the phase contrast microscope (Labovert, Leitz) coupled with a color camera (JVC TK 1280E) and a monitor (JVC TM-10E). The relative area of the samples occupied by the cells was determined with a Macintosh AV840 using the NIH1.54 Image Analyzer program. For each sample, 9 spots have been analyzed and averaged in order to get a representative surface occupation. The adhesion and proliferation studies have been realized also on TCPS (tissue culture polystyrene), used as a reference. Therefore, for each experiment, cells have been inoculated on TCPS in the same conditions as on PHB/HV. Results are expressed as ratio of the area occupied by the cells on the sample to the surface occupied on TCPS. The values reported are the average between 2 different experiments. A t-student test was used to compare the samples.

Results and discussion

Surface characterization of PHB/HV

XPS

The surface elemental composition of PHB/HV before and after hydrolysis is reported in table 1.

XPS analysis reveals that the chemical composition of the two faces of the native film are different. This is due to the corona treatment applied by the manufacturers on face 1. Decomposition of the C_{1s} peak of the native film indicates that the three kinds of expected carbon, namely C-(C, H) (at 284.8 eV), C-O (at 286.4 eV) and O=C-O (at 288.8 eV) are present. Curve fitting of the O_{1s} spectra reveals the presence of oxygen making two bonds (at 531.8 eV) and one bond (at 533.2 eV) with carbon, as expected. Nevertheless, the proportion of the different kinds of carbon and oxygen detected on the native polymer is different from the proportion expected on the basis of bulk chemical composition. Native face 2 shows a slight deficit of oxygen with respect to the expected value. The deficit is much larger for native face 1, suggesting that the corona treatment has destroyed ester functions, replacing some of them by functions in which one carbon atom is bound to one oxygen atom (alcohol). Elements other than carbon and oxygen are also present at the surface of the film. Besides an important contamination of both faces with silicium, face1 is contaminated by N, Na and a few other elements at less than 1%. Therefore the data from the native material peak decomposition should be considered with caution.

Curve fitting of the C_{1s} peaks of the PHB/HV film hydrolyzed for 4h indicates that the two faces are close to each other. The proportion of the different kinds of carbon and oxygen is closer to the expected one. The level of contamination is considerably lowered on face 1 and all traces of contaminants have been eliminated from face 2.

The surfaces obtained after treatment are almost pure PHB/HV or at least cleaner PHB/HV than before hydrolysis.

The C_{1s} peak shape does not allow any distinction between carboxylic and ester groups. However, if the number of -COOH functions present in the area exploited by XPS was important, the percentage of carbon singly bound to oxygen would be inferior to the expected values. As the experimental value is very close to the expected one, we assume that the percentage of carboxylic functions is very low compared to the amount of ester functions.

Tab 1 : Surface composition of native and 4h hydrolyzed films of PHB/HV (92/8) determined by XPS.

	atomic fraction (%)with respect to C+O					O/C	impurities %
	C-C	C-O	O=C	O-C	O=C		
native face1	50.5	15.5	9	7.3	17.7	0.31	Si (3.9), N(1.4), Na (1.2), S(0.4), Ca (0.5), Cl (0.6)
native face2	42.8	13.8	12.7	12.3	18.4	0.42	Si (5.6)
hydrolyzed 4h face1	35.5	19.4	14.1	16.1	14.9	0.42	Si (0.8), Cl (0.2)
hydrolyzed 4h face2	37.2	15.4	15.5	16.8	15.1	0.43	
expected value for native	34	16.5	16.5	16.5	16.5	0.49	

Water contact angle

Figure 1 shows the influence of hydrolysis on the water contact angle.

The two faces of the native film have slightly different water contact angles. Face 1 ($\theta = 58$) appears more hydrophilic than face 2 ($\theta = 69$). The contact angle of face 1 increases with the duration of hydrolysis and reaches an almost constant value after 2 h. The contact angle of the other face is not significantly modified by the treatment, taking into account the precision of the measurements. The water contact angles of both faces are not statistically different after 2h hydrolysis (student test at 5%).

Carboxylic chain -end functions

The surface concentrations of -COOH functions accessible to chemical grafting by [^{3}H]-lysine are shown in figure 1. It is important to note that the experimental procedure does not allow to discriminate between the two faces of the polymer. Variations by a factor of 2 have been noted between independent experiments; however all the experiments show the same trend and typical results are reported here.

