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SURFACE PROPERTIES AND TISSUE COMPATIBILITY OF NH_3-PLASMA MODIFIED POLYETHYLENTEREPHTALAT MEMBRANES

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Microwave surface plasma treatment was used to improve the tissue compatibility of polymeric membranes. Changes of surface properties, adhesion and spreading of human skin fibroblasts were studied on ammonia-plasma modified polyethylentetephtalat (PET) membranes. The variation of the microwave (MW)-plasma parameters - duration, feed-gas composition (Ar/NH_3) and microwave power – caused a significant change of the physicochemical surface properties. An enhanced cell-surface interaction was observed based on the number of adhering fibroblasts, but also on morphological criteria including overall cell morphology. It was found that fibroblasts adsorbed better on plasma modified hydrophilic surfaces with a relatively high amine content than on the original membrane.

Keywords: NH_3-plasma, surface functionalisation, wettability, tissue compatibility, cell morphology

Introduction

Polymeric membranes play a crucial role for the development of biohybrid organs in tissue engineering and also in biotechnological applications. In addition to specific bulk characteristics like permeability and selectivity they must possess an excellent biocompatibility. Substitutes for cell cultures have to support cell attachment and proliferation as well as to assure the delivery of nutrients and oxygen and the removal of metabolites [1, 2, 3]. There are several polymeric materials available for the production of synthetic membranes with diverse mechanical properties and permeation characteristic according to special needs, but most of them possess poor characteristics regarding to cellular interaction and function [4].

Attachment, spreading, and proliferation of anchorage dependent cells are highly dependent on the physicochemical properties of the biomaterial surface [5]. Several authors have already reported on enhanced cell adhesion on hydrophilic surfaces [1, 6, 7]. The presence of certain functional groups (eg. amines) [8, 9], or immobilised adhesive proteins [10], surface charge [11], and morphology [12] were also found to regulate cell adhesion.

A number of physical and chemical techniques have already been applied for the modification of the topmost layer of polymeric materials ranging from methods of conventional wet chemistry to novel methods of plasma chemistry [13]. In the last decade techniques using electromagnetic irradiation to induce chemical reactions are getting even more frequent. Such methods like plasma treatment, plasma polymerisation, and ion irradiation, are very attractive, and favourable in modifying samples with a chemically resistive surface or complex shape [6, 14, 15]. If a surface is exposed to a non-polymer forming plasma reactive plasma-species interact with the polymer surfaces and new functional groups are formed. As a result, such implantation reactions lead to remarkable changes of physical and chemical surface properties, and are frequently used to improve adhesion properties and wettability. Ammonia plasma treatment of polymeric surfaces leads to the incorporation of nitrogen containing groups – amine, amide, imin etc. – resulting a hydrophilic surface and supports cell adhesion and growth [9, 16, 17].

In frames of our present work polyethylene-terephtalate (PET) track-etched membranes were modified by low-temperature NH_3-plasma to improve the cell adhesive properties. Polyesters are widely used in the biomedical praxis for catheters, vascular grafts [15] or for joint protheses [18]. Because of the relatively low production costs, mechanical properties, uniform pore size distribution track-etched PET-membranes could be very attractive for tissue engineering applications such as the production of biohybrid organs. We investigated the influence of different process

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parameters on the amine concentration of polyester surfaces. Samples having the highest amine concentration were further characterised by means of X-ray photoelectron spectroscopy (XPS), and fluorescence labelling. The wettability of samples chosen for biocompatibility tests was also investigated by water-air contact angle (captive bubble) measurements. Biocompatibility was evaluated by studying adhesion and proliferation of human skin fibroblasts on modified PET surfaces. Cell adhesion was further characterised studying the overall cell morphology of adhering cells.

Methods and materials

PET-membrane

PET track-etched microfiltration membranes with an effective thickness of 20 µm and nominal pore diameter of 1 µm were purchased from Oxyphem (Großerkmannsdorf, Germany). All membranes were cleaned ultrasonically for 5 min in pure ethanol bath and dried in an exsiccator for 15 min before every treatment.

MW-plasma treatment

The plasma treatment was performed in a flow type cylindrical MW-plasma reactor operating at 2.45 GHz (modified Plaslan 500, JE PlasmaConsult, Wuppertal, Germany) [19].

Samples were placed in the post discharge region, 6 cm downstream from the plasma chamber. The reactor was evacuated down to the base pressure of 10^{-3} bar for 10 minutes, followed by a purge with Ar for 10 other minutes. The flow rate of the Ar/NH_3-mixture was then adjusted and the plasma ignited.