The amount of -COOH functions accessible to [^{3}H]-lysine increases with the duration of hydrolysis. The sharpest increase is observed at the very beginning of hydrolysis. The number of carboxylic functions detected after 30 min of hydrolysis is 100 times higher than before the treatment, whereas the increase is only 30% between 30 min and 4 h. The lower increase in -COOH functions between 30 min and 4 h might be attributed to the balance between the creation of new chain-ends and the rupture of oligomers which probably occurs when the chain-ends generated by hydrolysis reach a certain length.

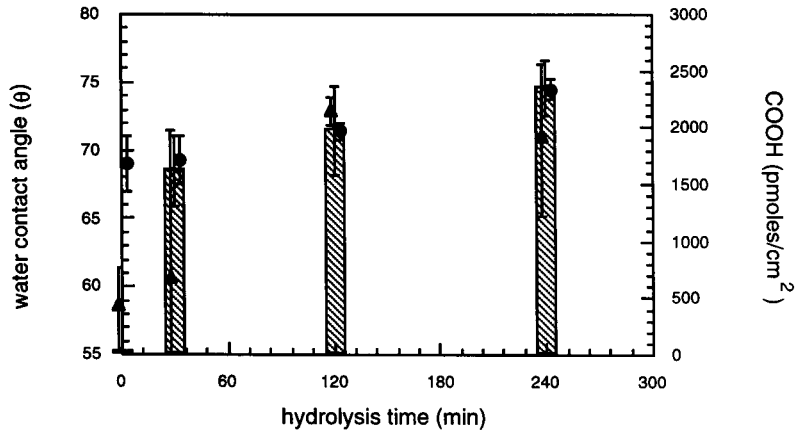

Fig. 1 : Evolution of the water contact angle θ (▲, face1 ; ●, face 2) and number of surface -COOH chain-ends (▨) of PHB/HV (92/8) films during hydrolysis

Assuming that all the polymer surface is covered by carboxylic chain-end functions and considering that the -COOH function is a sphere with a 4 nm diameter [26], the -COOH concentration would be of 1300 pmoles/cm^2. If we consider that the inelastic electron mean free path in the polymer substrate is equal to 3.8 nm [27], that the surface analysed by XPS is 600 μm^2, the density of the polymer is 1.2 g/cm^3 (personal communication from BIOPOL) and the molecular weight of PHB/HV determind by Gel Permeation Chromatography is

337 000, the amount of carboxylic functions estimated in the area analysed by XPS would be 44 pmoles. The amount of carboxylic functions detected on the hydrolyzed polymer is around 2000 pmoles/cm^2; it is of the same order than a densely packed layer of carboxylic functions. This means that the carboxylic chain-ends are formed and accessible over a certain depth. As most part of the -COOH functions result from the hydrolysis, it is concluded that hydrolysis affects not only on the surface but a certain depth of the polymer.

Surface morphology

The surface morphology of the PHB/HV films, native or hydrolyzed for 4h was first examined by Scanning Electron Microscopy (SEM). No difference was noticed between the two faces of the native PHB/HV film.

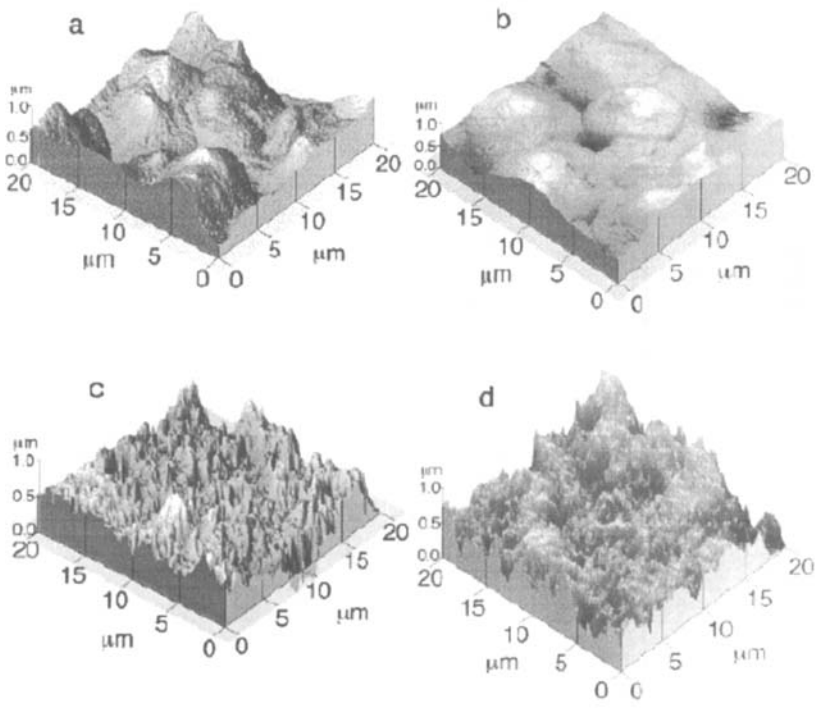

Fig 2 : AFM pictures of native (a-face1, b-face2) and 4 h hydrolyzed (c-face1, d-face2) PHB/HV surfaces.

Surface morphology was also investigated by Atomic Force Microscopy (AFM). In order to have access as close as possible to the surface accessible to the proteins, we used a tip with an approximate radius of curvature at the apex of the order of magnitude of the size of proteins (10 nm). Micrographs are illustrated at figure 2. Spherulites are clearly visible on the native film; the face that went through a corona treatment is more bumped than the other one. This observation is in agreement with other authors who noticed that corona treatment of low-density polyethylene in oxygen or oxygen-containing gases produced bumps on the surface [28]. It appears from the AFM pictures that the surface is etched by hydrolysis. Actually, the number of peaks and valleys seems to increase. A slight increase in the specific surface of PHB/HV with hydrolysis is calculated : the specific surface on face 1 increases from 1.02 ± 0.01 to 1.09 ± 0.01 and that of face 2 shifted from 1.01 ± 0.01 to 1.12 ± 0.01 after 4 h of hydrolysis. The theoretical calculations following from the AFM measurements do thus reveal a maximum increase in the specific surface of 10%. It might be judicious to investigate, if possible, other analytical methods to verify this parameter. No significant change in R_{rms} rugosity is detected with hydrolysis ($100 \text{ nm} \leq R_{rms} \leq 180 \text{ nm}$) eventhough the AFM pictures proves that hydrolysis decreases strongly the characteristic lateral dimension of the surface roughness.
The AFM pictures support the conclusion drawn from the quantification of the carboxylic chain-end functions, that hydrolysis affects the polymer on a certain depth and not only the extreme surface of the film.

Protein adsorption

The second part of the work consisted in studying the effect of the chemical treatment on the adsorption of proteins. In a first set of experiments, we determined the total amount of 3 radiolabelled proteins, namely collagen, albumin and fibronectin, adsorbed from solutions of different concentrations in the range of physiological concentrations on the native polymer and on the 4h hydrolyzed film. In a second set of experiments, the amount of immunoreactive albumin and fibronectin associated with the native and hydrolyzed surfaces were determined by ELISA.

Radiochemical Assay

Typical results are shown in figure 3 (average of two independent sets of experiments). Variations with a factor up to two were observed between different sets of experiments ; however the trend shown by figure 3 was always observed.
The adsorption behaviors on the native and modified film are drastically different : the amount of protein adsorbed on the hydrolyzed polymer was about one order of magnitude higher than on the native one.