Surface chemical characterisation

The chemical composition of the sample surfaces was determined by X-ray photoelectron spectroscopy (XPS) [20]. The total amine (primary, secondary, and ternary) concentration of surfaces was determined by colorimetric staining with Acid Orange II (AO) [21] and primary amines were labelled with Fluram® to obtain relative concentrations [22].

Surface physical characterisation

Wettability was determined by water air contact angle measurements using the captive bubble method as described elsewhere [3] Pore size distribution of PET-micro-filtration membranes was characterized by bubble point technique using a Coulter Porometer II (Beckman & Coulter Electronics, UK) and Porofil (Coulter) as displacement liquid [3].

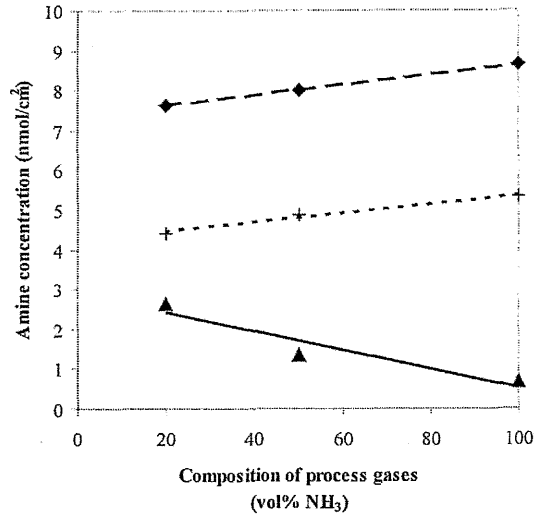

Fig.1 The influence of process gas composition on amine concentration. Samples were treated for 60 seconds. ◆ P_{MW} = 1.2 kW; + P_{MW} = 0.8 kW; ▲ P_{MW} = 0.4 kW

Tissue compatibility

Human skin fibroblasts were obtained from Cell lining GmbH, (Berlin, Germany) and used up to the ninth passage. Cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM) containing 10 % Fetal Bovine Serum (FBS; Sigma, Taufenkirchen, Germany) in a humidified incubator with 5 % CO_2 at 37.5 °C. Samples with a diameter of 13 mm were placed in 24-well tissue culture plates a cell suspension with $2.5x10^4$ cells/ml was filled into each well, and the samples were incubated at 37 °C for times indicated.

For the quantitative determination of cell proliferation LDH (lactate dehydrogenase, Boehringer Mannheim, Penzberg, Germany) tests were carried out, using the commercial PET as reference material. To accomplish biocompatibility studies overall cell morphology was studied by vital staining with fluorescein diacetate (FDA) (Sigma). 5 µl FDA stock solution (5 mg FDA dissolved in 1 ml acetone) was added to the samples with cells and incubated for 2 min at 37 °C, then the samples were washed twice with phosphate-buffered saline (PBS) fixed on a microscope slide and observed by a confocal laser scanning microscope LSM 510 (Carl Zeiss, Jena, Germany) with an excitation at 440...480 nm and emission at max. 520 nm.

Results and Discussion

Surface chemical characterisation

Treatment time and chemical composition of the plasma are the most important factors to determine the result of plasma processes [6, 14]. First the effect of process gas

Table 1 Chemical composition of samples obtained by colorimetric staining, fluorescent labelling and XPS. PET1 was treated with NH_3:Ar (1:5) plasma, at a MW-power of 0.4 kW for 600 sec; PET2 with pure NH_3-plasma, at a MW-power of 0.8 kW for 420 sec; PET3 with NH3-plasma at a MW power of 1.2 kW, for 240 sec

Sample	C	O	N	C_{amine}	$C_{p\text{-}amine}$	* C_{amine}/ N	$C_{p\text{-}amine}$/ N	$C_{p\text{-}amine}$/ C_{amine}
	cc at%			nmol/ cm^2	rel int.			
PET	72.1	27.9	-	-	-	-	-	-
PET1	85.0	13.6	1.4	14.2	135.1	10.1	96.4	9.5
PET2	85.8	12.3	1.9	15.7	100.8	8.3	53.1	6.4
PET3	84.9	11.5	3.6	15.7	81.5	4.4	22.6	5.2