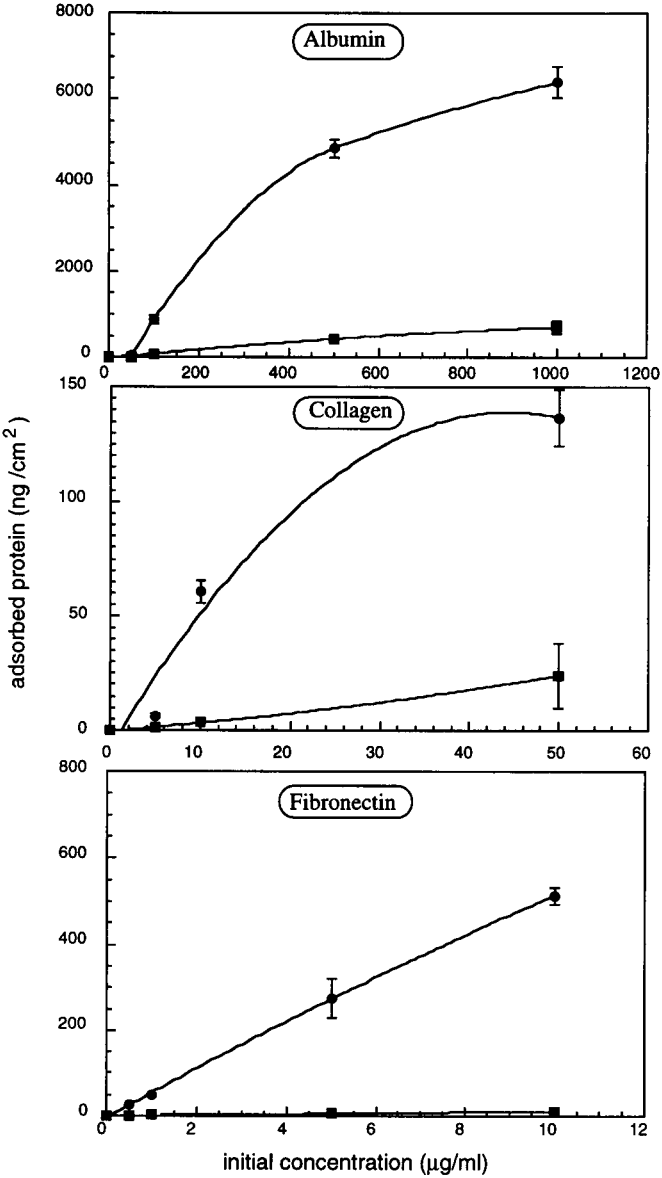

Fig 3 : Adsorption of [^{3}H]-albumin, [^{3}H]-collagen and [^{3}H]-fibronectin on native and hydrolyzed PHB/HV determined by radiochemical assay. Means of 2 experiments are given : ■ = native polymer ; ● = hydrolyzed polymer.

Whatever the substrate, the amount of adsorbed albumin increases linearly up to a concentration of *ca.* 500 µg/ml and then seems to reach a plateau. The amount of collagen adsorbed on the hydrolyzed polymer also tends to reach a plateau. The amount of fibronectin adsorbed on the hydrolyzed polymer increases linearly through the whole range of concentration studied. At a concentration of 10 µg/ml, the amount of radiolabelled fibronectin adsorbed on the modified polymer is one order of magnitude higher than the amount of adsorbed albumin or collagen.

The amount of albumin adsorbed on the native polymer is in the same range than the amount of bovine serum albumin (BSA) detected by Yoon *et al.* [29] on microspheres of PS/PMMA and on TiO_2 and of the same order of magnitude than the amount of HSA adsorbed on negatively charged PS and glass powder [30].

The amount of fibronectin adsorbed on the native polymer is much lower than what is usually reported in the litterature while the amount adsorbed on the hydrolyzed surface is close to the amount adsorbed on TiO_2 [31], PMMA and PET (unpublished data). The amount of albumin adsorbed on the hydrolyzed polymer is much higher than what is generally reported in the litterature.

Knowing the size (14 * 3.8 * 3.8 nm for albumin [29], 300 * 1.5 nm * 1.5 nm for collagen [27] and 70 * 6 * 3 nm for fibronectin [32]) and molecular weight (69 000 for albumin [29], 300 000 for collagen [27] and 440 000 for fibronectin [32]) of the proteins and assuming that the proteins are rectangular parallelepiped, it is possible to estimate the surface they would occupy either in an 'horizontal' (side-on adsorption) or in a 'vertical' (end-on adsorption) position and to deduce the amount corresponding to a closely packed monolayer. The results obtained are 214 ng/cm^2 for albumin, 100 ng/cm^2 for collagen and 175 ng/cm^2 for fibronectin in side-on position and 790 ng/cm^2, 28 µg/cm^2 and 4.1 µg/cm^2 respectively in end-on position.

Assuming, on the basis of the calculations made from the AFM measurements, that the specific surface of the films before and after hydrolysis are effectively very close to each other, the results might signify that albumin and fibronectin form multilayers at the surface of the hydrolyzed polymer and that collagen either form multilayers or adsorbs more or less perpendicular to the surface. On the opposite, adsorption of collagen and fibronectin on the native polymer might lead only to a partial coverage of the film. The current hypothesis explaining the adsorption of albumin and fibronectin in multilayers is that the different layers of proteins adsorbed are unsolubilized because they are trapped in the network formed by oligomers generated by hydrolysis. Nevertheless, on the view of the AFM pictures one should also consider that AFM might not be particularly suitable to evaluate the surface really accessible to the proteins. The important increase in the amounts of radiolabelled protein adsorbed on the hydrolyzed polymer could result from the accumulation of proteins in narrow and deep invaginations of the surface which are not accessible to the AFM tip.

ELISA

The determination by ELISA is based on the recognition of an antigen by specific antibodies coupled to an enzyme whose reaction leads to a color change quantitatively detectable by photometry. It is rather simple to perform but difficult to interpret quantitatively. The information obtained is the density of epitopes recognized by the antibodies and might give an indication on the protein conformation after adsorption on the polymer surface. It is therefore more suitable for relative comparison of the behavior of one particular protein than for the quantitative assay of several proteins on the same substrate. Results are shown in figure 4. It was not possible to perform these experiments on collagen because of the absence of any antibody specific for this protein.

The general tendency of the observed curves was comparable. The absorbance increases linearly with the protein concentration at first and then comes to a saturation. At low concentration, proteins spread on the surface owing to the absence of inhibition contact and their conformation becomes less and less extended while the concentration increases.

For albumin, the adsorption is almost the same on both substrates with a slight preference for the native one. On the native surface, the absorbance increases proportionally to the protein concentration up to a protein concentration of *ca.* 100 µg/ml with the half-saturation reached at *ca.* 30 µg/ml. On the hydrolyzed polymer, the absorbance increases proportionally to the protein concentration up to a protein concentration of *ca.* 30 µg/ml with the half-saturation reached at *ca.* 20 µg/ml. In the range of concentration studied, the surface is saturated very rapidly by albumin.

The adsorption of fibronectin is significantly more important on the hydrolyzed film. On the native surface, the absorbance increases proportionally to the protein concentration to a value of approximately 3 µg/ml and the half-saturation is reached at *ca.* 2 µg/ml. On the hydrolyzed surface, the absorbance increases proportionally to the protein concentration up to a protein concentration of *ca.* 4 µg/ml with the half-saturation reached at *ca.* 3 µg/ml.

The ELISA experiments show that the binding of anti-fibronectin antibodies to the adsorbed fibronectin is only about 5 times higher on the hydrolyzed polymer than on the native one whereas the amount of [^{3}H]-fibronectin detected on the native film at high concentration is almost 100 times lower than on the hydrolyzed one. In the case of albumin, the number of epitopes detected by anti-albumin antibodies on the native polymer is slightly higher than on the hydrolyzed polymer whereas the amount of radioactive albumin detected on the hydrolyzed polymer is an order of magnitude higher than on the native one.

In the hypothesis that fibronectin and albumin adsorb in multilayers on the hydrolyzed polymer, the immunological assay would react only with proteins of the upper layer which would be the most accessible whereas the radiochemical assay would estimate the total