* p-amine contents obtained by fluorescent labelling are only relative values

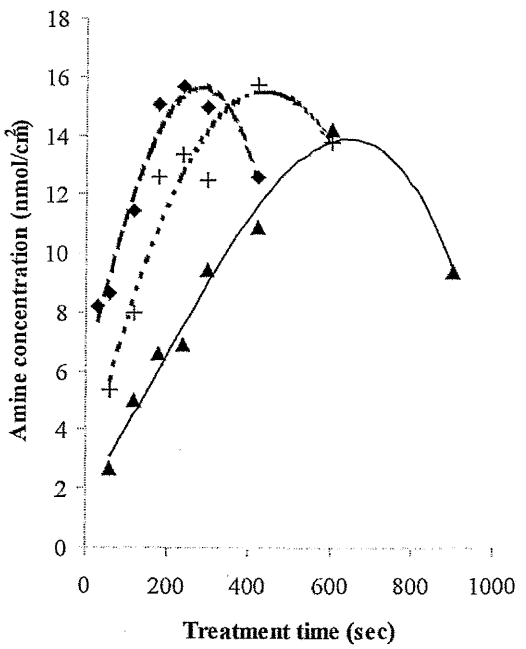

Fig.2 Effect of treatment time on amine concentration of NH_3 treated PET-membrane surfaces. Process parameters were ◆ P_{MW} = 1.2 kW; ϕ_{NH3} = 300 sccm + P_{MW} = 0.8 kW; ϕ_{NH3} = 300 sccm and ▲ P_{MW} = 0.4 kW; ϕ_{NH3} = 50 sccm ϕ_{Ar} = 250 sccm respectively. Deviation within 10 %

composition on the amine concentration of the polymer surface was studied. It was observed that at high MW-power (1.2 kW) the amine concentration increased proportionally to the NH_3 content in the process gas. The highest concentration was reached using a pure NH_3-plasma. In this case the coupled energy was high enough to assure an appropriate ion density and energy of the reactive gas ammonia, in the plasma.

On the contrary at low (0.4 kW) energies when plasma was operated with pure ammonia the coupled energy might not have been enough for an adequate ionisation of the reactive gas, indicated by the relatively low amine concentrations obtained. The mixing of

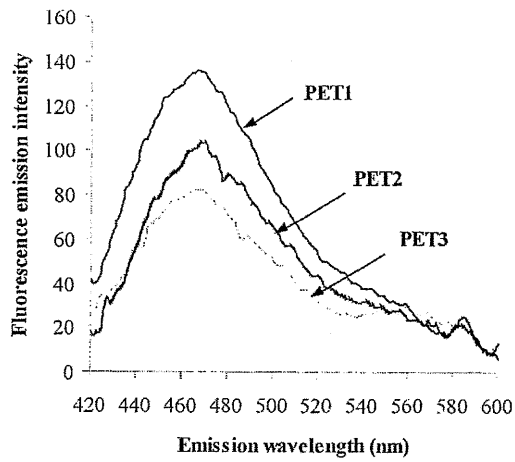

Fig.3 Fluorescence emission spectra of plasma modified PET membranes with Fluram® labelling of primary amines (excitation at 335 nm, emission at 467 nm)

argon to the process gas up to 80 % promoted the implantation of amine functionalities possibly resulted by a higher ion density in the plasma (*Fig.1*).

Treatment time dependency of amine concentration is depicted in *Fig.2*. Already after a short treatment amine groups were incorporated in the samples, and parallel with a longer treatment time an increase of the amine concentration was observed. It is clearly shown that irrespective of the plasma composition (MW-power, and process gas composition) a maximum (15 ± 0.8 nm/cm^2) concentration was reached. This occurred however earlier when samples were treated with high energetic plasma (1.2 kW) - containing more high energetic reactive species - than modified at "milder" conditions (0.4 kW). Nevertheless a further increase of the treatment time led to a loss of amine functionalities. Surface modification by low-pressure plasma can be described as a dynamic equilibrium of competing functionalisation and degradation processes [14]. Our results indicate that after a longer treatment surface degradation, while in the initial phase implantation was predominant.

Samples (PET1, PET2 and PET3) - treated under different plasma conditions – having a relatively high amine concentration were further characterised regarding their primary amine content (*Fig.3*) and elemental composition. The highest p-amine concentration was found on sample PET1, modified at 0.4 kW whereas treatments at higher energies led to a considerably lower p-amine content (*Table 1*).