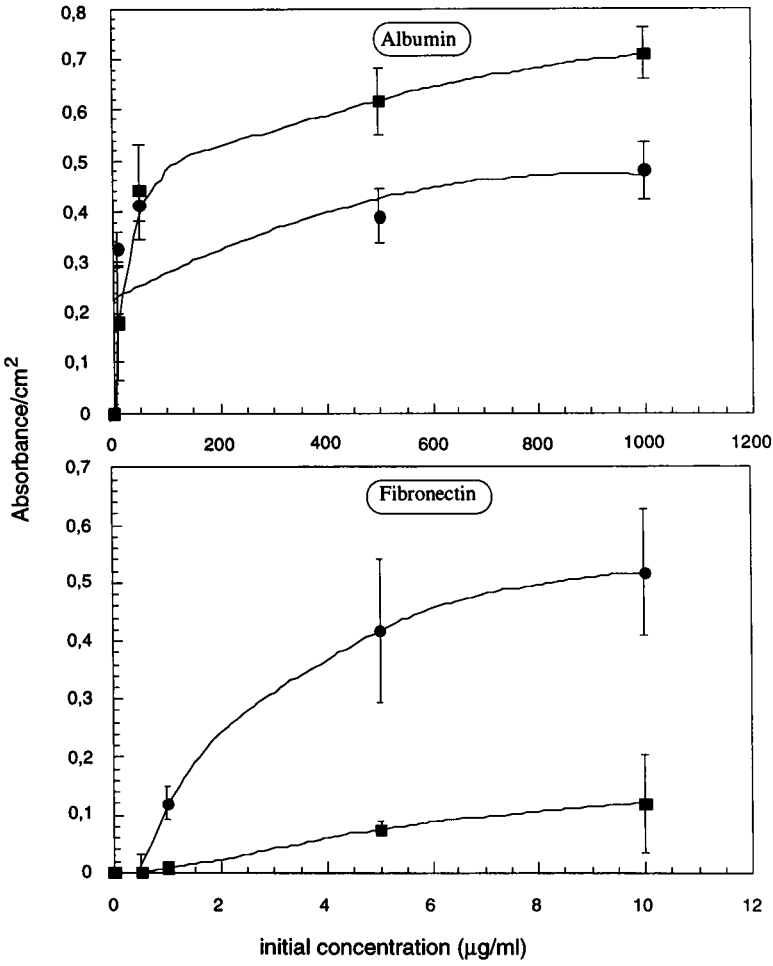

Fig 4 : Adsorption of albumin and fibronectin on native and hydrolyzed PHB/HV (92/8) determined by ELISA. Means of 2 experiments are given : ■ = native polymer : ● = hydrolyzed polymer.

amount adsorbed on the surface. This hypothesis would partly justify the differences observed between the two methods. Another hypothesis, which has already been reported by Dimilla *et al* [35] suggests that the adsorption of the proteins on the different surfaces occured in different conformations and/or orientations. By the way, all the molecules would not be

detected by the antibodies and the ELISA method would underestimate the amount of protein adsorbed. Changes in conformation is the most suitable justification for the divergent results observed by the two methods in the case of albumin.

Cell Culture

The third part of the work consisted in studying the effect of the alkaline hydrolysis on the adhesion and proliferation of monocytes-macrophages from the J774 continuous cell line. Therefore we determined the percentage of the polymer surface area covered by these cells, after inoculation on the native or on the hydrolyzed polymer either without pre-treatment or after a collagen, albumin or fibronectin coating. Results are reported in figure 5.
The only significant difference observed in the adhesion and proliferation of J774 on both faces of the native film is on the surface covered with albumin. The adsorption is slightly higher on the face that went through a corona pre-treatment.
The adsorption of fibronectin on the native film does not have any significant effect (student test at 5%) on the adhesion and proliferation of the cells. Collagen prevents any adhesion of J774 cells while albumin slightly decreases it, affecting by the way the subsequent proliferation of the cells. These observations are in opposition with experiments reported in the litterature with other cell types [5,19] where collagen and fibronectin are well known for its enhancing capacity in adhesion of anchorage dependant cells. Albumin decreases the adsorption of monocytes-macrophages from the J774 cell line, as already reported for other cell types [4,13].

The cells have a greater affinity for the hydrolyzed polymer than for the native one. It has already been reported in the litterature that cell growth may be correlated to the number of carboxyl functions [14] or to the number of hydroxyl functions [15,16] at the surface of the biomaterial, depending on the cell type. The hydrophilicity, the chemical composition and the surface morphology [21,33-35] of the support are also known to modify the cells behavior. Our results confirm these observations. However it is difficult to conclude which is the most important factor modifying the cells behavior due to the complexity of the system.
Contrary to what was observed on the native polymer, the adhesion of the cells is not significantly modified by the adsorption of collagen and albumin whereas fibronectin significantly increases the adsorption of the cells. Proliferation is not significantly modified by the different proteins adsorbed on the hydrolyzed polymer.

The repulsive effect of collagen and, to a lesser extent, of albumin, which is observed on the native polymer, is annihilated on the hydrolyzed film eventhough the amount of protein adsorbed is more important on the hydrolyzed polymer. There is thus no direct correlation

between an increase of the adsorption of proteins on the surface of PHB/HV and a higher sensitivity of macrophages inoculated on that substrate to the protein. These observations

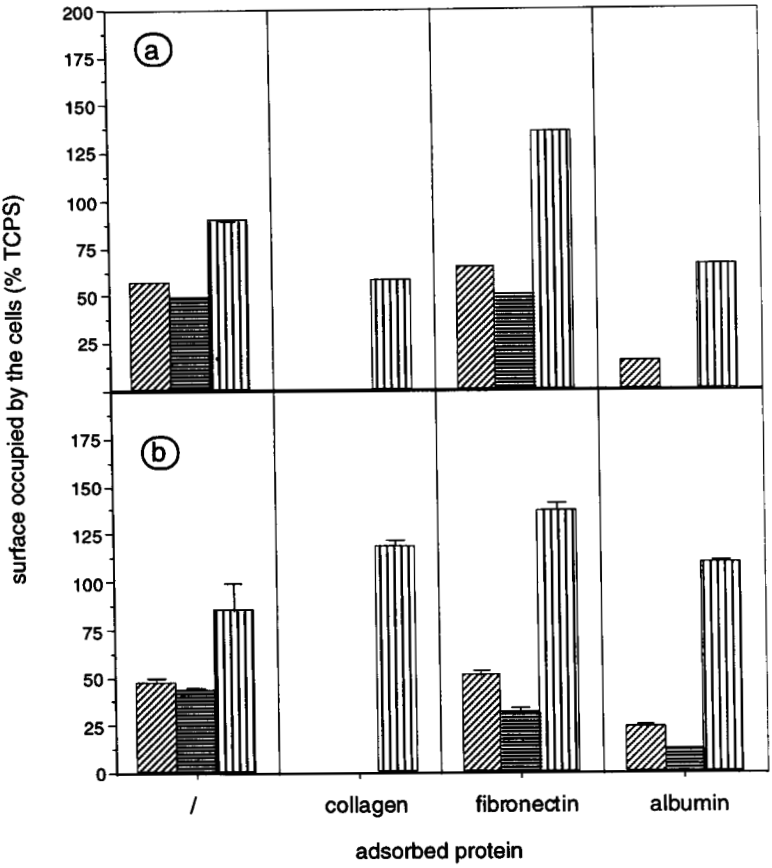

Fig 5 : Adhesion (a) and proliferation (b) of monocytes-macrophages J774 on the native and 4h hydrolyzed PHB/HV (92/8). Results are expressed in function of the area occupied by the cells on TCPS (tissue culture polystyrene) which is usually used as a reference polymer. The values reported are the average between 2 different samples : ▨ = face 1 of the native polymer ; ▤ = face 2 of the native polymer ; ▥ = hydrolyzed polymer.

suggest that, although proteins affect the biocompatibility of the polymer, the polymer substrate by itself and, more precisely, its surface properties, might also influence the cells behavior. This could result from a direct effect of the polymer surface on the conformation of the adsorbed proteins affecting by this way the cell behavior. Actually, the ELISA experiments showed a small difference between the amount of epitopes presented by albumin and fibronectin on the native and hydrolyzed polymer while the amount of radiolabelled proteins present on the hydrolyzed polymer is much more important than on the native one. The conformation of the proteins might be different on both supports and we would assume that the epitopes exposed by the proteins to cells do not induce any specific reaction. Another explanation is that proteins are eliminated from the surface of the hydrolyzed polymer by the macrophages or displaced by proteins secreted by the macrophages.The last hypothesis at this point of the work is that cells proteins would precipitate on the hydrogel formed by the oligomers generated by hydrolysis and collagen multilayers and therefore passivate the surface. These hypothesis are under investigation.

Conclusion

Comparing chemical and morphological surface properties of native and hydrolyzed PHB/HV films revealed that alkaline hydrolysis modifies the chemical as well as the morphological properties of PHB/HV. Alkaline hydrolysis increases the hydrophobicity of one face of the film. This process also eliminates most of the contaminants initially present at the surface of the polymer suggesting that the contamination originates from the corona treatment applied by the manufacturer. The number of carboxylic functions increases very rapidly during the hydrolysis to reach high concentrations. AFM pictures reveal an important change in surface morphology. These results suggest that hydrolysis attacks the polymer in depth.

Radiochemical and immunological experiments investigated the adsorption of proteins and pointed out that these surface modifications lead to an important increase in adsorption of albumin, fibronectin and collagen and probably to a partial change in conformation of these proteins.

The surface modifications also affect the adhesion and proliferation of monocytes-macrophages which are enhanced on the hydrolyzed polymer. The drastic repulsive effect of collagen on the adhesion and proliferation of the cells on the native polymer dissapears on the hydrolyzed one.

Accordingly, it clearly appears from this study that it is possible to modulate the biocompatibility of PHB/HV by either chemical hydrolysis or protein adsorption.

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