Results of XPS-measurements are shown in *Table 1*. After exposure to high energetic plasma a relatively high amount of nitrogen was found on PET3 (3.6 at%) and only 1.4 at% on sample PET1 (*Table 1*). Oxygen abstraction was also observed, the total oxygen content decreased drastically in all cases. From 28 at% of the untreated sample it was reduced to 11.4 at% on PET3 and to 13.6 at% on PET1.

It is well known when ammonia is exposed to a plasma, fragmentation, ionisation, excitation and radical

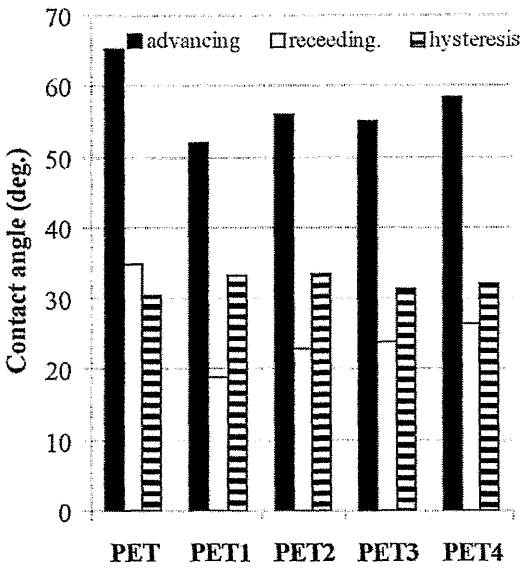

Fig.4 Contact angle measurement of untreated PET, and samples PET1, PET2 PET3, and PET4 (PET4 was exposed to the same plasma as PET1, treated however only for 300 sec and had an amine concentration of 9.45 nmol/cm^2). Deviation was ± 2.5 °

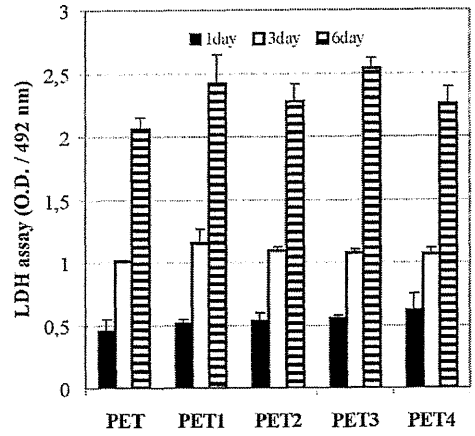

Fig.5 Cell proliferation of human fibroblasts adsorbed on plain PET and plasma modified PET-membranes. Results obtained by LDH-test

generation occurs. In general the fragmentation, ion density and energy of heavy ions is higher at higher energy input than at lower energies. As a consequence a variety of nitrogen containing groups could form depending on process parameters [23]. The higher p-amine concentration on PET1 could be described by the lower fragmentation of ammonia which means that more NH_3^+, NH_2^+ or NH_2^* were available in the plasma, thus the possibility to form $-NH_2$ functionality was also higher in contrast with PET3. Also the differences in plasma composition and ion energy could explain the observation that though amine concentrations were almost the same nitrogen content of PET3, and PET2 was higher than that of PET1. This means - as indicated also from results presented in *Table 1* – that a bigger part of nitrogen was involved to form N-containing groups - other than amines - on PET2 and PET3 than on PET1.

Surface physical characterisation

Fig.4 shows results of water contact-angle measurements of plain PET and differently modified membranes chosen for biocompatibility tests (PET1-4). It is clearly shown that the functional groups introduced by plasma treatment increased the wettability of the surfaces. Sample PET1 were found to be the most hydrophilic than the other samples, but the differences in contact angles between modified ones were only marginal. These results correlated with the results obtained from XPS and colorimetric measurements. Despite of the same amine concentration measured on PET1-3, the degradation of surface polymer and the loss of oxygen of PET2 and PET3 was higher, what could explain the observed deviation in wettability. PET4 was the most hydrophobic of all modified samples. There were no considerable differences in contact angle-hysteresis between the modified polymers, however there is a slight difference in comparison to the unmodified membrane. This could be explained by a change of roughness and/or by the heterogeneity of the samples [7].

No significant changes in permeation characteristics were observed after membrane characterisation. For the unmodified membrane the N_2-flux obtained was 7247 m^3/hm^{2}bar, the average pore size diameter (D(50)) 1.4 µm, and the cut-off (D(100)) 1.51 µm, while for PET3 7338 m^3/hm^{2}bar, 1.37 µm, and 1.51 µm respectively.

Cell proliferation

Metabolic activity and growth of adhering fibroblasts on plain and differently modified PET membranes were evaluated over a cultivation period of 6 days by LDH-assay, shown in *Fig.5*. In early cultures the number of adhered cells on modified membranes was only slightly higher than on plain PET. After 6 days however this effect became significant ($p < 0.05$), while no significant difference were observed between the differently modified membranes (PET1-4) over the entire cultivation period. The improved cell adhesivity of ammonia plasma modified membranes can be explained due to their hydrophilic character and the presence of nitrogen-containing functional groups [24].

Cell morphology

To supplement studies on cell proliferation, investigations studying the morphology of adhering

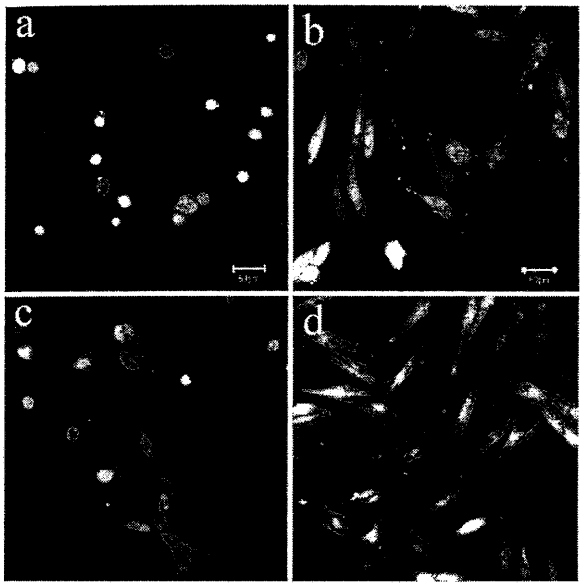

Fig.6 Overall cell morphology of fibroblasts adhering on plain PET (a, b) and PET3 (c, d) membranes; 4 h of cultivation (a, c), 24 h of cultivation (b, d)

fibroblasts were carried out. For cells from connective tissue extensive cell spreading on the foreign substrata indicates generally good cell-contacting properties, while rounding up of cells is a sign of weak cell-material interaction, which could be followed by cell death [1]. Overall cell morphology was studied by staining with FDA after 4h and 24 h of cell contact with the surface. Pictures obtained from plasma modified membranes were very similar thus we present only pictures made from PET3. A higher cell density was observed on modified surfaces, compared to plain PET. Furthermore after 4h cultivation the attached cells on the unmodified PET-surfaces were mostly round and only some spread. On the contrary better fibroblast adhesion and spreading was seen on modified surfaces where many cells were flattened and spread (*Fig.6/a,c*). After 24 h cells were spread on both – plain PET and modified PET - surfaces showing typical fibroblast like morphology. The higher density of fibroblasts however was obvious on PET3 (*Fig.6/b,d*).

Conclusions

Polyethyleneterephtalat membranes were modified by low temperature ammonia plasma with the objective to couple amine functional-groups on the surface and to improve their biocompatibility. It was shown that NH_3-plasma treatment can be effectively used to change the chemical and physical properties of PET membranes. Amine functionalities were coupled onto the membrane surfaces and a saturated stage seemed to exist. In this case the amine concentration was almost the same for all samples but the maximum was reached earlier for high energetic plasma. A high variety of N-containing functional groups were formed above all on PET3 as a result of fragmentation of ammonia caused by high plasma energy. All modified membranes become hydrophilic due to the presence of amine groups and wettability seemed to correlate with the primary amine content. Permeation characteristics remained unchanged after membranes were exposed to the plasma.

Cell proliferation on plasma treated surfaces was almost 125 % of the original membrane after 6 day of cultivation. In the initial stage of attachment more intensive cell spreading was also observed on modified membranes. Hence modified membranes provide better starting conditions for the immobilisation of cells and could be also easily modified by simple wet chemistry. On the other hand based on quantitative and qualitative measurements we presume that differences in plasma conditions (MW-power gas composition) did not influence substantially the adhesive properties of the treated samples. No significant connection between the kind of amine functionalities and biocompatibility was found.

Besides tissue culture a wide range of possible applications for amine containing hydrophilic membranes are imaginable. For instance enzymes or adhesive proteins can be immobilised on amine functionalities [7], blood compatibility can be improved by immobilisation of heparin [25], or anti-fouling properties can be improved by grafting of PEG on amine groups [26].

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